DISCUSSION
4. Discussion

4.1 Identification of Foc races

Reliable identification and genetic characterization of pathogens is necessary for appropriate management of plant diseases. Studying the variability of Foc pathogen from various agro-climatic zones is important in disease resistance breeding for the selection of chickpea cultivars resistant against specific races or for pyramiding resistant genes against multiple races for sustainable resistance. In this study, three DNA based marker system approaches were used for identification of the Indian pathogenic races of Foc namely, gene specific oligonucleotides (GSOs), ITS-RFLP and AFLP as well as proteomics approach.

4.1.1 DNA based approaches for Foc race identification

GSO approach

In the first approach gene specific oligonucleotides (GSOs) were used to detect PCR-polymorphism in the four standard Foc races. The eight pathogenic races of Foc all over the world differ from each other on the basis of their pathogenicity towards differential chickpea cultivars (Jiménez-Gasco et al., 2001). Thus, studying the virulence factors in Foc can conceptually aid the detection of the differences in the races. We, therefore, designed GSOs in the first approach, from the conserved regions of reported virulence related and metabolically essential genes of different plant pathogenic fungi like Magnaporthe, Colletotrichum, and Fusarium (Table 2.2). The GSOs were degenerate enough to amplify Foc genomic DNA although most of these genes were not reported from Foc. All the four standard Foc races prevalent in India were used for the analysis.

GSO approach: genes involved in carbon and nitrogen stress

Once the fungus is in contact with its host plant, it often initially faces nutrient starvation conditions, till it invades the host plant and uses its metabolic sources for growth and survival. Carbon stress in fungi leads to utilization of other sources of carbon for energy when glucose is not readily available. Under such situations acetate molecules can serve as energy rich and carbohydrate building reserve. The enzyme isocitrate lyase (ICL) is a key enzyme of glyoxylate cycle involved in conversion of acetate to other carbohydrates which can then be used for fungal growth and development in the host plant. Similarly, under glucose scarcity, sucrose is converted to
glucose by sucrose non-fermenting (SNF1) enzyme. The SNF1 gene has been shown to play a central role in carbon catabolite repression in *Saccaromyces cerevisiae* and is required for invasive growth during glucose starvation (Palecek *et al.*, 2002). In the absence of glucose, SNF1 protein kinase causes derepression of *SUC2* gene (encoding secreted invertase) that hydrolyzes sucrose to glucose and fructose. Trehalose is another disaccharide molecule widely existing in bacteria, plants, insects as well as fungi and is implicated in cellular responses to numerous environmental stresses such as heat-shock, starvation, hyperosmotic shock and dessication. Trehalose and its precursor- trehalose 6 phosphate (T6P) regulate fungal growth and development as well. T6P is known to inhibit hexokinase activity and therefore, acts as a means of regulating the entry of glucose into glycolysis. The enzyme responsible for T6P synthesis, that is trehalose 6 phosphate synthase (TPS1) has been shown to be essential for establishment of rice blast disease caused by the fungi *M. grisea* (Foster *et al.*, 2003). Also, TPS1 is a link between the cross talk occurring in carbon and nitrogen metabolism in fungi, for it acts as a derepressor via NMR1 and then *mut1* genes involved in nitrate utilization. It thus integrates carbon and nitrogen metabolism through glycerol 6 phosphate (G6P) sensing, resulting in increased NADPH production and induction of gene expression associated with nitrate utilization (Wilson *et al.*, 2007). Fungi, otherwise, are able to utilize a wide range of nitrogen containing compounds. But when preferred nitrogen sources like glutamine and ammonia are lacking (during stress, starvation and/or pathogenesis), other nitrogen containing sources are used. Global N2 regulatory genes like area/Nit2 and their orthologue *CLNR1* in *Colletorichum* enable uptake and catabolism of such secondary N2 sources. Other enzymes involved in fungal metabolism involve desaturases (Dst) which are not directly related to fungal virulence but are involved in cellular growth and differentiation. Fig. 4.1 depicts the genes involved during fungal stress and starvation conditions.

