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DETECTION OF PATHOGENESIS RELATED PROTEINS IN *HEVEA BRASILIENSIS* INFECTED BY *PHYTOPHTHORA MEADII*

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Eleven pathogenesis related proteins (PR-proteins) were detected in rubber (*Hevea brasiliensis*) tree infected with *Phytophthora meadii*. New anionic peroxidase (PR-9 proteins) appeared in the tolerant interaction. Induction of PR-proteins is correlated with resistant interactions between *H. brasiliensis* and *P. meadii*. Two proteins corresponding to molecular weights 29kD and 33kD were prominent in expression in the tolerant plants 24 h after inoculation with the fungal pathogen. *In vacua* infiltration studies revealed the presence of similar proteins in the intercellular fluids, obtained from leaves. New anionic peroxidase bands appeared in the resistant plants during interaction with the pathogen. Similar changes in the protein induction were observed on treatment with salicylic acid. The results indicate an induction of tolerance in tolerant *Hevea* plants when challenged with *P. meadii*.

Key words : Anionic peroxidase, *Hevea brasiliensis*, *Phytophthora meadii*, PR proteins.

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INTRODUCTION

Plants respond to pathogen attack by synthesizing a set of proteins termed pathogenesis related (PR) proteins (Antoniw *et al.*, 1980). Van Loon *et al.* (1994) defined PR-proteins as the proteins, which are newly expressed upon infection. The appearance of PR-proteins in uninfected control tissues confirmed by western blot analysis as well as using cDNA probes (Lawrence *et al.*, 1996), prompted Van Loon (1999) to define them as proteins, which are readily detected in infected tissues but not in uninfected ones. The PR-proteins are induced more in resistant interactions (Van Loon, 1985; 1999). Their involvement in disease resistance has generated more interest among molecular biologists to exploit them for developing disease resistant plants. Several transgenic plants over expressing PR-proteins show increased resistance to pathogens (Vidhyasekaran, 1997; 1998). Rubber tree (*Hevea brasiliensis*) is severely infected by *Phytophthora meadii* and the disease causes heavy yield losses. PR-proteins have been exploited to manage *Phytophthora* disease in other crops like tobacco, potato, *etc.* (Alexander *et al.*, 1993; Liu *et al.*, 1994). No previous reports are available on the detection of PR-proteins induced in *Hevea* during pathogen attack. However, reports are available on the detection of chitinase and β-1, 3-glucanase group of PR-proteins in *Hevea* latex. In this paper an attempt

was made to identify the PR-proteins in *Hevea*, induced during pathogen infection, which could be exploited further for disease management.

EXPERIMENTAL

Plant and fungal materials

Hevea brasiliensis, clone RRII 105, a tolerant one to *Phytophthora meadii* and the clone RRIM 600, highly susceptible to this pathogen, were used in the present study. Plants were grown in pots and maintained in a green house prior to fungal inoculation. Three-months old plants were used for the study. Sporangia of *P. meadii* were produced by culturing the fungus in oat broth for 48 h in dark and exposing the sterile water-washed mycelium for 16 h to the light (Rajalakshmi and Joseph, 1986). The sporangia were given cold shock for 15 min at 10°C and subsequently the temperature was raised to 26 to 28°C. The number of zoospores were adjusted to 30 per 10 ml. Fifteen droplets were placed on the lower surface of each leaflet for fungal infection. Inoculated plants were maintained at 95 to 100 per cent relative humidity at 18°C with an 8/16-dark/light cycle for three days before transplanting them to the green house.

