Proteomic and metabolomic analysis of chickpea-*Fusarium oxysporum* interactions

A thesis submitted to the

Savitribai Phule Pune University

For the degree of

DOCTOR OF PHILOSOPHY

In

BIOTECHNOLOGY

By

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February 2015

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CERTIFICATE

Certified that the work in the Ph. D. thesis entitled, '**Proteomic and metabolomic analysis of chickpea-*Fusarium oxysporum* interactions'** submitted by **Mr. Yashwant Kumar** was carried out by the candidate under my supervision. The material obtained from other sources has been duly acknowledged in the thesis.

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DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled, **"Proteomic and metabolomic analysis of chickpea-*Fusarium oxysporum* interactions"** submitted by me for the degree of Doctor of Philosophy to the University of Pune, is the record of work carried by me at Biochemical Sciences Division, CSIR-National Chemical Laboratory, Pune - 411008, Maharashtra, India, under the supervision of Dr. Vidya S. Gupta (research guide) and Dr. Ashok P. Giri (research co-guide). The work is original and has not formed the basis for the award of any degree, diploma, associateship, and fellowship titles in this or any other university or other institute of higher learning. I further declare that the material obtained from other resources has been duly acknowledged in the thesis.

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Dedicated to my
beloved brother
….Sanju

This is just for you…

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Acknowledgements

It is my pleasure to convey my gratitude and acknowledge the contribution of all, who have contributed to my Ph. D. thesis research and writing this dissertation. First of all, I owe sincere appreciation to my research advisor Dr. Vidya S. Gupta for her supervision, patience, motivation and guidance from the very early stage of this research. She has molded me into an independent researcher by giving me all the intellectual freedom to put my own thoughts in designing and conducting the experiments. She has been my oasis, whenever I was lost in the dessert of confusions. Without her guidance and persistent help, this dissertation would not have been possible. I am indebted to her more than she owes.

I would like to convey my sincere gratitude to my research co-advisor, Dr. Ashok P. Giri, for his constructive suggestions and criticism which helped me to focus my research objectives. He helped me unconditionally in all aspects and I had the privilege of learning many things from him. My special thanks to Dr. Narendra Kadoo for his support and insightful suggestions that helped me to troubleshoot technical difficulties in my research.

I am thankful to Prof. Huiru Tang and Prof. Limin Zhang (Wuhan Institute of Physics and Mathematics, CAS, China) for help in NMR data analysis. I am also very thankful to Dr. Mahesh Kulkarni, Dr. Rajamohanan and Mrs. Santa kumari for providing me the central facilities of CSIR-National Chemical Laboratory for proteomics and metabolite data acquisition. I gratefully acknowledge Dr. Paul Goulding (Nonlinear Dynamics,UK) for providing support for non-targeted metabolomic analysis with Progenesis QI software, Dr. Moskau Detlef (Bruker BioSpin, Switzerland) for the help rendered during data acquisition; Dr. Nicholas Heard (Imperial College London, UK) for help in Spline Cluster analysis. I cannot forget the continuous support of Dr. Jonathan Fox, Dr. Lee Gething, Dr. Alex Muck, Dr. Jessica Wang and Dr. Junhua Wang for proteomics and metabolomics data analysis.

I gratefully acknowledge Mr. Priyabrata Panigrahi for his support in data analysis using different programming languages and for teaching me the basics R and perl for proteomics and metabolomics data processing. I also thank Mr. Avinash Ghanate for support in data processing using MATLAB. My special thanks to Dr. Bhushan Dholakia for supporting me in research and personal problems. I gratefully thank Dr. Arvind, Dr. Yogesh, Dr. Prabhakar and Mr. Swamy for their company all the time. I also thank Ms Veena Dewangan for her support in my research work.

I enjoyed the company of my friends and labmates- Amey, Atul, Anand, Aditi, Amol K., Amol J., Ashish, Ashwini R, Manasi M., Neha M., Neha K., Priyanka S., Sachin, Sheon, Yojana, Deepika, Hemangi, Gopal, Gouri Pathak, Gayatri, Medha, Monika, Radhika, Nidhi, Priyanka J., Pranjali, Prushottam, Rakesh P., Rakesh J., Rahul, Ram, Ramesha, Rasika, Ramya, Ranjit, Saandip, Saleem, Sonal, Smiriti, Sucheta, swapnil, Tejas, Uma, Vishal, Vitthal, Yamini and other lab members. I

reserve special thanks to Indira mam, Sheetal and Yashshree who continue to manage the scientific requirements of the group. I would like to thanks all supporting staff including Jagtap kaka, Ramakant, Giri and Trehan. A special thanks to my best friend, Mr. Ajit.

I feel happy to express my thanks to Dr. Sourav Pal, Director, CSIR-NCL for providing me the research facilities. I must thank the office staff of Biochemical Sciences Division and Students Academic office, which was always ready to help whenever required. I thank CSIR, India for supporting my research work through Research fellowships.

Last but not least, I would like to pay high regards to my family. There are no words to acknowledge my family for their constant support and encouragement. I owe my deepest gratitude to my younger brother, Sanju for giving me freedom and supporting all the way, Maa, Papa, didi, Guddu, and the list is endless, for their support and love which made me to reach up to this level.

Besides this, several people have knowingly and unknowingly helped me in the successful completion of this project. I thank all of them for their assistance.

Yashwant Kumar

Abbreviations

% CV RT	Percent Coefficient Of Variance Of Retention Time
1-DE	One Dimensional Electrophoresis
^{1}H-NMR	Proton Nuclear Magnetic Resonance
2D PAGE	Two-Dimensional Polyacrylamide Gel Electrophoresis
2-DE	Two Dimensional Electrophoresis
ABA	Abscisic acid
ACN	Acetonitrile
Ala	Alanine
Asn	Asparagine
BEH	Bridged-Ethyl Hybrid
BiP	Luminal Binding Protein
BSA	Bovine Serum Albumin
CAGE	Cap Analysis of Gene Expression
CAOMT	Caffeic acid O-methyltransferase
CCoAMT	Caffeoyl-CoA O-methyltransferase
cDNA	Complementary deoxyribonucleic acid
CHI	Chalcone Isomerase
CHS	Chalcone Synthase
COSY	^{1}H-^{1}H Correlation Spectroscopy
CuAO	Copper amine oxidase
DAI	Days After Innoculation
DHAR	Dehydroascorbate Reductase
DRR	Disease Resistance Response
DTT	Dithiothreitol
DV	Digvijay
EDTA	Ethylenediamine tetraacetic acid
EIC	Extracted Ion Chromatogram
ER	Endoplasmic Reticulum
ESI-	Electrospray Ionization-Negative mode
ESI+	Electrospray Ionization-Positive mode
FAO	Food And Agriculture Organization Of The United Nations
FAOSTAT	Statistics department of FAO
FDR	False Discovery Rate
Fmol, mmole	Femtomole, millimole
µmole	micromole
Foc	Fusarium oxysporum f. sp. Ciceri
FTMS	Fourier Transform Mass Spectroscopy
g, mg, µg, ng	Gram, milligram, microgram, nanogram
GABA	Gamma-Aminobutyric Acid
GC-MS	Gas Chromatography Mass Spectroscopy
GFP-B	Glu-Fibrinopeptide B
Gln	Glutamine
Glu	Glutamic acid
GST	Glutathion S-transferase
HCL	Hydrochloric acid
HDMS	High Defination Mass Spectroscopy
HESI	Heated Electrospray Ionization Source
HMBC	^{1}H-^{13}C Heteronuclear Multiple-Bond Correlation

Hsp	Heat Shock Protein
HSQC	Heteronuclear Single Quantum Coherence Spectroscopy
IAA	Iodoacetamide
ICRISAT	International Crops Research Institute for Semi Arid Tropics
IF4α	Initiation Factor 4α
IFR	Isoflavone Reductase
IFS	Isoflavonoid Synthase
IIPR	Indian Institute of Pulse Research
Ile	isoleucine
JG	JG62
JRE	Jump-Return-Echo
KCL	Potassium chloride
KH2PO4	Potassium dihydrogen phosphate
LC-ESI-LTQ-Orbitrap	Liquid Chromatography-Electrospray Ionization Linear Ion Trap Quadrupole-Orbitrap
LC-MS	Liquid Chromatography Mass Spectrometry
LC-MS/MS	Liquid Chromatography tandem Mass Spectrometry
LC-QTOF	Liquid Chromatography-Quadrupole Time-Of-Flight
Leu	leucine
lincRNA	Long intergenic ncRNA
lncNAT	Long non-coding natural antisense transcripts
lncRNA	Long non-coding RNA
LTQ-FT MS	Linear Ion Trap- Fourier transform-Mass Spectroscopy
Lys	Lysine
M, mM, mM	Molar,millimolar, micromolar
MALDI-TOF/TOF	Matrix-assisted laser desorption/ionization-Time-Of-Flight/ Time-Of-Flight
MAS	Marker Assisted Selection
MLP	Major Latex Protein
MPKV	Mahatma Phule Krishi Vidyapeeth
MPSS	Massively Parallel Signature Sequencing
mRNA	Messenger RNA
MS	Mass Spectrometry
Multi-dim	Multi dimension
nESI-IT MS/MS	nano- Electron Ionization–Ion Trap– Tandem Mass Spectrometry
NF-Y	Nuclear Transcription Factor-Y
NMCEI	National Multi-Commodity Exchange of India
NMR	Nuclear Magnetic Resonance
OPLS-DA	Orthogonal Partial Least Squares-Discriminant Analysis
PCA	Principle Component Analysis
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDI	Protein Disulfide-Isomerase
PIP	Plasma membrane intrinsic protein
PLGS	ProteinLynxGlobal server
PME	Pectin Methyl Esterase
ppm	Parts per million
PR	Pathogenesis-Related
QC	Quality Control

QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RILS	Recombinant Inbred Lines
RNA	Ribonucleic acid
RNAi	RNA interference
RNA-Seq	sequencing of RNA
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
RR	Resistance Related
RT	Retention Time
SAGE	Serial Analysis Of Gene Expression
SBP	Streptavidin-Binding Peptide
SBP	Selenium Binding Protein
SSR	Simple Sequence Repeat
STH	Saitohin
TAR	Transcriptionally Active Region
TCA	Trichloroacetic acid
TOCSY	^{1}H-^{1}H Total Correlation Spectroscopy
Trp	Tryptophan
TSP	sodium3-trimethlysilyl [2,2,3,3-D4] propionate
UHPLC	Ultra High Performance Liquid Chromatography
UPR	Ubiquitin-Proteasome Pathway
UPR	Unfolded Protein Response
UV	Unit Variance
Val	Valine
VIP	Variable Importance in Projection

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Chapter 1 Introduction

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Annexure 2: List of all the identified metabolites which has highest loading influence (of VIP>1.0) using high resolution orbitrap LC-MS with their details.

Thesis Summary

Chickpea (*Cicer arietinum* L.) is the second most widely grown legume after soybean in the world. This legume has been considered as a beneficial source of carbohydrates, proteins, minerals, vitamins, b–carotene and health-promoting fatty acids. Chickpea seed, composed of approximately 21% protein, are a primary vegetarian source of human dietary protein for millions of people in developing countries, and thus, is nutritionally significant. As a top producer, India, along with neighboring countries, accounts for about 90% of global chickpea production (http://faostat.fao.org/site/339/default.aspx). However, the major constraints in chickpea production are biotic stresses like Fusarium wilt, Ascochyta blight, pod borer and abiotic stresses such as drought, heat, cold and high-salinity. The estimated collective yield loss due to biotic stresses is 4.8 million ton in spite of successful efforts of national and international breeding programs. Annual yield losses due to fungal wilt alone have been estimated to be at 10 to 90% (Jimenez-Diaz *et al.,* 1989).

Fusarium wilt is caused by about eight races of *Fusarium oxysporum* f. sp. *ciceri* (Foc) affecting all major chickpea growing areas and at least three Foc races are known to exist in India (Gurjar *et al.,* 2009). Foc infects plant roots and clogs the xylem, resulting in obstruction of nutrient supply, ultimately leading to plant death. It can survive many years in soil even without its host and hence, poses a serious challenge for disease management (Haware *et al.,* 1996). Development of Foc resistant chickpea cultivars through breeding programs is the most effective way to counter fungal attack. However, pathogen variability and mutability result in loss of host resistance and remain as main hurdles for plant breeders (Barve *et al.,* 2001).

In sharp contrast to the importance of chickpeas as staple food and industrial raw material, the biotic stress at the proteome and metabolite levels had only been dealt with at very low throughput until some years ago. In the last several years, many low throughput studies have been carried out to identify the molecular basis of chickpea resistance or susceptibility to Foc using various approaches including candidate gene identification, differential expression and biochemical analysis post-fungal infection (Giri *et al.,* 1998; Gupta *et al.,* 2013). Due to the variations in chromosomal rearrangements between non-model and model plants, differences in plant-pathogen interaction with respect to signaling and immunity events are expected

and evinced (Gupta *et al.,* 2013). Therefore, studies on non-model plants with combination of unbiased modern high-throughput technologies are required to improve our knowledge of dynamics of complex plant-fungus interaction (Kushalappa and Gunnaiah 2013). In recent years, few studies have been performed on plant-pathogen systems of Fusarium head-blight and cereal crops using functional genomics (Cho *et al.,* 2012), proteomics (Yang *et al.,* 2010) and metabolomics (Bollina *et al.,* 2011; Kumaraswamy *et al.,* 2011) approaches to understand the complex machineries involved in *Fusarium* infection process in the host tissues. Consequently, this study was aimed to identify the mechanistic basis for resistance/ susceptibility in chickpea against Foc by non-targeted high-throughput profiling of metabolites and proteins. Further, a targeted gene expression analysis was performed to confirm the findings of non-targeted metabolite/protein analyses. The following objectives were formulated to deal with the problem presented. 1) To understand the mechanisms of Foc resistance/ susceptibility in chickpea using non-targeted quantitative metabolomics using high resolution mass spectrometry and NMR based metabolomic profiling along with multivariate data analysis approach. 2) To identify the molecular mechanisms in chickpea for resistance/ susceptibility against Foc with label-free quantitative proteomics along with multivariate data analysis approach. 3) Validation of the identified proteins/metabolites with gene expression analysis using few representative candidate genes.

Phenotypic evaluations

The Fusarium wilt susceptible (JG) and resistant (DV) chickpea cultivars were inoculated with Foc. JG showed typical wilting phenotype (>95%) with yellowing on 2 days after inoculation (DAI), followed by drooping of leaves that finally caused plant death by 12 DAI. Whereas, the mock-inoculated resistant or susceptible cultivar and Foc inoculated resistant cultivar remained healthy till maturity (~80 DAI). The establishment of the pathogen within host vascular tissue, characterized by colonization in the xylem vessels, was observed after 2 DAI. Therefore, root tissues from these Foc and mock-inoculated chickpea plants were collected at 2, 4, 8 and 12 DAI. These were used to identify the global metabolic changes during chickpea-Foc interactions with high-throughput non targeted quantitative metabolomics approach.

Proteomic profiling of chickpea roots against Foc infection

Plant defense mechanisms, either innate or induced, involve various kinds of proteins such as pathogen/pattern recognition receptors, proteins produced by the R genes, enzymes mediating oxidative burst, hypersensitive response, PR proteins, signaling pathways and enzymes catalyzing the biosynthesis of secondary metabolites. A temporal protein expression of chickpea resistant and susceptible cultivar at 0, 2, 4, 8 and 12 days after inoculation with Foc along with their respective controls (mock inoculated) were analyzed using label free quantitative proteomics. The proteomics analysis identified a total of 811 proteins. Of the 811 identified proteins, 481 showed statistically significant differential accumulation in resistant and susceptible cultivars at every time point with $P< 0.05$ and fold change >1.2. We have observed a complex response from numerous interconnected metabolic pathways including glycolysis /gluconeogenesis, TCA cycle, phenylepropenoid pathway, and increased lignification. Other cellular processes altered during Foc infection include unfolded protein response and defense related proteins. Proteins involved in isoflavenoid and flavenoid pathway for example, chalcone reductase, chalcone isomerase, Isoflavone reductase, isoflavone 4'-O-methyltransferase, isoflavone-7-O-methyltransferase and 2-hydroxyisoflavanone synthase has been found more expressed in resistant cultivar than susceptible suggests that resistant plant synthesize phytolexins efficiently to combat Fusarium oxysporum infection. Intense lignin deposition on the cortex of the roots of Foc inoculated resistant cultivar was also confirmed by microscopy in the present study. Redox state alterations coupled to downstream defense signaling plays important role in Foc recognition and responses. Taken together, ROS generation and higher accumulation of copper amine oxidase in this study suggested that the Foc infection triggered hydrogen peroxide generation, lignosuberization process and initiation of defense response in the resistant cultivar of chickpea DV.

Plants respond to pathogen challenge by increased activation of phenylpropanoid pathway. In the present study, subsets of proteins participating in multiple branches of phenylepropanoid pathway including lignins, flavanoids and phenolics biosynthesis were identified in Foc inoculated chickpea cultivars. Caffeic acid O-methyltransferase (CAOMT) and caffeoyl-CoA O-methyltransferase (CCoAMT) lignin biosynthetic enzymes were upregulated in the resistant in comparison with the susceptible chickpea cultivar suggesting increased lignin

deposition thereby providing resistance against Foc in the present study. Enzymes involved in methionine metabolism, including methionine synthase, adenosine homocystenin hydrolase, adenosine kinase, and AdoMet synthetase showed higher accumulation in the Foc inoculated resistant cultivar at both, early and late stages, whereas these proteins were significantly down regulated at an early stage with increase in late stages in susceptible cultivar. Taken together, our results indicated the close association between pathogen defense and increased level of activated methyl groups.

During stress, accumulation of misfolded or unfolded proteins increases in ER that result in triggering unfolded protein response (UPR) to remove malfunction proteins by ubiquitin-proteasome pathway. In the present study, several UPR proteins including Hsp70, calnexin, luminal binding protein (BiP), calmodulin, translocon associated proteins (SEC61), component of SCF-for SKP1-Cullin-F box protein, ubiquitin ligase complex, 26s proteosome, calthrin and protein disulfide-isomerase (PDI) were identified in chickpea roots of both the cultivars upon Foc inoculation. The majority of these proteins were high in early, but low in late stage in the resistant cultivar while susceptible cultivar showed opposite accumulation pattern. During defense response, there is constant requirement for proteins stabilization in the process of folding, assembly, vesicle trafficking and secretion. Up-regulation of 14-3-3 transcripts was detected in Foc inoculated resistant chickpea root in previous study (Nimbalkar *et al.,* 2006). Thus, up-regulation of 14-3-3 and H+-ATPase suggested their role in activating hypersensitivity response during fungal infection in chickpea leading to resistance. Overall quantitative proteomics data suggested that there is orchestrate event that determine the defense response in chickpea against Foc.

NMR based metabolomics profiling of chickpea root against Foc infection

^{1}H-NMR was acquired for resistant and susceptible chickpea plant after Foc infections. A total of 52 metabolites were identified and dominating metabolites noted from NMR spectra included a range of amino acids (Ala, Val, Ile, Leu, Gln, Glu, Asn, Trp, Lys and GABA), sugars (Glu, Suc, Fru, trehalose and salicin), some organic acids (pyruvate, lactate, acetate, citrate, succinate, formate, fumarate, malate and guanidoacetate), nucleosides (adenosine, uridine, inosine, 5CMP and hypoxanthine), phytoalexins (genistein and luteolin), clotrimazole, cholesterol, lipids and few

unknown resonances. The overview of global metabolic alterations in Foc inoculated resistant and susceptible chickpea roots compared with their respective control were constructed by principal component analysis of all the data collected. The PCA trajectory obtained from the resistant inoculated groups followed a globally similar trajectory trend to control, but the susceptible inoculated group showed a significantly different process from the control, which showed dramatic trajectory movement after 8 days of inoculation. The metabolic changes induced due to Foc inoculation were further evaluated by constructing OPLS-DA models.

We also observed change in concentration of various amino acids as a result of deleterious effect of Foc infection. The amino acids including Val, Thr, Ala and Asn significantly decreased in Foc-inoculated resistant and susceptible cultivars in the early stage (till 8 days), which suggested that Foc probably utilized these amino acids for its establishment and proliferation inside the host. Interestingly, ^{1}H NMR metabolomic data showed rapid decrease in sugars such as sucrose and fructose in both but more predominantly in susceptible cultivars upon Foc infection as compared to the resistant cultivar. These results emphasized the regulatory role of sugars in the metabolic reprogramming. The accumulation of phytoalexins and phenolics such as genistein, luteoline and quinone are identified by metabolome analysis. Thus in our study, accumulation of isoflavonoid metabolites in Foc inoculated resistant chickpea cultivar suggested their potential involvement in Foc resistance.

High resolution mass spectrometer (LC-Orbitrap) based metabolomics

More comprehensive analysis of perturbed metabolome was done in chickpea cultivars infected with Foc using high resolution LC-MS. Untargated metabolomics is a powerful method for a comprehensive investigation of metabolite variations in biological systems. We have used non-targeted metabolomics approach using high resolution orbitrap mass spectrometer to systematically identify metabolic modulations in root during various stages of the interaction between resistant and susceptible chickpea varieties and wilt causing fungal pathogen Foc. Further, multivariate data analysis approach was employed to select the features that are differential expressed after Foc inoculation. This approach has enabled us to understand the Foc induced mechanism for resistance or susceptibility in chickpea and metabolic network associated during the disease development.

The non-targeted metabolomics investigation revealed total 5,018 and 6,508 metabolite ions in DV and JG62, respectively from ESI(+) mode, while ESI(-) analysis revealed total 4,909 and 5,058 metabolite ions in DV and JG62, respectively with significance of P< 0.005, FDR< 1% and more than two fold change. To ascertain the time course of metabolic variations that were induced in resistant and susceptible plant after Foc1 inoculation, a PCA model was constructed for LC-MS metabolomics data from the control and Foc1 inoculated DV and JG62 plants at all the stages. We observed clear separation between control and inoculated plants at all the stages. The OPLS-DA models were constructed to distinguish chickpea root metabolome between resistant and susceptible inoculated chickpea plant at various time points. It showed the discriminating metabolites in chickpea root tissue associated with Foc inoculation such as flavanoids, isoflavanoids, alkaloids, amino acids and sugars. Foc inoculated resistant plants were found to accumulate more flavanoids and isoflavanoids along with their malonyle conjugates. Many fungicides that induce after fungal infection for example, obstine beta glucosides and querecitin were observed elevated in resistant cultivar of chickpea. Overall this study suggested that genetic and biochemical mechanisms responsible for defense were different in Foc resistant cultivar from susceptible.

Candidate gene expression analysis

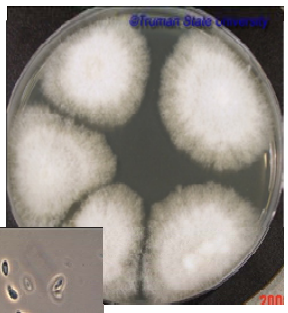
Expression levels of key genes involved in various metabolic pathways showed very good concurrence with proteomic and metabolomic results. These included genes from nitrogen mobilization (glutamate dehydrogenase-GDH, glutamate synthase, glutamine synthase and asparagine synthase), stress response (NF-Y and SKP1-like protein 1A), methionine metabolism (methionine synthase and AdoMet synthetase) and lignin and phytoalexin biosynthetic pathway (CCoAMT, CHS, CHI, isoflavone 4'-O-methyltransferase, IFS and IFR). Quantitative real time expression of these genes concurrence in the finding of overall study in resistant and susceptible inoculated as well as in their control suggests that these have potential role in defense against Foc.

This study concludes that Foc inoculation lead to activation of phenylpropanoid pathway plays a major role in chickpea defense against Fusarium oxysporum. These key phenylpropanoids/flavonoids could potentially be used as biomarkers for the development of disease resistant chickpea varieties. The

endoplasmic reticulum (ER) stress response plays an important role in determining the pathogenicity towards Foc. Efficient unfolded protein response (UPR) in resistant cultivar of chickpea helped to combat against Foc. Also, Successful pathogens compete with the host for essential metabolites and attempt to capture its primary metabolism. On the other hand, in an incompatible reaction the host utilizes its mass energy to protect its primary metabolism from the foreign invaders. Sugar and nitrogen metabolism occupies a pivotal position in plant life.

Chapter 1

Introduction



Chickpea is a cool season legume crop and is grown in several countries worldwide as a food source and is commonly known as Bengal gram, Chana, or Garbanzo. Seed is the main edible part of the plant and is a rich source of protein, carbohydrates and minerals especially for the vegetarian population. As in case of other legume crops, even chickpea can fix atmospheric nitrogen through its symbiotic association with *Rhizobium* sp.; thus helping in enhancing the soil quality for subsequent cereal crop cultivation. It belongs to the family Leguminosae and is a self-pollinating annual plant with a diploid genetic content of 2n=16. Classification of the chickpea is as follows:

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Fabales
Family: Fabaceae
Subfamily: Faboideae
Genus: *Cicer*
Species: *C. arietinum* L.

1.1 Origin, occurrence and domestication

Chickpea has been thought to originate in south-eastern Turkey, from where it has spread to other countries of the world. It was first grown in Turkey around 7,500 B.C. *Cicer arietinum*, the cultivated species of *Cicer*, has been domesticated from *C. reticulatum* Ladizinsky, a closely related wild species. After its domestication in Middle East this crop progressed further throughout the Mediterranean region, India and Ethiopia (Ladizinsky, 1975; van der Maesan, 1987). Lately, it has been introduced in Mexico, Argentina, Chile, Peru, Australia and the US (Duke, 1981). The present scenario indicates its plantation in countries like India, Pakistan, Turkey, Iran, Myanmar, Ethiopia, Mexico, Australia, Syria, Spain, Canada, United States, Bangladesh, Algeria, Ethiopia, Malawi, Sudan, Tanzania, Tunisia, and Portugal (NMCEI, India, 2009).

1.2 Growth conditions

Chickpea is a hardy, deep-rooted, dryland crop sown on marginal lands, which can grow to full maturity in conditions that would be unsuitable for most crops (Singh and 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. The yield is maximal when the legume crop is grown in sandy, loam soils possessing an appropriate drainage system; since it is very sensitive to excess water availability. Also, very cold conditions can greatly reduce the productivity of chickpea. It is basically a rabi crop, grown in months of September-November and harvested in the months of February-April. Maturity period ranges from 95-110 days after sowing.

1.3 Nutritional importance of chickpea

Chickpea acquires importance as it provides protein rich food for humans as well as for livestock. Furthermore, chickpea seed coats and pod covers can also be used as fodder. It is consumed as a fresh immature green seed, whole seed, dhal and flour (Fig 1.1). In grain legumes, proteins are an important seed component and are responsible for their relevant nutritional and socio-economic impact. The chickpea seed is a good source of carbohydrates and proteins, which together constitutes 80% of the total dry seed weight. On an average, chickpea seed contains 64% total carbohydrates, 23% protein, 47% starch, 6% crude fiber, 6% soluble sugar, 5% fat and 3% ash. The mineral component is high in phosphorus (340 mg/100 g), calcium (190 mg/100 g), magnesium (140 mg/100g), iron (7 mg/100 g) and zinc (3 mg/100 g). Chickpea protein has the highest digestibility when compared to other dry edible legumes. The lipid fraction is high in unsaturated fatty acids, primarily linoleic and oleic acids (Ibrikci *et al.,* 2003). Thus, chickpea serves as a main source of dietary protein for more than 80% of the Indian population which is vegetarian in nature. Since chickpea plays the pivotal role of supplying protein source in the vegetarian diet, it is also called as the ‘poor man’s meat’. Supplementation of cereals with high protein legume is potentially one of the best solutions to protein-calorie malnutrition, particularly in the developing countries.

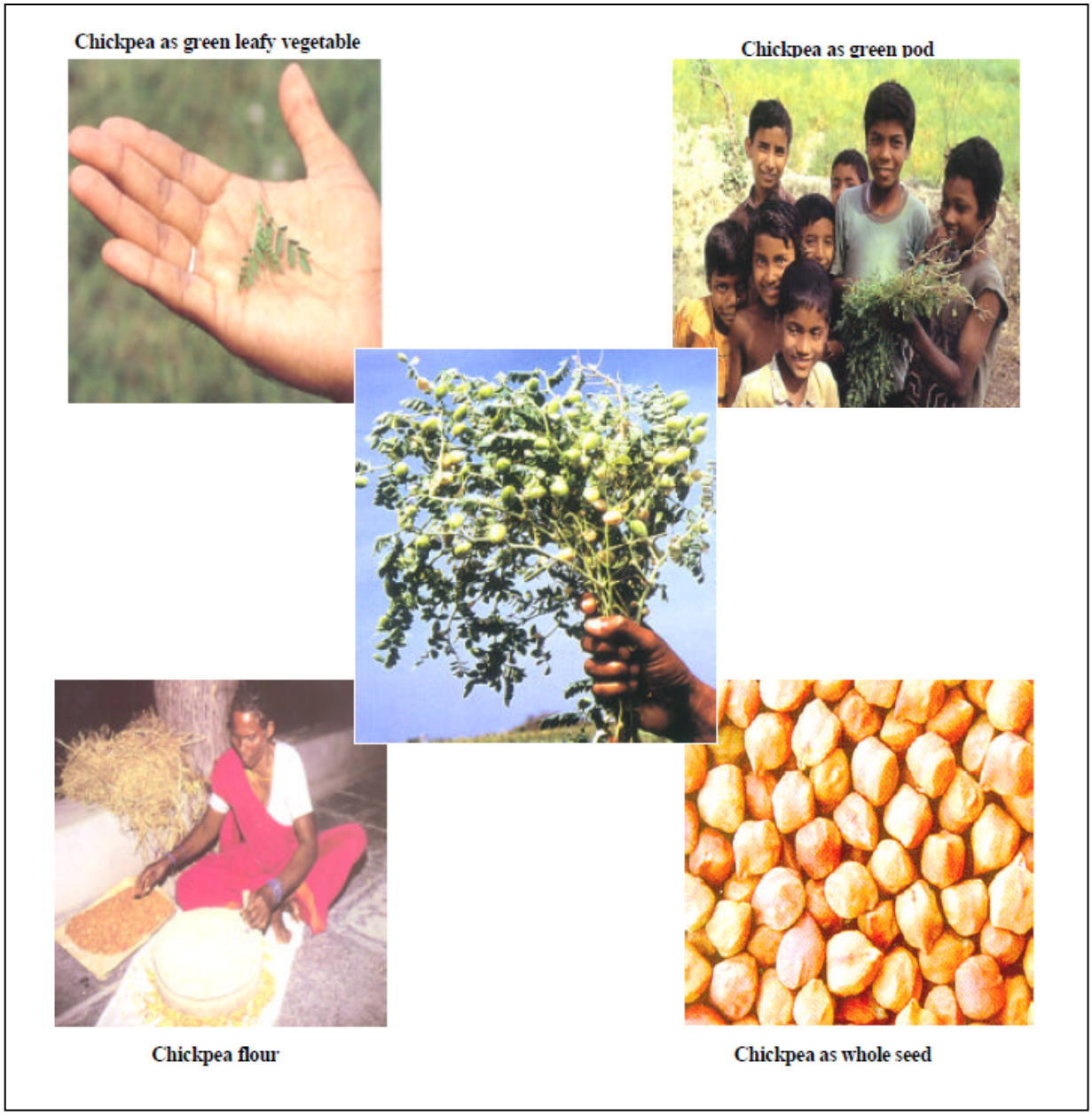
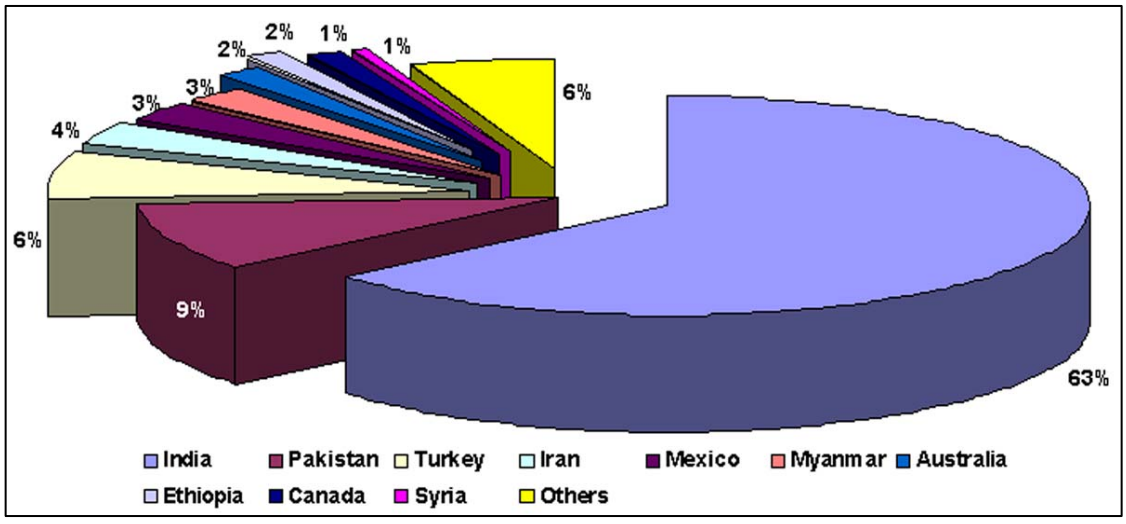


Fig 1.1: Different forms of chickpea used for consumption

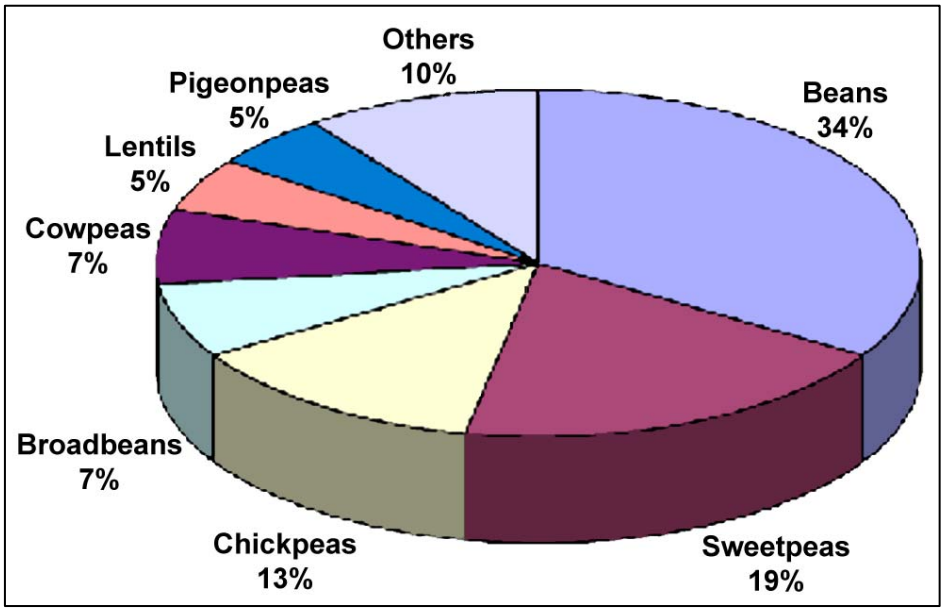
1.4 Chickpea production

ckpea is grown in at least 33 countries including Central and West Asia, South Europe, Ethiopia, North Africa, North and South America and Australia. It is the second most important cool season pulse crop in the world (Ladizinsky and Adler 1976, Singh 1997). India is the largest producer of chickpea accounting for 75% and 73%, respectively, of the world`s share in terms of the area under cultivation and production (Jodha and SubbaRao 1987) (Fig 1.2 A). Among various pulse crops, chickpea covers over 40 % of total pulse production followed by pigeonpea (18-20%), mungbean (11%), urdbean (10-12%), lentil (8-9%) and other legumes (20%) (IIPR Vision 2030) (Fig. 1.2 B). It covers 15% (10.2 million hectares) of the area and accounts for 14% (7.7 million ton) of the production of pulses in the world (FAOSTAT 2012) (Fig. 1.2 C). In India, `Kabuli' type with bold and cream coloured seeds is grown in around 10% area whereas `Desi' type of chickpea with small and brown seeds accounts for nearly 90% area under chickpea cultivation (Fig. 1.2 D).

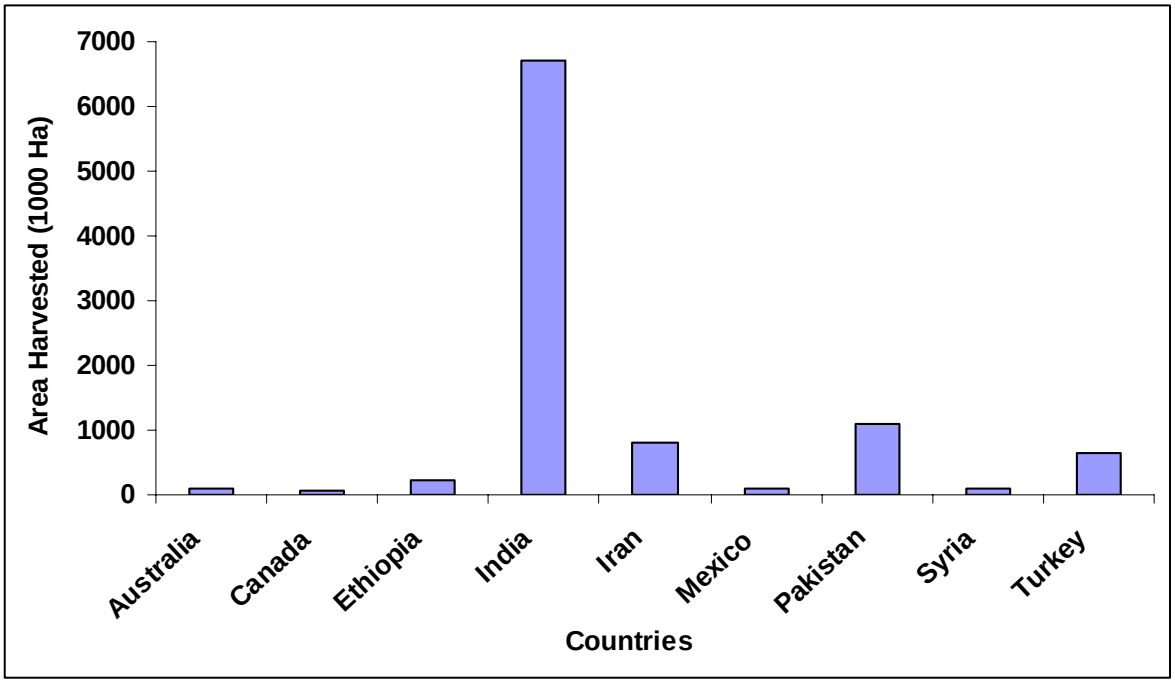
A



B


Others
10%
Pigeonpeas
5%
Beans
34%
Lentils
5%
Cowpeas
7%
Broadbeans
7%
Chickpeas
13%
Sweetpeas
19%

C


Area Harvested (1000 Ha)
7000
6000
5000
4000
3000
2000
1000
0
Australia
Canada
Ethiopia
India
Iran
Mexico
Pakistan
Syria
Turkey
Countries

D

Figure 1.2: Contribution of Indian agriculture to global production of chickpea. A) India is the world's largest producer of chickpeas, contributing to >60% of the total global produce. B) Global production of chickpea in comparison with other legume crops. C) Area under cultivation of chickpea. D) Desi and Kabuli type of chickpea. (Source: FAO Data (http://faostat.fao.org)).

