Chapter 3
RESULTS

1.3 Scopoletin (Scp)

1.3.1 Identification and analysis of scopoletin

The inoculum droplet collected from rubber leaves was analyzed by Thin Layer Chromatography (TLC). The chromatogram of TLC was observed under UV light (366 nm) and the blue fluorescent compound in lane 2, 3 and 4 were detected at R_f 0.75 which was the same R_f of standard scopoletin (sigma) in lane 1 (Fig. 25). The quantity of compound in lane 2, 3 and 4 were sequentially increased corresponded to the inoculation period of *P. palmivora* zoospore in BPM-24 (R) leaves. These findings confirmed that the compound was scopoletin, a hydroxycoumarin, which was synthesized by *H. brasiliensis*. Furthermore, this compound was ascertained to be Scp by using HPLC method and showed the same retention time (14.5 minutes) as the Scp marker. (Churngchow and Rattarasarn, 2001)

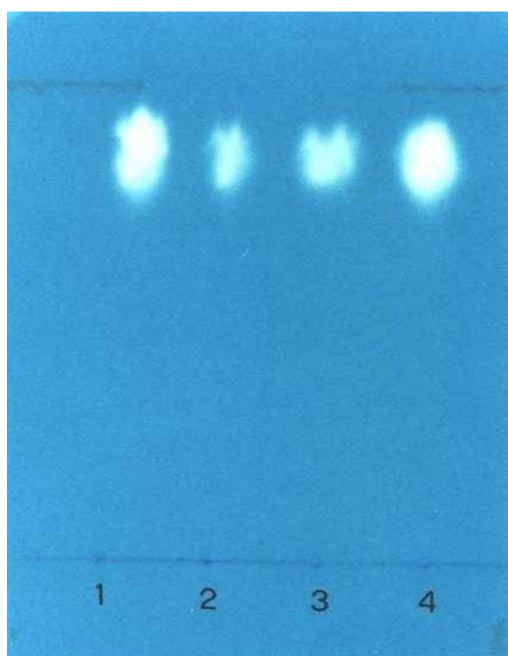


Fig. 25 Thin Layer Chromatography of standard scopoletin (lane 1), and induced scopoletin 6, 12 and 24 hours after inoculation of rubber leaves (BPM-24)

with high concentration of *Phytophthora palmivora* ($5x10^7$ zoospores/ml) (lane 2-4).

1.3.2 Scopoletin synthesis in rubber leaves

The inoculums were recovered 24, 48, 72, 96, 120 and 144 hours after inoculation. The Scp in the inoculum droplet ($5x10^6$ zoospores/ml) of four rubber clones were different in quantity. The Scp produced by the resistant and marked partially resistant clones peaked around 48 hours after inoculation, while the weak partially resistant and susceptible clones did not reach the highest levels until 24 hours later and slightly declined before the resistant and marked partially resistant clones did (Fig. 26). The Scp concentrations in inoculum droplets of resistant and partially resistant clones (BPM-24, PB-235 and RRIT251) were higher than that in the susceptible one (RRIM600). As the new sterile distilled water was reapplied at each time point after the inoculum were collected, the Scp was newly synthesized in the inoculum every 24 hours and reflected new Scp produced in the cells. Thus, the speed and extent of Scp biosynthesis in infected rubber leaflets were positively correlated with the degree of resistance of rubber to *P. palmivora.*

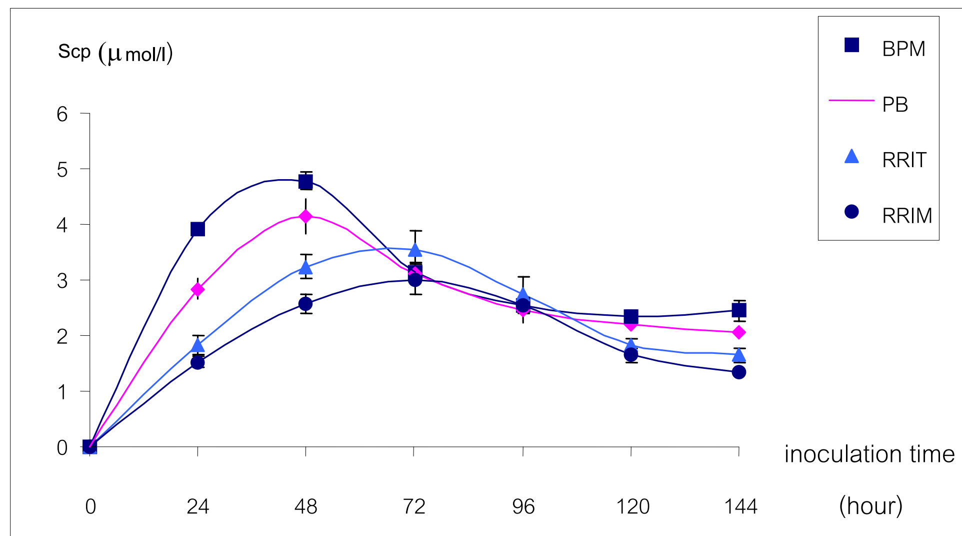


Fig. 26 Synthesis of scopoletin by different resistant rubber clones, BPM-24 : resistant, PB-235 : marked partially resistant, RRIT251 : weak partially resistant and RRIM600 : susceptible, after inoculation with *Phytophthora palmivora* ($5x10^6$ zoospores/ml). Values are means of three replicates.

1.3.3 Scopoletin biosynthesis after zoospore inoculation

Three different zoospore concentrations ($5x10^5$, $5x10^6$ and $5x10^7$ zoospores/ml) were used for further study on two rubber clones very different in degree of resistance to *P. palmivora,* BPM-24 (R) and RRIM600 (S). At low concentration of $5x10^5$ zoospores/ml), Scp production in both rubber clones were relatively the same (Fig. 27). At medium spore concentration ($5x10^6$ spores/ml), the resistant leaves, BPM-24, secreted significant amounts of Scp at 24 hours after inoculation (3.9 μmol/l) and attained a maximum around 24 hours later (4.7 μmol/l) while the susceptible leaves, RRIM600, did not peak until 72 hours after inoculation. At 48 hours after infection, the Scp concentration in droplets of resistant leaves was 4.7 μmol/l which was 1.8-folds higher than 2.5 μmol/l of the susceptible one. From 96-144 hours, the Scp in droplets of resistant leaves remained stable whereas a decreasing concentration was observed in the susceptible one (Fig. 27). At high zoospore concentration ($5x10^7$ zoospores/ml), only 4 hours after infection, the resistant leaves secreted detectable amounts of Scp (1.14 μmol/l) while traces of Scp (0.25 μmol/l) were produced in the susceptible, RRIM600 leaves. With the high zoospore concentration, $5X10^7$ zoospores/ml the Scp of these two clones reached the highest levels around 24 and 48 hours after infection, respectively. At 24 hours, the Scp concentration in droplets of resistant leaves was 8.0 μmol/l which was 1.8-folds higher than 4.3 μmol/l of the susceptible one. From 96-144 hours, the Scp in droplets of both clones were decreased similarly (Fig. 27). However, the Scp remained in the resistant leaves (BPM-24) was slightly higher than in the susceptible one (RRIM600).

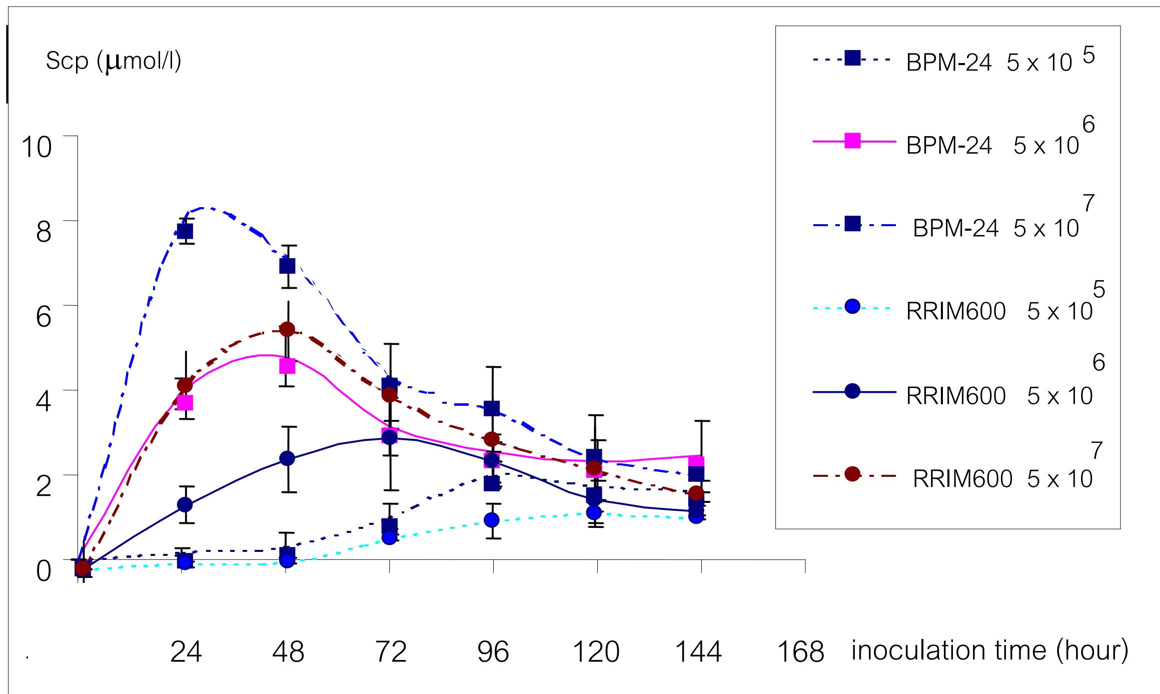


Fig. 27 Speed and extent of scopoletin biosynthesis in inoculum droplets of resistant (BPM-24) and susceptible (RRIM600) rubber clone leaflets inoculated with different concentrations of *Phytophthora palmivora* ($5x10^5$, $5x10^6$ and $5x10^7$ zoospores/ml). Values are means of three replicates.

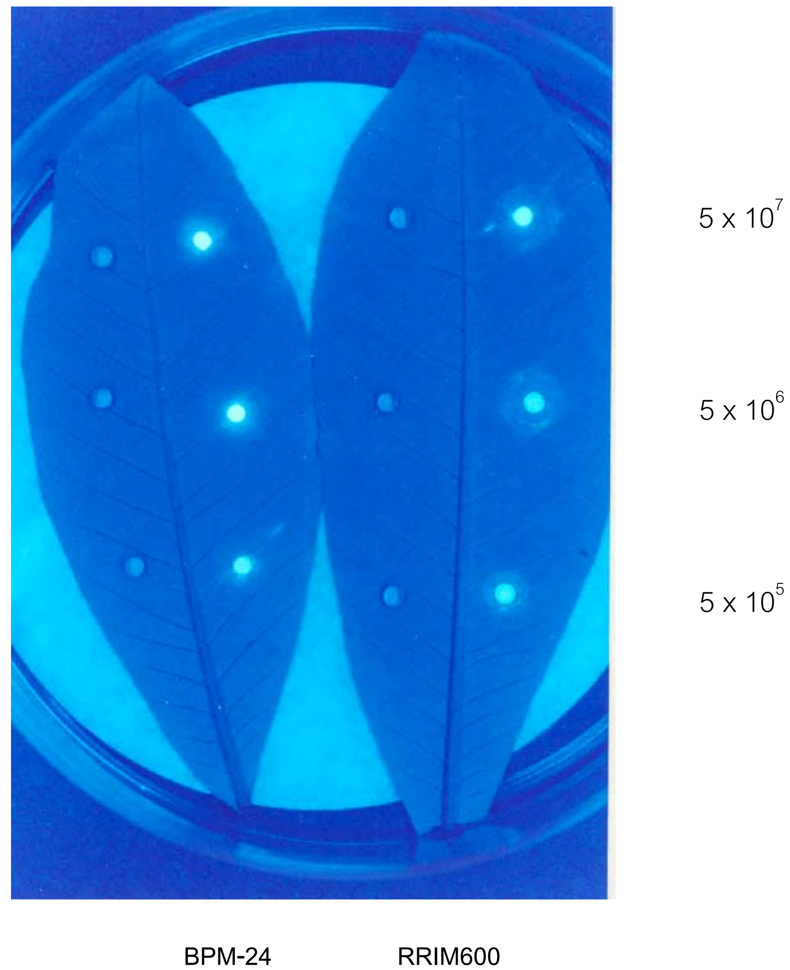


Fig. 28 Scopoletin biosynthesis in inoculum droplets of resistant (BPM-24) and susceptible (RRIM600) rubber clone leaflets inoculated with 20 µl of different zoospore concentrations of *Phytophthora palmivora* ($5x10^5$, $5x10^6$ and $5x10^7$ zoospores/ml). Water was used as control on the other site.

