Molecular characterization of chickpea-*Fusarium oxysporum* f. sp. *ciceri* interaction

A thesis submitted to the
Savitribai Phule Pune University

for the degree of
DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

BY
Medha Laxmikant Upasani

Research Guide

Dr. Vidya S. Gupta

PLANT MOLECULAR BIOLOGY GROUP
BIOCHEMICAL SCIENCES DIVISION
CSIR-NATIONAL CHEMICAL LABORATORY
PUNE- 411 008 (INDIA)

January 2017

राष्ट्रीय रासायनिक प्रयोगशाला
(वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद)
डॉ. होमी भाभा रोड, पुणे - 411 008. भारत
NATIONAL CHEMICAL LABORATORY
(Council of Scientific & Industrial Research)
Dr. Homi Bhabha Road, Pune - 411008. India

CERTIFICATE

This is to certify that the work presented in this thesis entitled, "**Molecular characterization of chickpea-*Fusarium oxysporum* f. sp. *ciceri* interaction**" by **Ms. Medha L. Upasani**, for the degree of **Doctor of Philosophy**, was carried out by the candidate under my supervision in the Division of Biochemical Sciences, CSIR-National Chemical Laboratory, Pune-411008, India. This work is original and has not been submitted for any other degree or diploma to this or any other university. Any material that has been obtained from other sources has been duly acknowledged in the thesis.

Date: **(Dr. Vidya S. Gupta)**

Place: **Research Advisor**

Communications Channels +91 20 25902000 +91 20 25893300 +91 20 25893400
Fax +91 20 25902601 (Director) +91 20 25902660 (Admin.) +91 20 25902639 (Business Development)
URL : www.ncl-india.org

DECLARATION

I hereby declare that the thesis **"Molecular characterization of chickpea-*Fusarium oxysporum* f. sp. *ciceri* interaction"** submitted for Ph.D. degree at the Savitribai Phule Pune University has not been submitted by me for a degree at any other university.

Date: (**Medha L. Upasani)**

Place: **CSIR-National Chemical Laboratory, Pune**

Dedicated to my beloved Parents and Husband

…..This is just for you

Contents

Acknowledgments

The journey to my thesis has been accompanied and supported by many people. This is the most pleasurable moment when I can express my gratitude for their love and support. I would try to include everyone but if your name is not listed, I assure you that my gratitude is not less than for those listed.

First and the most importantly, I would like to thank my guide Dr. Vidya Gupta. I still remember my first discussion with her after my interview. The way, she pointed out loopholes in my communication during interview, encouraged me to work hard improving my presentation skills. This really helped me throughout my research and teaching span as well which was also appreciated by her. I am indebted to her for the constant support throughout the course of my thesis work. She taught very minute things which helped in understanding the experimental flaws and wise ways to rectify them.

I sincerely thank Dr. Narendra Kadoo for his constant help in experimental work and troubleshooting. He always inspired me to learn new techniques especially about computers and helped me to improve my technical skills. His good wishes and company will always be cherished.

I am thankful to Dr. Ashok Giri for his overall guidance. I am grateful to Dr. Meena Lagu for her systematic referencing for SAGE. I am thankful to Drs. Mukund Deshpande, Suresh Bhat, Shirgukar and Sushma Gaikwad for their help. I also thank all my labmates for creating a pleasant atmosphere in the laboratory. Special thanks to Dr. Krishnanad Kulkarni and Arun Kancharla for helping me at the start of my thesis work. Krishna specifically taught me to excel in the techniques required. Many thanks to Dr. Gayatri Gurjar for guidance at many steps of my thesis work. We had fruitful discussions which helped me in manuscripts preparation and thesis writing. Special thanks to Meenakshi and Pooja for all their help in Bioinformatics related processing of my data. I thank a special friend Ajit Ranjekar for his support and help. It has also been a pleasure to interact with Dr. Bhushan, discussion with him has always helped me to think and write the research articles. Rebecca needs a special mention for her help in referencing using Endnote. I always had a great help from Sheetal and Yashashree.

I am also thankful to Jagtap, Trehan, Ramakant and Indira for their help. Many thanks to the summer trainees and project student who helped me with my experiments at various times.

I would like to thank the Director, NCL for providing the laboratory facilities during the tenure of my work. I acknowledge the financial support from CSIR (Council of Scientific and Industrial Research) New Delhi, India in the form of Junior and Senior Research fellowships.

Dr. R. L. Deopurkar, my M.Sc. teacher, needs a special mention for inspiring me to pursue doctoral program. He inculcated many small but important virtues like 'To think', 'Work on basics', etc which enriched me. All the teachers of my graduation from Nashik have a great share in shaping my career.

I am greatly indebted to my family for their unending love, support and sacrifice. A special mention goes for my Aai and Baba whose selfless and constant efforts made my Ph.D. thesis come true. I feel short of words to describe the help, support, love and encouragement they have always given me. Though they are very old but are still young at heart, in understanding my academic needs.

My husband Rahul needs a special mention since he has been by my side throughout my tough times. This thesis would not have been completed without his support. I have no words to express my gratitude for my inlaws, especially my mother in-law who has taken care of my daughter, when I was away from her.

Maithili, my daughter also has a lion's share in this thesis. She has been a source of love, inspiration for me.

Lastly I thank all my well-wishers and friends whose names are not mentioned here.

I deeply express my gratitude to God for being with me and helping me out every time.

Medha Laxmikant Upasani

List of abbreviations

ABA	Abscisic acid
Bp	Base pairs
BSA	Bovine Serum Albumin
CAOMT	Caffeic acid O-methyltransferase
CCoAMT	Caffeoyl-CoA O-methyltransferase
CCR 1	Cinnamoyl-CoA reductase 1
cDNA	Complementary deoxyribonucleic acid
CLSM	Confocal Laser Scanning Microscope
COG	Clusters of orthologous groups
CTAB	Hexadecyl-trimethyl-ammonium bromide
DEGs	Differentially Expressed Genes
DEPC	Diethyl pyrrocarbonate
DGE	Differential Gene Expression sets
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide 5' triphosphate
dpi	Days post inoculation
DV	Digvijay
DVC	Digvijay Control
DVI	Digvijay inoculated with fungus
eGFP	Enhanced Green Fluorescent Protein
EF 1α	Elongation factor 1α
FAO	Food and Agriculture Organization of the United Nations
FAOSTAT	Statistics department of FAO
FDR	False Discovery Rate
Foc	*Fusarium oxysporum* f. sp. *ciceri*
GFP	Green Fluorescent Protein

Hph	Hygromycin phosphotransferase
hpi	Hour post inoculation
ICRISAT	International Crops Research Institute for the Semi-Arid Tropics
IFR	Isoflavone Reductase
JG	JG62
JGC	JG 62 Control
JGI	JG 62 inoculated with fungus
LFC	Log2 fold change
µg	Microgram
µl	Microliter
µM	Micromolar
mg	Milligram
min	Minute
ml	Milliliter
MLP	Major Latex Protein
mM	Millimolar
M	Molar
MPKV	Mahatma Phule Krishi Vidyapeeth
mGFP	Modified Green Fluorescent Protein
ng	Nanograms
^{0}C	Degree Celsius
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
qRT-PCR	Quantitative reverse transcription PCR
qPCR	Quantitative polymerase chain reaction
RFU	Relative Fluorescence Units
RGR	Radial Growth Rate

RMG	Radial Mycelial Growth
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SAGE	Serial Analysis of Gene Expression
SCAR	Sequence characterized amplified region
NCBI	National Centre for Biotechnology Information

List of Figures

Chapter 1: Introduction and Review of literature

Chapter 2: Materials and methods

Chapter 3: Results

Chapter 4: Discussion

List of Tables

Chapter 1: Introduction and Review of literature

Chapter 2: Materials and methods

Chapter 3: Results

Note: Those tables which could not be accommodated in A4 size paper are provided as Annexure in the compact disk (CD) attached to the inside of the back cover of the thesis.

List of Annexure

Annexure:

Thesis Abstract

Cultivated chickpea, *Cicer arietinum L.*, is worldwide the second most widely grown food legume after soybean, grown in over 40 countries representing all the continents. Although India is the largest chickpea producing country accounting for 70% of the global chickpea production, its consumption is much higher than production requiring imports of chickpea from other countries (FAOSTAT 2014). The average chickpea productivity is low compared to its yield potential, which can be attributed to susceptibility of the crop to various biotic and abiotic stresses. One of the major constraints in realizing the genetic yield potential of chickpea is the wilt caused by the Deuteromycetes fungal pathogen *Fusarium oxysporum* f. sp. *ciceri* (Padwick) Matuo & K. Sato (Foc). The pathogen penetrates vascular bundles of roots of chickpea plants and stops or reduces water uptake to the foliage. The infected plants ultimately wilt and die. The disease is highly destructive and worldwide in occurrence (Kraft, 1994). Persistence of the pathogen in soil and its capacity to survive there for years even in the absence of host (Haware *et al.*, 1996) renders its control difficult.

Plant-pathogen interaction is a multifaceted process involving large number of both pathogen- and plant-derived molecules which mainly include proteins, sugars and lipopolysaccharides (Boyd *et al.*, 2013). Various 'omics' approaches have been employed to study the molecular basis of plant-pathogen interactions. Specifically in chickpea-Foc pathosystem, defense related genes like glucanases, *PAL*, *CHS* etc. involved during Foc 1 and Foc 0 infections have been studied (Cho & Muehlbauer, 2004; Arfaoui *et al.*, 2007). In our earlier studies, enzymes like glucanases, chitinases and proteases and upregulation of pathways like flavonoid, isoflavonoid, phenylpropanoid, ROS and lignosuberization in resistant challenged cultivar have been shown to be probably involved in chickpea defense against Foc infection (Giri *et al.*, 1998; Kumar *et al.*, 2015a; Kumar *et al.*, 2016).

On the other hand, the knowledge of virulence factors active in the host environment is required for understanding the pathogenicity mechanism. Till date, various candidate genes with prime roles in fungal pathogenesis have been identified

(Gurjar *et al.*, 2012). These fungal pathogenicity genes are categorized based on formation of infection structures, cell wall degradation, toxin biosynthesis, signaling and proteins suppressing plant defense (Mobius & Hertweck, 2009). In *F. oxysporum*, cell wall degrading enzymes (CWDE), involved in penetration and colonization in the host plant have been studied. Several signaling genes have also been shown to be necessary for virulence (Jain *et al.*, 2003).

Considering the prevalence of Foc in soil without host, multigenic and complex resistance to the pathogen and failure of classical breeding approaches to develop wilt resistant lines with genetic diversity, understanding the molecular mechanism underlying the interaction is important. Hence, the thesis work was initiated with the following objectives

1) To monitor and analyze morphological changes occurring as the vascular wilt pathogen progresses in wilt resistant and susceptible cultivars of chickpea upon inoculation.
2) To transform *Fusarium oxysporum* (Fo) with the Green Fluorescent Protein (*GFP*) gene and observe pathogen progression during infection.
3) To elucidate the colonization pattern of the pathogen in wilt resistant and susceptible cultivars using qPCR assay
4) To perform real time PCR analysis of chickpea defense genes and Foc virulence genes at stipulated time-points.
5) To analyze and compare gene expression profiles of wilt resistant and wilt susceptible chickpea genotypes upon pathogen infection using Serial Analysis of Gene Expression (SAGE)

Plant infection assays and phenotypic evaluations

Seedlings of chickpea (*Cicer arietinum* L.) cultivars JG62 and Digvijay were inoculated with standard Foc races 1, 2 and 4. Seedlings treated with sterile deionized water served as control. Root and shoot tissues were collected separately for each race and both cultivars at 11 time intervals namely 0 hours post inoculation (hpi), 8 hpi, 16

hpi, 24 hpi, 2 days post inoculation (dpi), 3 dpi, 4 dpi, 7 dpi, 14 dpi, 21 dpi and 28 dpi. Wilting symptoms started to appear at about 7 dpi in JGI and intensified with time. More than 90% of JGI plants were almost dead by 28 dpi, while the remaining plants were severely wilted. On the contrary, all the DVI plants were healthy even beyond 28 dpi and till maturity.

Microscopic monitoring of pathogen progression in chickpea plants

The LBA4404 strain of *Agrobacterium tumefaciens* containing modified pCAMBIA 1302 (*mGFP* cassette replaced with *eGFP* cassette from pCBdeltaXCE) was used for transformation of Foc 2 (Mullins *et al.*, 2001). A set of JG62 and Digvijay plants was inoculated with the transformant. The inoculated and control chickpea plants were sampled daily during 1 to 4 DPI and at a 2–3 day interval thereafter, up to 18 DPI. The entire surface of the tap and lateral roots of each plant was observed under a confocal laser scanning microscope (CLSM). Uniform green fluorescence was observed in mycelia and microconidia of the isolate under CLSM. During early infection stages (up to 4–6 dpi), both the cultivars showed surface colonization and entry of the pathogen in lower roots. However, substantial colonization of vascular region was thereafter observed only in JGI with the progressive time-points. This difference between JGI and DVI further intensified by 28 dpi; where JGI showed exhaustive colonization of both root and shoot tissues, while minimal fungal colonization was observed in DVI.

In planta pathogen quantification

The primer combination Foc 3F & Foc 3R amplified an 88-bp fragment from an internal portion of the 1.5-kb sequence characterized amplified region (SCAR) of *F. oxysporum* f.sp. *ciceri*. To generate standard curves for qPCR assays, 10-fold dilutions of Foc 2 DNA (10 ng/µl) were prepared (1:1, $1:10^1$, $1:10^2$, $1:10^3$, $1:10^4$ and $1:10^5$) in sterile deionized water. To determine pathogen load in susceptible (JG62) and resistant (Digvijay) chickpea plants, genomic DNA isolated from whole roots of inoculated plants was used as template for qPCR. Whole root analysis showed significant amount of Foc 2 DNA in both JGI and DVI till 16 hpi, followed by a decrease till 4 dpi. Thereafter, the

amount of Foc 2 DNA increased significantly only in JGI till 14 dpi. At 28 dpi, the fungal DNA content decreased in both JGI and DVI; however in DVI, the pathogen load itself was significantly less than that in JGI.

Candidate gene expression analysis

Root and stem tissues, challenged by Foc 2 and sampled at 8 time-points 0 hpi, 16 hpi, 24 hpi, 2 dpi, 4 dpi, 7 dpi, 14 dpi and 28 dpi along with the control plants mock-inoculated with sterile water at each of the corresponding time points, were used for qRT-PCR analysis. Three independent biological replications were performed for both inoculated and control plants. The amount of target gene transcripts was normalized over the constitutive abundance of chickpea *GAPDH* for plant genes and individual time-point basis of Foc *EF1α* for pathogen genes. In JGI, most of the Foc virulence genes followed a similar pattern as that of colonization i.e. expression of genes increased at initial establishment phase, decreased in the autophagy phase and was again elevated at late stage. However, in DVI, most of these genes were weakly expressed except the gene encoding cell wall extracellular matrix protein which showed an increasing trend with the disease progression. This was concluded to be repeated attempts of the pathogen trying to establish in the resistant host. In case of plant defense related genes, few enzymes acting on cell wall structural components of fungi, were up-regulated in the resistant cultivar compared to the susceptible. Key enzymes of phenylpropanoid pathway and certain stress management genes were also up-regulated in the resistant cultivar compared to the susceptible. Some genes of this category like *CYP450*, *PR10* and H_2O_2 scavenging enzymes were up-regulated in the susceptible cultivar compared to the resistant.

Construction of Long-SAGE libraries

Total RNA was extracted from 100 mg chickpea (both resistant and susceptible cultivars) root and shoot tissue each and was reverse transcribed. Total RNA from all the 11 time-points of individual Foc races (1, 2 and 4) inoculated plantswas normalized using the reference gene actin. Based on this normalization, RNA from all these time-points was pooled for control and pathogen challenged plants, separately for construction of

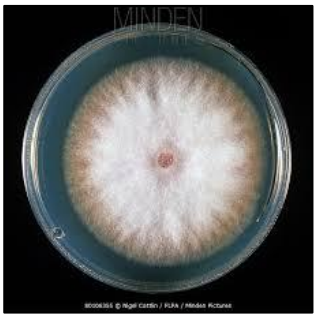
Long-SAGE libraries. Ditags obtained at the endpoint of Long SAGE protocol were sequenced using Ion-Torrent platform, were extracted from the raw reads and were converted into tag sequences ranging from 16 bp to 19bp using Bio-SAGE-Data Processing package. Tag mapping analysis was performed using SeqMap tool (with '1 mismatch' allowed) for 'unique match' and 'selected match'. The tags having frequency more than one were considered for further analysis. After mapping, the individual libraries with raw tag count were obtained. These raw counts were normalized and then analyzed for differential expression (DE) resulting into four datasets [Differential Gene Expression (DGE) sets]. The trimmed mean of M-values normalization method i.e. TMM was used for normalization of all the 4 datasets using edgeR package (Robinson & Oshlack, 2010). The up-regulated and down-regulated genes were selected by using cut-off of 2 fold (LFC >=1).The DEGs from plant species were processed for gene enrichment analysis using the Mercator tool. Further Mapman analysis (Usadel *et al.*, 2005) was performed for pathway enrichment of the differentially expressed genes. The DEGs from pathogen were processed for gene enrichment analysis using Blast2GO.

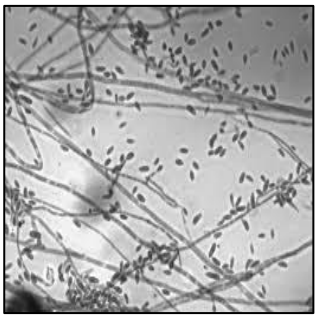
Transcriptome analysis revealed 3816 differentially expressed (DE) genes in DE_JGC_JGI set excluding the fungal sequences (unique to JGI library). Similarly, 3429 DEGs in DE_DVC_DVI set, 3640 DEGs in DE_JGI_DVI set and 2987 DEGs in DE_JGC_DVC set were obtained. The annotation tool 'Mercator' allowed the assignment of genes of all the four sets into 35 functional classes referred to as 'BINs'. A total of 400 DEGs (all having LFC≥1 in at least one of the sets) were clustered using Euclidean distance and complete linkage method. Six expression patterns (cluster 1-6) were obtained based on hierarchical clustering algorithms. Exclusively expressed genes represent the candidates reprogrammed by the pathogen for its own benefit in JGI (562) or those which activate the defense response against the pathogen in DVI (860). Uniquely expressed important genes in DVI included beta-D-xylosidase 7, rhamnogalacturonate lyase B, etc. while those in JGI were *MLO* like transcript, actin depolymerizing factor (*ADF*) 5 and tonoplast intrinsic protein (*TIP*) aquaporin type alpha, etc.

Comparative transcriptome analysis of JGI and DVI libraries revealed total 1569 genes showing high homology to Fo and *Fusarium graminarium* (Fg) and absence of these sequences in control library. This gene number was further reduced when statistical significance was considered. Total 18 Foc transcripts were significantly up-regulated in JGI and the same genes were down-regulated in DVI. Among these were the transcripts with similarity to serine rich protein, ubiquitin fusion protein, glucosidase, heat shock proteins, histone proteins and five of the transcripts with possible involvement in fungal growth such as tropomyocin 1, polarized growth protein rax2, woronin body major protein, etc. Only five Foc transcripts namely putative tartrate transporter, TKL protein kinase (Tyrosine kinase like) and three uncharacterized were expressed exclusively in DVI while total 533 Foc transcripts were expressed only in JGI out of which 382 (71.66%) transcripts could be annotated using available resources.

Chapter 1

Introduction and Review of literature



1.1 Chickpea, the host plant

1.1.1 Background

Chickpea is one of the earliest grain crops cultivated by man and has been found in Middle Eastern archaeological sites dated at 7500-6800BC (Zohary *et al.*, 2012). Chickpea (*Cicer arietinum* L.), the only cultivated species within the genus *Cicer*, is a self-pollinated diploid (2n=2x=16) crop with a relatively small genome size of 740 Mb (Arumuganathan & Earle, 1991). It ranks second among food legumes in terms of production after beans (*Phaseolus vulgaris*). It is grown in over 50 countries with 90% of its area in developing countries. Two types of chickpeas are recognized, the white-seeded "Kabuli" and the brown colored "Desi" types (Fig.1.1). Kabuli chickpeas are relatively bigger in size having a thinner seed coat while the Desi type seeds are relatively smaller in size having a thicker seed coat. The Desi type chickpea contributes to around 80% and the Kabuli type around 20% of the total production. Chickpea has been classified as

Table 1.1 Classification of chickpea

Kingdom	**:**	*Plantae* – Plants
Subkingdom	**:**	*Tracheobionta* – Vascular plant
Super division	**:**	*Spermatophyta* – Seed plants
Division	**:**	*Magnoliophyta* – Flowering plants
Class	**:**	*Magnoliopsida* – Dicotyledons
Subclass	**:**	*Rosidae*
Order	**:**	*Fabales*
Family	**:**	*Fabaceae* – Pea family
Genus	**:**	*Cicer*
Species	**:**	*C. arietinum* L. – chickpea

Fig. 1.1 Kabuli-type (left) and desi-type (right) chickpea seed
Source: (http://exploreit.icrisat.org/page/chickpea/685)

1.1.2 Nutritional importance of chickpea

Chickpea is consumed as a dry pulse crop or as a green vegetable commonly used in preparing *dal*, in vegetable combinations, or as a component of fresh salads mainly as a source of protein in vegetarian diets (Oplinger *et al.*, 1990). Carbohydrates and proteins in chickpea together constitute about 80 % of the total dry seed mass in comparison with other seed pulses. Starch is the major storage carbohydrate followed by dietary fiber, oligosaccharides and simple sugars such as glucose and sucrose. Proteins have significant amounts of all the essential amino acids except sulphur-containing amino acids. Chickpea is a good source of vitamins such as riboflavin, niacin, thiamin, folate and minerals, Ca, Mg, P and K (MacMichael, 2002; Wood & Grusak, 2007; Chibbar *et al.*, 2010). Although lipids are present in low amounts, chickpea is rich in nutritionally important unsaturated fatty acids such as linoleic and oleic acids. β-sitosterol, campesterol and stigmasterol are important sterols present in chickpea oil. As with other pulses, chickpea seeds also contain anti-nutritional factors which can be reduced or eliminated by different cooking techniques. Overall, chickpea is an important pulse crop with a diverse array of potential nutritional and health benefits (Jukanti *et al.*, 2012).

1.1.3 Growth conditions

Chickpea is a hardy, deep-rooted, dry land crop sown on marginal lands, which can grow to full maturity in conditions that would be unsuitable for most crops (Singh & Reddy, 1991). The deep-tap root system enhances its capacity to withstand drought conditions. It

is usually well suited for cultivation in cooler areas with low rainfall. Chickpea does best in sandy, loam soils possessing an appropriate drainage system; since it is very sensitive to excess water availability. Even short periods of flooded or waterlogged fields reduce growth and increases susceptibility to root and stem rots. Also, very cold conditions can greatly reduce the productivity of chickpea. It is basically a rabbi crop sown in months of September-November and harvested in the months of February- April. Maturity period ranges from 95-110 days after sowing. Heavier rainfall seasons show reduced yields due to disease outbreaks and stem lodging problems from the excessive vegetative growth. Areas with lighter, well distributed rainfall patterns have produced the highest yield and quality chickpea seed (Margheim, 2004).

1.1.4 Chickpea yield and losses

Chickpea ranks second in the world among pulses after beans with production of 14.23 million tons (Fig 1.2). During 2014, globally chickpea was grown on 10.74 million hectares (ha) to produce 9.88 Million Tonnes (MT) with an average yield of approximately 919.9 kg ha−1 (http://faostat.fao.org). India alone contributes to 69% of world's chickpea production (FAOSTAT, 2014 http://faostat.fao.org/site/567/default.aspx). However, the supply in terms of production is not able to meet the increasing demand due to demographic growth in India which led to import of about 0.58 MT in the year 2013. In spite of its economic importance and strong national breeding programs, the productivity of chickpea has not improved considerably over the years (Gowda *et al.*, 2009). Major constraints in realization of the full yield potential of chickpea are various abiotic and biotic factors.

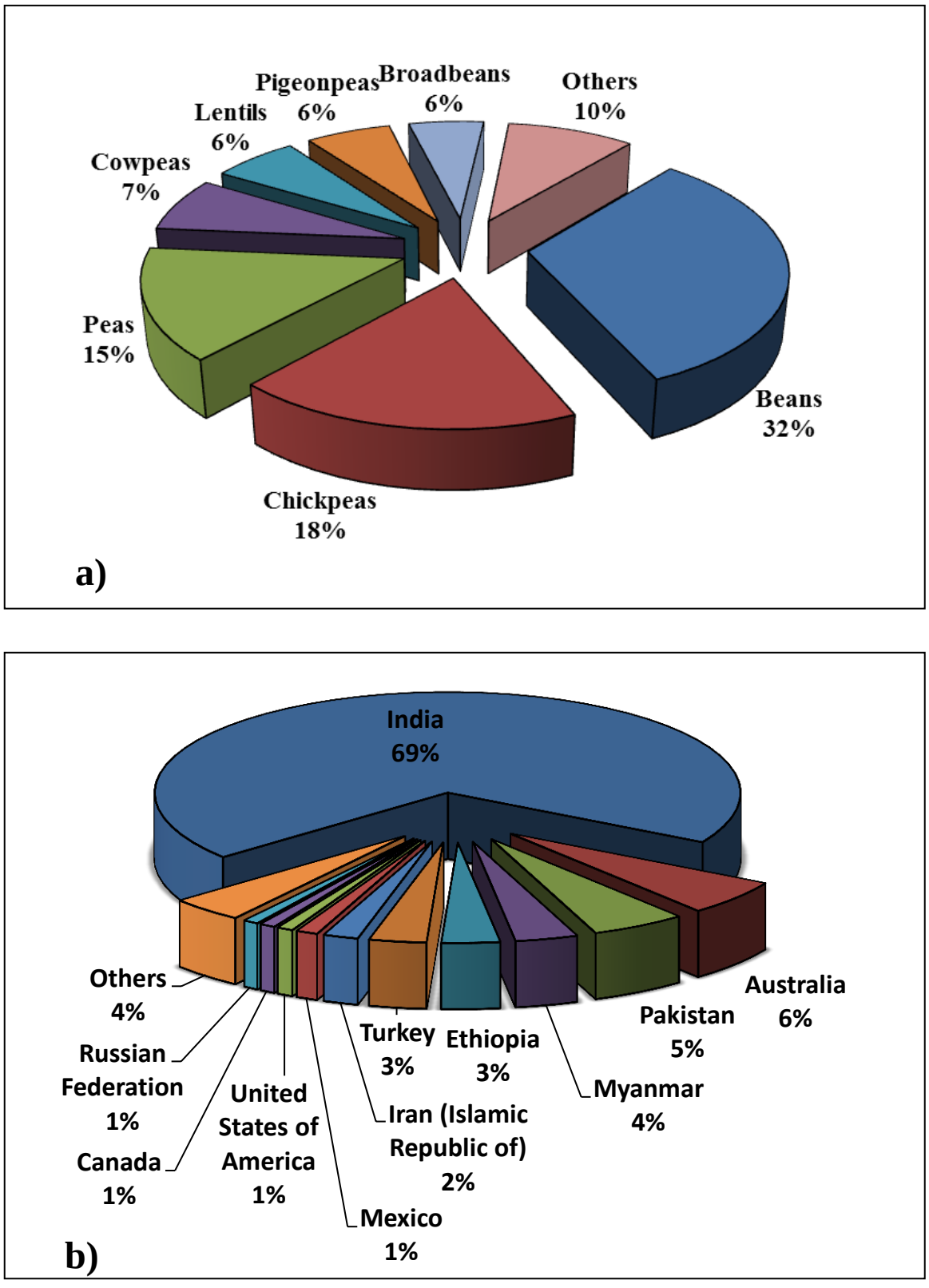


Fig. 1.2 Contribution of Indian agriculture to the global production of chickpea. a) Global production of chickpea in comparison with other legumes b) India is world's largest chickpea producer

The major abiotic constraints to productivity include drought, cold and salinity. Majority of chickpea (90%) is grown under rain-fed conditions. Hence, drought is a factor of major concern to the farmers (Kumar & Abbo, 2001).Injury from cold, chilling and freezing to the plant at all the developmental stages particularly at flowering and/or pod setting stages causes absolute loss of yield (Croser *et al.,* 2003). In addition, sensitivity to sodicity and salinity has also been reported to adversely affect germination, biomass and yield of chickpea crops (Ahmad *et al.*, 2005). Amongst the causative agents of biotic stresses, about 67 fungi, 3 bacteria, 39 viruses, 60 insect species and 80 nematodes have been reported on chickpea but only few of which proved to be extremely detrimental (Haware *et al.*, 1996; Li *et al.*, 2015). The key biotic constraints are Ascochyta blight (*Ascochyta rabii*), Fusarium wilt (*Fusarium oxysporum*), Dry root rot (*Rhizoctonia bataticola*), Botrytis grey mould (*Botrytis cinerea*), Collar rot (*Sclerotium rolfsii*), Root-knot nematode (*Meloydogyne incognita* and *M. javanica*), Stunt-virus, Pod borer (*Helicoverpa armigera*), and Cutworm (*Agrotis ipsilon*).

1.1.5 Fusarium wilt of chickpea

Fusarium wilt is one of the major constraints in realization of full yield potential of chickpea. This disease was first reported in India by Butler in 1918 but its etiology was not correctly determined until 1940 by Padwick (Jimenez-Diaz *et al.,* 2015).Now it is widespread in most chickpea growing areas of the world including the Indian subcontinent, Iran, Peru, Syria, Ethiopia, Mexico, Spain, Tunisia, Turkey and US but it was not reported in Australia till 2007 (Cunnington *et al.*, 2007). Annual yield losses due to wilt have been estimated to be 10%–90% (Jimenez-Diaz *et al.*, 1989; Singh & Reddy, 1993). The causative agent of this disease has been classified as *Fusarium oxysporum* f. sp. *ciceris* (Padwick) Synd. & Hans (Foc). Foc is internally seed borne and is found as chlamydospore-like structures in the hilum region of the seed (Haware *et al.*, 1978). Thus, infected seed plays an important role in long distance dispersal and in transmitting the disease to new areas. Once the inoculum is established in soil, it is difficult to eradicate as the chlamydospores survive in the soil for at least 6 years, and under favorable conditions germinate and infect the seedlings through tender roots (Haware *et*

al., 1996). Among other legumes, lentil, pea and pigeon pea are symptomless carriers of the chickpea wilt fungus (Haware & Nene, 1982).

1.2 *Fusarium oxysporum* f. sp. *ciceri*, the wilt pathogen

1.2.1 The genus *Fusarium*

Traditional classification and identification schemes for *Fusarium* are exclusively based on a morphological species concept derived from cultural characteristics of single-spore isolates grown on special media, shared morphological trait of the anamorph, host range, and to a lesser extent, teleomorph micromorphology (Booth, 1971). Due to the conflicting morphological species concepts employed in taxonomic treatments of this genus (Booth, 1971; Gerlach & Nirenberg, 1982; Nelson & Wfo Toussoun, 1983), the systematics of *Fusarium* remains controversial and confusing (Gams & Nirenberg, 1989), especially if more than one taxonomic treatment is consulted. Gerlach and Nirenberg's system (1982) is the most differentiated, including 73 species and 26 varieties; while 44 species and 7 varieties have been recognized by Booth (1971) and, 30 species by Nelson et al. (1983).

Fusarium is a large cosmopolitan genus of pleoanamorphic hyphomycetes whose members are responsible for a wide range of plant diseases (Farr *et al.*, 1989), mycotoxicoses and mycotic infections of humans and other animals (Nelson *et al.*, 1994). The species *Fusarium oxysporum* is well represented among the soil borne fungi, in every type of soil all over the world (Burgess *et al.*, 1981) and is considered to be a normal constituent of the rhizosphere of plants (Appel & Gordon, 1994). However, some strains of *Fusarium oxysporum* are pathogenic to different plant species; they penetrate into the roots and provoke either root rots or tracheomycosis when they invade the vascular system, causing severe damage on many plant species of economic importance. The vascular wilt causing forma speciales of *Fusarium oxysporum* typically invade only living root tissues, tend to be specialized, are host specific, and suppressed by saprophytes. Based on the plant species and plant cultivars infected, they are classified into more than 120 forma speciales and races (Armstrong, 1981). The presently accepted

classification for the fusarium wilt pathogen *Fusarium oxysporum* f. sp. *ciceri* is: Form - Class: Fungi Imperfecti, Form -order: Moniliales, Form-family: Tuberculariaceae, Form-genus: *Fusarium*, Form species: *oxysporum*, forma specialis *ciceri*. *Fusarium oxysporum* f. sp. *ciceri* (Foc) is reported from most of the chickpea growing areas all over the world. Variation in the virulence within isolates of a given forma specialis has led to the designation of pathogenic races. Accordingly, *Foc* has been classified into eight races worldwide on the basis of the pathogenic reaction of a particular Foc isolate to a standard set of differential chickpea cultivars. Races 0 and 1B/1C cause yellowing symptoms whereas races 1A, 2, 3, 4, 5 and 6 induce wilting symptoms (Del Mar Jimenez-Gasco *et al.*, 2001). Out of these, races 2, 3 and 4 have been reported only in India, races 0, 1B/1C, 5 and 6 have been mainly found in the Mediterranean region and in California, USA while race 1A is reported in India, the United States, and the Mediterranean (Landa *et al.*, 2006).

1.2.2 Growth and cultural characteristics

The fungus grows on potato dextrose agar at 25°C and appears as delicate, white and cottony growth becoming felted and wrinkled in older cultures (Nelson & Wfo Toussoun, 1983). Fungal hyphae are septate and profusely branched. Microconidia are borne on simple short conidiophores, arising laterally on the hyphae. Microconidia and macroconidia are generally sparse on solid media; however, they are formed abundantly in potato dextrose broth. Microconidia are oval to cylindrical, straight to curved and measure 2.5-3.5 x 5-11 µm. Macroconidia, which develop on the same conidiophores on which microconidia are formed (Nelson & Wfo Toussoun, 1983), are thin walled, 3-5 septate, fusoid, pointed at both ends, fewer in number than microconidia, and measure 3.5-4.5 x 25-65 µm. Chlamydospores, formed in 15-day-old cultures are smooth or rough walled, terminal or intercalary, and may form singly, in pairs, or in chains.

1.2.3. Life cycle of Foc

In a comprehensive review addressing the life cycle, disease progression and host pathogen interactions with respect to wilt causing pathogens, Beckman and Roberts

(1995) proposed a model, wherein, the pathogens have distinct saprophytic and parasitic phases in their life cycles (Beckman & Roberts, 1995). Fig. 1.3 depicts the life cycle of soil-borne, wilt causing fungi including their saprophytic and parasitic growth and successive phases of colonization and pathogenesis. There are three distinct phases in the pathogen lifecycle i) Determinative phase, ii) Expressive phase and iii) Saprophytic phase. In determinative phase the extent of colonization of host by the pathogen is determined; in the expressive phase mainly disease symptoms are developed while the survival of the pathogen by formation of long-lived resting structures mainly ensues in the saprophytic phase of the life cycle. Under disease prone conditions, the pathogen invades the root tissue and after it has acquired significant cortical colonization, readily enters the second phase of vascular invasion and spreads along with the transpiration pull. The plant defense response to pathogen invasion is offered mainly at two places (i) in the endodermis and xylem parenchyma, where the invading pathogen is restricted by infusion of phenolic compounds, progressive suberization and lignin deposition, and by hydrolytic enzymes like chitinases and glucanases, and (ii) in the vascular tissue, where the upward movement of the pathogen is arrested to compartmentalize the pathogen, by the formation of callose, gelgum and tyloses, which are mainly derivatives of celluloses and hemi-celluloses. The difference in resistant and susceptible cultivars lies in the speed with which they can activate the defense mechanisms and accumulate substances like callose to restrict the growth and spread of the pathogen; most of the disease symptoms seem to be caused by the plant response to infection.

1.2.4 Symptoms of Foc infection

In general, dull green colored foliage are seen when chickpea seedlings are infected with Foc along with rapid drooping of the leaves. The uprooted plants show uneven contraction at the collar (Fig. 1.4 a and b). Root and pith decaying are not externally visible; however, due to infection of the xylem tissues of the root and stem, internal discoloration may be visible upon vertical sectioning of such roots (Fig 1.5) (Nene, 1981). Further, the diagnosis of vascular wilt can be confirmed with the presence of hyphae and spores of the fungus in the xylem by observing the transverse sections of

such infected roots under the microscope (Fig. 1.6) (Nene, 1981). As seen in Fig. 1.7, seeds are lighter and duller from the late wilted plants compared to the healthy plants. Chickpea genotypes differ in rates of symptom expression after Foc infection. This can be classified as early and late wilting categories on the basis of days to wilting from sowing (Haware & Nene, 1980). Plants grown from infected seed wilt faster than the plants grown from clean seed. Accordingly, susceptible cultivar shows wilt symptoms within 25 days after sowing in infected soil that is known as 'early wilt' (Haware & Nene, 1980). Depending on the genotype, Foc isolates may provoke either fast wilting or a progressive yellowing syndrome, which develops after 15-40 days of inoculation. Plant may show wilting during reproductive growth phase which is termed as 'late wilt'.

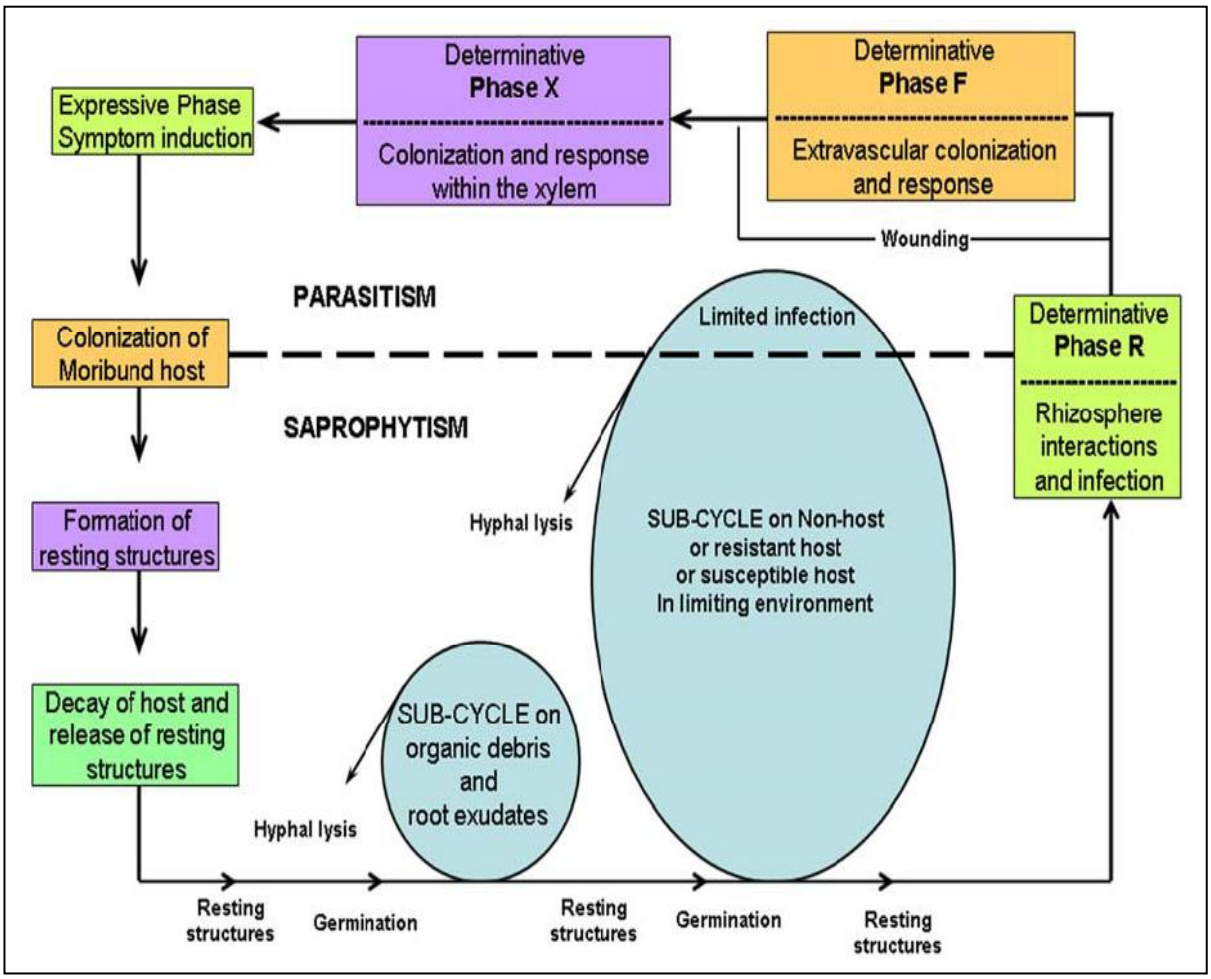


Fig. 1.3 Life cycle of vascular wilt pathogen '*Fusarium oxysporum* f.sp. *ciceri*'
(Source: Thesis of S. Nimbalkar with modifications)

1.2.5 Fungal colonization *in planta*

Studies of plant-pathogen interactions include the basic step of microscopic analysis which not only reveals colonization pattern of the pathogen but gives a useful direction for further analysis of the interactions. Visualization of GFP labeled organisms with Confocal Laser Scanning Microscope (CLSM) is an effective, fast, and noninvasive tool that allows the spatiotemporal analysis of interactions while preserving the integrity of the organisms under study. A large number of studies have utilized Green Fluorescent Protein (GFP) from the jellyfish *Aequorea victoria* (Chalfie & Kain, 1998; Tsien, 1998) to label the pathogen in order to visualize it in *in vivo* conditions. The GFP fluorescence is stable and species independent and does not require any cofactors or substrates. This marker has been used to study the colonization, infection and disease development (Li *et al.*, 2011; Lu *et al.*, 2011; Lakshman *et al.*, 2012) and to trace the fluorescent proteins for verifying their role in pathogenesis (Kim *et al.*, 2009; Michielse *et al.*, 2009; Kakoschke *et al.*, 2016).

1.2.6 Disease Management

Various approaches have been practiced to overcome losses due to Fusarium wilt in chickpea. They are detailed below.

1.2.6.1 Cultural practices

The chickpea wilt pathogen is monocyclic and the increase in the Foc population in soil is because of the longevity of reproductive units and the difficulty in eliminating them from wilt sick fields (Haware *et al.*, 1996). Chickpea wilt has been reported to increase with increasing levels of soil inoculum; initial high levels of Foc propagules causing 100% wilting much earlier than initial low levels of Foc propagules (Bhatti & Kraft, 1992). Disease potential can, therefore, be assessed from the knowledge of the initial pathogen population for early forecast of severity of diseases induced by soil-borne pathogens (Fry, 2012).

