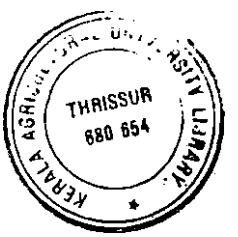
EVALUATION OF PATHOGENESIS RELATED PROTEINS IN RELATION TO *PHYTOPHTHORA* FOOT ROT IN BLACK PEPPER [*Piper nigrum* L.]

By

G. V. PARAB

(98-12-23)



THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Horticulture

Faculty of Agriculture

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Department of Plantation Crops and Spices

COLLEGE OF HORTICULTURE

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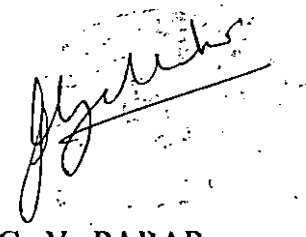
2000

DECLARATION

I hereby declare that this thesis entitled **"Evaluation of pathogenesis related proteins in relation to *Phytophthora* foot rot in black pepper (*Piper nigrum* L.)"** is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.



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Certified that this thesis entitled **"Evaluation of pathogenesis related proteins in relation to *Phytophthora* foot rot in black pepper (*Piper nigrum* L.)"** is a record of research work done independently by Mr. **G. V. Parab**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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I thank Dr. ***Jacob John****, Professor of Biochemistry, College of Horticulture, for his valuable suggestions and encouragement during the study.*

I am grateful to Dr. ***Babu Philip****, Professor of Biochemistry, Marine Biology Laboratory, Cochin University of Science and Technology for his suggestions during the research period.*

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I am grateful to Shri. Sreekumar, Farm Assistant, CCRP for his services in preparation of photographs.

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Junior Merit Fellowship awarded by the Kerala Agricultural University is also acknowledged.

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We, the undersigned members of the Advisory Committee of Mr. G.V. Parab, a candidate for the Degree of **Master of Science in Horticulture**, with major in **Plantation Crops and Spices**, agree that this thesis entitled "**Evaluation of pathogenesis related proteins in relation to *Phytophthora* foot rot in black pepper (*Piper nigrum* L.)**" may be submitted by **Mr. G. V. Parab**, in partial fulfilment of the requirement for the Degree.

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G. V. Parab

In memory of my beloved
grand father

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ABBREVIATIONS

BSA	-	Bovine serum albumine
CCRP	-	Cadbury Cocoa Research Project
CPBMB	-	Centre for Plant Biotechnology and Molecular Biology
cm	-	centimeter
g	-	gram
pH	-	hydrogen ion concentration
kD	-	kilo dalton
μl	-	micro litre
μg	-	micro gram
μM	-	micro molar
°C	-	degree celsius
mA	-	milli amphere
mM	-	milli molar
mg	-	milli gram
mm	-	milli meter
min	-	minutes
nm	-	nano meter
P. capsici	-	*Phytophthora capsici*
P. nigrum	-	*Piper nigrum*
P. colubrinum	-	*Piper colubrinum*
TEMED	-	N, N, N', N' Tetramethylene ethylene diamine
%	-	per cent
PAGE	-	Polyacrylamide gel electrophoresis
Rm	-	Relative mobility
rpm	-	revolutions per minute
SDS	-	Sodium dodecyl sulfate
v/v	-	volume in volume

Introduction

1. INTRODUCTION

Black pepper (*Piper nigrum* L.), originated in the Western Ghats of India, is an important spice crop commercially grown in India, Indonesia, Brazil, Malayasia, Madagascar, Sri-Lanka, Thailand and some parts of China. It is popularly known as "black gold" and is the major export earner among the Indian spices.

The foot rot disease of black pepper caused by *Phytophthora capsici* (Leonin emend, Alizadeh and Tsao) is one of the major constraints in production all over the world. In India, the disease was known as early as in 1902 when severe vine death was noticed in Wynad region of Kerala and then in Madras state (Menon, 1949). The fungus infect all parts of the plant. Preliminary screening studies showed that the cultivars like Narayakodi, Kalluvally, Balankotta, Uthirankotta and Cheriakanyakkadan were tolerant while cultivars like Panniyur-1 and Karimunda were highly susceptible to *Phytophthora* foot rot (Kuch and Khew, 1980 and Sarma and Nambiar, 1982). However, *Piper colubrinum* L., a wild species of pepper introduced from the Amazon basin is reported to be immune to *Phytophthora capsici*.

In order to thrive over the stress conditions, different defence mechanisms have been reported in crop plants. Various host proteins may accumulate in response to different biotic and abiotic stresses. These novel plant coded proteins remain at very low concentration in uninfected tissues (Antoniw *et al.*, 1985). The

occurrence of these proteins is not pathogen specific but determined by the type of reaction of the host plant indicating that these proteins are of host origin (Van Loon, 1985). Since these proteins are induced under specific pathological condition, they have been named as 'pathogenesis related proteins' (PR protein). They may be involved in the mechanism that leads to the localization of pathogen that confers some degree of resistance subsequent to infection.

Several fungitoxic substances like phenolics, phytoalexins, lytic enzymes and pathogenesis related proteins are synthesized in plant tissues upon infection by *Phytophthora* species. These toxic substances are accumulated both, in resistant and susceptible varieties. The major difference is in the delayed induction of these substances in the susceptible ones.

The present study is an attempt to evaluate pathogenesis related proteins and specific enzymes in *Piper* species induced or expressed during infection by *Phytophthora capsici*. Since this is the first attempt of its kind, the results would help to elucidate the expression of defense genes against *Phytophthora,* that can further be cloned and used to improve the defence mechanism of black pepper.

Review of Literature

2. REVIEW OF LITERATURE

Phytophthora foot rot disease induced by *Phytophthora capsici* is an ubiquitous disease of black pepper posing serious threat to its cultivation. The disease was reported in India as early as in 1902, from Wynad region of Kerala (Menon, 1949). There is no effective control measure to tackle this disease and all the cultivated types are found susceptible to the disease. However, some of the wild species like *Piper colubrinum* and *Piper obliqum* were found to be immune to the disease. A thorough evaluation of gene products in susceptible and resistant varieties would unfold the defence mechanism of the products of the plant defense genes against *Phytophthora*. So, an effort is made in this review to bring together the facts, interpretations and theories of evaluation of PR proteins and their role in the defense mechanism of the host.

2.1 Symptomatology of *Phytophthora* foot rot disease

Many authors reported symptomatological studies on *Phytophthora* foot rot of black pepper. Butler (1906) observed blackening of the diseased black pepper root which subsequently extended acropetally into the base of the stem. According to Muller (1936), leaves, stem and root of black pepper vine at all stages of growth were susceptible to the disease. He observed typical symptoms like leaf rot, collar rot and root rot in the field. Holliday and Mowat (1963) found more number of infected leaves on the lower part of the vine with uniform spread of brown lesions

with fimbriate edges. Sarma *et al.* (1994) have reviewed the symptomatology of black pepper upon infection with *Phytophthora capsici*. They reported that all parts of the plant viz., the tender runner shoots, leaves, spikes, aerial branches and roots were prone to pathogen infection. Foliar phase of the infection was characterized by production of small dark brown spots with a fast advancing margin. The infection spread rapidly towards the stem resulting in rotting of green tender stem. This in turn resulted in varying degrees of defoliation and finally led to reduced canopy.

2.2 Sources of resistance to *Phytophthora capsici*

The available genotypes of black pepper and related species of pepper were screened for resistance and/ or tolerance to the fungus by many workers (Holliday and Mowat, 1963; Ruppel and Almeydar, 1965; Leather, 1967; Alconero *et al.*, 1971; Turner, 1973; Kuch and Khew, 1980; Sarma *et al.*,1982 and Vilasini, 1982). They found that none of the cultivated types were resistant to the disease while wild species like *P. colubrinum* and *P. obliqum* showed certain resistance. The relative tolerance of Indian varieties, Kalluvally and Balankotta was reported by Kuch and Khew (1980) from Malaysia. The immunity of *P. colubrinum* a wild relative of pepper from Amazon basin, was also reported by Sarma *et al.* (1991).

2.3 Plant response to infection

Plants respond to pathogen attack in a variety of ways. Some plants are able to restrict the pathogen to a small region near the site of infection. Some

times, this resistance is accompanied by localized death of tissues. In addition to the localized response, plants respond to infection by activating the defense genes in all parts. As a result, pathogenesis related proteins and/or phytoalexins are accumulated in the plant. So also, liginification of cell wall was observed as a consequence of infection (Kuc, 1982; Dean and Kuc, 1985; Kuc and Rush, 1985 and Yalpani *et al.*, 1991). This renders the entire plant resistant to the disease.

The hypersensitive response leading to systemic acquired resistance (SAR) was first characterized in sweet william infected by carnation mosaic virus (Gilpatrick and Weintraub, 1952). Systemic acquired resistance is usually defined as resistance acquired upon subsequent pathogen attack and can be detected in both infected and uninfected parts of the plant (Ross, 1961). Association of hypersensitive response and SAR is related with the systemic synthesis of several families of serologically distinct pathogenesis related proteins as reviewed by Carr and Klessig, (1989). The phenomena of SAR imply the existence of a signal that spreads systemically from the site to hypersensitive response to rest of the plant. Though number of systemic signals like salicylic acid, ethylene, and jasmonates linking SAR and defense response have been identified by Shetty and Kumar (1999), at present, very little is known about the signal transduction pathways that are activated during the plant response to pathogen attack.

2.4 Pathogenesis related proteins

2.4.1 Occurrence

The occurrence of these new proteins is not pathogen specific but is determined by the type of reaction of host origin. Since these proteins are induced under specific pathological condition they are named as "Pathogenesis Related Proteins" (Van Loon, 1985). Houge and Asselin (1987) reported their predominant presence in the intercellular fluid of the leaves. They may also be localized in either extracellular fluid or in the vacuole (Vera *et al.*, 1989).

2.4.2 Properties

Symptom specific pathogenesis related protein have been reported in a number of plants like cucumber (Tas and Peter, 1977), cowpea (Coutts, 1978), tobacco (Ahl *et al.*, 1982), globe amaranth (Redolfi *et al.*, 1982), potato (Rahimi *et al.*, 1996) and pumpkin (Cheong *et al.*, 1997).

These proteins exhibit some common distinguishing properties. They are relatively low molecular weight proteins. Typical PR protein described for number of plant species is acid soluble (Pierpoint, 1986) and highly resistant to the endogenous plant protease but are not proteinase inhibitors (Pierpoint *et al.*, 1981).

2.4.3 Mode of action

Induction of pathogenesis related proteins in plants are associated with the hypersensitive reaction and systemic acquired resistance (Redolfi, 1983; Gianinazzi, 1984; Van Loon, 1985).

According to the nomenclature proposed by Van Loon *et al.* (1987), recently several PR proteins have been identified as hydrolytic enzymes. The PR proteins, PR-2, PR-N and PR-O have been identified as ß-1,3-glucanases (Kauffmann *et al.*, 1987), while PR proteins PR-P and PR-Q in tobacco as chitinases (Legrand *et al.* 1987). Chitinases and ß-1,3-glucanases are enzymes that hydrolyze the carbohydrate polymer chitin and ß-1,3-glucan respectively. These polymers are major components of the cell wall of many fungi, where as chitin is also present in the exoskeleton of insects. Mauch *et al.* (1988) suggested PR proteins to be enzymes of secondary metabolism, such as, phenylalanine ammonia lyase and chalcone synthease. Dassi *et al.* (1998) focussed on the putative role of pathogenesis related proteins, as bio-protection of mycorrhizal tomato roots towards *Phytophthora parasitica.*

2.4.4 Classification of PR proteins

The different groups working in this field have used several nomenclature systems. But in this review we refer to the nomenclature suggested by Van Loon *et al.* (1987). According to this, PR proteins have been grouped into five classes namely PR-1 to PR-5 type proteins.

2.4.4.1 PR-1 type proteins

These proteins have molecular weight of approximately 15 kD. Acidic PR-1 protein having iso-electric point (pI) of approximately 4.0 (Cornelissen *et al.*, 1897) and basic PR-1 type proteins analogous to acidic ones

have a pI of 10.7 (Payne *et al.*, 1989). Genes encoding for basic PR-1 proteins show tissue specific expression as compared to acidic ones where the maximum expression is in the roots. PR-1 proteins are induced not only during infection by pathogen but also by the treatment with certain chemicals.

2.4.4.2 PR-2 type proteins: ß-1,3-Glucanase

Kauffmann *et al.* (1987) found the ß-1,3-Glucanase activity of PR-2 type proteins. On contrary to the extracellular localization of the acidic glucanases, basic glucanases accumulate in the vacuole. Homologous to PR-1 protein, basic ß-1,3-glucanases are expressed at high level in the roots of non-infected plants (Memelink *et al.*, 1990).

As suggested by Mauch and Stachelin (1989), ß-1,3-glucanases play an important role in signalling, leading to the hypersensitive response by releasing elicitors from cell wall. Glucanase degrades ß-1,3-glucan substrate laminarin *in vitro*.

2.4.4.3 PR-3 type proteins: Chitinases of Group I & II

Legrand *et al.* (1987) characterized both acidic and basic chitinases. Additional lysozyme activity of chitinase responsible for hydrolysis of bacterial peptidoglycan has been reported by Trudel *et al.* (1989). Chitinases are polymers of ß 1-4 N acetylglucosamine (chitin) like basic PR-1 and PR-2 type proteins. Basic chitinase accumulates at high level in the roots. Chitinases and ß-1,3-glucanases are enzymes that hydrolyse the chitin and ß-1,3-glucan

respectively which constitute the major components of cell wall of many fungi where as chitin is also present in exoskeleton of insects.

2.4.4.4 PR-4 type proteins

These proteins, in non-denaturing gel system, migrate with molecular weight of 13 to 14.5 kD. Nassuth and Sanger (1986) reported their homologous nature with PR-1 type proteins.

2.4. 4.5 PR-5 type proteins

After comparison of aminoacid sequence of PR-5 type proteins from database, Cornelissen *et al.* (1986) and Richardson (1987) reported close similarity with thaumatin, a protein induced during pathogen attack or stress in *Thaumalococous daniellii*. PR-5 proteins accumulate in the extracellular fluid.

2.4.4.6 Other proteins induced during pathogen attack or stress

Shumway *et al.* (1970) reported proteinase inhibitors induced by the chewing insects in the leaves. These proteins have been found to be localised intracellularly in the vacuoles. Esquerre-Tugaye *et al.* (1979) reported the structural proteins in maintaining the structure of the cell wall and their importance in controlling growth, development and disease resistance. These structural proteins include: (1) Extensins and related hydroxyproline rich glycoproteins (2) proline rich proteins and (3) glycine rich proteins. Inhibitory nature of inhibitor of viral replication (IVR) was reported by Loebenstein and Gera (1981). These IVR

are located extracellularly. In parsely, cytosolic proteins have been reported by Sommsich *et al.* (1986). Wounded potato tubers induce proteins which are basic in nature and possess no similarity to any other known protein (Logemann *et al.*, 1988). Samac *et al.* (1990) reported chitinase of group III having lysozyme activity in the extracellular fluid of cucumber.

2.4.5 Induction of PR proteins

Pathogen after attacking a particular plant gives out elicitors, which are perceived by the receptors present in the plasma membrane and undergoes configurational changes. Receptors increase the activity of phenylalanine ammonia lyase (Legrand *et al.*, 1976), an enzyme dedicated in salicylic acid biosynthesis pathway which acts as a signal molecule. Salicylic acid in turn acts as an endogenous signal that triggers the local and systemic induction of PR proteins (Yalpani *et al.*, 1991). Synthesis of salicylic acid enhances the production of hydrogen peroxide and induces the activity of defense genes (Shirasu *et al.*, 1997).

Jasmonic acid is synthesised in plants from linolenic acid in an octadecanoid pathway (Vick and Zimmerman, 1983). Doares *et al.* (1996) suggested that this pathway mediates the induction of defence genes by the accumulation of proteinase inhibitor protein.

2.4.6 Induction of PR proteins during fungal infection

Gianinazzi *et al.* (1980) reported host protein appearance in response to *Thielaviopsis basicola*. Pegg and Young (1981) reported induction of PR proteins

in relation to fungal colonization in tomato during infection of *Verticillium albo-atrum*. Wolf (1983) studied the protein pattern of bean leaves during bean rust infection. Mazau and Esquerre-Tugaye (1986) reported accumulation of hydroxyproline rich glyco protein in the cell wall during *Colletotrichum lagenarium* and *C. lindemuthianum* infection in melon and bean, respectively. Hydrolytic enzymes like chitinase and ß-1,3-glucanase were induced in the immature pea pod during infection with *Fusarium solani* f sp *pisi* (Mauch *et al.*,1988). He has reported increase in antifungal hydrolases in pea during inoculation of *Fusarium solani*. Acidic ß-1,3-glucanase was detected in cucumber leaves during *C. lagenarium* infection (Ji and Kuc, 1995). Even the transgenic tobacco, constitutively expressing PR proteins against *Glomus mossae* was reported by Vierheilig *et al.*(1995). In the seedlings of sunflower, chitinase and ß-1,3-glucanase were induced in response to infection by *Plasmpora halstedii* (Cachinero *et al.*, 1996). Lusso and Kuc (1996) studied the sense and antisense expression of the PR-N gene that codes for ß-1,3-glucanase in tobacco upon infection by *Phytophthora parasitica*.

2.4.7 Characterization of PR proteins

Henriquez and Sanger (1984) carried out partial characterization of PR p 14 protein from tomato infected by potato spindle tuber viroid. Eight pathogenesis related proteins were characterized, in tobacco leaves hypersensitive to tobacco mosaic virus (Jamet and Fritig, 1986). Keller *et al.* (1989) suggested glycine rich protein to be associated with lignin deposition. Later on,

Condit *et al.* (1990) succeeded in examining expression of the petunia glycine rich protein (pt GRP-1) using an antibody. Three basic PR proteins from tomato were characterized and were reported to be homologous to that of tobacco (Joostein *et al.*, 1990). Genes encoding basic and acidic chitinases in *Arabidopsis thaliana* were characterized by Samac *et al.* (1990). Antifungal thaumatin like protein PR-5c was characterized from tobacco by Koiwa *et al.* (1997). Similar antifungal PR-5c protein was characterized from pumpkin leaves by Cheong *et al.* (1997).

2.5 Enzymes involved in defence response

2.5.1 ß-1,3-Glucanase activity

Bartnicki-Garcia (1969) reported that ß-1,3-glucanase hydrolyse the ß-1,3-glucan component in cell wall of pathogenic fungi. Various plants (Abeles *et al.* 1970) as well as phytopathogenic fungi (Holten and Bartnicki-Garcia, 1972 and Bell, 1981) produce glucanase.

Robenantoandro *et al.* (1976) reported that increased endo ß-1,3-glucanase activity in seedling was involved in the infective mechanism of melon to the *Colletotrichum lagenarium.*

Activity of ß-1,3-glucanase in Fusarium wilt resistant and susceptible near isogenic lines of muskmelon has been reported by Netzer *et al.* (1979). The study revealed that the activity of ß-1,3-glucanase was higher and increased rapidly in the resistant line than in susceptible ones. Pretreatment of susceptible muskmelon with laminarin prior to inoculation increased the enzyme activity and markedly reduced the disease.

Matta *et al.* (1988) inoculated the roots of tomato with a non pathogenic form of *Fusarium oxysporum* fungi. Inoculation resulted in an increase of ß-1,3-glucanase and chitinase activity. Activity of these enzymes was correlated with variation in peroxidase and polyphenol oxidase activity, concentration of phenols and released ethylene. Inoculation with pathogenic fungi, *F. oxysporum* f. sp. *lycopersici* caused further increase of enzyme activity in previously stressed plants. Similarly, Pan *et al.* (1991) found higher activity of 1-3 glucanase in tobacco leaves inoculated with *Peronospora tabacina* and tobacco mosaic virus compared to control. Upon subsequent challenge with *Perenospora tabacina,* the enzyme activity continued to increase in the plants inoculated by both TMV and *P. tabacina.* However, one day after inoculation, enzyme activity increased in the uninoculated plant but was lower in already induced plants. The author reported higher activity of ß-1,3-glucanase in the control plant six days after challenge. Inoculation might be too late to prevent the development of lesion and sporulation of *P. tabacina.*

Rouhier *et al.* (1995) isolated ß-D glucan from cell wall of *Phytophthora capsici*. Structural analysis revealed that ß-D glucan had common ß-1,3-linkage. Constitutive expression of ß-1,3-glucanase cDNA coding for PR isoform in tobacco plant increased the foliar resistance to *Perenospora tabacina* and *Phytophthora parasitica* var. nicotiana.

