Exploring wheat-*Bipolaris sorokiniana* interaction during spot blotch disease

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In

BIOLOGICAL SCIENCES

By

Ms. Deepika Somani

(Registration Number: 10BB13J26038)

Under the guidance of

Dr. Narendra Y. Kadoo

Biochemical Sciences Division
CSIR-National Chemical Laboratory
Pune - 411008, MH, India

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Dedicated to…

My inspiration, a divine soul……

Late Smt. Narayani Namdhar

सीएसआईआर - राष्ट्रीय रासायनिक प्रयोगशाला
(वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद)
डॉ. होमी भाभा मार्ग, पुणे - 411 008. भारत
CSIR - NATIONAL CHEMICAL LABORATORY
(Council of Scientific & Industrial Research)
Dr. Homi Bhabha Road, Pune - 411 008. India

Certificate

This is to certify that the work incorporated in this Ph.D. thesis entitled "**Exploring wheat-*Bipolaris sorokiniana* interaction during spot blotch disease**" submitted by **Ms. DEEPIKA SOMANI** to the Academy of Scientific and Innovative Research (AcSIR) in fulfillment of the requirements for the award of the Degree of **DOCTOR OF PHILOSOPHY,** embodies original research work under my supervision/ guidance. I/We further certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree or diploma. Research material obtained from other sources has been duly acknowledged in the thesis. Any text, illustration, table etc., used in the thesis from other sources, have been duly cited and acknowledged.

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Deepika Somani
Deepika Somani
(Student)

Date: Feb. 15, 2019

Place: PUNE

Kadoo
Dr. Narendra Y. Kadoo
(Supervisor)

Date: Feb. 15, 2019

Place: PUNE

Communications Channels ☎ +91 20 25902000 +91 20 25902600 +91 20 25902222 Fax +91 20 25902601 (Director) +91 20 25902660 (Admin.) +91 20 25902639 (Business Development) URL : www.ncl-india.org

Declaration by the Candidate

I hereby declare that the original research work embodied in this thesis entitled "**Exploring wheat-*Bipolaris sorokiniana* interaction during spot blotch disease**" submitted to the Academy of Scientific and Innovative Research (AcSIR) for the award of the degree of **Doctor of Philosophy** (Ph.D.) is the outcome of experimental investigations carried out by me under the supervision of **Dr. Narendra Y. Kadoo**, Principal Scientist, Biochemical Sciences Division, CSIR-National Chemical Laboratory, Pune. I affirm that the work incorporated is original and has not been submitted to any other academy, university or institute for the award of any degree or diploma.

Date: Feb. 15, 2019

Place: Pune

Deepika Somani

(PhD Candidate)

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Deepika Somani

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List of abbreviations

Abbreviation	Meaning
ATP	Adenosine triphosphate
AFLP	Amplified fragment length polymorphism
ABC	ATP binding cassette
BLAST	Basic Local Alignment Search Tool
CDR	Candida drug resistance
cm	Centimeter
CC	Chirya 3 control
CI	Chirya 3 inoculated with fungus
cDNA	Complementary decarboxynucleic acid
CPM	Counts per kilobase per million mapped reads
dpi	Days post inoculation
DC	DDK 1025 control
DI	DDK 1025 inoculated with fungus
°C	Degree Celsius
dNTP	Deoxynucleotide 5' triphosphate
DNA	Decarboxynucleic acid
DMI	Demethylation inhibitor
DEG	Differentially expressed genes
2-D PAGE	2-Dimensional Polyacrylamide Gel Electrophoresis
ESI	Electro spray ionization
EC	Enzyme code
ESTs	Expression Sequence Tags
ECM	Extracellular matrix
FAO	Food and Agricultural Organization of the United Nations
FPKM	Fragments per kilobase per million mapped reads
GC-MS	Gas Chromatography Mass Spectrometry
GO	Gene Ontology
g	Gram
EC_{50}	Half maximum effective concentration

Abbreviation	Meaning
HPLC	High Performance Liquid Chromatography
HST	Host selective toxin
h	Hour
hpt	Hours post treatment
IDM	Integrated disease management
ITS	Internal Transcribed Spacer
JGI	Joint Genome Institute
kg	Kilogram
KEGG	Kyoto Encyclopedia of Genes and Genomes
LFC	Log2 fold change
MFS	Major Facilitator Superfamily
MS	Mass Spectrometry
µl	Microlitre
µm	Micrometer
ME	Mega-environment
ME5H	Mega-environment 5 humid
ME1HT	Mega-environment 1 with heat
mg	Milligram
ml	Milliliter
min	Minute
M	Molar
MPSS	Multiple Parallel Signature Sequencing
ng	Nanogram
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
NMR	Nuclear Magnetic Resonance
ppm	Parts per million
PDR	Pleiotropic drug resistance
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction

Abbreviation	Meaning
QoI	Quinone outside inhibitors
RNA	Ribonucleic acid
RIN	RNA Integrity Number
rpm	Rotation per minute
SEM	Scanning electron microscopy
SRA	Sequence Read Archive
SAGE	Serial Analysis of Gene Expression
SDS-PAGE	Sodium Dodecyl Polyacrylamide Gel Electrophoresis
SSH	Subtractive Sequence Hybridization

Synopsis

Chapter 1: Introduction

Wheat is the second most widely grown and consumed food crop of the world after rice, and is the staple food of around 35% of the world's population. The present wheat production is about 749 million tons (FAO, 2016; http://www.fao.org/faostat) and to feed the world's ever-growing population with annual growth rate of 2.6%, there will be a requirement to produce about 1040 million tons of wheat in 2020. To reach this target, it is crucial to keep the crop free from various biotic as well as abiotic stresses. In recent years, spot blotch caused by *Bipolaris sorokiniana* has emerged as a serious threat for wheat cultivation in warmer and humid regions of the world. It causes foliar spot blotch, root rot, black point on grains, head blight and seedling blight of wheat and barley. Estimates of yield losses due to spot blotch are reported to vary from 30-80% and can reach up to 100% under severe infection conditions.

In spite of several efforts world over, no wheat variety highly resistant to spot blotch has been released for field cultivation. One of the main reasons for this is that the molecular mechanism behind resistance to spot blotch has not yet been fully understood. In order to develop measures to control plant diseases, it is very important to understand not only the characteristic features of the pathogen, but also the molecular mechanism behind the disease progression. With this purpose, the thesis encompasses the following objectives:

Objectives of the study

1. To explore the mechanism of plant-pathogen interaction during spot blotch in susceptible and moderately resistant wheat varieties
2. To understand the mechanism of survival of *Bipolaris sorokiniana* on exposure to the fungicide propiconazole

Chapter 2: Isolation and characterization of *Bipolaris sorokiniana* isolates from different geographical regions of India

B. sorokiniana is a phytopathogenic fungus causing diseases in wheat, barley and other winter cereals. Previous studies involving large numbers of strains collected from around the globe suggest that *B. sorokiniana* exist as numerous forms of isolates

varying in virulence and aggressiveness with specific and nonspecific interactions. *B. sorokiniana* has high morphological as well as pathological variations. We collected or isolated 12 strains of *B. sorokiniana* from three different wheat growing geographical regions of India. During microscopic examinations, some cultures were found to be polysporic and hence needed to be purified. Thus, monoconidial cultures were established for seven sporulating isolates of *B. sorokiniana*. These cultures were characterized at morphological level as well as by sequencing the ITS region of the isolates and confirmed to be *B. sorokiniana*.

Like previous reports, our results also showed high morphological variability among the isolates. However, the morphological variation had no relationship with the geographical background. No correlation was observed between genetic similarity of the isolates and their geographical origin, concluding that the morphological characteristics expression is not conditioned solely by genes. Light microscopy and scanning electron microscopy of the spores showed several variations in conidial size, level of melanization and number of septa.

For evaluation of disease reaction, the following reported methods of pathogen inoculation were attempted: leaf painting, sterile seed inoculation and inoculation at Zadok's scale 12 stage. All these methods had some or other limitations for pathogenicity testing and hence another method, inoculation of germinated seeds, was developed. This method was found to be the best method for high throughput evaluation of pathogenicity as well as screening of germplasms. Our study showed that isolates from the same geographic region and morphological group could show differences in virulence levels.

Chapter 3: Exploring the molecular interaction of wheat-*Bipolaris sorokiniana* during spot blotch disease

Triticum dicoccum (emmer wheat) has superior organoleptic, therapeutic and nutritional qualities. However, dominance by high yielding hexaploid wheat varieties has restricted its cultivation to some niche areas in Europe and other regions including the peninsular India. *T. dicoccum* is resistant to various biotic stresses and rust diseases but highly susceptible to stripe rust and spot blotch. Spot blotch has become a major constraint in *T. dicoccum* cultivation in India. The hemibiotrophic disease cycle of this pathogen is observed only in the susceptible host. Interactive transcriptome sequencing is gaining importance in plant-pathogen interaction studies

and has enabled simultaneous analysis of expression of plant as well as pathogen genes. Similarly, next generation sequencing has enabled genome sequencing of organisms to a great extent. With the availability of the reference genome sequences from plants as well as pathogen, it has become much easier to align the reads from RNA-seq data and hence expression quantification. In order to explore the interaction, we performed global transcriptome analysis of spot blotch susceptible variety, DDK 1025 and a moderate resistant variety, Chirya 3 upon pathogen inoculation using Illumina HiSeq platform.

To understand the infection process and mechanism of disease progression, we performed differential gene expression analysis of spot blotch susceptible variety, DDK 1025 upon pathogen inoculation. A time series comparative study was performed to understand the biotrophic (1 dpi, days post inoculation), early necrotrophic (4 dpi) and necrotrophic phase (6 dpi) responses. The numbers of differentially expressed genes (DEGs) from three stages were 1810, 1562 and 2908 individually. GO annotations were obtained using Blast2GO for 75.63%, 70% and 73.89% of these DEGs respectively. GO enrichment was performed using agriGo online tool (http://bioinfo.cau.edu.cn/agriGO/analysis.php) using *Triticum aestivum* transcript ID v2.2 as the reference. Biological processes associated with carbohydrate metabolic process, response to abiotic stress, photosynthesis, cell death, regulation of gene expression, secondary metabolic process and generation of precursor metabolites were enriched. Under molecular function category carbohydrate binding, catalytic activity, enzyme regulator activity, protein binding and hydrolase activity was enriched. Although cellular component distribution showed all cellular parts including endoplasmic reticulum, plastid etc., extracellular region was profoundly enriched.

Since acceptable annotation of *T. aestivum* genome was not available, insights into functional annotation were achieved using blast against *Oryza sativa* japonica group using the STRING platform v10.5 (https://string-db.org/). Pfam enrichment was performed to gain comprehensive understanding about the gene families involved in the infection process. Pathways intricate to this interaction mechanism were explored by KEGG enrichment of DEGs using ClueGo (cytoscape plugin) using *O. sativa* blast hits. After several enrichments and annotations, major components involved in the interaction were recognized as glycolysis, phenylpropanoid biosynthesis, protein processing in endoplasmic reticulum, photosynthesis, glyoxylate and dicarboxylate metabolism, heat shock proteins, protein kinases and defense response genes like

chitinases and hydrolases. Down-regulation of several defense responsive genes in biotrophic phase suggests the contribution of effector mediated susceptibility. Glutathione metabolism mediated regulation of glycolysis and pentose phosphate pathway was identified. Differential expressions of multiple components of ubiquitin mediated proteolysis emphasize their role in hormone signal transduction during spot blotch. Of these DEGs, 177 genes were differentially expressed across all the three time points irrespective of the phases. Co-expression analysis using k-means clustering showed six patterns. Annotations showed that these genes had activities like chitin catabolic process, defense response to fungus, Bowman-Birk proteinase inhibitor and phenylalanine ammonia-lyase. Further information about the significance of these genes during interaction with the pathogen needs to be revealed using over/under expression experiments.

Likewise, to recognize the resistance phenomenon, we sequenced the transcriptome of spot blotch resistant (*T. aestivum*) hexaploid variety, Chirya 3 upon *B. sorokiniana* inoculation. Differential expression analysis was performed for three stages i.e. 1 dpi, 4 dpi and 6 dpi, which depicts the biotrophic, early necrotrophic and necrotrophic phases in the spot blotch susceptible variety. Our results showed that the number of upregulated genes was higher than downregulated genes. GO annotation was obtained for 64.38%, 66.6% and 64.25% genes from the DEGs. A higher number of genes were unannotated and were found to have significantly higher fold change expression. This suggests that these genes with unknown function could be novel defense responsive genes from wheat. Comparison of gene ontology enrichment showed that biological processes like photosynthesis and cell death were affected in susceptible variety but not in the resistant variety. Whereas, enhanced activity of extracellular proteinase inhibitors and peroxidases was observed in the resistant variety. Thus, early recognition and activation of defense pathways in resistant variety appears to hinder pathogen growth, survival and hence infection.

The results from transcriptome sequencing analyses demand confirmation using other complementary techniques like the quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), which is an indispensable tool for gene expression analyses. The most adopted method for relative quantification of gene expression in qRT-PCR is based on the DDCt method. However, accurate quantification by this method requires an appropriate internal reference gene with stable expression across all or most of the experimental tissues. Selecting an

appropriate internal reference gene is very important to elucidate the target gene expression reliably. Several housekeeping genes including *18S rRNA*, *ACTIN*, *GAPDH* and *EF-1α* have been proposed as standard reference genes for qRT-PCR studies. However, in case of plant-pathogen interaction analyses, selection of an appropriate reference gene is even more crucial due to the presence of RNA from both the plant as well as the pathogen in the infected tissues. As several of these genes are also present in the fungal pathogen genome, this could result in unintended cross amplification; which can cause improper quantification of the target genes. Hence, we aimed to identify a wheat gene with the most stable expression and unique primers, which would selectively amplify only the wheat gene and not the pathogen gene, providing accurate quantification of the target genes. Hence, we evaluated six previously reported genes with expression stability under different conditions using the wheat-*Bipolaris sorokiniana* system. We employed various statistical analysis methods, based on which, we identified two most stable genes, ubiquitin conjugation enzyme (*ULE*) and phytochelatin synthase (*PCS*) as the best reference genes for qRT-PCR based quantification in wheat pathosystem. We further confirmed the expression of several candidate defense genes in wheat using *ULE* as the reference gene. However, both the genes can be used either individually or together as internal reference genes.

Chapter 4: Global gene expression analysis of *Bipolaris sorokiniana* after exposure to propiconazole

Integrated disease management has been proposed to control the spot blotch disease. However, due to the unavailability of spot blotch resistant wheat varieties, the application of foliar fungicide is the most widely practiced measure. Propiconazole is a commonly used azole fungicide to manage the spot blotch disease in the field. However, due to its fungistatic mode of action, there is a possibility of emergence of fungicide resistant pathogen strains. Several mechanisms are reported for azole resistance in fungi. However, the strategies vary in different fungi. Resistance to the fungicide could be attributed to multiple molecular components in the fungus. Moreover, azoles have multiple modes of action out of which few are not explored yet. Global transcriptomics analysis of the pathogen after exposure to sub-lethal doses of the fungicide can reveal the mechanism of survival as well as the mode of action of the azoles in the fungi. Hence, a time series gene expression analysis was performed

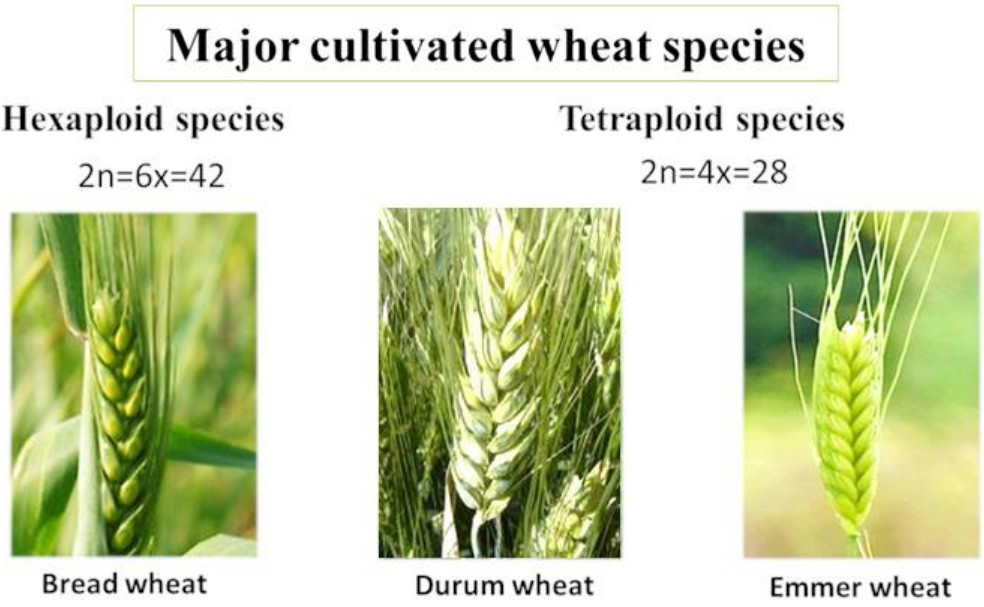
using RNA-seq. Transcriptome analysis using various tools showed overexpression of the target genes in the sterol biosynthesis pathway of the pathogen. In addition, this study also revealed altered expression of several metabolic pathways, transporters and stress regulators in the pathogen. The use of multiple analysis tools for transcriptomics analysis provided additional confidence on the observed results. The observed results were validated using qRT-PCR. We explored three strategies in *B. sorokiniana* against propiconazole stress: i) overexpression of target enzymes, ii) increased expression of transporter genes, and iii) expression modulation of stress responsive factors. This study revealed several novel putative targets such as ent-kaurene oxidase, ligninase lg6 precursor and spore germination protein. These genes help the fungi to overcome stresses and survive. Hence, the drugs targeting these genes can be developed, which are expected to impair the stress tolerance and hence survival of the pathogen. However, resistance is a polygenic phenomenon and to understand the functional contribution of each gene, knockout/knockdown studies are suggested.

Chapter 5: Conclusions and Future Prospects

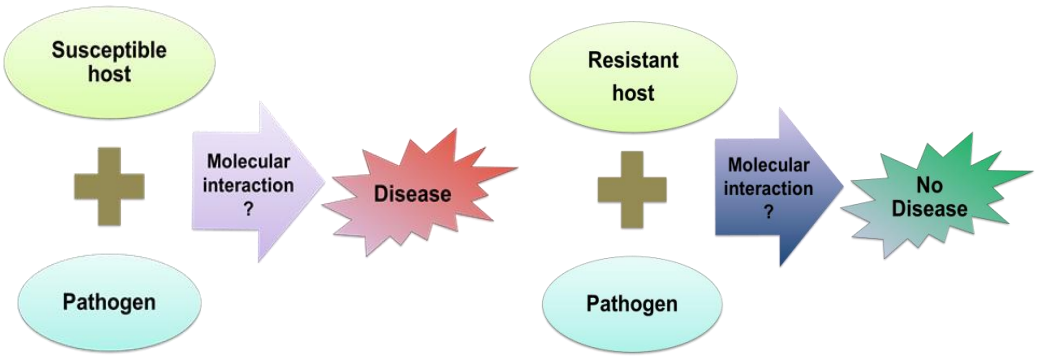
Spot blotch is an emerging disease causing yield losses of economically important cereals. The worldwide distribution of the causal agent, *B. sorokiniana* makes it of global concern. In the present study, we aimed to explore the mechanism of interaction of the pathogen with spot blotch susceptible and resistant wheat varieties. Initially, we established monoconidial cultures of seven isolates of *B. sorokiniana* collected from three different wheat growing regions of India and characterized them at morphological and molecular level. Although pathogenicity cannot directly be correlated with morphology on culture media, melanization level might be considered an important aspect in determining the level of virulence. Effector mediated downregulation of innate immunity and delayed response by plants leads to successful establishment of the pathogen in susceptible variety. We found that differential expression of ubiquitin mediated proteolysis played a key role in development of the disease in susceptible variety. On the contrary, proteinase inhibitors and peroxidase secretion led to effective elimination of pathogen in the resistant variety. Genotypes with higher expression of these genes are likely to provide improved resistance against the spot blotch disease. As integrated disease management is a sustainable

approach which also includes judicial use of fungicides to control the disease; we explored novel targets for developing efficient fungicides. However, essentiality of these genes for the pathogen survival needs to be confirmed through knock-out/down studies. Overall, this study helped in understanding the molecular paradigm of spot blotch disease in wheat. The outcome of this study will assist in advancement of controlling measures against spot blotch.

Chapter 1: Introduction



SPOT BLOTCH OF WHEAT



1.1 Wheat (*Triticum* spp.)

1.1.1 Background

Cereals like wheat, rice, and maize are agriculturally the most important crops being essential component of food all around the world. They together account for about 80% of total food grains production of the world. Wheat with ~13% protein content is a good source of vegetable protein in human food. Wheat is not only the staple food of nearly 35% population all over the world; it is also an important ingredient of processed foods (Paux et al., 2008). Wheat belongs to the grass family Poaceae, one of the largest families of Angiosperms. The detailed taxonomic classification of wheat is presented in **Table 1.1** (Key, 1954; Nakamura et al., 2009). Phylogenetic analysis revealed that wheat, rice, maize, and sorghum might have evolved from a common ancestor (**Figure 1.1**) (Zhao et al., 2013).

Table 1.1: Taxonomy of wheat

Kingdom	Plantae
Division	Magnoliophyta (Angiosperms)
Class	Liliopsida (Monocotyledon)
Subclass	Commelinidae
Order	Poales
Family	Poaceae
Subfamily	*Pooideae*
Tribe	*Triticeae*
Genus	*Triticum*

Cultivated wheat species are classified into three main groups on the basis of their genome complement: diploid (2*n*=2*x*=14), tetraploid (2*n*=4*x*=28) and hexaploid (2*n*=6*x*=42). The common or bread wheat (*Triticum aestivum*) is a hexaploid with AABBDD genomes and evolved through natural hybridization between domesticated tetraploid wheat (*T. turgidum*, AABB genomes) and diploid wild goat-grass (*Aegilops*

tauschii, DD genomes), followed by polyploidization of the hybrid (Salamini et al., 2002). The Einkorn wheat (single-grained wheat), *T. monococcum*, is considered as one of the most ancient cultivated species of wheat. The Emmer wheat (*T. dicoccum*), is also very ancient and the evidence for its early domestication has been found in the South-eastern Turkey, Jordan valley and the Damascus basin from about 10 thousand years ago (Ozkan et al., 2011). The durum wheat (*T. turgidum*) is prized for its high gluten content and widely used in preparation of pasta, bulgur, couscous, biscuits, and some bread flours. Durum is free threshable wheat, having little or no husk around each grain. By contrast, the ancient wheats, such as einkorn and emmer, have thick husks causing difficulty in extraction of edible parts of the grain, the endosperm and wheat germ.

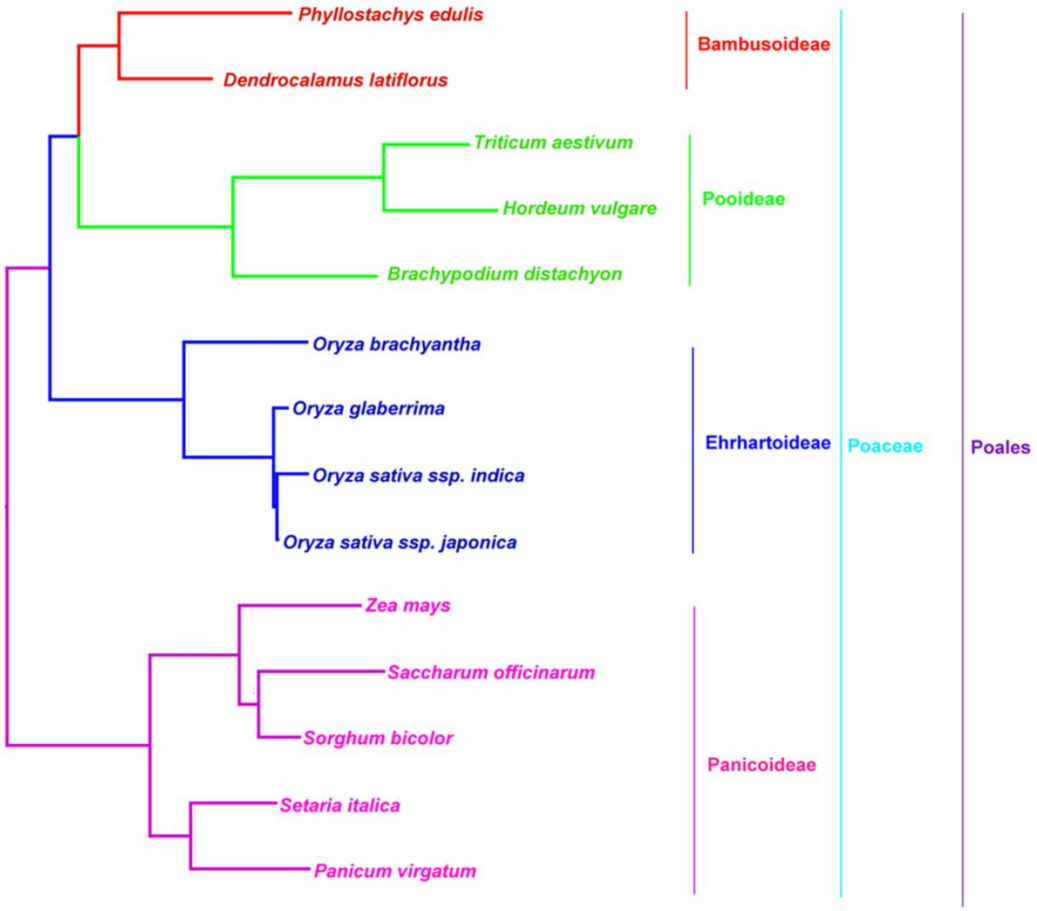


Figure 1.1 : Phylogenetic tree showing the evolutionary relationship between some of the major cereal grasses (Modified from (Zhao et al., 2013))

1.1.2 Nutritional importance of wheat

Wheat is considered as the primary source of the immediate energy because of its high carbohydrate content (75%). However, the nutritional value of wheat is much higher. Wheat contains significant amount of nutritional supplements including protein (13%) and dietary fibers (15%). Although wheat proteins lack the essential amino acids, a study revealed that wheat protein is better utilized by human body as compared to the milk protein (Millward et al., 2000). Its high dietary fiber content provides protection against cardiovascular diseases, type-2 diabetes, and certain forms of cancer. Beside these, wheat is a rich source of minerals like calcium, magnesium, phosphorus, and potassium. Several vitamins including niacin, folate, thiamin, riboflavin, vitamin B6, vitamin E, and vitamin K are also present in substantial concentration in wheat (https://ndb.nal.usda.gov/ndb/foods). Wheat occupies a central place for essential nutrients contribution in daily diet. Nutritional properties of different wheat genotypes vary widely in amount and composition, which is further influenced by the growth environment (Shewry and Hey, 2015). Wheat is consumed by all age groups in different forms and the most widely consumed form is bread.

1.1.3 Growth conditions required for wheat cultivation

Several factors affect wheat growth and productivity. Suitable temperature, rainfall, soil, and topography conditions provide optimum yield. The optimum temperature for wheat during growing season is 15-18°C. The weather should be warm and moist during the early stage of growth while sunny and dry during the seed maturation stages. For optimum vegetative growth of the crop, the average temperature should be less than 20°C. A frost-free period of 100 days is generally required; however, some early ripening varieties may mature only in 90 days. The rainfall required for wheat cultivation ranges between 30 and 100 cm. Wheat can also be grown in areas having lesser (25 cm) rainfall by adopting dry farming method. It is also cultivated in drylands where irrigation facilities are available. The soil suitable for wheat is either light clay or heavy loam. Wheat can grow in plain as well as in rolling topography, which provides adequate drainage and at the same time facilities of the use of machinery.

1.1.4 Wheat production

Wheat cultivation occupies more land area (220 million ha) than any other commercial food. The global annual production of wheat in 2016 was 734.6 million tonnes, making it the second most-produced cereal after maize. India ranks second in contribution (12.5%) to the world's total wheat production after China (http://www.fao.org/faostat/en/#data/QC) (**Figure 1.2**). It is one of the most routinely consumed cereals contributing 19% to the total daily calories intake. However, the demand for wheat is ever increasing with increasing human population. In addition, food industry also demands wheat because of its high gluten content, which allows the processing of wheat to produce bread, other baked goods, noodles, pasta, and a range of other functional ingredients. With the improved baking systems and industrialization, these processed foods are more convenient to produce, store, and consume. This is probably the major cause of increasing demand of wheat in new markets beyond the climatically adopted regions of wheat. As a result, wheat dominates the world trade compared to all other crops. The largest exporters of wheat in 2016 were European Union (33 million metric tonnes), followed by Russia (24.5 million metric tonnes), Canada (22.5 million tonnes), and United States (21.2 million metric tonnes). Asian countries are the major importers of wheat contributing to 40.9% of the global wheat import. Among these, Egypt, Indonesia, and Algeria are the top wheat importer countries.

1.1.5 Factors affecting wheat production

Wheat production is affected by multiple factors including grain yield, genotype quality, tolerance to abiotic stresses, including mineral, humidity, and thermal tolerance and various biotic stresses like diseases and insect feeding. Severe changes in climatic conditions pose a significant challenge for obtaining grain with consistent quality in the near future due to the complex interactions between atmospheric CO_2, increasing temperature, and changing rainfall patterns on yield and quality. The global wheat production is estimated to fall by 6% for each degree Celsius of rise and it would become more variable in due course of time (Asseng et al., 2014). Too short supply of food is anticipated by 2030 due to potential impacts of climate change on crop yield under the current scenario of population growth (Ritchie et al., 2018).

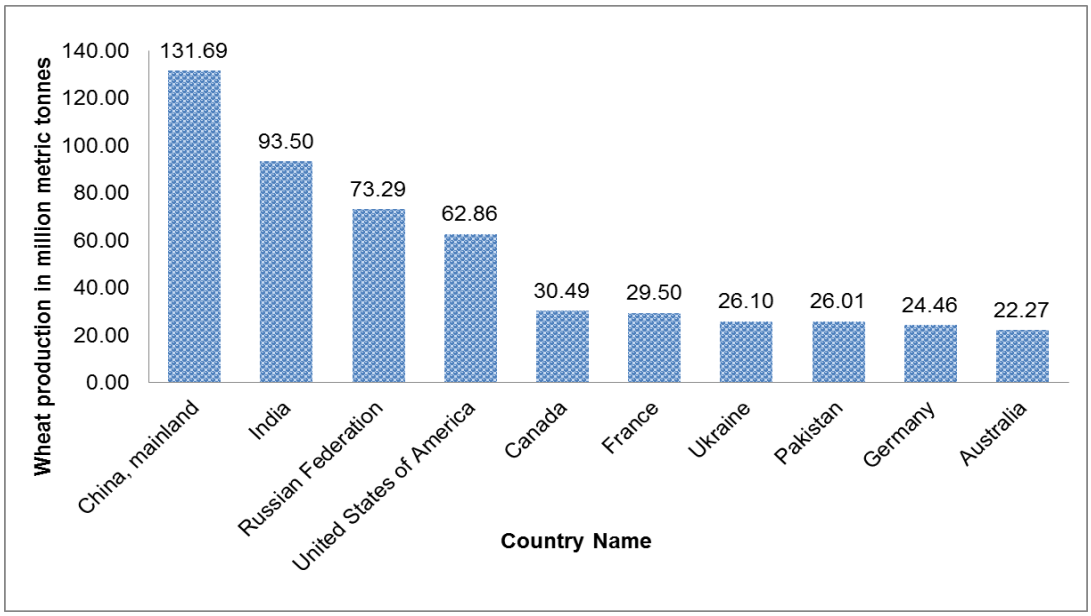


Figure 1.2: Top 10 wheat producing countries and their contribution in global wheat production in 2016 (http://www.fao.org/faostat)

Grain protein concentration and composition are important characteristics for nutritional and end-use properties of wheat affecting bread making process and quality of the product (Blumenthal et al., 1993; Jenner, 1994; Fernando et al., 2012). Physical and nutritional properties of grains are also influenced by climatic variations. The post-anthesis environment such as water availability and temperature strongly influences seed size (Wardlaw and Wrigley, 1994; Guttieri et al., 2001). Atmospheric CO_2 concentration and high temperature during the grain filling phase affects starch and protein deposition and their functional properties. Grain quality is also regulated by genetics, field management, and environmental conditions. There is strong genetic control over kernel characteristics such as shape, germ tissue, thickness of bran, and crease. Continuous breeding results in narrow genetic diversity of the elite wheat germplasm pool and reduced adaptability to abiotic stresses (Reif et al., 2005) and increased incidences of biotic stresses.

The global population is expected to rise by 2.3 billion people by 2050 from the present levels (7.3 billion). Furthermore, the demand for cereals, for both food and animal feed uses is projected to reach ~3 billion tonnes by 2050. According to the UN Food and Agricultural Organization (FAO), this means that feeding a world

population of 9.1 billion people in 2050 would require almost doubling the overall food production from the present levels (http://cordis.europa.eu/news/rcn/124823_en.html).

Wheat production is influenced by several biotic and abiotic factors. To achieve the targeted production, it is essential to manage the biotic stresses affecting wheat (Mehta, 2014). There are several wheat diseases, mainly caused by fungi, bacteria and viruses. The major fungal diseases in temperate environments include eyespot, Stagonospora nodorum blotch (also known as glume blotch), spot blotch, yellow or stripe rust, powdery mildew, Septoria tritici blotch (sometimes known as leaf blotch), brown or leaf rust, Fusarium head blight, tan spot, and stem rust.

1.2 *Bipolaris sorokiniana*

B. sorokiniana is a pervasive plant pathogen of grass family including cereals. Its presence is reported from all the cereal growing areas, giving it a world-wide distribution (**Figure 1.3**). The pathogen forms a continuous genetic pool of isolates differing in virulence and aggressiveness to various cereals and grasses. However, the mechanism behind this variation is poorly understood. *B. sorokiniana* is an anamorphic stage of *Cochliobolus sativus* (teleomorph). The sexual stage is very uncommon, thus the genetic variation might be due to heterokaryosis and parasexuality mechanisms.

Figure 1.3: Geographical distribution of *B. sorokiniana* on world map (Adapted from Gupta et al. (2018))

1.2.1 Taxonomic nomenclature

The taxonomic nomenclature of *B. sorokiniana* was in discussions for long. Earlier, the fungus was named as *Helminthosporium sativus*. The name *Helminthosporium sativum* (Pammel et al., 1910) was given to it without considering the earlier description of *H. sorokinianum* Sacco in Sorokin, Trans. Soc. Nat. Univ. Kazan 22:15 (1890) (Sivanesan, 1987). Shoemaker (1959) proposed the generic name *Bipolaris* for the *Helminthosporium* species with fusoid, straight or curved conidia showing two germination tubes i.e. one from each end/pole of spore. Thus, the pathogen was renamed as *Bipolaris sorokiniana* (Sacc.) Shoem. The fungus is differentiated from other members of the *Bipolaris* genus on the basis of morphological features of conidiophores and conidiospores.

In taxonomy, *B. sorokiniana* is classified as shown in **Table 1.2**. The synonyms include *H. acrothecioides* Lindfors, *H. californicum* Mackie & Paxton, *Drechslera sorokiniana* (Sacc.) Subram. & Jain, *B. californica*, *H. acrothicoides*, *H. sativum*, *H. sorokinianum* and *Ophliobolus sativus*.

Table 1.2: Scientific classification of *Bipolaris sorokiniana*

Kingdom	Fungi
Phylum	Ascomycota
Subphylum	Pezizomycotina
Class	*Dothideomycetes*
Order	*Pleosporales*
Family	*Pleosporaceae*
Genus	*Bipolaris*
Species	*Sorokiniana*

Although there is a convention to use a single nomenclature to maintain the uniformity in scientific world, the International Code of Botanical Nomenclature permits pleomorphic organisms to be given two names (Hawksworth, 2011). Numerous fungi are reported to show pleomorphism. Several times fungal isolates lose their capacity to switch to teleomorph and hence their anamorph stage remains prevailing (Gehlot et al., 2010). Several *Bipolaris* species have anamorph as dominant

stage and hence they are not linked with the genus *Cochliobolus* because of their inability to produce the sexual state. However, many *Cochliobolus* species also have their asexual states and consequently are assigned synonyms in either *Bipolaris* or *Curvularia*. The molecular analysis of different species of *Bipolaris* and *Curvularia* revealed that none was monophyletic (Berbee et al., 1999).

1.2.2 Morphology

B. sorokiniana shows high morphological and physiological variability. Various isolates have been evaluated for their morphological variability, considering mycelium color, sector formation, and growth rate. Some common features observed on culture plates are:

1. Mycelium is olive brown and produces light grey colonies on to PDA medium which later turn black to olivaceous black.
2. Conidiophores are 6-10 µm × 110 – 120 µm, brown, erect, un-branched, single or clustered.
3. Conidia are 1 –28 µm × 40 – 120 µm straight olive brown to dark brown.
4. Conidia are oval shaped covered with smooth walls thickened at septa.
5. Conidia are 4-7 septate.

1.2.3 Life cycle

B. sorokiniana being an anamorph reproduces by asexual cycle (**Figure 1.4**). Anastomosis between adjacent heterogenetic hyphae causes heterokaryosis and allows the pathogen to follow the parasexual cycle during asexual reproduction. This is considered as the primary source of genetic variability in anamorphic fungi (Day, 1974). This genetic diversity offers the evolution of highly virulent strains of phytopathogenic fungi to overcome host resistance (Guseva et al., 1979).

1.2.4 Disease cycle

B. sorokiniana has a wide host range (Iftikhar et al., 2009). It reproduces asexually to produce spores. The fungus is usually a saprophyte and survives primarily as thick walled conidia. However, the pathogenic strains penetrate both externally as conidia and internally as mycelium in the seeds. As the anamorph stage is sporulating and pathogenic, the sexual stage is not required for completion of the disease cycle.

Primary inoculum includes mycelium from infected seed, spores in the soil and on the kernel surface that transmits conidia via physical contact or rain splashes (Murray et al., 1998) (**Figure 1.5**).

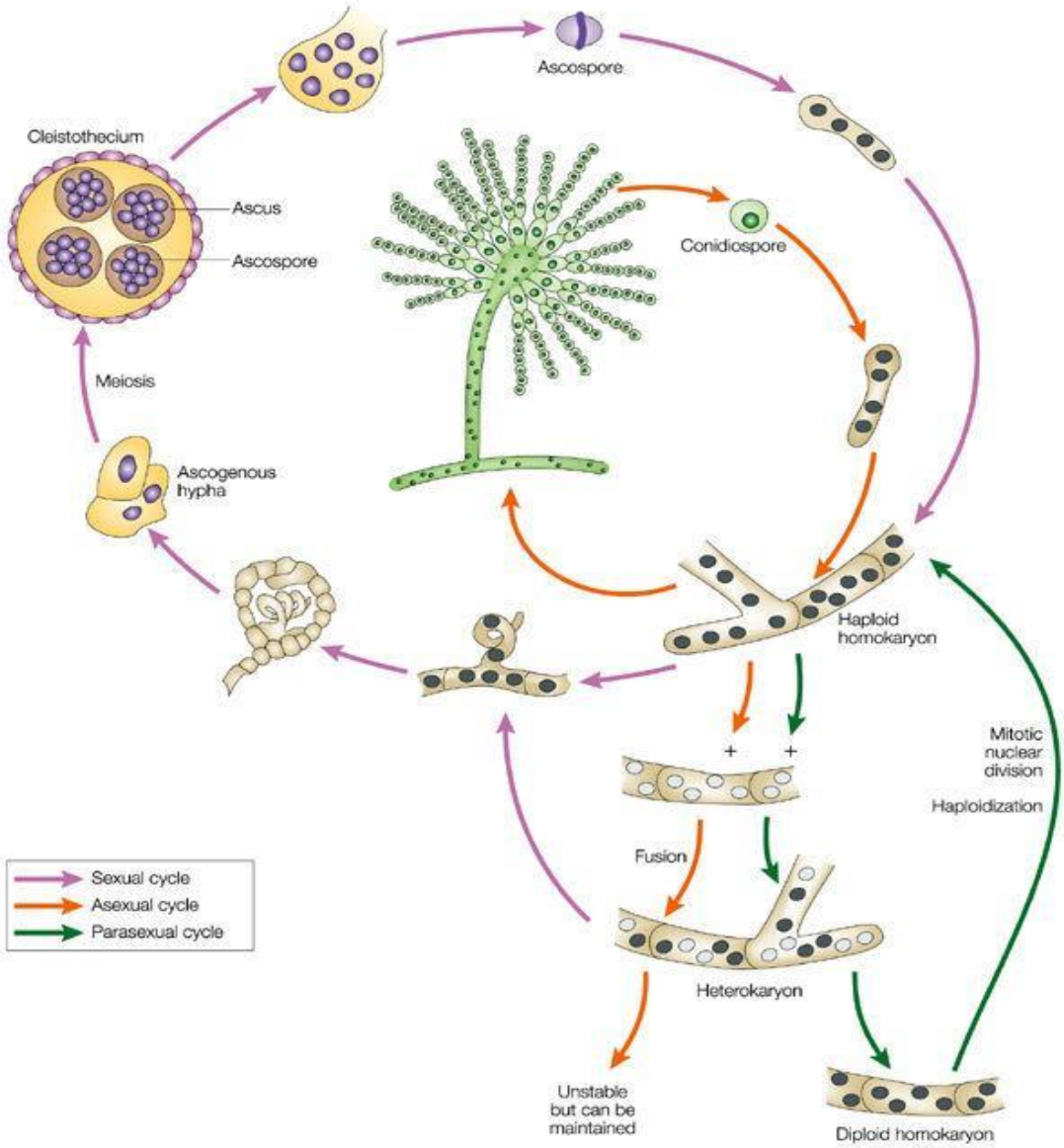


Figure 1.4: Life cycle of pleomorphic fungi, *B. sorokiniana*
(Adapted from Casselton and Zolan (2002))

In a virulent strain, about 90% of the conidia germinate and start to grow, forming hyphae within 3 h of favorable conditions. In the presence of susceptible hosts, primary infection is initiated on the coleoptile or primary roots. The germ tubes

and hyphae are surrounded by extra cellular matrix (ECM). The ECM provides beneficial environment for the growth and pathogenicity of the fungus by helping in adhesion to plants. The ECM also contains fungal toxins, which provide protection from cuticle degrading enzymes of plant origin. The infections on the leaves usually start through natural wounding, open stomata by infection hyphae or involves an appresorium-like structure through the cell wall attributing to the biotrophic life style.

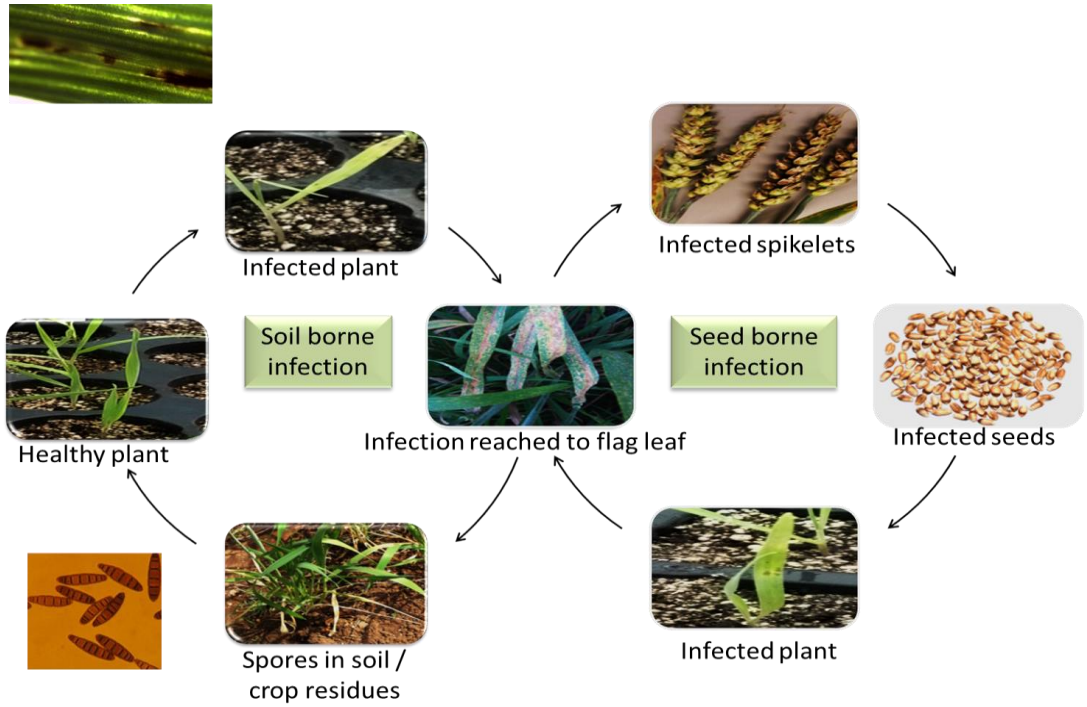


Figure 1.5: Disease cycle of *B. sorokiniana*. *B. sorokiniana* causes blotches on leaves (Centre). The spores survive on plant debris, soil and infected seeds, which are the primary sources of infection. The conidia (bottom left) are usually present in soil and infect healthy plants during watering or rain. Conidia also spread via infected seeds (centre right).

Virulence of the pathogen is reported to be correlated with the level of the toxin produced (Jahani et al., 2012). Different species of *Bipolaris* produce a variety of phytotoxic compounds, which include prehelminthosporol, helminthosporol, helminthosporic acid, and sorokinianin (Carlson et al., 1991) (**Figure 1.6**). Prehelminthosporol is the most abundant toxic compound which weakens and kills plant cells. It is also the precursor for Helminthosporol and helminthosporic acid.

These compounds are plant growth inhibitors and known to inhibit seed germination or hypocotyl/radicle growth (Qader et al., 2017). Sorokinianin also exhibits inhibitory effect on germination of conidia (Nakajima et al., 1994). Bipolaroxin produces yellow or necrotic lesions when put on the leaf of a wheat plant (Jahani et al., 2013).

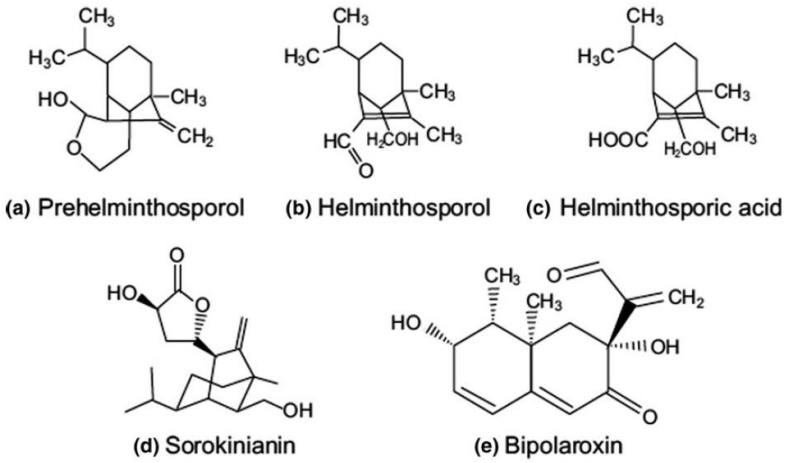


Figure 1.6: Chemical structures of the major toxins produced by *B. sorokiniana* (Adapted from Gupta et al. (2018))

B. sorokiniana can grow on all plant parts of winter cereals. The fungal disease has two distinct stages: first is the photosynthetic interference, when the aerial parts are infected and second is the interruption in water and nutrients absorption while infecting the roots (Minotto et al., 2014). It penetrates the host tissue either directly through the epidermis or through natural openings or wounds. Along the germination of diseased seeds, the perennating organs of the pathogen become active which is the starting point of the disease. Under favorable conditions, the fungus completes germination in 4 h, then appresoria form at the juncture of epidermal cell wall after 8 h prior to penetration. Infection pegs form beneath the appressoria and infection cushions. Hyphae from initial infected cells enter adjacent cells in 24 h which results in the granulation of host growing area. It shows that plants are under the pressure of the disease. Infection continues from the cortex and endodermis, causing physiological damages of the host tissue. Conidia from the infected plant parts spread on the rest of the plant and help in further fungal colonization and disease progression to the rest of the plant. Any movement in the soil due to water and

implements can move inoculum of the pathogen to the adjacent plants. However, infected seed can also serve as a means of dissemination of the fungi over long distances (Garcia and Mathre, 1987). On the basis of growth and statistical analysis, Minotto et al. (2014) concluded that polysporic isolates are more virulent (> 60%) as compared to monosporic isolates (43%) for all plant parts except coleoptiles.

Conducive environmental conditions also increase severity of the disease. Warm soil temperatures and humidity favor the growth of the pathogen. Although the disease incidences were reported to occur between 15-35°C, the optimum soil temperature for infection is 28-32°C. Watering during planting promotes the infection and colonization by soil borne inoculum due to increased humidity. Maximum symptoms appear when the leaves remain wet for more than 18 h with temperature above 18°C. The pathogen can complete the asexual reproduction and disease cycle without a secondary host. The spores surviving in soil or remnants of crop provide inoculum source for subsequent crops (Murray et al., 1998).

B. sorokiniana causes foliar spot blotch, root rot, black point on grains, head blight, and seedling blight of wheat and barley. It is a hemibiotrophic fungus having both biotrophic and necrotrophic phases. The biotrophic growth phase is confined to a single epidermal host cell that is invaded by a network of infected hyphae, while the necrotrophic growth phase is characterized by invasion of the mesophyll tissue and host cell death (Kumar et al., 2002).

1.2.5 Common root rot

Common root rot is one of the most widespread and persistent diseases of wheat in the western Canada (Harding, 1972) and Great Plains of North America (Stack, 1994). *B. sorokiniana* is the most important and dominating pathogen among other common root rot causing fungi in wheat all over the world (Fedel-Moen and Harris, 1987; Dubin and Bimb, 1994). All parts of the wheat plant can be attacked by the disease. Initially, dark necrotic lesions are seen at nodes. The classic symptom is a dark lesion on the sub-crown internode, which can reach up to the crown or even up to the lower internodes of the stems during severe infections (**Figure 1.7**). Plants with severe sub-crown internode symptoms have reduced root growth, especially of crown roots (Smiley et al., 2005). The disease can cause thinner crop stands, reduced numbers of fruiting tillers, fewer kernels, and low kernel weights (Machacek, 1943;

Verma et al., 1976; Duczek et al., 1985). Varied levels of susceptibility to common root rot exists in commercial wheat cultivars (Chaurasia et al., 1999). Yield loss of up to 35.3% has been reported in wheat due to the common root rot (Machacek, 1943). In Australia, percentage loss in yield ranged from 13.9 to 23.9% in a susceptible cultivar and 6.8 to 13.6% in a partially resistant cultivar (Wildermuth et al., 1992). The average yield loss reported due to common root rot is about 14% in South Asia (Saari, 1998). The disease is common and problematic in even in tropical environments (Mergoum et al., 1997). For disease management, strategies like removal of stubble, tillage, crop rotation, and seed treatment have been recommended. However, the use of resistant varieties is the only effective and durable tool for sustainable wheat production.