**GSO approach: cell wall degrading enzymes**
Fungi are known to produce an array of extracellular wall-degrading enzymes enabling invasion of host plant tissue. Xylan is the major hemicellulosic component of the plant cell wall and the most abundant renewable hemicellulose. The degradation of this complex polysaccharide requires the synergistic action of several hydrolytic enzymes for efficient and complete hydrolysis (Collins *et al.*, 2005), of which endo-β-1,4-xylanase is a crucial component that carries out the initial breakdown of the xylan backbone producing xylo-oligomers of different lengths.
Apart from their role in the degradation of xylan, family 11 fungal endo-β-1,4-xylanases are well-known proteinaceous elicitors of defense response reactions in plants in a way that is independent of its enzymatic activity (Enkerli et al., 1999). Endo-β-1,4-xylanase is clearly involved in the degradation of plant cell walls, which, therefore suggests that it may play an important role in pathogenesis (Walton, 1994). In many of the phytopathogenic fungi, like *Colletotrichum carbonum*, the extracellular enzyme activities are often subjected to catabolite repression (Van Hoof et al., 1991; Ransom and Walton, 1997). The SNF1 protein kinase has been predicted to release the catabolite repression, at least in yeast (Vincent and Carlson, 1998).

The plant cuticle forms a hydrophobic coating that covers nearly all above-ground parts of terrestrial plants and constitutes the interface between the plant and the environment. The main structural component of the plant cuticle is cutin, which is hydrolyzed by cutinase enzyme. Knockout of the cutinase gene cut1 in *F. solani* f. sp. *pisi* resulted in decreased virulence on pea (Rogers et al., 1994; Li et al., 2002). Fig. 4.2 shows involvement of cell wall degrading enzymes during fungal virulence.
GSO approach: signaling pathway enzymes

Chitin binding proteins (CBP) are lectin like extracellular proteins localized in the fungal cell wall probably involved in sensing of factors from solid surfaces that induce appressorium differentiation and signal triggering leading to transduction of such signals through complex pathways. Signaling cascades are known to process environmental cues and govern fungal virulence. Signal transduction cascades are also involved in regulation of filamentous growth and differentiation. A conserved MAP kinase pathway required for filamentous growth involves \textit{Fmk1 kinase} and further \textit{Mst12} like transcription factors which govern the formation of appressorium like specialized infection structures. This MAP kinase cascade is activated by $\beta$ and $\gamma$ subunits of the pheromone activated G proteins and is specialized to regulate fungal invasive growth. The second signal transduction pathway is the nutrient sensing cAMP pathway, which functions in a parallel way to MAP kinase pathway to regulate hyphal differentiation. It also involves G proteins for its activation, adenyl cyclase, cAMP and cAMP dependent protein kinase; which play a specialized role in filamentous growth. Gene \textit{clk1} encodes a putative serine/threonine protein kinase which is a part of signal transduction pathway involved in \textit{C. lindemuthianum} infection process. \textit{Clk1} involving cAMP signal transduction pathway is important in the development of \textit{Colletotrichum} on the plant surface, i.e., germination of conidia and appressorium formation. Fig. 4.3 shows the fungal genes essential in signaling cascades.
GSO approach: other genes
There are genes which play an indirect role in fungal pathogenesis. Tolerance to phytoalexins may be a key characteristic for fungal virulence. Enzymatic detoxification of phytoalexins is apparently an important mechanism contributing to tolerance, for example Kievitone hydratase (KVH) is an enzyme involved in such mechanisms (Turbek et al., 1990). Similarly, pisatin demethylase of Nectria haematococca is also known for inactivation of Pisatin, a phytoalexin produced in pea (Funnell et al., 2002). Other proteins like transposases are also suggested to be involved in fungal virulence. Transcription of fungal transposons is known to occur during carbon or nitrogen starvation condition; which mimics the pathogenesis conditions in fungi (Rep et al., 2005). Hop78 transposon was, therefore included in the study.