1.3.4 Fungitoxicity of scopoletin for growth inhibition

Four leaf pathogens found on *H. brasiliensis*, *P. palmivora, P. botryosa, C. cassiicola* and *C. gloeosporioides* were tested for their sensitivities to Scp. It was shown that 2 mmol/l of Scp had a marked inhibitory effect on the mycelium growth of *P. palmivora* and the I_{50} was about 1.0 mmol/l which was relatively the same as the effect observed on *P. botryosa* (Fig. 29, 30a). For the concentration 0.5 mM of Scp, the growth rate of *P. palmivora* and *P. botryosa* were inhibited only in the first two days then close to normal two days later. The I_{50} of 3.3 mmol/l scopoletin was obtained against *C. cassiicola,* but *C. gloeosporioides* was inhibited by only 41 % at concentration up to 4 mmol/l (Fig. 30b). Under the ultraviolet light, the unfluorescent zone was observed around mycelium of *C. cassiicola* and *C. gloeosporioides* but not of *P. palmivora* and *P. botryosa*.

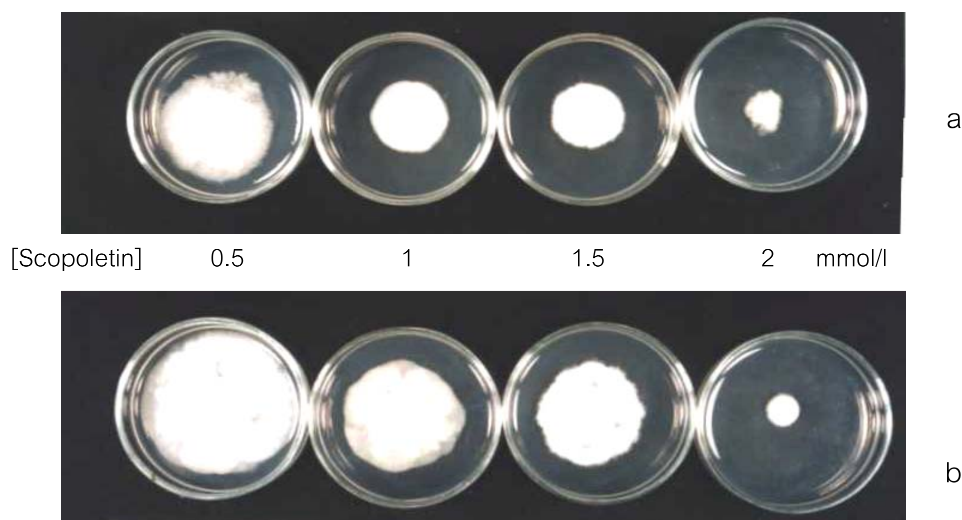


Fig. 29 Growth inhibition of various concentrations of scopoletin to *Phytophthora palmivora*

(a) and *Phytophthora botryosa* (b) at 5 days.

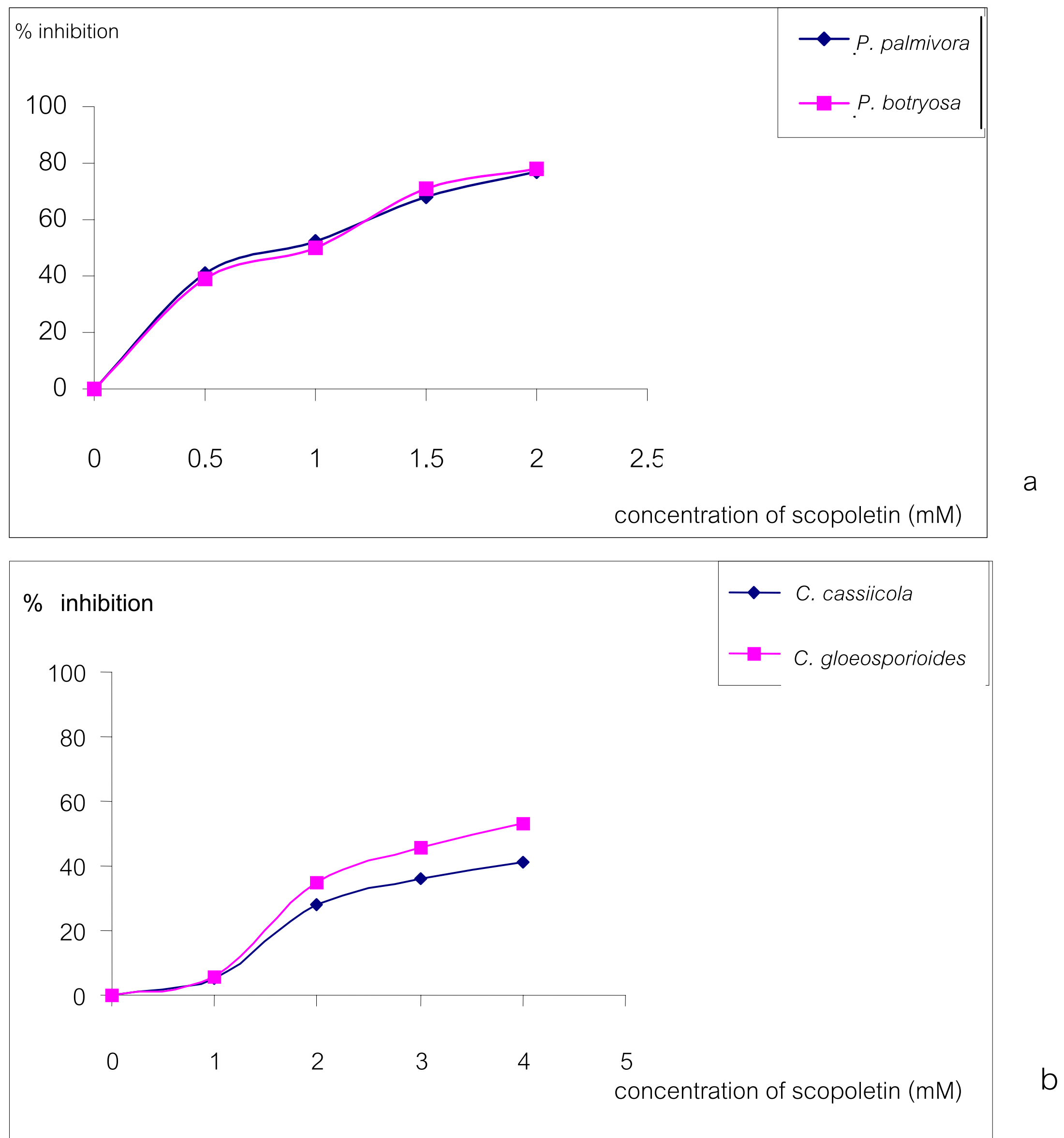


Fig. 30 The effect of scopoletin on mycelium growth (5 days incubation) of *Phytophthora palmivora* and *Phytophthora botryosa* (a) and the other leaf pathogens of rubber, *Corynespora cassiicola* and *Colletotrichum gloeosporioides* (b). Values are means of three replicates.

1.3.5 Fungitoxicity of scopoletin for zoospore germination

After incubation zoospores with different concentration of Scp solution as described in 1.4.6. The number of germinated zoospores at low concentration of Scp (100 μM) was less than in control and was further decreased with higher Scp concentration indicating the direct positive relationship between the amount of Scp and the inhibition of zoospore germination (Fig. 31). Furthermore, germ tube of *P. palmivora* was also shorter at high Scp concentration. It was demonstrated that the length of germ tube at Scp concentration of 1000 μM was 2-4 times shorter than that at Scp concentration of 100 μM at 2 hour after incubation (data not shown). From Fig. 31 the GI_{50} was extrapolated to 750 μM.

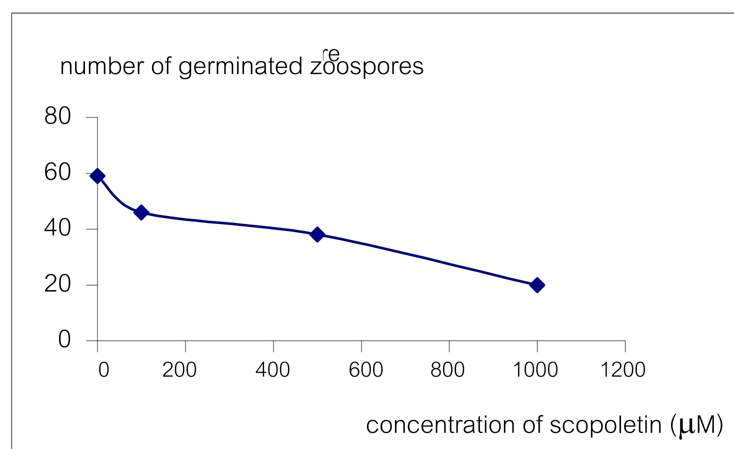


Fig. 31 The zoospore germination of *Phytophthora palmivora* after 2 hours incubation with each scopoletin concentration (100, 500 and 1,000 μM).

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

1.4.1 Measurement of crude leaf extract

The BPM-24 (R) and RRIM600 (S) leaves were inoculated with the high concentration of zoospores ($5x10^7$ zoospores/ml). The inoculated and control leaves were cut into strips and collected every 24 hours for 96 hours after inoculation as describe in 1.5.1. All were ground as described in 1.5.2 and protein in the quantity of the crude extracts were measured by Bradford method. The protein syntheses were increased 24 hours after inoculation and reached theirs peaks at 72 hours and slightly decreased thereafter in both rubber clones. Though the protein syntheses were increased in both rubber clones but the amount of protein in BPM-24 (R) was 2 folds higher than that of RRIM600 (s) (Fig. 32). It appeared that the rate of protein synthesis in the inoculated RRIM600 was decreased slightly quicker than that in BPM-24. In fact, the amount of protein in RRIM600 at 24 hours was slightly lower than that at 0 hour in control and treated leaves. The protein syntheses in the control of both clones were decreased after 24 hours, there were not any infections in the leaves prior to performing the experiments.

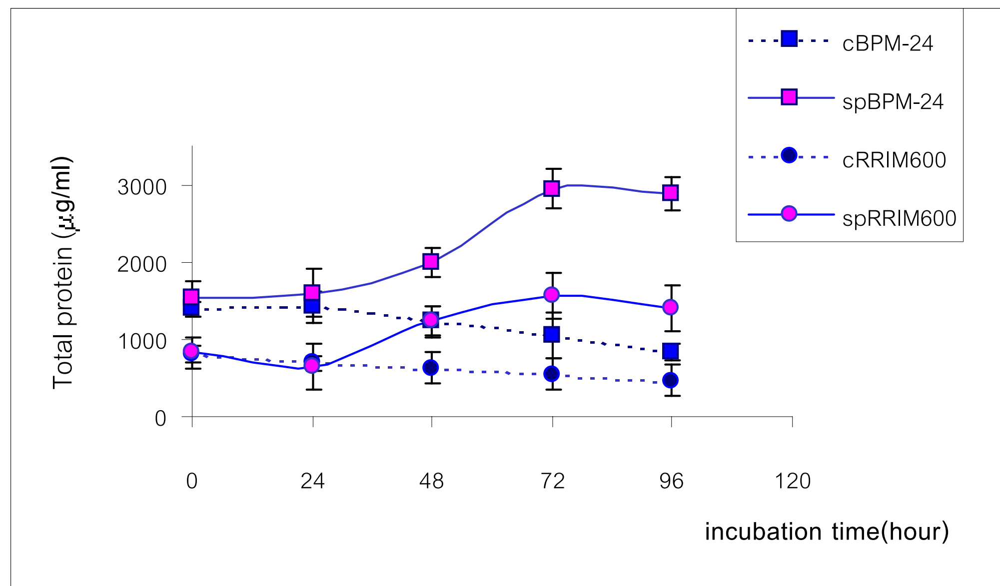


Fig. 32 Evolution of total protein of rubber leaves from a resistant (BPM-24) and a susceptible (RRIM600) clones inoculated with *Phytophthora palmivora* (5×10^7 zoospores/ml) at 0, 24, 48, 72 and 96 hours. Protein was measured by using Bradford method.

c : control, sp : zoospores

1.4.2 Semi-SDS polyacrylamide gel electrophoresis for protein

After zoospore inoculation at 24, 48, 72 and 96 hours, the leave extracts were analysed for the total proteins by Bradford's method (as shown in 1.4.1) and semi-SDS-PAGE. The number and staining intensities of the bands detected in the induced leaf extract of resistant and susceptible clones increased with time at comparable time period after inoculation. However, the intensities of bands in the induced-resistant leaves at 72 and 96 hours were greater than those detected in the induced susceptible leaves (Fig. 33a and b). In contrast, the band of control leaves in both rubber clones decreased with time. Thus results of semi-SDS-PAGE protein analysis were similar to the total protein qualification by Bradford's method.

