Chapter 4
DISCUSSION

1 *Hevea* defense responses against zoospores of *P. palmivora.*

P. palmivora is a ubiquitous pathogen of more than 100 plant species including *H. brasiliensis*. The cell wall of *Phytophthora* is composed of cellulose and β–glucan but not chitin (Bartnicki-Garcia, 1969). *Phytophthora* species do not synthesize sterols but require an exogenous source of β–hydroxy sterols for sporulation (Elliot, 1983). *P. palmivora* isolated from *H. brasiliensis* was identified in the workshop exhibited at Prince of Songkla University and reconfirmed by Professor André Drenth from the university of Queensland, Australia. The sporangium of *P. palmivora* is ovoid in shape and prominently papillate. *P. palmivora* grows rapidly and produces a lot of sporangium on V8 agar, upto $5x10^8$ zoospores/ml. Biflagellate zoospores of *P. palmivora* move fast therefore the zoospore suspension should be diluted to obtain the accurate number.

The plant-pathogen interactions lead to an incompatible (plant defense) and a compatible (disease) reactions. An incompatible reaction, for examples, ion effluxes, phytoalexin and PR-protein productions, lignification, hypersensitive cell death and SAR, occurs in the non-host or resistant host, whereas a compatible reaction, similar events as found in an incompatible reaction but poorer in rate and quantity which would not restrict fungal growth, happens in the susceptible host (Ebel, *et al*., 1995; Asada and Matsumoto, 1972; Garcia, *et al*., 1995; Stakman, *et al*., 1915; Kuć, 1995; Guest, 1997). Zoospores of *P. palmivora* were used to classify the degree of resistance of *H. brasiliensis* by studying necrosis,

dehydration, lignification and the production of phytoalexin (Scp) and PR-proteins (such as β-1,3-glucanase, chitinase).

1.1 Necrotic lesion and degree of resistance

The necrosis of plant caused by host and pathogen or elicitor interaction can be classified into hypersensitive cell death in the incompatible reaction and disease lesion in the compatible reaction. The rapid plant cell death at the infection site [hypersensitive (HR)] is the most common expression of incompatibility interaction (Heath, 1997). It has been suggested to be a form of programmed cell death (PCD) evolved specifically as an animal defense against microbial attack (De Wit PJGM, 1997). Using low zoospore concentration, the necrosis occurred in BPM-24 was characterized as hypersensitive cell death whereas disease was generated in RRIM600. The hypersensitive cell death was one of the important mechanisms of the defense responses that imprisoned the fungus in BPM-24 whereas RRIM600 failed to create this reaction. This phenomenon caused the fungal death in the resistant clone whereas the fungus was able to grow in the susceptible clone and developed the disease. Not only the macroscopic necrosis was different between two rubber leaves but the accumulation of brown substances in neighbouring cell around infection site was also significantly detected in the resistance. At the same time, the discolored of chlorophyll was clearly observed in the susceptible clone but not in the resistant one (data not shown). The hypersensitive cell death in the resistant cultivar was effective to restrict spreading of pathogen by cytoplasmic changes such as numerous electron-transparent vesicles, cell wall apposition and tubule-like structures and hypha was not clearly observed (Hu, *et al*., 2003). Our results were comparable to the