Primers designed for all the above mentioned genes, responsible for diverse functions were used (Fig. 4.4); to analyze race specific variation and fungal virulence. These GSOs were used for the amplification of genomic DNA of Foc races 1, 2, 3 and 4 in order to detect polymorphism, which could help in developing race specific markers. Foc race 3 was clearly identified using Hop78-2, Cut and Dst and race 4 using Xyl GSOs, respectively.
The GSO approach is a nascent technique and has been used previously only for identification of Cochliobolus carbonum race 1 using oligonucleotides directed towards Tox2 locus (Jones and Dunkle, 1993). These studies clearly demonstrate the potential of GSOs to discriminate between isolates which are genetically very similar and morphologically indistinguishable. The results are either indicative of minor differences in the primer binding site of the Foc 3 xylanase gene; absence of the xylanase gene or a particular isoform of xylanase gene in Foc 3, leading to the differences in the amplification profile. Since, the gene specific primers were designed for genes reported from other fungal genera apart from Fusarium; only a few of the primers worked well. This could be because of the degeneracy introduced in the primer sequence. Using primers specific for amplification in Fusarium spp. can give better amplification profiles.

GSO approach proved to be an efficient tool for the identification of Foc race 3 and 4 from each other and from other Foc races. This approach is comparatively less time consuming, robust, and more reliable, with the minimum chances of errors as compared to other techniques. However, more GSOs designed from a wider spectrum of
virulence-related genes need to be screened with \textit{Foc} races 1 and 2 to get differential amplification in these races. This approach can be further modified suitably to identify \textit{Foc} standard races from soil samples directly from the fields, which would provide practical utility of such race specific markers. In future, this approach can be used for identification of \textit{Foc} races 0, 5, 6 and 7 as well. Also, more isolates per race can be used for better validation of race specific markers.

**ITS-RFLP approach**

The second approach used to distinguish \textit{Foc} races was ITS-RFLP. The ribosomal DNA has sequences that evolve at different rates (Apples and Honeycutt, 1986) and can be used for systematic studies at different taxonomic levels (Hibbert, 1992). The ITS region within the rDNA unit evolves rapidly, but remains uniform in sequence within a particular species, and differs between species.

The Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA (rDNA) are highly variable sequence and of great importance in distinguishing fungal species by PCR analysis. "ITS1" and "ITS4" primers amplify the highly variable ITS1 and ITS2 sequences of the fungal ITS region, surrounding the 5.8S-coding sequence and situated between the Small SubUnit-coding sequence (SSU) and the Large Subunit-coding sequence (LSU) of the ribosomal operon (White \textit{et al.}, 1990) (Fig. 4.5). These primers amplify a wide range of fungal targets and work well to analyze DNA isolated from individual organisms.

![Fig. 4.5 The rRNA gene structure](image)

Sequence comparison of the ITS region is widely used in taxonomy and molecular phylogeny because (a) it is (due to the high copy number of rRNA genes) easy to amplify even from small quantities of DNA, and (b) has a high degree of variation even
between closely related species. The ITS region is now perhaps the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races). Because of its higher degree of variation than other genic regions of rDNA (for small- and large-subunit rRNA), variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions. In addition to the standard ITS1+ITS4 primers used by most labs, several taxon-specific primers have been described that allow selective amplification of fungal sequences (Gardes and Bruns, 1993). ITS region is nowadays being used to know the genetic diversity among different strains of bacteria by sequencing the ITS gene.

Restriction fragment patterns from PCR amplified ITS sequences are one of the major tools in pathological and taxonomic studies at species level in fungi, and a wide range of fungi have been examined with this method (Bridge et al., 1998). However, the ITS-RFLP method has not been used previously for race identification purposes. We analyzed the four Foc races using ITS-RFLP. The ITS amplification product when digested with HinfI, MboI, HaeIII and HhaI showed identical restriction patterns in case of race 1, 2 and 4 suggesting that these races have high ITS region sequence homology, except for race 3 which showed completely different restriction pattern with the above mentioned enzymes. This shows that Foc race 3 does not entirely share sequence homology with the other three Foc races.

**AFLP approach**

Since, the ITS-RFLP of the four Foc races showed no polymorphism for race 1, 2 and 4, AFLP technology was used to differentiate these races. Twelve combinations of EcoRI/MseI primers could distinguish race 1 and race 2 in our studies. AFLP markers simultaneously detect variations at numerous loci and have been frequently used in studies on fungi (Majer et al., 1996; Baayen et al., 2000a; Jurgenson et al., 2002; Zhong et al., 2002). Genetic variability among 43 isolates Foc, collected from nine states of India including the four well-characterized races of the pathogen were assessed using the molecular markers, RAPD and AFLP (Sivaramakrishnan et al., 2003). In a very recent study, forty-eight isolates of Foc collected from different chickpea growing regions in India were evaluated for genetic variations using AFLP (Sharma et al., 2009).