2.5.2 Chitinase activity

Among the proteins induced during fungal attack, chitinase and ß-1,3-glucanase are the lytic enzymes that catalyze the hydrolysis of the chitin and ß-1,3-glucan components respectively of the fungal cell wall. Chitinase have been purified from a variety of sources including a number of crop plants (Molano *et al.*, 1979; Pegg and Young, 1982; Boller *et al.*, 1983; Leah *et al.*, 1987 and Fink *et al.*, 1988).

Many authors have reported increase in chitinase activity during pathogen attack. Boller (1985) reported high chitinase activity in higher plants during pathogen attack. Combined higher activities of chitinase and beta 1,3-glucanase in pea pod infected with *Fusarium solani* f.sp. *phaseoli* was reported by Mauch *et al.* (1988). Enhanced chitinase activity was closely related with the onset and level of induced systemic resistance in tobacco to blue mould caused by *Peronospora tabacinà* (Ye *et al.*, 1989). Chitinase activity was markedly increased in the induced plants both locally and systemically, 12 days after infection with tobacco mosaic virus (Ye *et al.*, 1990). In response to *Plasmopara halstedii* in sunflower, marked increase in chitinase activity was reported by Cachinero *et al.* (1996). Increase in activity even during chemical treatment was reported by Dann *et al.* (1996). Munch *et al.* (1997) reported increased activity in stem rust affected , elicitor treated isogenic wheat lines. Resistant lines of pea showed increased activity during powdery mildew infection when compared to susceptible ones (Rakshit *et al.*, 1999). Krishnaveni *et al.* (1999) reported similar increase in chitinase activity in resistant cultivar of sorghum in response to fungal infection.

Though a number of authors have reported an increasing trend of chitinase activity, Kunio *et al.* (1990) reported no potent chitinolytic activity in samples of 87 plant families.

2.5.3 Phenylalanine ammonia lyase activity (PAL)

Apart from producing proteins involved in limiting the effect of pathogen attack, plants respond to infection by producing phytoalexins and also by fortification of the cell wall by the deposition of lignin (Lamb *et al.*, 1989). Most of the phytoalexins are aromatic compounds such as furanocoumarins and isoflavonoids that are synthesized via the metabolic pathway for phenyl propanoid biosynthesis. The important enzymes in this pathway are phenylalanine ammonia lyase (PAL), cinnamate-4 – hydrolase (C4H) and 4 – coumaroyl-CoA ligase (4CL).

Chen and Chen (1993) reported increasing trend of PAL activity in the case of rice resulting in decrease in blast incidence. PAL activity in susceptible and resistant cultivars of capsicum in response to *Leveilula turica* was compared by Chander (1994). He reported a higher activity of PAL in the resistant cultivar. Clark *et al.* (1994) suggested that there is no strong correlation between PAL induction and major gene resistance though PAL induction was asociated with general defense response to *Erysiphe gramini* in barley. Velazhahan and Vimala (1994) suggested the correlation between the extent and timing of increased PAL activity and the biosynthesis of phytoalexins in sunflower during infection with *Puccinia helianthi* infection. PAL activity peak was high in the case of resistant cultivars than the susceptible ones when they were infected with the fungus

Bipolaris sorotriniam · (Peltonen and Karjalainen, 1995). Apart from microorganisms, chemicals like salicylic acid can enhance PAL activity (Xiao *et al.*, 1996). Kumar (1997) noticed increased PAL activity in resistant varieties of brinjal than the susceptible ones after inoculation with reniform nematode *Rotylenchus reniformis*. Dynamic changes in the activity of PAL in resistant and susceptible cultivars of soyabean was reported by Shen *et al.* (1999), upon infection with soyabean mosaic virus.

Materials and Methods

3. MATERIALS AND METHODS

The present study was undertaken as a part of M.Sc. (Hort.) programme of the Department of Plantation Crops and Spices during the period from Nov. 1998 to May 2000. The work was carried out at the Centre for Plant Biotechnology and Molecular Biology(CPBMB) and Biochemistry Laboratory of the College of Horticulture. The details of the work carried out are presented in this chapter.

3.1 Maintenance of plants

One hundred and fifty rooted cuttings, each of *Piper colubrinum* and two varieties of *Piper nigrum* namely Panniyur-1 and Kalluvally were maintained in the green house condition for the study. The source material for the preparation of cutting was obtained from the Pepper Research Station, Panniyur.

3.2 Maintenance of the pathogen

Pure culture of *Phytophthora capsici* supplied from CPBMB was maintained on carrot agar medium and used for the infection.

3.3 Preparation of carrot agar medium

Two hundred grams of carrot was boiled upto half cooked condition in 500 ml water and filtered with muslin cloth. Ten grams of agar was dissolved

in 500 ml distilled water. Both solutions were later mixed and made upto one litre with distilled water. The medium was poured into conical flask and autoclaved at 121°C for 15 minutes. The medium was cooled and stored in culture room for further use.

3.4 Preparation of fungal culture for inoculation

The carrot agar medium was allowed to melt in a microwave oven and poured in sterile petriplate (25 ml per 64 cm^3) on a sterile table top of laminar flow. The medium was allowed to solidify and the pure culture of the fungus was inoculated in the medium. The culture was incubated at 28°C and 80 per cent relative humidity.

3.5 Infection with fungus

Rooted cuttings of all the three varieties were infected with seven-day old culture of *P. capsici* with culture disc method after giving 12 pinpricks per 7 mm^2 on the lower surface of the leaf lamina. High relative humidity (>70 %) was maintained by covering with polythene bags. Every day the cuttings were sprayed with water in order to maintain the humidity upto seven days. Foliar symptoms developed were recorded at daily intervals.

3.6 Sample preparation

Infected leaves were detached from the plant at various interval (6^{th} hr to 6^{th} day after inoculation). They were washed thoroughly and wiped with

absorbent paper. Sample tissue was collected from within 1 cm area around the necrotic region.

3.7 Extraction of PR proteins (pH 2.8)

Leaf tissue (0.5 g) was homogenised in a mortar in the presence of sodium citrate phosphate buffer at pH 2.8. In order to avoid polyphenol interference, mercaptoethanol (100μl g^{-1} tissue) was incorporated while grinding (Gianinazzi *et al.*, 1980). The homogenate was centrifuged at 15000 rpm for 10 minutes at 4°C. The protein was estimated using spectrophotometer at 660 nm following Lowry method (Lowry *et al.*, 1951). The sample was kept at 4°C for immediate use in polyacrylamide gel electrophoresis (PAGE). A fraction of the sample was treated with sodium dodecyl sulfate buffer and stored at 4°C for later use in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE). Quality chemicals procured from Sisco Research Laboratory Pvt. Ltd., Mumbai; E. Merck (India) Ltd., Mumbai; HiMedia laboratories Limited, Mumbai and Sigma Chem Co., USA were used throughout study.

3.7.1 Composition of sodium citrate phosphate buffer

Sodium citrate	:	85 mM
NaH_2PO_4	:	32 mM
pH	:	2.8
Stored at	:	4°C

3.8 Protein quantification

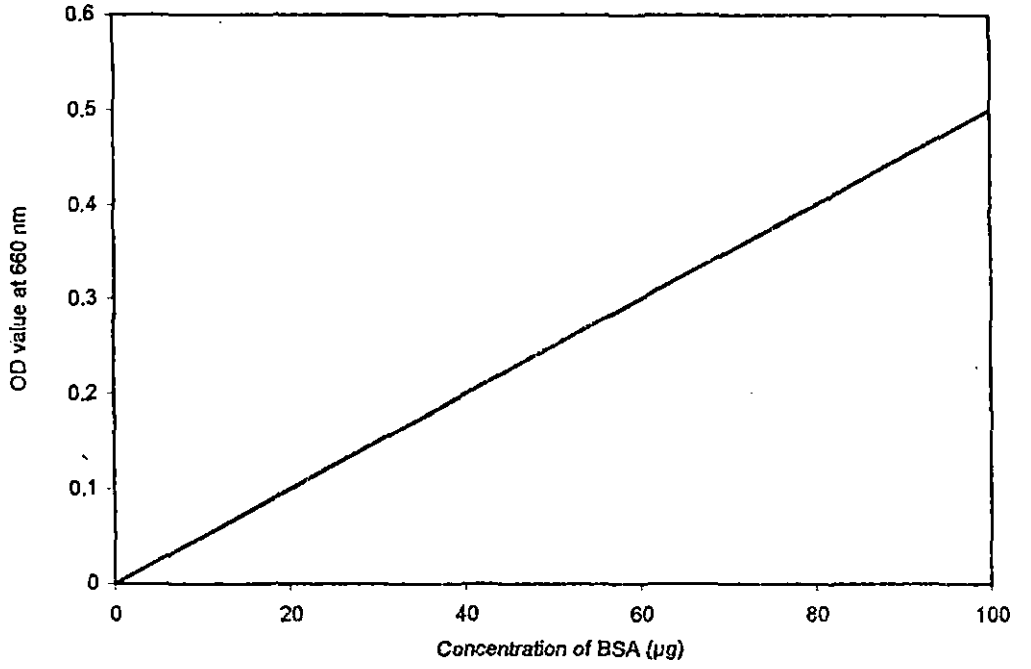
The method depends upon the reaction of phosphomolybdate in alkaline solution with tyrosine residues of protein. Copper ion is included to measure sensitivity of the reaction. This method is 200 to 1000 times more sensitive than biuret method.

3.8.1 Reagents for quantification of protein

Reagent A	:	2% Sodium carbonate in 0.1 N NaOH
Reagent B	:	5% Copper sulphate in 1% sodium tartarate
Reagent C	:	Mix 50 ml of reagent A with 1 ml reagent B
Reagent D	:	Commercial folin reagent diluted with water in a ratio of 1:1
Working standard	:	BSA 1 mg/ml

Standard curve was prepared using different concentrations of BSA (0 to 100 μg/ ml). The samples were diluted (1:100) and mixed with reagent C (1 ml) and incubated at room temperature for 10 min. It was then mixed with reagent D (100 μl) and incubated for 30 min at room temperature. The optical density was read at 660 nm. The graph was plotted with protein concentration on x-axis and OD at 660 nm on y-axis. The protein content in the unknown sample was determined by reading the OD at 660 nm and finding the corresponding value from the standard graph (Fig.1).

Fig. 1. Standard graph for protein quantification (Lowry, 1953)



3.9 Polyacrylamide gel electrophoresis (PAGE)

PAGE separate native proteins. Separation is based not only on protein size but also on protein charge and shape. This system is recommended only if the biological activity of a protein needs to be retained. In the present study both anionic and cationic systems were attempted to study the PR protein profile in black pepper.

Acrylamide is a monomer, which in presence of free radical and when stabilized by TEMED get polymerised into long chain. When the bifunctional reagent N, N, methylene bisacrylamide is included in the polymerisation reaction, the chain become cross linked to form a gel whose porosity is determined by the length of chain and degree of cross linking. Though they are difficult to prepare, it is often used to separate protein molecules due to their high resolution power.

3.9.1 Standardization of gel preparation

For native anionic system, the procedure reported by Laemmli (1970) was followed. Two different procedures described by Hames (1981) and Patel (1994) with modification were tried for the preparation of non denaturing polyacrylamide gel for cationic system.

3.9.1.1 The cationic system

3.9.1.1.1 Procedure No.I (Patel, 1994)

The stock solution suggested by the author used for the purpose.

A. Monomer solution (30% Acrylamide, 2.7% Bisacrylamide)

Composition

Acrylamide	:	30 g
Bis acrylamide	:	0.8 g
Distilled water to	:	100 ml

Solution was stored at 4°C in amber coloured bottle.

(Extreme care was taken while handling monomer solution to avoid spill over).

B. Resolving gel buffer (pH 4.3)

Composition

1 N KOH	:	24 ml
Acetic acid glacial	:	8.6 ml
TEMED	:	2 ml
Distilled water to	:	100 ml

C. Stacking gel buffer (pH 6.8)

Composition

1 N KOH	:	48 ml
Acetic acid glacial	:	2.87 ml
TEMED	:	0.46 ml
Distilled water to	:	100 ml

The above solutions were mixed and pH was adjusted either with acetic acid or KOH.

D. Catalyst components/100 ml distilled water

I. Ammonium per sulphate (APS 0.56%)

APS : 0.56 g

II. Riboflavin (0.004%) 4 mg

E. Sucrose solution (40%)

Sucrose : 40 g

F. Casting of gel unit

The Hoefer Mighty Small™ gel system of Hoefer Pharmacia Biotech California was used. The glass plates were cleaned with tissue paper soaked in 95 per cent ethanol. They were set apart by 0.75 mm thick spacer coated with sealing wax to avoid leak. The spacer and two glass plates were then assembled in the clamp. The clamp was tightened by aligning the glass plate spacer on the casting stand.

G. Separating gel

Aliquot of different solutions were mixed well and poured between the glass plates using the micropipette. The gel was allowed to solidify at different intervals as mentioned in different combinations so as to allow complete polymerization (Table 1).

H. Stacking gel

The required quantity of stock solutions for stacking gel was pipetted out, mixed well and poured above the separating gel between the glass plates and allowed to polymerise for specified time. Different combinations of separating and stacking gel tried are presented in Table 1.

Table 1. Details of different combinations tried for modifying PAGE analysis of basic proteins reported by Patel, 1994

Components	Separating gel				Stacking gel			
	I	II	III	IV	I	II	III	IV
1) Monomer solution*	2.49 ml (7.5%)	3.33 ml (10%)	2.49 ml (7.5%)	3.32 ml (10%)	1.34 ml (7.5%)	0.67 ml (7.5%)	0.67 ml (4%)	0.67 ml (4%)
2) Resolving gel buffer	4.98 ml	3.33 ml	1.24 ml	1.66 ml	-	-		-
3) Stacking gel buffer	-	-			0.67 ml	0.335 ml	0.335 ml	0.335 ml
4) Sucrose	-	-			2.68 ml	1.34 ml	1.34 ml	1.34 ml
5) Riboflavin	-	-			-	0.335 ml	0.335 ml	0.335 ml
6) APS	2.49 ml	3.33 ml	1.94 ml	1.66 ml	-	-	-	
7) Distilled water			5.04 ml	3.36 ml			2.32 ml	2.32 ml
Polymerisation Method	Chemical	Chemical	Chemical	Chemical	Chemical	Chemical	Chemical	Chemical
Time	1 hour	1 hour	1 hour	1 hour	20 minutes	20 minutes	20 minutes	20 minutes

* Value in paranthesis is the monomer concentration in final solution

3.9.1.1.2 **Procedure No. II.**

Method suggested by Hames, 1981 was adopted. Following stock solutions were prepared.

A. Monomer solution (30% Acrylamide, 2.7% Bisacrylamide)

As described in Section 3.9.1.1.1.A.

B. 8X Resolving gel buffer (pH 4.3)

Composition

1 N KOH	:	48 ml
Glacial acetic acid	:	17.2 ml
Distilled water to	:	100 ml

C. 8X Stacking gel buffer (pH 6.8)

Composition

1 N KOH	:	48 ml
Glacial acetic acid	:	2.9 ml
Distilled water to	:	100 ml

D. Riboflavin 5' phosphate (0.004%)

Composition

Riboflavin 5'phosphate	:	4 mg
Distilled water to	:	100 ml

E. Ammonium per sulphate (APS 10%)

Composition

Ammonium persulphate	:	0.1 g
Distilled water to	:	1 ml

F. Separating gel

Aliquot of different solutions were mixed well and poured between the glass plates using micropipette. The gel was kept 10 cm away from the light source for varying periods of time in different combinations so as to allow complete polymerisation (Table 2).

G. Stacking gel

The required quantity of stock solutions for stacking gel were pipetted out, mixed well and loaded over the separating gel between the glass plates and either photopolymerised or chemical polymerised for specified time as mentioned in the details of combinations presented in Table 2.

H. Sample preparation

Plant samples were prepared as described in 3.7. Each sample was then mixed with the treatment buffer in the ratio of 1:4. Electrophoresis was carried out immediately.

I. Electrophoresis

The gel unit was assembled as described earlier (3.9.1.1.1.F.). For stacking gel, the monomer concentration was kept constant to four per cent. Electrode buffer and treatment buffer were prepared as suggested by Hames (1981). Composition of various reagents used in electrophoresis unit is as follows:

IX Electrode buffer (pH 4.3)

Composition

Glacial acetic acid	:	8 ml
ß alanine	:	31.2 g
Distilled water to	:	1000 ml

Table 2. Details of different combinations tried for modifying PAGE analysis of basic proteins reported by Hames, 1981

Components	Separating gel											Stacking gel	
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	I	II
1) Monomer solution	2.49 ml (7.5%)	2.49 ml (7.5%)	2.49 ml (7.5%)	2.49 ml (7.5%)	2.49 ml (7.5%)	2.49 ml (7.5%)	2.49 ml (7.5%)	2.49 ml (7.5%)	2.49 ml (7.5%)	2.49 ml (7.5%)	3.33 ml (10%	0.67 ml (4%)	0.67 ml (4%)
2) Resolving gel buffer	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml	-	-
3) Stacking gel buffer	-	-	-	-	-	-	-	-	-	-	-	1.25 ml	1.25 ml
4) Riboflavin 5' phosphate	1.25 ml	1.25 ml	1.25 ml	1.25 ml	1.25 ml	1.25 ml	1.25 ml	1.25 ml	1.25 ml	1.25 ml	1.25 ml	0.67 ml	-
5) TEMED	10 µl	-	-	40 µl	40 µl	30 µll	20 µl	30 µl	30 µl	30 µl	30 µl	10 µl	5 µl
6) APS	-	-	-	-	-	-	-	-	25 µl	15 µl	25 µl	-	50 µl
7) Distilled water	3.75 ml	3.75 ml	3.76 ml	3.72 ml	3.72 ml	3.73 ml	3.74 ml	3.73 ml	3.70 ml	3.71 ml	2.91 ml	2.43 ml	3.0 ml
8) Polymerisation 1) Method	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	Chemical
2) Source	40W tube light	40W tube light (2)	40W tube light (3)	40W tube light	15W FL	15W FL	15W FL	15W FL	15W FL	15W FL	15W FL	40W Tube light	-
3) Time	One hour	Two hour	Four hour	One hour	One hour	One hour	Three hour	Three hour	Three hour	Three hour	Three hour	Thirty min.	Twenty min.

PC - Photochemical
FL - Flouorescent day light lamp
W - Watt

2X Treatment buffer

Composition

8X acetic acid-KOH(pH 6.8):		2.0 ml
Glycerol	:	1.6 ml
0.4% Methylene blue	:	0.4 ml
Distilled water to	:	4.0 ml

The upper and lower tanks were filled with pre-chilled electrode buffer and samples prepared were loaded in the well. Upper tank was connected to anode and lower to cathode of the power pack. A constant current of 10 mA per plate was maintained throughout the run till the tracking dye reaches the anode.

3.9.2 Staining

Coomassie blue staining procedure (Ausubel *et al.*, 1995) was followed for staining the gel. Staining and destaining solutions were prepared as follows:

A. **Coomassie solution**

Composition

Methanol	:	50% (v/v)
Coomassie brilliant blue	:	0.05% (v/v)
Acetic acid	:	10% (v/v)
Distilled water	:	10%

Dissolved Coomassie brilliant blue R-250 in methanol before adding acetic acid and water, diluted to required volume. The solution was stored at 4°C.

B. Destaining solution

Composition

Acetic acid	:	7 ml
Methanol	:	5 ml
Distilled water	:	88 ml

The solution was prepared fresh.

C. Staining of gel

After the electrophoresis, glass plates were taken out and the gel carefully transferred to staining solution (100 ml) and allowed to stain for six hours with gentle shaking. It was then destained until clear background was obtained. The gel was viewed in a transilluminator and documented immediately using the Alpha Innotech Image Analysis System.

3.10 SDS Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS gel electrophoresis allows the separation of proteins in denatured condition. Migration of polypeptides is based on the molecular weight. Sodium dodecyl sulfate is an anionic detergent that denatures protein by wrapping around the polypeptide backbone. In doing so, SDS confers a negative charge to the polypeptide in proportion to its length. As a result, polypeptides become rod of negative charge with equal densities or charge per unit length. The samples were electrophoresied in a vertical electrophoretic unit (Hoefer Mighty Small) according to the procedure described by Laemmli (1970).