Chickpea is a hardy deep-rooted dry land crop and can grow to full maturity despite conditions that would prove fatal for most crops. Nearly 90% of the crop is cultivated under rainfed conditions mostly on receding soil moisture. It is grown on marginal land and rarely receives fertilizers or protection from diseases and insect pests (Singh and Reddy 1991).

1.5 Production losses due to stresses

The yield output of chickpea at present surpasses 5.0 t/ha with about 0.8 t/ha of an average yield. This large difference between these two yield data could be attributed to mainly diseases and poor management practices. Various biotic and abiotic stresses are major factors for the reduction in chickpea production as shown in Fig. 1.3 A (Singh *et al.,* 1994). Drought is the most important factor in chickpea yield loss. Additionally, during the pod-filling phase, terminal drought stress affects frequently in chickpea leading to production loss (Nageshwara Rao *et al.,* 1985a and 1985b). During the late vegetative phase cold is another critical factor that can cause the reduction in total chickpea yield and as chickpea grows from germination to flowering stage the degree of cold tolerance generally reduces (Singh *et al.,* 1984b; Wery, 1990). Among the biotic stress, there are about three bacteria, 22 viruses, 67 fungi and 80 nematodes which are known to affect chickpea and few of them are considered to be economically important diseases (Nene *et al.,* 1996; Haware, 1998). There has also been an increase in various chickpea pathogens in the past two decades. The insect *Helicoverpa armigera* is the most threatening pest of chickpea that can survive on developing seeds, flowers and foliage. Similarly in virus induced diseases, the stunt is the most prevalent in majority of global regions of chickpea production. In tropical and temperate regions, diseases like Fusarium wilt, Ascochyta blight and botrytis gray mold are major economically important fungal diseases of chickpea. Tables 1.1 depict diseases caused by fungi, viruses and nematodes in chickpea. The potential seed yield of about 5 t/ha has been reported in chickpea. However, the realized seed yield hovers around 850 kg/ha (world average ≈ 0.8 t/ha, FAOSTAT, 2005) which has stagnated over the years (Fig 1.3 B). However, in this chapter, more focus has been given to Fusarium wilt and chickpea.

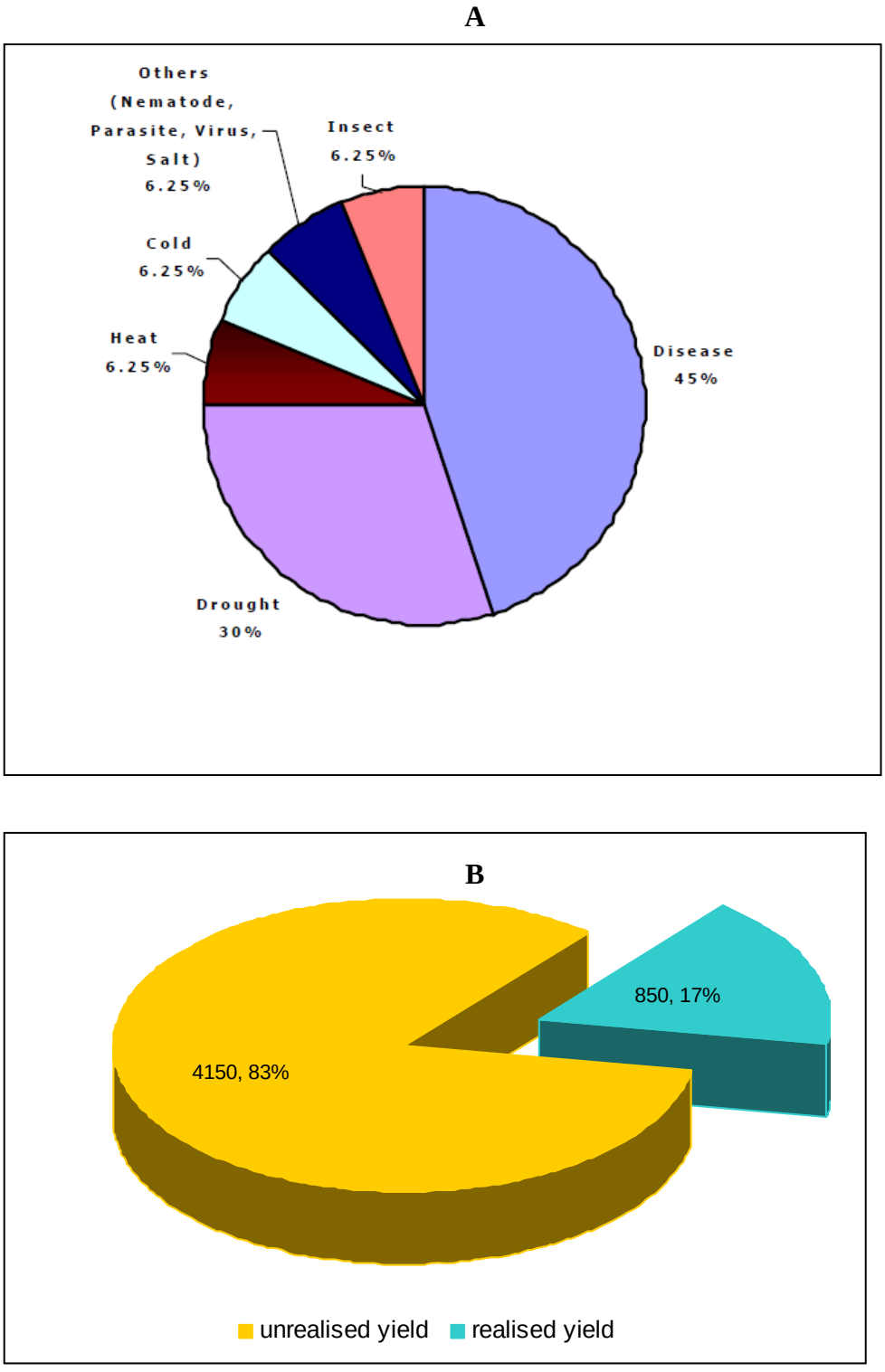


Figure 1.3: A) Yield loss in chickpea. B) Unrealized yield of chickpea due to various pests and disease

Table 1.1: Various diseases of chickpea and causative agent

Disease	Causative agent
Fungal diseases (Soil borne)	
Fusarium wilt	*Fusarium oxysporum* Schlecht.emend Snyd. & Hans. f.sp. *ciceri* (Padwick) Snyd. & Hans.
Verticillium wilt	*Verticillium dahliae* Reinke & Berth
Dry root rot	*Rhizoctonia bataticola* (Taub.) Butler [*Macrophomina phaseolina* (Tassi) Goid]
Collar rot	*Sclerotium rolfsii* Sacc.
Wet root rot	*Rhizoctonia solani* Khun
Black root rot	*Fusarium solani* (Mart.) sacc.
Phytophthora root rot	*Phytophthora megasperma* Drechs
Pythium root and seed rot	*Pythium ultimum* Trow.
Foot rot	*Operculella padwickii* Kheshwalla
Stem rot	*Sclerotinia sclerotiorum* (Lib) de Bary
Fungal diseases (Foliar)	
Ascochyta blight	*Ascochyta rabiei* (Pass.) Lab. (*Mycosphaerella rabiei* Kovachevski)
Botrytis gray mold	*Botrytis cinerea* Pers.ex Fr
Alternaria blight	*Alternaria alternata* (Fr) Kiessler
Stemphylium blight	*Stemphylium sarciniforme* (Cav.) Wills
Rust	*Uromyces ciceris-arietini* (Grogn.) Jacz & Beyer
Viral diseases	
Stunt	Bean (pea) leaf roll virus
Nematode diseases	
Root knot	*Meloidogyne incognita* (Kofoid and White) Chitw., *M. javanica* (Treub) Chitw.
Cyst	*Heterodera ciceri* Vovlas, greco, and Di Vito
Root Lesion	*Pratylenchus thornei* Sher and Allen

1.6 Fusarium wilt

Fusarium oxysporum f. sp. *ciceri*- (Foc) causes Fusarium wilt in chickpea growing areas between latitudes 30°N and 30°S that includes Asia, Africa, Southern Europe and USA with the dry and warm season appropriate for chickpea growth (Nene *et al.,* 1996). Transmittance of wilt disease by long distance seed dispersal into new regions generally happens due to the infected chickpea seeds. The fungal chlamydospores can survive in the soil for a minimum period of 5-6 years and therefore, it is very hard to eliminate such inoculum after its establishment in the soil. These spores can germinate upon suitable environmental conditions that lead to infection in tender roots at the seedlings stage (Haware *et al.,* 1996). Fig. 1.4 shows plants in a wilt sick plot as compared to a healthy chickpea field. Other *Cicer* species can also be affected under artificial inoculation conditions and other legume crops such as pigeonpea, pea and lentil act as the wilt carriers without any symptoms (Haware and Nene, 1982a).

Fig. 1.4: Wilt sick and healthy plot of chickpea

1.7 Fusarium wilt epidemiology

The epidemiology of wilt fungi is complex. Various factors such as inoculum density, pathotype, plant age, host resistance, plant density, soil moisture, soil nutrients, air and soil temperature may affect wilt development (Haware *et al.,* 1990). With the decrease of pH, intensity of wilt disease increases (Gupta *et al.,* 1986). Disease severity and *Foc* populations also increase with decrease in soil-matrix potential (Bhatti and Kraft, 1992). Further, maximum disease incidence occurs at 25°C in the pot tests which are conducted at 15-35°C and below 15°C no disease is developed.

1.8 Wilt pathogen life cycle

Understanding the life cycle of wilt pathogen is very important keeping in mind the relation to its survival, the causation of disease within a framework of time and space and host-parasite interactions with respect to disease resistance or susceptibility. Earlier, a model was proposed to explain the interactions between vascular wilt causing pathogens and their host plants (Fig. 1.5) that indicated discrete saprophytic and parasitic phases in life cycles of the pathogens (Beckman and Roberts, 1995). The extent of host vascular system colonization by fungus is controlled in the determinative phase. Further, in the expressive phase, disease symptoms appear, leading to the saprophytic phase of the life cycle ensuring the survival of the fungus. The wilt fungus attacks the root to acquire significant colonization in the root cortex. Along with the transpiration pull, pathogen spreads in the second phase of vascular invasion under conducive conditions for this disease. The chickpea plant defends against this pathogen invasion mainly at two levels. The first response from plant is by infusion of phenolic compounds, progressive suberinization and lignin deposition, and by hydrolytic enzymes like chitinases and glucanases at xylem parenchyma and the endodermis levels to restrict the invading fungus. Secondly, the derivatives of celluloses and hemi-celluloses namely, callose, gelgum and tyloses are synthesized in the vascular tissue to compartmentalize the

pathogen and prevent its further upward movement. Overall, the phenotypic difference as resistance and susceptibility of the chickpea cultivars is manifested by the pace of the activation of different defense mechanisms that can result into accumulation of crucial factors/metabolites to restrict the growth and spread of the *Fusarium oxysporum*.

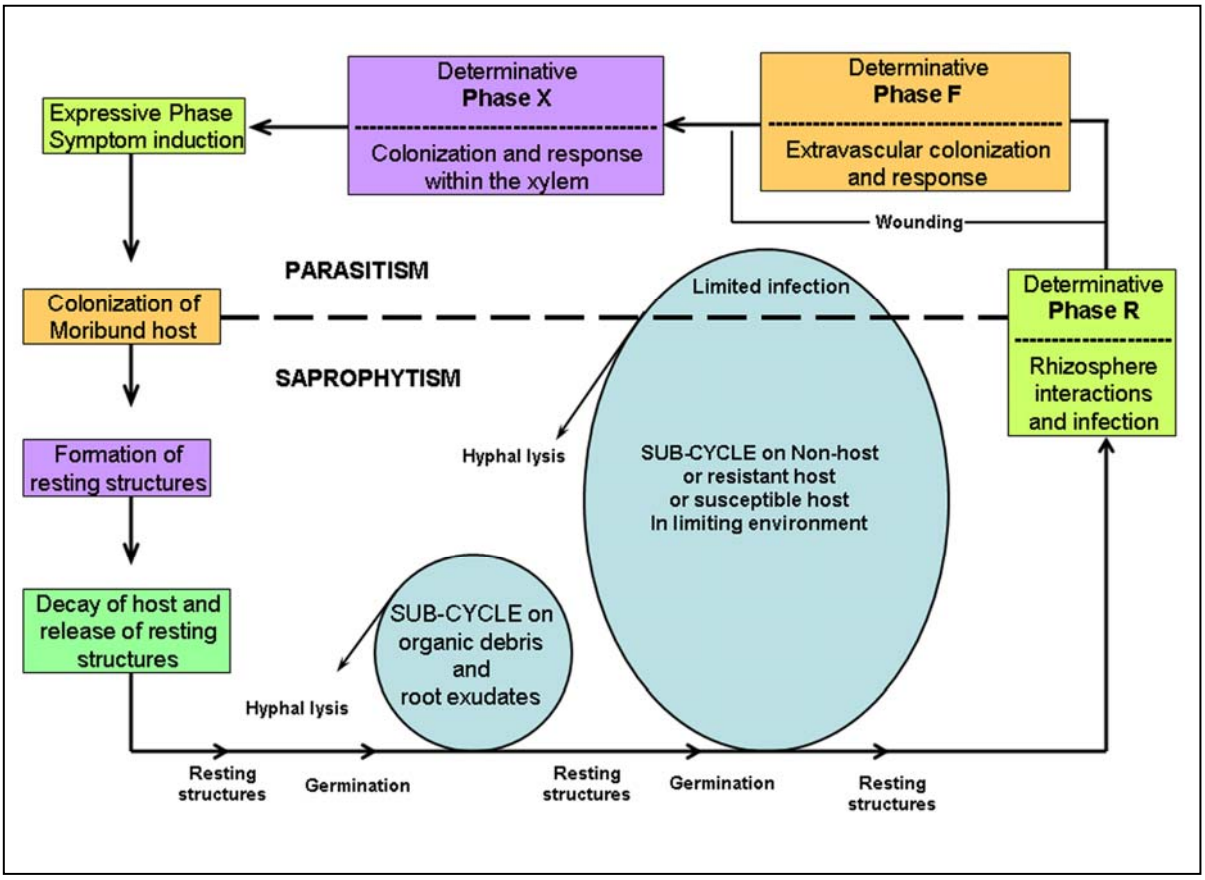


Fig. 1.5: Life cycle of *Fusarium oxysporum* (Source: Thesis of S. Nimbalkar with modifications)

1.9 Virulence and aggressiveness of Foc races

The pathogenic races are designated based on variation in the virulence within isolates of a given forma specialis. Thus, eight races of Foc are classified worldwide on the basis of the pathogenic reaction of a particular Foc isolate on the set of differential chickpea

cultivars. Jiménez-Gasco *et al.,* (2001) have reported that races 0 and 1B/1C show yellowing symptoms whereas other races 1A, 2, 3, 4, 5 and 6 prompt the wilting symptoms on these differential genotypes. Based on global occurrence, races1, 2, 3 and 4 have been shown to occur only in India (Fig. 1.6), while the Mediterranean region and in California, USA have races 0, 1B/1C and 5 (Jiménez-Gasco and Jiménez-Diaz, 2003). Similarly, USA, Morocco, Spain and India are known to be affected by race 1A whereas race 6 has been reported to be present in USA, Spain, Israel and Morocco (Jiménez-Gasco *et al.,* 2001). Recently, race 3 from India has been shown to be *Fusarium proliferatum*. Thus there are only three races Foc 1, 2 and 4 predominant in the country (Barve 2001).

Apart from the virulence Foc isolates can also differ in their aggressiveness to cause disease. The amount of disease caused by a pathotype on a given host plant genotype is defined as aggressiveness and indicated by the amount of inoculum needed to cause severe disease in a particular chickpea cultivar. For example, yellowing pathotype of Foc is less aggressive than the wilting pathotype and the aggressiveness further differed among the races belonging to a particular pathotype (Navas-Cortes *et al.,* 2000).

1.10 Recent approaches used to study Fusarium races

Morphological identification of *F. oxysporum f.* sp. *ciceris* races is difficult. The classical method of race identification involves inoculation of differential chickpea cultivars with a particular *F. oxysporum f.* sp. *cicer* isolate and determining its pathogenicity. This is a time-consuming procedure requiring at least 40 d for the analysis, and reactions can be influenced by environmental parameters (Haware and Nene 1982). Also several sets of cultivars are available and some of the differentiation is based on intermediate reactions (Sharma *et al.,* 2005). To overcome these problems molecular maker based identification has been developed. DNA-based approaches, namely gene-specific amplification, internal transcribed spacer region (ITS)-RFLP, intersimple sequence repeat (ISSR) amplification, amplified fragment length polymorphism (AFLP) and translation elongation factor 1 alpha (EF-1a) sequencing. A combination of these methods was found to be useful for the identification of Indian *F. oxysporum f.* sp. *cicer* races.

Fig. 1.6: Spread of Foc races in India

1.11 Symptoms of wilt pathogen infection

In general, dull green colored foliage are seen when chickpea seedlings are infected with Fusarium along with rapid drooping of the leaves. The uprooted plants show uneven contraction at the collar (Fig. 1. 7 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. 8) (Nene *et al.,* 1978). 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. 9) (Nene *et al.,* 1978). As seen in Fig. 1. 10, seeds are lighter and duller from the late wilted plants compared to the healthy plants. Chickpea genotypes differed 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 and 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 and 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. 1.7: (A) Dull green color of the foliage in a wilting chickpea plant (B) Single wilted plant

Fig. 1.8 Internal discoloration due to infection of the root xylem tissues when the roots are split vertically.

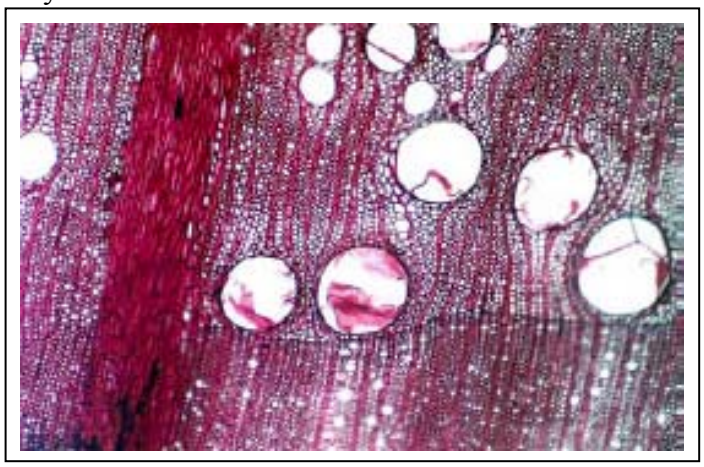


Fig 1. 9 T. S. of a wilted plant showing presence of fungal hyphae in the xylem

Fig 1.10 Seed harvested from the late wilted plants is lighter and duller than that harvested from healthy plants

1.12 Production loss due to *F. oxysporum ciceri*

Based on percentage incidence of the wilt disease, estimates of crop losses have been made mainly from the field estimates. Average 10% of loss in annual chickpea production has been reported in India (Singh and Dahiya, 1973). Similarly, annual loss of 12-15% due to both wilt and root rots have been estimated in other countries (Trapero-Casás and Jiménez-Díaz, 1985). About 77-94% yield loss occurs due to early wilting whereas 24-65% loss is due to late wilting (Haware and Nene, 1980). At times under specific conditions Foc is capable of completely destroying the crop (Halila and Strange, 1996). As a result, Foc resistance improvement and higher seed dry matter are major targets of global chickpea research efforts.

1.13 Approaches to overcome yield loss

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

1.14 Cultural practices

As chickpea wilt pathogen is monocyclic the FOC population in soil persists due to the longevity of reproductive units and thus makes it difficult to eliminate them from wilt sick fields (Haware *et al.,* 1986; 1996). Occurrence of wilt disease, its severity and disease progression is directly proportional to the density of the pathogen population. Presence of high levels of FOC propagules leads to 100% wilting much earlier than lower initial levels of FOC propagules (Bhatti and Kraft, 1992). Thus, it may be possible to early forecast the severity of diseases induced by soil-borne pathogens by assessing the initial pathogen population (Fry, 1982).

Avoidance of planting in heavily infested fields is advised to minimize the effects of wilt disease; however, availability of land is a limiting factor in Indian conditions. Moreover, as the pathogen can survive in soil for longer periods (Haware *et al.,* 1996) crop rotation is not an effective practice for reducing wilt incidence. On the other hand, cultural practices like deep ploughing during summer and removal of host debris from the field

can considerably reduce inoculum levels. Solarization (covering the soil with transparent polythene for 6-8 weeks during summer months) is known to effectively control wilt in chickpea (Chauhan *et al.,* 1988). However, it is not a practical option in India as the poor farmer is already strapped for resources. Control of seed transmission of wilt can be achieved by using disease free seed, obtained from plants grown in disease free areas.

1.15 Fungicides

The disease is primarily managed by resistance breeding programs. But high pathogenic variability and mutability limit the sustainability and effectiveness of any naturally selected resistance against the pathogen (Nimalkar *et al.,* 2006). 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 nontarget beneficial flora and evolving fungicidal resistance 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 susceptible host.

1.16 Biological control

As such in the present context, biological management of wilt with bioagents 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 and 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).

1.17 Resistance breeding

To overcome many of the above problems, the use of resistant and quality cultivars to control wilt is the best and cheapest way for breeders to adopt (Mahmood *et al.,* 2011) Chickpea production in India can be stabilized and improved by the development of suitable chickpea cultivars adaptable for all the environments (Bakhshi *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 (Choudhary *et al.,* 2008; Allahverdipoor *et al.,* 2011). 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).

1.18 Biochemical basis of resistance to *Fusarium oxysporum*

Understanding of the biochemical basis of host resistance can be of great use to explore the combined potential of induced and constitutive resistance mechanisms to combat virulent *Foc* races. Previously, zinc in culture solution at 3ppm has been reported to check the symptoms caused by *Foc*, but not the spread of the pathogen in the plant. The reduction in wilt symptoms has been attributed to inhibition of fusaric acid production and alteration of host metabolism. Cachinero *et al.,* (2002) have reported non-host Foc isolates to be more potent inducers of plant defense reactions in chickpea against Fusarium wilt as compared to the incompatible race 0 of *Foc.* Other reports have shown induced defense mechanisms in chickpea with the production of two pterocarpan phytoalexins, maackiain and medicarpin against pathogenic fungi (Clemens *et al.,* 1993; Ingham, 1976; Smith and Ingham, 1981). Root exudates containing phytoalexins exhibit *in vitro* anti-*Foc* activity that may contribute partly to wilt resistance (Stevenson *et al.,* 1994, 1995, 1997). Vogelsang and Barz (1993) have reported differentially expressed two chitinases and a β-1, 3-glucanase in chickpea. In another study, the induction of protease

and chitinase activity in a wilt resistant cultivar has been correlated with antifungal properties of the root extract against *Foc* race 1. In these studies, the decrease in β-1, 3-glucanase activity in roots upon fungal infection has been attributed to a higher rate of callose deposition in resistant cultivars (Giri *et al.,* 1998). However, the genetics of Fusarium resistance is complex and are governed by multigenes (van Rheenen, 1992).

1.19 Studies on chickpea Foc interactions using 'Omics' based technologies

Term "Omics" originally came from Genomics which refers to study of genome. It was then expanded to be used for proteins termed as proteomics, and similarly to other areas of biology like transcripts, metabolites, lipids and so on. This part provides an overview of host plant-*Fusarium* interactions using transcriptomics, proteomics and metabolomics approaches and discusses how these have contributed to understanding the chickpea-*Fusarium* interactions.

1.20 Transcriptomics

The transcriptome refers to total transcripts from a cell with their levels at a specific stage/ condition. Main aim of transcriptomics includes sorting all the types of transcripts such as, small RNAs, non-coding RNAs and mRNAs and to detect the variations in expression levels of each transcript. In order to identify qualitative and quantitative changes in transcriptome, different sequence or hybridization-based techniques have been developed. Microarray based transcriptomics uses hybridization approach which is relatively cheaper with capacity of high throughput. Nonetheless, there are numerous limitations such as cross-hybridization of related sequences, signal saturation and insensitivity because of high background (Okoniewski *et al.,* 2006; Royce *et al.,* 2007). On the contrary, differences at nucleic acid sequence level can be detected by earlier sequence-based approaches which were expensive, non-quantitative and low throughput (Boguski *et al.,* 1994; Gerhard *et al.,* 2004). With further technological improvements new techniques are developed that are tag-based sequencing methods such as cap analysis of gene expression (CAGE) (Shiraki *et al.,* 2003; Nakamura *et al.,* 2004; Kodzius *et al.,* 2006), massively parallel signature sequencing (MPSS) (Brenner *et al.,* 2000; Reinartz*et*

al., 2002; Peiffer *et al.,* 2008) and serial analysis of gene expression (SAGE) (Velculescu *et al., 1995;* Harbers *et al.,* 2005). Other approaches used in for transcriptomics study are, supression substractive hybridization is a technology that allows for PCR-based amplification of only cDNA fragments that differ between a control and experimental transcriptome (SSH). This technique has been used to identify *Medicago truncatula* putative defense genes in response to *Orobanche crenata* parasitization (Die *et al.,* 2007). Further, SSH has been applied to study chickpea-*Helicoverpa armigera* interaction study (Singh *et al.,* 2008). The potential of the AFLP technique for generating mRNA fingerprints was first recognized by Bachem *et al.,* (1996) for the study of differential gene expression during potato tuber formation. Nimbalkar *et al.,* (2006) compared transcript profiles generated from three chickpea root cDNA libraries, viz., uninfected WR-315 (WRC), WR-315 infected with *Foc*1 (WR-I) and JG-62 infected with *Foc*1 (JG-I), by subjecting them to cDNA-AFLP analysis. Differential expression of WRKY proteins, NBS-LRR type sequences and 14-3-3 proteins was observed in this study. Similarly, Gupta *et al.,* 2009, has also successfully utilized cDNA-AFLP to study differential expression of many defense responsive elements in chickpea-*Fusarium oxysporum* interactions. These approaches generate signature of exact transcript levels with high throughput. However, these techniques have some drawbacks, such as (a) relatively costly as classical Sanger sequencing methods are utilized, (b) many times comparison as well as mapping of short tags on reference genome is difficult to identify precise variations and (c) isoforms cannot be detected as only short part of transcripts is used.

Next generation sequencing provides powerful alternative strategies for analysis of transcriptomes by enabling unbiased quantification of transcript expression levels with broader genome coverage and higher sensitivity as compared to microarrays (Mortazavi *et al.,* 2008). Another novel approach that has reformed transcriptomics especially for eukaryotes is based on sequencing of RNA (RNA-Seq). Transcriptome sequencing has been applied in chickpea to analyze and compare transcripts from different varieties (Agarwal *et al.,* 2012). The reports on transcriptome analysis of Kabuli chickpea which has more nutritional and market price have generated SSRs of high intra-specific

polymorphic potential that can be used for genetic analysis (Agarwal *et al.,* 2012). Along with this the study also showed that functional markers crucial for crop improvement can be detected by comparing transcriptomes (Hiremath *et al.,* 2007). This approach has been also utilized in other plants such as Rice, Grapes, Soybean and Cotton (Nagalakshmi *et al.,* 2008; Wilhelm*et al.,* 2008; Mortazavi *et al.,* 2008; Lister *et al.,* 2008; Cloonan *et al.,* 2008; Marioni *et al.,* 2008; Morin *et al.,* 2008; Lu *et al.,* 2010; Zhang *et al.,* 2010; Zenoni *et al.,* 2010; Kim *et al.,* 2011; Xu *et al.,* 2011). Transcriptomic studies related to host pathogen interaction are enlisted in Table 1.2. *Fusarium oxysporum* induced defense transcriptome has been studied in Arabidopsis (Zhu *et al.,* 2013a) and banana (Li *et al.,* 2012) using RNA-Seq technology. Time course transcriptome response to the pathogen has been analyzed using this approach. The study discovered that only *AtROBHD* and *AtROBHF* out of 10 ROBH genes in Arabidopsis responded to *F. oxysporum* showing opposite effect on disease progression. Receptors like protein kinases and putative R genes were induced against Fusarium at various stages suggested new insights into their specific functions and role of NADPH oxidase genes in fungal resistance along with non-coding RNAs and other new disease resistance genes (Zhu *et al.,* 2013a). In another investigation in *Arabidopsis* using strand-specific RNA-seq approach upon *F. oxysporum* infection revealed new transcriptionally active regions (TARs) with many of *Foc*-responsive long non-coding RNA (lncRNA), natural antisense transcripts NATs (lncNATs) and long intergenic ncRNAs (lincRNAs). This study also proposed that for antifungal immunity, most of these lncRNAs have critical role during interaction of host and pathogen (Zhu *et al.,* 2013b). Defense transcriptome analysis in banana against *Fusarium oxysporum* contributed in identification of candidate genes related to plant resistance and understanding of plant-pathogen interplay. Moreover, presence of several novel genes and defense pathways in banana compared to model plants like rice and *Arabidopsis* suggested specific variation in host defense mechanisms in plants (Li *et al.,* 2012).

1.21 Proteomics

Proteomics refers to identification, quantification and characterization of protein complement of the genome. Proteomics has been employed to study plant-pathogen interactions since almost two decades with the help of traditional protein identification and electrophoretic techniques as well as current modern techniques. In recent years, there is a vast increase in analysis of proteomes. Although genome represents possible proteins and their sequence information, it fails to explain post-translational modifications and lacks the information about actual contributions to cellular function (Park *et al.,* 2004; Thurston *et al.,* 2005). Poor correlation between protein abundance and mRNA transcript levels compels the use of proteomic approaches (Carpentier *et al.,* 2008; Larson *et al.,* 2007). This might be due to the lack of information on post-translational modification of protein and their actual contribution to cellular function. Proteomics allows us to study the differentially expressed proteins under diverse biotic and abiotic stress conditions using technological advances making proteomics a method of choice to study plant pathogen interactions (Geddes *et al.,* 2008; Palomares Rius *et al.,* 2011; Rampitsch *et al.,* 2012). Protein functions essential for mediating susceptibility or resistance and underlying molecular mechanism of defense can be elucidated with the help modern proteomic approaches. Protein identification has been simplified by mass spectrometry technology, stains, software and progress in bioinformatics. Most of the proteomic studies depend upon tandem mass spectrometry, the method more precisely termed as bottom up proteomics. In spite of great potential, proteomics has only been recently applied to study protein modifications such as phosphorylation involved in plant pathogen interaction. Identification of proteins depends on genome databases along with mass spectrometry and next generation sequencing makes available such databases for non-model plants very cheaply (Quirino *et al.,* 2010). Although proteomic analysis for chickpea-*Fusarium* interaction is still lacking, the interactions of *Fusarium* with banana and tomato have been studied. Total 27 proteins were detected by conventional proteomic approach of two-dimensional PAGE (2D-PAGE) and mass spectrometry in xylem sap of tomato against *F. oxysporum* f. sp. *lycopersici*. This was the first study reporting xylem

sap protein changes upon *Fusarium* infection. Fungal infected samples had 13 proteins while the remaining were present in both, healthy and infected plants. The common proteins were peroxidase, chitinase, polygalacturonase and subtilisin-like protease. Xylem sap included pathogenesis-related PR-1 and PR-5 proteins in healthy *Brassica napus* and PR-5 and a PR-2/1, 3-beta glucanase in healthy maize, but in contrast these were found in *Fusarium* infected tomato. This indicated their critical role in *Fusarium* wilt resistance (Houterman *et al.,* 2007). Table 1.3 gives a brief account of proteomic approaches applied to various plant-Fusarium systems and their findings. Varied proteins were identified during these studies which have been enlisted in Table1.4. Proteome analyses of *Fusarium* infected banana roots revealed involvement of different proteins in vital metabolic pathways including defense. Differential expression pattern of *Fusarium* responsive elements such as PR proteins, antifungal compound synthesis proteins and lignin synthesis-related protein in susceptible and resistant banana (Li *et al.,* 2013). Interaction of Strawberry with *F. oxysporum* f. sp. *fragariae* identified 79 proteins through MALDI-TOF/TOF that were mainly involved in stress and defense responses, hormone biosynthesis, detoxification mechanisms, primary and secondary metabolism. Some of these proteins were related to resistance such as PR proteins and proteins involved in ethylene/jasmonic acid signalling pathways, reactive oxygen species detoxification, ubiquitin/26S proteasome and secondary metabolite biosynthesis (Fang *et al.,* 2013). Similarly, differential expression of several antioxidant proteins along with PR2 in a resistant wheat cultivar compared to the susceptible cultivar against *F. graminearum* with 2D-PAGE and liquid chromatography mass spectrometry (LC-MS) were also performed (Zhou *et al.,* 2005). Another effort to understand molecular basis of *F. graminearum* resistance in wheat using proteomics identified proteins from jasmonic acid signaling pathways, PR proteins such as ß-glucanases, chitinase and thaumatin-like protein, oxidative stress response, and proteins involved in amino acid synthesis and nitrogen metabolism, such as cysteine synthase, glutamate dehydrogenase and tryptophan synthase (Zhou *et al.,*2006).

1.22 Metabolomics

The metabolome represents the collection of all metabolites in a biological cell, tissue, organ or organism, which are the end products of various cellular processes (Jordan *et al.,* 2009). The large variations in the relative concentrations of metabolites also add to the complications of metabolite analysis. Therefore, comprehensive coverage of metabolites of an organism can be achieved by using multi-parallel complementary extraction and detection technologies with careful experimental design. Metabolic profile data are obtained using biochemical analytical platforms such as GC-MS, LC-MS, and NMR, each having its own advantages and limitations. Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) provide the richest metabolome information among various analytical techniques available so far. NMR spectroscopy enables simultaneous measurement of proton containing low molecular weight metabolites. Samples can be analyzed without metabolite extraction using high-resolution magic angle spinning technique (Waters *et al.,* 2000). To overcome low sensitivity, reversed-phase high-performance liquid chromatography coupled to sample enrichment and fractionation, can be combined with NMR spectroscopic analysis (Rezzi *et al.,* 2008). In contrast thousands of metabolites from a biological sample can be analyzed using MS. To separate metabolites with higher sensitivity and coverage liquid chromatography or gas chromatography is generally used along with MS (Dunn *et al.,* 2008). Comprehensive metabolic fingerprinting can be obtained using ultra high resolution MS like Fourier transform MS (FTMS) (Han *et al.,* 2008).

A novel MS platform, orbitrap, provides a higher mass resolution and mass accuracy over a wider dynamic range. It has higher potential to detect greater number of metabolites of similar accurate mass with a high level of confidence of metabolite identification. However, Very few examples including barley (Bollina *et al.,* 2010), *Arabidopsis* (Botanga *et al.,* 2012), potato (Aliferis *et al.,* 2012), rice (Jones *et al.,* 2011) etc. have been reported with study in plant pathogen interactions using metabolomics approach.

Table 1.2: Transcriptomics studies by Next generation sequencing during plant–pathogen interactions

Host-pathogen	**Sequencing platform**	**Important findings**
Arabidopsis–Fusarium oxysporum	SOLiD	Many novel disease responsive genes, including non-coding RNAs, were detected along with the network of plant-defense genes (Zhu *et al.*, 2013a)
Banana- *Fusarium oxysporum* f. sp *cubense*	Illumina	Large amount of transcripts from banana were analyzed. Comparison of resistant and susceptible Cavendish banana was performed for defense responses against Foc TR4. (Li *et al.*, 2013)
Banana - *Fusarium oxysporum* f. sp. *Cubense*	Illumina HiSeq 2000	Novel insights on the understanding of the compatible and incompatible interactions between banana and Foc4. (Bai *et al.*, 2013)
Banana - *Fusarium oxysporum* f. sp. *Cubense*	Illumina	Banana root transcriptome of Foc4. (Wang *et al.*, 2012)

Table 1.3: Proteomic studies of *Fusarium* - host plant interactions

***Fusarium* -host**	**Methods applied**	**Principal findings**
F. sporotrichioides*-*Arabidopsis	MALDI-TOF MS	Defence-related proteins showed higher accumulation, while proteins from metabolism and photosynthesis were decreased upon pathogen inoculation. (Asano *et al.*, 2012).
***F. verticillioides*-Maize**	2-DE/ MALDI-TOF MS & nESI-IT MS/MS	Germinating maize embryos showed variation in protein expressions in response to fungal infection (Campo *et al.*, 2004).
***F. graminearum*-Wheat**	2-DE /LC-MS/MS	Inoculated wheat spikelets had 15 differential proteins. (Zhou *et al.*, 2005)
***F. graminearum*-Wheat**	2-DE /LC-MS/MS	Detected 41 differentially expressed proteins in wheat spikelets during compatible interaction with fungus (Zhou *et al.*, 2006)
***F. graminearum*-Wheat**	1-DE/LTQ-FT MS	Presence of secreted proteins *in vitro* and *in planta* (wheat heads) (Paper *et al.*, 2007).
***F. oxysporum* f.sp. *lycopersici* - Tomato**	2-DE /MALDITOF LC-QTOF MS/MS	Detected specific proteins from tomato (21) and fungus (7) in the tomato xylem sap upon pathogen inoculation (Houterman *et al.*, 2007).
***F. oxysporum*-Sugar Beet**	Multi-dim. LC, MALDI-TOF MS	Identified 31 proteins from resistant variety and 48 from susceptible (Larson *et al.*,2007)
***F. graminearum*-Barley**	2-DE/LC-MS/MS	Differentially expressed proteins from spikelet of barley for fungal resistance (Geddes *et al.*, 2008).
***F. oxysporum* f. sp. *cubense*- Banana**	2 DE, MALDI-TOF/TOF	Detected 38 differentially accumulated proteins with role in cell metabolism (Li *et al.*, 2013).
***F. oxysporum* f. sp. *fragariae*-Strawberry**	MALDI-TOF TOF MS MS	Detected 79 proteins from stress responses, hormone biosynthesis, antioxidant and detoxification mechanisms, primary and secondary metabolism (Fang *et al.*, 2013).