Figure 1.7: Common root rot caused by *B. sorokiniana* in wheat
(Adapted from Kumar et al. (2002))

1.3 Spot blotch

Spot blotch, also known as Helminthosporium leaf blight, is an emerging wheat disease of growing concern especially in developing countries. The disease affects all plant parts of wheat and can cause up to 100% yield loss (Mehta, 1998). Currently, the disease has spread across almost all the wheat growing regions of the world (**Figure 1.8**). The areas affected by the disease are the non-traditional, subtropical lowland wheat growing areas in the Andean region of Latin America, including Bolivia and the warmer regions of Brazil, northeast Argentina, and Paraguay. In Africa, Tanzania and the rain-fed wheat growing areas of Zambia and Madagascar also favors the disease. In the Indian subcontinent, the warmer parts of eastern India,

Bangladesh, and in the terai region of Nepal, similar environmental conditions support the disease spread. Similar growing conditions are found in Southeast Asia, most small wheat growing areas in Thailand, the Philippines and Indonesia, and the high rainfall and warm wheat growing areas of southern China. Thus, spot blotch is now not only located in the earlier, narrowly defined non-traditional "tropical" wheat growing areas (Mega-environment, ME5), but has spread into the large, often adjacent areas of cooler, traditional irrigated rice-wheat production areas (ME1) with moderate climates.

Figure 1.8: Global occurrence of spot blotch disease caused by *B. sorokiniana* (https://www.plantwise.org/KnowledgeBank)

1.3.1 Symptoms

B. sorokiniana is a hemi-biotrophic pathogen and can infect all plant parts. It produces sesquiterpenoid toxins causing lesions on leaves. Early lesions on leaf are characterized by small, dark brown tiny spots without chlorotic margin and 1-2 mm size scattered throughout the leaves. Subsequently, these small lesions merge and form large areas of necrosis during disease development. In susceptible genotypes, these lesions extend very quickly in oval to elongated blotches, light brown to dark brown in color. They may reach several centimeters before coalescing and inducing the death of the leaf (**Figure 1.9**). During this late necrotrophic phase, the pathogen derives nutrients from dead host tissues, which enables pathogen growth and disease

progression. Fruiting structures develop readily under humid conditions and are generally easily observed on old lesions. If infection reaches to flag leaves spikelets are also affected. It results in shriveled grain and black point, a dark staining of the embryo end of the seed (Sharma and Dubin, 1996).

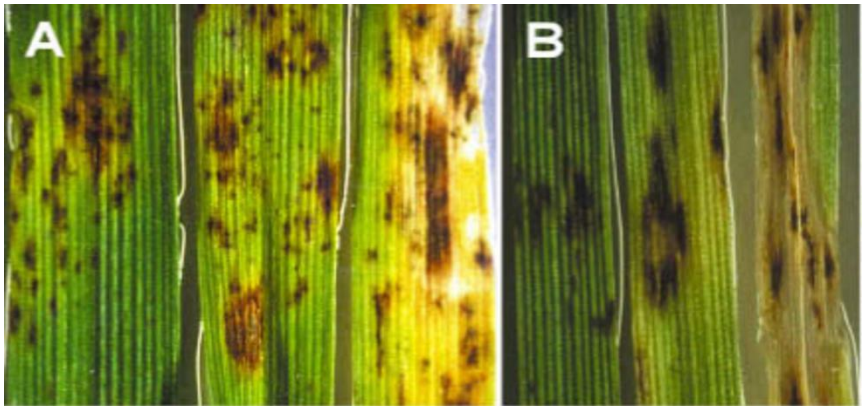


Figure 1.9: Spot blotch on primary leaves of barley and wheat. Necrotic lesions are light colored on barley (A), compared to darker lesions on wheat (B). Barley leaves develop more chlorosis as compared to wheat. (Adapted from (Kumar et al., 2002))

Eleven plant species including Poaceae and other plants have been confirmed as the hosts of this pathogen (Iftikhar et al., 2009). More than 29 species of Poaceae and other families from North-eastern China, 65 species of Poaceae from Yellow and Hai river region and 17 plant species from Guandong province were already reported to serve as the hosts of *B. sorokiniana* (Naitao and Yousan, 1998).

1.3.2 Economics of spot blotch

Spot blotch is a foliar disease and affects the plant health severely. Estimates of yield losses in wheat due to spot blotch are reported to vary from 15.5 to 19.6% (Dubin and Ginkel, 1991), 20 to 80% (Duveiller and Gilchrist, 1993), and may reach up to 100% under severe infection conditions (Mehta, 1998). Losses of up to 85% in Zambia (Raemaekers, 1988) and 40% in Philippines (Lapis, 1985) were reported. Hetzler et al. (1991) observed estimated yield loss to be up to 87% in highly susceptible varieties due to *B. sorokiniana*. In Australia up to 24% yield losses have been pronounced from susceptible varieties (Wildermuth et al., 1992). Fungicide protected plots provided 43% higher yield than diseased plots in Mexico (Villareal et al., 1995).

In South East Asia and Latin American countries, nearly 12 million hectares of lands under cultivation were under disease influence causing major constraint to wheat production (Nagarajan and Kumar, 1998). Spot blotch was reported to cause 15% (under natural conditions) and 23% (artificial epiphytotic condition) grain yield reduction in recommended varieties in Bangladesh (Alam et al., 1998; Saari, 1998). Wheat production is severely affected in China due to soil borne diseases and *B. sorokiniana* is the major pathogen in 80% of the cases. In Bolivia, depending on planting date, wheat varieties and climatic conditions, up to 57% yield losses have been observed (Toledo and Guzman, 1998). Wheat production is severely affected even in Paraguay due to spot blotch causing yield loss of up to 70% (including some other constraints). Yield loss was reported to vary in different locations for different varieties in Nepal. Disease incidence ranged from 40-90% causing yield losses up to 15.2% (Shrestha et al., 1998). The pathogen causes grain yield losses up to 10, 15 and 20% through common root rot and seedling blight in Scotland, Canada, and Brazil respectively. Yield loss from spot blotch ranged from 40-85% in Brazil and other non-traditional wheat growing areas (Murray et al., 2008).

1.3.3 Spot blotch in India

Total wheat production has increased in India with the introduction of high yielding varieties after green revolution. Being the staple food crop of majority of the population, both the policy makers and scientists have given high importance to wheat. As a result, both wheat productivity and production have increased by several folds since mid-1960s. However, the wheat yields vary widely from region to region. Punjab and Haryana are highly productive zones in the Indo-gangetic plains contributing about 69% of the total food harvest in the country because of which this region is called as the "food bowl of India" (Singh and Sidhu, 2014). However, several problems have cropped up in the region in the last four decades, threatening the sustainability of the system. Other than climatic constraints, spot blotch adversely affects wheat production, particularly under late sown conditions (Singh et al., 1998). Although spot blotch of wheat was noticed in India since 1924 (Kulkarni, 1924), the disease was not severe until recent. In India, the disease is reported to cause an annual yield loss of about 15.5% on an average (Saari, 1998).

The disease has prevailed after green revolution with the integration of rice-wheat cropping system in Indian agriculture. The best time for sowing of wheat in

India is mid-November. However, the sowing is delayed to mid-December because of delay in rice harvest in rice-wheat rotation cropping system. Crop cultivation coincides with optimum environmental condition for pathogen infection. Consequently, major yield losses are observed. *B. sorokiniana* is the predominant pathogen of foliar blight disease in the Indian states of Bihar, Delhi, Gujarat, Haryana, Karnataka, Maharashtra, Rajasthan, Uttar Pradesh, West Bengal and in the neighboring countries of Bangladesh and Nepal (Singh et al., 1998).

1.3.4 Disease management

Some of the commonly employed approaches for spot blotch control include an appropriate cropping system, soil solarization, use of fungicides, biocontrol agents, and use of resistant varieties, which are discussed below.

1.3.4.1 Soil solarization

Soil solarization is the solar heating of moistened soil by mulching with clear polyethylene sheets to trap solar energy and decontaminate the soil. It is an environment friendly method to control soil-borne plant pathogens, insects as well as weeds. Solarization causes physical, chemical, and biological changes in soil by affecting both biotic and abiotic processes and determines the micro-environment for plant growth. After soil solarization, improved plant growth was observed even in the presence of known pathogens in soil (Stapleton et al., 1985; DeVay and Katan, 1991). An increase in grain yield of 10-36% in protected and 5-25% in unprotected field was observed in Nepal (Ruckstuhl, 1998).

1.3.4.2 Chemical control

Disease control of black point in wheat grain, caused by *B. sorokiniana*, was successfully achieved using iprodione (an imidazole derivative), guazatine (guanidine), or triadimenol (triazole), antifungal agents for seed treatments. Foliar applications of systemic fungicides during heading and grain filling stages, is also effective for disease control. However, multiple applications of fungicide is required for grain yield increase under severe cases (de Viedma and KohlF, 1997). However, highly susceptible varieties may not respond to the fungicide application, resulting in major yield losses.

1.3.4.3 Cultural practices

Integrated wheat-rice cropping system increases the chances of spot blotch occurrence. Harvesting rice at appropriate time and tillage practices can provide a better control of the disease. As spot blotch is seed and soil-borne disease, the best way to control it is elimination of the sources of inoculum. Selection of disease free seeds for cultivation is an important measure for effective control. Removal of grass weeds and other secondary hosts and clearing the stubble reduces inoculum from field (Diehl et al., 1982). Use of seed lots with black point on more than 10% grains should be avoided to limit spot blotch incidence (Bhatta et al., 1998). Seeds are the main source of pathogen inoculum for the reappearance of spot blotch of wheat in crop rotation system (Pandey et al., 2005). Application of fertilizers containing essential macro and micro nutrients in balanced amount not only increases grain quality, but also reduces disease occurrence. Macro and micronutrients benefit plants to tolerate stress induced by pathogen attack. Potassium regulates metabolism of plants and prevents the establishment of pathogen (Reuveni and Reuveni, 1998). Sharma and Dietz (2006) showed that the appropriate application of macronutrients (nitrogen, phosphorous, and potassium) reduced the disease severity by 15%. Nutrient availability offers advantage to plant growth and hinders the pathogen endurance (Singh, 2015).

1.3.4.4 Crop rotations

Crop rotation is advantageous for yield improvement without application of external nutrients. It promotes plant growth by enrichment of beneficial microorganisms in soil. As *B. sorokiniana* is a major pathogen of grass family plants, crop rotation with non-host crops along with fungicidal application leads to pathogen eradication (Bailey and Duczek, 1996). However, *B. sorokiniana* exhibits a wide host range and can survive in secondary hosts too. Therefore, selection of non-suitable host crop is a challenging task. Crop rotation with legume crops like rape, soybean, cowpea, and chickpea can reduce the inoculum load, restricting the pathogen in the subsequent crop and enhancing its productivity. Cultivation with non-gramineae crops for three years, followed by wheat showed reduced spot blotch incidence (Reis et al., 1997). However, selection of suitable non-host crop requires further research.

1.3.4.5 Use of resistant varieties

Some Chinese wheat genotypes like SW895422, Chirya 1, Chirya 3, Chirya 7, NL781, and NL785 showed significant resistance to spot blotch. Thereafter, various other sources of spot blotch resistance from different countries were reported *viz*. Latin America (e.g. BH1146, CNT1), China (e.g., Shanghai, Suzhoe, and Yangmai), South Asia (Gautam), few Brazilian and Zambian wheat lines, and wild relatives of wheat or alien species (e.g., *Aegilops squarrosa*, *Thinopyrum curvifolium*) (Van Ginkel and Rajaram, 1998). In a study conducted in China, out of 9,400 evaluated wheat cultivars, only 29% showed some level of spot blotch resistance (Naitao and Yousan, 1998). The low heritability of resistance in cultivars, environmental changes, and unknown virulence components from the pathogen make breeding difficult for the resistance character. Hence, conventional breeding of spot blotch resistant genotypes of wheat has not achieved significant progress in past years (Sharma et al., 2007). Shuttle breeding and multi-location screening for different germplasms have been tried for identification of spot blotch resistant lines. Based on nursery trials at different locations in the Indian subcontinent, CHUKUI#1 (GID 6178575) and VAYI#1 (GID 6279248) lines were proposed as promising cultivars/donors for spot blotch resistance. However, confirmation of disease resistance under varying environmental conditions is essential for release of these lines as resistant varieties (Singh et al., 2015).

1.3.4.6 Use of biocontrol agents

Limited number of chemical control agents against the fungal pathogen has engrossed the researchers towards identification of biocontrol agents against the pathogen. *Streptomyces hygroscopicus* provided protection against leaf spot in barley (Hodges et al., 1993). Application of *Stenotrophomonas maltophilia* suspension hampers conidial germination and hyphal growth of *B. sorokiniana* (Zhang and Yuen, 1999). However, it is reported as a human pathogen and hence not suitable for agricultural application. Induction of systemic resistance in forage grass by *Lysobacter enzymogenes* is effective to control *B. sorokiniana* (Kilic-Ekici and Yuen, 2004). However, more experimentation is required to check the efficacy in wheat system. Common root rot of wheat has been successfully controlled by soil application of *Trichoderma* species. Additionally, *Trichoderma* serves as a plant growth promoting organism (Knudsen et al., 1995).

1.3.4.7 Integrated Disease Management

Integrated disease management (IDM) system was proposed for controlling spot blotch under field conditions (**Figure 1.10**). However, there are many constraints in filed implication of the proposed IDM. Major constraints in IDM can be categorized into political, technological, and socio-economic constraints. Few of the constraints to IDM are as: 1) insufficient resources and funding for research and agriculture advancement; 2) defective transfer of technology; 3) inadequate extension services; 4) inadequate biological monitoring systems; 5) IDM strategies based on commodity and not usually on production systems; 6) Unawareness about IDM; 7) lack of international collaboration on crop protection or quarantine problems; 8) interaction gap between government research organizations, cooperatives, industries, and farmers; 9) expensive IDM approaches; and 10) lack of interdisciplinary incorporation (Mehta, 1998).

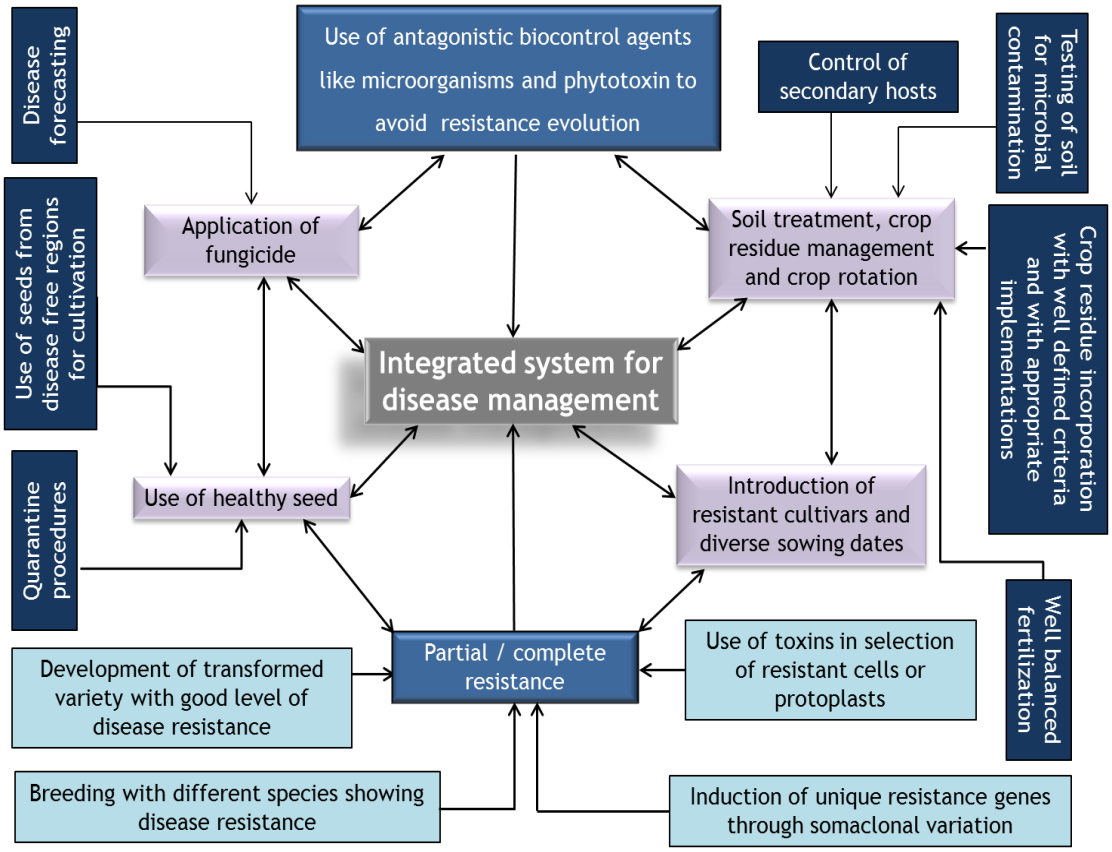


Figure 1.10: Potential tools for integrated management of spot blotch disease of wheat (Modified from (Mehta, 1998))

1.4 Plant-pathogen interactions

Disease development and progression is a multifaceted process involving virulence factor from pathogen and immune trigger by plants in response. Interface of these two factors plays an important role in susceptibility, a condition where pathogen overcomes the immune response and establishes itself, or resistance, when pathogen cannot evade the hostile environment and its growth is restricted. Susceptibility or resistance is attributed to interaction of multiple biomolecules from both the plant as well as the pathogen. Disease is a strictly regulated and highly specific phenomenon, restricting host diversity of the pathogen. The pathogen may utilize the nutrients only from living host cells without causing cell death, known as biotrophic pathogen. Resistance against such pathogen can be achieved by hypersensitive reaction causing the pathogen to strive for nutrient supply. Some pathogens secrete toxic molecules causing cell death and utilize the host cell supplements for own growth, referred as necrotroph. The pathogenic characteristics of *B. sorokiniana* differ from the above two mechanisms. It follows hemibiotrophic lifestyle, initially it grows on the living host cells and after sufficient colonization, kills the host cells and proliferates on necrotic tissue. This complex infection process makes the understanding of the interaction very difficult.

The role of a wide variety of biomolecules, *viz.* phytohormones and signaling (Sahu et al., 2016), phenolics (Eisa et al., 2013), transcription factors (Dong et al., 2010) and lignin (Yusuf et al., 2016) have been explored in separate studies. A comprehensive analysis is further required for insights into their significance. The *ToxA* gene from *B. sorokiniana* is suspected to cause the disease by triggering effector mediated susceptibility (McDonald et al., 2018), but the results are still obscure. Effectors are the products of pathogen virulence or avirulence genes, targeting specific gene product from the host. Molecular interaction studies are important for recognition of such gene-for-gene relationship between the host and the pathogen. Numerous techniques have been evolved to study these biomolecules with the realization that every category of biomolecules has its own contribution and their functions must be explored. With the advancement in knowledge and techniques, the prospects have shifted from exploring a single molecule to the entire pool. The "Omics" approaches, which may include the genomics, transcriptomics, proteomics, and metabolomics approaches, with the high throughput technologies, have commendatory potential for exploring such complex interaction mechanisms.

1.4.1 Genomics

Genomics is the study of the entire DNA constitution of an organism. Genomic composition of an organism remains the same at any growth stage of life. In contrast to genetics, which refers to study of a particular gene or character, genomics encompasses the overall characterization of structure, function, mapping and evolution of the whole genome including coding (gene) and non-coding (structural components) regions. Besides this, genomics can be applied to study the intra-genomic interactions like epistasis, heterosis, pleiotropy, and epigenetics etc. Before the genomics era, it was difficult to understand the differences in virulence for different isolates due to the inability to explore the pathogenicity associated characters at genomic level. The pathogens have comparatively smaller genomes than the host hence sequencing their genomes is easier and cheaper. The study of a pathogen's genome is referred as pathogenomics, which involves the utilization of genome sequencing data to understand the molecular diversity in the pathogen at DNA/gene level. Comparison of data from different isolates or strains can reveal the similarities and differences in their morphological and functional characters.

1.4.2 Transcriptomics

Transcriptomics is also referred to as functional genomics. It involves the quantification of mRNA pool of a given organism under certain set of conditions or growth. Transcriptome of an organism varies during its growth as well as various environmental cues and provides information about the significance of a gene at particular stage. Interactive transcriptome analysis has been the choice for many plant-pathogen interaction studies. With the ever increasing significance of transcriptome analysis, various approaches have been developed such as Subtractive Sequence Hybridization (SSH), cDNA-AFLP (Amplified fragment length polymorphism), Expressed Sequence Tags (ESTs), Serial Analysis of Gene Expression (SAGE), Multiple Parallel Signature Sequencing (MPSS), qRT-PCR (Quantitative Reverse Transcriptase Polymerase Chain Reaction), Microarray, and Next Generation Sequencing (NGS). Of these, microarray and NGS are currently the most widely used techniques for transcriptomics analysis.

The NGS based approach has significant advantages over microarrays because of its greater sensitivity and broad dynamic range as well as the ability to identify novel genes. Microarray can provide information only for the genes represented on the array chip; however, gene expression measurement using sequencing based approaches are unbiased. Unlike microarrays, prior information about the genes or

sequences is not required for NGS. However, such available preliminary information is useful during data analysis. Indeed, microarray based approach have gained a noteworthy interest for transcriptomics assays. Because NGS based approach also has some limitations, it requires dedicated storage devices for massive data generated from sequencing and bioinformaticians for correct and efficient data analysis.

1.4.3 Proteomics

Proteomics is the study of the entire set of proteins (proteome) of an organism under a given experimental setup. It includes profiling of the protein composition of an organism to understand the expression, function, and regulation of a single or multiple genes in response to specific environment. Based on the design of study and information required, various approaches have been under development for proteomics analyses. The most commonly employed techniques are immunoassay, western blotting, SDS-PAGE (Sodium Dodecyl Polyacrylamide Gel Electrophoresis), 2D-PAGE (2-Dimensional Polyacrylamide Gel Electrophoresis), Protein Chip, Reverse-phased protein microarray, and mass spectrophotometry. Mass spectrophotometry is the most recent and recognized technique for present high-throughput proteomics studies. In this technique, proteins after extraction are cleaved by proteases like trypsin and the resulting peptides are directly ionized using ESI (Electro spray ionization) and identified by a mass spectrophotometer. Advancements in bioinformatics tools and identification of the organelle specific signal sequences have provided substantial progress in protein biology (Kislinger et al., 2006). Although proteomics has been applied in many plant-pathogen interaction studies, identification of the proteins contributed by two different organisms during interaction is still a challenge. Secretory proteins play a major role in development of disease and pathogen establishment on host (Giraldo et al., 2013). Remarkable efforts need to be put forward for isolation and analysis of secretory proteins during plant-pathogen interactions, as these may be missed out from the intracellular proteome.

1.4.4 Metabolomics

Metabolomics is described as the study of the whole metabolic content/ metabolome of an organism under a given set of physiological conditions. Metabolomics is the latest technique of “Omics” era being applied for the upgradation of information in functional genomics. Although, metabolomics is viewed as a complimentary technique to transcriptomics and proteomics, it has several advantages in functional genomics. Firstly, metabolome profile is directly contributing to the phenotype and

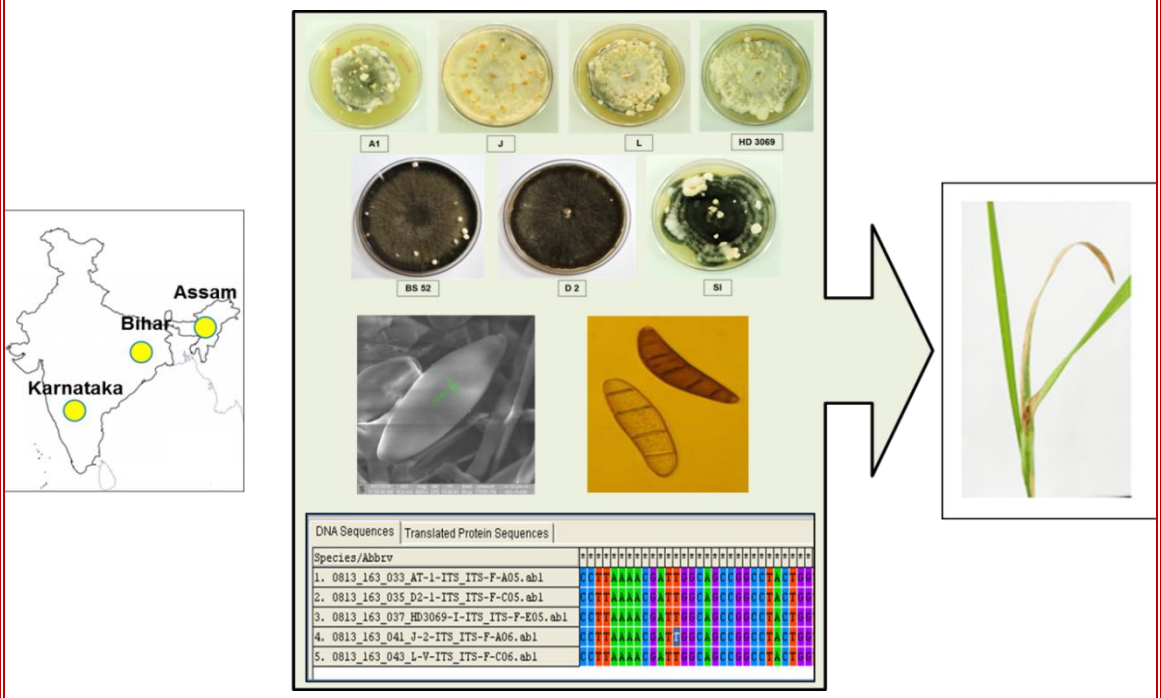
second, fluctuations in metabolite expression are much higher than its relative transcript or protein. Additionally, metabolites are often ubiquitous in nature except a few species specific secondary metabolites. This helps the researchers in studying the non-model organisms without prior facts of genome data. Untargeted metabolome profiling has recently been available and applied for elucidating the plant-pathogen interaction. Approaches available for metabolomics profiling includes Gas Chromatography Mass Spectrometry (GC-MS), High Performance Liquid Chromatography (HPLC), Nuclear Magnetic Resonance (NMR), and Direct-infusion electrospray ionization spray Mass spectrometry (ESI-MS). Metabolomics data alone can contribute to conclude for the biologically relevant hypothesis. ESI-MS has been elegantly applied for plant-pathogen interaction experiments (Kumar et al., 2015). However, integration of multiple techniques is suggested for reliable profiling of all compounds from a metabolome. Moreover, it is also desirable to focus on optimizing the extraction protocol, detection technologies (Tan et al., 2009) and analysis tools in order to characterize the complete metabolome.

1.5 Objectives of the thesis

In spite of several research and breeding efforts, only a few spot blotch resistant wheat varieties could be developed for field cultivation. The molecular mechanism underlying resistance to the disease is yet to be fully understood. In order to develop any measure for plant disease control, it is very important to understand not only the characteristic features of the pathogen, but also the molecular mechanism behind the plant-pathogen interaction. With this purpose, the main aim of this study was to investigate the molecular mechanism of wheat – *B. sorokiniana* interaction using transcriptomics approach, which could reveal new resistance genes that could be used to devise new controlling measures. Accordingly, the thesis encompasses the following objectives:

1. To explore the mechanism of plant-pathogen interaction during spot blotch in susceptible and moderately resistant wheat varieties
2. To understand the mechanism of survival of *Bipolaris sorokiniana* on exposure to the fungicide propiconazole.

Chapter 2:
Isolation and characterization of *Bipolaris sorokiniana* isolates from different geographical regions of India



2.1 Introduction

The filamentous ascomycetes genus *Cochliobolus* (anamorph *Bipolaris / Curvularia*) includes more than 40 closely related species. These are often highly aggressive and show high pathogenic specificity to their host plants. All species of the genus cause economically important crop diseases and form a close group in phylogenetic tree, suggesting a common ancestor giving rise to the diverse species over a relatively short period of time. Each species is distinguished by its selective pathogenicity to a specific type of cereal. These include *Cochliobolus heterostrophus* and *C. carbonum*, the corn pathogens; *Bipolaris sorghicola*, the sorghum pathogen; *B. sacchari*, the sugarcane pathogen; *C. victoriae*, the oat pathogen; and *C. miyabeanus*, the rice pathogen. A majority of the aggressive members of this genus are necrotrophic. The generalized cereal and grass pathogen, *C. sativus*, was earlier categorized as a necrotroph; however, it was later found to be a hemibiotroph (Kumar et al., 2002). The *Cochliobolus* species and their host interaction biology are presented in **Table 2.1**.

Comparative evaluation of six species causing the oat helminthosporiosis in Brazil revealed that *B. sorokiniana* was the most aggressive (de Farias et al., 2005). *B. sorokiniana* is a phytopathogenic fungus causing serious diseases like spot blotch, common root rot and black point of grains in wheat, oat, barley, and other winter cereals. *B. sorokiniana* is reported to have high morphological, physiological, and genetic variability, due to which it is often difficult to identify the fungus, or to take appropriate measures to control the disease (Poloni et al., 2009). *B. sorokiniana* is differentiated from other members of the genus based on morphological features of conidiophores and conidiospores. Conidia of the fungus are elliptical (60–120µm × 12–20µm), thick-walled and have four to eight septa. In agar culture plates, the mycelium is observed as a loose cottony mass of interwoven hyphae. The hyphae appear white or light to dark olive green depending on the isolate.

Various methods have been employed to analyze the variability of *B. sorokiniana*. Mycelial growth characteristics and virulence provides vital information about the pathogen, which also depends on the activity of enzymes with capacity to degrade plant cell wall. This method has been extensively used in characterization of

variability in various phytopathogenic fungi. Electrophoretic analysis of protein profile is a simple and practical method for identification of fungal species and in genetic variability studies. Christensen (1926) first described the physiological specialization at species level. He showed that the fungal isolates varied substantially in virulence to wheat and barley.

Table 2.1: *Cochliobolus* species host interaction biology

Species	Host/ tissue	Disease	HST/ Effector	Target	Pathogen lifestyle
Ch race O (strains C5, Hm540)	corn/leaves	Southern corn leaf blight	?	-	necrotroph
Ch race T (strains C4, Hm338, PR1x412)	corn with Tcmsb/leaves	Southern corn leaf blight	T-toxin	URF13 protein	necrotroph
Cc race 1 (strain 26-R-13) hm1c	corn/leaves	Northern leaf spot	HC-toxin	histone deacetylase	necrotroph
Cv (strain FI3) Vbd	oats/crown	Victoria blight	Victorin	LOV1	necrotroph
Cm (strain WK1C)	rice/leaves	Brown spot	?	-	necrotroph
Cs (strain ND90Pr)	barley, wheat, cereals/leaves	Spot blotch, Common root rot	?	?	hemibiotroph

Note: Ch: *C. heterostrophus*, Cc: *C. carbonum*, Cv: *C. victoriae*, Cm: *C. miyabeanus*, Cs: *C. sativus*

Studies involving large numbers of strains collected from across the globe suggest that *B. sorokiniana* exists as numerous forms of isolates varying in aggressiveness and virulence and with specific and nonspecific interactions (Maraite et al., 1998; Duveiller and Garcia-Altamirano, 2000). Several necrotrophic *Cochliobolus* spp. and related taxa (e.g. *Pyrenophora tritici-repentis*, *Stagonospora nodorum* etc.) have been reported for their ability to evolve into highly virulent and host selective toxin (HST) producing strains. The HSTs impart them the ability to infect cereal crops and cause diseases on them. A previously avirulent strain can evolve into highly virulent strain and result in major epidemic such as Southern leaf blight of corn in 1970 caused by *B. maydis* (Race T) (Ullstrup, 1972).

The mechanism of variability in *B. sorokiniana* is not well understood. Parasexual recombination and anastomosis have been reported as one of the causes of variability in natural populations of *B. sorokiniana* (Tinline, 1962; Pandey et al., 2008). Fusions between germinating hyphae from different conidia may result in somatic hybridization and can lead to the emergence of new fungal variants (Kumar et al., 2002). However, no systematic study has been performed for virulence characteristics of *B. sorokiniana* isolates from different parts of India. Hence, the main objective of the present work was to study the variability and virulence of *B. sorokiniana* isolates from different geographical regions of India through the analysis of mycelial morphology, microscopy, ITS sequence and pathogenicity assays.

2.2 Material and Methods

2.2.1 Plant material

Two wheat varieties were used for the experiment. Genetically pure breeder's seeds of DDK 1025 (*Triticum dicoccum*; 2*n*=4*x*=28), susceptible to spot blotch, was obtained from the University of Agricultural Sciences, Dharwad (Karnataka) and multiplied at CSIR-NCL, Pune. Similarly, the genetically pure breeder's seeds of Chirya 3 (*Triticum aestivum*; 2*n*=6*x*=42), moderately resistant to spot blotch, were obtained from Indian Agricultural Research Institute, New Delhi and multiplied at CSIR-NCL, Pune.

2.2.2 Collection of isolates

Source of inoculum was obtained from the University of Agricultural Sciences, Dharwad (Karnataka) in form of *B. sorokiniana* inoculated sorghum seeds. The seeds were surface sterilized using 2% NaOCl and transferred aseptically on water-agar petri-plates. The plates were observed regularly under a stereo-zoom microscope (Leica, Germany) for sporulation. The isolate DI was isolated from these samples. Similarly, spot blotch infected leaves of wheat were collected from the fields of University of Agricultural Sciences, Dharwad (Karnataka) and brought to CSIR-National Chemical Laboratory, Pune. The leaves were surface sterilized using 2% NaOCl and transferred aseptically on water-agar petri-plates. The plates were observed regularly under a stereo-zoom microscope (Leica, Germany) for sporulation. The isolate SI was isolated from these samples. The pathogen was sub-cultured on potato dextrose agar (PDA) at 28ºC with 12 h light and 12 h dark for 10 days. Sporulating culture was observed at 40X under a stereo-zoom microscope (Leica, Germany).

Subsequently, the isolates D2, HD3069, A, L, O1, O2, N1, Bp and J were obtained from Dr. Rajendra Prasad Central Agricultural University, Pusa (Bihar). These isolates had been isolated from infected wheat fields of Dr. Rajendra Prasad Central Agricultural University and transferred to PDA plates. Likewise, the isolate BS52 was obtained from Indian Agricultural Research Institute, New Delhi, which had been collected from infected wheat fields from Assam (India).

2.2.3 Establishment of monoconidial cultures of the isolates

All the above 12 isolates were grown on PDA plates in CSIR-National Chemical Laboratory, Pune, India and monoconidial cultures were established. The non-sporulating isolates were sub-cultured on different media (V8, Czapek Dox and yeast extract agar) to induce sporulation. Conidia were harvested by scraping the surface of the PDA plates with inoculation loop and suspended in sterile distilled water. Mycelia were separated from spores by filtering the suspension through a sterile muslin cloth. Spore count of the suspension was taken using a haemocytometer. The resulting conidial suspension was diluted to achieve the spore count of 50 spores/ml. The conidial suspension (100 µl) was spread-plated on water-agar containing streptomycin (150 mg/l). Five such plates were incubated for each isolate at 28ºC for 24 h with 12 h photoperiod and then observed for germination at 80X under the stereo-zoom

microscope. Individual germinating spores were marked on petri plates. The agar blocks/ discs containing a single germinating spore were lifted using a 5 mm cork borer and transferred to PDA plates containing streptomycin (150 mg/l). Multiple agar blocks were sub-cultured for each isolate in separate plates. These "master" plates were incubated at 28ºC with 12 h light and 12 h dark for 72 h, followed by further sub-culturing on PDA plates. Growing mycelia from these plates were spot inoculated on fresh PDA plates and incubated for one week.

The characteristics of all these monoconidial cultures grown on PDA and water-agar plates were monitored with respect to color and growth pattern of the fungal mycelium. Further, the cultures were stored on PDA slants at 4^{o}C and by inoculation of sterile wheat seeds. The cultures on slants were regularly sub-cultured after every three months.

2.2.4 DNA isolation and ITS sequencing

All the monoconidial cultures were grown in Potato Dextrose Broth (PDB) at 28^{o}C in a shaking incubator with 180 rpm and 12 h photoperiod. After five days, the mycelia were harvested, crushed and stored in -80^{o}C. DNA isolation was performed using Qiagen Miniprep DNA isolation kit (Qiagen, India) following the manufacturer's protocol. The yield of extracted DNA was quantified using Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). Genomic DNAs from all the monoconidial cultures were subjected to PCR using internal transcribed spacer (ITS) primers ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS4 (5′TCCTCCGCTTATTGATATGC 3′) (White et al., 1990). Amplified products were sent for sequencing. Sequences were further analyzed for identification of the isolates.

2.2.5 Culture morphology

The morphological characteristics of the cultures were observed on PDA plates visually, while the spores were visualized using a compound light microscope and a scanning electron microscope. For sporulation, all the monoconidial isolates (A, HD 3069, J, D2, L, BS-52 and SI) were sub-cultured onto water-agar. After sporulation stage, the spores were harvested in sterile distilled water containing 0.1% tween-20 and observed under light microscope at 600X (Leica, Germany).

2.2.6 Pathogenicity assay

Isolates from different geographical regions can vary in their virulence. Hence, virulence efficiency of all the isolates was compared by allowing them to infect plants under the same experimental conditions. The virulence of the seven sporulating isolates was assessed using DDK 1025 variety, which is considered susceptible to *B. sorokiniana*. For this purpose, seed inoculation method was followed for pathogenicity assay as described by Minotto et al. (2014) with some modifications. In brief, seeds were surface sterilized using 1% sodium hypochlorite (NaOCl) for 5 min and washed thrice with sterile distilled water. The disinfected seeds were placed on moist autoclaved filter paper in petri plates, wrapped in aluminum foil and incubated at room temperature for 48 h for germination. The seeds with aborted germination were discarded and the healthy germinated seeds were transferred to vials with spore suspension of *B. sorokiniana* isolates. The spore suspension was prepared from seven days PDA grown culture of *B. sorokiniana* isolates. Spore concentration was adjusted to 2×10^3 spores/ml. The seeds with spore suspension were incubated at 28°C for 48 h with 12 h photoperiod condition. After incubation, the seeds were washed with sterile distilled water to remove the spores adhering to the surface and the seedlings were transferred to sterile soil-rite in green-house having temperature range 28-32°C and relative humidity 80%. The seedlings were regularly monitored for 10 days for any disease symptoms. Healthy germinated seeds incubated without spore suspension were considered as control.

2.2.7 Characterization of spore morphology on leaf surface

Scanning electron microscopy (SEM) of spores was performed to observe spore morphology of the isolates. Monoconidial cultures were grown on water-agar to induce sporulation. The agar blocks containing the spores were used for SEM directly. Spores were also inoculated on leaves of wheat varieties. Leaves were observed for visible disease symptoms and under a stereo-zoom light microscope (Leica, Germany) for pathogen growth. Infected leaves were mounted on the aluminum stubs with the help of carbon conducting tape for SEM after 12 h and 48 h of inoculation with spore suspension.

2.2.8 Disease progression analysis

The combined effect of high temperature, high relative humidity and long leaf wetness period (more than 12 h in any 24 h period) caused primarily by daily dew provides conducive environment for spot blotch. The information on virulence diversity of plant pathogens is necessary to develop controlling measures against the prevalent pathotypes (Dubin and Bimb, 1994). To understand the disease progression and pathogen lifestyle under favorable environment, spot blotch susceptible (DDK 1025) and resistant (Chirya 3) varieties were inoculated at Zadok's scale 12 stage (Nagarajan and Kumar, 1998) of the wheat plants. For this, the seeds of DDK 1025 and Chirya 3 were sterilized by 1% NaOCl treatment for 5 min followed by three washes of sterile distilled water of 5 min each. The sterilized seeds were imbibed in sterile water for 6 h followed by *in vitro* germination under aseptic conditions. The germinated seeds were transferred to autoclaved soil-rite and allowed to grow under green-house conditions in temperature range 28-32°C. All the plants of the two varieties looked uniform as expected. At two leaf stage (Zadok's scale 12 stage (Nagarajan and Kumar, 1998)), half of the plants were inoculated with spore suspension (2×10^3 spores/ml) of *B. sorokiniana*, while the rest were mock inoculated with water having 0.1% tween-20 as controls. The plants were regularly monitored for growth and spot blotch symptoms.

2.3 Results

2.3.1 Culture characteristics of pathogen isolates

In all, 12 isolates of *Bipolaris sorokiniana* were obtained from the three wheat-growing regions of India (Assam, Bihar and Karnataka). The isolate BS52 was from Assam, while the isolates DI and SI were from Karnataka. The rest of the isolates (A, Bp, D2, HD3069, J, L, N1, O1, and O2) were from Bihar. Culture characters of all the isolates varied greatly. The mycelial morphology varied from cottony white to dark (melanized) on PDA medium as shown in **Figure 2.1**. The isolate 'Bp' showed cottony radial growth with orange pigmentation at hyphal base, while the isolate 'HD3069' showed cottony mycelia having melanized base with compact bunch of erect mycelia. Although the hyphal growth was white mass in case of 'O1', some melanization was observed at the center of the growth. Similar growth pattern was observed in case of isolate 'L' with some fruiting structure dispersed through the

radial mat. The isolate 'J' showed white mat growth with fur like mycelia, while the isolate 'N1' had dark green cottony erect mycelial mat. The isolate 'DI' showed the highest melanization and hence appeared black on PDA.

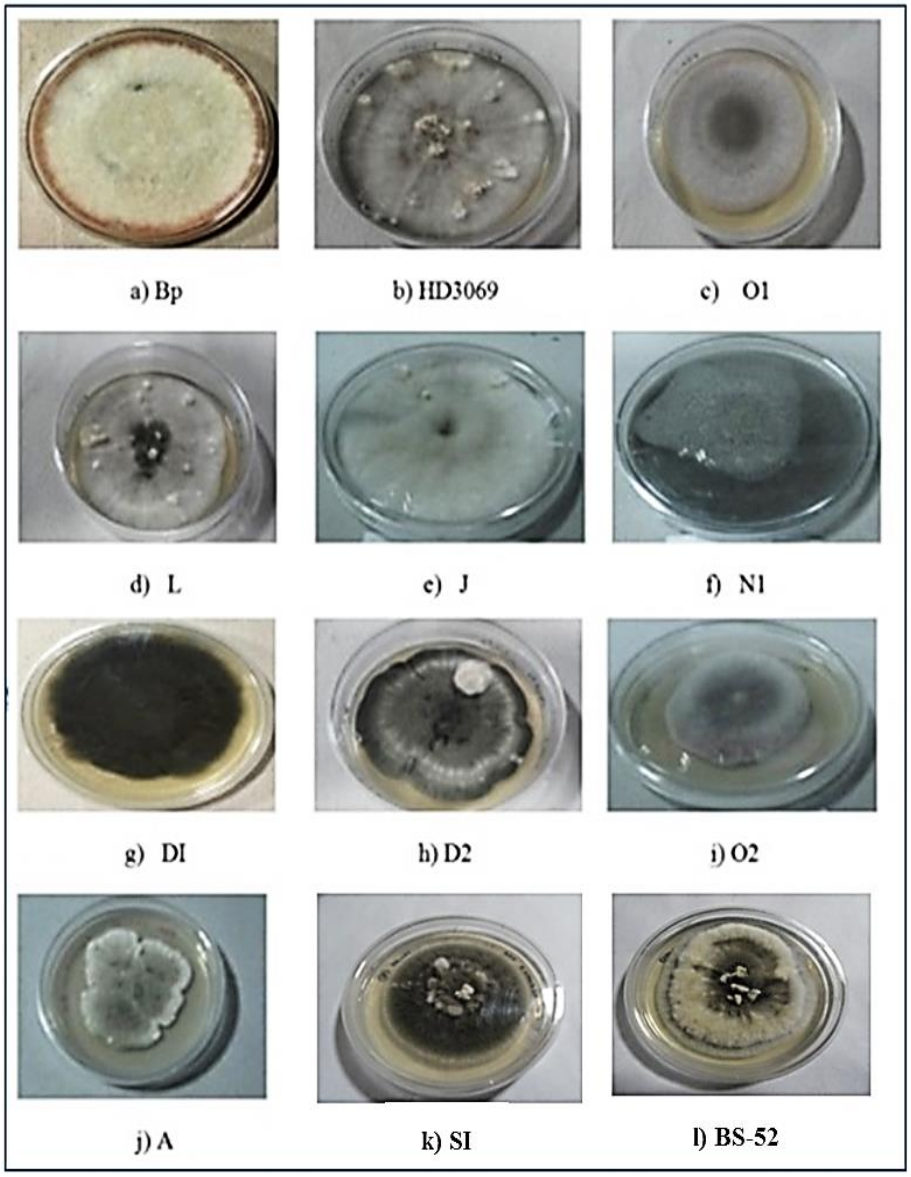


Figure 2.1: Morphology of 12 isolates of *B. sorokiniana* collected from different geographical regions

A mixture of white and olive green colored hyphae was observed in case of isolate 'D2' along with some white fruiting structures. While in case of 'O2' isolate, wooly growth was observed and the hyphae were rigid as compared to 'O1'. Isolate 'A' showed irregular radial growth as compared to other isolates and very little

melanization was seen, which was restricted to older mycelia. Cottony hyphal radial mat was observed in culture of 'SI' isolate with varied level of melanization. Contrary to this, white woolly mycelial mat was observed in case of isolate 'BS52', which was dense at the periphery. Melanization was observed in sporulating mycelia. Slow and restricted growth was seen in isolates A, L and HD3069 with dense fruiting structures. The fruiting structures were of orange color in isolate J; while high level of melanization was seen in monoconidial culture of BS52 and D2. The isolate SI showed mixed (both white and melanized) type of mycelia with white colored fruiting bodies.

2.3.2 Establishment of the monoconidial cultures

Sporulation was observed after seven days of inoculation of the cultures on water-agar plates in seven isolates of *B. sorokiniana*. Four of the twelve isolates did not sporulate even after growing on different media and under different culture conditions and hence these were not processed further. The eight sporulating isolates were grown on both PDA and water-agar plates and were found to be heterogenous in spore morphology. Hence, single spore (monoconidial) cultures from these eight isolates were established.

2.3.3 Pathogen characterization by ITS sequencing

To confirm the identity of the isolates and estimate the genetic variation among the eight isolates, primers for the ITS region were used to amplify their genomic DNA. Amplicons from the eight isolates with five replicates each (total 40 samples) were sequenced. For each isolate, all the replicates gave identical sequence, confirming that there was no variation among the replicates of the isolates. The BLAST searches revealed homology to *C. sativus* isolate D_D44. Seven of the eight isolates were *B. sorokiniana* (**Figure 2.2**), while one isolate (DI) was found to be *Setosphaeria rostrata* (a sorghum pathogen). Hence, the cultures of this isolate were excluded from further study. The ITS sequences of all the isolates of *B. sorokiniana* and *S. rostrata* have been deposited in NCBI database (Accession nos: KJ562714-KJ562720).

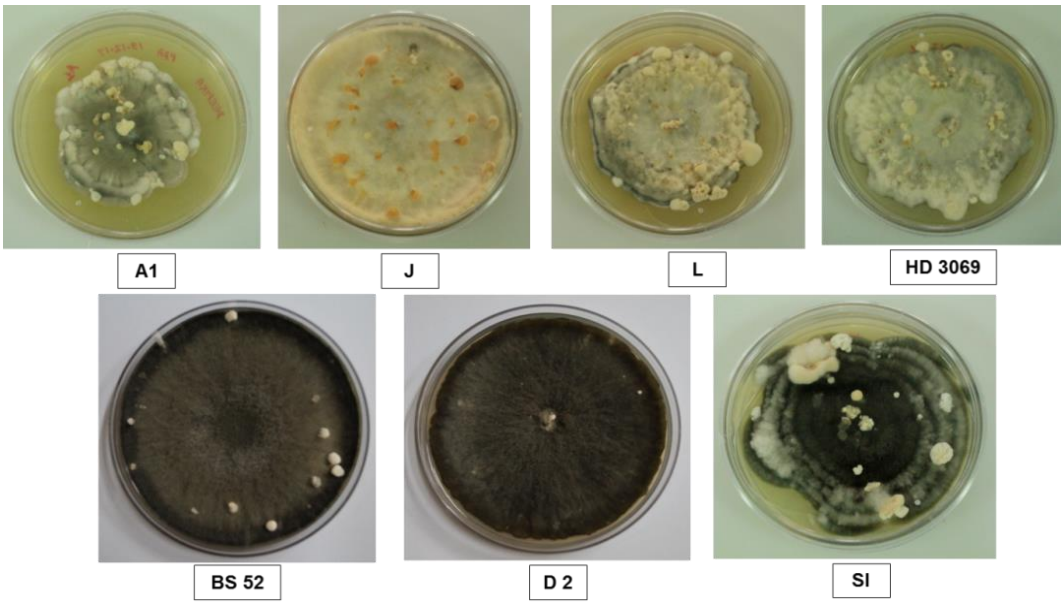


Figure 2.2: Monoconidial cultures of seven sporulating isolates of *B. sorokiniana* on PDA

2.3.4 Microscopic characterization of spores

Culture suspension was placed on glass slide and observed under light microscope. All the spores were brown in color. However, primary mycelia were white or transparent while secondary mycelia showed melanization and hence appeared brown. A bulbous or outgrowth like structure was observed on mycelia at the site of conidiation (**Figure 2.3**).

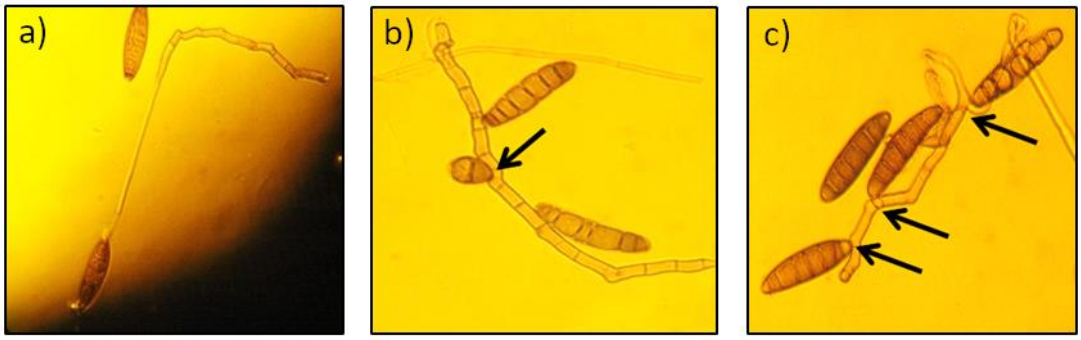


Figure 2.3: Spore structures observed under microscope. (a) Germinating spore showing primary and secondary mycelium (b) and (c) Site of conidiation

2.3.5 Spore morphology

About 50-100 spores from individual monoconidial cultures for the seven isolates were analyzed for spore morphology using a compound light microscope (Leica, Germany). The conidia varied in size and consisted of 2-13 septa. The conidia from all the isolates were slightly curvaceous; spindle shaped and appeared olive green (D2) to brown (A, HD 3069, L, J, SI, and BS52) (**Figure 2.4**).

