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The elicitin secreted by *Phytophthora palmivora*, a rubber tree pathogen

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Abstract

Palmivorein, a new member of the elicitin family, was purified from the culture filtrate of *Phytophthora palmivora* isolated from the rubber tree, *Hevea brasiliensis*. The elicitin was obtained by ammonium sulfate precipitation and further purified using ion-exchange and gel filtration. The molecular weight, isoelectric point, amino acid composition and N-terminal sequences of this molecule are reported and compared to other known elicitins. Palmivorein, as determined by SDS-PAGE, is a small protein of M_r ca. 10,000. It is classified as an α-elicitin according to its acidic pI and the valine residue at position 13. Like other elicitins, the *P. palmivora* elicitin causes tissue necrosis on tested tobacco leaves. It also causes severe wilting and necrosis of *Hevea* tissue, and leaves of the susceptible rubber clone (with respect to *P. palmivora*) are much more sensitive to this elicitin than those that are resistant.

Keywords: *Phytophthora palmivora*; Fungus; Elicitin; Toxin; *Hevea brasiliensis*; Euphorbiaceae; Rubber tree

1. Introduction

Phytophthora palmivora is a ubiquitous pathogen with a wide host range. It causes root, stem and fruit rot on more than 100 plant species, including pineapple, papaya, orange, tomato, tobacco and the rubber tree (*Hevea brasiliensis*, Euphorbiaceae). While *P. palmivora*, *P. botryosa*, *P. hevea*, *P. meadii* and *P. parasitica* have all been described as pathogens of the rubber tree, *P. palmivora* and *P. meadii* are the most frequently isolated and are described as the causal agents of black stripe, green pod rot and abnormal leaf fall. In Malaysia and Thailand these diseases are considered to be caused by *P. palmivora* and *P. botryosa* (Erwin and Ribeiro, 1996).

Plant defence reactions can be triggered by elicitors present in the cell wall of phytopathogenic microorganisms or in their extracellular media. They are responsible for the induction of either the hypersensitive reaction or susceptibility, serving as signals for the interaction between the plant and the pathogen (Dixon and Lamb, 1990). Such molecules are secreted in large amounts by most *Phytophthora* spp. except for *P. nicotianae*, and they are collectively called elicitins (Pernollet et al., 1993). *P. nicotianae*, the causal agent of tobacco black shank disease, can invade tobacco stem whereas other *Phytophthora* species cause limited colonization, and leaf necrosis at a distance from the inoculation site (Bonnet, 1985). When elicitins are applied to tobacco plants, they elicit leaf necrosis, cause the accumulation of pathogenesis-related proteins (Bonnet et al., 1986), and induce protection against a subsequent inoculation with tobacco pathogen *P. nicotianae* (Ricci et al., 1989). Some *Phytophthora* spp. also secrete a 32 kD glycoprotein into culture medium, which displays similar necrosis of tobacco leaf (Baillieul et al., 1996). In addition, a potent elicitor of phytoalexin accumulation in cultured parsley cells, a 42 kD glycoprotein, was purified from

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the culture filtrate of *P. megasperma* f. sp. *glycinea*. (Parker et al., 1991). Several elicitins have been isolated from the culture filtrates of various *Phytophthora* species: cryptogein, cinnamomin, capsicein and parasiticein from *P. cryptogea*, *P. cinnamomi*, *P. capsici* and *P. parasitica*, respectively (Ricci et al., 1989; Billard et al., 1988; Huet and Pernollet, 1989; Ricci et al., 1992). They are holoproteins devoid of glycosylation showing M_r ca. 10,000 (Nespoulous et al., 1992; Huet et al., 1992; Huet and Pernollet, 1993). The complete 98-amino acid sequences of all are known (Nespoulous et al., 1992; Huet et al., 1992; Huet and Pernollet, 1993; Huet et al., 1993; Mouton-Perronnet et al., 1995). They are classified into two groups: the α-class corresponds to acidic elicitins with a valyl residue at position 13, while the β-class is characterized by a hydrophillic residue at that position and a basic pI. These two classes are also distinguished by their abilities to cause necrosis on tobacco leaves, with β-elicitins being more toxic than α ones (Nespoulous et al., 1992; Huet et al., 1992).