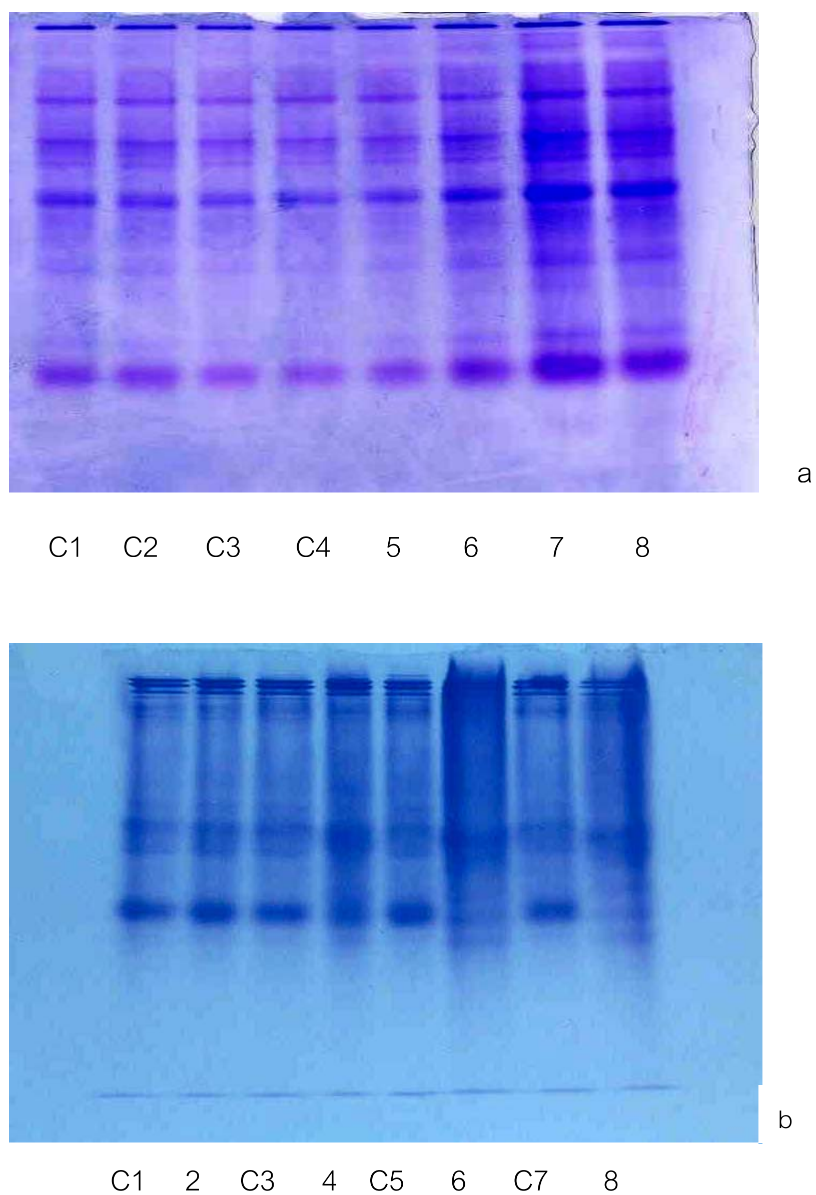


Fig. 33 Electrophoresis of proteins from crude leaf extracts with and without (control) inoculated with *Phytophthora palmivora* (5×10^{7} zoospores/ml) in BPM-24 (a) and RRIM600 (b).

(a) Lane C1 to C4 : control leaves at 6, 12, 24 and 48 hours.

Lane 5 to 8 : inoculated leaves at 6, 12, 24 and 48 hours.

(b) Lane C1, C3, C5 and C7 : control leaves at 6, 12, 24 and 48 hours.

Lane 2, 4, 6 and 8 : inoculated leaves at 6, 12, 24 and 48 hours.

C : control

1.4.3 Glucanase activity

One gram of control and inoculated leaf strips were ground with liquid N_2. Crude extract was quantified for protein content prior to eliminating of internal sugar. Samples, free from internal sugar, were reacted with laminarin at 35 °C and measured for amount of reducing sugar at OD 540 nm as described in 1.7.1. From Fig. 34, the activity of glucanase in inoculated resistant clone (BPM-24) was peaked at 72 hours which was higher than the susceptible and its control. In RRIM600, the activity of glucanase was slightly increased in comparison to its control. When comparing relative values (spore/control) between inoculated BPM-24 and RRIM600, the former was increased much more obviously. Furthermore, the amount and activity of glucanase in BPM-24 reached the highest levels at the same time point as protein content.

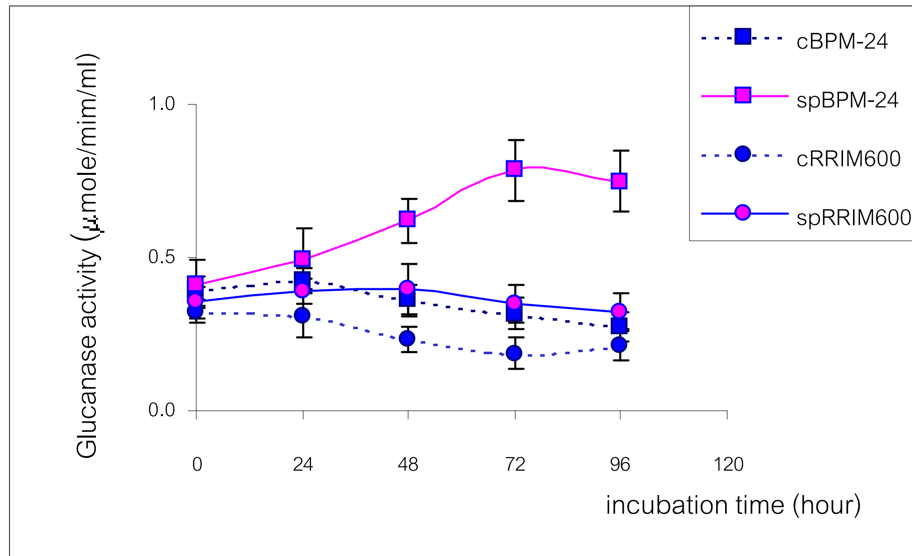


Fig. 34 Evolution of β–1,3-glucanase of rubber leaves from a resistant (BPM-24) and a susceptible (RRIM600) clones inoculated with *Phytophthora palmivora* (5×10^7 zoospores/ml).

c : control; sp : zoospores

1.4.4 Chitinase activity

Leaf strips of two rubber clones, BPM-24 (R) and RRIM600 (S), which were inoculated with zoospores of *P. palmivora* and sterile distilled water were kept at several times and ground with liquid N_2 as in 1.5.1. Crude extract was quantified as in 1.5.3. Measured the amount of reducing sugar which was the product of chitin cleavage by chitinase, the chitinase activity was peaked and increased approximately 3.25-fold in BPM-24 at 48 hours after zoospore inoculation as compared to its control and remained higher than control until 96 hours. The chitinase activity in BPM-24 (R) reached to peak before the total proteins and glucanase activity as well. The chitinase activity in RRIM600 was peaked at 72 hours after inoculation with zoospores and increased only 1.8-fold as compared with its control and started to decline to the same level of its control at 96 hours (Fig. 35). As compared with the resistant rubber clone (BPM-24), the chitinase activity in the susceptible rubber clone (RRIM600) was significantly lower since 48 hours until 96 hours.

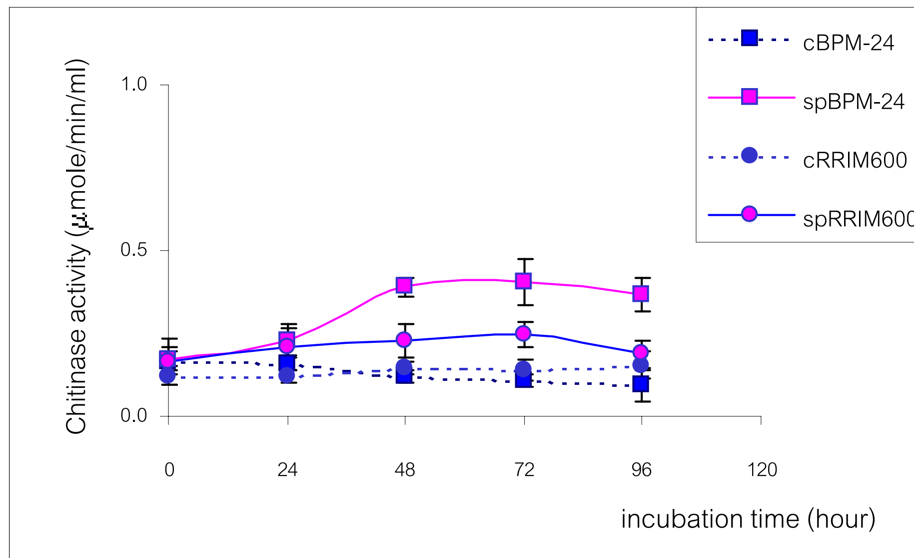


Fig. 35 Evolution of chitinase activity of rubber leaves from a resistant (BPM-24) and a susceptible (RRIM600) clones inoculated with *Phytophthora palmivora* (5×10^7 zoospores/ml)

c : control, sp : zoospores

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

Analysis of chitinase activity in the control leaf extracts by SDS-PAGE revealed 3 isozymes (x, y and z bands) in the resistant clone and 2 isozymes (x and y bands) in the susceptible clone (Fig. 36). After zoospores inoculation, the total activity of chitinase reached its peak at 48 hours in the resistant clone (Fig. 35) and the y and z bands were thicker (Fig. 36a lane 6). In the susceptible clone, the intensity of y bands slightly increased at 72 hours after zoospores inoculation (Fig. 36b lane 7). The x bands in BPM-24 decreased in intensity in both of control and treated leaves after the leaves were detached whereas the intensity of x band was faded in RRIM600.

