INHERITANCE OF AVIRULENCE IN *Sclerospora graminicola* (Schroet) AND RESISTANCE IN PEARL MILLET TO THE PATHOGEN

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M.Sc. (Ag.)

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(PLANT PATHOLOGY)

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BY

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M.Sc. (Ag.)

THESIS SUBMITTED TO THE PROFESSOR JAYASHANKAR TELANGANA STATE AGRICULTURAL UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF

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DECLARATION

I, **CHANDRAMANI RAJ**, hereby declare that the thesis entitled **"INHERITANCE OF AVIRULENCE IN *Sclerospora graminicola* (SCHROET) AND RESISTANCE IN PEARL MILLET TO THE PATHOGEN"** submitted to the **Professor Jayashankar Telangana State Agricultural University** for the degree of **DOCTOR OF PHILOSOPHY IN AGRICULTURE** is the result of original research work done by me. I also declare that no material contained in this thesis has been published earlier in any manner.

Place: Rajendranagar (**CHANDRAMANI RAJ**)

Date: **I. D. No. RAD/12-19**

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Mr. **CHANDRAMANI RAJ** has satisfactorily prosecuted the course of research and that thesis entitled **"INHERITANCE OF AVIRULENCE IN *Sclerospora graminicola* (SCHROET) AND RESISTANCE IN PEARL MILLET TO THE PATHOGEN"** submitted is the result of original research and is of sufficiently high standard to warrant its presentation to the examination. I also certify that neither the thesis nor its part thereof has been previously submitted by him for a degree of any University.

Date: **(B. PUSHPAVATHI)**

Chairperson

CERTIFICATE

This is to certify that the thesis entitled **"INHERITANCE OF AVIRULENCE IN *Sclerospora graminicola* (SCHROET) AND RESISTANCE IN PEARL MILLET TO THE PATHOGEN"** submitted in partial fulfillment of the requirements for the degree of 'Doctor of Philosophy in Agriculture' of the Professor Jayashankar Telangana State Agricultural University, Hyderabad is a record of the bonafide original research work carried out by Mr. **CHANDRAMANI RAJ** under our guidance and supervision.

No part of the thesis has been submitted by the student for any other degree or diploma. The published part and all assistance and help received during the course of the investigations have been duly acknowledged by the author of the thesis.

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LIST OF SYMBOLS AND ABBREVIATIONS

×	:	Cross
≥	:	More than or equal to
≤	:	Less than or equal to
+	:	Mixture
~	:	Approximately
µl	:	Micro liter
%	:	Per cent
<	:	Less than
>	:	Greater than
C	:	Celsius
h	:	Hour
cm	:	Centimeter
dNTP	:	deoxynucleotide 5' - triphosphate
EDTA	:	Ethylene Diamine Tetra Acetic acid
CTAB	:	Cetyl Trimethyl Ammonium Bromide
et. al	:	and others
Fig.	:	Figure
g	:	gram
ha	:	hectare
rpm	:	revolutions per minute
ml	:	milliliter
Sg	:	*Sclerospora graminicola*
Tris	:	tris-hydroxymethyl amino methane
ICRISAT	:	International Crops Research Institute for Semi-Arid Tropics
ICMB	:	ICRISAT Millet B-line
IP	:	Germplasm line
AMOVA	:	Analysis of Molecular Variance
PIC	:	Polymorphic Information Content
MISA	:	MIcroSAtellite identification tool
SSRs	:	Simple Sequence Repeats

Abstract

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ABSTRACT

Studies on development of simple sequence repeat markers and its validation, inheritance and molecular mapping of avirulence and inheritance and allelic study of resistance genes in pearl millet to the isolates of *S. graminicola* were undertaken at International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, Telangana, India. A total of 14481 sequences spanning 215561828 bp were screened using a microsatellite search tool, MISA, that identified 7453 SSRs from 3912 SSR containing sequences, of which 235 (3.3%) SSRs were of compound type and remaining 7218 (96.7%) were of perfect type. The overall relative abundance and density of SSRs in whole genome were 34.57 /Mb and 459.67 bp/Mb, respectively.

A total of 2702 Simple sequence repeat primers were developed. Out of 2702 markers, 106 were custom synthesized and screened for diversity analysis on 60 isolates of *S. graminicola*. The gel electrophoresis result showed 22 scorable polymorphic markers. Allele frequencies i.e 57 alleles with an average of 2.59 alleles per locus were detected. The frequency of major alleles (M_{AF}) per locus was 0.47-0.94, with an average of 0.72. In addition, the H_O values were 0.00-0.76 with an average of 0.26, and H_E values were 0.12- 0.62 with an average of 0.39. The polymorphic index content values were 0.11- 0.60, with an average of 0.33. The PCR product sizes were ranged from 110-320 bp. Analysis of molecular variance revealed 56.21% of the variance over 22 SSRs loci due to the differences among the isolates within states, 35.28 % due to within isolates and 8.51% was due to differences among the states. Dendrogram analysis revealed five major clusters across all isolates. Genetic analysis of 60 *S. graminicola* isolates at high delta K value provided evidence for the presence of two genetically distinct population structures with six admixtures. However, unstable graph of delta K verses K, suggested that there could be more differentiation of *S. graminicola* populations. Besides, *S. graminicola* primer pairs also amplified the fragments from other oomycetes *P. sorghi* (67.9%) and *Phytophthora* species (52.7%).