In this paper, we report purification of palmivorein, a new member of the elicitin family, and its comparison to other known elicitins. This elicitin was purified from the culture filtrate of *P. palmivora* which was isolated from rubber tree leaf. Physical characteristics (M_r, isoelectric point, amino acid composition and N-terminal amino acid sequences) and necrosis of detached tobacco leaves are reported. The toxicity of the purified elicitin on detached rubber leaves was also investigated. We are studying the interaction between rubber tree and *P. palmivora* in order to understand the main defence mechanism of *Hevea* against this fungus.

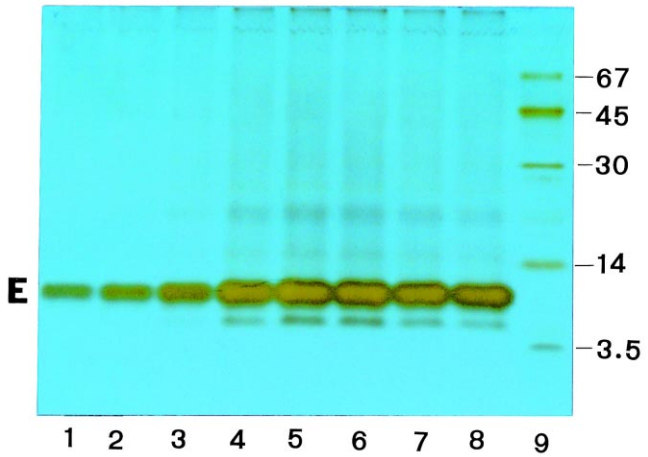

Fig. 1. Extracellular proteins secreted by *P. palmivora*, *Hevea* isolate, grown in PDB for 40 days. Every five days, culture filtrate was separated on a 16.5% polyacrylamide gel of Tricine-SDS-PAGE then stained with silver nitrate (lanes 1–8). Lane 9 is standard protein markers, LMW kit from Pharmacia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

Table 1
Yield during purification of *P. palmivora* elicitin from culture filtrate, 1 litre

Purification steps	BCA method	
	Protein (mg/l)	% yield
Filtrate	194.77	100
$(NH_4)_2SO_4$ precipitation	61.19	31.42
DEAE column	11.86	6.09
Sephadex G50 column	4.12	2.11

2. Results and discussion

The growing mycelium of *P. palmivora* from potato dextrose agar (PDA) was cut with a cork borer (diameter of 0.5 mm) into 15 pieces, and mixed with 150 ml of potato dextrose broth (PDB). The fungus was grown in a liquid medium for 40 days at 25°C in darkness, and the filtrate was aliquoted (0.5 ml) every five days for protein determination. Protein concentration was maximum between 20 and 35 days, which paralleled the levels of elicitin as detected by Tricine-SDS-PAGE using 16.5% polyacrylamide gel according to the method of Schagger and Jagaw (1987). (Fig. 1). Therefore, we chose to harvest culture filtrate after growing for 3 weeks.

Crude proteins from culture filtrate were precipitated with ammonium sulfate, then further purified by DEAE-cellulose ion-exchange and Sephadex G50 gel filtration. The elicitin peaks were identified by SDS-

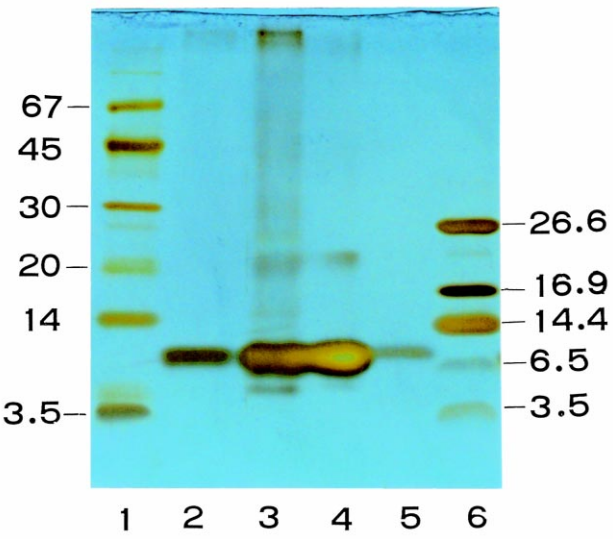

Fig. 2. Silver-stained Tricine-SDS-PAGE of purified elicitin. Lane 1: standard protein markers (LMW kit from Pharmacia plus insulin chain B: 3.5 kD); lane 2: crude culture filtrate (1.5 μg); lane 3: ammonium sulfate precipitated proteins (3.9 μg); lane 4: bound to DEAE cellulose (4.3 μg); lane 5: purified elictin from sephadex G50 (0.7 μg); lane 6: standard protein markers (26.6, 16.9, 14.4, 6.5 and 3.5 kD, polypeptide kit from Bio-RAD).

