Identification and molecular characterization of virulence related factors in *Fusarium oxysporum* f. sp *ciceris*

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BY
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CERTIFICATE

Certified that the work in this Ph.D. thesis entitled **'Identification and molecular characterization of virulence related factors in *Fusarium oxysporum* f. sp *ciceris*'** submitted by **Ms. Gayatri S. Gurjar** was carried out by the candidate under my supervision. The material obtained from other sources has been duly acknowledged.

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

Place: **Research Advisor**

DECLARATION

I hereby declare that the thesis **'Identification and molecular characterization of virulence related factors in *Fusarium oxysporum* f. sp *ciceris*'** submitted for Ph.D. degree at the University of Pune has not been submitted by me for a degree at any other university.

Date :
Place:

(**Gayatri S. Gurjar)**
National Chemical Laboratory,
Pune

Dedicated to my beloved parents

Dear Aai and Baba,

This is just for you . . . . . .

CONTENTS

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Gayatri S Gurjar

List of abbreviations

AFLP	amplified fragment length polymorphism
βME	beta-mercaptoethanol
bp	base pairs
$CHCl_3$	chloroform
CTAB	hexadecyl-trimethyl-ammonium bromide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide 5' triphosphate
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
EF 1α	elongation factor 1α
Foc	*Fusarium oxysporum* f. sp. *ciceris*
h	hour
IAA	isoamyl alcohol
ISSR	inter-simple sequence repeat
kb	kilo bases
µg	microgram
µl	microliter
µM	micromolar
M	molar
Mb	megabase
min	minute
ml	milliliter
mM	millimolar
mt DNA	mitochondrial DNA
nit	nitrate non-utilizing
ng	nanograms
^{0} C	degree celsius
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
pmoles	pico-moles
QTL	quantitative trait loci
RAPD	random amplified polymorphic DNA
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
SCAR	sequence characterized amplified region
SDS	sodium dodecyl sulphate
s	second
SSR	simple sequence repeat

STMS	sequence tagged microsatellite site
TAE	tris-acetate-EDTA
TBE	tris-borate-EDTA
TE	tris-EDTA buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0)
Tm	melting temperature
Tris-HCl	tris-hydroxymethyl amino methane
U	units of enzyme
UBC	University of British Columbia
UPGMA	unweighted pair group method using arithmetic averages
V	volt
VCG	vegetative compatibility group
$AgNO_3$	silver nitrate
Na_2CO_3	sodium carbonate
HCHO	formaldehyde
ITS	inter transcribes spacer
UV	ultra violet
GSO	gene specific oligonucleotide
NCBI	National centre for biotechnology information
dai	days after inoculation
DEPC	diethyl pyrrocarbonate
cDNA	complementary deoxyribonucleic acid
rRNA	ribosomal RNA
LB	Luria Bertani
IPTG	isopropyl-beta-thio galactopyranoside
X-gal	5-Bromo-4-chloro-indoly-β-D-galactoside
NaOH	sodium hydroxide
KOAc	potassium acetate
$(NH_4)_2SO_4$	ammonium sulphate
KCl	potassium chloride
PVP	polyvinyl pyrrolidone
$Na_2S_2O_3$	sodium thiosulphate
mA	milli ampere
ACN	acetonitrile

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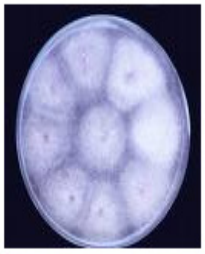



INTRODUCTION AND LITERATURE REVIEW





1. Introduction

1. 1 Chickpea (*Cicer arietinum* L.)

1.1.1 Introduction

Chickpea is a cool season legume crop and is grown in several countries worldwide as a food source. 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. Chickpea is the third most important food legume crop and India is the largest producer contributing to 65% of world's chickpea production (FAOSTAT, 2008). Even though India is the largest producer of chickpea; it still imports chickpea from other countries. Keeping in view, the ever-increasing demand for this legume crop; it is essential to improve the production and area under cultivation, at the same time minimizing the stress on this crop plant. Two types of chickpeas are recognized, the white-seeded "Kabuli" and the brown colored "Desi" types. Kabuli chickpeas are relatively bigger in size having a thinner seed coat while the Desi type seeds are relatively smaller in size having a thicker seed coat. The Desi type chickpea contributes to around 80% and the Kabuli type around 20% of the total production. Chickpea has been classified as follows:

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

1.1.2 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).

1.1.3 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) (Fig. 1.1). 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.

Fig. 1.1 Mature chickpea plants with flowers and pods

1.1.4 Ecology

Chickpea is a self-pollinated crop and cross-pollination is a rare event; only 0-1 % is reported (Singh, 1987; Smithson *et al.*, 1985). It is usually grown as a rainfed cool-weather crop or as a dry climate crop in semi-arid regions. Optimum conditions include 18-26^0 C day and 21-29^0 C night temperatures and annual rainfall of 600-1000 mm (Duke, 1981; Muehlbauer *et al.*, 1982; Smithson *et al.*, 1985). It is generally grown on heavy black or red soils (pH 5.5-8.6). Frost, hailstones and excessive rains damage the crop. Though sensitive to cold, some cultivars can tolerate temperatures as low as -9.5^0 C in early stages or under snow cover. Daily temperature fluctuations are desired with cold nights with dewfall. Relative humidity of 21-41% is optimum for seed setting. In virgin sandy soils or for the first planting in heavier soils, inoculation is said to increase yield by 10-62% (Duke, 1981). Chickpea is a quantitative long-day plant, but flowers in every photoperiod (Smithson *et al.*, 1985).

1.1.5 Taxonomy, morphology and floral biology

The genus *Cicer* includes 9 annuals and 34 perennial herbs (van der Maesen, 1972; Muehlbauer, 1993). Chickpea stems are branched, erect or spreading, sometimes shrubby much branched, 0.2-1m tall, glandular pubescent, olive, dark green or bluish green in color. Root system is robust, up to 2m deep, but major portion up to 60cm. Leaves imparipinnate, glandular-pubescent with 3-8 pairs of leaflets and a top leaflet (rachis ending in a leaflet); leaflets ovate to elliptic, 0.6-2.0cm long, 0.3-1.4cm wide; margin serrate, apex acuminate to aristate, base cuneate; stipules 2-5 toothed or absent. Flowers solitary, sometimes 2 per inflorescence, axillary; peduncles 0.6-3cm long, pedicels 0.5-1.3cm long, bracts triangular or tripartite; calyx 7-10mm long; corolla white, pink, purplish (fading to blue), or blue, 0.8-1.2cm long. The staminal column is diadelphous (9-1) and the ovary is sessile, inflated and pubescent (Duke, 1981; Cubero, 1987; van der Maesen, 1987). Pod rhomboid ellipsoid, 1-2 with three seeds as a maximum, and inflated, glandular-pubescent. Seed color cream, yellow, brown, black, or green, rounded to angular, seedcoat smooth or wrinkled, or tuberculate, laterally compressed with a median groove around two-thirds of the seed, anterior beaked; germination cryptocotylar (Duke, 1981; Cubero, 1987; van der Maesen, 1987).

1.1.6 Chickpea association with *Rhizobium*

Nitrogen (N) is a plant nutrient, which is highly important for crop productivity. Sustainability considerations suggest the need to search for alternatives to N fertilisers

such as biological nitrogen fixation, a microbial process that converts atmospheric nitrogen into a plant-usable form. Continuous cultivation for cereal cropping in the major cereal growing areas has led to depletion of soil organic carbon and N. This has resulted in decreased cereal crop yields and cereal grain protein concentrations in recent years, preventing farmers from obtaining maximal yields for their crop. Although chickpea (*Cicer arietinum* L.) is widely accepted in cereal rotations in the region, limited work has been conducted on management options to enhance its ability to fix atmospheric N. This dinitrogen (N_2) fixing pulse legume has been widely grown throughout South and West Asia and the Mediterranean region for centuries, usually in sequence with either summer or winter cereals (Smithson *et al.*, 1985; Beck *et al.*, 1991; Pala *et al.*, 1994; Lopez-Bellido *et al.*, 1996). Recently, N fixation inputs and N balances, i.e. the difference between N fixation inputs and N in harvested products (outputs), as well as rotational benefits of chickpea have been quantified revealing its importance in nitrogen fixation.

1.1.7 Chickpea nutrition and uses

Chickpea is an important source of protein in the diets of the poor, and is particularly important in vegetarian diets. Also, it is being used increasingly as a substitute for animal protein. Chickpeas are a helpful source of zinc, folate and protein. They are also very high in dietary fiber and hence a healthy source of carbohydrates for persons with insulin sensitivity or diabetes. Chickpeas are low in fat and most of this is polyunsaturated. One hundred grams of mature boiled chickpeas contains 164 calories, 2.6g of fat (of which only 0.27g is saturated), 7.6g of dietary fiber and 8.9g of protein. Chickpeas also provide dietary calcium (49–53mg/100g). According to the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) chickpea seeds contain on average- 23% protein, 64% total carbohydrates (47% starch, 6% soluble sugar), 5% fat, 6% crude fiber and 3% ash. High mineral content has been reported for phosphorus (340mg/100g), calcium (190mg/100g), magnesium (140mg/100g), iron (7mg/100g) and zinc (3mg/100g). Recent studies have also shown that they can assist in lowering of cholesterol in the bloodstream (Pittaway *et al.*, 2008). Among the food legumes, chickpea is the most hypocholesteremic agent; germinated chickpea was reported to be effective in controlling cholesterol level in rats (Geervani, 1991). Glandular secretion of the leaves, stems, and pods consists of malic and oxalic acids, giving a sour taste. Medicinal applications include use for aphrodisiac,

bronchitis, cholera, constipation, diarrhea, dyspepsia, flatulence, snakebite, sunstroke and warts. Acids are supposed to lower the blood cholesterol levels (Duke, 1981).

1.1.8 Chickpea yield

The total world production of pulses was 56.5 million tonnes (mt) in 2003/2004 (i.e. for the harvest of mid 2003 in northern hemisphere and of early 2004 for Southern hemisphere) (Fig. 1.2). India is the world's major producer of food grain legumes (about 13mt in 2003/2004): chickpeas (5.3mt), lentils (0.8), dry beans (about 3) and other pulses (pigeon peas, etc.). Chickpea ranks third in the world among pulses after pea and common bean with an area of 11.67 million hectares. The FAOSTAT, (2008) data indicates a chickpea production of 5.7mt in India, while the world chickpea production is 8.7mt indicating that India contributes to 65% of world's chickpea production (FAOSTAT Database, http://faostat.fao.org/site/567/default.aspx, 2008). However, the supply in terms of production plus imports is not keeping abreast of the increase in demand due to demographic growth. Pulses imports reached 1.8mt in by year 2004 (www.grainlegumes.com) (Fig. 1.3). Despite its economic importance and strong national and international breeding programs, the productivity of chickpea has not improved considerably over the years. Major constraints in realization of the full yield potential of chickpea are various abiotic and biotic factors.

1.1.9 Stresses: biotic and abiotic

The yield potential of present day chickpea cultivars exceeds 5.0t/ha, while the average yield is stagnating around 0.8t/ha. The wide gap between average yield and potential yield is mostly due to diseases, pests and poor management practices. Among abiotic stresses, drought is the most important stress in chickpea since the crop is mostly grown on rainfed marginal lands (Singh *et al.*, 1994). The crop invariably suffers from moisture stress at one or the other stages of development depending on water availability in the soil.

Terminal drought stress, which occurs during the pod-filling phase, is a common yield reducer in chickpea (Leport *et al.,* 2006). Cold is the second most important abiotic stress and susceptibility to cold is greater at the late vegetative stage than at the seedling stage (Singh *et al.*, 1984). Cold tolerance in chickpea plants tends to decrease from germination to flowering (Wery, 1990). The necessity of cold tolerant chickpea cultivars arises due to the advantages of winter-sown chickpea over traditionally spring sown chickpea (Singh *et al.*, 1994).

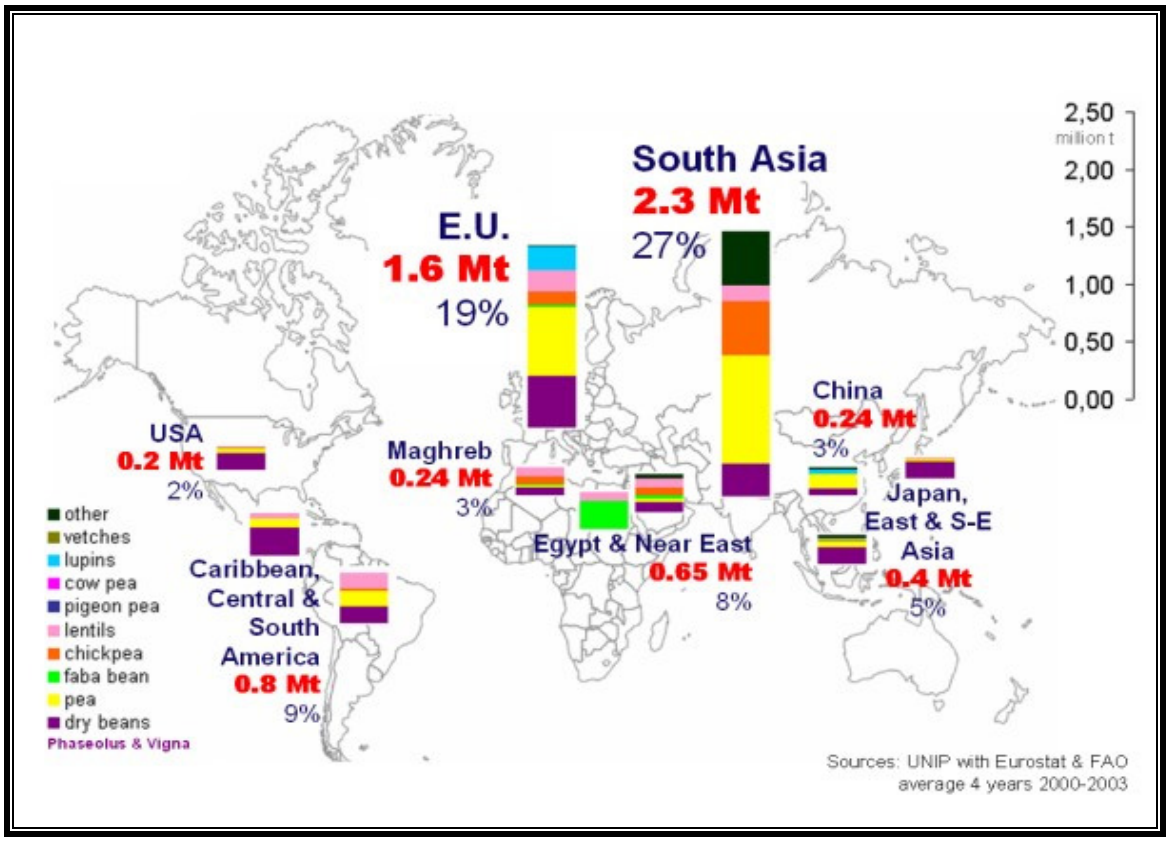


Fig. 1.2 Total world production of pulses (5 years average 2000-2004) (www.grainlegumes.com)

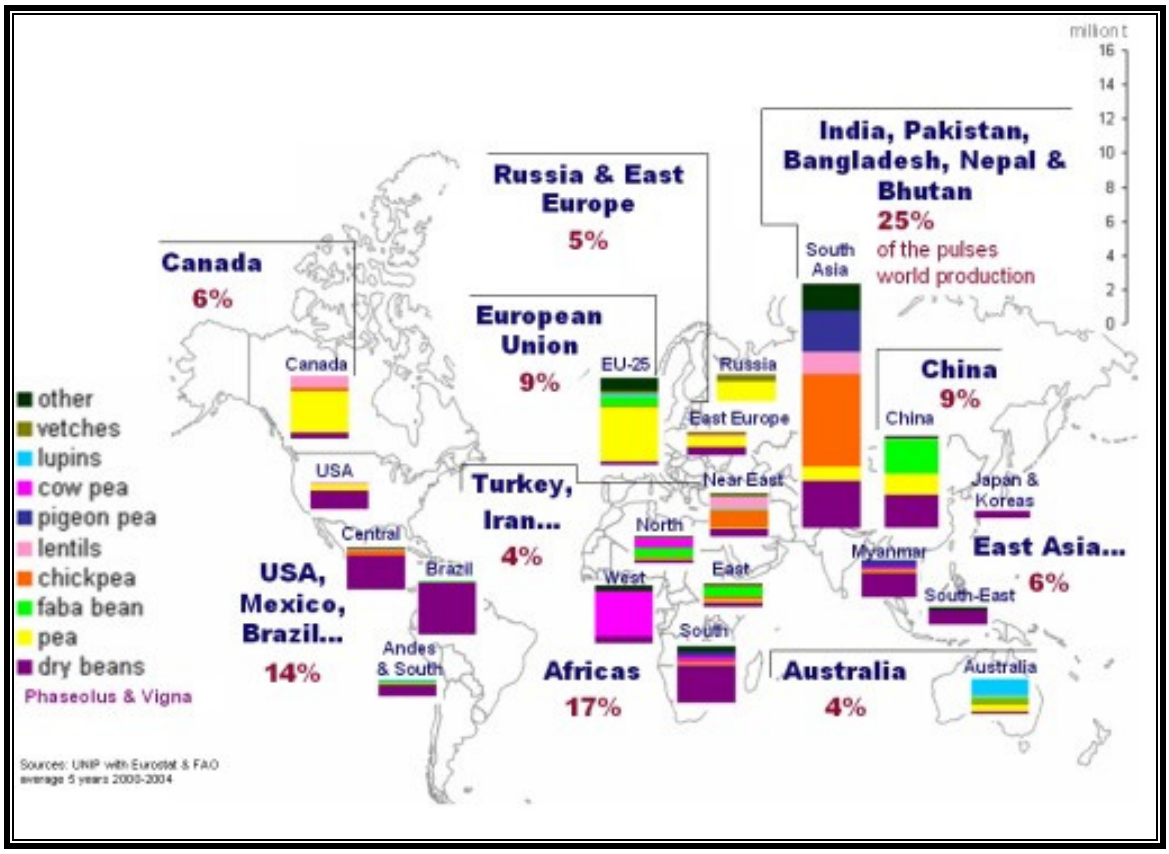


Fig. 1.3 Pulses imports (5 years average 2000-2004) (www.grainlegumes.com)

Amongst the causal agents of biotic stresses, about 67 fungi, 3 bacteria, 22 viruses and 80 nematodes have been reported on chickpea (Nene *et al.*, 1996) but only few of these cause economically important diseases (Haware, 1998). The maximum number of pathogens has been reported from India alone with the number rising to 89 pathogens in 1995 from 35 in 1978 (Nene *et al.*, 1996). The insect *Helicoverpa armigera* which feeds on foliage, flowers and developing seeds, is the most important pest of chickpea, while stunt is the most important and prevalent viral disease in most chickpea growing regions of the world. Among economically important fungal diseases of chickpea are root diseases like fusarium wilt and root rots caused by a complex of soil borne fungi, foliar diseases like ascochyta blight and botrytis grey mould, of which wilt and blight are the most devastating diseases affecting chickpea in tropical and temperate regions, respectively.

1.1.10 Fusarium wilt of chickpea

Fusarium wilt is the most important disease of chickpea and is wide-spread in chickpea growing areas of Asia, Africa, Southern Europe and the Americas between latitudes 30°N and 30°S, where the chickpea-growing season is dry and warm (Nene *et al.*, 1996). Annual yield losses due to wilt have been estimated at 10-90% (Jiménez-Diaz *et al.*, 1989). The causative agent of this disease has been classified as *Fusarium oxysporum* f. sp. *ciceris* (*Foc*). *Foc* is internally seed borne and is found as chlamydospore-like structures in the hilum region of the seed (Haware *et al.*, 1978). Infected seed plays an important role in long distance dispersal and in transmitting the disease to new areas. Once the inoculum is established in soil, it is difficult to eradicate as the chlamydospores survive in the soil for at least 6 years, and under favorable conditions germinate and infect the seedlings through tender roots (Haware *et al.*, 1996). The pathogen can also survive in infected crop residues buried in the soil and other *Cicer* species can also be affected under artificial inoculation conditions (Barve, 2003). Among other legumes, lentil, pea and pigeonpea are symptomless carriers of the chickpea wilt fungus (Haware and Nene, 1982).

1.2 *Fusarium oxysporum* f. sp. *ciceris*

1.2.1 Pathogen growth and cultural characteristics

The fungus grows on potato sucrose agar at 25^0 C and appears as delicate, white and cottony growth becoming felted and wrinkled in older cultures (Nelson *et al.*, 1983). Fungal hyphae are septate and profusely branched. Microconidia are borne on simple

short conidiophores, arising laterally on the hyphae. Microconidia and macroconidia are generally sparse on solid media, however, they are formed abundantly in potato sucrose broth. Microconidia are oval to cylindrical, straight to curved and measure 2.5-3.5 x 5-11 µm. Macroconidia, which develop on the same conidiophores on which microconidia are formed (Nelson *et al.*, 1983), are thin walled, 3-5 septate, fusoid, pointed at both ends, fewer in number than microconidia, and measure 3.5-4.5 x 25-65µm. Chlamydospores, formed in 15-day-old cultures are smooth or rough walled, terminal or intercalary, and may form singly, in pairs, or in chains.

1.2.2 Fusarium wilt- disease symptoms

Affected seedlings show a dull green color of the foliage; sudden drooping of the petioles, rachis and leaves (Fig. 1.4). The plants, when uprooted, show uneven shrinkage at the collar (Nene *et al.*, 1978). There is no external rotting of roots and pith, however, when the roots are split vertically, internal discoloration may be seen in such wilted plants (Nene *et al.*, 1978). Transverse sections of the infected roots examined under the microscope show the presence of hyphae and spores of the fungus in the xylem, thereby confirming the diagnosis of vascular wilt (Nene *et al.*, 1978). Isolates of *Foc* may induce either fast wilting or a progressive yellowing syndrome, which develops 15-40 days after inoculation depending on the cultivar. Wilting can be observed in a susceptible cultivar within 25 days after sowing in *Foc* infested soil and this is known as ‘early wilt’ (Haware and Nene, 1980) (Fig. 1.5 A, B). Wilting may also occur during reproductive growth and is known as ‘late wilt’. Early wilting is reported to cause 77-94% yield loss while late wilting causes 24-65% loss (Haware and Nene, 1980). Seed harvested from the late wilted plants is lighter and duller than that harvested from healthy plants. Plants grown from infected seeds wilt faster than the plants grown from healthy seeds.

1.2.3 Life cycle

In a comprehensive review addressing the life cycle, disease progression and host pathogen interactions with respect to wilt causing pathogens, Beckman and Roberts (1995) proposed a model, wherein, the pathogens have distinct saprophytic and parasitic phases in their life cycles. Fig. 1.6 depicts the life cycle of soil-borne, wilt causing fungi including their saprophytic and parasitic growth and successive phases of colonization and pathogenesis. The extent of colonization of the vascular system of the

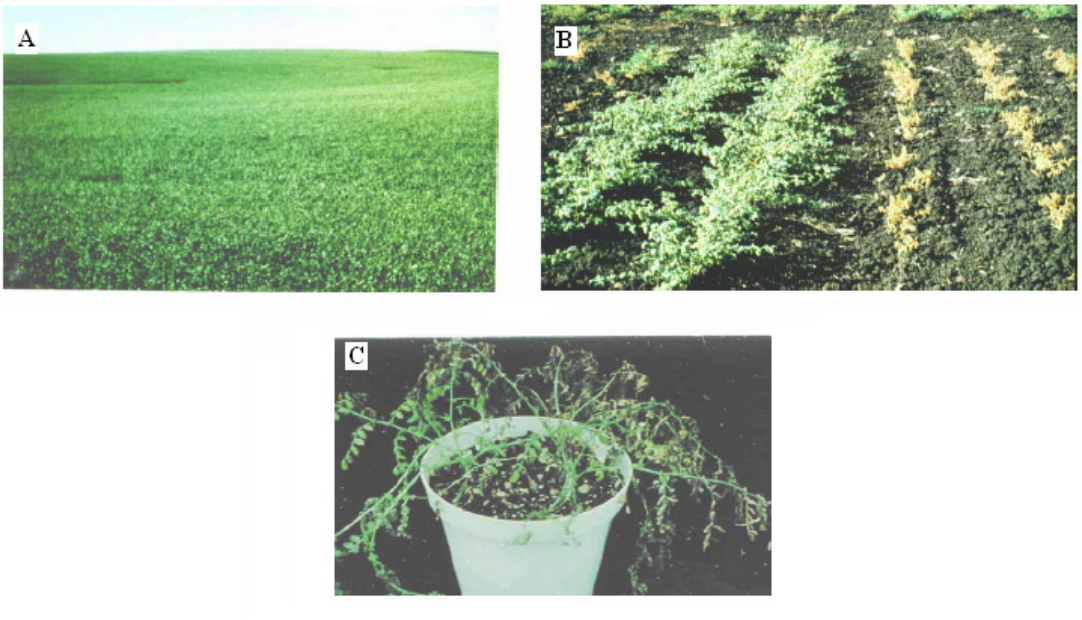


Fig. 1.4 (A) Chickpea field showing healthy plants (B) Healthy and wilt infested chickpea plants (C) Wilt infested chickpea plant showing wilting symptoms

Fig. 1.5 (A) Dried chickpea plant showing wilting symptoms (B) Transverse section of chickpea root showing browning of xylem

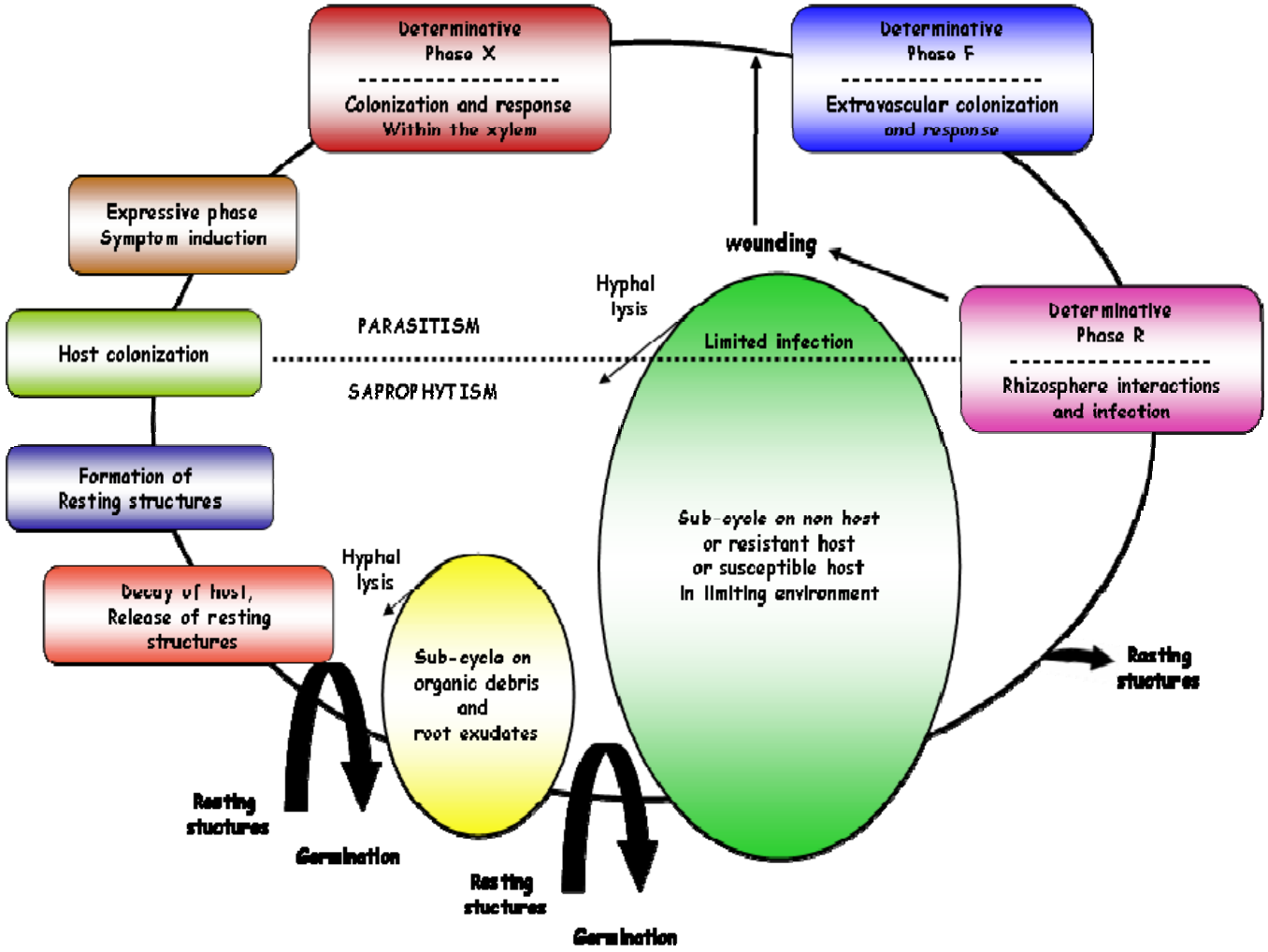


Fig. 1.6 Life cycle of vascular wilt pathogen- *Fusarium oxysporum* f. sp *ciceris*

host by the pathogen is determined in the determinative phase, while disease symptoms are developed mainly in the expressive phase, and the survival of the pathogen by formation of long-lived resting structures is mainly in the saprophytic phase of the life cycle. Under disease prone conditions, the pathogen invades the root tissue and after it has acquired significant cortical colonization, readily enters the second phase of vascular invasion and spreads along with the transpiration pull. The plant defense response to pathogen invasion is offered mainly at two places (i) in the endodermis and xylem parenchyma, where the invading pathogen is restricted by infusion of phenolic compounds, progressive suberinization and lignin deposition, and by hydrolytic enzymes like chitinases and glucanases (ii) in the vascular tissue, where the upward movement of the pathogen is arrested to compartmentalize the pathogen, by the formation of callose, gelgum and tyloses, which are mainly derivatives of celluloses and hemi-celluloses. The difference in resistant and susceptible cultivars lies in the speed with which they can activate the defense mechanisms and accumulate substances like callose to restrict the growth and spread of the pathogen; most of the disease symptoms seem to be caused by the plant response to infection.

1.2.4 *Foc* classification

Fusarium is a large cosmopolitan genus of pleoanamorphic hyphomycetes whose members can cause a wide range of plant diseases (Farr *et al.*, 1989), mycotoxicoses and mycotic infections of other animals as well as humans (Nelson *et al.*, 1994). *Fusariam* are noted for production of secondary metabolites such as plant growth hormones (gibberellin) as well as toxins (tricothecenes and fumosins).

Many species of this fungus are recognized, out of which *F. oxysporum* is the commonest one. *F. oxysporum* isolates display variation in chromosome number and total genome size, when analyzed by electrophoretic karyotyping (Momol and Kistler, 1992; Kim *et al.*, 1993; Boehm *et al.*, 1994). Deletion, duplication, translocation and the presence of dispersible chromosome, all have been suggested to be a source of such genomic variation in filamentous fungi like *Fusarium* (Kistler and Miao, 1992). The species is well represented among the soil borne fungi and can be observed in diverse soil types all over the world (Burgess, 1981) and is considered to be a normal constituent of the rhizosphere of plants (Appel and Gordon, 1994). However, only some strains of *F. oxysporum* are pathogenic to plants where they penetrate into the roots and provoke root rots causing severe damage. The vascular wilt causing forma speciales of *F. oxysporum* typically invade only living root tissues, tend to be

specialized, are host specific, and suppressed by saprophytes (Hillocks, 2001). Based on the plant species and plant cultivars infected, *F. oxysporum* is classified into more than 120 *forma speciales* and races (Armstrong and Armstrong, 1981). The presently accepted classification for the fusarium wilt pathogen *F. oxysporum* f. sp. *ciceris* is: Form-Class: Fungi Imperfecti, Form-order: Moniliales, Form-family: Tuberculariaceae, Form-genus: *Fusarium*, Form species: *oxysporum*, forma specialis: *ciceris*.

1.3 Phylogenetic studies of *F. oxysporum* f. sp. *ciceris*

1.3.1 Virulence pathotypes and races

Variation in the virulence within isolates of a given forma specialis has led to the designation of pathogenic races. Accordingly, *Foc* has been classified into eight races worldwide on the basis of the pathogenic reaction of a particular *Foc* isolate to a set of differential chickpea cultivars. Races 0 and 1B/1C cause yellowing symptoms whereas races 1A, 2, 3, 4, 5 and 6 induce wilting symptoms (Jiménez-Gasco *et al.*, 2001). Out of these, races 2, 3 and 4 have been reported only in India (Fig. 1.7), while races 0, 1B/1C and 5 have been mainly found in the Mediterranean region and in California, USA (Jiménez-Gasco and Jiménez-Diaz, 2003). Race 1A has been reported to occur in India, California, Morocco and Spain; while race 6 has been found in California (USA), Spain, Israel and Morocco (Jiménez-Gasco *et al.*, 2001).

1.3.2 Aggressiveness

Apart from the virulent phenotypes distinguished as pathotypes (yellowing and wilting) and races (0, 1A, 1B/1C, 2, 3, 4, 5 and 6); *Foc* isolates can also differ in their aggressiveness to cause disease. Aggressiveness is defined as the amount of disease caused by a pathogen genotype on a given host plant genotype. The aggressiveness is indicated by the amount of inoculum needed to cause severe disease in a particular chickpea cultivar. Differences in the stage of plant pathogen interaction can reflect upon the aggressiveness of a particular race. In studies conducted by Navas-Cortes *et al.*, (2000); it was observed that yellowing pathotype of *Foc* is less aggressive than the wilting pathotype and the aggressiveness further differed among the races belonging to a particular pathotype.

1.3.3 Phylogenetics

F. oxysporum is a consortium of asexually reproducing fungi capable of causing soil borne diseases in a variety of host plants; though several non-pathogenic soil borne isolates also do exist. The genetic variation arising in *F. oxysporum* results mainly from the accumulation of mutations; which are transmitted from one generation to the next (Jiménez-Gasco *et al.*, 2004).

Fig. 1.7 Geographic location of occurrence of *Foc* races in India

This clonal nature of *F. oxysporum* has been associated with the vegetative compatibility concept (Gordon and Martyn, 1997; Kistler, 1997). Vegetative compatibility can be described as ability of fungal isolates to establish stable heterokaryons by continued anastomosis between adjacent homokaryotic hyphae (Di Primo *et al.*, 2001; Gordon and Martyn, 1997; Klein and Correll, 2001). Thus, isolates from a particular vegetative compatibility group (VCG) are genetically secluded from, and are incompatible with isolates from other VCGs. The *forma speciales* of *F. oxysporum* are known to have different evolutionary patterns and can be monophyletic or polyphyletic depending upon their origin (O'Donnell *et al.*, 1998; Baayen *et al.*, 2000a). The high phenotypic and genetic diversity observed within the fungus *F. oxysporum* f. sp. *ciceri* raises a possibility that *Foc* might have polyphyletic nature. Despite the high degree of variability in *Foc* various studies show that all *Foc* isolates, regardless of their pathotype, race and geographic origin; belong to the same VCG (Pérez-Artés *et al.*, 1995; Nogales-Moncada, 1997; Jiménez-Gasco *et al.*, 2004). In the study conducted by Jiménez-Gasco *et al.*, (2004) *Foc* isolates representative of all races, pathotypes and geographic range, were analyzed for sequence variation in the EF1α gene. Parsimony analysis of the EF1α gene sequence available in the NCBI database with that of *Foc* isolates used in this study was accomplished. The results indicated that *Foc* isolates formed a group distinct from other *forma speciales* and non-pathogenic isolates; which shows that *Foc* is monophyletic in its origin. The monophyly in *Foc* and lack of multilocus sequence variation is suggestive of the fact that only a small population of *F. oxysporum* is capable of causing wilt in *Cicer* spp. and these virulent phenotypes have resulted from the accumulation of relatively recent genetic changes (Gorden and Martyn, 1997).

1.3.4 Phylogeny of *Foc* races based upon DNA fingerprinting

The phylogeny of the *Foc* isolates inferred from fingerprinting haplotypes in PAUP*4.0b4a (Sinauer Associated, Suderland, MA, USA) by neighbour-joining analysis (Saitou and Nei, 1987) indicated that pathogenic isolates grouped together in a clade clearly delineated from isolates non-pathogenic to chickpea. Within the pathogenic isolates, two clades clearly correlated with the yellowing and wilting pathotypes. Neither UPGMA nor neighbour joining trees showed any association between fingerprint lineages and geographic origin of isolates, except for grouping of

races 2, 3 and 4 which have been reported only from India (Fig. 1.8) (Jiménez-Gasco *et al.*, 2004).

Several studies indicate that the yellowing race 0 is probably ancestral to the wilting races because it is virulent on the fewest differential cultivars and is most wide spread in the Mediterranean region; although it has not been reported in the Indian subcontinent (Haware and Nene, 1982; Jalali and Chand, 1992; Jiménez-Diaz *et al.*, 1993). Moreover in *Foc*, which reproduces asexually, genetic variation results mainly from accumulation of mutations over time; indicating that greater diversity can be expected in the older lineages. Indeed previous RAPD analysis (Jiménez-Gasco *et al.*, 2001) is suggestive of race 0 being the ancestor of all races. Conversely, race 5 which shows the lowest diversity and is most virulent on many differential cultivars is the youngest amongst all races (Fig. 1.9) (Jiménez-Gasco *et al.*, 2002).

1.3.5 Genetic diversity studies among *Foc* isolates: Pathogenic races and their variability analysis

In the very initial studies using seven isolates of *Foc*, representing pathogenic races 1, 2, 3 and 4 from India and 0, 5 and 6 from Spain, restriction fragment length polymorphisms (RFLPs) in the mitochondrial DNA (mt DNA) using restriction endonucleases *Bam*HI, *Bgl*II, *Eco*RI, *Kpn*I, *Sac*I, *Sal*I, *Sma*I and *Xho*I, were conducted (Pérez-Artés *et al.*, 1995). But, no RFLP in the mt DNA was detected among the seven races of *Foc*. Next, studies using microsatellite markers to analyze genetic variability in four Indian races of the pathogen were accomplished. Hybridization patterns, revealed the presence of different repeat motifs in the *Foc* genome. (AGT)5, (ATC)5 and (GATA)4 were identified as the best fingerprinting probes for the *Foc* races. The distribution of microsatellite repeats in the genome revealed races 1 and 4 to be closely related at a similarity index value of 76.6%, as compared to race 2 at a similarity value of 67.3%; race 3 was very distinct at a similarity value of 26.7% (Barve *et al.*, 2001).

Honnareddy and Dubey (2006) further conducted a study to determine pathogenic and genetic variability of isolates of *Foc* collected from different parts of India. Pathogenic virulence study of 25 isolates of the pathogen on international set of differential cultivars was accomplished for characterization of new isolates in the known four races of the pathogen. Genetic variability within 24 isolates representing seven races of *Foc* was assessed by RAPD. At the same time, Singh *et al.*, (2006) analyzed thirty isolates of *Foc* obtained from rhizosphere soil of chickpea from different locations in Northern

India. The amount of genetic variation was evaluated by RAPD and IGS analysis. Genetic similarity between each of the isolates was calculated and results indicated that there was little genetic variability among the isolates collected from the different locations.

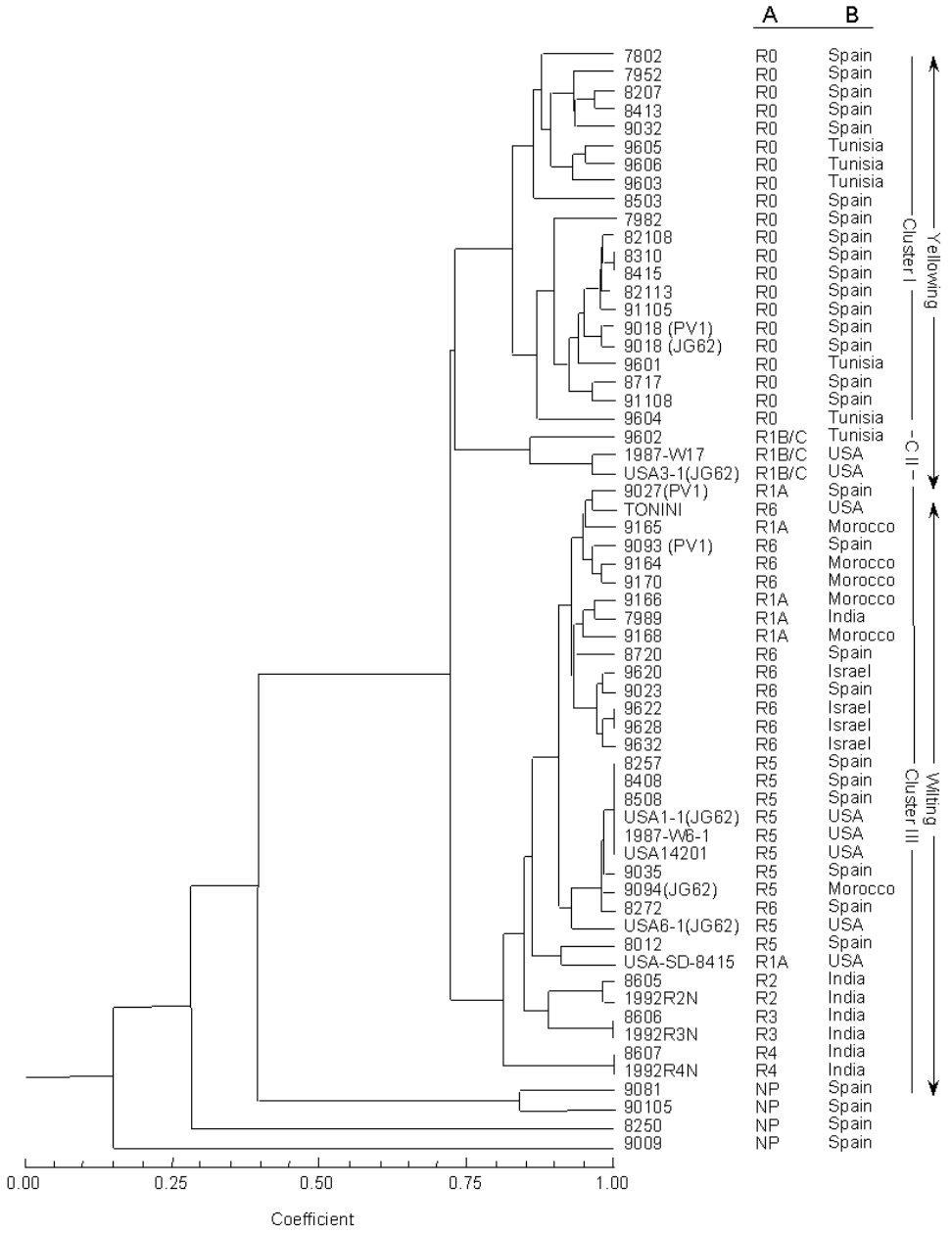


Fig. 1.8 Dendrogram derived from RAPD analysis of 57 *Fusarium oxysporum* f. sp. *ciceris* isolates and four *F. oxysporum* isolates nonpathogenic to chickpea using primers OPI-01, OPI-09, OPI-18, OPF-06, OPF-10, OPF-12, and OPF-16. The dendrogram was derived by UPGMA (unweighted paired group method with arithmetic averages). The bottom scale is the percentage of similarity by Jaccard's similarity coefficient. (A) Race assignment of an isolate: R0, race 0; R1A race 1A; R1/BC, race 1B/C; R2, race 2; R3, race 3; R4, race 4; R5, race 5; R6, race 6; NP, nonpathogenic *F. oxysporum*. (B) Geographic origin of isolates. (Jiménez-Gasco *et al.*, 2004)

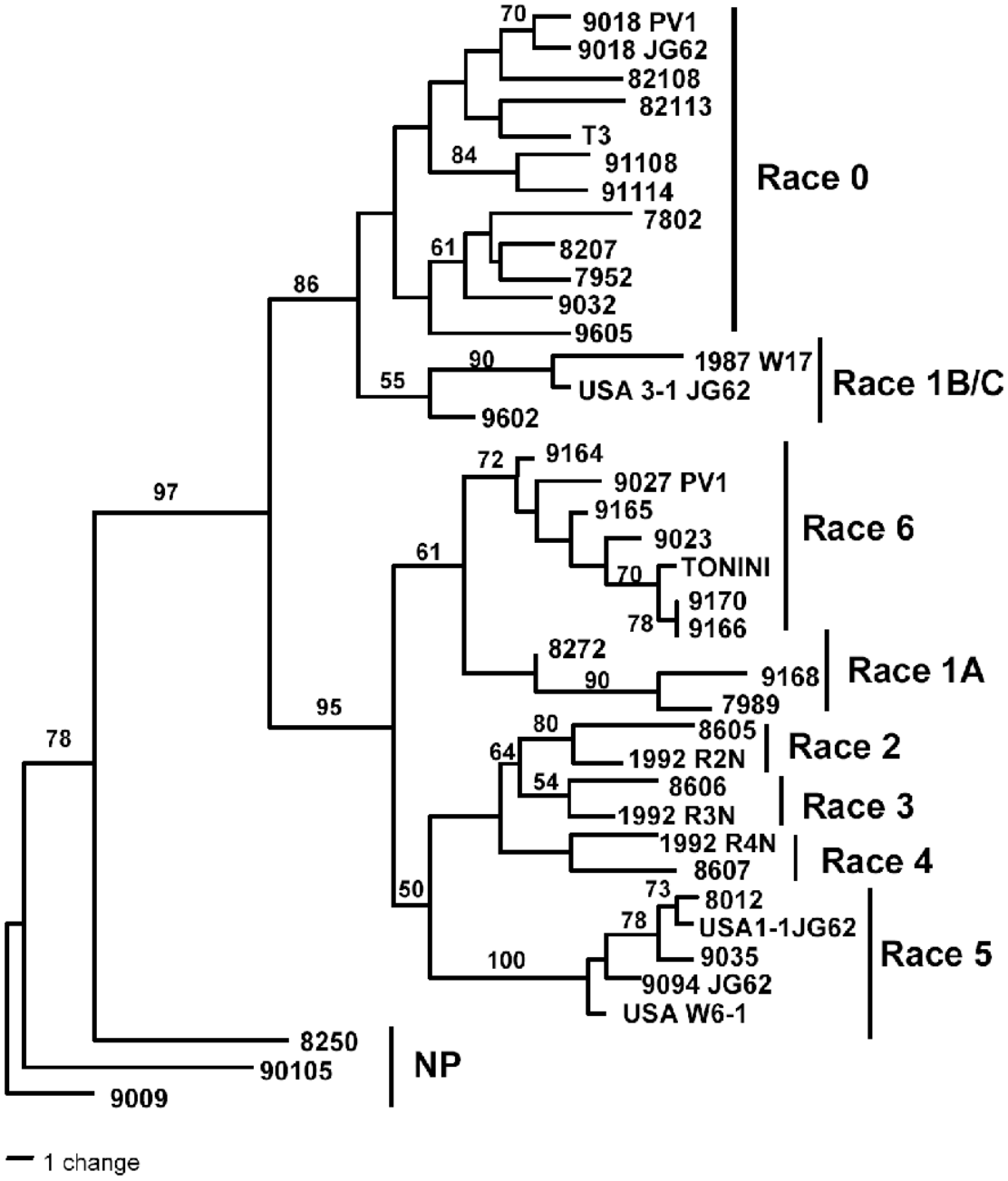


Fig. 1.9 Inferred phylogeny of races within *Fusarium oxysporum* f. sp. *ciceris* based neighbor-joining analysis of DNA fingerprint haplotypes. Fingerprints were generated by hybridization of genomic DNA to three repetitive probes: *Foc*B10, *Foc*O2 and *Foc*P18. Three *F. oxysporum* isolates (NP) nonpathogenic to chickpea were included for comparison. Numbers on branches represent bootstrap values >50% based on 1000 replicates.(Jiménez-Gasco *et al.*, 2002)

Similarly, genetic variability among 43 isolates of *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. These molecular markers established the distinctness of race 1 and race 2 pathogen isolates and the close similarity of pathogen isolates of race 3 with that of race 4. The high levels of DNA polymorphism observed with the molecular markers suggested the rapid evolution of new recombinants of the pathogen in the chickpea growing fields (Sivaramakrishnan *et al.*, 2003).