Table 1.4: Proteins identified in plant *Fusarium* interaction

Protein	Organism	Pathogen	Accession no.	Reference
Peroxidases (PR-9)	Tomato	*F. oxysporum*	–	Houterman (2007)
	A. thaliana	*Fusarium elicitor*	At1g07890	Chivasa *et al.*, (2006)
b-1,3-Glucanases (PR-2)	*Zea mays*	*F. verticillioides*	–	Campo *et al.*, (2004)
	Tomato	*F. oxysporum*	AAA03617	Houterman (2007)
Thaumatin-like protein (PR-5)	*T. aestivum*	*F. graminearum*	CAA66278	Zhou *et al.*, (2006)
	Tomato	*F. oxysporum*	AAM23272	Houterman (2007)
Chitinase (PR-3)	*T. aestivum*	*F. graminearum*	BAB82472	Zhou *et al.*, (2006)
	Tomato	*F. oxysporum*	CAA78845	Houterman (2007)
Glutathione S-transferase	*T. aestivum*	*F. graminearum*	CAC94005	Zhou *et al.*, (2006)
	Z. mays	*F. verticillioides*	2288968	Campo *et al.*, (2004)
	A. thaliana	*Fusarium elicitor*	At1g02930	Chivasa *et al.*, (2006)
Glyceraldehyde 3-phosphate dehydrogenase	*T. aestivum*	*F. graminearum*	XP493811	Zhou *et al.*, (2006)
	Z. mays	*F. verticillioides*	Q09054	Campo *et al.*, (2004)
Fructose-bisphosphate aldolase	*Z. mays*	*F. verticillioides*	P08440	Campo *et al.*, (2004)
	A. thaliana	*Fungal elicitor*	At3g52930	Chivasa *et al.*, (2006)
Adenosine kinase	*Z. mays*	*F. verticillioides*	AJ012281	Campo *et al.*, (2004)
Superoxide dismutase (Cu–Zn)	*Z. mays*	*F. verticillioides*	P23346	Campo *et al.*, (2004)
Glutamate dehydrogenase	*T. aestivum*	*F. graminearum*	AAB51596	Zhou *et al.*, (2006)
Thioredoxin	*T. aestivum*	*F. graminearum*	CAA06735	Zhou *et al.*, (2006)
Lyases, esterases, proteases, nucleases	Wheat	*F. graminearum*	BC1G_13367.1 BC1G_09000.1 BC1G_12517.1 BC1G_01617.1	Paper *et al.*, (2007)

1.23 Chemometric approaches in plant metabolomics

1.23.1 Multivariate data analysis

The modern analytical methods for comprehensive metabolite screening require computerized data collection for the rapid sampling of large amounts of data. These large data quantities have to be filtered by automatized techniques to separate information from noise. Of the thousands of resonances/peaks in a 1H NMR spectrum/LCMS spectrum of plant tissue extract, those which are altered as a consequence of a pathogen insult must be filtered. There are various approaches for multivariate data analysis in metabonomics (Trygg *et al.,* 2007), but the most common one, principal component analysis (PCA), is based on the projection of variance also known as unsupervised pattern recognition. The study can be taken one step further and a computer can be trained to recognize different classes and to sort samples according to which class they belong to. This is referred to as classification, and is done utilizing supervised pattern recognition; this includes PLSDA (Projections onto Latent Structures Discriminant Analysis) and OPLSDA (Orthogonal Projections onto Latent Structures Discriminant Analysis).

1.23.2 Unsupervised models: principal component analysis

PCA is a multivariate projection method, based on the identification of systematic variation and the maximization of variance. Many partially correlated variables are reduced to few independent, latent variables, the principal components (PCs). Every PC is a linear combination of the original parameters. Consecutive PCs are orthogonal to each other and thus independent. Every observation can, therefore, be plotted in a two- or three- dimensional coordinate system of latent variables, which still contains most of the spectral or chromatographic information (Fig. 1.11). Since the localization of the scores in the plot is based purely on variance and no other information is put into the model, PCA is called an unsupervised method. PCA is the first method of choice used for metabonomics, before any more complex data analysis methods are employed, since it is

robust, unsupervised and can be implemented quite easily (Trygg *et al.,* 2007). While PCA can be applied to gain a rapid insight into data structure, a problem associated with PCA-based variance analysis is its sensitivity to variables not correlated to the trends investigated, such as analytical variability, sample work-up, analytical artifacts, physiological influences (genotype, cultivar variations and day time). For the identification of discriminating markers between experimental groups, supervised approaches are superior.

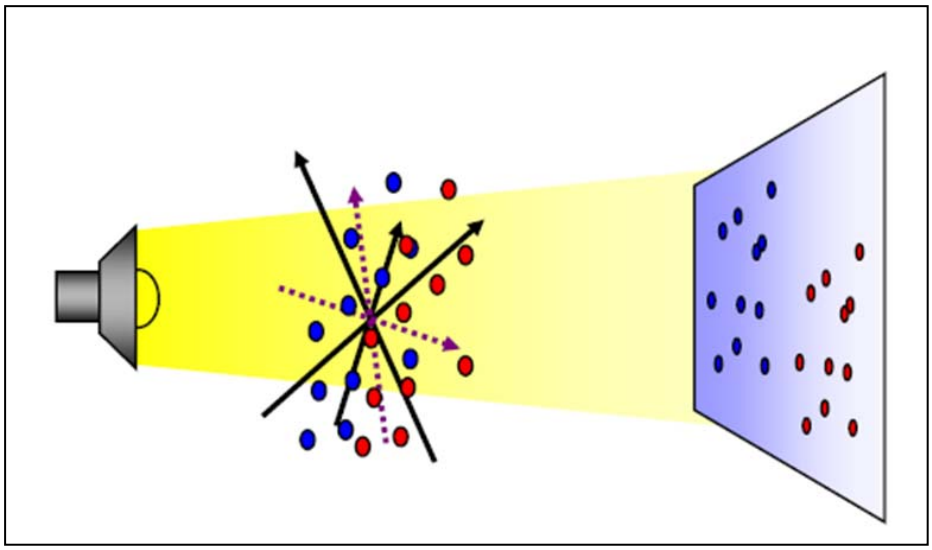


Fig. 1.11: Principal component analysis (PCA) is a data reduction method based on the projection of variance. The data, represented by a k-dimensional coordinate system is reduced to a two-dimensional coordinate system, spanned by the principal components (PCs). The first PC is chosen in such a way that the variance along it is maximized. The second PC is orthogonal to the first, and maximizes the variance as well. In this way, the complex data can be visualized two-dimensionally, while retaining a large part of the information which is contained in the data.

1.23.3 Supervised models: orthogonal projection to latent structures

For closer examination of such samples containing a large variance and possessing only very little discriminating information, a supervised approach often yields superior results. Supervised multivariate analysis means that information on class identity, such as

"control" versus "infected" is included in the statistical analysis. The orthogonal projection to latent structures (OPLS) can be seen as an extension of PCA with a regression element.

The OPLS-DA approach allows a better analysis of discriminating features, and allows the analysis and interpretation of information not contributing to class separation. Analytical variation, sampling sequence, genetic differences, daytime effects and physiological variation have been found by this approach. The OPLS-DA models can also be used to identify the variables responsible for class separation and therefore, they can be used for identification of potential biomarkers. One approach used is the S-plot (Wiklund *et al.,* 2007) which plots the covariance (p) against the correlation (pcorr). For a marker, both the contribution to the model expressed in p and the effect and reliability of this contribution expressed in p(corr) should be high. Thus the potential markers are located on the outer ends of the S-shaped extended arm (Fig. 1.12). Thus, in recent years, advancement in analytical technology has made it possible to understand the system wide responses. However, inadequate studies of global nature necessitate more such studies to understand plant pathogen interactions.

1.24 Genesis of the thesis:

Chickpea is a very important legume crop, economically as well as nutritionally, for the developing countries like India. It is essential to configure the mechanism of resistant to overcome the devastating damage caused by *Fusarium oxysporum*. Comparative “Omics” technologies have proven to be potential tools in identification of genes and deciphering gene functions. Differentially accumulating transcripts, proteins and metabolites can be linked back to the genome to identify genes responsible for their differential accumulation. This study was aimed to identify the mechanistic basis for resistance/ susceptibility in chickpea against Foc by non-targeted high-throughput profiling of proteins and metabolites. I have therefore, utilized high though put quantitative proteomics and metabolomics approaches to achieve the objective of my thesis. I have made an attempt to take a step further in using advance analytical technology to study the system wide interaction in a non-model plant, chickpea and Foc. Further, a targeted gene

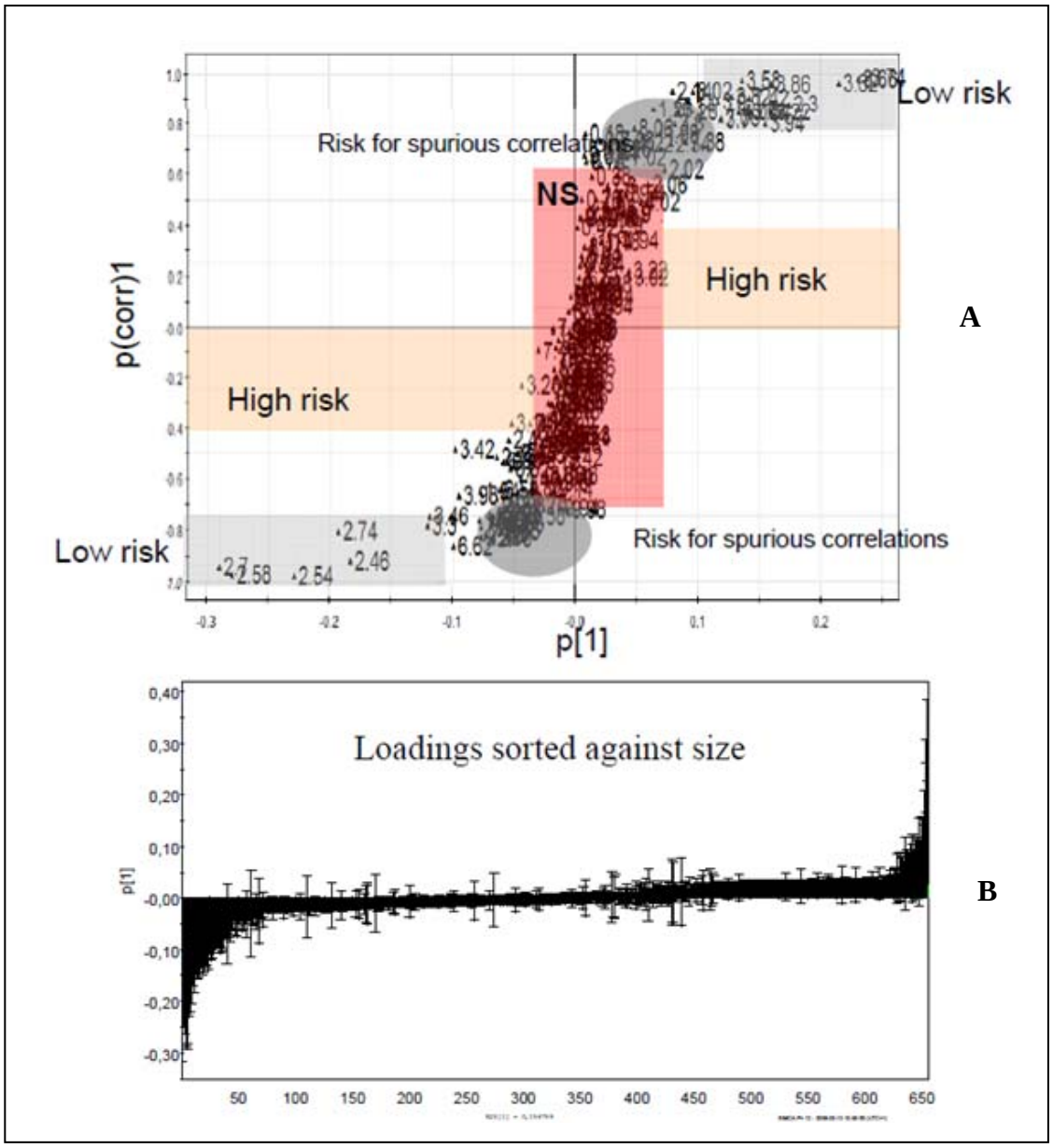


Fig: 1.12: S-plot (A) and column plot of extracted variables with jack-knifed confidence intervals (B) of an LC/MS data based OPLS-DA model to illustrate the process of marker identification. The S-plot shows the covariance p against the correlation p(corr) of the variables of the discriminating component of the OPLS-DA model. Cut-off values for the covariance of $p \geq 0.05$ and for the correlation of $p(corr) \geq 0.5$ were used, the variables thus selected are highlighted in the S-plots with red squares (a). In order not to over interpret the model, the markers are selected in a conservative manner by investigating only those variables showing a jack-knifed confidence interval less than half of the variable's value (b).

expression analysis was performed to confirm the findings of non-targeted metabolite/protein analyses. The following objectives were formulated to deal with the problem presented.

1.25 Objectives:

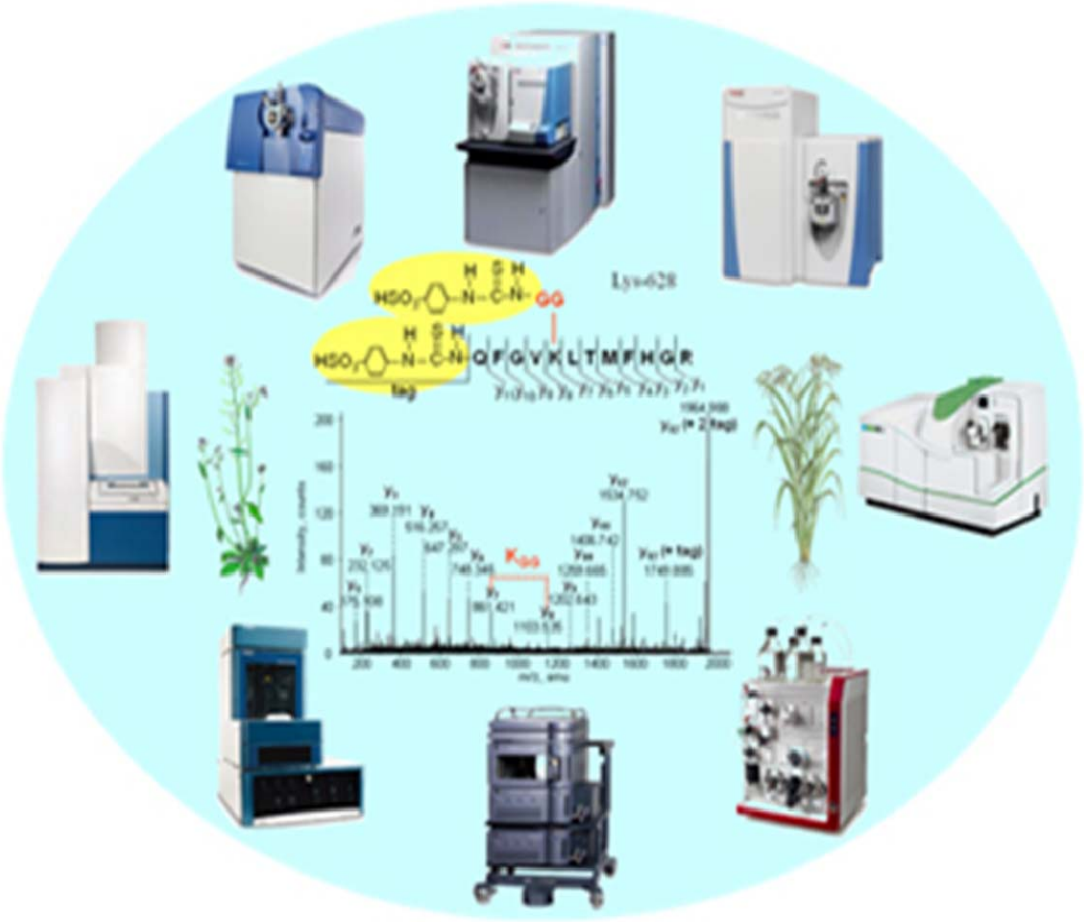
1. To identify the molecular mechanisms in chickpea for resistance/ susceptibility against Foc with label-free quantitative proteomics along with multivariate data analysis approach.
2. To understand the mechanisms of Foc resistance/ susceptibility in chickpea using non-targeted quantitative metabolomics using high resolution mass spectrometry and NMR based metabolomic profiling along with multivariate data analysis approach.
3. To confirm the finding of non-targeted proteomics and metabolomics study using a targeted gene expression analysis approach

1.26 Organization of thesis:

The thesis is organized into five chapters, the first being the introduction to plant and pathogen (Foc) which forms the basis of this study, and detailed review of techniques and relevant information currently available to study the interaction. Second chapter described the materials and methods used for the experimental design. The third chapter includes the results wherein I have used label free quantitative proteomics, UHPLC-Orbitrap and 1H-NMR based metabolomics to identify differentially expressed proteins/metabolites in chickpea root extract at different time points after Foc inoculation. Also, key gene expression involved in different pathways has been studied using real time quantitative PCR. The fourth chapter includes the discussion of results and the significance of this work. In the fifth and the final chapter I have summarized outcome of the work and mentioned the different possibilities to explore this chickpea-Fusarium interaction in future. Literature used in this study has been detailed at the end of the thesis.

Chapter 2

Materials and methods



2.1 Plant and seed materials

Cicer arietinum seeds of cultivar JG62 and Digvijay (Fig. 2.1) were obtained from Mahatma Phule Krishi Vidyapeeth (MPKV), Rahuri, Maharashtra, India. JG62 (pedigree-selection from germplasm) is highly susceptible to wilt, and shows features like twin podding and early maturity. It has medium sized seeds. Digvijay (pedigree-Phule G-91028 × Bheema) shows high average yields (19,00 q/ha; which is higher by 14.44% than Vijay and 17.81% than Vishal) and is highly resistant to Fusarium wilt as compared to the other varieties such as Vijay and Vishal. It has attractive yellowish brown coloured bold seeds (24.0g/100 seeds) and is suitable for optimum sowing, well irrigated and late sown conditions.

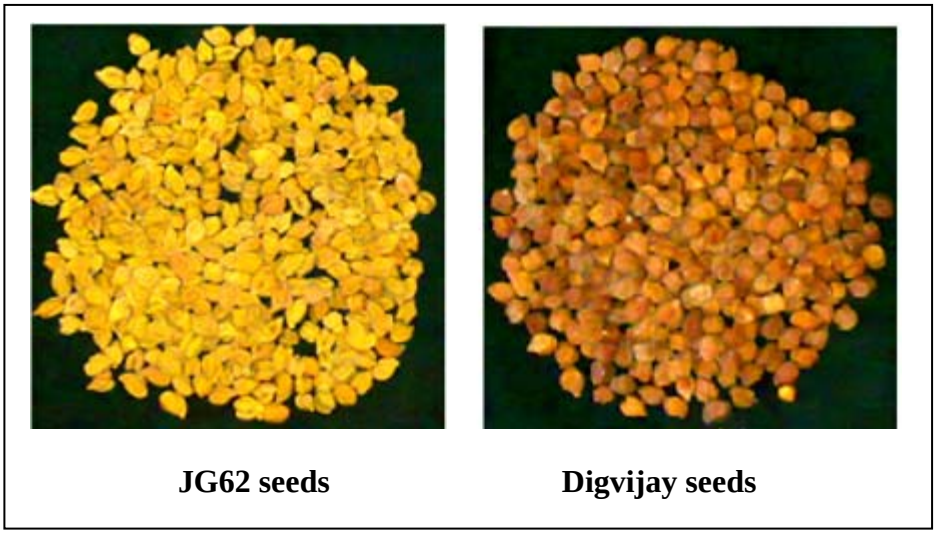


Fig. 2.1: Seeds of chickpea cultivars, JG62 and Digvijay

2.2 Fungal strain

F. oxysporum f. sp. *ciceri* (*Foc*) standard race 1 (NRRL 32153) was obtained from the International Crops Research Institute for Semi Arid Tropics (ICRISAT), Patancheru, India. The culture was maintained on Potato Dextrose Agar (PDA) slants with regular sub-culturing. Fig. 2.2 shows the growth patterns of *Foc* race 1 on PDA plates.

Fig. 2.2: Foc race 1 growth pattern

2.3 Methodologies involved in chickpea-*Fusarium* interaction

Seeds of Fusarium wilt resistant (Digvijay, DV) and susceptible (JG62, JG) chickpea cultivars were surface sterilized by sodium hypochlorite (4%) treatment prior to sprouting on wet filter paper and sowing in Soil-Rite. The DV and JG plants were further grown in greenhouse [25°C/16 h light (06:00-22:00) and 25°C/8 h dark (22:00-06:00) cycle] for seven days and divided into two groups (control or mock inoculated and Foc inoculated). The scheme for Foc inoculation and workflow for data collection/analysis is shown in Fig. 2.3. Foc inoculum (*F. oxysporum* f. sp. *ciceri* race 1) was used at a concentration of 10^6 spores/mL, while sterile water was used for mock inoculation. As the pathogen is reported to colonize the xylem vessels two days post inoculation (Gupta *et al.,* 2010, Jimenez-Fernandez *et al.,* 2013), the plant tissues (roots) were collected at various time interval such as 2, 4, 8 and 12 DAI. In each group, a pool of 10 plants comprised one biological replicate. Roots of Mock inoculated and Foc inoculated JG62 and DV roots of these time points were washed with sterile milli-Q water and cut at the hypocotyl region. The harvested root tissues were snap-frozen in liquid nitrogen and stored at -80°C till further use. Plant root tissue was ground well in liquid nitrogen using bead beater (Retsch GmbH, Germany). For proteomics analyses, tissues from three biological replicates were analyzed, for NMR based metabolomics analysis, tissues from ten biological replicates while for UHPLC-Orbitrap based untargeted metabolomics three independent biological replicates with three technical replicates of each were analyzed.

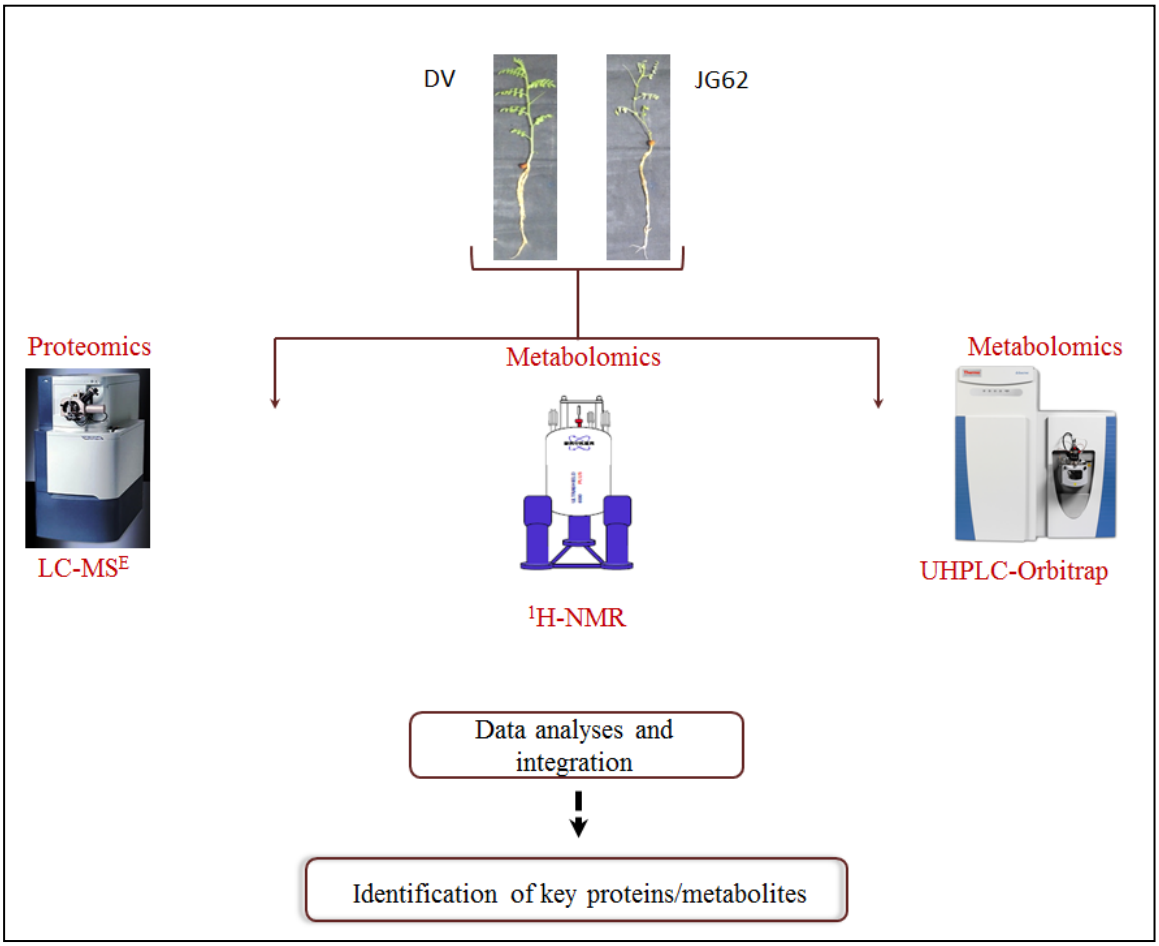


Fig. 2.3: Design of proteomics and metabolomics experiment with details of sample preparation, tissue collection stages, approaches and data analysis.

2.4 Protein extraction and Mass spectrometry analysis

Total proteome of root tissue was extracted as described by Isaacson *et al.,* (2006). In brief, this includes, removal of phenolic compounds by protein precipitation in 10% TCA/Acetone resuspention of pallet in 10 ml of extraction buffer containing 0.7 M sucrose; 0.1 M KCl; 0.5 M Tris-HCl, pH 7.5 and 50 mM EDTA. The reducing agent beta-mercaptoethanol should be added to a final concentration of 2% (vol/vol). Samples were vortexed and incubated by shaking for 10 min on ice. Afterwards, an equal volume of Tris-buffered phenol (pH = 8.0) was added, and solutions were incubated on a shaker for 10 min at room temperature. The phenol phase was carefully transferred to a new tube

and four volumes of precipitation buffer, consisting of ammonium acetate in ice-cold methanol and samples were incubated overnight at – 20 °C. After centrifugation, the pellets were washed three times with ice-cold precipitation buffer and finally, pellets were dried under air at room temperature (Fig 2.4). Further, protein pellets were solubilized in 50 mM ammonium bicarbonate buffer containing 0.1% Rapigest (Waters, USA). The dissolved proteins were reduced and alkylated by DTT and iodoacetamide, respectively followed by overnight tryptic hydrolysis at 37°C using Promega sequencing grade trypsin. The digested peptides were analyzed with LC-MS^E workflow using nano-ACQUITY online coupled to a SYNAPT HDMS system (Waters, USA). Nano-LC separation was performed with symmetry C18 trapping column (180 µm x 20 mm, 5 µm) and bridged-ethyl hybrid (BEH) C18 analytical column (75 µm x 250 mm, 1.7 µm). The binary solvent system comprised solvent A (0.1% formic acid in water), and solvent B (0.1% formic acid in acetonitrile). Each sample (500 ng) was initially applied to the trapping column and desalted by flushing with 1% solvent B for 1 minute at a flow rate of 15 µL/min. Elution of the tryptic digested sample was performed at a flow rate of 300 nL/min by increasing the solvent B concentration from 3% to 40% over 90 min. Before data acquisition, the mass analyzer was calibrated using Glu-fibrinopeptide B (Sigma-Aldrich, USA) from m/z 50 to 1990. The Glu-fibrinopeptide B (GFP-B) was delivered at 500 fmole/µL to the mass spectrometer via a NanoLockSpray interface using the auxiliary pump of the nano-ACQUITY system at every 30 second interval for lock mass correction during data acquisition. Data independent acquisition was performed (LC-MS^E) as described by Patel *et al.,* (2009).

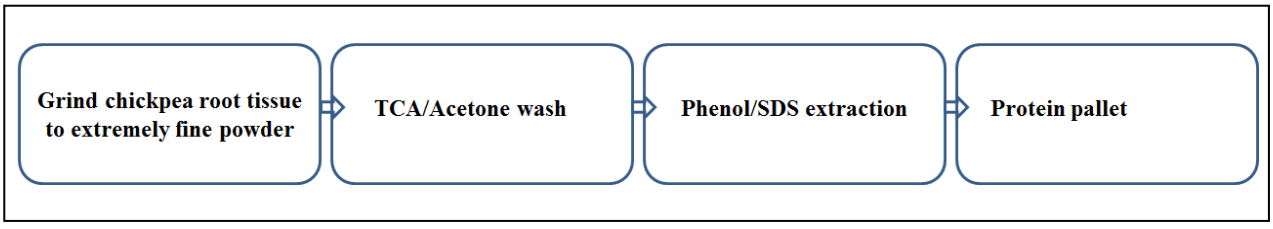


Fig. 2.4: Schematic presentation of plant proteome extraction

As accuracy and reproducibility in mass measurement are critical in data acquisition during large-scale proteomic experiments, Principle Component Analysis (PCA) was used to assess the quality of the measurement in terms of replicate similarity of unit variance (UV) scaled data using Metaboanalyst software. ProteinLynxGlobal server (PLGS) quality control outputs suggest that the replicates of each sample were clustered together reflecting inherent similarities between like samples (Fig. 2.5A). In addition, linear response and reproducibility of measurement of the quantitative proteomic data acquisition were tested by plotting two replicates (Fig. 2.5B), whereas data were acquired below 3 ppm mass accuracy (Fig 2.5C). Further, the percent coefficient of variance of retention time was calculated to assess the separation stability and coefficient of variance of 0.3 minutes, which also suggested stability in chromatographic separation (Fig. 2.5D).

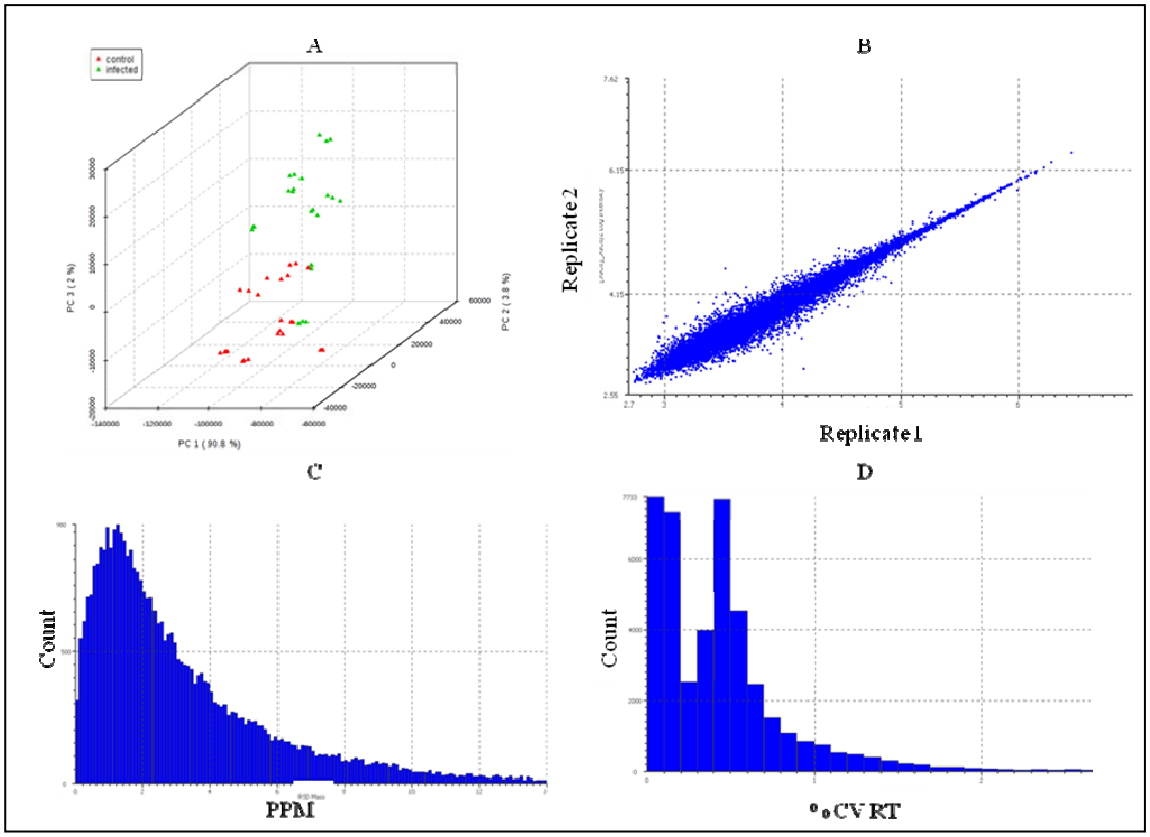


Fig. 2.5: Protein quality control measurements; PCA plot indicating clear separation between control and inoculated samples (A), reproducibility of intensity in replicates of samples (B), majority of ion counts with less than 3 ppm error (C) and percent coefficient of variance of retention time (% CV RT) (D).

2.5 Analysis of quantitative proteomics data

The acquired LC-MS^E data were processed using the ProgenesisQI for Proteomics software (Waters, USA). Protein identifications were obtained by searching the genomic databases of chickpea (http://www.icrisat.org/) and *Fusarium oxysporum* (http://www.broadinstitute.org/). LC-MS^E data were searched with a fixed carbamidomethyl modification for cysteine residues, along with a variable modification for oxidation of methionine, N-terminal acetylation, deamination of asparagine and glutamine and phosphorylation of serine, threonine and tyrosine. The ion accounting search algorithm within ProgenesisQI for Proteomics software was used which has been developed specifically for searching data-independent MS^E data sets and described in detail by Li *et al.,* (2009). The ion accounting search parameters were- precursor and product ion tolerance: automatic setting, minimum number of product ion matches per peptide: 3, minimum number of product ion matches per protein: 7, minimum number of peptide matches per protein: 1, and missed tryptic cleavage sites: 1. False positive rate was set at 1%. Search results of the proteins and the individual MS/MS spectra with a confidence level at or >95% were accepted. Label free quantitation of identified proteins was done on the basis of spiked bovine serum albumin (BSA) protein.

2.6 Clustering of identified proteins

Data were normalized by spiked BSA (50 fmoles) and relative accumulation differences were determined for proteins having differential expression. Sum of three replicates of inoculated samples was divided by that of the respective controls. This established a ratio of fold change of a protein in plants upon Foc infection in relation to that in mock-inoculated control plants. The $\log_2$ transformed ratio (susceptible/control and resistant/control) pairs were clustered by the application of SplineCluster (Heard *et al.,* 2006), a Bayesian model-based hierarchical clustering algorithm for time series data.

2.7 Gene ontology enrichment analysis

Protein functional annotation was determined using Blast2GO (Conesa *et al.,* 2005) and for each cluster, GO enrichment analysis was carried out using BiNGO 2.3 plugin tool in Cytoscape version 2.8 (Maere *et al.,* 2005). Overrepresented GO Biological Process categories were identified using a hypergeometric test with a significance threshold of 0.05 after Benjamini and Hochberg false discovery rate correction (Benjamini and Hochberg, 1995) using the annotated chickpea genome as the reference set. Whole chickpea genome GO term for biological process was extracted from Blast2go software and was used to make customized annotation file for chickpea as explained in Maere *et al.,* (2005).

2.8 Metabolite extraction and NMR measurement

Plant root tissue was ground well in liquid nitrogen by using bead beater (Retsch GmbH, Germany) and lyophilized. The powdered root tissue (~ 50 mg) was extracted with 0.75 mL of CD_3OD and 0.75 mL of 10 mM KH_2PO_4 buffer (pH 6.0) containing sodium3-trimethlysilyl [2,2,3,3-D_4] propionate (TSP) as described previously (Kim *et al.,* 2010). After ultrasonication for 20 min and centrifugation at 12,000*g* for 10 min at room temperature (~25°C), 0.5 mL of supernatant was collected for NMR detection (Fig. 2.6). ^{1}H NMR spectra of root extract were acquired at 25°C on a Bruker AV II 500 spectrometer (Bruker Biospin, Germany) operating at 500.13 MHz for ^{1}H. A standard water-suppressed one-dimensional NMR spectrum was recorded using *noesypr1d* pulse sequence (RD-90°-t_1-90°-*t*m-90°-acquisition) with the recycle delay of 6 s and the mixing time (t_m), of 50 ms. Typically, 90° pulse was set to about 15μs and 256 transients were collected into 48K data points for each spectrum with a spectral width of 16 ppm. All spectra were referenced to chemical shift of TSP (δ=0.00). For the metabolite assignment purpose, a range of 2DNMR spectra were recorded for selected samples including ^{1}H-^{1}H correlation spectroscopy (COSY), ^{1}H-^{1}H total correlation spectroscopy (TOCSY), ^{1}H-^{13}C heteronuclear single quantum coherence spectroscopy (HSQC), and ^{1}H-^{13}C heteronuclear multiple-bond correlation (HMBC). In COSY and TOCSY experiments, respective 64

and 32 transients were collected into 2 K data points for each of 256 increments with the spectral width of 2426 Hz for both dimensions. Magnitude mode was used with gradient selection for the COSY experiments whereas the *mlevgpphw5* pulse program was employed as the spin-lock scheme in the phase sensitive mode, with the mixing time of 60 ms, for TOCSY. Both HSQC and HMBC spectra were acquired using the gradient-selected sequences. In HSQC experiment, 80 transients were collected into 1k data points for each of 140 increments. In HMBC experiment, 160 transients were collected into 2k data points for each of 256 increments. The spectral widths were 2426 Hz for ^{1}H and 9809 Hz for ^{13}C in HSQC and HMBC experiments.