Extraction of acidic proteins

PR-proteins were detected by following the methodology of Henriquez and Sanger (1982). Leaf samples were ground in liquid nitrogen. Extraction buffer consisted of acetic acid (5%) and β–mercaptoethanol (1%), pH 2.8 (1 ml/g fresh leaf weight). Proteins were extracted at three time intervals, 0, 24 and 48 h after fungal inoculation. Plant leaf extract homogenates were centrifuged at 20000 xg for 20 min at 4°C. The supernatant was adjusted to pH 5.5 with 5N sodium hydroxide, passed through Sephadex G-25 column for desalting and then concentrated with five volumes of acetone. The induced proteins were identified through SDS-PAGE electrophoresis. Protein content was estimated using Bradford (1976) method, and equal concentration of protein was loaded on each well (20 µg). Isoelectric focusing followed by activity staining was done for peroxidase and aryl esterase to detect zones of enzyme activity (Vallejos, 1983). Salicylic acid, a chemical stimulant of PR-proteins and disease resistance, was injected (0.5 mM concentration) into veins of the third and fourth fully expanded leaves and the response was compared with that of the pathogen induced response. Control plants were injected with water.

Extraction of intercellular fluids

The procedure described by Parent and Asselin (1984) was used for extraction of proteins from intercellular fluids. Intact leaflets were submerged in 32 mM/84 mM, phosphate/citrate buffer (pH 2.8) containing 1% β-mercaptoethanol and infiltrated for 20 min under vacuum. The leaflets were dried with Kleenex paper, rolled into a cylindrical bundle and placed in 50 ml syringe inside a centrifuge tube. The intercellular fluid was obtained by centrifuging the tissue at 1000 xg for 15 min. The controls were infiltrated with water under the same conditions.

SDS-polyacrylamide gel electrophoresis

The proteins were separated on 15 per cent SDS gels according to Laemmli (1970). The buffer had 25 mM Tris, 190 mM glycine and 0.1% (w/v) SDS, pH 8.3. Samples were made up to 1 per cent and 0.0025% with SDS and bromophenol blue respectively, heated to 95°C for 3 min, spun

rapidly and loaded (a maximum 20 µg protein per slot) into 160x160x0.8 mm slab gels. The gels were fixed for 1 h in 40 per cent MeOH, 10 per cent acetic acid and stained with 0.1 % Coomassie brilliant blue R-250.

Isoelectric focusing of proteins was carried out using LKB-Pharmacia Multiphor II system using a pH gradient (ampholytes pH 3-10) to separate proteins. The electrode strips (Pharmacia) were soaked with electrode buffer. After a pre-run for 30 min. at low voltage (200 volts) the samples were applied using a sample application foil kept on the top of the 1.5 mm thick polyacrylamide gel. Samples were loaded near the cathode electrode strip for optimum separation of proteins. A voltage of 1500V was applied for 2 h. All enzyme-staining procedures were followed according to Vallejos (1983).

RESULTS AND DISCUSSION

Induction of proteins, anionic peroxidase and aryl esterase enzymes were specifically monitored in the resistant as well as susceptible plants. The host response stimulated by external application of salicylic acid was also monitored. *In vacua* infiltration studies were carried out to understand the accumulation pattern of proteins in the molecular fluids during pathogen infection. In the susceptible clone no new proteins were formed during 24 and 48 h after challenging with the pathogen (Fig. 1a).

