Insight into genomic architecture of *Fusarium oxysporum* f. sp. *ciceri* and *Ascochyta rabiei*, the two major fungal pathogens of chickpea (*Cicer arietinum* L.)

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BY

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CERTIFICATE

Certified that the work in this Ph.D. thesis entitled **'Insight into genomic architecture of *Fusarium oxysporum* f. sp. *ciceri* and *Ascochyta rabiei*, the two major fungal pathogens of chickpea (*Cicer arietinum* L.)'** submitted by **Ms. Maneesha P. Barve** was carried out by the candidate under my supervision. The material obtained from other sources has been duly acknowledged.

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

Research Advisor

DECLARATION

I hereby declare that the thesis entitled **'Insight into genomic architecture of *Fusarium oxysporum* f. sp. *ciceri* and *Ascochyta rabiei*, the two major fungal pathogens of chickpea (*Cicer arietinum* L.)'** submitted for Ph.D. degree at the University of Pune has not been submitted by me for a degree at any other university.

Date : (**Maneesha P. Barve)**

National Chemical Laboratory, Pune

Dedicated to my beloved parents…

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NCL	Dr. V. S. Gupta *(Research Advisor)*
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(Maneesha P. Barve)

List of Abbreviations

AFLP	amplified fragment length polymorphism
AMOVA	analysis of molecular variance
AR	*Ascochyta rabiei*
BME	beta-mercaptoethanol
bp	base pairs
$CHCl_3$	chloroform
cM	centimorgan
CTAB	hexadecyl-trimethyl-ammonium bromide
dATP	deoxyadenosine 5' triphosphate
dCTP	deoxycytidine 5' triphosphate
dGTP	deoxguanosine 5' triphosphate
dTTP	deoxythymidine 5' triphosphate
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. *ciceri*
h	hour
IAA	isoamyl alcohol
ISSR	Inter-simple sequence repeat
kb	kilo bases
µCi	microcurie
µg	microgram
µl	microliter
µM	micromolar

M	molar
Mb	megabase
min	minute
ml	milliliter
mM	millimolar
mt DNA	mitochondrial DNA
MYG	malt extract (0.3%), yeast extract (0.5%), glucose (1%) liquid medium
nit	nitrate non-utilizing
ng	nanograms
°C	degree centigrade
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDA	Potato dextrose agar
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
sec	second
SSC	sodium chloride sodium citrate
SSLP	simple sequence length polymorphism
SSPE	sodium chloride sodium dihydrogen phosphate EDTA

SSR	simple sequence repeat
STMS	sequence tagged microsatellite site
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
$T_{10}E_1$	Tris-EDTA buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0)
T_m	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
$(X_D)^n$ value	'Probability of identical match by chance'

Abbreviations of Institutes

IAC	ICRISAT Asia Center
ICARDA	International Center for Agricultural Research in Dry Areas
ICRISAT	International Crops Research Institute for the Semi-Arid Tropics
IMI	International Mycological Institute, U.K., formerly Commonwealth Mycological Institute (CMI)
MPKV	Mahatma Phule Krishi Vidyapeeth, Rahuri
NCL	National Chemical Laboratory
WSU	Washington State University, Pullman

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Thesis Abstract

Chickpea (*Cicer arietinum* L.) has been described as the world's third most important pulse crop, with India accounting for 75% of the world production. It is a major source of protein for the largely vegetarian Indian population. However, chickpea productivity is limited by various biotic and abiotic stresses. Economically important among the biotic stresses are fungal diseases and insect pests, which are the major yield reducers. Chickpea wilt, caused by *Fusarium oxysporum* Schl. emend. Snyd. and Hans. f. sp. *ciceri* (Padwick) and Ascochyta blight caused by *Ascochyta rabiei* (teleomorph *Didymella rabiei*) are the most important fungal diseases of chickpea all over the world.

Fusarium oxysporum f. sp. *ciceri* (*Foc*), the causal agent of chickpea wilt is both soil- and seed-borne and difficult to eradicate from the soil due to longevity of fungal chlamydospores. Pathogen genotypes can interact with specific host genotypes leading to 'breakdown of resistance' within very short periods of time (Brown, 1995). It is, therefore, desirable to determine the genetic variability existing within pathogen populations to breed chickpea cultivars with durable resistance and to formulate effective disease control strategies. Seven races of *Foc* have been reported from different chickpea growing areas of the world. In India, the races are geographically distinct, wherein race 1 is widespread in central and peninsular India and race 2 in northern India, while races 3 and 4 are restricted in their distribution. Subspecific division of *F. oxysporum* isolates of a given *forma speciales* is usually based on physiological race reactions to a set of differential cultivars. Although widely used, this method of race identification is time consuming, labour-intensive and sensitive to variations in the environment. Molecular markers can be useful in studying genetic diversity in the pathogen population and providing an assessment of pathogen variation for development of resistant varieties. If correlated with race, they can also be used for race diagnosis.

Ascochyta rabiei, the ascochyta blight pathogen, is a loculoascomycete fungus and affects all the above-ground parts of chickpea plants. The sexual stage of the fungus (*Didymella rabiei*) is thought to be important in disease epidemiology and in generating genetic diversity in the fungus. In ascomycete fungi, sexual reproduction is controlled by a single regulatory locus referred to as the mating type or *MAT* locus. Alternate alleles at this locus consist of completely dissimilar sequences referred to as 'idiomorphs'. The *MAT1-1* idiomorph encodes a DNA binding protein containing an alpha domain, while the *MAT1-2* idiomorph encodes a DNA binding protein containing a high mobility group (*HMG*) domain. Sexual reproduction requires the presence of both mating types in close proximity, and 1:1 ratio of mating types is expected in populations undergoing regular random mating (Milgroom, 1996). However, these ratios have been reported to be significantly different from 1:1 in several regions sampled so far. Such studies on distribution of mating types in population samples of *A. rabiei* from different parts of the world have largely relied on laboratory crosses with fertile mating-type tester strains on senescent chickpea stems under favourable environmental conditions. Performing such crosses is a tedious, time consuming and labour intensive process. A PCR assay for mating type would allow (i) rapid determination of mating type ratios in populations, (ii) the ability to track the introduction of a mating type into an area previously free of a particular mating type and (iii) the ability to make predictions about the mating system of *A. rabiei* (i.e. asexual versus sexual) populations.

In this context, I have addressed a few important aspects of genome analysis of these two fungal pathogens, and the specific outcomes of my study are presented in Chapter 2 and Chapter 3 of the present thesis.

Chapter 2 highlights the study of genetic diversity in Indian isolates of *Fusarium oxysporum* f. sp. *ciceri* (*Foc*), using multilocus hybridization-based, as well as polymerase chain reaction (PCR)-based fingerprinting approaches.

Section 2A: Potential of simple sequence repeat restriction fragment length polymorphism (SSR-RFLP) markers to distinguish four races of *Fusarium oxysporum* f. sp. *ciceri* prevalent in India.

In this section, I have examined the potential of SSR-RFLP markers to study genetic variability in four Indian races of *Foc*. Thirteen oligonucleotide probes complementary to microsatellite loci, in combination with 11 restriction enzymes, were used to generate hybridization profiles for the standard isolates of the four races. These hybridization patterns, which were dependent on both, the restriction enzyme and oligonucleotide probe used, revealed the presence of different repeat motifs in the *Foc* genome. Among the restriction enzymes used, hexa-cutting enzymes were more informative than tetra- and penta-cutting enzymes, whereas tetranucleotide and trinucleotide repeats yielded better hybridization patterns than dinucleotide repeats. Depending on the levels of polymorphism detected, I have identified $(AGT)_5$, $(ATC)_5$ and $(GATA)_4$ as the best fingerprinting probes for the Indian *Foc* races. The distribution of microsatellite repeats in the genome revealed the 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% while race3 was very distinct at a similarity value of 26.7%. This study demonstrates the potential of oligonucleotide probes for fingerprinting and studying variability in the *F. oxysporum* f. sp. *ciceri* races and represents a step towards the identification of potential race diagnostic markers.

The work described in this section has been published as a full-length paper in *Theoretical and Applied Genetics* (2001) 102:138–147.

Section 2B: Genetic diversity analysis of Indian isolates of *Foc* and other *Fusarium* spp. using PCR based ISSR and AFLP markers.

In this section, I have described the use of PCR based markers to study genetic diversity in Indian *Foc* isolates representative of different physiological races. I have used fourteen ISSR and fifteen AFLP markers to study genetic diversity and correlation with physiological race reactions in 31 Indian isolates of *Foc*, and two isolates of *F.*

solani and *F. udum*. Polymorphic and clear amplification patterns from 10 ISSR and 12 AFLP primer combinations, corresponding to 198 ISSR loci and 1009 AFLP loci, respectively, grouped the isolates of races 1, 2 and 4 into discrete clades corresponding to the physiological race reaction exhibited by the isolates within the clades. In my studies, race 3 and isolate Fu7 (a race 3 isolate) always grouped together and formed a distinct cluster from isolates of races 1, 2 and 4 with all the primers used, while the cluster of *Foc* race 1, 2 and 4 isolates always received strong bootstrap support with both ISSR and AFLP markers. The data obtained by ISSR and AFLP were highly congruent though theoretically different regions of the genome were analyzed with these marker systems. The ISSR and AFLP markers used in my studies could clearly differentiate isolates of *Foc* races 1, 2 and 4 from race 3 isolates and *F. solani* and *F. udum* (fungi of the wilt complex). Although, there are previous reports of differentiation of wilting pathotype from the yellowing pathotype in *Foc* isolates, this is the first study differentiating races of the highly virulent wilting pathotypes from India. In this section I have also analyzed individual ISSR and AFLP primers for their potential to differentiate the isolates of the four *Foc* races, wherein the ISSR primers UBC 834 and UBC 835 and AFLP primers AA-ca and AA-caa have been identified as potential candidates for development of race specific markers for races 1 and 2. However, race 4 isolates could not be differentiated by any of the primers used by me. Hence, more and different markers need to be identified for differentiating isolates of race 4.

The work described in this section has been communicated to *Phytopathology* as a full-length paper.

Chapter 3 deals with genetic diversity analysis and mating type locus identification from the chickpea blight pathogen *Ascochyta rabiei.*

Section 3A: Genetic diversity analysis of a worldwide collection of *Ascochyta rabiei* isolates using STMS markers.

In recent years microsatellites have emerged as powerful tools for genome analysis in many organisms including plant pathogenic fungi due to high levels of polymorphism

and the relative ease of scoring. They have found application in areas varying from genome mapping to population genetics. Compound microsatellite loci contain stretches of two or more different repeats, and may constitute up to about 10% of the microsatellite repeats. Alleles at such loci can vary in length at any of the repeats constituting the locus. *ARMS*1, a previously characterized compound microsatellite locus containing penta- and decameric repeat units has been reported to reveal genetic diversity in *Ascochyta rabiei* (Pass.) Labr. isolates (Geistlinger *et al*., 1997b). In this section, I have examined thirty-seven isolates of *Ascochyta rabiei* collected from different states of India and thirty-eight isolates from various countries in the world for their diversity at this locus. In my studies, twenty-six alleles on the basis of size (228 bp to 451 bp) were detected in the world isolates examined, while fifteen alleles (287 bp to 418 bp) were observed in isolates from the Indian subcontinent alone. Size of the alleles was not correlated to mating type or to geographic region. To the best of my knowledge, this study is the first to demonstrate diversity in representative *Ascochyta rabiei* isolates from different parts of the world at the *ARMS*1 locus.

Section 3B: Cloning and characterization of the mating type locus (*MAT*) of *Ascochyta rabiei* using PCR based approaches.

In this section, I have described cloning and characterization of the mating type (*MAT*) locus of *Ascochyta rabiei* (teleomorph: *Didymella rabiei*) using a combination of TAIL-PCR and inverse PCR. In this study, degenerate primers designed from the high mobility group (HMG) motif of *Cochliobolus heterostrophus*, *C. sativus* and *Alternaria alternata MAT1-2* genes were used as a starting point to amplify HMG from *A. rabiei*. I have used these gene sequences to design *MAT*-specific PCR primers for use in a rapid and convenient multiplex PCR assay in which I have demonstrated that *MAT*-specific PCR amplicons correlate perfectly to mating phenotype of (i) single ascospore progeny from a *MAT1-1* x *MAT1-2* genetic cross and of (ii) field-collected isolates from diverse geographic locations. In this section, a phylogeny has been estimated among legume-associated *Ascochyta* spp. and related loculoascocmycete fungi using sequence data from the nuclear ribosomal internal

transcribed spacer (ITS) and the HMG region. While the nuclear ribosomal internal transcribed spacer (ITS) demonstrated the monophyly of *Ascochyta*/*Didymella* spp. associated with legumes, it was insufficiently variable to differentiate isolates associated with different legume hosts. In contrast, data from the HMG region of *MAT1-2* were substantially more variable, revealing seven well-supported clades that correlated to host of isolation. *A. rabiei* on chickpea is phylogenetically distant from other legume-associated *Ascochyta* spp. and the specific status of *A. rabiei*, *A. lentis, A. pisi* and *A. fabae* was confirmed by the HMG phylogeny. The multiplex PCR assay described herein would help rapid and reliable detection of mating type in large samples of *Ascochyta rabiei* isolates and may also aid the differentiation of *A. rabiei* from closely related *Ascochyta* species.

The work described in this section has been published as a full-length paper in *Fungal Genetics and Biology* (in press).

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Chapter 1: Review of Literature

Two economically important fungal pathogens of chickpea- *Fusarium oxysporum* f. sp. *ciceri* and *Ascochyta rabiei*

1A Chickpea

1A.1 Chickpea: An important pulse crop

Of the four main edible cool season food legumes, faba bean (*Vicia faba* L.), chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris* L.) and dry pea (*Pisum sativum* L.); chickpea and lentil are predominantly produced in developing countries, and dry pea in developed countries, with faba bean being more evenly distributed (Oram and Agcaoili, 1994). Chickpea is grown in at least 33 countries in Central and West Asia, South Europe, North Africa, North and South America and Australia (Ladizinsky and Alder, 1976; Singh, 1997a, b), and is the third most important grain legume globally, after common bean and pea. Chickpea covers 15% (10.2 million ha) of the area and accounts for 14% (7.9 million tonnes) of the world's pulse production. Cultivation of chickpea has recently become important in certain developed countries like the USA (Wiese *et al.*, 1991), Canada (Armstrong *et al.*, 2001), and Australia (Clarke and Siddique, 2003). India is the largest chickpea producer accounting for about 73% of the world's share (Jodha and Subba Rao, 1987). In India, the *desi* type of chickpea with small brown seeds accounts for nearly 90% of the area under cultivation, while the *kabuli* type with bold and cream coloured seeds are grown in rest of the area.

Chickpea is a hardy, deep-rooted, dryland crop grown on marginal lands, which can grow to full maturity in conditions that would be unsuitable for most crops (Singh and Reddy, 1991). Being a legume, the plant produces nitrogen-fixing nodules, which can enrich the soil with at least 50 kg of N/ha every season. Chickpea root exudates, rich in citric acid, dissolve calcium phosphates and help in mobilization of phosphorous to readily usable forms. Chickpea seed is a good source of carbohydrates and proteins, which together constitute 80% of the total dry seed weight and is an important source of

protein for the largely vegetarian Indian population. The crude protein content of chickpea varies from 17% to 24%, containing essential amino acids like tryptophan, methionine and cysteine (Williams and Singh, 1987).

1A.2 Biotic and abiotic stresses

Thought the yield potential of present day chickpea cultivars exceeds 5.0 t/ha, the average yield is only about 0.8 t/ha. The wide gap between the average yield and potential yield is mostly due to diseases, pests and poor management practices. Losses in chickpea yield can be attributed to biotic and abiotic stresses as depicted in Fig. 1.1 (Singh *et al.*, 1994). 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 (Nageshwara Rao *et al.*, 1985a and 1985b). 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.*, 1984b). 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).

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). There has been an increase in different chickpea pathogens like fungi, bacteria and viruses over a period of past 17 years. 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

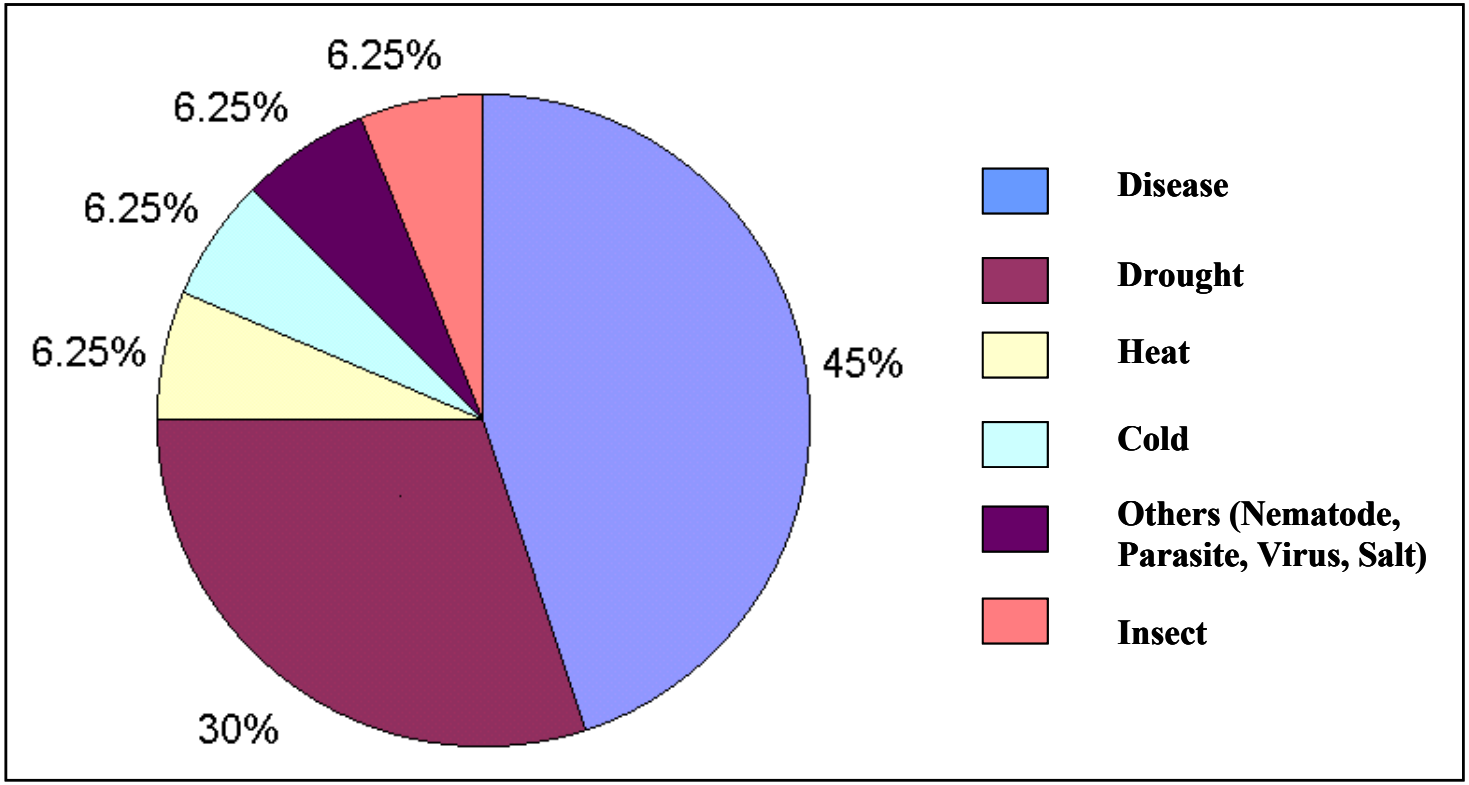


Fig. 1.1 Relative importance of abiotic and biotic stresses affecting chickpea crop (Singh *et al*., 1994).

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 gray mould, of which wilt and blight are the most devastating diseases affecting chickpea in tropical and temperate regions, respectively. Other diseases of local and minor importance caused by fungi, viruses and nematodes are listed in Tables 1.1, 1.2 and 1.3. In the following sections, I have attempted to give an account of the available literature on these two economically important fungal pathogens of chickpea, namely *Fusarium oxysporum* f. sp. *ciceri* (*Foc*) and *Ascochyta rabiei*.

1B *Fusarium oxysporum* f. sp. *ciceri*- the chickpea wilt pathogen

1B.1 Historical account

Chickpea wilt was first reported by Butler in 1918. McKerral (1923) considered the disease to be soil borne and the soil samples analyzed yielded *Fusarium* spp. Narsimhan (1929) and Dastur (1935) reported an association of *Fusarium* spp. and *Macrophomina phaseolina* (Tassi) Goid., with wilted plants. However, the latter could not prove pathogenicity of the isolated *Fusarium* spp. and concluded that wilt was due to abiotic factors (Dastur, 1935). In a detailed account, Prasad and Padwick (1939) isolated and classified 300 *Fusarium* isolates into non-pathogenic types, wilt causing types and seed rotting types, and reported *Fusarium* spp. to be the causal agent of chickpea wilt. Padwick (1940) named the fungus *Fusarium orthoceras* Appel and Wollenw. var. *ciceri*, while Erwin (1958) proposed the name *Fusarium lateritium* (Nees) Snyd. and Hans. f. sp. *ciceri* (Padw.) Erwin. Following the classification of Snyder and Hansen (1940), Chattopadhyay and Sen Gupta (1967) renamed the pathogen as *Fusarium oxysporum* Schl. f. sp. *ciceri* (Padw.) Snyder and Hansen. This was accepted as the correct name of the pathogen (Booth, 1971) and was revised to *F. oxysporum* Schl. f. sp. *ciceris* (Padw.) Matuo and Sato (Holliday, 1980). Investigations at ICRISAT revealed that what was earlier referred to as the 'wilt complex', was actually a number

Table 1.1

Table 1.1 Fungal diseases of chickpea of minor or local importance

Disease	Causal fungus	Distribution	Importance
Stem rots, root rots and Wilts			
Stem anthracnose	*Colletotrichum capsici* (Syd.) Butl. & Bisby	India	Occasional, minor
Stem rot, White mould	*Sclerotinia sclerotiorum* (Lib.) de Bary	Iran, India, Pakistan, Australia, Chile	Locally important, especially under cool wet conditions
Foot rot	*Operculella padwickii* Kheswalla	North India	Locally important
Rhizoctonia root rot	*Rhizoctonia solani* Kühn	Widespread	Minor
Root rot	*Phytophthora megasperma* Drechs.	India	Minor
Neocosmospora root rot	*Neocosmopora vasinfecta* Smith	India	Minor
Root rot	*Thielaviospsis basicola* (Berk. & Br.) Ferr.	USA	Minor
Acrophialophora wilt	*Acrophialophora fusispora* (Saksena) Samson	India	Minor
Verticillium wilt	*Verticillium dahliae* Kleb.	USA	Minor
Foliar diseases			
Phoma blight	*Phoma medicaginis* Malbr. & Roum.	Australia, India, Bangladesh, USA	Minor
Stemphylium blight	*Stemphylium sarciniforme* (Cav.) Wilts	India, Iran, Syria	Minor?
Alternaria blight	*Alternaria alternata* (Fr.) Keissler	India, Bangladesh, Nepal	Minor
Powdery mildew	*Leveillula taurica* (Lév.) Arn.	India, Ethiopia, Sudan	Minor
Rust	*Uromyces ciceris-arietini* Jacz. apud Boy. & Jacz.	Widespread in Mediterranean region, SE Europe and Asia: present also in E. Africa and Mexico	Locally important: regarded as the major factor affecting chickpea production in Central Mexico

Source: M. P. Haware, 1998

Table 1.2 Virus diseases of chickpea of minor or local importance

Disease	Casual virus	Distribution	Importance
Yellow mosaic, yellowing	Bean yellow mosaic potyvirus (BYMV)	USA, Iran, India	Locally important; yield loss estimated at 77-92% in Iran
Yellow mosaic	Beet western yellows luteovirus (BWYV)	Australia, India, Spain, Syria, USA	Minor
Mosaic, bud, necrosis, wilt	Alfalfa mosaic virus (AMV)	Widespread	Locally important in Iran where yield losses of 22-96% have been recorded
Mosaic	Cucumber mosaic cucumovirus (CMV)	Widespread	Locally important in Iran where yield losses of 52% have been recorded
Enation mosaic	Pea enation mosaic mosaic virus (PEMV)	USA, Italy	Minor?
Necrotic yellows	Lettuce necrotic yellows rhabdovirus (LNYV)	Australia	Potentially important in northern New South Wales and southern Queensland

Source: M. P. Haware, 1998

Table 1.3 Nematode diseases of chickpea of minor or local importance

Disease	Casual virus	Distribution	Importance
Root-Knot	*Meloidogyne artiellia* Franklin	India, Spain, Syria	Potentially serious in spring-sown crops
	M. incognita (Kofoid & White) Chitwood	Bangladesh, India, Nepal, Pakistan	Locally important
	M. javanica (Treub.) Chitwood	Widespread	Locally important
Cyst	*Heterodera ciceri volvas*, Greco & Dvito	Jordan, Lebanon, Syria	Severe damage in Syria
Root lesion	*H. rosii* Duggen & Brennan	Syria	
	Pratylenchus brachyurus (Godfrey) Goodey	Australia, Brazil	Potentially serious
	P. thornei Sher. & Allen	Australia, India, Myanmar, Syria	Locally important
Decline	*Rotylenchulus reniformis* Lindford & Oliveira	Ghana, India	Minor

Source: M. P. Haware, 1998

of distinct diagnosable diseases (Nene *et al.* 1978) wherein, fusarium wilt (*Foc*) and black root rot (*Fusarium solani*) are the main components.

1B.2 The genus *Fusarium*

Traditional classification and identification schemes for *Fusarium* are exclusively based on a morphological species concept derived from cultural characteristics of single-spore isolates grown on special media, shared morphological trait of the anamorph, host range, and to a lesser extent, teleomorph micromorphology (Booth, 1971). Due to the conflicting morphological species concepts employed in taxonomic treatments of this genus (Booth, 1971; Gerlach and Nirenberg, 1982; Nelson *et al.* 1983), the systematics of *Fusarium* remains controversial and confusing (Gams and Nirenberg, 1989), especially if more than one taxonomic treatment is consulted. Gerlach and Nirenberg's system (1982) is the most differentiated, including 73 species and 26 varieties; while 44 species and 7 varieties have been recognized by Booth (1971) and, 30 species by Nelson *et al.* (1983). Molecular systematics, based on discrete DNA sequence data, on the other hand, offers an objective, phylogenetically based system of classification for *Fusarium* and its teleomorphs (Bruns *et al.* 1991). Specific regions of the genome from a large number of taxa can be sequenced conveniently due to the advent of polymerase chain reaction (PCR) methodology (Mullis and Faloona, 1987) and non-radioactive, automated DNA sequencing. Previous investigations of *Fusarium* species by cladistic analysis of DNA sequences from multiple unlinked loci have revealed the utility of gene phylogenies inferred from mitochondrial small subunit (mtSSU) rDNA, nuclear 28S rDNA, β-tubulin gene and nuclear translation elongation factor 1α (Baayen *et al.*, 2000; O'Donnell *et al.* 1998b), however, nuclear rDNA ITS gene tree was found to be composed of non-orthologous sequences (O'Donnell and Cijelnik, 1997).

Fusarium is a large cosmopolitan genus of pleoanamorphic hyphomycetes whose members are responsible for a wide range of plant diseases (Farr *et al.*, 1989), mycotoxicoses and mycotic infections of humans and other animals (Nelson *et al.*, 1994). In addition to producing a wide range of toxins including tricothecenes and

fumosins, the fusaria are noted for their production of other secondary metabolites such as gibberellin plant growth hormones, named after *Gibberella fujikuroi* from which they were first discovered. The species *Fusarium oxysporum* is well represented among the soil borne fungi, in every type of soil all over the world (Burgess, 1981) and is considered to be a normal constituent of the rhizosphere of plants (Appel and Gordon, 1994). However, some strains of *Fusarium oxysporum* are pathogenic to different plant species; they penetrate into the roots and provoke either root rots or tracheomycosis when they invade the vascular system, causing severe damage on many plant species of economic importance. The vascular wilt causing forma speciales of *Fusarium oxysporum* typically invade only living root tissues, tend to be specialized, are host specific, and suppressed by saprophytes (Hillocks, 2001). Based on the plant species and plant cultivars infected, they are classified into more than 120 *forma speciales* and races (Armstrong and Armstrong, 1981). The presently accepted classification for the fusarium wilt pathogen *Fusarium oxysporum* f. sp. *ciceri* is: Form -Class: Fungi Imperfecti, Form -order: Moniliales, Form-family: Tuberculariaceae, Form-genus: *Fusarium*, Form species: *oxysporum,* forma specialis *ciceri*.

1B.3 Fusarium wilt of chickpea

1B.3.1 Prevalence and yield losses

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). *Foc* is internally seed borne and the fungus 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 fungal 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). Figure 1.2 represents plants in a wilt sick field as compared to a healthy chickpea field. 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 (P. Stevenson, ICRISAT, India, 1995, personal communication). Among other legumes, lentil, pea and pigeonpea are symptomless carriers of the chickpea wilt fungus (Haware and Nene, 1982a).

Assessments of crop losses caused by wilt have mainly been made from field estimates based on percentage incidence of the disease. In India, an annual loss of 10% has been reported (Singh and Dahiya, 1973), while in Spain annual losses of 12-15% due to both wilt and root rots have been estimated (Trapero-Casás and Jiménez-Díaz, 1985). An annual loss of US$ 1 million was reported from Pakistan in 1953 (Sattar *et al.*, 1953). In the drier areas of North Africa, wilt of chickpea is a serious disease and is especially common in Tunisia (Haware *et al.*, 1990). At ICRISAT, an attempt was made to estimate the yield losses on a single plant basis. Early wilting caused 77-94% yield loss while late wilting caused 24-65% loss (Haware and Nene, 1980).

1B.3.2 Epidemiology of wilt

The epidemiology of root-infecting fungi in the soil is complex, and factors such as inoculum density, pathotype, plant age, host resistance and its genetic potential, air and soil temperature, soil moisture, soil nutrients and plant density may affect wilt development (Haware *et al.*, 1990). Wilt severity and populations of *Foc* have been reported to increase with decreasing soil-matrix potential (Bhatti and Kraft, 1992). Disease intensity was reported to increase with the lowering of pH, being considerably low at pH 9.2 (high pH) (Gupta *et al.*, 1986).s Also, pot tests conducted at 15-35°C have revealed maximum disease incidence at 25°C and no disease incidence at 15°C.

1B.3.3 Symptoms

Affected seedlings show a dull green color of the foliage; sudden drooping of the petioles, rachis and leaves. The plants, when uprooted, may show uneven shrinkage at the collar (Fig. 1.3A and B) (Nene *et al.*, 1978). There is no external rotting of roots and pith (Nene *et al.*, 1978), however, when the roots are split vertically, internal discoloration may be seen extending to the stem, due to infection of the xylem tissues of the root and stem as can be seen in Figure 1.3C. Transverse sections of the infected

Fig. 1.2 (A) Healthy chickpea field (B) Fusarium wilt infected plants in a chickpea field (Source Dr. F. J. Muehlbauer, W. S. U)

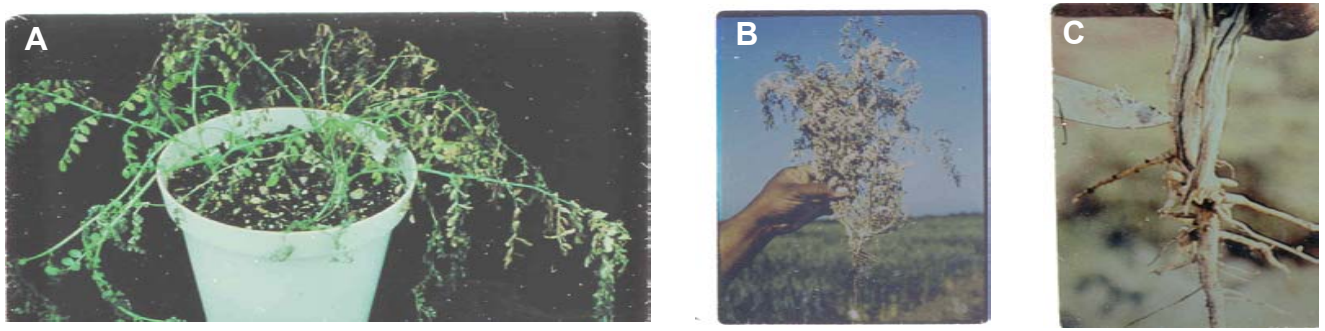


Fig. 1.3 (A) Dull green color of the foliage in a wilting chickpea plant (B) Single wilted plant
(C) Internal discoloration due to infection of the root xylem tissues when the roots are split vertically
(Source- Dr. M. P. Haware, ICRISAT)

roots examined under the microscope show the presence of hyphae and spores of the fungus in the xylem (Nene *et al.*, 1978), thereby confirming the diagnosis of vascular wilt (Fig. 1.4A and B). Seed harvested from the late wilted plants is lighter and duller than that harvested from healthy plants (Fig. 1.5). Chickpea genotypes show different rates of symptom expression after *Foc* infection and can be classified in early and late wilting categories on the basis of days to wilting from sowing (Haware and Nene, 1980). Wilt can be observed in a susceptible cultivar within 25 days after sowing in infected soil and this is known as 'early wilt' (Haware and Nene, 1980). 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 may also occur during reproductive growth and is known as 'late wilt'. Plants grown from infected seed wilt faster than the plants grown from clean seed.

1B.4 Life cycle of vascular wilt pathogens

Understanding of the life cycle of wilt pathogens in relation to their survival, the causation of disease within a framework of time and space, host-parasite interactions with respect to disease resistance or susceptibility is very important. In a comprehensive review addressing these topics, Beckman and Roberts proposed a model to explain the interactions between vascular wilt causing pathogens and their host plants, wherein, the pathogens have distinct saprophytic and parasitic phases in their life cycles (Beckman and Roberts, 1995). Figure 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 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

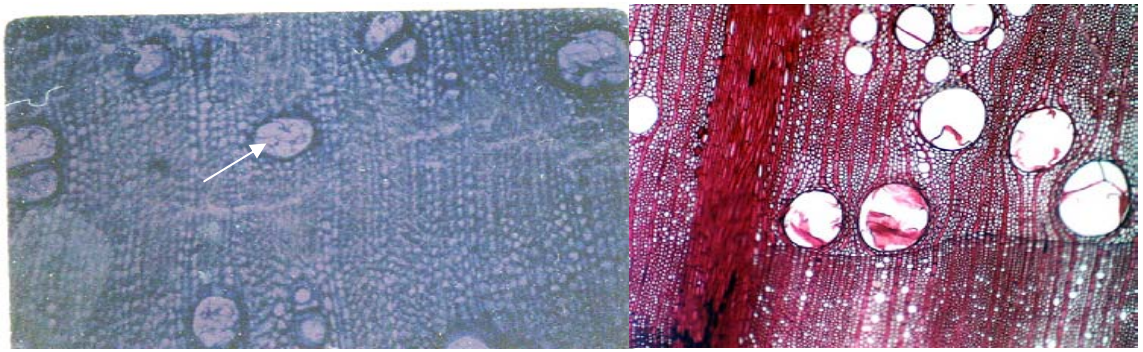


Fig. 1.4 (A) Transverse section (T. S.) of an infected chickpea root examined under the microscope showing the presence of hyphae of the fungus in the xylem (B) T. S. of a wilted plant showing presence of fungal hyphae in the xylem. (Source- Dr. M. P. Haware and downloaded from the Internet)

Fig. 1.5 Seed harvested from the late wilted plants is lighter and duller than that harvested from healthy plants (Source-ICRISAT information bull. No. 34)

response to pathogen invasion is offered mainly at two places (i) 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. However, there is still a debate about the role of fungal toxins in vascular wilt diseases. *Fusarium oxysporum* is known to produce the toxin fusaric acid in culture filtrates, but most of the disease symptoms seem to be caused by the plant response to infection.

1B.5 Description of the fungus and fungal structures

The fungus grows on potato sucrose agar at 25°C and appears as delicate, white and cottony growth becoming felted and wrinkled in older cultures (Nelson *et al.*, 1983) Figure 1.7 is a photograph of ten-day old cultures of four Indian *Foc* races incubated at 25°C on laboratory media. 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 25-65 μm. Chlamydospores, formed in 15-day-old cultures are smooth or rough walled, terminal or intercalary, and may form singly, in pairs, or in chains. Figures 1.8, 1.9, 1.10 represent photographs of micro- and macrocondia and *Foc* chlamydospores, respectively.

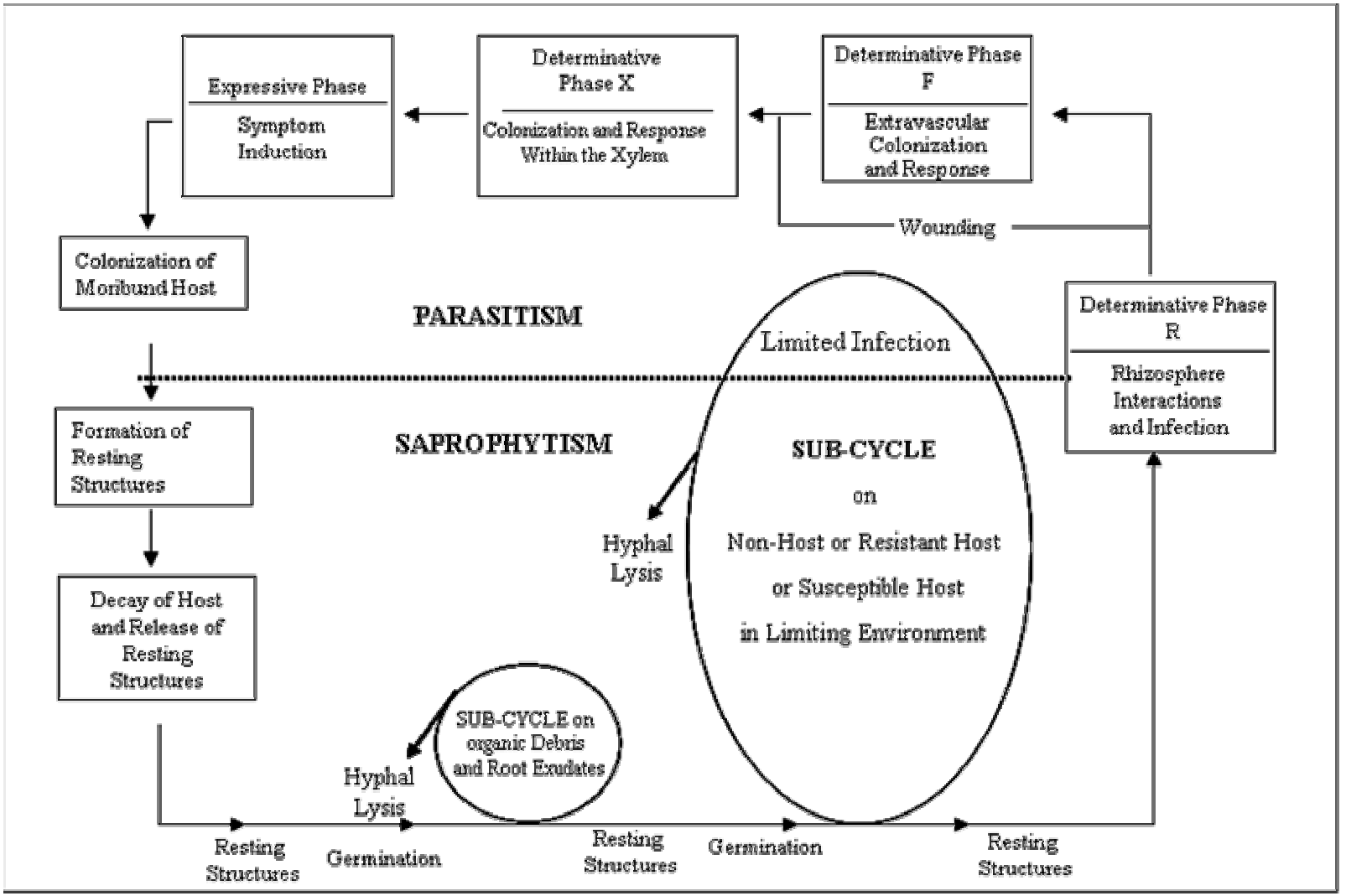


Fig. 1.6 Life cycle of soil-borne, wilt causing fungi including their saprophytic and parasitic growth and successive phases of colonization and pathogenesis (Beckman and Roberts, 1995)

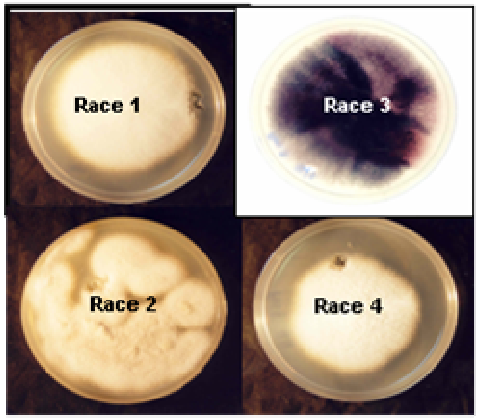


Fig. 1.7 Growth patterns of four Indian *Foc* races on laboratory media.