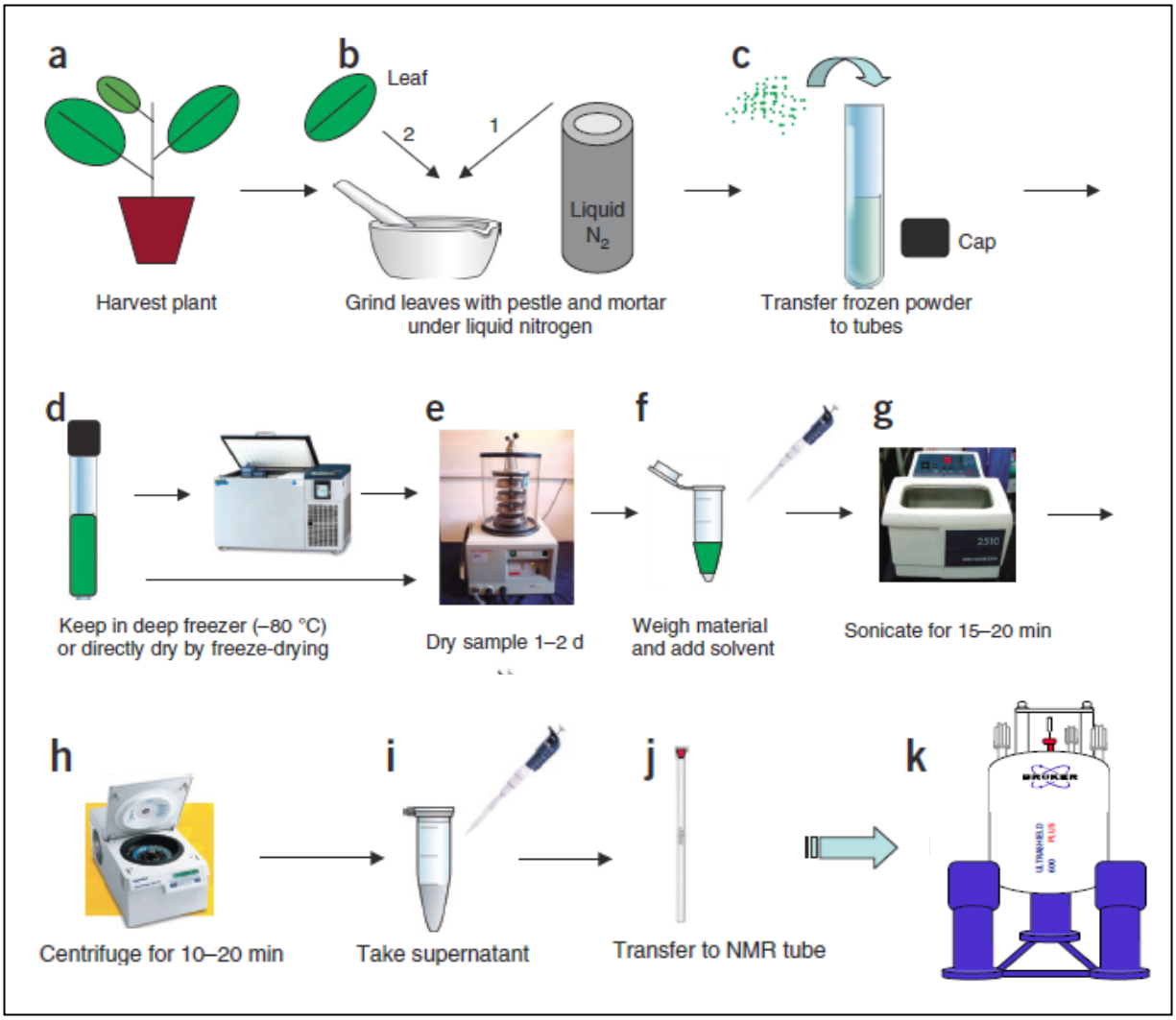


Fig. 2.6: Metabolite extraction protocol and data acquisition methodology; (Source: modified from Kim *et al.,* 2010)

2.9 NMR spectra processing and multivariate data analysis

All the ^{1}H NMR spectra were manually corrected for phase and baseline distortions using TOPSPIN (v2.1, Bruker Biospin), and calibrated for chemical shift drifting by in-house developed script for MATLAB (The Mathworks, USA). The spectral region δ 0.5-9.5 was divided into bins with width of 0.002 ppm (1.0 Hz) using AMIX software (v3.8.3, Bruker Biospin GmbH, Germany). The region δ 4.727-5.089 ppm was discarded to remove the effects of imperfect water pre-saturation. The areas of the remaining bins were normalized to total sum of intensity for each spectrum to compensate for the overall concentration differences prior to statistical data analysis. Multivariate data analyses were carried out with SIMCA-P+ v 12.0 software package (Umetrics, Sweden). PCA was performed on the mean-centered NMR data to inspect overall data distributions and possible outliers. Using the NMR data as the X-matrix and group information as Y-matrix, orthogonal projection to latent structures discriminant analysis (OPLS-DA) was carried out with unit variance scaling (Tyrgg 2002; Xiao *et al.,* 2008). The OPLS-DA models were 7-fold cross-validated and the quality of the model was described by the parameters R^2X, representing the total explained metabolic variables, and Q^2, indicating the model predictability. The models were further evaluated with a CV-ANOVA approach (p<0.05) and permutation tests. To facilitate interpretation of the results, back-transformation (Cloarec *et al.,* 2005) of the loadings generated from the OPLS-DA was performed prior to generating the loadings plots, which were color-coded with the Pearson linear correlation coefficients of variables (or metabolites) using an in-house developed script for MATLAB (The Mathworks, USA) (Wang *et al.,* 2007). The color-coded correlation coefficient indicates the significance of the metabolite contribution to the class separation, with hot colors (e.g., red) being more significant of the metabolite contributions to the group classification than the cold ones (e.g., blue). In this study, a correlation coefficient cutoff value of 0.602 (i.e., N=10, $|r| > 0.602$) was used for the statistical significance based on the discrimination significance at the level of P<0.05, which was determined according to the discriminating significance of the Pearson's product-moment correlation coefficient (Cloarec *et al.,* 2005).

2.10 Methodology and experimental design for non-targeted metabolomics with UHPLC

Foc inoculated and mock inoculated chickpea tissues were collected in similar way as described in section 2.3. For all stages, ten plants per pot were considered as an experimental unit, and three independent biological replicates of all the samples consisting of resistant and susceptible cultivars inoculated with Foc1 and their respective controls as described in Fig. 2.7 were used.

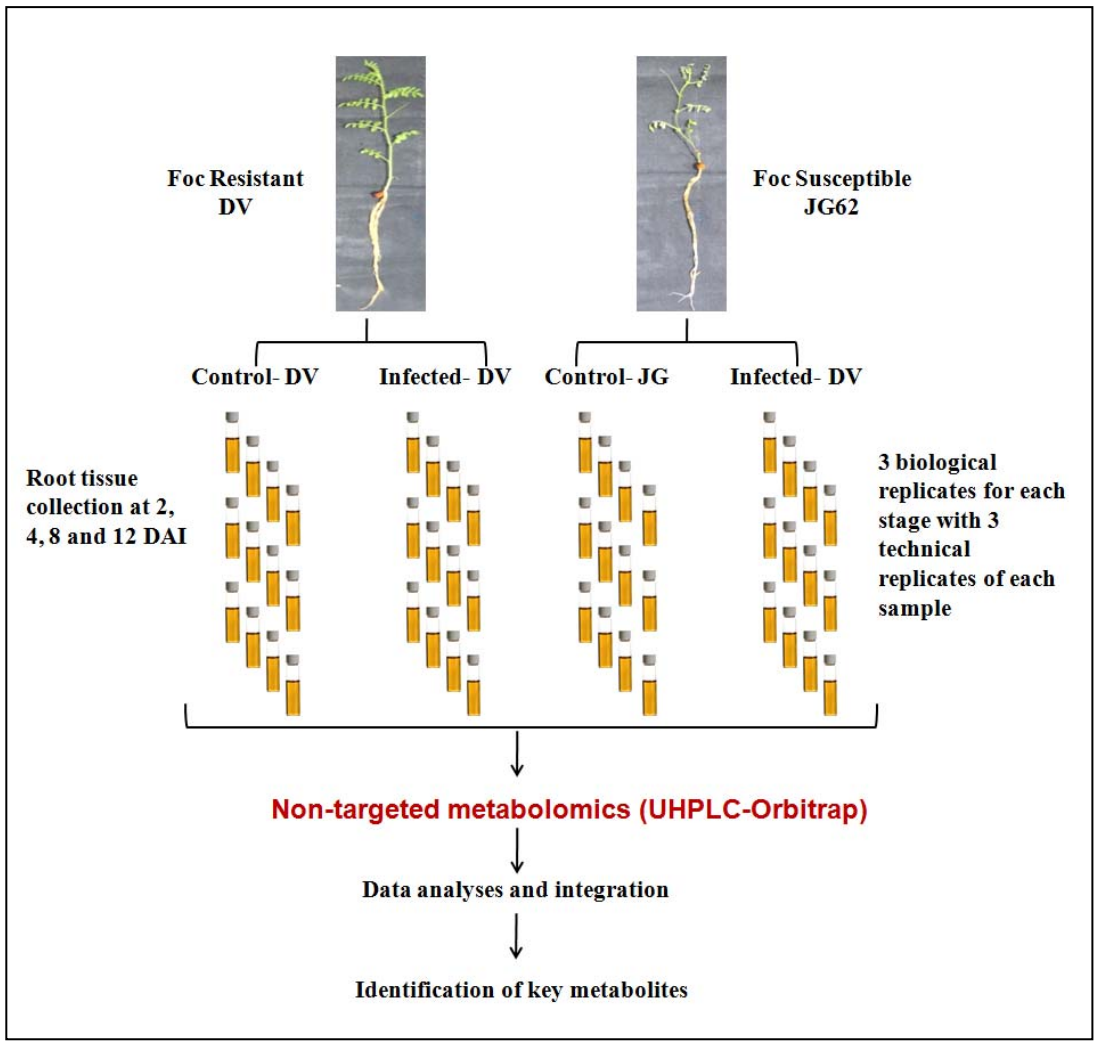


Fig. 2.7: Experimental design for untargeted metabolomics (UHPLC-Orbitrap)

2.11 Extraction of metabolites and UHPLC profiling

Metabolites from 100 mg of each root tissue sample were extracted with 1 mL of 60% ice cold methanol and 0.1% formic acid followed by sonication for 20 min and centrifugation at 4°C at 15,000g for 30 min. Supernatant was filtered with 0.2 micron amicon filter (Millipore, Hessen, Germany) and stored at -80°C until further use. An Accela™ ultra high performance liquid chromatography (UHPLC) system (ThermoFisher, Waltham, USA), coupled online via heated electrospray ionization source (HESI) with a Q-Exactive-Orbitrap mass spectrometer (ThermoFisher), was employed for non-targeted metabolomics profiling with 3 µL sample injection volume. The metabolites were profiled using a C18 Hypersil Gold column (1.9 µm, 2.1 mmX150, ThermoFisher). The temperature of column oven was set at 40°C and the sample manager was maintained at 4°C. The eluents A (water containing 0.1% formic acid) and B (acetonitrile containing 0.1% formic acid) were employed in the electrospray ionization-positive (ESI+) mode and electrospray ionization-negative (ESI−) mode. The flow rate was adjusted at 0.6 mL/min with a linear gradient elution over 15 min. From the start to 0.3 min, eluent B was held at 2%, linearly increased to 30% till 2 min, to 45% during next 5 min, and then to 98% in 12 min. Subsequently, eluent B was returned to 2% in 13.4 min and held for an additional 1.2 min before returning to the initial conditions. The sample sequence was random. In the ESI+ mode, the MS spray voltage was 3.7 KV while it was 2.8 KV in the ESI− mode. The capillary temperature was set at 300°C with the sheath gas at 45 arbitrary units and the aux gas at 5 arbitrary units. The tube lens was set to 45V and the mass scan range was set from 100 to 1000 m/z. The resolution of the Orbitrap was set at 70,000. The tandem mass spectrometry (MS/MS) data were collected with the collision energy between 10 and 35 eV.

2.12 LC-MS stability and reproducibility

Pooled quality control (QC) samples were prepared by mixing all the samples to ensure the quality of metabolic profiling data. Five QC samples were run before analyzing the sample sequence. In addition, one QC sample was run after every 10 sample injections to

monitor the stability of the system during the analysis of sample sequence. To verify the ability of the mass spectrometer system to accurately detect changes in metabolite abundance, a set of parameters was calculated to assess the reproducibility of the system using pooled QC of all the tissue samples. Extracted ion chromatograms (EICs) of leucine (Leu) and isoleucine (Ile) were selected to verify the resolution of the mass spectrometer, intensity deviation, ppm error and retention time shift. Moreover, the retention times, mass accuracies and peak areas of these two selected EICs in the QC samples were also determined to validate the system stability.

2.13 Data analysis

The raw data alignment and peak picking were performed using the Progenesis QI software (Waters and Nonlinear Dynamics) for positive (ESI+) and negative (ESI−) ionization modes separately. All the detected ions in each sample with ANNOVA $p<0.005$, FDR <1% and minimum fold change of 2 between control and their respective inoculated samples, were normalized using total intensity before importing into SIMCA-P v. 13.0 software (Umetrics) for multivariate data analysis. PCA was performed with mean centered data to check the overall pattern and trend in data. Further, OPLS-DAwas performed on pareto scaling data to identify the discriminating metabolites between control and inoculated samples. The default 7-round cross-validation was applied with $1/7^{th}$ of the samples being excluded from the mathematical model in each round. The parameters of the models, such as the R^2X, R^2Y, Q^2Y and the R^2Y-, Q^2Y-intercepts, were analyzed to ensure the quality of the multivariate models and to avoid the risk of over-fitting using 200 iterations. Further, model validity was assessed with CV-ANOVA (with the *p* value indicating the probability that the model is the result of chance alone). The VIP values of all the peaks from the 7-fold cross-validated OPLS-DA model were considered as a coefficient for peak selection. Discriminating variables were selected according to their highest influence on loading, VIP values (VIP>1.0), S-plot and jack-knifed-based confidence intervals of OPLS-DA model. Additionally, univariate method and the Student's *t*-test were applied to determine the significance of each metabolite in separating the pathogen inoculated and mock inoculated samples. Identification of metabolites was carried out by searching the available databases such as KEGG

(http://www.kegg.com), Massbank (http://massbank.imm.ac.cn/MassBank), KNApSAcK (http://kanaya.naist.jp/KNApSAcK) and METLIN (http://metlin.scripps.edu) using exact mass and MS/MS fragmentation patterns. *In silico* prediction of the mass fragmentation of the candidate structures was also performed using Mass Frontier™ software (ThermoFisher) and compared with MS/MS fragmentation pattern of identified metabolites. Commercially available standards were adopted to confirm the structures of some metabolites. Four-way comparison among the identified metabolites was performed using VENNY (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

2.14 RNA extraction, cDNA synthesis and quantitative Real-Time PCR analysis

Total RNA was extracted from 100 mg root tissue by using TRI Reagent (Sigma-Aldrich, USA). First strand cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, USA) with 3 µg of DNase1 treated total RNA using oligo (dT) primer following manufacturer's protocol. Gene specific primers were designed using Primer Express (v2.0) software and listed in Table 2.1. Primer concentration and annealing temperature were optimized before the final analysis. Real time PCR was carried out using 7900HT Fast real-time PCR system (Applied Biosystems, USA) using FastStart universal SYBR green master mix (Roche, USA) with the following conditions: 95°C denaturation for 10 min, followed by 40 cycles of 95°C for 3 s, with primer annealing and extension at 60°C for 30 s. Following amplification, a melting dissociation curve was generated using a 62-95°C ramp with 0.4°C increment per cycle in order to monitor the specificity of each primer pair. The Initiation factor 4α (*IF4α*) gene was used as internal standard or reference gene (Garg *et al.,* 2010). The reactions were performed in triplicate and the results were averaged. PCR conditions were optimized such that the PCR efficiencies of the reference gene and the gene of interest were close to 2.0. PCR efficiencies were calculated using LinRegPCR (Ramakers *et al.,* 2003) software. Relative transcript abundance calculations were performed using the comparative CT (ΔCT) method described by Schmittgen *et al.,* (2008).

2.15 Lignin staining

Lignin accumulation within root tissue after Foc inoculation was detected using the phloroglucinol/ hydrochloric acid stain as described by Mauch-Mani and Slusarenko (1996). The control and Foc inoculated chickpea roots were subjected to transverse sections with a scalpel and immersed in 1mL of 1% pholoroglucinol in 6N HCl for 5 min and the lignin staining was visualized under light microscope.

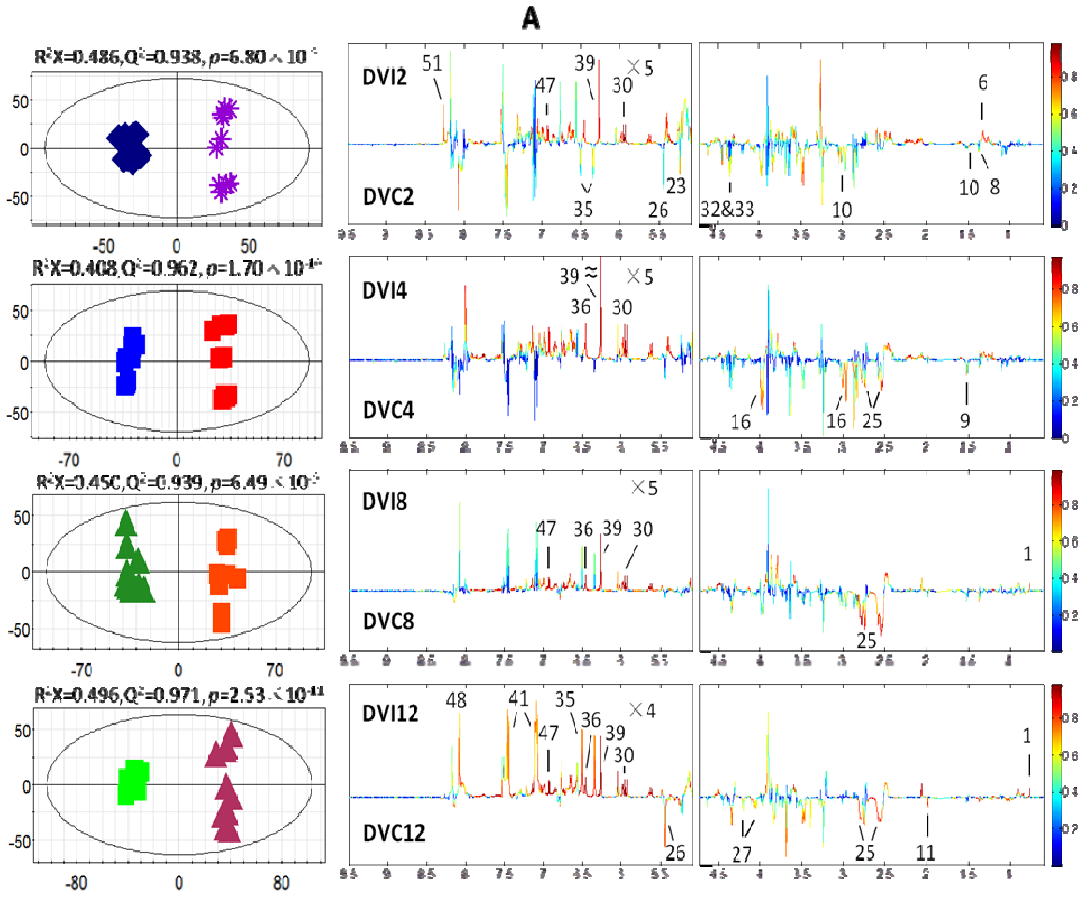
Table 2.1: List of primers used in quantitative real-time PCR

Accession	Gene name	Primer(5' to 3')	Product length (bp)
XM_004498429	Glutamate synthase	F: TGATGGGGAGGAGGACTGA R: GGGAGCAAAACCTGGTGAAG	115
XM_004512714	Glutamate dehydrogenase	F: CAAATCACCCAACTGACCCA R: GCTAACTGTAACACCTCCTG	101
XM_004490556	Asparagine synthetase	F: TGGTGTCAAATGGGTAATCTCTG R: TTCTCTGTGAAACTCTTCCTTGTC	100
XM_004512714	Glutamine synthetase	F: AGTCACCCAGATGTTGTTGC R: CCAGGAAAGCCACCAATAGG	113
CAA10131.1	Chalcone synthase	F: TATGTCAAGTGCGTGTGTATTGTTT R: CTCCTGTTGTCTTCAGTCCATCTTT	80
XP_004497326.1	Chalcone isomerase	F: GTTGCTCCAGACCCTTGATTTCTAC R: TGCCACACAATTCTCCATTACCTTC	130
XP_004505108.1	Isoflavanoid synthase	F: GGGTCTTGTTGTGGATTTCTTCTCT R: GCTTTCTTCATCACTCTTGGGTTGT	105
NP_001266030.1	Isoflavone reductase	F: TACCATAAGAGCAGCAAATGACCC R: TTTCTCCCACAAGGATACAACTTCA	107
XP_004505107.1	Isoflavone 4'-O-methyltransferase	F: CATCCTCCATCGTTTCTTACGCC R: TGCTATTTCTCCTTCTCCACCTTCT	94
XP_004505242.1	Caffeoyl-CoA O-methyltransferase	F: CACCTCCTGATGCTCCTCTC R: CGACGGCAGATAGTGATTCCA	139

XP_004501704.1	Nuclear factor Y subunit C-1	F: AACTCACCATTCGTTCTTGGCTT R: TATCAGTCCTCGTAATAGCAGCAG	93
XM_004512108.1	SKP1-like protein 1A	F: TCGCAGACAATCAAGCACAT R: TAGGTTTATCATCAGAGGTCGCAG	96
NM_001282317.1	AdoMet synthetase	F: AAGTTTCTTATGCTATTGGTGTTCC R: CTTATCAGGTATCTTTCCAGTTCCA	80
NM_001279076.1	Methionine synthase	F: GCTGGAGTGGTTGATGGAAGGA R: AGTGTGAAGAAGTGAGCAGGAGG	132
XM_004513381	Eukaryotic initiation factor 4A	F: TGTGCTAGATGAGGCTGATG R: GCAGAGAAAACTCCCACTTG	105

Chapter 3

Results



3.1 Phenotypic evaluation of chickpea cultivars upon Foc inoculation

The Fusarium wilt susceptible (JG) and resistant (DV) chickpea cultivars were inoculated with Foc1. JG showed typical wilting phenotype (>95%) with yellowing on 2 DAI, followed by drooping of leaves that finally caused complete wilting by 12 DAI. Whereas, the mock-inoculated resistant or susceptible cultivar and Foc inoculated resistant cultivar remained healthy. The establishment of the pathogen within host vascular tissue, characterized by colonization in the xylem vessels, was observed after 2 DAI in chickpea (Gupta *et al.,* 2010; Jimenez-Fernandez *et al.,* 2013). Therefore, root tissues from these Foc and mock-inoculated chickpea plants were collected at 2, 4, 8 and 12 DAI (Fig. 3.1). These root tissues were used to identify the global metabolic changes during chickpea-Foc interactions with high-throughput quantitative proteomic as well as metabolomics approaches.

3.2 Protein identification and quantification in Foc inoculated chickpea roots

The phenotypic analysis of resistant (DV) and susceptible (JG cultivar upon Foc inoculation) indicated till 4 DAI as early stage; while 8 and 12 DAI as late stages, respectively (Fig 3.1). We conducted high throughput label free quantitative proteomics analysis with Foc and mock-inoculated chickpea root tissues at various time points from 2 to 12 DAI. This analysis identified a total of 811 proteins (Annexure 1, List of all the proteins which are identified with their details). The proteins of fungal origin were excluded from this analysis. From the total 811 proteins identified, 481 had statistically significant differential expression ($P< 0.05$ and fold change >1.2) across cultivars and over the course of infection (Annexure 1). The ratio of normalized intensity of proteins from the inoculated plant samples vis-a-vis respective controls revealed increased or decreased expression in the Foc inoculated plant roots. The $\log_2$-transformed values of differentially expressed proteins were clustered using SplineCluster (Heard *et al.,* 2006), a Bayesian model-based hierarchical clustering algorithm for time series data. This generated total eight clusters using a prior precision of 1×10^{-4} (Fig. 3.2).

Fig. 3.1: The Foc resistant-DV and susceptible-JG62 (JG) chickpea plants at early and late stages after inoculation. The letters 'C' or 'I' after the names of the cultivars indicate Control (mock-inoculation) or Foc inoculation, respectively, while the numbers 2 or 12 indicate the number of days after inoculation.

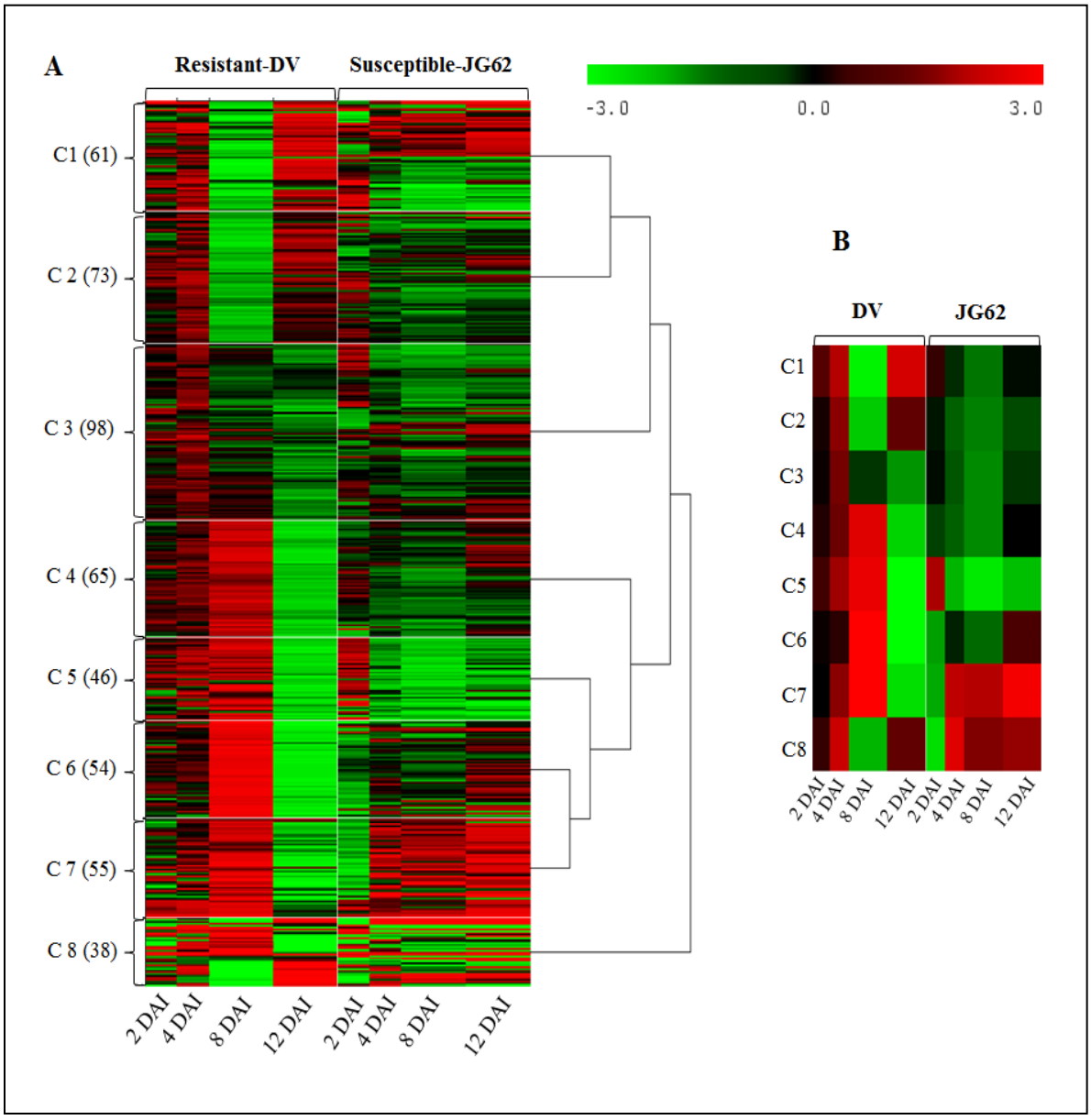


Fig 3.2: (A) Clusters (C1 to C8) of 481 proteins based on differential accumulation trend in root of resistant and susceptible cultivars at various stages (2 to 12 DAI) after Foc inoculation. For each protein, the ratio of log2 normalized expression of Foc inoculated with its respective control and represented by a color, according to the color scale at the top. The number of proteins in a given cluster with similar accumulation trend is indicated in parentheses. (B) Differential expression pattern based on mean expression value of proteins grouped in each cluster in resistant and susceptible cultivars at 2 to12 DAI.

3.3 Protein expression patterns in Foc inoculated resistant and susceptible chickpea roots

The number of proteins ranged from 36 to 98 in the above mentioned eight clusters. The potential biological function for each cluster of proteins was deduced based on gene ontology enrichment analysis using BiNGO. Cluster 1 (C1) had 61 proteins enriched for isoflavonoids biosynthesis and response to oxidative stress (Fig. 3.2 A) while there were 73 proteins enriched in lignin biosynthesis, s-adenosyl methionine biosynthetic process and glycolytic process in C2. Based on the mean expression values (Fig. 3.2 B), all these proteins from C1 and C2 revealed higher expression at all the stages except 8 DAI in the resistant DV plant compared to that in the susceptible genotype JG. The C3 cluster with 98 proteins was enriched for stress response, malate metabolism, oxidation-reduction process and glycolytic pathway. All the proteins from C3 had increased expression at early stages (2 and 4 DAI) which decreased at later stages in the resistant DV plant. However, the susceptible JG had decreased expression at all the stages (Fig. 3.2 B). Total 63 proteins from C4 cluster were enriched in response to misfolded proteins, microtubule polymerization processes, osmotic stress response, proteosome core complex assembly and gluconeogenesis. The C5 cluster had 44 proteins enriched for ubiquitin based protein degradation, ATP biosynthesis, photorespiration and active proton (H^+) transport. Proteins from C4 and C5 clusters showed general trend of higher expression in all the stages except 12 DAI in the resistant DV roots. On the contrary, the susceptible genotype JG showed lower expression in all the stages from C4 while the proteins from C5 increased in JG at 2 DAI and reduced considerably as the stage progressed from 4 to 12 DAI (Fig. 3.2 B). There were 54 proteins present in C6 with high expression at all the stages except 12 DAI in the resistant genotype compared to the susceptible one and did not show enrichment to any specific process through BiNGO. The C7 cluster had 52 proteins that were enriched in defense response and oxidative stress response. This cluster showed mean protein expression trend of increase till 8 DAI and subsequently reduction by 12 DAI in the resistant DV plants. Interestingly, the susceptible JG showed increased mean expression of C7 proteins at all the stages except 2 DAI. The lowest number of proteins (36) was present in C8 cluster which were enriched in response to

abiotic stress, fatty acid biosynthesis and nucleosome assembly processes. These proteins revealed higher mean expression at all the stages except 8 DAI in the resistant DV roots; however, susceptible plants had increased expression from 4 to 12 DAI even though the initial stage (2 DAI) showed lower expression.

Fig. 3.2 B depicts the overall differential mean expression of these proteins in each cluster upon Foc inoculation to DV and JG chickpea plants from 2 to 12 DAI. The resistant DV plant had most of the proteins up-regulated from all the eight clusters by 2 and 4 DAI stages. Some of these proteins (especially from C4 to C7) showed further higher expression by 8 DAI and reduced by 12 DAI. Interestingly, expression of some of these proteins (from C1, C2 and C8) increased again by 12 DAI, though they were low at 8 DAI in DV plants while C3 proteins showed decrease in expression pattern at 8 and 12 DAI. On the contrary, the susceptible JG62 plant revealed decreased expression in majority of proteins (except C5) at early stage of 2 DAI as well as stages of 4 to 12 DAI (especially C1 to C5). Some of the proteins (from C7 and C8) exhibited higher expression from 4 to 12 DAI while those from C6 showed higher expression at 12 DAI only in the JG plants.

3.4 Quantitative variation in proteins from important metabolic pathways upon Foc inoculation

We observed a complex response from numerous interconnected metabolic pathways including primary amino acid metabolism, glycolysis/ gluconeogenesis, TCA cycle, phenylepropenoid pathway and increased lignifications in chickpea upon Foc inoculation (Fig. 3.3). Other cellular processes altered during Foc infection included unfolded protein response (UPR) and defense related proteins. Enzymes such as, sucrose synthase, phosphoglucomutase, transaldolase, enolase, pyruvate dehydrogenase, citrate synthase, succinyle-coA ligase, fructose bis-phosphate aldolase, phosphogluco kinase, phosphoglyceratemutase, fumaratedehydratase and malate dehydrogenase were up-regulated up to 3.0-fold during early and late stages following Foc infection in resistant plants. However, they showed up to 2.0-fold decrease in the roots of susceptible plant.

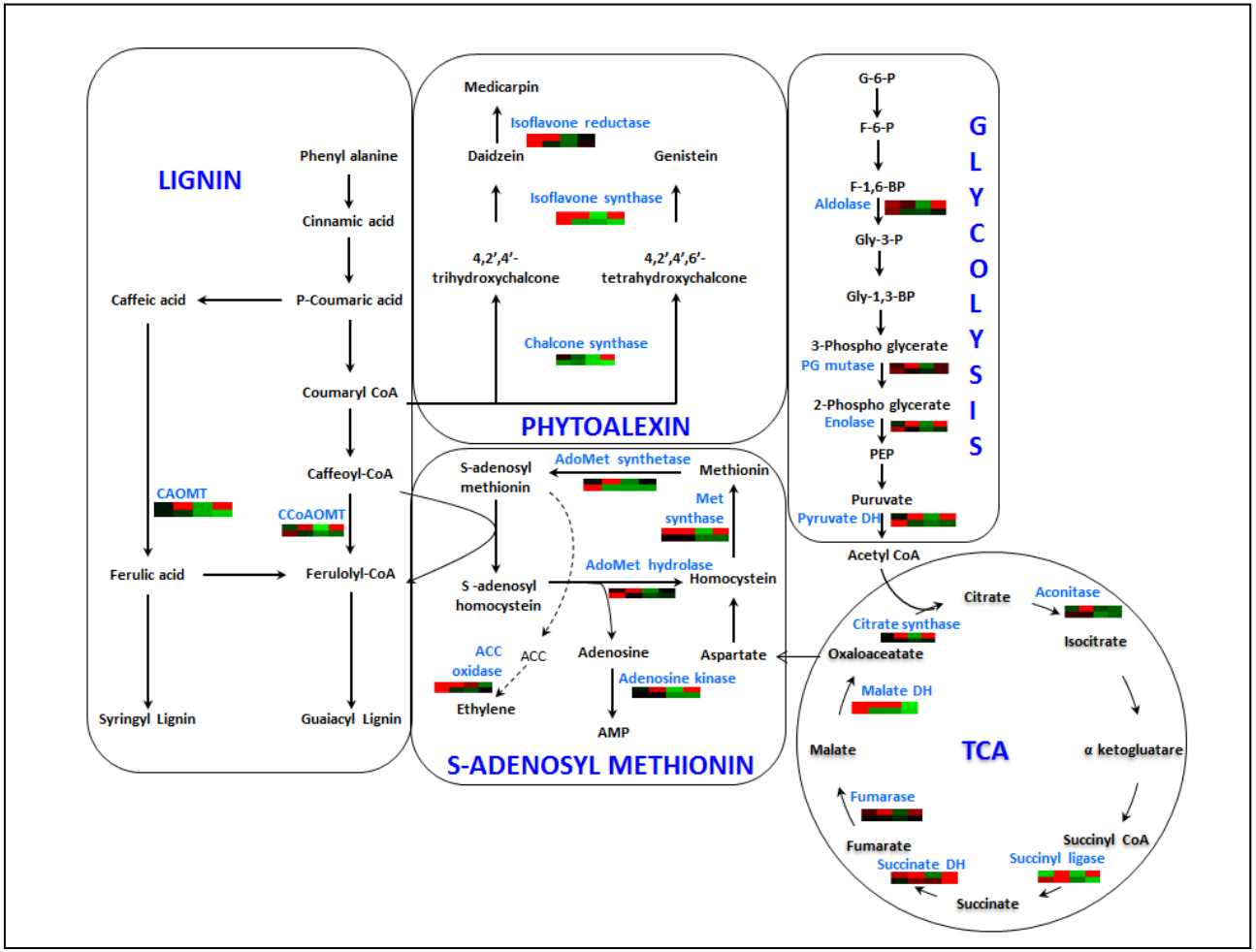


Fig 3.3: Inter connection between various metabolic processes showing modulation in proteins/ metabolites in chickpea roots after Foc inoculation. The differential accumulation patterns based on Fig. 1A in resistant (upper) and susceptible (lower) plants are indicated with four blocks representing four stages after Foc inoculation (2 to12 DAI) are also indicated for respective enzymes.