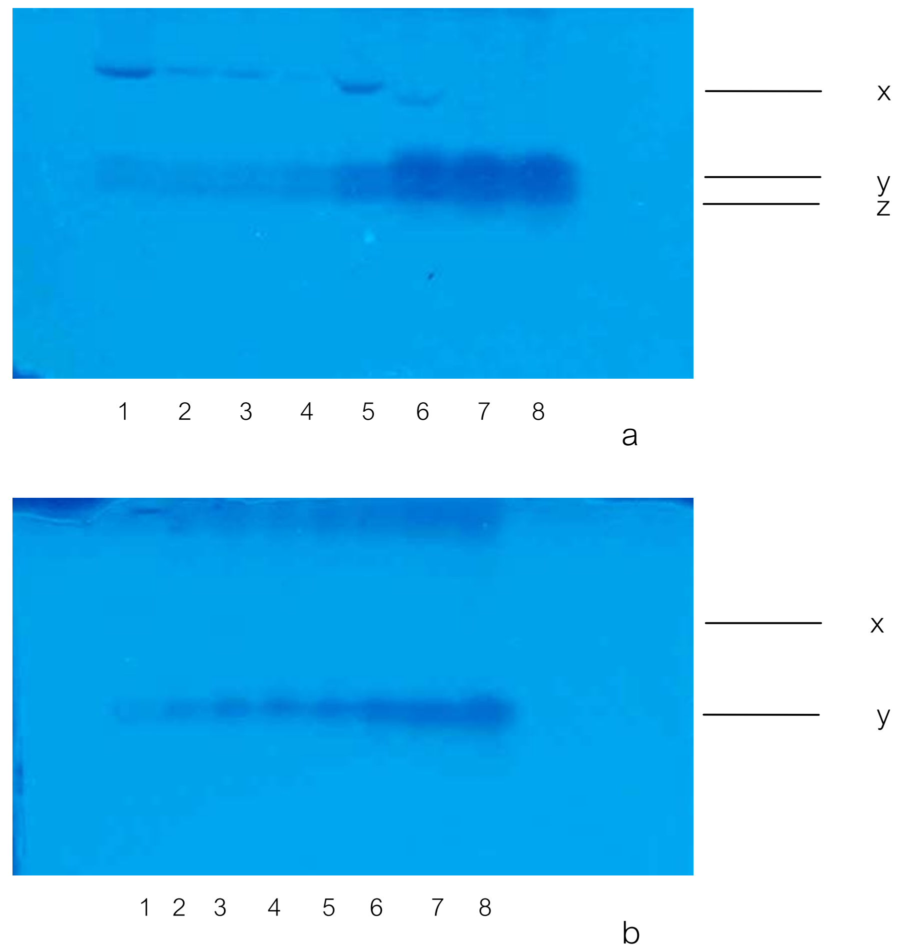


Fig. 36 Time course of induction of chitinase in (a) BPM-24 and (b) RRIM600 after inoculation with *Phytophthora palmivora* ($5x10^7$ zoospores/ml). X, Y and Z were *Hevea* chitinase isozymes.

Lane 1 to 4 were crude extracts of control leaves at 24, 48, 72 and 96 hours.

Lane 5 to 8 were crude extracts of inoculated leaves at 24, 48, 72 and 96 hours.

2 Interaction between elicitin and rubber leaves

2.1 *Phytophthora palmivora*

2.1.1 The culture filtrate of *Phytophthora palmivora*

The mycelium of *P. palmivora* that was transferred from PDA grew like a cotton ball in PDB. The color of culture filtrate was clear and slightly yellow. (Fig. 37)

Fig. 37 The 20-day old in 300 ml culture filtrate of *Phytophthora palmivora.*

2.1.2 The extracellular protein production curve of *P. palmivora*

The protein quantification by BCA method was calculated by comparing with standard curve of BSA. *P. palmivora* which was cultured in PDB, produced extracellular protein including elicitin which peaked at 20 days after subcultured from PDA. The amount of extracellular proteins was stable for a short period and slowly declined after 40 days which paralleled the level of elicitin as detected by Tricine–SDS–PAGE using 16.5% polyacrylamide gel according to the method in Schagger and Jagow, 1987 (Fig. 38, 39). This phenomenon indicated that *P. palmivora* actively produced proteins when it

was growing and stopped producing proteins when the population of mycelium in PDB was crowded. There were some tiny bands after the 20th day of culture. Therefore, the suitable time for harvesting the high yield of elicitin and low contamination from other proteins is 20 days.

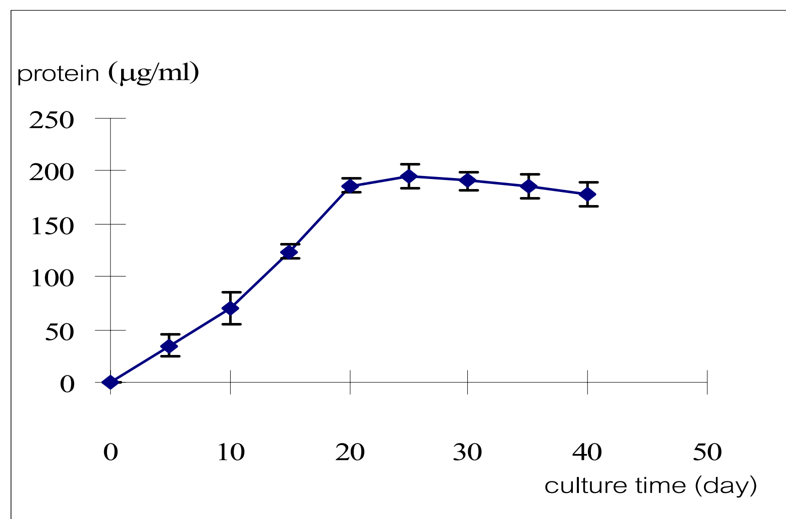


Fig. 38 Extracellular proteins secreted by *Phytophthora palmivora*, *Hevea* isolate, grown in Potato Dextrose Broth for 40 days. Every five days, culture filtrate was measured for protein concentration.

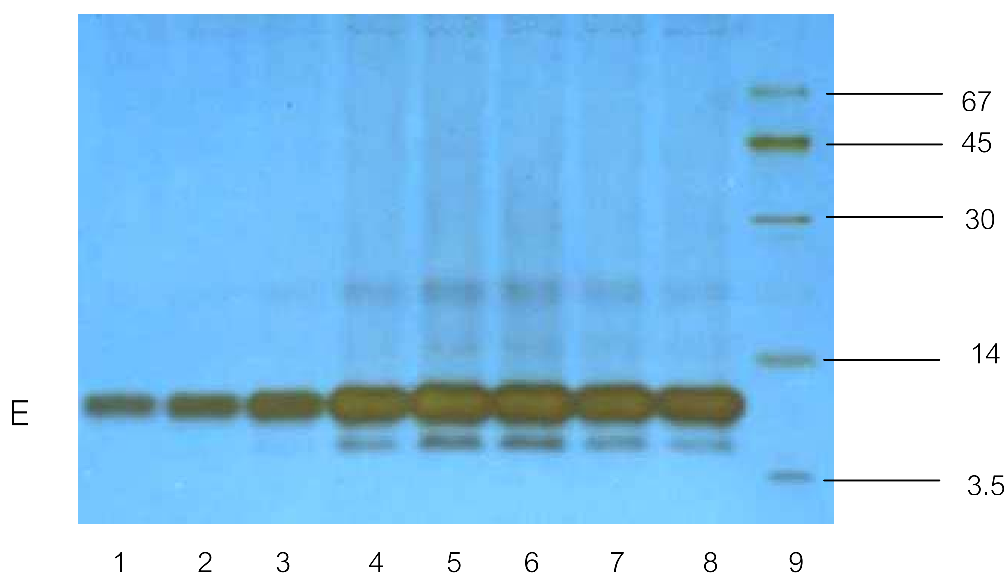


Fig. 39 Extracellular proteins secreted by *Phytophthora palmivora*, *Hevea* isolate, grown in Potato Dextrose Broth for 40 days. Every five days, culture filtrate was separated on a 16.5 % polyacrylamide gel of Tricine-SDS-PAGE then stained with silver nitrate (lanes 1-8). Lane 9 is standard protein markers, LMW kit *Phytophthora* from Pharmacia plus insulin chain B : 3.5 kDa; elicitin bands are indicated by E in the figure.

2.1.3 Purification and percent yield of elicitin

Crude proteins from culture filtrate were precipitated with ammonium sulfate, then further purified by DEAE-cellulose ion-exchange and Sephadex G-50 gel filtration (Fig. 40,41). The elicitin peaks were identified by SDS-PAGE and also by toxicity tests on detached tobacco leaves. Active fractions (#35-50) from the DEAE-cellulose column were pooled and applied on a Sephadex G-50 column (Fig. 41). By Bradford method, we obtained only 0.41 mg of elicitin thus named palmivorein (Pal) per 1 litre of filtrate; however, the protein was not sensitive to Bradford reagent because it possesses a very low content of basic amino acids (Table 6). For this reason, a bicinchoninic acid (BCA) method was used instead to calculate yields (Table 5). As seen in table 5, the Pal concentration was much higher than that suggested by the Bradford method. The Pal appears to be pure because no any contaminant proteins were revealed through the silver staining of Tricine-SDS-PAGE and its M_r was found to be ca 10,000. (Fig. 43, lane 5).

The elicitin possesses a very low content of basic amino acids, therefore the amount of protein determined by Bradford method was not accurate. For this reason, the BCA method was used to quantify the amount of elicitin which will be described in 2.2.6. By this latter method, 8 mg of elicitin was purified from 1 litre of culture filtrate and equivalent to 2% yield of purification (Table 5).

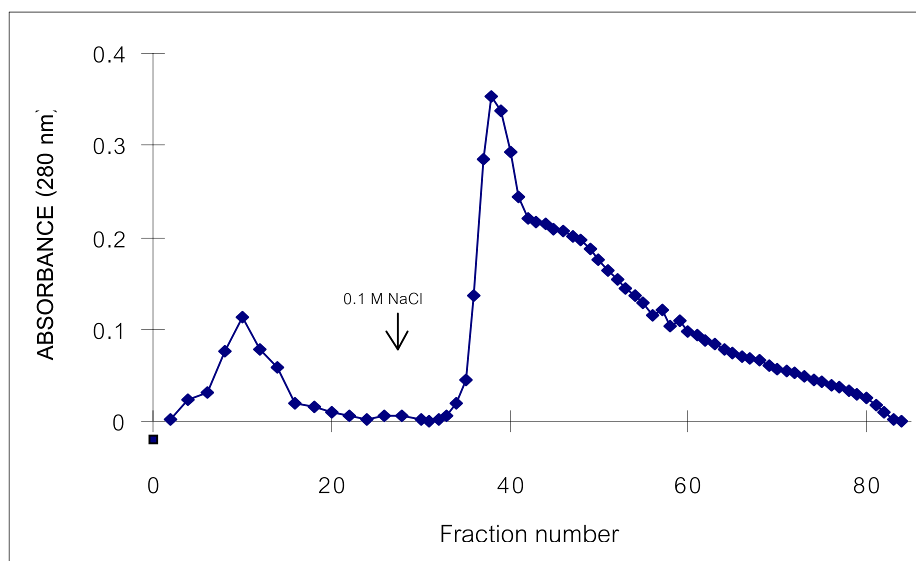


Fig. 40 DEAE-cellulose chromatography of the culture filtrate of *Phytophthora palmivora*. The first peak was unbound proteins. The bound protein was shown by the second peak after eluted with 0.1 M NaCl in 20 mM Tris-HCl pH 7.0 (flow rate 12 ml/hr).

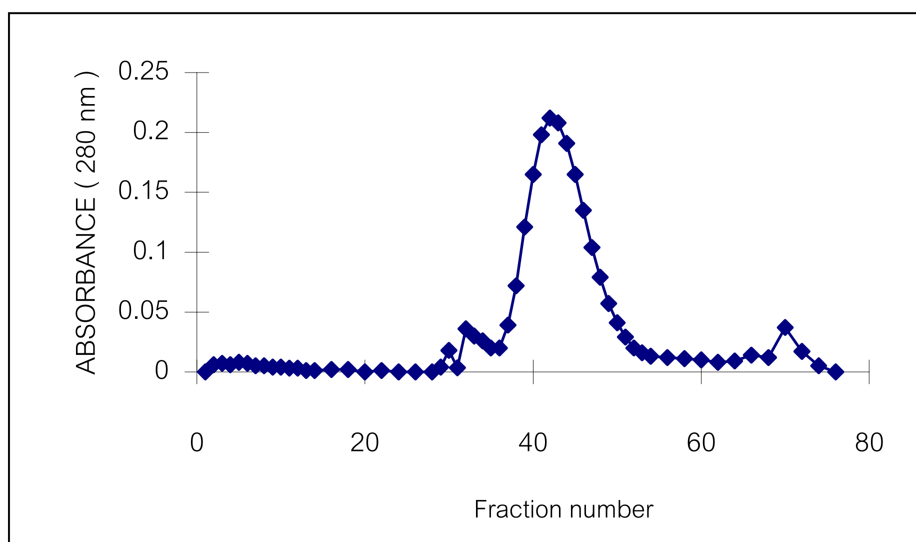


Fig. 41 Sephadex G-50 chromatography of bound proteins from DEAE column. The column was equilibrated and eluted with 20 mM Tris-HCl pH 7.0 (flow rate 18 ml/hr).