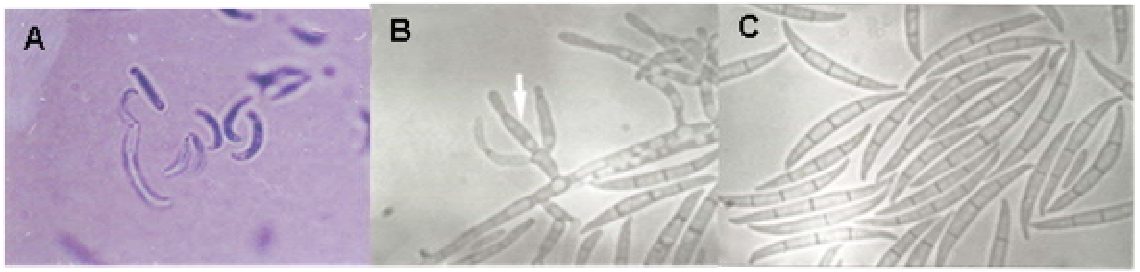


Fig. 1.9 (A) *Foc* macroconidia (Source-Dr. M.P. Haware) (B) *Foc* macroconidia on microconidiophores (arrowed), x1130 (C) macroconidia, x1130 (Source- ICRISAT information bull. No. 34)

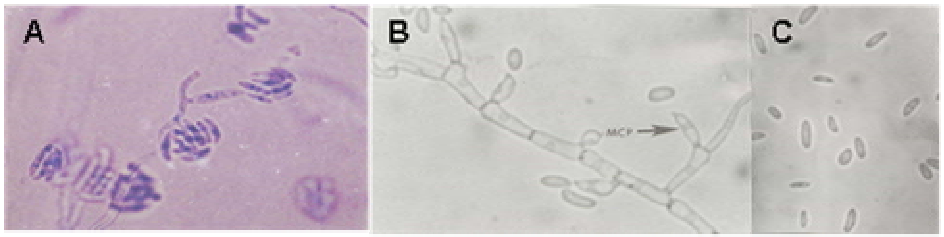


Fig. 1.8 (A) *Foc* microconidia (Source-Dr. M.P. Haware) (B) *Foc* microconidia on microconidiophores (MCP), x1130 (C) micro-conidia x1130 (Source ICRISAT information bull. No. 34)

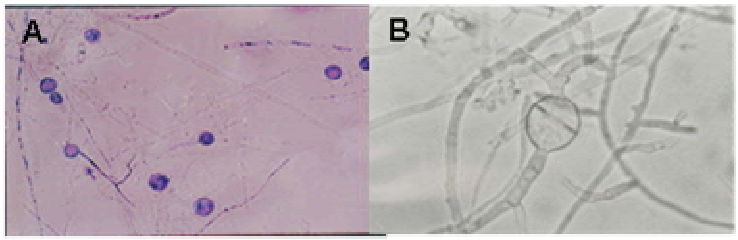


Fig. 1.10 (A) *Foc* chlamydospores (Dr. M.P. Haware) (B) *Foc* chlamydospores (arrowed), x1130 Source ICRISAT information bull. No. 34

A temperature of 25°C and pH 6.0 are optimum growth conditions for *Foc*. Most experiments on pathogenic variability require the use of single spore isolates of the wilt fungus. Pure cultures are routinely obtained from wilted chickpea plant specimens following surface sterilization with a 2.5% sodium hypochlorite solution. After pathogenicity of the isolated fungus is tested by root dip inoculation of seven-day-old chickpea seedlings of a susceptible variety, for example JG 62, seedlings with typical wilt symptoms are used for re-isolation of the fungus from single germinating spores on 2% water agar medium using the method of Tousson and Nelson (1976). Single spore cultures thus obtained can be maintained by routinely subculturing on PDA plates and stored temporarily at 4°C, however, soil stocks are preferred for long term storage of *Fusaria*.

1B.6 Race concept in *Foc*

Race is a term commonly used in biology to denote a group of individuals possessing common features that distinguish them from other groups of the same kind within formally recognized species or sub-species. Physiological race is an accepted term for a sub-specific group of parasites characterized by specialization to different cultivars of one host (Johnson and Booth, 1983). Pathogen populations are characterized primarily by virulence analysis on cultivars carrying differential resistance genes. The potential number of races that can be recognized is, therefore, determined by the number of different sources of resistance included in the differential host and the precise grouping of isolates into races is completely dependent upon the set of differentials. Pathogenicity, on the other hand, is defined as the capacity of the fungus to cause disease; however, the environment, inoculation technique, and subjective judgment of the scientist can change the disease score.

Pathogenic variability in *Foc* has been reported and seven races have been identified based on their differential reactions to chickpea cultivars (Haware *et al.*, 1990, Table 1.4).

Table 1.4 Reaction of ten differential lines of chickpea to different races* of *Fusarium oxysporum* f. sp. *ciceri*

Line	Race 0	Race 1	Race 2	Race 3	Race 4	Race 5	Race 6
JG 62	R[2]	S	S	M	S	S	S
C 104	M	S	S	R	S	S	S/M
JG 74	R	R	M	R	R	S	R
CPS 1	R	R	S	S	M	S	R
BG 212	R	R	S	M	M	R	R
WR 315	R	R	R	M	R	R	R
Annigeri	-	S	S	S	S	-	R
Chafa	-	S	S	M	S	-	R
L 550	-	S	S	M	S	-	S/M
850-3/27	-	S	M	M	M	-	R

[2]R = Resistant (0-20% mortality); M = Moderately Susceptible (21-50% mortality); S = Susceptible (>50% mortality)

Source: M. P. Haware, 1998

Figure 1.11 depicts the classical method of race identification by inoculating ten differential chickpea cultivars with inoculum from a single spore culture of *Foc*. Races 1, 2, 3 and 4 were first described in India by Haware and Nene (1982c), races 0 and 5 in Spain by Jiménez Díaz *et al.* (1989, 1991, 1993) and race 6 from California by Phillips (Phillips, 1988). However, some workers have recognized eight races, namely, 0, 1B/C, 1A, 2 through 6, and the yellowing and the wilting pathotypes in *Foc* (Jiménez-Díaz *et al.*, 1993; Kelly *et al.*, 1994). According to them, isolates of races 0 and 1B/C cause the yellowing syndrome, while isolates of races 1A, 2 though 6 cause the wilt syndrome (Jiménez-Diaz *et al.*, 1993; Kelly *et al.*, 1994). Races 1, 2, 3 and 4 are generally geographically distinct in India, and only race 1, and to a lesser extent, race 2 are widespread and appear to be more virulent than others (Haware and Nene, 1982c). In India, race 1 is present in the central and peninsular region, and race 2 is reported from northern region, while races 3 and 4 are location specific to some northern states of India. Race 1 was originally isolated from Hyderabad (A. P.), race 2 from Kanpur

(U.P.), and races 3 and 4 from Gurdaspur (Punjab) and Hissar (Haryana), respectively. Races 2, 3 and 4 have not been reported from countries besides India, while races 0, 1A, 1B/C, 5 and 6 have been found in California (USA) and Spain; races 0 and 1B/C in Syria, Tunisia and Turkey; races 0, 1A, and 6 in Israel; races 1A and 6 in Morocco; and race 0 in Lebanon (Jiménez-Gasco *et al.*, 2001).

1B.7 Variability studies in *Foc* races

The classical method of race identification (Haware and Nene, 1982c) requires at least forty days for complete analysis, is labour intensive and sensitive to variations in the environment. Several workers have investigated the potential of alternative biochemical and molecular methods to differentiate the *Foc* races. Serological and electrophoretic variability has been investigated in the four Indian physiological races of *Foc* by Desai *et al.* (1992b). These races have shown close antigenic relationships and one prominent cross-reaction band. Furthermore, race 4 has been shown to lack most of the higher molecular weight protein bands (approx. 63kD) present in races 1, 2 and 3. In another study, investigations on biochemical variability of the four Indian *Foc* races have revealed that race 3 was 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). Following a different approach, Nogales-Moncada *et al.* (1993) have reported that all the seven races of *Foc* are represented by a single vegetative compatibility group. DNA based molecular markers have also been used for variability analysis in *Foc*. Variability studies in seven representative isolates of the seven reported *Foc* races, using mitochondrial DNA RFLPs, have revealed identical restriction patterns (Pérez-Artés *et al.*, 1995). In another study, Kelly *et al.* (1994) have used RAPD primers based on known ribosomal DNA sequences or sequencing primers to characterize and differentiate the yellowing and wilting pathotypes of *Foc*. The results from these studies have also been used for *in-planta* (Kelly *et al.*, 1998) and in-soil (García-Pedrajas *et al.*, 1999) detection of the wilt inducing pathotype of *Foc*. In another study using RAPD primers, Jiménez-Gasco *et al.* (2001) have concluded that RAPD primers can be used to characterize *Foc* races 0, 1B/C, 5 and 6, wherein, the isolates were subdivided such that the yellowing isolates grouped into two clusters

corresponding to races 0, 1B/1C and the wilt isolates formed a third cluster including races 1A, 2, 3, 4, 5 and 6. In addition to variability studies, phylogenetic analysis in *Foc* has been carried out in another study based on sequence data from conserved regions of EF-1α in seventeen *Foc* isolates, wherein, Jiménez-Gasco *et al.* (2002) have arrived at a conclusion of monophyletic origin of this *forma speciales.*

1B.8 Disease management

1B.8.1 Cultural practices

The chickpea wilt pathogen is monocyclic and the increase in the *Foc* population in soil is because of the longevity of reproductive units and the difficulty in eliminating them from wilt sick fields (Haware *et al.*, 1986, Haware *et al.*, 1996). Chickpea wilt has been reported to increase with increased levels of soil inoculum, high initial levels of *Foc* propagules causing 100% wilting much earlier than low initial levels of *Foc* propagules (Bhatti and Kraft, 1992). Disease potential can, therefore, be assessed from the knowledge of the initial pathogen population for early forecast of severity of diseases induced by soil-borne pathogens (Fry, 1982).

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

1B.8.2 Biological control

Soils harbor large populations of non-pathogenic *Fusarium oxysporum*, which play an important role in soil microbial ecology, especially in soils suppressive to fusarium wilt (Alabouvette *et al.*, 1993; Larkin *et al.*, 1996; Paulitz *et al.*, 1987; Smith and Snyder, 1971). Currently there is a lot of interest in studying genetic diversity in non-pathogenic strains of *F. oxysporum*, the interactions between pathogenic and the non-pathogenic strains, and the mechanisms of soil suppressiveness to fusarium wilt by other fungi sharing the same ecological niche. Such interactions form the basis of the strategy for biological control, although no clear examples for *Foc* races are available. Previous studies have revealed that incidence and severity of fusarium wilt of chickpeas are influenced by multiple factors like virulence and inoculum density of the pathogen, cultivar susceptibility and their interactions (Hervás *et al.*, 1997; Navas-Cortés *et al.* 2000). The degree of disease suppression by antagonistic bacteria varies with the strain used, the chickpea cultivar under consideration, as well as with the race and inoculum concentration of *Foc* (Landa *et al.*, 1997). The potential of the chickpea rhizosphere environment has also been explored as a source of microbial strains as potential biocontrol agents for use in the same environment, wherein 32% of 74 bacterial strains isolated were found to be effective (Landa *et al.*, 2001). The progress made in characterizing the diversity in populations of *Fusarium oxysporum* has provided tools for assessing the effects of the abiotic soil characteristics, cultural practices and plant species and cultivars on the establishment and activity of different strains of non-pathogenic *Fusarium oxysporum* in soil and in the rhizosphere of cultivated plants. It may be possible to modify microbial communities through environment friendly techniques to control fusarium diseases of cultivated plants.

In addition to interactions with fungi, *Foc* can interact with nematodes in soil, among which, the most damaging root-knot nematodes are *Melidogyne spp*. in the warmer regions. *Foc* and *M. javanica* occur together in many chickpea growing regions, and the wilt susceptible cultivars have been reported to die earlier from wilt when co-infected with *M. javanica* (Sharma *et al*., 1992). Non-uniformity of host

response to co-infection by these two pathogens has been reported in that, *M. javanica* increased the susceptibility of some but not all of the fusarium wilt resistant chickpea genotypes (Maheshwari *et al*., 1995).

1B.8.3 Resistant cultivars

Due to the difficulty of widespread application of available cultural and chemical control measures for wilt, especially for the resource poor farmers and the limitations associated with the use of biocontrol methods, considerable emphasis has been placed on the development of resistant cultivars (Haware *et al.*, 1992b; Nene and Haware, 1980).

1B.8.3.1 Screening for resistant lines

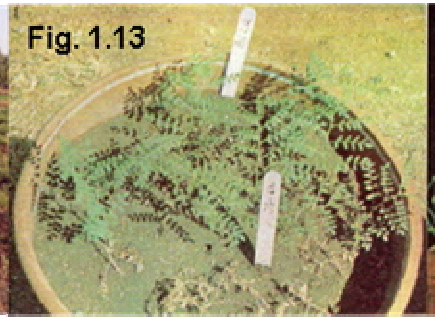
Effective field and laboratory procedures are available to screen for resistant lines while developing a wilt resistant variety (Nene *et al.*, 1981; Nene and Reddy, 1987). Uniform disease nurseries or wilt sick plots can be developed by incorporating chopped wilted plants in the soil by growing susceptible cultivars (ICRISAT information bull. 10). Susceptible check rows planted in the fields ensure (i) uniformity of the inoculum and (ii) maintain a sick plot with *Foc* as the predominant pathogen, and must show more than 90% wilt for the results of field screening to be considered reliable. Figure 1.12 is a photograph of wilt sick plot to screen for wilt resistant chickpea lines at MPKV, Rahuri. However, the presence of other soil-borne pathogens cannot be totally avoided and to verify the results of field screening, pot screening of promising lines needs to be performed in sterile sand-maize medium inoculated with pure cultures of *Foc* as described previously (ICRISAT information bull. 10). Screening in pots ensures homogeneity of the inoculum, and requires up to only 60 days at any time of the year as compared to 4-6 months of the season in a sick plot. Figure 1.13 is a photograph of pot screening for wilt resistant chickpea lines. In comparison to these two techniques, the water culture technique is particularly well suited for race studies of *Foc*. This technique involves inoculation of ten-day-old chickpea seedlings with a spore suspension of a pure culture of *Foc* ensuring a concentration of $6.5x10^5$ spores/ml, and

the data are recorded within 15 days of inoculation (ICRISAT information bull. 10). Figure 1.14 is a picture representing the water culture technique for studying susceptibility of different chickpea varieties to *Foc* races.

1B.8.3.2 Development of wilt resistant lines and deployment of resistant varieties

At ICRISAT center, India, more than 13,500 chickpea germplasm accessions from 40 countries have been evaluated for their resistance to race 1 of *Foc*, and over 160 wilt resistant sources have been identified (Singh *et al.*, 1987a, b; Haware *et al.*, 1990; 1992b). Many of these accessions were obtained from India and Iran, and were *desi* types as resistance to wilt is less common in *kabuli* types (Singh *et al.*, 1987a, b; Haware *et al.*, 1992b). Despite the existence of geographically isolated races of the pathogen, high levels of resistance, operational over wide areas, have been identified (Haware and Nene, 1982b, c) and wilt resistance genes have been incorporated into high yielding *desi* and *kabuli* backgrounds (Kumar *et al.*, 1985). Many lines with resistance to *Foc* races 1 and 2 have been developed, such as ICCV2-10, ICCC4 and ICC 37, whereas ICC12237 and ICC12269 have additional resistance to dry rot and black root rot (Nene, 1988). In Mahatma Phule Krishi Vidyapeeth (Rahuri, Maharashtra State, India), significant efforts are being made to breed wilt resistant cultivars and these have resulted in several resistant cultivars, namely, Vijay, Vishal, Virat etc. Chickpea breeding programs elsewhere in the world have released highly resistant cultivars like Suratato 77 in Mexico (Morales, 1986), UC15 and UC27 in California (Buddenhagen *et al.*, 1988) and Amdoun-1 in Tunisia in 1986 (Haware *et al.*, 1992b). There is no evidence of breakdown of resistance to *Foc*. Accessions of several wild species including *C. bijugum*, *C. echinospermum*, *C. judaicum*, and *C. pinnatifidum* are resistant to wilt and can be tapped as sources of wilt resistance genes (Haware *et al.* 1992a; P. Stevenson, ICRISAT, India, 1995, personal communication).

Fig. 1.11 Classical method of race identification by inoculating ten chickpea differential lines with inoculum from a single spore culture of *Foc* (Source- Dr. M. P. Haware)



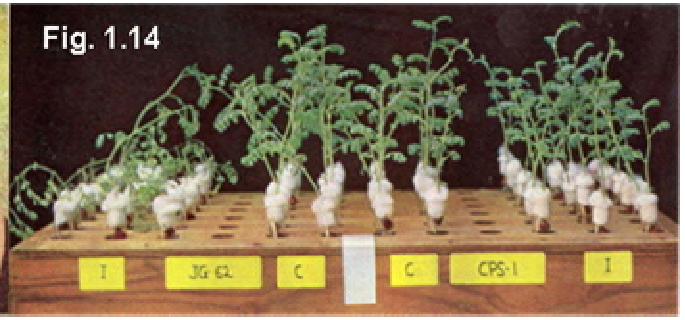


Fig. 1.12 Wilt sick plot at MPKV, Rahuri for screening wilt resistant chickpea lines (Source- V.J. Sant)
Fig. 1.13 Pot screening for wilt resistant chickpea lines (Source- Dr. M. P. Haware)
Fig. 1.14 Water culture technique for studying susceptibility of chickpea varieties to *Foc* races (Source- Dr. M. P. Haware)

The development of wilt resistant cultivars has to be effectively followed by region specific deployment of resistance in accordance with prevalence of specific races. For example, chickpea cultivars to be used in northern plains of India should have resistance to race 2 (Jagdish Kumar, ICRISAT, India, 1996, personal communication). For this purpose, extensive sampling to assess the races of *Foc* needs to be done from regions where the resistant cultivars are to be deployed.

1B.8.3.3 Basis of resistance

(A) Biochemical basis of resistance

Elucidation of the biochemical basis of host resistance can be of considerable value to explore the combined potential of induced and constitutive resistance mechanisms to combat virulent *Foc* races. This is mainly because integration of different resistance mechanisms into cultivars in ongoing breeding programs has previously been stressed as an important contribution to effective durable resistance (Lamb *et al.*, 1992).

Previously, zinc in culture solution at 3ppm has been reported to check the symptoms caused by *Foc*, but not the spread of the pathogen in the plant, and the reduction in wilt symptoms has been attributed to inhibition of fusaric acid production and alteration of host metabolism (Prasad, 1979). Induced resistance to wilt, using nonpathogenic isolates of *Foc* races 0 and 1, has been reported by Hervás *et al.* (1995). Recently, Cachinero *et al.* (2002) have reported non-host *Fusarium oxysporum* isolates to be more potent inducers of plant defense reactions in chickpea against fusarium wilt as compared to the incompatible race 0 of *Foc.* Other reports on induced defense mechanisms in chickpea to pathogenic fungi have demonstrated the production of two pterocarpan phytoalexins, maackiain and medicarpin (Clemens *et al.*, 1993; Ingham, 1976; Smith and Ingham, 1981). Although there is no direct evidence implicating elicitation of phytoalexin synthesis in chickpea to be the basis of resistance to fusarium wilt, root exudates of chickpea plants containing phytoalexins exhibit *in vitro* anti-*Foc* activity, and may contribute at least partly, to wilt resistance (Stevenson *et al.* 1994, 1995, 1997). Vogelsang and Barz (1993) have reported two differentially expressed chitinases and a β-1,3-glucanase in chickpea. In another study from our laboratory, the

induction of protease and chitinase activity in a wilt resistant cultivar Vijay has been correlated with antifungal properties of the root extract, and with resistant reaction to *Foc* race 1. In these studies, the decrease in β-1,3-glucanase activity in roots of Vijay upon pathogen infection has been attributed to a higher rate of callose deposition in resistant cultivars (Giri *et al.*, 1998).

(B) Genetics of host resistance

The genetics of fusarium wilt in chickpea has been studied by Kumar and Haware (1982) suggesting that resistance may be conferred due to the existence of major genes or polygenic complexes acting as modifiers for wilt resistance. Upadhyaya *et al.* (1983a) have reported that the difference in the number of days to wilt JG 62 (early wilter) and C104 (late wilter) was controlled by a single gene with early wilting partially dominant over late wilting. Similar studies by Smithson *et al.* (1983) have indicated the presence of three recessive genes for resistance to race 1, and have identified genes for very late wilting, late wilting and early wilting in different genotypes. Subsequent studies have suggested that resistance to race 1 is controlled by at least three independent loci designated as H_1, H_2 and H_3 (Singh *et al.*, 1987a, Upadhyaya *et al.*, 1983b), and the phenomenon of early wilting is caused by two dominant genes at the first two loci and a recessive gene at the third locus. Partially recessive alleles in homozygous form at either the H_1 or H_2 locus or a dominant allele at the third locus separately delay wilting but any of these two loci together confer resistance to race 1 of *Foc* (Upadhyaya *et al.*, 1983b). Recently, the resistance in cultivar Vijay has been shown to be due to 4 recessive genes (Girase, 1999). A few chickpea genotypes have been shown to differ in their times of wilting (Haware and Nene, 1980), which may indicate different degrees of resistance, and may reflect on the differential behaviour in crosses (Upadhyaya *et al.*,1983a).

Molecular methods have been used to identify markers linked in coupling or in repulsion to genes conferring resistance to fusarium wilt caused by race 1 and race 4 (Mayer *et al.*, 1997; Muehlbauer *et al.*, 1994b; Ratnaparkhe *et al.*, 1998; Tullu *et al.*,

1999). Later Cs27, a marker linked to one of the two recessive genes conferring resistance to race 4, has been mapped on linkage group 2 of the integrated chickpea map by concerted efforts from India, USA and Germany (Winter *et al.*, 2000). Thus genes conferring resistance to *Foc* races 1, 4 and 5 have been mapped on linkage group 2 and are clustered together. On the other hand, Zote *et al.* (1996) have demonstrated that the resistant cultivar WR 315 also promoted *Foc* multiplication in soil, and hence it may not be possible to eliminate the wilt pathogen from infested soil by growing resistant cultivars alone, thus demonstrating the need for other management practices to reduce the inoculum density in soil.

1C *Ascochyta rabiei*- the chickpea blight pathogen

1C.1 Historical account

Ascochyta blight, caused by *Ascochyta rabiei* (Pass.) Labrousse (teleomorph: *Didymella rabiei*) is the most serious disease of chickpea worldwide, particularly in South/West Asia, the Middle East, the Mediterranean region and North Africa (Kaiser and Muehlbauer, 1988; Nene, 1982; Nene and Reddy, 1987) and has been reported from several chickpea growing countries (Nene *et al.*, 1996). Historically, the causal fungus of ascochyta blight was first named *Zythia rabiei* by Passerini in France in 1867 on the basis of unicellular and hyaline pycnidiospores (Khune and Kapoor, 1980). The disease has always been considered economically important, and one of the best-documented accounts of blight epidemic exists for the former Punjab province of British India, where the disease was first observed in 1911 (Butler, 1918). Since then, much research has been carried out on the pathogen, its biology, spread, survival and control. Kovachevski, in 1936, recorded *Mycosphaerella rabiei* (syn. *Didymella rabiei* (Kovachevski) Arx) on overwintered straw, and obtained cultures producing pycnidia when ascospores were plated (Holliday, 1980). However, Punithalingam and Holliday in 1972 suggested that until further clarification of the perfect-imperfect stage association, the chickpea pathogen should be retained under *Ascochyta rabiei* (Holliday, 1980). Later, Trapero-Casás and Kaiser (1992) clearly proved the relationship between

the perfect state and the imperfect state of *A. rabiei* under both, field and laboratory conditions, and confirmed the identity of the perfect state as *Didymella rabiei*. In Spain, Navas-Cortés *et al.* (1995) reconfirmed the relationship through similar studies. Since then, the perfect stage has been found in a number of other countries (Nene, 1982; Nene and Reddy, 1987). Cool-season food legumes are believed to have originated in the Fertile Crescent of the Near East (Smartt, 1984) from where these crops and their associated *Ascochyta* pathogens may have been transported to many countries. Although taxonomists differ in their opinion about its ordinal and family classification, *Didymella rabiei* (the perfect stage of fungus) is classified by many taxonomists as follows: Class: Ascomycetes, Order: Pleosporales, Family: Phaeosphaeriaceae, Genus: *Didymella,* Species: *rabiei*. *Ascochyta rabiei* is the anamorph of *Didymella* and is classified as: Form Class: Fungi Imperfecti, Form-order: Sphaeropsidales, Form-family: Sphaeropsidaceae, Form-genus: *Ascochyta*, and Form-species: *rabiei*.

1C.2 The disease: Ascochyta blight

Ascochyta blight is one of the most serious foliar diseases of chickpea causing significant yield losses, especially in regions where the winter-sown chickpea technology has been adopted to increase crop yields. There are many reports of serious losses caused by blight (Nene, 1982; Nene and Reddy, 1987) wherein yield losses of 25-70%, 20-50%, upto 100%, 10-20%, 40% and 5-30% have been reported from Pakistan, Bulgaria, the former USSR, Greece, Tunisia and Syria, respectively (as reviewed by Haware, 1998). Appropriate temperature, humidity, rainfall, and wind conditions are important for disease development and spread (Nene and Reddy, 1987; Trapero-Casás and Kaiser, 1992). The optimum conditions for seedling infection are a temperature of 20°C with a leaf wetness period of at least seven hours under artificial conditions, and an upper and lower temperature limit for infection and disease development being 5°C and 30°C, respectively (Trapero-Casás and Kaiser, 1992). In the field, ascochyta blight first appears on small groups or patches of plants, low in the crop canopy. Under weather conditions conducive to disease development, patches of

diseased plants in the field may rapidly increase in size, and lesions may develop higher in the crop canopy on leaves and pods. Figure 1.15 A is a photograph of a healthy chickpea field as compared to Fig. 1.15B, that of a blighted chickpea.

The ascochyta blight pathogen is an ascomycete fungus, which can infect all the aboveground parts of the chickpea plant, giving rise to round or elongated, dark sunken lesions. The symptoms on leaflets are round spots with brown margin and a gray center, while stem and petioles usually have elongated necrotic lesions which often girdle stems, weaken and break branches and petioles (leaf stems), killing all plant parts above the lesion (Gowen *et al.*, 1989; Nene and Reddy, 1987). Fully developed lesions on pods are usually round, up to 0.5 cm in diameter, and pod infection ultimately leads to seed infection. Figure 1.16 A, B, C, D and E represent ascochyta blight lesions on leaflets, petioles, branches and pods, respectively. Minor seed contamination is not always visible, and the fungus in or on the surface of the seed is difficult to detect in the laboratory, while heavily infected seeds bear visible blight symptoms, which induce small wrinkles, lesions, and/or dark discoloration. The fungus reproduces asexually via conidia or sexually via ascospores (Nene and Reddy, 1987; Wiese *et al.,* 1991). It produces tiny (65-245 μm in size), black, raised asexual fruiting bodies called pycnidia, which are embedded in concentric rings within lesions on stems, leaves, pods and seed. Figures 1.17A and 1.17B represent *A. rabiei* pycnidia on chickpea stem and a close-up view of a single pycnidium, respectively. Pycnidia are also formed on artificial media in concentric rings within 4-5 days of incubation at 20°C under fluorescent lights (12 hours), while the growth of the fungus on PDA medium at 20-25°C is initially creamy to pinkish in color turning darker with age. The pycnidial wall is composed of 1-2 layers of pseudoparenchymatous cells, and the ostiole is 30-40μm wide. Pycnidiospores (or conidia) contained within pycnidia are formed on hyaline, ampulliform phialides, are hyaline, oval to oblong, rounded at each end, slightly constricted at the septum, 0-1 septate, and measure 10-16 x 3.5μm (Fig. 1.17C).

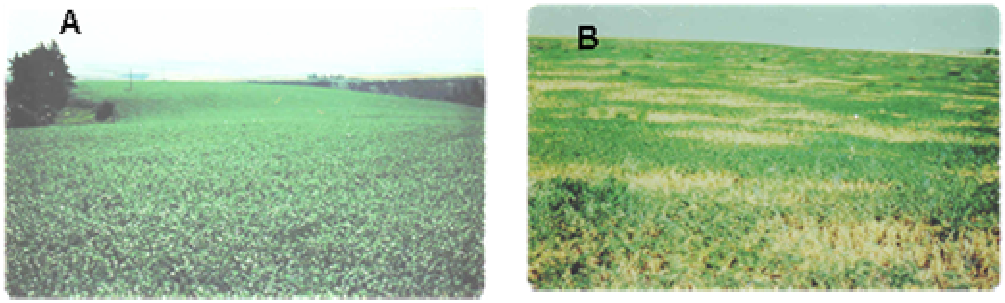


Fig.1.15 Photographs of (A) a healthy chickpea field (B) a blighted chickpea field (Source- Dr. W.J. Kaiser and Dr. F. J. Muehlbauer).

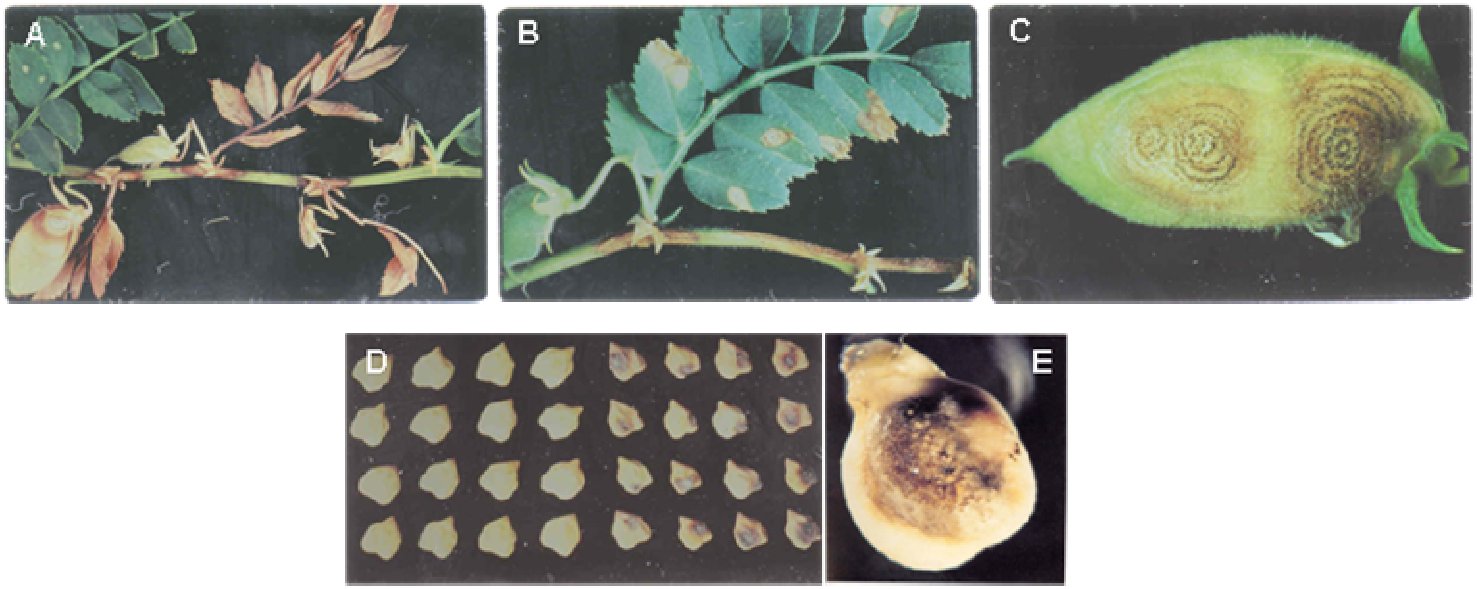


Fig. 1.16 Ascochyta blight lesions on (A and B) chickpea stem, central axis, leaflets, (C) chickpea pod with concentric arrangement of pycnidia (D) healthy versus infected chickpea seeds (Fig. 1.16 A-D, Source-Dr. F. J. Muehlbauer) (E) single seed (x13) (Source-ICRISAT information bull. No.34).

1C.3 The teleomorph of *A. rabiei*

Under the stereomicroscope (50 x magnification), pseudothecia of *Didymella rabiei* (the teleomorph of *A. rabiei*) are not easily distinguishable from pycnidia, however, they have a comparatively larger size (70-150 x 120-250 μm), darker wall, are markedly subglobose, have an inconspicuous ostiole and split open in response to excessive moisture (Nene, 1982). They are never observed in blight lesions on living plants and are usually arranged in rows on chickpea straw. In immature pseudothecia, pseudoparaphyses are conspicuous, filamentous, hyaline and septate; however, they disappear at maturity. Asci contained within pseudothecia are 48-70 x 9-14 μm, cylindrical to subclavate, eight spored, and have a bitunicate wall. Ascospores contained within the asci are 12.5-19 x 6.7-7.6 μm in size, irregularly distichous, hyaline, ellipsoidal to biconic, bicelled (with the upper cell broader than the lower cell), and strongly constricted at the septum (septum below the middle). Figures 1.18 A, B, and C depict photographs of centrum crush of a pseudothecium of *D. rabiei*, asci containing ascospores, and a single ascospore, respectively.

Under artificial conditions, pseudothecia can develop on chickpea straw following inoculation with compatible mixtures of single ascospore or conidial isolates; however, they do not develop on tissue inoculated with isolates obtained from a single ascospore or a single conidium, or with mixtures of incompatible isolates (Trapero-Casás and Kaiser,1992). Pseudothecia fail to develop on normal or low nutrient media (artificial media) in petridishes. After several unsuccessful attempts on different media, Trapero-Casás and Kaiser (1987) used senescent chickpea stems to perform these crosses, with standard tester strains (ATCC 76501 and ATCC 76502), thereby elucidating the specific requirement of chickpea stems for development and maturation of the teleomorph stage of *A. rabiei*. In their studies, low temperature, high moisture and a relatively long incubation period were also reported as requirements for development of pseudothecia; with temperature having a major influence on their maturation, though it had a limited role in their induction. Abundant asci and ascospores

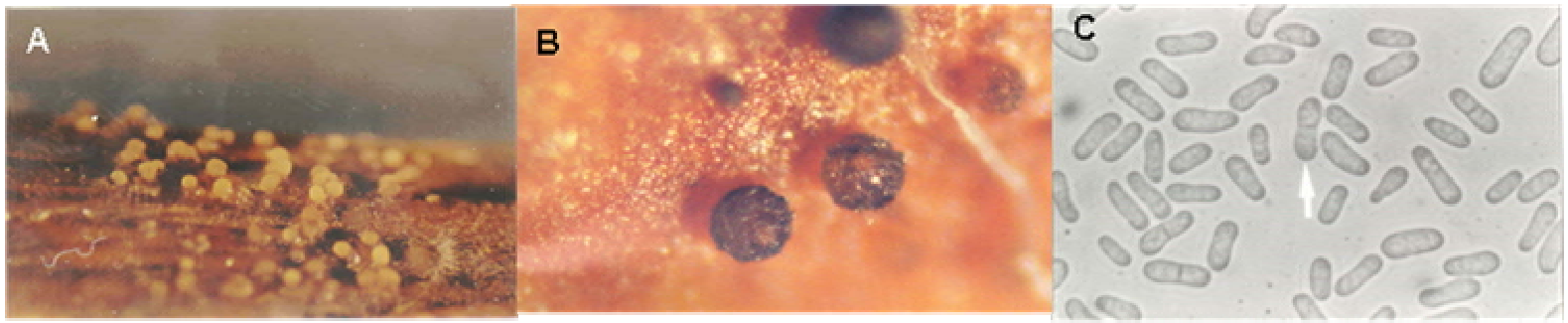


Fig. 1.17 (A) *A. rabiei* pycnidia on chickpea stem (Source- Dr. T. L. Peever), (B) A single pycnidium (x113) (C) Pycnidiospores (or conidia) (x1130) (Source Fig. 1.17B,C - ICRISAT information bull. No. 34).

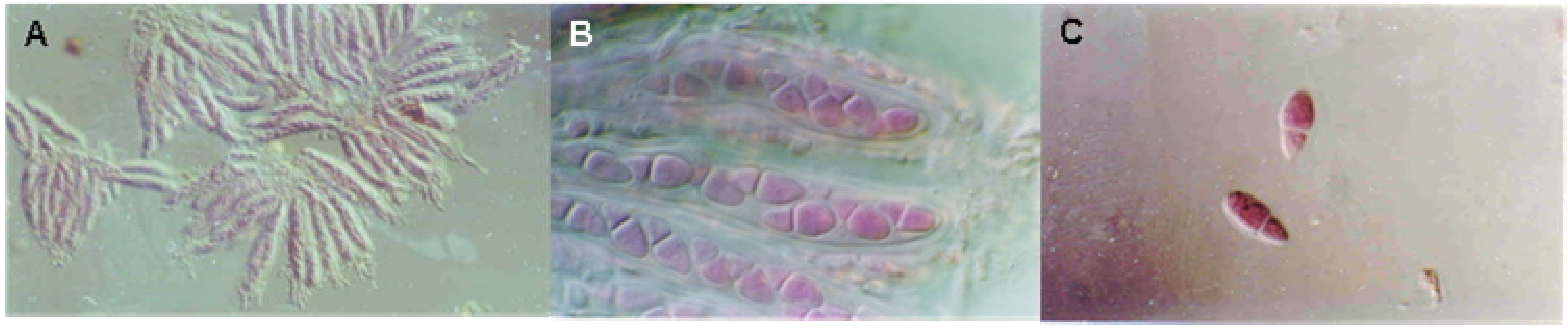


Fig. 1.18 (A) Centrum crush of a *D. rabiei* pseudothecium with asci and remnants of apical paraphyses (x200) (B) bi-tunicate asci containing bi-celled ascospores (x400) (C) single ascospores (x1000) (Source- Dr. J. D. Rogers)

only developed at 5-10°C, whereas most pseudothecia aborted at 15°C, and all of them failed to produce asci and ascospores at 20-25°C. These requirements for sexual reproduction are common in many plant pathogenic members of Ascomycotina (Müeller, 1979).

1C.4 Disease cycle and spread

The fungus is well adapted for survival, multiplication, and dispersal in infested plant residue on the soil surface, and has previously been isolated from the foliage of 'volunteer plants' from a field where debris from a previous blight infected chickpea crop was left on the soil surface (Wiese *et al*. 1991). Alternative hosts, such as pea, alfalfa, certain other legumes, and some weeds may be rarely attacked, and the infections may remain latent or may result in mild disease symptoms, yet may play a role in the local survival of the pathogen (Wiese *et al*. 1991, Kaiser, 1990). As described above, asexual spores (conidia) are produced abundantly in pycnidia in diseased tissues, while sexual spores (ascospores) are produced in similar looking, dark fruiting bodies (pseudothecia) on overwintered chickpea residues in contact with moist soil. Figure 1.19 represents the disease cycle of *A. rabiei* on chickpea in temperate regions where the teleomorph formation is possible in the field. In general, crop residues and seeds are primarily responsible for season-to-season survival of the pathogen.

Primary infections may arise from seed borne inoculum, from windborne ascospores released from infected chickpea residue, or from alternative weed or legume hosts in addition to thriving on volunteer chickpea plants. Because trace quantities of *A. rabiei* in and on seed are difficult to detect, apparently symptom-less seeds may still harbor and readily disseminate the fungus. The role of *A. rabiei* as a seed-borne pathogen and introduction of the fungus into areas previously free of the disease by this mode has been well documented (Kaiser, 1997a). Once primary infections are established, asexual conidia produced in pycnidia on blighted plants, may be dispersed by rain-splash and sometimes by wind, or on contaminated machinery to cause secondary spread of the disease to uninfected plants within the field (Wiese *et al.*,

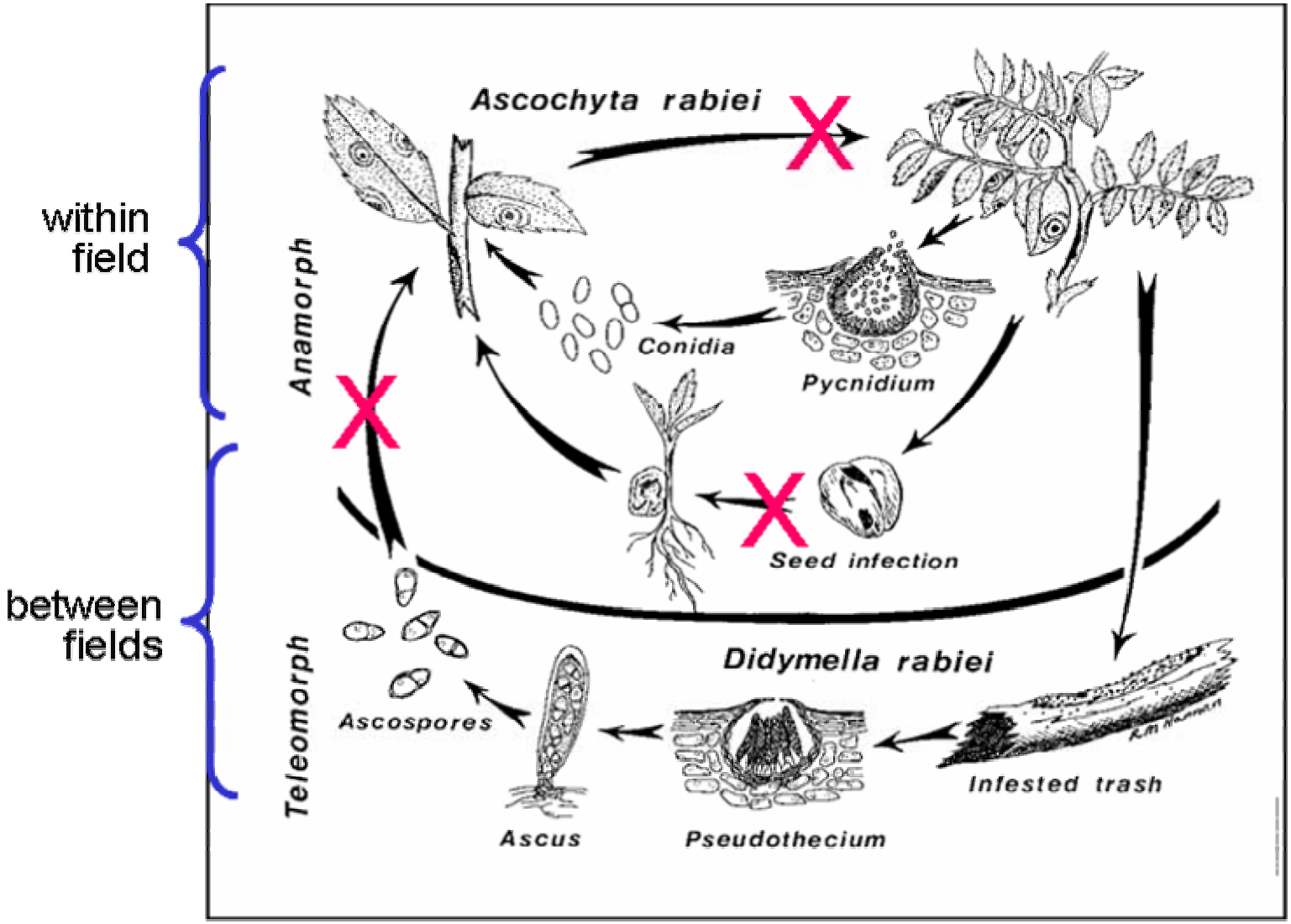


Fig. 1.19 The disease cycle of *A. rabiei* on chickpea in temperate regions (Source- Dr. R. M. Hannan). The red crosses indicate stages in the life cycle of *A. rabiei* where the disease can be controlled (Source- Dr. T. L. Peever).

