# Transcriptome analysis of *Phytophthora capsici* tolerance in black pepper (*Piper nigrum* L.)

By

**BASIL BABU PAUL**

**(2015-11-100)**

**THESIS**

***Submitted in partial fulfilment of the requirement for the degree of***

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I hereby declare that the thesis entitled **"Transcriptome analysis of *Phytophthora capsici* tolerance in black pepper (*Piper nigrum* L.)"** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

**Vellanikkara**
**Date:** 05.07.2017

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## CERTIFICATE

Certified that the thesis entitled **"Transcriptome analysis of *Phytophthora capsici* tolerance in black pepper (*Piper nigrum* L.)"** is a record of research work done independently by **Mr. Basil Babu Paul (2015-11-100)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship, fellowship to him.

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*Dedicated to family and friends*

# CONTENTS

# LIST OF TABLES

## LIST OF TABLES (contd.)

# LIST OF PLATES

## LIST OF FIGURES

## LIST OF ANNEXURES

| ANNEXURE NO. | PARTICULARS |
|---|---|
| I | Composition of Carrot Agar Medium |
| II | Chemicals and buffers for RNA isolation |
| III | Chemical composition of the buffers, gels and solutions used for Urea-PAGE |

## ABBREVATIONS

| | | |
|---|---|---|
| APS | : | Ammonium per sulphate |
| Avr | : | avirulence |
| bp | : | Base pair |
| BLAST | : | Basic local alignment search tool |
| β | : | Beta |
| CO | : | Carbon monoxide |
| CPBMB | : | Centre for Plant Biotechnology and Molecular Biology |
| CCR | : | Cinnamoyl CoA reductase |
| C-DNA | : | Complementary Deoxyribonucleic Acid |
| °C | : | Degree Celsius |
| dNTPs | : | Deoxy ribo Nucleoside Triphosphate |
| DNase | : | Deoxy ribonuclease |
| DNA | : | Deoxy ribonucleic Acid |
| DEPC | : | Diethyl pyrocarbonate |
| DDRT PCR | : | Differential Display Reverse Transcriptase PCR |
| ETI | : | Effector Triggered Immunity |
| EDTA | : | Ethylene Diamine Tetra Acetic acid |
| EST | : | Expressed Sequence Tags |
| F5H | : | Ferrulate 5- hydroxylase |
| g | : | Gram |
| ha | : | Hectare |
| hai | : | Hours after inoculation |
| pH | : | Hydrogen ion concentration |
| IISR | : | Indian Institute of Spice Research |
| KAAS | : | KEGG Automatic Annotation Server |

| | | |
|---|---|---|
| Kb | : | Kilo basepairs |
| KEGG | : | Kyoto Encyclopedia for Genes and Genomes |
| L | : | Liter |
| LOH | : | Loss of Heterozygosity |
| $MgCl_2$ | : | Magnesium chloride |
| Mb | : | Mega base pair |
| mRNA | : | Messenger RNA |
| µl | : | Micro liter |
| µg | : | Microgram |
| mg | : | Milli gram |
| ml | : | Milli liter |
| mM | : | Milli molar |
| M | : | Molar |
| MOPS | : | 3-(*N*-morpholino) propane sulfonic acid |
| ng | : | Nano gram |
| NLS | : | Nuclear Localisation Signal |
| OD | : | Optical Density |
| PTI | : | PAMP Triggered Immunity |
| PR | : | Pathogenesis-related |
| % | : | Percentage |
| PAL | : | Phenylalanine ammonia lyase |
| pM | : | Pico molar |
| PAGE | : | Polyacrylamide gel electrophoresis |
| PCR | : | Polymerase Chain Reaction |
| PVP | : | Polyvinylpyrrolidone |
| ROS | : | Reactive Oxygen Species |
| RDA | : | Representational Difference Analysis |
| R gene | : | Resistance gene |

| | | |
|---|---|---|
| RT-PCR | : | Reverse transcriptase polymerase chain reaction |
| rpm | : | Revolutions per minute |
| RNase | : | Ribonuclease |
| RNA | : | Ribonucleic acid |
| RNA-Seq | : | RNA Sequencing |
| SAGE | : | Serial Analysis of Gene Expression |
| SSH | : | Suppressive Subtractive Hybridisation |
| TEMED | : | Tetramethyl ethylene diamine |
| TDFs | : | Transcript derived fragments |
| TBE | : | Tris Borate EDTA |
| DON | : | Deoxynivalenol |
| FHB | : | Fusarium head blight |
| PGIP | : | Polygalacturonase inhibiting proteins |
| STS | : | Stilbene synthase |
| ToMV | : | Tomato mosaic virus |
| PRS | : | Pepper Research Station |
| RAPD | : | Randomly Amplified Polymorphic DNA |
| SSR | : | Simple Sequence Repeats |
| CAM | : | Carrot agar medium |
| MEGA | : | Molecular Evolutionary Genetics Analysis |
| NCBI | : | National Center for Biotechnology Information |
| CYP | : | Cytochrome |
| UPL | : | Ubiquitin protein ligase |

# *Introduction*

## 1. Introduction

Black pepper (*Piper nigrum* L.) regarded as the 'king of spices' is one of the most important spices in India. Black pepper was originated from the monsoon forests along the coasts of Malabar in Southwestern India. Black pepper is a woody climber which is cultivated in more than 30 countries and the major growers are India, Brazil, Indonesia, Malaysia, Thailand, Sri Lanka and Vietnam (Ahmad *et al.*, 2010). Black pepper is a traditional spice crop which holds predominant role in the world spice market because of its medicinal and culinary properties apart from its taste, pungent smell and piperine content (Ravindran *et al.*, 2000).

1922 marked the new era of a deadly phytopathogen *Phytophthora capsici,* described by Leonian in chile pepper. To date, the understanding of pathogen's biology, host range, dissemination, and management has been made in many crops. This pathogen costs many symptoms including foliar blighting, damping-off, wilting, and root, stem, and fruit rot in a large spectrum of plants (Hausbeck and Lamour, 2004). In Black pepper, *Phytophthora capsici* occurs on all parts of the plant and cause severe economic damage. The expression of disease symptoms depend upon the site of infection and extent of damage. *Phytophthora* infections in black pepper is broadly classified into aerial and soil infections. Aerial infection observes on the runner shoots, foliage, spikes and branches causing blight, spike shedding, defoliation and die back and at times death of plants. Infection on the runner shoots often reaches the collar causing foot rot (Mammootty, 1978).

The long term survivability of *Phytophthora* oospores in soil (Lamour and Hausbeck, 2003), a wide host range (Erwin and Rebiero, 1996), long-distance movement of the pathogen in surface water used for irrigation (Gevens *et al.*, 2007), the presence of fungicide-resistant pathogen populations (Lamour and Hausbeck, 2000), and a lack of commercially acceptable resistant host varieties (Thabuis *et al.*, 2003, 2004) make this the most deadly disease in black pepper. Understanding of molecular basis of *Phytophthora* resistance can help control the disease by gene stacking, molecular breeding and exploiting the natural resistance prevailing in wild species of crop plants.

To combat the invasion of fungus and for its survival, the plant inherently adopts many morphological and biochemical defense mechanism. Biotic stress induces the overproduction of the antimicrobial compounds and immune proteins or the down-regulation of genes which

promotes pathogenesis (Rojas *et al.*, 2014; Geisler *et al.*, 2012; Kumari *et al.*, 2016; De Coninck *et al.*, 2015). Apart from Pathogenesis-related (PR) proteins, defense related genes, signal transduction pathways, R-genes, transport and cellular functions, secondary metabolites like antifungal proteins *etc.* are induced during pathogen attack (van Loon *et al.*, 2006). Elicitation of most PRs and related proteins are through the action of the signaling compounds such as, salicylic acid, jasmonic acid, or ethylene, and possess antimicrobial activities *in vitro* by means of hydrolytic activities on cell walls, contact toxicity, and feasibly an involvement in defense signaling (Kunkel and Brooks, 2002).

Till date, none of the black pepper cultivars including the varieties and clonal selections are resistant to *Phytophthora* foot rot disease. IISR Shakthi and IISR Thevam released from IISR, Kozhikode, are tolerant to *Phytophthora capsici* (Ravindran *et al.*, 2000; Krishnamoorthy and Parthasarathy, 2010). In this scenario, comparison of transcriptome profile of susceptible and tolerant cultivars before and after infection will reveal the differential expression of defense related genes and metabolic and signal transduction pathways, which will function against the pathogen invasion and thereby providing resistance to the plant. The information generated could be used for the identification of gene responsible for resistance and improvement of high yielding susceptible varieties by genetic engineering. The identified gene or the marker can also be used for the detection of resistance and susceptibility through the application of marker technology.

The present study "Transcriptome analysis of *Phytophthora capsici* tolerance in black pepper (*Piper nigrum* L.)" was carried out with the following objective.

- To understand the differential expression of genes during *Phytophthora* infection in susceptible and tolerant black pepper varieties through DDRT-PCR analysis on mRNA.

# *Review of Literature*

## 2. REVIEW OF LITERATURE

Black pepper (*Piper nigrum* L., Piperaceae) the 'king of spices' is a traditional and historic spice crop which has been under cultivation since ancient times in India. Black pepper is a woody climber and is a native of Western Ghats of South India. This crop is cultivated in more than 30 countries and the major growers are India, Brazil, Indonesia, Malaysia, Thailand, Sri Lanka and Vietnam (Ahmad *et al.*, 2010). In India, black pepper is cultivated in Kerala (largest producer), Karnataka and to a lesser extent, in Maharashtra, Andhra Pradesh, Tamil Nadu and North-Eastern regions, in an area of 0.13 million ha with a production of 48,500 tonnes. Export has been 28,100 tonnes worth ₹ 1.73 billion (Spice Board, 2016). Indian black pepper is preferred in the international market due to its proper combination of pleasant flavour, taste, piperine content and essential oil. Black pepper is not only used as a condiment but also in culinary preparations, food processing, perfumery and as an important ingredient in most of the Ayurvedic medicine preparations (Gordo *et al.*, 2012).

Till 1951, India was the leading producer but presently Malaysia, Vietnam and Brazil are the leaders. The increased mortality of vines due to dreaded foot rot disease caused by *Phytophthora capsici*, attributed to the radical drop in the black pepper production in India (Thomas, 2017). Tropical climates favour the development of foot rot disease wherein the survival of pathogen is exceptionally high (Ahmad *et al.*, 2010). Due to the epiphytotic appearance of this disease, most of the gardens has been wiped out since 1978 (Sastry, 1982; Dutta and Hedge, 1987).

Transcriptome analysis of tolerant and susceptible black pepper cultivars for *Phytophthora capsici* tolerance may throw light into the identification of resistance genes, metabolite pathway and therefore the molecular mechanism of defense. Hence, an effort to excavate the ruling genes controlling resistance to *Phytophthora* foot rot disease is of utmost importance in the current scenario.

### 2.1 Cultivars of black pepper used in this study

#### 2.1.1 IISR Shakthi

IISR Shakthi is adapted to both plains and high ranges under rainfed conditions, developed by IISR, Kozhikode having a yield of 2253 kg dry pepper/ha. It is an open pollinated progeny of

Perambramundi. Berries are bold and dry recovery is more, hence, acceptable to farmers and Industry and is tolerant to *Phytophthora* foot rot (Ravindran *et al.*, 2000).

### 2.1.2 IISR Subhakara

IISR Subhakara is widely adapted to growing tracts of Kerala and Southern Karnataka, developed by IISR, Kozhikode (released in 1990) having a yield of 2352 kg dry pepper/ha. It is a selection from Karimunda (KS 27) and it has a leaf length of 12.3 cm and breadth 6.5 cm with ovate shape and spike length of 7.7 cm. Subhakara is susceptible to *Phytophthora* foot rot (Ravindran *et al.*, 2000).

## 2.2 Resistance of black pepper varieties to *Phytophthora*

Till date, none of the black pepper cultivars including the varieties and clonal selections are completely resistant to *Phytophthora* foot rot disease. Mammotty *et al.* (2008) screened fifty genotypes of black pepper against *Phytophthora capsici*, none showed resistance. Kalluvally, Panniyur 5, Balankotta, Cheriyakaniakadan and Shimoga were suffered relatively lower motality rates which implies some tolerance against *Phytophthora capsici*. IISR Shakthi and IISR Thevam released from IISR, Kozhikode, are reported tolerant to *Phytophthora capsici* (Ravindran *et al.*, 2000; Krishnamoorthy and Parthasarathy, 2010). Intervention of biotechnological tools gave appraisal to black pepper breeding like, successful interspecific hybrids from the crosses of *Piper nigrum* x *Piper attenuatum* and *Piper nigrum* x *Piper barberi* (Sasikumar *et al.*, 1999). Vanaja *et al.* (2008) produced a partly fertile interspecific hybrid resistant to *Phytophthora* foot rot disease by using cultivated *Piper nigrum* and *Piper colubrinum* (wild exotic species which is resistant to foot rot) as parents. The wild species of black pepper, resistant to *Phytophthora* foot rot disease are excellent source of genes for the incorporation into cultivated popular varieties through molecular breeding.

## 2.3 Resistance to fungal diseases

Chitin constitutes the major component of most fungal cell wall and it can be hydrolyzed by chitinase. On the other hand, β-1,3-glucanase is known to degrade glucans which are also present in the fungal cell walls. The synthesis of chitinases and glucanases are elicited in response to pathogen infection and fungal growth is more effectively inhibited when both enzymes are expressed simultaneously (Neuhaus, 1999).

In recent years, transgenic development of plants with genes encoding β-1,3-glucanase and chitinase of plant origin to enhance resistance to fungal diseases such as grapevine (Yamamoto *et al.*, 2000), peanut (Rohini *et al.*, 2000) and cotton (Tohidfar, 2005; Tohidfar, 2012). Melchers and Stuiver (2000) reported the effective prevention of fungal disease development by the combined expression of chitinase and glucanase in transgenic carrot, tomato and tobacco.

Polygalacturonase inhibiting proteins (PGIP) are glycoproteins present in the plant cell wall, inhibits the activity of fungal endopolygalacturonases (Oelfose *et al.*, 2006). The products of wheat are usually contaminated with mycotoxins, thrichotoxins and deoxynivalenol (DON), which are appeared due to *Fusarium* head blight (FHB) threaten human and animal health. Recently, a L3 gene (N-terminal fragment of yeast ribosomal protein) was transferred to wheat and the transgenic plants showed resistance to *Fusarium* disease and improved level of DON in transgenic wheat kernel (Di *et al.*, 2010).

Activation of phytolaexins are yet another strategy to confer resistance to plants. Transformation of rice with stilbene synthase gene (STS) of *Vst1*, a key enzyme in synthesis of phytoalexin in grape, could improve its resistance to *Piricularia orizae* (Coutos-Thevenot *et al.*, 2001). Similarly, barley was improved to resist to powdery mildew (Liang *et al.*, 2000). More recently, the role of Mitogen-activated protein kinase (MAPK) cascade, especially OsMKK6, in the regulation of genes responsible for phytoalexin synthesis in rice in response to UV and blast infestation was reported by Wankhede *et al.* (2013). In their investigation, the expression of phytoalexin in rice was increased specifically under UV radiation. Moreover, the authors reported that the mitogen-activated protein kinase kinase (MAPKK) is a key component of MAPK cascade. They also identified OsMKK6 through studying the expression profile of rice MAPKKs under UV stress and eventually, achieved transgenic rice lines containing OsMKK6 gene showing an over-expression of phytoalexins.

Heat tolerance and resistance to tomato mosaic virus (ToMV) conferred by *Arabidopsis* NPR1 (nonexpresser of PR genes) gene was introduced into a tomato cultivar. The transgenic lines expressing NPR1 were normal as regards overall morphology and horticultural traits for at least four generations. Disease screening of various tropical diseases unraveled the enhanced resistance to bacterial wilt (BW) and Fusarium wilt (FW), and moderate degree of enhanced resistance to gray leaf spot (GLS) and bacterial spot (BS) and were stably inherited.

### 2.3.1 Development of *Phytophthora* resistant varieties in other crops

Four resistance genes are identified in tomato against late blight *viz.,* Ph-1, Ph-2, Ph-3, Ph-4 and Ph-5, derived from *Solanum pimpinellifolium,* a wild relative of tomato (*Solanum lycopersicum*). Majorly, Ph-2 and Ph-3 are conventionally bred into hybrids. They are being bred singly or in pairs. Using pairs with either different genes (e.g. Ph-2 and Ph-3) or two copies of the same gene are strategies to achieve higher levels of resistance (McGrath *et al.,* 2013).

Late blight caused by *Phytophthora infestans*, a devastating disease to members of solanaceae family, especially potato. To overcome this infection, several *R* genes (resistance) have been identified and isolated from various sources (Ballvora *et al.*, 2002; Vossen *et al.*, 2005; Pel *et al.*, 2009). Late blight resistant genes, *Rpi-sto1* and *Rpi-pta1* were identified from the transient co-expression of *LpiO* (as effector) and *Rpi-blb1* (as resistance gene) in *Nicotiana benthamiana* (Vleeshouwers *et al.*, 2008). Stacking of three broad spectrum potato *R* genes (*Rpi*), *Rpi-sto1* (*Solanum stoloniferum*), *Rpi-vnt1.1* (*Solanum venturii*) and *Rpi-blb3* (*Solanum bulbocastanum*) was transformed into susceptible cultivar 'Desiree'. Nearly, 4 % of the transformed plants showed hypersensitive response against pathogenic effects of *Phytophtora* (Zhu *et al.*, 2012).

### 2.4 Black pepper foot rot fungus *Phytophthora capsici*

*Phytophthora capsici,* a filamentous oomycete, account for root, crown, foliar and fruit rot on a number of critical vegetables (Erwin and Ribeiro, 1996). *Phytophthora capsici* was first labelled in 1922 after it was redeemed from chilli pepper at the New Mexico Agricultural Experiment Station field plots in 1918 (Leonian, 1922). It was primitively believed to be host specific to pepper, but was soon narrated on tomato, eggplant, cucurbits (cucumber, melon, pumpkin and others) and green and lima beans (Davidson *et al.*, 2002; Gevens and Hausbeck, 2004; Kreutzer, 1937; Kreutzer and Bryant, 1946; Kreutzer *et al.*, 1940; Tompkins and Tucker, 1937[a]; Tompkins and Tucker, 1937[b]; Wiant and Tucker, 1940; Crossan *et al.*, 1954). Although it is not assertive how it is disseminated, *P. capsici* has been reported at locations worldwide, including North and South America, Asia, Africa and Europe (Erwin and Ribeiro, 1996; Hwang and Kim, 1995; Sun *et al.*, 2008). Attempts have been exerted on revealing the epidemiology, genetics and mechanisms of infection and virulence with the emanation of *P. capsici* on diverse plant families (Hausbeck and Lamour, 2004; Quesada-Ocampo *et al.*, 2011; Ristaino, 1990; Hord

and Ristaino, 1991; Ristaino and Johnston, 1999). Studies imply that both sexual outcrossing and rapid asexual reproduction captivated a unique life history, at many locations, for the propagation and survival (Lamour and Hausbeck, 2003).

**2.5 Symptomatology of *Phytophthora* foot rot disease**

In Kerala, the *Phytophthora* foot rot disease appears mainly amid the South-West monsoon period, where weather conditions are benign (Anandaraj *et al.*, 1988). A well distributed high rainfall (2,500 mm) with less sunshine (1 to 3 h) during monsoon is highly congenial for the development of the disease (Anandaraj *et al.*, 1988; Sarma *et al.*, 1988). The pathogen is versatile in nature to infect all parts of the plant i.e. root, collar, stem, leaves, inflorescence and berries. The black pepper vine succumbs to infection at any of its growth stages. Since the pathogen is soil-borne, the spread of the pathogen is mainly through soil and water (Sarma and Nambiar, 1982). Soil-borne inoculum plays an important role in the onset of epiphytotic of *Phytophthora* diseases (Thorold, 1959; Turner, 1967; Evans, 1973).

*Phytophthora capsici* infects whole black pepper vine, *i.e.*, leaves, stem, inflorescence, collar and roots that bring on leaf rot, stem rot, dropping of inflorescence, collar rot and root rot, respectively. Amidst these infections, collar rot and root rot cause severe and abrupt mortality of the vines. Leaf infections proceed with grey centres, surrounded by interspersing dark and light brown zones with peripheral water-soaked margins (Muller, 1936). This bifold zonation appeared in alternate wet and dry weather but not in continuous wet condition. Leaves are also noted with homogenous brown lesions with fimbriate margins (Holliday and Mowat, 1963). Wilting and rapid defoliation primarily developed as an early symptom of the disease (Alconero *et al.*, 1972), the tender leaves were highly susceptible than mature ones wherein, the incidence in the lower surface of the leaf seems more than upper surface (Turner, 1969). Tender shoot tips or leaves are first infected and the infection quickly spreads from the base of the vines (Sarma *et al.*, 1988). Spike sheds by abscission upon infection at the distal end. Black lesions are developed at the distal end of the spike and few berries are hardly infected (Holliday and Mowat 1963; Nambiar and Sarma 1977). Holliday and Mowat (1963) reported that fruit-bearing plants were considerably more susceptible than younger plants. Treatment with a manifold of inoculum greatly increases the pathogen penetration of stem from roots and shoots. Stem infection as dark brown lesions on the lateral branches results in wilting and shedding. The tender berries on infected spikes appear

shrivelled and the spikes are shed. The cracking of the nodal region of the stem is a distinct symptom. The collar and root infection are fatal and advance to the death of vines within 10-20 days, hence, so-called "quick wilt'. The collar rot infection occurs either at the collar or just above or below the soil level. Collar and root infection go unnoticed unless marked by foliar yellowing. The infection at initial stage starts as water soaked, later progressed to brown to dark brown within 2-3 days and appear as a slimy dark patch. Young leaves become flaccid followed by yellowing and defoliation. Vascular discolorations observed in many cases but not consistently (Nambiar and Sarma, 1977). During cessation, the cortex gets disintegrated and peeled off. The collar infection steadily progress downwards and transmitted to the root system resulted in rotting of the root (Holliday and Mowat, 1963). Root infection of the vines goes unnoticed without any visible aerial symptoms. At the end of the line, foliar yellowing of the vine and shedding of leaves, spikes and lateral branches are noticed. Root damage and the amount of defoliation are directly correlated which determines the spread of the decline and death of the vine. During the post-monsoon season, the entire vine collapse due to the depletion of soil moisture which leads to wilting and drying of leaves. Foliar yellowing, flaccidity, defoliation, breaking off the stems at nodal regions and spike shedding are the characteristic aerial symptoms of root rot and collar rot infections (Muller, 1936 and Holliday and Mowat, 1963). *Phytophthora* infection in black pepper is weather dependent and is polycyclic and polyetic. The inoculum is carried from one season to another. The aerial spread is rapid when weather conditions are favourable. A temperature of 23-29°C, relative humidity of 81-99%, daily rainfall of 15.8 - 23.0 mm and sunshine of 3.5 hours per day favour aerial spread (Sarma *et al.* 1988; Ramachandran *et al.*, 1990). Losses of up to 100 per cent can occur in fields due to the incidence of this pathogen (Granke *et al.*, 2012).

## 2.6 Taxonomy of *Phytophthora*

*Phytophthora* species can be classified into 10 major clades, with *P. capsici* falling into Clade 2 based on the genus-wide phylogenetic analysis (Blair *et al.*, 2008) (Fig. 2.1). Morphological characters as well as isozyme data shows that *P. capsici* is closer to *P. tropicalis* (Aragaki and Uchida, 2001). Both species produces deciduous sporangia which are oblong shaped with prominent apical papillae on long pedicels. Oospores germinate to produce germ tubes that branch into typical mycelium and/or produce sporangia on long pedicels. The sporangia are caducous (deciduous at maturity) and have a prominent papilla (lateral thickening of the sporangial wall) (Mchau and Coffey, 1995).

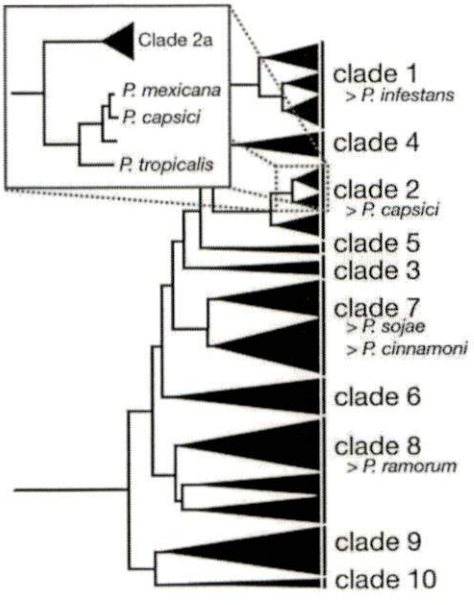



Fig. 2.1: Ten clades of *Phytopthora* species based on genome-wide phylogenetic analysis (Blair *et al.*, 2008)

*Phytophthora capsici* produces a massive number of sporangia on the surface of the infected tissue when the conditions become favourable. Mature sporangia are easily dislodged during irrigation or rain and when immersed in water, can quickly release 20–40 biflagellate motile zoospores. Since the zoospores are negatively geotropic, they will swim towards plants chemotactically. Zoospores shed their flagella onto the plant surface, encyst and adhere to the surface and form a germ tube. The germ tube can penetrate the plant cuticle directly and colonise host tissues (Feng *et al.*, 2010; Li *et al.*, 2011).

The host is a prerequisite for the successful completion of the life cycle of *P. capsici* and this fungus survives in soil for extended periods only as thick-walled oospores. The infection starts when the oospores germinate forming hypha which penetrates the plant cuticle and gets hold of host cells. In many cases, infection site predominates with appressoria. Growth and colonisation of host tissues mark the successful invasion which ultimately results in tissue collapse and sporulation. Within a period of 2-3 days, sporulation occurs if the optimum conditions of temperature (25-30 °C) and relative humidity (65-90 %) are in vogue. During the early phase, a direct host-pathogen interface is fashioned as a result of the encumbrance of hyphal protrusions (haustoria) which pushes the host cell membrane inwards (Schornack *et al.*, 2010). At early stages of infection, cells are not affected (biotrophy) but announce native suppression of defense responses (Huitema *et al.*, 2006). Later on access, *P. capsici* swaps to necrotrophy, elicit symbolic tissue collapse and necrosis by killing infected cells (Fig. 2.2) (Huitema *et al.*, 2006; Lamour *et al.*, 2012). Tissue collapse ensued by the emergence of sporangia, aids the dispersal and a new infection cycle.

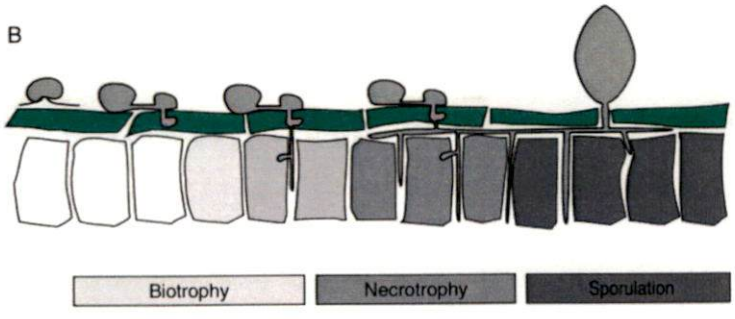



Figure 2. 2: Representation of *Phytophthora* infection process (adopted from Lamour *et al.*, 2012)

### 2.7 *Phytophthora* genomics

The *P. capsici* genome is 64 Mb long, a significantly larger compared to the more distant oomycetes like *Pythium* (42.8 Mb) and more similar in size to *S. parasitica* (63 Mb) and *P. ramorum* (68 Mb), though not as large as *P. sojae* (80 Mb). The numbers of predicted protein coding genes, however, do not differ as much between the *Phytophthora* species and range from around 14,500 for *P. sojae* to 21,000 for *P. parasitica*, and 18,000 and 20,000 for *P. infesans* and *P. capsici,* respectively (Haas *et al.*, 2009; Siedl *et al.*, 2012).

It quite seems that all oomycete species originate from a common stramenophile ancestor with approximately 10,000 core genes. Studies of genome expansion in the *Phytophthora* genus

resulted from the gene duplications arose before speciation and more interestingly, the duplicated gene groups are strongly associated with pathogenicity (Seidl *et al.*, 2012). Raffaele *et al.* (2010) describe so-called gene dense and gene sparse regions. Typically *Phytophthora* genomes consist of gene dense blocks with conserved order and high levels of synteny between species. These blocks contain 90% of all core ortholog genes and are separated by gene sparse regions. The gene sparse regions have no conserved order and contain predominantly pathogenicity associated genes. These regions also have a higher non synonymous over synonymous SNP rate, indicating that indeed these regions are more rapidly evolving than the rest of the genome. This phenomenon where some regions of the genome are evolving faster than others, was dubbed the 'two speed genome' and although it is less evident in *P. capsici* than it is to *P. infestans* and other members of clade 1, there is still evidence for rapid evolution of *P. capsici* pathogenicity related genes (Lamour *et al.*, 2012). *Phytophthora capsici* shows a much larger SNP rate than any other oomycete, creating large variation in the population. Additionally, large stretches of the genome have been identified that show Loss of Heterozygosity (LOH). LOH has also been observed in *P. ramorum* and can possibly account for the rapid evolution and high genomic diversity in *P. capsici* populations as beneficial mutations can be fixed rapidly in LOH stretches (Goss *et al.*, 2011; Lamour *et al.*, 2012).

### 2.8 Effector Biology of *Phytophthora* and other fungi

Fungi, like most plant pathogens, go through several developmental stages in the establishment of an infection. A plant pathogen must, for example, recognise and adhere to the plant surface before penetration and disease development commences. These steps are characterised by chemotaxis (recognition), glycoprotein interactions (adhesion), enzymatic degradation (penetration), and biotrophic or necrotrophic colonisation of the host plant (infection). Several enzymes are involved in each of these stages and some have been extensively studied in ascomycetes. These include cutinases, adhesins, cellulases, and polygalacturonases (Kolattukudy, 1985; Scott-Craig *et al.*, 1990; Talbot *et al.*, 1993; Annis and Goodwin, 1997; Rogers *et al.* 1994).

Plant pathogens use effector proteins to facilitate infection and reproduction (Birch *et al.*, 2006; Chisholm *et al.*, 2006; Tyler *et al.*, 2006). Early stages (biotrophy) seem to be healthy but a class of effectors, termed RXLRs, is secreted and delivered to the haustorial host–pathogen interface (translocation), where they are assumed to aid infection. Secretion and

translocation stand in need of a signal peptide, followed by a conserved N-terminal RXLR motif (Birch *et al.*, 2008; Win, *et al.*, 2007; Whisson *et al.*, 2007), features which grant the hasty recognition of effector candidates from oomycete (genome) sequences.

Besides the RXLR-type effectors, another class of cytoplasmic effectors, referred to as 'crinklers', has been defined recently in *Phytophthora* (Kunjeti *et al.*, 2012). The Crn1 and Crn2 proteins were first identified (Kamoun, 2007) in a high-throughput functional screen from the *P. infestans* proteins secretions and designated after their 'crinkling and necrosis' phenotypes (CRN) inspected on systemic expression in plants (Torto *et al.*, 2003). CRN proteins share a conserved N-terminal region that anchorage an anticipated signal peptide and a sustainable LQLFLAK motif needed for translocation. The family demeanour a diverse repository of C-terminal effector domains, which are assumed augmented virulence (Torto *et al.*, 2003).Despite intracellular effectors, recent studies have implicated other secreted proteins as virulence factors. Lately, 18 PcNpp (necrosis-inducing *Phytophthora* protein) and nine pectin methylesterase (Pme) coding genes have been described in *P. capsici* strain SD33 (Feng *et al.*, 2011; Li *et al.*, 2011). NPP coding genes formerly identified in other oomycetes were found to induce host cell death, suggesting a role in host cell distress (Fellbrich *et al.*, 2002; Kanneganti *et al.*, 2006; Qutob *et al.*, 2002; Veit *et al.*, 2001).

NPP-like protein (NLP) have shown to be actively participating in the cytosolic activity during pore formation in infection, have a structural analogy with cytosolic actinoporins (Ottmann *et al.*, 2009). NLP's high levels of expression and death-inducing activity contribute to the transition from biotrophy to necrotrophy. Although 12 PcNpp genes were found to be expressed during infection, their operative roles in *P. capsici* virulence remain mystery. In addition to the NPP proteins, PcPme genes were also found to be expressed during infection which speculates the role in tissue collapse and cell death. Functional studies of NLP's, PcNPP's and PcPme's during the host-pathogen interaction should illuminate the roles played by these proteins in the virulence of *P. capsici* (Li *et al.*, 2011).