Other proteins from the same pathway such as fructokinase, succinate dehydrogenase and aconitase showed up to 2.56-fold higher level in the resistant DV cultivar while the susceptible JG root exhibited up to 1.4-fold reduction in these proteins (Annexure 1). Similarly in the present study, several proteins from the UPR pathway including Hsp70, luminal binding protein (BiP), calmodulin, component of SCF-for SKP1-Cullin-F box protein, and protein disulfide-isomerase (PDI) (1.5-fold) increased up to 2.6-fold in chickpea roots of the resistant plant while they showed 2-fold reduction in the susceptible cultivar upon Foc inoculation. The majority of these proteins were high in the early stage, but low in the late stage in the resistant cultivar while the susceptible plant showed opposite expression. Further, proteins induced by abiotic stress such as profilin and aquaporin PIP-type 7a revealed 1.7 to 2.5-fold increase in the resistant plants upon Foc inoculation compared to the susceptible one. However, nuclear transcription factor-Y (NF-Y) had more than 30-fold increase at both the stages in the susceptible chickpea plants compared to the resistant cultivar (Annexure 1).

Proteomic analyses further depicted that proteins from reactive oxygen species (ROS) generation such as ascorbate peroxidase, peroxiredoxin, dehydroascorbate reductase (DHAR), hydroxyacyl glutathione hydrolase, glutathione peroxidase, glutaredoxin, glutathion S-transferase (GST), quinone-oxidoreductase and copper amine oxidase (CuAO) showed 3.0 to 6.0-fold increased expression in the resistant chickpea roots compared to that in the susceptible plants highlighting that Foc induced significant oxidative stress (Annexure 1). Additionally, enzymes involved in methionine metabolism, including methionine synthase, adenosine homocystenin hydrolase, adenosine kinase, and AdoMet synthase showed up to 1.6-fold higher levels in the Foc inoculated resistant cultivar at both, early and late stages, whereas these proteins were significantly down regulated by more than 1.3-fold at an early and late stages in the susceptible one (Annexure 1). In the present study, subsets of proteins participating in multiple branches of phenylepropanoid pathway including lignins, flavanoid, isoflavonoid and phenolics biosynthesis were identified in Foc inoculated chickpea plants. Caffeic acid O-methyltransferase (CAOMT) showed up to 2.0-fold increase in the resistant plant compared to that in the susceptible one. Other enzyme of the

phenylepropanoid pathway, caffeoyl-CoA O-methyltransferase (CCoAMT) also increased by up to 3.85-fold in the resistant DV roots compared to the susceptible cultivar. Similarly, enzymes involved in isoflavonoid biosynthetic pathway such as chalcone synthase (CHS) showed significant increase with more than 5.0-fold in the resistant roots in comparison to that in the susceptible plants. Chalcone isomerase (CHI), isoflavonoid synthase (IFS) and isoflavone reductase (IFR) revealed up to 2.7-fold increase in the resistant plants compared to the susceptible one (Annexure 1).

Additionally, pathogenesis related (PR) proteins such as endo β-1,3-glucanase, major latex protein (MLP), major latex allergen hev b5 and Bet v1 showed up to 2.1-fold increased expression in the resistant plants compared to the susceptible one. Similarly, β-gulcosidase, disease resistance response (DRR) protein-206 and DRR-49 had more than 5.0-fold higher accumulation in the resistant DV roots than that in JG. Proteolytic chitinases offer antifungal properties and confer resistance to fungal pathogens. Chitinase, selenium binding protein (SBP) and glycine rich proteins revealed up to 1.8-fold increased expression in the roots of the resistant than the susceptible chickpea plants. Interestingly, some PR proteins such as PR protein STH-2 (2-fold), thaumatin-like protein PR-5b (more than 5-fold) and PR-4A (more than 10-fold) were very high at early stage in the resistant chickpea plant compared to the susceptible one. However, these proteins exhibited reverse trend with 2.5 to 10-fold increase in the susceptible chickpea roots at late stages of infection indicating their response to heavy wounding. Some of the proteins involved in the stress signaling process such as ABA-responsive protein, auxin-binding protein ABP19a and Ran-binding protein showed 2.1-fold increase in the resistant DV plants compared to the susceptible one. Additionally, 14-3-3 and H^+-ATPase showed up to 2.0-fold higher accumulation in the roots of the resistant plants compared to that in the susceptible one, suggesting its association with defense response against Foc in chickpea roots (Annexure 1).

3.5 NMR-based metabolic profiling in chickpea root

A typical annotated ^{1}H NMR spectrum of chickpea root extract is depicted in Fig. 3.4. The metabolite resonances were assigned according to the in-house databases and

previous publications (Fan *et al.*, 1996 and Eisenreich *et al.*, 2007) and further confirmed with a series of 2D NMR spectra including COSY, TOCSY, JRES, HSQC and HMBC with both ^{1}H and ^{13}C chemical shifts and signal multiplicities shown in Table 3.1. A total

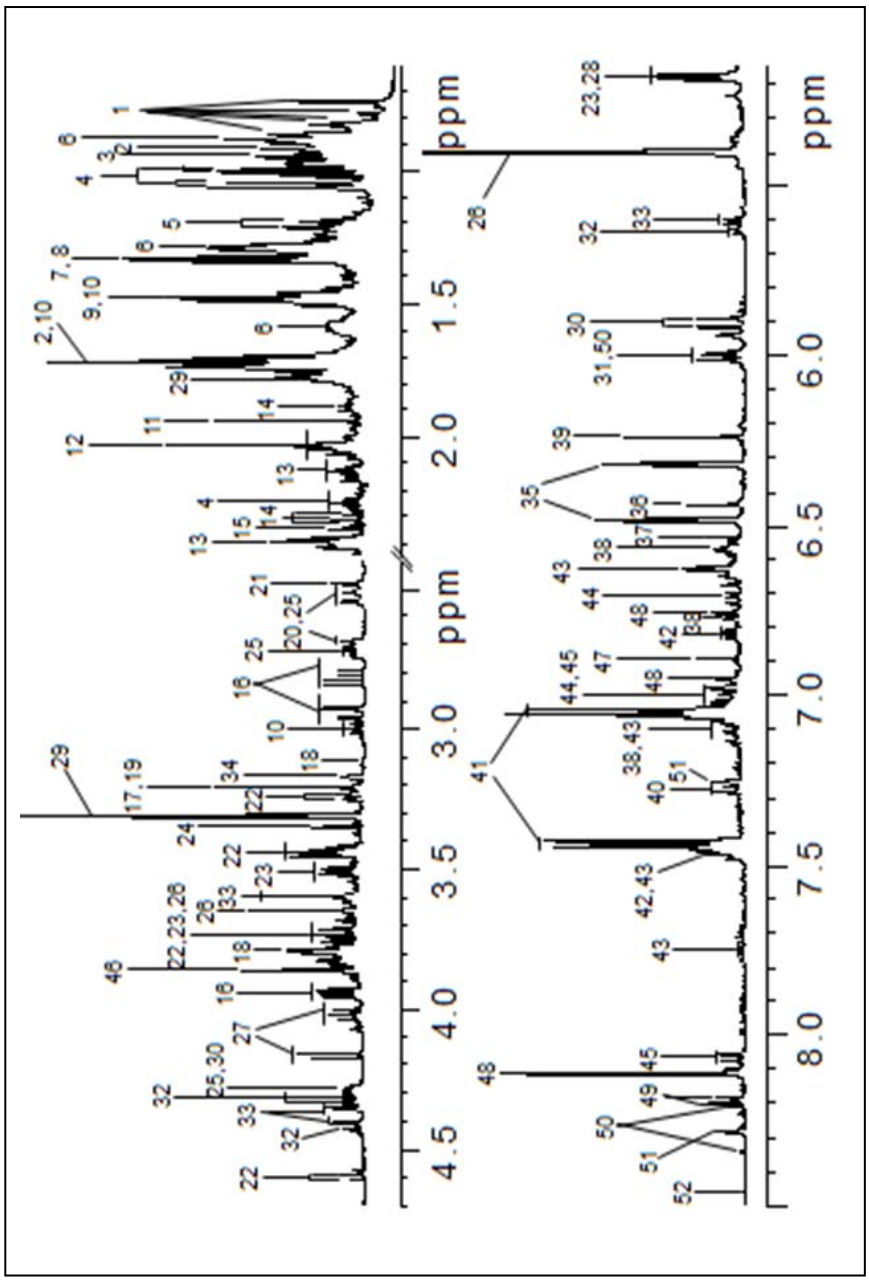


Fig 3.4: Typical NMR spectrum of chickpea root extract: Annotation with number and details provided in Table 3.1.

Table 3.1: Assignments of metabolites from ^{1}H-NMR analysis

Key	Metabolites	Assignment	δ^{1}H (multiplicity)[a]	δ^{13}C	Assigned with
1	Cholesterol	CH_3	0.74(s), 0.78(s), 0.83(s), 0.86 (s)	23.1, 21.9, 17.7, 15.8	HSQC, HMBC
2	Isoleucine	αCH	3.68(d)	62.6	JRE, COSY, TOCSY, HSQC, HMBC
		βCH	1.98(m)	39.4	
		γCH_2	1.24(m), 1.45(m)	27.4	JRE, COSY, TOCSY, HMBC
		$\gamma'CH_3$	1.02(d, 7.0Hz)	17.6	JRE, COSY, TOCSY, HSQC, HMBC
		δCH_3	0.94(t, 7.3Hz)	13.9	
3	Leucine	βCH_2	1.73(m)	42.4	JRE, COSY, TOCSY, HMBC, HSQC
		γCH	1.69(m)	27.4	
		δCH_3	0.98(d, 6.5Hz)	24.5	JRE, COSY, TOCSY, HSQC
		$\delta'CH_3$	0.96(d, 5.9Hz)	24.5	
4	Valine	αCH	3.62(d, 4.3Hz)	63.4	JRE, COSY, TOCSY, HSQC, HMBC
		βCH	2.28(m)	32.1	JRE, COSY, TOCSY, HSQC, HMBC
		γCH_3	1.01(d, 6.9Hz)	19.6	JRE, COSY, TOCSY, HSQC, HMBC
		$\gamma'CH_3$	1.06(d, 6.9Hz)	20.9	
		COOH		177.3	HMBC
5	D-3-hydroxybutyrate	CH	4.16(dt)	68.4	HMBC
		CH_2	2.41(dd)	46.5	COSY, TOCSY
		CH_2	2.31(dd)	46.5	
		γCH_3	1.20(d)	24.6	JRE, COSY, TOCSY, HSQC
6	Lipid	CH_3	0.88(s)	19.3	TOCSY, HSQC, HMBC
		$(CH_2)_n$	1.29(m), 1.59(m)	20.1	
7	Lactate	αCH	4.13(q)	#	JRE, COSY, TOCSY
		βCH_3	1.34(d)	22.9	JRE, COSY, TOCSY, HSQC

		COOH		183.1	HMBC
8	Threonine	α-CH	3.53(d)	63.5	
		β-CH2	4.23(m)	69.1	
		γ-CH3	1.34(d)	22.9	
		COOH		#	
9	Alanine	αCH	3.79(q)	53.4	JRE, COSY, TOCSY,HSQC, HMBC
		βCH_3	1.49(d, 7.3Hz)	19.1	
		COOH		#	HMBC
10	Lysine	αCH	3.73(t)	57.4	JRE, COSY, TOCSY, HSQC, HMBC
		βCH_2	1.91(m)	#	
		γCH_2	1.49(m)	25.8	
		εCH_2	1.73(m)	29.0	
		εCH_2	3.01(t, 7.5Hz)	42.2	
		COOH		#	HMBC
11	Acetate	CH_3	1.94(s)	25.7	JRE, HSQC, HMBC
		COOH		#	
12	Acetamide	CH_3	2.03(s)	17.6	JRE, HSQC
		CO		#	HMBC
12	Glutamate	αCH	3.77(m)	57.6	JRE, COSY, TOCSY, HSQC, HMBC
		βCH_2	2.06(m), 2.1(m)	29.8	
		γCH_2	2.34(dt, 3.8, 11.6Hz)	36.4	
		δCO		184.3	HMBC
		COOH		177.2	
13	Glutamine	αCH	2.15(m)	29.6	
		βCH_2	2.40(m)	36.9	
		γCH_2	3.77(m)	57.1	
14	γ-Aminobutyrate	αCH_2	2.19(t, 7.6Hz)	32.2	JRE, COSY, TOCSY, HSQC
		βCH_2	1.8(m)	26.0	JRE, COSY, TOCSY, HSQC, HMBC
		γCH_2	3.12(t)	44.2	
		COOH		186.1	HMBC

15	Pyruvate	CH_3	2.35(s)	28.3	HSQC
		CO		#	
		COOH		#	
16	Aspargine	αCH	3.94(dd)	54.7	JRE, COSY, TOCSY, HSQC, HMBC
		βCH_2	2.84(dd) 2.94(dd)	37.5	
		CO		176.5	HMBC
		COOH		176.5	
17	Choline	$N(CH_3)_3$	3.21(s)	56.5	JRE, COSY, TOCSY, HSQC, HMBC
		OCH_2	#	#	
		NCH_2	3.51(m)	70.4	HMBC
18	Ethanolamine	αCH_2	3.12 (t)	44.3	JRE, COSY, TOCSY, HSQC, HMBC
		βCH_2	3.81(t)	63.6	
19	phosphocholine	$N(CH_3)_3$	3.21(s)	56.5	TOCSY, HSQC
		OCH_2	#	#	
		NCH_2	3.61(m)	#	
20	Citrate	CH_2	2.54(d)	47.1	TOCSY, HSQC, HMBC
		CH_2	2.68(d)	78.2	
21	Succinate	CH_2	2.49(s)	35.2	JRE, HSQC
		COOH		182.2	HMBC
22	β-Glucose	C_1H	4.67(d, 7.8Hz)	99.2	JRE, COSY, TOCSY, HSQC, HMBC
		C_2H	3.25(t, 9.3Hz)	77.6	
		C_3H	3.49(dd)	79.0	
		C_4H	3.38(dd)	56.1	
		C_5H	3.41(t)	72.8	
		C_6H	3.73(dd), 3.83(dd)	63.1	
23	α-Glucose	C_1H	5.19(d, 3.9Hz)	95.4	JRE, COSY, TOCSY, HSQC
		C_2H	3.54(#)	72.2	COSY, TOCSY, HSQC, HMBC
		C_3H	3.71(#)	76.0	
		C_4H	3.43(#)	72.8	
		C_5H	3.84(#)	74.5	
		C_6H	3.73(#)	64.2	
24	Methanol	CH_3	3.37(s)	52.1	JRE, HSQC

25	Malate	CH_2	2.50(dd)	44.4	JRE, TOCSY, HSQC, HMBC
		CH_2	2.73(dd)	44.4	
		CH	4.29(dd)	72.5	
26	Sucrose	C_1H	3.66(s)	64.9	JRE, TOCSY, HSQC, HMBC
		C_2H	3.80(m)	63.6	
		C_3H	3.81(m)	84.9	
		C_4H	#	79.6	
		C_5H	5.41(d)	95.4	
			#	106.7	HMBC
27	Fructose	C_1H	3.80(m)	63.6	JRE, TOCSY, HSQC, HMBC
		C_2H	3.84(m)	84.6	
		C_3H	4.03 (dd)	77.2	
		C_4H	4.17(d)	79.8	
28	Trehalose	C_1H	3.48(d)	#	TOCSY, HSQC, HMBC
		C_2H	3.69(d)	#	
		C_3H	3.80 (m)	75.4	
		C_4H	5.18(d)	95.4	
30	Uridine	C_1		169.4	HMBC
		C_2		155.0	
		C_3	7.88(d, 8.1Hz)	145.1	JRE, COSY, TOCSY, HSQC, HMBC
		C_4	5.91(d, 3.9Hz)	104.7	
		C_5	5.93(d)	104.7	JRE, HMBC
31	5’-CMP	C_1H	7.85(d, 7.5Hz)	144.9	JRE, COSY, TOCSY, HSQC, HMBC
		C_2H	6.06(d, 6.9Hz)	99.5	
		C_3		169.1	HMBC
		C_4		160.5	
32	U		5.64, 3.70, 4.34	81.6, 66.7	
33	U		5.61, 3.66, 4.32	81.8	
34	U		3.18	45.4, 178.6	
35	U		6.32, 6.48	102.3, 97.5	HSQC, HMBC

36	Genistein[b]	CH	6.44	96.2	HSQC
37	Fumarate	CH	6.53(s)	138.7	JRE, HSQC
		COOH		177.5	HMBC
38	Luteolin[b]	C_4H	6.56	108.6	JRE, TOCSY,HSQC
		C_3H	6.78	#	
		C_2H	7.09	#	
		C_1H	7.27	#	
39	Orotate[b]	CH	6.24(s)	101.7	HSQC
40	Salicin[b]	CH	7.28(d)	128.3	JRE, HSQC
41	Clotrimazole[b]	CH	7.05(d)	117.1	JRE, TOCSY,HSQC
		CH	7.44(d)	133.5	
42	U		6.82(m)	113.9	
			7.46	#	
43	Tryptophan	C4H,ring	7.74(d,8.28Hz)	#	JRE, COSY, TOCSY,
		C5H,ring	#	#	
		C6H,ring	7.09(t,7.37)	#	
		C7H,ring	7.46(d)	#	
45	Nicotinate[b]	4CH	8.06(d)	#	
46	Guanidoacetate[b]	CH_2	3.86(s)	160.2	JRE, HSQC
47	Quinone[b]	CH	6.88(s)	#	JRE, TOCSY
48	4-Nitrophenol	CH	8.116(s)	126.0	JRE,TOCSY, HMBC
49	Hypoxanthine	CH	6.86(s)	#	
		C_2H	8.19(s)	#	JRE, TOCSY, HSQC, HMBC
50	Adenosine	C_7H	8.21(s)	139.7	
		C_1H	8.34(s)	143.4	HSQC, HMBC
51	Inosine[b]	C_4H	8.25(s)	#	HSQC
		C_6H	6.07(d, 5.9Hz)	91.4	JRE, COSY, TOCSY, HSQC, HMBC
		CH	8.26(s)	#	JRE
52	Formate	CH	8.46(s)	#	JRE

[a]Multiplicity: s-singlet; d- doublet; dd, doublet of doublets; t- triplet; q- quartet; U- unidentified signal; #- signals or multiplicities were not determined.[b] tentatively assigned

of 52 metabolites were identified as dominant metabolites including a range of amino acids (Ala, Val, Ile, Leu, Gln, Glu, Asn, Trp, Lys and GABA), sugars (glucose, sucrose, fructose, trehalose and salicin), organic acids (pyruvate, lactate, acetate, citrate, succinate, formate, fumarate, malate and guanidoacetate), nucleosides (adenosine, uridine, inosine, 5CMP and hypoxanthine), phytoalexins (genistein and luteolin), clotrimazole, cholesterol, and lipids (Table 3.1).

3.6 Metabolic alterations induced by Foc infection as observed by NMR analysis

The overview of global metabolic alterations in Foc inoculated resistant and susceptible chickpea roots compared with their respective controls was constructed by principal component analysis (PCA) of the NMR data. The averaged PCA scores were calculated for the first two PCs (Fig. 3.5). The PCA trajectory illustrated clear separation between chickpea root metabolite profiles of the control and inoculated samples of the susceptible and resistant cultivars as visible after 2 DAI (Fig. 3.3). The metabolic profiles obtained from the resistant inoculated groups followed a globally similar trajectory trend to control; however, the susceptible inoculated group revealed a significantly different process from the control, which showed dramatic trajectory movement after 8 days of inoculation (Fig. 3.3). The metabolic changes induced by Foc inoculation were further evaluated by constructing OPLS-DA models. The quality of models is indicated by the values of R^2 and Q^2 and cross-validated with a CV-ANOVA approach ($P< 0.05$) (Fig. 3.4, A and B) and permutation tests (Fig. 3.7). The significantly changed metabolites associated with the chickpea roots infected with Foc are annotated in the OPLS-DA coefficient plots summarized in Table 3.2.

Compared to the respective control, Foc inoculation induced marked reduction in the levels of amino acids including Thr, Ala, Lys and Asn at early stage (2 and 4 days) in the resistant and susceptible cultivars whereas significant elevation of their levels in the susceptible cultivar at later stage (8 and 12 days) and remained unchanged in the resistant cultivar (Table 3.2). The decreased levels of Ile and Leu were observed at early stage and remained unchanged at late stage in the susceptible cultivar after Foc inoculation (Table 3.2). Interestingly, Val and Trp levels increased dramatically in the late stage of

susceptible cultivar but remained unchanged in both the stages of the resistant cultivar after Foc inoculation (Table 3.2). Similarly, two of the most important metabolites from nitrogen metabolism, such as glutamate and glutamine were upregulated only at early stage of Foc inoculation in the resistant cultivar DV. However, the susceptible plant revealed significantly higher accumulation of both of these amino acids at early and late stages after Foc inoculation. In addition, Foc inoculation resulted in decreased level of glucose at early stage in the resistant, however, no significant change was observed in the later stage of both the cultivars. The levels of sucrose and fructose were decreased in early stage, followed by significant reduction at the later stage in the susceptible cultivar after Foc inoculation (Table 3.2).

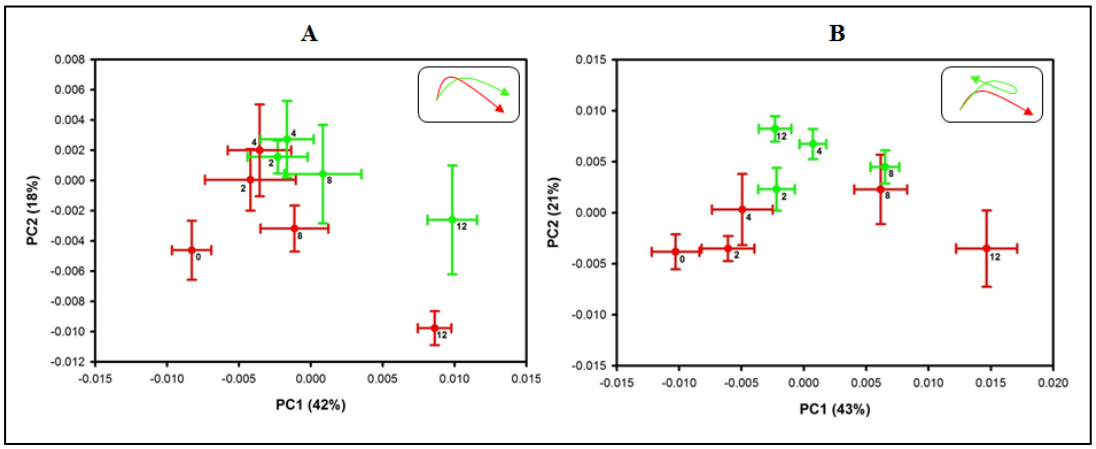


Fig 3.5: PCA trajectory plot of (A) resistant-DV and (B) susceptible-JG62 plants with their respective controls obtained from mean of PC1 and PC2 values at 2 to 12 DAI with error bars representing two standard deviations. Inoculated samples are in green while respective controls in red. Top right corner box subset indicates overall pattern.

It is of particular interest that the levels of some nucleotides including uridine and orotate were significantly decreased in the later stage of the susceptible cultivars compared with the resistant cultivar after Foc inoculation. However, the opposite trend was observed in the changed levels of adenosine and inosine at the later stage of susceptible cultivar. Compared to the controls, Foc inoculation induced significant

reduction in the levels of malate and acetate in the later stage of the susceptible cultivar (Table 3.2). As for phytoalexins, the elevated level of genistein was also observed in the later stage of the resistant cultivar and remained unchanged in the susceptible cultivar, whereas the level of luteolin decreased in the later stage of the susceptible cultivar and remained unchanged in the resistant cultivar. An elevation in the level of clotrimazole, a typical antifungal compound, at the later stage of the resistant cultivar but marked reduction in its level in the susceptible cultivar was also observed (Table 3.2). In addition, Foc infection caused elevated level of cholesterol at the later stage of the resistant cultivar. On the contrary, total lipid contents were significantly increased at the later stage of the susceptible cultivar while quinone increased in the early stage of the resistant one (Table 3.2).

3.7 UPHPLC-based global metabolic profiling after Foc inoculation in chickpea roots

The stability and reproducibility of the LC-MS system were validated using several parameters before the samples were run. The stability of the system was confirmed based on the retention times (RT), mass accuracies and peak areas of the two selected extracted ion chromatograms (EICs) in the QC samples. Intensity coefficient variation of Leu and Ile was 0.148 and 0.145, respectively, in all the QC runs. Alignment of the EIC of Leu and Ile revealed the least deviation in RT shift (Fig. 3.8 A). Mass accuracy was ~2 ppm during data acquisition (Fig. 3.8 B). These results demonstrated that the reliability and stability of the system were qualified for running the samples and data acquisition.

The non-targeted metabolomics investigation revealed total 5,018 and 6,508 compounds in DV and JG, respectively, from ESI(+) mode, while ESI(-) analysis revealed total 4,909 and 5,058 compounds in DV and JG, respectively, with significance of $P< 0.005$, FDR< 1% and more than two fold change. In ESI(+) mode, 1,297 metabolites were shared among the four stages (2 to 12 DAI) in DV, whereas in JG, 1,112 were common among them (Fig. 3.9 A and B). Similarly in ESI(-), 968 metabolites were common in DV as compared to 890 in JG. However, stage specific metabolites were

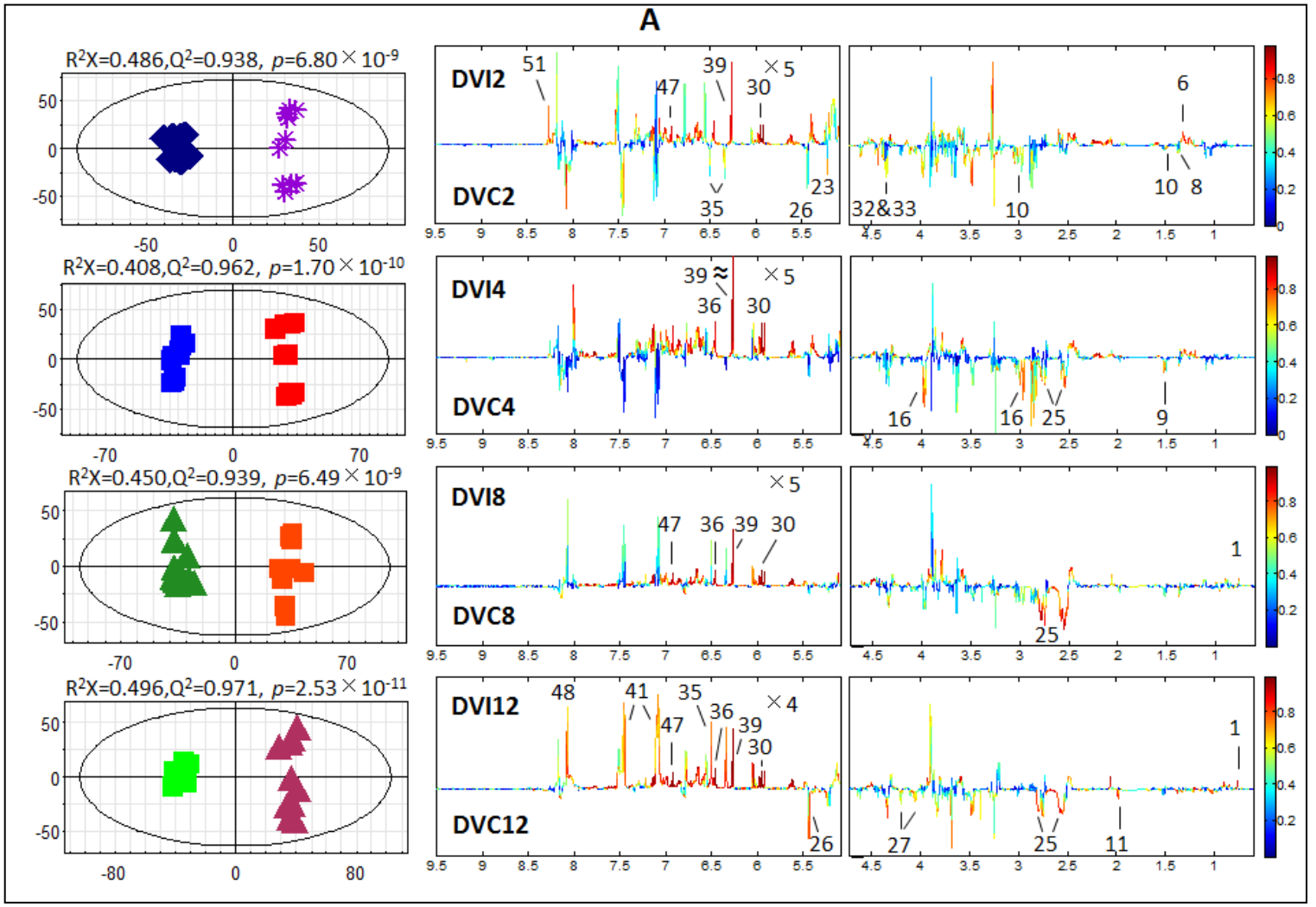


Fig 3.6: Pairwise comparison via OPLS-DA at various stages of Foc inoculation. OPLS-DA scores plots (left) and corresponding coefficient-coded loadings plots (right) obtained from metabolic profiles of *A*, resistant-DV with respective controls at 2 to 12 DAI. The colored scale in correlation coefficient (|r|: absolute values) plots shows the significance of metabolite variations discriminating between the Foc inoculated and control plants.

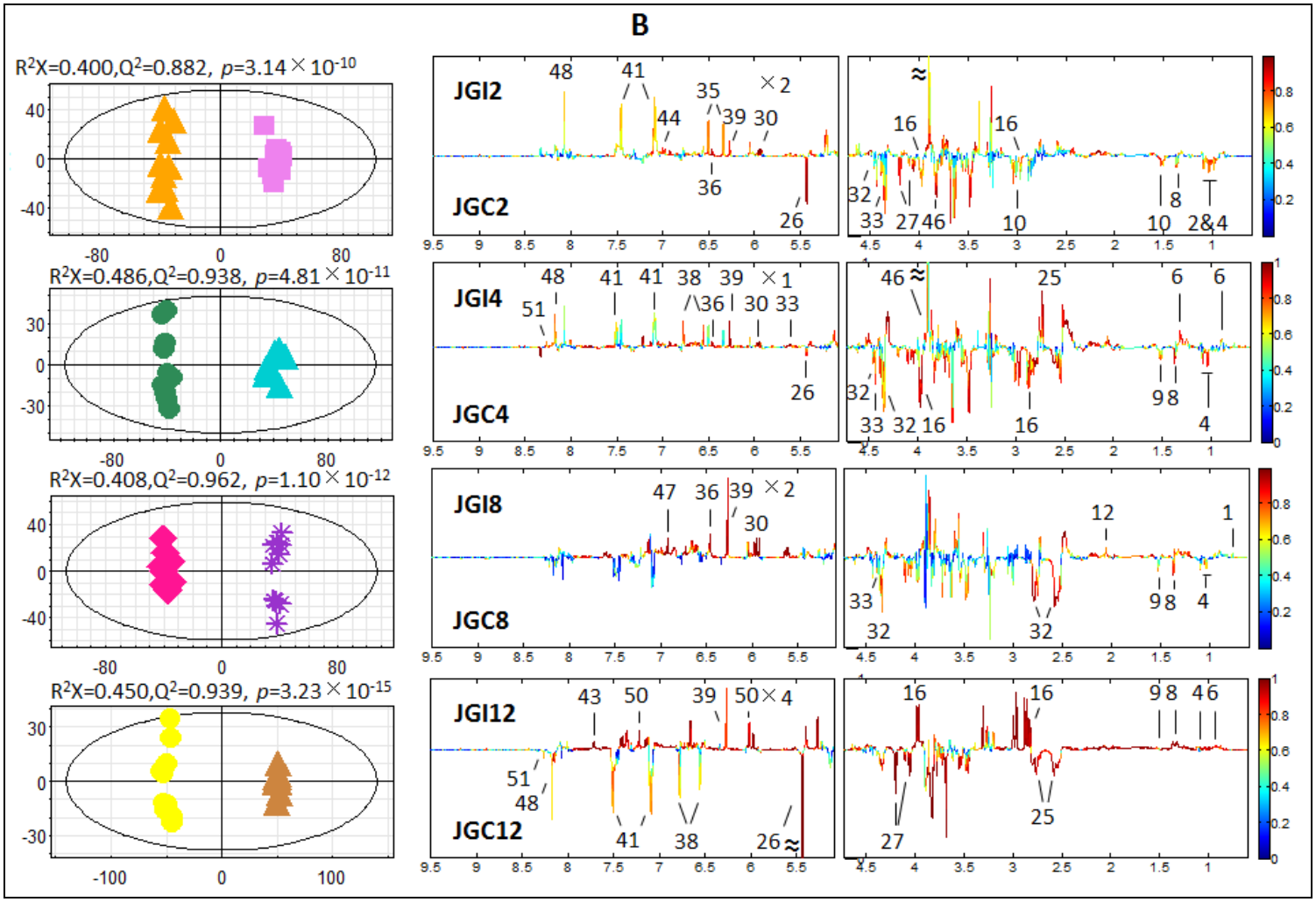


Fig. 3.6: Pairwise comparison via OPLS-DA at various stages of Foc inoculation. OPLS-DA scores plots (left) and corresponding coefficient-coded loadings plots (right) obtained from metabolic profiles of B, susceptible JG62 with respective controls at 2 to 12 DAI. The colored scale in correlation coefficient (|r|: absolute values) plots shows the significance of metabolite variations discriminating between the Foc inoculated and control plants.

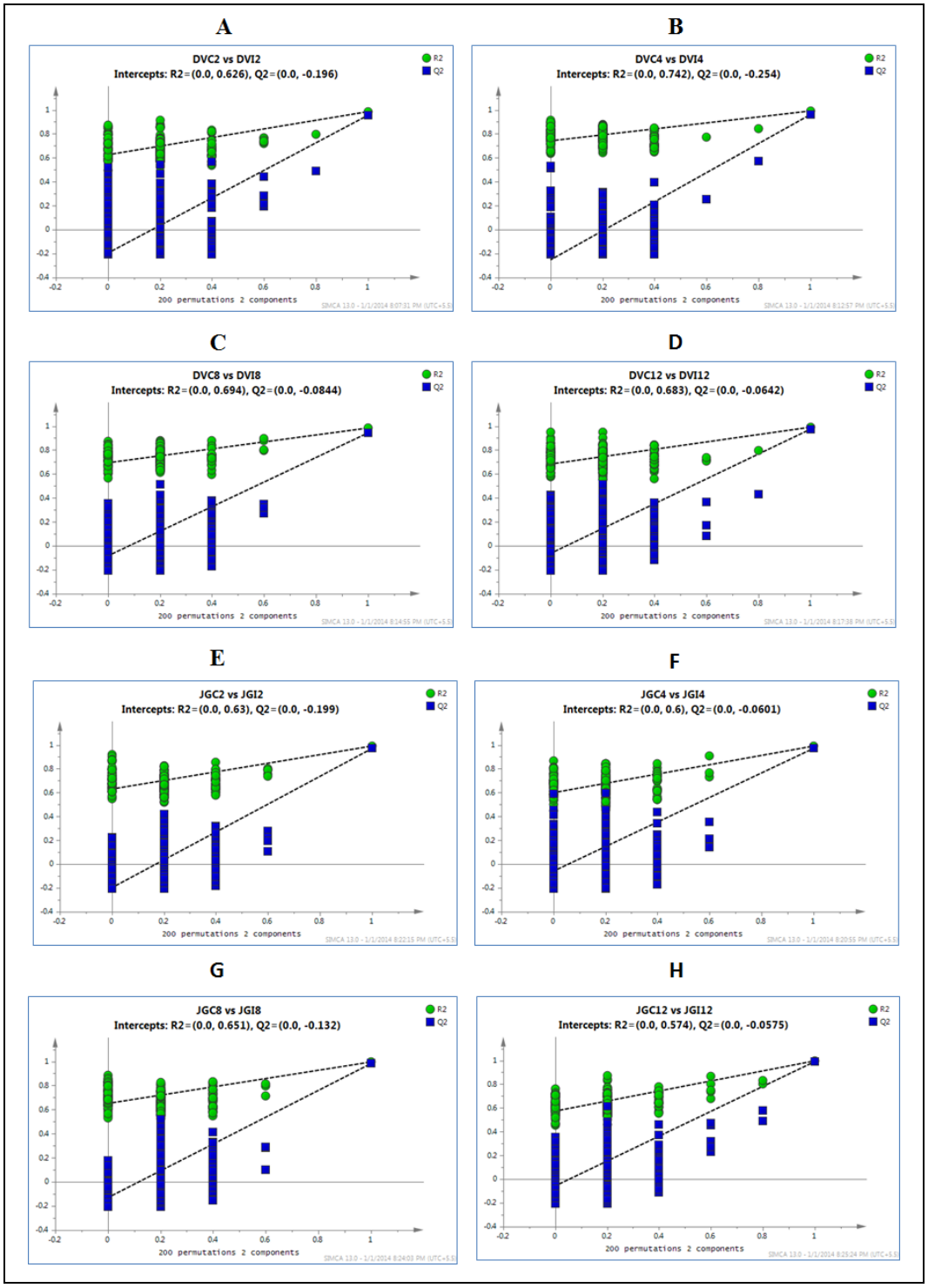


Fig 3.7: Permutation test results for OPLS-DA models with two components and 200 permutations. Models of resistant (DV) chickpea plants are shown at 2, 4, 8 and 12 DAI (A-D) and susceptible (JG62) of same stages (E-H).

Table 3.2: Metabolites with significant contribution to the discriminations between inoculated and control plants based on NMR with OPLS-DA analysis.