1991). The sexual stage of *A. rabiei* is again formed on chickpea debris colonized by the fungus in the previous season and requires approximately two months at cool temperatures (5-10°C) to mature and produce ascospores. Once mature, these are released by specific environmental conditions, become windborne and can potentially infect chickpea plants several hundred meters from the source (Trapero-Casás and Kaiser, 1992). Due to their potential for long-distance movement, ascospores may be the most likely route by which the pathogen is transferred from infected to uninfected fields, while conidia are splash-dispersed only to short distances (Kaiser, 1992).

There are varying opinions about the role of the perfect stage of *A. rabiei* in the disease epidemiology of ascochyta blight. The sexual stage is thought to play an important role as (i) a survival mechanism between chickpea crops, (ii) as a source of primary inoculum for blight epidemics and (iii) in generating genetic diversity in the fungus. Previous studies, where the sexual stage was sampled periodically and tested for ascospore release in the laboratory, indicate that ascospores produced in a given season are released during a limited period of time (Trapero-Casás and Kaiser, 1992), and maximum disease severity coincides with time of ascospore release from diseased debris (Trapero-Casás *et al.*, 1996). The ascospores, which are genetic recombinants, may be significant from an evolutionary perspective by generating genetic diversity and giving rise to potentially more virulent forms of the fungus (Kaiser and Kusmenoglu, 1997; Trapero-Casás and Kaiser, 1992; Wilson and Kaiser, 1995).

1C.5 Mating type in *A. rabiei*

Ascochyta rabiei is a heterothallic loculoascomycete fungus with a bipolar mating system (Wilson and Kaiser, 1995) typical of Ascomycete fungi, where sexual reproduction is governed by a single regulatory locus referred to as a *MAT* locus (Coppin *et al.*, 1997; Turgeon, 1998). Two compatible mating types, namely MAT1 and MAT2 are required for formation of fertile pseudothecia; however, mere presence of both mating types in close proximity does not necessitate sexual reproduction (Trapero-Casás and Kaiser, 1992; Wilson and Kaiser, 1995). A 1:1 ratio of mating types is expected in populations undergoing regular random mating (Milgroom, 1996) and

equal ratios of mating types suggest that the sexual stage of the pathogen is important in the life cycle and disease epidemiology. However, the ratio of mating types in particular areas may deviate significantly from 1:1, raising a possibility that the sexual stage may not form regularly in the life cycle of the pathogen in that area (Kaiser and Kusmenoglu, 1997). In previous studies, both mating types were present in equal ratios in field samples obtained across Canada (Armstrong *et al.*, 2001), Turkey (Kaiser and Kusmenoglu, 1997), and the Pacific North West, USA (Kaiser, 1997b; Kaiser and Hannan, 1987; Trapero-Casás and Kaiser, 1992) where the sexual stage is thought to form regularly. However, several samples of chickpea debris infested with *A. rabiei* from individual fields within these countries failed to develop the sexual stage upon incubation in conducive conditions, and study of mating type ratios in these regions suggested that both mating types may not be present in all these locations, or they may not be distributed evenly within populations (Armstrong *et al.*, 2001; Kaiser, 1995; Kaiser, 1997b; Kaiser and Kusmenoglu, 1997). In extreme cases of deviation from the 1:1 ratio, a single mating type of *A. rabiei* has been reported from several areas of the world and *A. rabiei* populations from India, Israel, Egypt, Australia, and California, USA currently consist of a single mating type (Kaiser, 1995; Kaiser, 1997a; Kaiser, 1997b; Kaiser and Kusmenoglu, 1997; Khan *et al.*, 1999), namely, MAT1-1 from India and Australia, and MAT1-2 from California, USA. This prevents the formation of the sexual stage and eliminates ascospores as a potential inoculum source (Kaiser, 1997b). Therefore, a large number of isolates need to be screened for mating type (i) from regions where both mating types are present, to help understand the precise role of the sexual stage in disease epidemiology and the existing genetic diversity in pathogen populations and, (ii) from regions where only one mating type has been reported so far to continuously monitor these regions for the appearance of the other mating type (Kaiser, 1997a).

1C.6 Variability studies in *A. rabiei*

Knowledge of variation in pathogen populations is important for disease control strategies in screening for resistance and resistance gene deployment (Milgroom and Fry, 1997; Peever *et al.*, 2000). Numerous studies have been carried out in *A. rabiei* to determine morphological, pathogenic and genotypic variation. In *A. rabiei*, morphological variation has been reported by several workers (Grewal, 1984; Kaiser 1973; Luthra *et al.*, 1939) while, Aujla (1964) reported the presence of strains/ races of *A. rabiei* in Punjab based on symptomatology, manner of pycnidial formation on host and pathogenic behavior. Since then, pathogenic variability has been demonstrated in *A. rabiei* by several workers (Bedi and Aujla, 1969; Grewal, 1984; Jamil *et al.* 2000; Jan and Wiese, 1991; Kaiser, 1973; Nene and Reddy, 1987, Reddy and Kabbabeh, 1985; Vir and Grewal, 1974). Such studies have been further retarded due to the inability to reliably estimate quantitative differences in virulence of *A. rabiei* isolates in controlled assays (Akem, 1999; Hamza *et al.*, 2000). In separate studies, *A. rabiei* isolates from Indian states have been grouped on the basis of morphological and cultural characteristics and differential reactions to chickpea lines (Ambardar and Singh, 1996; Singh, 1990). However, till date, there is no consensus as to whether variability in *A. rabiei* isolates is due to 'race' or 'aggressiveness' in a single race. The main argument against variability due to race has been that there are no significant reversals in the ranking order of cultivars in their reaction to different races. Previous studies have suggested variability in aggressiveness of *A. rabiei* isolates, rather than in their virulence (Haware, 1987b; Mmbaga *et al.*, 1997). Although in some studies reverse ranking of genotype reaction to different *A. rabiei* isolates has been observed (Jan and Wiese, 1991; Porta-Puglia, 1992), the information available is insufficient for using precise terminology such as 'race'. *A. rabiei* isolates have been reported to differ in their aggressiveness, the most aggressive pathotypes being associated with areas where the selection pressure is high (Mmbaga *et al.*, 1997). A high adaptability of *A. rabiei* to the chickpea host has been indicated as the possible reason for resistance instability in chickpeas (Mmbaga *et al.*, 1997; Hamza *et al.*, 2000). The influence of environmental

factors on disease expression affects the reproducibility of results of variability studies, and has been well documented in *A. rabiei*, as is the case for many foliar pathogens (Mmbaga, 1997). Phytotoxin production has been documented (Alam *et al.*, 1989; Höhl *et al.*, 1991), and *A. rabiei* has been reported to produce toxins in response to aqueous extracts from chickpea seed (Alam *et al.*, 1989).

Various molecular markers are available to study genetic variation in plant pathogens (Brown, 1996). Isozyme profiles of *A. rabiei* isolates from Algeria, Tunisia and Syria are reported to be nearly uniform and differ very little from other species of the genus *Ascochyta* (Udupa and Weigand, 1997). Several DNA based molecular markers, like hybridization-based simple sequence repeats, have been used for variability studies in *A. rabiei* populations (Geistlinger *et al.*, 1997a; Morjane *et al.*, 1994; Udupa *et al.*, 1998; Weising *et al.*, 1991b). In their studies, Morjane *et al.*, (1994) have used a hierarchical sampling strategy to demonstrate genetic variation in *A. rabiei* isolates in Tunisia on a microgeographic scale, wherein, multiple genotypes were recovered from single lesions on infected plants. PCR based RAPD markers have been used by several workers to characterize genetic diversity in *A. rabiei* populations (Fischer *et al.*, 1995; Jamil *et al.*, 2000; Kaemmer *et al.*, 1992; Navas-Cortés *et al.*, 1998; Santra *et al.*, 2001; Udupa *et al.*, 1998). In the study on Italian isolates of *A. rabiei* by Fischer *et al.* (1995), no pathotype-characteristic amplification was observed. Similarly, in another study by Navas-Cortés *et al.* (1998) with isolates collected from India, Pakistan, Spain and the USA., no correlation was observed between RAPD genotype groups, pathotypes or mating type, although some evidence of clustering based on geographic origin was detected. Similar results were obtained in a study using *A. rabiei* isolates collected from different states of India using RAPD markers, wherein the isolates were grouped according to their geographic origin (Santra *et al.*, 2001). However, all the above-mentioned studies were unsuccessful in correlating pathotype variation with RFLP or RAPD genotype. In addition to RFLP and RAPD markers, several codominant sequence-tagged microsatellite site (STMS) markers have been

generated for *A. rabiei* (Geistlinger *et al.*, 1997b, Geistlinger *et al.*, 2000). *A. rabiei* is an excellent experimental organism for population genetic studies because of the ability to perform laboratory crosses, and to determine the inheritance of alleles and linkage relationships. Mendelian inheritance of DNA fingerprint markers has been demonstrated using progeny from a laboratory cross of *A. rabiei* (Geistlinger *et al.*, 1997a).

1C.7 *Ascochyta* species on related legume hosts

Ascochyta spp. infect a number of legumes besides chickpea and represent serious biotic constraints to legume production in many parts of the world. These fungi are host-specific but the genetic basis of this specificity is not known. The ascochyta blight pathogens of faba bean (broad bean) (*Vicia faba*), and lentil (*Lens culinaris* Medik.) *Ascochyta fabae* Speg., and *Ascochyta fabae* f. sp. *lentis* Gossen *et al.* (= *A. lentis* Vassilievsky), respectively, are economically important seed borne pathogens of their respective hosts. These pathogens are reported to be heterothallic, based on pairings of compatible isolates within each species. The ability to perform laboratory crosses in these fungi has made it possible to delimit biological species. In previous studies, crosses between *A. rabiei* and either *A. lentis* or *A. fabae* consistently failed to produce pseudothecia (Kaiser *et al.*, 1997). In contrast, crosses between *A. lentis* and *A. fabae* produced pseudothecia with viable ascospores, however, asci produced in these crosses contained fewer than eight ascospores which grew poorly in culture and failed to infect either lentil or faba bean. The magnitude of these fertility barriers suggested that *A. rabiei* was phylogenetically distantly related to *A. lentis* and *A. fabae*, while *A. fabae* and *A. lentis* were closely related; *A. rabiei, A. lentis* and *A. fabae* each being phylogenetic species. Furthermore, RAPD banding patterns clearly differentiated *A. fabae* and *A. lentis*, and the banding patterns of the progeny from a cross between *A. fabae* and *A. lentis* confirmed hybridity (Kaiser *et al.*, 1997).

1C.8 Disease management

The most important component of blight management is to reduce or prevent the entry of primary inoculum to the field. Figure 1.19 highlights the stages in the life cycle of the ascochyta blight pathogen where the disease can be controlled.

1C.8.1 Cultural practices and chemical control

Disease control includes use of disease free seeds (Kaiser and Muehlbauer, 1988; Kovachevski, 1936), burning of infected plant residues, deep ploughing, crop rotation, use of proper nitrogen, phosphorous and potassium in the field, and exposure of seeds to direct sunlight (Tripathi *et al.*, 1987). Pathogen-free seed, procured from fields and areas free of ascochyta blight, with high germinability and the ability to produce vigorous plants, is a prerequisite of an effective disease control program (Nene, 1982; Sattar, 1933). Deep ploughing of infested chickpea stubble into the soil following harvest is one of the most effective measures to minimize primary ascospore inoculum as it reduces the viability of the pathogen from several years if left on the soil surface to a few months if ploughed into the soil (Kaiser, 1973; Navas-Cortés *et al.*, 1995). However, over the past few years, there has been a shift in cropping systems, in many parts of the world from regular tillage to reduced tillage in an effort to reduce soil erosion (Cook, 1992). This promotes inoculum build-up on the soil surface creating conditions conducive for sexual reproduction, and producing progeny, possibly with increased virulence due to sexual recombination (Peever and Muehlbauer, personal communication).

Chemical seed treatment and prophylactic sprays suppress the amount of initial inoculum induced by polycyclic pathogens like *A. rabiei*. Furthermore, the chickpea seed to be sown can be treated with fungicides, for instance, specific formulations of metalaxyl, captan, thiabenzadole, and benomyl can be used to significantly limit fungal pathogens that may be present on the seed or in the soil, but they may not eliminate *A. rabiei* from the seed completely (Hanounik and Reddy, 1984; Kaiser and Hannan, 1985; Kovachevski 1936). Protectant fungicides like chlorothalonil can be used,

however, their usefulness in controlling blight is limited because of lack of post-infectional activity, and protection of the plant surface only (Illyas and Bashir, 1984; Reddy and Kabbabeh, 1984; Tripathi *et al.*, 1987). Application of post-infectional, systemic fungicides according to a weather-based model may be more useful to control ascochyta blight (Shtienberg *et al.*, 2000) as they are able to control fungal diseases of plants after an infection event has occurred, and are effective at lower rates. However, disease control options such as the application of fungicides may not be economical for many farmers in developing countries.

1C.8.2 Host resistance

1C.8.2.1 Screening for resistant lines

Due to the limitations of cultural and chemical methods of control of ascochyta blight, priority has been given to the exploitation of host-plant resistance as a means of controlling the disease. Selecting blight resistant varieties is economically and environmentally the most sound means of controlling ascochyta blight. Several techniques have been proposed to screen for host plant resistance under field, greenhouse and controlled growth chamber conditions (Haware *et al.*, 1995b; Pieters and Tahiri, 1985; Reddy *et al.*, 1984; Singh *et al.*, 1989). In view of the polycyclic nature of ascochyta blight and association between age of the plant and disease susceptibility, field evaluation of the lines exposing all stages of the crop to the disease is necessary. Screening for resistant lines in the field is a comparatively cheap means for testing a large number of individuals, during the whole plant cycle under conditions similar to those in which the resistant cultivars are expected to perform. Planting the crop in a period when the average minimum and maximum temperatures are between 10-20°C with high relative humidity (which can artificially be maintained by sprinkler or perfo-irrigation) ensures blight development. Inoculations are done either by spraying plants with spore suspensions of the fungus multiplied in the laboratory or by scattering diseased debris in the field; the advantage of the latter being that inoculation can be done at anytime and blight develops when conditions become favorable for the

disease. The former method can be used when natural conditions are favorable for disease development or by providing such conditions soon after inoculation. Blight epidemics can be encouraged by inter-planting rows of susceptible genotypes (spreaders). However, prevailing environmental conditions, the nature of the blight inoculum available in the area, and the interactions with other organisms, can affect the expression of resistance and consequently, in such screening techniques disease escape may be confused with resistance. These problems associated with field screening can be avoided by screening chickpea germplasm for resistance to ascochyta blight under controlled glass house conditions. Furthermore, the plants can be inoculated at different stages of development with varying concentrations of inoculum of one or more purified isolates of the pathogens, and the inoculum can be distributed to ensure uniform infection. One major disadvantage of glass house screening, however, is that space is often a constraint, and the results obtained need to be again validated in the field.

For rapid evaluation of a large number of lines in the field/glasshouse, it is necessary to have a simple rating scale, however, more precise studies involving-components of resistance, genetics of resistance, pathogenic variability etc. require a more detailed scale taking into consideration disease severity and sporulation of the fungus. Different rating scales have been proposed and used by researchers for scoring ascochyta blight severity under field and controlled conditions (Morrall and McKenzie, 1974; Riahi *et al.*, 1990; Singh *et al.*, 1981; Vir and Grewal, 1974). However, there is a need to simplify the rating procedure and adopt a common scale acceptable to most researchers. A nine points scale judgment for disease severity in chickpea ascochyta blight has been found to be very useful (Reddy *et al.*, 1984). It is rapid, repeatable, covers a wider range of disease severities and is suitable for scoring progeny rows and yield plots. It is important to establish a relation between disease severity scores and extent of yield loss to help selecting lines in resistance breeding programs. In case of chickpea ascochyta blight, the relationship between 1-9 scores and yield loss has been estimated, and the yield loss in lines with a disease score between 2 to 4 was less than

10%, in those with a score of 5 was 16%, with a score of 6-7 was 26-27%, while the yield loss in lines with an 8-9 score was 81-98% (Reddy and Singh, 1990).

1C.8.2.2 Ascochyta blight resistant cultivars

Systematic evaluation of chickpea for ascochyta blight resistance started with the initiation of the ICRISAT-ICARDA *kabuli* chickpea program in 1978, which was further intensified after the epiphytotics of 1981 and 1982 (Nene and Reddy, 1987; Reddy and Singh, 1984). At ICARDA, over 19,000 germplasm accessions of chickpea have been evaluated for resistance to six isolates of *A. rabiei* between 1979 and 1991, of which only three *desi* accessions (ICC 4475, ICC 6328, ICC12004) and two *kabuli* accessions (ILC 200 and ILC 6482) showed resistance during repeated greenhouse and field screening (Singh and Reddy, 1993). Evaluation of chickpea germplasm and breeding lines for resistance to ascochyta blight in 48 locations throughout 20 countries has indicated that *kabuli* germplasm generally shows higher resistance to blight than *desi* germplasm (Haware *et al.* 1995a, 1995b; Reddy *et al.*, 1992). From the material generated at ICARDA, 29 blight resistant cultivars have been released in 14 countries (Singh, 1993). In India, lines with moderate resistance have been identified (Singh, 1989). However, there is a need to quantify the level of yield gained by the use of these lines in comparison to susceptible lines.

Although complete immunity to this disease is not known, the highest level of resistance has been reported in a perrenial wild accession of *Cicer anatolicum*, PI383626 (personal communication, Dr. Walter J. Kaiser). High levels of resistance are also available in other wild *Cicer* species including accessions of *C. bijugum*, *C. judaicum*, and *C. pinnatifidum* (Haware *et al.*, 1992a; Singh *et al.*, 1992). Although there have been efforts to identify and transfer resistance genes from wild resistant *Cicer* species and landraces, problems associated with interspecific hybridization still persist (Akem, 1999). With biotechnological tools, the utilization of resistance from wild *Cicer* species to improve the resistance in cultivated chickpea could be possible if

the genetic basis of resistance is different to that in the crop and once transformation techniques for chickpea have been perfected.

Breakdown of blight resistance has been reported time to time from many countries due to appearance of new virulent pathotypes of *A. rabiei* (Aujla, 1964; Dolar and Gurcan 1992; Grewal, 1984; Luthra *et al.* 1939; Nene 1984; Peever and Muehlbauer (personal communication); Singh *et al.*, 1981; Vir and Grewal, 1974). It is well established that the pathogen is highly variable and the pathotypes present in Pakistan and India appear to be more aggressive than those prevalent in Mediterranean regions (Singh *et al.*, 1984b).

Populations of pathogens inciting disease in the aboveground parts of plants, like *A. rabiei*, vary in time and space because of the airborne or seed-borne nature of the inoculum, which facilitates long-distance dispersal of their variants. The changes in frequency of virulence genes among these populations are very frequent, hence, breeding for resistance to foliar pathogens is, in general, more difficult as compared to the case of more stable and less mobile pathogens, for example soil borne fungi (Porta-Puglia *et al.*, 1994).

1C.8.2.3 Basis of resistance- Genetic and biochemical

Resistance to blight is partial and the existence of immunity has not been confirmed (Allen, 1983). Studies reviewed by Nene (1982) suggested that resistance was governed by a single dominant gene, while subsequent studies reported that blight resistance is quantitatively inherited (Boorsma, 1980; Pieters, 1984; Van Rheenan and Haware, 1993). Dey and Singh (1993) have further observed that resistance is governed by different genes in different cultivars. In another study, a correlation of blight resistance with seed-coat color has been established and resistant genotypes have been reported to be predominantly black seeded suggesting that pigment may be associated with blight resistance (Allen, 1983). Due to the varied and often contradictory results obtained to date, further work on inheritance of resistance to ascochyta blight appears essential to develop a sound breeding strategy.

Very little is known about the mechanisms, underlying blight resistance. Hafiz (1952) noted that the penetration of the pathogen in two resistant cultivars was delayed possibly due to the greater secretion of malic acid from the dense covering of glandular trichomes in them, but these findings need to be verified by further work (Nene, 1982). No linkage was detected between isozyme loci and ascochyta blight resistance in a study where eight F_2 populations were scored for allozymes of four polymorphic isozymes loci (Kusmenoglu *et al.*, 1992). In other studies, accumulation of pterocarpan phytoalexins (Barz *et al.*, 1990), and formation of several defense related enzymes in chickpea after *A. rabiei* infection were analyzed in detail (Daniel *et al.*, 1990; Tiemann *et al.*, 1993). On the other hand, reductive conversion of such defense related compounds (medicarpin and maackiain in chickpea), in a stereospecific manner by *A. rabiei* enzymes has also been reported (Hohl and Barz, 1987; Weltring *et al.*, 1995). Histology of the infection process of *A. rabiei* in resistant and susceptible chickpea cultivars has been studied by Hohl *et al.* (1990), wherein, several biochemical factors such as early metabolic activity (autofluorescence), active cell death (necrotic spots) and perhaps detoxification of fungal toxins were reported to contribute to resistance (Hohl *et al.*, 1990). Vogelsang *et al.* (1994) reported rapid hypersensitive reaction like browning in chickpea cell cultures of only the resistant cultivar, similar to the leaves of the same.

1C.8.3 Integrated disease management

Disease management by integration of cultural practices with host resistance and chemical applications may provide a good control for ascochyta blight, and help improve chickpea yields, while reducing pesticide load into the environment. Such disease management strategies specific to areas of chickpea cultivation need to be formulated and their feasibility needs to be tested in field experiments.

1D Genesis of the thesis

Since 1994, our group at the National Chemical Laboratory, Pune, India has been collaborating with Washington State University, Pullman, USA under the McKnight Foundation supported project on 'Increasing the efficiency of production of chickpea'. Three main aspects of research include molecular mapping of the chickpea genome, mapping and tagging of economically important yield traits and disease resistance genes for marker assisted selection in chickpea breeding programs and development of *Helicoverpa armigera* resistant chickpea using a genetic engineering approach.

'*Know the foe well before combat*' is the philosophy, which has inspired us to include the study of chickpea pathogens and pests as a segment in the holistic approach towards improvement of this economically important crop. Fusarium wilt and ascochyta blight are the two widespread fungal diseases of chickpea in the tropical and temperate chickpea growing regions of the world, respectively, caused by *Fusarium oxysporum* f. sp. *ciceri* and *Ascochyta rabiei*. Both these diseases are economically significant because they limit the productivity of chickpea, which has been described as the world's third most important pulse crop. The nature of the diseases caused by *Fusarium oxysporum* f. sp. *ciceri* and *Ascochyta rabiei*, and the geographical regions, in which the diseases caused by them assume significance, are very different, as has been detailed in the chapter on review of literature. The study of both these pathogens is especially relevant in the Indian context because India accounts for 75% of the world chickpea production and both diseases occur in different chickpea growing regions of the country. Furthermore, *Foc* is a monocyclic root pathogen, while *A. rabiei* (teleomorph: *Didymella rabiei*) in contrast, is a polycyclic, necrotic foliar pathogen. The biology of these two pathogens is also very different in that, *Foc* is clonally propagated and no sexual stage has been identified to date, while *A. rabiei* is a loculoascomycete with a well-defined sexual stage in its life cycle, which is probably very significant in its disease epidemiology at least in some parts of the world.

In the pursuit of research towards a Ph.D. degree, I joined the group at National Chemical Laboratory, Pune under the supervision of Dr. Vidya Gupta in 1996. As a research fellow equipped with a basic degree in Microbiology and Biotechnology, I found it interesting to pursue the molecular analysis of both these fungal pathogens. In addition to the training in molecular biology techniques, I was deputed for a short training program in the legume pathology unit at ICRISAT Asia center, Patancheru, Hyderabad, India under the guidance of Dr. M. P. Haware to acquire familiarity with the plant pathological aspect of my research objective. Later, I got an opportunity to work in the laboratories of Dr. Tobin Peever (Plant Pathology, W.S.U.) and Dr. Fred Muehlbauer (Crop and Soil Sciences, USDA, W.S.U) as a visiting scientist from January 2001 to February 2002 under the McKnight foundation fellowship. I was also fortunate to be able to interact with Dr. Walter Kaiser, and several eminent scientists in W.S.U., which was an enriching experience during my Ph.D.

1E Organization of thesis

I have organized my thesis in the following three chapters in addition to the review of literature (Chapter 1) entitled 'Two economically important fungal pathogens of chickpea- *Fusarium oxysporum* f. sp. *ciceri* and *Ascochyta rabiei*'.

My thesis addresses the following specific objectives organized under two chapters:

Chapter 2: Genome analysis and study of genetic diversity in *Fusarium oxysporum* f. sp. *ciceri* (*Foc*), the wilt-causing agent of chickpea using hybridization and PCR based approaches

Chapter 2 comprises two sections:

Section 2A) Potential of simple sequence repeat - restriction fragment length polymorphism (SSR-RFLP) to distinguish four races of *Fusarium oxysporum* f. sp. *ciceri* prevalent in India

Section 2B) Genetic diversity analysis of Indian isolates of *Foc* and other *Fusarium* spp. using PCR based ISSR and AFLP markers

Chapter 3: Genetic diversity and mating type locus identification in the chickpea blight pathogen *Ascochyta rabiei*

Chapter 3 comprises two sections:

Section 3A) Genetic diversity analysis of a world-wide collection of *Ascochyta rabiei* isolates using STMS markers

Section 3B) Cloning and characterization of the mating type (*MAT*) locus from *Ascochyta rabiei* (teleomorph: *Didymella rabiei*) and a *MAT* phylogeny of legume-associated *Ascochyta* spp.

Chapter 4: General Discussion - Potential of molecular markers for *Fusarium oxysporum* f. sp. *ciceri* and *Ascochyta rabiei* for disease diagnostics and resistance breeding in chickpea

These chapters are followed by bibliography and my résumé.

Section 2A

Potential of simple sequence repeat - restriction fragment length polymorphism (SSR-RFLP) to distinguish four races of *Fusarium oxysporum* f. sp. *ciceri* prevalent in India

2A.1 Abstract

Thirteen oligonucleotide probes complementary to microsatellite loci, in combination with eleven restriction enzymes were used to assess potential of such markers to study genetic variability in four Indian races of the chickpea wilt pathogen *Foc*. Hybridization patterns, which were dependent upon the restriction enzyme as well as the oligonucleotide probe used, revealed the presence of different repeat motifs in the *Foc* genome. Among the restriction enzymes used, hexa-nucleotide cutting enzymes were more informative than tetra- and penta-nucleotide cutting enzymes, whereas, tetranucleotide and trinucleotide repeats yielded better hybridization patterns as compared to dinucleotide repeats. Depending on the level of polymorphism detected, I identified $(AGT)_5$, $(ATC)_5$, and $(GATA)_4$ 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 similarity index value 76.6%, as compared to race 2 at similarity value 67.3%, and race 3 to be very distinct at similarity value 26.7%. My study demonstrates the potential of oligonucleotide probes for fingerprinting and studying variability in the *Foc* races, and represents a step towards identification of potential race diagnostic markers**.**

2A.2 Introduction

Fusarium oxysporum Schlechtend.: Fr. is a complex of economically important soil borne plant pathogenic fungi worldwide. More than 150 host-specific *forma speciales* have been described in the *F. oxysporum* complex, many of them subdivided into distinct pathogenic races (Baayen *et al.*, 2000). *F. oxysporum* isolates of a given *forma speciales* are morphologically indistinguishable. Therefore, sub-specific division is usually based on physiological race reactions to a set of differential cultivars (Snyder and Hansen, 1940) or on vegetative compatibility groups (VCGs) as determined by heterokaryon formation between anastomosing, nitrate non-utilizing (*nit*) mutants (Katan and Katan, 1999; Puhalla, 1985).

Seven races of the chickpea-wilt pathogen *F. oxysporum* f. sp. *ciceri* (*Foc*) have been reported worldwide on the basis of differential disease reactions of ten chickpea cultivars to pathogen isolates (Haware *et al.*, 1990) and are represented by the same VCG (Nogales-Moncada, 1993). Of these, races 1 to 4 have been reported from India (Haware and Nene, 1982c), where they are geographically distinct. Race 1 is widespread in central and peninsular India and race 2 in northern India. Races 3 and 4 are location specific and are prevalent in Punjab and Haryana states of India (Haware *et al.*, 1992b).

The classical method of race identification based on inoculation of differential chickpea cultivars (Haware and Nene, 1982c) is time consuming, labour intensive and requires expensive glasshouse conditions, as it is sensitive to variations in environment. Earlier studies on serological, electrophoretic and biochemical variability have also revealed that the four Indian physiological races of *Foc* have close antigenic relationship, common isozyme patterns for catalases, esterases, peroxidases and similar proteins; hence these may not be very useful for characterizing individual races (Desai *et al.,* 1992a, 1992b).

Molecular markers can be potentially useful in overcoming the above limitations and providing additional information for fungal characterization (Ouellett

and Seifert, 1993). Previous studies by Pérez-Artés *et al.* demonstrated that all *Foc* races had the same mitochondrial DNA RFLP pattern (Pérez-Artés *et al.* 1995). Recently, Jiménez-Gasco *et al.* (2001) clustered *Foc* isolates into three groups using RAPD markers, wherein isolates with the yellowing pathotype grouped into two clusters corresponding to races 0 and 1B/1C, while the wilt causing isolates constituted a third cluster including races 1A, 2, 3, 4, 5 and 6. However, Indian *Foc* races, which have the wilting pathotype and are highly virulent, were not differentiable by these RAPD markers (Jiménez-Gasco *et al.*, 2001; Kelly *et al.*, 1994). A monophyletic origin for *Foc* races has been suggested based on sequencing of conserved regions from introns of the genes for translation elongation factor 1α (EF1α), β-tubulin, histone 3, actin, and calmodulin. In these studies, all isolates pathogenic to chickpea shared an identical EF1α sequence, which differed from that shared by three *Fusarium oxysporum* isolates nonpathogenic to chickpea, hence *Foc* isolates formed a group distinct from other *formae speciales* and nonpathogenic isolates (Jiménez-Gasco *et al.*, 2002).

The utility of DNA markers in detecting polymorphism is well established in several animal, plant, and fungal systems (Weising *et al.*, 1991a). DNA fingerprinting approach has been exploited in the studies of population structure, epidemiology, and systematics of fungi pathogenic to plants and animals (Rosewich and McDonald, 1994). In addition to RFLP and RAPD markers (Hamer *et al.*, 1989; Kistler *et al.*, 1991; Levy *et al.*, 1991; Milgroom *et al.*, 1992), which have been successfully used to estimate the genetic diversity in fungal pathogens, microsatellite based markers have found application in variability analysis in many fungi (DeScenzo and Harrington, 1994) like *Ascochyta rabei* (Geistlinger *et al.*, 1997a); *Sclerospora graminicola* (Sastry *et al.*, 1995); and *Letosphaeria maculans* (Meyer *et al.*, 1992). Several techniques have been developed to exploit the variability in microsatellites for DNA profiling and molecular marker generation such as (i) Simple sequence repeat RFLP technique which involves the use of synthetic oligonucleotides complementary to microsatellite motifs as hybridization probes for mutilocus DNA fingerprinting (Weising *et al.*, 1995) (ii) as

STMS (sequence-tagged microsatellite markers) markers to detect length variation of individual microsatellite repeats (Powell *et al.*,1996) and (iii) single oligonucleotide primers complementary to mini- or microsatellite repeats for amplification of inter-simple sequence repeat (ISSR) regions in genomic DNA (Heath *et al.*, 1993; Zietkiewicz *et al.*, 1994). In this and the following sections of the present thesis, I have used all the above-mentioned types of markers for analysis of *Foc* and *A. rabiei*. However, as described earlier in this section, DNA marker based studies in *Foc* races have mainly focused on the use of RAPD-PCR and mitochondrial DNA-restriction fragment length polymorphism approaches.

In this section, I have described the use of simple sequence repeats (di-, tri-, and tetranucleotide repeats) complementary to microsatellite loci in the genome to distinguish the four *Foc* races predominant in India, and to assess their potential for DNA fingerprinting.

2A.3 Materials and Methods

2A.3.1 Sampling of isolates

Foc isolates, cultured from roots of chickpea plants showing typical wilt symptoms from different regions of India where chickpea is cultivated, were obtained from the Legumes Pathology Unit at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India. The cultures were obtained from single germinating conidia on water-agar medium and subcultured on PDA medium (Potato Dextrose Agar: 200g peeled potatoes, 20g dextrose, 20g agar per liter) as described in section 1B.5. These isolates were characterized by conventional methods at ICRISAT (as described in section 1B.5 and 1B.6 of review of literature) and their identity was confirmed by was confirmed by CABI Biosciences, U.K., formerly Commonwealth Mycological Institute (C.M.I.). Single conidial isolates of the four Indian races of *Fusarium oxysporum f.* sp. *ciceri* (races 1, 2, 3, and 4) were used for the microsatellite based studies.

2A.3.2 DNA isolation

The fungal cultures were maintained on Potato Dextrose Agar (PDA slants) at 5°C and multiplied on MYG liquid medium (0.3% malt extract, 0.5% yeast extract, 1% glucose) at 28°C, 160 rpm for 48 h. The mycelial mass was harvested by vacuum filtration on sterile Whatmann No.1. filter discs, and washed thoroughly by passing two to three volumes of sterile water. Either fresh or frozen tissue was used for DNA extraction using a modified CTAB DNA extraction protocol. The fungal mycelium, ground to a fine powder using mortar-pestle was suspended in a buffer containing 25 mM Tris-HCl pH 8.0, 10 mM EDTA, and 1% BME. EDTA and SDS were added at final concentrations of 60mM and 1%, respectively and incubated at 57°C for 10 min with intermittent mixing. The slurry was reincubated at 57°C for 10 min with NaCl and CTAB (final concentrations of 1.4 M 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, 1 mM EDTA, 1 M NaCl) and reprecipitated with 2.5 volumes of absolute ethanol. The pellet was washed with 70% ethanol, dried under vacuum, dissolved in TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0) and incubated with RNase A at a concentration of 100μg/ml at 37°C for 1.0 h. Concentration of DNA was estimated by visual comparison with known concentration of standard bacteriophage lambda DNA (Bangalore Genei, India) by agarose gel electrophoresis.

2A.3.3 Microsatellite hybridization

Fungal DNA (10μg) was digested to completion with several hexanucleotide and tetranucleotide cutting restriction enzymes, and one pentanucleotide cutting restriction enzyme according to manufacturers' instructions (Promega, USA, Bangalore Genei, India). DNA fragments, thus obtained were separated by electrophoresis in 0.8% or 1.2% agarose gels submerged in 1x TAE buffer (0.04M Tris acetate; 0.001M EDTA, pH 8.0) depending on the restriction enzyme used. DNA was transferred from agarose gels to Hybond N membrane (Amersham, U.K.) using vacuum blotting apparatus

(LKB) as described by Sambrook *et al.* (1989). In few cases, gels were dried *in vacuo*. Dinucleotides $(AT)_{10}$, $(CT)_{10}$, and $(TG)_{10}$, trinucleotides- $(ACA)_5$, $(ACC)_5$, $(ACG)_5$, $(ACT)_5$, $(AGC)_5$, $(AGG)_5$, $(AGT)_5$, $(ATC)_5$, and tetranucleotides $(GACA)_4$, and $(GATA)_4$, synthesized on a gene assembler plus (Pharmacia, U.K.), desalted on NAP-5 column and purified on 20% denaturing polyacrylamide gels were used for hybridization studies. Oligonucleotide probes were end-labelled as described by Sambrook *et al.* (1989) using γ-^{32}P dATP. The DNA blots were used for pre-hybridization and hybridization according to manufacturers' instructions, while dry gels were used for hybridization as described by Ali *et al.* (1986). Hybridization was carried out overnight in a hybridization oven at Tm-5°C (Miyada and Wallace, 1987) by adding labelled probe (10^7-10^8 cpm/μg DNA) directly to the prehybridization solution. The blots/dry gels after hybridization were washed at various stringent conditions, exposed to X-ray films in presence of intensifying screens and incubated at -70°C. After adequate exposure, autoradiograms were developed.

2A.3.4 Analysis of molecular data

Band positions on the autoradiograms were visually determined and pair-wise comparisons of degree of band sharing were made. Fingerprint patterns were transformed into a binary matrix (1 for presence and 0 for absence of a band at a particular position). Similarity index values were calculated by Nei's method (Nei and Li, 1979), as S.I.= 2Nab /Na+Nb, where Na= total number of bands present in lane ‘a’, Nb = total number of bands present in lane ‘b’, and Nab= number of bands common to lanes ‘a’ and ‘b’. Computer software TAXAN, version 2.0 (Swartz D., University of Maryland, USA 1980), was used to calculate genetic distance values from simple matching of bands and a phenogram was constructed by the unweighted pair group method using arithmetic averages (UPGMA) (Sneath and Sokal, 1973). To determine robustness of the phenogram, the data were bootstrapped with 1000 replications using the computer program Winboot (IRRI, Manila, Philippines). Probability of identical match by chance (for each enzyme-probe combination) by which the two genotypes would show identical band pattern was calculated as $(X_D)^n$, where X_D represents

average similarity index value for all pair-wise comparisons and 'n' represents the number of total bands shared per probe (Wetton *et al.*, 1987). $(X_D)^n$ values were then compared to categorize the oligonucleotide probes used.

2A.4 Results

2A.4.1 General features of restriction endonuclease digestion of genomic DNA of *Foc* races and hybridization with oligonucleotide probes

Eleven restriction enzymes including seven hexanucleotide cutting (*Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, and *Pvu*II), one pentanucleotide cutting (*Hinf*I) and three tetranucleotide cutting enzymes (*Alu*I, *Hae*III, and *Taq*I) were selected out of several restriction enzymes used for initial trials, as they revealed good DNA digestion for the four races. Genomic DNA digests in the size range 23.0 to 0.4 kb were obtained with hexanucleotide cutting enzymes, whereas tetra- and pentanucleotide cutting restriction enzymes yielded a smear in the size range 6.0 to 0.4 kb. Genomic DNA digests with *Eco*RV, *Hin*dIII and *Pvu*II depicted bands superimposed on smears in agarose gels, indicating the presence of repetitive DNA. Digestion smear below 2.0 kb with *Alu*I reflected the abundance of *Alu*I restriction sites in the *Foc* genome. However, genomic DNA digests of the four *Foc* races revealed discrete bands in the size ranges 6.0 to 1.8 kb for *Hae*III, 2.8 to 1.3 kb for *Hin*fI, and 4.2 to 2.5 kb for *Taq*I (Fig. 2.1). In general, the patterns obtained for race 3 were always different from those for the other three races as exemplified in Fig. 2.1. For assessing the potential of oligonucleotides as fingerprinting probes, sixty enzyme-probe combinations, including three dinucleotide, eight trinucleotide and two tetranucleotide repeats as probes, were used for hybridization.

However, the similarity matrix and dendrogram were constructed using data obtained from twenty-one such combinations, which exhibited distinct and scorable hybridization bands. Intense signals were obtained with almost all oligonucleotide probes used, indicating the abundance of these repeat motifs in the *Foc* genome. It was

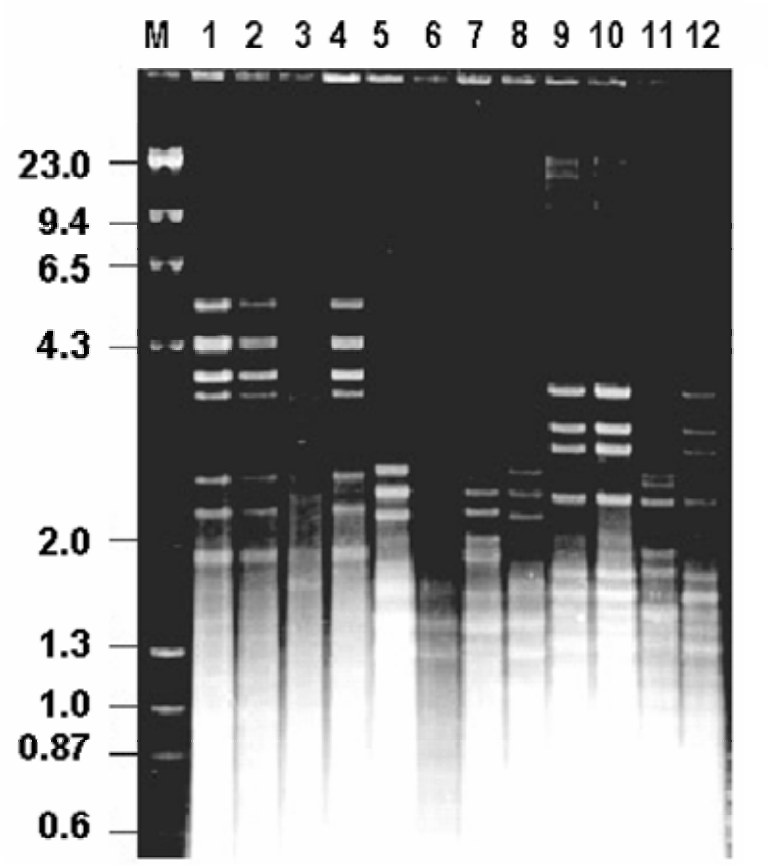


Fig. 2.1 Agarose gel electrophoresis of genomic DNA digests of *Fusarium oxysporum* f. sp. *ciceri* races 1, 2, 3 and 4 with restriction enzymes *Hae*III, *Hin*fI and *Taq*I. *Lanes 1–4* Pattern for *Hae*III with races 1, 2, 3 and 4, respectively, *lanes 5–8* and *9–12* patterns for *Hin*fI and *Taq*I, respectively, for races 1, 2, 3 and 4 in the same order, *lane M* λ/*Hin*dIII molecular-weight marker (in kilobases).

interesting to note that some di-, and trinucleotide repeats revealed bands superimposed on a smear; whereas other dinucleotide repeats and most of the tri-, and tetranucleotide repeats exhibited discrete bands.