interaction of soybean / *P. megasperma* which exhibited less necrosis in the resistant cultivars than in the susceptible cultivars. (Ward, *et al*., 1989). The BPM-24 is classified as the most resistant, PB-235 and RRIT251 as the partially resistant and RRIM600 as the susceptible clones according to the degree of necrosis. This result also corresponds to the degree of resistance of *H. brasiliensis* classified by the Rubber Research Institute of Thailand (Annual report, 1999). Inoculation of *Corynespora cassiicola* onto rubber leaves, stage B_2-C, caused the hypersensitive cell death in GT1, the resistant clone, and disease in RRIC100, the susceptible clone (Breton, 1997). After classifying the degree of resistance, the most resistant and susceptible clones which are BPM-24 and RRIM600, respectively, were inoculated with three zoospores concentrations. With medium and high zoospore concentration, the necrotic lesions in the resistant clone, although were enlarged, developed at a slower rate than those detected on the susceptible leaflets. The concentration of zoospores directly effects the size of necrosis. Actually, even in the resistance clone, the development of necrosis had a potential to change from hypersensitive cell death to disease lesion when the high concentration of zoospores were applied. These data demonstrated that the degree of resistance was decreased. Fortunately, the concentration of zoospore in the nature was not as high as in our experiment so that the BPM-24 is the most resistant to *P. palmivora*. Therefore, a certain zoospore concentration is required to classify degree of clonal resistance. In addition, the age of leaf used for the experiment is also important to this study since the younger leaf with less cutin is more susceptible than the older one of the same clone (data not shown). Breton (1997) reported that the

different stage of rubber leaves produced the different results for the same type of experiment. Hence, both of rubber clones, BPM-24 and RRIM600 displayed different necroses at every zoospore concentration and were classified as the incompatible and compatible reactions, respectively. The various zoospore concentration ($5x10^6$ and $5x10^7$ zoospores/ml) and leaves of B_2-C stage (as described in Materials and Methods) were used to demonstrate the correlation between the degree of resistance and other defense responses.

1.2 Lignification

Lignin reinforces the cell wall to restrict the penetration of pathogen including pathogen enzymes and toxins. Lignification of *H. brasiliensis* infected with *Microcyclus ulei* is related to the degree of resistance (Garcia, *et al*., 1995a). In this study, lignification in the resistant rubber clone, BPM-24, was higher than that in the susceptible one, especially at the young petiole, at 4 hours after inoculation. The rapid deposition of lignin restricted the invasion of mycelium to neighbouring cells, which could be demonstrated in the resistant clone by the smaller size of necrosis and no further spreading. On the other hand, the slower deposition of lignin in the susceptible clone after inoculated with zoospores of *P. palmivora* caused mycelium growth and spreading of necrosis. The small amount of lignin was observed in the control leaves of both clones. This phenomenal occurred in the other plant-fungal interaction. Asada and Mutsumoto (1972) found that the lignification occurs very rapidly in the resistant radish root-downy mildew interaction. The lignin which was deposited in the healthy plant had similar structure as the disease lignin but differed in the rate of production and accumulation between cultivars and degree of resistance which compared in

the same cultivar. Therefore, lignification served as a physical barrier for further growth of fungus.

1.3 Scopoletin (Scp)

1.3.1 Scopoletin analysis

Plants resist to infection by pathogens in various ways. One of these protective mechanisms is the production of antimicrobial compounds, namely phytoalexins, that kill the pathogen or restrict its intracellular development (Darvill and Albersheim 1984). *H. brasiliensis* was demonstrated to synthesize phytoalexin after fungal infection and was characterized as hydroxycoumarin namely scopoletin, Scp (Tan and Low, 1975; Giesemann, *et al*., 1986). The accumulation of Scp has been reported in herbaceous plants following fungal (Tal and Roberson, 1986a, Zeringue, 1984), bacterial (Sequeira, 1969) and viral (Clarke and Baines, 1976) infections. This hydroxycoumarins has been found as well in a tree, *Platanus acerifolia*, after infection with the canker stain disease (El Modafar, *et al*., 1993). In higher plants, Scp is induced in the interactions between *H. brasiliensis* / *M. ulei* and *H. brasiliensis* / *C. gloeosporioides,* (Garcia, *et al*., 1995 b; Breton, *et al*., 1994). In this study, Scp analyzed by TLC was also found in the inoculum droplets over rubber leaves infected by zoospores of *P. palmivora*. This compound was confirmed to be Scp by using HPLC method (Churngchow and Rattarasarn, 2001).