Keys	Metabolites	Changes in resistant (DV) cultivar inoculated vs control				Changes in susceptible (JG) cultivar inoculated vs control			
		2 DAI	4 DAI	8 DAI	12 DAI	2 DAI	4 DAI	8 DAI	12 DAI
11	Acetate	-	-	-	-0.88	-	-	-	-
50	Adenosine	-	-	-	-	-	-	-	0.91
9	Alanine	-	-0.77	-	-	-	-0.92	-0.74	0.84
16	Aspargine	-	-0.64	-	-	-0.69	-0.78	-	0.98
1	Cholesterol	-	-	0.71	0.93	-	-	0.61	-
41	Clotrimazole	-	-	-	0.71	0.65	0.69	-	-0.69
27	Fructose	-	-	-	-0.66	-0.94	-	-	-1.00
36	Genistein	-	0.93	0.98	0.99	0.98	0.98	0.98	-
22,23	Glucose	-0.62	-	-	-	-	-	-	-
12	Glutamate	0.81	0.78	-	-	0.78	0.81	0.75	0.86
13	Glutamine	0.83	0.80	-	-	0.81	0.83	0.64	0.91
46	Guanidoacetate	-	-	-	-	0.96	0.70	-	-
51	Inosine	0.75	-	-	-	-	0.76	-	-0.74
2	Isoleucine	-	-	-	-	-0.80	-	-	-
3	Leucine	-	-	-	-	-0.79	-	-	-
6	Lipid	-	-	-	-	-	0.87	-	0.98
38	Luteolin	-	-	-	-	-	0.82	-	-0.66
10	Lysine	-0.78	-	-	-	-0.78	-	-	-
25	Malate	-	-0.82	-0.92	-0.67	-	0.97	-0.97	-0.92
39	Orotate	0.88	0.91	0.95	0.94	0.83	0.96	0.88	-
47	Quinone	0.95	-	0.98	0.97	-	-	0.97	-
21	Succinate	-	-	-	-	-	-	-	-
26	Sucrose	-0.63	-	-	-0.78	-0.95	-0.89	-	-1.00
8	Threonine	-0.67	-	-	-	-0.86	-0.93	-0.92	0.99
53	TMAO	-	-	-	-	0.82	-	-	-
43	Tryptophan	-	-	-	-	-	-	-	0.99
30	Uridine	0.98	0.95	0.98	0.98	0.96	0.99	0.99	0.82
4	Valine	-	-	-	-	-0.72	-0.87	-0.70	0.88
48	4-Nitrophenol	-	-	-	0.71	0.67	0.77	-	-0.65
32	1-Unknown	-0.80	-	-	-	-0.88	-0.85	-0.68	-
33	2-Unknown	-0.71	-	-	-	-0.82	-0.86	-0.69	-

35	3-Unknown	-0.70	-	-	0.71	0.73	-	-	-
44	4-Unknown	-	-	-	-	0.84	-	-	-

The coefficient of 0.6 was used as a cutoff value for the significant difference evaluation ($P< 0.05$). Positive values: up-regulated metabolites in inoculated with respect to control. Negative values: down-regulated metabolites in inoculated with respect to control; -: No significant change observed

also detected in both the chickpea varieties. Total 1229, 213, 168 and 495 stage specific metabolites were accumulated in DV roots at 2, 4, 8 and 12 DAI, respectively (Fig. 3.9A) while JG had 324, 147, 409 and 2,157 such metabolites specifically in 2, 4, 8 and 12 DAI, respectively (Fig. 3.9 B) in ESI(+) mode. Identification with ESI(-) mode showed 169, 124, 1,752 and 335 stage specific metabolites in DV plants at 2, 4, 8 and 12 DAI, respectively (Fig. 3.9 C); whereas 215, 248, 218 and 1,665 metabolites were specific to 2, 4, 8 and 12 DAI stages, respectively, in JG (Fig. 3.9 D).

An unsupervised pattern recognition technique was used to observe a potential clustering behavior and pattern in the metabolite data set. For this purpose, principal component analysis (PCA) was employed on whole data set to establish the presence of any Foc induced metabolic change. UV scaled PCA score plots showed fairly clear differences between control and infected chickpea root tissue in the resistant and the susceptible cultivars, indicating significant changes in metabolite profile after Foc1 inoculation (Fig. 3.10). Further, PCA analysis of metabolites from each time point (2, 4, 8 and 12 DAI) separately also revealed clear separation between control and Foc inoculated samples with good R^2X and Q^2 values (Fig. 3.11). Moreover, to ascertain the time course of metabolic variations that were induced in the resistant and the susceptible plants after Foc1 inoculation, a PCA model was built for resistant and susceptible plants separately using trajectory plot. The average of PCA score for the control and the Foc1 inoculated resistant and susceptible plants were calculated for the first two PCs. The PCA trajectory

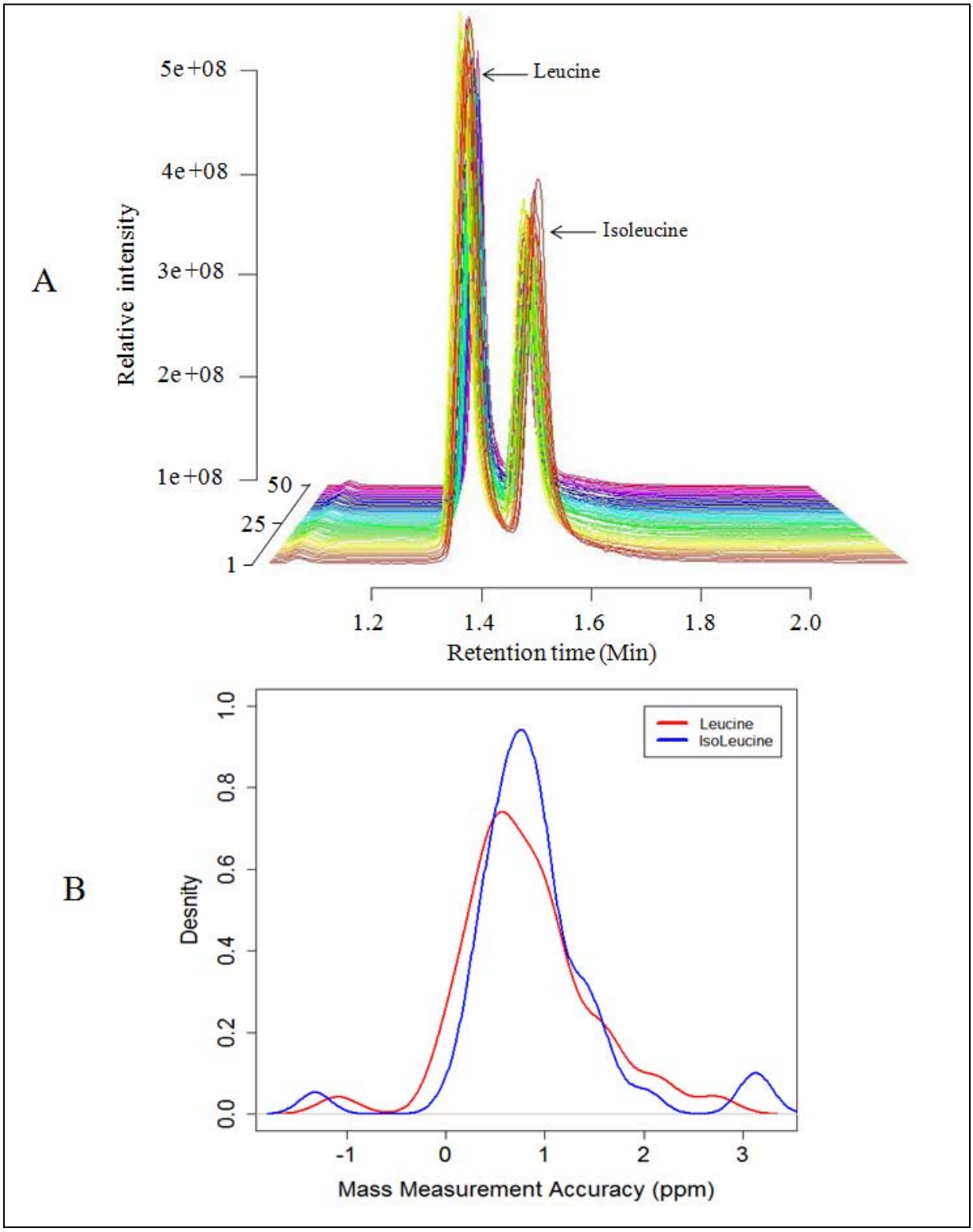


Fig. 3.8: (A) Overlaid EIC of Leu and Ile from 48 pooled QC samples (B) Density plot of mass accuracy calculated for Leu and Ile

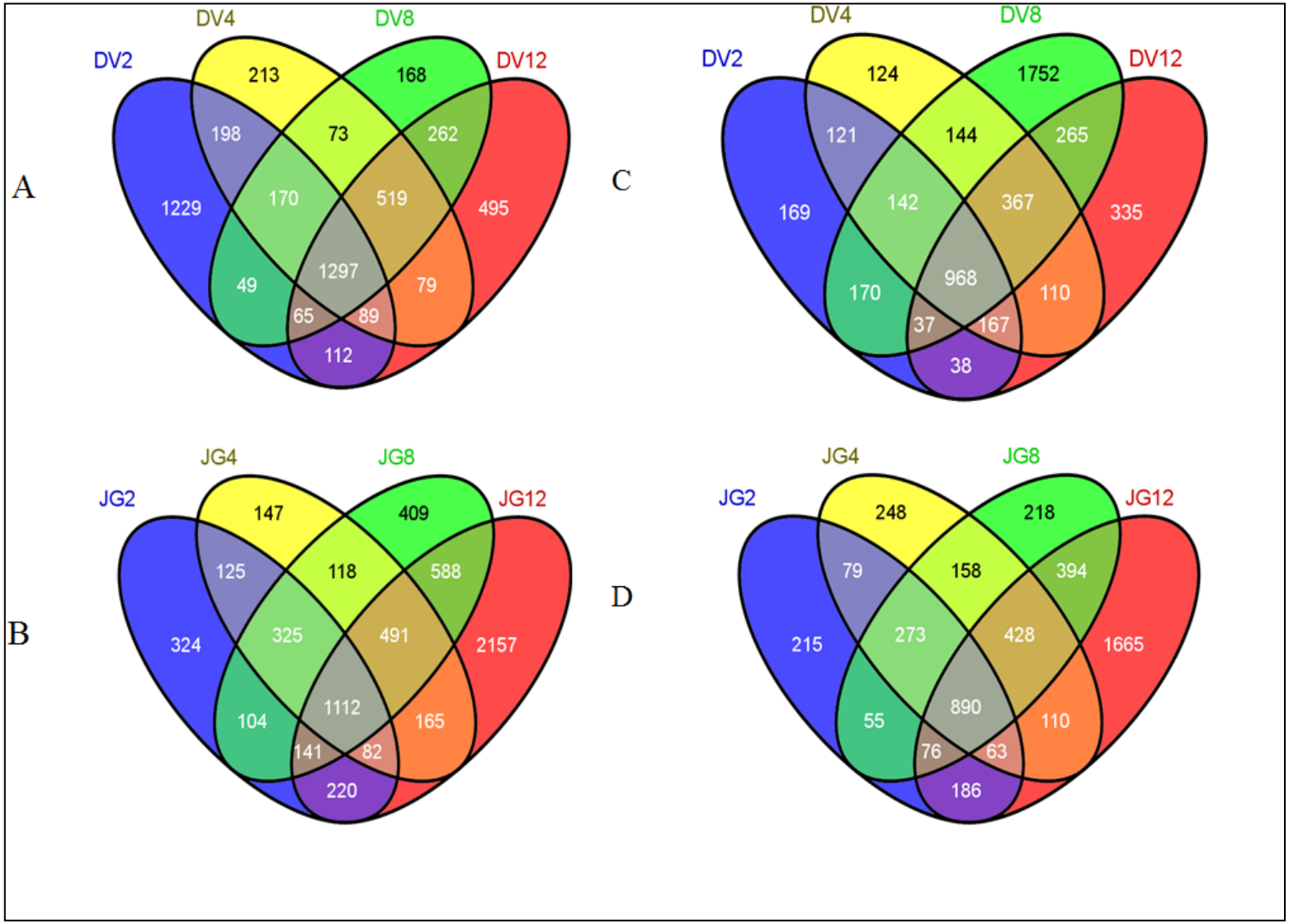


Fig. 3.9: Four-way Venn diagram showing the number of metabolites in DV (A and C) and JG62 (B and D) in ESI (+) and ESI (-) respectively

plots illustrated the time dependence of alterations in the metabolic profiles of DVI and JGI plants induced by Foc1 inoculation (Fig. 3.12). This revealed similar metabolite accumulation patterns for both the control chickpea cultivars in the trajectory plots; however, significant difference was observed in trajectory plots of the Foc1 inoculated DV and JG plants. Total metabolic profile of JGI samples showed rapid upward metabolic shift after 8 DAI, in contrast to that in DV. The PCA trajectory plots clearly illustrated time and infection progression dependent pattern with significant metabolic deviations.

3.8 Metabolic alteration in chickpea roots after Foc inoculation using LC-MS approach

First, the orthogonal partial least squares-discriminant analysis (OPLS-DA) model was constructed for three groups, control of resistant-DVC and susceptible-JGC plant together as one group and Foc1 inoculated resistant-DVI and susceptible-JGI plants as two separate groups to uncover any metabolic changes associated with fungal infection. OPLS-DA score plot (Fig. 3.13) revealed complete separation between the control and the infected plants with three distinguished clusters. Similarly, OPLS-DA model of these three comparison groups separately at each time points (2, 4, 8 and 12 DAI) also indicated clear separation between the control and the infected chickpea plants (Fig. 3.14).

Further, OPLS-DA analysis between Foc1 inoculated resistant-DVI and susceptible-JGI plants of all time points together was performed to identify important metabolites responsible for the variation in phenotypic response between the resistant and the susceptible genotypes (Fig. 3.15). OPLS-DA score plot showed clear separation between all DVI and JGI plants. Moreover, CV-ANOVA validated model parameters in the permutation test for the explained variation (R^2X=0.588) and the predictive capability (Q^2=0.812) were significantly high (Fig. 3.15A)

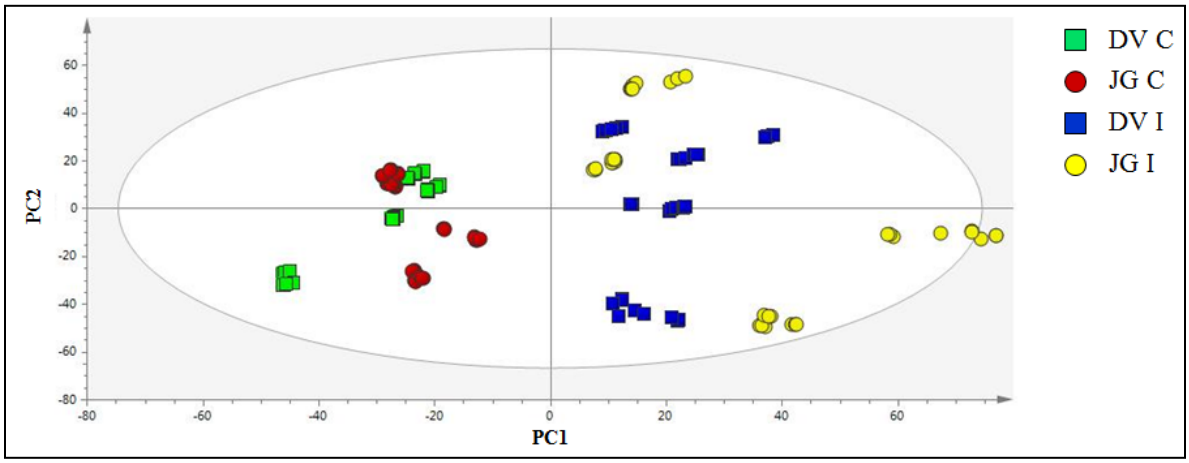


Fig. 3.10: Combined PCA considering all the samples of control and Foc inoculated resistant (DV) and susceptible (JG) plants from four different stages

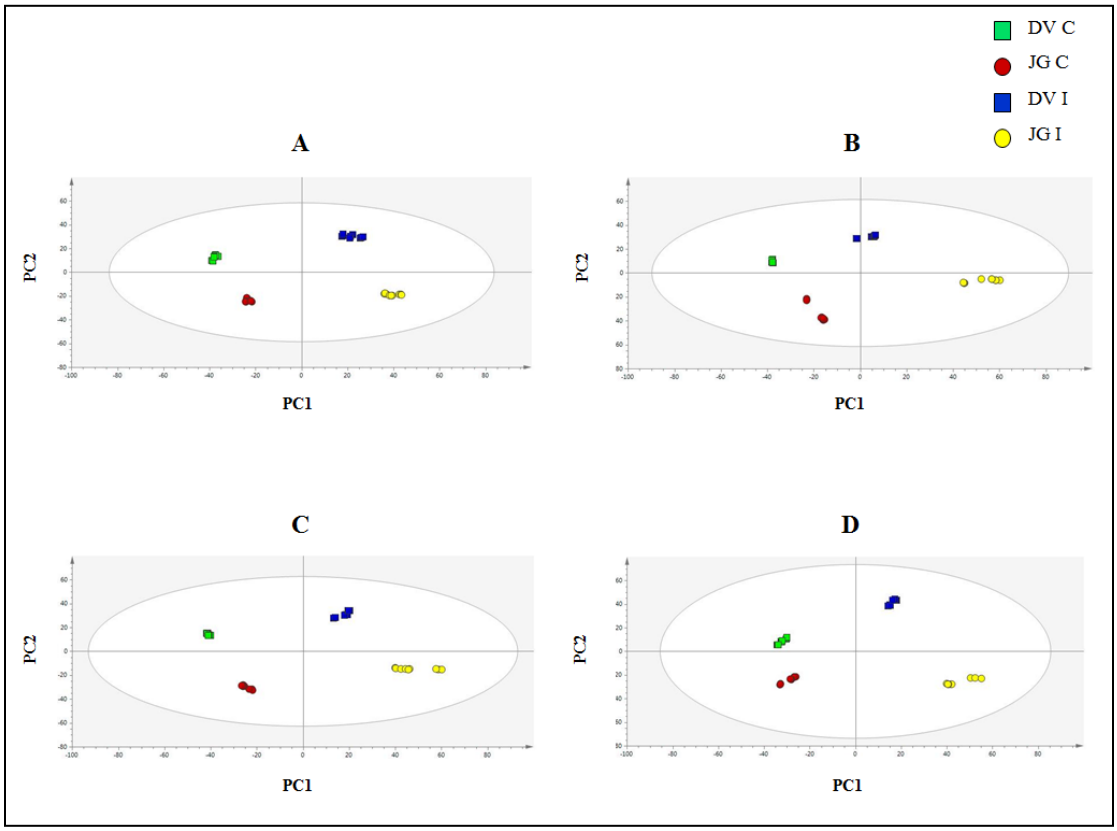


Fig. 3.11: Combined PCA considering all the samples of control and Foc inoculated resistant (DV) and susceptible (JG) plants at 2 DAI (A), 4 DAI (B), 8 DAI (C) and 12 DAI (D)

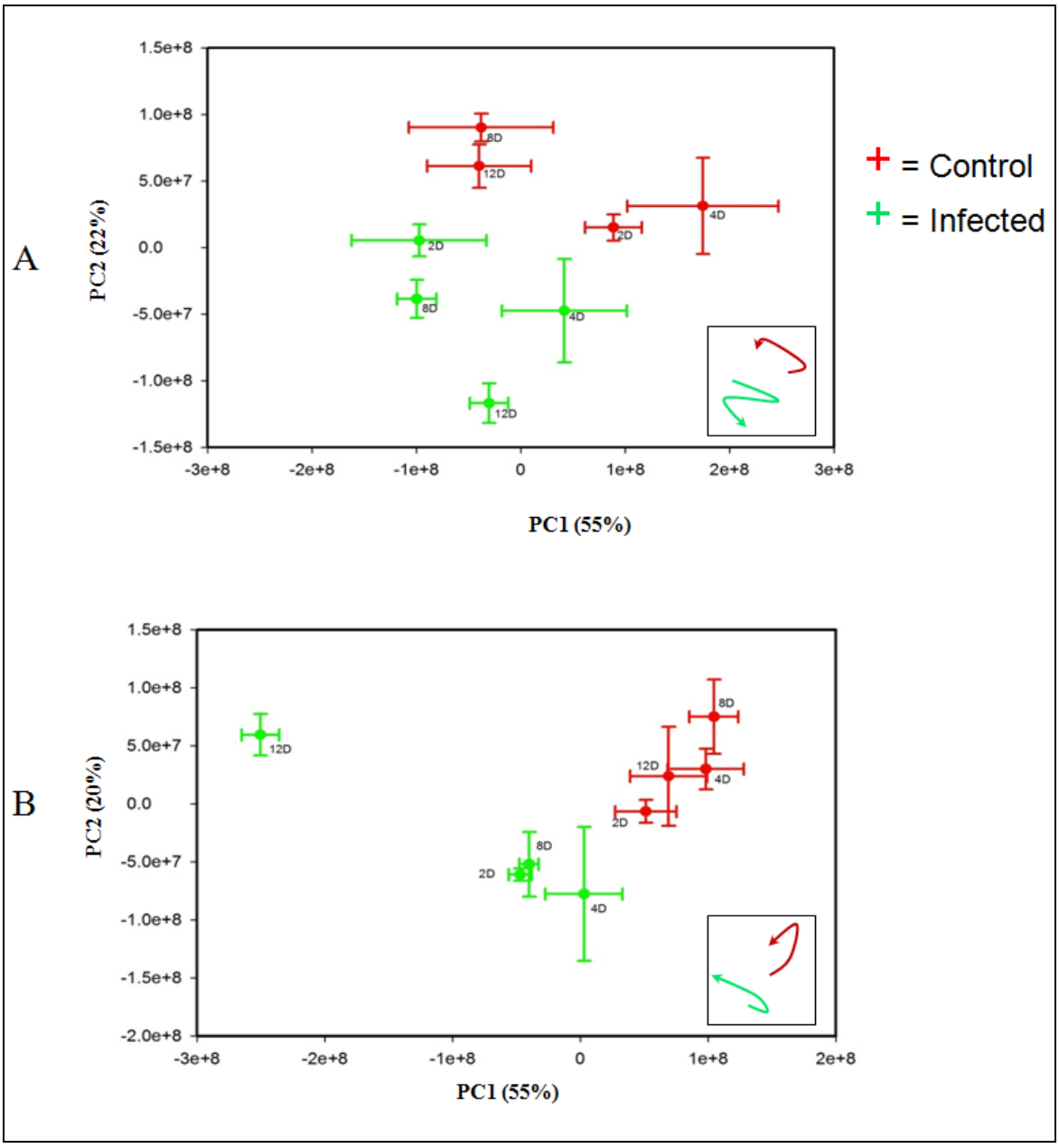


Fig. 3.12: PCA trajectory plot of resistant-DV (A) and susceptible-JG (B) chickpea plants with their respective controls obtained from mean of PC1 and PC2 values at 2, 4, 8 and 12 DAI with error bars representing two standard deviations. Inoculated samples are in green while respective controls in red. Variation in overall trajectory pattern between control and infected samples are shown in lower right corner box.

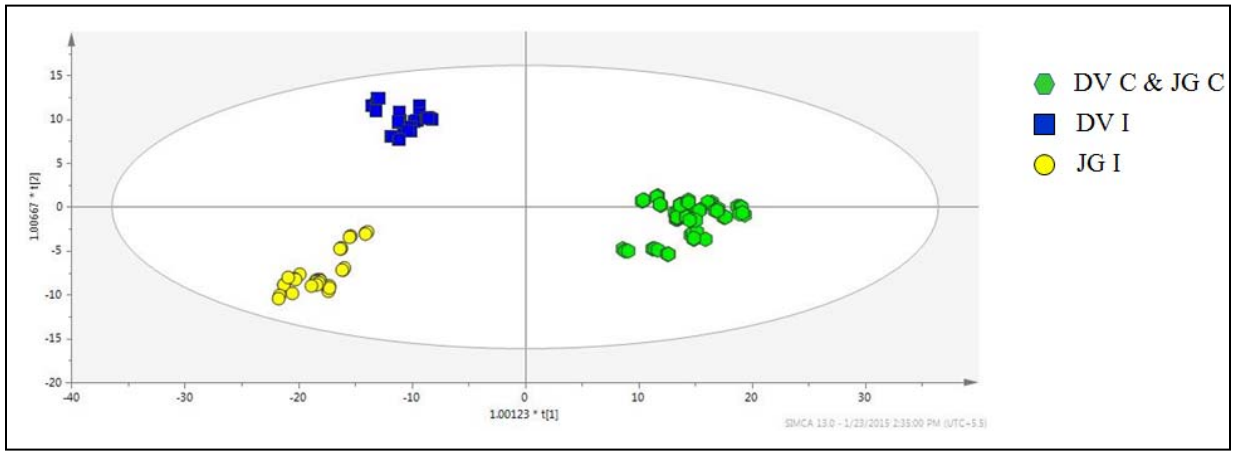


Fig 3.13: Pairwise comparison via OPLS-DA considering all the samples of controls of resistant (DV) and susceptible (JG) plants together, Foc inoculated resistant (DV I) and susceptible (JG I) plants from all the four stages of Foc treatments

S-plot from the above generated model between all the Foc1 inoculated resistant-DVI and susceptible-JGI genotypes identified key intermediate metabolites of flavonoid and isoflavenoid biosynthesis pathway which lead into phytoalexin biosynthetic pathway such as formononetin 7-O-glucoside, formononetin 7-O-glucoside-6"-O-malonate, malonylgenistin, isoformononetin; 4'-hydroxy-7-methoxyisoflavone, pseudobaptigenin, afrormosin, 4',6-Dihydroxy-5,7-dimethoxyflavanone, biochanin A 7-O-beta-D-glucoside 6"-O-malonate, malonyldaidzin and (-)-maackiain-3-O-glucosyl-6"-O-malonate, soyasaponin I and dehydrosoyasaponin. Other metabolites responsible for separation between these groups were amino acids and sugars (Annexure 2).

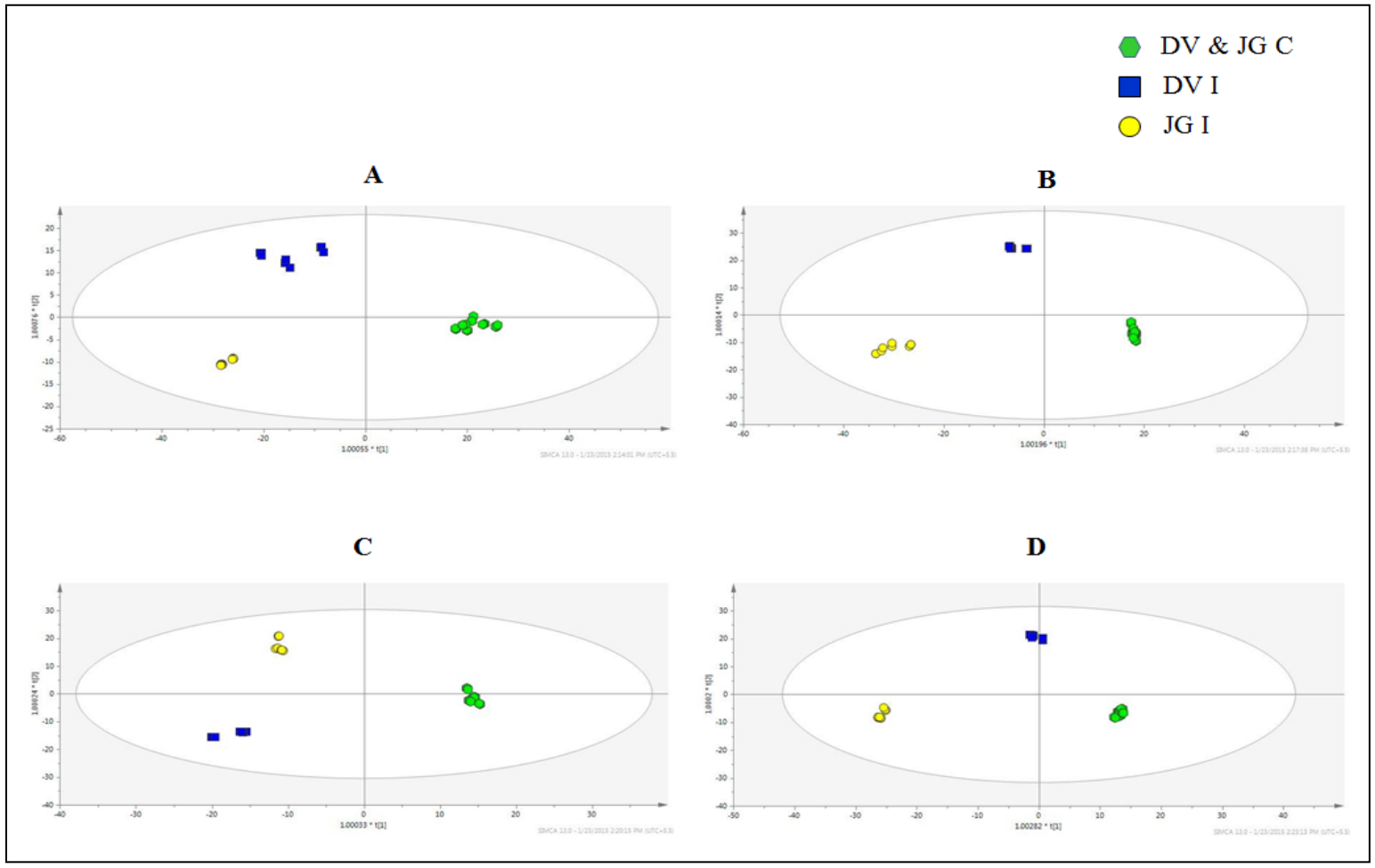


Fig. 3.14: Pairwise comparison via OPLS-DA considering all the samples of controls of resistant (DV) and susceptible (JG) plants together, Foc inoculated resistant (DV I) and susceptible (JG I) plants at 2 DAI (A), 4 DAI (B), 8 DAI (C) and 12 DAI (D)of Foc treatments

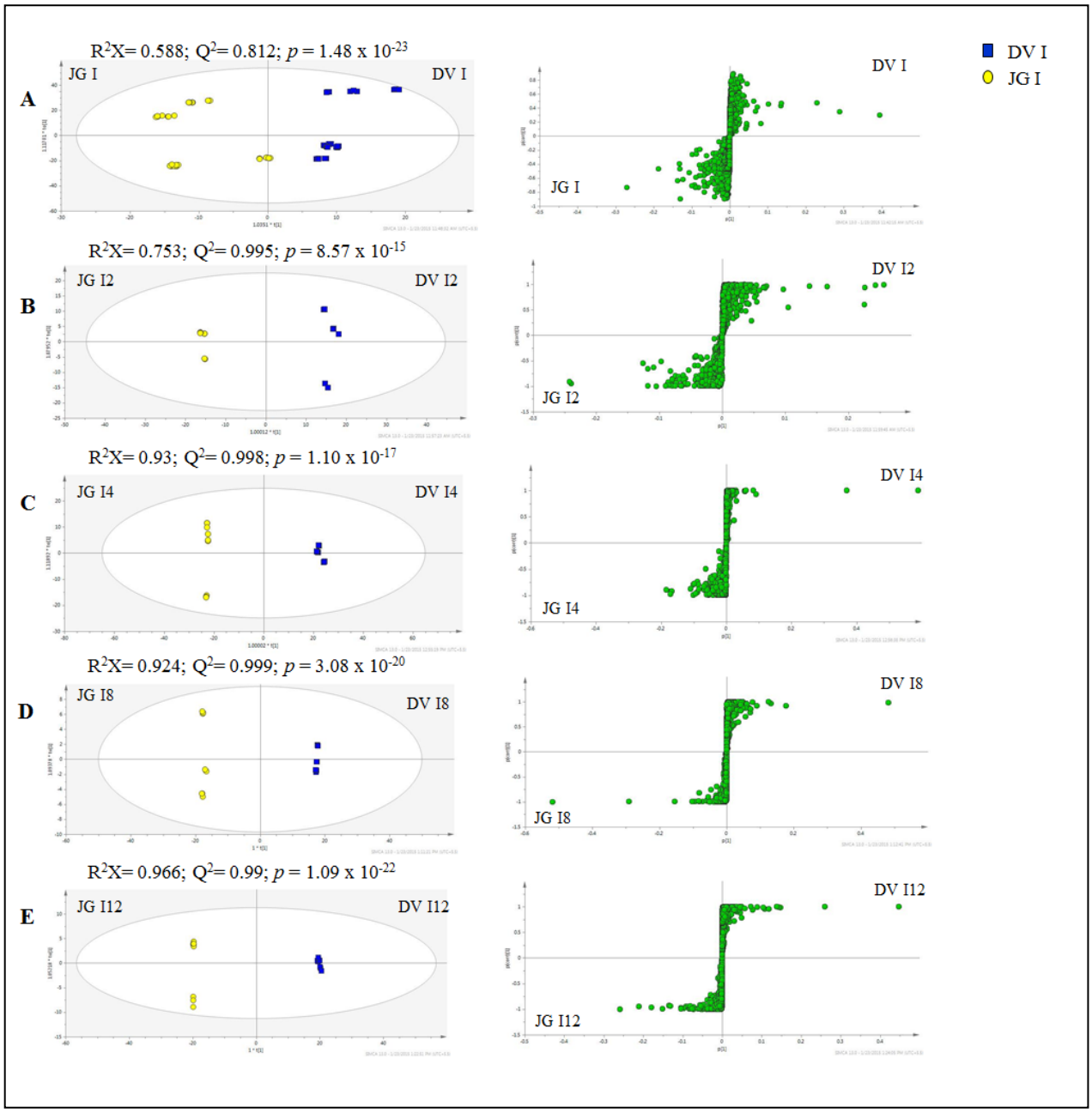


Fig: 3.15: Pairwise comparison via OPLS-DA at various stages upon Foc inoculation. OPLS-DA scores plots (left) and corresponding S-plots (right) obtained from metabolic profiles considering all the samples of Foc inoculated resistant (DV I) and susceptible (JG I) plants from all the four stages of Foc treatments together (A), separately at 2 DAI (B), 4 DAI (C), 8 DAI (D) and 12 DAI (E)

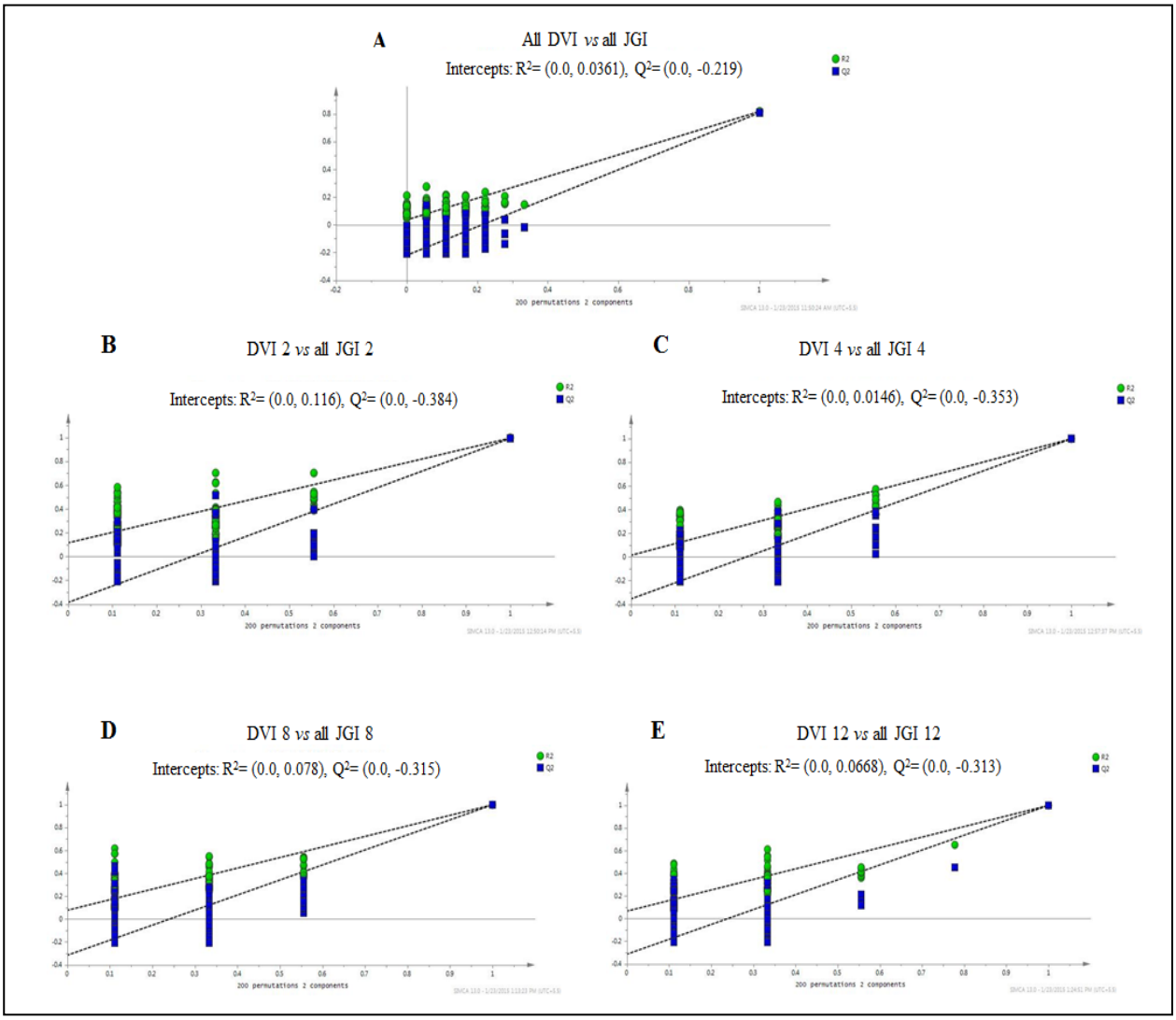


Fig 3.16: Permutation test results for OPLS-DA models with two components and 200 permutations. Models of all Foc inoculated resistant (DVI) and susceptible (JGI) chickpea plants at four stages together (A) as well as separately at 2 DAI (B), 4 DAI (C), 8 DAI (D) and 12 DAI (E) of Foc treatments.