2A.4.2 Hybridization patterns with specific enzyme-probe combinations

Hybridization of genomic DNA digested with *Bam*HI, *Eco*RV, *Hin*dIII, *Pst*I, and *Pvu*II with different oligonucleotide probes yielded bands from 21.0 to 0.7 kb range, whereas lower molecular weight fragments ranging from 2.0 to 0.6 kb were seen with tetra-, and pentanucleotide cutting restriction enzymes. However, in case of *Hin*fI/$(GATA)_4$, hybridization bands were obtained in the range of 5.0 to 0.6 kb. Figure 2.2A depicts hybridization of $(TG)_{10}$ with *Bam*HI digested *Foc* genomic DNA revealing discrete bands (21.0 to 1.7 kb) superimposed on a smear, which persists even after stringent washes. A higher number of scorable bands are observed in races 1, 2, and 4 (Fig. 2.2A, lanes 1, 2 and 4), as compared to race 3 (Fig. 2.2A, lane 3). The banding pattern is almost monomorphic for races 1, 2, and 4, whereas bands in race 3 are highly polymorphic. Figure 2.2B is a representative hybridization profile of trinucleotide repeat $(AGT)_5$ with *Eco*RV digests of genomic DNA of races 1 to 4 (lanes 1 to 4). Prominent hybridization bands are detected in the size range 9.4 to 0.7 kb for *Eco*RV digests.

Polymorphic bands obtained in *Foc* races indicate the informativeness of such combinations for their fingerprinting. Figures 2.3A and 2.3B represent hybridization patterns of *Bam*HI digested genomic DNAs of four races with $(GATA)_4$ and $(ATC)_5$, respectively, where the same dry gel was used in both the cases. Intense signals obtained for race 3/$(ATC)_5$ (Fig. 2.3B, lane 3) as compared to race 3/$(GATA)_4$ (Fig. 2.3A, lane 3) reflect the higher frequency of $(ATC)_n$ repeats as compared to $(GATA)_n$ repeats in its genome. Table 2.1 summarizes the data on number of bands obtained on hybridization with various enzyme-probe combinations in four *F. oxysporum* f. sp. *ciceri* races. In most cases, the number of bands obtained in race 3 is equal or less than

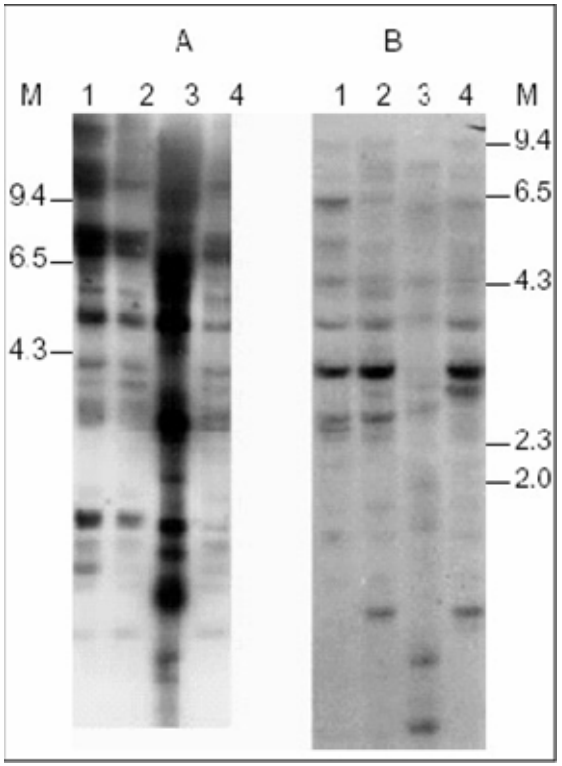


Fig. 2.2 A, B Hybridization profiles of genomic DNA of *Fusarium oxysporum* f. sp. *ciceri*. *Lanes 1–4* Races 1, 2, 3 and 4 digested with (**A**) restriction enzyme *Bam*HI and probed with $(TG)_{10}$ and (**B**) digested with *Eco*RV and probed with $(AGT)_5$. *Lane M* λ/*Hin*dIII molecular-weight marker (in kilobases).

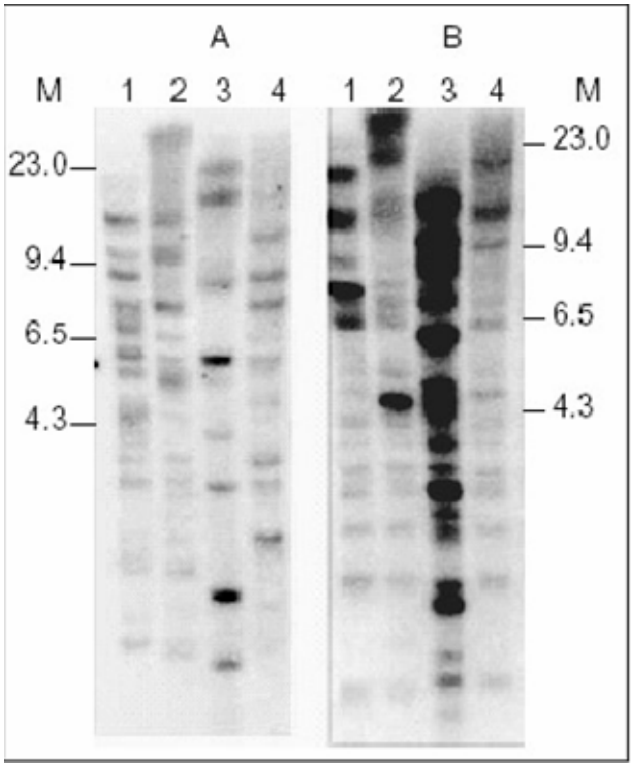


Fig. 2.3 A, B Hybridization profiles of genomic DNA of *Fusarium oxysporum* f. sp. *ciceri. Lanes 1–4* Races 1, 2, 3 and 4 digested with (**A**) restriction enzyme *Bam*HI and probed with $(GATA)_4$ and (**B**) digested with *Bam*HI and probed with $(ATC)_5$. *Lane M* λ */Hin*dIII molecular-weight marker (in kilobases).

that obtained for the other three races, with only two exceptions, *Bam*HI/$(ATC)_5$ and *Bam*HI/$(AGC)_5$ (Table 2.1).

2A.4.3 Fingerprinting with microsatellites identifies presence of unique bands in the *Foc* races

The 'Probability of identical match by chance' $(X_D)^n$ value is an indicator of the degree of informativeness of an enzyme-probe combination for fingerprinting the races. In general, lower the value, less is the chance that any two races exhibit identical banding patterns with that combination, and hence, greater is the potential of that combination to identify each race discretely. Twenty-one combinations, which yielded clear and scorable banding patterns were used for calculation of $(X_D)^n$ values. Based on these values detailed in Table 2.2, enzyme-probe combinations for fingerprinting of *Foc* races are arbitrarily categorized as (i) informative $\{(X_D)^n$ value below $10^{-5})\}$, (ii) less informative $\{(X_D)^n$ values between 10^{-3} and $10^{-5}\}$, and (iii) least informative $\{(X_D)^n$ values above $10^{-3}\}$. Oligonucleotides $(TG)_{10}$, $(AGT)_5$, $(ATC)_5$, and $(GATA)_4$ reveal low $(X_D)^n$ values with many enzymes used in the present study, and hence qualify as the best probes for fingerprinting of *Foc* races (Table 2.2). $(AGC)_5$ reveals intermediate fingerprinting potential while $(CT)_{10}$ and $(ACC)_5$ are uninformative as fingerprinting probes. Although the $(X_D)^n$ values are low for *Bam*HI/$(TG)_{10}$ and *Hae*III/$(TG)_{10}$ combinations ($6.32x10^{-6}$ and $8.3x10^{-4}$, respectively), $(TG)_{10}$ is not a good fingerprinting probe for the *Foc* races due to the grossly monomorphic banding pattern obtained for races 1, 2 and 4 (Fig. 2.2A). However, the low $(X_D)^n$ values for *Bam*HI/$(TG)_{10}$ and *Hae*

Section 2B

Genetic diversity analysis of Indian isolates of *Foc* and other *Fusarium* spp. using PCR based ISSR and AFLP markers

2B.1 Abstract

Fusarium oxysporum f. sp. *ciceri* (*Foc*) is a serious yield reducer and an impediment to chickpea productivity in several chickpea growing regions of the world. Studying variability in *Foc* isolates from various agro-climatic zones represents the first step in the overall objective of breeding fusarium wilt resistant chickpea cultivars. Genetic diversity in 31 Indian isolates of *Foc* and two isolates of *F. solani* and *F. udum* was studied using inter-simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP) markers. The two marker systems clearly differentiated *Foc* isolates from *F. solani* and *F. udum*. Among *Foc* isolates, race 1, 2 and 4 isolates formed a strongly bootstrapped group, while race 3 isolates formed a distinct cluster. The data at 196 ISSR and 1009 AFLP loci further grouped race 1 and 2 isolates into discrete clades corresponding to their physiological race reaction. ISSR primer UBC-835 and AFLP primer combination E+AA/m+caa were identified as potential candidates for developing race specific markers for *Foc* races 1 and 2, which are widespread in India.

2B.2 Introduction

Molecular markers are useful in identifying isolates, monitoring disease demographics, or investigating evolutionary relationships, especially if the relationship is maintained over time and with increased sample size (Elias *et al.*, 1993). Neutral DNA markers can be exploited to identify races for pathogens, especially with asexual reproduction where no recombination occurs and the entire pathogen genome is effectively linked (Milgroom and Fry, 1997). If correlated to races, they can be developed into race specific markers. DNA markers are useful in studying genetic diversity in pathogen populations (Santra *et al.*, 2001), which is required to identify shifts in race or population structure (McDonald, 1997) and to breed for cultivars with durable resistance to known variants of the pathogen. Two types of DNA markers, namely hybridization based and PCR based have been used for such studies (Brown, 1996; McDonald, 1997; Sunnucks, 2000). PCR based multilocus DNA markers have become increasingly popular for studying populations of plant pathogenic fungi because of their ease of handling and small amounts of DNA sufficient for PCR amplifications as compared to hybridization based markers (Sunnucks, 2000). Among many PCR based marker systems, ISSR markers have been widely used in plants (Pujar *et al.*, 2002; Wolfe and Liston, 1998, and references therein) and to a somewhat lesser extent in fungi (Arenal *et al.*, 1999; Han *et al.*, 2002; Zhou *et al.*, 1999). The AFLP technique (Vos *et al.*, 1995) has also 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 (Baayen *et al.*, 2000; Jurgenson *et al.*, 2002; Majer *et al.*, 1998; Zhong *et al.*, 2002).

My studies demonstrating the potential of simple sequence repeat hybridization profiles to detect polymorphism in the four races from India (Section 2A) encouraged me to use microsatellite based PCR primers (ISSR primers) for diversity analysis in a larger set of *Foc* isolates sampled from various agro-climatic regions in India where chickpeas are grown. Studies on these *Foc* isolates, including standard races, *F. solani*

and *F. udum* using ISSR and AFLP markers are presented in this section. *F. udum* and *F. solani* were included as outgroup species in my studies with *Foc*.

2B.3 Materials and Methods

2B.3.1 Sampling of *Foc* isolates and DNA isolation

Foc isolates from different chickpea cultivation regions of India were procured from the Legumes Pathology Unit at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India. The single condial cultures were characterized by conventional methods at ICRISAT as described in section 2A.3.1. Their identity was confirmed by CABI Biosciences, U.K., formerly Commonwealth Mycological Institute (C. M. I.). Thirty-three such isolates including four standard races of *Foc* (races 1, 2, 3, and 4), and two isolates representative of *F. solani* and *F. udum* were used in this study (Table 2.4). Map locations of the places of collection of isolates listed in Table 2.4, are indicated on the map of India (Fig. 2.5). DNA was isolated from the mycelia of each of these isolates using a modified CTAB DNA extraction protocol as detailed in section 2A.3.2.

2B.3.2 PCR with ISSR and AFLP primers

Genomic DNA of *Foc* races 1 and 4 was used for initial screening of eighty ISSR primers (UBC series 9, UBC, Vancouver) to select polymorphic primers. Ten primers, which yielded reproducible polymorphic bands (Table 2.5), were used to amplify template genomic DNA from twenty-seven of the thirty-three *Foc* isolates (indicated by 'a' in Table 2.4). Each amplification reaction contained 25-30ng genomic DNA, 200µM dNTPs, 1X PCR buffer, 0.8u *Taq* DNA Polymerase (Perkin-Elmer, AmpliTaq) and 0.3µM UBC primer in a 25µl reaction volume. The reactions were thermocycled using the Perkin-Elmer GeneAmp 9700 PCR System. PCR cycles were performed for 5 min at 94°C, followed by forty cycles of denaturation at 94°C, annealing at 50°C to 54°C (depending on the T_m of the primer), and extension at 72°C for one min each, and final extension at 72°C for 5 min. Amplification products were separated by

Table 2.4 *Foc* isolates used for ISSR and AFLP studies and their location of collection

Isolates used and their physiological race designations	**Location of Collection**
Race 1[a](Standard isolate), *Foc* 6[a](1), *Foc* 10(1), *Foc* 13[a](1), *Foc* 14[a](1), *Foc* 15[a](1), *Foc* 16[a](1), *Foc* 17[a](1), *Foc* 18[a](1), *Foc* 35[a](1), *Foc* 36[a](2), *Foc* 37(1), *Foc* 38[a](4),	ICRISAT, Hyderabad (Andhra Pradesh)
Race 2[a] (Standard isolate), Fu7[a](3), *Foc* 39 (race not identified), *Foc* 40[a](2)	Kanpur (Uttar Pradesh)
Foc 30[a](1), *Foc* 32(2), *Foc* 31[a] (Independent race reaction)	Durg (Madhya Pradesh)
Foc 4[a](2), *Foc* 11[a](1)	Hisar (Haryana)
Foc 9(2), *Foc* 8(2)	Ludhiana (Punjab)
Foc 7[a](2)	Faridkot (Punjab)
Race 3[a](Standard isolate)	Gurdaspur (Punjab)
Race 4[a](Standard isolate)	Jabalpur (Madhya Pradesh)
Foc 41[a](1)	Rahuri (Maharashtra)
Foc 33[a](1), *Foc* 34[a](1)	Raipur (Madhya Pradesh)
Foc 2[a](2)	Sri Ganganagar (Rajasthan)
F. udum [a]	Dharwad (Karnataka)
F. solani [a]	Durgapura (Rajasthan)

'a': Isolates used for ISSR analysis. The numbers/comments in parentheses indicate the physiological race designation of *Foc* isolates on the basis of reactions to ten chickpea differentials in glass house studies (Rahman, 1997).

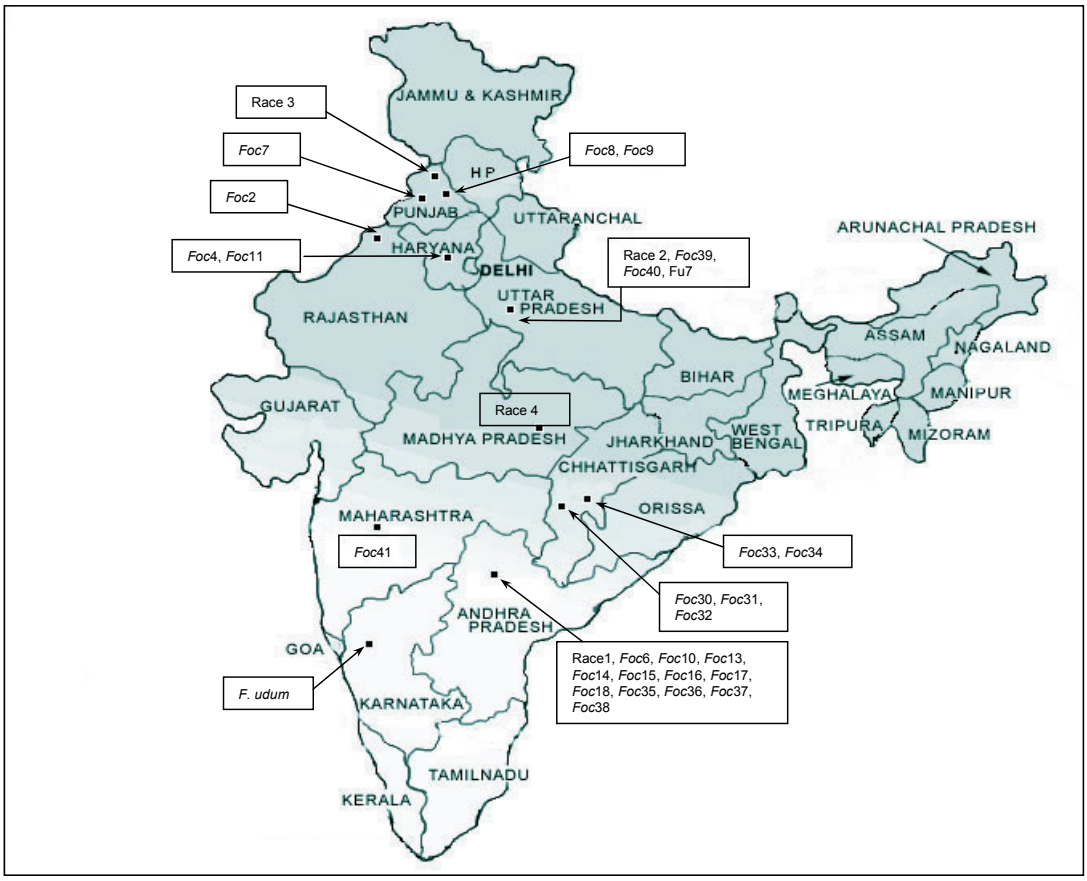


Fig. 2.5 Map locations of *Fusarium* isolates used in the study. Physiological race description of the isolates is given in Table 2.4.

electrophoresis in 2% agarose gels with phage ϕX-174 DNA/*Hae*III as molecular weight marker. Minimum three replications were performed for each primer.

AFLP™ Analysis System I (AFLP Starter Primer Kit, Life Technologies Inc.) was used for performing AFLP reactions. 500ng-1µg genomic DNA from the 33 isolates listed in Table 2.4 was digested to completion with restriction enzymes *Eco*RI and *Mse*I and ligated to adapters provided in the kit as per the supplier's instructions. Subsequent pre-amplification reactions were performed using *Eco*RI+A and *Mse*I+C primers. Pre-amplified products were used as template DNA in 20µl amplification reactions, each containing a γ-33 P end-labelled *Eco*RI +2/+3 primer and a cold (unlabelled) *Mse*I +2/+3 primer (with dNTPs provided in the kit), 0.8u of *Taq* DNA polymerase (AmpliTaq) and 1X reaction buffer. Pre-amplification and amplification reactions were performed in the Perkin-Elmer GeneAmp 9700 PCR System as per the prescribed instructions. Control reactions contained sterile water instead of template DNA. Amplified products were separated on 6% denaturing polyacrylamide gels with γ-33 P labelled phage ϕX-174/*Hae*III digest as a size marker, and the bands were visualized by autoradiography on Konica X-ray film.

2B.3.3 Analysis of molecular data

DNA bands of the same electrophoretic mobility (molecular weight) were assumed to be identical and pair-wise comparisons of degree of band sharing were made. Fingerprint patterns were transformed into a binary matrix (1 for presence and 0 for absence of a band). Computer software TAXAN, version 2.0 (D. Swartz, University of Maryland, USA, 1980) was used to calculate similarity index values and to cluster the isolates by an unweighted pair group method with arithmetic averages (UPGMA) (Sneath and Sokal, 1973) based on Jaccard's similarity coefficient. The data were bootstrapped with 1000 repetitions using the computer program WINBOOT (IRRI, Manila, Philippines) to determine robustness of clusters. Clusters obtained in the phenograms were compared with groups based on physiological race reactions with ten host differentials.

Analysis of molecular variance of the data (AMOVA) was performed using the software 'Arlequin' version 2 (Schneider *et al.*, 2000). AMOVA estimates the different molecular variance components among populations and among individuals within a population. The populations were defined in three ways: (i) by physiological reaction only (race 1, race 2, race 3, race 4, unknown/different reaction, other species) (ii) by geographic location of collection only (North India (N.I.), Central India (C.I.) and South India (S.I.)) and (iii) by race and geographical location of collection (for example 'race 1 isolates from N.I.', 'race 1 isolates from C.I.' etc.). These populations were further grouped either by 'physiological reaction' or by 'geographic location of collection'.

2B.3.4 DNA amplification and sequencing for elongation factor in *Foc* isolates

Genomic DNA of the four Indian standard races, namely *Foc* races 1, 2, 3 and 4, isolates Fu-7 and *Foc*39, *F. solani*, and *F. udum* was subjected to PCR amplification with primers EF-1 and EF-2, which prime within conserved exons of nuclear elongation factor 1α (EF-1α) and nucleotide sequences were obtained as described previously (O'Donnell *et al.*, 1998b). The sequence data was subjected to phylogenetic analysis using PAUP* version 4.0b1 (Swofford, 1998). Unweighted parsimony analyses were performed with the heuristic search option and 1,000 random addition sequences with the MULTIPARS function on, and with tree bisection-reconnection branch swapping. A sequence of *F. udum* (NRRL 22114) was used to root the gene tree by the outgroup method. Clade stability was assessed by 1, 000 parsimony bootstrap replications. Sequences from reference strains *Fusarium oxysporum* f. sp. *vasinfectum* race 1 (NRRL 25420 *Fov* 1) and *Fusarium oxysporum* f. sp. *lycopersici* (NRRL 26034), and *F. proliferatum* (accessions AF160280 and AF291058) were included in the phylogenetic analyses.

Table 2.5 Resolving power of ISSR and AFLP primers used for the study

ISSR Primers		***AFLP Primers***	
Primer Name	**Number of loci**	**Primer pair combination**	**Number of loci**
Primers which differentiate *F. udum*, *F. solani* and *Foc* race 3 from *Foc* races 1, 2 and 4			
UBC 807	18	E+AC/m+cc	82
UBC 841	21	E+AA/m+ct	114
UBC 842	29	E+AG/m+ct	145
UBC 849	17	E+AG/m+cat	73
UBC 850	16	E+ACT/m+cat	64
UBC 856	11	E+AAG/m+caa	46
		E+ACG/m+ctg	54
Primers which differentiate (i) *Foc* race 1 and race 2 isolates and/or (ii) isolates from Central and North Vs Central and South India			
UBC 868	22	E+AC/m+cg	86
UBC 881	11	E+AG/m+cg	77
UBC 834	24	E+ACA/m+ctt	45
UBC 835#	27	E+AA/m+ca	132
		E+AA/m+caa#	91

Best primers to differentiate *Foc* race1 and 2 isolates

2B.4 Results

2B.4.1 Selection of primers and isolates for ISSR and AFLP analysis

When eighty ISSR primers were screened with race 1 and race 4 standard isolates of *Foc*, ten polymorphic primers distinguished the two races (Table 2.5). These ten primers were more likely to be polymorphic among other isolates based on previous reports of microsatellite hybridization patterns of *Foc* races 1 and 4, which were very similar (as described in sections 2A.4.4, 2A.5.2). Twenty-seven isolates used for the ISSR study were representative of the range of pathogenicity reactions obtained with ten chickpea differentials, and were a subset of the 33 isolates listed in Table 2.4

For AFLP reactions, the complete set of 33 isolates was used (Table 2.4). Initially the data were obtained using sixty-four *Eco*RI+3/*Mse*I+3 and sixteen *Eco*RI+2/*Mse*I+2 combinations with pre-amplified DNA of the four races. Of these, six *Eco*RI+2/*Mse*I+2, two *Eco*RI+2/*Mse*I+3 and four *Eco*RI+3/*Mse*I+3 primer combinations yielded polymorphic banding patterns among the four races, and hence were selected for use with all the 33 isolates (Table 2.5).

2B.4.2 Amplification profiles using ISSR and AFLP primers

Amplification patterns with ISSR and AFLP primers were highly reproducible among replications. On an average, 20 loci were scored per ISSR primer, and the bands obtained were in the size range 0.2-2.0kb. In case of AFLP analysis, on an average, 106 bands were obtained per isolate with the *Eco*RI+2/*Mse*I+2 combinations, 82 bands/isolate with *Eco*RI+2/*Mse*I+3 combinations, and 52 bands/isolate with *Eco*RI+3/*Mse*I+3 combinations. Amplification products were in the size range 0.1-1.0kb. *F. udum* and *F. solani* yielded unique amplification profiles irrespective of the ISSR/AFLP primer used. *Foc* race 3 standard isolate and Fu7 (a *Foc* isolate obtained independently and pathotyped as race 3 at ICRISAT), resulted in characteristic banding patterns very similar to each other but different from those of race 1, 2, and 4 isolates with both the marker systems, while *Foc*39 revealed a amplification profile different from the profiles of other isolates of races 1, 2 and 4 with AFLP primers.

When only *Foc* isolates of the four races were considered for ISSR analysis, polymorphism was observed to be 55.40%. Interestingly, exclusion of race 3 isolates resulted in a drastic reduction in polymorphism to 19.17% implying a high level of similarity within the *Foc* isolates of races 1, 2, and 4. The higher value of percentage polymorphism (55.40%) in all the four isolates was thus due to polymorphic bands in the race 3 isolates. A similar trend was also seen using AFLP markers, thereby providing support to my observations with ISSR markers.

2B.4.3 Individual ISSR and AFLP primers for differentiation of *Foc* races

Phenograms obtained by cluster analysis of binary data from 10 ISSR and 12 AFLP primers were individually analyzed to evaluate their resolving power and the resolution obtained with individual ISSR and AFLP primers is summarized in Table 2.5.

All ISSR primers clearly distinguished *F. udum, F. solani,* and *Foc* race 3 isolates from isolates of races 1, 2 and 4. ISSR primers UBC 834, 835, 868 and 881 clustered the isolates into groups generally corresponding to race and/or geographic distribution (Table 2.5). In particular, the primers UBC 834 and 835 clearly resolved isolates of races 1 and 2 into separate clades. Figure 2.6 represents the clustering pattern of *Foc* isolates obtained with UBC 835, wherein the cluster of isolates with a race 1 like reaction received bootstrap support of 71%, whereas isolates with a race 2 like reaction clustered together with 28.4% bootstrap support with the exception of grouping of *Foc*36 and *Foc*11 in the race 1 and race 2 clades, respectively. However, race 4 standard isolate and isolate *Foc*38 (race 4 like reaction) could not be grouped together with any of the primers used. Furthermore, the race 1 clade contained isolates from central and southern India while the race 2 clade included isolates from central and north India.

Phenograms from individual AFLP combinations revealed that all the primers used clearly differentiated *F. udum, F. solani, Foc* race 3, Fu7, and *Foc*39. Primer combinations E+AC/m+cg, E+AA/m+ca, E+AG/m+cg, E+AA/m+caa and E+ACA/m+ctt clustered the *Foc* isolates into groups generally corresponding to race

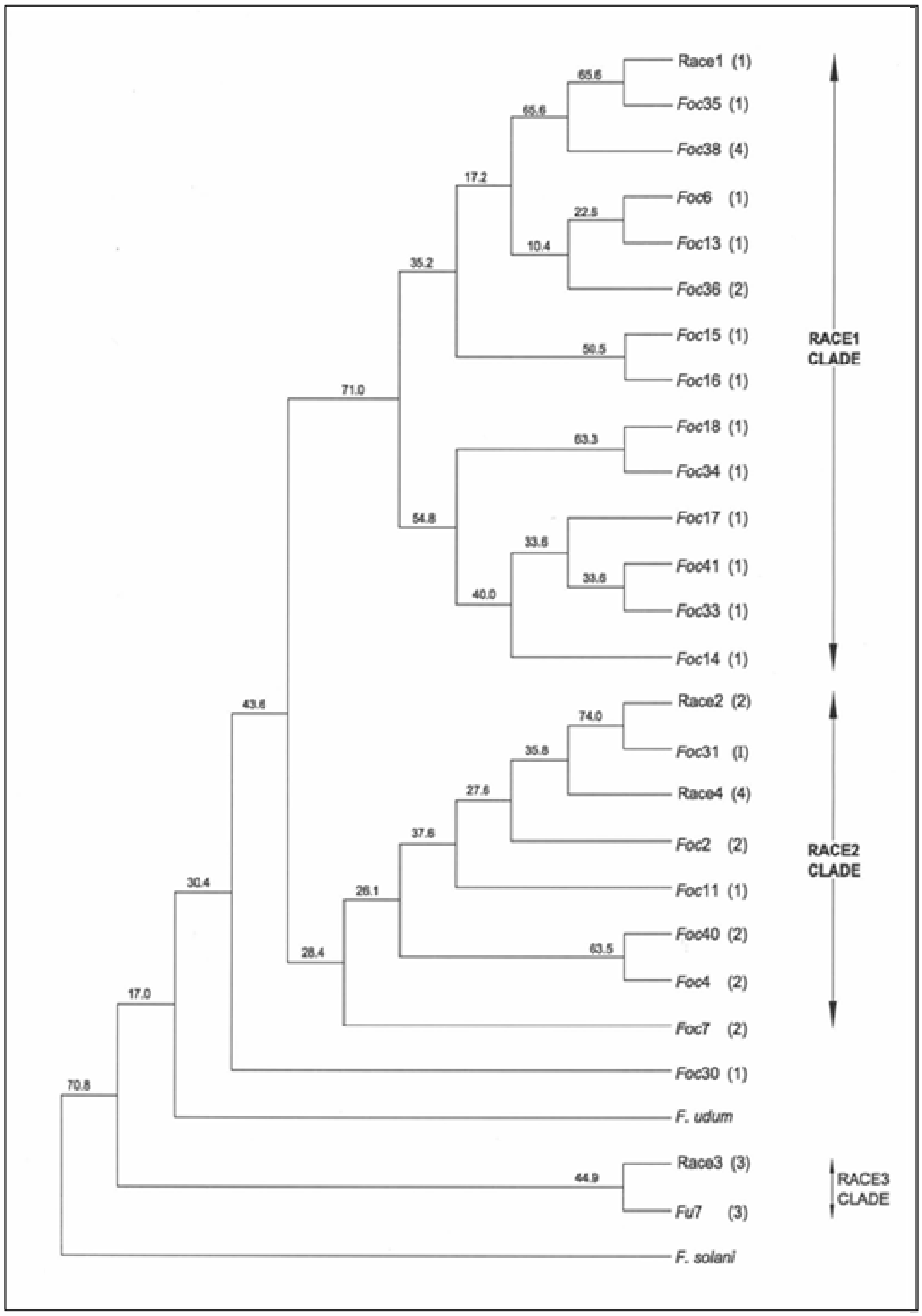


Fig. 2.6 Phenogram of *Fusarium oxysporum* f. sp. *ciceri* (*Foc*) isolates obtained with ISSR primer UBC 835 using Jaccard's coefficient. Bootstrap values are indicated on the internodes. Physiological race reactions of the *Foc* isolates using ten host differentials are presented as numbers/comments in parentheses.

and/or geographic distribution (Table 2.5). Of these, E+AA/m+ca and E+AA/m+caa combinations clearly resolved the isolates into two clades according to their physiological race. Figure 2.7 represents the clustering pattern of *Foc* isolates with AFLP primer combination E+AA/m+caa depicting a clear differentiation of two clades, which together received 100% bootstrap support. The race 1 clade with a bootstrap support of 61% grouped isolates collected from central and southern India, and the race 2 clade with a support of 54.7% grouped isolates collected from central and northern India. However, isolates *Foc*11 and *Foc*36 remained exceptions in this case too, in that they did not group as per their race designation (Table 2.4). It was interesting to note that within the race 1 clade, *Foc*38 (race 4) was separate from other race 1 isolates, while the ICRISAT isolates *Foc*6, *Foc*10 and *Foc*13 formed a robust cluster. Within the race 2 clade, *Foc*31 and *Foc*32 collected independently from a single location (Durg) clustered together (Fig. 2.7). *Foc*2, *Foc*7, and *Foc*9 (isolates from Punjab and Sriganganagar/Haryana) formed a sub-cluster with 74% support. Further, the race 4 standard isolate remained separate from race 2 isolates within the race 2 clade (Fig. 2.7). Although *Foc*39 (an isolate from Kanpur) always remained separate from the race 1 and 2 clades, it received a strong bootstrap support (100%) with other isolates of race 1, 2 and 4. However, none of the AFLP primer combinations used by us clustered isolate *Foc*38 (showing a race 4 like reaction) with race 4 standard isolate.

2B.4.4 Race 1 and 2 specific bands with AFLP markers

Analysis of the AFLP autoradiograms revealed the presence of race specific bands in AFLP primer combinations E+AA/m+ca, E+AA/m+caa and E+AAG/m+caa (Fig. 2.8). In Fig. 2.8(A), representing the AFLP combination E+AA/m+ca, a 229 bp band was observed in all race 1 isolates including standard race 1, while a band of 187 bp was present only in the race 1 isolates. Two race 1 specific bands of the same size were also observed in AFLP profiles of the primer pair E+AA/m+caa wherein, the 229 bp band was present in standard race 1 and race 1 isolates while the 187 bp band was present only in the *Foc* race 1 isolates. In case of the E+AAG/m+caa combination, only one

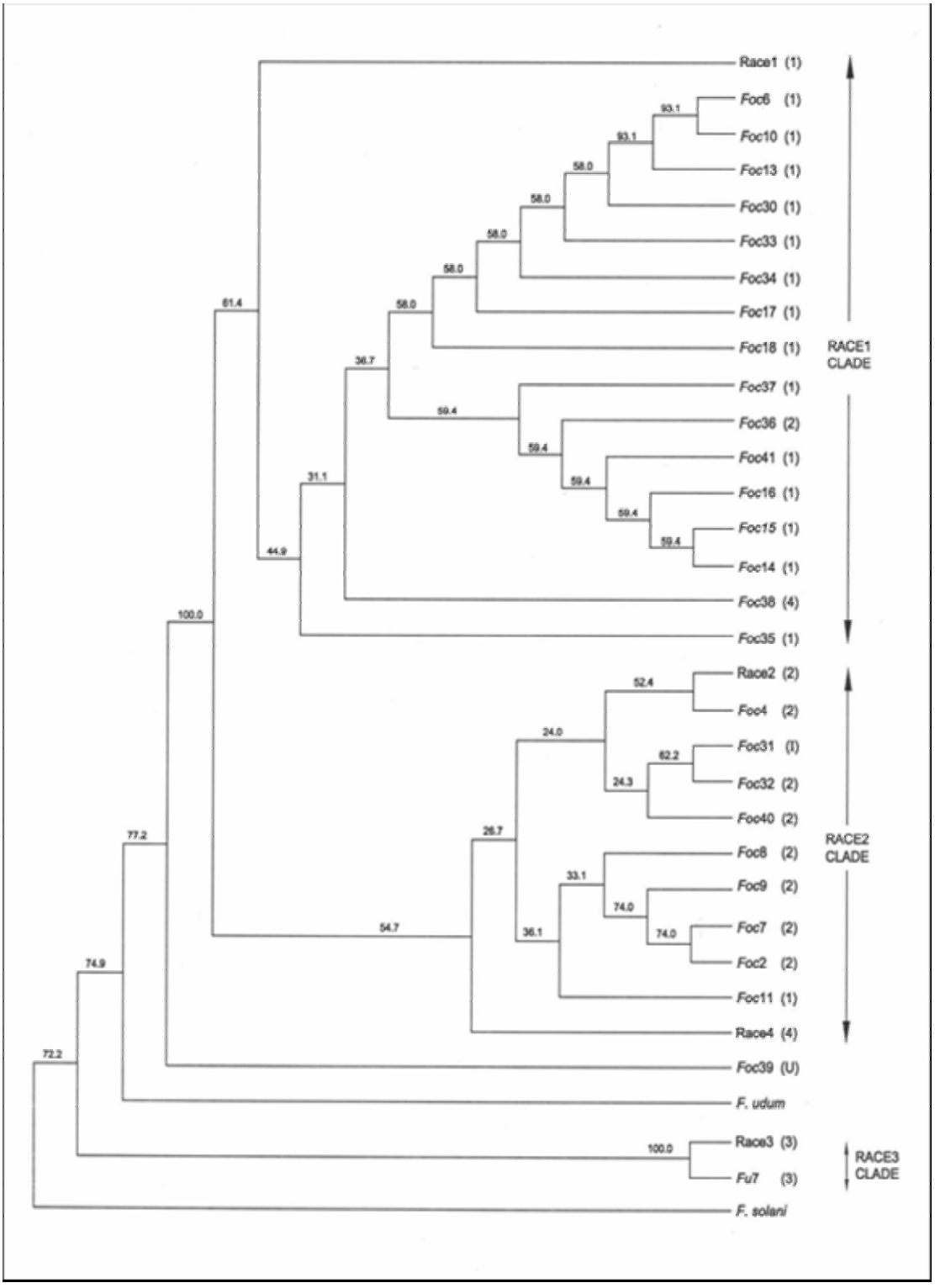


Fig. 2.7 Phenogram of *Fusarium oxysporum* f. sp. *ciceri* (*Foc*) isolates obtained with AFLP primer pair E-AA/m-caa using Jaccard's coefficient. Bootstrap values are indicated on the internodes. Physiological race reactions of the *Foc* isolates using ten host differentials are presented as numbers/comments in parentheses.

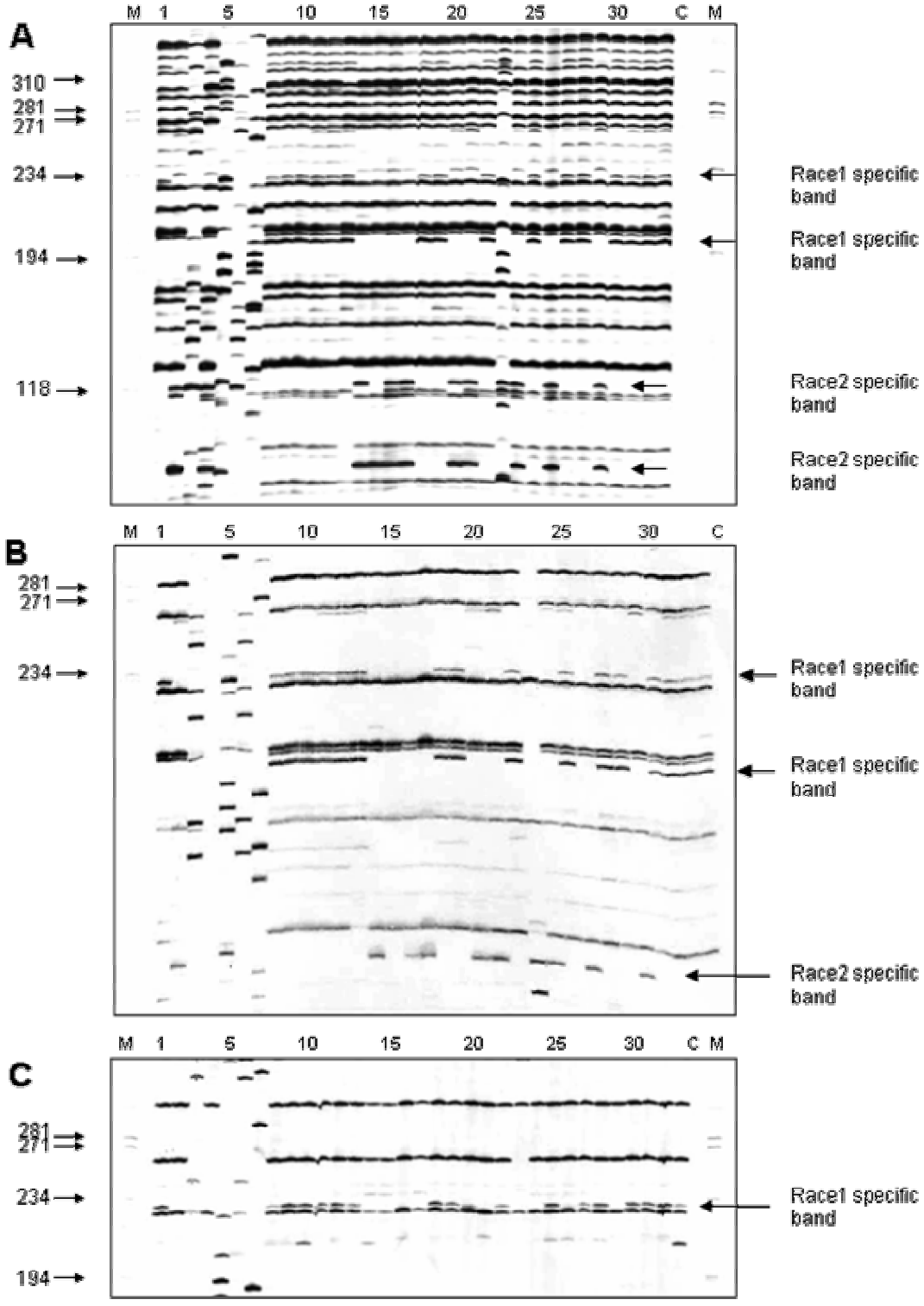


Fig. 2.8 Race 1 and 2 specific bands in *Fusarium oxysporum* f. sp. *ciceri* (*Foc*) isolates using AFLP primer pair (**A**) E-AA/m-ca (**B**) E-AA/m-caa and (**C**) E-AAG/m-caa. Lane M: molecular weight marker ΦX-174/*Hae*III (in base pairs), Lane C: negative control. Lanes 1-33: standard race1 (1), standard race2 (2), standard race3 (3), standard race 4 (4), *F. udum* (another species), Fu7 (3), *F. solani* (another species), *Foc*6 (1), *Foc*10 (1), *Foc*13 (1), *Foc*14 (1), *Foc*15 (1), *Foc*16 (1), *Foc*2 (2)**,** *Foc*7 (2), *Foc*9 (2), *Foc*11 (1), *Foc*30 (1), *Foc*33 (1), *Foc*4 (2), *Foc*32 (2), *Foc*34 (1), *Foc*39 (Race not identified), *Foc*40 (2), *Foc*41 (1), *Foc*8 (2), *Foc*17 (1), *Foc*18 (1), *Foc*31 (Independent race reaction), *Foc*35 (1), *Foc*36 (2), *Foc*37 (1), and *Foc* 38 (4). Physiological race reactions of the *Foc* isolates using ten host differentials are presented as numbers/comments in parentheses.

band corresponding to 229 bp was observed in *Foc* race 1 isolates and the standard race 1. Furthermore, two bands corresponding to 118 bp and 92 bp were specifically present in all isolates of race 2 with primer pair E+AA/m+ca, whereas only the 118 bp band was observed in case of the E+AA/m+caa combination. *Foc*11 (race 1) and *Foc*36 (race 2) (lanes 17 and 31, respectively in Fig. 2.8A, B and C), remained exceptions to the presence of race specific bands, in each case. However, bands specific to races 1 and 2 could not be identified in profiles from ISSR primers.