1.3.2 Scopoletin production in rubber clones

There are evidences that phytoalexins accumulate faster and to higher concentrations in resistant cultivars. These events occur more slowly and more diffuse in susceptible plants (Mayama, *et al*., 1981; Doke 1982;

Hahn, *et al*., 1985, David and Brown 1997). The speed and extent of Scp accumulation were correlated to the degree of resistance of *Hevea* to *M. ulei* and *C. gloeosporioides* (Garcia *et al*., 1995 b; Breton *et al*., 1994). In contrast, the Scp accumulation in *Hevea* resistant leaves was expressed at a lower level after infection with *C. cassiicola*. The authors suggested that the inverse relation may be due to. THe higher degradation of Scp in the resistant clone (Breton, *et al*., 1997). After inoculation of *H. brasiliensis* with *P. palmivora*, the Scp levels were positively linked to the degree of rubber resistance of *Hevea* to *P. palmivora* and the Scp concentration in droplets (micromolar range) was comparable to other rubber patho-systems (*M. ulei* and *C. gloeosporioides*). The time course of Scp biosynthesis (Fig. 27) suggested that the fungitoxic effect of Scp was not related only to its concentration around the penetration site, but also to the rapidity of its biosynthesis after infection. In my opinion, for *H. brasiliensis* / *C. cassiicola* (Breton, *et al*., 1997), the low amount of Scp observed in the inoculum over the resistant clone might be due to the insufficient concentration of conidia, as shown by small necroses. Eventhough, the Scp production in the resistant cultivar was higher than that in the susceptible cultivar but there are less exits in the resistant cultivar due to less penetration sites. Therefore, lower Scp was obtained in the inoculum droplets. In our hand, even with low zoospore concentration, the amount of Scp in the inoculum droplet which placed over the scrap was higher than that in the resistant clone (data not shown). As mentioned above, proper concentration of zoospores (low and high concentration) should be used for the experiments and some other techniques

such as using confocal microscope, for investigating amount of Scp in mesophyll cell might be considered for further study.

1.3.3 Effect of zoospore concentration on the production of scopoletin

Scp could be detected in abundant amount immediately after inoculation of rubber leaflets with medium and high zoospore concentration. With low zoospore concentrations, smaller numbers of cells were infected, thus longer time was required for Scp to reach its peak. Although, we could not find any references to support these phenomena but these results are a good explanation for the less excretion in the *H. brasiliensis* / *C. cassiicola* interaction that we mentioned above. These results revealed that the higher zoospore concentration was used, the more penetration site was occurred and the higher Scp in the excretion was observed especially in the same leaf as shown in Fig. 28. Thereafter, Scp levels declined suggesting that the infection was under control. In contrast, the rapid decrease of Scp levels observed in infection with high zoospore concentration indicated the destruction of infected tissues as shown by disease lesion as previously discussed and Scp was therefore no longer produced.

1.3.4 Fungitoxicity of scopoletin for growth inhibition

Phytophthora is a member of Oomycetes, of which major structure of its cell wall is cellulose-glucan instead of chitin-glucan. Coumarin itself which was reported to inhibit cellulose biosynthesis in higher plants, is significantly more toxic to three oomycetous species than those members of the higher fungi tested (Dietrich and Valio, 1973). In this investigation, Scp (hydroxycoumarin) was more toxic to *Phytophthora* spp. (*P. palmivora* and *P. botryosa*) than the other two fungi, *C. cassiicola* and *C. gloeosporioides* (Fig. 26 ; a and b). The