PAGE and also by toxicity tests on detached tobacco leaves. Active fractions (35–50) from the DEAE cellulose column were pooled and applied to a Sephadex G50 column. By Bradford method (Bradford, 1976), we obtained only 0.41 mg palmivorein per 1 litre of filtrate; however, the protein is not sensitive to Bradford reagent because it possesses a very low content of basic amino acids (Table 2). For this reason, a Bicinchoninic acid (BCA) method (Smith et al., 1985) was used instead to calculate yields (Table 1) demonstrating that the palmivorein concentration is 4.12 mg per 1 litre of filtrate, much higher than that suggested by the Bradford method. The palmivorein appears pure because no contaminant protein was revealed through the silver staining of Tricine-SDS-PAGE and its M_r was found to be ca. 10,000. (Fig. 2, lane 5).

At least 10 elicitins have been sequenced and it was found that all contained 98 amino acids (Mouton-Perronnet et al., 1995; Billard et al., 1988; Huet and Pernollet, 1989; Ricci et al., 1992; Nespoulous et al., 1992; Huet et al., 1992; Huet and Pernollet, 1993; Huet et al., 1993). The integer number of each amino acid in palmivorein was deduced based on their mole percents as determined by amino acid analysis (Table 2), and as compared to other α-elicitins: Cacto (*P. catorum*) elicitin and MgMα (*P. megasperma* var. *megasperma*) elicitin (Huet and Pernollet, 1993; Huet et al., 1993). Since these two elicitins were completely sequenced, the number of each amino acid was obtained directly from the sequences (Table 2). Like other elicitins, palmivorein contains 10 Leu, 6 Cys and 3 Met, and lacks Trp, His and Arg. In *P. palmivora* elicitin, Leu, Ser, Thr and Ala account for nearly 50% of the residues, as found for other known elicitins (Table 2).

The N-terminal end of palmivorein was sequenced up to Gln 26, allowing the identification of Val 13, which is characteristic of acidic α-elicitins. Fig. 3 shows the N-terminal sequences of known α-elicitins (Huet et al., 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 to 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. The N-terminal 26 residues of palmivorein 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 et al., 1992). Huet et al. (1993) also reported the occurrence of two isoforms in another *Phytophthora* species, *P. megasperma* var.

Table 2
The amino acid compositions of palmivorein and the proximal integer values deduced from the determined numbers are compared to other α-elicitins: Cacto (*P. cactorum*) and MgMα (*P. megasperma* var. *megasperma*). The amino acid compositions and the numbers deduced from the sequence of these two elicitins were obtained from Huet and Pernollet (1993) and Huet et al. (1993)

Amino acid	MgMα		Cacto		Palmivorein	
	Determined	Deduced from sequence	Determined	Deduced from sequence	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

Cacto	A	TCT	SS	QQT	A	AYV	A	LVSILSD	T	SFNQC	ST
Cap	A	TCT	TT	QQT	A	AYV	A	LVSILSD	S	SFNQC	AT
Dreα	T	TCT	ST	QQT	A	AYV	T	LVSILSD	S	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. 3. Comparison of the N-terminal sequences of *P. palmivora* elictin to other α-elictins: Cacto (*P. cactorum*); Cap (*P. capsici)*; Dreα (*P. drechsleri* α elicitin); Inf (*P. infestans)*; MgMα (*P. megasperma* var. *mesgasperma* α elicitin); Para (*P. parasitica)* and Pal (*P. palmivora*). Boxes show the conserved consensus regions. Known N-terminal amino acid sequences were obtained from Huet et al. (1994).

megasperma, one acidic and one basic but no species has yet been found to secrete only a basic isoform.

