CHARACTERIZATION OF PR PROTEINS IN SELECTED CALLICLONES OF BLACK PEPPER IN RELATION TO *PHYTOPHTHORA* FOOT ROT DISEASE

By

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THESIS

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DECLARATION

I, hereby declare that the thesis entitled **'Characterization of PR proteins in selected calliclones of black pepper in relation to *Phytophthora* foot rot disease'** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.

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CERTIFICATE

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ABBREVIATIONS

°C	:	Degree Celsius
cm	:	Centimeter
CPBMB	:	Centre for Plant Biotechnology and Molecular Biology
cDNA	:	Complementary Deoxyribose Nucleic Acid
g	:	Gram
KAU	:	Kerala Agricultural University
kDa	:	Kilo Dalton
M	:	Molar
MALDI-TOF	:	Matrix Assisted Laser Desorption Ionization-Time of Flight Spectrophotometry
mg	:	Milligram
min	:	Minute
ml	:	Mililiter
mm	:	Milimeter
µm	:	Micrometer
mM	:	Milimole
M_r	:	Molecular Mass
MS	:	Mass Spectrometry
nm	:	Nanometer
OD	:	Optical Density
pI	:	Isoelectric Point
PR	:	Pathogenesis Related
%	:	Per cent
µg	:	Microgram
µl	:	Microliter
µM	:	Micromole
min	:	Minute
pH	:	Hydrogen ion concentration
ppm	:	Parts per million
qRT-PCR	:	Quantitative Real Time-Polymerase Chain Reaction

rpm	:	Revolution per minute
SDS	:	Sodium Dodecyl Sulphate
V	:	Volt
2DE	:	Two Dimensional Gel Electrophoresis

Introduction

1. INTRODUCTION

Black pepper (*Piper nigrum* L.) belonging to the family Piperaceae is an important spice crop of humid tropics popularly known as "King of Spices". It was originated in the Western Ghats of India, and is widely cultivated in the southern states of India such as Kerala, Karnataka and Tamil Nadu.

In India, black pepper cultivation is spread over an area of 1,28,870 ha with an annual production of 48,500 tonnes as per 2015-2016 statistics. Among the three southern states, Kerala holds the topmost position in black pepper cultivation both in terms of area (85,430 ha) and production (22,000 tonnes) (SBI, 2016).

Though black pepper is a crop of high economic importance among the growers, the major delimiting factor in production is the incidence of *Phytophthora* foot rot disease caused by *Phytophthora capsici*, which accounts for 20 to 30 per cent of vine mortality and crop loss. A recent survey report by Thomas and Naik (2017) for the incidence of *Phytophthora* foot rot disease in Karnataka state revealed a highest incidence of this disease in Mathodu village in Shivamogga district (65 %) followed by Kabilaseathve village of Chickmagaluru district (50 %). The severity of this disease is not only localized to one part of the plant, but the entire part of vine from the roots upto the leaves of the upper branches are affected, leading to decline in berry yield and finally the entire vine is succumbed to death within a period of two to three weeks (Manohara and Rizal, 2002).

Study conducted by Sarma *et al.* (1982) revealed that cultivars *viz.* Cheriakanyakkadan, Kalluvally, Balankotta, Uthirankotta, Narayakodi were tolerant to *Phytophthora* foot rot infection, and Panniyur-1 and Karimunda were susceptible genotypes. Electrolyte leakage studies and *in vitro* callus screening using toxic metabolites of *P. capsici* done by Shylaja *et al.* (1996a; 1996b) reported tolerance of Cheriakanyakkadan and Kalluvally calliclones to *Phytophthora* foot rot disease. Dagade (1999) performed anatomical and biochemical investigations in *Piper* spp. for tolerance to *Phytophthora* foot rot. A wild relative of black pepper, *Piper colubrinum* L., was reported resistant to *Phytophthora* foot rot infection (Nazeem *et al.*, 2008). Sanchu *et al.* (2002)

reported three black pepper calliclones of Cheriakanyakkadan *viz.* CKCC 43, CKCC 58 and CKCC 60 as tolerant to *Phytophthora* foot rot infection when they assessed the variability for disease reaction in the field grown calliclone. Mammootty *et al.* (2008) reported tolerance of Kalluvally cultivar to *Phytophthora* foot rot infection in the studies conducted in black pepper nursery.

The above studies gave rise to a new direction of research with an objective to elucidate the underlying defense mechanism in the tolerant genotypes and derive out the probable molecular candidates inflicting defense response against *Phytophthora* foot rot infection in black pepper. Jebakumar *et al.* (2001) and Nazeem *et al.* (2008) reported the role of pathogenesis related (PR) proteins in black pepper especially the β-1,3-glucanases (PR-2 protein) in the defense mechanism for *Phytophthora* foot rot in black pepper.

Development in proteomic technologies such as enzyme bioassays, total protein profiling through SDS-PAGE and two-dimensional gel electrophoresis, protein identification through MALDI-TOF / MS and MASCOT, *in silico* tools for protein characterization such as BLAST2GO and several structural and functional databases have boosted the proteome research. Hence, the current study was undertaken to characterize pathogenesis-related proteins in selected calliclones of black pepper against *Phytophthora* foot rot disease by using the advanced proteomic technologies. The current study will enlighten the overall scenario of defense response in black pepper against *Phytophthora* foot rot infection that can supplement in future for transgenic / cisgenic research or metabolic engineering of the pathways triggering transcription of PR-genes.

Review of literature

2. REVIEW OF LITERATURE

The current study entitled "Characterization of PR proteins in selected calliclones of black pepper in relation to *Phytophthora* foot rot disease" was intended to portray role of β-1,3-glucanases in defense mechanism and to analyze the proteome of tolerant and susceptible genotype against *Phytophthora* foot rot disease. The production of hydrolytic enzymes such as β-1,3-glucanases and chitinases, localized in the vacuole or directly secreted into the extracellular spaces of the plant cells, are involved in the plant defense action against the plant pathogens. The higher activity renders enhanced resistance in resistant cultivars against the diseases (Netzer *et al.*, 1979). These molecular armaments against the disease are acknowledged as pathogenesis-related (PR) proteins (Meins *et al.*, 1992; Simmons, 1994).

2.1. IMPORTANCE OF BLACK PEPPER (*Piper nigrum* L.), *PHYTOPHTHORA* FOOT ROT DISEASE AND CROP LOSS

The black pepper (*Piper nigrum* L.) or the 'Black Gold' (Nair, 2004) is ruling the entire spice market around the globe as "King of Spices" and its popularity is immensely widespread such that it is known by different names. Even though the black pepper production is providing sufficient remuneration to the pepper producers thus boosting the socio-economic development of farm economy, the major hinderance confronted by the farmers is the incidence of *Phytophthora* foot rot disease or quick wilt of black pepper (Anandaraj, 2000) in the pepper gardens that result in severe mortality of vines. The severity of the disease is not only localized to only a part of plant, but the entire vine from root region to collar, to the leaves of the upper branches are affected and finally leads to decline in berry yield. The causal organism for this deadly disease is the soil borne fungus *Phytophthora capsici* which is a common pathogen infecting other crops also (Fernandez *et al.*, 2004).

The historic biological relationship of the pathogen with the black pepper plant has been reported long back. During 1885, the Lampung province of Indonesia reported an accelerated collapsing of black pepper vines (Erwin and Ribeiro, 1996). The earliest prevalence of the disease in India was reported from Wayanad district of Kerala in 1902 (Barber, 1902). Butler (1906) entitled the disease as 'wilt' as there was

rapid wilting of the vines leading to death. Rao (1929) made the foremost attempt of isolating the *Phytophthora* sp. from a diseased vine in Karnataka. Muller (1936) later reported a similar disease occurrence in the Dutch East Indies caused by *Phytophthora palmivora* pv. *piperis*. He first coined the term 'foot rot' for the disease. Samraj and Jose (1966) made the first bonafide report on the disease in Kerala. Sarma *et al.* (1988) proposed that the term 'wilt' in black pepper is loose and not the correct representative of vascular wilt in firm phytopathological sense. On the other hand the term 'foot rot' is also associated with the *Fusarium* species which brings out confusion about legitimate terminology (Watanabe *et al.*, 1996; Duarte *et al.*, 1999). International Community Workshop on Joint Research for the Control of Black Pepper Diseases held at Goa, India in 1988 proposed a more convincing term '*Phytophthora* foot rot' in precedence over 'foot rot' or 'quick wilt' of black pepper (Nair and Sarma, 1988). Since then, the terminology adopted as '*Phytophthora* foot rot' in all the literatures published.

The outbreak of *Phytophthora* foot rot disease has acquired the status of an evil spirit haunting all the pepper growers around the globe due to its deadliest implications arising from collar infection to entire vine death perpetually leading to low productivity. In certain pepper growing nations of the globe, around 95 per cent vine loss has been reported from individual farms (Anandaraj *et al.*, 1989). Shamarao and Siddaramaiah (2002) reported that, in India the loss of vines due to infection is significantly higher than 5-20 per cent range as proposed by Manohara *et al.* (2004). The report of Vietnam Pepper Association (VPA, 2014) mentioned about 73,000 ha of estimated pepper growing area out of which 53,000 ha are productive in Vietnam. From the total global exports made for pepper products in the year the 2013, the Vietnam accounted for 58 per cent *i.e.* 1,56,396 tonnes that generated revenue of about 1.2 billion dollars. Between 2011 and 2014, the pepper area reported increment on an average of 2800 ha $year^{-1}$ which will be further rising in future. But according to the statistical report of VPA, between the year 2012 and 2014, the pepper area declined by about 2 per cent $year^{-1}$ (*i.e.* 1,200 ha). According to Gia Lai Statistical office, in the Gia Lai province the pepper yield drastically reduced to 30 per cent in 2014 compared to 2013 due to *Phytophthora* foot rot disease.

In aspiration to attain higher strands in black pepper cultivation, the growers have amended several advanced technologies in cultivation. For example, increased use of organic materials, use of chemical and biological methods for disease control, use of live supports and drainage systems and moreover in a broad sense use of Integrated Disease Management (IDM) techniques in black pepper are the common traditions practiced by the farmers in the field level. But the efficacy of all the control measures adopted has not yielded satisfactory results. No effective, single hit control measure has been developed so far as all the cultivable black pepper varieties are susceptible to *Phytophthora* foot rot disease.

The challenge accepted by the breeders to develop genetically resistant lines by screening the already available germplasm, open pollinated and irradiated seedling progenies through conventional breeding programmes have proved futile (Cheriyan, 2000). Natural sources of resistance to the disease were not reported in black pepper cultivars (Holliday and Mowat, 1963; Turner, 1973; Sarma *et al.*, 1982 and Vilasini, 1982). Still the knowledge about the fungus, its pathogenic relationship with the black pepper plant and defense reaction is in its infancy. Some of the wild genotypes of black pepper such as *Piper colubrinum* and *P. obliqum* have been reported to show resistance against the disease (Purseglove *et al.*, 1981). Tissue culture techniques have been adopted to develop disease tolerant lines by raising plants *in vitro* (Shylaja *et al.*, 1996). But now it has been revealed that there are seven decisive factors governing resistance to *Phytophthora* foot rot infection in black pepper. They are total phenols, orthodihydroxy (OD) phenols, change in membrane conductance, lignin and defense related enzymes such as peroxidase, β-1,3-glucanase and β-1,4-glucanase (Vandana *et al.*, 2014).

So in order to develop an effective management strategy against the disease, the primary requisite is to thoroughly comprehend the existent defense mechanism present within the plant and host-pathogen interactions. With this intent an earnest effort is made in this review to associate all the specifics, interpretations and theories regarding pathogenesis related (PR) proteins and its role in resistance to biotic stresses with special reference to biochemical defense response of black pepper through β-1,3-glucanase enzyme against the *Phytophthora* foot rot infection. Also a thorough

spotlight is made upon why and how essential is to characterize PR-proteins in black pepper through integration of different proteomic technologies.

2.2. SPECIFIC CHARACTERISTICS OF *Phytophthora capsici* AND SYMPTOMATOLOGY OF *PHYTOPHTHORA* FOOT ROT

In a span of 60 years or more, several *Phytophthora* species have been accredited as contributory agents for *Phytophthora* foot rot disease such as *Phytophthora nicotianae*, *P. palmivora* pv. *piperina*, *P. palmivora*, *P. palmivora* MF4, *P. capsici* and *P. tropicalis*) based upon results of morphological and molecular characterization (Muller, 1937; Tsao, 1988; Aragaki and Uchida, 2001; Zhang *et al.*, 2004).

Muller (1937) made preliminary attempt to isolate *Phytophthora* sp. from both the soil and stem of the infected pepper vine in Java and categorized it as *P. palmivora* under the varietal appellation 'piperis'. Holliday and Mowat (1963) conducted a meticulous research and attributed the causal organism as *P. palmivora* that arose more perplexity and controversies later. Zentmyer *et al.* (1977) reclassified the pathogen under *P. palmivora* MF_4 group on the basis of Malaysian and Thailand isolates which was further re-described by Tsao (1988) as *P. capsici sensu lato* based upon morphological distinctiveness. Tsao and Alizadeh (1988) amalgamated the two species *i.e P. palmivora* MF_4 isolate and *P. capsici* into a single nomenclature as *Phytophthora capsici* Leonian emend A. Alizadeh and P.H. Tsao. *Phytophthora* foot rot disease in black pepper in many countries is reported and accepted as to be caused by *P. capsici* (Erwin and Ribeiro, 1996; Anandaraj, 2000).

2.2.1. Characteristics of the pathogen

Apart from the above reviewed researches, a comprehensive research had also been carried out by Sastry and Hegde (1987) and Santhakumari (1987) to study credible reasons of variability existing within the *P. capsici* isolates and to gather profound knowledge about the fascinating biology of the fungus. Tsao and Alizadeh (1988) and Zentmeyer (1988) gave imperative characteristic features of *P. capsici* enlisted in Table 2.1. Mammootty (2003) reported that *Phytophthora* isolates produced petalloid, uniformly dense cottony aerial mycelium, rarely stellate or radiating when

grown in carrot agar medium. The growth was more in central portion. The pathogen produced hyaline hyphae, non-septate and smooth. Long caduceus sporangia were produced on sporangiophore in an umbellate fashion. The sporangia produced were ovoid to pyriform and ellipsoid with a tapered base toward the stalk end. The sporangial length ranged from 20.5 to 92.3 µm and breadth from 15.8 to 104.0 µm.

Table 2.1. Characteristic features of *P. capsici*

Sl. No.	Characteristics	Description
I	**Colony Morphology**	Petalloid pattern with diffuse edge; uniform dense aerial mycelium over entire colony on carrot agar medium
II	**Sporangial characters**	
1	Shape	Spherical, ovoid, obovoid, ellipsoidal, fusiform, pyriform, rounded/ tapered base
2	Papilla	Prominent occasionally
3	Sporangial size	40-52 x 20-31 µm
4	L/B ratio	1.6-2.0
5	Caducity	Caducous
6	Pedicel type	Narrow, long and not occluded
7	Pedicel length	20-150 µm, sometimes upto 250 µm
8	Ontogeny	Umbellate/irregular
III	Chlamydospores	Rarely produced

2.2.2. Symptomatology of *Phytophthora* foot rot disease

The *Phytophthora* foot rot disease has been widely explored by the researchers around the globe for its devastating symptoms on the entire parts of the vine especially at the collar region of the vine, in which the plant succumbs to death.

Mammootty *et al.* (2003) reported three types of symptoms *i.e.* leaf rot, stem / collar rot and root rot. Small, irregular, water soaked brown lesion appeared initially within 24-48 hours on the leaves with smooth and fimbricate margins, which advanced rapidly later. A yellow halo region developed around the fully developed lesion with a necrotic centre. Initially, the symptoms developed in the lower leaves which later

spread to upper leaves. Immature leaves were severely damaged than mature leaves. Appearance of greyish brown lesions with concentric zonations near the tip as well as on other portions of the infected leaves were found in certain cases.

The severe and fatal infection was inflicted due to stem / collar rot in which complete vine was succumbed to death. The stem developed dark brown to black linear lesions initially within 48 hours which later turned wet and slimy to touch. In the affected pepper cuttings, the general aerial symptoms noticed were foliar yellowing, defoliation and breaking off of stem at the nodal region. The collar region of the cuttings were discoloured and rotted. Complete decaying and disintegration of internal tissues were noticed as the infection advanced further. The progression of rotting was both upwards and downwards. Internal discolouration at the site of infection was also noticed. The entire cuttings were succumbed to death within 10-15 days after appearance of first symptom.

Dark brownish discolouration in the fine roots was initial characteristic of root rot infection. The infection then progressed to major roots and then to underground portion.

2.3. SCREENING FOR RESISTANCE / TOLERANCE TO *PHYTOPHTHORA* FOOT ROT

2.3.1. Existing germplasm

Many researchers (Holliday and Mowat, 1963; Ruppel and Almeyda, 1965; Leather, 1967; Alconero *et al.*, 1971; Turner, 1973; Kueh and Khew, 1980; Sarma *et al.*, 1982 and Vilasini, 1982) have made attempts for thorough screening of existing black pepper genotypes for their resistance / tolerance to *P. capsici*. None of the cultivated genotypes were found disease resistant except some of the wild *Piper* species such as *Piper colubrinum* and *Piper obliqum* that showed greater disease resistance.

Alconero *et al.* (1971) conducted field evaluation studies in Puerto Rico and Brazil for disease resistance to *P. palmivora* and graft compatibility of cultivated genotypes as scion and the resistant *Piper colubrinum* as root stocks. They found three

genotypes as less susceptible *i.e* Kalluvally, P.I. 214301 and Balankotta, whereas *Piper colubrinum* as resistant genotype. Kueh and Khew (1980) from Malaysia reported Kalluvally and Balankotta as tolerant black pepper varieties from India. Sarma *et al.* (1991) reported similar results about *Piper colubrinum* that this wild relative of genus *Piper* from Amazon forests is a resistant genotype known until now.

Sarma *et al.* (1982) confirmed Panniyur-1 and Karimunda as highly susceptible genotypes for *Phytophthora* foot rot infection, whereas Balankotta, Uthirankotta, Kalluvally, Cheriyakaniakadan and Narayakodi as cultivated tolerant genotypes. Similar results were reported by Shylaja *et al.* (1996b) based upon electrolyte leakage studies conducted by treating black pepper callus cultures with toxic metabolites from *P. capsici.*

Mammootty *et al.* (2008) screened 50 varieties / cultivars of black pepper against the *P. capsici* infection in the nursery to identify resistant genotypes. They found none of the screened genotypes immune to the infection but 'Kalluvally II', 'Panniyur-5' and 'Kalluvally IV' had less than 60 per cent leaf infection that were statistically on par with cultivars such as 'Cheriyakaniakadan', 'Balankotta' and 'Shimoga'. They reported that 'Kalluvally II' showed relatively lower mortality rates conferring tolerance to *Phytophthora* foot rot disease.

Currently some of the tolerant genotypes developed by Indian Institute of Spice Research (IISR), Calicut, Kerala are IISR Shakthi and IISR Thevam (IISR, 2007). Panniyur-8 is a hybrid black pepper variety obtained by crossing Panniyur-6 with Panniyur-5 is a tolerant variety developed at Pepper Research Station, Panniyur, Kerala (KAU, 2013).

2.3.2. Biotechnological interventions

Shylaja *et al.* (1996b) developed 158 calliclones of black pepper by inducing somaclonal variation through callus mediated organogenesis using explants from axenic seedlings of two cultivars *viz.* Cheriakanyakkadan and Kalluvally. The induced calli were subjected to *in vitro* screening with toxic metabolites of *Phytophthora capsici* and the regenerants were screened *in vivo* against *Phytophthora* foot rot infection. On the basis of *in vitro*, *in vivo* screening results and field evaluation from

2000 to 2008, 13 calliclones with improved performance were selected for subsequent multiplication and evaluation in future extensive experiments (Shylaja *et al.*, 2012).

Lissamma (2007) performed genetic transformation in black pepper for *Phytophthora* foot rot resistance / tolerance. *Agrobacterium tumefaciens* strains EHA 105, AGL 1.1303, GV 2260 and LBA 4404 were used for transformation. Strain EHA 105 contained plasmid p35SGUSINT with *gus A* gene and *npt II* gene. Strain AGL 1.1303 contained plasmid harbouring selectable marker genes (*npt II* and *hPf IV*), GUS and GFP reporter genes. The GV 2260 strain contained the plasmid pGV2260 having osmotin gene and *npt II* gene. The LBA 4404 contained plasmid pBZ100 having alfalfa glucanase gene, rice chitinase gene and *npt II* gene. Transformed zygotic embryo, leaf and cotyledonary node explants were obtained in the screening medium.

2.4. DEFENSE MECHANISM IN PLANTS

2.4.1. Anatomical defense mechanisms

2.4.1.1. Cuticle as defense barrier

Presence of a thicker cuticle over the leaf epidermis acts as defensive armour against the intruding pathogen. The cuticle acts as chemical and physical barrier to the germination and penetration of fungi. Level of resistance in infected tissues against the fungal pathogen is correlated with cuticular thickness (Wang and Pinckard, 1973; Bell, 1974).

2.4.1.2. Mesophyll as defense barrier

Anatomical investigation carried out by Kaur *et al.* (1992) in the leaves of groundnut varieties against the tikka leaf spot pathogen (*Cercospora arachidicola*) suggested that the resistant varieties had thicker epidermis, palisade tissues and thinner spongy parenchyma and susceptible varieties had maximum thickness of spongy parenchyma.

2.4.1.3. Vascular system as defense barrier

Jennings and Ullstrup (1957) studied the histopathology of corn leaf blight pathogen (*Heliminthosporium* spp.) and reported that lesion development in chlorenchyma became necrotic followed by extensive xylem plugging and then hyphae invaded into parenchyma. In susceptible inbreds, about 80 – 90 per cent of the xylem area of larger bundles was plugged with mycelium within 14 days after inoculation, whereas in resistant inbreds, the plugging was observed within 21 to 28 days.

2.4.2. Biochemical defense mechanisms

The presence of toxic chemical factor inhibits the growth and activity of pathogen in the host for *eg.* Mycolaminarins against Phytophthora infection (Marcan *et al.*, 1979) and Helminthosporin against *Helminthosporium maydis* (Angara-Sharma and Sharma, 1994).

2.4.2.1. Phenols

2.4.2.1.1. Total phenols

Matern and Kneusel (1988) elucidated two way defense strategy of phenols in plants *viz.* rapid accumulation of phenols at necrotic site and activation of *de novo* synthesis of phytotoxins or other stress related compounds. Sindhan *et al.* (1996) reported higher levels of total phenols in resistant wheat varieties than compared to susceptible ones at pre-infection stage.

2.4.2.1.2. Ortho dihydric phenol

Mukherjee and Kundu (1973) reported antibiotic and enzyme denaturing activity of monomeric dihydroxy phenols. Veermohan *et al.* (1994) reported high level of ortho dihydric phenol and total phenols in plants infected with *Alternaria solani*.

2.4.2.2. Sugars

Prasada *et al.* (1972) reported that bacterial leaf blight resistant variety of rice had lesser quantities of reducing, non-reducing and total sugars than compared to

susceptible and moderately susceptible varieties. Abraham (1986) attributed higher level of accumulation of reducing, non reducing and total sugars to resistance of betelvine cultivars to bacterial leaf spot pathogen.

2.4.2.3. Amino acids in defense mechanism

Rohringer (1957) reported increase in amino acid content in the wheat leaves infected with rust fungi (*Puccinia graminis tririci*). Pronounced increase in glutamine content was recorded in the infected leaves. Also the uredospores of the host pathogen also had higher glutamine content. Chile and Vyas (1983) reported greater amino acid content in the less susceptible cultivar than highly susceptible cultivar infected with *Phytophthora* leaf spot. The fungal infection caused depletion in free amino acid content.

2.4.3. Molecular defense mechanisms

The capability of a living organism to escape, to prohibit or to absolutely surmount the causal effects by the pathogen or other detrimental factors of the disease is known as resistance (Agrios, 1997).

The initiation of disease resistance in plants is manifested as restricted development of symptoms, the consequence of which is that the growth or multiplication of the pathogen becomes intricated. This limits the pathogen to remain restrained to the necrotic patches in the proximity of infection site which is known as hypersensitivity response (HR). In hypersensitive response, one or few localized cells endure rapid death at site of infection in order to restrict further incursion by the pathogen into the healthy plant tissues in the proximity. It is otherwise known as programmed cell death (PCD). The necrotrophic pathogens utilize the dead cells to obtain their energy, whereas the biotrophic pathogens exploit energy from the living cells (Kumar *et al.*, 2002). Against this further defense action by the plant commences with the activation of various regulatory pathways involved in specific defense mechanism against a particular type of pathogen confronted (Garcia-Brugger *et al.*, 2006). Jasmonic acid (JA) pathway and ethylene dependent responses are triggered by the necrotrophic pathogens whereas salicylic acid (SA) pathway is initiated by infection with biotrophic pathogens.

The plant pathogens mainly target the leaves and the root system. The plant undergoes substantial changes in gene expression as a consequence of signal conveyed by the triggered defense related pathways in order to combat against biotic stresses (Yang *et al.*, 1997). Induced responses and the preformed barriers are the two bases for plant defense response against the pathogen (Bryngelsson and Collinge, 1992). Presence of thick cuticular envelope, composition and thickness of host cell wall and production of antimicrobial compounds by the host are instances of preformed barriers. But once the pathogen adheres onto the host surface, it initiates induced responses. The primary requisite of induced response is production of specific signal molecules or elicitors either by the pathogen or by the degraded products of the host cell wall. These signal molecules can be classified as exogenous or endogenous elicitors on the basis of source of derivation (Collinge *et al.*, 1993; Fujita *et al.*, 2004). These elicitors trigger downstream signaling cascade thus coordinating a subsequent expression of genes concerned with plant defense response (Yang *et al.*, 1997).

The mechanism of plant defense response is reported as creation of ionic instability across the plasma membrane due to opening of specific gated ion channels or by rapid accumulation of toxic reactive oxygen species (ROS) for *eg.* hydrogen peroxide (H_2O_2) and superoxide (O_2^-) or by cascade of proteins undergoing activation and deactivation through regulated phosphorylation and dephosphorylation reaction. According to Doke *et al.* (1996) and Conrath *et al.* (1997) the ROS production is responsible for suicidal activity of the infected host cells mediated by oxidative burst. It is toxic for both the pathogen and infected host cell thus causing substantial damage to initiate further defense action and forms the first signal for hypersensitive response (HR). Leon *et al.* (1995) reported that increased levels of H_2O_2 is associated with increased expression of enzymes regulating salicylic acid (SA) pathway such as glutathione-s-transferase (GST) (Levine *et al.*, 1994) and glutathione peroxidase (GPX) (Avsian- Kretchmer *et al.*, 2004).

The oxidative burst later enhances several other metabolic and structural changes in the host such as strengthening of the plant cell wall due to enzymatic activation and accumulation of toxic secondary metabolites such as phytoalexins and phytoanticipins. The cell wall undergoes lignification and suberization in order to act

as an armor or structural barrier against pathogen entry. They also accumulate thionins, hydroxyproline-rich glycoproteins (HGP), complex polysaccharides such as callose, cellulose and pectins that enhance further thickening of cell wall and specific papilla at the penetration site of pathogen into the host (Collinge *et al.*, 1993; Brisson *et al.*, 1994; Baker and Orlandi, 1995).

Apart from above cited defense responses the plants also develop systemic acquired resistance (SAR) following HR. The SAR is mediated by the synthesis of pathogenesis related (PR) proteins such as glucanases (PR-2) and chitinases (PR-3) having antifungal or antibacterial functionality which renders disease resistance to uninfected distal parts of plant (Ryals *et al.*, 1996; Sticher *et al.*, 1997). Another kind of systemic resistance known as induced systemic resistance (ISR) is conciliated by jasmonate and ethylene dependent pathways which do not involve synthesis of PR proteins (Pieterse *et al.*, 1998; Van loon *et al.*, 1998). According to Tazum (2001), the ISR has non-specific activity against a broad group of plant pathogens. Multiple gene products like peroxidases, hydrolases or defense-related gene products timely accumulate which is responsible for ISR.

All the defense related pathways cited above are regulated by enhanced transcription of defense related genes and their translated products like enzymes or proteins. These translated protein products acknowledged as pathogenesis-related (PR) proteins form the major class of defense related proteins that are localized around the infection site or is systemically induced in the uninfected regions of the plant (Van Loon *et al.*, 2006).

2.5. PATHOGENESIS RELATED (PR) PROTEINS

Antoniw *et al.* (1980) first coined the term "pathogenesis related (PR) proteins" and defined it as proteins synthesized by the host plant when induced during pathological and related situations. These proteins are systemically induced, accumulate locally in the infected region of the plant and are responsible for the progression towards systemic acquired resistance (SAR) against future development or occurrences of infection by bacteria, fungi and virus (Van Loon and Van Strien, 1999). The pathological situations refer both the hypersensitive response towards plant

pathogenic bacteria, fungi, virus attack and the parasitic attack by insect pests, nematodes and herbivores (Van Loon, 1999).

The initial testament of PR proteins was proved by two autonomous groups Gianinazzi *et al.* (1970) and Van Loon and Van Kammen (1970) in tobacco leaves infected with tomato mosaic virus (TMV). Since then the research interests were diverted towards the possible involvement of different PR proteins in plants involved in resistance mechanism against pathogen attack. The initial findings arose assumptions that these proteins were expressed only in resistant plants that produce a hypersensitive necrotic response (HR) against plant pathogenic bacteria, virus and fungus. But later, Van Loon (1985) found that PR proteins are expressed in both susceptible and resistant plants when subjected to both biotic and abiotic stresses. The PR proteins are present at very low concentrations in the healthy plants or might be absent but upon HR, these proteins accumulate in higher quantities around the necrotic regions. On an average, these proteins may account 10 per cent of the total soluble proteins synthesized in the leaves within initial few hours of infection (Van Loon *et al.*, 1987).

Based upon the amino acid sequence, enzymatic or biological activity and serological relationship, about 17 families of PR proteins have been reported until now (Van Loon *et al.*, 2006) (Table 2.2). Each PR family is further categorized into several classes based upon presence of varied isoforms with either high or low isolelectric point (pI) values. Each PR protein family is designated by an arabic numeral and named by lower case letters (Van Loon *et al.*, 1994). According to the recommendations of Commission on Plant Gene Nomenclature, the PR genes having conserved sequences and that are not designated based on function are designated as *Ypr*. Based upon the order of their discovery, the PR families have been numbered and any new PR proteins reported from different species are assigned to the existing categorized family if similarity exists or else if there exists no similarity, then a novel PR family is created (Santen, 2007). Most of the PR families have been reported from tobacco followed by other crop plants such as rice, wheat, maize, barley, tomato, radish, cucumber, *etc.* in which few other PR families have been reported.

Table 2.2. Recognized families of pathogenesis related proteins (modified from Van Loon *et al.*, 2006)

Family	Type member	Properties	Gene symbols
PR-1	Tobacco PR-1a	Unknown	*Ypr1*
PR-2	Tobacco PR-2	β-1,3-glucanase	*Ypr3* [*Gns2*(‘*Glb*’)]
PR-3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII	*Ypr3, Chia*
PR-4	Tobacco ‘R’	Chitinase type I, II	*Ypr4, Chid*
PR-5	Tobacco S	Thaumatin-like	*Ypr5*
PR-6	Tomato inhibitor I	Proteinase-inhibitor	*Ypr6, Pis* (‘*Pin*’)
PR-7	Tomato P_{69}	Endoproteinase	*Ypr7*
PR-8	Cucumber chitinase	Chitinase type III	*Ypr8, Chib*
PR-9	Tobacco “lignin forming peroxidase”	Peroxidase	*Ypr9, Prx*
PR-10	Parsley “PR-1”	Ribonuclease-like	*Ypr 10*
PR-11	Tobacco “Class V” chitinase	Chitinase type I	*Ypr11, Chic*
PR-12	Radish Rs-AFP3	Defensin	*Ypr12*
PR-13	Arabidopsis THI2.1	Thionin	*Ypr13, Thi*
PR-14	Barley LTP4	Lipid-transfer protein	*Ypr14, Ltp*
PR-15	Barley OxOa (germin)	Oxalate oxidase	*Ypr15*
PR-16	Barley OxOLP	Oxalate-oxidase-like	*Ypr16*
PR-17	Tobacco PRp27	Unknown	*Ypr17*

2.5.1. Significance of various PR proteins in plant defense

2.5.1.1. Tobacco PR-1 protein

White *et al.* (1987) reported that 14-18 kDa molecular weight PR proteins were produced in infected plants or salicylic acid-treated plants of potato, tomato, maize, barley, *Gomphrena globosa*, *Chenopodium amaranticolor* and *Solanum demissum* that serologically responded to affinity-purified antibodies of PR 1a protein from *Nicotiana tabacum* cv. *Xanthi* in immunoelectroblots. Tahiri-Alaoui *et al.* (1993) reported that upon infection by black root rot fungus, *Chalara elegans*, in roots of tobacco, the intercellular space material, over cell walls, over secondary thickenings of xylem vessels and inter- and intracellular fungal hyphae penetrating the root tissues were significantly enriched with PR-1 proteins. Lee *et al.* (2000) demonstrated through immunogold labeling studies that the PR-1 proteins were predominantly expressed in the intercellular spaces of the pepper (*Capsicum annuum* L.) stem cells at 24 hours after inoculation with *Phytophthora capsici*.

2.5.1.2. PR-2 protein (β-1,3-glucanase)

Linthorst *et al.* (1990) challenge inoculated the leaves of healthy tobacco plants with tobacco mosaic virus and found that both acidic and basic β-1,3-glucanases were induced after infection. Southern blot analysis showed that there are approximately eight genes coding for β-1,3-glucanases. Wrobel-Kwiatkowska *et al.* (2004) developed a transgenic flax (*Linum usitatissimum* L.) genetically modified with potato β-1,3-glucanase cDNA that showed about threefold higher resistance against *Fusarium oxysporum* and *F. culmorum* compared to non-transformed plants. Wei *et al.* (2005) isolated β-1,3-glucanase from *Jatropha curcas* using Sephadex G-75 FF and HiPrepSephacrayS-100HR chromatography columns and subjected to *in vitro* testing for antifungal activity and they found that β-1,3-glucanase was responsible for hydrolysis of cell walls of *Rhizoctonia solani* kuha. and *Gibberelle zeae* (Schw.). Liu *et al.* (2009) performed Immunogold labeling assays in wheat to study the defense response of the plant after inoculation with stripe rust fungus (*Puccinia striiformis* f. sp. *tritici*). They found that β-1,3-glucanases were predominantly localized in the host cell wall and over the extrahaustorial matrix. Wan *et al.* (2011) elucidated the role of

Osg1 gene encoding β-1,3-glucanase for callose degradation in process of tetrad dissolution thus supplementing pollen development in rice. Nayyar *et al.* (2017) developed transgenic sugarcane genetically modified with β-1,3-glucanase gene from Trichoderma *spp.* that showed resistance to red rot fungus (*Colletotrichum falcatum* Went.) by coding β-1,3-glucanase responsible for cleaving β-1,3-glucan bond in the fungal cell wall further leading to hyphal lysis.

Intense researches have been made to investigate possible roles of β-1,3-glucanases and chitinases in plant defense mechanism against pathogenic fungus (Abeles *et al.*, 1971; Pegg, 1977; Mauch *et al.* 1988). β-1,3-glucanases are the most interesting and focused defense related proteins among all the proteins with known enzymatic function because they are regulated hormonally and developmentally in healthy plants, involved in coordinating various physiological and developmental processes (Mohnen *et al.*, 1985; Felix and Meins, 1987) and also protect the plants from attack by pathogenic fungus (Mauch *et al.*, 1988). The more detailed information and general aspects associated with β-1,3-glucanases are given under section 2.5.3.

2.5.1.3. PR-3 protein (Chitinase type I, II, IV, V, VI, VII)

Ebrahim *et al.* (2011) evaluated 12 mango cultivars for the resistance reaction to floral malformation caused by *Fusarium* spp. The results revealed higher chitinase activity in the leaves during the flowering period in the resistant mango cultivars Bhadauran and Elaichi. Tobias *et al.* (2017) identified about 67 gene models for chitinase in *Eucalyptus grandis* belonging to two families known as glycosyl hydrolase 18 and 19 within the genome assembly. They investigated that, in *E. grandis* a single gene codes for putative vacuolar targeted chitinase type-I that is strongly up-regulated in response to attack by eucalypt gall wasp (*Leptocybe invasa*) and fungal stem canker caused by *Chrysoporthe austroafricana* in both susceptible and resistant plants.

2.5.1.4. PR-4 protein (Chitinase type I, II)

Bertini *et al.* (2003) reported in *Triticum aestivum* that upon infection with soil-borne pathogenic fungus *Fusarium culmorum*, PR-4 transcripts are induced in the wheat coleoptiles and the roots that correspond to production of PR-4 proteins. They

also reported that upon treatment with chemical inducers such as salicylic acid (SA), methyl jasmonate (MeJA) and benzo (1,2,3) thiodiazole-7-carbothionic acid S-methyl ester (BTH), the PR-4 genes are activated following SA- and JA-dependent defense response pathways. Guevara-Morato *et al.* (2010) investigated that in *Capsicum chinense* upon infection by pepper mild mottle virus, led to induction of necrogenic reaction at primary infection sites of the leaves and restriction of virus which was due to accumulation of PR-4 proteins around the necrotic local lesions. Upon purification of these proteins it was found that it did not have chitinase activity rather it had both DNase and RNase activity. Khaliluev *et al.* (2011) developed a transgenic tomato plant encoding chitin-binding proteins (PR-4) belonging to *Amaranthus caudatus* and *A. retraflexus* that showed enhanced resistance to *Phytophthora infestans* compared to untransformed control tomato plants. Bai *et al.* (2013) identified a new PR-4 gene (*MdPR-4*) from *Malus domestica* that is highly up-regulated by *Botryosphaeria dothidea* infection coding for MdPR-4 protein that exhibited ribonuclease activity for single strand RNA specifically and inhibited hyphal growth of three apple pathogenic fungi *B. dothidea*, *Valsa ceratosperma* and *Glomerella cingulata* in *in vitro* assays.

2.5.1.5. PR-5 protein (Thaumatin-like)

Piggott *et al.* (2004) reported that a 23 kDa protein corresponding to thaumatin like (PR-5) protein was accumulated in the canker margins in the bark of western white pine (*Pinus monticola* D. Don) seedlings infected with blister rust pathogen (*Cronartium ribicola*) as a defense response. Mahdavi *et al.* (2012) genetically engineered banana plants with rice thaumatin-like protein (*tlp*) gene using particle bombardment method and further challenge inoculation by Fusarium wilt pathogen (*Fusarium oxysporum* pv. *cubensec* (race 4)) showed enhanced resistance in transgenic banana plants compared to control plants. He *et al.* (2017) identified gene (*VaTLP*) coding for thaumatin-like protein from the downy mildew-resistant grapevine "Zuoshan-I" and transformed somatic embryogenic calli of *Vitis vinifera* with *VaTLP* gene. Leaf discs and whole plant inoculation with downy mildew fungus (*Plasmopara viticola*) in transformed plants showed inhibition of both hyphal growth and asexual reproduction.