## 2.9 Plant defense response to *Phytophthora capsici*

### 2.9.1 Biochemical defense response

The analysis of black pepper plants infected by *P. capsici* has revealed changes in the host cell membrane conductivity (measure of membrane permeability), in enzyme activities and in metabolite concentrations within the infected host tissues (Vandana *et al.*, 2014). However, due to the intricate nature of host-pathogen interactions, it is difficult to delineate the individual change caused by the plant or pathogen.

Vandana *et al.* (2014) have reported significantly higher content of total phenols, lignin and peroxidase activity in the roots of *Phytophthora* resistant line compared to those of susceptible line. In stem, total phenols, lignin, β-1, 3-glucanase and β-1, 4-glucanase activity were significantly high. Phenols are resistant factors because they become highly reactive upon oxidation by polyphenol oxidase and peroxidase to the corresponding quinines, which are toxic to the pathogen or which inactivate enzymes including hydrolytic enzymes produced by plant pathogenic fungi.

Plants respond to the presence of microbial pathogens by *de novo* synthesis of defense related enzymes. Cell wall peroxidase belongs to one of the important enzyme systems in reactive oxygen species (ROS) metabolism, generating $H_2O_2$ (Bolwell *et al.*, 1995; Gross *et al.*, 1977) which in turn leads to the development of an antimicrobial environment within the apoplast (Peng and Kuc, 1992). Upon *P. capsici* infection, enhancement of peroxidase activity was observed in the roots of resistant black pepper lines (Vandana *et al.,* 2014). Enhanced peroxidase generation is also reported in muskmelon (*Cucumis* melo) upon infection with *Pseudoperonospora cubensis* (Reuveni *et al.*, 1992), in sugarcane upon *Colletotrichum falcatum* invasion (Sundar *et al.*, 1998), in *Cucumis sativus* upon inoculation with cucumber downy mildew *P. cubensis* (Lebeda and Dolezal, 1995; Lebeda *et al.*, 2001), in green bean upon infection with *Uromyces appendiculatus* (Siegrist *et al.*, 1997), in taro upon inoculation with *P. colocasia* (Misra *et al.*, 2008) and in black pepper upon inoculation with *P. capsici* (Vandana *et al.*, 2014).

### 2.9.2 Molecular defense response

Knowledge of plant defense is important for the development of any varieties with enhanced resistance to biotic and abiotic stresses. The best strategy to prevent disease in plants is the development of resistant varieties because it is less expensive, environmentally friendly, and a

sustainable alternative to chemical applications such as fungicides. In addition, planting disease resistant varieties does not require alteration to cultivation practices. Disease resistance manifests itself in two forms: host and non-host resistance. Non-host resistance is defined as the resistance shown by a plant species toward many pathogens for which it is not considered to be a host, and it is effective against all known isolates of those given pathogens. The mechanisms of non-host resistance are not well understood but likely vary on a case-by-case basis and rely upon one or more mechanisms, such as the strengthening of the plant cytoskeleton to provide a physical barrier or the production of secondary metabolites with antimicrobial activity that present a pre- and post-invasion defense mechanism. The second form of resistance is host resistance, which is expressed when a plant that is considered a host can resist the infection of a specific pathogenic strain. Race-specific resistance operates within host resistance and is defined as resistance to specific isolates of a pathogen but not to all. This type of resistance is associated with the gene-for-gene mechanism. Basically, a protein product or the effector, produced by an avirulence gene in the pathogen is recognised by a resistance (*R*) gene in the host (Vleeshouwers and Oliver, 2014). Successful recognition results in the induction of a signal transduction in the host that initiates the host defense responses and the inhibition of pathogen growth.

Apart from the host-translocated (cytoplasmic) effectors oomycete fungi such as *Phytophthora* spp., secretes apoplastic effectors (Fawke *et al*., 2015). Plant pathogenic fungi can circumvent host immunity throwing effectors that suppress R- gene mediated resistance. *e.g.*, the resistance response conferred by the *R*-genes *I-2* and *I-3* are quenched by the Avr1 effector of *Fusarium oxysporum* f. sp. *lycopersici* (Parmar and Subramanian, 2013).

### 2.9.3 Host-disease resistance

Invading pathogens trigger various immune responses and the strategies adopted are part of plants innate immune system. During infection, plant pathogen secretes effector proteins called elicitors that recondition host physiology and immunity for their growth and development. Thus, the pathogen finds its way to counteract the basic defence responses [PAMP-triggered immunity (PTI)] (Jones and Dangl, 2006). Effector-triggered immunity (ETI) creates the forward barrier which is highly specific and triggered if and only when the plant have the so called *R* genes or plant resistance genes (Jones and Dangl 2006; Ingle *et al*. 2006).*Phytophthora capsici* is virulent on plants conferring the *R3a* resistance gene unless *PiAvr3a* is expressed in transgenic strains. The functionality of *Avr3a*-triggered immunity is determined by reinstating *PiAvr3a* RXLR region with

candidate translocation signals and verified *in vivo* which requires its delivery inside host cells (Kemen *et al.*, 2011; Schornack *et al.*, 2010).

**2.10 Work done at KAU**

Investigations were made to evaluate somaclonal variations in black pepper cultivars, Kalluvally, Cheriyakanyakkadan, Balankotta, Karimunda and Panniyur-1 under the selection pressure of *Phytophthora capsici in vitro* and the calliclones of Cheriyakanyakkadan showed greater degree of tolerance to *Phytophthora* foot rot disease (Shylaja, 1996).

Jubina (1997) reported the biocontrol activity of antagonistic bacteria from the black pepper rhizosphere *in vitro* which were highly effective in inhibiting sporangial production by the *P. capsici* and disease suppression in rooted cuttings of susceptible black pepper varieties and provided continued protection for longer period.

Paul (2000) attempted parasexual hybridisation of *Piper nigrum* and *Piper colubrinum* through protoplast fusion but all the protoplasts died due to the heterogeneous size of protoplasts of two interspecific species.

Vijayaraghavan (2003) reported the management of *Phytophthora* foot rot in black pepper nursery by general soil solarisation, application of antagonists and Ridomil MZ spray and observed favourable effect in checking the incidence and severity of the disease.

Mammootty (2003) also studied the management of *Phytophthora* diseases in black pepper and reported the effectiveness of general soil solarisation, application of *Trichoderma* spp. and fungicides like Akomin 40 or Ridomil MZ against *Phytophthora* foot rot disease.

Nair (2003) investigated the effect of plant growth promoting rhizobacterial strains belonging to fluorescent *pseudomonas* and *Bacillus* spp. and the root endophytic fungus, *Piriformospora indica* on the suppression of nursery wilt of black pepper incited by *Phytophthora capsici*. and observed that *P. putida* strain 89B61 showed disease suppression *in vivo* apart from its role of plant growth promotion.

Shankar (2009) studied the role of β- 1,3 glucanase in disease tolerance against *Phytophthora* foot rot in black pepper at molecular level and analysed the expression in

comparison with resistant and susceptible genotypes. The elevated expression profile in resistant genotype unraveled the role of β- 1,3 glucanase in defense mechanism.

Jagtap (2012) characterised the partially fertile interspecific hybrid (Culture P5PC-1) from the cross *P. nigrum* x *P. colubrinum* tolerant to *Phytophthora* foot rot and tested the hybridity of putative F1 hybrids developed at Pepper Research Station (PRS), Panniyur, using Randomly Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeats (SSR) markers which revealed significantly high similarity of interspecific hybrids to respective female parents.

Joseph (2007) genetically transformed black pepper with rice *chitinase* and *npt II* genes through *Agrobacterium* mediated transformation against *Phytophthora* foot rot resistance and the integration has been confirmed by PCR.

The *in planta* transformation via pollen tube pathway was done in the black pepper variety Panniyur-2, using the total exogenous DNA of *Piper colubrinum*, a wild relative species of Piper resistant to the dreaded foot rot disease caused by *Phytophthora capsici*. These cultures were screened *in vitro* in the rooting phase by incorporating the toxic culture filtrate of the pathogen *P. capsici* in the rooting media and the plantlets showed variation in banding pattern compared to the DNA recipient parent *P. nigrum* variety Panniyur-2 (Asha and Rajendran, 2009).

### 2.11 Approaches for analysis of gene expression

Pioneer methods in analysis of gene expression includes the analysis of known genes and unknown or uncharacterized genes and also the analysis of transcripts of varied expression profiles (Green *et al.*, 2001).

#### 2.11.1 Complementary DNA (cDNA) libraries

cDNA libraries are developed by converting RNA to cDNA by reverse transcriptase, for use in gene expression studies based on complementarity and the coding genes are identified aided by messenger RNA (mRNA) as the template for the identification of a coding gene and the study of its regulation and function (Klickstein, 2008; Nascimento *et al.*, 2010). The synthesis of cDNA library makes use of an mRNA template, dNTPs, reverse transcriptase and oligo dT primers, which filters mRNA by exploiting the poly-A tail of the mRNA. The fragments obtained by this process are then linked to vectors, cloned and sequenced. These sequences are evaluated for homology

search in databases and microarrays or differential display techniques are adopted for the assessment of differential gene expression (Malone *et al.*, 2006). A peptide library can also be constructed availing these libraries (Rays *et al.*, 1996; Ying, 2004). Availability of sequence information can be considered for design specific primers which can anneal to specific regions of the transcript (Klickstein *et al.*, 2008). Gene structure studies and the elucidation of molecular mechanisms are made possible by transcript (Wise *et al.*, 2007). The spatial and temporal distribution of gene products in both the pathogen and the host can be analysed from the differential expression of transcripts (Guigo Serra *et al.*, 2000).

Ma *et al.* (2009) reported the identification of 2743 unique sequences out of total 5793 expressed sequences in cDNA library related to the wheat-Pst compatible interaction by expression analysis of the compatible and incompatible interactions between wheat and *Puccinia striiformis* (Pst) and the expression profile varies with different stages of infection.

cDNA libraries obtained by mRNA sequencing (mRNASeq) are investigated for the differential gene expression in soybean infected with *Phakopsora pachyrhizi* and observed the increased expression of *Clostridium stercorarium* subsp. *stercorarium* (CSS) copper chaperones, cytochrome P450, O-methyltransferases and reductases, class IV chitinase, β-1,3-glucanases, glutathione S-transferase, lipoxygenase 2, ATP-binding cassette transporters (ABC transporters), dienelactone hydrolases and EF-hand proteins (Tremblay *et al.*, 2012).

### 2.11.2 cDNA-Amplified Fragment Length Polymorphism (cDNA-AFLP)

The total RNA is converted to cDNA and subsequently digested with two restriction enzymes, one with rare and the other with the frequent cutter. The ends of cDNA are attached with synthetic linkers and the fragments are amplified using primers complementary to these synthetic linkers, which are then visualised and compared on a gel. Differentially expressed fragments which are of different size can then be isolated and sequenced (Bachem *et al.*, 1996).

cDNA-AFLP produced 255 wheat transcripts with expression changes after infection with *P. striiformis*, of which 161 classified as constitutive since they were induced in both compatible and incompatible interactions while 94 were preferentially expressed in the incompatible interaction. These sequences exhibits homology to genes regulating metabolism and

photosynthesis, defense and signal transduction, transcription, transport, protein metabolism and cell structure. (Wang *et al.*, 2010).

cDNA-AFLP produced 763 transcript derived fragments (TDFs) from the challenged susceptible (H24) and resistant (Anagha), genotypes of tomato against Bacterial wilt disease incited by *Ralstonia solanacearum,* of which 58 fragments were differentially expressed at the transcript level. The cloned sequences exhibits homology to plant retrotransposons, aspartate kinase/homoserine dehydrogenase, secretin proteins, which are well known genes involved in plant defense mechanism and the results satisfactorily pointed out the genes that can be targeted for incorporating bacterial wilt resistance (Nazeem *et al.*, 2011).

### 2.11.3 Microarrays

Microarray hybridization technology allows the expression analysis of a large number of genes from different species (Guindalini and Tufik, 2007). Specific gene probes (DNA or RNA) are immobilized in a glass, plastic or nylon matrix in such a way that the target labelled with fluorescent probe complementarily bind to the probe, low intensity or high-intensity fluorescence indicating the low or high expression of a particular gene within a pathosystem or in plants undergone specific stress (Kuo *et al.*, 2002; 2004). Thousands of genes can be simultaneously analysed and identification of expression profile reveals the possible function of specific genes (Al-Taweel and Fernando, 2011).

Microarray technique evaluated 185 up-regulated and 16 down-regulated genes in wheat with respect to *Fusarium graminearum* infection, and the sequences showed homology to stress and the defense responses such as β-1,3-glucanase and class I chitinase as well as oxidative reactions, regulatory functions, protein synthesis and the phenylpropanoid pathway (Golkari *et al.*, 2007).

### 2.11.4 Suppressive Subtractive Hybridization (SSH)

SSH separates differentially expressed genes by hybridization between sequences taken from a sample under study (the "tester") as compared to a control sample (the "driver"). In other words, tester sequences with no homology with the driver are separated from the pool as differentially expressed (Diatchenko *et al.*, 1999).

Goswami *et al.*, (2006) have reported the genes involved in the pathogenicity mechanisms of *F. graminearum* in wheat and identified four genes (*Abc*2, *Lyp*1, *Rrr*1 and *Zbc*1) likely involved in the pathogenesis and development of *Fusarium*.

### 2.11.5 Expressed Sequence TAG (EST) sequencing and Serial Analysis of Gene Expression (SAGE)

In SAGE the RNA is converted to cDNA using biotin-linked 3' oligodT primers, which are latterly cleaved with restriction enzymes. The fragments thus generated are attached to adapters, linked and amplified using PCR. The fragments generated are concatamers, as a result of the joining of various fragments, which are then cloned and sequenced for the analysis of differential expression (Velculescu *et al.*, 1995; Yamamoto *et al.*, 2001).

The infection of wheat with *Blumeria graminis* f. sp. *tritici* showed differential expression of genes coding for the enzymes ferulate 5-hydroxylase (F5H), phenylalanine ammonia lyase (PAL), cinnamoyl-CoA reductase (CCR), caffeic acid O-methyltransferase (CAOMT) and caffeoyl-CoA3-O-methyltransferase (CCOAMT), plus the multifunctional protein with carbamoylphosphate synthetase, aspartate carbamoyltransferase and dihydroorotase activity (CAD) which assumed to be important in defense mechanism (Bhuiyan *et al.*, 2009).

### 2.11.6 Representational Difference Analysis (RDA)

The RDA technique combines the subtractive libraries (Lisitsyn and Wigler, 1993) with PCR amplification, emanate the enrichment of differentially expressed fragments i.e. RNA is converted to cDNA and digested by restriction enzymes. Adapters are attached to the fragments and amplified by PCR. The tester sample is again digested and linked to new adapters which are complementary former adapter and proceeded with digestion of driver sample with restriction enzymes. The two samples are placed in contact and the tester-tester hybrids are exponentially amplified (Bowler *et al.*, 1999; Hubank and Schatz, 1994).

McGrann *et al.* (2007) have evaluated the non-host interaction of *Polymyxa graminis* on beet confirmed 17 genes related to metabolism (e.g. *NADP-isocitrate dehydrogenase*), synthesis and protein processing (e.g. ubiquitin extension protein), oxidative stress (e.g. class VII chitinase precursor), cell wall structure and development (e.g. glycine-rich protein) and signal transduction (e.g. serine/threonine protein kinase) are up-regulated.

### 2.11.7 RNA Sequencing (RNA-Seq)

The sequencing of RNA enables the entire transcriptome of a species to be studied using only small amounts of RNA. The data obtained by RNA-Seq analysis can be analysed using bioinformatics tools and can be coupled with real-time PCR (RT-PCR), is one of the most effective strategies to discover new genes (Howald *et al.*, 2012).

Cheng *et al.* (2013) have identified a large number of genes associated with specific stages and pathogenicity, including 98 predicted effector genes and validated transcriptional levels of 19 effector genes during the developmental and host infection stages of *P. capsici* using RT-PCR. *P. capsici* RXLR and CRN effectors are annotated for suppression of host cell death triggered by elicitins and NLP effectors.

### 2.11.8 Differential display reverse transcriptase PCR (DDRT- PCR)

Differential display technique concentrated on detection of differentially expressed genes. Liang *et al.* (1992) have first showed that combinations of anchored and arbitrary cDNA primers can be used effectively to produce a collection of expressed transcripts resulting from the total mRNA of a cell (Liang *et al.*, 1992; Liang *et al.*, 1993; Liang and Pardee, 1992). The banding pattern thus obtained from differentially expressed transcripts can be used to compare two or more cell types, stress intensities and durations which include both biotic and abiotic stresses which unravels the genes responsible for the above said condition (Bauer *et al.*, 1993; Ito *et al.*, 1994; Medini *et al.*, 2009; Xu *et al.*, 1996). This technique is apt and cost effective unlike differential hybridisation.

Differential display technique has been successfully employed in studying differentially expressed genes in fishes (Bowman *et al.*, 2002), threw light into the genes and transcription factors regulating the drosophila's circadian rhythm (Blau and Young, 1999) and among plants, this technique has been used in *Aneurolipidium chinense* for PIP kinase (Shi *et al.*, 2002), in other plants at different stages like fruit ripening stage (Wilkinson *et al.*, 1995); flower senescence (Hajizadeh *et al.*, 2011); seed development (Huang *et al.*, 2015) or by treatment with ozone (Sharma and Davis, 1995) or hydrogen peroxide (Desikan *et al.*, 2000); induction of touch regulated genes in *Arabidopsis* (Chotikacharoensuk *et al.*, 2006), expression of CA7 and NCED genes in common bean genotypes under drought stress (Khodambashi *et al.*, 2013) and for gene

expression associated with floral transition in orchids (Yu and Goh, 2000). The differentially expressed genes or cDNAs during heat stress (Joshi and Nguyen, 1996), senescence (Kleber-Janke and Krupinska, 1997) and dormancy (Johnson *et al.*, 1995) have also been reported. More than half of the crop loss is caused by abiotic stress worldwide (Rasool *et al.*, 2013; Rodziewicz *et al.*, 2014). A wide and extensive research using the DDRT-PCR technique has been carried out in the field of biotic and abiotic plant stress.

**Table 2.1: Application of differential display in stress biology of various crops**

| Sl. No. | Crop | Findings | Reference |
|---|---|---|---|
| 1 | Rice (*Oryza sativa*) | Expression of *SAMDC1* gene positively correlates with salt tolerance in rice. | Li and Cheng, (2000) |
| 2 | Wheat (*Triticum aestivum*) | The full-length cDNA or Root Hair Defective- 3 gene (*RHD3*) from the salt tolerant wheat variety Shanrong No. 3 (Za3) was cloned and upregulation of the gene under salinity was reported | Shan *et al.* (2005) |
| 3 | Barley (*Hordeum vulgare*) | Better expression of salt-inducible nuclease activity possibly analogous to the *Bnuc1* gene identified | Muramoto *et al.* (1999) |
| 4 | Chickpea (*Cicer arietinum* L.) | The differential display technique in two Iranian chickpea cultivars showed upregulation of twenty five gene fragments responding to salt stressed condition | Angaji *et al.* (2013) |
| 5 | Tomato (*Lycopersicon esculentum*) | The study revealed the induction, promotion or repression of several gene expression by salt treatment and cloned salt-induced mRNAs | Wei *et al.* (2001) |
| 6 | *Brassica campestris* L. ssp. *chinensis* (L.) | Makino Seven out of 78 cDNA sequences obtained were greatly homologous to certain identified expression genes linked to the signalling pathways under different abiotic stress | Qiu *et al.* (2009) |
| 7 | Sunflower (*Helianthus annuus*) | Cloned and sequenced 12 salinity-regulated cDNAs of which certain genes were reported to respond to both the salinity and drought stressed conditions. | Liu and Baird, (2003) |

| | | | |
|---|---|---|---|
| 8 | *Eucalyptus microcorys* | Down-regulation in genes noticed during adaptation to salt stress. A cDNA which was greatly related to α-tubulin readily got repressed under salinity | Chen and De Filippis (2001) |

### 2.11 Data mining tools- expression browser

#### 2.11.1 Botany Array Resourse (BAR)

The Botany Array Resource (BAR) Expression Browser program encounters hierarchical clustering, automatic averaging, and automatic treatment/control calculation capabilities and enables user to interpret expression profiles of a particular set of genes (Toufighi *et al.,* 2005).

#### 2.11.2 Expression browser tool

The Expression Browser is a tool for performing electronic Northern. More than 400 genes can be queried simultaneously making the expedition of large gene families possible and it regulates the automatic calculation of response ratios to the corresponding to the signals with respect to control. Various dimensions of the information like functional classifications, annotations, gene aliases are added to the output (Toufighi *et al.*, 2005).

#### 2.11.3 Expression Browser – Database

The Expression Browser allows you to query different expression databases. In Bio-Analytic Resource (BAR) Database, which contains expression levels from the Affymetrix ATH1 Whole Genome GeneChip for ca. 22810 genes across approximately 175 samples in *Arabidopsis*. Several data sets can be queried from AtGenExpress Consortium, which is coordinated by Lutz Nover (Frankfurt), Thomas Altmann (Potsdam) and Detlef Weigel (Tubingen), and supported by funds from the DFG, the Max Planck Society, and from other individual researchers. The AtGenExpress developmental data set was produced by Schmid and coworkers helps the user to compare sample with data sets available (Schmid *et al.*, 2005).

# Materials and Methods

# 3. MATERIALS AND METHODS

The study on "Transcriptome analysis of *Phytophthora capsici* tolerance in black pepper (*Piper nigrum* L.)'' was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University during the period 2015-2017. The materials used and methodologies adopted are discussed in this chapter.

## 3.1 Planting materials

Rooted cuttings of varieties were procured from Indian Institute of Spice Research (IISR), Kozhikode and raised in earthen pots in the glass house (Plate 4.1).

### 3.1.1 Salient features of black pepper varieties used in the study

#### 3.1.1.1 IISR Shakthi

IISR Shakthi developed by Indian Institute of Spices Research, Kozhikode, Kerala, is adapted to both plains and high ranges under rainfed conditions. It is an open pollinated progeny of Perambramundi and tolerant to *Phytophthora* foot rot. The yield potential of this variety is 2253 kg dry pepper/ha.

#### 3.1.1.2 IISR Subhakara

IISR Subhakara is also developed by Indian Institute of Spices Research, Kozhikode and is widely adapted to the growing tracts of Kerala and Southern Karnataka. It is a selection from Karimunda (KS 27) and it has a leaf length of 12.3 cm and breadth 6.5 cm with ovate shape and spike length of 7.7 cm. Subhakara is susceptible to *Phytophthora* foot rot. The yield potential of this variety is 2352 kg dry pepper/ha.

## 3.2 Laboratory chemicals, equipment and machinery

The chemicals used in the present study (Analytical grade) were procured from Merck India Ltd., Amresco, Genetix Biotech Asia Ltd., HIMEDIA, Sigma-Aldrich., Thermo-Scientific, and SISCO Research Laboratories. DNA ladder and PCR chemicals were supplied by Bangalore GeNei Ltd. All the plasticware were obtained from Tarson India Ltd. Borosilicate glassware were used.

Pathology works were carried out at the Department of Plant Pathology, College of Horticulture, Vellanikkara under Laminar Air Flow Hood. Microwave oven (Morphy Richards), hot air oven and autoclave (Kdt) were used for media preparation and autoclaving. High precision

electronic balance (Shimadzu), pH meter (EuTech Instruments PC 510), Icematic (F100 compact), micropipettes (Eppendorf, Accupipet), RNA ZAP™ and nuclease free water (Ambion, Inc, USA) and high speed refrigerated centrifuge (KUBOTA 3500, Japan) were used for RNA isolation. Samples were stored for the long term at -80 °C (Haier Bio Medical, China) and -20 °C (VESTFROST). For short term storage of 0-4 °C, refrigerators were used. Nanodrop® ND-1000 spectrophotometer was used for the estimation of RNA and cDNA concentration. For RNA quality estimation, Electrophoresis (BioRad), UV transilluminator (WEALTEC) and documentation (Gel Doc XR+) were used. ProFlex Veriti (Life Technologies) and Tarson Spinwin MC-00 were used for sample treatment. PCR reactions were carried out in Agilent Technologies SureCycler 8800. Urea PAGE anlaysis was performed in Sequi-Gen GT Sequencing Cell (Bio-Rad) along with PowerPac HV 5000. Staining was aided by the Spinix Reciprocating Shaker (Tarsons). The Urea PAGE gels were scanned using a scanner (hp Scanjet 4500c, 2400 dpi/48 bit).

**3.3 Collection of infected leaf samples**

Black pepper plants showing typical symptoms of *Phytophthora* foot rot disease were collected from Pepper Nursery, Vellanikkara (Plate 4.4).

**3.4 Isolation and maintenance of pathogen**

The pathogen, *Phytophthora capsici* was isolated from the infected leaf samples of black pepper. The infected leaves were collected and washed thoroughly with mild detergent and tap water, air dried and blotted with sterile tissue paper. Isolation of pathogen was done using standard protocol. The infected leaf portion was cut into small bits with a sterile blade and incubated in sterile distilled water for 24 hours in an AC chamber for the development of spoangia (Plate 4.5a). The leaf bits were observed under a microscope for hyphae and lemon shaped sprangia (Plate 4.5b). The pathogen was isolated on Carrot Agar Medium (CAM) from this leaf bits which showed the development of mycelia and sporangia (Annexure I). The isolation was carried out aseptically in Laminar Air Flow Hood and the Petri plates were incubated at low temperature (24±2 °C). The Petri plates were examined for the growth of pathogen from the next day onwards. The fungal culture was further purified by hyphal tip method.

**3.5 Inoculum preparation**

Seven day old pathogen culture on Carrot Agar medium was made into 5 mm diameter disc using a cork borer and placed in Petri plates containing autoclaved sterile water.

### 3.6 Artificial inoculation on plants

Artificial inoculation was carried out on abaxial side of the tender leaves of two black pepper cultivars at 6 pm in glasshouse. The tender leaves were wiped with distilled water to remove dust particles and pin prick was given using sterile needle. Culture disc was transferred using an inoculation needle and placed over the prick and covered with thin layer of sterile wet cotton. The whole plant was covered with polythene bag after sprinkling with distilled water to provide high humidity. The inoculated plants were incubated under low temperature to develop symptoms.

### 3.7 Collection of leaf sample

The inoculated leaf samples of two varieties were collected using DEPC treated scalpel on to the ice at 0, 2, 4, 6, 12, 24, 48 hours after inoculation. Leaf samples were also collected from control plants. The samples were packed in labelled aluminium foil and proceeded with RNA isolation.

### 3.8 Total RNA extraction

#### 3.8.1 General precautions for RNA isolation

To obtain intact and high quality RNA, precautions are strictly followed to avoid contamination and degradation during RNA isolation. All the materials like glassware, mortar and pestle, microtips and microcentrifuge tubes were treated overnight with DEPC treated water and then double autoclaved. Solutions like 75 per cent ethanol and MOPS buffer were prepared with double autoclaved DEPC treated water. Electrophoresis unit was first wiped with 75 per cent ethanol or RNAZap$^{TM}$ and washed thoroughly with DEPC treated water.

#### 3.8.2 Isolation of total RNA

Isolation of good quality RNA in sufficient quantity is a prerequisite for the RT-PCR analysis. The total RNA was isolated from healthy and inoculated leaves using Trizol method (Ambion, USA). The detailed procedure for the RNA isolation is given below.

**Reagents used**

1. Trizol-reagent
2. Chloroform
3. Ice-cold Isopropanol (100 %)
4. PVP
5. β-mercaptoethanol
6. 75 per cent ethanol (DEPC treated)

7. Autoclaved DEPC treated water

**Procedure**

1. Tender leaf (100 mg) was pulverised well in liquid nitrogen using DEPC treated mortar and pestle.
2. One ml of Trizol was added and the homogenate was transferred to a 2 ml centrifuge tube.
3. Homogenate was incubated at room temperature till the solution become brown.
4. The content was centrifuged at 12000 g for 10 min at 4 °C.
5. The supernatant was transferred to a 2 ml centrifuge tube.
6. To the supernatant, 200 µl of chloroform was added and the content was shaken vigorously for 15 sec and then kept at room temperature for 10 min.
7. The content was then centrifuged at 12000 g for 10 min at 4 °C.
8. The supernatant was transferred into 1.5 ml microcentrifuge tube and 500 µl of ice-cold isopropanol was added.
9. The contents were mixed by slight inversion and incubated in ice for 10 min. and then centrifuged at 12000 g for 10 min. at 4 °C.
10. The supernatant was discarded and the pellet was washed with 1 ml of 75 % ethanol by centrifuging at 5000 g for 2 min. at 4 °C.
11. The supernatant was discarded and the pellet was dried under laminar air flow.
12. The pellet was dissolved in 20 µl of DEPC treated water.

**3.9 Quality and quantity of analysis of total RNA**

**3.9.1 Quality analysis by Formaldehyde-agarose gel electrophoresis**

One per cent formaldehyde agarose gel electrophoresis was performed to check the quality of total RNA by using the protocol given by Baburao (2012).

**Chemicals used for gel electrophoresis**

1. Agarose (HIMEDIA)
2. 10 X MOPS (pH-7) (SRL)
3. 6 X gel RNA Loading dye (GeNei)
4. *Hind*III/*Eco*RI double digest ladder/100bp DNA ladder (GeNei)
5. Ethidium bromide solution (0.5 µg/ml)

**Procedure**

One litre 1X MOPS buffer was prepared by diluting 100 ml of 10X MOPS buffer in 900 ml of double autoclaved DEPC treated water. This buffer was used for the preparation of gel and filling the electrophoresis tank. The open end of casting tray was sealed with cello tape and kept on a horizontal platform and comb was placed at the one end. Agarose (0.5 g) was dissolved in 5 ml of 10X MOPS and 47.3 ml of autoclaved DEPC treated water by boiling in the microwave oven and allowed to cool for few minutes at room temperature. After that 3µl of ethidium bromide followed by 2.7 ml of formaldehyde was added and poured into the tray. The gel was allowed to solidify at room temperature. After the solidification of gel, the cello tap and the comb were removed and the gel was kept in the electrophoresis tank containing 1X MOPS buffer (with the wells facing towards the cathode) in such a way that it submerged to a depth of 1 cm. Five µl of total RNA was taken and denatured in PCR at 96 °C for 3 min. and immediately cooled on ice. Denatured RNA was mixed with 1µl of 6X RNA loading dye and loaded onto the gel and 3 µl of DNA ladder was run on the side to mark the molecular weight. The cathode and anode were connected to the power pack and the gel was run at 50 volts. The power was turned off when the tracking dye moved 5 cm from the wells. The gel was visualised under UV-Light and gel image was documented.

**3.9.2 Spectrophotometric analysis of total RNA**

The samples which were giving 3 intact bands in the formaldehyde agarose gel were further analysed to determine the quality and quantity of the isolated total RNA using Nanodrop® ND-1000 (Nanodrop Technologies Inc., USA). Before taking the reading in the Nanodrop, the paddle stand of the instrument was wiped properly and then 1µl of double autoclaved DEPC treated water was used as blank to initialize the instrument to zero and after that 1µl of the sample was loaded. The absorbance of the total RNA was measured at a wavelength of $OD_{260}/OD_{280}$ and the $OD_{260}/OD_{230}$ ratio was observed to determine the purity of the isolated total RNA. A sample with 1.8 to 2.0 $OD_{260}/OD_{280}$ ratio and above 1.0 $OD_{260}/OD_{230}$ ratio was considered as good quality RNA with no DNA or protein contamination.

**3.10 Differential Display Reverse Transcriptase Polymerase Chain Reaction (DDRT-PCR)**

**3.10.1 First strand cDNA synthesis**

The isolated total RNA predominantly consists of rRNA, tRNA and mRNA. The isolation of intact mRNA is essential for successful first strand synthesis and subsequent PCR amplification.

Mature mRNA molecules carry poly (A) at their 3' termini, to which oligo dT primers anneal. Reverse transcriptase enzyme can synthesise cDNA from isolated RNA under favourable conditions (Malek *et al.*, 2000). The single strand cDNA was used immediately for PCR without further purification (Thermo Scientific).

**Reagents used**

1. RNA sample
2. Oligo $(dT)_{18}$ primer (500 µg/ml)
3. First strand buffer (5X)
4. 10 mM dNTP mix (2.5 mM each)
5. DTT (0.1 M)
6. M-MuLV reverse transcriptase (200 units/µl)
7. RNase OUT Recombinant Ribonuclease Inhibitor (40 units/µl)
8. Autoclaved DEPC treated water

**Procedure**

1. Three µl of total RNA (~1 µg), 1 µl of oligo-dT primer and 8 µl nuclease free water were added to 0.2 ml of microcentrifuge tubes.
2. The mixture was incubated at 65 °C for 5 min. in the thermal cycler and then quick-chilled on ice. The mixture was then spun briefly to collect the content at the bottom of the tube.
3. Four µl of 5X first strand buffer, 2 µl of 10 mM dNTPs, 1 µl RNAse OUT recombinant ribonuclease inhibitor and 1 µl M-MuLV reverse transcriptase were added one by one to the tube in the order.
4. The contents were mixed gently and incubated at 42 °C for 10 min.
5. The reaction was inactivated by heating the contents at 70 °C for 5 min. and stored at -80 °C.