Table 5 Protein yield during purification of elicitin from 1 litre of culture filtrate of *Phytophthora palmivora*. The culture filtrate was precipitated with ammonium sulfate, then further purified by DEAE-cellulose ion-exchange and Sephadex G-50 gel filtration chromatography.

Purification steps	Bradford method		BCA method	
	Protein (mg/L)	% yield	Protein (mg/L)	% yield
Filtrate	33.00	100	194.77	100
$(NH_4)_2SO_4$ precipitation	11.27	34.14	61.19	31.42
DEAE colu mn	0.58	1.75	11.86	6.09
Sephadex G-50 column	0.41	1.23	4.12	2.11

2.1.4 Amino acid compositions of *Phytophthora palmivora* elicitin

At least ten elicitins have been sequenced and it was found that all contained 98 amino acids (Parker *et al*., 1991; Billard *et al*., 1988; Huet and Pernollet, 1989; Ricci *et al*., 1992; Nespoulous, Huet & Pernollet, 1992; Huet, Nespoulous and Pernollet, 1992; Huet and Pernollet, 1993; Huet, Mansion and Pernollet, 1993). The integer number of each amino acid in Pal was deduced based on their mole percents as determined by amino acid analysis (Table 6), and compared to other α-elicitins : Cacto (*P. catorum* elicitin) and MgMα (*P. megasperma* var. *megasperma* elicitin) (Huet and Pernollet, 1993; Huet, Mansion and Pernollet, 1993). Since these two elicitins were completely sequenced, the number of each amino acid was obtained directly from the sequences (Table 6). Like other elicitins, Pal contains 10 Leu, 6 Cys and 3 Met, and lacks Trp, His and Arg. In *P. palmivora* elicitin, Leu, Ser, Thr and Ala are accounted for nearly 50% of the residues, as found in other known elicitins (Table 6).

Table 6 Approximated and determined amino acid compositions of *Phytophthora palmivora* elicitin and comparison to other α- elicitins : Cacto, *P. cactorum* elicitin; MgMα, *P. megasperma* var. *megasperma* α elicitin (Huet and Pernollet, 1993; Huet, Mansion and Pernollet, 1993).

Amino acid	MgMα		Cacto		Palmivorein	
	determined	deduced from seq.	determined	deduced from seq.	determined	deduced from the determined no.
Leu	10.2	10	10.1	10	9.7	10
Ser	12.2	12	14.7	16	13.1	13
Thr	16.8	17	16.6	16	17.0	17
Ala	10.3	11	8.3	8	10.2	10
Cys	5.7	6	5.8	6	5.9	6
Met	2.7	3	2.9	3	3.0	3
Phe	2.1	2	2.0	2	2.1	2
Tyr	5.0	5	4.9	5	5.1	5
Lys	2.1	2	2.1	2	1.9	2
Ile	3.0	3	2.9	3	3.5	4
Val	5.9	6	6.0	6	4.9	5
Pro	4.2	4	4.1	4	4.9	5
(Asp+Asn)	9.7	9	8.1	8	8.3	8
(Glu+Gln)	4.8	5	5.3	5	5.4	5
Gly	3.1	3	4.0	4	3.3	3
Trp	0	0	0	0	0	0
His	0	0	0	0	0	0
Arg	0	0	0	0	0	0
Total		98		98		98

2.1.5 N-terminal sequencing and isoelectric point of purified elicitin

The N-terminal end of Pal was sequenced up to Gln 26, allowing the identification of Val 13, which is characteristic of acidic α-elicitins. Fig. 42 shows the N-terminal sequences of known α-elicitins (Huet, Salle′-Tourne and Pernollet, 1994) : Cacto (*P. cactorum* elicitin); Cap *(P. capsici* elicitin); Dre α (*P. drechsleri* α elicitin); Inf (*P. infestans* elicitin); MgMα (*P. megasperma* var. *megasperma* α elicitin); Para (*P. parasitica* elicitin) as compared with Pal (*P. palmivora* elicitin). They are homologous to each other and no deletion is necessary for aligning their sequences. The N-terminal sequences with Val at position 13 and the measured isoelectric point of 4.0 ± 0.2 clearly classifies this protein as an acidic α-elicitin (Churngchow and Rattarasarn, 2000). The N-terminal 26 residues of Pal are identical to those of the elicitin from *P. parasitica*; however, they are not the same protein, since they differ in their overall amino acid composition (the elicitin of *P. parasitica* was completely sequenced by Mouton-Perronnet, *et al*.,1995). Some *Phytophthora* species secrete only an acidic elicitin as observed in our experiment; however, it was demonstrated that *P. drechsleri* simultaneously secreted three elicitin isoforms representing both acidic and basic classes (Huet, Nespoulous and Pernollet, 1992). Huet, *et al.* (1993) also reported the occurrence of two isoforms in another *Phytophthora* species, *P. megasperma* var. *megasperma*, one acidic and one basic, but none has yet been found to secrete only a basic isoform.

Cacto	A	TCT	SS	QQT	A	AYV	A	LVSILSD	T	SFNQC	ST
Cap	A	TCT	TT	QQT	A	AYV	S	LVSILSD	A	SFNQC	AT
Dreα	T	TCT	ST	QQT	T	AYV	S	LVSILSD	A	SFNQC	AT
Inf	T	TCT	TS	QQT	V	AYV	A	LVSILSD	T	SFNQC	ST
MgMα	T	TCT	ST	QQT	A	AYV	T	LVSILSD	S	SFNQC	AT
Para	T	TCT	TT	QQT	A	AYV	A	LVSILSD	T	SFNQC	ST
Pal	T	TCT	TT	QQT	A	AYV	A	LVSILSD	T	SFNQ-	--

Fig. 42 Comparison of the N-terminal sequences of *Phytophthora palmivora* elictin to other α - elictins : Cacto, *P. cactorum* elictin; Cap, *P. capsici* elicitin; Dreα, *P. drechsleri* elicitin; Inf, *P. infestans* elictin; MgMα, *P. megasperma* var. *mesgasperma* α elicitin; Para, *P. parasitica* elicitin and Pal, *P. palmivora* elicitin. The boxes show the conserved consensus regions. Known N-terminal amino acid sequences were obtained from Huet, *et al.* (1994).

2.1.6 Characterization of elicitin by Tricine-SDS-PAGE

After staining the PAGE gel with silver staining method, the electrophoresis profile exhibited the major band of protein, molecular weight of which was about 10 kDa compared with the markers in Fig. 43 lane 1 and 6. One band of protein from the culture filtration lane 2 was identified and supposed to be elicitin this is the major extracellular protein. In lane 3 (the precipitate of culture filtrate), there were other bands of protein in addition to the one observed in lane 2. After purification of the precipitate of culture filtrate with DEAE chromatography, most of proteins were removed. The fraction obtained through DEAE column was repurified by Sephadex G-50 column and the electrophoresis profile of purified protein was clearly exhibited as elicitin in lane 4. The molecular weight of elicitin was about 10 kDa and it was named palmivorein (Churngchow and Rattarasarn, 2000).

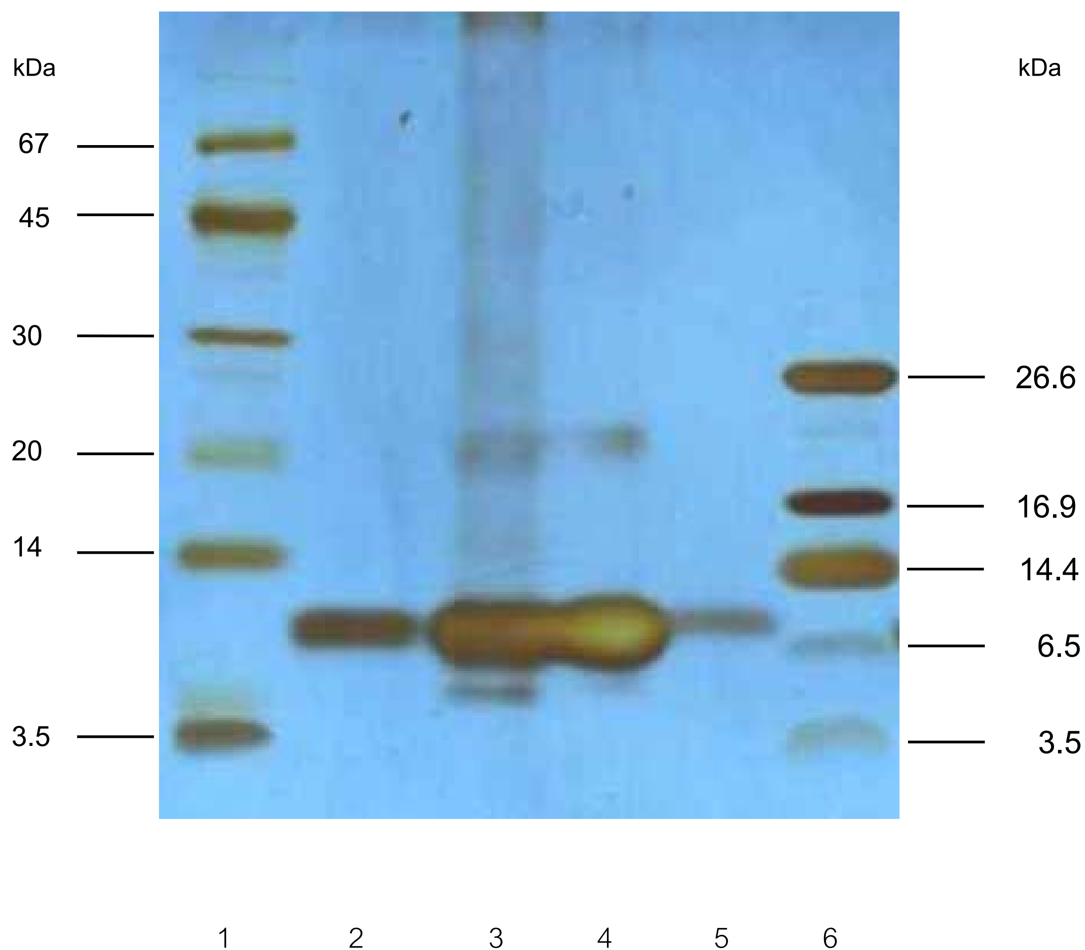


Fig. 43 Silver stained Tricine-SDS-PAGE of purified elicitin.

Lane 1: standard protein markers (LMW kit from Pharmacia plus insulin chain B : 3.5 kDa).

Lane 2: crude culture filtrate (1.5 μg).

Lane 3: ammonium sulfate precipitated proteins (3.9 μg).

Lane 4: bound to DEAE cellulose (4.5 μg).

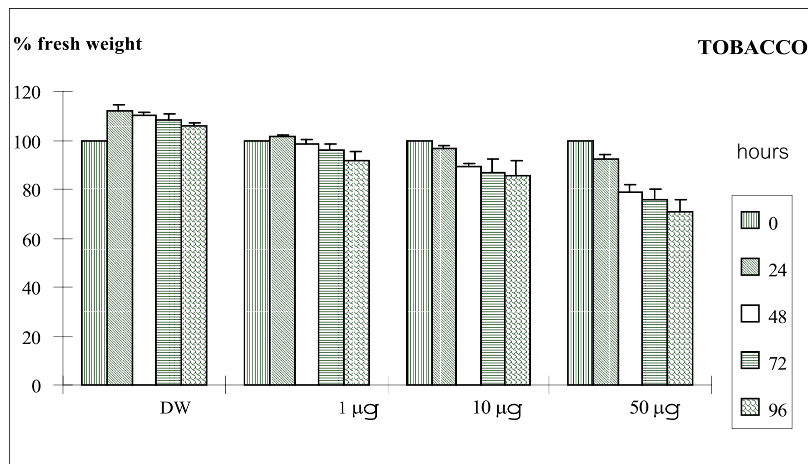
Lane 5: purified elictin from sephadex G-50 (0.7 μg).

Lane 6: standard protein markers, polypeptide kit from Bio-Rad.