2.3.6 Pathogenicity assay

Mortality rate of seedlings after incubation with spore suspension was considered for assessing the virulence of isolates. All the isolates were found to be virulent. However, the isolates varied for their degree of aggressiveness. The mortality rate for isolate D2 was found to be highest followed by SI and BS52 **(Table 2.2)**. The spot blotch symptoms appeared in the inoculated plants for all the seven isolates. Infected plants displayed spot blotch symptoms on first and second leaves by 10^{th} day post inoculation. Spot blotch appeared as a brown spindle shaped blotches either on tip of the leaves or at the center as well as on the entire margin of the leaves (**Figure 2.5**).

Table 2.2: Pathogenicity assay for the seven sporulating isolates

Isolate	Mortality percentage
A	26.66
HD3069	20.00
L	26.66
J	23.33
D2	56.66
SI	46.66
BS52	43.33

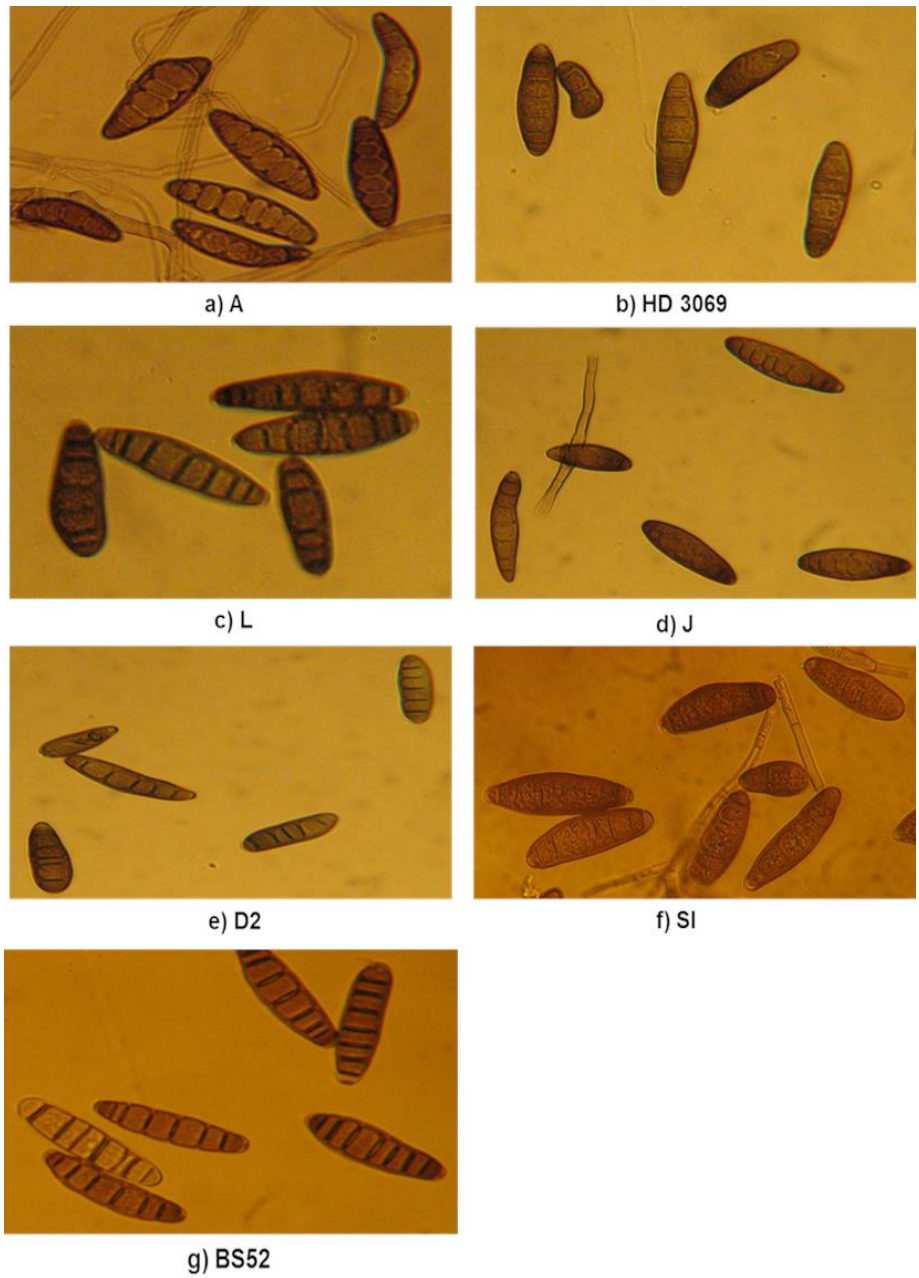


Figure 2.4: Spores from different isolates observed under compound light microscope

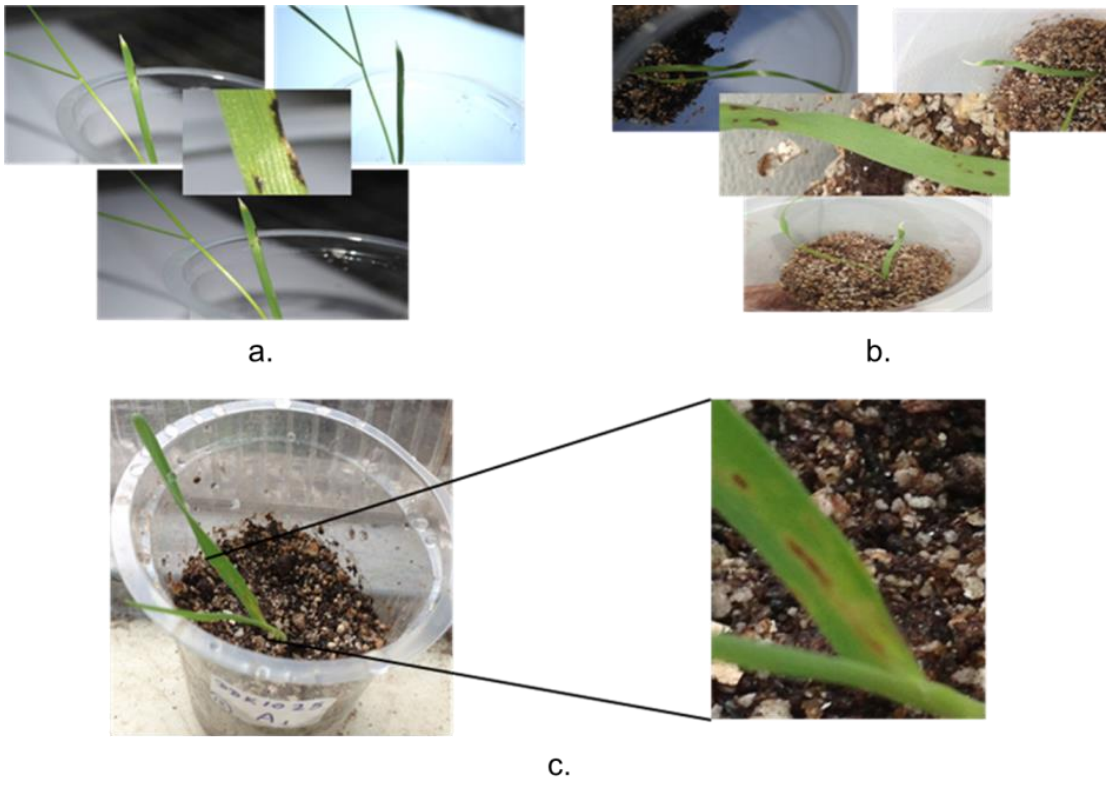


Figure 2.5: Spot blotch symptoms observed in seedlings after incubation with spore suspension. a. Leaf margins b. Mid regions of leaf, and c. Near coleoptile

2.3.7 Spore characterization on leaf

Spores of a representative isolate (D2) were inoculated on wheat leaf and observed under microscope. During SEM, spores lost the septation under the vacuum condition and showed smooth surfaces (**Figures 2.6a** and **2.6b**). Bipolar germination was spotted after 12 h of inoculation on leaf (**Figure 2.6c**). The mycelia observed after 48 h of spore inoculation using a stereo-zoom microscope showed a dense network in susceptible variety (**Figure 2.6d**) as compared to the resistant variety (**Figure 2.6e**). SEM also showed that the mycelial network was compact in DDK 0125 (**Figure 2.6f**) as compared to Chirya 3 (**Figure 2.6g**).

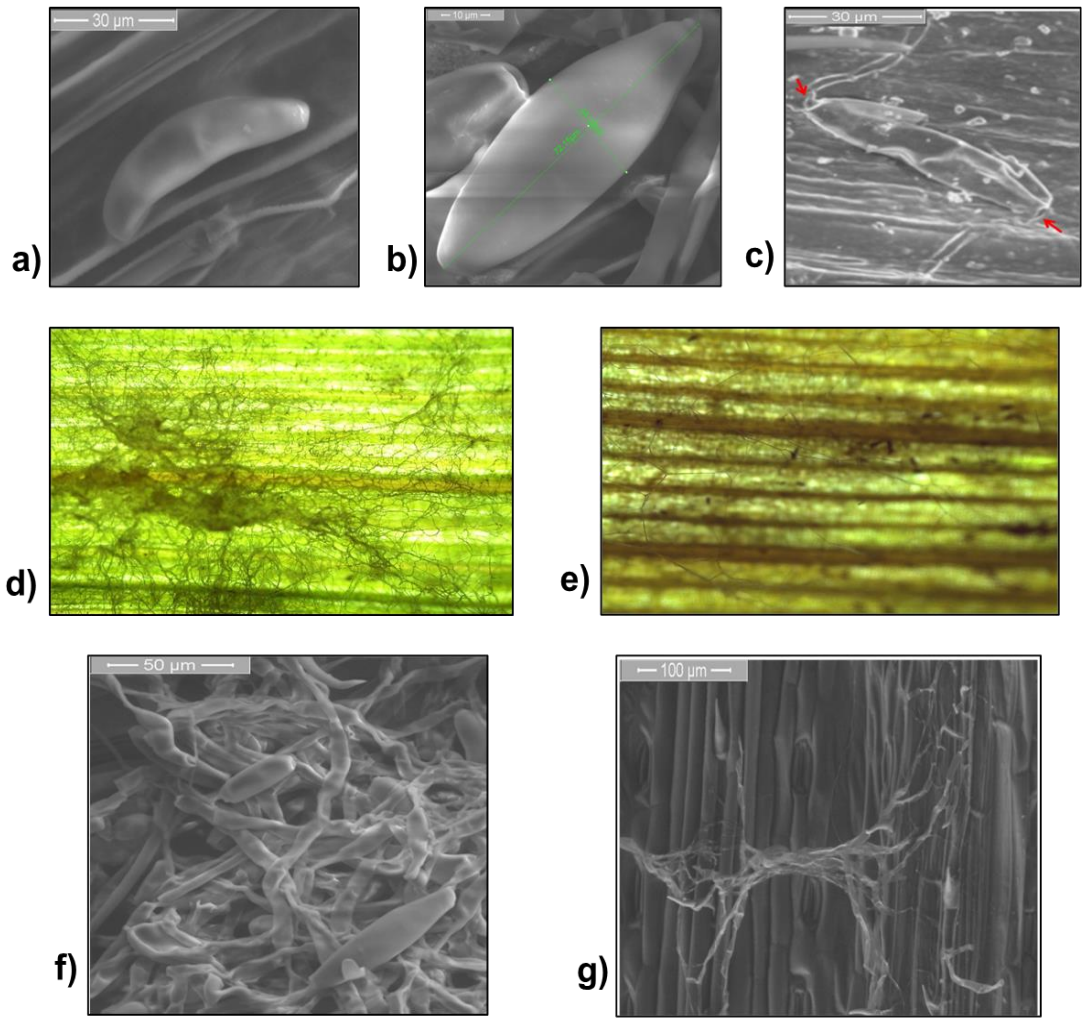


Figure 2.6: Spores characterization using scanning electron microscope and stereozoom microscope. Please refer text for details.

2.3.8 Disease progression and lifestyle analysis

Spraying the spore suspension with appropriate temperature and high humidity conditions led to development of spot blotch symptoms on wheat plants. After inoculation, the plants under greenhouse condition were monitored daily till 10 days for growth as well as disease symptoms. No disease symptoms were observed in plants till 1 dpi in either of the variety. In case of Chirya 3 (spot blotch resistant variety), mock inoculated as well as fungal inoculated plants did not show any typical spot blotch symptoms. However, whitening of leaves was observed in fungal inoculated plants of Chirya 3 after 8 days post inoculation (**Figure 2.7**).

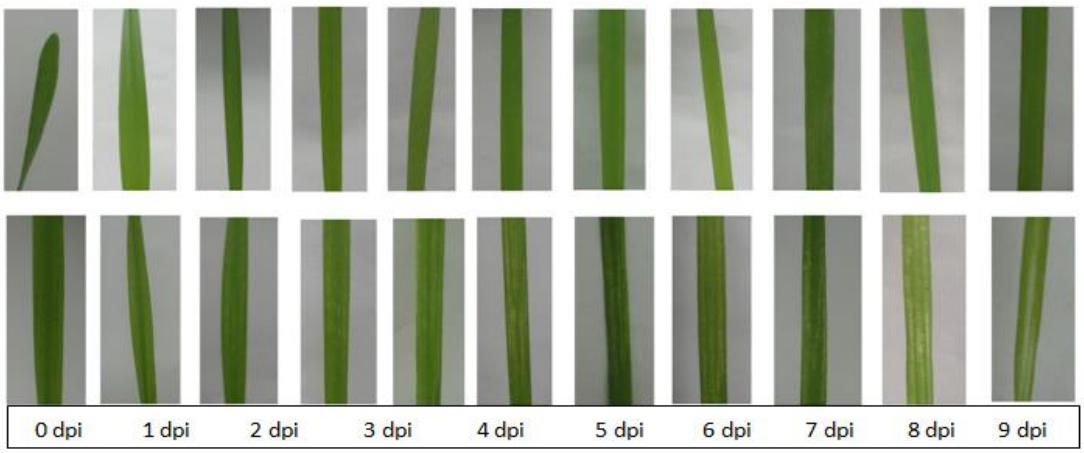


Figure 2.7: First leaf from Chirya 3 plants, 0-9 dpi control (upper) and fungal inoculated (lower) (dpi- days post inoculation)

In case of fungal inoculated plants of DDK 1025 (spot blotch susceptible variety), tiny brown spots were observed at 2 dpi on the first leaf. The spots enlarged in size later to give blotch like appearance. The number of spots increased by 5 dpi, converging in spindle shaped blotches. Control plants of DDK 1025 did not show any disease or stunted growth symptoms either. Pathogen inoculated plants of DDK 1025 showed severe infection by 7 dpi and died by 9 dpi (**Figure 2.8**); while the fungal inoculated plants of Chirya 3 remained healthy and grew like control plants of Chirya 3.

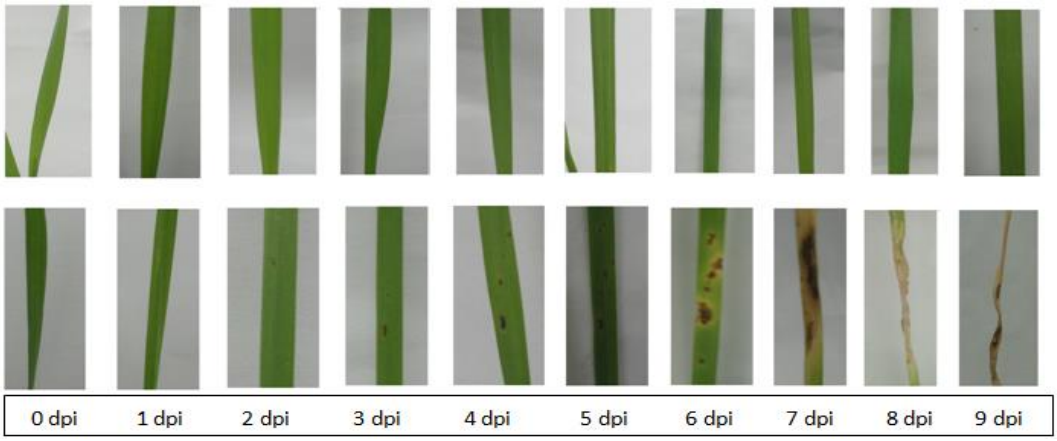


Figure 2.8: First leaf from DDK 1025 plants, 0-9 dpi control (upper) and fungal treated (lower) (dpi-days post inoculation)

2.4 Discussion

In case of fungal populations, it is said that "diversity is the norm while uniformity is the exception" (Poloni et al., 2009). The phytopathogenic fungus *Bipolaris sorokiniana* is also no exception to this and has high morphological as well as pathological variations (Arabi and Jawhar, 2007). In this study, we also observed high morphological variability among the isolates. Three of the seven isolates, *viz*. A, L, and HD3069 had similar culture morphology and white mycelia with some level of melanization. However, the isolates varied in their growth patterns. Similarly, the isolates BS52 and D2 had similar growth pattern on PDA, but showed differences in the level of melanization. We found that the morphological variations observed in the fungal isolates had no relationship with their geographical background. In an earlier study too, no correlation was observed between genetic similarity of groups and geographical origin in *B. sorokiniana* isolates, concluding that the morphology characteristics is not conditioned solely by genetic composition (de Moura Nascimento and Van Der Sand, 2008). The isolates from the same species differ from one another in their morphology as well as genetic compositions. Hence, this variability must be attributed to the interactions between genetic information and environmental conditions, such as the ecology and climatic variations in the areas from where the isolates have been obtained.

B. sorokiniana isolates with white hyphae are reported to be melanin deficient (Mishra and Singh, 2015). Melanin is essential for survival of all loculoascomycetes, some pyrenomycetes and many deuteromycetes fungi (Sussman, 1968; Rotem and Aust, 1991). Significance of melanin pigment is well recognized in spore dormancy, survival and in protection against microbial lysis in soil (Bell and Wheeler, 1986). These studies support the fact that melanin rich subpopulations are present in high frequencies in natural population as compared to the white melanin deficient population; probably due to their increased fitness. We also observed that the three melanin-rich isolates i.e. D2, BS52, and SI, produced more spores. As spores enable better persistence under stress, these isolates might have better survival ability than other strains. We observed that the isolates with only white mycelia did not produce reproductive structures in culture medium. Hence, these isolates can be used to induce systemic resistance in plants as they can colonize the plant without causing the

disease. However, this would activate plant defense systems and provide triggered immunity. They may also compete with the virulent isolates for the ecological niche.

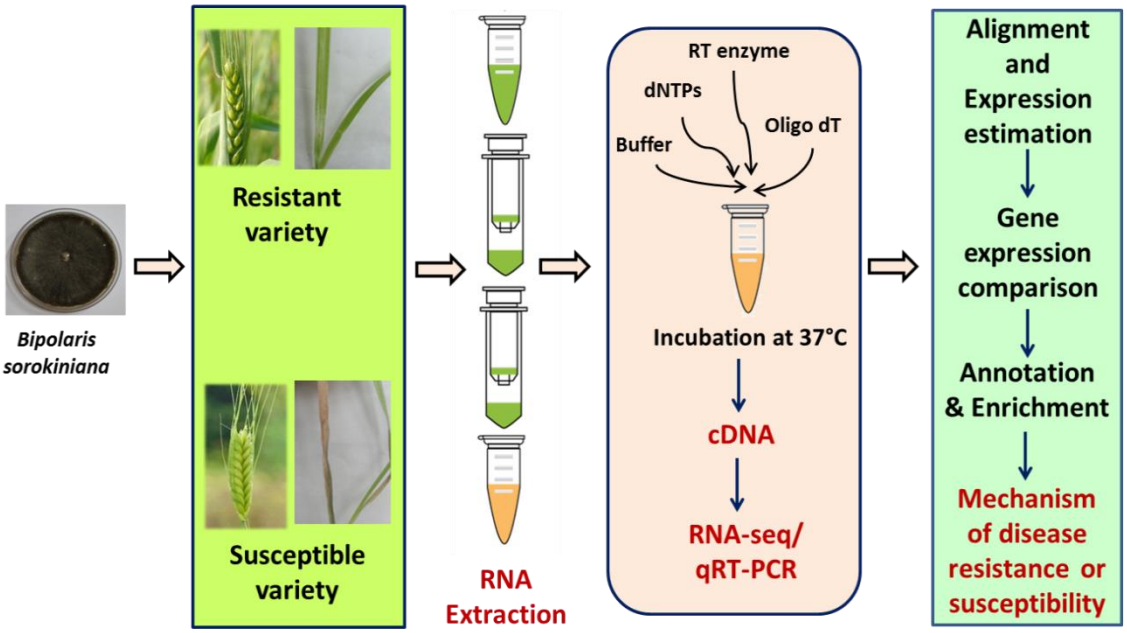
Based on morphology, *B. sorokiniana* isolates are classified into three morphological groups, i) White mycelia, ii) Black mycelia and iii) Mixed colony. All morphological groups include isolates with high, moderate, and low infectivity. Arabi and Jawhar (2007) reported three different pathotypes of *C. sativus*, one of which was extremely virulent. Mahto et al. (2012) found that *B. sorokiniana* isolates from the same geographic region and morphological group exhibited different degrees of aggressiveness on wheat cultivars. Seed inoculation is the most widely used method for assessing pathogenicity of the isolates. However, we found that this method did not discriminate between unviable and infected seeds. This can lead to ambiguous perception of mortality rate. Painting the leaf with spore suspension is another approach. However, it was found to be time consuming. Hence, we developed the germinated seed inoculation method. We found this method to be the best for high-throughput pathogenicity and mortality assays. Using this method, we found that the D2 isolate was extremely virulent. This isolate also showed very high level of melanization and sporulation. Oliveira et al. (2002) reported variance in pathogenicity among the *B. sorokiniana* isolates. However, virulence was not correlated to the morphological characteristics. On the other hand, Jaiswal et al. (2007) reported correlation between virulence and the colonial morphology. According to them, the isolates with black mycelia were most virulent. Similarly, Pandey et al. (2008) also studied the variability in *B. sorokiniana* and reported the association of virulence character with morphological variations. We found high correlation in the level of melanization and virulence. Nonetheless, the isolates with white mycelia were also pathogenic, although they did not produce reproductive structures.

The variability in virulence as well as morphology suggests that extensive genetic exchange happens in this species. As the pathogen, *B. sorokiniana* chiefly follows asexual or haploid reproduction. The diversity may be attributed to the presence of multiple nuclei in the mycelium and conidia. However, the variation can also be caused through the exchange of nuclei followed by nuclear fusion, somatic recombination and the subsequent chromosomal rearrangement for the haploidization.

Earlier the pathogen was reported as necrotrophic however, it was later found to be a hemibiotroph (Kumar et al., 2002). We confirmed the hemibiotrophic nature of

the pathogen on wheat species by following the inoculation at Zadok's scale 12 stage with the isolate D2. The results were consistent when tests were repeated over time or in multiple experiments under green-house condition. The number and size of lesions per leaf varied; however, the biotrophic and necrotrophic phases were observed in all plants irrespective of the disease score. There was significant difference in the disease progress and symptoms observed in both the genotypes. Spot blotch was not observed in the resistant variety. The results confirmed that *B. sorokiniana* infection is highly variable and very sensitive to the environmental factors.

Chapter 3:
Exploring the molecular interaction of wheat-*Bipolaris sorokiniana* during spot blotch disease



Publications based on this Chapter:

1. **Somani Deepika**, Prashant Ramya, Kadoo Narendra (2019) Evaluation of reference genes in wheat-*Bipolaris sorokiniana* pathosystem for precise expression profiling of plant defense genes using qRT-PCR (Journal of Plant Pathology, in Review)
2. **Somani Deepika**, Prashant Ramya, Kadoo Narendra (2019) Transcriptional paradigm of wheat-*Bipolaris sorokiniana* interaction (In preparation)

3.1 Introduction

The increasing threat of spot blotch demands serious efforts to understand various dimensions of the plant-pathogen interaction including molecular basis of disease resistance mechanism to develop resistant varieties. As spot blotch is an emerging disease, the molecular mechanisms underlying disease resistance or susceptibility have not yet been elucidated. Molecular events in plant-pathogen interaction can be understood by analyzing the expression levels of pathogen responsive genes across various stages of infection. Several bioinformatics tools and databases are now available for identifying the genes encoding specific classes of enzymes based on the conserved domain sequences. However, the information about gene expression is substantial in order to reduce the number of candidate gene clones for functional characterization. Hybridization approaches, microarrays, or transcriptome sequencing can provide significant insights into the functional role of the set of genes under a given condition (Dixon, 2001). Among these, transcriptomics provides a highly reliable platform for gene expression and quantification analyses. Molecular studies about candidate disease resistance genes along with their differential expression pattern in response to a specific pathogen can provide valuable information for better understanding of the resistance mechanism.

With the ease in availability and advancement of real time PCR machines, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) has gained wide acceptance for gene expression profiling. qRT-PCR is the most favored method for diverse gene expression analyses as it requires very less amount of starting material and is highly sensitive, specific and relatively inexpensive. Although the technique is routinely employed for validation of the results of high-throughput studies employing microarrays, RNA-Seq, Chip-Seq, proteomics, etc., multiple parameters need to be optimized for accurate quantification of gene expression. In qRT-PCR, quantification is performed using either standard curve or reference gene based methods. The standard curve method is normally not suitable for the vast variety of biological samples due to the unavailability of appropriate standard cDNA. Because in most gene expression studies, the samples are usually obtained from a variety of experiments, considering multiple factors like different species, genotypes,

individuals, growth conditions, treatments, time points, and tissues. Alternatively, the reference gene based method employs normalization of the expression of a target gene with that of a selected internal reference gene. Livak's DDCt (also known as $2^{-\Delta\Delta}$Ct or delta-delta-Ct) method (Livak and Schmittgen, 2001) is the most accepted approach for such relative quantifications in qRT-PCR. Data normalization with validated reference genes minimizes the variations resulting due to RNA extraction, reverse transcription, and amplification efficiency. Ideally, the expression level of the reference gene should be as stable as possible among the various tissues, treatments, and time points under consideration. This allows precise assessment of target mRNA concentrations and hence gene expression across various samples (Bustin et al., 2009). Genome wide expression analysis has shown that the expression of most genes is affected by growth stage and environmental conditions (Long et al., 2010). However, unlike environmental or abiotic stresses, the samples from pathogen infection studies contain the DNA, RNA, proteins, and metabolites from two or more organisms i.e. the plant and its pathogen(s). This poses difficulty in choosing an appropriate internal reference gene, as cross-amplification of the reference gene from the pathogen can lead to ambiguous perception of plant target gene expression. Hence, the selection of an appropriate reference gene and the primers that would specifically amplify only the host cDNA, are the crucial aspects.

Hence in this study, we performed interactive transcriptome analysis of wheat-*Bipolaris sorokiniana* interactions using resistant and susceptible wheat genotypes to document differential gene expression pattern among them. We employed the Illumina HiSeq 2000 platform to generate the transcriptional profiles through RNA-seq. We detected important alterations intervened by pathogen evasion in both the wheat genotypes. We also evaluated some of the previously reported reference genes for qRT-PCR analysis; however, few of them showed cross-amplification with pathogen cDNA, which could have led to improper quantification of expression of plant defense genes. Hence, we screened eight previously reported candidate reference genes to identify the reference genes, which specifically amplify wheat cDNA and not the pathogen cDNA, and are highly stable and efficient in expression across treatments. Using the identified reference gene, we evaluated the expression pattern of previously reported candidate plant defense genes.

3.2 Material and Methods

3.2.1 Preparation of fungal inoculum

Pure (monoconidial) culture of *B. sorokiniana* strain D2 was used for the experiment. The strain was isolated from the wheat fields of Dr. Rajendra Prasad Central Agricultural University (Pusa, Bihar, India) and the monoconidial culture was established at CSIR-National Chemical Laboratory, Pune. For inoculation purpose, 10 days old culture grown on potato dextrose agar (PDA) medium was used. Sporulating culture was observed at 40× under a stereo-zoom microscope (Leica, Germany). Conidia were harvested by scraping surface of the petri-plate with inoculation loop and suspended in 0.1% Tween20 prepared in sterile distilled water. The mycelia were separated from spores by filtering the suspension through a sterile muslin cloth. The resulting conidial suspension was used to measure the spore count using a haemocytometer under a light microscope. To count the number of viable spores in the suspension, 0.1 ml of the spore suspension was spread onto sterile water-agar plates by spread plate technique. The plates were incubated for 12 h at 28°C, and were observed under stereo-zoom microscope for germinating spores. The spores were counted by plate count method.

3.2.2 Plant materials and growth conditions

Two wheat varieties, DDK 1025 (*Triticum dicoccum* L.; tetraploid; 2*n*=4*x*=28) and Chirya 3 (*Triticum aestivum* L.; hexaploid; 2*n*=6*x*=42) (obtained from the University of Agricultural Sciences (UAS), Dharwad, Karnataka, India), were selected for this study. DDK 1025 is susceptible to spot blotch caused by *B. sorokiniana*, while Chirya 3 is resistant. Seeds of DDK 1025 and Chirya 3 were surface-sterilized by 1% NaOCl treatment for 5min followed by three washes of sterile distilled water of 5min each. The surface-sterilized seeds were imbibed in sterile water for 6h followed by *in vitro* germination under aseptic conditions and then transferred to greenhouse. Autoclaved Soil Rite (mixture of 75% Irish peatmoss and 25% horticulture grade expanded perlite; obtained from M/s Naik Krushi Udyog, Pune, India), was filled into plastic trays arranged in humidity chambers and germinated seedlings were sown and allowed to grow. At two leaf stage (Zadok's scale 12 stage (Nagarajan and Kumar, 1998)), half of the plants were inoculated with spore suspension (10^3 spores/ml) of *B. sorokiniana*, containing 0.1% Tween20; while the rest were mock inoculated with

sterile distilled water having 0.1% Tween20 as control. Thus, the plants were grouped into four groups on the basis of genotype and treatments as: (i) Chirya 3 control (CC), (ii) Chirya 3 inoculated (CI), (iii) DDK 1025 control (DC) and (iv) DDK 1025 inoculated (DI). The plants were regularly monitored for growth and disease symptoms. Tissue was collected at three time points as 1, 4 and 6 days post inoculation (dpi). The tissue collection was performed in three biological replicates and each replicate consisted of 30 plants. The collected leaf tissue was immediately frozen in liquid nitrogen and stored at -80°C till further processing.

3.2.3 Photosynthetic efficiency measurement

As spot blotch is characterized by the appearance of light brown to dark brown elongated blotches on leaves, we measured the effect of the disease on plant health in terms of photosynthetic efficiency after pathogen inoculation. For this, we measured photosynthetic efficiency of leaves of five different control and inoculated plants after 20 min pre-darkening using Handy PEA chlorophyll fluorimeter (Hansatech, England). The maximum fluorescence dark yield (*Fv*/*Fm*) of photosystem II (PSII) is defined as $Fv/Fm = (Fm - Fo)/Fm$ (Maxwell and Johnson, 2000) and provides information about the overall photosynthetic performance of the plant. *Fm* represents the maximum fluorescence after a strong flash of light (all PSII reduced), *Fo* the minimum (all PSII oxidized) and *Fv* the variable fluorescence. The ratio *Fv*/*Fm* is considered for calculating the photosynthetic efficiency of plants (Genty et al., 1989) and reduced *Fv*/*Fm* indicates that the plant is under stress.

3.2.4 High performance liquid chromatography (HPLC) pigment analyses

Immediately after photosynthetic efficiency measurements, the leaves were cut and snap-frozen in liquid nitrogen and stored at -80°C for further analyses. For pigment extraction, the leaves were crushed to fine powder in liquid nitrogen using mortar and pestle. To each sample, 3ml of 90% acetone were added; the mixture was homogenized, and centrifuged at 10,000rpm for 10 min to separate the debris. The supernatant was collected and stored for 12 h in dark at 4°C, while the debris was discarded. After incubation, the supernatant was removed, gently centrifuged at 3000 rpm for 2min and taken for analysis of lipophilic pigments. HPLC identification and quantification for chlorophyll pigments was performed using the protocol described by Edelenbos et al. (2001).

3.2.5 RNA extraction, cDNA synthesis and sequencing

Total RNA was isolated from 100 mg of the finely ground leaf tissue using the Spectrum™ Plant Total RNA kit (Sigma, USA), followed by DNase treatment (Promega, USA). The DNase treated samples were purified using RNA binding column provided in the Spectrum™ Plant Total RNA kit. The quality and concentration of the isolated RNA were evaluated using Nanodrop 1000 (ThermoFisher Scientific, USA) and 1% agarose gels. The integrity of the RNA samples was evaluated by running on Bioanalyzer 1000 (Agilent, USA) and the samples with RNA integrity number (RIN) value >8.0 were used for library preparation for next generation sequencing. Paired-end sequencing was performed using HiSeq 2000 sequencing system (Illumina, USA), by following the standard Illumina RNA-Seq protocol. Two micrograms of RNA from each extract was also reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, USA) for qRT-PCR.

3.2.6 Bioinformatics analysis

3.2.6.1 Sequence data analysis

We eliminated low-quality ($q < 30$) sequence reads from the analysis. Further, low quality bases ($q < 30$) were also trimmed from the remaining reads and only the high quality (HQ) reads were retained for further analysis. The mitochondrial/chloroplast genome sequences, ribosomal RNAs, transfer RNAs, adapter sequences were excluded from the analysis. The contamination removal step was performed using bowtie2 (version 2.2.2), in-house Perl scripts (SciGenom, Kerala, India) and Picard tools (version 1.119). The pre-processed reads were aligned to the *Triticum aestivum* reference genome and gene models downloaded from the ENSEMBL database (ftp://ftp.ensemblgenomes.org/pub/release-32/plants/gtf/triticum_aestivum/Triticum_aestivum.TGACv1.32.gtf.gz). The alignment was performed using Tophat2 (version 2.1.0) with default parameters (Ragiba et al., 2004; Trapnell et al., 2009; Kim et al., 2013) and maximum number of hits required to consider the alignment was set as 20 (Trapnell et al., 2009). Gene expression levels were calculated using Cufflinks (version 2.2.1) and the maximum intron length was set to 1000. The differentially expressed genes (DEGs) were estimated using Cuffdiff with statistical significance p value ≤0.01 and q value ≤0.01.

Differential expression was measured as log2 fold change in FPKM (fragments per kilobase per million mapped reads) values. The log2 fold change cutoff for the differentially expressed genes (DEGs) was set ±1.

3.2.6.2 Gene Ontology annotations and pathway analysis

All the differentially expressed transcripts were annotated. Protein sequences for all the DEGs were retrieved from the annotations downloaded from ftp://ftp.ensemblgenomes.org/pub/plants/release-38/fasta/triticum_aestivum. These protein sequences were mapped to the plant proteins in the NCBI (http://www.ncbi.nlm.nih.gov/) nr protein database, using standalone blastp. The blast results were imported in Blast2GO (version 3.1.3) (Conesa et al., 2005) for Gene Ontology (GO) and pathway analysis. The mapping module of Blast2GO retrieved the GO annotations and the annotation module was employed to select reliable functions. The InterPro module was used to generate annotations like motif details, protein domains and protein families from various protein databases. DEGs with log2 fold change (LFC) ≥ ±1, p-value ≤ 0.01 and q-value/false discovery rate (FDR) ≤ 0.01 from three time points were used for stage specific comparisons. To explore the key manipulation in host cells in response to pathogen inoculation, gene ontologies were enriched using singular enrichment analyses (SEA) employing the agriGO (http://bioinfo.cau.edu.cn/agriGO/analysis.php) using *Triticum aestivum* annotations as background reference.

As *Triticum aestivum* annotations are not well established yet and annotations for several DEGs could not be retrieved, protein sequences for the DEGs were searched for homologous sequences in *Oryza sativa* using the STRING database (https://string-db.org; version 10.5) for better insights into the functional properties of the DEGs. Pfam enrichment was obtained from string protein-protein interaction (PPI) analysis tool. The blast hits identifiers obtained from *O. sativa* were used for KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment and network analysis in Cytoscape (version 3.2.1) using the ClueGo plugin (Bindea et al., 2009). The Multiple Experiment Viewer (MEV) main data visualization and analysis tool was used for hierarchical and k-means clustering of the DEGs, common to three stages in both genotypes.

3.2.7 Selection of reference genes and primer designing

Housekeeping genes have been frequently used as internal reference genes for normalization as they are expected to express uniformly in all cells and tissues of the organism under varied developmental, environmental as well as biotic and abiotic stress conditions. Several housekeeping genes like *ACTIN*, *TUBULIN*, *18S rRNA* and Elongation factor (*EF-1α*) have been reported to express constitutively in various plant tissues under varied conditions and hence are being used as internal reference genes in qRT-PCR for years. For this study, we acquired the primer sequences for *EF-1α* (Zhu et al., 2012), *ACTIN* (Dong et al., 2010) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Beccari et al., 2010) from the reported literature. Additionally, five genes reported by Long et al. (2010), which showed stable expression across biotic and abiotic stresses as well as in various developmental stages, were selected. These genes were: phytochelatin synthase (*PCS*), heterogeneous nuclear ribonucleoprotein Q (*hnRNP*), scaffold-associated regions DNA binding protein (*SAR*), ubiquitin-conjugating enzyme 2 (*ULE*) and eukaryotic translation initiation factor-5α1 (*eIF-5α1*). Further, these five genes showed higher potential as the internal reference genes since they were among those with stable expression in nine microarray experiments involving 333 datasets and further validated by qRT-PCR with cDNA samples from different tissues, stages of development and environmental conditions. The probes for these genes had been designed from wheat expressed sequence tags (ESTs) or full-length cDNAs (FL-cDNA) (Long et al., 2010).

To design primers for these five genes, we used the respective ESTs/FL-cDNAs as blastn query against the *T. aestivum* non-redundant nucleotide sequences in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The best hits were subjected to prediction of open reading frame (ORF) (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and the longest ORF was used for marking the start and end positions of the coding sequence (CDS). Multiple primers were designed for each gene using Primer3 (http://bioinfo.ut.ee/primer3/), targeting the 5' and 3' untranslated region (UTR) of the mRNA transcript, so that wheat-specific amplification would be expected. The transcript sequences were then used as query for blastn against the recently published draft genome sequence of hexaploid wheat (http://plants.ensembl.org/Triticum_aestivum/Tools/Blast) to detect their chromosomal locations. All the genes, except *eIF-5α1*, indicated three homoeologous

positions. The multiple primer sequences that were designed for each gene were also subjected to blastn in similar manner and confirmed that they were targeted to the respective genes. More importantly, to eliminate the possibility of cross-amplification of the pathogen genes, the primers designed for all the genes were also used for blastn against the *B. sorokiniana* gene models in the Mycocosm database at the Joint Genome Institute (JGI; http://genome.jgi.doe.gov/pages/blast-query.jsf?db=Cocsa1) and the primers that did not show match with the pathogen transcripts or showed mismatch at the 3' end were selected for further analysis. Of the eight candidate reference genes selected for the initial evaluation, *EF-1α* and *ACTIN* were rejected for showing either cross-amplification with fungal cDNA or non-specificity with wheat *ACTIN* gene sequence. Hence, only six genes were evaluated further as the candidate reference genes (**Table 3.1**). The primers for the genes (**Table 3.2**) were synthesized by Eurofins (India).

3.2.8 PCR screening and qRT-PCR analysis

The primers were initially tested for amplification by end-point PCR using cDNA from the four treatments *viz*., susceptible control (DDK 1025 control; designated as 'DC'), susceptible inoculated (DDK 1025 inoculated; designated as 'DI'), resistant control (Chirya 3 control; designated as 'CC') and resistant inoculated (Chirya 3 inoculated; designated as 'CI') from 7 dpi stage as well as the mycelial cDNA of the PDA-grown culture of *B. sorokiniana* strain D2. After successful amplification of a single sharp band only with wheat cDNA samples (DC, DI, CC and CI), but not with the pathogen cDNA, the primers were used for standardizing the qRT-PCR conditions using the 'SYBR Green' chemistry (Roche, Switzerland) and the ABI 7900HT Fast Real Time PCR System (Applied Biosystems, USA). We found that the *EF-1α* primers cross-amplified fungal cDNA even at higher annealing temperatures (>62°C). In contrast, the wheat *ACTIN* gene primers amplified only the wheat cDNA and not the fungal cDNA. However, the Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) analysis did not show match of the primers with the wheat *ACTIN* gene sequence, resulting in ambiguity. Due to these reasons, we did not evaluate the *EF-1α* and *ACTIN* gene primers further and only six genes were evaluated.

Table 3.1: Wheat genes selected as candidates for reference genes for qRT-PCR

Sr No	**Gene name**	**Probe (Affymetrix)**	**GenBank Accession no.**	**Best hit for *T. aestivum***	**Chromosomal position on the IWGSC draft genome sequence**	**Reference**
1	Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	-	-	EF592180.1	Group 6; Long arm	Beccari et al. (2010)
2	Phytochelatin synthase (*PCS*)	Ta.24713.1.A1_at	BJ274652	AF093752.1	Group 7; Short arm	Long et al. (2010)
3	Heterogeneous nuclear ribonucleoprotein Q (*hnRNP*)	Ta.10105.1.S1_at	CA603317	AK331502.1	Group 2; Long arm	Long et al. (2010)
4	Scaffold-associated regions (*SAR*) DNA binding protein	Ta.14126.1.S1_at	BE429982	AK330303.1	Group 7; Long arm	Long et al. (2010)
5	Ubiquitin-conjugating enzyme 2 (*ULE*)	Ta.23834.1.S1_at	CD900040	AY952317.1	Group 1; Long arm	Long et al. (2010)
6	Eukaryotic translation initiation factor-5α1 (*eIF-5α1*)	Ta.1532.1.S1_a_at	BJ231467	DQ167203.2	2DL/3B	Long et al. (2010)

Table 3.2: Primer details for the candidate reference genes for qRT-PCR

Sr No	Gene name	Forward (F) and Reverse (R) primer sequences (5'-3')	Melting Temperature (°C)	Annealing Temperature (°C)	Amplicon size (bp)	Amplicon location
1	Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	F: CCTTCCGTGTTCCCACTGTTG	61.8	60	124	3' region of CDS
		R: ATGCCCTTGAGGTTTCCCTC	59.3	60		
2	Phytochelatin synthase (*PCS*)	F: GCGACAATCTGCTCACTTGGT	59.8	60	119	3' UTR
		R: AGTATGTCCTCCTACCTCACGAA	60.6	60		
3	Heterogeneous nuclear ribonucleoprotein Q (*hnRNP*)	F: TGGATCTGGACAAGTGGCAAGT	60.3	60	91	3' UTR
		R: CACAAGACTACTGCTTCAACCA	60.6	60		
4	Scaffold-associated regions (*SAR*) DNA binding protein	F: TGTCGCGCTTCACTCTCTGT	59.4	60	102	3' UTR
		R: GCTGCTGACATGCCATAGGT	59.4	60		
5	Ubiquitin-conjugating enzyme 2 (*ULE*)	F: TTGAAGACCTGCATCGTGGACA	60.3	60	100	3' UTR
		R: GATTGGATTACACACAACCAGC	60.6	60		
6	Eukaryotic translation initiation factor 5α-1 (*eIF-5α1*)	F: TAGTGCTTCTGGGTGTTTGAGA	62.1	60	117	3' UTR
		R: ACTAGCAGCACCTCGAGCAT	61.4	60		

The PCR efficiency for each gene was ensured to be between 1.8 and 2.0, and the dissociation curves were examined to ascertain that there were no non-specific amplifications and primer dimers. However, in case of *eIF-5α1*, the primer dimers were prominent even after multiple levels of optimization, and hence this gene was excluded from further analysis. The primer pairs for the remaining five genes namely, *GAPDH*, *PCS*, *hnRNP*, *SAR* and *ULE*, which showed specified single band with desired amplicon size and good amplification efficiency, were profiled across the seven time points (6 and 12 hpi, 1, 4, 5, 6 and 7 dpi). The reactions were performed with three biological replicates and three technical replicates. Each reaction contained 5 ng cDNA template (diluted to 1:20), 0.33 µM of each primer and 5 µl SYBR Green PCR master mix in 10 µl reaction volume. The thermocycling profile was: initial denaturation at 95ºC for 10 min followed by 40 cycles of 95ºC for 3 sec and 60ºC for 30 sec. Following amplification, a melting dissociation curve was generated using a 60–95°C ramp to monitor specificity of the primers. Using LinRegPCR (version 2017.0) (Ramakers et al., 2003), the exponential phase of the reaction was identified by plotting the fluorescence on a log scale and linear regression analysis was performed to estimate the efficiency of each reaction.

3.2.9 Statistical analysis for stable gene expression

The expression of an ideal reference gene should remain stable under all or most of the experimental conditions. The suitability of a selected gene as the reference gene is calculated in terms of its expression stability score (M). Multiple algorithms (GeNorm (Vandesompele et al., 2002), Normfinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004), and comparative delta-Ct (Silver et al., 2006) can be used to screen the candidate reference genes based on their Ct values for a given data set. The four algorithms vary in their method of calculation of M. The RefFinder suite (http://150.216.56.64/referencegene.php?type=reference) was used for evaluation of stability of the candidate reference genes. RefFinder integrates the four methods and produces a comprehensive stability score of each gene to indicate the most stable gene among the evaluated ones. These algorithms were applied separately to different data sets: i) all experimental samples, ii) DDK 1025 (control and inoculated), iii) Chirya 3 (control and inoculated), iv) DDK 1025 control, v) DDK 1025 inoculated, vi) Chirya 3 control, vii) Chirya 3 inoculated, viii) Control (DDK 1025 and Chirya 3) and

ix) Inoculated (DDK 1025 and Chirya 3) to evaluate the stability of the five genes across different datasets.

3.2.10 Evaluation of gene expression modulation in candidate defense-related genes

Eight candidate genes that had previously been reported to be associated with plant defense and/ or expression modulation in response to fungal pathogens were evaluated for expression variation in response to *B. sorokiniana* in this study. These were, actin depolymerization factor (*TaADF-7*), allene oxide synthase (*TaAOS*), heat shock protein (*TaHSP-70*), lethal leaf spot-1 (*TaLLS-1*), metacaspase-4 (*TaMCA-4*), monodehydroascorbate reductase (*TaMDHAR-4*), non-expresser of pathogenesis related protein 1 (*TaNPR-1*) and SNARE (*TaSYP-132*). The details of these selected genes are provided in **Table 3.3**.

3.3 Results and Discussion

3.3.1 Plant disease progression and tissue collection

After inoculation, all the plants were monitored for disease symptoms and photographed daily till 11 days (**Figure 3.1**). The mock inoculated plants of DDK 1025 (DC) grew healthy under green-house conditions; whereas the pathogen inoculated plants of DDK 1025 (DI) showed severe infection by 7 dpi and died by 9 dpi. However, the fungal inoculated plants of Chirya 3 (CI) remained healthy and grew like control plants of Chirya 3 (CC). Joshi et al. (2007) proposed that the stay green trait can be used for screening susceptible and resistant varieties. We too found that the Chirya 3 plants were green and healthy even after pathogen stress, supporting the reported resistance character of the variety (Ragiba et al., 2004; Neupane et al., 2007).

Table 3.3: Candidate plant defense genes evaluated in the spot blotch pathosystem

Sr No.	**Gene**	**GenBank Accn. No.**	**Role in cellular function**	**Participation in plant defense**	**Reference**
1	Actin depolymerization factor (Ta*ADF-7*)	JX486723.1 (nucleotide)	Actin deplymerization-cytoskeletal modulation	Knock-down wheat showed susceptibility to avirulent *Puccinia striiformis*	Fu et al. (2014)
2	Allene oxide synthase (Ta*AOS*)	AY196004.1 (nucleotide)	Jasmonic acid biosynthesis	Upregulated in response to leaf rust infection in wheat	Chauhan et al. (2015)
3	Heat shock protein (Ta*HSP-70*)	GQ280382 (nucleotide)	70-kD heat shock protein	Upregulated in response to the virulent race of the pathogen	Duan et al. (2011)
4	Lethal leaf spot-1 (Ta*LLS-1*)	AFC61176.1 (protein)	Pheophorbide a oxygenase (PaO); cell death suppressor	Increased tolerance of *Puccinia striiformis* infected transgenic wheat over-expressing lls-1	Tang et al. (2013)
5	Metacaspase-4 (Ta*MCA-4*)	JN807891 (nucleotide)	Protease, participates in programmed cell death	Knockdown of the gene expression increased hyphal extension and branching and reduced leaf necrosis	Wang et al. (2012)

Sr No.	Gene	GenBank Accn. No.	Role in cellular function	Participation in plant defense	Reference
6	Monodehydroascorbate reductase (Ta*MDHAR-4*)	JX034702 (nucleotide)	Regulation of the ROS level via the ascorbate-glutathione (AsA-GSH) cycle	Modulated during infection with *P. striiformis*	Feng et al. (2014)
7	Non-expresser of pathogenesis related protein 1 (Ta*NPR-1*)	KM017012 (nucleotide)	Cross-talk between hormone signaling pathways	Expression modulation in *Fusarium* head blight; colocation with resistance gene	Diethelm et al. (2014)
8	SNARE (Ta*SYP-132*)	JX104550 (nucleotide)	Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) vesicle fusion	Modulated upon inoculation with *P. striiformis*; knockdown induces susceptibility to avirulent strain	Wang et al. (2014)

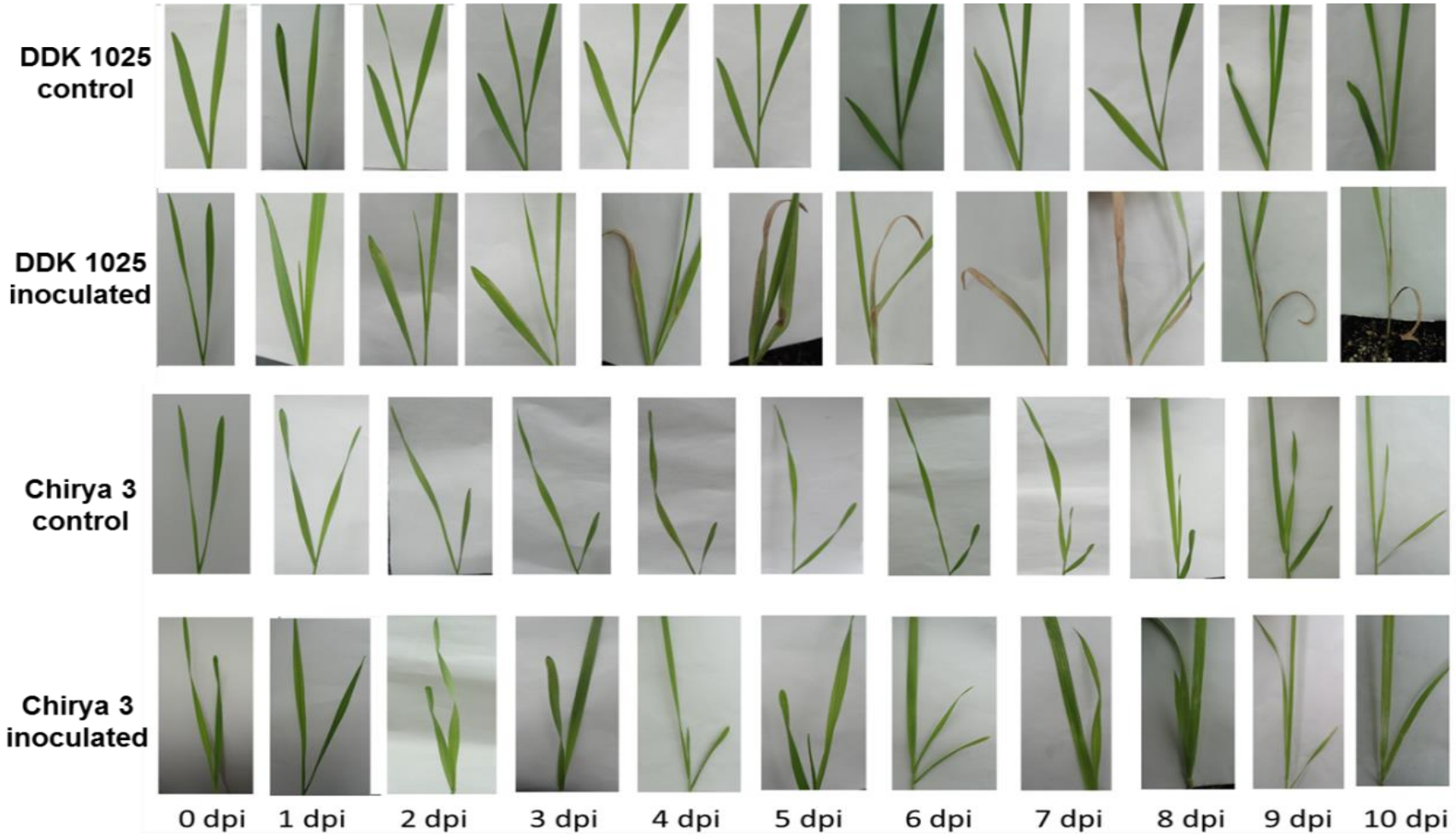


Figure 3.1: Photographs of the first leaf of plants from the four experimental sets for 11 days (0 – 10 dpi).