other two rubber pathogens, *C. cassiicola* and *C. gloeosporioides,* were less sensitive to Scp in the bioassays. We detected that the *C. cassiicola* and *C. gloeosporeoides* were able to degrade Scp which were shown as clear zones while the two *Phytophthora* spp. were not. This observation corresponded to the result obtained in the liquid culture studied by Breton, *et al*. (1997) with *C. cassiicola*. Eventhough the I_{50} on *C. cassiicola* was much higher than previously reported by Breton, *et al*. (1997), this may be due to the vilurence difference of each isolate. Differences in germination rates according to the origin of isolates have been observed in *C. cassiicola* (Garcia, *et al.,* 1995 b). The concentration of Scp in the inoculum droplets (7.5 μM) was much lower than that required to inhibit growth in vitro (I_{50} was about 1.0 mM). However, this amount of Scp was able to restrict fungal growth in this experiment (Fig. 21). It implied that the concentration of Scp at the sites of fungal penetration should be 10-20 times higher than that in the inoculum droplets (the concentration in droplets depended on the volume applied, 20 μl, which was obviously higher than that of the intercellular fluid). The same observations with other rubber pathogen such as *M. Ulei* and *C. cassiicola* were also reported by Garcia, *et al*., (1995 b) and Breton, *et al*. (1997). Ahl Goy, *et al*. (1993) studied the accumulation of Scp in tobacco against its pathogens such as *Cercospora nicotianae*, *Phytophthora parasitica* var. nicotianae and tobacco mosaic virus and suggested that the local concentration, e.g. near the leaf surface which is the first contact point for the pathogens, could be 10-50 times higher than that in the whole leaf. It was found that concentrations about 0.5 mM and 1 mM effectively inhibited growth of this fungus but hardly inhibited at the concentration about 0.25 mM. These data

indicated that 2 times increase in the rate of initial Scp synthesis would make a difference which supported the results shown in Fig. 27. It also implied that the inhibition level of Scp in the tissue should relatively be the same as the I_{50} value. Since Scp produced by rubber leaves after infection was able to restrict growth of this pathogen, therefore the induction of Scp is one of the main defense mechanisms in rubber against *Phytophthora* spp.

1.3.5 Fungitoxicity of scopoletin for zoospore germination

Scp directly effects rate of zoospore germination at the concentration of 100 μM. Ahl Goy, *et al.* (1993) suggested that the local concentration of Scp could be 10-50 times higher than that of the whole leaf as mentioned earlier. The Scp concentration in the 20 μl inoculum on BPM-24 leaf was about 8 μM, therefore intracellular Scp concentration was expected to be at least 400 μM which was 50 times higher than that in the inoculum, the concentration of which is high enough to inhibit zoospore germination and hyphal growth. The higher the concentration of Scp, the lower the rate of zoospore germination and hyphal growth. Other phytoalexins could inhibit the germination and growth of pathogen such as phytoalexin in sweet potato could inhibit *Rhizopus stolonifer* germination and growth (Stange Jr, *et al.,* 2001). Rishitin, lubimin and phytuberol, the phytoalexins of tobacco leaf, also inhibited spore germination and fungal growth in the medium (Xie and Kúc, 1997). These evidences support that the rate of Scp production indicates degree of resistance in the rubber clone.

1.4 Total protein and PR-proteins after inoculation with zoospores

Plants synthesize organic and inorganic substances to inhibit the invasion and destruction from the pathogens; the pathways of which require several enzymes. Therefore, it is reasonable to expect the increase in total protein content in those invaded plants. The protein content in the resistant rubber clone inoculated with *C. cassiicola* was demonstrated to be higher than that in the susceptible clone (Breton, 1997). This is consistent with our findings where the inoculation of rubber clone with zoospores of *P. palmivora* resulted in a greater increase in total protein content in BPM-24 (R) compared with RRIM600 (S). Furthermore, the protein content tended to decline more slowly in the resistant clone. These findings indicate that the resistant clone has greater capacity for protein synthesis than the susceptible one. However, protein which was lost in the first 24 hours might be a reserve protein using as a precursor in emergency situation such as synthesis of phytoalexins or other substances corresponding to defense mechanism.

One of several plant defense mechanisms is the synthesis of PR-proteins, for example, β-1,3-glucanase and chitinase. These proteins have hydrolytic activities and act as enzymes to digest cell walls of the pathogens which consists of glucan and chitin.