Although race-specific amplification was obtained in our studies for races 1 and 2, successful conversion of AFLP fragment into a SCAR was difficult. The eluant often
contains a consortium of fragments, which is probably the result of co-isolation of background amplification products (Brugmans et al., 2003).

Similarly, as reported earlier, the amplified fragments of AFLP patterns often include heterogenous sequences of same size; hence clones of a single fragment often contain inserts of different sequences, which makes conversion of AFLP fragment into SCAR a difficult task (Martins-Lopes et al., 2001). Sequence analysis of clones of a single race specific fragment in our study also revealed variation in the sequence of clones of each AFLP band. These fragments revealed their homology to ESTs and fungal genes. Most of the fragments showed homology to metabolically essential genes.

Thus in our study, race 3 specific GSOs were developed, namely Hop78-2, Cut and Dst; and Xyl GSO for race 4. Also ITS-RFLP could differentiate race 3 from races 1, 2 and 4. Finally, AFLP markers could distinguish between race 1 and 2.

**Foc 3 is actually F. proliferatum**

Our results of gene specific approach and ITS-RFLP analysis, showing race 3 being entirely different from race 1, 2 and 4, correspond well with previous studies of microsatellite analysis (Barve et al., 2001) and the RE digestion patterns (Chakrabarti et al., 2000) suggesting the same unique characteristics of Foc race 3. In the present study as well; the GSOs and ITS-RFLP molecular markers, which theoretically examine different regions of the genome, unanimously indicated the same. The unique characteristics of Foc race 3 isolates necessitated phylogenetic characterization of this race.

Evolution of phenotypic traits in asexual plant pathogenic fungi, such as host specificity or relatedness among pathogenic races, can be studied by analyzing genealogies of genes that do not have a direct functional relationship to the phenotypes of interest (O’Donnell et al., 1998; Taylor et al., 1999; Steenkamp et al., 2000). Sequencing of introns from EF-1α has been extensively used for phylogenetic analysis in Fusarium spp. (Baayen et al., 2000b; O’Donnell et al., 1998; O’Donnell, 2000). As a step toward phylogenetic characterization of race 3 with respect to standard races 1, 2 and 4, we compared sequence data from EF-1α of the standard isolates of Indian Foc races, F. solani and F. udum, obtained from ICRISAT, India. Phylogenetic analysis using PAUP (Swofford, 1998) revealed that EF1α sequence of Foc race 1, 2 and 4 standard isolates was similar to F. oxysporum (25420Fov1, 26034Fol), whereas, Foc race 3 had
translation EF sequence similar to *F. proliferatum* indicating that *Foc* race 3 is actually the *F. proliferatum*.

### 4.1.2 Proteomic characterization of *Foc* races

Improvements in key proteomics technologies such as protein separation by two-dimensional gel electrophoresis (2-DE) and peptide analysis by mass spectrometry (MS) have allowed efficient characterization and identification of a large number of proteins from microbial origin (Jungblut and Hecker, 2004). However, only a few reports on filamentous fungi characterization are available (Lim et al., 2001; Nandakumar and Marten, 2002; Grinyer et al., 2004; Kim et al., 2004; Ebstrup et al., 2005; Medina et al., 2005; Shimizu and Wariishi, 2005). A recent report by Fernández-Acero et al., (2006) describes the first analysis of *B. cinerea* proteome by 2-DE and MS.

In our studies five proteins, differentially expressed in *Foc* race 1, 2 and 4 were analyzed using 1D-SDS PAGE followed by MALDI-TOF analysis of PMF of these proteins. Among these, mycelial protein (m4.1) showed homology to the hypothetical protein of *Gibberella zeae* PH-1 similar to glucosamine 6-phosphate N-acetyltransferase from *Neurospora crassa* OR74A. UDP-N-acetylglucosamine (UDP-GlcNAc) is an essential precursor of chitin and glycoproteins in yeast (Cabib et al., 1982; Herscovics and Orlean, 1993). The yeast GNA1 gene encodes for glucosamine-6-phosphate acetyltransferase which catalyzes the reaction of glucosamine 6-phosphate with acetyl-CoA to form N-acetylglucosamine 6-phosphate, a fundamental precursor in UDP-N-acetylglucosamine biosynthesis. *C. albicans* GNA1 is required for survival of the fungus in host animals, probably because an insufficient level of N-acetylglucosamine is available from the host tissues (Mio et al., 2000).