3.3.2 Photosynthetic efficiency

Foliar pathogens disturb the plant leaf physiology through membrane damage. We observed that pathogen inoculation negatively affected leaf health and reduced photosynthetic efficiency of the infected plants. The photosynthetic efficiency did not change much till 4 dpi in fungal inoculated plants of both DDK 1025 and Chirya 3. However, after 4 dpi, the photosynthetic efficiency started to progressively decrease in fungal inoculated plants of DDK 1025 till the leaves were dead (**Figure 3.2**). In contrast, in fungal inoculated Chirya 3, a transient drop was seen at 5 dpi; however, the plants recovered to the normal level by 7 dpi. Positive correlation between photosynthetic efficiency and spot blotch resistance was reported by Rosyara et al. (2010). The recovery of photosynthetic efficiency by Chirya 3 plants can be attributed to the resistance phenomenon (Ragiba et al., 2004; Neupane et al., 2007) as well as the stay green trait (Joshi et al., 2007).

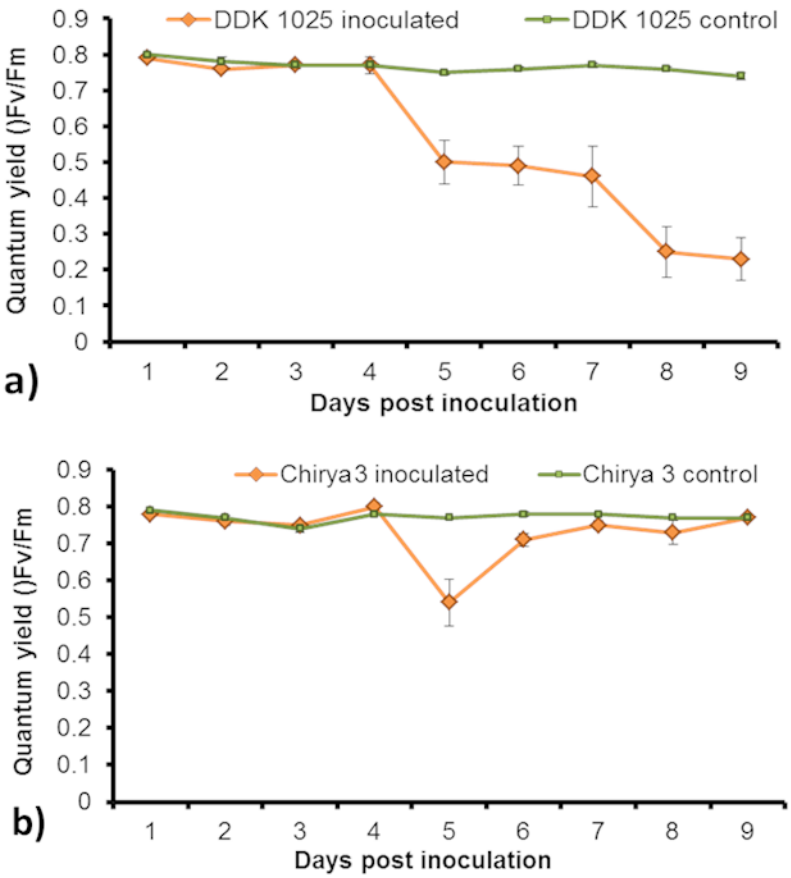


Figure 3.2: Photosynthetic efficiency of leaves in control and *B. sorokiniana* inoculated plants of a) DDK 1025 and b) Chirya 3

3.3.3 Chlorophyll estimation using HPLC

Chlorophyll content is highly affected by *B. oryzae* infection in rice (Dallagnol et al., 2011). Relative quantification of total chlorophyll content (chlorophyll a and b) of leaves depicted decrease in chlorophyll levels upon pathogen inoculation. Initially, Chirya 3 showed about 10% drop in total chlorophyll content, which was slowly and progressively reduced thereafter. In contrast, progressive decrease in total chlorophyll content was observed in case of *B. sorokiniana* inoculated plants of DDK 1025 and over 35% decrease in total chlorophyll content was observed by 6 dpi (**Figure 3.3**). Our findings match the results obtained under field condition on adult wheat plants infected with *B. sorokiniana* (Rios et al., 2017). This further assures the implication of our findings for field application too.

Activation of defense responses requires modulation of regular plant processes like photosynthesis and primary metabolism (Scharte et al., 2005). Downregulation of several growth and development related processes was observed in CI. However, even though initial drop in total chlorophyll content was observed in CI, inhibition of photosynthesis was not discernible during incompatible interaction. Our finding supports the hypothesis that a decrease in photosynthetic activity is not necessarily associated with the expression of photosynthetic genes during pathogen infection (Bonfig et al., 2006; Swarbrick et al., 2006).

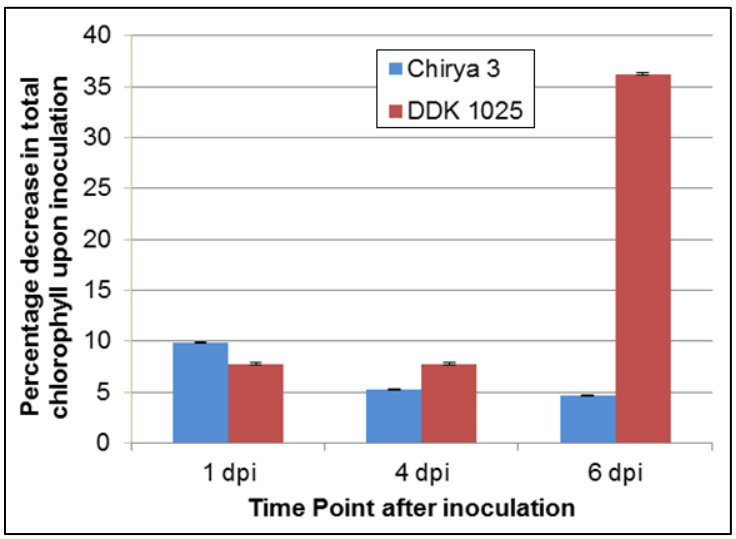


Figure 3.3: Percentage decrease in total chlorophyll content of leaves in control and *B. sorokiniana* inoculated plants of Chirya 3 and DDK 1025.

3.3.4 RNA isolation and cDNA synthesis

Total RNA was isolated from all the four experimental sets and an average of 700 ng/µl RNA was obtained from 100mg wheat leaf tissue. cDNA was synthesized from 2 µg RNA from each sample. Good quality cDNA was obtained as determined by amplification using the *GAPDH* gene primers (**Figure 3.4**).

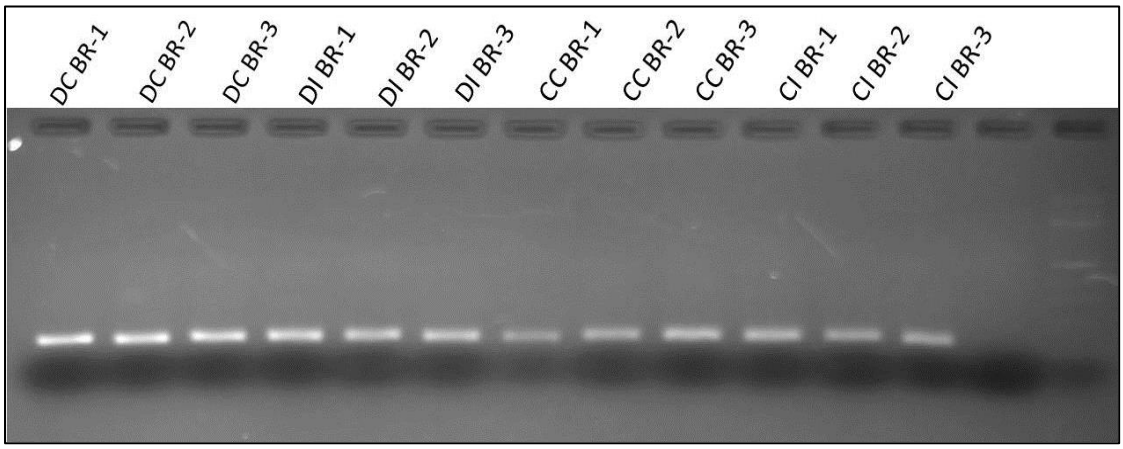


Figure 3.4: Gel electrophoresis of cDNA from 1 dpi samples using PCR with *GAPDH* gene primer (BR - biological replicate)

3.3.5 Bioinformatics analysis

3.3.5.1 Gene expression profiles under pathogen stress

To investigate the effects of infection of the pathogen on wheat plants and its response at molecular level, transcriptomics approach was opted. As RNA-Seq provides far more precise measurement of levels of transcripts and their isoforms than other methods, we performed interactive transcriptome analysis using Illumina HiSeq 2000 (SciGenom, Kerala, India). Total 36 RNA-Seq libraries were generated and sequenced, with two genotypes (Chirya 3 and DDK 1025), two treatments (pathogen inoculated and mock inoculated/control), three time points (1 dpi, 4 dpi and 6 dpi) and three biological replicates for each sample. The number of reads per sample ranged from 60 million to 137 million, with an average of 78 million.

3.3.5.2 Identification of differentially expressed genes

The sequence reads obtained from Illumina HiSeq 2000 were filtered to obtain high quality reads. Filtering involved trimming of low quality bases, adapter removal and

contamination removal. On average, ~99.04% of total reads from all the samples passed the q value threshold of ≥30. These high quality (HQ) reads obtained after filtering were used for reference-based pair-wise alignment using Tophat2 for expression quantification and gene structure prediction. The average read count of the HQ reads from all the samples was 76.97 million. Of which, 92.18% reads were aligned on the *T. aestivum* reference genome (ftp://ftp.ensemblgenomes.org/pub/release-32/plants/gtf/triticum_aestivum/Triticum_aestivum.TGACv1.32.gtf.gz). The average no. of aligned read count was 70.95 million. The overall alignment summary is presented in **Table S3.1**.

3.3.5.3 Gene expression analysis

The normalized expression levels (FPKM: fragments per kilobase per million mapped reads) of wheat transcript were calculated using uniquely mapped reads onto the genomes. FPKM values for the three biological replicates for each sample were averaged for differential gene expression analysis. The analysis was performed using Cuffdiff program of the Cufflinks package with default settings. **Table 3.4** shows the numbers of up- and down-regulated genes found using Cuffdiff analysis with p value ≤0.01, q value ≤0.01 and log2 fold change ≥1. The overview of the total number of DEGs at the three time points showed downregulation of majority of genes in DDK 1025 at initial stages after inoculation, followed by upregulation at later stages. While, Chirya 3 showed overall higher numbers of upregulated genes as compared to downregulated genes at all stages (**Table 3.4** and **Figure 3.5**).

Table 3.4: Numbers of differentially expressed genes at three stages in DDK 1025 and Chirya 3

Genotype	Time point					
	1 dpi		4 dpi		6 dpi	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated
DDK 1025	101	1709	968	594	2604	304
Chirya 3	659	305	1187	933	1075	86

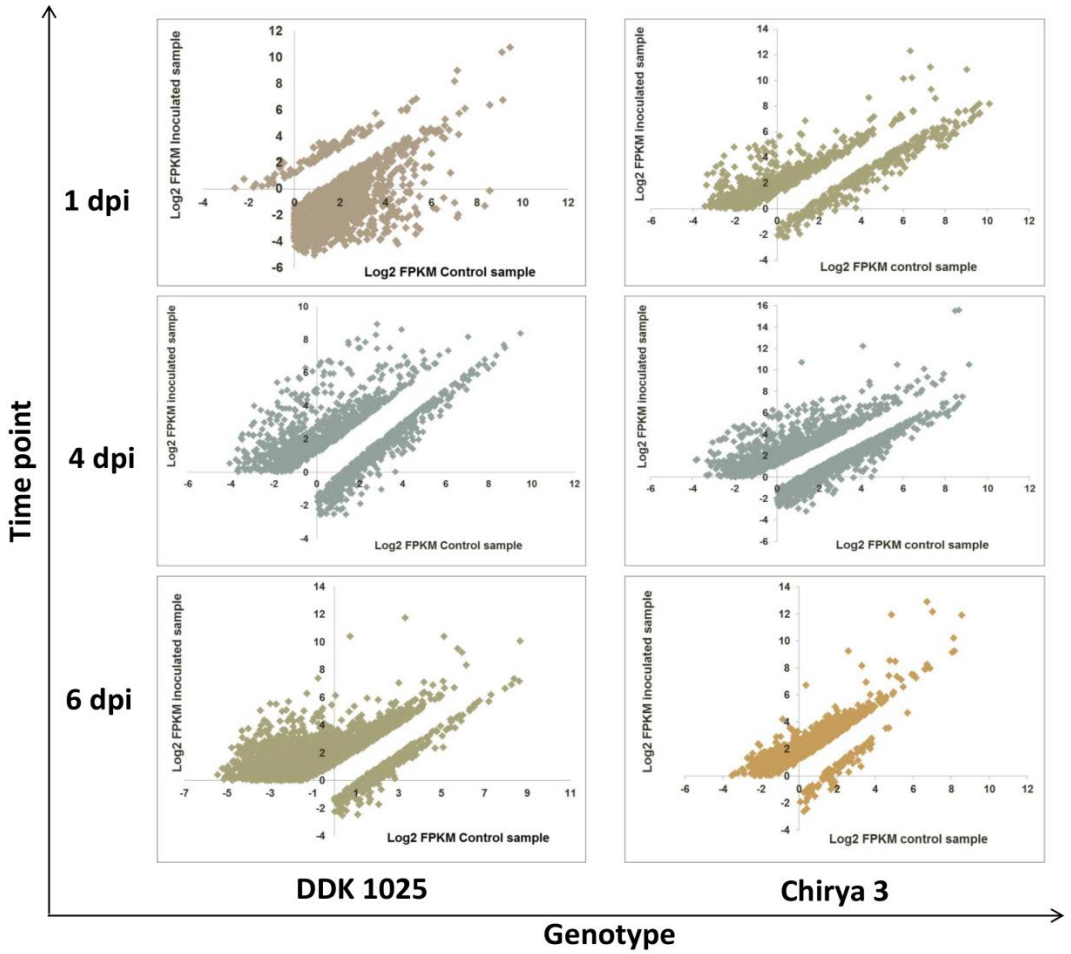
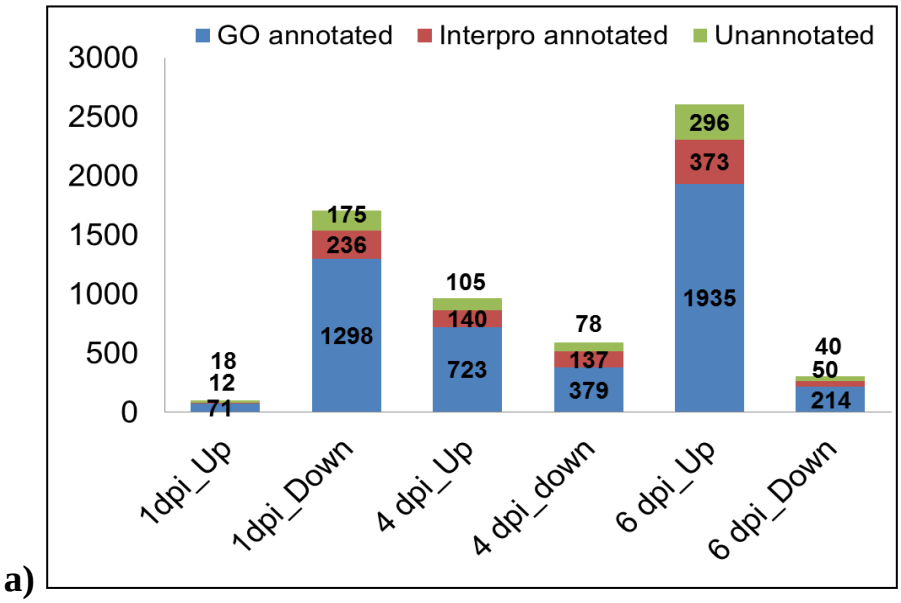


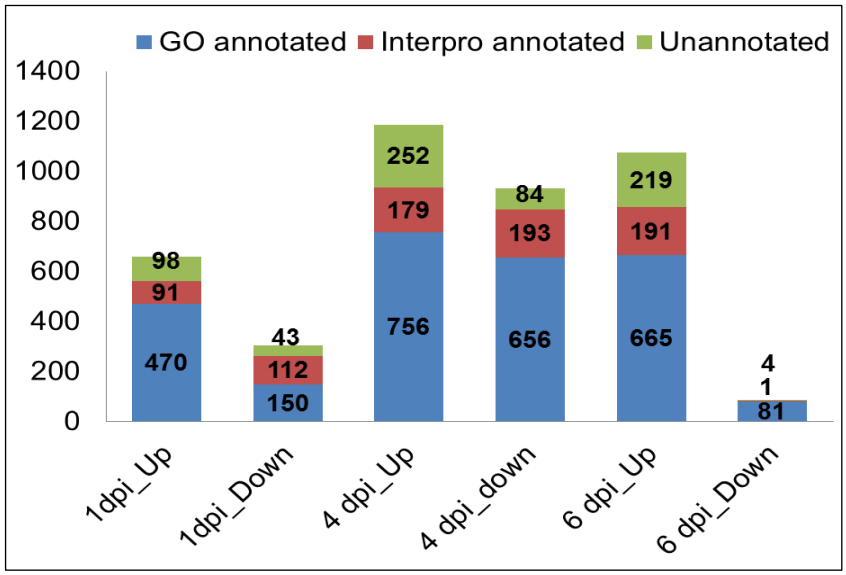
Figure 3.5: Comparison plot of FPKM value of DEGs in control and inoculated varieties

3.3.5.4 Gene ontology annotation

Blast2GO mapping retrieved the GO and InterPro annotations from various protein databases to deduce dependable functions of the differentially expressed transcripts. The transcripts were first checked for associated GO annotations. The transcripts not having any GO term were further looked for their InterPro annotations. The transcripts not mapped with either of the annotations were tagged as unannotated. The statistics of the annotation is presented in **Figure 3.6**.



a)



b)

Figure 3.6: Annotation statistics of differentially expressed genes: a) DDK 1025, b) Chirya 3

3.3.6 Transcriptome analysis during compatible interaction

As stated before, the *B. sorokiniana* inoculated plants of DDK 1025 (DI, spot blotch susceptible) developed severe infection by 7 dpi and died by 9 dpi. Analysis of transcriptomics data from this compatible interaction was performed and the results are discussed below.

3.3.6.1 Gene ontology enrichment

Gene ontology enrichment provides an overview about the biologically significant mechanisms underlying the event. GO term categorizes the gene terms into biological processes, molecular function and cellular component. Upregulated and downregulated genes were individually analyzed for GO enrichment. Carbohydrate metabolic process was the dominant term for upregulated genes in DI at 1 dpi; while the response to pathogen stress and stimulus were enriched in downregulated genes group. The gene ontologies associated with response to pathogen were enriched in upregulated group at 4 dpi onwards. Other enriched ontologies were secondary metabolic process, anatomical structure development and cell death (**Figure 3.7**). In the molecular function category, carbohydrate binding, protein binding and enzyme regulator activity were upregulated (**Figure 3.8**). The extracellular constituents were dominant in upregulated set of cellular component category (**Figure 3.9**).

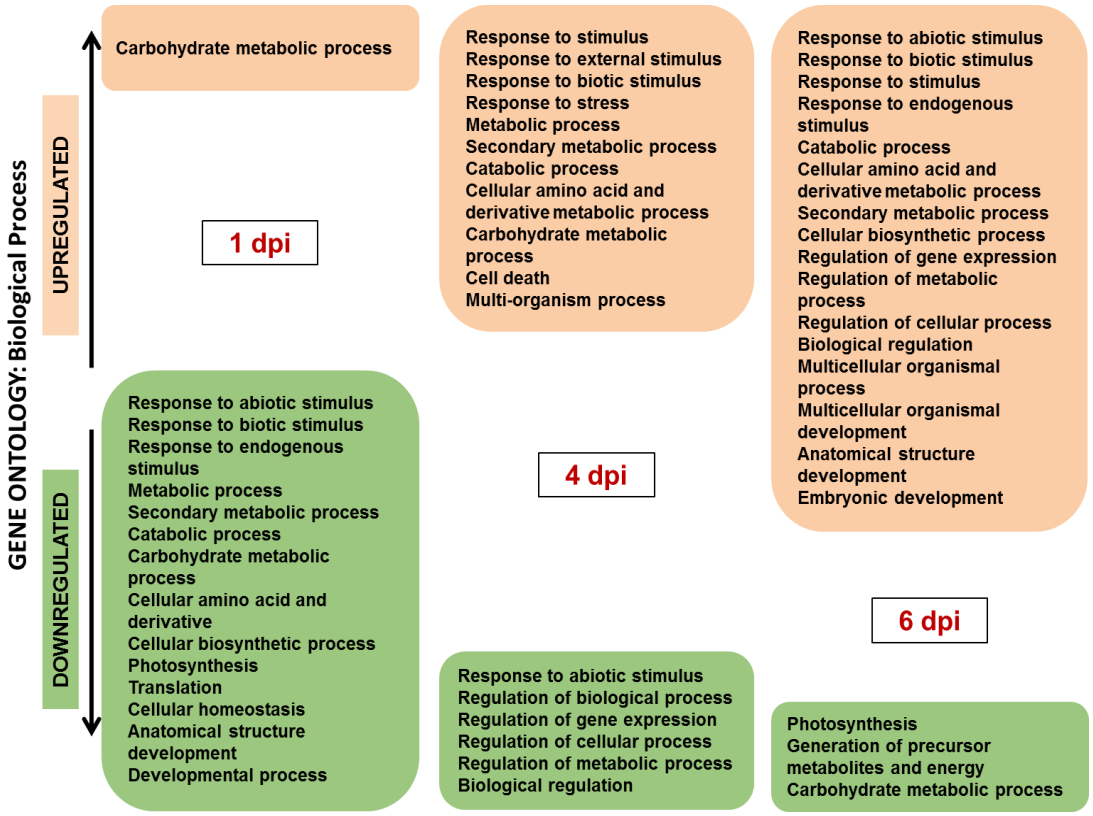


Figure 3.7: Gene ontology enrichment for DEGs in DI under Biological Process category

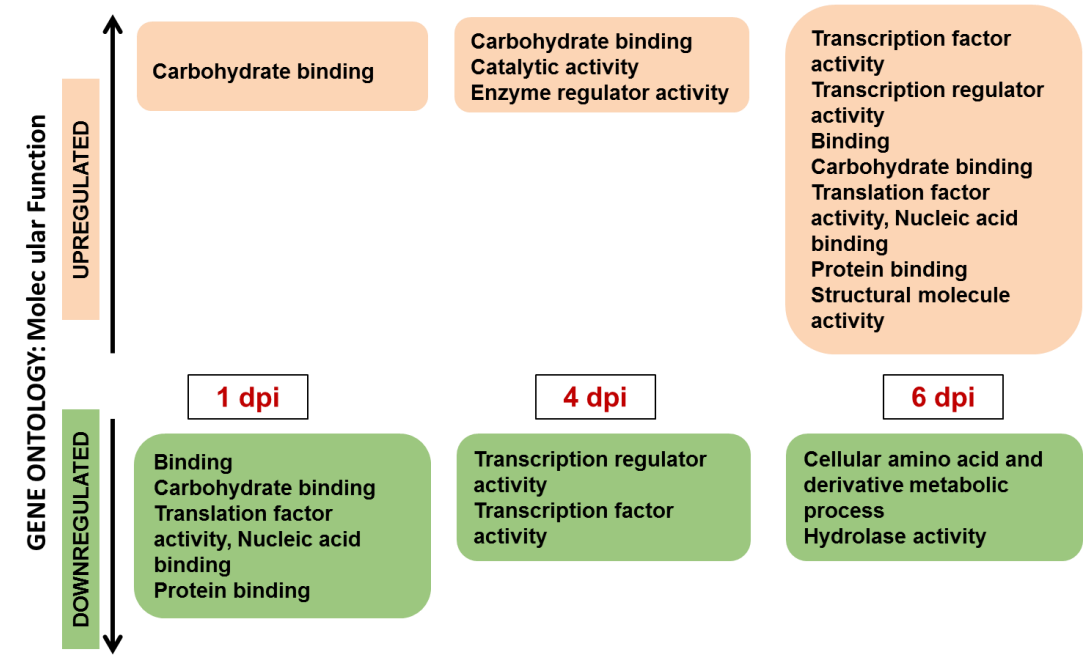


Figure 3.8: Gene ontology enrichment for DEGs in DI under Molecular Function category

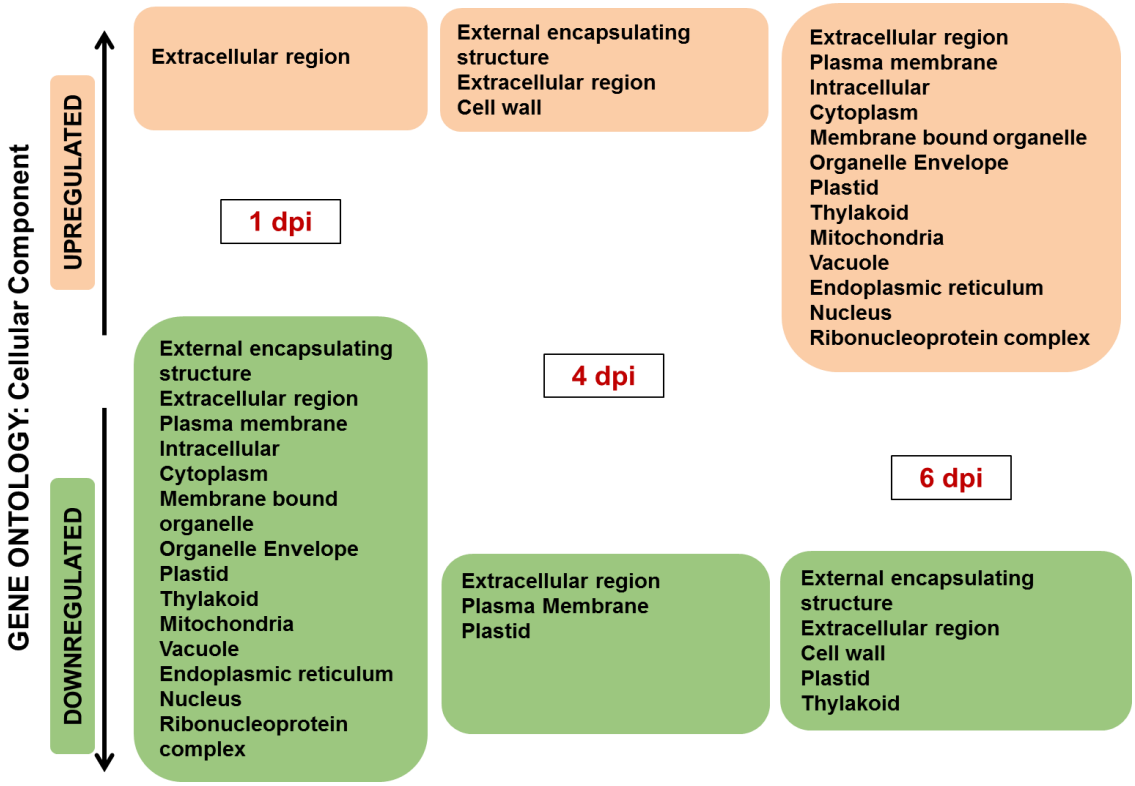


Figure 3.9: Gene ontology enrichment for DEGs in DI under Cellular Component category

3.3.6.2 Modulation in metabolic processes

DEGs from each time point from the up- and down-regulated sets were mapped individually to get insights into the metabolic pathways affected by pathogen invasion. As depicted below, photosynthesis and protein processing in endoplasmic reticulum were the principal components to be downregulated at 1 and 4 dpi. In contrast, glutathione metabolism, phenylpropanoid biosynthesis and plant-pathogen interaction were the key processes in the upregulated group (**Figure 3.10**).

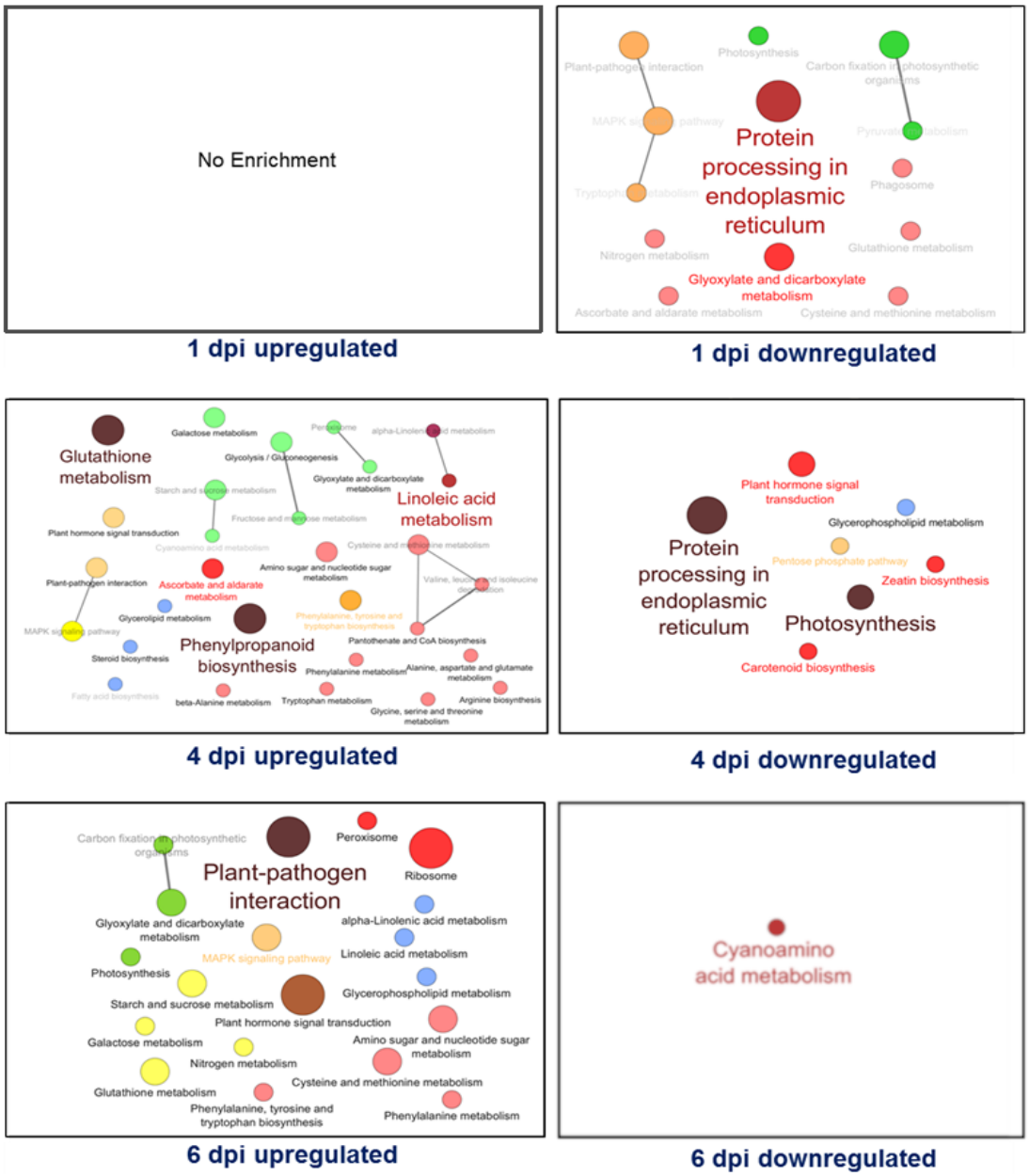


Figure 3.10: Modulation in metabolic processes and KEGG pathway network of DEGs in DI

Analysis of the DEGs showed induction of glycosyl hydrolase 32 and expansin genes and suppression of protein kinases, chitin binding protein, cellulose synthase, glyceraldehyde phosphate dehydrogenase, Hsp90, lipoxygenase, amino acid lyase and several transporters at 1 dpi. Likewise, glutathione-s-transferase, phenyl ammonia lyase, peroxidase, glycosyl hydrolase family 17 and 19 were overexpressed and chlorophyll a/b binding protein, expansin, chalcone synthase, and RNA polymerase were repressed at 4 dpi. However, at 6 dpi several protein kinases, glyceraldehyde 3-phosphate dehydrogenase, ubiquitin, transcription factors, and transporters were upregulated while several chlorophyll a/b binding protein, leucine rich repeat, glycosyl hydrolase family 16, peptidases, and peroxidases were downregulated.

3.3.6.3 DEGs common to DI in three stages

DEGs common to the three stages were extracted using Venny 2.0 (http://bioinfogp.cnb.csic.es/tools/venny) (Oliveros, 2007-2015). About 177 genes were differentially expressed in all the three stages considered together (**Figure 3.11**). Gene ontology enrichment was performed for these 177 genes using agriGo (http://bioinfo.cau.edu.cn/agriGO/analysis.php) (Du et al., 2010) (**Figure 3.12**). Co-expression analysis of DEGs was performed based on K-means clustering using MEV (version 4.9.0; http://mev.tm4.org/#/welcome). These 177 DEGs were classified in six groups according to their log2 fold change expression values (**Figure 3.13**).

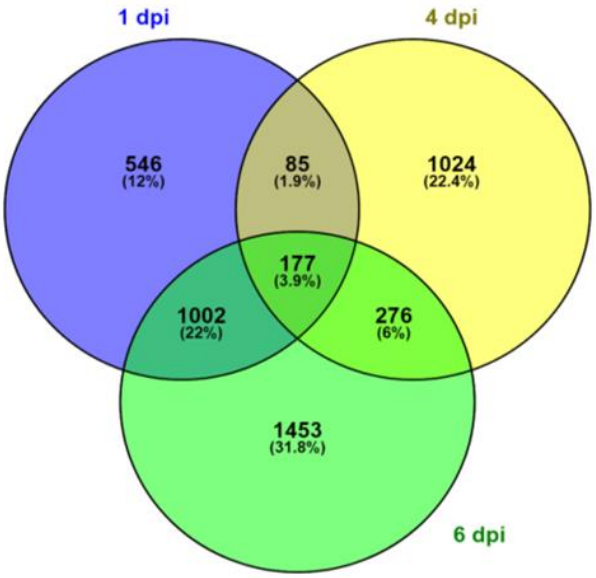


Figure 3.11: Venn diagram showing the DEGs from DI common in three time points

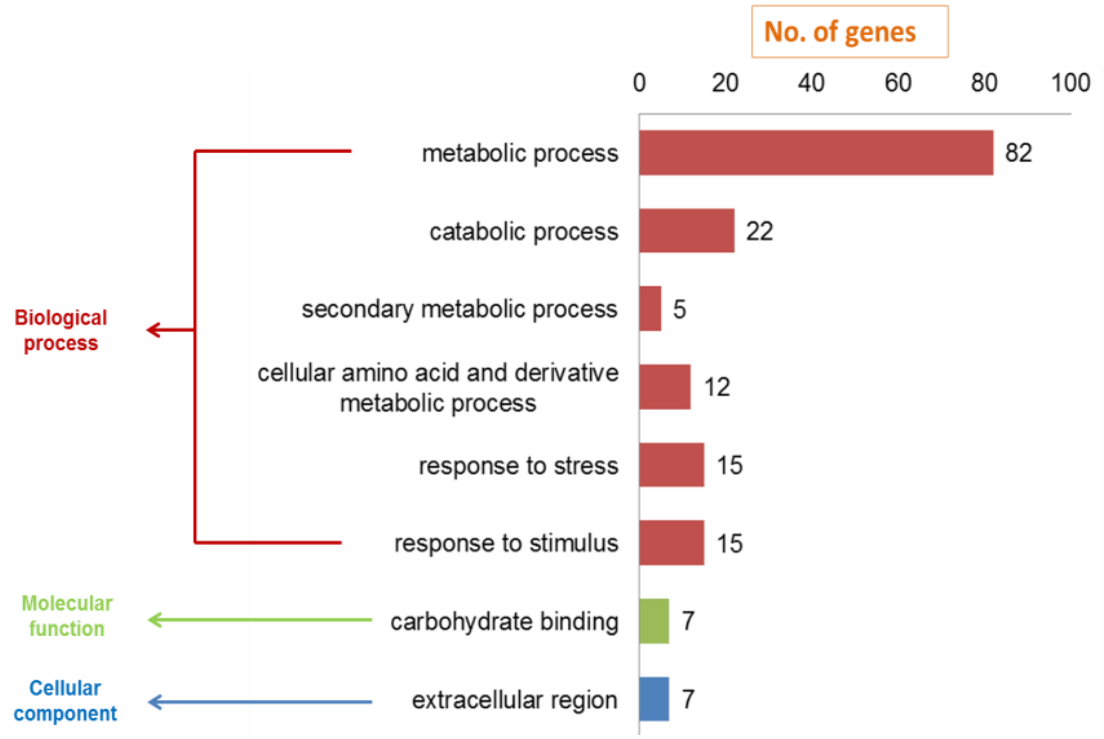


Figure 3.12: Gene ontology enrichment of DEGs common in three time points from DI

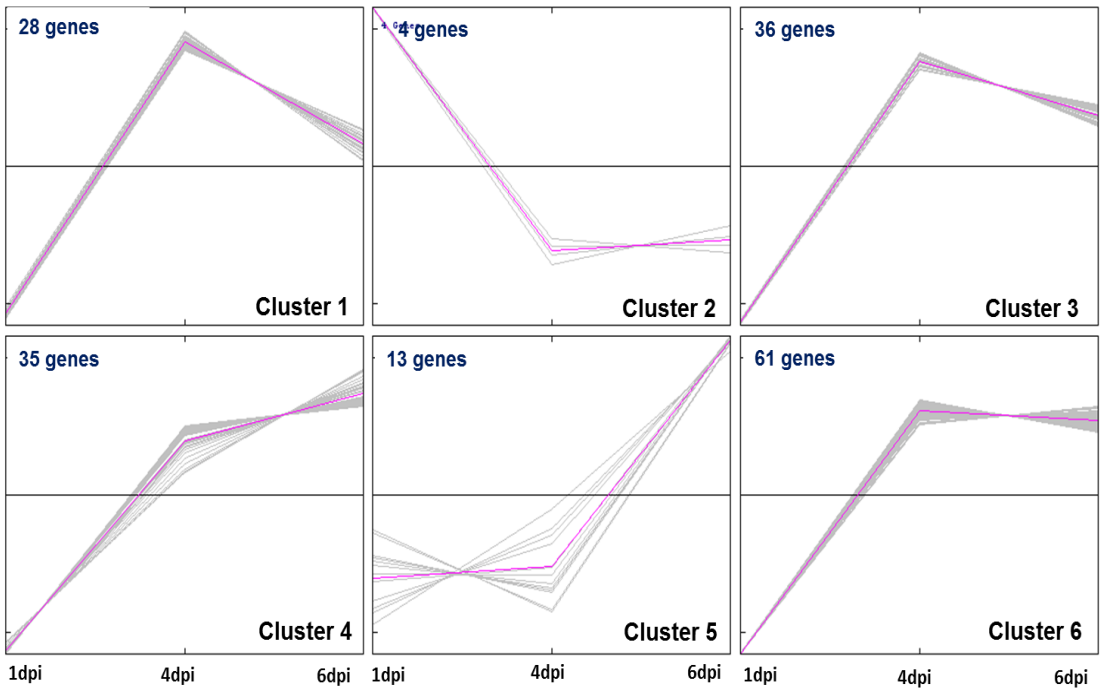


Figure 3.13: K-means clustering of 177 DEGs common to the three stages in DI

3.3.6.4 Functional enrichment analysis of clusters

Functional enrichment analysis was performed using the STRING database (https://string-db.org/) by retrieving *O. sativa* homologs for genes from each cluster. Biological process enrichment was observed in two clusters i.e. Cluster 1 and Cluster 6 (**Figure 3.14**). Both cluster 1 and cluster 6 were downregulated at 1 dpi, upregulated at 4 and 6 dpi and showed differences in their fold change levels. Cluster 1 included catabolic process while cluster 6 included different metabolic and biosynthetic processes.

InterPro annotated two genes from Cluster 6 as phenylalanine ammonia-lyase (PAL) (**Table S3.2**). In plants, PAL is encoded by a multi-gene family with few to more than a dozen copies in species like potato and tomato (Chang et al., 2008). PAL is the first enzyme in biosynthesis of phenylpropanoid. This pathway generates a wide variety of secondary metabolites such as alkaloids, flavanoids, phytoalexins, lignin and other cell wall components. These compounds are important for growth under normal and various stress conditions (Xu et al., 2010; Cass et al., 2015; Zhang and Liu, 2015). As shown by the $\log_2$ fold change pattern of Cluster 6, PAL activity was reduced at 1 dpi followed by accumulation at later stages. Peltonen and Karjalainen (1995) found increased PAL activity in case of fungal inoculation. However, its inhibition had no effect on susceptibility of the plants.

3.3.6.5 Effect on glycolysis pathway upon pathogen infection

Several genes from the glycolysis, pentose phosphate and tricarboxylic acid pathways were altered during *B. sorokiniana* infection. Phosphohexose isomerase and one isoform of transketolase were upregulated at 1 dpi, while other genes were downregulated at this stage and upregulated at other stages (**Figure 3.15**). Phosphoglucose isomerase was also highly upregulated in *Glycine max* upon nematode infection (Ibrahim et al., 2011). Sugar molecules are important molecules not only for primary metabolism but also serve as stress regulators (Ehness et al., 1997). Pathogen interference causes reduction in endogenous sugar levels and accumulation of soluble sugars like hexose during biotrophic interaction (Chou et al., 2000; Berger et al., 2004). Initial suppression of hexokinase activity is responsible for sugar accumulation and then overexpression at later stages causes growth reduction and senescence (Xiao et al., 2000).

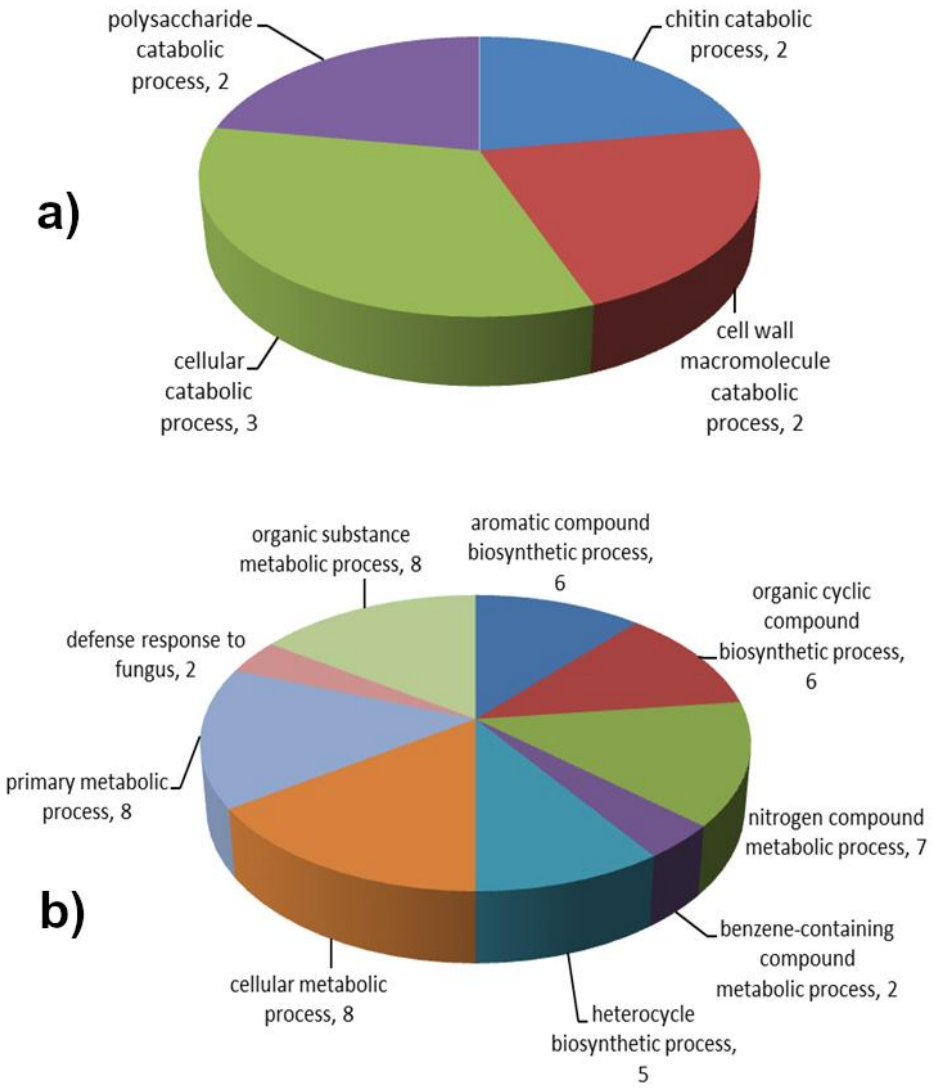


Figure 3.14: Gene ontology enrichment of Biological Process: (a) Cluster 1; (b) Cluster 6

3.3.6.6 Effect on ubiquitin mediated proteolysis pathway

Ubiquitin mediated proteolysis plays an important role in regulation of hormone signaling and receptor control. Regulatory mechanisms ought to ensure a fast response after recognition of the pathogen. Consequently, rapid attenuation of this response is also crucial for resuming normal growth when the threat has passed. Transcriptional repressors of auxin signaling AUX/IAA are degraded through ubiquitin mediated proteolysis, allowing expression of auxin responsive genes (Peer, 2013). The inhibition of auxin responses is an integral part of the salicylic acid mediated disease resistance. According to the F-box hypothesis (Skowyra et al., 1997), F-box proteins in association with Skp1 and cullin regulate the ubiquitin

mediated protein degradation. We also observed differential expression of several components of ubiquitin mediated proteolysis pathway (**Figure 3.16**).

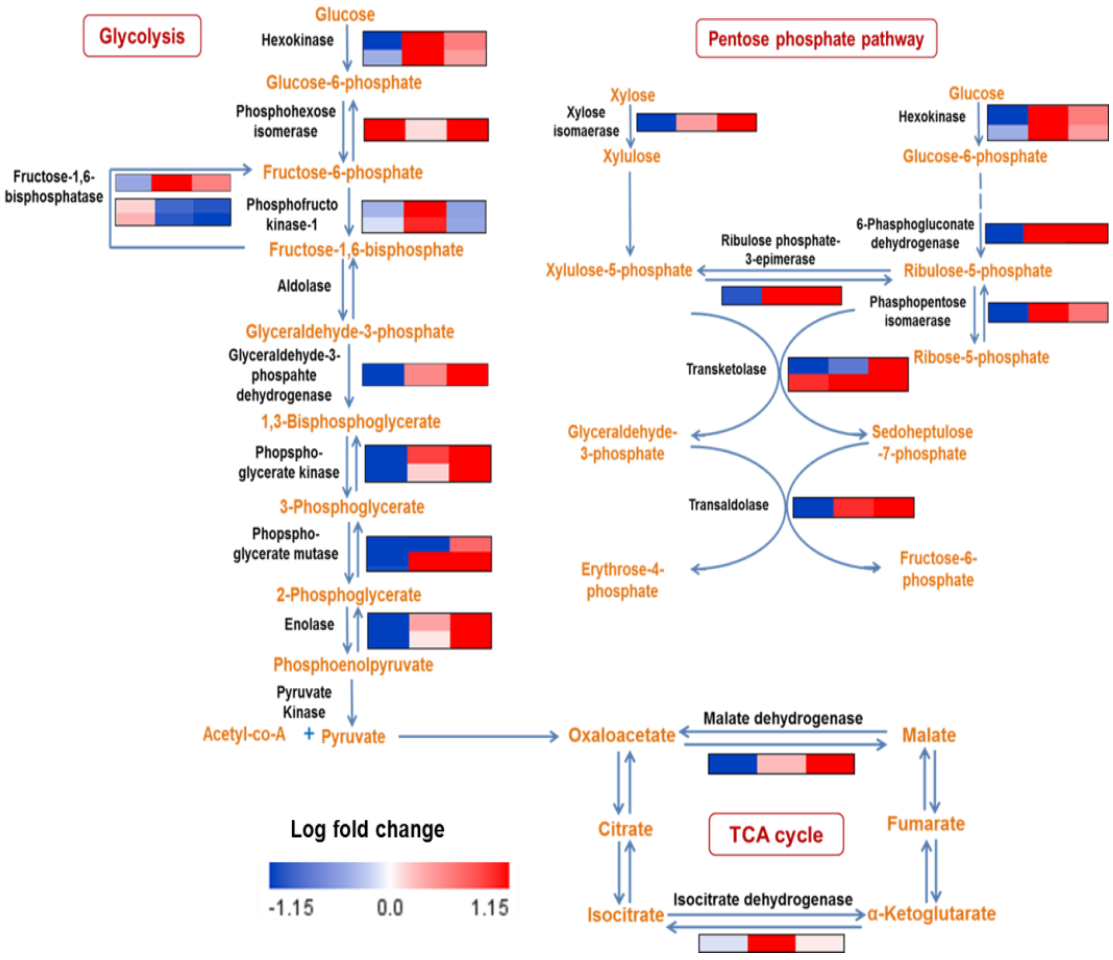


Figure 3.15: Differential expression of glycolysis, pentose phosphate pathway and TCA cycle genes at three stages in DI. The mini-heatmap for each gene shows expression of the detected isoforms as rows in three stages (1, 4 and 6 dpi) as columns.