3.10.1 Preparation of reagents

The reagents used for sodium dodecyl sulfate polyacrylamide gel electrophoresis include monomer solution, resolving gel buffer, stacking gel buffer, electrode buffer, treatement buffer, initiator and SDS solution. The procedure followed for preparation of various reagents are as follows:

A. Monomer solution (30% Acrylamide, 2.7% Bisacrylamide)

As described in Section 3.9.1.1.1.A.

B. 4X Resolving gel buffer (1.5 M Tris-Cl, pH 8.8)

Composition

Tris base	:	18.5 g
Distilled water to	:	100 ml

Dissolved the tris base in about 50 to 60 ml distilled water, the pH was adjusted to 8.8 with 1 N HCl and stored at 4°C.

C. 4X Stacking gel buffer (0.5 M Tris-Cl, pH 6.8)

Composition

Tris base	:	6 g
Distilled water to	:	100 ml

Dissolved tris base in about 50 to 60 ml distilled water, the pH was adjusted to 6.8 with 1 N HCl and stored at 4°C.

D. Electrode buffer (0.025 M Tris, pH 8.3, 0.192 M Glycine)

Composition

Tris base	:	1.525 g
Glycine	:	7.2 g
SDS	:	0.5 g
Distilled water to	:	500 ml

The solution was stored at room temperature.

E. 2X Treatment buffer (0.125 M Tris-Cl)

Composition

4X Tris-Cl, pH 6.8	:	2.5 ml
Glycerol	:	2.0 ml
2-Mercaptoethanol	:	0.2 ml
Bromophenol blue	:	0.2 g
SDS (10%)	:	4.0 ml
Distilled water to	:	10 ml

F. Initiator (10% APS)

Composition

Ammonium persulphate	:	0.1 g
Distilled water	:	1 ml

This solution was prepared fresh, immediately before use.

G. SDS (10%)

Composition

Sodium dodecyl sulfate	:	10 g
Distilled water to	:	100 ml

H. Casting of the gel units

The gel unit was assembled as described in section 3.9.1.1.1.F.

I. Preparation of the sample

The sample prepared as described in 3.7 and mixed with the treatment buffer in the ratio 1:1 and was heated at 100°C for two minutes for denaturation of the protein. The sample was stored at 4°C.

J. Preparation of gel

1. Resolving gel

Different combinations of resolving gel were tried out to select the optimum combination. The composition include.

Components	7.5%	10%	15%
Monomer	2.49 ml	3.33 ml	4.99 ml
4 x Resolving gel buffer	2.5 ml	2.5 ml	2.5 ml
SDS 10%	0.1 ml	0.1 ml	0.1 ml
Distilled water	4.85 ml	4 ml	2.36 ml
APS 10%	50μl	50μl	50μl
TEMED	5μl	5μl	5μl
Total	10 ml	10 ml	10 ml

2. Stacking gel

Composition

Monomer	:	0.67 ml
4 x Stacking gel buffer	:	1.25 ml
SDS 10%	:	0.05 ml

Distilled water	:	3 ml
APS 10%	:	25 μl
TEMED	:	30 μl

Various stock solutions of resolving gel were mixed serially in a conical flask. The mixture was then poured between glass plates with the help of micro pipette upto desired height (leaving 2 cm at the top). It was then overlayed with distilled water to allow polymerization to take place for 30 minutes. After complete polymerization of resolving gel, distilled water was drained and stacking gel solution was carefully poured. The comb was placed properly and allowed to polymerize for 15 minutes.

After allowing complete polymerization (15 min.), the comb was removed and the gel slab was carefully taken out from the casting unit, washed with the electrode buffer and then transferred to electrophoretic apparatus. The upper and lower tanks were filled with pre-chilled electrode buffer. The sample (50μg) prepared as described earlier (3.10.1.I.) was loaded in the well. Protein molecular weight marker (Cat-PMWL GENEI) of 3000 to 43000 kD range was loaded as standard. The upper tank buffer was connected to cathode and lower to anode of the power pack (Hoefer Scientific Instruments, San Francisco). A constant current of 20 mA per plate was maintained throughout the run till the tracking dye reaches the anode.

3.10.2 Fixing the gel

After the run, the gel was separated carefully and transferred to fixing solution (300 ml) and gently shaken at 40 rpm in a slow speed shaker. The composition of the fixing solution was as follow:

A. Fixing solution (30% ethanol 10% acetic acid)

Composition

Ethanol	:	270 ml
Glacial acetic acid	:	90 ml
Distilled water to	:	900 ml

The gel was retained in the fixing solution initially for 10 minutes after which it was replaced with fresh fixing solution. Three such changes were allowed for fixing the polypeptides. The gel was taken out and washed free of fixative by gently shaking it in distilled water allowing three changes at intervals of 10 minutes each. The gel was then transferred to staining solution.

3.10.3 Staining

Silver staining procedure was followed for staining the gel. Silver stain method depends on the reduction of ions to metallic silver to provide metallic silver images. Selective reduction of silver ions to metallic silver at gel sites occupied by protein depends on difference in the oxidation reduction potentials in the sites occupied by the protein in comparison with adjacent sites in the gel which do not contain proteins.

3.10.3.1 Preparation of staining solutions

Silver staining kit supplied by Sigma Chemical Company was used for preparing the stain. Different solutions were prepared as per the direction given in the kit. The details are as follows:

A. Silver equilibrating solution

Diluted 1.5 ml silver nitrate concentrate to 300 ml with distilled water, mixed well and stored in dark coloured bottle at room temperature.

B. Developer solution

The developer solution was prepared fresh at the time of experiment. Diluted 30 ml of developer No. I to 300 ml with distilled water. To this solution added 0.17 ml developer No. II reagent and mixed gently.

C. Stop solution

Diluted 3 ml of glacial acetic acid to 300 ml with distilled water and stored at room temperature.

D. Reducer solution

Mixed together 2 ml of reducer solution A with 4 ml reducer solution B and 0.7 ml reducer solution C, supplied by Sigma Chemical Co.,USA. The mixture was then diluted to 300 ml with distilled water.

3.10.3.2 Staining the gel

The fixed gel was transferred to silver equilibrating solution taken in a plastic acrylic tray sufficient enough to completely immerse the gel. It was then kept at room temperature for 30 minutes with gentle shaking. Later on the gel was rinsed with distilled water for 10 to 20 seconds and then placed in the developer solution (300 ml). Gentle shaking was continued for 5 to 8 minutes till protein bands were properly developed. The gel was then immediately transferred to the stop solution (300 ml) and allowed to stay for five minutes. The excess stain was

removed by placing the gel in reducer solution for 10 to 30 seconds. The gel was then washed in running tap water and later rinsed with distilled water. The protein profile was viewed in transilluminator and documented using the Alpha Imager™ 1200 (Alpha Innotech Coroporation, USA).

3.11 Partial purification of protein

3.11.1 Preparation of saturated ammonium sulphate solution

Crystal ammonium sulphate was added slowly to about 100 ml water while shaking to its maximum solubility. Heated the solution and added more ammonium sulphate till the crystal become insoluble. Cooled the solution to room temperature, removed the excess crystals and neutralized with the ammonia solution.

3.11.2 Precipitation of protein using $(NH_4)_2SO_4$

The procedure suggested by Chaykin (1966) was followed for the purpose. Ammonium sulphate solution was added drop by drop to the protein solution at 4°C so as to get a 20 per cent saturation. The mixture was allowed to stand at 4°C for two hours and later on centrifuged at 20000 rpm for 15 minutes. The precipitate was saved and dissolved in extraction buffer.

The remaining protein solution having 20 per cent $(NH_4)_2SO_4$ was further increased to 40 percent $(NH_4)_2SO_4$. Salted out protein was separated after centrifugation. The supernatant was further saturated to 60 and 80 per cent with $(NH_4)_2SO_4$ and proteins were salted out.

3.11.3 Removal of $(NH_4)_2SO_4$ and protein assay

In order to avoid interference of $(NH_4)_2SO_4$, the proteins were dialysed. Dialysis tubing supplied by Sigma Chemical Co., USA was used for the purpose. The sample was loaded in the tube, both end tied and immersed in distilled water for two hours. In order to concentrate the protein after dialysis, the tube along with sample was immersed in sucrose for imposing osmotic gradient. The protein separated in each fraction was subjected to SDS- PAGE analysis.

3.12 Pathogenesis related enzymes

3.12.1 Estimation of Beta-1,3-glucanase activity

Beta-1,3-glucanase enzyme activity was estimated as per the method suggested by Pan *et al.* (1991) with slight modification.

3.12.1.1 Preparation of stock solutions

A. Sodium acetate buffer (0.05 M, pH 5)

Composition

Sodium acetate	:	0.68 g
Distilled water to	:	100 ml

Dissolved the required amount in 50 to 70 ml of water and adjusted the pH with 0.1 N HCl.

B. Laminarin (4%)

Composition

Laminarin	:	4 g
Distilled water to	:	100 ml

C. *Sodium hydroxide (4.5%)*

Composition

Sodium hydroxide	:	4.5 g
Distilled water to	:	100 ml

D. **Dinitrosalicylic acid (DNS)**

Composition

Dinitrosalicylic acid	:	8.8 g
Na^+K^+ tartarate	:	2.55 g
Sodium hydroxide	:	300 ml
Distilled water	:	800 ml

Dissolved required DNS and Na^+K^+ tartarate in 800 ml of water and later on added 300 ml of 4.5% sodium hydroxide.

E. **Cystein HCl (0.05 M)**

Composition

Cystein HCl	:	0.394 g
Distilled water to	:	50 ml

F. **Phenyl methane sulphonyl flouride (PMSF 0.1 M)**

Composition

PMSF	:	20 mg
Isopropanol	:	1 ml

This solution was prepared fresh before use.

G. Ascorbic acid (5 mM)

Composition

Ascorbic acid	:	0.05 g
Distilled water to	:	100 ml

3.12.1.2 Preparation of crude enzyme extract

Recently matured physiologically active leaves were collected over ice for enzyme assay. The tissue (500 mg) around the necrotic patch was separated and macerated with 1 ml of sodium acetate buffer, 50 μl each of ascorbic acid, PMSF and mercaptoethanol and 100μl of cystein HCl in a chilled mortar and pestle. The homogenate was centrifuged at 15000 rpm for ten minutes in refrigerated centrifuge at 4°C. The supernatant was used as enzyme source.

3.12.1.3. Assay for Beta-1,3-glucanase

The enzyme extracted (62.5 μl) was mixed with an equal quantity of laminarin and incubated at 40°C for ten minutes. The reaction was stopped by adding 375 μl of DNS reagent and heated for five minutes in a boiling water bath. The coloured solution was diluted with 4.5 ml of water and the absorbance was measured at 500 nm using a spectrophotometer.

One unit of enzyme activity is defined as the quantity of reducing sugar produced at 40°C for 10 minutes.

3.12.2 Estimation of chitinase activity

Chitinase enzyme activity was assayed according to the method described by Jeuniaux (1966) with some modifications. The enzyme activity was the measure of acetylglucosamine (AG) released from chitin in the assay system.

3.12.2.1 Preparation of stock solutions

A. Chitin suspension

A stock of chitin was prepared to act as the substrate. Two grams of pure chitin was dissolved in 64 ml of prechilled concentrated H_2SO_4 with constant stirring at 4°C. It was allowed to stand at 4°C for one hour with occasional stirring. This viscous acid solution was transferred to 90 ml prechilled distilled water with continuous stirring and kept over night at 4°C. The precipitate formed was centrifuged and washed with distilled water, until pH reaches to 5. Chitin suspension was brought to 5 mg/ml with distilled water for the assay.

B. Citrate-phosphate buffer (pH 5.1)

Composition

Citric acid (0.6 M)	:	11.5 g
Na_2HPO_4 (1.2 M)	:	21.3 g
Distilled water to	:	100 ml

Required quantities of above reagents were dissolved in 50 to 60 ml of distilled water, adjusted the pH with 0.1 N HCl and the solution made upto 100 ml.

C. P-Dimethylamino benzaldehyde (DMAB)

Dissolved one gram of DMAB in 100 ml of glacial acetic acid containing 1.25% (v/v) 10 N HCl. This solution was prepared fresh before use.

D. Potassium tetraborate ($K_2B_4O_7$) 0.8 M

Composition

Potassium tetra borate	:	24.4 g
Distilled water	:	100 ml

Dissolved required quantity of potassium tetraborate in 100 ml of distilled water.

E. Cystein HCl (0.05 M)

(As described in section 3.12.1.1.E)

F. Phenyl methane sulphonyl flouride

(As described in Section 3.12.1.1.F)

G. Ascorbic acid

(As described in Section 3.12.1.1.G)

3.12.2.2 Preparation of crude enzyme extract

Recently matured physiologically active leaves were used for enzyme assay. One gram of leaf tissue was macerated with 2 ml of buffer, 50 μl each of ascorbic acid, PMSF and mercaptoethanol and 100 μl of cystein HCl in chilled mortar using pestle. This homogenate was centrifuged at 15000 rpm for 15 minutes at 4°C. The supernatant collected was used as enzyme source.

3.12.2.3 Assay for chitinase activity

Procedure suggested by Jeuniaux (1966) was followed with modification (Table 3) for detecting chitinase activity.

Procedure

Mixed 1 ml of chitin suspension (5 mg/ml) with 1 ml buffer and 0.5 ml of crude enzyme extract and made upto 4 ml with distilled water. This mixture was incubated for 90 minutes at 37°C. Pipetted out 1ml of this extract into centrifuge tube containing 1 ml distilled water, boiled for 10 minutes and centrifuged at 1000 rpm. Took 0.5 ml of supernatent in the tube containing 0.1 ml potassium tetraborate and boiled for 3 minutes in boiling water bath. After cooling, 3 ml DMAB solution was added, mixed and allowed to stand for 20 minutes at 37°C. The sample was cooled and the optical density read within 10 minutes at 585 nm.

3.12.3 Estimation of phenylalanine ammonia lyase (PAL) enzyme activity

Phenylalanine ammonialyase enzyme activity was assayed by the method suggested by Sadasivam and Manikam (1992) with slight modification. PAL activity was determined spectrophotometrically by following the formation of trans-cinnamic acid which exhibit increase in absorbance at 290 nm (crude enzyme)/270 nm (purified enzyme).

Table 3. Details of different combinations tried for asssessing chitinase activity at different levels of substrate and incubation

Components	*Combinations*							
	1	2	3	4	5	6	7	8
Leaf sample (g)	1	1	1	1	1	4	4	4
Extraction buffer (ml)	2	2	2	2	2	2	2	2
Crude extract (ml)	2	2	2	2	1.5	2	2	2
Assay buffer (ml)	1	1	1	1	1	1	1	1
Chitin (mg/ml)	5	5	30	30	30	30	30	30
Incubation time (*min*) at 37°C	90	180	90	180	180	90	180	180

3.12.3.1 Preparation of stock solutions

A. Borate buffer (0.2 M, pH 8.7)

Composition

Boric acid	:	1.2 g
Distilled water to	:	100 ml

Boric acid was dissolved in 50 to 70 ml water, adjusted pH with 0.2 M sodium hydroxide.

B. L-Phenylalanine (0.1M)

Composition

L-Phenylalanine	:	1.65 g
Distilled water to	:	100 ml

Dissolve L-Phenylalanine in 50 to 60 ml of water and adjusted the pH with 0.1N KOH.

C. Trichloroacetic acid solution (1M)

Composition

Trichloroacetic acid	16.3 g
Distilled water to	100 ml

D. Cystein HCl (0.05 M)

As described in Section 3.12.1.1.E.

E. Phenyl methane sulphonyl flouride (PMSF 0.1 M)

As described in Section 3.12.1.1.F.

3.12.3.2 Preparation of enzyme extract

Recently matured, physiologically active leaves were collected over ice for enzyme assay. The tissue (500 mg) was macerated with 5 ml of borate buffer, 25 μl mercaptoethanol, 50 μl PMSF, 50 μl cystein HCl and 50 μl ascorbic acid in chilled mortar using pestle. The homogenate centrifuged at 12000 rpm for 20 minutes at 4°C. Supernatant was saved for the enzyme assay.

3.12.3.3 Assay for PAL activity

Initiated the reaction by the addition of one ml of L-phenylalanine solution to the mixture containing 0.5 ml borate buffer, 0.2 ml of enzyme solution and 1.3 ml of distilled water. Incubation was carried out for one hour at 32°C. Reaction was stopped by the addition of 0.5 ml of trichloroacetic acid in the reaction system. Control was run by adding phenylalanine solution after trichloroacetic acid and measured the absorbance at 290 nm.

Enzyme activity was expressed as micromole trans-cinnamic acid formed per mg protein/minute.

Results

4. RESULTS

The results generated during evaluation of pathogenesis related (PR) proteins in black pepper in relation to infection with *Phytophthora capsici* are presented in this chapter.

4.1 Induction of disease symptoms

Symptoms were induced on healthy mature leaves of black pepper with pure culture of fungus, *Phytophthora capsici* maintained on carrot agar medium. The procedure followed for maintaining the fungus was found ideal and growth of fungal mycelia when incubated at 28°C on carrot agar medium is shown in Plate 1. Seven-day-old culture was used for infecting healthy plants. The foliar symptoms were observed in *Piper nigrum* varieties from second day onwards while *P. colubrinum* did not express any symptom (Plate 2). The response of *Piper* species and varieties studied was found to vary considerably when artificially inoculated with *Phytophthora capsici*. The results are presented in Table 4 and Fig. 2.