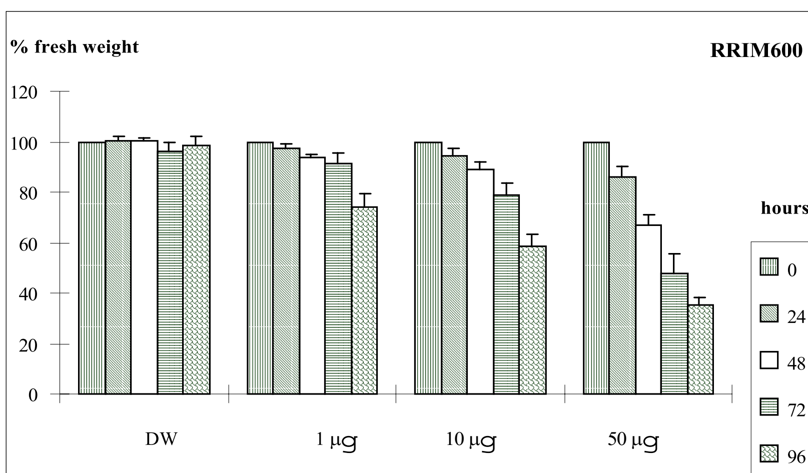
2.2 Toxicity test

2.2.1 Toxicity of elicitin on tobacco and rubber leaves.

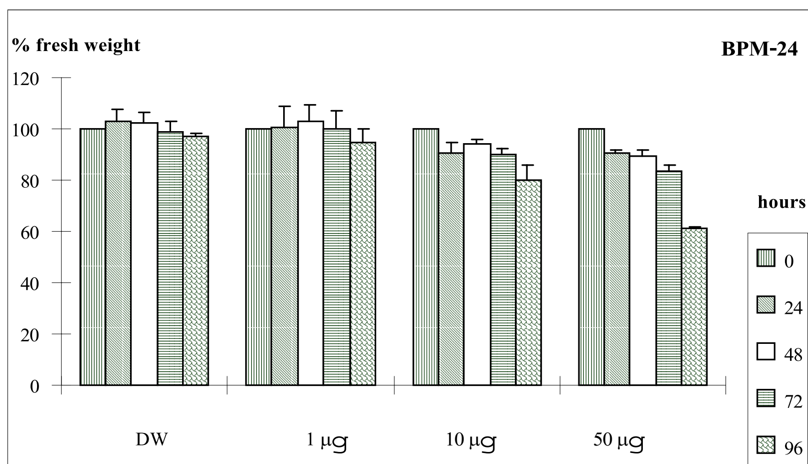
When detached leaves of *N. tabacum* were tested with purified elicitin, they partly dried up and necrosis developed first in the apical part (Fig. 45). A decrease in leaf fresh weight was both time-and dose-dependent (Fig. 44a). The excised rubber leaves were studied in parallel; Pal also showed severe wilting and necrosis of these leaves (Fig. 45), as indicated by a decrease in percent of fresh weight (Fig. 44b and 44c). The reasonable physiologic doses for detecting necrosis in tobacco and BPM-24 leaves in Fig. 44a and 44c were about 12.5 μg/gram leaf fresh weight. The lowest of elicitin caused necrosis in RRIM600 leaves was at about 2.5 μg/gram fresh weight (Fig. 4B).



a

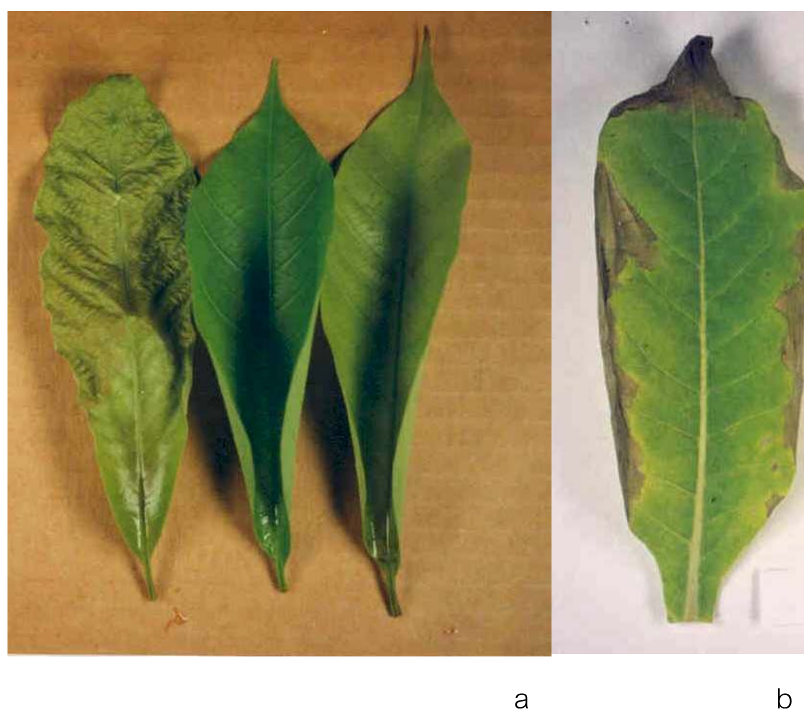


b



c

Fig. 44 Induction of leaf necrosis by palmivorein in *Nicotiana tabacum* (a), RRIM600 (b) and BPM-24 (c). A 100 µl of palmivorein (1, 10 and 50 µg by the bichoninic acid method) was adsorbed through the petioles of excised leaves. At various times leaf fresh weights were measured. Results are expressed as percentages of the initial values of the fresh weight.



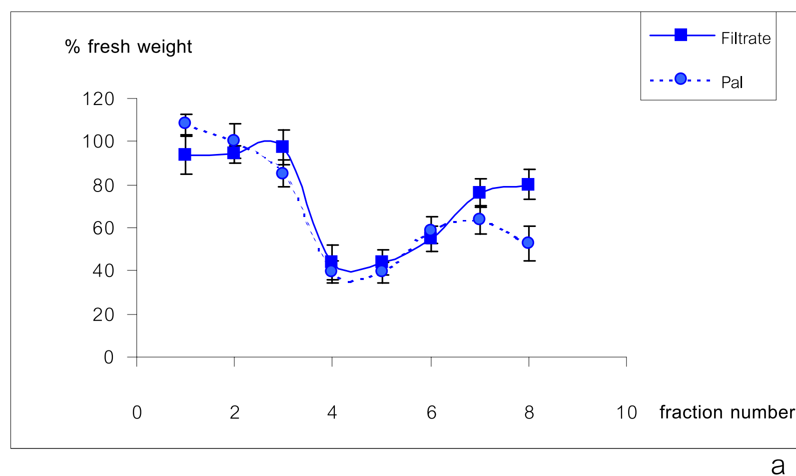
a b

Fig. 45 Distal necrosis caused by palmivorein (a); treated RRIM600, control RRIM600 and treated BPM-24 leaves, from left to right, and treated tobacco (b). Leave were dipped, through petioles, with palmivorein at 12.5 μg/g fresh weight and obserbed the necrosis at 72 hours after elicitation.

2.2.2 The toxicity tests of the culture filtrate and purified fractions of *Phytophthora palmivora* on rubber leaves

Culture filtrate of *P. palmivora* was checked for the toxicity on every step of purification using the dehydration of the susceptible rubber clone (RRIM600) as the indicator. At the first step of purification, the precipitate of filtrate was resuspended and loaded onto PD–10 column. Fractions no. 4-6 strongly caused dehydration of rubber leaves (Fig. 46a). The fractions no. 7-8 showed less effect than fractions number 4-6 but their proteins (300 μg/100 μl in no.8) were comparatively more than those in fractions no. 4-6 (42 μg/100 μl in no. 4). Proteins in the fractions no. 4-6 might be effective than those in the fractions no. 7-8 (Fig. 46b)

The fractions no. 4-6 were pooled and purified by using DEAE and Sephadex G-50 chromatography following lyophilyzation, passing PD-10 and finally resuspened in distilled water. The yield of Pal loss was about 50% after passing through PD–10 column, its toxicity was clearly exhibited in the fractions no 4-6 like the fraction of filtrate (Fig. 46a). This result showed that the fungus could produce proteins which were also toxic to the rubber leaves and remained their biological activities throughout every step of purification.



a

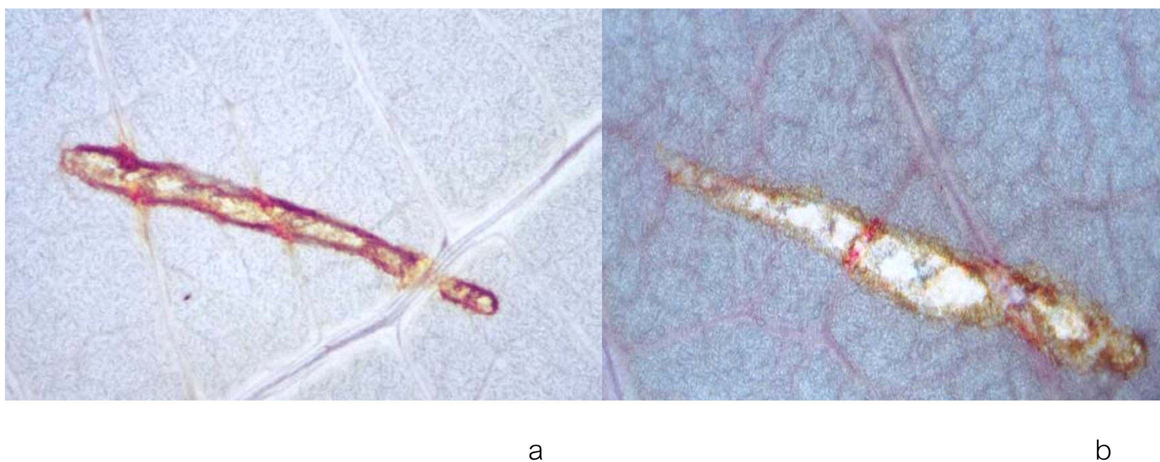
number of fraction	protein (μg/100 μl)	
	precipitate of culture filtrate	purified palmivorein
1	0	0
2	18	0.50
3	18	3.75
4	42	10.50
5	152	8
6	198	3.20
7	88	2.25
8	300	2.50
9	58	2

b

Fig. 46 Toxicity of the PD-10 fractions from the ammonium sulfate precipitate of *Phytophthora palmivora* culture filtrate and purified palmivorein after passing through Sephadex G-50 column. Results are expressed as percentages of the fresh weight of RRIM600 leaves after elicited with 20 μl of each fraction (a). The amount of protein from culture filtrate and purified palmivorein were shown in table (b). Values are mean of three replicates.

2.2.3 Lignification

The lignification was observed in the resistant and susceptible rubber clones after elicitation at 24 hours and the result was in the same direction as in the zoospore inoculation described in 1.2.3. The red color represented the stained lignin that started to deposit around the elicited site (Fig. 47). In the resistant clone, the lignin was significantly intense than that in the susceptible at every peroid. Moreover, the lignification at the lesion was clearly observed but less condensed form was detected at the site far from the lesion.



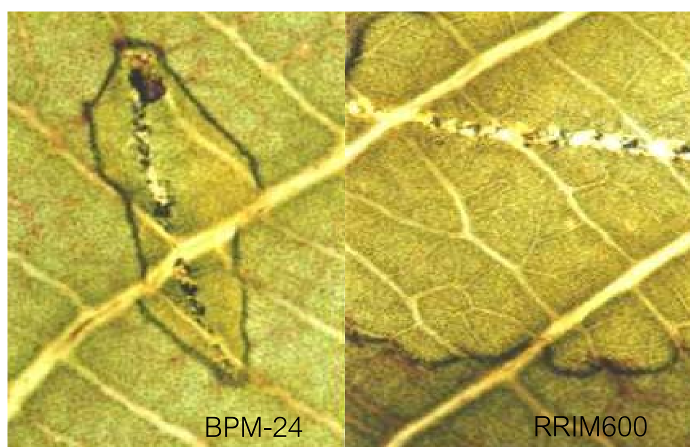
a b

Fig. 47 Lignification were obtained in BPM-24 (a) and RRIM600 (b) at 24 hours after elicited with palmivorein. Leaves were wounded with needle and placed with 10 μl of palmivorein (10 μg) then the leaves were cut into 1×1 cm. before immersing in 2% phloroglucinol in 95% ethanol. The lignin was shown as red color under light microscope (x40).