Negative regulation of F-box auxin receptor enhances susceptibility to biotrophic pathogens (Navarro et al., 2006). Hence, downregulation of several F-box proteins at 1 and 4 dpi, observed in the present study, might account for the susceptibility of DDK 1025 to *B. sorokiniana*. However, overexpression of these proteins at later stage could be attributed to the efforts of the plants for systemic acquired resistance. The ubiquitin pathway is also commonly used to control receptor levels. Pathogen virulence/ avirulence genes are recognized through receptor binding.

Altered expression of receptors has been observed in wheat due to fungal attack (Xiao et al., 2013). To our surprise, overexpression of several components of the ubiquitin pathway was detected at later stage in DI. We suggest that rapid protein degradation occurring in DDK 1025 might be in responsible for cell death and leaf senescence. Further, identification of the targets for the differentially expressed F-box proteins can reveal the regulatory mechanism of pathogen susceptibility.

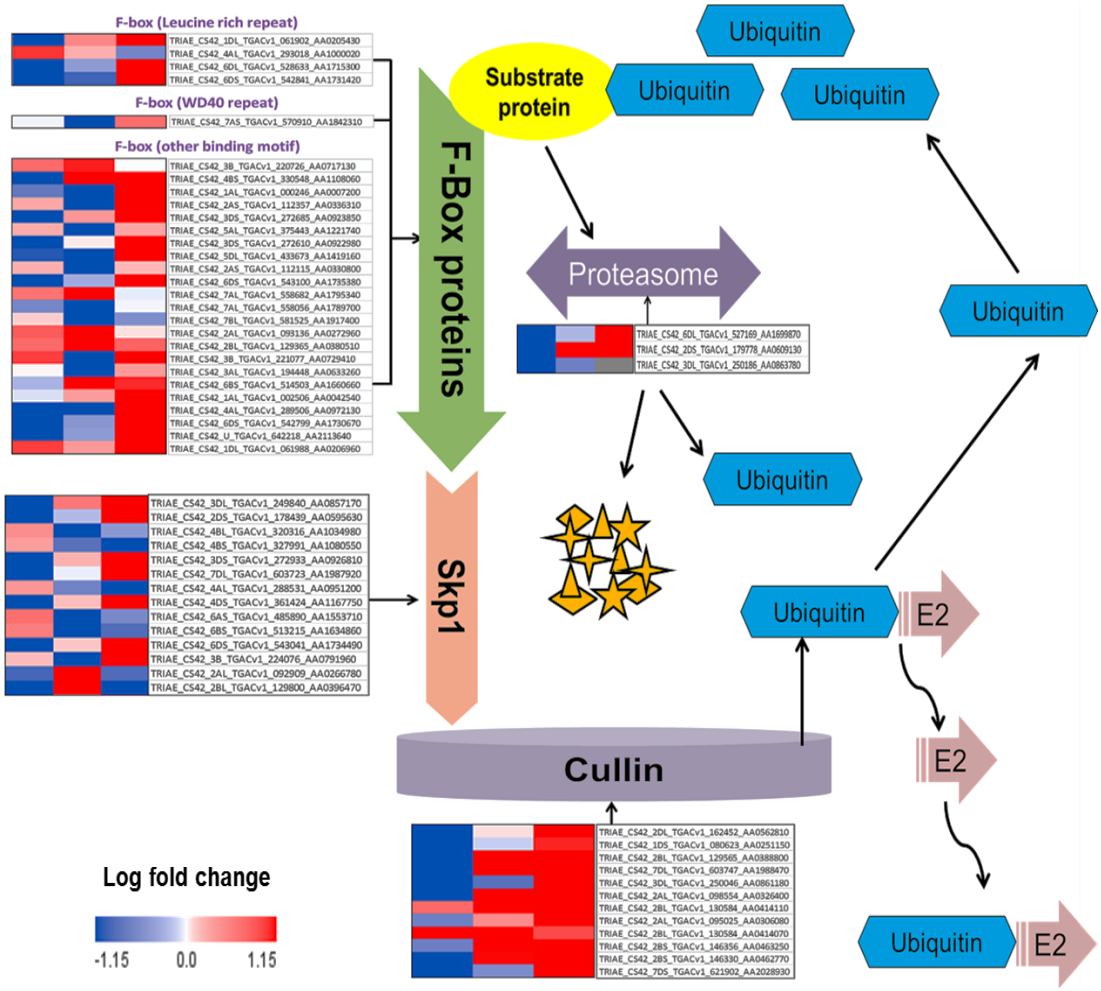


Figure 3.16: Differential expression of ubiquitin mediated proteolysis pathway in DI

3.3.7 Transcriptome analysis during incompatible interaction

In case of Chirya 3 (resistant to spot blotch), the *B. sorokiniana* inoculated plants (CI) did not show any spot blotch symptoms, except mild whitening of leaves at 9 dpi. Analysis of transcriptomics data from this incompatible interaction was performed and the results are discussed below.

3.3.7.1 Gene ontology enrichment

The DEGs from the resistant variety Chirya 3 were also enriched and categorized into biological processes, molecular function and cellular component. In CI, response to biotic stimulus was upregulated, while response to abiotic stimulus was downregulated at 1 dpi (**Figure 3.17**). However, metabolic process associated GO terms were enriched in downregulated genes group at 4 dpi; while upregulation of the same was observed at 6 dpi along with initiation of transcriptional regulation. In the molecular function category, in addition to carbohydrate binding (as observed in DI), catalytic activity, enzyme regulator activity and transcriptional activity were also upregulated (**Figure 3.18**). The extracellular constituents were dominant in upregulated set of cellular component (**Figure 3.19**).

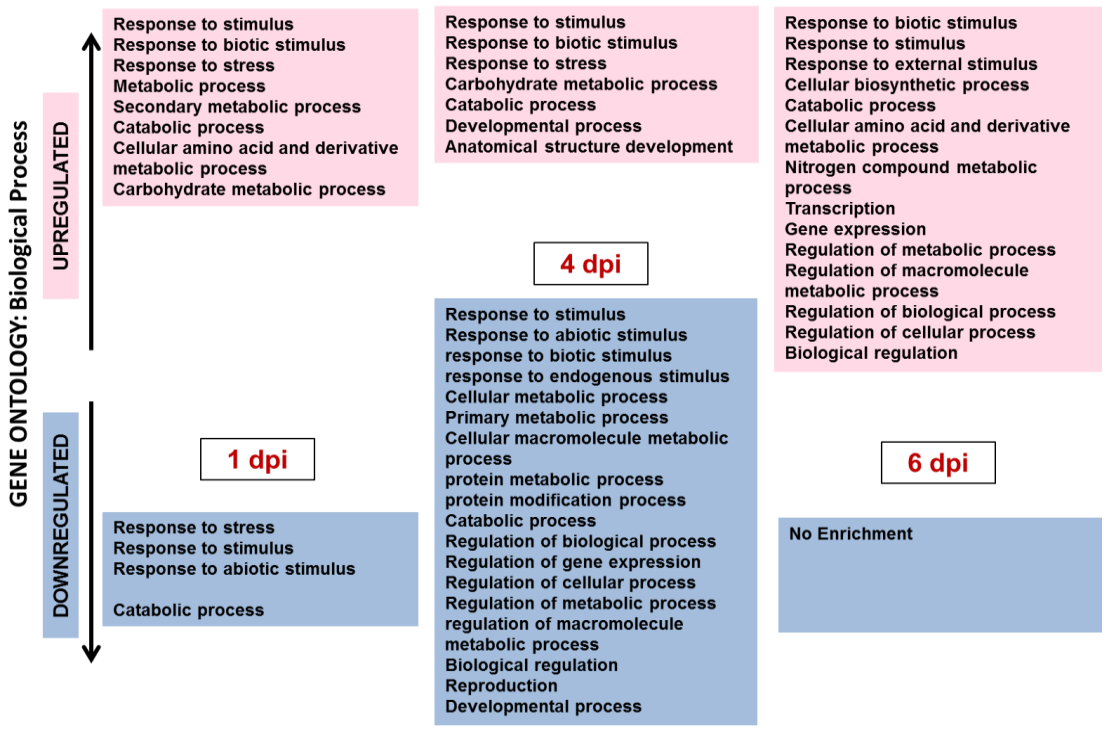


Figure 3.17: Enriched gene ontology for DEGs from CI under Biological Process category

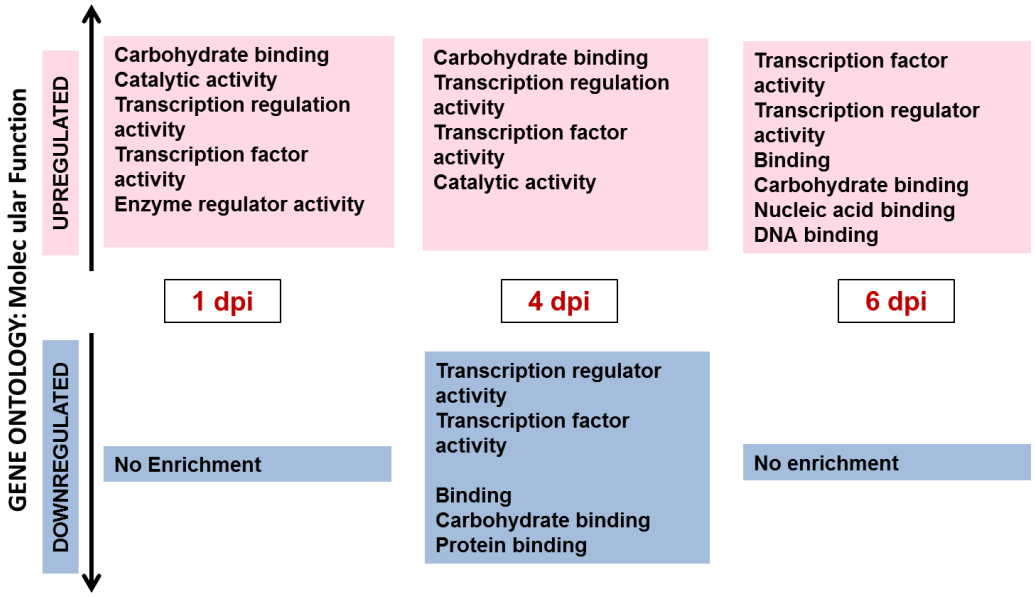


Figure 3.18: Enriched gene ontology for DEGs from CI under Molecular Function category

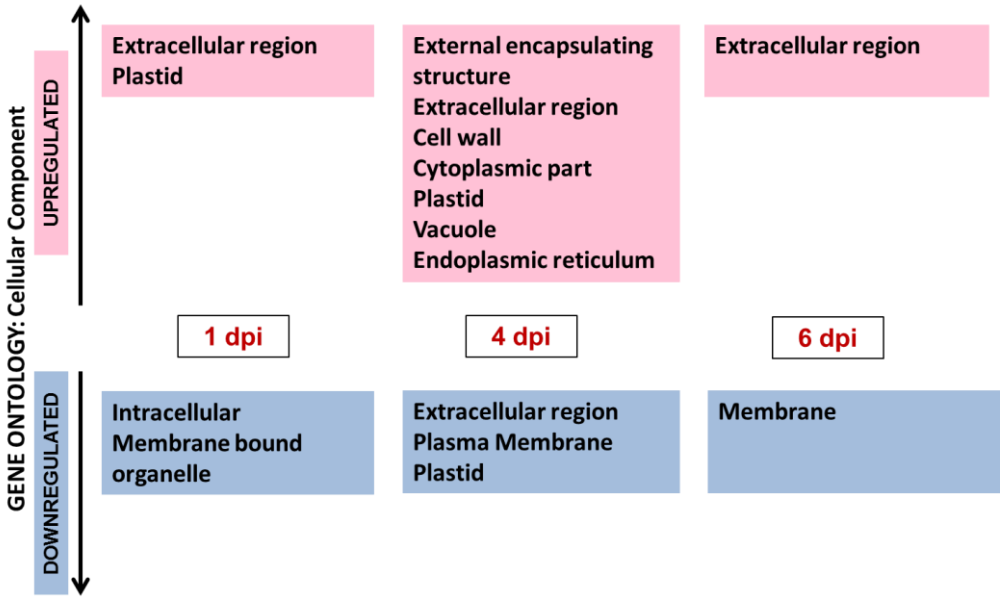


Figure 3.19: Enriched gene ontology for DEGs from CI under Cellular Component category

3.3.7.2 Comparison of GO enrichment of DEGs from DI and CI

Gene ontology of DEGs from the two varieties was compared for enriched GO terms. A comparative observation of this data showed differential pattern in their expression (**Figure 3.20**). For instance, response to biotic stimulus was downregulated at 1 dpi in DI but upregulated in CI. However at 4 dpi, it was upregulated in DI. In contrast, in CI, some genes involved in the response to biotic stimulus were upregulated, while other genes from the same GO term were downregulated. This differential recognition and regulation of gene expression could be the basis for pathogen entry and disease establishment in susceptible plants; while blocking these genes results in resistance.

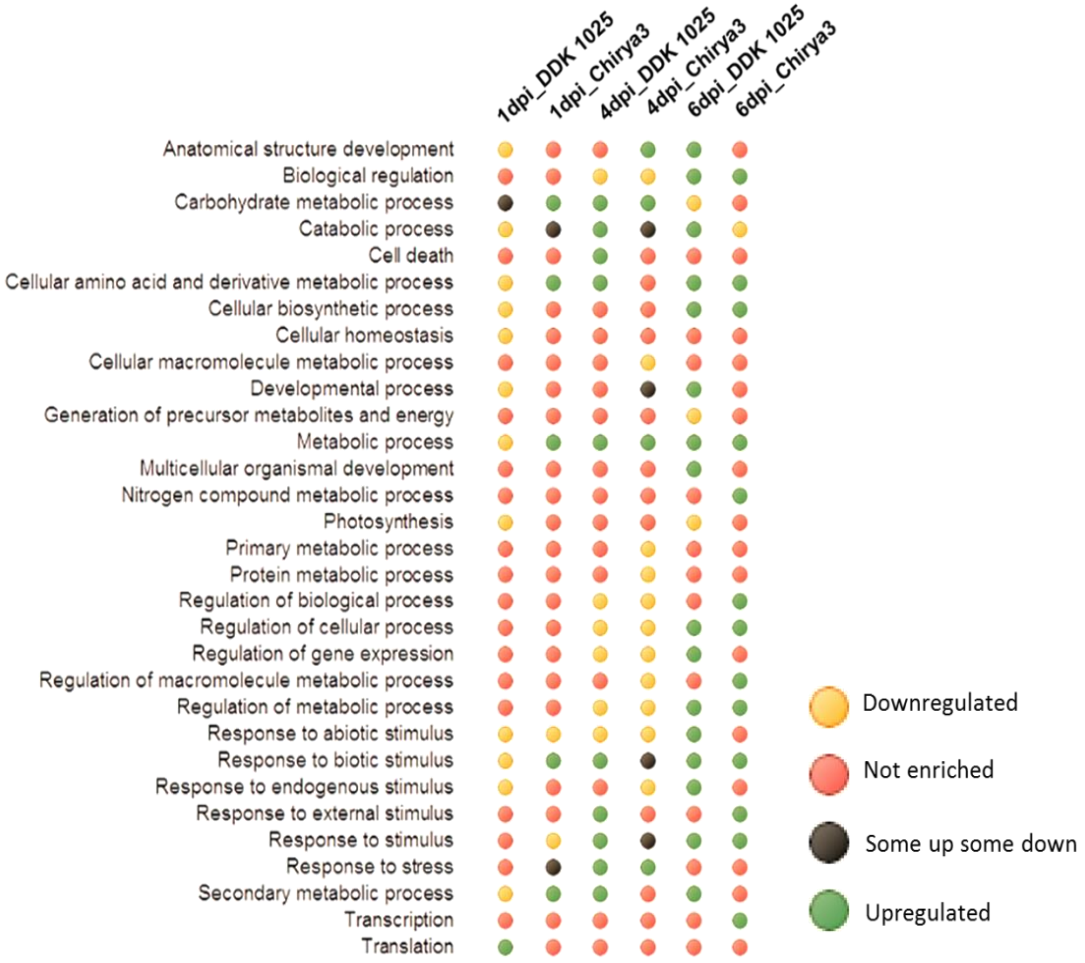


Figure 3.20: Comparison of Gene ontology enrichment for DEGs from DDK 1025 and Chirya 3

3.3.7.3 Modulation in metabolic processes

DEGs from each time point from up- and down-regulated sets of CI were mapped individually to get insights into the metabolic pathways affected by pathogen invasion. As shown in **Figure 3.21**, phenylpropanoid biosynthesis, MAPK signaling pathway and plant pathogen interaction processes were the primary upregulated processes. Protein processing in endoplasmic reticulum was the principal component to be downregulated at 1 and 4 dpi, and upregulated at 6 dpi.

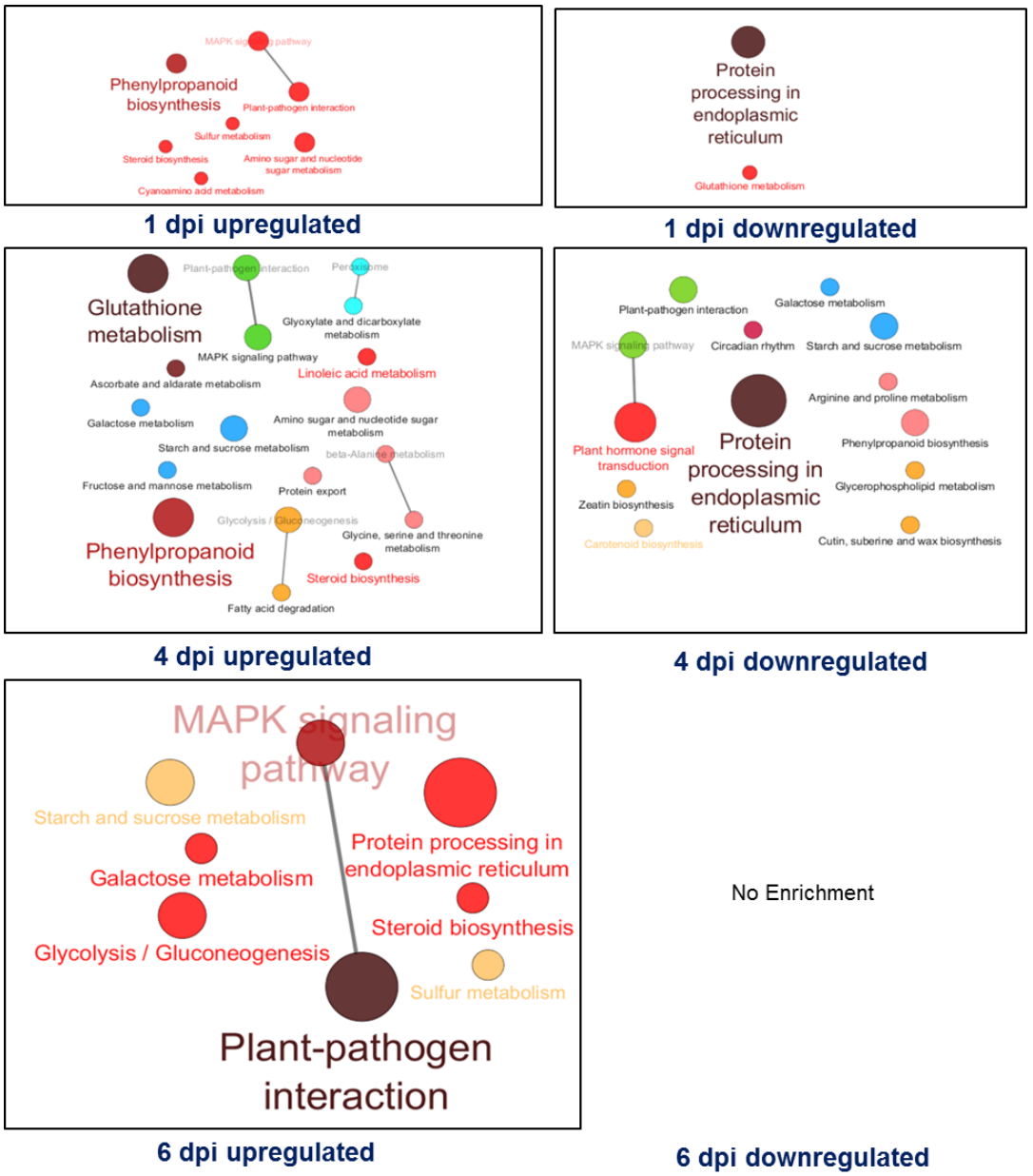


Figure 3.21: Modulation in metabolic processes and KEGG pathway network of DEGs in CI

3.3.7.4 DEGs common to CI in three stages

DEGs common to the three stages were extracted using Venny 2.0 (http://bioinfogp.cnb.csic.es/tools/venny) (Oliveros, 2007-2015). There were 175 DEGs common to the three selected stages (**Figure 3.22**). Gene ontology enrichment of these common genes using the agriGo online tool showed response to stress and carbohydrate metabolic process terms in biological process, enzyme regulation and catalytic activity in molecular function and extracellular region in cellular component category (**Figure 3.23**).

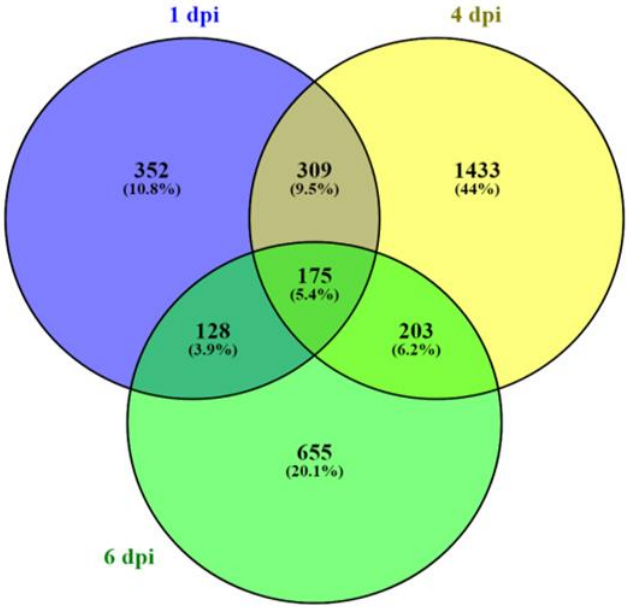


Figure 3.22: Venn diagram showing the DEGs from CI common in three time points

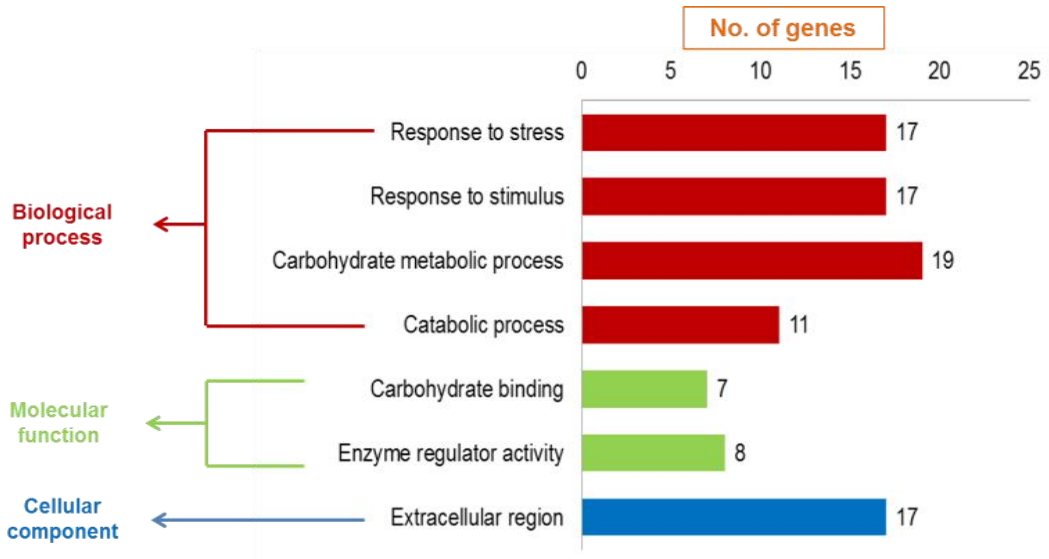


Figure 3.23: Gene ontology enrichment of 175 common DEGs from three time points in CI

Co-expression analysis of the DEGs was performed based on K-means clustering using MEV (version 4.9.0). These 175 DEGs were classified in six groups according to their log_2 fold change expression values (**Figure 3.24**).

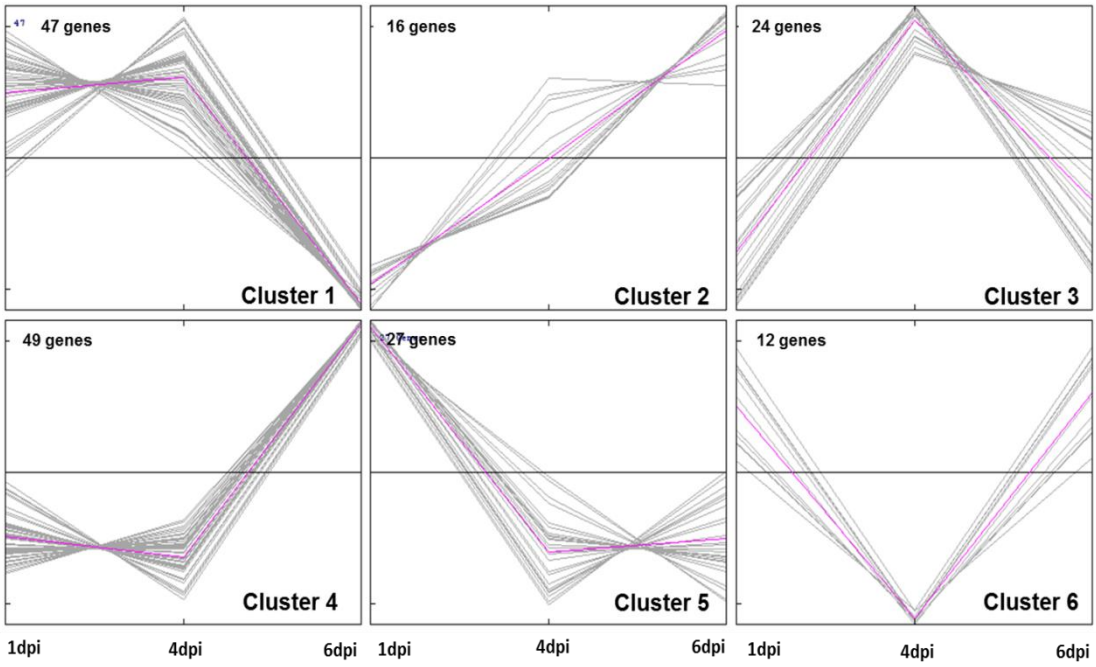


Figure 3.24: K-means clustering of 175 DEGs common in three stages in CI

3.3.7.5 Functional enrichment analysis of clusters

Functional enrichment analysis was performed for the DEGs from each cluster. Biological process enrichment was observed in three clusters i.e. Cluster 1, Cluster 2 and Cluster 4 (**Figure 3.25**). Cluster 1 comprised of genes with metabolic process including carbohydrate metabolic and chitin catabolic process. Cluster 2 and 4 showed stress responsive genes. InterPro annotation showed three thaumatin genes from cluster 1. Two genes of Bowman-Birk inhibitors were displayed in each Cluster 1 and Cluster 3. Small heat shock proteins Hsp20 belonged to Cluster 4 (**Table S3.3**).

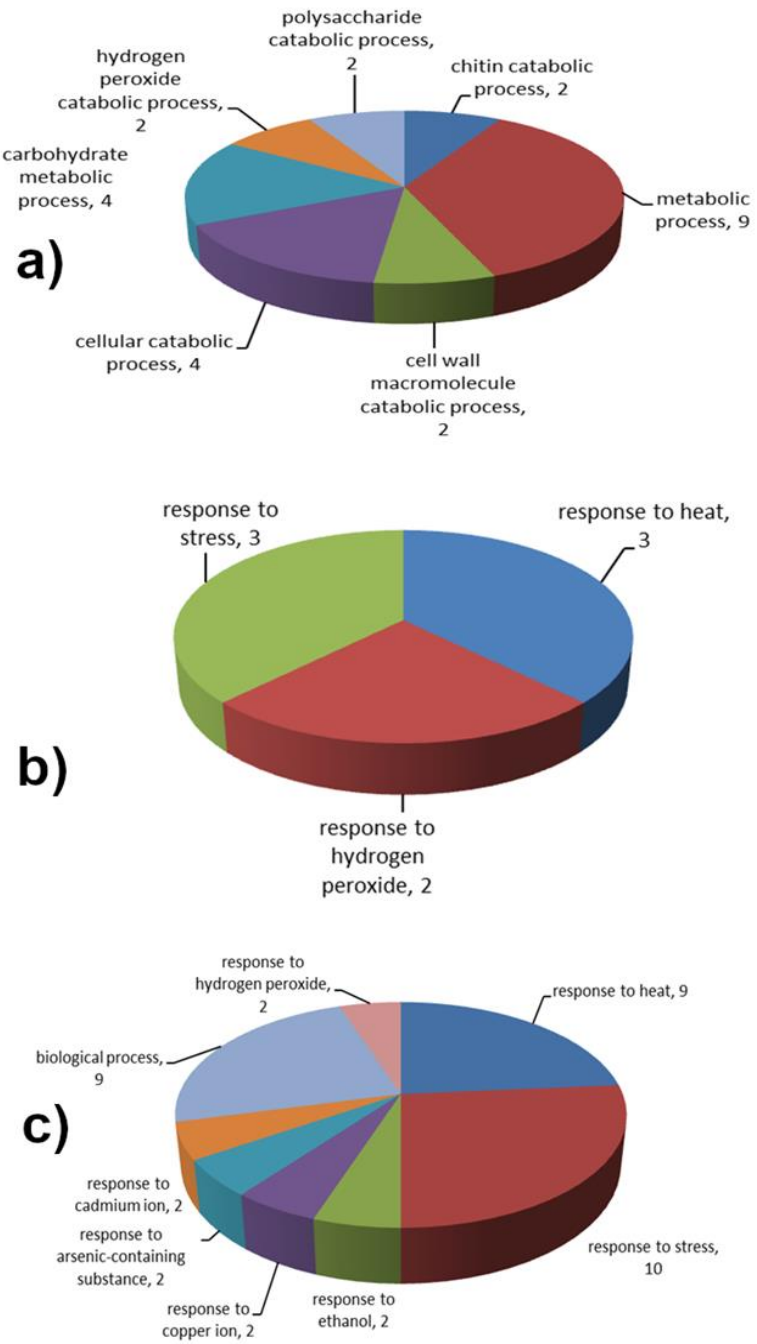


Figure 3.25: Gene ontology enrichment of Biological Process a) Cluster 1, b) Cluster 2, and c) Cluster 4

Among the DEGs annotated using Blast2GO, we classified the genes based on gene ontology into various categories. Some of the important categories that showed highly altered expression upon *B. sorokiniana* infection in Chirya 3 are discussed below.

3.3.7.6 Carbohydrate metabolic process

Analysis of carbohydrate metabolic process showed differential expression of several glycosyl hydrolase (GH) genes belonging to the gene families GH 1, GH 3, GH 8, GH 16, GH17 and GH 19 (**Figure 3.26**). Chitinases are important enzymes for fungal pathogen defense. These enzymes bind to chitin present in the cell wall of the pathogen and inhibit pathogen growth by degradation of the polymer. As chitinases from wheat are not well characterized, we searched for the ontologies showing chitin binding activity as putative chitinases and checked their differential expression. Three of the five isoforms showed high expression fold change in Chirya 3 inoculated plants at 1 and 4 dpi. Besides these, Glucose-6-phosphate epimerase enzyme expression was also high. This enzyme is involved in conversion of alpha-D-glucose-6-phosphate to beta-D-glucose-6-phosphate, which is the precursor for glycolysis. Moreover, upregulation of phosphoglucose isomerase also suggests that degradation of the fungal cell wall by chitinase could result in release of sugar, which could be utilized in glycolysis or gluconeogenesis pathway by the plant. Thus, in resistant plants, the chitinases might degrade the fungal cell wall and utilize the degradation products in its own growth.

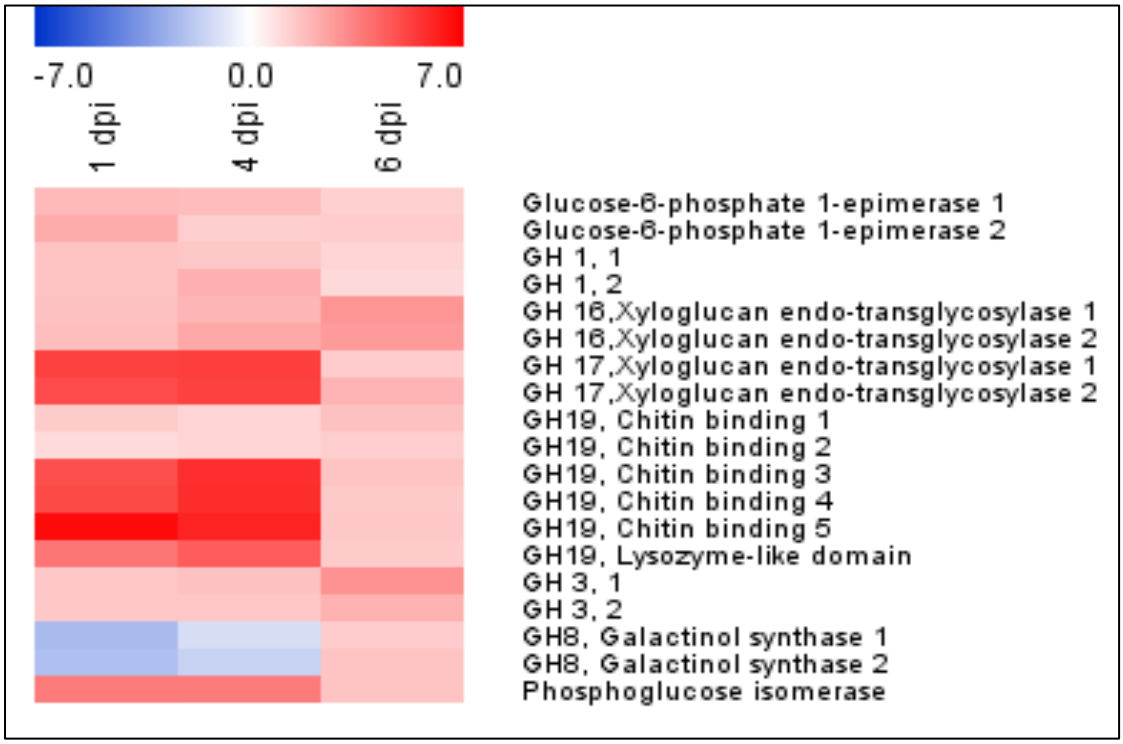


Figure 3.26: Heatmap of log2 fold change gene expression from carbohydrate metabolic process in CI

3.3.7.7 Stress responsive genes

It is well known that pathogen invasion creates stress on plants because of anatomical breaching as well as the resulting nutritional scrap (Chou et al., 2000; Eisa et al., 2013). The plant senses pathogen invasion and expresses defense genes to restrict the pathogen entry, growth, colonization or reproduction. In this study too, the RNA-seq analysis showed altered expression of stress responsive genes (**Figure 3.27**). The genes included Barwin domain; RlpA-like protein 1, BetV I/major latex protein, catalase, Hsp90, Hs1 pro-1, haem peroxidase and proteinase inhibitor I 13. Most of these genes overexpressed in Chirya 3 in all the three time points.

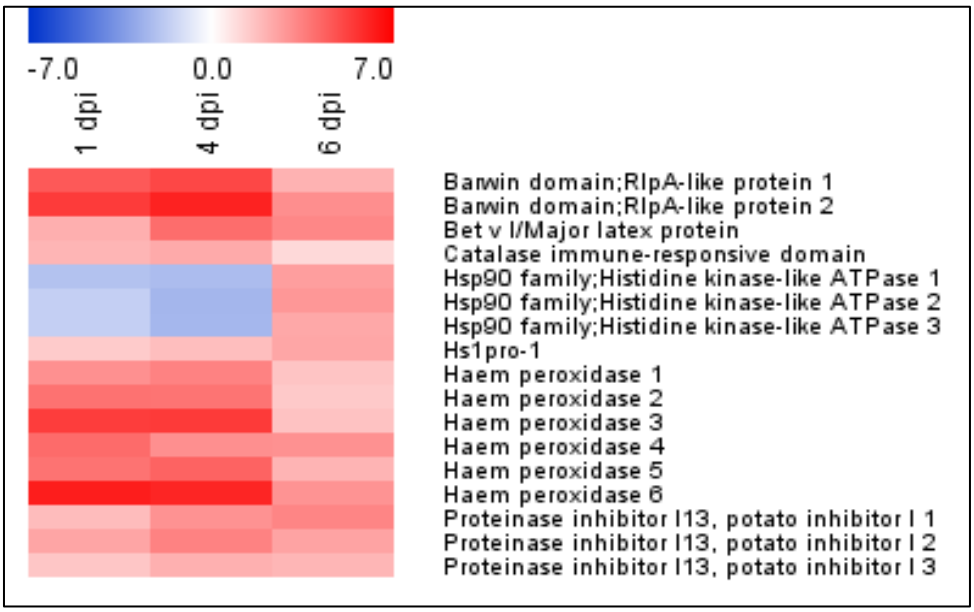


Figure 3.27: Heatmap of $\log_2$ fold change expression of stress responsive genes in CI

3.3.7.8 Extracellular genes

After initial infection, most pathogens reside in extracellular spaces of host plant. Nutrition is obtained either through haustoria or infection hyphae (Kumar et al., 2002). Moreover, some effectors are also released by the plant in extracellular regions which are governing factors for either susceptibility or resistance. Secretion of extracellular defense or antifungal molecules is important for plant cells in order to restrict the pathogen growth. The role of extracellular invertase has been extensively studied in plant pathogen interaction (Geissmann et al., 1991; Wright et al., 1995; Chou et al., 2000; Voegele et al., 2006). However, there are several other extracellular enzymes

involved in plant defense. Hence, we searched other genes showing extracellular activity among the DEGs, which includes the genes allergen V-5, expansin, xyloglucan endotransglucosylase, haem peroxidase and proteinase inhibitor I 12. Surprisingly, all of them were highly upregulated at all the three stages **(Figure 3.28)**. This indicates that the plant is continuously expressing these genes to restrict the pathogen in intracellular spaces only and not allowing it to grow any further.

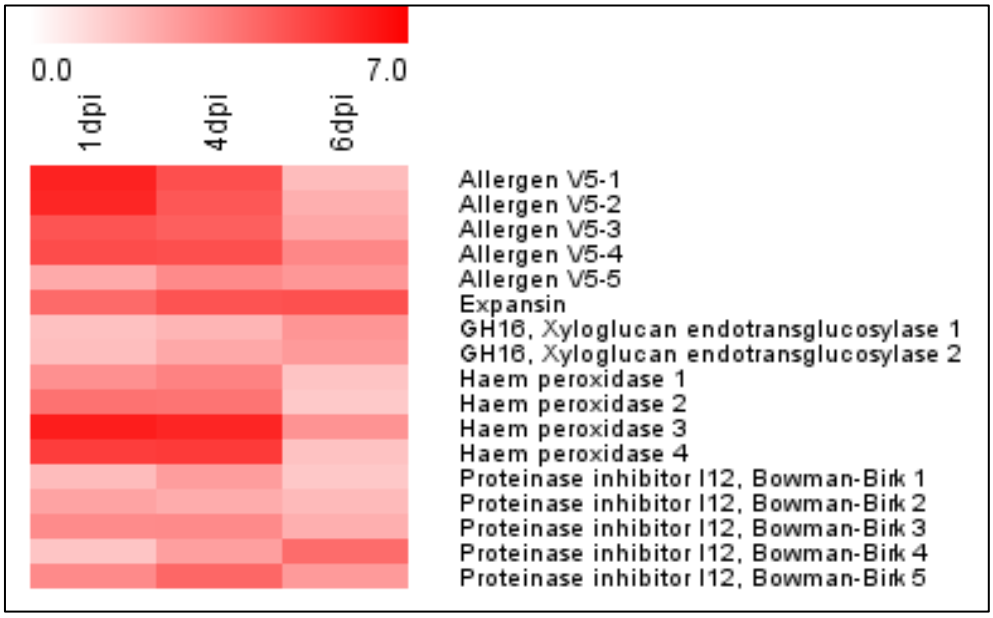


Figure 3.28: Heatmap of log2 fold change expression of genes with extracellular activity in CI

3.3.7.9 Proteinase inhibitor activity

Proteinase inhibitors are intrinsic to the plant defense mechanism. We found overexpression of five genes from this class of genes, *viz*. proteinase inhibitor I 12, Bowman-Birk inhibitor (BBI) and three from proteinase inhibitor I 13, potato inhibitor I (Pin I) family (**Figure 3.29**). The detail of these proteinase inhibitors is provided in **Table S3.4.**

Overexpression of a BBI in rice provided increased resistance to the blast causing fungal pathogen *Magnaporthe grisea* (Qu et al., 2003). Expression of potato inhibitor I is induced by wounding (Ryan, 1990), jasmonic acid treatment (Farmer et

al., 1992) and insect attack (Dunse et al., 2010). Overexpression in response to *B. sorokiniana* suggests its role in hormone regulation mediated defense mechanism.

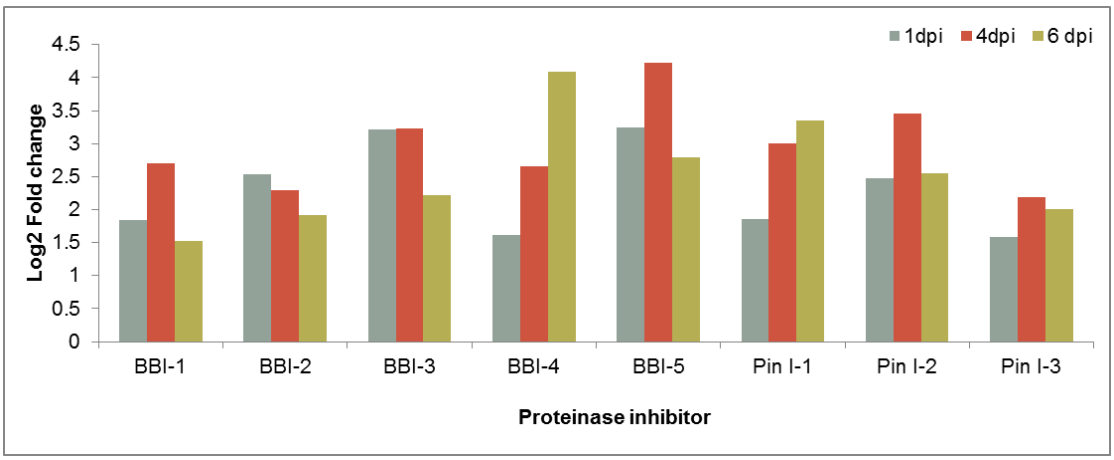


Figure 3.29: Log2 fold change expression of genes with proteinase inhibitor activity in CI

3.3.7.10 Unidentified defense responsive genes

As expected, the global transcriptomics analysis revealed altered expression of thousands of genes in response to the pathogen inoculation. Gene Ontology and enrichment categorization of the DEGs provided valuable information about the functional role of genes based on reported annotations. However, one of our objectives of the transcriptome analysis was to identify novel or previously unidentified genes exhibiting major impact during plant pathogen interaction. We looked for the significantly differentially expressed unannotated transcripts with a very high fold change in all the three stages. The top 10 genes with highest $\log_2$ fold change from unannotated genes list are shown (**Figure 3.30**). The details of all these overexpressed transcripts are provided in **Table S3.5**. Such a highly induced expression of these transcripts at all three stages underscores their role in resistance mechanism. In spite of this, BLAST or homology based search did not show match with any reported gene. Hence, their functional role could not be elucidated. *In vitro* or transient expression of these genes in plants could provide deeper insights about their roles in plant defense against biotic stresses.

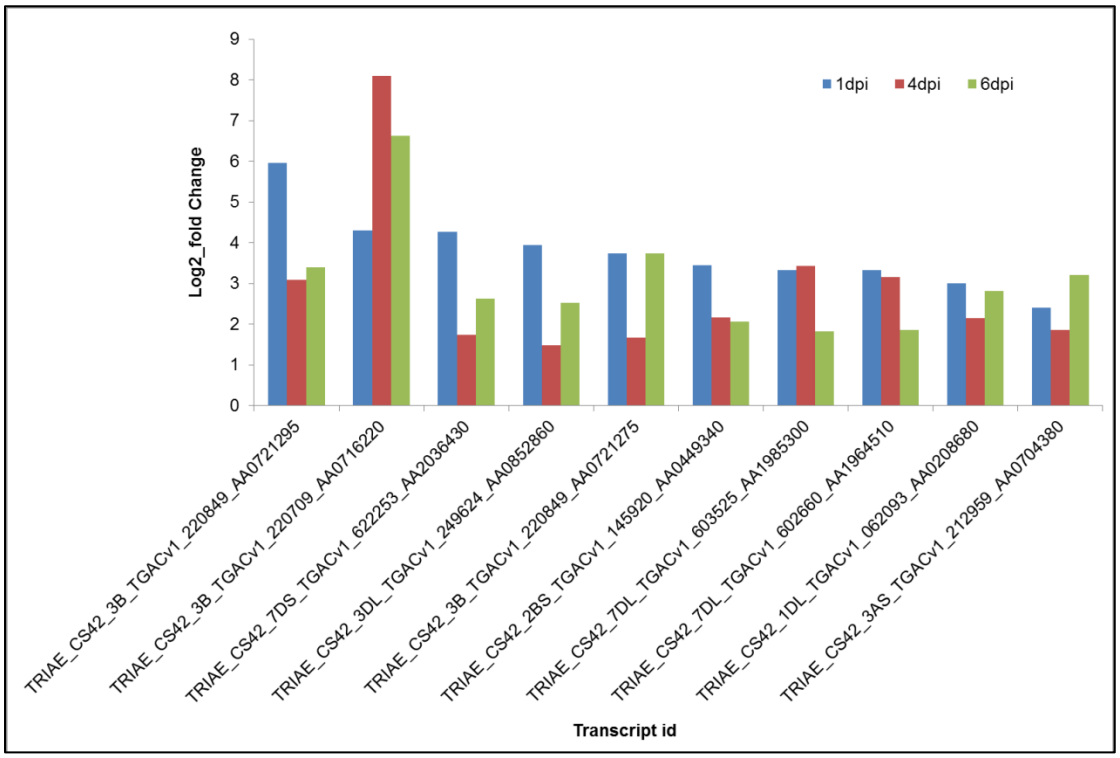


Figure 3.30: Log2 fold change expression of transcripts with unknown function in CI

3.3.8 Homology based protein family enrichment analysis for DEGs from DI and CI

Gene ontology (GO) offers classification of genes at superficial level. However, protein family (Pfam) analysis provides information about the functional roles of the genes at molecular level. Hence, Pfam enrichment was performed for the homologues of the wheat DEGs in rice using the STRING database. Annotations from rice were used, as *Triticum aestivum* annotations are not well established yet and annotations for several DEGs could not be retrieved. Up- and down-regulated gene groups from both DI and CI at three time points were assessed for Pfam enrichment (**Figures 3.31, 3.32, 3.33, 3.34, 3.35** and **3.36**).

The characteristics of protein families of the wheat homologs annotated using the *O. sativa* var. *japonica* genome that showed differential expression upon *B. sorokiniana* inoculation are discussed below.

Four transcripts from pollen allergen family and rare lipoprotein A-like-double-psi-beta-barrel were upregulated at 1 dpi in DI. These families include various

expansin genes. The expression of expansin genes is important for growth of cells and silencing of expansin genes causes growth inhibition; whereas their excessive expression leads to uncontrolled or abnormal growth (Sampedro and Cosgrove, 2005). Expansins also play a role in leaf primordium initiation (Reinhardt et al., 1998) as well as in ethylene induced abscission (Belfield et al., 2005). Ubiquitin mediated proteolysis plays important roles in regulation. Upregulation of these genes at early stage might be responsible for alteration in leaf physiology.

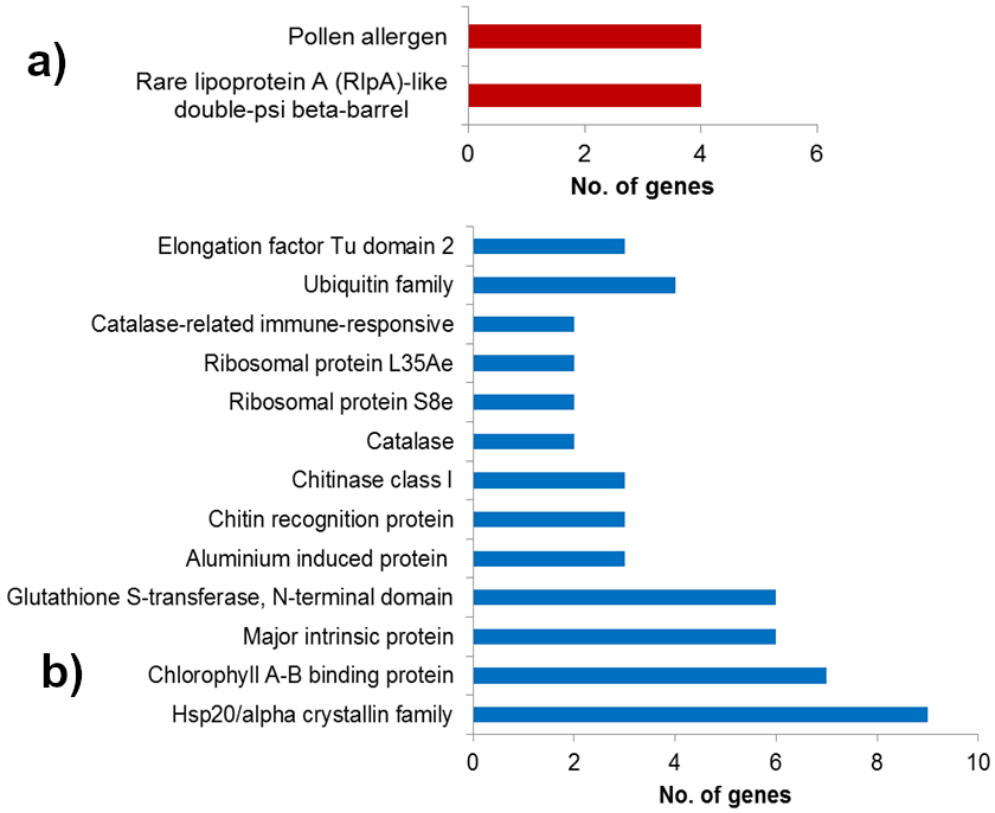


Figure 3.31: Pfam enrichment of DEGs from DI at 1 dpi (a) Upregulated (b) Downregulated

Four transcripts from ubiquitin family were downregulated at 1 dpi in DI. Chito-oligosaccharides released from fungal cell wall disrupt the ubiquitin interaction with lysine motif receptor kinase causing its accumulation and induction of plant innate immunity (Liao et al., 2017). The pathogen might interfere in innate immunity stimulation by reducing cellular ubiquitin.