A score of 45 was obtained for the f1.3 digested protein, which showed homology to the hypothetical protein of *Gibberella zeae* PH-1 similar to glycosyltransferase from *Bacteroides* sp. _2_ _2_ _4_. A sterol glycosyltransferase from *Colletotrichum* was found to demonstrate an important role in pathogenesis, suggesting a novel biological function for this transferase (Kim et al., 2002). Disruption of *chip6* glycosyltransferase gene markedly reduced the UDP-glucose:sterol glycosyltransferase activity of *C. gloeosporioides*. These results indicated that sterol glycosyltransferase of *C. gloeosporioides* is a pathogenesis-related protein (Kim et al., 2002).

Similarly, a score of 47 was obtained for the m1.1 digested protein, which showed homology to the hypothetical protein of *Gibberella zeae* PH-1 similar to DEAD-box
RNA helicase from *Aspergillus oryzae*. The DEAD-box RNA helicases are enzymes involved in many critical aspects of RNA metabolism within both eukaryotic and prokaryotic organisms and are classically defined as ATP-dependent enzymes that separate DNA and/or RNA duplexes. Several studies have shown that these proteins may have important functions in mediating microbial pathogenesis. Several studies suggest that DEAD-box proteins are crucial to signaling pathways that mediate host-pathogen interactions (Heung and Poeta, 2005). DEAD-box RNA helicase in the pathogenic fungus *Cryptococcus neoformans* has been proposed to play novel roles in the development and progression of cryptococcosis (Panepinto *et al.*, 2004).

m4.4 and f 4.1 fragments showed homology to the hypothetical protein of *Gibberella zeae* PH-1 but their roles in fungal virulence have not yet been determined. This is the first study where these proteins have been reported to be present in *Foc*. The homologies of these proteins to hypothetical proteins of *Gibberella zeae* (which is the sexual stage of *F. gramiearum*) reveals the importance of this study.

Our results indicate that proteomic analysis seems to be an important tool for identifying new pathogenicity related factors, therapeutic targets and for basic research on this plant pathogen in the postgenomic era. Of course, the present work is only a preliminary step and in-depth studies are essential for better understanding of this phytopathogen.

4.2 Studying the chickpea defense and *Fusarium* pathogenesis system

This study was performed to understand chickpea-*Fusarium* interactions at transcriptional level for both, plant defense as well as fungal virulence related genes. Not many studies have been attempted to determine the defense related gene expression in chickpea against biotic stresses like Fusarium wilt. A few studies which have been previously conducted specify the defense transcript accumulation only during the first few hours of plant infection process, when the pathogen has merely entered the host plant and has started to establish itself. Analysis of defense related genes like basic glucanase, ascorbate peroxidase, glutathione reductase (Cho and Muehlbaur, 2004) or phenylalanine ammonium lyase, *CHS, IFR* (Arfaoui *et al.*, 2007) revealed only slight increase in the expression of these genes between resistant and susceptible accessions indicating that no significant differential expression of these genes correlated with Fusarium wilt resistance. Comparatively, in our studies imperative variations were observed which could be due to the time scale chosen for gene expression analysis. Also, continuous exposure to the pathogen load (mimicking the field conditions) could
be a reason for such dramatic differences. We included a stretched time scale (2-16 dai), throughout the course of fungal infection and disease development; for determining the defense and virulence gene expression. Earlier, individual studies using \( Foc \) race 0 and race 1 have been conducted for profiling defense gene expression in chickpea (Arfaoui \textit{et al.}, 2007; Cho and Muehlbaur, 2004), however; this is the first report of a comparative study exploring the gene specific transcript accumulation against three different \( Foc \) races causing wilt in the Indian subcontinent. In previous studies conducted using \( Foc \) inoculated cultivars, the expression level of defense related genes was found to be much higher in moderately resistant accession than the susceptible accession (Arfaoui \textit{et al.}, 2007). Similar results were reported by others (Wang \textit{et al.}, 2006) who showed that the level of accumulation of transcripts could be correlated with the differences in resistance/susceptibility of the host plant. Overall, in this study, an enhanced expression of plant defense related genes was observed in case of resistant inoculated cultivar as compared to susceptible inoculated cultivar. Additionally, genes like glycosyltransferse, \textit{Msr}, \textit{Betvl} and \textit{GroES2} which have not been previously harnessed for their roles in plant defense; especially in chickpea, were found to express upon pathogen attack. Fungal virulence genes like \textit{Fgb1}, \textit{Gas1}, \textit{Chs7} and \textit{Fow1} which have been previously reported to be essential for various cellular functions including fungal pathogenesis; showed an elevated expression during 9-13 dai indicating the window period of disease progression. Earlier studies conducted for the above mentioned virulence genes established their role in fungal pathogenesis and development in several systems (Delgado-Jarana \textit{et al.}, 2004; Iori \textit{et al.}, 2002), while in our study the temporal expression and race specific behavior of the suite of these virulence genes against both resistant and susceptible cultivars has been analyzed.