2.5.1.6. PR-6 protein (Proteinase-inhibitor)

Shin *et al.* (2001) reported a cDNA clone encoding putative proteinase inhibitor II (*CaPinII*) in pepper (*Capsicum annuum* L. cv. Bugang). They found that upon infection by tobacco mosaic virus (TMV) pathotype P_0, the accumulation of *CaPinII* transcript was maximum at 72 hours post-infection that declined afterwards. They proved that that the *CaPinII* gene was expressed as defense response to viral attack. Lopes *et al.* (2009) isolated three Kunitz-type serine protease inhibitors (APTIA, APTIB and APTIC) from *Acacia plumose* that had antifungal activity against *Aspergillus niger*, *Colletotrichum* sp. and *Thielaviopsis paradoxa.*

2.5.1.7. PR-7 protein (Endoproteinase)

Vera *et al.* (1988) purified an alkaline endoproteinase P-69 that was induced as a defense response against Citrus Exocortis viroid in tomato (*Lycopersicon esculentum* Mill, cv. "Rutgers"). Jorda *et al.* (1999) demonstrated in tomato that, two endoproteinases P69B and P69C were induced systemically after post-infection with *Pseudomonas syringae* and treatment with SA. Ekchaweng *et al.* (2017) isolated three genes (*HbSPA, HbSPB* and *HbSPC*) coding for subtilisin-like proteases belonging to endoproteinase family from *Hevea brasiliensis* and performed qRT-PCR. The results revealed that only *HbSPA* was over-expressed in *Hevea* stem, leaves, hypocotyl and roots upon infection with *Phytophthora palmivora* and slight wounding.

2.5.1.8. PR-8 protein (Chitinase type III)

Kishimoto *et al.* (2004) reported induction of class III chitinase gene (*CHI2*) in cucumber plants (*Cucumis sativa* L.) coding for chitinase III upon infection by gray mold fungus *Botrytis cinerea.* They developed transgenic cucumber that over-expressed the *CHI2* gene and found that the symptoms by *B. cinerea* infection were reduced for four days after inoculation.

2.5.1.9. PR-9 protein (Peroxidase)

In cucumber, Hammerschmidt *et al.* (1982) observed enhanced peroxidase activity in leaf-2 when first true leaf was challenged with *Colletotrichum lagenarium* that proved the association of enhanced peroxidase activity with ISR. Peng and Kue

(1992) reported generation of hydrogen peroxide (H_2O_2) *in vitro* as stimulated due to peroxidase production in horseradish. The H_2O_2 totally inhibited the germination of three fungal spores *in vitro i.e Peronospora tabacina*, *Cladosporium cucumerinum* and *Colletotrichum lagenarium* at 2.61 x 10^{-5} *M* concentration. Choi *et al.* (2007) reported an extracellular peroxidase gene (*CaPO2*) from pepper (*Capsicum annum*) which when transferred in *Arabidopsis thaliana* was responsible for generation of ROS which lead to enhanced disease resistance accompanied by cell death and *PR* gene induction against infection by *Pseudomonas syringae* pv. *tomato.* Oliveira *et al.* (2017) purified an antifungal class III peroxidase (Mm-POX) from *Marsdenia megalantha* latex using DEAE-cellulose and gel filtration chromatography on Superose 12 HR 10/30 column. The Mm-POX protein was responsible for permeabilization of the conidial membrane of *Fusarium oxysporum* and *F. solani* and induction of oxidative stress in *F. solani.*

2.5.1.10. PR-10 protein (Ribonuclease-like)

Lo *et al.* (1999) developed cDNA library from mesocotyls of sorghum cultivar DK18 inoculated with fungal pathogens. They isolated a cDNA clone by differential screening and found it as coding sequence for PR-10 protein family with ribonuclease function. Further analyses showed over-expression of *PR-10* and chalcone synthase (*CHS*) genes in the mesocotyl along with accumulation of fungicidal phytoalexins upon post-inoculation with *Cochliobolus heterostrophus*. McGee *et al.* (2001) reported increment in expression of *RPR10a* gene coding for PR-10 protein in rice (*Oryza sativa*) from a lower basal level at 12 hours after inoculation, second higher level at 48 hours after inoculation and until 144 hours after inoculation with rice blast fungus (*Magnaporthe grisea*). Park *et al.* (2004a) isolated a cDNA clone encoding PR-10 protein (CaPR-10) from hot pepper (*Capsicum annuum*), the transcript of which was induced upon infection by tobacco mosaic virus and *Xanthomonas campestris* pv. *vesicatoria.*

2.5.1.11. PR-11 protein (Chitinase type I)

Heitz *et al.* (1994) first isolated PR-11 protein (named as Pz protein) from tobacco leaves (*Nicotiana tabacum* cv. Samsun NN) inoculated with tobacco mosaic

virus that was found similar to chitinases / lysozymes and was responsible for initiation of hypersensitive response.

2.5.1.12. PR-12 protein (Defensin)

Lai *et al.* (2002) isolated and characterized a new gene family coding for defensin protein (DRR230-c) in pea (*Pisum sativum*). They found elevated expression of this protein in the young pods and mature foliar tissues upon infection with *Ascochyta pinodes* and *Pseudomonas syringae*. Aerts *et al.* (2007) isolated an anti-fungal plant defensin protein (RsAFP2) from *Raphanus sativus* that induced production of ROS in *Candida albicans* in a dose dependent manner leading to membrane permeabilization and yeast cell death. Mello *et al.* (2011) isolated a new defensin protein ($P_{\nu}D_1$) from *Phaseolus vulgaris* (L.) seeds and purified it through anion exchange and phase-reverse chromatography. The SYTOX green uptake assay showed membrane permeabilization activity of this protein at 100 μg ml^{-1} concentration in *Fusarium solani*, *F. oxysporum*, *F. laterithium*, *C. parapsilosis*, *C. tropicalis*, *C. albicans*, *Pichia membranifaciens*, *Kluyveromyces marxiannus* and *Saccharomyces cerevisiae*.

2.5.1.13. PR-13 protein (Thionin)

Carmona *et al.* (1993) expressed α-thionin gene from barley endosperm in tobacco plant which showed antibiotic properties *in vitro* against *Pseudomonas syringae* pv. *tabici* 153 and *P. syringae* pv. *syringae*. Lee *et al.* (2000) reported strong expression of a thionin coding gene *CATHION1* in pepper (*Capsicum annuum*) leaves infected with *Xanthomonas campestris* pv. *vesicatoria* and *Phtophthora capsici* in separate assays. Hoshikawa *et al.* (2012) isolated five novel genes coding for thionin in three Brassicaceae species (*Brassica oleracea* var. *acephala*, *Nasturtium officinale* and *Barbarea vulgaris*). These genes were transferred into potato genome followed by subjecting transgenic potato plants to antifungal assay using detached leaves showed enhanced resistance to gray mold fungus (*Botrytis cinerea*) compared to non-transformed control plants.

2.5.1.14. PR-14 protein (Lipid-transfer protein)

Regente and Canal (2000) purified and characterized a 10 kDa antifungal protein (Ha-AP10) from sunflower (*Helianthus annuus*) seeds. They reported 50 per cent inhibition in germination of spores of *Fusarium solani* f. sp. *eumartii* (40 μg ml^{-1} concentration) at 0.65 μ*M* concentration of this antifungal protein. Yang *et al.* (2006) purified a novel heat-stable antimicrobial protein (LJAMP2) from the seeds of a medicinal herb *Leonurus japonicus* Houtt. The partial N-terminal sequence showed similarity with plant non-specific lipid transfer protein (nsLTP) and *in vitro* bioassays showed toxic properties of LJAMP2 against *Alternaria brassicae*, *Botrytis maydis*, *Rhizoctonia cerealis*, *Bacillus subtilis*, *Aspergillus niger*, *Fusarium oxysporum*, *Penicillium digitatum* and *Saccharomyces cerevisiae*. Nawrot *et al.* (2017) isolated and characterized a nsLTP from the latex of *Chelidonium majus* L. (Papaveraceae) named CmLTP9.5. This isolated fraction showed strong antimicrobial activity against gram-negative bacterium *Campylobacter jejuni* and gram-positive bacteria *Listeria greyi* and *Clostridium perfringens* in diffusion method and critical dilution assay.

2.5.1.15. PR-15 protein (Oxalate oxidase)

Dumas *et al.* (1995) detected the presence of oxalate oxidase in mature region of primary roots and coleorhizae of three day old barley seedlings infected with *Erysiphe graminis* f. sp. *hordei* as confirmed through immunoblotting using antibodies raised against wheat oxalate oxidase in *Escherichia coli*. Hu *et al.* (2003) analyzed transgenic sunflower (*Helianthus annuus* cv. SMF3) plants expressing wheat oxalate oxidase (*OXO*) gene. In their study, they found that upon infection with oxalic acid (OA) - generating fungus *Sclerotinia sclerotiorum*, lead to high level expression of *OXO* gene that converted OA to hydrogen peroxide (H_2O_2) which further activated expression of three defense related genes coding for PR-5, carbohydrate oxidase and defensin, respectively as confirmed through RNA-blot analysis. Livingstone *et al.* (2005) developed transgenic peanut plants from embryogenic cultures of three Virginia peanut cultivars (Wilson, Perry and NC-7) genetically modified with barley oxalate oxidase gene. Detached leaflet assay revealed significant reduction in lesion size (by 75-97 %) in transgenic plants infected with Sclerotinia blight pathogen (*Sclerotinia minor* Jagger) compared to non-transformed controls.

2.5.1.16. PR-16 protein (Oxalate-oxidase-like)

Park *et al.* (2004b) identified a 660 bp cDNA clone (*CaGLP1*) from the cDNA library constructed with mRNA isolated from hot pepper plants inoculated with tobacco mosaic virus (TMV) coding for germin-like protein (GLP). Results revealed rapid accumulation of *CaGLP1* transcripts after TMV infection and SA application. Godfrey *et al.* (2007) isolated seven novel cDNA clones from grapevine (*Vitis vinifera* cv. Chardonnay) coding for GLP that exhibited both diverse and specific expression patterns when challenged with abiotic and biotic treatments. One gene *VvGLP3* was specifically induced upon *Erysiphe necator* infection and the expression was closely linked to infection site. This gene coded for GLP with superoxide dismutase activity. Beracochea *et al.* (2015) identified a novel sunflower gene *HaGLP1* coding for GLP which they introduced into Arabidopsis genome and assessed the transformed plants by inoculation with *Sclerotinia sclerotiorum* and *Rhizoctonia solani*. Results revealed significant protection against both the pathogens *in vitro* and elevated levels of ROS production.

2.5.1.17. PR-17 protein (Tobacco PRp27)

Christensen *et al.* (2002) isolated two barley (*Hordeum vulgare* L.) cDNA clones (pBH6-12 and pBH6-17) from the library prepared from the leaves inoculated with *Blumeria graminis* f. sp. *hordei* at six hours after inoculation. The two transcripts strongly accumulated with peaks at 6, 15-24 and 48-96 hours after inoculation. These transcripts encoded two proteins *Hv*PR-17a and *Hv*PR-17b of molecular weight 26 and 24 kDa, respectively belonging to PR-17 group from tobacco. These proteins were found to accumulate in the mesophyll apoplast, leaf epidermis and in the tissues invaded by the fungal pathogen. Zhang *et al.* (2012) confirmed the importance of PR17c protein in penetration resistance against powdery mildew fungus (*Blumeria graminis* f. sp. *hordei*) encoded by *CSEP0055* transcript in barley. The protein was reported to accumulate in the apoplastic region, localized to the papillae. The transcript didn't accumulate until 24 hours after inoculation suggesting too late response against primary penetration by the fungus but rather appeared to accumulate at the secondary penetration sites of the fungus.

2.5.2. Defense related proteins in black pepper and related species

Dagade (1999) performed biochemical investigations in black pepper against *Phytophthora* foot rot disease. Activity of indole-3 acetic acid oxidase showed maximum decrease in Panniyur-1, whereas increase in activity in case of Kalluvally and *Piper colubrinum*. The peroxidase activity was recorded higher *Piper colubrinum*. Activity of polyphenol oxidase was found to decrease maximum in case of Kalluvally followed by Panniyur-1 and *P. colubrinum*.

Jebakumar *et al.* (2001) reported phenylalanine ammonia lyase (PAL) and β-1,3-glucanase activity in the leaf and root tissues of three black pepper (*Piper nigrum* L.) varieties (tolerant P24, susceptible Panniyur-1 and Subhakara) inoculated with *Phytophthora capsici*. Both the enzymes were found expressed in both healthy and infected plants. Western-blotting with anti-tobacco β-1,3-glucanase polyclonal antibody confirmed the presence of β-1,3-glucanase isoforms. Among the three varieties investigated, tolerant P24 showed significantly higher enzyme activity in both healthy and infected plants.

Nazeem *et al.* (2008) studied change in protein profiles of susceptible variety 'Panniyur-1', relatively tolerant 'Kalluvally' and resistant wild species *Piper colubrinum* upon infection with *Phytophthora capsici*. They found a protein, that was over-expressed after challenge inoculation with *P. capsici* which was later characterized as β-1,3-glucanase. They also performed enzyme bioassays for phenylalanine ammonia lyase, chitinase, β-1,3-glucanase and peroxidase and confirmed the role of these enzymes in defense mechanism against *P. capsici*. The wild species *Piper colubrinum* reported higher enzyme activities followed by relatively tolerant 'Kalluvally'.

Varma *et al.* (2009) reported enhanced chitinase activity as induced by inoculation with *P. capsici* in the leaves of *Piper colubrinum* and *Piper nigrum*. The results revealed that the older leaves showed maximum chitinase activity with maximum after 60 hours of inoculation which decreased gradually thereafter. The activity was also found substantially higher in *Piper nigrum* when compared to *Piper colubrinum* after inoculation with *P. capsici*.

Mani and Manjula (2010) reported and characterized two osmotin isoforms and an anti-fungal *PR-5* gene homologue in *Piper colubrinum* as induced by salicylic acid treatment. The larger form *PcOSM2* (693 bp) encoded 21.5 kDa basic protein and smaller form *PcOSM1* (543 bp) encoded 16.4 kDa acidic protein.

2.5.3. β-1,3-glucanases

The PR-2 proteins are known as "β-1,3-glucanases" or "glucan endo-1,3-β-glucosidases" (E.C. 3.2.1.39). These are the group of enzymes that catalyze the hydrolytic cleavage of endo-type 1,3-β-D-glucosidic linkages in β-1,3-glucans. These are widely distributed in seed-plant species as highly regulated enzymes (Meins *et al.*, 1992; Stone and Clarke, 1992; Simmons, 1994; Hoj and Fincher, 1995). Though major divergence of researches in β-1,3-glucanases is inclined towards their possible roles in defense response against microbial pathogens, but there are strong evidences implicating the roles played by these enzymes in diverse physiological and developmental processes such as cell division (Waterkeyn, 1967; Fulcher *et al.*, 1976), microsporogenesis (Worrall *et al.*, 1992; Bucciaglia and Smith, 1994), pollen germination and pollen tube growth (Roggen and Stanley, 1969; Meikle *et al.*, 1991), fertilization (Lotan *et al.*, 1989; Ori *et al.*, 1990), embryogenesis (Dong and Dunstan, 1997; Helleboid *et al.*, 1998), fruit ripening (Hinton and Pressey, 1980), seed germination (Vogeli-Lange *et al.*, 1994; Leubner-Metzger, 1995), mobilization of stored reserves in the endosperm of cereal grains (Fincher and Stone, 1993), bud dormancy (Krabel *et al.*, 1993) and responses to wounding, cold, ozone and UV-B (Brederode *et al.*, 1991; Linthorst, 1991; Ernst *et al.*, 1996; Thalmair *et al.*, 1996; Hincha *et al.*, 1997).

2.5.3.1. Occurrence

Felix and Meins (1986) reported accumulation of β-1,3-glucanases in higher concentrations (up to four per cent of soluble proteins) in lower leaves and roots of healthy plants. Keefe *et al.* (1990) reported these enzymes to be located in specialized tissue such as leaf epidermis and in stylar tissues reported by Lotan *et al.* (1989).

Accumulation of β-1,3-glucanases was found in the vacuoles of lower epidermal cells, parenchyma cells adjacent to vascular bundles, and over the middle

lamella in the intercellular spaces as induced by ethylene in bean leaves (Mauch and Stachelin, 1989; Mauch *et al.*, 1992).

Hu and Rikenberg (1998) recovered the β-1,3-glucanases from the domains of the cell wall near to plasmalemma, guard cells and secondary thickening of the xylem vessels, intercellular spaces of wheat leaves as well as in the hyphal cytoplasm and cell wall against infection by wheat leaf rust fungus (*Puccinia recondita* f. sp. *tritici*).

Localization of PR-2 proteins predominantly exists in the cell walls and vacuoles of the tomato roots infected with *Fusarium oxysporum* as well as in the cell wall and septa of this fungus (Benhamou *et al.*, 1989).

Liljeroth *et al.* (2005) reported accumulation of PR-2 proteins in barley floral organs such as anthers, pistil tissues, stigmatic hairs and stylar cells where it is developmentally regulated.

2.5.3.2. Classes of β-1,3-glucanases

Characterization of β-1,3-glucanases have been reported from many different plant species (Castresana *et al.*, 1990; Beerheus and Kombrink, 1994). Meins *et al.* (1992) proved the existence of β-1,3-glucanases in multiple structural isoforms differing from one another in size, primary structure, isoelectric point, cellular localization and pattern of regulation.

Kauffmann *et al.* (1987) and Sela-Buurlage *et al.* (1993) further classified β-1,3-glucanases into three structurally distinct classes based upon presence of acidic and basic counterparts governing specific enzymatic and anti-fungal activity.

2.5.3.2.1. Class I β-1,3-glucanases

They are basic isoforms accumulating primarily in vacuoles as induced by microbial attacks and are thought to have positive influence on normal development of healthy plants such as flower development, seed germination and growth (Simmons, 1994). Many researchers have performed *in vitro* assays and demonstrated anti-fungal activity of these basic isoforms against a broad range of plant pathogenic fungus,

either contributing alone or in combination with chitinases in fungicidal activity (Mauch *et al.*, 1988).

2.5.3.2.2. Class II β-1,3-glucanases

These are acidic extracellular proteins further classified into three subgroups, PR-2a, 2b and 2c. SP41a and SP41b are style specific, acidic extracellular proteins (Ori *et al.*, 1990) while PR-2c, GL153 and GL161 are either basic or neutral vacuolar proteins (Ward *et al.*, 1991).

2.5.3.2.3. Class III β-1,3-glucanases

These acidic, extracellular β-1,3-glucanases that are induced after pathogenic attack.

Apart from the above three mentioned classes, an another class (Class IV) of β-1,3-glucanase isoform has been observed that is not involved in plant defense against pathogen invasion (Van Eldik *et al.*, 1998; Thanseem *et al.*, 2003).

2.5.3.3. Mode of action of β-1,3-glucanases in plant defense

Fungal cell walls are chiefly composed of β-1,3-glucans (Wessels and Sietsma, 1981). The β-1,3-glucanases are believed to act upon the β-1,3-glucan component of cell wall of the pathogenic fungus to release oligosaccharides (Mauch and Staehelin, 1989). The β-1,3-D-glucosidic linkages in β-1,3-glucans are hydrolyzed by β-1,3-glucanases leading to cleavage of these linkages thus releasing oligosaccharide fragments that might act as elicitors for initiation of hypersensitive response against the pathogen (Leubner-Metzger and Meins, 1999).

Hwang *et al.* (1989) observed that, pepper plants inoculated with *Phytophthora capsici*, the fungus grew into the intercellular spaces that further came in contact with the β-1,3-glucanases and chitinases that were probably located in the middle lamella. When the outer cell wall of the fungus came in contact with β-1,3-glucanases, triggered a release of oligosaccharides from the fungal cell wall that further acted as elicitors for production of fungicidal phytoalexins (Kim and Hwang, 1994).

2.6. ASSAY TO DETECT β-1,3-GLUCANASE ACTIVITY

The β-1,3-glucanases hydrolyze β-1,3-glucans by two different mechanisms of action. The glucosyl residue of the nonreducing terminal of the glucan chain is hydrolyzed by Exo-β-1,3-glucanase (EC 3.2.1.58) resulting in release of glucose molecule (Kulminskaya *et al.*, 2001; Bara *et al.*, 2003; Fukuda *et al.*, 2008). Endo-β-1,3-glucanase (EC 3.2.1.39) hydrolyzes the glucan chain randomly and releases small oligosaccharide fragments (Erele and Teather, 1991; Nobe *et al.*, 2003; Yang *et al.*, 2008). Most of the Endo-β-1,3-glucanases hydrolyse all the β-1,3-D-glucosidic linkages in the glucan chain to release large amount of glucose molecules as by-product.

Laminarin (otherwise named as Laminaran) is a complex carbohydrate food reserve of all brown algae and was first reported in *Laminaria* sp. The same reserve is also stored in the sinks as alginates and fucoidans. Mayer *et al.* (1987) observed that, during the fast growth period in the spring, laminarin was absent in the fronds of algae but during autumn and winters, laminarin was representing up to 35 per cent of the dry weight of the fronds. Laminarin is a glucan existing in two different forms, the cold form is water soluble and the other is water insoluble. Laminarin from *Laminaria digitata* is a neutral polysaccharide with 20-30 glucosyl residues linked together *via.* β-1,3-D-glucosidic linkages in the linear chain and cross-linked *via.* β-1,6-glucosidic linkages in the branched chain (Kim *et al.*, 2006). Two types of terminal units are present in laminarin *i.e.* one mannitol unit and the other reducing glucosyl unit present in 3 : 1 ratio (Read *et al.*, 1996).

During *in vitro* β-1,3-glucanase assays using laminarin-dinitrosalicylic acid method (Pan *et al.*, 1991), laminarin is used as a common substrate (glucan source) to estimate β-1,3-glucanase activity in the test biological samples. This enzyme hydrolyzes the β-1,3-D-glucosidic linkages in the laminarin polymer thus releasing free glucose molecules in the solution. More the enzyme activity, more is the quantity of free glucose units produced. The amount of reducing sugar produced can be precisely and efficiently detected spectrophotometrically after reaction with 3,5-Dinitrosalicylic acid (DNS) reagent (Silva *et al.*, 2003). Dinitrosalicylic acid reagent developed by Sumner *et al.* (1921) reacts with the reducing sugar glucose and get

reduced to 3-amino-5-nitrosalicylic acid. The aldehyde group in glucose is oxidized to carboxyl group (Hostettler *et al.*, 1951). The resulting reaction (yellow-coloured solution) when stopped by boiling in water-bath for 10-15 minutes, results in change in colour of solution to reddish-brown the intensity of which can be measured using spectrophotometer set at 500 nm and the optical density (O.D) values obtained can be used to estimate β-1,3-glucanase activity.

Dagade (1999) performed β-1,3-glucanase assay in susceptible variety Panniyur-1, tolerant 'Kalluvally' and resistant *Piper colubrinum*. Results revealed maximum percentage increase in Panniyur-1 (60 %) than compared to Kalluvally and *Piper colubrinum*. Maximum β-1,3-glucanase activity was recorded in the leaves, whereas decrease in β-1,3-glucanase activity was recorded in the stem.

Nazeem *et al.* (2008) used laminarin-dinitrosalicylic acid method to detect β-1,3-glucanase activity in the leaves of three month old rooted cuttings of susceptible *Piper nigrum* variety Panniyur-1, tolerant 'Kalluvally' and resistant wild species *Piper colubrinum* when challenged with *Phytophthora capsici*. Enzyme bioassay results showed highest enzyme activity in resistant wild species *Piper colubrinum* at all times. Tolerant 'Kalluvally' showed an elevated β-1,3-glucanase activity at 3-5 days after inoculation (1.70 µmol / mg protein / 10 minutes) compared to susceptible variety Panniyur-1 (0.262 µmol / mg protein / 10 minutes).

2.7. PROTEOME PROFILING

Every organism is a result of complex regulation and expression of different proteins that determine a particular phenotype or a trait. The environmental signals or stress conditions bring about vast transformations in the proteome of an organism that alter the expression pattern of a particular trait in order to respond or counteract the effect of external conditions. For every organism or a cell, there exists a single genome coding for infinite number of proteins. The entire protein complement of a genome is known as proteome (Wasinger *et al.*, 1995). Hence, a comprehensive study of entire proteome of an organism under a defined condition will provide more precise information about proteins encoded by the genome, the quantitative details regarding level of expression of proteins, details of differentially expressed proteins compared to

the proteome profile of control organism, occurrence of post-translational modifications and distribution of specific proteins in the cell (Anderson and Anderson, 1998). Giorgianni (2003) stated that, proteomics is an essential aspect in biological science to thoroughly comprehend varied biological systems.

The lack of whole genome sequence in black pepper is the most challenging objective for biotechnology research. The advances in proteomic technologies have simplified the study of different protein candidates playing role as direct executors of physiological and biochemical reactions and has mobilized the researchers to explore more about this crop.

2.7.1. Protein sample preparation

Black pepper is a crop rich in essential oils, oleoresin, piperin and polyphenol profiles. The protein isolation and sample preparation from such crops with higher fractions of secondary metabolites becomes a great challenge for researchers. In quantitative proteomics, desire for well resolved proteins and reproducible data, demands efficient sample preparation protocol. Carpentier *et al.* (2005) observed that, presence of proteases and interfering compounds such as phenols, carbohydrates and lipids, affect the protein yield and reproducibility of results. In tropical plant species, the greater part of cell mass is occupied by cell wall and vacuoles and least part from cytosol. Islam *et al.* (2004) studied that, bacterial and animal cells contain higher protein content than compared to plant tissues as cytosolic part is more. Hence, standardization of an effective protein isolation protocol in plant specimens becomes utmost requirement before further analyses, as plant species and its tissues vary from one another in amount and types of non-protein interfering compounds present (Shaw and Riederer, 2003; Gorg *et al.*, 2004; Carpentier *et al.*, 2005).

Umadevi and Anandaraj (2015) isolated total leaf protein from *Piper nigrum* using five different extraction methods (Method I- Modified TCA / Acetone method (Damerval *et al.*, 1986) with some modifications; Method II- Dense SDS / Phenol method (Wang *et al.*, 2003); Method III- PVP / TCA acetone method (Shen *et al.*, 2002) with some modifications; Method IV- Phenol method (Hurkman and Tanaka, 1986) with slight modification; Method V- Lysis buffer extraction (O'Farrell, 1975)

with some modifications). Of the different methods evaluated, modified lysis buffer method and phenol method yielded good quality protein required for SDS-PAGE and Two-dimensional gel electrophoretic analysis. Among the two methods, the phenol method was time consuming and hazardous as it involved use of toxic phenol. They concluded that, modified lysis buffer method of protein isolation was found superior, rapid and yielded protein with negligible polyphenol contamination.

The lysis buffer converts all proteins into individual conformations, prevent formation of protein aggregates, prevent protein oxidation, dissolve hydrophobic proteins in the solution, inactivate proteases and cleave disulphide and hydrogen bonds. The buffer is composed of a concentrated urea medium (urea and a stronger denaturing chaotrope like thiourea) that facilitates conversion of proteins into single conformation, dissolve hydrophobic proteins and avoid protein-protein interactions. The use of zwitterionic or non-ionic detergents (CHAPS, Triton X-100 or NP-40) increases the solubility of hydrophobic proteins. The use of DTT / DTE prevents protein oxidation and related oxidative damages. The use of IPG buffers and carrier ampholytes enhance solubility of differentially charged proteins from the mixture into the solution. Use of protease inhibitors while tissue homogenization such as phenylmethylsulphonyl fluoride (PMSF) or broad-range protease inhibitor cocktail prevents protein degradation by the proteases and enhance recovery of proteins.

2.7.2. Separation of proteins by SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is the commonly used analytical method to resolve different components of a protein mixture and allows qualitative estimation of a given protein sample. This technique developed by Laemmli (1970) is a powerful tool to estimate different molecular weight proteins in a protein mixture (Weber *et al.*, 1971; Chambach and Rodbard, 1971) based upon their difference in electrophoretic mobility through a resolving polyacrylamide gel matrix (Scopes, 1994). The use of discontinuous buffer systems (Tris-HCL / Tris-glycine and pH 6.8 / 8.3, respectively) facilitated loading of larger volumes of treated protein samples while maintaining good resolution of sample components because proteins are focused as thin bands before entering the resolving gel (Ornstein, 1964; Davis, 1964). Use of sodium dodecyl sulphate (SDS) and

reducing agents cause protein denaturation that facilitates better separation of proteins (Shapiro, 1969). SDS being an anionic detergent binds strongly with amino acids of the protein at an approximate ratio of one dodecyl sulphate molecule per two amino acid residues thus imparting net negative charge to the proteins (Reynolds and Tanford, 1970) and facilitating electrophoretic separation in the resolving gel. The use of tris-glycine-SDS (Laemmli, 1970), tris-borate (Neville, 1974) and tris-tricine (Shagger, 1987) electrode buffer systems have improved the resolving power.

The use of two types of polyacrylamide gel system, upper one-third stacking gel (pH 6.8) and the lower two-third resolving gel (pH 8.8) in SDS-PAGE provides scope for efficient resolution of protein bands. The initial electrophoresis of proteins through the stacking gel allows passing of all the protein fractions from the mixture through the gel and linearize the protein movement before they enter into the resolving gel where the actual separation of proteins take place.

Nazeem *et al.* (2008) performed SDS-PAGE analysis with the total leaf protein isolated from the leaves of susceptible variety 'Panniyur-1', relatively tolerant 'Kalluvally' and resistant wild species *Piper colubrinum* inoculated with *Phytophthora capsici*. Analysis revealed that two polypeptides of 8 kDa and 16.5 kDa corresponding to β-1,3-glucanase were expressed on the second day after inoculation in Kalluvally and on the fifth day in Panniyur-1. Unique protein bands of 14.3, 8.8 and 7.0 kDa were expressed in Kalluvally whereas varietal specific proteins of 90 kDa and 5.5 kDa were expressed in Panniyur-1.

2.7.3. Proteome profiling by two-dimensional gel electrophoresis

The proteome of plant cells is extremely complex, comprising of thousands of proteins expressed at a time. Hence, separation of protein fractions only based upon molecular weight might not give a relevant protein profile. In an SDS-PAGE resolving gel, a single protein band might correspond to numerous proteins of similar molecular weight. With the introduction of modern proteome profiling methodology of two-dimensional gel electrophoresis (2DE or 2D gel electrophoresis) by O'Farrell (1975), has revolutionized the proteomic research. 2D gel electrophoresis has been adopted by many proteomic researchers around the globe because of its enhanced resolution,

advantage of storing proteins in the gel until further analyses and is unrivalled by any other alternative technique.

Two-dimensional gel electrophoresis independently separates proteins based upon two parameters *i.e.* isoelectric point (pI) in first dimension gel electrophoresis or isoelectric focusing and molecular mass (M_r) in the second dimensional gel electrophoresis or SDS-PAGE (O'Farrell, 1975). Maximum upto 10000 proteins can be resolved simultaneously with an average of 2000 proteins routinely and protein spots of one nanogram can be detected and quantified.

2.7.3.1. First dimensional gel electrophoresis (Isoelectric focusing)

Proteins in the mixture have amphoteric behavior containing varied proportions of acidic and basic groups that makes each protein different from others based on net charge on them. Net charge of a protein is sum total of all negative or positive charges of the amino acid side chains. These proteins can become protonated or deprotonated depending upon pH environment. The acidic groups become negatively charged in basic environment and the basic groups become positively charged in the acidic environment. In presence of electric field, the proteins migrate towards respective electrodes of opposite sign of its net charge. At a point, when the proteins reach their respective isoelectric points, the net charge on them becomes zero and they become immobilized. This principle is used to separate proteins from mixtures based on isoelectric point in first dimensional gel electrophoresis.

Earlier, first dimensional gel electrophoresis was done in thin polyacrylamide gel rod in glass or plastic tubes containing urea, detergent, reducing agent and carrier ampholytes to form pH gradient in the electric field. There were disadvantages using gel rods for isoelectric focusing as they required great experimental skills to handle and the focusing patterns were not reproducible enough. Hence, modern methods have replaced gel rods with immobilized pH gradient (IPG) strips with a broad pH gradient (3-10, 4-7) that can focus both acidic and basic proteins in a single gel. The advantages of using IPG strips are that it offers greater reproducibility and allows loading of greater amount of protein sample.

Length of the IPG strip used, purity of the protein sample loaded, IEF voltage parameters applied and the temperature used determine the successful IEF run.

2.7.3.2. Second dimensional gel electrophoresis (SDS-PAGE)

The second dimensional electrophoretic run is similar to basic SDS-PAGE protocol, but here the difference is that only resolving gel is used for protein separation and the IPG strip containing the focused proteins is directly placed in contact at top of the resolving gel. Due to presence of SDS, all the proteins separated by isoelectric focusing get masked with net negative charge imparted by SDS hence here the separation of proteins in the electric field is purely based on molecular weight. Proteins with higher molecular weights migrate to smaller distances in the resolving gel whereas the low molecular weight proteins migrate to larger distances. The critical difference that exists here is that, here in the resolving gel all the proteins appear as individual distinct spots whereas in basic SDS-PAGE protocol, the separated proteins appear as thin protein bands with corresponding molecular weight.

2.7.4. Visualization of protein profile in the resolving gel

Visualization of the resolved proteins in the gel is by three methods: Coomassie Brilliant Blue (CBB) staining, Silver staining and Fluorescent staining with SYPRO dyes.

Coomassie Brilliant Blue staining is the widely adopted procedure to visualize protein profile as it is reproducible, gives clear background, reasonable sensitivity (30 ng per band), cheaper and has excellent compatibility with mass spectrometry (Candiano *et al.*, 2004). The principle of this staining is the strong affinity of CBB dye with the proteins. So when the entire gel is destained, the background becomes transparent except for the protein bands or spots that remain stained purple or violet.

Silver staining is the most sensitive, non-radioactive protein visualization method that can detect proteins at nanogram level (Yan *et al.*, 2000; Candiano *et al.*, 2004). Adoption of this staining procedure is quite limited as it involves laborious multiple steps, high background and not compatible with mass spectrometry (Candiano *et al.*, 2004).

The use of SYPRO family dyes either alone or combined with other fluorescent dyes are incorporated in staining procedures due to their convenience and high sensitivity (Steinberg *et al.*, 1996; Lopez *et al.*, 2000; Patton, 2000; Steinberg, 2000). But due to rapid quenching and highly expensive affair, the uses of SYPRO family dyes are limited. Also it demands use of sophisticated instruments and software to visualize the protein profile (Patton, 2000).

2.7.5 Significance of 2DE in assessment of PR-proteins

Yamchi (2017) studied variation in proteome of model legume *Medicago truncatula* in response to infection by *Ralstonia solanacearum*. The study was conducted in susceptible and resistant recombinant inbred lines (RILs). Total protein isolated from the roots and aerial parts of artificially challenged plants were subjected to 2DE analysis. Root proteome analysis revealed expression of two putative isoforms of cyclophilin (spot-2 and -3) and Kunitz proteinase inhibitor 20 (spot-1) in the resistant RILs. Four protein spots showed specific changes in resistant RILs. At one day post infection (dpi), a protein spot identified as PR-10 protein (spot-7) was expressed and another spot was identified as cold-stress responsive protein (spot-8). Increased expression of peroxidase (spot-9 and PR-5 proteins (spot-10) was recorded at 3 dpi in resistant RILs. Proteome analysis with aerial parts revealed increased expression of 14-3-3-like protein (spot-12) at one dpi in susceptible and resistant plants. Expression of a putative ankyrin-repeat protein (spot-13) was found to increase at 3 dpi in inoculated resistant plants.

Zhang *et al.* (2017) performed proteome analysis with roots of watermelon seedlings infected with *Fusarium oxysporum*. 2D gel electrophoresis results revealed expression of 690 spots in the gel out of which 32 showed significant changes in expression. Among 32 spots, ten protein spots were involved in metabolism, out of which vacuolar H^+-ATPase catalytic subunit A (spot s1), pyruvate dehydrogenase E1 component subunit beta (spot s29) and phosphoglycerate kinase (spot s29) regulated energy metabolism pathway. Three proteins, succinate dehydrogenase flavoprotein subunit (spot s2), isocitrate dehydrogenase (spots s22) and NADP-dependent malic enzyme (spot s21) participated in carbohydrate metabolism. About 11 spots corresponded to the stress and defense related proteins that included heat shock protein

(spot s4) and two peroxidases (spots s11 and s31); two jasmonate induced proteins NAD(P)H dehydrogenase (quinine) FQR1-like 2 (spot s15) and chaperonin CPN60-2 (spot s5). Seven protein spots regulated amino acid biosynthesis and corresponded to 3 S-adenosylmethionine synthases (spots s7, s8 and s9). Gibberellin receptor GID1L2 (spot s14) involved in signal transduction and tyrosine-protein kinase (spot s24) were down-regulated after *F. oxysporum* infection.

2.8. PROTEIN IDENTIFICATION AND CHARACTERIZATION

2.8.1. Edman degradation

Edman degradation method or N-terminal sequencing method for proteins was developed by Edman (1949). In this method, the chemical such as phenyl isothiocyanate is used for degradation of labeled peptide into individual amino acid entity starting from the N-terminal of the peptide chain typically up to 20 amino acids in a cycle dependent manner (Graves and Haystead, 2002). The derivative of the terminal amino acid is cleaved as thiazolinone derivative under acidic condition which is selectively extracted with an organic solvent. Treatment with acid forms phenylthiohydanthion (PTH)-amino acid derivative that is identified by subjecting it to chromatography or electrophoresis. The peptide length sequenced at a time is limited due to cyclical derivatization. The protein identification through this method is extremely laborious, tedious and not applicable for proteins having non-α-amino acid (*eg.* isoaspartic acid) residue in the peptide chain and with chemically modified N-terminal region. Due to availability and development of protein and DNA databases such UniProt, PDB, PIR, DDBJ, EMBL, GenBank, *etc.* this method has become obsolete.

2.8.2. Matrix assisted Laser-Desorption-Ionization Time-of-Flight / Mass Spectrometry (MALDI-TOF/MS)

MALDI-TOF/MS method developed by Hillenkamp and Karas *et al.* (1985) is precise and rapid method of peptide identification based upon peptide mass and time of flight values. In this method, the trypsin digested peptides are converted from solid phase to gaseous phase and this process is competitive. The tryptic peptide sample mix is combined with matrix compound in a solution. The matrix compound used here is

capable of absorbing UV-spectrum (Karas and Hillenkamp, 1988). The matrix is composed of small energy absorbing molecules such as 2,5-dihydroxy benzoic acid or α-cyano-4-hydroxycinnamic acid and sinapinic acid. The above tryptic peptide-matrix mixture is mixed with appropriate solvent and spotted on the sample probe of the metal plate or MALDI plate. The solvent is allowed to evaporate resulting in crystal formation. The metal plate is placed in MS at high vacuum. A laser beam of selected wavelength is targeted to the spotted area on the plate. Matrix molecules get ionized after absorbing photons from the incident laser beam. These molecules transfer excess energy to the peptides in the sample thus transforming it to gaseous phase. Each peptide is charged by a single photon thus peptide ions are singly charged (Graves and Haystead, 2002). Application of high voltage (+20 V to +30 V) on the MALDI plate induce acceleration of positively charged peptides towards the orifice of the flight tube. As the ionized peptides enter the flight tube, they traverse along the length of the tube maintained under high vacuum inside and finally hit the detector. The ionized peptides in MALDI are equally charged hence they traverse with different velocities. The time of flight of an ionic peptide is inversely proportional to its mass hence the ionic peptides hit the detector at different time intervals (Rodwell and Barnes, 2000). To enhance the resolution, a reflector device is placed in the path of ionic peptides that reflect the striking ions at greater intensities (Kaufmann *et al.*, 1996).