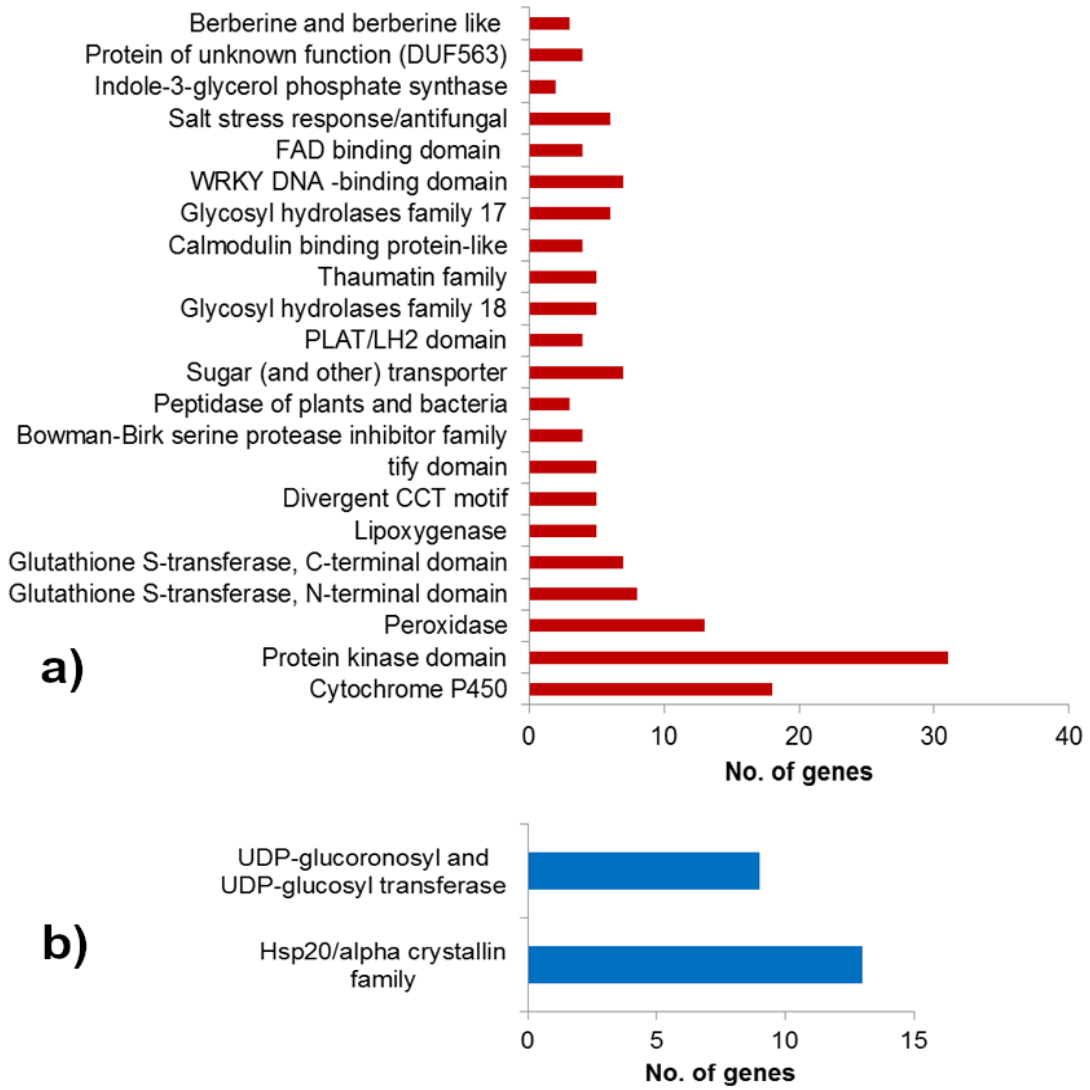


Figure 3.32: Pfam enrichment of DEGs from DI at 4 dpi (a) Upregulated (b) Downregulated

Catalase is an anti-oxidative enzyme protecting cell through programmed cell death. Downregulation of catalase and catalase-related immune-responsive genes was observed at 1 dpi in DI. Catalase enzyme acts upstream to immunity triggered autophagy and induces autophagy dependent cell death upon ROS accumulation (Hackenberg et al., 2013). Inhibition of innate immunity response might cause suppression of catalase.

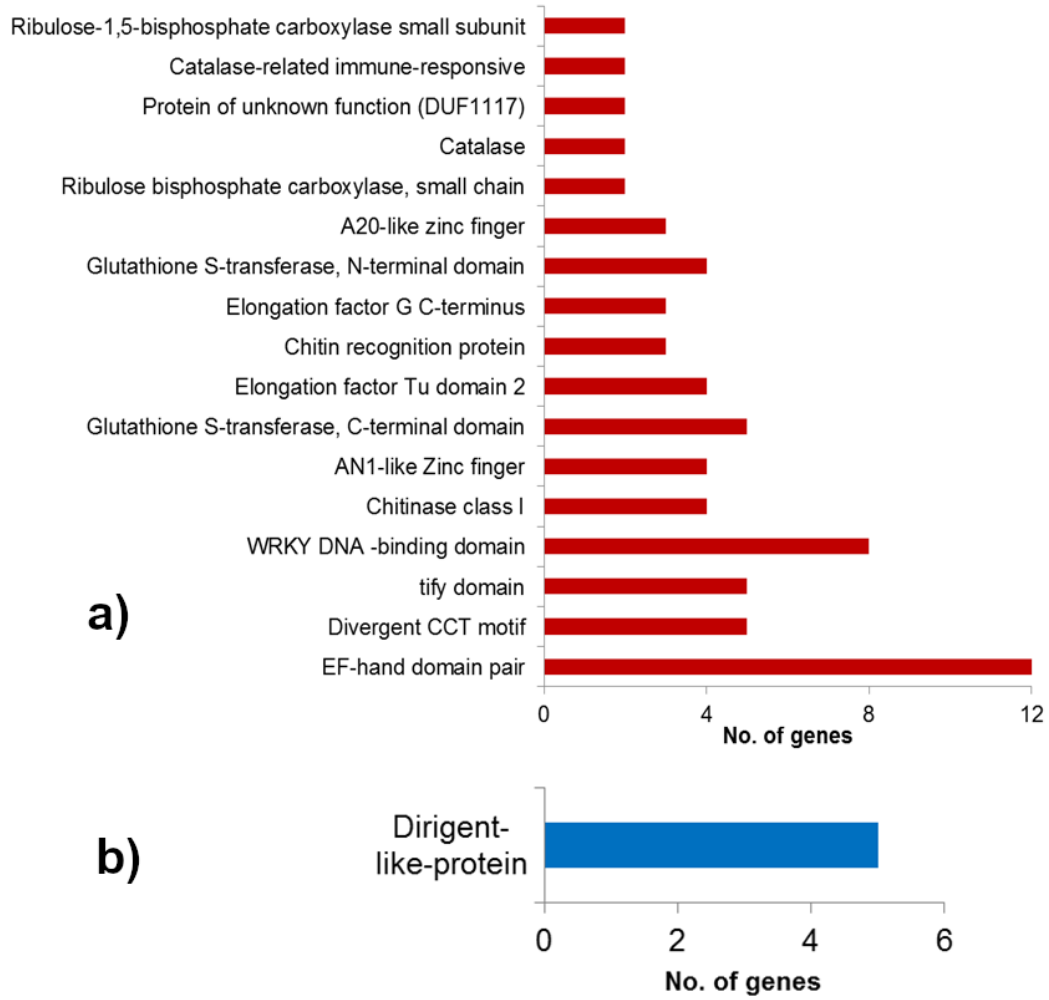


Figure 3.33: Pfam enrichment of DEGs from DI at 6 dpi (a) Upregulated (b) Downregulated

Chlorophyll A-B binding proteins are integral components of light-harvesting complex in plants. At 1 and 6 dpi, eight and seven transcripts respectively, from this family were significantly downregulated in DI. This family includes the photosystem II protein PsbS, which plays a role in heat dissipation of excess light energy absorbed by the photosystem through energy-dependent quenching (Li et al., 2004). Deficiency of these proteins is responsible for the disease severity.

Berberine/berberine like domain is present in berberine bridge enzymes, which are involved in alkaloid and cannabinoid biosynthesis. Although this family is conserved in bacteria, fungi and plants, the number of genes has increased in monocots (Daniel et al., 2017). At 4 dpi, three genes were upregulated from this

family in DI. Indole-3-glycerol phosphate synthase enzyme is a component of aromatic amino acid biosynthesis and was upregulated at 4 dpi. This enzyme catalyzes the synthesis of indole-3-glycerol phosphate, which is the branch point for tryptophan biosynthesis. The synthesis of several plant defense molecules is dependent on tryptophan pathway (Frey et al., 1997). Hence, increase in these two pathways at 4 dpi would have delayed the host response to pathogen stress in DI.

Divergent CCT motif includes the proteins having CCT motif at their N-termini, including CONSTANS (CO), CONSTANS-LIKE (COL) and TOC1. Five transcripts having this domain were upregulated at both 4 dpi and 6 dpi stages. These genes play an important role in plant development, seedling growth, dormancy, tuberization and regulation of flowering transition in photoperiod-responsive plants (Putterill et al., 1995; Strayer et al., 2000; Wu et al., 2017). Rise of these transcripts at later stages indicates their putative role in dormancy during pathogen infection.

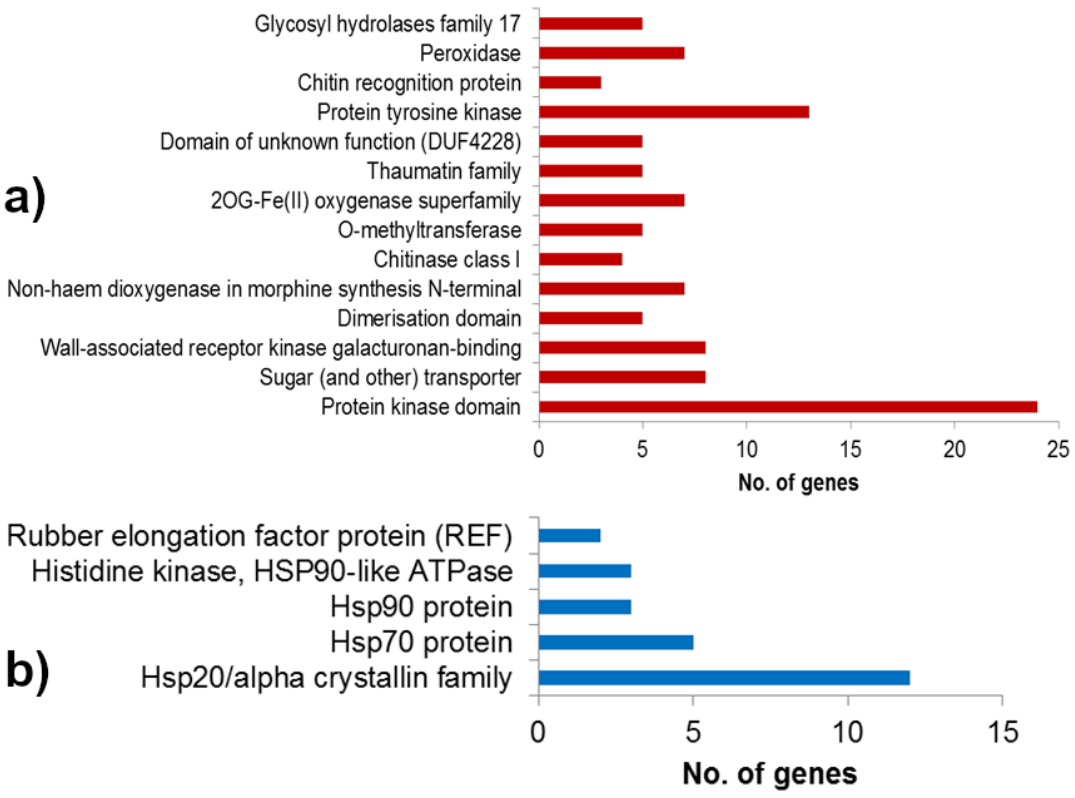


Figure 3.34: Pfam enrichment of DEGs from CI at 1 dpi (a) Upregulated (b) Downregulated

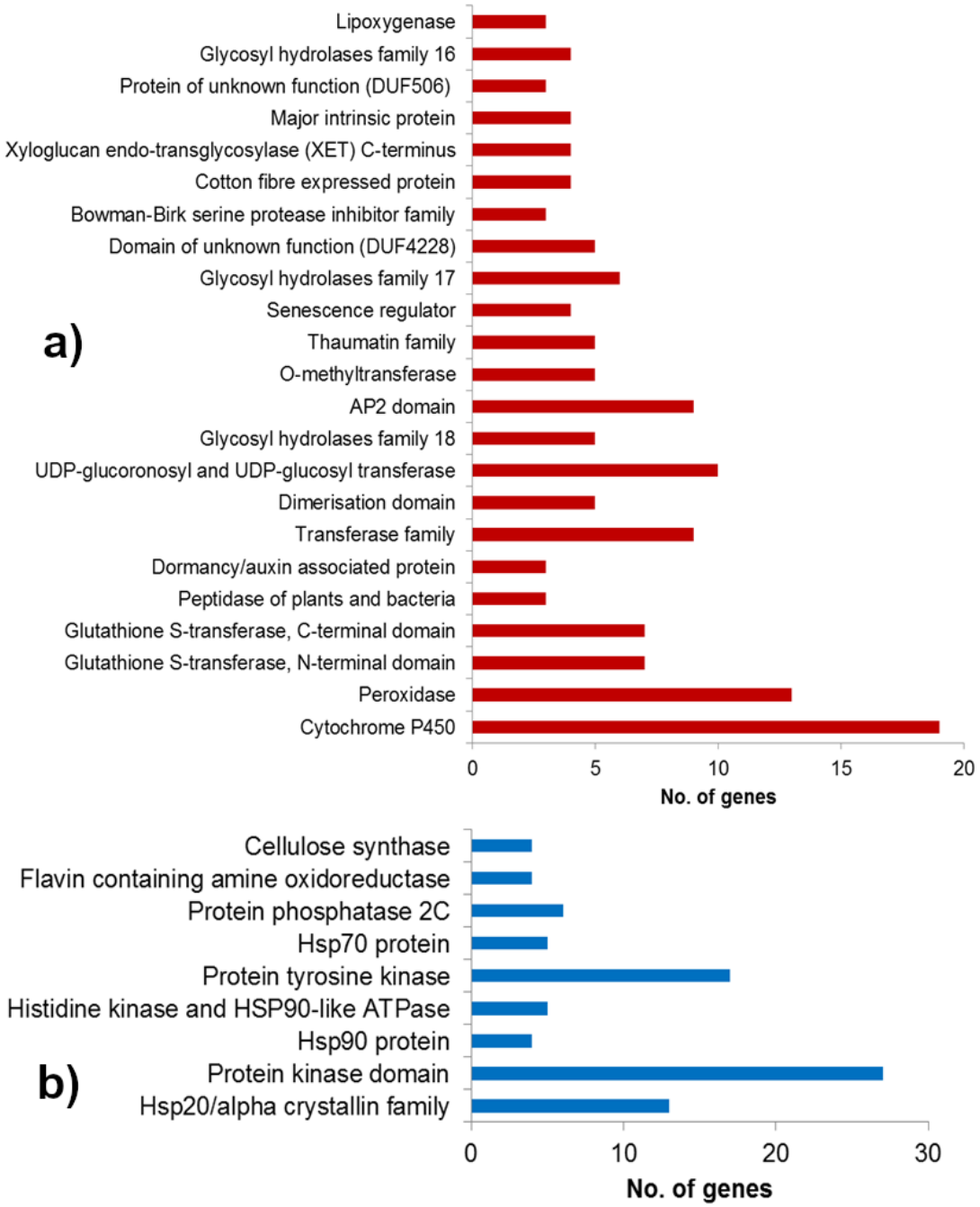


Figure 3.35: Pfam enrichment of DEGs from CI at 4 dpi (a) Upregulated (b) Downregulated

Dirigent proteins are also involved in lignin and lignan biosynthesis. Plants with overexpression of dirigent like protein from wheat showed increased pathogen resistance (Ma and Liu, 2015). Five transcripts with such domain showed decreased

expression in response to pathogen inoculation at 6 dpi in DI. Dirigent proteins control cell wall metabolism and production of antibacterial compounds and also help plant to adapt to dynamically changing environmental responses (Paniagua et al., 2017). Absence of these proteins in DI might have led to leaf damages enabling pathogen entry and hence disease severity.

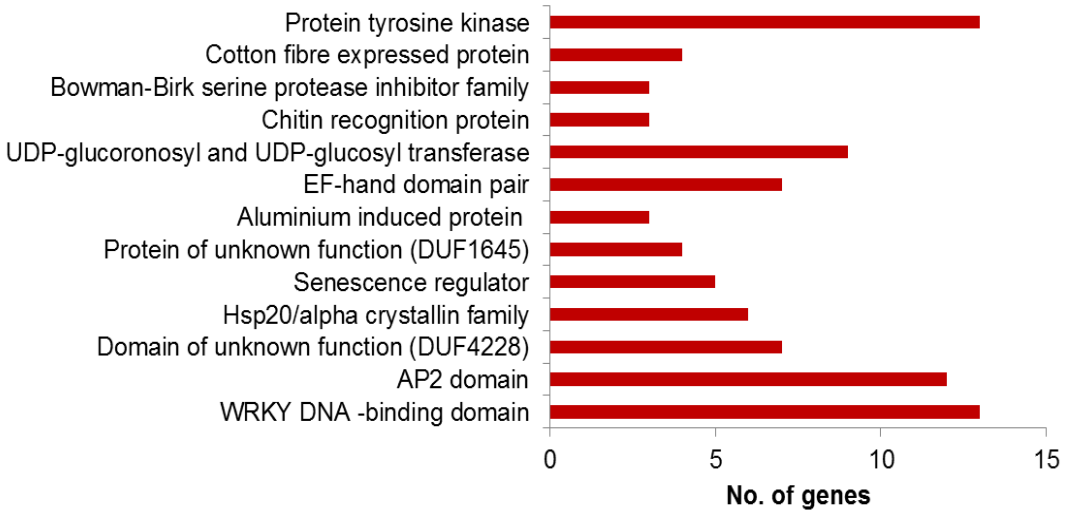


Figure 3.36: Pfam enrichment of DEGs upregulated from CI at 6 dpi

Class I chitinase belonging to the glycosyl hydrolase family 19 are plant specific. Chitinases are pathogenesis related proteins and induced during fungal infection (Zur et al., 2013). At 1 dpi, chitinase class I and chitin recognition protein were downregulated in DI in contrast to CI. However, chitinase activity cannot be directly related to the resistance phenomenon (Rybka et al., 1998). Nevertheless, upregulation of chitinases in resistant variety indicates its significance in defense. The aluminum induced protein family comprises of several glutamine amidotransferase enzymes (GATase). Three of these transcripts were downregulated at 1 dpi in DI and upregulated at 6 dpi in CI. DUG3, a protein comprising GATase domain is reported in the degradation of glutathione (GSH) bound to peptides in *Saccharomyces cerevisiae*. Glutathione-s-transferase (*GST*) transfers glutathione group (GSH) to a variety of substrates, majorly xenobiotic compounds. GST is a cytosolic protein involved in glutathione mediated cellular detoxification in response to stress. GSH binds to the N-terminal domain, while the hydrophobic substrate occupies a pocket in the C-terminal domain. GST family also includes the proteins with no enzymatic activity which regulates cell proliferation, differentiation and death. GST acts in cell signaling

modulation by binding to cascade components like MAPK in non-enzymatic manner (Laborde, 2010). Differential expression of GST was detected in both DI and CI. In case of DI, downregulation of six GST-N-terminal domain transcripts was observed at 1 dpi; while eight and five genes were upregulated at 4 and 6 dpi respectively. Similarly, seven GSH transcripts with C-terminal domain were upregulated at 4 dpi. In CI plants, seven transcripts from both GST-N-terminal domain and GST-C-terminal domain family were upregulated at 4 dpi. Repression of glutathione mediated detoxification causes accumulation of pathogen released toxins inside host cells promotes cell death.

The major intrinsic proteins (MIPs) are the key regulators in osmoregulation, reactive oxygen species signaling, and intracellular transport and storage processes during stress in plants. MIP's were downregulated in response to pathogen inoculation at 1 dpi in DI but upregulated at 4 dpi in CI. The MIP family comprises aquaporins, which function not only as water channels but are also documented for their role in the transport of other small neutral molecules (e.g. glycerol, urea, boric acid, silicic acid, etc.), gases (e.g. CO_2, ammonia, etc.) and even ions under certain circumstances (Wong et al., 2018). Suppression of these proteins might be responsible for reduced gas exchange and hence impaired photosynthesis after *Bipolaris* inoculation (Rios et al., 2017).

The WRKY domain is present in one of the plant specific families of transcription factors. They regulate diverse biological functions in plants including disease resistance, stress response, nutrient deprivation, senescence, seed and trichome development and embryogenesis among others (Bakshi and Oelmuller, 2014). In tobacco, silencing of multiple *WRKY* genes resulted in inactivation of effector triggered immunity of AVR3a (Adachi et al., 2015). Similarly, the blast resistance in rice requires *WRKY* genes for transduction of defense signals (Liu et al., 2016). Overexpression of *WRKY* in tomato resulted in enhanced resistance against *A. solani* (Shinde et al., 2018). In *Arabidopsis*, WRKY18 and WRKY40 act independently as negative regulators of flg22-induced genes (Birkenbihl et al., 2016). At 6 dpi, induced expression of 10 and 13 *WRKY* domain containing transcripts was observed in DI and CI respectively. Although the role of several WRKY transcription factors is well reported, the expression of some of the WRKY elements might have negative effect on plant defense; whereas others may positively regulate the expression of plant defense genes through involvement with distinct regulatory

complexes (Eulgem and Somssich, 2007). The WRKY candidates identified in this study can be used for discovery of disease responsive genes.

The glycosyl hydrolases (GH) family have been categorized into more than 100 sub-families based on their substrates, function and structure. The GH17 family has callose degrading enzymes localized in plasmodesmata (Gaudioso-Pedraza and Benitez-Alfonso, 2014). Callose is important in vascular maturation and symplastic trafficking in phloem (Slewinski et al., 2012). Eight transcripts from this family showed upregulation in DI at 4 dpi stage. In contrast, five and six genes were upregulated in CI at 1 and 4 dpi, respectively. The plasmodesmata-localized GH17 proteins play a role in plant development and response to viral pathogens (Levy et al., 2007; Zavaliev et al., 2011; Burch-Smith and Zambryski, 2012). The GH 18 family is unique with reference to structure and function, and includes enzymes with class III/V chitinases as well as catalytically inactive proteins. The role of chitinases is well known as antifungal agent (Amian et al., 2011; Kalinina et al., 2011; Ahmed et al., 2012). Beside chitin degrading activity, chitinases are involved in kinase interaction and signaling mediated cell death, reactive oxygen species and nitrous oxide burst (Kim et al., 2015). Catalytically inactive proteins from this family were characterized as xylanase inhibitors which were reported to be induced upon fungal attack (Coutinho et al., 2003; Durand et al., 2005). We also observed four and six transcripts from this family to be upregulated at 4 dpi in DI and CI respectively, in response to the pathogen attack. Although, both varieties are able to produce GH, the number of genes and response time varied attributing to different levels of disease severity.

Heat shock proteins (HSPs) are essential for viability of cells. The Hsp20 family of proteins are heat inducible energy independent chaperones preventing protein aggregation (McHaourab et al., 2009). Stress conditions lead to the generation of protein aggregates inside the cells. The refolding of aggregated proteins is facilitated by these small HSPs (Tyedmers et al., 2010). Five and twelve transcripts from Hsp20/alpha crystallin family were downregulated in DI at 1 and 4 dpi respectively. In case of CI, 12 and 13 transcripts were downregulated at 1 and 4 dpi, while six transcripts were upregulated at 6 dpi. Hsp90s mediate both abiotic and biotic stress resistance in plants. Kinases and transcription factors are the chief targets of Hsp90 and hence it functions indirectly in signal transduction to activate or suppress defense response as well as in the regulation of different signaling pathways (Xu et

al., 2012). Three transcripts from Hsp90 family were downregulated in DI as well as CI at 1 dpi. Furthermore, four transcripts were downregulated in CI at 4 dpi also. In addition to these Hsp90 proteins, three and four genes from Histidine kinase and Hsp-90 like ATPase family were also downregulated in CI at 1 and 4 dpi respectively. These heat shock proteins might collaborate with ubiquitin mediated proteolysis for regulation of hormone signaling during pathogen stress.

The Bowman-Birk inhibitors (BBI) are plant serine protease inhibitors found only in Fabaceae and Poaceae families. Induced expression of these inhibitors offers a broad spectrum immune resistance in response to pest or pathogen attack. The increased gene expression of BBI in wounded tissue helps to resist pathogen invasion by inhibiting food digestive enzymes (Qi et al., 2005). In CI, three transcripts were upregulated at both 4 and 6 dpi stages. However, the number of upregulated BBI transcripts was more (4) in DI at 4 dpi stage; but no upregulation was observed at 6 dpi. Upregulation of BBI in rice in response to jasmonic acid treatment and wounding emphasizes its role in plant defense (Rakwal et al., 2001; Qu et al., 2003). The peptidase family includes basic secretory proteins. Three transcripts from this family were upregulated in response to pathogen inoculation in both DI and CI at 4 dpi. The expression of these secretory proteins was induced by abscisic acid treatment in wheat suspension culture. These peptidases are reported to be involved in defense response (Kuwabara et al., 1999). Overexpression of these inhibitors can be used as a strategy to provide improved resistance.

The protein kinase domain is a conserved catalytic site in protein kinases. These enzymes are involved in phosphorylation of several classes of proteins resulting in their activation or inactivation. Protein kinases are broadly classified into three classes, serine/ threonine kinase, tyrosine kinase and dual specificity kinases. At 4 dpi, 43 transcripts with this domain were upregulated in DI. Similarly in CI, upregulation of 24 genes from this family was observed at 1 dpi, while 27 genes were downregulated at 4 dpi. In upregulated group, 13 genes from protein tyrosine kinase family were detected. Mitogen activated protein kinases (MAPKs) belong to protein tyrosine kinase and are reported for melatonin mediated activation of defense responsive genes (Lee et al., 2016). Calcium dependent protein kinases (CPKs) are conserved in plants; however, although their role in development and abiotic stress is well established; their importance in biotic stress is not well recognized (Valmonte et

al., 2013). Receptor like kinases triggers pattern triggered immunity upon pathogen recognition instigating the first role of defense against pathogens (Saintenac et al., 2018). However, the exact mechanism of interaction could not be elucidated, involvement of protein kinases during fungal infection is evident from our transcriptome data.

Peroxidases are the apoplast localized enzymes catalyzing oxidation of various substrates using H_2O_2 as electron acceptor. Pfam enrichment showed that 13 transcripts from this family were upregulated in CI at 1 dpi. An equal number of genes were also upregulated in CI at 4 dpi stage. Peroxidases play a crucial role in growth and development by polymerization of lignin and suberin eventually contribute to the rigidity of cell walls, an important phenomenon of the primary defense as well as dimerization of antimicrobial phenols in plants (Ghosh, 2006; Karkonen and Kuchitsu, 2015; Minibayeva et al., 2015). Peroxidases are cross linked with wall proteins and callose and callose synthase. Peroxidase dependent oxidative burst is essential for activation of pattern triggered immunity (Mammarella et al., 2015). Peroxidase from *Marsdenia* (Oliveira et al., 2017) and *Triticum* (Caruso et al., 2001) are reported to inhibit germ tube elongation of fungi. The number of genes increased in upregulated group in CI at 4 dpi counting 13 like that of DI. Our findings of increased peroxidase activity in resistant variety correlate with previous reports (Kristensen et al., 1999; Liu et al., 2015; Silva et al., 2018).

Lipoxygenase (LOX) are recognized for contribution towards pathogen resistance in plants through hypersensitive response mediated cell death (Kolomiets et al., 2000). We observed upregulation of five and three lipoxygenase genes in DI and CI at 4 dpi respectively. Induction of the LOXl, a lipoxygenase gene is reported for regulatory activity in the synthesis of jasmonic acid or other octadecanoids in response to pathogen attack (Melan et al., 1993). LOX are highly conserved and essential enzymes for hypersensitive response and provide broad spectrum resistance against plant pathogens (Hwang and Hwang, 2010). Discernibly altered expression by both varieties points the involvement of hypersensitive response and jasmonic acid during *B. sorokiniana* infection.

Cytochrome p450 Group IV comprises the CYP7 (cholesterol 7-alpha-hydroxylase), CYP8 (prostacyclin synthase) family and CYP51 (lanosterol 14-alpha-demethylase) families. In response to pathogen attack, 18 and 19 transcripts were

overexpressed at 4 dpi in DI and CI, respectively. Cytochromes P450 domain is also reported in enzymes catalyzing the generation of basic skeletons of terpenoid biosynthesis pathway (Weitzel and Simonsen, 2015). Overexpression of such a high number of genes from the family highlights the contribution of cytochromep450 enzymes in fungal diseases.

AN1-zinc finger domain is reported in several stress associated proteins (Dixit et al., 2018). Induced expression of four transcripts from this family was observed at 6 dpi. Constitutive expression of *OsSAP1*, an AN1 gene from rice provided improved disease resistance against bacterial pathogen through upregulation of defense genes in tobacco (Tyagi et al., 2014). Overexpression of *ZFP185I*, another A20/AN1 zinc finger type protein in rice, led to decrease in gibberellic acid production and increase in drought, cold and salt stress sensitivity (Zhang et al., 2016). Hydrogen peroxide induced the expression of *CsZfp*, an A20/AN1 domain containing protein in tea (Paul and Kumar, 2015). Induced expression of four transcripts from this family was observed at 6 dpi in DI. This result emphasize that delayed response by DDK 1025 allows the lifestyle switching by the pathogen and hence disease progression.

EF-hand domain is a helix-loop-helix motif having calcium binding site and integral component of several intracellular Ca^{2+} receptors (Grabarek, 2011). Pfam enrichment showed 12 and 7 transcripts from this family to be upregulated at 6 dpi in DI and CI respectively. Calcium level is altered in response to several stimulus or stresses in plants like drought, salt, cold, UV, heat, abscisic acid or pathogen infection (Lecourieux et al., 2006). The role of calcium homeostasis and signaling is well established in cell survival and growth (Bagur and Hajnoczky, 2017). Besides these, EF-hand motifs are also reported in NADPH oxidases, which are involved in production of reactive oxygen species during innate immune response (Oda et al., 2009).

The sugar (and other) transporters are the subfamily of Major Facilitator Superfamily (MFS). The primary function of these MFS proteins is the uptake of sugars. Upregulation of eight genes from this family was detected in CI at 1 dpi. Pathogens are dependent on plant for sugar and other nutrition and sugar transporters are essential in both symbiotic as well as pathogenic interactions of fungi with plants. As far as possible, pathogens avoid the plant defense system by utilizing extracellular sugar (Doidy et al., 2012); however, if extracellular sugar is exhausted, the fungal

hyphae invade cells and derive nutrition. The pathogen, in turn, might induce the overexpression of plant sugar transporters, harvesting more sugar from the plants. In fact, overexpression of such sugar transporters has been reported in *Arabidopsis* during infection with the fungal powdery mildew pathogens *Golovinomyces cichoracearum* and *Botrytis cinerea*; while inhibition of *OsSWEET11*, a sugar transporter from *O. sativa* using RNA interference, conferred resistance to the bacterial pathogen *Xanthomonas oryzae* (Chen et al., 2010). Hence, upregulation of these transporter genes in case of the resistant variety indicates restriction of the pathogen to extracellular spaces. Subsequent production of defense genes would have further diminished the growth of the pathogen.

Thaumatins are structurally diverse and ubiquitous proteins in plants. Thaumatin family comprises of several thaumatins and thaumatin-like proteins induced by various stress conditions. Overexpression of five genes from thaumatin family was observed in CI at 1 and 4 dpi stages. Pathogen induced thaumatin like proteins have been characterized from wheat, rice, peanut and many other species (Rebmann et al., 1991; Reimmann and Dudler, 1993; Liu et al., 2010; Singh et al., 2013). Transgenic plants with thaumatin like proteins displayed improved survival under biotic and abiotic stresses (Rajam et al., 2007; Misra et al., 2016). Induction of these thaumatins in response to fungus only in the resistant variety intensifies their role in defense against *B. sorokiniana*.

2-oxoglutarate (2OG)-Fe(II)-dependent oxygenase superfamily genes are responsible for oxygenation/hydroxylation of phytohormones in plants causing their inactivation. Seven transcripts from this family were upregulated at 1 dpi in CI. Similarly, higher upregulation of these oxygenases is reported in incompatible rather than compatible interactions at early time points (Van Damme et al., 2008). In plants, the 2OG-Fe (II) oxygenase protein participates in the synthesis of diverse compounds, such as flavones, flavonoids and β-1,3-glucanase, which has antimicrobial properties (Coqueiro et al., 2015). Enhanced β-1,3-glucanase activity is linked with fungal resistance.

Wall-associated receptor kinases (WARK) are the enzymes, which activate plant immune system by perceiving the pathogen associated molecular patterns (PAMP) or host cell derived elicitors. Overexpression of such receptor kinases provides enhanced resistance against fungal pathogens in maize against head smut

(Zuo et al., 2015) and leaf blight (Hurni et al., 2015). Besides this, rice plants having *Xa4*, a WARK gene showed improved agronomic trait and resistance to bacterial infection due to increased mechanical strength of cell wall (Hu et al., 2017). Eight genes from this family were upregulated in CI at 1 dpi i.e. biotrophic phase of the pathogen. Hijacking these receptor kinases mediated resistance by necrotrophic pathogen leads to disease progression in plants (Shi et al., 2016). WARK are reported with gene-for-gene disease resistance without causing hypersensitive response in wheat against *Zymoseptoria tritici* (Saintenac et al., 2018). These receptor kinases can serve as the candidates for genetic engineering against spot blotch. Further, exploring these receptor kinases can help in identification of effector mediated interaction during spot blotch.

During pathogen infection, the plant tries to defend itself, while the pathogen tries to evade the plant defense and establish itself. Large-scale transcriptional re-programming and adjustments during the early phase of pathogen invasion govern the disease progression in plants. The interactive transcriptomics aspect of our study revealed novel candidate genes from wheat genome, which are highly differentially expressed. We identified the function for several of these important genes either through Gene ontology or on the basis of protein sequence homology, showing some important roles in pathogen recognition, signaling and secondary metabolite synthesis. However, a large set of genes remain unannotated and demands further analysis. All the above described disease-related genes can serve as the candidates for development of new markers and further characterization of the spot blotch resistance in wheat.

3.3.9 Evaluation of reference genes for qRT-PCR analysis

The qRT-PCR is an integral tool for gene expression analyses in host-pathogen interactions, usually performed using the delta-delta-C_t (DDC_t) method. Accurate relative quantification by this method requires an internal reference gene with stable expression across experimental conditions and tissues. In case of plant-pathogen interaction, selection of a suitable reference gene is even more crucial task due to the presence of plant- as well as pathogen- mRNA in infected tissues. It must be ensured that there is no unintended cross-amplification of the reference gene; i.e. the reference gene selected to evaluate the expression of plant target genes, should specifically amplify only the plant reference gene and not the pathogen reference gene, and *vice*

versa. Additionally, as the infection progresses, the fungal load (and hence its RNA) in the host tissue increases, which could contribute to variation in mRNA concentration and hence influence the normalization. Therefore, selection of an appropriate reference gene is a prerequisite in plant pathogen interaction studies involving qRT-PCR for normalization. Housekeeping genes are supposed to express stably in all cells of all tissue types during entire growth cycle of the organisms.

In a study performed with 'Chinese Spring' wheat, *60S rRNA* and *EF-1α* were reported as the most stable genes; while *Beta-TUBULIN*, *26S rRNA*, *ACTIN*, *GAPDH* and *Alpha-TUBULIN* were the most unstable ones (Long et al., 2010). rRNA genes are not favored in qRT-PCR involving oligo-dT mediated reverse transcription and mRNA expression study. Hence, we initially evaluated the primers for wheat *EF-1α* gene used earlier to profile the expression of *TaLTP5* in wheat-spot blotch pathosystem (Zhu et al., 2012). However, these primers produced cross-amplification from the fungal mycelium-derived cDNA even at higher annealing temperatures (> 62 °C). In contrast, the wheat *ACTIN* gene primers used previously to profile the expression of *TaPIEP1* in hexaploid wheat-spot blotch pathosystem (Dong et al., 2010), specifically amplified the wheat cDNA and not the fungal cDNA in the present study. However, the Primer-BLAST analysis of the primers did not show match with the wheat *ACTIN* gene sequence. Due to this ambiguity, we did not evaluate this gene further. These factors necessitated identification of a suitable reference gene for the gene expression quantification in the wheat-spot blotch pathosystem. Hence, for this study, we selected five genes reported by Long et al. (2010), which showed stable expression across biotic and abiotic stresses as well as in developmental stages of wheat. These genes were: phytochelatin synthase (*PCS*), heterogeneous nuclear ribonucleoprotein Q (*hnRNP*), scaffold-associated regions DNA binding protein (*SAR*), ubiquitin-conjugating enzyme 2 (*ULE*) and eukaryotic translation initiation factor-5α1 (*eIF-5α1*). These five genes showed higher potential as the internal reference genes since they were among those with stable expression in nine microarray experiments involving 333 datasets and further validated by qRT-PCR with cDNA samples from different tissues, stages of development and environmental conditions. In addition, another gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), reported by Beccari et al. (2010) was also selected.

It is known that the UTRs are highly conserved elements of a gene (Siepel et al., 2005). They are species specific and play important role in regulation of mRNA stability, splicing, transport and translation (Zhao et al., 2011). Therefore, we

designed primers from 3' and 5' UTRs of wheat transcripts with the aim of achieving maximum species specificity with wheat. We found that the primers designed from 3' UTR showed better amplification than those designed from 5' UTR (data not shown). This may be attributed to the fact that 3' UTRs are followed by polyadenylation signal and reverse transcription starts from the 3' end. In case of incomplete reverse transcription, the 5' UTR region of the CDS might not get amplified.

3.3.9.1 Amplification specificity and PCR efficiency of reference gene primers

Amplification specificity of the primers for the six reference genes was evaluated by electrophoresis of end-point PCR products onto 2% agarose gel as well as dissociation curve analysis of the qRT-PCR products. After electrophoresis, the primers showing the presence of a single intense band were selected as specific primers. It was confirmed that there was no cross-amplification from the fungal cDNA and the observed amplicon size from the wheat cDNA was comparable to the expected product length for each gene (**Figure 3.37**).

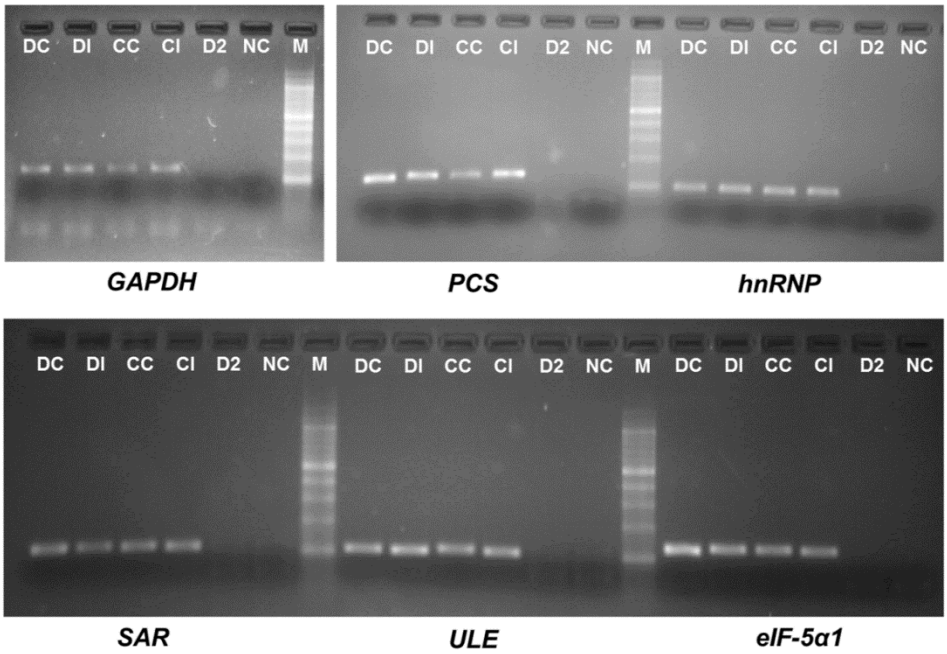


Figure 3.37: Amplification profiles of the candidate reference genes in wheat and fungal cDNA. DC: DDK 1025 control, DI: DDK 1025 inoculated, CC: Chirya 3 control, CI: Chirya 3 inoculated, D2: *B. sorokiniana* strain D2, NC: no template control and M: DNA size marker (100bp ladder)

Dissociation curve analysis for PCR products using qRT-PCR also confirmed specificity of the primers. A single peak was observed for the samples, while no signals were detected in the no-template controls (**Figure 3.38**). However, in case of *eIF-5α1*, even after multiple levels of optimization, the primer dimers were still prominent and hence this primer pair was excluded from further analysis and only five genes were evaluated further.

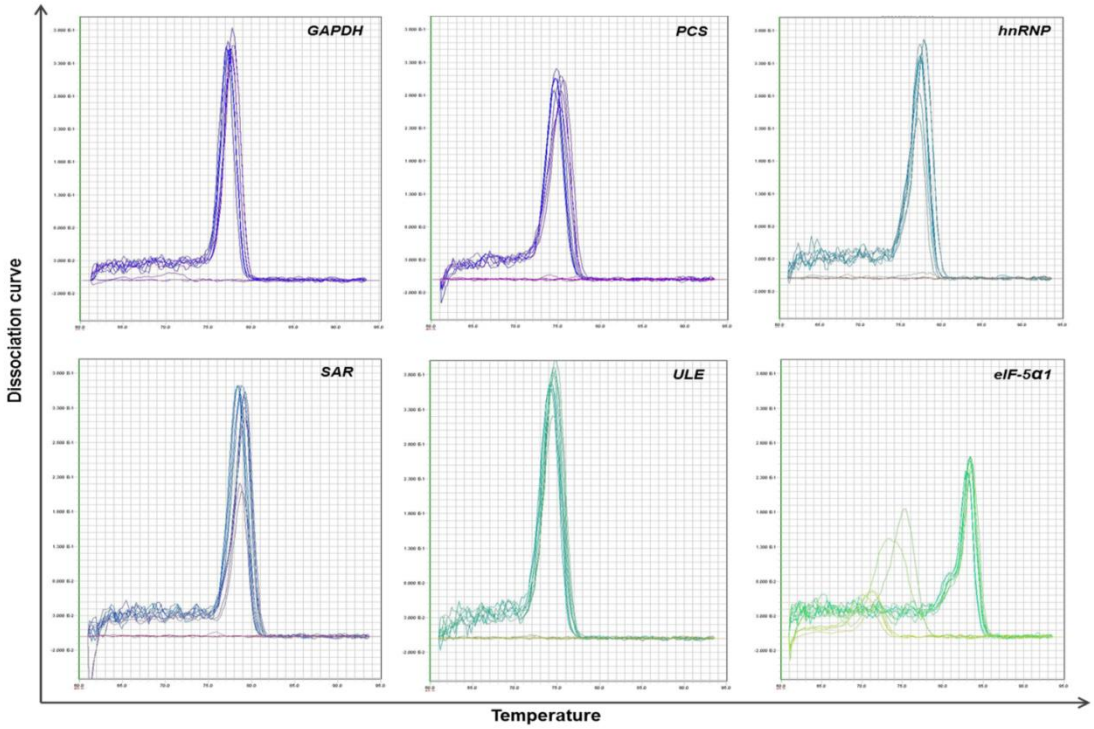


Figure 3.38: Dissociation curves of the candidate reference genes in qRT-PCR

The amplification efficiency was calculated for individual qRT-PCR reaction using the LinRegPCR software (Ramakers et al., 2003). The average efficiency of all the reactions (3 biological and 3 technical replicates) was considered as the PCR efficiency of the primer-pair, which ranged from 1.80 (*GAPDH*) to 1.94 (*hnRNP*). The correlation coefficient (R^2) of linear standard curve varied between 0.994 and 0.999 to the plotted data points of the amplification curve (**Table 3.5**).

Table 3.5: PCR efficiencies (E) and correlation coefficient (R^2) of the qRT-PCR evaluation of the candidate reference genes

Gene Name	PCR efficiency (E)	Correlation coefficient (R^2)
GAPDH	1.80	0.994
PCS	1.91	0.999
hnRNP Q	1.94	0.999
SAR	1.93	0.999
ULE	1.92	0.999

The presence of a single band with no visible primer-dimer formation in end-point PCR, as well as a single peak in the qRT-PCR dissociation curve, confirmed the specific amplification for each gene. The correlation coefficient (R^2) of the linear standard curve (0.9) indicated good quality of amplification. All the primers produced good amplicons and showed specificity to both genotypes of wheat. None of these primers produced PCR product with the genome of *B. sorokiniana* analyzed using the NCBI Primer-BLAST, nor they amplified the fungal genomic or cDNA using end-point PCR or qPCR. These results confirmed that the primers selected for the reference genes of wheat-spot blotch pathosystem are specific to wheat and highly efficient. Further, as desired, the average C_t values of the selected reference genes did not vary between control and inoculated samples. This indicates that the fungal inoculation had no detectable effect on the expression of these five candidate reference genes.

3.3.9.2 Statistical analysis for stable expression

Descriptive statistics and the coefficients of variation (Cv) for the five genes across the time points within treatments, genotypes and across all samples for all the datasets are presented in **Table 3.6**. *SAR* showed the minimum Ct value, while *PCS* showed the highest Ct values in all samples. *GAPDH* showed the maximum Cv (4.89), while *PCS* showed the least Cv (2.00). In addition, a box-plot was drawn depicting the variations in Ct values of the five genes across the datasets and replicates, which also indicated minimum spread of Ct values for *ULE* and *PCS* (**Figure 3.39**).

Table 3.6: Descriptive statistics of the qRT-PCR evaluation of the candidate reference genes

Gene	Min C_t					Max C_t					Average C_t					Coefficient of variation C_t				
	GAPDH	***PCS***	***hnRNP***	***SAR***	***ULE***	***GAPDH***	***PCS***	***hnRNP***	***SAR***	***ULE***	***GAPDH***	***PCS***	***hnRNP***	***SAR***	***ULE***	**GAPDH**	**PCS**	**hnRNP**	**SAR**	**ULE**
DC	22.2	25.6	23.0	20.0	21.4	28.0	28.8	27.6	24.4	24.4	24.2	27.0	24.5	21.9	23.2	4.89	2.00	4.29	4.59	2.75
DI	22.2	26.0	22.7	20.1	22.0	25.7	28.5	28.5	23.2	24.5	24.1	27.0	24.4	21.6	23.2	3.94	2.07	3.88	3.97	2.64
CC	21.6	25.7	21.9	18.2	20.6	25.6	28.7	25.8	21.3	24.5	23.6	27.1	23.9	20.0	22.8	3.94	2.07	3.88	3.97	2.64
CI	22.2	26.1	22.6	18.7	21.3	25.2	28.9	27.6	21.4	24.4	23.8	27.3	24.4	20.2	22.8	3.15	2.24	3.99	2.99	2.95
DDK 1025	22.2	25.6	22.7	20.0	21.4	28.0	28.8	28.5	24.4	24.5	24.2	27.0	24.5	21.7	23.2	4.43	2.03	4.09	4.34	2.69
Chirya 3	21.6	25.7	21.9	18.2	20.6	25.6	28.9	27.6	21.4	24.5	23.7	27.2	24.2	20.1	22.8	3.87	2.30	3.73	3.55	3.34
Control	21.6	25.6	21.9	18.2	20.6	28.0	28.8	27.6	24.4	24.5	23.9	27.0	24.2	20.9	23.0	4.87	2.17	4.00	6.35	3.39

Gene	Min C_t					Max C_t					Average C_t					Coefficient of variation C_t				
	GAPDH	***PCS***	***hnRNP***	***SAR***	***ULE***	***GAPDH***	***PCS***	***hnRNP***	***SAR***	***ULE***	***GAPDH***	***PCS***	***hnRNP***	***SAR***	***ULE***	**GAPDH**	**PCS**	**hnRNP**	**SAR**	**ULE**
Inoculated	22.2	26.0	22.6	18.7	21.3	25.7	28.9	28.5	23.2	24.5	24.0	27.1	24.4	20.9	23.0	3.61	2.22	3.92	4.93	2.91
ALL	21.6	25.6	21.9	18.2	20.6	28.0	28.9	28.5	24.4	24.5	23.9	27.1	24.3	20.9	23.0	4.28	2.20	3.96	5.68	3.12

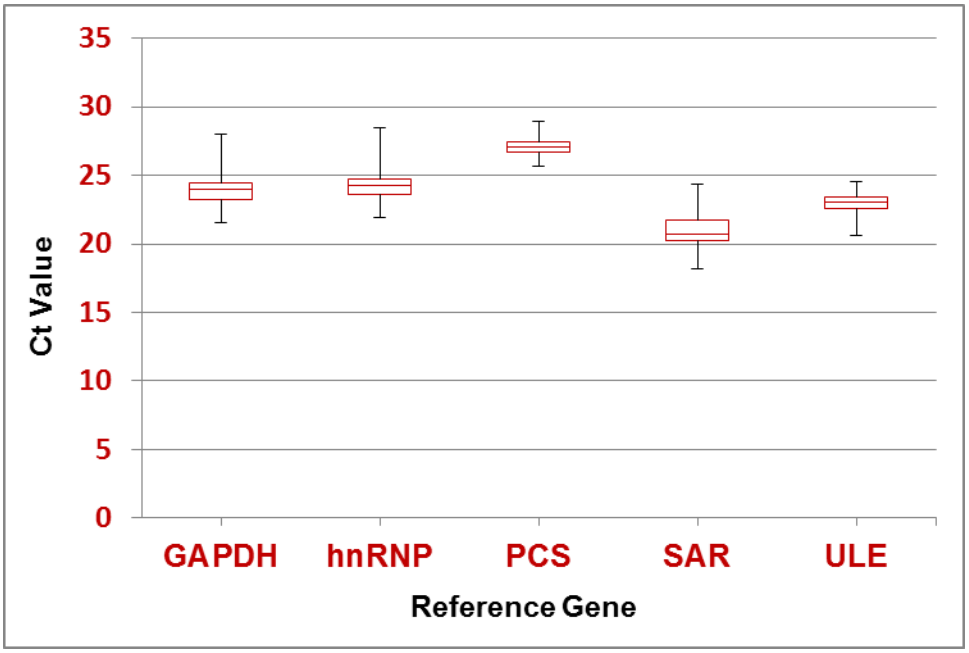


Figure 3.39: Box-plots of C_t values for the four treatments across the time-points for the five candidate reference genes. The lower box indicates first quartile, upper box indicates third quartile, Centre line indicates median and box height indicates interquartile range. Vertical bars indicate minimum (lower) and maximum (upper) C_t values.