In a very recent study, 48 isolates of *Foc* collected from different chickpea growing regions in India were evaluated for genetic variations using amplified fragment length polymorphism (AFLP). Out of 48 isolates, 41 were found pathogenic and seven non-pathogenic. Pathogenic isolates differed in their virulence however; there was no apparent correlation between geographical origin and virulence of the isolates. UPGMA cluster analysis and principle coordinate analysis distinctly classified 48 isolates into two major groups; pathogenic and non-pathogenic. The pathogenic isolates could be further clustered into six major groups at 0.77 genetic similarities. Region specific grouping was observed with in few isolates (Sharma *et al.*, 2009).

1.3.6 Race concept in *Foc* and identification

Morphological identification of *Foc* into races is difficult. The classical method of race identification involves inoculation of differential chickpea cultivars with a particular *Foc* isolate and determining its pathogenicity (Fig. 1.10 and Table 1.1). This is a time consuming procedure requiring at least 40 days for the analysis and reactions can be influenced by various environmental parameters such as temperature and humidity (Haware and Nene, 1982). Also, there are several sets of differential cultivars available and some of the differentiation is based on intermediate reactions (Sharma *et al.*, 2005). To overcome these problems, several other approaches have also been attempted. Previously, serological and electrophoretic variability of proteins isolated from Indian *Foc* races has been studied by Desai *et al.*, (1992a). Based on antigens, they identified close relationships between races 1, 2 and 3, while race 4 was different. Biochemical analysis of the four *Foc* races has revealed variation in total sugar and amino acid content for race 3 as compared to races 1, 2 and 4 (Desai *et al.*, 1992b). DNA-based molecular markers have also been used for studying the variability in *Foc* races. Races 0, 1B, 1C, 5 and 6 were distinguished by RAPD fingerprinting (Jiménez Gasco *et al.*, 2001) and sequence characterized amplified regions (SCAR) markers have been

Fig. 1.10 Classical method of *Foc* race identification using chickpea differential cultivars

Table 1.1 Disease reaction of differential chickpea lines to inoculation with pathogenic races of *Fusarium oxysporum* f. sp *ciceris*

	Pathogenic race							
Diiferential chickpea line	**0**	**1A**	**1B/C**	**2**	**3**	**4**	**5**	**6**
12-071/10054	S	M	S	R	R	R	R	M
JG-62	R	S	S	S	S	S	S	S
C-104	M	M	R/M	S	S	S	S	M
JG-74	R	R	R	S	R	R	M	R
CPS-1	R	R	R	S	M	M	M	R
BG-212	R	R	R	S	M	M	R	R
WR-315	R	R	R	R	S	R	R	R
ICCV-2	R	R	R	S	S	S	S	M
ICCV-4	R	R	R	S	S	S	S	M
P-2245	S	S	S	S	S	S	S	S

Disease was evaluated on a 0-4 severity scale depending upon the percentage of affected foliar tissue (0=0%, 1=1-33%, 2=34-66%, 3=67-100%, 4=dead plant) at 40 days after sowing in an infested soil. Average disease reactions of <1 and >3 were considered resistant (R) and susceptible (S), respectively. Intermediate disease reactions were considered moderately susceptible (M) (Jiménez-Gasco *et al.,* 2004).

developed for races 0 and 6, while a race 5 specific identification assay has been developed using touchdown PCR (Jiménez-Gasco and Jiménez-Diaz, 2003). The *Eco*RI restriction patterns of nuclear ribosomal DNA of Indian *Foc* races has suggested that races 1 and 4 are more similar to each other than races 2 and 3 (Chakrabarti *et al.*, 2000). In recent years, variation in phytotoxicity of representative isolates of *Foc* has been examined (Gopalakrishnan and Strange, 2005). Although DNA marker based variability for Indian *Foc* races has been assessed to a great extent, however, molecular markers have not been developed as yet for these races.

1.4 Marker based approaches for identification of fungal races

RAPD markers have been used with success for the intraspecific characterization of several plant pathogens (Silva *et al.,* 1995; Talamini *et al.,* 2006; Saharan *et al.,* 2007). Attempts have also been made to identify specific races of *Fusarium oxysporum* in cotton (Fernandez *et al.,* 1994; Abd-Elsalam *et al.,* 2004). In studies conducted by Mesquita *et al.*, (1998) a strategy to identify race specific DNA bands for *C. lindemuthianum* was developed, in which DNA bulks containing samples of at least six isolates from each of three races of *C. lindemuthianum* (races 73, 65, and 64) were PCR amplified with random primers. Some of these primers revealed bands that were specific for each bulk.

Jiménez-Gasco *et al.*, (2001) concluded that *Foc* races 0, 1B/C, 5, and 6 can be characterized by the RAPD markers. Ninety-nine isolates of *Foc*, representative of the two pathotypes (yellowing and wilt) and the eight races described (races 0, 1A, 1B/C, 2, 3, 4, 5, and 6), were used in this study. Further, sequence characterized amplified regions (SCAR) markers were developed for races 0 and 6, while a race 5 specific identification assay was developed using touchdown PCR (Jiménez-Gasco and Jiménez-Diaz 2003). Among many PCR based marker systems, AFLP (Vos *et al.*, 1995) has found wide applications in analyses of genetic variation, particularly at and below the species level in investigations of population structure and differentiation (Müeller and Wolfenbarger, 1999). AFLP markers simultaneously detect variations at numerous loci and have been frequently used in studies on fungi (Majer *et al.*, 1996; Baayen *et al.*, 2000b; Jurgenson *et al.*, 2002; Zhong *et al.*, 2002). RFLP patterns have also proved useful for analyzing closely related species. In a study conducted previously, ITS region of rDNA of ectomycorrhizal fungi was examined using RFLP method and intraspecific polymorphisms in the ITS region were found in seven species (Kårén *et al.*, 1997). Further, ITS-RFLP studies conducted in *Cenococcum geophilum*

and *Pisolithus arhizus* confirmed intraspecific variation in these species, confirming the usefulness of this method (Farmer and Sylvia, 1998). In a very recent study, RFLP have also been used to distinguish variation amongst ITS region of isolates of *F. solani* f. sp *cucurbitaceae* race1, indicating their usefulness for identification of intra-specific as well as intra-racial variation (Alymanesh *et al.*, 2009). They characterized 33 pathogenic isolates of *F. solani* f. sp *cucurbitaceae* all of which belonged to race1. Amplification using ITS1-ITS4 primers with the race1 isolates followed by RFLP revealed intra-racial variation; and specific primers which were derived from the restriction digestion profile, furnished race1 specific amplification product of 505bp in all the isolates.

Development of SCAR markers from the race specific AFLP or ITS-RFLP derived fragments enables to discriminate *Foc* races. Also single nucleotide polymorphisms (SNPs) detected using any of these marker systems can greatly enhance the correct identification of these races without leaving any scope for misinterpretation. Further, use of gene targeted oligonucleotides which are specific for fungal genes can help in tracing the closely related fungal races. As the name suggests, these primers are specifically designed to amplify the gene of interest in the fungal genome. Variation in the primer binding region of two closely related fungal isolates (fungal races) can be traced using such primers. Since these primers are intended to amplify a single specific gene; they are useful in generating a reproducible, non-ambiguous, race specific and single band product. This technique though used previously for identification of *C. carbonum* race 1 (Jones and Dunkle, 1993) has not been widely used; but can prove to be the most targeted approach to identify very minor variations amongst fungal races.

1.5 Host pathogen interaction

A complex interaction between plant and its fungal pathogen is an outcome of expression of both, plant defense genes as well as fungal pathogenesis related genes. The result of such a relationship is projected either as host resistance or disease development in the plant. Plants generally reject attacking phytopathogenic fungi. A majority of plants are not readily colonized and parasitized by most pathogens. Such plants are non-host plants for the pathogen and exhibit immunity against them; since the pathogen is unable to surmount the barriers that prevent plant colonization. These various barriers comprise the basic resistance or basic incompatibility of the plant and are the first level of pathogen defense in a plant. The underlying mechanism for basic incompatibility may depend either on the plant, on the phytopathogenic fungus, or on

both. The successful parasitism by the fungus depends on the production of pathogenicity factors which it has acquired during its co-evolution with the plant. During the process of colonization the pathogen withdraws nutrients from its host plant and lives and multiplies at its expense; leading to biochemical changes in the plant causing disease symptoms like yellowing of leaves, wilting, necrosis or distortion, reducing the vitality of plant ultimately causing plant death. Basic compatibility is a highly specific phenomenon referring to only a particular plant species and the appropriate pathogen species or forma speciales (Niks, 1988).

In spite of the basic compatibility between plant and its pathogen; the host plant may deploy different defense strategies to limit pathogen attack. Firstly, in the hypersensitive reaction (HR) - the plant cells injured by the invading pathogen die very rapidly causing necrosis of the adjacent tissue; ensuring the pathogen to be cut off from the plant. In the second strategy, the plant cells survive pathogen attack and are induced to build up new defense barriers. This is a non-hypersensitive response often led by HR occurring in adjacent tissues (Klement and Goodman, 1967).

Presence of gene for resistance against a pathogen in a particular host plant during a compatible reaction can turn it into an incompatible reaction. Such kind of host resistance comes into action because the infecting pathogen induces defense reaction in the host plant and represents as the second level of pathogen defense. These defense mechanisms are highly specific for the pathogen since only certain races of the pathogen are rejected by the resistant cultivar. Hence this kind of defense is also termed as cultivar specific resistance. These block or restrain growth and reproduction of the pathogen thereby restricting the colonization in the plant. Host resistance presupposes the existence of basic compatibility between the plant and its pathogen (Flor, 1971).

In cases where a compatible interaction does not result in the appearance of disease symptoms on the plant, or does not evoke any resistance against the pathogen; the plant is said to exhibit tolerance against the pathogen. Many fungi exhibit biotrophy in the initial phase of the life cycle – obtaining nutrients from the metabolically active plant cells; and saprophytism in the later stages, when they acquire nutrients from the dead plant tissue. Cultivar specific resistance is often exclusively observed against attack by biotrophic pathogens (Prell and Day, 2001).

Basic resistance is directed against all pathogens in general, while cultivar specific resistance or host resistance is highly selective against only one pathogen species or forma speciales or race. Two types of host resistance genes are recognized, race-non-specific resistance (horizontal/uniform or generalized resistance) and race-specific

resistance (vertical/specific or differential resistance). Race-non specific resistance is directed against all members or races of a pathogen species, while race-specific resistance is mounted against only a particular pathogen race. In case of race-specific resistance; a specific mutation in the gene enables specific recognition/discrimination of a particular pathogen race from the other races. Thus, a phytopathogen must first be able to conquer basic resistance and then negate the race specific resistance of its host plant. Virulence is thus the ability of a phytopathogen to overcome race specific resistance and is acquired through gene mutation (Prell and Day, 2001).

A study enabling to determine the race specific resistance in a cultivar or cultivar specific resistance in a pathogenic race is essential. Such kind of study would help to understand the mutations necessary in both plant defense and fungal virulence genes for such an association/interaction in the host and its pathogen.

1.5.1 Plant defense

There are multiple events involved that lead to successful plant defense during pathogen attack. Further, these defense mechanisms are governed by an array of genes, which either singly or synergistically, are involved in plant resistance traits. Many defense related genes have been cloned and characterized in an attempt to elucidate the mechanism of defense upon *Fusarium* attack in various plant species, including chickpea. For example, defense related genes like basic glucanases, *PAL*, *CHS* etc., involved during *Foc* 1 and *Foc* 0 infections have been previously studied in chickpea (Cho and Muehlbaur, 2004; Arfaoui *et al.*, 2007). In our earlier studies, enzymes like glucanases, chitinases and proteases have been shown to be probably involved in chickpea defense against *Foc* infection (Giri *et al.*, 1998). Further, various up-regulated transcript derived fragments like 14-3-3, WRKY and NBS-LRR type sequences as well as transposable elements, were identified using cDNA-RAPD and cDNA-AFLP techniques (Fig. 1.11) (Nimbalkar, 2007). However, exact molecular mechanisms involved in chickpea wilt resistance are still unexplored.

1.5.2 Fungal pathogenesis

To colonize and parasitize a plant, fungi must be able to invade the plant successfully; combating all the barriers presented by the host plant. For the same, fungi express pathogenicity genes/factors which help the fungi to establish successfully in the host plant. These pathogenicity factors are essential only for infecting the plant and are not required for normal growth of the fungi; being distinct from the “house keeping” genes

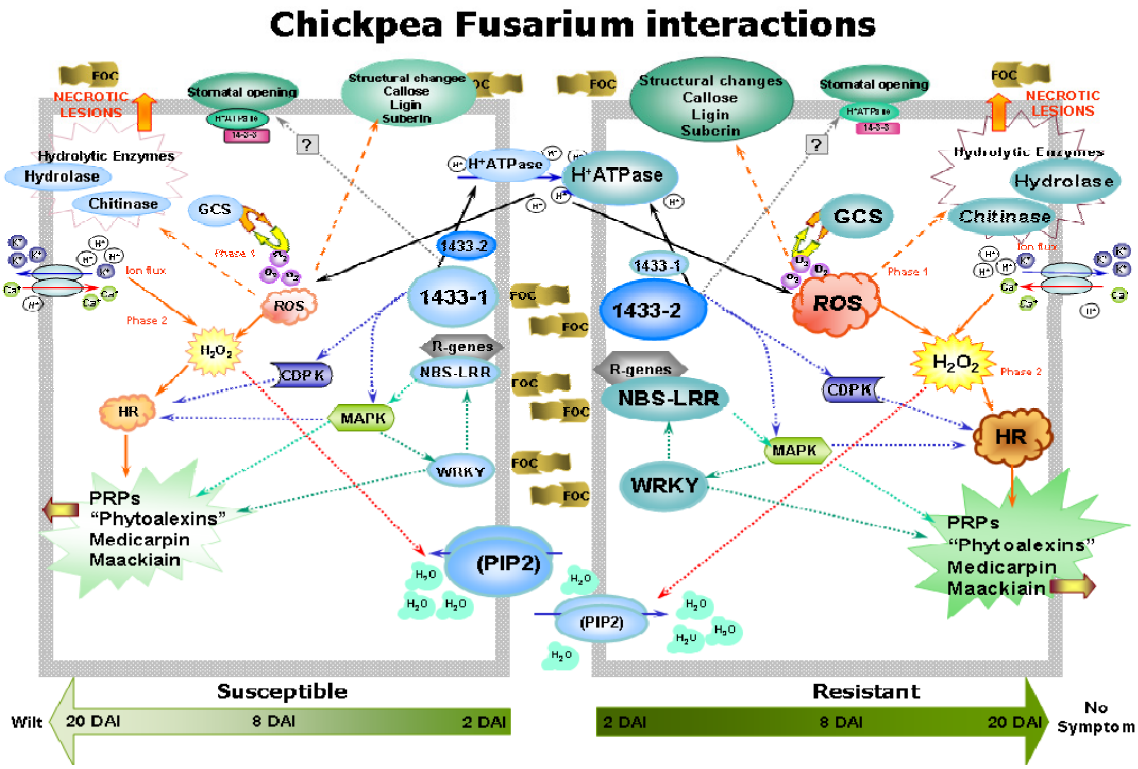


Fig. 1.11 Host pathogen interactions (Nimbalkar, 2007, Ph.D. thesis)

of the fungal pathogen. The pathogenicity genes can quote for varied functions such as penetration, formation of specialized invasion structures and nutrition availability. Thus more than one pathogenicity factors are essential for mounting disease in the host plant. Expression of pathogenicity factors can often lead to counter defense from the plant and can thus prevent the pathogen from successfully colonizing the host plant. Generally, a pathogen's virulence/pathogenicity genes do not turn off the expression of host cell defense genes; rather they enable the pathogen to negate the host defense genes. For example, *Xyl3* gene encoding for xylanase enzyme helps the phytopathogenic fungi in plant cell wall degradation (Ruiz-Roldan *et al.,* 1999), but still the plant can synthesize xylan enabling it to re-build its cell wall.

Understanding the pathogenicity mechanism of fungi, demands knowledge of the virulence factors, which are active in the host environment. Till date many pathogens have been studied in context with their virulence aspects and various genes have been identified, which have a prime role to play in fungal pathogenesis. Various G protein subunits have been reported to be necessary for fungal morphogenesis, development as well as virulence (Seo *et al.*, 2005). Idnurm and Howlett, (2001) have tabulated 79 genes of phytopathogenic fungi which are specific for virulence. Since then many more genes have been identified, specific for the virulence of *Fusarium* spp. Genes like *Rho1* and *Frp1* encoding GTPase and F-box protein, respectively, are required for

pathogenicity of *Fusarium* spp (Duyvesteijn *et al.*, 2005; Martínez-Rocha *et al.*, 2008). Also, chitin synthase genes (Madrid *et al.*, 2002) and transcription factors like *Ftf1* (Ramos *et al.*, 2007) and *PacC* (Caracuel *et al.*, 2003) are shown to be essential for virulence of *Fusarium*. Since then, few more genes have been studied and their contribution in *F. oxysporum* pathogenesis has been established. Specifically, in *F. oxysporum* f. sp. *lycopersici* genes like *ARG1*, *Fow1* and *Fow2* (Namiki *et al.*, 2001; Iori *et al.*, 2002 and 2007), *Six1*, *Six2*, and *SSH1* have been found to be involved in its virulence (van der Does *et al.*, 2008). However, in-depth search of mechanisms and the genes involved in pathogenicity is essential in wilt causing root pathogens. There have been a few efforts, using gene disruption and RNAi technologies, to throw light on the mechanisms of pathogenicity. In earlier studies conducted, cutinase gene disruption resulted in a significant decrease in the pathogenicity of *F. oxysporum* f. sp *pisi* on pea (Rogers *et al.,* 1994). Similarly, targeted inactivation of *Fmk1* gene in *F. oxysporum* f.sp *lycopersici* lead to loss of virulence (Di Petro *et al.,* 2001). The *chsV* gene was identified in an insertional mutagenesis screen for pathogenicity mutants.

The *chsV* insertional *F. oxysporum* mutant displayed morphological abnormalities such as hyphal swellings, indicative of alterations in cell wall structure. The mutants were unable to infect and colonize tomato plants or to grow invasively on tomato fruit tissue. Reintroduction of a functional *chsV* copy into the mutant restored the growth phenotype of the wild-type strain (Madrid *et al.,* 2002). In a transformation experiment conducted, two Aspergilli (*A. flavus* and *A. parasiticus*) and a Fusarium (*F. graminearum)*, with inverted repeat transgenes (IRT) containing sequences of mycotoxin-specific regulatory genes, suppressed mycotoxin production in all three plant-pathogenic fungi. *F. graminearum* IRT strains were less virulent and did not produce toxin on wheat as opposed to the wild type. These results indicate the existence of RNAi mechanism in fungi and its importance in understanding fungal pathogenicity (McDonald *et al.,* 2005).

1.6 Approaches to study plant defense and fungal virulence

Plant-pathogen interactions play an important role in agriculture and a lot of effort has been dedicated to analyze these interactions in detail. Varoius transcriptomic, proteomic as well as metabolomic approaches have helped in achieving the target of understanding this complex interaction (Fig. 1.12). With the advent of the large-scale genomic sequencing and expressed sequence tag (EST) projects, and with the development of DNA microarray technologies, it is now possible to monitor the expression of hundreds or thousands of genes simultaneously. This can be done under

different defense-related treatments and over different time points. The technologies open up tremendous opportunities to identify new pathogenesis- related genes, to identify co-regulated genes and the associated regulatory systems, and to reveal interactions between different signaling pathways (Harmer *et al.,* 2001; Kazan *et al.*, 2001). Cellular or organismal roles can be proposed for gene products with no previously identified function, or added functions can be proposed for previously studied genes. Similarly, using proteomic as well as metabolomic approaches, spatial and temporal expression of proteins as well as metabolites during host-pathogen interactions can be analyzed.

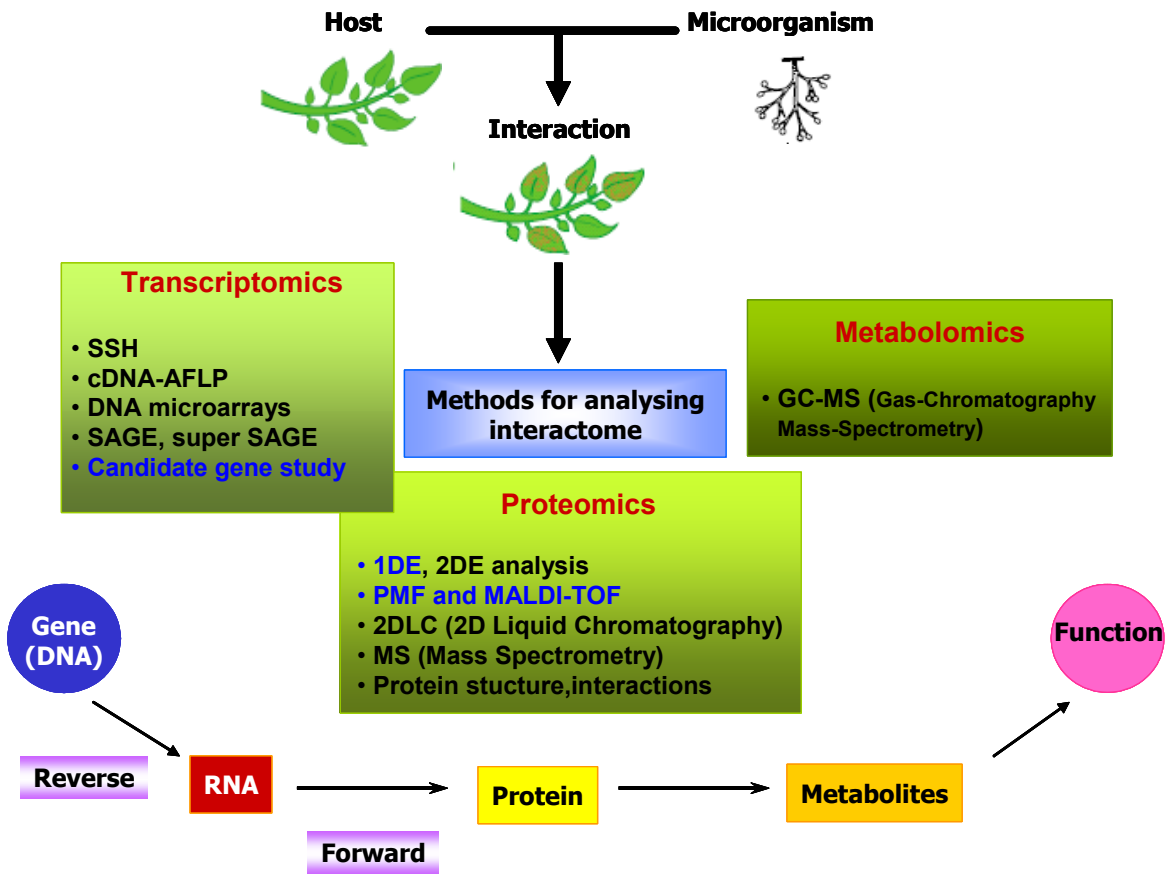


Fig. 1.12 Various approaches to study the Chickpea-*Fusarium oxysporum* interactome

1.6.1 SSH

Substractive hybridization is a technology that allows for PCR-based amplification of only cDNA fragments that differ between a control and experimental transcriptome. Differences in relative abundance of transcripts are highlighted. The technique relies on the removal of dsDNA formed by hybridization between a control and test sample, thus eliminating cDNAs or gDNAs of similar abundance, and retaining differentially

expressed, or variable in sequence, transcripts or genomic sequences. The technique can be efficiently used to determine the upregulated transcripts in the pathogen infected host plant tissue as compared to the uninfected one. This technique has been used to identify *Medicago truncatula* putative defense genes in response to *Orobanche crenata* parasitization (Die *et al.*, 2007). Recently, *Cicer arietinum* L. defense responses against *Helicoverpa armigera* feeding, transcript patterns elicited by both herbivore and mechanical wounding were profiled and compared, and the application of defense regulators was assessed using various techniques, including SSH (Singh *et al.*, 2008) and upregulation of salicylic acid and methyl-jasmonic acid was observed.

1.6.2 cDNA-AFLP

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. Since then it has been used to profile genes in a range of different systems including humans (Egert *et al.*, 2006), animals (Fukuda *et al.*, 1999; Vandeput *et al.*, 2005), plants (Carmona *et al.*, 2004; Kemp *et al.*, 2005; Diegoa *et al.*, 2006) and microbes (Qin *et al.*, 2000; Decorosi *et al.*, 2005). cDNA-AFLP remains a useful technique for several reasons; it is versatile, easy, inexpensive, robust and quantitative (Reijens *et al.*, 2003). Nimbalkar *et al.*, (2006) compared transcript profiles generated from three chickpea root cDNA libraries, viz., uninfected WR-315 (WR-C),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.

1.6.3 Serial Analysis of Gene Expression (SAGE)

The complex interaction between a microbial pathogen and a host is the underlying basis of infectious disease. By understanding the molecular details of this interaction, we can identify virulence-associated microbial genes and host-defense strategies and characterize the cues to which they respond and mechanisms by which they are regulated. SAGE (Velculescu *et al.*, 1995) is one of the most powerful techniques for quantitative and comprehensive transcriptome analysis of host-pathogen interaction and to identify the transcripts and genes involved in resistance mechanism. Hence, it is an ideal tool to study host-pathogen interactions, particularly where a number of resistance genes involved and their products or their individual phenotypic effects are not known. Thomas *et al.*, (2002) applied SAGE for transcript profiling in the barley mildew

pathogen *Blumeria graminis* and assigned 1,274 tags to EST clones. Matsumura *et al.*, (2003) used SuperSAGE to perform gene expression analysis of host–pathogen interactions in *Magnaporthe grisea* (blast)-infected rice leaves.

1.6.4 DNA microarrays

First described in 1995 (Schena *et al.*, 1995), high-density DNA microarray methods have already made a marked impact on many fields, including host pathogen interaction studies. The key unifying principle of all microarray experiments is that labeled nucleic acid molecules in solution hybridize, with high sensitivity and specificity, to complementary sequences immobilized on a solid substrate, thus facilitating parallel quantitative measurement of many different sequences in a complex mixture (Brown and Botstein, 1999; Southern *et al.*, 1999). DNA microarray techniques are particularly suitable for monitoring gene expression changes in plants during plant-pathogen interactions, due to their relative simplicity, comprehensive sampling capacity and high throughput (Kazan *et al.*, 2001). The most attractive feature of DNA microarray techniques is that they allow researchers to examine the responses of hundreds or thousands of genes simultaneously during a given treatment. Using these expression profiles, it is possible to identify differentially present mRNA species and to hypothesize potential defense-associated function based on this differential expression. Recently, a small number of DNA microarray experiments have identified an amazing number of potential defense-related genes. Although some of these genes have previously been implicated in plant defense responses, most have not. The derived amino-acid sequences of some of these genes have significant similarity to known proteins, but many of the genes encode hypothetical or unknown products. Using a maize DNA microarray representing 1,500 maize genes, Baldwin *et al.*, (1999) identified 117 genes that consistently showed altered mRNA expression in maize 6h after various treatments with the fungal pathogen *Cochliobolus carbonum*. *Arabidopsis* is a particularly well-developed experimental system that has been utilized in a number of studies. In an important early study that examined gene expression changes in *Arabidopsis* under 14 different SAR-inducing or repressing conditions, including a notable focus on plant mutants, Maleck *et al.*, (2000) identified 413 ESTs that appeared to be associated with SAR. This study used a cDNA microarray containing 10,000 ESTs representing approximately 7,000 genes, or 25–30% of all *Arabidopsis* genes.

1.6.5 Candidate gene study for analyzing host-pathogen transcriptome

The above mentioned methodologies help in unrevealing the novel transcripts involved during host pathogen interaction. Further studies are directed towards understanding the role of such novel genes during the interaction. Analyzing the expression of plant defense related genes or fungal pathogenesis genes which have been known to be involved in such an interaction; as well as characterizing new genes for their role in the host pathogen interaction can enable the in-depth search of functions of such genes in the interactome.

1.6.6 Metabolomics

Constituents of the metabolome reflect the cellular processes that control the biochemical phenotype of the cell, tissue or whole organism. Measurements of intracellular metabolites, whether qualitative or quantitative, reveal the biochemical status of an organism and in turn can be used to monitor and assess gene function (Fiehn *et al.*, 2000). Within functional genomics, metabolomics has emerged as a robust approach to predict gene activity. At present, it is impossible to quantify all the metabolites within a cell, tissue or organism of any kingdom in any system. The problem lies within the fact that not any single extraction technique or analytical instrument can isolate and detect every metabolite within a biological sample (Sumner *et al.*, 2003). These problems are further compounded by issues such as human error in sample preparation and extraction, sample storage and instrument reproducibility (Dunn and Ellis, 2005). Metabolomics is further complicated by the huge diversity of metabolites in any given species. As a result of the huge variation between the interactions of plants with various interacting organisms (pathogenic or non-pathogenic), there is a huge variance in the biochemical responses of the plant (i.e. initial signaling cascades leading to either a mutually beneficial response with a symbiont or a targeted biochemical defense against a pathogen or grazer). Thus, plant–pathogen interactions are not only extremely interesting in terms of metabolite richness but also extremely challenging with regard to the huge diversity of different chemical classes (requiring differing technologies for their comprehensive coverage) and also in terms of identifying the individual metabolite changes associated with the host and interacting organism within a dynamic system.

Bednarek *et al.*, (2005) used *A. thaliana* wild-type and mutant root cultures infected by the root-pathogenic oomycete *Pythium sylvaticum* to investigate aromatic metabolite profiles. Sixteen indolic, one heterocyclic and three phenylpropanoid compounds were

screened. The relative levels of most of the indolics greatly increased on infection, whereas the three phenylpropanoids decreased. These data indicated that roots differed greatly from leaves with regard to the nature and abundance of major soluble phenylpropanoid metabolites. However, for indolics, by contrast, the data indicated a close similarity between roots and leaves.

1.6.7 Proteomics

Proteomic analysis of differential proteins activated upon pathogen attack can be important to determine the role of such molecules in plant defense. Previously, alterations in the proteome of Arabidopsis (*Arabidopsis thaliana*) leaves during responses to challenge by *Pseudomonas syringae* pv *tomato* DC3000 have been analyzed using two-dimensional gel electrophoresis (Jones *et al.*, 2006). Similar studies in chickpea in response to fusarium wilt, ascochyta blight or other pathogens can highlight the potential targets, which can be used for pyramiding into the elite cultivars or for developing transgenic chickpea. Further the approach is suitable not only for studying defense related proteins but can also track the virulence related proteins associated with fungal pathogenesis. Recently, through comparative proteomics analysis identification of 134 differentially expressed proteins that include predicted and novel dehydration-responsive proteins was accomplished in chickpea. The study highlighted that over a hundred extra-cellular matrix proteins, involved in a variety of cellular functions, like cell wall modification, signal transduction, metabolism, cell defense and rescue, are essential for the molecular mechanism of dehydration tolerance in plants (Bhushan *et al.,* 2007).

1.7 Objectives of the thesis

The devastating damage caused by fungal pathogen *Fusarium oxysporum* f. sp. *ciceri* (*Foc*) to the economically and nutritionally important chickpea crop, projects the need to understand the pathogen in detail. At the same time it becomes essential to configure the host pathogen interactions so as to combat the disease in a better way. The thesis work was initiated with the following objectives:

1) Identification of Indian pathogenic races of *Foc* using various molecular marker approaches:

- Use of Gene specific oligonucleotides (GSOs), ITS-RFLP and AFLP markers to differentiate *Foc* races 1, 2, 3 and 4

2) Proteome analysis of *Foc* races

3) Differential expression of fungal virulence genes during chickpea wilt

- Temporal expression of virulence genes during infection of resistant and susceptible cultivars
- Race specific differential expression of fungal virulence genes

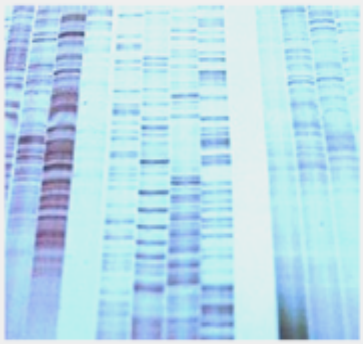
4) Transcriptome analysis of chickpea for differential expression of plant defense related genes during Fusarium wilt:

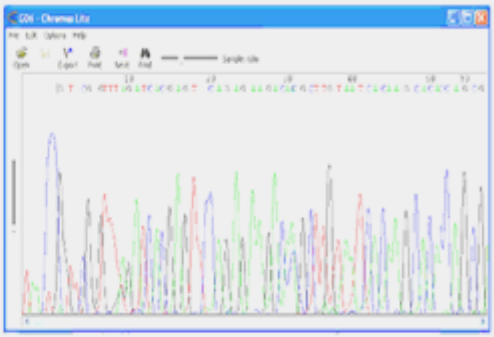
- Use of random primers for revealing plant defense related genes from chickpea
- Analyzing the temporal expression of defense related genes in resistant and susceptible cultivars upon infection with *Foc* races 1, 2 and 4

1.8 Organization of the thesis

In pursuit of achieving these objectives, in my thesis, I have tried to explore both the pathogen as well as the host plant systems. The thesis is organized into five chapters, the first being the introduction to the chickpea plant and the pathogen (*Foc*) which form the basis of this study; and a detailed review of the techniques currently available to study their interactome. Next, I have described the materials and methods used for the experimental design, which comprises the second chapter of the thesis. The third chapter elucidates the results wherein I have used three approaches namely; AFLP, ITS-RFLP and gene specific oligonucleotides to identify the closely related Indian pathogenic races of *Foc*. In this work, proteomics approach has also been used to differentiate *Foc* races at the proteome level. Also, I have used candidate gene approach for unveiling the functional role of plant defense related as well as fungal pathogenesis related genes during chickpea-*Foc* interaction.

The fourth chapter includes the discussion of my results and the significance of this work. In the fifth and final chapter I have summarized the gist of the work and set directions for the future work; laying a foundation to further explore this interesting host-pathogen relationship. Literature used in this study has been detailed at the end of the thesis.





MATERIALS AND METHODS

2. Materials and methods

2.1 Fungal cultures

F. oxysporum f. sp. *ciceris* (*Foc*) standard races 1 (NRRL 32153), 2 (NRRL 32154), 3 (NRRL 32155 characterized as *F. proliferatum* in this thesis work) and 4 (NRRL 32156), isolates 10, 16 (race1), isolates 9, 4 (race2), isolate Fu-7 (race 3 like NRRL 32157) and isolate 39 (isolate not typed for race, NRRL 32158) and *F. solani*, and *F. udum* isolates were obtained from International Crops Research Institute for Semi Arid Tropics (ICRISAT), Patancheru, India. *Foc* isolates were collected from various hot-spot regions in the country by ICRISAT, India (Fig. 2.1). *Foc* isolates were characterized and classified according to the conventional method of race identification using cultivar specificity (Haware and Nene, 1982) at ICRISAT, India. In addition, the identity of the cultures was confirmed by CABI Biosciences U.K., formerly Commonwealth Mycological Institute. These cultures were a kind gift from Dr. M.P. Haware, pathologist, ICRISAT, India. The cultures were maintained on Potato Dextrose Agar (PDA) slants with regular sub-culturing. Fig. 2.2 shows the growth patterns of four *Foc* races on PDA plates.

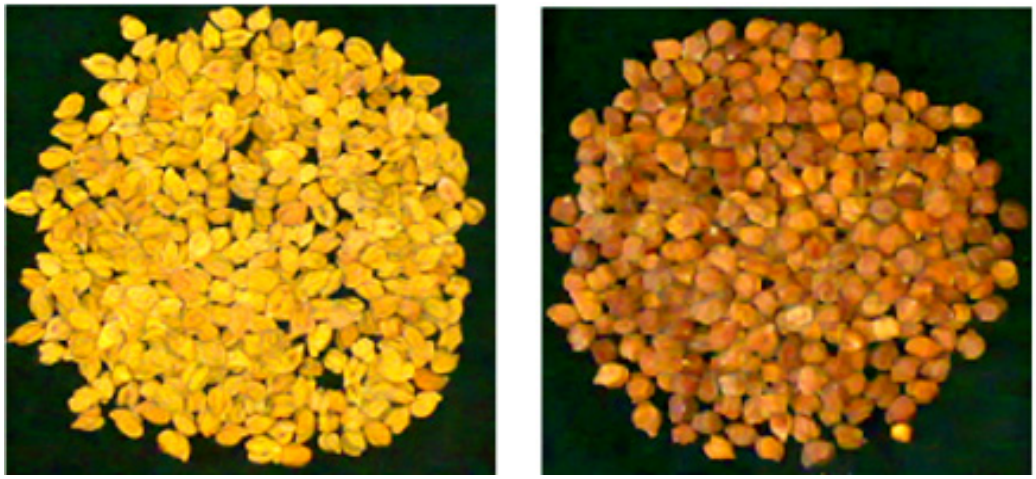
2.2 Chickpea seed

Cicer arietinum seeds of cultivar JG62 and Digvijay (Fig. 2.3) were obtained from Mahatma Phule Krishi Vidyapeeth (MPKV), Rahuri.

JG62 is highly susceptible to wilt, and shows features like twin podding and early maturity. It has medium sized seeds. Genotype Phule G 9425-5 was developed at Mahatma Phule Krishi Vidyapeeth (MPKV), Rahuri, Maharashtra in year the 2005 from a cross of Phule G- 91028 x Bheema. Due to its high performance and superior quality when compared to standard cultivar, Vijay, it has been named as Digvijay. It shows high average yields (19,00g/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 check varieties 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. Its performance under rainfed conditions is equivalent to that of Vijay.

Fig. 2.1 Collection sites of various *Foc* races across India

Fig. 2.2 Growth patterns of Indian *Foc* races on PDA plates



JG62 seeds **Digvijay seeds**

Fig. 2.3 *C. arietinum* cultivars JG62 and Digvijay used in the present study

2.3 Methodologies used in the present study towards identification of *Foc* races

2.3.1 DNA isolation and quantification

Each individual *Foc* race was inoculated in Potato Dextrose Broth (PDB) and grown at 25^0 C for four days with sufficient shaking at 200 rpm and aeration. The mycelial mass was harvested by vacuum filtration on sterile Whatmann No.1 filter discs, washed thoroughly by passing two to three volumes of sterile water, frozen in liquid nitrogen and crushed to a fine powder. Genomic DNA was extracted from each culture using a Cetyl trimethylammonium bromide (CTAB) method with slight modifications (Barve *et al.*, 2001). The fungal mycelial powder was suspended in a buffer containing 25mM Tris-HCl pH 8.0, 10mM EDTA, and 1% βME. EDTA and SDS were added at final concentration of 60mM and 1%, respectively and incubated at 57^0 C for 10 min with intermittent mixing. The slurry was reincubated at 57^0 C for 10 min with NaCl and CTAB (final concentrations of 1.4M and 2%, respectively), followed by two rounds of chloroform:isoamylalcohol (24:1) extractions. The aqueous supernatant was aspirated and precipitated with 0.6 volumes of isopropanol. The pellet obtained after centrifugation was dissolved in high salt TE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA, 1M NaCl) and reprecipitated with 2.5 volumes of absolute ethanol. DNA samples were dried under vacuum and dissolved in TE buffer (10mM Tris-HCl, 1mM

EDTA, pH 8.0), followed by incubation with RNase A at a concentration of 100µg/ml at 37^0 C for 1h. The concentration and purity of DNA were estimated spectrophotometrically at 260nm (and at 230, 280 and 300nm), as well as by agarose gel electrophoresis, in which the samples were compared with known concentrations of λ DNA.

2.3.2 AFLP analysis

AFLP analysis was carried out using the AFLP^{TM} Analysis System I (Gibco-BRL, Life Technologies, USA) as per the manufacturer's instructions with slight modifications as stated below. 500ng of *Foc* genomic DNA of individual race and isolates was digested with *Eco*RI and *Mse*I enzymes at 37^0 C for 6h and ligated overnight to respective adaptors. The ligated products were diluted 1:25 in TE buffer, of which 2µl was used as a template in a pre-amplification reaction with *EcoR*I+A and *Mse*I+C oligonucleotides. The pre-amplification reaction was diluted 1:20 and 5µl of it was used as a template for further selective amplification reactions. The PCR products (10µl) were electrophoresed on 6% denaturing polyacrylamide gel containing urea. Fragments were detected by the silver staining technique of AFLP fragment visualization (Chalhoub *et al.*, 1997). All AFLP PCRs were carried out on PTC-200 thermocycler (MJ Research, USA). Only those oligonucleotide combinations, which yielded polymorphic bands, were selected for further analysis. These combinations were used with two isolates of each of races 1 and 2. Oligonucleotides that were polymorphic in either of the standard races as well as their respective isolates were selected for further cloning and sequencing.

2.3.3 Silver staining

The polyacrylamide gel was silver stained after fixing in 10% acetic acid for 1 hour followed by 2 washes with single distilled water. Then the gel was transferred to silver stain (2g $AgNO_3$, 800µl of 10mg/ml Na_2CO_3 and 3ml of 37% HCHO per 2L of silver stain) for 1h in dark followed by 10 s wash with single distilled water. The gel was developed in chilled developer (800µl of 10mg/ml Na_2CO_3 and 6ml of 37% HCHO per 2L of developer). Once the DNA bands were clearly visible, the reaction was stopped with 10% acetic acid (Chalhoub *et al.*, 1997).

2.3.4 Excision and elution of race specific DNA fragments from polyacrylamide gels

The race-specific bands were eluted from the silver stained polyacrylamide gels by crushing the specific gel pieces in 40µl water and heating at 95^0 C for 5 min (Smith, 1980). Part of this reaction (5µl) constituted the template for a reamplification reaction with the 10µM of same selective oligonucleotides previously used for identification of the race-specific bands and 10mM of each dNTP, 1X PCR buffer, 0.8U *Taq* DNA Polymerase (Bangalore Genei, India) on PTC-200 thermocycler (MJ Research, USA). The PCR conditions were as follows: initial denaturation at 94^0 C for 5 min, followed by 25 cycles of 94^0 C for 1 min denaturation, 56^0 C for 30 s annealing and 72^0 C for 30 s extension. Final extension was carried out at 72^0 C for 10 min. The reamplified product was precipitated with absolute ethanol and the pellet was washed with 70% ethanol and air-dried. The pellet was dissolved in 10µl of water and then cloned in pGEM-T easy vector system I (Promega, USA).