2B.4.5 Clustering of *Foc* isolates with ISSR and AFLP primers

Data from 10 ISSR and 12 AFLP primer combinations corresponding to 196 ISSR and 1009 AFLP loci were combined into two phenograms (Fig. 2.9 and Fig. 2.10) representing diversity of *Foc* isolates at these loci, respectively.

Figure 2.9 represents the clustering pattern obtained using the binary data at ISSR loci wherein, *Foc* race 3 and the race 3 isolate Fu7 formed a robust cluster. This group along with *F. solani* and *F. udum* isolates was outgrouped from other *Foc* isolates. Further, *Foc* race 1, 2 and 4 isolates received a strong bootstrap support and were grouped into two distinct clades, although bootstrap support for the two clades was poor. Isolates with a race 1 like physiological reaction clustered together in the race 1 clade. *Foc*38, a race 4 isolate, was also grouped in race 1 clade (Fig. 2.9), while *Foc*36 was the only exceptional race 2 isolate, which grouped with race 1 isolates. The race 2 clade contained isolates showing a race 2 like physiological reaction where *Foc*11 was the single race 1 like isolate. Furthermore, the race 1 clade contained isolates from central and southern India, while the race 2 clade contained isolates from central and northern India.

The clustering pattern of *Foc* isolates obtained using binary data at 1009 AFLP loci is represented in Fig. 2.10. In this phenogram, *Foc* race 3 and isolate Fu7 (race 3 isolate) clustered together with bootstrap support of 100%. Further, *F. udum*, *F. solani* and *Foc* race 3 cluster were outgrouped from other *Foc* isolates (Fig. 2.10). *Foc*39,

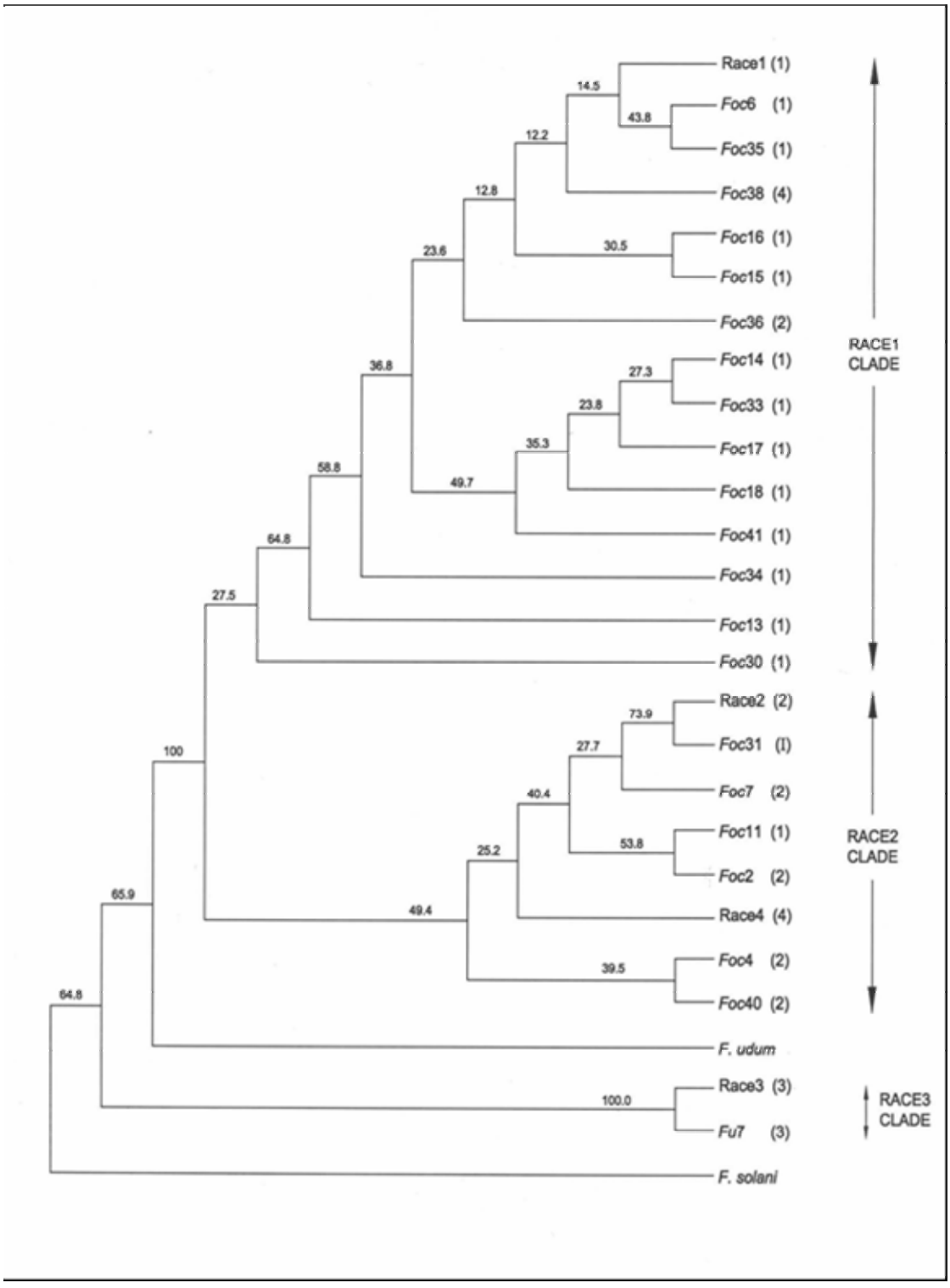


Fig. 2.9 Phenogram of *Fusarium oxysporum* f. sp. *ciceri* (*Foc*) isolates obtained with 196 ISSR loci using Jaccard's coefficient. Bootstrap values are indicated on the internodes. Physiological race reactions of the *Foc* isolates using ten host differentials are presented as numbers/comments in parentheses.

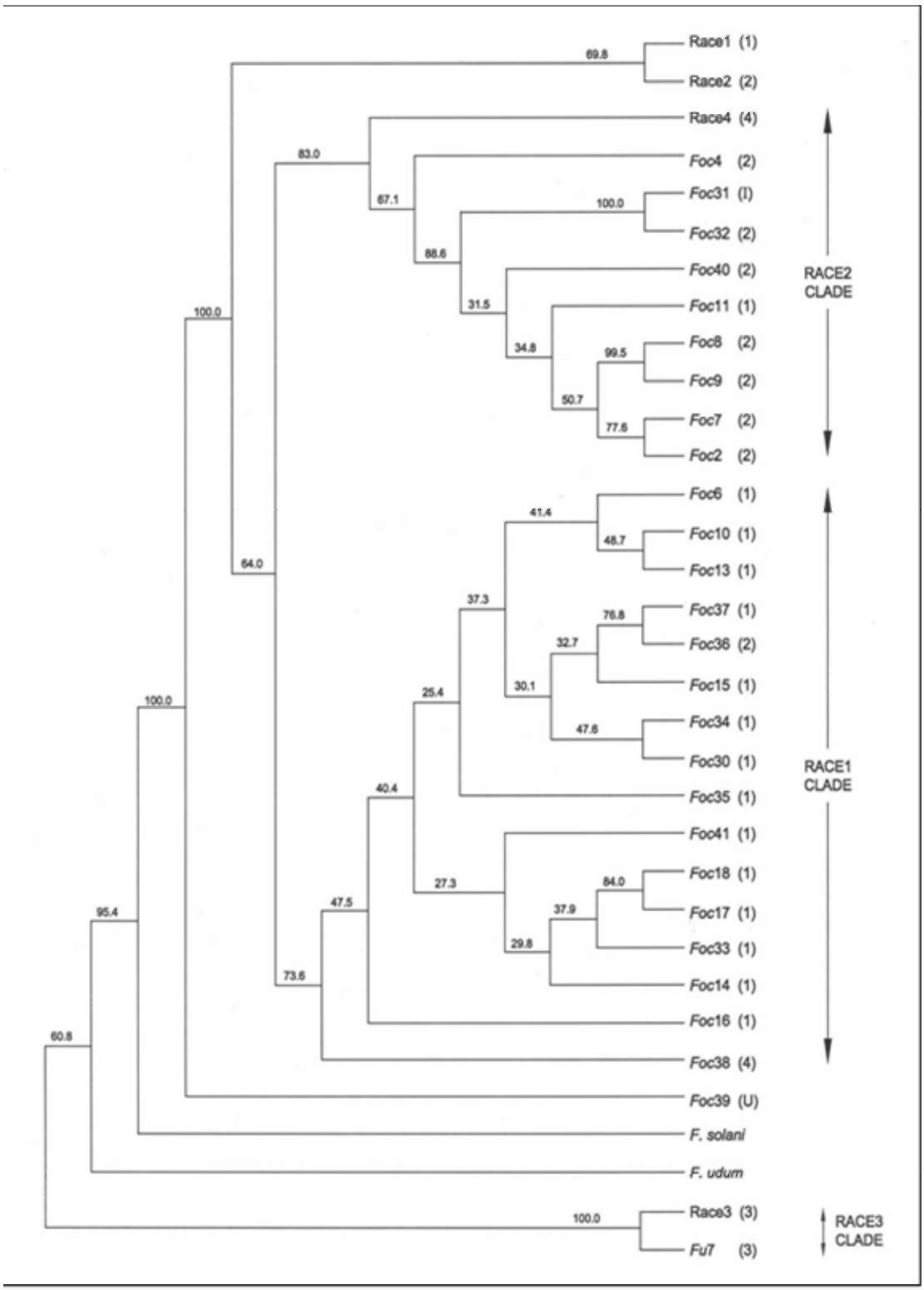


Fig. 2.10 Phenogram of *Fusarium oxysporum* f. sp. *ciceri* (*Foc*) isolates obtained with 1009 AFLP loci using Jaccard's coefficient. Bootstrap values are indicated on the internodes. Physiological race reactions of the *Foc* isolates using ten host differentials are presented as numbers/comments in parentheses.

though an outlier, was strongly bootstrapped (100%) to the cluster formed by *Foc* race 1, 2 and 4 isolates. *Foc* race 1, 2 and 4 isolates (100% bootstrap support) formed two clades primarily according to their race designation. The race 2 clade contained isolates, which exhibited a race 2 like physiological reaction, and clustered together with 83% bootstrap support except *Foc*11, which exhibited a race 1 reaction. Within this clade, *Foc*8 and *Foc*9 (from Ludhiana) and *Foc*31 and *Foc*32 (from Durg, MP) formed groups with bootstrap values of 100%. The standard race 4 isolate also grouped in this clade. Interestingly, isolates from this clade were from the northern and central India. However, standard race 1 and race 2 isolates did not cluster with isolates showing race 1 or race 2 reactions, and were grouped together with 69.8 % bootstrap support. Furthermore, isolates with a race 1 like physiological reaction clustered together in the race 1 clade with bootstrap support of 73.6%. This clade exclusively contained isolates from Central or Southern India and did not include any isolates from North India. *Foc*36 (ICRISAT) was the only exception in this clade showing a race 2 like reaction. Among the race 1 isolates, *Foc*17 and *Foc*18, isolated from IAC Hyderabad, grouped with 84% support. *Foc*38 (a race 4-like physiological reaction) also grouped with the race 1 isolates in this clade, though its position was distant as compared to the other race 1 isolates.

2B.4.6 AMOVA analysis

The genetic differentiation of the populations within and between races and within and between geographic regions was estimated by an analysis of molecular variance (AMOVA). The number of isolates used in ISSR and AFLP studies were different, hence the degrees of freedom for the data were different. When six populations (representing isolates of races 1, 2, 3, 4, 'unknown/different race reactions' and 'other species') within a single group '*Fusarium* isolates from India' were analyzed, 'among populations' and 'within-population' variation was 58.76% and 41.24% with the ISSR and 66.21% and 33.79% with the AFLP data, respectively (Table 2.6). AMOVA analysis of the same set of isolates, alternatively defined by 'geographic location of

Table 2.6 AMOVA analysis of *Foc* isolates with ISSR and AFLP markers

Method of grouping	Source of variation	Degrees of freedom		Variance components		Percentage variation		FST (p=0.05)		P[a]
		ISSR	AFLP	ISSR	AFLP	ISSR	AFLP	ISSR	AFLP	
1) All isolates including *F. solani* and *F. udum*										
1a) By physiological reaction										
	Among populations	5	5	12.50	66.70	58.76	66.21	0.587	0.662	<0.001
	Within populations	21	27	8.78	34.04	41.24	33.79			
1b) By geographical location of collection										
	Among populations	2	2	2.33	5.48	12.77	6.64	0.127	0.066	<0.001
	Within populations	24	30	15.89	76.98	87.23	93.36			
1c) By geographical location of collection and physiological reaction										
	Among groups	5	5	8.64	52.33	40.54	51.69	0.740	0.794	<0.001
	Among populations within groups	5	7	7.31	28.07	34.29	27.73			
	Within populations	16	20	5.36	20.82	25.17	20.57			
2) Isolates of *Foc* races 1, 2, 3 and 4										
2a) By physiological reaction										
	Among populations	3	3	10.25	53.52	60.42	69.71	0.604	0.697	<0.001
	Within populations	20	25	6.71	23.26	39.58	30.29			
2b) By geographical location of collection										
	Among populations	2	2	3.84	8.79	26.62	14.84	0.266	0.148	<0.001
	Within populations	21	26	10.58	50.45	73.38	85.16			
2c) By geographical location of collection and physiological reaction										
	Among groups	3	3	8.51	50.28	50.22	65.48	0.683	0.728	<0.001
	Among populations within groups	4	5	3.07	5.69	18.12	7.41			
	Within populations	16	20	5.36	20.82	31.66	27.12			

3) Isolates of *Foc* races 1, 2 and 4									
3a) By physiological reaction									
Among populations	2	2	1.88	5.90	22.58	20.27	0.225	0.202	<0.001
Within populations	19	24	6.46	23.21	77.42	79.73			
3b) By geographical location of collection									
Among populations	2	2	3.81	7.02	42.55	24.11	0.425	0.241	<0.001
Within populations	19	24	5.15	22.11	57.45	75.89			
3c) By geographical location of collection and physiological reaction									
Among groups	2	2	0.15	2.79	1.85	9.56	0.407	0.293	<0.001
Among populations within groups	4	5	3.25	5.78	38.92	19.79			
Within populations	15	19	4.95	20.63	59.23	70.65			
4) Isolates of *Foc* races 1 and 2									
4a) By physiological reaction									
Among populations	1	1	2.24	6.91	26.61	23.63	0.266	0.236	<0.001
Within populations	18	23	6.18	22.33	73.39	76.37			
4b) By geographical location of collection									
Among populations	2	2	3.69	7.11	42.91	25.13	0.429	0.251	<0.001
Within populations	17	22	4.90	21.19	57.09	74.87			
4c) By geographical location of collection and physiological reaction									
Among groups	1	1	0.57	4.58	6.82	15.68	0.407	0.294	<0.001
Among populations within groups	3	4	2.83	4.02	33.9	13.75			
Within populations	15	19	4.95	20.63	59.27	70.58			

P^a : Probability of having equal or more extreme variance component and F statistic than the observed values by chance alone. Tested by non-parametric randomization analysis, using 1,000 repetitions.

collection of isolates' {*Fusarium* isolates from North India (N.I), Central India (C.I) and South India (S.I)} partitioned variance components 'among populations' and 'within populations' as 12.77% and 87.23% for the ISSR and 6.64% and 93.36% for the AFLP data, respectively (Table 2.6). AMOVA analysis of populations defined by 'race and geographic location' yielded values of 40.54% and 51.69% for 'among group variation' with the ISSR and AFLP data, respectively, while 'variation among populations within groups' was 34.29% and 27.73%, respectively. The values 25.17% and 20.57% indicated variation 'within populations' for the ISSR and AFLP data, respectively (Table 2.6).

When isolates of *Foc* races 1, 2, 3 and 4 only were analyzed based on 'physiological reaction', 'geographical location of collection' and 'physiological reaction and geographical location', the pattern of partitioning of variance remained the same (Table 2.6). However, elimination of race 3 isolates from the dataset of '*Foc* races' flipped the pattern of variance partitioning in favor of 'within population variation' as compared to 'among population variation' in both the marker systems (Table 2.6). The partitioning of variance 'among populations' was 22.58% and 20.27% and 'within population' variation was 77.42% and 79.73% with the ISSR and AFLP data, respectively (Table 2.6). The pattern remained the same when only isolates of races 1 and 2 were considered for analysis.

Further, AMOVA analysis of isolates of *Foc* races 1, 2, 3 and 4, based on AFLP primer combination E+AA/m+caa alone, revealed that 'among population variation' was 83.76% and 'within population variation' was 16.24%. However, the partitioning of variance 'among populations' and 'within populations' with isolates of *Foc* races 1, 2 and 4 only was 48.20% and 51.80%, respectively while the value for 'among populations' variation further increased to 56.25% when only isolates of races 1 and 2 were used for analysis (AMOVA Table for E+AA/m+caa not shown).

2B.4.7 Comparison of Elongation factor (EF-1α) sequences from the *Foc* races and related species

Foc race 3 isolates (standard race 3 and Fu-7) clustered with each other, forming a distinct group from isolates of *Foc* races 1, 2, and 4 by both the molecular methods used in the present studies. These genomic differences in race 3 isolates as compared to *Foc* race 1, 2, and 4 isolates led us to sequence conserved regions of the nuclear elongation factor 1α for the four standard races, isolate Fu7 (a race 3 like physiological reaction) and *Foc*39 (unknown race reaction). Related species, *F. solani* and *F. udum* were also sequenced as outgroup species, and *F. udum* sequence was used to root the gene tree. The aligned translation elongation factor (EF-1α) sequences, which totaled 696 bp, were used to construct a phylogenetic tree. Fig. 2.11 represents one of 27 most-parsimonious trees obtained from the analysis, revealing that *Foc* race 1, 2, and 4 standard isolates and *Foc*39 formed an exclusive group of *F. oxysporum* complex isolates, while *Foc* race 3 and Fu7 isolates grouped with *F. proliferatum*. Elongation factor sequences of *F. udum* and *F.solani* were similar to reported sequences from *F. udum* and *F. solani* f.sp. *pisi*, respectively (O'Donnell, 2000; O'Donnell *et al.*, 1998a).

2B.5 Discussion

2B.5.1 Usage of ISSR and AFLP markers for *Foc* genome analysis

Foc isolates representing the range of genetic and physiological diversity were collected from different geographic locations in India, and were analyzed for their physiological race reaction at ICRISAT centre, Patancheru using 10 chickpea differentials (Table 2.4). Two marker systems, namely ISSR and AFLP, were used in parallel for genetic variability analysis in these isolates. ISSR markers reveal variation in regions of the genome flanked by microsatellite repeats, while AFLP markers analyze the genome based on the variation in restriction sites and primer binding sites (Vos *et al.*, 1995). In the present study, correlation of molecular and pathogenicity data revealed that although theoretically ISSR and AFLP markers examined different regions of the *Foc* genome, overall clustering patterns for isolates obtained with both the methods remained similar. Comparatively lower bootstrap values obtained for clades in ISSR

analysis may be due to only 196 ISSR loci analyzed as compared to 1009 loci in AFLP analysis. Thus, AFLP is a powerful tool for molecular fingerprinting as it simultaneously yields information at a large number of loci as compared to ISSR; however, ISSR markers are technically much simpler to handle.

2B.5.2 Potential of ISSR and AFLP markers to differentiate *Foc* races

Individual phenograms obtained with ISSR and AFLP primers (representative Fig. 2.6 and Fig. 2.7, respectively), and the combined ISSR and AFLP phenograms constructed with 196 ISSR and 1009 AFLP loci (Fig. 2.9 and Fig. 2.10, respectively), revealed that *F. solani* and *F. udum* isolates were not a part of the *F. oxysporum* complex. Thus, ISSR and AFLP markers clearly distinguished *Foc* (wilt pathogen) and *F. solani* (black root rot pathogen); fungi of the so-called wilt complex in chickpea (Nene *et al.*, 1978).

Further, in the present study both the marker systems used always grouped the standard race 3 isolate with Fu7 (race 3 reaction), but distinct from isolates of *Foc* races 1, 2 and 4. *Foc* race 3 and Fu7 were purple pigmented and caused vascular wilt in chickpea differentials (Rahman, 1997). These results are in agreement with earlier reports on race 3. *Foc* race 3 was unique because of its peculiar physiological reaction to four of the ten chickpea differentials used for race identification (Haware and Nene, 1982c) and had localized distribution in certain pockets of North India (Haware *et al.*, 1992b). 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). As described in Section 2A, simple sequence repeats as probes for DNA fingerprinting, have also demonstrated unique hybridization profiles for *Foc* race 3.

Comparisons of phenograms constructed with data from all ISSR and AFLP loci subdivided the robust cluster of *Foc* race 1, 2 and 4 isolates into two clades, generally corresponding to physiological race (Fig. 2.9 and Fig. 2.10) Nevertheless, *Foc*11 and *Foc*36 did not group as per their race designation with any primer used in this study.

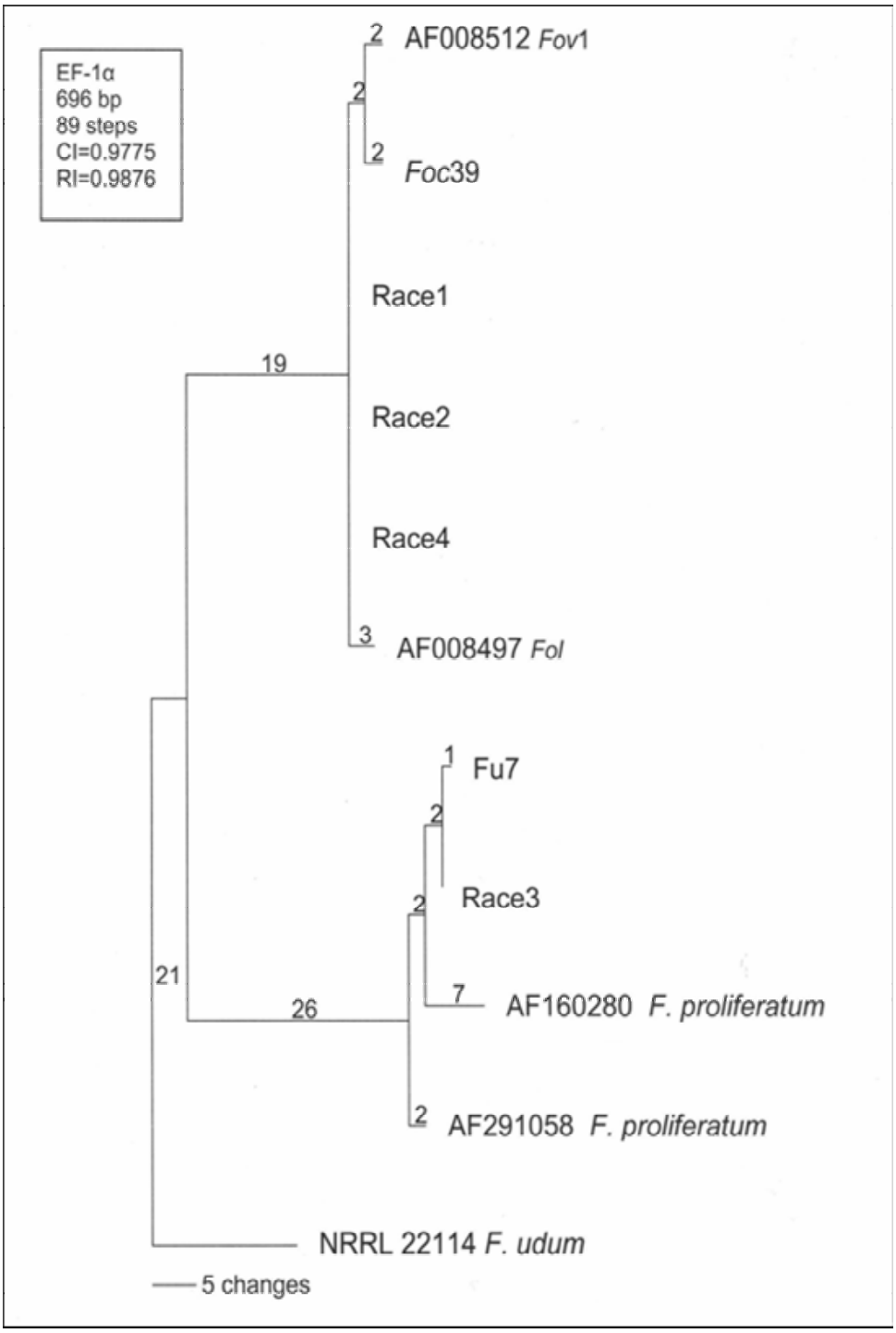


Fig. 2.11 One of 27 most-parsimonious trees inferred from the partial EF-1α DNA sequence data of *Fusarium oxysporum* f. sp. *ciceri* (*Foc*) races 1, 2, 3 and 4. The phylogram was rooted with a sequence of *Fusarium udum* (NRRL 22114). Reference strains NRRL 25420 *Fov*1 (*Fusarium oxysporum* f. sp. *vasinfectum* race1, accession AF008512), NRRL 26034 *Fol* (*Fusarium oxysporum* f. sp. *lycopersici*, accession AF008497), *F. proliferatum* (accession AF160280), and *F. proliferatum* (accession AF291058).

Isolate *Foc*11 (from Thupa, Hissar), the only exceptional isolate from North India showing a race 1 reaction, consistently grouped with race 2 isolates. On the other hand, *Foc*36, the only isolate from South India (Hyderabad, Andhra Pradesh) showing a race 2 reaction, always grouped with race 1 isolates irrespective of the primer, or the marker system used (Fig. 2.9 and Fig. 2.10). *Foc*31, an isolate from Durg, (Madhya Pradesh), which showed an independent reaction in glass house studies grouped with race 2 isolates by both the marker systems. *Foc*39, an isolate from Kanpur (Uttar Pradesh) with slight purple pigmentation in culture, showed AFLP amplification profiles different from *Foc* isolates of races 1, 2 and 4, but a grouping of this isolate with them received 100% bootstrap support (Fig. 2.7, Fig. 2.8, and Fig. 2.9). However, physiological race data for this isolate is not available.

A clustering pattern variation observed in the AFLP analyses was that the standard isolates of races 1 and 2 formed a group among themselves with 69.8% support, unlike in the ISSR phenogram (Fig. 2.10 and Fig.2.9, respectively). This may be because laboratory maintained standard cultures (isolated almost 20 years ago) are periodically re-isolated by plant inoculations, and are consequently 'frozen in time' with respect to more recently collected field isolates which are evolving in nature as a result of exposure to various environmental conditions and varietal selection. However, genomic changes in these field-collected isolates may or may not reside in the virulence loci as random markers like ISSR and AFLP detect polymorphism in the genome, irrespective of their phenotypic implications (physiological race).

2B.5.3 Revealing *Foc* race 1 and 2 specific markers and their application

Evaluation of individual ISSR and AFLP primers for their resolving power revealed that four ISSR and five AFLP primers clustered the isolates into groups generally corresponding to race and/or geographic distribution (Table 2.5). Of these, UBC 835 and AFLP primer pair E+AA/m+caa were identified as the best combinations for differentiation of *Foc* race 1 and 2 isolates (Table 2.5). Both the combinations clearly resolved *Foc* isolates (except *Foc*36 and *Foc*11) into two clades according to their physiological race. Although, previous studies based on Random Amplified

Polymorphic DNA (RAPD) markers by Jiménez-Gasco *et al.* (2001) and Kelly *et al.* (1994) successfully differentiated the yellowing and wilting pathotypes in *Foc* races, they could not differentiate the wilting pathotypes from India. In the present study, I have demonstrated the potential of ISSR and AFLP markers, together as well as individually, to group wilting pathotypes belonging to races 1 and 2 from India.

Three AFLP primer pairs revealed bands unique to race 1 and race 2 isolates as depicted in Fig.2.8. These bands need to be cloned, characterized and their co-segregation with race 1 or race 2 isolates needs to be further confirmed with a larger sample of race 1 and race 2 isolates. Interestingly, the AMOVA data from E+AA/m+caa combination revealed the partitioning of variance 'among populations' and 'within populations' to be 48.20% and 51.80%, respectively, when isolates of *Foc* races 1, 2, and 4 only were considered for analysis by physiological race. The value for 'among populations' variation further increased to 56.25%, when only isolates of *Foc* races 1 and 2 were included. These values for 'among populations' variation were much higher than the corresponding values of 20.27% and 23.63% (Table 2.6) using the data from all AFLP combinations with isolates of *Foc* races 1, 2 and 4, and *Foc* races 1 and 2, respectively.

Thus, the present studies represent an initial step towards development of race-specific markers for races 1 and 2, which are widespread in India. If linked with race, these markers may also offer potential for development of markers linked to virulence genes. Race identification by pathogenicity tests requires considerable time (50-60 days), effort, extensive facilities and controlled environment. Race specific markers can provide a means to study distribution of races, facilitate monitoring of introduction of a new race in a particular area, and to identify shifts in population structure (McDonald, 1997). Early detection of introduced races can help in effective deployment of resistant cultivars in accordance with region specific races, prevent yield losses and also prevent inoculum build-up in soils by avoiding planting of susceptible cultivars.

2B.5.4 Genetic variation in *Foc* isolates is independent of their geographic distribution

AMOVA analysis of thirty-three isolates (including *Foc* isolates, *F. udum* and *F. solani*) revealed a significantly higher value for 'among-population variation' as compared to variation 'within populations'. A similar pattern of partitioning of variance was obtained when only isolates of the four *Foc* races were analyzed, wherein partitioning was strongly in favor of 'inter-racial variation' as compared to 'intra-racial variation' (Table 2.6). However, when race 3 isolates were eliminated from this analysis, 'intra-racial variation' in isolates of races 1, 2 and 4 exceeded the 'inter-racial variation' with both marker systems (Table 2.6). The higher value for 'inter-racial variation' obtained when race 3 was included for AMOVA analysis might be due to the different molecular patterns obtained for race 3 isolates. It is possible that *Foc* isolates of races 1, 2 and 4 collected from different geographical locations belong to different physiological races but are genetically similar to each other at the loci analyzed by ISSR and AFLP markers used in this study. Overall genetic similarity of *Foc* isolates, revealed in the grouping with ISSR and AFLP markers, is reflective of the asexual nature of the pathogen as a result of which the entire genome is passed onto progeny as a unit. Asexually reproducing fungi do not undergo regular recombination and genetic variation results mainly from the accumulation of mutations. Consequently, in these fungi the whole genome is linked, and transmitted, as a unit from one generation to the next and different regions in the genome share the same evolutionary history (Taylor, 1999b).

In our molecular data, partitioning of variance was according to physiological reaction and not as per geographical distribution of isolates. The 'within populations variation' component was significantly higher than the 'among populations variation' component with both marker systems when all the isolates used in the present study were categorized into 'populations' defined by 'geographic location of their collection' (isolates of North India, Central India and South India). The pattern remained the same even when only isolates of races 1 and 2 from different geographical locations were considered (Table 2.6). Higher values of 'within population variation' are reflective of variation in populations from a single geographic region, while lower values of 'among population variation' indicate that isolates from different geographical locations are not very different from each other genetically. Thus the genetic variation in *Foc* isolates is

probably not influenced by different agro climatic conditions. It is well known that *F. oxysporum* is a cosmopolitan species complex that exists in many pathogenic forms (Armstrong and Armstrong, 1981).

2B.5.5 *Foc* race 3 is distinct from *Foc* races 1, 2 and 4 in India

Two isolates namely, standard race 3 and Fu7, independently isolated from chickpea plants showing vascular wilt, and characterized as race 3 at ICRISAT based on their physiological reactions to chickpea differentials were distinct from isolates of *Foc* races 1, 2, 3 and 4 based on previous molecular data using hybridization based microsatellite markers (described in Section 2A) and the present data with ISSR and AFLP markers, necessitating further characterization by phylogenetic methods.

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, 1998b; Steenkamp, 2000; Taylor *et al.*, 1999a). Sequencing of conserved regions from elongation factor 1α (EF-1α) has been extensively used for phylogenetic analysis in *Fusarium* spp. (Baayen *et al.*, 2000; O'Donnell, 2000; O'Donnell *et al.*, 1998b). Although Jiménez-Gasco *et al.* have arrived at a conclusion of monophyletic origin of this *forma speciales*, based on sequence data from conserved regions of EF-1α in 17 *Foc* isolates, only 7 isolates from India were used (Jiménez-Gasco *et al.*, 2002). Sequencing of conserved regions from a large number of isolates of races 1, 2, 3 and 4, collected from India would elucidate this concept more clearly with respect to isolates from the Indian subcontinent.

As a step toward phylogenetic characterization of these race 3 isolates with respect to standard races 1, 2 and 4, I compared sequence data from EF-1α of the standard isolates of four Indian *Foc* races, Fu7, *Foc*39, *F. solani*, and *F. udum*. Phylogenetic analysis using PAUP (Swofford, 1998) revealed that EF-1α sequence of *F. solani* was similar to reported *F. solani* sequences from *Pisum sativum* (O'Donnell, 2000), while the *F. udum* sequence was similar to the reported *F. udum* sequence from (O'Donnell *et al.*, 1998a). *Foc* race 1, 2, and 4 standard isolates and *Foc*39, a wilt causing isolate for which race data is not available, revealed EF sequences similar to *F. oxysporum* (NRRL 25420 *Fov*1, NRRL 26034 *Fol*). Furthermore, in the AFLP studies, profiles for *Foc*39 were different from those of other *Foc* isolates (Fig. 2.8); however a

grouping of *Foc*39 with the *Foc* isolates received 100% bootstrap support (Fig. 2.10). Thus, clusters obtained with the AFLP fingerprinting data supported the gene-genealogy based phylogram for Indian *Foc* races, which is in agreement with previous studies wherein AFLP fingerprint data supported a gene-genealogy based phylogram (Baayen *et al.*, 2000). However, in that study, AFLP based phylogenies were considerably more homoplasious than the gene genealogies. AFLP analysis, in theory, samples loci throughout the entire genome, while the gene-genealogies in Baayen *et al.* examined the evolution of nuclear and mitochondrial gene sequences (Baayen *et al.*, 2000).

Molecular data from simple sequence repeat hybridization based markers (detailed in Section 2A) and the present data with ISSR and AFLP markers indicated that race 3 was distinct from the *Foc* races 1, 2 and 4. This finding was consistent with the sequence data, which clearly demonstrated that this race actually represents *F. proliferatum* (accessions AF160280 and AF291058). Further analysis of this race (using more isolates) by morphological and phylogenetic methods should help characterize it more fully. Furthermore, *F. udum*, *F. solani*, and *Foc* race 3 (*F. proliferatum*) appeared to have independent evolutionary origins from the *Foc* isolates of races 1, 2 and 4. *F. proliferatum* has been reported to be pathogenic on a wide range of plants, mostly monocots (*Asparagus officinalis*, *Cattleya* species, *Sorghum bicolor*, *Triticum* species, *Zea mays*), dicots (*Ficus carica*, *Morus alba,* legumes like *Arachis hypogaea*, *Trifolium vesiculosum*, *Trigonella foecum*), and a gymnosperm (*Ephedra nebrodensis*) (http://nt.ars-grin.gov/fungaldatabases/fungushost/fungushostframe.cfm). However, to the best of my knowledge, it has never been described as a pathogen of chickpeas.

In conclusion, these studies represent an important step towards developing race specific markers and characterizing the genetic diversity in pathogen populations of this economically important chickpea wilt pathogen *Fusarium oxysporum* f. sp. *ciceri*, thereby assisting breeding for wilt resistance, which is the best available control strategy for fusarium wilt of chickpea. These studies also strongly support the need for development of phylogenetic markers for identification of *Fusarium* species as previously emphasized by O'Donnell *et al.* (O'Donnell *et al.*, 1998b).

Section 3A

Genetic diversity analysis of a world-wide collection of *Ascochyta rabiei* isolates using STMS markers

3A.1 Abstract

A previously characterized compound microsatellite locus *ARMS*1, containing penta- and decameric repeat units, has been reported to reveal genetic diversity in *Ascochyta rabiei* (Pass.) Labr. isolates. Therefore, thirty-seven isolates of *Ascochyta rabiei* collected from different states of India and thirty-eight isolates from various countries in the world were examined for their diversity at this locus. Twenty-six alleles on the basis of size (228 bp to 451 bp) were detected in the world isolates examined, while fifteen alleles (287 bp to 418 bp) were observed in isolates from the Indian subcontinent. To the best of my knowledge, this study is the first to demonstrate diversity in representative *Ascochyta rabiei* isolates from different parts of the world at the *ARMS*1 locus.

3A.2 Introduction

Ascochyta blight of chickpea has occurred worldwide in chronic epidemic cycles despite frequent introduction of resistant germplasm (Peever and Muehlbauer, personal communication) and periodic appearances of new and more virulent strains of the pathogen are a major problem in resistance breeding (Grewal *et al.*, 1984; Singh *et al.*, 1981). However, no systematic studies to determine the mechanisms of development of diverse virulent pathotypes have been conducted. Pathogen populations show considerable variations in aggressiveness but 'races' or 'pathotypes' are poorly defined (Udupa *et al.*, 1998). Also no genetic map or virulence loci have been reported for this pathogen till date.

Suitable genetic markers are needed for analyzing genetic diversity of the pathotypes, population structure and mapping pathogenicity genes (Brown, 1996). The increasing popularity of microsatellite based PCR markers (STMS) is because they are abundant, co-dominant, distributed over the euchromatic part of the genome, require small amounts of DNA and are highly polymorphic (Schlötterer, 1998; Zane *et al.*, 2002). Furthermore, STMS (sequence-tagged microsatellite markers) markers detect length variation of individual microsatellite repeats (Powell *et al.,* 1996). However, the main drawback is that they have to be generated *de novo* for every system they are to be used in (Zane *et al.*, 2002).

Using simple sequence repeats as primers has been reported to be a less sensitive marker technique for *A. rabiei* genome analysis as compared to using the same oligonucleotides as probes for RFLP fingerprinting (Morjane *et al.*, 1994). In another study, the extent of polymorphism among fungal isolates collected from different chickpea growing localities of Syria and Tunisia by microsatellite primers in microsatellite primed PCR (MP-PCR) was rather low, and most of the isolates could not be differentiated from each other (Geistlinger *et al.*, 1997b). However, a PCR amplification fragment obtained with a combination of primers $(GTTTGG)_3$ and $(GGAT)_4$ revealed the presence of a compound microsatellite (*ARMS1*). The locus was

composed of comparably large size arrays of four continuous penta- and decanucleotide motifs surrounded by extremely AT rich DNA and combined typical features of microsatellites (compound repeats of pentameric motifs), minisatellites (total length of 200-500 bp) and satellites (occurrence at a few genomic loci only) (Gesitlinger *et al.*, 1997b). The *ARMS1* locus was reported to have a high mutation rate and direct sequencing revealed one newly mutated allele in the progeny of a cross (Gesitlinger *et al.*, 1997b). At least 25 different microsatellite motifs have now been detected in the *A. rabiei* genome by extensive oligonucleotide fingerprint analyses (Geistlinger *et al.*, 1997a; Morjane *et al.,* 1994; Weising *et al.,* 1991; Weising *et al.,* 1995) and several such microsatellite based STMS markers have been generated (Geistlinger *et al.*, 2000).

In this section, I have carried out diversity analysis with respect to allelic size variation at the *ARMS1* locus in (1) *A. rabiei* isolates of both the mating types from different countries across the world and (2) *A. rabiei* isolates from different states of India, where only one mating type is reported to occur.

3A.3 Materials and Methods

A. rabiei isolates (thirty-seven isolates from India and thirty-eight isolates from different parts of the world) were obtained from the culture collection established by Dr. Walt Kaiser (USDA-ARS) at the Washington State University in Pullman, USA (details presented in Table 3.1). *Ascochyta rabiei* isolates from peas, lentils, amaranthus and an isolate from wheat were also included in the study (Table 3.1).