The TOF unit measures the mass / charge ratio (m/z value) of each ionic peptide by determining the time required by each ionic peptide for traversing from orifice of the flight tube to the detector (Graves and Haystead, 2002). The m/z values of all the peptides obtained from MALDI-TOF peak data plot is used as input for protein identification by loading it into a database for further analysis using bioinformatics tools (Eng *et al.*, 1994; Quadroni and James, 1999).

2.8.3. Peptide mass fingerprinting database search for protein identification

Peptide mass fingerprinting refers to protein identification using *in silico* tools and the analyses and database searches are fully automated (Mann *et al.*, 1993; Mann and Wilm, 1994). In this method, mass of each peptide obtained from the MALDI-TOF analysis of an unknown protein sample is matched against the theoretical or predicted mass of peptide obtained by *in silico* digestion of all protein amino acid

sequences in the database using same protease of particular restriction site as used for digesting unknown protein sample (Pappin *et al.*, 1993; Jensen *et al.*, 1997). If the predicted mass hit from the database matches with the unknown peptide mass, then protein identification can be easily done, as the protein corresponding to the predicted mass hit from the database will be the identity of unknown protein. The database is a repository of proteins, positioned based upon their peptide masses matching their sequence within a given mass error tolerance. The online tools used for peptide mass fingerprinting are PepSea (Mann *et al.*, 1993), MS-Fit (Clauser *et al.*, 1999); PeptIdent / MultiIdent (Wilkins *et al.*, 1999) and ProFound (Zhang and Chait, 2000). MASCOT server on the website of 'Matrix Science' developed by Perkins *et al.* (1999) is a widely used search engine used for peptide mass fingerprinting using mass spec data. Rapid processing and completely free automation are the two biggest advantages associated with MASCOT server.

Rep *et al.* (2002) performed mass spectrometric identification of isoforms of detected PR proteins using MALDI-TOF in the xylem sap of tomato plants infected with vascular wilt fungus *Fusarium oxysporum*. Results revealed accumulation of PR-1 isoforms (PR-1a, PR-1b), β-1,3-glucanase (PR-2a) and thaumatin-like proteins (PR-5x).

Li *et al.* (2014) performed 2D-gel electrophoresis and MALDI-TOF analysis to characterize defense related proteins in apple against a deadly pathogen *Marssonina coronaria*. Proteome analysis by 2D-gel electrophoresis with total protein isolated from the infected leaves at 3 to 6 days post-infection revealed accumulation of 59 differentially expressed proteins in inoculated sample as compared to non-inoculated control. Identification by MALDI-TOF and alignment of 35 different proteins revealed their roles in photosynthesis, transport, energy metabolism, amino acid synthesis, carbohydrate metabolism, binding, antioxidant, defense and stress. PR proteins identified to be expressed against *M. coronaria* infection were class III endo-chitinase, β-1,3-glucanase and thaumatin-like proteins.

Mahadevan *et al.* (2016) attempted an integrated transcriptome-assisted label-free quantitative proteomics approach to study the underlying immune components present in black pepper involved in interaction against fungal pathogen *Phytophthora*

capsici infection. They had adopted an improved protein isolation strategy to isolate total protein form the infected leaves which was digested using trypsin before the tryptic peptides were used for mass spec analysis using nanoUPLC-MS approach for peptide identification. The generated peptide mass data were analyzed using Protein Lynx Global SERVER v2.5.3 (PLGS, Waters) for peptide identification and label-free quantitative proteomics analysis. The raw data obtained from nanoUPLC-MS/MS analysis of all peptide fractions from un-inoculated leaf sample (mock control) and inoculated leaf sample (test sample) were processed in assistance with transcriptome derived black pepper protein database and merged it as a single file. About 151,189 peptides were identified in the label-free proteome profile with individual PLGS score over 20 out of which 5870 were unique peptides of which about 532 peptides corresponded to the novel protein hits. Out of 532 proteins about 194 proteins showed significant differences compared to test sample. About 134 proteins were significantly down-regulated and at least 22 proteins were significantly up-regulated.

The first limitation of this technique is ambiguity in protein identification *i.e.* in a peptide of ten amino acids, mass will remain unchanged if its constitutive amino acids are reorganized (Mirza and Olivier, 2008). Second limitation is that, this technique is only effective for analyzing proteins isolated from those organisms whose genome size is smaller and completely sequenced before (Qin *et al.*, 1997). Thirdly, the use of this technique for identifying an unknown protein that is extensively modified by post-translational modifications will reduce the accuracy of results as due to non-matching of peptides produced with the unmodified proteins in the database.

2.8.4. Characterization of proteins using BLAST2GO tool

The tool "BLAST2GO" developed by Conesa *et al.* (2005) is a comprehensive bioinformatics tool for the purpose to functionally annotate and analyze genome scale sequence and protein sequence datasets. It is user-friendly and single window interface for gene ontology (GO) (Gene ontology consortium, 2008) and annotation (Gotz *et al.*, 2008). Additionally a wide collection of graphical, analytical and statistical tools is offered by BLAST2GO tool for data manipulation and mining.

The key steps involved in BLAST2GO analysis are: BLASTing, Mapping, Annotation, Statistical analysis and Visualization of data. Basically the tool finds similar sequences to one or many query sequences (both nucleotide and amino acid sequences) by using local or remote BLAST searches. GO terms are extracted associated with each BLAST hits by the program and returns an evaluated GO annotation for the query sequences. Mapping analysis derives associated enzyme codes (EC) from the equivalent GOs. The InterProScan web service characterizes the query sequences by scanning the sequences for the presence of kind of InterPro motifs and domains and also assign name of corresponding gene or protein family. The GO annotation results can be visualized by reconstructing the structure of GO relationships. The EC codes associated help to derive corresponding KEGG pathway maps from the database in KEGG analysis.

The advantages associated with the tool are simplicity in usage, minimal set up requirements, automatic updates, visual-oriented information display and minimal requirement of computational background.

Mahadevan *et al.* (2016) did functional annotation of 532 protein sequences with BLAST2GO against *Viridiplantae* database and *Arabidopsis thaliana* TAIR10 protein database revealed that the proteins were involved in at least 49 biochemical pathways involved directly or indirectly in plant defense response against *P. capsici* infection. Majority of the proteins were found to be regulating various pathways such as glyoxylate and dicarboxylate mechanism, carbon-fixation in photosynthetic organisms, glycolysis / gluconeogenesis pathways, amino acid metabolism (Cysteine, methionine, glycine, serine and threonine), methane metabolism, phenylpropanoid biosynthesis, fructose and mannose metabolism, purine metabolism, glutathione metabolism and pentose phosphate pathway. The GO analysis results revealed 52 biological process terms associated with 532 proteins of which 13 per cent of the sequences were involved in stress response. The results of proteome study were validated with real-time qRT-PCR analysis and they derived complete coding sequences of all the proteins.

Materials and methods

3. MATERIALS AND METHODS

An intensive study entitled "Characterization of PR proteins in selected calliclones of black pepper in relation to *Phytophthora* foot rot disease" was embarked at Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture (CoH), Vellanikkara, Department of Plant Pathology, College of Horticulture, Vellanikkara and Distributed Information Centre, College of Horticulture, Vellanikkara during March 2016 to August 2017.

3.1. MATERIALS

3.1.1. Plant materials

Eleven black pepper calliclones showing better performance as reported by Shylaja *et al.* (2012) formed the experimental material for the study which included six calliclones of Cheriakanyakkadan (CKCC 5, CKCC 41, CKCC 60, CKCC 61, CKCC 25 and CKCC 27) and five calliclones of Kalluvally (KLCC 119, KLCC 89, KLCC 133, KLCC 134 and KLCC 86). The variety Panniyur-1 susceptible to *Phytophthora* foot rot was the check used in the study. Mother plants of calliclones and Panniyur-1 were maintained in CPBMB nursery.

3.1.2. Chemicals, glass wares and plastic wares

The chemicals utilized for the study were of good quality (Extrapure grade) procured from different manufacturing firms like Sigma Aldrich, USA; Sisco Research laboratories (SRL), India; Himedia, India and Merck, Germany. Plastic wares and glass wares used were obtained from Borosil and Tarsons India Ltd.

3.1.3. Equipment facilities

Total protein profiling, two dimensional gel electrophoresis, imaging and documentation of protein were done by utilizing the already established instrumentation facility at CPBMB. The facility for performing MALDI-TOF / MS analysis with selected protein spots was accomplished at Rajiv Gandhi Centre for Biotechnology,

Thiruvananthapuram, Kerala. Further *in silico* analyses with raw peptide sequences were done at the Distributed Information Centre (DIC), CPBMB.

The details regarding procedures and protocols followed during the course of work are elaborately projected in this chapter.

3.2. METHODS

3.2.1. Raising of rooted cuttings

Runners were collected during March 2016 from the mother plants of all the selected calliclones and Panniyur-1 maintained at departmental nursery of CPBMB. The runners were cut into two noded cuttings and were dipped in 1000 ppm solution of Indole-3-butyric acid (IBA) for 45 seconds for early and better rooting. The treated cuttings were shade dried and planted directly in polybags (2-3 cuttings per bag) containing soil, sand and cow dung in the ratio 1:1:1. The cuttings were mulched after irrigation and were maintained in the nursery. The cuttings were kept mulched until sprouting. Irrigation was provided daily during morning and evening hours. No chemical pesticides or fungicides were applied as it will interfere with disease progression upon challenging with *Phytophthora capsici* and might tamper with the accuracy of results during further analyses.

The observations on date of sprouting, number of cuttings sprouted per calliclone and establishment percentage were recorded for all the calliclones. Three month old rooted cuttings were used for challenge inoculation with *Phytophthora capsici*.

3.2.2. Isolation of *Phytophthora capsici* and studying the disease reaction

3.2.2.1. Growing medium for P. capsici

For the current study, carrot agar medium was selected for isolation, multiplication and subculturing the fungus. The method of preparation of carrot agar medium is given in Annexure-I.

3.2.2.2. Isolation of P. capsici from infected leaf sample

Infected leaf samples were collected from a black pepper nursery during the month of June 2016 when the incidence of *Phytophthora* foot rot disease was at its peak. The infected leaves with round dark brown to black water soaked lesions with fimbricate margins were selected for isolation of *P. capsici* (Anandaraj *et al.*, 1994). The infected leaves were brought to the lab and rinsed with mild soapy water and then with running tap water to remove the adhering dust particles. The leaves were then dried by gentle pressing in between folds of blotting paper. With the help of a sterile blade, small square bits (approx. 5 mm) were cut out from the leaves such that each bit contained half healthy and half infected region. The excised leaf bits were placed in a Petri plate containing 10-15 ml of sterilized distilled water and kept at 25°C for 24 hours to induce sporangial formation. The leaf bits were observed under the 100X magnification of the microscope for the formation of lemon-shaped sporangia and only after ensuring the sporangial formation, the leaf bits were further inoculated into carrot agar medium. Care was taken not to keep the leaf bits for too long in water otherwise the sporangium might release all the zoospores before inoculation.

For inoculation into carrot agar medium, the leaf bits were first surface sterilized by dipping it in one per cent sodium hypochlorite solution followed by washing with three changes of sterile distilled water. The leaf bits were blot dried on sterile blotting paper to remove all the traces of water. With the help of flame sterilized forceps, the leaf bits were transferred over the solidified carrot agar medium in Petri plates (3 leaf bits per Petri plate) containing streptomycin sulphate @ 200 mg L^{-1}. The inoculated plates were placed in air conditioned room maintained at 25°C, kept inside a bell jar for the mycelial growth. The plates were observed daily for mycelial growth of the pathogen. A dense white mat of mycelial growth developed over the media within 24 hours of inoculation.

3.2.2.3. Purification of the culture by hyphal tip method and maintenance

The pure culture of *P. capsici* was made by repeated subculturing of the hyphal tip into fresh carrot agar medium. Once the mother culture plate grew one day old, the tip of

mycelial growth along with media was excised out using flame sterilized inoculation needle and subcultured into Petri plate containing fresh carrot agar medium with antibiotic. The inoculated plates were sealed with parafilm tape and placed in air conditioned room maintained at 25°C, kept inside a bell jar for the mycelial growth. The subculturing was done repeatedly until the pure culture of *P. capsici* was obtained.

It was primary requisite for maintaining the pure culture of *P. capsici* in order to preserve for long without other microbial contamination, to maintain virulence and viability of the pathogen and in order to perform future inoculations. The culture was maintained in test tube slants containing carrot agar medium. From the pure culture, a small mycelial bit along with the media was excised out and transferred to test tube slants containing medium with the help of flame sterilized inoculation needle. The slants were kept in air conditioned room maintained at 25°C, kept inside a bell jar for the mycelial growth. After 5-7 days when the medium is completely covered by the fungal mycelial mat, the slants were transferred to refrigerator and maintained at 4°C. After every 15 days, this process of subculturing into fresh slants and culture maintenance was repeated.

3.2.2.4. Challenge inoculation with Phytophthora capsici

The selected black pepper calliclones along with susceptible variety Panniyur-1 were challenge inoculated with *P. capsici*. Detached leaves and three months old rooted cuttings were selected for inoculation. The net house of Department of Plant Pathology was used for pathogen inoculation studies.

3.2.2.4.1. Preparation of seven day old culture for leaf inoculation

From the already maintained pure culture in test tube slants, subculturing was done to Petri plates containing carrot agar medium and antibiotic. The inoculated plates were kept in air conditioned room maintained at 25°C kept inside a bell jar for seven days. When the culture became seven day old, a dense white mycelial mat of *P. capsici* covered the entire surface of plated media. This seven day old culture of *P. capsici* was used for preparation of 5 mm mycelial discs for inoculation.

3.2.2.4.2. Preparation of 5 mm diameter mycelial discs for inoculation

With the help of flame sterilized 5mm diameter cork borer, mycelial discs were excised out and transferred to a Petri plate containing 10-15 ml of sterile distilled water for sporangial development. After 24 hours, the discs were observed under 100X magnification of microscope for the development of lemon-shaped sporangia. Once it was certained for the sporangial formation, the mycelial discs were used for inoculation.

3.2.2.4.3. Inoculation in detached leaves with 5 mm mycelial discs

Leaves from respective calliclones and Panniyur-1 were detached from the mother plant and brought to the lab for inoculation. The leaves were washed gently with soap under running water and then air dried at room temperature. With the help of a sterile needle, pin pricking was done in the abaxial surface of the selected leaves at three locations @ six pin pricks per location. With the help of sterile forceps, mycelial disc was placed over the pricked area and then sterile moist cotton was placed over the disc to hold the disc in position. The inoculated leaves were kept under high relative humidity maintained inside respective labeled polythene covers kept in air conditioned room maintained at 25°C. The inoculated leaves were observed daily at 0, 24, 48 and 72 hours after inoculation for the development of symptoms and lesion diameter was recorded for all the calliclones including Panniyur-1 at 0, 24, 48 and 72 hours after inoculation.

3.2.2.4.4. Leaf inoculation with 5 mm mycelial discs in three-month old rooted cuttings

Three month old rooted cuttings with fully expanded green to dark green leaves were selected for inoculation. Leaves that were not too young (light green) and not too old (lower dark green leaves) *i.e.* second and third leaf from the top were selected for inoculation. Moist cotton was used to swab the abaxial surface of the selected leaves of all cuttings to remove the adhering dust and mud particles. Using sterile forceps, 5 mm mycelial discs were gently transferred and placed at the centre of the abaxial surface of the leaves (one disc per leaf) without giving any mechanical injury to the leaves. Careful attention was taken while inoculation in leaf as any kind of abrasion or mechanical injury

to the leaves might trigger abrupt synthesis of PR proteins and release of phenols at the wounded site that will tamper with the results during further analyses. After placing the mycelial discs, the discs were covered with sterile moist cotton to fix the disc in position and also for giving moist condition for pathogen to induce infection. The inoculated plants were irrigated judiciously. Water was sprinkled inside big polythene covers and used to cover all the inoculated cuttings in order to create high humid conditions required for pathogen invasion and symptom development. Also care was taken to keep the inoculated cuttings in a cool atmospheric condition by placing it in a closed shady location in the nursery and by sprinkling water inside the polythene covers thrice daily. The inoculated leaves at 0, 24, 48 and 72 hours after inoculation, from three month old cuttings were the experimental material for β-1,3-glucanase assay, SDS-PAGE analysis and 2D-gel electrophoresis.

3.2.3. Assay of β-1,3-glucanase activity

The enzyme activity of β-1,3-glucanase at 0, 24, 48 and 72 hours after inoculation in calliclones and variety Panniyur-1 were assayed by laminarin dinitrosalicylic acid method of colourimetric detection as devised by Abeles and Forrence (1970) along with certain refinements suggested by Pan *et al.* (1991).

3.2.3.1. Chemicals required

1. Sodium acetate buffer (0.05 *M*, pH 5.0)
2. Laminarin (4 %)
3. Sodium hydroxide (4.5 %)
4. Dinitrosalicylic acid (DNS)
5. Cystein HCL (0.05 *M*)
6. Phenyl methane sulphonyl fluoride (PMSF) (0.1 *M*)
7. Ascorbic acid (5 m*M*)

The detailed preparation scheme for the reagents required for β-1,3-glucanase assay is given in Annexure-II.

3.2.3.2. Extraction of crude enzyme from inoculated leaves

The inoculated leaf samples were collected in labeled aluminium foils over ice at 0, 24, 48 and 72 hours interval from all the calliclones including susceptible Panniyur-1 and brought to the lab for isolation of crude enzyme. Green leaf tissue surrounding the lesion was taken for isolation. About 0.5 g of this leaf tissue was weighed and transferred to a pre-chilled sterilized pestle and mortar. Then homogenization of the leaf tissue was carried out by crushing the leaf tissue after adding 1 ml of chilled sodium acetate buffer followed by addition of 50 µl each of ascorbic acid, PMSF, β-mercaptoethanol and finally 100 µl of cystein HCL. Care was taken to provide proper chilling conditions althrough the enzyme isolation procedure by carrying out isolation in air conditioned room or cold chamber, by crushing the sample in pestle and mortar kept over the ice, transferring of samples in ice and by periodic changes of ice whenever ice started to melt. The homogenate was centrifuged at 15000 rpm for 15 minutes pre-cooled at 4°C. After centrifugation the supernatant was collected in fresh microfuge tubes over ice and required volume from the resultant supernatant was used directly as enzyme source for β-1,3-glucanase assay.

3.2.3.3. β-1,3-glucanase assay

About 62.5 µl of crude enzyme was pipetted in 5 ml glass tube followed by addition of equal volume of laminarin (4 %). The reaction mixture was incubated in water-bath maintained at 40°C for 15 minutes to facilitate enzyme-substrate reaction. About 375 µl of DNS was added to each glass tube and then boiled in water bath for seven minutes at 100°C to stop the reaction. The coloured solution obtained was diluted with distilled water (4 ml) and the absorbance of the solution was measured by using spectrophotometer at 500 nm. The blank used was the heat killed enzyme (heat denaturing the enzyme by boiling in water bath at 100°C) added with equal volume of laminarin (4 %), 375 µL of DNS and distilled water (4 ml). The optical density (O.D) values obtained were used as input for calculating enzyme activity. From a standard curve plotted (straight line plot) with known glucose concentrations (µmol) on X-axis and corresponding O.D

values on the Y-axis, the values of unknown glucose concentrations (µmol) were extrapolated. This way gave the values of quantity of reducing sugar (µmol) produced during 15 minutes of enzyme-substrate reaction at 40°C.

The specific activity of enzyme was defined as the amount of enzyme required to produce reducing sugar equivalent per 15 minutes at 40°C per quantity of total protein in milligrams (mg).

The β-1,3-glucanase assay was performed immediately after isolation of crude enzyme as the enzyme will be most active during initial few hours after isolation and looses its activity as the storage time is prolonged.

3.2.3.4. Quantification of total protein (by Lowry's method)

Quantification of the total protein (mg ml^{-1}) in the enzyme extract was done by the protocol devised by Lowry *et al.* (1951).

3.2.3.4.1. Precipitation of total protein

The total protein from the enzyme extract was precipitated by trichloroacetic acid (TCA) method of protein precipitation. About 1.5 volume of freshly prepared 10 per cent TCA containing 0.07 per cent of 2-mercaptoethanol in ice cold acetone was added to the enzyme extract. The mixture was mixed gently by inversion and then incubated overnight undisturbed at -20°C. The next day the white precipitated protein at the bottom was recovered by centrifugation at 12000 g for 20 minutes at 4°C. Then the supernatant was discarded and the white pellet of protein at the bottom was air dried over blotting paper followed by dissolution of pellet in 50 µl of 0.1 *N* NaOH.

3.2.3.4.2. Protein quantification (by Lowry's method)

The chemicals required were prepared freshly before use.

3.2.3.4.2.1. Chemicals required

1. Bovine serum albumin (BSA) stock solution (1 mg ml^{-1})

2. Sodium carbonate (2 %) solution
3. NaOH solution (0.1 *N*)
4. Copper sulphate (1.56 %) solution
5. Sodium potassium tartarate (2.37 %) solution
6. Folin-Ciocalteau reagent (1 *N*)

The preparation method for the alkaline copper reagent, its composition and BSA stock preparation are detailed in Annexure-III.

3.2.3.4.2.2. Procedure

Different working stocks of 1 ml of BSA solution (0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mg ml^{-1}) were prepared from the master stock by pipetting required volumes into fresh microfuge tubes and diluting it with required volume of 0.1 *N* NaOH solution. Experimental blank was taken as 0.1 ml of 0.1 *N* NaOH solution. From the different working stocks, 0.1 ml of BSA protein of different concentrations were pipetted into separate microfuge tubes and added with 1 ml of alkaline copper sulphate reagent including the blank. The solution was mixed properly and incubated at room temperature for 10 minutes. Then 0.1 ml of Folin-Ciocalteau reagent was added to each tube followed by mixing and then incubated for 30 minutes in room temperature. After 30 minutes, the spectrophotometer was set zero with blank and the intensity of coloured solution obtained was measured by using spectrophotometer at 660 nm. The optical density values obtained for all BSA working standards were plotted on Y-axis with the corresponding BSA concentrations on the X-axis and a standard plot was obtained. Similar reaction was done with precipitated protein samples also and O.D values obtained were used to extrapolate their respective values of protein concentration (mg ml^{-1}) from the standard plot.

3.2.4. Protein profiling by SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) protocol standardized by Laemmli (1970) was attempted in current research to generate a protein profile containing different molecular weight proteins resolved in a

polyacrylamide gel, to study the number and quality of resolved protein bands and to validate the presence or absence of β-1,3-glucanase protein from the total profile. The principle of SDS-PAGE is that the SDS being an anionic detergent imparts net negative charge to all the proteins in the loaded sample, hence since the charge on proteins is negative, the separation of proteins is purely based on molecular weight. Lower molecular weight proteins resolve towards the bottom of the resolving gel and the larger molecular weight proteins resolve at the top of the resolving gel.

3.2.4.1. Chemicals required

1. Monomer solution (30 % acrylamide, 27 % bis-acrylamide)
2. 4X resolving gel buffer (1.5 *M* Tris-Cl, pH 8.8)
3. 4X stacking gel buffer (0.5 *M* Tris-Cl, pH 6.8)
4. Electrode buffer (0.025 *M* Tris, 0.192 *M* glycine, pH 8.3)
5. 2X treatment buffer (0.125 *M* Tris-Cl)
6. Ammonium persulphate (APS) (10 %)
7. Sodium dodecyl sulphate (SDS) (10 %)
8. Tetramethylethylenediamine (TEMED)
9. Fixer solution
10. Coomassie brilliant blue staining solution
11. Destaining solution
12. Prestained protein molecular weight ladder

The details of chemicals used, and method of preparation of solutions used are given in Annexure – IV.

3.2.4.2. SDS-PAGE protocol

3.2.4.2.1. Treatment of protein samples

On the basis of proteins quantified by Lowry's method, calculated volume of protein was taken in microfuge tubes containing about 15-20 µg of total protein. Treatment buffer of about one-third of the volume of protein was added to the protein and

mixed by vortexing. The mixture was boiled in water bath at 100°C for 3-4 minutes for complete denaturation of proteins. This heat denatured protein contained in treatment buffer was used for loading in the gel.

3.2.4.2.2. Components of resolving gel

Components	7.5 per cent gel	10 per cent gel	15 per cent gel
Monomer solution	2.49 ml	3.33 ml	4.99 ml
4X resolving gel buffer	2.5 ml	2.5 ml	2.5 ml
SDS (10 %)	0.1 ml	0.1 ml	0.1 ml
Distilled water	4.85 ml	4 ml	2.36 ml
APS (10 %)	50 µl	50 µl	50 µl
TEMED	10 µl	10 µl	10 µl
Total	10 ml	10 ml	10 ml

3.2.4.2.3. Components of stacking gel (5 %)

Components	5 per cent gel
Monomer solution	1.0 ml
4X stacking gel buffer	0.75 ml
SDS (10 %)	60 µl
Distilled water	4.1 ml
APS (10 %)	60 µl
TEMED	20 µl

3.2.4.2.4. Procedure for SDS-PAGE

The base plate and the bind plate were cleaned thoroughly in running water and then air dried. Both the plates were swiped with cotton soaked in 70 per cent ethanol. The plates were then assembled in the gel casting apparatus. First in a small 25 ml beaker, all the components of resolving gel buffer were added and mixed properly except the APS

and TEMED which were added simultaneously at last. Then with the help of pipette, the resolving gel was poured into the gap of base plate bind plate assembly leaving one-third of the head space. The head space was filled with distilled water to prevent air contact with the gel and dissolve air bubbles if any. The resolving gel was kept undisturbed for it to solidify for 30 minutes. After solidification was complete, the water over the resolving gel was discarded by tilting the stand and getting water absorbed by tissue paper. Then the components of stacking gel were mixed properly in a small 25 ml beaker except the APS and TEMED which were added simultaneously at last. The stacking gel was then poured in the head space completely till the top and comb was placed immediately such that there was 1 cm gap between the bottom of wells formed by the comb and top of the resolving gel. The assembly was kept undisturbed for 30 minutes for the stacking gel to solidify.

After the complete polymerization of the gel, the plate assembly with solidified gel was removed from the casting apparatus and fixed in the gel running gasket vertically. A dummy plate was fixed on the other side of the running gasket. This assembly was tightened properly to eliminate any gaps for preventing the leakage of inner electrode buffer. Then this was placed inside the electrophoresis tank after the comb was gently removed clearly demarcating distinct wells formed. Immediately electrode buffer was poured completely inside the inner tank till the top and some buffer was poured outside the inner tank upto the level marked in the electrophoresis tank.

The protein samples in treatment buffer were loaded into the respective wells with the help of pipette. Also 3 µl of broad range prestained protein molecular weight ladder (11 kDa to 245 kDa range) was loaded into one of the wells to track the movement of proteins while electrophoresis. The lid was placed over the electrophoresis tank and the chords were connected to electrophoresis power pack. The initial running voltage was set at 80 V and the electrophoresis was allowed to run. After first 15 minutes of the electrophoretic run when the samples have travelled through stacking gel completely and reached just above the level of resolving gel, then the voltage was changed to 100 V and kept the same voltage condition until the tracking dye reaches the bottom of the gel plate. To prevent the protein samples from degradation due to heat generation while

electrophoresis, ice packs were kept on both sides of the tank or the electrophoresis was carried out in air-conditioned room maintained at 20°C.

When the tracking dye reached the bottom end of the plate, then the electrophoresis was stopped by switching off the power pack. The chords were unplugged from the power pack and the lid of the electrophoresis tank was opened. The gel running gaskets containing the plates were removed out from the tank and the locks were relaxed. The dummy plate was removed first followed by the base plate bind plate assembly. With the help of gel releasers, the base plate and the bind plate was separated and with the same tool the resolving gel was cut separated from the stacking gel. Care was taken while detaching the gel from the glass plate as there may be chance of breakage of resolving gel if mishandled. Hence if there was fragile gel sticking to one of the plates then it was detached patiently by squeezing with distilled water. The resolving gel was transferred to a staining dish containing distilled water. With mild shaking, the gel was washed in distilled water to remove the adhering electrode buffer. After two washes with distilled water, fixer solution was added into the staining dish containing the resolving gel to fix the proteins in the gel. The fixing step was carried out for 30 minutes over a shaker.

3.2.4.2.5. Coomassie brilliant blue staining of resolving gel

After fixation of proteins in the resolving gel, the fixer solution was drained off and coomassie brilliant blue staining solution was added into the staining dish. The staining of the gel was done overnight by constant shaking over a shaker in order to attain uniform and saturated staining of the gel.

3.2.4.2.6. Destaining of the stained resolving gel

After staining was over, the staining solution was transferred into a glass bottle and destaining solution was added into the staining dish. The destaining was carried out in constant shaking over a shaker. Periodic changes with fresh destaining solution were given when the solution turned dark blue. The destaining was carried out until the background of the gel became completely transparent and colourless and the proteins

were prominent with dark bluish-purple stained bands. Property of coomassie brilliant blue stain is that only proteins hold up the stain and appear as dark bluish-purple bands in the resolving gel even after complete destaining. The destained gel was preserved in distilled water containing few drops of glacial acetic acid at 4°C to prevent fungal contamination in the gel.

3.2.4.2.7. Documentation

The destained gel was documented by scanning the gel using GS-800™ calibrated densitometer that scans the gel with highest resolution and converted it into digital image file required for further image analyses.

3.2.5. Two-dimensional gel electrophoresis of total protein from selected tolerant calliclone and susceptible Panniyur-1

Based upon the above analyses, a tolerant clone and susceptible variety Panniyur-1 were selected in order to carry out an authenticated study about the differentially expressed proteins and novel proteins expressed upon challenging with *Phytophthora capsici*. For this objective, the two-dimensional gel electrophoresis (2DE or 2DGE) was attempted with total leaf protein isolated from inoculated leaves of selected tolerant calliclone and susceptible Panniyur-1 to generate a complete protein profile containing unique protein signatures that were differentially expressed in both tolerant and susceptible plants. 2DE is a reliable and irreplaceable method to analyze complex protein mixtures.

3.2.5.1. Chemicals required

1. Lysis buffer
2. Liquid nitrogen
3. Phenyl methane sulphonyl fluoride (PMSF) (1 m*M*)
4. Trichloroacetic acid (TCA) (10 %)
5. 2-mercaptoethanol (0.07 %)
6. Acetone

7. Rehydration buffer
8. Equillibration buffer-I
9. Equillibration buffer-II
10. 1X Tris Glycine SDS (TGS) buffer (Electrode buffer)
11. Overlay agarose
12. Mineral Oil for molecular biology
13. Proteomics grade water
14. Monomer solution (30 % acrylamide, 27 % bis-acrylamide)
15. 4X resolving gel buffer (1.5 *M* Tris-Cl, pH 8.8)
16. Ammonium persulphate (APS) (10 %)
17. Sodium dodecyl sulphate (SDS) (10 %)
18. Tetramethylethylenediamine (TEMED)
19. Fixer solution
20. Coomassie brilliant blue staining solution
21. Destaining solution

The details of constituents of chemicals used, its method of preparation are given in Annexure –V.

3.2.5.2. Isolation of total leaf protein for 2DE

For isolation of the total leaf protein from selected tolerant calliclone and susceptible variety Panniyur-1, the inoculated leaf samples were collected in aluminium foil over ice and brought to the laboratory. The collected leaves were immediately subjected to protein isolation protocol for 2DE. The leaf tissue of about one gram around the lesion was crushed in pre-chilled pestle and mortar using liquid nitrogen. The groun powder (about 0.2 g) was weighed and each 0.2 g powder stored in different microfuge tubes. The ground powder can be used directly for protein isolation or can be stored in -80°C until protein isolation. To 0.2 g of ground leaf powder, about 200 µl of lysis buffer and 1 m*M* of PMSF were added. Then it was vortexed briefly before incubating in ice for 15 minutes. After incubation, it was centrifuged at 12000 g for 20 minutes at 4°C to

remove debris. After centrifugation, the supernatant containing the protein was transferred into fresh pre-chilled microfuge tube.

3.2.5.3. Precipitation of total protein by TCA-acetone method

The supernatant collected was added with 1.5 volumes of freshly prepared 10 per cent TCA solution in ice cold acetone containing 0.07 per cent 2-mercaptoethanol. The contents were vortexed gently and then incubated at -20°C for overnight precipitation.

3.2.5.4. Recovery of precipitated protein and protein purification

After overnight precipitation, a white precipitate of protein was seen at the bottom which was recovered by centrifugation at 12000 g for 20 minutes at 4°C. The supernatant was then discarded and the protein pellet was purified by giving two washes with ice cold acetone containing 0.07 per cent 2-mercaptoethanol. For the each washing step, the pellet was first completely resuspended in acetone by vortexing, then incubated at -20°C for 15 minutes and finally centrifuged at 12000 g for 15 minutes at 4°C to recover the full protein pellet. The protein pellet was air dried to remove traces of acetone. The recovered protein pellet was used directly or it can be stored in -80°C until its next usage.

3.2.5.5. Protein solubilization

The protein pellet was first dissolved in minimum quantity of rehydration buffer by vortexing. The undissolved impurities were removed by carrying out centrifugation at 10000 rpm for 15 minutes at 4°C. The impurities settled at the bottom of tube and the supernatant was collected in fresh pre-chilled microfuge tube. This was further advanced for protein quantification protocol by Bradford's method.

3.2.5.6. Protein quantification by Bradford's method

Bradford method is the most reliable and widely adopted method of protein quantification for 2DE analysis. It is simpler, faster and compared to Lowry's method, it is least subjected to interference by components of 2DE reagents, zwitterionic buffers, thiol compounds, ammonium ions, *etc*. This quantification assay is resorted on a principle

that the dye Coomassie Blue G250 in Bradford reagent, binds with the protein and more is the bound dye with the protein, more blue the solution is and more is the protein content (Bradford, 1976). The intensity of blue coloured solution can be measured spectrophotometrically and hence the protein can be quantified further.

3.2.5.6.1. Chemicals required

1. Bovine serum albumin (BSA) stock solution (1 mg ml^{-1})
2. Bradford reagent
3. 0.1 *N* NaOH

The details of constituents of chemicals used, its method of preparation are given in Annexure –VI.

3.2.5.6.2. Procedure

Several BSA protein standards (0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg ml^{-1}) of about 1 ml final volume were prepared by pipetting required volume in test tubes separately and diluting it with required volume of 0.1 *N* NaOH. Test tube with 1 ml of 0.1 *N* NaOH was taken as blank. About 5 ml of Bradford reagent was added in each tube and the contents were mixed properly. It was left undisturbed for 30 minutes. After 30 minutes, the absorbance of the coloured solutions were measured by using spectrophotometer set at 595 nm and zero blank. The O.D values obtained for all the protein standards were plotted on Y-axis with their respective protein concentration on X-axis to obtain a standard plot (straight line plot). Similar reaction was progressed with the protein test samples and the standard plot was used to extrapolate protein concentration (mg ml^{-1}) for unknown O.D values of the protein test sample.

3.2.5.7. Passive rehydration of Immobilised pH gradient gel (IPG) strip

For performing Isoelectric focusing (IEF or One-dimensional gel electrophoresis), the basic requirement was of IPG strips that was procured from Bio-Rad laboratories, U.S.A. The IPG strip comprises of a pH gradient polyacrylamide gel overlayed on a

plastic strip and covered by a cover strip. For the rehydration step, a rehydration tray was taken and 320 µl of rehydration buffer containing 700 µg of solubilized protein (quantified by Bradford method and calculated volume of protein diluted with rehydration buffer) was pipetted as a line along the back edge into one of the channels of the 17 cm rehydration tray. Pipetting was done carefully so as to prevent insertion of any air bubbles. A 17 cm IPG strip was taken out from the packaging kept in freezer and then kept outside at room temperature. With the help of forceps supplied along with the 2DE kit, the cover strip was gently removed and the strip side containing the gel was gently lied (gel-side down configuration) over the protein solubilized rehydration buffer in the channel. Care was taken while lowering the strip into the channel to avoid trapping of any air bubbles beneath the strip as it may affect the uniform loading of protein solubilized buffer while rehydration. The rehydration tray was covered using plastic lid and the tray was left undisturbed on a leveled bench for one hour to facilitate uniform rehydration of the IPG strip.

3.2.5.8. Isoelectric focusing of rehydrated IPG strip

The 17 cm PROTEAN IEF focusing tray was cleaned by washing with mild soap solution and then by rinsing with millipore water. The tray was kept for complete drying on the lab bench. After drying, the positive and negative electrode assemblies were fixed in the focusing tray in their respective terminals. With the help of forceps the paper wicks were dipped in Bio-Rad proteomics grade water for 45 seconds and then placed into a channel over the wire electrode at both terminals. Moistened wicks were placed over the electrodes to prevent direct contact of the gel in the strip with the electrodes and also for facilitating conduction of current. After an hour of rehydration, the lid of the rehydration tray was removed and the remaining protein solubilized rehydration buffer was pipetted into the focusing tray at the centre without transferring any air bubbles. With the help of forceps, the IPG strip was taken out and then transferred into the channel of focusing tray (gel-side down configuration). The strip was placed such a way that it just rests over the wicks at two ends and also while placing, the strip was lowered carefully to avoid trapping of air bubbles below. After this, the entire setup was transferred and fixed over

the metal plate in the PROTEAN® i12™ IEF cell. Bio-Rad mineral oil for molecular biology (7 ml) was overlayed by pipetting into the channel containing the IPG strip. The mineral oil was added to prevent the buffer from getting evaporated during the entire IEF process. The weights were placed at both the ends of the strip to prevent the strip from floating in oil and keep its position fixed. The lid of the IEF cell was closed and a seven step IEF protocol was programmed in the IEF cell, the details of which are given in the Table 3.1. Also the protocol for rehydration step was set for 10-12 hours at 20°C default temperature and maximum current of 50 μA per strip. The protocol was initiated by clicking on 'START' option. Initially the IEF cell will run the rehydration protocol and then after completion the machine will automatically switch on to programmed seven step IEF protocol. The PROTEAN® i12™ IEF cell was kept in an air-conditioned room maintained at 16°C during IEF run to prevent formation of water droplets inside the IEF cell due to condensation.

3.2.5.9. Equillibration of IPG strip

After all the steps of IEF protocol were completed, the entire focusing tray assembly was removed from the IEF cell after clicking on 'END RUN'. By holding at one end with the help of forceps, the IPG strip was taken out of the channel of focusing tray and then excess oil sticking to the IPG strip was drained out by gently pressing the IPG strip in between the folds of blotting paper without causing any damage to the gel. It was then transferred into a channel in the rehydration tray (gel-side up configuration). Equilibration buffer-I (approx. 4 ml) was pipetted into the channel with IPG strip. The tray was then kept on shaker for 20 minutes. After 20 minutes, with the help of pipette, the equilibration buffer-1 solution was decanted. Then Equilibration buffer-II (approx. 4 ml) was pipetted into the channel. The tray was then kept on shaker for 20 minutes. After 20 minutes, with the help of pipette, the equilibration buffer-II solution was decanted. Tray was kept covered with lid.