Additionally, to find out the stage specific metabolites using OPLS-DA, the time course change in metabolic variations between Foc1 inoculated resistant and susceptible plants was also analyzed separately at 2, 4, 8 and 12 DAI (Fig. 3.15 B-E). These OPLS-DA score plots showed clear separation between the DVI and JGI plants at all the time points with CV-ANOVA validated model parameters with R^2X and Q^2 (Fig. 3.15 B-E, left side). Permutation tests with 200 iterations further confirmed that all the OPLS-DA models were robust (Fig. 3.16). Additionally, potential metabolites among thousands of variables from the resistant and the susceptible plants were determined based on the S-plots depicting covariance and correlation between the metabolite variables and the model (Fig. 3.15, right side). Selected metabolites preferentially showed a high covariance combined with a high correlation (Wiklund *et al.,* 2008). The effect of every variable on the classification was reflected from their loading and variable importance in projection (VIP) value (Annexure 2). Metabolic variables with high influence on loading and VIP >1 were taken for further analysis (Eriksson 2006). To remove the variables with low reliability, raw data plots and jack-knifed-based confidence interval were subsequently used. Additionally, an independent *t*-test ($p<0.05$) was applied to confirm the significance of the difference in concentration between metabolites identified by these methods in the tested groups. OPLS-DA S-plots indicated that the most discriminative metabolites separating the DVI and JGI were isoflavanoids, flavanoids, alkaloids derived from terpenoids and fatty acid derivatives (Fig 3.17). Additionally, we observed high proportion of amino acids and sugars at later stages of infection in the susceptible plants. The fungal infection led to increase in intermediates of medicarpin and (-)maackiain biosynthesis pathways in the resistant plants compared to those in the susceptible plants. Moreover, malonated conjugates of most of the isoflavanoids and flavanoids level increased in the resistant cultivar. A marked increase in the level of daidzein and pseudobaptigenin in the resistant cultivar compared to the susceptible cultivar was apparent (Fig 3.17). Also, fungal infection resulted in increase in (-)maackiain accompanied by increase in malonated conjugates of daidzein, genistin, formononetin, apigenin, biochanin along with (-)maackiain (Fig 3.17). However, we observed malonated medicarpin upregulated in JG at all the stages compared to DV. Interestingly,

sharp decrease of 3.4 and 3.9-folds was evident in medicarpin and (-)maackiain, respectively, at 12 DAI in the susceptible compared to the resistant cultivar. Various metabolites from flavone and flavonol biosynthesis such as 3,7-di-O-methylquercetin, 3-O-methylquercetin, and quercetin 3-O-[β-D-xylosyl-(1->2)-β-D-glucoside], apigenin, apiin and isovitexin 2"-O-β-D-glucoside,with potent anti-fungal activities increased significantly in DVI as the infection progressed. Methylated isoflavones and afrormosin were also accumulated more in the resistant cultivar; however, they increased by 22-fold in the susceptible cultivar at 12 DAI. Chitin induced anthraquinones such as aurantio-obtusinβ-D-glucoside exhibited higher accumulation in DV. The expression of aurantio-obtusinβ-D-glucoside displayed similar trends in both the inoculated varieties; however, DV showed an increase of 5.99-fold at 2 DAI, 2.2-fold at 4 DAI and 1.9-fold at 12 DAI. Surprisingly, the accumulation of this metabolite was 1.2-fold more in the susceptible cultivar compared to that in DV (Table 3.3).

Various saponins decreased in Foc inoculated chickpea cultivars, compared to their respective controls. We identified soyasaponin I, soyasaponin III, soyasapogenol C and dehydrosoyasaponin I in the present study (Table 3.3). The accumulation of almost all detected saponins illustrated similar trend in both the cultivars; however, the susceptible cultivar showed significant lower levels at all the time-points except 12 DAI. Interestingly, two metabolites namely, sucrose and isocitrate from energy metabolism, were lower at only 12 DAI in JG plants and were not detected at any stages in DV (Table 3.3).

Table 3.3: Significantly differentially accumulated metabolites identified from UPHPLC in chickpea root upon FOC inoculation

Metabolites	Formula	$(M+H)^+$	RT [a] (min)	MS/MS Fragments [b]	Fold change DV (Resistant)				Fold change JG (Susceptible)			
					2 DAI	4 DAI	8 DAI	12 DAI	2 DAI	4 DAI	8 DAI	12 DAI
Isoscoparine [R-1]	$C_{22}H_{22}O_{11}$	463.12	2.73	445, 397, 379, 353, 343	9.63	7.38	7.29	5.07	5.17	4.65	4.27	7.05
3,7-Di-O-methylquercetin	$C_{17}H_{14}O_7$	331.08	3.70	-	23.94	48.89	16.41	14.61	4.83	21.49	20.00	7.73
Apigenin [S]	$C_{15}H_{10}O_5$	271.05	3.31	271, 243, 163, 153, 145, 121	9.30	12.38	6.39	5.11	4.72	7.96	8.39	2.96
Isovitexin 2"-O-beta-D-glucoside	$C_{27}H_{30}O_{15}$	595.16	4.83	-	3.07	20.04	7.46	10.24	-	6.62	15.03	33.35
3-O-Methylquercetin [S]	$C_{16}H_{12}O_7$	317.06	3.62	317, 302, 285, 245, 229, 153	133.86	376.23	120.10	47.44	74.92	142.46	136.24	36.26
3,7,4'-Tri-O-methylquercetin	$C_{18}H_{16}O_7$	345.09	7.10	-	-	10.08	3.10	5.97	-	4.14	6.26	18.17
Apiin	$C_{26}H_{28}O_{14}$	565.15	4.98	-	5.73	15.34	10.62	14.00	5.73	14.35	13.05	13.56
Biochanin A 7-O-beta-D-glucoside [S]	$C_{22}H_{22}O_{10}$	447.12	2.86	447, 285, 271, 261, 235, 193	6.93	7.72	7.39	5.91	4.92	5.39	6.83	3.29
Apigenin 7-(6"-malonylglucoside)	$C_{24}H_{22}O_{13}$	519.11	2.84	-	27.52	21.06	9.96	6.20	11.27	15.14	13.38	4.80
Luteolin 7-O-(6"-malonylglucoside)	$C_{24}H_{22}O_{14}$	535.42	2.85	-	87.86	79.29	69.18	37.73	27.55	46.66	54.80	104.01
Afrormosin [S]	$C_{17}H_{14}O_5$	299.09	5.74	285, 270, 132	-	4.16	2.00	7.16	2.75	2.62	3.46	22.40
Daidzein [S]	$C_{15}H_{10}O_4$	255.06	3.72	237, 227, 199, 137	4.45	10.45	10.07	8.46	2.89	6.40	13.96	7.80
(-)-Medicarpin [S]	$C_{16}H_{14}O_4$	271.09	4.65	254, 163, 147, 135, 122, 108	-	-	2.87	4.94	-	-	3.25	0.29
Formononetin 7-O-glucoside [S]	$C_{22}H_{22}O_9$	431.13	3.64	431, 269, 254, 237, 213	2.09	5.29	6.89	9.96	2.68	5.16	11.87	8.72
(-)-Maackiain [R-2]	$C_{16}H_{12}O_5$	285.07	7.3	239, 175, 151, 147, 138	-	-	-	-	-	-	-	0.25
Malonyldaidzin [R-3]	$C_{24}H_{22}O_{12}$	503.11	3.0	485, 405, 329, 255, 233	7.22	7.17	3.69	3.41	4.98	4.31	5.26	2.50
Malonylgenistin [R-4]	$C_{24}H_{22}O_{13}$	519.11	3.06	517, 315, 271, 269, 227	5.43	8.25	8.15	2.68	5.40	4.81	6.99	7.06
(-)-Maackiain-3-O-glucosyl-6"-O-Malonate [R-2]	$C_{25}H_{24}O_{13}$	533.12	3.75	471, 354, 287, 283, 269	18.58	21.66	9.60	10.38	15.76	19.63	22.60	6.66
Formononetin 7-O-glucoside-6" -O-malonate [R-4]	$C_{25}H_{24}O_{12}$	517.13	3.63	517, 269	-	11.33	7.74	8.99	-	8.87	15.70	3.84
Medicarpin 3-O-glucoside-6'-Malonate [R-5]	$C_{25}H_{26}O_{12}$	519.14	4.64	519, 431, 271, 269	5.77	9.52	20.24	14.28	6.47	9.96	25.40	16.96
Pseudobaptigenin [R-2]	$C_{16}H_{10}O_5$	283.06	5.36	255, 238, 237, 146, 137	27.12	29.52	12.84	10.93	15.99	16.43	15.72	4.35
Aurantio-obtusin beta-D-glucoside	$C_{23}H_{24}O_{12}$	493.12	3.85	-	29.74	51.29	18.35	15.75	4.96	22.61	23.28	8.10
Soyasaponin I [R-6]	$C_{48}H_{78}O_{18}$	943.52	7.63	797, 617, 599, 441	0.35	0.22	0.45	0.41	0.25	0.16	0.22	-
Soyasapogenol C	$C_{30}H_{48}O_2$	441.37	7.61	-	0.31	0.19	0.42	0.40	0.22	0.13	0.20	-
Soyasaponin III [R-6]	$C_{42}H_{68}O_{14}$	797.46	8.06	599, 411, 142	0.26	0.13	0.33	0.25	0.17	0.08	0.13	-
Dehydrosoyasaponin [R-6]	$C_{48}H_{76}O_{18}$	941.50	8.65	795, 615, 439	0.24	0.11	0.27	0.16	0.41	0.07	0.09	-

[a] RT: retention time [b] Fragmentation pattern matched through either reference (R) or standard (S) [1: Wojakowska *et al.,* (2013); 2: Arman (2011); 3: Wu *et al.,* (2008); 4: Farag *et al.,* (2007); 5: Lin *et al.,* (2000); 6: Baiocchi *et al.,* (2013)]

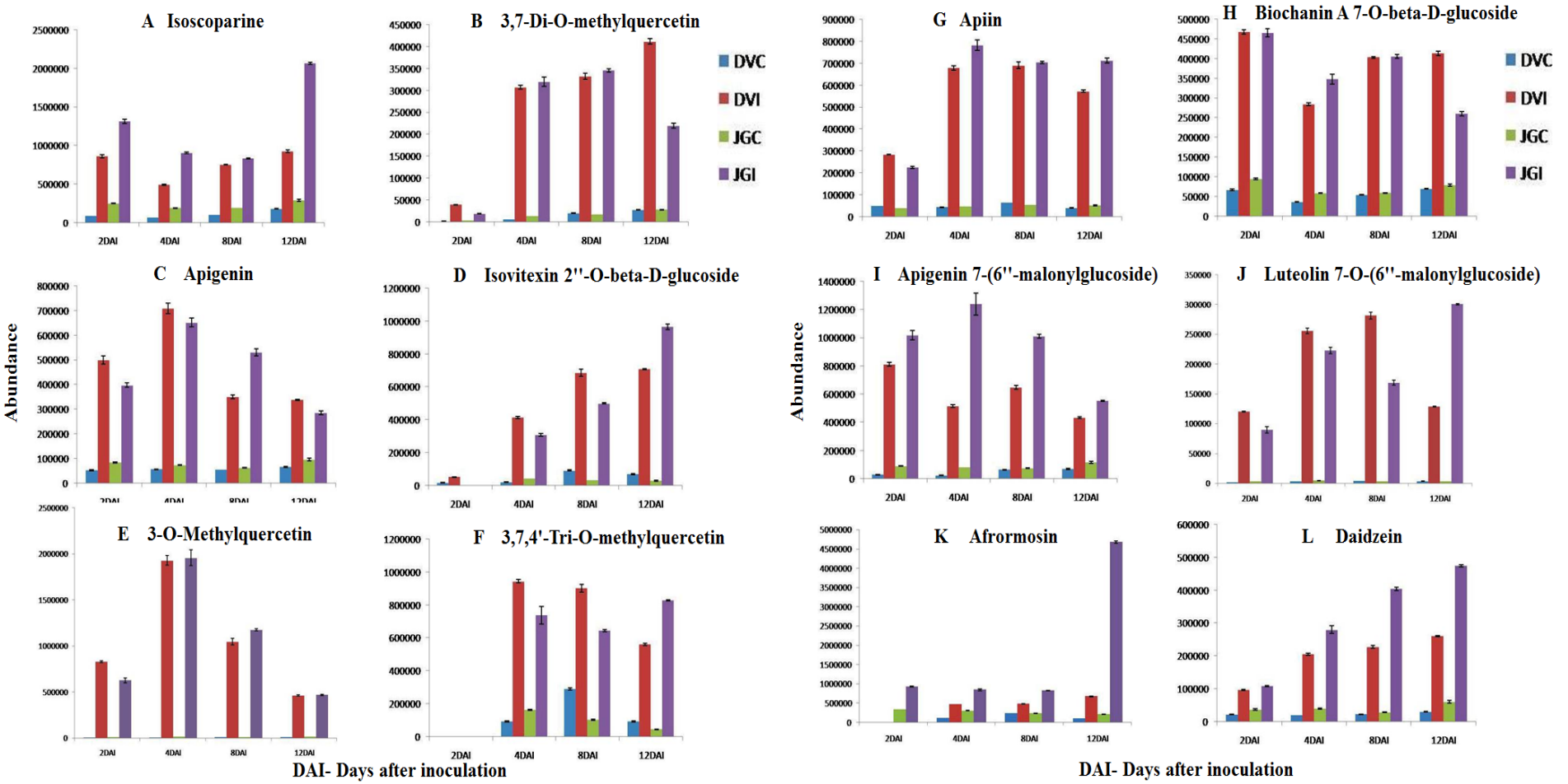

A Isoscoparine
B 3,7-Di-O-methylquercetin
G Apiin
H Biochanin A 7-O-beta-D-glucoside
C Apigenin
D Isovitexin 2''-O-beta-D-glucoside
I Apigenin 7-(6''-malonylglucoside)
J Luteolin 7-O-(6''-malonylglucoside)
E 3-O-Methylquercetin
F 3,7,4'-Tri-O-methylquercetin
K Afrormosin
L Daidzein
DVC
DVI
JGC
JGI
2DAI
4DAI
8DAI
12DAI
Abundance
DAI- Days after inoculation

Fig. 3.17

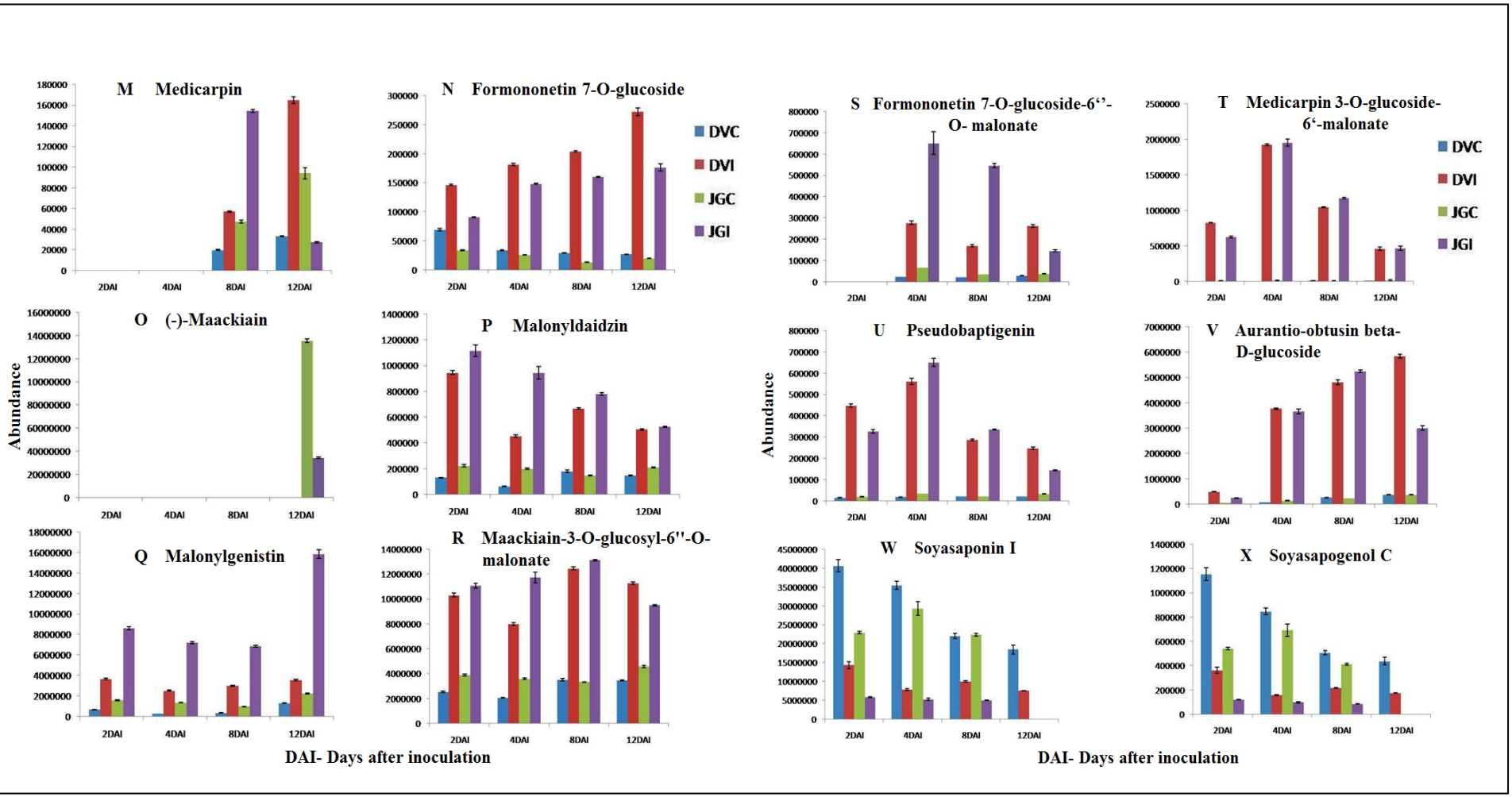

M Medicarpin
N Formononetin 7-O-glucoside
S Formononetin 7-O-glucoside-6''-O- malonate
T Medicarpin 3-O-glucoside-6'-malonate
DVC
DVI
JGC
JGI
O (-)-Maackiain
P Malonyldaidzin
U Pseudobaptigenin
V Aurantio-obtusin beta-D-glucoside
Q Malonylgenistin
R Maackiain-3-O-glucosyl-6''-O-malonate
W Soyasaponin I
X Soyasapogenol C
Abundance
2DAI
4DAI
8DAI
12DAI
DAI- Days after inoculation

Fig. 3.17

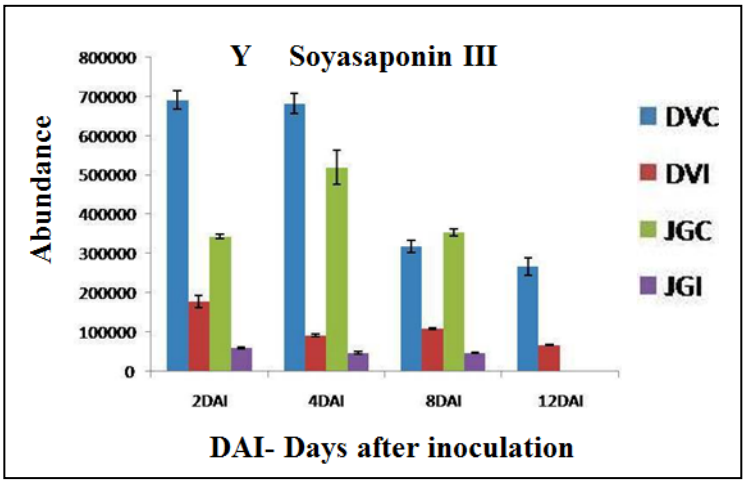


Fig. 3.17: Bar graphs of **s**ignificantly differentially accumulated metabolites identified in resistant-DV and susceptible -JG chickpea root in upon Foc inoculation at 2, 4, 8 and 12 DAI.

3.9 Comparative Expression of Candidate Genes in Root

To obtain complementary information of transcriptional variations induced by Foc inoculation in correlation to the quantitative proteomic and metabolic changes, we examined expression levels of key genes (Fig. 6, supplemental Table S1) involved in various metabolic pathways based on our above proteomic and metabolomics results in the resistant and susceptible chickpea plants. These included genes from nitrogen mobilization (glutamate dehydrogenase-GDH, glutamate synthase, glutamine synthase and asparagine synthase), stress response (NF-Y and SKP1-like protein 1A), methionine metabolism (methionine synthase and AdoMet synthetase), lignin and phytoalexin biosynthetic pathway (CCoAMT, CHS, CHI, isoflavone 4'-O-methyltransferase, IFS and IFR). *GDH* expression increased >200 fold in the later stage of infection in the susceptible inoculated plants. Similarly, glutamine synthetase, asparagine synthetase and glutamate synthase were significantly up-regulated up to 4-fold in the susceptible inoculated cultivar than the resistant inoculated cultivar (Fig. 6A-6D). Gene expression pattern of these enzymes showed increased expression in the Foc inoculated susceptible

cultivar as compared to that in the resistant inoculated cultivar. Further, expression of other important genes involved in lignin and phytoalexin biosynthetic pathway revealed increased expression in the resistant cultivar as compared to the susceptible one (Fig. 6I-6N). Similarly, candidate genes from methionine metabolism such as AdoMet synthetase had >3-fold increase while methionine synthase showed 10-fold higher expression in the resistant plant as compare to the susceptible one (Fig. 6E and 6F). Abiotic stress induced NF-Y gene showed >8-fold higher expression in the susceptible plant compared to the resistant inoculated cultivar (Fig. 6H). However, SKP1 gene had higher expression in resistant chickpea cultivar in comparison to the susceptible plant (Fig. 6G).

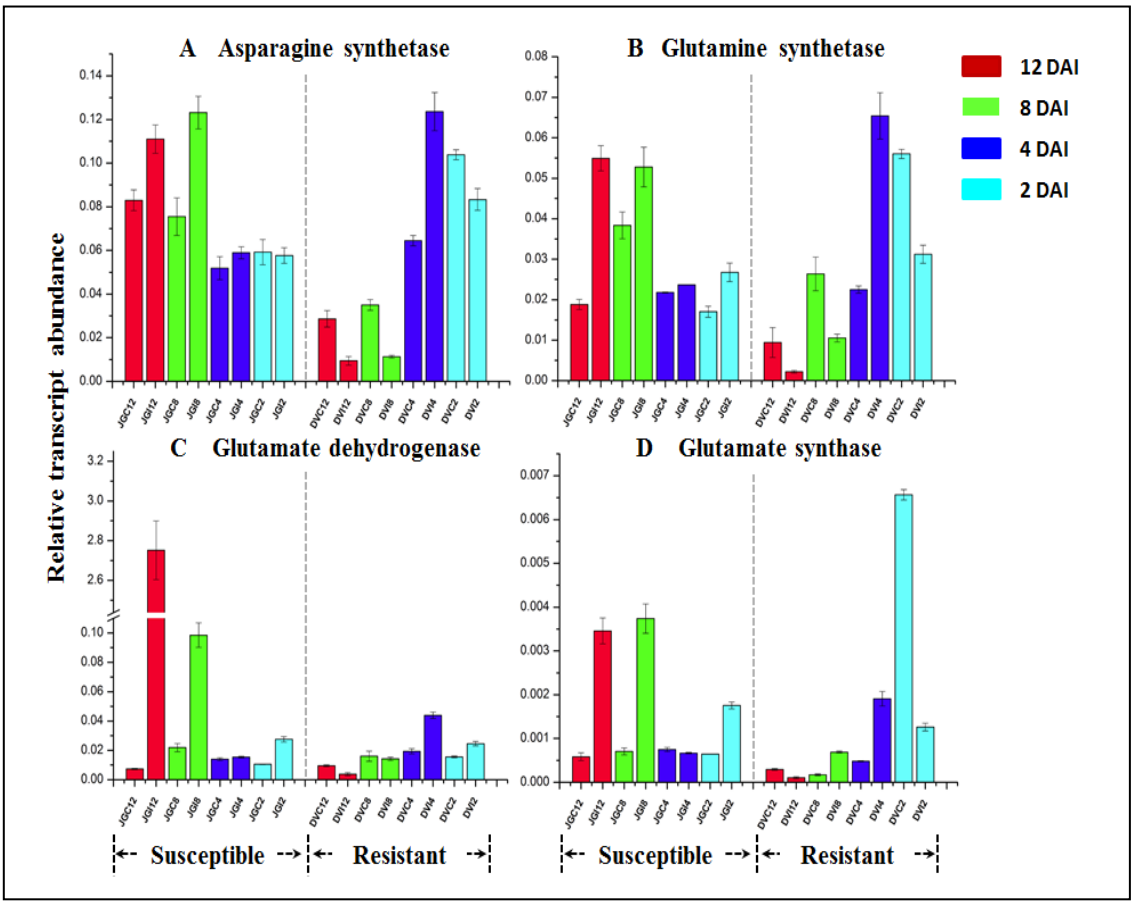


Fig. 3.18

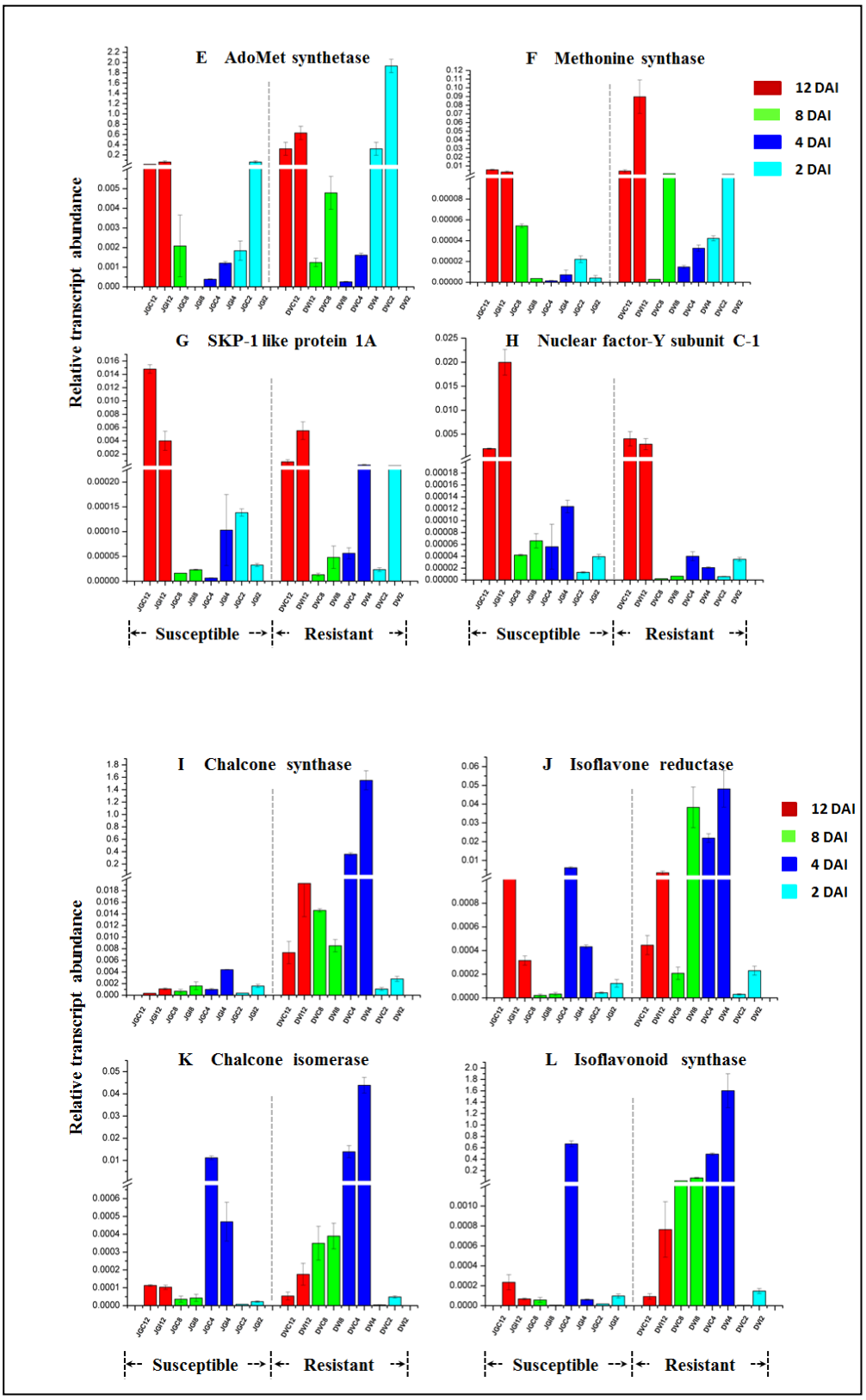

E AdoMet synthetase
F Methonine synthase
G SKP-1 like protein 1A
H Nuclear factor-Y subunit C-1
I Chalcone synthase
J Isoflavone reductase
K Chalcone isomerase
L Isoflavonoid synthase
12 DAI
8 DAI
4 DAI
2 DAI
Relative transcript abundance
Susceptible
Resistant

Fig. 3.18

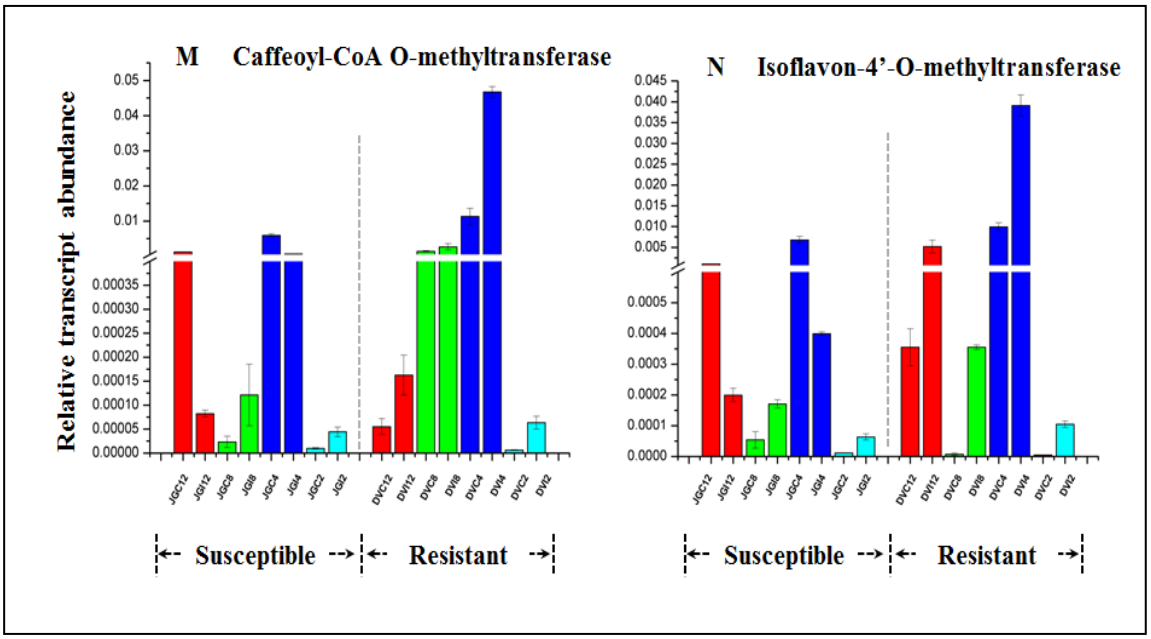


Fig. 3.18: Quantitative real-time PCR for the mRNA expression levels of candidate genes in root tissue of Foc inoculated resistant-DV and susceptible-JG62 chickpea cultivars compared to their respective controls at 2 to 12 DAI. *A*, asparagine synthetase; *B*, glutamine synthetase, *C*, glutamate dehydrogenase, *D*, glutamate synthase; *E*, AdoMet synthetase; *F*, methionine synthase; *G*, SKP-1 like protein 1A; *H*, Nuclear factor-Y subunit C-1; I, Chalcone synthase; *J*, Isoflavone reductase; *K*, Chalcone isomerase; L, Isoflavonoid synthase; *M*, Caffeoyl-CoA O-methyltransferase and *N*, Isoflavon-4'-O-methyltransferase.

3.10 Intense lignification is associated with Foc resistant phenotype

After inoculation, as the time progressed, differential lignin deposition in the root tissue of the resistant and the susceptible cultivars was observed; wherein the resistant cultivar exhibited intense lignification as compared to that in the susceptible cultivar (Fig. 3.19). The fungal pathogen invaded the susceptible cultivar and blocked the vascular tissue completely by 12 DAI, leading to wilting of JG62 plants.

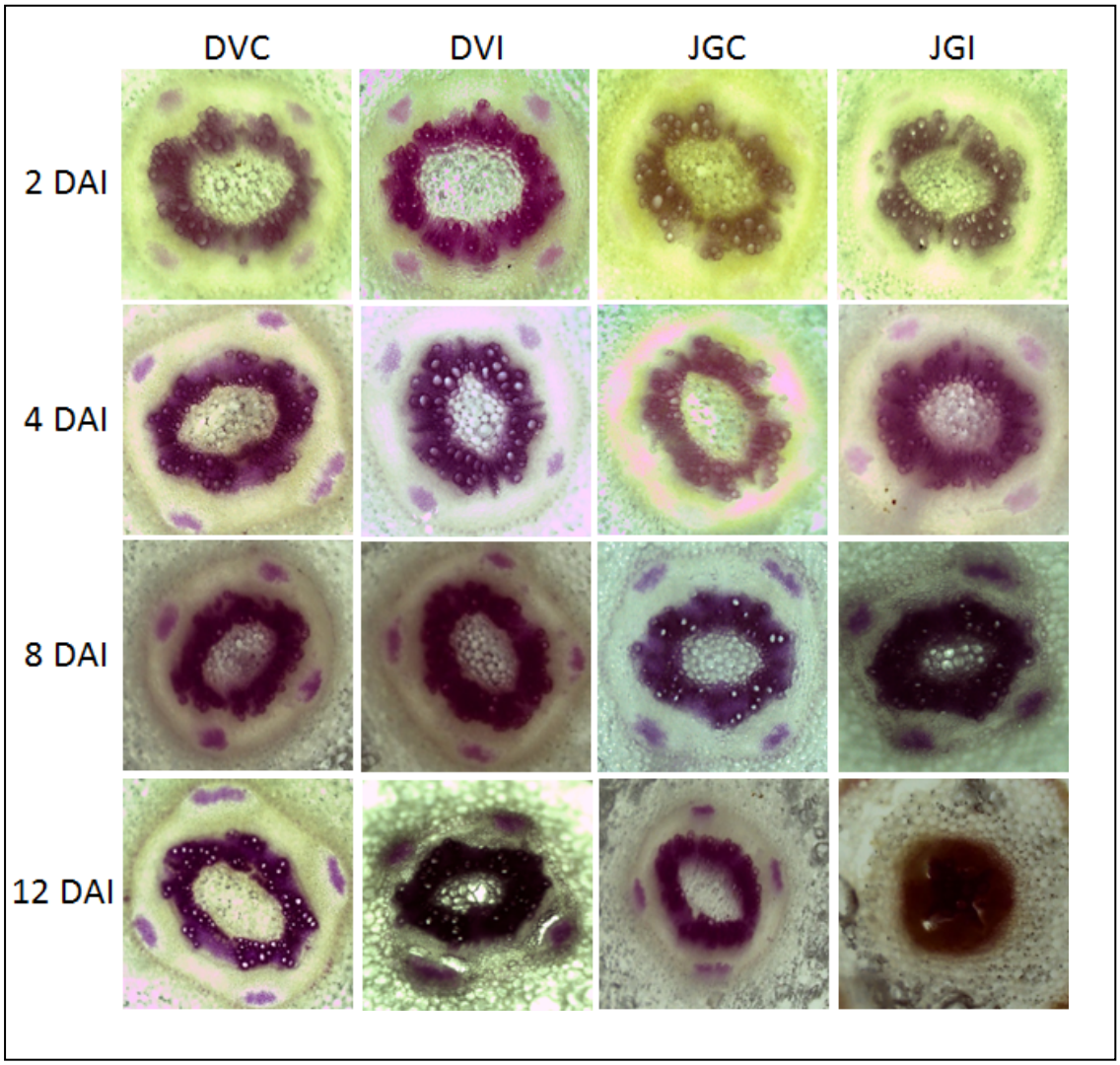
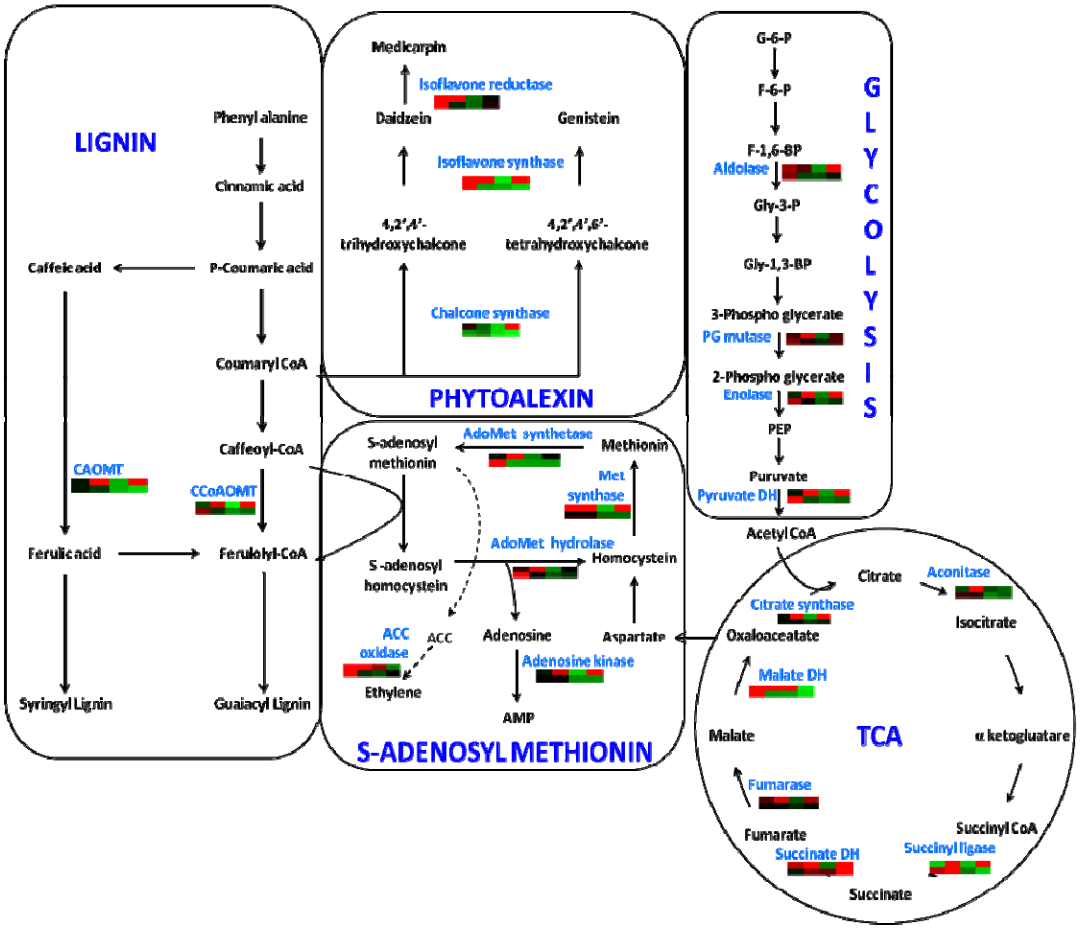


Fig 3.19: Pattern of lignification in cross sections of chickpea root tissue from resistant-DV and susceptible-JG at different Foc inoculation stages using phloroglucinol/ hydrochloric acid stain.