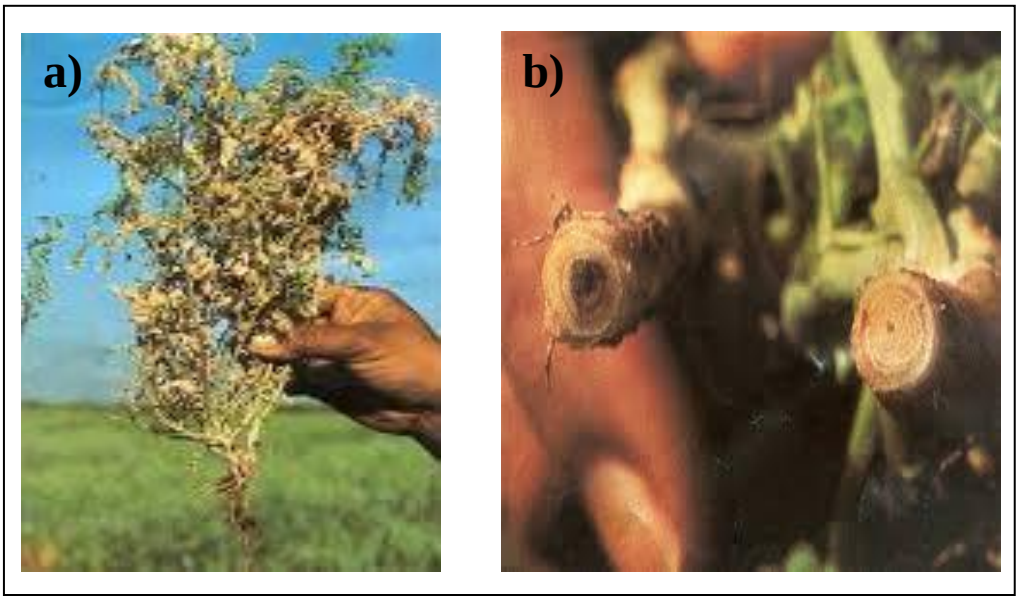


Fig. 1.4 A. Dull green foliage of wilted chickpea plant B. Comparison of wilted chickpea root with healthy

Fig. 1.5 Internal discoloration of root xylem in vertically split root of chickpea

Fig. 1.6 T.S. of a wilted plant showing presence of hyphae in xylem

Fig. 1.7 Seed harvested from the late wilted plants is lighter and duller than that harvested from healthy plants

Where land is not limiting, avoidance of planting in heavily infested fields can minimize the effects of wilt disease on yield. Crop rotation, however, is not an effective practice for reducing wilt incidence as the pathogen can survive in soil for long periods (Haware *et al.*, 1996). On the other hand, deep ploughing during summer and removal of host debris from the field can considerably reduce inoculum levels. Solarization, by covering the soil with transparent polythene sheet for 6-8 weeks during summer months, effectively controls wilt in chickpea and improves plant growth and yield (Chauhan *et al.*, 1988). Although this method is useful in commercial production, it is not a practical option for the resource poor farmer. Seed transmission can be avoided by using disease free seed, obtained from plants grown in disease free areas. Seed can also be treated with appropriate fungicides (mixture of 30% benomyl + 30% Thiram at 1.5g/kg seed) for eradication of seed borne inoculum (Haware *et al.*, 1978).

1.2.6.2 Fungicides

The chemical fungicides that are most effective are Benomyl Captan and Carbendazim. However, management of Fusarium wilt with chemical fungicides is uneconomical and difficult to achieve because of the soil and seed-borne nature of the pathogen (Ahmad *et al.*, 2010). Moreover, the application of fungicides causes groundwater pollution, loss of non-target beneficial flora and evolving fungicidal resistant variants of the pathogen. The recontamination of the pathogen in the fungicide-treated soil often flourishes faster due to

the absence of competitive microflora leading to higher incidence of disease in the susceptible host.

1.2.6.3 Biological Control

Soils harbor large populations of non-pathogenic *Fusarium oxysporum*, which play an important role in soil microbial ecology, especially in soils suppressive to fusarium wilt (Alabouvette *et al.*, 1993; Larkin & Fravel, 1999). Currently there is a lot of interest in studying genetic diversity in non-pathogenic strains of *F. oxysporum*, the interactions between pathogenic and non-pathogenic strains and the mechanisms of suppression to fusarium wilt by other soil fungi sharing the same ecological niche. Such interactions form the basis of the strategy for biological control. As such in the present context, biological management of wilt offers a great promise. *Trichoderma harzianum* is one efficient biocontrol agent that is successfully used to suppress Fusarium wilt (Khan *et al.*, 2004; Dubey *et al.*, 2007). Similarly, amending soil with plant extracts significantly reduces Fusarium wilt in the field (Chand & Singh, 2005). However, biological suppression of plant disease is often subjected to ecological limitations and is not sufficient alone to escape the pathogen under field conditions. Instead, biological control when used in combination with other management strategies offer potential for suppression of disease. Therefore, management of Fusarium wilt of chickpea should be based on strategies that combine the use of additive or synergistic combinations of biotic, cultural, and chemical control measures (Landa *et al.*, 2004).

1.2.6.4 Use of resistant cultivars

Due to the difficulty of widespread application of available cultural and chemical control measures for wilt, especially for the resource poor farmers and the limitations associated with the use of biocontrol methods, considerable emphasis has been placed on the development of resistant cultivars (Haware & Nene, 1980; Haware *et al.*, 1992). Chickpea production in India can be stabilized and improved by the development of wilt resistant chickpea cultivars adaptable for all the environments (Bakhsh *et al.*, 2011). The selection and inheritance of the desirable traits has now become possible with the

advancement of Marker Assisted Selection (MAS) which provides a beneficial source to exploit the potential of genes for resistance and agronomic traits (Collard & Mackill, 2008). DNA marker based tagging of resistance genes for FOC 1– 5 races has been established using various RAPD and SSR markers in recombinant inbred lines (RILS) populations generated from various resistant and susceptible parental combinations (Winter *et al.*, 2000; Sharma *et al.*, 2004; Iruela *et al.*, 2007; Gowda *et al.*, 2009).

1.3 Host-pathogen interaction

Plants and pathogens are continuously engaged in co-evolutionary struggle for their dominance and their interactions represent extremely complex biological phenomena. Plant cells perceive and integrate signals from their pathogens and respond by modulating defense response through activation or repression of a large array of genes. Similarly, microbial pathogens develop different strategies to cope with the plant responses. Plants generally reject attacking phytopathogenic fungi. A majority of plants are not readily colonized and parasitized by most pathogens. Such plants are non-host plants for the pathogen and exhibit immunity against them; since the pathogen is unable to surmount the barriers that prevent pathogen colonization. Such barriers comprise the basic resistance or basic incompatibility of the plant and are the first level of pathogen defense. Basic compatibility is a highly specific phenomenon referring to only a particular plant species and the corresponding pathogen species or forma speciales (Niks, 1988). In spite of the basic compatibility between plant and its pathogen; the host plant may deploy different defense strategies to limit pathogen attack such as hypersensitive response, built-up of new defense barriers etc.

Basic resistance is directed against all pathogens in general, while cultivar specific resistance or host resistance is highly selective against only one pathogen species or forma speciales or race. Among two types of host resistance genes, race-nonspecific resistance is directed against all members or races of a pathogen species, while race-specific resistance is mounted against only a particular race of pathogen (Prell & Day, 2001). A study enabling to determine the race specific resistance in a cultivar or cultivar

specific resistance in a pathogenic race is essential. Such kind of study would help to understand the mutations necessary in both plant defense and fungal virulence genes for such an association/interaction in the host and its pathogen. It would also facilitate development of wilt resistant cultivars effectively followed by region specific deployment of resistance in accordance with prevalence of specific races.

1.3.1 Plant defense

Multiple events are involved that lead to successful plant defense during pathogen attack. Further, these defense mechanisms are governed by an array of genes, which either singly or synergistically, are involved in plant resistance traits. Many defense related genes have been cloned and characterized in an attempt to elucidate the mechanism of defense upon Foc attack in various plant species, including chickpea. For example, defense related genes like basic glucanases, *PAL, CHS* etc., involved during Foc 1 and Foc 0 infections have been previously studied in chickpea (Cho & Muehlbauer, 2004; Arfaoui *et al.*, 2007). In our earlier studies, enzymes like glucanases, chitinases and proteases have been shown to be probably involved in chickpea defense against Foc infection (Giri *et al.*, 1998). Further, various upregulated transcript derived fragments like *14-3-3, WRKY* and *NBS-LRR* type sequences as well as transposable elements, were identified using cDNA-RAPD and cDNA-AFLP techniques (Nimbalkar *et al.*, 2006). A study using cDNA-AFLP approach predicted that hypersensitivity and active species generation failed to impart host defense in compatible interaction between chickpea and Foc. On the contrary, the defense related gene(s) played a critical role in conferring natural resistance to the resistant host (Gupta *et al.*, 2010). Another study from our lab determined the transcripts involved in chickpea-Foc interactions using cDNA-RAPD approach which revealed an enhanced expression of plant defense related genes namely, *GroES2*, *60srp*, *BetvI*, *CHS* and *IFR* in case of resistant inoculated cultivar as compared to susceptible inoculated cultivar (Gurjar *et al.*, 2012). Additionally, a study has identified several conserved and novel miRNAs in chickpea that are associated with gene regulation following exposure to wilt and salt stress (Kohli *et al.*, 2014). Recently in our lab, proteomic and metabolomic approaches have been used to reveal the chickpea-Foc interaction at molecular level. The

metabolomic approach highlighted up-regulation of various metabolic pathways related to flavonoids, isoflavonoids, amino acids and sugars in the resistant cultivar as potential defense strategy. The proteomics analysis further indicated up-regulation of several stress responsive proteins, phenylpropanoid and ROS pathway, and lignosuberization in resistant cultivar upon infection (Kumar *et al.*, 2015a; Kumar *et al.*, 2016). However, exact molecular mechanisms involved in chickpea wilt resistance are still unexplored.

1.3.2 Fungal virulence

Fungi express pathogenicity genes/factors which help them to establish successfully in the host plant defeating the barriers. These pathogenicity factors are essential only for infecting the plant, not required for normal growth of the fungi and quote for varied functions such as penetration, formation of specialized invasion structures and nutrition availability. Understanding the pathogenicity mechanism of fungi demands the knowledge of the virulence factors active in the host environment. Till date many pathogens have been studied in context with their virulence aspects and various genes have been identified, which have a prime role to play in fungal pathogenesis (Gurjar *et al.*, 2012). These fungal pathogenicity genes are categorized based on formation of infection structures, cell wall degradation, toxin biosynthesis, signaling and proteins suppressing plant defense (Idnurm & Howlett, 2001; de Wit *et al.*, 2009; Mobius & Hertweck, 2009). In *F. oxysporum*, cell wall degrading enzymes (CWDE), involved in penetration and colonization in the host plant have been studied. Pectate lyase, a CWDE, has been suggested as a Foc pathogenicity factor in chickpea wilt (Jorge *et al.*, 2006). Signaling genes expressed during pathogenesis such as fmk1 (a mitogen-activated protein kinase) in *F. oxysporum* f.sp. *lycopersici* (Di Pietro *et al.*, 2001) and G protein subunits including G protein α subunit (fga1) and β subunit (fgb1) in *F. oxysporum* f.sp. *cucumerinum* have reported to be necessary for fungal morphogenesis, development and virulence (Jain *et al.*, 2002; Jain *et al.*, 2003). In addition, the genes *Fow1* and *Fow2* (Namiki *et al.*, 2001; Imazaki *et al.*, 2007), Six1, Six2 and Six3 (van der Does *et al.*, 2008) and Frp1 F-box protein (Duyvesteijn *et al.*, 2005) in *F. oxysporum* f.sp. *lycopersici* (Fol) have been suggested to play essential role during pathogenesis in tomato.

1.4 Approaches to study plant defense and fungal virulence

Plant-pathogen interaction is a multifaceted process involving large number of both pathogen- and plant-derived molecules. These interactions play an important role in agriculture and a lot of effort has been dedicated to analyze them in detail. High-throughput technologies of genomics, transcriptomics, proteomics and metabolomics have shifted the focus from single gene research to a holistic understanding of gene function. None of these methods used in isolation provide enough information to infer function for an unknown gene, instead, combined data from different functional genomics tools help to achieve this goal. (Fig. 1.8)

1.4.1 Transcriptomics/Gene expression studies

Transcriptomics is the quantification of the transcriptome, the complete set of transcripts in a cell, and their abundance, for a specific developmental stage or physiological condition (Wang *et al.*, 2009). It provides an ability to compare qualitative and quantitative differences in gene expression under various conditions by measuring multiple mRNA populations in the cell. Transcriptomics has a significant role in improving the understanding of fungal plant diseases. Microarrays have been a choice for many transcriptomic studies although various sequence based techniques such as Serial Analysis of Gene expression (SAGE), cDNA-AFLP, Multiple Parallel Signature Sequencing (MPSS), Subtractive Sequence hybridization (SSH) libraries, Generation of ESTs and RNA-seq are attractive as Next Generation Sequencing technologies are continuously being developed.

1.4.1.1 Generation of ESTs

A common first step in functional genomics studies is EST generation, which involves large-scale single-pass sequencing of randomly selected clones from cDNA libraries constructed from mRNA isolated at a particular developmental stage and in response to a particular stress. Functional identification of sequenced clones is being made easier by the availability of rapidly growing sequence databases, such as GenBank, and the full

sequencing of model species genomes. As of December, 20, 2016, about 53,333 EST sequences of chickpea and 25,213 EST sequences of *Fusarium oxysporum* are available in GenBank. Although ESTs don't represent full length gene sequences, EST analysis has been a popular method for gene discovery and mapping in many organisms. The first report of large-scale EST generation in chickpea was published in 2005 wherein >500 unigenes were isolated from the stems and leaves of an Ascochyta-blight- resistant

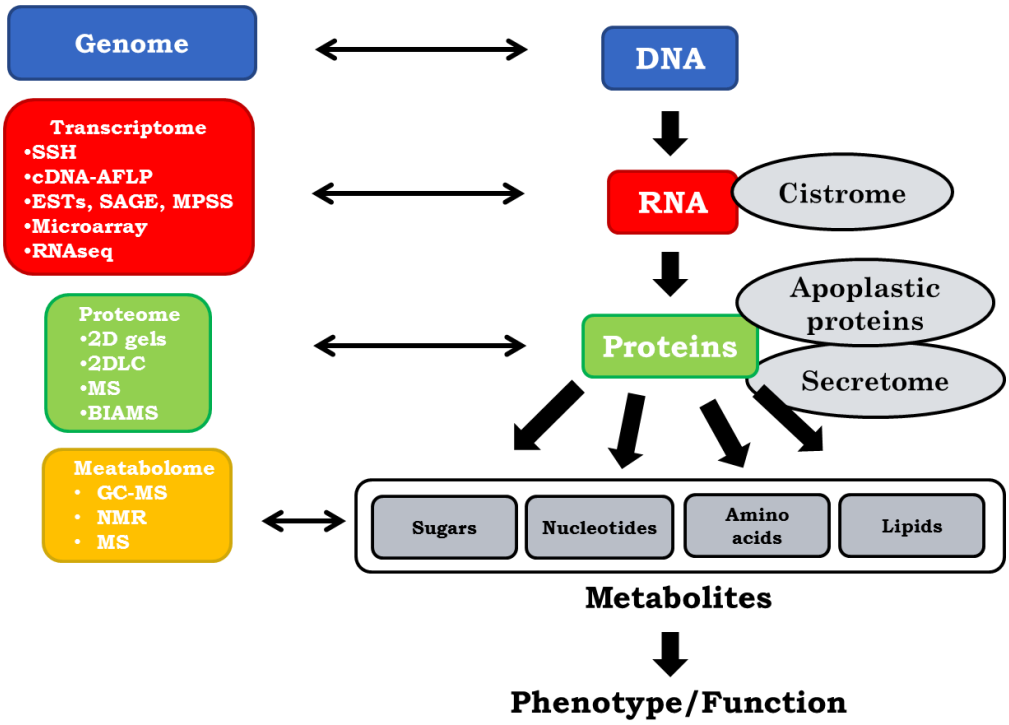


Fig 1.8 Various approaches used to study plant-pathogen interactions

genotype after pathogen inoculation (Coram & Pang, 2005). An EST library of chickpea root tissue was also made available in the same year (Jayashree *et al.*, 2005). It has also been used for Flowering Gene Discovery and SSR Marker Development in Upland Cotton (Lai *et al.*, 2011), marker development in yam (*Dioscorea alata* L.) (Narina *et al.*, 2011), identification of abiotic stress-responsive genes from salt-stressed roots of *Jatropha curcas* (Eswaran *et al.*, 2012)and to explore Salt-Responsive Related Genes from Halophyte *Atriplex canescens* (Li *et al.*, 2014).

1.4.1.2 Suppression Subtractive hybridization

One of the problems, linked to random sampling of ESTs, is the lack, or the under-representation of genes that are weakly expressed, but might still be key regulators of defense activation, such as those encoding transcription factors. The suppression subtractive hybridization (SSH) technique can address this drawback. It allows for PCR-based amplification of only cDNA fragments that differ between a control and experimental transcriptome. This method has often been used to study molecular mechanisms of plants in complex relationships with different pathogens and a variety of abiotic stresses. Recently this technique has been used to profile defense related genes in tomato in response to *T. longibrachiatum, B. cinerea* or both (De Palma *et al.*, 2016). Additionally, differentially regulated genes in abscisic acid (ABA) pretreated pepper plants under chilling conditions were identified using this technique (Guo *et al.*, 2013).

1.4.1.3 cDNA-AFLP

cDNA amplified fragment length polymorphism (cDNA-AFLP) is one of the few genome-wide expression profiling methods to explore genes that have not yet been cloned or even predicted from sequence but have interesting expression patterns under the studied conditions (Kivioja *et al.*, 2005). Another advantage of cDNA-AFLP is its high sensitivity, i.e. the ability to detect low-abundance transcripts as well (Fukumura *et al.*, 2003), which can be difficult with the microarray technology (Evans *et al.*, 2002).The potential of the AFLP technique for generating mRNA fingerprints was first recognized by Bachem, Hoeven *et al.*, (1996) for the study of differential gene expression during potato tuber formation (Bachem *et al.*, 1996). Since then it has been used to profile genes in several different systems including humans (Egert *et al.*, 2006), animals (Cappelli *et al.*, 2005; Vandeput *et al.*, 2005; Pareek *et al.*, 2012), plants (Juana *et al.*, 2006; Nimbalkar *et al.*, 2006; Gupta *et al.*, 2009; Sestili *et al.*, 2011) and microbes (Decorosi *et al.*, 2005; Booijink *et al.*, 2010; Bove *et al.*, 2011).

1.4.1.4 Microarrays

First described in 1995 (Schena *et al.*, 1995), high-density DNA microarray methods have already made a marked impact on many fields, including host pathogen interaction studies. DNA microarray techniques are particularly suitable for monitoring gene expression changes in plants during plant-pathogen interactions, due to their relative simplicity, comprehensive sampling capacity and high throughput (Kazan *et al.*, 2001). Microarray technologies allow the parallel hybridization of hundreds to thousands of carrier-bound DNA probes (Nowrousian, 2007). Microarrays have been used in studying gene expression profiling in fungal biology with *Magnaportha oryzae* as a pioneer pathogen (Oh *et al.*, 2008). In addition, gene expression of the biotroph *Blumeria graminis* f. sp. *hordei* during infection on barley (Both *et al.*, 2005), the model basidiomycete pathogen *Ustilago maydis* (Molina & Kahmann, 2007) have also been determined. Recently, microarray analysis of gene expression profiling in ripening pineapple fruits revealed molecular basis of pineapple fruit ripening and non-climacteric fruit ripening in general (Koia *et al.*, 2012). Additionally, profiling the expression of root genes after infection with *F. oxysporum* by microarray analysis revealed new regulators to confer resistance (Chen *et al.*, 2014).

1.4.1.5 Serial Analysis of Gene Expression (SAGE)

Serial Analysis of Gene Expression (SAGE) is a powerful technique that allows the identification of multiple transcripts simultaneously (Velculescu *et al.*, 1995). The technique is based on three principles, i) A sequence tag obtained from a defined region within each transcript contains sufficient information to uniquely identify a transcript, ii) Sequence tags can be linked together to form long DNA molecules (concatemers) that can be cloned and sequenced. Sequencing of the concatemer clones results in the identification of individual tags and iii) The expression level of the transcript is quantified by the number of times a particular tag is observed. Multiple variants of SAGE, such as MicroSAGE (Datson *et al.*, 1999), SAGE-lite (Peters *et al.*, 1999), SADE (Serial Analysis of Downsized Extracts) (Virlon *et al.*, 1999), Long-SAGE (Saha *et al.*, 2002), Robust-LongSAGE (RL-SAGE) (Gowda *et al.*, 2004) and SuperSAGE (Matsumura *et*

al., 2003), were later developed to increase efficiency of the various enzymatic reactions and permit analysis of gene expression from small amounts of sample (Anisimov, 2008). Some key features of this technique are it can be used to obtain a comprehensive gene expression profile for a specific tissue or cell type, identify novel genes, identify more sequence information to facilitate full-length cloning of novel targets and provide sufficient sequence information for direct mapping to genomic DNA. In plants, it has been mainly used for studying plant-pathogen interactions (Hamada *et al.*, 2008; Molina *et al.*, 2008; Matsumura *et al.*, 2010; Molina *et al.,* 2011).

1.4.1.6 Massively Parallel Signature Sequencing (MPSS)

MPSS is a unique and proprietary technology invented by Sydney Brenner (Brenner *et al.,* 2000), which has been established as one of the most powerful methods for identifying polyadenylated transcripts (and recently non-polyadenylated RNAs such as micro- and small interfering RNAs) (Reinartz *et al.*, 2002). It provides a complete, accurate and permanent digital record of every mRNA molecule in the cell (Zhou *et al.*, 2006). MPSS captures and sequences a 20-base pair signature including and adjacent to the 3'-most *Dpn* II restriction site in the cDNA reverse-transcribed from its corresponding mRNA. It thus surveys virtually all mRNAs in a sample, and provides direct sequence-based identification of transcripts. MPSS technology has been used for whole genome analysis of transcripts in *Arabidopsis* (Meyers *et al.*, 2004) and to study genes expressed during the plant defense response (Meyers *et al.,* 2007). Additionally, MPSS databases for four species *viz. Arabidopsis*, rice, grape and *Magnaporthe grisea*, the rice blast fungus have been created, which measure the expression level of most genes under defined conditions and provide information about potentially novel transcripts (Nakano *et al.*, 2006).

1.4.1.7 Next generation sequencing (NGS)

Next generation sequencing is the most recent technology that appeared in the last decade and has revolutionized the field of biological research. It has provided tremendous applications in genomics, transcriptomics, and epigenomics, including whole genome

genotyping, RNA sequencing (RNA-seq), mitochondrial genome sequencing, chromatin immunoprecipitation coupled to DNA microarray (ChIP-chip) or (ChIP-seq), detection of mutations and genetic disorders, clinical research, personal genome sequencing, and establishment of DNA libraries (Moustafa & Cross, 2016). Principally it is similar to previous generation sequencing (Sanger sequencing) except in the first sequencing generations, only one DNA fragment is sequenced while in NGS the process is extended to millions of fragments in parallel. Recently, NGS has been applied to study salt response in radish (Sun *et al.*, 2016), heat stress response in wheat (Kumar *et al.*, 2015b), cold stress response in sugar beet (Moliterni *et al.*, 2015), transcriptional changes in maize in response to water deficit, drought and salinity stress response in cotton (Xie *et al.*, 2015), etc.

1.4.1.8 Quantitative Reverse Transcriptase PCR (qRT-PCR)

Real time PCR is the advanced variant of traditional endpoint PCR which has substantially increased the applications and versatility of classical PCR method. It is a robust method with several applications like gene expression quantitation, pathogen quantification, molecular diagnosis, validation of data etc. It is based on the exactly same principle as that of traditional PCR comprising denaturation, annealing and extension. The detection of amplified products is achieved by two methods: 1) double-stranded DNA-intercalating dyes such as SYBR Green (Wittwer *et al.*, 2003) that bind and fluoresce when bound to double-stranded DNA, 2) fluorescent probes carrying a fluorescent reporter at one end and a quencher at the opposite end (Heid *et al.*, 1996) (Moustafa & Cross, 2016). Owing to its great sensitivity, reproducibility, and specificity, since its first documentation (Higuchi *et al.*, 1993; Heid *et al.*, 1996), it has been widely used in molecular biology experiments in humans (Alcoser *et al.*, 2011; Song *et al.*, 2012; Moreau *et al.*, 2013), plants (Exner, 2010; Delporte *et al.*, 2015) and animals (Cawthraw *et al.*, 2009; Pegels *et al.*, 2012; Golinelli *et al.*, 2016).

1.4.2 Proteomics

As transcriptome provides an overview of global gene expression, proteomics is a global technique which provides a complementary insight into protein profile of an organism. Proteomics is a term coined to encompass a field that attempts to understand the expression, function and regulation of the entire set of proteins, or 'proteome', encoded by an organism (Zhu *et al.*, 2003). In the last few years, the analysis of plant proteomes has drastically expanded which is significantly contributing to plant biology. For example, the assignment of proteins to particular organelles, the development of better algorithms to predict sub-cellular localization and occurrence of protein phosphorylation and degradation during plant defense. Biological variation and complexity in a situation involving two organisms in intimate contact, the existence of complete genome sequence databases for comparison are some of the intrinsic challenges in area (Quirino *et al.*, 2010). However, with constantly growing number of completely sequenced genomes and cheaper sequencing technologies, proteomics has been applied to understand various pathosystems till date (Gupta *et al.*, 2015; Zhang *et al.*, 2015). Additionally, in solving the mystery of plant-pathogen interaction, remarkable efforts have been done to analyze the secreted proteins in response to pathogen attack, in the last few years (Krause *et al.*, 2013; Delaunois *et al.*, 2015; Wang *et al.*, 2016).

1.4.3 Metabolomics

Metabolomics is the term used to describe the non-targeted identification and quantification of the metabolome defined as the quantitative complement of low-molecular weight metabolites present in a cell under a given set of physiological conditions (Kell *et al.*, 2005). It is a complementary technique to transcriptomics and proteomics. However, it also offers distinct advantages when attempting to understand a pathogen or dissect an interaction (Hollywood *et al.*, 2006). First, the metabolome more directly influences the phenotype than do either transcripts or proteins. Second, changes in the metabolome are often amplified relative to changes in the transcriptome or proteome (Tan *et al.*, 2009). Various techniques have been used to study metabolomes of plants and their fungal pathogens. GC-MS, Direct-infusion electrospray mass

spectrometry (ESI-MS) and nuclear magnetic resonance (NMR)-based fingerprinting are the most commonly used techniques for metabolomics. These have been utilized to elucidate plant-pathogen interactions from both plant defense and pathogen virulence point of view (Mazzei *et al.*, 2016; Webb *et al.*, 2016).

1.5 Objectives of the thesis

Considering the prevalence of Foc in soil without host, multigenic and complex resistance to Foc and failure of classical breeding approach to develop wilt resistant lines with genetic diversity, understanding the molecular mechanism beneath the interaction is important. Hence, the thesis work was initiated with the following objectives

1) Elucidating the colonization pattern of the pathogen in wilt resistant and susceptible chickpea cultivars
    - Tracing the pathogen *in planta* using CLSM
    - Assessing pathogen load using qPCR
2) Candidate gene expression analysis using qRT-PCR
    - Chickpea defense related genes
    - Foc virulence related genes
3) Analyzing gene expression profiles of wilt resistant and wilt susceptible chickpea genotypes upon pathogen infection using Serial Analysis of Gene Expression (SAGE)

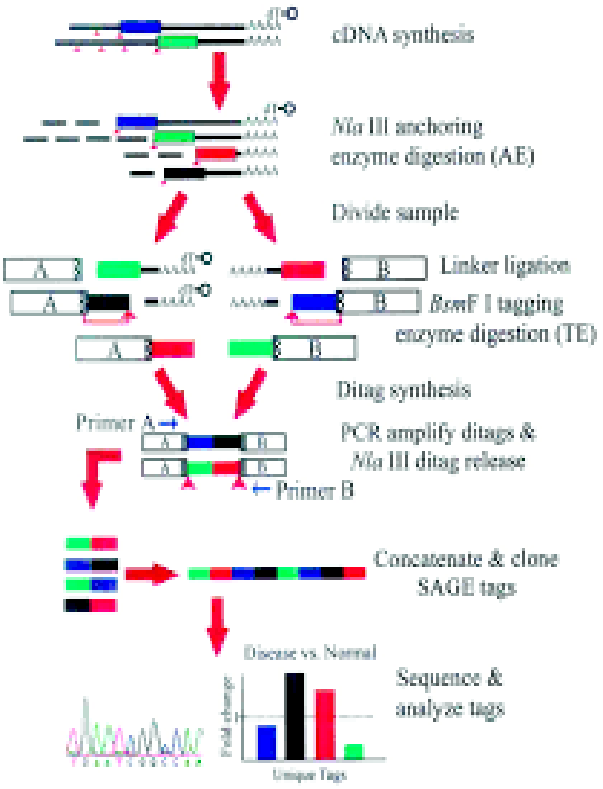
1.6 Organization of the thesis

Based on the above mentioned objectives, execution of experiments in a stepwise manner has led to the formation of this thesis. The thesis is organized into five chapters, the first being an introduction to the plant, the pathogen under study and a detailed review of the techniques currently available to study pathogen colonization *in planta*, expression analysis of candidate plant defense and pathogen virulence genes and the interaction transcriptome. Materials and methods of the experiments executed are described in the

second chapter. The third chapter elucidates results wherein two approaches namely CLSM and qPCR have been used to study progression and colonization of Foc in chickpea. qRT-PCR has also been used to study candidate gene expression analysis with respect to plant defense and pathogen virulence. Additionally, transcriptome analysis using SAGE is used to study the chickpea-Foc interaction transcriptome. The fourth chapter includes discussion of the results obtained and significant findings. In the fifth and the final chapter, the total work has been summarized and attempted to set directions for the future work. At the end of the thesis, literature used in this study has been included.

Chapter 2

Materials and Methods



2. Materials and Methods

2.1 Chickpea genotypes

The chickpea (*Cicer arietinum*) cultivars JG62 and Digvijay were obtained from Mahatma Phule Krishi Vidyapeeth (MPKV), Rahuri, Maharashtra state, India. JG62 (selection from germplasm) is highly susceptible to wilt, and shows features like twin podding and early maturity. It has medium sized seeds (15.8g/100seeds) (Gaur *et al.*, 2006). Genotype Phule G 9425-5 (later named as Digvijay) was developed at MPKV in year the 2005 from a cross of Phule G- 91028 x Bheema. It has high average yield (19,00q/ha; which is higher by 14.44% than Vijay and 17.81% than Vishal, respectively) and is highly resistant to Fusarium wilt compared to the check varieties Vijay and Vishal (http://www.ccrp.org/sites/default/files/chickpea___year_4__increasing_the_efficiency_of_chickpea_production.pdf). It has attractive yellowish brown colored bold seeds (24.0g/100 seeds) and is suitable for optimum sowing, well irrigated and late sown conditions. (Fig. 2.1)

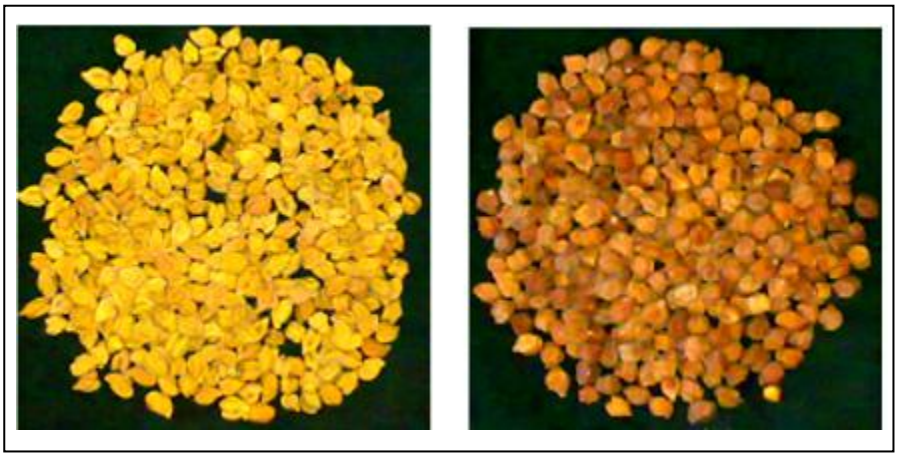


Fig 2.1 Seeds of chickpea cultivars JG62 and Digvijay

2.2 Fungal cultures

Fusarium oxysporum f. sp. *ciceri* (Foc) standard races 1 (NRRL 32153), 2 (NRRL 32154) and 4 (NRRL 32156) were obtained from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India. Foc isolates were characterized and classified according to the conventional method of race identification using cultivar specificity (Haware & Nene, 1982) at ICRISAT, India and confirmed by CABI Biosciences U.K. These cultures

were a kind gift from Dr. M.P. Haware, pathologist, ICRISAT, India. Further single spore cultures for each race were obtained and maintained on Potato Dextrose Agar (PDA) slants with regular sub-culturing. (Fig 2.2)

Fig. 2.2 Growth pattern of Indian Foc races 1, 2 and 4

2.3 Methodologies towards Foc colonization in chickpea

2.3.1 Plant infection assays

The seeds of chickpea cultivars JG 62(wilt-susceptible, JG) and Digvijay (wilt-resistant, DV) were surface-sterilized using 1% sodium hypochlorite solution and soaked overnight in sterile deionized water. They were wrapped in wet sterile muslin cloth till sprouting and transferred to surface-sterilized plastic cups containing autoclaved Soil Rite (mixture of 75% Irish Peatmoss and 25% horticulture grade Expanded Perlite; obtained from M/s Naik Krushi Udyog, Pune, India). The plants were grown for one week in growth chamber (14 h light/10 h dark, 22–25°C, 50–60% relative humidity) and inoculated by root clipping (Tullu *et al.*, 1998) with freshly prepared spore suspension (1×10^6 spores/ml) of Foc 2. Tips of tap and lateral roots were cut and the entire root system was dipped in spore suspension for 5 min. Plants mock-inoculated with sterile deionized water served as control. Thus four treatments *viz*. JG62 inoculated (JGI); JG62 control (JGC); Digvijay inoculated (DVI), and Digvijay control (DVC); comprising 10 plants per time-point at eight time-points, *viz*. 0 hpi (hours post inoculation), 16 hpi, 24 hpi, 2 dpi (days post inoculation), 4 dpi, 7 dpi, 14 dpi and 28 dpi in three replicates each were raised in growth chamber. The plants were lightly watered using autoclaved tap water every 2–3 days. (Fig 2.3)

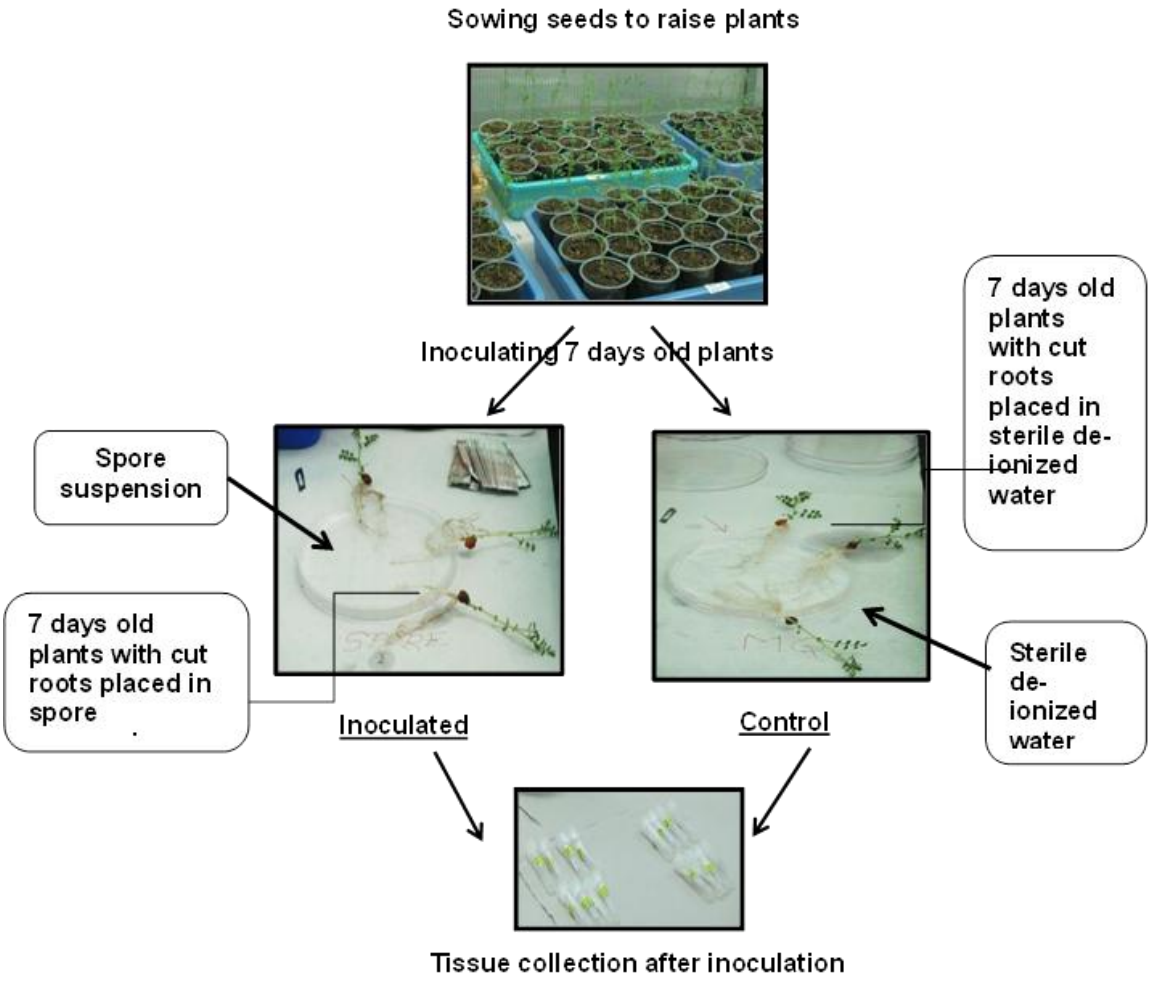


Fig. 2.3 Plant infection assay

2.3.2 Evaluation of disease symptoms and tissue collection

The plants were evaluated for morphological changes and development of wilting symptoms daily after inoculation. Tissues of all the four treatments were collected at all the eight time-points mentioned above. The time scale of the infection process was divided as: a) early stage (0 hpi to 7 dpi), b) middle stage (7 to 14 dpi), and c) late stage (14 to 28 dpi) based on the morphological symptoms observed. Two types of tissues were collected: whole roots and root fractions of approximately 2 inches in length, as well as two fractions of shoot till 2nd internode (Fig 2.4). The tissues were flash frozen in liquid nitrogen and stored at -80°C till further analysis.

Figure 2.4 Schematic presentation of root and shoot fractions

2.3.3 eGFP transformation of Foc 2

A kill curve was initially set up for Foc using the poisoned food technique (Sinclair & Dhingra, 1995) to determine the minimum inhibitory concentration of hygromycin B. For this, potato dextrose agar (PDA) was supplemented with 25, 50, 75 and 100 µg/ml of hygromycin B and the fungus was grown at 28°C for up to 21 days in dark in triplicates. For transformation of Foc 2, the pathogen was grown in potato dextrose broth (PDB) without hygromycin B at 26–28°C for 10 days with shaking at 180 rpm and conidia were harvested in sterile water. Spore count was recorded using a hemocytometer (McDonnell, 1962).

E.coli with plasmid pCBdeltaXCE containing *eGFP* cassette was a kind gift from Prof. Punekar, IIT,Mumbai (Dave & Punekar, 2011). Plasmid DNA was isolated and the cassette containing *eGFP* with *citA* promoter in plasmid pCBdeltaXCE was amplified using primers resin F (5'-AAAAAAGGTGACCCTCGAGCAAGTATGAGAGA-3') and resin R (5'-AAAAAATCTAGAACTAGTGGATCCCTGTCTGGTCTTCTAC-3') containing a restriction site of *BstE* II (G'GTGACC) to introduce it at the start of the cassette. This amplified cassette and pCAMBIA 1302 (containing *mGFP* cassette) were then double digested with *Xba* I (T'CTAGA) and *BstE* II (G'GTGACC) and the sticky end products were ligated using T4

DNA ligase (Fig. 2.5). Thus, a modified pCAMBIA 1302 vector with *mGFP* cassette replaced by *eGFP* cassette was transformed in LBA4404 strain of *Agrobacterium tumefaciens*.

To express *eGFP* in Foc 2, Agrobacterium mediated transformation was performed according to Mullins, Chen et al (Mullins *et al.*, 2001) with some modifications (Lagopodi *et al.*, 2002; Lakshman *et al.*, 2012). An overnight grown single colony of modified *A. tumefaciens* LBA4404 was transferred to a flask containing minimal medium supplemented with rifampicin (50μg/ml) and kanamycin (50μg/ml) for two days at 25°C with shaking at 180 rpm. At 0.6 OD (600λ), the cells were diluted in induction medium containing 200 μM acetosyringone and were allowed to grow for additional 5–7 hrs at 26–27°C at 200 rpm. Microconidia (1.0×10^6/ml) from a 10 days old Foc 2 culture were mixed in equal proportion with acetosyringone-induced *A. tumefaciens* cells (0.3 OD), incubated for 10 min at 22°C and plated on Hybond N+ membrane spread over the co-cultivation medium (Wang, 2006) containing 200 μM acetosyringone. The plates were incubated at 23°C for 48 to 60 hrs. The membranes were transferred to PDA selection medium containing 100 μg/ml hygromycin B and 200 μM cefotaxime, the latter antibiotic was used to kill *Agrobacterium*. Hygromycin B resistant (HygR) putatively transformed colonies of Foc 2 were observed following 7–9 days of incubation at 23–25°C. These colonies were transferred in the selection medium five times consecutively to confirm stability, growth and morphology of the transformants. Hygromycin resistant single conidial cultures of the transformants were preserved in 25% glycerol at −80 °C for long term storage and analysis.

2.3.4 Phenotypic characterization of wild type and transformed Foc 2

The Foc 2 transformants were transferred to PDA (containing hygromycin B) to observe colony morphology and cultural characteristics vis-à-vis wild-type. Slides for both were prepared using a drop of sterile water and hyphae for microscopic observations. Mycelial growth rate was evaluated in triplicates by placing a PDA plug of actively growing culture on PDA plate and incubating at 26–28°C for a week. The radial mycelial growth (RMG) was determined by measuring the length of four radii (each radius in one direction) daily.