Table 3.1: Programme set for Isoelectric focusing (IEF) of leaf protein isolated from inoculated leaves of black pepper

Step	Voltage (V)	Gradient	Current (µA)	Time / VHr	Units
1	300	Linear	50	200	Volt Hr
2	1000	Rapid	50	300	Volt Hr
3	5000	Rapid	50	400	Volt Hr
4	5000	Rapid	50	1250	Volt Hr
5	5000	Rapid	50	3000	Volt Hr
6	5000	Rapid	50	5800	Volt Hr
7	0	Hold	50		

3.2.5.10. Second dimensional focusing

3.2.5.10.1 Casting of resolving gel

For second dimensional run of isoelectric focused proteins, a 17 cm wide and 1.5 mm thick polyacrylamide resolving gel containing 10 per cent SDS was casted. For this, first the two glass plates (beveled inner plate and outer plate) were thoroughly rinsed with millipore water to remove adhering dust and then wiped with cotton moistened with 70 per cent ethanol to eliminate greasy layer on the glass plates. The two glass plates were then assembled in the gel casting apparatus. About 10 ml of distilled water was poured in between the plates to check leakage if any which was later poured out by inverting and absorbing it in blotting paper. If there is leakage, then the plates were reassembled in the gel casting apparatus. A resolving gel mixture was prepared in a 100 ml beaker with APS and TEMED added at the last moment. The components used for making 12 per cent resolving gel buffer is as shown below.

Monomer solution	:	24 ml
4X resolving gel buffer	:	15 ml
SDS (10 %)	:	0.6 ml
Distilled water	:	19.8 ml
APS (10 %)	:	0.3 ml
TEMED	:	0.06 ml
Total	**:**	**60 ml**

With the help of a 50 ml syringe without needle, the resolving gel mixture was poured in between the glass plates leaving a head space of the width of IPG strip on the top. A small quantity of distilled water was poured in the head space to remove air bubbles if any. The gel was allowed to solidify for an hour.

3.2.5.10.2. Loading of IPG strip over the resolving gel and electrophoretic run

Before loading the equilibrated IPG strip, the IPG strip was taken out from the rehydration tray and dipped in 1X TGS (Tris-glycine-SDS) buffer for 45 seconds. Carefully with the help of forceps, the IPG strip was placed over the outer plate (gel-side up configuration and then gently lowered into the space in between the glass plates such that the strip with the gel touches the upper surface of the solidified resolving gel without any introduction of air bubbles at the point of contact. Melted overlay agarose was pipetted in the head space and allowed to solidify for 10 minutes such that the strip gets completely immovable and sealed in the head space. This glass plate assembly was fixed in the gel running gasket vertically with dummy plate on the opposite side. This entire assembly was then placed in the electrophoresis tank. The electrode buffer was poured both into the inner chamber and outer chamber. After pouring the electrode buffer upto the marked level, the electrophoresis tank was covered by lid and the chords were connected to the electrophoresis power pack. A voltage of 100 V was set in the power pack and then the vertical electrophoretic run was initiated by pressing the 'RUN' button. The running was carried out in air conditioned room maintained at 16°C to prevent the proteins from getting degraded due to heating of buffer.

As the tracking dye reached the bottom of the resolving gel, the power pack was switched off and the chords were unplugged from the power pack. The lid of the electrophoresis tank was opened. The gel running gaskets containing the plates were removed out from the tank and the locks were relaxed. The dummy plate was removed first followed by the glass plate assembly. With the help of gel releasers, the two glass plates were separated and with the same tool the resolving gel was detached from the glass plate. Care was taken while detaching the gel from the glass plate as there may be chance of breakage of resolving gel if mishandled. Hence if there was fragile gel sticking to one of the plates then it was detached patiently by squeezing with distilled water. The resolving gel was transferred to a staining dish containing distilled water. With mild shaking, the gel was washed in distilled water to remove the adhering electrode buffer. After two washes with distilled water, fixer solution was added into the staining dish

containing the resolving gel to fix the proteins in the gel. The fixing step was carried out for one hour over a shaker.

3.2.5.11. Coomassie brilliant blue staining of resolving gel

After fixation of proteins in the resolving gel, the fixer solution was drained off and coomassie brilliant blue staining solution was added into the staining dish. The staining of the gel was done for 36 hours by constant shaking over a shaker in order to attain uniform and saturated staining of the gel.

3.2.5.12. Destaining of the stained resolving gel

After staining was over, the staining solution was transferred into a glass bottle and destaining solution was added into the staining dish. The destaining was carried out in constant shaking over a shaker. Periodic changes with fresh destaining solution were given when the solution turned dark blue. The destaining was carried out until the background of the gel became completely transparent and colourless and the proteins were prominent with dark bluish-purple stained spots. The destained gel was preserved in distilled water containing few drops of glacial acetic acid at 4°C to prevent fungal contamination in the gel.

3.2.5.13. Documentation

The destained gel was documented by scanning the gel using GS-800™ calibrated densitometer that scans the gel with highest resolution and converts it into digital image file required for further image analyses.

3.2.5.14. Analysis of scanned gel images with Bio-Rad PDQUEST software

The Bio-Rad PDQUEST software is a tool that utilizes the scanned 2DE gel images as input file to provide us with enormous data about total number of protein spots in the gel, presence of differentially expressed spots in the gel, presence of novel spots, comparative expression details of a same protein in two different samples and varied kinds of analytical reports such as quantity table, quantity graph, scatter plot, analysis set

reports *etc.* For the current study, this software tool was fed with the required scanned 2DE gel image files to perform a comparative analysis on protein profile change in susceptible Panniyur-1 and tolerant calliclone upon challenging with *Phytophthora capsici.*

3.2.6. Peptide mass fingerprinting by MALDI-TOF / MS analysis and *In silico* analysis with raw peptide sequences

Using PDQuest software data reports, the differentially expressed spots were positioned. These spots (only the stained area) were cut from the gel and transferred into microfuge tubes. The tubes were filled with 50 per cent methanol, labeled properly and sent for peptide sequencing by Matrix Assisted Laser Desorption / Ionization – Time of Flight Mass Spectrophotometry (MALDI-TOF / MS) at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram.

The *In silico* analysis with the raw peptide sequence data and peak plot data were done at Distributed Information Centre (DIC), CoH, Vellanikkara. The peak values obtained from the peak plot generated by MALDI-TOF / MS of all the selected protein spots were used as input for peptide identification in MASCOT server. The complete amino acid sequences of all the protein hits corresponding to the identified peptides were downloaded in 'fasta' format from the UNIPROT database by using the UNIPROT-IDs listed in the peptide summary report. All the amino acid sequences obtained in fasta format were clubbed into one file and the clubbed file was used as input for BLAST2GO analysis. The analyses done for all the hit sequences through BLAST2GO tool were, Blast analysis, Enzyme Code (EC) distribution analysis, InterPro analysis, Gene Ontology (GO) analysis and KEGG pathway analysis. The results obtained from all the analyses were saved in '.png', '.txt' and '.pdf' formats for interpretation of results.

Results

4. RESULTS

The results obtained in the investigations on characterization of pathogenesis related proteins in selected calliclones of black pepper in relation to *Phytophthora* foot rot disease are presented in this chapter.

4.1. RAISING OF ROOTED CUTTINGS

Two-noded vine cuttings were planted after dipping in 1000 ppm IBA solution for 45 seconds in March 2016. Sprouting was observed in the two noded cuttings two weeks after planting. Details on rooting and establishment of two-noded cuttings of black pepper calliclones and Panniyur-1 are presented in Table 4.1. The calliclones of Cheriakanyakkadan recorded better establishment in the nursery than calliclones of Kalluvally and Panniyur-1. The mean establishment percentage of calliclones belonging to the Cheriakanyakkadan group was 88.96 per cent that was higher compared to mean establishment percentage of Kalluvally calliclones (70.58 %) and Panniyur-1 (83.87 %). Among the Cheriakanyakkadan calliclones, CKCC 60 showed highest establishment percentage (96.66) followed by CKCC 27 (93.33 %) and the lowest was shown by CKCC 5 (83.33 %). Among the five selected calliclones belonging to the Kalluvally group, KLCC 89 showed higher establishment percentage (90.63 %) followed by KLCC 134 (83.87 %) and the lowest was recorded by KLCC 133 (53.33 %). Panniyur-1 recorded an establishment percentage of 83.87 per cent. Three month old rooted cuttings of calliclones and Panniyur-1 were used for challenge inoculation with *Phytophthora capsici* (Plate 4.1).

4.2. ISOLATION OF *Phytophthora capsici* AND STUDYING THE DISEASE REACTION IN BLACK PEPPER CALLICLONES

4.2.1 Isolation and maintenance of pathogen culture

The fungus *Phytophthora capsici* was isolated and maintained in carrot agar medium. The fungus grew profusely in the medium in the form as uniform dense, white cottony mycelium. The mycelial growth was luxurious when the culture plates were maintained at a room temperature of 25°C. For purification of the fungal culture,

Table 4.1: Rooting and establishment in two-noded cuttings of black pepper calliclones and variety Panniyur-1

Calliclone / Variety	Cuttings planted (No.)	Cuttings rooted and established (No.)	Establishment (%)	Group establishment (%)
CKCC 5	30	25	83.33	88.96
CKCC 25	34	29	85.29	
CKCC 27	30	28	93.33	
CKCC 41	33	28	84.85	
CKCC 60	30	29	96.66	
CKCC 61	31	28	90.32	
KLCC 86	34	21	61.76	70.58
KLCC 89	32	29	90.63	
KLCC 119	30	19	63.33	
KLCC 133	30	16	53.33	
KLCC 134	31	26	83.87	
Panniyur-1	31	26	83.87	83.87
Mean			**80.88**	
Standard Deviation			**13.751**	

CKCC - Cheriakanyakkadan calliclone

KLCC - Kalluvally calliclone

85

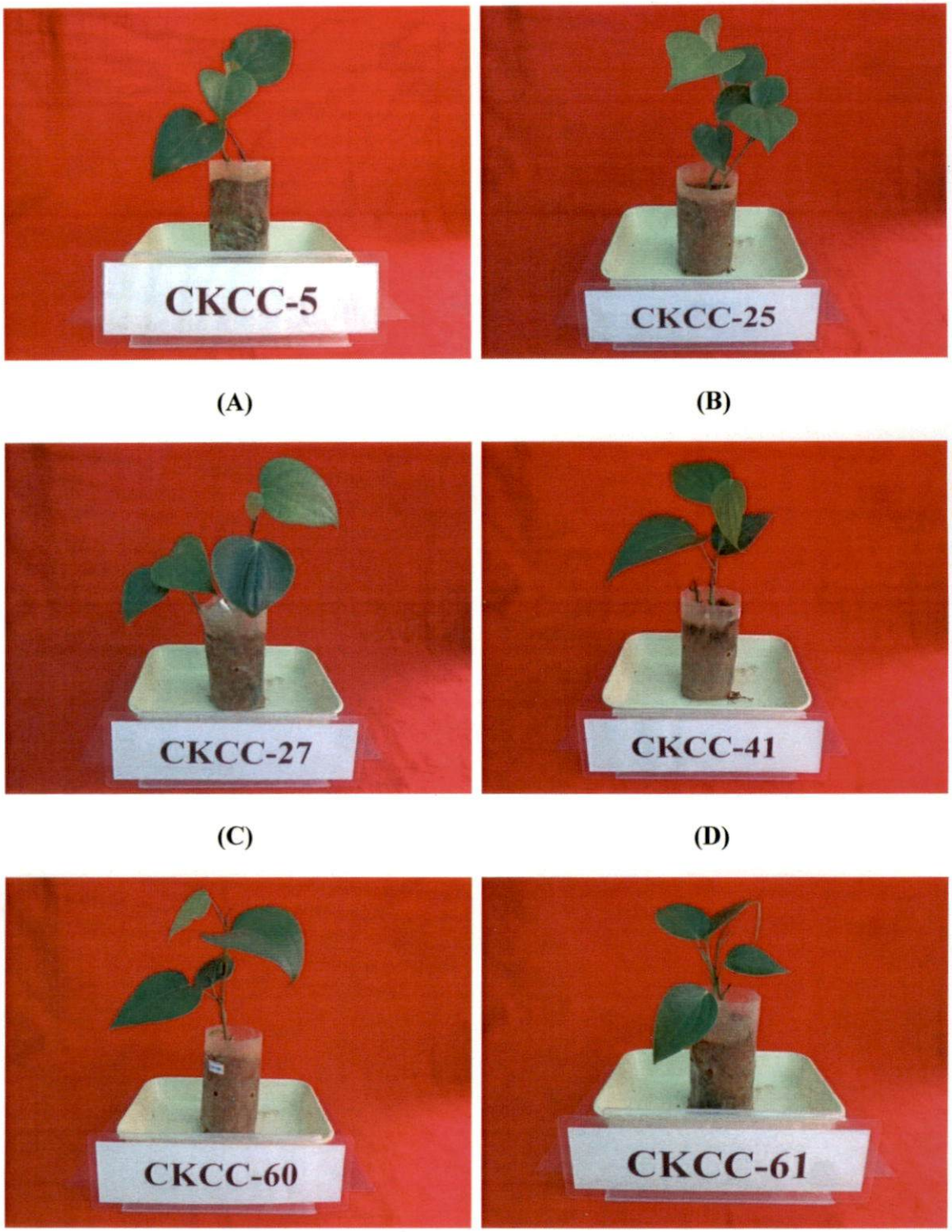


Plate 4.1 (a): Three month old rooted cuttings of calliclones of Cheriakanyakkadan group for challenge inoculation (A) to (F)

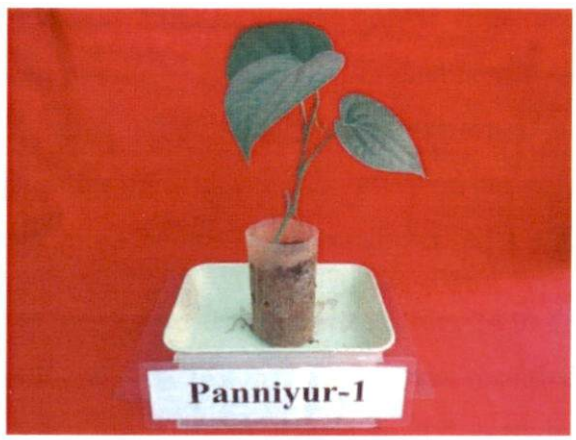
(G)

(H)

(I)

(J)

(K)



(L)

Plate 4.1 (b): Three month old rooted cuttings of calliclones of Kalluvally group (G) to (K) and susceptible variety Panniyur-1 (L) for challenge inoculation

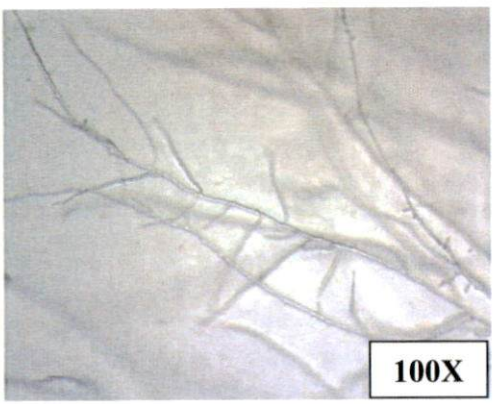
hyphal tip method was followed. The mycelial growth was observed within 24 hours of inoculation and it took seven days to cover the entire surface of the carrot agar medium in the petri plate. Seven day old culture was used for performing detached leaf symptom bioassay and leaf inoculation studies on three month old rooted cuttings (Plate 4.2).

4.2.2. Screening of black pepper calliclones for *Phytophthora* foot rot by detached leaf symptom bioassay

Disease reaction varied significantly in all the calliclones when inoculated with seven day old culture of *Phytophthora capsici* in detached leaves (Plate 4.3). The lesions developed in the calliclones were round black, water soaked with fimbricate margins. As compared to Cheriakanyakkadan and Kalluvally calliclones, Panniyur-1 was found susceptible to *P. capsici* recording higher lesion diameter at 24, 48 and 72 hours after inoculation. At 24 hours after inoculation, the highest lesion diameter was recorded by KLCC 89, which was on par with Panniyur-1 and KLCC 133 (Table 4.2). Calliclones such as CKCC 41, CKCC 60, CKCC 61, CKCC 25 were on par with each other whereas CKCC 27 recorded least lesion diameter. At 48 hours after inoculation, CKCC 41 recorded significantly highest lesion diameter. Panniyur-1 and KLCC 89 were on par with each other. The least lesion diameter at 48 hours was recorded by CKCC 27. At 72 hours after inoculation, the highest lesion diameter was recorded by CKCC 41 which was on par with Panniyur-1. Calliclones such as KLCC 133 and KLCC 89 were on par with each other whereas least lesion diameter was recorded by CKCC 27. The lesion diameter in all the calliclones is significantly higher at 72 hours after inoculation. Irrespective of inoculation period, CKCC 41 recorded the highest lesion diameter which was on par with Panniyur-1 and KLCC 89, whereas CKCC 27 recorded the least lesion diameter.

The size of lesion diameter was found to increase as time increased from 24 to 48 and 48 to 72 hours after inoculation. Per cent increase in lesion diameter was recorded maximum for Kalluvally calliclones (332.93 %) from 24 to 48 hours followed by Cheriakanyakkadan calliclones (311.88 %) (Table 4.3). From 48 to 72 hours after inoculation, Cheriakanyakkadan calliclones recorded maximum percentage increase

(A)



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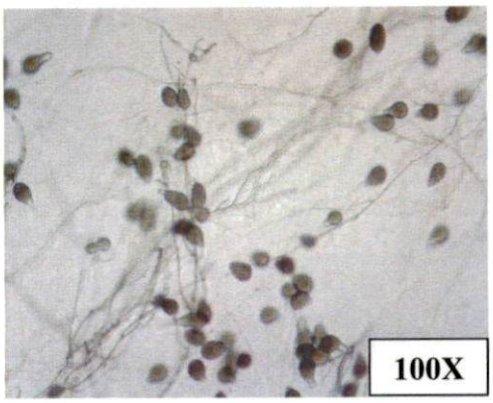
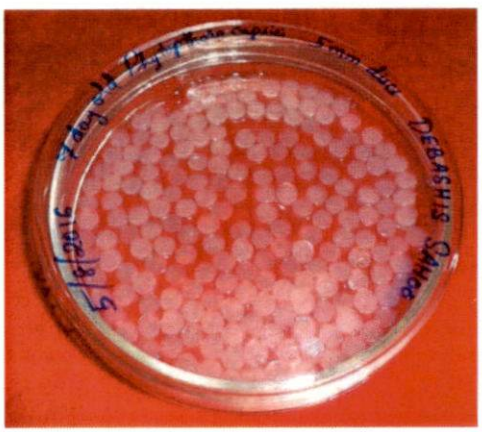
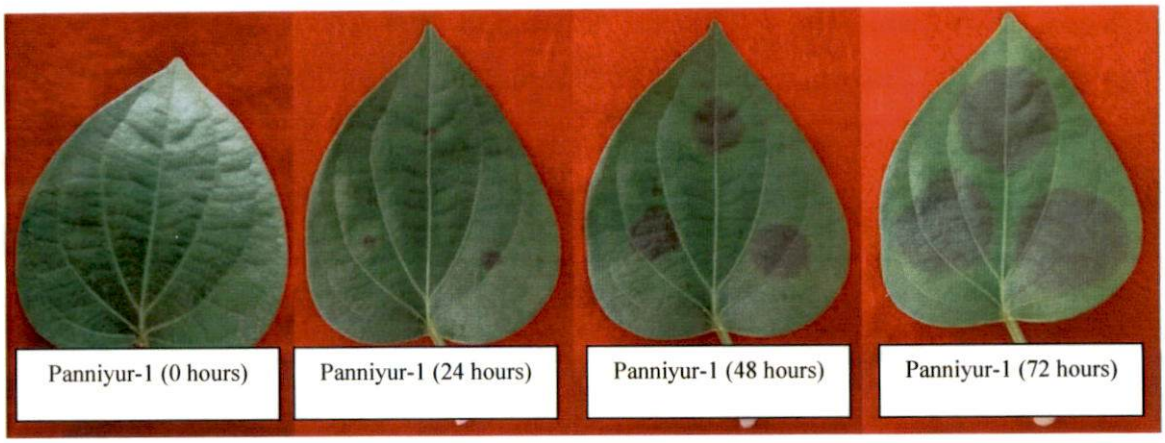


(C)

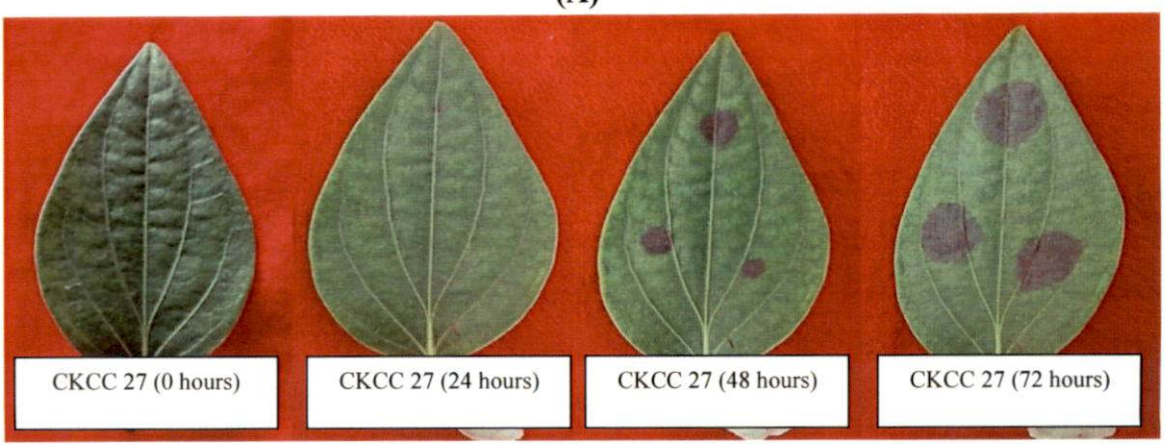


(D)

Plate 4.2: (A) Seven day old culture of *P. capsici* (B) hyaline aseptate hyphae (100X) (C) lemon shaped sporangia (100X) (D) 5mm mycelial discs kept in sterile water for inoculation



(A)



(B)

Plate 4.3: Lesion development in (A) Susceptible variety Panniyur-1 and (B) CKCC 27

Plate 4.4: Maintenance of inoculated leaves for detached leaf symptom bioassay

Table 4.2: Lesion diameter (cm) in detached leaf symptom bioassay (with pinprick) at 24, 48 and 72 hours after inoculation with *Phytophthora capsici*

Calliclone / Variety	Lesion diameter (cm)			Mean	Group mean
	24 hours after inoculation	48 hours after inoculation	72 hours after inoculation		
CKCC-5	0.205	0.907	1.852	0.988	1.137
CKCC-25	0.293	0.985	2.413	1.231	
CKCC-27	0	0.478	1.307	0.595	
CKCC-41	0.338	1.51	2.85	1.566	
CKCC-60	0.315	0.992	2.04	1.116	
CKCC-61	0.313	1.157	2.513	1.328	
KLCC-86	0.15	0.888	1.912	0.983	1.182
KLCC-89	0.438	1.353	2.69	1.494	
KLCC-119	0.137	0.875	1.848	0.953	
KLCC-133	0.353	1.273	2.727	1.451	
KLCC-134	0.152	0.937	2.002	1.03	
Panniyur-1	0.415	1.41	2.838	1.554	1.554
Mean	**0.259**	**1.064**	**2.249**		

Factors	C.D.
Factor(A)	0.043
Factor(B)	0.086
Factor(A X B)	0.149

A - Inoculation interval

B - Calliclone / Variety

(115.22 %) in lesion diameter than Kalluvally calliclones (109.95 %). Panniyur-1 recorded minimum percentage change in lesion diameter at both 24 to 48 (239.76 %) and 48 to 72 hours after inoculation (101.28 %) compared to Cheriakanyakkadan calliclones and Kalluvally calliclones.

Table 4.3: Increase in lesion diameter (%) in detached leaves of Cheriakanyakkadan, Kalluvally calliclones and Panniyur-1

Calliclone group	Increase from 24 to 48 hours (%)	Increase from 48 to 72 hours (%)
Cheriakanyakkadan calliclones	311.88	115.22
Kalluvally calliclones	332.93	109.95
Panniyur-1	239.76	101.28

4.2.3. Intact leaf inoculation without pinprick in three month old rooted cuttings

Inoculation in intact leaves of three month old rooted cuttings at different inoculation periods was done for sampling leaf tissue for β-1,3-glucanase assay, SDS-PAGE analysis and 2D-gel electrophoresis. The inoculated cuttings were maintained in the shade net house under high moisture conditions for proper symptom development (Plate 4.6). The lesion developed in the leaves of calliclones and Panniyur-1 was round black, water soaked with fimbricate margins (Plate 4.5 (A) and 4.5 (B)). The inoculated intact leaves were collected at 0, 24, 48 and 72 hours after inoculation over ice and brought to laboratory for isolation of crude enzyme.

4.3. ASSAY OF β-1,3-GLUCANASE ACTIVITY

The β-1,3-glucanase activity was assayed from the crude enzyme extract isolated from intact inoculated leaves of all the calliclones and Panniyur-1 at 0, 24, 48 and 72 hours after inoculation. The specific activity of the enzyme was expressed as µmol of glucose equivalents formed / mg of protein / 15 minutes (Table 4.4).

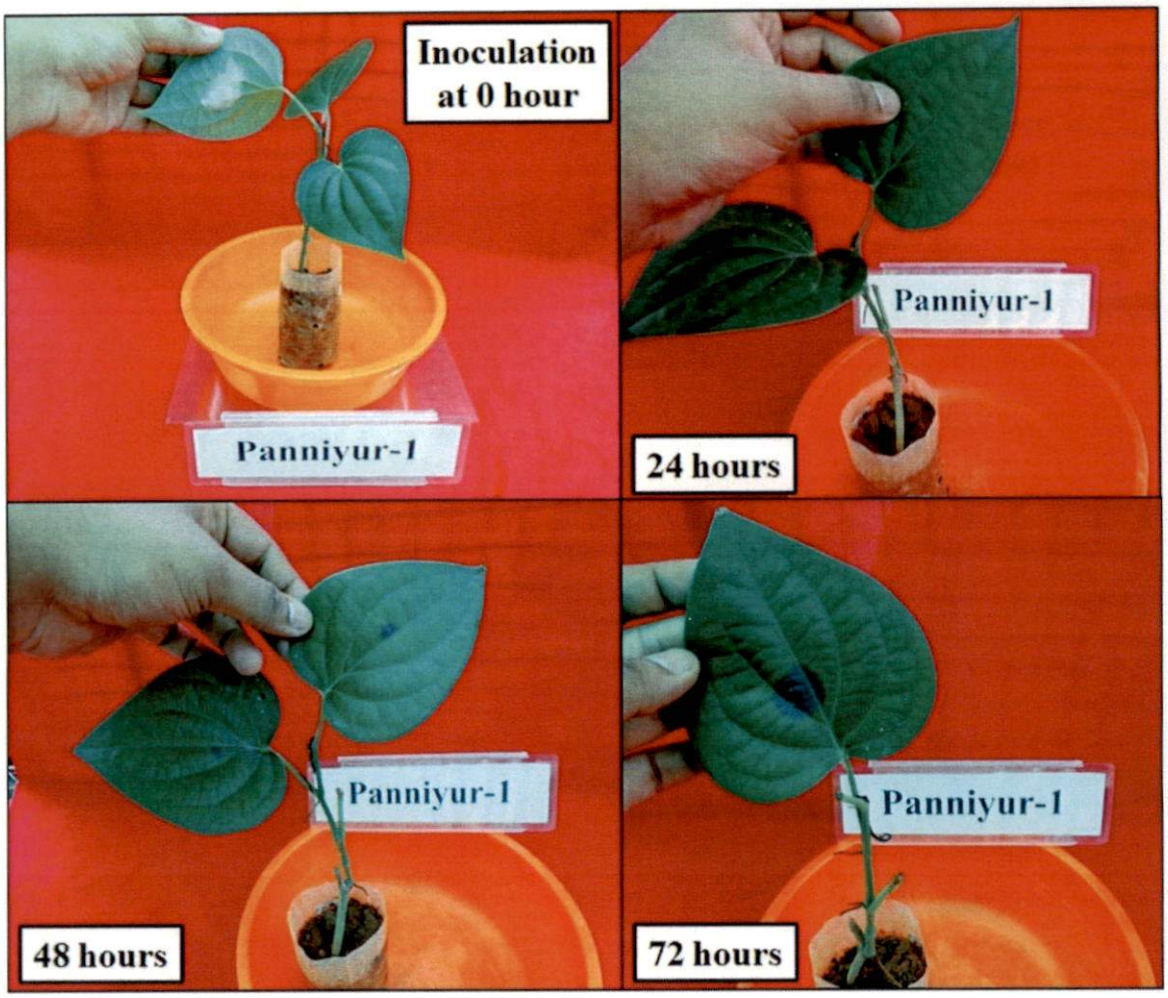


Plate 4.5 (A): Lesion development in three month old rooted cutting of Panniyur-1 at 0, 24, 48 and 72 hours after inoculation by *P. capsici*

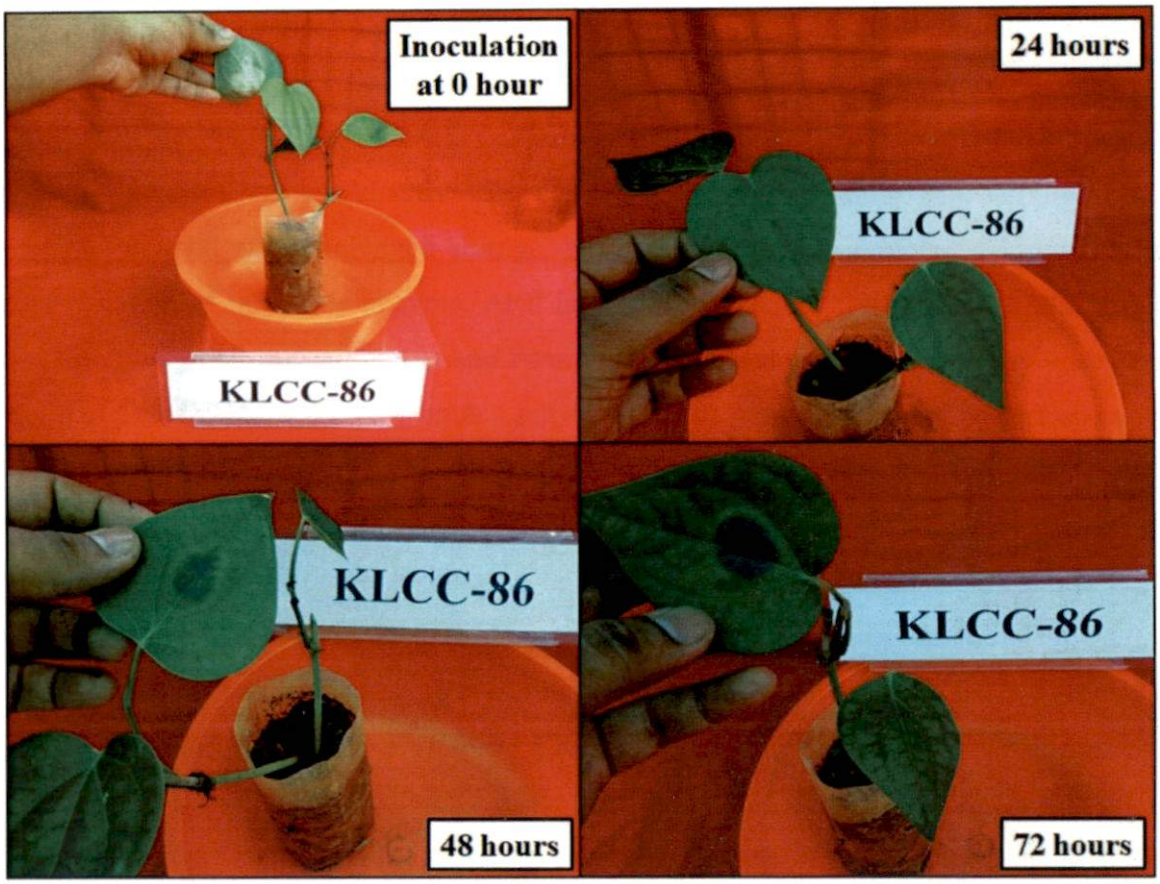


Plate 4.5 (B): Lesion development in three month old rooted cutting of calliclone KLCC 86 at 0, 24, 48 and 72 hours after inoculation by *P. capsici*

Plate 4.6: Maintenance of three month old rooted cuttings inoculated with *P. capsici* in the net house

The maintenance of ice cold temperature throughout the enzyme isolation procedure and addition of chemicals such as PMSF, ascorbic acid and β-mercaptoethanol along with ice cold sodium acetate buffer resulted in greater enzyme yield. The β-1,3-glucanase enzyme was found active only for few hours after isolation. The activity of the enzyme declined after prolonged storage, hence the assay was performed immediately after isolation of the crude enzyme. At 0 hours, the specific activity of β-1,3-glucanase was observed in all the calliclones and Panniyur-1 with highest specific activity observed in CKCC 60 (1918.92 enzyme units / mg of protein / 15 minutes) and lowest specific activity recorded by CKCC 5 (1119.6 enzyme units / mg of protein / 15 minutes). At 24 hours after inoculation, an elevated and highest specific activity of β-1,3-glucanase was recorded by KLCC 89 (2509.15 enzyme units / mg of protein / 15 minutes) and lowest activity recorded by KLCC 134 (825.28 enzyme units / mg of protein / 15 minutes). At 48 hours after inoculation, CKCC 41 recorded highest specific activity of β-1,3-glucanase (1786.33 enzyme units / mg of protein / 15 minutes) and the lowest activity recorded by KLCC 134 (891.65 enzyme units / mg of protein / 15 minutes). At 72 hours after inoculation, KLCC 134 recorded an elevated specific activity of β-1,3-glucanase (2670.67 enzyme units / mg of protein / 15 minutes) and CKCC 25 recorded the least (940.05 enzyme units / mg of protein / 15 minutes).

The fold change in β-1,3-glucanase activity from 0 to 24, 24 to 48 and 48 to 72 hours after inoculation is illustrated graphically in Figure 4.1.