2.3.5 Cloning of amplified products

Cloning of desired products in pGEM-T easy vector system-I was accomplished, maintaining the insert to vector ratio as 3:1 as per the manufacturer's instructions (Promega, USA). The ligation reaction was carried out using 1µl vector, 3µl product, 5µl 10X ligation buffer and 1µl T4 DNA ligase. The reaction was kept at 16^0 C overnight.

2.3.6 Transformation

5µl of ligation mixture was added to the competent cell solution and incubated on ice for 30 min. The tubes were transferred to a preheated 42^0 C circulating water bath for 90 s. The tubes were then quickly transferred to ice bath and the cells were allowed to cool for 15 min. 800µl of LB medium was added to each tube and the cultures were warmed to 37^0 C in a water bath, and then transferred to shaking incubator set at 37^0 C. The cultures were incubated for 45 min to allow the bacteria to recover and to express the antibiotic (ampicillin) resistance marker encoded by the plasmid. 200µl of transformed competent cells were then transferred onto LB agar plates containing X-gal, IPTG and ampicillin. The plates were incubated at 37^0 C in inverted position for 12-16 hours. The transformed white colonies were picked up and sub cultured, as well as used for colony PCR, to confirm the presence of insert, followed by plasmid isolation (Maniatis *et al.*, 1982).

2.3.7 Colony PCR

The transformed colonies were picked up with the aid of a sterile toothpick or pipette tip, added to each reaction tube and mixed well. The PCR conditions were set to 94^0 C, 5 min of initial denaturation, 35 cycles of 94^0 C for 1 min, 45^0 C for 45 s, and extension at 72^0 C for 1 min, followed by final extension at 72^0 C for 5 min. The PCR products were visualized on a 1.5% agarose gel. The gels were documented using Geldoc system (Amersham, USA or Syngene, USA).

2.3.8 Plasmid preparation (alkaline lysis method)

Positive transformants, which appeared as white colonies and which showed presence of the desired gene fragment in colony PCR were selected for plasmid isolation (Maniatis *et al.*, 1982). A single white colony was inoculated in 5 ml LB broth containing 100µg/ml ampicillin. The culture was incubated overnight at 37^0 C with vigorous shaking at 250 rpm. The cells were harvested into a 1.5 ml microfuge tube by centrifugation at 5000 rpm for 2 min at room temperature. To the bacterial pellet, 100µl of ice cold Solution 1 (25mM Tris-8.0, 10mM EDTA, and 50mM glucose) was added and the cells were resuspended by vortexing for 10 s. To the dispersed cells, 200µl of freshly prepared Solution 2 (0.2M NaOH, 1% SDS) was added and mixed well by inversion, followed by the addition of 150µl of Solution 3 (3M KOAc, pH 5.5) and mixing by inversion. The mixture was vortexed for 10 s. Bacterial cell debris was pelleted by centrifugation of the sample at 12,000 rpm for 10 min at 4^0 C. The supernatant was transferred to a fresh microfuge tube and 0.3ml of phenol-isoamyl alcohol-chloroform was added and vortexed. This was then centrifuged at 4^0 C with 14,000 rpm for 5-10 min and the supernatant was transferred to a new tube. Care was taken not to pipette out the phenol phase. Further, 0.7ml of isopropanol was added. The mixture was kept at 4^0 C for 15 min. The plasmid DNA was pelleted by centrifuging at 12,000 rpm for 25 min at 4^0 C. The supernatant was discarded and to the pellet 500µl of 70% ethanol was added and centrifuged at 10,000 rpm for 5 min at 4^0 C. The pellet was air-dried and 40µl of sterile Milli-Q water was added to dissolve the pellet. The sample was then treated with 2µl of RNase A and incubated at 37^0 C for 1h to remove the RNA. The plasmids were visualized on 0.8% agarose gels.

2.3.9 DNA sequencing

Cloned cDNA fragments were bidirectionally sequenced by dideoxy termination method using the DYEnamic ET sequencing kit and the MegaBACE 1000 DNA Analysis

System (GE Healthcare, USA). The nucleotide sequences were compared with the reported nucleotide sequences of Genbank nonredundant database using the BLAST sequence alignment program (Altschul *et al.*, 1990).

2.3.10 Development of SCAR markers from AFLP derived race specific fragments

Using the sequence information generated from race specific AFLP fragments homologous sequences were aligned by CLUSTALX 1.83 software (Thompson *et al.,* 1997). Race specific primers were designed from such sequences (Table 2.1). These primers were used for amplification of *Foc* races 1 and 2 and their respective isolates. The PCR conditions were as follows: initial denaturation at 94^0 C for 5 min, followed by 25 cycles of 94^0 C for 1 min denaturation, 60-65^0 C for 1 min annealing and 72^0 C for 1 min extension. Final extension was carried out at 72^0 C for 10 min. PCR reagents used were as follows: 40ng of *Foc* genomic DNA, 10mM of each dNTP, 1X PCR buffer, 0.8U *Taq* DNA Polymerase (Bangalore Genei, India) and 10µM of each primer (forward and reverse). The annealing temperature was varied according to the T_m of the oligonucleotide used. The PCR products were visualized on 1.5% agarose gels by electrophoresis in TAE buffer and documented.

Most of these primer pairs either furnished multiple banding profiles with all *Foc* races as well as isolates or one band amplified in both the races and their isolates. Thus, the SCAR primers were not used in further studies.

Table 2.1 SCAR primers designed from AFLP fragements

Primer name	Primer sequence (5'-3')
2D18F	GAC ACT CCT CTT ACA CAC TTC CAC
2D18R	CAG TGT TTC TGT ACC ATA TGC AAC
2D-22F Hop78	CTT TTG GCA TGA GAT TGT AGC CTC
Hop78R	CGT GGG GTT ATA CCT CTA GGC TA
2D-22R Hop78	AGT CAG GGA TAT AAC GCT TCT TG
Hop78F	ATG GAC TCA ATT GGC ATT CAC CG
2D-22R	AGG TTT CTG TGC CAT ATG CAA CGG
2D-23F	CAG GTT TCT GTA CCA TAT GCA ACG
2D-23R	GAG TTC CTG AGA TGA GAG TTC TCG
2D-33F	GGT AAA GAT GCC ATT TTT AGA AGG
2D-33R	AGT TCC TGA GAT GAG AGT TCC TGG

2.3.11 ITS-PCR

PCR amplification of ITS regions using universal ITS oligonucleotides (ITS1- 5′ TCCGTAGGTGAACCTGCGC 3′ and ITS4- 5′ TCCTCCGCTTATTGATATGC 3′, which flank the ITS1, 5.8S and ITS2 region) (White *et al.*, 1990) was carried out in a PTC-200 thermocycler (MJ research, USA) with initial denaturation of 94^0 C for 5 min, followed by 30 cycles of 94^0 C for 1 min denaturation, 55^0 C for 30 s annealing and 72^0 C for 30 s extension. Final extension was carried out at 72^0 C for 10 min. PCR reagents used were as follows: 40ng of *Foc* genomic DNA, 10mM of each dNTP, 1X PCR buffer, 0.8U *Taq* DNA Polymerase (Bangalore Genei, India) and 10µM of each primer (forward and reverse). The amplified products were separated on 2% agarose gel in TAE buffer and visualized after staining with ethidium bromide (EtBr 0.5µg/ml) and documented.

2.3.12 ITS-RFLP

The PCR product (5µl) amplified using ITS primers, was digested in 10µl reaction buffer (as per manufacturer's instructions, Promega, USA) overnight at 37°C with 2U of *Hinf*I, *Hae*III, *Mbo*I and *Hha*I enzymes, respectively. Restriction enzyme-digested DNA fragments were analyzed on 2.5% Nuseive agarose gel run in TBE buffer, stained with ethidum bromide, visualized under UV light and documented.

2.3.13 Candidate gene studies: designing of gene specific oligonucleotides (GSOs) and PCR

The oligonucleotide sequences of metabolically essential and virulence related genes of fungal genera, were obtained using NCBI Genbank database (http://www.ncbi.nlm.nih.gov). Sequences of a class were then aligned using CLUSTALX 1.83 (Thompson *et al.*, 1997). Based on the conserved regions of a specific class, fourteen pairs of GSOs were designed (Fig. 2.4 and Table 2.2). Oligonucleotide primers were synthesized from Sigma, USA. These GSOs were used for amplification of *Foc* genomic DNA. The PCR conditions were as follows: initial denaturation at 94^0 C for 5 min, followed by 25 cycles of 94^0 C for 1 min denaturation, 50-58^0 C for 30 s annealing and 72^0 C for 30 s extension. Final extension was carried out at 72^0 C for 10 min. PCR reagents used were as follows: 40ng of *Foc* genomic DNA, 10mM of each dNTP, 1X PCR buffer, 0.8U *Taq* DNA Polymerase (Bangalore Genei, India) and 10µM of each primer (forward and reverse).

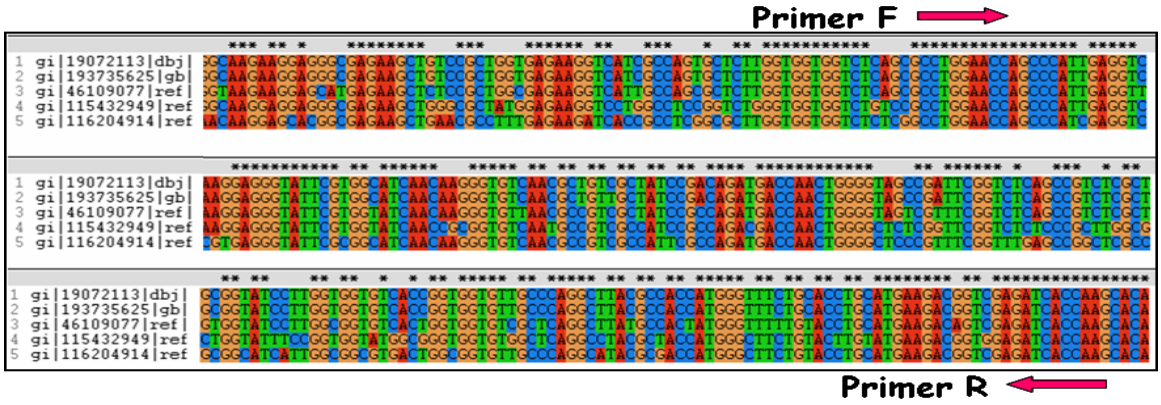


Fig. 2.4 Designing gene specific oligonucleotides

Table 2.2 Oligonucleotide pairs used for gene specific amplification			
Gene name	**Primer abbreviations**	**Primer sequence (5'-3')**	**Expected Size (kb)**
Isocitrate lyase	ICL – F	GGC TGG CAG TCN TCY TCT ACM GC	1.0
	ICL – R	TKG TAN CGG TAG TAN CCC TCR CG	
Transcription factor	MST12 – F	GCS CCW GTN GAC TGG CAA CCC GA	1.1
	MST12 – R	CCS GTC TCC TCG TTG GCN ATG TA	
Sucrose non-fermenting protein	SNF – F	GGA GGH GAG CTD TTT GAC TAT AT	0.5
	SNF – R	CTR TCG TCR AAA GGD AGR TAG CCA	
Serine/threonine kinase	CLK – F	GAG AAT TTG TAC GGC GAR ATY M	0.6
	CLK – R	GCC ATR TAB AAT GGH GAD CCA CA	
Chitin binding protein	CBP – F	TTG CGC TYA CYT TYG ATG AYG GTC	0.7
	CBP – R	AAT TAC CTY TYC KTC CGC TTT GRM G	
Global nitrogen regulator	CLNR – F1	TKC GCA ARA CGA GYA TCG AYG A	1.4
	CLNR – R1	CGR TTT CGY TTC TTR ATC ACR T	
	CLNR – F2	CAG TCN ACK GYC TCG ACS CC	0.6
	CLNR – R2	GCR TTR CAC AGN GGC TGT CCY TC	
Cutinase	CUT – F	GAA RTT CTT YGC TCT YAC CAC	0.9
	CUT – R	AGC AGA RCC ACG SAC AGC	
Xylanase	XYL - F	GAC AAY AGC ATG AAG TGG GAT	0.7
	XYL – R	ACA CCC CAD ACR GTR ATD CC	
Keivitone hydratase	KVH – F	AGC GRS TCG TGG TGB AHC A	0.6
	KVH – R	CCC AGT CVA GCG GGT ASA	
Trehalose phosphate synthase	TPS – F	TCC AYT AYC AYC CNG GNG AR	1.0
	TPS – R	CAN RRT TCA TNC CRT CNC G	
MAP kinase	FMK – F	TCY CGA KCG AAC CCC CCT A	0.8
	FMK – R	CAT RAT CWC CTG GTA GAT SAR	
Transposon	HOP78 – F2	CTT TTG GCA TGA GAT TGT AGC CTC	1.5
	HOP78 – R2	CGT GGG GTT ATA CCT CTA GGC TA	
Desaturase	DST - F	ATG GTT AAA GAC ACA AAG CC	0.6
	DST - R	GTT TGA AAC TCA GTC TCG TTG CG	

The annealing temperature was varied according to the T_m of the oligonucleotide used. The PCR products were visualized on 1.5% agarose gels by electrophoresis in TAE buffer and documented.

2.4 Methodologies involved in *Foc* characterization using proteomic approach

2.4.1 *Fusarium* culture filtrate protein purification

200ml of Potato dextrose broth was inoculated with *Foc* races individually and kept for incubation at 28^0 C, 200 rpm for 5 days. The broth was filtered under vacuum using Whatman paper number 1 and the filtrate was precipitated with 90% ammonium sulphate (60.3 g of ammonium sulfate was added to 100ml of filtrate and kept at 4^0 C overnight for protein precipitation). This was centrifuged at 10,000 rpm for 30 min at 4^0 C. The precipitate was dissolved in deionized water and dialyzed against deionized water at 4^0 C using dialysis tubing (Sigma, USA) of 1kDa cutoff value. The sample was concentrated (SpeedVac, Labconco, USA) and then assayed using protein estimation kit by Bradford Macro method (Bangalore Genei, India). Equal concentration of the individual protein sample was loaded on 15% SDS-PAGE.

2.4.2 Mycelial protein extraction

The extraction protocol was followed as suggested by Schuster and Davies, (1983). 1g of mycelial tissue was ground in liquid nitrogen into fine powder using mortar and pestle. The tissue was macerated in 10ml of extraction medium containing 0.7M sucrose, 0.5M Tris, 50mM EDTA, 0.1M KCl, 2% βME and 10% insoluble poly vinyl pyrrolidone; and vortexed 3-4 times for 15 s. The crude extract was centrifuged at 4^0 C at 10,000 rpm for 30 min. The supernatant was transferred to a fresh tube, equal volume of water saturated phenol (room temperature) was added, vortexed for 10 min and centrifuged at 4^0 C at 10,000 rpm for 20 min. The upper phenol phase was transferred to a fresh tube, re-extracted with equal volume of cold extraction medium (without insoluble PVP), vortexed at room temperature for 5-10 min and centrifuged at 4^0 C at 10,000 rpm for 10 min.

The phenol phase was transferred to a fresh tube; the protein was precipitated by adding 3-5 volumes of cold methanol containing 0.1M ammonium acetate and 10mM βME. The protein sample was incubated overnight at -20ºC and centrifuged at 4^0 C at 5000 rpm for 20 min. The pellet was washed by vortexing with 1ml of cold methanol

containing 0.1M ammonium acetate and 10mM βME. The sample was transferred to 1.5 ml tubes and centrifuged at 5000 rpm for 5 min. The supernatant was removed and the pellet was washed by vortexing with 1 ml of cold 100% acetone containing 10mM βME; and centrifuged at 5000 rpm for 1 min. Pellet was air dried for 10-15 minutes and resuspended in 100-200 µl of SDS. Protein concentration was assayed and 5 to 10µl was loaded on 15% SDS-PAGE. Protein estimation was done using protein estimation kit by Bradford Macro method (Bangalore Genei, India). Also Nanodrop equipment was used to quantitate protein by observing the absorbance at 280 nm.

2.4.3 1D-SDS polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel was prepared consisting 15% resolving gel and 4% stacking gel, as described by Maniatis *et al.*, (1982). Tris glycine buffer (25mM Tris, 250mM glycine) containing 10% SDS at pH 8.3 was used to run the gel at 25mA and 200 volts on "minigel" apparatus (Biorad, USA). The samples and BSA were heated along with sample buffer containing 0.5M Tris-HCl, pH 6.8, glycerol, 10% SDS, 2-βME, and 0.05% (w/v) bromophenol blue for 5 min at 95^0 C. Equal concentrations of all the samples were loaded on SDS-PAGE. The gel was kept overnight in staining solution containing Coomassie Brilliant Blue R-250 dye (500mg), methanol (250ml), glacial acetic acid (50ml) and distilled water (200ml). Destaining was done using destainer containing 3:1 methanol: glacial acetic acid and volume was made up using distilled water.

2.4.4 Silver staining

For silver staining, the gel was first kept in fixing solution containing 50% methanol and 10% glacial acetic acid for 45 min with gentle shaking. It was then transferred to a solution containing 5% methanol and 1% acetic acid. The gel was then washed with deionized water for 50 min, 3 times. The gel was then incubated in solution containing 0.02g/100ml of freshly made $Na_2S_2O_3$ for 90 s. Deionized water wash was given for 5 min followed by incubation in solution containing 0.2g/100ml of $AgNO_3$ and HCHO (70µl/100ml) with gentle shaking for 20-25 min. Deionized water wash was given for 3 min and developed with solution containing 2g/100ml of Na_2CO_3, 50µl/100 ml of 37% HCHO and 2ml of 0.02g/100ml of $Na_2S_2O_3$ with gentle shaking for 7 min. The gel was stored in solution containing 6% acetic acid.

2.4.5 Manaul trypsin in-gel digestion (Yamaguchi *et al.*, 2003)

2.4.5.1 Excision of protein bands from 1D-SDS polyacrylamide gel

Silver stained protein band was excised and, if greater than 1mm^2, chopped into small pieces with a scalpel on a glass plate. Enough water was used to stop the pieces from drying out. These were then transferred to a 1.5ml eppendorf tube and excess water was removed. This was further washed briefly with 100mM ammonium acetate, to make sure that the pieces were at the correct pH.

2.4.5.2 Destaining (For silver stained gels)

200µl of destaining solution (0.2g potassium ferricyanide in 100ml of 100 mg/L of $Na_2S_2O_3$) was added to each tube and incubated on shaker until no stain was visible, typically 15 min or 30 min for darker spots. The gel pieces were bright yellow. Gel pieces were washed twice with 100mM ammonium acetate, for 5-10 min and twice with deionized water for 5-10 min, until the gel was no longer bright yellow. Excess liquid was removed and the gel pieces were washed for 5 min with 200ul 50% ACN in 50% 50mM ammonium acetate, then 5 min with 100% acetonitrile to dehydrate gel pieces and vortexed during incubations. ACN was removed, and was evaporated briefly in SpeedVac (Labconco, USA), without heating. The gel pieces were noticeably shrunken and white.

2.4.5.3 Reduction and alkylation

Reduction and alkylation step is essential for cysteine containing peptides. Gel pieces were covered with 50µl of 10mM DTT in 100mM ammonium acetate. This was vortexed, given a short spin and incubated for 45-60 min at 56^0 C. This was then cooled to room temperature, and DTT solution was removed. 50µl of 55mM iodoacetamide in 100mM ammonium acetate was added. This was vortexed, centrifuged briefly and incubated for 45 min in dark place at room temp. Iodoacetamide was removed and discarded. Gel pieces were washed with 100µl of 100mM NH_4HCO_3 for 5 min with vortexing, then twice with 50% ACN/50% 50mM ammonium acetate for 5 min with vortexing and dehydrated with 100µl of 100% ACN as above. The remaining liquid was removed and dried in SpeedVac. The gel pieces were shrunken and white.

2.4.5.4 Trypsin digestion

20µg of trypsin per 1ml of 25mM NH_4HCO_3 was prepared in 5% CAN (250µl of 100mM ammonium acetate + 50µl ACN + 700µl deionized water). Digestion was

accomplished at a ratio of 1:10, 1:20, 1:30 (Trypsin: Protein) depending on the concentration of the protein band eluted from the gel (for 10ng of protein, 1ng of trypsin was added). Enough trypsin solution (around 20µl) was added to just cover the gel pieces. The gel pieces were rehydrated at 4^0 C for 30 min in buffer containing 50mM ammonium acetate and trypsin. The procedure was performed at 4^0 C to get as much active trypsin sucked into the gel as possible before and to avoid auto digesting of trypsin. Brief spin was given and more ammonium acetate was added to cover gel pieces, (typically another 25µl). Overnight digestion at 37^0 C (or at least for 4h) was accomplished.

2.4.5.5 Peptide extraction

The digest solution supernatant (if any) was transferred to a clean polypropylene tube. To the gel pieces, 30µl (or enough to cover) of 50% ACN in 2% formic acid was added, incubated and vortexed for 20 min, centrifuged briefly and sonicated for 5 min in a water bath with no heat. Supernatant was removed and combined with initial digest solution supernatant. The above step was repeated, to give a combine peptide extract volume of around 60µl. For larger gel slices, extraction step was repeated again and the supernatant was combined. The extracted digests were vortexed and dried using SpeedVac to reduce volume to 5-10µl. The remaining 5-10µl sample was centrifuged at 14,000 rpm for at least 10 min to remove any micro particulates. The supernatant was transferred very carefully to a fresh polypropylene tube. The sample was further used for MALDI/TOF analysis or stored at -20^0 C until further use.

2.4.5.6 Sample preparation for MALDI-TOF analysis

Matrix-assisted laser desorption ionization (MALDI) produces gas phase protonated ions by excitation of the sample molecules from the energy of a laser transferred via an ultraviolet (UV) light-absorbing matrix. The matrix is a conjugated organic compound (normally a weak organic acid such as a derivative of cinnamic acid and dihydroxybenzoic acid) that is mixed with the sample. The sample (1-10pmol/mm^3) is mixed with an excess of the matrix and dried onto the target plate, where sample and matrix co-crystallize on drying. Pulses of laser light of a few nanoseconds duration cause rapid excitation and vaporization of the crystalline matrix and the subsequent ejection of matrix and analyte ions into the gas phase, this generates a plume of matrix and analyte ions that were analyzed in a TOF mass analyzer. The advantage of MALDI

is the ability to produce large mass ions, with high sensitivity. It is a soft ionization technique that produces molecular ions with little fragmentation.

10mg/ml of CHCA (α-cyano-4-hydroxy-cinnamic acid) matrix was prepared by dissolving it in 50% ACN in water and was vortexed for 5 minutes. 2µl of peptide sample was mixed with 8µl of the matrix. 0.8µl of the sample was loaded on 96 well Teflon coated plate. Standard mixture of protein was used for calibration and the peaks were detected using MALDI-TOF (Applied Biosystems, USA).

Tryptic peptides usually have basic groups at the N and C termini. Trypsin cleaves after lysine and arginine residues, both of which have basic side-chains (an amino and a guanidine group, respectively). This results in a large proportion of high energy doubly charged positive ions that are more easily fragmented. The digestion of the protein into peptides is followed by identification of the peptides by (m/z), either as very accurate masses alone or by using a second fragmentation that gives ladders of fragments cleaved at the peptide bonds. Although a wide variety of fragmentation may occur, there is a predominance of peptide bond cleavage, which gives rise to peaks in the spectrum that differ sequentially by the residue mass. The mass differences are further used to reconstruct the amino acid sequence of the peptide.

The identification of proteins obtained by peptide mass fingerprinting was carried out using freely available software 'MASCOT' from Matrix Science (http://www.matrixscience.com/cgi/index.pl?page=/search_form_select.html). Fig. 2.5 shows the web page of Mascot peptide mass fingerprint.

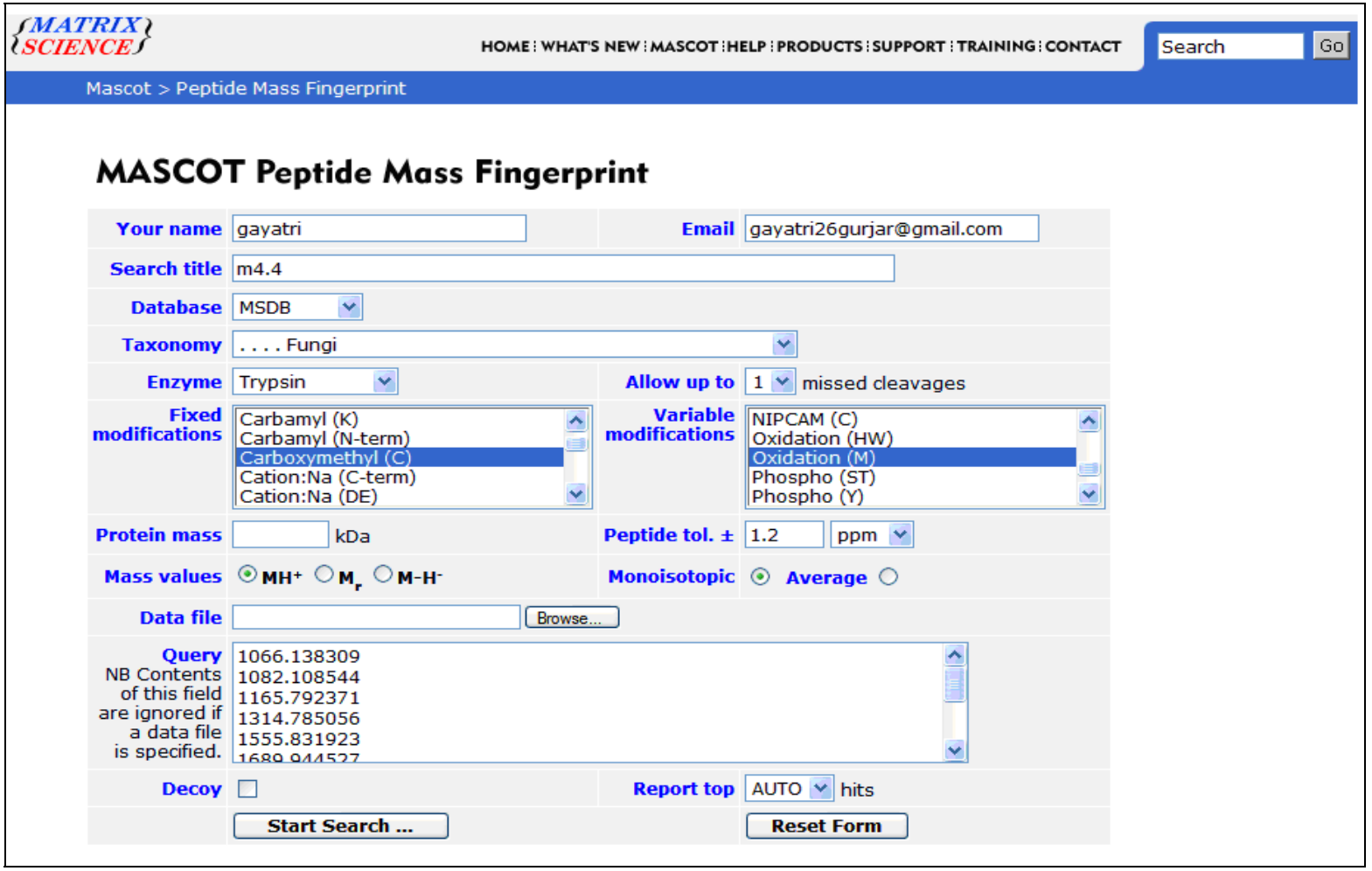


Fig. 2.5 Mascot peptide mass fingerprint web page

2.5 Methodologies involved in plant pathogen interaction studies at molecular level

2.5.1 Hydroponic culture of chickpea plants

Surface sterilized seeds of JG62 and Digvijay varieties were wrapped in wet sterile muslin cloth and stored at room temperature (24-26^0 C) till sprouting (Fig. 2.6A). The sprouted seeds were transferred into trays containing sterile water including macro- and micro- nutrients (half strength Hoagland's nutrient medium) as suggested by Nimbalkar *et al.*, (2006), and kept in controlled conditions at 22^0 C and 60% relative humidity under normal day conditions (14 h light/10 h dark) (Fig. 2.6B).

A

JG62 seeds

Digvijay seeds

B

Fig. 2.6 Hydroponic culture of chickpea plants

2.5.2 Inoculation with *Foc* races

Freshly prepared spore suspension ($1x10^6$spores/ml) of individual *Foc* race was added to the sterile hydroponic trays containing seven days old chickpea plants. Seedlings grown in similar trays with no pathogen (non-inoculated plants) served as control.

2.5.3 Tissue collection

Total RNA was extracted from chickpea root samples collected at various time intervals such as 2, 6, 9, 13 and 16 days after infection (dai) and immediately frozen in liquid N until further use. Later, the tissue was crushed and 100mg of tissue was used for RNA extraction using either TRIzol reagent (Invitrogen, USA) or TRI Reagent (Sigma Aldrich, USA).

2.5.4 RNA extraction and cDNA synthesis

C. arietinum root tissue (100mg) was pulverized in liquid nitrogen and transferred to 1 ml of TRIzol reagent. After vortexing, the lysate was stored for 5 min at room temperature and 0.2 ml chloroform was added. The mixture was shaken vigorously for 15 s and stored at room temperature for 10 min before centrifugation at 4^0 C for 15 min at 12,000 rpm. The aqueous phase was transferred to a new tube and 0.5 ml isopropanol was added to precipitate RNA. The sample was stored at room temperature for 10 min and centrifuged at 13,000 rpm for 10 min at 4^0 C. The RNA pellet was retained and washed with 1ml 75% ethanol, pelleted by centrifugation, air-dried, and dissolved in 30µl RNase-free water. To remove contaminating DNA, the total RNA (10µg) was treated with RNase free DNaseI (0.1U per µg RNA) at 37^0 C for 1 h in the presence of RNasin (0.4U) and terminated by heating at 65^0 C for 15 min. The RNA was precipitated with 0.1 volume of 3M sodium acetate buffer, pH 5.2 and 3 volumes of absolute ethanol at -70^0 C for 1h. The RNA pellet was collected by centrifugation at 12,000 rpm for 10 min at 4^0 C, dried under vacuum, and resuspended in 5µl of DEPC-treated water. The RNA samples were quantified by spectrophotometry at 260-280 nm or stored at -80^0 C until used. The DNase treated RNA was used for first strand cDNA sysnthesis using RT-PCR kit (Promega, USA) as per the manufacturer's instructions.

2.5.5 cDNA-RAPD

cDNA isolated from *Foc* infected and uninfected JG62 and Digvijay plant roots was used as template for amplification with RAPD primers (OPAD and OPAE series, Operon technologies, USA). RAPD PCR conditions were as follows: 34 cycles of 94^0 C

for 30 s denaturation, 35^0 C for 1 min annealing and 72^0 C for 1.5 min extension. Final extension was carried out at 72^0 C for 5 min. PCR reagents were as follows: 1µl cDNA, 10mM of each dNTP, 1X PCR buffer, 0.8U *Taq* DNA Polymerase (Bangalore Genei, India) and 20µM of RAPD primer (forward and reverse). The amplification product (greater than 200bp) from cDNA of resistant infected cultivar Digvijay was cloned in pGEM-T easy vector (Promega, USA) and fragments were sequenced using an automated sequencer (MegaBACE1000, formerly Amersham Biosciences, USA now GE healthcare, USA). Primers were designed from random primer amplified sequences and were used for transcript expression analysis of infected and non-infected cDNA pool of Digvijay cultivar root tissue previously normalized using 18s rRNA primers or ITS primers.

2.5.6 Normalization of RNA

For analysis of plant defense response, 1:100 diluted cDNA was normalized using 18s rRNA primers, while *F. oxysporum* specific ITS primers were used in separate experiment, for the normalization of undiluted fungal cDNA pool for analysis of fungal virulence genes. PCR conditions were as follows: initial denaturation at 94^0 C for 5 min, followed by 25 cycles (30 cycles in case of ITS primers) of 94^0 C for 1 min denaturation, 55^0 C for 30 s annealing and 72^0 C for 30 s extension. Final extension was carried out at 72^0 C for 10 min. PCR reagents were as follows: normalized concentration of cDNA, 10mM of each dNTP, 1X PCR buffer, 0.8U *Taq* DNA Polymerase (Bangalore Genei, India) and 10µM of each primer (forward and reverse) (Table 2.3).

2.5.7 Designing fungal pathogenesis related and plant defense related primers; and PCR using these primers

Specific primers against fungal virulence related (Table 2.4) and plant defense (Table 2.5) genes were designed in a similar fashion as described in section **2.3.13** These primers were used to study the expression of respective genes in infected and non-infected root tissue using semi-quantitative RT-PCR method.

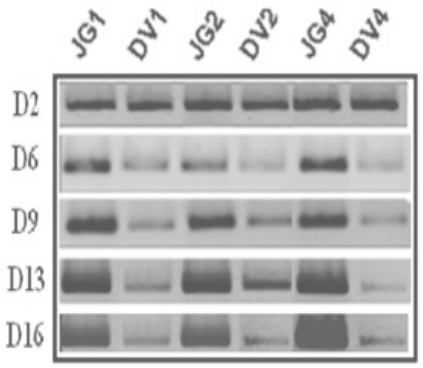
Table 2.3 Primers used for normalization of cDNA

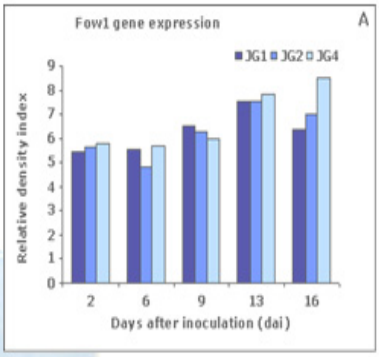
Primer name	Primer sequence (5'-3')	Specificity
18s rRNA F	AAACGGCTACCATCCAAG	Chickpea
18s rRNA R	TCATTACTCCGATCCCGAAG	
ITSFu-F	CAACTCCCAAACCCCTGTGA	*Fusarium oxysporum*
ITSFu-R	GCGACGATTACCAGTAACGA	

Table 2.4 Primers used for expression analysis of pathogenesis related genes in chickpea-*Foc* transcriptome

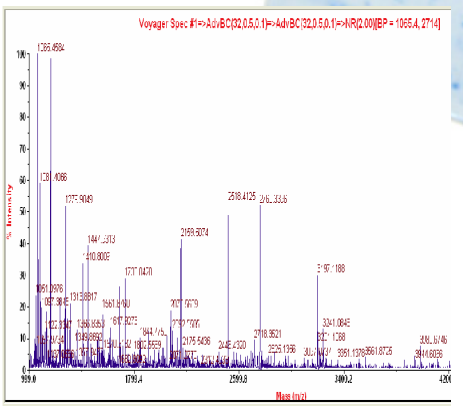
Gene	Primer (5'-3')
Tri-F	CTGTCGGTAGCCGAAGACTC
Tri-R	CGTGAGTTTGCGACAAAGAA
Fo-transposase-F	TTGGCATGAGATTGTACCCAG
Fo-transposase-R	GGGGTTTTACGTCTTGGC
Gas1F	GACTCCGACCTCTGCGACT
Gas1R	CTTTTACAGAAGGACCATACCG
Chs7F	GCTGGGCGTTATGATGGT
Chs7R	GCGAGTAAGGCAGATCATAG
Fgb1F	GTGATGAGCATCAGCCTCAA
Fgb1R	CAACGAGAGAGCCAACCTTC
Fow1F	GATTGAAGCTTCCAGCAAGG
Fow1R	GCCGTTGGTCTCGTAAATGT

Table 2.5 Primers used for expression analysis of defense related genes in chickpea-*Foc* transcriptome	
Gene	**Primer (5'-3')**
H2A-F	CTGCTACAACCAAGGGAGGA
H2A-R	AGATCCCAAAAGCTTGCTGA
60srp-F	GATCAGGGGAATCGGAAAAT
60srp-R	GACTGATTCACACGCCTCAA
GroES2-F	CTGAGGTGGAGTTCGATGGT
GroES2-R	GCCATGACATCTGAGGCTCT
glyt-F	CTGCACTTGACCGGTGTG
glyt-R	TCAAAGCATGAAGTCGCATC
Msr-F	ACCTGCCAAGGTCCAGAAAT
Msr-R	AAAACCTTCCCCCTTGAAGA
Rrp-F	CAAGCTCTTCGATGGAGACA
Rrp-R	ACCGACACGCATATCCAAAT
Betv1-F	TCACGATGTGCAAAACCATT
Betv1-R	TCCATGTATCCATTTGGAGGA
CHS-F	GAGCTGCTGCATTGATTGTTGG
CHS-R	CACCCCATTCAAGTCCTTCTCC
IFR-F	GCTGCTGCTAACCCTGAAAG
IFR-R	GTTCCCACATCAGCCTCAGT


JG1
DV1
JG2
DV2
JG4
DV4
D2
D6
D9
D13
D16


Fow1 gene expression
A
JG1 JG2 JG4
Relative density index
0
1
2
3
4
5
6
7
8
9
2
6
9
13
16
Days after inoculation (dai)

RESULTS



3. Results

3.1 Identification of Indian pathogenic races of *Foc* using various DNA based molecular markers

Foc races 1, 2, 3 and 4 are prevalent pathotypes occurring in India. Race 1 is the most virulent of all the four races. The classical method of identification involves pathogenicity assays using differential chickpea cultivars; and is a time consuming process. Under field conditions the outcome can be dependent upon environmental parameters (Sharma and Muehlbaur, 2007). As mentioned previously, use of DNA based marker systems can eliminate the possibilities of erroneous results; at the same time, these approaches are robust, reproducible and less time consuming. With the aim of accurate discrimination of *Foc* races in the present study, three DNA based molecular markers namely gene specific, ITS-RFLP and AFLP were attempted for race identification.

3.1.1 Gene specific marker analysis

Gene specific oligonucleotides (GSOs) were designed from the conserved regions of metabolically essential and virulence related genes of various fungi. Fourteen genes which were eventually reported to play some role in fungal virulence were chosen for marker analysis.

Out of these fourteen GSOs, only four primer pairs, namely *Hop78*F2/R2, *Cut*F/R, *Dst*F/R and *Xyl*F/R, yielded polymorphic patterns with genomic DNA of *Foc* races; while other primer pairs did not show either any amplification, or race specific and reproducible amplification profiles. Races 1, 2 and 4 yielded amplification products of 1.5kb with oligonucleotides *Hop78*F2/R2 designed from Hop78 transposon sequence (GenBank accession number AY267761) (Fig. 3.1A), while a 900bp amplicon was obtained with oligonucleotides *Cut*F/R designed from the cutinase sequence reported in NCBI database (GenBank accession number K02640) (Fig. 3.1B). However, no amplification was observed for race 3 using the same oligonucleotides. *Dst*F/R oligonucleotides amplified a 600bp product specifically in race 3 and not in other standard *Foc* races (Fig. 3.1C). Furthermore, *Xyl*F/R oligonucleotides designed from *F. lycopersici Xyl3* gene (GenBank accession number AF052582) amplified 700bp fragments in *Foc* races 1 and 2 and race 3, while the consistent absence of this 700bp band was specific for race 4 only (Fig. 3.1D). Thus, *Foc* race 4 was clearly distinguished by oligonucleotides *Xyl*F/R. The amplified products, 1.5kb, 900bp, 600bp and 700bp as

described above, were cloned and sequenced. Fig. 3.1E depicts a uniform amplification profile of all four *Foc* races using *Mst12*F/R primers.

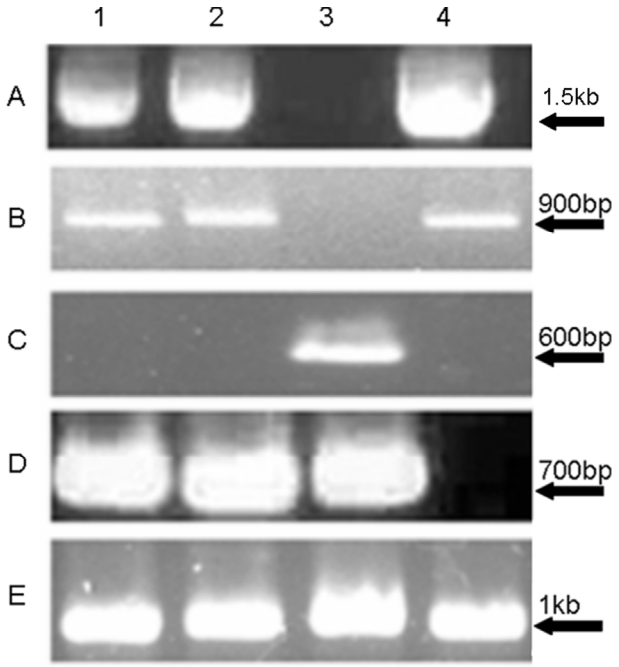


Fig. 3.1 Identification of *Foc* races using gene specific oligonucleotides

Gene specific oligonucleotide amplification with *Foc* genomic DNA. (A): Amplification with *Hop78*F2/R2, (B): Amplification with *Cut*F/R, (C): Amplification with *Dst*F/R, (D): Amplification with *Xyl*F/R and (E): Amplification with *Mst12* F/R. Lanes 1, 2, 3 and 4 represent genomic DNA of *F. oxysporum* f. sp. *ciceri* races 1, 2, 3 and 4, respectively.

3.1.2 Cloning and sequencing of GSO derived fragments

The amplification products obtained using GSOs were cloned (from race 1 in case of *Hop78*, *Cut* and *Xyl* primers; and race 3 in case of *Dst* primers). Ten clones of each were sequenced and the sequences were aligned using CLUSTALX 1.83 (Thompson *et al.,* 1997) software. The sequencing errors were removed and a consensus sequence was deposited in the Genbank database. Fig. 3.2A-E represent *Hop78*, *Cut*, *Xyl* and *Dst* gene sequences.