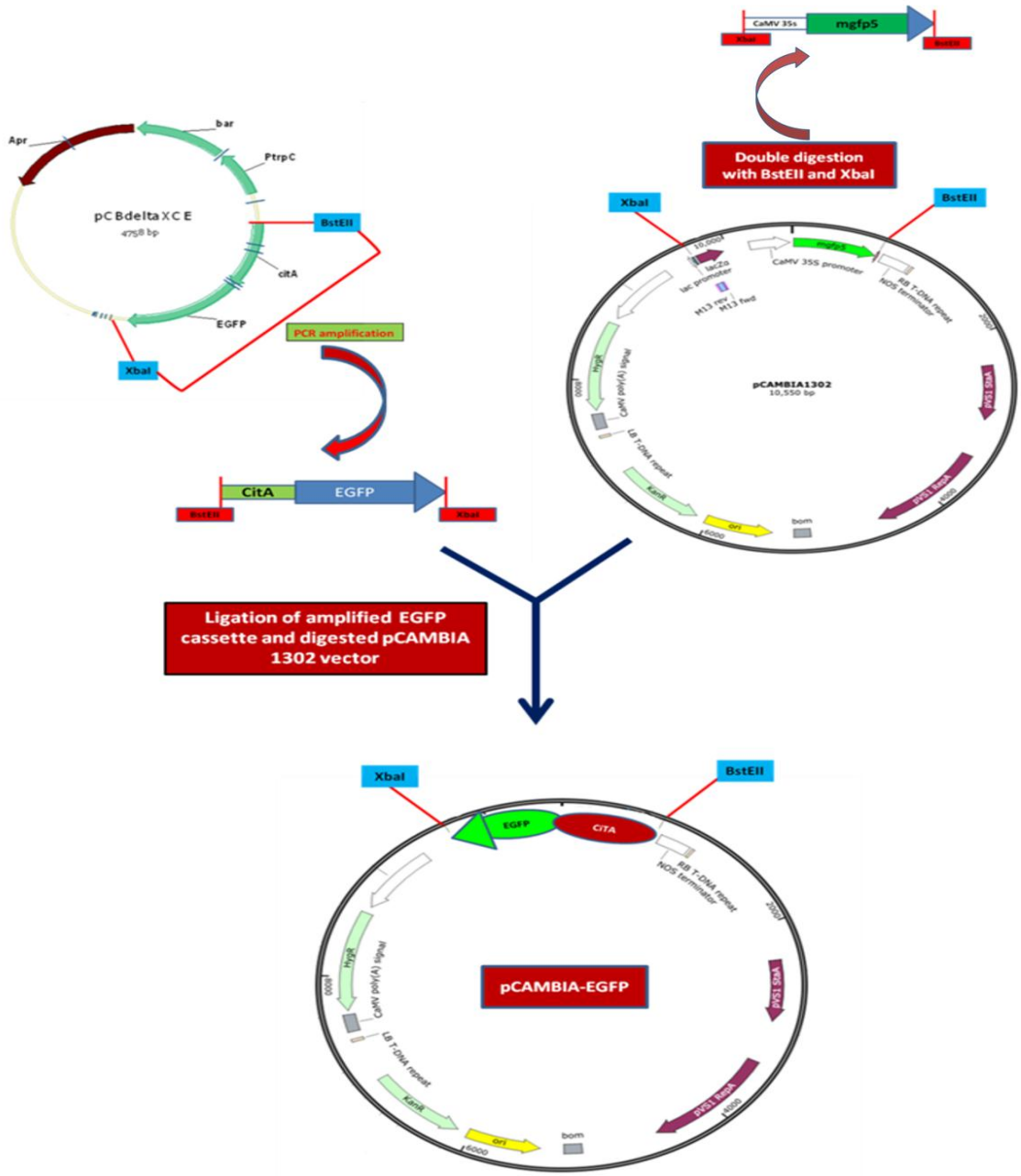


Fig 2.5 Steps in construction of modified pCAMBIA 1302 vector, pCAMBIA-EGFP

The radial growth rate (RGR) was calculated by slope of a linear regression of the mean colony radius over time (Jimenez-Fernandez *et al.*, 2013). In addition, pathogenicity of the transformants was evaluated in comparison to the wild type. Microconidial suspensions of wild-type Foc 2 and five transformants were used to inoculate the susceptible (JG62) and resistant (Digvijay) chickpea cultivars as described earlier. Three replicates of five seedlings

per cultivar per transformant as well as the wild-type were inoculated. The seedlings mock inoculated with sterile deionized water served as control.

2.3.5 Measurement of GFP fluorescence

GFP fluorescence in five selected eGFP transformed Foc 2 isolates was measured using luminescence spectrometer LS-5 (PerkinElmer, USA). The transformants were grown in triplicate at 26–28^0 C for 72 hrs in 10 ml PDB culture at 180 rpm. The cultures were centrifuged at 4000 rpm and the pelleted mycelia were crushed under liquid nitrogen and transferred to 5 ml extraction buffer (10 mM Tris pH 7.4, 1 mM$CaCl_2$). Mycelial debris was removed by another round of centrifugation. The resulting supernatant was assayed for fluorescence at 488 nm and 512 nm wavelengths for excitation and emission, respectively. Protein concentration was measured using Bradford assay with bovine serum albumin as standard (Bradford, 1976; Aboul-Soud *et al.*, 2004). Relative fluorescence units (RFU) were then normalized with respect to protein concentration.

2.3.6 Molecular characterization of eGFP transformed Foc 2

Five selected eGFP transformed Foc 2 isolates were grown in 100 ml PDB containing hygromycin B (75 µg/ml) at 28°C and 180 rpm for 4–5 days. Mycelial mass collected by filtration through muslin cloth was crushed to fine powder under liquid nitrogen and DNA was isolated using modified CTAB protocol (Cubero *et al.*, 1999). PCR was used to confirm the presence of hygromycin (*hph*) and *eGFP* genes in transformed isolates. Both transformed as well as wild-type Foc 2 DNA were used for PCR amplification with *hph* and *eGFP* specific primers viz. Hph F (5'-TC CTGCAAGCTCCGGATGCCC-3') and Hph R (5'-CGTGCACAGGGTGTCACGTTGC-3'); hph new F (5'-CTCGGACGAGTGCTGGGGCGT-3') and hph new R (5'-AAGCCTGAACT CACCGCGACGTCTG-3'); eGFP1F (5'-ACGTAAACGGCCACAAGTTC-3') and eGFP1R (5'TGCTCAGGTAGTGGTTGTCG-3') to confirm the presence of *hph* and *eGFP* genes in the transformants.

2.3.7 Microscopic monitoring of pathogen progression in chickpea plants

Another set of JG62 and Digvijay plants was inoculated with the specifically selected transformant (D4). The inoculated and control chickpea plants were sampled daily from 1 to 4 DPI and at a 2–3 days interval thereafter, up to 18 DPI. During each sampling, four plants were collected from each treatment. The entire surface of the tap and lateral roots of each plant was observed under a confocal laser scanning microscope (CLSM). Images were acquired by excitation at 488 nm argon laser (515–530 nm) for detection of fluorescence emitted by the pathogen using a Zeiss™ 710 CLSM system (Carl Zeiss Inc., USA). In addition, auto fluorescence of chickpea plants was assessed at wavelengths of 550–590 nm. The images were observed using a Zeiss Axio Observer™ inverted microscope with a 20×1.3 NA Plan-Apochromat objective.

2.3.8 *In planta* pathogen quantification

Three sets of primers *viz*. IV-SP & IV-ASP; Foc 1F & Foc 1R and Foc 3F & Foc 3R were designed to specifically amplify an internal portion of the 1.5-kb sequence characterized amplified region (SCAR) (GenBank accession no. AF492451) of *F. oxysporum* f.sp. *ciceri* (Jimenez-Fernandez *et al.*, 2011) using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). PCR conditions were optimized for each primer pair and all the reactions were performed at least twice. Also a positive control (Foc 2 DNA) and negative controls (DNA from un-inoculated chickpea root and no template DNA) were included. The amplification products were electrophoresed and visualized using a gel documentation system (Syngene, USA). The primer combination finally selected was Foc 3F & Foc 3R which amplified an 88-bp fragment (Table 2.1). To generate standard curves for qPCR assays, 10-fold dilutions of Foc 2 DNA (10 ng/µl) were prepared (1:1, $1:10^1$, $1:10^2$, $1:10^3$, $1:10^4$ and $1:10^5$) in sterile deionized water. The samples were amplified in triplicate using Foc 3F & 3R primers and three independent standard curves were established.

To determine pathogen load in susceptible (JG62) and resistant (Digvijay) chickpea plants, genomic DNA isolated from whole roots of inoculated plants was used as template for qPCR. Non-template control as well as DNA from un-inoculated chickpea root served as

negative controls. All qPCR amplifications were performed in triplicate using FastStart Universal SYBR Green Master mix (Roche, Germany) and 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). Each reaction contained 30 ng DNA, 0.33 µM of each primer and 15 µl SYBR Green master mix in 30 µl reaction. DNA concentration was adjusted to10 ng/µl before use in qPCR. The thermocycling profile consisted of initial denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 3 sec and 60°C for 30 sec. Following amplification, a melting dissociation curve was generated using a 60–95°C ramp in order to monitor the specificity of the primers. 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 using Linreg software (Ramakers *et al.*, 2003).

The amount of pathogen DNA was estimated in the roots of inoculated cultivars at different time points from the standard curves established earlier. This revealed the biomass of the pathogen at respective time-points. Similar procedure was followed for estimating the biomass of the pathogen in the fractions of chickpea root and shoot tissues (2 inch fractions from the root tip clipped for inoculation) at different time points. The lowest 2 inch fraction of the root was named as R1, while the topmost 2 inch fraction was named as R5. Similarly, S1 was the lowest shoot fraction, followed by S2. JG62 has very short root-length and root mass compared to Digvijay. Hence, the fraction R5 was absent in JG62, while R4 could be collected 7 DPI onwards. Whereas, in Digvijay R4 could be collected from 2 DPI onwards and R5 only at 28 DPI (Refer to Fig. 2.4 from section 2.3.2).

2.4 Methodologies in candidate gene expression analysis

2.4.1 Analysis of expression of plant defense related genes

To analyze the expression of defense related candidate genes of chickpea, total RNA was isolated from 100 mg ground chickpea root tissue from inoculated plants using the Spectrum Plant Total RNA isolation kit (Sigma-Aldrich, USA), followed by treatment with RNase-free DNase. 1 µg total RNA was reverse transcribed using a high capacity cDNA reverse transcription kit (Sigma-Aldrich, USA).

Table 2.1: Primer sequences specific to *Fusarium oxysporum* f.sp. *ciceri* 1.5-kb sequence characterized amplified region (SCAR) (GenBank accession no. AF492451) used for quantification of the pathogen in chickpea roots using qRT-PCR.

Primer Name	Sequence (5' - 3')	Amplicon size	Tann[a]	Tm[b]
IV-SP	TACGGTACCAGATCATGGCGT	160 bp	60 ºC	-
IV-ASP	CGCTTTCGATCGTGGCTATG		60 ºC	
Foc 1F	CATTCGATTCAGGCAAACCT	88 bp	60 ºC	75.3 ºC
Foc 1R	TTTCGACCTACGCCAACTCT		60 ºC	
Foc 3F	AAATGACTGCACCCATGAGAAA	88 bp	60 ºC	74.9 ºC
Foc 3R	TGAACCGTAGACCGGAAGGA		60 ºC	

[a]Annealing temperature (°C)

[b]Melting temperature (°C) at which a specific dissociation peak of increased fluorescence is generated in the melting curve analysis.

Gene specific primers (Table 2.2) were designed from conserved regions of plant defense related genes using the sequences available in NCBI database (database-Fabaceae) (http://www.ncbi.nlm.nih.gov). Glyceraldehyde 3 phosphate dehydrogenase (*GAPDH*) was used as a reference gene. QRT-PCR was performed as described earlier and the data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

2.4.2 Analysis of expression of Foc 2 virulence related genes

Similarly, the expressions of virulence related genes of Foc 2 *in planta* were analyzed by designing gene specific primers (Table 2.3) from the conserved regions of fungal virulence related genes using the sequences available in NCBI database (database-fungi). The specificity of the primers was determined by NCBI Primer BLAST. The elongation factor alpha (*EF1α*) was used as a reference gene.

Table 2.2 qRT-PCR primer sequences of defense related genes and *GAPDH* (as a reference gene)

Target gene	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Amplicon size
β-Glucosidase	CGGATCCAACGTGTCTAGGTGGA	GCACGGCATGTGCATGAGCA	87 bp
glucan-endo-1,3-beta-glucosidase	GAATCGGTTGGGTGAATGTC	TACGCGCGTTATCATACGAA	84 bp
glycosyltransferase	TGGGAAGCTCTTACGAATGG	CTGCAGCAGCCAGTAAATCA	75 bp
Phenylalanine ammonia-lyase	ACGCATGGTGGAAGAGTACC	CCGCAGCCACTTGAGATATT	77 bp
Superoxide dismutase	GCCTGGTCTCCATGGCTTCCA	CATTAGGATTGAAATGTGGTCCGG	86 bp
60 S ribosomal protein	CCCAAACCTAAGCCTGAAGCTCCC	TTGCGAAGTGGCTTCTGGACATC	80 bp
Chalcone synthase	GGCTGAGAACAACAAAGGTGCACG	GGACCACGAAATGTGACTGCAGTG	72 bp
Chitinase	GCCAGAGTCAATGTGGAAGCGGTG	TCTCGATGCTTCAGCATCTGGTTG	85 bp
Isoflavone reductase	CTGCTGCTAACCCTGAAAGC	CAACTTGCTTGATTGCTTTAACA	120bp
Histone protein A	CTGCTACAACCAAGGGAGGA	TTTCCGGCCTTTAGGAATCT	118 bp
Bet V I family protein	CACGATGTGCAAAACCATTG	CAGACACATGCCAATCATCA	73 bp
Metallothionin	GGAGCTGAAATGAGTGTTGCAG	TCACTTGCAGTTGCAAGGGT	84 bp
LRR	TGATTGGATACTGTGATGAAG	GTGATCTCTTAGTGTTCCATT	77bp
Pathogenesis related protein PR 10	CCCTTGGCTTTGCCACTTTT	GATTGCAGGCCCAAATGGAG	108 bp
Cytochrome P 450	TGGTTCAATGCTTTGATTGG	ACGAATCCATCTCGTTCTGC	70 bp
GAPDH	CCAAGGTCAAGATCGGAATCA	CAAAGCCACTCTAGCAACCAAA	65 bp

EF1α primers were designed to specifically amplify the Foc 2 cDNA and not the chickpea cDNA. QRT-PCR was performed as described earlier and the data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Table 2.3: qRT-PCR primer sequences of the virulence related genes and *EF1α* (as a reference gene)

Target gene	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Amplicon size
Chitin synthase	GGCACAAGGATGAACA ACTGGGA	GGAGCTGGATTGAGCA TAGGGATC	99 bp
Cell wall protein	GGCAAGCCTTACACCAT CCGCTAC	TGTAGTCAGAGAGATC ATCGGAGG	91 bp
Mitochondrial carrier protein	CCGCGTTGAGATGCAGA GCAAGAA	GACGCCGTTGGTCTCG TAAATGTAC	97 bp
Glucanosyltransferase	GGCTACATCTGCGGCCA AGACAAG	GAGCACATGCTGTAGG CACCATAG	89 bp
G protein β subunit	GGTCGACCGATAGGAG GCACC	GTGGACCTTGTTGGTG GTATAGGC	86 bp
Xylanase	CCGGCGACGATGTGATG CGA	CCCAGGTGTGGTTGCT CGCT	82 bp
Pectate lyase	GCGGTGCTTTCCATGCT AGCG	GACGAGCTTTCCGTAG TCTTCGGC	98 bp
Polygalacturonase	CTCGCCACTCGACTTGA CCTGG	TGAAGCTGTGGTCTGC CCAGTAG	101 bp
EF1α	AGCTCGGTAAGGGTTCC TTC	TCCAGAGAGCAATATC GATGG	93 bp

2.5 Methodologies in transcriptomic study of chickpea-Foc interaction

2.5.1 Construction of Long SAGE libraries

Total RNA was isolated from 100 mg chickpea root and shoot tissue from both resistant and susceptible cultivars, separately for each time-point using the Spectrum Plant Total RNA isolation kit (Sigma-Aldrich, USA) and was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Total RNAs from all the 11 time-points of individual Foc races (1, 2 and 4) were normalized using the chickpea *Actin* as a reference gene. Based on this normalization, RNAs from all these time-points were pooled for control and pathogen challenged plants, separately for construction of four LongSAGE

libraries (JGC, JGI, DVC and DVI) using the I-SAGE^{TM} Long Kit (Invitrogen, USA) (Fig.2.6). The ditags obtained at the endpoint of LongSAGE protocol were sequenced using the 318 chip on the Ion-Torrent PGM platform (Genotypic Technologies, Bangalore, India). (Figs. 2.6 and 2.7)

2.5.2 Transcriptome analyses

Following the Ion-Torrent PGM sequencing, the raw data in the fastq format was processed with the fastqc toolkit to remove shorter and low-quality reads. The high quality reads were used for extracting the ditags. A ditag is defined as the stretch of nucleotides flanked between two 'CATG' sequences, having length of 32-38 bp. The ditags thus obtained were split into individual tags of 16-19 bp using in-house developed Perl scripts. Reverse complements of tags ending with 'CATG' were also considered in the final dataset of tags. Tag mapping was assigned to the transcript as a measure of its expression value, while the frequencies of all 'selected match' tags mapping on single position were summed up and the frequency values thus obtained were assigned to the transcript. The tags having frequency of more than one were considered for further analysis. This entire tag extraction and mapping procedure was performed separately for both plant and fungal tags using the respective reference transcriptomes. The databases considered for mapping are indicated in Table 2.4.

Table 2.4: Databases used for mapping SAGE tags in transcriptome analysis

SAGE tags from	**Reference transcriptomes used for mapping (Designation)**	**Source**
Chickpea	*Cicer arietinum* L (CA)	NCBI
	Medicago truncatula (MT)	Ensembl Plants
	Glycine max (GM)	NCBI
	Cajanus cajan (CC)	NCBI
	Lotus japonicas (LJ)	NCBI
Fusarium oxysporum	*Fusarium oxysporum* (FO)	Ensembl Fungi
	Fusarium graminearum (FG)	Broad Institute

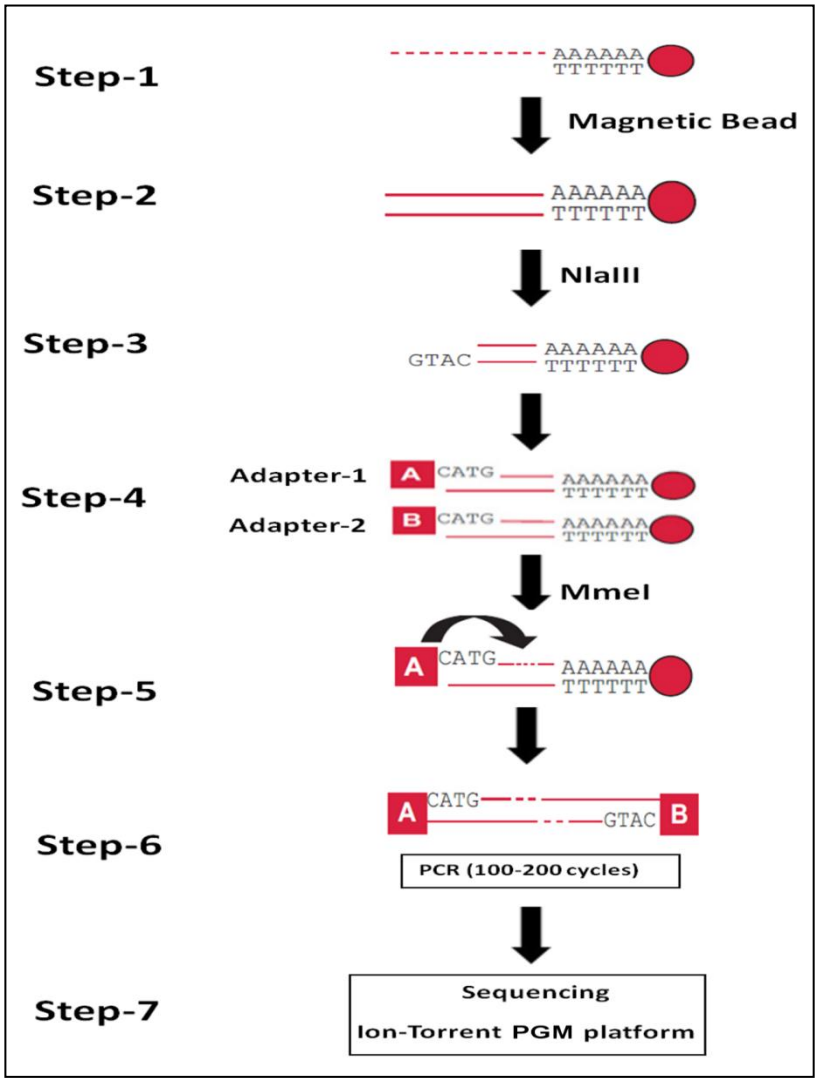


Figure 2.6: Schematic representation of LongSAGE library construction protocol. The Figure shows seven consecutive steps for the construction of LongSAGE library starting with mRNA. mRNA captured on magnetic beads is reverse transcribed into cDNA. An anchoring enzyme *NlaIII* cleaves cDNA generating 4 bp overhang 'CATG'. Adapters A and B are ligated to this cleaved cDNA pool while another restriction enzyme *MmeI* cleaves cDNA generating an adapter-tag pool in supernatant. These are then ligated to form ditags which are PCR amplified and sequenced using an Ion-Torrent platform.

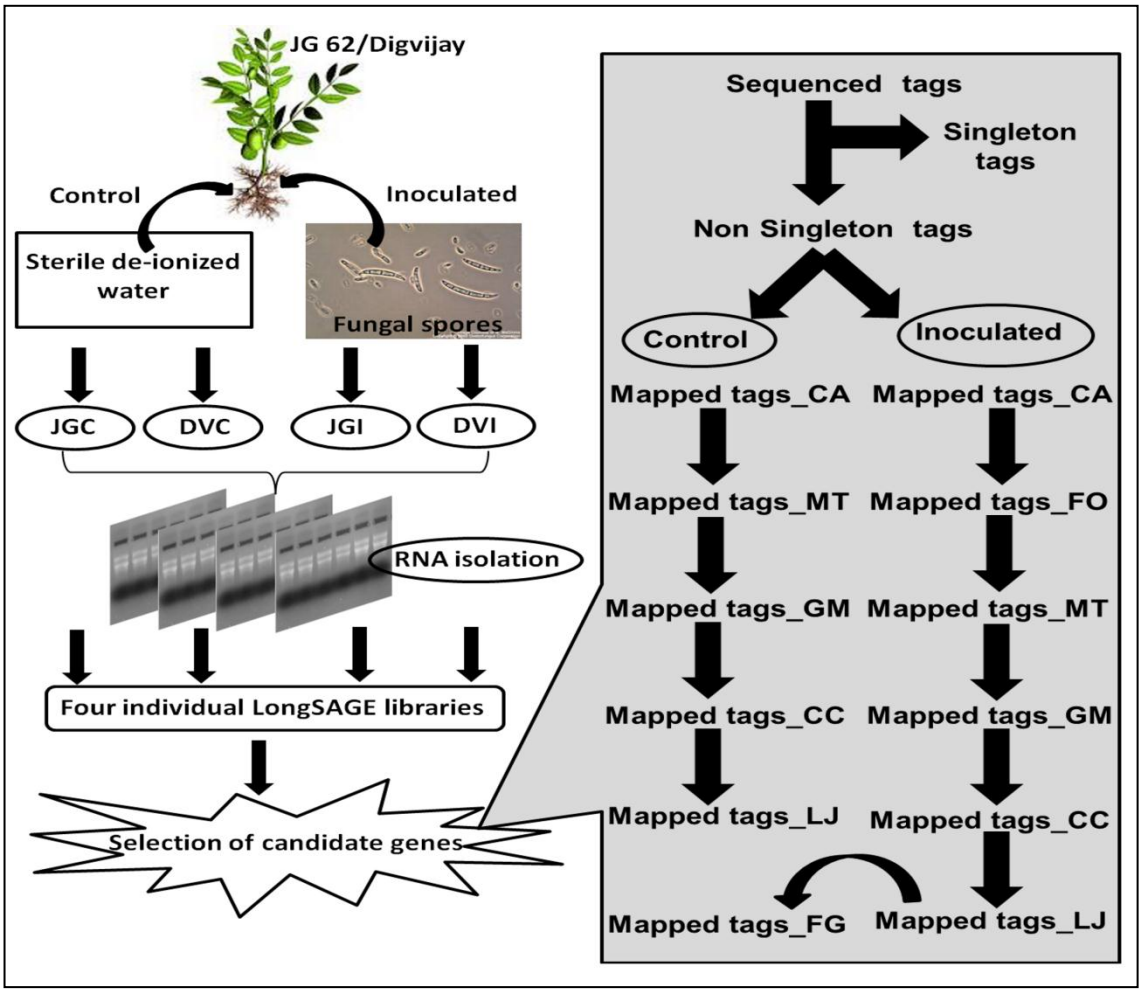


Figure 2.7 Experimental design of the present study. Fusarium wilt susceptible (JG62) and resistant (Digvijay) cultivars of chickpea were raised in greenhouse. Seedlings treated with sterile deionized water (un-inoculated plants) served as control, while those inoculated with Foc 1, 2 and 4 served as treated. Total four SAGE libraries were constructed for each treatment: susceptible control (JGC), resistant control (DVC), susceptible inoculated (JGI) and resistant inoculated (DVI). Analysis of all four libraries with the respective available transcriptomes led to the identification of candidate chickpea and Foc genes. CA- *Cicer arietinum*; MT- *Medicago truncatula*; GM- *Glycine max*; CC- *Cajanus cajan*; LJ-*Lotus japonicas*; FO- *Fusarium oxysporum*; FG- *Fusarium graminearum*.

2.5.3 Screening of Differentially Expressed Genes (DEGs)

After mapping, raw tag counts for the individual libraries were obtained. These raw counts were normalized and analyzed for differential expression (DE); resulting into four datasets [Differential Gene Expression (DGE) sets] namely DE_JGC_JGI, DE_DVC_DVI, DE_JGC_DVC and DE_JGI_DVI. For example, DE_JGC_JGI represents the DEGs between JGC and JGI libraries. The trimmed mean of M-values normalization method i.e. TMM (Robinson & Oshlack, 2010) was used for normalization of all the four datasets using the edgeR package (Robinson *et al.*, 2010). Gene expression analyses were performed using the combined approach of the Audic and Claverie test (ACT) and Chi-square test (Chi) (Audic & Claverie, 1997). The p-value of significance obtained was adjusted to reduce the false discovery rate (Benjamini & Hochberg, 1995). The cutoffs used for significantly expressed genes were p-value ≤ 0.05 and False Discovery Rate (FDR) ≤ 0.05. The normalized values were used to find the log2fold change (LFC) for each transcript in all the four datasets. The DEGs were obtained by comparing all the four libraries as shown in Table 2.5. The up-regulated and down-regulated genes were selected by using cut-off of 2 fold (LFC ≥ 1). The DEGs from plant species were processed for gene enrichment analysis using the Mercator tool (Lohse *et al.*, 2014). Further, MapMan analysis (Thimm *et al.*, 2004; Usadel *et al.*, 2005) was performed for pathway enrichment of the DEGs. Cluster analysis of the DEGs was performed by employing the Euclidean distance method over a complete linkage using the Multi-experiment viewer (Saeed *et al.*, 2003). The DEGs from pathogen i.e. *Fusarium* species were processed for gene enrichment analysis using Blast2GO (Conesa & Gotz, 2008).

2.5.4 Bioinformatics analysis of chickpea and Foc DEGs

About 400 DEGs of chickpea were obtained across comparisons of all the four datasets and used for BLASTX analysis using the National Center for Biotechnology Information (NCBI) nr database to retrieve the corresponding protein sequences based on E-value and bit score. The best assigned clusters of orthologous groups (COGs) for these proteins were selected from *Glycine max* and used them for protein-protein interactions (PPI) analysis using the Search Tool for Retrieval of Interacting Genes/Proteins (STRING) database (version 10.0, http://string-db.org) (COG mode).

Table 2.5: Differential gene expression analysis. Differential Gene Expression (DGE) Sets represent comparisons among the four SAGE libraries. For example, DE_JGC_JGI shows DEGs up- or down-regulated in JGI compared to JGC. CA stands for *Cicer arietinum* (chickpea) and FO stands for *Fusarium oxysporum*.

DGE sets	**No. of differentially expressed genes**	**Upregulated genes**	**Downregulated genes**	**Significantly expressed genes in only one Library**
DE_JGC_JGI (CA genes)	3816	1230	1647	695 (JGC) , 256 (JGI)
DE_DVC_DVI (CA genes)	3429	1390	1100	312 (DVC), 349 (DVI)
DE_JGC_DVC (CA genes)	2987	937	1092	77 (JGC), 68 (DVC)
DE_JGI_DVI (CA genes)	3622	1694	1106	480 (JGI), 904 (DVI)
DE_JGI_DVI (FO genes)	18	0	18	533 (JGI), 5 (DVI)

The assigned COG descriptions were obtained from Egg NOG 4.5 database (http://eggnogdb.embl.de/#/app/home). The interactions, only based on co-expression and experiment conditions and had a confidence score of at least 0.7, were used to construct the network, displayed using Cytoscape (version 3.3.0) (http://www.cytoscape.org/). Annotated genes of Foc were translated using BLASTX and analyzed using InterProScan5 (Jones *et al.*, 2014) and SignalP 4.1 (Petersen *et al.*, 2011). To supplement these analyses, Pathogen–Host Interaction (PHI) database (version 4.0, http://www-phi4.phibase.org/) (Winnenburg *et al.*, 2008) was used to reveal the involvement of these genes in pathogen virulence.

2.5.5 Validation of sequencing data

The results of LongSAGE transcriptomics and comparative analysis were validated using randomly selected eight plant and eight pathogen genes, respectively by verifying their expression patterns. Root and stem tissues, challenged by Foc 2 and sampled at eight time-

points along with the control plants at each of the corresponding time points, were used for this analysis. Three independent biological replications were performed for inoculated and control plants. Primer design, (Tables 2.6 and 2.7) reverse transcription and qRT-PCR were conducted as described in section 2.3.8. The amount of target gene transcript was normalized for plant genes over the constitutive abundance of chickpea *GAPDH* while for pathogen genes over the abundance of Foc *EF1α* at individual time-points, respectively.

2.5.6 Accession numbers

LongSAGE sequencing data are available in Gene Expression Omnibus in NCBI under accession numbers GSM2301186, GSM2301187, GSM2301188 and GSM2301189.

Table 2.6: QRT-PCR primer sequences of the defense related genes of chickpea and *GAPDH* (as a reference gene) used for LongSAGE validation

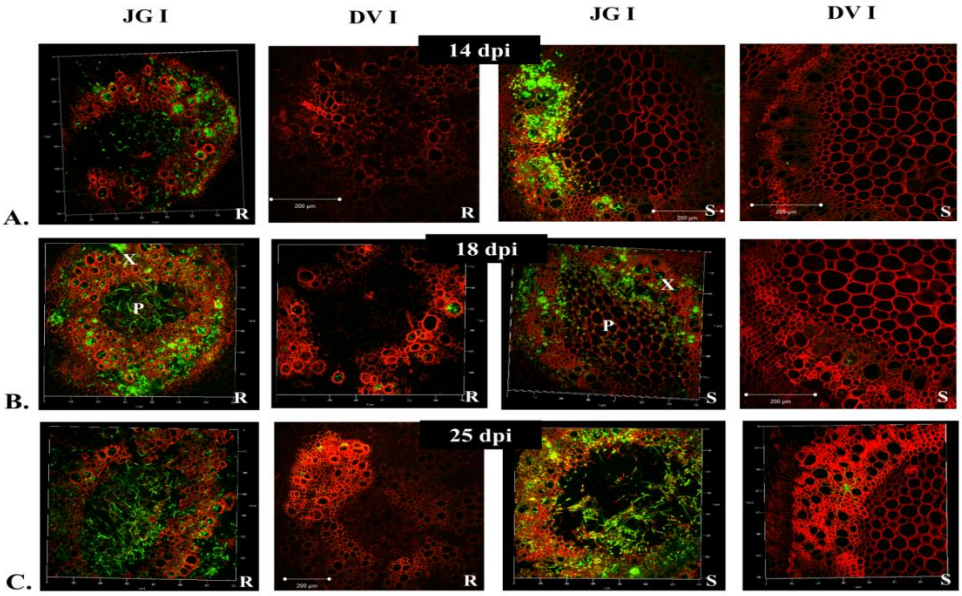
Target gene	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Amplicon size
Mitogen Activated protein kinase	GGAAGACGTGCGAGAGCTTA	AATCCTGTTGGCTCTGCTCC	93 bp
14-3-3 like protein	TGTGCTGTCTTTGTAAGACTCCT	AAAGGGCATGTCACCTTGCT	89 bp
UDP-glycosyltransferase	GTTGGAAGAGCCGTTTGAGC	TAGCAACATCAACGGGCCAT	98 bp
Auxin binding protein ABP19a	GGCTACCACTGCAAACCTCT	TGCGGCGTTGAATGTGTTTT	96 bp
Linoleate 9S-lipoxygenase	CCCGGTGGTATAATCGGTGG	CCCAAGAAAGAAGTGGCGGT	83 bp
Cystein protease	ATGTGCGGAGGGCTTACAAA	TTTGGGTCTGGTGGTTCAGG	85 bp
DELLA protein	GCAGGAAGCGAATCACAACG	CCAACGAGTCAAACAGCGTC	86 bp
NAC transcription factor	TCCTGTTGGCTTCCAATAACCA	GGTAGAGCTTTGGCTGAGGG	96 bp
Glyceraldehyde phosphate dehydrogenase	CCAAGGTCAAGATCGGAATCA	CAAAGCCACTCTAGCAACCAAA	93 bp

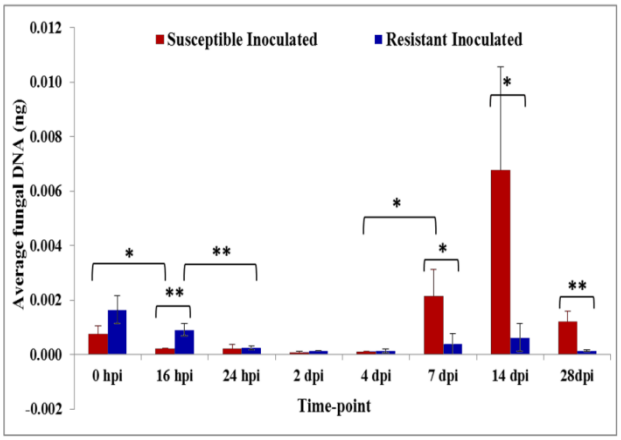
Table 2.7: QRT-PCR primer sequences of the virulence related genes of Foc and *EF1α* (as a reference gene) used for LongSAGE validation

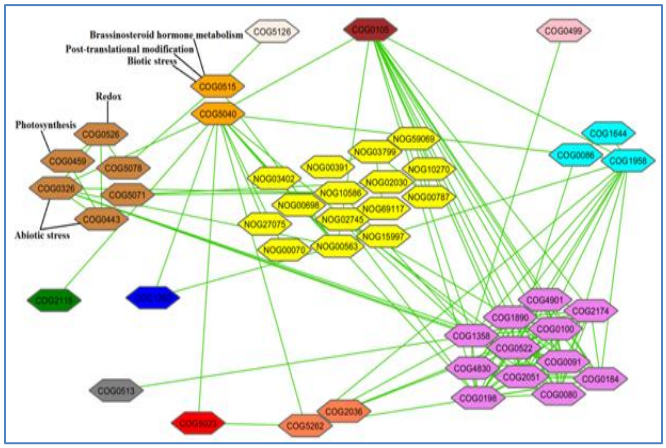
Target gene	**Forward primer sequence (5' to 3')**	**Reverse primer sequence (5' to 3')**	**Amplicon size**
Class V Chitin synthase	GGCCTACATCAACTCTGCAAC	GGGCATTATAACGACCGTCTCAA	96 bp
Ubiquitin fusion protein	CAACCCCAATTCGCACCATC	CCGTGAGGGTCTTGACGAAA	96 bp
Chitin synthase 4	CGGATTATGGGGGAAACCATGT	TTGGCCTCAAGAATGTTACCCCTT	99 bp
Worronin body major protein	ACCCGCTCCCCAATTCTATT	GGTTGTACTGAGGGCGAGAT	86 bp
ABC transporter CDR4	GATTCACCCCTTAACCCGCA	CTGTCGAAACCCAGAGCCAT	99 bp
ATP synthase	CAATGTTTGCATGCCCGTCT	CGTTGACACCAGCGAAGATG	98 bp
B-glucosidase	CTGTTCACCGAGTGCATCCT	AAATCACCGTTGCCATTGCC	91 bp
60S ribosomal protein	GTGCCCTCAAGTACGTCGAA	ATTGACGGAGTTCCCAGCAG	93 bp
EF1α	AGCTCGGTAAGGGTTCCTTC	TCCAGAGAGCAATATCGATGG	93 bp

Chapter 3

Results







3. Results

3.1 Colonization of Foc in chickpea using CLSM and qPCR

3.1.1 Effect of Foc 2 inoculation on susceptible and resistant chickpea cultivars

Seven days old plants of wilt-resistant (Digvijay) and wilt-susceptible (JG62) chickpea cultivars were individually inoculated with fungal spores (DVI and JGI, respectively); while the control plants were mock-inoculated using sterile deionized water (DVC and JGC, respectively).Wilting symptoms started appearing at about 7 dpi in JGI and intensified with time. More than 90% of JGI plants were almost dead by 28 dpi, while the remaining plants were severely wilted. On the contrary, all the DVI plants were healthy even beyond 28 dpi (Fig. 3.1) and till maturity. Similarly, the control plants of both the cultivars (JGC and DVC) were healthy throughout the experimental period.

3.1.2 Generation and characterization of transformants

The minimum inhibitory concentration of hygromycin B for wild-type Foc 2 was 75µg/ml. Hence, the stability of eGFP Foc 2 transformants was confirmed by serially transferring them five times to fresh selection medium containing 75µg/ml of hygromycin B. The presence of hygromycin B phophotransferase (*hph*) and *eGFP* genes in the transformants was confirmed by PCR amplicons of sizes 495 bp(Hph F and R, internal) and 1 kb (Hph new F and R, full-length as mentioned in Materials and Methods chapter) for *hph* and 546 bp for *eGFP*, respectively; while no amplification was observed in wild-type Foc 2 (Fig. 3.2). These transformants and the wild type Foc 2 also showed nearly similar final radial mycelial growth (RMG) and radial growth rate (RGR) values (Table 3.1). No morphological changes in size or shape of vegetative structures were observed. The transformants retained the colony morphology characteristics of the wild type including white cottony growth of aerial mycelia. Similarly, virulence of all the five transformants was comparable to that of the wild type. The level of GFP fluorescence in the five transformants was variable, while the wild-type Foc 2 showed negligible fluorescence. The transformant ‘D4’ showed the highest fluorescence (60 RFU/mg of protein) (Fig. 3.3), hence was chosen for further studies.

Fig. 3.1 Phenotypic changes in chickpea cultivars, JG62 and Digvijay, control (JGC & DVC) and Foc 2 inoculated (JGI & DVI), 14 days and 28 days after inoculation (DPI). Red arrows indicate typical wilting symptoms in susceptible inoculated cultivar (JGI).

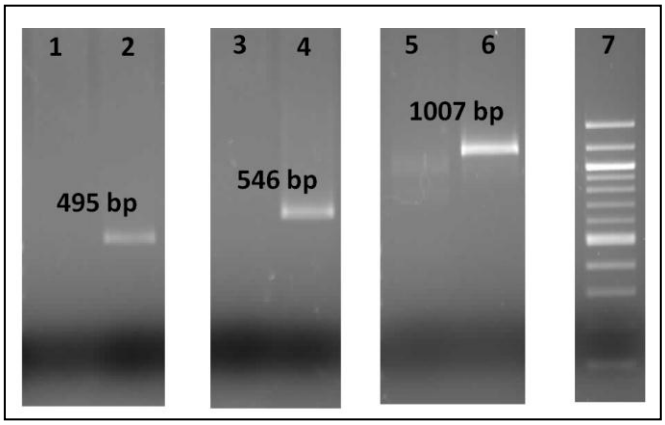


Fig 3.2. PCR amplification of wild type and D4 transformant of Foc 2. Lane 1- wild type Foc 2 DNA with no amplification, Lane 2- D4 DNA with *hph* amplification (495 bp), Lane 3- wild type Foc 2 DNA with no amplification, Lane 4- D4 DNA with *eGFP* amplification (546 bp), Lane 5- wild type Foc 2 DNA with no amplification, Lane 6- D4 DNA with *hph* amplification (1007 bp), Lane 7–100 bp ladder.

Table 3.1: Phenotypic characterization of *eGFP* transformants of Foc

***F. oxysporum* f.sp. *ciceri* race 2** **Wild type and Transformant**	**Mycelial growth**	
	RMG_F (mm)	**RGR (mm/h)**
Wild type	20.2	0.15
A1	18.9	0.13
A3	19.3	0.13
C2	19.4	0.13
C4	19.1	0.13
D4	18.7	0.13

A1, A3, C2, C4 and D4 are the isolates transformed with the *eGFP* gene

RMG_F: Radial mycelial growth final, assessed by the average value of the fungal colony radius reached after 9 days growth at 25°C under light.

RGR: Radial growth rate; calculated by slope of linear regression of the mean colony radius over time. Each value is the mean of three replicates (petri dishes).

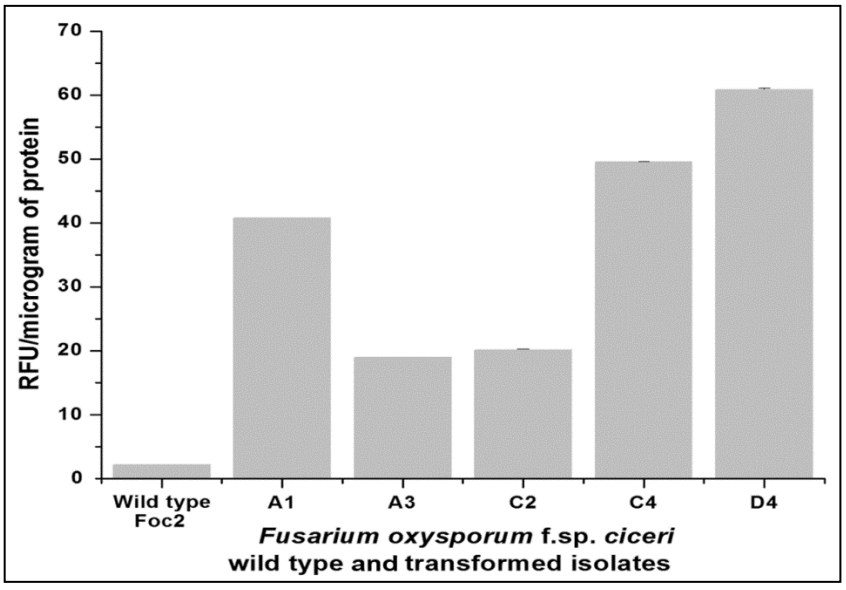


Fig. 3.3 GFP fluorescence in protein extracts of selected Foc 2 transformants. Fluorescence values were expressed as relative fluorescence units (RFU) and normalized against the protein concentration of each sample.

