CHARACTERIZATION OF PATHOGENESIS RELATED PROTEINS FOR ANTHRACNOSE RESISTANCE IN VEGETABLE COWPEA, *Vigna* spp.

By

A. AGATHA SHINY

(2011-11-174)

THESIS

Submitted in partial fulfillment of the requirement

for the degree of

MASTER OF SCIENCE IN AGRICULTURE

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

Kerala Agricultural University, Thrissur

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR - 680 656

KERALA, INDIA

2013

DECLARATION

I, hereby declare that this thesis entitled **"Characterization of pathogenesis related proteins for anthracnose resistance in Vegetable Cowpea, *Vigna* spp."** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

Agatha Shiny

Vellanikkara,
17-08-2013.

A. Agatha Shiny
(2011-11-174)

CERTIFICATE

Certified that this thesis entitled **"Characterization of pathogenesis related proteins for anthracnose resistance in Vegetable Cowpea, *Vigna* spp."** is a bonafide record of research work done independently by **Ms. A. Agatha Shiny** (2011-11-174) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

Vellanikkara,
17-08-2013.

Dr. Deepu Mathew
(Major Advisor)

Assistant Professor
Centre for Plant Biotechnology
and Molecular Biology
College of Horticulture
Vellanikkara
Thrissur-680 656.

CERTIFICATE

We, the undersigned members of the Advisory Committee of **Ms. A. Agatha Shiny (2011-11-174)** a candidate for the degree of **Master of Science in Agriculture** with major field in **Plant Biotechnology,** agree that the thesis entitled **"Characterization of pathogenesis related proteins for anthracnose resistance in Vegetable Cowpea, *Vigna* spp."** may be submitted by **Ms. A. Agatha Shiny** in partial fulfillment of the requirements for the degree.

18/10/13

Dr. Deepa Mathew
(Major Advisor)
Assistant Professor
Centre for Plant Biotechnology
and Molecular Biology
College of Horticulture, Vellanikkara

18/10/13

Dr. P.A. Valsala
(Member, advisory committee)
Professor and Head
Centre for Plant Biotechnology
and Molecular Biology
College of Horticulture, Vellanikkara

18/10/13

Dr. P.A. Nazeem
(Member, advisory committee)
Professor
Centre for Plant Biotechnology
and Molecular Biology
College of Horticulture, Vellanikkara

18/10/13

Dr. Abida P. S.
(Member, advisory committee)
Associate professor (Sel. Gr.)
Centre for Plant Biotechnology
and Molecular Biology
College of Horticulture, Vellanikkara

18/10/13

Dr. Sally K. Mathew
(Member, advisory committee)
Professor
Department of Plant Pathology
College of Horticulture, Vellanikkara

EXTERNAL EXAMINER.
Dr. S. Backiyarani
Principal Scientist. NRCB
Thirunelveli. T.Nadu.

Acknowledgement

I thank the ***Lord God Almighty*** *whose grace has protected, strengthened, guided and blessed me with knowledge and understanding during this course of study.*

I am ineffable in expressing my deep sense of gratitude to ***Dr. Deepu Mathew,*** *Assistant Professor, CPBMB, College of Horticulture, Vellanikkara, my major advisor, for his inspiring guidance, critical comments, timely support, motivation and encouragement throughout the course of study and in the preparation of this thesis.*

My sincere thanks are due to ***Dr. P.A. Valsala,*** *Professor and Head, CPBMB, College of Horticulture, Vellanikkara, member of my advisory committee for the kind support and suggestions given for the improvement of the thesis.*

I take this opportunity to express my indebtedness to ***Dr. P.A. Nazeem*** *Professor, CPBMB, member of my advisory committee for the untiring support, motherly care, inspiring teaching and valuable guidance during my course of study. It would have been difficult for me to complete the research work without her guidance, encouragement and efforts.*

I am highly obliged to express my gratefulness to ***Dr. Abida P.S.,*** *Associate professor, CPBMB, member of my advisory committee for her constant caring advices, timely support, encouragement and guidance for the successful completion of my research work.*

I also owe my deep sense of gratitude and sincere thanks to ***Dr. Sally K. Mathew,*** *Professor, Department of Plant Pathology, College of Horticulture, Vellanikkara, member of my advisory committee for the valuable guidance and support*

rendered in the conduct of the experiment. I am also grateful to her for the critical evaluation of thesis.

I sincerely thank ***Dr.M.R. Shylaja,*** *Professor, CPBMB, my year coordinator for the careful guidance, constant support and valuable advices during my course of study.*

My sincere thanks to ***Dr. R. Kesavachandran,*** *Professor, CPBMB and all the other course teachers of the MSc degree programme for their support and the great knowledge delivered during course work.*

I thank ***Mr.P.K. Sreekumar,*** *Farm Assitant, CPBMB, for the help in photography. I am also thankful to* ***Dr. Haseena Bhaskar,*** *Associate Professor, Agricultural Entomology, CoH for providing the microscopic facility.*

My heartfelt thanks to Ms.Jisha, research associate, Department of Plant Pathology who sincerely guided me in the pathology work. I am very much thankful to the workers, Balan chaetan, Remmi chaechi, Shobha chaechi, Densi chaechi and Sumathi chaechi for assisting me in raising the crop.

I also wish to express my sincere and heartful thanks to all the non-teaching staff members, research associates and workers of CPBMB, Mrs.Shylaja, Mrs.Simi, Mrs.Usira, Vipin,, Anu, Geethu, Amritha, Divya, Sinjula, Rajee, Tina, Nithin, Sarah chaechi, Savitri chaechi, Sugaratha chaechi, Priya chaechi and Shoba chaechi for their whole-hearted cooperation, timely assistance and constant support.

I appreciate and acknowledge the facilities provided at CPBMB and Bioinformatics Centre for the successful completion of my research work. I also acknowledge all the other facilities, research grant and Junior Research Fellowship provided by the University.

I take this opportunity to thank all my batchmates, senior and junior friends especially Saadhana, Pranita, Parimal, Ravindra, Pujaitha, Subhashini, Manibala, Sharda, Rohini, Jomily, Manjusha, Smitha, Neethu, Anusha, Meera, Renju, Reshmi, Ashok, Amar, Jusna, Shreeja, Vishali, Shital, Renuka, Sachin, Audumbar, Datta, Meena, Shriram, Deemanth, Yashwanth, Devi and Judi whose warm concern, constant support, encouragement, love and affection rendered during my stay in KAU was instrumental for my progress.

Above all, I am forever beholden to my loving parents, brother, relatives and other friends their unfathomable love, boundless affection, personal sacrifice, incessant inspiration and constant prayers, which supported me to stay at tough tracks.

A. Agatha Shiny

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

List of figures contd...

LIST OF PLATES

List of tables contd...

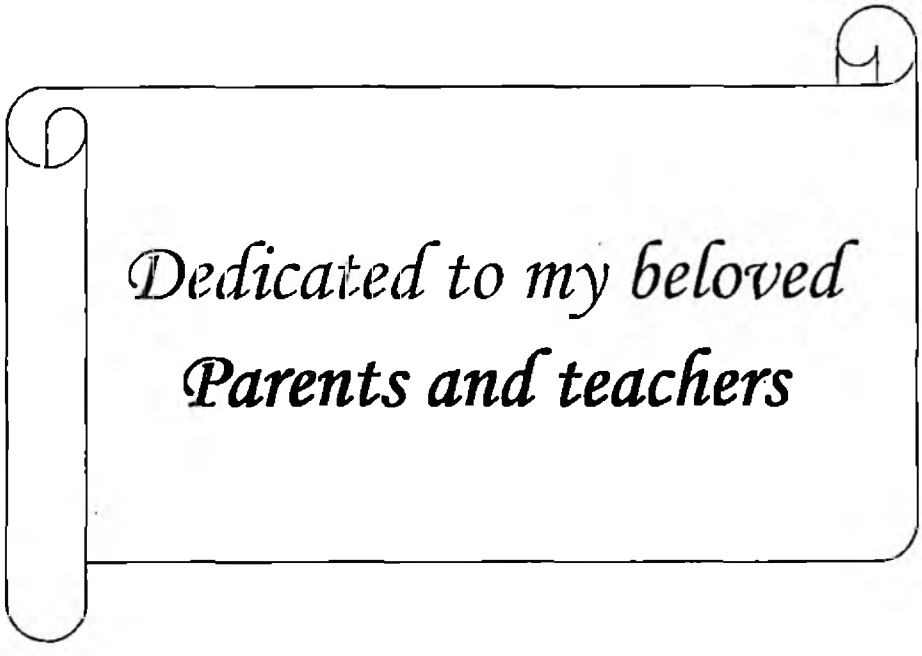
LIST OF ANNEXURES

Annexures	Title
I	Details of laboratory equipment items used for the study
II	Media preparation
III	Buffers for protein extraction
IV	Chemicals for SDS-PAGE analysis
V	Defense enzyme assay

ABBREVIATIONS

APS	-	Ammonium Per Sulphate
C.lindemuthianum	-	*Colletotrichum lindemthianum*
CPBMB	-	Centre for Plant Biotechnology and Molecular Biology
cm	-	Centimeter
°C	-	Degree celsius
g	-	Gram
hr	-	Hour
HCl	-	Hydrochloric acid
pH	-	Hydrogen ion concentration
IARI	-	Indian agricultural research institute
kD	-	Kilo dalton
KAU	-	Kerala agricultural university
KVK	-	Krishi vigyan kendra
L	-	Litre
MALDI-TOF/ MS	-	Matrix Assisted Laser Desorption/ Ionization - Time of Flight Mass Spectrometry
m	-	Meter
µg	-	Micro gram
µl	-	Micro litre
µM	-	Micro molar
mA	-	Mili amphere

mg	-	Mili gram
ml	-	Mili litre
mm	-	Mili meter
mM	-	Mili molar
min	-	Minutes
M	-	Molar
nm	-	Nano meter
NGA	-	Neopeptone Glucose agar
TEMED	-	N, N, N', N' Tetramethylene ethylene diamine
PR	-	Pathogenesis related
PAGE		Polyacrylamide gel electrophoresis
pm	-	Post meridiem
PDA	-	Potato dextrose agar
RARS	-	Regional agricultural research station
rpm	-	Revolutions per minute
Sacc. And Magn.	-	Saccardo and Magnus
sec	-	Second
SDS	-	Sodium dodecyl sulfate
t/ha	-	Tonnes per hectare
V. unguiculata	-	*Vigna unguiculata*
V	-	Volt
v/v	-	Volume in volume
Walp. and Verdc.	-	Walper and Verdcourt


Dedicated to my beloved
Parents and teachers

Introduction

1. INTRODUCTION

Cowpea (Family: Fabaceae) is one of the highly nutritious pulse as well as vegetable crop with 22.9-77.6 per cent proteins, 9.4-64.3 per cent carbohydrates and 0.1-0.3 per cent fats (Abdelatief, 2010). It is an important component of ancient farming system and believed to have originated in West Africa between five to six thousand years ago. Cowpea was introduced to the Indian subcontinent approximately 2000 to 3500 years ago (Padulosi, 1993). This tropical and sub-tropical legume is drought tolerant and best suited for the semi-arid regions with temperatures varying between 20 to 35°C. In the 21st century with threats of global warming and climate change, cowpea forms an important component in multiple cropping systems by fixing the atmospheric nitrogen, increasing the phosphorus availability and revitalizing the degraded soils.

Among the 20,000 germplasm samples collected and evaluated worldwide, India stands second in the cowpea diversity with 2075 accessions, second only to Nigeria with 3221 accessions, which is the world's largest producer of cowpea (Ng, 1990). In India, cowpea is cultivated in 0.5 million hectares and has a productivity of only 600 – 670 kg/ha (Ahlawat and Shivakumar, 2005). Even though the crop is tolerant to heat, drought, moderate shade and low fertile soil, it has little tolerance to salinity and intolerant to frost and waterlogged or flooded conditions. Having soft and succulent tissue with extrafloral nectaries on its petioles and leaflets, it is attractive to many insects. Under wet humid conditions, cowpea is also susceptible to many diseases that affect legumes (Valenzuela and Smith, 2002).

Cowpea is one of the main hosts for the fungal disease, anthracnose caused by the fungus, *Colletotrichum lindemuthianum* (Sacc. and Magn.) Br. and Cav. under warm to cool and moist conditions. The cowpea anthracnose locally known as vine blackening is a severe disease in vegetable cowpea especially affecting the pole types, leading to yield loss of 35 -50 per cent in the mature

plants and total crop loss in seedlings (Varma and Langerak, 1988). The disease also hampers the grain quality and marketability of the crop.

To combat the invasion of the fungus and for its survival, the plant inherently adopts many morphological and biochemical defense mechanisms. Pathogenesis-related proteins (PR) are structurally diverse groups of antimicrobial plant proteins induced by the pathogen attack in the plant and are toxic to the pathogens. They are widely distributed in plants in trace amounts and are produced in much greater concentration following pathogen attack or stress. Earlier study by Kumar (1999) has shown that the cowpea variety Kanakamony is immune and Pusa Komal is highly susceptible to the anthracnose disease. Comparing the protein profiles of the resistant and susceptible varieties after artificial infection may unfold the presence of any PR proteins involved in the resistance mechanism of the plant. One highly susceptible variety Pusa Komal and immune variety Kanakamony, both belonging to *Vigna unguiculata* ssp. *cylindrica* and one highly susceptible variety Lola and resistant variety Arimbra Local, both belonging to V. u. ssp. sesquipedalis are compared in the present study to understand the differentially expressed PR proteins and to get a cross verification among the different sub-species. The information generated could be further utilized for the identification of the gene responsible for the resistance and improvement of the high yielding susceptible varieties by genetic engineering.

In this context, the following objectives are formulated for the present research entitled, "Characterization of pathogenesis related proteins for anthracnose resistance in vegetable cowpea, *Vigna* spp."

1. To develop the protein profiles of resistant and susceptible bush and pole genotypes through SDS-PAGE at different stages of infection
2. To identify the differentially expressed proteins by MALDI-TOF through outsourcing followed by *in-silico* analysis for protein annotation.

Review of Literature

2. REVIEW OF LITERATURE

Anthracnose is a severe disease in vegetable cowpea caused by *Colletotrichum lindemuthianum* (Sacc. and Magn.) Br. and Cav. especially affecting the pole types by principally infecting the stem of the plant. This disease is reported to cause 35 -50 per cent crop loss in mature plants and if occurs at seedling stage, the loss will be complete (Varma and Langerak, 1988). In India, the incidence of the disease was first reported from Maharashtra in 1966 (Rao, 1966). From Kerala a yield loss up to 58.8 per cent was reported due to this disease (Kumar, 1999). The causal agent may induce up to 100 per cent yield loss when a susceptible variety is grown in a region with cool to moderate temperatures and abundant moisture (Shao and Teri, 1985). Hence as an effort to tackle the situation, cowpea genotypes were screened for resistance to this disease by Kumar (1999). Comparative studies on the protein profile of resistant and susceptible varieties may throw light on the identification of pathogenesis related protein, which is one of the main factors controlling the resistance.

2.1. General background

2.1.1. Salient features of cowpea varieties used in the study

2.1.1.1. Pusa Komal

As reported by Dharm and Patel (1990) Pusa Komal is a widely adaptable, high yielding, early flowering and photo insensitive cowpea variety developed by IARI in 1986 for vegetable purpose. It is a product of P-85-2 and P-426 cowpea strains and resistant to bacterial blight. It bears non-fibrous, long, cylindrical, bright green and tender pods with an average length of 18-22 cm. The plant is compact and bushy without vines or trailers yielding 17.2 t/ha. Duration of the crop is around 60-65 days. Kumar (1999) observed that Pusa Komal is highly susceptible to anthracnose disease with cent per cent disease incidence and severity.

2.1.1.2. Kanakamony

Rajan and Prameela (2004) documented that Kanakamony is a high yielding dual purpose variety developed by RARS, Pattambi by the pure line selection of Kunnamkulam Local in 1977. The plant is bushy with medium long and dark green pods with an average length of 15-18 cm and yields 18-20 t/ha. Duration of the crop is 70-80 days and it is best suited for Kharif and summer seasons. It is found to be immune to anthracnose disease with no disease incidence by Kumar (1999).

2.1.1.3. Lola

Rajan and Prameela (2004) documented that Lola is a high yielding (20 t/ha) vegetable cowpea developed at KAU through the pure line selection of the landrace Kanakari Local in the year 2000. It has smooth, glossy and light green coloured extra long pods (50-57 cm) with purple tip. The plant is pole type with duration of 120-125 days and best suited for cultivation in Kerala during June - September and December - March seasons. The variety is observed to be highly susceptible to anthracnose disease.

2.1.1.4. Arimbra Local

Arimbra local is a local variety of cowpea from the Arimbra village of Malappuram district. This landrace has been previously reported to be resistant to anthracnose from farmer's fields and was subsequently tested at KVK Malappuram (Kerala Agricultural University) through On Farm Trials (OFTs) (KAU, 2011). It has light green, non-fibrous, slender and extra long pods (45-50 cm). The growth habit of the plant is pole type and grows for a long duration of 140-150 days producing a regular yield of 18-20 t/ha.

2.1.2. Symptomatology of anthracnose disease

Anthracnose disease of cowpea was reported for the first time in Islamabad by Qureshi *et al.*, (1985).

Onesirosan and Barker (1971) and Williams (1975) observed individual lenticular to circular, brown to tannish pink sunken lesions with dark red margins on the stem. Later, the lesions developed in to large, spreading, dark lesions which griddled stem, branches and petioles. Coalescence of lesions led to chlorosis and death of the leaves. Brown sunken lesions also appear on pods.

Kumar (1999) studied the detailed symptomatology of the anthracnose disease and has observed that in wet conditions, reddish brown streaks appear on stem. In seedlings, the streaks coalesce spreading over stem resulting in wilting of the plant. In mature plants, individual lenticular to oval light brown lesions with dark reddish brown margins appear. Reddish brown streaks appear initially on lower surface of leaves and later on extend to upper surface also. In few plants, numerous minute black dots (mildew appearance) appear on leaf lamina. As the disease advanced, these lesions coalesced and extended all over the stem and vines leading to drying up of the stem and veins. Finally leaves become chlorotic and defoliates. The whole plant appears chlorotic and stunted. Reddish brown streaks on the pods results in rotting of the pods and the seeds become shriveled and discolored. Black fruiting bodies of the fungus were also observed on the dried pods.

2.1.3 Morphological and cultural characters of *C.lindemuthianum*

Anthracnose disease of cowpea caused by the fungus, *Colletotrichum lindemuthianum* belongs to Melanconiales of class Coelomycetes under Deuteromycotina. Its perfect stage is *Glomerella lindemuthianum* (Sacc. and Magn.) in family Polystigmataceae, order Sphaeriales and class Pyrenomycetes of Ascomycotina (Agrios, 2006).

Watanabe (2010) has documented, *C.lindemuthianum* produce acervuli only on natural media containing bean straws and agar. The acervuli in culture is pale brown, hemi-spherical composed of conidiophores, conidia and indistinct setae. Conidiophores are hyaline, simple or branched, erect, bearing 2-3 conidia at phialicles. The conidia phialosporous, hyaline, single celled and cylindrical. Setae are brown, thick walled and sharp at the apex having 2-3 septae. The dimension of the conidia is 13-17.5 x 4.7-5.3 µm. The acervuli is 200-330 µm in diameter and the setae is 65 – 107.5 X 3.7-6 µm.

The fungus is seed borne and seed transmitted. Mordue (1971) reported that the fungus can survive for at least 2 years in seed. The longevity in infected pods and seeds varies considerably depending on environmental conditions. He also suggested that temperature and humidity are the important factors in the expression of bean anthracnose. Infection is favoured by warm wet condition with a temperature of 15-26°C and humidity greater than 92%.

Tu (1983) observed that the pathogen was able to survive for at least 5 years on pods and seeds that were air-dried and kept in storage at 4°C or on dry infected plant materials left in the field in sealed polyethylene envelopes. Humidity of more than 92% or free moisture is required during all stages of conidium germination, incubation and subsequent sporulation.

Zaumeyer and Meiners (1975) reported, *C. lindemuthianum* survives as dormant mycelium within the seed coat, sometimes even within cells of cotyledons, as spores between cotyledons or elsewhere in the seed. The fungus survives in the seed as long as the seed remains viable. It also survives in infected crop residues.

Ferrante and Bisiach (1976) also reported that the infection is favoured by moderate temperatures between 13 and 26°C while Tu and Aylesworth (1980) reported that infection is favoured by an optimum temperature of 17-24°C.

Zaumeyer and Thomas (1957) reported that the dissemination and spread of the conidia and the development of severe anthracnose epidemics is favoured by wind or rain. *C. lindemuthianum* required about 10 mm of rain to establish the infection (Tu, 1981). Conidia spread may be dispersed within the crop by insects, animals and man, especially when foliage is moist.

2.2. Process of disease infection by *C. lindemuthianum*

Perfect *et al.* (2002) studied that the species of *Colletotrichum* use diverse strategies for invading host tissue, ranging from intracellular hemibiotrophy to subcuticular intramural necrotrophy. In addition, these pathogens develop a series of specialized infection structures, including germ tubes, appressoria, intracellular hyphae, and secondary necrotrophic hyphae. *Colletotrichum* species provide excellent models for studying the molecular basis of infection structure differentiation and fungal–plant interactions.

Dada *et al.* (1994) reported that, biotrophic phase is characterized by unusual, large, multilobed, multiseptate infection vesicles with elongated neck region whereas the necrotrophic phase by rapid development of invasive secondary hyphae.

Leach (1923) observed that the conidia of *C. lindemuthianum* which are round or elongated are borne on acervuli which may be present on pods, leaves, stems and branches. The mycelium is hyaline, branched and septate. A conidium takes 6-9 hours to germinate under favourable environmental conditions. The pathogen penetrates the cuticle and epidermis mechanically. Following penetration of host cells, infectious hyphae enlarge and grow between the cell wall and protoplast for 2-4 days without apparent damage to host cells.

2.3 Plant responses to fungal pathogen

When the fungus, *C. lindemuthianum* is established in a susceptible plant, yellowish spots occur as the first noticeable symptom and they later on develop into ulcerous necrotic wounds affecting all plant structures as a generalized infection in the plant (Martínez-Pacheco *et al.*, 2009).

Plants have evolved sophisticated and efficient mechanisms to prevent the invasion of their tissues by pathogens, and disease rarely occurs. One common feature of disease resistance is the rapid development of cell death at and immediately surrounding infection sites, called the Hypersensitive Response, or HR (Agrios, 2006; Goodman and Novacky, 1994).

Elliston *et al.* (1976) observed that *C. lindemuthianum* causes hypersensitive response in resistant bean cultivars with the appearance of red-brownish wounds of different sizes that are produced by the plant to delimit the spread of the pathogenic fungus and to kill it. Goodman and Novacky (1994) also explained that hypersensitive reaction is the most effective method in which the cells around the infection site rapidly necroses.

As reported by Van Loon (1997) hypersensitive response is associated with a coordinated and integrated set of metabolic alterations that are instrumental in impeding further pathogen ingress, as well as in enhancing the capacity of the host to limit subsequent infection by different types of pathogens.

Sequeira (1983) reported that in the resistant cultivars, damage caused by the pathogen also remains restricted as a result of the inducible defense mechanisms such as the accumulation of host-synthesized phytoalexin antibiotics, deposition of lignin-like material, accumulation of hydroxyproline-rich glycoproteins (HRGPs) and proteinase inhibitors, and increases in the activity of certain hydrolytic enzymes like chitinase.

Mauch and Staehehn (1989) proposed the following model for plant defense against fungal pathogens. Early in the course of infection, β-1, 3 glucanase release β -1, 3-glucans from fungal hyphae, which serve as elicitors and induce plant defense genes, such as those for extracellular chitinase. Cell lysis due to fungal invasion releases large quantities of in vacuolated chitinase. The large outflux of chitinase result in formation of a hypersensitive reaction in resistant hosts that effectively limits infection damage.

Yang *et al.* (1997) has observed that altered ion fluxes across the plant cell membrane, generation of active oxygen species, changes in the phosphorylation state of regulatory proteins and transcriptional activation of plant defense systems culminate in cell death at the site of infection, local accumulation of phytoalexins and cell wall rigidification as a result of callose, lignin and suberin deposition.

2.4 Pathogenesis related proteins (PR proteins)

Plants when exposed to pathogens such as fungi and viruses produce low-molecular-weight antimicrobial compounds called phytoalexins, antimicrobial peptides, and small proteins (e.g., thionins, defensins, heveinlike proteins, and knottin-like peptides) and up-regulate a number of antimicrobial proteins. These antifungal proteins are mainly involved in the inhibition of the synthesis of the fungal cell wall or disrupt cell wall or fungal membrane structure and/or function resulting in fungal cell lysis (Selitrennikoff, 2001).

In order to unify the naming of these proteins, to describe the full set of novel proteins in the reaction of a plant to a pathogen, and to facilitate their study and explore their role in pathology and host defense, the term pathogenesis-related proteins (PRs) was introduced in 1980 to designate "proteins coded for by the host plant but induced only in pathological or related situations" (Antoniw *et al.*, 1980)

2.3.1 Occurrence and properties

Pathogenesis-related proteins were first observed as new protein components induced by tobacco mosaic virus (TMV) in hypersensitively reacting tobacco by Gianinazzi *et al.* (1970) and recognition of the accumulation depends on biochemical properties, induction mechanism, genetic diversity, and functional significance in infected plants.

Generally, PR proteins were induced by different types of pathogens and abiotic stresses, were most often of relatively low molecular weight, preferentially extracted at low pH, highly resistant to proteolytic degradation, and localized predominantly in the intercellular space of the leaf (Redolfi *et al.*, 1983; Van Loon, 1989).

Niderman *et al.* (1995) reported these proteins occur in the vacuolar compartment or cell wall or intercellular spaces and have physicochemical properties that enable them to resist to acidic pH and proteolytic cleavage and thus survive in the harsh environments.

Van Loon (1999) discussed that localization of the major, acidic PRs in the intercellular space of the leaf seems to guarantee contact with invading fungi or bacteria before these are able to penetrate. However, in spite of numerous investigations involving injection of plants with purified proteins, in vitro tests for inhibition of pathogen growth or in vivo infection of transgenic plants, few of the inducible acidic PRs associated with SAR have been shown to possess significant anti-pathogenic activity (Lawton *et al.*, 1993; Van Loon, 1997). In contrast, hypersensitive resistance to viruses is a cellular phenomenon, acting not at the level of virus entry but limiting viral multiplication in the cytoplasm, and viral spread through symplastic connections (Van Loon, 1983).

Linthorst (1991) proposed the term PR-like proteins to accommodate proteins homologous to PRs as deduced from their amino acid sequence or on the basis of the nucleotide sequence of their corresponding cDNA or gene, but

induced principally in a developmentally controlled, tissue-specific manner. In contrast to the classical PRs, which are mostly acidic and extracellular proteins, the homologous counterparts are mostly basic and localized intra-cellular in the vacuole.

PRs and PR-like proteins may be expressed constitutively in some organs and are inducible by pathogens in other organs, i.e., acidic and basic glucanase and chitinase in floral organs (Lotan *et al.*, 1989) and leaves (Memelink *et al.*, 1990) respectively.

Leung (1992) observed these new protein components were found in small amounts in senescing leaves of flowering plants and in progressively larger quantities when necrosis was more severe. The occurrence of almost all types of PRs in various floral tissues also suggests specific physiological functions during flower development.

2.3.2 Induction and Expression

When plants are locally infected with a pathogen inducing hypersensitive necrosis, they acquire systemically enhanced resistance to subsequent infection by various types of pathogens, and this systemic acquired resistance (SAR) is associated with the induction of PRs in tissues distant from the original inoculation site (Van Loon *et al.*, 1970; Kassanis, 1974).

PRs are stress proteins directed to alleviate harmful effects of cellular degradation products on hitherto untouched neighboring cells. The cellular damage and death occurring during a hypersensitive reaction is a major stress to the plant, as exemplified by high increases in abscisic acid (ABA) and ethylene. Both acidic and basic PRs may be induced by high concentrations of ethylene (Brederode, 1991) or physiological necrosis (Edreva *et al.*, 1990) plasmolysis (osmotic stress) (Wagih *et al.*, 1982) or wounding (Szczepanski *et al.*, 1985).