A

>*Foc* race 1 *Xyl* sequence Genbank accession no. EU520473

AGAACCACGTCAAGACCATGGTCACCCGCTACAAGGGCAAGATCCTCCAGTGGGATGTCGTCAACGAGAT
CTTCGCTGAGGACGGTAACCTCCGCGACAGTGTCTTCAGCCGAGTTCTCGGTGAGGACTTTTGTCGGTAT
TGCTTTCCGCGCTGCCCGCGCCGCTGATCCCGCTGCCAAGCTCTACATCAACGATTACAATCTCGACAAG
TCTGACTATGCTAAGCTGACCCGCGGGAATGGTCGCTCACGTTAACAAGTGGATTGCTGCCGGTATTCCC
ATCGACGGTATTGGATCTCAGGGCCATCTTGCTGCTCCTAGTGGCTGGAATCCTGCCTCTGGTGTTCCTG
CTGCTCTCCGAGCTCTTGCTGCCTCAGACGCCAAGGAGATTGCCATCACTGAGCTTGATATCGCTGGCTG
CCAGTGCTAACGATTACCTTACTGTCATGAACGCTTGCCTTGCCGTTCCTAAGTGTGTCTGGAATCACTG
TCTGGGGTGT

B

>*Foc* race 1 *Hop78*-2 sequence Genbank accession no. EU520472

ACCTATCCTCCCCTAGTTTCTACAGCTCACGGGAGGAACTACCTTCAGCATTTAATGCTTGGGCAGCGCC
ACGAGGTTATGCCTTTGTGATCAAGAGATCTTCGAAGACTGCCAATGGAGAGAACTCACGTTATCTTCAA
TGTGACCGTGGAGCAGGGCGTATTCCCTCCCTTTCAGACCGTCGGCAAACTACAACACGTCGTACAGGAT
GCCTCTTCTCTGTATTAGCAAAGGAAAGCCTGGTGTAAGACCATATGGAGTCTCAGGCATCGTCCCGGAC
CTCATTTCAGTCAGCACAATCATGAACCAAGCTTCAGTGAAGTGGCACATCCAACACTTCGTCAGCTATC
ACGCCAGAGGAAATAACAGTCAATCAACTCGCCAATGCCGGCATTGCCGCCAAAGGAGATTGGATCCTTC
TTACGCATTACCTCAAATACACTTGCTACGCAGCAAGATATCTATAATTGCATTGCGAAGGGCAGACGAG
ATCTCTCTAAGGGCCAGAGTAACATTCATGCCCTTGCAGATCAGCTCAATGAGGAGGGCTTCTGGAAGTC
GAATATTGCTTGGACGAGACGCGTAAAGTTACAGCAGTATTATTTTGCACCATCCCGAATGTCCCCTGGA
ATCCCTTTAAACTTTCCTGAAGGTGGTTATATGGGGACCTCCTCTTTTCCCCCTTGGGTTCGGATGCCTC
TCCTTGAATTTATTGGGGTTAATGCTGCCCAGGGGACCTTCTGTTGCTTT

C

>*Foc* race 1 *Hop78*-1 sequence Genbank accession no. EU520471

ATAGACGCCGTTGATTAATGCAAGAAACGTTATATCCCCCCACTATATTAATGAGGTGGGCTACATCCTG
GAAACCTGGGGCTAGATCTCTATAAGAAGAGCTTCGTCAAGGCTTGGGTCAACACTCACCTTCACTTCGA
GCAATATGCTACATCACGGGTTGAGGGCATTCATTCGCTTATCAAATTACATTTAAACCACTCGCAAGTT
GATCTCTTTGAGGCCTGGAGGATCATCAAGCTTGTTCTGATGAACCAGCTTAGTCAACTTGAGGCAAACC
AAGCCAGGCAGCATATTGGCAACCCTATTCGCGAATCTAGGGTATTATACAGCAATATCCGTGGTTGGAT
ATCACATGAAGCCCTGCGGAAGGTTGAGACTCAACGGGAACGACTATTGAAAGAGGTTCCTGTGTGTACA
GGGGTATTCACTAGGACTCTTGGTCTGCCTTGTGCTCATAGCCTTCAGCCCTTACTGAAGCAGAATCAGC
CCCTTCTACTGAATCATTTCCACTCACATTGGCATCTTCGACGCCCAGGAAGCCCCCGGTTCCTTATTGA
GCCTCGTAAAGCAGTTGATCGTCTAACAGCTAGTCAACGCTAACAACAAAGAGCACACAACGTGAGCCTT
TCTCACGTTTGAGCGTATTGAGAAGGGCACTTACAACCAAAGGCACCGGCCAAAGGTGTTCCAGATGTCA
TCAGGAAGGGCAACATGGATGGAACCTCTA

D

>*Foc* race 3 *Dst* sequence

CTCGATGAGTTTAACATACCCGTTTGGTAAGCTCAGACCATCGGTACAAGATATAGAATGCTGCGTATGC
GGATGTGTCATATCCGCGATACATGTAGCAATTCCAGTGAGATCAGAGTTCGCCATCGATCAACCAGAGC
CGGACCATCAACACCAGCGAATCGAGAGGACCCTGGCATCTCGCAGAGCTCCCTGCCTTGAAGGGGCTGT
GCATTGCAACTATTTATGGTCTAGTATACAGCACAACCAATGGAGTGCAATTATATCTTGCAACACGATG
CACACTCTATGATCTGCAGGTTTCTTGCTCATGCCAGGCAGGCCAGCCATGCAGCAGTCCAATATATCAT
TGGCTTGCTACCAGTTACTAGCGCGCATTATAAAGGTCTACAGCTGCCAGATAATCCCTTATCACAAGAC
ACAGCAGATCACCACCACTGACATACGCTAGACTTTGGCGCTATATGGGTAAGGCTATATTTAGAGCAAG
TAACTGTCAAATCCAGGTCAGGTATATGGGGTTCGAAATTCCACCAGGGTCACGAGCACCGCCAACGGAG
CACTGTGA

E

>*Foc* race 1 *Cut* sequence

AACTATACACGTATAGTCAGAGGTCGGACATGCTGTGTCTATGTGAGAAAGGGATAACTAGGCAGCTAGAG
ACCTATTATAAGACGCAGCTAACAAACTAGGAAGACAAGCAAGAAATATGACTCTCATGGACTAGCCTACC
ATGCATACAGAAACCTACAGATCAAGAGATACACTGTAATCAAGAAAACTTCCACTCATTCGCTCTTCTAA
CAGACCATAATGTATCAATCACAGACCCCATTAAGGCAGGGAAGCCTACGCGAGATCCAGAGGTCCTACCG
GATCCCCCCTAGCATGATACAGACGAGCCGGGCTCAGGTTACGCACAGAATGCGAGAAACCCCGAATCCTC
AACCTTGGCACACTGCAAACCACATGGTATCCGCATGCAATGAACAAAAATACCTCATTACAGGCAGCCAC
ACTCCTCCCTTATTACCCTGGCGGAACCCACTTACACGGAGTACTAGTGGAGAGAATCAACGGGTGAACCC
CCGGGGGCTGCTTCCACTCCCACAACACTTTACACTTCCAACACACCTATCAACCACACATTGAAGCGGGC
TATCACGGGAGGGCTCATTAACACCACCCCAATTAAATGCTCCCCACCAGTGCCGCGCGCCACTAACCACG
GCGCACCGC

Fig 3.2 DNA sequences of *Hop78*, *Cut*, *Xyl* and *Dst* genes

(A) *Foc* race 1 *Xyl* sequence (B) *Foc* race 1 *Hop78*-2 sequence (C) *Foc* race 1 *Hop78*-1 sequence (D) *Foc* race 3 *Dst* sequence and (E) *Foc* race 1 *Cut* sequence

Fig. 3.3A shows the TELD motif in light grey shading, which is a characteristic for the catalytic domain of family F-xylanase. The TELD motif was present at the 410bp of the *Foc Xyl* sequence.

BlastN (Altschul *et al.*, 1990) search yielded significant homology with *Hop78* transposon of *F. oxysporum* f. sp. *melonis* (Chavlet *et al.*, 2003). Further, using the BlastX program the putative translation product of *Foc Hop78* showed similarities with *Hop78* transposon of *F. oxysporum* f. sp. *melonis* and other putative proteins. A conserved domain of approximately 100 amino acids, which was previously identified in plant MULEs, bacterial IS sequence and *Hop78* sequence of *F. oxysporum* f. sp. *melonis* (Chavlet *et al.*, 2003) was also present in the deduced *Foc* Hop78 protein sequence (Fig. 3.3B). Internal oligonucleotides were designed from the reported *Hop78* transposon sequence of *F. oxysporum* f. sp. *melonis* (*Hop78* F1-5′ ATGGACTCAATTGGCATTCACCG 3′ and *Hop78* R1- 5′ AGTCAGGGATATAACGCTTCTTG 3′) to obtain the N-terminal sequence of the transposase, which was also deposited in the NCBI database (Hop 78-1, Genbank accession no. EU520471). The deduced amino acid sequence of Hop 78-1 showed the presence of CCHC motif (Fig. 3.3C). Such a motif has been reported in MURA transposase of maize and in many MURA related proteins in *Arabidopsis thaliana* (Chavlet *et al.*, 2003). This motif also exists in many nucleic acid binding proteins and has been shown to interact with RNA and single-stranded DNA. It has been suggested that this domain interacts with transposon DNA or RNA and host genome interaction sites (Chavlet *et al.*, 2003).

The DNA sequences of amplification products of *Dst*F/R and *Cut*F/R oligonucleotides were not significantly homologous to any of the reported gene sequences in the NCBI database.

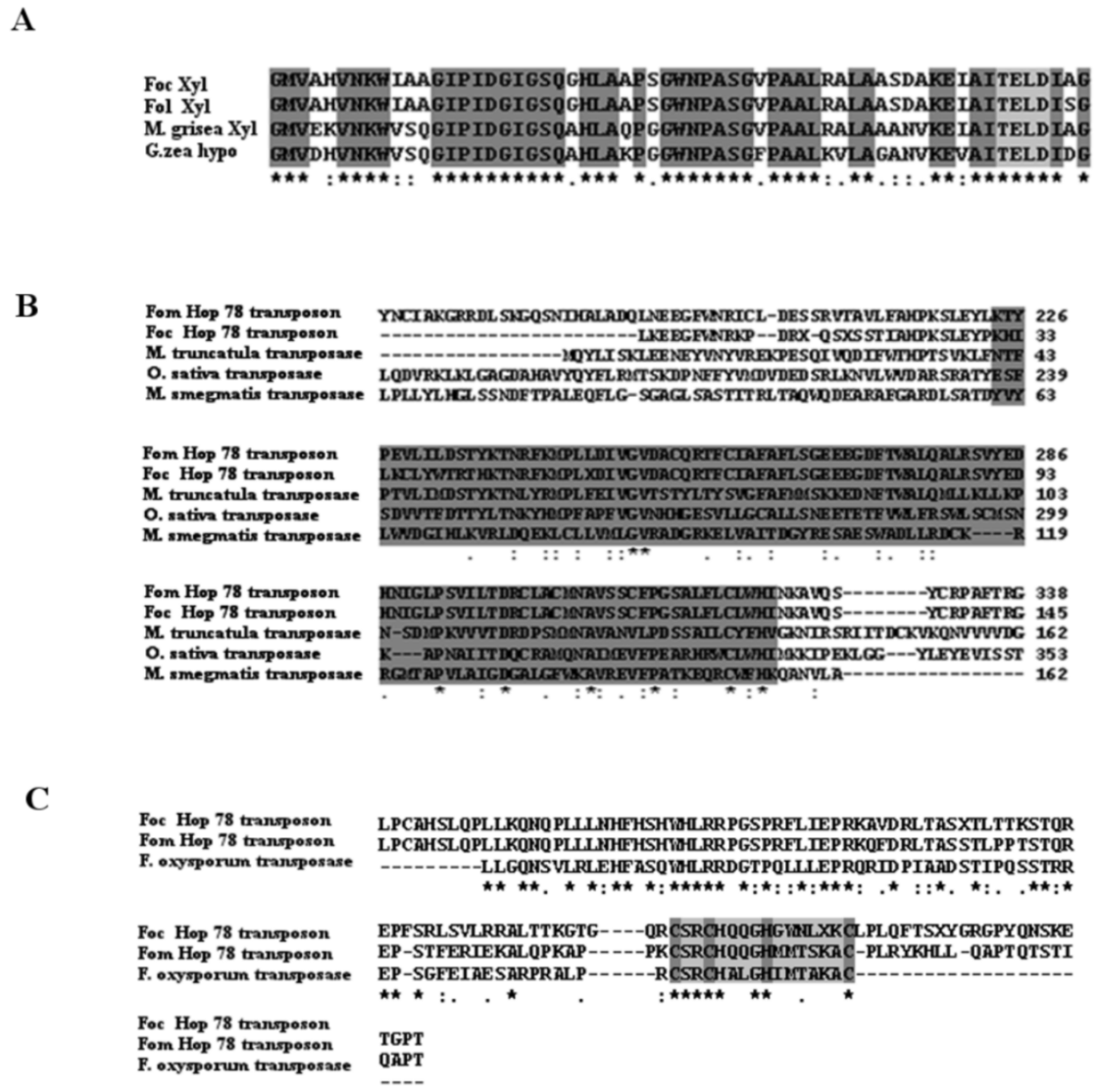


Fig 3.3 Conserved domains of *Xyl* gene and Hop78 transposon (A): *F. oxysporum* f. sp. *ciceri Xyl* (*Foc Xyl* - in the present study) amino acid sequence alignment depicting the TELD motif, Family F xylanase *F. oxysporum* f.sp. *lycopersici* (Fol *Xyl*, Genbank accession no.AAC06239), endo-beta-1,4-D-xylanase (*M. grisea Xyl*, Genbank accession no.XP_365543), hypothetical protein (*G. zeae* hypo, Genbank accession no.XP_391480). Dark gray shading represents homologous sequences, light gray shading denotes TELD motif amino acid sequence. (B): 100 amino acid conserved domain. *F. oxysporum* f. sp *melonis* (Fom Hop 78 transposon, Genbank accession no. AY267761.1), *F. oxysporum* f. sp *ciceri* (*Foc* Hop 78 transposon - in the present study), *M. truncatula*, putative transposase (Genbank accession no. ABN04845.1), *Oryza sativa*, transposon protein (Genbank accession no. ABA95414.1) and *Mycobacterium smegmatis*, transposase (Genbank accession no. AAA25034.1) sequences were aligned to identify the conserved 100 amino acid domain in *F. oxysporum* f. sp. *ciceri* Hop78 using ClustalX software. Gray shading represents homologous sequences. (C): Putative CCHC domain. *F. oxysporum* f. sp. *ciceri* sequence (*Foc* Hop 78 transposon - in the present study), *F. oxysporum* f. sp. *melonis Hop78* transposase (Fom Hop78 transposon, Genbank accession no. AY267761.1) and *F. oxysporum* transposon (Genbank accession no. AAP31248.1) were aligned to identify the CCHC conserved domain in *Foc Hop*78 using ClustalX software. Light gray shading represents homologous sequences, dark gray shading denotes CCHC domain amino acid sequence.

3.1.3 ITS-RFLP distinguishes *Foc* race 3 from race 1, 2 and 4

An attempt was made to distinguish *Foc* races using PCR-RFLP with universal oligonucleotides of ITS region. The ITS forward (ITS1) and reverse (ITS4)

oligonucleotide pairs furnished a single amplified DNA fragment of approximately 550bp in all the four *Foc* races. Sequencing of ITS amplification product from *Foc*1 was accomplished (Fig. 3.4A) and this sequence was used to determine the restriction enzyme digestion sites present (Fig. 3.4B) so as to generate RFLP profiles with a few enzyme combinations, using freely available NEBcutter V.02 software (http://tools.neb.com/NEBcutter2/).

A

ITS- *Foc*1 sequence

CTAGTGAACTGCGGAGGGATCATTACCGAGTTTACAACTCCCAAACCCCTGTGAACATACCACTTGTTGC
CTCGGCGGATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATA
TGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT
GAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGC
ACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACAGCTTG
GTGTTGGGACTCGCGTTAATTCGCGTTCCTCAAATTGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGT
AGTAAAACCCTCGTTACTGGTAATCGTAAAGCCACGGGGGTAACCCCACTTCTGATAACCTCGGG

B

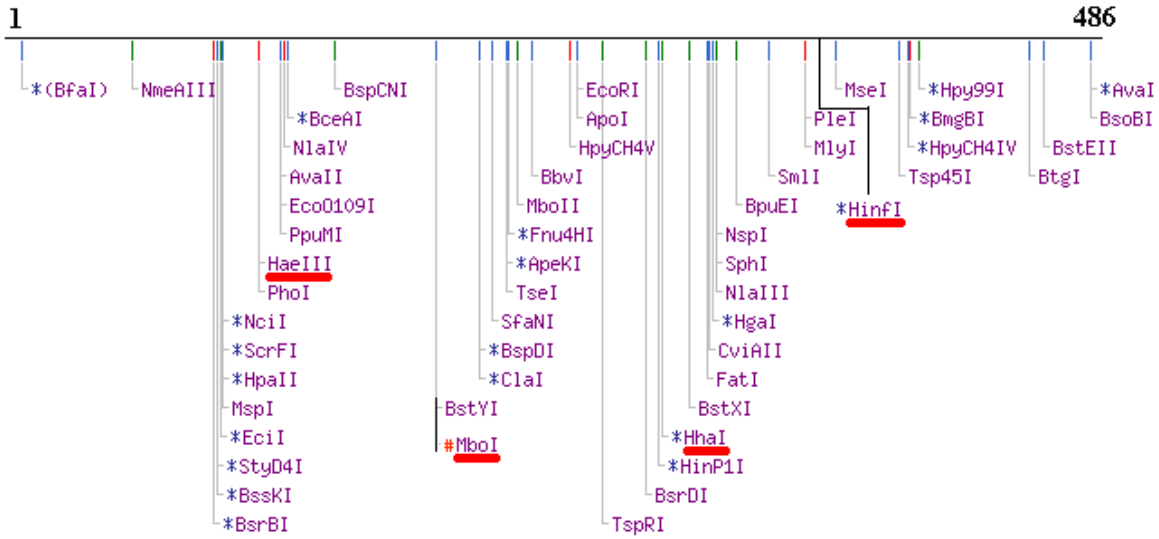


Fig. 3.4 ITS region of *Foc* race 1 (A) DNA sequence of ITS region of *Foc* race 1 (B) in-silico restriction digestion profile of ITS-*Foc*1 generated using NEBcutter V.02 software

An amplification product of 550bp amplified in the four *Foc* races (Fig. 3.5A) was further used for PCR-RFLP analysis. Four enzymes namely, *Hinf*I, *Hha*I, *Mbo*I and *Hae*III were chosen, which yielded a race specific and reproducible profile. Figs. 3.5B, 3.5C, 3.5D and 3.5E depict the restriction endonuclease digestion patterns of ITS

amplification product with restriction enzymes *Hinf*I, *Hha*I, *Mbo*I**,** and *Hae*III, respectively. As evident from the Fig. 3.5, race 1, 2 and 4 exhibit identical restriction digestion profiles while race 3 shows a different profile with each restriction endonuclease used in the present study. Thus, it was possible to identify race 3 easily using ITS-RFLP approach.

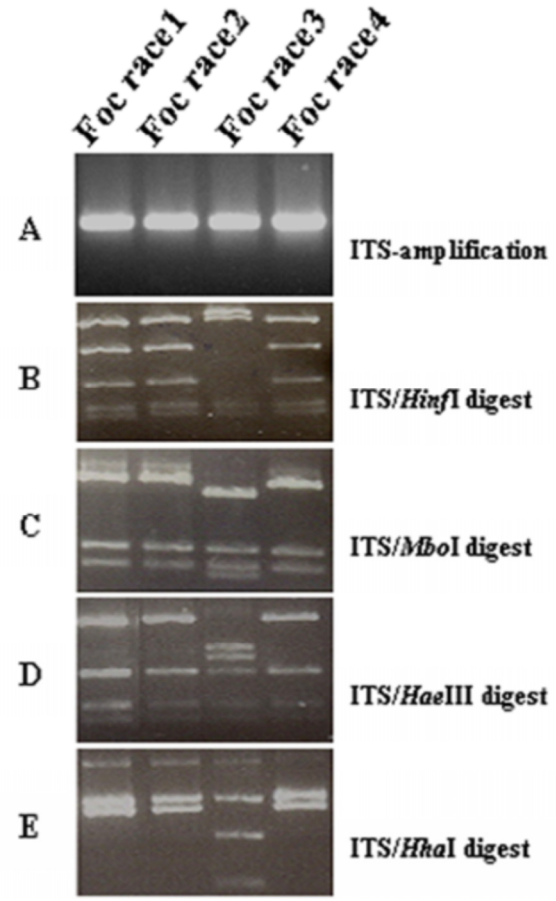


Fig. 3.5 ITS-RFLP patterns of *Foc* races (A): *Foc* genomic DNA amplified using ITS oligonucleotides 1/4, (B): ITS amplification product of 550bp digested with restriction endonuclease *Hinf*I, (C): ITS amplification product digested with restriction endonuclease *Mbo*I, (D): ITS amplification product digested with restriction endonuclease *Hae*III and (E): ITS amplification product digested with restriction endonuclease *Hha*I. Lanes 1, 2, 3 and 4 represent genomic DNA of *Foc* races 1-4 as labelled.

3.1.4 AFLP mediated differentiation of *Foc* races 1 and 2

Foc races 1 and 2 could not be distinguished using either GSOs or ITS-RFLP technique. Hence, AFLP technology was employed to distinguish *Foc* races. Several race 1 or race 2 specific AFLP primers were identified using silver staining method of fragment detection. AFLP oligonucleotide combinations and their corresponding race specificities have been detailed in Table 3.1.

Table 3.1 AFLP screening of *Foc* race 1, race 2 and their isolates		
Number of oligonucleotides screened	**Race specific oligonucleotide combination**	**Race specificity and approximate fragment size (bp)**
64 (E+3/m+3)	E-ACA/m-ctt	Race1 (400) and 2 (350)
16 (E+2/m+2)	E-AA/m-ca	Race1 (230) and 2 (118)
	E-AC/m-cg	Race1 (194)
	E-AG/m-cg	Race2 (118)
24 (E+2/m+3)	E-AA/m-caa	Race1 (230) and 2 (194 and 118)
	E-AA/m-cac	Race2 (234)
	E-AA/m-cag	Race1 (236) and 2 (290)
	E-AA/m-ctt	Race2 (400)
	E-AC/m-cac	Race1 (118)
	E-AC/m-cta	Race2 (230)
	E-AG/m-cac	Race1 (194)
	E-AG/m-cat	Race1 (294)

All these oligonucleotide combinations were further used to analyze races 1 and 2, and two isolates of each race (as represented in Fig. 3.6). In all, 64 (E+3/m+3) combinations were screened, of which only one oligonucleotide pair, namely, E-ACA/m-ctt showed race-specific polymorphisms. Out of the 16 (E+2/m+2) oligonucleotide combinations screened, three oligonucleotides exhibited race-specific polymorphisms between races 1 and 2, while out of the 24 (E+2/m+3) combinations, eight oligonucleotide combinations were polymorphic for races 1 and 2. Thus, out of total 104 oligonucleotide pairs screened, only 12 oligonucleotide pairs were polymorphic for races 1 and 2. These oligonucleotide combinations yielded 17 polymorphic DNA fragments (race-specific) in the size range of 100-400bp, which were present in either standard *Foc* race 1 or race 2 and also in their respective isolates. A representative AFLP profile of *Foc* DNA with oligonucleotides E-AG/m-cg and E-ACA/m-ctt has been depicted in Fig. 3.7A and B.

3.1.5 Cloning and sequencing of AFLP derived fragments

The race specific AFLP fragments were cloned and sequenced. At least 10-15 clones were picked up representing each fragment. The sequence alignment of clones of a single fragment revealed variations. Homologous sequences were aligned, errors were removed and then the sequences were used for designing SCAR markers. The

sequences were deposited in NCBI Genbank database. Fig. 3.8 (A-M) represents sequences of race specific AFLP fragments.

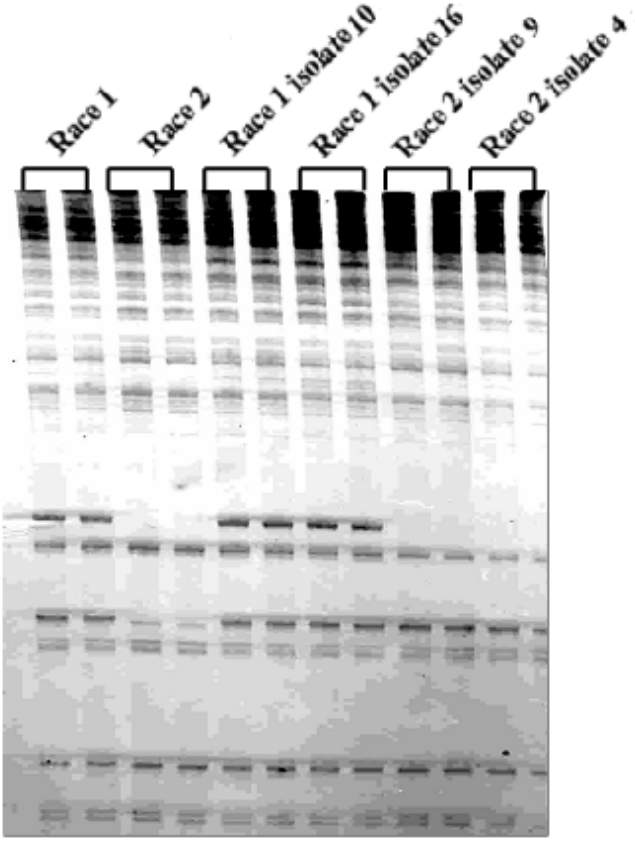


Fig. 3.6 AFLP profile of *Foc* races and isolates using primer combination E-aa/m-caa

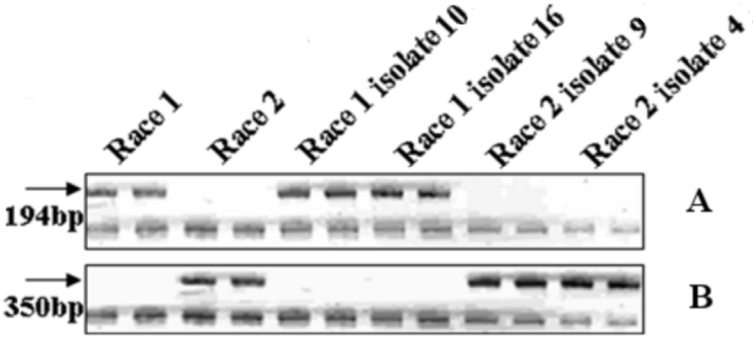


Fig. 3.7 AFLP profile of *Fusarium oxysporum* f. sp. *ciceri* DNA (A): AFLP profile of *Foc* DNA with oligonucleotide E-AG/m-cg (B): AFLP profile of *Foc* DNA with oligonucleotide E-ACA/m-ctt. Lanes 1 and 2 represent *Foc* race 1 genomic DNA, lanes 3 and 4 represent *Foc* race 2 genomic DNA while remaining lanes represent other isolates either classified as race 1 (lanes 5-8) or race 2 (lanes 9-12) all in replicates with equal amount of genomic DNA used for the initial digestion reaction.

A

>gi|193090083|gb|EU556726.1| *Foc* race 1 AFLP fragment fragment 1 genomic sequence

TCGCTCTGCCTCTGATATCGTTTTCCTTGCCCCCGGTCTTGGTGCTATCATTGACGCTTTGAAAACTTCA
AGACAGATTTTCCATCGAATGTATGCTTACGTCGTCTACCGTATTGCTCTGTCTCTCCACATGGAGATCT
TCCTTGGTCTCTGGATCGCCATCCTCAACAGGAGCTTGAACATTGAGCTTGTCGTTTTCATTGCCATTTT
CGCTGATATTGCTACTCTTGCCATTGCTTACGACAACGCTCCCTTCTCCCAGACTCCCGTCAAGTGGAAC
CTCCCCAAGCTCTGGGGTATGTCCGTCCTTCTCGGTGTTGTCCTGGCTGT

B

>gi|193090074|gb|EU520466.1| *Foc* race 1 AFLP fragment fragment 2 genomic sequence

AATACTAAGGTGAACATGCTACGGGACCAAGCTGGTGGGACTAACCATCCAATAAGCTTCGCTGCGCAAG
CGCCCGGAATTTCGGATAGATGTAAGCGCTTGACTGCGTACCAATTCAACGGAAATAGAGTAATTGTCGA
CGTGACAAAGAGTGATGGGTTACAAGGCGGCAACGCGACTGTAGATGGTTAGAC

C

>gi|193090084|gb|EU556727.1| *Foc* race 1 AFLP fragment fragment 3 genomic sequence

CAATTCCCTTGTATCTCTACACACAGGCTCAAATCAATAAGAAGAACGGTTCGTCTTTTTCGTTTATATC
TTGCATCGTCGTTTAAGCTATTGGCGGGATATTCTGTCCGCAGTTGGCTGACTTGAAGTAATCTCTGCAG
AGTAGCACCGTCGTCGACCGGCATTCATCGCGGCCTGGAGACGTTCTCGCAGAATCTTCTTCCAACACAT
CTCCGGCACCGCCTGGTACACGCAGCTTGCGCCGGTTTCC

D

>gi|193090085|gb|EU556728.1| *Foc* race 2 AFLP fragment fragment 4 genomic sequence

TCGAGTATATCATGGCTGAGCAGCAGATCTCCAACCTTCTTTCAATGTTTGATGCTTTTCACGCAAGCCA
GAAGTTGGAGAATACGGAACAGGTGATGGAATCCTTCCATTACAGAGACACTCGCACCAGACTCTTCCTC
TTCGGAAGGTGTTCCATATCTCGCTATGATGAGCGACGAGTCTCCCTTGGTCTC

E

>gi|193090086|gb|EU556729.1| *Foc* race 1 AFLP fragment 5 genomic sequence

CTACTCTAGGACTGCTTGGTGATTCTGGCTGCCTTTTCTCTGCAGGAAACTTCCAGGTGGTCACTCCCCG
TCAAGGATCATCAGCAGTCCGTCGCCTACACCTCGAGGCCACCGTAATATGGGCACCTCTTTTGCTAGAG
ACGGACTCTGACAGGAGTTCTAGTAGCTGCCATCGACGGGCCATTCAGCATACCCATGTTAGAGTTGAAC
CTTATTGCTTGGTCTGTGGGCGACACTTCACTCCAGTGGGCAAATATGCTATGTAGTGTACCCACCCTGG

AGATGGACAATCGC

F

>**gi|193090082|gb|EU520474.1|** ***Foc*** **race 1 AFLP fragment 6 genomic sequence**

AGGAACAAGCACCTGCAGCCGTCGCTCGTCACGGATGAGTATGAACGATATGCAACTCTGAACGGGTTTA
CGGGTTCACAAGCGCCCTTGCATGGTGGCTAGAGGAGACACAGCAGAAAAACTACCCAAACTTGAGTAAA
ATGGCGGTGGACATACTGTCAATTCCCGCAATGTCTGCTGAGCCCGAACGACTCTTCTCCGGGGCCAAGA
TAACCATTACAGATCATCGATGCTCGTCTTGCGCAAATCG

G

>**gi|193090078|gb|EU520470.1|** ***Foc*** **race 1 AFLP fragment 7 genomic sequence**

GAGGATGTCTTTACCTCGAGTAAATGCAGGCCTGCAATAGCTCTGAACTGCTTTATTGATATGCCATAGG
CATAAGAATAGGGCTGAACCTGGGAAACAGGAGGAAACGGCATTCATACAAGCGAGGCACCGATCTGTAA
GTATTACAGATGGAAGGCCTATGTTATGATCCTCATATACAGATCGTAACGCTTGAAGAGCCCAGGTAAA
GTCACCCTCTTCCTCACCACTGAGGAATGCAAATGCAATACAGAAGGTCCGTTGGCAAGCATCAACTCCA
ACTATATCAGGAGAGGCATCTTGAACCTATTGGTCTTATGTGTCCGAGTCCAATATAAGCACTTCAGGAT

H

>**gi|193090075|gb|EU520467.1|** ***Foc*** **race 1 AFLP fragment 8 genomic sequence**

TTCGCAAAACGAGTATCGATGAATACGATTGCAATGGACCCTTACGATGATGGCACCGTCGCCAATTGAT
TGATGTATGGGAGCTAATTGCCGCACCGCAGTCTATCTACAAGTCCCAGGATGTCGTCCAGGTCAACATC
CCCGCCGAGTCTGGAGAGATGGGTGTCCTCGCCAACCACGTTCCTTCCATTGAGCAGCTGAAGTCTGGTC
TG

I

>**gi|193090076|gb|EU520468.1|** ***Foc*** **race 1 AFLP fragment 9 genomic sequence**

CTCGCCAACCACGTTCCTTCCATTGAGCAGCTGAAGTCTGGTCTGGTTGAGGTTGTCGAGGAGTCCGCTG
GCTCCAAGCAGTTCTTCCGTACGTCATTAGTTATAGATCCAGATCCAAGATCCAATGCCCGAGGTCGACA
AATCGCTGATGGAATTATGTCTTAGTCTCTGGTGGATTCGCTACCGTTCAGCCGAACTCCGTCCTCAGCA
TTAACGCTGTTGAGGGATACCCCCTTGAGGACTTCAGCGCCGAGGCCATCCGAGCCCAGATCGCTGAGGC
CCAGAAGGTTGCCA

J

>**gi|193090087|gb|EU556730.1|** ***Foc*** **race 2 AFLP fragment 10 genomic sequence**

AGGGAGACAGTGGTGGATCCGATTGGTATATTAATCAAACTACTTTACTGAAATTGATGAGGCAATAAAG
AAGAGAGCCCATAAGATCATTGACTATGAGCAATGTAAATCGAAAGTAAGAAGATTGGTTGATATAGGTG
CCATAGACGCTGCCATGTTGCCCCGGATCGAAAAGGAATTGTCAATGGCGAAGAGATATATGATGAATTG

AATGATCAATTAAAGGCAGAGTTGCCACAGTTGATTGCATTAAGAGTGCCCTTCTATGATCCTTCATTTG
AGGCATTGGATCTATTCAATTGAGGTTT

K

>**gi|193090077|gb|EU520469.1|** ***Foc*** **race 2 AFLP fragment 11 genomic sequence**

CGTTGGGTGACGAAGTGAGGCATCTAGAGAAGTTAGTGAGTGTTGGACGTAGGGGGAGTAAGGAGGACTA
ACCTGCATCTGAACCAATTGACCAAAGACTGTGAGGACCTGGAAGATGGCAAACATCTGGTTCTGCAAGC
CCTGAATTGAAAGAGGAGCGTTGAGGAAGACGAGACCGATAAGAGACCAACTTGGATACACAGAGCAGCC
TTGGAGTAGATGTATGAAGGAGTGCGCCAGTACTGCTGGAAGACACGGAGACTAGCAATGCGAAGCTGCT
CCCAGAAAGGCGCAGCGAATTCCCGGTACGACTCAGAGTTCTTGTCGTGAGGTTCATTGTGAGCACTGCC

L

>**gi|193090088|gb|EU556731.1|** ***Foc*** **race 2 AFLP fragment 12 genomic sequence**

TGGATGACAACGGTTCGGCCAATGATTCTGTGAGGGCCAGTCAGCTTGATAAGAGAATCAGTGACGGAAC
CCTTGGCATTCCCTGGCCCAGTCTCAACGTTTCCGAGATCACCAACGTGACGGGTCTCGTCAGAAGGAGC
ACCGTGGGTC

M

>**gi|193090089|gb|EU556732.1|** ***Foc*** **race 2 AFLP fragment 13 genomic sequence**

TCCTCCCCTTTCCTCTACCCCTTACTCTCTCCCGCCTGTTTTCTTCCGTGCCCTACTTCCATACCGTCTT
TTTCCCGCCCGCCACCCACTCCTCCTCTCTACCCATCTCCTG

Fig. 3.8 (A-M) represent sequences of race specific AFLP fragments

3.1.6 DNA sequence characterization of AFLP fragments

All race-specific bands obtained were cloned and sequenced. Clones of a single AFLP fragment revealed multiple sequences of the same size. Sequence homology was determined using the BlastN program in the NCBI database (database- fungi). Few of the AFLP fragments showed homology to metabolically essential genes of fungi (Table 3.2). AFLP fragments 3, 9 and 11 showed homology to *gpd*A, ATP synthase subunit delta precursor and ABC transporter genes, respectively, suggesting their probable role in metabolism.

Table 3.2 Homologies of race specific AFLP fragments to reported sequences in NCBI database					
Fragment no.	**Accession no.**	**AFLP combination**	**Race specificity & insert size**	**Sequence homology observed with**	**Accession no.**
1	EU556726	AA/cag	Race1, 350 bp	FOL10E3 Fol2_NS_SSH Fol cDNA clone FOL10E3, mRNA sequence	CK615560.1
2	EU520466	AA/cag	Race1, 236 bp	Asexual conidial life-cycle of *Foc* cDNA clone FU099a11 5'	EC598090.1
3	EU556727	AA/cag	Race1, 250 bp	*A.nidulans gpdA* gene	Z32524.1
4	EU556728	AA/caa	Race2, 194 bp	*F. proliferatum* for geranylgeranyl diphosphate gene, ggs2 (D)	AJ810803.1
5	EU556729	AC/cg	Race1, 294 bp	*C.cinereus clp1* gene	AB034196.1
6	EU520474	ACA/ctt	Race1, 250 bp	Transposase-like protein, *F. oxysporum*	BAA32244.1
7	EU520470	ACA/ctt	Race1, 350 bp	*F.oxysporum* f. sp. *melonis* clone Hop78 transposon	gi30421203
8	EU520467	AA/cag	Race1, 230 bp	*G. zeae* PH-1 strain PH-1; NRRL 31084 chromosome 4	XM_386824.1
9	EU520468	ACA/ctt	Race1, 294 bp	*M. grisea* 70-15 ATP synthase subunit delta precursor	XP_360368.2
10	EU556730	ACA/ctt	Race2, 308 bp	*C. dubliniensis* partial *rvs161* gene	AJ634665.1
11	EU520469	ACA/ctt	Race2, 350 bp	*G. pulicaris abc1* gene for ABC-transporter	AJ306607
12	EU556731	AA/caa	Race2, 150 bp	*G. zeae* cDNA from subtracted cDNA library of infected wheat heads	DT527791.1
13	EU556732	AA/caa	Race2, 112 bp	*P. triticina* cDNA clone Ptg.1.R--Ptg053102.1.R	EC415940.1

Fragment 5 of asexual fungus *Foc* showed similarity to *clp1* gene involved in A-regulated sexual development of *Coprinus cinereus*. Fragment 6 and 7 exhibited similarity to transposase like protein in *F. oxysporum* and *Hop78* transposon of *F. oxysporum* f. sp *lycopersici*. Fragment 8 was found to be homologous to hypothetical protein present on chromosome 4 of *G. zeae*. Fragment 4 revealed homology to geranylgeranyl diphosphate *ggs2(D)* gene of *F. proliferatum*. Fragment 2 and 13 showed homology to ESTs of *F. oxysporum* under asexual stage of its life cycle and *Puccinia triticana* clone of cDNA obtained from mixed stages of growth. Interestingly, fragment 10 was similar to *rvs161* gene of *Candida dubliniensis.* This gene encodes a protein that is expressed only during starvation conditions. Similarly, fragment 1 showed homology to EST of *F. oxysporum* f.sp *lycopersici* under nitrogen starvation conditions. Hence fragment 1 and 10 along with fragment 12 which is similar to *G. zeae* clone of subtracted cDNA library of infected wheat heads; could be appropriate candidates to decipher the virulence mechanism of *Foc* as many of the virulence genes are known to express during nutrient stress (Talbot *et al.*, 1993; Coleman *et al.*, 1997).

3.1.7 *Foc* race 3 is distinct from the other *Foc* races

The genetic profile of *Foc* race 3 was found to be different than the other three races using gene specific markers, ITS-RFLP as well as AFLP markers. Previous studies conducted in other labs as well as in our lab indicated the unique nature of race 3. It has been reported that race 3 was biochemically different from races 1, 2 and 4 with respect to total sugar, and content of individual amino acids glycine, arginine, methionine and histidine (Desai *et al.*, 1992a). Simple sequence repeats as probes for DNA fingerprinting, have also demonstrated unique hybridization profiles for *Foc* race 3 (Barve *et al.*, 2001). Therefore, amplification, cloning and sequencing of elongation factor-1α (EF-1α) for all the four standard *Foc* races was performed in the laboratory of Dr. O'Donnell (USDA, ARS, Washington DC)

Sequence data from EF-1α of the standard isolates of four Indian *Foc* races, Fu7, *Foc*39, *F. solani*, and *F. udum* was compared. Phylogenetic analysis using PAUP (Swofford, 1998) revealed that EF-1α sequence of *Foc* race 1, 2, and 4 standard isolates was similar to *F. oxysporum* (NRRL 25420 *Fov*1, NRRL 26034 *Fol*) and that of *Foc* race 3 actually represented *F. proliferatum* (accessions AF160280 and AF291058) (Fig. 3.9).

Since *Foc* race 3 was characterized to be *F. proliferatum*, it was excluded from the further proteomic and transcriptomic studies, in this thesis work.

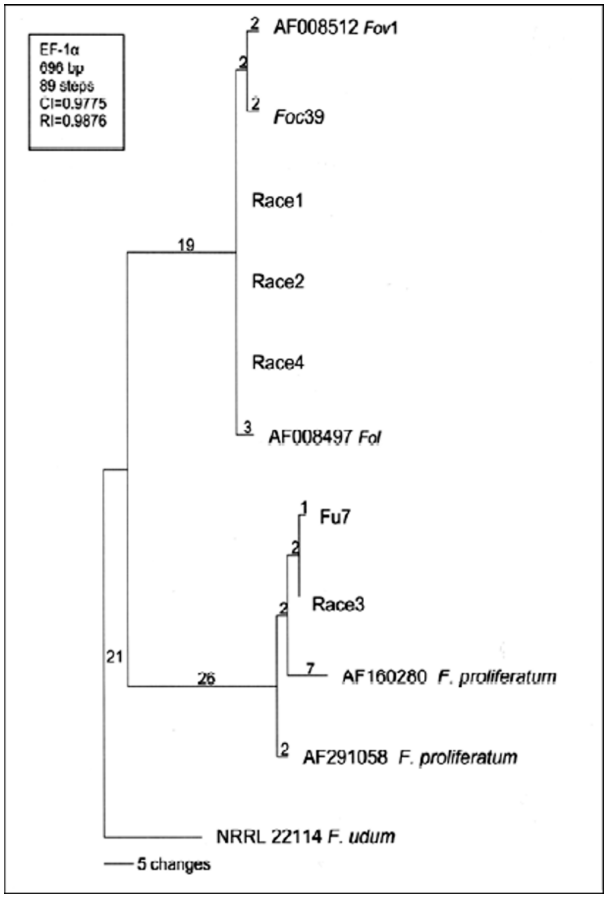


Fig. 3.9 Most-parsimonious tree inferred from the partial EF-1α DNA sequence data of *Foc* races 1, 2, 3 and 4. The phylogram was rooted with a sequence of *F. udum* (NRRL 22114). Reference strains NRRL 25420 *Fov*1 (*F. oxysporum* f. sp. *vasinfectum* race1, accession AF008512), NRRL 26034 *Fol* (*F. oxysporum* f. sp. *lycopersici*, accession AF008497), *F. proliferatum* (accession AF160280), and *F. proliferatum* (accession AF291058) (Barve, 2003)

3.2 Characterization of Indian pathogenic races of *Foc* using proteomic approach

In an attempt to identify and distinguish putative fungal virulence factors, one-dimensional gel electrophoresis (1-DE) protein profiles from *Foc* races differing in virulence were compared. Protein extracts from fungal filtrate as well as mycelium, obtained by tissue homogenization were analyzed. The protein profile revealed differences between the analyzed races. Due to the absence of protein and DNA databases containing *Foc* sequences, protein identification was achieved by peptide fragmentation fingerprinting (PFF) followed by either database search or sequence alignment.

For comparative proteome analysis, filtrate and mycelial protein extracts from *Foc* races 1, 2 and 4 were used. After 1-DE separation and Coomassie Blue staining of the protein extracts of *Foc* races, 10-12 protein bands were resolved and detected by digital image analysis and visual confirmation (Fig. 3.10).

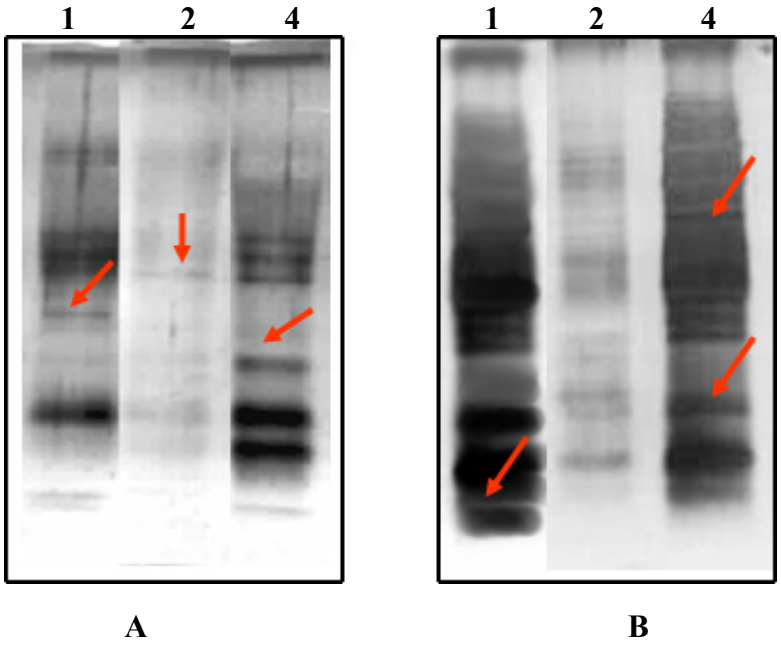


Fig. 3.10 Proteomic profiles of *Foc* filtrate and mycelial proteins

(A) 1D-SDS PAGE profile of *Foc* filtrate proteins (B) 1D-SDS PAGE profile of *Foc* mycelial proteins. Lanes 1, 2 and 3 represent protein profiles of *Foc* races 1, 2 and 4, respectively

3.2.1 Peptide mass fingerprinting

The peaks were observed for all the peptide samples obtained by manual in-gel trypsin digestion using MALDI-TOF. Fig. 3.11 represents the MALDI-TOF profile of one of the trypsin digested mycelial proteins m4.4. The peaks obtained for the samples were calibrated using a standard mixture of peptides containing insulin chain B oxidized (3,493.6513 daltons), bradykinin fragment 1-7 (756.3997 daltons), ACTH fragment 18-39 (2464.1989 daltons) and angiotensin II (1045.5423 daltons) (ProteoMassTM, MALDI-MS standard, Sigma). These calibrated peaks were then analysed using freely available 'MASCOT' software from Matrix Science (http://www.matrixscience.com) database. Not all proteins could be identified since only a few showed relevant homologies with reported proteins in the database. Table 3.3 indicates the proteins with significant homologies to hypothetical G. zeae proteins. Further, these were found to be similar to fungal proteins essential for virulence like glucosamine 6-phosphate N-acetyltransferase, glycosyltransferase and DEAD-box RNA helicase.