The growth of *Trichoderma reescei, Alternaria alternaria, Phycomyces blakesleesasus* and *Neurospora crassa* in Barley were inhibited by chitinase (Robert and Selitrennikoff, 1986). Infection by *Pseudomonas tabacci* and *P. parasitica* in tobacco increased both β-1,3-glucanase and chitinase levels (Meins, 1989). There are also evidences that, after invasion by pathogens, the levels of both enzymes increased in greater amount in resistant cultivars than

those in susceptible cultivars. Likewise, β-1,3-glucanase activity in leaves of a resistant cultivar of melon increased more rapidly than that in susceptible cultivar after infected with *Sphaerotheca fusca* (Rivera, 2002).

β-1,3-glucanase and chitinase were induced in *Nicotiana tabacum* which exhibited hypersensitive reaction when infected by TMV (Vögeli-Lange, *et al.*, 1988). The rubber-*Phytophthora* interaction also resulted in greater increase of β-1,3-glucanase and chitinase in BPM-24 (R) than in RRIM600 (S). Not only the increase of glucanase and chitinase in the resistant clone is greater than that in the susceptible clone, but their levels in the resistant clone also decline more slowly. This makes the resistant clone more capable in pathogen destruction. The declination of enzyme activities in the susceptible clone may be the result of cell destruction. Similarly, both enzyme and total protein content after inoculation of zoospores increase in greater amount and are more stable in the resistant than in the susceptible. Furthermore, the study of enzyme activities by SDS-PAGE demonstrated the significant increase of 2 chitinase isozymes (y, z) in BPM-24 (R) and only small increase in one chitinase isozyme (y) in RRIM600 (S). Therefore, the x band is not a pathogenesis related-isoform. Only the y and z bands are related to the defense response. More isoform of chitinase observed in the resistant may cause a wider range of pathogen destruction than the susceptible and also found in the other plant-pathogen interaction. Ji and Kúc (1997) reported enhancement of chitinase activity in leaves of host (cucumber) and non-host (pumpkin and squash) inoculated with conidia suspension of *Colletotrichum lagenarium* by infiltration whereas the activity was only observed in host leaves, but not in non-host leaves, inoculated with conidia suspension by

placing over leaf surface. The isoform pattern of chitinase in squash leaves was similar to that of cucumber but different from that of pumpkin. This report suggests that chitinase is not a primary initial defense compound but involves in post-infection defense. The first phenomenon for plant defense could be physical barrier. Infiltration of conidia reduced the effective of physical barrier of non-host which was shown by appearance of anthracnose symptoms. Furthermore, fungal infection, insect infestation and mechanical wounding in sorghum plants resulted in elevation of chitinase and β-1,3-glucanase. The number of isozymes induced, or their relative concentrations or the duration of the response depended on the differences of treatments. The induction pattern of chitinases and β-1,3-glucanases of resistant and susceptible differed in the number of isozymes induced and their relative concentrations (Krishnaveni, *et al.,* 1999).

2 *Hevea* defense responses against elicitin of *Phytophthora palmivora*

2.1 Elicitin

All *Phytophthora* species except for *P. parasitica* var. *nicotianae*, the virulent strain isolated from tobacco, produce and secrete protein called elicitin (Huet and Pernollet, 1989; Kamoun, *et al*., 1994). Elicitin is the extracellular protein with molecular weight of 10 kDa. It consists of 98 amino acid residues which are conserved in amino acid sequences. (Nespoulous, *et al*., 1992; Huet and Pernollet, 1993). Elicitin is named according to species by which it is produced such as parasiticein, Para, from *P. parasitica*; capsicein, Cap, from *P. capsici*; cryptogein, Cry, from *P. cryptogea*, etc. Elicitin is considered to be avirulent agent as it induces hypersensitive responses in the resistant cultivars, for example cryptogein and capsicein induce hypersensitive reaction in *Nicotiana*