Two distinct mating types Sg 019 (*Mat 2/A*$_2$) and Sg 445-1/Sg 018 (*Mat 1/A*$_1$) were selected for generation of segregating population. The 121 F_1 progenies were established separately from oospores on the susceptible line 7042S. In order to decipher the nature of genes responsible for inheritance of avirulence, Parents and 121 F_1 progenies were screened on five differential lines along with susceptible check 7042 S. The phenotyping results suggested the presence of two genes (AaBb) for avirulence in Sg 019 on IP 18292 and 852B, and one gene (Aa) on ICMB 01333 and ICML 22. The segregation in F_1 progenies on differential lines implied the heterozygous nature of avirulence gene. The complete loss of avirulence in F_1 progenies against 81B-P6 suggested complex inheritance. Therefore, the probable genotypes for avirulence and virulence in parents Sg 019 and Sg 445-1 might be Aa and aa, respectively on ICMB 01333 and ICML 22, respectively.

Bulk segregants analysis (BSA) revealed three polymorphic primers Sg_SSR 49, Sg_SSR 68 and Sg_SSR 99 between two parents and four bulks. Genetic linkage among three markers revealed markers Sg_SSR 49 and Sg_SSR 68 to be linked with a LOD value of 22.5 and genetic distance of 7.4 cM. The association of markers with avirulence trait showed tight linkage (0 cM) of Sg_SSR 49 to the avirulence trait on IP 18292 and ICMB 01333 with LOD value of 6.96 and 7.43, respectively. Linkage of Sg_SSR 68 with avirulence trait was observed at 7.4 cM distance with LOD value of 9.66 to 9.79 for on ICMB 01333 and IP 18292. The significant R^2 values higher than 10 for the trait of avirulence on three host differentials *viz*., IP 18292, ICMB 01333 and ICML 22 with Sg_SSR 49 and two host differentials IP 18292, ICMB 01333 with Sg_SSR 68 indicated the linkage of these markers with avirulence loci in Sg 019. In order to determine the diagnostic value of avirulence linked microsatellite marker, a set of 60 isolates of *S. graminicola* were phenotyped on pearl millet lines IP 18292 and genotyped by linked SSR marker Sg_SSR 49. Isolates expected to carry avirulence gene showed high level of avirulence to the cultivar IP 18292. Genotyping with avirulence linked marker Sg_SSR 49 showed presence of avirulent gene in 44 isolates that were shown avirulent reaction on IP 18292.

To study the inheritance and allelic relationship among resistance genes to this disease, three resistant pearl millet lines (834 B, IP 18294-P1 and IP 18298-P1) and one susceptible line (81B) were selected on the basis of disease reaction in tests conducted under greenhouse conditions with two isolates of *S. graminicola* (Sg 526-1 and Sg 542-1). Three resistant parents was crossed with susceptible parent to generate three populations, each consisting of susceptible × resistant F_{1s}, F_{2s} and their backcrosses with resistant and susceptible parents for inheritance study. To carry out allelism study, three resistant × resistant F_{1s} and F_{2s} were generated. These populations were evaluated for disease reaction with two isolates by artificial inoculation under both greenhouse conditions. The segregation patterns of resistance in the F_{2s}, corresponding backcross generations and allelic study revealed that resistance to downy mildew in pearl millet is controlled by a single dominant gene, designated as *Rsg2* in 834B, *Rsg3* in IP 18294-P1, and two dominant genes, *Rsg2* and *Rsg3* in IP 18298-P1.

Introduction

Chapter I

INTRODUCTION

Downy mildew (DM), a major threat to pearl millet [(*Pennisetum glaucum* L.) R. Br.] production throughout the world, is caused by a biotrophic, oomycete *Sclerospora graminicola* [(Sacc). Schroet].The disease causes significant yield losses (upto 80%) mainly in single-cross (F_1) hybrids, which are being cultivated on about 60% of the total nine million ha area in India (Yadav and Rai, 2013). There are several reports of downy mildew epidemics during 1970s-80s that resulted in considerable yield losses and withdrawal of several hybrids from cultivation in India (Thakur *et al*., 1999 and Yadav *et al.,* 2002). One of the factors in these epidemics was the evolution of new races /pathotypes of *S. graminicola* due to sexual recombination and rapid spread of virulent phenotypes by prolific asexual cycle. The pathogen is largely heterothallic (Michelmore *et al*., 1982 and Idris and Ball, 1984), but homothallism also exists (Michelmore *et al*., 1982). These characteristics of the pathogen make it highly variable like its host, pearl millet, which is a highly out crossing crop species. The F_1 hybrids, which are produced primarily through the crossing of two inbred lines, became more vulnerable to the pathogen if grown on same field continuously for 3-5 years (Thakur *et al*., 2003., Rao *et al*., 2005 and Thakur *et al*., 2006).