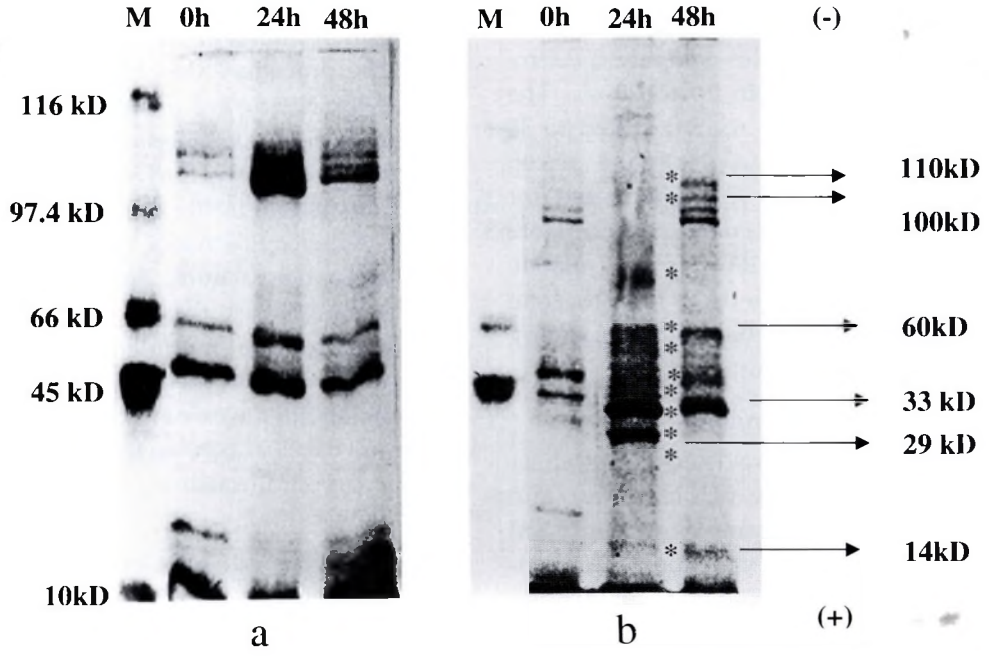

Fig. 1. SDS-Polyacrylamide gel electrophoresis of acid soluble proteins extracted from leaves infected with *Phytophthora meadii*. a. Susceptible plant; b. Tolerant plant; M : Marker proteins (Sigma) with molecular masses in kD. The numbers on the right hand side indicate the bands according to their apparent molecular masses. A 0 h, B 24 h and C 48 h after inoculation with the fungal pathogen. * indicate newly induced proteins.

Eleven acid extractable proteins were induced in the resistant clone during pathogen infection (Fig. 1b). Two bands corresponding to 29 kD and 33 kD were prominent. The 33 kD protein was present even 48 h after inoculation, while the 29 kD protein band disappeared after 24 h. Two other high molecular weight bands corresponding to 100 kD and 110 kD appeared 48 h after inoculation. A 14 kD protein band was also detected at 24 h as well as 48 h after inoculation. But these protein bands were not so prominent as the 29 kD and 33 kD proteins. The accumulation and disappearance of the protein bands during the development of defence response can be regarded as the result of the pathophysiological response of *Hevea* plants to infection. Induction of proteins in *Hevea* was comparable to that reported in leaves of tobacco, tomato and cowpea when challenged with tobacco mosaic virus (TMV), tobacco necrosis virus (TNV) and the fungal pathogen *Thielaviopsis basicola* (Antoniw *et al.*, 1985; Christ and Mosinger, 1989; Nassuth and Sanger, 1987). In *Hevea*, proteins similar to chitinase group of PR-proteins have already been detected in the latex. This protein has been termed as Heavamine (molecular weight 29 kD) (Rozeboom *et al.*, 1990; Potter *et al.*, 1993; Lee and Raikhel, 1995; Osmark *et al.*, 1998; Navrath and Mltraux, 1999). Appearance of proteins in the intercellular fluid has been previously reported in tomato plants on challenging with *Cladosporium fulvum* (Christ and Mosinger, 1989). On analysis of intercellular fluids using the procedure described by Van Loon *et al.* (1985) on resistant and susceptible plants of *Hevea*, the presence of two proteins corresponding to 29 kD and 33 kD were observed 24 h after inoculation with the pathogen (Fig. 2).

Induction of 29 kD and 33 kD proteins were observed on injecting salicylic acid into the leaves (Fig. 3). Salicylic acid plays an important role in the activation of defence responses in plants (White, 1979; Klessig *et al.*, 1989; Yalpani and Raskin, 1993). Exogenous application of salicylic acid was found to induce the accumulation of acidic proteins in cucumber (Rasmussen *et al.*, 1995).