2.2.4 Effect of elicitin on necrosis of rubber leaves

Elicitin application promoted the necrotic lesions on the resistant, BPM-24, and the susceptible, RRIM600, rubber leaves. In the resistant rubber clone, the necrosis was significantly limited with intense border whereas the border of necrosis in the susceptible rubber clone was not clearly observed. Furthermore the necrosis lesion in the resistant clone ceased to spread whereas it was continuously enlarged in the susceptible clone after 48 hours of elicitin treatment (Fig. 48a). Observations by light microscope with the magnification of x40 in non-induced rubber leaves demonstrated that the cell around the scrape turned to black color which was the result of cell death. Most cases, the clear zones occurred around the scrapes and were encircled with dark-green border. The color of the clear zone around each wound in the resistant rubber clone was more intense than that in the susceptible rubber clone (Fig. 48b). The size of necrotic lesion around the scrape in BPM-24 was about 4 times smaller than in RRIM600.

a



b

c

Fig. 48 The necrosis effect of palmivorein on the resistant (BPM-24) and susceptible (RRIM600) rubber leaves. Leaves were scraped with fine needle and placed with 10 μl of palmivorein (10 μg). Distilled water was used as control. Control and elicited leaves were observed at 48 hours after elicitation.

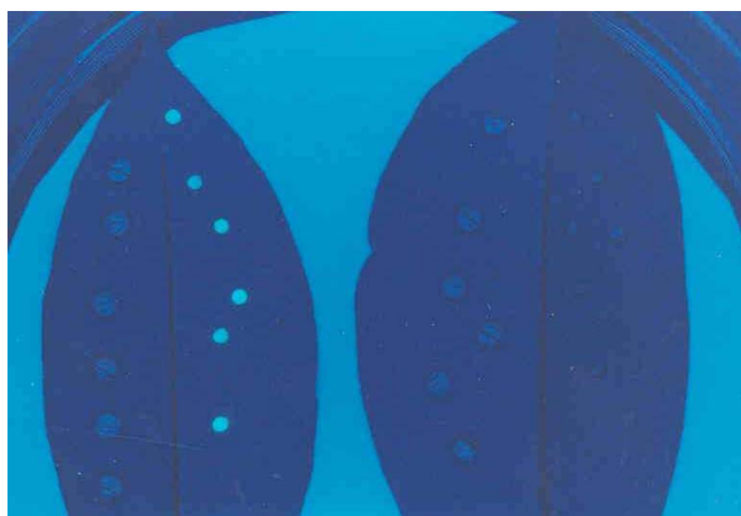
(a) Photograph. The elicitation leaves of BPM-24 and RRIM600.

(b) Micrograph : The elicitation leaves of BPM-24 and RRIM600. (x40)

(c) Micrograph : Controled leaves of BPM-24 and RRIM600. (x40)

2.3 Scopoletin synthesized by elicitin induction

Scopoletin, a hydroxycoumarin, was synthesized in the rubber clone after plant-fungal interaction. Scp was clearly exhibited in both the resistant and susceptible rubber clones, which were inoculated with zoospores of *P. palmivora*. The elicitin, the extracellular protein produced by *Phythopthora palmivora* was tested for the toxicity on their hosts. After the elicitin was applied on rubber leaves, the Scp appeared inside the leaves within 0.5 to 1 hour and in the droplet over the wound within 1 to 2 hours later. The amount of Scp in the treated resistant clone was 5-folds higher than that in the susceptible clone. It peaked at 16 hours after being treated and rapidly declined until the amount of Scp was equal to the control (Fig. 50). In fact, the Scp in RRIM600, the susceptible rubber clone, was slightly increased after treatment of elicitin compared with its control eventhough it was hardly detected in the photograph (Fig. 49). A little amount of Scp in control was observed and steady until 24 hours after treatment.



a. BPM-24　　　　　　b. RRIM600

Fig. 49 The scopoletin production in the inoculum droplet over the resistant (a) and susceptible (b) rubber leaves which were treat with water (leaf side of each leaf) with palmivorein (12.5 µg/30 µl/1 g leaf weight) (right side). Scopoletin was observed under ultraviolet light at 12 hours after elicitation.

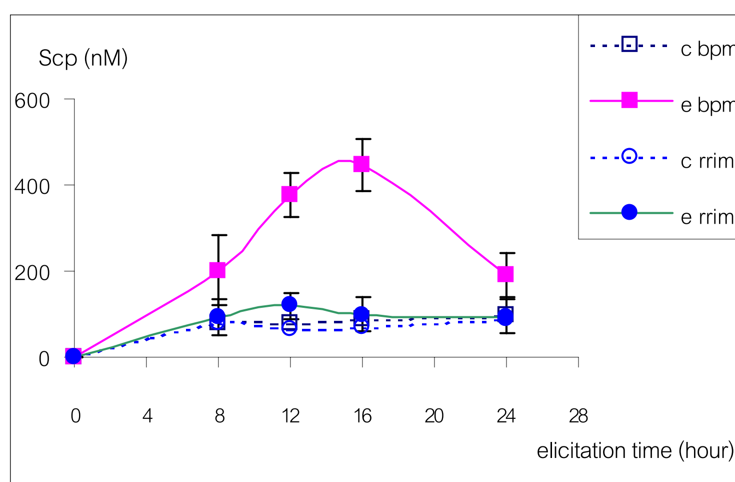


Fig. 50 The effect of palmivorein (12.5 µg/30 µl/1 g leaf weight) on scopoletin production of the resistant and susceptible rubber clone. The amount of scopoletin was measured at 8, 12, 16 and 24 hours after elicitation using spectrofluorophotometer at excitation and emission wavelength at 340 and 440 nm, respectively. c : control, e : elicitin.

2.4 Total protein quantification and enzyme activities of crude leaf extract

2.4.1 Total protein quantification

The crude extract of leaf treated with 1.5 µg / 5 µl of elicitin per 1 gram fresh weight was measured for the total protein with Bradford method. For uninfected rubber leaves (control), the protein contents slowly declined and reached a half of the initial amount at 72 hours after elicitation. The highest level of protein could be detected at 12 hours in BPM-24, the level of which was 3–folds higher than that in RRIM600 and subsequently declined to the same level as its control at 72 hours after elicitin treatment (Fig. 51).

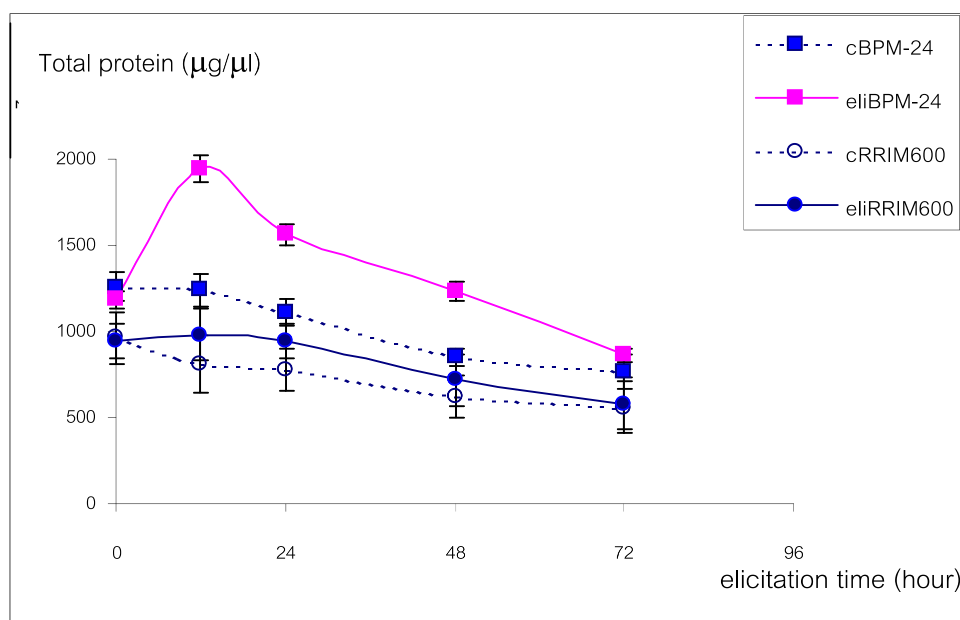


Fig. 51 Evolution of total protein contents of rubber leaves from the resistant (BPM-24) and the susceptible (RRIM600) clones treated with 1.5 µg / 5 µl of palmivorein at 0, 12, 24, 48 and 72 hours. Protein was measured by using Bradford method. c : control, e : elicitin (palmivorein).

2.4.2 Glucanase activity

Glucanase was induced in the resistant and susceptible rubber clones upon the elicitation, but the response of the resistant clones was significantly different from that of the susceptible clone. The activity of glucanase in BPM-24 (R) reached its maximum at 12 hours after the elicitation, the level of which was about 3-folds higher than that in RRIM600 (S). After 12 hours of elicitation, the activity of glucanase was started to decline in both of rubber clones. At 72 hours after elicitation, the activity of glucanase in the resistant clone was still higher than that in the susceptible clone (Fig. 52). The enzyme activities in the control of both rubber clones were slightly decreased with time.

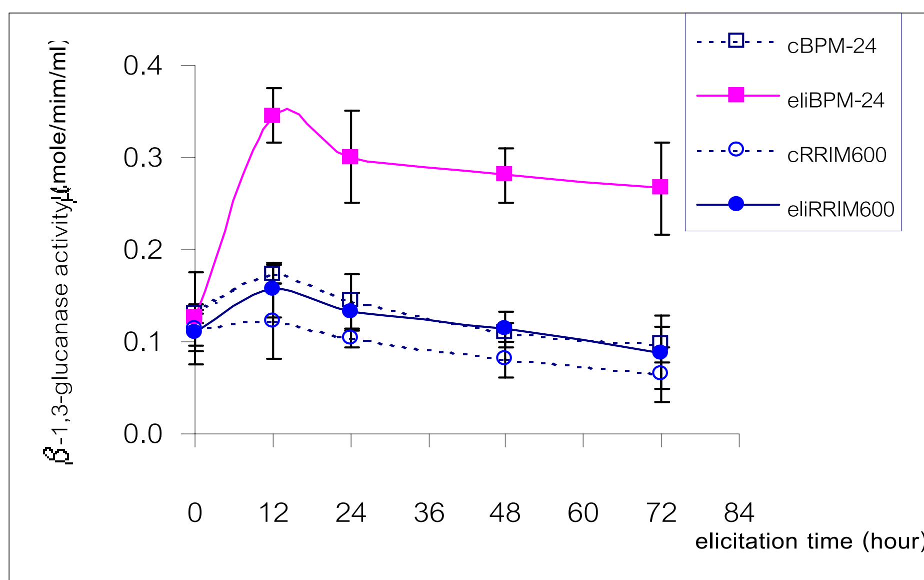


Fig. 52 Evolution of β–1,3-glucanase activity of rubber leaves from the resistant (BPM- 24) and the susceptible (RRIM600) clones treated with 1.5 μg/5 μl of palmivorein at 0, 12, 24, 48 and 72 hours. The activity was measured as the amount of glucose which is produced in 1 minute using laminarin as a substrate. c : control, e : elicitin (palmivorein).

2.4.3 Chitinase activity

Reducing sugars from chitin were assayed for the chitinase activity after eliciting both of the rubber leaves, BPM-24 (R) and RRIM600 (S). In the resistant clone, BPM-24, chitinase activity which is commonly expressed during plant-pathogen interaction, was risen 2 and 2.2-folds as compared to the susceptible clone, RRIM600, at 12 and 48 hours, respectively. The level of chitinase activity in the resistant clone were slightly decreased after it was reached to its peak whereas, in the susceptible clone, it was decreased closely to the same level as its control (Fig. 53).