**3.10.2 Control PCR amplification**

In order to verify the results of first strand cDNA synthesis, a control reaction was carried out using Actin forward and reverse primers (Malek *et al.*, 2000).

1. The cDNA generated with the control first strand cDNA reaction was diluted to 1:1000 in nuclease free water
2. PCR reagents were thawed and briefly spun
3. The following PCR reagents were added into a PCR tube on ice (Table 3.1)

**Table 3.1. Composition of mixture for control reaction**

| Sl. No. | Components | Volume (µl) |
|---|---|---|
| 1 | cDNA from control RT reaction (1:1000 dilution) | 2 |
| 2 | 10X PCR buffer | 5 |
| 3 | 10 mM dNTP mix | 1 |
| 4 | 25 mM $MgCl_2$ | 3 |
| 5 | Forward actin primer | 1.5 |
| 6 | Reverse actin primer | 1.5 |
| 7 | *Taq* DNA polymerase (5 U/µl) | 0.5 |
| 8 | Water, nuclease free | 35.5 |
| Total volume | | 50 |

4. PCR programme is presented in Table 3.2.

**Table 3.2 Temperature profile for control reaction**

| Step | Temperature (°C) | Time | No. of cycles |
|---|---|---|---|
| Initial denaturation | 94 | 3 min | 1 |
| Denaturation | 94 | 30 sec | 35 |
| Annealing | 58 | 30 sec | |
| Extension | 72 | 45 sec | |

5. Twenty µl of the RT-PCR product was loaded on 1 % agarose gel. A distinct 450 bp was observed after ethidium bromide staining.

### 3.10.3 Second strand cDNA synthesis

Each fragment of single strand cDNA was amplified by PCR with the anchored and respective arbitrary primers (Table 3.3).

**Table 3.3 List of Anchor (T11C) and Arbitrary (AP) primers used in DDRT PCR**

| Sl. No. | Primer | Primer sequence (5' - 3') | No. of bases |
|---|---|---|---|
| 1 | T11C | AAGCTTTTTTTTTTT | 16 |
| 2 | AP1 | AAGCTTGATTGCC | 13 |
| 3 | AP2 | AAGCTTCGACTGT | 13 |
| 4 | AP3 | AAGCTTTGGTCAG | 13 |
| 5 | AP4 | AAGCTTCTCAACG | 13 |
| 6 | AP5 | AAGCTTAGTAGGC | 13 |
| 7 | AP6 | AAGCTTGCACCAT | 13 |
| 8 | AP7 | AAGCTTAACGAGG | 13 |
| 9 | AP8 | AAGCTTTTACCGC | 13 |

### 3.10.4 Procedure

Master mix was prepared as indicated in Table 3.4 and the mixture was spun properly. Master mix was added to each tube and cDNA template was added separately.

1. A negative control was maintained to ensure that no non-specific amplification has occurred due to contamination in any reagents
2. Special thin walled tubes of uniform thickness were used to ensure rapid and equal temperature changes throughout the reaction volume.
3. PCR reaction was standardised for second strand synthesis (Table 4).

**Table 3.4: Composition of reaction mixture for PCR**

| Sl. No. | Components | Volume per reaction (µl) |
|---|---|---|
| 1 | 10X reaction buffer A | 2 |
| 2 | dNTP mix (25 µM) | 1.6 |
| 3 | T11C Anchored primer (2 µM) | 2 |
| 4 | AP (1-8) Arbitrary primer (2 µM) | 2 |
| 5 | cDNA (25 ng) | 2 |
| 6 | Taq DNA polymerase (5U/µl) | 0.2 |
| 7 | Nuclease-free water | 10.2 |
| Final volume | | 20 |

4. PCR programme followed is presented in Table 3.5.

**Table 3.5 Temperature profile for the second strand cDNA synthesis**

| Sl. No. | Step | Temperature (°C) | Time |
|---|---|---|---|
| 1 | Initial denaturation | 94 | 2 min |
| 2 | Denaturation | 94 | 30 sec |
| 3 | Annealing | 40 | 2 min |
| 4 | Extension | 72 | 1 min |
| 5 | Step 2-4 | 40 cycles | |
| 6 | Final extension | 72 | 10 min |
| 7 | Hold | 4 | 10 min |

PCR product was electrophoresed using 6 % Polyacrylamide Gel Electrophoresis (PAGE) and visualized by silver staining.

### 3.11 Urea-PAGE

The cDNA or transcript derived fragments from eight different treatments of two varieties were visualised in 6 % denaturing Urea-PAGE gel by electrophoresis and silver staining (Liang and Pardee, 1992) to detect the polymorphic bands in resistant and susceptible genotypes.
The composition of Urea-PAGE gel is presented in Annexure III.

#### 3.11.1 Protocol

The work area was cleaned with distilled water followed by absolute alcohol. Cleaned IP plate and bind plate were wiped thoroughly with distilled water, dried and wiped with absolute alcohol followed by 10 % NaOH and allowed to dry for 30 minutes. Bind silane and Repel Silane (Sigmacote) were applied onto the dried bind plate and IP plate, respectively. Few µl of distilled water was dropped over the spacer sheet on both the sides and placed on the sides of the IP plate and covered with bind plate (bind silane applied surface towards the IP plate). The clamping unit was placed along the sides of the plates and locked by folding the lever towards the plates. The entire unit was placed vertically in the bottom frame. Locked it using the knots on both sides in the bottom frame. The entire unit was placed parallel to the work area and the level was adjusted using level balancer. Acrylamide-bis-acrylamide monomer solution was injected slowly through the cavity in the bottom frame without forming bubbles and comb was placed at the top of the gel

in an inverted position and allowed for solidification. After solidification, the bottom frame was removed and the plates were transferred to electrophoresis tank containing 0.5X TBE buffer. The inner chamber of IP plate was filled with 0.5X TBE buffer, comb was reverted with shark tooth towards the gel and purged the wells with a pipette. The wells were loaded with 1 µl of Formamide dye and connected the power pack, allowed for a pre-run at 1200 V for 30 minutes. Power pack was switched off and wells were purged again to avoid air bubbles.

**3.11.2 Sample preparation and electrophoresis**

Samples were prepared by mixing 4 µl of sample with 2 µl loading dye (10 µl Formamide, 0.5 M EDTA, pH 8.0, 10 mg of Xylene cyanol and 10 mg of bromophenol blue). Samples were denatured at 96 °C for 5 minutes and immediately dipped in ice and then loaded into the well. Power pack was restarted and run (1200 V for ~3 h) till three-fourth of the gel was covered. Power pack was switched off, the plates were dismantled from the unit and applied ice on the plates and allowed the plates to cool. Plates were separated with the help of spatula.

**3.11.3 Silver staining**

The Urea gel plate was immersed in the fixer solution and kept under shaking condition for 10 minutes. The plate was washed twice in DW. The plate was then transferred into a tray containing 0.1 % silver nitrate solution and shaken intermittently for 10 minutes. Plate was washed quickly in DW and transferred to tray containing developer solution and shaken till bands were developed. Further the gel was transferred to a tray containing 10 % acetic acid for 3 minutes. The plate was kept in DW for 5 minutes and transferred the plate into sequencing gel casting stand for drying.

**3.12 Isolation of differential cDNA bands from polyacrylamide gel**

Differentially expressed cDNA fragments were rehydrated for 20 min. (Does it mean that water was placed over the differentially expressed spots for 20 minutes?) with 10 µl of distilled water and scooped out from the polyacrylamide gel with a sterile surgical blade, avoiding much exposure to UV on a transilluminator. Each gel slice was incubated in 40 µl of distilled water for 2 h at room temperature (Wu, 2006).

**3.13Elution of DNA from the sliced gel fragments**

1. cDNA fragments were eluted from the gel slices using GeneJET Gel Extraction Kit (Thermo Scientific) as per manufacturer's guidelines (Vogelstein Gillespie, 1979). Gel slice was weighed in a 1.5 ml microcentrifuge tube.

2. Added 3X gel volume of gel solubilization buffer (DEA buffer).
3. The gel was resuspended in gel solubilization buffer by vortexing. The mix was heated at 75 °C until the gel was completely dissolved. Intermittent vortexing was given every 2 to 3 min to enhance gel solubilization.
4. Added 0.5X gel solubilization volume of binding buffer (DEB buffer) and mixed properly.
5. A spin column was placed in a 2 ml collection tube. The solubilized gel slice was transferred into the spin column that was assembled in the 2 ml collection tube and centrifuged at 12,000 g for 1 min.
6. The filtrate was discarded. Added 500 µl of wash buffer (W1) to the spin column and centrifuged at 12,000 g for 30 sec.
7. The filtrate was discarded and 700 µl of desalting buffer (W2) was added and centrifuged at 12,000 g for 30 sec.
8. A second wash was given by adding 700 µl of desalting buffer (W2), followed by centrifugation at 12,000 g for 30 sec to ensure that complete removal of salt. The filtrate was discarded and the spin column was again placed in the collection tube.
9. The column was again centrifuged for 1 min at 12,000 g to remove any residual buffer.
10. Spin column was transferred to a fresh 1.5 ml microcentrifuge tube. The eluent was pre-warmed at 65 °C to improve the elution efficiency.
11. 50 µl of eluent was added to the center of the spin column and column was allowed to stand for 1 min at room temperature. Then centrifuged at 12,000 g for 1 min.

The eluted cDNA fragments were checked on 1 per cent agarose gel and stored at -20 °C for further analysis. Ten µl of eluent was used for selective PCR amplification with modified dNTP concentration of 2mM and same primer set. The product was electrophoresed using 1.5% agarose gel, DNA was eluted from the marker and sent for sequencing.

### 3.14 *In silico* analysis of sequences

The sequences obtained were analyzed with the following online bioinformatics tools and validated.

#### 3.14.1 Reverse complement of sequences

To merge the sequences generated by forward and reverse primer, one of the sequences has to be reverse complemented. This was done by using online tool 'Reverse complement' available online.

### 3.14.2 Merging of sequences

Sequences generated by forward primer and reverse complemented sequence of reverse primer merged by using online tool 'Emboss Merger' available online at http://www.bioinfo.nhri.org.tw/gui/

### 3.14.3 Search for Homology

The nucleotide sequence of all the sequences was compared with the sequences available in nucleotide database using BLAST tool (www.ncbi.nlm.nih.gov/Blast/) provided by NCBI (Altschul *et al.*, 1997). Nucleotide-Nucleotide sequence comparison was done using BLASTn tool. While nucleotide-protein sequence comparison was done using BLASTx. The best sequence alignment results were noted and saved.

### 3.14 Validation of gene expression by Electronic Northern (eNorthern)

Electronic Northern (eNorthern) is a useful way to analyse how genes of interest are being expressed; using the gene expression data sets accumulated from publically available microarray data. The Arabidopsis Genome Initiative (AGI) codes of the *Arabidopsis* homologues were used for eNorthern analysis at the eNorthern/ Expression browser tool of Botany Array Resource (BAR) at http://bbc.botany.utoronto.ca/. the expression levels of genes in shoot and root of the *Arabidopsis* homologues under different abiotic stresses like osmotic, drought, cold, salinity, radiation, genotoxic, oxidative, wounding and heat stress at 0.5, 1, 3, 6, 12, 24 hours of stress induction were analysed and maintained in the *Arabidopsis thaliana* gene expression database.

Initially, the respective AGI codes for the sequence of cDNA or the transcript derived fragment of wet lab results were found by translated BLAST analysis at (www.ncbi.nlm.nih.gov/Blastx). From the BLAST results, the AGI codes or the *Arabidopsis* homologues of the genes for the sequence were obtained. Then eNorthern analysis using Botany Array Resource (http://bbc.botany.utoronto.ca/). was performed by selecting expression browser option from the BAR homepage (Plate 3.1- 3.4). The fluorescence value was obtained from the above output. The log2 values obtained were transformed to actual fluorescence values by the formula 2^log value. The expression levels were indicated as whole number scores after eNorthern analysis. A score of 3.0 and above indicates that the genes are highly expressive. The fluorescence value obtained for each stress was plotted separately on the graph as fold increase over control against the different time periods. The graphs were studied for the expression pattern. Genes with consistently high expression levels were selected for further analysis.

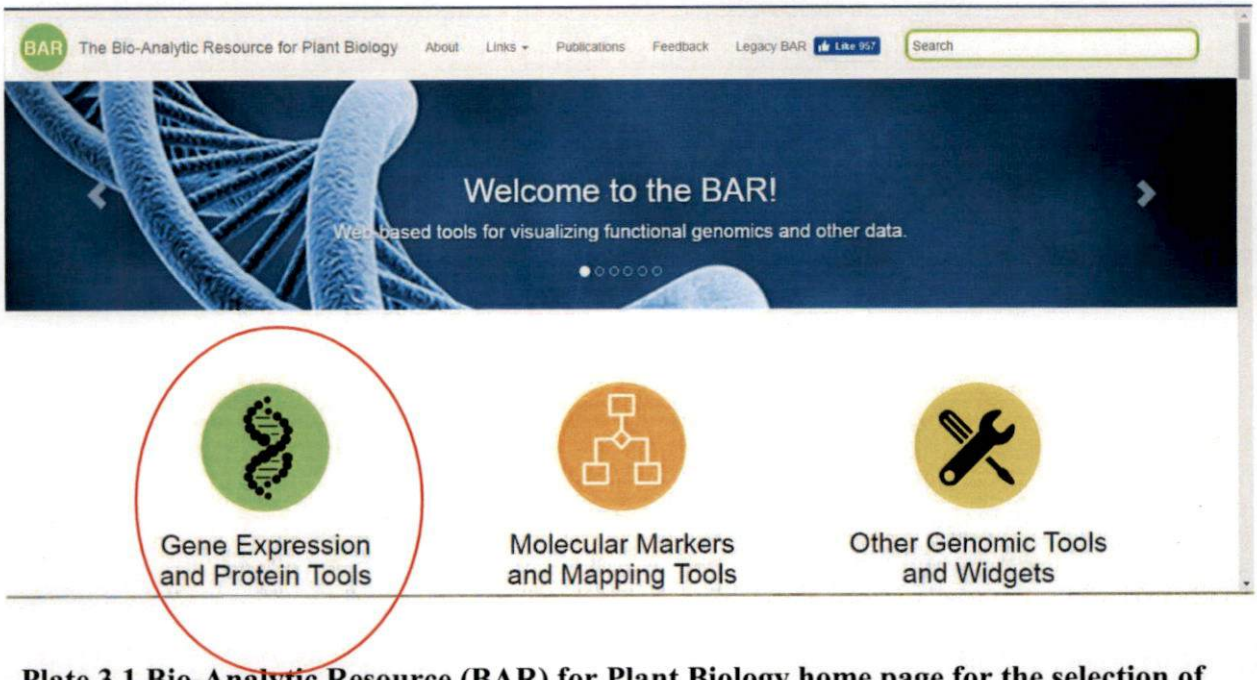



**Plate 3.1 Bio-Analytic Resource (BAR) for Plant Biology home page for the selection of required browser option**

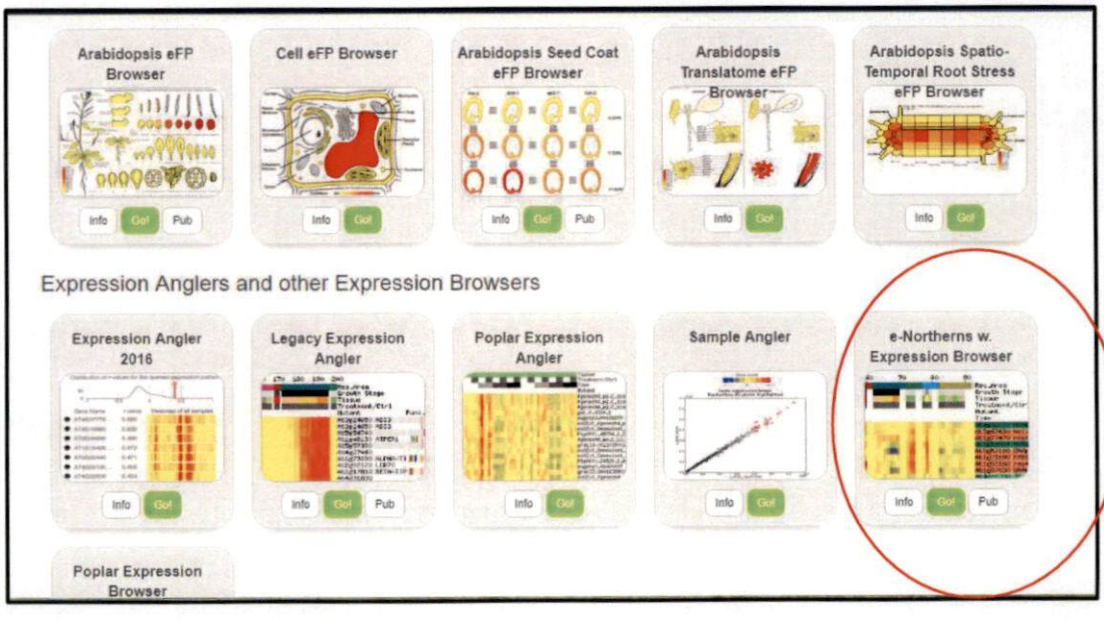



**Plate 3.2 Expression browsers available in BAR Gene expression tool**

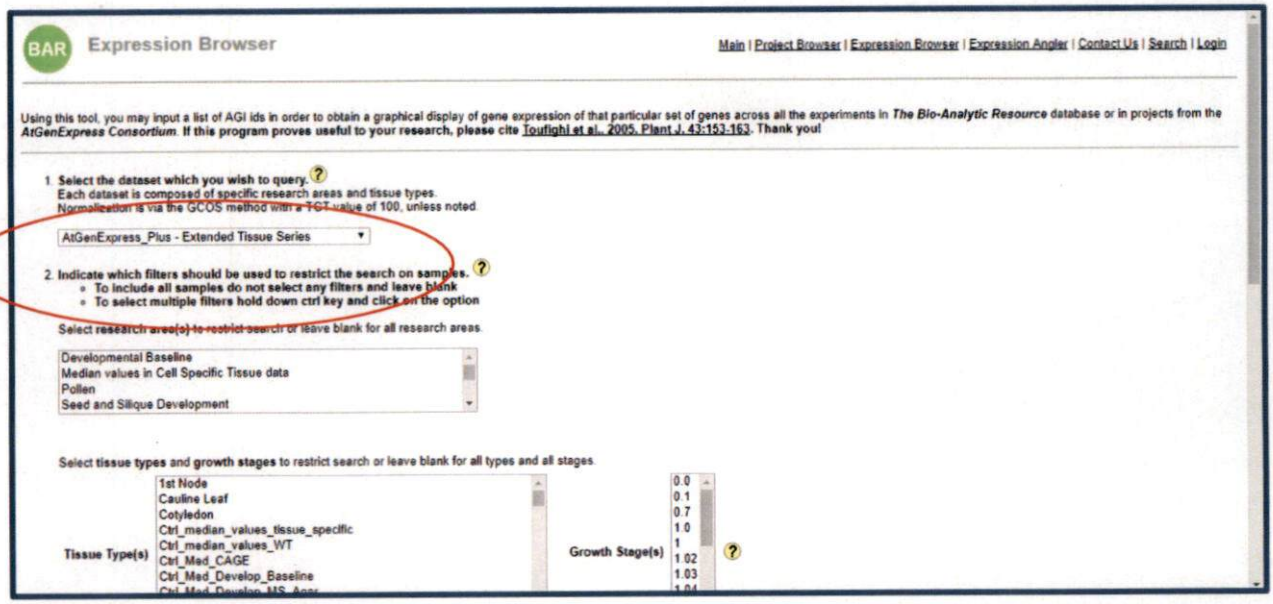



**Plate 3.3 Home screen of the eNorthern expression browser**

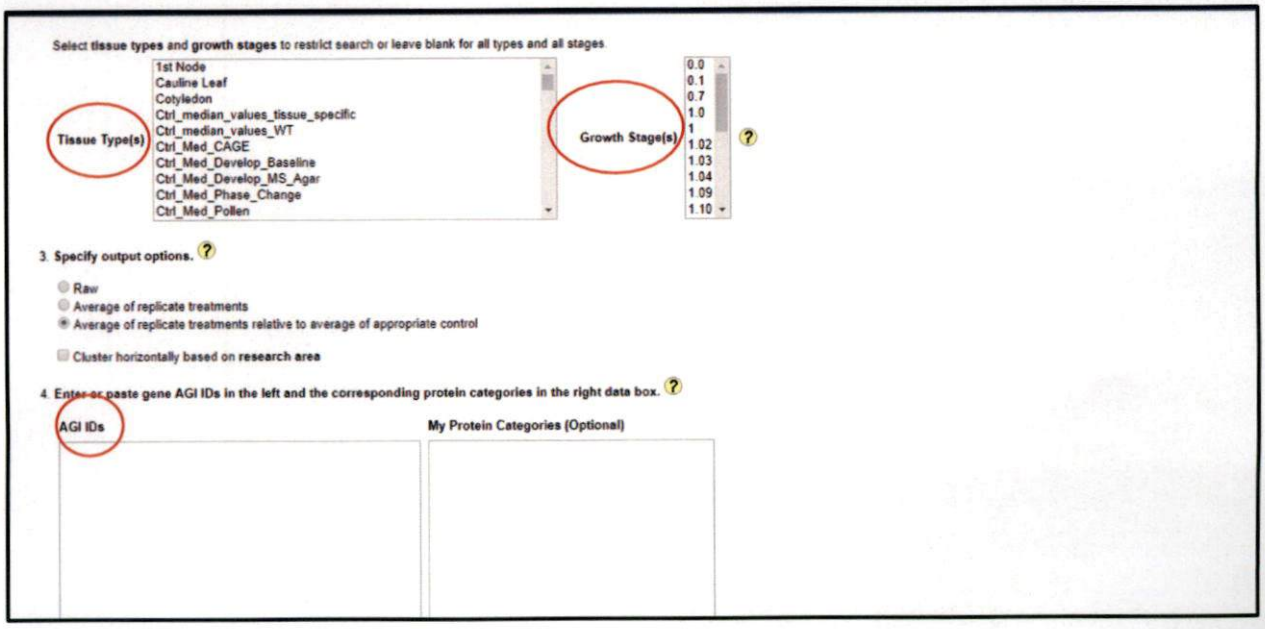



**Plate 3.4 Home page for feeding required information (includes database, AGI codes, protein categories, tissue type, growth stage, type of stress *etc.*)**

# Results

# 4. RESULTS

The results of the different experiments carried out to understand the differential expression of genes during *Phytophthora* infection in susceptible and tolerant black pepper cultivars through DDRT-PCR analysis on mRNA are presented in this chapter.

## 4.1 Collection and maintenance of black pepper cultivars

Rooted cuttings of varieties procured from IISR, Kozhikode were planted (Plate 4.1) and maintained in the polyhouse at CPBMB (Plate 4.2). Tender leaves of seven months old plants (Plate 4.3) were subjected to artificial inoculation followed by total RNA isolation.

## 4.2 Identification and isolation of pathogen

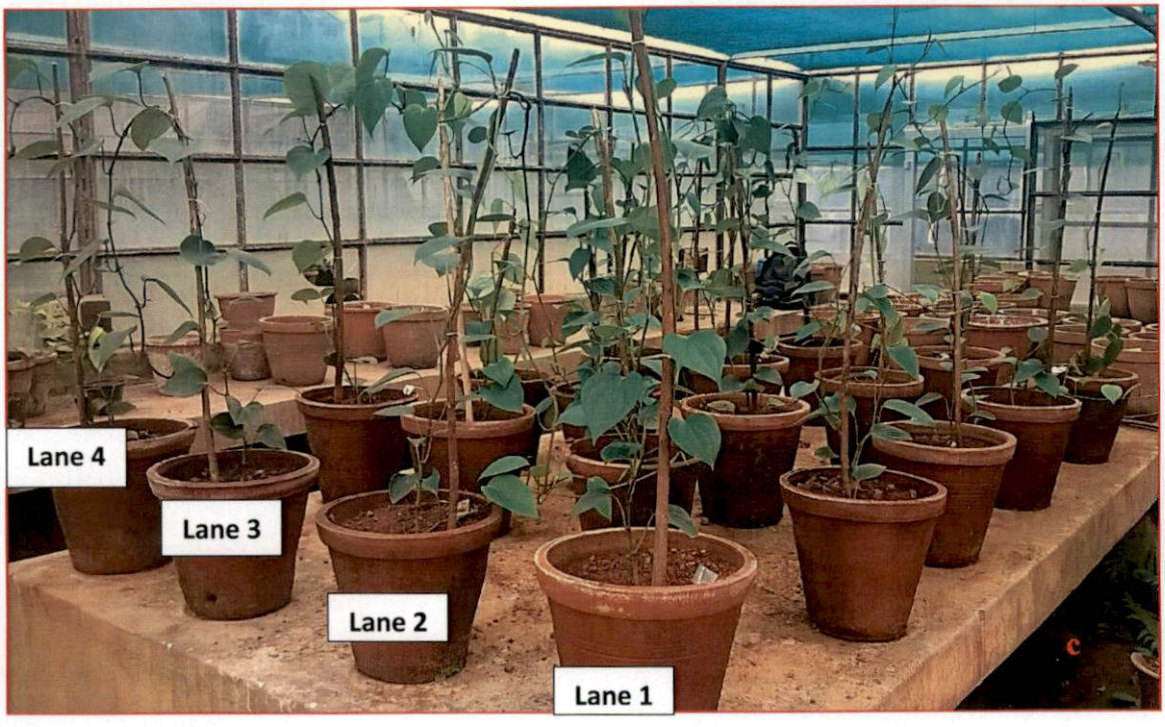
The fungal pathogen associated with foot rot disease, *Phytophthora capsici* was isolated from the leaf sample collected from Pepper Nursery, Central Nursery, Vellanikkara (Plate 4.4) on carrot agar medium. Mycelia and sporangia were observed after 24 hours of incubation at low temparature (Plate 4.5a, 4.5b). The initial growth was very slow in antibiotic medium (Streptomycin- 10 mg/ml). The growth was observed after 24 hrs in CAM and later sub-cultured in antibiotic-free medium for the fast growth, followed by antibiotic medium and finally, hyphal tip method was adopted to develop the pure culture (Plate 4.6). *Phytophthora capsici* marked with the appearance of whitish cottony growth with hyaline, aseptate hyphae and lemon shaped sporangium (Plate 4.7).

## 4.3 Artificial inoculation of plants

Artificial inoculation was carried out under controlled conditions of temperature (24 $\pm$2 °C) and relative humidity (65-90 %) for the infection to occur. Artificial inoculation was undertaken by placing 5 mm pre-incubated culture disc (24 hr incubation in distilled water) after pinprick was given at the abaxial side during the late evening 6 pm so that, the temperature is maintained cool throughout the infection time. After inoculation, the inoculated discs were covered with a wet cotton and the whole plant was covered with polybag and sprayed distilled water to maintain the humidity (Plate 4.8).


**IISR SUBHAKARA**
**(Susceptible)**


**IISR SHAKTHI**
**(Tolerant)**




**Lane 1 & 2 (IISR Subhakara) andLane 3 & 4 (IISR Shakthi)**

**Plate 4.2 Plant populations raised for the experiment (Label a,b and c)**

**Plate 4.1 Transplanted rooted cuttings in earthen pots**

**Plate 4.3 Seven months old plants for inoculation**

**Plate 4.4 Infected leaf samples collected from Pepper Nursery, Central Nursery, Vellanikkara**

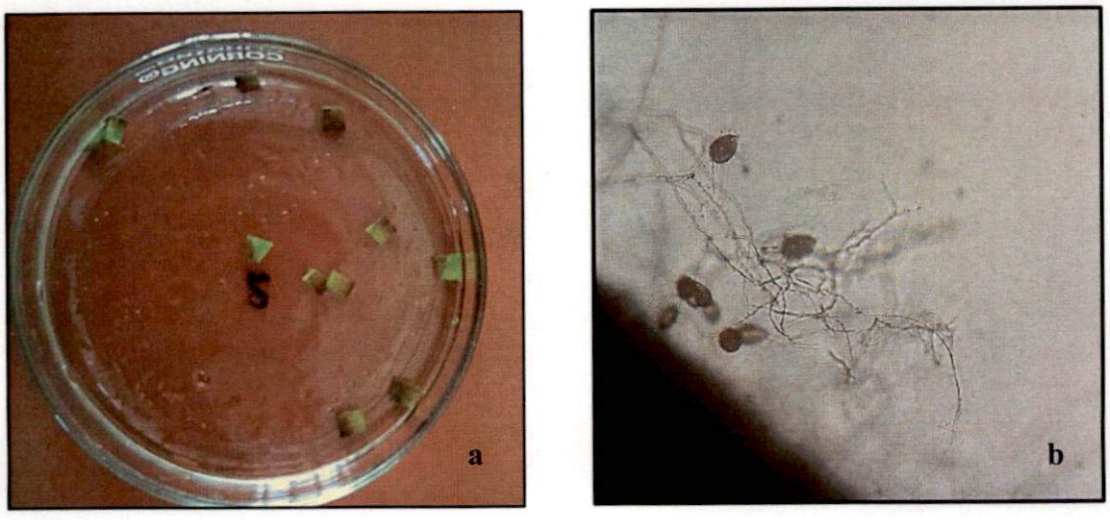



**Plate 4.5 (a) Incubation of leaf bits in distilled water for mycelial development and (b) developed mycelium observed in microscope after 24 hours of incubation (100X)**

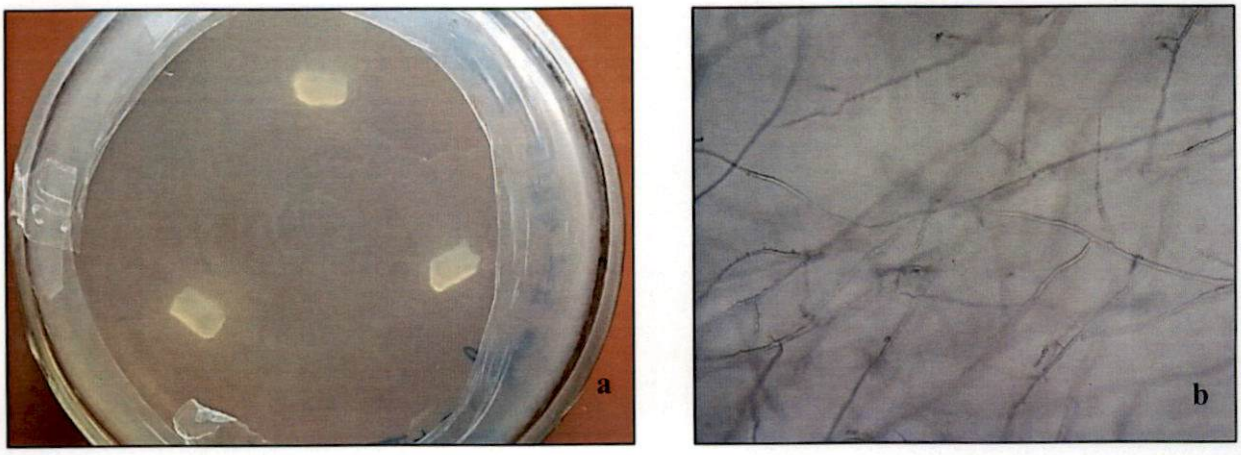



**Plate 4.6 Pathogen pure culture was made by hyphal tip method (a) subculture and (b)hyphal growth in Carrot Agar medium**

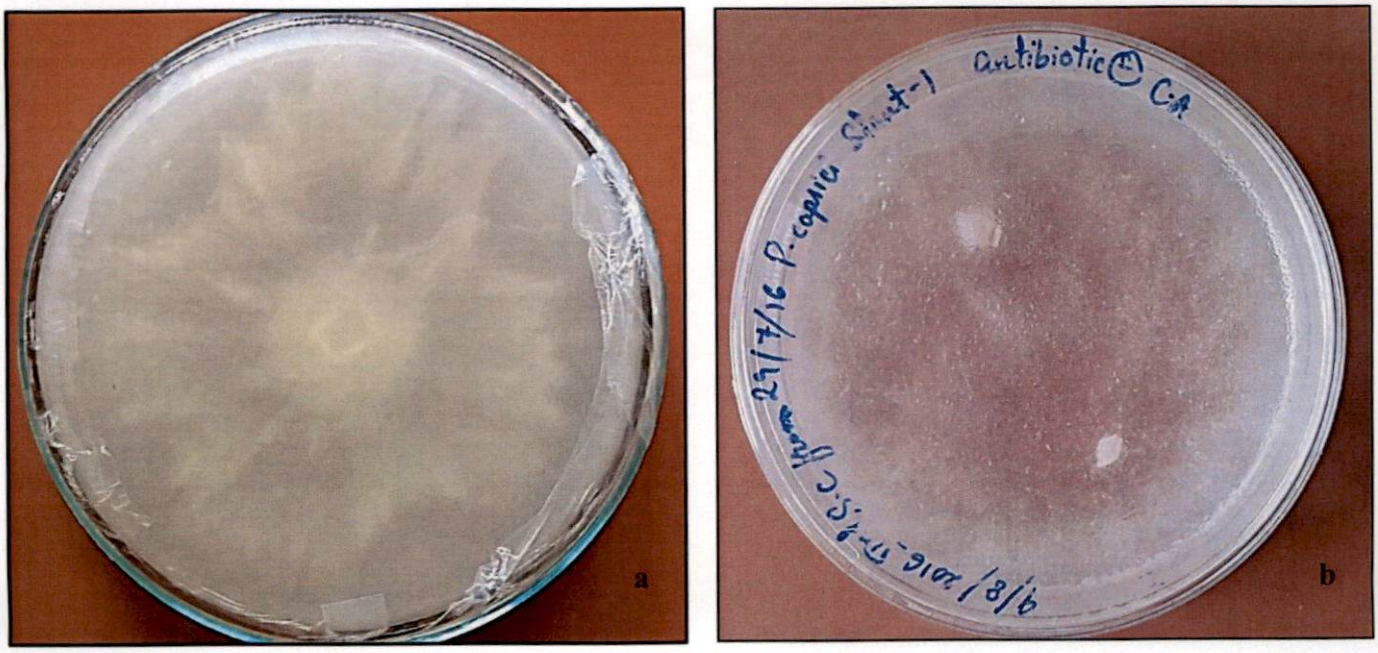



**Plate 4.7 (a) *Phytophthora capsici* pure culture and (b) maintenance of culture**


**Plate 4.8 (a) Artificially inoculated IISR Subhakara and (b) IISR Shakthi with *Phytopthora capsici* culture discs and maintained at 24±2 °C (AC chamber) and high relative humidity (80-90 %)**

**4.4 Symptomatology of the disease under artificial inoculation**

The artificially inoculated leaves showed yellow discoloration on the infected area. Later it enlarged with and became water soaked and black in colour. The dark lesions gradually turned to grey and exhibit light and dark zonation. Leaf samples were collected at different time periods of 0, 2, 4, 6, 12, 24, and 48 hours after inoculation (hai), kept in ice and continued with total RNA isolation.

