Chapter 5
CONCLUSION

Although the defense responses of *Hevea brasiliensis* against zoospores and elicitin of *P. palmivora* which include necrosis, lignification, Scp and PR-proteins production as well as transcription level of PR-proteins are in the same direction but the elicitin is more effective than zoospores in stimulation of defense responses in rubber. Such responses differ between the resistant and the susceptible clones; they occurred with a faster rate and stayed longer in the former. Thus, these responses can be used as parameters for investigating the degree of resistance of rubber clones. Since the elicitin concentration can be easier controlled and the results are more accurate than those of zoospores, therefore elicitin is more preferable in this regard.

Applications

1. Low concentration of elicitin can induce SAR, therefore the modified elicitin may be used as a plant vaccine.
2. The degree of necrosis and other defense mechanisms induced by elicitin can be used as parameters in selection of rubber resistant clones.

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Appendix

1 Protein Measurement

1.1 Bradford Method (Bradford, 1976)

Bradford reagent : 100 mg of Coomassie brilliant blue G-250 was dissolved in 50 ml of 95% ethanol, then 100 ml of 85% phosphoric acid was added, mixed and diluted with distilled water to total volume of 1 liter. The solution was filtered prior using.

Standard protein 0.5 mg/ml of Bovine Serum Albumin (BSA) : 0.5 mg of BSA was dissolved in 1 ml distilled water, then diluted with distilled water to concentration of 5, 10, 15, 20 and 25 μg /100 μl, respectively.

1.2 Bicinchoninic acid method or BCA method (Smith, *et al.,* 1985)

Solution A :

1. BCA-Na_2 1 % (w/v)
2. sodium carbonate 2 % (w/v)
3. sodium tartrate 0.16 % (w/v)
4. sodium hydroxide 0.4 % (w/v)
5. sodium bicarbonate 0.95 % (w/v)

The mixture is adjust to pH 11.25.

Solution B :

Copper sulphate ($CuSO_4.5H_2O$) 4 % (W/V)

Solution C :

Solution A	20 ml
Solution B	400 μl

The mixture is stable for 1 week at room temperature.

1.3 V8 agar

V8 juice	300 ml
$CaCO_3$	3 g
Agar	15 g
distilled water	800 ml

V8 juice was mixed with distilled water and $CaCO_3$ was added and gently stirred. The media was steriled prior pouring into steriled plate.

2 Enzyme Activity

2.1 Glucanase activity

Laminarin 2 mg / ml : 5 mg of laminarin was dissolved in 2.5 ml of 0.1 M sodium acetate buffer pH 5.0 and kept at 4°C

Dinitrosalicylic acid (DNS) solution : 5 grams of DNS was dissolved in 100 ml of 2 M sodium hydroxide at 80 – 90°C. And then 150 grams of sodium potassium tartrate in 250 ml distilled water was then added and stirred, after which distilled water was added to the total volume of 500 ml and kept at room temperature.

2.2 Glucanase staining on Native-PAGE

0.15 % 2, 3, 5–triphenyltetrezolium chloride : The amount of 0.15 gram of 1 M sodium hydroxide. Only newly prepared solution was used for the study.

2.3 Chitinase activity

2.3.1 colloidal chitin : 10 grams of chitin powder was dissolved in 200 ml of concentrated HCl, stirred with glass rod until completely dissolved and left overnight at 4°C prior to pouring in 600 ml of 50 % deeply cold methanol with vigorous stirring, chitin was gradually precipitated, filtered through filtered paper by using suction flask and washed with ion-free water until washing water reached pH ≅ 7. The chitin precipitates was sucked, then weighed and prepared for 1% concentration (10 mg/ml).

2.3.2 Schales reagent : The amount of 5.295 grams sodium carbonate was dissolved in 90 ml of distilled water : 0.05 gram of potassium ferricyanide was then added and mixed. Distilled water was later added to the total volume of 100 ml.