From 0 to 24 hours post-inoculation, the highest increment in specific activity of β-1,3-glucanase was recorded by KLCC 89 (increased by 1.03 times) and minimum increment was recorded by CKCC 5 (increased by 0.13 times) and CKCC 61 (increased by 0.048 times). Rest all the calliclones and Panniyur-1 showed decrement in specific activity of β-1,3-glucanase from 0 to 24 hours. The sharp downfall in specific activity of β-1,3-glucanase was recorded by Panniyur-1 (decrement by 0.497 times). From 24 to 48 hours post-inoculation, KLCC 89 recorded sharp downfall in specific activity of β-1,3-glucanase (decrement by 0.528 times). CKCC 27 recorded an elevation in specific activity of β-1,3-glucanase by 0.47 times followed by CKCC 41 (increased by 0.462 times) from

Table 4.4: Specific activity of β-1,3-glucanase (μmol of glucose equivalents formed / mg of protein / minute) at 0, 24, 48 and 72 hours after inoculation

Calliclone / Variety	0 hours after inoculation	24 hours after inoculation	48 hours after inoculation	72 hours after inoculation
CKCC 5	1119.60	1263.74	1436.37	1813.45
CKCC 25	1534.70	1263.36	809.23	940.05
CKCC 27	1334.72	1134.86	1667.98	1302.06
CKCC 41	1845.90	1222.00	1786.33	1770.21
CKCC 60	1918.92	1396.80	1709.85	1835.24
CKCC 61	1383.21	1449.61	1324.94	2091.24
KLCC 86	1468.82	1029.62	1476.77	1122.05
KLCC 89	1236.23	2509.15	1183.81	1368.23
KLCC 119	1806.36	1707.66	1728.98	1355.33
KLCC 133	1443.62	1130.53	1557.89	1873.51
KLCC 134	1436.59	825.28	891.65	2670.67
Panniyur-1	1842.46	926.36	1287.55	1802.69

Figure 4.1 (A): Native β-1,3-glucanase activity in different calliclones and susceptible variety Panniyur-1

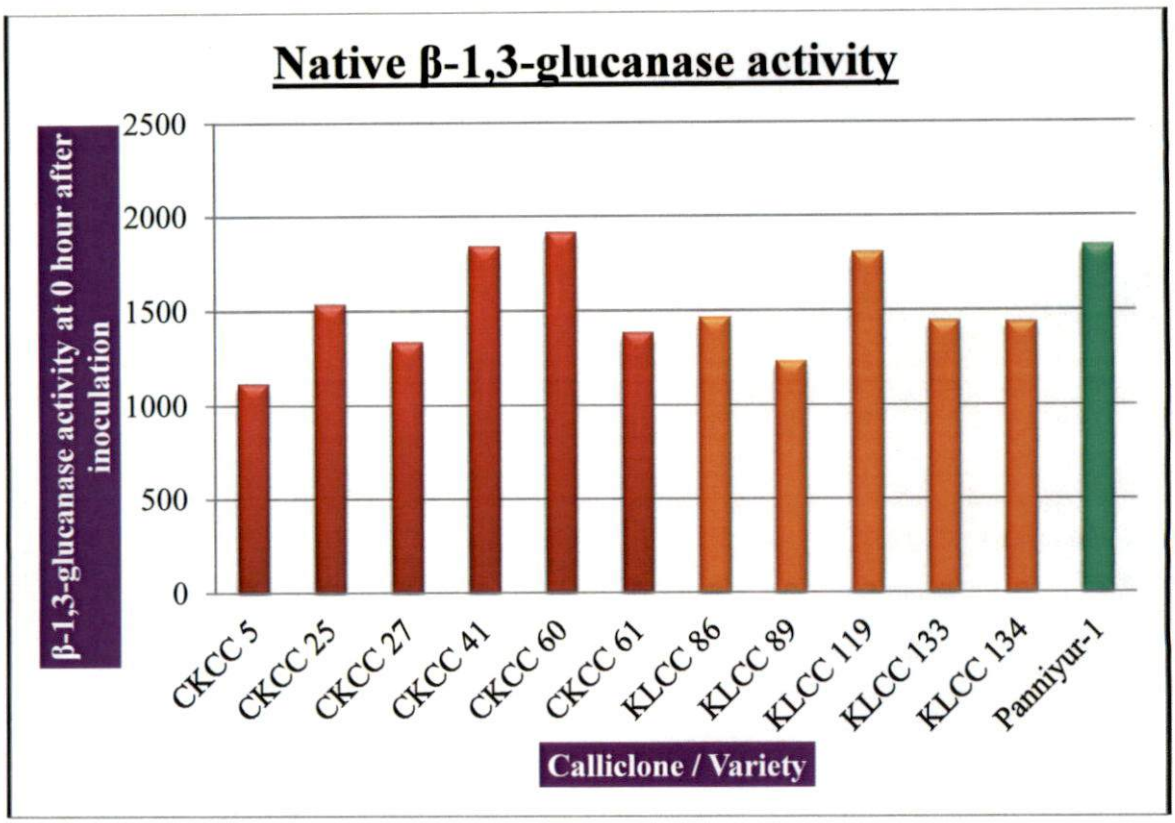


Figure 4.1 (B): Variation in β-1,3-glucanase activity in different calliclones and susceptible variety Panniyur-1 at different inoculation periods

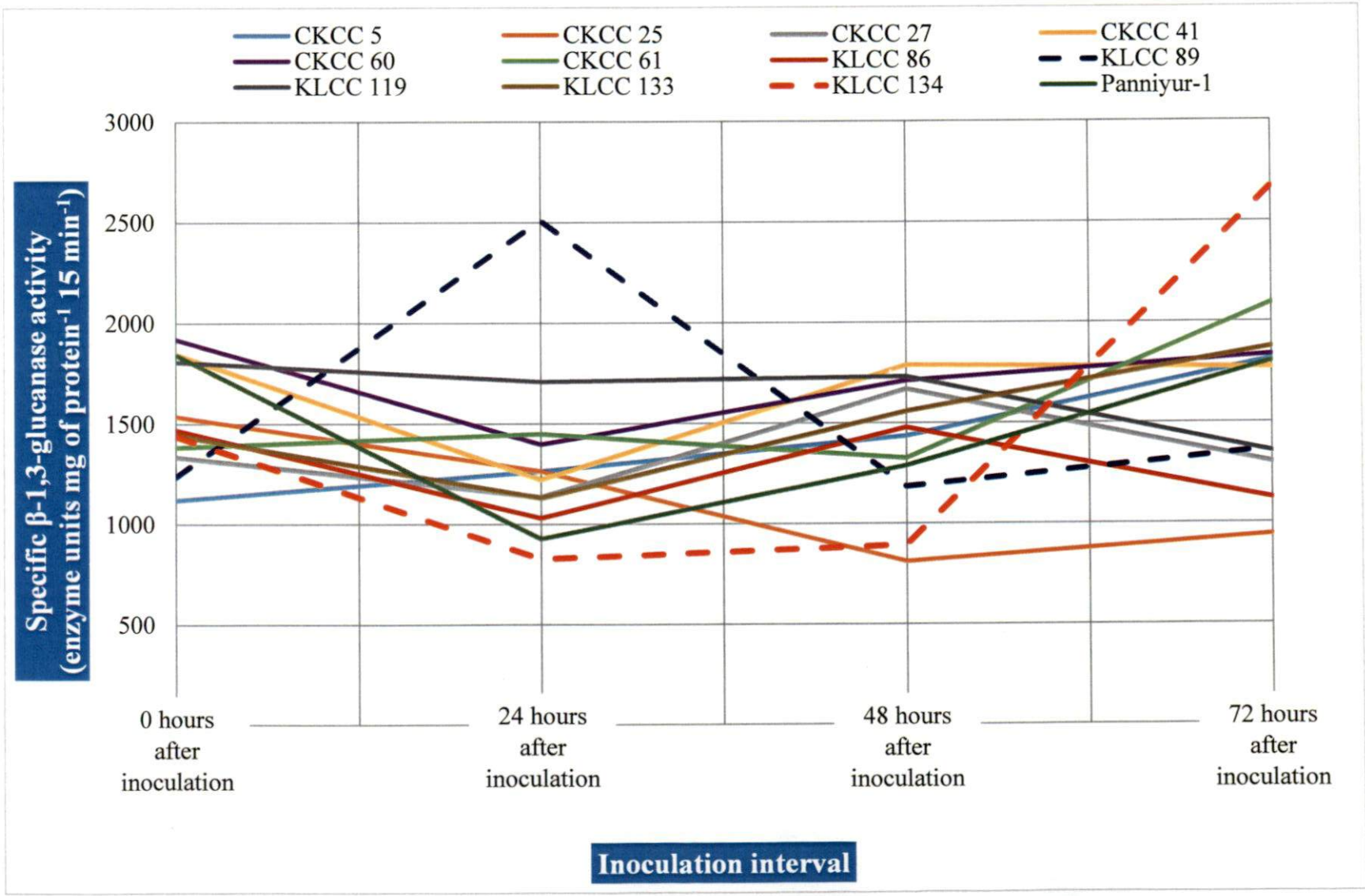


Figure 4.1 (C): Fold change in β-1,3-glucanase activity in calliclones and Panniyur-1 at different inoculation intervals

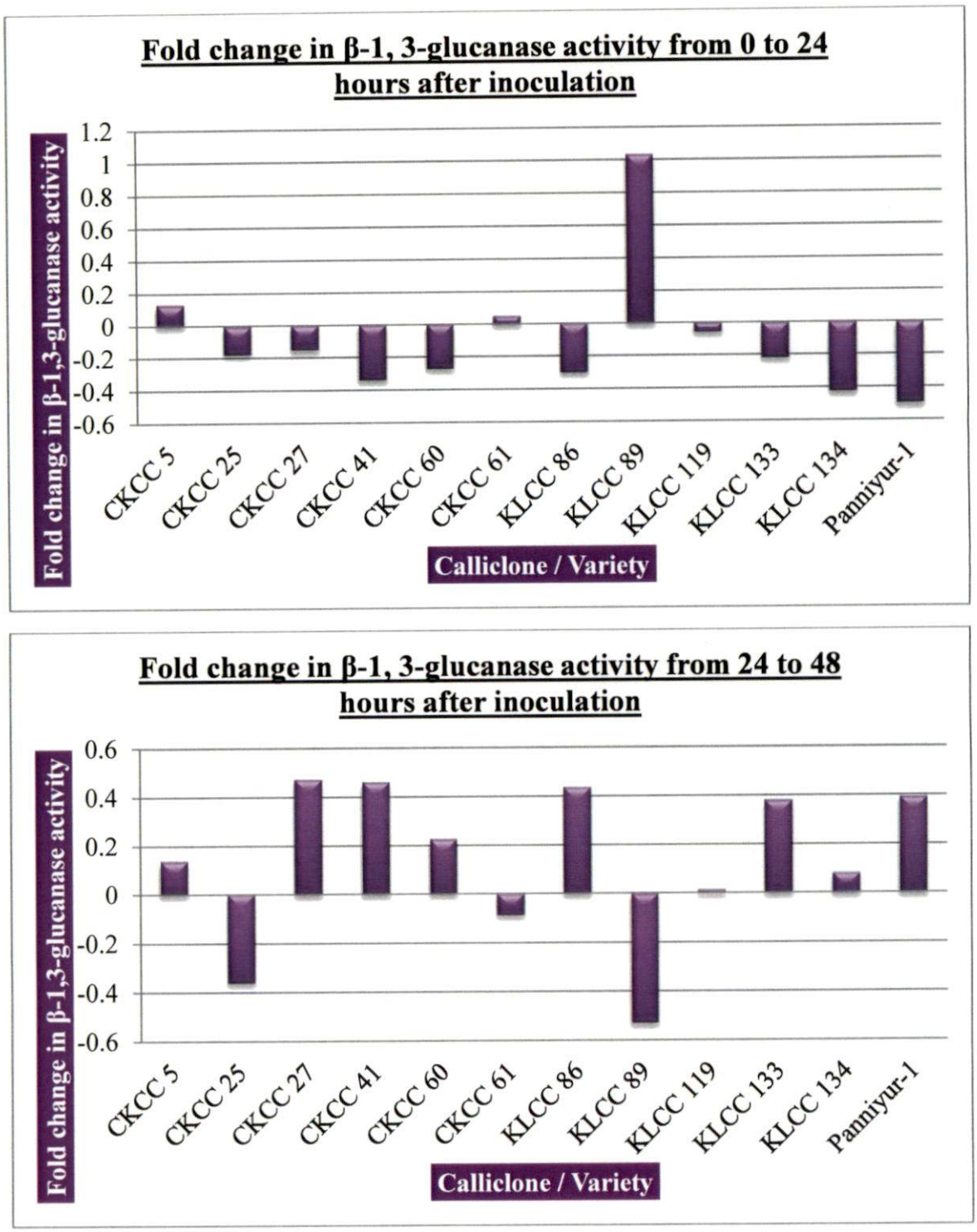
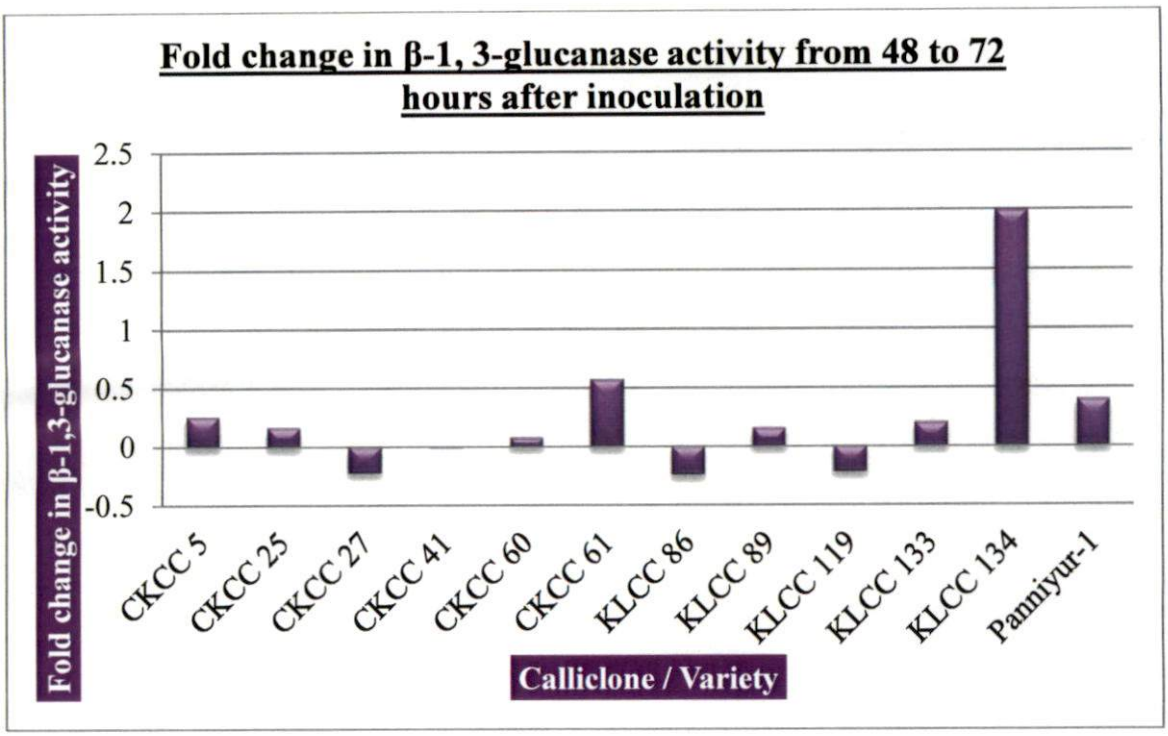


Figure 4.1 (C) contd.: Fold change in β-1,3-glucanase activity in calliclones and Panniyur-1 at different inoculation intervals



24 to 48 hours after inoculation. From 48 to 72 hours post-inoculation, KLCC 134, recorded highest increment in specific activity of β-1,3-glucanase (increased by 1.995 times) compared to all other calliclones. CKCC 27, CKCC 41, KLCC 86 and KLCC 119 recorded decrement in specific activity from 48 to 72 hours post-inoculation.

Overall, KLCC 89 recorded the earliest (at 24 hours after inoculation) and highest specific activity of β-1,3-glucanase among all the 11 calliclones and susceptible variety Panniyur-1 studied while enzyme bioassay.

4.4. PROTEIN PROFILING BY SDS-PAGE

The SDS-PAGE analyses of total leaf protein isolated from all the calliclones and Panniyur-1 at different periods were attempted as per the procedure reported by Laemmli (1970) with slight modification. Modifications were made in the concentration of APS and TEMED, percentage of resolving gel, protein concentration technique and duration of gel staining. Concentration of APS and TEMED standardized as 50 µL and 10 µL respectively in the resolving gel gave satisfactory solidification. Increasing the concentration of monomer solution in the resolving gel *i.e.* from 10 per cent to 15 per cent gave more distinctly resolved, stained intact protein bands. Precipitation of total protein from the crude enzyme extract by TCA-Acetone method of precipitation followed by SDS-PAGE also gave more number of distinctly resolved, intact protein bands which was not evident when the crude enzyme extract was directly loaded in SDS-PAGE gel. Overnight staining with coomassie brilliant blue staining solution under constant shaking over a shaker enhanced clarity and visibility to the bluish-purple stained protein bands.

SDS-PAGE analysis of precipitated leaf protein in all the calliclones and Panniyur-1 showed distinct expression of protein bands at 0, 24, 48 and 72 hours after inoculation. On an average, 15 bluish-purple stained protein bands were seen in 11 calliclones at different time intervals while in the variety Panniyur-1, on an average of 17 protein bands were resolved at different time period of inoculation. The analysis showed that, the 16.5 kDa protein band corresponding to β-1,3-glucanase was found expressed in all the calliclones but variation in level of expression of 16.5 kDa band was observed in

different calliclones and within calliclone at different inoculation period. For KLCC 89 at 24 hours after inoculation, the 16.5 kDa protein band was found over expressed compared to all others that is evident from the gel image as bluish-purple stained thickest band. The calliclones CKCC 5, CKCC 60, CKCC 61 and KLCC 134 showed 16.5 kDa band expressed at all times 0, 24, 48 and 72 hours after inoculation with maximum expression at 72 hours after inoculation. In KLCC 133 and Panniyur-1, the same band was observed more prominently at 72 hours after inoculation. In CKCC 25, the expression of 16.5 kDa band was more at 24 hours then reduced at 48 hours that was visible as less intense band. In the calliclones CKCC 27, CKCC 41, KLCC 86 and KLCC 119, the expression of 16.5 kDa band was found increased from 24 to 48 hours with maximum expression at 48 hours but decreased at 72 hours, as evident with the less intense band.

Apart from variable expression of 16.5 kDa band in all the calliclones and Panniyur-1, other molecular weight bands were also found differentially expressed, the details of which is given in Table 4.5. Protein band corresponding to 54 kDa size was overexpressed in three calliclones *viz.* CKCC 27, KLCC 89 and KLCC 133. Over expression of 18 kDa protein band in CKCC 25 at 72 hours and 25.5 kDa protein band in CKCC 27 at 48 hours was also observed. The SDS-PAGE gel pictures of all the resolving gels showing β-1,3-glucanase band (16.5 kDa) and differentially expressed bands are given in Plates 4.7 A to 4.7 L.

Table 4.5: Expression of β-1,3-glucanase band (16.5 kDa) and differentially expressed protein bands in different calliclones at different inoculation intervals

Calliclone / Variety	Number of bands			Expression of 16.5 kDa β-1,3-glucanase band			Molecular weight of differentially expressed bands (kDa)	Expression of differential bands		
	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours		24 hours	48 hours	72 hours
CKCC 5	15	14	15	+	+	+	12.0	-	-	+
							48.0	+	-	-
CKCC 25	13	14	14	++	+	+	26.5	+	-	-
							28.5	+	+	+
							18.0	-	+	++
CKCC 27	15	14	14	+	++	+	25.5	-	++	+
							54.0	++	+	+
CKCC 41	16	16	16	-	++	+				
CKCC 60	14	14	14	+	+	++				
CKCC 61	14	14	14	+	+	++				

Table 4.5: Continued

KLCC 86	15	15	15	+	++	+				
KLCC 89	15	15	15	+++	+	+	54.0	++	+	+
KLCC 119	15	15	15	-	++	+				
KLCC 133	14	14	14	-	-	+	12.0	+	+	+
							54.0	++	+	+
KLCC 134	14	14	14	+	+	+++				
Panniyur-1	16	17	15	+	+	++	8.0	+	+	-
							9.5	-	+	-

Band present (+)

Moderately expressed band (++)

Over-expressed band (+++)

Band absent (-)

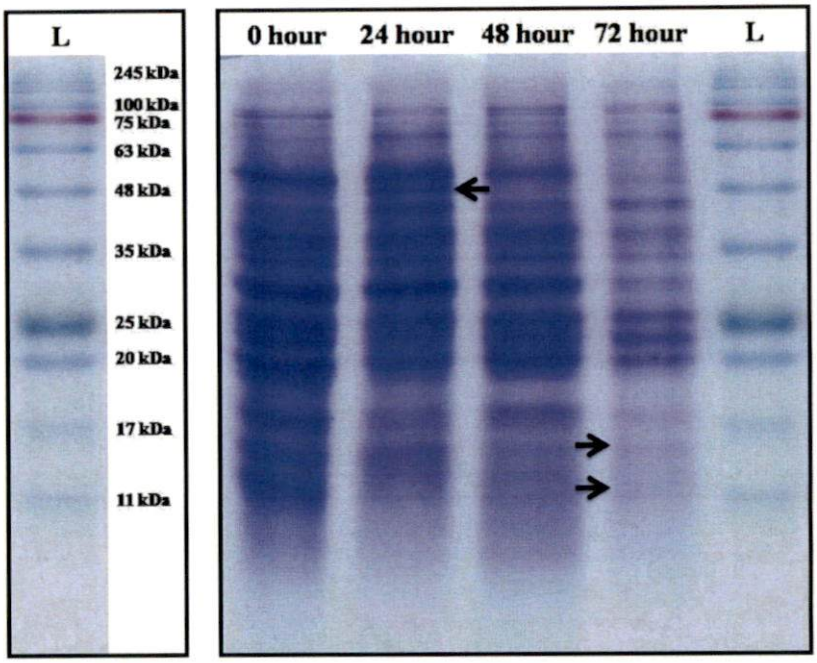


Plate 4.7 (A): SDS-PAGE protein profile of CKCC 5 at different inoculation periods

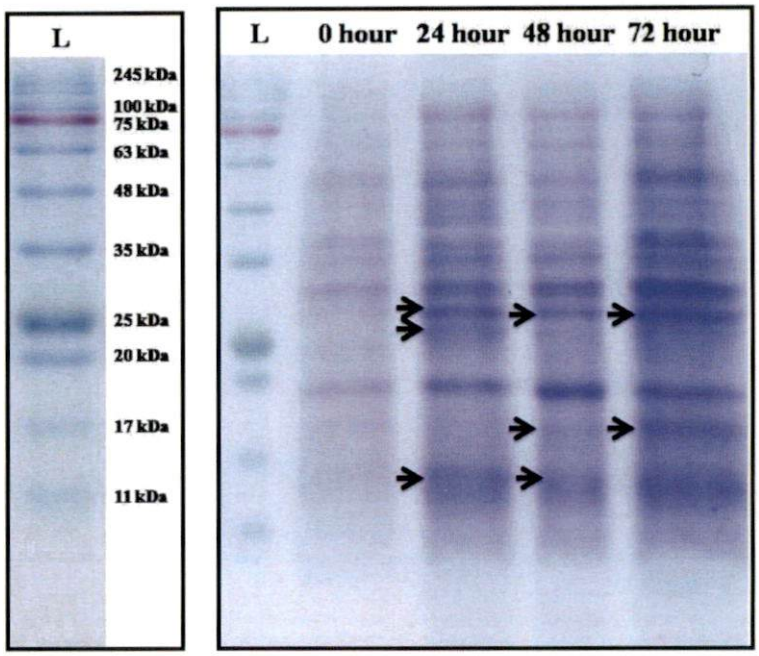


Plate 4.7 (B): SDS-PAGE protein profile of CKCC 25 at different inoculation periods

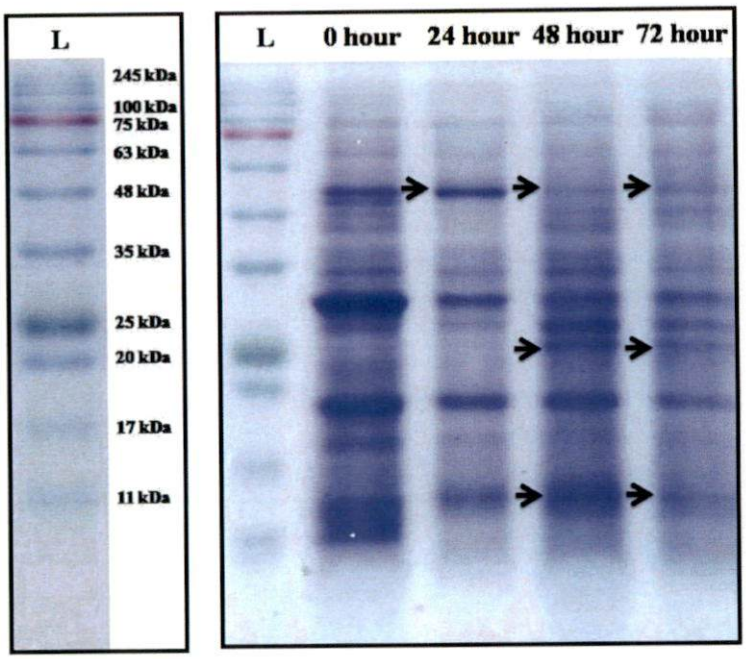


Plate 4.7 (C): SDS-PAGE protein profile of CKCC 27 at different inoculation periods

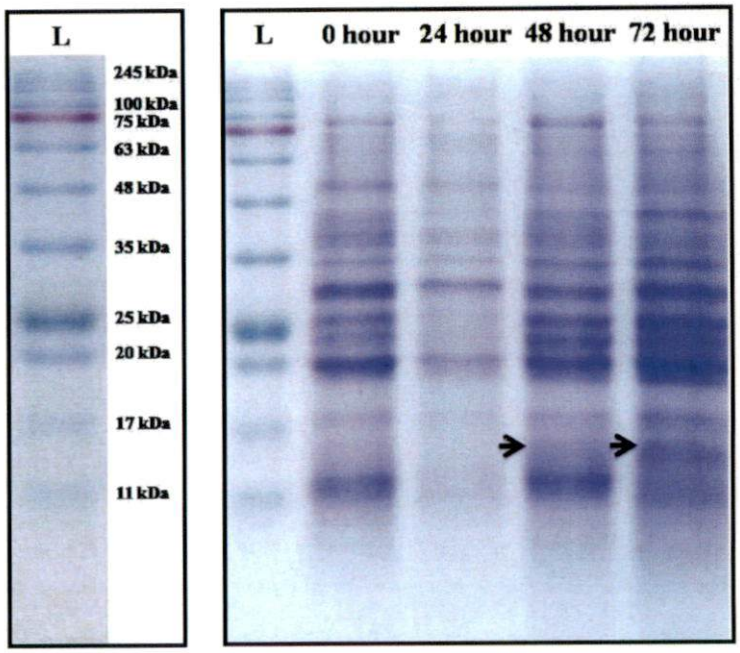


Plate 4.7 (D): SDS-PAGE protein profile of CKCC 41 at different inoculation periods

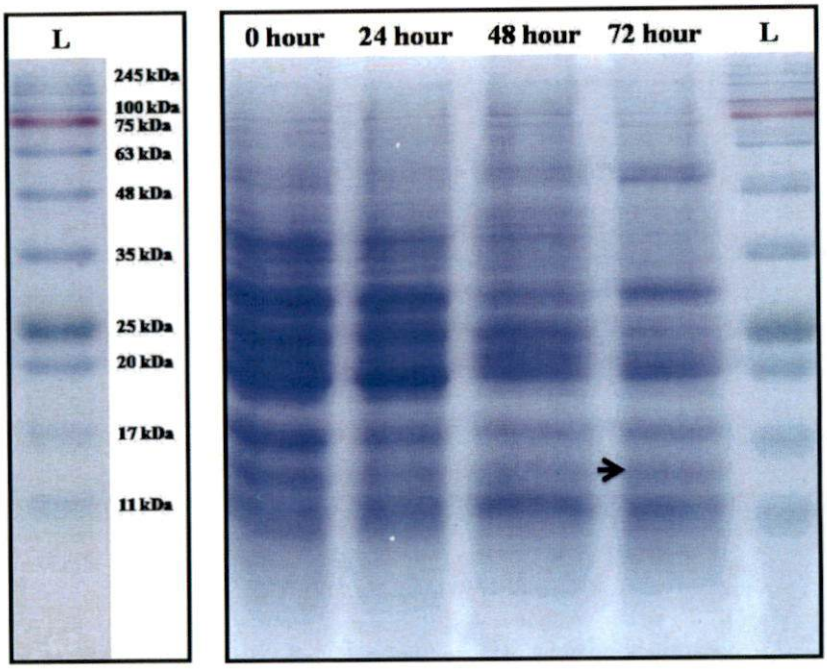


Plate 4.7 (E): SDS-PAGE protein profile of CKCC 60 at different inoculation periods

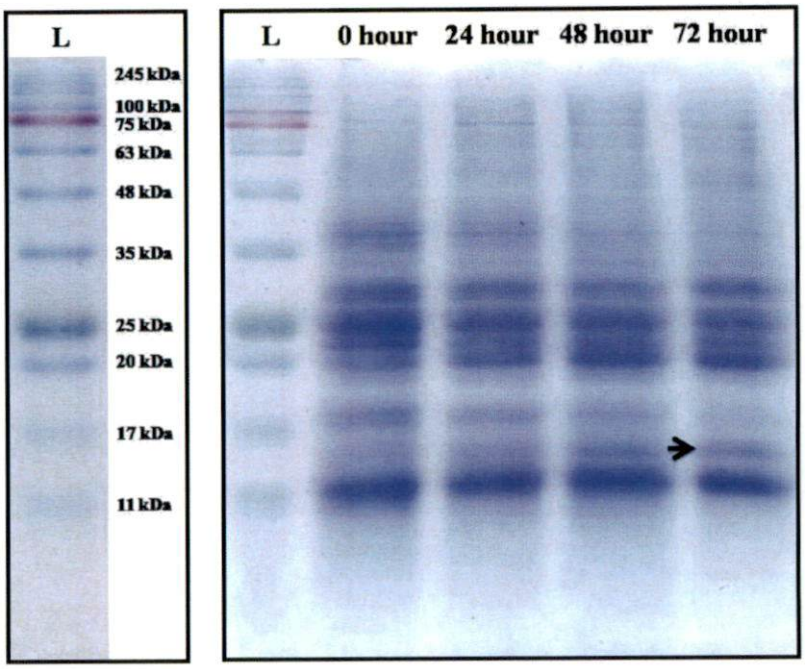


Plate 4.7 (F): SDS-PAGE protein profile of CKCC 61 at different inoculation periods

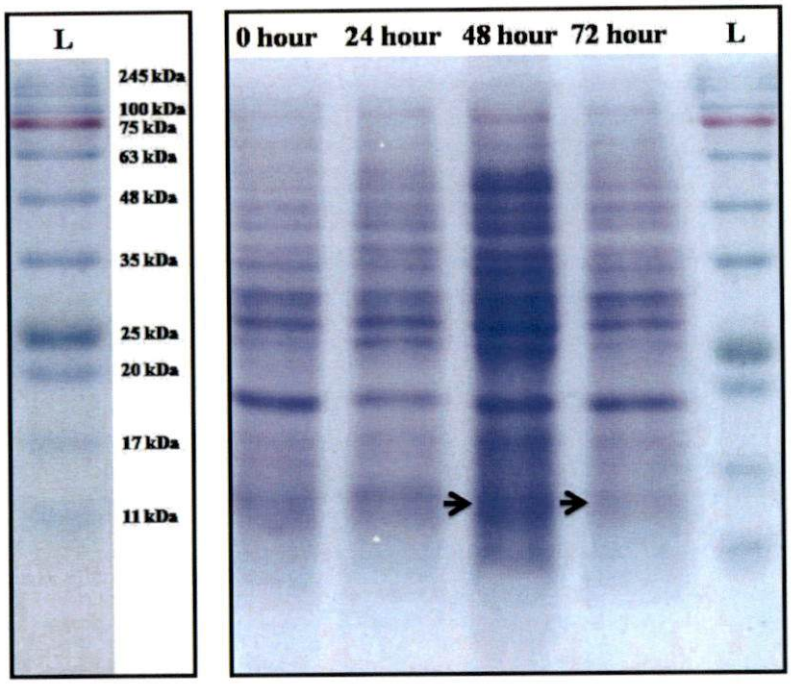


Plate 4.7 (G): SDS-PAGE protein profile of KLCC 86 at different inoculation periods

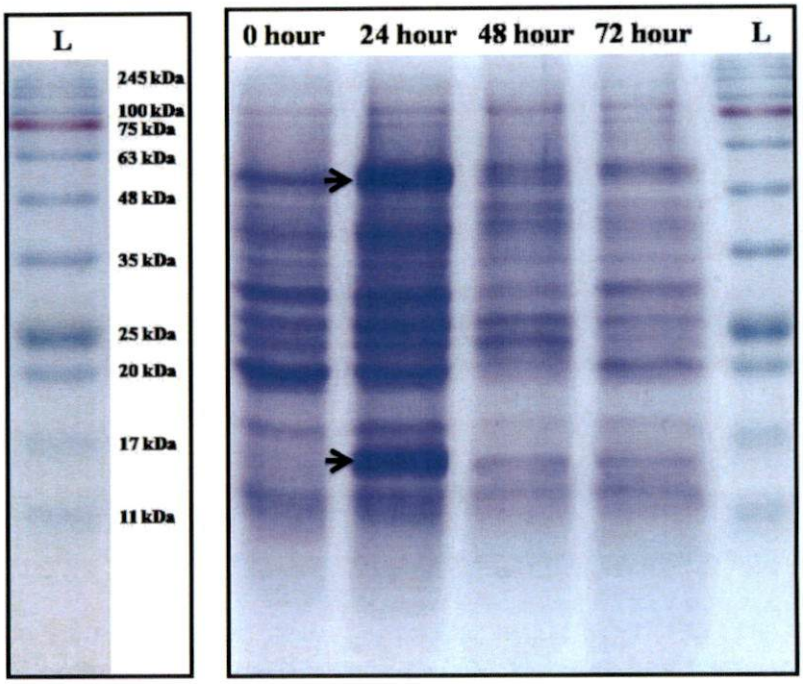


Plate 4.7 (H): SDS-PAGE protein profile of KLCC 89 at different inoculation periods

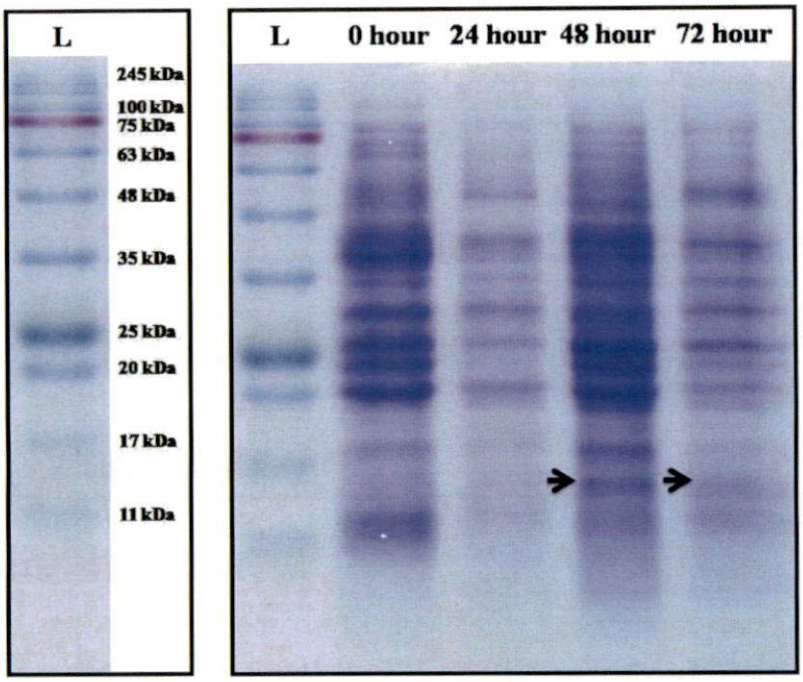


Plate 4.7 (I): SDS-PAGE protein profile of KLCC 119 at different inoculation periods

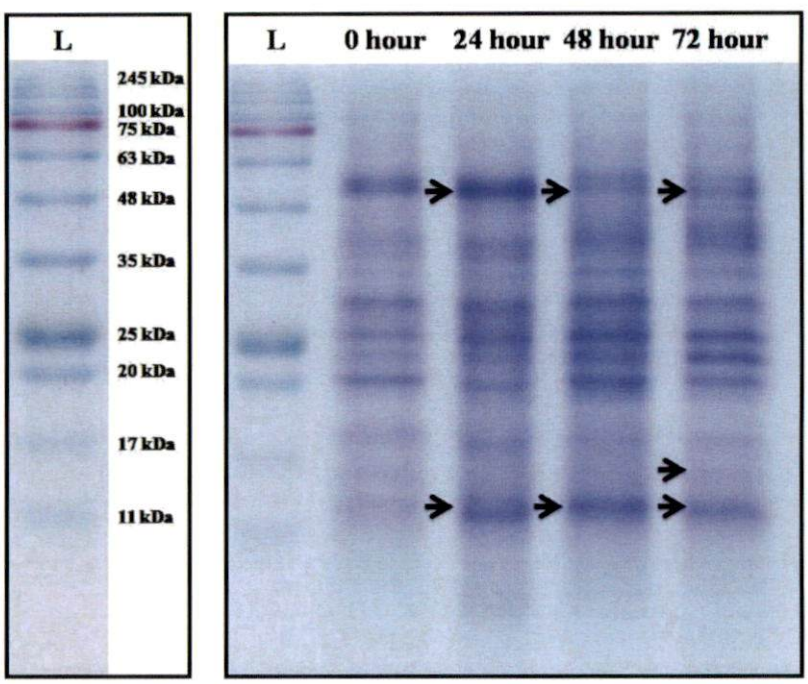


Plate 4.7 (J): SDS-PAGE protein profile of KLCC 133 at different inoculation periods

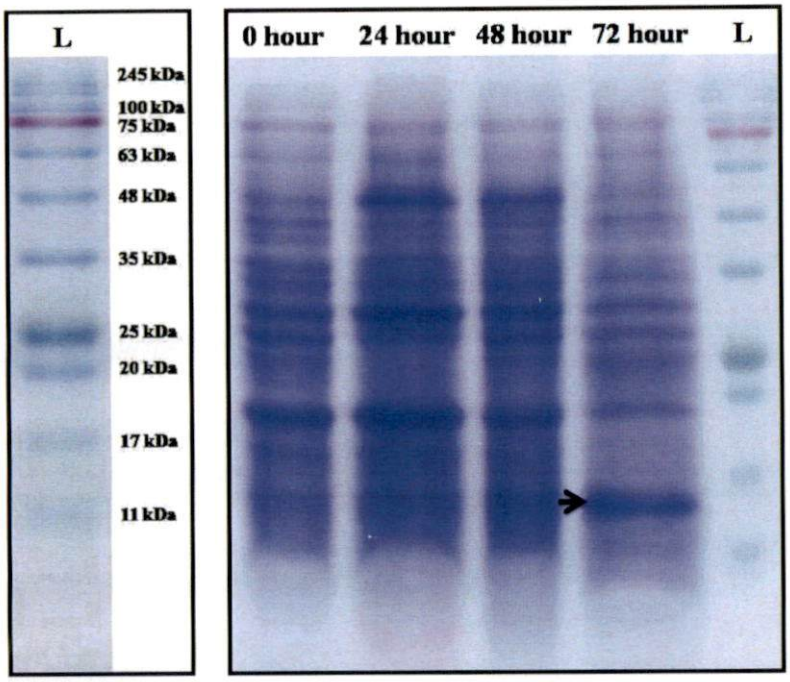


Plate 4.7 (K): SDS-PAGE protein profile of KLCC 134 at different inoculation periods

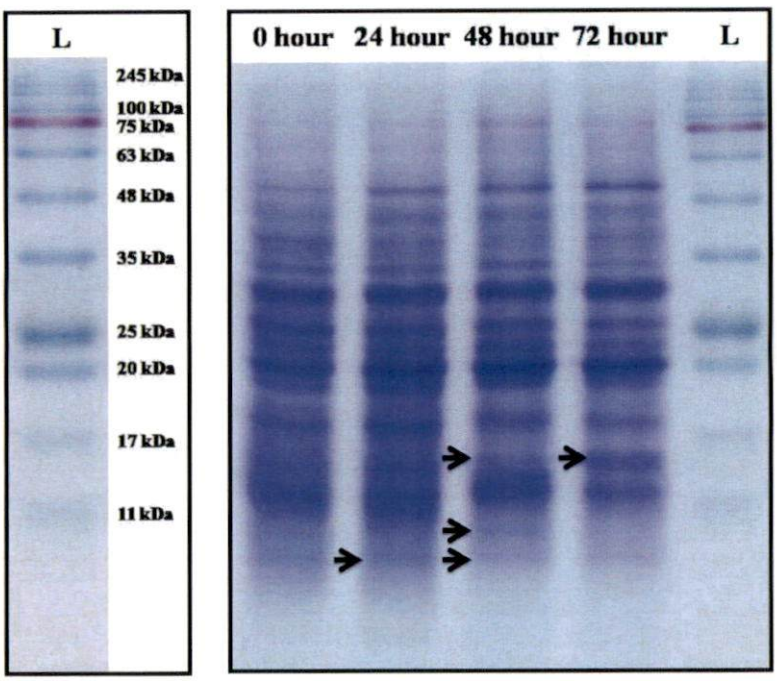


Plate 4.7 (L): SDS-PAGE protein profile of Panniyur-1 at different inoculation periods

4.5. PROTEOME ANALYSIS OF MOST TOLERANT AND SUSCEPTIBLE GENOTYPE BY 2D-GEL ELECTROPHORESIS

2D-gel electrophoresis was carried out in most tolerant calliclone alongwith susceptible variety Panniyur-1 to generate a complete protein profile containing unique protein signatures that were differentially expressed in susceptible genotype and tolerant calliclone. The calliclone KLCC 89 showed the maximum specific activity of β-1,3-glucanase at 24 hours after inoculation among all other calliclones and Panniyur-1. Presence of an over-expressed protein band of 16.5 kDa in the SDS-PAGE gel was also observed in the calliclone KLCC 89. Hence, KLCC 89 was selected as tolerant calliclone and Panniyur-1 as susceptible genotype.

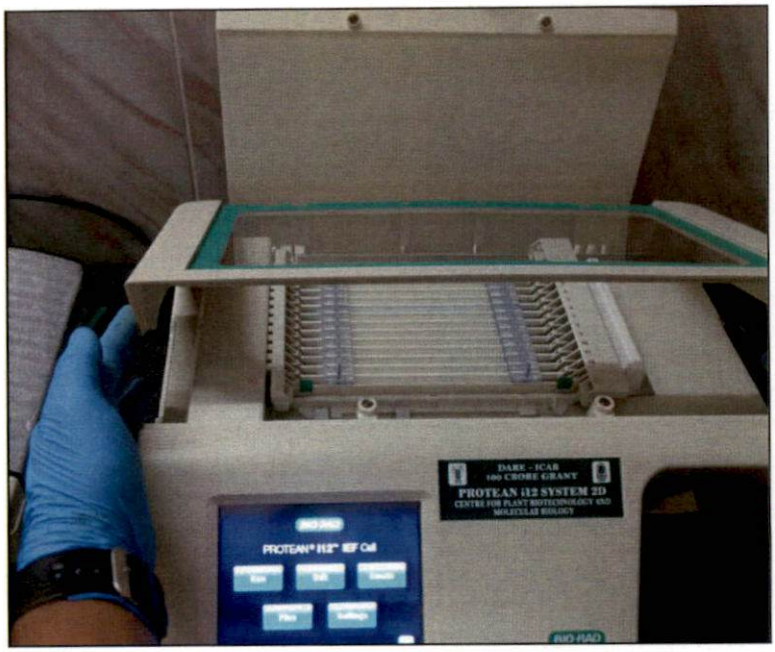
As disease progression was highest at 24 hours after inoculation, inoculated leaf samples of tolerant calliclone KLCC 89 and susceptible Panniyur-1 at 0 hours and 24 hours after inoculation were selected for total protein isolation protocol for 2D-gel electrophoresis. Lysis buffer (containing CHAPS, DTT, Thiourea and Urea) method of extraction of protein for 2DE analysis gave successful results with maximum number of protein spots in the resolving gel. Size of the protein pellet obtained was also more when 200 µL of lysis buffer was used for 0.2 g sample. Twice washing of TCA precipitated protein pellet with ice cold acetone containing 0.07 per cent 2-mercaptoethanol gave good quality, pure white protein pellet. After dissolution of protein pellet in rehydration buffer, centrifugation at 10000-12000 rpm for 5 minutes at 4°C separated insoluble impurities from the mixture, the supernatant of which when used for 2DE analysis gave ample number spots in the gel. The moistening of electrode wicks in Bio-Rad Proteomics grade water gave maximum number of protein spots which was not evident when autoclaved millipore water was used for moistening electrode wicks. Using 17 cm Bio-Rad IPG strips for isoelectric focusing showed distinct visualization of individual protein spots in the gel. Attainment of successful 2DE results was achievable when the entire process was performed in a room maintained at 16-20°C temperature.