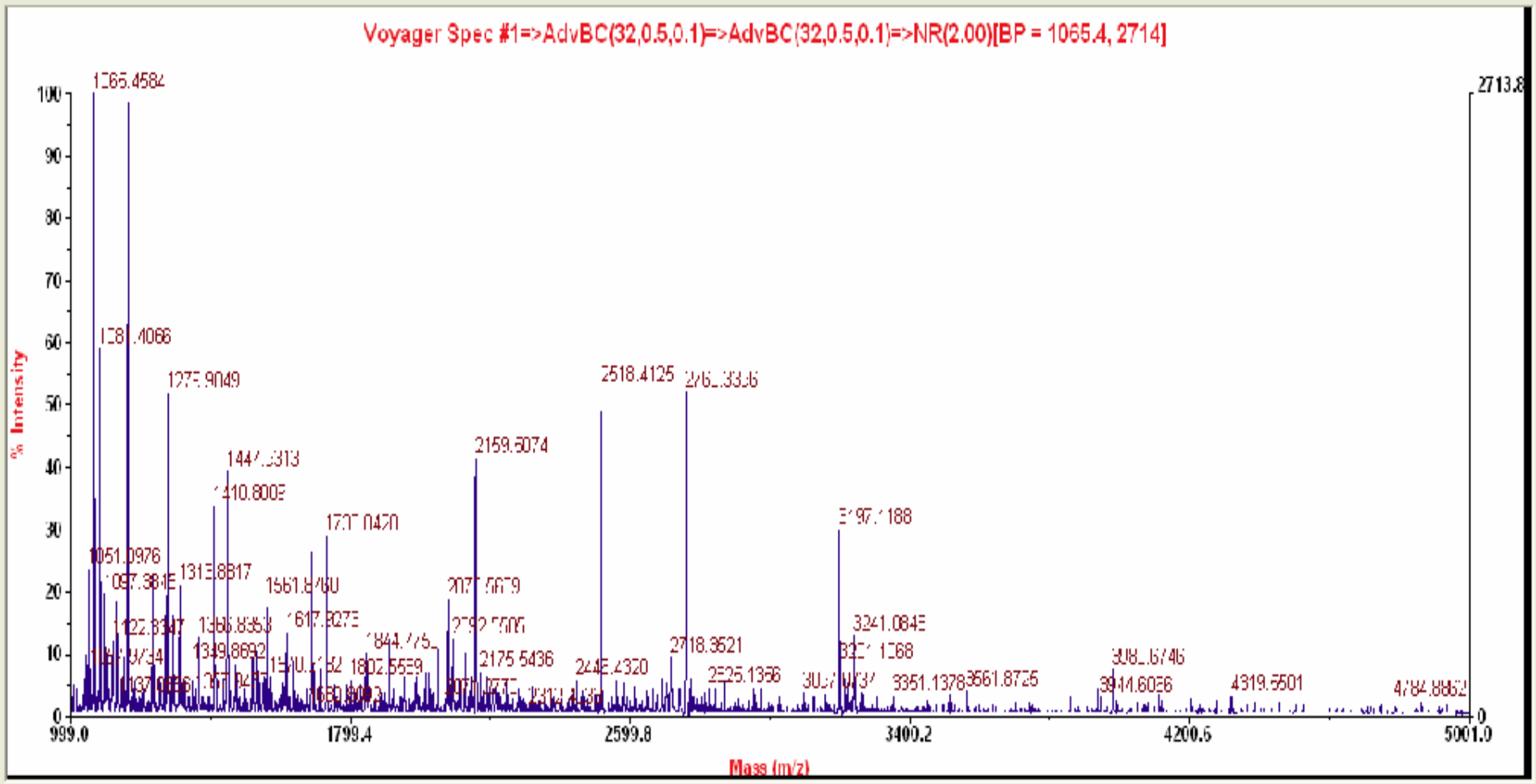


Fig. 3.11 MALDI/MS spectra obtained with CHCA matrix for the race 4 mycelial protein sample (m4.4) located on 1DE gel

Table 3.3 Homologies of trypsin digested *Foc* proteins analyzed using MASCOT software			
Protein fragment	**Top score**	**Homologous to**	**Similar to**
m4.1	41	GENE ID: 2789782 FG08944.1 | hypothetical protein [*Gibberella zeae* PH-1]	GENE ID: 3881679 NCU01902 | glucosamine 6-phosphate N-acetyltransferase [*Neurospora crassa* OR74A]
m4.4	46	GENE ID: 2786390 FG04677.1 | hypothetical protein [*Gibberella zeae* PH-1]	GENE ID: 854293 IAH1 | Iah1p [*Saccharomyces cerevisiae*]
f1.3	45	Q4IM62_GIBZE, Hypothetical protein.- i (*Fusarium graminearum*)	GENE ID: ZP_04552657 glycosyltransferase [*Bacteroides* sp. 2_2_4]
f4.1	63	GENE ID: 2789149 FG06580.1 | hypothetical protein [*Gibberella zeae* PH-1]	Q43NC5_SOLUS, Biotin/lipoyl attachment.- *Solibacter usitatus* Ellin6076 GENE ID: 7030229 PMAA_006650 | 6-phosphofructokinase alpha subunit [*Penicillium marneffei* ATCC 18224]
m1.1	47	GENE ID: 2783729 FG01357.1 | hypothetical protein [*Gibberella zeae* PH-1]	Q2TZP7_ASPOR, DEAH-box RNA helicase.- *Aspergillus oryzae* GENE ID: 4700165 CLA_081040 | ATP-dependent RNA helicase (Hrh1), putative *Aspergillus clavatus* NRRL 1]

3.3 Chickpea-*Fusarium oxysporum* interactions at transcriptomic level during Fusarium wilt progression

Plant pathogen interactions are complex relationships governed by an array of genetic and metabolic pathways. *Foc*, one of the most important biotic stresses of chickpea, exists as four races predominant in the different regions of Indian subcontinent. In this study transcriptome analysis of chickpea roots during *Foc* infection was performed using two approaches. In the first approach, the cDNA obtained from total mRNA during infection process was amplified using random primers, cloned and sequenced. Sequence characterization revealed homology to various fungal genes and plant genes essential for diverse metabolic functions, including defense. Specific expression patterns of candidate transcripts were analyzed in the infected and uninfected resistant chickpea cultivar, Digvijay, at around day 6 of infection as compared to the susceptible cultivar, JG62. In the second approach, semiquantitative RT-PCR analysis of fungal virulence genes, namely *Fgb1*, *Gas1*, *Chs7* and *Fow1*; and plant defense related genes; namely *BetvI*, Chalcone synthase, Isoflavone reductase, *GroES2* and 60s ribosomal protein was performed using gene specific primers in resistant (Digvijay) and susceptible (JG62) chickpea cultivars upon exposure to *Foc* races 1, 2 and 4 at 2, 6, 9, 13 and 16 days after infection (dai).

3.3.1 Hydroponic culture of chickpea plants

Seven days old chickpea plants were inoculated with fungal spores of *Foc* races individually. Uninoculated plants served as control. In general, Digvijay variety was found to grow at a slower pace as compared to JG62 variety. Upon infection JG62 showed wilting symptoms by 10 dai, while no wilting was observed in case of Digvijay even by 25 dai (till the plants were allowed to grow hydroponically). Within *Foc* infected JG62; race 1 infected JG62 showed wilting symptoms earlier than race 4 followed by race 2. Race 4 was observed to show more aggressive wilting than race 1 and race 2. Fig. 3.12 shows the uninfected and race 1, 2 and 4 infected (showing wilting symptoms) chickpea varieties, JG62 and Digvijay.

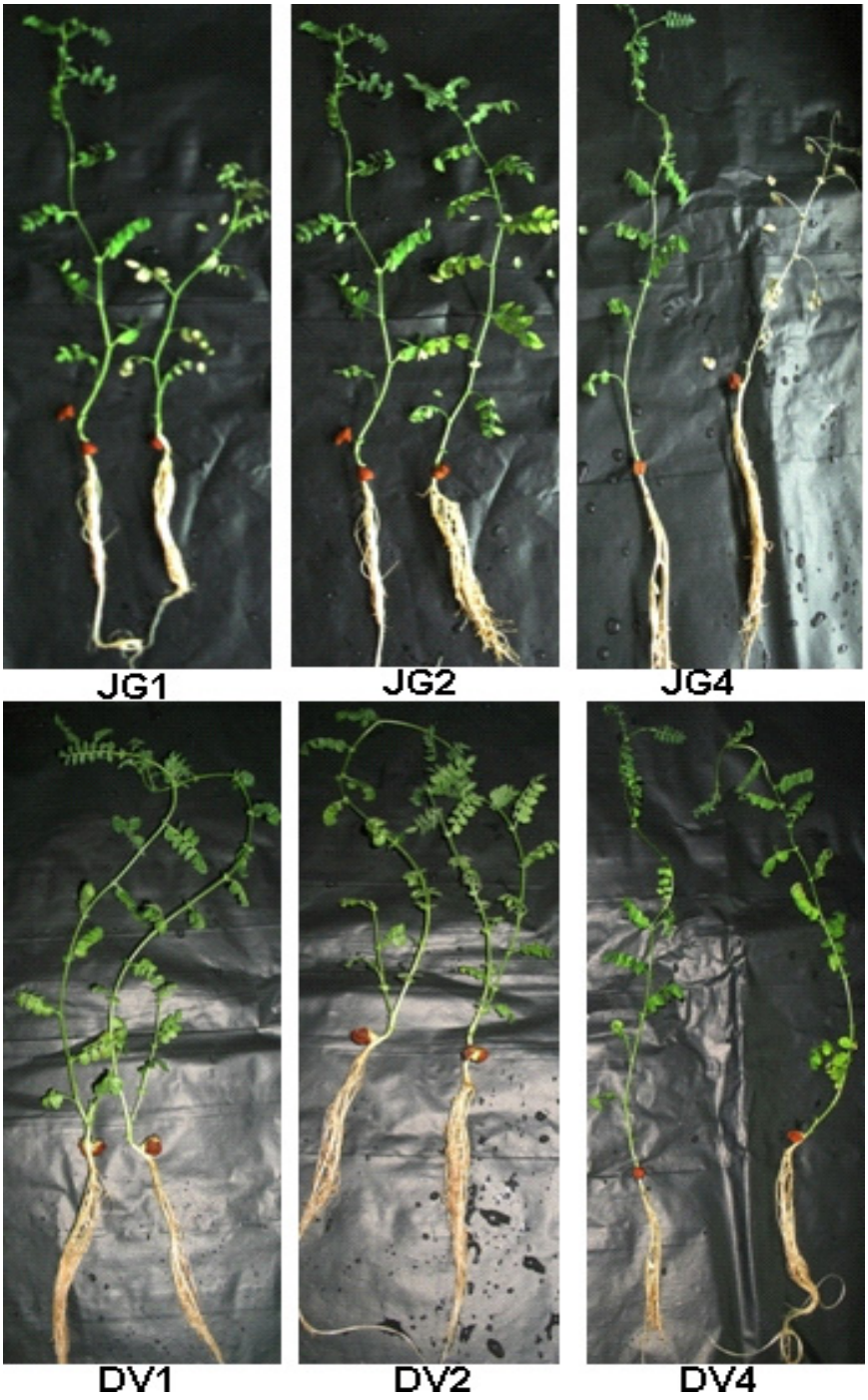


Fig 3.12 *Foc* race infected and uninfected JG62 and Digvijay chickpea varieties

JG1, JG2 and JG4 represent *Foc* race1, 2 and 4 inoculated JG62 plants (right) in comparison to control plants (left), respectively. DV1, DV2 and DV4 represent *Foc* race1, 2 and 4 inoculated Digvijay plants (right) in comparison to control plants (left), respectively.

3.3.2 cDNA-RAPD approach to study differential chickpea-*Fusarium oxysporum* interactions

Using RAPD primers of OPAD and OPAE series (20 primers each), transcript profiling of root tissue cDNAs obtained from *Foc*1 inoculated JG62 and Digvijay cultivars at 6 dai, was accomplished (Fig. 3.13). Differentially expressed and reproducible 134 transcripts exhibiting >200bp size were selected for cloning and 117 clones thus obtained, were further sequenced. Based on homology search, these clones represented 65% and 18% genes of plant and fungal origin, respectively; and 17% were novel in nature (Fig. 3.14). A few of these representative class of genes are tabulated in Table 3.4.

One of these clones was homologous to C3HC4 type zinc finger protein of *A. thaliana*. Though the exact function of zinc finger protein in plant pathogen interactions is yet unknown; in a few host-pathogen interaction studies conducted till date, a constant upregulation of this gene has been observed (Chen *et al.,* 2002). Three fungal genes represented in this study were homologous to Trichothecene 3-O-acetyltransferse (*Tri*) gene, fungal transposase and fungal regulatory protein, respectively. 27% of the clones were homologous to DNA/protein synthesis related proteins like histone proteins and ribosomal proteins; while 7% showed high homology to chaperonin molecules namely *GroES2* and *Hsp70*. Further, a class of clones obtained from inoculated Digvijay roots revealed significant homologies to glycosyltransferase and methionine sulphoxide reductase (*Msr*) genes. A strong homology of one of the transcripts was observed towards a ripening related protein of chickpea. All this sequence information was used to design primers, which were then deployed for expression analysis of the identified genes in inoculated and control resistant cultivar-Digvijay.

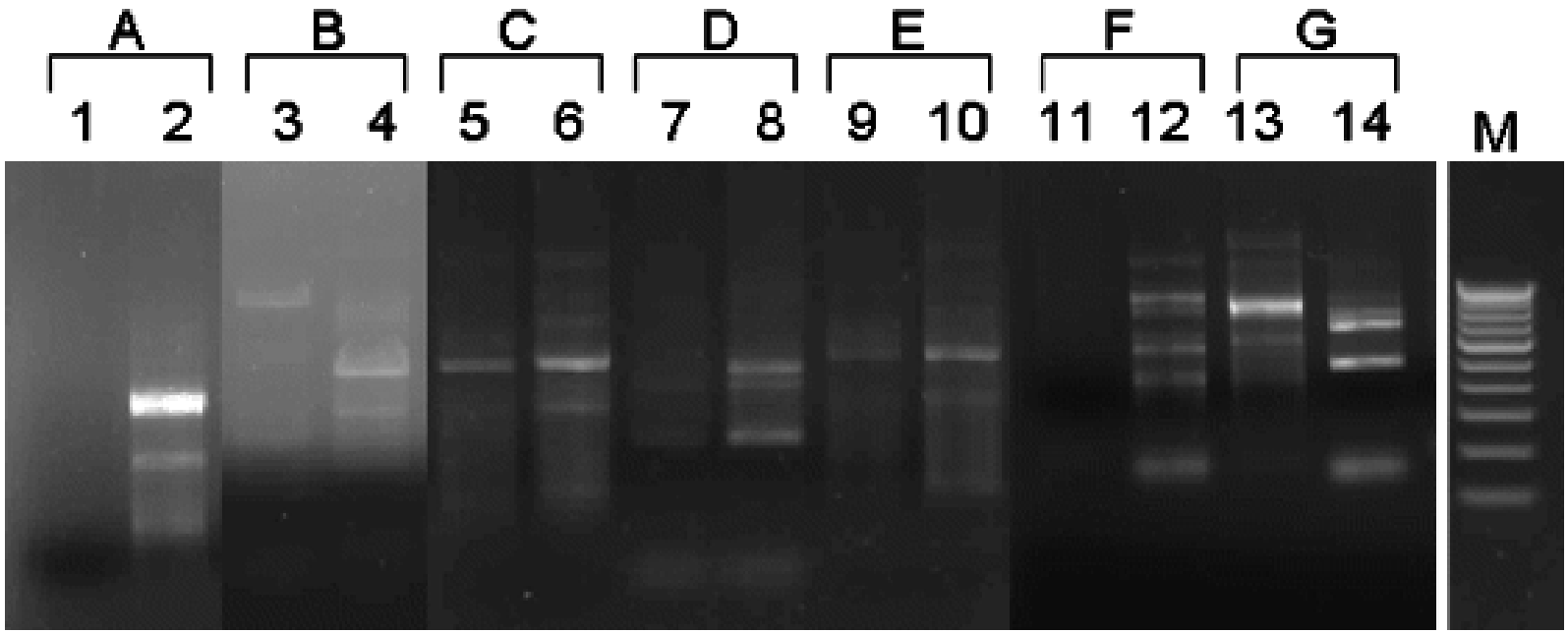


Fig. 3.13 cDNA-RAPD of *Foc* inoculated JG62 (susceptible) and Digvijay (resistant) chickpea cultivars. Lanes 1, 3, 5, 7, 9, 11 and13 represent *Foc* inoculated JG62 cDNA; lanes 2, 4, 6, 8, 10, 12 and 14 represent *Foc* inoculated Digvijay cDNA amplified with RAPD primers (A): OPAD1, (B): OPAD3, (C): OPAD7, (D): OPAD8, (E): OPAE3, (F): OPAE14 and (G): OPAE19

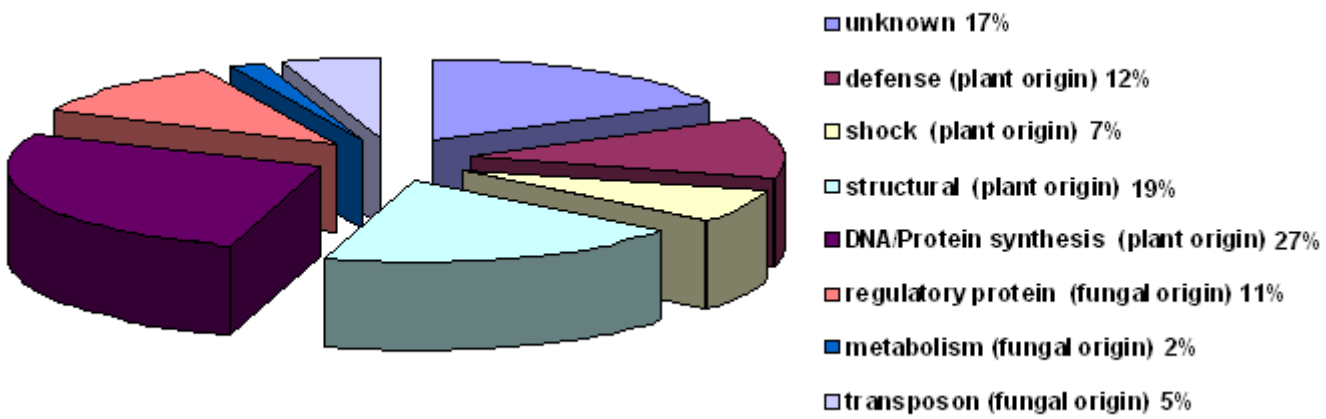


Fig. 3.14 Pie diagram showing the percentage of cDNA-RAPD clones representing functional class of genes

Table 3.4 Homology of cDNA clones generated using random primers to reported sequences in NCBI database		
Homology	**Genebank acc. no.**	**Function**
C. arietinum mRNA for putative ripening related protein	AJ487040	Stuctural
Hypoxia-responsive family protein / zinc finger (C3HC4-type RING finger) family protein *A. thaliana*	NP_190386	Unknown function
C.arietinum 60s ribosomal protein L6	AJ276271.1	Protein synthesis
Histone H2A C. *arietinum*	O65759	DNA synthesis
Transposase *F. oxysporum* f. sp. *melonis*	AAP31248	Fungal
M. truncatula clone mth2-18n7, complete sequence	AC152751	Unknown function
Pentatricopeptide (PPR) repeat-containing protein *A. thaliana*	NP_175985	Unknown function
Regulatory protein *F. culmorum*	AAN08452	Fungal
Ribosomal protein S21e *M. truncatula*	ABD28456	Protein synthesis
Glycosyltransferase *G. max*	CAI94900	Defense related
Fiber protein Fb11 *G. barbadense*	AAN77150	Stuctural
30% homologous to Tricothecene 3-O-acetyltransferase *F. acaciae-mearnsii*	ABE03832	Fungal
Chloroplast methionine sulphoxide reductase B2 precursor *N. tabacum*	ABU49226	Defense related
GroES like protein *A. hypogaea*	ACF74274.1	Chaperonin
M. sativa heat shock protein 70	AY830127.1	Chaperonin

3.3.2.1 Involvement of important fungal and plant specific genes during Fusarium wilting in chickpea

All the primers designed from the sequence information in the previous point were used for semiquantitative RT-PCR in JG62 and Digvijay infected with *Foc* race 1. Fig. 3.15 shows the differential expression pattern of plant defense related and fungal specific transcripts of Digvijay and JG62 only at 6 dai. Although resistant variety Digvijay showed no wilting symptoms upon *Foc* inoculation, fungal genes were found to express; indicating the fungal growth and proliferation in the resistant cultivar. In this study, fungal *Tri101* gene was found to be upregulated during *Foc*-chickpea interaction (Fig. 3.15), although not all the species of the genus *Fusarium* express trichothecene. However, some species have been reported to harbour both functional and non-funtional copies of *Tri* genes (Tokai *et al.*, 2005). Semi quantitative RT-PCR revealed the expression of transposase gene during fungal invasion of chickpea roots (Fig. 3.15). 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).

Apart from the above mentioned fungal genes, several plant genes were found to express in Digvijay upon *Foc* inoculation. Histone proteins are involved in chromosome duplication of eukaryotic genome and are shown to be overexpressed in plants during pathogen attack (Jeong *et al.*, 2005). In the present study, expression of H2A histone protein was found to be unaffected upon infection (Fig. 3.15), while previous reports showed an increase in histone protein levels upon fungal and viral infection in *Petroselinum crispum* and *Glycine max* (Logemann *et al.,* 1995; Iqbal *et al.*, 2005). 60s ribosomal proteins (*60srp*) on the other hand were found to be more abundantly expressed in the inoculated Digvijay plants, suggesting active changes in metabolism of inoculated chickpea roots (Fig. 3.15). It is likely, since the higher need of protein synthesis arises during plant defense (Giri *et al.*, 1998). Similar results were obtained by McFadden *et al.*, (2004), where they showed an increase in ribosomal protein transcript levels during cotton root infection by *F. oxysporum* f. sp. *vasinfectum*.

Glycosyltransferase gene was found to be upregulated in inoculated Digvijay plants as compared to its expression in healthy plants, suggesting its role in mounting defense against pathogen attack (Fig. 3.15), which is concordant with the information available from tomato and tobacco plants wherein, glycosyltransferases have been shown to respond rapidly to signals from wounds and pathogen attack (O'Donnell *et al.*, 1998; Roberts *et al.*, 1999). In inoculated roots of chickpea plants, slight upregulation in the

expression of *Msr* gene was observed (Fig. 3.15). In earlier reports also, Cauliflower mosaic virus (CMV) exposed *Arabidopsis* leaves showed a strong induction of the plastidic *Msr* gene (*c-pmsr*) after 2 to 3 weeks of chronic pathogen infection (Sadanandom *et al.*, 2000).

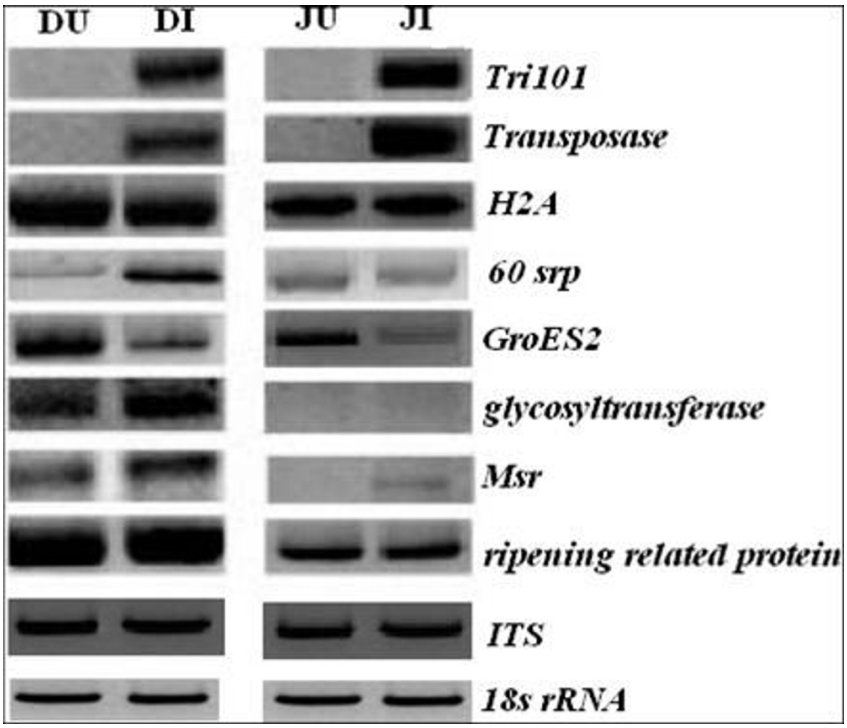


Fig. 3.15 Expression of transcripts in non-inoculated and *Foc* 1 inoculated Digvijay (DU and DI) and JG62 (JU and JI) chickpea cultivars at 6 dai. *Tri101* (tricothecene 3-O-acetyltransferase), *transposase*, *H2A* (histone protein 2), *60srp* (gene for 60s ribosomal protein), *GroES2* (chaperonin), *glcosyltransferase*, *Msr* (methionine sulfoxide reductase), ripening related protein, *ITS* (Intertranscribed spacer region) and *18s rRNA* (for cDNA normalization)

Ripening related proteins are involved during fruit development and ripening. However, these proteins are also thought to protect the developing fruit from pathogen attack and are expressed during symbiotic relationships in plants (De Beer and Vivier, 2008). Slight increase in the expression of ripening related protein in the *Foc* inoculated chickpea cultivars as compared to uninoculated one, was observed, projecting its involvement in plant defense (Fig 3.15); though the exact role of this protein during pathogen attack needs to be justified. Chaperonin molecules are reported to be essential for the plants during biotic and abiotic stress conditions (Rutherford, 2003). However, in our studies *GroES2* chaperonin was found to be downregulated in *Foc* inoculated chickpea plants as compared to the uninoculated plants (Fig. 3.15).

3.3.3 Candidate gene approach to study fungal pathogenesis in *Fusarium oxysporum* f. sp *ciceris* – chickpea system

An array of fungal pathogenesis related genes have been reported till date which are involved in cell signaling, adhesion and appressoria formation, production of cell wall degrading enzymes (CWDEs) and toxins, etc. (Tudzynski and Sharon, 2003). In the present study, expression of many of these genes during *Foc* infection to chickpea (both resistant and susceptible cultivars), was attempted but only a few genes could be successfully analyzed throughout the course of infection (2-16 dai). For example, genes encoding for CWDEs did not show a prolonged expression, while genes involved in appressoria formation could not be amplified in *Foc*. However, few important genes essential for *Foc* infection were extensively studied, at various time points, throughout the course of infection. *Foc* 1, 2 and 4 inoculated JG62 and Digvijay root cDNA samples were normalized using ITS primers, prior to candidate gene studies with fungal gene specific primers. All the cDNA samples showed uniform amplification with ITS primers (Fig. 3.16). The primers being specific to *F. oxysporum*, they did not show amplification in uninoculated chickpea cultivars.

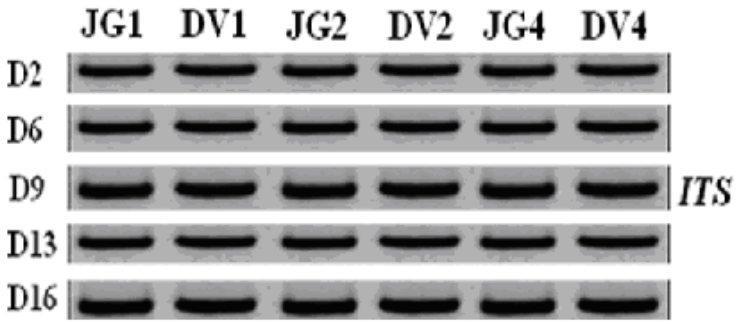


Fig. 3.16 cDNA normalization using ITS primers. JG1, DV1, JG2, DV2, JG4 and DV4 represent *Foc* 1, 2 and 4 inoculated JG62 and Digvijay root cDNA samples. D2-D16 represent days after infection.

3.3.3.1 Fungal pathogenesis related gene expression in *Fusarium oxysporum* f. sp *ciceris* inoculated chickpea during wilt progression

Fgb1 gene

F. oxysporum G protein β subunit (*Fgb1*) has been reported to be essential for cell signaling, hyphal growth, conidiation as well as virulence. In this study race specific response of *Fgb1* was observed in *Foc* race 1, 2 and 4 inoculated resistant and susceptible cultivars (Fig. 3.17).

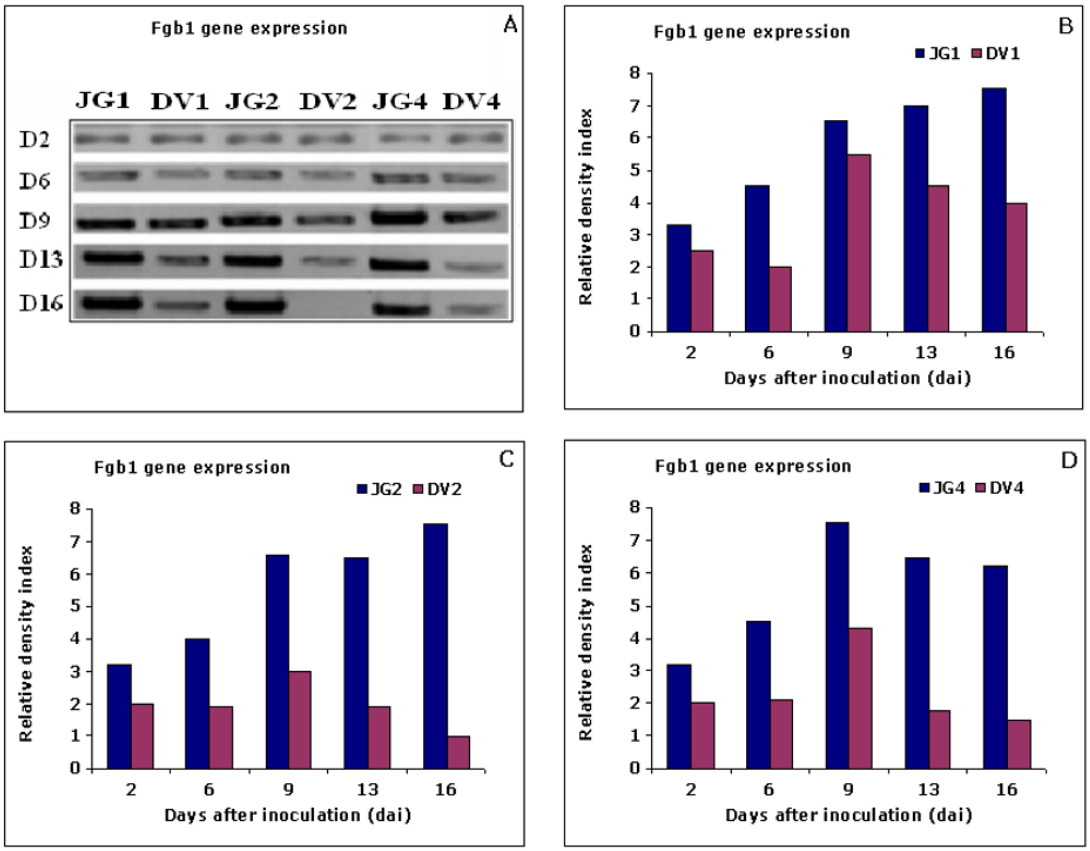


Fig. 3.17 *Fgb1* gene expression profile in *Foc* races 1, 2 and 4 inoculated JG62 and Digvijay. A: *Fgb1* gene expression profile. JG1, DV1, JG2, DV2, JG4 and DV4 represent *Foc* 1, 2 and 4 inoculated JG62 and Digvijay root cDNA samples. D2-D16 represent days after inoculation. B: A comparative graphical representation of *Fgb1* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 1. C: A comparative graphical representation of *Fgb1* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 2. D: A comparative graphical representation of *Fgb1* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 4. (Days after inoculation (dai) are represented on X axis while relative density index values are represented on Y axis which indicate the image density, measured using Syngene tools.)

Foc 1 and *Foc* 2 showed progressively intense expression of *Fgb1* gene till 16 dai as compared to *Foc* 4 infecting JG62, which showed lower expression at 16 dai. A reduced gene expression response was observed in case of Digvijay, especially where no expression of *Fgb1* gene was observed at 16 dai in case of *Foc* 2 infecting Digvijay (Fig. 3.17A).

As seen in the graphical representation of *Fgb1* gene expression, *Foc* 1 infecting JG62 showed higher expression of *Fgb1* gene increasing gradually from 2 dai to 16 dai; while in Digvijay the gene expression was maximum at 9 dai (Fig. 3.17B). Similar observation was made in case of *Foc* 2 and 4 infecting Digvijay, which showed the highest expression of *Fgb1* gene at 9 dai (Fig. 3.17 C and D). The response of JG62

inoculated with race 2 was similar to that inoculated with race 1, but in case of race 4 inoculated JG62, maximum expression of *Fgb1* gene was observed at 9 dai which decreased gradually as the disease progressed to 16 dai (Fig. 3.17D).

Comparative analysis of expression of *Fgb1* in case of susceptible (JG62) and resistant (Digvijay) cultivars of chickpea with *Foc* races 1, 2 and 4 indicated that in general *Fgb1* gene expression was high in case of race 1 and 2 infecting JG62 as compared to race 4 infecting JG62 (Fig. 3.18A). In case of Digvijay the expression levels were manifold low, still the highest expression was observed in case of *Foc* 1 infecting Digvijay at 9 dai (Fig. 3.18B).

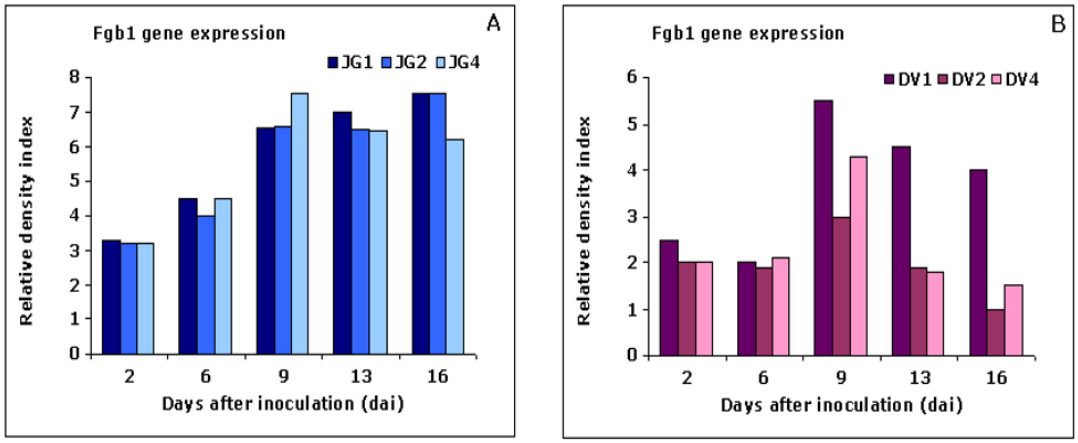


Fig. 3.18 Graphical representation of *Fgb1* gene expression profile in chickpea cutivars inoculated with *Foc* races 1, 2 and 4. A: A comparative graphical representation of *Fgb1* gene expression profile in JG62 inoculated with *Foc* race 1, 2 and 4. B: A comparative graphical representation of *Fgb1* gene expression profile in Digvijay inoculated with *Foc* race 1, 2 and 4. (Days after inoculation (dai) are represented on X axis while relative density index values are represented on Y axis which indicate the image density, measured using Syngene tools.)

Gas1 gene

Glucanosyltransferases are essential for growth and morphogenesis of fungi and thus are involved in fungal pathogenesis as well, when the fungus comes in contact with the host tissue (Mouyna *et al.,* 2005). The temporal expression of *Gas1* revealed involvement of this gene throughout the course of disease development and establishment. *Gas1* was found to be expressing in *Foc* 1, 2 and 4 infecting JG62 as well as Digvijay (Fig. 3.19A). Intense expression was observed in *Foc* 4 followed by *Foc* 1 infecting JG62 till 13 dai. *Gas1* gene expression in *Foc* 2 infecting JG62 was observed only till 9 dai after which there was a drop in the expression at 13 dai. *Foc* 1, 2 and 4 showed *Gas1* expression only till 9 dai when infecting Digvijay. In general

expression of *Gas1* was intense and prolonged in *Foc* when infecting susceptible cultivar as compared to the resistant cultivar, Digvijay (Fig. 3.19A).

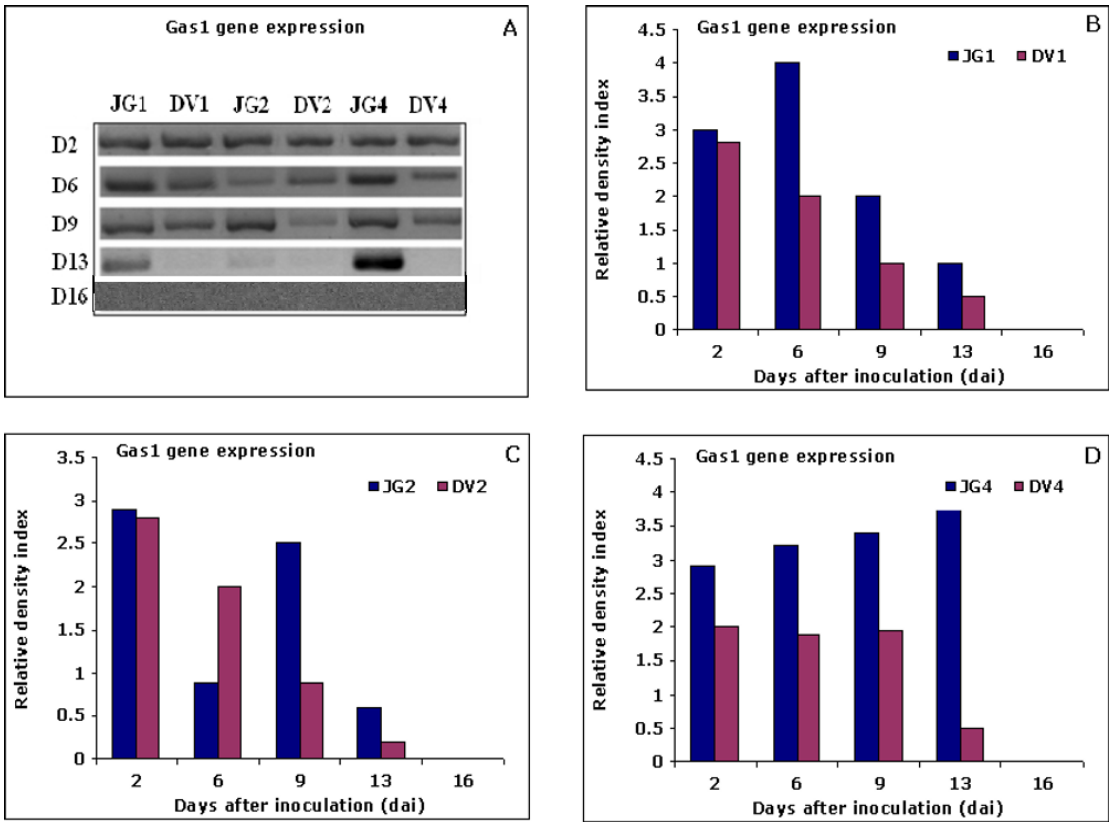


Fig 3.19 *Gas1* gene expression profile in *Foc* races 1, 2 and 4 inocualed JG62 and Digvijay. A: *Gas1* gene expression profile. JG1, DV1, JG2, DV2, JG4 and DV4 represent *Foc* 1, 2 and 4 inoculated JG62 and Digvijay root cDNA samples. D2-D13 represent days after inoculation. B: A comparative graphical representation of *Gas1* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 1. C: A comparative graphical representation of *Gas1* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 2. D: A comparative graphical representation of *Gas1* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 4. (Days after inoculation (dai) are represented on X axis while relative density index values are represented on Y axis which indicate the image density, measured using Syngene tools.)

In graphical representation *Foc* 1 infecting JG62 showed higher expression of *Gas1* gene diminishing gradually from 2 dai to 13 dai, the expression level being maximum at 6 dai (Fig. 3.19B). In case of *Foc* 4 infecting JG62, higher expression of *Gas1* gene was observed from 2 dai to 13 dai, with maximum level of gene expression detected at 13 dai (Fig. 3.19 D). However, in case of *Foc* 2 infecting JG62 a drop in gene expression was observed at 6 dai followed by a sudden rise in expression level at 9 dai and then again a gradual decrease till 13 dai (Fig. 3.19C). In case of *Foc* 1 and 2 infecting Digvijay gradual decrease in *Gas1* gene expression level was observed from 2-13 dai,

while in case of *Foc* 4, expression was steady till 9 dai after which it declined steeply by 13 dai (Fig. 3.19A, B, C).

In comparative analysis, *Gas1* gene expression was most significant in case of race 1 at 6 dai and for race 4 at 13 dai infecting JG62 (Fig 3.20A). However, in case of Digvijay infected with *Foc* races, *Gas1* gene expression was intense till 6 dai after which it decreased noticeably by 13 dai (Fig. 3.20B).

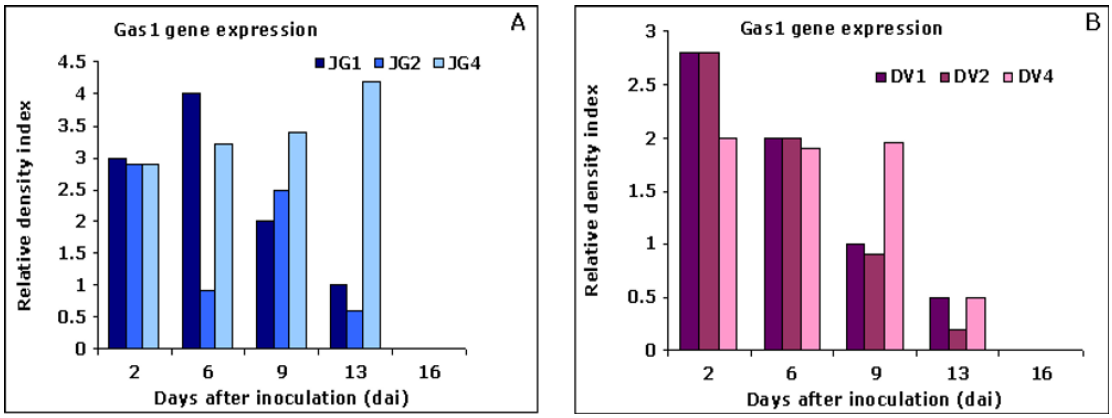


Fig. 3.20 Graphical representation of *Gas1* gene expression profile in chickpea cutivars inoculated with *Foc* races 1, 2 and 4. A: A comparative graphical representation of *Gas1* gene expression profile in JG62 inoculated with *Foc* race 1, 2 and 4. B: A comparative graphical representation of *Gas1* gene expression profile in Digvijay inoculated with *Foc* race 1, 2 and 4. (Days after inoculation (dai) are represented on X axis while relative density index values are represented on Y axis which indicate the image density, measured using Syngene tools.)

Chs7 gene

Chitin synthases (CSs) are integral membrane bound proteins that participate in the biosynthesis of chitin and are important for cell wall synthesis, hyphal growth and differentiation. Fungal CSs occur in specialized vesicles in cytosole called chitosomes (Bartnicki-Garcia and Bracker, 1984) which are responsible for their transport from ER to the cell surface. Chitin synthase VII (Chs7) is a chaperonin like ER protein involved in export of CS from ER to cell surface.

In the present study *Chs7* gene expression was weak around 2 dai which enhanced by 6 dai. At 6 dai the expression was stronger in *Foc* 1 and *Foc* 4 infecting JG62 as compared to Digvijay. *Chs7* gene expression in general was very weak in *Foc* 1 and 4 infecting Digvijay. By 13 dai *Chs7* gene expression was seen only in *Foc* 1, 2 and 4 infecting JG62 and was absent in *Foc* infecting Digvijay. Also, the gene expression was most intense around 9 dai (Fig. 3.21A).

In graphical presentation, *Foc* 1 infecting JG62 showed a higher level of *Chs7* gene expression as compared to Digvijay; the expression being maximum at 9 dai (Fig. 3.21B). Similarly, in case of race 2 infecting JG62, maximum gene expression was observed at 9 dai, but in case of Digvijay initially no gene expression was observed at 2 dai (Fig. 3.21C). While in case of *Foc* 4 infecting Digvijay, maximum gene expression was observed at 13 dai incase of JG62 and a steady but low expression was observed in Digvijay till 13 dai (Fig. 3.21D).

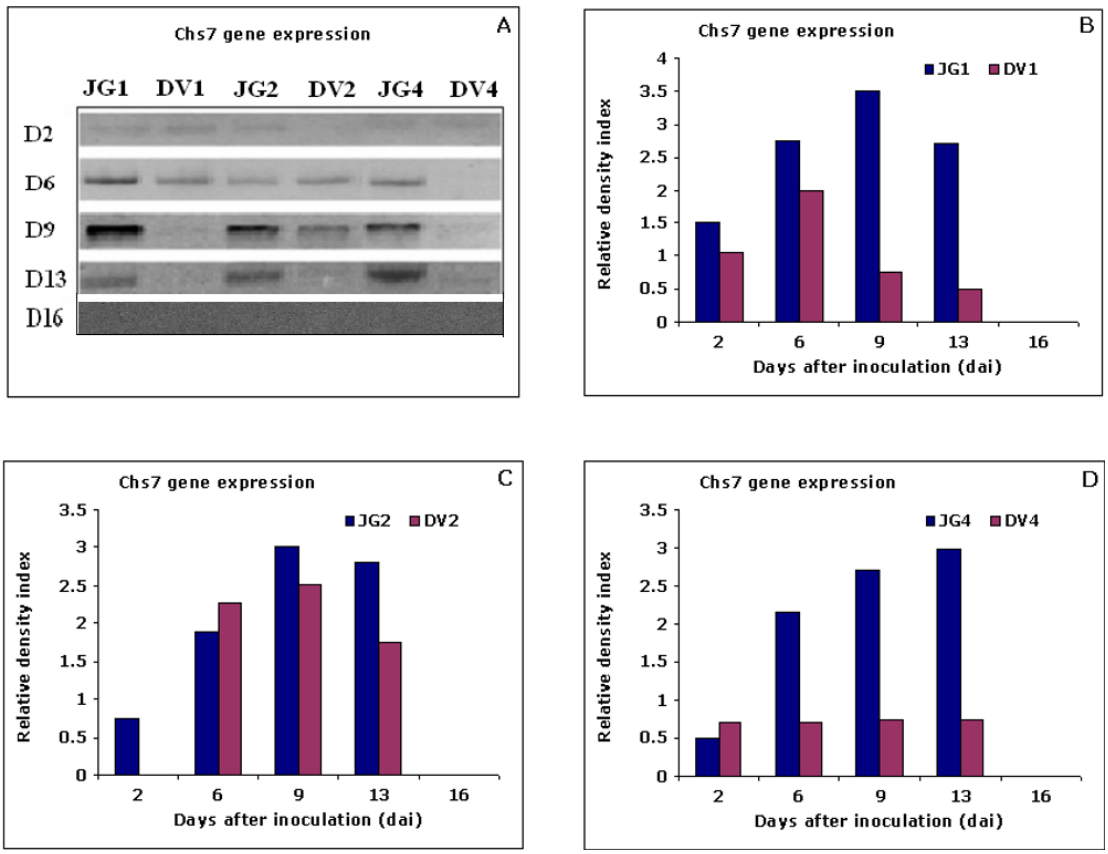


Fig. 3.21 *Chs7* gene expression profile in *Foc* races 1, 2 and 4 inoculated JG62 and Digvijay. A: *Chs7* gene expression profile. JG1, DV1, JG2, DV2, JG4 and DV4 represent *Foc* 1, 2 and 4 inoculated JG62 and Digvijay root cDNA samples. D2-D13 represent days after inoculation. B: A comparative graphical representation of *Chs7* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 1. C: A comparative graphical representation of *Chs7* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 2. D: A comparative graphical representation of *Chs7* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 4. (Days after inoculation (dai) are represented on X axis while relative density index values are represented on Y axis which indicate the image density, measured using Syngene tools.)