Among the varieties studied, Panniyur-1 took infection earlier and showed initial foliar symptoms within 24 hours after infection while Kalluvally took 48 hours for expressing initial symptoms. The infected area was more in the case of Panniyur-1 indicating susceptibility to disease. In this variety, the necrotic spots on leaf lamina were very distinct and spread faster than in variety Kalluvally. The

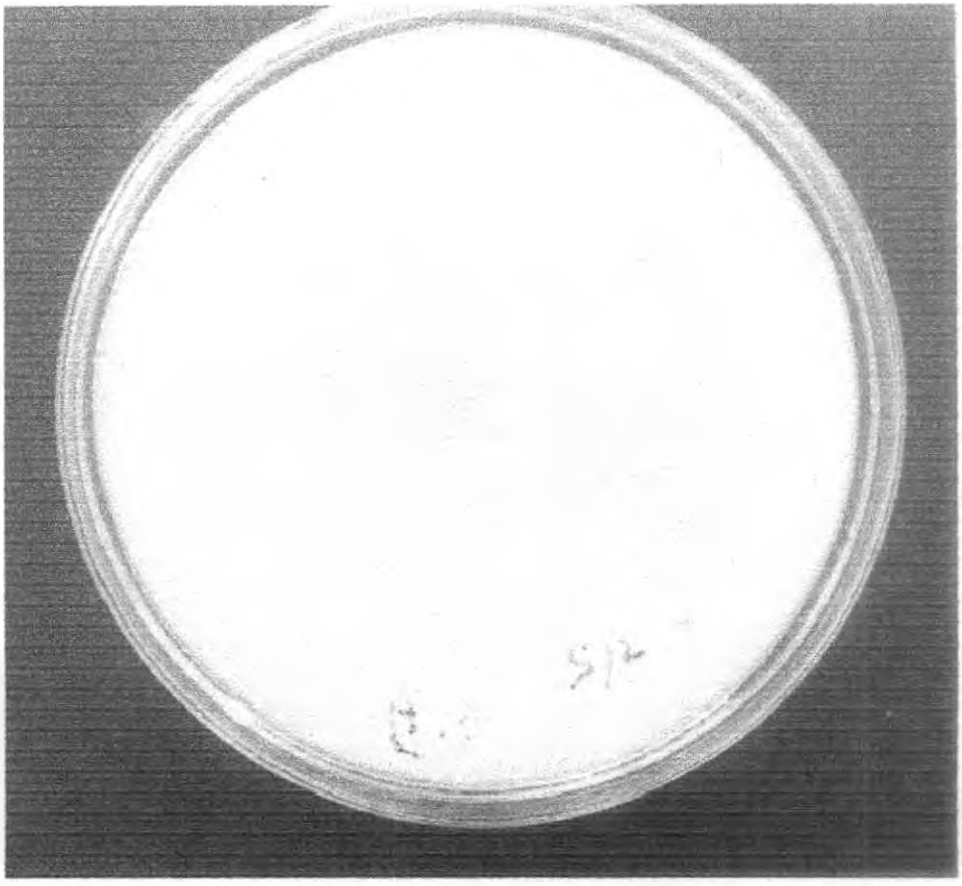


Plate 1. Seven day old culture of *Phytophthora capsici* with luxuriant growth of fungal mycelia on carrot agar medium

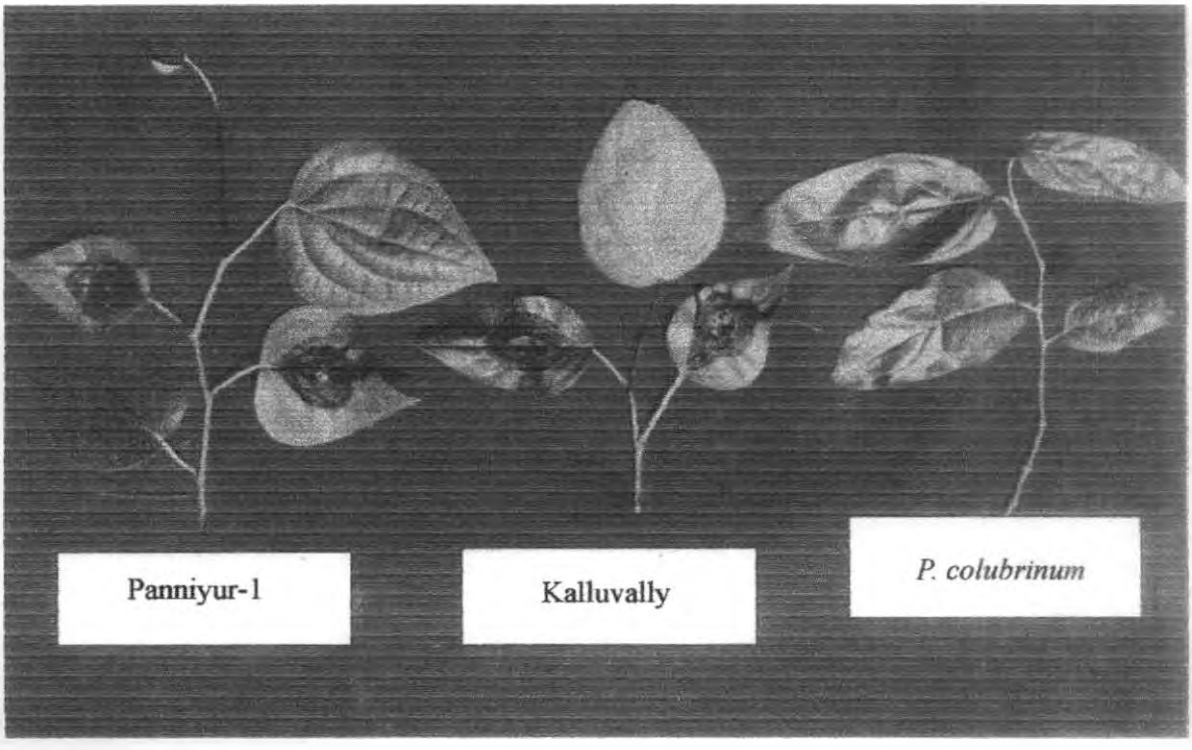


Plate 2. Response of *Piper* species upon infection with *Phytophthora capsici* (foliar symptom developed on *Piper nigrum* varieties, Panniyur-1 and Kalluvally while *Piper colubrinum* did not respond)

Table 4. Response of *Piper nigrum* varieties (Panniyur-1 and Kalluvally) and *P. colubrinum* to infection with *P. capsici*

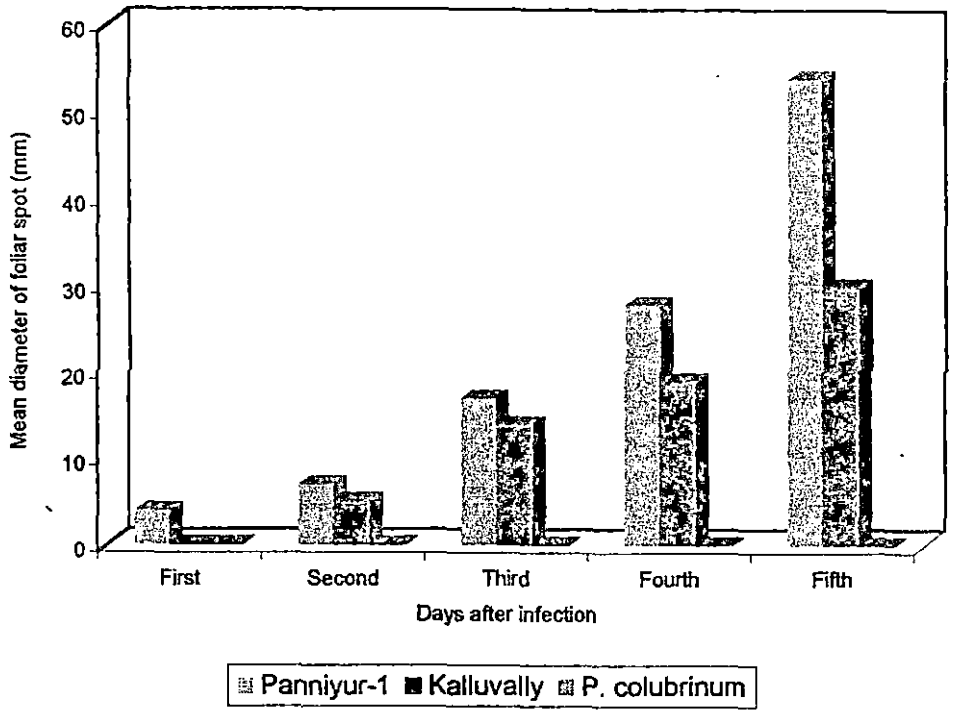
Days after inoculation	Panniyur-1		Kalluvally		*P. colubrinum*	
	Intensity of infection	Necrotic area dia/spot (mm)	Intensity of infection	Necrotic area dia/spot (mm)	Intensity of infection	Necrotic area dia/spot (mm)
First	Poor	4 (±1.0)	Nil	Nil	Nil	Nil
Second	Medium	7 (±1.5)	Poor	5 (±2.0)	Nil	Nil
Third	Fair	17 (±2.5)	Medium	14 (±1.5)	Nil	Nil
Fourth	Severe	28 (±3.0)	Fair	19 (±3.0)	Nil	Nil
Fifth	Severe	54 (±5.0)	Severe	30 (±3.5)	Nil	Nil

Poor - <5 mm Fair - 16 to 25 mm

Medium - 6 to 15 mm Severe - >26 mm

(±) are the SD values

Fig. 2. Response of *P. nigrum* varieties (Panniyur-1 & Kalluvally) and *P. colubrinum* to infection with *P. capsici*



mean diameter of the necrotic spot was 54 mm by fifth day while it was only 30 mm for Kalluvally. The infection was so severe in Panniyur-1 that leaves were detached from the stem within seven days after infection under artificial condition.

4.2 Isolation and quantification of proteins extracted at pH 2.8

The method followed for isolation of PR proteins in black pepper was found satisfactory. The protein extracted at pH 2.8 in relation to infection with *P. capsici* was quantified and presented in Table 5.

The protein content was found to be high in *P. nigrum* samples compared to *P. colubrinum*. The values ranged between 16.8 and 22.0 mg/ ml of extract in the *P. nigrum* varieties studied, while it ranged between 12.9 and 14.0 mg/ ml of extract in *P. colubrinum*. In the variety Panniyur-1, the protein level was relatively more than that estimated in the variety Kalluvally under healthly condition. Slight variation observed for healthy samples analyzed on different days.

When pepper plants were infected with live culture of *Phytophthora capsici*, the response with respect to basic protein level was different for *P. nigrum* and *P. colubrinum*. In variety Panniyur-1, the protein level was found reduced upon the expression of foliar symptoms by the fifth day after infection. The healthy region in the leaf lamina showed a reduction in protein content to the extent of 20 per cent by the fifth day after infection. The same trend was observed also in the case of variety Kalluvally. However, the extent of depletion of protein was

Table 5. Foliar protein (basic) content (mg/ml of extract) in *Piper nigrum* varieties (Panniyur-1 and Kalluvally) and *P. colubrinum* as influenced by infection with *Phytophthora capsici*

	Days after infection					
	0 day	First	Second	Third	Fourth	Fifth
I. *P. nigrum*						
Panniyur-1						
Healthy	20.4 (±0.15)	19.6 (±0.09)	22.0 (±0.17)	18.0 (±0.17)	20.8 (±0.17)	18.7 (±0.11)
Infected	20.4 (±0.15)	19.2 (±0.30)	18.1 (±0.25)	17.7 (±0.10)	16.0 (±0.20)	14.8 (±0.15)
Kalluvally						
Healthy	17.1 (±0.20)	17.2 (±0.20)	17.5 (±0.21)	17.2 (±0.19)	16.8 (±0.20)	17.7 (±0.21)
Infected	17.1 (±0.20)	16.6 (±0.17)	15.8 (±0.22)	15.9 (±0.14)	15.2 (±0.19)	15.0 (±0.15)
II. *P. colubrinum*						
Healthy	13.5 (±0.09)	13.4 (±0.26)	13.2 (±0.15)	14.0 (±0.19)	13.0 (±0.19)	12.9 (±0.15)
Infected	13.5 (±0.09)	13.4 (±0.09)	13.5 (±0.09)	13.2 (±0.20)	13.4 (±0.25)	13.2 (±0.25)
CD(0.05) for comparing						
1. Genotypes	2.5	2.5	3.5	2.4	2.6	1.6
2. Healthy/ infected	2.0	2.1	2.8	1.9	2.1	1.3

(±) are the SD values

less severe in this variety and a reduction to the extent of 15 per cent was observed. In *Piper colubrinum,* no significant difference was observed for the protein level when inoculated with *Phytophthora capsici*.

The protein level in *P. colubrinum* and *P. nigrum* as influenced by infection with *P. capsici* is depicted in Fig. 3.

4.3 PAGE analysis of PR proteins

4.3.1 Standardisation of the procedure

The procedure followed for the detection of anionic proteins in the plant extract gave good polymerization. However, no anionic protein was found separated in the system. Results of different combinations tried for the separation of basic proteins and the banding pattern observed are presented in Table 6.

The combination number IX was found ideal for the PAGE analysis of basic proteins in black pepper. The polymerization was poor in the other methods tried. Photochemical polymerisation in the presence of riboflavin 5' phosphate gave the best gelling and separation. Details of the best combination identified for PAGE analysis is as follows:

Monomer solution (7.5%)	2.49 ml
Resolving gel buffer	2.5 ml
Riboflavin 5' phosphate	1.25 ml
TEMED	30μl

Fig. 3. Comparison of foliar protein (PR) content in *P. nigrum* varieties (Panniyur-1 & Kalluvally) and *P. colubrinum* in relation to infection with *P. capsici*

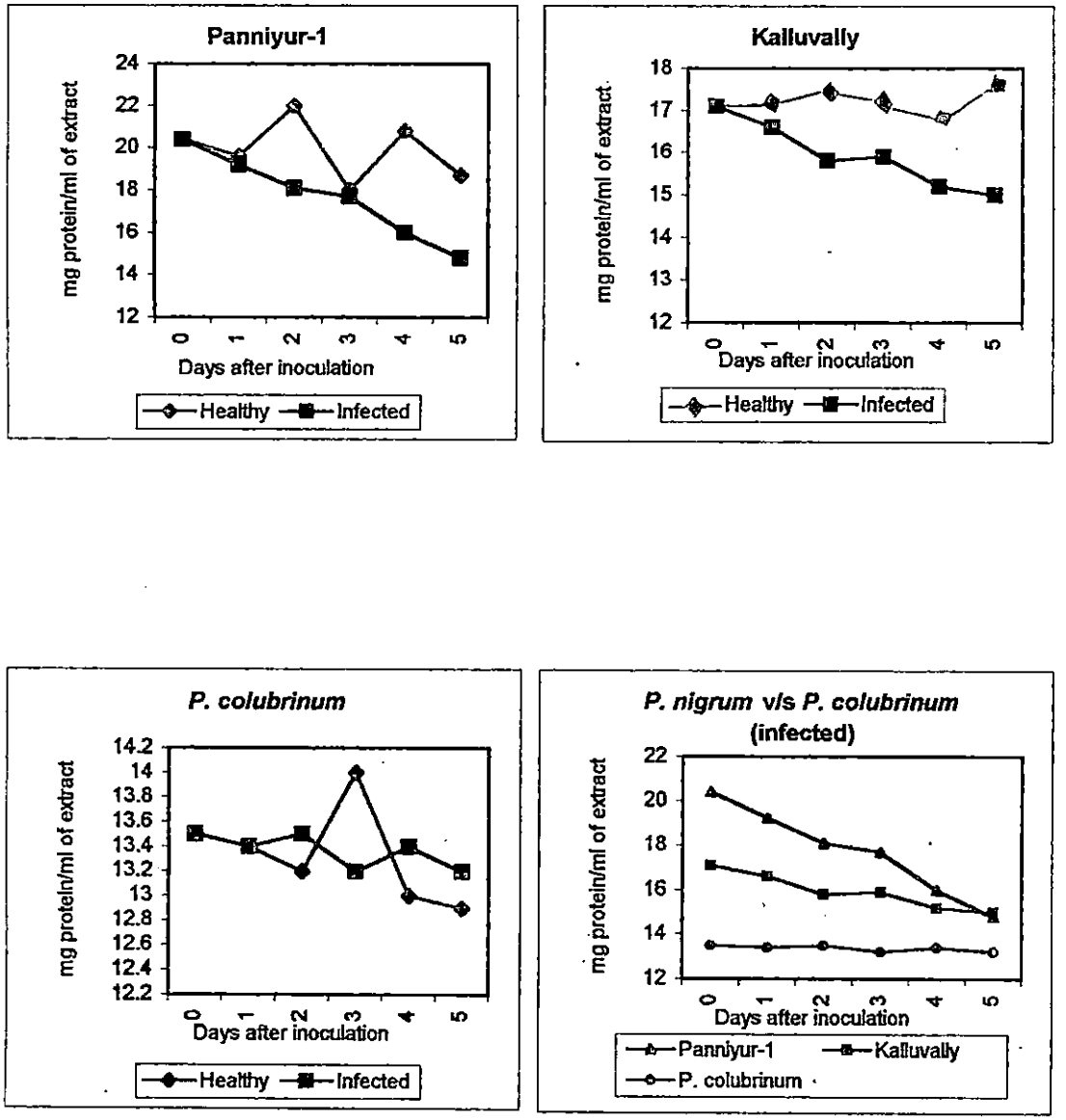


Table 6. Effect of different combinations tried for PAGE analysis of basic protein

Sl.No.	Treatment No.	Level of polymerisation	Separation of protein
1	I	Very poor	No
2	II	Poor	No
3	III	Poor	No
4	IV	Poor	No
5	V	Poor	No
6	VI	Poor	No
7	VII	Good	No
8	VIII	Good	No
9	IX	Good	Good
10	X	Good	No
11	XI	Good	No

APS	25μl
Distilled water	3.75 ml

Photopolymerisation was allowed by keeping 10 cm away from 15 W fluorescent day light lamp for three hours.

4.3.2 Protein profile in healthy and infected plants

The polyacrylamide gel electrophoresis carried out for the cationic proteins extracted at pH 2.8 showed distinct banding pattern for *P. nigrum* and *P. colubrinum*. No separation was observed in the anionic system attempted.

4.3.2.1 Cationic protein profile for *P. nigrum*

The zymogram prepared based on Rm value for basic proteins expressed as distinct bands upto fifth day after infection is shown in Figs. 4 to 8. The *P. nigrum* varieties (Panniyur-1 and Kalluvally) expressed six protein bands out of which four were shared by both the varieties. The variety Panniyur-1 was distinct from Kalluvally with the expression of a protein with Rm value 0.43, while variety Kalluvally showed expression of a specific protein with Rm value 0.40.

Expression of basic protein as observed in the polyacrylamide gel electrophoresis on the sixth day after infection is shown in Plate 3 and Fig.9. The banding pattern observed for the infected plants of Panniyur-1 was different from that of healthy one with respect to a unique band of Rm value 0.56. The variety Kalluvally also behaved in similar pattern. An additional band with Rm value 0.56 was found expressed in this variety also by the sixth day after infection.

Fig.4. Zymogram showing PAGE (cationic) banding pattern in *P. nigrum* varieties (Panniyur-1 and Kalluvally) and *P. colubrinum* on the first day after infection with *Phytophthora capsici*

Band No.	Rm. Value	HP	IP	HK	IK	HC	IC
1	0.08					–	–
2	0.10	–	–	–	–		
3	0.20	–	–	–	–		
4	0.24					–	–
5	0.30	–	–	–	–		
6	0.35					–	–
7	0.40			–	–		
8	0.43	–	–				
9	0.50	–	–	–	–	–	–
10	0.56					–	–

HP Healthy **Panniyur-1** HK Healthy Kalluvally

IP Infected **Panniyur-1** IK Infected Kalluvally

HC Healthy *P.colubrinum*

IC Infected *P.colubrinum*

Fig.5: Zymogram showing PAGE (cationic) banding pattern in *P. nigrum* varieties (Panniyur-1 and Kalluvally) and *P. colubrinum* on the second day after infection with *Phytophthora capsici*

Band No.	Rm. Value	HP	IP	HK	IK	HC	IC
1	0.08					–	–
2	0.10	–	–	–	–		
3	0.20	–	–	–	–		
4	0.24					–	–
5	0.30	–	–	–	–		
6	0.35					–	–
7	0.40			–	–		
8	0.43	–	–				
9	0.50	–	–	–	–	–	–
10	0.56					–	–

HP Healthy Panniyur-1 HK Healthy Kalluvally

IP Infected Panniyur-1 IK Infected Kalluvally

HC Healthy *P.colubrinum*

IC Infected *P.colubrinum*

Fig.6. Zymogram showing PAGE (cationic) banding pattern in *P. nigrum* varieties (Panniyur-1 and Kalluvally) and *P. colubrinum* on the third day after infection with *Phytophthora capsici*

Band No.	Rm. Value	HP	IP	HK	IK	HC	IC
1	0.08					–	–
2	0.10	–	–	–	–		
3	0.20	–	–	–	–		
4	0.24					–	–
5	0.30	–	–	–	–		
6	0.35					–	–
7	0.40			–	–		
8	0.43	–	–				
9	0.50	–	–	–	–	–	–
10	0.56					–	–

HP Healthy Panniyur-1 HK Healthy Kalluvally

IP Infected Panniyur-1 IK Infected Kalluvally

HC Healthy *P.colubrinum*

IC Infected *P.colubrinum*

Fig.7. Zymogram showing PAGE (cationic) banding pattern in *P. nigrum* varieties (Panniyur-1 and Kalluvally) and *P. colubrinum* on the fourth day after infection with *Phytophthora capsici*

Band No.	Rm. Value	HP	IP	HK	IK	HC	IC
1	0.08					–	–
2	0.10	–	–	–	–		
3	0.20	–	–	–	–		
4	0.24					–	–
5	0.30	–	–	–	–		
6	0.35					–	–
7	0.40			–	–		
8	0.43	–	–				
9	0.50	–	–	–	–	–	–
10	0.56					–	–

HP Healthy **Panniyur-1** HK Healthy Kalluvally

IP Infected **Panniyur-1** IK Infected Kalluvally

HC Healthy *P.colubrinum*

IC Infected *P.colubrinum*

Fig.8. Zymogram showing PAGE (cationic) banding pattern in *P. nigrum* varieties (Panniyur-1 and Kalluvally) and *P. colubrinum* on the fifth day after infection with *Phytophthora capsici*

Band No.	Rm. Value	HP	IP	HK	IK	HC	IC
1	0.08					–	–
2	0.10	–	–	–	–		
3	0.20	–	–	–	–		
4	0.24					–	–
5	0.30	–	–	–	–		
6	0.35					–	–
7	0.40			–	–		
8	0.43	–	–				
9	0.50	–	–	–	–	–	–
10	0.56					–	–

HP Healthy **Panniyur-1** HK Healthy Kalluvally

IP Infected **Panniyur-1** IK Infected Kalluvally

HC Healthy *P.colubrinum*

IC Infected *P.colubrinum*

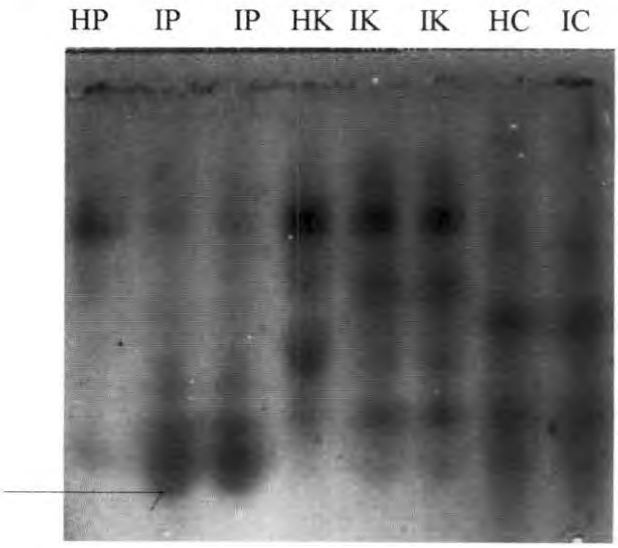


Plate 3. Cationic protein profile for PAGE analysis in *Piper* species, in relation to infection with *Phytophthora capsici*

HP - Healthy Panniyur-1, IP - Infected Panniyur-1, HK - Healthy Kalluvally, IK - Infected Kalluvally, HC - Healthy *P. colubrinum*, IC - Infected *P. colubrinum*

Fig.9. Zymogram showing PAGE (cationic) banding pattern in *P. nigrum* varieties (Panniyur-1 and Kalluvally) and *P. colubrinum* on the sixth day after infection with *Phytophthora capsici*

Band No.	Rm. Value	HP	IP	HK	IK	HC	IC
1	0.08					–	–
2	0.10	–	–	–	–		
3	0.20	–	–	–	–		
4	0.24					–	–
5	0.30	–	–	–	–		
6	0.35					–	–
7	0.40			–	–		
8	0.43	–	–				
9	0.50	–	–	–	–	–	–
10	0.56		–		–	–	–

HP Healthy **Panniyur-1** HK Healthy Kalluvally

IP Infected **Panniyur-1** IK Infected Kalluvally

HC Healthy *P.colubrinum*

IC Infected *P.colubrinum*

4.3.2.2 Cationic protein profile for *P. colubrinum*

P. colubrinum samples showed expression of five basic proteins with Rm value ranging from 0.09 to 0.56 of which only one (Rm 0.50) was found shared with *P. nigrum* varieties. Samples from healthy and infected plants did not show any variation in the expression of basic proteins in this species. However, the additional band of Rm value (0.56) found expressed in varieties Panniyur-1 and Kalluvally at the later stages of infection was found present in *P. colubrinum* throughout the experiment.