The two major steps of 2D gel electrophoresis are visualized in Plate 4.8 A and Plate 4.8 B. The gel images scanned by calibrated densitometer of all the 2D gels are given in Plates 4.9 A to 4.9 D.

The analysis of scanned 2DE gels using Bio-Rad PDQUEST software showed high variability in the number of protein spots expressed in tolerant calliclone KLCC 89 and susceptible variety Panniyur-1 at 0 hours and 24 hours after inoculation (Table 4.6). The maximum protein spots were detected in the KLCC 89 at 24 hours (939 spots). Panniyur-1 recorded a number of 313 spots expressed at 0 hours after inoculation which later increased to 772 spots at 24 hours. Similarly for KLCC 89 that recorded 590 spots at 0 hours after inoculation which later increased to 939 spots at 24 hours. At 0 hours after inoculation, 277 spots were differentially expressed in KLCC 89 as compared to Panniyur-1. At 24 hours after inoculation, 167 spots were differentially expressed in KLCC 89 as compared to Panniyur-1. In Panniyur-1, compared to 0 hours after inoculation, about 459 spots were differentially expressed at 24 hours after inoculation with *Phythophthora capsici*. In case of KLCC 89, compared to 0 hours after inoculation, about 349 spots were differentially expressed at 24 hours after inoculation. About 141 protein spots were found commonly expressed in susceptible variety Panniyur-1 and tolerant calliclone KLCC 89 at 24 hours after inoculation with *P. capsici*.

Table 4.6: Number of expressed protein spots detected by the PDQUEST software

Protein sample	**Total no. of protein spots (in 12 % resolving gel)**		**No. of spots differentially expressed at 24 hours after inoculation**
	0 hours after inoculation	**24 hours after inoculation**	
Panniyur-1	313	772	459
KLCC 89	590	939	349
No. of spots differentially expressed in KLCC 89 as compared to Panniyur-1	277	167	



(A)

(B)

Plate 4.8: Two dimensional gel electrophoresis: (A) Isoelectric focusing (First dimensional gel electrophoresis) (B) Second dimensional gel electrophoresis

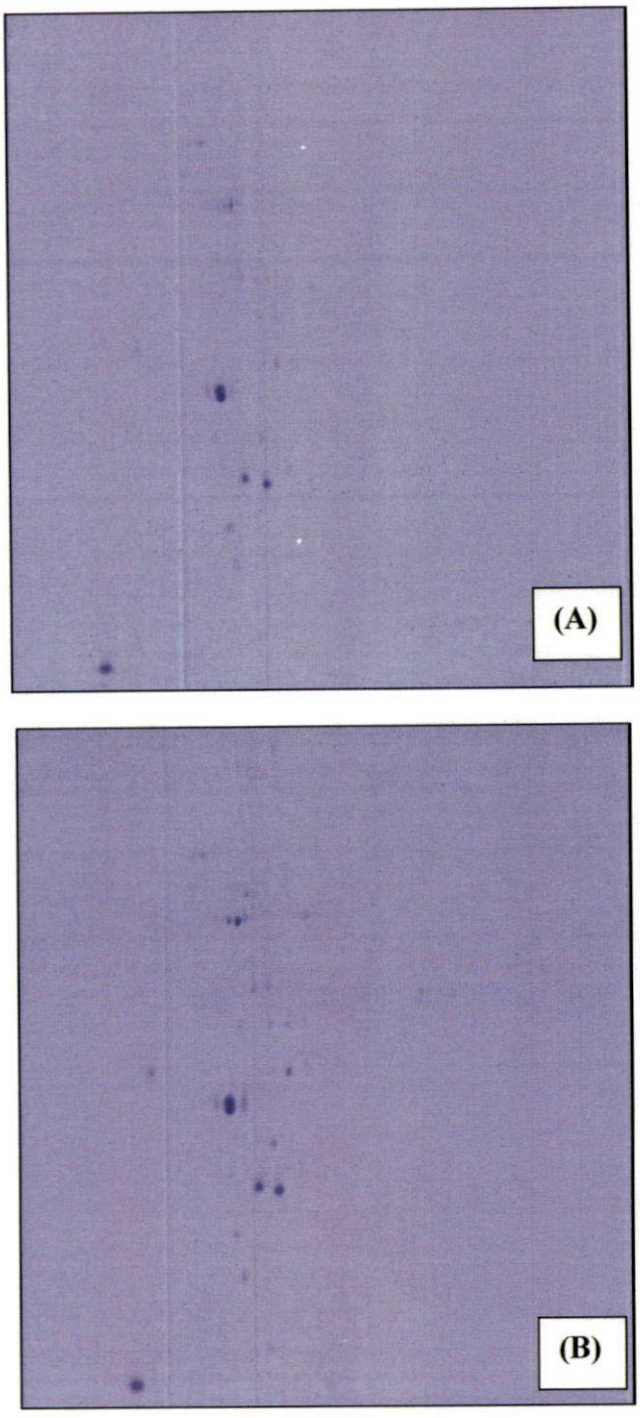


Plate 4.9: Two dimensional gel electrophoretic profile of total protein in Panniyur-1 at (A) 0 hour (B) 24 hours after inoculation

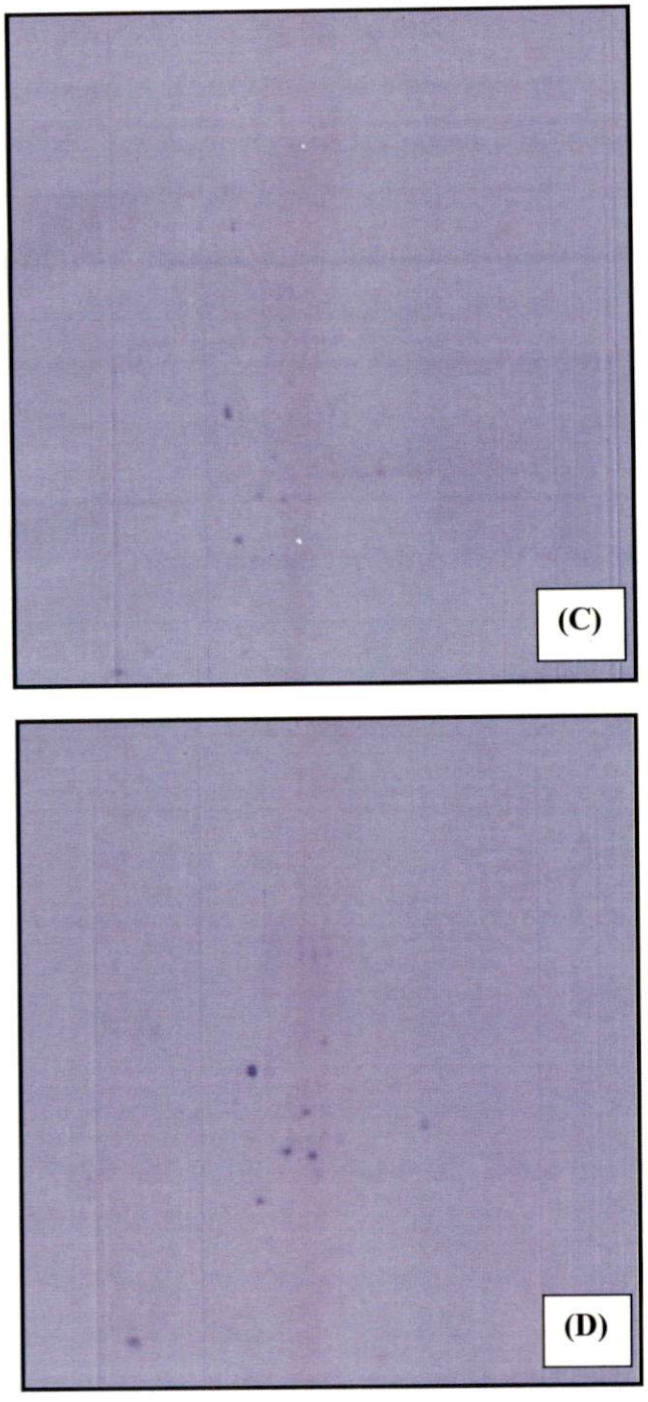


Plate 4.9 contd.: Two dimensional gel electrophoretic profile of total protein in KLCC 89 at (C) 0 hour (D) 24 hours after inoculation

On the basis of detected protein spots by PDQUEST software, 167 protein spots were differentially expressed in KLCC 89 at 24 hours after inoculation. Further analysis using PDQUEST software was made to generate quantity table report tabulating all the protein spots with their respective SSP number allotted by the software and the degree of change in expression of these proteins. From the quantity table report, four protein spots were selected as differentially expressed in tolerant calliclone KLCC 89 with higher degree of change in expression and spots were designated as Spot 1, Spot 2, Spot 3 and Spot 4 (Table 4.7). These four protein spots were selected for MALDI-TOF analysis (Plate 4.10).

Table 4.7: Details of protein spots selected for MALDI-TOF analysis

Spot name	SSP number	Ratio for differential expression
Spot 1	1003	44.0
Spot 2	4202	23.7
Spot 3	3104	23.1
Spot 4	5301	14.5

4.6. PEPTIDE MASS FINGERPRINTING BY MALDI-TOF / MS ANALYSIS

For MALDI-TOF analysis, the four selected protein spots were excised from the gel and sent to Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram. The centre performed mass spectrometric analysis and provided with a set of peptide molecular masses with their corresponding peaks of the proteins from all the four spots. The mass spectrometric data were used as input file for MASCOT server developed by Matrix Science that helped to identify top protein hits from a protein database, characterize and quantify proteins based upon peak data. The peak data plots for four spots based on MALDI-TOF / MS analysis are given in Plates 4.11 A to 4.11 D. The MASCOT search results are given in Table 4.8. The details of identified proteins are enlisted in Table 4.9.

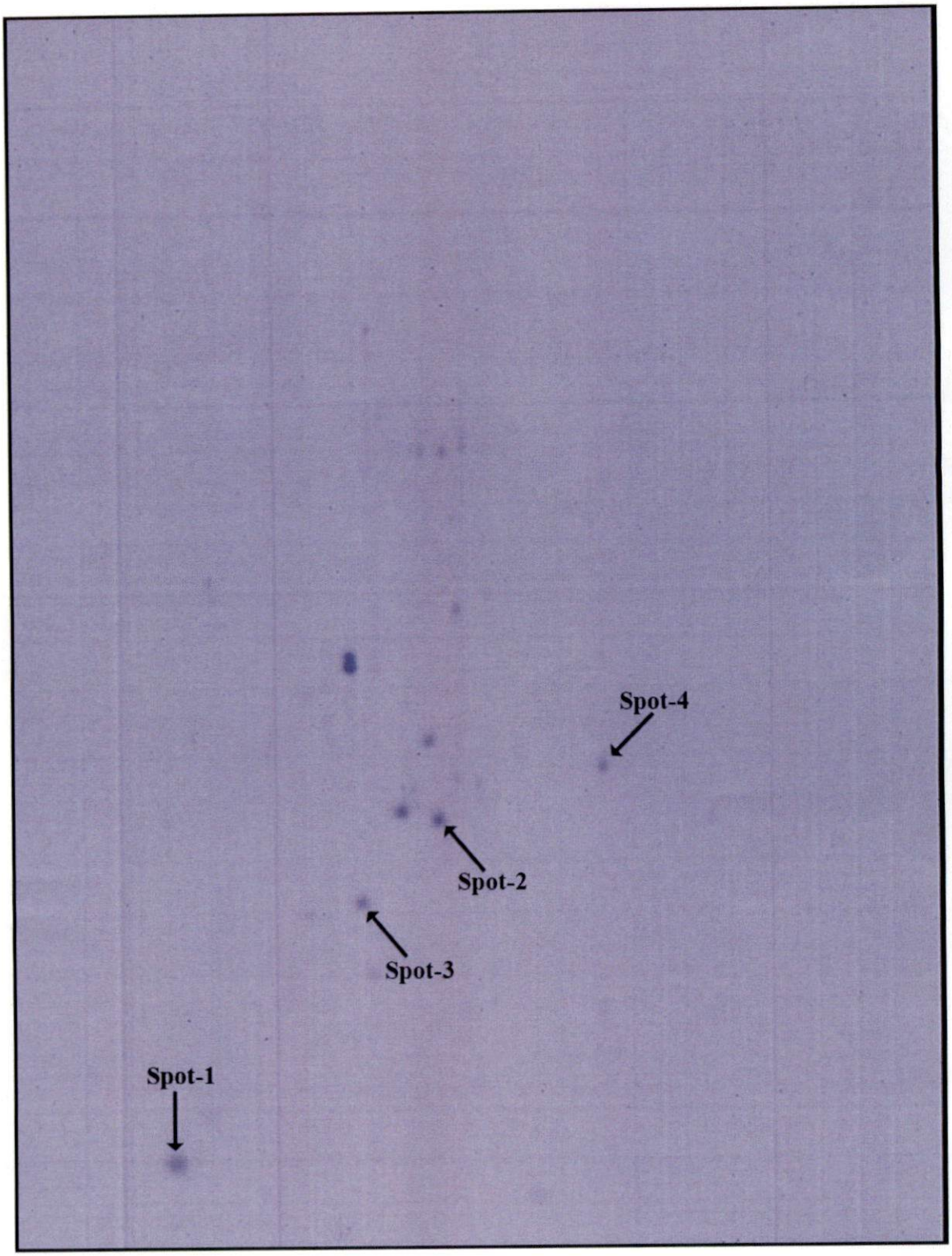


Plate 4.10: Protein spots selected for MALDI-TOF analysis as analyzed by Bio-Rad PDQUEST software

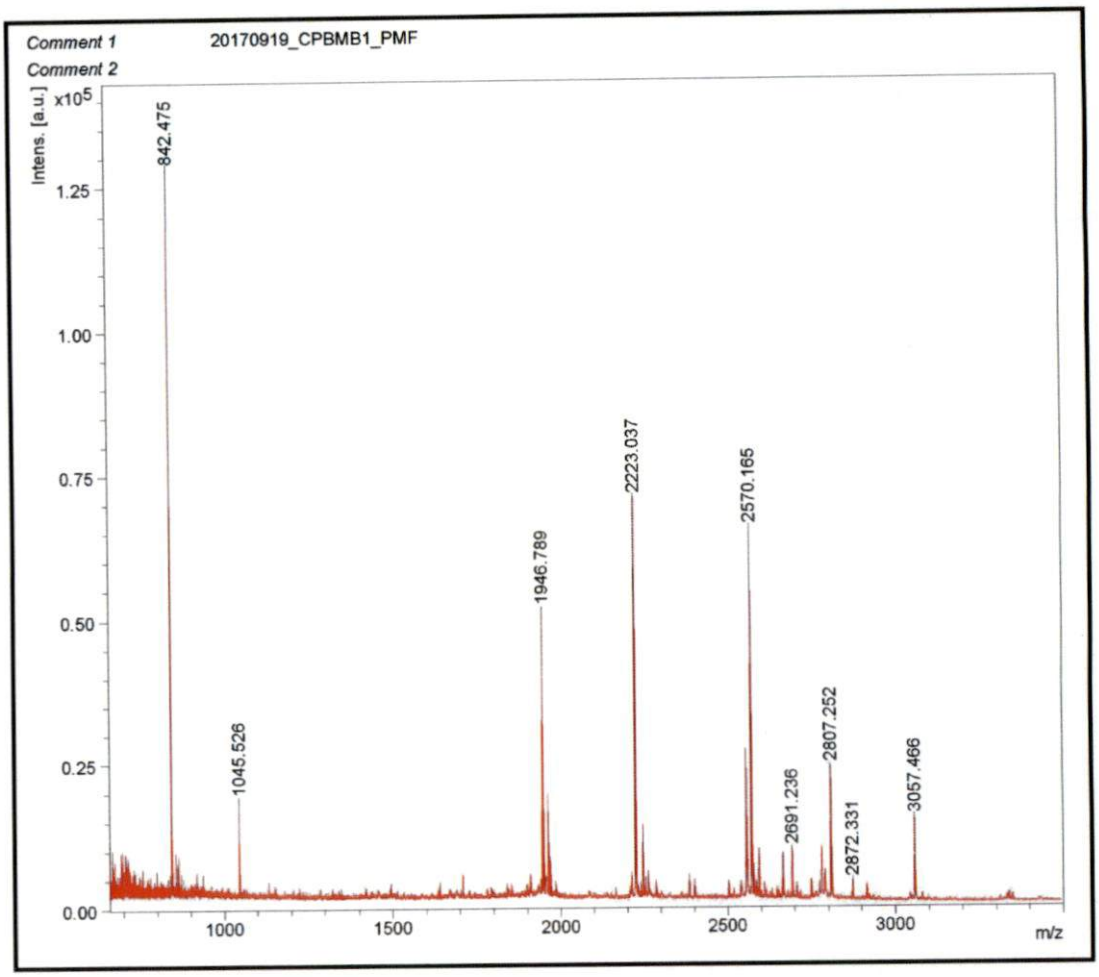


Plate 4.11 (A): Peak plot of Spot 1 based on MALDI-TOF / MS analysis

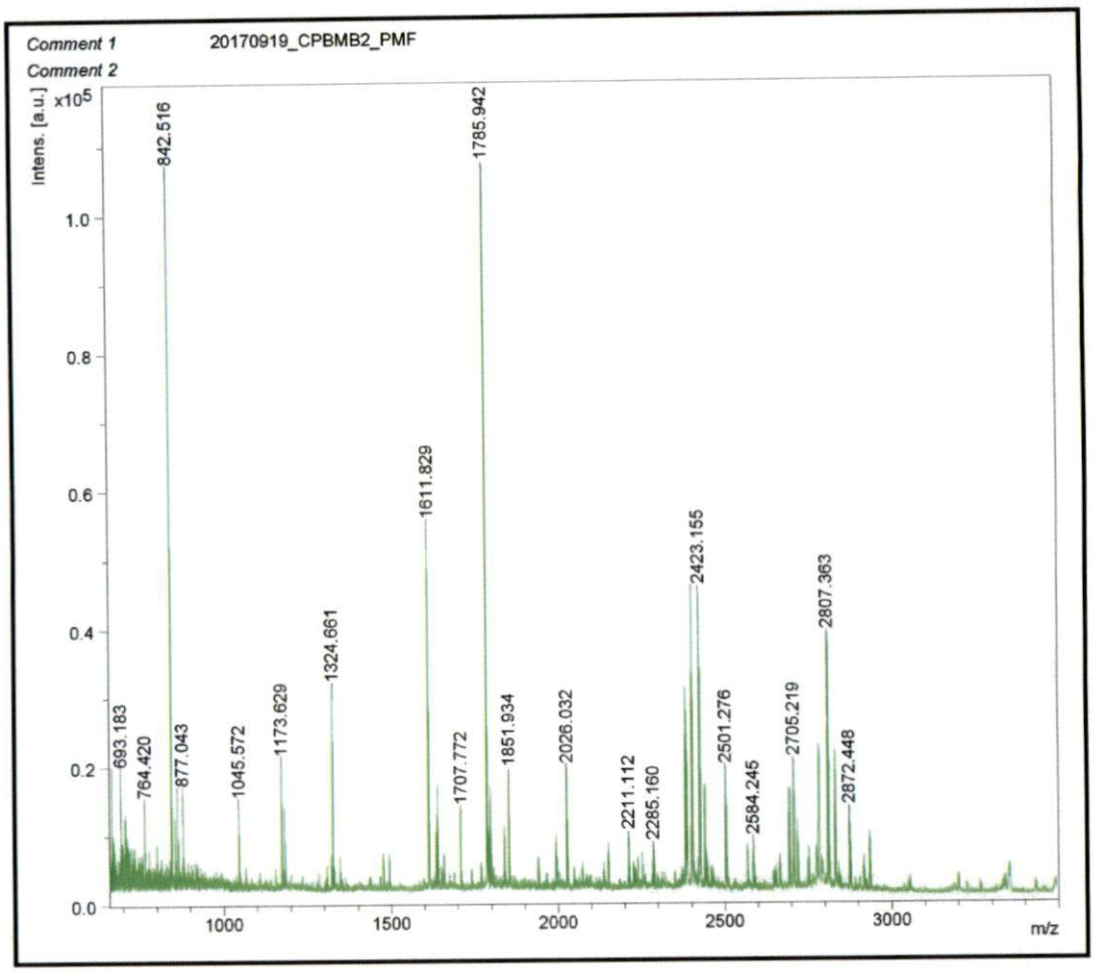


Plate 4.11 (B): Peak plot of Spot 2 based on MALDI-TOF / MS analysis

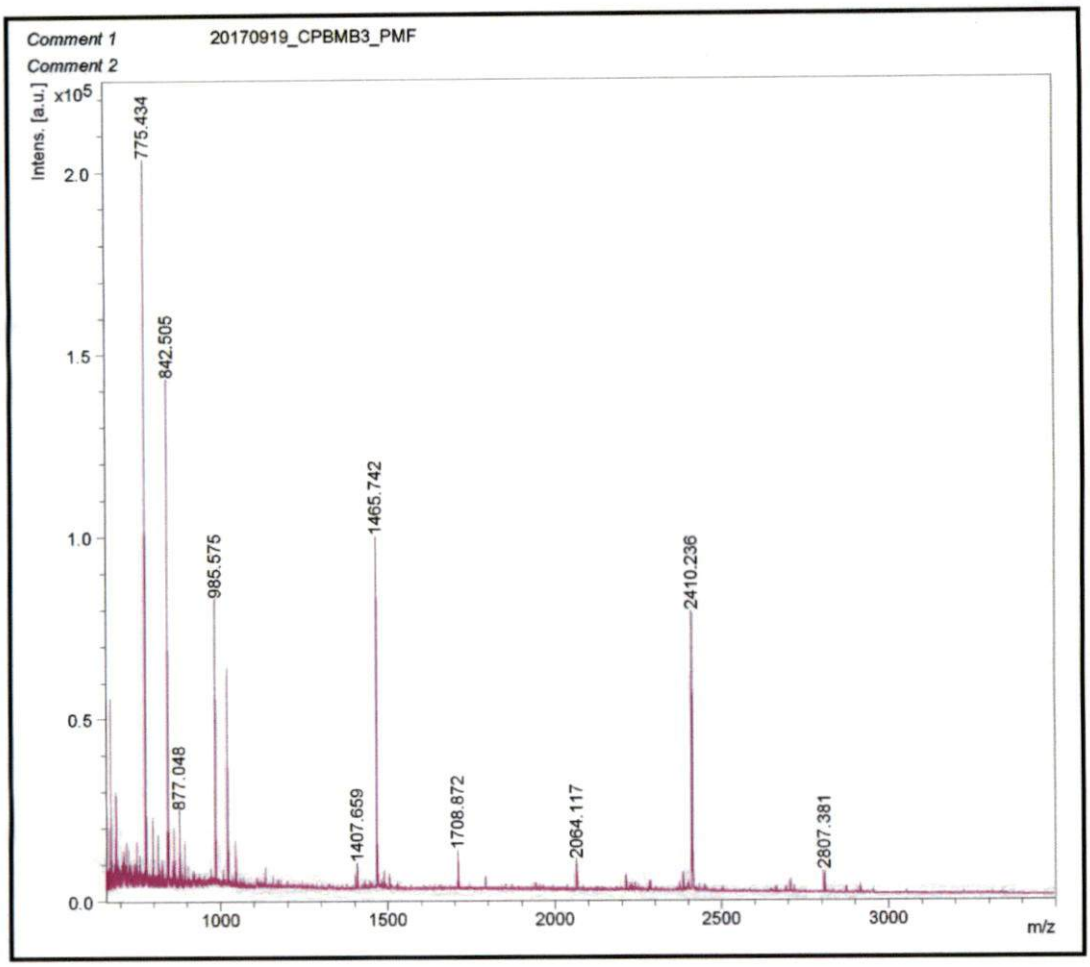


Plate 4.11 (C): Peak plot of Spot 3 based on MALDI-TOF / MS analysis

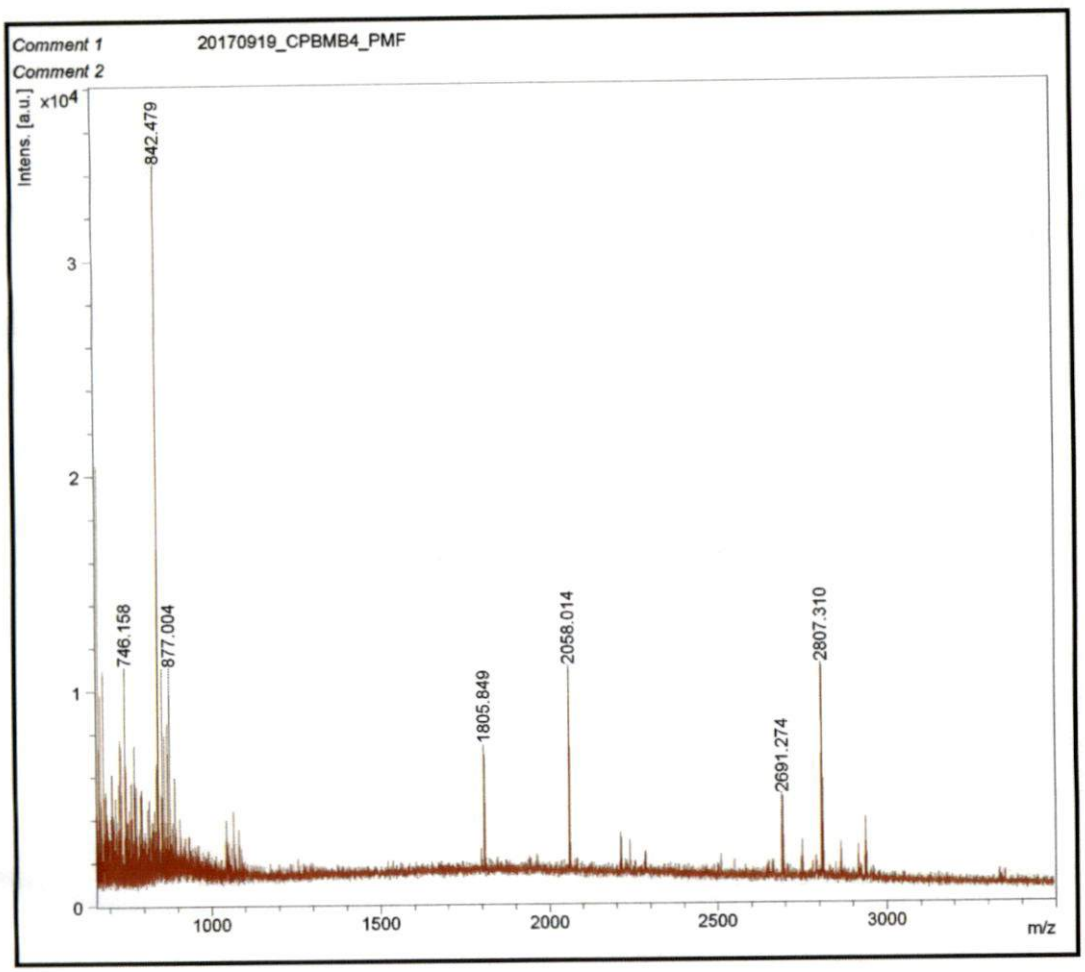


Plate 4.11 (D): Peak plot of Spot 4 based on MALDI-TOF / MS analysis

Table 4.8: MALDI-TOF / MS analysis of four protein spots and peptides identified

Name of protein spot	Number of peaks identified	Number of hit peptides	Hit number	Peptide sequence	Peptide mass	Score for protein hits
Spot 1 (SSP 1003)	20	3	1	GTYSFYCSPHQGAGMVGK	17045	55
			2	NNAGFPHNVVFDEDEIPAGVDVSK	17112	54
			3	EQDTGTFGAEIYIVKPAQQK	Not assigned	32
Spot 2 (SSP 4202)	43	6	1	WNPSKEVEFPGQVLR	28733	78
			2	WPPSTASSSAACPGRVR	61467	55
			3	SPKELDAVEQDIPTDK	72637	53
			4	VLQMRDQTNNVVDPR	24935	48
			5	RGDIQWLEESDILGR	54659	44
			6	ESPKQDSTVAETELRP	15139	44
Spot 3 (SSP 3104)	21	5	1	TFQGPPHGIQVER	51934	247
			2	LTYYTPEYETKDTDILAAFR	21358	242
			3	ENMAPNFLANISK + Oxidation (M)	Not assigned	53
			4	GVAGVEALLR	36567	47
			5	GVTKTVLIR	Not assigned	44
Spot 4 (SSP 5301)	16	1	1	LGAMHICFADDLLMFCK + Oxidation (M)	Not assigned	24

Table 4.9: MASCOT search analysis of hit protein sequences for the four protein spots

Name of protein spot	Hit no.	Accession no. of protein hit	Protein	Organism	Name of gene	Function
Spot 1 (SSP 1003)	1	A0A1S4A1K3	Plastocyanin	*Nicotiana tabacum*	*LOC107792758*	Transfer electron between P700 and cytochrome b6-f complex in PS-I
	2	A0A1S4BSK7	Plastocyanin	*Nicotiana tabacum*	*LOC107811439*	
	3	F4JZS4	TRAF-like family protein	*Arabidopsis thaliana*	*At5g26290*	Mediate ABA-related drought stress signaling
Spot 2 (SSP 4202)	1	A0A1S3XWU9	Oxygen-evolving enhancer protein 2-1, chloroplastic like	*Nicotiana tabacum*	*LOC107769475*	Optimize water splitting in PS II by optimizing Ca^{2+} and Cl^- levels
	2	Q0J1F6	Os09g0443000 protein	*Oryza sativa* subsp. *japonica*	*Os09g0443000*	Mediate protein ubiquitination for degradation by 26S proteasome, associated with signal transduction and cell cycle regulation
	3	C7J6M3	Os09g0382120 protein	*Oryza sativa* subsp. *japonica*	*Os09g0382120*	Transposase like protein with DNA binding function, regulate plant growth and development. Regulate gene expression by recruiting other cellular factors

Table 4.9: Continued

	4	A0A1P8B2C3	L-aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase	*Arabidopsis thaliana*	*At2g02765*	Catalyze post-translational modification of target proteins by phosphopantetheinyl pathway
	5	Q7EY28	Os07g0231900 protein	*Oryza sativa* subsp. *japonica*	*Os07g0231900*	Uncharacterized
	6	Q0JHH8	Os01g0863100 protein	*Oryza sativa* subsp. *japonica*	*Os01g0863100*	Mediate retrotransposition
Spot 3 (SSP 3104)	1	A0A075M4T0	Ribulose bisphosphate carboxylase large chain (Fragment)	*Nicotiana tabacum*	*rbcL*	Regulate photorespiration and pentose-phosphate cycle
	2	A0A075E2T3	Ribulose bisphosphate carboxylase large chain (Fragment)	*Piper nigrum*	*rbcL*	
	3	A0A1S4AP71	Proteasome-associated protein ECM29 homolog	*Nicotiana tabacum*	*LOC107799855*	Inhibit 26S proteasome, thus preventing protein degradation

Table 4.9: Continued

	4	A0A1S4B4X8	Uncharacterized protein LOC107804580	*Nicotiana tabacum*	*LOC107804580*	Uncharacterized
	5	A0A1S4CNL8	Uncharacterized protein LOC107820978 isoform X3	*Nicotiana tabacum*	*LOC107820978*	Uncharacterized
Spot 4 (SSP 5301)	1	A0A1S3XP38	Uncharacterized protein LOC107767279	*Nicotiana tabacum*	*LOC107767279*	Uncharacterized

4.7. ANALYSIS OF IDENTIFIED PROTEINS THROUGH *IN SILICO* APPROACH

For *in silico* analysis of the identified protein hits of the four protein spots, the amino acid sequences of the protein hits were retrieved from the UNIPROT database in "fasta" format. The 15 sequences in fasta format were used as input file to perform BLAST2GO analysis. The sequences identified by BLAST2GO, sequence names, amino acid sequence lengths, blast similarity means and blast top hits taxonomy names are detailed in Table 4.10. The various results report obtained from the BLAST2GO analysis are detailed in following paragraphs.

For the 15 sequences analyzed through BLAST2GO tool, blast hits and InterPro scan results were obtained for all the 15 sequences. Out of 15 sequences, only ten sequences were mapped and eight sequences were GO (Gene Ontology) annotated by the software (Figure 4.2). The elaborative explanation of all the results *i.e.* Blast results, Enzyme Code distribution results, InterPro scan results, Gene Ontology (GO) results and the KEGG pathway results are given in the following paragraphs.

In the Blast result, the species distribution of 15 sequences showed that, eight sequences belong to the *Nicotiana tabacum* followed by four sequences to *Oryza sativa* japonica group and three sequences to *Nicotiana sylvestris*. One sequence belonged to *Nicotiana benthamiana* and *Piper nigrum* each (Figure 4.3).

The Enzyme code (EC) distribution results showed that, Hit-4 from Spot 2 belonged to EC class transferases involved in transfer of 4-phosphopantetheine moiety from coenzyme A (CoA) to an invariant serine in an acyl carrier protein (ACP) involved in fatty acid biosynthesis. Hit-1 and Hit-2 from Spot 3 belonged to EC class lyases involved in carboxylation of ribulose-1,5-biphosphate during carbon dioxide fixation by green plants (Figure 4.4).

The InterPro scan results revealed functional domains present in the input protein hit sequences (Table 4.11) and the family of proteins these sequences belong to (Table 4.12). The Spot 1 corresponded to functional domains such as blue (type-1) copper

Figure 4.2: Protein hit sequences analyzed using BLAST2GO

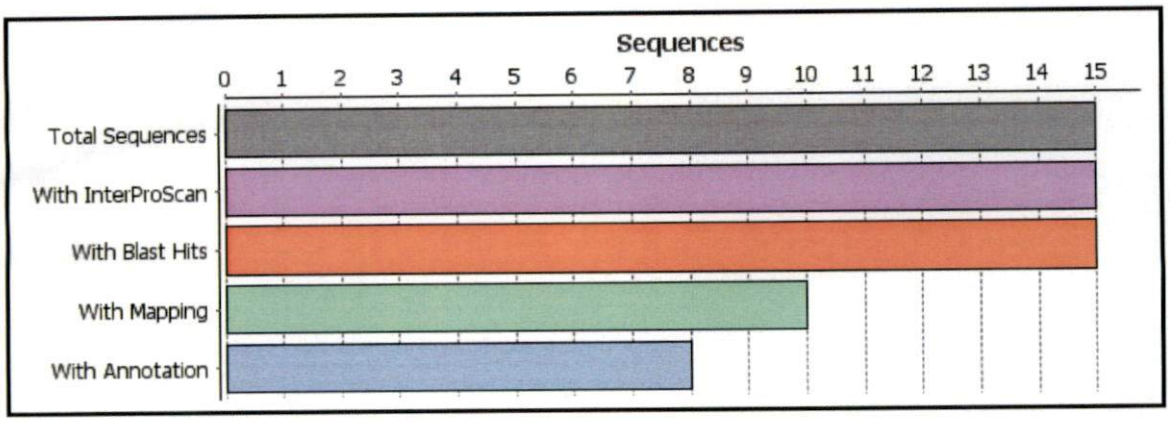


Figure 4.3: Species distribution of hit protein sequences analyzed using BLAST2GO

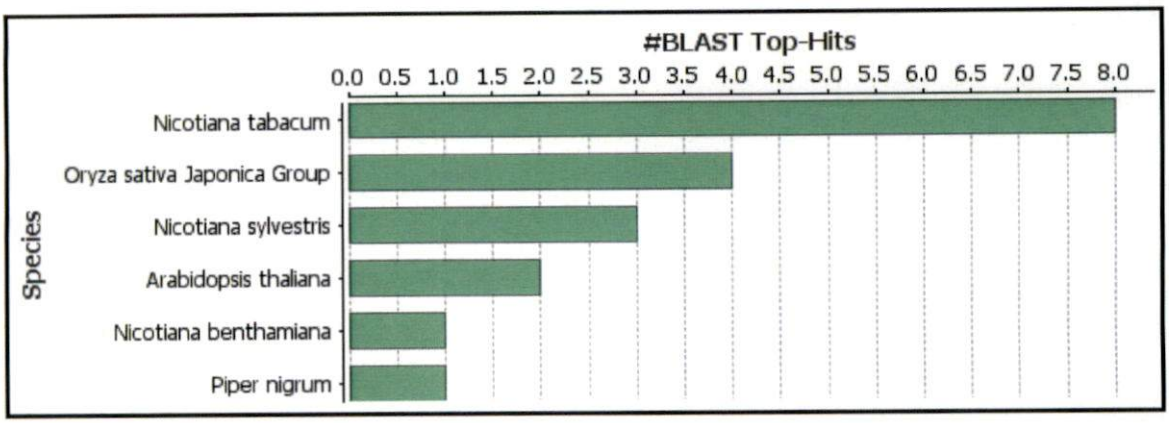
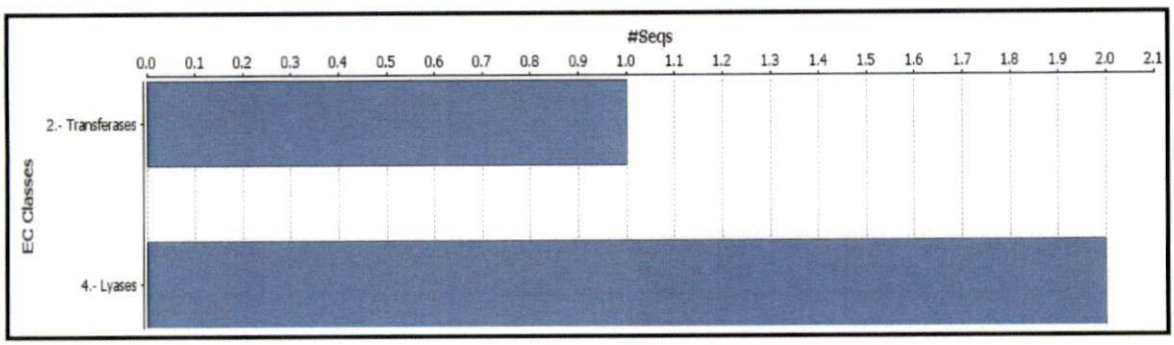


Figure 4.4: Enzyme Code (EC) allotted for sequences analyzed using BLAST2GO



domain, cupredoxin domain, TRAF-like and MATH / TRAF domain. Spot 2 corresponded to domains such as zinc finger BED-type domain and C2H2 type domain, HAT domain, PDZ domain, leucine-rich repeat domain, 4,-phosphopantetheinyl transferase super family, peptidase S1 domain, ribonuclease H-like domain, F-box domain and Mog1/PsbP, alpha/beta/alpha sandwich domain. Spot 3 corresponded to domains such as RUBISCO large subunit C-terminal domain, RUBISCO large subunit ferrodoxin-like N-terminal domain and Armadillo-type fold domain. Spot 4 corresponded to Reverse transcriptase domain. The protein family distribution showed that Spot 1 corresponded to plastocyanin protein family, Spot 2 corresponded to PsbP family and Spot 3 corresponded to RUBISCO and Proteasome component Ecm 29 protein families.