(Rusterucci, *et al*., 1996). Cotyledons of *Brassica napus* cultivars expressed a hypersensitive reaction after either inoculation with an avirulent isolate of *Leptosphaeria maculans (L. maculans*), the fungus responsible for blackleg of crucifers, or infiltration with an elicitin, cryptogein whereas HR was not observed in plants infected with a virulent isolate of *L. maculans* (Roussel, *et al*., 1999). Elicitin is classified into two classes, acidic and basic, according to its pI. *P. palmivora* isolated from rubber also produces elicitin, palmivorein (Pal) which is the extracellular protein in the media culture (Churngchow and Rattarasarn 2000). Only acidic form of Pal is produced by *P. palmivora* and its pI is 4.0$\pm$0.2 (Churngchow and Rattarasarn 2000). Pal, was purified from PDB (potato dextrose broth) and by using Bradford method (Bradford, 1976), its concentration was estimated at only 0.41 mg/L of filtrate. However, since the protein is not sensitive to Bradford reagent because it possesses a very low content of basic amino acid, bicinchoninic acid (BCA) method was used instead and demonstrated that the concentration of Pal was 4.12 mg/L of filtrate, much higher than that obtained by the Bradford method. The silver staining was also used instead of the Coomasie blue staining because of the low content of basic amino acid. Since this protein is a small peptide which moves immediately behind glycine and the band of proteins cannot clearly be illucidated, therefore the Tricine-SDS-PAGE was used instead of glycine-SDS-PAGE, (Janson and Rydén, 1989). The silver staining of Tricine-SDS-PAGE revealed the purified form of Pal by using DEAE and Sephadex G-50 column chromatography, respectively and MW of Pal was found to be ca. 10,000. Pal was the major extracellular protein of *P. palmivora* since only one band on each lane was observed for the first 2

weeks. Some of tiny bands seen in the following week might be the protein from cell death or cell lysis. Pal exhibits the similarity of amino acid compositions as in other α-elicitins such as Cacto, Dreα, Inf and MgMα (Huet, *et al.,* 1994). It lacks Trp, His and Arg, conserves consensus region of N-terminal end and has Val at position 13 of the N-terminal end. Eventhough, 26 residues of the N-terminal region of Pal are identical to those of Para (Perronnet, *et al*., 1995), the difference of amino acid compositions confirms that they are not the same protein. The degree of responsiveness such as necrosis, phytoalexin and PR-proteins productions for the plant-elicitin interaction depends on both cultivars and biological activity of elicitins (Kamoun, 1993; Ricci, *et al.,* 1989; Blein, *et al*., 2002).

2.2 Tests for necrotic activities in tobacco and rubber leaves

Toxin from *C. cassiicola* caused leaf wilting and vein blackening (disease symptoms) in the susceptible clones. By two bioassays (wilting and necrosis), the toxin can be used to classify the degree of resistance of rubber clones (Breton, *et al*., 1997). Elicitin, the avirulent agent from *Phytophthora*, causes necrosis in non-host, tobacco, the reaction of which is different between the resistant and the susceptible cultivars (Kamoun, *et al*., 1998; Keizer, *et al*., 1998). Pal causes hypersensitive reaction in the resistant and disease lesion in the susceptible rubber leaves. The appearance of distal necrosis or dehydration of tobacco leaves indicates the existence of Pal biological activity. The dehydration of tobacco which is non-host and BPM-24 which is the resistant clone show an incompatible reaction whereas the more severe dehydration of RRIM600 indicates compatible reaction. RRIM600 is sensitive to the low concentration of Pal in the development of dehydration. Elicitin has been proposed to act as avirulence

factor in the *N. tabacum-P. parasitica* interaction (Kamoun *et al*., 1994). Our finds, Pal was able to induce both incompatible and compatible reactions in the resistant and susceptible clones, respectively. These results indicate that Pal might be a toxin for rubber leaves but depend on the resistance of plant and the amount of applied elicitin. The low amount of elicitin was able to induce plant defense reponse or SAR similar to low dose of toxin from human pathogens, *Clostridium botulinum*, was used to stimulate human immunity. For immunization, we have to, however, find out the optimal amount of elicitin corresponding to each clonal resistance.