Latunde-Dada and Lucas (2001) identified acibenzolar-S-methyl to be the plant defense activator against the *Colletotrichum* infection in cowpea.

In case of resistance acquired against cowpea banding mosaic virus, salicylic acid was found effective in inducing PR protein accumulation by Malamy and Klessig (1992).

(Belkhadir *et al.* (2004) identified the presence of leucine-rich repeats, a nucleotide-binding site and a putative amino-terminal signaling domain to be initiators for plant disease resistant protein signaling

Vera-Estrella *et al.* (1994) demonstrated that activation of plasma membrane redox reactions support the role of CTP-binding proteins in the transduction of signals leading to the activation of the defense response mechanisms of tomato against fungal pathogens.

2.3.3 Classes

The PR proteins have been classically divided into five groups, PR-1, 2, 3, 4, and 5, based on serological and amino acid sequence analyses (Van Loon, 1985). Recently, another 6 groups of proteins have been suggested for inclusion as PR proteins, bringing the total to 11 groups. Each of the five classical groups of PR proteins has two subclasses: a basic subclass found in the plant cell vacuole and an acidic subclass usually found in the extracellular space (Kitajima and Sato, 1999). Each group has members with antifungal activity, but the mechanisms have been clearly identified for only PR-2 and PR-3 groups of proteins.

As reported by Van Loon (1999) the criteria used for the inclusion of new families of PRs were that firstly, protein(s) must be induced by a pathogen in tissues that do not normally express the protein(s), and secondly, induced expression must have been shown to occur in at least two different plant-pathogen

combinations, or expression in a single plant-pathogen combination must have been confirmed independently in different laboratories.

2.3.3.1 PR-1 proteins

PR-1 proteins are accumulated to high levels after pathogen infection and are antifungal in nature. They have been found in rice, wheat, maize, tobacco, *Arabidopsis thaliana,* barley, and many other plants (Cote *et al.*, 1991). Although these proteins are from diverse sources, they are remarkably similar (at least 35 per cent identity). PR-1 proteins have antifungal activity at the micro molar level against a number of plant pathogenic fungi, including *Uromyces fabae, Phytophthora infestans*, and *Erysiphe.*

2.3.3.2 PR-2 proteins

PR-2 proteins have (1, 3) β-endoglucanase activity *in vitro* and have been grouped into three classes on the basis of amino acid sequence analysis. Class I glucanases are basic proteins of 33 kD and are found in the plant vacuole. Classes II and III include acidic, extracellular proteins of about 36 kD. The major structural difference between class I proteins and the other two classes is that class I proteins are synthesized as prepro-proteins that are processed prior to being enzymatically active.

PR-2 proteins have been found in a wide variety of plants, including tobacco, *A. thaliana,* peas, grains, and fruits. The proteins are active *in vitro* at micro molar levels (50 mg/ml) against a wide number of fungi including *Rhizoctonia solani, C. albicans*, and *Aspergillus fumigatus.* The antifungal activity of plant (1,3) β-glucanases is thought to occur by PR-2 proteins hydrolyzing the structural (1,3) β-glucan present in the fungal cell wall, particularly at the hyphal apex of filamentous molds where glucan is most

exposed, resulting in a cell wall that is weak. This weakened cell wall results in cell lysis and cell death.

2.3.3.3 PR-3 proteins

A number of enzymatic assays have shown PR-3 proteins to have *in vitro* chitinase activity. Most PR-3 proteins have molecular masses of between 26 and 43 kD (Nielsen *et al.*, 1997). Chitinases have been divided into five groups. Class I chitinases contain an N-terminal cysteine-rich domain of about 40 amino acids (also known as the wheat germ agglutinin domain), a chitin-binding hevein-like domain, a highly conserved central portion, and a hinge region; most class I proteins have molecular masses of 32 kD. Class II proteins are similar in amino acid sequence to class I proteins, but they lack the N-terminal cysteine-rich domain and have molecular masses of 27 to 28 kD. Class IV proteins resemble class I chitinases but are significantly smaller due to four major deletions. Class III proteins do not share amino acid sequence homology to any other class and have molecular masses of 28 to 30 kD. Class V chitinases show sequence similarities to bacterial exochitinases and have molecular masses of 41 to 43 kD.

In addition to chitinases, a chitosanase (chitosan is deacetylated chitin) from *Streptomyces* strain N174 with antifungal activity has been isolated (Money and Harold, 1992), and its X-ray structure has been determined. Chitinases have been isolated from fungi (Kang *et al.*, 1999), plants such as tobacco (Melchers *et al.*, 1994), cucumber, beans (Ye *et al.*, 2000a) peas, grains (Huynh *et al.*, 1992), and bacteria (Chernin *et al.*, 1997) and have potent antifungal activity against a wide variety of human and plant pathogens, including *Trichoderma reesei, Alternaria solani, A. radicina, Fusarium oxysporum, Rhizoctonia solani, Guignardia bidwellii, Botrytis cinerea*, and *Coprinus comatus*. By analogy with β-glucanases, the mode of action of PR-3 proteins is relatively straightforward; PR-3 proteins are endochitinases that cleave cell wall chitin polymers in situ, resulting in a weakened cell wall and rendering fungal cells osmotically sensitive.

2.3.3.4 PR-4 proteins

PR-4 proteins are chitin-binding proteins with molecular masses of 13-14.5 kD, and have been classified into two groups. Class I proteins have amino acid sequence similarities to Hevein (a chitin-binding polypeptide and belong to the superfamily of chitin-binding lectins. Class II proteins lack the chitin-binding domain. PR-4 proteins have been isolated from potato, tobacco, barley, tomato, and many other plants. Both classes of proteins have potent antifungal activity against a wide variety of human and plant pathogens (e.g., *Trichoderma harzianum, Fusarium culmorum, F. graminearum*, and *B. cinerea*). The antifungal activity of class I proteins is likely due to protein binding to nascent fungal cell wall β-chitin resulting in disrupted cell polarity, with concomitant inhibition of growth (Bormann *et al.*, 1999). The mechanism of action of class II proteins which lack the chitin-binding hevein domain but are antifungal nonetheless is not understood. Fungi inhibited by these proteins include plant and human pathogens, e.g., *Paecilomyces variotii, Aspergillus* spp., *F. oxysporum, N. crassa, B. cinerea*, and *Alternaria brassicola*. It is likely that these proteins act by a mechanism similar to that of the class I PR-4 proteins, namely, binding to cell wall chitin and disrupting cell polarity, thus leading to inhibition of fungal growth.

2.3.3.5 PR-5 proteins

PR-5 proteins share significant amino acid homology to thaumatin protein from the South African ketemfe berry bush and are known as TL proteins. TL proteins have been isolated from *A. thaliana*, corn, soybeans, rice, wheat, tobacco, tomato, pumpkin, beans, barley, flax, and many other plants. Majority of the PR-5 proteins have molecular masses of 22 kD and are stabilized by eight disulfide bonds. This highly stabilized structure allows PR-5 proteins to be very resistant to protease degradation (Roberts and Selitrennikoff, 1990). Although the precise mechanism of action of PR-5 proteins is not completely understood, there are a number of interesting observations that may eventually lead to a unified

hypothesis for how these proteins function to kill fungi. First, several TL proteins cause cell permeability changes in fungal cells with a cell wall but have no or little effect on protoplasts. Second, a number of PR-5 proteins bind β-glucan and have detectable *in vitro* β -glucanase activity. Third, zeamatin inhibits insect a-amylase and mammalian trypsin activities *in vitro*. Fourth, osmotin, a TL protein from tobacco, causes perturbations in the regulation of fungal cell wall assembly. Fifth, zeamatin and nikkomycin act in synergy, reducing the amount of zeamatin required for cell killing up to 1,000-fold. These discrete observations are difficult to assimilate into one mechanism of action. Regardless of the precise mode of action of TL proteins, they are fungicidal against a wide number of plant and human pathogens *in vitro*.

2.4 Antifungal proteins of leguminous plants

Graham and Sticklen (1994) isolated an antifungal protein, dolichin of 28 kD demonstrating N-terminal sequence similarity to chitinases from the field bean *Dolichos lablab* having activity against *Rhizoctonia solani*.

From the pinto bean (*Phaseolus vulgaris* cv. 'Pinto') a 32 kD antifungal protein with a novel N-terminal sequence and prominent macrophage stimulating activity has been isolated by Ye and Ng (2002 a).

A cyclophilin-like 18 kD antifungal protein has also been purified by Ye and Ng, (2001 a). The protein demonstrated stronger antifungal activity toward *Mycosphaerella arachidicola*. A cyclophilin-like18 kD protein, Mungin with antifungal activity has been isolated from mungbean by Ye and Ng (2000 a) and chickpea seeds by Ye and Ng (2002 b).

A homodimeric 67 kD antifungal protein with moderate activity against *F. oxysporum* and *R. solani* has been found in red kidney bean (*P. vulgaris*) seeds by Ye *et al.* (2001). A peroxidase, with a molecular mass of 37 kD and antifungal

activity against *M. arachidicola*, *F. oxysporum*, and *B. cinerea*, has been isolated from french bean (*P. vulgaris*) by Ye and Ng (2002 c).

An antifungal protein of molecular mass 38 kD, designated as sativin and with an N-terminal sequence resembling Miraculin, has been purified from sugar snap (*Pisum sativum* var. *macrocarpon*) by Ye and Wang (2000 b). Another antifungal protein designated as pisumin and distinct from sativin has also been isolated by Ye and Ng (2003).

A 7.5 kD protein capable of inhibiting mycelial growth in *M. arachidicola*, *F. oxysporum*, and *B. cinerea* has been isolated from broad bean (*Vicia faba*) seeds by Ye and Ng (2001 b). Fabin is an antifungal protein from the broad bean with N-terminal sequence similarity to glucanase and calcyon.

A thaumatin-like antifungal protein has been isolated from french bean exhibiting a molecular mass of 20 kD by Ye and Wang (1999). Regalado and Ricardo (1996) characterized a chitinase and a thaumatin-like protein from intercellular fluid of *Lupinus albus* leaf, stem and root tissues. Thaumatin-like proteins which can be induced by infection with *Ascochyta rabiei* have been purified from intercellular washing fluid of chickpea (*Cicer arietinum* L.) leaves by Hanselle and Ichinoseb (2001). A glucanase and two chitinases have been isolated from chickpea (*C. arietinum*) by Regalado and Ricardo (1996).

Novel antifungal peptides designated as cicerin and arietin have been prepared from seeds of the chickpea by Ye *et al.* (2002). From the seeds of the green chickpea an antifungal peptide cicerarin was isolated by Chu *et al.*, (2003).

Peptides, with a molecular mass of 5 kD and an N-terminal sequence with remarkable resemblance to those of cowpea 10 kD protein precursor and garden pea disease response protein, have been isolated from pinto beans and red beans by Ye and Ng (2001 a). The peptides exhibit, in general, potent antifungal activity toward *M. arachidicola*, *B. cinerea*, and *F. oxysporum*. Another antifungal peptide designated angularin, 8 kD in molecular mass, and exhibiting antifungal

activity against *M. arachidicola* and *B. cinerea*, has been isolated from red beans by Ye and Ng (2002 d).

From yunnan bean *Gymnocladus chinensis,* a 6.5 kD peptide with antifungal activities was identified by Wong and Ng (2003). A 7.2 kD peptide, with an N-terminal sequence resembling peanut allergen Ara H1 and antifungal activity against *M. arachidicola*, *Coprinus comatus*, and *F. oxysporum* has been isolated from seeds of the peanut *Arachis hypogea* by Ye and Ng (2000 b). Ngai and Ng (2003) has isolated a 25 kD antifungal protein from soybean *Glycine soja* having N-terminal sequence resembling to a segment of chitin synthase.

2.5 PR proteins identified in cowpea

A chitinase-like protein with a molecular mass of 28 kD and another antifungal protein with a lower molecular mass (12 kD) and a novel N-terminal sequence were isolated from cowpea (*Vigna unguiculata*) seeds (Ye *et al.*, 2000b).

Chilling tolerance at seedling emergence stage of cowpea was identified due to dehydrin LEA proteins (Ismail *et al.*, 1999). Carvalho *et al.* (2001) isolated two cysteine-rich antimicrobial peptides (6.8 and 10 kD) from cowpea (*Vigna unguiculata*) seeds which were found to deter the development of *Fusarium oxysporum, F. solani* and *Saccharomyces cerevisiae*.

2.6 SDS-PAGE for detection of PR proteins

The discovery of PR protein was a direct consequence of the introduction of polyacrylamide gel electrophoresis, which allowed complex mixtures of proteins to be separated with unprecedented resolution on the basis of their combination of size and charge (Ornstein, 1964; Davis, 1964).

Accumulation of β-fructosidase was observed by Benhamou *et al.* (1991) in the cell walls of tomato roots following infection by fungal wilt pathogen by analyzing the intercellular fluid from in 15 per cent (w/v) polyacrylamide gels.

Vigers *et al.* (1991) identified a new class of plant antifungal proteins, zeamatin of 22 kD mass from corn seeds by analyzing the total protein extracted at neutral pH in using 12 per cent polyacrylamide gels.

Yan *et al.* (2008) characterized a pathogenesis-related class 10 protein from *Astragalus mongholicus* with ribonuclease activity using SDS-PAGE and the protein was found to be dimeric in nature with 17.2 kD and 32.8 kD.

Shivashankar *et al.* (2010) reported higher levels of polygalacturonase inhibitor protein with 37 kD mass from fruits of anthracnose resistant varieties of chilli by analyzing on 10 per cent polyacrylamide gels.

2.7 Peptide mass fingerprinting using MALDI-TOF/MS

MALDI-TOF peptide mass fingerprinting (PMF) is the fastest and cheapest method of protein identification (Sommerer, 2007).

Campo *et al.* (2004) identified GST and glyceraldehyde 3-phosphate dehydrogenase are the proteins responsible for resistance in *Zea mays* against *Fusarium verticillioides* by 2-DE and MALDI-TOF/MS techniques.

Kim *et al.* (2004) reported the induction of 8 PR protein including PR-2, 5, 9, 10, PBZ induced protein and receptor-like protein kinase in *Oryza sativa* against *Magnaporthe grisea* by using 2DE and MALDI-TOF/ MS.

Colditz *et al.* (2004) found the induction of 12 PR proteins including PR-10, Disease-resistance-response protein pi 49, HSP, ABA-responsive protein ABR17, proline rich protein, isoliquiritigenin and 2-O-methyltransferase in

Medicago truncatula in response to *Aphanomuces euteiches* by 2 DE and MALDI-TOF/MS analyses.

2.8 Defense related enzymes

The plant generates defense responses to resist disease when infected by the pathogen, which include the release of various reactive oxygen species, expression of defense genes and hypersensitive responses (Leister, 2004; Takakura *et al.*, 2008). Some substances that are contained in the plant host itself, such as saponins, glucosinolates and cyanogenic glycosides also have disease resistance properties (Asgary *et al.*, 2008; Kim *et al.*, 2009; Xinzhang, 2012).

2.8.1 Peroxidase activity

Vera-Estrella *et al.* (1992) reported an increase in active oxygen species and extracellular peroxidase activity upon incubation of tomato *(Lycopersicon esculentum)* cell suspensions with one race-specific elicitor, the putative product of the avirulence gene *avr5* of the fungus *Cladosporium fulvum.* This observed increase in active oxygen species suggested that changes in the redox processes at the plasma membrane may be involved in the plant defense response against pathogens.

Two isoperoxidases were detected in cowpea leaves in response to 10 mM salicylic acid (Fernandes *et al.*, 2006). Leina *et al.* (1996) have demonstrated a direct correlation between the variation in peroxidase activity in the soluble fraction of inoculated leaves and resistance to infection in cowpea cultivars. Fink *et al.*, (1991) has studied the early defense reactions of *V. sinensis* and found the induction of extracellular peroxidase activity as early as 24h after inoculation.

2.8.2 Polyphenol oxidase activity

Campos *et al.*, (2004) observed positive correlations among peroxidase and polyphenol oxidase activity, phenolic compound levels and anthracnose resistance. Zhang *et al.*, (2006) have evaluated resistant and susceptible cultivars of cowpea (*Vigna sesquipedalis*) and found a higher activity of PO, PPO, PAL, chitinase, β-1,3-glucanase and catalase enzymes against *Fusarium oxysporum* infection. Chandra *et al.*, (2007) observed that the inoculation with Rhizoctonia solani resulted in a quantitative change in polyphenol oxidase, peroxidase isoforms and increase in PAL activities from 4.38 to 19.48 unit g−1 Bundel-1, UPC-4200 and IFC-902 cowpea genotypes. Vanitha *et al.* (2009) observed a temporal pattern of induction of PPO enzyme reaching maximum activity at 15 hrs after the bacterial wilt inoculation in resistant cultivars of tomato.

2.8.3 Phenylalanine ammonia lyase activity

Lawton and Lamb (1987) observed that elicitor treatment of bean (*P. vulgaris*) with *C. lindemuthianum* culture caused marked transient stimulation of transcription of genes encoding apoproteins of cell wall hydroxyproline-rich glycoproteins (HRGP), phenylpropanoid biosynthetic enzymes phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS). Fink *et al.*, (1991) has studied the early defense reactions of *V. sinensis* and found the induction of phenylalanine ammonia-lyase at 10h after inoculation. Chandra *et al.*, (2007) observed the decline in disease progress caused by *Rhizoctonia solani* in cowpea due to phenylalanine ammonia lyase activities. Mechanism of resistance in cowpea to the root-knot nematode in *Meloidogyne incognita* was found to occur by the early induction of phenylalanine ammonia lyase and chlorogenic acid (Anil and Dasgupta, 1993). Vanitha *et al.* (2009) observed a temporal pattern of induction of PAL enzyme reaching maximum activity at 12 h after the bacterial wilt inoculation in resistant cultivars of tomato.

Materials and Methods

3. MATERIALS AND METHODS

The study on "Characterization of pathogenesis related proteins for anthracnose resistance in vegetable cowpea, *Vigna* spp." was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University during the period 2011-2013. The materials used and methodologies adopted are discussed in this chapter.

3.1 Plant materials

The four Cowpea varieties *viz*, Pusa Komal, Kanakamony, Lola and Arimbra Local were selected for the study (Plate1). Of these, two were bush type (*Vigna unguiculata* (L.) Walp. subsp. *sesquipedalis* (L.) Verdc.) and other two were pole type (*Vigna unguiculata* (L.) Walpers subsp. *cylindrica* (L.) Verdc.) with one being susceptible and the other being resistant. The seeds were collected from different places as listed in Table 1 and stored at -20 °C.

Table 1. Salient features of four vegetable cowpea varieties used in the study

Sl. No.	Cowpea variety	Source of seed collection	Plant growth habit	Reported response to anthracnose disease
1	Pusa Komal	IARI, New Delhi	Bush type	Highly susceptible
2	Kanakamony	KVK, Thavanur	Bush type	Immune
3	Lola	Dept. of Olericulture, CoH, KAU, Vellanikkara	Pole type	Highly susceptible
4	Arimbra Local	Farmer's field, Arimbra, Mallapuram district, Kerala	Pole type	Resistant

a. Seeds of different cowpea variteies selected for the study

b. Pusa komal (Bush type - Suceptible)

c. Kanakamony (Bush type - Resistant)

d. Lola (Pole type - Suceptible)

e. Arimbra local (Pole type - Resistant)

Plate 1. Different varieties of vegetable cowpea (*V. unguiculata*) selected for the study

3.2 Laboratory chemicals, equipments and machinery

The chemicals used in the present study (AR grade) were procured from Merck India Ltd., HIMEDIA and SISCO Research Laboratories. The protein molecular weight marker - medium range and acrylamide were supplied by Bangalore Genei Ltd. All the plastic wares used were obtained from Axygen and Tarson India Ltd. Borosilicate glass wares were used.

Pathology works were carried out under laminar air flow chamber. Olympus research microscope and phase contrast microscope (Leica DM 500) having image analyzer software were used for studying the morphology of the pathogen. For the media preparation and autoclaving, Microwave oven (Samsung), Hot air oven and Autoclave (Kdt) were used. High precision electronic balance (Shimadzu), pH meter (EuTech Instruments PC 510), micropipettes (Eppendorf, Accupipet), Icematic (F100 compact) and high speed refrigerated centrifuge (KUBOTA 6500 and Heyich Zentrifugen – MiRo 22R) were utilized for protein extraction. Samples were stored for long term at -80°C and -20°C (SANYO Medical freezer). For short term storage of 0-4°C refrigerators were used. NanoDrop^{R} ND-1000 spectrophotometer was used for the estimation of the protein concentration. Sample treatment was done in Cylindrical water bath (Rotex) and spinned using Mini spin (Eppendorf). SDS PAGE analysis was performed in the small and large units (Protean^{R} II xi cell - BioRad) along with Power PAC 1000 and Power Pac 300 (BioRad). Staining was aided by the Rocker 25 (Labnet). A circulating cooling system (Colora) was also attached with the unit to prevent heating of the sample. The SDS-PAGE gels were photographed using the gel documentation unit (BioRad).

3.3 Collection of infected samples

Lola cowpea plants showing typical symptoms of anthracnose disease (Plate 2) were collected from the seed production field of Central Nursery, Vellanikkara.

a. Reddish brown lesions on stem, petioles and leaf veins

c. Advanced stage of infection of stem. Leaves having black dots (mildew symptom)

Plate 2. Symptoms of anthracnose disease under natural condition

3.4 Isolation, identification and maintenance of the pathogen

The collected samples were brought to the laboratory, washed under tap water and dried with blotting paper. Isolation of the pathogen was done using standard protocol. The infected stem portions were cut into small bits of 5 mm size and then surface sterilized with one per cent sodium hypochlorite for a minute and washed with three changes of sterile water. The bits were then transferred aseptically to sterile Petriplates containing potato dextrose medium (PDA) and neopeptone glucose agar medium (NGA) amended with streptomycin sulphate. Petriplates were incubated at room temperature (26±2°C) and examined daily for the growth of the pathogen for 7 days. The fungus developed on PDA and NGA were purified by hyphal tip method. Pure cultures were then maintained in the refrigerator for further studies. The culture and morphological characteristics of the fungus on different media were recorded for the identification of the fungus.

3.5 Pathogenicity test

The susceptible varieties Pusa Komal and Lola were used for the pathogenicity study. Three seeds of each variety were sown in five earthen pots. The plants were sprayed with 20 ml of spore suspensions having a concentration of 10^6 spores/ml after pin prick injury at 30 days after sowing. Cotton soaked in inoculum was also tied around stem after wounding. Inoculated plants were covered with moistened polythene cover to maintain humidity. Inoculated plants were observed daily for the disease appearance and the symptoms produced were noted. Plants inoculated with sterile water served as control. Pathogen was reisolated from the infected plants and then compared with the original culture.

3.6 Pot culture experiments

The pot culture experiment was conducted twice, the first experiment during July-August and the second during August-September, 2011 in a rain shelter polyhouse. The potting mixture was prepared with soil, sand and vermicompost at the ratio of 2:1:1. Fifty pots were maintained for each variety of which 25 were kept for inoculation and 25 as control with 4 m distance between them (Plate 3). Three seeds were sown per pot and the plants were properly maintained for further studies.

3.7 Inoculum preparation

Method 1

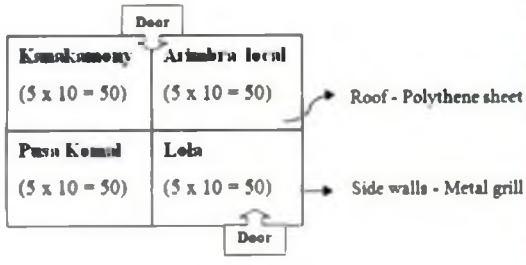
Hundred ml of neopeptone glucose broth was taken in each 250 ml flask and sterilized by autoclaving. All the flasks were inoculated with four uniform mycelium discs of 5 mm diameter taken from the actively growing seven day old culture of *Colletotrichum lindemuthianum*. After seven days of incubation, the inoculum was serially diluted to the concentration of 10^6spores/ml.

Method 2

Seven day old pathogen was grown on neopeptone glucose agar in two Petriplates and incubated for seven days. The inoculum was prepared by scrapping off the mycelium and spores from the medium using a sterile glass slide and suspended in 100 ml sterile distilled water. The concentration of the inoculum was then adjusted to 10^6spores/ml.

3.8 Artificial inoculation on plants

Artificial inoculation was carried out at 5-6 pm in the rain shelter polyhouse itself in the first experiment whereas the 30 day old plants were



a. Sketch of the rain shelter polyhouse

b. Cowpea plants after 30 days of sowing

c. Artificial inoculation of the pathogen

Plate 3. Pot culture experiment and artificial inoculation

transferred to a greenhouse with mist facility in the second experiment (Plate 3). Each of the plants maintained for treatment were sprayed with 20 ml of spore suspension after pin prick injury in the first experiment. In the second experiment, pin prick injury was avoided, the inoculated plants were covered with moistened polythene covers and the pots were kept in basins containing water to maintain high humidity. Plants inoculated with sterile distilled water served as control.

Observations on disease incidence and disease severity were assessed at 3 weeks after inoculation. Per cent disease incidence was calculated using the formula,

$$\text{Per cent disease incidence} = \frac{\text{Number of plants infected}}{\text{Total number of plants observed}} \times 100$$

Disease severity was assessed using 0-5 scale as mentioned below (Dada, 1990).

0 - No infection

0.5 - Hypersensitive spots on main stem only

1 - Trace of infection - small anthracnose lesions on main stem and petioles of lower leaves only

2 - Slight infection - lesions on stem, petioles and branches

3 - Moderate infection - Advanced anthracnose lesions on stem, petioles, branches, veins on the abaxial surface of leaves

4 - Severe - Advanced anthracnose lesions on stem, petioles, branches, leaves, veins and peduncles

5 - Very severe - Advanced lesions on stem, petioles, branches, leaf veins spreading lesions on peduncles and pods.

Based on the above score, per cent disease severity was calculated using the following formula (Wheeler, 1969).

$$\text{Per cent disease severity} = \frac{\text{Sum of all numerical ratings}}{\text{Total number of plants taken for observation}} \times \frac{100}{\text{Maximum disease category}}$$

Based on the percent disease severity, the cowpea genotypes were grouped into five categories as suggested by Rajkumar *et al.* (1995).

Percent disease severity	Category
0	Immune
1 – 10	Highly resistant
10.1 – 25	Moderately resistant
25.1 – 50	Moderately susceptible
Above 50	Highly susceptible

3.9 Collection of the leaf sample

Mature leaf samples from treated and control plants of the four varieties were collected on ice at 0, 6, 12, 18, 24, 48, 72, 96, 120, 144, 168, 192 hours after inoculation. The samples were packed in labeled aluminum foil and polythene sheets and stored at -80°C for further studies.

3.10 Total protein extraction

One gram of leaf tissue was ground into fine power using autoclaved and prechilled pestle and mortar in liquid N_2. The samples were homogenized in 3 ml of Tris-HCl buffer pH-7.5 (0.25 M Tris-HCl and 0.5 M NaCl) (Zhang, 2006). The homogenate was then filtered using muslin cloth and centrifuged at 20,000g for 30 min at 4°C. The supernatant was collected as crude extract containing the total protein. The protein extract was aliquoted using Tris-HCl buffer pH-7.0 (50 mM Tris-HCl and 0.5 M NaCl). One set of the sample was stored for long term storage at -80°C and the working sample was stored at -20°C (Annexure III).

3.11 Protein Quantification

The aliquoted protein samples were quantified using NanoDrop^{R} ND-1000 spectrophotometer with the option Protein A280. For loading in the SDS-PAGE gel, the proteins were normalized to a concentration of 1500 µg/ml such that the final concentration in the gel while loading reached 50-100 µg/ml.

3.12 Protein profiling

3.12.1 Sample treatment

Twenty µl of protein samples having 50 µg concentration from different treatments were mixed well with 5 µl of sample loading buffer (Annexure IV) in a microfuge tube, boiled for 5 min and quickly snap cooled on ice. Similarly, 20 µl of ready to use markers was boiled for 2 min and quickly snap cooled on ice.

3.12.2 Standardization of SDS-PAGE protocol

SDS PAGE (Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis) analysis was done by the standardized protocol (Laemmli, 1970) with minimum modifications by adjusting the APS and Tris concentrations.