A 33 kD protein was observed both in the susceptible and resistant plants. However, the 29 kD protein was observed only in the resistant cultivar. The water-injected controls did not show any accumulation of the proteins after 24 h. Activation of defence responses may occur through the interaction of salicylic acid with other cellular factors. Like catalase or peroxidase. This contention is supported by the responses of different tissues of *Oryza sativa* on treatment with salicylic acid (Chen *et al.*, 1995). Peroxidase involved in many plant developmental and environmental responses (Gaspar *et al.*, 1982; Scott-Craig *et al.*, 1995) plays a key role in the successful defence of the host against both bacterial and fungal pathogens. Expression of anionic peroxidase which belong to PR-9 group of PR-proteins (Vidhyasekaran, 1997; 1998), in transgenic tobacco plants, resulted in 20-fold increase in the lignin content, which has a direct role in preventing the entry of the pathogen. Differential activation and suberization associated with anionic peroxidase genes were observed in near isogenic resistant lines of tomato when treated with elicitors of *Verticillum alboatrum* (Roberts and Kolattukudy, 1989; Mohan and Kolattukudy, 1990). Similarly accumulation of anionic peroxidase was observed in soyabean tissues on inoculation with the

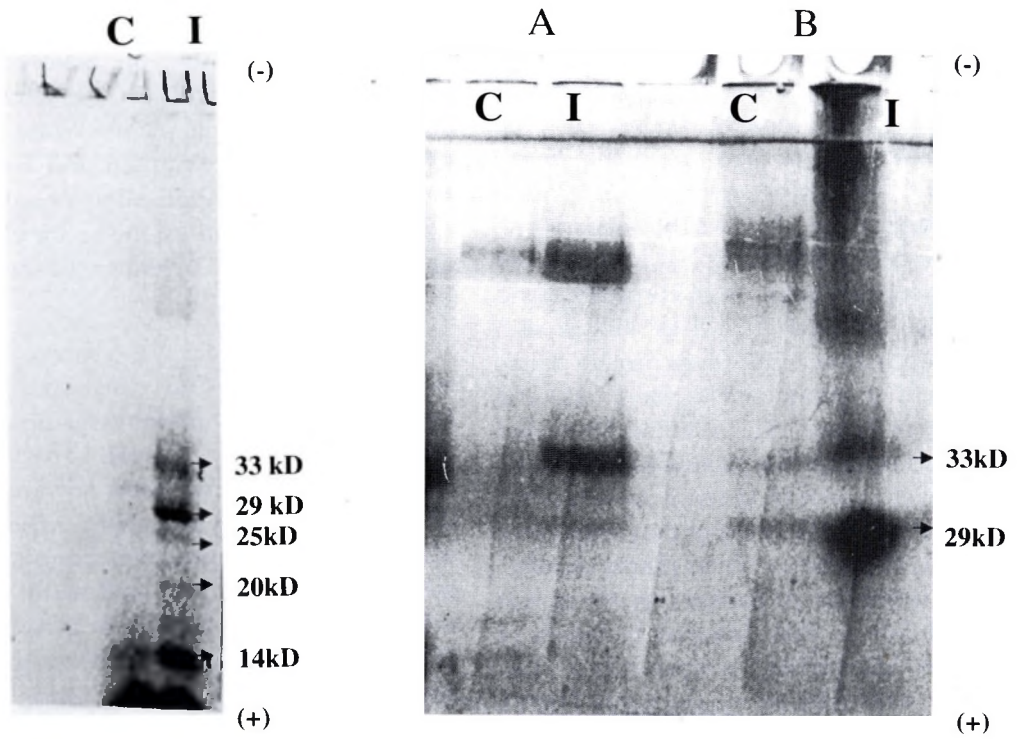

Fig. 2. SDS-Polyacrylamide gel electrophoresis analysis of acid soluble proteins extracted from intercellular spaces after *in vacua* infiltration (24 h post inoculation with the fungal pathogen). C control injected with water and I inoculated with pathogen on tolerant plants.

Fig. 3. SDS-Polyacrylamide gel electrophoresis analysis of salicylic acid induced proteins in tolerant and susceptible plants. A susceptible, and B tolerant plant samples. C and I represent proteins extracted from control with water injected and salicylic acid injected leaf tissues respectively.

fungal pathogen *Phytophthora meagasperma* (Graham and Graham, 1991). Isoelectric focusing studies on samples extracted from leaves of *Hevea* showed the appearance of several new anionic peroxidase bands in the resistant plants (Fig. 4).