2.4 Chitinase staining on Native-PAGE

2.4.1 0.1 % Glycol chitin : 5 grams of glycol chitosan was ground in 100 ml of 10 % acetic acid, left overnight at 22°C and added with 450 ml of methanol prior to filtering with filtered paper. Thereafter, 7.5 ml of acetic anhydride was added with continuous stirring. The solution was left for about 30 minutes until gel formation, after which it was cut into small pieces, immersed in methanol and ground for 4 minutes prior to centrifugation at 12,000 rpm for 15 minutes at 4°C. Gel was collected, then methanol was added at 1:1 ratio by volume prior to re-spinning and re-centrifugation. The precipitate was collected, added with 0.02 % sodium azide in 500 ml of distilled water and ground for 4 minutes in order to give 1 % glycol chitin solution.

2.4.2 0.01 % Fluorescent bringhtener 28 : The amount of 0.01 gram of fluorescent brightener (Calcoflur white M2R) was dissolved in 100 ml of 0.5 M Tris–HCl pH 8.5 only newly prepared solution was used for the study.

3. DNA probe preparation

3.1 LB broth : 10 grams of tryptone, 5 grams of yeast extract and 5 grams of NaCl were dissolved in 1 liter of distilled water.

3.2 Lysis solution : Lysozyme was dissolved in 50 mM glucose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA pH 8.0 and made to a final concentration of 5 mg/ml (lysozyme was added before use).

3.3 TE buffer solution : consisted of 10 mM Tris-HCl and 1 mM EDTA pH 8.0.

3.4 NaOH / SDS solution : consisted of 0.2 M NaOH and 1 % sodium dodecyl sulphate. The solution could not be stored, only newly prepared solution was used.

3.5 3 M potassium acetate pH 4.8 solution : The amount of 29 grams potassium acetate was dissolved in 115 ml of glacial acetic acid, then adjusted to pH 4.8 and added with distilled water to total volume of 1 liter.

3.6 Phenol / chloroform / isoamyl alcohol : Phenol, chloroform and amyl alcohol were mixed in 25:24:1 ratio by volume and kept at 4°C in dark room to prevent phenol oxidation. The solution was re-prepared if it turned to orange-red color.

Remark : All solution except phenol / chloroform / isoamyl alcohol was autoclaved at 100°C for 15 minutes to destroy nuclease enzyme before use.

4. Transfer of RNA to nylon

4.1 10 X MOPS /EDTA buffer : consisted of 0.5 M MOPS [3-(N-Morpholino) propanesulfonic acid] pH 7.0 and 10 mM EDTA pH 7.5 diluted to 1X when prepared for electrophoresis buffer.

4.2 Formaldehyde / formamide : consisted of 178 μl of 37 % formaldehyde (12.3 M), 500 μl of deionzied formamide and 322 μl of DEPC (dietyl pyrocarbonate) treated water. The final concentration were 2.2 M and 50 %, respectively.

4.3 Buffer A : consisted of 294 μl of 10 X MOP / EDTA buffer, 706 μl of water and 339 μl of formaldehyde / formamide.

4.4 Gel loading buffer : consisted of 322 μl of buffer A, 5 mg of xylene cyanol, 5 mg of bromophenol blue, 400 mg of sucrose, 178 μl of 37% formaldehyde and 500 μl of formamide.

4.5 1.5% Agarose : consisted of 1.5 gram agarose dissolved in 10 ml of 10 X MOPS / EDTA buffer and 72 ml of water, left to ≅ 60°C and then added 18 ml of 37% formaldehyde prior to pouring on tray. Thirty-minutes pre-run was required before RNA loading.

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Churngchow, N. and **Rattarasarn, M.** 2001. Biosynthesis of scopoletin in *Hevea brasiliensis* leaves inoculated with *Phytophthora palmivora.* J. Plant Physiol. 158, 875-882.