3.1.3 Microscopic evaluation of pathogen infection in chickpea cultivars

Uniform green fluorescence was observed in mycelia and microconidia of the isolate D4 under Confocal Laser Scanning Microscope (CLSM) (Fig. 3.4A). Pilot experiments were performed to study the colonization patterns of D4 in JGI and DVI roots. In early stage of infection, colonization on root surface was observed in both the cultivars by forming the primary mycelia at the root apex (Fig. 3.4B–D). Surface colonization was followed by direct penetration of hyphae into epidermal cells without forming any specialized structures (Fig. 3.4B Inset). The pathogen then entered root cortex region by 2 dpi (Fig. 3.4E). It readily reached the vascular region of lower roots of JGI within 1–3 dpi (Fig. 3.4F and G), whereas it remained restricted within the cortex region in DVI (Fig. 3.4H).

Based on these pilot studies, in-depth analysis of pathogen infection in JGI and DVI was performed throughout the disease progression. During early infection stages (up to 4–6 dpi), both the cultivars showed surface colonization and entry of the pathogen in lower roots (Fig. 3.5A and B). However, substantial colonization of vascular region was thereafter observed in lower and middle root zone of only JGI at 8 dpi (Fig. 3.5C). Further, the appearance of wilting symptoms in JGI was marked with heavy colonization of lower, middle and upper root zones along with the lower stem region at 10–12 dpi (Fig. 3.5D). However, in DVI initially the pathogen was restricted to root cortex region (Fig. 3.5C and D) and reached xylem vessels very late (by 18 dpi) and that too in very less numbers. In JGI the pathogen reached as far as the fifth internode by 14 dpi (Fig. 3.6A). By 25 dpi, both the root and the stem of JGI were heavily colonized resulting in disruption of normal architecture, complete wilting and death of most of the plants; while root and shoot architectures of DVI plants were nearly normal (Fig. 3.6B and C). As depicted in Fig. 3.7A–C, these differences between JGI and DVI further intensified by 28 dpi; where JGI showed exhaustive colonization of both root and shoot tissues, while minimal fungal colonization was observed in DVI (Fig. 3.7D–G).

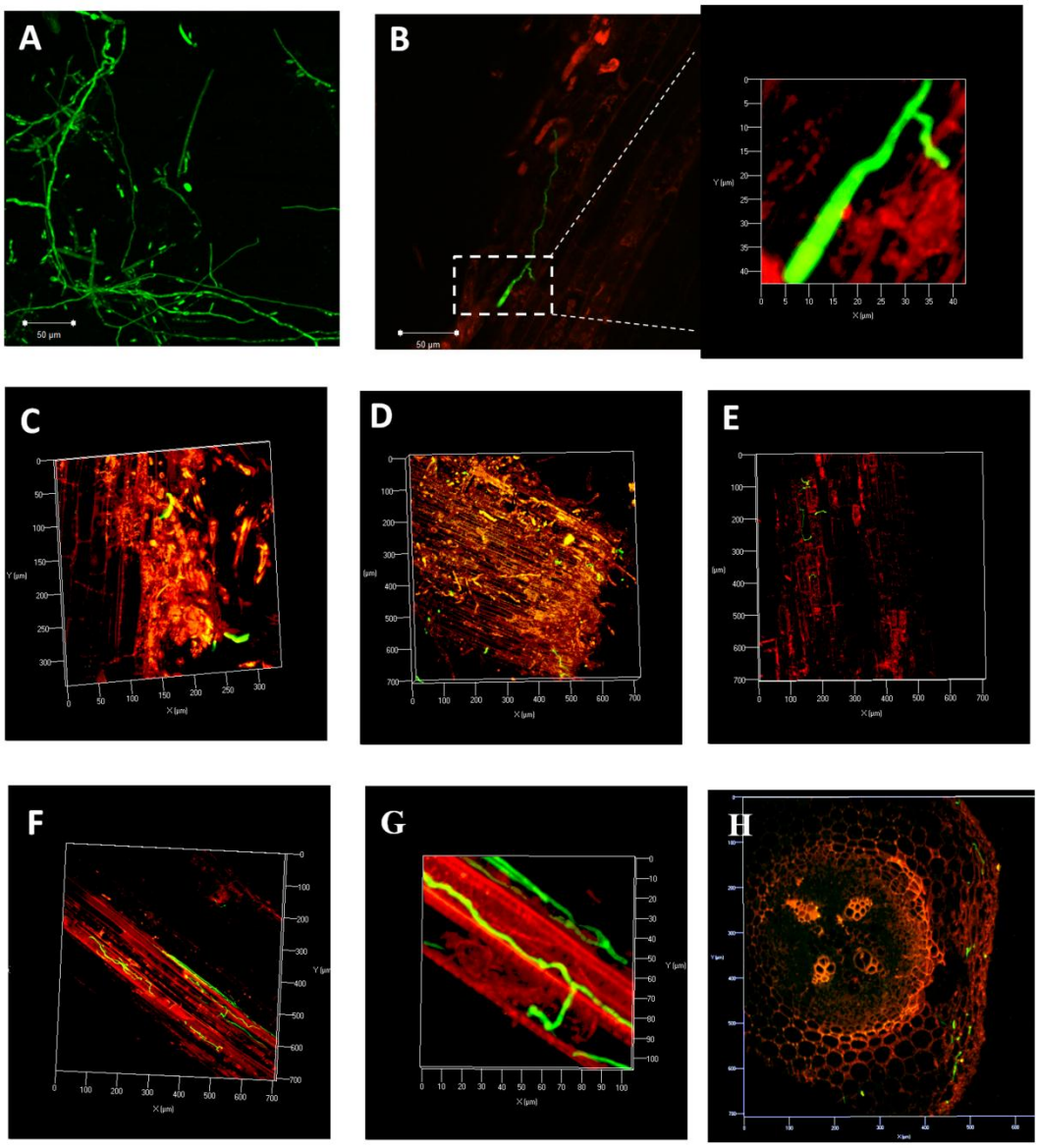


Fig 3.4. Early stages of chickpea root colonization by Foc 2 marked with eGFP in susceptible (JG62) cultivar by Confocal Laser Scanning Microscopy. A. Uniform expression of eGFP in hyphae and spores of transformed isolate D4. B. Germinating conidium with primary mycelium in contact with root apex at 24 hpi. C-D. Initial hyphal colonization in lower root zone at 2 dpi. E. Intermediate root zone showing hyphal colonization extending from epidermis to cortical cells at 2 dpi. F-G. Vascular region of root colonized at 3 dpi. H. Fungal colonization in cortex region of DVI.

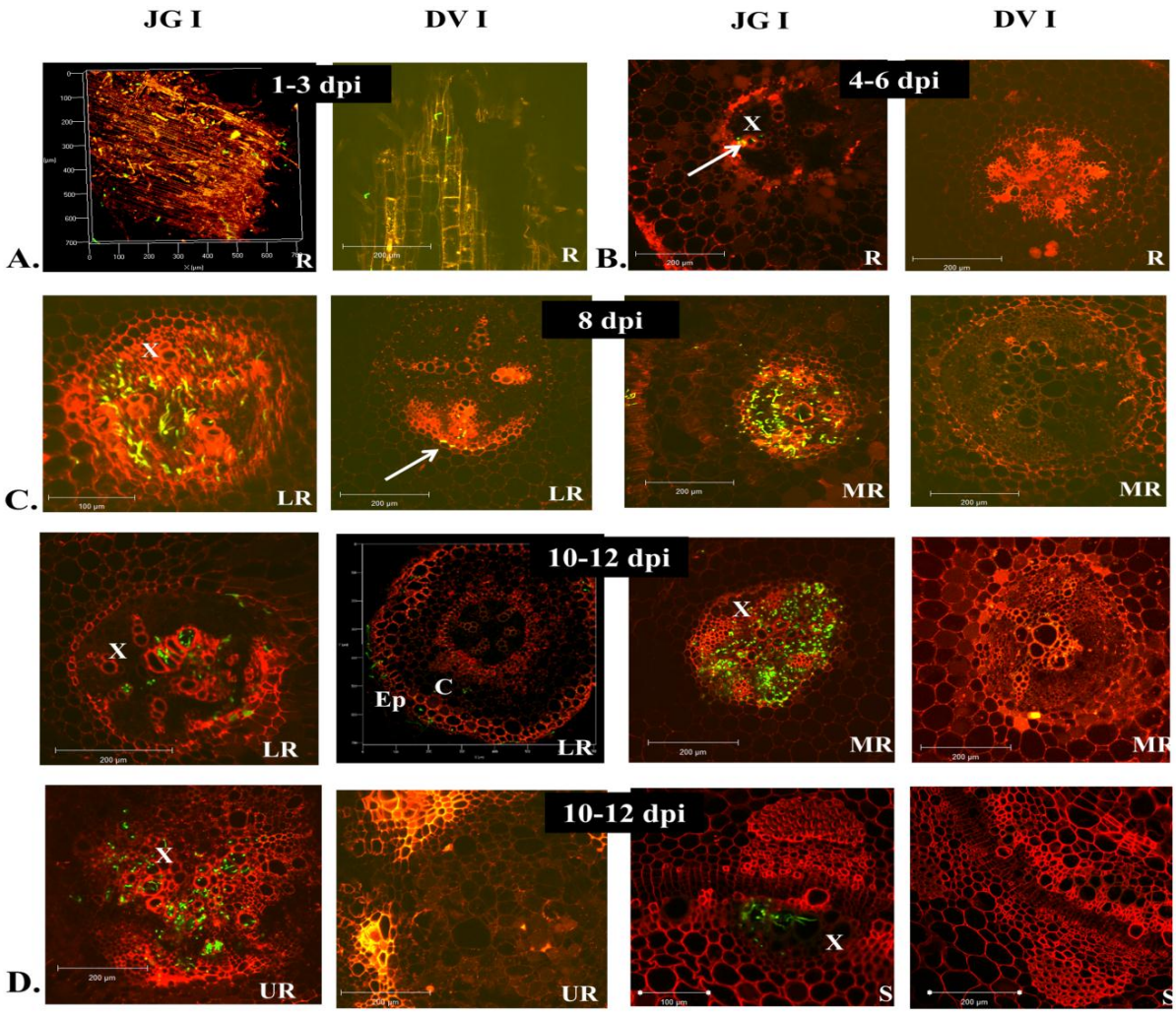


Fig 3.5. Temporal pattern of colonization of Foc 2 in both susceptible (JG62) and resistant (Digvijay) cultivars of chickpea. R- Root, S- Shoot, LR- Lower Root, MR- Middle Root, UR- Upper Root. A. (1–3 dpi) Surface colonization in both susceptible and resistant cultivars. B. (4–6 dpi) Transverse sections (TS) of root depicting entry of fungus in vascular bundle only in JGI. C. (8 dpi) Transverse sections of lower and middle roots showing increasing amount of fungus in vasculature of JGI while very less fungal mass seen in DVI only in LR. D. (10–12 dpi) Transverse sections of all lower, middle, upper roots of JGI reveal higher fungal mass in vascular tissue with few fungal mycelia reaching even stem. However, less fungal mass can be seen till cortex region of TS of LR in DVI with remaining TSs of MR, UR and Stem are clear.

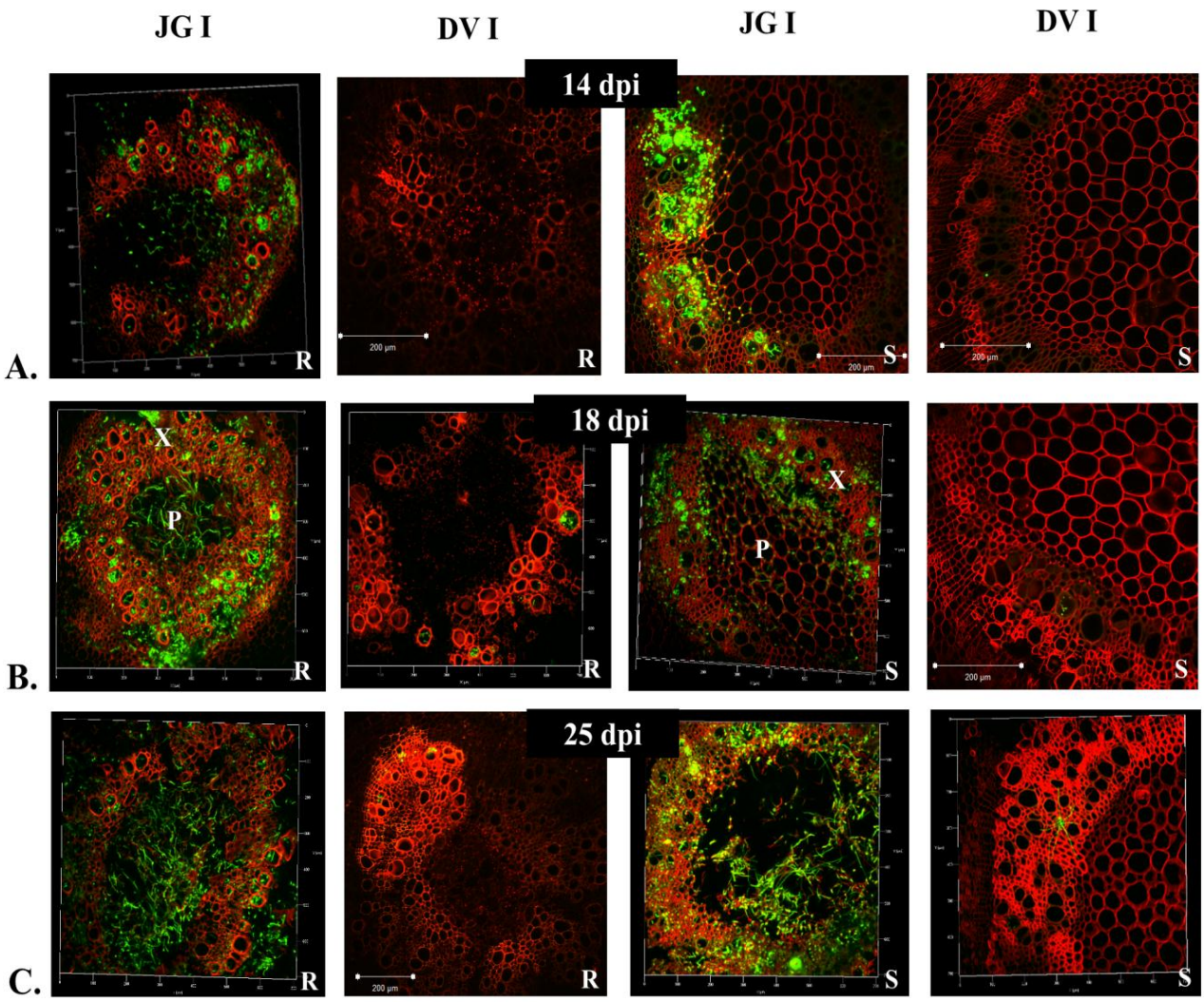


Fig 3.6. Temporal pattern of colonization of Foc 2 in both susceptible (JG62) and resistant (Digvijay) cultivars of chickpea. R- Root, S- Shoot, LR- Lower Root, MR- Middle Root, UR- Upper Root. A. (14 dpi) Transverse section of root and shoot reveals still higher fungal mass in JGI while nearly absence of fungus in DVI. (slight fluorescence marked in the figure). B. (18 dpi) Transverse section of root and shoot showing vasculature completely flooded with fungal mass in JGI and very few fungal hyphae in DVI. C. (25 dpi) Transverse section of root and shoot reveals distortion of vasculature because of heavy fungal colonization in JGI whereas plant tissues are near normal in DVI with sporadic fungal mass.

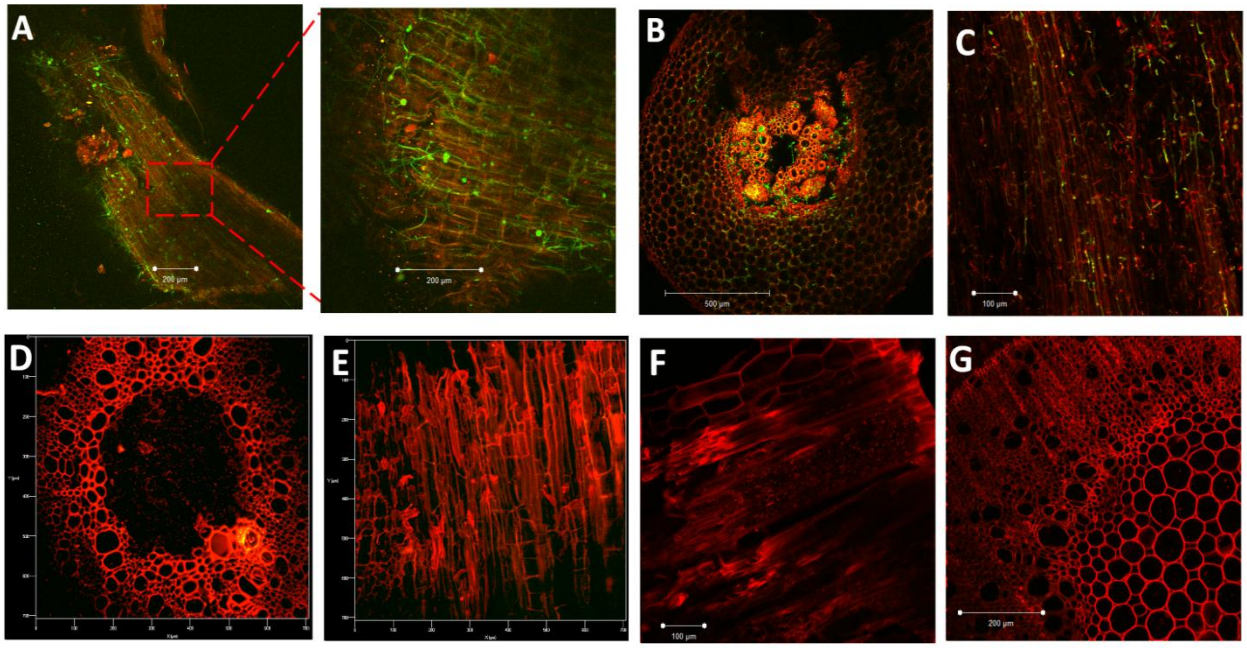


Fig 3.7. Late stages of chickpea root and shoot colonization by *Fusarium oxysporum* f.sp. *ciceri* race 2 marked with eGFP in susceptible (JG62) and resistant (Digvijay) cultivars at 28 DPI. A. Heavy colonization of root of susceptible (JG62) plant in longitudinal section. B. Cross section of root of susceptible (JG62) plant showing complete colonization of fungus in cortex (C) as well as xylem vessels (X) and deformation of root architecture. C. Longitudinal section of stem of susceptible (JG62) plant with the extensive presence of conidia as well as mycelia of fungus. D-E. Cross and longitudinal sections of root of resistant plant (Digvijay) with normal architecture and absence of any fungus in it. F-G. Longitudinal and cross section of stem of resistant (Digvijay) plant without any fungal infection.

3.1.4 *In planta* pathogen quantification using qPCR

Whole root analysis. A nearly perfect (R2 ~0.999) linear regression between the logarithm of known concentrations of fungal DNA and qPCR threshold cycles (Cts) was established (Fig. 3.8). Using these standard regression lines, significant amount of Foc 2 DNA was detected in both JGI and DVI till 16 hpi, followed by a decrease till 4 dpi. Thereafter, the amount of Foc 2 DNA increased significantly only in JGI till 14 dpi. At 28 dpi, the fungal DNA content decreased in JGI and DVI both; however in DVI, the pathogen load itself was significantly less than that in JGI (Fig. 3.9).

Progressive colonization of Foc 2 in chickpea root and shoot fractions. Genomic DNA was isolated from root (R1-R4 in JGI and R1-R5 in DVI) and shoot fractions (S1 and S2 in both JGI and DVI) to study the pathogen progression in JGI and DVI plants (Fig. 3.10), using qPCR based pathogen DNA quantification as described in section 2.3.8 of Materials and Methods. The pathogen DNA detected at 0 hpi in R1 and R2 fractions of both JGI and DVI was higher compared to the subsequent time points till 4 dpi in JGI and 7 dpi in DVI (Fig. 3.10A and B). At later time points (16 hpi to 14 dpi), the pathogen DNA increased gradually in DVI in R1 and R2 fractions, which reached the maxima at 14 dpi (Fig. 3.10B). At this time point, the maximum amount of pathogen DNA was detected in all the four root fractions (R1-R4) and both the shoot fractions (S1-S2). However, at 28 dpi, the pathogen load significantly decreased in all the fractions. Interestingly, the pathogen load was below the detectable limit in R3 and R4 fractions at 28 dpi in DVI. The fraction R5 could be assigned only at this time point in DVI, as the root-length increased due to growth, and showed minor amount of pathogen DNA. In JGI, pathogen load remained high in R1 till 24 hpi and dipped during 2 to 4 dpi. Thereafter, pathogen DNA was detected in the entire root and shoot fractions with maximum at 14 dpi. All the root fractions of JGI had nearly 10 times higher pathogen load compared to that of DVI (Fig. 3.10). In JGI, the 28 dpi root tissue was completely wilted and fragmentation was not possible.

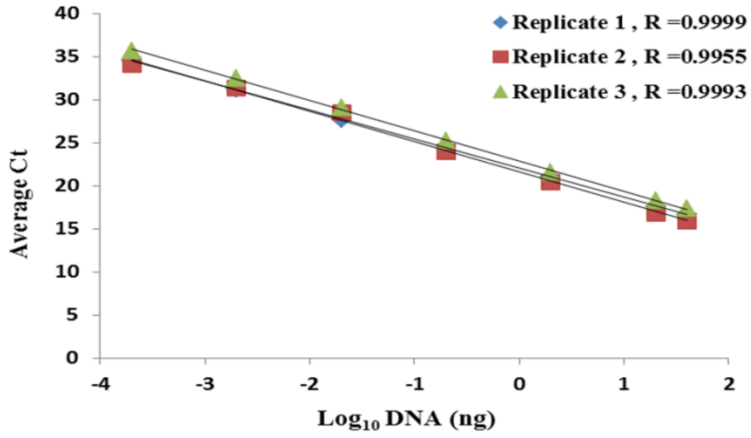


Fig 3.8. Standard regression lines of three replicates of 10-fold serial dilution of Foc 2 DNA (10 ng/µl). Threshold cycles (Ct) were plotted against the log of known concentrations of Foc 2 genomic DNA.

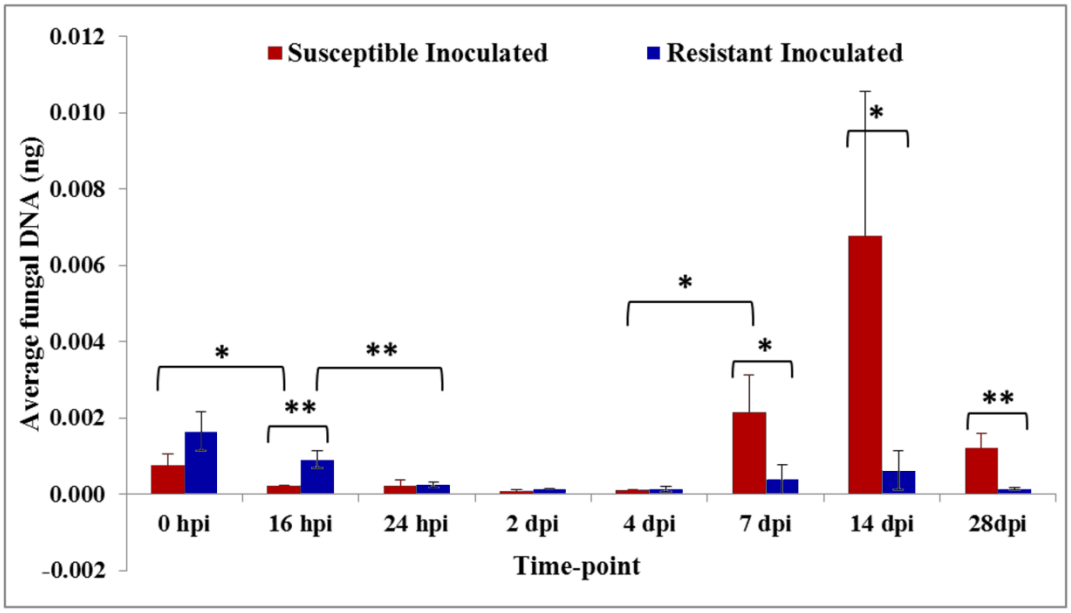


Fig 3.9. Proportion of Foc 2 DNA in whole root of chickpea cultivars, JG62 (susceptible) and Digvijay (resistant), at various time-points after inoculation. Amount of pathogen DNA was estimated using qPCR. Bars with asterisk (*) indicate level of significance of the amount of pathogen between JGI and DVI as per T-test performed between the two groups. '*'- P<0.05; '**'- P<0.01; '***'-P<0.001.

3.2 qRT-PCR analysis of Foc virulence and chickpea defense related genes

3.2.1 Expression of Foc virulence genes

The expression of several pathogen virulence related genes was evaluated in all the four treatments using qRT-PCR, however, no expression was detected in JGC and DVC. Hence in further studies, the expression of those genes was analyzed only in JGI and DVI. Few of these genes could be successfully traced throughout the disease progression (Fig. 3.11). The expression of chitin synthase VII (*Chs7*), a chaperonin like ER protein was initially weak in JGI and DVI; however in JGI, the expression increased at 16 hpi followed by decrease till 14 dpi and again increase at 28 dpi. In DVI, it was elevated at later stages reaching to maximum at 28 dpi. However, the expression was much higher in JGI compared to that in DVI at 28 dpi. The expression of G protein β subunit gene was very high at 16 hpi in JGI, which decreased

with disease progression and again increased at 28 dpi. In DVI, the expression was initially weak but was elevated at 7 dpi.

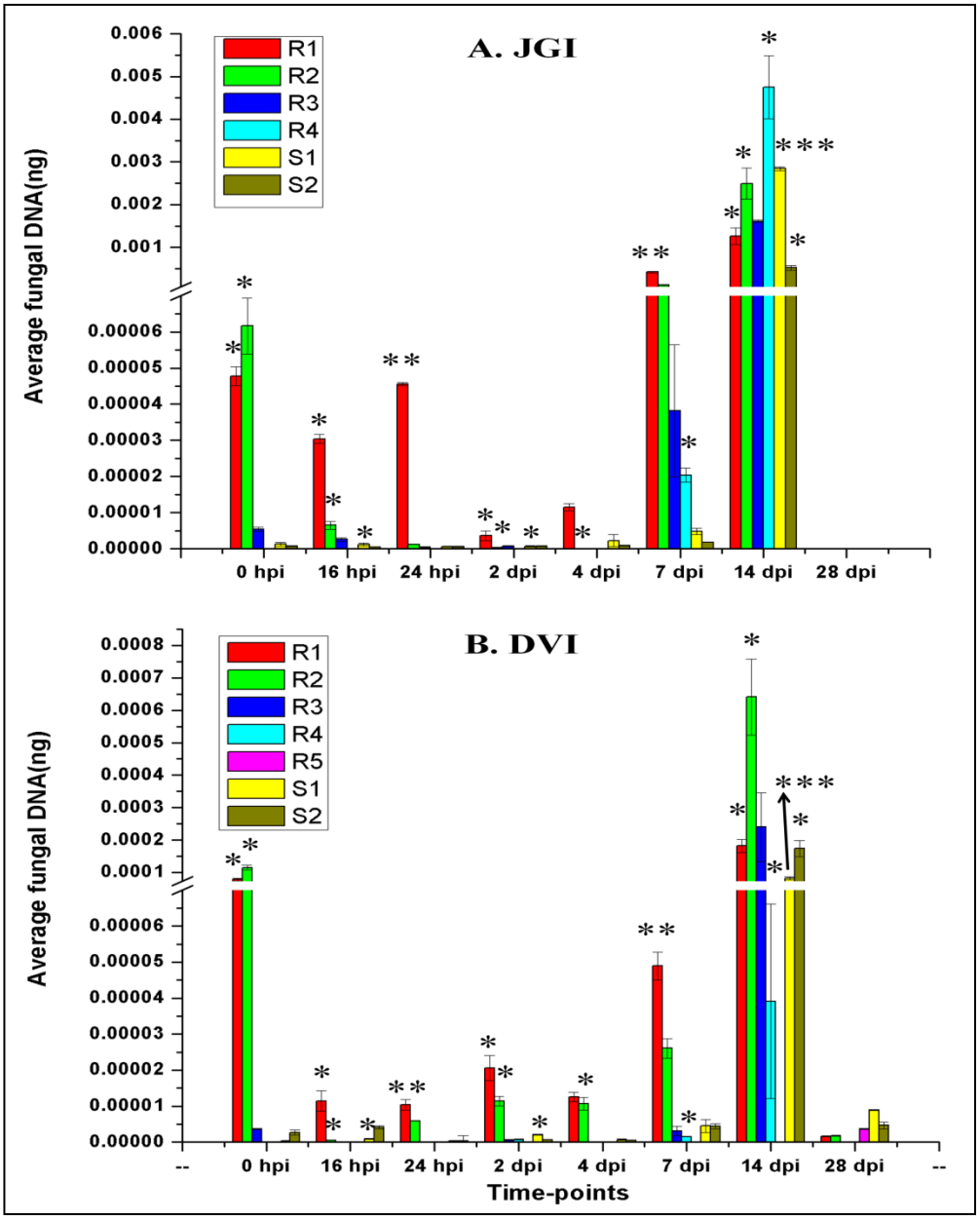


Fig.3.10 Proportion of Foc 2 DNA in fractions of root and shoot from the inoculated root tip of (A) susceptible (JG62) and (B) resistant (Digvijay) chickpea cultivars at various time-points after inoculation. In case of JG62, 28 DPI root fractions could not be collected as the plant was completely wilted. Bars with asterisk (*) indicate level of significance of the amount of pathogen between JGI and DVI as per T test performed between the two groups. '*'- P<0.05; '**'- P<0.01; '***'-P<0.001.

We found that the expression of this gene was significantly high in JGI at early stage of colonization, which can be correlated with hyphal growth and establishment. Similarly, the expression of mitochondrial carrier protein (*Fow1*), which is responsible for the transfer of tricarboxylates across the mitochondrial inner membrane, was initially high in JGI, decreased till 4 dpi, and thereafter gradually increased reaching its peak at 28 dpi. In DVI, the expression was almost nil with only weak detection at 28 dpi. Glucanosyltransferases are the key enzymes involved in fungal cell wall synthesis that are required for fungal growth and morphogenesis. The expression of glucanosyltransferase gene was high till 16 hpi, then decreased and again increased when plants showed wilting symptoms in JGI. While in DVI, the expression was throughout very low. Cell wall extracellular matrix proteins (CWEMPs) are the glycoproteins covalently linked to cell wall matrix and are major pathogen virulence factors. The expression of the CWEMP showed gradual increase with disease progression in DVI; whereas in JGI, initial increase in the expression of the gene followed a gradual decrease and the final elevation at the end of the infection.

Production of cell wall degrading enzymes (CWDEs) like xylanases (*XYL*), polygalacturonases (*PG*) and pectate lyase (*PL*) is vital for pathogen establishment in plants. High expression of *XLY* gene was observed only in JGI, particularly in late stages; while the expression was not detected in DVI. On the other hand, elevated expression of *PG* was found in initial hours of disease progression in JGI, which then decreased further. However, gene expression was evident only at 28 dpi in DVI. The expression of *PL* gene was higher at the start of infection in both JGI and DVI, which then declined at 16 hpi and again increased in DVI at 24 hpi and in JGI at 2 dpi, respectively. Thus, the gene mainly expressed during initial surface colonization and up to 2 dpi, when the fungus invaded root cortex and vascular region in JGI, as also observed by confocal microscopy.

3.2.2 Expression of chickpea defense genes

To analyze the expression pattern of defense related genes, cDNAs generated from root tissues of various stages of disease development in JGI and DVI and corresponding time-points in

JGC and DVC were used (Fig. 3.12). Glyceraldehyde 3 phosphate dehydrogenase (*GAPDH*) was used as a reference gene.

3.2.2.1 Expression of enzymes acting on major structural components of fungal cell wall

In this study, chitinase and glucanase (family of PR 2 proteins) were evaluated for their expression over a prolonged time-scale (0 hpi to 28 dpi). Glucanase expression was pronounced at later stage of infection in both the cultivars but this increase was significantly higher in case of JGI. Expression of chitinase was consistently low in both the cultivars except in case of JGI where the expression significantly increased in the later stage of infection and reached maxima on 28dpi. Thus both chitinase and glucanase were found to be induced coordinately only in JGI at later stages but in case of DVI there was marginal increase in the expression that too at later stage of disease progression. The expression of B glucosidase and Glycosyltransferase was also analyzed. B glucosidase expression was significantly highest at the start of infection which declined with the progress of disease. JGI plants showed overall reduced expression with significant reduction at all time-points except at 16 and 24 hpi compared to JGC. However, DVI plants showed significantly elevated expression than DVC plants except at 0 hpi, 14 and 28 dpi. Glycosyltransferase gene showed almost consistent expression throughout the disease progression with statistically higher expression in DVI than DVC at 16 hpi and 2 dpi.

3.2.2.2 Expression of key enzymes of phenylpropanoid pathway

In this study the expression of phenylalanine ammonia-lyase (*PAL*) was significantly higher in DVI as compared to the remaining treatments at the start of infection. However, it alternately increased and decreased at initial, middle, late and later time-points of the inoculation. On the contrary, steady level of *PAL* expression was seen in JGI. The expression of chalcone synthase gene was significantly higher in DVI than that in JGI at majority of the time-points and followed the similar pattern of expression as that for *PAL*. Interestingly, JGI showed significantly less expression of chalcone synthase than that in JGC at least till 2 dpi and

thereafter showed significant increase at 14 dpi. The expression of Isoflavone reductase gene was almost comparable between JGI and DVI at initial time-points but decreased significantly in DVI from 7 dpi onwards. On the other hand, its expression in JGI compared to that in JGC significantly increased after 2 dpi.

3.2.2.3Expression of key genes involved in stress management in chickpea

In this study, cytochrome P450 expression was increased gradually with disease progression in JGI reaching to the highest level at 14 dpi with a sharp decrease thereafter. In DVI, the expression was higher at later stages but with significantly less increase as compared to that in JGI. The expression of *60 SRP* was significantly higher in DVI compared to JGI at almost all the time-points. Expression of this gene was significantly reduced in JGI compared to JGC till 7 dpi while the expression was comparable in DVC and DVI. The expression of Histone Protein A was found to be comparable in all the treatments with increasing trend till 14 dpi. Thereafter expression of this gene significantly decreased in DVI compared to that in JGI. In this study, expression of both *PR10* and *BetVI* (known to show significant homology to *PR 10*) was also analyzed. In case of *PR10*, the expression enhanced significantly with the disease progression in JGI compared to that in DVI. Similarly, expression of *BetVI* showed significant increase at several time-points in JGI compared to that in DVI. The expression of LRR was significantly higher in DVI at majority of the time-points compared to that in JGI. Further in JGI, the expression of *SOD* gene was found to be steady across the disease progression but was significantly lower as compared to JGC. DVI, on the other hand, showed significantly less expression of *SOD* compared to JGI. In case of metallothionein, the expression significantly increased with disease progression in JGI compared to that in DVI with a comparable expression at 14 dpi.

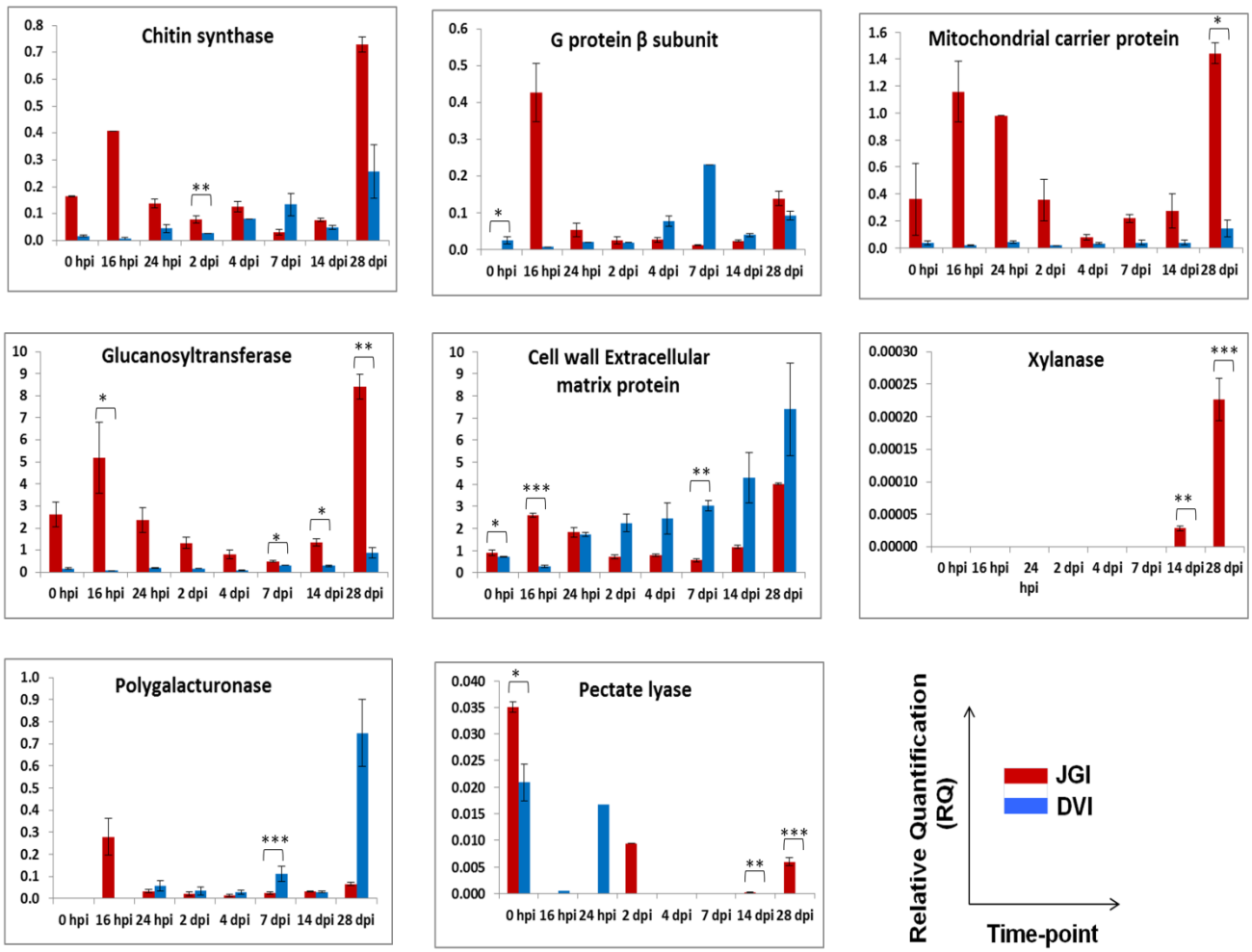


Fig. 3.11 Expression patterns of eight fungal virulence related genes in JGI and DVI at eight time-points using quantitative reverse transcriptase PCR. Bars with asterisk (*) indicate level of significance of the expression of particular gene between JGI and DVI as per the Student's't' test performed between the two groups. '*'- P<0.05; '**'- P<0.01; '***'- P<0.001.

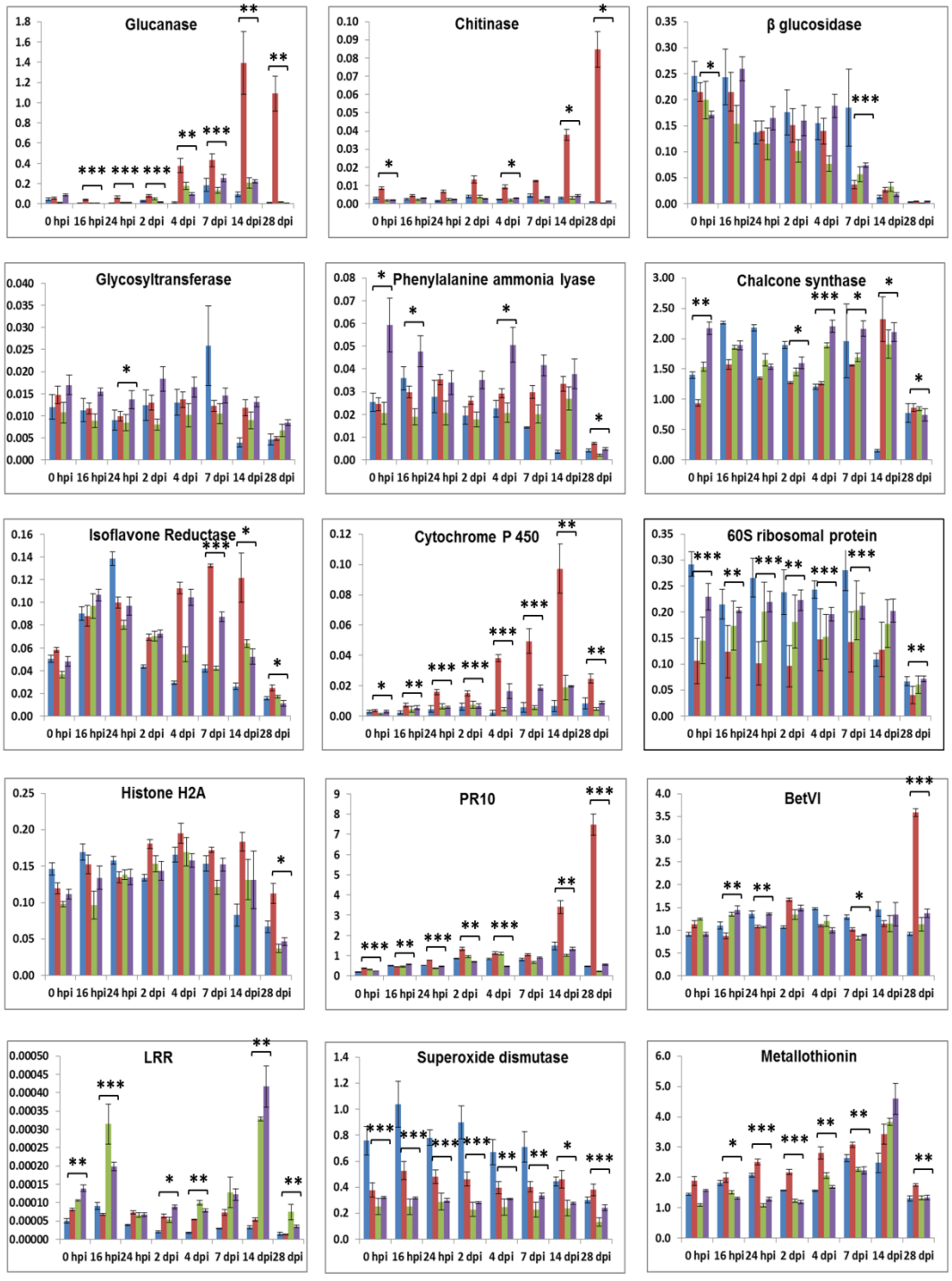


Fig. 3.12 Expression patterns of eighteen plant defense related genes in JGC, JGI and DVC, DVI at eight time-points using quantitative reverse transcriptase PCR.