Despite the importance, *S. graminicola* and the recognized variability in the pathogen (Sastry *et al*., 1995., Thakur *et al*., 1999., Singru *et al*. 2003., Sivaramakrishnan *et al*. 2003., Thakur *et al*. 2004., Pushpavathi *et al*., 2006b., Sudisha *et al*., 2008 and Sharma *et al*., 2010), studies on molecular diversity have been constrained due to limitations of markers. However, work carried out in different institutes revealed the presence of diverse populations at genetic level, but the mechanism responsible for this variability has not been studied in detail. To understand the biology and ecology of *S. graminicola* populations, fully characterize powerful markers, such as microsatellites also known as simple sequence repeats (SSRs), are essentially required.

SSRs are tandemly repeated motifs of one to six bases found in the nuclear genomes of all eukaryotes and are often highly abundant and evenly distributed throughout the genome (Tautz and Renz, 1984). Microsatellite sequences are usually characterized by a high degree of length polymorphism, and are ideal single-locus co-dominant markers for genetical studies. Co-dominance offers a greater resolving power

and the data can be used to determine population genetic structure, kinship, reproductive mode and the extent of genetic isolation (Ashley and Dow, 1994). Microsatellites have been used to characterize the oomycetes *Plasmopara viticola* (Gobbin *et al*., 2003), *Phytophthora cinnamomi* (Dobrowolski *et al*., 2002), *P. ramorum* (Prospero *et al*., 2004), *P. infestans* (Ivors *et al*., 2006 and Lees *et al*., 2006) and *Pernosclerospora sorghi* (Perumal *et al*., 2008). However, a major limitation to their wider exploitation is the need for prior knowledge of the DNA sequence of the SSR flanking regions to which specific primers have to be designed. Such regions are usually conserved within a species but the likelihood of primers successfully working between species decreases with increasing genetic distance and in practice, primers are usually developed a new for each species (Prospero *et al*., 2004 and Schena *et al*., 2008). Conventional methods for the discovery of SSR loci based on constructing genomic DNA libraries enriched for SSR sequences are time-consuming and expensive (Dobrowolski *et al*., 2002 and Prospero *et al*., 2004). The availability of whole genome sequences for an increasing number of species including oomycetes has proved novel opportunities to identify and evaluate potential SSR markers identified by computational tools (Garnica *et al*., 2006 and Robinson *et al*., 2004). Apart from their application as molecular markers, determining the abundance and density of SSRs may help to understand the functional and evolutionary significance of the pathogen (Toth *et al*., 2000). To date, with the exception of a publication on the cross-transferability of *P. sorghi* SSR markers to *S. graminicola* (Perumal *et al*., 2008), there is no report on the development and application of SSRs for this pathogen.

Understanding the function and expression of genes controlling avirulence in a pathogen population is important for determining genetic changes that allow strains to overcome resistance in the host. The gene-for-gene relationship between host and pathogen has been described in its simplest form as each locus specifying specific host resistance or susceptibility in host is matched by a complementary locus controlling specific avirulence or virulence in the pathogen (Flor, 1956). The downy mildew patho-system is also believed to exhibit gene-for-gene interactions and serves the basis of determination of compatibility and incompatibility between host and pathogen (Thakur *et al*., 1992). Determination of avirulence in *S. graminicola* by a single dominant gene was reported by Pushpavathi *et al*. (2006c). However, all results from genetic studies on gene-for-gene relationships cannot be fully agreeable due to oversimplification of the basic genetic principles of the gene-for-gene theory (Ilott *et al*., 1989). In some pathogens the expression of an avirulence allele were suppressed due to the presence of

an inhibitory allele at a second locus in the pathogen (Lawrence *et al*., 1981 and Ellis *et al*., 2007). So far, genetic mechanisms of governing resistance in pearl millet and a/virulence in *S. graminicola* have not been determined, and in the absence of genetic interpretation of both host and pathogen, neither breeders nor pathologists can confirm that a particular line or lines will provide effective resistance to the race or races of pathogen prevalent in a particular area. Unavailability of inbreed strains of *S. graminicola* pose additional problems for further studies because crossing has to be carried out between individuals taken from natural populations which are expected to be in heterozygous states for various alleles. The gene-for-gene system study can be a good choice in *S. graminicola* because of diploid and predominant heterothallic nature and due to potential of generating large numbers of progenies after sexual crosses between parents of opposite mating types (Pushpavathi *et al*., 2006a).