4.4 SDS-PAGE analysis for PR proteins

In SDS-PAGE, the protein mixture is denatured by heating at 100°C in the presence of excess SDS and thiol reagent is employed to break the disulfide bond. Thus protein get denatured into polypeptides and form a complex with SDS.

4.4.1 SDS-PAGE profile for *Piper* spp.in relation to infection with *P. capsici*

4.4.1.1 24 hours after infection

The SDS-PAGE analysis of plant extract isolated at pH 2.8 has shown distinct banding pattern for *P. nigrum* (Panniyur-1 and Kalluvally) and *P. colubrinum*. The banding pattern observed 24 hours after infection with *P. capsici* is shown in Plate 4 and 5. The zymogram relating to the expression of polypeptides in *P. nigrum* and *P. colubrinum* is given in Fig.10. The healthy and infected plants gave the same banding pattern in both the species. All

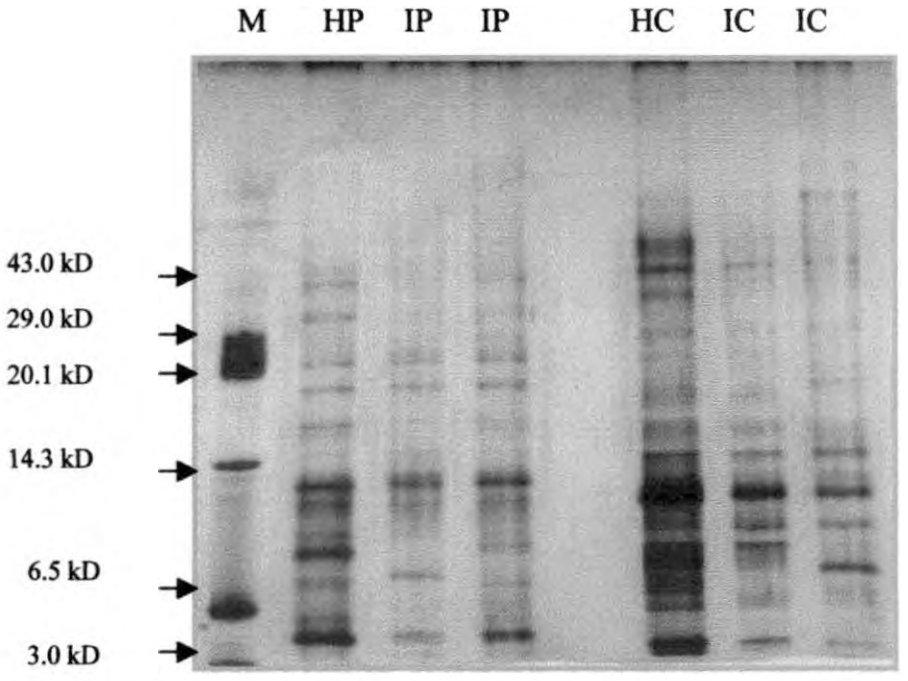


Plate 4: SDS-PAGE profile of PR proteins in *P. nigrum* (Var:Panniyur 1) and *P. colubrinum* 24 hrs after infection with *Phytophthora capsici*

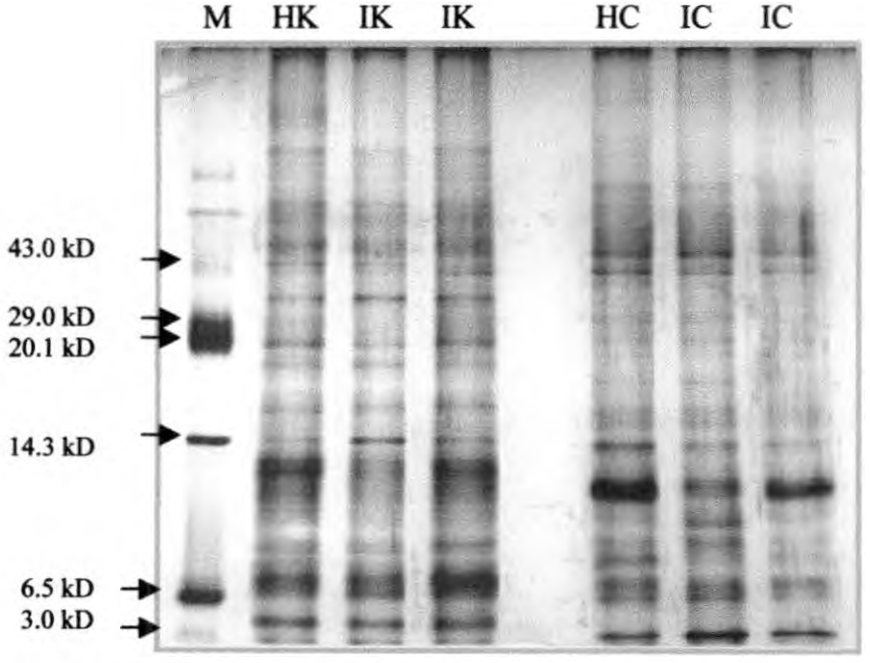


Plate 5 : SDS-PAGE profile of PR proteins in *P. nigrum* (Var: Kalluvally) and *P. colubrinum* 24 hrs after infection with *Phytophthora capsici*

M – Marker, HP- Healthy Panniyur I , IP- Infected Panniyur 1, HC- Healthy *P. colubrinum*, IC- Infected *P. colubrinum* , HK- Healthy Kalluvally, IK- Infected Kalluvally

Fig.10 Zymogram showing SDS-PAGE banding pattern in *P. nigrum* varieties (Panniyur-1 and Kalluvally) and *P. colubrinum* on first day after infection with *P. capsici*

Band No	Molecular wt (kD)	HP	IP	HK	IK	HC	IC
1	43.0	–	–	–	–	–	–
2	33.0	–	–	–	–		
3	29.0					–	–
4	24.0					–	–
5	22.0	–	–	–	–	–	–
6	19.5	–	–	–	–		
7	19.0					–	–
8	17.0	–	–	–	–		
9	16.5					–	–
10	14.3			–	–		
11	13.0	–	–	–	–	–	–
12	11.5	–	–	–	–	–	–
13	10.0	–	–	–	–	–	–
14	9.0	–	–			–	–
15	8.8			–	–	–	–
16	8.0					–	–
17	7.5	–	–	–	–		
18	7.0			–	–	–	–
19	6.5					–	–
20	5.5	–	–				
21	4.3					–	–
22	4.0	–	–	–	–	–	–

HP Healthy Panniyur-1

IP Infected Panniyur-1

HK Healthy Kalluvally

IK Infected Kalluvally

HC Healthy *P. colubrinum*

IC Infected *P. colubrinum*

together 15 bands were observed for the two varieties of *P. nigrum* and all except five were shared by both the varieties. The expression in *P. nigrum* varieties, Panniyur-1 and Kalluvally were almost similar except for those bands observed at 9.0 kD, 5.5 kD, 14.3 kD, 8.8 kD,and 7.0 kD. The first two of these were found expressed only in Panniyur-1 while the others were observed only in Kalluvally.

An entirely different banding pattern was expressed by *P. colubrinum*. Sixteen distinct bands were observed of which only six (43.0 kD, 22.0 kD, 13.0 kD, 11.5 kD, 10.0 kD and 4.0 kD) were found shared with *P. nigrum*

4.4.1.2 48 hours after infection

Expression of polypeptide in plant sample on the second day after infection is presented in Plates 6 and 7 and the zymogram related to the clear specific bands is shown in Fig.11. The banding pattern observed was almost similar to that expressed on the first day. All the bands expressed by the healthy plants of Panniyur-1was also found expressed by the infected one. However in variety Kalluvally, one additional band of molecular weight 16.5kD (band No. 9) was found expressed in the infected one. The profile observed for *P. colubrinum* on the second day was exactly the same as on the previous day.

4.4.1.3 72 hours after infection

Similar banding pattern was observed in both the varieties of *P. nigrum* (Panniyur-1 and Kalluvally) and in *P. colubrinum* as on the second day. (Plates 8 and 9 and Fig.12). The newly expressed band found expressed in the infected Kalluvally (band No. 9) on the second day persisted on the third day also.

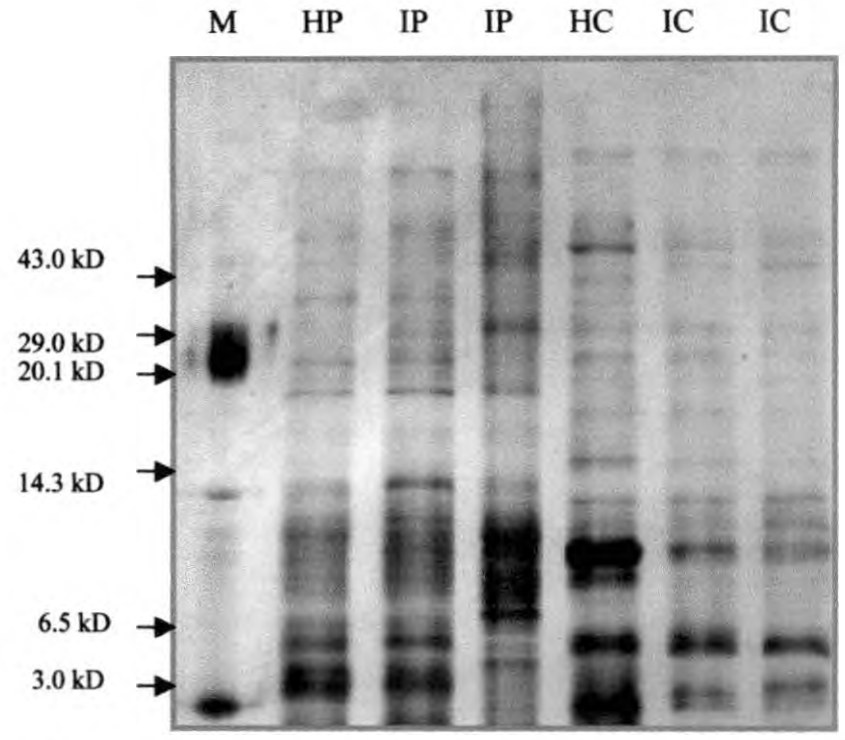


Plate 6: SDS-PAGE profile of PR proteins in *P. nigrum* (Var: Panniyur 1) and *P. colubrinum* 48 hrs after infection with *Phytophthora capsici*

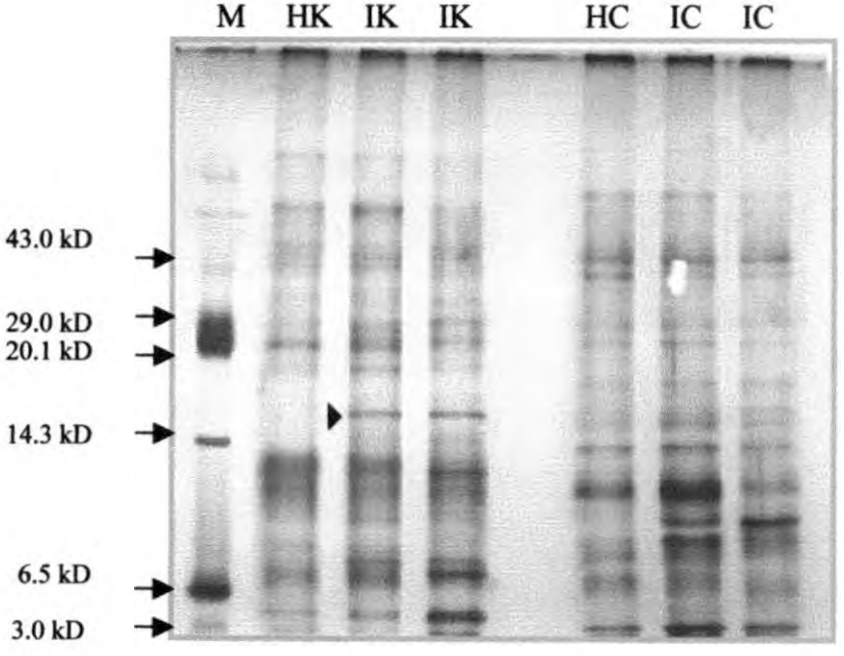


Plate 7: SDS-PAGE profile of PR proteins in *P. nigrum* (Var: Kalluvally) and *P. colubrinum* 48 hrs after infection with *Phytophthora capsici*

M – Marker, HP- Healthy Panniyur I , IP- Infected Panniyur 1, HC- Healthy *P. colubrinum*, IC- Infected *P. colubrinum* , HK- Healthy Kalluvally, IK- Infected Kalluvally

Fig. 11 Zymogram showing SDS-PAGE banding pattern in *P. nigrum* varieties (Panniyur-1 and Kalluvally) and *P. colubrinum* on second day after infection with *P. capsici*

Band No	Molecular wt (kD)	HP	IP	HK	IK	HC	IC
1	43.0	–	–	–	–	–	–
2	33.0	–	–	–	–		
3	29.0					–	–
4	24.0					–	–
5	22.0	–	–	–	–	–	–
6	19.5	–	–	–	–		
7	19.0					–	–
8	17.0	–	–	–	–		
9	16.5				–	–	–
10	14.3			–	–		
11	13.0	–	–	–	–	–	–
12	11.5	–	–	–	–	–	–
13	10.0	–	–	–	–	–	–
14	9.0	–	–			–	–
15	8.8			–	–	–	–
16	8.0					–	–
17	7.5	–	–	–	–		
18	7.0			–	–	–	–
19	6.5					–	–
20	5.5	–	–				
21	4.3					–	–
22	4.0	–	–	–	–	–	–

HP Healthy Panniyur-1

IP Infected Panniyur-1

HK Healthy Kalluvally

IK Infected Kalluvally

HC Healthy *P. colubrinum*

IC Infected *P. colubrinum*

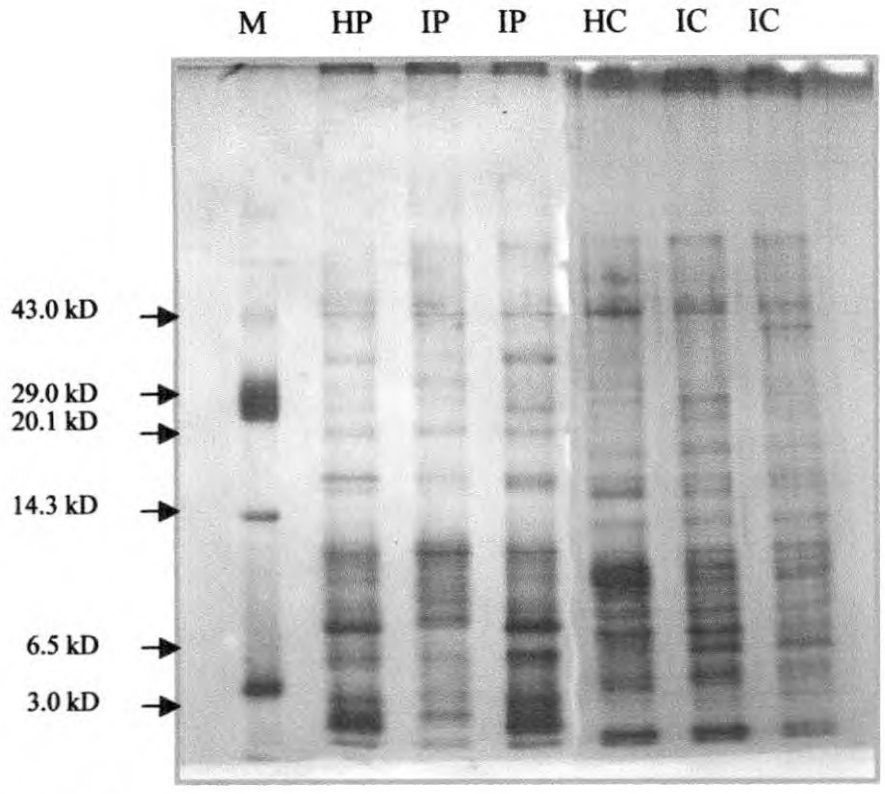


Plate 8: SDS-PAGE profile of PR proteins in *P. nigrum* (Var: Panniyur 1) and *P. colubrinum* 72 hrs after infection with *Phytophthora capsici*

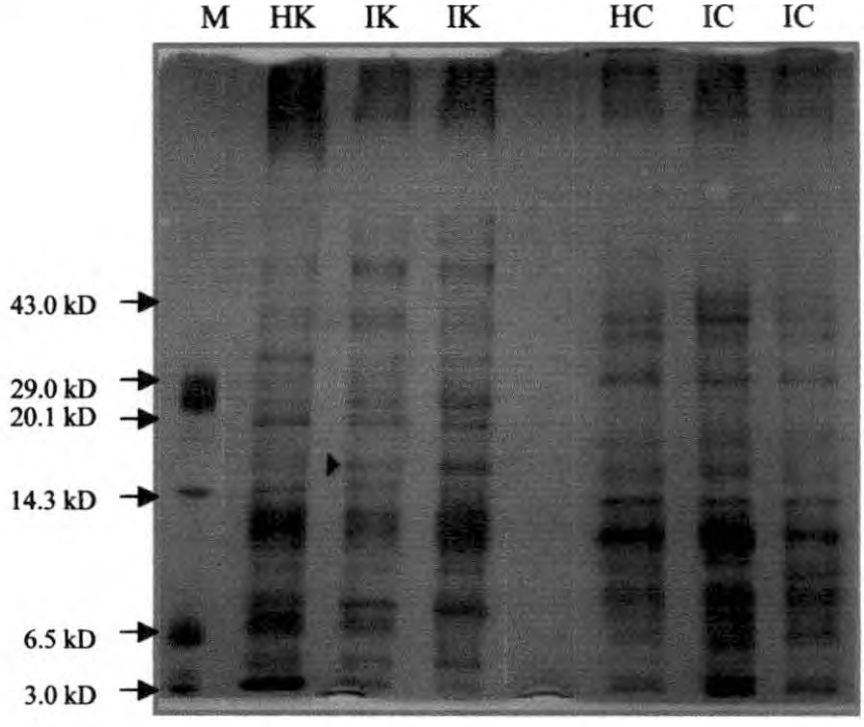


Plate 9: SDS-PAGE profile of PR proteins in *P. nigrum* (Var: Kalluvally) and *P. colubrinum* 72 hrs after infection with *Phytophthora capsici*