3.3 SAGEseq analysis of chickpea-Foc interaction

3.3.1 Analysis of LongSAGE libraries

Four LongSAGE libraries were generated from wilt-susceptible and wilt-resistant chickpea cultivars following inoculation with pathogen spores and mock inoculation with sterile deionized water to both the cultivars (JGC, JGI, DVC and DVI). The Ion Torrent PGM® platform (Thermo Fisher Scientific, USA) was used for sequencing and the raw data obtained was processed to extract tags. The statistics of tag mapping of the four libraries is presented in Table 3.2. Among the four LongSAGE libraries, the highest number of tags was obtained in DVI library followed by JGC. However, the maximum number of mapped tags was observed in JGC followed by DVI library. Considering the number of mapped transcripts, abundance was the highest in DVI followed by JGC library. Interestingly, the JGI library showed the least number of tags, mapped tags and mapped transcripts.

Table 3.2 Statistics of tag mapping of the four LongSAGE libraries

Library	Total no. of tags	No. of mapped tag	Total no. of mapped transcripts	Total no. of transcripts with unique matches*	Total no. of transcripts with selected matches^
JGC	189947	37253	14658	5872	8786
JGI	149785	28825	13949	7180	6769
DVC	189703	33497	14534	6270	8264
DVI	386458	34658	15048	6112	8936

*: 'Unique' match represents those tags mapped only on single transcript

^: 'Selected' match represents multiple tag sequences mapped on the same transcript.

3.3.2 Characteristic DEGs and their functional classification

Differential gene expression (DGE) sets defined by comparisons across all the four LongSAGE libraries revealed both differentially expressed as well as uniquely expressed genes (p-value <= 0.05 and FDR <= 0.05) within the compared pairs of libraries (Table 3.3). Annotations and expression values of mapped transcripts either differentially or uniquely

expressed in the above comparisons are compiled in the form of Tables S1, S2, S3, S4 and are also included in the annexure in CD at the end of the thesis. Transcriptome analysis revealed the highest number (3816) of DEGs in DE_JGC_JGI set excluding the fungal sequences (unique to JGI library) wherein 43.16% genes were down-regulated (1647) and 32.23% genes (1230) were up-regulated. On the other hand, in DE_JGC_DVC set, 2987 DEGs were obtained with 36.55% down-regulated genes accounting slightly higher than the 31.36% up-regulated ones. However, only 18 Foc DEGs were obtained in DE_JGI_DVI set all of which were up-regulated in JGI (Table 3.3).

The annotation tool 'Mercator' (http://www.plabipd.de/portal/mercator-sequence-annotation) allowed assignment of genes of all the four sets into 35 functional classes referred to as 'bins'(Thimm *et al.*, 2004). Four comparisons were analyzed based on the most frequent bins assigned and their regulation. Fig. 3.13 shows the distribution of functional classes among DEGs in the four comparisons. Based on these assignments, an attempt was made to understand the role of DEGs in modulating the cellular mechanisms in chickpea in response to Foc stress. The MapMan annotation software was further used to display DEGs from three comparisons namely DE_JGC_JGI, DE_DVC_DVI and DE_JGI_DVI sets with respect to stress (Thimm *et al.*, 2004) (Fig. 3.14). Overall up-regulated genes were, although more in DVI, stress responsive transcripts were abundant in JGI, while the transcripts associated with abiotic stress, heat-shock proteins, secondary metabolism and PR proteins were noticeably up-regulated in DVI. Interestingly, reticulon-like protein B2 (RTNLB2), shown to regulate intracellular trafficking and activity of the FLS2 immune receptor (Lee *et al.*, 2011), showed the highest expression (15.36 fold) in DVI. The PR protein category particularly included disease resistance response proteins namely, (DRR) SR1, 206 and TMV resistance protein. Likewise, several heat shock proteins (HSPs) were highly induced in DVI. Moreover, lignin biosynthetic enzymes such as 4-coumarate--CoA ligase (*4CL*), cinnamoyl-CoA reductase 1 (*CCR1*) and caffeoyl-CoA O-methyltransferase (*CCoAOMT*) were also up-regulated in DVI. MapMan revealed a disease resistance protein belonging to nucleotide-binding site leucine-rich repeat (NBS-LRR) gene family in DE_DVC_DVI set. Overall, MapMan highlighted various up-regulated metabolic processes (represented under stress) leading to a multilevel

defense response in resistant cultivar endowing it with the capacity to arrest the pathogen colonization.

Table 3.3 Differential gene expression analysis

Differential Gene Expression (DGE) Sets represent comparisons among the four SAGE libraries. For example, DE_JGC_JGI shows DEGs up- or down-regulated in JGI compared to JGC. CA stands for *Cicer arietinum* (chickpea) and FO stands for *Fusarium oxysporum*.

DGE sets	**No. of differentially expressed genes**	**Up-regulated genes**	**Down-regulated genes**	**Significantly expressed genes in only one Library**
DE_JGC_JGI (CA genes)	3816	1230	1647	695 (JGC), 256 (JGI)
DE_DVC_DVI (CA genes)	3429	1390	1100	312 (DVC), 349 (DVI)
DE_JGC_DVC (CA genes)	2987	937	1092	77 (JGC), 68 (DVC)
DE_JGI_DVI (CA genes)	3622	1694	1106	480 (JGI), 904 (DVI)
DE_JGI_DVI (FO genes)	18	0	18	533 (JGI), 5 (DVI)

3.3.3 Clustering of core DEGs across the comparisons

To delineate the genes responsive to pathogen inoculation either in resistant or susceptible cultivar without neglecting the basal gene expression level between the varieties, significant DEGs between all the four aforementioned DGE sets were compared and the genes with LFC<1 in all the four sets were omitted. A total of 400 DEGs (all having LFC≥1 in at least one of the sets) were clustered using Euclidean distance and complete linkage method (Fig. 3.15). Six expression patterns (cluster 1-6) were obtained based on hierarchical clustering algorithms. Cluster 1 included genes mainly belonging to protein metabolism, RNA-regulation of transcription, hormone metabolism (Gibberellin and Jasmonate), calcium signaling and stress (biotic and abiotic). These genes were highly up-regulated in DVI and might be involved in

resistance mechanism such as ubiquitin-conjugating enzyme E2 28 (~20 fold), peroxidase 42 (~18 fold), Glutathione S-transferase (~18 fold), ADP ribosylation factor (ARF) (~15 fold) etc.

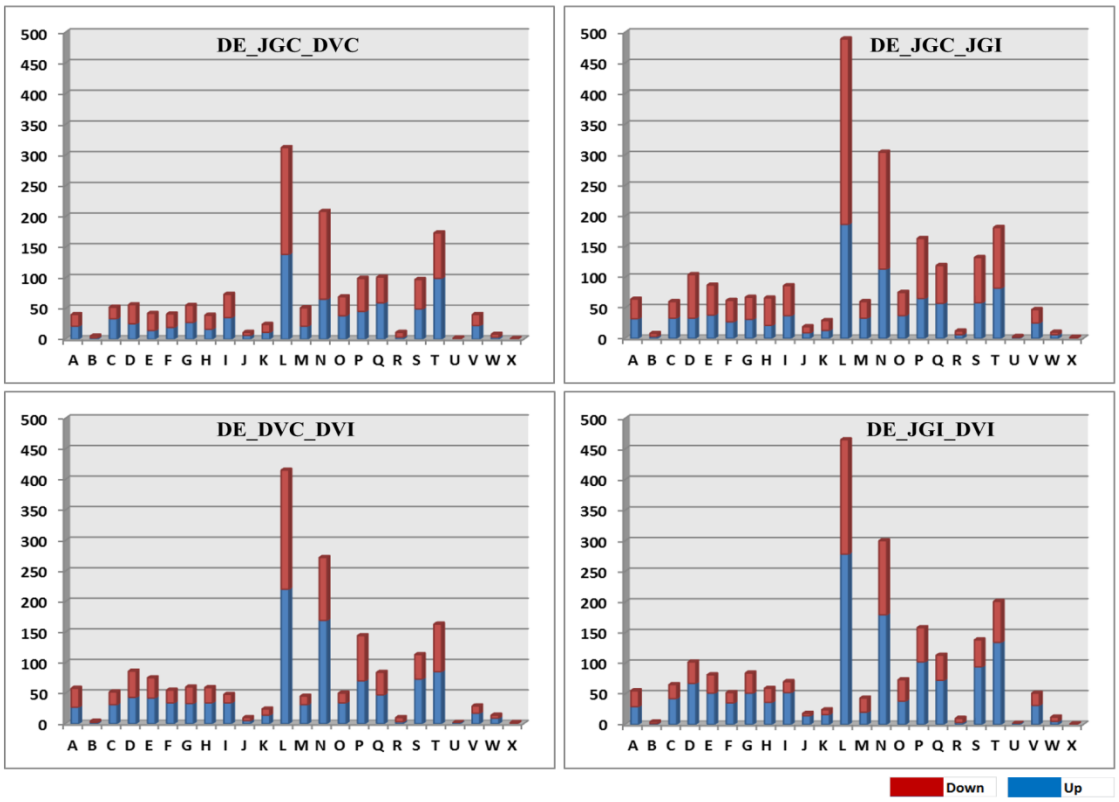


Fig. 3.13. Distribution of functional classes among up- and down-regulated chickpea genes in four Differential Gene Expression (DGE) datasets. 'Mercator' based assignment of the genes to different functional classes (bins) is represented on X axes (A - Amino acid metabolism; B - Biodegradation of Xenobiotics; C - Cell wall related; D - Cell E - Development; F - DNA metabolism; G - Hormone metabolism; H - Lipid metabolism; I - CHO metabolism; J - ATP synthesis; K - Nucleotide metabolism; L - Protein metabolism; M - Photosynthesis; N - RNA metabolism; O - Secondary metabolism; P - signaling; Q - Stress; R - Tetrapyrrole synthesis; S - Transport; T - Miscellaneous; U - Polyamine metabolism; V - Redox; W - Vitamin metabolism; X - S-assimilation). The Y axes denote the number of transcripts belonging to a particular bin.

Cluster 2a represented an array of genes belonging to protein synthesis and degradation, RNA-regulation of transcription, signaling (calcium, G-proteins, MAP kinases and LRR) and stress. This group was down-regulated in JGI while up-regulated in DVI and included 14-3-3-like protein B, heat shock proteins, calcineurin, serine-threonine protein kinase etc.

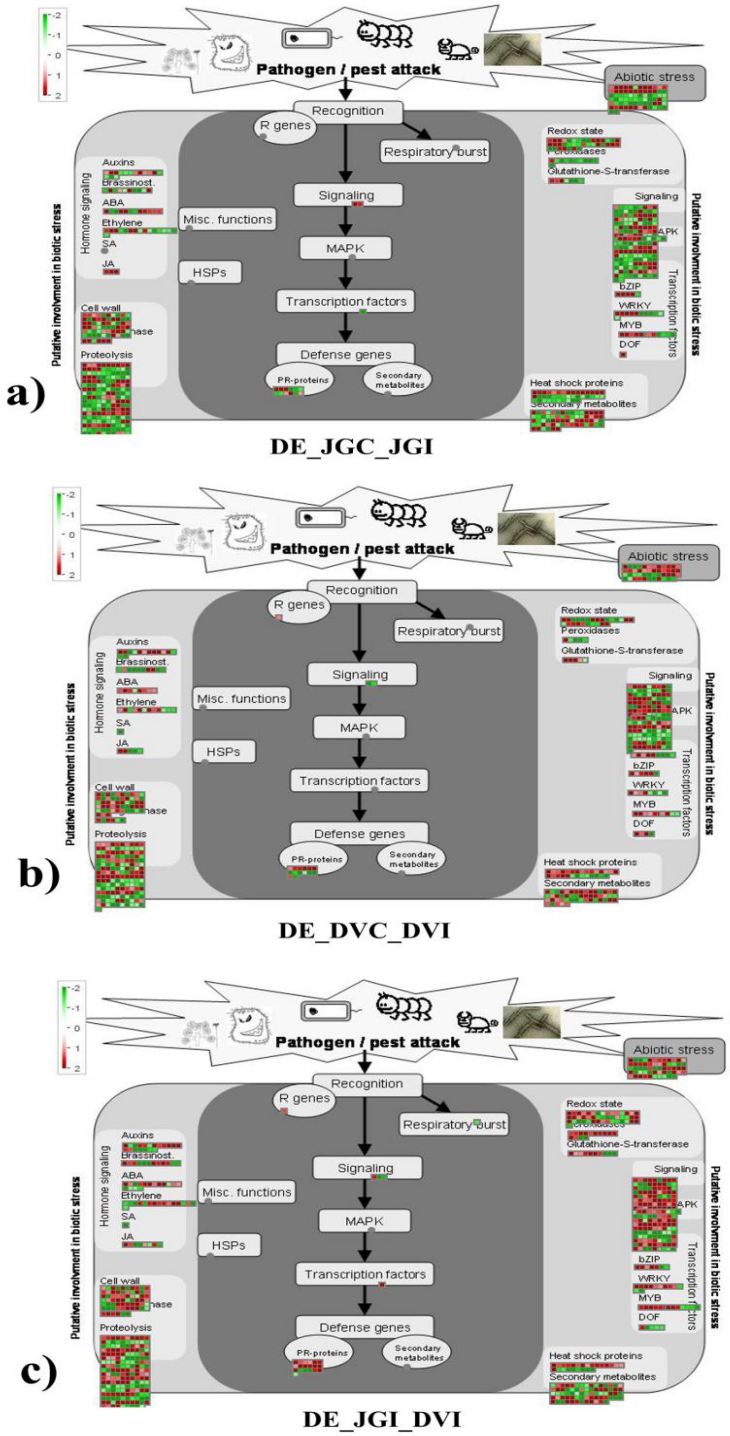


Fig. 3.14 'MapMan' presentation of DEGs (chickpea) of the datasets DE_JGC_JGI (A), DE_DVC_DVI (B) and DE_JGI_DVI (C) under 'Stress' category. 'Mapman' was used to display DEGs of the three datasets represented during stress. The color change from dark red (+2 LFC) to dark green (-2 LFC) through white (0 LFC) indicates the differential expression ranging from 4 fold up-regulation to 4 fold down-regulation.

Alternatively the genes in cluster 2b showed opposite trend i.e. up-regulated in JGI compared to DVI. This included transcripts pertaining to protein synthesis and degradation, secondary metabolism and hormone metabolism such as ethylene ACC oxidase, ethylene responsive transcription factor RAP 2-1, NAD(P)H-dependent 6'-deoxychalcone synthase, expansin like genes etc. Cluster 3 exhibited an interesting expression profile depicting candidates with reduced basal gene expression in DVC as compared to JGC. Few of these genes were highly down-regulated in JGI as compared to JGC while up-regulated in DVI with respect to DVC. This finally reflected as much higher up-regulation of these transcripts in DVI as compared to JGI. The genes belonging to this cluster represented protein metabolism, RNA-regulation of transcription, G-protein signaling, aromatic amino acid synthesis and stress such as auxin-binding protein ABP19a, phospho-2-dehydro-3-deoxyheptonate aldolase, chitinase, glucanase etc.

The genes belonging to cluster 4 were up-regulated in DVC compared to JGC and in JGI compared to JGC, respectively. These involved processes like protein metabolism, brassinosteroid hormone metabolism, C3H zinc finger regulation of transcription and stress, and genes like aquaporin PIP-type 7a, magnesium protoporphyrin IX monomethyl ester [oxidative] cyclase (MPP) etc. Cluster 5 had much higher upregulation in DVC and JGI than JGC while downregulation in DVI as compared to JGI and DVC. These mainly included the genes belonging to photosynthesis, protein synthesis and hormone metabolism such as sedoheptulose-1,7-bisphosphatas, ferredoxin, Linoleate 9S-lipoxygenase etc. Finally, cluster 6 had the genes showing similar pattern as that of cluster 5 and belonging to N-metabolism and photosynthesis such as chlorophyll a-b binding protein, ferredoxin--nitrite reductase etc. The evaluation of DEGs by BLASTP search against the PRGdb, a database of plant resistance genes (Sanseverino *et al.*, 2010), revealed presence of 15 R genes (associated with resistance against pathogen) from the set of 400 DEGs. A majority (10) of these belonged to cluster 2; while three R genes belonged to cluster 4 and two belonged to cluster 5. Surprisingly, JGI expressed higher number of R genes than DVI. (Table S5 included in the annexure in CD at the end of the thesis)

3.3.4 Interaction network of DEGs

To determine the interactions of these DEGs, protein–protein interaction (PPI) analysis was performed using STRING. Fifty-seven best assigned COGs, representing 62 unique DEGs, obtained based on most significant E-value using *Glycine max* as the organism (nearest neighbor legume in the STRING database) were used to construct an interaction network (Fig. 3.16). The PPI network of all the DEGs was extracted from the whole interaction network and reconstructed using Cytoscape. The PPI network highlighted several protein functional groups interacting with each other. Majority of the COGs (21.05%) belonged to 'translation, ribosome structure and biogenesis' which showed maximum interactions with other groups followed by 'Post-translational modification, protein turnover and chaperones'. Mercator terms assigned to the DEGs and the 57 COGs associated with these DEGs shared the same biological functions (Table S6 included in the annexure in CD at the end of the thesis). However, as depicted from the figure, a group of COGs represented some additional biological functions apart from the COG descriptions. For example, 'Signal transduction mechanisms' COG contained an additional set of DEGs assigned to Mercator terms like brassinosteroid hormone metabolism, posttranslational modification and biotic stress. Similarly, 'Post-translational modifications' COG possessed DEGs with additional Mercator terms like photosynthesis, redox and abiotic stress. Thus, indicating the interconnectivity of all these biological processes plays an important role in biotic stress resistance.

3.3.5 Exclusively expressed genes in JGI and DVI

The genes expressed uniquely in either of the cultivars only on inoculation were analyzed. In JGI, these genes (562, Table S7 included in the annexure in CD at the end of the thesis) might represent the candidates reprogrammed by the pathogen for its own benefit; or those which activate the defense response against the pathogen in DVI (860; Table S8 included in the annexure in CD at the end of the thesis). The uniquely expressed important genes in DVI included beta-D-xylosidase 7, rhamnogalacturonate lyase B, pectate lyase 12, thiamine pyrophosphokinase, dirigent proteins, etc.; while the unique ontologies included cell wall and LRR (Leucine rich repeat) proteins, cofactor and vitamin metabolism, thermospermine (TSpm) synthesis, abscisic acid and cytokinin metabolism. Similarly, the uniquely expressed

important genes in JGI were MLO like transcript, actin depolymerizing factor (ADF) 5, tonoplast intrinsic protein (TIP) aquaporin type alpha, etc.; while other ontologies were shared with DVI. The uniquely expressed genes were also evaluated for the presence of R genes using the PRGdb database, which identified 52 and 45 R genes in DVI and JGI, respectively. Assessment of distribution of R protein types indicated higher proportion of NBS-LRR types (19.23%) in DVI dataset, whereas the JGI dataset depicted higher (31.11%) RLK (receptor like kinase) type R proteins (Tables S5 included in the annexure in CD at the end of the thesis).

3.3.6 General features of the Foc transcriptome

Comparative transcriptome analysis of JGI and DVI libraries revealed a total of 1569 genes showing high homology to *Fusarium oxysporum* (Fo) and *Fusarium graminearum* (Fg) genes. These genes were not detected in control libraries. However, only 18 of these Foc transcripts were significantly up-regulated in JGI (Table S9 included in the annexure in CD at the end of the thesis) and the same genes were down-regulated in DVI. Among these were the transcripts with similarity to heat shock proteins, histone proteins and five transcripts with possible involvement in fungal growth such as tropomyocin 1, polarized growth protein rax2, woronin body major protein, fimbrin and phosphatidate cytidylyltransferase. Similarly, only five Foc transcripts were expressed exclusively in DVI while a total of 533 Foc transcripts were expressed only in JGI, of which 382 (71.66%) transcripts were annotated using the available resources. These 382 transcripts were categorized into 41 functional groups belonging to the three gene ontology (GO) categories: Cellular Components (CC), Molecular Functions (MF) and Biological Processes (BP) (Fig. 3.17). In the CC category, maximum transcripts were from ribosome and protein complex, while few were also localized to nucleus, integral component of membrane and mitochondrial part. In the MF category, the highest number of transcripts was also from structural component of ribosome followed by those showing ATP binding and metal ion binding activities. Few transcripts were also involved in transferase activity, protein binding and nucleoside-triphosphatase activity. DNA binding, GTP binding, cofactor binding, oxido-reductase activity and translation initiation factor activity were also presented by some transcripts.

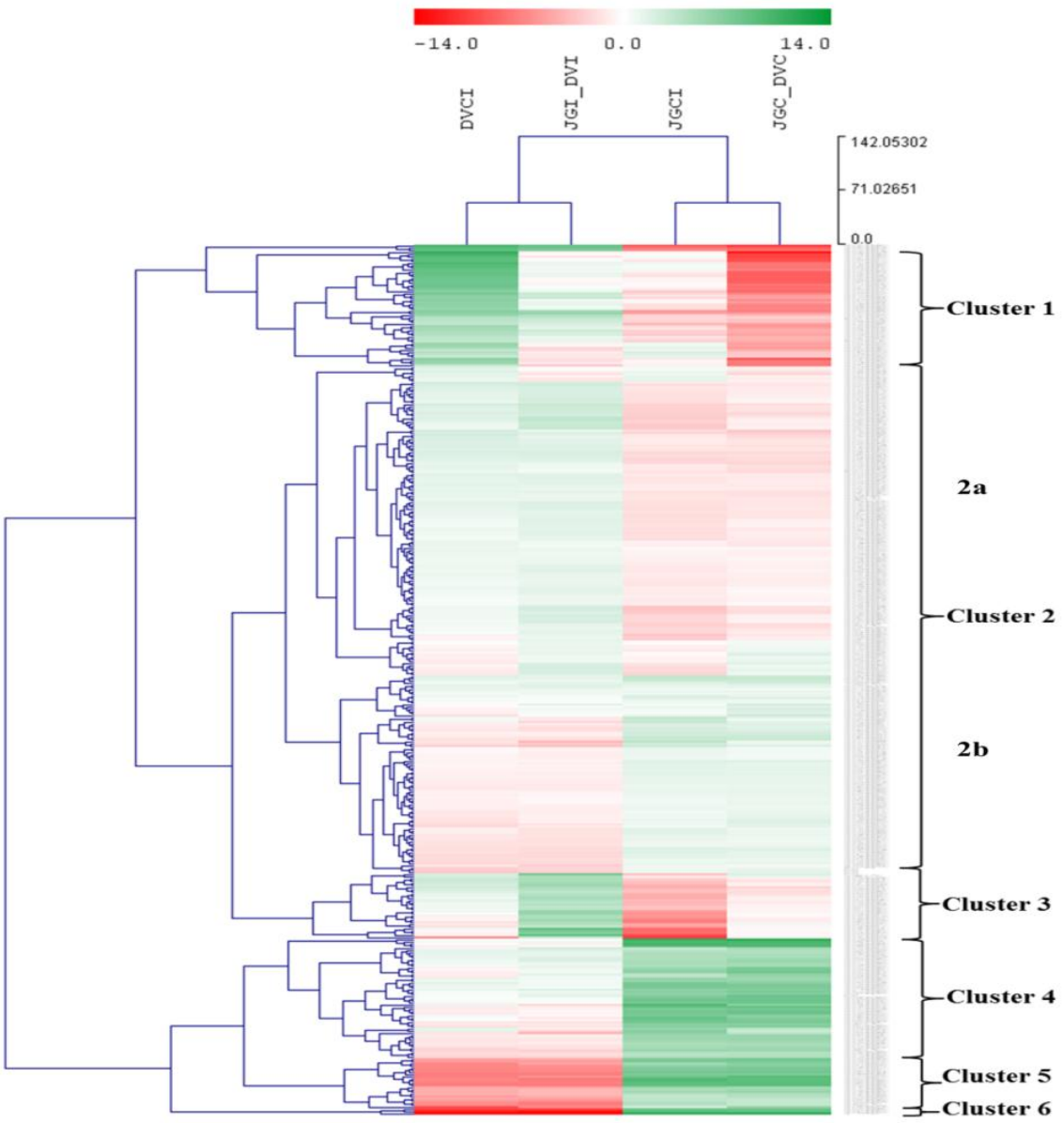


Fig. 3.15: Heatmap and cluster analysis of core DEGs (chickpea) across four datasets. Comparison of significant DEGs among DGE sets resulted in 400 core DEGs (all having LFC≥1 in at least one of the sets). Heatmap was generated with the Log 2 fold change values (LFC). Column 1: DE_DVC_DVI (DVCI), column 2: DE_JGI_DVI (JG I_DV I), column 3: DE_JGC_JGI (JGCI) and column 4: DE_JGC_DVC (JG C_DV C). Each row represents corresponding genes with their identities. Up and down-regulation are indicated by color change from dark red (-14 LFC) to green (+14 LFC). These genes were clustered using Euclidean distance and complete linkage method.

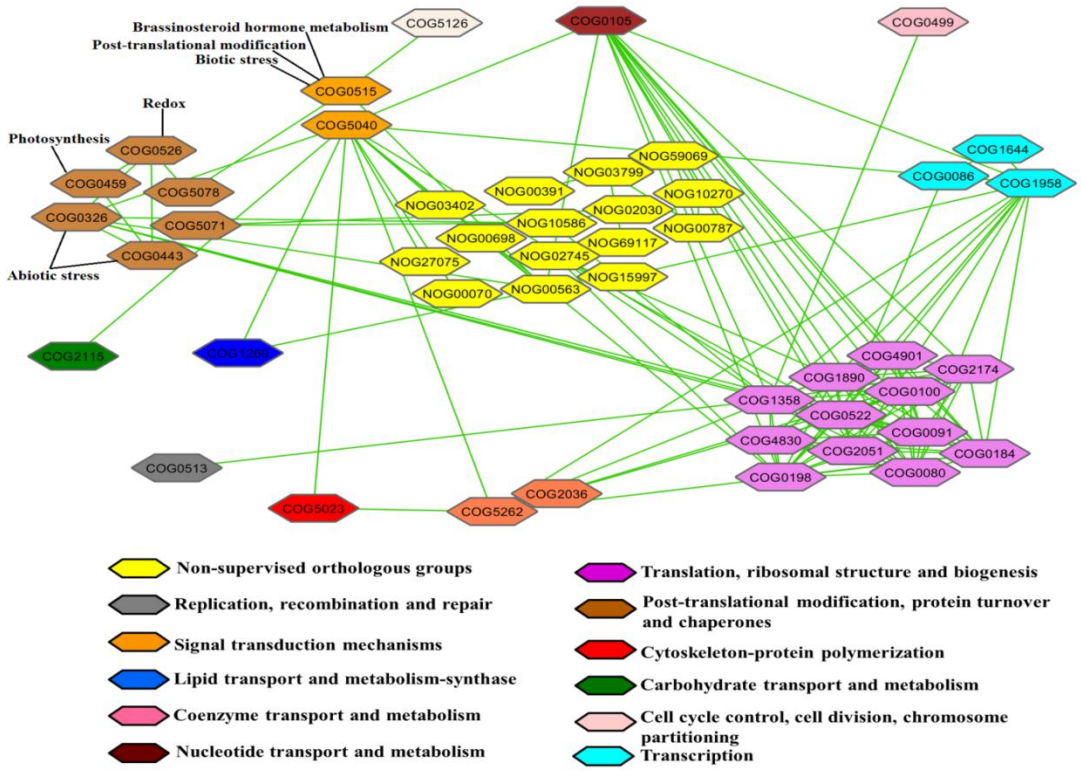


Fig. 3.16: Protein-protein interaction network analysis (PPI) of core DEGs. PPI analysis was conducted using STRING (version 10.0, http://string-db.org, COG mode) and *Glycine max* as an organism (nearest neighbor legume in the organism list present in STRING database). The confidence score was set at ≥0.70 and co-expression and experiment parameters were chosen. COG descriptions along with color codes are mentioned in the figure.

In the BP category, the largest number of transcripts was involved in oxidation-reduction process and organonitrogen compound metabolic process. The remaining transcripts were involved in various cellular, metabolic processes, response to stimulus, stress, intracellular transport as well as biological regulation. KEGG pathway enrichment analysis of these genes was also performed. A total 37 transcripts were allocated to 24 KEGG pathways. The pathways involving highest number of transcripts were TCA cycle (4, 10.81%), carbon fixation in photosynthetic organisms (4, 10.81%), fructose and mannose metabolism (3, 8.1%), pyruvate metabolism (3, 8.1%) and carbon fixation pathways in prokaryotes (3, 8.1%). (Table S10 included in the annexure in CD at the end of the thesis)

Fungal transcripts identified in this study were searched against the PHI database, which is a collection of fungal pathogenicity genes validated using gene knockout studies. Homologues of Foc transcripts which had an effect on pathogenicity in other fungal systems were identified. One Foc transcript (identified as TKL protein kinase) expressed only in DVI was recognized as a virulence factor. Three out of 18 differentially expressed transcripts, such as ATP synthase subunit alpha and two hypothetical, while total 85 Foc transcripts (expressed only in JGI) showed homology to experimentally proven virulence factors. In addition InterProScan analysis was performed to gain an insight into specific functions of genes and to support the functional annotation (Table S9 included in the annexure in CD at the end of the thesis).

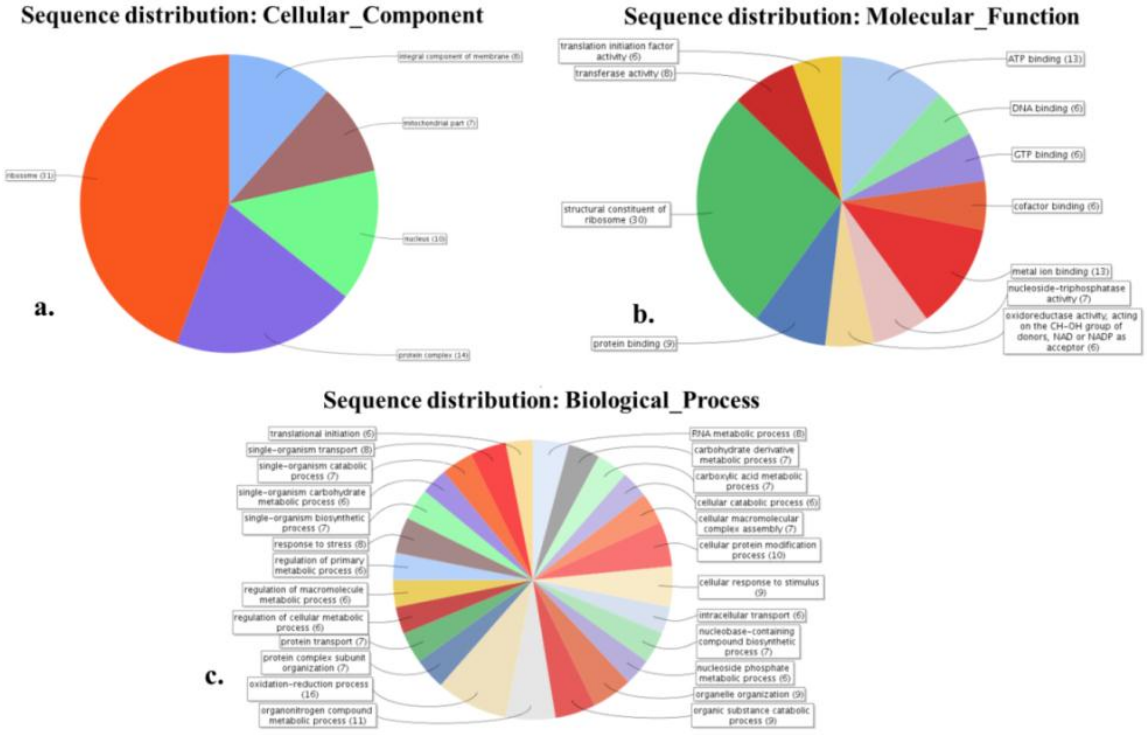


Fig. 3.17 Gene enrichment analysis of Foc genes expressed exclusively in JGI. Distribution of 533 Foc genes expressed only in JGI based on Blast2GO analysis as A. Cellular Component (CC), B. Molecular Function (MF) and C. Biological Process (BP).

3.3.7 Validation of SAGE data by qRT-PCR

As detailed in section 2.5.5 of Materials and Methods, validation of important genes identified in LongSAGE analysis was performed by qRT-PCR wherein the relative expression levels indicated by LongSAGE results were reflected in qRT-PCR. For example, chickpea genes like 14-3-3, auxin binding proteins ABP19a and mitogen activated protein kinase which showed higher expression in DVI in LongSAGE analysis also showed higher fold changes at several time-points in DVI in qRT-PCR analysis. Similarly, many up-regulated Foc genes in JGI showed higher fold change in qRT-PCR, especially all selected Foc genes showed exclusive expression in stem tissue of JGI (Figs. 3.18, 3.19, 3.20 and 3.21).

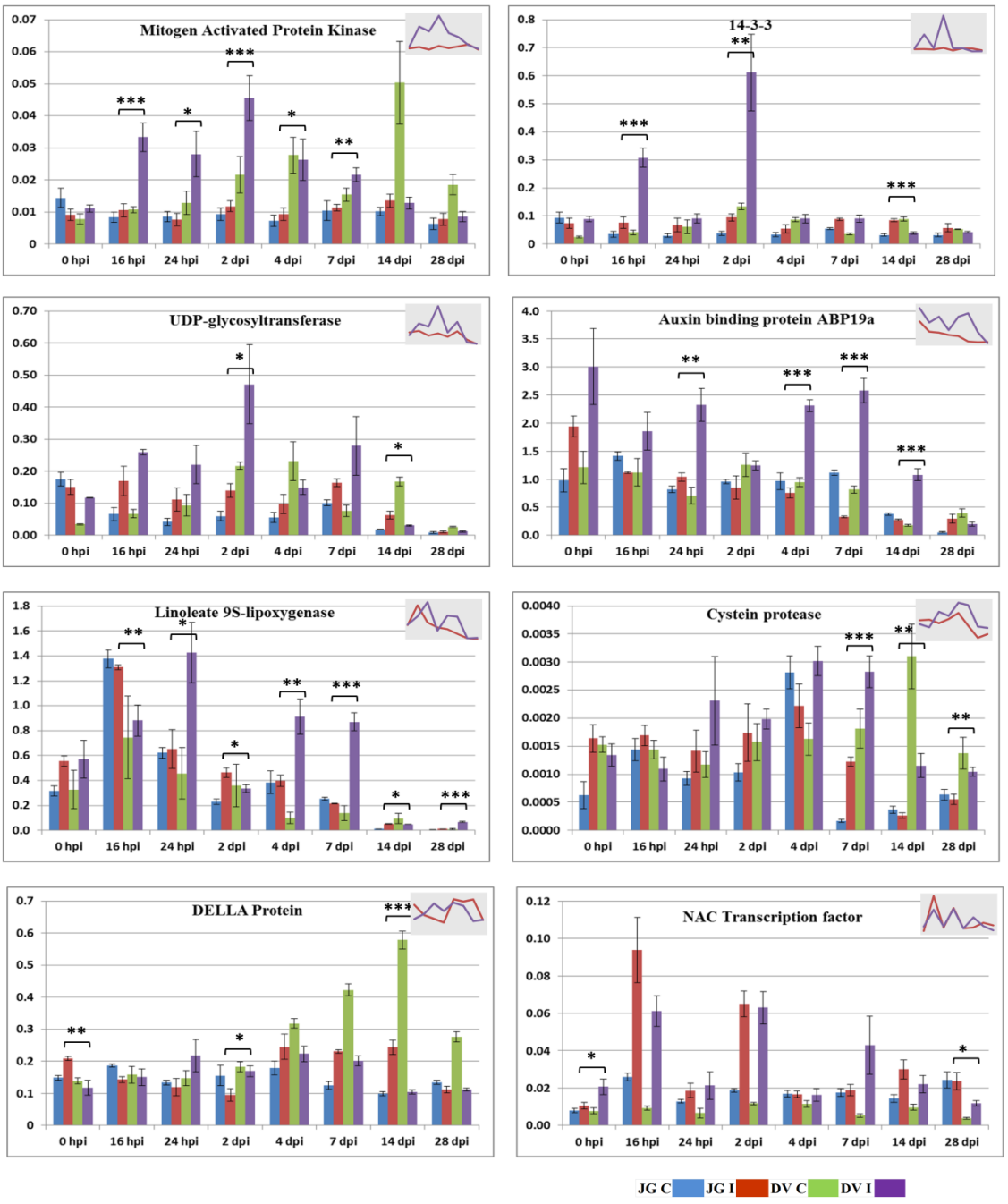


Fig. 3.18 Expression patterns of eight chickpea defense related genes in root tissue at eight time-points using qRT-PCR. The inset in each graph depicts expression pattern of the gene in JGI and DVI across the time-points.

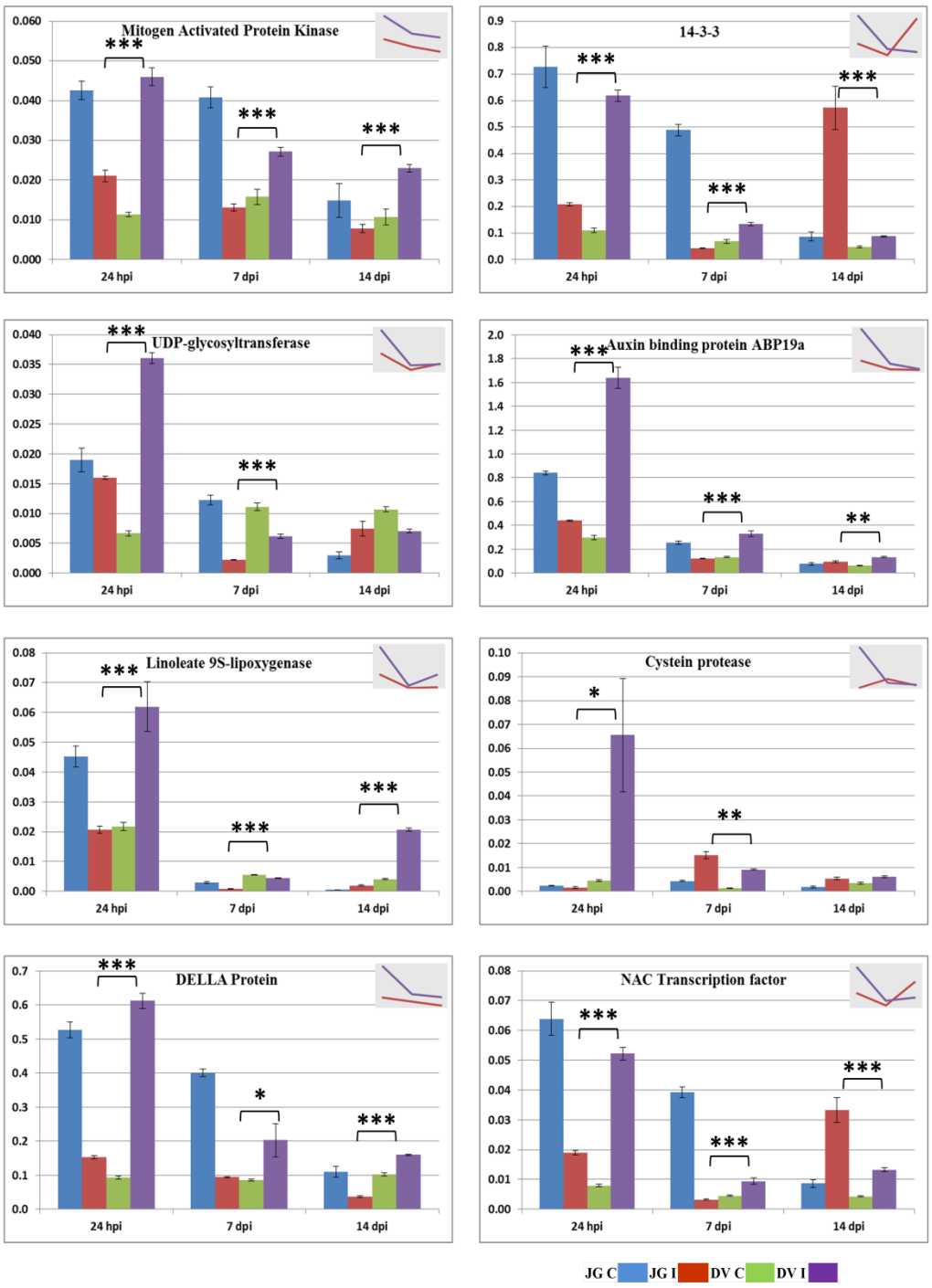


Fig. 3.19 Expression patterns of eight chickpea defense related genes in shoot tissue at three time-points using qRT-PCR. The inset in each graph depicts expression pattern of the gene in JGI and DVI across the time-points.

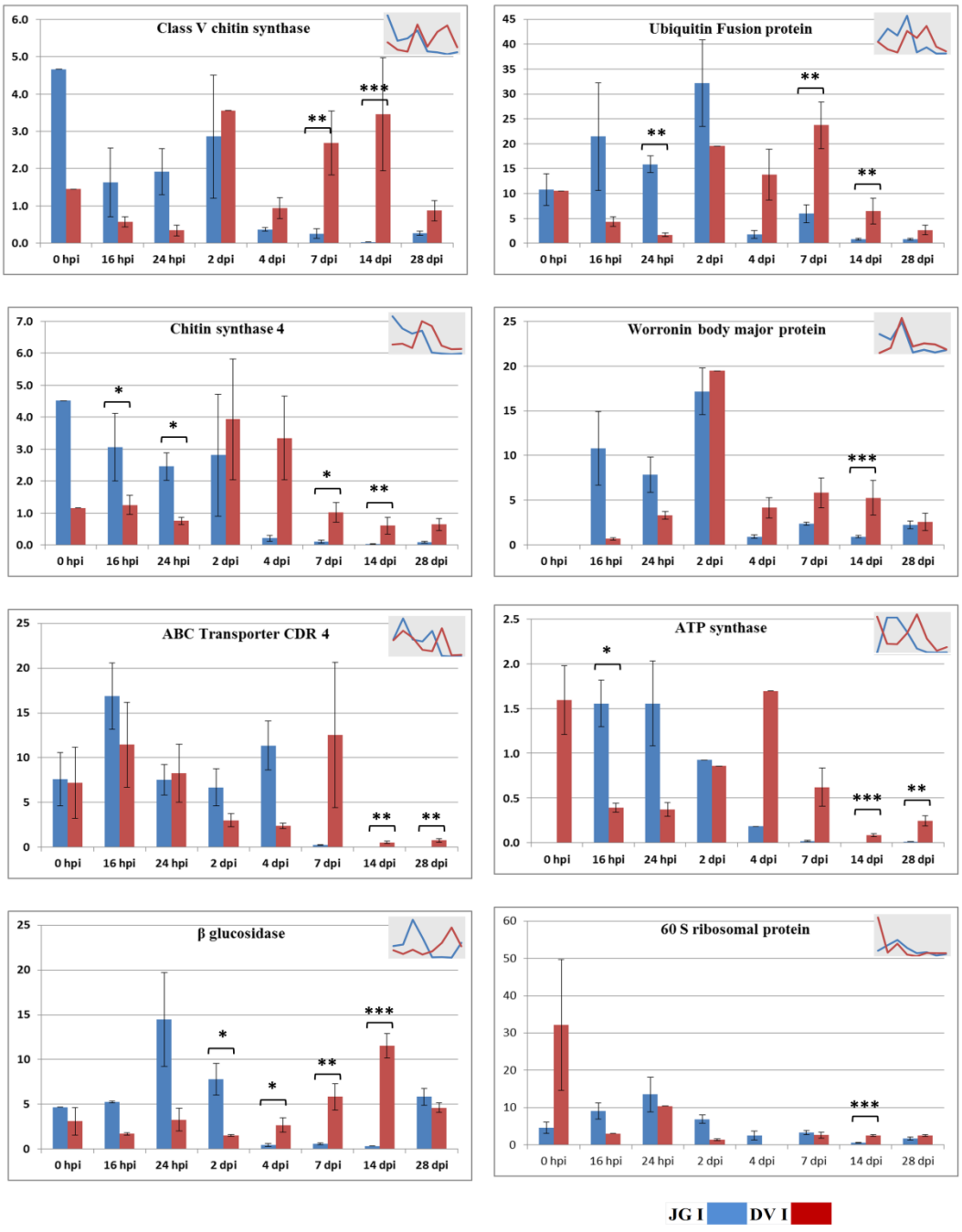


Fig. 3.20 Expression patterns of eight Foc virulence related genes in root tissue at eight time-points using qRT-PCR. The inset in each graph depicts expression pattern of the gene in JGI and DVI across the time-points.