3.12.3 SDS-PAGE analysis

The plates were washed well with tap water and wiped with distilled water followed by ethanol and acetone. The plates were then assembled in a gel casting apparatus. Separating gel mixture of 12 per cent was prepared (volume according to the thickness of spacer and plate size used) and poured into the plates. A layer of water saturated ethanol of about one cm was poured over the gel mix and allowed to solidify for 45 min with the presence of light and in the absence of air. After solidification, the layer of water saturated ethanol was removed by tilting,

washed and dried with filter paper. Stacking gel mixture of 4 per cent (volume according to the thickness of spacer and plate size used) was then prepared and poured above. Appropriate comb was placed at the top immediately and allowed to solidify for 30 min.

After complete polymerization, the plates were separated from the casting apparatus and fixed in the gel running gasket vertically. The inert plate was fixed on the other side and the setup was tightened before placing inside the buffer tank containing 1X tank buffer. The combs were then removed carefully and the wells were washed thoroughly to remove the unbound acrylamide (Annexure IV).

Treated samples containing equal volume and concentration of proteins derived from different treatments were loaded into the wells. The medium range molecular weight marker was then loaded and electrophoresis was carried out at constant voltage of 80 V until the samples travel through the stacking gel. Then the voltage was adjusted to 120 V until the samples reach the bottom of the separating gel.

To prevent the sample from degradation due to the heating of the buffer while running, polar packs were kept on both sides of the buffer tank for the small unit. The large unit was connected to a cooling water circulator unit (Colora).

When the tracking dye reaches the bottom of the plates, the power was switched off and the chords were removed. The gel running gasket containing the plates was taken out and the lock was relaxed. The inert plate was taken first followed by the glass plate assembly. The two plates were then separated carefully using the tool provided with the set up and the fragile gel sticking to one of the plates was removed patiently with the help of water squeeze without breaking the gel.

3.12.4 Staining by using Coomassie brilliant blue stain

The gel was then transferred to staining solution and kept overnight with uniform shaking on rocker shaker. On next day the gel were immersed into the destaining solution with uniform shaking for one and half hours and the process was repeated at least twice until the background of the gel became colorless (Annexure IV).

3.12.5 Silver staining

The gels were immersed in the fixing solution and kept under shaking for one hour. The step was repeated by changing the solution and fixing for overnight. The gel was then washed twice in 30 per cent ethanol for 10 min each and washed again with deionized water. The gel was transferred to the pretreatment solution and taken out within one min exactly. It was again washed in deionized water thrice for 30 sec and impregnated in 0.2 per cent silver nitrate for 20 min in shaker followed by washing twice for 10 sec. It was then dipped in developing solution for 5-10 min until clear band appeared with appropriate intensity. During the above step, the brown/ grey precipitate developed was redissolved by shaking the developer gel containing box. The gel was quickly transferred into stop solution for 30 min followed by washing twice for 30 min (Blum *et al.*, 1987; Chevallet *et al.*, 2006), (Annexure IV).

3.12.5 Gel documentation

The protein profile was viewed in white light transilluminator and documented by placing over a conversion screen in gel documentation unit connected to the computer having *Quantity 1* software (BioRad) using epi-white option.

3.13 Peptide mass fingerprinting by MALDI-TOF/ MS and *In-silico* analysis

The differentially expressed protein bands were identified, cut from the gel and send for peptide sequencing by using Matrix Assisted Laser Desorption/ Ionization - Time of Flight Mass Spectrometry (MALDI-TOF/MS). The peaks obtained were analyzed with the online bioinformatics tool, MASCOT/ MS peptide search engine for the characterization of the proteins (Outsourcing from Sandor Proteomics, Hyderabad).

3.14 Protein precipitation, dialysis and SDS-PAGE analysis

To purify the total proteins extracted at neutral pH and to characterize the acid soluble PR protein by suspending them in acidic buffer, the following steps were adopted. Two ml of crude extract of the protein was added with ammonium sulphate to obtain 90 per cent saturation (61.1 g in 100 ml) and incubated overnight at 4°C for the proteins to get precipitated. The protein containing tubes were centrifuged at 8000 rpm for 15 min and the supernatant was removed. The pellet was dissolved in 0.1 M Sodium acetate buffer (pH 5.2) (Legrand *et al.*, 1987).

The dialysis tube (width - 29.31 mm, diameter - 17.5 mm, capacity - 2.41 ml/cm) was cut into appropriate length and placed in 2 L beaker containing 1 L of preheated distilled water. Then it was drained and boiled in 1 L solution of 2 per cent sodium carbonate and 1 mM EDTA (pH-5.0) for 30 min. It was drained again and rinsed twice in distilled water followed by boiling in distilled water for 30 min. It was drained and rinsed twice in distilled water using a sterilized forceps and again rinsed in 25 per cent ethanol. After draining the treated tubing was stored in 25 per cent Ethanol at 4°C until use.

Just before use, the treated dialysis tube was washed in distilled water. Leaving an inch or two from one of the open end, dialysis tubing-specific closure was used to close that end and the part was cut off from the remaining tubing. The

precipitated protein sample was pipette out into the tubing and the other end was closed off with another closure. It was then inserted in a large beaker containing water and the volume of water was 100 times of that of the protein sample. The beaker was kept in a shaking incubator with temperature control, set at 4°C and stirred for 1.30 hours. Water was then discarded and beaker was filled with same amount of water and dialyzed for overnight. Next day tubing was removed from beaker and one end was carefully opened. The protein was pipetted out and stored at -20 °C.

The dialyzed protein was made to run in SDS-PAGE gel made with 12 per cent separating gel and 4 per cent stacking gel, stained with coomassie brilliant blue dye R-250 and documented in gel documentation unit (BioRad) connect with *Quantity 1*, image analyzer software.

3.15 Defense enzyme assay

3.15.1 Assay of Peroxidase (PO)

One g of fresh plant tissue was ground in 3 ml of 0.1 M phosphate buffer (pH - 7.0) using a pre-cooled mortar and pestle. The homogenate was centrifuged at 18,000 g at 5°C for 15 min and the supernatant was used as enzyme source within 2-4 hours by storing them on ice. 3ml of 0.1 M phosphate buffer (pH - 7.0) at 25°C was added to 50 µl of 20 mM guaiacol solution, 100 µl of enzyme extract and 30 µl of 0.042 per cent H_2O_2 in a cuvette and mixed well. The cuvette was then placed in spectrophotometer and the absorbance was read at 470 nm. The enzyme activity was expressed in units/µl (Putter, 1974; Annexure V).

3.15.2 Assay of Polyphenol Oxidase (PPO)

The enzyme extract for the assay was prepared by grinding 1 g of leaves with a pre-chilled pestle and mortar in about 4 ml of buffer solution containing 50

mM Tris-HCl (pH -7.2), 0.4 M sorbitol and 10 mM NaCl. The homogenate was centrifuged at 20,000 g for 10 min and the supernatant was used for the assay. 2.5 ml of 0.1 M sodium phosphate buffer (pH - 6.0) was added to 0.5 ml of 0.01 M catechol and 100 µl of enzyme extract. The reactants were mixed well and read at 495 nm in spectrophotometer (Esterbaner *et al.*, 1977; Annexure V).

3.15.3 Assay of Phenylalanine Ammonia Lyase (PAL)

Five hundred mg of leaf sample was homogenized in 5 ml of cold 25 mM borate-HCl (pH-8.8) buffer solution containing 5 mM mercaptoethanol. The homogenate was centrifuged at 120,000 g for 20 min and the supernatant was used for the assay. 0.5 ml of 0.1 M borate buffer (pH - 8.8) was added with 200 µl enzyme extract, 1.3 ml of water and 0.5 ml of 0.1 M L-phenylalanine. All the components are mixed well and incubated for 30 min at 30°C. The reaction was stopped by adding 0.5 ml of 1 M trichloroacetic acid and the absorbance value was read at 290nm (Brueske, 1980; Annexure V).

Results

4. RESULTS

The results of different experiments carried out to characterize the pathogenesis related proteins for anthracnose resistance in vegetable cowpea, *Vigna* spp. are presented in this chapter.

4.1 Isolation and identification of the pathogen

The pathogen associated with the anthracnose disease of cowpea isolated on potato dextrose agar (PDA) and neopeptone glucose agar (NGA) medium showed different culture and spore characteristics as shown in the Plate 4.

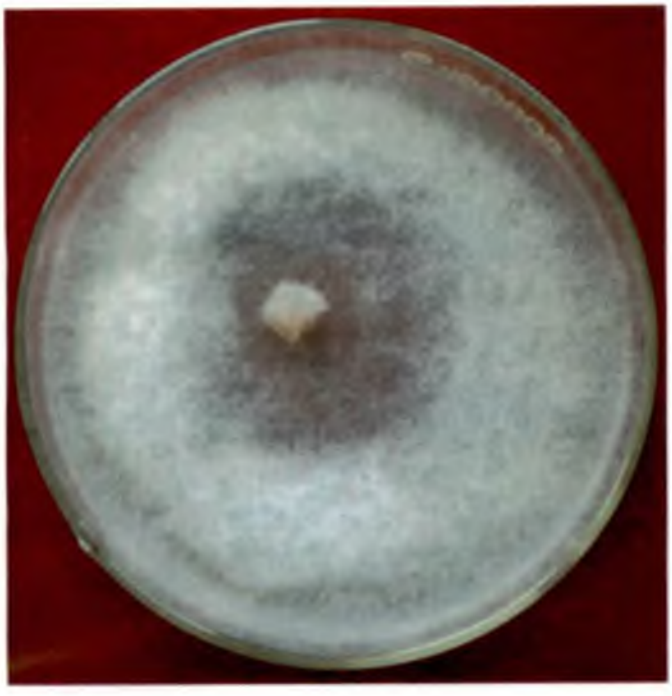
On PDA medium, the fungus showed slow growth, colonies appeared whitish grey first turned to darker with compact aerial mycelium and the reverse of the colony almost black, less sporulation, no pink pigmentation and setae were present. Dark, hard and spherical sclerotia like bodies were abundant in old cultures.

On NGA medium, colonies were fast growing, sparse whitish mycelium, high sporulation, light pink pigmentation, abundant and very few sclerotia like bodies.

Conidia were hyaline, cylindrical with both ends obtuse, aseptate and uninucleate. Based on cultural and morphological characteristics the pathogen was identified as *Colletotrichum lindemuthianum*.

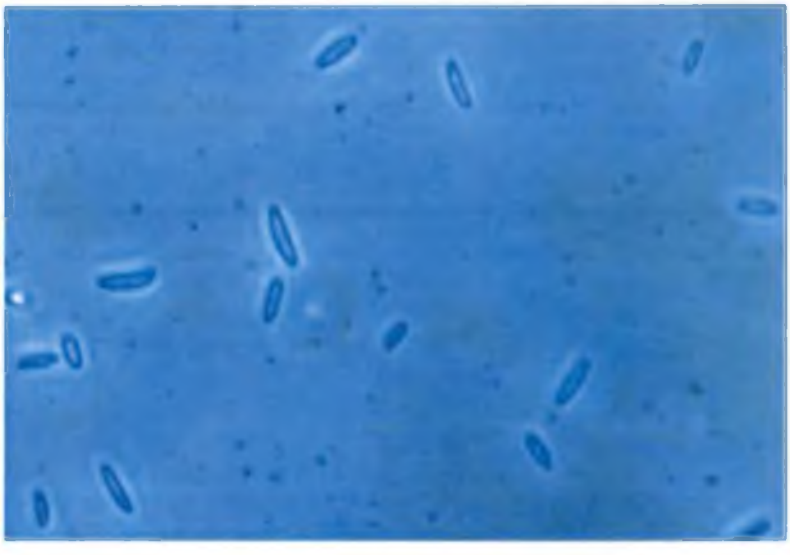
4.2 Pathogenicity test

The plants showed symptoms after 3-4 days of inoculation. Both Pusa Komal and Lola showed typical symptoms of anthracnose disease as shown in Plate 5. The pathogen was reisolated and found similar to that of the original



a. Culture of *C. lindemuthianum* on potato dextrose agar medium

b. Culture of the pathogen o
neopeptone glucose agar medi



c. Conidia of *C. lindemuthianum* at 10 x 10X magnification

Plate 4. Culture and conidia of *C. lindemuthianum*

a. Pusa Komal showing anthracnose symptom

b. Lola showing anthracnose symptom

c. Symptoms appearing on stem and along the veins of leaves

Plate 5. Symptoms of anthracnose on artificial inoculation

culture. The pathogenicity test thus indicated *C. lindemuthianum* as the causal organism of anthracnose disease in cowpea.

4.3 Pot culture experiments

The first experiment was carried out during July-August. In this experiment symptoms did not appear even with pinprick injury as the congenial conditions for the disease development was not met due to less rainfall and less humidity. The second pot culture experiment was carried out during August-September and the symptoms appeared on 3-4 days after inoculation even without pin prick injury.

4.3.1 Symptomatology of the disease under artificial condition

Artificial inoculation with *C. lindemuthianum* spore suspension of 10^6 spores/ml concentration resulted in the development of symptoms corresponding to anthracnose disease in the four cowpea varieties taken for study (Plate 6, 7).

Reddish brown streaks appeared on the stem, petioles, vines and along the veins of the leaves. Mildew symptoms appeared on the leaf lamina. Leaves became chlorotic and defoliated by rotting of petiole. The pods were rottened and turned to grey, covered with black fruiting bodies of fungus. After 10 days of symptom appearance rotting of the top portion of stem was also observed due to high humidity.

4.3.2 Reaction of genotypes to *C. lindemuthianum*

The per cent disease incidence was found to be cent per cent in the Pusa Komal and Lola whereas it is 22 per cent in Arimbra Local and no disease incidence was observed in Kanakamony.

Susceptible variety

a. Control - Pusa Komal **b. Treated - Pusa Komal**

Resistant variety

c. Control - Kanakamony **d. Treated - Kanakamony**

Plate 6. Symptoms of anthracnose on bush type varieties at three weeks after artificial inoculation

Susceptible variety

a. Control - Lola **b. Treated - Lola**

Resistant variety

c. Control - Arimbra Local **d. Treated - Arimbra Local**

Plate 7. Symptoms of anthracnose on pole type varieties at three weeks after artificial inoculation

The per cent disease severity was observed to be cent per cent in Pusa Komal and 68.8 per cent in Lola which are above 50 per cent. Hence Pusa Komal and Lola were categorized as highly susceptible varieties. The per cent disease severity of Arimbra local was 8.8 per cent which is less than 10 per cent and hence categorized as highly resistant variety whereas Kanakamony was found to be immune with no disease incidence (Table 2).

4.4 Extraction and quantification of total protein

A high concentration of protein ranging between 18 to 74 mg/g of leaf sample was extracted using Tris-HCl buffer (pH-7.5) from the control and the treated samples collected at different time interval from 0 to 192 hr. An appropriate concentration of 1.5 mg/ml was obtained by normalizing the aliquoted protein samples using NanoDrop spectrophotometer so that 50-100 ng can be loaded into the SDS-PAGE gel.

4.5 Protein profiling

4.5.1 Standardization of the SDS-PAGE protocol

Laemmli (1970) protocol for the SDS-PAGE analysis was further appropriated to the laboratory conditions by increasing the APS concentration to promote the polymerization of the SDS-PAGE gel. To reduce the fragility of the gel, the molarities of Tris in the separating and stacking buffer are slightly increased from 1.5M to 1.875M and from 0.5M to 0.6 M, respectively.

4.5.2 Protein profiling in relation to *C. lindemuthianum* infection

The SDS-PAGE analysis of the protein isolated at pH 7.5 has shown distinct expression of the bands for the resistant and suceptible cowpea varieties.

Table 2: Reaction of cowpea genotypes to *C. lindemuthianum* under artificial condition

Sl.No.	Cowpea variety	Per cent Disease Incidence	Per cent Disease Severity	Category
1	Pusa komal	100	100	Highly susceptible
2	Lola	100	68.8	Highly susceptible
3	Arimbra local	22	8.8	Highly resistant
4	Kanakamony	0	0	Immune

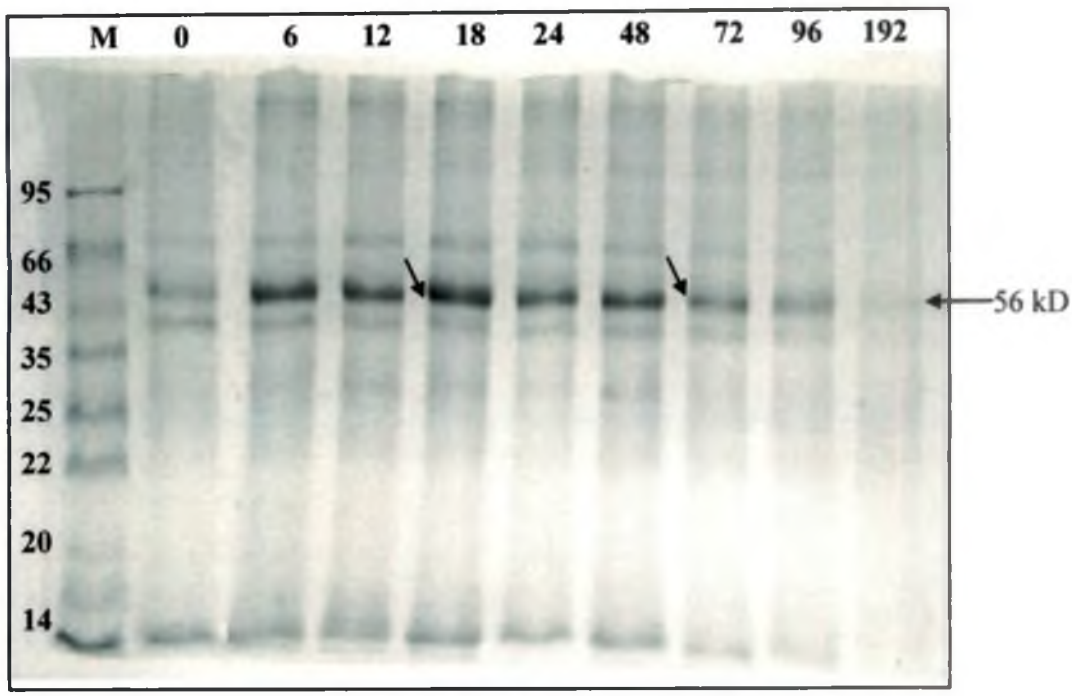
Totally 7 bands were observed in each lane in all the four varieties and there was difference in the expression of the proteins at different time intervals. The molecular weight of the bands are elucidated by comparing with a medium range marker (95-14kD). Among them two bands were observed with more than 100kD whereas other bands are observed in the size range of 70, 56, 40, 27and 14 kD.

4.5.2.1 SDS-PAGE profile of proteins in bushy varieties

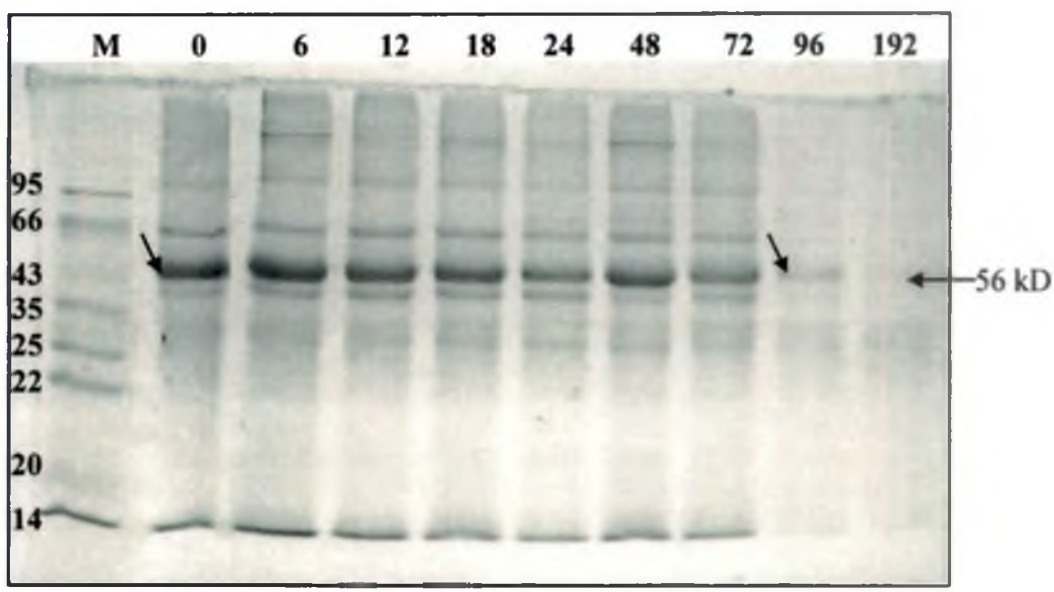
The difference in the expression of proteins of the bushy varieties Kanakamony (immune) and Pusa Komal (suceptible) were obseved as shown in the Plate 8. The difference was mainly observed in two prominent bands with molecular weight of 56 kD and 14 kD. Higher level of expression of these proteins for Pusa Komal was observed at 18 hrs after artificial inoculation with *C. lindemuthianum* wheras in Kanakamony after inoculation also the same higher level of expression was maintained. The 56kD protein was observed as faint band in Pusa Komal at 0 hr and reached its peak at 18 hr, and maintained a high level of expression up to 48 hr and fainted from 72 hr onwards. But the 56kD protein of Kanakamony has maintained a high level of expression from 0 hr to 72 hr and fainted from 96 hr onwards. This protein which already has a high level in the immune variety might have profound role in the immunity mechanisms of the plant.

4.5.2.2 SDS-PAGE profile of proteins in pole type varieties

The difference in the expression of proteins for the pole type varieties Arimbra Local (resistant) and Lola (suceptible) are presented in Plate 9. Difference was mainly observed in two prominent bands with molecular weight of 56 kD and 14 kD. Higher level of expression for both Lola and Arimbra Local were observed at 18 hrs after artificial inoculation with *C. lindemuthianum*. The



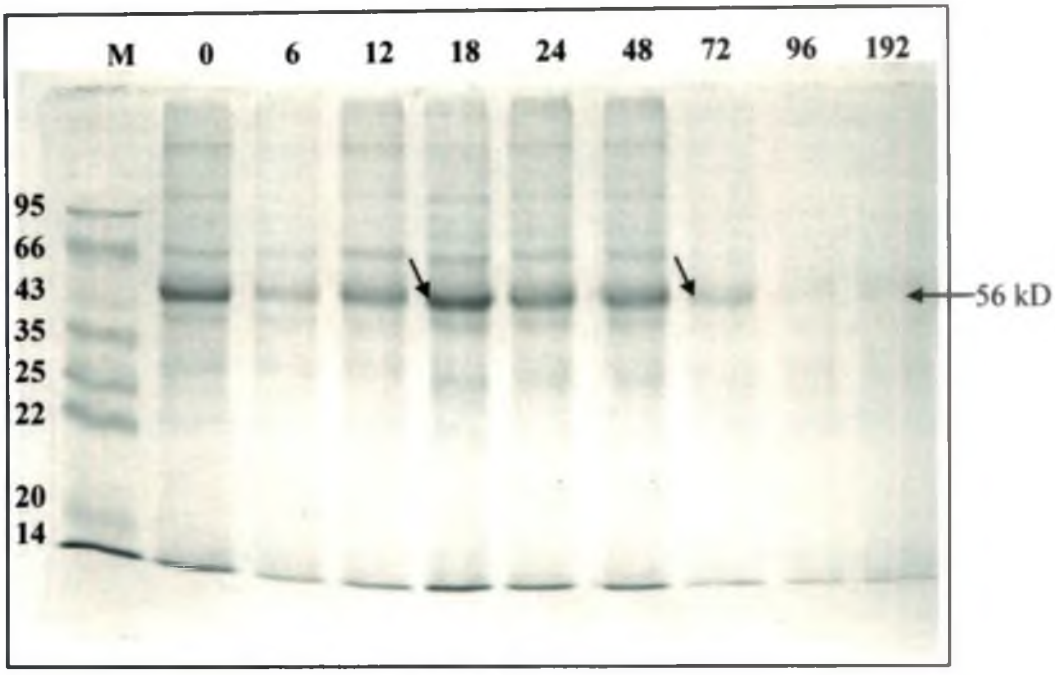
a. Pusa Komal



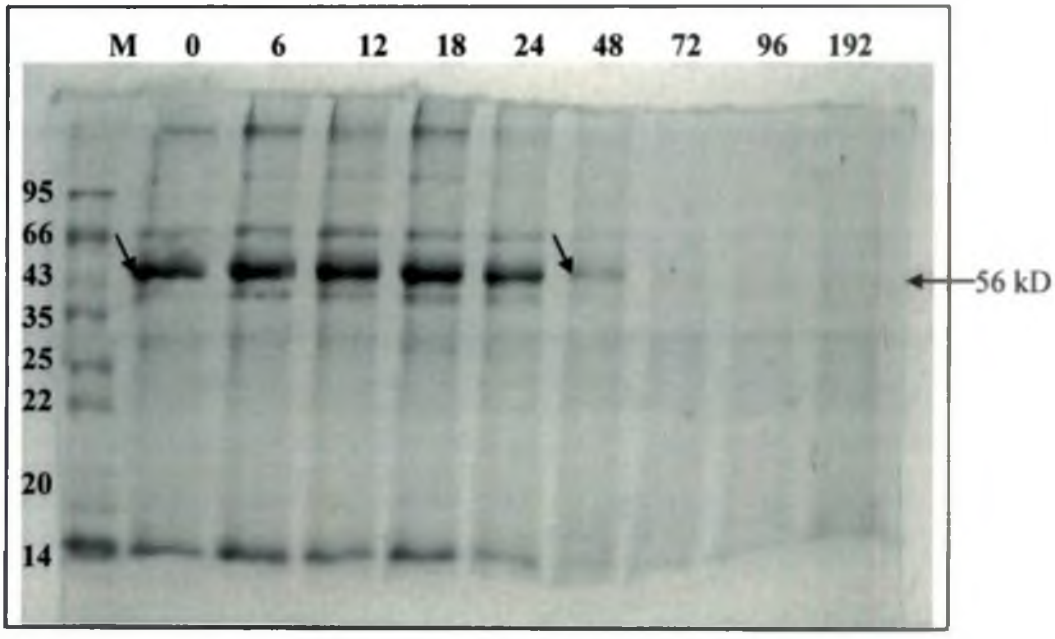
b. Kanakamony

Plate 8. Protein profile of bush type varieties at different time intervals after inoculation with *C. lindemuthianum*

M – Midrange Protein Marker (14-95kD)



a. Lola



b. Arimbra local

Plate 9. Protein profile of pole type varieties at different time intervals after inoculation with *C. lindemuthianum*

M – Midrange Protein Marker (14-95kD)

56kD protein was highly expressed in Lola at 0 hr and then fainted after 6 hr of inoculation, increased again at 12 hr and reached the maximum at 18 hr. That further maintained a high evel of expression up to 48 hr and fainted from 72 hr onwards. In Arimbra Local the 56kD protein has maintained a high level of expression from 0 hr to 24 hr and becomes fainter from 48 hr onwards. Thus the susceptible variety could not maintain the protein level after inoculation wheras a higher level of expression was observed in the resistant variety from the initial hours.

4.5.2.3 Comparison of SDS-PAGE profile of proteins in different time intervals

Comparison of SDS-PAGE profile of proteins from four varieties at 6, 12, 18 and 24 hour after infection with *C. lindemuthianum* was shown in the Plate 10, 11, 12 and 13 respectively. All the hours had shown the same banding pattern in the four varieties. Pusa Komal had shown decrease in the expression of both 56kD and 14 kD proteins after infection whereas in Lola it was a slightly higher expression. Both Arimbra Local and Kanakamony which are the resistant varieties had shown higher level of expression in both control and treatment.

Comparison of SDS-PAGE profile of proteins from the four varieties at 18 hour after infection with *C. lindemuthianum* by silver staining was also done and the result is presented in the Plate 14. The bands were visualized more clearly by the sensitive silver staining method. But no difference in the banding pattern was observed against the previous staining method. No new proteins of lower molecular weight in the range of already reported PR proteins of less concentration were observed. But the fainter band in the range of 29 kD was shown to be highly expressed in the resistant varieties Kanakamony and Arimbra Local compared to the susceptible varieties Pusa Komal and Lola. Thus the resistance mechanism was found to be controlled by the protein of 29kD.