M – Marker, HP- Healthy Panniyur I , IP- Infected Panniyur 1, HC- Healthy *P. colubrinum*, IC- Infected *P. colubrinum* , HK- Healthy Kalluvally, IK- Infected Kalluvally

Fig.12 Zymogram showing SDS-PAGE banding pattern in P. nigrum varieties (Panniyur-1and Kalluvally) and *P. colubrinum* on third day after infection with *P. capsici*

Band No	Molecular wt (kD)	HP	IP	HK	IK	HC	IC
1	43.0	—	—	—	—	—	—
2	33.0	—	—	—	—		
3	29.0					—	—
4	24.0					—	—
5	22.0	—	—	—	—	—	—
6	19.5	—	—	—	—		
7	19.0					—	—
8	17.0	—	—	—	—		
9	16.5				—	—	—
10	14.3			—	—		
11	13.0	—	—	—	—	—	—
12	11.5	—	—	—	—	—	—
13	10.0	—	—	—	—	—	—
14	9.0	—	—			—	—
15	8.8			—	—	—	—
16	8.0					—	—
17	7.5	—	—	—	—		
18	7.0			—	—	—	—
19	6.5					—	—
20	5.5	—	—				
21	4.3					—	—
22	4.0	—	—	—	—	—	—

HP Healthy Panniyur-1

IP Infected Panniyur-1

HK Healthy Kalluvally

IK Infected Kalluvally

HC Healthy *P. colubrinum*

IC Infected *P. colubrinum*

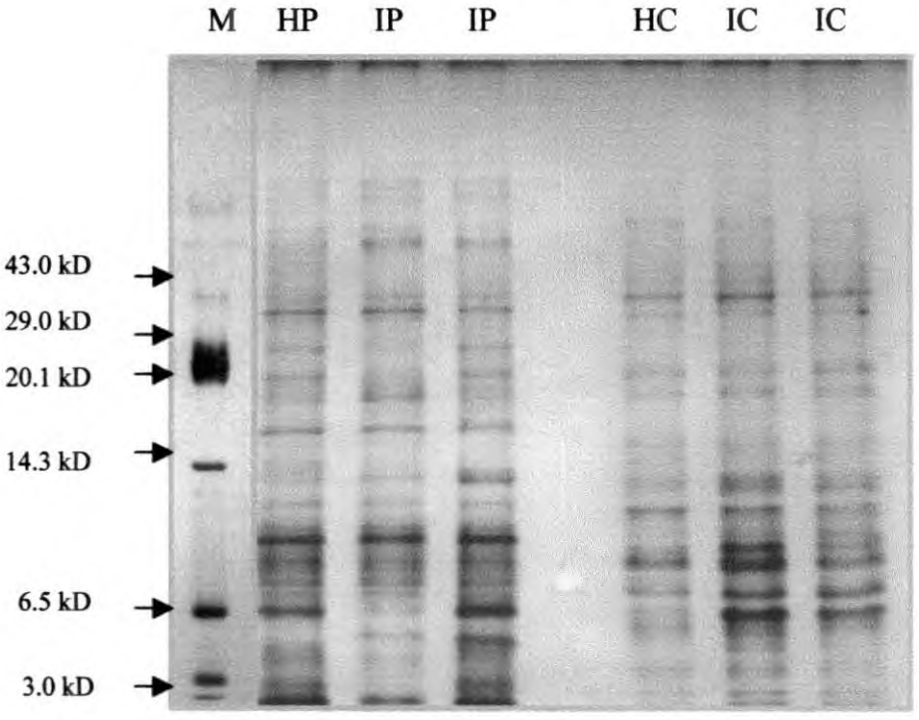


Plate10: SDS-PAGE profile of PR proteins in *P. nigrum* (Var: Panniyur 1) and *P. colubrinum* 96 hrs after infection with *Phytophthora capsici*

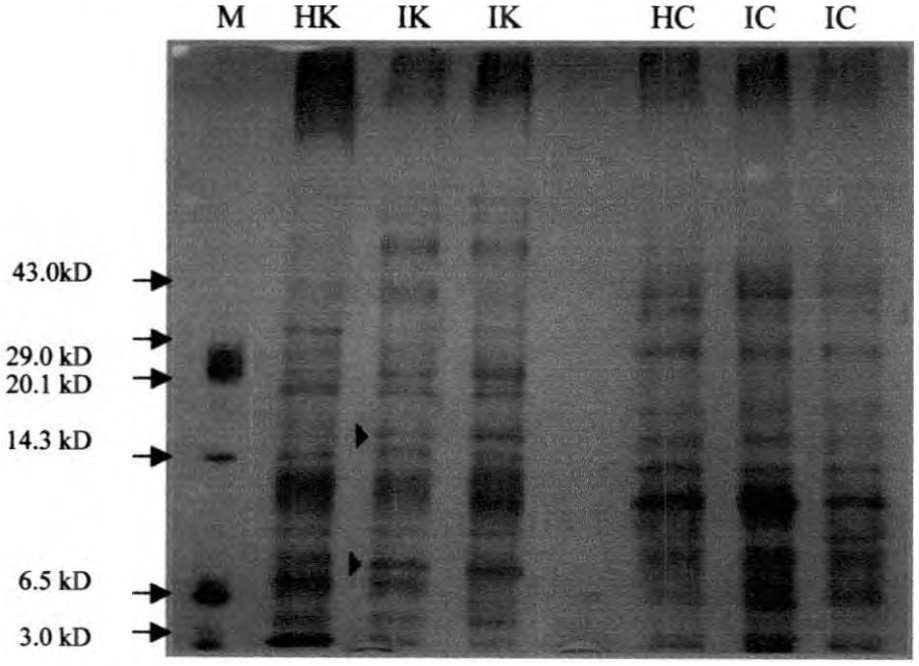


Plate 11: SDS-PAGE profile of PR proteins in *P. nigrum* (Var: Kalluvally) and *P. colubrinum* 96 hrs after infection with *Phytophthora capsici*

M – Marker, HP- Healthy Panniyur I , IP- Infected Panniyur 1, HC- Healthy *P. colubrinum*, IC- Infected *P. colubrinum* , HK- Healthy Kalluvally, IK- Infected Kalluvally

4.4.1.4 96 hours after infection

The banding pattern observed for the polypeptides on the fourth day after infection is showed in Plates 10 and 11. The zymogram related to banding pattern is presented in Fig.13. In the variety Panniyur –1, the pattern was same as that on the previous day while in the variety Kalluvallly, one more additional band of 8kD (band No. 16) was observed in infected plants. However, the second additional band observed in Kalluvally was also found shared with *P. colubrinum*. The *P. colubrinum* behaved in the same manner as on the previous day.

4.4.1.5 120 hours after infection

Expression of polypeptides on the fifth day after infection is presented in Plates 12 and 13 and their zymogram in Fig.14. All the protein bands observed in healthy plants were shared by the infected ones also. The infected plants of variety Kalluvally continued to express the additional bands (band No. 9 and 16) on the fifth day. These additional bands were found newly expressed in the infected plants of variety Panniyur-1 on the fifth day. The pattern observed for *P. colubrinum* was similar to that on the previous day.

4.5 Partial purification of proteins

Protein samples were salted out with different concentrations of $(NH_4)_2SO_4$ so as to precipitate the proteins. Ammonium sulphate precipitation was carried out for the samples collected on the fifth day after infection in variety Panniyur-1. Electrophoretic results of different fractions obtained after salting out is shown in

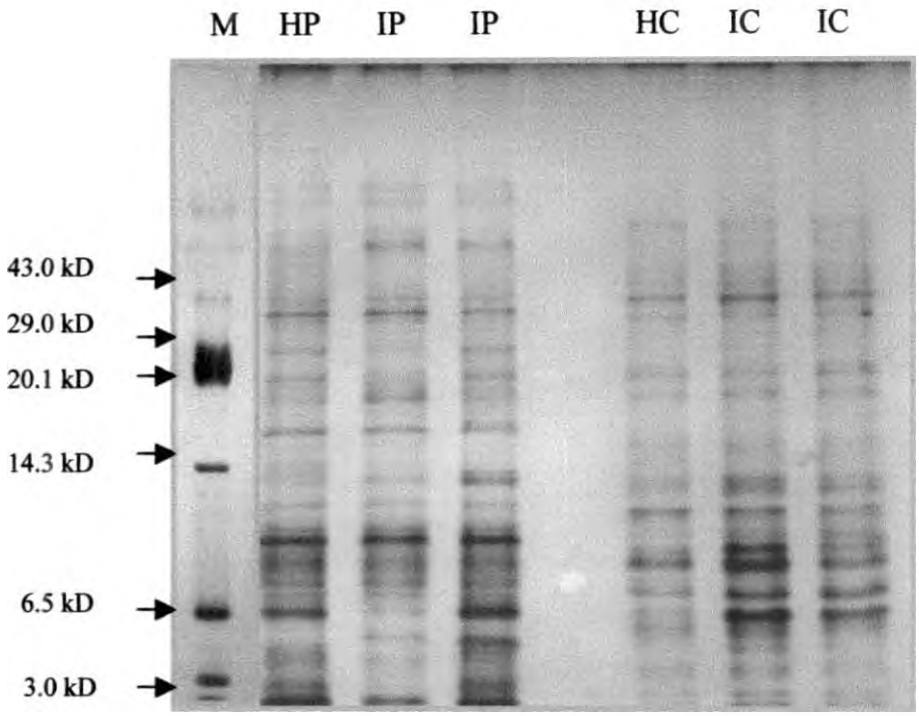


Plate10: SDS-PAGE profile of PR proteins in *P. nigrum* (Var: Panniyur 1) and *P. colubrinum* 96 hrs after infection with *Phytophthora capsici*

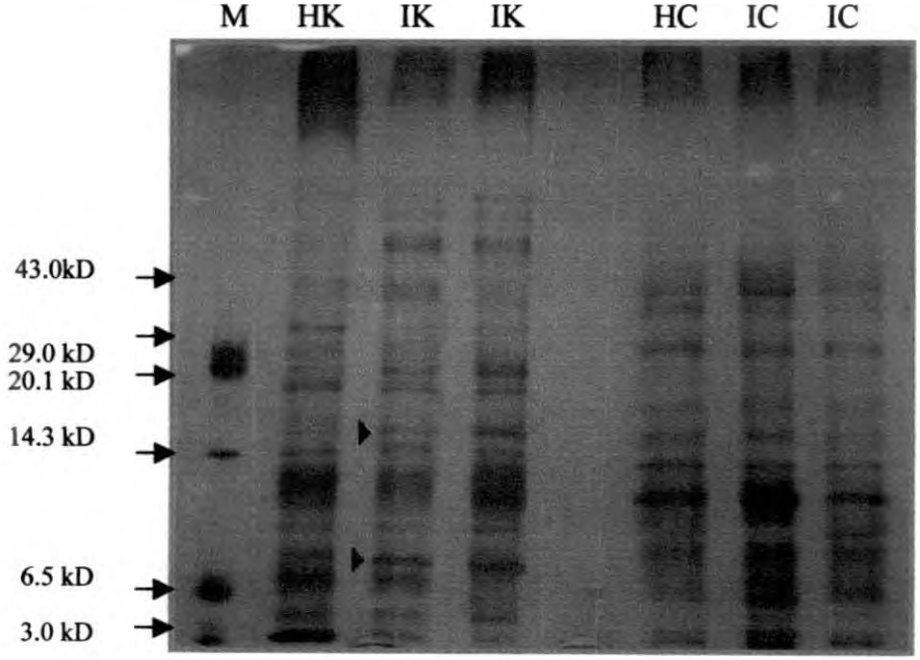


Plate 11: SDS-PAGE profile of PR proteins in *P. nigrum* (Var: Kalluvally) and *P. colubrinum* 96 hrs after infection with *Phytophthora capsici*

M – Marker, HP- Healthy Panniyur I , IP- Infected Panniyur 1, HC- Healthy *P. colubrinum*, IC- Infected *P. colubrinum* , HK- Healthy Kalluvally, IK- Infected Kalluvally

Fig. 13 Zymogram showing SDS-PAGE banding pattern in *P. nigrum* varieties (Panniyur-1and Kalluvally) and *P. colubrinum* on fourth day after infection with *P. capsici*

Band No	Molecular wt (kD)	HP	IP	HK	IK	HC	IC
1	43.0	–	–	–	–	–	–
2	33.0	–	–	–	–		
3	29.0					–	–
4	24.0					–	–
5	22.0	–	–	–	–	–	–
6	19.5	–	–	–	–		
7	19.0					–	–
8	17.0	–	–	–	–		
9	16.5				–	–	–
10	14.3			–	–		
11	13.0	–	–	–	–	–	–
12	11.5	–	–	–	–	–	–
13	10.0	–	–	–	–	–	–
14	9.0	–	–			–	–
15	8.8			–	–	–	–
16	8.0				–	–	–
17	7.5	–	–	–	–		
18	7.0			–	–	–	–
19	6.5					–	–
20	5.5	–	–				
21	4.3					–	–
22	4.0	–	–	–	–	–	–

HP Healthy Panniyur-1

IP Infected Panniyur-1

HK Healthy Kalluvally

IK Infected Kalluvally

HC Healthy *P. colubrinum*

IC Infected *P. colubrinum*

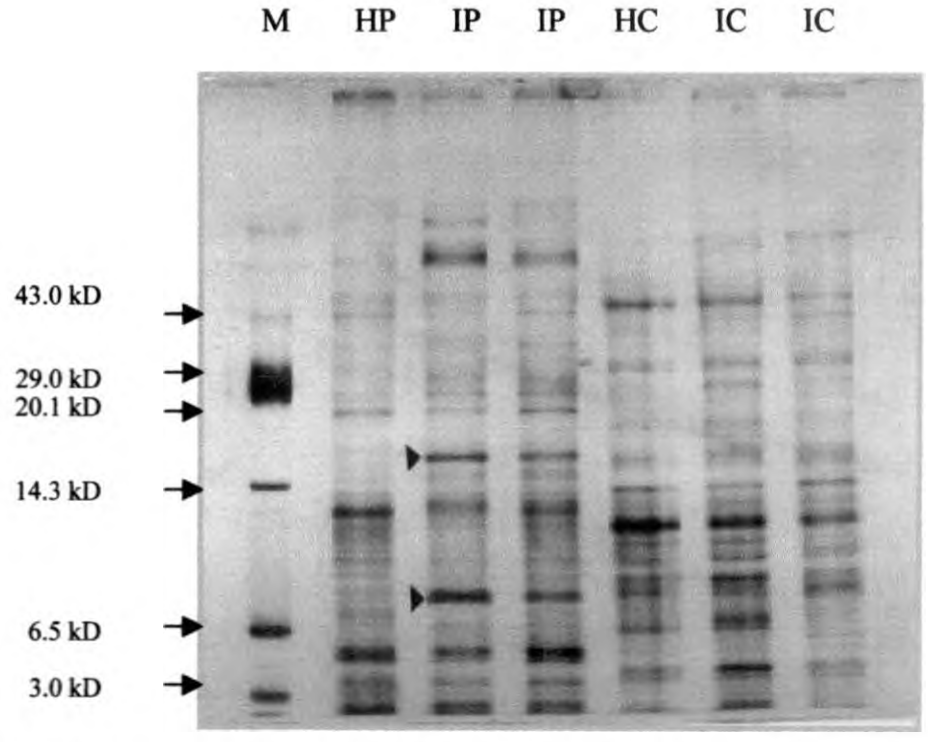


Plate12: SDS-PAGE profile of PR proteins in *P. nigrum* (Var: Panniyur 1) and *P. colubrinum* 120 hrs after infection with *Phytophthora capsici*

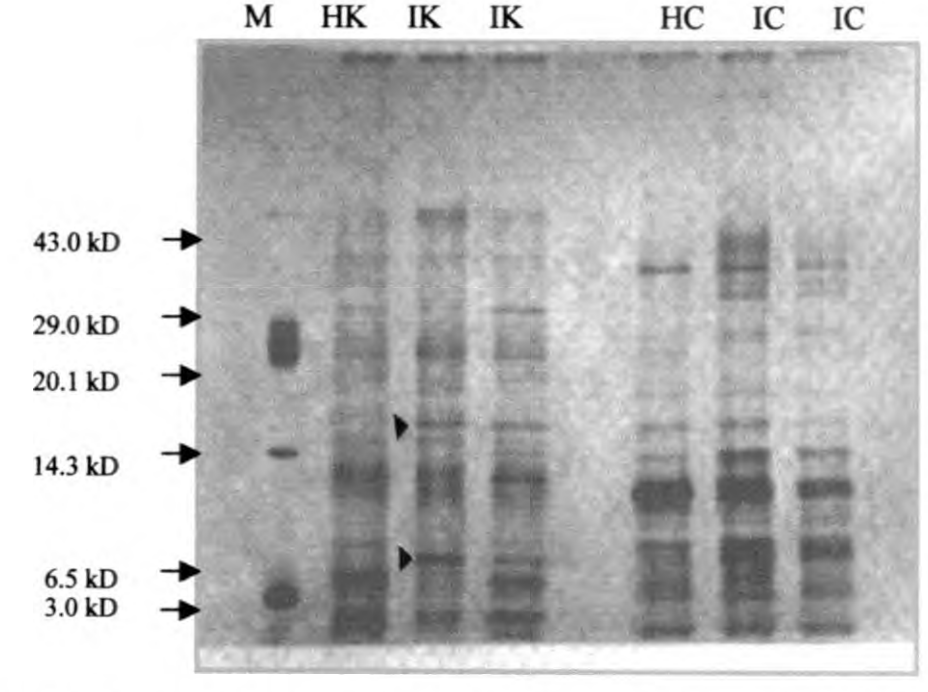


Plate 13: SDS-PAGE profile of PR proteins in *P. nigrum* (Var: Kalluvally) and *P. colubrinum* 120 hrs after infection with *Phytophthora capsici*

M – Marker, HP- Healthy Panniyur I , IP- Infected Panniyur 1, HC- Healthy *P. colubrinum*, IC- Infected *P. colubrinum* , HK- Healthy Kalluvally, IK- Infected Kalluvally

Fig.14 Zymogram showing SDS-PAGE banding pattern in *P. nigrum* varieties (Panniyur-1 and Kalluvally) and *P. colubrinum* on fifth day after infection with *P. capsici*

Band No	Molecular wt (kD)	HP	IP	HK	IK	HC	IC
1	43.0	—	—	—	—	—	—
2	33.0	—	—	—	—		
3	29.0					—	—
4	24.0					—	—
5	22.0	—	—	—	—	—	—
6	19.5	—	—	—	—		
7	19.0					—	—
8	17.0	—	—	—	—		
9	16.5		—		—	—	—
10	14.3			—	—		
11	13.0	—	—	—	—	—	—
12	11.5	—	—	—	—	—	—
13	10.0	—	—	—	—	—	—
14	9.0	—	—			—	—
15	8.8			—	—	—	—
16	8.0		—		—	—	—
17	7.5	—	—	—	—		
18	7.0			—	—	—	—
19	6.5					—	—
20	5.5	—	—				
21	4.3					—	—
22	4.0	—	—	—	—	—	—

HP Healthy Panniyur-1

IP Infected Panniyur-1

HK Healthy Kalluvally

IK Infected Kalluvally

HC Healthy P. colubrinum

IC Infected *P. colubrinum*

Fig.15. All the fractions precipitated with 40, 60 and 80 per cent $(NH_4)_2SO_4$ showed the presence of newly expressed protein. The banding pattern observed for the partially purified samples was not sharp enough to clearly detect the separation of protein.

The method followed in the present study for fractionation of proteins was not highly effective for the purpose. The concentration of sample after removal of $(NH_4)_2SO_4$ was not found perfect.