Studies on inheritance of molecular markers in oomycetes have ascertained that nuclear loci generally segregate in a Mendelian fashion. For instance, Hulbert *et al*. (1988) in *Bremia lactucae* and Van der Lee *et al*. (1997) in *Phytophthora infestans* observed segregation of RFLP and AFLP loci, respectively, in the Mendelian genetics. Microsatellite markers certainly could be a better choice for segregation studies due to their inheritance in a Mendelian fashion as co-dominant markers. In addition, high polymorphism rates and even distribution throughout the genome renders microsatellites as marker of choice (Tautz and Renz, 1984). Kaye *et al*. (2003) reported segregation of simple sequence repeat (SSR) loci in the ratio of 1:1 in 58 F_1 progenies of *Magnaporthe grisea* and further added SSR loci in integrated genetic map of *M grisea*. However, sufficient numbers of markers have to be identified to develop detailed genetic maps which are powerful tools for studying variation and cloning genes of interest, such as avirulence genes. Mapping of avirulence genes helps in deciphering the molecular events involved in pathogenesis and in understanding plant-pathogen interactions in general. First step in this direction is the identification of DNA markers linked to *Avr* genes that can be used as a starting point for map based cloning. So far, there are no reports on the molecular mapping of avirulence gene in *S. graminicola.*

The continuous emergence of host specific virulences in *S. graminicola*, due to high degree of pathogenic and genetic variability, lead to frequent breakdown of disease resistance, and poses a continuous challenge to pearl millet resistance breeding (Werder and Ball, 1992., Thakur *et al*., 1992., Sastry *et al*., 2001 and Pushpavathi *et al*., 2006b), therefore, pyramiding of resistance genes in one single genotype or identification of

single genotype with different resistant genes and resistant to multiple pathotypes would be the possible solutions to increase the durability of resistance. Attempts have been made to identify several sources of resistance against one or multiple pathotypes and based on resistant sources, several resistant hybrids and varieties were released for general cultivation in India (Hash *et al*., 2006 and Sharma *et al*., 2015). In addition, the inheritance of DM resistance in pearl millet has been interpreted by various workers in terms of simple and complex interactions. The DM resistance has generally been reported to be governed by a dominant gene (Appadurai *et al.,* 1975 and Singh and Talukdar, 1998), recessive gene (Singh *et al*., 1980 and Pethani *et al*. 1980), single, two or more genes (Appadurai *et al.,* 1975., Gill *et al.,* 1978 and Joshi and Ugale, 2002) and multiple genes with epistatic effect (Deswal and Govilla, 1994). However, allelic relationship or difference between genes of resistance is still not very clear. The inconsistency of gene effects over crosses could be clarified with the analysis of allelic diversity of resistance gene in pearl millet to downy mildew and it would be helpful in understanding molecular interaction between host and pathogen that will further help in development of management strategies.

Hence, in consideration of above mentioned gap in the understanding of pearl millet-downy mildew system, the present study was planned with the following objectives:

Objectives of investigation

1. Development of Simple Sequence Repeat markers (SSR) for diversity analysis of *Sclerospora graminicola.*
2. Development of segregating population from a cross between avirulent and virulent isolates of *S. graminicola.*
3. To study inheritance and molecular mapping of avirulence genes in *S. graminicola.*
4. To study inheritance of downy mildew resistance in pearl millet genotypes and allelic relationships among the resistance genes.

Review of Literature

Chapter II

REVIEW OF LITERATURE

A brief review of literature relevant to the objectives of the present investigation is presented under the following heads:

2.1 General

2.2 Variability in *Sclerospora graminicola*

2.3 Inheritance and mapping of avirulence gene

2.4 Inheritance of resistance and allelic relationship

2.1 GENERAL

2.1.1 Pearl millet - the host

Pearl millet is a hardy warm-season, dryland cereal grain crop largely grown in the drought-prone regions of Africa and Asia where it performs quite better than other cereals (Yadav and Rai, 2013). It ranks fifth among cereal crops after rice, wheat, maize and sorghum and grown as an important staple food in the drier regions of Sub-Saharan Africa, especially in Nigeria, Niger, Mali, and Burkina Faso. In India, about 50 million people mostly in the states of Rajasthan, Maharashtra, Gujarat, Haryana and Uttar Pradesh depend upon pearl millet as a staple food (Harvest Plus, 2015). It has also been recognized as a valuable forage crop, because of its robust and quick growth with high fodder yield, in the South-eastern USA and dry areas of Australia (Yadav, 1996).