3A.3.1 Fungal material

Single spore cultures of the isolates were used to inoculate chickpea stem pieces, which were then stored in dry state till further use. Isolates from chickpea stems were revived as follows: a small section of chickpea stem was scraped from the surface with a sterile knife and cultured on 2% water agar (WA) media in 9-cm diameter petri dishes. These dishes were incubated at 21-23°C under fluorescent light for 12h. A 1-cm^2 piece of WA containing the fungal culture was cut out from the plate of each isolate with a sterile

Table 3.1 List of *A. rabiei* isolates collected from different states of India and isolates collected from different countries in the world and the size in (bp) of the *ARMS1* alleles amplified.

Indian isolates				World isolates			
Isolate code	**State of collection**	**Mating - type**	**Allele size (bp)**	**Isolate code**	**Country of collection**	**Mating-type**	**Allele size (bp)**
AR54	Punjab	1	294	AR89	Pakistan	1	305
AR209	,,	1	392	AR82 I	,,	2	247
AR216	,,	1	392	AR19	Iran	1	367
AR219	,,	1	418	AR192	,,	2	357
AR220	,,	1	300	AR126	Turkey	1	305
AR674	,,	1	392	AR125	,,	2	314
AR208	,,	1	400	AR74	Tunisia	1	301
AR212	,,	1	392	AR114	,,	2	317
AR217	,,	1	392	AR655	Syria	1	314
AR210	,,	1	400	AR628	,,	2	314
AR213	,,	1	396	AR136	Greece	1	267
AR58	Jammu & Kashmir	1	296	AR110 II	,,	2	370
AR211	Jammu &Kashmir	1	396	AR160	Bulgaria	2	305
AR224	Himachal Pradesh	1	392	AR107 I	Portugal	1	326
AR60	,,	1	396	AR105	,,	2	326
AR658	,,	1	396	AR117	Spain	1	291
AR225	,,	1	404	AR115	,,	2	451
AR226	,,	1	287	AR181	France	1	354
AR57	?	1	300	AR170	,,	2	245
AR222	Haryana	1	400	AR143	Italy	1	321
AR673	,,	1	302	AR97	,,	2	228
AR56	New Delhi	1	302	AR184	Morocco	1	384
AR221	,,	1	392	AR46	,,	2	308
AR218	Rajasthan	1	409,388	AR365	Algeria	1	384
AR135	,,	1	413	AR191	,,	2	354
AR223	,,	1	413	AR607	Canada	1	312
AR59	Uttar Pradesh	1	304	AR147	,,	2	299
AR659	,,	1	304	4B (AR20)	ATCC 76501	1	451
AR214	Unknown	1	404	6 (AR21)	ATCC76502	2	308
AR215	,,	1	409	AR588	USA,ID	1	337
AR815	Gurdaspur	1	404	AR616	USA,WA	1	413
AR816	Jammu	1	418	AR229	USA, ND	1	310
AR817	Ganganagar	1	418	AR232	USA, ND	2	301
AR818	Hisar	1	306	AR237	USA,ID Amaranthus	1	351
AR819	Haryana	1	306	AR630	Lentil	2	337
AR820	Haryana	1	306	AR275	Amaranthus	1	253
AR821	Palampur	1	418	AR38	Pea	2	312
				AR281	Wheat	2	295

knife and used to inoculate 50-ml of potato-dextrose broth (PD-broth, Difco Laboratories, Detroit, Mich.) in 250-ml conical flasks, which were then kept in an incubator shaker at 21-23°C at 150 rpm for 7 days. Mycelia were harvested by filtration through four layers of muslin cloth, lyophilized, stored at –70°C and later used for DNA extraction.

3A.3.2 DNA isolation

DNA was isolated from lyophilized mycelia using the miniprep method of Doyle and Doyle (1987) with a few modifications. Two to three grams of mycelium from each isolate were submerged in liquid nitrogen and ground to a fine powder. The powder was quickly transferred to a tube containing 7.5 ml of ice-cold extraction buffer (0.35 M sorbitol, 0.1M Tris, 5mM EDTA, pH 7.5). After briefly shaking the tube, 7.5 ml of nuclei lysis buffer (2M NaCl, 0.2M Tris, 50mM EDTA, 2% CTAB, pH 7.5) was quickly added, followed by 3ml of 5% sarkosyl solution. Sample sets were incubated in a 65°C water bath for 20min, allowed to cool for a few minutes, and 18ml of chloroform/isoamyl alcohol (24:1) was added to each tube. The tubes were centrifuged at 5000g for 15 min; the aqueous layer was removed and re-extracted with 15 ml of chloroform/isoamyl alcohol mixture. The aqueous layer was transferred to a fresh tube and DNA was precipitated with double volume of chilled ethanol. The dry DNA pellet was then suspended in 500 µl of TE buffer (10mM Tris-HCl and 1mM EDTA, pH 8) and DNA was quantified on a minigel by comparing band intensities with that of a standard lambda/*Hin*dIII DNA marker (GIBCO BRL, U.S.A.) in ethidium bromide stained gels (Sambrook *et al.*, 1989).

3A.3.3 Microsatellite primers and PCR conditions

PCR primers: L (**5'-GTAGAGTAGTTGTAGCTACT-3'**) and R (**5'-GGATCAATTACTAGGTTGCT-3'**) reported by Geistlinger *et al.* (1997b) were used to amplify alleles at the compound microsatellite locus from *Ascochyta rabiei* isolates (Table 3.1). PCR amplifications were carried out in 20ul reaction volume containing 200uM dNTPs, 1x reaction buffer (containing 1.5mM $MgCl_2$),

1unit/reaction *Taq* DNA polymerase (Amplitaq, PE, USA), 5-10ng genomic DNA as template and 5 picomoles each of primers L and R. Control reactions did not contain any DNA. PCR products were amplified on a Perkin Elmer-9700 thermal cycler with slight modification of the protocol of Geistlinger *et al.* (1997b). Initial denaturation was carried out at 94°C for 3 min, followed by 37 cycles of denaturation, annealing and extension at 94°C for 30 sec, 52°C for 1 min and 72°C for 1 min, respectively. Final extension was at 72°C for 5 min. Amplification products were resolved on 2% agarose gels in 1X TAE buffer and bands were visualized by ethidium bromide staining. For better resolution of the amplification products, reactions were repeated with addition of 2μCi of α^{32}PdATP and 25μM dATP per 20μl reaction, keeping the other conditions constant and thermocycled as per the protocol described above. Amplification products were resolved in 6% denaturing PAGE gels in 1x TBE buffer with 32P end-labeled ΦX-174/*Hae*III digest as a size marker and bands were visualized by autoradiography. Band size of each allele amplified was calculated in base pairs, using the program Sequaid II (tm) version 3.5 (Schaffer and Sederoff, 1981) wherein, the distance migrated by the bands in the marker lane was considered as a standard, against which the sizes of fragments of interest in the remaining lanes were calculated.

3A.4 Results

3A.4.1 Genetic diversity in Indian isolates of *A. rabiei*

PCR amplified products were resolved initially in agarose and later in denaturing polyacrylamide gels. Resolution in denaturing PAGE gels revealed strong amplification bands and variation in band sizes at the *ARMS1* locus in *A. rabiei* isolates from different parts of the world as compared to resolution in agarose gels, hence, denaturing PAGE gels were used for all further studies as represented in Figures 3.1 and 3.2. Fifteen size alleles ranging from 287 bp to 418 bp were detected in thirty-seven isolates collected from various states in India alone, while twenty-six alleles varying in size from 228 bp to 451 bp were detected in thirty-eight isolates from all over the world (Tables 3.1 and 3.2). Among the Indian isolates studied, the smallest size allele corresponding to a band

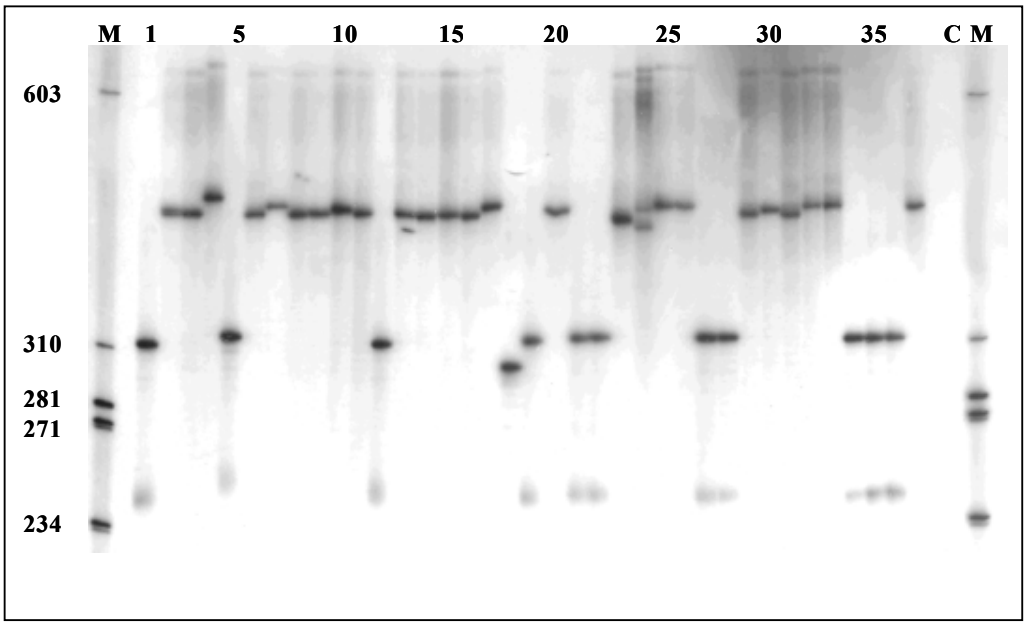


Fig 3.1 Polyacrylamide gel electrophoresis of *ARMS1* alleles in *Ascochyta rabiei* (AR) isolates from different states of India (Table 3.1). *Lanes 1-37* Alleles in *Ascochyta rabiei* isolates AR54, AR209, AR216, AR219, AR220, AR674, AR208, AR212, AR217, AR210, AR213, AR58, AR211, AR224, AR60, AR658, AR225, AR226, AR57, AR222, AR673, AR56, AR221, AR218, AR135, AR223, AR59, AR659, AR214, AR215, AR815, AR816, AR817, AR818, AR819, AR820, and AR821, respectively. *Lane M* ΦX-174/*Hae*III molecular weight marker (in bases). *Lane C* Sterile water control.

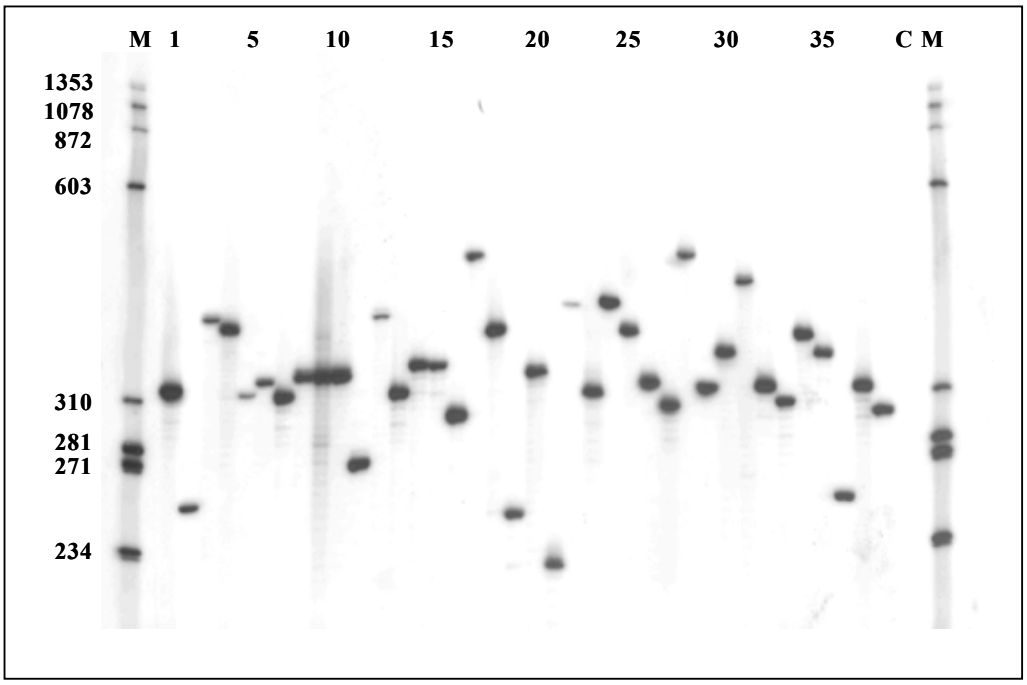


Fig 3.2 Polyacrylamide gel electrophoresis of *ARMS1* alleles in *Ascochyta rabiei* (AR) isolates from different countries (Table 3.1). *Lanes 1-38* Alleles in *Ascochyta rabiei* isolates AR89, AR82I, AR19, AR192, AR126, AR125, AR74, AR114, AR655, AR628, AR136, AR110 II, AR160, AR107I, AR105, AR117, AR115, AR181, AR170, AR143, AR97, AR184, AR46, AR365, AR191, AR607, AR147, 4B(AR20), 6(AR21), AR588, AR616, AR229, AR232, AR237, AR630, AR275, AR38, and AR281, respectively. *Lane M*- ϕX 174/*Hae*III molecular weight marker (in bases), *Lane C* Sterile water control.

of 287 bp was recorded in AR226 from Himachal Pradesh (Lane 18, Fig. 3.1), while the largest size allele corresponding to a band of 418 bp was amplified in four isolates, AR219 (Punjab), AR816 (Jammu), AR817 (Ganganagar) and AR821 (Palampur) (Fig. 3.1, lanes 4, 32, 33, 37, respectively). Table 3.2 represents the frequency distribution of the size alleles at the *ARMS*1 locus for the Indian and world isolates used in the present study. Among Indian isolates, the most frequent allele obtained was 392 bp and seven isolates; five from the state of Punjab and one each from Himachal Pradesh and New Delhi revealed co-migrating bands (Fig. 3.1, Table 3.1). One isolate from Rajasthan, India AR218 consistently amplified two bands corresponding to molecular weights 388 bp and 409 bp even making the PCR conditions more stringent (Fig. 3.1, lane 24).

3A.4.2 Genetic diversity in world isolates of *A. rabiei*

In case of the world isolates studied, the smallest allele of 228 bp was amplified in AR97 from Italy (Fig. 3.2, lane 21), while the largest allele of 451 bp was recorded in AR115 from Spain and isolate AR20 (ATCC 76501, a strain routinely used as a mating-type tester strain for crosses among *A. rabiei* isolates) (Fig. 3.2, lanes 17 and 28, respectively). In my studies strains of both the mating types from each country (wherever available) were used; however, there was no correlation observed between mating-type, and the size of the band amplified from isolates from different countries. The Indian isolates used were of the same mating specificity (*MAT*1). Isolates AR237 and AR735 (from *Amaranthus*), AR630 (from lentils), AR38 (from pea) and AR 281(from wheat) also showed amplification of single bands ranging in size from 253 bp to 351 bp.

3A.5 Discussion

3A.5.1 Allelic variation at *ARMS*1 locus in *A. rabiei* isolates

Microsatellites or simple sequence repeats are tandemly repeated motifs of 1-6 bases found in all eukaryotic genomes (Epplen, 1988; Tautz and Renz, 1984) as well as some prokaryotic genomes (Field and Wills, 1996; Hancock 1996a), while compound

microsatellite loci contain stretches of two or more different simple sequence repeats,

Table 3.2 Frequency distribution table for *A. rabiei* isolates amplifying a particular size allele at the *ARMS1* locus

Indian Isolates		**World Isolates**	
Allele size (bp)	**Frequency of the size allele**	**Allele size (bp)**	**Frequency of the size allele**
287	1	228	1
294	1	245	1
296	1	247	1
300	2	253	1
302	2	267	1
304	2	291	1
306	3	295	1
388	1	299	1
392	7	301	2
396	4	305	3
400	3	308	2
404	3	310	1
409	2	312	2
413	2	314	3
418	4	317	1
		321	1
		326	2
		337	2
		351	1
		354	2
		357	1
		367	1
		370	1
		384	2
		413	1
		451	2

and may constitute up to about 10% of the microsatellite repeats (Weber, 1990). Alleles at such loci can vary in length at any of the repeats constituting the locus (Bull *et al.*, 1999 and references therein). Microsatellite markers have been generated for a number of fungi ranging from plant pathogens (Arenal *et al.*, 1999; Delmotte *et al.*, 1999; Hantula *et al.*, 1998; Kim *et al.*, 2000; Sirjusingh and Kohn, 2001; Tenzer *et al.*, 1999) to endophytes (Moon *et al.*, 1999) and mycorrhizal fungi (Longato and Bonfante, 1997). In my study, PCR amplification at the *ARMS*1 locus revealed the presence of numerous size alleles in *A. rabiei* isolates from different parts of the world as well as from India. Fifteen size alleles (287 bp to 418 bp) detected in thirty-seven Indian isolates had a frequency distribution pattern different from those of the twenty-six size alleles (228 bp to 451 bp) detected in thirty-eight isolates from other countries, implying that the size alleles at *ARMS*1 locus in *A. rabiei* populations from India are different from those in other parts of the world except for only one allele of size 413 bp.

From the frequency distribution table of alleles among world isolates of *A. rabiei*, only two size alleles (305 and 314 bp) were represented more than twice, which may be because only two samples from each country were used in most cases. However, in a few cases, the same size allele was shared in isolates from different countries; for example, 305 bp allele was common to isolates AR89 (*MAT*1, Pakistan), AR126 (*MAT*1, Turkey), and AR160 (*MAT*2, Bulgaria). Similarly, the size allele, corresponding to 314 bp was represented in isolates AR125 (*MAT*2, Turkey), AR655 (*MAT*1, Syria) and AR628 (*MAT*2, Syria). The same size alleles, however, do not ensure that they are identical in sequence, as changes like base substitutions do not change the size of the amplicon (Bull *et al.*, 1999). Also, comparative sizing of alleles accomplished by comparing the electrophoresed distance or time, respectively, of a DNA fragment with a gauge curve of distances or times of co-electrophoresed fragments and their known, sequence based sizes (size standard) are not necessarily accurate to the single nucleotide level (Haberl and Tautz, 1999; Schwengel *et al.* 1994).

In my study, among Indian isolates, the most frequent size allele obtained was 392 bp and seven isolates namely; AR209, AR212, AR216, AR217, AR221, AR224

and AR674 shared the same size allele (392 bp). In an earlier report, Santra *et al.* (2001) studied the genetic diversity in thirty-seven Indian, five American (USA), three Syrian, and two Pakistani *A. rabiei* isolates using RAPD primers wherein, most of the Indian isolates used were common to those used in the present study. In this study, the isolates were grouped into two major clusters (A and B), and the subclusters within these clusters contained isolates according to their geographic origin. In comparison with the present study, the isolates AR209, AR216, AR217, and AR224 in Santra *et al.* (2001) clustered in group B, while only AR212 clustered with the A group isolates. AR 674 was not included in their studies; therefore a comparison cannot be drawn for this isolate. Similarly, among Indian isolates sharing a 396 bp size allele in my study, only AR213 (Punjab) grouped along with the A group isolates in the study by Santra *et al.* (2001), while AR60, AR211, AR658 grouped with the group B. In case of isolates with the 418 bp size allele, AR817 was the only isolate which grouped in the A group as compared to AR816 and AR821, which were in the B group. In my studies, eleven isolates from Punjab state of India revealed six size alleles at the *ARMS1* locus in range 294 bp to 409 bp. These results are consistent with earlier reports where isolates from Punjab state were observed to be more diverse than isolates from other states in northwestern India (Santra *et al.*, 2001).

It would be interesting to sequence the amplification products corresponding to the same size and different size alleles at the *ARMS*1 locus in Indian and world *A. rabiei* isolates to reveal the diversity in alleles of the same size (co-migrating bands) as well as to reveal the basis of size variation in alleles of different sizes. Geistlinger *et al.* (1997b) have reported that the variation in the number of TATTT repeats at the *ARMS*1 locus resulted in the generation of a new allele. Sequence comparison of alleles amplified in my study would reveal if variation in repeat-number of some other microsatellite (in the compound microsatellite *ARMS*1) is responsible for generating different size alleles at this locus in different regions of the world, and their significance, if any, could be elucidated.

3A.5.2 Correlation of allelic variation with mating type, geographical distribution and virulence

In the present study, I used one isolate of each mating type from different countries and could not find any correlation between size of the allele amplified and the mating type. Similarly, geographic correlation was also not observed with any of the specific alleles. In a previous report, (UBC $756_{1.6kb}$), a DNA marker specific to Indian isolates was identified; however, a lack of correlation between pathogenicity of eleven Indian isolates and their grouping using RAPD markers was indicated in the same report (Santra *et al.*, 2001). Unfortunately, reliable quantitative virulence assays for *A. rabiei* are not available and this limits the correlation of virulence phenotype with molecular markers.

Six *A. rabiei* isolates from Washington State, Idaho, and North Dakota (USA) in the present study had six different size alleles at the *ARMS*1 locus reflecting upon diversity at this locus as has been previously reported for the Tunisian isolates where both mating types are present and the sexual stage is known to occur (Kaiser, 1997b). However, the sample size in my study is inadequate to make comments about population structure of the pathogen in these areas, and larger samples and more microsatellite loci need to be analyzed to have a better understanding of the generation of genetic diversity in areas where the sexual stage is observed regularly and in areas where the sexual stage is not found. Determination of the existing genetic diversity makes it necessary that the ascochyta blight resistant chickpea cultivars developed for a certain region need to be tested against all the different genotypes of the pathogen prior to their release.

Section 3B

Cloning and characterization of the mating type (*MAT*) locus from *Ascochyta rabiei* (teleomorph: *Didymella rabiei*) and a *MAT* phylogeny of legume-associated *Ascochyta* spp.

3B.1 Abstract

The mating type (*MAT*) locus of *Ascochyta rabiei* (teleomorph: *Didymella rabiei*) was cloned and sequenced using a combination of TAIL-PCR and inverse PCR. Degenerate primers designed from the high mobility group (HMG) motif of *Cochliobolus heterostrophus*, *C. sativus* and *Alternaria alternata MAT1-2* genes were used to amplify HMG from *A. rabiei*. TAIL and inverse PCR amplifications extended the *MAT1-2* sequence into the conserved flanking DNA and primers designed from the *MAT1-2* flanking DNA were used to amplify the entire *MAT1-1* idiomorph plus flanking DNA. *MAT1-1* and *MAT1-2* idiomorphs were 2294 and 2693 bp in length, respectively, and each contained a single putative open reading frame (ORF) and intron similar to other loculoascomycete fungi. The *MAT1-1* ORF was 1152 bp in length and coded for a putative 366 amino acid (aa) protein with an α motif. The *MAT1-2* ORF was 1111 bp long and coded for a putative 351 aa protein with an HMG motif. Putative start and stop codon positions and intron splice sites were highly conserved when compared to *C. heterostrophus*, *A. alternata* and *Mycosphaerella graminicola*. *MAT* genes were expressed at high levels in rich media and intron splicing was confirmed by sequencing *MAT* cDNAs. *MAT*-specific PCR primers were designed for use in a multiplex PCR assay and *MAT*-specific PCR amplicons correlated perfectly to mating phenotype of 35 ascospore progeny from a genetic cross of *MAT1-1* and *MAT1-2* isolates and to the mating phenotype of field-collected isolates from diverse geographic locations. *MAT*-specific PCR was used to rapidly determine the mating type of isolates of *A. rabiei* sampled from chickpea fields in the US Pacific Northwest. Mating type ratios were not significantly different from 1:1 among isolates sampled from two commercial chickpea fields consistent with the hypothesis that these *A. rabiei* populations are randomly

mating. Mating type ratios among isolates sampled from an experimental chickpea field where asexual reproduction was enforced differed significantly from 1:1. A phylogeny estimated among legume-associated *Ascochyta* spp. and related loculoascocmycete fungi using sequence data from the nuclear ribosomal internal transcribed spacer (ITS) demonstrated the monophyly of *Ascochyta*/*Didymella* spp. associated with legumes but was insufficiently variable to differentiate isolates associated with different legume hosts. In contrast, data from the HMG region of *MAT1-2* was substantially more variable, revealing seven well-supported clades that correlated to host of isolation. *A. rabiei* on chickpea is phylogenetically distant from other legume-associated *Ascochyta* spp. and the specific status of *A. rabiei*, *A. lentis, A. pisi* and *A. fabae* was confirmed by the HMG phylogeny.

3B.2 Introduction

Ascochyta rabiei (Pass.) Labr. [teleomorph: *Didymella rabiei* (Kovacheski) v. Arx (=*Mycosphaerella rabiei* Kovacheski)] is a heterothallic loculoascomycete fungus with a bipolar mating system (Trapero-Casás and Kaiser, 1992; Wilson and Kaiser, 1995) typical of ascomycete fungi, where sexual reproduction is controlled by a single regulatory locus referred to as the mating type or *MAT* locus (Coppin *et al.*, 1997; Nelson, 1996; Turgeon, 1998). In filamentous Ascomycete fungi, alleles at the single *MAT* locus consist of completely dissimilar sequences that have been referred to as 'idiomorphs' (Metzenberg and Glass, 1990). The idiomorph of one mating type is, by definition and by function, completely unlike that of the other mating type, though small islands of *MAT*-1/*MAT*2 identity, reflecting a common origin, do exist between the two idiomorphs (Coppin *et al.*, 1997). As the two idiomorphs are highly dissimilar in sequence, they do not recombine with each other (Sharon *et al.*, 1996) and are inherited uniparentally (Turgeon, 1998). The idiomorphs are flanked by regions of DNA, which have high sequence similarity between both mating types (Christiansen *et al.* 1997; Turgeon *et al.* 1998). All Ascomycete idiomorphs encode proteins with confirmed or putative DNA binding motifs, which suggest that *MAT* genes encode transcriptional regulators and control the expression of some genes required for sexual reproduction (Turgeon *et al.*, 1998). The *MAT1-1* idiomorph encodes a DNA binding protein containing an alpha (α) domain while *MAT1-2* idiomorph encodes a DNA binding protein containing a high mobility group (HMG) domain (Coppin *et al.*, 1997; Turgeon *et al.*, 1993; Turgeon, 1998). The alpha and HMG domains are highly conserved across the Ascomycetes studied to date (Turgeon *et al.*, 1998).

Mating type loci have been cloned and characterized from several ascomycete fungi using a variety of cloning strategies. These have ranged from functional complementation (Glass *et al.*, 1988; Turgeon *et al.*, 1993), substractive hybridization (Kang *et al.*, 1994), genomic library screening with heterologous probes (Picard *et al.*, 1991; Waalwijk *et al.*, 2002), to PCR-based approaches (Arie *et al.*, 1997; Arie *et al.*, 1999; McGuire *et al.*, 2001; Zhong and Steffenson, 2001). Cloning and characterization

of *MAT* genes from filamentous ascomycetes has revealed similar structural organization and expression (Coppin *et al.*, 1997; Nelson, 1996; Turgeon, 1998). *MAT* genes have also been cloned and characterized from putatively asexual fungi and these genes are functional in closely related sexual species (Arie *et al.*, 2000; Sharon *et al.*, 1996).

Development of the teleomorph of *A. rabiei* is dependent upon the presence of both mating types in close physical proximity. In most areas of the world, the teleomorph is produced during the winter and functions as a survival stage between chickpea crops (Navas-Cortés *et al.*, 1995). Primary inoculum for Ascochyta blight epidemics in most chickpea-producing areas is thought to be ascospores which are forcibly ejected from pseudothecia, dispersed by wind and are able to infect chickpea plants several hundred meters from the source (Navas-Cortés *et al.*, 1998; Trapero-Casás and Kaiser, 1992; Trapero-Casás *et al.*, 1996). Although the mating system of *A. rabiei* has not been determined directly in the field, it is assumed that recombinant *A. rabiei* ascospore progeny could contribute to increased genotypic diversity in *A. rabiei* populations. This variation is potentially adaptive, allowing the pathogen to evolve increased virulence on resistant cultivars or to develop resistance to fungicides. Mating types ratios of *A. rabiei* within a given country, region, or field have revealed that they may significantly differ from 1:1, precluding formation of the sexual stage thereby adversely affecting long term survival and eliminating an important source of primary inoculum. The absence of the sexual stage could have a major impact on disease epidemiology. More research is needed to determine the role of the sexual stage in the biology of *A. rabiei* populations and to determine the mating system of the fungus in the field.

Pseudothecia of *D. rabiei* develop on chickpea debris and require approximately two months of high humidity and 5-10°C temperatures to mature and release ascospores (Navas-Cortés *et al.*, 1998; Trapero-Casás and Kaiser, 1992). The teleomorph can also be induced to form on senescent chickpea stems in laboratory crosses of compatible mating types of the fungus by simulating these environmental conditions (Trapero-

Casás and Kaiser, 1992; Wilson and Kaiser, 1995), and such crosses have been used extensively to determine mating type in population samples of the fungus (Armstrong *et al.*, 2001; Kaiser and Kusmenoglu, 1997). The mating type of several hundred *A. rabiei* isolates from different locations around the world have been determined through conventional laboratory crosses with mating type tester strains (Armstrong *et al.*, 2001; Kaiser and Kusmenoglu, 1997; Wilson and Kaiser, 1995). This is a tedious and labor-intensive process and evolutionary studies of *A. rabiei* and other *Ascochyta* spp. would be greatly facilitated by the development of a mating type-specific PCR assay. This has already been accomplished with several other fungi (Dyer *et al.*, 2001; Foster *et al.*, 1999; Waalwijk *et al.*, 2002). A PCR assay for mating type in *A. rabiei* would allow rapid determination of mating type ratios in populations and provide a means to track the introduction of a second mating type into an area. Such an assay would also allow predictions to be made regarding the prevalence of asexual versus sexual reproduction in populations and its effect on Ascochyta blight epidemiology.

Host-specific *Ascochyta* spp. cause disease on a number of legume hosts and laboratory crosses among some of them has allowed the delimitation of biological species (Kaiser *et al.*, 1997). Ascospore progeny from crosses between the morphologically similar *A. fabae* (faba bean pathogen) and *A. lentis* (lentil pathogen) were morphologically abnormal and could not infect either host. This indicated the presence of significant fertility barriers between these fungi. This data, in combination with molecular markers, was used as evidence that these two host-specific forms should be considered distinct biological species (Kaiser *et al.*, 1997). A phylogeny of legume-associated *Ascochyta* spp. and a more thorough understanding of the genetic control of sexual processes in these fungi would provide a framework for further studies of evolution, speciation and the genetics of host specificity among this group of organisms. *MAT* genes have been proposed as potentially useful regions of the genome for phylogenetic reconstruction (Turgeon, 1998) but have seen only limited use in fungal systematics studies to date (Poeggeler, 1999; Schoch *et al.*, 2000; Witthuhn *et al.*, 2000). *MAT* genes appear to evolve more quickly than other regions of the

genome but are highly conserved within species making them useful for phylogenetic analysis of closely related species (Poeggeler, 1999; Turgeon, 1998).

The specific objectives of this study were: 1) to clone and characterize the *MAT* locus from *A. rabiei* using PCR-based techniques, 2) to develop mating type-specific primers for use in a multiplex PCR assay for mating type, and 3) to use *MAT1-2* HMG sequence data from *A. rabiei* and related *Ascochyta* spp. to estimate a phylogeny among legume-associated *Ascochyta* spp.

3B.3 Materials and Methods

3B.3.1 Isolates, culturing and DNA isolation

Ascochyta rabiei isolates AR20 (ATCC 76501, mating type 2) and AR21 (ATCC 76502, mating type 1) and 22 additional isolates of *A. rabiei*, *A. fabae, A. lentis*, *A. pisi*, *Ascochyta* spp., *Didymella* spp., *Phoma medicaginis* and *Mycosphaerella pinodes* from various legumes were obtained from the USDA Western Region Plant Introduction Station, Pullman, WA (Table 3.3). *A. rabiei* isolates AR20 and AR21 are mating type tester strains which have been used extensively in population studies of *A. rabiei* (Kaiser and Kusmenoglu 1997, Trapero-Casás and Kaiser 1992, Wilson and Kaiser 1995). All isolates were routinely cultured on V8 juice agar (200 ml V8 juice, 3 g $CaCO_3$, 20 g agar per liter) on lighted growth shelves at 22°C with a 12 hour alternating photoperiod and stored on sterile filter paper at 20°C as previously described (Peever *et al.*, 1999). Liquid cultures were initiated with 4 mm^2 pieces of filter paper in 2-YEG medium (2 g yeast extract, 10 g glucose per liter) and incubated at 20°C on a rotary shaker at 175 rpm for 5-6 days. Mycelia were harvested by vacuum filtration and lyophilized in sterile 2 ml centrifuge tubes. Freeze-dried mycelia were powdered and DNA extracted using a modified CTAB extraction protocol as described in section 2A.3.2 of the present thesis.

Table 3.3 Isolates of *Ascochyta*, *Didymella*, *Mycosphaerella*, and *Phoma* spp. from legumes used in *MAT* cloning and phylogenetic analysis

Species	ATCC #	Code	Mating type[a]	*MAT* genotype [b]	Host	Location	Collector	Date
Ascochyta rabiei	24891	AR19	2	2	*Cicer arietinum*	Iran	W.J. Kaiser	1990
Ascochyta rabiei	76501	AR20	2	2	*Cicer arietinum*	Idaho, USA	W.J. Kaiser	1986
Ascochyta rabiei	76502	AR21	1	1	*Cicer arietinum*	Idaho, USA	W.J. Kaiser	1986
Ascochyta rabiei		AR150	1	1	*Cicer arietinum*	Saskatoon, Canada	R.A.A. Morrall	1991
Ascochyta rabiei		AR231	2	2	*Cicer arietinum*	North Dakota, USA	F. Muehlbauer	1993
Ascochyta rabiei		AR248	2	2	*Cicer arietinum*	Oregon, USA	W.J. Kaiser	1993
Ascochyta rabiei		AR250	2	2	*Cicer arietinum*	Idaho, USA	W.J. Kaiser	1993
Ascochyta rabiei		AR329	1	1	*Cicer arietinum*	California, USA	I. Buddenhagen	1994
Ascochyta rabiei		AR428	2	2	*Cicer arietinum*	Idaho, USA	W.J. Kaiser	1987
Ascochyta rabiei		AR445	1	1	*Cicer arietinum*	Washington, USA	W.J. Kaiser	1987
Ascochyta rabiei		AR600	2	2	*Cicer arietinum*	Idaho, USA	W.J. Kaiser	1995
Ascochyta rabiei	201622	AR628	1	1	*Cicer arietinum*	Malikiye, Syria	J. Geistlinger	1995
Ascochyta rabiei		AR830	2	2	*Cicer arietinum*	Victoria, Australia	N. Mahmood	1997
Ascochyta fabae	96418	AF1	2	2	*Vicia faba*	Saskatoon, Canada	B. Vandenberg	1992
Ascochyta fabae	96409	AF4	1	1	*Vicia faba*	Iran	C. Bernier	1992
Didymella fabae	201613	AF8	2	2	*Vicia faba*	Cambridge, UK	R.A.A. Morrall	1992
Ascochyta lentis	96419	AL1	2	2	*Lens culinaris*	Australia	W.J. Kaiser	
Ascochyta lentis	96420	AL2	1	1	*Lens culinaris*	Brazil	W.J. Kaiser	
Ascochyta lentis		AL5	2	2	*Lens culinaris*	Morocco	W.J. Kaiser	
Ascochyta lentis		AL391	2	2	*Lens culinaris*	Australia	W.J. Kaiser	1997

Cont…

Ascochyta pisi	201618	AP2	2	2	*Pisum sativum*	Poznan, Poland	E. Swiecicki	
Ascochyta pisi	201620	AP4	2	2	*Pisum sativum*	Saskatoon, Canada	B. Gossen	
Ascochyta pisi		AP9	2	2	*Pisum sativum*	Monteagudo, Bolivia	W.J. Kaiser	1999
Ascochyta spp.		AV-01-1	2	2	*Vicia villosa*	Washington, USA	T.L. Peever	2001
Ascochyta spp.		AV-11	nt	nt	*Vicia grandiflora*	Connecticut, USA	Ken Leath	1994
Ascochyta spp.		AV22	1	1	*Vicia villosa*	Aprilci, Bulgaria	W. J. Kaiser	1996
Didymella spp.		AV23	2	2	*Vicia villosa*	Washington, USA	W.J. Kaiser	1996
Phoma medicaginis		PMP2	2	2	*Pisum sativum*	Washington, USA	W.J. Kaiser	1996
Phoma medicaginis		PMP9	2	2	*Pisum sativum*	Australia	D. Webster	1995
Mycosphaerella pinodes	201628	MP1	2	2	*Pisum sativum*	Oregon, USA	J. Baggett	1995
Mycosphaerella pinodes		MP11	2	2	*Pisum sativum*	New Zealand	J. Kraft	1996
Ascochyta spp.		AS1	2	2	*Medicago sativa*	Washington, USA	T.L. Peever	2001
Ascochyta spp.		AS4	2	2	*Medicago sativa*	Washington, USA	T.L. Peever	2001

[a] mating type as determined by pairing with mating-type testers and PCR amplification of *HMG* motif. *HMG*-positive isolates are classified as *MAT1-2* following the recommendations of Turgeon and Yoder (2000) but is opposite the original mating type classification scheme of W.J. Kaiser (Trapero-Casás & Kaiser 1992). **nt** = not tested

[b] mating type as determined with *MAT*-specific PCR primers

3B.3.2 Thermal asymmetric interlaced (TAIL)-PCR

High mobility group (HMG) motifs of *MAT1-2* sequences of *Alternaria alternata* (GenBank accession no. AB009452), *Cochliobolus heterostrophus* (GenBank accession no. X68398), and *C. sativus* (GenBank accession no. AF275374) were aligned using Clustal-X (Thompson *et al.*, 1997) and degenerate primers HMG-L and HMG-R (Table 3.4) were designed to the HMG motif using the primer design software Primer 3 (http://www.genome.wi.mit.edu/genome_software/other/primer3.html). HMG-L and HMG-R were used to amplify the HMG motif (270 bp) from *A. rabiei* mating type 2 isolates AR231, AR248, AR250, and AR600 (Fig. 3.3A, Table 3.3). Twenty-five µl PCR amplifications contained 1X PCR buffer (Promega, USA), 2.5 mM MgCl2 (Promega, USA), 200 µM dNTPs (Idaho Technologies, USA), 2 mM each HMG-L and HMG-R degenerate primers (Operon Technologies, USA), 20 ng of DNA, and 1 unit *Taq* polymerase (Promega, USA). Cycling conditions consisted of a 2 min initial denaturation at 95°C followed by 40 cycles of: 1 min denaturation at 95°C, 1 min annealing at 55°C, and 1 min extension at 72°C. These cycles were followed by a final 5 min elongation cycle at 72°C. Reactions were performed in an Omn-E thermal cycler (Hybaid, UK) and amplicons were separated in 1.8% agarose gels with *Hind* III/*Eco*R1 digested lambda DNA (Promega, USA) as size standards and visualized with a digital imaging system (UltraViolet Products, USA). Amplicons were purified using Qiaquick PCR purification columns (Qiagen, USA) following the manufacturer's instructions and column-purified PCR amplicons were direct-sequenced with HMG-L or HMG-R primers. *A. rabiei* HMG sequences were aligned using Clustal-X and a nested set of four *A. rabiei*-specific HMG primers (HMG-4L, HMG-34L, HMG-203R, HMG-239R) (Fig. 3.3A, Table 3.4) were designed using Primer 3. These primers were paired in TAIL-PCR amplifications with arbitrary degenerate random primers AD1, AD5 and AD6 (Table 3.4) to generate TAIL-PCR amplicons containing the HMG motif (Fig. 3.3B). TAIL- PCR amplifications were as described previously in (Arie *et al.*, 1997, 2000).