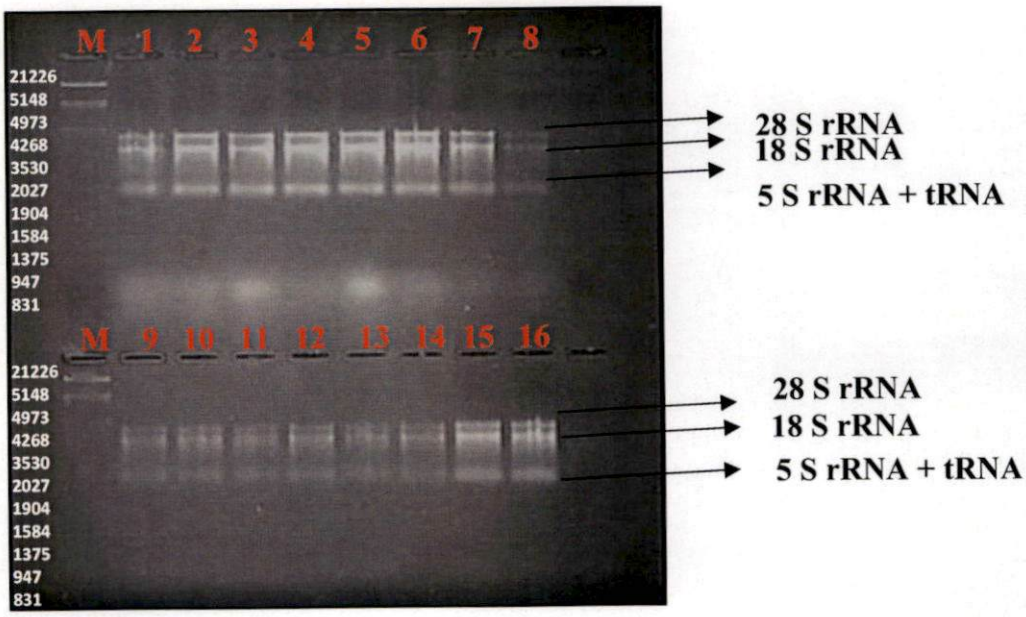
**4.5 Isolation and quantification of RNA**

A high concentration of total RNA was isolated using TRIzol reagent (Ambion, USA) from the control and treated leaf samples collected at different time periods of 0, 2, 4, 6, 12, 24, 48 hours after inoculation (hai) respectively. The RNA samples were quantified spectrophotometrically in NanoDrop® spectrophotometer ND 1000. The ratio of $OD_{260}/OD_{280}$ for the samples were quantified spectrophotometrically and was greater than 1.8 indicating pure RNA which is free from DNA and protein contaminations. The ratio of $OD_{260}/OD_{230}$ was greater than 1.0 indicating that the samples were free from polysaccharides and polyphenols (Table 4.1) and quality was confirmed through 1 % Formaldehyde Agarose gel electrophoresis. Three intact bands corresponding to 28S, 18S and 5S rRNA + tRNA were obtained which marked the quality of isolated RNA (Plate 4.9).

**4.6 Synthesis of cDNA first strand**

The mRNA from total RNA was first selectively withheld using oligo-dT primer and further proceeded for the synthesis of cDNA using H minus First strand cDNA synthesis kit (Thermo Scientific), aided by the addition of M-MuLV reverse transcriptase. The RNA strand was protected from degradation by the addition of RNase inhibitor and reaction was terminated at 70 °C for 5 minutes.

The conformation of the cDNA was done using the actin primer and PCR protocol, provided in the kit for the control reaction (Refer Table 3.1 and 3.2). The amplified product was analysed on 1.5 per cent agarose gel and presence of 450 bp band in agarose gel electrophoresis confirmed the synthesis of cDNA from total RNA (Plate 4.10).




**Lane 1 (M) – EcoRI/HindIII double digest.**

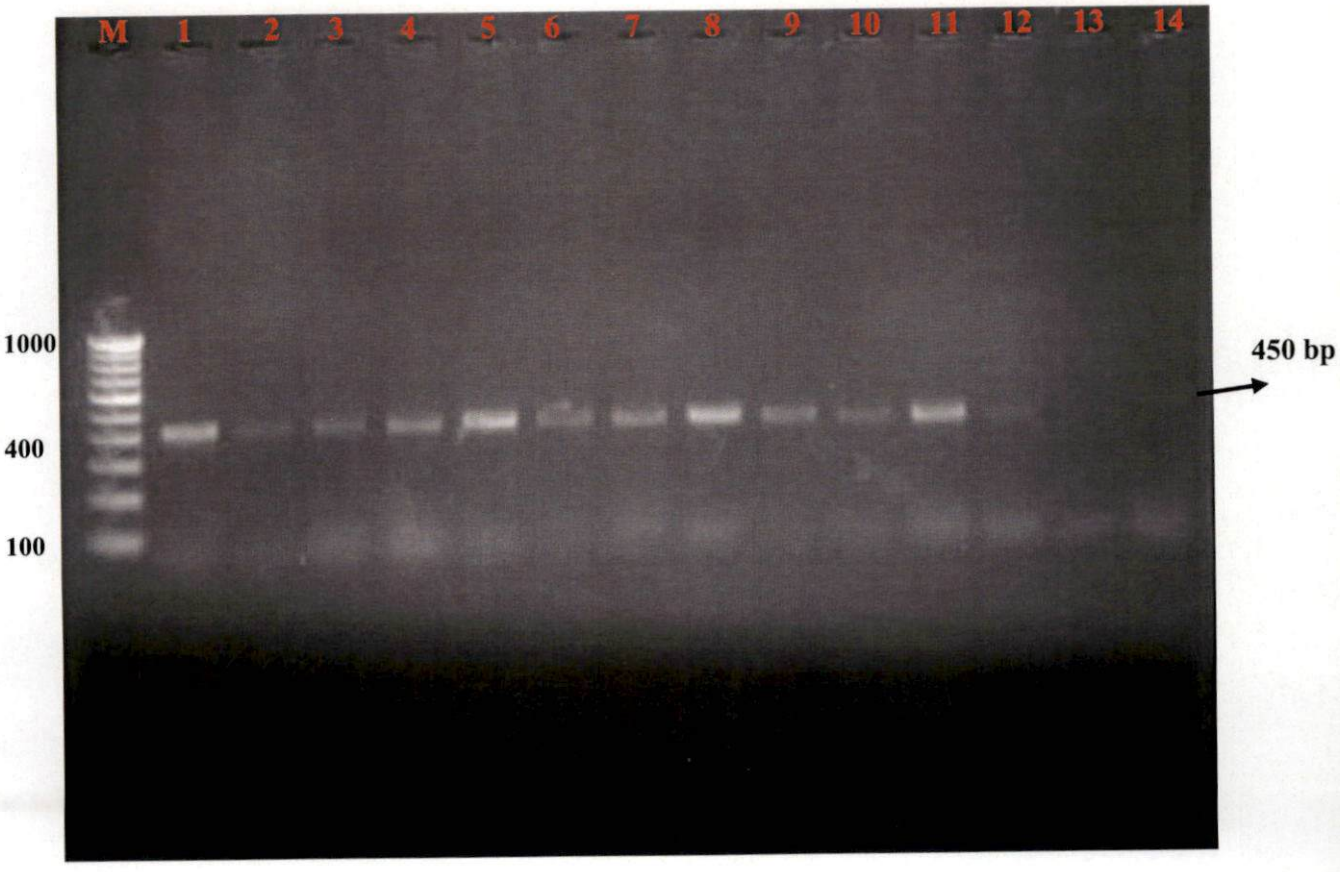
**Lane 1-8 -- IISR Subhakara (C, 0, 2, 4, 6, 12, 24, 48 hours post inoculation)**

**Lane 1-8 -- IISR Shakthi (C, 0, 2, 4, 6, 12, 24, 48 hours post inoculation)**

**Plate 4.9 Formaldehyde agarose gel electrophoresis of total RNA from two samples of control and seven treatments.**

**Table 4.1 Quality and quantity of total RNA isolated from leaves of two cultivars at different time periods.**

| Sl No. | Sample name | Concentration (ng/µl) | $OD_{260}/OD_{280}$ | $OD_{260}/OD_{230}$ |
|---|---|---|---|---|
| 1 | Subhakara (Control) | 1674.6 | 1.92 | 1.15 |
| 2 | Shakthi (Control) | 1735.7 | 1.83 | 1.05 |
| 3 | Subhakara (0 h) | 1994.6 | 1.92 | 1.62 |
| 4 | Shakthi (0 h) | 2157.3 | 1.87 | 1.47 |
| 5 | Subhakara (2 h) | 2339.1 | 1.89 | 1.68 |
| 6 | Shakthi (2 h) | 2146.8 | 1.98 | 1.75 |
| 7 | Subhakara (4 h) | 1885.9 | 1.73 | 1.14 |
| 8 | Shakthi (4 h) | 1026.1 | 1.91 | 1.05 |
| 9 | Subhakara (6 h) | 2305.7 | 1.82 | 1.25 |
| 10 | Shakthi (6 h) | 1572.7 | 1.86 | 0.86 |
| 11 | Subhakara (12 h) | 1204.0 | 1.93 | 1.04 |
| 12 | Shakthi (12 h) | 1340.4 | 1.91 | 0.91 |
| 13 | Subhakara (24 h) | 1178.2 | 1.88 | 0.80 |
| 14 | Shakthi (24 h) | 1643.5 | 1.84 | 1.18 |
| 15 | Subhakara (48 h) | 1414.0 | 1.93 | 1.03 |
| 16 | Shakthi (48 h) | 1316.7 | 1.88 | 0.90 |




**M- 100bp marker, Lane 1-14 – cDNA of RNA after infection**

**Plate 4.10 cDNA first strand confirmation of converted mRNA with Actin primers**

**Table 4.2 Quality and quantity of first strand cDNA synthesized from the isolated RNA**

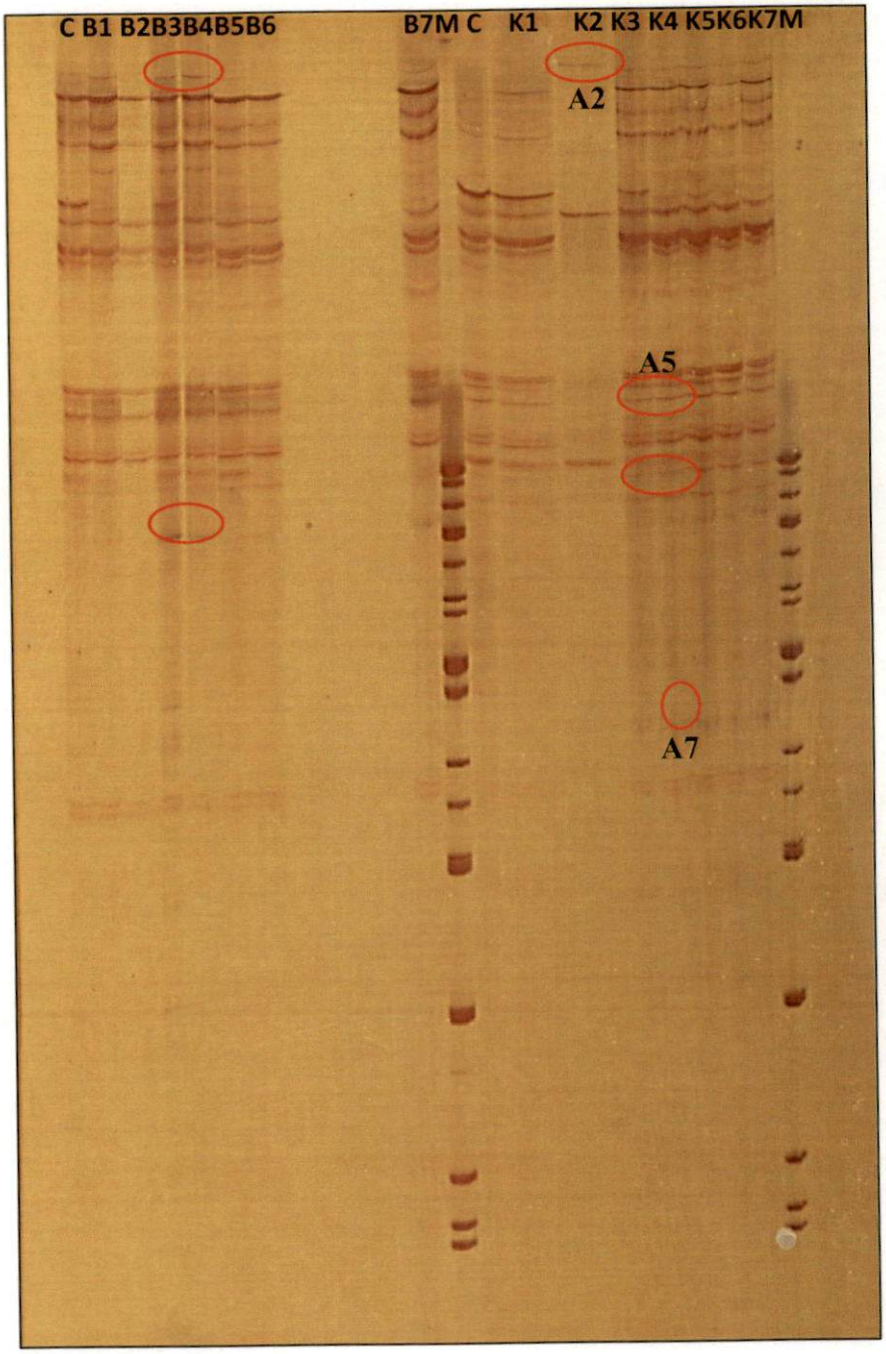
| Sl No. | Sample name | Concentration (ng/µl) | $OD_{260}/OD_{280}$ | $OD_{260}/OD_{230}$ |
|---|---|---|---|---|
| 1 | Subhakara (Control) | 1452.7 | 1.68 | 1.82 |
| 2 | Shakthi (Control) | 1451.3 | 1.64 | 1.70 |
| 3 | Subhakara (0 h) | 1205.0 | 1.67 | 1.55 |
| 4 | Shakthi (0 h) | 959.1 | 1.68 | 1.41 |
| 5 | Subhakara (2 h) | 854.2 | 1.71 | 1.49 |
| 6 | Shakthi (2 h) | 1168.4 | 1.78 | 1.63 |
| 7 | Subhakara (4 h) | 847.9 | 1.67 | 1.27 |
| 8 | Shakthi (4 h) | 282.4 | 1.25 | 1.35 |
| 9 | Subhakara (6 h) | 1397.3 | 1.70 | 1.28 |
| 10 | Shakthi (6 h) | 1054.4 | 1.71 | 1.17 |
| 11 | Subhakara (12 h) | 872.8 | 1.74 | 1.47 |
| 12 | Shakthi (12 h) | 1007.5 | 1.81 | 1.16 |
| 13 | Subhakara (24 h) | 569.7 | 1.67 | 1.05 |
| 14 | Shakthi (24 h) | 979.1 | 1.76 | 1.15 |
| 15 | Subhakara (48 h) | 1475.37 | 1.74 | 1.62 |
| 16 | Shakthi (48 h) | 1199.18 | 1.75 | 1.54 |

### 4.7 Differential Display (DD) Reverse Transcriptase PCR

The first strand cDNA synthesized from the above RNA samples were subjected to PCR amplification using one anchored (T11C) and eight arbitrary primers (AP) as mentioned in the Table 3.3. The PCR product was resolved on 6 % denaturing urea polyacrylamide gels and visualised after silver staining (Plates 4.11, 4.12, 4.13, 4.14, 4.15, and 4.16). The urea gel was dried overnight and differentially expressed, up-regulated and down-regulated genes were marked, and bands are eluted and stored at -20 °C for further analysis (Table 4.1).

**Table 4.1 Description of differentially expressed fragments (DEFs) in the gel**

| Sl No. | Sample | Primers | Plate No. | Description |
|---|---|---|---|---|
| 1 | A2 | AP1-T11C | 4.11 | Differentially expressed at 2nd hour after inoculation in resistant lane |
| 2 | A5 | AP1-T11C | 4.11 | Differentially expressed at 4th hour after inoculation in resistant lane |
| 3 | A7 | AP1-T11C | 4.11 | Upregulation from 6th hour after inoculation in resistant lane |
| 4 | C2 | AP4-T11C | 4.16 | Expressed only in control and 0th hour at resistant lane |
| 5 | I2 | AP5-T11C | 4.13 | Differentially expressed at 4th hour after inoculation in resistant lane |
| 6 | M1 | AP3-T11C | 4.15 | Differentially expressed at 12th hour after inoculation in resistant lane |
| 7 | M3 | AP3-T11C | 4.15 | Expressed only in resistant control |
| 8 | M4 | AP3-T11C | 4.15 | Upregulated at 48th hour after inoculation |
| 9 | K4 | AP7-T11C | 4.14 | Downregulated in resistant line from control to 48 hour |
| 10 | J2 | AP6-T11C | 4.12 | Differentially expressed at 2nd hour after inoculation in resistant lane |
| 11 | J3 | AP6-T11C | 4.12 | Differentially expressed at 48th hour after inoculation in resistant lane |
| 12 | J4 | AP6-T11C | 4.12 | Differentially expressed at 2nd hour after inoculation in resistant lane |

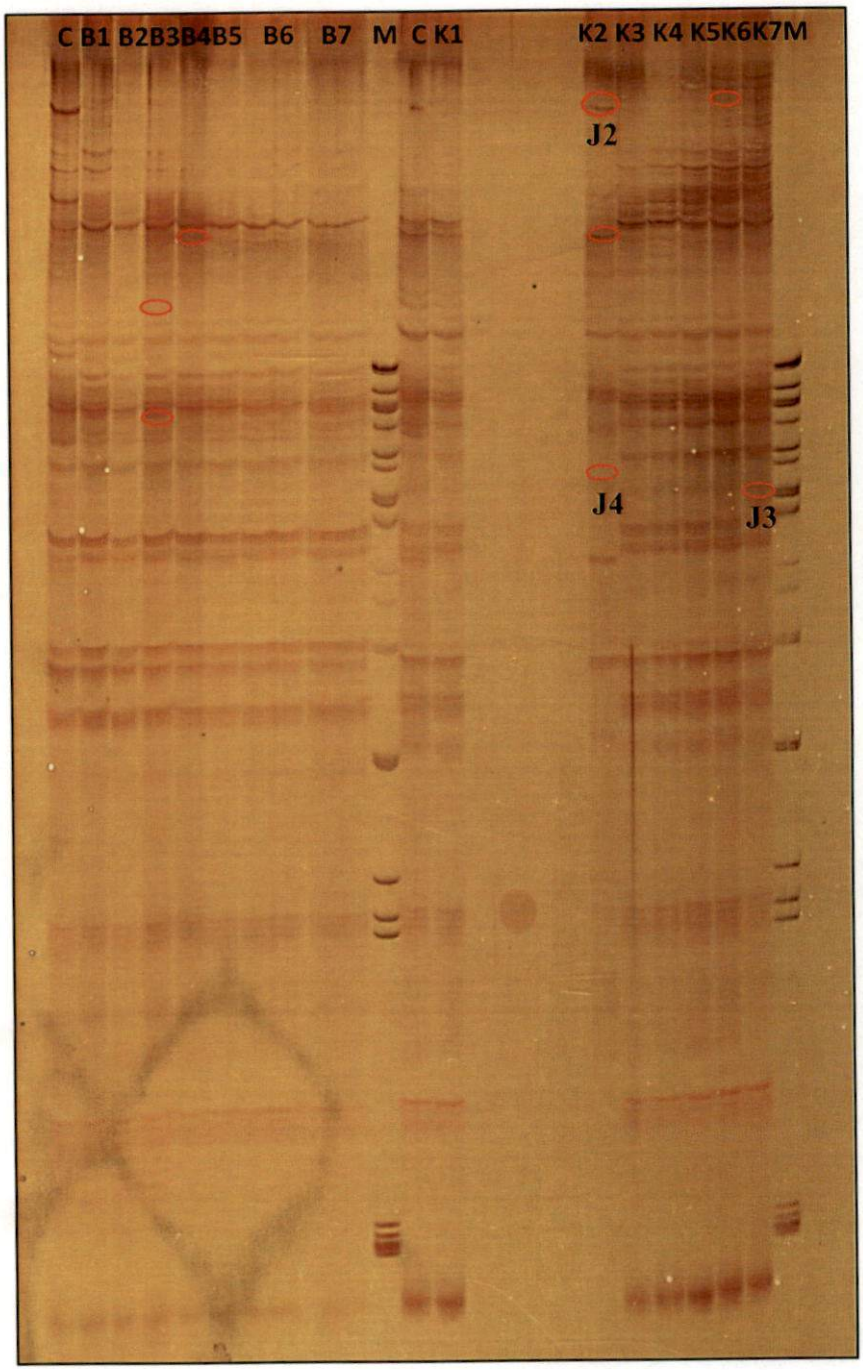



**M – GeNei 100 bp ladder**

**Lane C-B7 – AP1-T11C transcriptome pattern of IISR Subhakara**

**Lane C-K7 – AP1-T11C transcriptome pattern of IISR Shakthi**

**Plate 4.11 Differential display pattern of transcript derived fragments from seven treatments and control using anchored (T11C) and arbitrary primer (AP1).**

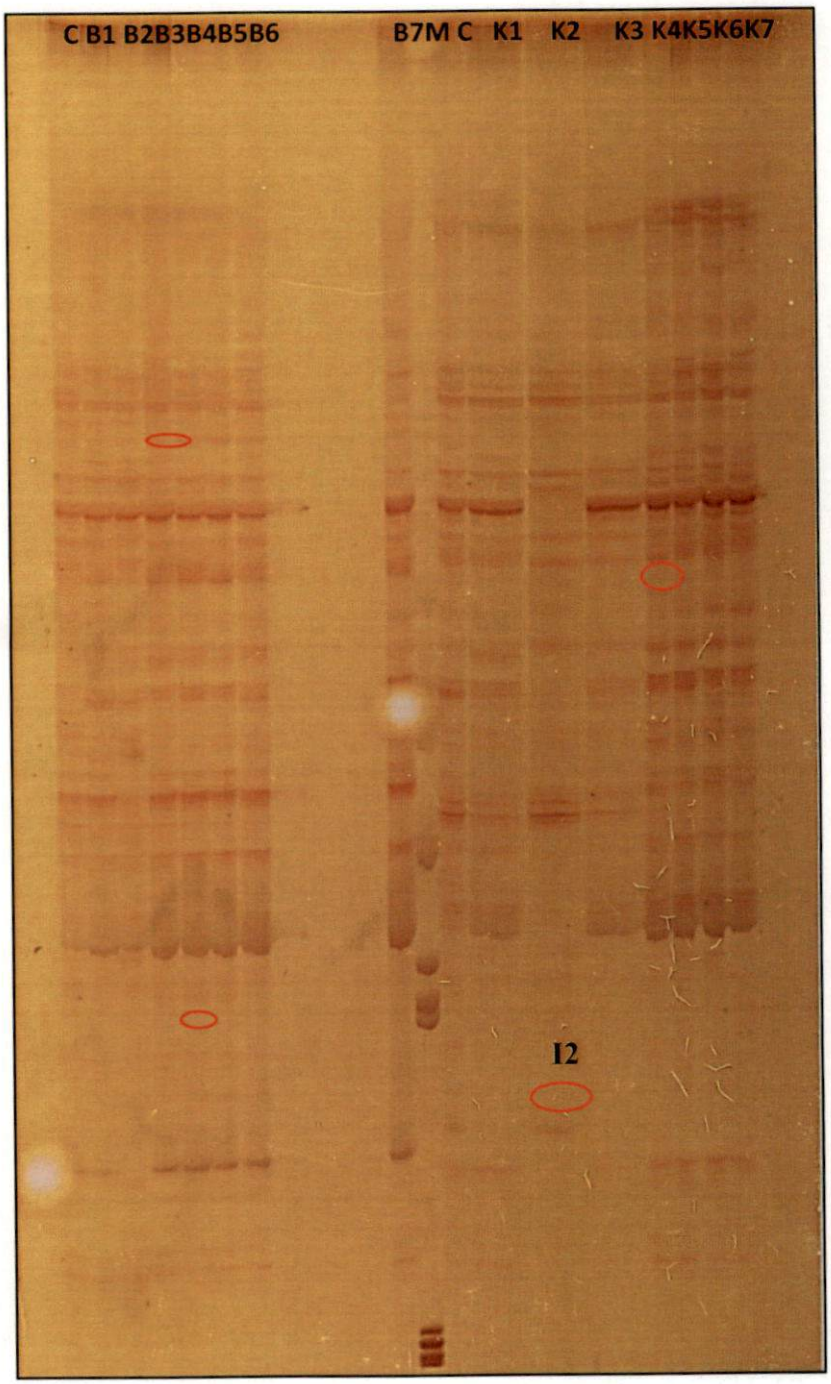



**M – GeNei 100 bp ladder**

**Lane C-B7 – AP6-T11C transcriptome pattern of IISR Subhakara**

**Lane C-K7 – AP6-T11C transcriptome pattern of IISR Shakthi**

**Plate 4.12 Differential display pattern of transcript derived fragments from seven treatments and control using anchored (T11C) and arbitrary primer (AP6).**

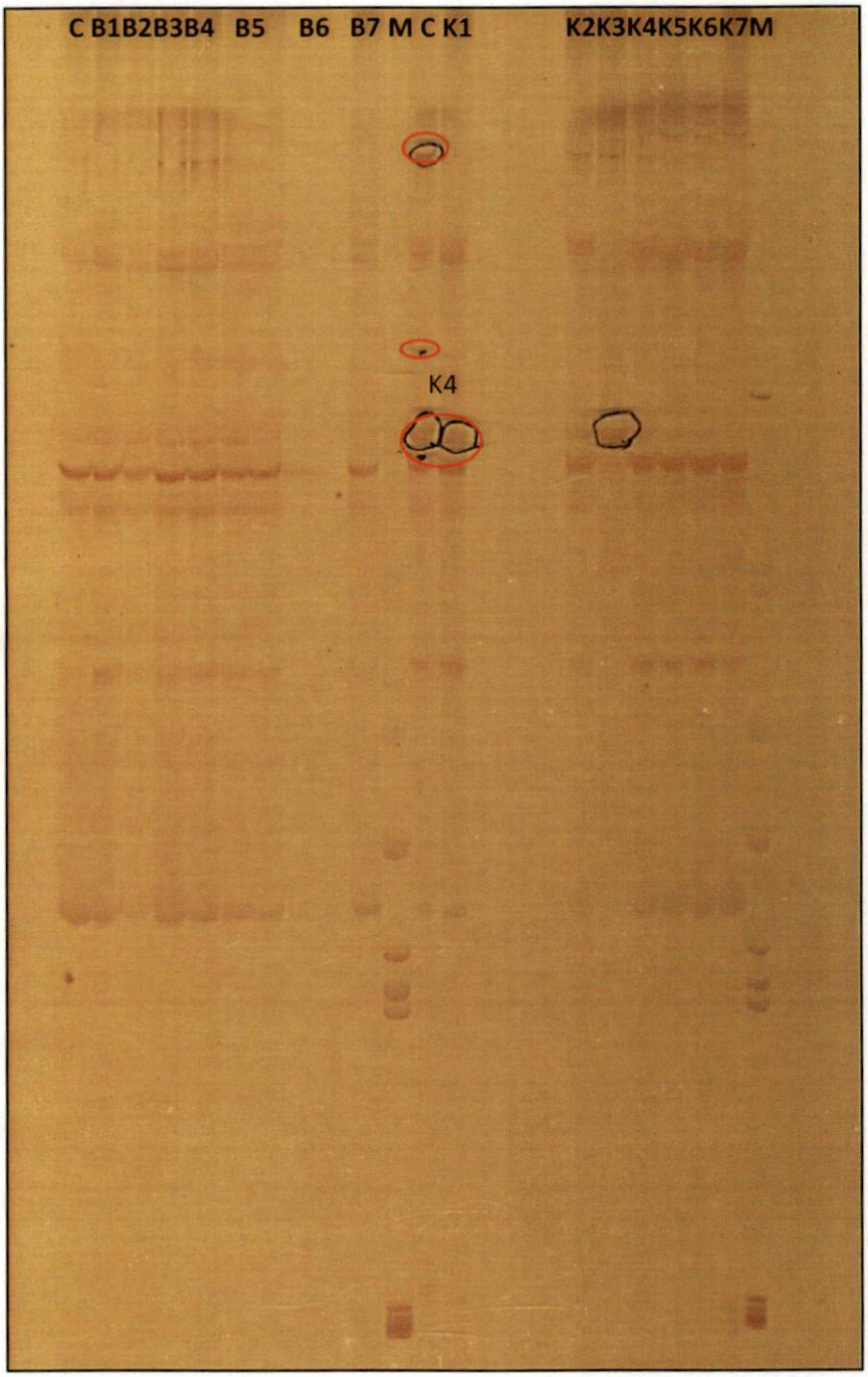



**M – GeNei 100 bp ladder**

**Lane C-B7 – AP5-T11C transcriptome pattern of IISR Subhakara**

**Lane C-K7 – AP5-T11C transcriptome pattern of IISR Shakthi**

**Plate 4.13 Differential display pattern of transcript derived fragments from seven treatments and control using anchored (T11C) and arbitrary primer (AP5).**