When detached leaves of *N. tabacum* were tested with purified elicitin, leaves partly dried up and necrosis developed first in the apical part of the leaves. A decrease in leaf fresh weight was both time- and dose-dependent (Fig. 4a). The excised rubber leaves were studied in parallel; palmivorein also showed severe wilting and necrosis of these leaves, as indicated by a decrease in % fresh weight (Fig. 4b and c). The typical physiological doses for detecting necrosis in tobacco and BPM-24 leaves in Fig. 4a and c are about 12.5 μg/g fresh weight of leaf. A typical dose for detecting necrosis in RRIM600 leaves is lower, about 2.5 μg/g fresh weight (Fig. 4b). From field tests at several Rubber Research Centers in Thailand, RRIM600 is the most susceptible clone with respect to *P. palmivora*, and BPM-24 is the most resistant (Rubber Research Institute of Thailand, 1997). The sensitivity of the rubber clone RRIM600 to elicitin was much higher than that of rubber clone BPM-24. The limited tissue necrosis of BPM-24 leaves caused by the elicitin is a hypersensitive response of a resistant host to a pathogen, indicating incompatibility. In contrast, the severe and extensive necrosis of RRIM600 leaves indicates a compatible reaction. A decreased leaf fresh weight for tobacco is also less severe than that observed in the susceptible rubber clone, RRIM600, indicating incompatible reaction of non-host against a factor from the pathogen. Elicitins have been proposed to act as avirulence factors in the *N. tabacum–P. parasitica* interaction (Kamoun et al., 1994). In the case of *Hevea*, the question of whether the elicitin is able to act as a pathogenicity factor, which kills tissue ahead of invasion and thus enables *Phytophthora* species to infect, has yet to be investigated.

3. Experimental

3.1. Cultures

Phytophthora palmivora isolated from rubber tree grown in Krabi (South of Thailand), was kindly provided by the Songkla Rubber Research Center. This isolate (No. KBNM 9) was identified as *P. palmivora* by the PCR based diagnostic assay using *Phytophthora* genus primers, generously provided by Prof. André Drenth of the University of Queensland, Australia. It was maintained on potato dextrose agar (PDA) before transferring into 2.4% of potato dextrose broth (PDB). After shaking (100 rpm for three weeks at 25°C in the dark), the mycelium was filtered and the medium was kept for elicitin purification.

3.2. Elicitin purification

Proteins were precipitated by addition of 61.1 g of $(NH_4)_2SO_4$ per 100 ml of medium at 4°C. The pellet was resuspended in water and desalted by loading through a PD-10 column, and then purified by chromatography on DEAE cellulose in 20 mM Tris–HCl (pH 7.0). Elicitin was eluted with 0.1 M NaCl in the same buffer. Pooled fractions (35–50) were concentrated by precipitation with ammonium sulfate and further purified by chromatography on Sephadex G50 in 20 mM Tris–HCl (pH 7.0). The elicitin was identified throughout the purification by Coomassie blue R250 and/or silver staining of 16.5% polyacrylamide gel (Tricine-SDS-PAGE), according to Schagger and Jagaw (1987). Its concentration was measured by the Bicinchoninic acid method (BCA) (Smith et al., 1985).