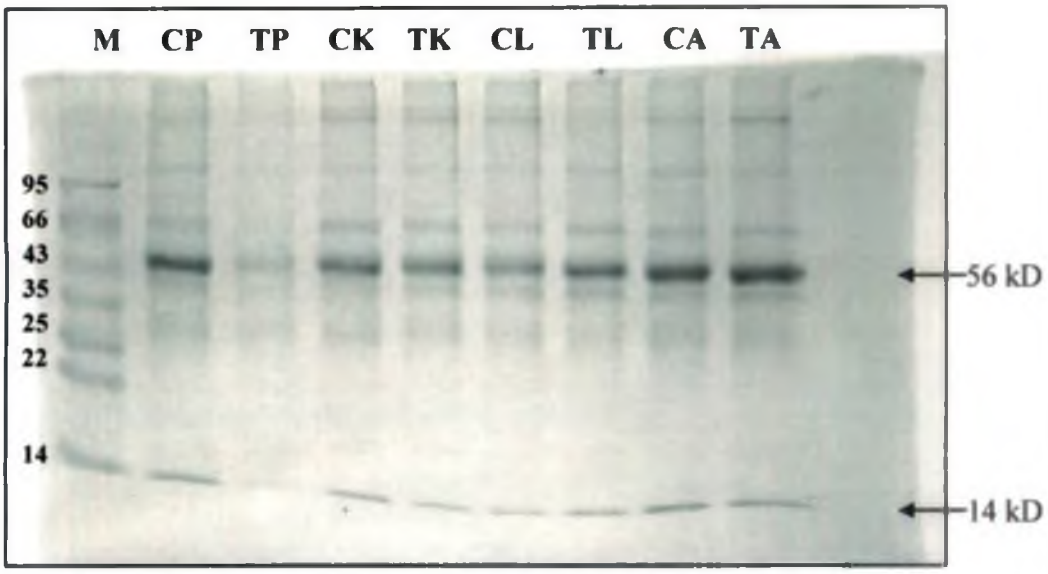


Plate 10. Comparison of protein profile of the four varieties at 6th hour after inoculation with *C. lindemuthianum*

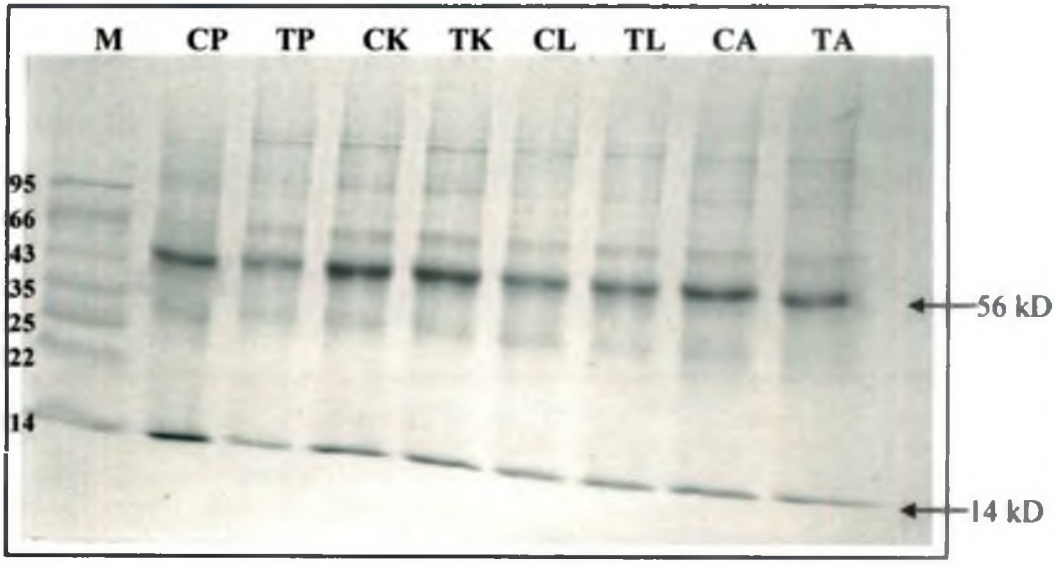


Plate 11. Comparison of protein profile of the four varieties at 12th hour after inoculation with *C. lindemuthianum*

M-Midrange Protein Marker (14-95kD), CP - Control Pusa Komal, TP - Treated Pusa Komal, CK - Control Kanakamony, TK - Treated Kanakamony, CL - Control Lola, TL - Treated Lola, CA - Control Arimbra Local, TL - Treated Arimbra Local

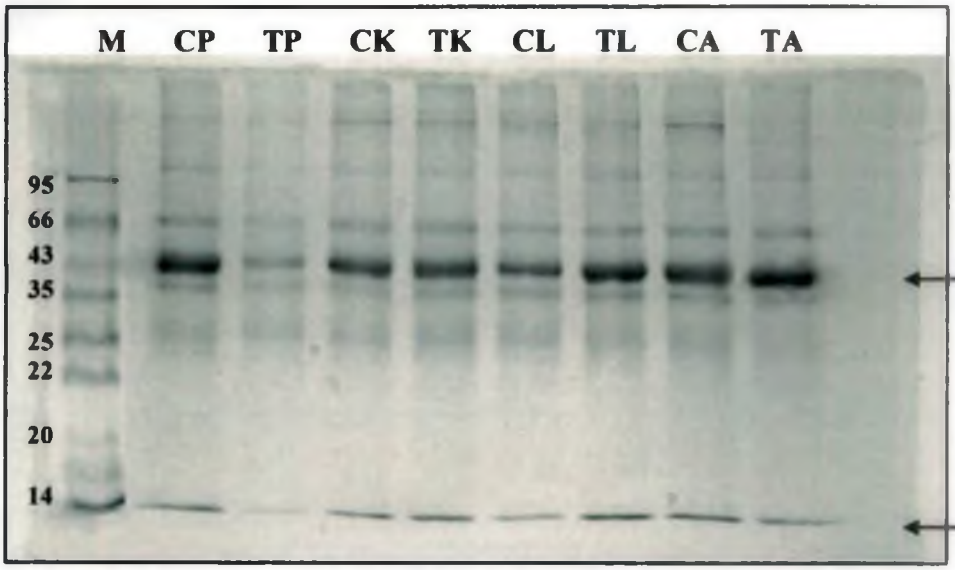


Plate 12. Comparison of protein profile of the four varieties at 18th hour after inoculation with *C. lindemuthianum*

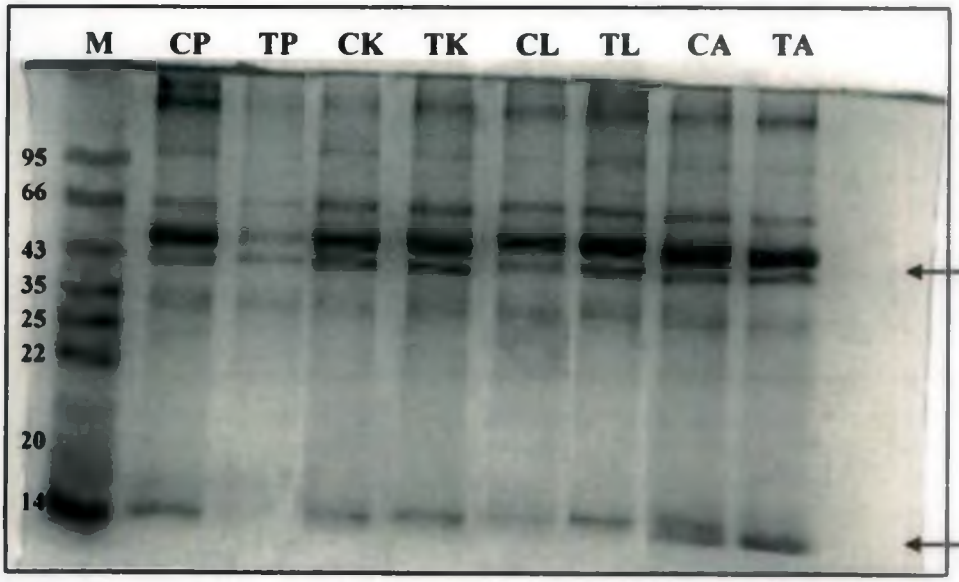


Plate 13. Comparison of protein profile of the four varieties at 24th hour after inoculation with *C. lindemuthianum*

M- Midrange Protein Marker (14-95kD), CP - Control Pusa Komal, TP - Treated Pusa Komal, CK - Control Kanakamony, TK - Treated Kanakamony, CL - Control Lola, TL - Treated Lola, CA - Control Arimbra Local, TL - Treated Arimbra Local

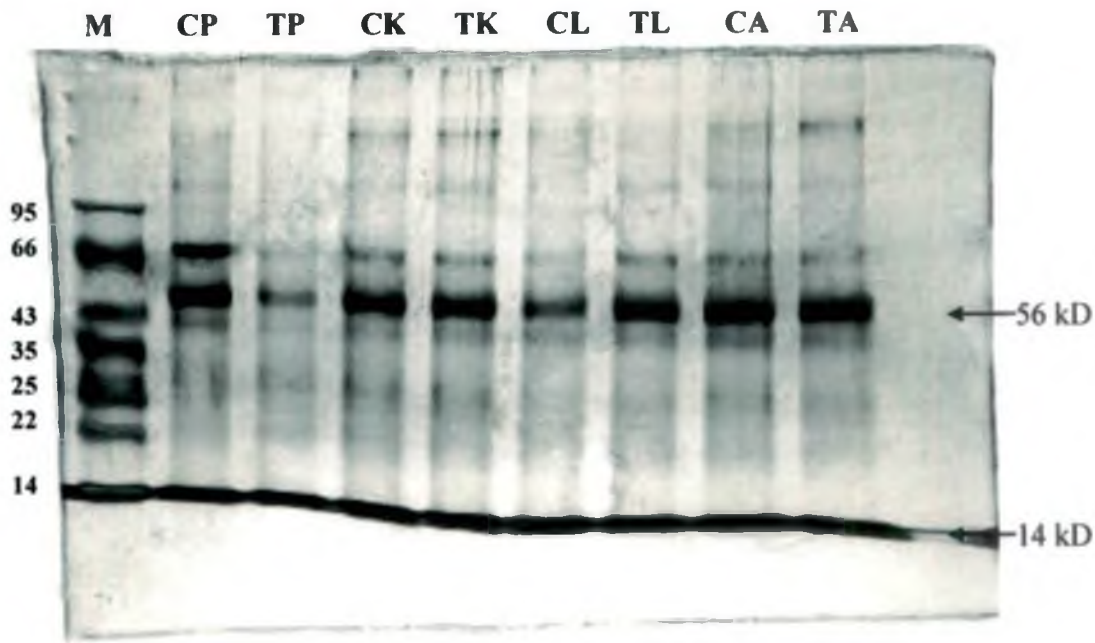


Plate 14. Comparison of protein profile of the four varieties at 18 hour after inoculation with *C. lindemuthianum* as developed by silver staining

M- Midrange Protein Marker (14-95kD), CP - Control Pusa Komal, TP - Treated Pusa Komal, CK - Control Kanakamony, TK - Treated Kanakamony, CL - Control Lola, TL - Treated Lola, CA - Control Arimbra Local, TA - Treated Arimbra Local

4.6 Peptide mass fingerprinting by MALDI-TOF/ MS

The two prominent bands at 56 kD and 14 kD were cut from the gel and sent for peptide mass fingerprinting by Matrix Assisted Laser Desorption and Ionization – Time of Flight Mass spectrometry (MALDI-TOF/MS) (Outsourcing from Sandor sequencing, Hyderabad) for the identification of the differentially expressed protein bands. The protein from the bands are eluted from the gel, digested with trypsin and analyzed by MALDI-TOF mass spectrometry. Mass spectrometric analysis of the intact digest mixture from a band thus provided a set of peptide molecular masses with the corresponding peaks separately for the 56 kD and 14 k D proteins as shown in the Figure 1 and 3 respectively.

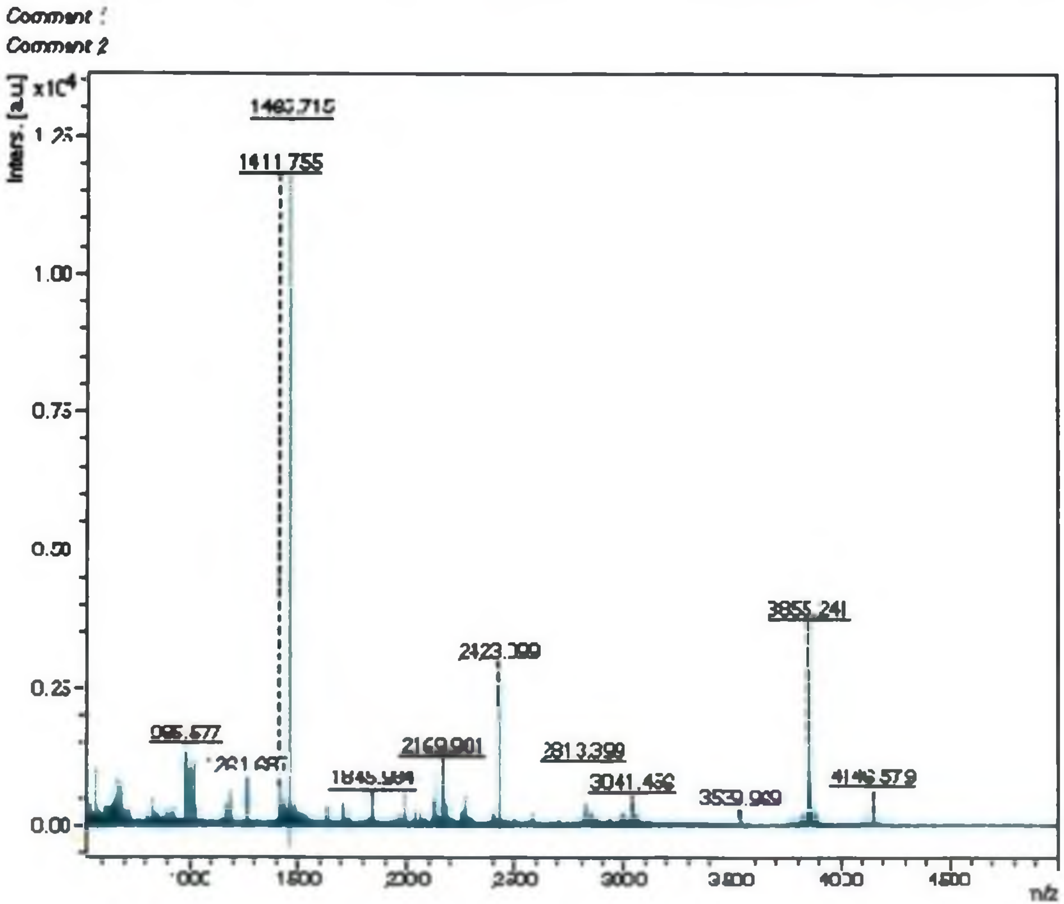
4.7 *In-silico* analysis

The mass spectrometry data having the peak values of peptide mass fingerprint of the 56 kD and 14 kD were analyzed with the Mascot Server software from Matrix Science which is basically utilized for the identification, characterization and quantification of proteins using mass spectrometry data. By comparing the peak values of different peptides with the all available protein databases, the 56 kD and 14 kD proteins were identified to have shown higher similarity with ribulose-1-5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) and ribulose-1-5-bisphosphate carboxylase/oxygenase small subunit, partial (chloroplast) respectively as shown in the Figure 2 and 4.

4.8 SDS-PAGE analysis of purified protein

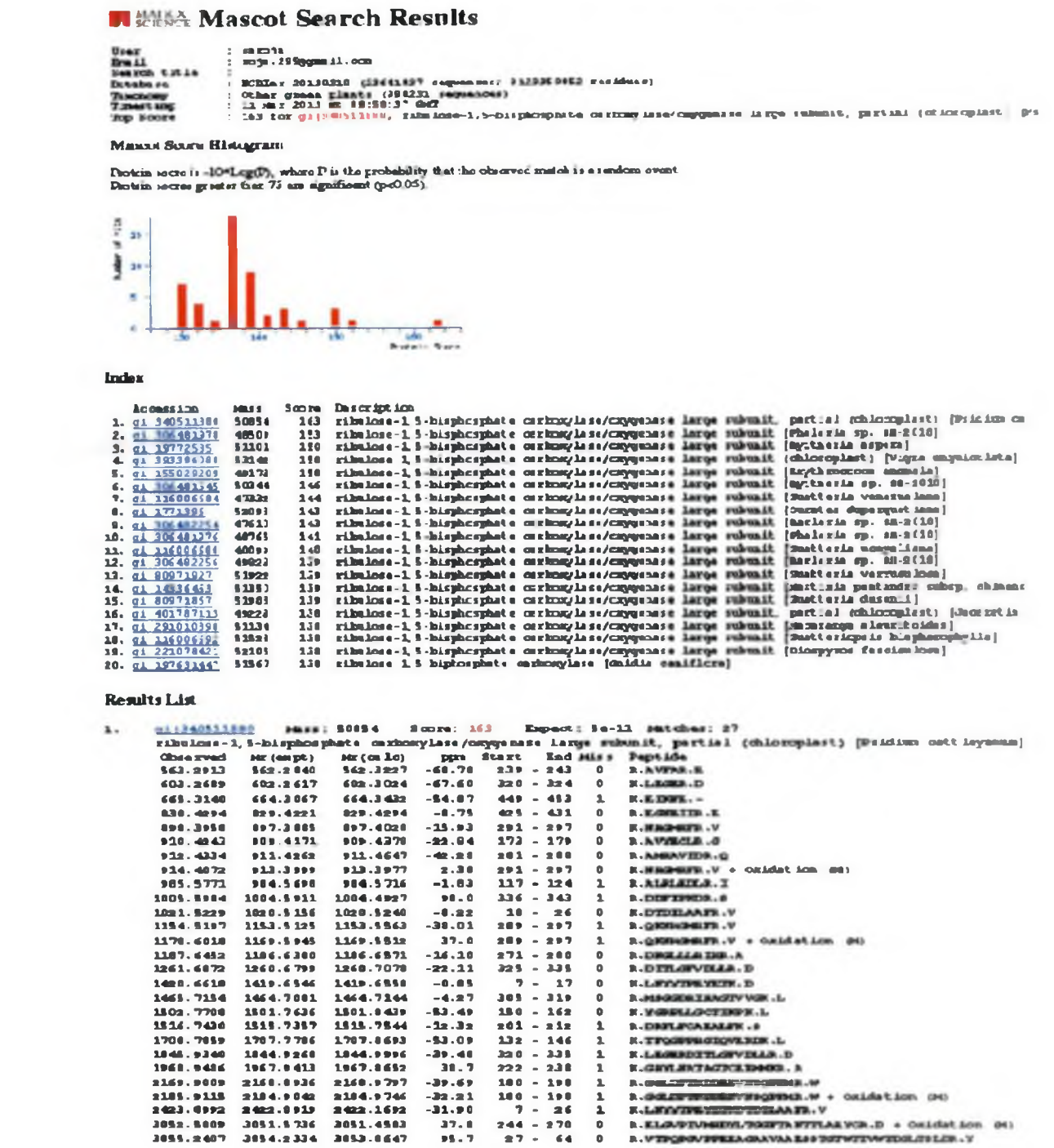
By the quantification of the purified protein in Nanodrop spectrophotometer after precipitation and dialysis, good concentration of protein in the range of 0.75 to 4.8 mg/ g of leaf sample was observed as shown in Table 4.3. The SDS-PAGE profile of the bush and pole type cowpea varieties taken for the study are shown in the Plate 15 and 16. Totally 8 bands were observed in Kanakamony and Arimbra Local whereas only 4 bands were observed in Pusa

Fig 1: Peptide mass fingerprint of the 56 kD protein obtained by MALDI-TOF Mass spectrometry



m/z	S/N	Quality Fac.	Res.	Intens.	Area
[illegible]	11.[illegible]	499	2725	[illegible]	141
539.077	7.4	373	2148	326.16	123
583.291	24.2	2211	3874	1045.89	236
611.340	7.5	200	4235	320.34	73
621.338	8.5	350	4936	361.34	74
637.348	6.4	235	3020	271.52	54
644.385	8.4	304	4706	350.84	76
653.336	9.6	339	4714	405.27	91
[illegible]	16.1	[illegible]	[illegible]	[illegible]	216
665.965	7.5	375	3654	331.72	96
681.947	10.1	148	4645	420.50	105
683.226	13.5	391	3514	571.77	120
830.439	13.0	1803	5342	512.13	146
929.417	8.3	464	4955	321.99	114
985.577	39.8	12126	5868	1411.06	420
1005.508	29.3	20412	5387	1083.73	380
1021.553	32.6	11364	5703	1141.16	412
1170.682	10.1	[illegible]	3360	[illegible]	125
1187.645	18.2	9897	5950	671.63	294
1261.687	24.5	7834	7236	894.66	366
1411.755	19.2	3855	5114	681.16	415
1420.662	7.8	189	5184	276.82	127
1451.761	12.2	307	3505	441.18	256
1450.728	6.2	329	3072	221.03	268
1465.715	362.0	39577	7529	12762.15	6593
1487.698	9.1	1164	5441	346.03	237
1638.800	9.6	6512	8017	332.71	195
1708.706	10.1	1300	6510	331.09	349
1845.954	16.1	20450	7846	564.98	429
1994.949	19.6	16190	8242	525.52	418
2038.930	7.2	1641	7539	190.57	167
2121.916	13.4	1398	5761	331.00	307
2130.124	13.1	1216	3203	324.00	655
2169.901	38.5	24142	7384	931.39	905
[illegible]	15.1	6445	7194	[illegible]	[illegible]
2251.131	7.2	2507	4584	164.28	224
2266.086	18.4	13218	7857	418.78	404
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
2279.228	6.1	373	6325	121.56	183

Fig 2. *In-silico* analysis of the 56 kD protein using Mascot Server software



Mascot Search Results

User : [illegible]
Email : [illegible]
Search title :
Database : NCBInr 20130218 (29441837 sequences; 9123836952 residues)
Taxonomy : Other green plants (38231 sequences)
Timestamp : 11 Mar 2013 at 08:58:3? GMT
Top Score : 163 for gi|[illegible], ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) [Pr

Mascot Score Histogram

Protein scores = -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 73 are significant (p<0.05).

Index

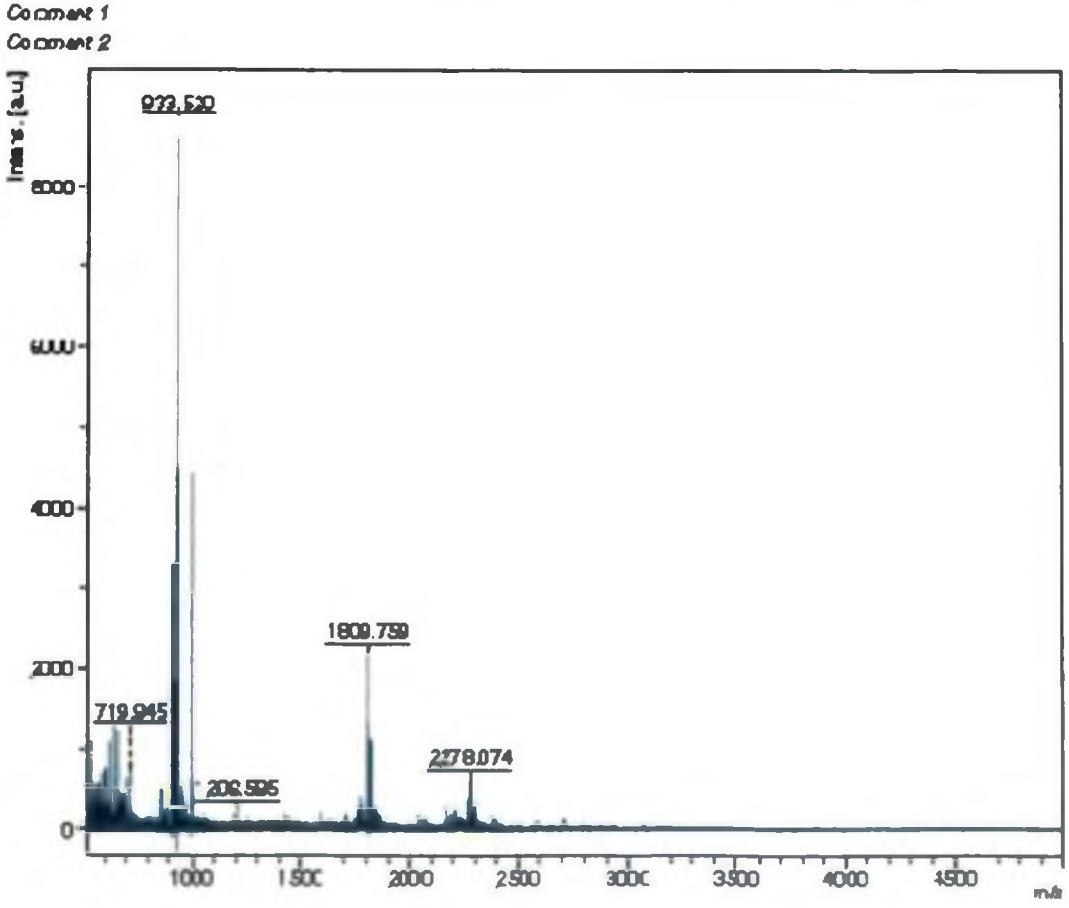
	Accession	Mass	Score	Description
1.	gi 340511880	50854	163	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) [Pyridium ca
2.	gi 306481378	46509	153	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Phaeria sp. SS-2(10]
3.	gi 19772535	51101	150	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Aphanothece sacrum]
4.	gi 293306381	53168	150	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (chloroplast) [Vigna unguiculata]
5.	gi 155020209	48172	150	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Erythrocoon anomala]
6.	gi 306481345	50044	146	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Aphanothece sp. SS-2010]
7.	gi 326006584	47822	144	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Xanthoria ventrosiana]
8.	gi 1771395	52093	143	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Cuscuta chapmanii ana]
9.	gi 306482254	47613	143	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Bacteria sp. SS-2(10]
10.	gi 306481276	46765	141	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Phaeria sp. SS-2(10]
11.	gi 326006581	46809	140	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Xanthoria wasmuthiana]
12.	gi 306482256	49822	139	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Bacteria sp. SS-2(10]
13.	gi 80971927	51929	139	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Xanthoria verrucosa]
14.	gi 14836461	51185	139	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Portulaca pratensis subsp. chamae
15.	gi 80972857	51983	139	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Xanthoria dasmi i]
16.	gi 401787133	49228	138	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) [Jacaratia
17.	gi 291010398	51134	138	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Amaranthus alexandrinus]
18.	gi 326006582	51821	138	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Xanthoriopsis hispanophylla]
19.	gi 22107842?	52105	138	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Diospyros fasciculosa]
20.	gi 197631447	51967	138	ribulose 1,5 biphosphate carboxylase [Gnidia caniflora]

Results List

1. gi|340511880 Mass: 50854 Score: 163 Expect: 5e-11 Matches: 27
ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) [Pyridium cattleyanum]