The RefFinder suite (http://150.216.56.64/referencegene.php?type=reference) was employed to evaluate the five candidate reference genes using the four algorithms (GeNorm, Normfinder, BestKeeper and comparative delta-Ct). Ranking of the genes varied depending on the algorithm used. The GeNorm analysis indicated that the expression stability score (M value) for all the studied genes was below the threshold limit (1.5). Comparative delta Ct, GeNorm, NormFinder and comprehensive ranked *ULE* as the best reference gene with minimum M values for the 'All' samples dataset. BestKeeper showed minimum M value for *PCS*. Comprehensive ranking for DDK 1025 control and inoculated data set gave the minimum value of M to *PCS* followed by *ULE* (**Table 3.7**). However in other datasets, *ULE* was selected as the best reference gene (**Table 3.8**). These results suggest *ULE* and *PCS* as the most stable reference genes. Between these two, *ULE* had moderate expression, while *PCS* had lower expression (higher Ct values).

Table 3.7: Gene stability score of the candidate reference genes for all samples based on different evaluation methods

Method	***ULE***	***PCS***	***GAPDH***	***hnRNP***	***SAR***
Delta CT	**1.16**	1.21	1.26	1.35	*2.14*
BestKeeper	0.56	**0.48**	0.78	0.71	*1.10*
Normfinder	**0.41**	0.65	0.67	0.89	*2.04*
GeNorm	**0.68**	**0.68**	0.85	0.94	*1.43*
Comprehensive	**1.19**	1.41	3.22	3.72	*5.00*

Note: The lower the value, more stable is the gene expression. For a given method, the values in 'bold' are the lowest and those in 'italics' are the highest.

Table 3.8: Comprehensive gene stability score of the five candidate reference genes within the genotypes and the treatments

Gene	**DDK 1025**		**Chirya 3**		**Control**		**Inoculated**	
	Comprehensive	**GeNorm**	**Comprehensive**	**GeNorm**	**Comprehensive**	**GeNorm**	**Comprehensive**	**GeNorm**
ULE	2.06	<u>0.61</u>	<u>1.19</u>	<u>0.59</u>	<u>1.19</u>	<u>0.54</u>	<u>1.19</u>	<u>0.78</u>
PCS	<u>1.00</u>	<u>0.61</u>	1.41	<u>0.59</u>	1.41	<u>0.54</u>	1.57	<u>0.78</u>
GAPDH	4.23	0.83	3.22	0.70	3.22	0.84	2.91	0.84
hnRNP	4.40	0.96	3.72	0.81	4.23	0.95	3.72	0.91
SAR	2.63	0.69	5.00	1.53	4.40	1.03	5.00	1.67

Note: The underlined are the most stable genes within each genotype/ treatment

Descriptive analysis of C_t values revealed the best reference gene for quantitative expression analysis of wheat. According to GeNorm, both *ULE* and *PCS* were the best stable genes for all the data sets with the same M score. However, based on comprehensive stability value, *ULE* was the most stable among the five genes. *ULE* also showed higher expression (lesser C_t value) and stable (lesser M value) across all the samples and conditions evaluated in this study. Hence, we propose *ULE* as the most stable internal reference gene for evaluating candidate defense related genes in wheat.

3.3.9.3 Differential expression analysis of candidate defense genes

Differential expression of the eight candidate defense responsive genes (**Table 3.3**) was evaluated based on the DDCt method using *ULE* as the reference gene and significance of the results was tested using the Student's t-test (P≤0.05). Differential gene expression was observed for all the genes at different time points in the four samples; however, the fold change level varied. Among all the genes, *TaMCA4* indicated significant expression difference between the control and inoculated conditions at 4 dpi. *TaADF7*, *TaMDHAR-4*, *TaLls-1*, *TaSYP132* and *TaAOS* showed differential expression in response to pathogen inoculation in the susceptible genotype (**Figure 3.40**).

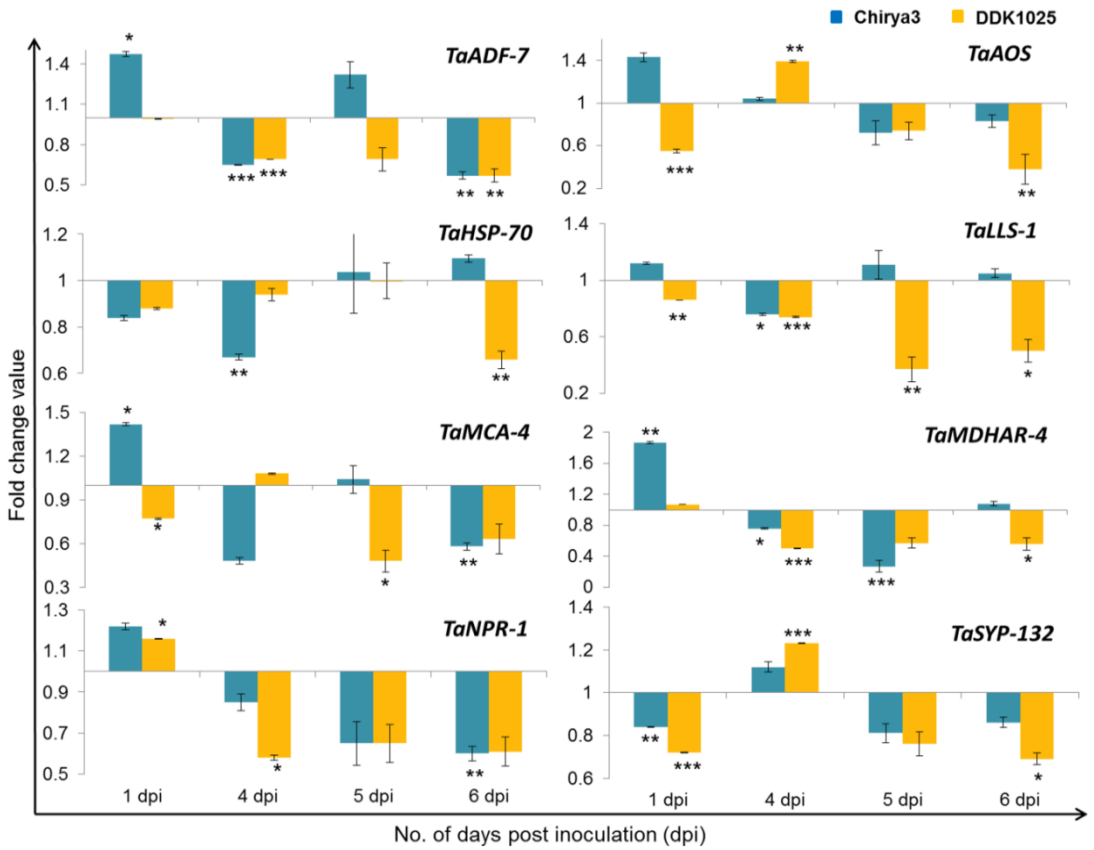


Figure 3.40: Gene expression modulation of the candidate defense responsive genes across the time-points in the spot blotch pathosystem. (* indicates the significance of Delta C_t values between the control and the inoculated samples based on t-test; *- P≤0.05, **- P≤0.01, ***- P≤0.001)

Upon pathogen attack, plants modulate the expression of several defense related genes. We found altered expression of few such candidate defense genes in wheat-*B. sorokiniana* pathosystem using qRT-PCR with *ULE* as the internal reference gene. These genes were reported to have distinguished role in pathogen defense in other pathosystems. The actin depolymerization factor-7 gene variants were upregulated during avirulent *Puccinia* infection in wheat and knock down of this gene in wheat led to increased hyphal growth of the pathogen (Fu et al., 2014). In contrast, upregulation of this gene at early time-point in resistant variety illustrated signal for pathogen recognition and defense for pathogen clearance. Hence, upregulation of this gene in spot blotch susceptible variety after pathogen inoculation might help in plant defense. Jasmonic acid biosynthesis is upregulated upon biotrophic pathogen recognition. Jasmonic acid mediated defense gene system inhibits necrotrophic pathogens (Mei et al., 2006). Early overexpression of *AOS*, a gene involved in jasmonic acid biosynthesis in resistant variety, showed pathogen mediated activation of the defense mechanism and restriction of necrotrophic phase. Downregulation of *LLS-1*, the gene responsible for homeostasis and cell death regulation (Tang et al., 2013), might contribute in development of necrotrophic lesions in susceptible variety, DDK 1025.

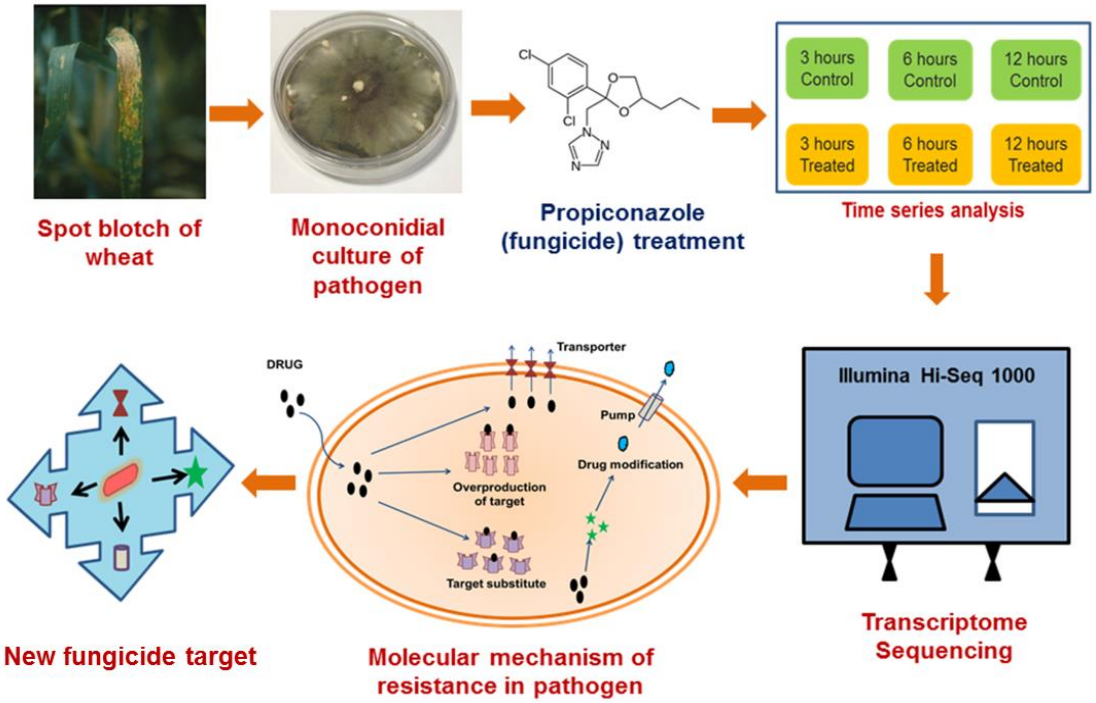
Metacaspases are responsible for programmed cell death mediated removal of diseased cells and hence inhibition of pathogen progression (Wang et al., 2012). The pathogenesis channeled downregulation of *MCA-4* might be responsible for successful pathogen progression in case of susceptible variety. In contrast, timely upregulation of *MCA-4* and hence removal of pathogen affected cells in Chirya 3 could have led to restricting pathogen growth and disease progression. Monohydroascorbate reductase is upregulated in biotrophic infection (Feng et al., 2014). Upregulation of *MDHAR*-4, the stress regulating gene in Chirya 3 soon after inoculation, is indicative of biotrophic phase of the pathogen. However, downregulation of *MDHAR-4* in both the genotypes during next stages suggests the activation of defense through H_2O_2 accumulation and activation of PR genes.

The crosstalk between various plant hormones is responsible for the defense response resulting in restricting the pathogen and protecting the plant under stress. The non-expressor of pathogenesis related protein (*NPR-1*) is involved in activation of salicylic acid mediated systemic acquired resistance. *NPR-1* has been reported to

play an important role in early stages of *Fusarium graminearum* infection in susceptible wheat genotypes (Diethelm et al., 2014). The overexpression of *NPR-1* in response to *Bipolaris sorokiniana* at 1 dpi in DDK 1025 indicates similarity with *Fusarium* infection. *TaSYP-132* has been reported to provide resistance against *Puccinia striiformis*, the fungal pathogen causing stripe rust in wheat. Knocking down *TaSYP-132* expression caused enhanced hyphae growth (Wang et al., 2014). Hence, the suppressed expression of *TaSYP-132* in both the varieties at early time-point in this study might indicate effector mediated downregulation strategy of the pathogen to cause the disease. However, the increase in expression of the gene was observed after the pathogen switched to the necrotrophic phase in DDK 1025.

In case of Chirya 3, the defense system was activated through jasmonic acid mediated signaling and the pathogen could not establish itself; hence no significant change in gene expression was observed. We did not detect very high fold change expression of the defense genes selected from previous studies. Nevertheless, the observation from our differential gene expression study showed results comparable to previous literature. *Dicoccum* genotypes are resistant to rust and other fungal pathogens, but are highly susceptible to spot blotch (Smurova and Mikhailova, 2007). Hence, the genes responsible for the defense against spot blotch might be different from the genes involved in resistance to rusts and other fungal diseases, as we identified significant differential expression of several genes that with no known function. This underlines the need for exploring this pathosystem further for identification of resistance genes.

Chapter 4:
Global gene expression analysis in response to propiconazole in *Bipolaris sorokiniana*



Publications based on this Chapter:

Somani Deepika, Adhav Ragini, Prashant Ramya, Kadoo Narendra (2019) Transcriptomics analysis of propiconazole-treated *Cochliobolus sativus* reveals new putative azole targets in the plant pathogen. *Functional & Integrative Genomics*, DOI: 10.1007/s10142-019-00660-9.

4.1 Introduction

Cochliobolus sativus (S. Ito & Kurib.) Drechsler ex Dastur (teleomorph) [Bipolaris sorokiniana (Sacc.) Shoemaker (anamorph)] is a pervasive plant pathogen of grasses including cereals like wheat, barley, and rice. Its potential to infect almost all crops belonging to the Poaceae (Gramineae) family makes it a pathogen of major concern. B. sorokiniana causes foliar spot blotch, root rot, black point on grains, head blight, leaf blight and seedling blight of wheat and barley (Kumar et al., 2002). The hemi-biotrophic fungal pathogen is becoming an increasing threat to wheat growing areas particularly in the Southeast Asian and Latin American countries because of the disease-conducive warm and humid climate in these areas during the cropping seasons. The estimates of yield losses due to spot blotch vary from 15% (Dubin and Ginkel, 1991) to 80% (Duveiller and Gilchrist, 1993), and might reach up to 100% under severe infection conditions in individual fields (Mehta, 1998).

An integrated approach involving resistant varieties, suitable cropping systems, cultural practices and timely application of fungicides can effectively control the disease under field conditions (Joshi and Chand, 2002). Germplasm evaluation against spot blotch as well as breeding new resistant varieties is under progress in several wheat breeding programs. However, very few wheat genotypes with confirmed resistance to spot blotch have been developed, while most of the existing wheat germplasm has not yet been systematically evaluated against the disease. As a result, the application of fungicides is currently the most adopted method to manage the disease. Two major classes of fungicides (strobilurins and triazoles) having broad spectrum activity against fungal pathogens are used to manage foliar as well as seed-borne fungal diseases of wheat. The strobilurins are named after their original source Strobilurus tenacellus, a mushroom that grows on the decaying cones of Pinus (Anke et al., 1977). They are quinone outside inhibitors (QoI) and have a specific-site mode of action i.e. interference with energy production by binding to cytochrome bc1 complex in yeast and fungi. They act by inhibiting fungal spore germination and early infection, and are highly effective when applied preventively (Balba, 2007). The triazoles have a five-membered ring of two carbon atoms and three nitrogen atoms. They hamper fungal growth by inhibiting the biosynthesis of sterols, which are the

building blocks of fungal cell membranes. They are curative and move systemically through the plant xylem. Triazoles act as demethylation inhibitor (DMI) and inhibit the sterol 24-c-methyltransferase and lanosterol 14 alpha-demethylase enzymes in steroid biosynthesis (Mullins et al., 2011; Hargrove et al., 2017). Triazoles are highly reliable because of their effectiveness against early fungal infections and the ability to reach to the site of infection in the plant. Metconazole, propiconazole, prothioconazole, and tebuconazole are some of the most commonly used and effective triazoles for field application (Wegulo, 2012). Propiconazole is widely used to manage spot blotch in wheat fields. However, indiscriminate use of fungicides can cause evolution of fungicide resistant strains in fungal pathogen population, which is an acute issue due to the limited number of potent antifungal compounds that are currently available.

In general, fungi can develop resistance to antifungal compounds through several possible mechanisms like overproduction of the target enzyme, alteration of the target enzyme, inactivating the anti-fungal compound either through degradation or inhibition, efflux of the compound or preventing its uptake (Ghannoum and Rice, 1999). The known mechanisms of azole resistance include overexpression or modification of the binding sites of the target enzyme, lanosterol 14 alpha-demethylase (encoded by ERG11) (Sanglard and Odds, 2002) and/or constitutive overexpression of multidrug transporters (Cannon et al., 2007), which efflux the fungicide out of the cell. In Saccharomyces cerevisiae, microarray analysis provided useful information to understand the specific/ independent changes in response to antifungals (Agarwal et al., 2003). High-throughput techniques like transcriptomics, proteomics, and metabolomics can reveal the targets, mechanisms of action, and off-target effects of small molecules (Dos Santos et al., 2016). In this study, we investigated the molecular response of C. sativus to sub-lethal doses of propiconazole through a time-series transcriptome analysis, with the objective of exploring the molecular response of the pathogen to the azole fungicide stress that would help in understanding its mode of action in this pathogen and reveal additional fungicide targets. We observed differential expression of not only the known azole target genes, but also those from other metabolic pathways, transporters, and stress regulators. Overall, this study has identified novel targets to develop new fungicides that could

potentially be used either separately or in combination for better disease management in field.

4.2 Materials and Methods

4.2.1 Fungal culture and fungicide sensitivity assay

Monoconidial culture of *C. sativus* isolate D2 was used for the experiment. The isolate had been previously isolated from infected wheat fields at Dr. Rajendra Prasad Central Agricultural University (Pusa, Bihar, India) and monoconidial culture was established at CSIR-National Chemical Laboratory, Pune. For this purpose, the pathogen was sub-cultured on potato dextrose agar (PDA) at 28ºC with alternating 12 h light and 12 h dark period for 10 days. Sporulating culture was observed at 80X under stereo-zoom microscope (Leica, Germany). Conidia were harvested by scraping the surface of the medium with inoculation loop and suspended in sterile distilled water. Mycelia were separated from spores by filtering the suspension through a sterile muslin cloth. The resulting conidial suspension was diluted to achieve the spore count of 10^2 /ml, and 100 µl of the suspension was spread-plated on water agar containing streptomycin (150 mg/l). Five such plates were incubated at 28ºC for 24 h with 12 h light period and observed for germination at 80X under the stereo-zoom microscope. Individual germinating spores were marked on petri-plates. The agar blocks containing a single germinating spore each were transferred to individual PDA plates containing streptomycin (150 mg/l) using a 5 mm cork borer. These "master" plates were incubated at 28ºC for 72 h with alternating 12 h light and 12 h dark periods, followed by sub-culturing on PDA plates. The culture was confirmed to be *C. sativus* based on the spore, mycelial and culture characteristics as well as by sequencing the fungal ITS region (NCBI Accession No.: KJ562716). Koch's postulates were verified by the appearance of typical spot blotch disease symptoms upon inoculation of the pathogen on 15-day old seedlings of spot blotch susceptible wheat variety "Agra Local" and re-isolation of the pathogen from the infected plants.

The sensitivity of *C. sativus* strain D2 to the triazole compound propiconazole was measured in terms of EC_{50} (effective concentration to give half maximal response) value. Initially Tilt® (Syngenta, USA), the commercially available emulsifiable concentrate of propiconazole, was used for this study. However, as the composition of the formulation of Tilt® was unknown, setting up of a suitable control

(formulation without propiconazole) for the experiment was not possible. Moreover, there was a possibility of having confounding effect on the experiment by constituents of the formulation itself. Hence, analytical grade propiconazole (Sigma-Aldrich, USA; Catalog No: N13576-250MG) was used for the experiment. As propiconazole is insoluble in water, 10 mg/ml stock of the compound was prepared by dissolving it in 1:9 mixture of methanol and sterile de-ionized water. To determine the EC_{50} /sub-lethal concentration of propiconazole for the D2 strain of *C. sativus*, the propiconazole stock solution was mixed with PDA to achieve final concentrations of 2, 4, 6, 8, 16, 32, 48, 60, 72 and 100 µg/ml of the medium. Spore suspension (10 µl of 5×10^6 spores/ml) of *C. sativus* D2 strain was inoculated in the center on these PDA plates and grown at 28ºC for seven days. Additionally, two controls were also set up: (i) PDA plates with equal volume of 1:9 stock solution of methanol and water, without propiconazole, and (ii) PDA plates with neither methanol nor propiconazole. Fungal growth was observed regularly till seven days post-inoculation and the EC_{50}/ sub-lethal concentration of propiconazole was determined.

4.2.2 RNA isolation and sequencing

For transcriptome analysis, 500 µl of spore suspension (5×10^6 spores/ml) was inoculated separately into eighteen 50 ml tubes containing 10 ml PDB and incubated at 28°C with shaking at 180 rpm. After five days, the growing cultures were fed with 9 ml of fresh PDB medium followed by 12 h incubation. After incubation, nine cultures were infused with 1 ml of PDB containing propiconazole, with the final pre-determined half maximal effective concentration/sub-lethal concentration of 16 ppm. For controls, the remaining nine tubes were fed with 1 ml of PDB containing only the solvent (1:9 solution of methanol in water without propiconazole). The cultures were further incubated for 3, 6 and 12 h in three biological replicates and harvested. At the end of each time point (3, 6 and 12 h for different samples), the tubes were immediately kept on ice to prevent further metabolic activity and the mycelia from each tube were harvested separately. Residual medium was removed from the mycelia by washing thrice with ice-cold sterile de-ionized water followed by centrifugation. The mycelia were immediately crushed to fine powder in liquid nitrogen and stored at -80ºC till RNA isolation.

Total RNA was isolated from 100 mg of the finely ground mycelia using the Spectrum™ Plant Total RNA kit (Sigma-Aldrich, USA), followed by DNase treatment (Promega, USA). The DNase treated samples were purified using RNA binding column provided in the Spectrum™ Plant Total RNA kit. The quality and concentration of the isolated RNA were evaluated using Nanodrop 1000 (ThermoFisher Scientific, USA) and 1% agarose gels. The integrity of the RNA samples was evaluated by running on Bioanalyzer 1000 (Agilent, USA) and the samples with RNA Integrity Number (RIN) >6.0 were used for library preparation for next generation sequencing. Paired-end sequencing was performed using HiSeq 1000 sequencing system (Illumina, USA), by following the standard Illumina RNA-Seq protocol.

4.3.3 Sequence data analysis

The basecall files obtained from the sequencer were converted into fastq format and demultiplexed using CASAVA ver. 1.8.2 software (Illumina, USA). Reads with quality score higher than 20 for >70% of the bases were filtered using the IlluQC module of NGSQC ver. 2.3.3 toolkit (Patel and Jain, 2012). The reference genome of *C. sativus* ND90Pr (North American strain) and its annotations were downloaded from the Mycocosm database (http://genome.jgi.doe.gov/Cocsa1/Cocsa1.home.html). The filtered reads were submitted to the alignment tool TopHat2 (Trapnell et al., 2009; Kim and Salzberg, 2011; Kim et al., 2013) and maximum number of hits required to consider the alignment was set as 20. The gene expression levels were calculated for two different units: i) FPKM (Fragments per kilobase million) and ii) CPM (Counts per million) reads. For FPKM estimation, Cufflinks ver. 2.2.1 (Trapnell et al., 2012) was used and the maximum intron length was set to 1000; whereas HTSeq ver. 0.6.1 (Anders et al., 2015) was used for CPM estimation. The differentially expressed genes (DEGs) were estimated by Cuffdiff and NOIseq ver. 2.16.0 (Tarazona et al., 2012) for FPKM and CPM methods, respectively. Minimum and maximum cutoff for $\log_2$ fold change (LFC) was kept as -1 and +1 respectively. DEGs common from both protocols were extracted using Venny ver. 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/) (Oliveros, 2007-2015) and were considered for further analysis.

4.3.4 Gene Ontology annotations and pathway analysis

Gene Ontology annotation for GO terms, Pfam and Interpro motifs of differentially expressed transcripts provide insights into their functional properties. For this, protein sequences for all the DEGs were downloaded from Mycocosm database (http://genome.jgi.doe.gov/programs/fungi/index.jsf) and were mapped to the fungal proteins in the NCBI (http://www.ncbi.nlm.nih.gov/) nr protein database using standalone blastp on an in-house server. The BLAST results were imported in Blast2GO ver. 3.1.3 (Conesa et al., 2005) platform for GO (Gene Ontology; www.geneontology.org/) and KEGG (Kyoto Encyclopedia of Genes and Genomes; https://www.genome.jp/kegg/) pathway analysis. The mapping module of Blast2GO retrieved the GO annotations and the annotation module was employed to select reliable functions. The annotation module generated information like motif details, protein domains and protein families from various protein databases. The Blast2GO analysis also mapped the proteins with Enzymes Codes (EC) to their respective KEGG pathways. Fold change in expression was calculated using both FPKM as well as CPM values, while the NOIseq data were used for other analyses as described later.

4.3.5 Functional protein association networks

Differentially expressed transcripts were checked for their functional correlation using STRING ver. 10.5 (https://string-db.org/) (Szklarczyk et al., 2014). As the *C. sativus* interaction information was not available in the STRING database, protein sequences from *C. sativus* were BLAST against *Pyrenophora tritici-repentis* (which was the closest Dothideomycetes member available in the STRING database) to identify similar sequences. One gene with the highest similarity with respect to each sequence was selected for network analysis.

4.3.6 Relative gene expression quantification using qRT-PCR

qRT-PCR was performed to validate the results of differential gene expression obtained through transcriptome analysis in response to propiconazole treatment for the 12 DEGs shared by the three experimental time points. For this, total RNAs were isolated from propiconazole treated and control cultures for three biological replicates as described previously. Two micrograms of RNA from each sample was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, USA). The cDNAs (diluted to 1:20) were assessed for the transcript levels

of the selected genes (**Table 4.1**) using the SYBR Green PCR master mix (Roche, Switzerland) and the ABI 7900HT Fast Real Time PCR System (Applied Biosystems, USA). Each reaction contained 5 ng cDNA templates, 0.33 µM of each primer and 5 µl SYBR Green master mix in 10 µl reaction volume in 384 well format PCR plate. The thermal cycling profile consisted of initial denaturation at 95ºC for 10 min followed by 40 cycles of 95ºC for 3 sec and 60ºC for 30 sec. Following amplification, a melting dissociation curve was generated using a 60–95°C ramp to monitor specificity of the primers.

The exponential phase of the reaction was identified using LinReg ver. 2.0 (Ramakers et al., 2003) by plotting the fluorescence on a log scale and linear regression analysis was performed to estimate the efficiency of each reaction. The fungal gene glyceraldehyde phosphate dehydrogenase (*GAPDH*) was used as the reference gene for normalization. The relative expression fold change of each gene in propiconazole induced sample versus control was calculated using the DDCt method (Livak and Schmittgen, 2001).

Table 4.1: The genes and the respective primers used for qRT-PCR validation

S No	**Gene Name**	**JGI protein ID**	**Forward primer sequence (5'-3')**	**Reverse primer sequence (5'-3')**	**Amplicon size**
1	Glyceraldehyde 3-phosphate dehydrogenase (Reference gene)	41141	GGCAACGCTTAGGAGTCAGGA	GCCTAGCCAGAAGTTCCGAGAAT	103
2	Cellulose binding protein	245819	CGTCTCGCGCTCTGGTATGA	AGCCCGGAAGCCAAAGAAGG	107
3	Dolichol phosphate mannosyltransferase	190143	ATGGGTGGATGCGATGCCAA	TCCATGAAAGACCTAGTGCGACC	105
4	Ent-kaurene oxidase	181322	AATGATAGTCGCCCGCCTCG	AGCGTCTTTGTGGGATCAGGC	117
5	Sterol 24-C-methyltransferase	39890	CCAGATTGAGCGTGCGACCA	GGCATCGAAGGAGTTGTCAGGG	112
6	Sphingoid Base Transporter rsb1	192685	TCCTCCACGAAAACCCCTGGA	CGACGTGCTTGAGTGTCAGGT	111
7	Spore germination protein	201010	GGTGTGCAAGAATCCACCCC	AGCCCCAGGATTGTCAAGTGT	109

S No	Gene Name	JGI protein ID	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Amplicon size
8	Ligninase lg6 precursor	152826	AGAAGGCGTGGGATGATGCG	TTCGCCGCACTACTCTTCGG	115
9	Glycosyltransferase	33275	AGCTGCGGAAGTCATGTGGA	ACAAAAGGACGGCACAAGAGC	104
10	Phospholipase C	86152	AGCTTGTCTCATCGCATCCAGA	CCAACAACACTCCCTCCCCA	105
11	Splicing factor 3B	183584	TGCCACTTTCCAGCCACAAG	AGTCCGCTGTCTCAACTCTGG	104
12	α-β barrel domain protein 1	97244	GCAGGCCATTGAATACACCACT	GCAGCCTCGTCCTTGAACAC	94
13	α-β barrel domain protein 2	33076	CCTCATGGCTCGCTGTGTCT	GCCTCAAACCTGTGCAGCAA	108

4.3 Results

4.3.1 Fungicide sensitivity assay

The EC50 value of propiconazole for the D2 strain of *C. sativus* was determined using inhibition of radial growth method (Secor and Rivera, 2012). The final concentration of propiconazole at 16 ppm (in 1:9 mixture of methanol and sterile de-ionized water) was found as the EC50 value (**Figure 4.1** and **4.2**). Hence, this concentration was considered for further experimentation.

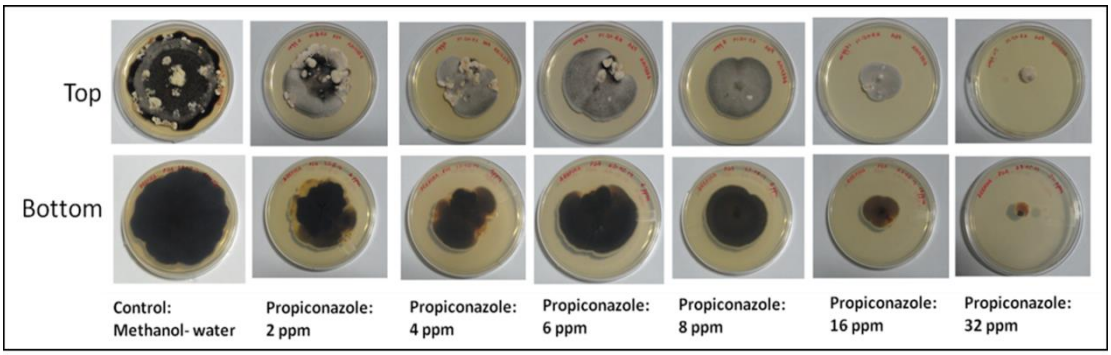


Figure 4.1: Radial growth inhibition assay using PDA plates for 2-32 ppm concentration. (Top and bottom views of culture plates in 2 ppm – 32 ppm concentration of propiconazole)

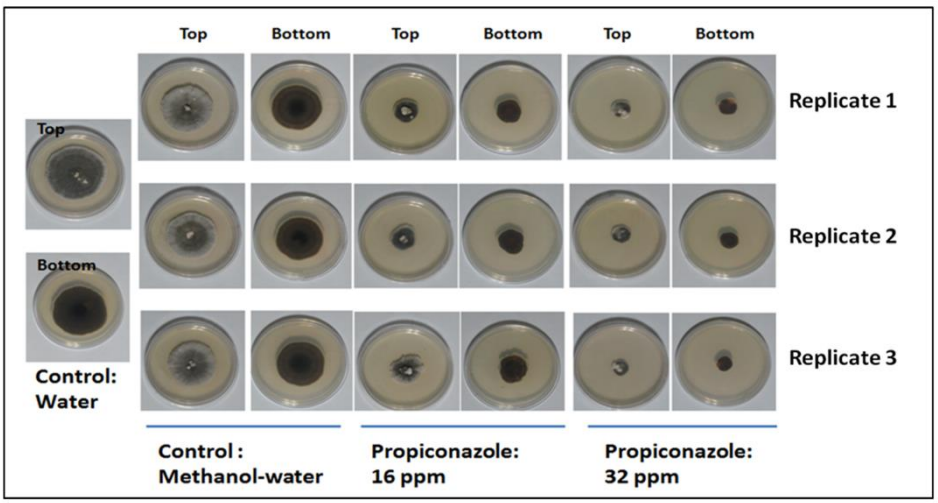


Figure 4.2: Comparison of culture in 16 ppm and 32 ppm concentrations of propiconazole with respect to control using radial growth inhibition assay

4.3.2 Gene expression in response to propiconazole

4.3.2.1 Quality and statistics

The average number of reads from propiconazole treated and control libraries were about 45 million. The raw data from the next generation sequencing run was submitted to NCBI sequence read archive (SRA) under the Bioproject accession no. SRP124410. Eighty nine percent of the high quality filtered reads could be aligned to the reference genome (*Cochliobolus sativus* ND90Pr; http://genome.jgi.doe.gov/Cocsa1/Cocsa1.home.html) using TopHat2. For reliable documentation of the DEGs, we employed two protocols instead of one viz. Cuffdiff (FPKM based) and NOIseq (Count based HTseq). The Cuffdiff protocol revealed 103, 359 and 355 DEGs for the three time points: 3 h, 6 h and 12 h respectively; whereas the numbers of DEGs through the NOIseq protocol were 598, 1029 and 823 for the same time points (**Table S4.1**). The common DEGs from Cufflinks (FPKM) and NOIseq (CPM) protocols were 61 for 3 hpt, 252 for 6 hpt and 251 for 12 hpt (**Figure 4.3** and **Table S4.2**). Of the 564 DEGs, 280 DEGs were upregulated at various stages, while 284 DEGs were downregulated (**Table S4.3**).

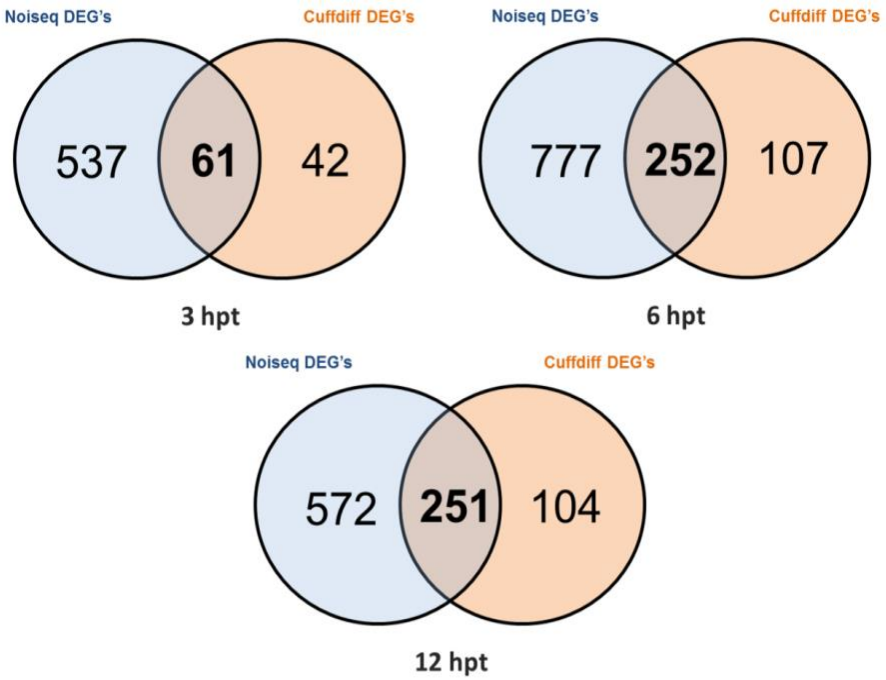


Figure 4.3: The numbers of DEGs identified in propiconazole treated culture of *B. sorokiniana* strain D2 for 3 h, 6 h and 12 h respectively from RNA-Seq analysis

4.3.2.2 Annotation of differentially expressed genes

DEGs common to both the NOIseq and Cuffdiff protocols were annotated using Blast2GO. Transcripts were classified according to their roles as Cellular Component, Biological Process or Molecular Function (**Tables S4.4** and **S4.5**). The DEGs without any known function were classified as hypothetical proteins. Among the annotated DEGs, only 54.46% (32/61), 48.02% (121/252) and 56.57% (142/251) DEGs at 3 hpt, 6 hpt and 12 hpt respectively, were classified to any of the three categories (**Figure 4.4**).

Among those classified under Cellular Component, the genes associated with integral component of cell membrane were enriched. Similarly, oxido-reductase activity related genes were enriched among those associated with Biological Process throughout the three time points. The distribution of the DEGs to Cellular Component and Biological Processes across the 3 hpt, 6 hpt and 12 hpt is depicted in **Figures 4.5** and **4.6**. Zinc and heme ion binding activity associated genes were dominated in the Molecular Function category of DEGs.

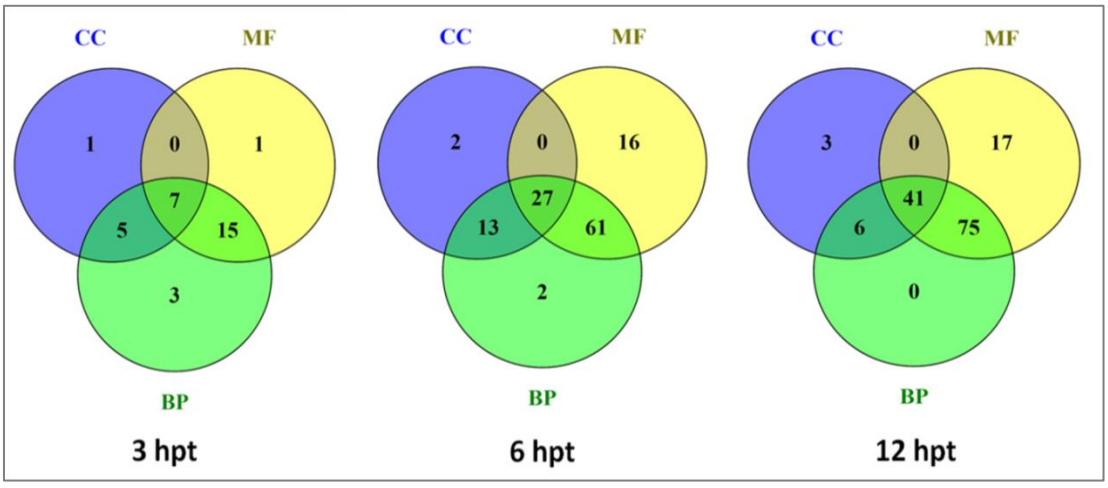


Figure 4.4: Functional classification of differentially expressed transcripts (CC: Cellular Component, MF: Molecular Function, BP: Biological Process)

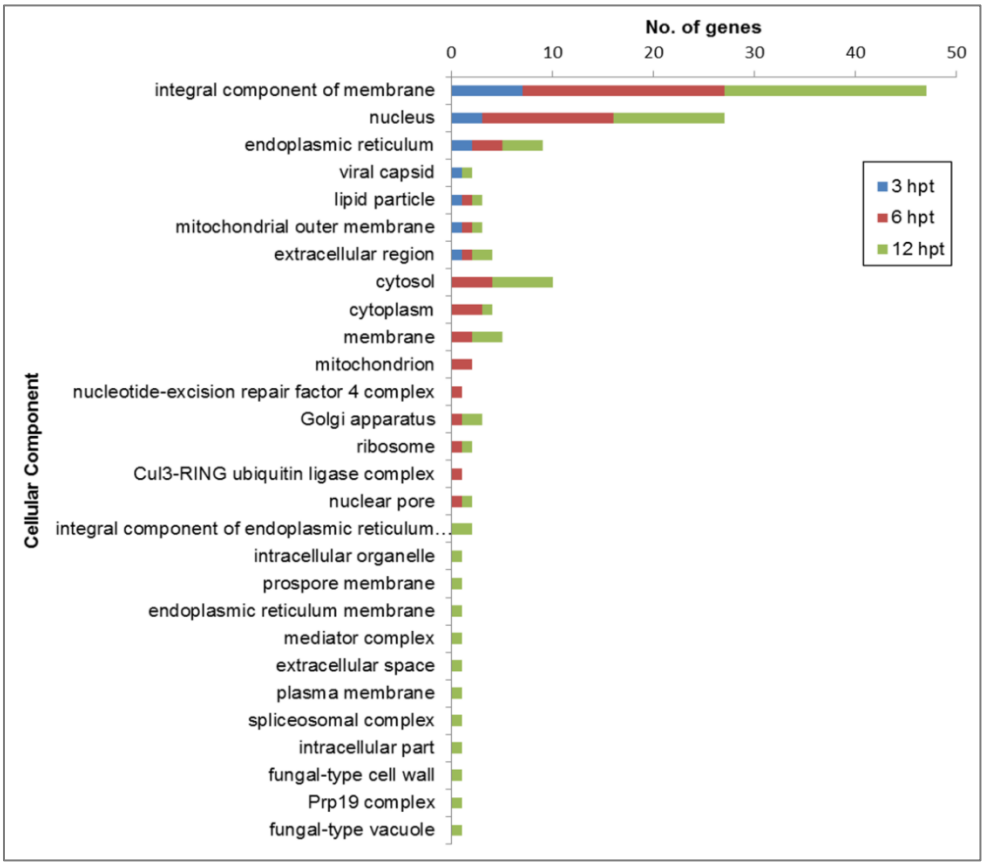


Figure 4.5: Enrichment analysis of DEGs for Cellular Components at 3 hpt, 6 hpt and 12 hpt from propiconazole treated fungal culture

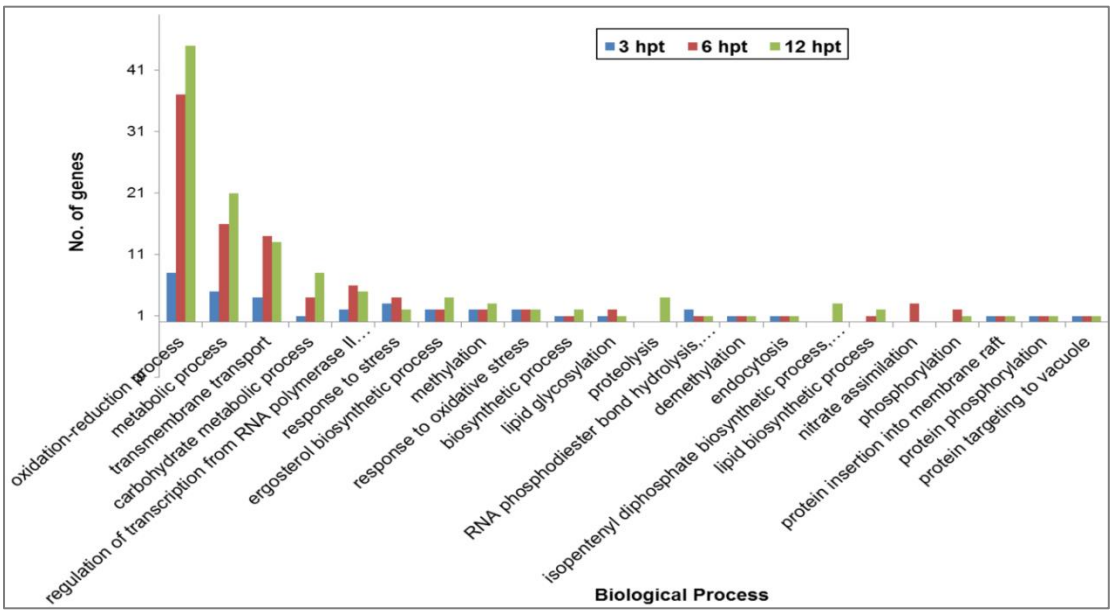


Figure 4.6: Enrichment analysis of DEGs for Biological Process at 3 hpt, 6 hpt and 12 hpt from propiconazole treated fungal culture

4.3.2.3 Molecular functions of the DEGs

A majority (133) of the genes were upregulated at 6 hpt stage; while only 15 genes were upregulated at 3 hpt stage. Of the upregulated genes, majority (48) of the DEGs were biosynthesis associated genes, while only seven genes belonged to the electron transport system. Transcription and translation related (77) and biosynthesis associated (66) genes included the maximum number of DEGs (**Table S4.3**). KEGG annotation mapped 5 of 61 genes at 3 hpt to eight pathways, 14 of 252 genes at 6 hpt to 23 pathways and 14 of 251 genes at 12 hpt to 35 pathways (**Table S4.6**).

4.3.2.4 Expression modulation at 3 hpt

Of the 61 genes that were differentially expressed at this time point, 15 genes were upregulated (**Table S4.7**), while 46 genes were downregulated (**Table S4.8**). Biosynthesis of antibiotics and steroids were particularly altered in response to propiconazole treatment (**Figure 4.7**). The gene ent-kaurene oxidase was highly upregulated (LFC 3.65) followed by sterol 24-C-methyltransferase (LFC 3.22). Similarly, the expression of duf 1749-domain-containing protein of alpha-beta hydrolase superfamily was enhanced, while aromatic ring hydroxylase was suppressed. The downregulated genes also included tyrosine and arginine biosynthesis associated enzymes 4-hydroxyphenylpyruvate dioxygenase and acetylornithine aminotransferase, vitamin D catabolizing enzyme, retinol dehydrogenase, polyketide synthase (involved in synthesis of secondary metabolites), oligopeptide transporter (involved in import of various substances), MFS-1 transporters (associated with transport of molecules like amino acid and phosphate), and hypersensitive response inducing protein. In contrast to the spore germination protein that was upregulated, the spore development regulator, vosA was downregulated. Likewise, naphthalene triol reductase (responsible for darkening of mycelia due to melanin synthesis) (Liu et al., 2011), splicing factor 3b (responsible for ribonuclease activity), and gag-like protein (responsible for RNA packaging activity) were also suppressed. Similarly, small cysteine rich protein (presumed to play an important role in pathogenicity as effector molecule) was also downregulated.

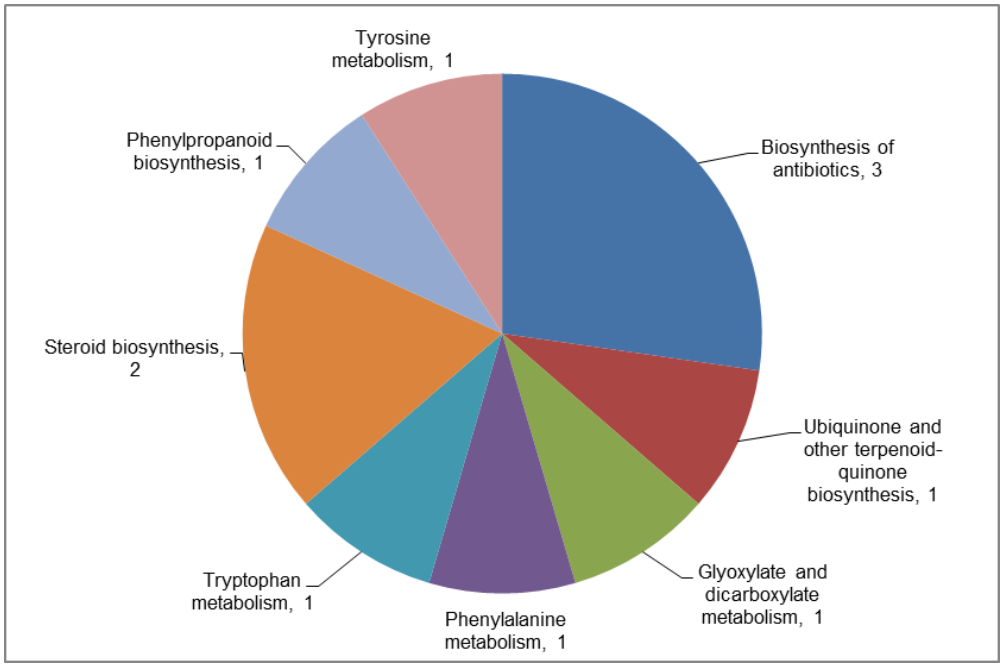


Figure 4.7: KEGG annotation of enzymes from DEGs to respective pathways at 3 hpt

4.3.2.5 Expression modulation at 6 hpt

The metabolism of carbohydrates along with steroid biosynthesis was prominent at this stage. Diverse secondary metabolite biosynthesis pathways, alkaloid biosynthesis, phenyl propanoid biosynthesis, sesqueterpenoid biosynthesis, gibberellic acid biosynthesis, and cobalamine biosynthesis showed differential expression (**Figure 4.8**). Of the 252 DEGs, 133 genes were upregulated (**Table S4.9**), while 119 were downregulated (**Table S4.10**) at this stage. The genes from glycolysis (aldehyde dehydrogenase), glycerol-phospholipid metabolism (glycerol-3-phosphate-O-acyltransferase, glycoside hydrolases), and gluconeogenesis (UDP-glucose 4-epimerase) were upregulated. Similarly, several stress responsive genes such as protease ftsh (Fischer et al., 2002), glutathione-s-transferase (Strange et al., 2001), epoxide hydrolase (Fretland and Omiecinski, 2000), and RTA-1 domain protein were overexpressed, depicting fungicidal stress on the pathogen. Membrane transporter proteins, including MFS-1 (an antibiotic resistance protein and benomyl methotrexate protein), were enhanced in response to propiconazole, while MFS (general substrate transporter), sulphate permease (anion transporter), amino acid permease (amino acid exchanger), small oligopeptide transporter, and MFS monocarboxylate transporter were repressed. The overexpression of various cell cycle and growth associated

proteins such as spc7, tf2-11, ribonuclease H and prz-1 indicated alteration in the growth process of the fungus. In contrast, the expression of polyketide synthase and norsolorinic acid reductase involved in polyketide synthesis and important for pathogenicity, were hindered. Similarly, nitrate and nitrite reductase enzymes, essential for protein synthesis, were also downregulated.