Previous studies on cucumber has shown the presence of a 33 kD apoplastic peroxidase induced during inoculation with *Pseudomonas syringae* pv *syringae* (Rasmussen *et al.*, 1995). Similar observations were made in our studies for the appearance of the 33 kD protein both in the intercellular fluids as well as leaf extracts. Appearance of new aryl esterase bands was observed in the tolerant cultivar during pathogen attack (Fig. 5).

Aryl esterase enzymes produced by host plants are known to play a major role in the dissolution of the fungal cell walls causing an electrolytic leakage of the cell contents finally leading to the death of the pathogen (Vallejos, 1983).

The present studies have shown the induction of new proteins in tolerant cultivar of *Hevea* on challenging with *Phytophthora meadii*. Results presented in this paper show a systemic induction of a group of acidic proteins, along with anionic peroxidase and esterase in tolerant *Hevea* plants.

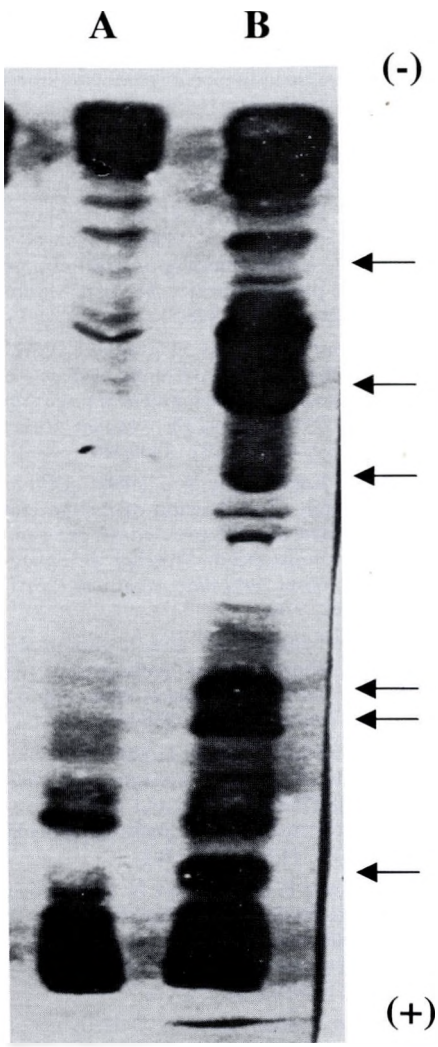

Fig. 4. Anionic peroxidase activity in tolerant plants of *Hevea*. **A** 0 h and **B** 24 h after fungal inoculation. Arrows indicate newly induced bands.

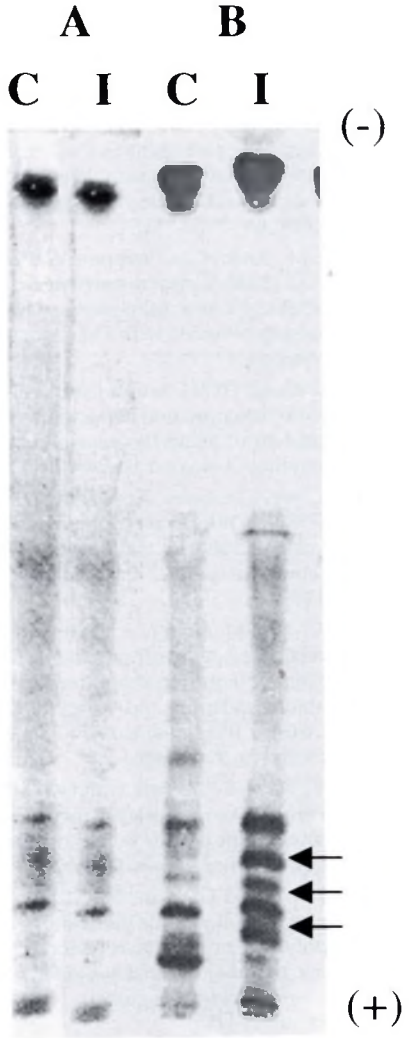

Fig. 5. Aryl esterase activity (24h post inoculation with the fungal pathogen) in resistant and susceptible plants following pathogen inoculation. **A** Susceptible plants and **B** tolerant plants, **C** injected with water, and **I** pathogen induced. Arrows represent newly formed bands.