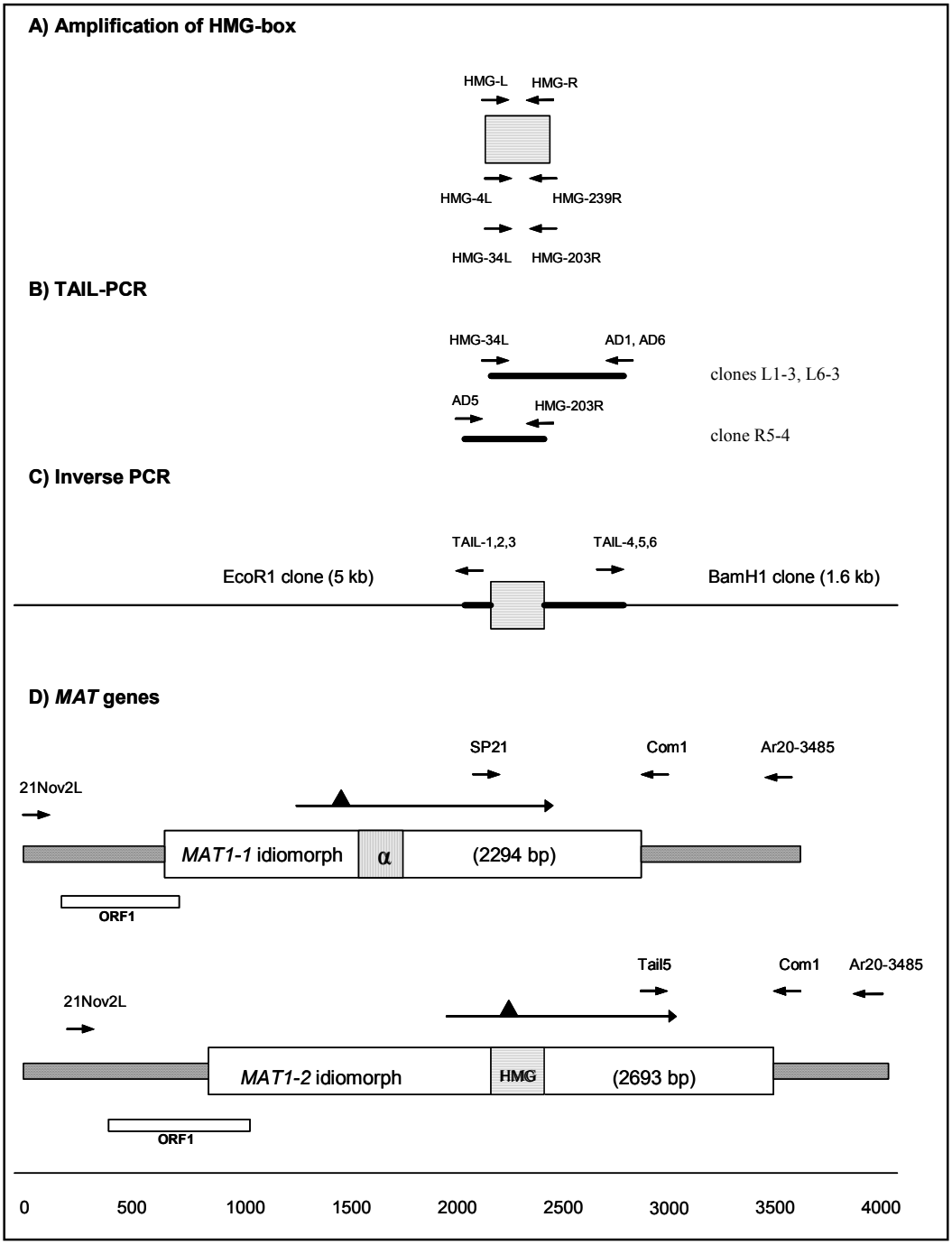


Fig. 3.3 PCR cloning of *MAT* loci from *Ascochyta rabiei*. A) Amplification of the *HMG* motif from *A. rabiei* using degenerate primers HMG-L and HMG-R. Design of *A. rabiei MAT*-specific primers HMG-4L, HMG-34L, HMG-203R, and HMG-239R and amplification and sequencing of HMG motifs from *A. rabiei*. B) TAIL-PCR to generate a 1.1 kb consensus sequence of 3 clones amplified with HMG-34L plus AD1 and AD6 primers and HMG-203R plus AD5 primers. C) Inverse PCR from five monomeric, ligated restriction fragments. Only *Eco*R1 and *Bam*H1 clones are shown. D) Structural organization of *A. rabiei MAT1-1* and *MAT1-2*. Primers 21Nov2l and Ar20-3485 designed to the flanking DNA of *MAT1-2* used to amplify the entire *MAT1-1* idiomorph plus flanking sequences. Primers Com 1 (common flanking primer), SP-21 (*MAT1-1*-specific), and Tail 5 (*MAT1-2*-specific) used in multiplex PCR reactions to amplify different sized *MAT*-specific PCR amplicons. Approximate nucleotide positions are indicated by scale at bottom of figure.

3B.3.3 Inverse PCR

Three sets of nested, outward-facing primers were designed to the 5' and 3' ends of the 1.1 kb TAIL-PCR sequence and used in inverse PCR amplifications (Arie *et al.*, 2000). Tail 1, 2 and 3 were designed to the 5' end and Tail 4, 5 and 6 were designed to the 3' end of TAIL-PCR clone, respectively (Fig. 3.3C, Table 3.4). Approximately 2 µg of genomic DNA from mating type 2 isolate AR20 was digested to completion using 12 restriction enzymes (*Bam*HI, *Bgl*I, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Not*I, *Nco*I, *Pvu*II, *Sal*I, *Sma*I, *Xho*I) (Promega, USA) according to the manufacturer's instructions. Digests were precipitated using standard protocols (Sambrook *et al.*, 1989) and ligation reactions were set up in 100 µl volumes consisting of 1X T4 DNA ligase buffer (Invitrogen, USA), 10 mM ATP (Invitrogen, USA), 12 U T4 DNA ligase (Invitrogen, USA), and 200-300 ng of digested DNA to promote the formation of monomeric ligation products. Ligated products were precipitated and dissolved in 20µl sterile distilled water. Forty µl PCR amplifications consisted of 2 µl ligated product (approx. 20 ng DNA), 200 nM each of primers Tail 1 and 4, 1X LA *Taq* Buffer (Panvera, USA), 200 µM dNTP, 2 U LA *Taq* Polymerase (Panvera, USA). Reactions were carried out in a PE 9700 thermal cycler (Applied Biosystems, USA). Cycling conditions consisted of an initial denaturation at 94°C for 3 min, followed by 45 cycles of: 94°C denaturation for 30 sec, 60°C annealing for 45 sec, 72°C extension for 2.5 min and a final 72°C extension for 10 min. Inverse PCR amplicons were resolved in 1% agarose gels with *Hin*dIII/*Eco*R1- digested lambda DNA (Promega, USA) as the size standard, stained with ethidium bromide and digitized as described above.

Table 3.4 Primers used in cloning, sequencing, and *MAT*-specific PCR from *Ascochyta rabiei* and amplification of *HMG* from related species

Name	**Nucleotide sequence**	**Comments**
HMG-L	5'-GVCCKATGAAYTGYTGGAT-3'	degenerate HMG-specific forward primer
HMG-R	5'-CMGGYYTYCKBGGRSWRTACTT-3'	degenerate HMG-specific reverse primer
HMG-4L	5-'CCGATGAATTGCTGGATCAT-3'	*A. rabiei* HMG-specific forward primer
HMG-34L	5'-GCTATGCACAAGCAGCTGAA-3'	*A. rabiei* HMG-specific forward primer
HMG-203R	5'-TTGCCAAGGCTTCTTCTCTG-3'	*A. rabiei* HMG-specific reverse primer
HMG-239R	5'-GCGCAGATGTTCTGCCTTC-3'	*A. rabiei* HMG-specific reverse primer
AD1	5'-NGTCGASWGANAWGAA-3'	Arbitrary degenerate primer used in TAIL-PCR
AD5	5'-TGYCCNAAYMGNYT-3'	Arbitrary degenerate primer used in TAIL-PCR
AD6	5'-TTRTTNCCRTANCCDAT-3'	Arbitrary degenerate primer used in TAIL-PCR
Tail 1	5'-GAGTGGCTGTGGAAAAGTTAATG-3'	*A. rabiei MAT1-2*-specific primer used in inverse PCR
Tail 2	5'-CTGTACAGTAAGGTGTGGGCTCT-3'	*A. rabiei MAT1-2*-specific primer used in inverse PCR
Tail 3	5'-GTTTTCAGCTGCTTGTGCATAG-3'	*A. rabiei MAT1-2*-specific primer used in inverse PCR
Tail 4	5'-ATGCGACTCTCATCGATCTACTC-3'	*A. rabiei MAT1-2*-specific primer used in inverse PCR
Tail 5	5'-CGCTATTTTATCCAAGACACACC-3'	Multiplex MAT-specific PCR primer
Tail 6	5'-GAACGCACTTTGAGATTTGGTCT-3'	*A. rabiei MAT1-2*-specific primer used in inverse PCR
Ar20-2-1	5'-GTCGTCGGGTCAGGAAGTT-3'	*MAT*1-2 sequencing
Ar20-2-2	5'-GTGAACGCAAGCATCGAGT-3'	*MAT1*-2 sequencing
Ar20-2-3	5'-AATCCCACTCCCAGATGCTT-3'	*MAT1-2* sequencing

Ar20-2-4	5'-GTGGAGACGGGCTTGATTT-3'	*MAT1-2* sequencing
Ar20-2-5	5'-TGGTTCCGGAATCGACAGTA-3'	*MAT1-2* sequencing
Ar20-2-6	5'-ATGGTGCCTTCGAGATTGAG-3'	*MAT1-2* sequencing
MB81401	5'-TGGTCCAAGGTGAAAGAGACTT-3'	*MAT1-2* sequencing
Ar20-Up	5'-ATGATTCGTGTGCGCTAGG-3'	*MAT1-2* sequencing
Ar20-Down	5'-CCAACACCCTGGTCTTGAAC-3'	*MAT1-2* sequencing
Ar20-3485	5'-GGAAAGGATCGTTGGTGAAC-3'	3’ flanking primer used to amplify MAT1-1 idiomorph
21Nov2L	5'-GCTCCTTCCCACTGCACTTA-3'	5’ flanking primer used to amplify MAT1-1 idiomorph
ArCL6-1F	5'-AAGGAGACACTCGGGAAGGT-3'	*MAT1-1* sequencing
ArCL10-1R	5'-AGTGCCTGGTTGGCAAAG-3'	*MAT1-1* sequencing
ArCL10-2R	5’-TCAGTGTTGTGGGCATTAGC-3’	*MAT1-1* sequencing
ArCL10-2F	5’-GACCATTGGTGAATGCACTG-3’	*MAT1-1* sequencing
Com1	5'-GCATGCCATATCGCCAGT-3'	For multiplex *MAT*-specific PCR - common flanking primer
SP-21	5'-ACAGTGAGCCTGCACAGTTC-3'	For multiplex *MAT*-specific PCR - MAT1-1-specific primer
AR20-2085L	5’-ATGGCACAATTTCAGAGTGC-3’	*MAT1-2* ORF – forward primer
AR20-3105R	5’-ATCGATGGAAGAGTCGCATT-3’	*MAT1-2* ORF – reverse primer
AR21-1393L	5’-TGACGTTTCCAAGAGATCCA-3’	*MAT1-1* ORF – forward primer
AR21-2485L	5’-GTGTGGCATTTTCGTCTGC-3’	*MAT1-1* ORF – reverse primer
HMG-8L	5’-GAAYTGCTGGATCATCTTTCG-3’	HMG-specific degenerate forward primer for *Ascochyta* spp.
HMG-236R	5’-GCAAATGCTCYGCTTTCG-3’	HMG-specific degenerate reverse primer for *Ascochyta* spp.

3B.3.4 Cloning and sequencing

TAIL-PCR and inverse PCR products were cloned into pCR4-TOPO (Invitrogen, USA) or pGEM-T Easy (Promega, USA) vectors following the manufacturer's instructions. TOP 10 competent *E. coli* cells (Invitrogen, USA) were chemically transformed and grown overnight in 2 ml LB cultures with 100µg/ml ampicillin. Plasmid DNA was purified using Bio 101 RPM spin columns (Q-BIOgene, USA) or Qiaprep Spin Minprep columns (Qiagen, USA) and sequenced using T7, SP6 (inverse PCR clones), M13F or M13R (TAIL-PCR clones) universal primers. Sequence reactions contained 40-90 ng DNA, 320 nM primer, 4 µl BigDye Terminator Cycle Sequencing Ready Reaction Mix (Applied Biosystems, USA), and sterile distilled water in 10 µl volumes. Cycle sequence reactions were performed in an Omn-E (Hybaid, U. K.) or PE 9700 (Applied Biosystems, USA) thermal cycler and cycling conditions consisted of 25 cycles of 15 s at 96°C, 15 s at 50°C, and 4 min at 60°C. Products were purified using Centriflex Gel Filtration Cartridges (Edge BioSystems, USA) and sequenced on a PE Biosystems Model 377 Automated DNA Sequencer (Applied Biosystems, USA). All sequencing was performed in the Laboratory for Biotechnology and Bioanalysis, School of Molecular Biosciences, Washington State University.

3B.3.5 *MAT* sequence analysis

MAT1-2 sequence data obtained from three TAIL-PCR clones (L1-3, L1-6, R5-4) and two inverse PCR clones (Fig. 3.3C) were assembled using Vector NTI Suite v.7.0 (InforMax, USA). The assembled sequence (4.4 kb) was aligned with *MAT1-1* of *A. alternata* (GenBank accession no. AB009451) to identify putative flanking regions and to design primers that would amplify the entire alternate idiomorph (*MAT1-1*) from *A. rabiei*. Primers 21Nov2L (forward) and Ar20-3485 (reverse) (Fig. 3.3D, Table 3.4) were designed to amplify the entire *MAT1-1* idiomorph. PCR amplifications were carried out as described above with 200 µM each of 21Nov2L and Ar20-3485 primers (Operon, USA) and a 60°C annealing temperature. *MAT1-1* amplicons were cloned and sequenced as described above. Assembled *A. rabiei MAT1-1* sequences were aligned

with *MAT1-1* sequences from *A. alternata* (GenBank accession no. AB009451) and *C. heterostrophus* (GenBank accession no. X68399) and assembled *A. rabiei MAT1-2* sequences were aligned with *MAT1-2* sequences from *A. alternata* (GenBank accession no. AB009452) and *C. heterostrophus* (GenBank accession no. X68398). Assembled sequences were analyzed for putative open reading frames and introns using Genetyx Mac v.11.2 software (Genetyx, Japan). Putative introns were spliced from the open reading frames, conceptually translated using Jellyfish software (Lab Velocity, USA) and aligned in Clustal-X. BLAST (Altschul *et al.*, 1990) searches for similar nucleotide and protein sequences were carried out against the National Center for Biotechnology Information (NCBI) databases.

3B.3.6 *MAT* expression

Mycelia of isolates AR20 and AR21 were separately filtered from 4 day-old cultures grown in 2-YEG medium, lyophilized and powdered in liquid nitrogen. Total RNA was extracted from each isolate using Trizol (Invitrogen, USA) according to the manufacturer's instructions. First strand cDNA synthesis was performed using the SuperScript First-Stand Synthesis System (Invitrogen, USA) according to the manufacturer s instructions and cDNAs were used in PCR amplifications with primers AR21-1393L and AR21-2485R (to amplify *MAT1-1-1*), and AR20-2085L and AR20-3105R (to amplify *MAT1-2-1*) (Table 3.4). Templates for PCR amplifications included genomic DNA, cDNAs and RNA controls from each isolate. Control PCR amplifications also included the same templates amplified with primers gpd-1 and gpd-2 for the glyceraldehyde-3-phosphate-dehydrogenase gene (Berbee *et al.*, 1999). PCR amplifications were carried out as described above with a 65°C annealing temperature and 30 s cycles and *Hae*III-digested ϕ*X174 RF* DNA (Invitrogen, USA) was used as a size standard. Amplicons were sequenced with HMG-239R (*MAT1-2-1*-specific) or AR21-1393L (*MAT1-1-1*-specific) primers. Sequences from AR20 cDNAs were aligned with *A. rabiei* genomic *MAT1-2-1* sequences, *A. alternata* (GenBank accession no. AB009452), and *C. heterostrophus* (GenBank accession no. X68398) *MAT1-2-1* sequences to confirm intron splice sites. AR21 cDNAs were aligned with *A. rabiei*

MAT1-1-1 genomic sequences, *A. alternata* (GenBank accession no. AB009451) and *C. heterostophus* (GenBank accession no. X68399) *MAT 1-1* sequences to confirm intron splice sites.

3B.3.7 Southern analysis

Isolates AR21, AR150, AR329, AR445, and AR628 (mating type 1) and AR19, AR20, AR428, AR600 and AR830 (mating type 2) (Table 3.3) were cultured separately in flasks containing liquid V8 juice medium. Four day-old cultures were vacuum filtered and lyophilized. Dry mycelium was ground to fine powder and genomic DNA was extracted following the method of Lee and Taylor (1990). DNA concentration was estimated visually by gel electrophoresis with known concentrations of phage lambda DNA (Invitrogen, USA) as a standard. Two to 3 µg of DNA from each isolate was digested separately at 37°C overnight with restriction enzyme *Hin*dIII (Invitrogen, USA) following the manufacturer's suggestions. Digested DNA was separated through 0.8% agarose gels and transferred to Hybond N+ membranes (Amersham Biosciences, USA) following the protocol of Salimath *et al.* (1995). *MAT*-specific probes were prepared by PCR amplification of *MAT1-1-1* and *MAT1-2-1* from isolates AR21 and AR20, respectively, using primers AR21-1393L and AR21-2485R (to amplify *MAT1-1-1*), and AR20-2085L and HMG239R (to amplify *MAT1-2-1*). PCR amplicons were column-purified and approximately 1 µg of DNA was random prime labeled with digoxigenin-11-dUTP using the DIG DNA Labeling and Detection Kit (Roche Diagnostics, USA) according to the manufacturer's instructions. Pre-hybridization, hybridization, washing and chemiluminescent detection with CSPD were carried out with the same kit (Roche Diagnostics, USA). Hybridization was detected by exposing the membranes to Kodak X-OMAT film (Kodak, Rochester, USA) for 15-30 minutes and developed under standard conditions.

3B.3.8 Genetic analysis of *MAT*-specific PCR products

Thirty-five progeny from an AR20 x AR21 cross were backcrossed to both parents to determine mating phenotype. Crosses were set up following the protocol previously

described (Trapero-Casás and Kaiser, 1992; Wilson and Kaiser, 1995) and mating phenotype was scored based on the production of ascospores when paired with a parent of opposite mating type as previously described (Kaiser and Kusmenoglu, 1997). Primers SP-21, Tail 5, and Com 1 were designed to amplify different size PCR amplicons from *A. rabiei MAT1-1* and *MAT1-2* strains using Primer 3 software (Fig. 3.3D, Table 3.4). Multiplex PCR amplifications with primers SP-21, Tail 5 and Com1 (Table 3.4) were performed with DNA extracted from the same progeny set to infer mating type based on the production of a *MAT*-specific amplicon. Twenty-five µl PCR amplifications were carried out as described above with 10-30 ng template, 400 nM of each primer, a 60°C annealing temperature and 45 s cycles. Multiplex *MAT*-specific PCR was also used to infer mating type among a worldwide collection of fourteen mating type 1 and ten mating type 2 isolates whose mating types were previously determined. Control PCR amplifications contained genomic DNA from *Fusarium oxsporum f. sp. ciceri* races 2, 3 and 4 (described in Section 2A), *Ascochyta fabae* (AF1 and AF4), *Ascochyta lentis* (AL1 and AL 2), and *Ascochyta* spp. from wild vetch (AV22 and AV23), and primers SP-21, Tail 5 and Com1 (Table 3.3).

3B.3.9 Mating type ratios in *A. rabiei* populations

Multiplex *MAT*-specific PCR with the SP-21, Tail 5 and Com1 primers was used to infer the mating type of field-collected isolates of *A. rabiei* obtained from different geographic locations in the US Pacific Northwest in 1998. Samples consisted of thirty-one and twenty isolates, respectively, from two commercial chickpea fields in Nez Perce Co., ID (DAN-98, PKS-98) and one sample of twenty-one isolates from the USDA-ARS Ascochyta blight resistance-screening nursery at Washington State University's Spillman Research Farm near Pullman, WA (Table 3.5). Single conidial isolates were obtained from leaves with Ascochyta blight lesions (one isolate per leaf) and the isolates were cultured and DNA extracted as described above. Multiplex PCR amplifications were performed as described above.

Table 3.5 Mating type ratios in field samples of *Ascochyta rabiei* determined with *MAT*-specific PCR

Sample [a]	*N* b	Ratio [c]	χ2 d	*P* e
DAN98	31	14:17	0.290	0.590
PKS98	20	12:8	0.800	0.371
SPL98	21	4:17	8.048	0.005

[a] samples obtained from commercial chickpea fields (DAN98, PKS98) and a chickpea resistance screening nursery (SPL98) in 1998
[b] number of isolates analyzed
c *MAT1-1*:*MAT1-2*
[d] χ^2 value for test of 1:1 ratio
[e] probability of a greater χ^2 value under the null hypothesis of 1:1 ratio (1d.f.)

3B.3.10 Phylogenetic analysis of ITS and HMG

The nuclear ribosomal internal transcribed spacer region (ITS) was amplified from two or more isolates from each legume host (Table 3.3) using ITS1 and ITS 4 primers (White *et al.*, 1990). PCR amplifications were carried out in 25 µl volumes as described and cycling conditions consisted of 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min followed by 5 min at 72°C. ITS amplicons were direct-sequenced on both strands as described above and additional loculoascomycete ITS sequences were retrieved from GenBank to facilitate comparison. Primers HMG-L and HMG-R (Fig. 3.3A, Table 3.4) were used to amplify 270 bp of the HMG motif from isolates AL1, AF1, AP2, and AV1 (Table 3.3). PCR amplifications were carried out as described above with 2 mM each HMG-L and HMG-R degenerate primers (Operon, USA), an annealing temperature of 55°C, and 15 s cycles. Sequences were aligned using Clustal-X and *Ascochyta*-specific HMG primers HMG-8L and HMG-236R (Table 3.4) were designed. These primers were used to amplify HMG from additional *Ascochyta*, *Didymella*, *Phoma* and *Mycosphaerella* spp. (Table 3.3) using the same PCR conditions except for a 60°C annealing temperature. Amplicons were

sequenced using HMG-8L and HMG-236R primers (Table 3.4). Sequences were aligned using Clustal-X and a maximum likelihood phylogeny was estimated for each genomic region using the DNAML program in PHYLIP (Phylogeny Inference Package, version 3.6a2. University of Washington, Seattle). Only unique sequences were used to infer the phylogenies. Prior to phylogenetic analysis, models of sequence evolution were tested and model parameter estimates obtained for each sequence alignment using MODELTEST 3.06 (Posada and Crandall, 1998) as implemented in PAUP 4.0b10 (Swofford, 2002). Statistical support for phylogram topologies was estimated using 100 bootstrapped datasets generated in the SEQBOOT program of PHYLIP. One hundred phylograms were estimated using DNAML with transition/transversion ratios, gamma distribution parameters and base frequencies estimated using MODELTEST, random input order of taxa with 3 jumbling steps. A majority-rule consensus tree was produced by the program CONSENSE and the consensus phylograms were visualized in TREEVIEW (Page, 1996). Maximum likelihood branch lengths for the consensus trees were estimated by using the consensus tree as a user tree in DNAML. Clades were inferred based on phylogram branches with bootstrap values greater than 80%.

3B.4 Results

3B.4.1 PCR cloning of *A. rabiei MAT*

PCR amplification of the HMG motif using degenerate primers HMG-L and HMG-R revealed that the mating type designations previously employed with *A. rabiei* (Kaiser and Kusmenoglu, 1997; Trapero-Casás and Kaiser, 1992) classified HMG-containing isolates as mating type 1. This is opposite the convention proposed by Turgeon and Yoder (2000) for ascomycete *MAT* genes and all *MAT* designations have been changed herein to be consistent with this standard. PCR amplicons of 262, 554, and 983 bp were amplified from genomic DNA of isolate AR20 *MAT1-2*) using TAIL-PCR (Fig. 3.3B). One amplicon (262 bp) 5' of the HMG motif was generated with arbitrary degenerate primer AD5 and HMG-specific primer HMG-203R (clone R5-4). Two amplicons (554 bp and 983 bp) 3' of the HMG motif were generated with HMG-specific primer HMG-34L and arbitrary degenerate primers AD1 and AD6, respectively (clones L1-6,

L6-3). Assembly of the sequences resulted in a 1.1 kb consensus sequence containing the *MAT1-2* HMG motif (Fig. 3.3B). Primers Tail 1 and Tail 4 were designed to the 5' and 3' ends of the consensus TAIL-PCR sequence, respectively, and used in additional TAIL-PCR amplifications. These subsequent TAIL-PCR amplifications were not successful in extending the *MAT-1-2* sequence. Primers Tail 1 and Tail 4 were used in inverse PCR amplifications and PCR amplicons were obtained from AR20 genomic DNA, which was digested with restriction enzymes *Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Not*I, re-ligated and amplified with primers Tail 1 and Tail 4 (Fig. 3.3C). DNA digested with seven additional restriction enzymes and re-ligated failed to amplify. The *A. rabiei* HMG probe hybridized to all five inverse PCR amplicons in Southern blots demonstrating that all contained the HMG motif (data not shown). Inverse PCR products were cloned and sequenced using a series of primers (Table 3.4) and assembled to obtain the consensus sequence of the *MAT1-2* idiomorph plus flanking DNA (Fig. 3.3D). Flanking primers 21Nov2L (forward) and Ar20-3485 (reverse) were designed to the consensus *MAT1-2* sequence and used to amplify a 3472 fragment from *MAT1-1* isolate AR21. The amplicon was cloned and sequenced using several primers (Table 3.4) and assembled to yield the complete sequence of the *MAT1-1* idiomorph plus flanking DNA (Fig. 3.3D).

3B.4.2 *MAT* structural organization

Analysis of the assembled sequences identified a *MAT1-1* idiomorph of 2294 bp (nucleotide positions 694-2988) and a *MAT1-2* idiomorph of 2693 bp (nucleotide positions 893-3586) (Fig. 3.3D). ORF analysis revealed a single putative ORF, which started and terminated within each idiomorph. The *MAT1-1* ORF (*MAT1-1-1*) is 1152 bp (1365 to 2517) with a putative 51 bp intron (1609-1660) and the *MAT1-2* ORF (*MAT1-2-1*) is 1111 bp (2013-3124) with a putative 55 bp intron (2506-2561) (Fig. 3.3D). Alignment of *A. rabiei MAT1-1-1* and *MAT1-2-1* with orthologous sequences from *A. alternata*, *C. heterostrophus* and *M. graminicola* revealed that putative start and stop codon positions varied slightly but that putative intron splicing sites were identical among the four species (Fig. 3.4). Conceptual translation of *MAT1-1-1* and *MAT1-2-1*

resulted in putative 366 amino acid and 351 amino acid proteins, respectively. The *A. rabiei* MAT1-1-1 protein was 46% identical to *C. heterostrophus* and *A. alternata* MAT1-1-1 proteins and 17% identical to the *M. graminicola* MAT1-1-1 protein (data not shown). These identities rose to 80%, 81% and 37%, respectively, through the α motif (Fig. 3.4A). The *A. rabiei* MAT1-2-1 protein was 43% identical to *C. heterostrophus* MAT1-2-1 protein, 45% identical to the *A. alternata* MAT1-2-1 protein, and 17% identical to the *M. graminicola* MAT1-2-1 protein, respectively (data not shown). These identities rose to 80%, 81% and 32%, respectively, through the *HMG* motif (Fig. 3.4B). An additional 494 bp ORF was found 51 bp upstream of the *MAT1-2* idiomorph and a slightly larger version of this ORF (563 bp) was found to overlap the *MAT1-1* idiomorph by 16 bp (Fig. 3.3D). Conceptual translation of these ORFs produced 164 and 187 aa proteins, respectively which were homologous to the putative ORF1 protein of *C. heterostrophus* (GenBank accession nos. AAB82944 and AAB84003) and hypothetical protein YLR456w of *Saccharomyces cerevisiae* (GenBank accession no. AAB64720). ORF1 proteins of *MAT1-1* strain AR21 and *MAT1-2* strain AR20 were 68% and 74% similar, respectively to *C. heterostrophus* ORF1 (Fig. 3.5). Alignment of putative ORF1 proteins from AR20 and AR21 revealed approximately 81% similarity (Fig. 3.5).

A

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A. alternata-α        KKALNAFVGFRCYYISIPHFKSWPMKKLSNLIGLLWETDPNKSLWSLMTKAWSAIRDQIGKDRAPLDQFF
C. heterostrophus-α   RKALNAFVGFRCYYVTIPMFKSWPMKKLSNLIGLLWEADPNKSLWSLMAKAWSTIRDQIGKDQAPLDQFF
A. rabiei-α           RKGLNAFVGFRCYYICIPTFKPWPMKKLSNPMGVMWESDPNKPLWSLMTKAWSVIRDQIGKDKAPLDQFF
M. graminicola-α      KRPLNSWMAFRNFY--IPLMLGVPQKCVSKTMTMLWSNDLFKAKWALLSKAYSVARGTRAKIDVPLSGFF
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B