In the comparative analysis, *Foc* infected susceptible cultivar JG62 showed high level of *Chs7* gene expression at 9 and 13 dai (Fig. 3.22A), while in case of Digvijay gene

expression was maximum around 6-9 dai especially upon race 1 and 2 inoculation (Fig 3.22B).

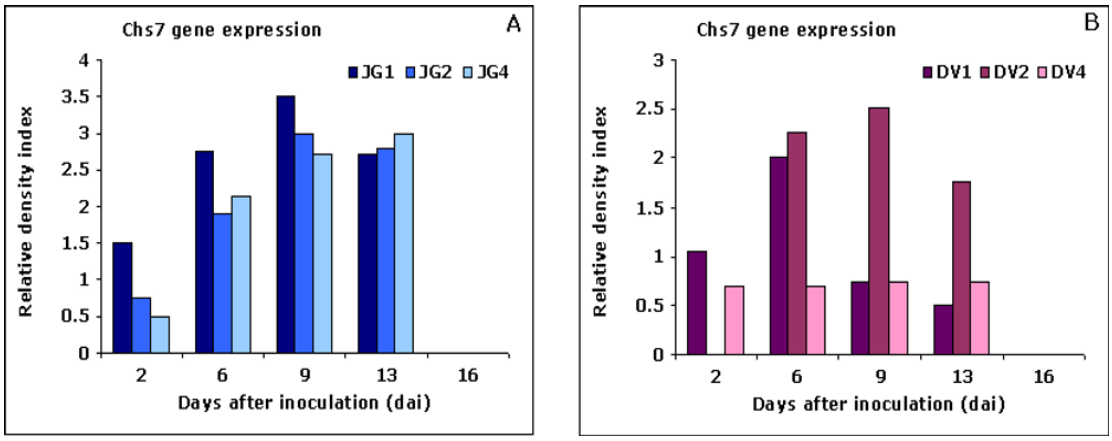


Fig. 3.22 Graphical representation of *Chs7* gene expression profile in chickpea cutivars inoculated with *Foc* races 1, 2 and 4. A: A comparative graphical representation of *Chs7* gene expression profile in JG62 inoculated with *Foc* race 1, 2 and 4. B: A comparative graphical representation of *Chs7* gene expression profile in Digvijay inoculated with *Foc* race 1, 2 and 4. (Days after inoculation (dai) are represented on X axis while relative density index values are represented on Y axis which indicate the image density, measured using Syngene tools.)

Fow1 gene

Mitochondrial carrier protein Fow1, which is responsible for the transfer of tricarboxylates (Mayer *et al.,* 1997), has been reported to be essential for pathogenesis of *F. oxysporum* (Iori *et al.,* 2002). In the present study, *Fow1* gene expression was strong initially in *Foc* 1, 2 and 4 infecting both JG62 and Digvijay. Gradually, with the disease progression; the expression level enhanced in case of *Foc* infecting susceptible cultivar and diminished in resistant cultivar (Fig. 3.23A).

In graphical presentation, *Foc* 1 infecting JG62 showed a maximum level of gene expression at 13 dai, while in Digvijay the response was the highest at 2 dai after which it diminished by 16 dai (Fig. 3.23B). In case of race 2 infecting JG62 similar observation was made, however, for Digvijay inoculated with *Foc* 2, the expression was not uniform (Fig. 3.23C). In case of race 4 infecting JG62, the highest expression was observed at 16 dai, and in Digvijay at 2 dai after which the expression level declined gradually by 16 dai (Fig. 3.23D).

In comparative analysis, *Fow1* gene expression was found to be higher in case of race 4 infecting JG62 especially at 13 and 16 dai (Fig. 3.24A). In case of *Foc* infected Digvijay, though the expression was high initially at 2 dai, it declined by 16 dai and was the lowest in case of *Foc* 4 infecting Digvijay (Fig. 3.24B).

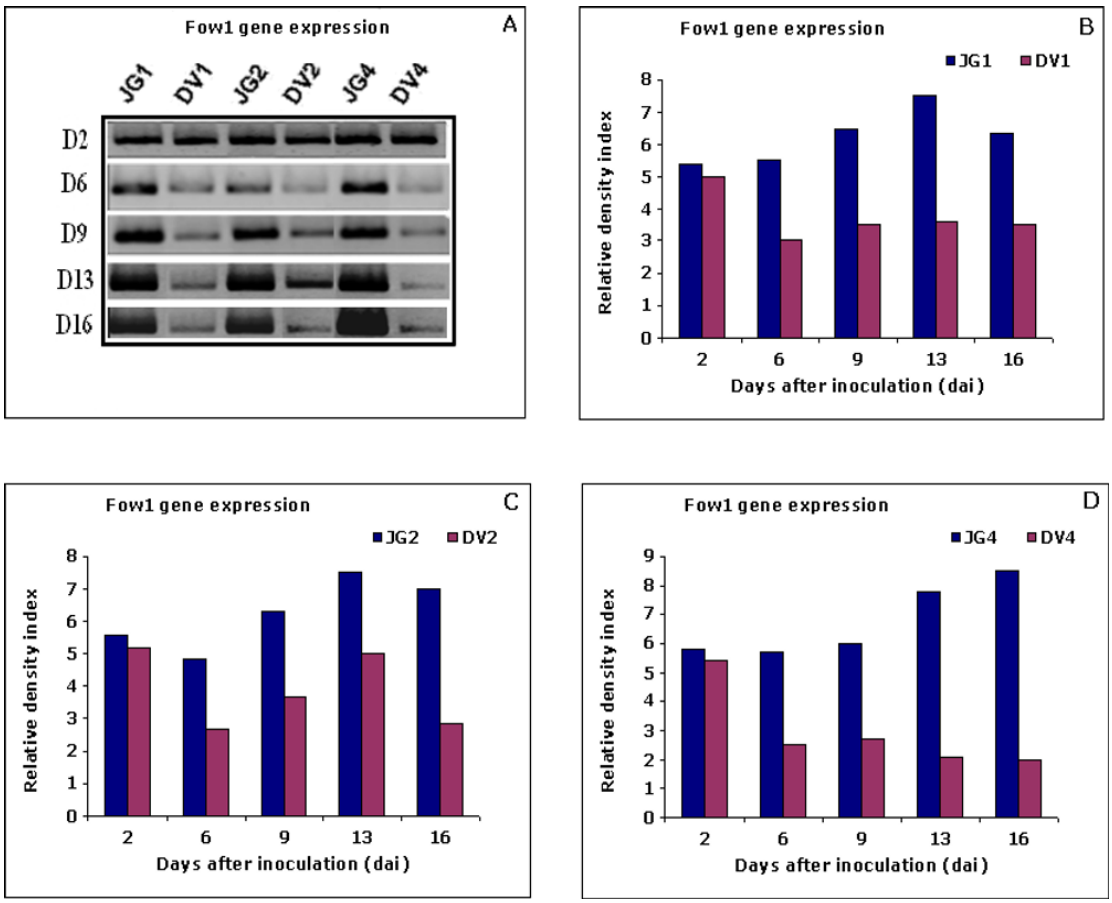


Fig. 3.23 *Fow1* gene expression profile in *Foc* races 1, 2 and 4 inoculated JG62 and Digvijay. A: *Fow1* gene expression profile. JG1, DV1, JG2, DV2, JG4 and DV4 represent *Foc* 1, 2 and 4 inoculated JG62 and Digvijay root cDNA samples. D2-D16 represent days after inoculation. B: A comparative graphical representation of *Fow1* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 1. C: A comparative graphical representation of *Fow1* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 2. D: A comparative graphical representation of *Fow1* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 4. (Days after inoculation (dai) are represented on X axis while relative density index values are represented on Y axis which indicate the image density, measured using Syngene tools.)

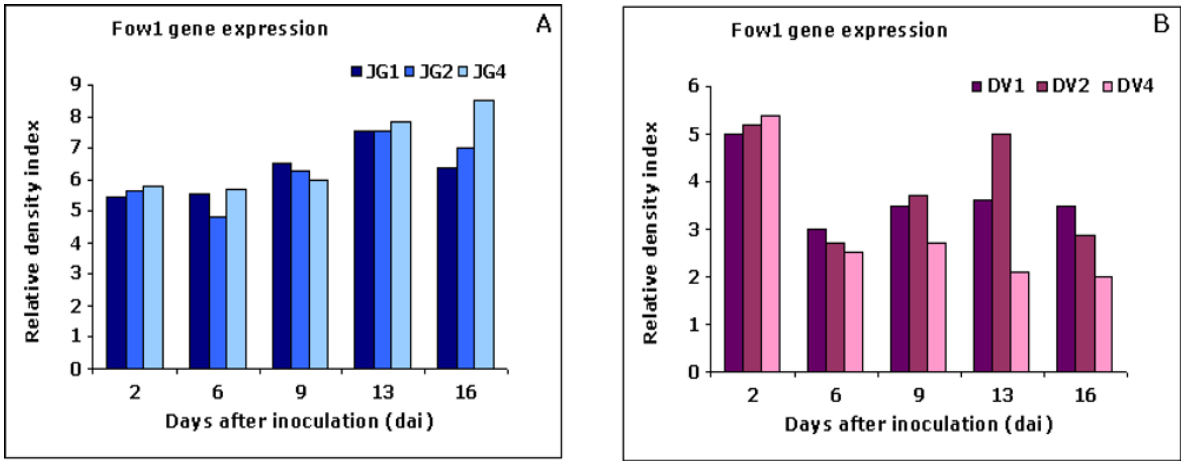


Fig. 3.24 Graphical representation of *Fow1* gene expression profile in chickpea cutivars inoculated with *Foc* races 1, 2 and 4. A: A comparative graphical representation of *Fow1* gene expression profile in JG62 inoculated with *Foc* race 1, 2 and 4. B: A comparative graphical representation of *Fow1* gene expression profile in Digvijay inoculated with *Foc* race 1, 2 and 4. (Days after inoculation (dai) are represented on X axis while relative density index values are represented on Y axis which indicate the image density, measured using Syngene tools.)

3.3.3.2 Highlighting race specific response of *Foc*

At a glance, the response of fungal virulence related genes, namely *Fgb1*, *Gas1*, *Chs7* and *Fow1*, in *Foc* 1, 2 and 4 inoculated JG62 and Digvijay from 2-13 dai has been represented in Fig. 3.25 (16 dai time point has been excluded in this figure since all the genes do not show expression at this time point). In case of race 1 inoculated JG62, all the four pathogenesis related genes showed higher level of expression in comparison to Digvijay. *Gas1* and *Chs7* showed variable response in both, JG62 and Digvijay inoculated with race 2. *Fgb1* and *Fow1* showed higher expression in *Foc* 2 inoculated JG62 as compared to Digvijay, throughout the course of infection. A similar finding was observed in case of race 4 inoculated JG62 and Digvijay, where *Fgb1*, *Gas1* and *Fow1* gene expression was higher in JG62 as compared to Digvijay. However, race specific response in *Chs7* gene expression was observed, expression being higher in *Foc* 4 inoculated Digvijay initially, as compared to that in JG62.

Fungal pathogenesis related genes

	2 dai						6 dai						9 dai						13 dai					
GENE	**JG1**	**DV1**	**JG2**	**DV2**	**JG4**	**DV4**	**JG1**	**DV1**	**JG2**	**DV2**	**JG4**	**DV4**	**JG1**	**DV1**	**JG2**	**DV2**	**JG4**	**DV4**	**JG1**	**DV1**	**JG2**	**DV2**	**JG4**	**DV4**
Fgb1	3.2	2.8	3.1	1.9	3	2	4.5	1.9	3.9	1.9	4.5	2	6.5	5.5	6.5	3	7.8	4.5	7	4.9	6.9	1.9	6.6	1.8
Gas1	3	2.8	2.8	2.7	2.9	2	4	2	0.9	1.9	3.3	1.9	2	1	2.5	0.9	3.4	2	1	0.5	0.65	0.25	3.7	0.5
Chs7	1.5	1	0.75	0	0.5	0.75	2.75	2	1.9	2.3	2.2	0.65	3.5	0.7	3	2.5	2.7	0.7	2.7	0.5	2.9	1.8	3	0.7
Fow1	5.5	5	5.5	5	5.9	5.5	5.5	3	4.9	2.8	5.8	2.5	6.5	3.5	6.3	3.8	6.1	2.8	7.8	3.8	7.5	5.5	8	2

Fig. 3.25 Response of fungal virulence related genes, namely *Fgb1*, *Gas1*, *Chs7* and *Fow1*, in *Foc* 1, 2 and 4 inoculated JG62 and Digvijay from 2-13 dai

3.3.3.3 Sequence characterization of fungal virulence related genes

Partial sequences of *Fgb1*, *Gas1*, *Chs7* and *Fow1* genes were determined and compared to the reported sequences in the NCBI database. Fig 3.26 represents the partial sequences of *Fgb1*, *Gas1*, *Chs7* and *Fow1* genes. Partial sequence of *Foc Fow1* when analyzed revealed 99% homology to reported *Fow1* sequence of *F. oxysporum* f. sp. *latuceae* and showed significant homologies to YMR241w mitochondrial carrier protein (MCP) of *S. pombe*; which has been suggested to play an important role in transport of citrate or tricarboxylates in the mitochondria (Mayer *et al.,* 1997).

Fow1 just like MCPs in general consists of conserved PX(D/E)XX(K/R) domain (Palmieri, 1994; Nelson *et al.,* 1998). The partial sequence of *Fow1* used in this study, indicated the presence of PLEVVK conserved motif and TVIETK which is similar to the conserved motif in MCPs (Fig. 3.27A). Partial sequence of *Gas1* (Fig. 3.26B) when analyzed revealed 100% homology to *F. oxysporum Gas1* sequence (Caracuel *et al.,* 2005) deposited in NCBI database. Partial sequence analysis of *Foc Fgb1* sequence revealed 97% homology to *F. oxysporum* f. sp. *lycopersici* (Delgado-Jarana *et al.,* 2005) and *G. moniliformis*; 95% similarity to *Chaetomium globosum*; 87% identity to *Neosartorya fischeri*, *Aspergillus clavatus* and *Aspergillus flavus* and 77% homology to *Rhizoctonia solani*, G protein β subunit sequences deposited in the Genbank. The deduced amino acid sequence of partial *Foc Fgb1* showed the presence of five WD repeats containing a GH dipeptide around 26-28 residues away from the WD repeat. Flexibility in the most conserved position of WD repeat regions is also seen, for example, in repeat 2, YN appears instead of WD. A conserved aspartic acid was observed preceding the WD dipeptide (Fig. 3.27B). Partial gene characterization of *Foc chs7* revealed 99% homology to *F. oxysporum chs7* gene reported in NCBI database. However, no conserved domains were observed in either the *Foc chs7* deduced amino acid sequence or in other homologous sequences deposited in the Genbank (Martin-Udiroz et al., 2004).

A

>*Foc-Fgb1* (Genbank acc no.GW342900)

GTCCCAACCTAATGGTTGCGTTAGATCACGAGTTCCAGAGAAGACAAGCTTGTAATCACAAGCACA
AATATGCCTTACCTTGCACCTCAAAGTCATCGTAACCAGCGAAAAGAAGACGACCTGATACAGATG
TAGCTACCGAGGTGATACCACAGAGAATAGACTCGGACTGCCAATTGCGTTAGCCTGGGTCAAAGA
ACCAATTTGCAAAAGTCCACACTTACTCCGTAGAGGTTAAGTTCGCGATCCGCGCGGATATCGAAG
AGACGGCAAGTAGCATCATCCGATCCGGTCACGAAAGAGTGGCCGTCAGGGAAGAATTGGATGGCG

TTAATATCGGACTCATGGCCAGCAAAGGTCTGGACAGCCTTTCCAGCGCGAATATCCCAAAGCTTG
GCGAAAGCATCACAAGCACCAGAGATGAAGGTGTTTTGATTGGTAGGGTTGAGG

B

>*Foc*-Gas1 (Genbank acc no.GW342901)

CTGTGTCCCGCTGATAACCTGAAGGCTAAGGACTTTAACGACATCTTTGGCTACATCTGCGGCCAG
GACAAGAAGATCTGCACTGCTATCAACGCCAACGCCACTGCCGGTATCTATGGTGCCTACAGCATG
TGCTCCAACGAGGCTAAGCTTGCTTACATCCTCGACGCCTACTACACCTCCCAGAAGTCCGCTGCC
GATGCTTGTGACTTCAAGGGCAAGGCCACCACCCAGAAGGCCGAGAGCCAGGACTCTTGCAAGTCT
GCTCTCGCCTCCGCCAGCAAGATCAACGAGGAGGTTGCCACTGCCACCCACGCCGTCGCTTCTTCC
TCCACCGGTGGTTCCAACAGCAGCAGCGAGGACGACGAGAACTTTGGTCTCCAGGCTGCCTCCATC
GCCCGCGTCTTCTCCCTCGGTGACTTCGCCGTTGGCGCCTACATGGCCGTTGCTGGTGTTGTCGGT
GCCGGTTGGTC

C

>*Foc*-Chs7 (Genbank acc no.GW342902)

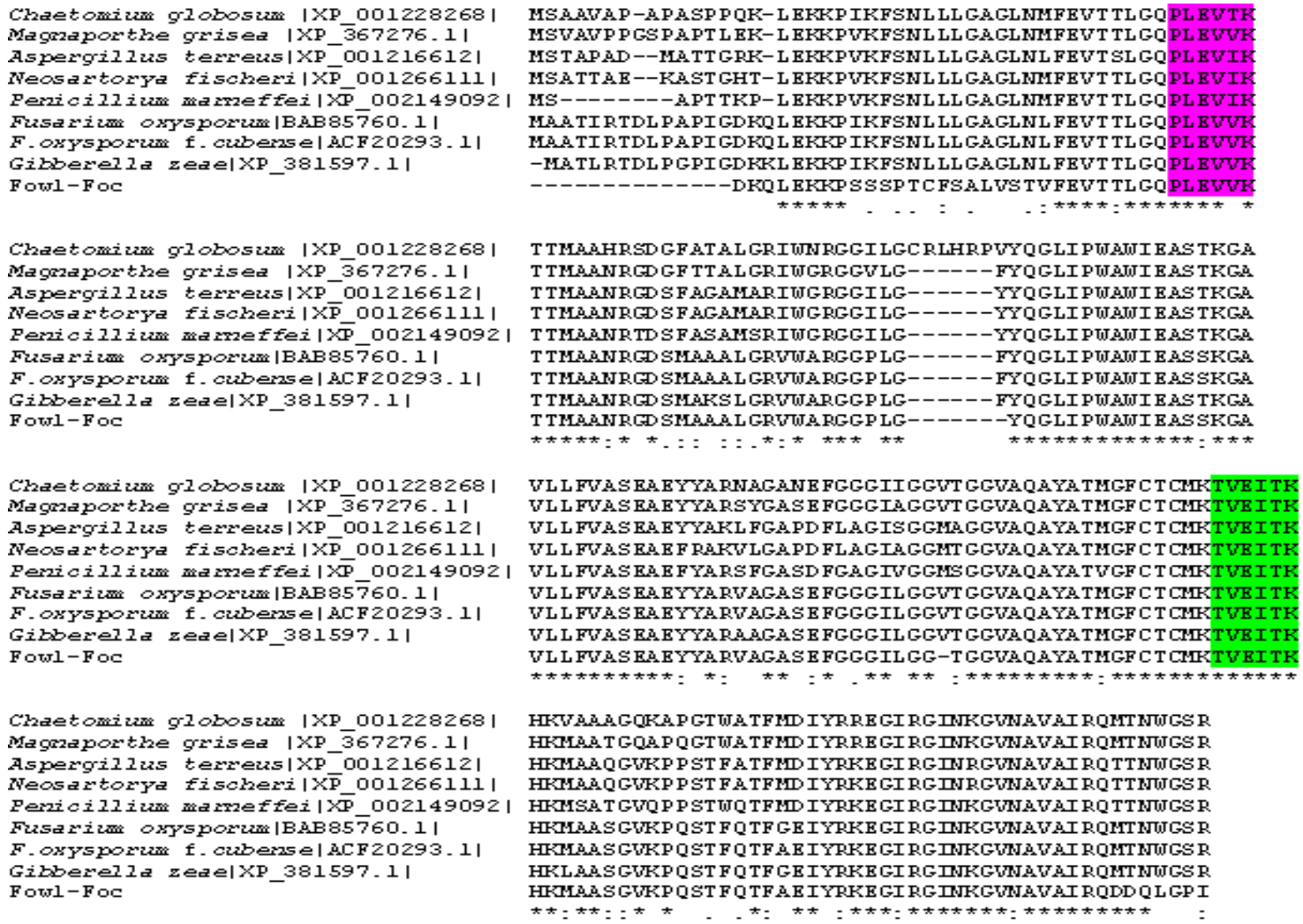
TTACAAGGTATGTCAAATATGATTCCGAACCACTGCGGTTAGTTATTAATATATTAACAGTACTG
GGATTCGATCACCAAGGAACATCTTGAGTTTTCAGTTGGCACAAGGATGAACAACTGGGAGGTCA
AGGAGCTGCTGCCAGAGGAAGACCGAAGCGCGACTGTCTTTTCGGACGATCCCTATGCTCAATCC
AGCTCC

D

>*Foc*-Fow1 (Genbank acc no.GW342903)

GCAGCTTTAGAAGAAGCCATCAGTTCTCCAACTTGCTTCTCGGCGCTGGTCTCAACCTCTTCGAAG
TGACCACCCTCGGACAGCCTCTCGATGGTCGTCAAGACCACCATGGCTGCCAACCTGAGGTGACAG
CATGGCTGCGGCTTTGGGACGCGTCTGGGCCCGCGGTGGTCCCCTGTGGCTTCTACCAATGGTCTC
ATCCCCTGGGCTTGGATTGAAGCTTCCAGCAAGGGCGCCGTCCTTCTCTTCGTCTGCCTCCGAGGC
CGAGTACTACGCCCGTGTCGCTGGTGCCTCTGAGTTTGGCGGCGGTATCCTTGGTGGTGTCACCGG
TGGTGTTGCCCAGGCTTATGCCACCATGGGTTTCTGCACCTGCATGAAGACGGTCGAGATCACCAA
GCACAAGATGGCTGCCTCCGGTGTCAAGCCCCAGTCTACCTTCCAGACCTTTGCTGAAAATCTACC
GCAAGGAGGGTAATCCGTGGGCATCAACAAGGTGTGTCAACGCTGTCGCTATCCGACAGATTGACC
AACTGGGGGTAGCCGATTCGGCTCAGCCGTTCTCGACTGAAGGGGTTGGATCAGTCCTCTCACTTG
GCAAGAAGGAGGGGAAAAAGCTTGTACCGTGGTGAAAGGTCA

Fig. 3.26 Partial sequences of fungal pathogenesis related genes. A: *Fgb1*, B: *Gas1*, C: *Chs7* and D: *Fow1* genes


Chaetomium globosum |XP_001228268|
Magnaporthe grisea |XP_367276.1|
Aspergillus terreus|XP_001216612|
Neosartorya fischeri|XP_001266111|
Penicillium marneffei|XP_002149092|
Fusarium oxysporum|BAB85760.1|
F.oxysporum f.cubense|ACF20293.1|
Gibberella zeae|XP_381597.1|
Fowl-Foc

A

KIYAMHWSTDRRHLVSASQDGKLIIWDAYTTNKVHAIPLRSSWVMTCAYAPSGNFVACGGLDNICSIYNLNQQRDGPTRVARELSGH
AGYLSCCRFINDRSILTSSGDMTCMKWDIETGQKVTEFADHLGDVMSISLNPTNQNTFISGACDAFAKLWDIRAGKAVQTFAGHESD
INAIQFFPDGHSFVTGSDDATCRLFDIRADRELNLYGVSVDFCSESILCGITSVATSVSGRLLFAGYDDFE

B

Fig. 3.27 A: Fow1 deduced amino acid sequence alignment depicting conserved motifs. B: Fgb1 partial amino acid sequence showing GH (blue colour) and WD (yellow colour) domains (conserved domains)

3.3.3.4 Mapping of *Foc* virulence related genes on *Gibberella zeae* genome

The partial sequences of *Foc* virulence related genes, namely *Fgb1*, *Gas1*, *Chs7* and *Fow1* were mapped on *Gibberella zeae* genome (www.ncbi.nlm.nih.gov) (Fig. 3.28). *Gibberella zeae* is the sexual stage of *Fusarium graminearum* and is a pathogen of wheat. *Fow1*, *Chs7* and *Gas1* genes were located on chromosome 1, while *Fgb1* was mapped on chromosome 2 of *Gibberella zeae* genome. Figure 3.29 A, B, C and D depict the exact chromosomal locations of *Fgb1*, *Gas1*, *Chs7* and *Fow1* genes, respectively.

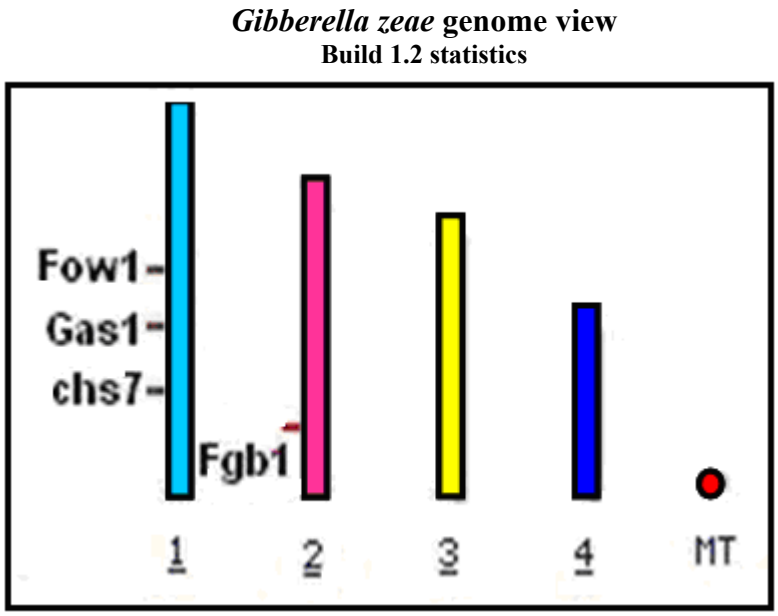
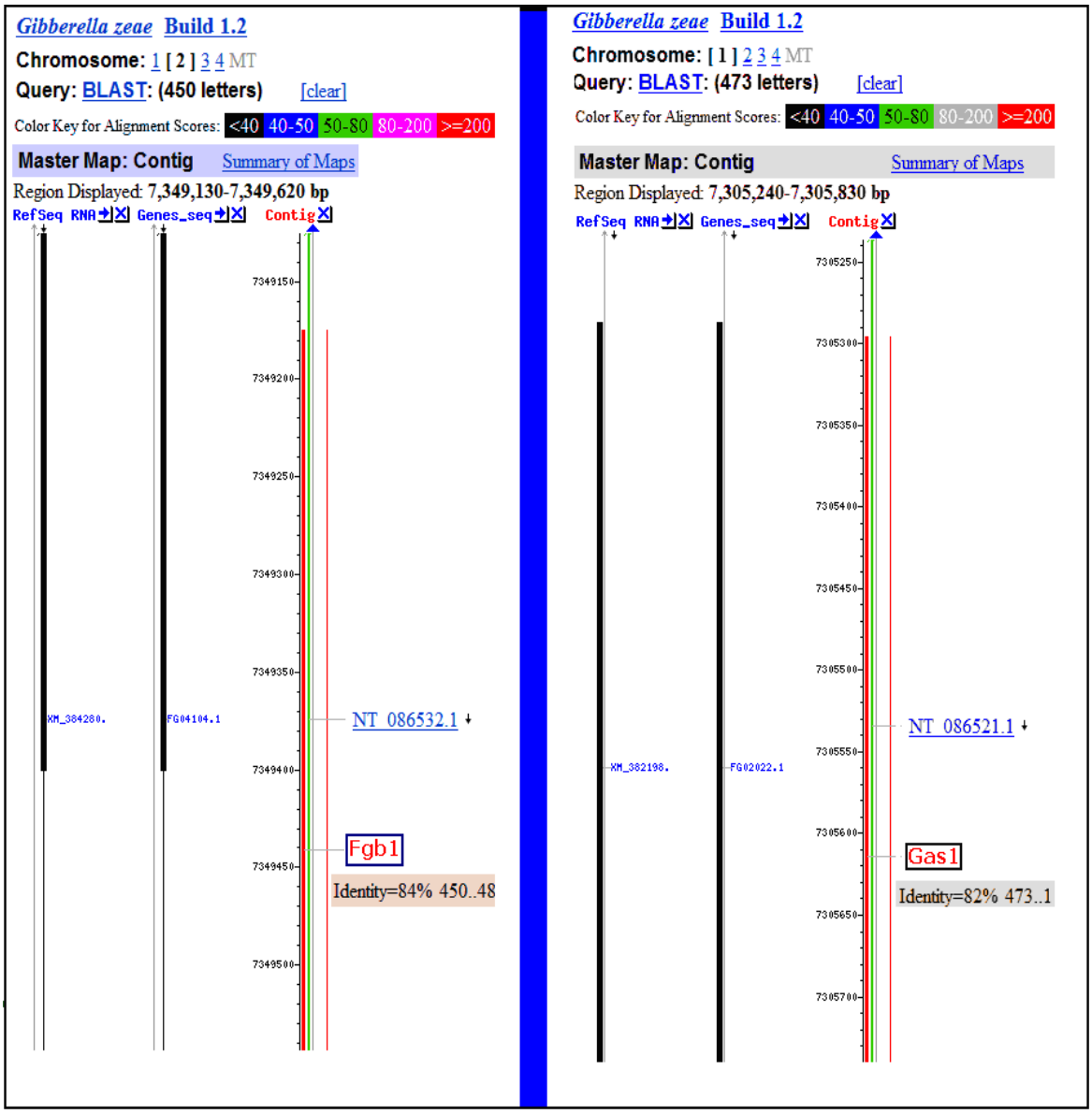


Fig. 3.28 Mapping of *Foc* virulence related genes on *G. zeae* genome


Gibberella zeae Build 1.2
Chromosome: 1 [2] 3 4 MT
Query: BLAST: (450 letters) [clear]
Color Key for Alignment Scores: <40 40-50 50-80 80-200 >=200
Master Map: Contig Summary of Maps
Region Displayed: 7,349,130-7,349,620 bp
RefSeq RNA Genes_seq Contig
7349150
7349200
7349250
7349300
7349350
7349400
7349450
7349500
XM_384280.
FG04104.1
NT_086532.1
Fgb1
Identity=84% 450..48
Gibberella zeae Build 1.2
Chromosome: [1] 2 3 4 MT
Query: BLAST: (473 letters) [clear]
Color Key for Alignment Scores: <40 40-50 50-80 80-200 >=200
Master Map: Contig Summary of Maps
Region Displayed: 7,305,240-7,305,830 bp
RefSeq RNA Genes_seq Contig
7305250
7305300
7305350
7305400
7305450
7305500
7305550
7305600
7305650
7305700
XM_382198.
FG02022.1
NT_086521.1
Gas1
Identity=82% 473..1

A **B**

Cont…

C **D**

Fig. 3.29 Exact chromosomal locations *Foc* virulence genes on *G. zeae* genome. A: *Fgb1*, B: *Gas1*, C: *Chs7* and D: *Fow1*

3.3.4 Candidate gene approach to study chickpea defense against Fusarium wilt

Variation in the expression pattern of specific genes observed in the above studies led us to perform detailed analysis of expression of candidate plant defense genes during various stages of disease development in susceptible and resistant chickpea cultivars, JG62 and Digvijay, respectively, inoculated with all the three races of *Foc*, namely *Foc* 1, 2 and 4 in order to understand the contribution of these genes in plant defense. As mentioned previously, chickpea root cDNA samples, control as well as *Foc* race 1, 2 and 4 inoculated, were normalized using 18s rRNA primers. Fig 3.30 shows the uniform amplification profile of inoculated and control chickpea root cDNA samples using these primers. The normalized cDNA samples were then used for candidate gene study using plant defense related gene primers.

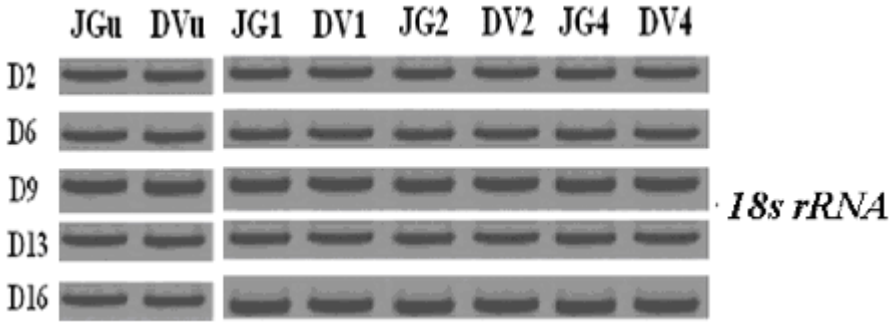


Fig. 3.30 cDNA normalization using 18s rRNA primers. JGu and DVu represent control JG62 and Digvijay root samples. JG1, DV1, JG2, DV2, JG4 and DV4 represent *Foc* 1, 2 and 4 inoculated JG62 and Digvijay root cDNA samples. D2-D16 represent days after inoculation.

3.3.4.1 *GroES2, 60srp* and *BetvI* expression in chickpea upon inoculation with *Foc* races 1, 2 and 4

GroES2 gene

As mentioned previously, chaperonins like GroES2 are essential for plant function during stress conditions including pathogen attack. In this study, control JG62 exhibited higher expression of *GroES2* at 2 dai than Digvijay which reversed at 6 dai and 9 dai. However, when inoculated with *Foc* races chickpea *GroES2* chaperonin gene was found to express only till 6 dai after which the transcripts' level declined drastically (Fig 3.31A). However, at 2 dai and 6 dai the *GroES2* expression level was found to be higher in *Foc* 1 inoculated Digvijay, while *Foc* 4 inoculated Digvijay showed no expression initially at 2 dai.

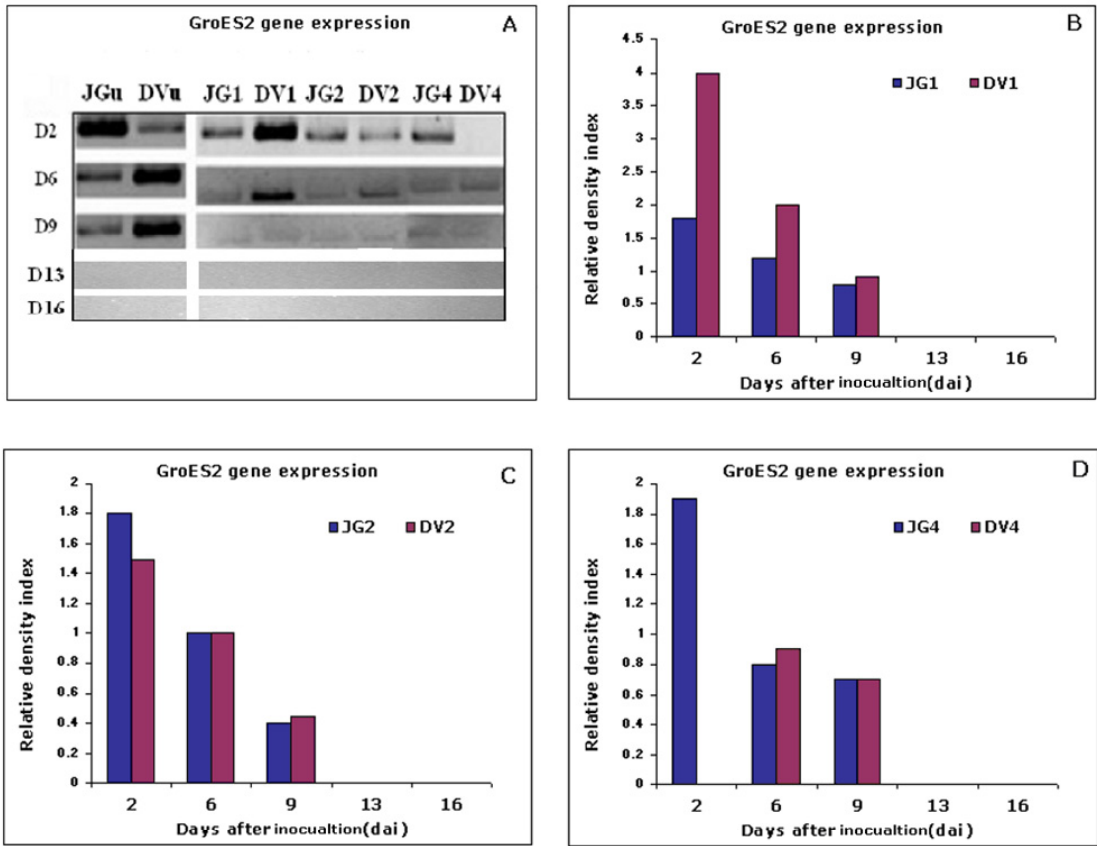


Fig. 3.31 *GroES2* gene expression profile in chickpea inoculated with *Foc* races 1, 2 and 4. A: *GroES2* gene expression profile. JGu and DVu represent control JG62 and Digvijay root samples. JG1, DV1, JG2, DV2, JG4 and DV4 represent *Foc* 1, 2 and 4 inoculated JG62 and Digvijay root cDNA samples. D2-D9 represent days after inoculation. B: A comparative graphical representation of *GroES2* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 1. C: A comparative graphical representation of *GroES2* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 2. D: A comparative graphical representation of *GroES2* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 4. (Days after inoculation (dai) are represented on X axis while relative density index values are represented on Y axis which indicate the image density, measured using Syngene tools.)

In graphical representation, in *Foc* 1 inoculated JG62, *GroES2* gene expression was low as compared to resistant cultivar Digvijay till 9 dai, beyond which no expression was observed (Fig. 3.31B). However, in case of *Foc* 2 and 4 inoculated JG62 high levels of *GroES2* gene expression were observed in comparison to Digvijay at early stage of inoculation, i.e, around 2 dai (Fig. 3.31C, D).

In comparative analysis, as compared to control susceptible cultivar, *Foc* inoculated JG62 showed weak *GroES2* expression profile. In control JG62, the expression level was the highest at 2 dai which decreased by 9 dai (Fig. 3.32A). In case of control Digvijay, *GroES2* expression was low initially at 2 dai but increased by 9 dai in

comparison to the inoculated Digvijay (Fig. 3.32B). The highest expression level of *GroES2* gene was observed in case of *Foc* inoculated JG62 initially, but at 6 and 9 dai, it decreased gradually (Fig. 3.32A). In case of *Foc* 1 inoculated Digvijay, more *GroES2* gene expression was observed from 2 to 9 dai as compared to *Foc* 2 and 4 inoculated Digvijay (Fig. 3.32B).

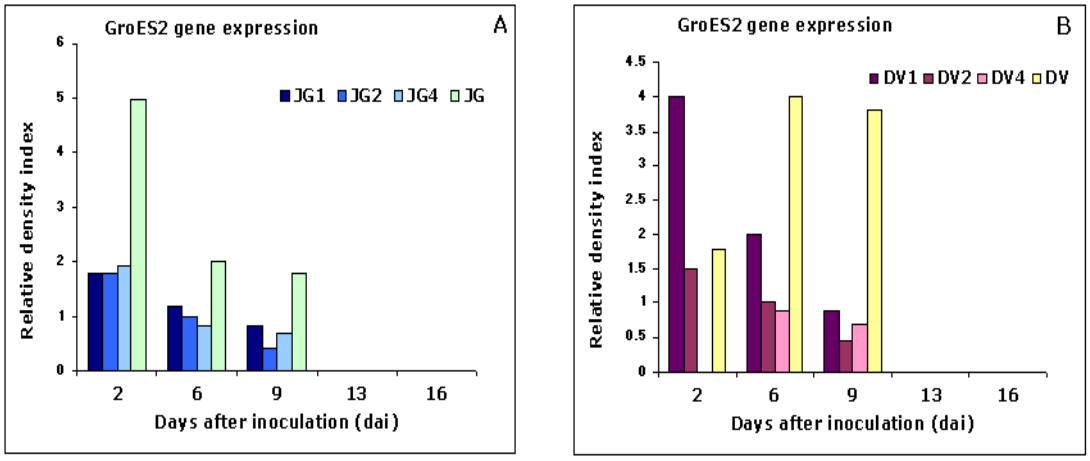


Fig. 3.32 Graphical representation of *GroES2* gene expression profile in chickpea cultivars inoculated with *Foc* races 1, 2 and 4. A: A comparative graphical representation of *GroES2* gene expression profile in JG62 inoculated with *Foc* race 1, 2 and 4 vis-à-vis uninoculated JG62. B: A comparative graphical representation of *GroES2* gene expression profile in Digvijay inoculated with *Foc* race 1, 2 and 4 vis-à-vis uninoculated Digvijay. (Days after inoculation (dai) are represented on X axis while relative density index values are represented on Y axis which indicate the image density, measured using Syngene tools.)

Gene for 60s ribosomal protein (60srp)

Constitutive expression of chickpea *60srp* gene was observed in roots of control chickpea plants (Fig 3.33A). In *Foc* 1, 2 and 4 inoculated chickpea expression of *60srp* gene was the highest during early stage of infection i.e. around 2 dai followed by a gradual decline as the disease progressed. By 13 dai, *60srp* gene expression was seen only in the resistant cultivar inoculated with *Foc* race 4 (Fig 3.33A). In earlier reports also, a constant upregulation of *60srp* gene transcripts was observed within mycorrhizal root tissues of tomato plants (at 10 dai) and during arbuscular mycorrhizal symbiosis of *Medicago truncatula* (Taylor and Harrier, 2003; Weidmann *et al.*, 2004).

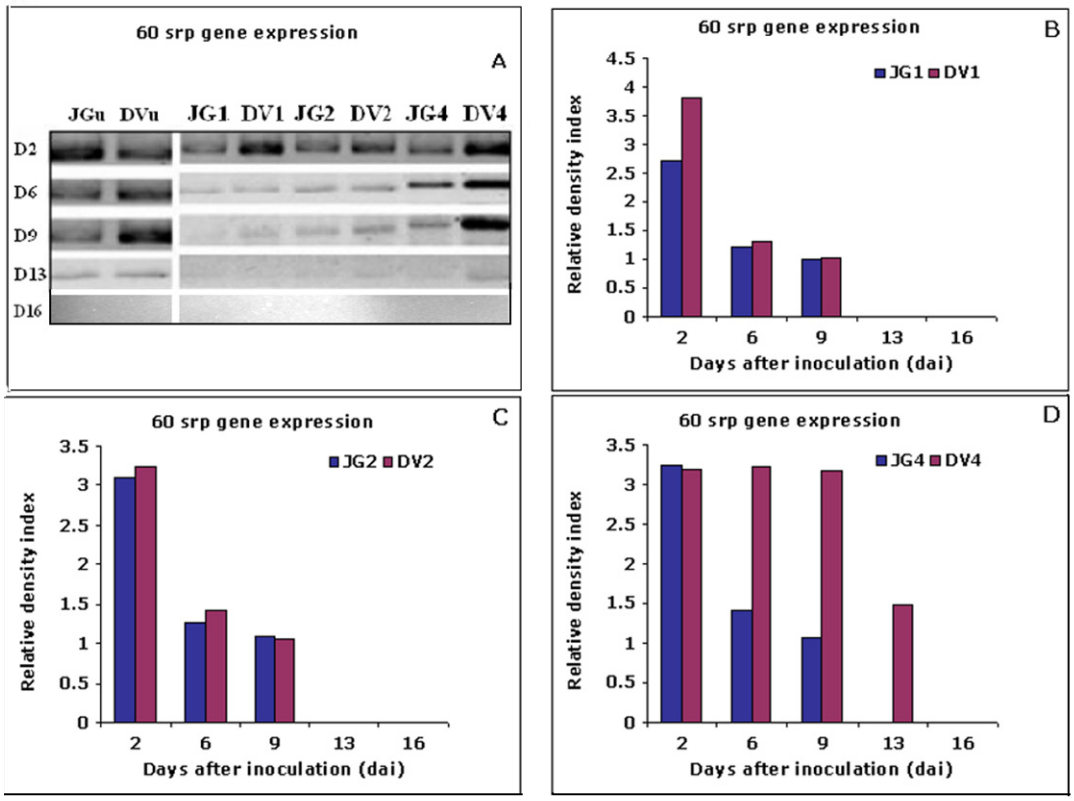


Fig. 3.33 *60srp* gene expression profile in chickpea inoculated with *Foc* races 1, 2 and 4. A: *60srp* gene expression profile. JGu and DVu represent control JG62 and Digvijay root samples. JG1, DV1, JG2, DV2, JG4 and DV4 represent *Foc* 1, 2 and 4 inoculated JG62 and Digvijay root cDNA samples. D2-D13 represent days after inoculation. B: A comparative graphical representation of *60srp* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 1. C: A comparative graphical representation of *60srp* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 2. D: A comparative graphical representation of *60srp* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 4. (Days after inoculation (dai) are represented on X axis while relative density index values are represented on Y axis which indicate the image density, measured using Syngene tools.)