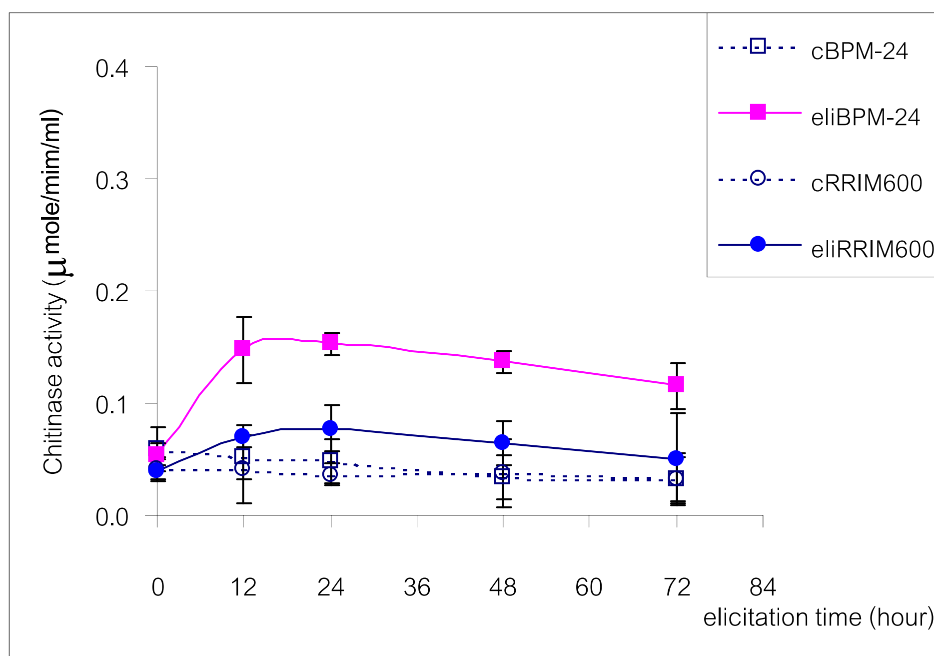


Fig. 53 Evolution of chitinase activity of rubber leaves from the resistant (BPM-24) and the susceptible (RRIM600) clones treated with 1.5 μg/5 μl of palmivorein at 0, 12, 24, 48 and 72 hours. The activity was measured as the amount of N-acetyl-D-glucosamine which is produced in 1 minute using colloidal chitin as a substrate.

c : control, e : elicitin (palmivorein).

2.4.4 Native SDS-PAGE of chitinase activity staining

The chitinase activity was determined by Native SDS-PAGE as described in 1.8.3. Chitinase levels increased from 6 hours after elicitation, reached a peak at 12 hours and persisted until 48 hours which was the end of the experiment in the resistant clone. The two isoforms of chitinase, y and z bands, in the elicited resistant clone clearly appeared and increased in intensity. The y band was slightly increased in the elicited susceptible leaves at 24 and 48 hours after elicitation (Fig. 54b lane 7 and 8). A slight decrease in the x band, one isoform of chitinase, was observed in both resistant and susceptible clones after the leaves were detached. Therefore, the x band is not the pathogenesis related-isozyme of chitinase (Fig. 54a lane 7 and 8; Fig. 54b lane 4 and 8).

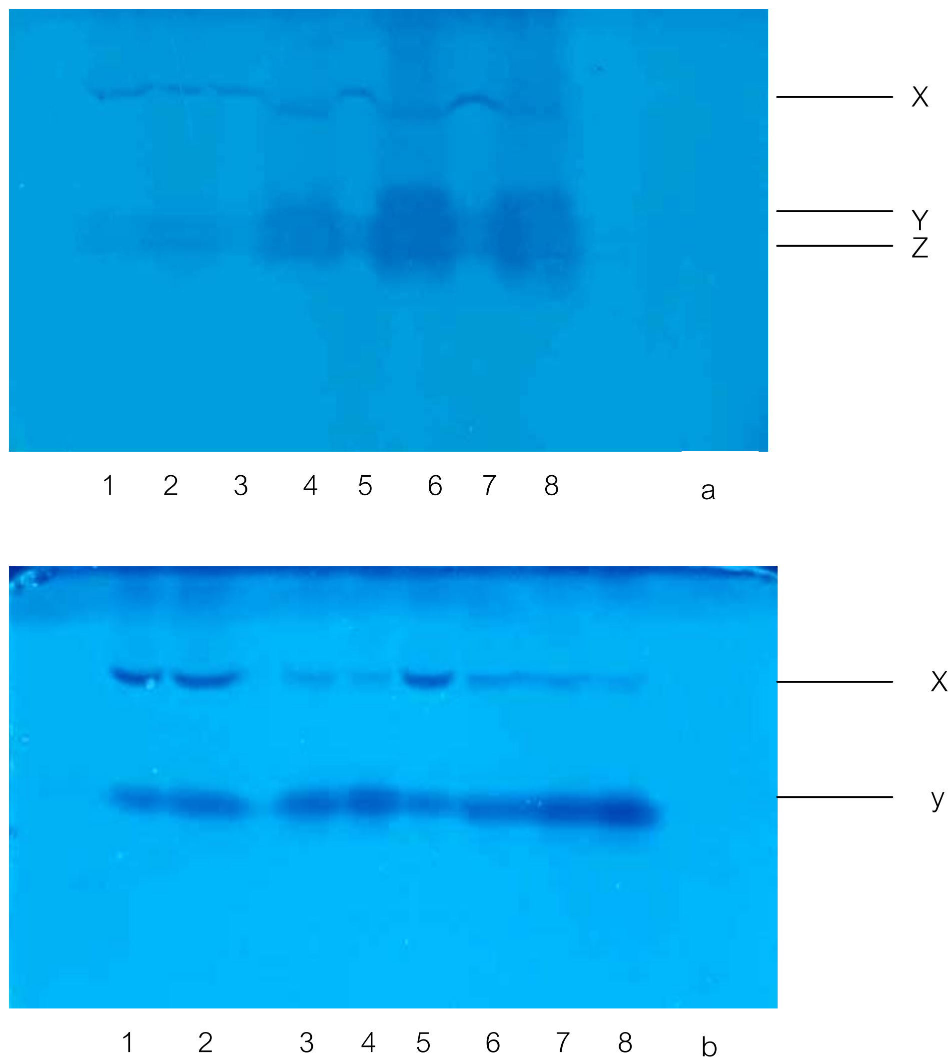


Fig. 54 Chitinase activity staining of crude leaf extracts from the resistant (a) and susceptible (b) rubber clones. The leaves were treated with 1.5 μg/5 μl of palmivorein and water was used as control.

(a) Lane 1, 3, 5 and 7 : control leaves at 6, 12, 24 and 48 hours.

Lane 2, 4, 6 and 8 : elicited leaves at 6, 12, 24 and 48 hours.

(b) Lane 1 to 4 : control leaves at 6, 12, 24 and 48 hours.

Lane 5 to 8 : elicited leaves at 6, 12, 24 and 48 hours.

2.5 Transcription of chitinase gene after elicitin treatment

2.5.1 Result of Northern blot hybridization

Plasmid DNAs (Hglu and QHb-16) were extracted from *E. coli* and digested with the restriction enzymes, *Hin*dIII and *Bam*HI. The sizes of β-1,3-glucanase and chitinase cDNAs were 976 and 160 bp compare with standard DNA (Lamda / *Hin*dIII) in 0.8 % agarose gel (Fig. 19). The cDNAs which were recovered from agarose gel were labeled with biotin and used for hybridization with the prepared RNA on membrane. After hybridization, the membrane was brought to detect by using North2South Chemiluminescent Nucleic Acid Detection Kit (Pierce). Finally the exposured film was developed and resulted the black spot only the position which was corresponded to the labelled probe. This result revealed that the cDNAs were properly labelled with biotin and detected by North2South Chemiluminescent Detection Kit. But all of the positions which were corresponding to RNAs from treated and control leaves were clear which mean the failure of hybridization between RNAs and probes. Actually, all of RNAs which prepared from treated and control leaves were not degraded according to the preparing process as they were shown as discrete band of rRNAs, 18S and 28S (Fig. 55). Whether the conditions of hybridization were changed such as temperature and hybridization time, the results were shown the same characteristic as described above.

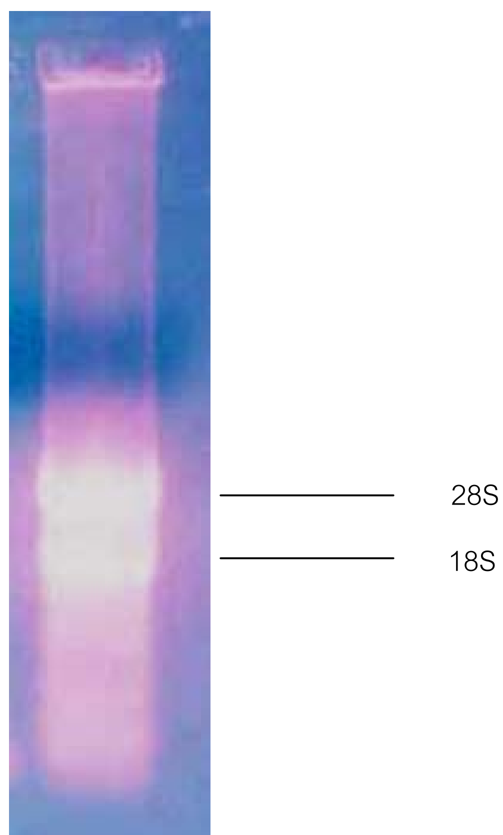


Fig. 55 Agarose gel electrophoresis of total RNA isolated from BPM-24 leaves which were elicited with 5 μg/10 μl of palmivorein at 48 hours. The gel was stained with ethidium bromide and observed under Ultraviolet light. 28S and 18S are ribosomal RNA of rubber leaves.

2.5.2 RT-PCR

In order to study an expression of *Hevea* chitinase gene, the chitinase transcript accumulation in leaves of BPM-24 clone were examined using designed primers and the product of 525 bp cDNAs were obtained from the RT-PCR. Rubber leaves were harvested at 4 hours after elicitation with Pal; RNA were extracted and the experiment was performed as described in 2.6.2.1. As shown in Fig. 56, chitinase gene significantly expressed in treated leaves which confirmed by the thick band of 525 obtained in lane 3. The low level of chitinase gene expression was observed in control leaves (lane 2). The negative control of this experiment was shown in lane 4. Since the same RNA was used in this analysis. It was confirmed that the RNA used in Northern analysis was intact. The failure was not due to the degradation of RNA or/and failure of detection by North2South Chemiluminescent Detection. In addition, the chitinase of *H. brasiliensis* responds to different stimuli by producing differrent isoforms, each of is encoded by the corresponding mRNAs so the mRNA of chitinase expressed after an induction might be encoded from mRNAs which were not corresponding to QHb-16 in Northern hybridization.

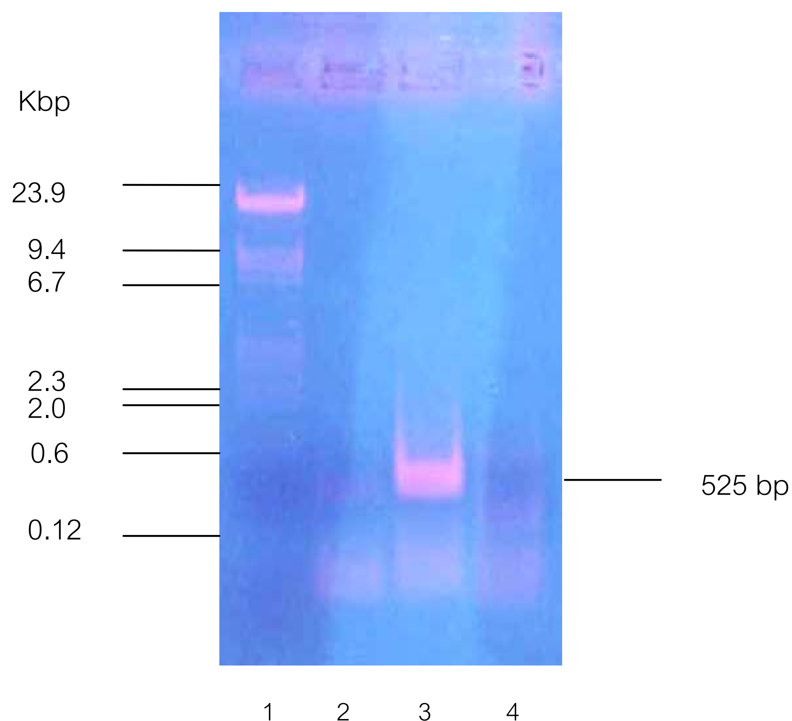


Fig. 56 RT – PCR analysis of chitinase gene expression at 4 hours after elicitation BPM-24 leaves with palmivorein 1 μg/5 μl/1 g leaf weight and distilled water was used as control. Amplification of 525 bp cDNA was clearly observed in lane 3.

lane 1 marker (Lamda + *Hin*dIII)

lane 2 control leaves

lane 3 treated leaves

lane 4 forward and reverse primer (as negative control)