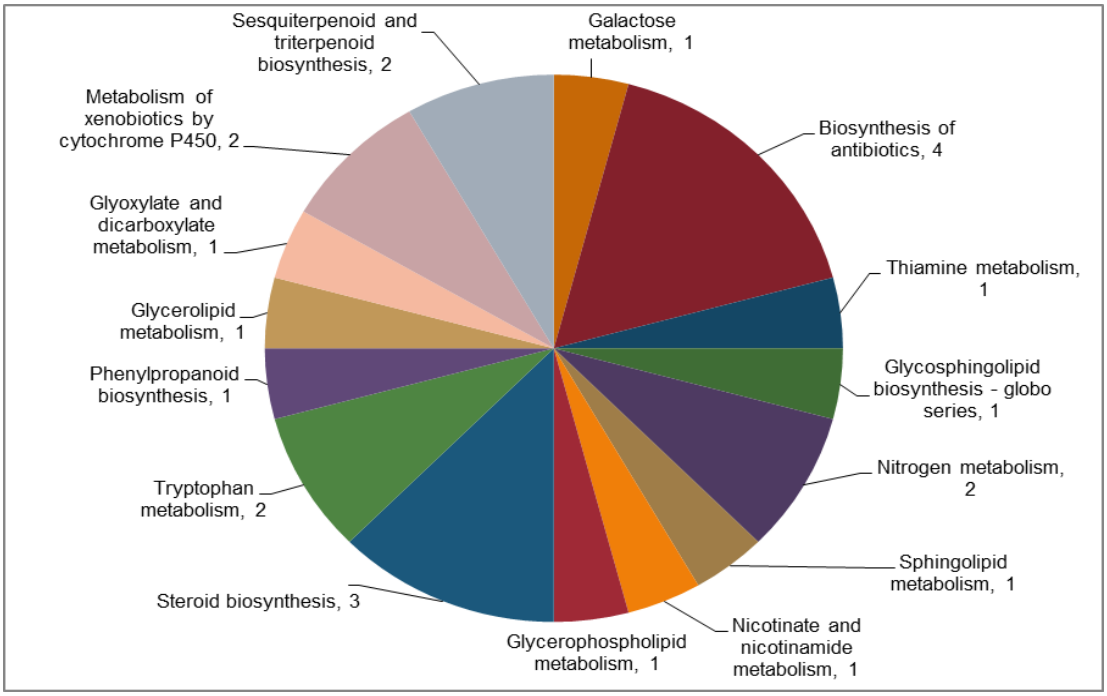


Figure 4.8: KEGG annotation of enzymes from DEGs to respective pathways at 6 hpt

4.3.2.6 Expression modulation at 12 hpt

Of the 251 DEGs identified at this time point, 132 were upregulated (**Table S4.11**) and 119 were downregulated (**Table S4.12**). A majority of the metabolic pathways linked to ergosterol synthesis i.e. steroid biosynthesis (acyl-sterol acyltransferase, lanosterol 14 alpha-demethylase and sterol 24-C-methyltransferase), mevalonate pathway (diphosphomevalonate decarboxylase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase), phenylpropanoid biosynthesis (ligninase lg6 precursor protein), and sesqueterpene biosynthesis (isotrichodermin c-15 hydroxylase, longiborneol synthase) were differentially modulated (**Figure 4.9**). Several enzymes from gibberellic acid biosynthesis such as farnesyl pyrophosphate synthetase, geranylgeranyl pyrophosphate synthase, ent-kaur-16-ene synthase, and ent-kaurene

oxidases were upregulated, while the genes involved in appressorium formation and sporulation, electron transport, oxido-reducatse activity, and TCA cycle were downregulated. In contrast, equisetin n-methyltransferase, valacyclovir hydrolase, beta-lactamase family protein, and oxidase-like protein (involved in detoxification of drug) were overexpressed, while nucleases, peptidases, and lyase enzymes were downregulated. Enhanced expression of ABC-G family proteins was also observed.

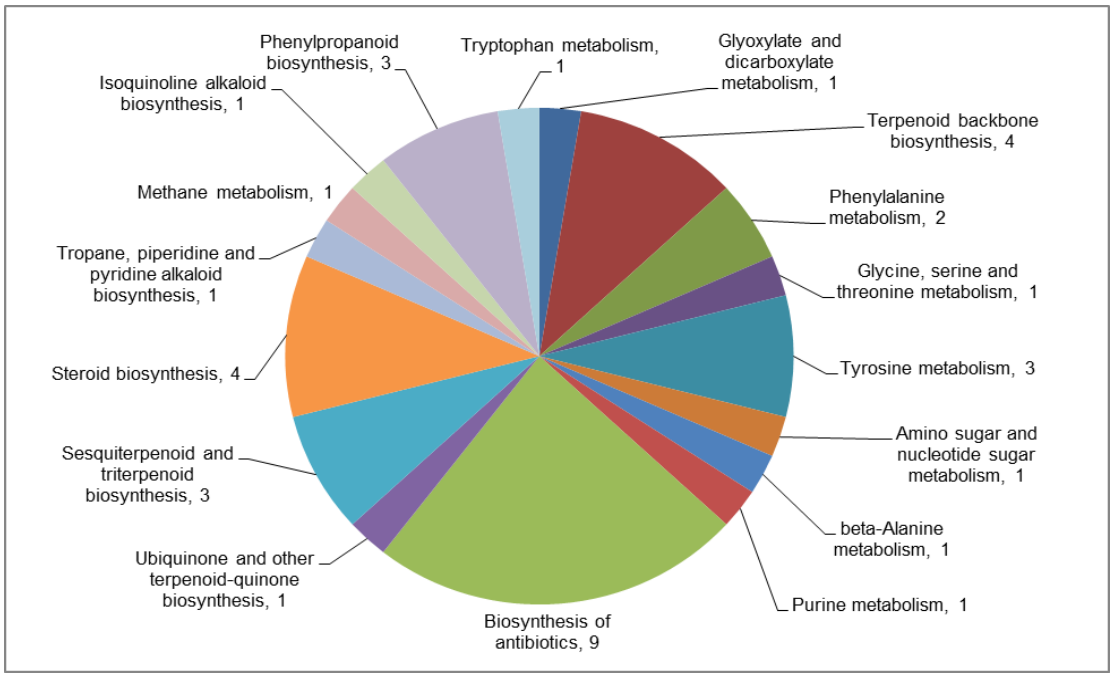


Figure 4.9: KEGG annotation of enzymes from DEGs to respective pathways at 12 hpt

4.3.3 Overview of differentially expressed genes

Based on the GO terms, 564 DEGs were organized into nine categories viz. transmembrane transporters, transcription and translation related genes, biosynthesis associated genes, cellular metabolism associated genes, stress responsive genes, carbohydrate metabolism associated genes, electron transport related genes, others, and hypothetical proteins (**Table S4.13**). Several transcripts encoding membrane transporters were differentially expressed on propiconazole treatment. These transporters are reported to be involved in general substrate transport as well as antibiotic resistance. Among the differentially expressed transporters, five transporters

from major facilitator superfamily (MFS) and two from ATP binding cassette (ABC) family were expressed at one or more time points. The expression of ankyrin repeat containing transporters, which play important roles in targeting proteins to plasma membrane and Ca+2 homeostasis in endoplasmic reticulum (Bennett and Healy, 2008), was altered at 6 hpt and 12 hpt.

Several transcription and translation related genes were also differentially expressed in propiconazole treated culture. GO annotation indicated their roles in nucleic acid binding and modification activity. Pre-RNA maturation and splicing associated genes were upregulated; while DNA replication and repair mechanism associated genes were downregulated. The genes involved in ergosterol biosynthesis were significantly upregulated. Besides these, oxidative stress responsive genes like ligninase lg6 precursor protein and 4-hydroxyphenylpyruvate dioxygenase were overexpressed; while the enzymes with reducing activity like naphthalene triol reductase, tropinonereductase 1, nitrate reductase, nitrite reductase, and norsolorinic acid reductase were downregulated. At early time points, 3 hpt and 6 hpt, several genes with oxido-reductase activity were downregulated; while they were upregulated at 12 hpt. Notably, increased expression of cytochrome P450 containing gene other than the known targets of azoles was also observed (**Table S4.13**).

4.3.4 Functional protein association networks

Identical protein search for differentially expressed transcripts yielded 23 of 61, 85 of 252 and 85 of 251 homologous proteins in *Pyrenopora tritici* at three time points respectively. These identical proteins were mapped to the functional network interaction. All networks showed significantly more interactions than expected for a random set of proteins (**Figure 4.10**).

4.3.5 DEGs common throughout the time series

The expression of 442 genes was altered in response to propiconazole stress at the three time points considered together. Of these 21, 151 and 162 genes were unique to 3 hpt, 6 hpt and 12 hpt time points respectively; while 19 DEGs were common between 3 hpt and 6 hpt, 68 DEGs were common between 6 hpt and 12 hpt, and only seven DEGs were common between 3 hpt and 12 hpt (**Table S4.14**). Among the DEGs, 14 genes showed differential expression across all the time points; of these eight genes were upregulated and six genes were downregulated due to propiconazole

treatment (**Figure 4.11**). The eight genes belonged to steroid biosynthesis pathway, gibberellic acid biosynthesis pathway, sphingolipid transport, terpenoid backbone biosynthesis, and glycogen metabolism. In contrast, the repressed genes belonged to stress responsive elements, glucose transferase pathway and ribonucleases family. The expression of ligninase lg6 precursor protein (reported to have peroxidase activity) was induced. This enzyme was strikingly overexpressed in propiconazole treated culture. Sphingoid long chain base transporter also showed enhanced expression. It contains RTA1 domain and is an integral component of cell membrane. Among the downregulated genes, the glycosyltransferase family 1 protein (involved in glycosylation of several substrates) was observed. The stress responsive alpha-beta barrel domain containing genes were also downregulated; however, their function remains yet unknown. The expression of UVi-1 gene (reported to be induced by UV light) was decreased.

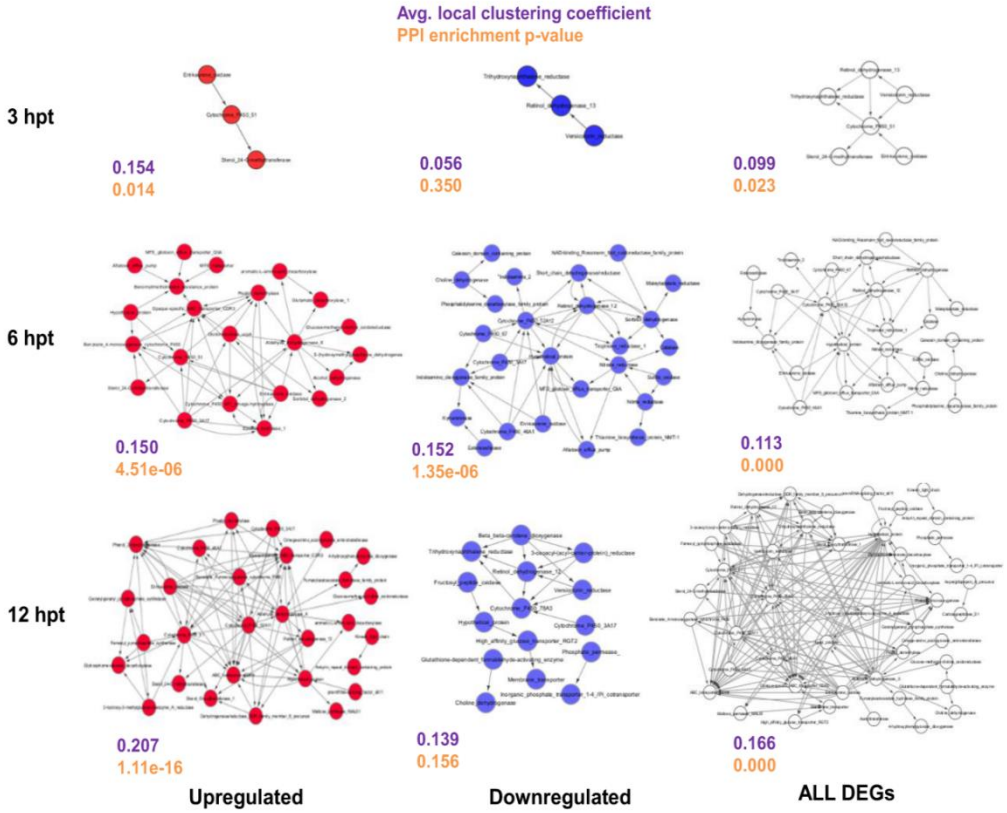


Figure 4.10: Functional protein association network of DEGs at 3 hpt, 6 hpt and 12 hpt from propiconazole treated culture (hpt: hour post treatment; NOIseq Analysis only)

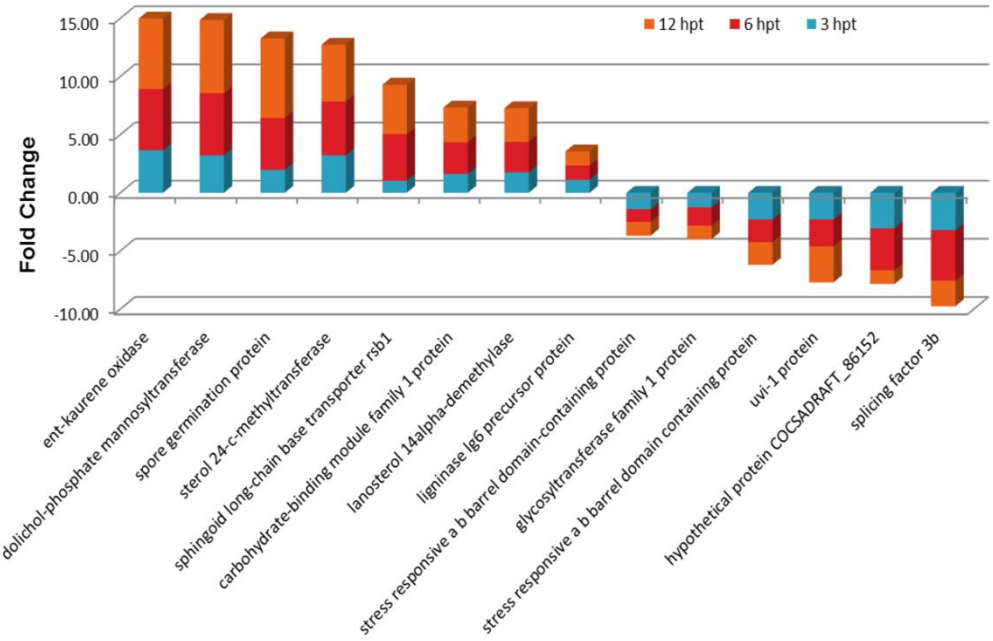


Figure 4.11: Expression modulation in propiconazole treated culture of *B. sorokiniana* of 14 common differentially expressed transcripts from RNA-Seq analysis at 3 hpt, 6 hpt and 12 hpt (hpt: hours post treatment)

4.3.6 Steroid biosynthesis pathway

The exposure to propiconazole led to overexpression of steroid biosynthesis genes (*ERG2*, *ERG6*, *ERG11*) in culture of D2 strain of *C. sativus* (**Table S4.15**). Sterol 24-C-methyltransferase (*ERG6*) and lanosterol 14alpha-demethylase (*ERG11*) are the known targets of the azole and were significantly upregulated throughout the time series. The LFC values were more for *ERG6* (2.96, 4.55 and 5.33) than *ERG11* (1.49, 2.57 and 3.32) for all the three time points. However, significant upregulation of *ERG2* was observed only after 12 hpt (**Figure 4.12**).

4.3.7 Expression modulation of ABC transporters

As transporters play a dynamic role in fungicidal resistance, the expression profile of the ABC transporters was assessed in propiconazole treated culture for the three time points from NOIseq data. Remarkably, expression modulation was observed for 41 ABC transporter genes (**Table S4.16**). Propiconazole treatment resulted in overexpression of ABC-B and ABC-G family transporter proteins (**Figures 4.13** and **4.14**, **Tables S4.16** and **S4.17**). ABC-G family proteins viz. JGI protein ID 183394

and 37813 were upregulated across the time series. BLAST analysis of the protein showed similarity with cdr1 of *C. albicans* and pdr5 of *S. cerevisiae*. The CDR (Candida drug resistance) and PDR (Pleiotropic drug resistance) genes have been well-studied for their roles in drug efflux (Nuruzzaman et al., 2014; Arendrup and Patterson, 2017). These transporters have been reported as glutathione-s conjugate pumps (de Waard et al., 2006). KEGG annotation of the protein sequence identified it as a flippase, which requires utilization of ATP molecules to efflux the toxic molecules out of the cell.

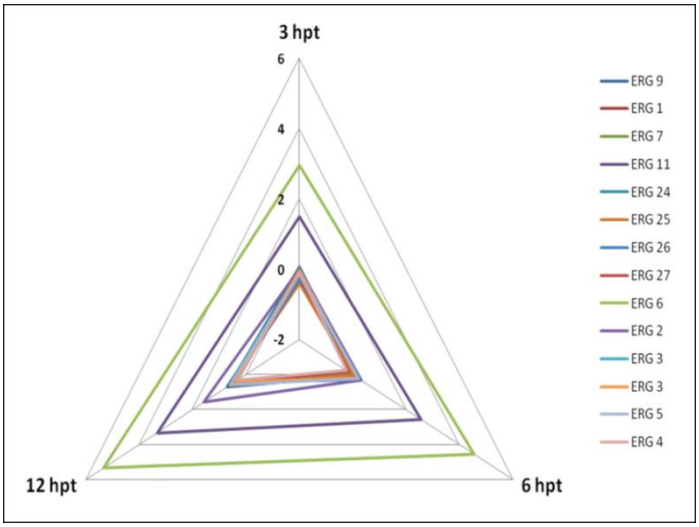


Figure 4.12: Fold change pattern of genes of steroid biosynthesis pathway

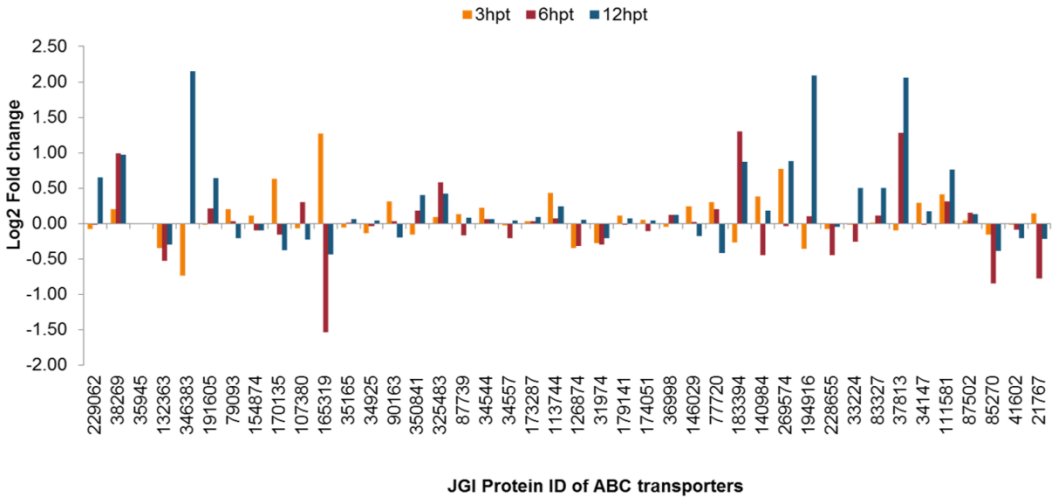


Figure 4.13: Relative transcript level (log_2 fold change) of ABC transporters from RNA-Seq data in propiconazole treated culture of *B. sorokiniana* (NOIseq data)

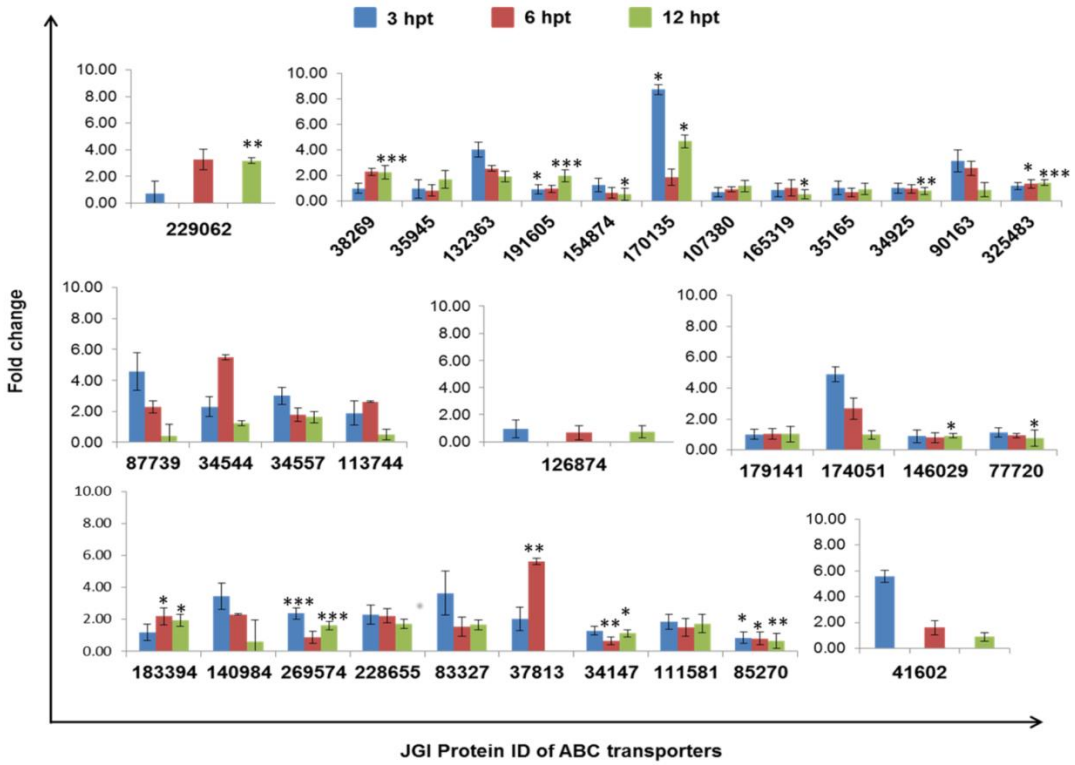


Figure 4.14: Relative transcript level (fold change) of ABC transporters from qRT-PCR in propiconazole treated culture of *B. sorokiniana* (Error bar – standard error; ∗∗∗ – p-value < 0.001, ∗∗– p-value <0.01, ∗–p-value <0.05)

4.3.8 Relative gene expression quantification using qRT-PCR

We verified the results of differential gene expression analysis from transcriptomics approach using qRT-PCR. In general, qRT-PCR showed relatively higher LFC values, probably because of its higher sensitivity (**Figure 4.15**, **Table S4.18**). Nonetheless, the correlation coefficients for the LFC values between qRT-PCR and RNA-seq (NOIseq) data were 0.96, 1.0 and 0.99 for 3 hpt, 6 hpt and 12 hpt propiconazole treated fungal cultures, respectively.

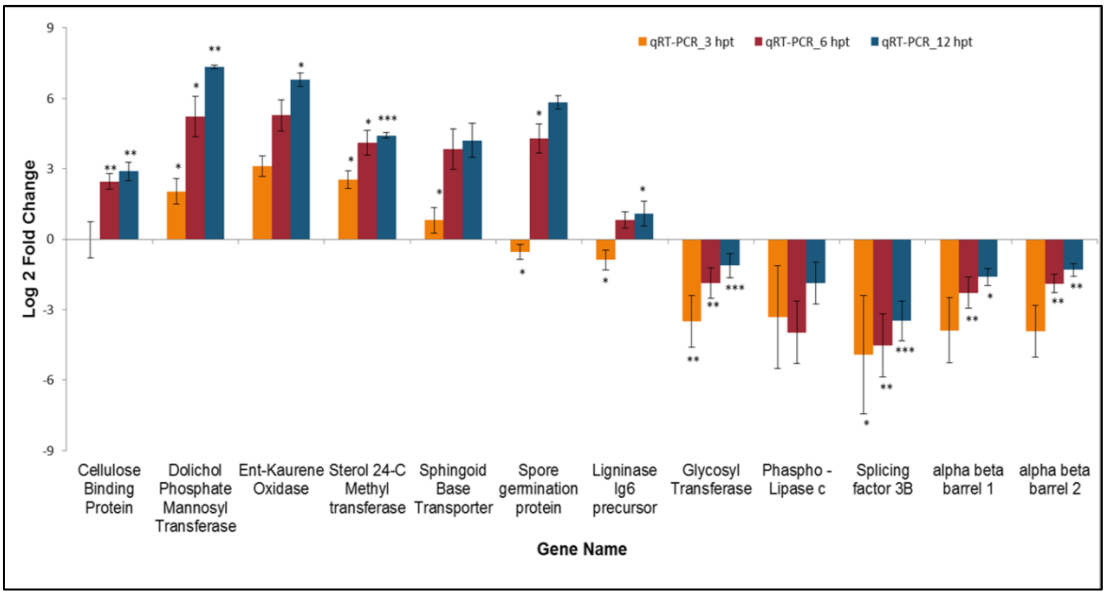


Figure 4.15: qRT-PCR validation for log2 fold change values from RNA-Seq (Error bar – standard error; ∗∗∗ – p-value < 0.001, ∗∗– p-value <0.01, ∗–p-value <0.05)

4.4 Discussion

Application of the foliar fungicide, propiconazole, is widely adopted control measure to manage spot blotch caused by *C. sativus*. However, widespread and imprudent use of a single fungicide might lead to evolution of fungicide resistant pathogen strains, which can lead to massive crop failures. Hence, identification of new molecular targets in the fungus and development of new fungicides (or fungicidal formulations containing more than one fungicide) targeting multiple genes or pathways is crucial.

In this study, our aim was to identify the molecular reciprocation acquired by the pathogen to survive and sustain growth under sub-lethal fungicidal stress. Differential gene expression analysis of propiconazole treated culture revealed the strategies adopted by the pathogen to sustain the stress and the putative new drug targets, which can be exploited to develop better fungicides.

We observed that various genes including the reported azole target genes were upregulated. During the three time points 3 hpt, 6 hpt and 12 hpt, the expression of steroid biosynthesis and transferase enzymes was enhanced, while the expression of hydrolase and oxido-reductase activity enzymes was decreased initially and increased later. Lanosterol 14 alpha-demethylase and sterol 24-C-methyltransferase are the enzymes from the steroid biosynthesis pathway. Lanosterol 14 alpha-demethylase (*ERG11*) catalyzes demethylation of lanosterol in steroid biosynthesis and is the CYP51 family target of triazoles in fungi (Becher and Wirsel, 2012). Its abundance indicated that the fungicide stress was perceived by the pathogen as early as 3 hours post treatment. Sterol 24-C-methyl transferase (*ERG6*) is another target enzyme for propiconazole and requires Mg2+ and glutathione as cofactors and converts zymosterol into fecosterol (Moore and Gaylor, 1969; Newman et al., 2005). Upregulation of ERG6 enzyme with higher fold change as compared to *ERG11* suggested its more efficient binding affinity and hence upregulation in response to propiconazole. This also reveals the first strategy adopted by the pathogen to combat the fungicidal stress by overproduction of the target enzymes.

The enzyme with the highest fold-change, ent-kaurene oxidase, is an integral component of membrane and is a cytochrome P450 family protein. It is also involved in gibberellic acid biosynthesis pathway (Helliwell et al., 1999). Another upregulated gene, dolichol-phosphate mannosyl transferase is involved in glycosylation of dolichol-phosphate. This enzyme is an important component of ER lumen and is involved in mannosylation of several proteins (Babczinski et al., 1980). The transcript level of spore germination protein was also high. It does not illustrate any conserved domain and hence its role remains obscure. InterPro motif search revealed the presence of both the cellulose binding domain and cellulase activity in the carbohydrate binding module family 1 protein, suggesting its role in cellulose degradation in cell. The expression of UVi-1 gene (reported to be induced by UV light) was decreased. Accumulation of this gene transcript was reported during

appresorium formation in *Bipolaris oryzae* (Kihara et al., 2001). The hypothetical protein (JGI protein id 86152) was downregulated upon propiconazole treatment and showed similarity to phosphatidylcholine-hydrolyzing phospholipase C enzyme using BLAST analysis. Phospholipases are attributed to the pathogen virulence and responsible for host cell damage and lysis. Additionally, these genes are also reported to play roles in signal transduction (Ghannoum, 2000).

Interference in ergosterol biosynthesis leads to cell membrane disruption (Bammert and Fostel, 2000). This can result in deficiency in nutrient uptake and oxidative stress. As a result, several stress responsive genes were up- or downregulated in the pathogen. Ligninase lg6 precursor protein that requires heme and calcium as cofactors was upregulated. Its putative role is to overcome the oxidative stress in the presence of hydrogen peroxide (Zhu et al., 2016). Surprisingly, the stress responsive alpha-beta barrel domain containing proteins were downregulated. These proteins have been reported to be upregulated under salt stress in the plant *Populus* (Gu et al., 2004); however, their functions in fungi are still unknown. As propiconazole treatment resulted in downregulation of these proteins, insights into their functional role can reveal the mechanism of azole tolerance in fungi.

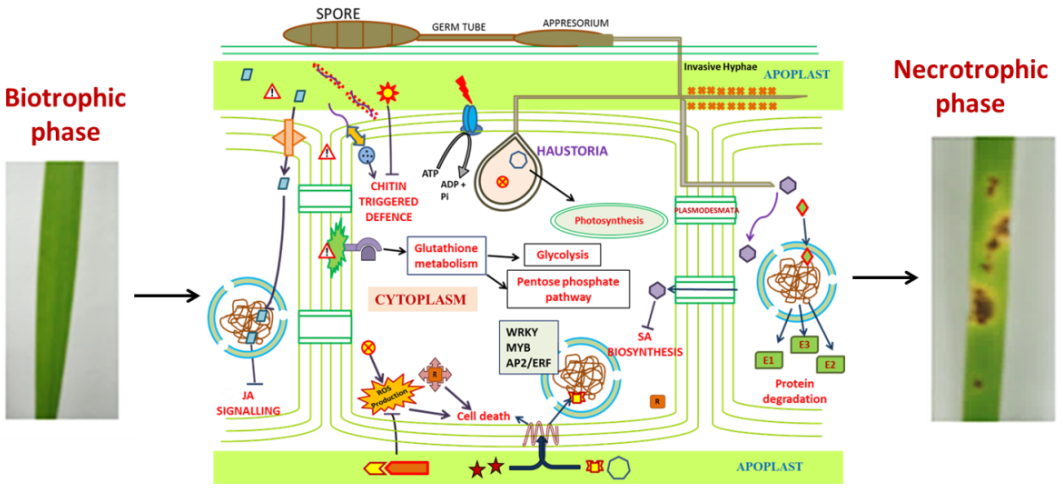
Downregulation of splicing factor 3b observed in the present study, indicated a reduction in U2 snRNP mediated pre-spliceosomal assembly (Wang et al., 2005). Similarly, the loss of splicing factor ASF/SF2 has been reported to induce cell cycle arrest in G2 phase and apoptosis in eukaryotic cells (Li et al., 2005). Hence, it is likely that downregulation of splicing factor 3B might account for the same in this pathogen. Similarly, glutathione dependent enzyme system compensates for azole induced susceptibility to oxidative stress (Rogers and Barker, 2003) and glutathione dependent drug efflux by ABC transporters (Renes et al., 1999). Thus, increase in the glutathione-s-transferase transcript level in the present study emphasizes its importance in azole stress tolerance.

Transporters play a dynamic role in fungicide resistance (Revie et al., 2018). ABC transporters from the yeasts, *Candida albicans* and *Saccharomyces cerevisiae* (Prasad and Goffeau, 2012) and the fungi, *Beauvaria bassiana* and *Mycosphaerella graminicola* have been reported for their role in antifungal and oxidative stress

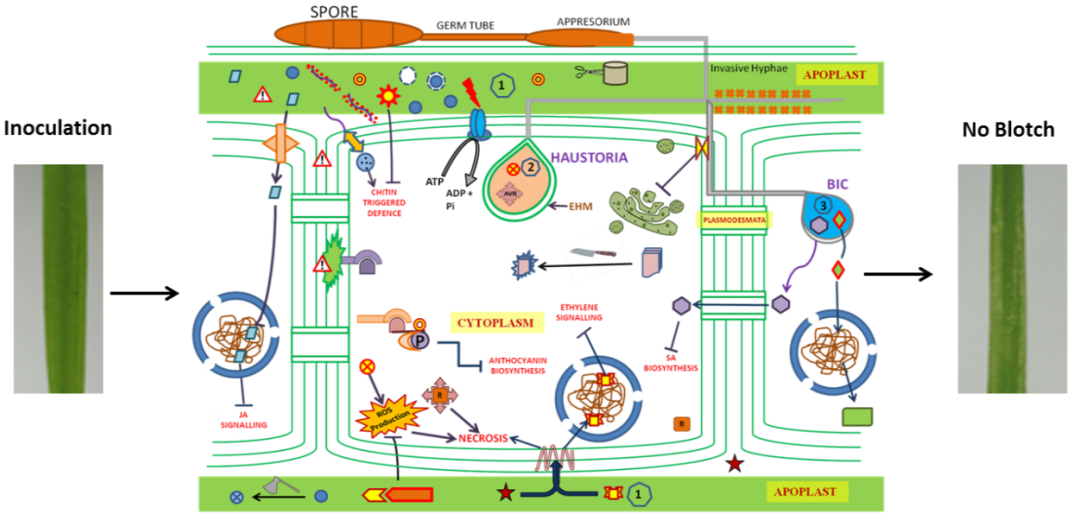
conditions (Song et al., 2013). ABC-G transporters have been reported for pleiotropic drug resistance (PDR) (Gardiner et al., 2013). These transporters are involved in export of toxic metabolites, sterol uptake, and translocation of membrane phospholipids, ion transport, and quorum sensing (Prasad and Goffeau, 2012). The increased expression levels of ABC-G transporter transcripts indicate their fungicide resistance associated roles. Such ABC transporters were highly overexpressed in resistant strain of *Cercospora beticola* in response to tetraconazole (Bolton et al., 2016). Sphingoid long chain base transporter, an integral component of cell membrane, was also found to be upregulated. It contains RTA1 domain, which binds to toxic molecules and abolishes their cytotoxicity effects (Soustre et al., 1996). Blocking of these transporters may lead to intense stress on the pathogen.

Other than the target enzymes from steroid biosynthesis, ent-kaurene oxidase showed the highest fold change among the 14 differentially expressed genes in all the three stages. Ent-kaurene oxidases are integral to gibberellic acid biosynthesis. Palcobutrazol, a plant growth promoting azole, has binding affinity to P450 form of ent-kaurene oxidase (Ashman et al., 1990). Functional network analysis stated its association with lanosterol 14 alpha-demethylase enzyme. Additionally, ent-kaurene oxidase comprises cytochrome P450 domain (Helliwell et al., 1999) and hence, might also be a target for azoles or serve as drug scavenger under fungicide stress. Molecular docking of this protein with propiconazole was attempted to evaluate their binding affinity. However, no significant direct interaction could be explored due to the less than 30% similarity of ent-kaurene oxidase with the reported enzyme structures in Protein Data Bank (https://www.rcsb.org/). Hence, functional validations like *in vitro* enzyme assay or knock-out studies can reveal its significance in azole tolerance in fungal pathogens.

Chapter 5:
Conclusions and Future prospects



DDK 1025: Susceptible variety



Chirya 3: Resistant variety

5.1 Conclusions

Wheat is the most important cereal with 66% utilization of total production for human consumption. A big population mass (2.5 billion) is dependent on wheat for their daily food requirements. Wheat occupies a prime place among cereals in poor's diet because of its protein and essential amino acids contents. However, wheat production needs to be increased by 60% of the present levels by 2050 to feed the ever growing population. Three types of wheats are commercially grown. The hexaploid or bread wheat (*Triticum aestivum*) is the most widely grown. The Emmer wheat (*T. dicoccum*) is well recognized for its high fiber and protein content; while the Durum wheat (*T. durum* or *T. turgidum* subsp. *durum*) is valued for its high gluten content and widely used in preparation of pasta, bulgur, etc. The Dicoccum wheat has several nutritional benefits and hence is therapeutically recommended. Although it possesses superior qualities than the most cultivated bread wheat, its cultivation is limited due to its susceptibility to diseases like spot blotch, which is an emerging disease causing yield losses of economically important cereals. Furthermore, the worldwide distribution of the causal agent, *Bipolaris sorokiniana* makes the disease of global concern.

5.1.1 Isolation and characterization of *Bipolaris sorokiniana* isolates from different geographical regions of India

The main aim of our study was to explore the molecular mechanism of plant pathogen interaction during the spot blotch disease. To understand the interaction, first it is important to know the characteristics of the pathogen. Hence, we collected 12 pathogen isolates from different spot blotch affected regions of India including the North-Himalayan region (Assam and Bihar) and peninsular region (Karnataka). Monoconidial cultures were established for seven sporulating isolates of *B. sorokiniana*. These isolates were characterized in terms of mycelial growth pattern, spore morphology as well as ITS region sequencing. Relatively high diversity was observed among the seven isolates and almost every isolate was different from the rest. Multiple methods for pathogen inoculation were attempted for suitability under green-house condition for high-throughput disease screening. Spray inoculation of pathogen at Zadok's scale 12 stage was identified as the best method for spot blotch study at seedling stage, while germinated seed inoculation can be used as a rapid

method for screening genotypes against spot blotch. The hemibiotrophic lifestyle of the pathogen was confirmed on the spot blotch susceptible wheat cultivar. Disease progression and severity were compared in susceptible emmer and resistant bread wheats.

5.1.2 Exploring the molecular interaction of wheat- *Bipolaris sorokiniana* during spot blotch disease

Two wheat varieties were used for exploring the molecular mechanism of disease susceptibility and resistance using the transcriptomics approach. DDK 1025 (dicoccum wheat, tetraploid) was susceptible to spot blotch, while Chirya 3 (hexaploid wheat) was employed as the resistant variety; as resistant variety from dicoccum was not available. We analyzed the transcriptomes of wheat and *B. sorokiniana* interaction through RNA-seq using reference based transcriptome assembly at three time-points, *viz*. 1, 4 and 6 days post inoculation, corresponding to the biotrophic, necrotrophic and late necrotrophic phases in the susceptible variety.

We identified several differentially expressed genes (DEGs) between the control and inoculated plants of DDK 1025 and Chirya 3, which suggested a complex regulatory network of genes involved in susceptibility or resistance to the fungal pathogen. Biotrophic to necrotrophic phase conversion of the pathogen was supported by reduced and delayed immune response of the susceptible plants. In contrast, timely activation of enzyme inhibitors, peroxidases, chitinases, several signaling, and defense molecules by Chirya 3 restricted the pathogen to extracellular space in its biotrophic phase. Sugar sensing and ubiquitin mediated proteolysis were the key processes during compatible interaction. Although a large number of genes were identified in our differential expression study, a high percentage of these genes showed no similarity with the reported proteins and remain unannotated. Homology based analysis and similarities in gene expression pattern with known genes provided information about the potential functions for novel genes. Further functional characterization of these genes will help in understanding the molecular basis of spot blotch resistance in wheat. This study will serve as a basis for development of spot blotch resistant varieties in near future.

The quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) is an integral tool for gene expression analyses in plant-pathogen interactions, usually

performed using the delta-delta-C_t method. Accurate relative quantification by this method requires an internal reference gene with stable expression across experimental tissues. In case of plant-pathogen interaction, selection of a suitable reference gene is even more crucial task due to the presence of plant- as well as pathogen- mRNA in infected tissues. We evaluated previously reported eight reference genes in wheat-*B. sorokiniana* pathosystem. However, some of them cross-amplified the pathogen genes, which can lead to incorrect quantification of plant defense gene expression. Hence, we designed primers from 3' UTR (untranslated region) of the wheat genes, which specifically amplified only the wheat genes and not the pathogen genes. We also identified the reference genes that are highly stable in expression in the pathosystem. Based on the results of various statistical tests, *ULE* was identified as the most stable internal reference gene for evaluating the gene expression of candidate defense genes in wheat. We also report suitable reference gene primers for accurate quantification of defense gene expression in wheat-pathogen interaction studies.

5.1.3 Global gene expression analysis of *Bipolaris sorokiniana* on exposure to propiconazole

Evolution of drug resistant fungal pathogen is a serious issue due to the limited number of antifungal compounds that are available. Azoles are most widely used antifungal agents. The effects of various azoles and the mechanism of azole resistance are being studied in yeast and fungi for long. Though drug resistance is a well-known phenomenon in fungi, the exact mechanism by which fungi evolve resistance is not fully understood yet. Overexpression of target, efflux of the compound, and modification of the drug are few mechanisms by which fungi can develop resistance against azoles. Propiconazole is recommended for field control of spot blotch. Among various azoles, the effect of propiconazole on mycelial fungal system has not yet been studied. Therefore, we explored the global gene expression using the transcriptomics analysis approach for *in vitro* propiconazole treated culture of *B. sorokiniana*.

We obtained high resolution gene expression profiles for the mycelia for three time points, corresponding to three distinct growth stages of the fungal pathogen. The transcriptome approach revealed three major strategies by *B. sorokiniana* to deal with the propiconazole stress: i) overexpression of target enzymes, ii) increased efflux of the drug by overexpression of transporters, and iii) expression modulation of certain

stress responsive factors. This study revealed several novel putative targets such as ent-kaurene oxidase, ligninase lg6 precursor, alpha beta barrel domain containing proteins, splicing factor ASF/SF2, glutathione-s-transferase, and sphingoid base transporters. These genes helped the fungus to overcome the fungicide stress and survive. Hence, these genes could be considered for development of new antifungal agents.

5.2 Future prospects

As spot blotch is an emerging disease, still much remains to be elucidated about the pathogen, disease progression and control measures. There are several technical future directions which could be chased for better insights of the interaction mechanism:

1. Comparative genomics of multiple isolates from different regions would reveal the genetic diversity and virulence characteristics of the pathogen.
2. Inoculation with a GFP-tagged pathogen can elucidate the growth and pathogen progression inside the host plant; thereby revealing potential resistance associated features.
3. Exploring other aspects of gene expression regulation such as protein and secondary metabolite levels is suggested. Combining other "Omics" approaches like proteomics and metabolomics with our findings will enrich the understanding about molecular interaction.
4. Overexpression or knock-down of some of the putative resistance genes could reveal their function in either susceptibility or resistance.
5. Functional and structural characterization of putative novel azole target in fungi will direct the development of new fungicides.

Most of the interaction studies focus either on the plant or the pathogen aspects only for several reasons. However, a combination of research from both host as well as pathogen including functional approaches is required to understand the molecular, cytological, and histo-chemical basis for plant–pathogen interactions. Such comprehensive understanding can only be used for field application of the research knowledge.

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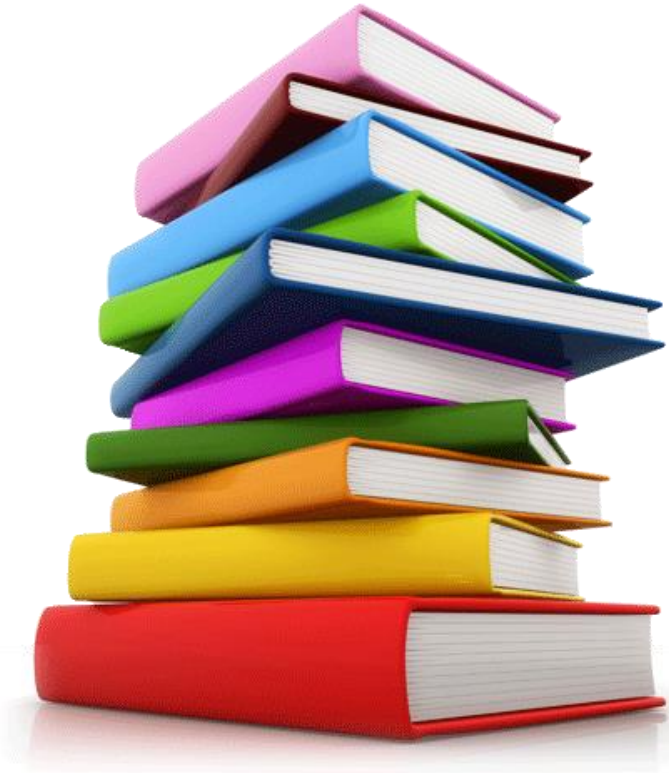

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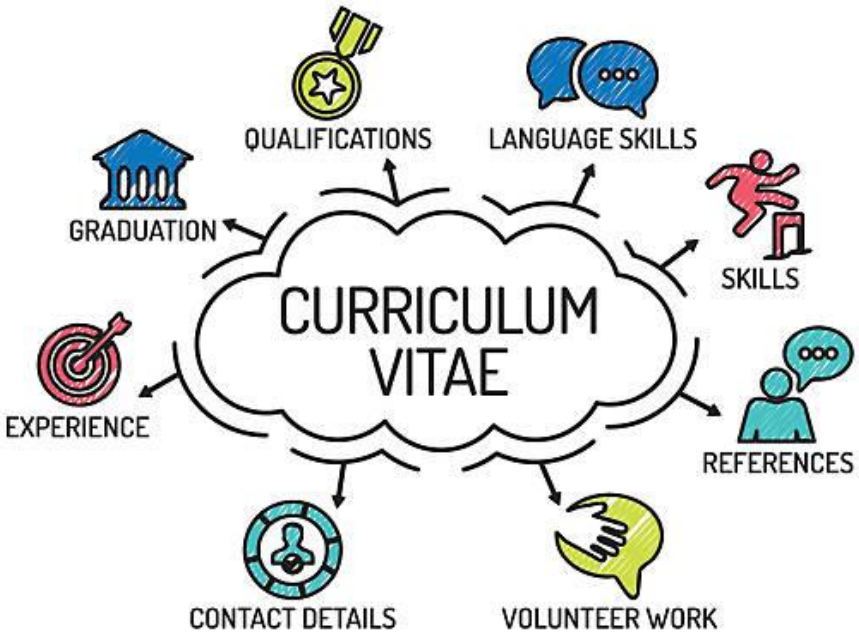
Curriculum vitae



DEEPIKA SOMANI

Personal details:

Date of Birth : 1st October 1990

Adress for correspondence : C/O MurliDhar Namdhar
Somani Mohalla, Kapasan P.O. 312202
Dist: Chittorgarh, Rajasthan, India

Email : somanideepika90@gmail.com

Contact No. : +919766070503

KEY STRENGTHS

1. Hard working, well organized and able to manage numerous projects simultaneously.
2. Excellent interpersonal skills, dynamic and highly team-oriented.
3. Flexibility and willingness to work on a broad variety of matters.
4. Superior communication and advanced Research skills.
5. Know how to get the things done, Reliable, Dependable, and Proactive.
6. Ability to manage daily work without losing focus on long-term objectives.

ACADEMIC QUALIFICATIONS

Examination Passed	Board/ University	Year of passing	Subject	% Marks	Rank
M.Sc.	Rashtrasant Tukadoji Maharaj University, Nagpur	2012	Biotechnology	**84.1%**	2nd Rank in University
B.Sc.	Mohan Lal Sukhadia University, Udaipur	2010	Biotechnology	**81.6%**	3rd rank in University
Higher Secondary	RBSE Board	2007	English, Hindi, Physics, Chemistry, Biology	**74%**	-
Secondary	RBSE Board	2005	English, Hindi, Science, Maths, Social Science	**86.33%**	-

AWARDS AND ACHIEVEMENTS

Academic

1. **Secured rank 68 under UGC-JRF category** in **CSIR-UGC-NET June 2012.**

2. **Qualified ICMR JRF examination** held on **22 july 2012** for **ICMR Funded research.**

3. **Secured 2nd rank in M.Sc. Biotechnology, session 2012 in RTM Nagpur University, Nagpur.**

4. **Secured rank-601Gen.** Category in **GATE for Life sciences in 2012** conducted by IIT, Delhi.

5. **Secured rank-93 Gen.** Category in **GATE for Biotechnology in 2011** conducted by IIT, Madras.

6. **Secured rank-405 A.I.R** Gen. Category in Combined Entrance Exam for Biotechnology conducted by Jawaharlal Nehru University, New Delhi in 2010.

7. **Secured 3rd rank in B.Sc. Biotechnology, session 2010 in MLS University, Udaipur.**

8. Won "Gargi Award" by 'Girls Education Foundation' for class X in 2005 and class VIII District topper amongst girls in class VIII Board exam in 2003.

Conferences and workshops attended

1. Participated and presented poster entitled "Exploring molecular paradigm of compatible interaction during spot blotch disease in emmer wheat using RNA-seq" during 4th International Plant Physiology Congress organised by CSIR-National Botanical Research Institute, Lucknow in 2018.
2. Participated and presented poster entitled "Global gene expression analysis in *Bipolaris sorokiniana* in response to Propiconazole" during National Science Day celebration at CSIR-National Chemical Laboratory in 2017.
3. Participated and presented poster entitled "Exploring plant pathogen interaction in wheat during spot blotch" during National Science Day celebration at CSIR-National Chemical Laboratory in 2015.

4. Participated and won 3rd prize for poster presentation and promoted to oral presentation, during National Seminar on anti-immunology organised by Hislop School of Biotechnology, Hislop College, Nagpur in Oct. 2011.
5. Participated and presented poster in UGC sponsored National level seminar on Recent Advances In Medical Microbiology and Immunology organised by Department Of Microbiology, Arts, commerce and Science College, Koradi, Nagpur in Oct. 2011.
6. Participated in Edu-workshop on High Melting Technology and Flow Cytometry organised by University department of Biochemistry, RTM Nagpur University, Nagpur in collaboration with Genetix, New Delhi and BD Biosciences, Mumbai in July 2011.
7. Participated in International Conference On "Recent Advances In Cancer Research: Bench To Bedside" held at School Of Life Sciences, Central University Of Gujrat, Gandhinagar, Gujrat in Feb. 2011.
8. Participated in Workshop On **"**Microbial Technology and Its applications**"** organised by Dolphin Institute of Biomedical and Natural Sciences in 2009.
9. Participated in "National Workshop on Bioinformatics" hands on practical training organised by Centre for Converging technologies, University Of Rajasthan, Jaipur in 2009.

Training / project experience

1. Working as research fellow at CSIR-National Chemical laboratory (January, 2013 onwards)
2. Successfully completed a workshop on "R for basic life sciences", organised by Bio-Sakshat in 2017.
3. Worked as Senior Research Fellow under project entitled "Validation of important Crop varieties through DNA finger printing (RKVY project) at Dept. Of MBBT, RCA, Udaipur in 2012 (Oct.-Dec 2012).
4. Successfully completed dissertation work on Isolation and characterization of Lactic acid bacteria and preparation of *Ocimum sanctum* fortified yoghurt from Rajiv Gandhi Biotechnology centre, R.T.M. Nagpur University, Nagpur in 2012.
5. Successfully completed training on Basic Flow cytometry course on BD FACS Calibur at BD Biosciences Training Centre, Gurgaon in 2011.

6. Successfully completed project training on various Advanced Biotechnological Techniques from S.P. Institute of Science and Research, Jaipur (RAJ) in 2009.

List of publications

1. Somani Deepika, Adhav Ragini, Prashant Ramya, Kadoo Narendra (2019) Transcriptomics analysis of propiconazole treated *Cochliobolus sativus* reveals new putative azole targets in the plant pathogen. *Functional and Integrative Genomics*: DOI: 10.1007/s10142-019-00660-9.
2. Somani Deepika, Prashant Ramya, Kadoo Narendra (2019) Evaluation of reference genes in wheat-*Bipolaris sorokiniana* pathosystem for precise expression profiling of plant defense genes using qRT-PCR (Journal of Plant Pathology, in Review)
3. Somani Deepika, Prashant Ramya, Kadoo Narendra (2019) Transcriptional paradigm of wheat-*Bipolaris sorokiniana* interaction (In preparation)

Field of interest

Plant pathology, Genetic engineering, Molecular Biology, Biochemistry and Microbiology

References

Dr. Narendra Kadoo

Principal Scientist,
Biochemical Sciences Division,
CSIR-National Chemical Laboratory,
Pune-411008
Email: ny.kadoo@ncl.res.in
Ph. +91-20-25902724

Dr. Vidya Gupta

Emeritus Scientist,
Biochemical Sciences Division,
CSIR-National Chemical Laboratory,
Pune-411008
Email: vs.gupta@ncl.res.in
Ph. +91-20-25902247