Chapter 4

Discussion

[Diagram of metabolic pathways involving lignin, phytoalexin, and glycolysis.]
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 SKP1 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 (Scalalet 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 flavonoids, 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-O-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 phyтоalexin (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 apigenin 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.