Chapter 4

Disscussion



In the present work an attempt has been made to highlight compatible and incompatible plant pathogen interactions using chickpea- *Fusarium oxysporum* f.sp. *ciceri* as the system. Chickpea being the most important legume in India and Fusarium wilt being a yearly threat to the crop, these studies are relevant to Indian agriculture. The inference drawn in our studies can be extrapolated or validated for other Foc races prevalent in rest of the world. For this, time series profiling of proteome and metabolome of Foc inoculated resistant and susceptible chickpea roots has been carried out using label free quantitative proteomics and untargeted metabolomics. Almost 95% of Foc inoculated susceptible JG62 chickpea plants showed yellowing symptom after 2DAI, followed by drooping of leaves and completed wilting sign after 12DAI. Whereas, Foc inoculated resistant, Digvijay and mock inoculated susceptible and resistant chickpea cultivar remained healthy throughout the experimental time points. Other signs of wilting such as retardation of root growth and root browning to blackening was also observed in Foc inoculated susceptible cultivar. This indicated that the changes in the infected plant samples were the consequences of pathogen attack. Thus, plant resistance/susceptibility to fungal pathogens is a highly orchestrate event involving various metabolic processes, significant modulation of such events was observed in chickpea roots. Particularly, the resistant plants could successfully inhibit Foc invasion and colonization inside the roots using various strategies that are discussed below vis-a-vis other plant-pathogen interactions reported so far.

4.1 Foc induced remodeling in energy metabolism and nitrogen mobilization

Obligate biotrophs depend on host metabolism for nutrient uptake, which in turn is known to determine their pathogenicity within the host. In the present study, proteins involved in glycolysis and TCA cycle were up-regulated in the root tissue of Foc resistant cultivar (DV) while down regulated in the susceptible roots of JG62 (Annexure 1). We also observed steep alteration in primary metabolites (amino acids and sugars) specifically in the susceptible cultivar JG62 (Table 3.2). Sugars affect disease susceptibility often favoring disease development while playing a critical role in innate defense pathways involving metabolic regulation (BolouriMoghaddam and Van den

Ende, 2012). In consistent with this, metabolomic results using both the approaches namely NMR and LC-MS showed rapid decrease in sugars such as sucrose and fructose in both the cultivars but more predominantly in the susceptible cultivar upon Foc infection as compared to the resistant one. Similar rapid reduction in the levels of these sugars was also observed in sunflower upon infection with *Botrytis cinerea* (Dulermo *et al*., 2009b). These results emphasized the regulatory role of sugars in the metabolic reprogramming, while higher expression of glycolysis and TCA cycle proteins endowed resistant plants to successfully combat the pathogen. Moreover, earlier supportive evidence derived from *F. oxysporum* infection induced up-regulation of various ESTs from sugar metabolism in chickpea (Ashraf *et al*., 2009; Gupta *et al*., 2010; Gupta *et al*., 2013).

Nitrogen plays an essential role in the nutrient relationship between plants and pathogens. It has been reported that changes in amino acid concentration are because of nitrogen mobilization after pathogen infection in plant (Tavernier *et al*., 2007; Dulermo *et al*., 2009a). Proteomics studies on wheat- *F. graminearum* identified up-regulation in the proteins from amino acid, carbon and nitrogen metabolism (Wang *et al*., 2005; Zhou *et al*., 2006). Moreover, such alteration was also confirmed with significant decreased amino acid content in tomato leaves and sunflower cotyledons after infection by *B. cinerea* and *Sclerotinia sclerotiorum*, respectively (Berger *et al*., 2004; Jobic *et al*., 2007). We also observed significant decrease in the concentration of various amino acids as a result of deleterious effect of Foc infection at the early stage, which suggested that the fungus probably utilized these amino acids for its establishment and proliferation inside the host. However, due to probable sporulation of Foc in the later stages (12 DAI) of infection in the susceptible cultivar, we observed increased levels of amino acids (Table 3.2). Hence, such role of nitrogen mobilization in chickpea-Foc interaction was further scrutinized by gene expression analysis of four representative enzymes from nitrogen mobilization *viz*. glutamate synthase, glutamate dehydrogenase, glutamine synthetase and asparagine synthetase. Significant up-regulation in these genes in the susceptible chickpea cultivar compared to the resistant plant upon Foc inoculation correlated well with our metabolomics results (Fig 3.18). Consistently, proteomic

analysis also revealed higher level of sucrose synthase and glutamine synthase following infection with Foc. Thus, proteomic, metabolomic and gene expression results together indicated that the process of nitrogen mobilization in the form of amino acid utilization by the fungus is critical in the establishment of Foc infection in the susceptible chickpea plants.

4.2 Foc induced stress responsive proteins in chickpea root

During stress, accumulation of misfolded or unfolded proteins increases in ER and results in triggering the UPR pathway to remove the malformed proteins by ubiquitin-proteasome pathway. Thus, UPR not only helps to avert the cytotoxic impact of malformed proteins, but also assists to relieve stress and reinstate normal functions in ER (Ye *et al*., 2011). In *Arabidopsis thaliana* roots, swelling of ER and vacuolar collapse resulting in ER stress and cell death were observed during fungal colonization (Qiang *et al*., 2012). Similarly, PDI expression level was higher in wheat plants upon stripe rust inoculation (Maytalman *et al*., 2013). Up-regulation of critical proteins such as Hsp70, BiP, calmodulin, SKP1 and PDI in pathogen resistant chickpea cultivar in the present study could suggest their coordinate response (Annexure 1). In consistent with proteomics data, increased expression of *SKP*1 gene in the resistant cultivar as compared to the susceptible plants in the present study also supported that during defense response, there was constant requirement for proteins stabilization in the process of folding, assembly, vesicle trafficking and secretion (Fig 3.18). Collectively, these outcomes point out the importance of an efficient utilization of UPR pathway in the resistant chickpea for plant defense against Foc.

Moreover during stress conditions, aquaporins such as PIP are involved in water transport in plant. Current investigation revealed significant increase in PIP-7a levels at both the stages in the resistant DV roots while drastic reduction was observed in the susceptible JG plant. This could have resulted in better water conductance in DV roots and helped the resistant plant against fungal attack. On the other side, impaired water transport might have led to the wilting symptoms in the susceptible plant infected with

Foc. Furthermore, nuclear factor Y (NF-Y) family and ABA-responsive protein have shown to be upregulated in Arabidopsis under water-limited conditions (Nelson *et al*., 2007). Similarly, we observed very high expression of NF-Y in proteomic as well as transcriptomic studies in the susceptible cultivar compared to the resistant plant (Fig 3.18). Above findings indicated differential response from DV and JG plants against Foc invasion and minimal water uptake due to clogged xylem after fungal invasion in the susceptible plants.

4.3 Early recognition of Foc leads to ROS generation and lignosuberization

Generation of ROS is one of the earliest cellular responses to pathogen recognition and/or infection. ROS mediates signaling pathways and plays a central role in defense mechanism against pathogens. The enzymes involved in ROS production such as peroxidase, DHAR, hydroxyacyl glutathione hydrolase, glutathione peroxidase, glutaredoxin, GST, quinone oxidoreductase and CuAO were significantly increased in the roots of resistant cultivar than the susceptible one in the current investigation (Annexure 1). Previously, it has been reported that CuAO and peroxidases functionally correlate in lignosuberization process (Scalet *et al*., 1991; Angelini *et al*., 1993) while the inhibitors of CuAO result in decreased defense response (Rea *et al*., 1998; Rea *et al*., 2002). More lignification in the resistant chickpea cultivar compared to the susceptible one upon Foc infection has been reported earlier (Raju *et al*., 2008). In the present study also, intense lignin deposition on the cortex of the roots of Foc inoculated resistant cultivar was observed (Fig. 3.19). Taken together, ROS generation and higher expression of CuAO suggested Foc triggered hydrogen peroxide generation and lignosuberization process leading to initiation of defense response in the resistant chickpea cultivar. Secondly, monolignol biosynthesis also plays critical role in host defense mechanism through lignification making cell wall more resistant to the pathogen penetration. Reduction in the monolignol biosynthesis in wheat through co-silencing of *CAOMT* and *CCoAMT* led to the higher penetration efficiency of a pathogen (Bhuiyan *et al*., 2009). We also found upregulation of these lignin biosynthetic enzymes at protein and transcript expression

level in the resistant cultivar than the susceptible chickpea plant, suggesting increased lignin deposition thereby providing resistance against Foc.

4.4 A critical role of methionine metabolism in Foc resistance in chickpea

In Yang cycle, methionine synthase converts homocysteine to methionine contributing in synthesis of Adomet by AdoMet synthetase. AdoMet can also lead to ethylene by 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase. Our proteomic and transcriptomic results consistently indicated higher levels of Adomet synthetase and methionine synthase in the roots of resistant plant compared to the susceptible cultivar upon Foc inoculation (Annexure 1). However, downregulation of ACC oxidase by 1.5-fold in roots of both the cultivars indicated that preferential AdoMet pool was not channelized towards ethylene production. A recent proteomic study has shown increased expression of AdoMet synthetase and lower level of ACC oxidase resulting in higher methionine recycling and lower ethylene biosynthesis in rice roots infected with *Herbaspirillum seropedicae* (Alberton *et al*., 2013). Silencing of AdoHcy hydrolase in transgenic tobacco plants confirmed its role in defense mechanism against pathogens (Masuta *et al*., 1995). Similarly, Kawalleck *et al*., (1992) identified mRNAs for AdoMet synthetase and AdoHcy hydrolase in parsley plant upon fungal infection. Altogether, current study indicated close association between pathogen defense and increased level of activated methyl groups during chickpea-Foc interplay.

Further, highly methylesterified pectin is required for normal plant cell wall while it is de-esterified by pectin methyl esterase (PME) which leads to increased vulnerability of plant cell wall to pathogen attack. Our proteomics data showed increased expression of PME at both the stages in the susceptible cultivar compared to the resistant one (Annexure 1). Earlier studies with either silencing of PME or overexpression of PME inhibitors in plants demonstrated negative role in the pathogen resistance (An *et al*., 2008; Ma *et al*., 2013). Likewise, plant sterols are structurally related to cholesterol and control mechanical property of cell membrane (Hodzic *et al*., 2008) and also serve as a

substrate for several metabolic pathways (Itkin *et al*., 2013). Interestingly, our metabolomic analysis based on NMR approach also revealed high cholesterol at later stages (8 and 12 DAI) in the roots of the inoculated resistant cultivar (Table 3.2). However, there was no significant change in the roots of susceptible cultivar except at 8 DAI. Similarly other sterols were also observed in susceptible inoculated during non-targeted LC-MS metabolomics study (Annexure 2). The stability of plant cell wall is also maintained by actin binding cytoskeleton protein such as Profilin, which showed higher expression in the resistant DV roots compared to the susceptible one at late stage. Overall, altered methionine metabolism affected methyl esterification of pectin in Foc infected susceptible roots, while stronger plant cell wall with normal pectin and cytoskeleton proteins helped the resistant chickpea with better defense against Foc.

Uridine and orotate (pyrimidinecarboxylic acid) are utilized for UDP-glucose formation. They serve as a precursor for the synthesis of cellulose and additional polysaccharides, glycoproteins and phospholipids; and act as a glucosyl donor (Lim *et al*., 2004). Decreased levels of uridine and orotate at later stage of infection in the susceptible chickpea cultivar while increased levels at both the stages in the resistant cultivar in our study indicated critical role of pyrimidine nucleotide metabolism for plant defense activity (Table 3.2).

4.5 Up-regulation of phenylepropenoid pathway in chickpea root against Foc

Plants respond to pathogen challenge by increased activation of phenylpropanoid pathway leading to flavanoids, isoflavonoids and phenolics biosynthesis. They play multiple roles in plant pathogen interaction including precursors for the defense related phytoalexins and signal molecules in response to pathogen infection. Based on the proteomic and transcriptomic results, enzymes involved in this pathway such as CHS, CHI, IFR and IFS were upregulated in the resistant DV cultivar as compared to the susceptible plant JG62 (Annexure 1 and Fig 3.18). In the transgenic soybean roots, RNAi silencing of *CHS* gene showed large decline in total isoflavonoids as well as reduced resistance to fungal pathogens (Subramanian *et al*., 2005; Lozovaya *et al*., 2006).

Additionally, Naoumkina *et al*. (2007) and Farag *et al*. (2008) showed that fungal extract induced higher levels of CHI, IFS and IFR leading to the production of phytoalexin in Medicago cell suspension culture to combat the infection. Moreover, earlier evidence suggested that isoflavone synthesis and accumulation in legumes is affected by pathogen elicitation (Dakora and Phillips 1996; Dixon and Sumner 2003). Massive accumulation of transcripts encoding isoflavonoids biosynthesis has been reported in yeast extract elicited *M. truncatula* cell suspension cultures (Naoumkina *et al.,* 2007). Moreover, in Leguminosae family, isoflavones are predominantly stored as 7-0-glucoside-6"-O-malonates (Koster *et al.,* 1983; Kessmann *et al.,* 1990). Further, these pterocarpan conjugates might represent the precursor pool for phytoalexin biosynthesis (Mackenbrock *et al.,* 1993). In the present study, dramatic changes were observed in phytoalexins and their conjugates in the chickpea roots after Foc infection. A representative figure of pathway leading to phytoalexin biosynthesis has been shown as Fig 4.1 including differential metabolite in the resistant and susceptible chickpea cultivars. Foc caused rapid accumulation in pterocarpan malonylglucosides at early stage in the resistant plants compared to the susceptible ones (Table 3.3 and Fig. 3.17). Malonylglucosides of maackiain, fomantin, apigenine daidzein, genistin, luteolin, quercetin and biochanin increased in susceptible cultivar as the disease progressed from 2 to 12 DAI. Kessmann and Barz (1986) also demonstrated that isoflavone moiety of these malonylglucosides might be consumed for phytoalexin biosynthesis. Concurrently in the present study, Foc inoculation led to the accumulation of malonylglucosides in both the resistant and the susceptible cultivars; however, the resistant plants had significantly higher accumulation than that in the susceptible cultivars (Table 3.3 and Fig. 3.17). Earlier studies showed that malonyl conjugates of isoflavone and pterocarpan were deposited in vacuoles, which were effluxed after fungal elicitation and subsequently accumulated as phytoelexin (Matern *et al.,* 1986; Mackenbrock *et al.,* 1992). Our result suggests that increased accumulation of isoflavone and pterocarpan conjugates in the resistant plants could act as precursor for synthesis of phytoalexin to combat the Foc infection. Additionally, medicarpin and maackiain, the known antifungal metabolites (Kessmann and Barz 1987, Kessmann *et al.,* 1988) were induced after Foc elicitation at later stages of infection. Our

data indicated that pronounced mobilization of malonylglucosides conjugates of medicarpin and maackiain into their unconjugated active form in the resistant plants enabled them to efficiently defend against Foc. We also observed that accumulation of isoflavonoid conjugates was regulated in response to Foc inoculation, which led into successful defense against the pathogen in the resistant plants.

The biosynthetic branch pathway leading to isoflavones (Fig. 4.1) was upregulated in the resistant plants as compared to the susceptible ones. Daidzein and genistein, which are the products of liquiritigenin and naringenin degradation, are involved in defense against pathogens in leguminous plants (Wegulo *et al.,* 2005). High accumulation of these two metabolites in the resistant plants compared to the susceptible plants in this study could potentially suggest that the resistant chickpea cultivar utilized them to wade off the Foc infection. Moreover, the metabolites from flavon and flavonol biosynthesis pathways such as apigenine and isovitexin 2"-O-β-D-glucoside were also abundant in the resistant cultivar compared to the susceptible one in the present study. These compounds have been reported in leguminous plants' defense against pathogen attack (Marinova *et al.,* 2007). Recently, Wang *et al.,* (2012) showed that 3-O-methylquercetin and 3,3'-di-O-methylquercetin from *H. halodendron* act as antimicrobial compounds. We observed increased accumulation of 3-O-methylquercetin and 3,7,4'-tri-O-methylquercetin in the resistant cultivar, suggesting that these quercetin derivatives might act as defense compounds in response to Foc. Together, these results suggested that the metabolites from flavon and flavonol biosynthesis pathway cumulatively strengthen the defense mechanism against Foc in the resistant cultivar. Consistently, the accumulation of phytoalexins and their precursor compounds identified by metabolome analysis using NMR and UHPLC-Orbitrap in the present study correlated well with the proteomic and transcriptomic analyses. Thus, the accumulation of isoflavonoid biosynthetic proteins and metabolites in Foc inoculated resistant chickpea cultivar suggested their potential involvement in Foc resistance.

4.6 Modulation of defense related proteins and metabolites in chickpea root upon Foc inoculation

In the present study we observed quantitative variation in many defense related proteins such as endo β-1,3-glucanase, MLP, hev b5 and Bet v1, β-gulcosidase, DRR-206, DRR-49, chitinases, SBP, PR10, STH-2, PR4a, PR-5b, 14-3-3 and H^+-ATPase in the resistant and the susceptible chickpea cultivars upon Foc inoculation (Annexure 1). Many previous studies in other plant pathogen interactions have also demonstrated the importance of these proteins in plant defense. Lytle *et al*. (2009) and Gurjar *et al*. (2012) have reported that Bet v1 protein is responsible for resistance towards pathogen infection. Similarly, β-1,3-glucans are involved in plant defense response against pathogen (Ward *et al*., 1991; Shetty *et al*., 2009). Previously two independent studies have shown that β-glucosidase, DRR-206 and DRR-49 proteins contribute in lignification process (Hosel *et al*., 1975; Burlat *et al*., 2001). Increased chitinase activity was reported earlier after Foc inoculation in the resistant chickpea cultivar (Giri *et al*., 1998). Similarly, overexpressed SBP in rice provided more resistance against rice blast fungus (Sawada *et al*., 2004). Another important defense related protein, 14-3-3 has known to be associated with hypersensitive cell death in pathogen incompatible cultivars (Roberts 2003). In our earlier study up-regulation of 14-3-3 transcripts was detected in Foc inoculated resistant chickpea roots (Nimbalkar *et al*., 2006). Thus, up-regulation of 14-3-3 and H^+-ATPase suggested their roles in activating hypersensitivity response during fungal infection in chickpea leading to resistance. Apart from the above-mentioned proteins, metabolite analysis in the present study showed antifungal compounds associated with plant defense such as Clotrimazole and 4-Nitrophenol increased in Foc inoculated resistant cultivar at late stage whereas decreased in the susceptible one (Table 3.2). Furthermore, induced anthraquinones synthesis was reported after invasion of microorganisms in plant cells. The polysaccharides (chitosan, chitin and pectin), involved in interaction between plant and microorganism, were most effective in inducing anthraquinones synthesis (Dornenburg *et al.,* 1994). Kim *et al.,* (2004) reported that anthraquinone isolated from leguminous plant's (*Cassia tora*) seed showed antifungal property against phytopathogenic fungi. We

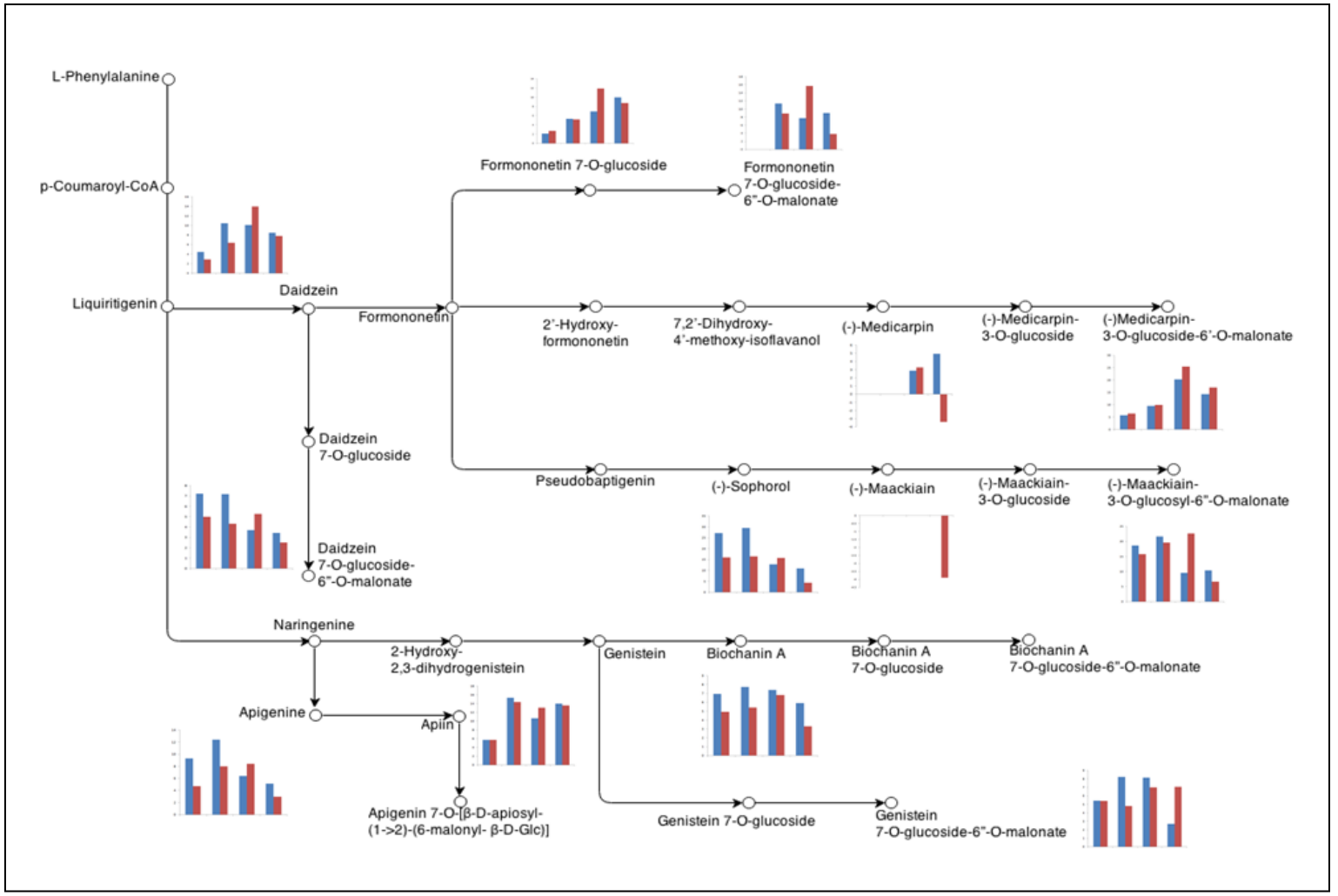


Fig. 4.1: Biosynthetic pathway of flavonoids and isoflavonoids with fold changes in metabolites that were identified in resistant DV (blue) and susceptible JG62 (light red) chickpea plants after Foc1 inoculation.

found increased accumulation of anthraquinones, aurantio-obtusin β-D-glucoside in the resistant chickpea cultivar compared to the susceptible plants in untargeted metabolomics study, especially at early stage of 2 and 4 DAI; and this might indicate that chitin from Foc induced rapid accumulation of anthraquinones in the resistant plants to overcome the fungal attack (Table 3.3). Overall, outcome suggested that various metabolites from biosynthesis pathways of phytoalexin, triterpenoid, flavon, flavonol and anthraquinones cumulatively strengthen the defense mechanism in roots of the resistant cultivar against Foc. Thus, the pathogen attack triggered expression of defense-related genes, secondary metabolites with antimicrobial nature and PR proteins in the chickpea-Foc interactions.

4.7 Role of glycosylated metabolites in Foc control

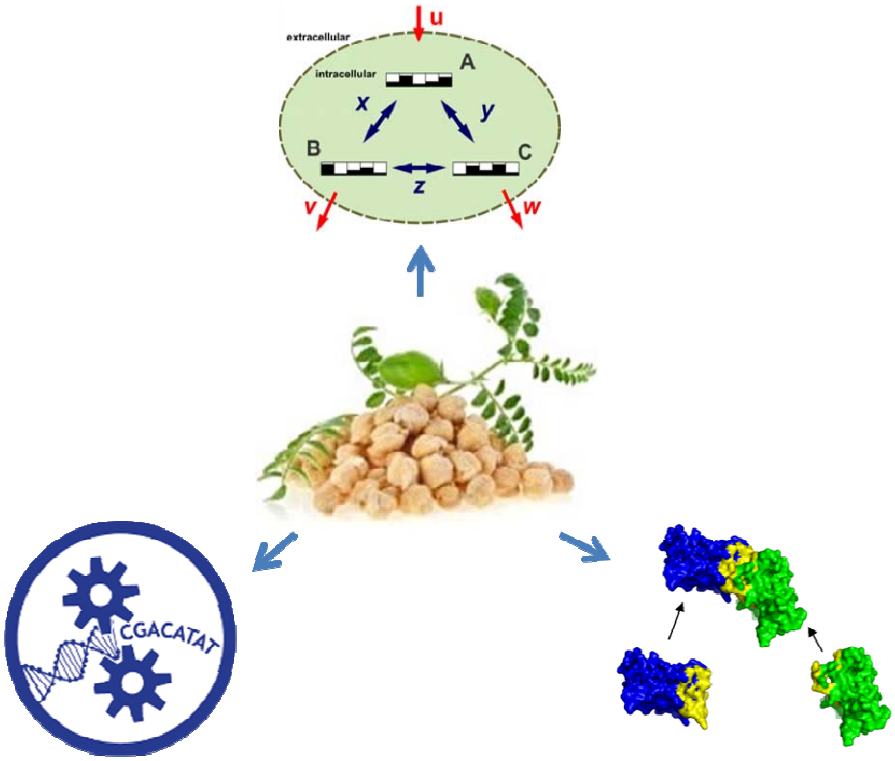
Saponins are glycosylated plant secondary metabolites and might assist as chemical barrier to fungal attack. However, some fungal pathogens can enzymatically detoxify the host plant's saponins (Bouarab *et al.,* 2002). Our analysis showed decreased accumulation of soyasaponin I, soyasaponin III, dehydrosoyasaponin I, soyasapogenol C, chikusetsusaponin V and soyasapogenol B 3-O-D-glucuronide in the Foc inoculated resistant and susceptible plants in comparison to their respective controls with progression of the disease (Table 3.3 and Fig. 3.17). Moreover, Kerem *et al.,* (2005) observed that saponins isolated from chickpea had the least fungicidal response against Foc as compared to the other tested fungi. This could suggest that, increased Foc load at later stage in the susceptible plants was probably responsible for degradation of these saponins to counteract the defense machinery of plants. Surprisingly, we did not observe significant change between the control and the infected samples at 12 DAI of the susceptible cultivar. This result might also suggest that Foc hydrolyzed the antifungal saponins and suppressed the host defense response; however, further studies would be required to confirm this result.

4.8 Conclusion

The present investigation successfully uncovered concurrent Foc1 induced proteomics and metabolic variations in the roots of the resistant and the susceptible chickpea cultivars at different stages of disease progression. The multivariate statistical analysis approach could successfully detect potential significance of the subtle metabolic differences between experimental groups of the resistant and the susceptible plants. These proteins and metabolites were primarily involved in, energy metabolism, unfolded protein response, lignin and phytoalexin biosynthesis, fungal chitin induced compounds and altered methionine biosynthesis pathway. Current investigation is the first report to show comprehensive alterations in the protein and metabolites after Foc inoculation. Targeted gene expression study was also in concurrence with the large-scale proteomics and metabolomics findings. Thus, this study successfully uncovers the mechanistic basis of wilt resistance in non-model plant like chickpea.

Chapter 5

Summary and future directions



Fusarium wilt of chickpea caused by *Fusarium oxysporum* f. sp. *Ciceri* (Foc) is the most destructive diseases of chickpea throughout the world and has emerged as disease of global significance. 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. “Omics” have been well utilized in elucidating host resistance mechanisms against diseases of *Fusarium* such as Fusarium head blight (Kushalappa and Gunnaiah, 2013). The present thesis work was planned to identify factors that govern wilt resistance in chickpea, by non-targeted profiling of proteins and metabolites using high resolution mass spectrometer. Further, validation of important candidate genes was performed by transcript expression analysis for identified proteins and metabolites from above study. Proteomic and metabolomic changes in the host plant due to pathogen attack can be traced back to their molecular level of defense mechanism and developing resistant genotype will be the most effective, viable and environmentally friendly approach for management of Fusarium wilt. Characterization of proteins and metabolites after Foc inoculation will help in understanding the host pathogen interaction and the defense responses in chickpea.

5.1 Proteomic profiling of chickpea roots against Foc infection

The dynamics in root metabolism was studied during compatible and incompatible interactions between chickpea and Foc using quantitative label free proteomics. The proteomics analysis identified a total of 811 proteins from the chickpea root. Of the 811 identified proteins, 481 showed statistically significant differential accumulation in resistant and susceptible cultivars at every time point with $P< 0.05$ and fold change >1.2. Massive reprogramming of metabolism and in particular plant defense response molecules was observed in the resistant chickpea plants. This study revealed a major shift in central metabolic processes which might be due to increased demand of energy and phenylepropenoid biosynthesis pathways in the resistant plants to combat the pathogen attack. These results also demonstrated the involvement of methionine metabolism with changes in transmethylation during chickpea-Foc interaction. Further, elevated levels of some of the proteins from the UPR pathway during incompatible reaction in the resistant

roots indicated that proper protein folding and transport might be crucial in the plant's survival after Foc invasion. Overall, the present study revealed complex molecular interplay in chickpea-Foc interactions and critical immediate response to defy Foc infection.

Proteins from isoflavanoid and flavanoid pathway were found more in the resistant cultivar than the susceptible suggested that resistant plant synthesize phytolexins efficiently to combat Foc infection. We also observed that ROS generation and higher accumulation of copper amine oxidase aid in lignification process that restrict pathogen to enter inside the xylem in resistant cultivar. Our data also showed increased expression of proteins from lignin biosynthetic pathway. Thus this study suggested that the Foc infection triggered hydrogen peroxide generation, lignosuberization process and initiation of defense response in the resistant cultivar of chickpea DV. Methionine metabolism play crucial role in methyl transfer. Many know cellular reactions that are involved in defense mechanism are dependent on them. We observed many proteins upregulated including methionine synthase, adenosine homocystenin hydrolase, adenosine kinase and AdoMet synthetase showed higher accumulation in the Foc inoculated resistant cultivar at both, early and late stages, whereas these proteins were significantly down regulated at an early stage with increase in late stages in susceptible cultivar. Taken together, these results indicated the close association between pathogen defense and increased level of activated methyl groups. In the present study, several UPR proteins including Hsp70, calnexin, luminal binding protein (BiP), calmodulin, translocon associated proteins (SEC61), component of SCF-for SKP1-Cullin-F box protein, ubiquitin ligase complex, 26s proteosome, calthrin and protein disulfide-isomerase (PDI) were identified in chickpea roots of both the cultivars upon Foc inoculation. The majority of these proteins were high in early, but low in late stage in the resistant cultivar while susceptible cultivar showed opposite accumulation pattern. Induction of these proteins is the fundamental adaptive cellular responses to ER stress in plants. Data suggested that resistant plant efficiently check quality control (QC) of secretory protein where susceptible plant failed to do so. Up-regulation of 14-3-3 and H^+-ATPase suggested their role in activating hypersensitivity response during fungal infection in chickpea leading to resistance which

has been earlier reported by Nimbalkar *et al.,* (2006). Overall quantitative proteomics data suggested that there is orchestrate events that determine the defense response in chickpea against Foc.

5.2 NMR based metabolomics profiling of Foc inoculated chickpea root

^{1}H-NMR was acquired from resistant and susceptible chickpea plants after Foc infections in this time series experiment to unrevealed the plant pathogen interaction. Total 52 metabolites were identified that included a range of amino acids, sugars, some organic acids, nucleosides, phytoalexins (genistein and luteolin), clotrimazole, cholesterol and lipids. The PCA trajectory obtained from data showed that controls of resistant and susceptible cultivars show similar trajectory however distinct change in trajectory of susceptible plant observed after eight of *Fusarium oxysporum* inoculation. This suggests that resistant plant has distinct defense mechanism from susceptible plant. The metabolic changes induced due to Foc inoculation were further evaluated by constructing OPLS-DA models. OPLS-DA is supervised data modelling techniques that has been successfully utilized for biomarker discovery.

Comparison of control and inoculated samples by OPLS-DA model showed change in concentration of various amino acids was observed as a result of deleterious effect of Foc infection during the infection progression. Significantly decreased in various amino acids in Foc-inoculated susceptible cultivars in the early stage (till 8 DAI), which suggested that Foc probably utilized these amino acids for its establishment and proliferation inside the host. Interestingly, ^{1}H NMR metabolomic data showed rapid decrease in sugars such as sucrose and fructose in both but more predominantly in susceptible cultivars upon Foc infection as compared to the resistant cultivar. These results emphasized the regulatory role of sugars in the metabolic reprogramming. The accumulation of phytoalexins and phenolics was also observed in metabolome analysis which is already reported for their function in defense response. Thus in our study, accumulation of isoflavonoid metabolites in Foc inoculated resistant chickpea cultivar suggested their potential involvement in Foc resistance.

5.3 LC-Orbitrap based metabolic profiling of Foc inoculated chickpea root

NMR based metabolomics has limitation in sensitivity and specificity of metabolites. Therefore, more comprehensive analysis of metabolome was performed in chickpea cultivars infected with Foc. This approach has enabled us to identify metabolic network associated during the disease development and understand the Foc induced mechanism for resistance or susceptibility in chickpea. PCA model was constructed for LC-MS metabolomics data from the control and Foc1 inoculated DV and JG62 plants at all the stages to ascertain the time course of metabolic variations that were induced in resistant and susceptible plant after Foc1 inoculation. Clear separation was observed between control and inoculated plants at all the stages. PCA trajectory plot also suggested that over all metabolic change between controls of both the cultivar were similar. However, Foc inoculation caused significant change in trajectory between resistant and susceptible plants. The OPLS-DA models showed the discriminating metabolites in chickpea root tissue associated with Foc inoculation such as flavanoids, isoflavanoids, alkaloids, amino acids and sugars. Foc inoculated resistant plants were found to accumulate more flavanoids and isoflavanoids along with their malonyle conjugates. Many fungicides that induce after fungal infection for example, obstine beta glucosides and querecitin, were observed elevated in resistant cultivar of chickpea. Overall outcome suggested that biochemical mechanisms responsible for defense were different in Foc resistant cultivar from the susceptible cultivars.

5.4 Targeted gene expression analysis

Expression levels of key genes involved in various metabolic pathways showed very good concurrence with proteomic and metabolomic results. These included genes from nitrogen mobilization (glutamate dehydrogenase, glutamate synthase, glutamine synthase and asparagine synthase), stress response (NF-Y and SKP1-like protein 1A), methionine metabolism (methionine synthase and AdoMet synthetase) and lignin and phytoalexin biosynthetic pathway (CCoAMT, CHS, CHI, isoflavone 4'-O-methyltransferase, IFS and

IFR). Quantitative real time expression of these genes in resistant and susceptible inoculated as well as in their control suggested that these have potential role in defense against Foc.

5.5 Future directions

Major aim of this study was to understand chickpea-*Fusarium* interaction using high throughput quantitative proteomics and metabolomics approaches. System wide utilization of this high-throughput technology has uncovered the complex event of chickpea-*Fusarium* interaction. However, few leads need to be followed further. The future directions are as follows:

- RNA-Seq study of chickpea-*Fusarium* interaction to understand the transcriptional response
- Resistance/susceptibility has to be validated by either gain or loss of function studies of key proteins/ metabolites/gene identified from this study.
- Protein-protein interaction study after chickpea-*Fusarium* interaction to understand the functional module involved in pathogenicity.
- Genome scale metabolic pathway reconstruction of chickpea-*Fusarium* interaction.

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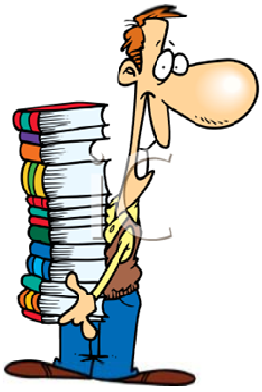

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Curriculum vitae

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- **Department of Biotechnology (Govt. of India) fellowship** to pursue Master's in Biotechnology
- **Awarded best oral presentation on "Proteomic analysis of plant pathogen interactions using High throughput mass spectrometry",** at National symposium on "Molecular approaches for management of fungal diseases of crop plant" held at IIHR, Bangalore, 23-30 Dec. 2010
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- **Yashwant Kumar**, Bhushan B. Dholakia, Priyabrata Panigrahi, Shrikant M. Kunjir, Pattuparambil R. Rajmohanan, Narendra Y. Kadoo, Ashok P. Giri, Vidya S. Gupta. Label free differential quantitative proteomic of Fusarium oxysporum inoculated resistant and susceptible chickpea cultivars. Poster presentation during National science day at CSIR-National Chemical Laboratory, Pune on Feb. 28, 2011.
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