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Peptide
563.2913	562.2840	562.3227	-68.78	239 - 243		0	R.AVFAR.E
603.2689	602.2617	602.3024	-67.60	320 - 324		0	R.LEGE.D
665.3140	664.3067	664.3432	-54.87	449 - 453		1	R.KIDFK.-
830.4094	829.4021	829.4294	-8.75	425 - 431		0	R.KGNKIR.E
898.3958	897.3885	897.4028	-15.93	291 - 297		0	R.HNGHSF.V
910.4843	909.4771	909.4378	-22.94	172 - 179		0	R.AVVKLR.G
912.4334	911.4262	911.4647	-42.28	281 - 288		0	R.AMAVIDR.G
914.4872	913.4799	913.4977	-2.38	291 - 297		0	R.HNGHSF.V + Oxidation (M)
985.5771	984.5698	984.5716	-1.83	117 - 124		1	R.AIRLEKLR.I
1005.5984	1004.5911	1004.4927	98.0	336 - 343		1	R.DNFPNDR.S
1021.5229	1020.5156	1020.5240	-8.22	18 - 26		0	R.DTDILAAFR.V
1154.5197	1153.5125	1153.5563	-38.01	289 - 297		1	R.QHNGHSFR.V
1170.6018	1169.5945	1169.5512	37.0	289 - 297		1	R.QHNGHSFR.V + Oxidation (M)
1187.6482	1186.6380	1186.6571	-16.10	271 - 280		0	R.DRGLLAGIDR.A
1261.6872	1260.6799	1260.7078	-22.11	325 - 335		0	R.DTLGHVDLLA.D
1420.6618	1419.6546	1419.6858	-0.85	7 - 17		0	R.LAYVTPEYETR.D
1465.7154	1464.7081	1464.7164	-4.27	301 - 319		0	R.HAGGERIAGIVVGK.L
1502.7708	1501.7636	1501.8439	-83.49	150 - 162		0	R.VGKLLQCTIKPK.L
1516.7436	1515.7363	1515.7844	-12.32	201 - 212		1	R.DNFLPCAIALSK.S
1708.7859	1707.7786	1707.8693	-53.09	132 - 146		1	R.TPQSPNGIQVLEDK.L
1846.9260	1844.9268	1844.9996	-39.48	320 - 335		1	R.LRGRDITLGYVDLLR.D
1968.9486	1967.9413	1967.8652	38.7	222 - 238		1	R.GIVLRYTAGTCEEMMK.A
2169.9009	2168.8936	2168.9797	-39.69	180 - 198		1	R.[illegible].W
2185.9115	2184.9042	2184.9746	-32.21	180 - 198		1	R.[illegible].W + Oxidation (M)
2423.0992	2422.0919	2422.1692	-31.90	7 - 26		1	R.LAYVTPEYETRDILAAFR.V
3052.5809	3051.5736	3051.4583	37.8	244 - 270		0	R.KLGAPIVMHEVLTGGFTANTTLAAYCR.D + Oxidation (M)
3055.2407	3054.2334	3053.8647	95.7	27 - 64		0	R.VTPQGVPPEEAQAAVAAESTGTWTTVWTDGLTSLDR.Y

Fig 3: Peptide mass fingerprint of the 14 kD protein obtained by MALDI-TOF Mass spectrometry



m/z	S/N	Quality Fac.	Res.	Intens.	Area
721.026	6.7	19[illegible]	29.00	3[illegible].65	137
525.104	9.0	276	1771	501.85	220
359.183	17.7	2[illegible]	29.63	5[illegible].62	285
546.194	6.1	526	21.28	393.97	89
554.098	8.0	617	29.87	456.53	120
569.130	9.1	768	25.15	492.90	168
584.394	11.8	1225	25.03	658.18	146
599.777	15.3	1[illegible]15	45.22	710.22	144
515.291	19.8	2892	47.95	1059.92	238
307.253	25.4	4[illegible]82	49.07	1[illegible]4.02	285
533.324	21.7	2265	45.23	1169.40	256
563.938	8.8	1[illegible]99	0.[illegible]79	3[illegible].52	128
587.903	6.4	426	26.23	341.10	104
703.964	9.3	856	28.74	491.74	146
719.945	15.8	434	38.98	729.88	220
366.417	10.0	1[illegible]5	31.95	484.55	140
214.[illegible]	73.4	22[illegible]43	57.15	2[illegible]419.17	1044
921.580	39.5	12[illegible]58	59.20	1[illegible]850.08	521
950.[illegible]90	191.1	20[illegible]72	59.[illegible]5	6[illegible]973.30	2759
952.568	11.5	1589	52.75	523.04	186
933.942	6.6	565	53.40	294.63	149
971.405	6.5	744	58.31	280.64	58
998.516	71.7	8[illegible]54	62.40	3[illegible]161.20	1011
999.404	62.2	10009	55.18	2[illegible]965.85	981
1206.595	7.9	1979	65.69	320.48	120
1961.739	7.3	258	53.05	2[illegible]84.81	272
1770.219	9.0	1042	27.69	290.40	508
1809.759	40.2	4[illegible]258	70.85	3[illegible]9.27	1570
1825.737	34.2	74449	61.27	1[illegible]01.44	759
1891.351	6.5	15[illegible]	0.[illegible]9	[illegible]81.37	175
2167.108	8.2	1678	58.45	203.20	298
2205.058	6.5	412	61.48	151.26	172
2211.048	8.3	4071	50.67	199.81	164
2267.035	10.0	621	61.58	221.85	239
2278.074	24.5	6[illegible]93	75.45	551.22	344
2294.086	9.9	308	61.07	220.42	238

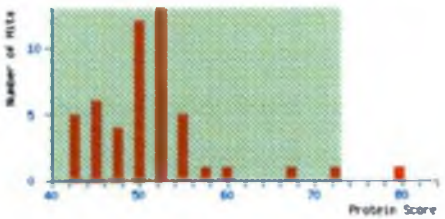
Fig 4. *In-silico* analysis of the 14 kD protein using Mascot Server software

MATRIX SCIENCE Mascot Search Results

User	: saroja
Email	: roja.295@gmail.com
Search title	:
Database	: NCBInr 20130310 (23641037 sequences; 8123359852 residues)
Taxonomy	: Other green plants (990231 sequences)
Timestamp	: 11 Mar 2013 at 10:03:22 GMT
Top Score	: 80 for gi|354460737, ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit, partial [Vigna unguiculata

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 73 are significant (p<0.05).



Index

	Accession	Mass	Score	Description
1.	gi|354460737	12279	80	ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit, partial [Vigna unguiculata]
2.	gi|326478223	20765	72	Ribulose 1,5-bisphosphate carboxylase small subunit precursor [Coffea canephora]
3.	gi|15374841?	20230	61	Rubisco SSU [Plantago major]
4.	gi|102375904	14121	59	plastid ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit [Coffea arabica]
5.	gi|402877330	20607	56	Cristal-Glass] protein [Capsicum annuum]
6.	gi|3914586	21814	55	RecName: Full=Ribulose bisphosphate carboxylase small chain, chloroplastic; Short=RuBisCO small
7.	gi|39000169	20526	55	RecName: Full=Ribulose bisphosphate carboxylase small chain, chloroplastic; Short=RuBisCO small
8.	gi|12010640	16361	54	ribulose 1,5 bisphosphate carboxylase [Pisum sativum]
9.	gi|227804858	48584	54	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Leucomalos narbonense]
10.	gi|30013663	20496	54	putative ribulose bisphosphate carboxylase small subunit protein precursor [Nicotiana tabacum]
11.	gi|194473475	52987	53	ribulose-1,5-bisphosphate carboxylase/oxygenase [Collania taxiphylloides]
12.	gi|410509313	52870	53	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (chloroplast) [Macromitrium ferrie
13.	gi|410509315	52890	53	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (chloroplast) [Macromitrium gymnos
14.	gi|410509317	52842	52	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (chloroplast) [Macromitrium proloo
15.	gi|372480283	53311	53	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (chloroplast) [Drimia altissima]
16.	gi|1332120	20508	53	RecName: Full=Ribulose bisphosphate carboxylase small chain 2A, chloroplastic; Short=RuBisCO sma
17.	gi|372480287	53680	52	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (chloroplast) [Ornithogalum tenuif
18.	gi|374973143	53734	52	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Albuca kirkii]
19.	gi|24840128	20720	52	ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit [Coffea arabica]
20.	gi|3914590	20164	52	RecName: Full=Ribulose bisphosphate carboxylase small chain, chloroplastic; Short=RuBisCO small

Results List

1. gi|354460737 **Mass**: 12279 **Score**: 80 **Expect**: 0.011 **Matches**: 6

ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit, partial [Vigna unguiculata]

Observed	mr(expt)	mr(calc)	ppm	Start	End	Miss	Peptide
914.4028	913.3955	913.3930	2.77	38 -	45	0	R.SPGYYDGR.Y
921.4896	920.4823	920.4967	-4.77	9 -	15	0	K.EIDYLLR.H
933.5189	932.5126	932.5080	5.02	81 -	88	0	R.IIGFDNVR.Q
998.5163	997.5091	997.5094	-0.31	72 -	80	0	R.IAEPNGFVR.I
1809.7595	1808.7522	1808.7981	-25.35	38 -	51	1	R.SPGYYDGKYWTPDK.L
1825.7573	1824.7500	1824.7930	-23.56	38 -	51	1	R.SPGYYDGKYWTPDK.L + Oxidation (M)

No match to: 521.0365, 523.1339, 539.0452, 586.1941, 654.0775, 569.1300, 584.3737, 599.7574, 615.3912, 637.3554, 653.3239, 665.9281, 687.9825, 703.9637, 719.9446, 864.4167, 952.3681, 955.4820, 971.4632, 999.4836, 1206.5953, 1761.7589, 1770.2191, 1841.7506, 2167.1075, 2205.0384, 2211.0485, 2267.0345, 2278.0738, 2294.0957

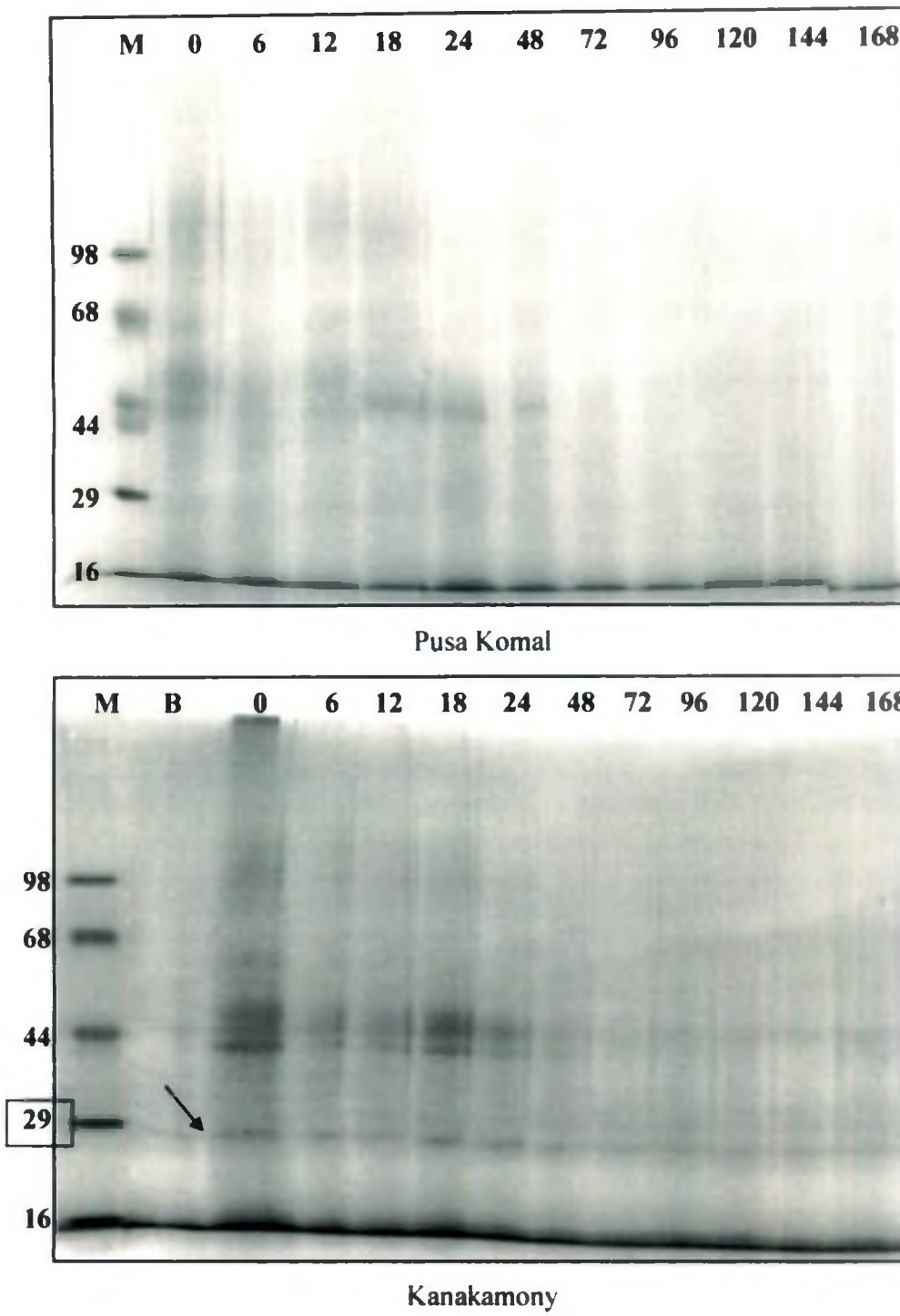


Plate 15. SDS-PAGE profile of purified proteins of bush type varieties at different time intervals after inoculation with *C. lindemuthianum*

M – Medium range protein marker (16-97 kD), B – Blank

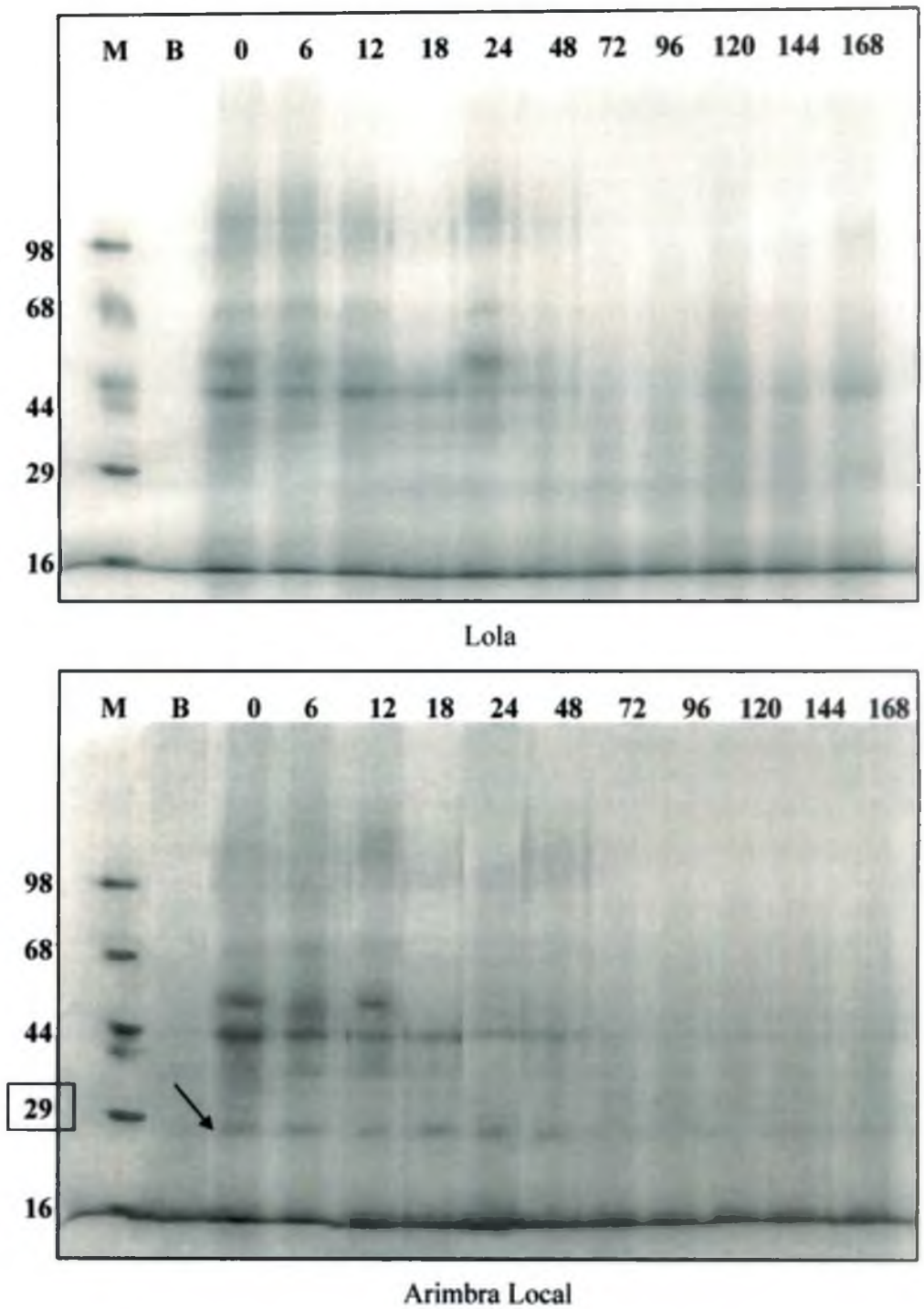


Plate 16. SDS-PAGE profile of of purified proteins of pole type varieties at different time intervals after inoculation with *C. lindemuthianum*

M – Medium range protein marker (16-97 kD), B – Blank

Komal and Lola. When compared to the protein profile of the cowpea varieties with crude protein extract as shown in plates 8 and 9, a distinct protein band at 29 kD was observed with the purified proteins. The resistant varieties Kanakamony and Arimbra Local have shown a higher expression of the 29 kD protein whereas it was absent in the susceptible varieties Pusa Komal and Lola.

4.9 Defense enzyme assay

4.9.1 Peroxidase (PO)

The peroxidase activity in the four cowpea varieties at different time intervals after artificial inoculation with the pathogenic fungi is presented in Table 3 and Figure 5. The PO activity increased with time after artificial inoculation, reached the maximum of 0.027, 0.041, 0.043 and 0.055 units/min in Pusa Komal, Lola, Arimbra Local and Kanakamony respectively at 18 hrs in all the varieties. Then gradually the PO levels were reverted to the initial levels. It is very clear from the Fig. 5 that immune variety Kanakamony maintains the highest level of PO, followed by the resistant variety Arimbra Local. In the susceptible variety Lola, PO level was lower and in most susceptible variety Pusa Komal, the PO level was the lowest. From this Figure it is very clear that PO assay directly indicate the level of resistance in any accession and hence could be recommended as a biochemical marker for anthracnose resistance in *Vigna* spp.

4.9.2 Polyphenol oxidase (PPO)

The PPO activity in the four cowpea varieties at different time intervals after artificial inoculation with the pathogenic fungi was given in Table 4 and depicted in the Fig. 6. The activity values in the four varieties were found to be higher in the range between 0.4 and 0.6 units/min. But there is no significant difference in the activity between the four varieties. The analysis failed to establish a direct relation between the level PPO and anthracnose infection in vegetable cowpea.

Table 3. Peroxidase (PO) activity of cowpea varieties in relation to infection with *C. lindemuthianum*

Sl.No	Sampling interval (Hours)	Enzyme activity (units/min)			
		Pusa Komal	Lola	Arimbra Local	Kanakamony
1	0	0.018	0.021	0.037	0.037
2	6	0.018	0.023	0.035	0.037
3	12	0.024	0.032	0.038	0.042
4	18	0.027	0.041	0.043	0.055
5	24	0.022	0.038	0.039	0.044
6	48	0.023	0.028	0.038	0.038
7	72	0.025	0.027	0.038	0.035
8	96	0.026	0.027	0.038	0.038
9	120	0.022	0.024	0.037	0.036
10	144	0.022	0.024	0.036	0.038
11	168	0.021	0.023	0.037	0.035
12	192	0.021	0.023	0.038	0.035

Fig 5: Standard graph showing pattern of peroxidase enzyme (PO) activity of the four cowpea varieties at different time intervals after inoculation with *C. lindemuthianum*

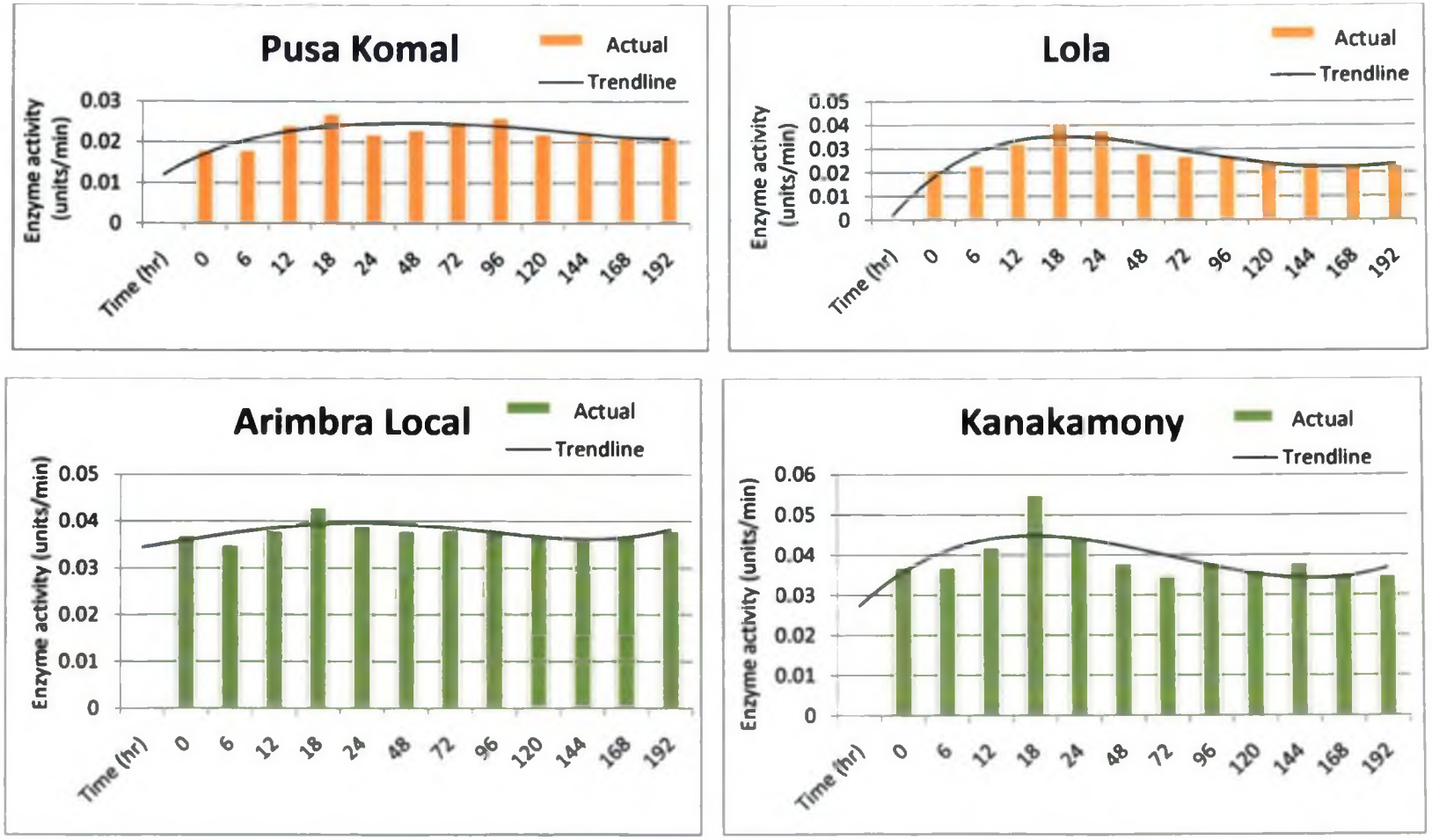
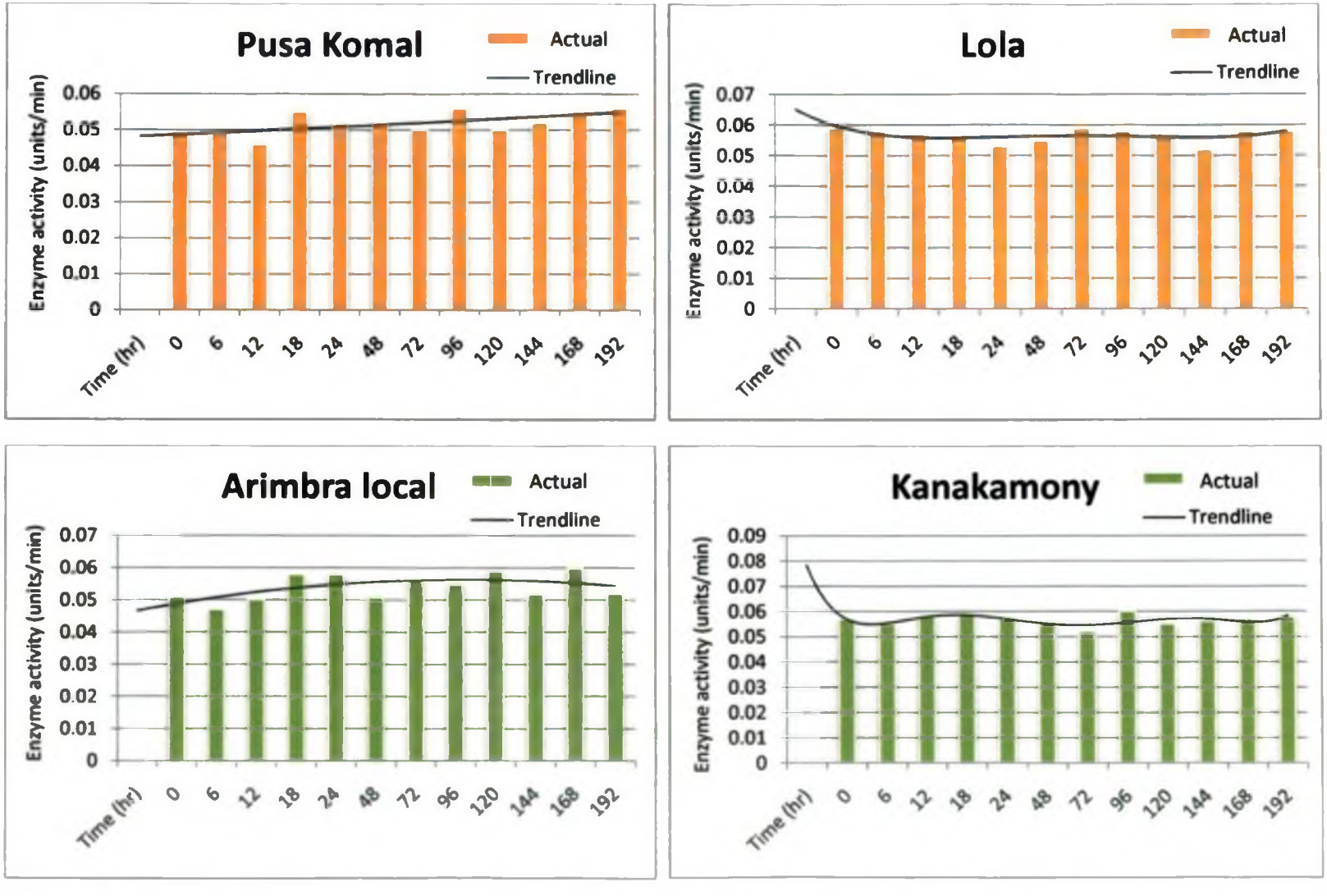


Table 4. Polyphenol oxidase (PPO) activity in cowpea varieties in relation to infection with *C. lindemuthianum*

Sl.No	Sampling interval (Hours)	Enzyme activity (units/min)			
		Pusa Komal	Lola	Arimbra Local	Kanakamony
1	0	0.049	0.059	0.051	0.057
2	6	0.049	0.058	0.047	0.055
3	12	0.046	0.057	0.05	0.058
4	18	0.055	0.056	0.058	0.059
5	24	0.051	0.053	0.058	0.057
6	48	0.052	0.055	0.051	0.055
7	72	0.05	0.059	0.056	0.052
8	96	0.056	0.058	0.055	0.06
9	120	0.05	0.057	0.059	0.055
10	144	0.052	0.052	0.052	0.056
11	168	0.055	0.058	0.06	0.057
12	192	0.056	0.058	0.052	0.058

Fig 6: Standard graph showing pattern of polyphenol oxidase enzyme (PPO) activity of the four cowpea varieties at different time intervals after inoculation with *C. lindemuthianum*



4.9.3 Phenylalanine ammonia lyase (PAL)

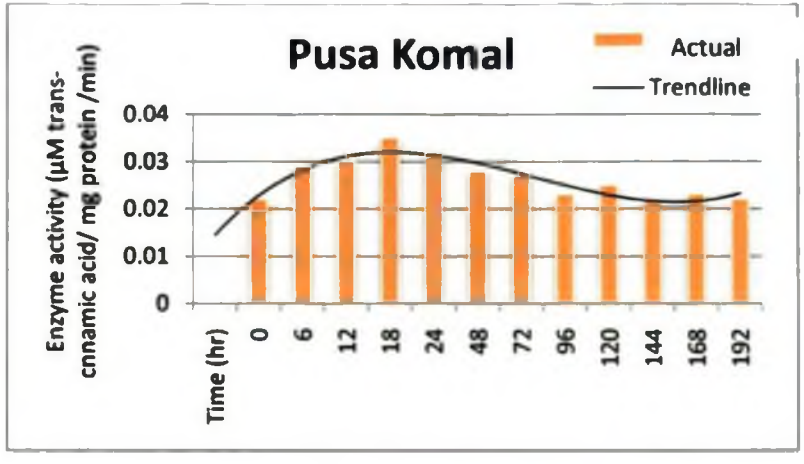
The PAL activity in the four cowpea varieties at different time intervals after artificial inoculation with the pathogenic fungi was given in Table 5 and Fig. 7. The activity reached the maximum value of 0.35, 0.44, 0.48 and 0.53 µM trans-cinnamic acid/ mg protein /min in the cowpea varieties Pusa Komal, Lola, Arimbra Local and Kanakamony respectively. The activity increases abruptly reaching the maximum at 18 hrs incase of Pusa Komal, Arimbra Local and Kanakamony whereas it reaches the maximum at 24 hrs incase of Lola variety and gradually decreased. As in case of PO, the immune Kanakamony and resistant Arimbra Local have maintained high levels of PAL against the susceptible varieties Lola and Pusa Komal. Thus the PAL levels in *Vigna* spp. are directly correlated with the level of resistance and hence, along with PO, PAL could also be recommended as a biochemical marker for understanding the level of anthracnose resistance in breeding lines.