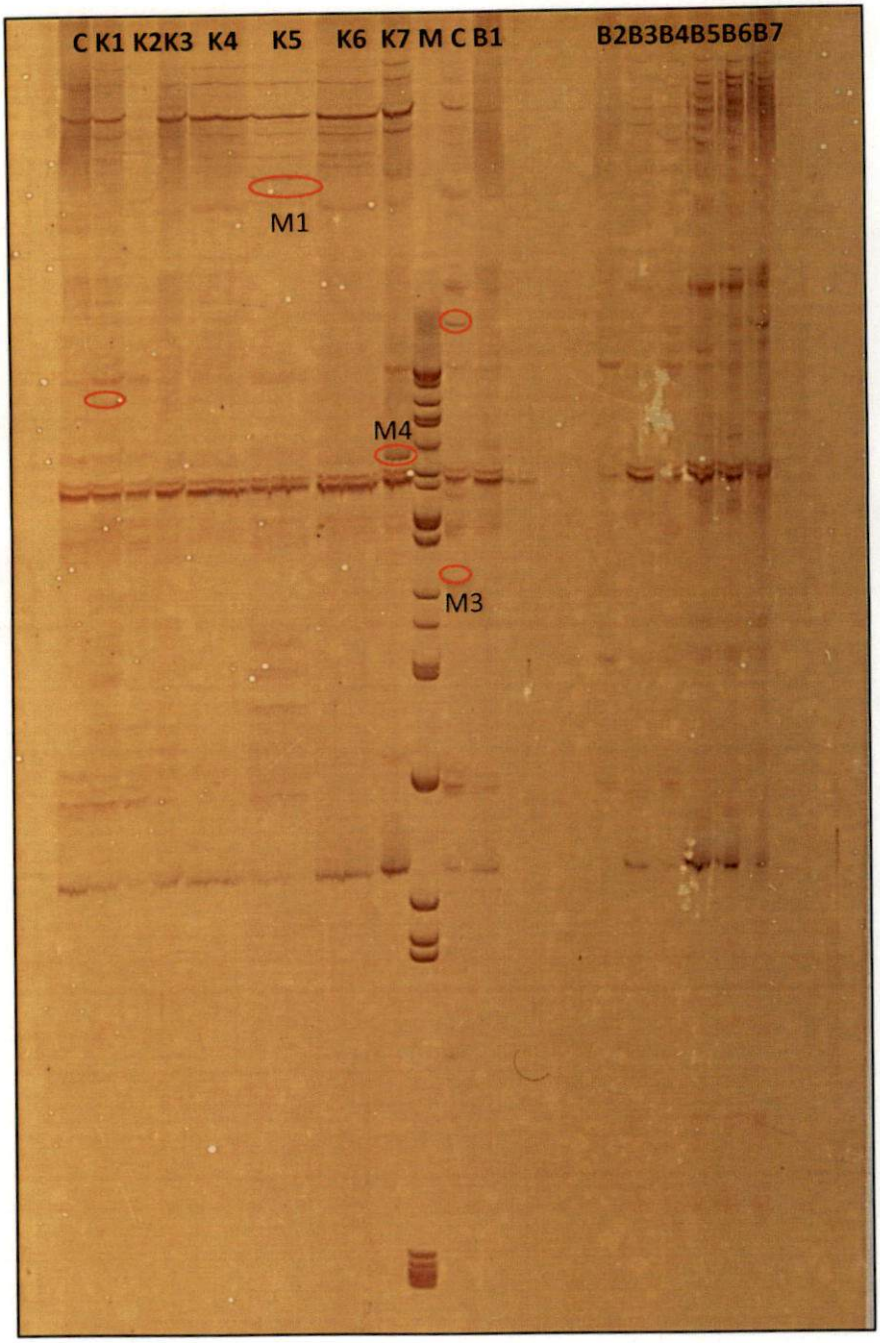



**M – GeNei 100 bp ladder**

**Lane C-B7 – AP7-T11C transcriptome pattern of IISR Subhakara**

**Lane C-K7 – AP7-T11C transcriptome pattern of IISR Shakthi**

**Plate 4.14 Differential display pattern of transcript derived fragments from seven treatments and control using anchored (T11C) and arbitrary primer (AP7).**

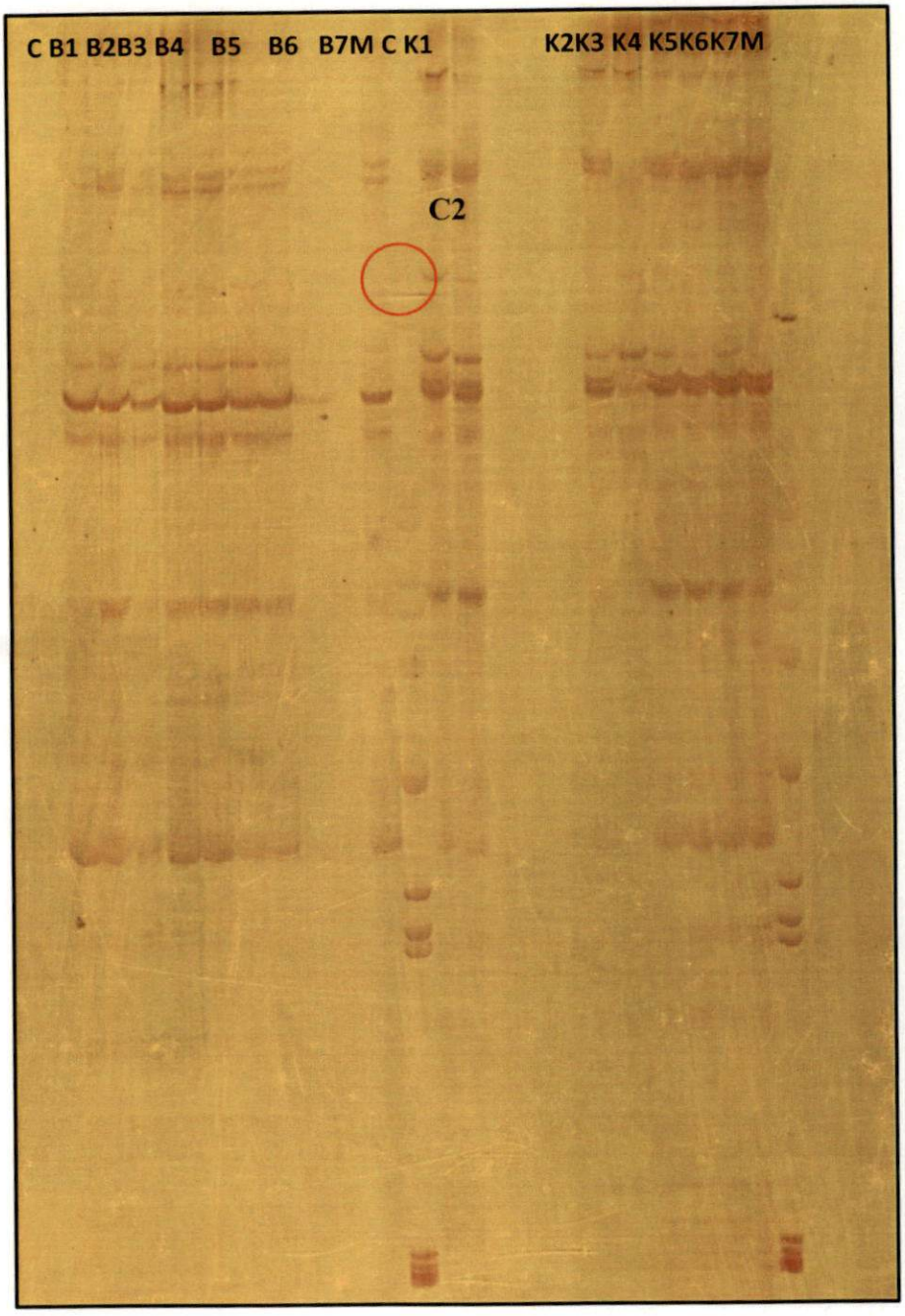



**M – GeNei 100 bp ladder**

**Lane C-B7 – AP3-T11C transcriptome pattern of IISR Subhakara**

**Lane C-K7 – AP3-T11C transcriptome pattern of IISR Shakthi**

**Plate 4.15 Differential display pattern of transcript derived fragments from seven treatments and control using anchored (T11C) and arbitrary primer (AP3).**




**M – GeNei 100 bp ladder**

**Lane C-B7 – AP4-T11C transcriptome pattern of IISR Subhakara**

**Lane C-K7 – AP4-T11C transcriptome pattern of IISR Shakthi**

**Plate 4.16 Differential display pattern of transcript derived fragments from seven treatments and control using anchored (T11C) and arbitrary primer (AP4).**

### 4.8 Elution and re-amplification of differentially expressed cDNAs

The upregulated, down regulated and differentially expressed cDNA fragments were retrieved from gel and reamplified with the same primer combination (selective PCR) and analysed electrophoretically in 1.5 per cent agarose.

### 4.9 Gel elution of PCR amplified fragments

cDNA fragments obtained after PCR amplification on agarose gel was eluted using GeneJET Gel Extraction Kit (Thermo scientific, USA). The eluted products were stored at -20 °C and were outsourced for sequencing (SciGenom, Kochi).

### 4.10 *Insilico* analysis of Sequences

#### 4.10.1 Sequence A2

5'gatatgcagttcttcaacttcttttccctttccttttgtgtcattcctcttttatttatgaaatggaagaattccaatagccaaaccaaaagattg
cctccaggtccatggaaattacctattcttggaagcatgcttcatatgctaggtggactgccacatcatgtccttagagatttagccaaaaaatat
ggaccaattatgcaccttcaactaggcgaattttctctagttgttattaattctcctgagatggcaaaagaagtactaaaaactcatgacctcgctt
ttgcaaataggcctttacttgtatctgccaagattttatgttataatgggacggacattacctttcatcttatggcaattactggagacaattgcgt
aaaatttgtctcttggaattgctcagtgccaaaactgtcaagtcattcaactccattagacaaga3'

The sequence obtained from sample 1 (A2) from the primer AP1 was 455 bp in length and was differentially expressed in resistant lane at 4$^{th}$ hour after inoculation and can be differentiated clearly in the resistant lane. BLAST analysis showed similarity to premnaspirodiene oxygenase and cytochrome P450 from *Nicotiana tabacum*. Several conserved domains were present in this sequence *viz*., flavonoid 3' monooxygenase, cytopchrome P450 and RNA Recognition Motif (RRM) from BLASTp and Interpro analysis (Fig. 4.7). The longest ORF (Fig. 4.6) obtained from the first frame having 450 bp (149 aa). Phobius predicted the presence of a signal peptide and transmembrane topology in the sequence (Fig. 4.8).

The details of the BLASTn result are given in Fig. 4.1 and Table 4.4 Details of BLASTx result are given in Fig. 4.2 and Table 4.5. The nucleotide sequence was translated into protein sequence using 'Emboss Transeq' tool (Fig. 4.3) and BLASTp analysis (Fig. 4.4) was carried out to find out conserved domains in the sequence and the results are mentioned in Fig. 4.5.

**Sequence 1 - A2_AP1- 455 bp**

gatatgcagttcttcaacttcttttccctttttccttttttgtgtcattcctctttttatttatgaaatggaagaattccaatagccaaaccaaaagattgcct
ccaggtccatggaaattacctattcttggaagcatgcttcatatgctaggtggactgccacatcatgtccttagagatttagccaaaaaaatatgg
accaattatgcaccttcaactaggcgaattttctctagttgttattaattctcctgagatggcaaaagaagtactaaaaactcatgacctcgctttt
gcaaataggcctttacttgtatctgccaagattttatgttataatgggacggacattacctttcatcttatggcaattactggagacaattgcgtaa
aatttgtctcttggaattgctcagtgccaaaactgtcaagtcattcaactccattagacaaga

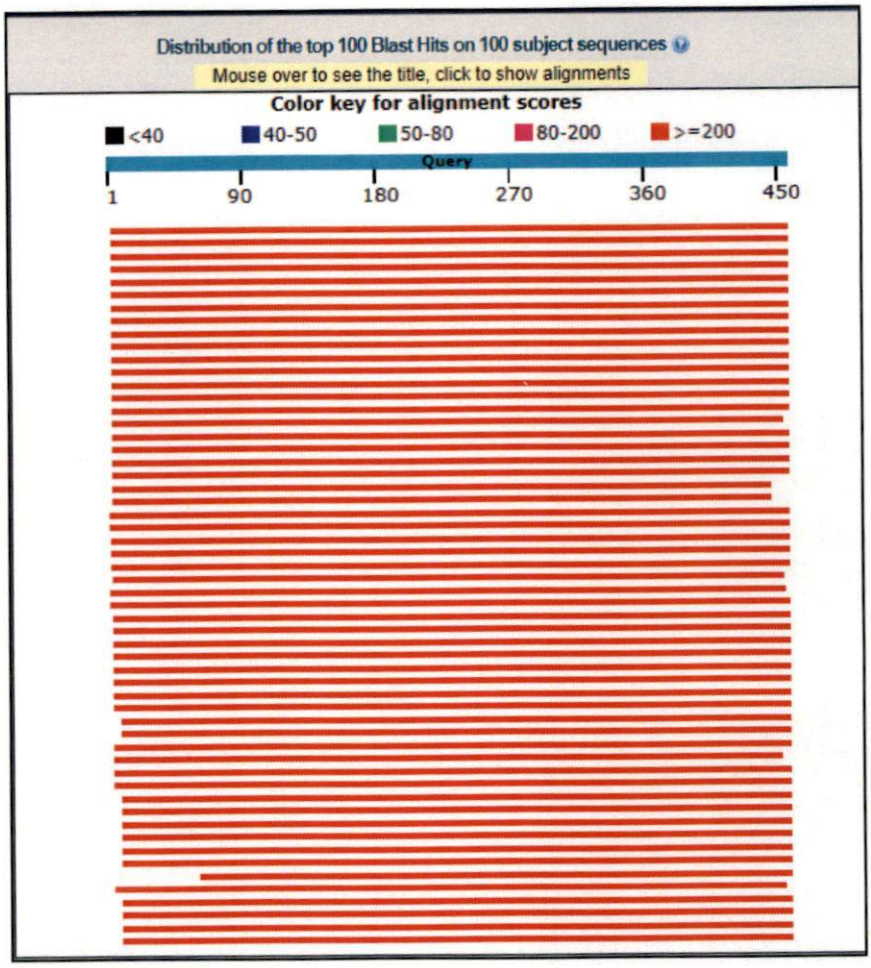



**Fig. 4.1 BLASTn analysis of sequence 1**

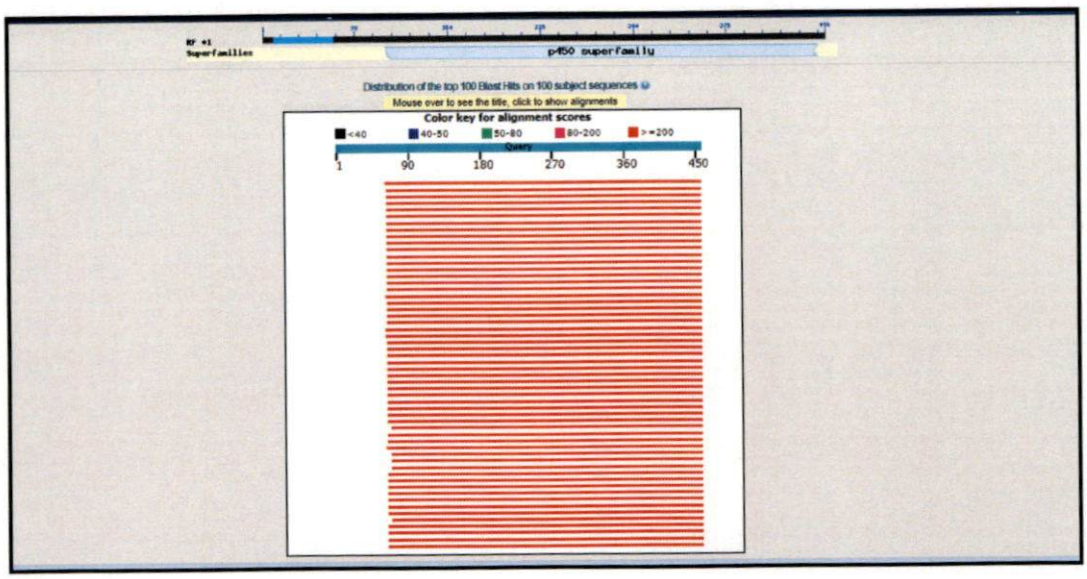



**Fig. 4.2 BLASTx analysis of sequence 1**

**Table 4.4 BLASTn analysis of sequence 1**

| Accessions | Description | Max. score | Query coverage (%) | E value | Max. identity |
|---|---|---|---|---|---|
| XM_009781727.1 | premnaspirodiene oxygenase-like (LOC104229139), Mrna ( *Nicotiana sylvestris*) | 791 | 99 | 0.0 | 98 |
| XM_016654206.1 | premnaspirodiene oxygenase-like (LOC107827130), mRNA ( *Nicotiana tabacum*) | 769 | 99 | 0.0 | 97 |
| AF166332.1 | cytochrome P450 gene, complete cds ( *Nicotiana tabacum*) | 725 | 99 | 0.0 | 96 |

**Table 4.5 BLASTx analysis of sequence 1**

| Accessions | Description | Max. score | Query coverage (%) | E value | Max. identity |
|---|---|---|---|---|---|
| XP_009780029.1 | premnaspirodiene oxygenase-like (*Nicotiana sylvestris*) | 263 | 85 | 1e-83 | 95 |
| XP_016509692.1 | premnaspirodiene oxygenase-like (*Nicotiana tabacum*) | 258 | 85 | 9e-82 | 94 |
| AAD47832.1 | cytochrome P450 gene (*Nicotiana tabacum*) | 244 | 85 | 3e-76 | 90 |

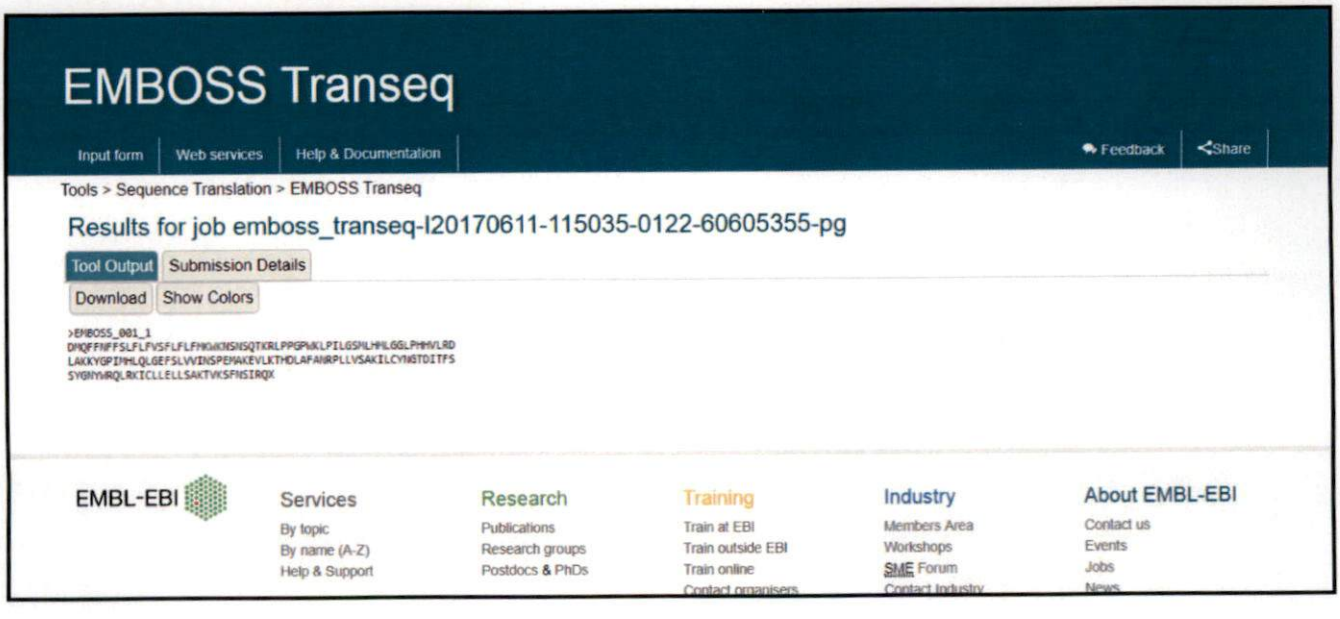



**Fig. 4. 3 Nucleotide sequence translation to amino acid sequences using Emboss Transeq**

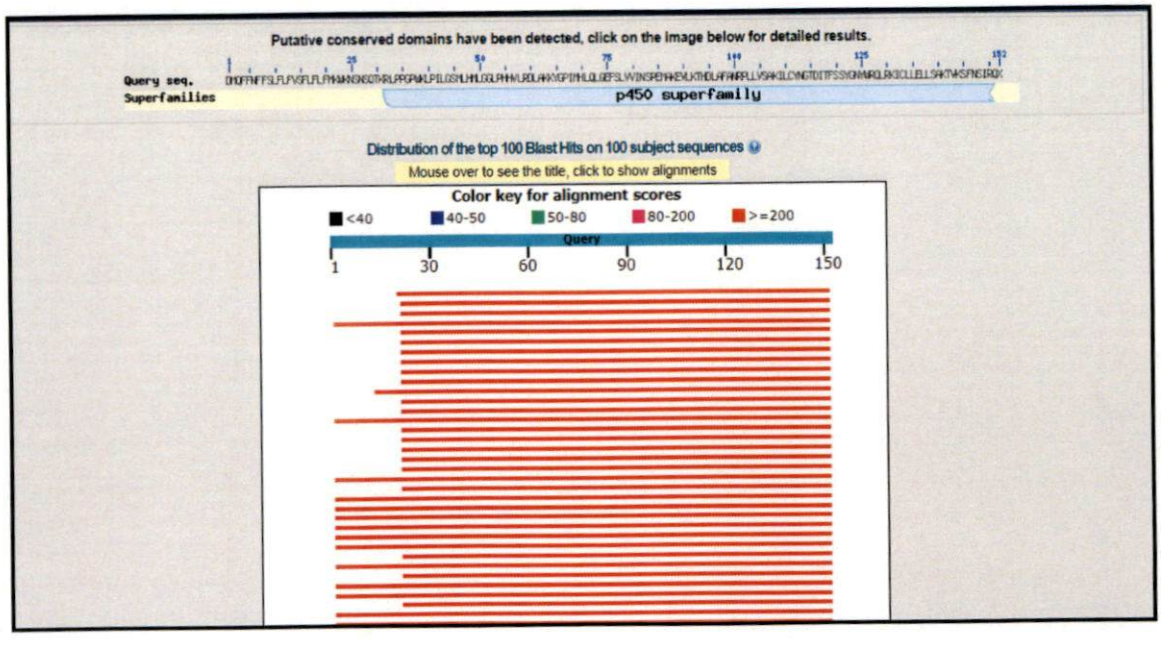



**Fig. 4.4 BLASTp analysis of sequence 1**

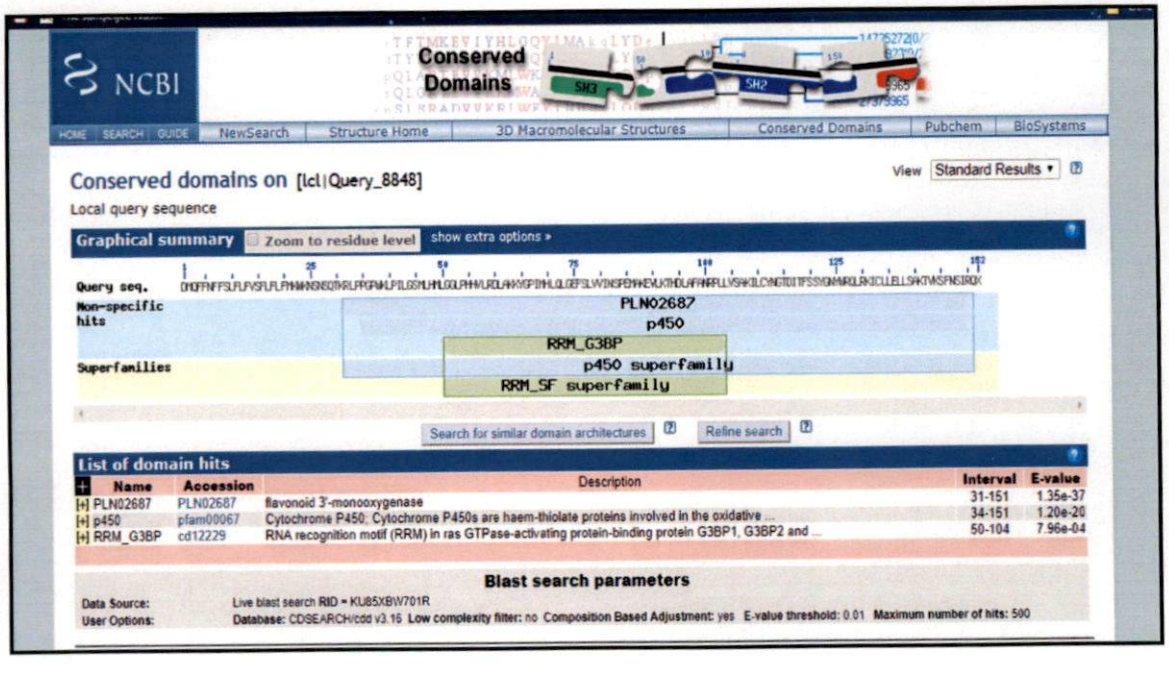



**Fig. 4.5 Conserved domains of sequence 1**

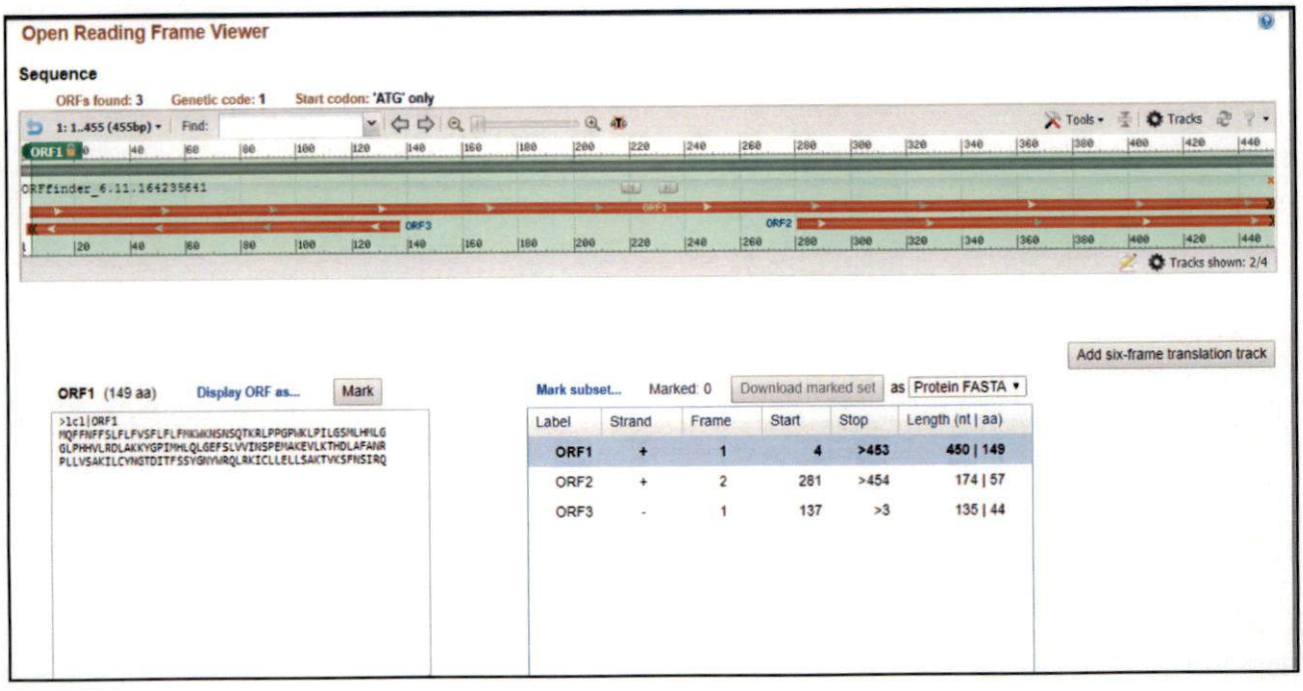



**Fig. 4.6 Search of open reading frames (ORF) in the sequence**

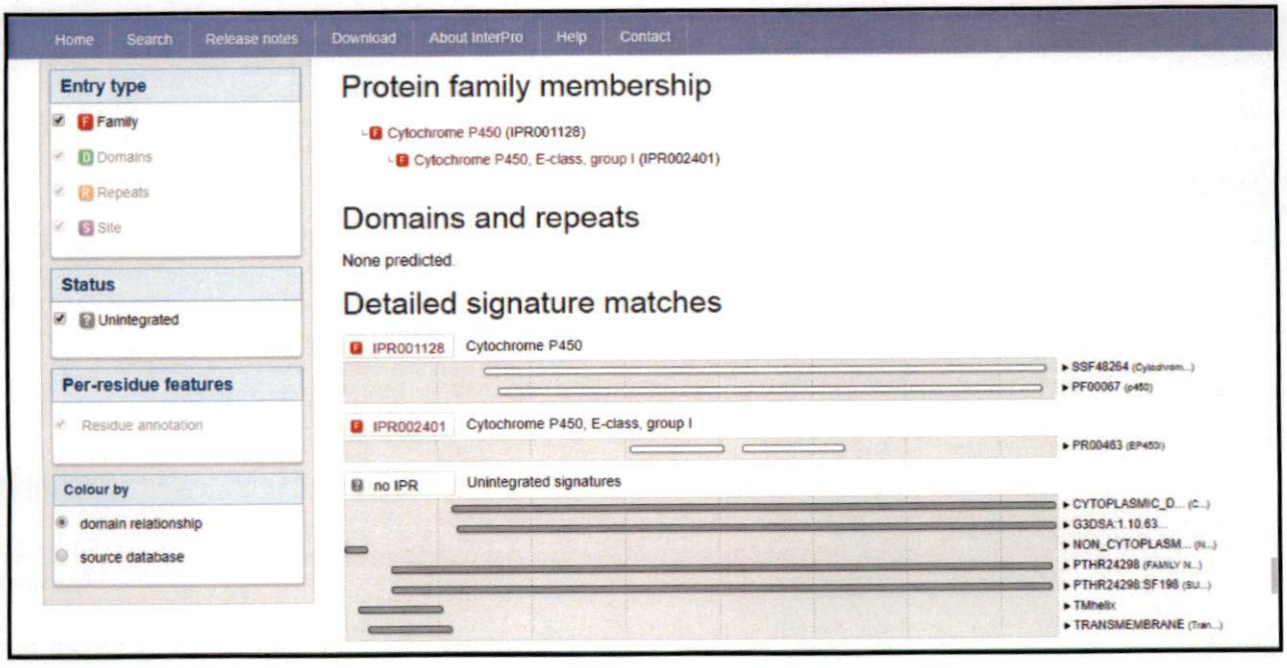



**Fig. 4.7 Functional analysis of protein sequences for identification of domains and families**

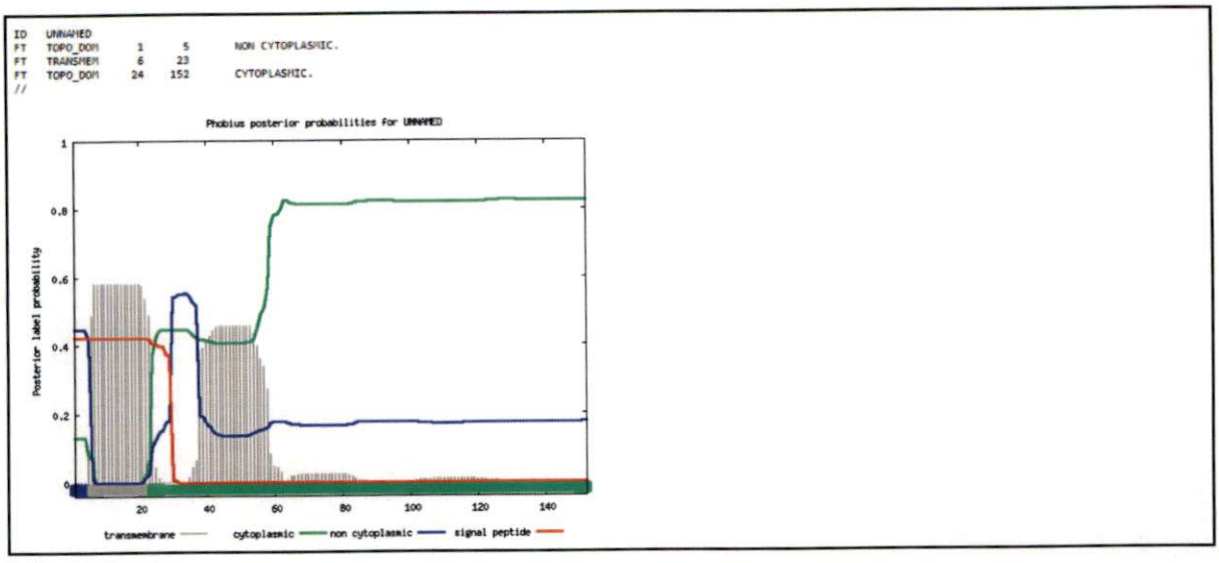



**Fig. 4.8 Prediction of transmembrane topology and signal peptide using Phobius**

**Sequence 2 - A5_AP1 (25bp)**

tctccttatggcaattactggagac

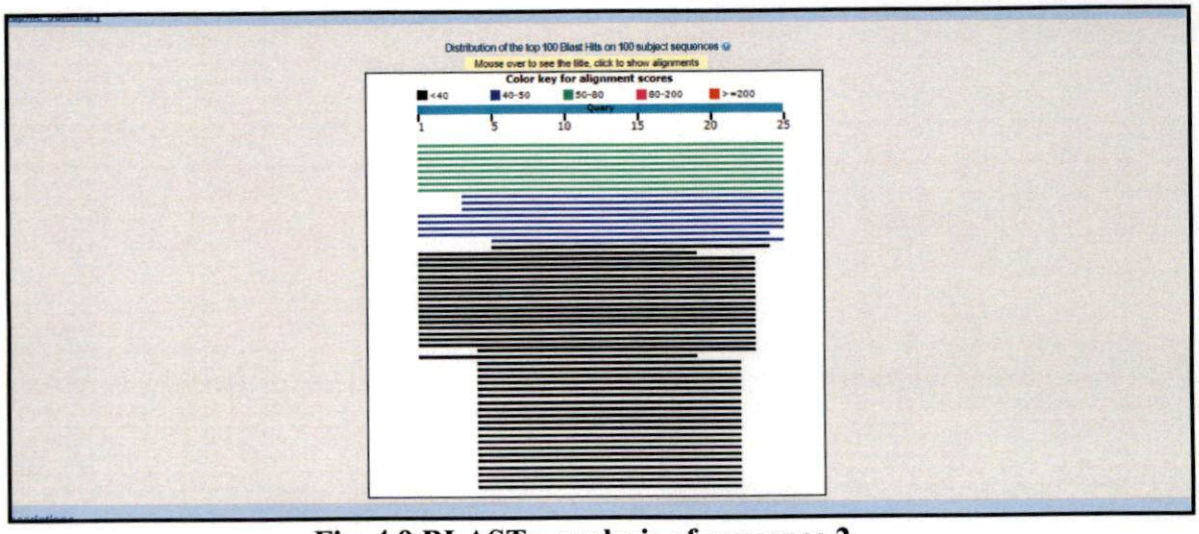



**Fig. 4.9 BLASTn analysis of sequence 2**

### 4.10.2 Sequence 2

tctccttatggcaattactggagac

The sequence obtained from the sample 2 (A5) from the primer AP1 was extremely short length (25) and is differentially expressed in resistant lane after inoculation. It showed similarity to the same premnaspirodiene oxygenase gene and cytochrome P450 fragment from *Nicotiana* species.