Pearl millet is cultivated on about 30 million ha in more than 30 countries of five continents viz., Asia, Africa, North America, South America and Australia (Yadav and Rai, 2013). Though the majority of crop area is in Asia (10 million ha) and Africa (about 18 million ha), cultivation is being expanded in some of the non-traditional areas like Brazil having the largest area of about 2 million ha. It is also being experimented as a grain and forage crop in the USA, Canada, Mexico, the West Asia and North Africa (WANA), and Central Asia. In India, pearl millet is the third most widely cultivated food crop after rice and wheat (Yadav and Rai, 2013). It is grown on 9 million ha with an average productivity of 1,000 kg ha^{-1} (Yadav and Rai, 2013). The major pearl millet growing states are Rajasthan, Maharashtra, Gujarat, Uttar Pradesh and Haryana which

account for more than 90 % of pearl millet acreage in the country. Most of pearl millet in India is grown in rainy season (June–September). It is also being increasingly cultivated during the summer season (February–May) in parts of Gujarat, Rajasthan and Uttar Pradesh; and during the post-rainy (rabi) season (November–February) at a small scale in Maharashtra and Gujarat.

2.1.2 *Sclerospora graminicola* - the pathogen

Sclerospora graminicola belong to the group Chromista; phylum Oomycota; class Oomycetes; order Sclerosporales; family Sclerosporaceae. The genus *Sclerospora* is generally heterothallic in nature, however, homothallism have also been reported (Michelmore *et al*., 1982). The pathogen undergoes both asexual (sporangia, zoospores) and sexual phase (oospores).

Asexual phase: Sporangia are hyaline, thin-walled, ephemeral, ellipsoid and papillate, with dimensions of 15 to 22 x 12 to 21 µm. In nature, sporangia are produced during the night hours between 1 am to 4 am and in artificial conditions, it can be harvested after 6 hours of incubation of cotton wiped infected leaf at 20°C. The optimum sporangial production coincides at 20-25°C with 95 to 100% RH. Sporangia are actively ejected and germinate immediately by producing germ tubes or by releasing 1 to 12 zoospores, or else die within a few hours if no host is available. However, germination by zoospore release is most common. The sporangial infectivity is limited by seedling age, with the greatest susceptibility from the time of seed germination to the 1-2 leaf stage. Thereafter, the susceptibility decreases sharply (Singh and Gopinath, 1985). The profuse sporangial growth can be observed on the under surface of the diseased leaf (and, also on the upper surface when conditions are favorable) under suitable conditions, giving an appearance of distinct white 'downy' growth (Francis and Williams, 1998).

Sexual phase: Oospores are sexual spores; appear as brownish yellow thick-walled spherical resting spores measuring 32 µm (22 to 35 µm**)** in diameter. Under laboratory conditions it can survive for 14 years, but its viability gets reduced after 4 years of storage. Four different types of oospores germination in *S. graminicola* have been reported; germination by vesicle-like structures, by both vesicles and germ tubes, by typical irregular structures different from germ tubes and vesicles, by germ tubes and germination by extrusion of small round bodies/sporangia-like structures (Lukose and Dave, 1995). However germ tube germination is most commonly accepted. Singh and Navi (1996) have reported that the optimum temperature for oospore germination is

28±2°C. It is generally agreed that one year old oospores give more infection (98%) than fresh or more than one than year old oospores (Nene and Singh, 1976).

2.1.3 Economic importance

Downy mildew (DM) of pearl millet, sometimes called as 'green ear' disease, is a major limiting factor in the production of crops across the cultivated area in the world (Williams, 1984). As disease coexists with the crop, it causes immense loss of economic yield at farmer's field. It was first reported by Butler (1907) in India and described as disease of ill-drained lands where it developed into severe form. The magnitude of grain yield reduction largely depends on disease severity levels and the stage of crop growth during infection. Total loss may occur in plants exhibiting downy mildew in seedling stage and green-ear infection at earhead formation (Thakur, 1987). Nene and Singh (1975) reported loss estimates of six to 60 per cent in different part of the world. It is also reported that the worldwide annual pearl millet grain yield losses due to downy mildew do not exceed 20 per cent (Khairwal *et al.,* 1999). However, this disease can assume an alarming level when a single genetically uniform pearl millet cultivar is repeatedly and extensively grown in a region where the pathogen is present (Andrews, 1987 and Singh *et al.,* 1987a). This was clearly demonstrated in HB 3, a popular hybrid in India in the early seventies, when pearl millet grain production was reduced from 8.2 million t in 1970-71 to 3.3 million t in 1971-72 (AICMIP, 1972). This reduction was to a large extent, due to downy mildew epidemic, in which yields in some fields were reduced by 60-70 per cent. Subsequent to this epidemic, grain yield losses continued to occur quite frequently due to downy mildew epidemics in India (Singh *et al.,* 1987b). Thakur *et al.* (1999) during a survey recorded disease incidence of 80 to 100 per cent on a few hybrids in states of Maharashtra, Rajasthan and Gujarat in India, accounting for a considerable yield loss. The economic loss due to a single epidemic of DM has been estimated to be £7.8 million (Hash *et al*., 2003)