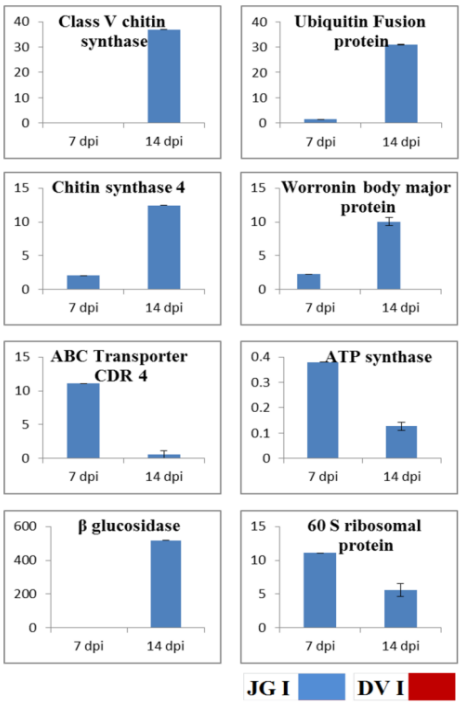
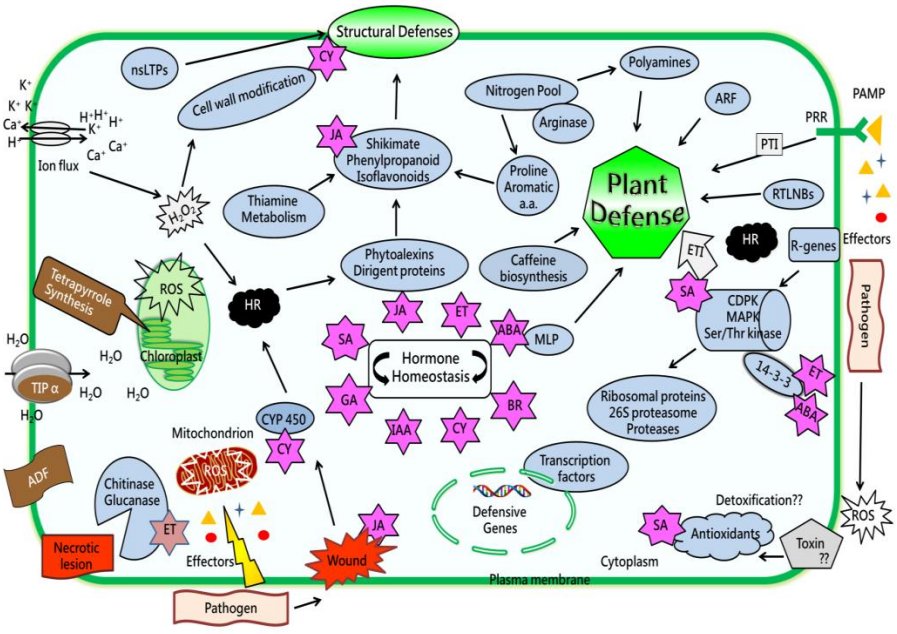


Fig. 3.21 Expression patterns of Foc virulence related genes in shoot tissue at two time-points using qRT-PCR.

Chapter 4

Discussion



4. Discussion

4.1 Differential colonization of Foc in chickpea

Plant-microbe interaction studies have been revolutionized by the high throughput 'Omics' methodologies. However, specific aspects of plant-pathogen interactions namely pathogen entry, localization and colonization in the host and, spatial and temporal behavior of the pathogen in compatible and incompatible reactions are not answered by these methods (Cardinale, 2015). Such aspects could be understood using Confocal Laser Scanning microscopy (CLSM) and targeted gene expression analysis using qRT-PCR. The present study employed CLSM along with fungal mass estimation using qPCR to study the infection process of Foc 2, a highly virulent race of *Fusarium oxysporum* f.sp. *ciceri* active in Indian peninsula.

4.1.1 CLSM approach

To understand the possible mechanism of invasion of Foc in susceptible and resistant chickpea cultivars, transformation of the pathogen and its localization *in planta* paired with quantification in various plant tissues were performed. The five eGFP transformed Foc isolates did not show any altered phenotypic or virulence characteristics compared to the wild type; however, variation in GFP fluorescence was observed. The transformant D4 having the highest and uniform GFP fluorescence and virulence comparable to that of wild type was selected for studying the *in planta* pathogen progression using CLSM. Attachment and germination of fungal spores on to epidermal cells was observed within 3 dpi followed by fast penetration of root epidermis, cortex and xylem of the susceptible cultivar by 4 dpi. These processes were, however, impeded in the resistant cultivar. Further, no specialized structure during penetration of Foc 2 in plant epidermis was observed. It was simply done by growing hyphal branch but not the germ tube. These findings are in accordance with previous studies in case of *Fusarium oxysporum* f.sp. *radicis-lycopersici* (Lagopodi *et al.*, 2002), *Fusarium oxysporum* f.sp. *melonis* (Zvirin *et al.*, 2010) and *Fusarium oxysporum* f.sp. *fragariae* (Fang *et al.*, 2012). Interestingly, swellings were seen at penetration site of the penetrating hypha in the susceptible cultivar. This observation is also consistent with previous reports on other formae speciales of *F.*

oxysporum in case of tomato and strawberry (Lagopodi *et al.*, 2002; Bolwerk *et al.*, 2005; Fang *et al.*, 2012).

The transformed Foc 2 could be detected throughout the inspected plant parts during disease progression in susceptible inoculated cultivar (JGI), particularly with increasing fungal load. However, the pathogen could be seen only in root cortex region of inoculated resistant cultivar with very few mycelia escaping to vascular tissue. Similar colonization patterns for Foc races 0 and 5 in compatible and incompatible interactions in chickpea have been reported earlier (Jimenez-Fernandez *et al.*, 2013). Likewise, it has been demonstrated that *F. oxysporum* f.sp. *fragaria*e was confined in the epidermal layer of roots in the resistant strawberry cultivar (Fang *et al.*, 2012). Even in resistant pea cultivar, *F. oxysporum* f.sp. *pis*i was restricted to the initially infected root vessels in asymptomatic reactions (Zvirin *et al.*, 2010). These differences of the colonization in the susceptible and resistant cultivars could be correlated with the differential defense mechanisms harbored in the chickpea genotypes as highlighted in our previous studies (Gurjar *et al.*, 2012; Kumar *et al.*, 2015a).

4.1.2 qPCR approach

Further, quantification of fungal colonization in various tissues of the resistant and susceptible chickpea cultivars was performed by qPCR. Based on this, four distinct phases of fungal proliferation can be put forth. In phase 1 (0 hpi) high amount of fungal DNA was observed in both the cultivars, indicating adherence and germination of the fungal spores. Phase 2 (16 hpi to 4 dpi) was marked by the decrease in the fungal DNA suggesting the degradation of a fraction of the hyphae due to autophagy. Similar phenomenon was reported in *F. graminearum* during colonization in wheat (Josefsen *et al.*, 2012). This could be attributed to the fact that, before the pathogen colonizes the plant to the extent that it can derive nutrients from the host, it undergoes intracellular degradation to supply nutrients to the non-assimilating fungal structures. Steep increase in fungal DNA was observed in phase 3 (4 dpi—14 dpi) indicating widespread colonization of the fungus in JGI compared to that in DVI. During this phase, JGI also showed the typical wilting symptoms like drooping of petioles etc. Lastly, the phase 4 (14 dpi—28 dpi) was marked by decrease in fungal DNA content in JGI (Fig. 3.9). This indicated that the fungus proliferated massively in JGI till nutrients from the host were available (phase

3), resulting in high mycelial mass and pathogen DNA. After the nutrients from the host were exhausted (due to wilting), the pathogen switched to conidiation, leading to reduced mycelial mass and as a result, pathogen DNA. Alternatively in DVI, successful activation of defense responses early in the infection process (before phase 3) might have restricted the fungal proliferation throughout the course of infection.

4.2 Foc virulence strategy - a candidate gene expression based study

Successful infection of the pathogen to the host plant requires a number of important steps like recognition and adhesion to host tissue, degradation of host tissue and resistance to host antimicrobials etc. In the present study, some of the genes involved in these processes were analyzed by qRT-PCR. It is known that hyphae of phytopathogenic fungi navigate the host surface topography for identifying the vulnerable sites of invasion where they mechanically penetrate by expansion of growing hyphal tip (Gow *et al.*, 1993; Hardham, 2001). Furthermore, fungal hyphae are predicted to resist opposing forces at their tips during such penetration (Cardinale, 2015). Thus fungal morphogenesis is an essential component of host invasion (Gow *et al.*, 2002). Chitin is considered as a structurally important component of fungal cell walls and chitin synthases, the enzymes implicated in chitin synthesis, belonging to different divisions and classes are found in fungi. In the present study, chitin synthase 7 (*Chs7*) was preferentially expressed in the JGI suggesting strong defense response in DVI. Our results correlated well with the earlier reports wherein chitin synthases are reported to be essential for virulence and invasive growth during plant infection in fungi like *Magnaporthe grisea* (Kong *et al.*, 2012) and *Fusarium oxysporum* (Madrid *et al.*, 2003).

As communications between the pathogen and the plant are critical for disease development, signaling pathways that mediate these communications are also important. Several proteins like G proteins, MAP kinases, protein kinases A are known to be involved in such pathways and studies have shown their importance in fungal development and virulence (Kasahara & Nuss, 1997; Sagaram & SHIM, 2007; Charoensopharat *et al.*, 2008; Yu *et al.*, 2008; Tzima *et al.*, 2012). Our results were in accordance with the above mentioned studies where G protein β subunit expression was the least in DVI in which pathogen could not

establish the infection. Mitochondrial carrier proteins (MCPs) are small transport proteins of the mitochondrial inner membrane that catalyzes the transport of metabolites across the inner membrane with a high degree of substrate specificity (Palmieri, 1994; Nelson *et al.*, 1998; Belenkiy *et al.*, 2000). The present study also revealed the expression of MCP *Fow1* preferentially in JGI. This highlights the importance of this gene during establishment in the host. Our findings correlate with earlier reports where *Fow1* was shown to be essential for colonization during infection (Inoue *et al.*, 2002; Gurjar *et al.*, 2012). We also detected higher expression of the enzyme glucanosyltransferase, which is essential for fungal morphogenesis, and reported to be required for virulence (Caracuel *et al.*, 2005; Zhang *et al.*, 2011). The present study validated the expression of these genes (Gurjar *et al.*, 2012) using qRT-PCR over a wider range of duration, from 0 hpi to 28 dpi. Apart from these genes, several others were observed for their expression in the present study. Cell wall glycoproteins of the fungus are involved in species specific adhesion processes and also increase resistance of fungi to antimicrobial proteins produced by plants (Narasimhan *et al.*, 2003; Lee *et al.*, 2010). Interestingly, the expression pattern of the cell wall extracellular matrix protein (CWEMP) showed gradual increase in resistant cultivar upon inoculation. This might be due to the pathogen making constant attempts to establish itself in the resistant cultivar, while protecting itself from the strong defense response of the resistant cultivar. In JGI however, initial increase in CWEMP expression accounted for successful establishment of the pathogen in the plant, followed by a transient decrease, which corresponded to phase 2; i.e. autophagy of the fungus for its own growth. When the fungus attains sufficient biomass to derive nutrients from the plant, the increase in CWEMP expression again indicates the attempts of the pathogen to colonize newer plant tissues.

Phytopathogenic fungi produce an array of extracellular hydrolytic cell wall degrading enzymes (CWDE) that enable them to penetrate and infect the host tissue. Plant cell wall degradation is essential in pathogenesis owing to the fact that the pathogen invades epidermis and grows through cortex to finally reach at xylem (Jorge *et al.*, 2006). Therefore, the expression of CWDEs like PG, PL and *XLY* was evaluated in the present study. The expression of *XLY* was detected only in JGI and only at late stage of disease, when the pathogen entered the necrotrophic phase. In this phase, it probably played role in plant cell wall degradation.

Our results are in accordance with the published reports indicating increased *XLY* activity with the disease progression (Jorge *et al.*, 2006) and contribution of *XLY* to the infection process by inducing necrosis of the infected plant tissue (Noda *et al.*, 2010).

Interestingly, *PG* expressed initially only in JGI and its expression decreased with the disease progression and symptom development in JGI, in accordance with the results reported previously (Jorge *et al.*, 2006). On the contrary, the expression of *PG* was detected only at late stages in DVI. These enzymes loosen the pectin network in plant cell wall and help the fungi to secrete the digestive enzymes for nutrient acquisition. Thus, their expression in late stages in resistant plants suggests attempts of the fungus to acquire nutrients in growth limiting environment, where the fungus is present in minimal number owing to the strong defense response of the resistant host. Furthermore, as evidenced by confocal studies, plant architecture of DVI remained almost normal throughout the disease progression, due to which intact pectin of the plant cell wall might have induced the production of PGs. Such expression of PGs in nutrient depriving condition as well as in the presence of pectin has been reported earlier (Di Pietro & Roncero, 1998; Wubben *et al.*, 2000). Another CWDE, PL was found to express at three time-points covering initial invasion and colonization, invasion from cortical cells to xylem and necrotic phases. Along with PG, PL has also been postulated to be involved in plant penetration and colonization by phytopathogens (Shih *et al.*, 2000).Our results are in accordance with the study depicting abundant expression of PL gene early in the infection process and required for full virulence in *Alternaria brassicicola* (Cho *et al.*, 2015).

4.3 Chickpea defense response - a candidate gene expression based study

Multiple events are involved in a successful plant defense during pathogen attack. In addition these defense mechanisms are governed by an array of genes which either singly or synergistically function in mounting the response promptly. In an attempt to elucidate defense in chickpea against Foc attack, candidate genes known to be involved in various processes active against pathogen colonization were evaluated using qRT-PCR approach.

Among the first category, enzymes acting on major structural components of fungal cell wall, chitinases and glucanases showed increased expression in the susceptible cultivar after

substantial increase in fungal invasion in xylem vessels. This reveals virulence strategy of the pathogen i.e. the pathogen successfully establishes in the susceptible host plant till the host plant recognizes the pathogen and then after this stage elevated expression of these enzymes lead to hypersensitivity and self-tissue degradation in the host. In accordance with this, previous reports showed that degradation products of fungal chitin and glucan function as PAMPs and trigger the hypersensitive response in host plants (Jones & Dangl, 2006). Chitinases and β-1, 3 glucanases have been reported as the key enzymes in the defense mechanism of chickpea against Foc1 and Foc 0 by earlier studies (Arfaoui *et al.*, 2007). Similarly, β-glucosidases in plants are involved in a variety of processes including defense against herbivores and fungi by release of toxic compounds from inactive glycosides, activation of lignin precursors (Cairns & Esen, 2010) and plant chemical defense (Morant *et al.*, 2008; Pankoke *et al.*, 2013). Glycosyltransferases are also known to glycosylate specialized metabolites in plants that have important functions in defense against biotic and abiotic stresses (Owatworakit *et al.*, 2013; Singh *et al.*, 2016). In accordance with these reports, both β-glucosidase and glycosyltransferases showed elevated expression in resistant inoculated cultivar in the present study.

In leguminous plants, various classes of phenylpropanoids are synthesized and accumulated in response to pathogen attack. (Bednarek *et al.*, 2001; Nimbalkar *et al.*, 2006; Gurjar *et al.*, 2012). They were reported as phytoanticipins, phytoalexins, structural barriers, modulators of pathogenicity, and/or activators of plant defense genes (Dakora & Phillips, 1996; Stafford, 1997; Mansfield, 2000). The expression of all the three key enzymes in resistant/susceptible scenario, in our study positively correlates with earlier reports. These genes were expressed at higher levels in resistant inoculated plants as compared to the susceptible inoculated ones. Interestingly, healthy (control) susceptible plant showed higher expression of chalcone synthase and isoflavone reductase as compared to inoculated indicating possible effect of pathogen virulence in shutting down of defense related expression to the level desirable for successful pathogen establishment. Thus, resistant reaction can be characterized by an early defense response by the plant to oppose establishment of pathogen whereas, susceptible reaction by late activation of this system upon entry of the pathogen in the vasculature.

Among the category of stress management genes, genes like CYP 450 and PR 10 showed enhanced expression in JGI. Plant cytochrome P450 monooxygenases (CYP) mediate synthesis and metabolism of many physiologically important primary and secondary compounds that are related to plant defense against a range of pathogenic microbes and insects (Bednarek *et al.,* 2009; Clay *et al.,* 2009; Liu *et al.,* 2010; Kidd *et al.,* 2011). In our study, significant induction of CYP450 expression in susceptible plant shows an attempt of the plant to oppose pathogen establishment with the disease progression. Similarly, pathogenesis related proteins are induced as systemic acquired resistance in plants in relation to pathogen attack. In chickpea, production of these proteins has been reported against various pathogens including Foc (Saikia *et al.,* 2005; Gurjar *et al.,* 2012). Thus increase in expression of these proteins specifically *PR10* in susceptible plant indicates attempt to combat the pathogen colonization. Histone proteins are involved in chromosome duplication of eukaryotic genome and are shown to be overexpressed in plants during pathogen attack (Jeong *et al.,* 2005). However, in the present study, expression of H2A histone proteins seemed to be unaffected. Reactive Oxygen Species (ROS) accumulation is associated with plant defense against pathogens (Huckelhoven & Kogel, 2003). However, ROS resulting from biotic and abiotic stresses can cause severe cellular damage and are thus tightly regulated and detoxified by complex enzymatic and non-enzymatic mechanisms (Mittler, 2002). *SOD*, an enzymatic protein and Metallothionein, a non-enzymatic protein both are H_2O_2 scavenging enzymes. Both were expressed significantly higher in JGI compared to DVI suggestive of reduced elicitor-induced hydrogen peroxide formation. Similar results were obtained in transgenic rice plants overexpressing *OsMT2b,* a metallothionein gene where overexpression increased susceptibility of plant to bacterial blight and blast fungus (Wong *et al.,* 2004).

The genes like *60 SRP* and *LRR* showed significantly elevated expression in DVI. Translation, post translational modifications and protein turn over play important role in survival of cell under different developmental stages and environmental conditions while ribosomal proteins play important role in translation. Thus it is likely that higher amount of proteins are synthesized during plant defense, which is a complex process demanding expression and interaction of several proteins at a time in DVI. The majority of disease resistance genes in plants encode nucleotide-binding site leucine-rich repeat (NBS-LRR)

proteins. Plant NBS-LRR proteins act through a network of signaling pathways and induce a series of plant defense responses, such as activation of an oxidative burst, calcium and ion fluxes, mitogen-associated protein kinase cascade, induction of pathogenesis-related genes, and the hypersensitive response (Hammond-Kosack & Parker, 2003; Belkhadir *et al.*, 2004). Our results are in accordance with these reports.

4.4 Chickpea defense response- a global transcriptome approach using LongSAGE methodology

Gene expression profiling upon biotic stresses has been broadly studied in a number of plant species using variety of transcriptomic tools (Casassola *et al.*, 2013). In the present study, we performed a comparative transcriptome analysis of susceptible and resistant cultivars of chickpea in response to Foc inoculation using a SAGEseq approach. Serial Analysis of Gene Expression (SAGE) (Velculescu *et al.*, 1995) gene expression profiling method that can be used to characterize the transcription levels of thousands of genes simultaneously. Since its description in 1995, it has been used in a number of conditions and organisms with robustness and substantial improvements (Anisimov, 2008). In plants, it has been mainly used for studying host-pathogen interactions as well as abiotic stress response (Jung *et al.*, 2003; Matsumura *et al.*, 2003; Fregene *et al.*, 2004)

The comparison of four SAGE libraries in the present study has elucidated key factors involved in chickpea resistance mechanisms upon Foc inoculation. As presented in results, number of DEGs in resistant and susceptible cultivars under control condition depicted the genotypic difference that might affect the plant's response upon fungal inoculation. Overall the four DGE sets revealed many biological processes induced in resistant cultivar upon inoculation which otherwise were inactive in absence of pathogen. On the contrary, many biological processes were repressed in susceptible cultivar upon fungal inoculation indicating that the pathogen might govern the host metabolic machinery for its own survival. Among these, important biological processes as highlighted in the PPI network as well as represented in Mercator terms of the DGE sets are discussed below.

4.4.1 Protein metabolism contributing to chickpea defense

An interesting feature in the transcriptome analyses was "protein synthesis, degradation and post-translational modifications" representing the top functional class among the DEGs and unique genes. Network analysis also showed higher abundance of these proteins with both intra and inter connections with other Foc induced proteins. In our study, in resistant cultivar, significant up-regulation of ribosomal proteins (60S, 40S and 50S), ubiquitin-conjugating enzyme E2 and protein kinases suggests that protein synthesis plays an important role in disease resistance wherein ubiquitination has already been suggested to be crucial contributor of plant innate immune response (Marino *et al.,* 2012; Pollier *et al.,* 2013). Furthermore, 26 proteasome subunits and F-box proteins known to contribute to both basal defense as well as R gene mediated defense; several proteases (serine, cysteine, aspartate) and metacaspases shown to be important for R gene mediated defense in *Arabidopsis* were up-regulated in DVI indicating their importance in chickpea defense against Foc. Increasing evidences have shown that many key components of plant disease resistance undergo protein degradation in response to pathogen infection for mounting defense hypersensitive response (HR) and systemic acquired resistance (SAR) (Suty *et al.,* 2003; Yao *et al.,* 2012; Piisila *et al.,* 2015; Pogany *et al.,* 2015).

4.4.2 Signaling required for quick defense response

"Signaling" was another functional class represented with the highest transcript abundance in resistant inoculated cultivar. It mainly included calcium Ca (2+), G-proteins and light induced signaling, followed by receptor like kinases and MAP kinases based signaling. Recent studies have revealed that mechanism of Ca (2+)-mediated signaling could be regulated by other cell signaling systems such as ubiquitin-proteasome system to mount precise and prompt plant defense responses. As an important secondary messenger in plant cells, changes in Ca^{2+} concentration have been detected during effector-triggered immunity (ETI), specifically in the incompatible interactions between *Pseudomonas syringae pv. tomato* containing *avrRpm1*; and *RPM1* in Arabidopsis (Grant *et al.,* 2000; Zhang *et al.,* 2014). Moreover, calcium dependent

protein kinases (CDPKs) are the key players translating the pathogen signal induced Ca^{2+} concentration into plant defense reactions like synthesis of ROS, altered gene expression and synthesis as well as signaling of phytohormones like salicylic acid (SA) (Schulz *et al.*, 2013). Thus, up-regulation of Ca (2+) mediated signaling and ubiquitin-conjugating enzyme E2, a component of ubiquitin-proteasome system in our study indicates possible strengthening of defense response in resistant cultivar in accordance with the above-mentioned studies.

Heterotrimeric G-proteins, well known in stress signaling (Suharsono *et al.*, 2002), have been recently discussed as activators of plant cell death, mediators of stomatal closure signaling (Zhang *et al.*, 2012), involved in cell wall biogenesis/metabolism and ABA signaling (Klopffleisch *et al.*, 2011; Nitta *et al.*, 2015). In the present study, induction of G protein signaling in resistant inoculated cultivar suggests their function in early defense response against the pathogen. Light induced signaling was also observed in up-regulated transcripts of resistant inoculated plant. In addition to providing life sustaining energy for growth and developmental processes, light also plays a role in plant defense against pathogens and is required for activation of several defense genes and regulation of the cell death response (Fryer *et al.*, 2003; Chandra • Shekara *et al.*, 2006). Importance of light and photoreceptors in precise resource allocation between growth and defense in plants is an emerging concept (Ballare, 2014).

Receptors like kinases (RLKs) in plants have diverse functions including development, growth, hormone perception and the response to pathogens. In addition to general elicitor recognition, RLKs with LRR motifs participate in the recognition of pathogen avirulence factors (Avr genes) produced by specific strains of plant pathogens (Lee *et al.*, 2006). In the present study, 27 transcripts pertaining to RLKs with varied motifs were upregulated in DVI compared to JGI. RLK regulation has also been linked to ubiquitination as a means of targeting receptors for degradation to mitigate plant immune response (Goff & Ramonell, 2007). In previous studies (Kanzaki *et al.*, 2008; Singh & Zimmerli, 2013) RLKs with lectin motif have been reported to be involved in plant resistance to pathogens. Our analysis also indicated the up-regulation of these kinases in DVI compared to JGI. Thus, in our study the role of receptor kinases in conjunction with ubiquitin-conjugating enzyme E2 is suggestive of providing the resistant cultivar an advantage in mounting defense response. Role of Mitogen

Activated Protein Kinases in plant defense upon pathogen associated molecular pattern (PAMP) treatment (Nitta *et al.*, 2014) and in response to insect pests (Hettenhausen *et al.*, 2015) has also been reported. Up-regulation of MAP kinases 3, 5, 16 and RALF (Rapid alkalization factor) like 33 in resistant inoculated plant in our study, affirms their role in plant defense endorsing earlier reports.

4.4.3 Hormone metabolism- Key players in chickpea defense

Plant hormones, ethylene, jasmonic acid (JA), and salicylic acid (SA) play crucial role in plant growth and response to environmental cues. Similarly the role of other plant hormones, namely auxins, abscisic acid (ABA), cytokinins, gibberellins, and brassinosteroids in plant immunity has recently been reported (Denance *et al.*, 2013). In the present study, the transcripts related to several phytohormones, particularly the genes related to auxin induction (especially GH 3.6 like) were up-regulated in resistant cultivar. However, association of activation of *OsGH3.2* or *OsGH3.8* with the inhibition of cell wall-loosening protein expansins in rice and GH3.5 expression in *Arabidopsis* have been reported to enhance broad-spectrum resistance to phytopathogens (Ding *et al.*, 2008; Fu *et al.*, 2011) while GH 3.6, the closest family member of GH 3.5, has been shown to impart disease susceptibility in *Arabidopsis* (Zhang *et al.*, 2007).

In the present study, the genes related to cytokinin synthesis and signal transduction, namely adenylate isopentenyltransferase 5 (IPT) and UDP glycosyltransferases (UDPGs 85A5 and 73C3) were up-regulated, while cytokinin dehydrogenase and IPT 3 were exclusively detected in DVI. This indicated cytokinin homeostasis having a great share in structuring the plant defense response. Previous reports in *Arabidopsis* have shown the involvement of cytokinin homeostasis (cytokinin synthases, dehydrogenases and glycosyltransferases) in resistance to *Verticillium longisporum* (Siemens *et al.*, 2006; Reusche *et al.*, 2013). Abscisic acid (ABA) is another phytohormone which has emerged as a complex modulator of plant defense responses as shown in *Arabidopsis* (Hu *et al.*, 2008; Feng *et al.*, 2012). In the present study, several transcripts associated with ABA synthesis, degradation and signal transduction were up-regulated in DVI, which indicates their key role in defense against the pathogen.

Further, a transcript encoding theobromine synthase 2, involved in synthesis of caffeine, was detected only in DVI. Caffeine acts as a protectant from pathogen (Kim & Sano, 2008) and also in combating fungal infections (Kim *et al.*, 2011). As the role of caffeine is being deciphered in plant defense mechanisms, effects of SA and JA, the key players of plant defense mechanisms, on expression of caffeine biosynthetic enzymes is also emerging (Kim & Sano, 2008; Kim *et al.*, 2011).

4.4.4 Biotic and abiotic stress response enriching chickpea immunity

The transcriptome analyses in the present study revealed both biotic and abiotic stress responsive transcripts; with higher abundance of characteristic candidates in resistant cultivar compared to susceptible. As presented in Results section, auxin binding proteins show high similarity to germin like proteins, implicated to play decisive roles in plant defense and possess antioxidant enzyme activities (Wang *et al.*, 2013). Chitinases, being able to degrade the structural components of fungal pathogens, are important for plant defense (Sela-Buurlage *et al.*, 1993). Previous studies have demonstrated the relationship between accumulation of extensins, the hydroxyproline rich glycoproteins (HRGPs) in plant cell wall and increased resistance to disease (Raggi, 2000; Ribeiro *et al.*, 2006). Recently, overexpression of these proteins has been shown to restrict pathogen invasion and enhance the tolerance to *Clavibacter* in transgenic tomato (Balaji & Smart, 2012). Thus higher expression of these genes in resistant inoculated cultivar in our study depicts their importance in plant defense.

Major latex proteins (MLPs), observed with higher expression in DVI belong to the Bet v 1 family, also known as the pathogenesis related 10 (*PR10*)-like protein family (Radauer *et al.*, 2008). Earlier studies from our lab have also reported the role of Bet v 1 protein in chickpea resistance to Foc (Gurjar *et al.*, 2012; Kumar *et al.*, 2016). Recent studies have shown MLPs to be important for plant defense in *Medicago* (Kiirika *et al.*, 2014) as well as a positive regulator of ABA conferring drought tolerance in *Arabidopsis* (Wang *et al.*, 2015); highlighting their significance in biotic and abiotic stress response. Transcripts for several other pathogenesis related proteins were also detected in stress response category with higher abundance in resistant inoculated cultivar. These proteins provided R gene mediated response,

strong lignification, proteinase inhibitory activities and chaperon like functions enriching the defense response of resistant cultivar against the pathogen.

4.4.5 Overview of defense responses in chickpea

A schematic representation of transcriptome comparisons and PPI network of cell responses contributing to plant defense in resistant cultivar is depicted in Fig. 4.1. It appears that Foc inoculation in resistant chickpea cultivar triggers ROS production mainly in chloroplast and mitochondria, making them important contributors of ROS during defense responses. While, SA production triggered due to ROS (H_2O_2 bursts) plays a crucial role in maintaining redox homeostasis through antioxidant activity by increasing ROS scavenging and minimizing host tissue damage. Several factors like non-specific lipid transfer proteins (nsLTPs), CYP450, dirigent proteins and phytoalexins are important in generating defensive shield over plant surfaces and thus contributing to successful structural defense. In addition, expression of signaling components and pathogenesis related proteins in resistant cultivar gives the plant an upper hand in mounting prompt defense. Conversely certain factors such as *ADF, TIPα* and tetrapyrrole synthesis add to susceptibility of the plant. Thus, overall the key difference between resistant and susceptible plants was timely detection of invading pathogen and rapid and immediate activation of defense responses in the resistant cultivar in response to pathogen effectors.

4.5 Interacting genes of Foc- as revealed in LongSAGE analysis

Comparative analysis of transcriptomes of both cultivars challenged by Foc revealed a large number of Foc genes expressed only in JGI (533). Only five Foc genes (three of which were uncharacterized) were expressed only in DVI substantiating the strong defense strategy of the cultivar.

4.5.1 Foc genes up-regulated in JGI

Only 18 genes were differentially expressed in both the cultivars, all of which were up-regulated in JGI. Among these genes were the candidates essential for invasion, growth and

establishment during plant infection. One of the transcripts shared similarity with serine rich protein domain of which has been shown to be important part of adhesins in human fungal pathogens (Siboo *et al.*, 2005). Adherence of microorganisms to host tissue is a prerequisite

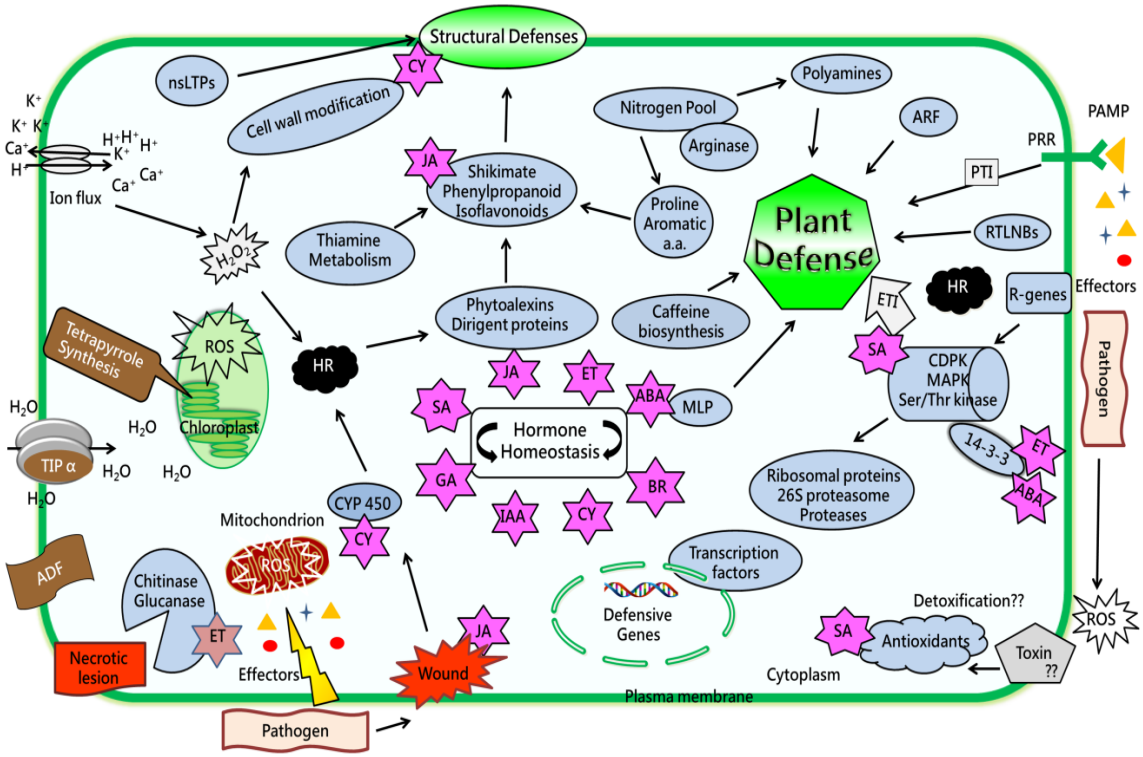


Figure 4.1: Schematic representation of interconnections of various biological processes induced in chickpea. Representation shows cell processes contributing to defense response in resistant cultivar (Blue and green color) with few processes that might render weakened response in susceptible cultivar (brown color).

for tissue invasion and infection. Another up-regulated transcript was glucosidase, mainly required for degradation of cellulose in plant cell wall, making it an essential enzyme for invasion of fungus (Glass *et al.*, 2013).

Heat shock proteins (HSP) are known to prevent apoptosis and cellular damage by inhibiting protein aggregation, in addition to function as endogenous danger signals (Osterloh & Breloer, 2008). In case of *Fo* these proteins have been shown to be produced in response to phytoalexin like defense responses of host plant (Thanonkeo *et al.*, 2000). Specifically HSP30

is known to be involved in energy conservation by inhibiting ATPase during stress conditions (Tereshina, 2005; Tiwari *et al.*, 2015). Thus up-regulation of these genes in JGI indicates that the pathogen can sustain in the host fighting against the host's defense but fails to do so in DVI, the resistant host. Histones are associated with chromatin alterations which induce genome-wide and local changes in gene expression and affect a variety of processes in response to internal and external signals. Thus elevated expression of histones H2A and H3 (of pathogen) in JGI supports pathogen colonization by reprogramming self-gene expression (and hence metabolism).

As presented in the results, few transcripts functionally associated with fungal cytoskeleton were also up-regulated in JGI. The literature survey highlighted their importance in fungal survival as discussed below. Tropomyosin is an important actin filament-stabilizing protein and fimbrin is a cytoskeletal protein associated with microfilament core bundles. The balance of fimbrin and tropomyosin was shown to be important for endocytosis and cytokinesis in fission yeast (Skau & Kovar, 2010), *Aspergillus nidulans* (Upadhyay & Shaw, 2008) and *Fusarium graminearum* reducing the rate of mycelial growth and conidiation in host tissue (Zheng *et al.*, 2015). A polarized growth protein rax 2 is required for establishment of growth sites as shown in *Candida albicans* (Gonia *et al.*, 2013). Woronin body major protein is described as highly refractile particle associated with the septa of filamentous fungi (Markham & Collinge, 1987) and functions as a septal plug after hyphal injury to prevent excessive loss of cytoplasm (Momany *et al.*, 2002). Phosphatidate cytidylyltransferase is one of the enzymes required for synthesis of phospholipids, essential components of cell membrane. Besides the role of building blocks of lipid bilayer, it influences the cellular behavior such as endocytosis (Bohdanowicz & Grinstein, 2013).

4.5.2 Foc genes uniquely expressed in JGI

Several Foc genes revealing diverse metabolic processes were expressed only in JGI. As mentioned in results, S-adenosylmethionine decarboxylase (*SAMD*), one of the enzymes involved in polyamine biosynthesis was among them. Polyamines have been determined to be important for virulence in several human bacterial pathogens (Wortham *et al.*, 2010; Russo *et*

al., 2011; Di Martino *et al.*, 2013). The *SAMD* mutants of *Penicillium marneffei* (Kummasook *et al.*, 2013) and *U.maydis* (Valdes-Santiago *et al.*, 2012) showed defects in pathogenesis in their specific hosts. In our study, we could observe polyamine synthesis only in JGI but not in DVI indicating successful establishment of pathogen possibly through polyamine metabolism playing a role in virulence.

In the present study, several CWDEs of Foc were detected only in JGI suggesting that synthesis of these enzymes facilitated the tissue invasion and establishment of Foc in JGI. CWDEs have previously been shown to be glucose-repressed and showed elevated expression under glucose-derepressing conditions in *M. oryzae* (Fernandez *et al.*, 2012). In our study, we have identified the transcripts of Foc during the course of host colonization which encompasses the metabolism of alternative carbon sources such as plant cell wall polysaccharides. For example, Beta-1, 6-galactanase was shown to be an important component of secretome of vascular wilt pathogen *Verticillium albo-atrum* (Mandelc & Javornik, 2015) while knock out mutants of 1,3-beta-glucanosyltransferase have reduced virulence in tomato infecting *Fusarium* (Caracuel *et al.*, 2005).Few other transcripts like β-1,3- and β-1,6-endoglucanases have also been detected in the secretome as well as gene expression profiling of many phytopathogenic fungi during host colonization (Williams *et al.*, 2014; Mandelc & Javornik, 2015).

Several studies have identified peroxidases as workhorses of fungal antioxidant defense system (Missall *et al.*, 2004; Dietz *et al.*, 2006; Boyd *et al.*, 2013) and demonstrated that host-driven ROS detoxification is an essential virulence determinant in many fungal pathogens (Enjalbert *et al.*, 2007; Molina & Kahmann, 2007; Chi *et al.*, 2009; Lin *et al.*, 2009; Samalova *et al.*, 2014). Thus expression of peroxiredoxin PRX1, peroxiredoxin HYR, superoxide dismutase, peroxidase/catalase 2, monothiol glutaredoxin-5 exclusively in JGI might indicate their important role in host-driven ROS detoxification culminating in successful pathogen colonization.

4.5.3 Overview of Foc metabolism during pathogenesis in the susceptible host

Based on the transcriptome analysis and further functional categorization, a schematic overview of Foc metabolism that might be operational during pathogenesis and successful disease establishment in JG62 has been shown in Fig. 4.2. It includes the genes expressed only in JGI and are homologues of known virulence factors based on PHI database. As depicted in the figure, almost all biological processes, required for fungal invasion, growth and pathogenesis, that govern Foc metabolism in chickpea were active in JGI. Among these processes, plant cell wall degradation mediated by cutinase, endoglucanase, 1, 3-beta-glucanosyltransferase, glucosidase and aspartic proteinase has been shown to be an important virulence mechanism.

We also identified several Foc transcripts related to signal transduction. Since signal transduction cascades mediate communication between environmental signals and the cellular machinery controlling growth and differentiation, expression of various kinases along with serine/threonine protein kinases and protein phosphatases only in JGI might have accelerated the fungal colonization. Further, several Foc transcripts were identified only in JGI under cell rescue, defense and virulence category. This reveals an attempt of the fungus to evade the plant defense responses and circumvent challenging environment in the host thereby assisting fungal proliferation. In addition to these, all basic metabolic processes of fungus including carbohydrate, protein, lipid, energy and cytoskeleton related metabolism were functional indicating stable pathogen establishment hijacking the host metabolic machinery.