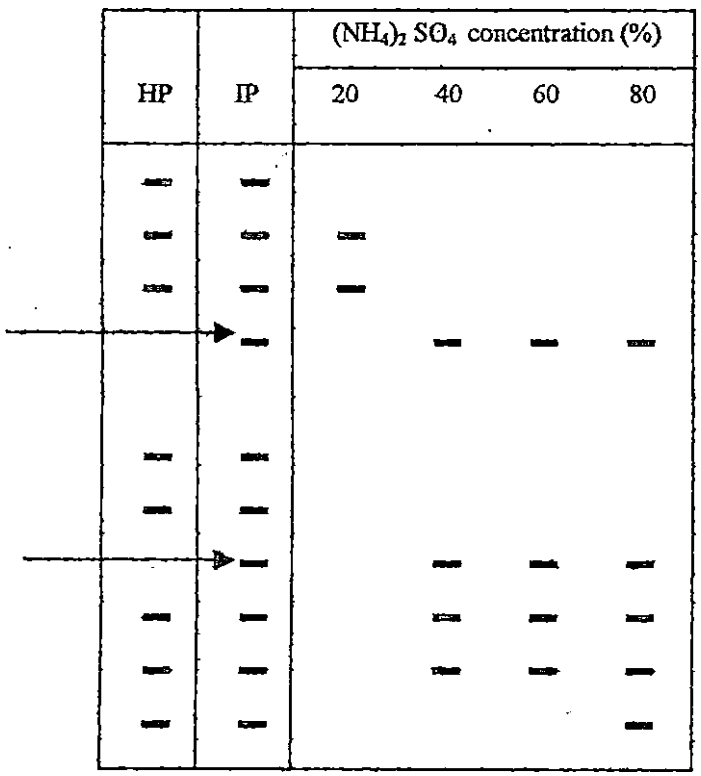
4.6 Assay of pathogenesis related enzymes

4.6.1 Glucanase activity in relation to Phytophthora infection

Beta-1,3-glucanase activity assessed in leaf samples of *P. nigrum* (Panniyur-1 and Kalluvally) and *P. colubrinum* are presented in Table 7 and Fig.16. The healthly plants of variety Panniyur-1 indicated glucanase activity ranging between 0.100 and 0.151 enzyme unit/ mg protein/ 10 min. The data indicated clear, significant difference in enzyme activity when infected with *P. capsici*. The foliar enzyme activity in infected plants was found elevated within one day after infection and an increasing trend was observed till fifth day by which time the necrotic patches were spread all over the leaf lamina. Two fold increase in the enzyme activity was recorded within five days for the variety Panniyur-1.

In the variety Kalluvally, the foliar activity of ß-1,3-glucanase was found to be relatively higher than variety Panniyur-1. The value assessed over six days ranged between 0.200 to 0.242 unit/mg protein/10 min. Upon infection with the fungus, the enzyme activity was increased to the extent of 1.700 enzyme unit/mg protein/10 min (seven fold) by third day after infection.

Fig. 15 Zymogram showing separation of PR proteins in variety Panniyur-1when precipitated with different concentration of $(NH_4)_2 SO_4$



HP Healthy Panniyur- 1

IP Infected Panniyur- 1

Arrow indicate additionally expressed proteins

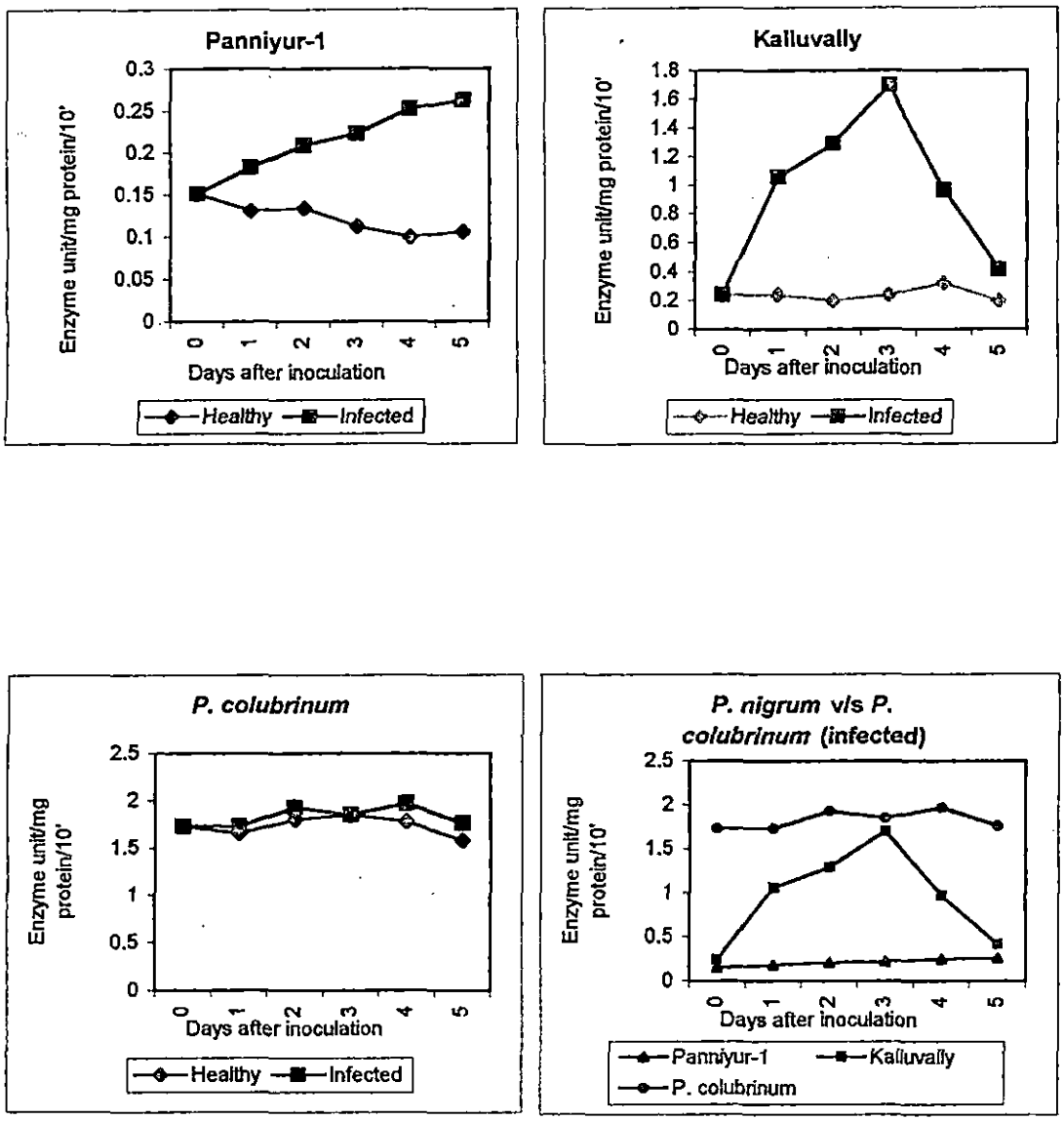
Table 7. Beta-1, 3 glucanase activity (enzyme unit/mg protein/10 min) in *Piper* species in relation to infection with *Phytophthora capsici*

	Days after infection						Mean
	0 day	First	Second	Third	Fourth	Fifth	
I. P. nigrum							
Panniyur-1							
Healthy	0.151	0.131	0.134	0.112	0.100	0.106	0.116
Infected	0.151	0.183	0.203	0.223	0.252	0.262	0.225
Kalluvally							
Healthy	0.242	0.240	0.200	0.240	0.220	0.200	0.22
Infected	0.242	1.060	1.290	1.700	0.965	0.414	1.080
II. *P. colubrinum*							
Healthy	1.73	1.65	1.80	1.85	1.78	1.58	1.72
Infected	1.73	1.73	1.93	1.85	1.97	1.76	1.84

CD (0.05) for comparing genotypes = 0.052

CD (0.05) for comparing healthy and infected plants = 0.042

Fig. 16. Comparison of Beta 1-3 glucanase enzyme activity in *P. nigrum* varieties (Panniyur-1 and Kalluvally) and *P. colubrinum* in relation to infection with *P. capsici*



In case of *P. colubrinum,* the glucanase activity in healthy and infected plants ranged between 1.58 and 1.93 unit/mg protein/10 min. The *P. colubrinum* did not show any variation in enzyme activity upon inoculation with the pathogen. It was interesting to observe that the enzyme activity in this species was relatively high as compared to *P. nigrum* varieties. The healthy plants of *Piper colubrinum* expressed higher (ten fold) glucanase activity than *P. nigrum* varieties

4.6.2 Chitinase activity

Chitinase activity was assessed based on the method suggested by Jeuniaux (1966). The activity read at different levels of standard N-acetyl glucosamine with chitin as substrate is depicted in Fig.17.

The procedure followed was found ideal for detecting chitinase activity using the standard chemical and a standard graph could be plotted against the assessed value. However, the activity of chitinase in healthy and infected samples of *P. nigrum* and *P. colubrinum* was found to be almost nil. The results obtained with different levels of crude extract, substrate and incubation time are presented in Table 8. Chitinolytic activity was not detected in any of the samples.

4.6.3 Phenylalanine ammonia lyase (PAL) activity

PAL activity assessed in leaf samples of *P. nigrum* varieties (Panniyur-1and Kalluvally) and *P. colubrinum* are presented in Table 9 and Fig.18. The healthy plants of variety Panniyur-1 showed PAL activity ranging between 0.238 and 0.269 μg cinnamic acid/ mg protein/ min. The data indicated significant difference

Fig. 17. Standard graph for determination of chitinase activity

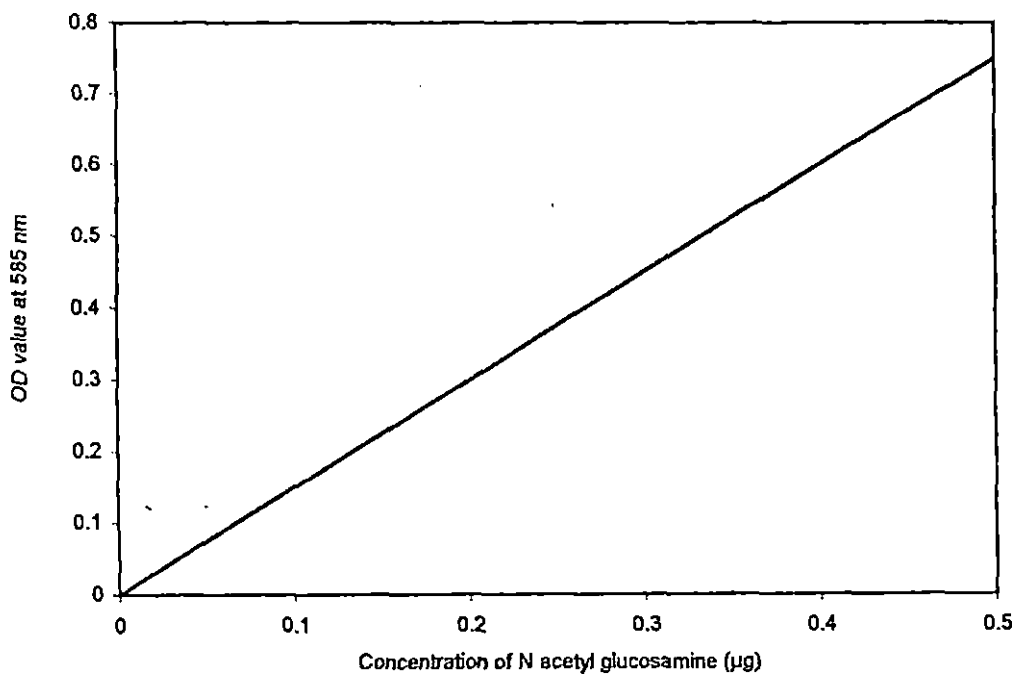


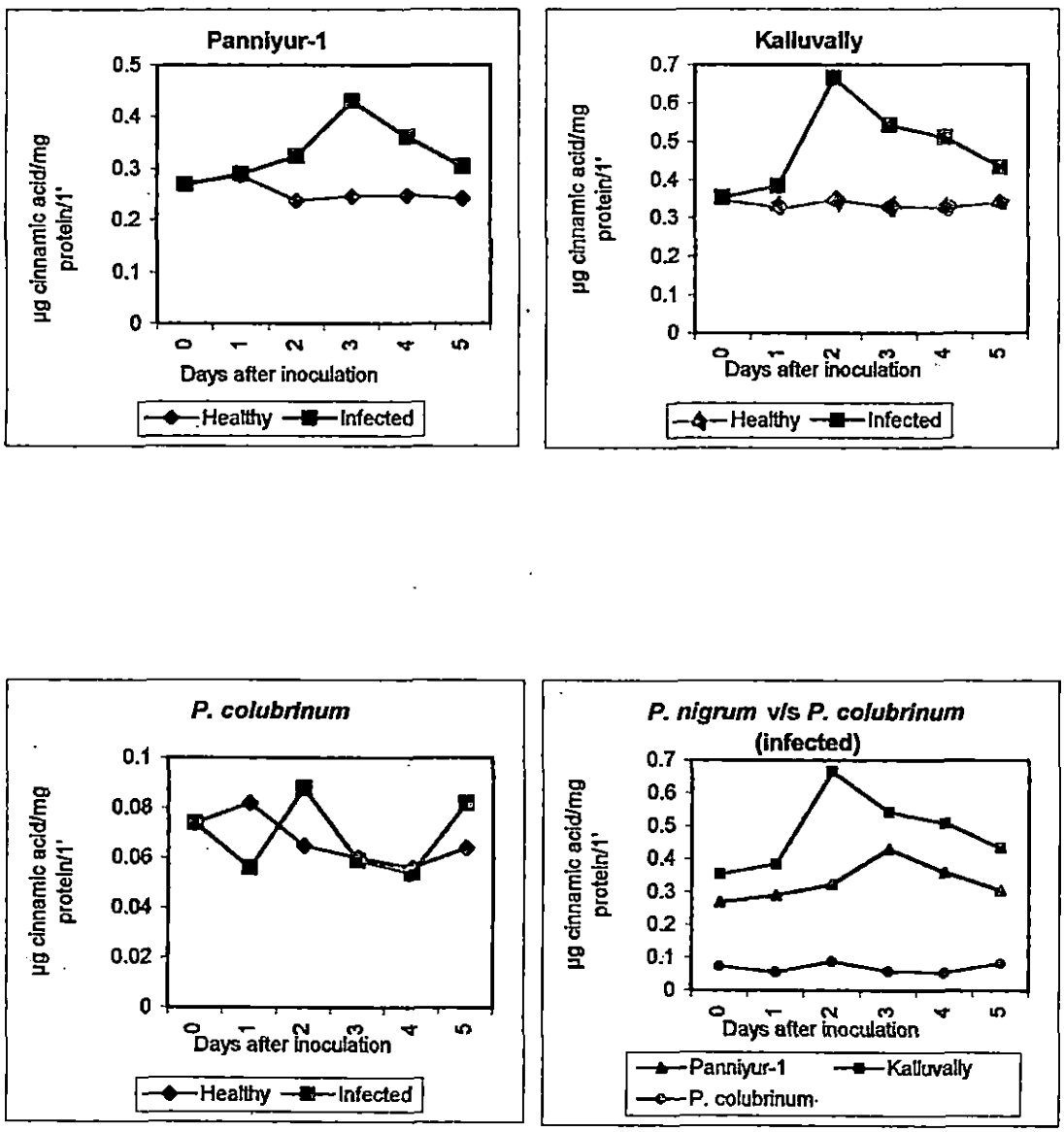
Table 8. Chitinase activity detected in *Piper nigrum* varieties and *Piper colubrinum* at different levels of substrate and incubation time.

Components	Combinations							
	1	2	3	4	5	6	7	8
Leaf samples (g)	1	1	1	1	1	4	4	4
Extraction buffer (ml)	2	2	2	2	2	2	2	2
Crude extract (ml)	2	2	2	2	1.5	2	2	2
Assay buffer (ml)	1	1	1	1	1	1	1	1
Chitin (mg/ml)	5	5	30	30	30	30	30	30
Incubation time (min) at 37°C	90	180	90	180	180	90	180	180
Enzyme activity	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

Table 9. PAL enzyme activity (µg cinnamic acid/mg protein/min) in *Piper* species in relation to infection with *P. capsici*

	Days after infection						Mean
	0 day	First	Second	Third	Fourth	Fifth	
I. P. nigrum							
Panniyur-1							
Healthy	0.269	0.287	0.238	0.246	0.248	0.242	0.252
Infected	0.269	0.290	0.324	0.431	0.361	0.304	0.342
Kalluvally							
Healthy	0.354	0.329	0.351	0.332	0.330	0.344	0.337
Infected	0.354	0.385	0.666	0.542	0.510	0.433	0.507
II. *P. colubrinum*							
Healthy	0.074	0.082	0.065	0.060	0.056	0.064	0.065
Infected	0.074	0.056	0.088	0.054	0.054	0.082	0.067
CD (0.05) for comparing							
1. Genotypes	0.047	0.079	0.047	0.056	0.063	0.073	
2. Healthy/ infected	0.038	0.064	0.039	0.045	0.051	0.059	

Fig. 18. Comparison of PAL enzyme activity in *P. nigrum* varieties (Panniyur-1 & Kalluvally) and *P. colubrinum* in relation to infection with *P. capsici*



in enzyme activity when infected with *P. capsici*. The foliar enzyme activity in infected plants was found elevated within one day after infection. The enzyme activity was found elevated by two fold within three days and decreased thereafter.

In the variety Kalluvally, the activity of PAL was found to be relatively higher than in variety Panniyur-1. The value assessed over six days ranged between 0.330 and 0.354 μg cinnamic acid/mg protein/min. Upon infection with the fungus, the enzyme activity was found to increase substantially. The PAL activity recorded on the second day after infection was 0.66 μg cinnamic acid/mg protein/min. This was two fold increase over healthy plants. The activity showed a decreasing trend from third day onwards.

In *P. colubrinum*, the PAL activity in healthy and infected plants did not show any significant difference and it ranged between 0.054 and 0.088 μg cinnamic acid/ mg protein/ min. It was interesting to observe that the PAL activity in *P. colubrinum* was comparatively low and was not affected by inoculation with *P. capsici*

Thus the results of the present study highlight the expression of PR proteins and specific PR enzymes in two varieties of *Piper nigrum* in relation to infection with the fungal pathogen, *Phytophthora capsici*. Behaviour of the resistant species *Piper colubrinum* was also revealed.

Discussion

5. DISCUSSION

The results obtained in the present study are discussed in this chapter based on the earlier reports and possible interpretations.

5.1 Induction of the disease symptom

Disease symptoms were induced on the healthy foliage of *Piper* spp. with live culture of *Phytophthora capsici* maintained at 28°C in carrot agar medium. Seven-day old culture was used due to its high potential for infection. The foliar symptoms developed after infection varied considerably among the varieties and species studied. Expression of foliar symptoms in the variety Panniyur-1 was found early (within 24 hrs) as compared to the variety Kalluvally (within 48 hrs). *P. colubrinum* did not take any foliar symptom throughout the experimental period. With respect to the severity of disease, it was much severe in the case of variety Panniyur-1 than the variety Kalluvally. Development of necrotic area was comparatively distinct in Panniyur-1 and the mean diameter was 54 mm by fifth day while it was only 30 mm for the variety Kalluvally. Detachment of leaves from the stem within seven days after infection was observed in Panniyur-1. Severity of infection and much faster development of necrotic area suggest the susceptibility of Panniyur-1 and the relative tolerance of variety Kalluvally to the fungal pathogen.

Susceptibility of *P. nigrum* varieties to *Phytophthora* foot rot has been reported by earlier workers (Sarma and Nambiar, 1982 and Vilasini, 1982). The relatively better tolerance of variety Kalluvally has been reported by Alconero *et al.* (1971) and Dagade (1999). The immunity of *P. colubrinum,* a wild relative of *P. nigrum* introduced from Amazon basin has been highlighted in the reports of Alconero *et al.* (1971) and Sarma *et al.* (1991). In the present study also, the relative tolerance of Kalluvally and immunity of *P. colubrinum* were confirmed.