In general, the Gene Ontology (GO) results revealed involvement of identified proteins in different biological processes, metabolic activities and differentially located in the cell (Table 4.13). Proteins in Spot 1 were analyzed to be involved in metabolic process and single-organism process. It was involved in electron carrier activity and binding activity. It was localized at cell, cell part, organelle, organelle part, membrane, membrane part, cell junction, extracellular region and the symplast. Proteins in Spot 2 were involved in metabolic process, single-organism process, cellular process and biological regulation process. It was involved in nucleic acid binding transcription factor activity and catalytic activity. It was localized at cell, cell part, organelle, organelle part, membrane, membrane part and macromolecular complex. Proteins in Spot 3 were involved in metabolic process, single-organism process and cellular process. It was involved in binding and catalytic activity. It was localized at cell, cell part and organelle.

Gene Ontology annotation results critically revealed about involvement of identified proteins in different biological processes (Table 4.14) their cellular localization (Table 4.15) and their metabolic functions (Table 4.16). Proteins in Spot 1 is responsible for oxidation-reduction process. It is localized in chloroplast, thylakoid membrane of chloroplast, plasmodesma and extracellular region. It regulates copper ion binding and electron carrier activity. Proteins in Spot 2 is responsible for regulation of transcription from RNA polymerase II promoter, photosynthesis, lysine biosynthetic process *via*

aminoadipic acid and protein phosphopantetheinylation. It is localized in mitochondrion, chloroplast thylakoid membrane at PS-II oxygen evolving complex, nucleus and as extrinsic component of membrane. It mediates calcium and magnesium ion binding, transcription factor activity, DNA binding activities and protein dimerization activity. Proteins in Spot 3 is responsible for regulation of oxidation-reduction process, reductive pentose-phosphate cycle and photorespiration. It is localized in the chloroplast. It is responsible for magnesium ion binding, ribulose-biphosphate carboxylase activity and monooxygenase activity.

The KEGG pathway analysis results with three sequences having assigned enzyme codes showed that two sequences (Hit-1 and Hit-2) corresponding to Spot 3 are involved in three different biological pathways *i.e* Glyoxylate and Dicarboxylate metabolism, carbon fixation in photosynthetic organisms and biosynthesis of antibiotics (Table 4.17). Hit-4 sequence corresponding to Spot 2 is involved in the Pantothenate and CoA biosynthesis pathway. The KEGG pathways for the three sequences are given in Plates 4.12 A to 4.12 D. KEGG pathway analysis showed that Spot 3 was involved in conversion of D-Ribulose-1,5-bisphosphate into Phosphoglycolate in Glyoxylate and Dicarboxylate metabolic pathway and carbon fixation process in photosynthetic organisms. This reveals that Spot 3 was involved in regulation of photorespiration that is a source of light dependent reactive oxygen species (ROS) production responsible for plant defense. Spot 3 was involved in conversion of D-Ribulose-1,5-bisphosphate to glycerate-3-phosphate in carbon fixation mechanism by green plants indicating that the temporary occurrence of enhanced photosynthesic activity to meet the nutrient requirements due to competition from the pathogen. Spot 3 was also found regulating antibiotic biosynthesis and biosynthetic pathway of secondary metabolites through KEGG pathway analysis. Spot 2 was involved in conversion of Coenzyme A (CoA) to Acyl-carrier protein in Pantothenate and CoA biosynthetic pathway which might be responsible for production of antimicrobial metabolites.

Table 4.10: Protein identification of 15 hit sequences analyzed using BLAST2GO

Assigned Name	Protein Description	Sequence Length (Number of amino acids)	Blast Similarity Mean (%)	Blast Top Hit Taxonomy Name
Spot 1 (SSP 1003) Hit 1	Plastocyanin B B	167	90.20	*Nicotiana sylvestris* / *Nicotiana tabacum*
Spot 1 (SSP 1003) Hit 2	Plastocyanin A A	169	91.80	*Nicotiana tabacum*
Spot 1 (SSP 1003) Hit 3	TRAF-like family	336	83.75	*Arabidopsis thaliana*
Spot 2 (SSP 4202) Hit 1	Oxygen-evolving enhancer 2-chloroplastic	268	93.25	*Nicotiana sylvestris* / *Nicotiana benthamiana* / *Nicotiana tabacum*
Spot 2 (SSP 4202) Hit 2	F-box FBD LRR-repeat At1g13570-like	554	70.65	*Oryza sativa* Japonica Group
Spot 2 (SSP 4202) Hit 3	Zinc finger BED domain-containing RICESLEEPER 1-like	634	70.20	*Oryza sativa* Japonica Group
Spot 2 (SSP 4202) Hit 4	4-phosphopantetheinyl transferase superfamily	217	84.95	*Arabidopsis thaliana*
Spot 2 (SSP 4202) Hit 5	PREDICTED: Uncharacterized protein LOC4342779	481	66.65	*Oryza sativa* Japonica Group
Spot 2 (SSP 4202) Hit 6	Retrotransposon Ty3-gypsy subclass	129	68.40	*Oryza sativa* Japonica Group
Spot 3 (SSP 3104) Hit 1	Ribulose 1,5-bisphosphate carboxylase oxygenase large subunit (chloroplast)	464	99.60	*Nicotiana sylvestris* / *Nicotiana tabacum*
Spot 3 (SSP 3104) Hit 2	Ribulose-1,5-bisphosphate carboxylase oxygenase large (chloroplast)	191	99.30	*Piper nigrum*

Table 4.10: Continued

Spot 3 (SSP 3104) Hit 3	Proteasome-associated ECM29 homolog isoform X1	1740	95.40	*Nicotiana tabacum*
Spot 3 (SSP 3104) Hit 4	PREDICTED: Uncharacterized protein LOC107804580	318	70.55	*Nicotiana tabacum*
Spot 3 (SSP 3104) Hit 5	PREDICTED: Uncharacterized protein LOC107820978 isoform X3	184	85.65	*Nicotiana tabacum*
Spot 4 (SSP 5301) Hit 1	PREDICTED: Uncharacterized protein LOC107767279	283	68.45	*Nicotiana tabacum*

Table 4.11: Protein domain distribution in hit sequences as analyzed by InterPro scan using BLAST2GO

Hit sequences	IPS domain ID with domain name
Spot 1 (SSP 1003) Hit 1, Spot 1 (SSP 1003) Hit 2	(IPR000923) Blue (type 1) copper domain
Spot 3 (SSP 3104) Hit 2, Spot 3 (SSP 3104) Hit 1	(IPR000685) Ribulose bisphosphate carboxylase, large subunit, C-terminal
Spot 1 (SSP 1003) Hit 1, Spot 1 (SSP 1003) Hit 2	(IPR008972) Cupredoxin
Spot 3 (SSP 3104) Hit 2, Spot 3 (SSP 3104) Hit 1	(IPR017443) Ribulose bisphosphate carboxylase, large subunit, ferrodoxin-like N-terminal
Spot 2 (SSP 4202) Hit 3	(IPR003656) Zinc finger, BED-type
Spot 2 (SSP 4202) Hit 3	(IPR013087) Zinc finger C2H2-type
Spot 2 (SSP 4202) Hit 5	(IPR001478) PDZ domain
Spot 3 (SSP 3104) Hit 3	(IPR011989) Armadillo-like helical
Spot 1 (SSP 1003) Hit 3	(IPR008974) TRAF-like
Spot 2 (SSP 4202) Hit 2	(IPR032675) Leucine-rich repeat domain, L domain-like
Spot 3 (SSP 3104) Hit 3	(IPR016024) Armadillo-type fold
Spot 4 (SSP 5301) Hit 1	(IPR000477) Reverse transcriptase domain
Spot 2 (SSP 4202) Hit 4	(IPR008278) 4'-phosphopantetheinyl transferase superfamily
Spot 2 (SSP 4202) Hit 5	(IPR009003) Peptidase S1, PA clan
Spot 2 (SSP 4202) Hit 3	(IPR012337) Ribonuclease H-like domain
Spot 2 (SSP 4202) Hit 1	(IPR016123) Mog1/PsbP, alpha/beta/alpha sandwich
Spot 2 (SSP 4202) Hit 2	(IPR001810) F-box domain
Spot 1 (SSP 1003) Hit 3	(IPR002083) MATH/TRAF domain
Spot 2 (SSP 4202) Hit 3	(IPR008906) HAT, C-terminal dimerisation domain

Table 4.12: Protein family distribution in hit sequences as analyzed by InterPro scan using BLAST2GO

Hit sequences	IPS family ID with family name
Spot 1 (SSP 1003) Hit 1, Spot 1 (SSP 1003) Hit 2	(IPR001235) Blue (type 1) copper protein, plastocyanin-type
Spot 1 (SSP 1003) Hit 1, Spot 1 (SSP 1003) Hit 2	(IPR002387) Plastocyanin
Spot 3 (SSP 3104) Hit 1	(IPR033966) RuBisCO
Spot 2 (SSP 4202) Hit 1	(IPR002683) PsbP family
Spot 3 (SSP 3104) Hit 1	(IPR020888) Ribulose bisphosphate carboxylase large subunit, type I
Spot 3 (SSP 3104) Hit 3	(IPR024372) Proteasome component Ecm29

Table 4.13: GO distribution for the protein hit sequences analyzed using BLAST2GO

GO ID	GO term	Hit sequences
GO:0008152	Metabolic process	Spot 2 (SSP 4202) Hit 1, Spot 1 (SSP 1003) Hit 1, Spot 2 (SSP 4202) Hit 4, Spot 1 (SSP 1003) Hit 2, Spot 3 (SSP 3104) Hit 2, Spot 2 (SSP 4202) Hit 3, Spot 3 (SSP 3104) Hit 1
GO:0009987	Cellular process	Spot 2 (SSP 4202) Hit 1, Spot 2 (SSP 4202) Hit 4, Spot 3 (SSP 3104) Hit 2, Spot 2 (SSP 4202) Hit 3, Spot 3 (SSP 3104) Hit 1
GO:0044699	Single-organism process	Spot 1 (SSP 1003) Hit 1, Spot 2 (SSP 4202) Hit 4, Spot 1 (SSP 1003) Hit 2, Spot 3 (SSP 3104) Hit 2, Spot 3 (SSP 3104) Hit 1
GO:0065007	Biological regulation	Spot 2 (SSP 4202) Hit 3

GO ID	GO term	Hit sequences
GO:0005488	Binding	Spot 2 (SSP 4202) Hit 1, Spot 1 (SSP 1003) Hit 1, Spot 1 (SSP 1003) Hit 2, Spot 2 (SSP 4202) Hit 4, Spot 2 (SSP 4202) Hit 3, Spot 3 (SSP 3104) Hit 2, Spot 3 (SSP 3104) Hit 1
GO:0003824	Catalytic activity	Spot 2 (SSP 4202) Hit 4, Spot 3 (SSP 3104) Hit 2, Spot 3 (SSP 3104) Hit 1
GO:0009055	Electron carrier activity	Spot 1 (SSP 1003) Hit 1, Spot 1 (SSP 1003) Hit 2
GO:0001071	Nucleic acid binding transcription factor activity	Spot 2 (SSP 4202) Hit 3

Table 4.13: Continued

GO ID	GO term	Hit sequences
GO:0044464	Cell part	Spot 2 (SSP 4202) Hit 1, Spot 1 (SSP 1003) Hit 3, Spot 1 (SSP 1003) Hit 1, Spot 2 (SSP 4202) Hit 4, Spot 1 (SSP 1003) Hit 2, Spot 3 (SSP 3104) Hit 2, Spot 2 (SSP 4202) Hit 3, Spot 3 (SSP 3104) Hit 1
GO:0043226	Organelle	Spot 2 (SSP 4202) Hit 1, Spot 1 (SSP 1003) Hit 3, Spot 1 (SSP 1003) Hit 1, Spot 2 (SSP 4202) Hit 4, Spot 1 (SSP 1003) Hit 2, Spot 3 (SSP 3104) Hit 2, Spot 2 (SSP 4202) Hit 3, Spot 3 (SSP 3104) Hit 1
GO:0005623	Cell	Spot 2 (SSP 4202) Hit 1, Spot 1 (SSP 1003) Hit 3, Spot 1 (SSP 1003) Hit 1, Spot 2 (SSP 4202) Hit 4, Spot 1 (SSP 1003) Hit 2, Spot 3 (SSP 3104) Hit 2, Spot 2 (SSP 4202) Hit 3, Spot 3 (SSP 3104) Hit 1
GO:0016020	Membrane	Spot 2 (SSP 4202) Hit 1, Spot 1 (SSP 1003) Hit 3, Spot 1 (SSP 1003) Hit 1, Spot 1 (SSP 1003) Hit 2
GO:0044422	Organelle part	Spot 2 (SSP 4202) Hit 1, Spot 1 (SSP 1003) Hit 1, Spot 1 (SSP 1003) Hit 2
GO:0044425	Membrane part	Spot 2 (SSP 4202) Hit 1, Spot 1 (SSP 1003) Hit 3
GO:0032991	Macromolecular complex	Spot 2 (SSP 4202) Hit 1
GO:0030054	Cell junction	Spot 1 (SSP 1003) Hit 3
GO:0005576	Extracellular region	Spot 1 (SSP 1003) Hit 3
GO:0055044	Symplast	Spot 1 (SSP 1003) Hit 3

Table 4.14: GO annotation for the protein sequences involved in various biological processes analyzed using BLAST2GO

Hit sequences	Biological process
Spot 1 (SSP 1003) Hit 1, Spot 1 (SSP 1003) Hit 2, Spot 3 (SSP 3104) Hit 2, Spot 3 (SSP 3104) Hit 1	Oxidation-reduction process
Spot 3 (SSP 3104) Hit 2, Spot 3 (SSP 3104) Hit 1	Reductive pentose-phosphate cycle
Spot 2 (SSP 4202) Hit 3	Regulation of transcription from RNA polymerase II promoter
Spot 3 (SSP 3104) Hit 1	Photorespiration
Spot 2 (SSP 4202) Hit 1	Photosynthesis
Spot 2 (SSP 4202) Hit 4	Lysine biosynthetic process via aminoadipic acid
Spot 2 (SSP 4202) Hit 4	Protein phosphopantetheinylation

Table 4.15: GO annotation for the protein sequences for cellular localization analyzed using BLAST2GO

Hit sequences	Cellular localization
Spot 1 (SSP 1003) Hit 3, Spot 3 (SSP 3104) Hit 2, Spot 3 (SSP 3104) Hit 1	Chloroplast
Spot 2 (SSP 4202) Hit 1, Spot 1 (SSP 1003) Hit 1, Spot 1 (SSP 1003) Hit 2	Chloroplast thylakoid membrane
Spot 2 (SSP 4202) Hit 1	Photosystem II oxygen evolving complex
Spot 1 (SSP 1003) Hit 3	Anchored component of membrane
Spot 1 (SSP 1003) Hit 3	Plasmodesma
Spot 1 (SSP 1003) Hit 3	Extracellular region
Spot 2 (SSP 4202) Hit 3	Nucleus
Spot 2 (SSP 4202) Hit 1	Extrinsic component of membrane
Spot 2 (SSP 4202) Hit 4	Mitochondrion

Table 4.16: GO annotation for the protein sequences for the metabolic functions analyzed using BLAST2GO

Hit sequences	Metabolic function
Spot 2 (SSP 4202) Hit 4, Spot 3 (SSP 3104) Hit 2, Spot 3 (SSP 3104) Hit 1	Magnesium ion binding
Spot 3 (SSP 3104) Hit 2, Spot 3 (SSP 3104) Hit 1	Ribulose-bisphosphate carboxylase activity
Spot 1 (SSP 1003) Hit 1, Spot 1 (SSP 1003) Hit 2	Copper ion binding
Spot 3 (SSP 3104) Hit 2, Spot 3 (SSP 3104) Hit 1	Monooxygenase activity
Spot 1 (SSP 1003) Hit 1, Spot 1 (SSP 1003) Hit 2	Electron carrier activity
Spot 2 (SSP 4202) Hit 3	Transcription factor activity, sequence-specific DNA binding
Spot 2 (SSP 4202) Hit 4	Holo-[acyl-carrier-protein] synthase activity
Spot 2 (SSP 4202) Hit 3	RNA polymerase II regulatory region sequence-specific DNA binding
Spot 2 (SSP 4202) Hit 3	Protein dimerization activity
Spot 2 (SSP 4202) Hit 1	Calcium ion binding

Table 4.17: KEGG pathway analysis of three protein hit sequences with EC codes analyzed using BLAST2GO

Hit sequences	Name of Pathway	Pathway ID	Enzyme code (EC) – Enzyme name
Spot 3 (SSP 3104) Hit 2, Spot 3 (SSP 3104) Hit 1	Glyoxylate and dicarboxylate metabolism	map00630	EC: 4.1.1.39 - carboxylase
Spot 3 (SSP 3104) Hit 2, Spot 3 (SSP 3104) Hit 1	Carbon fixation in photosynthetic organisms	map00710	EC: 4.1.1.39 - carboxylase
Spot 2 (SSP 4202) Hit 4	Pantothenate and CoA biosynthesis	map00770	EC: 2.7.8.7 - synthase
Spot 3 (SSP 3104) Hit 2, Spot 3 (SSP 3104) Hit 1	Biosynthesis of antibiotics and secondary metabolites	map01130	EC: 4.1.1.39 - carboxylase

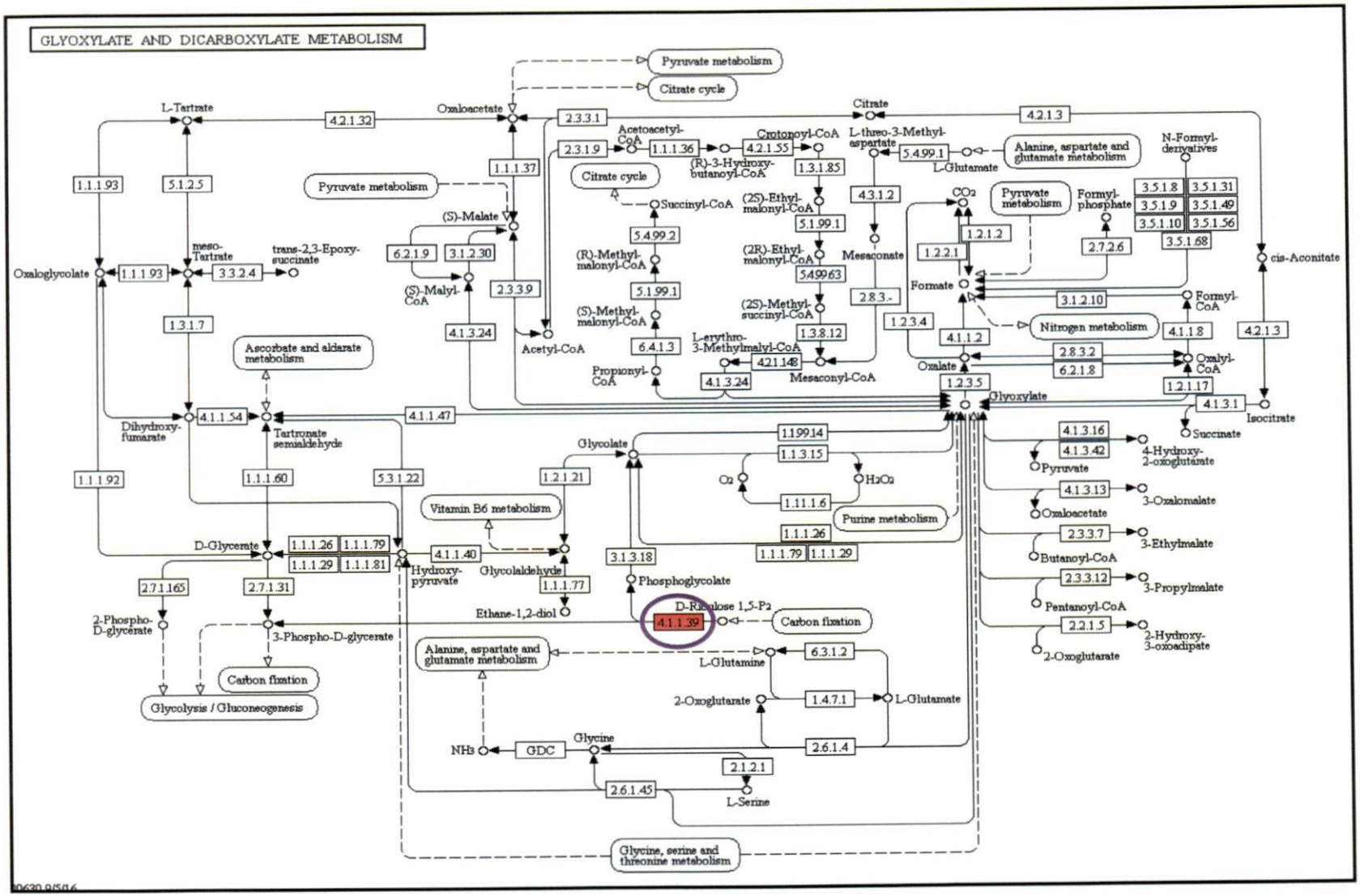


Plate 4.12 (A): KEGG pathway obtained in BLAST2GO analysis: Glyoxylate and Dicarboxylate metabolic pathway

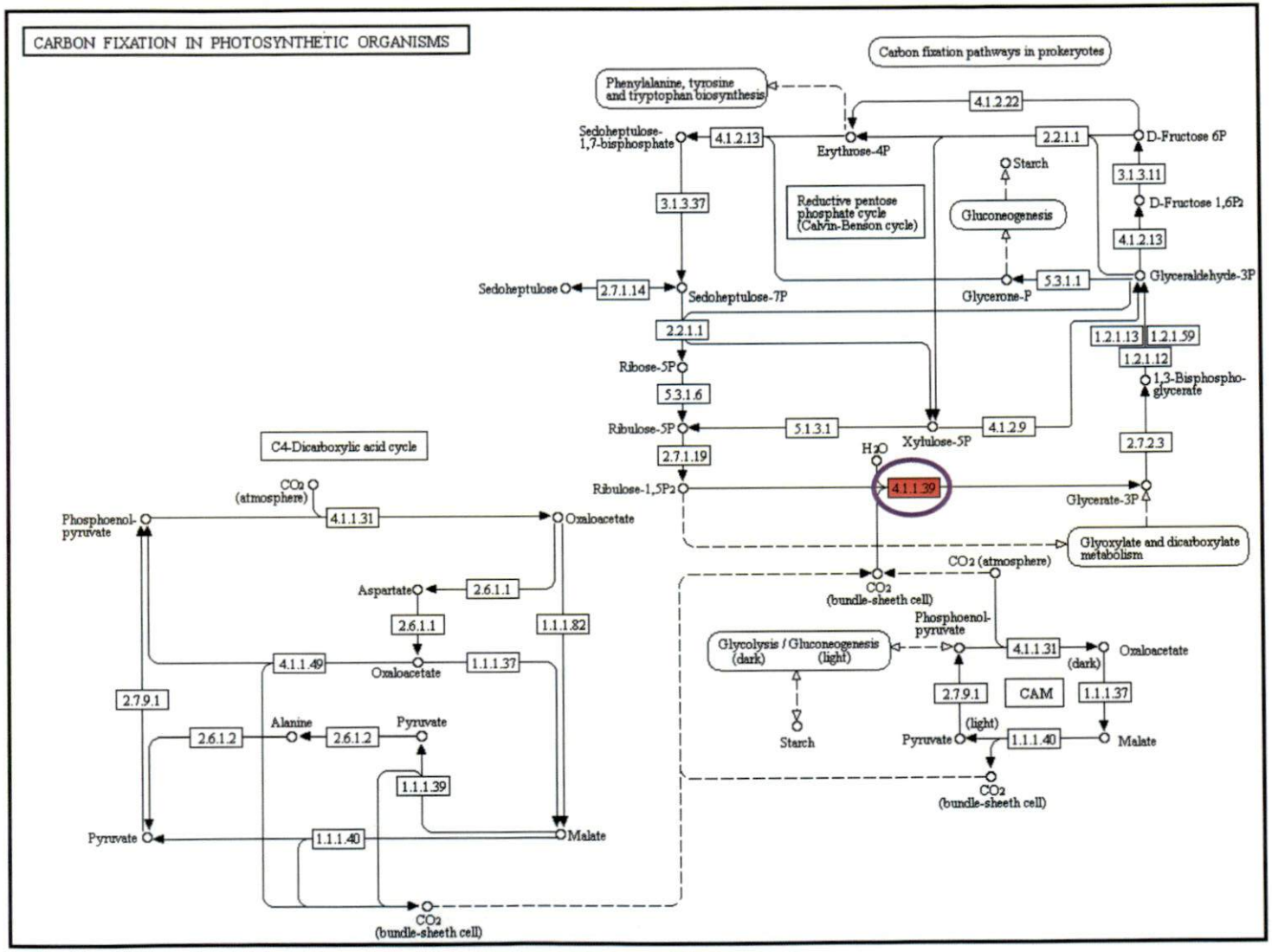


Plate 4.12 (B): KEGG pathway obtained in BLAST2GO analysis: Carbon fixation mechanism in green plants

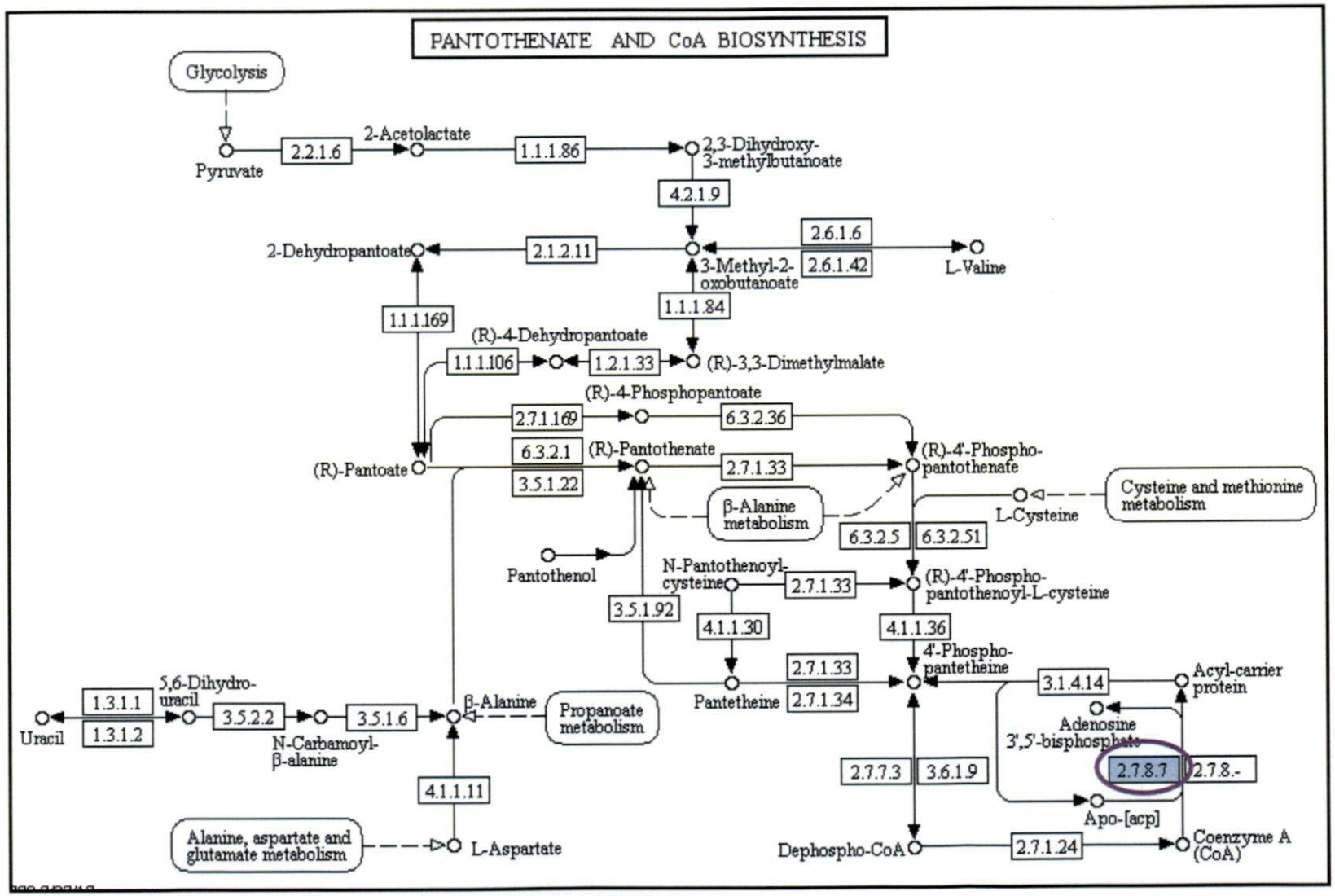


Plate 4.12 (C): KEGG pathway obtained in BLAST2GO analysis: Pantothenate and CoA biosynthesis pathway

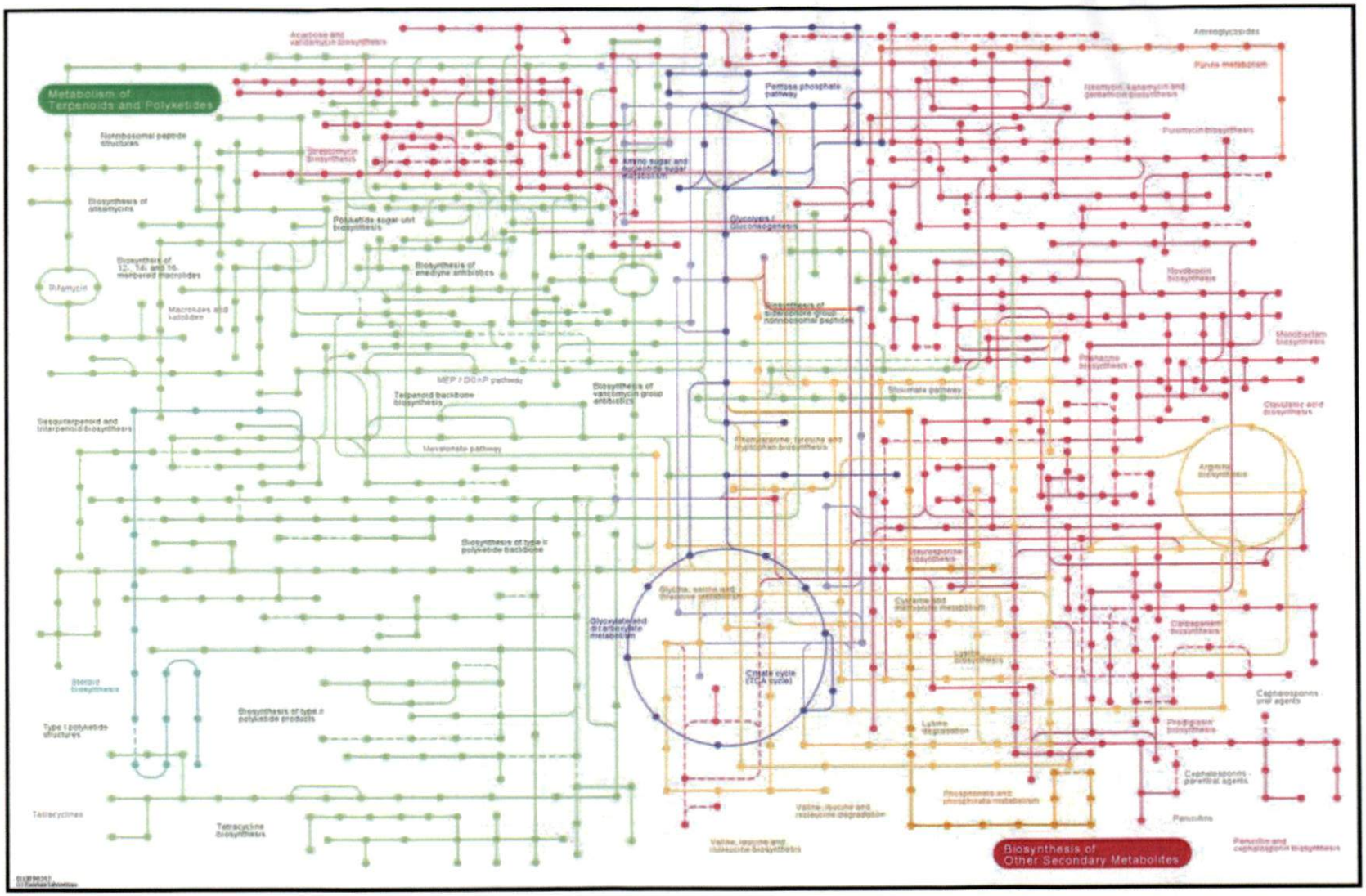


Plate 4.12 (D): KEGG pathway obtained in BLAST2GO analysis: Biosynthesis of antibiotics and secondary metabolites

Discussion

5. DISCUSSION

The investigations performed to characterize pathogenesis related proteins in selected calliclones of black pepper in relation to *Phytophthora* foot rot disease provided authenticated results that are discussed comprehensively with best supportive interpretations in this chapter.

5.1. RAISING OF ROOTED CUTTINGS

5.1.1. Basis for selection of calliclones for raising rooted cuttings

Two groups of black pepper (*Piper nigrum* L.) calliclones *i.e.* Cheriakanyakkadan group and Kalluvally group and susceptible variety Panniyur-1 were selected for raising rooted cuttings. Shylaja *et al.* (1996) performed electrolytic leakage studies and reported lowest leakage of electrolytes in Kalluvally and Cheriakanyakkadan cultivars than compared to Karimunda and Balankotta cultivars. Panniyur-1 registered 78 per cent more leakage of electrolytes when treated with concentrated culture filtrates (CCF) of *Phytophthora capsici*. This report revealed the tolerance reaction of Kalluvally and Cheriakanyakkadan cultivars against *Phytophthora* foot rot infection and confirmed the findings of Kuch and Khew (1980) and Sarma *et al.* (1988) that, Panniyur-1 and Karimunda show highly susceptible reaction to this disease. Kalluvally and Cheriakanyakkadan reported higher percentage of growing calli, confirming better performance compared to other cultivars *in vitro*. Shylaja *et al.* (2012) carried out *in vitro*, *in vivo* screening for tolerance to toxic metabolites from *P. capsici* and clonal evaluation, and selected 13 best performing calliclones *i.e.* seven from Cheriakanyakkadan group and six from Kalluvally group.

Based upon the above reports, six calliclones from Cheriakanyakkadan (CKCC 5, CKCC 41, CKCC 60, CKCC 61, CKCC 25 and CKCC 27) and five calliclones from Kalluvally (KLCC 119, KLCC 89, KLCC 133, KLCC 134 and KLCC 86) were selected for raising rooted cuttings for characterization of PR-proteins against *Phytophthora* foot rot disease.

5.1.2. Establishment of rooted cuttings

The calliclones of Cheriakanyakkadan proved to have better establishment in the nursery than calliclones of Kalluvally group and Panniyur-1. Shylaja *et al.* (2012) also reported better field establishment in Cheriakanyakkadan calliclones (52.5 %) as compared to Kalluvally calliclones (42.3 %) which might be due to presence of better root system in Cheriakanyakkadan calliclones.

Higher extent of variation was noticed in the Kalluvally calliclones with respect to rooting and establishment as compared to Cheriakanyakkadan calliclones. All Cheriakanyakkadan calliclones had higher establishment percentage above 83 per cent, whereas in Kalluvally group, only KLCC 89 recorded the highest establishment percentage (90.63 %) and other calliclones had establishment percentage values ranging from 53 to 84 per cent. The higher variability in Kalluvally calliclones might be due to the effect of genotype (Shylaja *et al.*, 1996). Daub and Jenns (1989) also reported similar elucidation in tobacco somaclones that showed higher extent of variability which was decided by the parental genotype. Iwamoto *et al.* (2007) also observed high extent of variation among the 50 calliclones of Japanese butterbur (*Petasites japonicus*) as compared to original parental line in terms of morphological characters and field performance. Another reason for higher variability in Kalluvally calliclones may be due to higher proliferation rate of the callus. Smith and Drew (1990) reported more variation in the cultures that proliferated at excessive rates.

5.2. ISOLATION OF *Phytophthora capsici* AND STUDYING THE DISEASE REACTION

For isolation of *P. capsici*, carrot agar medium was used as it is a selective medium for *Phytophthora* spp. The fungus grew profusely in the medium in the form as uniform dense, white cottony mycelium. The mycelial growth was luxurious when the culture plates were maintained at a room temperature of 25°C. Zentmyer *et al.* (1979) reported that, oospores of *P. cinnamomi* were produced abundantly in carrot agar medium at 20-25°C temperature. The oospores germinate and produce either mycelium or

sporangium that hastens the growth of fungus in the medium. Hendrix (1964) reported that sterols such as stigmasterol, β-sitosterol, phytosterol and ergosterol induce reproduction and stimulate growth of the *Pythium* and *Phytophthora* spp. The carrot contains plant sterols such as stigmasterol and β-sitosterol (Dutta, 2003). The profuse growth of *P. capsici* in the carrot agar medium as uniform dense, white cottony mycelium might be due the presence of stigmasterol and β-sitosterol in the carrot agar medium.

The pure culture of *P. capsici* was maintained by subculturing into fresh medium on every 15 days. To maintain the viability of the pathogen, the culture was inoculated on the leaves of susceptible Panniyur-1, and the pathogen was again re-isolated from the infected leaves of Panniyur-1 on carrot agar medium. Kashino *et al.* (1990) reported similar approach of preserving the virulence of the pathogen for a longer period by re-inoculating the old culture in susceptible plants and re-isolating the same after virulence is regained.

5.2.1. Detached leaf symptom bioassay (with pinpricking)

Detached leaf symptom bioassay has been used as a rapid screening tool to pre-screen the genotypes for resistance or susceptibility to plant pathogens in many crops such as wheat (Arraiano *et al.*, 2001), rice (Jia *et al.*, 2003), *etc.* In detached leaf symptom bioassay, leaf puncturing with sterile needle was adopted before inoculation of mycelial disc at the punctured site of the leaf. The puncturing was done to facilitate the early penetration of *P. capsici* inside the leaves and early initiation of the lesion development. Kunkeaw *et al.* (2010) found that when the detached cassava leaves were punctured in the mid-rib followed by inoculation with mycelial plugs of *Colletotrichum gloeosporiodes* f. sp. *manihotis*, lesion development was recorded early (on the fourth day after inoculation).

Screening of calliclones for tolerance / susceptibility against *Phytophthora* foot rot infection through detached leaf symptom bioassay revealed that, CKCC 27 from Cheriakanyakkadan showed the least lesion diameter at different inoculation intervals *i.e.* 24, 48 and 72 hours after inoculation. Lesion diameter of calliclones of

Cheriakanyakkadan group was less at 24 and 72 hours than calliclones of Kalluvally group. This suggests the more tolerance reaction of Cheriakanyakkadan calliclones than compared to Kalluvally calliclones. Shylaja *et al.* (2012) studied disease reaction of *P. capsici* in Kalluvally and Cheriakanyakkadan calliclones and reported similar results that Cheriakanyakkadan calliclones exhibited less symptom development and were more tolerant to *Phytophthora* foot rot disease. Lesion diameter was found maximum for Panniyur-1 as compared to Cheriakanyakkadan and Kalluvally calliclones at 24, 48 and 72 hours after inoculation thus confirming the susceptible reaction of Panniyur-1 to *Phytophthora* foot rot infection as reported by Kuch and Khew (1980).