Distal necrosis induced by Pal in BPM-24 and RRIM600 was similar to the reaction occurred after inoculation with zoospores, i.e. incompatible reaction in the resistant clone, BPM-24, and the compatible reaction in the susceptible clone, RRIM600.

2.3 Toxicity tests for the fractions of culture filtrate

Biological activity of elicitin depends on its structure, the change in elicitin structure can affect biological activity. When culture filtrate was purified for elicitin by using DEAE and Sephadex G-50 columns and tested for its activity by determining the development of dehydration in the susceptible rubber clone, it was found that the culture filtrate had elicitin activity confirmed by Tricine-SDS-PAGE. Moreover, the other 40 kDa protein was also found in the culture filtrate which was able to induce dehydration in RRIM600 (This protein was not elicitin by testing with Tricine-SDS-PAGE). An extracellular 42 kDa glycoprotein from *P. megasperma* was found to elicit phytoalexin, H^+/Ca^{2+} influxes, K^+/Cl^- effluxes and oxidative burst in culture parsley cells (Parker, *et al*., 1991; Nürnberger, *et al*.,

1994). A 42 kDa glycoprotein from *P. sojae* also stimulated the defense responses in parsley cells (Fellbrich, *et al*., 2000).

2.4 Lignification

Lignification of *H. brasiliensis* can be induced by inoculation with *M. ulei* zoospores and latex tapping. The extent of lignification depends on the degree of resistance to fungus (Garcia *et al.,* 1995; Thomas, *et al.,* 1995). The rate as well as the amount of lignification around the scrap in resistant rubber clone, BPM-24 induced by Pal was significantly greater those that in the susceptible clone, RRIM600. These results consistent with those of inoculation with *P. palmivora* zoospores.

2.5 Necrosis

The study of necrosis under light microscope demonstrated smaller size of necrosis occurred in the resistant clone, BPM-24 than in the susceptible clone. The color of cells surrounding scraped sites was lighter in the susceptible clone due to disorganiz chlorophyll and cell desiccation . These results were the same as the study of action of radish-elicitin interaction which showed small dark spot on the underside of the leaves in the resistant cultivar, but wilting was not shown for eliciting with 3 μg elicitin • g^{-1} FW. Lower amount, 1 μg elicitin • g^{-1} FW was able to cause cell death and desiccation in the susceptible cultivar. At very lower levels of elicitin (0.3 μg elicitin • g^{-1} FW), there was no difference between elicitin-treated leaves and controls in the resistant cultivar whereas type II response (such as cell desiccation) was still observed in the susceptible cultivar (Keizer, *et al.,* 1998). Furthermore, the border of necrotic lesion was limited and was not extended in the resistant clone whereas in the susceptible clone, it was

widespread and had no distinct border. The findings of infiltration by elicitin is similar to that occurred by the inoculation with zoospores.

2.6 Scopoletin synthesis

Phytoalexin synthesis is one of the responses of elicitation. Elicitor from *P. parasitica* and *P. sojae* increases the production of phytoalexin in parsley protoplast (Fellbrich, *et al.,* 2000). Cry induces phytoalexin production in a cellular suspension of tobacco and tobacco leaves. (Milat, *et al.,* 1991). Pal induced Scp synthesis at a faster rate in BPM-24 (R) than in RRIM600 (S) and in higher amount. Scp synthesis induced by elicitin in the resistant rubber clone was similar to that induced by zoospores but the rate of Scp synthesis was faster in the former with its peak was obtained at 16 and 30 hours, respectively. This result was effected by the defense response was induced by zoospore penetration in macroscopic level whereas elicitin induced the defense response in molecular level which was directly interacted with receptor (Blein, 2002). Furthermore, the zoospore need time for their growing and development for awhile before producing elicitin.