Result of BLASTn is given in Fig. 4.9 and Table 4.6. Details of BLASTx result are given in Fig. 4.10.

### 4.10.3 Sequence 3

5'tatcacttaaaggaaggagaagtcgtaacaaggtatctgtaggtgaacctgcagatggatcattaacacatccttatttacctaacacacttt
ttatttttaaaacctcagcgatggatatctcggctcttgtaacgatgaagaacgcagccaactgcgaaaagcactgcgaattgcagaacaccg
tgagtcagtagatctttgaacgcaaccggcgccctctgggcatgtttgcttcagtgcctcctttgctaccagctgcgagtgcagtgctctcgcc
aagttctcctatccagatctggcaggttggatctgg3'

The sequence obtained from the sample 3 (A7) from the primer AP1 which is upregulated after inoculation, was having 317 bp length. It showed similarity to 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, partial sequence from *Blepharisma undulans* and *Blepharisma japonicum* and uncultured *Heterotrichida* clone. Translated BLAST showed similarity with hypothetical protein from *Baudoinia panamericana, Bipolaris maydis and Kuraishia capsulate.*

The details of the BLASTn result are given in Fig. 4.11 and Table 4.7. Details of BLASTx result are given in Fig. 4.12 and Table 4.8.

### 4.10.4 Sequence 4

5'caattttaattgattattctgattccacttttattcatttgagttattttaattatcttttctattgtttattctttttaaagtatgttattgcataatttataaaa
gaataaaattgcacttttaaacctctcttctaccttaaaatgtaaaacaaaaatgtaatgatcataagtctaaataaatgaagtatttctcactcattg
caagtatatctttttggttatcactgatacccacatgtttacattgatcatgactaggtagaacaatacaaagtatttttttagtcatgtgtttcacattt
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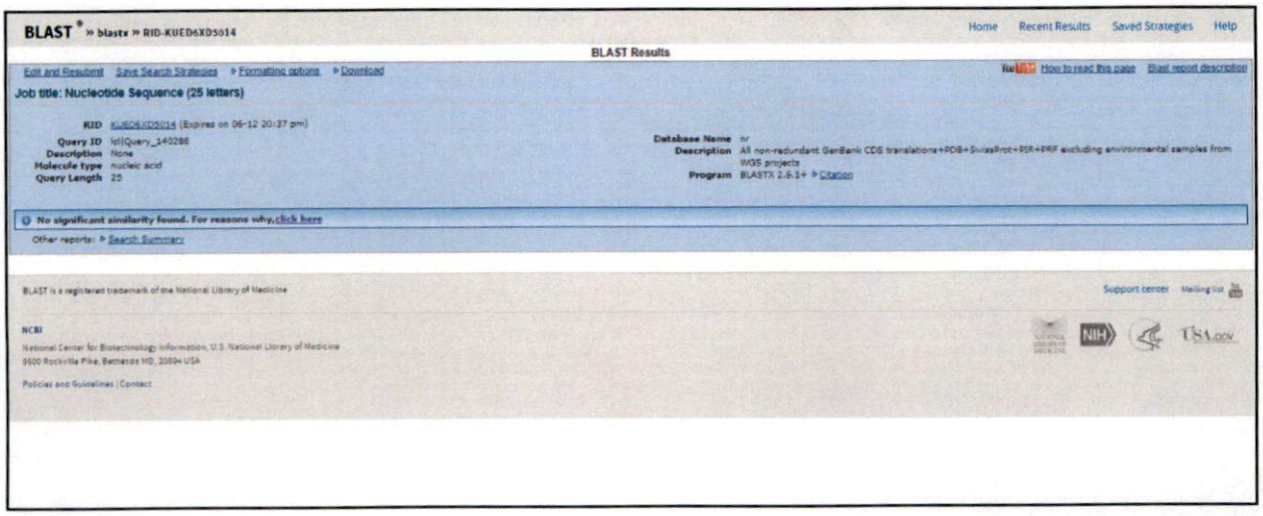



**Fig. 4.10 BLASTx analysis of sequence 2**

**Table 4.6 BLASTn analysis of sequence 2**

| Accessions | Description | Max. score | Query coverage (%) | E value | Max. identity |
|---|---|---|---|---|---|
| XM_019397224.1 | premnaspirodiene oxygenase-like (*Nicotiana attenua*) | 50.1 | 100 | 6e-04 | 100 |
| XM_016645376.1 | premnaspirodiene oxygenase-like (*Nicotiana tabacum*) | 50.1 | 100 | 6e-04 | 100 |
| XM_016699355.1 | cytochrome P450 complete cds (*Nicotiana tabacum*) | 50.1 | 100 | 6e-04 | 100 |

**Sequence 3 A7-AP1 (317 bp)**

tatcacttaaaggaaggagaagtcgtaacaaggtatctgtaggtgaacctgcagatggatcattaacacatccttatttacctaacacacttttta
ttttaaaacctcagcgatggatatctcggctcttgtaacgatgaagaacgcagccaactgcgaaaagcactgcgaattgcagaacaccgtg
agtcagtagatctttgaacgcaaccggcgccctctgggcatgtttgcttcagtgcctcctttgctaccagctgcgagtgcagtgctctcgccaa
gttctcctatccagatctggcaggttggatctgg

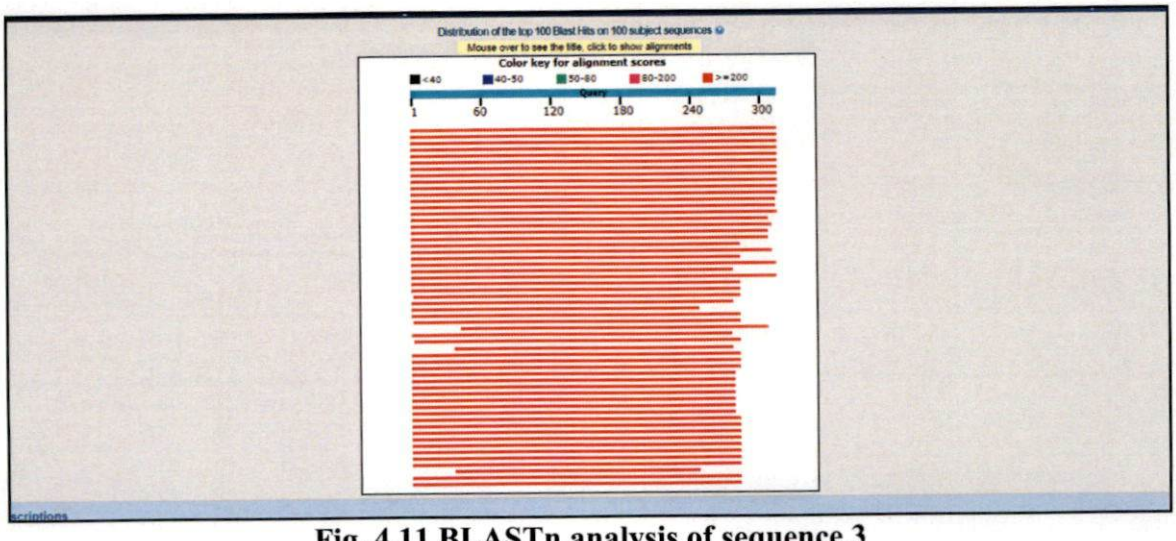



**Fig. 4.11 BLASTn analysis of sequence 3**

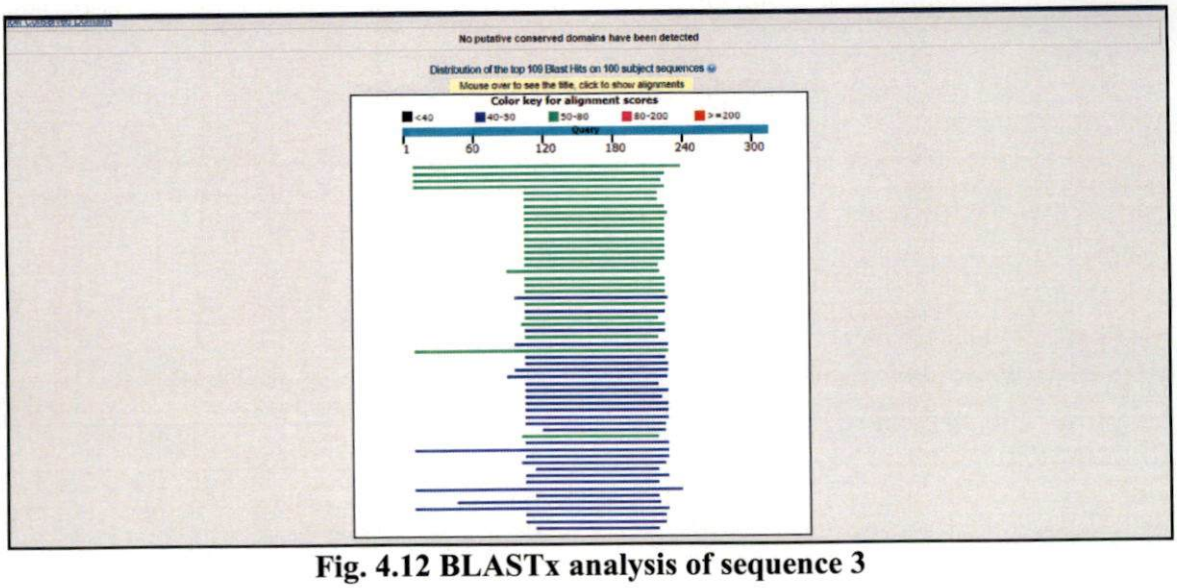



**Fig. 4.12 BLASTx analysis of sequence 3**

**Table 4.7 BLASTn analysis of sequence 3**

| Accessions | Description | Max. score | Query coverage (%) | E value | Max. identity |
|---|---|---|---|---|---|
| KP970286.1 | *Blepharisma undulans* isolate BU 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence | 571 | 100 | 3e-159 | 99 |
| KP970281.1 | *Blepharisma japonicum* 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence | 571 | 100 | 3e-159 | 99 |
| FJ554186.1 | Uncultured *Heterotrichida* clone LTSP EUKA P5NO1 18S ribosomal RNA/intergenic spacer, partial sequence | 523 | 99 | 9e-145 | 97 |

**Table 4.8 BLASTx analysis of sequence 3**

| Accessions | Description | Max. score | Query coverage (%) | E value | Max. identity |
|---|---|---|---|---|---|
| XP_007682038.1 | Hypothetical protein BAUCODRAFT 127553 (*Baudoinia panamericana*) | 60.5 | 73 | 4e-10 | 49 |
| EMD91248.1 | Hypothetical protein COCHEDRAFT 1103804 (*Bipolaris maydis*) | 60.1 | 68 | 6e-10 | 51 |
| CDK30069.1 | Unnamed protein product (*Kuraishia capsulate*) CBS 1993 | 57.8 | 67 | 1e-08 | 42 |

**Sequence 4 C2- T11C (468 bp)**

caattttaattgattattctgattccacttttattcatttgagttattttaattatcttttctattgtttattctttttaaagtatgttattgcataatttataaaag
aataaaattgcacttttaaacctctcttctaccttaaaatgtaaaacaaaaatgtaatgatcataagtctaaataaatgaagtatttctcactcattgc
aagtatatctttttggttatcactgatacccacatgtttacattgatcatgactaggtagaacaatacaaagtatttttttagtcatgtgtttcacatttg
gatattttgaacatcaacgttttattattaccaaagtattaggtttccaaatcttcactagctcaatactgttgtccttttggtttcaggaaaggaaata
aaatgctcaccaaaaaaaggggggcataaagtggtccgaaacttgaaaaagtatttgcccc

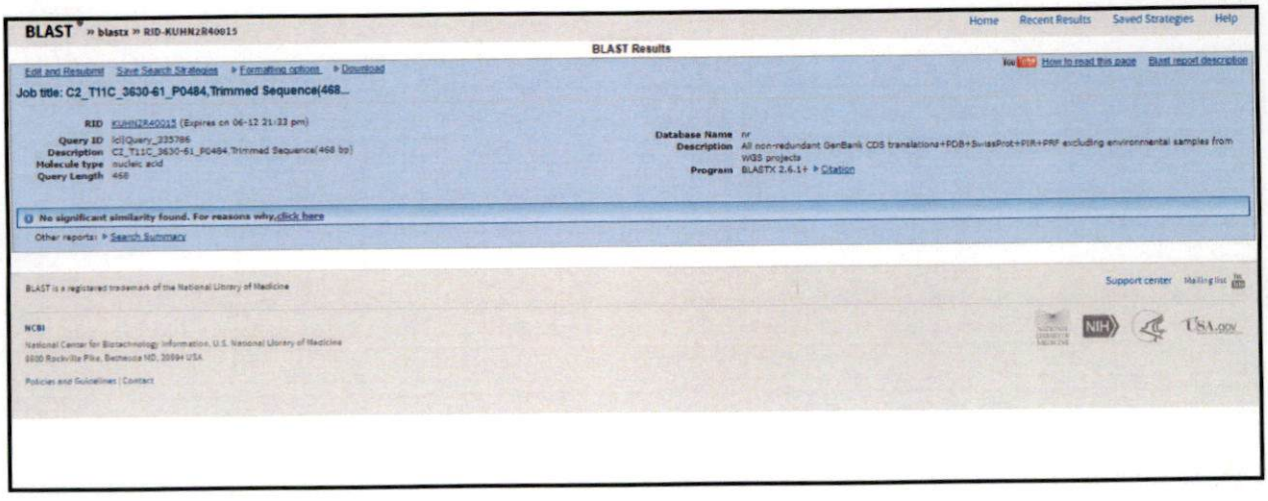



**Fig. 4.13 BLASTx analysis of sequence 4**

The sequence obtained from the sample 4 (C2) from the primer T11C was differentially expressed in resistant lane post inoculation, having 468 bp length. The sequence showed no similarity with the database and found uncharacterized. Coding regions are identified using MEGA 7.0 and found numerous stop codons in between and the longest coding protein sequence (40 aa) (Table 4.10) did not find any similarity with existing protein database.

**Table 4.10 List of possible protein sequences from sequence 4 obtained from MEGA 7.0**

| Sl No. | Frame | Longest possible protein sequence |
|---|---|---|
| 1 | 1 | INEVFLTHCKYIFLVITDTHMFTLIMTR |
| 2 | 2 | LGRTIQSIFLVMCFTFGYFEHQRFIITKVLGFQIFTSSILLSFWFQERK |
| 3 | 3 | VSKSSLAQYCCPFGFRKGNKMLTKKRGHKVVRNLKKYLPX |

### 4.10.5 Sequence 5

5'aaaattgtgataatgaacaaacatatgtgctgtgttacgcttgatcagaatgctaatgaagcctgtggtgtaccataatctgtaaattagtgaa ttctagagaatggtatatccatttttcatgtttgtattcttttgcattctctttgtttgtgtatttttttctcgtagcaaattcttcgacataactgatcttgtag cctcttggaagccagttgcactgcagctcgatgttccactagaaaacatatttgccaaccaactactgtttggaagctctggtgaatattgcgg gtttgataaaaacgaaccaacttccagaagcgggggaaaagctactgctgcccaaactataaaaaagacccatggatacaagtctttggtca tgattggggatggtgcaagc3'

The sequence obtained from the sample 5 (J2) from the primer T11C was differentially expressed during forth hour after inoculation, having 405 bp length. BLAST analysis showed similarity with phosphoserine phosphatase protein from *Arabidopsis thaliana* and *Malus domesticus*. The protein sequence contain domains such as HAD like super family and phosphoserine component as shown in Fig. 4.15. The details of the BLASTx result are given in Fig. 4.14 and Table 4.9. BLASTp details are mentioned in Fig. 4.19 and conserved domain information in Fig. 4.20. KEGG pathway analysis is mentioned in Fig. 4.21.

### 4.10.6 Sequence 6

5'aagcattgcctctgacctccctacactggcgacctcactgccagccagcgccgtcatcgccccacttcccctcctccttcctctcccagctt cttcttcctcctactttgttcaccaccaccaccggccacttttcaccaccgcctccctcatcctggccagctccctccctgacca3'

**Sequence 5 J2 – T11C (405 bp)**

aaaattgtgataatgaacaaacatatgtgctgtgttacgcttgatcagaatgctaatgaagcctgtggtgtaccataatctgtaaatt
agtgaattctagagaatggtatatccatttttcatgtttgtattcttttgcattctctttgtttgtgtatttttttctcgtagcaaattcttcgac
ataactgatcttgtagcctcttggaagccagttgcactgcagctcgatgttccactagaaaacatatttgccaaccaactactgtttgg
aagctctggtgaatattgcgggtttgataaaaacgaaccaacttccagaagcgggggaaaagctactgctgcccaaactataaaa
aagacccatggatacaagtctttggtcatgattggggatggtgcaagc

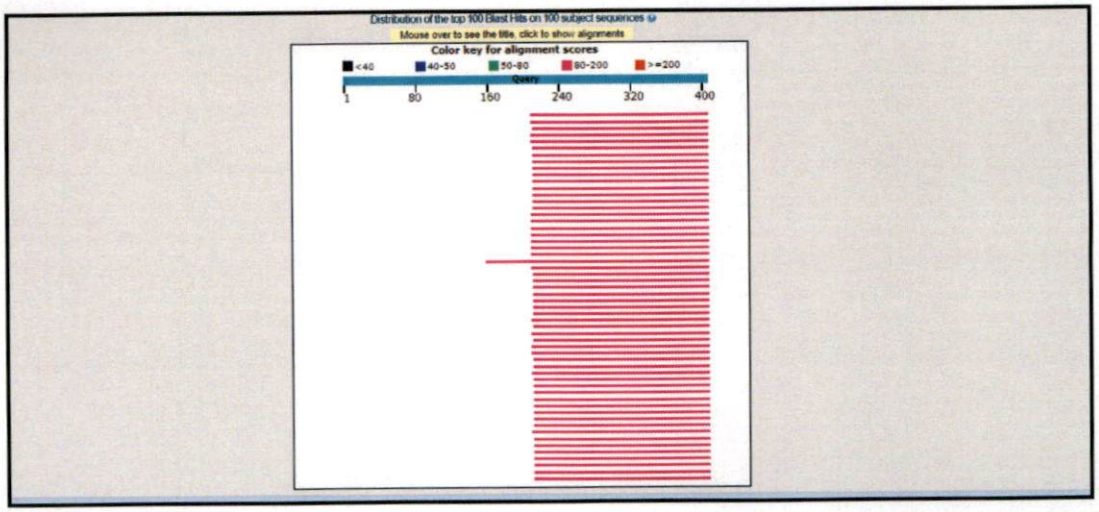



**Fig. 4.14 BLASTx analysis of sequence 5**

**Table 4. 9 BLASTx analysis of sequence 5**

| Accessions | Description | Max. score | Query coverage (%) | E value | Max. identity |
|---|---|---|---|---|---|
| XP_008346782.2 | Phosphoserine phosphatase, chloroplastic like (*Malus domesticus*) | 112 | 48 | 4e-29 | 77 |
| OAY77951.1 | Phosphoserine phosphatase, chloroplastic like (*Ananus comosus*) | 113 | 48 | 5e-28 | 79 |
| XP_016577735.1 | Phosphoserine phosphatase, chloroplastic like (*Capsicum annum*) | 112 | 48 | 1e-27 | 80 |

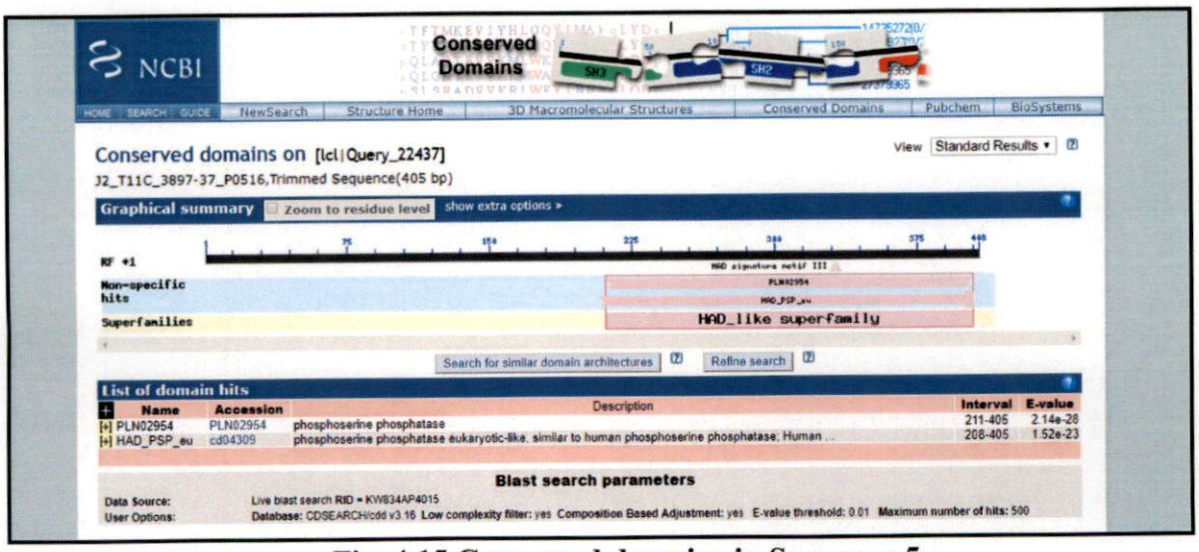



**Fig. 4.15 Conserved domains in Sequence 5**

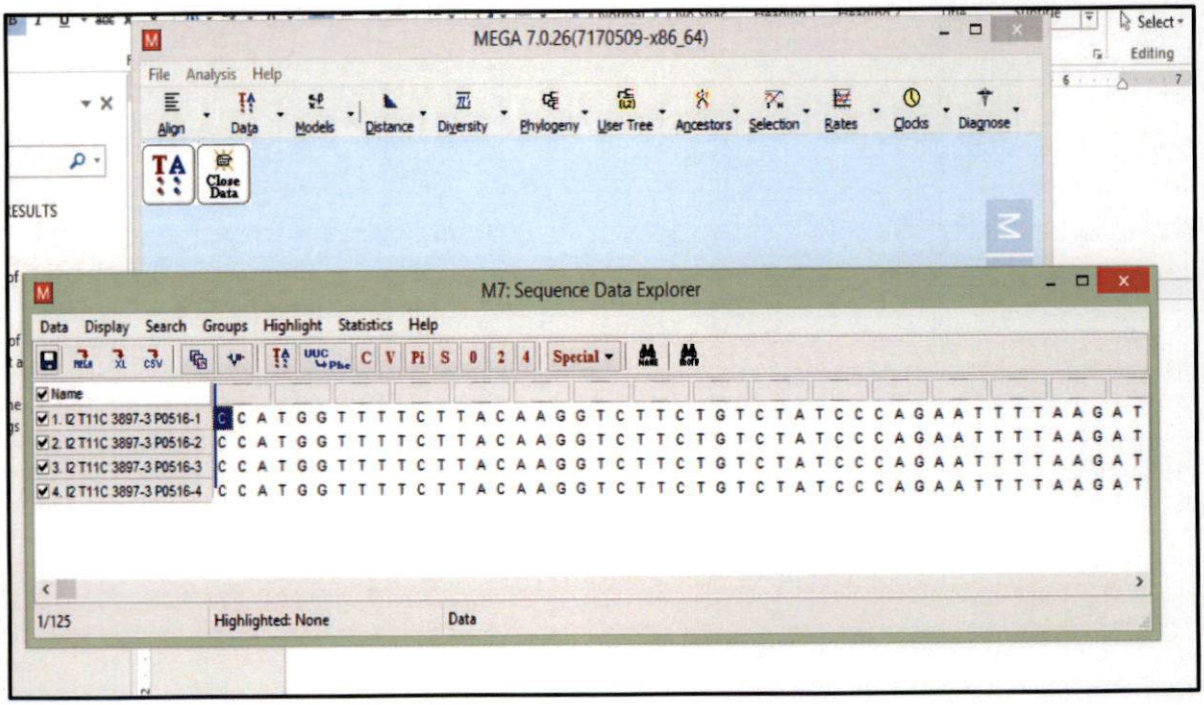



**Fig. 4.16 Analysis of sequence for coding regions**

**Table 4.11 List of protein coding regions of sequences from MEGA**

| Sl No. | Nucleotide sequence Sample code | Frame No. | Protein coding sequences |
|---|---|---|---|
| 1 | M4 | 2 | SIASDLPTLATSLPASAVIAPLPLLLPLPASSSSY<br>FVHHHHHRPLFTTASLILASSLPDX |
| 2 | M3 | 3 | LLFTMNSIPHLMVKVIYTSFLCNTDSFILGVNGCD<br>LFFYLHRFRVLLLIKYVFYMAF |
| 3 | M1 | 2 | IASDLPTLATSLPASADIAPLPLLLPLPASSSSYFV<br>HHHHRPLFTTASLILASSLPDX |
| 4 | J4 | 3 | TLLGRSTYIFILHLALLLLYMMELLIYISWCKX |
| 5 | J3 | 1 | PNNVSRFVFSGRSSDHHQICIQKFRTTKSSWETSFHHR<br>ANTGPPLPTRSPTHRKINRQWCX |
| 6 | J2 | 1 | ILENGISIFHVCILLHSLCLCIFSRSKFFDITDLVASWKP<br>VALQLDVPLENIFANQLLFGSSGEYCGFDKNEPTSRSGG<br>KATAAQTIKKTHGYKSLVMIGDGAS |
| 7 | I2 | 2 | HGFLTRSSVYPRILRSNRFTRVIKTFTSISL |
| 8 | A2 | 1 | DMQFFNFFSLFLFVSFLFLFMKWKNSNSQTKRLPPGPWKL<br>PILGSMLHMLGGLPHHVLRDLAKKYGPIMHLQLGEFSLVV<br>INSPEMAKEVLKTHDLAFANRPLLVSAKILCYNGTDITFSSY<br>GNYWRQLRKICLLELLSAKTVKSFNSIRQX |
| 9 | A7 | 3 | SLKGRRSRNKVSVGEPADGSLTHPYLPNTLFIFKTSAMDISAL<br>VTMKNAANCEKHCELQNTVSQ |
| 10 | C2 | 2 | LGRTIQSIFLVMCFTFGYFEHQRFIITKVLGFQIFTSSILLSFWFQERK |

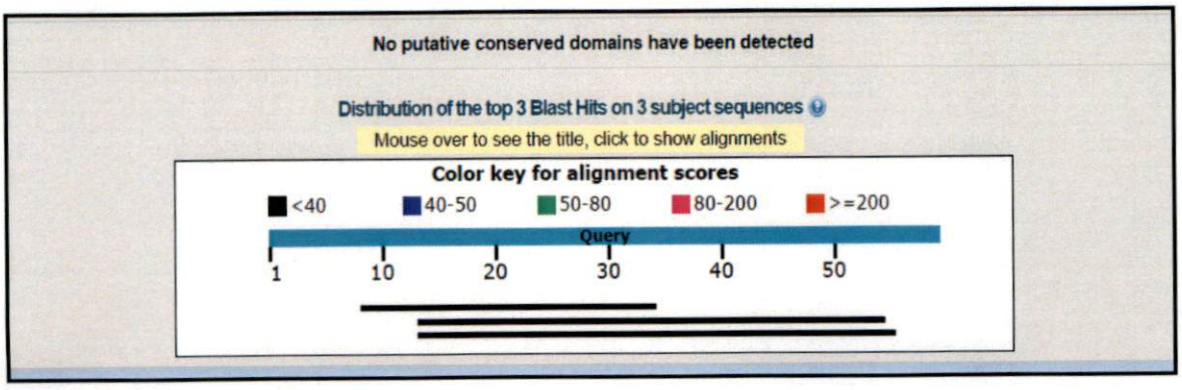



**Fig. 4.17 BLASTp analysis of sequence M4**

**Table 4.12 BLASTp analysis of sequence M4**

| Accessions | Description | Max. score | Query coverage (%) | E value | Max. identity (%) |
|---|---|---|---|---|---|
| KDQ06355.1 | Hypothetical protein BOTBODRAFT (*Botrybasidium botryosum*) | 35 | 44 | 2.8 | 54 |
| WP_080570564.1 | Hypothetical protein (*Janthinobacterium ividum*) | 33.9 | 69 | 7.9 | 47 |
| NP_192994.1 | Ubiquitin protein ligase 5 (*Arabidopsis thaliana*) | 26.6 | 69 | 2.4 | 34 |
| OAP01054.1 | UPL5 (*Arabidopsis thaliana*) | 26.6 | 69 | 2.5 | 34 |

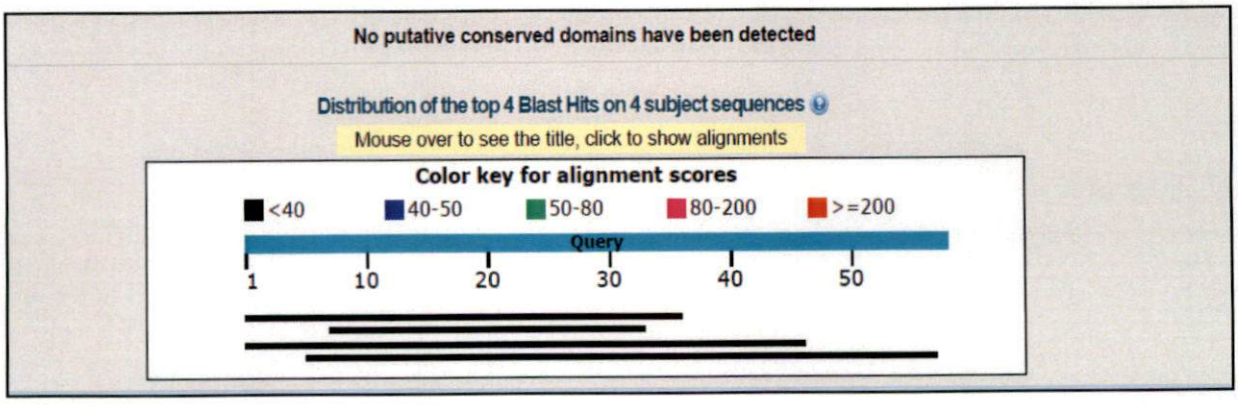



**Fig. 4.18 BLASTp analysis of sequence M1**

**Table 4.13 BLASTp analysis of sequence M1**

| Accessions | Description | Max. score | Query coverage (%) | E value | Max. Identity (%) |
|---|---|---|---|---|---|
| XP_012691221.1 | Predicted ankyrin repeat and KH domain containing protein 1 like isoform X2 (*Clupea herengus*) | 38.9 | 47 | 0.93 | 45 |
| WP_024890432.1 | Heme oxygenase (*Luteimonas huabeiensis*) | 35.8 | 31 | 7.9 | 54 |
| CAD44271.1 | Map 4 kinase alpha 1 (*Arabidopsis thaliana*) | 26.9 | 40 | 9.0 | 30 |
| NP_175724.2 | Protein kinase super family (*Arabidopsis thaliana*) | 26.9 | 40 | 9.0 | 30 |
| XP_018671421.1 | Predicted adhesion G-protein coupled receptor G7 like (*Ciona intestinalis*) | 33.9 | 89 | 8.0 | 33 |