The lesion diameter of individual calliclones at 24, 48 and 72 hours after inoculation was found to be highly varying within the respective group as it is due to presence of genotypic variations in the calliclones. Shylaja *et al.* (1996) developed calliclones of black pepper by inducing somaclonal variation through callus mediated organogenesis using explants from axenic seedlings of two cultivars *viz.* Cheriakanyakkadan and Kalluvally and subjecting it to *in vitro* mutagenesis using gamma-irradiation. Clone to clone variability in lesion development was recorded higher, thus reflecting broader differences in susceptible and tolerance reaction to *Phytophthora* foot rot infection.

5.2.2. Intact leaf inoculation without pinprick in three month old rooted cuttings

Inoculation with *P. capsici*, in intact leaves of three month old rooted cuttings of all calliclones and Panniyur-1, at different inoculation periods was done for sampling leaf tissue for β-1,3-glucanase assay, SDS-PAGE analysis and 2D-gel electrophoresis. Here, leaf puncturing was not adopted as any kind of mechanical injury to the leaves will trigger defense reaction in plants and might alter the leaf proteome. The pre-initiation of hypersensitive response due to leaf puncturing before challenge inoculation will inhibit the pathogen invasion and lesion development that will tamper the accuracy of results. Chassot *et al.* (2008) observed similar response in Arabidopsis against grey mould fungus (*Botrytis cinerea*) when the leaves were subjected to physical injury or wounding. The

growth of fungal hyphae in the wounded leaves got inhibited strongly than unwounded controls when both the wounded leaves and unwounded controls were challenged with *B. cinerea* infection.

5.3. ASSAY OF β-1,3-GLUCANASE ACTIVITY

The β-1,3-glucanase activity was assayed from the crude enzyme extract isolated from inoculated leaves of different calliclones and Panniyur-1 at 0, 24, 48 and 72 hours after inoculation. The specific activity of the enzyme was expressed as µmol of glucose equivalents formed / mg of protein / 15 minutes. The β-1,3-glucanase enzyme was found active only for few hours after isolation. The activity of the enzyme declined after prolonged storage, hence the assay was performed immediately after isolation of the crude enzyme.

The specific activity of β-1,3-glucanase was highly variable among the calliclones. In KLCC 89, highest increment in specific activity of β-1,3-glucanase was recorded from 0 to 24 hours whereas in Panniyur-1 and most of the calliclones except CKCC 5 and CKCC 61, decrement in specific activity of β-1,3-glucanase was recorded from 0-24 hours. But, from 24 to 48 hours after inoculation, KLCC 89 recorded steep downfall in specific activity of β-1,3-glucanase whereas CKCC 27 and CKCC 41 recorded an elevation in specific activity. From 48 to 72 hours after inoculation, KLCC 134 recorded highest increment in specific activity of β-1,3-glucanase but most of the calliclones such as CKCC 27, CKCC 41, KLCC 86 and KLCC 119 recorded decrement in specific activity of β-1,3-glucanase. This variability in β-1,3-glucanase activity might be due to genetic variations present in the calliclones that might inflict variable levels of β-1,3-glucanase expression in the calliclones. The sharp increment in β-1,3-glucanase activity observed in KLCC 89 is due to enhanced expression of β-1,3-glucanase enzyme that was induced as hypersensitive response against the *P. capsici* infection. The fungal invasion triggered signaling pathways that further triggered enhanced expression of gene coding for β-1,3-glucanase. The enhanced expression of β-1,3-glucanase enzyme was to hydrolyze the β-1,3-glucan component of cell wall of *P. capsici* and to prevent further invasion of the

fungus inside the inoculated leaves (Erwin and Ribeiro, 1996). McDowell and Dangal (2000) also reported high glucanase activity in the potato leaves inoculated with *P. infestans*. The enzyme was reported to solubilize elicitor active glucan molecule in the fungal cell wall and induce other defense related enzymes.

When a pathogen attacks a plant, if it is a resistant variety / plant, then, the plant will induce all types of defense mechanisms against the pathogen (structural and biochemical). So the prevalence of defense-related enzymes, anti-microbial compounds, phenolic compounds *etc.* will be maximum in resistant plants and thus the plant will prevent the entry of pathogen. Once, it is succeeded, then the quantity of all these compounds will be reduced suddenly. But in susceptible varieties, the level of defense-related enzymes and their production is very low and remain in low level for a period, but here the pathogen overcomes these defense mechanisms and can spread the infection.

Overall, KLCC 89 recorded the earliest (at 24 hours after inoculation) and highest β-1,3-glucanase activity among the 11 calliclones and susceptible variety Panniyur-1 thus elucidating the tolerance reaction of KLCC 89 and role of β-1,3-glucanase in plant defense against *Phytophthora* foot rot disease. Mammootty *et al.* (2008) also reported tolerance reaction of Kalluvally genotype in the nursery. Nazeem *et al.* (2008) performed β-1,3-glucanase assay in susceptible 'Panniyur-1', moderately tolerant cultivar 'Kalluvally' and resistant genotype *Piper colubrinum*. Results revealed higher β-1,3-glucanase activity in tolerant cultivar 'Kalluvally' (1.70 µmol / mg protein / 10 min) than the susceptible Panniyur-1 (0.262 µmol / mg protein / 10 min) thus confirming the tolerance of Kalluvally against *P. capsici* due to higher β-1,3-glucanase activity.

5.4. PROTEIN PROFILING BY SDS-PAGE

SDS-PAGE analysis was done with total leaf protein isolated from the inoculated leaves of calliclones and Panniyur-1 at 0, 24, 48 and 72 hours after inoculation. SDS-PAGE analysis of precipitated leaf protein in the calliclones and Panniyur-1 showed distinct expression of protein bands at 0, 24, 48 and 72 hours after inoculation. The banding pattern was found to vary from clone to clone due to variation in genetic

configuration of the calliclones. The 16.5 kDa band corresponding to β-1,3-glucanase was found expressed in all the calliclones but variation in level of expression of 16.5 kDa band was observed in different calliclones and within the calliclone at different inoculation period. This is evident from the results of β-1,3-glucanase assay that, all the calliclones including Panniyur-1, reported β-1,3-glucanase activity at all time periods and the enzyme activity values were highly fluctuating at four time periods. In KLCC 89 at 24 hours after inoculation, the 16.5 kDa protein band was found over expressed compared to other calliclones and also β-1,3-glucanase activity was also higher at 24 hours after inoculation. The calliclones, CKCC 5, CKCC 60, CKCC 61, KLCC 133, KLCC 134 and Panniyur-1 showed maximum expression of 16.5 kDa band at 72 hours after inoculation and these clones showed higher β-1,3-glucanase activity at 72 hours. The calliclones CKCC 27, CKCC 41, KLCC 86 and KLCC 119 reported higher in β-1,3-glucanase activity from 24 to 48 hours and higher expression of 16.5 kDa band at 48 hours in the SDS-PAGE gel. Thus, the higher activity of β-1,3-glucanase and higher expression of 16.5 kDa band in SDS-PAGE separation was found to correlate.

Jebakumar *et al.* (2001) performed SDS-PAGE analysis with total protein isolated from leaves and roots of three black pepper (*Piper nigrum* L.) varieties (tolerant P24; susceptible Panniyur-1 and Subhakara) inoculated with *Phytophthora capsici*. SDS-PAGE analysis revealed expression of 27, 34 and 38 kDa proteins in the infected leaves of tolerant P24 variety. Similarly, in the roots, 20, 26 and 31 kDa proteins were expressed in tolerant P24 variety and 22, 24, 31 and 33 kDa proteins in Panniyur-1 infected with *P. capsici*. Western-blotting with anti-tobacco β-1,3-glucanase polyclonal antibody confirmed the presence of β-1,3-glucanase isoforms.

Nazeem *et al.* (2008) also observed through SDS-PAGE analysis, that in case of resistant genotype *Piper colubrinum*, the 16.5 kDa band was over-expressed at all the times that was evident as negligible change in the activity pattern of β-1,3-glucanase in enzyme bioassay. But in case of Kalluvally, the 16.5 kDa band was expressed after 48 hours post-infection and continued to express until fifth day after inoculation which was evident as increasing trend in β-1,3-glucanase activity over a period of three to five days.

In susceptible variety Panniyur-1, the expression of 16.5 kDa band was delayed up to fifth day and symptom developed faster. This elucidated the role of 16.5 kDa protein in defense reaction against *P. capsici* which was characterized as β-1,3-glucanase through western blot analysis using tobacco β-1,3-glucanase polyclonal antibodies.

Apart from variable expression of 16.5 kDa band in the calliclones and Panniyur-1, other molecular weight bands were also found differentially expressed that might be expressed as a consequence of defense mechanism against *P. capsici* infection or might have no roles in defense thus suggesting the further characterization of these polypeptides in future to know its nature and biological function.

5.5. PROTEOME ANALYSIS OF MOST TOLERANT AND SUSCEPTIBLE GENOTYPE BY 2D-GEL ELECTROPHORESIS

For obtaining a broader protein profile as consequence of *P. capsici* infection in tolerant and susceptible genotype, two-dimensional gel electrophoresis was attempted. Based upon the above analyses, KLCC 89 showed higher β-1,3-glucanase activity at 24 hours after inoculation and over-expressed protein band of 16.5 kDa in the SDS-PAGE gel. Hence, KLCC 89 was selected as tolerant calliclone and Panniyur-1 as susceptible genotype for 2D-gel electrophoresis. Inoculated leaf samples of tolerant calliclone KLCC 89 and susceptible Panniyur-1 at 0 hours and 24 hours after inoculation were selected for total protein isolation for 2D-gel electrophoresis. The results of 2D-gel electrophoresis revealed that the number of protein spots, increased in both KLCC 89 and Panniyur-1 at 24 hours after inoculation from 0 hour showing that numerous proteins were expressed as defense related proteins against *P. capsici* infection. From the quantity table report, four protein spots were selected as differentially expressed in tolerant calliclone KLCC 89 with higher degree of differential expression and spots were designated as Spot 1, Spot 2, Spot3 and Spot 4. These four protein spots were selected for MALDI-TOF analysis.

5.6. PEPTIDE MASS FINGERPRINTING BY MALDI-TOF / MS ANALYSIS AND *IN SILICO* ANALYSIS OF IDENTIFIED PROTEINS

The results of MASCOT search and BLAST2GO analysis showed 15 protein hits corresponding to four protein spots that were analyzed by MALDI-TOF / MS. The biological roles in plant defense mechanism by the 15 protein hits corresponding to four protein spots are discussed in detail in the following paragraphs.

5.6.1. Spot 1

The Spot 1 corresponded to the three protein hits namely Plastocyanin AA, Plastocyanin BB and TRAF-like family proteins.

First two protein hits corresponded to Plastocyanin, a blue copper protein that plays a major role in photosynthesis as agent involved in electron-transfer from cytochrome f of cytochrome b_6f complex from photosystem-II and P700+ from photosystem-I (Gross, 1993). Cytochrome f acts as electron donor while P700+ acts as electron acceptor from reduced plastocyanin. Both cytochrome b_6f complex and P700+ has residues exposed on the lumen-side of thylakoid membrane of chloroplasts. Expression of plastocyanin reflects the temporary occurrence of enhanced photosynthetic activity. During initial infection stage in the host, *P. capsici* utilizes the stored nutrients and proteins as major energy source (Hosseini *et al.*, 2012). This might create temporary competition for nutrients between the host plant and the fungal pathogen. To meet the requirements of nutrient competition, the plant may enhance photosynthesis activity. As the pathogen invades further there is decrease in photosynthetic activity thus affecting source-sink dynamics leading to cell death and necrosis (Mahadevan, 2015).

Third protein hit corresponding to Spot 1 is Tumour necrosis factor (TNF) receptor associated factors (TRAF) like family proteins which are adaptor proteins that regulate cellular apoptosis or programmed cell death (Inoue *et al.*, 2000). Apoptosis is an inherent defense mechanism in plants in which the localized tissue death around the infection site prevents further invasion of the pathogen into healthy tissues (Reape *et al.*,

2008). Presence of TRAF-like family proteins reveals the fact that the plant developed hypersensitive response against *P. capsici* infection.

5.6.2. Spot 2

The Spot 2 corresponded to six protein hits *viz.* Oxygen-evolving enhancer 2-chloroplastic protein, F-box FBD LRR-repeat At1g13570-like protein, Zinc finger BED domain-containing RICESLEEPER 1-like protein, 4-phosphopantetheinyl transferase protein superfamily, Uncharacterized protein LOC4342779 and Retrotransposon protein Ty3-gypsy subclass.

First hit corresponding to oxygen-evolving enhancer 2-chloroplastic protein is associated with photosystem-II complex, involved in regulation of photorespiration process by regulating proteolysis of water molecule in the presence of Mg^{2+} ions and release of photosynthetic oxygen (Mayfield *et al.*, 1987). Photorespiration is a source of light dependent reactive oxygen species (ROS) production through peroxisomal glycolate oxidase reaction that implicates ROS mediated stress response (Noctor *et al.*, 2002). The ROS production is involved in oxidative damage to invading fungus and mediates downstream signaling mechanism regulated by antioxidant enzymes and molecules (Sharma *et al.*, 2013).

Second protein hit corresponding to Spot 2 is F-box FBD LRR-repeat At1g13570-like protein. F-box proteins are reported to be involved in regulation of various developmental processes for example, photomorphogenesis, circadian clock regulation, self-incompatibility, floral meristem and floral organ identity determination (Sullivan *et al.*, 2003; Moon *et al.*, 2004; Smalle and Viestra, 2004). But in plant defense response, the role of and F-box protein COI1 was reported as initial players in stimulation of jasmonate-regulated defense related pathways (Xie *et al.*, 1998; Xu *et al.*, 2002; Devoto *et al.*, 2005).

Third protein hit corresponded to Zinc finger BED domain-containing RICESLEEPER 1-like protein. It is a transposase-like protein required for regulation of plant growth and development in rice. It is also responsible for regulating global gene

expression by recruiting other cellular factors (Knip *et al.*, 2012). It is a DNA binding protein encoded by *RICESLEEPER 1* gene which was reported to be formed after retro-transposition process in gene coding for hAT transposase during evolution of first angiosperms.

The fourth protein hit corresponded to 4-phosphopantetheinyl transferase protein superfamily that is involved in catalyzing reaction involved in transfer of a 4'-phosphopantetheine (4'-PP) moiety from coenzyme A (CoA) to an invariant serine in an acyl carrier protein (ACP) (Plate 4.12 (C)) involved in synthesis of wide range of compounds such as amino acids, fatty acids, polyketides, non-ribosomal peptides and secondary metabolites (Lambalot and Walsh, 1995). Glazebrook *et al.* (1997) reported accumulation of antimicrobial metabolites such as phytoalexin and camalexin in arabidopsis plants inoculated with the pathogenic fungus *Cochliobolus carbonum*. Hence, the protein hit 4-phosphopantetheinyl transferase might regulate production of antimicrobial metabolites in black pepper against *P. capsici* infection.

The fifth protein hit corresponded to an uncharacterized protein LOC4342779 showing presence of protein domains *i.e* peptidase S1 domain or PDZ domain. Peptidase S1 domain is found in proteases belonging to MEROPS peptidase family S1 (clan PA). This clan PA contains two groups of proteases cysteine proteases and serine proteases (Bazan and Fletterick, 1988). Cysteine proteases have multi-faceted roles, but in plant defense against biotic and abiotic stresses, they are involved regulating signaling pathways (Grudkowska and Zagdańska, 2004). Serine proteases are mostly involved in peptide bond cleavage in proteins (Hedstrom, 2002).

The sixth protein hit corresponded to retrotransposon Ty3-gypsy subclass. It is a category of LTR retrotransposons that are abundantly found in plant genomes. SanMiguel and Bennetzen (1998) reported that 49-78 per cent of the maize genome is composed of retrotransposons. These retrotransposons are involved in transposition of genetic elements from one place to other in a chromosome *via.* formation of an RNA intermediate. The cDNA formed by reverse transcription of transcripted RNA intermediate, get inserted into

the genome at target sites. The function of this retrotransposon protein in plant defense is unknown, but it is a highly regulated process occurring within the nucleus that is induced in response to diverse stress conditions (Tapia *et al.*, 2005). Chen *et al.* (2013) also reported stimulation of retrotransposon Ty3-gypsy subclass in rice plants infected with spotted leaf disease.

5.6.3. Spot 3

The Spot 3 corresponded to five protein hits *i.e.* two for Ribulose 1,5-bisphosphate carboxylase oxygenase large subunit (chloroplast), one each for Proteasome-associated ECM29 homolog isoform X1, Uncharacterized protein LOC107804580 and Uncharacterized protein LOC107820978 isoform X3.

The first two protein hits corresponded to ribulose-1,5-bisphosphate carboxylase oxygenase (RUBISCO) large subunit. It is a protein involved primarily in fixation process of atmospheric carbon dioxide in photosynthetic organisms (Plate 4.12 (B)) by catalyzing carboxylation of ribulose-1,5-bisphosphate (RuBP) during calvin cycle (Cooper and Geoffrey, 2000). In plant defense mechanism, RUBISCO plays an indirect role through glycolate and glyoxylate metabolism. It converts RuBP into phosphoglycolate that is converted into glycolate by phosphoglycolate phosphatase. Metabolism of glycolate into glyoxylate involves an intermediate pathway where hydrogen peroxide (H_2O_2) is synthesized (Plate 4.12 (A)). The H_2O_2 generation creates oxidative stress in the invading pathogen thus inhibiting the growth of pathogen by initiating oxidative burst (Shetty *et al.*, 2008). Eloy *et al.* (2015) reported direct antimicrobial effect of H_2O_2 against infection by *Colletotrichum gloeosporiodes* in cowpea. Apart from antimicrobial effect, H_2O_2 generation regulates cross-linking of cell walls, signaling, induction of gene expression, hypersensitive cell death and induced systemic acquired resistance.

The third protein hit corresponded to proteasome-associated Ecm29 homolog isoform X1. This protein acts as 26*S* proteasome inhibitor (De La Mota-Peynado *et al.*, 2013). 26*S* proteasomes complexed with ATP and ubiquitin play an important role in protein degradation (Goldberg, 2003). During oxidative stress induced by H_2O_2

generation, recruits Ecm29 bind to proteasome holozyme, inhibiting the ATPase activity and thus preventing the activity of proteasomes (Mota-Peynado *et al.*, 2013). This is necessary for proteasome stabilization (Leggett *et al.*, 2002; Kleijnen *et al.*, 2007) and proteasome remodeling (Wang *et al.*, 2010; Park *et al.*, 2011). Wang *et al.* (2011) suggested that disassembly of 26*S* proteasome may increase abundance of 20*S* proteasome, that will enhance plant defense against oxidative stress. Also the inhibition of 26*S* proteasomes during acute oxidative stress may be regulated to provide sufficient time for protein repair to reduce demand on cellular synthesis of proteins. The role of proteasome-associated Ecm29 homolog isoform X1 in plant defense against fungal pathogens is not elucidated so far.

The fourth and fifth protein hit corresponded to uncharacterized protein LOC107804580 and uncharacterized protein LOC107820978 isoform X3 from *Nicotiana tabacum* for which GO annotations and InterPro scan for presence of protein domains and corresponding protein families were not characterized by BLAST2GO tool.

5.6.4. Spot 4

The Spot 4 corresponded to uncharacterized protein LOC107767279 from *Nicotiana tabacum* that was analyzed through InterPro scan, and presence of reverse transcriptase domain was detected. GO annotation could not be performed with this protein as it was uncharacterized. The characteristic of this spot was that it is novel and was expressed only in tolerant KLCC 89 at 24 hours after inoculation. Presence of reverse transcriptase domain from *Nicotiana tabacum* suggests for the presence of retrotransposon activity that might have initiated as a consequence of attack by *P. capsici*. Reverse transcriptase is involved in reverse transcription of the RNA intermediate transcribed by retrotransposon back to complementary DNA fragment which later integrates at new position in the genome (Dombroski *et al.*, 1994). Grandbastein *et al.* (1989) first reported transcription of predominantly characterized retrotransposon, Tnt1 in tobacco, and the same in Arabidopsis (Moreau-Mhiri *et al.*, 1996) and tomato (Mhiri *et al.*, 1999) when subjected by many factors such as external stresses, biotic stresses of bacterial eg. *Erwinia*

chrysanthemi (Moreau-Mhiri *et al.*, 1996), fungal eg. *Cladosporum fulvum* (Mhiri *et al.*, 1999) and viral origin. They found that the expression of Tnt1 retrotransposon is linked with biological responses of plant to elicitor or pathogen attack in particular that is involved in regulation of metabolic pathways leading to activation of PR-proteins. Hence, there is a possibility that Tnt1 retrotransposon might also be involved regulating defense responses against *P. capsici* in black pepper which requires further characterization. Proposition of Tnt1 retrotransposons will be the first ever report of retrotransposition activity mediating defense mechanisms in black pepper against *Phytophthora* foot rot infection .

The defense response in tolerant calliclone KLCC 89 against *Phytophthora* foot rot disease is a consequence of complex regulation of different biological processes such as temporary increment in expression of photosynthetic proteins to increase photosynthetic activity to meet the requirements of nutrient competition by the *P. capsici*, expression of TRAF-like family proteins involved in regulation of programmed cell death (PCD), RUBISCO dependent glycolate and glyoxylate metabolism in cytotoxic H_2O_2 generation, light dependent ROS production during photorespiration, activation of F-box proteins to stimulate jasmonate regulated defense-related pathways, synthesis of antimicrobial metabolites, enhanced β-1,3-glucanase activity and enhanced retrotransposition activity within the genome.

The study could characterize PR proteins in the selected calliclones and investigate in depth the *Phytophthora capsici* interaction in black pepper at proteome level. Future research should focus on validation of identified proteins in defense mechanism, characterization of novel protein in KLCC 89, in-depth investigations on retrotransposons in defense mechanism of *Phytophthora* foot rot tolerance in black pepper, metabolic engineering of the pathways triggering transcription of PR-genes and transgenic / cisgenic research for *Phytophthora* foot rot resistance.

Summary

6. SUMMARY

The current study entitled "Characterization of PR proteins in selected calliclones of black pepper in relation to *Phytophthora* foot rot disease" was conducted with the objective to portray role of β-1,3-glucanases and other defense related proteins in defense mechanism against *Phytophthora* foot rot disease. Six calliclones of Cheriakanyakkadan group (CKCC 5, CKCC 41, CKCC 60, CKCC 61, CKCC 25 and CKCC 27), five calliclones of Kalluvally group (KLCC 119, KLCC 89, KLCC 133, KLCC 134 and KLCC 86) reported as better performing and a susceptible variety Panniyur-1 were selected for the study. The experiments were conducted at Centre for Plant Biotechnology and Molecular Biology (Spot), College of Horticulture (CoH), Vellanikkara and Department of Plant Pathology, CoH, Vellanikkara and Distributed Information Centre, CoH, Vellanikkara during March 2016 to August 2017.

The salient findings of the study are summarized as follows

1. The calliclones of Cheriakanyakkadan group recorded better establishment in the nursery than calliclones of Kalluvally group and Panniyur-1. CKCC 60 recorded highest establishment percentage among the calliclones followed by CKCC 27 and KLCC 89.
2. Detached leaf symptom bioassay at 24, 48 and 72 hours after inoculation revealed that, CKCC 27 showed the least lesion diameter depicting tolerance reaction. Percentage increase in lesion diameter was recorded maximum for Kalluvally calliclones from 24 to 48 hours. Cheriakanyakkadan calliclones recorded maximum percentage increase in lesion diameter from 48 to 72 hours after inoculation. Panniyur-1 recorded minimum percentage change in lesion diameter at both time periods.
3. Intact leaf inoculation with *Phytophthora capsici* was performed in the leaves of three month old rooted cuttings. All the calliclones and Panniyur-1 developed water soaked lesions with fimbricate margins. The inoculated leaves were collected at 0, 24, 48 and 72 hours after inoculation for performing β-1,3-glucanase assay and SDS-PAGE analysis.

4. β-1,3-glucanase activity assayed from the crude enzyme extract isolated from inoculated leaves of calliclones and Panniyur-1 at 0, 24, 48 and 72 hours after inoculation revealed that, KLCC 89 recorded the earliest (at 24 hours after inoculation) and highest specific activity β-1,3-glucanase among the 11 calliclones and susceptible variety Panniyur-1.
5. Protein separation by SDS-PAGE revealed that 16.5 kDa protein band corresponding to β-1,3-glucanase was found expressed in all the calliclones at different intensities. In KLCC 89 at 24 hours after inoculation, the 16.5 kDa protein band was found over expressed as compared to other calliclones and Panniyur-1, which is in accordance with the activity of β-1,3-glucanase. Apart from this, other molecular weight bands were also found differentially expressed in CKCC 5, CKCC 25, CKCC 27, KLCC 89, KLCC 133 and Panniyur-1.
6. Proteome analysis by two-dimensional gel electrophoresis revealed that, in KLCC 89, 349 spots were differentially expressed at 24 hours after inoculation with *P. capsici*. In Panniyur-1, 459 spots were differentially expressed at 24 hours after inoculation. About 141 protein spots were found commonly expressed in susceptible variety Panniyur-1 and tolerant calliclone KLCC 89 at 24 hours after inoculation with *P. capsici*. From the 167 protein spots differentially expressed in KLCC 89 at 24 hours after inoculation, four protein spots with higher degree of differential expression were selected and designated as Spot 1, Spot 2, Spot3 and Spot 4 that were further selected for MALDI-TOF analysis.
7. MALDI-TOF analysis followed by MASCOT search revealed three hit peptides for Spot 1, six hit peptides for Spot 2, five hit peptides for Spot 3 and one hit peptide for Spot 4.
8. *In silico* analysis of the 15 protein hits using BLAST2GO tool identified eight sequences of *Nicotiana tabacum*, four sequences of *Oryza sativa* japonica group, three sequences of *Nicotiana sylvestris* and one sequence each belonged to *Nicotiana benthamiana* and *Piper nigrum*.
9. Spot 1 corresponded to Plastocyanin AA, Plastocyanin BB and TRAF-like family proteins. Expression of plastocyanin reflects the temporary occurrence of enhanced

photosynthesic activity to meet the nutrient requirements due to competition from the pathogen. TRAF-like family proteins are involved in regulation of cellular apoptosis or programmed cell death as hypersensitive response against *P. capsici* infection.

10. Spot 2 corresponded to Oxygen-evolving enhancer 2- chloroplastic protein, F-box FBD LRR-repeat At1g13570-like protein, Zinc finger BED domain-containing RICESLEEPER 1-like protein, 4-phosphopantetheinyl transferase protein superfamily, Uncharacterized protein LOC4342779 and Retrotransposon protein Ty3-gypsy subclass. Oxygen-evolving enhancer 2-chloroplastic protein is associated with photosystem-II complex, involved in regulation of photorespiration that is a source of light dependent reactive oxygen species (ROS) production responsible for plant defense. F-box proteins in plant defense response play role in triggering jasmonate-regulated defense related pathways. 4-phosphopantetheinyl transferase protein superfamily might regulate production of antimicrobial metabolites in black pepper against *P. capsici* infection.
11. Spot 3 corresponded to Ribulose 1,5-bisphosphate carboxylase oxygenase large subunit (chloroplast) (RUBISCO), Proteasome-associated ECM29 homolog isoform X1, Uncharacterized protein LOC107804580 and Uncharacterized protein LOC107820978 isoform X3. RUBISCO plays indirect role in H_2O_2 generation that creates oxidative stress in the invading pathogen thus inhibiting the growth of pathogen by initiating oxidative burst.
12. Spot 4 is novel and was expressed only in tolerant KLCC 89 at 24 hours after inoculation. Presence of reverse transcriptase domain from *Nicotiana tabacum* suggests for the presence of retrotransposon activity that might have initiated as a consequence of attack by *P. capsici*.

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Annexure

ANNEXURE-I

Preparation of carrot agar medium

Composition

Carrot	:	200 g
Agar	:	20 g
Distilled water	:	1000 ml

Preparation

Carrot was peeled off and cut into small bits and weighed. It was boiled in 500 ml of distilled water to soften the tissues and then the extract was squeezed out through a muslin cloth. Agar was boiled in 500 ml distilled water with constant stirring and then was added to carrot extract. The final volume of solution was made up to 1000 ml and autoclaved at 121.6°C for 20 minutes at 15 psi before used for inoculation.

ANNEXURE-II

Reagents required for β-1,3-glucanase assay

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

Composition

Sodium acetate	:	0.41 g
Distilled water	:	100 ml

Sodium acetate of about 0.41 g was dissolved in 50 to70 ml of distilled water followed by adjusting the pH to 5 with 0.1 *N* HCl. The final volume was made to 100 ml with distilled water.

B. Laminarin (4 per cent)

Composition

Laminarin	:	40 mg
Distilled water	:	1000 µL

The laminarin was dissolved in distilled water by heating briefly in a boiling water bath just before use.

C. Sodium hydroxide (4.5 per cent)

Composition

Sodium hydroxide	:	4.5 g
Distilled water	:	100 ml

D. Dinitrosalicylic acid (DNS)

Composition

Dinitrosalicylic acid	:	8.8 g
Sodium potassium tartarate	:	2.55 g
Sodium hydroxide	:	300 ml
Distilled water	:	800 ml

Dissolve required DNS in sodium potassium tartarate in 800 ml of water and later on added 300 ml of 4.5 per cent sodium hydroxide.

E. Cystein HCl (0.05 *M*)

Composition

Cystein HCl	:	0.44 g
Distilled water	:	50 ml

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

Composition

PMSF	:	20 mg
Dimethylsulphonyl fluoride	:	1 ml

The solution was prepared fresh before use.

G. Ascorbic acid (5m*M*)

Composition

Ascorbic acid	:	0.088 g
Distilled water	:	100 ml

ANNEXURE-III

Reagents required for Lowry's method of protein quantification

A. Bovine serum albumin (BSA) stock solution

Composition

BSA	:	1 mg
Distilled water	:	1 ml

B. Analytical reagents

1. *Reagent A*

50 ml of 2 per cent sodium carbonate mixed with 50 ml of 0.1 N NaOH solution (0.4 g in 100 ml distilled water.)

2. *Reagent B*

10 ml of 1.56 per cent copper sulphate solution mixed with 10 ml of 2.37 per cent sodium potassium tartarate solution. Prepare analytical reagents by mixing 2 ml of (B) with 100 ml of (A).

C. Folin - Ciocalteau reagent solution (1*N*)

Dilute commercial reagent (2*N*) with an equal volume of water on the day of use (2 ml of commercial reagent + 2 ml distilled water)

ANNEXURE-IV

Chemicals required for SDS-PAGE

A. Monomer solution (30 per cent Acrylamide, 27 per cent Bis-acrylamide)

Composition

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

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

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

Composition

Tris base	:	18.5 g
Distilled water	:	100 ml

Tris base was dissolved in about 50 to 60 of distilled water, the pH was adjusted to 8.8 with 1 *N* HCl and stored at 4°C.

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

Composition

Tris base	:	6 g
Distilled water	:	100 ml

Tris base was dissolved in about 50 to 60 of distilled water, the pH was adjusted to 6.8 with 1 *N* HCl and stored at 4°C.

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

Composition

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

The solution was stored at room temperature.

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

Composition

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

F. Initiator (10 per cent Ammonium persulphate (APS))

Composition

APS	:	0.1 g
Distilled water	:	1 ml

Solution was prepared freshly before use.

G. Sodium dodecyl sulphate solution (10 per cent)

Composition

SDS	:	10 g
Distilled water	:	100 ml

H. Fixer solution

Composition

Methanol	:	50 ml
Distilled water	:	50 ml

Solution was prepared freshly before use.

I. Coomassie brilliant blue staining solution

Composition

Coomassie Brilliant Blue R250	:	0.1 g
Methanol	:	40 ml
Acetic acid	:	10 ml
Distilled water	:	50 ml

First the dye was dissolved in methanol and then was mixed with water and acetic acid.

J. Destaining solution

Composition

Methanol	:	40 ml
Acetic acid	:	10 ml
Distilled water	:	50 ml

Solution was prepared freshly before use.

ANNEXURE-V

Chemicals required for Two-dimensional gel electrophoresis

A. Lysis buffer (10 ml solution in millipore water)

Composition

7 *M* Urea	:	4.2 g
2 *M* Thiourea	:	1.52 g
2 per cent CHAPS	:	0.2 g
40 m*M*	:	0.062 g

The above solution was stored in aliquots of 200 µL volume in eppendorf tubes and stored in -20°C. The solution once thawed cannot be stored in -20°C again.

B. Rehydration buffer (25 ml solution prepared in millipore water)

Composition

8 *M* Urea	:	12.01 g
2 per cent CHAPS	:	0.5 g
18 m*M* DTT	:	0.069 g
0.5 per cent Ampholytes, pH 3-11	:	312.5 µL
0.002 per cent Bromophenol blue	:	0.0005 g

The above solution was stored in aliquots of 400 µL volume in eppendorf tubes and stored in -20°C. The solution once thawed cannot be stored in -20°C again.

C. Equillibration buffer-I (50 ml solution prepared in millipore water)

Composition

50 m*M* Tris-Cl, pH 8.8	:	0.394 g
6 M Urea	:	18 g
30 per cent V/V glycerol	:	15 ml
2 per cent W/V SDS	:	1 g
0.002 per cent Bromophenol blue	:	0.001 g
0.5 per cent W/V DTT	:	0.25 g

The above solution was stored in aliquots of 2 ml volume in eppendorf tubes and stored in -20°C. The solution once thawed cannot be stored in -20°C again.

D. Equillibration buffer-II (50 ml solution prepared in millipore water)

Composition

50 m*M* Tris-Cl, pH 8.8	:	0.394 g
6 *M* Urea	:	18 g
30 per cent V/V glycerol	:	15 ml
2 per cent W/V SDS	:	1 g
0.002 per cent Bromophenol blue	:	0.001 g
0.5 per cent W/V DTT	:	0.25 g
2.5 per cent W/V Iodoacetamide	:	1.25 g

The above solution was stored in aliquots of 2 ml volume in eppendorf tubes and stored in -20°C. The solution once thawed cannot be stored in -20°C again.

E. Overlay agarose (100 ml prepared)

Composition

0.25 m*M* Tris	:	0.003 g
192 m*M* Glycine	:	1.44 g
0.1 per cent W/ V SDS	:	0.1 g
0.002 per cent Bromophenol blue	:	0.002 g
0.5 per cent Agarose	:	0.5 g

F. 1X TGS electrode buffer (0.025 *M* Tris, pH 8.3, 0.192 *M* Glycine, 5 L prepared)

Composition

Tris base	:	15.25 g
Glycine	:	72 g
SDS	:	5 g
Distilled water	:	5 L

G. Monomer solution (30 per cent Acrylamide, 27 per cent Bis-acrylamide)

Same as in Annexure-IV

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

Same as in Annexure-IV

I. Initiator (10 per cent Ammonium persulphate (APS))

Same as in Annexure-IV

J. Sodium dodecyl sulphate solution (10 per cent)

Same as in Annexure-IV

K. Fixer solution

Same as in Annexure-IV

L. Coomassie brilliant blue staining solution

Same as in Annexure-IV

M. Destaining solution

Same as in Annexure-IV

ANNEXURE-VI

Chemicals required for Bradford method of protein quantification

A. Bradford reagent

Preparation

The assay reagent was made by dissolving 100 mg of Coomassie Blue G250 in 50 ml of 95 per cent ethanol. The solution was then mixed with 100 ml of 85 per cent phosphoric acid and made up to 1 L with distilled water. The reagent was filtered through Whatman no. 1 filter paper and then stored in an amber bottle at room temperature. It was stable for several weeks. The solution was used from the next day.

B. Bovine serum albumin (BSA) stock solution

Composition

BSA	:	1 mg
Distilled water	:	1 ml

CHARACTERIZATION OF PR PROTEINS IN SELECTED CALLICLONES OF BLACK PEPPER IN RELATION TO *PHYTOPHTHORA* FOOT ROT DISEASE

By
DEBASHIS SAHOO
(2015-11-002)

ABSTRACT OF THE THESIS

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ABSTRACT

Black pepper (*Piper nigrum* L.), the king of spices is severely affected by *Phytophthora* foot rot disease caused by *Phytophthora capsici*. The disease results in severe crop loss, and valuable genotypes are lost every year due to this dreadful disease. The local cultivars and released varieties of black pepper are susceptible to the pathogen, but variation exists in genotypes in the degree of tolerance and mechanism of defense to the disease. The plants resist the pathogen infection by accumulating a number of defense related proteins in the intercellular spaces which are collectively known as pathogenesis related (PR) proteins. The study was aimed to characterize PR proteins in selected eleven calliclones of black pepper along with susceptible variety Panniyur-1 after challenge inoculation with *Phytophthora capsici* so as to get more insight on the defense mechanism of *Phytophthora* foot rot.

Investigations on disease reaction of the selected calliclones and variety Panniyur-1 after challenge inoculation with *Phytophthora capsici*, β-1,3-glucanase activity and protein analysis by SDS-PAGE was carried out at 0, 24, 48 and 72 hours after inoculation. Protein profiling by 2D-gel electrophoresis and protein identification by MALDI-TOF MS / MS in the most tolerant and susceptible calliclone and *in silico* analysis for characterization of proteins were also attempted in the present study.

In leaf symptom bioassay, variety Panniyur-1 showed susceptible reaction as compared to calliclones of Cheriakanyakkadan and Kalluvally. Based on β-1,3-glucanase activity and expression of 16.5 kDa band in SDS-PAGE, clone KLCC 89 was selected as the tolerant calliclone. 2D-gel electrophoresis was attempted in the selected tolerant calliclone, KLCC 89 and susceptible variety Panniyur-1. Proteome analysis by 2D-gel electrophoresis could locate 167 differentially expressed protein spots in KLCC 89, 24 hours after inoculation. Analysis by PDQUEST software could select four protein spots (Spot 1, Spot 2, Spot 3 and Spot 4) from 167 spots based on higher degree of differential expression. The selected spots were sent to Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram for protein identification by MALDI-TOF. Analysis by MALDI-TOF and MASCOT search could identify 15 hit peptides from the selected four protein spots. Analysis and functional characterization of the 15 hit peptides by

BLAST2GO revealed the defense response of tolerant calliclone KLCC 89 against *Phytophthora* foot rot disease.

The enhanced expression of plastocyanin protein, TRAF-like family proteins, RUBISCO dependent glycolate and glyoxylate metabolism, light dependent ROS production during photorespiration, F-box proteins, synthesis of antimicrobial metabolites and retrotransposition activity were observed in the tolerant calliclone KLCC 89 as defense related responses. Plastocyanin is involved in regulation of photosynthesis to meet the requirements of nutrient competition by the *P. capsici* whereas the TRAF-like family proteins is involved in regulation of programmed cell death. The increment in RUBISCO, regulates the glycolate and glyoxylate metabolism for H_2O_2 production. F-box proteins are involved in regulation of jasmonate regulated defense-related pathways.

The study could characterize PR proteins in the selected calliclones and investigate in depth the *Phytophthora capsici* interaction in black pepper at proteome level. The future research should focus on validation of identified proteins in defense mechanism, characterization of novel protein in KLCC 89, in-depth investigations on retrotransposons in defense mechanism of *Phytophthora* foot rot tolerance in black pepper, metabolic engineering of the pathways triggering transcription of PR-genes and transgenic / cisgenic research for *Phytophthora* foot rot resistance.