2.1.4 Diagnostic symptoms

The characteristic symptoms of the disease differ with the age of plant. At the seedling stage (after 10 days of inoculation), three different types of symptoms are produced: 1. Systemic chlorosis moves upward from the base of leaf blade (normally 2^{nd} and 3^{rd} leaf, may come on 1^{st} leaf also) to leaf apex, sometimes it goes along the side of margin in certain varieties of pearl millet; 2. Under conditions of high humidity and moderate temperature, whitish growth of the pathogen in the form of sporangiophores and sporangia appear, generally on the abaxial leaf surface, giving them downy

appearance. 3. At the time of panicle emergence, green ear symptoms appear on ear heads due to transformation of floral parts into leafy structures, which can be total or partial. This is sometimes referred to as virescence. These leafy structures can also be chlorotic. In certain cases, green ear is the only manifestation of the disease. The infected old plant leaves subsequently turn reddish brown and dry due to oospore production when both mating types are present or homo/heterothallism is operative. In acute cases, shredding of leaves can also occur (Butler, 1907).

At the time of panicle emergence, green ear symptoms appears on ear head with all possible degrees of proliferations and malformations. In ear head, florets are converted into leafy structures of diverse appearance. Generally four types of malformations can be observed: a) transformation of entire inflorescence into green leafy tuft i.e. 'green ear'; b) proliferation of lower half of ear-head with normal flowers on the upper half; c) hypertrophy of bristles into tough and spiny structures without malformation of leaves and d) leafy tufts at the top where shoots remain stunted (Singh, 1993).

2.2. Variability in *Sclerospora graminicola*: Pathological to genotypic diversity

2.2.1.1 Variability in *Sclerospora graminicola*-the beginning with race concept

The evidence of variation in *S. graminicola* was first reported by Uppal and Desai (1932) as existence of two physiological forms based on cross inoculation experiment on Setaria and Pearl millet. Tasugi (1934) observed similar results and supported the idea of existence of physiological forms in *S. graminicola*. The concept of multilocational screening of one cultivar to the respective regional pathotypes has been explored by several researchers to know about the variability in the pathogen. Bhat (1973) found resistant and susceptible reaction of pearl millet cultivars HB 3 to the pathotypes at Mysore location and some other locations in India respectively. Nene and Singh (1976) interpreted this variation as existence of races in the pathogen. Furthermore, Rasheed *et al*. (1978) and Shetty *et al*. (1980) experienced differences in the size of asexual structures, number of nuclei, seed borne nature and soluble protein in *S. graminicola* and affirmed the existence of two pathogenic races of the pathogen.

2.2.1.2 Variation in pathogenicity

Pathogenic variation in natural populations, single zoospore and single oospore derived isolates of S. *graminicola* was demonstrated by several researchers (Ball, 1983.,

Ball and Pike, 1983., Ball and Pike, 1984., Ball *et al.,* 1986., Thakur and Shetty 1993., Thakur *et al*, 1998a., Thakur *et al*, 1998b., Sharma *et al*., 2010 and Sharma *et al*., 2011). Samples (pathogen collections) from different geographic regions differed in pathogenicity when tested on a set of host cultivars. Populations from sub-sahelian regions of West Africa were more pathogenic than the Indian ones on Indian cultivars (Ball and Pike, 1984). Similarly, populations form Burkina Faso, Nigeria and Niger were generally more aggressive than those from Senegal, Zambia or India, but no differences in aggressiveness were found between Indian and Zambian populations (Ball *et al.*, 1986). In India, variations in pathogenicity of *S. graminicola* populations were reported from Mysore and Gulbarga on the pearl millet cultivar HB 3 (Shetty and Ahmed, 1981) and from Patancheru and Durgapura on NHB 3 (Singh and Singh, 1987). Werder and Ball (1992) reported differential response of Pearl millet lines to different sources of inoculums from West Africa and concluded that the variability exist both in pearl millet lines and in *S. graminicola*. Thakur *et al.* (2001) observed highly significant variability in downy mildew incidence across the 46 genetically diverse male sterile lines and reported that the variability was due to genetic divergence among the lines, the pathotypes and their interaction. In the similar line, Sharma *et al*. (2010) observed significant variation for downy mildew incidence, latent period and virulence index and classified 46 *S. graminicola* isolates into 21 pathogenic groups/pathotypes and eight groups based on disease reaction and cluster analysis of virulence index, respectively. They also reported the temporal variation in pathogenicity among the isolates collected from the same location and same host over the years.