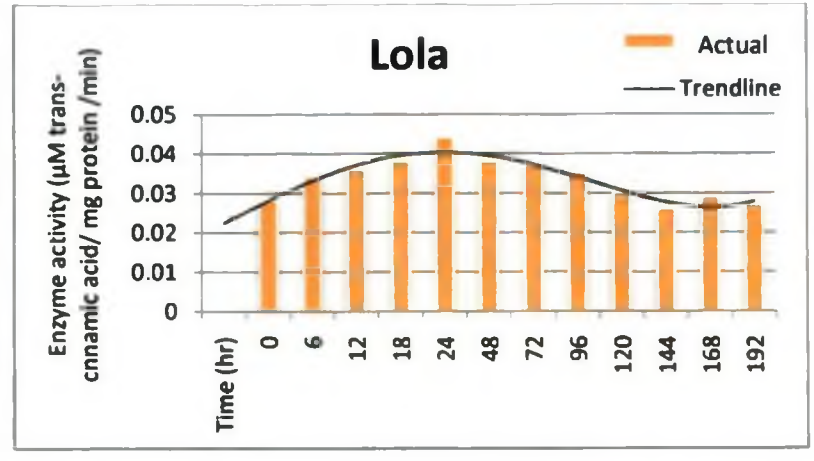
In all the three assays, considering the large sample size there could be deviations in the values when taken at different time intervals. So the absorbance values of a single assay were taken directly without any replications.

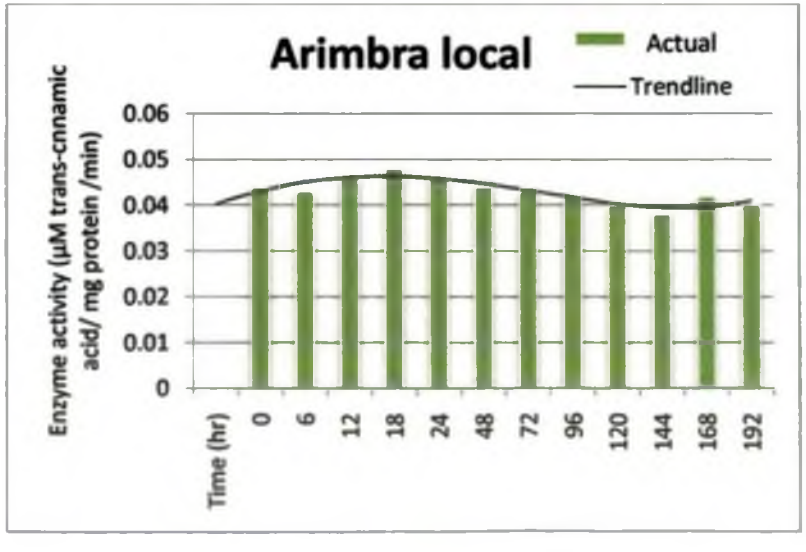
Table 5. Phenylalanine ammonia lyase (PAL) activity in cowpea varieties in relation to infection with *C. lindemuthianum*

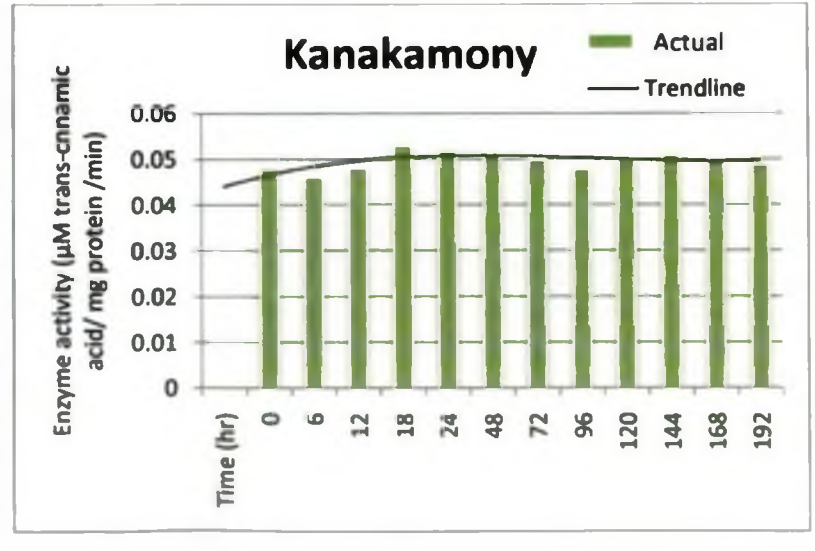
S.No	Sampling interval (Hours)	Enzyme activity (µM trans-cnnamic acid/ mg protein /min)			
		Pusa Komal	Lola	Arimbra Local	Kanakamony
1	0	0.022	0.028	0.044	0.048
2	6	0.029	0.034	0.043	0.046
3	12	0.03	0.036	0.046	0.048
4	18	0.035	0.038	0.048	0.053
5	24	0.031	0.044	0.046	0.052
6	48	0.028	0.038	0.044	0.051
7	72	0.027	0.037	0.043	0.05
8	96	0.023	0.035	0.042	0.048
9	120	0.025	0.03	0.04	0.05
10	144	0.022	0.026	0.038	0.051
11	168	0.023	0.029	0.042	0.05
12	192	0.022	0.027	0.04	0.049

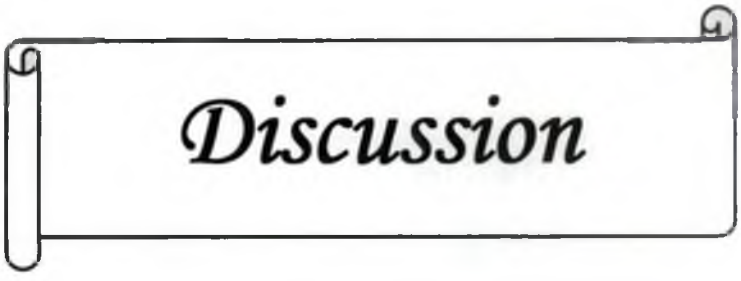
Fig 7: Standard graph showing pattern of phenylalanine ammonia lyase enzyme (PAL) activity of the four different varieties at different time intervals after inoculation with *C. lindemuthianum*










Discussion

5. DISCUSSION

The results obtained in the present study "Characterization of pathogenesis related proteins for anthracnose resistance in vegetable cowpea, *Vigna* spp." are discussed in this chapter based on the earlier reports and best possible interpretations.

5.1 Isolation and identification of the pathogen

The typical anthracnose symptom showing reddish-brown streaks on the stem was used for the isolation of the pathogen plating on PDA medium which is a commonly used fungal growth medium. But as the sporulation was found to be very less, a selective medium (Neopeptone-Glucose-Agar) was used for inoculating the fungus which was already standardized by Kumar (1999). The NGA medium showed fast growth and high sporulation as compared to PDA and chosen for maintenance of the pure culture of the pathogen.

5.2 Pathogenicity test

The pathogenicity of the organism was established by artificial inoculation on susceptible varieties Pusa Komal and Lola which produced typical symptoms of anthracnose disease at 3-4 days of inoculation. Reisolation of the pathogen yielded same as that of original culture. Based on cultural and morphological characters as reported by Kumar (1999), the fungal pathogen was identified and confirmed as *Colletotrichum lindemuthianum* causing stem anthracnose disease in cowpea. The inoculation procedure followed by wrapping of the wounded stem with inoculm was found to the best method of inoculation of *C. lindemuthianum* in cowpea by Adebitan *et al.* (1992). The pathogenicity test was also carried out by Sicard *et al.* (1997) with the spore suspension having the same concentration of 10^6 spores per ml. Geffroy *et al.* (2008) have also conducted the pathogenicity test under more than 90 per cent relative humidity while studying the resistance to *Colletotrichum lindemuthianum* in *Phaseolus vulgaris*.

5.3 Pot culture experiments

The initial pot culture experiment carried out during July - August, 2012 had failed to induce disease symptom with artificial inoculation of the pathogen even with pin prick injury due to the unfavorable conditions prevailed during the experimental period where the high humidity required for the disease development was not met due to sparse rainfall. The pin prick injury given before artificial inoculation can itself induce the pathogenesis related proteins which are responsive to wounding as reported by Tao (2003). The experiment was repeated again during August – September 2012 with measures taken to ensure the high humidity and favorable temperature by keeping the pots in a greenhouse with mist facility. High humidity was maintained by placing the potted plants in a basin of water and covering them with a moistened plastic cover. Goncalves-Vidigal and Kelly (2006), Marin *et al.* (2007), Suarez *et al.* (2008) and Goncalves-Vidigal *et al.* (2011) have also maintained high humidity in mist chamber after artificial inoculation while studying the genes responsible for anthracnose resistance in the common bean cultivar in different cultivars.

5.3.1 Symptomatology of the disease under artificial condition

Typical symptoms of anthracnose disease were observed on artificial inoculation similar to that described by Onesirosan and Barker (1971), Williams (1975) and Kumar (1999).

5.3.2 Reaction of genotypes to *C. lindemuthianum*

Pusa Komal was found to be highly susceptible with cent per cent disease incidence and severity. Kanakamony was found to be immune to anthracnose disease as no infection was observed. The results confirmed earlier findings of Kumar (1999). Furthermore, Lola was also found to be highly susceptible recorded cent per cent disease incidence and 68.8 per cent severity whereas

Arimbra Local was found to be highly resistant with 22 per cent disease incidence and 8.3 per cent severity.

Kanakamony is a dual type cowpea i.e., it is suited as a grain type and also as a vegetable type. Generally the grain types are resistant to anthracnose (Kumar, 1999) and this may be the reason for the immunity observed in Kanakamony.

Anthracnose disease is a very severe problem in vegetable cowpea in Kerala, where the climatic conditions, with two continuous monsoons during the cropping phase, are highly suitable for the proliferation of this debris borne fungus. Arimbra Local is a landrace which is reported to be resistant to the anthracnose disease. This landrace was identified from the Arimbra village of Malappuram district in Kerala, where vegetables are extensively grown for the purpose of export to Gulf countries, since the village falls close to the Kozhikode airport. Few years back the vegetable cowpea cultivation in this village had come to a halt with all the available pole type varieties falling highly susceptible to the anthracnose disease. Subsequently, farmers have identified this particular landrace which shows the resistance to anthracnose disease and the cultivation was revived. This accession is being reported as the first pole type vegetable cowpea in the world which holds resistance to anthracnose and will have immense breeding value. The results of the present study are confirmatory to the claims from the farmer's fields.

5.4 Extraction and quantification of total protein

Pathogenesis related (PR) proteins occur in the vacuolar compartment or cell wall or intercellular spaces. According to Van Loon (1999) localization of the major, acid soluble PRs in the intercellular space of the leaf seems to guarantee contact with invading fungi or bacteria before these are able to penetrate. However, in spite of numerous investigations involving injection of plants with purified proteins, in vitro tests for inhibition of pathogen growth or in vivo infection of transgenic plants, few of the inducible acidic PRs associated with

SAR have been shown to possess significant anti-pathogenic activity. Hence Tris-HCl buffer of neutral pH (7.5) was used for the total protein extraction from the treated and control leaf samples as already reported by Gomes (1996) for the isolation of chitinase and β-1, 3-glucanase from cowpea seeds which have shown bioactivity against *C. lindemuthianum*. Thus a high concentration of protein ranging between 18 to 74 mg/g of leaf sample was obtained. Quantification of the protein was carried out by A280 method in NanoDrop Spectrophotometer as already performed by Shivashankar *et al.* (2010).

5.5 Protein profiling by SDS-PAGE analysis

The SDS-PAGE profile of the proteins extracted at neutral pH (pH-7.5) from the four varieties collected at different time intervals were compared. In all the four varieties, a maximum of 7 bands/ lane were observed in the size range of more than 100 kD, 70 kD, 56 kD, 40 kD, 29 kD and 14 kD. The susceptible varieties, Pusa Komal and Lola were found to have similar expression profile in which there was lesser expression of proteins in the initial hours then increased gradually up to 18 hours after infection and started to decrease drastically after 72 hours whereas the resistant varieties, Kanakamony and Arimbra Local where found to have the similar expression profile where there was higher level of expression in the initial hours which was maintained up to 72 and 48 hours respectively and further decreases.

The reduction in the protein expression during later hours can be explained due to the hemibiotrophic nature of infection which includes a biotrophic and necrotrophic phase of infection as indicated by Mendgen and Hahn (2002). In the biotrophic phase, large, unusual, multilobed, multiseptate infection vesicles with elongated neck region occur inside the host cells but does not kill any cells and induce the plant for the defense response. In the necrotrophic phase, rapid development of invasive secondary hyphae damage and kills the host cell leading to the degradation of the protein during later hours.

Comparison of the SDS-PAGE profile of the proteins obtained from control and treated samples of the four varieties in the early hours (6, 12, 18, 24 hours) had shown a similar banding pattern which confirms the reproducibility of the SDS-PAGE analysis. Two protein bands of size 56 kD and 14 kD showed prominent difference in expression in the cowpea varieties selected for study. In Pusa Komal, there is a downregulation of the proteins after infection whereas in Lola there is a slight upregulation and the resistant varieties Kanakamony and Arimbra local maintained the same higher level of expression even after the infection. The pattern of protein expression indicates that the resistance behavior was found to be associated with the 56 kD and 14 kD bands which occurred in the size range of PR proteins.

The results were also found in accordance with those obtained by Benhamou *et al.*(1991) in which the resistant tomato plants showed high level of accumulation of β-fructosidase earlier than the susceptible plants. The early process of infection in resistant plants triggers the rapid synthesis of β-fructosidase, which accumulates in all tissues and does not increase thereafter. By contrast, in susceptible plants, the enzyme seems to be gradually synthesized in newly infected cells only, indicating that cell surface interactions between plant and pathogen may be of key importance for the induction of β-fructosidase synthesis.

5.6 Peptide mass fingerprinting by MALDI-TOF/ MS

The two prominent protein bands of 56 kD and 14 kD in Lola variety which clearly depicted the difference in the expression before and after inoculation with the pathogenic fungus were selected for peptide sequencing. The protein bands were eluted and digested with trypsin into peptides before embedding into a matrix made of aromatic compounds. A laser beam ionized the matrix along with the peptides, evaporated them and made to travel along a tube. The time of flight of each peptide is directly proportional to the molecular mass of the peptides, the data was fed to the computer and the peptide mass fingerprint

was generated. The 56 kD band size protein was digested into peptides with size ranging from 9 kD to 41 kD and the 14 kD band size protein was digested into peptides in the size range between 5 kD and 22 kD.

MALDI-TOF peptide mass fingerprinting (PMF) is the fastest and cheapest method of protein identification promoting the characterization of PR proteins in many plants like *Zea mays* (Campo *et al.*, (2004), *Oryza sativa* (Kim *et al.*, 2004) and *Medicago truncatula* (Colditz *et al.*, 2004).

5.7 *In-silico* analysis

Analysis of the peptide mass fingerprint of the two prominent bands of size 56 kD and 14 kD in the Mascot Server Software compared the data with all other protein sequences in NCBI database. Thus the two prominent bands of size 56 kD and 14 kD were found to be highly similar to the large and small subunit of Ribulose-1-5-bisphosphate carboxylase/ oxygenase which is the main enzyme in the photosynthesis that fixes the atmospheric carbon dioxide in the cells (Hopkins and Hüner, 2009). High expression of these enzymes directly correlated with the photosynthetic efficiency of the plant. In mesophyll cells of C_3 plants, CO_2 binding to its primary acceptor, ribulose-1,5-bisphosphate (RuBP), is catalyzed by RuBP carboxylase/oxygenase (RuBisCo), and the product of this carboxylation process, 3-phosphoglycerate (PGA), is converted to other carbohydrates. In addition to the usual carboxylation reaction, Rubisco catalyzes an oxygenase reaction in which O_2 reacts with RuBP to give PGA and phosphoglycolate, a process known as photorespiration (Pessarakli, 2001). Pathogen attacks affect the photosynthesis of plant and the leaves and stem turn chlorotic. In this study, it is proven that the susceptible variety, Pusa Komal has shown a downregulation of RuBisCo after infection whereas the resistant varieties, Kanakamony and Arimbra Local were able to maintain the higher level of expression even after the infection with the pathogenic fungi. This pattern of enzyme expression suggests that the high demand in energy and carbon sources during pathogenesis could be met by the maintenance of higher levels of RuBisCo enzyme in the resistant varieties.

5.8 SDS-PAGE analysis of purified protein

In search of the most abundant intracellular pathogenesis related proteins which are acid soluble, the protein extracted in the neutral pH was precipitated using 90 per cent saturation of ammonium sulphate, suspended in the 5.5 pH buffer, purified in dialysis tubing and analyzed by SDS-PAGE. Thus the protein was purified and the buffer system was also changed. The protein concentration when quantified in Nanodrop spectrophotometer was found in the range of 0.75 to 4.8 mg/g of leaf sample which was lesser in comparable to the total protein extracted with neutral pH but sufficient for the SDS-PAGE analysis. The SDS-PAGE profile of the resistant and susceptible varieties were found to be distinct in case of a 29 kD band which was present in the resistant varieties, Arimbra Local and Kanakamony whereas it was absent in the susceptible varieties, Pusa Komal and Lola. The similar kind of PR protein was also detected in leaves and roots of potato plants infected with pathotypes of *Globodera pallida* (Rahlmi *et al.*, 1993). A PR-2 class protein of 29 kD was also found in *Capsicum annuum* leaves induced by cucumber mosaic virus infection (Candela *et al.*, 1993). A 29 kD protein was also found to be accumulated as a developmentally controlled defense response during fruit ripening in grapes having endochitinase activity (Salzman *et al.*, 1998). Thus the 29 kD protein observed in the protein profile of the resistant varieties could be a member of the class 2 or class 3 PR protein.

5.9 Defense enzyme assay

5.9.1 Peroxidase (PO)

Many previous studies have previously established that peroxidase is a defense enzyme in plants and is extensively used for understanding the stress responses. They oxidizes phenolics to quinines and generate hydrogen peroxide They participate in a broad range of physiological processes, such as lignin and suberin formation, cross-linking of cell wall components, and synthesis of phytoalexins, or participate in the metabolism of reactive oxygen species (ROS)

and reactive nitrogen species (RNS), both switching on the hypersensitive response, a form of programmed host cell death at the infection site associated with limited pathogen development (Almagro *et al.*, 2008).

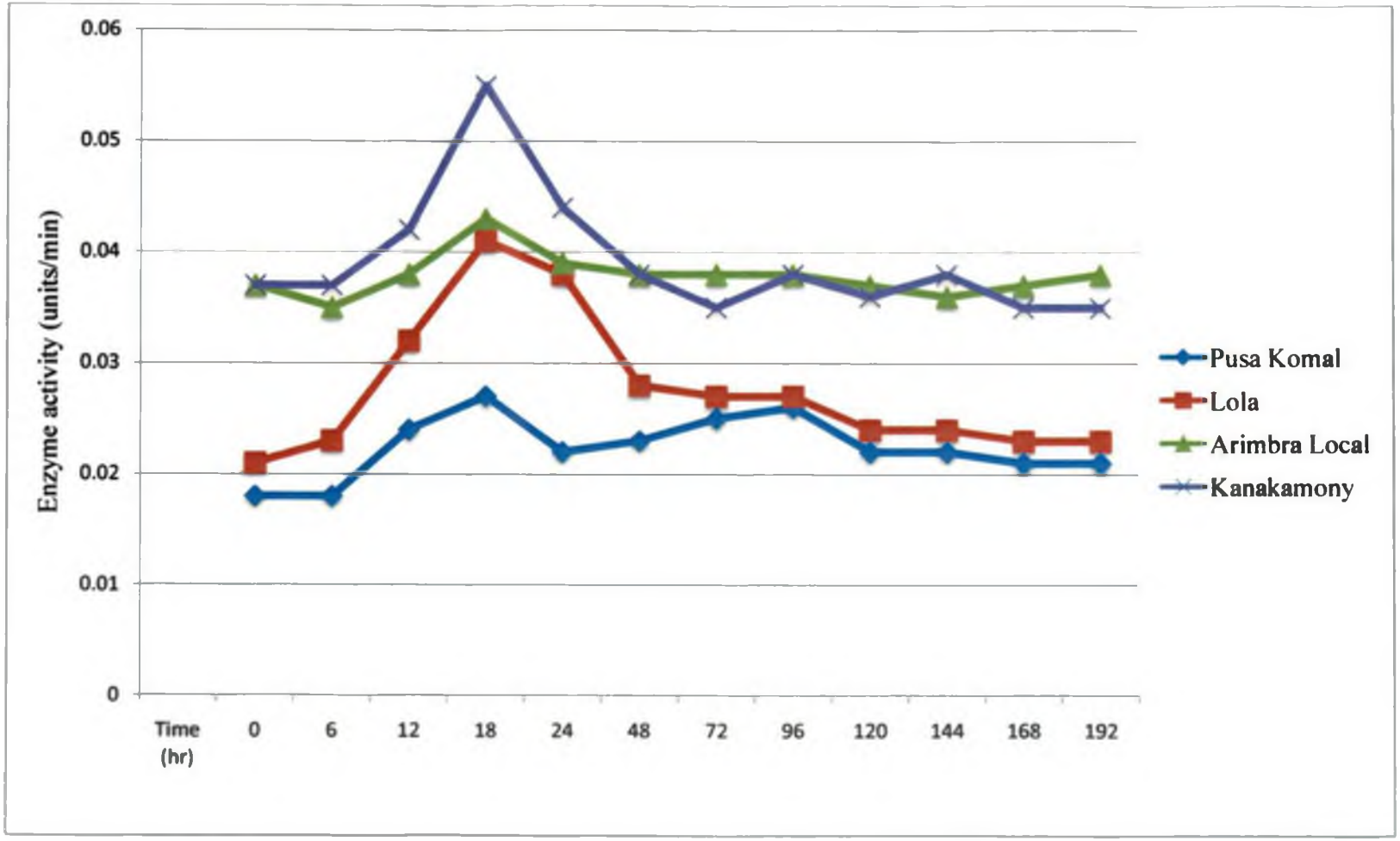
Comparison of the PO activity of the four cowpea varieties under study at different time intervals was presented in the Fig. 8. The PO activity in the resistant varieties, Arimbra Local and Kanakamony were found to be higher than that of the susceptible varieties, Pusa Komal and Lola. The activity reached the maximum at 18 hours in all the four varieties and gradually fallen back. By comparing the PO activity in all the four varieties, Kanakamony was found to have the high activity followed by Arimbra Local, Lola and then Pusa Komal. The values were found to be proportionate with the resistance behavior as Kanakamony is immune to anthracnose whereas Arimbra Local is highly resistant with only 8.8 per cent disease severity, Lola is highly susceptible with 68.8 per cent disease severity and Pusa Komal is highly susceptible with no disease incidence.

Leina *et al.* (1996) also have demonstrated a direct correlation between the variation in peroxidase activity in the soluble fraction of inoculated leaves and resistance to infection in cowpea cultivars. Two isoperoxidases were detected in cowpea leaves in response to 10 mM salicylic acid (Fernandes *et al.*, 2006). Further, Fink *et al.* (1991) has studied the early defense reactions of *V. sinensis* and found the induction of extracellular peroxidase activity as early as 24h after inoculation. All these results are in line with the present finding that the PO is proportionate to the level of resistance in vegetable cowpea and hence it is recommended that PO could be used as a biochemical marker for the anthracnose resistance in cowpea.

5.9.2 Polyphenol oxidase (PPO)

PPO are ubiquitous copper-containing enzymes which oxidize phenolic compounds into more toxic quinines which are highly reactive and may cross-link or alkylate proteins, leading to the commonly observed brown pigments in

Fig 8: Comparison of the peroxidase enzyme (PO) activity of the four cowpea varieties at different time intervals after inoculation with *C. lindemuthianum*



damaged plant tissues and plant extracts. An increased activity of PPO results in greater degrees of resistance to infection (Constabel and Barbehenn, 2008).

Comparison of the PPO activity of the four cowpea varieties under study at different time intervals was presented in the Fig. 9. Higher activity of the enzyme was observed in all the four varieties in a linear model and no consistent peak could be found. The varieties were also not able to get distinguished based on the resistance behavior.

Zhang *et al.* (2006) have evaluated resistant and susceptible cultivars of cowpea (*Vigna sesquipedalis*) and found a higher activity of PO, PPO, PAL, chitinase, β-1,3-glucanase and catalase enzymes against *Fusarium oxysporum* infection. Chandra *et al.* (2007) observed that the inoculation with *Rhizoctonia solani* resulted in a quantitative change in polyphenol oxidase, peroxidase isoforms and increase in PAL activities from 4.38 to 19.48 unit g^{-1} in UPC-4200 and IFC-902 cowpea genotypes. Vanitha *et al.* (2009) observed a temporal pattern of induction of PPO enzyme reaching maximum activity at 15 hours after the bacterial wilt inoculation in resistant cultivars of tomato.

5.9.3 Phenylalanine ammonia lyase (PAL)

The role of PAL as a defense enzyme is well established. Stressed plants increase the production of PAL which is involved in the production the antimicrobial phenolic compounds such as phytoalexins (Agrios, 2006).

Comparison of the PAL activity of the four cowpea varieties under study at different time intervals was presented in the Fig. 10. The PPO activities of the resistant varieties were observed to be higher than that of the susceptible varieties. The activity reached maximum at 18 hours after inoculation in Pusa Komal, Arimbra Local and Kanakamony whereas it reaches the maximum at 24 hours in Lola. The values are found to highest in the immune variety, Kanakamony followed by highly resistant Arimbra Local and the susceptible varieties, Lola and Pusa Komal.