In graphical representation, both race 1 inoculated Digvijay and JG62 showed the highest expression of *60srp* gene at 2 dai which decreased gradually by 9 dai, though the response in case of Digvijay was more as compared to that of JG62 at all the time points of the study (Fig. 3.33B). Fig. 3.33C represents the graphical view of the *60srp* gene expression in both the genotypes, JG62 and Digvijay inoculated with race 2. The profiles for race 1 and 2 were almost the same; however expression of *60srp* gene was higher in JG62 inoculated with race 2 as compared to race 1, while that was more in Digvijay inoculated with race 1 as compared to race 2 (Fig. 3.33B, C).

The *60srp* gene expression pattern changed in response to *Foc* 4 (Fig. 3.33D). The expression was high in Digvijay from 2-9 dai which lowered by 13 dai and was not

detectable beyond; but in case of JG62 the expression profile was similar to Digvijay at 2 dai; and drastically reduced by 13 dai.

In comparative analysis, *Foc* challenged Digvijay showed an elevated expression of *60srp* gene as compared to that in JG62. However, *Foc* 4 challenged JG62 showed higher *60srp* gene response as compared to *Foc* 2 followed by *Foc* 1 (Fig. 3.34A, and 3.34B).

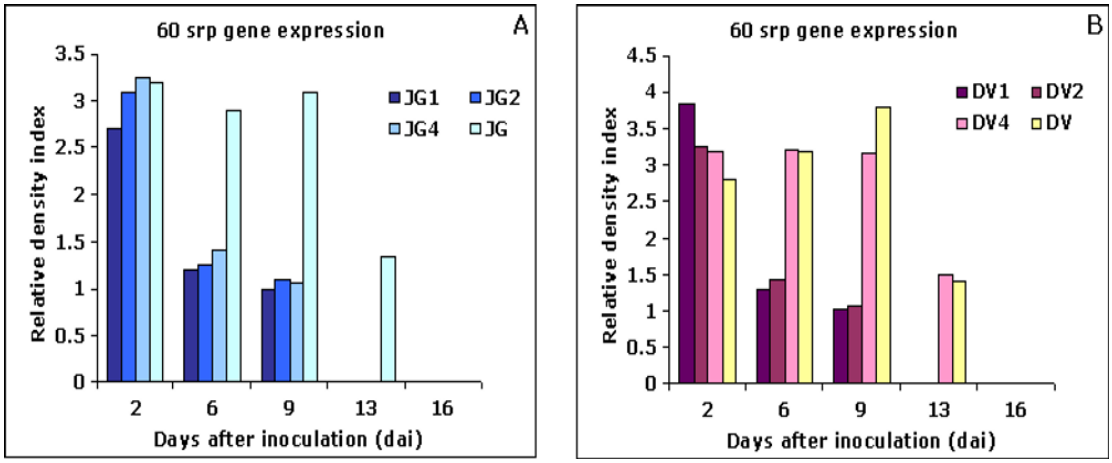


Fig. 3.34 Graphical representation of *60srp* gene expression profile in chickpea cultivars inoculated with *Foc* races 1, 2 and 4. A: A comparative graphical representation of *60srp* gene expression profile in JG62 inoculated with *Foc* race 1, 2 and 4 vis-à-vis uninoculated JG62. B: A comparative graphical representation of *60srp* gene expression profile in Digvijay inoculated with *Foc* race 1, 2 and 4 vis-à-vis uninoculated Digvijay. (Days after inoculation (dai) are represented on X axis while relative density index values are represented on Y axis which indicate the image density, measured using Syngene tools.)

As compared to control susceptible cultivar, *Foc* inoculated JG62 showed weak *60srp* expression profile. In control JG62, the expression level was the highest at 2 dai and gradually decreased by 13 dai (Fig. 3.34A). In case of control Digvijay, *60srp* expression was lower as compared to *Foc* 4 inoculated Digvijay especially at 2 dai and 13 dai. In comparison to *Foc* 1 and 2 inoculated Digvijay, the expression in control plants was low at 2 dai but eventually increased especially at 6-13 dai (Fig. 3.34B).

BetvI gene

Pathogenesis related proteins (PR proteins) are reported to be extensively involved in chickpea defense against various pathogens (Saikia *et al.*, 2005). In our study, *PR2*, *PR5* and *PR10* as well as *BetvI* which is known to show significant homologies to *PR10* proteins and is a major pollen allergen of several plant species (Borch *et al.,* 1987;

Luttkoph *et al.,* 2002; Bohle *et al.,* 2003; Neudecker *et al.,* 2003) were screened for their reproducible, race specific and prolonged expression (2-16 dai) in both the chickpea cultivars. Except *BetvI*, all other PR proteins could not meet the above mentioned selection criteria. Chickpea *Betv1* gene showed constitutive expression in the control chickpea cultivars, JG62 and Digvijay, expression level being higher in case of Digvijay as compared to JG62 (Fig. 3.35A).

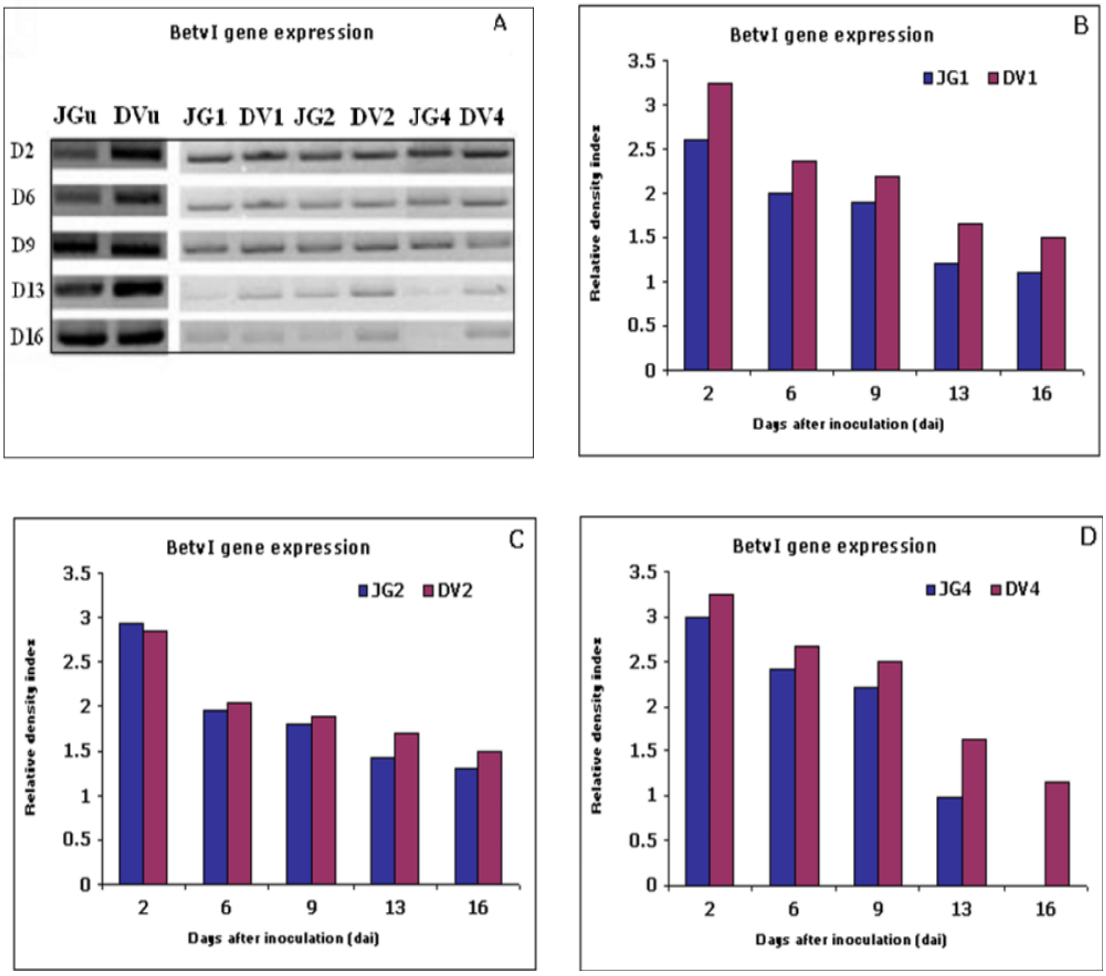


Fig. 3.35 *BetvI* gene expression profile in chickpea inoculated with *Foc* races 1, 2 and 4. A: *BetvI* gene expression profile. JGu and DVu represent control JG62 and Digvijay root samples. JG1, DV1, JG2, DV2, JG4 and DV4 represent *Foc* 1, 2 and 4 inoculated JG62 and Digvijay root cDNA samples. D2-D16 represent days after inoculation. B: A comparative graphical representation of *BetvI* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 1. C: A comparative graphical representation of *BetvI* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 2. D: A comparative graphical representation of *BetvI* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 4. (Days after inoculation (dai) are represented on X axis while relative density index values are represented on Y axis which indicate the image density, measured using Syngene tools.)

In inoculated plants, *BetvI* expression was pronounced around early and mid stages of infection i.e. 2 to 9 dai in both the resistant and susceptible cultivars, and the expression level dropped in both the cases by 16 dai, although the drop was more prominent in JG62 as compared to Digvijay, the resistant cultivar. This is in accordance with previous studies conducted by Foster-Hartnett *et al.*, (2007) where, during infection by *Erysiphe pisi*, both resistant and susceptible cultivars of *Medicago truncatula* showed that the transcript accumulation was higher in the resistant cultivar as compared to the susceptible one.

In graphical presentation, in general, *BetvI* gene expression was higher in case of *Foc* 1 inoculated Digvijay as compared to JG62 at all the time points of the study (Fig. 3.35B). Similar observation was made in case of *Foc* 2 and 4 inoculated Digvijay as compared to JG62 (Fig. 3.35C, D).

In comparative analysis, the *BetvI* gene expression in *Foc* inoculated JG62 plants was lower at all time points of the study when compared to control JG62 plants, maximum being at 9 dai (Fig. 3.36A). *Foc* 4 inoculated JG62 showed a higher level of gene expression till 9 dai, i.e in the earlier stages of infection, while in the later stages (13-16 dai) *Foc* 2 inoculated JG62 showed a higher expression of *BetvI* (Fig. 3.36A) as compared to *Foc* 1 and 4 inoculated JG62. Interestingly, no expression of *BetvI* was observed in *Foc* 4 inoculated JG62 at 16 dai.

In the *Foc* 4 inoculated resistant cultivar, Digvijay a higher level of gene expression was observed till 9 dai, i.e in the earlier stages of infection, which reduced by 16 dai when compared to *Foc* 1 and 2 inoculated Digvijay (Fig. 3.36B). Interestingly, *BetvI* showed the highest level of expression in early stages of infection (2-9 dai) and the lowest level at later stages (13-16 dai) in both the cultivars inoculated with all the three races of *Foc*. Also, the *BetvI* gene expression in *Foc* inoculated Digvijay plants was lower at all time points of the study when compared to control Digvijay plants, maximum being at 13 dai followed by 16 dai (Fig. 3.36B).

3.3.4.2 Expression of major genes of phenylpropanoid pathway in chickpea upon inoculation with Foc races 1, 2 and 4

Phenolic compounds like phytoanticipins and phytoalexins are important plant secondary metabolites; which have been suggested to play diverse roles in plant defense against phytopathogens (Daayf *et al.*, 1997; Ramos-Valdivia *et al.*, 1997). Medicarpin and Maakianin are the major phytoalexins involved in chickpea defense and are produced through phenylpropanoid pathway (Kessmann and Barz, 1987; Kessmann *et*

al., 1988). Chalcone synthase (*CHS*) and Isoflavone reductase (*IFR*) are the two key enzymes that are involved in isoflavonoid production against biotic stresses.

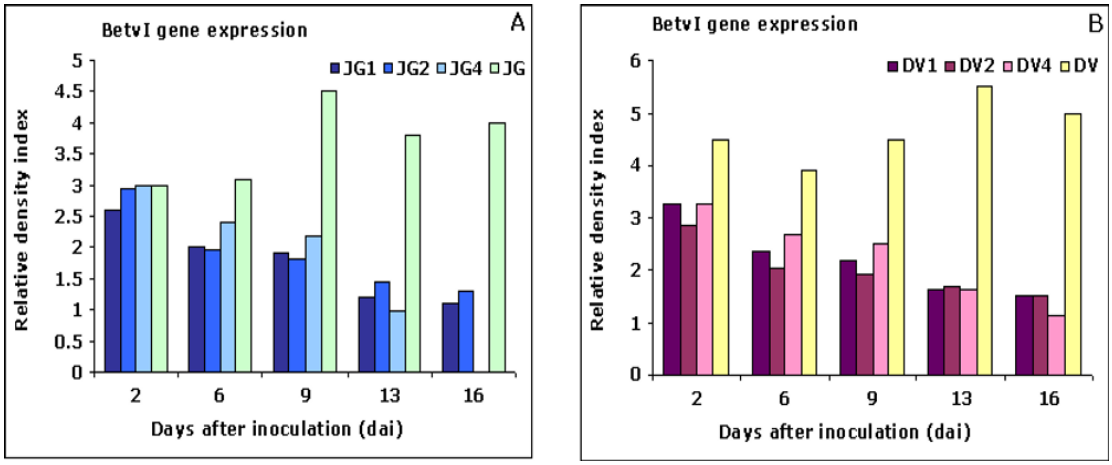


Fig. 3.36 Graphical representation of *BetvI* gene expression profile in chickpea cultivars inoculated with *Foc* races 1, 2 and 4. A: A comparative graphical representation of *BetvI* gene expression profile in JG62 inoculated with *Foc* race 1, 2 and 4 vis-à-vis uninoculated JG62. B: A comparative graphical representation of *BetvI* gene expression profile in Digvijay inoculated with *Foc* race 1, 2 and 4 vis-à-vis-uninoculated Digvijay. (Days after inoculation (dai) are represented on X axis while relative density index values are represented on Y axis which indicate the image density, measured using Syngene tools.)

Chalcone synthase (CHS) gene

In this study the expression of both *CHS* and *IFR* was analyzed against *Foc* inoculation in chickpea. A constitutive expression of *CHS* gene was observed in control plants, though a significant difference was evident among JG62 and Digvijay (Fig. 3.37A). When JG62 and Digvijay inoculated with *Foc* 1, 2 and 4 were compared for *CHS* expression, it was either enhanced or maintained in the resistant cultivar, while the expression steadily decreased with the progression of disease in the susceptible cultivar. In general, expression levels declined in a pronounced manner as the disease progressed to 9 dai, where *CHS* was found to be expressing only in race 1 and 2 inoculated Digvijay; and by 13 dai *CHS* level was marked only in *Foc* 1 inoculated Digvijay.

In graphical presentation, *Foc* 1 inoculated Digvijay showed a higher level of *CHS* gene expression than JG62. At 9 and 13 dai, *CHS* gene expression was found to be very high in Digvijay while no response was observed in case of race 1 inoculated JG62 (Fig. 3.37B).

Foc 2 inoculated Digvijay showed a higher level of *CHS* gene expression than JG62, especially at 6 dai when the expression level was the highest (Fig. 3.37C). A similar observation was made in case of *Foc* 4 inoculated Digvijay. At 13 dai, *CHS* gene was found to be expressed in *Foc* 2 inoculated Digvijay while no response was observed in case of race 4 inoculated JG62 and Digvijay (Fig. 3.37D).

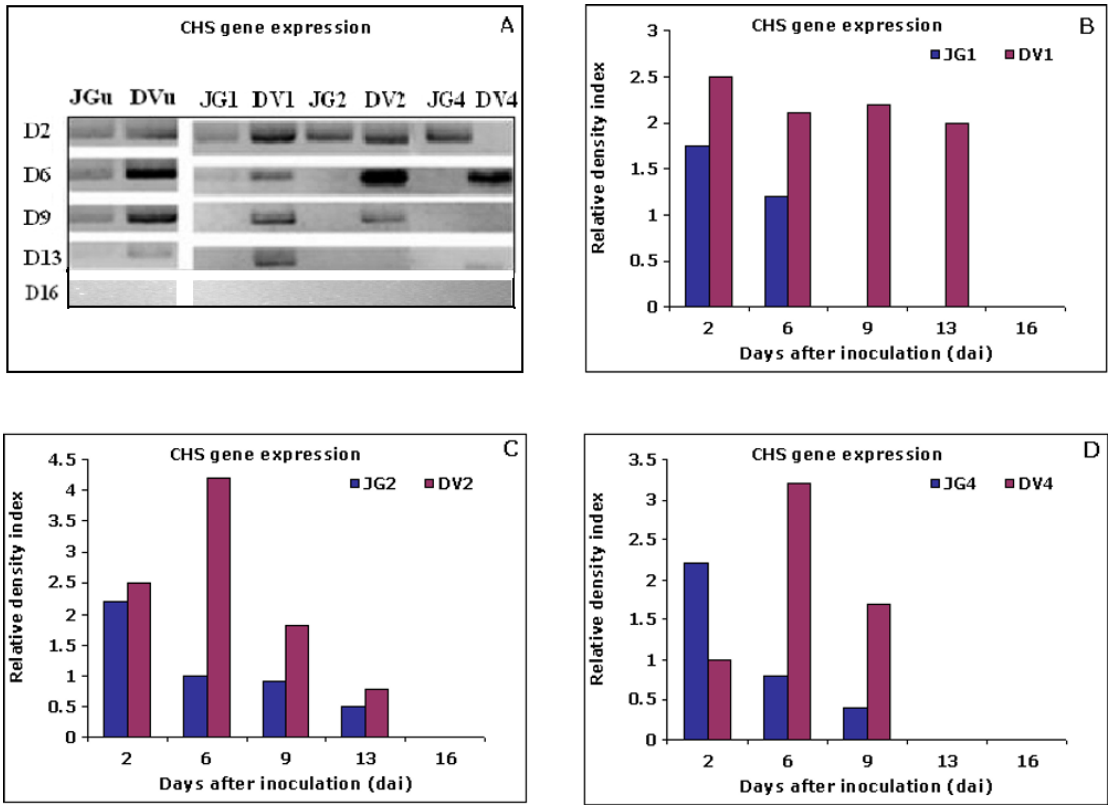


Fig. 3.37 *CHS* gene expression profile in chickpea inoculated with *Foc* races 1, 2 and 4. A: *CHS* gene expression profile. JGu and DVu represent control JG62 and Digvijay root samples. JG1, DV1, JG2, DV2, JG4 and DV4 represent *Foc* 1, 2 and 4 inoculated JG62 and Digvijay root cDNA samples. D2-D13 represent days after inoculation. B: A comparative graphical representation of *CHS* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 1. C: A comparative graphical representation of *CHS* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 2. D: A comparative graphical representation of *CHS* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 4. (Days after inoculation (dai) are represented on X axis while relative density index values are represented on Y axis which indicate the image density, measured using Syngene tools.)

In the comparative analysis, in case of race 1, 2 and 4 inoculated JG62, *CHS* gene expression was high in the early stages of infection while the expression diminished by 13 dai where only race 2 inoculated JG62 showed expression (Fig. 3.38A). When compared to control JG62 plants, *Foc* inoculated JG62 showed high *CHS* gene expression especially at 2 dai; while the case reversed from 6-13 dai (Fig. 3.38A). In

case of Digvijay, high level of *CHS* gene expression was observed till 13 dai, except in race 4 inoculated Digviajy where no expression was observed at 13 dai (Fig. 3.38B). *Foc* 1 inoculated Digvijay showed higher *CHS* gene expression; interestingly *Foc* 2 inoculated Digvijay showed the highest *CHS* gene expression at 6 dai (Fig. 3.38B).

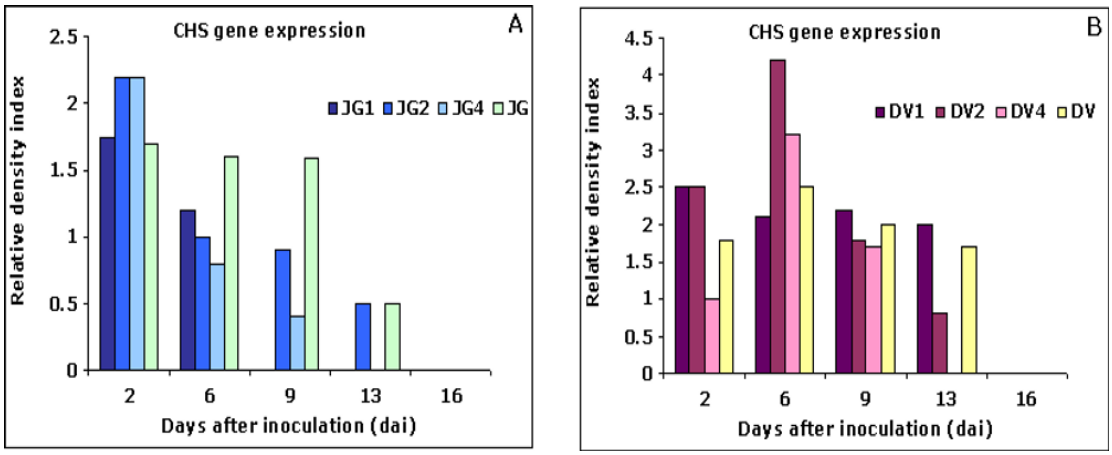


Fig. 3.38 Graphical representation of *CHS* gene expression profile in chickpea cultivars inoculated with *Foc* races 1, 2 and 4. A: A comparative graphical representation of *CHS* gene expression profile in JG62 inoculated with *Foc* race 1, 2 and 4 vis-à-vis uninoculated JG62. B: A comparative graphical representation of *CHS* gene expression profile in Digvijay inoculated with *Foc* race 1, 2 and 4 vis-à-vis uninoculated Digvijay. (Days after inoculation (dai) are represented on X axis while relative density index values are represented on Y axis which indicate the image density, measured using Syngene tools.)

Isoflavone reductase (IFR) gene

A constitutive expression of *IFR* gene was detected in all the analyzed root tissues (Fig. 3.39A). In general, expression level was higher in the resistant cultivar as compared to that in the susceptible one, in the inoculated tissues. Previous results obtained by Arfaoui *et al.*, (2007) support this data; where they also showed an increase in *IFR* transcripts in resistant line suggesting the role of fungicidal isoflavones in the restriction of pathogen infection.

In graphical representation, *Foc* 1 inoculated Digvijay showed a higher level of *IFR* gene expression than JG62 upto 9 dai. At 9 dai no amplification was observed in case of race 1 inoculated JG62. By 13 dai no amplification was observed in either Digvijay or in JG62 (Fig. 3.39B). *Foc* 2 inoculated Digvijay and JG62 showed similar *IFR* gene expression at 2 dai while JG62 and Digvijay showed higher expression at 6 dai and 9 dai, respectively (Fig. 3.39C). In case of *Foc* 4 inoculated Digvijay, expression was maximum at 6 dai, while at 9 dai and 13 dai, *IFR* gene expression was found only in Digvijay (Fig. 3.39D).

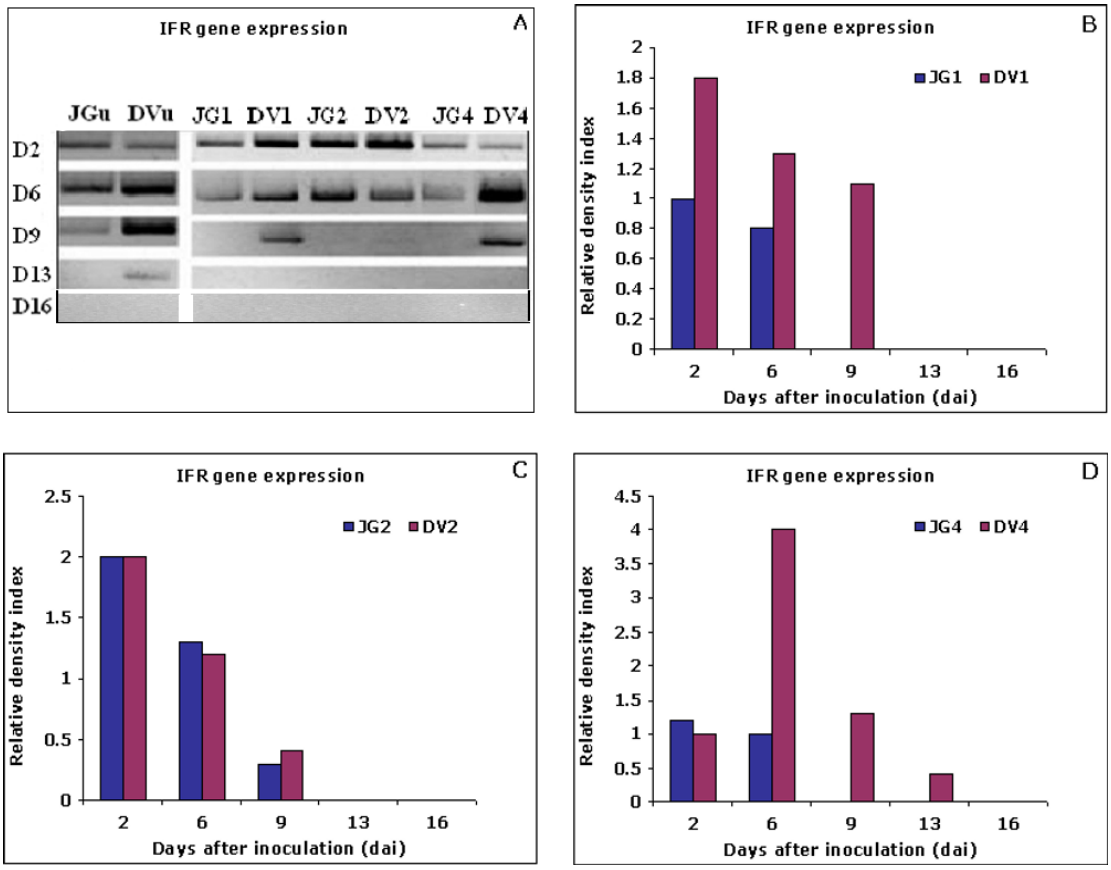


Fig. 3.39 *IFR* gene expression profile in chickpea inoculated with *Foc* races 1, 2 and 4. A: *IFR* gene expression profile. JGu and DVu represent control JG62 and Digvijay root samples. JG1, DV1, JG2, DV2, JG4 and DV4 represent *Foc* 1, 2 and 4 inoculated JG62 and Digvijay root cDNA samples. D2-D13 represent days after inoculation. B: A comparative graphical representation of *IFR* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 1. C: A comparative graphical representation of *IFR* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 2. D: A comparative graphical representation of *IFR* gene expression profile in JG62 and Digvijay inoculated with *Foc* race 4. (Days after inoculation (dai) are represented on X axis while relative density index values are represented on Y axis which indicate the image density, measured using Syngene tools.)

In the comparative analysis, in case of race 1, 2 and 4 inoculated JG62, *IFR* gene expression was high in the early stages of infection (2-6 dai) while the expression diminished by 9 dai where only race 2 inoculated JG62 showed response. Control JG62 plants showed high *IFR* gene expression from 6-9 dai when compared to *Foc* inoculated JG62 plants (Fig. 3.40A). In case of Digvijay inoculated with all the races, maximum level of *IFR* gene expression was observed in race 2 inoculated plants at 6 dai; which prevailed up-to 13 dai only incase of race 4 inoculated Digvijay (Fig. 3.40B). In control Digvijay plants *IFR* gene expression was the highest at 9 dai, but in comparison to inoculated plants, response was low initially at 2-6 dai and then it enhanced from 9-13 dai (Fig. 3.40B).

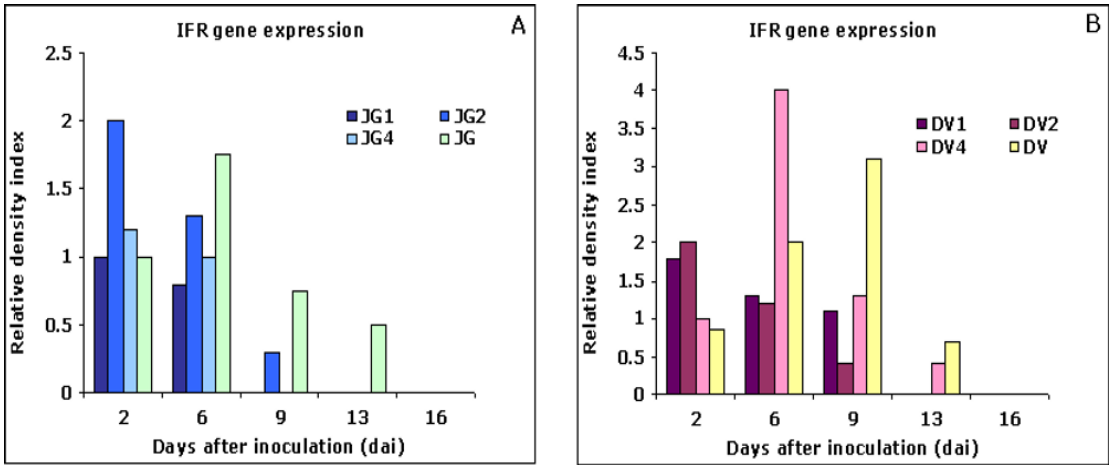


Fig. 3.40 Graphical representation of *IFR* gene expression profile in chickpea cultivars inoculated with *Foc* races 1, 2 and 4. A: A comparative graphical representation of *IFR* gene expression profile in JG62 inoculated with *Foc* race 1, 2 and 4 vis-à-vis uninoculated. B: A comparative graphical representation of *IFR* gene expression profile in Digvijay inoculated with *Foc* race 1, 2 and 4 vis-à-vis uninoculated. (Days after inoculation (dai) are represented on X axis while relative density index values are represented on Y axis which indicate the image density, measured using Syngene tools.)

3.3.4.3 Highlighting chickpea defense response to *Foc* races

Fig. 3.41 depicts at a glance defense response of susceptible and resistant chickpea cultivars, JG62 and Digvijay towards, *Foc* race 1, 2 and 4 from 2-13 dai (16 dai time point has been excluded in this figure since all the genes do not show expression at this time point). It was observed that race 1 inoculated, resistant cultivar, Digvijay showed higher expression of defense related genes namely, *GroES2*, *60srp*, *BetvI*, *CHS* and *IFR*, as compared to susceptible cultivar JG62, throughout the study. In case of race 2 inoculated plants, expression of *CHS* gene was higher in Digvijay as compared to JG62 throughout the course of disease development, indicating its importance in defense against race 2. However, *GroES2* and *BetvI* showed low expression initially at 2 dai in Digvijay as compared to JG62, which enhanced at 6 and 9 dai where the expression of these two genes was higher in the resistant cultivar as compared to the susceptible cultivar JG62. Since *60srp* and *IFR* showed variation in their expression, the role of these genes is difficult to explain. In case of race 4 inoculated cultivars, *BetvI* showed higher expression in the resistant cultivar Digvijay throughout from 2-13 dai when compared to JG62. While *60srp*, *CHS* and *IFR* showed low expression in Digvijay as compared to JG62 initially at 2 dai which reversed at 6 and 9 dai. *GroES2* showed variation in expression and its role is thus difficult to understand with respect to race specificity.

Plant defense related genes

	2 dai						6 dai						9 dai						13 dai					
GENE	**JG1**	**DV1**	**JG2**	**DV2**	**JG4**	**DV4**	**JG1**	**DV1**	**JG2**	**DV2**	**JG4**	**DV4**	**JG1**	**DV1**	**JG2**	**DV2**	**JG4**	**DV4**	**JG1**	**DV1**	**JG2**	**DV2**	**JG4**	**DV4**
GroES2	1.8	4	1.8	1.5	1.9	0	1.2	2	1	1	0.8	0.9	0.8	1	0.4	0.5	0.7	0.7	0	0	0	0	0	0
60srp	2.7	3.7	3.1	3.3	3.3	3.2	1.3	1.4	1.3	1.49	1.4	3.2	1	1.1	1.25	1.1	1.1	3.3	0	0	0	0	0	1.49
BetvI	2.5	3.25	2.9	2.8	3	3.2	2	2.3	1.8	2	2.4	2.6	1.9	2.3	1.9	2	2.4	2.6	1.3	1.7	1.5	1.75	0.9	1.7
CHS	1.7	2.5	2.2	2.5	2.25	0.95	1.2	2.5	1	4	0.8	3.2	0	2.4	0.9	1.9	0.4	1.6	0	2.1	0.4	0.7	0	0
IFR	1	1.8	2	2	1.3	0.9	0.8	1.3	1.3	1.2	1	4	0	1.15	0.3	0.45	0	1.45	0	0	0	0	0	0.35

Fig. 3.41 Defense response of susceptible and resistant chickpea cultivars, JG62 and Digvijay, towards *Foc* race 1, 2 and 4

3.3.4.4 Sequence characterization of major plant defense genes

Amplicons of size 400bp, 650bp, 450bp, 450bp and 400bp obtained using *GroES2*, *60srp*, *BetvI*, *CHS* and *IFR* gene specific primers, respectively, from cDNA of control Digvijay plant were sequenced. Fig. 3.42A-E depict the partial sequences of *GroES2*, *60srp*, *BetvI*, *CHS* and *IFR* genes.

A

>***Cicer arietinum GroES2*** **(Genbank acc. no.GW342895)**

AGAATAGGAAAAGCCTCACGGCTTCCAATGGATGGTTTCTCCTTTGGTTGCCTCTGTGAGCAACA
AACCACCAGCAGTTTTTCCCTCAGCATCTGCAACCTGGCGGCCATCGATAGTTTAAGTATGTAAG
ATCAAGCATAAATCAATTTGAGGTGTACACCTTGATGCAATAATATGGTTTAAACAAATAATTCA
AGAGTGGGAAACTCGTCAGGACAACTGGAGCATTAGACTACTAAACATAGTGATAACAAATGATC
CAGTGCCATTATCCAATTATAATAAATTAATCAAAGTCTCTGAAAAGTTTGCCCTTTAGTCAATA
CTCTATCGATTCCAAGGGGTTAAGATCCTGACCTTCCTTGGGTTTCAAGGGTCCATGATTCCTCT
CCGTTCA

B

>***Cicer arietinum 60srp*** **(Genbank acc. no. GW342896)**

ATTCAGGCAAACACGGTCGGCGCTTTCCCTTAAACATGATCCCAAACCTAAGCCTGAAGCTCCCGC
ATGAGAAGGCTCCTAAATTCTACCCCGCCGATGATGTCAAGAAGCCACTTCGCAAACAAGCACAAA
CCTAAGCCTACTAAGCTCAGGTTCGTTACGGTCGTTCATTTCTATCCACACTCTTTTTTGTTAGCA
TATGTGTAGGAAATTAGGTTTTGCTTTACTGCGTTTTAATTGGGATCTGATAAATGTCGTTTATTT
AAGTAGTAAATTATTTATAATAAACGATTATTTAATAGTTTTTTTTGTTGATTTACTTATAGTCAA
AGATTACTGTAGTTGAATGGTTTTATATTGTACTGTTTTTGTTTCAGGGCTAACATTACTCCAGGG
ACAGTGTTGATTCTTCTTGCCGGTAGATTCAAGGGAAAGAGGGTTGTGTTTTGAAACAGCTTCCTT
CTGGCCTTCTTCTTGTACTGGTAACTATTTTCCCCTATAATAAATATTGTGGTGGGGCCGACTTCT
GTGTGTTTAAGTTAGTGAAATTATACTATGGTTCACATCCCAAAATAAAAATTGCGCTTGTAATGC
ACGTTCCCTATTGACTATTCATTGTTGGTAAATGCTCACACAACCCGGGGGA

C

>***Cicer arietinum BetvI*** **(Genbank acc. no. GW342897)**

AAAGATTTCATGATACCATATTGCATGAAGGTGATGAATGGCATGTGTCTGATTCTGTTAAACAA
TGGACTTATGTCATAGATGGCAAGGTACACACATGTAAAGAGAGTGTTGAAGAAATTGATGAAGA
GAACAAAAAACTCACTTTCAAGCTCTATGGTGGAGATATTGATGAGCACTATAAGATCTTTAAAC
TCATCATTGAAGTTATAGATAAGGCTGATGGTAGTGCTTCCGTTAAATGGACTATAGAATATGAG
GAGATCCGTGAGCATTATGATCCTCCAAATGGATACATGGACTACTTTGCCAAATGCCCTAAAGA
TATGGATGCTCATCTTGTCAATGCATCACTCCAAAAGTGATATAGAAGATAAAATAAAAAAATAG
AGTGTAATAAAGGATAATGGCTCAATGGGGTTGCCTTACT

D

>***Cicer arietinum CHS*** **(Genbank acc. no. GW342898)**

ATTTCTTTCTCATTTCATCCAATATAAACAATACACACGCACTTGACCATATTTCCATATTCACTC
AACACTTCCCTAGTTGCCCTCATCTTTTCAGGCTTCAAGGCTAACTTTTCCTCTACTTGGTCTAGA
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AAAGCTTCAATCAAAGCTTTATCAATGTTCTTTGAGACAATCCCAGGAACATCTTTAAGAAGGTGA
AATGTGAGTCCTGCTTCACGAAGGTGACCATCAATAGCACCTTCACTATCTGGAGCAATTGTTTGT
GCAGTCCAAACCATCTCAAATATTGGTTTCTCAATTTCAGGAATTGGATCAGATCCAACAATCAAT
GCAGCAGCTCAGGGATGGTCTTGATGG

E

>***Cicer arietinum IFR*** **(Genbank acc. no. GW342899)**

AGAATGATACCTTGTCCCTGGAGTTCAGTGGCGTCGAATTGAGCCAAGTTACGTAAGGAGTAACCA
GTGAAGGCATGGCACAGAGGTAAGTGTAAGGAACTCCTTCAGCTTCAACTACTCTTCGAATACTTG
CTTTTTCGTCAAAAACTGGTCTAACAGGGTCAACTGCATCGTGACGGTCCACATCTAGACCAAATT
CAGATGGGAAAAATCTCTTGACATTTCCAGCTTCTTTAATAGCTTTAATGATCTTACTTGATCCAA
AATTAGTAATCTACCAAAGTGCAGATCACAGTATCAACTTGCTTTATTGCTTTACAAGAGCTTCAT
GATCCTTCATATCACCTTCAAGTAGAATAACTCCTGCTGCTGGAAACTCTGAAGAAGCTCTTCCTT
GCT

Fig. 3.42 Partial sequences of *GroES2*, *60srp*, *BetvI*, *CHS* and *IFR* genes. A: Partial sequence of *GroES2* gene. B: Partial sequence of *60srp* gene. C: Partial sequence of *BetvI* gene. D: Partial sequence of *CHS* gene. E: Partial sequence of *IFR* gene.

The chickpea *GroES2* partial sequence characterized in this study showed homology to *Arabidopsis thaliana* and *Gossypum hirsutum* chapenonin 10; while the *60srp* sequence was found to show 99% similarity to the reported *Cicer arietinum* 60s ribosomal protein deposited in the NCBI database. The *BetvI* sequence was found to show 84% homology to *Cicer arietinum* partial mRNA for putative *BetvI* family protein (*Bet* gene). The *CHS* and *IFR* partial nucleotide sequences showed 100% homology to *Cicer arietinum CHS* and *IFR* sequences available in the Genbank.

3.3.4.5 Mapping of chickpea defense related genes on *Medicago truncatula* genome

The partial sequences of *GroES2*, *60srp*, *BetvI*, *CHS* and *IFR* genes were mapped on *Medicago truncatula* genome using CViT-blast utility available at website http://www.medicago.org/genome/cvit_blast.php.

CViT is a whole-genome mapping tool that places features on chromosomes. CViT-blast displays blast results on the *Medicago truncatula* genome and hits for each sequence are assigned a different color. Thus, using this facility chromosomal location of chickpea defense related genes, which showed homology to corresponding *Medicago*

genes, was assigned. Fig. 3.43 depicts mapping of chickpea defense related genes on *Medicago truncatula* genome.

GroES2 was localized on chromosome 8, while *60srp* was placed on chromosome 3, 4 and 5. The *BetvI* gene was mapped at multiple locations on chromosome 7 and 8. Similarly, *CHS* gene was localized on chromosome 3, 5, 7 and 0, while *IFR* gene was mapped only on chromosome 5. Figs. 3.44A-E depict the exact locations of *GroES2*, *60srp*, *BetvI CHS* and *IFR* genes on *Medicago truncatula* genome.