2.2.1.3 Host directed selection of virulence in the pathogen

The emergence of a new race/pathotype from the asexual generations of an isolate is not solely an outcome of genetic recombination, but could be the effect of host genotype - directed selection for specific virulence in the pathogen population. In this context, Thakur *et al.* (1992) isolated *S. graminicola* from NHB 3 or 7042S, which was less virulent on MBH 110 and 852 B, was passed through several asexual generations on MBH 110 and 852 B. Within 12 generations of selection on MBH 110 and 5 generations of selection on 852 B, highly host-specific virulence, comparable to their respective field pathotypes, were identified. The result indicated the genetic variation for host genotype specific virulence exists within field populations of the pathogen, and that selection through asexual generations can rapidly increase the quantitative virulence of the population to the specific host genotypes.

2.2.1.4 Genetic variation in the pathogen

Genetic studies in any organism require precise and easily scoreable heritable characters or markers. Fungi are often microscopic and have few phenotypic markers, such as vegetative compatibility, mating types or specific virulence (Leslie, 1993., McDonald and McDermot, 1993 and Michelmore and Hulbert, 1987). The advent of highly versatile, molecular markers, which are based on differences in DNA sequences, has made it possible to conduct basic studies on population and evolutionary biology in fungi. These techniques include DNA hybridization methods, such as endogenous genomic and mitochondria1 Restriction Fragment Length Polymorphisms (RFLP), DNA fingerprinting and PCR-based DNA markers. These techniques have been widely used to estimate the genetic diversity in several fungal species of zygomycetes, ascomycetes and deuteromycetes (Weising *et al.,* 1995). Variations in fungi or oomycetes denote the change in genetic structure and it arises largely due to sexual recombination, heterozygosity, somatic recombination, mutation and hybridization (Singh, 1986).

The advents of molecular markers, which are based on DNA sequences, were well received by Sastry *et al*. (1995) for the first time in *S. graminicola* and they demonstrated the use of DNA fingerprinting to detect genetic variation in the pathogen populations. The simple repetitive DNA sequences (microsatellites) $(GATA)_4$, $(GACA)_4$, and $(GAA)_6$ have been found effective in detecting polymorphism among pathotypes of *S. graminicola*. In general, these microsatellite probes detected more polymorphism in *S. graminicola* when restriction enzymes with four-base specificity were used than with hexacutter-specific enzymes. Microsatellite $(GATA)_4$-derived fingerprinting was stable even after ten asexual generations of *S. graminicola*, indicating the utility of this probe in DNA fingerprinting. Thakur *et al*. (1999) confirmed similar results on six pathotypes of *S. graminicola* with $(GATA)_4$ probe.

Sivaramakrishnan *et al*. (1996) utilized 50 random amplified polymorphic DNA (RAPD) primers to detect the genetic variation in six host specific pathotypes of *S. graminicola* and confirmed the existence of distinct host genotype-specific virulence in the pathogen. In the similar study, Zihad (1997) noticed significant genetic variation within and between four populations of *S. graminicola* from Africa and India using 65 arbitrary oligonucleotide primers. Polymorphism among 20 isolates of *S. graminicola*

was also observed from different geographic locations using 34 RAPD primers. The dendrogram plotted for relatedness among the isolates revealed two distinct clusters of which one indicated African isolates and the other indicated Indian isolates. Sastry *et al*. (2001) examined genetic variation in the host-specific pathotypes due to shift to other cultivars of pearl millet and maintaining them for 10 asexual generations on same cultivars through RAPD and microsatellite probe $(GATA)_4$ and observed the differences in banding pattern of RAPD and microsatellite probe in the two extreme generations. A comparison of molecular markers between RAPD and Inter Simple Sequence repeats (ISSR) was carried out by Sudisha *et al*. (2008) to classify six *S. graminicola* pathotypes collected from different agro climatic regions of India and found that the ISSR are more efficient and suitable marker for detection of genetic variation than RAPD based on high number of total polymorphic, discriminant and presence of unique bands. Later, Sudisha *et al*. (2009) assessed the polymorphism at DNA level among 22 isolates of *S. graminicola* using 20 ISSR primers and revealed significant level of genetic diversity among the isolates.

Singru *et al*. (2003) employed 14 AFLP primers to detect the extent of genomic variation among 19 isolates of *S. graminicola* collected from different cultivars of pearl millet in India and demonstrated geographical relatedness or gene pool among the isolates from neighboring geographical area, high level of genetic variation in natural populations due to pyramided virulence selection against pearl millet populations in planned breeding programme and variation in isolates with similar host origin because of stepwise selection for virulence against the influence of new cultivars followed by mutation or genetic reassortment in the genetic structure of *S. graminicola*. The similar trend of genetic variations obtained by AFLP patterns among the *S. graminicola* isolates in the study of Sivaramakrishnan *et al.* (2003), Thakur *et al.* (2004), Pushpavathi *et al*. (2006b) suggest that the isolates collected from specific genotype may adapt to new host genotype through several cycles of asexual growth on the host and the genetic changes in the isolate may or may not be reflected in virulence changes. Sharma *et al*. (2010) examined genotypic diversity among 46 isolates of *S. graminicola* collected from seven states of India during 1992–2005 through AFLP analysis and found high degree of genetic variation in the isolates within the states. The reason behind this high genotypic variability within states was speculated due to frequent recombination events occurring between different sexual compatibility types in the natural populations of *S. graminicola* and region specific clustering of the isolates.