Fig 9: Comparison of the polyphenol oxidase enzyme (PPO) activity of the four cowpea varieties at different time intervals after inoculation with *C. lindemuthianum*

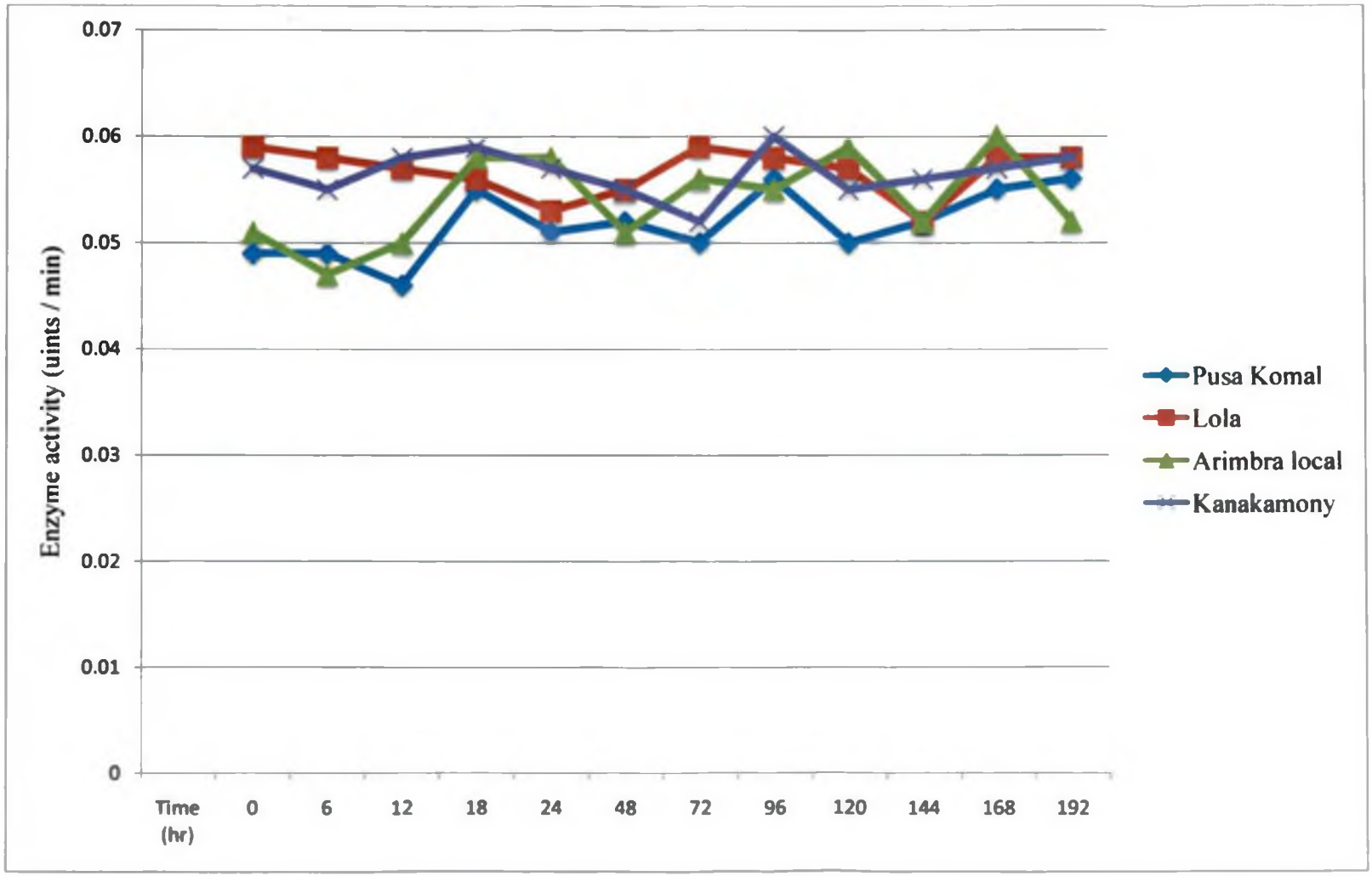
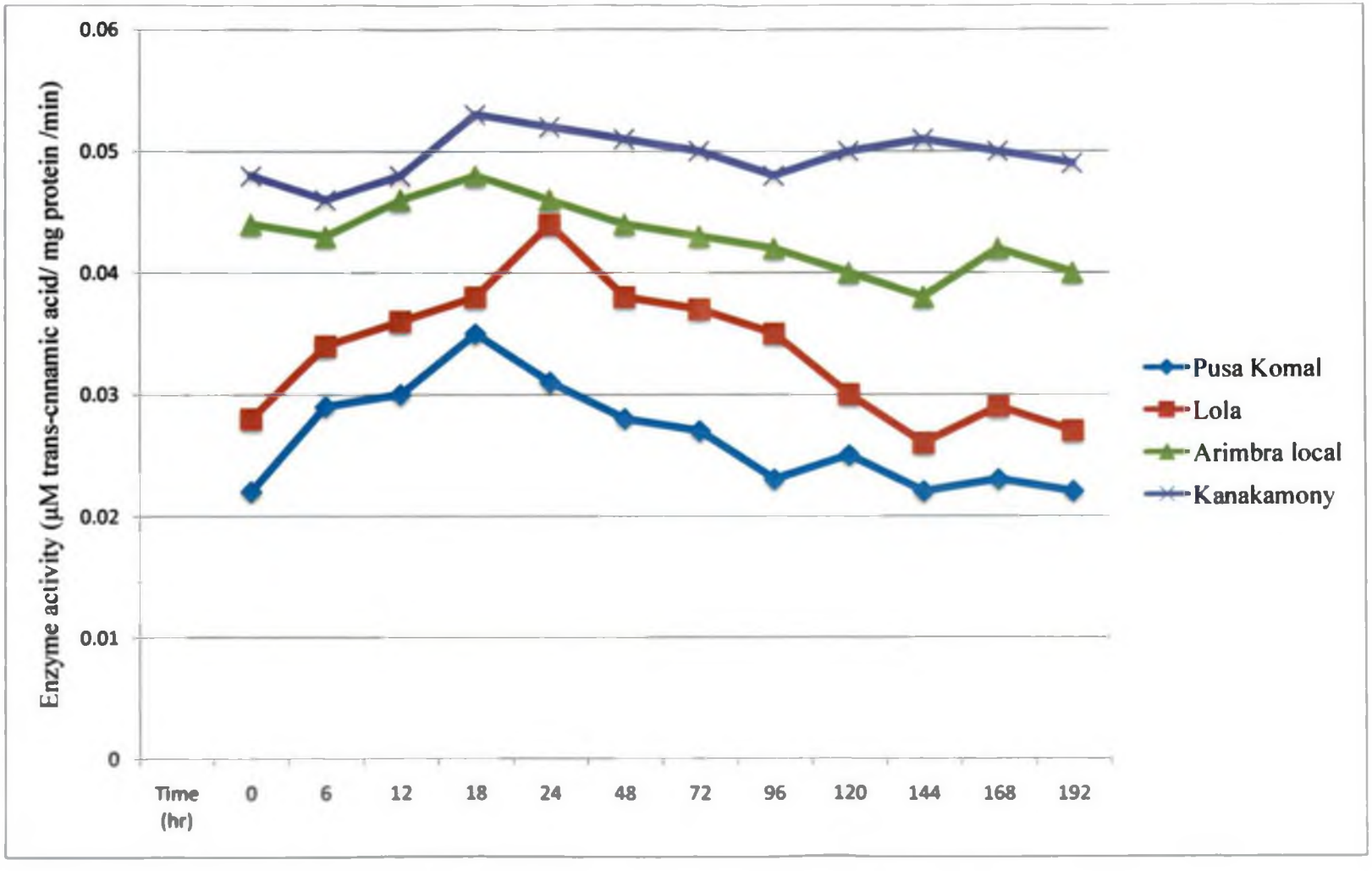


Fig 10: Comparison of the phenylalanine ammonia lyase enzyme (PAL) activity of the four cowpea varieties at different time intervals after inoculation with *C. lindemuthianum*



Fink *et al.* (1991) has studied the early defense reactions of *V. sinensis* and found the induction of phenylalanine ammonia-lyase at 10hours after inoculation. Chandra *et al.* (2007) observed the decline in disease progress caused by *Rhizoctonia solani* in cowpea due to phenylalanine ammonia lyase activities. Mechanism of resistance in cowpea to the root-knot nematode in *Meloidogyne incognita* was found to be the early induction of Phenylalanine ammonia lyase and chlorogenic acid (Anil and Dasgupta, 1993). Vanitha *et al.* (2009) observed a temporal pattern of induction of PAL enzyme reaching maximum activity at 12 h after the bacterial wilt inoculation in resistant cultivars of tomato. All these results were confirmatory to the present findings and based on these it could be recommended that along with PO, PAL could also be recognized as a biochemical marker to identify the level of anthracnose resistance in cowpea.

5.10. Conclusions

The present study has identified Arimbra Local as the world's first V. unguiculata ssp. sesquipedalis variety with resistance to anthracnose disease and provided the proof for the resistance scientifically. From the present studies, it was clear that the resistance to anthracnose in cowpea is highly dependent on the ability of a variety to maintain the cellular level of RuBisCo, after infection. The susceptible varieties had shown a drastic fall in the level of this enzyme whereas the resistant varieties maintained the levels consistently. The levels of defense enzymes PO and PAL were directly proportional to the level of resistance in the accessions and are recommended as the biochemical markers for the anthracnose resistance in cowpea. These markers could be directly used to identify the resistant segregating lines in any breeding programme.

Summary

6. SUMMARY

The study entitled "Characterization of pathogenesis related proteins for anthracnose resistance in vegetable cowpea, *Vigna* spp." was conducted as part of the MSc programme at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, during the period 2011-2013. Part of the work was carried out in the Department of Plant Pathology, College of Horticulture. The objectives of the study were to develop the protein profiles of resistant and susceptible bush/semi-trailing and pole genotypes through SDS-PAGE at different stages of infection and to identify the differentially expressed proteins by MALDI-TOF/MS through outsourcing followed by *in-silico* analysis for protein annotation.

Pusa Komal and Lola, the susceptible varieties along with Arimbra Local and Kanakamony, the resistant varieties were evaluated by SDS-PAGE for their response to *C. lindemuthianum* causing anthracnose disease in cowpea. Among them, bush type Pusa Komal and semi-trailing Kanakamony belong to the subsp. *cylindrica* whereas pole type Lola and Arimbra Local belong to the subsp. *sesquipedalis*. The salient findings of the study are summarized as follows:

The pathogen associated with anthracnose disease was isolated from the infected plant sample on neopeptone glucose agar medium and pathogenicity of the organism was established by artificial inoculation. The organism was identified as *Colletotrichum lindemuthianum* based on cultural and morphological characters.

Symptoms appeared at 3-4 days after artificial inoculation on susceptible varieties, Pusa Komal and Lola. With respect to the reaction of *C. lindemuthianum* on different cowpea varieties, Pusa Komal and Lola were found to highly susceptible with cent per cent and 68.8 per cent disease severity

respectively whereas Arimbra Local was found to be highly resistant with only 8.8 per cent disease severity and Kanakamony was found to be immune with no disease incidence.

Total protein was extracted from the leaf samples collected at regular time intervals from 0 to 192hrs using Tris-HCl buffer (pH-7.5). Concentration of the extracted proteins was high in the range of 18 to 74 mg/g as quantified by Nanodrop spectrophotometer. The normalized proteins of 1.5 mg/ml concentration were analyzed by SDS-PAGE technique to obtain the protein profile of different varieties with samples collected at different time intervals after artificial inoculation.

The protein profiles were distinct for each of the four different varieties with a maximum of 7 bands/ lane. Immediately after infection, the susceptible varieties had shown a downregulation of total protein profile, but reached a higher level at 18 hrs and all the proteins were downregulated after 72 hrs, due to the initiation of the necrotic phase. The protein profile of resistant varieties had shown a higher level of expression in the initial stage itself and maintained the same even after the inoculation. While comparing the protein profiles of control and treated plants for the initial hours separately, two prominent bands were found to show clear difference in the expression such that Pusa Komal had shown a downregulation after inoculation, whereas Lola had a slight upregulation and the resistant varieties maintained the higher level of expression continuously.

The two prominent bands at 56 kD and 14 kD size were cut from the gel and sequenced by MALDI-TOF/MS (outsourcing) to obtain the peptide mass fingerprint. The data was further analyzed by Mascot server software and the protein bands were found to be homologous to the large and small subunits of RuBisCo which is the main photosynthetic enzyme. Hence it was evident that the capability of the plant to maintain the higher level of RuBisCo is a key factor in the resistance behavior of the plant.

The activities of the defense enzymes Peroxidase and Phenylalanine ammonia lyase were found to be proportionate to the degree of resistance. Kanakamony which is immune to anthracnose disease had shown the highest activity followed by the highly resistant Arimbra Local and then the highly susceptible varieties Lola and Pusa Komal. Polyphenol oxidase also had shown a higher level of activity in all the varieties but with no significant difference among the susceptible and resistant varieties can be found. Hence the defense enzymes, PO and PAL could be used as biochemical markers for identifying the resistant varieties.

SDS-PAGE profile obtained after the protein purification by precipitation and dialysis had indicated the upregulation of a 29 kD protein band in the resistant varieties Kanakamony and Arimbra Local whereas it was absent in the susceptible varieties Pusa Komal and Lola. This protein band is in the size range of the already reported PR proteins.

Future line of the work includes

- ✓ Characterization of the PR proteins in acidic and basic pH by 2D-PAGE system.
- ✓ Identification of the genes responsible for the resistance mechanism which are present in the resistant varieties.
- ✓ Improvement of the high yielding varieties of vegetable cowpea with the incorporation of resistance to anthracnose disease.

References

REFERENCES

Abdelatief, S.H. El-Jasser. 2010. Chemical and biological properties of local cowpea seed protein grown in gizan region. *Int. J. Agri. Biol. Sci.* **1**:2.

Adebitan, S.A, Ikotun, T., Dashiell, K.E., Singh, S.R. 1992. Use of three inoculation methods in screening cowpea genotypes for resistance to two *Colletotrichum* species. *Plant diseases* **76**(10):1025-1028.

Agrios, G.N. 2006. *Plant Pathology* (5th Ed.). Elsevier Academic Press, New Delhi, 922p.

Ahlawat, I.P.S. and Shivakumar, B.G. 2005. Kharif pulses. In: Prasad, R. (Ed.), *Field Crops Production.* Directorate of information and publication of Indian Council of Agricultural Research, Pusa, New Delhi, pp. 252-270.

Almagro1, L., Gómez Ros1, L.V., Belchi-Navarro1, S., Bru, R., Ros Barceló, A. and Pedreño, M.A. 2008. Class III peroxidases in plant defence reactions. *J. Exp. Bot.* **60**(2):377-390.

Anil, S. and Dasgupta, D.R. 1993. Mechanism of resistance in cowpea to the root-knot nematode, Meloidogyne incognita Race 1: I early induction of phenylalanine ammonia lyase (E.C. 4.3.1.5.) and chlorogenic acid. *Indian J. Nematology.* **23**(1): 31-41.

Antoniw, J.F., Ritter, C.E., Pierpoint, W.S., and Van Loon, L.C., 1980. Comparison of three pathogenesis-related proteins from plants of two cultivars of tobacco infected with TMV, *J. Gen. Virol.,* **47**:79.

Asgary, S., Moshtaghian, J., Hosseini, M. and Siadat, H. 2008. Effects of alfalfa on lipoproteins and fatty streak formation in hypercholesterolemic rabbits. *Pak. J. Pharm. Sci.* **21**:460-464.

Belkhadir, Y., Subramaniam, R. and Dangl, J.L. 2004. Plant disease resistance protein signaling: NBS–LRR proteins and their partners. *Curr. Opinion Pl. Biol.* 7:391-399.

Benhamou, A., Grenier, J. and Chrispeels, M.J. 1991. Accumulation of β-fructosidase in the cell walls of tomato roots following infection by a fungal wilt pathogen. *Pl. Physiol.* 97:739-750.

Blum, H., Beier, H. and Gross, H.J. 1987. Silver staining of proteins in polyacrylamide gels. *Electrphoresis.* 8:93-99.

Bormann, C., Baier, D., Horr, I., Raps, C., Berger, J., Jung, G. and Schwartz, H. 1999. Characterization of a novel, antifungal, chitin-binding protein *Streptomyces tendae* Tu901 that interferes with growth polarity. *J. Bact.* 181:7421-7429.

Brederode, F.T., Linthorst, H.J.M. and Bol, J.F. 1991. Differential induction of acquired resistance and PR gene expression in tobacco by virus infection, ethephon treatment, UV light and wounding. *Pl. Mol. Biol.* 17:1117.

Brueske, C.H. 1980. Phenylalanine ammonia lyase activity in tomato roots infected and resistant to the root-knot nematode, *Meloidogyne incognita. Physiol. Pl. Path.* 16: 409-414.

Campo, S., Carrascal, M., Coca, M., Abian, J., and Segundo S.B. 2004. The defence response of germinating maize embryos against fungal infection: a proteomics approach. *Proteomics* 4:383-396.

Campos, A.D., Ferreira, A.G., Hampe, M.M.V., Antunes, I.F., Brancao, N., Silveira, E.P.da, Osorio, V.A. and Augustin, E. 2004. Peroxidase and polyphenol oxidase activity in bean anthracnose resistance. *Pesquisa Agropecuaria Brasileira.* 39(7): 637-643.

Candela, M.E., Espin, A. and Alcazar, M.D. 1993. Soluble pathogenesis-related proteins of Capsicum annuum leaves induced by cucumber mosaic virus infection. *Biologia Plantarum* **35**(4): 609-615.

Carvalho, A.O., Machado, O.L.T., Cunha, M.D., Santosa, I.S. and Gomesa V.M. 2001. Antimicrobial peptides and immunolocalization of a LTP in *Vigna Unguiculata* seeds. *Pl. Physiol. Biochem.* **39**: 137-149.

Chandra, A., Saxena, R., Dubey, A. and Saxena P. 2007. Change in phenylalanine ammonia lyase activity and isozyme patterns of polyphenol oxidase and peroxidase by salicylic acid leading to enhance resistance in cowpea against *Rhizoctonia solani*. *Acta Physiologiae Plantarum.* **29**(4): 361-367.

Chernin, L.S., De la Fuente, L., Sobolev, V., Haran, S., Vorgias, C.E., Oppenheim, A.B. and Chet, I. 1997. Molecular cloning, structural analysis, and expression in *Escherichia coli* of a chitinase gene from *Enterobacter agglomerans*. *Appl. Environ. Microbiol.* **63:** 834-839.

Chevallet, M., Luche, S., and Rabilloud, T. 2006. Silver staining of proteins in polyacrylamide gels. *Nat. Protoc.* **1**(4): 1852–1858.

Chu, K.T., Liu, K.H. and Ng, T.B. 2003. Cicerarin, a novel antifungal peptide from the green chickpea. *Peptides* **24**:659-63.

Colditz, F., Nyamsuren, O., Niehaus, K., Eubel, H., Braun, H.P., and Krajinski, F. 2004. Proteomic approach: identification of Medicago truncatula proteins induced in roots after infection with the pathogenic oomycete Aphanomyces euteiches. *Plant Mol Biol* **55**:109-120.

Constabel, C.P. and Barbehenn, R. 2008. Defensive roles of polyphenol oxidase in plants. In: Schaller, A. (ed.), Induced Plant Resistance to Herbivory, Springer Science and Business Media. B.V, London, 480p.

Cote, F., Cutt, J.R., Asselin, A. and Klessig, D.F. 1991. Pathogenesis related acidic β-1,3-glucanase genes of tobacco are regulated by both stress and developmental signals. *Mol. Pl. Microbe Interact.* **4**: 173-181.

Dada, L.A.O., Connell, R.J., Nash, C., Pring, R.J., Lucas, J.A. and Bailey, J.A. 1994. Infection process and identity of the hemibiotrophic anthracnose fungus (*Colletotrichum destructivum*) from cowpea (*Vigna unguiculata*). *Mycol. Res.* **100**(9): 1133-1141.

Datta, S.K. and Muthukrishnan, S. 1999. Occurrence and properties of plant Pathogenesis-Related Proteins. In: Van Loon, L.C. (ed.), *Pathogenesis-Related Proteins in plants*. CRC Press LLC, 303p.

Davis, B.J., 1964. Disc electrophoresis II. Method and application to human serum proteins, *Ann. N. Y. Acad. Sci.*121: 404p.

Dharm, S. and Patel, R.N. 1990. Pusa Komal - a cowpea for vegetable. *Indian Hort.* **35**(3): 23-25.

Edreva, A. M. 1990. Induction of "pathogenesis-related" proteins in tobacco leaves by physiological (non-pathogenic) disorders. *J. Exp. Bot.* **41**:701

Elliston, J., Kuc, J. and Williams, E. 1976. A comparative study of the development of compatible, incompatible and induced incompatible interactions between *Colletotrichum* species and *Phaseolus vulgaris. Phytopathol. Z.* **87**:289-303.

Esterbaner, H., Schwarzl, E. and Hayn, M. 1977. A rapid assay for catechol oxidase and laccase using 2-nitro-5-thiobenzoic acid. *Anal. Biochem.* **77**:486-494.

Fernandes, C.F., Moraesb, V.C.P., Vasconcelos, I.M., Silveira, J.A.G. and Oliveira, J.A. 2006. Induction of an anionic peroxidase in cowpea leaves by exogenous salicylic acid. *J. Pl. Physiol.* **163**: 1040-1048.

Ferrante, G.M. and Bisiach, M. 1976. Comparison of methods for experimental infection of bean with *Colletotrichum lindemuthianum. Rivista di Patologia Vegetale* IV **12**(3/4): 99-118.

Fink, W., Haug, M., Deising, H. and Mendgen K. 1991. Early defence responses of cowpea (*Vigna sinensis* L.) induced by non-pathogenic rust fungi. *Planta* **185**: 246-254.

Geffroy, V., Sevignac, M., Billant, P., Dron, M., and Langin, T. 2008. Resistance to Colletotrichum lindemuthianum in Phaseolus vulgaris: a case study for mapping two independent genes.*Theor Appl Genet.* **116**:407-415.

Gianinazzi, S., Martin, E. and Vallee, J.E. 1970. Gianinazzi, S., Martin, e. and Vallee, J.e. 1970. Hypersensitivity to virus, temteratures and soluble proteins in *Nicotiana xanthi.* In: *Appearance of New Macromolecules at the Repression of Viral Synthesis.* Comptes Rendus de I' Academie des Sciences de, Paris, pp. 2383-2386.

Gomes, V.M., Oliveira, A.E.A and Xavier-Filho, J. 1996. A Chitinase and a β-1,3 glucanase isolated from the seeds of cowpea (Vigna unguiculata L Walp) inhibit the growth of fungi and insect pests of the seed. *J. Cci. Food Agric.*72:86-90.

Gonclaves-Vidigal and Kelly, J.D. 2006. Inheritance of anthracnose resistance in the common bean cultivarWidusa. *Euphytica* **151**:411-419.

Gonçalves-Vidigal, M.C., Cruz, A.S., Garcia, A., Kami, J., Filho, P.S.V., Sousa, L.L., McClean, P., Gepts, P. and Pastor-Corrales, M.A. 2011. Linkage mapping of the Phg-1 and Co-14 genes for resistance to angular leaf spot and anthracnose in the common bean cultivar AND 277. *Theor Appl Genet.* **122**(5): 893-903.

Goodman, R.N. and Novacky, A.J. 1994. The Hypersensitive Reaction in Plants to Pathogens: A Resistance Phenomenon. APS Press, St. Paul, M.N., USA, 244p.

Graham, L.S. and Sticklen, M.B. 1994. Plant chitinases. *Can. J. Bot.* **72**: 1057-83.

Hanselle, T. and Ichinoseb, Y. 2001. Biochemical and molecular biological studies on infection (*Ascochyta rabieie*)-induced thaumatin-like proteins from chickpea plants (*Cicer arietinum*). *Z Naturforsch* **56**:1095-107.

Hopkins, W.G. and Hüner, N.P.A. 2009. *Introduction to Plant Physiology* (4th Ed.). John Wiley and Sons Inc., New York, 140p.

Huynh, Q.K., Hironaka, C.M., Levine, E.B., Smith, C.E., Borgmeyer, J.R. and Shah, D.M. 1992. Antifungal proteins from plants. Purification, molecular cloning, and antifungal properties of chitinases from maize seed. *J. Biol. Chem.* **267:** 6635-6640.

Ismail, A.M., Hall, A.E. and Close, T.J. 1999. Purification and partial characterization of a dehydrin involved in chilling tolerance during seedling emergence in cowpea. *Pl. Physiol.* **120:** 237-244.

Kang, S.C., Park, S. and Lee, D.G. 1999. Purification and characterization of a novel chitinase from the entomopathogenic fungus, *Metarhizium anisopliae. J. Invert. Pathol.* **73:**276-281.

Kassanis, B., Gianinazzi, S., and White, R.F. 1974. A possible explanation of the resistance of virus-infected plants to a second infection. *J. Gen. Virol.* **23**: 11.

KAU [Kerala Agricultural University]. 2011. *Annual Report* submitted to Zonal Project Directorate, Zone VIII, Indian Council of Agricultural Research, Bangalore. KVK Malappuram, Kerala Agricultural University, Vellanikkara.

Kim, M.H., Lee, J., Yoo, D.S., Lee, Y.G., Byeon, S.E., Hong, E.K. and Cho, J.Y. 2009. Protective effect of stress-induced liver damage by saponin fraction from Codonopsis lanceolata. *Arch. Pharm. Res.* **32**:1441-1446.

Kim, S.T., Kim, S.G., Hwang, D.H., Kang, S.Y., Kim, H.J., Lee, B.H., Lee, J.J., and Kang, K.Y. 2004. Proteomic analysis of pathogen-responsive proteins from rice leaves induced by rice blast fungus, Magnaporthe grisea. *Proteomics* **4:** 3569-3578.

Kitajima, S. and F. Sato. 1999. Plant pathogenesis-related proteins: molecular mechanisms of gene expression and protein function. *J. Biochem.* **125:** 1-8.

Kumar, D. and Singh, N.B. 2004. *Cowpea in India.* Scientific Publishers, India. 282p.

Kumar, M.P. 1999. Anthracnose disease of vegetable cowpea [*Vigna unguiculata* ssp. *sesquipedalis* (L.) Verdcourt]. M. Sc. Thesis, Kerala Agricultural University, 106p.

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

Latunde-Dada, A.O. and Lucas, J.A. 2001. The plant defence activator acibenzolar-S-methyl primes cowpea seedlings for rapid induction of resistance. *Physiol. Mol. Pl. Path.* **58**: 199-208.

Lawton, K., Uknes, S., Friedrich, L., Gaffney, T., Alexander, D., Goodman, R., Metraux, J. P., Kessmann, H., Goy, A. P., Rella, G. M., Ward, E., and Ryals, J. 1993. In: Fritig, B. and Legrand, M. (eds.), *The Molecular Biology of Systemic Acquired Resistance*, in Mechanisms of Plant Defense Responses, Kluwer Acad. Publ., Dordrecht, 422p.

Lawton, M.A. and Lamb, C.J. 1987. Transcriptional activation of plant defense genes by fungal elicitor, wounding, and infection. *Mol. Cell Biol.* 7(1): 335-341.

Leach, J.G. 1923. *The parasitism of Colletotrichum lindemuthianum*. Agricultural Experiment Station Technical Bulletin No. 14, University of Minnesota, Minneapolis, US, 41 p.

Legrand, M., Kauffmann, S., Geoffroy, P., and fritig, B. 1987. Biological function of pathogenesis-related proteins: Four tobacco pathogenesis-related proteins are chitinases. *Proc. Nati. Acad. Sci.* USA. **84**:6750-6754.

Leina, M.J., Tan, T.K. and Wong, S.M. 1996. Resistance of *Hibiscus esculentus* L and *Vigna sinensis* Endl to *Pseudocercospora* and plant peroxidase activity in relation to infection. *Ann. Appl. Biol.* **129**(2): 206.

Leister D. 2004. Tandem and segmental gene duplication and combination in the evolution of plant disease resistance gene. *Trends Genet.* **20**: 116-122.

Leung, D.W.M. 1992. Involvement of plant chitinase in sexual reproduction of higher plants. *Phytochemistry* **31**: 1899.

Linthorst H. J. M. 1991. Pathogenesis-related proteins of plants. *Crit. Rev. Pl. Sci.*, **10**: 123.

Lotan, T., Ori, N. and Fluhr, R. 1989. Pathogenesis-related proteins are developmentally regulated in tobacco flowers. *Pl. Cell* **1**: 881.

Malamy, J. and Klessig, D.F. 1992. Salicylic acid and plant disease resistance. *Pl. J.* **2**: 643-654.

Marin, L.A., Souza, K.A., Morais, M.G., Oliveira, E.J., Moreira, M.A., and Barros, E. G. 2007. Genetic characterization of anthracnose resistance genes. *Euphytica.* **154**:1-8.

Martínez-Pacheco, M.M., Saucedo-Luna, J., Flores-García, A., Martínez-Muñoz, R.E. and Campos-García, J. 2009. *Colletotrichum lindemuthianum* (Sacc. & Magn.) Scrib. is a potential cellulases producer microorganism. Rev. Latinoam Microbiol. **51**(1-2): 23-31.