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A. alternata-HMG      KAPRPMNCWIIFRDAMHKQLKAEFPNLTVQEISTRCSEIWRSLTPEGKKPWQAAAQSAKEEHLRQHPDYKYTPRKPGEKKKRQSRK
C. heterostrophus-HMG KAPRPMNCWIIFRDAMHKHLKAEFPHLTIQEISTRCSHIWHNLSPEAKKPWQDAAQSAKEEHLRQHPNYKYTPRKPGEKKKRQSRK
A. rabiei-HMG         KAPRPMNCWIIFRDAMHKQLKTESPHLTVQQISTRCSQMWHDLSPAEKKPWQAAAKSAKAEHLRAHPDYKYCPRKPGEKKKRRSRK
M. graminicola-HMG    KIKRPKNAFLIYRLEHHALTAALNPDMHNNDISKVIGKRWSSESQEVRDQYKQKAEEEKRQHAIEHPGYQYKPRKPSEKKRRMTKK
```

Fig. 3.4 Amino acid alignment of the conserved domains of putative mating type proteins of *Ascochyta rabiei* and other loculoascomycete fungi (**A**) MAT1-1 α domain proteins and (**B**) MAT1-2 HMG domain proteins. Amino acid residues in black are identical while those shaded in red are similar. Arrows indicate positions of conserved introns.

Fig. 3.5 Amino acid alignment of putative ORF1 proteins of *Ascochyta rabiei* isolates AR21 (*MAT1-1*) and AR20 (*MAT1-2*) and isolate C4 of *Cochliobolus heterostrophus* (GenBank accession no. AAB82944). ORF1 sequences from *MAT1-1* and *MAT1-2* strains of *C. heterostrophus* are identical and only ORF1 of the *MAT1-1* isolate is shown. Amino acid residues colored in red are identical while those in blue are similar.

3B.4.3 *MAT* Expression

MAT genes were expressed when isolates AR20 and AR21 were grown in 2-YEG, a standard rich growth medium at 25°C (Fig. 3.6). A 1093 bp PCR product was amplified from genomic DNA of isolate AR21 with *MAT1-1-1*-specific AR21-1393L and AR21-2485R primers (Fig. 3.6A). A slightly smaller PCR product was amplified from AR21 cDNA using the same primers (Fig. 3.6A). A 1021 bp PCR product was amplified from genomic DNA of isolate AR20 with *MAT1-2-1*-specific AR20-2085L and AR20-3105R primers (Fig. 3.6B). A slightly smaller PCR product was amplified from AR20 cDNA using the same primers (Fig. 3.6B). No products were amplified from any of the RNA controls (Fig. 3.6A, B,C). A 610 bp PCR fragment was amplified from genomic DNA of both isolates using the gpd-1 and gpd-2 primers, which was approximately 160 bp smaller when cDNA was used as the PCR template (Fig. 3.6C). This 160 bp consists of two introns, which have been described, in other loculoascomycete species (Berbee *et al.*, 1999). *MAT1-1-1* and *MAT1-2-1* amplicons from cDNA templates were sequenced with the AR21-1393L and HMG-239R primers, respectively, to confirm putative intron splicing. Alignment of *MAT* sequences amplified from genomic templates with those from cDNA templates revealed that 51 and 55 bp were spliced from the *MAT1-1-1* and *MAT1-2-1* cDNAs, respectively. Intron splice sites were identical to those of *A. alternata* and *C. heterostrophus* (Fig. 3.4A,B).

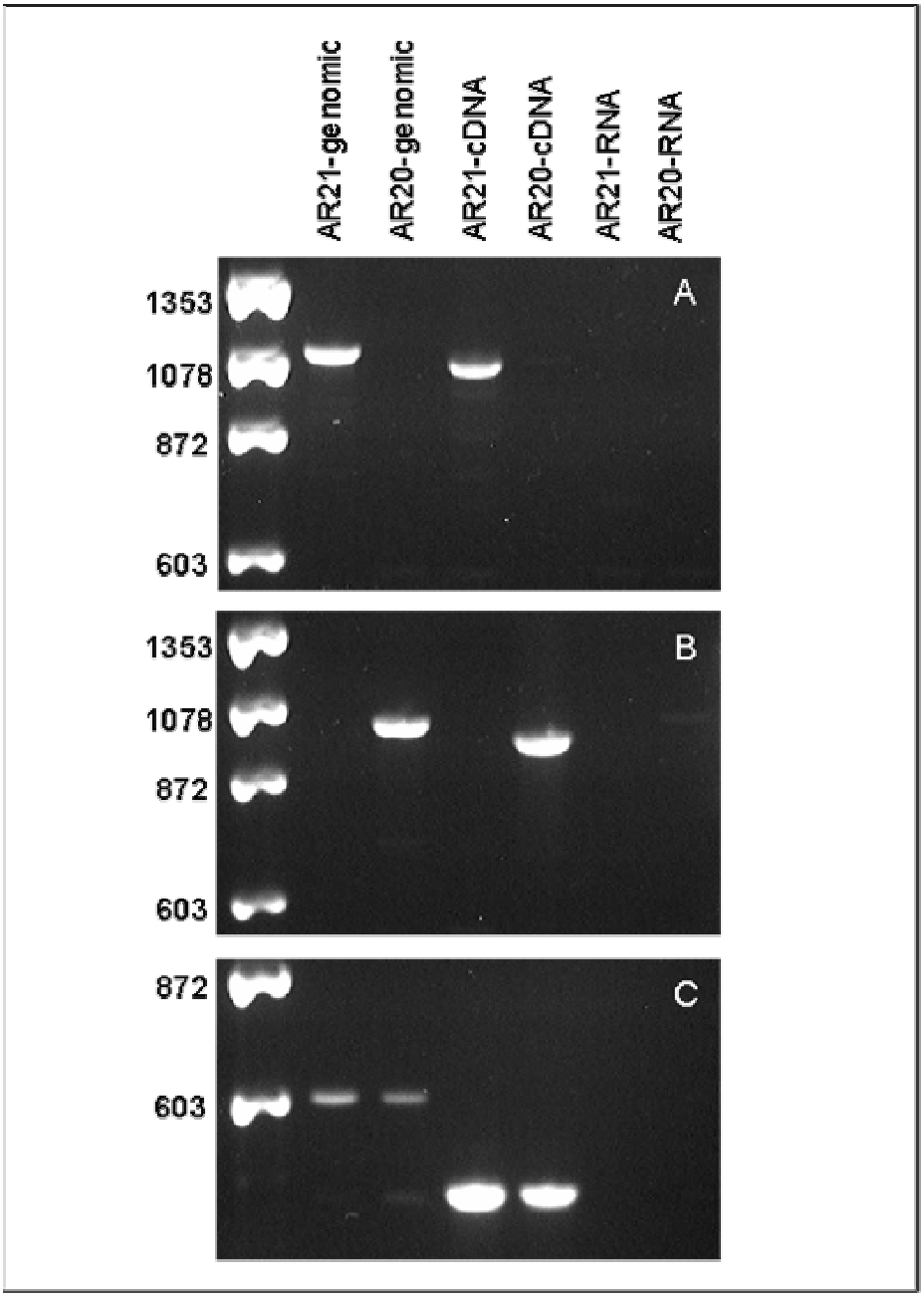

Fig 3.6 *A. rabiei MAT* gene expression. PCR amplification of *MAT1-1-1* and *MAT1-2-1*-specific amplicons in an ethidium bromide-stained 1.5% agarose gel using primers designed to *A. rabiei MAT1-1-1* and *MAT1-2-1*. (**A**) Amplification *of MAT1-1-1*-specific products from AR21 genomic DNA (lane 2) and AR21 cDNA (lane 4) using AR21-1393L and AR21-2485R primers (**B**) Amplification *of MAT1-2-1*-specific products from AR20 genomic DNA (lane 3) and AR20 cDNA (lane 5) using AR20-2085L and AR20-3105R primers. (**C**) Amplification of the glyceraldehyde-3-phosphate-dehydrogenase gene using gpd-1 and gpd-2 primers. Lanes 1 through 7 contain *Hae*III-digested ϕ*X174 RF* DNA as a size standard, AR21 (*MAT1-1*) genomic DNA, AR20 (*MAT1-2*) genomic DNA, AR21 cDNA, AR20 cDNA, AR21 total RNA, AR20 total RNA.

3B.4.4 Genetic analysis of *MAT*-specific PCR products

A Southern blot of *HindIII*-digested genomic DNA from five mating type 1 and five mating type 2 isolates of *A. rabiei* of diverse geographic origin was probed with *MAT1-1-1*-specific and *MAT1-2-1*-specific probes (Fig. 3.7). The mating phenotype of these isolates was determined previously by performing genetic crosses with AR20 and AR21 tester strains. The *MAT1-1-1* probe hybridized exclusively to DNA from mating type 1 strains (Fig. 3.7A) while the *MAT1-2-1* probe hybridized exclusively to DNA from mating type 2 strains (Fig. 3.7B). A single hybridizing restriction fragment was found in all isolates demonstrating that *MAT1-1* and *MAT1-2* sequences are present in a single copy in each genome (Fig. 3.7A,B). Amplification of *MAT*-specific PCR products from thirty-five progeny isolates from a cross of *A. rabiei* isolates AR20 and AR21 revealed 1:1 segregation of mating type (Fig. 3.8). Primers Com 1, Tail 5 and SP-21 were combined in a multiplex PCR reaction and a 700 bp and a 400 bp fragment was amplified from each mating type 1 and mating type 2 isolate, respectively (Fig. 3.8). Mating phenotype, as determined by backcrossing each progeny isolate to AR20 and AR21, was absolutely correlated to *MAT*-specific PCR amplicon (Fig. 3.8). Amplification of the 700 and 400 bp amplicons was also completely correlated to mating phenotype of field-collected isolates from different geographic locations (Fig. 3.9). Furthermore, *A. rabiei MAT*-specific primers did not amplify any PCR products from *A. fabae*, *A. lentis* or *Fusarium oxysporum* f. sp. *ciceri* (Fig. 3.9).

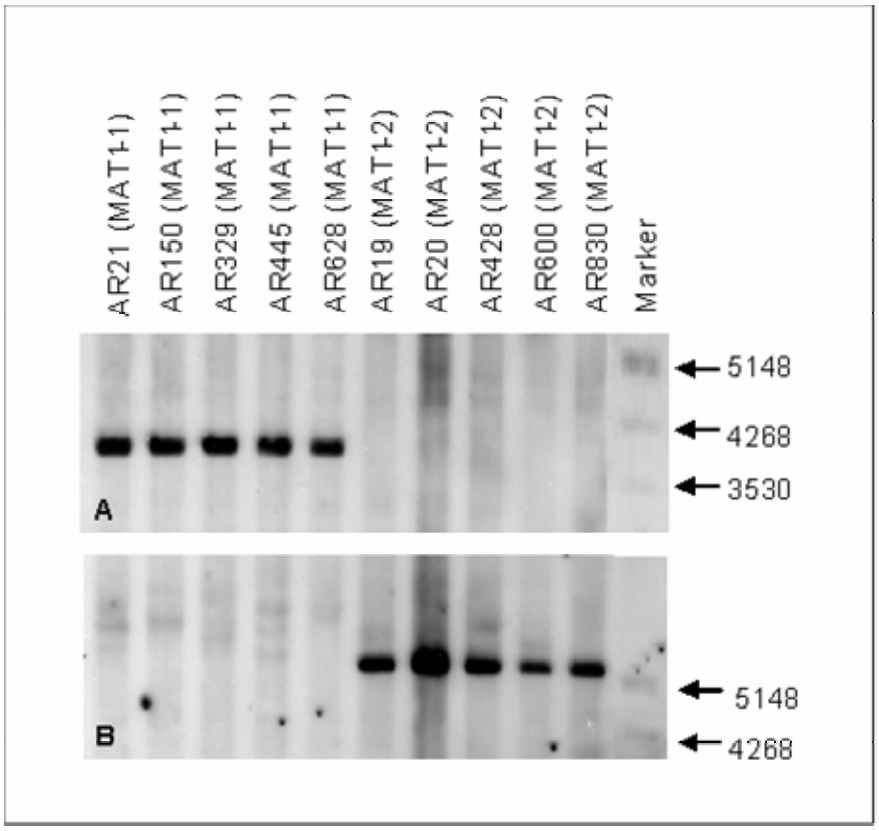

Fig. 3.7 Southern hybridization of *MAT*-specific PCR amplicons to *HindIII*-digested genomic DNA of mating type 1 and mating type 2 isolates of *A. rabiei*. A) membrane probed with the DIG-labeled 1093 bp *MAT1-1-1*-specific fragment amplified by AR21-1393L and AR21-2485R primers. B) membrane probed with the DIG-labeled 1021 bp *MAT1-2-1*-specific fragment amplified by AR20-2085L and AR20-3105R primers. Lanes 1-5 were loaded with *Hind*III-digested genomic DNA of mating type 1 isolates from different parts of the world, lanes 6-10 were loaded with *Hind*III-digested genomic DNA of mating type 2 isolates from different parts of the world, and lane 11 contained *HaeIII*-digested Φ*X174 RF* DNA as a size standard.

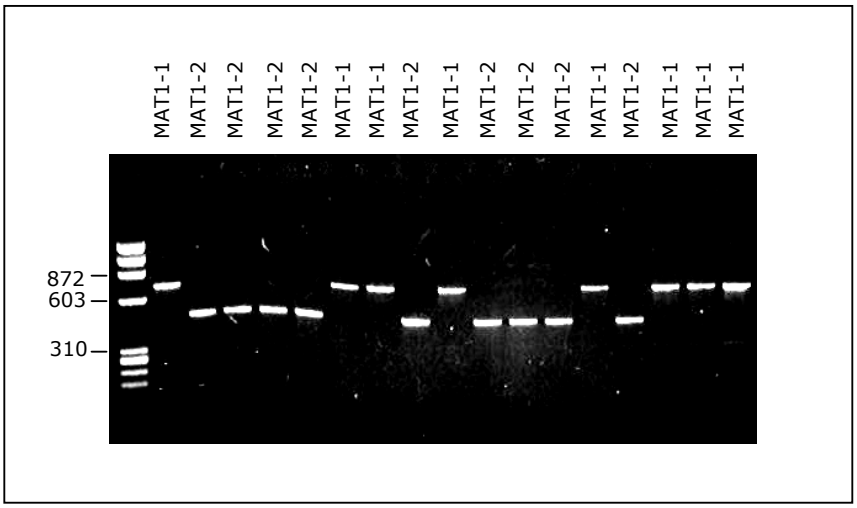

Fig. 3.8 Amplification of *MAT*-specific PCR products among seventeen random ascospore progeny of a cross between AR20 (mating type 2) and AR21 (mating type 1) of *Ascochyta rabiei*. Mating phenotype of the isolates, as determined by backcrossing to AR20 or AR21, is shown across the top of the figure. Lane 1 contains *Hae*III-digested Φ*X174 RF* DNA as a size standard and the sizes of relevant bands in base pairs are indicated. Only 17 of 35 progeny tested are shown and parental isolates (AR20 and AR21) are not shown.

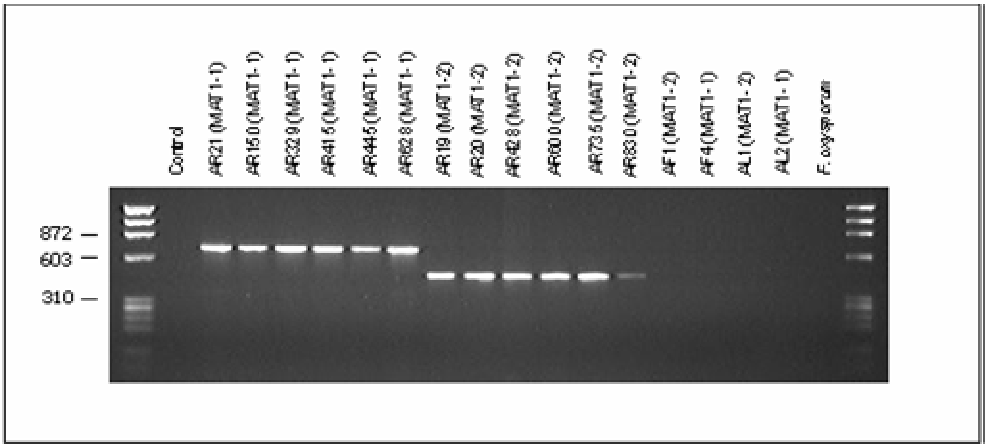

Fig. 3.8 Amplification of *MAT*-specific PCR products among seventeen random ascospore progeny of a cross between AR20 (mating type 2) and AR21 (mating type 1) of *Ascochyta rabiei*. Mating phenotype of the isolates, as determined by backcrossing to AR20 or AR21, is shown across the top of the figure. Lane 1 contains *Hae*III-digested Φ*X174 RF* DNA as a size standard and the sizes of relevant bands in base pairs are indicated. Only 17 of 35 progeny tested are shown and parental isolates (AR20 and AR21) are not shown.

3B.4.5 Mating type ratios in *A. rabiei* populations

MAT-specific PCR using DNA from field-collected *A. rabiei* isolates revealed mating type ratios that were not significantly different from 1:1 among isolates from two commercial chickpea fields (DAN98, PKS98) (Table 3.5). In contrast, the mating type ratio in a sample collected from the Ascochyta blight-screening nursery at Spillman Farm was 4:17 (*MAT1-1: MAT1-2*), which was significantly different from 1:1 (P = 0.005) (Table 3.5).

3B.4.6 Phylogenetic analysis of ITS and HMG

Isolates from the same legume host had identical ITS sequences and only one sequence was used in the phylogenetic analysis. Estimation of the ITS phylogeny among *Didymella*, *Ascochyta, Phoma and Mycosphaerella* spp. sampled from various legume hosts demonstrated two well-supported clades (Fig. 3.10). One clade contained *Mycosphaerella* spp. including *M. graminicola* and another clade contained *Ascochyta*, *Didymella*, *Phoma* and *Mycosphaerella* spp. from legumes plus *D. bryoniae* from cucumber. No significant differentiation was detected among isolates from legumes. The HMG phylogeny revealed substantially more variation among the legume-associated fungi than did the ITS phylogeny. With the HMG data, six well-supported clades were obtained that correlated to the host of isolation and to described morphological and biological species (Fig. 3.11). The exception was Clade 3 which contained three isolates of *A. lentis* from lentil (*Lens culinaris*) and two isolates of an undescribed *Ascochyta* spp. from hairy vetch (*Vicia villosa*), all of which had identical HMG sequence. Clade 1 contained isolates of *Mycosphaerella pinodes* (MP) and *Phoma medicaginis* var. *pinodella* (PMP), respectively, from pea (*Pisum sativum*). Clade 2 contained isolates of an undescribed *Ascochyta* species from alfalfa (*Medicago sativa*), Clade 3 contained *A. lentis* isolates from lentil (*Lens culinaris*) and

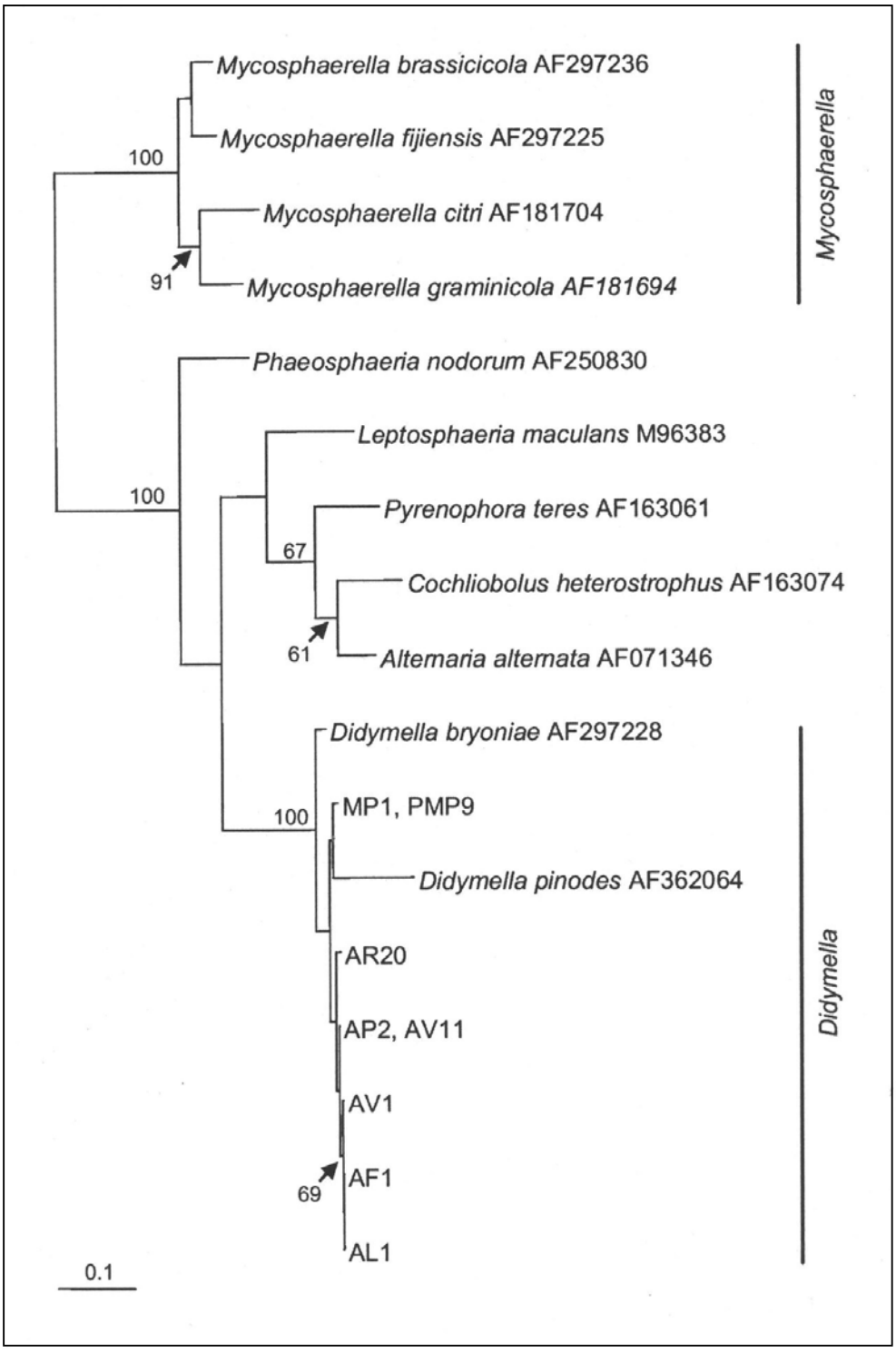

Fig. 3.10 Unrooted ITS phylogeny estimated among *Ascochyta*, *Didymella*, *Phoma*, and *Mycosphaerella* spp. from legumes plus several additional loculoascomycete fungi from other hosts. Sequences of additional loculoascomycetes were retrieved from GenBank. Phylogeny was estimated using maximum likelihood with the DNAML program in PHYLIP. Numbers at the major branches indicate the percentage occurrence of the clade to the right of the branch in 100 bootstrapped datasets. Only bootstrap values greater than 60% are shown. Branch lengths are proportional to the inferred amount of evolutionary change and the scale represents 0.1 nucleotide substitutions per site. Clades corresponding to *Mycosphaerella* spp. and *Didymella* spp. are indicated.

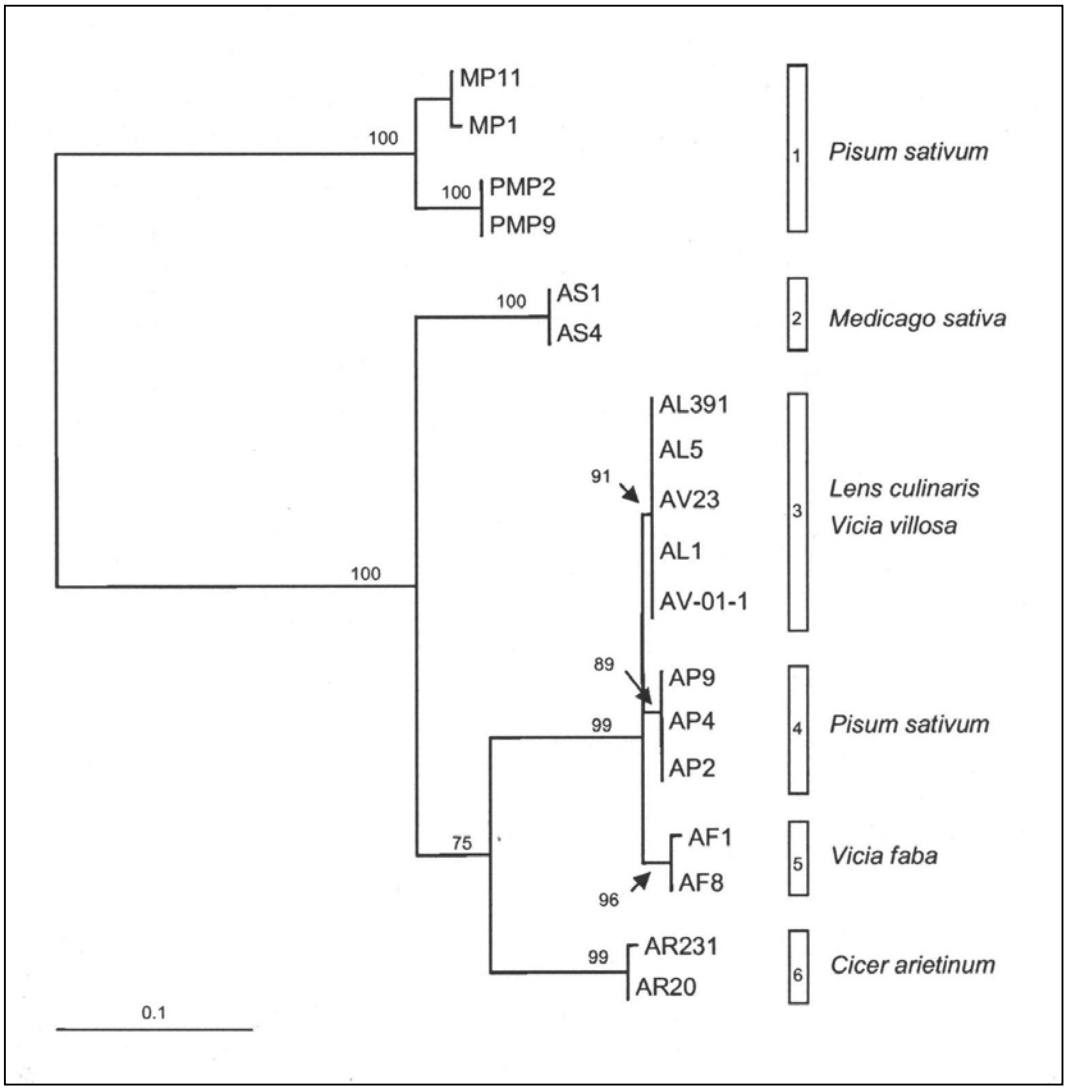

Fig. 3.11 Unrooted *HMG* phylogeny estimated among *Ascochyta*, *Didymella*, *Phoma*, and *Mycosphaerella* spp. from legumes. Phylogeny was estimated using maximum likelihood with the DNAML program in PHYLIP. Numbers at the major branches indicate the percentage occurrence of the clade in 100 bootstrapped datasets. Only bootstrap values greater than 60% are shown. Branch lengths are proportional to the inferred amount of evolutionary change and the scale represents 0.1 nucleotide substitutions per site. Significant clades were inferred from bootstrap values greater than 80% and are indicated by open boxes numbered 1 through 6. Host plants from which isolates were obtained are indicated.

an undescribed *Ascochyta* spp. from hairy vetch (*Vicia villosa*), and Clade 4 contained *A. pisi* isolates from pea (*P. sativum*). Clade 5 contained two *A. fabae* isolates from faba bean (*V. fabae*) and Clade 6 contained two *A. rabiei* isolates from chickpea (*Cicer arietinum*). Little or no sequence divergence was detected among isolates sampled from the same host even though they were obtained from diverse geographic locations (Fig. 3.11, Table 3.3). Attempts to root the phylogeny with HMG sequences from *Alternaria alternata* (GenBank accession no. AB009452), *A. brassicae* (GenBank accession no. AY042091), or *Cochliobolus heterostrophus* (GenBank accession no. X68398) (the closest matches in the databases) resulted in ambiguous alignments due to extensive sequence divergence.

3B.5 Discussion

3B.5.1 The mating locus of *A. rabiei*: structural organization

The mating locus of *A. rabiei* (teleomorph: *Didymella rabiei*) was cloned and sequenced using a combination of TAIL-PCR and inverse PCR. The approach taken to clone the mating locus in *A. rabiei* was similar to that used with *Alternaria alternata* and *Fusarium oxysporum* (Arie *et al.*, 1997; Arie *et al.*, 2000). In those studies, primers designed from the HMG motif of *C. heterostrophus* were successful in amplifying HMG from *A. alternata* (Arie *et al.*, 2000). Alignment of HMG sequences of *A. alternata*, *C. heterostrophus* and *C. sativus* allowed the design of degenerate primers which successfully amplified HMG from *A. rabiei* and provided a starting point for cloning. In contrast, primers designed to the *C. heterostrophus* HMG motif were unsuccessful in amplifying HMG from another loculoascomycete, *Mycosphaerella graminicola*, presumably due to extensive sequence divergence (Waalwijk *et al.*, 2002). The alignments of HMG and α domain proteins and the results of the phylogenetic analysis of ITS revealed that *C. heterostrophus*, *A. alternata*, *A. rabiei* are closely related to each other, but distantly related to *M. graminicola*. Thus, the *MAT* locus of *M. graminicola* was cloned by screening a genomic library with a heterologous probe from a discomycete fungus, *Tapesia yallundae*. However, PCR-based strategies are a very

straightforward way to obtain *MAT* sequences from any fungus if sequences are available from closely related fungi, thought other strategies such as library screens with heterologous probes may have to be employed for more distant taxa or taxa which are underrepresented in the databases.

The structural organization of the *A. rabiei MAT* locus is very similar to that of *MAT* loci in other loculoascomycete fungi studied to date (Arie *et al.*, 2000; Turgeon, 1998; Turgeon *et al.* 1993, Waalwijk *et al.*, 2002). *MAT1-1* and *MAT1-2* idiomorphs each contained a single open reading frame that started and terminated in the idiomorph. The *MAT* idiomorphs of *A. rabiei* are almost double the size of those found in *C. heterostrophus* (Turgeon *et al.*, 1993), slightly larger than those of *A. alternata* (Arie *et al.*, 2000), and slightly smaller than those of *M. graminicola* (Waalwijk *et al.*, 2002). The *MAT1-1* ORF of *A. rabiei* occupies approximately 50% of the idiomorph while the *MAT1-2* ORF occupies approximately 41% of the idiomorph. These values are slightly higher than those of *M. graminicola* (36% and 43%, respectively) and slightly lower than those of *A. alternata* (64% and 49% respectively). However, they are markedly different from *C. heterostrophus* where the *MAT* ORFs occupy more than 90% of each idiomorph. Despite these differences in idiomorph size, the sizes of the open reading frames are almost identical in all these fungi. The transition between 5' flanking sequence and idiomorph was much more gradual in *A. rabiei* compared to *C. heterostrophus*, *A. alternata* and *M. graminicola* (Arie *et al.* 2000, Turgeon *et al.* 1993, Waalwijk *et al.* 2002). The mating loci in these latter fungi display a very sharp transition between the 5' flanking sequences, which are 95% to 99% similar and the idiomorphs where sequence similarity drops to random levels. Gradual transitions were also observed between the flanking sequences and idiomorphs of *Cryphonectria parasticia* when compared to other pyrenomycetes (McGuire *et al.* 2001). Sequences flanking the idiomorphs in the 5' direction were only 78% similar between the *MAT1-1* and *MAT1-2* strains of *A. rabiei*, which is substantially more variable than other loculoascomycete fungi studied to date (Arie *et al.* 2000, Turgeon *et al.* 1993, Waalwijk *et al.* 2002). Alignment of 5' flanking sequences revealed 99% similarity between

MAT1-1 and *MAT1-2* strains of *C. heterostrophus*, 98% similarity in *A. alternata* and 95% similarity in *M. graminicola*. Sequences flanking the *A. rabiei* idiomorphs in the 3' direction were 97% similar which is comparable to other loculoascomycetes (86% in *A. alternata*, 98% *in C. heterostrophus* and 95% in *M. graminicola*). Intron splice sites in both *MAT1-1-1* and *MAT1-2-1* were identical to those found in *A. alternata* (Arie *et al.*, 2000) and *C. heterostrophus* (Turgeon *et al.*, 1993) but were shifted approximately 70 bp downstream of the splice sites in *M. graminicola* (Waalwijk *et al.*, 2002). Comparisons of conceptually translated MAT proteins among loculoascomycete fungi revealed that the *A. rabiei* MAT1-1 and MAT1-2 proteins were approximately 43-46 % identical to *A. alternata* and *C. heterostrophus*. In contrast, *A. alternata* and *C. heterostrophus* MAT proteins were 53-55% identical and *A. rabiei* MAT proteins were only 17% similar to *M. graminicola*. This pattern of protein sequence divergence is consistent with phylogenies estimated from the ITS region which have demonstrated that *Didymella* spp. are distantly related to *Mycosphaerella* spp. (Goodwin *et al.* 2001, Crous *et al.* 2001).

An additional ORF was observed immediately upstream of the idiomorphs in both *MAT1-1* and *MAT1-2* strains of *A. rabiei*. These ORFs are homologous to ORF1 of *C. heterostrophus* (GenBank accession nos. AAB82944, AAB84003) and hypothetical protein YLR456w of *Saccharomyces cerevisiae* (GenBank accession no. AAB64720). In contrast to the *MAT* locus of *C. heterostrophus* where ORF1 is found more than 1 kb upstream of both idiomorphs (Wirsel *et al.,* 1998), ORF1 in *A. rabiei* was found only 51 bp upstream of the idiomorph in the *MAT1-1* strain and overlapped the idiomorph in the *MAT1-2* strain. ORF1 also differed in length between *MAT1-1* and *MAT1-2* strains and conceptually translated proteins were only 81% similar. In contrast, *C. heterostrophus* ORF1 proteins were identical between the two mating types. An ORF1 homolog was also identified 800 bp upstream from the start of *MAT1-2-1* in *A. alternata* (GenBank accession no. AB005039), which was also approximately 70% similar to *C. heterostrophus* ORF1. It could not be determined if *MAT1-1* strains of *A. alternata* also carry the ORF1 homolog as insufficient sequence is currently available 5'

of *MAT1-1-1* for this strain. No evidence for sequences homologous to ORF1 was found at the *MAT* locus of *M. graminicola* (Waalwijk *et al.* 2002). ORF1 has no known function in *C. heterostrophus* and is not required for mating (Wirsel *et al.* 1998).

Each *A. rabiei* isolate carries a single *MAT1-1* or *MAT1-2* sequence at the *MAT* locus and HMG sequences were invariant among several *A. rabiei MAT1-2* isolates from diverse geographic locations. This result is consistent with a single-locus, two-allele (bipolar) mating system model, which has been supported by the results of genetic crosses, made with this species (Trapero-Casás and Kaiser, 1992; Wilson and Kaiser, 1995). The *MAT* locus of *A. rabiei* appears to be very similar to that of most heterothallic ascocmycetes described to date (Coppin *et al.*, 1997; Nelson, 1996; Turgeon, 1998). *MAT*-specific PCR products were perfectly correlated to segregation of *MAT* phenotype of among progeny from a cross of the standard mating type tester strains. They were also perfectly correlated to *MAT* phenotype of several field isolates of *A. rabiei*. In addition, hybridization of *MAT*-specific sequences to genomic DNA demonstrated a perfect correlation between mating phenotype and the presence of a single *MAT* idiomorph. All of this data provides strong genetic evidence that the sequences cloned in this study were *MAT* sequences.

3B.5.2 *MAT* gene expression

Ascochyta rabiei MAT genes are expressed during growth in standard liquid media at laboratory temperatures (20-23°C). This is similar to patterns of *MAT* expression observed in *A. alternata* (T. Arie, *unpublished*) but distinct from *C. heterostrophus* which expresses *MAT* in minimal medium but not in complete medium (Leubner-Metzger *et al.*, 1997). Successful laboratory matings of *A. rabiei* can only been accomplished on senescent chickpea stems at 5-10°C and not at 15°C or above (Trapero-Casás and Kaiser, 1992). *MAT* genes have DNA binding motifs and are thought to be regulatory genes (Coppin *et al.*, 1997; Turgeon and Berbee, 1998) but the targets of these genes are not known. The present data indicate that *A. rabiei MAT* genes are expressed at high levels during vegetative growth in rich media and at temperatures that are not conducive for mating. More research is needed to understand how the

downstream targets are regulated by *MAT* genes and how these additional genetic loci control temperature perception and sexual development.

3B.5.3 Mating type assessment in *A. rabiei* isolates

Cloning and sequencing the *A. rabiei* mating locus has allowed the development of a multiplex PCR assay for mating type. This assay will prove extremely useful for population genetic studies of this fungus as has been demonstrated with other species (Dyer *et al.*, 2001; Foster *et al.*, 1999; Waalwijk *et al.*, 2002). Extensive mating type testing has been performed with *A. rabiei* and related *Ascochyta* species using conventional tests (Ahmed *et al.*, 1996; Armstrong *et al.*, 2001; Kaiser and Kusmenoglu, 1997), which involve mating each isolate individually with fertile tester strains and screening the crosses for the production of ascospores. These tests are extremely labor intensive, require the production, harvesting and enumeration of conidia, inoculation of sterile, senescent chickpea stems and an extended incubation at 10°C with high humidity (Trapero-Casás and Kaiser, 1992; Wilson and Kaiser, 1995).

Although, the multiplex assay for mating type described in this section of my thesis requires isolate culturing, DNA extraction and PCR; DNA extractions from large numbers of *A. rabiei* isolates can be easily performed in the laboratory. Further, preliminary data suggests that it may also be possible to bypass the fungal culturing and DNA extraction steps altogether and amplify directly from autoclaved conidial suspensions or infected chickpea leaves (T.L. Peever, *unpublished*). A multiplex PCR assay for mating type in *A. rabiei* will complement and extend the data obtained from other molecular markers and will allow rapid estimation of mating type ratios in populations of this fungus. The assay can also be efficiently used to detect introduction of the second mating type in regions of the world where populations of *A. rabiei* have been reported to consist of a single mating type to date (Kaiser, 1995; Kaiser, 1997a; Kaiser, 1997b; Kaiser and Kusmenoglu, 1997; Khan *et al.*, 1999).

Application of the multiplex PCR assay to samples of *A. rabiei* isolates from two commercial chickpea fields revealed that the null hypothesis of a 1:1 ratio of

mating types could not be rejected in either population. A 1:1 ratio of mating types is expected in populations undergoing regular random mating (Milgroom, 1996) as each mating type is subject to frequency-dependent selection. Equal ratios of mating types in these populations suggest that the sexual stage of the pathogen is important in the life cycle and epidemiology of ascochyta blight of chickpea in the US Pacific Northwest. Application of additional molecular markers to larger samples of isolates is needed to confirm these results. However, the ratio of mating types was significantly different from 1:1 in a sample of isolates obtained from a chickpea resistance-screening nursery, which was consistent with a predominantly asexual mode of reproduction in this population. Chickpea germplasm in this nursery was inoculated by spreading infested chickpea debris between rows of emerging plants and allowing conidia to be splashed via irrigation to initiate infection. The debris used for inoculation was collected from heavily infected, senescent plants at the end of each season, stored dry through the winter and spread into the plots the following spring. Storing the debris dry through the winter is predicted to preclude formation of the sexual stage and this prediction was consistent with the skewed mating type ratio observed at this site.

Further, primers SP-21, Tail 5, and Com 1 amplified *MAT*-specific amplicons from *A. rabiei* but not from other *Ascochyta* spp. indicating a potential additional use for these primers in an *A. rabiei*-specific PCR assay. Such an assay could be used to quantitatively detect the fungus in chickpea seed and used as a quarantine tool to prevent migration of the fungus into areas currently free of the fungus.

3B.5.4 Phylogeny of legume associated *Ascochyta* species

Previous phylogenetic studies of loculoascomycete fungi have shown that *A. alternata* and *C. heterostrophus* are closely related members of the Pleosporales (Berbee, 1996; Berbee *et al.*, 1999; Zhang and Berbee, 2001). The similarity in MAT1-1 and MAT1-2 proteins among *C. heterostrophus*, *A. alternata* and *A. rabiei* relative to that between these species and *M. graminicola* suggests that *A. rabiei* is more closely related to *A. alternata* and *C. heterostrophus* than to *M. graminicola*. This result was supported by the ITS phylogeny which demonstrated that *Mycosphaerella* spp. are distantly related to

C. heterostrophus, *A. alternata* and *A. rabiei*. Only a few *Didymella* species have been included in phylogenetic studies of loculoascomycetes to date but these studies have consistently shown that *Didymella* spp. are distantly related to *M. graminicola* and other *Mycosphaerella* spp. (Crous *et al.*, 2001; Goodwin *et al.*, 2001). The ITS phylogeny provided strong support for a monophyletic *Ascochyta*/*Didymella* clade which included all the legume-associated fungi sampled in this study and *D. bryoniae* from cucumber, although the ITS data were not sufficiently variable to allow further discrimination among the fungi associated with specific legume hosts within this clade. In contrast, the HMG phylogeny was considerably more variable and delimited clades that were highly correlated to host of isolation and to previously described morphological and biological species. Although ITS has been the preferred region of the genome used for fungal molecular systematics to date, other regions of the genome such as *MAT* loci may provide much better resolution among closely related taxa. These data demonstrated that the species comprising the 'Ascochyta complex' on pea (*Pisum sativum*) are phylogenetically diverse and that *Mycosphaerella pinodes* and *Phoma medicaginis* are closely related to each other but distantly related to *A. pisi*. The ITS datas*et* also suggest that *M. pinodes* from pea is very likely not a *Mycosphaerella* spp. and this result needs to be confirmed with additional data and with careful morphological examination of the teleomorph. The present data support the results of Faris-Mokaiesh *et al.* (1996) who demonstrated with ribosomal intergenic spacer (IGS) RFLP data that *A. pisi* is distinct from both *M. pinodes* and *P. medicaginis* var. *pinodella*.

The *Ascochyta* spp. isolated from alfalfa (*Medicago sativa*) was distinct from all other *Ascochyta* spp. isolated from legumes. The HMG phylogeny correlated well with the results of previous genetic crosses among *A. rabiei*, *A. lentis* and *A. fabae* (Kaiser *et al.*, 1997) and indicated that the phylogenetic species defined in this study were consistent with previously described biological species. In a previous study (Kaiser *et al.*, 1997), isolates from chickpea (*A. rabiei*) could not be successfully mated with isolates from either faba bean (*A. fabae*) or lentil (*A. lentis*). In contrast, *A. fabae* could be mated with *A. lentis* but the crosses resulted in aberrant numbers of ascospores,

misshapen ascospores and ascospore progeny which failed to infect either host. These genetic incompatibilities, coupled with molecular marker data, were used as evidence for the species status of these isolates (Kaiser *et al.*, 1997). Their results (Kaiser *et al.*, 1997) are supported by the HMG phylogeny from the present data, which revealed *A. fabae* and *A. lentis* to be much more closely related to each other than either is to *A. rabiei*. A teleomorphic stage has never been described for *A. pisi* or the unidentified *Ascochyta* spp. from wild vetch but it is clear from their close evolutionary relationship and their similarity to *A. lentis* and *A. fabae* that all of these fungi will likely be connected to *Didymella* teleomorphs in the future.

Chapter 4: General Discussion

Potential of molecular markers for *Fusarium oxysporum* f. sp. *ciceri* and *Ascochyta rabiei* for disease diagnostics and resistance breeding in chickpea

I have carried out genetic analysis of two important fungal pathogens of chickpea namely *Fusarium oxysporum* f. sp. *ciceri* and *Ascochyta rabiei* in this thesis and the salient achievements of my study are as follows:

The work described in Chapter 2 has resulted in enhancement in the knowledge of genetic diversity in physiological races of *Foc* from diverse geographic regions in the Indian subcontinent using multilocus profiles generated by hybridization-based microsatellite probes and PCR based ISSR and AFLP markers. The markers used, especially AFLPs are informative for differentiation of the wilt causing Indian *Foc* races from *F. solani* and *F. udum*, and also among the *Foc* races. Race specific bands for *Foc* races 1 and 2 have been identified with AFLP primers as detailed in Section 2B. These are potential candidates for development of race specific markers for these races, which are widespread in India. I have further demonstrated that *Foc* race 3, which causes vascular wilt symptoms in chickpea, has elongation factor 1α sequence similar to *Fusarium proliferatum*, which has never been reported earlier as a pathogen of chickpea.

The work included in Section 3A of the thesis deals with allelic variation revealed by diversity in allele size at a previously characterized compound microsatellite locus in representative *A. rabiei* isolates from countries all over the world and in isolates from different states of India. I have also carried out the cloning and characterization of mating type genes from *A. rabiei*. A significant contribution of this work is the development of mating type specific primers for both the mating types of *A. rabiei*, and design of a convenient multiplex PCR assay for their identification. Furthermore, these primers are specific to *A. rabiei* isolates, do not amplify even from

closely related species of the genus *Ascochyta* and represent potential candidates for development of *A. rabiei* specific markers.

4A Impact and significance of the work with reference to *Foc*

For better control and management of fusarium wilt, research is needed in the following areas including identifying good sources of resistance, breeding resistant lines which need to be screened in disease nurseries and multilocation trials, followed by region specific deployment of these resistant cultivars. For the purpose of screening for wilt resistance, it is necessary to have uniformly sick plots containing the specific race of *Foc* against which breeding lines need to be screened. The soil may simultaneously harbor other pathogens such as *F. solani*, *F. udum*, *Rhizoctonia bataticola*, *Sclerotium rolfsii*, in addition to having more than one race of *Foc*. The ability to differentiate *Foc* from other soil-borne pathogens can be useful in such cases.

Race specific bands identified for *Foc* races 1 and 2 need to be cloned, developed into simple SCAR markers, and validated using a larger number of *Foc* race 1 and 2 isolates and other *Foc* races. Such markers can be used for diagnostic purposes to check introduction of a particular race of *Foc* to areas previously devoid of that race. The advantage of race specific markers is that they can aid extensive sampling and a large number of isolates can be simultaneously screened using fewer resources, in a shorter time period. On the other hand, current methods for race identification, which rely on physiological reactions to a set of chickpea differentials, are extremely labour intensive and time consuming in addition to being influenced by the environment and consequently have to be carried out under regulated glasshouse conditions. Use of race specific markers can facilitate race identification from a single field and also from several fields. This would be of particular interest to plant breeders to ensure homogeneity of the inoculum in terms of (i) presence of a specific race and (ii) uniform distribution of the race in plots used for screening fusarium wilt resistant lines while developing a wilt resistant cultivar. Moreover, the development of race specific markers in *Foc* is also significant for effective deployment of wilt resistant chickpea lines because race specific markers can be used in extensive multilocation trials of such

cultivars or lines. This can enhance the knowledge of spatial and temporal distribution of various *Foc* races and assist decisions on the release of resistant varieties in accordance with region specific prevalence of *Foc* races. Such studies would be especially relevant to *Foc* races, which are localized in their distribution, for example races 3 and 4, which are reported to be restricted to Punjab and Haryana states of India; although specific markers for races 3 and 4 are not presently available with me. Such molecular methods further provide a tool for the fine level detection of variation within a race, which cannot be efficiently picked up with the classical methods for race identification. These variations may be important in understanding the process of resistance breakdown. In order to detect variation at a finer level by classical methods, the number of host differentials to be used for each test may have to be significantly increased, which would make the existing technique for race identification increasingly demanding.

It is very important to detect seed borne *Foc* infection at an early stage and remove this source of inoculum because once the soil in a field becomes wilt sick, it is hard to eradicate the pathogen due to the longevity of the *Foc* chlamydospores. Race specific markers would find potential application in detection of seed borne *Foc* infection, and in preventing the long distance transfer of *Foc* races through infected seedlots. This can have a significant impact on seed testing from the point of view of national and international quarantine regulations. Such studies can be of significance in case of *Foc* races with restricted geographical distribution. Furthermore, in case of *Foc* and other clonally reproducing organisms, where not much variation is expected, race specific bands identified by me may specifically represent physiological race variants, and may be tightly linked to virulence genes. This could open an interesting possibility for further characterization of such genes in *Foc* and the project undertaken by various researchers on genome sequencing, for instance, of *Fusarium graminearum* (*Gibberella zeae*) (http://www.nhgri.nih.gov/NEWS/sequencing.html), might be helpful in such characterizations.

Apart from the impact on agriculture, the results presented in Section 2B would contribute to evolutionary studies. It has been demonstrated that race 3 has translation elongation factor sequence similar to *F. proliferatum*. This resemblance is significant in that although the races cause similar symptoms (vascular wilt) on chickpeas, *Foc* races 1, 2 and 4 have probably evolved independent of race 3. The present studies strongly support the need for development of phylogenetic markers for identification of *Fusarium* species as previously emphasized by O'Donnell *et al.* (O'Donnell *et al.*, 1998b). In a similar report from Egypt disease symptoms on chickpea caused by a previously unknown pathogen, belonging to anamorph genus *Colletotrichum*, were confused with those of the well known pathogen *Ascochyta rabiei,* emphasizing the need for molecular markers to support morphological data (http://www.plbio.kvl.dk/plpat/gruppeprofil/eigel.htm).

4B Impact and significance of the work with specific reference to *A. rabiei*

In order to better control ascochyta blight worldwide, research is urgently needed in several areas including 1) the role of the sexual stage of the pathogen in disease epidemiology 2) better understanding of spatial and temporal distribution of genetic variation in *A. rabiei* populations, and how this relates to breeding blight-resistant cultivars 3) improved seed testing technologies to detect low rates of *A. rabiei* infection in seed, and 4) improved chemicals and timing of fungicide applications. The sexual stage of *A. rabiei* is thought to play an important role in disease epidemiology (i) as a source of primary inoculum for blight epidemics in the form of wind borne ascospores, which can infect chickpea plants several hundred meters away from the source (Kaiser and Kusmenoglu, 1997; Trapero-Casás *et al.*, 1996) (ii) as a survival mechanism between chickpea crops and (iii) in generating genetic diversity in the fungus and giving rise to potentially more virulent forms. To address these questions, it is necessary to determine mating type ratios in field populations of *A. rabiei* by mating-type assessment of isolates collected from single fields and/or several fields.

The work embodied in Section 3B on *A. rabiei* mainly deals with the cloning and characterization of the mating type locus of *A. rabiei.* Mating type specific primers designed from the sequences of *MAT1-1* and *MAT1-2*, and the multiplex PCR assay developed in this study can simultaneously screen a large number of fungal isolates and facilitate reliable and rapid identification of both mating types in a simple and convenient assay. This assay for mating type does require isolate culturing, DNA extraction and PCR, however, preliminary data suggests that it may as well be possible to bypass the fungal culturing and DNA extraction steps altogether and amplify directly from microwaved conidial suspensions or infected chickpea leaves (T. L. Peever, unpublished). This multiplex method can reliably replace the currently available methods for mating-type assessment in *A. rabiei*, which involve tedious and time-consuming mating assays with established tester strains performed on senescent chickpea stems (Trapero-Casás and Kaiser, 1992; Wilson and Kaiser, 1995). In previous studies, mating-type specific PCR primers have been shown to be very useful to rapidly identify mating types in field samples of fungi as exemplified in the cases of *Tapesia yallundae* and *Tapesia acuformis* (Dyer *et al.*, 2001), *Mycosphaerella graminicola* (Waalwijk *et al.*, 2002) and *Pyrenopeziza brassicae* (Foster *et al.*, 1999).

The PCR assay for mating type will allow rapid determination of mating type ratios in populations and help to track the introduction of a mating type into an area previously free of a particular mating type. Such an assay is especially relevant for regions of the world where blight epidemics occur regularly, but the sexual stage has never been reported. As described in Section 3B, the sexual stage of *A. rabiei* in the form of pseudothecia of *Didymella rabiei* has been observed in several parts of the world with cool, wet winters (Kaiser, 1997; Kaiser, 1995; Trapero-Casás and Kaiser, 1992); however, it has never been reported in India, Israel, Egypt, Australia, and California, USA (Kaiser, 1995; Kaiser, 1997a; Kaiser, 1997b; Kaiser and Kusmenoglu, 1997; Khan *et al.*, 1999). Large-scale sampling from these chickpea growing regions needs to be done, followed by testing for mating types to check if the other mating type is present/ has been introduced, and if present, what are the reasons for lack of

formation of the sexual stage? In case the sexual stage is not formed (or not important as primary inoculum for blight epidemics) in certain fields or regions, disease control options need to be targeted at other potential sources of inoculum (eg. infected seed or windblown stubble). Similarly, in regions where the sexual stage is very important in disease epidemiology, thrust areas for research need to be towards possible ways of inhibition of development of the sexual stage and/or prevention of dissemination of inoculum from the sexual stage. Elucidation of basic biology of the pathogen will, therefore, be of importance in giving direction to further research for effective disease management depending on the conditions prevailing in a particular region.

The International Seed Testing Association accredited test for *A. rabiei* in chickpea seed involves plating 400 seeds and counting the number of seeds, which produce colonies of the fungus. The test is further complicated by the large size of *kabuli* seeds, which limits the number of seeds per plate, and the slow growth of the fungus, making the assay time consuming. There are further concerns about the sensitivity of the test to detect *A. rabiei* infection which necessitates the requirement of a simple, accurate and sensitive test for seed borne *A. rabiei*, readily applicable to additional seeds per sample. The mating type primers developed in my studies are highly specific to *A. rabiei* and do not amplify from closely related *Ascochyta* species like *A. lentis*, *A. pisi* etc. This situation is very significant for development of *A. rabiei* specific molecular markers to detect seed borne *A. rabiei* in addition to determination of the mating type. Further work can be initiated to devise convenient methods to detect *A. rabiei* from chickpea flour. Similar assays can be developed for seed borne pathogens for other cool season legumes. The sequence data from *A. rabiei* MAT genes can be used to clone and characterize mating type genes from closely related *Ascochyta* species, which are also economically important but not as well studied as *A. rabiei*. Such studies would be very useful for quarantine measures where it is crucial to reliably detect low levels of pathogen infection.

A. rabiei MAT genes are expressed in standard liquid media at laboratory temperatures (20-23^{0}C), while successful laboratory crosses of *A. rabiei* have only been

accomplished on senescent chickpea stems at 5-10^{0}C and not at 15^{0}C or above (Trapero-Casás and Kaiser, 1992), as compared to *C. heterostrophus MAT* genes which are expressed only in nutrient poor media (Leubner-Metzger *et al.*, 1997). *MAT* genes have DNA binding motifs and are thought to be regulatory genes (Coppin *et al.*, 1997; Turgeon and Berbee, 1998) indicating that these genes may have additional functions apart from controlling mating type. The present study forms a base to understand the regulatory roles of these genes and opens a new avenue for further research.

Résumé

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Publications:

M.P. Barve, M.P. Haware, M.N. Sainani, P.K. Ranjekar and V.S. Gupta (2001) Potential of microsatellites to distinguish four races of *Fusarium oxysporum* f.sp. *ciceri* prevalent in India. *Theoretical and Applied Genetics* **102**:138-147

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M.P. Barve, D. K. Santra, V.C. Sanjay, M.P. Haware, P.K. Ranjekar and V.S. Gupta. ISSR and AFLP markers distinguish races of *Fusarium oxysporum* f. sp. *ciceri* prevalent in India (communicated).

T.L. Peever, **M.P. Barve,** W.J. Kaiser Molecular systematics of *Ascochyta* spp. infecting legumes (manuscript under preparation).

M. P. Barve, D. K. Santra, P.K. Ranjekar and V.S. Gupta Genetic diversity analysis of a world-wide collection of *Ascochyta rabiei* isolates using STMS markers (manuscript under preparation).

Poster presentations:

Molecular Variability Studies in the Indian isolates of *Fusarium oxysporum* f.sp. c*iceri* using microsatellites : **Maneesha Barve,** Aparna Patankar, Vidya S. Gupta, Mohini Sainani, Prabhakar K. Ranjekar, and M.P. Haware at the International Conference on "Integrated Plant Disease Management for Sustainable Agriculture",10-15 November, 1997, IARI, New Delhi.

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