4.2.1 Race specific interaction of chickpea-\textit{Fusarium oxysporum}

Interestingly, in the present study, the response of plant defense genes has been observed to be specific to \( Foc \) races (Fig. 3.41, in results section). All the five genes, namely \textit{GroES2}, \textit{60srp}, \textit{Betvl}, \textit{CHS} and \textit{IFR}, showed higher expression in resistant chickpea cultivar, Digvijay vis-à-vis susceptible cultivar JG62, when exposed to race 1. However, only \textit{CHS} and \textit{Betvl} genes gave similar response in case of race 2 and race 4 inoculated chickpea cultivars, respectively. This clearly indicated that, to establish resistance against \( Foc \) race 1 collective response of all the five genes under present study was essential whereas for remaining two races (\( Foc \) 2 and \( Foc \) 4) differential
upregulation of some of these genes was sufficient to give complete resistance in Digvijay.

Similarly out of four fungal genes assessed in the present study, all the four genes revealed higher expression in the susceptible chickpea cultivar, JG62 than in the resistant chickpea cultivar, Digvijay when inoculated with race 1 (Fig. 3.25, in results section). While only Fgb1 and Fow1 genes were upregulated in JG62 upon race 2 infection; and Fgb1, Gas1 and Fow1 genes were upregulated in JG62 upon race 4 infection. The study indicated that amongst these four genes, Fowl and Fgb1 are the most essential genes for prolonged virulence in Foc during its infection to chickpea; followed by Gas1 and then Chs7, confirming race specific involvement of these genes in establishing pathogenicity of Foc in chickpea.

Thus, it is noteworthy that, though a higher level of fungal virulence gene expression is essential for disease development in chickpea; comparatively a low level of plant defense related gene expression is sufficient to allow complete disease resistance in resistant chickpea cultivar- Digvijay (as shown in the result section). Protecting Digvijay from pathogen attack can be accredited to the enhanced expression of the defense related genes; however, there is a need to further explore the exact role of these genes and their interaction with others during defense, for the confirmation of this hypothesis.

4.3 Model proposed for chickpea-Fusarium oxysporum interactions

In the previous studies accomplished by Nimbalkar, (2007) genes involved during Fusarium wilt of chickpea, caused by Foc race 1, were studied for their expression in both resistant and susceptible cultivar. A gene network was then suggested wherein 14-3-3 and WRKY genes were implicated to be involved in plant defense, alongwith other genes like NBS-LRR, chitinase, hydrolase, ATPase and gamma-glutamyl synthatase. Updating this model suggested by Nimbalkar, (2007) we incorporated genes responsible for fungal pathogenesis while attacking chickpea, at the same time depicting the genes essential for mounting defense reaction in chickpea counteracting the fungus attack (Fig. 4.6).
Fig. 4.6 Schematic representation of *Fusarium* virulence genes and chickpea defense genes involved in the host-pathogen studies accomplished in this research work.