Mauch, F. and Staehelin, L. A. 1989. Functional implications of the subcellular localization of ethylene-induced chitinase and β-1,3-glucanase in bean leaves, *Pl. Cell* **1**: 447.

Melchers, L.S., Apotheker-de Groot, M., van der Knaap, J.A., Ponstein, A.S., Sela-Buurlage, M.B., Bol, J.F., Cornelissen, B.J., van den Elzen, P.J. and Linthorst, H. J. 1994. A new class of tobacco chitinases homologous to bacterial exo-chitinases displays antifungal activity. *Pl. J.* **5:** 469-480.

Memelink, J., Linthorst, H. J. M., Schilperoort, R. A. and Hoge, J. H. C. 1990. Tobacco genes encoding acidic and basic isoforms of pathogenesis-related proteins display different expression patterns. *Pl. Mol. Biol.* **14**: 119.

Mendgen, K and Hahn, M. 2002. Plant infection and the establishment of fungal biotrophy. *Trends Pl. Sci.* 7: 352-356.

Money, N., and Harold, F. 1992. Two water molds can grow without measurable turgor pressure. *Planta* **190:**426-430.

Mordue, J.E.M. 1971. *Colletotrichum lindemuthianum - Descriptions of Pathogenic Fungi and Bacteria*. Commonwealth Mycological Wallingford, UK Institute, Set 32, Sheet No. 316, 2pp.

Ng, N.Q. 1990. Recent developments in cowpea germplasm collection, conservation, evaluation and research at the genetic resources unit, IITA. In: Ng, N.Q. and Monti, L.M. (ed.), *Cowpea Genetic Resources*. International Institute of Tropical Agriculture, Nigeria, pp. 13-28.

Ngai, P.H.K. and Ng, T.B. 2003. Purification of glysojanin, an antifungal protein, from the black soybean *Glycine soja*. *Biochem. Cell Biol.* **81**: 387-94.

Niderman, T., Genetet, I., Bruyere, T., Gees, R., Stintzi, A., Legrand, M., Fritig, B., and Mosinger, E. 1995. Pathogenesis-Related PR-1 Proteins Are Antifungal: Isolation and Characterization of Three 14-Kilodalton Proteins of Tomato and of a Basic PR-1 of Tobacco with Inhibitory Activity against *Phyfophfhora infestans*. *Plant Physiol.* **108**: 17-27.

Nielsen, K.K., Nielsen, J.E., Madrid, S.M. and Mikkelsen, J.D. 1997. Characterization of a new antifungal chitin-binding peptide from sugar beet leaves. *Plant Physiol.* **113:** 83-91.

Onesirosan, P.T. and Barker, L.N. 1971. Stem anthracnose of cowpea in Nigeria. *Pl. Dis. Rep.* **55**: 820-827.

Ornstein, L., 1964. Disc electrophoresis I. Background and theory, *Ann. N. Y. Acad. Sci.* **121**: 321p.

Padulosi, S. 1993. Genetic diversity, taxonomy, and ecogeographic survey of the wild relatives of cowpea (*V. unguiculata*). PhD thesis. University of Louvain La Neuve, Belgium.

Perfect, S.E., Hughes, H.B., O'Connell, R.J. and Greena, J.R. 2002. Colletotrichum: A model genus for studies on pathology and fungal–plant interactions. *Fungal Genet. Biol.* **27**(2-3): 186-198.

Pessarakli, M. 2001. *Handbook of Plant and Crop Physiology* (2nd Ed.). Marcel Dekker, Inc., New York, p. 59

Putter, J. 1974. Peroxidases. In: Bergmeyer (ed.), *Methods of Enzymatic Analysis* (2nd Ed.). Verlag Chemie-Academic Press, Newyork, pp. 685-690.

Quereshi, S.H., Bashir, M. and Alam, S.S. 1985. Anthracnose of cowpea – a new disease record in Pakistan. *Trop. Grain Legume Bull.* **30**: 26.

Rahimi, S., Preey, R.N. and Wright, D. 1993. Induction and detection of pathogenesis-related proteins in leaves and roots of potato plants infected with pathotypes of *Globodera pallida*. *Fundam. appl. Nemawl.*, 16(6):549-556.

Rajan, S. and Prameela, K.P. 2004. Cowpea Varieties Released from KAU. Kerala Agricultural University, Vellaikkara, 256p.

Rajkumar, G., Kalloo and Pandey, P.K. 1995. Resistance in cowpea to Pseudocercospora. In: Paper, National symposium on Recent evelopments in Vegetable Improvement; 2-5 February, 1995, Raipur, 23p.

Rao, V.G. 1966. An account of the market and storage disease of fruits and vegetables in Bombay, Maharastra. *Mycopath. et Mycol. appl.* **28**: 165-176.

Redolfi, P., Occurrence of pathogenesis-related proteins and similar proteins in different plant species, *Neth. J. Plant Pathol.*, **89**: 245, 1983.

Regalado, A.P. and Ricardo, C.P. 1996. Study of the intercellular fluid of healthy *Lupinus albus* organs. Presence of a chitinase and a thaumatin-like protein. *Pl. Physiol.* **110**: 227–32.

Roberts, W. and Selitrennikoff, C.P. 1990. Zeaman, an antifungal protein from maize with membrane-permeabilizing activity. *J. Gn. Microbiol.* **136**: 1771-1778.

Salzman, R.A., Tikhonova, I., Bordelon, B.P., Hagawa, P.M. and Bressan, R.A. *1998.* Coordinate accumulation of antifungal proteins and hexoses constitutes a developmentally controlled defense respo during fruit ripening in grape. *Plant Physiol.* **117**:465-472.

Selitrennikoff, C.P. 2001. Antifungal proteins. *Appl. Environ. Microbiol.* **67**(7): 2883-2894.

Sequeira, L. 1983. Mechanisms of induced resistance in plants. *Ann Rev Microbiol.* **37**:51-79.

Shao, F.M. and Teri, J.M. 1985. Yield losses in *Phaseolus* beans induced by anthracnose in Tanzania. *Trop. Pest Manage.* **31**: 60-62.

Shivashankar, S., Thimmareddy, C. and Roy, T.K. 2010. Polygalacturonase inhibitor protein from fruits of anthracnose resistant and susceptible varieties of chilli (*Capsicum annuum*). *Indian J. Biochem. Biophy.* **47**: 243-248.

Sicard, D., Michalakis, Y., Dron, M., and Neema, C. 1997. Genetic Diversity and Pathogenic Variation of *Colletotrichum lindemuthianum* in the Three Centers of Diversity of Its Host, *Phaseolus vulgaris*. *The American Phytopathol. Soc.* **87**(8): 807.

Sommerer, N., Centeno, D., and Rossignol, M. 2007. Peptide mass fingerprinting: identification of proteins by MALDI-TOF. *Methods Mol Biol.* **355:** 219-234.

Suarez, C.R., Ferreira, J.J., Campa, A., Paneda, A., Giraldez, R. 2008. Molecular mapping and intra-cluster recombination between anthracnose race-specific resistance genes in the common bean diVerential cultivars Mexico 222 and Widusa Theor Appl Genet. **116**:807–814.

Szczepanski, M. and Redolfi, P. 1985. Changes in the proteins of bean leaves infected with tobacco necrosis or alfalfa mosaic viruses. *Phytopath. Z.* **113**: 57.

Takakura, Y., Che, F.S., Ishida, Y., Tsutsumi, F., Kurotani, K., Usami, S., Isogai, A. and Imaseki, H. 2008. Expression of a bacterial flagellin gene triggers plant immune

responses and confers disease resistance in transgenic rice plants. *Mol. Pl. Pathol.* **9**: 525-529.

Tao, X., Qiang Z., Wei, C., Guren, Z., Guofeng, H., Dexiang, G. & Wenqing, Z. 2003. Involvement of Jasmonate signaling pathway in the herbivore-induced rice plant defense. *Chinese Sci. Bull.* 48(18): 1982-1987.

Tu, J.C and Aylesworth, J.W. 1980. An effective method of screening white (pea) bean seedlings (Phaseolus vulgaris L.) for resistance to *Colletotrichum lindemuthianum*. *Phytopathologische Zeitschrift* **99**(2): 131-137

Tu, J.C. 1981. Anthracnose (*Colletotrichum lindemuthianum*) on white bean (Phaseolus vulgaris L.) in southern Ontario: spread of the disease from an infection focus. *Pl. Dis.* **65**(6): 477-480.

Tu, J.C. 1983. Epidemiology of anthracnose caused by *Colletotrichum lindemuthianum* on white bean (*Phaseolus vulgaris*) in southern Ontario: survival of the pathogen. *Pl. Dis.* **67**(4): 402-404

Valenzuela, H. and Smith, J. 2002. Cowpea. *Sustainable Agriculture - Green Manure Crops* **6**: 1-3

Van Loon, L. C. 1985. Pathogenesis-related proteins. *Pl. Mol. Biol.* **116**: 111-116.

Van Loon, L.C. 1989. Proteins in infected plants. In: Kosuge, T. and Nester, E. W. (eds.), Plant - Microbe Interactions, Molecular and Genetic Perspectives, McGraw-Hill Book Co., New York, 198p.

Van Loon, L.C. 1997. Induced resistance in plants and the role of pathogenesis-related proteins. *Eur. J. Pl. Path.* **103**: 753-765.

Van Loon, L.C. and Strien, E.A. 1999. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiological and Molecular Plant Pathology* .**55**: 85-97.

Van Loon, L.C. and Van Kammen, A. 1970. Polyacrylamide disc electrophoresis of the soluble leaf proteins from Nicotiana tabacum var. "Samsun" and "Samsun NN" II. Changes in protein constitution after infection with tobacco mosaic virus. *Virology* **40**: 199.

Van Loon, L.C., 1983. Mechanisms of resistance in virus-infected plants. In: Bailey, J.A. and Deverall, B.J. (eds.), *The Dynamics of Host Defense,* Academic Press, North Ryde, NSW, Australia, 123p.

Vanitha, S.C., Niranjana, S.R. and Umesha, S. 2009. Role of Phenylalanine Ammonia Lyase and Polyphenol Oxidase in Host Resistance to Bacterial Wilt of Tomato. *J. Phytopathology.* **157** (9): 552-557.

Varma, B.K. and Langerak, C.J. 1988. Seed-transmitted pests and diseases of legumes in rice-based cropping systems. In: *Rice Seed Health*. Proceedings of an international workshop, 16-20 March 1987, Los Baños, Laguna. International Rice Research Institute, Manila, Philippines, pp. 189-201.

Vera-Estrella, R., Blumwald, E. and Higgins, V.J. 1992. Effect of Specific Elicitors of Cladosporium fulvum on Tomato Suspension Cells : Evidence for the Involvement of Active Oxygen Species. *Pl. Physiol.* **99**(3):1208-15.

Vera-Estrella, R., Higgins, V.I. and Blumwald, E. 1994. Plant Defense Response to Fungal Pathogens - II. C-Protein-Mediated Changes in Host Plasma Membrane Redox Reactions *Pl. Physiol.* **106**: 97-102.

Vigers, A.J., Roberts, W.K. and Selitrennikoff, C.P. 1991. A new family of plant antifungal proteins. *Mol. plant microbe interactions* 4(4):315-323.

Wagih, E. E. and Coutts, R. H. A. 1982. Comparison of virus-elicited and other stresses on the soluble protein fraction of cucumber cotyledons. *Phytopath. Z.*, **104**: 364.

Watanabe, T. 2010. *Pictorial Atlas of Soil and Seed Fungi – Morphologies of Cultured Fungi and Key to Species* (3rd Ed.). Taylor and Francis Group, Boca Raton. 404p.

Wheeler, B.E.J. 1969. *An introduction of Plant Disease*. Jhon Wiley and Sons Ltd., London, 301p.

Williams, R. J. 1975. An international testing programme for resistance to cowpea diseases. *Trop. Grain Legume Bull.* **2**: 5-7

Wong, J.H. and Ng, T.B. 2003. Gymnin, a potent defensin-like antifungal peptide from the yunnan bean (*Gymnocladus chinensis* Baill). *Peptides* 24: 963-8.

Xinzhang, Yang, G., Yang J. and Qinglong S. 2012. Physiological mechanism of resistance to anthracnose of different *Camellia* varieties. *J. Biotech.* **11**(8): 2026-2031.

Yan, Q., Qi, X., Jiang, Z., Yang, S. and Lujia, H. 2008. Characterization of a pathogenesis-related class 10 protein (PR-10) from *Astragalus mongholicus* with ribonuclease activity. *Plant Physiol Biochem.* **46**:93-99.

Yang, Y., Shah, J. and Klessig, D.F. 1997. Signal perception and transduction in plant defense responses, *Genes Dev.* **11**: 1621.

Ye, X.Y and Ng, T.B. 2002b. Isolation of a new cyclophilin-like protein from chickpeas with mitogenic, antifungal and anti-HIV-1 reverse transcriptase activities. *Life Sci.* **70**: 1129-38.

Ye, X.Y. and Ng, T.B. 2000a. Mungin, a novel cyclophilin-like antifungal protein from the mungbean. *Biochem. Biophys. Res. Commun.* **273**: 1111-5.

Ye, X.Y. and Ng, T.B. 2000b. Hypogin, a novel antifungal peptide from peanuts with sequence similarity to peanut allergen. *J. Peptide Res.* **57**: 330–6.

Ye, X.Y. and Ng, T.B. 2001a. Peptides from pinto bean and red bean with sequence homology to cowpea 10-kDa protein precursor exhibit antifungal, mitogenic, and HIV-1 reverse transcriptase-inhibitory activities. *Biochem. Biophys. Res. Commun.* **285**: 424-9.

Ye, X.Y. and Ng, T.B. 2001b. Isolation of unguilin, a cyclophilin-like protein with anti-mitogenic, antiviral, and antifungal activities, from black-eyed pea. *J. Protein Chem.* **20**: 353-9.

Ye, X.Y. and Ng, T.B. 2002a. A new antifungal protein and a chitinase with prominent macrophage-stimulating activity from seeds of *Phaseolus vulgaris* cv. *pinto*. *Biochem. Biophys. Res. Commun.* **290**: 813-9.

Ye, X.Y. and Ng, T.B. 2002c. Isolation of a novel peroxidase from French bean legumes and first demonstration of antifungal activity of a non-milk peroxidase. *Life Sci.* **71**: 1667-80.

Ye, X.Y. and Ng, T.B. 2002d. A new peptide protease inhibitor from *Vicia faba* seeds exhibits antifungal, HIV-reverse transcriptase inhibiting and mitogenic activities. *J. Peptide Sci.* **8**: 656-62.

Ye, X.Y. and Ng, T.B. 2002e. Purification of angularin, a novel antifungal peptide from adzuki beans. *J. Peptide Sci.* **8**: 101-6.

Ye, X.Y. and Ng, T.B. 2003b. Isolation of pisumin, a novel antifungal protein from legumes of the sugar snap *Pisum sativum* var. *macrocarpon*. *Comp. Biochem. Physiol. Part.* **134**: 235-40.

Ye, X.Y., Ng, T.B. and Rao, P.F. 2002. Cicerin and arietin, novel antifungal proteins from chickpea. *Peptides* **23**: 817-22.

Ye, X.Y., Ng, T.B., Tsang, P.W.K. and Wang J. 2001. Isolation of a homodimeric lectin with antifungal and antiviral activities from red kidney bean (*Phaseolus vulgaris*) seeds. *J. Protein Chem.* **20**: 367-75.

Ye, X.Y., Wang, H.X. and Ng, T.B. 1999. First chromatographic isolation of an antifungal thaumatin-like protein from French bean legumes and demonstration of its antifungal activity. *Biochem. Biophys. Res. Commun.* **263**: 130-4.

Ye, X.Y., Wang, H.X. and Ng, T.B. 2000a. Sativin, a novel antifungal miraculin-like protein isolated from the legumes of the sugar snap *Pisum sativum* var. *macrocarpon*. *Life Sci.* **67**: 775-81.

Ye, X.Y., Wang, H.X. and Ng, T.B. 2000b. Structurally dissimilar proteins with antiviral and antifungal potency from cowpea (*Vigna unguiculata*) seeds. *Life Sci.* **67**: 3199-207.

Zaumeyer, W.J. and Meiners, J.P. 1975. Disease resistance in beans. *Annu. Rev. Phytopathol.* **13**:313-334.

Zaumeyer, W.J. and Thomas, H.R. 1957. A monographic study of bean diseases and methods for their control. United States Department of Agricultural Technical Bulletin, Washington, US, 868p.

Zhang, Y., Zhang, X., Wen, F. and Wang, X. 2006. Enzymatic and histopathological changes during resistance of cowpea to Fusarium oxysporum f. sp. tracheiphilum. *Can. J. Pl. Pathol.* **28**:501-508.

Annexures

ANNEXURE-I

Details of laboratory equipment items used for the study

Research microscope	: Olympus
Phase contrast microscope, DM 500	: Leica
Microwave oven	: Samsung
Autoclave	: Kdt
Icematic	: SIMAG
Micropipettes	: Eppendorf, Accupipet
Balance	: Shimadzu
pH meter, PC 510	: EuTech Instruments
Medical freezer	: SANYO
Refrigerator	: Godrej, LG, Samsung
High speed refrigerated centrifuge	: Kubota, Japan
Centrifuge, MiRo 22R	: Heyich Zentrifugen
Minispin	: Eppendorf
Cylindrical water bath	: Rotex
Vertical electrophoresis system	: BIO-RAD
Power PAC 1000 and Power Pac 300	: BIO-RAD
Circulating cooling system	: Colora
Rocker 25	: Labnet
Shaking incubator	: Dk-sioio – Dai Ki Scientific Co)
Gel documentation system	: BIO-RAD, USA
NanoDrop^{R} ND-1000 Spectrophotometer	: NanoDrop^{R} Technologies Inc.USA

ANNEXURE-II

Media preparation

Potato Dextrose Agar medium (PDA)

Potato - 200 g
Dextrose - 20 g
Agar - 20 g
Distilled water - 1L

Selective medium (NGA)

Neopeptone - 20 g
Glucose - 28 g
$MgSO_4.7H_2O$ - 12.3 g
KH_2PO4 - 27.2 g
Agar - 20 g
Distilled water - 1L

ANNEXURE-III

Buffers for protein extraction

0.25 M Tris HCl

1 M Tris HCl = 157.6 g in 1L
0.25 M Tris HCl was obtained by making up 3.94 g of Tris HCl in 100 ml of distilled water.

50 mM Tris HCl

1 M Tris HCl = 157.6 g in 1L
50 mM Tris HCl was obtained by making up 0.78 g of Tris HCl in 100 ml of distilled water.

0.5 M NaCl

1 M NaCl = 58.44 g in 1L
0.5 M NaCl was obtained by making up 2.92 g of NaCl in 100 ml of distilled water.

ANNEXURE-IV

Chemicals for SDS-PAGE analysis

Acrylamide-Bisacrylamide stock

29.2 g of acrylamide and 0.8 g of N' N' Bismethlene acrylamide was dissolved in 80 ml of distilled water and made up to 100 ml. The solution was the filtered and stored at 4°C in dark up to 30 days.

Separating gel buffer (pH - 8.8)

22.7 g of 1.875M Tris base was dissolved in 80 ml of distilled water, the pH was adjusted to 8.8 with 1N HCl and the volume was made up to 100 ml with distilled water. The solution was stored at 4°C.

Stacking gel buffer (pH - 6.8)

7.26 g of 0.6M Tris base was dissolved in 60 ml of distilled water, the pH was adjusted to 6.8 with 1N HCl and the volume was made up to 100 ml with distilled water. The solution was then stored at 4°C.

10 per cent Sodium Dodecyl Sulphate (SDS)

1 g of SDS was dissolved in distilled water and the volume was made up to 10 ml with distilled water. The solution was stored at room temperature.

6.6 per cent Ammonium Persulphate (APS)

50 mg of APS was dissolved in 750 µl of distilled water to obtain 6.6 per cent of APS.

Tank buffer

192mM Glycine - 14.4 g

25mM Tris base - 3.0 g

0.1 per cent SDS - 1.0 g

Distilled water - 1 L

All the components are mixed and made up to 1L. The buffer can be stored at 4 °C and warmed to 37°C before use. The same buffer can be used 2-3 times for running the gel.

Sample buffer

0.125 M Tris HCl (pH-6.8) - 1.25 ml

Glycerol - 1.0 ml

2-mercaptoethanol - 0.1 ml

Bromophenol blue - 0.1 g

10 per cent SDS - 2 ml

Made up to 10 ml with distilled water.

Preparation of the gel mixture

Components	**12 per cent Separating gel**	**4 per cent Stacking gel**
Distilled water	3.32 ml	1.7 ml
Acrylamide stock	4.0 ml	0.425 ml
1.875 M Tris HCl buffer	2.52 ml	-
0.6 M Tris HCl buffer	-	0.31 ml
10 per cent SDS	100µl	25 µl
10 per cent APS	100µl	25 µl
TEMED	4 µl	2.5 µl
Total	**10 ml**	**2.5 ml**

Protein staining solution

Coomassie brilliant blue R 250 dye - 0.1 g

Methanol - 40 ml

Acetic acid - 10 ml

Distilled water - 50 ml

The dye was first dissolved in methanol and all other components were added. Every time fresh preparation of the dye solution was prepared.

Destaining solution

Methanol - 40 ml

Acetic acid - 10 ml

Distilled water - 50 ml

Solutions for silver staining

Fixing solution

Add 30 per cent ethanol and 10 per cent acetic acid.

Pretreatment solution

0.02 per cent sodium thiosulphate prepared by adding 0.02 per cent sodium thiosulphate in 100 ml of distilled water.

Developing solution

250 µl of formaldehyde was added with 3 g of sodium carbonate and o.5 mg of sodium thiosulphate. The volume was made up to 100 ml with distilled water.

Stop solution

4 per cent Tris was prepared and added with 2 per cent acetic acid.

Annexure V

Defense enzyme assay

1. Assay of Peroxidase (PO)

0.1 M phosphate buffer (pH – 7.0)

A: 1.39 g of sodium dihydrogen phosphate was weighed and made up to 100 ml with distilled water.

B: 2.6825 g of disodium hydrogen phosphate weighed and made up to 100 ml with distilled water.

39 ml of A and 61 ml of B were mixed well and made up to 200 ml with distilled water to obtain 7.0 pH buffer.

20 mM guaiacol

0.25 ml was made up to 100 ml with distilled water to obtain 20mM guaiacol.

2. Assay of Polyphenol oxidase (PPO)

50 mM Tris-HCl (pH -7.2) buffer containing 0.4 M sorbitol and 10mM NaCl

0.605 g of Tris (hydroxymethyl) aminomethane, 0.1168 g of NaCl and 14.5736 ml of Sorbitol were dissolved in 100 ml of distilled water and the pH was adjusted to 7.2 using 1 N HCl. Then the total volume was made up to 200 ml with distilled water to obtain 7.2 pH buffer.

0.1 M sodium phosphate buffer (pH – 6.5)

A: 1.56 g of sodium dihydrogen phosphate was weighed and made up to 100 ml with distilled water.

B: 1.42 g of disodium hydrogen phosphate weighed and made up to 100 ml with distilled water.

68.5 ml of A and 31.5 ml of B were mixed well and made up to 200 ml with distilled water to obtain 6.5 pH buffer.

0.01 M catechol

1M = 110.11 g in 1L

0.11g of catechol was made up to100 ml with distilled water to obtain 0.01M catechol.

3. Assay of Phenylalanine ammonia lyase (PAL)

25 mM borate-HCl (pH -8.8) buffer solution containing 5 mM mercaptoethanol

A: 0.953 mg of sodium borate was weighed and made up to 100 ml with distilled water.

B: 0.1 M solution of HCl was prepared by adding 0.83 mL of conc. HCl in 100 ml of distilled water.

50 mL of A and 9.4 ml of B were added and diluted to 100 mL with distilled water to obtain buffer with 8.8 pH. 0.04 ml of mercaptoethanol was also added to the above solution to obtain 5 mM concentration.

0.1 M borate buffer (pH – 8.8)

A: 1.24 g of boric acid was weighed and made up to 100 ml with distilled water.

B: 1.42 g of sodium borate was weighed and made up to 100 ml with distilled water.

50 ml of A and 30 ml of B were mixed well and made up to 200 ml with distilled water to obtain 8.8 pH buffer.

0.1 M L-phenylalanine

1M = 165.19 g in 1L

1.65g of L-phenylalanine was made up to100 ml with distilled water to obtain 0.1M L-phenylalanine.

1 M trichloroacetic acid

36.6 g trichloroacetic acid wade made up to 100 ml with distilled water to obtain 1M trichloroacetic acid.

CHARACTERIZATION OF PATHOGENESIS RELATED PROTEINS FOR ANTHRACNOSE RESISTANCE IN *VIGNA* SPP.

By

A AGATHA SHINY
(2011-11-174)

ABSTRACT OF THE THESIS

Submitted in partial fulfillment of the requirement for the degree of

Master of Science in Agriculture

(Plant Biotechnology)

Faculty of Agriculture

Kerala Agricultural University, Thrissur

Centre for Plant Biotechnology and Molecular Biology
COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR - 680 656
KERALA, INDIA

2013

ABSTRACT

Cowpea (Family: Fabaceae) is an important pulse cum vegetable crop of suitable for the tropical and sub-tropical regions of the world. The grain type cowpeas better tolerates the biotic and abiotic stresses against the vegetable types. Under humid conditions, vegetable types, especially the pole types are susceptible to many diseases and among them, anthracnose caused by *Colletotrichum lindemuthianum* (Sacc. & Magn.) Br. and Cav. is very severe. In Kerala, complete yield loss in vegetable cowpea is reported due to anthracnose during monsoons. The study entitled "Characterization of pathogenesis related proteins for anthracnose resistance in vegetable cowpea, *Vigna* spp." was carried out with objective to develop the protein profiles of resistant and susceptible bush and pole genotypes through SDS-PAGE analysis at different time intervals of infection and to characterize the differentially expressed proteins by MALDI-TOF followed by *in-silico* analyses.

Two bush type varieties Pusa Komal and Kanakamony, the former reported to be highly susceptible and the latter immune to anthracnose and two pole type varieties Lola and Arimbra Local, of which the former susceptible and the latter resistant were used in the study. Pure culture of the pathogenic fungus was developed and maintained on selective medium (Neopeptone-Glucose-Agar) at the Dept. of Plant Pathology. The identity of *Colletotrichum lindemuthianum* has been established from the spore characteristics observed under phase contrast microscope and the pathogenicity was confirmed through artificial inoculation under controlled conditions. The pot culture experiment was conducted with 50 pots per variety. Artificial inoculation of pathogenic fungus was done and the leaf samples were collected at 0, 6, 12, 18, 24, 48, 72, 96, 120, 144,168 and 192 hours after artificial inoculation. The total protein was extracted using Tris-HCl buffer (pH-7.5), quantified using spectrophotometer and analyzed by SDS-PAGE method. The

defense enzymes like peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) were assayed.

By artificial inoculation, disease responses for anthracnose were confirmed to be highly susceptible in Pusa Komal and Lola; highly resistant in Arimbra Local and immune in Kanakamony. Protein expression was found to be higher from the initial hours in resistant varieties whereas in susceptible varieties, the expression was reduced immediately after infection then peaked at 18hr and gradually decreased later on. Two prominent and differentially expressed protein bands at 56 kD and 14 kD were sequenced in MALDI-TOF to obtain the peptide mass fingerprint. Through *in-silico* analyses using Mascot server software, they were identified to be the large and small subunits of the chloroplastic enzyme RuBisCo. Thus the capability of a variety to maintain high levels of RuBisCo was found to be the deciding factor for anthracnose disease resistance. Further, protein profiles developed after purification of proteins by dialysis have clearly identified the differentially expressed band at 29 kD in the resistant varieties which is in the size range of already reported PR proteins.

PO and PAL activities were proportionate to the resistance behavior, with the peak values at 18 and 24 hr after inoculation. With the results of this study, these defense enzymes are recommended as biochemical markers for identifying the resistance in the accessions. Capability to maintain higher levels of RuBisCo, PO and PAL enzymes is the characteristic of anthracnose resistant vegetable cowpeas and the future breeding programmes could be oriented in this direction.