The presence of acidic proteins in the intercellular fluids as well as induction of PR-proteins following salicylic acid treatment indicate the involvement of a possible systemic induced resistance mechanism in the resistant plants to ward off pathogen.

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REFERENCES

Alexander, D., Goodman, R.M., Gut-Rella, M., Glascock, c., Weymann, K., Friedrich, L., Maddox, D., Ahl-Goss, P., Lunitz, T., Ward, E. and Ryals, J. (1993). Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related proteins. *Proceedings of the National Academy of Sciences of the USA*, **90** : 7327-7331.

Antoniw, J.F., Ritter, C.E., Pierpoint, W.S. and Van Loon, L.C. (1985). Comparison of three pathogenesis-related proteins from plants of two cultivars of tobacco infected with TMV. *Journal of General Virology*, **47** : 79-87.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72** : 248-254.

Chen, Z., Iyer, S., Caplan, A., Klessig, D.F. and Fan, B. (1995). Differential accumulation of salicylic acid and salicylic acid sensitive catalase in different rice tissues. *Plant Physiology*, **108** : 1379-1385.

Christ, U. and Mosinger, E. (1989). Pathogenesis related proteins of tomato : 1. Induction by *Phytophthora infestants* and the biotic and abiotic inducers and correlations with resistance. *Physiological and Molecular Plant Pathology*, **35** : 53-65.

Gaspar, T., Penel, C., Thorpe, T. and Greppin, H. (1982). Peroxidases : A survey of their biochemical and physiological roles in higher plants. University of Geneva Press, Geneva.

Graham, M.Y. and Graham, T.L. (1991). Rapid accumulation of anionic peroxidases and phenolic polymers in soybean cotyledon tissues following treatment with *Phytophthora megasperama* f. sp. *glycinea* w: glucan. *Plant Phygiology*, **97** : 1445-1455.

Henriquez, A.C. and Sanger, H. (1982). Analysis of acid extractable tomato leaf proteins after infection with a viroid, two viruses and a fungus and partial purification of the pathogenesis related proteins, P14. *Archives of Virology*, **74** : 181-195.

Klessig, D.F., Cutt, J.R., Dixon, D.C., Carr, J.P., Malamy, J., Metzler, M., Harpster, M.H., Dunsmuir, P., Beachy, R.N. and Raskin, I. (1989). The PR-1 proteins : Their synthesis, location and possible function (Abstract) In : *Workshop on Pathogenesis Related Proteins in Plants*, (Eds. V. Conejero and L.C. Van Loon). Fundacion Juan March, Madrid, Spain, 1989, p. 61.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227** : 680-685.

Lawrence, C.B., Joosten, M.H.A.J. and Tuzun, S. (1996). Differential induction of pathogenesis-related proteins in tomato by *Alternaria solani* and the association of a basic chitinase isozyme with resistance. *Physiological and Molecular Plant Pathology*, **48** : 361-377.

Lee, H.I. and Raikhel, N.V. (1995). Prohevein is poorly processed but shows enhanced resistance to a chitin-binding fungus in transgenic tomato plants. *Brazilian Journal of Medical and Biological Research*, **128**(7) : 743-750.

Liu, D., Raghothama, K.G., Hasegawa, P.M. and Bressan, R.A. (1994). Osmotin over-expression in potato delays development of disease symptoms. *Proceedings of the National Academy of Sciences of the USA*, **91** : 1888-1892.

Mohan, R. and Kolattukudy, P.D. (1990). Differential activation of expression of a suberization-associated anionic peroxidase gene in near isogenic resistance and susceptible tomato lines by elicitors of *Verticillium alboatrum*. *Plant Physiology*, **92** : 276.