5.2 Isolation and quantification of proteins extracted at pH 2.8

In the present study, the protein was extracted with sodium citrate phosphate buffer at pH 2.8 and quantified using Lowry's method. Intention of isolating protein at pH 2.8 was to isolate PR proteins in black pepper and the significance of acidic pH (2.8 to 3.0) has been reported by various workers (Gianinazzi *et al.*, 1977; Fraser, 1981 and Linthorst, 1991). At this pH, most of the other proteins get denatured while PR proteins are highly resistant to proteolytic enzymes and are predominantly present in intercellular fluid of the leaves. The intercellular fluid of tissue surrounding necrotic region would have a relatively low pH as a result of tissue collapse and release of vacuolar acids and acidic protease activity. The PR proteins appears to be well adapted to function in this environment. These PR proteins are selectively extractable at low pH where they remain soluble and majority of the other leaf proteins are not (Gianinazzi *et al.*, 1977; Fraser, 1981 and Van Loon, 1985).

Fluctuation was observed in the protein content recorded for *P. nigrum* and *P. colubrinum* even in the healthy plants. In Panniyur-1, the protein level in healthy plants ranged from 18 to 22 mg/ ml of extract while it ranged between 16.8 and 17.7 mg/ ml of extract in variety Kalluvally. *P. colubrinum* recorded protein level in between 12.9 and 14.0 mg/ ml of extract (Table 5). This difference among species and varieties may be due to their genetic make up. The high phenol content in the plants of *Piper* species (Dagade, 1999) might have also contributed to the fluctuation.

After infection with *P. capsici,* the protein content was found reduced for *P. nigrum* varieties. Reduction in protein content to the extent of 20 per cent was noticed in the variety Panniyur-1 while in Kalluvally, protein depletion was to the extent of 15 per cent. Reduction in the amount of protein content can be explained by assuming that the transcription mechanism of the cell was altered due to infection by the organism. In the present study, samples were collected from the near infected area and hence it is also possible that part of the total amount released from the cell organelle may be denatured in the near infected area. In *P. colubrinum*, the protein content did not show much variation even after infection with *P. capsici*. This data along with the absence of necrotic area in *P. colubrinum* favour the statement that it is immune to infection.

5.3 Protein profile in relation to infection with *P. capsici*

5.3.1 PAGE analysis

Distinct banding pattern was observed in PAGE analysis of basic proteins extracted at pH 2.8 for varieties of *P. nigrum* and *P. colubrinum* and there was no separation of protein bands in the anionic system attempted. Eariler workers

have also reported better separation of PR proteins extracted at pH 2.8 in the cationic system. Granell *et al.* (1987) could obtain separation of 10 PR proteins in tomato following cationic system. In the present study, the zymogram indicate that the variety Panniyur-1was distinct from variety Kalluvally with respect to two proteins of Rm value 0.40 and 0.43. Both the healthy and infected ones showed same banding pattern upto fifth day after infection.

The banding pattern observed for basic proteins in *P. colubrinum* was entirely different from that of *P. nigrum* except for a single band of Rm value 0.50 (Figs. 4 to 8). Since *P. colubrinum* is a wild relative of *P. nigrum,* the difference in protein profile observed is quite natural. The protein profile observed in *P. colubrinum* did not show any change in relation to infection with *P. capsici* throughout the experiment.

Expression of an additional band with Rm value 0.56 was observed in *P. nigrum* varieties (Panniyur-1 and Kalluvally) on the sixth day after infection with fungus (Fig.9 and Plate 3). The foliar symptoms were well spread within six days and any senescence related protein that is resistant to proteolytic enzyme may also get expressed here. However, it was interesting to note the expression of this particular protein in *P. colubrinum* throughout the experiment irrespective of infection with organism. Expression of this new protein of low molecular weight in the infected plants indicate its relation with pathogenesis. This is further confirmed by its expression in *P. colubrinum*, the resistant species irrespective of inoculation with the organism. Various workers have reported the expression of such PR proteins in different crop species. (Pierpoint, 1986; Joostein and Dewit, 1989; Cutt and Klessig, 1992; Hertz *et al.*, 1994 and Bera and

Purkayastha, 1997). Expression of such PR proteins in infected plants and in resistant species confirm their relation with pathogenesis.

Several PR proteins have been reported so far and many of them get accumulated in healthy plants also (Fraser, 1981). The PR protein accumulation may be triggered by biotic stress and the infection with *Phytophthora* in black pepper might have stimulated the signal for expression of an additional protein. (Fig. 9 and Plate 3). In the present study, the additional protein was found expressed in *P. nigrum* varieties only on the sixth day after infection. This might be due to the delayed / lesser amount of such protein synthesis in *P. nigrum* owing to its susceptibility to disease. The low sensitivity of Coomassie stain in the methodology adopted for PAGE analysis might have also contributed to the poor detection as compared to the SDS - PAGE analysis. The pathogen is so virulent that it had infected the whole leaf lamina by the time the specific PR protein was transcribed by the plant in sufficient quantity. This suggests that the susceptibility of *P. nigrum* could be minimised through better expression of this protein through genetic manipulation. Detection and characterisation of PR poteins and their later exploitation for genetic manipulation of crop plants have been reported by various workers (Cutt *et al.*, 1989; Samac *et al.*, 1990; Dewit, 1992; Ji and Kuc, 1995; Chamnongpol *et al.*, 1996 and Koiwa *et al.*, 1997).

5.3.2 SDS- PAGE analysis

The SDS-PAGE analysis carried out for detecting the expression of polypeptides at pH 2.8 in the plant samples has given significant results. The silver

staining procedure adopted for SDS-PAGE analysis helped easy detection of additional protein bands in the present study.

The varieties of *P. nigrum* (Panniyur-1 and Kalluvally) expressed distinct banding pattern in SDS-PAGE analysis. Among the 15 bands expressed in these two varieties, 10 were found shared by healthy plants of both the varieties.

The variety Kalluvally was distinct from Panniyur-1 with respect to three bands of molecular wt. 14.3 kD, 8.8 kD, 7.0 kD while the variety Panniyur-1 was unique with the absence of these bands and presence of a unique low molecular weight (5.5 kD) band (Fig. 10 and Plates 4 and 5). *P. colubrinum* showed an entirely different pattern as expected due to the difference in genetic configuration. Sixteen distinct bands were observed for *P. colubrinum* out of which only six were shared with the varieties of *P. nigrum.*

The results obtained for SDS-PAGE analysis for infected plants that have shown foliar symptoms were quite interesting. Infected plants of Panniyur-1 showed presence of two additional bands (Plate 12 and Fig.14) within five days after infection and were totally absent in healthy plants. It was also observed that one of the new bands (No. 9) got expressed in infected plants of Kalluvally even on the second day after infection (Fig.11). The second additional band (Plate 11 and Fig.13) appeared in Kalluvally on the fourth day after infection. This indicates that some additional polypeptides/ proteins are induced under infected condition in *P. nigrum.*

In the present study, the newly expressed proteins in *P. nigrum* varieties was evidenced as two polypeptide bands of molecular weight 16.5 kD and 8.0 kD.

These additional/modified polypeptides might have a role in the development of symptom or in the disease resistance mechanism. The behavior of *P. colubrinum* confirms their role in the defence mechanism of *Piper* species. The results indicate that two additional bands found expressed in the infected plants of *P. nigrum* were already present in *P. colubrinum* irrespective of infection with the pathogen. No specific induction was found necessary for *P. colubrinum* for expression of these polypeptides. Since *P. colubrinum* is the only one species of *Piper* showing absolute resistance to infection with *P. capsici*, the presence of these polypeptide in natural condition and induction of the same in *P. nigrum* only under infected condition indicate the relevance of these additional bands in the resistance mechanism. In the present study, plants of *P. colubrinum* did not show any symptom when infected with live culture of the organism and incubated under optimum condition.

The mechanism for resistance may be encoded in the genome of living organism. The variation in the expression of genes may be the difference in defence mechanism and tolerance level among individuals. Several molecules may be involved in the activation of transcription of genes encoding the PR proteins (Vera and Conjero 1989; Enyedi *et al.*, 1992; Nielsen *et al.*, 1994; Meuwly *et al.*, 1995; Kauss and Jeblick, 1996; Du and Klessig, 1997)

Among the two varieties of *P. nigrum*, expression of additional polypeptide/ protein was observed earlier in the variety Kalluvally. The first protein got expressed on the second day itself, while it was expressed only on the fifth day in variety Panniyur-1. Better tolerance of Kalluvally (Plate 7 and Fig.11) over

Panniyur-1 was also observed in our earlier studies (section 5.1) wherein the foliar symptoms developed faster in variety Panniyur-1. The better expression of PR proteins in variety Kalluvally might have delayed the development of foliar symptom.

5.4 Pathogenesis related enzymes in black pepper

In the present study three pathogenesis related enzymes were assayed in *Piper* spp. in relation to infection with *Phytophthora capsici*.

5.4.1 Beta-1,3-glucanase activity

The glucanase activity in *P. nigrum* varieties ranged between 0.10 and 0.24 enzyme unit/ mg protein/ 10 min under healthy condition. The activity recorded was more in variety Kalluvally than in variety Panniyur-1 (Table 7 and Fig. 16). The enzyme activity was found elevated in both the varieties upon infection with *P. capsici*. However, at later stages of infection when symptoms were severe on the leaf, the rate of enzyme activity was found to decline. The wild relative *P. colubrinum* recorded a significantly higher activity for ß-1,3-glucanase. These results indicate the relation of this PR enzyme with foot rot disease in black pepper. Among the *P. nigrum* varieties studied, the less susceptible variety Kalluvally recorded higher enzyme activity and the elevation in enzyme activity upon infection indicates its role in the defense mechanism of black pepper against the foot rot disease.

The fungal pathogen *P. capsici* has ß-1,3-glucan as its cell wall component. Glucanase activity in black pepper might be defending the plant against the pathogen by degrading its cell wall. Such a host pathogen interaction has been reported in different crop species (Pan *et al.*, 1991; Vierheilig *et al.*, 1995; Cachinero *et al.*, 1996; Guzzo and Martins, 1996 and Rehimi *et al.*, 1996). It is suggested that a signal is produced which systemically regulates glucanase activity and the level of enzyme encountered by the fungus at the time of challenge and shortly there after may be critical to fungal development in the plant.

P. colubrinum, the wild relative of *P. nigrum* recorded a significantly higher activity for beta-1,3- glucanase even under healthy condition. It was also not possible to induce foliar symptom in this species by repeated inoculation with the live culture of *P. capsici*. This again confirm the role of ß-1,3-glucanase activity in the defence mechanism of *Piper* species. In a recent study, Dagade (1999) has reported lower activity of this enzyme in *P. colubrinum*. The contradictory result might be due to difference in the incubation temperature adopted in the study and this highlights the importance of standardizing optimum condition for assessing the enzyme activity.

5.4.2 Chitinase activity

The chitinase activity recorded for *Piper* spp. in the present study was almost nil irrespective of infection with the fungal pathogen. No activity was recorded even at substrate level (chitin) of 5 mg/ml. Thus, in the present study, chitin is not found figured in the defense mechanism of black pepper. Kunio *et al.* (1990) have studied the potent chitinolytic activity in 329

samples from 87 families and reported the absence of chitinase activity in many plant species.

5.4.3 Phenylalanine ammonia lyase activity

The PAL activity in *P. nigrum* varieties studied was found influenced by infection with *P. capsici*. Among the two varieties studied, Kalluvally recorded higher PAL activity. In both the varieties, the enzyme activity got elevated upon infection with the fungus. However, on the later days of infection, the rate of activity showed a decreasing trend. In *P. colubrinum,* the PAL activity recorded was lower than that in *P. nigrum* and it was found not affected by the interference of *P. capsici*

The results indicate the influence of fungal infection on the PAL activity in *P. nigrum* varieties. The hyphal wall or its product can act as an elicitor that stimulates PR enzymes. Phenylalanine ammonia lyase is identified as a key enzyme in biochemical pathway involved in formation of phytoalexin which help the plant to respond to biotic stress. The role of PAL activity in the defence mechanism of plants has been reported by various workers. It has been reported to have an involvement in the calcium signal pathway in elicitor mediated response in tobacco. (Vurro and Ellis, 1997; Peters *et al.*, 1998 and Sharan *et al.*, 1998)

In the present study, the PAL activity was found elevated immediately after infection and after two to three days, it showed a decreasing trend. Such a response has been reported for PR enzymes in various other crops also. (Matsuda *et al.*, 1996). They have reported high PAL activity in more resistant varieties of sunflower and marked increase in enzyme activity soon after inoculation which was more pronounced in resistant cultivars.

The high level of PAL activity in post infection period resulting in more effective protection of plants against pathogen has been reported (Yarulina *et al.*, 1997). Phenylalanine ammonia lyase is reported to have positive role in lignification and there by enhancing effective plant protection. He has also reported a higher PAL activity on early days after infection, which is in conformity with the response observed in black pepper.

Difference in varietal response to *Phytophthora capsici* has been reported earlier in capsicum species (Mozzetti *et al.*, 1997). Peltonen (1998) has reported the role of PAL in active defence in barley and wheat in response to pathogen attack but not in plants with induced resistance and in those when infected with a non-pathogen. Such a host pathogen relationship is also obsered in *Piper* species in relation to *Phytophthora* infection. The variety Kalluvally with better tolerance recorded higher PAL activity than the susceptible variety Panniyur-1. The organism is a non-pathogen with respect to *P. colubrinum* and thus the PAL activity in this species did not respond to infection with the fungus.

Among the three PR enzymes studied, ß-1,3-glucanase and PAL activity in black pepper were found influenced by infection with the fungus. Glucanase activity was found elevated upon infection in both the varieties studied and reflected as an increase in the end product. This may influence the normal metabolism in which the PAL activity is directly involved. In the present study, the PAL activiy was found to increase initially and then reduced in both the varieties. This could be related to glucanase activity detected as an end product inhibition in the production of trans cinnamic acid, which is having an important role in normal metabolism of the plant. The lower concentration of protein (Table 5) after infection and higher activity of glucanase and lower activity of PAL at later stages of infection can thus be related.

Summary

6. SUMMARY

The study on 'Evaluation of pathogenesis related (PR) proteins in relation to *Phytohthora* foot rot in black pepper (*Piper nigrum* L.)' was conducted as part of the MSc programme in the Department of Plantation Crops and Spices at the College of Horticulture, Vellanikkara. The work was carried out at the Centre for Plant Biotechnology and Molecular Biology. Facilities available with the Biochemistry laboratory of the College was also utilised.

Two species of *Piper* namely, *P. nigrum* and *P. colubrinum* were evaluated for their response to infection with the fungal pathogen, *Phytophthora capsici*. The salient findings of the study are summarised here under.

P. nigrum varieties (Panniyur-1 and Kalluvally) and *P. colubrinum* responded differently to infection with *P. capsici*. The infection was severe and earlier in Panniyur-1 than in the variety Kalluvally. *P. colubrinum* did not show any foliar symptom upon infection with the fungal pathogen.

Electrophoretic studies and enzyme assays were carried out to evaluate expression of pathogenesis-related proteins. The two *species* differed significantly in the electrophoretic banding pattern and enzymes studied.

Upon infection with the fungus, the protein content extracted at pH 2.8 was found reduced in *P. nigrum* varieties. The variety Kalluvally showed 15 per cent reduction while it was to the extent of 25 per cent in Panniyur-1. In *P. colubrinum,* the difference in protein content observed was insignificant when inoculated with *P. capsici*.

Out of the two systems (anionic and cationic) attempted for PAGE analysis of PR proteins, only cathodic system showed distinct banding pattern .Both the species showed distinct banding pattern in PAGE analysis. Upon infection with the fungus, the banding pattern in *P.nigrum* differed on the sixth day and showed expression of an additional band of Rm value 0.56. This additional protein was found natively present in *P. colubrinum* irrespective of infection with the pathogen.

SDS-PAGE analysis of the proteins extracted at pH 2.8 also showed distinct banding pattern in *P. nigrum* and *P. colubrinum*. On the second day of infection, one additional band of 16.5 kD was found expressed by the infected plants of Kalluvally. The same protein was also found expressed in healthy *P. colubrinum*. This protein once expressed was persistent upto sixth day after infection. Second additional band (8.0 kD) was expressed on the fourth day in Kalluvally and was also shared by *P. colubrinum*. Variety Panniyur-1 did not show expression of any additional protein upto fourth day. But on the fifth day after infection, Panniyur-1 expressed both the additional bands, which were already expressed by the infected plants of Kalluvally. Thus these two proteins/polypeptides (16.5 kD and 8 kD) are detected as the PR proteins in black pepper with respect to *Phytophthora* foot rot infection.

Influence of three pathogenesis related enzymes was studied in relation to *Phytophthora capsici* in *Piper* species.

The glucanase activity in variety Kalluvally was found to be relatively higher than in variety Panniyur-1 and showed an increasing trend up to third day after infection. Increasing trend was observed in variety Panniyur-1 upto fifth day.

Beta-1,3-glucanase activity in healthy and infected plants of *P. colubrinum* did not vary significantly but enzyme activity in this species was higher than that expressed by *P. nigrum.*

Chitinase activity was observed to be almost nil in healthy and infected plants of *P. nigrum* and *P. colubrinum.*

PAL activity assessed in leaf samples of *P. nigrum* and *P. colubrinum* showed interesting results. In variety Panniyur-1, PAL activity was increased upto third day while in Kalluvally it showed an increasing trend upto second day. In *P. colubrinum,* the PAL activity was lower as compared to *P. nigrum* and was found not influenced by inoculation with pathogen.

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* Originals not seen

EVALUATION OF PATHOGENESIS RELATED PROTEINS IN RELATION TO *PHYTOPHTHORA* FOOT ROT IN BLACK PEPPER [*Piper nigrum* L.]

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ABSTRACT OF THE THESIS

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ABSTRACT

Black pepper, the most valued and popular spice of India is often severely infected by the fungal pathogen, *Phytophthora capsici* causing 'foot rot' disease. Almost all the cultivated species and varieties of *Piper* are found susceptible to the disease while *P. colubrinum* is the only species expressing resistance to infection. Little is known about the host-pathogen interaction and is the main limiting factor in formulating strategies for disease management in this valuable crop. In the present study, an attempt was made to unravel the defense mechanism in *Piper* spp. through studying the pathogenesis related proteins in relation to Phytophthora foot rot disease.

Two species of *Piper* viz, *P. nigrum* and *P. colubrinum* were infected with the fungal pathogen and the reaction of the host was studied in relation to development of disease symptom, expression of PR proteins and activity of PR related enzymes. Both the varieties of *P.nigrum* studied, namely Panniyur–1 and Kalluvally expressed foliar symptoms within three days after inoculation with the fungus while *P. colubrinum* remained healthy throughout the experiment. The variety Panniyur–1 was found to be more susceptible to the infection.

Significant difference was observed in the expression of PR proteins and activity of PR enzymes assayed. Upon infection with the fungus, the protein extracted at pH 2.8 was found reduced in *P. nigrum* varieties (Panniyur-1 and Kalluvally) while no significant difference was observed for *P. colubrinum*. In PAGE analysis, five basic proteins were found expressed in the healthy plants and

the banding pattern was unique for *P. nigrum* varieties and for *P. colubrinum*. An additional protein of Rm value 0.56 was found expressed in the infected plants of *P. nigrum* on the sixth day after infection with *P. capsici*. This additonal protein was also found expressed in the healthy plants of resistant species, *P. colubrinum* indicating its role in the defence mechanism.

SDS-PAGE analysis of the PR proteins extracted at pH 2.8 also showed distinct banding pattern in *P. nigrum* varieties and *P. colubrinum*. Two additional bands (16.5 kD and 8.0 kD) were found expressed in *P. nigrum* varieties upon infection with the fungal pathogen. The relatively tolerant variety, Kalluvally recorded early expression of these specific PR proteins. It was also interesting to note the expression of these proteins in the resistant species *P.colubrinum* indicating their significance in the defence mechanism.

The activity of the PR enzyme, ß-1,3-glucanase was found to be relatively high in *P. colubrinum*. Among the *P.nigrum* varieties, Kalluvally recorded better activity related to its better tolerance. The PAL activity increased substantially in *P. nigrum* varieties, immediately after infection with the fungal pathogen and the rate of increase later declined. The resistant species *P. colubrinum* recorded low PAL activity. Chitinase activity was almost nil in healthy and infected plants of *P. nigrum* and *P. colubrinum*. Thus among the pathogenesis related enzymes studied, ß-1,3-glucanase and phenylalanine ammonia lyase were found to have a positive role in the defence mechanism of black pepper in relation to *Phytophthora* foot rot disease.