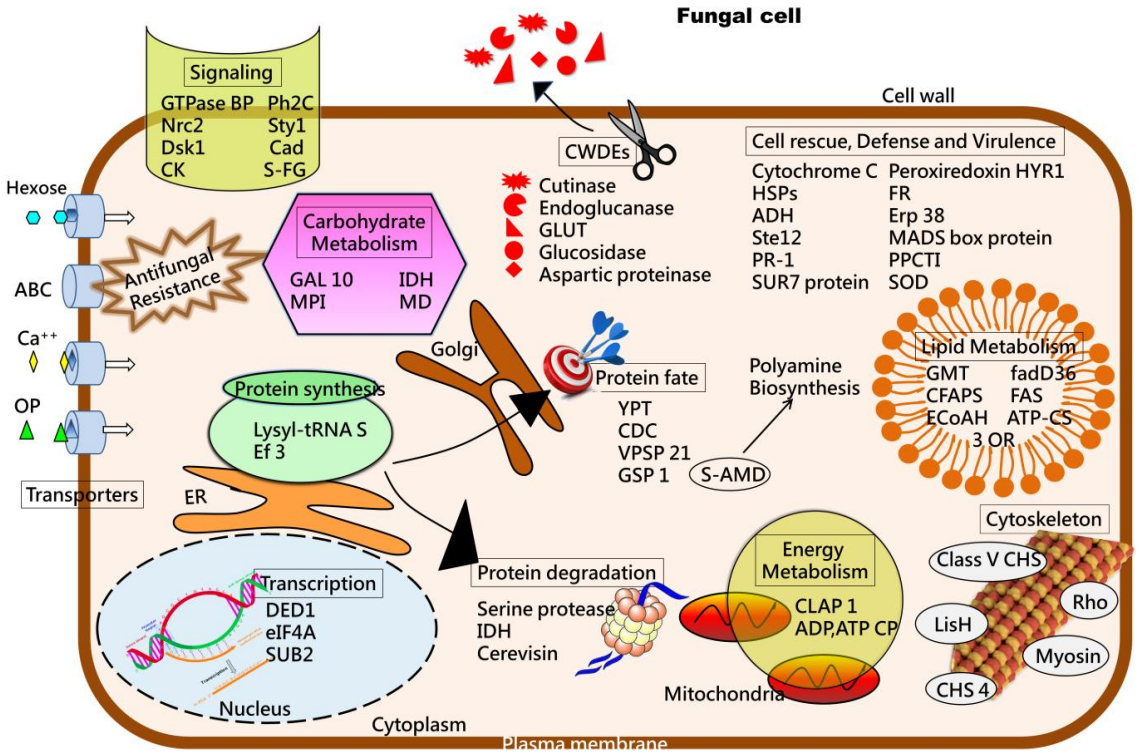
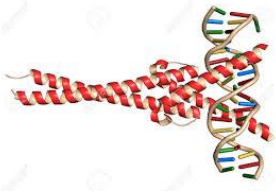
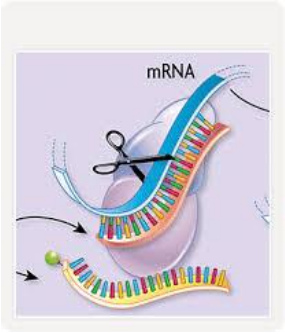


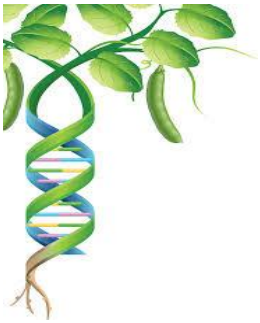
Fig. 4.2 Schematic overview of Foc metabolism during pathogenesis in the susceptible host. The overview shows several aspects of Foc metabolism operational in the susceptible host based on the transcriptomics and its functional classification. The presentation mainly includes the genes, expressed only in JG I, homologues of which are proven virulence factors in PHI database (Pathogen-Host Interaction).

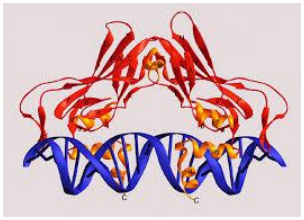
Chapter 5

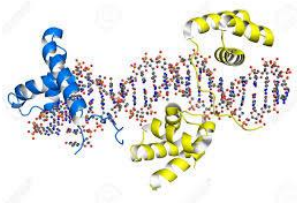
Summary and Future directions











5.1 Summary

Chickpea is an important source of dietary proteins, especially for the vegetarian population and is the most abundantly grown legume in India which contributes to 70% of the world production of this legume crop (FAOSTAT, 2014). Fusarium wilt, caused by *Fusarium oxysporum* Schlenchtend.: Fr. f. sp *ciceris* (Foc) (Padwick) Matuo and Sato, is one of the most destructive diseases of chickpea throughout the world and a major limiting factor of chickpea productivity. Foc is a root pathogen, which causes blockage of xylem vessels upon infection, preventing the uptake of water from the soil finally resulting in severe wilt and death of plants. The present thesis work was planned to characterize the chickpea-Foc interaction at molecular level using approaches like Confocal Laser Scanning Microscopy, quantitative reverse transcription PCR and transcriptome profiling using LongSAGE.

5.1.1 Differential colonization of Foc in chickpea cultivars

Possible mechanism of invasion of Foc in wilt resistant and susceptible chickpea cultivars was studied using CLSM of eGFP-transformed Foc 2. The transformant D4 having the highest and uniform GFP fluorescence and virulence comparable to that of wild type was selected for studying the *in planta* pathogen progression. Attachment and germination of fungal spores on to epidermal cells within 3 dpi followed by fast penetration of root epidermis, cortex and xylem of the susceptible cultivar by 4 dpi. These processes were, however, impeded in the resistant cultivar. The transformed Foc 2 could be detected throughout the inspected plant parts during disease progression in susceptible inoculated cultivar (JGI), particularly with increasing fungal load. However, the pathogen could be seen only in root cortex region of inoculated resistant cultivar with very few mycelia escaping to vascular tissue.

Quantification of fungal colonization in various tissues of the resistant and susceptible chickpea cultivars by qPCR uncovered four distinct phases of Foc proliferation in susceptible cultivar In phase 1 (0 hpi) high amount of fungal DNA was observed in both the cultivars, indicating adherence and germination of the fungal spores. Phase 2 (16 hpi-4 dpi) was marked by the decrease in the fungal DNA suggesting the degradation of a fraction of the hyphae due to autophagy. This could be attributed to the fact that, before the pathogen colonizes the plant

to the extent that it can derive nutrients from the host, it undergoes intracellular degradation to supply nutrients to the non-assimilating fungal structures. Steep increase in fungal DNA was observed in phase 3 (4 dpi—14 dpi) indicating widespread colonization of the fungus in JGI compared to that in DVI. During this phase, JGI also showed the typical wilting symptoms like flaccidity of leaves, drooping of petioles etc. Lastly, the phase 4 (14 dpi—28 dpi) was marked by decrease in fungal DNA content in JGI. This indicated that the fungus proliferated massively in JGI till nutrients from the host were available (phase 3), resulting in high mycelial mass and pathogen DNA. After the nutrients from the host were exhausted (due to wilting), the pathogen switched to conidiation, leading to reduced mycelial mass and as a result, pathogen DNA. Alternatively in DVI, successful activation of defense responses early in the infection process (before phase 3) might have restricted the fungal proliferation throughout the course of infection.

5.1.2 **Gene expression dynamics of chickpea and Foc during the interaction**

Expression of chickpea defense related genes revealed differential response of the cultivars to Foc challenge. Expression of chitinase and glucanase intensified only in the late stages of disease in susceptible inoculated cultivar while these genes were steadily expressed right from the beginning in resistant inoculated cultivar suggesting clever programming of plant itself to oppose the pathogen establishment. While the expression of β-glucosidase**,** glucan endo β-1,3 glucosidase and glycosyltransferase elevated at initial stage of colonization are indicative of their role in defense against the pathogen. Similarly phenylalanine ammonia lyase, Chalcone synthase and isoflavone reductase, three key enzymes of phenylpropanoid pathway expressed very high in resistant inoculated cultivar. Interestingly, these genes expressed high in susceptible control plants compared to susceptible inoculated ones indicating the effect of Foc virulence in shutting down the defense gene expression in the cultivar. Several other genes like WRKY, chaperonin, Pathogenesis related proteins, metallothionein and NBS-LRR were observed to be expressed more in resistant inoculated cultivar highlighting their role in defense mechanism.

Quantitative real time expression of Foc genes were assessed in both chickpea cultivars during the disease progression. Good concurrence was observed in the expression of genes with the pathogen proliferation phases obtained prior. Expression of genes involved in fungal morphogenesis, signaling, plant cell wall degradation in susceptible inoculated cultivar was in accordance with the colonization pattern. Many of these genes expressed least and at later stage of disease progression in resistant inoculated cultivar. The genes like chitin synthase, glucanosyltransferase, G protein β subunit and mitochondrial carrier protein, which play important role in fungal growth and morphogenesis, were expressed significantly during the initial colonization period, when the pathogen tried to establish in the host environment. This was followed by the decrease in expression of these genes pertaining to autophagy phase. The last phase was again the rise in expression revealing successful invasion and further proliferation of pathogen in the susceptible host. This rise was significantly high in JGI compared to that in DVI as the pathogen could colonize to great extent only in JGI.

5.1.3 Transcriptomic outcome of chickpea-Foc interaction

Transcriptome analysis of chickpea-Foc interaction revealed several chickpea defense related genes and Foc virulence related genes with differential as well as unique expression in both the cultivars. The comparison of four LongSAGE libraries in the present study elucidated key factors involved in chickpea resistance mechanisms upon Foc inoculation. Mercator term assignment and protein-protein interaction network analysis revealed important biological processes like protein, hormone metabolism, signaling and biotic-abiotic stress that might be playing a role in successful defense in resistant chickpea cultivar. In addition, certain transcripts related to tetrapyrrol synthesis, aquaporins and actin depolymerization factor, observed to be up-regulated in susceptible cultivar upon Foc challenge, could be associated with susceptibility based on literature evidences.

On the other hand, comparative analysis of transcriptomes of both cultivars challenged by Foc revealed a large number of Foc genes expressed only in JGI (533). Only five Foc genes (three of which were uncharacterized) were expressed only in DVI substantiating the strong defense strategy of the cultivar. Blast2GO analysis and comparison with PHI database

revealed a complete Foc metabolism functional only in susceptible inoculated cultivar. All the differentially expressed genes of Foc were up-regulated in susceptible and down-regulated in resistant inoculated cultivar.

5.2 Future directions

The present study mainly highlighted chickpea-Foc interaction using molecular tools like CLSM, qRT-PCR and LongSAGE. The aspects of Foc colonization in chickpea, defense and virulence gene expression and interaction transcriptomics have been elucidated in detail. A rich resource of data generated in this work can be followed further to address control of Fusarium wilt and development of sustainable resistant cultivars. Thus this study provides the following future directions.

- Studying the race specific chickpea defense response
- Functional characterization of chickpea defense genes
- Tracing the role of chickpea transcription factors in susceptibility and resistance reactions and identifying their downstream genes
- Searching the race specific chickpea transcription factors and their significance
- Targeted disruption, RNAi mediated inhibition and overexpression of specific genes to confirm the function of Foc virulence genes
- Identification of Foc transcription factors during pathogenesis

Chapter 6

Bibliography

Aboul-Soud MA, Yun B-W, Harrier LA, Loake GJ. 2004. Transformation of *Fusarium oxysporum* by particle bombardment and characterisation of the resulting transformants expressing a GFP transgene. *Mycopathologia* **158**(4): 475-482.

Ahmad F, Gaur P, Croser J. 2005. Chickpea (*Cicer arietinum* L.). *Genetic Resources, Chromosome Engineering and Crop Improvement-Grain Legumes* **1**: 185-214.

Ahmad MA, Iqbal SM, Ayub N, Ahmad Y, Akram A. 2010. Identification of resistant sources in chickpea against Fusarium wilt. *Pak. J. Bot* **42**(1): 417-426.

Alabouvette C, Lemanceau P, Steinberg C. 1993. Recent advances in the biological control of Fusarium wilts. *Pesticide Science* **37**(4): 365-373.

Alcoser SY, Kimmel DJ, Borgel SD, Carter JP, Dougherty KM, Hollingshead MG. 2011. Real-time PCR-based assay to quantify the relative amount of human and mouse tissue present in tumor xenografts. *BMC Biotechnology* **11**(1): 124.

Anisimov SV. 2008. Serial Analysis of Gene Expression (SAGE): 13 years of application in research. *Current pharmaceutical biotechnology* **9**(5): 338-350.

Appel D, Gordon T. 1994. Local and regional variation in populations of Fusarium oxysporum from agricultural field soils. *Phytopathology* **84**(8): 786-791.

Arfaoui, El Hadrami A, Mabrouk Y, Sifi B, Boudabous A, El Hadrami I, Daayf F, Cherif M. 2007. Treatment of chickpea with Rhizobium isolates enhances the expression of phenylpropanoid defense-related genes in response to infection by *Fusarium oxysporum* f. sp. *ciceris*. *Plant Physiology and Biochemistry* **45**(6-7): 470-479.

Armstrong G. 1981. Formae speciales and races of Fusarium oxysporum causing wilt diseases. *Fusarium: diseases, biology, and taxonomy*.

Arumuganathan K, Earle E. 1991. Nuclear DNA content of some important plant species. *Plant molecular biology reporter* **9**(3): 208-218.

Audic S, Claverie J-M. 1997. The Significance of Digital Gene Expression Profiles. *Genome Research* **7**(10): 986-995.

Bachem CW, Hoeven RS, Bruijn SM, Vreugdenhil D, Zabeau M, Visser RG. 1996. Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development. *The Plant Journal* **9**(5): 745-753.

Bakhsh A, Akhtar LH, Malik SR, Masood A, Iqbal SM, Qurashi R. 2011. Grain yield stability in chickpea (*Cicer arietinum* L.) across environments. *Pak J Bot* **43**(5): 2947-2951.

Balaji V, Smart CD. 2012. Over-expression of snakin-2 and extensin-like protein genes restricts pathogen invasiveness and enhances tolerance to *Clavibacter michiganensis* subsp. *michiganensis* in transgenic tomato (*Solanum lycopersicum*). *Transgenic Research* **21**(1): 23-37.

Ballare CL. 2014. Light regulation of plant defense. *Annual Review of Plant Biology* **65**: 335-363.

Beckman CH, Roberts E. 1995. On the nature and genetic basis for resistance and tolerance to fungal wilt diseases of plants. *Advances in Botanical Research* **21**: 35-77.

Bednarek P, Franski R, Kerhoas L, Einhorn J, Wojtaszek P, Stobiecki M. 2001. Profiling changes in metabolism of isoflavonoids and their conjugates in Lupinus albus treated with biotic elicitor. *Phytochemistry* **56**(1): 77-85.

Bednarek P, Pislewska-Bednarek M, Svatos A, Schneider B, Doubsky J, Mansurova M, Humphry M, Consonni C, Panstruga R, Sanchez-Vallet A. 2009. A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. *Science* **323**(5910): 101-106.

Belenkiy R, Haefele A, Eisen MB, Wohlrab H. 2000. The yeast mitochondrial transport proteins: new sequences and consensus residues, lack of direct relation between consensus residues and transmembrane helices, expression patterns of the transport protein genes, and protein–protein interactions with other proteins. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **1467**(1): 207-218.

Belkhadir Y, Subramaniam R, Dangl JL. 2004. Plant disease resistance protein signaling: NBS-LRR proteins and their partners. *Current Opinion in Plant Biology* **7**(4): 391-399.

Benjamini Y, Hochberg Y. 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B (Methodological)* **57**(1): 289-300.

Bhatti M, Kraft J. 1992. Effects of inoculum density and temperature on root rot and wilt of chickpea. *Plant Disease* **76**(1): 50-54.

Bohdanowicz M, Grinstein S. 2013. *Role of Phospholipids in Endocytosis, Phagocytosis, and Macropinocytosis*.

Bolwerk A, Lagopodi AL, Lugtenberg BJ, Bloemberg GV. 2005. Visualization of interactions between a pathogenic and a beneficial Fusarium strain during biocontrol of tomato foot and root rot. *Molecular Plant-Microbe Interactions* **18**(7): 710-721.

Booijink CC, Boekhorst J, Zoetendal EG, Smidt H, Kleerebezem M, De Vos WM. 2010. Metatranscriptome analysis of the human fecal microbiota reveals subject-specific expression profiles, with genes encoding proteins involved in carbohydrate metabolism being dominantly expressed. *Applied and environmental microbiology* **76**(16): 5533-5540.

Booth C. 1971. The genus fusarium. *The genus Fusarium.*

Both M, Eckert SE, Csukai M, Muller E, Dimopoulos G, Spanu PD. 2005. Transcript profiles of Blumeria graminis development during infection reveal a cluster of genes that are potential virulence determinants. *Molecular Plant-Microbe Interactions* **18**(2): 125-133.

Bove C, Lazzi C, Bernini V, Bottari B, Neviani E, Gatti M. 2011. cDNA-amplified fragment length polymorphism to study the transcriptional responses of Lactobacillus rhamnosus growing in cheese-like medium. *J Appl Microbiol* **111**(4): 855-864.

Boyd LA, Ridout C, O'Sullivan DM, Leach JE, Leung H. 2013. Plant–pathogen interactions: disease resistance in modern agriculture. *Trends in genetics* **29**(4): 233-240.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry* **72**(1-2): 248-254.

Brenner S, Johnson M, Bridgham J, Golda G, Lloyd DH, Johnson D, Luo S, McCurdy S, Foy M, Ewan M. 2000. Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays. *Nature biotechnology* **18**(6): 630-634.

Burgess L, Dodman R, Pont W, Mayers P. 1981. Fusarium diseases of wheat, maize and grain sorghum in eastern Australia. *Fusarium: diseases, biology, and taxonomy. The Pennsylvania State University Press, University Park*: 64-76.

Cairns JRK, Esen A. 2010. β-Glucosidases. *Cellular and Molecular Life Sciences* **67**(20): 3389-3405.

Cappelli K, Porceddu A, Verini-Supplizi A, Capomaccio S, De Marchis F, Falcinelli M, Gaiti A, Silvestrelli M. 2005. cDNA AFLP-based techniques for studying transcript profiles in horses. *Research in veterinary science* **79**(2): 105-112.

Caracuel Z, Martinez-Rocha AL, Di Pietro A, Madrid MP, Roncero MIG. 2005. *Fusarium oxysporum* gas1 encodes a putative beta-1, 3-glucanosyltransferase required for virulence on tomato plants. *Molecular Plant-Microbe Interactions* **18**(11): 1140-1147.

Cardinale M. 2015. Scanning a microhabitat: plant-microbe interactions revealed by confocal laser microscopy. *The plant microbiome and its importance for plant and human health*: 14.

Casassola A, Brammer SP, Chaves MS, Ant J, Grando MF. 2013. Gene expression: A review on methods for the study of defense-related gene differential expression in plants. *American Journal of Plant Sciences* **2013**.

Cawthraw S, Saunders GC, Martin TC, Sawyer J, Windl O, Reaney SD. 2009. Real-time PCR detection and identification of prohibited mammalian and avian material in animal feeds. *Journal of Food Protection®* **72**(5): 1055-1062.

Chalfie M, Kain S 1998. GFP: Green Fluorescent Protein Strategies and Applications: Wiley, New York.

Chand H, Singh S. 2005. Control of chickpea wilt (*Fusarium oxysporum* f sp. *ciceri*) using bioagents and plant extracts. *Indian Journal of Agricultural Sciences* **75**(2): 115-116.

Chandra-Shekara AC, Gupte M, Navarre D, Raina S, Raina R, Klessig D, Kachroo P. 2006. Light-dependent hypersensitive response and resistance signaling against Turnip Crinkle Virus in Arabidopsis. *The Plant Journal* **45**(3): 320-334.

Charoensopharat K, Aukkanit N, Thanonkeo S, Saksirirat W, Thanonkeo P, Akiyama K. 2008. Targeted disruption of a G protein α subunit gene results in reduced growth and pathogenicity in *Rhizoctonia solani*. *World Journal of Microbiology and Biotechnology* **24**(3): 345-351.

Chauhan Y, Nene Y, Johansen C, Haware M, Saxena N, Singh S, Sharma S, Sahrawat K, Burford J, Rupela O. 1988. *Effects of Soil Solarization on Pigeonpea and Chickpea. Information Bulletin No. 11*: International Crops Research Institute for the Semi-Arid Tropics.

Chen YC, Wong CL, Muzzi F, Vlaardingerbroek I, Kidd BN, Schenk PM. 2014. Root defense analysis against *Fusarium oxysporum* reveals new regulators to confer resistance. *Scientific Reports* **4**.

Chi M-H, Park S-Y, Kim S, Lee Y-H. 2009. A novel pathogenicity gene is required in the rice blast fungus to suppress the basal defenses of the host. *PLoS Pathog* **5**(4): e1000401.

Chibbar RN, Ambigaipalan P, Hoover R. 2010. Review: molecular diversity in pulse seed starch and complex carbohydrates and its role in human nutrition and health. *Cereal chemistry* **87**(4): 342-352.

Cho S, Muehlbauer FJ. 2004. Genetic effect of differentially regulated fungal response genes on resistance to necrotrophic fungal pathogens in chickpea (*Cicer arietinum* L.). *Physiological and Molecular Plant Pathology* **64**(2): 57-66.

Cho Y, Jang M, Srivastava A, Jang J-H, Soung N-K, Ko S-K, Kang D-O, Ahn JS, Kim BY. 2015. A Pectate Lyase-Coding Gene Abundantly Expressed during Early Stages of Infection Is Required for Full Virulence in *Alternaria brassicicola*. *Plos One* **10**(5): e0127140.

Clay NK, Adio AM, Denoux C, Jander G, Ausubel FM. 2009. Glucosinolate metabolites required for an Arabidopsis innate immune response. *Science* **323**(5910): 95-101.

Collard BC, Mackill DJ. 2008. Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Philosophical Transactions of the Royal Society B: Biological Sciences* **363**(1491): 557-572.

Conesa A, Gotz S. 2008. Blast2GO: A Comprehensive Suite for Functional Analysis in Plant Genomics. *International Journal of Plant Genomics* **2008**: 619832.

Coram TE, Pang EC. 2005. Isolation and analysis of candidate ascochyta blight defence genes in chickpea. Part I. Generation and analysis of an expressed sequence tag (EST) library. *Physiological and Molecular Plant Pathology* **66**(5): 192-200.

Croser J, Clarke H, Siddique K, Khan T. 2003. Low-temperature stress: implications for chickpea (Cicer arietinum L.) improvement. *Critical Reviews in Plant Sciences* **22**(2): 185-219.

Cubero OF, Crespo A, Fatehi J, Bridge PD. 1999. DNA extraction and PCR amplification method suitable for fresh, herbarium-stored, lichenized, and other fungi. *Plant Systematics and Evolution* **216**(3-4): 243-249.

Cunnington J, Lindbeck K, Jones RH. 2007. National Diagnostic Protocol for the Detection of Fusarium Wilt of Chickpea (*Fusarium Oxysporum* f. sp. *ciceris*). *Plant Health Australia, Camberra, Australia*.

Dakora FD, Phillips DA. 1996. Diverse functions of isoflavonoids in legumes transcend anti-microbial definitions of phytoalexins. *Physiological and Molecular Plant Pathology* **49**(1): 1-20.

Datson N, Van Der Perk-de Jong J, Van den Berg M, De Kloet E, Vreugdenhil E. 1999. MicroSAGE: a modified procedure for serial analysis of gene expression in limited amounts of tissue. *Nucleic Acids Research* **27**(5): 1300-1307.

Dave K, Punekar NS. 2011. Utility of *Aspergillus niger* citrate synthase promoter for heterologous expression. *Journal of biotechnology* **155**(2): 173-177.

De Palma M, D'Agostino N, Proietti S, Bertini L, Lorito M, Ruocco M, Caruso C, Chiusano ML, Tucci M. 2016. Suppression Subtractive Hybridization analysis provides new insights into the tomato (*Solanum lycopersicum* L.) response to the plant probiotic microorganism *Trichoderma longibrachiatum* MK1. *Journal of Plant Physiology* **190**: 79-94.

de Wit PJ, Mehrabi R, van den Burg HA, Stergiopoulos I. 2009. Fungal effector proteins: past, present and future. *Molecular Plant Pathology* **10**(6): 735-747.

Decorosi F, Viti C, Mengoni A, Bazzicalupo M, Giovannetti L. 2005. Improvement of the cDNA-AFLP method using fluorescent primers for transcription analysis in bacteria. *Journal of microbiological methods* **63**(2): 211-215.

Del Mar Jimenez-Gasco M, Perez-Artes E, Jimenez-Diaz RM. 2001. Identification of pathogenic races 0, 1B/C, 5, and 6 of *Fusarium oxysporum* f.sp. *ciceris* with random amplified polymorphic DNA (RAPD). *European journal of plant pathology* **107**(2): 237-248.

Delaunois B, Jeandet P, Clement C, Baillieul F, Dorey S, Cordelier S. 2015. Uncovering plant-pathogen crosstalk through apoplastic proteomic studies. *Plant cell wall in pathogenesis, parasitism and symbiosis*: 133.

Delporte M, Legrand G, Hilbert J-L, Gagneul D. 2015. Selection and validation of reference genes for quantitative real-time PCR analysis of gene expression in *Cichorium intybus*. *Frontiers in Plant Science* **6**(651).

Denance N, Sanchez-Vallet A, Goffner D, Molina A. 2013. Disease resistance or growth: the role of plant hormones in balancing immune responses and fitness costs. *Frontiers in Plant Science* **4**.

Di Martino ML, Campilongo R, Casalino M, Micheli G, Colonna B, Prosseda G. 2013. Polyamines: emerging players in bacteria-host interactions. *International Journal of Medical Microbiology* **303**(8): 484-491.

Di Pietro A, García-Maceira FI, Meglecz E, Roncero MIG. 2001. A MAP kinase of the vascular wilt fungus *Fusarium oxysporum* is essential for root penetration and pathogenesis. *Molecular Microbiology* **39**(5): 1140-1152.

Di Pietro A, Roncero MIG. 1998. Cloning, expression, and role in pathogenicity of pg1 encoding the major extracellular endopolygalacturonase of the vascular wilt pathogen *Fusarium oxysporum*. *Molecular Plant-Microbe Interactions* **11**(2): 91-98.

Dietz K-J, Jacob S, Oelze M-L, Laxa M, Tognetti V, de Miranda SMN, Baier M, Finkemeier I. 2006. The function of peroxiredoxins in plant organelle redox metabolism. *Journal of Experimental Botany* **57**(8): 1697-1709.

Ding X, Cao Y, Huang L, Zhao J, Xu C, Li X, Wang S. 2008. Activation of the indole-3-acetic acid-amido synthetase GH3-8 suppresses expansin expression and promotes salicylate-and jasmonate-independent basal immunity in rice. *The Plant Cell* **20**(1): 228-240.

Dubey SC, Suresh M, Singh B. 2007. Evaluation of Trichoderma species against *Fusarium oxysporum* f. sp. *ciceris* for integrated management of chickpea wilt. *Biological control* **40**(1): 118-127.

Duyvesteijn RG, Van Wijk R, Boer Y, Rep M, Cornelissen BJ, Haring MA. 2005. Frp1 is a *Fusarium oxysporum* F-box protein required for pathogenicity on tomato. *Molecular Microbiology* **57**(4): 1051-1063.

Egert M, De Graaf AA, Smidt H, De Vos WM, Venema K. 2006. Beyond diversity: functional microbiomics of the human colon. *Trends in microbiology* **14**(2): 86-91.

Enjalbert B, MacCallum DM, Odds FC, Brown AJP. 2007. Niche-specific activation of the oxidative stress response by the pathogenic fungus *Candida albicans*. *Infection and Immunity* **75**(5): 2143-2151.

Eswaran N, Parameswaran S, Anantharaman B, Raja Krishna Kumar G, Sathram B, Sudhakar Johnson T. 2012. Generation of an expressed sequence tag (EST) library from salt-stressed roots of *Jatropha curcas* for identification of abiotic stress-responsive genes. *Plant Biology* **14**(3): 428-437.

Evans SJ, Datson NA, Kabbaj M, Thompson RC, Vreugdenhil E, De Kloet ER, Watson SJ, Akil H. 2002. Evaluation of Affymetrix Gene Chip sensitivity in rat hippocampal tissue using SAGE analysis. *European Journal of Neuroscience* **16**(3): 409-413.

Exner V. 2010. Quantitative real time PCR in plant developmental biology. *Plant Developmental Biology: Methods and Protocols*: 275-291.

Fang X, Kuo J, You MP, Finnegan PM, Barbetti MJ. 2012. Comparative root colonisation of strawberry cultivars Camarosa and Festival by *Fusarium oxysporum* f. sp. *fragariae*. *Plant and soil* **358**(1-2): 75-89.

Farr DF, Bills GF, Chamuris GP, Rossman AY. 1989. *Fungi on plants and plant products in the United States*: APS press.

Feng DX, Tasset C, Hanemian M, Barlet X, Hu J, Tremousaygue D, Deslandes L, Marco Y. 2012. Biological control of bacterial wilt in *Arabidopsis thaliana* involves abscissic acid signalling. *New Phytologist* **194**(4): 1035-1045.

Fernandez J, Wright JD, Hartline D, Quispe CF, Madayiputhiya N, Wilson RA. 2012. Principles of Carbon Catabolite Repression in the Rice Blast Fungus: Tps1, Nmr1-3, and a MATE-Family Pump Regulate Glucose Metabolism during Infection. *PLoS Genetics* **8**(5): e1002673.

Fregene M, Matsumura H, Akano A, Dixon A, Terauchi R. 2004. Serial analysis of gene expression (SAGE) of host–plant resistance to the cassava mosaic disease (CMD). *Plant Molecular Biology* **56**(4): 563-571.

Fry WE. 2012. *Principles of plant disease management*: Academic Press.

Fryer MJ, Ball L, Oxborough K, Karpinski S, Mullineaux PM, Baker NR. 2003. Control of Ascorbate Peroxidase 2 expression by hydrogen peroxide and leaf water status during excess light stress reveals a functional organisation of Arabidopsis leaves. *The Plant Journal* **33**(4): 691-705.

Fu J, Liu H, Li Y, Yu H, Li X, Xiao J, Wang S. 2011. Manipulating broad-spectrum disease resistance by suppressing pathogen-induced auxin accumulation in rice. *Plant Physiology* **155**(1): 589-602.

Fukumura R, Takahashi H, Saito T, Tsutsumi Y, Fujimori A, Sato S, Tatsumi K, Araki R, Abe M. 2003. A sensitive transcriptome analysis method that can detect unknown transcripts. *Nucleic Acids Research* **31**(16): e94-e94.

Gams W, Nirenberg H. 1989. A contribution to the generic definition of Fusarium. *Mycotaxon* **35**(2): 407-416.

Gaur P, Pande S, Upadhyaya H, Rao B. 2006. Extra-large kabuli chickpea with high resistance to Fusarium wilt. *Journal of SAT Agricultural Research* **2**(1): 1-2.

Gerlach W, Nirenberg H. 1982. The genus Fusarium--a pictorial atlas. *Mitteilungen aus der Biologischen Bundesanstalt fur Land-und Forstwirtschaft Berlin-Dahlem*(209).

Giri A, Harsulkar A, Patankar A, Gupta V, Sainani M, Deshpande V, Ranjekar P. 1998. Association of induction of protease and chitinase in chickpea roots with resistance to *Fusarium oxysporum* f. sp. *ciceri*. *Plant Pathology* **47**: 693-699.

Glass NL, Schmoll M, Cate JHD, Coradetti S. 2013. Plant Cell Wall Deconstruction by Ascomycete Fungi. *Annual Review of Microbiology* **67**(1): 477-498.

Goff KE, Ramonell KM. 2007. The Role and Regulation of Receptor-Like Kinases in Plant Defense. *Gene Regulation and Systems Biology* **1**: 167-175.

Golinelli LP, Silva JT, Carvalho AC, Paschoalin VMF. 2016. Detection of Animal Products in Ruminant Feeds by Microscopy and Real Time PCR. *Journal of Veterinary Science & Technology* **2016**.

Gonia S, Norton J, Watanaskul L, Pulver R, Morrison E, Brand A, Gale CA. 2013. Rax2 is important for directional establishment of growth sites, but not for reorientation of growth axes, during *Candida albicans* hyphal morphogenesis. *Fungal genetics and biology : FG & B* **0**: 116-124.

Gow N, Perera T, Sherwood-Higham J, Gooday G, Gregory D, Marshall D. 1993. Investigation of touch-sensitive responses by hyphae of the human pathogenic fungus *Candida albicans*. *Scanning microscopy* **8**(3): 705-710.

Gow NA, Brown AJ, Odds FC. 2002. Fungal morphogenesis and host invasion. *Current opinion in microbiology* **5**(4): 366-371.

Gowda M, Jantasuriyarat C, Dean RA, Wang G-L. 2004. Robust-LongSAGE (RL-SAGE): a substantially improved LongSAGE method for gene discovery and transcriptome analysis. *Plant Physiology* **134**(3): 890-897.

Gowda S, Radhika P, Kadoo N, Mhase L, Gupta V. 2009. Molecular mapping of wilt resistance genes in chickpea. *Molecular breeding* **24**(2): 177-183.

Grant M, Brown I, Adams S, Knight M, Ainslie A, Mansfield J. 2000. The RPM1 plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. *The Plant Journal* **23**(4): 441-450.

Guo W-L, Chen R-G, Gong Z-H, Yin Y-X, Li D-W. 2013. Suppression Subtractive Hybridization Analysis of Genes Regulated by Application of Exogenous Abscisic Acid in Pepper Plant (Capsicum annuum L.) Leaves under Chilling Stress. *Plos One* **8**(6): e66667.

Gupta R, Lee SE, Agrawal GK, Rakwal R, Park S, Wang Y, Kim ST. 2015. Understanding the plant-pathogen interactions in the context of proteomics-generated apoplastic proteins inventory. *Frontiers in Plant Science* **6**: 352.

Gupta S, Chakraborti D, Rangi RK, Basu D, Das S. 2009. A molecular insight into the early events of chickpea (*Cicer arietinum*) and *Fusarium oxysporum* f.sp. *ciceri* (Race 1) interaction through cDNA-AFLP analysis. *Phytopathology* **99**(11): 1245-1257.

Gupta S, Chakraborti D, Sengupta A, Basu D, Das S. 2010. Primary Metabolism of Chickpea Is the Initial Target of Wound Inducing Early Sensed Fusarium oxysporum f. sp. ciceri Race I. *Plos One* **5**(2): e9030.

Gurjar G, Giri A, Vidya S G. 2012. Gene expression profiling during wilting in chickpea caused by *Fusarium oxysporum* f. sp. *ciceri*. *American Journal of Plant Sciences* **2012**.

Hamada H, Matsumura H, Tomita R, Terauchi R, Suzuki K, Kobayashi K. 2008. SuperSAGE revealed different classes of early resistance response genes in Capsicum chinense plants harboring L-3-resistance gene infected with Pepper mild mottle virus. *Journal of General Plant Pathology* **74**(4): 313-321.

Hammond-Kosack KE, Parker JE. 2003. Deciphering plant-pathogen communication: fresh perspectives for molecular resistance breeding. *Current Opinion in Biotechnology* **14**(2): 177-193.

Hardham AR 2001. Cell biology of fungal infection of plants. *Biology of the Fungal Cell*: Springer, 91-123.

Haware M, Nene Y. 1980. Influence of wilt at different growth stages on yield loss in chickpea. *Tropical Grain Legume Bulletin*.

Haware M, Nene Y. 1982. Races of *Fusarium oxysporum* f. sp. *ciceri*. *Plant Disease* **66**(9): 809-810.

Haware M, Nene Y, Natarajan M. 1996. The survival of Fusarium oxysporum f. sp. ciceri in the soil in the absence of chickpea. *Phytopathologia mediterranea*: 9-12.

Haware M, Nene Y, Pundir R, Rao JN. 1992. Screening of world chickpea germplasm for resistance to fusarium wilt. *Field Crops Research* **30**(1): 147-154.

Haware M, Nene Y, Rajeshwari R. 1978. Eradication of *Fusarium oxysporum* f. sp. *ciceri* transmitted in chickpea seed. *Phytopathology* **68**(9): 1364-1367.

Heid CA, Stevens J, Livak KJ, Williams PM. 1996. Real time quantitative PCR. *Genome Research* **6**(10): 986-994.

Hettenhausen C, Schuman MC, Wu J. 2015. MAPK signaling: A key element in plant defense response to insects. *Insect science* **22**(2): 157-164.

Higuchi R, Fockler C, Dollinger G, Watson R. 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology* **11**: 1026-1030.

Hollywood K, Brison DR, Goodacre R. 2006. Metabolomics: current technologies and future trends. *Proteomics* **6**(17): 4716-4723.

Hu J, Barlet X, Deslandes L, Hirsch J, Feng DX, Somssich I, Marco Y. 2008. Transcriptional responses of *Arabidopsis thaliana* during wilt disease caused by the soil-borne phytopathogenic bacterium, *Ralstonia solanacearum*. *Plos One* **3**(7): e2589.

Huckelhoven R, Kogel K-H. 2003. Reactive oxygen intermediates in plant-microbe interactions: Who is who in powdery mildew resistance? *Planta* **216**(6): 891-902.

Idnurm A, Howlett BJ. 2001. Pathogenicity genes of phytopathogenic fungi. *Molecular Plant Pathology* **2**(4): 241-255.

Imazaki I, Kurahashi M, Iida Y, Tsuge T. 2007. *Fow2*, a Zn (II) 2Cys6-type transcription regulator, controls plant infection of the vascular wilt fungus *Fusarium oxysporum*. *Molecular Microbiology* **63**(3): 737-753.

Inoue I, Namiki F, Tsuge T. 2002. Plant colonization by the vascular wilt fungus *Fusarium oxysporum* requires *FOW1*, a gene encoding a mitochondrial protein. *The Plant Cell* **14**(8): 1869-1883.

Iruela M, Castro P, Rubio J, Cubero J, Jacinto C, Millan T, Gil J. 2007. Validation of a QTL for resistance to ascochyta blight linked to resistance to fusarium wilt race 5 in chickpea (*Cicer arietinum* L.). *European journal of plant pathology* **119**(1): 29-37.

Jain S, Akiyama K, Kan T, Ohguchi T, Takata R. 2003. The G protein β subunit FGB1 regulates development and pathogenicity in *Fusarium oxysporum*. *Current genetics* **43**(2): 79-86.

Jain S, Akiyama K, Mae K, Ohguchi T, Takata R. 2002. Targeted disruption of a G protein α subunit gene results in reduced pathogenicity in *Fusarium oxysporum*. *Current genetics* **41**(6): 407-413.

Jayashree B, Buhariwalla HK, Shinde S, Crouch JH. 2005. A legume genomics resource: the chickpea root expressed sequence tag database. *Electronic Journal of Biotechnology* **8**(2): 8-13.

Jeong R-D, Lim W-S, Kwon S-W, Kim K-H. 2005. Identification of Glycine max genes expressed in response to Soybean mosaic virus infection. *Plant Pathol* **21**: 47-54.

Jimenez-Diaz R, Trapero-Casas A, De La Colina JC 1989. Races of *Fusarium oxysporum* f. sp. *ciceri* infecting chickpeas in southern Spain. *Vascular wilt diseases of plants*: Springer, 515-520.

Jimenez-Diaz RM, Castillo P, del Mar Jimenez-Gasco M, Landa BB, Navas-Cortes JA. 2015. Fusarium wilt of chickpeas: Biology, ecology and management. *Crop Protection* **73**: 16-27.

Jimenez-Fernandez D, Landa BB, Kang S, Jimenez-Diaz RM, Navas-Cortes JA. 2013. Quantitative and microscopic assessment of compatible and incompatible interactions between chickpea cultivars and *Fusarium oxysporum* f. sp. *ciceris* races. *Plos One* **8**(4): e61360.

Jimenez-Fernandez D, Montes-Borrego M, Jimenez-Diaz RM, Navas-Cortes JA, Landa BB. 2011. In planta and soil quantification of *Fusarium oxysporum* f. sp. *ciceris* and evaluation of Fusarium wilt resistance in chickpea with a newly developed quantitative polymerase chain reaction assay. *Phytopathology* **101**(2): 250-262.

Jones JDG, Dangl JL. 2006. The plant immune system. *Nature* **444**(7117): 323-329.

Jones P, Binns D, Chang H-Y, Fraser M, Li W, McAnulla C, McWilliam H, Maslen J, Mitchell A, Nuka G, et al. 2014. InterProScan 5: genome-scale protein function classification. *Bioinformatics*.

Jorge I, Navas-Cortes JA, Jimenez-Diaz RM, Tena M. 2006. Cell wall degrading enzymes in fusarium wilt of chickpea: correlation between pectinase and xylanase activities and disease development in plants infected with two pathogenic races of *Fusarium oxysporum* f. sp. *ciceris*. *Botany* **84**(9): 1395-1404.

Josefsen L, Droce A, Sondergaard TE, Sorensen JL, Bormann J, Schafer W, Giese H, Olsson S. 2012. Autophagy provides nutrients for nonassimilating fungal structures and is necessary for plant colonization but not for infection in the necrotrophic plant pathogen *Fusarium graminearum*. *Autophagy* **8**(3): 326-337.

Juana G, Rodriguez FD, Lorenzo JLR, Grappin P, Cervantes E. 2006. cDNA-AFLP analysis of seed germination in *Arabidopsis thaliana* identifies transposons and new genomic sequences. *Journal of Plant Physiology* **163**(4): 452-462.

Jukanti AK, Gaur PM, Gowda CLL, Chibbar RN. 2012. Nutritional quality and health benefits of chickpea (Cicer arietinum L.): a review. *British Journal of Nutrition* **108**(S1): S11-S26.

Jung S-H, Lee J-Y, Lee D-H. 2003. Use of SAGE technology to reveal changes in gene expression in *Arabidopsis* leaves undergoing cold stress. *Plant Molecular Biology* **52**(3): 553-567.

Kakoschke TK, Kakoschke SC, Zeuzem C, Bouabe H, Adler K, Heesemann J, Rossier O. 2016. The RNA chaperone Hfq is essential for virulence and modulates the expression of four adhesins in *Yersinia enterocolitica*. *Scientific Reports* **6**.

Kanzaki H, Saitoh H, Takahashi Y, Berberich T, Ito A, Kamoun S, Terauchi R. 2008. NbLRK1, a lectin-like receptor kinase protein of *Nicotiana benthamiana*, interacts with *Phytophthora infestans* INF1 elicitin and mediates INF1-induced cell death. *Planta* **228**(6): 977-987.

Kasahara S, Nuss DL. 1997. Targeted disruption of a fungal G-protein β subunit gene results in increased vegetative growth but reduced virulence. *Molecular Plant-Microbe Interactions* **10**(8): 984-993.

Kazan K, Schenk PM, Wilson I, Manners JM. 2001. DNA microarrays: new tools in the analysis of plant defence responses. *Molecular Plant Pathology* **2**(3): 177-185.

Kell DB, Brown M, Davey HM, Dunn WB, Spasic I, Oliver SG. 2005. Metabolic footprinting and systems biology: the medium is the message. *Nature Reviews Microbiology* **3**(7): 557-565.

Khan MR, Khan SM, Mohiddin FA. 2004. Biological Control of Fusarium Wilt of Chickepa through Seed Treatment with the Commercial Formulation of Thricoderma harzianum and/or Pseudomonas fluorescens. *Phytopathologia mediterranea* **43**(1): 20-25.

Kidd BN, Kadoo NY, Dombrecht B, Tekeoglu Mc, Gardiner DM, Thatcher LF, Aitken EAB, Schenk PM, Manners JM, Kazan K. 2011. Auxin signaling and transport promote susceptibility to the root-infecting fungal pathogen *Fusarium oxysporum* in Arabidopsis. *Molecular Plant-Microbe Interactions* **24**(6): 733-748.

Kiirika LM, Schmitz U, Colditz F. 2014. The alternative *Medicago truncatula* defense proteome of ROS-defective transgenic roots during early microbial infection. *Frontiers in Plant Science* **5**.

Kim K-H, Willger SD, Park S-W, Puttikamonkul S, Grahl N, Cho Y, Mukhopadhyay B, Cramer Jr RA, Lawrence CB. 2009. TmpL, a transmembrane protein required for intracellular redox homeostasis and virulence in a plant and an animal fungal pathogen. *PLoS Pathog* **5**(11): e1000653.

Kim Y-S, Lim S, Yoda H, Choi Y-E, Sano H. 2011. Simultaneous activation of salicylate production and fungal resistance in transgenic *Chrysanthemum* producing caffeine. *Plant signaling & behavior* **6**(3): 409-412.