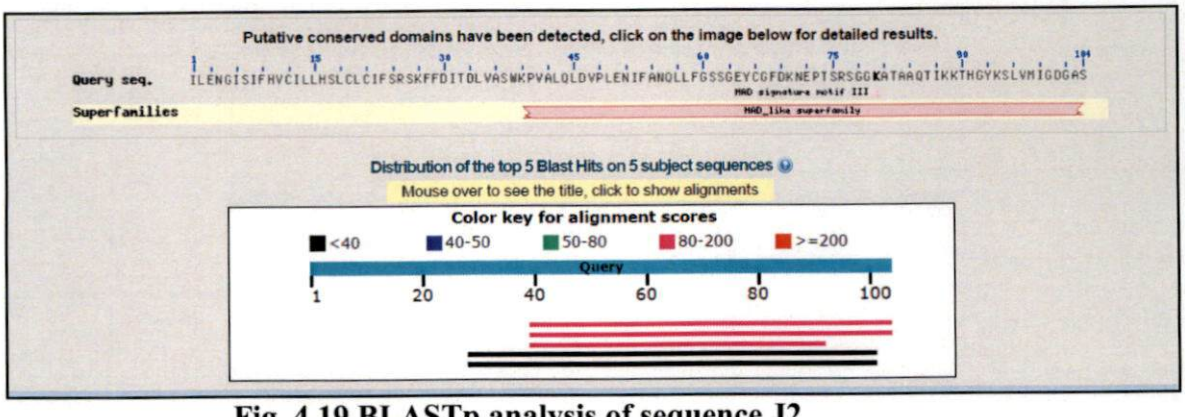



**Fig. 4.19 BLASTp analysis of sequence J2**

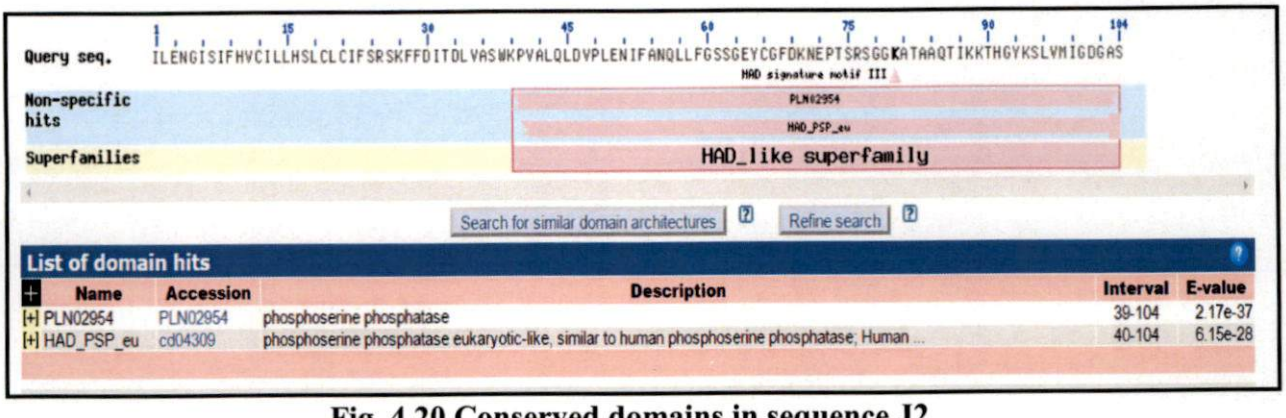



**Fig. 4.20 Conserved domains in sequence J2**

**Table 4.14 BLASTp analysis of sequence J2**

| Accessions | Description | Max. score | Query coverage (%) | E value | Max. Identity (%) |
|---|---|---|---|---|---|
| XP_008346782.2 | Predicted phosphoserine phosphatase, chloroplastic-like (*Malus domesticus*) | 113 | 63 | 6e-30 | 77 |
| BAA33806.1 | 3-phosphoserine phosphatase (*Arabidopsis thaliana*) | 94.7 | 62 | 2e-24 | 68 |
| NP_973858.1 | 3-phosphoserine phosphatase (*Arabidopsis thaliana*) | 94.7 | 62 | 2e-24 | 68 |

The sequence obtained from the sample 3 (M4) from the primer T11C which is upregulated 48th hour after inoculation in resitant lane, was having 177 bp length. BLASTn and BLASTx analysis did not find similarity in the database. Coding regions are identified using MEGA 7.0 and found the longest coding protein sequence (from frame 2) (Table 4.11) and showed similarity with hypothetical protein of *Botrybasidium botryosum, Janthinobacterium ividum* and *Arabidopsis thaliana.* The details are mentioned in the Fig. 4.17.

**4.10.7 Sequence 7**

**5'cattgcctctgacctccctacactggcgacctcactgccagccagcgccgacatcgccccacttcccctcctccttcctctcccagc ttcttcttcctcctactttgttcaccaccaccaccggccacttttcaccaccgcctccctcatcctggccagctccctccctgacca3'**

The sequence obtained from the sample 3 (M1) from the primer T11C which is differentially expressed at the 12th hour after inoculation in susceptible lane, was having 174 bp length. BLASTn and BLASTx analysis did not find similarity in the database. Coding regions are identified using MEGA 7.0 and found the longest coding protein sequence (from frame 2) (Table 4.11) and showed similarity with ankyrin repeat and KH domain containing protein 1 like isoform X2 from *Clupea herengus*, Map 4 kinase alpha 1 and kinase super family from *Arabidopsis thaliana*, heme oxygenase of *Luteimonas huabeiensis* and adhesion G-protein coupled receptor G7 like of *Ciona intestinalis, Janthinobacterium ividum* and *Arabidopsis thaliana.* The details of BLAST are mentioned in the Fig. 4.18.

**4.11 Functional annotation of differential genes**

Analysis of cDNA sequences against NCBI database and KEGG pathway database revealed that cDNAs were found to belong to the category of antifungal genes, pathogenesis and signaling molecules like serine *etc.* There is always functional cross talk between pathways *i.e.* the proteins involved in metabolism is also involved in cellular activities and between signaling pathways and some are uncharacterized yet.

KAAS (KEGG Automatic Annotation Server) was used to find the linked pathway of the identified protein through BLAST analysis. Phosphoserine phosphatase was found to be involved in the conversion of Phosphoserine to serine and premnaspirodiene oxygenase for the conversion of reververetrol to piceatannol (Fig. 4.21).

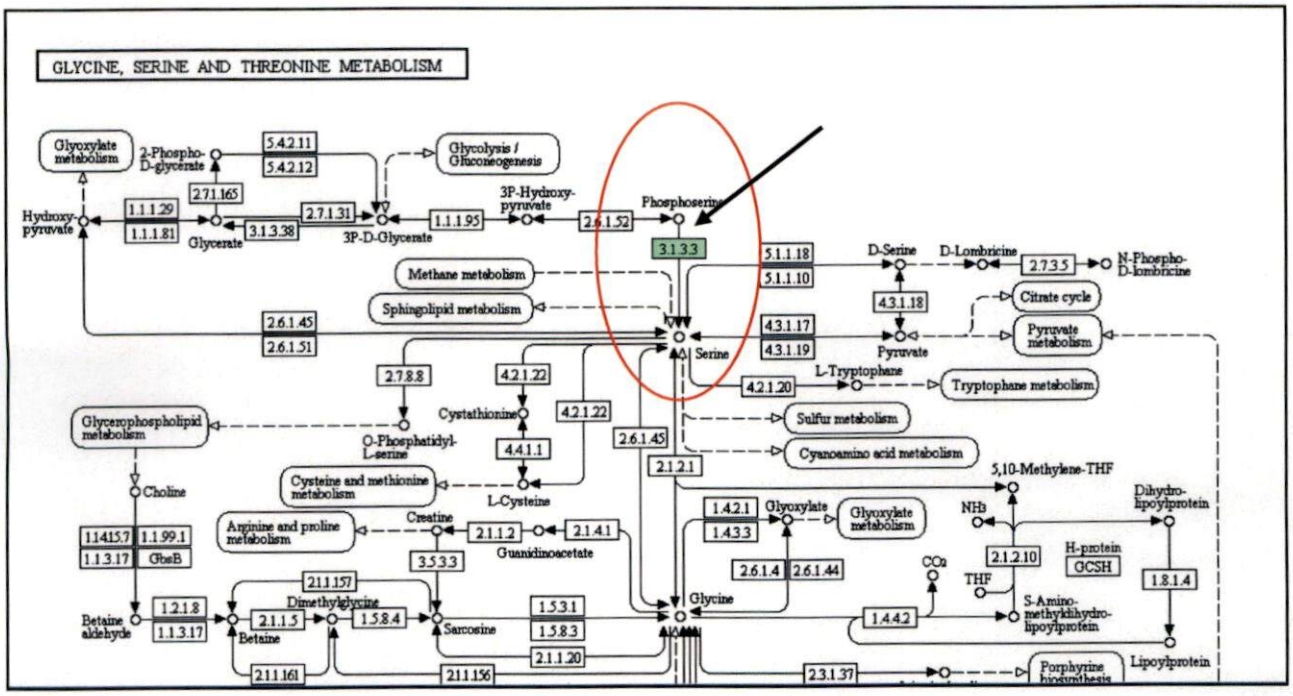



**Fig. 4.21 KEGG Pathway analysis of Sequence 5 (J2)**

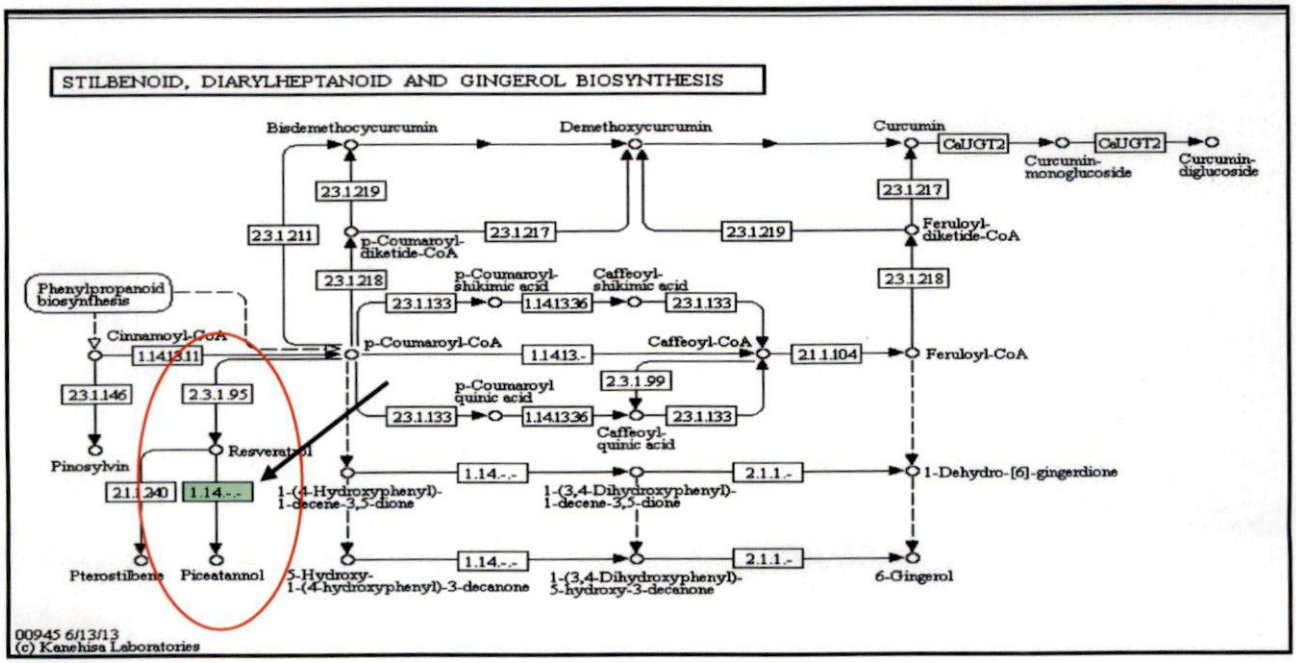
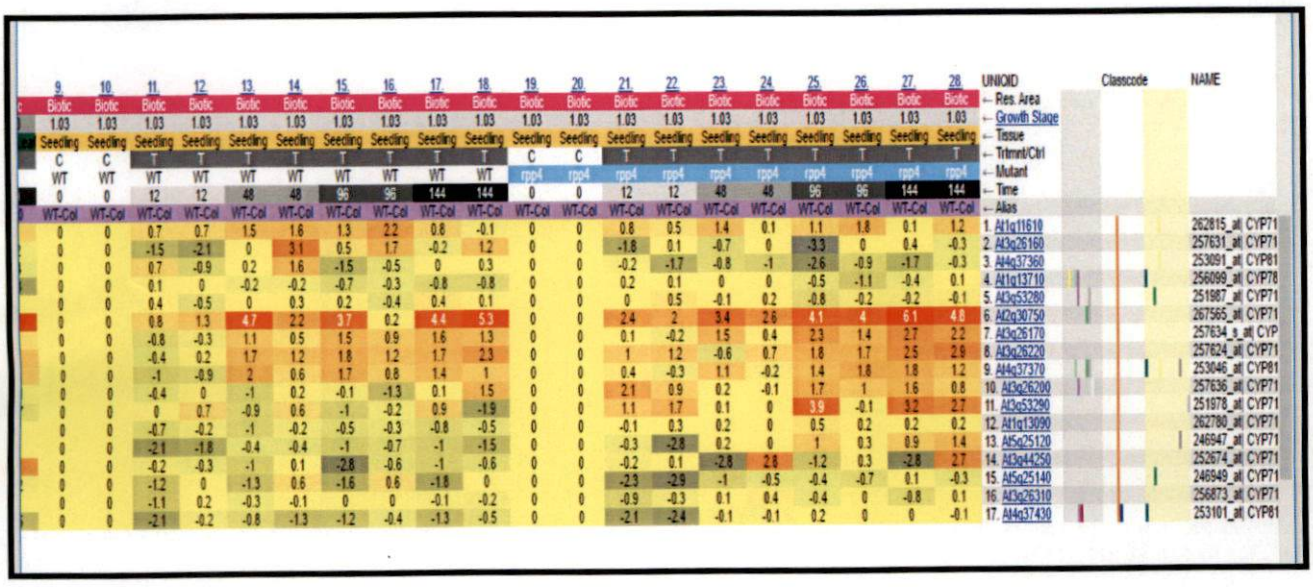



**Fig. 4.22 KEGG Pathway analysis of Sequence 1 (A2)**

**Table 4.15 List of AGI IDs used for eNorthern expression analysis**

| Sl No. | AGI IDs | Reference sequence |
|---|---|---|
| 1 | AT1G11610 | Cytochrome P450 |
| 2 | AT1G13710 | Cytochrome P450 |
| 3 | AT3G26160 | Cytochrome P450 |
| 4 | AT3G26200 | Cytochrome P450 |
| 5 | AT3G26310 | Cytochrome P450 |
| 6 | AT3G53280 | Cytochrome P450 |
| 7 | AT4G37360 | Cytochrome P450 |
| 8 | AT5G25120 | Cytochrome P450 |
| 9 | AT5G25140 | Cytochrome P450 |
| 10 | AT1G13090 | Cytochrome P450 |

The sequence 1 (A2) which shows similarity with cytochrome P450 of Arabidopsis thaliana was analysed for AGI IDs (Table 4.15)by BLASTx analysis against *Arabidopsis* genome and also from the identified KEGG pathway (Fig. 4.22) and the IDs were fed into eNorthern expression analysis portal by selecting (http://bbc.botany.utoronto.ca/.) expression browser option from BAR homepage. Biotic stress was analysed in different time periods with respect to *Arabidopsis* stress database and the level of expression was compared with different gene sequences of Arabidopsis cytochrome P450 (CYP) (Fig. 4.23). The flurescence value obtained from each stress was plotted separately on the graph as fold increase over control against the different time periods (Fig. 4.24). The expression level more than three indicates the genes are highly expressed. Genes with constitutively high levels of expression can be selected for further studies.



| | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | UNIOID | Classcode | NAME |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Biotic | Biotic | Biotic | Biotic | Biotic | Biotic | Biotic | Biotic | Biotic | Biotic | Biotic | Biotic | Biotic | Biotic | Biotic | Biotic | Biotic | Biotic | Biotic | Biotic | ← Res Area | | |
| | 1.03 | 1.03 | 1.03 | 1.03 | 1.03 | 1.03 | 1.03 | 1.03 | 1.03 | 1.03 | 1.03 | 1.03 | 1.03 | 1.03 | 1.03 | 1.03 | 1.03 | 1.03 | 1.03 | 1.03 | ← Growth Stage | | |
| | Seedling | Seedling | Seedling | Seedling | Seedling | Seedling | Seedling | Seedling | Seedling | Seedling | Seedling | Seedling | Seedling | Seedling | Seedling | Seedling | Seedling | Seedling | Seedling | Seedling | ← Tissue | | |
| | C | C | T | T | T | T | T | T | T | T | C | C | T | T | T | T | T | T | T | T | ← Trtmnt/Ctrl | | |
| | WT | WT | WT | WT | WT | WT | WT | WT | WT | WT | rpp4 | rpp4 | rpp4 | rpp4 | rpp4 | rpp4 | rpp4 | rpp4 | rpp4 | rpp4 | ← Mutant | | |
| | 0 | 0 | 12 | 12 | 48 | 48 | 96 | 96 | 144 | 144 | 0 | 0 | 12 | 12 | 48 | 48 | 96 | 96 | 144 | 144 | ← Time | | |
| | WT-Col | WT-Col | WT-Col | WT-Col | WT-Col | WT-Col | WT-Col | WT-Col | WT-Col | WT-Col | WT-Col | WT-Col | WT-Col | WT-Col | WT-Col | WT-Col | WT-Col | WT-Col | WT-Col | WT-Col | ← Alias | | |
| | 0 | 0 | 0.7 | 0.7 | 1.5 | 1.6 | 1.3 | 2.2 | 0.8 | -0.1 | 0 | 0 | 0.8 | 0.5 | 1.4 | 0.1 | 1.1 | 1.8 | 0.1 | 1.2 | 1. At1g11610 | | 262815_at\| CYP71 |
| | 0 | 0 | -1.5 | -2.1 | 0 | 3.1 | 0.5 | 1.7 | -0.2 | 1.2 | 0 | 0 | -1.8 | 0.1 | -0.7 | 0 | -3.3 | 0 | 0.4 | -0.3 | 2. At3g26160 | | 257631_at\| CYP71 |
| | 0 | 0 | 0.7 | -0.9 | 0.2 | 1.6 | -1.5 | -0.5 | 0 | 0.3 | 0 | 0 | -0.2 | -1.7 | -0.8 | -1 | -2.6 | -0.9 | -1.7 | -0.3 | 3. At4g37360 | | 253091_at\| CYP81 |
| | 0 | 0 | 0.1 | 0 | -0.2 | -0.2 | -0.7 | -0.3 | -0.8 | -0.8 | 0 | 0 | 0.2 | 0.1 | 0 | 0 | -0.5 | -1.1 | -0.4 | 0.1 | 4. At1g13710 | | 256099_at\| CYP78 |
| | 0 | 0 | 0.4 | -0.5 | 0 | 0.3 | 0.2 | -0.4 | 0.4 | 0.1 | 0 | 0 | 0 | 0.5 | -0.1 | 0.2 | -0.8 | -0.2 | -0.2 | -0.1 | 5. At3g53280 | | 251987_at\| CYP71 |
| | 0 | 0 | 0.8 | 1.3 | 4.7 | 2.2 | 3.7 | 0.2 | 4.4 | 5.3 | 0 | 0 | 2.4 | 2 | 3.4 | 2.6 | 4.1 | 4 | 6.1 | 4.8 | 6. At2g30750 | | 267565_at\| CYP71 |
| | 0 | 0 | -0.8 | -0.3 | 1.1 | 0.5 | 1.5 | 0.9 | 1.6 | 1.3 | 0 | 0 | 0.1 | -0.2 | 1.5 | 0.4 | 2.3 | 1.4 | 2.7 | 2.2 | 7. At3g26170 | | 257634_s_at\| CYP |
| | 0 | 0 | -0.4 | 0.2 | 1.7 | 1.2 | 1.8 | 1.2 | 1.7 | 2.3 | 0 | 0 | 1 | 1.2 | -0.6 | 0.7 | 1.8 | 1.7 | 2.5 | 2.9 | 8. At3g26220 | | 257624_at\| CYP71 |
| | 0 | 0 | -1 | -0.9 | 2 | 0.6 | 1.7 | 0.8 | 1.4 | 1 | 0 | 0 | 0.4 | -0.3 | 1.1 | -0.2 | 1.4 | 1.8 | 1.8 | 1.2 | 9. At4g37370 | | 253046_at\| CYP81 |
| | 0 | 0 | -0.4 | 0 | -1 | 0.2 | -0.1 | -1.3 | 0.1 | 1.5 | 0 | 0 | 2.1 | 0.9 | 0.2 | -0.1 | 1.7 | 1 | 1.6 | 0.8 | 10. At3g26200 | | 257636_at\| CYP71 |
| | 0 | 0 | 0 | 0.7 | -0.9 | 0.6 | -1 | -0.2 | 0.9 | -1.9 | 0 | 0 | 1.1 | 1.7 | 0.1 | 0 | 3.9 | -0.1 | 3.2 | 2.7 | 11. At3g53280 | | 251978_at\| CYP71 |
| | 0 | 0 | -0.7 | -0.2 | -1 | -0.2 | -0.5 | -0.3 | -0.8 | -0.5 | 0 | 0 | -0.1 | 0.3 | 0.2 | 0 | 0.5 | 0.2 | 0.2 | 0.2 | 12. At1g13090 | | 262780_at\| CYP71 |
| | 0 | 0 | -2.1 | -1.8 | -0.4 | -0.4 | -1 | -0.7 | -1 | -1.5 | 0 | 0 | -0.3 | -2.8 | 0.2 | 0 | 1 | 0.3 | 0.9 | 1.4 | 13. At5g25120 | | 246947_at\| CYP71 |
| | 0 | 0 | -0.2 | -0.3 | -1 | 0.1 | -2.8 | -0.6 | -1 | -0.6 | 0 | 0 | -0.2 | 0.1 | -2.8 | 2.8 | -1.2 | 0.3 | -2.8 | 2.7 | 14. At3g44250 | | 252674_at\| CYP71 |
| | 0 | 0 | -1.2 | 0 | -1.3 | 0.6 | -1.6 | 0.6 | -1.8 | 0 | 0 | 0 | -2.3 | -2.9 | -1 | -0.5 | -0.4 | -0.7 | 0.1 | -0.3 | 15. At5g25140 | | 246949_at\| CYP71 |
| | 0 | 0 | -1.1 | 0.2 | -0.3 | -0.1 | 0 | 0 | -0.1 | -0.2 | 0 | 0 | -0.9 | -0.3 | 0.1 | 0.4 | -0.4 | 0 | -0.8 | 0.1 | 16. At3g26310 | | 256873_at\| CYP71 |
| | 0 | 0 | -2.1 | -0.2 | -0.8 | -1.3 | -1.2 | -0.4 | -1.3 | -0.5 | 0 | 0 | -2.1 | -2.4 | -0.1 | -0.1 | 0.2 | 0 | 0 | -0.1 | 17. At4g37430 | | 253101_at\| CYP81 |

**Fig. 4.23 Fluorescence values of gene expression in *Arabidopsis thaliana* from eNorthern analysis (sequence 1 –A2)**

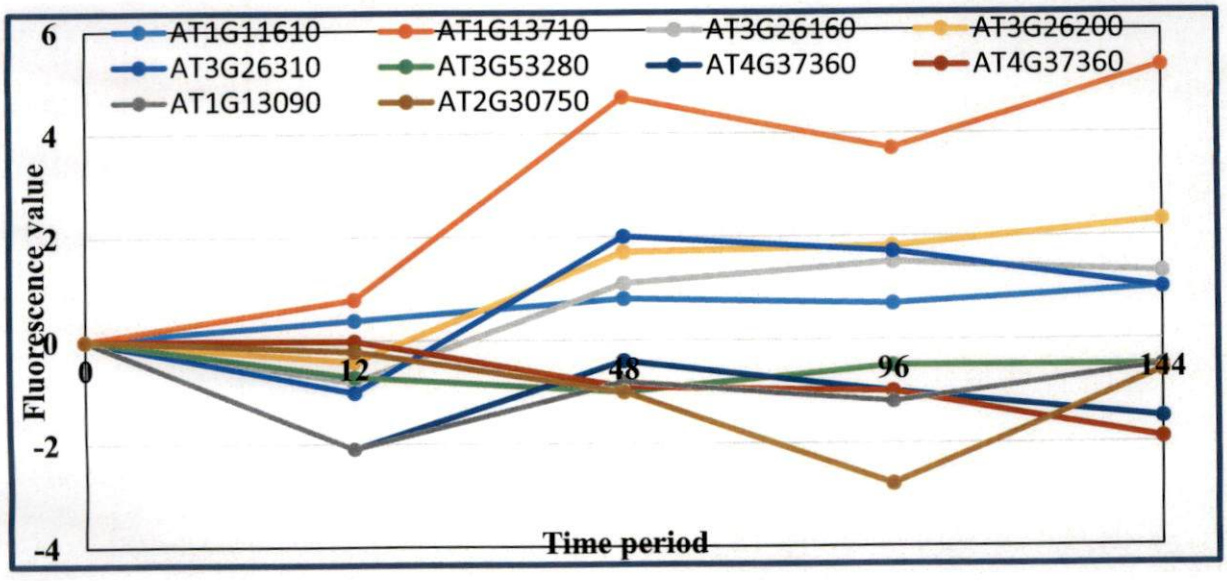



**Fig. 4.24 Fluorescence value for biotic stress plotted on the graph as fold increase over control**

# *Discussion*

# 5. DISCUSSION

The results obtained in the study "Transcriptome analysis of *Phytophthora capsici* tolerance in black pepper (*Piper nigrum* L.)'' are discussed in this chapter based on the earlier reports and best possible interpretations.

## 5.1 Isolation and identification of pathogen

Fungal pathogen, *Phytophthora capsici* was isolated from the infected leaf samples of black pepper during rainly season (July-August) since it required low temperature and high humidity (Granke and Hausbeck, 2010). Samples were collected from a Pepper Nursery, Cental Nursery Vellanikkara, Mannuthy. Isolation of pathogen was done using a standard protocol as proposed by Griffin, 1997 in Carrot Agar medium (Al-Hedaithy and Tsao 1979; Stelfox and Herbut, 1979). The infected leaf portion was cut into small bits using a sterile blade and incubated in distilled water for 24 hours for the development of sporangia (Narayanaswamy, 2011). The leaf bits were observed in a microscope for hyphae and lemon shaped sporangia and leaf bits with mycelia were then inoculated in Carrot Agar medium (Appendix I) aseptically in Laminar Air Flow Hood aseptically and Petri plates were incubated at low temperature (24±2 °C) and examined daily for the growth of the pathogen. Initially, the fungal growth was lethargic but later attained the momentum in antibiotic-free medium. The culture was purified by hyphal tip method (Narayanaswamy, 2011). Seven days old pathogen culture, on incubation in distilled water, produced numerous sporangia and were used for plant inoculation artificially.

## 5.2 Artificial inoculation of plants

For the comparison of differential expression of *Phytophthora capsici* selective genes in tolerant and susceptible cultivars, artificial inoculation was carried out under controlled conditions of temperature (24±2 °C) and relative humidity (65-90 %) for the effective infection to occur. The plants were well irrigated prior to and days before the inoculation to avoid the unwanted expression of abiotic stress related genes in plants. The leaves were wiped with distilled water thoroughly using a sterile tissue paper. Artificial inoculation was undertaken by giving a pin prick with sterile needle and five mm diameter pre-incubated culture disc (24 hr incubation in distilled water) was placed at the abaxial side of the leaf (Turner, 1969; Sarma *et al.*, 1988) in three different locations during the late evening 6 pm so that the temperature is maintained cool throughout the infection

time. *Phytophthora* sporulate abundantly at an optimum temperature of 25 °C (Rao, 1970). Evening hours was preferred for inoculation because of the environmental factors and the ease of RNA isolation. After inoculation, the inoculated discs were covered with a wet cotton and the whole plant was wrapped with polybag and sprayed distilled water to maintain relative humidity and the pot was kept in a tray of water.

**5.3 Symptomatology of the disease under artificial inoculation**

The artificially inoculated leaves showed yellow discoloration on the infected area. Later it enlarged with and became water soaked and black in colour. The dark lesions gradually turned to grey and exhibit light and dark zonation (Muller, 1936; Holliday and Mowat, 1963).

**5.4 Isolation and quantification of RNA**

The molecular mechanisms are activated at the initial stages of pathogen infection and hence the time periods selected for the isolation of total RNA may contain the expressed gene differentially at the time of pathogen attack (Palloix *et al.,* 1988). The inoculated leaf samples were collected at different time periods of 0, 2, 4, 6, 12, 24, 48 hours after inoculation (hai). (Shiny *et al.*, 2015). The mycelial disc on the inoculated leaf was wiped with sterile tissue paper and using a sterile scalpel the whole leaf was peeled off and immediately placed in ice after covering with an aluminium foil. 100 mg of the leaf sample from the area of infection are cut and weighed and proceeded with RNA isolation. The time gap between the collection of leaf sample and crushing of sample is crucial in getting a good quality RNA especially for black pepper where the phenolic compounds are exceptionally high (Meghwal and Goswami, 2012). Isolation of good quality RNA from plant tissues is challenging because they are rich in secondary metabolites such as phenolic compounds and polysaccharides that co-precipitate with nucleic acids and they interact irreversibly with nucleic acids and proteins, leading to their oxidation and degradation and renders RNA unsuitable for downstream purposes. (John, 1992; Chan *et al.*, 2007; Pinto *et al.*, 2009). Apart from protocol followed by Baburao (2012), PVP and β-ME were added before crushing to rule out the phenolic oxidation during RNA isolation. PVP removes polyphenols by the formation of hydrogen bonds and β-ME is a reducing agent which inhibits the polyphenolic oxidation. Samples were crushed in liquid nitrogen and proceeded with TRIzol reagent method as followed by Baburao (2012), Biju *et al.* (2010) and Siljo *et al.* (2014) in crops such as rice and cardamom. Trizol is a mixture of phenol and guanidine isothiocyanate which maintains the

integrity of RNA during the process of cell disruption and lysis. TRI reagent solubilizes the biological sample while denatures the protein and this made it most efficient method of isolating the total RNA from the leaf over phenol-chloroform extraction method (Simms *et al.,* 1993). Double chloroform wash was not necessarily needed if the time gap between collection and crushing is minimised. Chloroform was helps in removal of polysaccharides and traces of phenolic contaminants in the extract. If the time gap is reduced, single chloroform wash itself yields a good quality RNA. Over-drying of the pellets, after ethanol wash has reduced the integrity of RNA. Vigorous tapping of the Eppendorf tubes during the dissolution of pellets can shear the RNA. Black pepper RNA got sheared in vigorous tapping and observed sheared bands in formaldehyde agarose gel. Wiping of pestle, mortar, scalpel, gloves with RNaseZap™ can substitute the use of DEPC. Utensils were wiped well with RNaseZap™ using sterile tissue paper and used for RNA isolation and the quality and quantity of the RNA was comparable with that of RNA from DEPC treated ones.

The overall quality of total RNA was assessed by 1 per cent denaturing formaldehyde agarose gel electrophoresis. The denaturing condition was used because under normal condition RNA can form different types of secondary structure that prevent its mobility in the gel under native condition and the separation under normal agarose gel is affected (Bryant and Manning, 2000). In denaturing gel formaldehyde is used to maintain the denatured condition of RNA and finally, the bands were visualised using ethidium bromide. This is the most common method of analysing the quality of total RNA but the use of formaldehyde which is a carcinogenic chemical which requires the proper handling (Mansour and Pestov, 2013) is a constraint. The RNA analysed in the denaturing gel was having 3 intact (28S, 18S and 5S + t-RNA) bands which confirm the purity of RNA without any DNA or protein contamination. Babaurao (2012) and Jacob *et al.* (2003) reported the use of denaturing formaldehyde agarose gel electrophoresis for the estimation of the quality and quantity of total RNA.