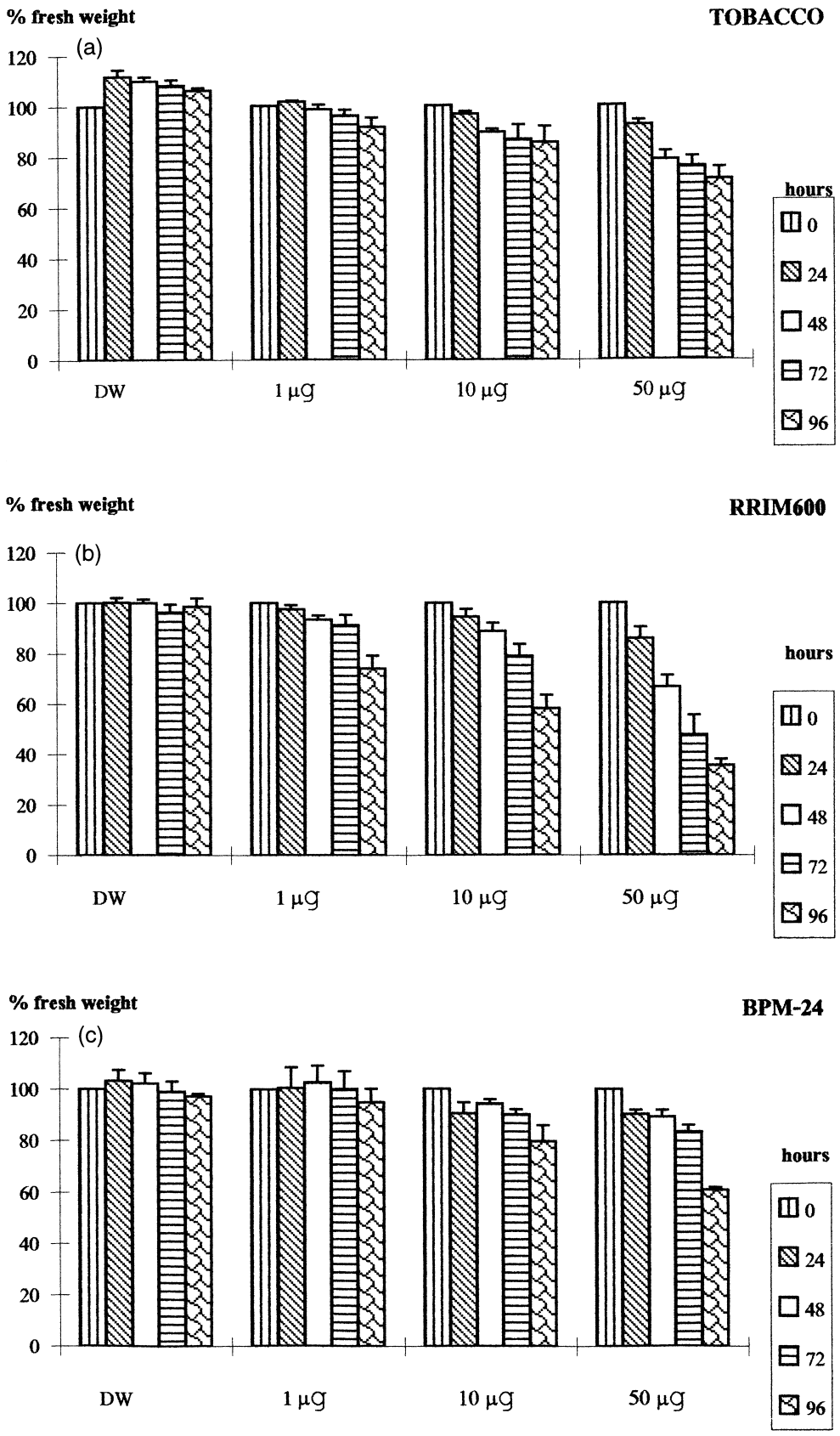

Fig. 4. Induction of leaf necrosis by palmivorein in *N. tabacum* (a), *Hevea* RRIM600 (b) and *Hevea* BPM-24 (c). A 100 µl drop of an aqueous solution of palmivorein (1, 10 and 50 µg, by the BCA method) was adsorbed through the petioles of excised leaves. At various times leaf fresh weights were measured. Results are expressed as percentages of the initial values of the fresh weight. DW: distilled water.

3.3. Amino acid analysis

Elicitin was analysed in microgram quantities using a System 6300 High Performance Analyser following the methods of the manufacturer. The values in Table 2 are the means 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.

3.4. 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 476 A) and phenylthiohydantoin (PTH) amino acid derivatives were automatically identified by data analysis apparatus (Applied Biosystems model 610 A).

3.5. Isoelectric point

Polyacrylamide gels (5%) with 3% cross-linkages containing 6.33% Pharmalyte were used. Elicitin was assayed over a 3–9 pH range. The pI was determined using Pharmacia IEF pI calibration kit (pH 3.6–9.6). The gel was stained with PhastGel Blue R.

3.6. Test for necrotic activity on tobacco leaves

Purified elicitin was tested on detached leaves from approximately 60 day old tobacco plants. Young leaves weighing about 4 g each were selected for elicitin treatment. Once the elicitin solution (1, 10 and 50 μg in 100 μl of pure water, by BCA method) had been taken up by the petioles, they were dipped into a sterile water at room temperature in the dark to allow necrosis to develop. Necrotizing effects were assessed from changes in leaf fresh weights and expressed as percentages of initial leaf weights, modified according to the method of Rustérucci et al. (1996).

3.7. Toxicity to rubber leaves

Toxicity of palmivorein was also assayed on detached leaves of the rubber tree (*Hevea brasiliensis*) in experiments carried out in the same way as for tobacco leaves. Six to eight day old leaflets, stage B_2-C, as described by Hallé and Martin (1968), weighing about 4 g each, were used for this investigation. Two rubber clones were used for this study, RRIM600 and BPM-24, representing strains susceptible and resistant to *P. palmivora*, repectively. RRIM600 was obtained from the Rubber Research Institute of Malaysia and BPM-24 (Balai Penelitian Perkebunan) was obtained from, Medan, Indonesia.

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