Kim Y-S, Sano H. 2008. Pathogen resistance of transgenic tobacco plants producing caffeine. *Phytochemistry* **69**(4): 882-888.

Kivioja T, Arvas M, Saloheimo M, Penttila M, Ukkonen E. 2005. Optimization of cDNA-AFLP experiments using genomic sequence data. *Bioinformatics* **21**(11): 2573-2579.

Klopffleisch K, Phan N, Augustin K, Bayne RS, Booker KS, Botella JR, Carpita NC, Carr T, Chen J-G, Cooke TR. 2011. Arabidopsis G-protein interactome reveals connections to cell wall carbohydrates and morphogenesis. *Molecular systems biology* **7**(1): 532.

Kohli D, Joshi G, Deokar AA, Bhardwaj AR, Agarwal M, Katiyar-Agarwal S, Srinivasan R, Jain PK. 2014. Identification and characterization of wilt and salt stress-responsive microRNAs in chickpea through high-throughput sequencing. *Plos One* **9**(10): e108851.

Koia JH, Moyle RL, Botella JR. 2012. Microarray analysis of gene expression profiles in ripening pineapple fruits. *Bmc Plant Biology* **12**(1): 240.

Kong L-A, Yang J, Li G-T, Qi L-L, Zhang Y-J, Wang C-F, Zhao W-S, Xu J-R, Peng Y-L. 2012. Different chitin synthase genes are required for various developmental and plant infection processes in the rice blast fungus *Magnaporthe oryzae*. *PLoS Pathog* **8**(2): e1002526.

Kraft J. 1994. Fusarium wilt of peas (a review). *Agronomie-Sciences des Productions Vegetales et de l'Environnement* **14**(9): 561-568.

Krause C, Richter S, Knoll C, Jurgens G. 2013. Plant secretome—from cellular process to biological activity. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics* **1834**(11): 2429-2441.

Kumar, Dholakia B, Panigrahi P, Kadoo N, Giri A, Gupta V. 2015a. Metabolic profiling of chickpea-Fusarium interaction identifies differential modulation of disease resistance pathways. *Phytochemistry* **116**(Complete): 120-129.

Kumar, R R, Goswami S, Sharma SK, Kala YK, Rai GK, Mishra DC, Grover M, Singh GP, Pathak H, et al. 2015b. Harnessing next generation sequencing in climate change: RNA-seq analysis of heat stress-responsive genes in wheat (*Triticum aestivum* L.). *OMICS: A Journal of Integrative Biology* **19**(10): 632-647.

Kumar J, Abbo S. 2001. Genetics of flowering time in chickpea and its bearing on productivity in semiarid environments. *Advances in Agronomy* **72**: 107-138.

Kumar Y, Zhang L, Panigrahi P, Dholakia BB, Dewangan V, Chavan SG, Kunjir SM, Wu X, Li N, Rajmohanan PR, et al. 2016. *Fusarium oxysporum* mediates systems metabolic reprogramming of chickpea roots as revealed by a combination of proteomics and metabolomics. *Plant Biotechnology Journal*: n/a-n/a.

Kummasook A, Cooper CR, Sakamoto A, Terui Y, Kashiwagi K, Vanittanakom N. 2013. Spermidine is required for morphogenesis in the human pathogenic fungus, *Penicillium marneffei*. *Fungal Genetics and Biology* **58-59**: 25-32.

Lagopodi AL, Ram AF, Lamers GE, Punt PJ, Van den Hondel CA, Lugtenberg BJ, Bloemberg GV. 2002. Novel aspects of tomato root colonization and infection by *Fusarium oxysporum* f. sp. *radicis-lycopersici* revealed by confocal laser scanning microscopic analysis using the green fluorescent protein as a marker. *Molecular Plant-Microbe Interactions* **15**(2): 172-179.

Lai D, Li H, Fan S, Song M, Pang C, Wei H, Liu J, Wu D, Gong W, Yu S. 2011. Generation of ESTs for Flowering Gene Discovery and SSR Marker Development in Upland Cotton. *Plos One* **6**(12): e28676.

Lakshman DK, Pandey R, Kamo K, Bauchan G, Mitra A. 2012. Genetic transformation of *Fusarium oxysporum* f. sp. *gladioli* with *Agrobacterium* to study pathogenesis in Gladiolus. *European journal of plant pathology* **133**(3): 729-738.

Landa BB, Navas-Cortes JA, del Mar Jimenez-Gasco M, Katan J, Retig B, Jimenez-Diaz RM. 2006. Temperature response of chickpea cultivars to races of *Fusarium oxysporum* f. sp. *ciceris*, causal agent of Fusarium wilt. *Plant Disease* **90**(3): 365-374.

Landa BB, Navas-Cortes JA, Jimenez-Diaz RM. 2004. Integrated management of Fusarium wilt of chickpea with sowing date, host resistance, and biological control. *Phytopathology* **94**(9): 946-960.

Larkin RP, Fravel DR. 1999. Mechanisms of action and dose-response relationships governing biological control of Fusarium wilt of tomato by nonpathogenic *Fusarium spp*. *Phytopathology* **89**(12): 1152-1161.

Lee H, Damsz B, Woloshuk CP, Bressan RA, Narasimhan ML. 2010. Use of the plant defense protein osmotin to identify *Fusarium oxysporum* genes that control cell wall properties. *Eukaryotic cell* **9**(4): 558-568.

Lee HY, Bowen CH, Popescu GV, Kang H-G, Kato N, Ma S, Dinesh-Kumar S, Snyder M, Popescu SC. 2011. Arabidopsis RTNLB1 and RTNLB2 reticulon-like proteins regulate intracellular trafficking and activity of the FLS2 immune receptor. *The Plant Cell* **23**(9): 3374-3391.

Lee S-W, Han S-W, Bartley LE, Ronald PC. 2006. Unique characteristics of *Xanthomonas oryzae* pv. *oryzae* AvrXa21 and implications for plant innate immunity. *Proceedings of the National Academy of Sciences* **103**(49): 18395-18400.

Li C, Chen S, Zuo C, Sun Q, Ye Q, Yi G, Huang B. 2011. The use of GFP-transformed isolates to study infection of banana with *Fusarium oxysporum* f. sp. *cubense* race 4. *European journal of plant pathology* **131**(2): 327-340.

Li H, Rodda M, Gnanasambandam A, Aftab M, Redden R, Hobson K, Rosewarne G, Materne M, Kaur S, Slater AT. 2015. Breeding for biotic stress resistance in chickpea: progress and prospects. *Euphytica* **204**(2): 257-288.

Li J, Sun X, Yu G, Jia C, Liu J, Pan H. 2014. Generation and analysis of expressed sequence tags (ESTs) from halophyte atriplex canescens to explore salt-responsive related genes. *International Journal of Molecular Sciences* **15**(6): 11172-11189.

Lin C-H, Yang SL, Chung K-R. 2009. The YAP1 homolog-mediated oxidative stress tolerance is crucial for pathogenicity of the necrotrophic fungus *Alternaria alternata* in citrus. *Molecular Plant-Microbe Interactions* **22**(8): 942-952.

Liu F, Jiang H, Ye S, Chen W-P, Liang W, Xu Y, Sun B, Sun J, Wang Q, Cohen JD. 2010. The Arabidopsis P450 protein CYP82C2 modulates jasmonate-induced root growth inhibition, defense gene expression and indole glucosinolate biosynthesis. *Cell research* **20**(5): 539-552.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *methods* **25**(4): 402-408.

Lohse M, Nagel A, Herter T, May P, Schroda M, Zrenner R, Tohge T, Fernie AR, Stitt M, Usadel B. 2014. Mercator: a fast and simple web server for genome scale functional annotation of plant sequence data. *Plant, Cell & Environment* **37**(5): 1250-1258.

Lu G, Guo S, Zhang H, Geng L, Song F, Fei Z, Xu Y. 2011. Transcriptional profiling of watermelon during its incompatible interaction with *Fusarium oxysporum* f. sp. *niveum*. *European journal of plant pathology* **131**(4): 585-601.

MacMichael G. 2002. Chickpeas: situation and Outlook. *Agriculture and Agri-food Canada* **15**(16): 1-6.

Madrid MP, Di Pietro A, Roncero MIG. 2003. Class V chitin synthase determines pathogenesis in the vascular wilt fungus *Fusarium oxysporum* and mediates resistance to plant defence compounds. *Molecular Microbiology* **47**(1): 257-266.

Mandelc S, Javornik B. 2015. The secretome of vascular wilt pathogen *Verticillium alboatrum* in simulated xylem fluid. *Proteomics* **15**(4): 787-797.

Mansfield JW 2000. Antimicrobial compounds and resistance. *Mechanisms of resistance to plant diseases*: Springer, 325-370.

Margheim JF. 2004. Chickpea production in the High Plains.

Marino D, Peeters N, Rivas S. 2012. Ubiquitination during Plant Immune Signaling. *Plant Physiology* **160**(1): 15-27.

Markham P, Collinge AJ. 1987. Woronin Bodies of Filamentous Fungi. *Fems Microbiology Letters* **46**(1): 1-11.

Matsumura H, Reich S, Ito A, Saitoh H, Kamoun S, Winter P, Kahl G, Reuter M, Krüger DH, Terauchi R. 2003. Gene expression analysis of plant host–pathogen interactions by SuperSAGE. *Proceedings of the National Academy of Sciences* **100**(26): 15718-15723.

Matsumura H, Yoshida K, Luo S, Kimura E, Fujibe T, Albertyn Z, Barrero RA, Kruger DH, Kahl G, Schroth GP. 2010. High-throughput SuperSAGE for digital gene expression analysis of multiple samples using next generation sequencing. *Plos One* **5**(8): e12010.

Mazzei P, Vinale F, Woo SL, Pascale A, Lorito M, Piccolo A. 2016. Metabolomics by Proton High-Resolution Magic-Angle-Spinning Nuclear Magnetic Resonance of Tomato Plants Treated with Two Secondary Metabolites Isolated from Trichoderma. *Journal of agricultural and food chemistry* **64**(18): 3538-3545.

McDonnell K. 1962. Relationship of pectic enzymes and pathogenicity in the Fusarium wilt of tomatoes. *Transactions of the British Mycological Society* **45**(1): 55-62.

Meyers BC, Haudenschild CD, Vemaraju K. 2007. Use of massively parallel signature sequencing to study genes expressed during the plant defense response. *Plant-Pathogen Interactions: Methods and Protocols*: 105-119.

Meyers BC, Tej SS, Vu TH, Haudenschild CD, Agrawal V, Edberg SB, Ghazal H, Decola S. 2004. The use of MPSS for whole-genome transcriptional analysis in *Arabidopsis*. *Genome Research* **14**(8): 1641-1653.

Michielse CB, van Wijk R, Reijnen L, Manders EM, Boas S, Olivain C, Alabouvette C, Rep M. 2009. The nuclear protein Sge1 of *Fusarium oxysporum* is required for parasitic growth. *PLoS Pathog* **5**(10): e1000637.

Missall TA, Pusateri ME, Lodge JK. 2004. Thiol peroxidase is critical for virulence and resistance to nitric oxide and peroxide in the fungal pathogen, *Cryptococcus neoformans*. *Molecular Microbiology* **51**(5): 1447-1458.

Mittler R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science* **7**(9): 405-410.

Mobius N, Hertweck C. 2009. Fungal phytotoxins as mediators of virulence. *Current Opinion in Plant Biology* **12**(4): 390-398.

Molina C, Rotter B, Horres R, Udupa SM, Besser B, Bellarmino L, Baum M, Matsumura H, Terauchi R, Kahl G, et al. 2008. SuperSAGE: the drought stress-responsive transcriptome of chickpea roots. *Bmc Genomics* **9**: -.

Molina C, Zaman-Allah M, Khan F, Fatnassi N, Horres R, Rotter B, Steinhauer D, Amenc L, Drevon JJ, Winter P, et al. 2011. The salt-responsive transcriptome of chickpea roots and nodules via deepSuperSAGE. *BMC Plant Biol* **11**: 31.

Molina L, Kahmann R. 2007. An *Ustilago maydis* gene involved in H2O2 detoxification is required for virulence. *The Plant Cell* **19**(7): 2293-2309.

Moliterni VMC, Paris R, Onofri C, Orru L, Cattivelli L, Pacifico D, Avanzato C, Ferrarini A, Delledonne M, Mandolino G. 2015. Early transcriptional changes in *Beta vulgaris* in response to low temperature. *Planta* **242**(1): 187-201.

Momany M, Richardson EA, Van Sickle C, Jedd G. 2002. Mapping Woronin body position in *Aspergillus nidulans*. *Mycologia* **94**(2): 260-266.

Morant AV, Jorgensen K, Jorgensen C, Paquette SM, Sanchez-Perez R, Moller BL, Bak S. 2008. β-Glucosidases as detonators of plant chemical defense. *Phytochemistry* **69**(9): 1795-1813.

Moreau F, Fetouchi R, Micalessi I, Brejeon V, Bacon N, Jannes G, Le Pendeven C, Lekbaby B, Kremsdorf D, Saint Guily JL. 2013. Detection and genotyping of human papillomavirus by real-time PCR assay. *Journal of Clinical Virology* **56**(3): 328-333.

Moustafa K, Cross JM. 2016. Genetic Approaches to Study Plant Responses to Environmental Stresses: An Overview. *Biology* **5**(2): 20.

Mullins ED, Chen X, Romaine P, Raina R, Geiser D, Kang S. 2001. Agrobacterium-mediated transformation of *Fusarium oxysporum*: an efficient tool for insertional mutagenesis and gene transfer. *Phytopathology* **91**(2): 173-180.

Nakano M, Nobuta K, Vemaraju K, Tej SS, Skogen JW, Meyers BC. 2006. Plant MPSS databases: signature-based transcriptional resources for analyses of mRNA and small RNA. *Nucleic Acids Research* **34**(suppl 1): D731-D735.

Namiki F, Matsunaga M, Okuda M, Inoue I, Nishi K, Fujita Y, Tsuge T. 2001. Mutation of an arginine biosynthesis gene causes reduced pathogenicity in *Fusarium oxysporum* f. sp. *melonis*. *Molecular Plant-Microbe Interactions* **14**(4): 580-584.

Narasimhan ML, Lee H, Damsz B, Singh NK, Ibeas JI, Matsumoto TK, Woloshuk CP, Bressan RA. 2003. Overexpression of a cell wall glycoprotein in *Fusarium oxysporum* increases virulence and resistance to a plant PR-5 protein. *The Plant Journal* **36**(3): 390-400.

Narina SS, Buyyarapu R, Kottapalli KR, Sartie AM, Ali MI, Robert A, Hodeba MJ, Sayre BL, Scheffler BE. 2011. Generation and analysis of expressed sequence tags (ESTs) for marker development in yam (*Dioscorea alata* L.). *Bmc Genomics* **12**(1): 100.

Nelson DR, Felix CM, Swanson JM. 1998. Highly conserved charge-pair networks in the mitochondrial carrier family. *Journal of molecular biology* **277**(2): 285-308.

Nelson P, Juba J, Ross P, Rice L. 1994. Fumonisin production by Fusarium species on solid substrates. *Journal of AOAC International* **77**(2): 522-525.

Nelson PM, Wfo Toussoun T. 1983. *Fusarium Species: an Illustred Manual for Identification*: The Pennsylvania State University.

Nene YLHMPRMV. 1981. Diagnosis of some wilt-like disorders.

Niks R. 1988. Nonhost plant species as donors for resistance to pathogens with narrow host range. II. Concepts and evidence on the genetic basis of nonhost resistance. *Euphytica* **37**(1): 89-99.

Nimbalkar SB, Harsulkar AM, Giri AP, Sainani MN, Franceschi V, Gupta VS. 2006. Differentially expressed gene transcripts in roots of resistant and susceptible chickpea plant (Cicer arietinum L.) upon *Fusarium oxysporum* infection. *Physiological and Molecular Plant Pathology* **68**(4-6): 176-188.

Nitta Y, Ding P, Zhang Y. 2014. Identification of additional MAP kinases activated upon PAMP treatment. *Plant signaling & behavior* **9**(11): e976155.

Nitta Y, Ding P, Zhang Y. 2015. Heterotrimeric G proteins in plant defense against pathogens and ABA signaling. *Environmental and Experimental Botany* **114**: 153-158.

Noda J, Brito N, Gonzalez C. 2010. The Botrytis cinerea xylanase Xyn11A contributes to virulence with its necrotizing activity, not with its catalytic activity. *Bmc Plant Biology* **10**(1): 1.

Nowrousian M. 2007. Of patterns and pathways: microarray technologies for the analysis of filamentous fungi. *Fungal Biology Reviews* **21**(4): 171-178.

Oh Y, Donofrio N, Pan H, Coughlan S, Brown DE, Meng S, Mitchell T, Dean RA. 2008. Transcriptome analysis reveals new insight into appressorium formation and function in the rice blast fungus *Magnaporthe oryzae*. *Genome Biology* **9**(5): 1.

Oplinger E, Hardman L, Oelke E, Kaminski A, Schulte E, Doll J. 1990. Chickpea (garbanzo bean): Alternative Field Crop Manual. *Jointly Published by the University*.

Osterloh A, Breloer M. 2008. Heat shock proteins: linking danger and pathogen recognition. *Medical Microbiology and Immunology* **197**(1): 1-8.

Owatworakit A, Townsend B, Louveau T, Jenner H, Rejzek M, Hughes RK, Saalbach G, Qi X, Bakht S, Roy AD. 2013. Glycosyltransferases from oat (Avena) implicated in the acylation of avenacins. *Journal of Biological Chemistry* **288**(6): 3696-3704.

Palmieri F. 1994. Mitochondrial carrier proteins. *FEBS letters* **346**(1): 48-54.

Pankoke H, Buschmann T, Muller C. 2013. Role of plant β-glucosidases in the dual defense system of iridoid glycosides and their hydrolyzing enzymes in *Plantago lanceolata* and *Plantago major*. *Phytochemistry* **94**: 99-107.

Pareek CS, Pierzchała JM, Szymanska S, Smoczynski NJ, Gołebiewski JT, Urbanski DG, Oprzadek LZ, Siriluck KW. 2012. Gene expression profiling by cDNA-AFLP and identification of differentially expressed transcripts of bovine pituitary gland in growing bulls of dairy breeds. *Animal Science Papers and Reports* **30**(2): 103-119.

Pegels N, Gonzalez I, Lopez-Calleja I, Fernandez S, Garcia T, Martin R. 2012. Evaluation of a TaqMan real-time PCR assay for detection of chicken, turkey, duck, and goose material in highly processed industrial feed samples. *Poultry Science* **91**(7): 1709-1719.

Peters DG, O'Hare EH, Ferrell RE, Kassam AB, Yonas H, Brufsky AM. 1999. Comprehensive transcript analysis in small quantities of mRNA by SAGE-lite. *Nucleic Acids Research* **27**(24): e39-e44.

Petersen TN, Brunak S, von Heijne G, Nielsen H 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions

Piisila M, Keceli MA, Brader G, Jakobson L, Joesaar I, Sipari N, Kollist H, Palva ET, Kariola T. 2015. The F-box protein MAX2 contributes to resistance to bacterial phytopathogens in *Arabidopsis thaliana*. *Bmc Plant Biology* **15**(1): 1-17.

Pogany M, Danko T, Kaman-Toth E, Schwarczinger I, Bozso Z. 2015. Regulatory Proteolysis in *Arabidopsis*-Pathogen Interactions. *International Journal of Molecular Sciences* **16**(10): 23177-23194.

Pollier J, Moses T, Gonzalez-Guzman M, De Geyter N, Lippens S, Bossche RV, Marhavy P, Kremer A, Morreel K, Guerin CJ, et al. 2013. The protein quality control system manages plant defence compound synthesis. *Nature* **504**(7478): 148-152.

Prell HH, Day P. 2001. *Plant-fungal pathogen interaction: a classical and molecular view*: Springer Science & Business Media.

Quirino BF, Candido ES, Campos PF, Franco OL, Kruger RH. 2010. Proteomic approaches to study plant–pathogen interactions. *Phytochemistry* **71**(4): 351-362.

Radauer C, Lackner P, Breiteneder H. 2008. The Bet v 1 fold: an ancient, versatile scaffold for binding of large, hydrophobic ligands. *BMC evolutionary biology* **8**(1): 286.

Raggi V. 2000. Hydroxyproline-rich glycoprotein accumulation in tobacco leaves protected against *Erysiphe cichoracearum* by potato virus Y infection. *Plant Pathology* **49**(2): 179-186.

Ramakers C, Ruijter JM, Deprez RHL, Moorman AF. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neuroscience letters* **339**(1): 62-66.

Reinartz J, Bruyns E, Lin J-Z, Burcham T, Brenner S, Bowen B, Kramer M, Woychik R. 2002. Massively parallel signature sequencing (MPSS) as a tool for in-depth quantitative gene expression profiling in all organisms. *Briefings in functional genomics & proteomics* **1**(1): 95-104.

Reusche M, Klaskova J, Thole K, Truskina J, Novak O, Janz D, Strnad M, Spichal L, Lipka V, Teichmann T. 2013. Stabilization of cytokinin levels enhances Arabidopsis resistance against *Verticillium longisporum*. *Molecular Plant-Microbe Interactions* **26**(8): 850-860.

Ribeiro JM, Pereira CS, Soares NC, Vieira AM, Feijas JA, Jackson PA. 2006. The contribution of extensin network formation to rapid, hydrogen peroxide-mediated increases in grapevine callus wall resistance to fungal lytic enzymes. *Journal of Experimental Botany* **57**(9): 2025-2035.

Robinson M, Oshlack A. 2010. A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biology* **11**(3): R25.

Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**(1): 139-140.

Russo BC, Horzempa J, O'Dee DM, Schmitt DM, Brown MJ, Carlson PE, Xavier RJ, Nau GJ. 2011. A *Francisella tularensis* Locus Required for Spermine Responsiveness Is Necessary for Virulence. *Infection and Immunity* **79**(9): 3665-3676.

Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M. 2003. TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* **34**(2): 374.

Sagaram US, SHIM WB. 2007. *Fusarium verticillioides* GBB1, a gene encoding heterotrimeric G protein β subunit, is associated with fumonisin B1 biosynthesis and hyphal development but not with fungal virulence. *Molecular Plant Pathology* **8**(4): 375-384.

Saha S, Sparks AB, Rago C, Akmaev V, Wang CJ, Vogelstein B, Kinzler KW, Velculescu VE. 2002. Using the transcriptome to annotate the genome. *Nature biotechnology* **20**(5): 508-512.

Saikia R, Singh BP, Kumar R, Arora DK. 2005. Detection of pathogenesisrelated proteins-chitinase and-1, 3-glucanase in induced chickpea. *Curr Sci* **89**(4): 659-663.

Samalova M, Meyer AJ, Gurr SJ, Fricker MD. 2014. Robust anti-oxidant defences in the rice blast fungus *Magnaporthe oryzae* confer tolerance to the host oxidative burst. *New Phytologist* **201**(2): 556-573.

Sanseverino W, Roma G, De Simone M, Faino L, Melito S, Stupka E, Frusciante L, Ercolano MR. 2010. PRGdb: a bioinformatics platform for plant resistance gene analysis. *Nucleic Acids Research* **38**(suppl 1): D814-D821.

Schena M, Shalon D, Davis RW, Brown PO. 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* **270**(5235): 467.

Schulz P, Herde M, Romeis T. 2013. Calcium-Dependent Protein Kinases: Hubs in Plant Stress Signaling and Development. *Plant Physiology* **163**(2): 523-530.

Sela-Buurlage MB, Ponstein AS, Bres-Vloemans SA, Melchers LS, Cornelissen BJC. 1993. Only Specific Tobacco (*Nicotiana tabacum*) Chitinases and 8-1,3-Clucanases Exhibit Antifungal Activity.

Sestili S, Polverari A, Luongo L, Ferrarini A, Scotton M, Hussain J, Delledonne M, Ficcadenti N, Belisario A. 2011. Distinct colonization patterns and cDNA-AFLP transcriptome profiles in compatible and incompatible interactions between melon and different races of *Fusarium oxysporum* f. sp. *melonis*. *Bmc Genomics* **12**(1): 1.

Sharma KD, Winter P, Kahl G, Muehlbauer FJ. 2004. Molecular mapping of *Fusarium oxysporum* f. sp. *ciceris* race 3 resistance gene in chickpea. *Theoretical and Applied Genetics* **108**(7): 1243-1248.

Shih J, Wei Y, Goodwin PH. 2000. A comparison of the pectate lyase genes, pel-1 and pel-2, of *Colletotrichum gloeosporioides* f. sp. *malvae* and the relationship between their expression in culture and during necrotrophic infection. *Gene* **243**(1): 139-150.

Siboo IR, Chambers HF, Sullam PM. 2005. Role of SraP, a Serine-Rich Surface Protein of *Staphylococcus aureus*, in Binding to Human Platelets. *Infection and Immunity* **73**(4): 2273-2280.

Siemens J, Keller I, Sarx J, Kunz S, Schuller A, Nagel W, Schmulling T, Parniske M, Ludwig-Muller J. 2006. Transcriptome Analysis of *Arabidopsis* Clubroots Indicate a Key Role for Cytokinins in Disease Development. *Molecular Plant-Microbe Interactions* **19**(5): 480-494.

Sinclair JB, Dhingra OD. 1995. *Basic plant pathology methods*: CRC press.

Singh G, Tiwari M, Singh SP, Singh S, Trivedi PK, Misra P. 2016. Silencing of sterol glycosyltransferases modulates the withanolide biosynthesis and leads to compromised basal immunity of *Withania somnifera*. *Scientific Reports* **6**.

Singh K, Reddy M. 1991. Advances in disease-resistance breeding in chickpea. *Advances in Agronomy* **45**: 191-222.

Singh K, Reddy M. 1993. Resistance to six races of *Ascochyta rabiei* in the world germplasm collection of chickpea. *Crop Science* **33**(1): 186-189.

Singh P, Zimmerli L. 2013. Lectin receptor kinases in plant innate immunity. *Frontiers in Plant Science* **4**: 124.

Skau CT, Kovar DR. 2010. Fimbrin and Tropomyosin Competition Regulates Endocytosis and Cytokinesis Kinetics in Fission Yeast. *Current Biology* **20**(16): 1415-1422.

Song P, Xie Z, Guo L, Wang C, Xie W, Wu Y. 2012. Human genome-specific real-time PCR method for sensitive detection and reproducible quantitation of human cells in mice. *Stem Cell Reviews and Reports* **8**(4): 1155-1162.

Stafford HA. 1997. Roles of flavonoids in symbiotic and defense functions in legume roots. *The Botanical Review* **63**(1): 27-39.

Suharsono U, Fujisawa Y, Kawasaki T, Iwasaki Y, Satoh H, Shimamoto K. 2002. The heterotrimeric G protein α subunit acts upstream of the small GTPase Rac in disease resistance of rice. *Proceedings of the National Academy of Sciences* **99**(20): 13307-13312.

Sun X, Xu L, Wang Y, Luo X, Zhu X, Kinuthia KB, Nie S, Feng H, Li C, Liu L. 2016. Transcriptome-based gene expression profiling identifies differentially expressed genes critical for salt stress response in radish (*Raphanus sativus* L.). *Plant Cell Reports* **35**(2): 329-346.

Suty L, Lequeu J, Lancon A, Etienne P, Petitot A-S, Blein J-P. 2003. Preferential induction of 20S proteasome subunits during elicitation of plant defense reactions: towards the characterization of plant defense proteasomes. *The International Journal of Biochemistry & Cell Biology* **35**(5): 637-650.

Tan K, Ipcho S, Trengove R. 2009. Challenges for molecular plant pathology over the next ten years assessing the impact of transcriptomics, proteomics and metabolomics on fungal phytopathology. *Molecular Plant Pathology* **10**: 703-715.

Tereshina VM. 2005. Thermotolerance in fungi: the role of heat shock proteins and trehalose. *Microbiology* **74**(3): 247-257.

Thanonkeo P, Akiyama K, Jain S, Takata R. 2000. Targeted disruption of sti35, a stress-responsive gene in phytopathogenic fungus *Fusarium oxysporum*. *Current Microbiology* **41**(4): 284-289.

Thimm O, Blasing O, Gibon Y, Nagel A, Meyer S, Kruger P, Selbig J, Muller LA, Rhee SY, Stitt M. 2004. MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. *The Plant Journal* **37**(6): 914-939.

Tiwari S, Thakur R, Shankar J. 2015. Role of Heat-Shock Proteins in Cellular Function and in the Biology of Fungi. *Biotechnology Research International* **2015**: 11.

Tsien RY. 1998. The green fluorescent protein. *Annual Review of Biochemistry* **67**(1): 509-544.

Tullu A, Muehlbauer F, Simon C, Mayer M, Kumar J, Kaiser W, Kraft J. 1998. Inheritance and linkage of a gene for resistance to race 4 of fusarium wilt and RAPD markers in chickpea. *Euphytica* **102**(2): 227-232.

Tzima AK, Paplomatas EJ, Tsitsigiannis DI, Kang S. 2012. The G protein β subunit controls virulence and multiple growth-and development-related traits in *Verticillium dahliae*. *Fungal Genetics and Biology* **49**(4): 271-283.

Upadhyay S, Shaw BD. 2008. The role of actin, fimbrin and endocytosis in growth of hyphae in *Aspergillus nidulans*. *Molecular Microbiology* **68**(3): 690-705.

Usadel B, Nagel A, Thimm O, Redestig H, Blaesing OE, Palacios-Rojas N, Selbig J, Hannemann J, Piques MC, Steinhauser D. 2005. Extension of the visualization tool MapMan to allow statistical analysis of arrays, display of coresponding genes, and comparison with known responses. *Plant Physiology* **138**(3): 1195-1204.

Valdes-Santiago L, Cervantes-Chavez JA, Winkler R, Leon-Ramirez CG, Ruiz-Herrera J. 2012. Phenotypic comparison of samdc and spe mutants reveals complex relationships of polyamine metabolism in *Ustilago maydis*. *Microbiology* **158**(Pt 3): 674-684.

van der Does HC, Duyvesteijn RG, Goltstein PM, van Schie CC, Manders EM, Cornelissen BJ, Rep M. 2008. Expression of effector gene SIX1 of *Fusarium oxysporum* requires living plant cells. *Fungal Genetics and Biology* **45**(9): 1257-1264.

Vandeput F, Zabeau M, Maenhaut C. 2005. Identification of differentially expressed genes in thyrotropin stimulated dog thyroid cells by the cDNA-AFLP technique. *Molecular and cellular endocrinology* **243**(1): 58-65.

Velculescu VE, Zhang L, Vogelstein B, Kinzler KW. 1995. Serial Analysis of Gene Expression. *Science* **270**: 484 - 487.

Virlon B, Cheval L, Buhler J-M, Billon E, Doucet A, Elalouf J-M. 1999. Serial microanalysis of renal transcriptomes. *Proceedings of the National Academy of Sciences* **96**(26): 15286-15291.

Wang J, Shi L, He X, Lu L, Li X, Chen B. 2016. Comparative Secretome Analysis Reveals Perturbation of Host Secretion Pathways by a Hypovirus. *Scientific Reports* **6**: 34308.

Wang K. 2006. *Agrobacterium protocols*: Springer.

Wang T, Chen X, Zhu F, Li H, Li L, Yang Q, Chi X, Yu S, Liang X. 2013. Characterization of Peanut Germin-Like Proteins, AhGLPs in Plant Development and Defense. *Plos One* **8**(4): e61722.

Wang Y, Yang L, Chen X, Ye T, Zhong B, Liu R, Wu Y, Chan Z. 2015. Major latex protein-like protein 43 (MLP43) functions as a positive regulator during abscisic acid responses and confers drought tolerance in *Arabidopsis thaliana*. *Journal of Experimental Botany*.

Wang Z, Gerstein M, Snyder M. 2009. RNA-Seq: a revolutionary tool for transcriptomics. *Nature reviews genetics* **10**(1): 57-63.

Webb KM, Freeman C, Broeckling CD. 2016. Metabolome profiling to understand the defense response of sugar beet (*Beta vulgaris*) to *Rhizoctonia solani* AG 2-2 IIIB. *Physiological and Molecular Plant Pathology*.

Williams H, Sturrock R, Islam M, Hammett C, Ekramoddoullah AK, Leal I. 2014. Gene expression profiling of candidate virulence factors in the laminated root rot pathogen *Phellinus sulphurascens*. *Bmc Genomics* **15**(1): 603.

Winnenburg R, Urban M, Beacham A, Baldwin TK, Holland S, Lindeberg M, Hansen H, Rawlings C, Hammond-Kosack KE, Kohler J. 2008. PHI-base update: additions to the pathogen-host interaction database. *Nucleic Acids Research* **36**(suppl 1): D572-D576.

Winter P, Benko-Iseppon A-M, Huttel B, Ratnaparkhe M, Tullu A, Sonnante G, Pfaff T, Tekeoglu M, Santra D, Sant V. 2000. A linkage map of the chickpea (*Cicer arietinum* L.) genome based on recombinant inbred lines from a *C. arietinum*× *C. reticulatum* cross: localization of resistance genes for fusarium wilt races 4 and 5. *Theoretical and Applied Genetics* **101**(7): 1155-1163.

Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. 2003. High-resolution genotyping by amplicon melting analysis using LCGreen. *Clinical chemistry* **49**(6): 853-860.

Wong HL, Sakamoto T, Kawasaki T, Umemura K, Shimamoto K. 2004. Down-Regulation of Metallothionein, a Reactive Oxygen Scavenger, by the Small GTPase OsRac1 in Rice. *Plant Physiology* **135**(3): 1447-1456.

Wood J, Grusak M. 2007. 5 Nutritional Value of Chickpea. *Chickpea breeding and management*: 101.

Wortham BW, Oliveira MA, Fetherston JD, Perry RD. 2010. Polyamines are required for the expression of key Hms proteins important for *Yersinia pestis* biofilm formation. *Environmental Microbiology* **12**(7): 2034-2047.

Wubben JP, ten Have A, van Kan JA, Visser J. 2000. Regulation of endopolygalacturonase gene expression in *Botrytis cinerea* by galacturonic acid, ambient pH and carbon catabolite repression. *Current genetics* **37**(2): 152-157.

Xie F, Wang Q, Sun R, Zhang B. 2015. Deep sequencing reveals important roles of microRNAs in response to drought and salinity stress in cotton. *Journal of Experimental Botany* **66**(3): 789-804.

Yao C, Wu Y, Nie H, Tang D. 2012. RPN1a, a 26S proteasome subunit, is required for innate immunity in Arabidopsis. *The Plant Journal* **71**(6): 1015-1028.

Yu H-Y, Seo J-A, Kim J-E, Han K-H, Shim W-B, Yun S-H, Lee Y-W. 2008. Functional analyses of heterotrimeric G protein Gα and Gβ subunits in *Gibberella zeae*. *Microbiology* **154**(2): 392-401.

Zhang C-x, Tian Y, Cong P-h. 2015. Proteome Analysis of Pathogen-Responsive Proteins from Apple Leaves Induced by the *Alternaria Blotch*, *Alternaria alternata*. *Plos One* **10**(6): e0122233.

Zhang H, Gao Z, Zheng X, Zhang Z. 2012. The role of G-proteins in plant immunity. *Plant signaling & behavior* **7**(10): 1284-1288.

Zhang L, Du L, Poovaiah BW. 2014. Calcium signaling and biotic defense responses in plants. *Plant signaling & behavior* **9**(11): e973818.

Zhang S, Xia Y, Keyhani NO. 2011. Contribution of the gas1 gene of the entomopathogenic fungus *Beauveria bassiana*, encoding a putative glycosylphosphatidylinositol-anchored β-1, 3-glucanosyltransferase, to conidial thermotolerance and virulence. *Applied and environmental microbiology* **77**(8): 2676-2684.

Zhang Z, Li Q, Li Z, Staswick PE, Wang M, Zhu Y, He Z. 2007. Dual regulation role of GH3. 5 in salicylic acid and auxin signaling during *Arabidopsis-Pseudomonas syringae* interaction. *Plant Physiology* **145**(2): 450-464.

Zheng Z, Gao T, Zhang Y, Hou Y, Wang J, Zhou M. 2015. FgFim, a key protein regulating resistance to the fungicide JS399-19, asexual and sexual development, stress responses and virulence in *Fusarium graminearum*. *Molecular Plant Pathology* **15**(5): 488-499.

Zhou D, Rao MS, Walker R, Khrebtukova I, Haudenschild CD, Miura T, Decola S, Vermaas E, Moon K, Vasicek TJ. 2006. Massively parallel signature sequencing. *Human Embryonic Stem Cell Protocols*: 285-311.

Zhu H, Bilgin M, Snyder M. 2003. Proteomics. *Annual Review of Biochemistry* **72**(1): 783-812.

Zohary D, Hopf M, Weiss E. 2012. *Domestication of Plants in the Old World: The origin and spread of domesticated plants in Southwest Asia, Europe, and the Mediterranean Basin*: Oxford University Press on Demand.

Zvirin T, Herman R, Brotman Y, Denisov Y, Belausov E, Freeman S, Perl-Treves R. 2010. Differential colonization and defence responses of resistant and susceptible melon lines infected by *Fusarium oxysporum* race 1· 2. *Plant Pathology* **59**(3): 576-585.

CURRICULUM VITAE

MEDHA L.UPASANI (Mrs. Medha R. Dange)

Personal details:

Nationality **:** Indian

Date of birth **:** 30 March 1986

Marital status **:** Married

Contact address **:** 569/B-5, 'Sangam', Behind Congress house, Shivajinagar, Pune-411005, Maharashtra, India

Email **:** medhaupasani3@gmail.com

Academic qualifications:

- **CSIR-NET** (2007 and 2008), **Junior Research Fellowship** and **Lectureship** through NET conducted by Council of scientific and Industrial Research (CSIR), New Delhi.
- **Masters of Science in Microbiology** (2006-2008), from Dept. of Microbiology, Savitribai Phule Pune University (SPPU), Pune.
  Secured: **GPA-5.92/6, First rank in SPPU**
- **Bachelor of Science in Microbiology** (2003-2006), from Dept. of Microbiology, HPT Arts and RYK Science college, Nashik.
  Secured: **86 %, First rank in SPPU**
  Major subjects included: **Chemistry, Zoology and Microbiology**

Academic Awards

- **Best poster award** promoted to **oral presentation** by The Centre for Development of Advanced Computing (CDAC), Pune, for the poster presented in "Accelerating Biology, 2014", Pune during January 17-19, 2014: **Medha L. Upasani**, Gayatri S. Gurjar, Bhakti M. Limaye, Amit k Singh, Sunitha Manjari K, Dr. Rajendra R. Joshi, Dr. Vidya Gupta, Dr. Narendra Kadoo.

'Transcription dynamics of chickpea-Fusarium interaction explored using serial analysis of gene expression studies'.

- **Best student Poster Award** by **American Society of Microbiology (ASM)**, United States of America, for the poster presented in the 50th Annual Conference of Association of Microbiologists of India, held at CSIR-National Chemical Laboratory, Pune, India during December 15-18, 2009: **M Upasani**, A Kancharla, N Kadoo and V Gupta. 'Serial Analysis of Gene Expression-A molecular approach to analyze plant-pathogen interactions for improving chickpea productivity'.

- Qualified Graduate Aptitude Test in Engineering (GATE) in 2008 in Life Sciences with score 422, percentile 95.26 and all India Rank 623.

- Cleared national level examination (for PhD) for Indian Institute of Science, India (in 2008) and National Institute of Immunology, India (in 2008).

- 14^{th} rank in Nasik divisional board in HSC, 2002.

Research Experience

- Pursuing **Ph.D. in Biotechnology** (August, 2008 onwards)

  Project: Molecular analysis of chickpea-*Fusarium* interactions using Serial Analysis of Gene Expression (SAGE)

  Guide: Dr. Vidya S. Gupta and Dr. Narendra Y. Kadoo

  Institute: CSIR-National Chemical Laboratory, Pune, India

- **M.Sc. Microbiology** (2006-2008)

  Project: Intra and intergeneric transfer of antibiotic resistance between *Acinetobacter* sp. and other Gram-negative bacteria in hospital environment

  Guide: Dr. B.A. Chopde

  Institute: Department of Microbiology, University of Pune, Pune, India.

Teaching experience

- Working as **Assistant Professor** in the Department of Microbiology, Savitribai Phule Pune University, Pune from 24^{th} September 2016 onwards.

- Worked as **Assistant Professor** in Department of Microbiology in MES Garware College, Pune from July to September, 2015-16.

- Worked as **Assistant Professor** in Department of Microbiology in Haribhai V. Desai college, Pune from December to April,2014-15.

- Conducted few biology practicals in IISER, Pune in 2008.

Conferences / Workshops attended

- One day workshop on "Fundamentals of HPLC" organized by Venture Center, Pune in November 2016.

- "Accelerating Biology 2014: Computing Life" organized by The Centre for Development of Advanced Computing (CDAC) at YASHADA, Pune in February 2014.

- National Symposium on 'Molecular Approaches for Management of Fungal Diseases of Crop Plants' organized by Indian Institute of Horticultural Research, Bangalore in December,2010.

- 50^{th} Annual Conference of Association of Microbiologists of India (AMI) organized at CSIR-National Chemical Laboratory, India in Dec. 2009.

- Workshop on "Management of Intellectual Property Rights in Biotechnology" organized by Biotech Consortium India limited, New Delhi at YASHADA, Pune in Feb. 2009.

- National Workshop in "Teaching and Learning Biology through a Problem-Solving Approach" in January, 2008.

Publications

1. Gurjar G, Mishra M, Kotkar H, **Upasani M**, Pradeep Kumar, Tamhane V, Kadoo N, Giri A and Gupta V (2009) Major biotic stresses of chickpea and strategies for their control. ***In***: Pests and Pathogens: Management Strategies.

Editors: - D.V. Reddy, P.N. Rao, K.V.Rao BS Publications ISBN: 978-81-7800-227-9.

2. **Upasani ML**, Gurjar GS, Kadoo NY, Gupta VS (2016) Dynamics of Colonization and Expression of Pathogenicity Related Genes in *Fusarium oxysporum* f.sp. *ciceri* during Chickpea Vascular Wilt Disease Progression. PLoS ONE 11(5): e0156490. doi:10.1371/journal.pone.0156490.
3. **Medha L. Upasani**, Bhakti M Limaye, Gayatri S. Gurjar, Sunitha Manjari K., Rajendra R. Joshi, Narendra Y. Kadoo, Vidya S. Gupta. Chickpea-*Fusarium* interaction transcriptome reveals differential modulation of plant defense and fungal virulence strategies (Nature Scientific Reports, in Review)
4. **Medha L. Upasani,** Meenakshi B. Tellis, Tanvi Phaltane, Gayatri Gurjar, Narendra Y. Kadoo, Vidya S. Gupta. Transcriptional regulations in Chickpea-*Fusarium* interaction. (Under Preparation)

References

Dr. Vidya S. Gupta

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

Dr. Narendra Y. Kadoo

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

Dr. R. L. Deopurkar

Emeritus Professor,
Department of Microbiology,
Savitribai Phule Pune University,
Pune-411007, India.
Email : writetodeopurkar@gmail.com
Ph. +91-9371026111125