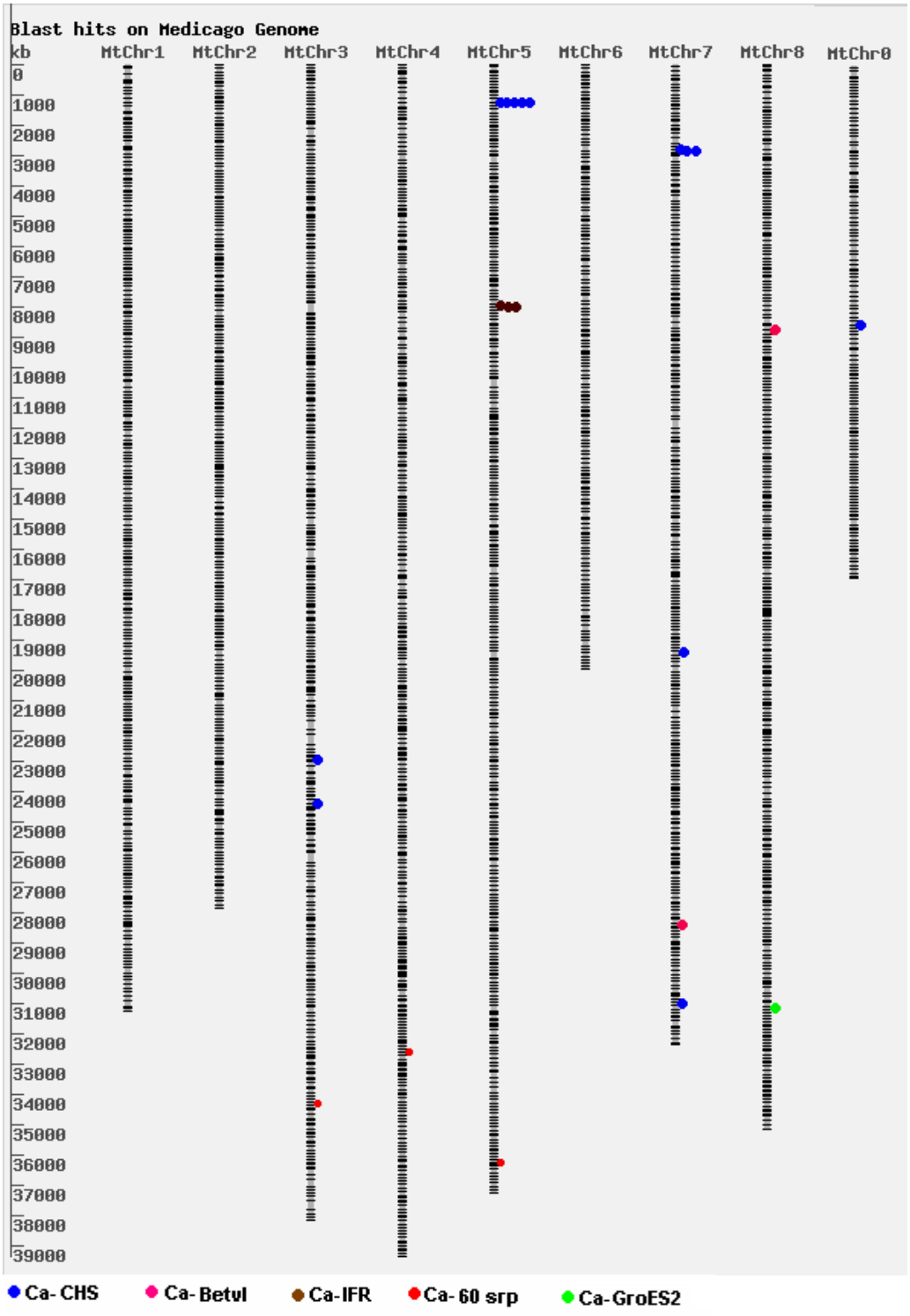
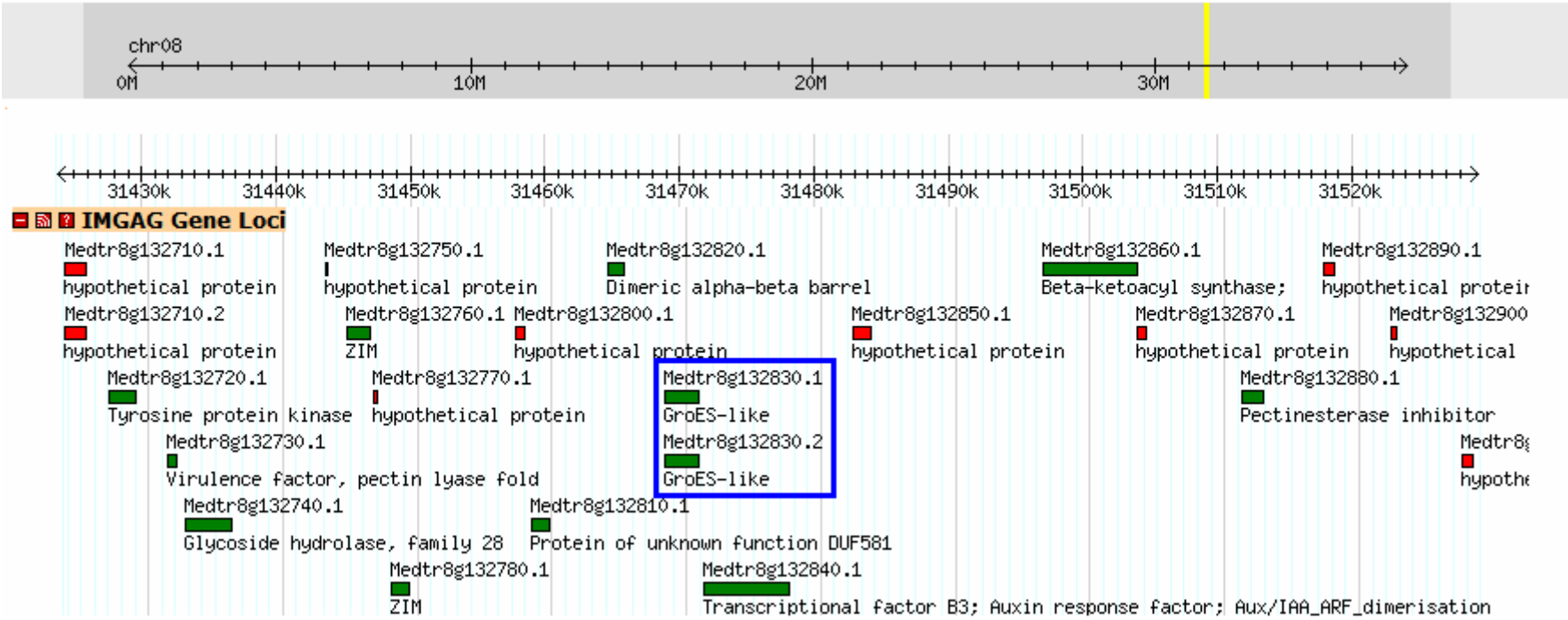


Fig. 3.43 Mapping of chickpea defense related genes on *Medicago truncatula* genome. The gene names along with the colour codes are displayed below the figure.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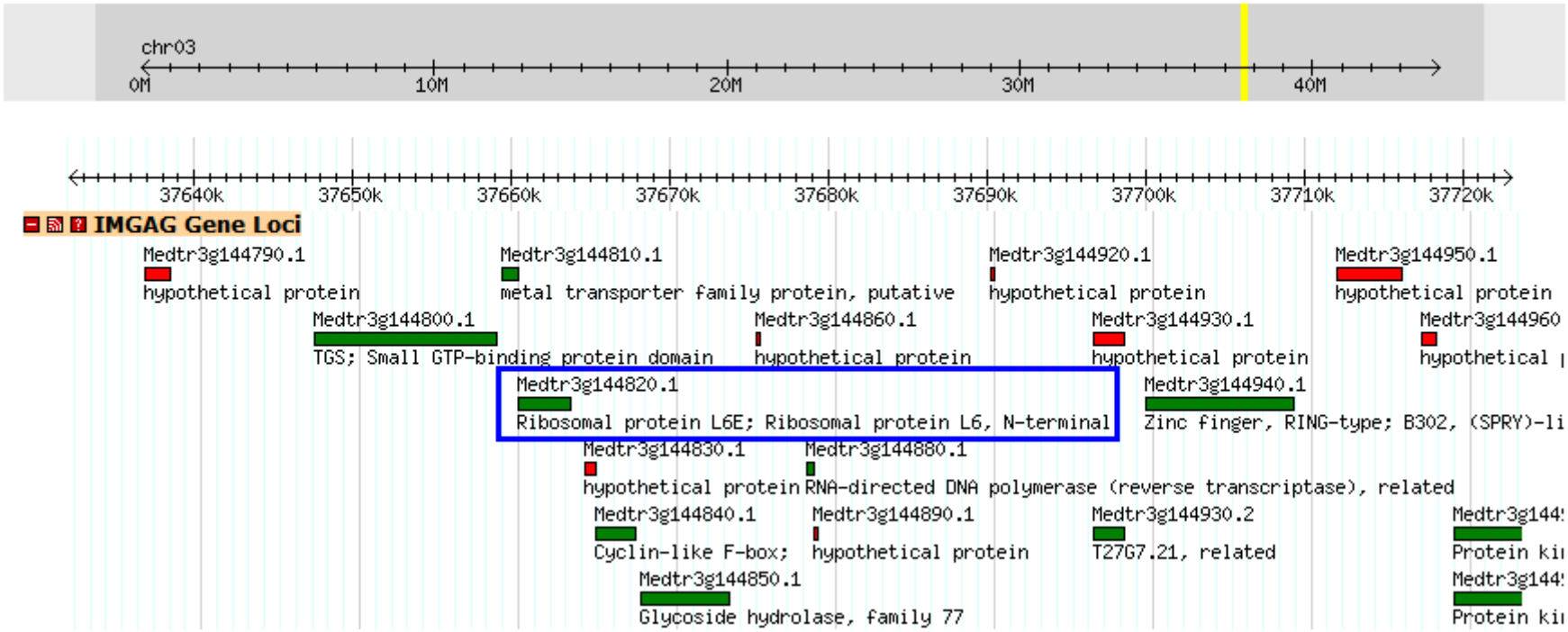
0M
10M
20M
30M
31430k
31440k
31450k
31460k
31470k
31480k
31490k
31500k
31510k
31520k
IMGAG Gene Loci
Medtr8g132710.1
hypothetical protein
Medtr8g132750.1
hypothetical protein
Medtr8g132820.1
Dimeric alpha-beta barrel
Medtr8g132860.1
Beta-ketoacyl synthase;
Medtr8g132890.1
hypothetical protein
Medtr8g132710.2
hypothetical protein
Medtr8g132760.1
ZIM
Medtr8g132800.1
hypothetical protein
Medtr8g132850.1
hypothetical protein
Medtr8g132870.1
hypothetical protein
Medtr8g132900
hypothetical
Medtr8g132720.1
Tyrosine protein kinase
Medtr8g132770.1
hypothetical protein
Medtr8g132830.1
GroES-like
Medtr8g132880.1
Pectinesterase inhibitor
Medtr8g132730.1
Virulence factor, pectin lyase fold
Medtr8g132830.2
GroES-like
Medtr8g132740.1
Glycoside hydrolase, family 28
Medtr8g132810.1
Protein of unknown function DUF581
Medtr8g132780.1
ZIM
Medtr8g132840.1
Transcriptional factor B3; Auxin response factor; Aux/IAA_ARF_dimerisation

A

Cont…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0M
10M
20M
30M
40M
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37650k
37660k
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37720k
IMGAG Gene Loci
Medtr3g144790.1
hypothetical protein
Medtr3g144810.1
metal transporter family protein, putative
Medtr3g144920.1
hypothetical protein
Medtr3g144950.1
hypothetical protein
Medtr3g144800.1
TGS; Small GTP-binding protein domain
Medtr3g144860.1
hypothetical protein
Medtr3g144930.1
hypothetical protein
Medtr3g144960
hypothetical
Medtr3g144820.1
Ribosomal protein L6E; Ribosomal protein L6, N-terminal
Medtr3g144940.1
Zinc finger, RING-type; B302, (SPRY)-li
Medtr3g144830.1
hypothetical protein
Medtr3g144880.1
RNA-directed DNA polymerase (reverse transcriptase), related
Medtr3g144840.1
Cyclin-like F-box;
Medtr3g144890.1
hypothetical protein
Medtr3g144930.2
T27G7.21, related
Medtr3g144850.1
Glycoside hydrolase, family 77

B

Cont…


chr07
0M
10M
20M
30M
32210k
32220k
32230k
32240k
32250k
32260k
32270k
32280k
32290k
32300k
32310k
32320k
IMGAG Gene Loci
Medtr7g135440.1
hypothetical protein
Medtr7g135450.1
Helicase, C-terminal
Medtr7g135460.1
ATP binding ^, putative
Medtr7g135470.1
hypothetical protein
Medtr7g135480.1
hypothetical protein
Medtr7g135490.1
Cyclin-like F-box
Medtr7g135500.1
hypothetical protein
Medtr7g135510.1
hypothetical protein
Medtr7g135520.1
Leucine-rich repeat; Leucine-rich repeat, cysteine-containing, putative
Medtr7g135530.1
Leucine-rich repeat; Leucine-rich repeat, cysteine-containing, putative
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hypothetical protein
Medtr7g135560.1
Ribosomal protein L33
Medtr7g135570.1
Cytochrome b6/f complex, subunit 5
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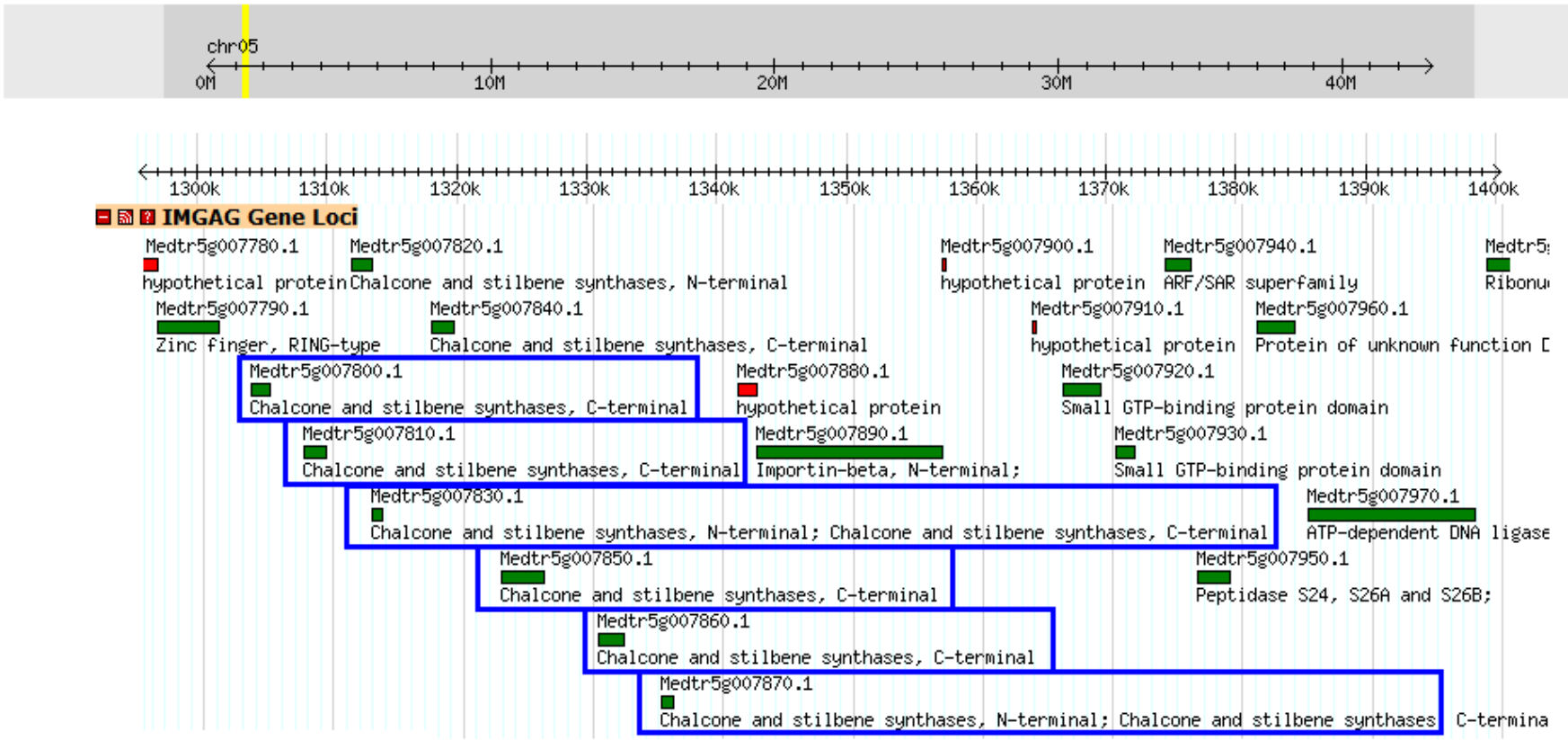
Bet v I allergen
Medtr7
hypoth
Medtr7g13561
hypothetical

C

Cont…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hypothetical protein
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Zinc finger, RING-type
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hypothetical protein
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Protein of unknown function D
Medtr5g007800.1
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Medtr5g007880.1
hypothetical protein
Medtr5g007920.1
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Medtr5g007970.1
ATP-dependent DNA ligase
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Chalcone and stilbene synthases, C-terminal
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Chalcone and stilbene synthases, N-terminal; Chalcone and stilbene synthases

D

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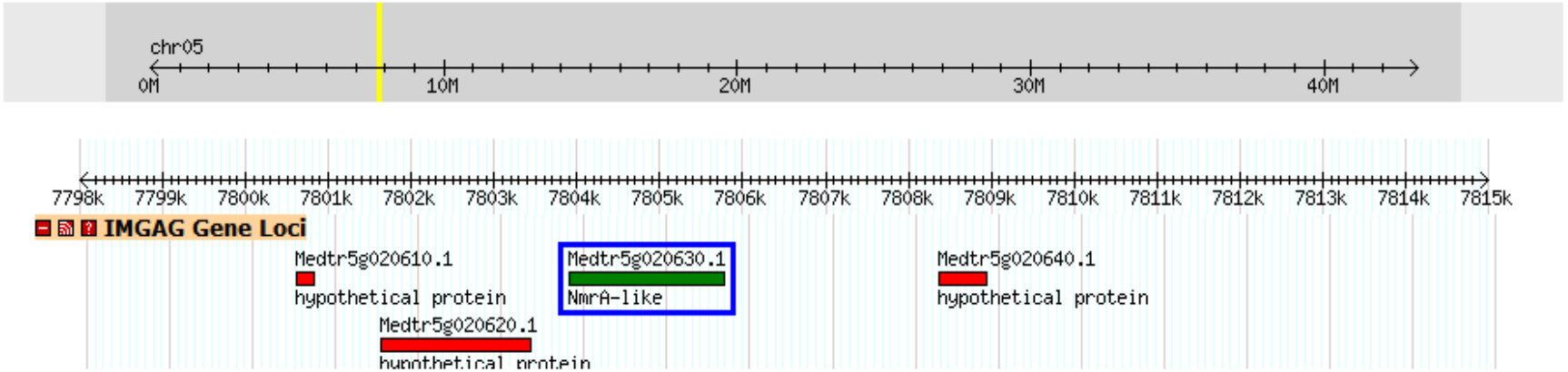


E

Fig. 3.44 Exact location of chickpea defense related genes on *Medicago truncatula* genome. A: *GroES2* gene. B: *60srp* gene. C: *BetvI* gene D: *CHS* gene and E: *IFR* gene.

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 *nut1* 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 N_2 regulatory genes like *area/Nit2* and their orthologue ***CLNR1*** in *Colletorichum* enable uptake and catabolism of such secondary N_2 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.

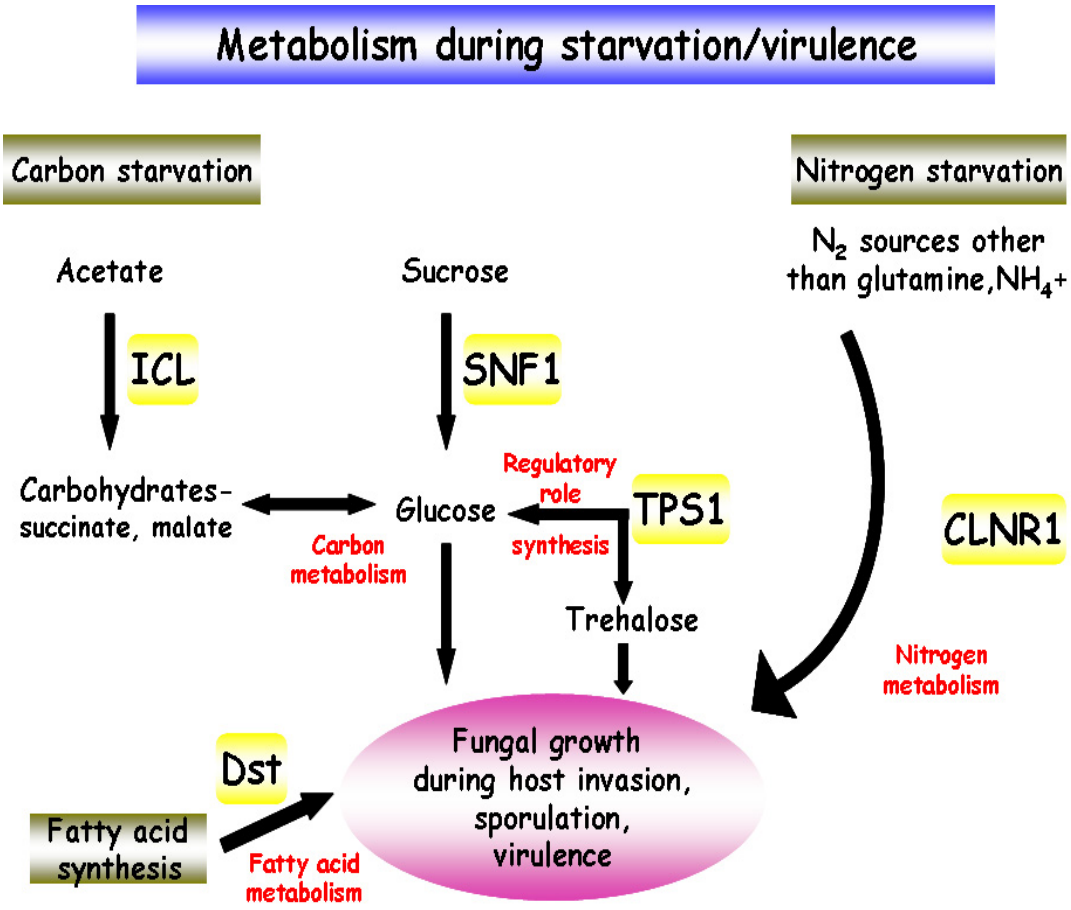


Fig. 4.1 Fungal metabolism during stress and starvation conditions

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.

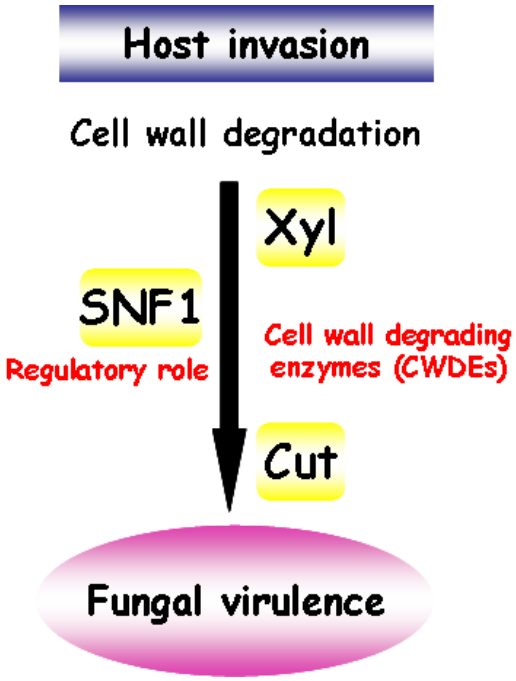


Fig. 4.2 Fungal genes involved in plant cell wall degradation

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 **Fmk1 kinase** and further **Mst12** like transcription factors which govern the formation of appressorium like specialized infection structures. This MAP kinase cascade is activated by β and γ 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, adenylyl cyclase, cAMP and cAMP dependent protein kinase; which play a specialzed role in filamentous growth. Gene ***clk1*** encodes a putative **serine/ threonine protein kinase** which is a part of signal transduction pathway involved in *C. lindemuthianum* infection process. *Clk1* involving cAMP signal transduction pathway is important in the development of *Colletotrichum* on the plant surface, i.e., germination of conidia and appressorium formation. Fig. 4.3 shows the fungal genes essential in signaling cascades.

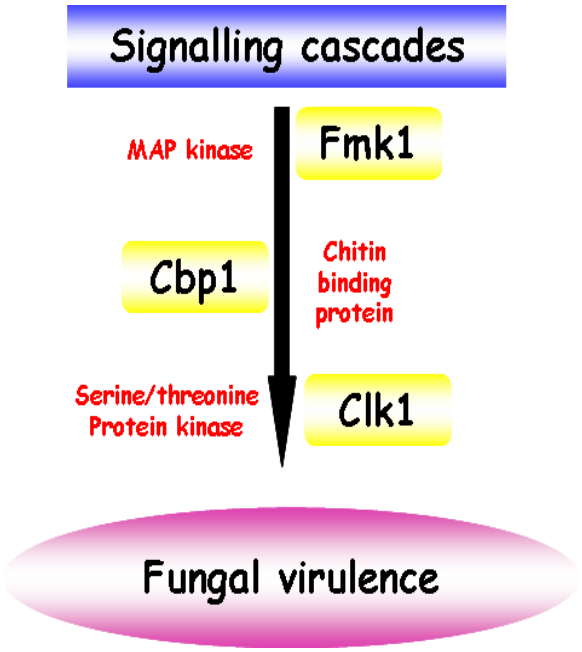


Fig. 4.3 Fungal gene involved in signaling cascades

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* races1, 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.

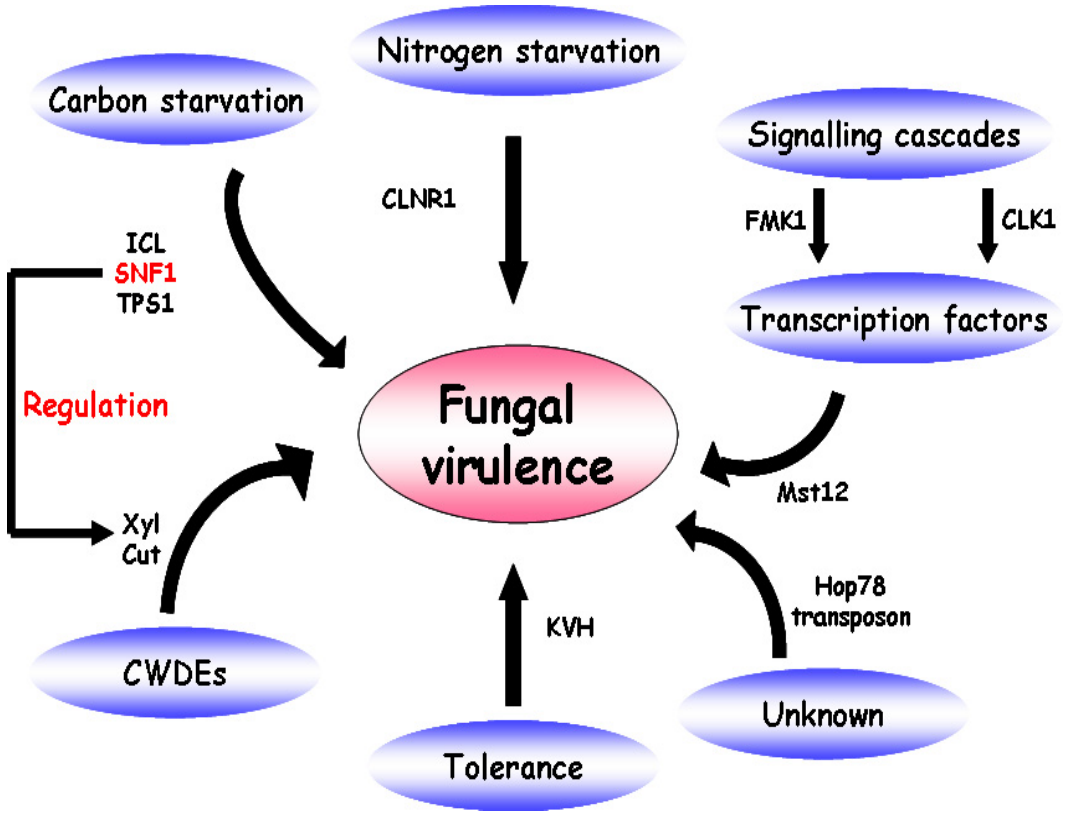


Fig. 4.4 Different pathways leading to fungal virulence

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 *Foc* races 1 and 2 to get differential amplification in these races. This approach can be further modified suitably to identify *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 *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 *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 *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.

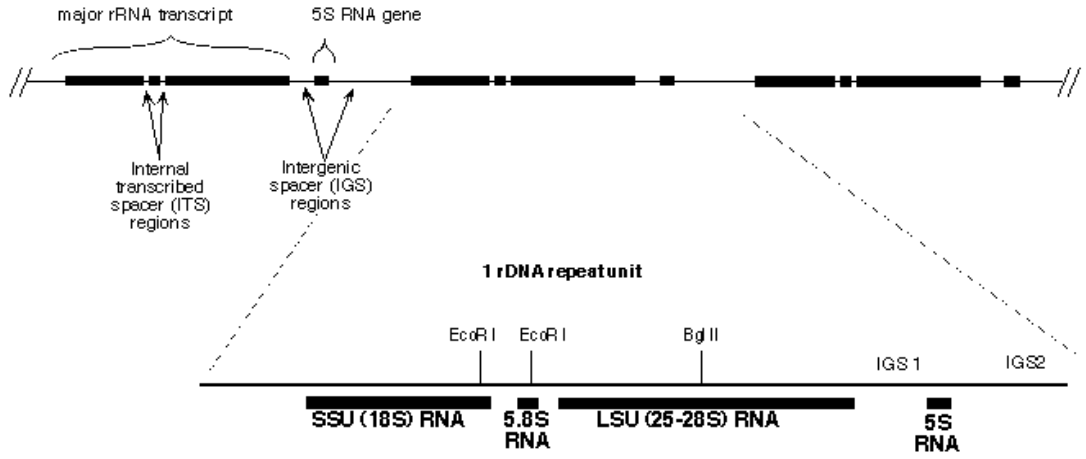


Fig. 4.5 The rRNA gene structure

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 *Hinf*I, *Mbo*I, *Hae*III and *Hha*I 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 *Eco*RI/*Mse*I 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* (25420*Fov*1, 26034*Fol*), 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 Ferna´ndez-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 catalyses 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 *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 *et al.,* 2007). Similar results were reported by others (Wang *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, *Msr*, *BetvI* and *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 *Fgb1*, *Gas1*, *Chs7* and *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 *et al.,* 2004; Iori *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-*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 *GroES2*, *60srp*, *BetvI*, *CHS* and *IFR*, showed higher expression in resistant chickpea cultivar, Digvijay vis-à-vis susceptible cultivar JG62, when exposed to race 1. However, only *CHS* and *BetvI* 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

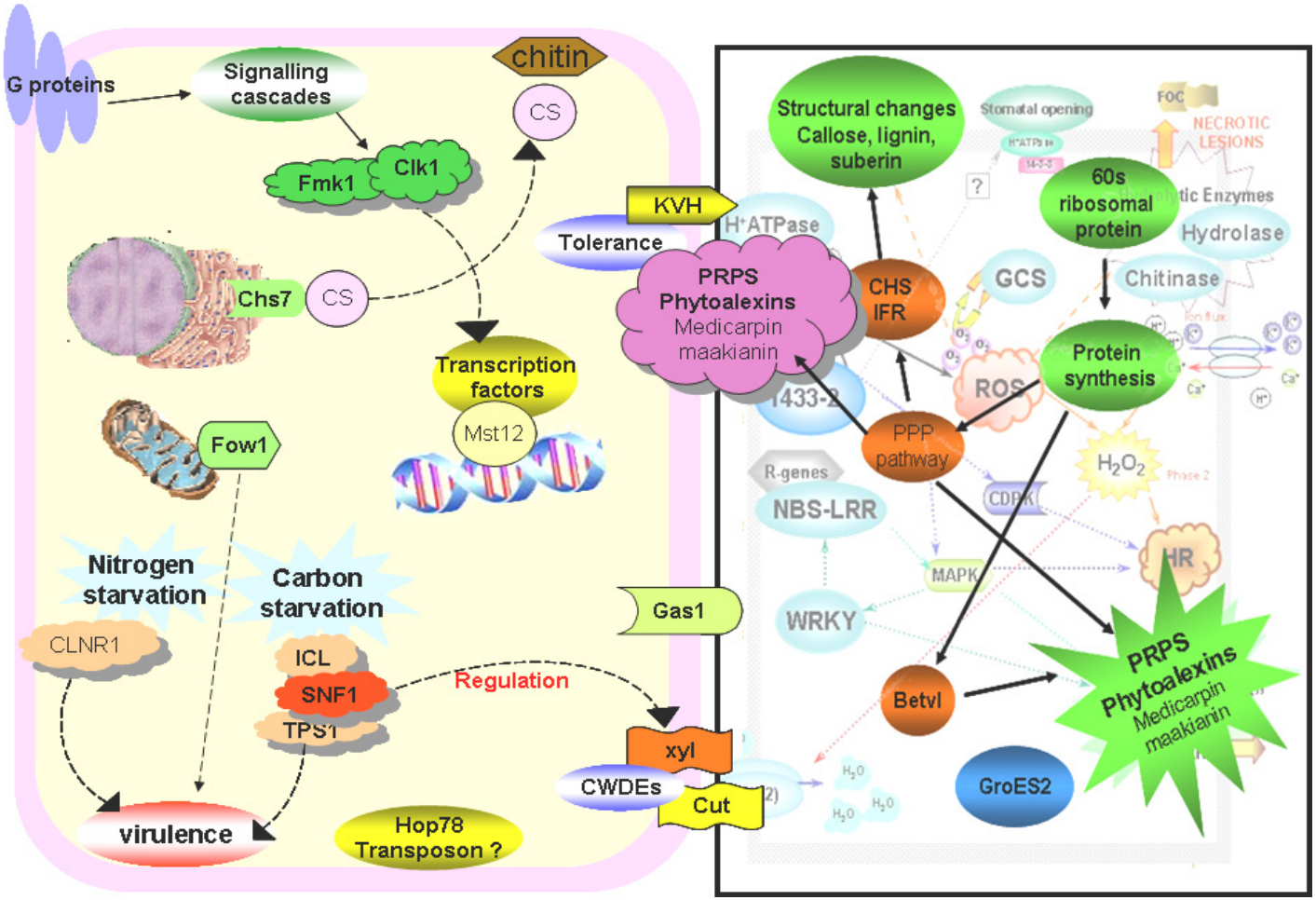
upregulation of some of these genes was sufficient to give complete resistance in Digvijay.

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, *Fow1* 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).



***Fusarium* cell** **Chickpea cell**

Fig. 4.6 Schematic representation of *Fusarium* virulence genes and chickpea defense genes involved in the host-pathogen studies accomplished in this research work

SUMMARY AND FUTURE DIRECTIONS

5 Summary and future directions

5.1 Summary

Race identification and variability in *Foc*

Chickpea is an important source of dietary protein, especially for the vegetarian population and is the most abundantly grown legume in India which contributes to 65% of the world production of this legume crop (FAOSTAT, 2007). Fusarium wilt, caused by *Fusarium oxysporum* Schlenchtend.: Fr. f. sp *ciceris* (*Foc*) (Padwick) Matuo and Sato, is one of the most destructive diseases of chickpea throughout the world (Holliday, 1980). *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. In severe cases of wilt, disease symptoms can be seen within 20-25 days post infection. Individual pathogenic strains within the species; having an ability to infect a particular host range have been assigned to intraspecific groups - forma speciales (Armstrong and Armstrong, 1981). Some of the forma speciales are further divided into races based on virulence to a set of differential cultivars within the same host plant species as in *Foc* (infecting chickpea- *Cicer arietinum*) (Armstrong and Armstrong, 1981) which has been classified into eight races worldwide. Cultivar specificity and disease reaction is the only methodology to assess the pathogenicity of a *Foc* isolate enabling it to be designated as a specific race.

To address these problems concerning *Foc* race identification, in-depth studies were performed. During these studies, various techniques like GSOs, ITS-RFLP, and AFLP for identification of *Foc* races 1, 2, 3 and 4, were used.

Using GSO approach, races 1, 2 and 4 yielded amplification products of 1.5kb with oligonucleotides *Hop78*F2/R2 (designed for *Hop78* transposon gene), while a 900bp amplicon was obtained with oligonucleotides *Cut*F/R (designed for *cutinase* gene). However, no amplification was observed for race 3 using the same oligonucleotides. *Dst*F/R oligonucleotides (designed for *desaturase* gene) amplified a 600bp product specifically in race 3 and not in other standard *Foc* races. Similarly, *Xyl*F/R oligonucleotides (designed for *xylanase* gene) amplified 700bp fragments in *Foc* races 1 and 2 and race 3, while the consistent absence of this 700bp band was specific for race 4 only. Thus, *Foc* race 3 was clearly distinguished using *Hop78*F2/R2, *Cut*F/R and *Dst*F/R primers; while race 4 could be identified by oligonucleotides *Xyl*F/R.

Further, the 550bp ITS amplification product when digested with restriction enzymes *Hinf*I, *Hha*I, *Mbo*I**,** and *Hae*III, respectively, yielded identical restriction digestion profiles in case of race 1, 2 and 4; while race 3 showed a different restriction pattern with each restriction endonuclease used in the present study. Thus, it was possible to identify race 3 easily using ITS-RFLP approach.

Race 1 and 2, which were not distinguished using GSOs and ITS-RFLP approaches, were resolved using AFLP technique. Twelve *EcoR*I/*Mse*I primers combinations yielded race specific profiles and were useful for identifying races 1 and 2. Few of these race specific AFLP fragments showed homology to metabolically essential genes of fungi. Some fragments were homologous to sequences expressed during stress conditions in fungi which might serve as candidates to decipher the virulence mechanism of *Fusarium*.

The genetic profile of *Foc* race 3 was found to be different than the other three races using gene specific markers, ITS-RFLP as well as AFLP markers. Sequence data from EF-1α of the standard isolates of four Indian *Foc* races and the phylogenetic analysis using PAUP (Swofford, 1998) revealed that EF-1α sequence of *Foc* race 1, 2, and 4 standard isolates was similar to *F. oxysporum* (NRRL 25420 *Fov*1, NRRL 26034 *Fol*) and that of *Foc* race 3 actually represented *F. proliferatum*, hence it was deleted from further analysis.

Proteome analysis of *Foc* races 1, 2 and 4 was attempted for better understanding of their race specificity at the proteomic level. Proteomic analysis indicated the significant homologies of *Foc* proteins to hypothetical *G. zeae* proteins. Further, these were found to be similar to fungal proteins essential for fungal virulence like glucosamine 6-phosphate N-acetyltransferase, glycosyltransferase and DEAD-box RNA helicase.

Chickpea-*Fusarium* interaction studies

A complex interaction between plant and its fungal pathogen is an outcome of expression of both, plant defense genes as well as fungal pathogenesis related genes. The result of such a relationship is projected either as host resistance or disease development in the plant. There are multiple events involved to bring about successful plant defense during pathogen attack. Further, these defense mechanisms are governed by diverse genes which either singly or synergistically bring about the resistance in plants. On the other hand, understanding the pathogenicity mechanism of a pathogen demands knowledge of the virulence factors which are active in the host environment. Till date, many fungal pathogens have been studied in context with their pathogenicity

aspects. Various virulence genes have been identified which have a prime role to play in fungal pathogenesis. However, indepth search of mechanisms and the genes involved in chickpea defense against pathogenicity mechanism of wilt causing root pathogens is essential.

In this study, transcripts involved in chickpea-*Fusarium* interactions were determined using cDNA-RAPD approach, which revealed their homology to important genes of plant origin like chaperonin, glycosyltransferase, *Msr*; and fungal origin like *Tri101* and transposase.

Expression of fungal pathogenesis related genes was assessed during infection of resistant and susceptible cultivars, JG62 and Digvijay, respectively with *Foc* race 1, 2 and 4. Race specific expression of fungal virulence genes like *Fgb1*, *Gas1*, *Chs7* and *Fow1* which have been previously reported to be essential for various cellular functions including fungal pathogenesis; was observed with an elevated expression during 9-13 dai indicating the window period of disease progression. The partial sequences of *Foc* virulence related genes, namely *Fgb1*, *Gas1*, *Chs7* and *Fow1* were further mapped on *G. zeae* genome.

Variation in the expression pattern of specific genes observed in the cDNA-RAPD studies led us to perform detailed analysis of expression of candidate plant defense genes during various stages of disease development in susceptible and resistant chickpea cultivars, JG62 and Digvijay, respectively inoculated with all the three races of *Foc*, namely *Foc* 1, 2 and 4 in order to understand the contribution of these genes in plant defense. Semi quantitative RT-PCR approach was used to analyze the temporal expression of plant defense related genes during chickpea-*Foc* interaction.

Overall, in this study, an enhanced expression of plant defense related genes namely, *GroES2*, *60srp*, *BetvI*, *CHS* and *IFR* was observed in case of resistant inoculated cultivar as compared to susceptible inoculated cultivar. The partial sequences of *GroES2*, *60srp*, *BetvI*, *CHS* and *IFR* genes were also mapped on *M. truncatula* genome.

5.2 Future directions

Major aim of this study is to understand the chickpea pathogen *Fusarium oxysporum* f. sp *ciceris* (*Foc*) with respect to its pathogenicity leading to its classification into various races. At the same time chickpea-*Fusarium* interactions were also studied with respect to plant defense and fungal pathogenesis. Based on the findings obtained in this work a few leads can be followed further. The future directions are as follows:

Studying the race specific variation in Foc

Development of race specific markers for race 1 and 2 as well as races outside India i.e, race 0, 5, 6 and 7, is essential. Screening more GSOs for race specificity and validation of existing GSOs using more number of isolates per race can help in better identification of races. Characterization of *Foc* races on the basis of their proteomic differences can be accomplished using 2D-PAGE and MALDI. Further, the novel and race specific proteins, if obtained can be analyzed. Studying the metabolome of the *Foc* races can reveal the race specific differences amongst their metabolomes (comparative metabolomics).

Studying the chickpea-Foc interactome

Since transcripome approach has lead us to identify putative plant defense and pathogen virulence genes, using proteomics and/or metabolomics a better understanding of identified gene network is possible. Also new proteins as well as metabolites essential for this interaction can be identified. The sequencing of several plant and fungi genomes has spurred major advances in comparative genomics field. In this study, using the information available in *Medicago* or *Gibberella* genome we were able to find and predict the presence of homologues of chickpea defense related and *Fusarium* pathogenesis related genes on these genomes. Further, genome sequences of more plant and fungal species can be used for comparative genomics.

Confirmation of the role of fungal virulence factors

The exact role of genes, their mode of action during wilt progression as well as their effect on fungal virulence is yet to be defined. Gene disruption is a powerful genetic tool that can define pathogenic or virulence factors and has been extensively used to identify fungal virulence genes (Kwon-Chung, 1998). Gene disruption, gene inactivation and/or gene silencing can prove to be extremely valuable methods for understanding fungal pathogenesis. The introduction of multiple gene disruption and RNAi technologies may help resolving situations that involve multiple, highly similar gene families. Development of knockout *Foc* mutants for virulence related genes would confirm the role of identified genes in fungal pathogenesis at the same time it will enable us to develop strategies to design antifungal targets. RNAi based approach can also be established for understanding the role of pathogenicity genes in *Foc*.

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Academic qualifications:

- **Masters of Science in Marine biotechnology** (2000-2004), from Dept. of Marine biotechnology, Goa University, Goa
  Secured: **73.2%, First class with distinction**
- **Bachelor of Science in Microbiology** (1997-2000), from Dept. of Microbiology, Abasaheb Garware College, Pune
  Secured: **70.6 %, First class with distinction**
  Major subjects included: **Chemistry, Botany and Microbiology**

Academic achievements:

- **Awarded Junior Research Fellowship**, through highly competitive national examination conducted by Council of Scientific and Industrial Research (CSIR, India) to pursue Ph.D.
- Secured **first position at M.Sc final year** of post graduation in Dept. of Marine Biotechnology, Goa University
- Awarded **Dr. Barros gold medal** for securing **first position in M.Sc final year**
- Selected through the All India combined entrance examination for biotechnology, conducted by Jawaharlal Nehru University, New Delhi, for M.Sc Biotechnology
- Secured **first position at B.Sc final year** of graduation in Abasaheb Garware College, Pune
- Secured **second position in "Chemiad" competition** (an entrance examination in chemistry, conducted by Pune University)

Research experience:

- Presently working as a Senior Research Fellow, in National Chemical Laboratory, Pune; on: **Identification and molecular characterization of virulence related factors in *Fusarium oxysporum* f.sp *ciceri***
- Worked as a project assistant with National Chemical Laboratory, Pune; on project entitled: **National facility for virus diagnosis and quality control of tissue culture raised plants- DNA based quality control of plants produced at MTP, NCL: A value addition to TC raised plants**

- Dissertation project at Dept. of Marine Biotechnology, Goa University entitled: **Persistence of genetically engineered DNA sequences in seawater and its transfer to marine isolate *Pseudomonas putida* strain GU109 (MTCC 3316) using a sea water recirculatory system**
- Summer training at National Centre for Cell Sciences, Pune; on project which included: **Isolation of recombinant complement control protein from *Pichia pastoris*, its purification and western blotting**

Research skills:

- Basic biochemical, microbiological and molecular techniques.
  DNA extraction, PCR, Cloning and Transformation in bacterial system, AFLP, RFLP, ISSR and RAPD marker analysis, agarose and polyacrylamide gel electrophoresis, silver staining of PAGE gels
- Gene expression studies- Infection assay of *Fusarium*-chickpea system, RNA extraction and semi-quantitative RT-PCR analysis
- Protein extraction and purification, SDS-PAGE, western blotting, ELISA
- Knowledge of different computational softwares- Nucleotide and protein sequence database search using GenBank, EMBL, DDBJ, PDB, Swiss Prot; Nucleotide and amino acid sequence analysis using various Bioinformatics tools such as BLAST and Mega blast tools; Nucleotide and amino acid sequence alignments using BLAST and primer designing, ClustalX/W, MEGalign, BioEdit softwares.

Workshops and Conferences attended:

- Participated in workshop under the Max Planck Society-India partnership program, entitled: **Proteomics insights into plant-insect interactions, December 12-15, 2006**; conducted at National Chemical Laboratory, India
- Participated (Poster presentation) in **National seminar on: Prospecting Fungal Diversity in Tropical India, its Conservation and Applications in Biotechnology, April 28, 2006**; conducted at Agharkar Research Institute, Pune
  Obtained the best poster award
- Participated (Oral presentation) in: **Fourth International Food Legumes Research Conference, October 18-22, 2006**; held at Indian Agricultural Research Institute, New Delhi

Publications:

- Identification of Indian pathogenic races of *Fusarium oxysporum* f. sp *ciceris* with gene specific, ITS and random markers. **Gayatri S. Gurjar,** Maneesha P. Barve, Ashok P. Giri and Vidya S**.** Gupta. **Mycologia**, 101(4), 2009, pp. 484–495. DOI: 10.3852/08-085.
- Gene expression profiling during progress of wilting in chickpea (*Cicer arietinum* L.) caused by *Fusarium oxysporum* f. sp *ciceri*. **Gayatri S. Gurjar,** Ashok P. Giri and Vidya S**.** Gupta. (communicated to European journal of plant pathology).
- Major biotic stresses of chickpea and strategies for their control. **Gurjar G**, Mishra M, Kotkar H, Upasani M, Soni P, Tamhane V, Kadoo N, Giri A and Gupta V. (Book chapter, In: Biology and management of pests and pathogens).

Other publications

- Identification and molecular characterization of virulence related factors in *Fusarium oxysporum* f.sp *ciceri.* **Gayatri S. Gurjar,** Maneesha P. Barve, Ashok P. Giri and Vidya S. Gupta. In the proceedings of **Fourth International Food Legumes Research Conference** held in Indian Agricultural Research Institute, New Delhi
- Molecular characterization of virulence related factors in *Fusarium oxysporum* f.sp *ciceri.* **Gayatri S. Gurjar,** Maneesha P. Barve, Ashok P. Giri and Vidya S. Gupta. In the proceedings of National seminar on: **Prospecting Fungal Diversity in Tropical India, its Conservation and Applications in Biotechnology**; conducted in Agharkar Research Institute, Pune

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