2.7 Total protein

The amount of total protein elicited by Pal was greater in BPM-24 (R) than in RRIM600 (S). Inoculation with zoospores increased the total protein of the resistant clone with the greater amount and faster rate than that of the susceptible clone. It should be noted that the total protein of the resistant clone induced by elicitin peaked at 16 hours after elicitation but its peak was delayed to 72 hours after inoculation with zoospores.

The responses of β–1,3-glucanase and chitinase activity elicited by Pal were in the same direction as in the total protein, their amounts were greater in

BPM-24 (R) than in RRIM600 (S) as well as the chitinase activity by SDS-PAGE. The y and z bands which were chitinase isozymes in the resistant clone were significantly increased in lesser amount. In the susceptible clone, only the y band was observed and increased. These results revealed the phathogenic relationship associated to chitinase isozymes. The isozyme x was decreased in both resistant and susceptible clones after treated with pal and also was decreased in later time point of control suggesting that this isozyme might not be involved in plant defense. In contrast, the significant increase of isozymes y and z in the resistant clone, especially the z isozyme which was abundantly increased in BPM-24, indicated that both were strongly involved with the plant defense. β–1,3-glucanase was increased after wounding and fungal infection through the tapping site (Churngchow, *et al.,* 1995). In potato, two β–1,3-glucanases and six chitinases were accumulated following the infection by pathogen (Guest, 1997). These findings could explain the effectiveness PR-proteins against the pathogens. The response rate of β–1,3-glucanase and chitinase to elicitin was faster than that of inoculation with zoospores and with greater amount in the resistant clone. This result revealed that the effect of Pal induced PR-protein induction was stronger and quicker as previously discusses.

2.8 Transcriptional level of chitinase expression

Elicitors and/or elicitins induced the expression of defense-related genes including βglu (β-1,3-glucanase gene). βglu was expressed in the resistant cultivars with more intense and faster rate than those in susceptible cultivars (Kauffmann, *et al.,* 1993; Rivera, *et al*., 2002). Para strongly induced encoding genes of 20S proteosome subunits in tobacco compared with Cry (Suty, *et al.,* 2003). RNA prepared from rubber clones was significantly increased in the

resistant clone, BPM-24, after elicited with Pal. Eventhough our preparation of RNA was not degraded which was confirmed by discrete bands of ribosomal RNAs, it could not be hybridized to cDNA probes demonstrated by Northern blot analysis. This failure might be due to using the uncorresponding probes. In addition, the ratio of OD260 and OD280 obtained was less than 1.8, indicating that some residues of phenolic compound may be left in the preparation RNA. Therefore, the RT-PCR technique was employed instead to analyse gene expression in rubber which was elicited with Pal. The observation of much higher amount of RT-PCR product indicates the induction of chitinase mRNA after Pal elicitation. These findings and the finding of the increase in chitinase amount in BPM-24 after being treated with Pal and zoospores suggested that the response of the resistant rubber clone to elicitin was at the transcriptional level. Since the pattern of the increase in β–1,3-glucanase was similar to those of total protein and chitinase, therefore it is conceivable that the response of β–1,3-glucanase to elicitin is also at the transcriptional level. The similar pattern of the responses of total protein, β-1,3-glucanase and chitinase activities after inoculation with zoospores and stimulation with elicitin in the resistant rubber clone indicate that the expression of β-1,3-glucanase and chitinase in response of zoospores should also be at the transcriptional levels. In the susceptible rubber clone, the expression of chitinase after being stimulated with elicitin was not different from that of control.

Both enzymes are not only restricted to pathogen infected tissue but it has also been observed under other conditions of stress as well (Kombrink, *et al*., 1988). β-glucanase was able to hydrolyse glucan either alone or in combination

with chitinase in bean leaves (Mauch and Staëhelin, 1989). Besides the hydroglucanolytic activities, β-1,3-glucanase may play an important but indirect role in defense mechanism by releasing elicitors of other defense responses (Keen and Yoshikawa, 1983).