Nassuth, A. and Sanger, H.I. (1987). Immunological relationships between 'pathogenesis related' leaf proteins from tomato, tobacco and cowpea. *Virus Research*, **4** : 2745-2749.

Navrath, C. and Mltraux, J.P. (1999). Salicylic acid induction-deficient mutants of *Arabidopsis* express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. *Plant Cell*, **11**(8) : 1393-1404.

Osmark, P., Boyle, B. and Brisson, N. (1998). Sequential and structural homology between intracellular pathogenesis-related proteins and a group of latex proteins. *Plant Molecular Biology*, **38**(6) : 1243-1246.

Parent, J.G. and Asselin, A. (1984). Detection of pathogenesis related proteins (PRb) and of other proteins in the intercellular fluid of hypersensitive plants infected with tobacco mosaic virus. *Canadian Journal of Botany*, **62** : 564-569.

Potter, S., Ukens, S., Lawton, K., Winter, A.M., Chandler, D., DiMaio, J., Novitzky, R., Ward, E. and Ryals, J. (1993). Regulation of a hevin-like gene in *Arabidopsis*. *Molecular Plant Microbe Interactions*, **6**(6) : 680-685.

Rasmussen, J.B., Smith, J.A., Williams, S., Burkhart, W., Ward, E., Somerville, S.C., Ryals, J. and Hammershimdt, R. (1995). CDNA cloning and systemic expression of acidic peroxidase associated with systemic acquired resistance to disease in cucumber. *Physiological and Molecular Plant Pathology*, **46** : 389-400.

Rajalakshmi, V.K. and Joseph, A. (1986). Production of sporangia by *Phytophthora meadii*. *Indian Phytopathology*, **39** : 470-471.

Roberts, E. and Kolattukudy, P.E. (1989). Molecular cloning, nucleotide sequence and abscicic acid induction of a suberization-associated highly anionic peroxides. *Molecular Genera Genetics*, **217** : 223-228.

Rozeboom, H.J., Budiani, A., Beintema, J.J. and Dijkstra, B.W. (1990). Crystallization of hevamine, an enzyme with lysozyme/chitinase activity from *Hevea brasiliensis* latex. *Journal of Molecular Biology*, 5 : **212**(3) : 441-443.

Scott-Craig, J.S., Kerby, K.B., Stein, B.D. and Somerville, S.C. (1995). Expression of an extracellular peroxidase that is inducted in barely (*Hordeum vulgare*) by the powdery mildew pathogen (*Erysiphe graminis* f. sp. *hordei*). *Physiological and Molecular Plant Pathology*, **47** : 407-418.

Vallejos, C.E. (1983). Enzyme activity staining. In : *Developments in Plant Genetics and Breeding : 1. Isozymes in Genetics and Breedings, Part A* (Eds. S.D. Tanksley and T.J. Orton). Elsevier Publishers, Amsterdam.

Van Loon, L.C. (1985). Pathogenesis related proteins. *Plant Molecular Biology*, **4** : 111-116.

Van Loon, L.C. (1999). Occurrence and properties of plant pathogenesis-related proteins. In : *Pathogenesis Related Proteins in Plants* (Eds. S.K. Datta and S. Muthukrishnan).CRC Press, Boca Raton, pp. 1-9.

Van Loon, L.C., Pierpoint, W.S., Boller, T. and Conejero, V. (1994). Recommendations for naming plant pathogenesis related proteins. *Plant Molecular Biology Reporter*, **12** : 245-264.

Vidhyasekaran, P. (1997). Fungal pathogenesis in plants and crops : Molecular biology and host defence mechanism. Marcel Decker, New York, 568 p.

Vidhyasekaran, P. (1998). Molecular biology of pathogenesis and induced systemic resistance. *Indian Phytopathology*, **51** : 111-120.

White, R.F. (1979). Acetyl salicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. *Virology*, **99** : 410-412.

Yalpani, N. and Raskin, I. (1993). Salicylic acid : A systemic signal in induced plant disease resistance. *Trends in Microbiology*, **1**(3) : 88-92.