**5.5 Synthesis of cDNA first strand**

The mRNA from total RNA was first selectively withheld using oligo-dT primer and further proceeded for the synthesis of cDNA by using H minus First strand cDNA synthesis kit (Thermo Scientific), aided by the addition of M-MuLV reverse transcriptase. Most of the RNA transcript has polyA tail at 3' end, effect the use of oligo-dT primer which can bind to polyA tail

of RNA transcript and M-MuLV RT act as polymerase and synthesizes DNA using RNA template (Malek *et al.*, 2000). The RNase was inhibited using the Ribolock RNase inhibitor to protect RNA template from degradation. The synthesis cDNA was confirmed by the synthesis of the second strand using actin primer, a house-keeping gene present in most of the eukaryotes forms a specific band at 450bp region in 1.5 per cent agarose gel. A similar method of cDNA synthesis was reported by Baburao (2012).

**5.6 Differential display (DD) Reverse Transcriptase PCR**

The first strand cDNA synthesized from the above RNA samples were subjected to PCR amplification using one anchored (T11C) and eight arbitrary primers (AP) as mentioned in Table 5 (Liang *et al.*, 2007). The protocol adopted from Baburao (2012), has been subjected to a slight modification of increased amount of *Taq* polymerase (0.4 µl) and made the reaction conditions favourable for getting clear bands. $MgCl_2$ was not separately added apart from included in GeNei Taq buffer A. The PCR product was stored in -20 °C and the product was confirmed by native PAGE and that product was resolved on 6 % denaturing urea polyacrylamide gels and visualised by silver staining.

Urea PAGE analysis was carried out in BioRad Sequi-Gen GT Sequencing Cell. The analysis was followed as per the guidelines mentioned in Baburao (2012), Liang and Pardee (1992) and Summer *et al.* (2009). The initial screening of each sample with respective primer combinations where T11C (anchored primer) was common and coupled with arbitrary primers (AP 1-8) one at a time was achieved. Mostly Urea PAGE plates were sticky, reasons contributing majorly due to the age of instrument and temperature fluctuations, both ambient and internal. Best results were obtained at an ambient temperature of 20 °C and hence carried out in an air conditioned room. Lack of a temperature probe in the instrument also made the gel vulnerable to stick and very high temperature in the plate during run caused the degradation of the sample and dispersed bands were visualised in the gel after staining. It was also observed that mounting of ice packs around the plates is not good because it causes a nonuniform run of bands in the gel. It implies that the proper run required a uniform temperature throughout the plates and can be accomplished by using a metal plate tied to the bind plate. Run time also played a crucial role in the visualisation of clear bands. As the run time increased, the samples got diffused in the gel. So the run time was restricted maximum to 4 hours so that sufficient amount of resolution was

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103

achieved. The IP plate and bind plate were thoroughly washed with detergent and rinsed in running tap water followed by distilled water. IP plate was wiped with 10 % NaOH solution using Kimwipe (facial tissue wipe) followed by absolute ethanol. Unlike IP plate, the bind plate was given separate treatment by shaking the plates overnight in 1N NaOH solution in rocker followed by shaking in distilled water for at least 2 hours. NaOH solution is used as a powerful degreaser on glasswares. The plate was dried completely in room temperature and bind silane (0.5 %) was applied thrice to the bind plate using Kimwipe and dried well. Repel silane (100 %) was applied once to IP plate to make it suitable to repel the gel after the run. After the run, the plates are kept on ice for a while to cool the plates and then dismantled. The clean plates are then subjected to silver staining. The gel was shaken in distilled water, fixer, stainer and developer solution in a serial manner with alternate washing in distilled water. Care was taken at the time of staining to avoid penetration of light to the area, especially during stainer and developer addition. The urea gel was dried overnight at room temperature and differentially expressed, up-regulated and down-regulated genes were marked, and bands are eluted and stored at -20 °C for analysis. Elution is carried out by scraping the gels carefully into the nuclease-free water with a sterile scalpel after dehydrating in nuclease-free water for 10 minutes (Minsheng, 2006).

### 5.8 *Insilico* analysis of the sequences

Differentially expressed, upregulated and down-regulated fragments were eluted from the Urea-PAGE gel and the sequences obtained were subjected to *insilico* analysis tools like BLASTn, BLASTx, MEGA, BLASTp, KAAS (KEGG Automatic Annotation Server) and eNorthern. The sequences are annotated to identify the genes and the pathways responsible for the resistance. Eleven out of 34 sequences have shown similarity to various genes and proteins related to defense pathways and signalling cascades.

#### 5.8.1 Sequence A2

The analysis of sequence 1 (A2) which was 455 bp in length and differentially expressed in *Phytophthora* infected tolerant cultivar. The annotation of the sequence by BLASTn showed similarity with *Nicotiana tobaccum* premnaspirodiene oxygenase like mRNA. BLASTx analysis also showed the same annotation above mentioned. The nucleotide sequences were translated to protein sequences by using 'Emboss Transeq' and the protein sequence thus obtained were subjected to BLASTp analysis and several conserved domains and motifs within the sequence are

obtained includes Flavanoid 3' monooxygenase, cytochrome P450 and RNA Recognition Motif (RRM). ORF finder, Phobius transmembrane and signal peptide predictor and Interpro confirmed the presence of suitable protein sequence and conserved domains.

Solavetivone, a potent antifungal phytoalexin which is derived from the sesquiterpene, premnaspirodiene, is catalysed in a single reaction by premnaspirodiene oxygenase enzyme (Takahashi *et al.*, 2007). The expression of premnaspirodiene oxygenase during *Phytophthora* infection differentially in tolerant cultivar implies the importance of gene. KEGG pathway analysis showed that premnaspirodiene oxygenase is involved in Stilbenoid, Diarylheptanoid and Gingerol biosynthetic pathway in the conversion of Resveratrol to Piceatannol which is 3, 3', 4' 5-Tetrahydroxystilbene, is a phenolic stilbenoid.

Brinker and Seigler (1991) reported the isolation of Piceatannol from sugarcane (*Saccharum* sp.) infected with *Colletotrichum falcatum* but not from healthy or wounded sugarcane. The compound inhibited both spore germination and germ tube growth of *C. falcatum*. This is the first report of a stilbene phytoalexin in the Poaceae.

The ectopic production of resveratrol, which is the precursor of piceatannol can lead to broad-spectrum resistance against fungi in transgenic lines, and to the enhancement of the antioxidant activities of several fruits, highlighting the potential role of this compound in health promotion and plant disease control (Delaunois *et al.*, 2009).

Cytochrome P450s are haem-thiolate proteins involved in the oxidative degradation of various compounds. The cytochrome P450 (CYP) superfamily plays vital roles in promoting plants growth and development and protecting plants from stresses via multiple biosynthetic and detoxification pathways (Ohkawa *et al.*, 1998; Li *et al.*, 2012).

The flavonoid 3' monooxygenase (F3'M) domain present in the sequence is of atmost importance in flavonoid biosynthesis of phenylpropanoid pathway. Flavanoid 3' monooxygenase catalyzes the 3'-hydroxylation of the flavonoid B-ring to the 3',4'-hydroxylated state. Convert naringenin to eriodictyol and dihydrokaempferol to dihydroquercetin. Flavanoids participate in plant protection against biotic (herbivores, pathogens) and abiotic stresses (UV radiation, heat), due to their antioxidative properties. The antioxidative activity of flavonoids is connected with the structure of the molecule: the presence of conjugated double bonds and the occurrence of

functional groups in the rings (Rice *et al.*, 1996; Seyoum *et al.*, 2006; Lattanzio *et al.*, 2006). Second domain present in the sequence was RRM (RNA Recognition Motif) in ras GTPase activating protein-binding protein G3BP1. G3BP acts at the level of RNA metabolism in response to cell signaling, possibly as RNA transcript stabilizing factors or an RNase. G3BP1 is stress granule-nucleating protein, purely antiviral by regulation of RNA granule formation (Lloyd, 2016).

### 5.8.2 Sequence A5

The analysis of sequence 2 (A5) which was 25 bp in length (very short fragment) and differentially expressed in *Phytophthora* infected tolerant cultivar. The annotation of the sequence by BLASTn showed similarity with *Nicotiana tobaccum* premnaspirodiene oxygenase like mRNA and *Nicotiana tobaccum* cytochrome P450, complete cds with 100 % query coverage and 100 % identity.

### 5.8.3 Sequence A7

The analysis of sequence 3 (A7) which was 317 bp in length and differentially expressed in *Phytophthora* infected tolerant cultivar. The annotation of the sequence by BLASTn showed similarity with 18S ribosomal RNA, internal transcribed spacer, 5.8S ribosomal spacer and 28S ribosomal RNA gene of organisms like *Blepharisma undulans, Blepharisma japoncum* and uncultured *Heterotrichida* clone with 100 % query coverage and 99 % identity. BLASTx analysis annotated the sequence to hypothetical protein from *Baudoinia panamericana* with 73 % query coverage and 49 % identity. The role of ribosomal RNA genes especially plant ribosomal proteins are reflected in non-host resistance mechanism against pathogens. The mutation of ribosomal protein encoding genes RPL 12 and RPL 19 delayed the non-host pathogen derived hypersensitivity (HR) reaction in *Nicotiana benthamiana* implies the role in inducing non-host resistance in plants (Nagaraj *et al.*, 2015).

### 5.8.4 Sequence C2

The analysis of sequence 4 (C2) which was 468 bp in length and differentially expressed in *Phytophthora* infected tolerant cultivar. It is an uncharacterized novel fragment. Three alternate proteins sequences were obtained from MEGA. Three alternative protein sequences were obtained from MEGA from first, second and third ORF having lengths 28, 49 and 40 aa respectively.

BLASTp analysis of these protein sequences yields showed any similarity with the existing protein database and are purely uncharacterized sequences.

### 5.8.5 Sequence J2

The analysis of sequence 5 (J2) which was 405 bp in length and upregulated in *Phytophthora* infected tolerant cultivar. The annotation of the sequence by BLASTx showed similarity with Phosphoserine phosphatase, chloroplastic like protein from *Malus domesticus* (48 % query coverage and 77 % identity). The presence of haloacid dehydrogenase superfamily (HAD super family) includes the phosphatases helps in amino acid biosynthesis especially serine, an important amino acid in signaling cascade. Protein phosphatases are small protein network like cascades in MAPK kinase against pathogen-host defense mechanism. Serine/threonine protein kinases are the foremost in this category which signals the MAP Kinases which generates the Nuclear Localisation Signals (NLS) and grant the expression of defense related genes (Pais *et al.*, 2009; Ebrahim *et al.*, 2011; Carrasco *et al.*, 2014). Therefore, in black pepper, the expression facilitates the signaling cascades of defense related genes along with MAPK cascade.

BLASTp analysis of sequence J2 annotated to predicted phosphoserine phosphatase, chloroplastic like of *Malus domesticus* with 63 % query coverage and 77 % identity, *Arabidopsis thaliana* with 62 % query coverage and 68 % identity. The role and function of serine metabolism by phosphoserine phosphatase was explained earlier.

### 5.8.6 Sequence M4

Longest coding sequences of sequence 6 (M4) was obtained from MEGA 7.0 and were subjected to BLASTp analysis. BLASTp analysis of M4 sequence was annotated to a hypothetical protein from *Botybasidium botyosum* with 44 % query coverage and 54 % identity and *Janthinobacterium ividum* with 69 % query coverage and 47 % identity. It also shows identity with ubiquitin protein ligase 5 (UPL5) of *Arabidopsis thaliana* with 69 % query coverage and 34 % identity. It is reported that about 5 % of Arabidopsis genome comprised of protein related to ubiquitination pathway. The protein UPL5 can degrade the target protein when it is polyubiquitinyled, mediated by 26S proteasome machinery (Mazzucotelli *et al.*, 2006). In black pepper, UPL5 proposed to act on the fungal elicitor proteins by ubiquitin mediated protein degradation machinery.

### 5.8.7 Sequence M1

BLASTp of sequence 7 (M1) annotated to predicted Ankyrin repeat and KH domain containing protein like 1 isoform 2 (ANKHD1) from *Clupea herengus* with 47 % query coverage and 45 % identity, heme oxygenase from *Luteimonas huabeiensis* with 31 % query coverage and 54 % identity, MAP Kinase alpha 1 and Protein kinase super family from *Arabidopsis thaliana* with 40 % query coverage and 30 % identity and predicted adhesion G-protein coupled receptor G7 like protein from *Ciona intestinalis* with 89 % query coverage and 33 % identity. ANKHD1 Isoform 2 may possess an antiapoptotic effect and protect cells during normal cell survival through its regulation of caspases (Miles *et al.*, 2005) and was reported to be overexpressed during flower senescence (Xu *et al.*, 2007). In black pepper, ANKHD1 might have a role in protecting the normal cells from degradation by the expressed apoptotic factors during the complex defense mechanism.

In plants, heme oxygenase (HO) is localized to the chloroplast and the biliverdin it produces is a precursor for the synthesis of the phytochrome chromophore, phytochomobilin and carbon monoxide as a byproduct (Muramoto *et al.*, 2002). The phytochromobilin attaches to the phytochrome apoprotein, which is synthesised in the nucleus, to form the heme catabolism pathway in plants and animals. Recent studies in plants suggests that CO regulates ROS levels in plant cells (Lin *et al.*, 2014). In plants, expression of many of the nuclear genomes are regulated by chloroplast genome. The communicating signals from the chloroplast to the nucleus, known as retrograde signaling is required for the transcriptional regulation of nuclear genomes (Nott *et al.*, 2006). Heme oxygenase (HO) is one of those signaling molecules that influence transcription of the nuclear genome (Surpin *et al.*, 2002). So, heme oxygenase had a proposed role of extra chromosomal transcription regulation of nuclear genes in black pepper.

The mitogen-activated protein kinase (MAP kinase) signal transduction cascades are routes through which eukaryotic cells deliver extracellular messages to the cytosol and nucleus. MAP kinase pathway was utilised by ABA signaling, ethylene-mediated signaling and even cytokine-mediated signaling cascades also (Cheong and Kim, 2010; Morris, 2001). During interaction between phytopathogenic fungi and plants, fungal MAPKs help to promote penetration of host tissues, while plant MAPKs are required for activation of plant immunity. MAPK cascades in both organisms are mutually contribute to a highly interconnected molecular dialogue between the plant and the fungus. As a result, signaling pathway in plants got activated including plant MAPK

cascades. Plant MAPKs promote defense mechanisms that threaten the survival of fungal cells, leading to a stress response mediated in part by fungal MAPK cascades (Hamel *et al.*, 2012). The same role of signaling might have worked out in black pepper during *Phytophthora* infection.

The functions of Adhesion G protein-coupled receptors are not yet characterised well in plants. In animals, it had prominent roles in development and produce immune response against pathogen infection (Chang *et al.*, 2016). Adhesion GPCRs are characterized by an extended extracellular region often possessing N-terminal protein modules that is linked to a TM7 region via a domain known as the GPCR-Autoproteolysis INducing (GAIN) domain. The GPCRs GAIN domains involved in the cleavage of signaling peptides from inactive proteins. In other way, autoproteolysis of inactive proteins to form active proteins are undertaken by GPCR Auto-proteolysis inducing domain (Araç *et al.*, 2012). Activation of Adhesion G-protein-coupled receptors in black pepper might leads to the activation differentially translated gene products at the time of infection by activation of proteins responsible for resistance.

Sequence information of few eluted fragments were not able unravel due to its larger fragment size (>3 kb). Description of the differentially expressed fragments and their proposed roles in defense mechanism are listed in Table 4.16.

**5.9 Validation of signaling and metabolic pathways using KAAS**

cDNAs isolated from differential expression studies were fed to KAAS database to track the pathway of action. Premnaspirodiene oxygenase is involved in the conversion of Resveratrol to Piceatannol which is an antifungal protein, of stilbenoid pathway belongs to phenylpropanoid signaling mechanism. The conversion of glucose to resveratrol is accomplished through glycolysis, phenylalanine biosynthesis, phenylpropanoid biosynthesis and stilbenoid pathways (Che *et al.*, 2016). Phosphoserine phosphatase is involved in the conversion of phosphoserine to serine which belongs to Serine/threonine metabolic pathway which is involved in cell signaling mechanism. Knock down of *Phytophthora capsici* serine/threonine protein kinase (PcSTPK) by virus induced gene silencing (VIGS) enhanced the susceptibility to *P. capsici* infection, as evidenced by the occurance of foliar necrotic lesions and increased proliferation and sporulation of *P. capsici* on the leaf surface in *Piper colubrinum*. PcSTPK is a prospective candidate gene identified from *P. colubrinum* for future genetic improvement of *P. nigrum* (Krishnan *et al.*, 2015).

**Table 5.1 Summary of differentially expressed fragments (DEFs) and its role in resistance**

| Sl. No. | Sample name | Name of gene or domain | Size of the eluted fragment (bp) | Length of the sequence (bp) | Period of expression (hai) | Type of expression in resistant variety | Functional role |
|---|---|---|---|---|---|---|---|
| 1 | A2 | Premnaspirodiene oxygenase | 1000 | 455 | 2 | Differential expression | Biosynthesis of antifungal phytoalexins |
| | | Cytochrome P450 | | | | | |
| 2 | A5 | Premnaspirodiene oxygenase | 150 | 25 | 2 | Differential expression | Biosynthesis of antifungal phytoalexins |
| | | Cytochrome P450 | | | | | |
| 3 | A7 | rRNA genes | 600 | 317 | 6 | Upregulation | Hypersensitivity reactions |
| 4 | C2 | uncharacterized | 800 | 468 | 48 | downregulation | - |
| 5 | J2 | Phosphoserine phosphatase | 700 | 405 | 2 | Differential expression | Serine metabolism and signaling |
| 6 | M4 | Ubiquitin protein ligase 5 | 450 | 177 | 48 | Upregulation | Ubiquitin mediated protein degradation |
| 7 | M1 | Ankyrin repeats and KH domain | 650 | 174 | 12 | Differential expression | Anti-apoptotic |
| | | Heme oxygenase | | | | | Regulation of reactive oxygen species |
| | | MAP kinase | | | | | Extra nuclear regulation of nuclear genes |
| | | Adhesion G coupled protein receptor like | | | | | Activation of signal peptides |

**5.10 Validation of expression by eNorthern analysis**

eNorthern expression browser looks through the expression profiles of a particular set of genes. It enables us to input a list of upto 125 genes, which will then be selected across all the experiments in the database and hierarchically clustered and displayed graphically. Additional facility to query the AtGenExpress Developmental Series data has also been implemented by expression browser. Once the user input the desired list of gene (Arabidopsis Genome Initiative) AGI codes and specify the appropriate options, the application retrieves the expression profiles, processes them and displays the results as a list of output options in the result page. AGI codes were developed by BLASTx analysis of sequences with Arabidopsis genome (Baburao, 2012) and KEGG pathway database, only sequence A2 corresponds to cytochrome P450 gene showed AGI code analogue for *Arabidopsis* genome. The related codes were input into BAR database for biotic stress was evaluated and graph was plotted. The level of expression was determined from the log transformed fluorescence values of clustered data in Arabidopsis database. If the fluorescence value is greater than 3.0, it implies the gene is highly expressive. The differentially expressed sequence from tolerant pepper cultivar during *Phytophthora* infection, showed higher expression in Arabidopsis stress database exceptionally high at 48 hours post infection. Thus the level of expression of the sequence A2 corresponds to cytochrome P450 gene was validated through Arabidopsis genome database.

# *Summary*

## 6. Summary

The study entitled "Transcriptome analysis of *Phytophthora capsici* tolerance in black pepper (*Piper nigrum* L.)" was carried out at Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, during the period 2015-2017. The objective of the study was to understand the differential expression of genes during *Phytophthora* infection in susceptible and tolerant black pepper cultivars through DDRT-PCR analysis on mRNA.

IISR Subhakara, the susceptible cultivar along with IISR Shakthi, the tolerant cultivar were used in this study for the evaluation of transcriptome profile in response to *Phytophthora capsici* causing foot rot disease in black pepper. The salient findings of the study are summarized hereunder:

The fungal pathogen *Phytophthora capsici* associated with foot rot of black pepper was isolated from the infected leaf sample and cultured on Carrot Agar Medium (CAM). The identity of the pathogen was established by examining the cultural and morphological characteristics.

Artificial inoculation was carried out in susceptible and tolerant cultivars using 5 mm mycelial disc from 7 days old *Phytophthora capsici* pure culture. Inoculation was done at the abaxial side of the leaves and covered with wet cotton. Brown spots were observed at the inoculated area 12 hours after inoculation.

Total RNA was isolated from the leaf samples at different time periods, *viz.*, 0, 2, 4, 6, 12, 24 and 48 hours after inoculation using TRIzol method. Concentration of the isolated RNA was high in the range of 1000 to 2400 ng/µl as quantified by NanoDrop® spectrophotometer. High quality RNA was obtained by using TRIzol reagent, PVP, β- mercaptoethanol and reduction of time gap between sample collection and RNA isolation. This procedure increased the stability and storability of RNA.

Using RevertAid H minus first strand cDNA synthesis kit (Thermo Scientific) high quality cDNA with 500-1500 ng/µl concentration was synthesized from the total RNA. The concentration of cDNA was normalized to 25 ng/µl for DDRT-PCR.

The transcriptome profiles were distinct for each of the primer combinations in tolerant and susceptible cultivars. A total of twelve differentially expressed bands were identified, of which two were upregulated and the rest down-regulated in resistant cultivar.

The band A2 (455 bp) obtained using the primer combination AP1-T11C which was upregulated two hours after inoculation in tolerant cultivar, shown similarity with premnaspirodiene oxygenase gene. Premnaspirodiene oxygenase is involved in the conversion of premnaspirodiene to solavetivone, a potential antifungal phytoalexin in a single reaction. So this gene is activated when the host plant is attacked by fungal pathogen. This gene is also involved in the stilbenoid biosynthetic pathway for the conversion of resveratrol to piceatannol, yet another antifungal phytoalexin which inhibits the spore germination and germ tube growth. Three conserved domains are present in the translated protein sequence *viz.*, Flavanoid 3-monooxygenase (F 3'M), cytochrome P450 and RNA Recognition Motif (RRM) domain. F 3'M is involved in the phenyl propanoid pathway for the conversion of naringenin to eriodictoyl and dihydroquercetin which is an active compound in defense against biotic and abiotic stresses. Cytochrome P450 regulates the oxidative degradation of various toxins and it might have a role in detoxification of fungal toxins during the time of infection. The cytochrome P450 (CYP) gene analogue in *Arabidopsis thaliana* shown high level of expression at 48 hours during biotic stress through eNorthern analysis.

The band J2 (405 bp) obtained using the primer combination AP6-T11C and upregulated/downregulated two hours after inoculation in the tolerant cultivar had shown similarity with phosphoserine phosphatase. Phosphoserine phosphatase is involved in the conversion of phosphoserine to serine, which is a potent signaling molecule marked the role in MAPK cascade mechanism for Nuclear Localisation Signal (NLS) for the activation of defense related genes in nucleus.

The band M4 (177 bp) generated using the primer combination AP3-T11C and upregulated 48 hours after inoculation in the tolerant cultivar had shown similarity with hypothetical protein and ubiquitin protein ligase 5 (UPL5) from *Arabidopsis thaliana.* UPL5 is involved in degradation of target protein by 26S proteasome machinery and is proposed to have a role in degradation of fungal elicitor proteins in black pepper.

The band M1 (174) generated using the primer combination AP3-T11C and downregulated 12 hours after inoculation in tolerant cultivar shown similarity with ankyrin repeat and KH domain containing protein like isoform 2 (ANKHD2), heme oxygenase, map 4 kinase alpha 1 and adhesion G protein coupled receptor G7 like proteins (Adhesion GPCRs). ANKHD2 possess an anti-apoptotic effect and protects cells during normal cell survival through regulation of caspases. It protects the plant tissue from damage when confronted with fungal attack in black pepper. Heme oxygenase gene is localized in chloroplast and CO, the byproduct of heme metabolism regulates ROS levels in plant cell and also it acts as a signal for the transcriptional activation of nuclear genes. MAPK pathway is utilised by ABA, ethylene mediated and cytokine mediated signaling pathways promotes the defense against pathogen attack. Adhesion GPCRs involved in the cleavage of signal peptides for the activation of proteins in the system with the help of GPCR Autoproteolysis INducing (GAIN) domain.

The fragments which are differentially expressed and upregulated in tolerant cultivar during the time of pathogenesis implies the resistance mechanism by which the black pepper tackles *Phytophthora capsici* attack when compared to the susceptible cultivar. Resistance attains momentum when the component mechanisms are acting together to tackle the stress.

Future line of work includes

- Design of SCAR primers using the sequences from differentially expressed bands, for distinguishing tolerant and susceptible cultivars
- Identification of more candidate genes and pathways responsible for the *Phytophthora capsici* tolerance in black pepper
- Use of the identified genes for the development of high yielding tolerant cultivars through genetic transformation

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# Annexures

## Annexure I

**Composition of Carrot Agar Medium**

Carrot – 250 g

Agar – 7.5 g

Make up volume to 500 ml

For preparation of Carrot Agar medium, 250g of raw carrot was peeled off for the dead cells from the outer skin. Further, the carrot was chopped into pieces. Carrot was half cooked in 250 ml water and the essence was squeezed using a muslin cloth. Agar (7.5 g) was melted separately in 150 ml distilled water and both the contents were mixed properly and volume was madeup to 500 ml. The homogenous medium was poured into 250 ml conical flasks and test tubes (for slant) and tied properly with autoclaved clean cotton and wrapped with blotting paper on the top and tied up with rubber bands. Test tubes were bundled in a set of 8-9 tubes and wrapped with blotting paper and rubber band. The media was autoclaved at 121 °C for 20 minutes at 15 psi. The autoclaved media was stored in clean dry area.

## Annexure II

## Chemicals and buffers for RNA isolation

1. DEPC treated autoclaved water

   DEPC – 1 ml

   Distilled water – 2 L

   Overnight stirring and double autoclave in amber bottle

2. 10X MOPS buffer (pH 8.0)

   MOPS – 20.93 g

   Sodium Acetate – 0.205 g

   Sodium EDTA – 1.861 g

   Make up the volume to 500 ml

3. 1X MOPS running buffer

   10X MOPS – 100 ml

   DEPC treated water – 900 ml

4. 75 % Ethanol (DEPC treated)

   Absolute ethanol – 75 ml

   DEPC treated water – 25 ml

## Annexure III

### Chemical composition of the buffers, gels and solutions used for Urea-PAGE

1. Bind Silane
   995 µl of Ethanol
   5 µl of Bind silane
   5 µl of Acetic acid
2. Repel Silane
   995 µl of Ethanol
   500 µl of Repel silane (Sigma cote)
   5 µl of acetic acid
3. 40 % monomer solution
   Acrylamide – 38 g
   Bis-acrylamide – 2 g
   Distilled water – 100 ml
4. 10 % Ammonium per sulphate
   APS – 0.1 g
   Distilled water- 1 ml
5. 6 % Urea Acrylamide solution
   Urea – 25.2 g
   10X TBE – 6 ml
   40 % Monomer solution – 9 ml
   Make up the volume to 60 ml
   APS – 600 µl
6. 10 % NaOH
   NaOH – 10 g
   Distilled water – 100 ml
7. 10X TBE (pH 8.0)
   Tris base – 27 g
   Boric Acid – 13.75 g
   EDTA – 2.325 g

Make up volume – 250 ml

**8.** 0.5X TBE (1 L)

10X TBE – 50 ml

Distilled water – 950 ml

**9.** Fixer solution

Absolute ethanol- 150 ml

Acetic acid – 7.5 ml

Make up the volume to 1.5 L with distilled water

**10.** Stainer solution

Silver nitrate - 2.25 g

37% Formaldehyde - 2.25 ml

Make up the volume to 1.5 L with distilled water and store

**11.** Developer solution

Sodium hydroxide - 22.5 g

37 % Formaldehyde - 2.25 ml

Make up the volume to 1.5 L with distilled water

**12.** 6 % Urea Acrylamide solution

Urea – 25.2 g

10X TBE – 6 ml

40 % Monomer solution – 9 ml

Make up the volume to 60 ml

APS – 600 µl

TEMED – 60 µl

**13.** Formamide dye (10 ml)

Formamide – 9.5 ml

Bromophenol blue – 2.5 mg

Xylene cyanol – 2.5 mg

0.5 M EDTA (pH 8.0) – 100 µl

145

# Transcriptome analysis of *Phytophthora capsici* tolerance in black pepper (*Piper nigrum* L.)

By

**BASIL BABU PAUL**

**(2015-11-100)**

**ABSTRACT OF THE THESIS**

***Submitted in partial fulfilment of the requirement for the degree of***

## Master of Science in Agriculture

**(PLANT BIOTECHNOLOGY)**

**Faculty of Agriculture**

**Kerala Agricultural University, Thrissur**


**CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY**

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# Abstract

# Abstract

Black pepper (*Piper nigrum* L.) regarded as the 'king of spices' is one of the most important spices in the world, cultivated in more than 30 countries. Foot rot disease caused by the fungal pathogen *Phytophthora capsici* Leonian is the major production constraint since 1951. Tropical climates favour the development of foot rot disease wherein the survival of pathogen is exceptionally high, leading to complete crop losses. The long term survivability of oospores in soil, a wide host range, long-distance movement of the pathogen in surface water, presence of fungicide-resistant pathogen population, and a lack of resistant high yielding cultivars make this the most deadly disease. Understanding of molecular basis of *Phytophthora* resistance can help control the disease by gene stacking, molecular breeding and exploiting the natural resistance prevailing in wild species of black pepper. The study entitled "Transcriptome analysis of *Phytophthora capsici* tolerance in black pepper (*Piper nigrum* L.)" was carried out with the objective to understand the differential expression of genes during *Phytophthora* infection in susceptible and tolerant black pepper cultivars through DDRT-PCR analysis on mRNA.

IISR Subhakara, the susceptible cultivar along with IISR Shakthi, the tolerant cultivar were used for the evaluation of transcriptome profiles in response to the infection. *Phytophthora capsici* pure culture has been isolated and maintained on Carrot Agar Medium (CAM). Artificial inoculation with mycelial discs (5 mm) was done on the tender leaves of two black pepper cultivars and total RNA was isolated at 0, 2, 4, 6, 12, 24 and 48 hours after inoculation (hai) using TRIzol reagent. The RNA was quantified using NanoDrop® spectrophotometer and electrophoresed on 1 per cent formaldehyde agarose denaturing gel. cDNA first strand was synthesized from the total RNA using RevertAid H minus first strand synthesis kit and second strand was synthesized using anchored and arbitrary primers (DDRT-PCR). The DDRT-PCR product was electrophoresed on 6 per cent urea polyacrylamide sequencing gel. The transcriptome profiles were distinct for each of the primer combinations in tolerant and susceptible cultivars. A total of twelve differentially expressed bands were identified, of which two were upregulated and one was down-regulated in tolerant cultivar. The bands are eluted and amplified selectively using the same primer combination. The selective-PCR product was sequenced and the sequences were analysed *in-silico* for characterisation of fragments and functional annotation of genes and metabolic pathways.

Sequence of the differentially expressed band (455 bp) A2 generated with the primer combination AP1-T11C was similar to that of Premnaspirodiene oxygenase gene-like, which is

involved in the biosynthesis of solavetivone and piceatannol, potent antifungal phytoalexins, which inhibits the spore germination and germ tube growth. Sequence of band A2 (455 bp) generated with the primer combination AP1-T11C was similar to Cytochrome P450 gene is involved in detoxification pathway which detoxifies the fungal protein elicitors and native toxins. Sequence of band J2 (405 bp) generated with the primer combination AP6-T11C was similar to Phosphoserine phosphatase. This super family of proteins stands for the activation of defense related genes in the nucleus through extra chromosomal transcription activation, reactive oxygen species (ROS) regulation and signaling pathways by the generation of nuclear localization signals (NLS) and it acts through ABA mediated, ethylene mediated or cytokine mediated signaling pathways. Similarly, BLASTp analysis showed similarity with UPL5 from M4 sequence (177 bp) and ANKHD1, MAPK alpha 1, protein kinase superfamily and GPCR from M1 sequence (174 bp). GPCR (G protein coupled protein receptor) cleaves the signal peptides thereby activating the protein molecules. G3BP1 (GTPase activating protein binding protein) is stress- granule nucleating protein which stabilizes the RNA transcript and helps in the formation of RNA granule mainly against viruses. ANKHD1 is an anti-apoptotic regulating domain which protects the normal cell from degradation. UPL5 (Ubiquitin protein ligase 5) is involved in 26S proteasome mediated protein degradation whereas the rRNA genes induces non host pathogen derived hypersensitivity reactions (HR).

The fragments which are differentially expressed, upregulated and downregulated in tolerant cultivar during the time of pathogenesis implies the resistance mechanism by which the black pepper tackles *Phytophthora capsici* attack. The sequence information could be further used to design SCAR primers which would be useful in distinguishing tolerant and susceptible cultivar. It can also enrich the genetic base by incorporation of identified genes through genetic transformation for disease tolerance.

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