Simple sequence repeats (SSRs) or microsatellites are PCR-based molecular markers are more desirable for population genetic analysis as they can detect accurate polymorphism due to co-dominance. Besides, these markers are highly reproducible, locus-specific, multi-allelic and abundant in animal, plant and microbial genomes. In oomycetes like *Phytophthora* and *Plasmopara*, SSRs have been used for diagnosis and determination of mating type, studying genetic structure and population genetics. However, there is no report of development of PCR based SSR markers from *S. graminicola* genomic DNA. Perumal *et al*. (2008) observed cross transferability of 37 primer pairs developed from *Peronosclerospora sorghi* to *S. graminicola* and opined that these primers can be used in the genetic diversity studies of pearl millet downy mildew. Sharma *et al*. (2009) screened these 37 microsatellite markers using 23 Indian isolates of *S. graminicola* and found that only 54% of the primers could amplify reproducible fragments. However, none of them was polymorphic.

2.3 Inheritance and mapping of avirulence gene

A gene-for-gene relationship between host and pathogen has been described in its simplest form as each locus specifying specific host resistance or susceptibility in host is matched by a complementary locus controlling specific avirulence or virulence in the pathogen (Flor, 1956). The determination of race-cultivar specificity by gene-for-gene interactions has been proposed for several diseases caused by oomycetes and the downy mildew patho-system is also believed to exhibit gene-for-gene interactions and serves the basis of determination of compatibility and incompatibility between host and pathogen. The concept of gene for gene relationships in pearl millet-downy mildew system was started with the work of Thakur *et al*. (1992) with the concept of host specific virulence on different cultivars in asexual populations of *S. graminicola*. They expected the existence of two different sets of virulence genes in *S. graminicola* populations corresponding to resistant genes in two different pearl millet genotypes.

However, till now, only one study on inheritance of avirulence and no study on molecular mapping of avirulence gene have been reported. Hence, the more relevant studies in other well studied oomycetes are also reviewed hereunder.

Pushpavathi *et al*. (2006c) hybridized an avirulent (Sg 110-9) and a virulent isolate (Sg 139-4) on a susceptible genotype 7042S of pearl millet and developed 33 F_1, 230 F_2, 46 BC_1 (F_1 x Sg 110-9) and 62 BC_2 (F_1 x Sg 139-4) progenies on the maintenance host 7042S. The evaluation of parents, F_1, F_2, BC_1 and BC_2 progenies on

the resistant cultivar IP 18292 revealed no segregation of avirulence in F_1 and BC_1, 3:1 segregation in F_2 and 1:1 segregation of avirulence and virulence in BC_2 generations, that suggested the dominance of avirulence over virulence and monogenic nature of governance of avirulence in the avirulent isolate Sg 110-9 to a corresponding resistance gene in IP 18292.

In case of lettuce downy mildew, at least 11 specific resistance factors conferring resistance in lettuce to *B. lactucae* were identified and the extensive genetic studies revealed that many of these resistance factors were inherited as dominant alleles at single loci (Crute and Johnson, 1976., Johnson *et al.,* 1977., Johnson *et al.*, 1978 and Norwood and Crute, 1980). Complementary studies on the inheritance of avirulence in the pathogen, however, were not possible until *B. lactucae* was shown to exhibit heterothallism (Michelmore and Ingram, 1980), which allowed controlled crosses between isolates of characterized virulence phenotype. Preliminary investigations with limited number of F_1 isolates form several crosses indicated that avirulence was dominant to virulence (Michelmore and Ingram, 1981 and Blok, 1981).

More detailed studies on inheritance in avirulence in *B. lactucae* was conducted by hybridizing two isolates which differed in their virulence on host cultivars carrying the resistance factors R_1, R_2, R_4, R_6 and R_{11}. The results demonstrated that the avirulence to match resistance factors R_1, R_2, R_4, and R_{11} segregated as single loci with avirulence dominant to virulence; however, the inheritance of avirulence to R_6 was complex in which the ratios obtained could not readily be interpreted in Mendelian terms (Norwood *et al.*, 1983). There seemed, therefore, to be a locus for avirulence/virulence specific and complementary to each of the host resistance factors except R_6, and this corroborated the proposal of a gene-for-gene interaction between *B. lactucae* and lettuce (Crute and Johnson, 1976) of the type first described by Flor (1956).

Michelmore *et al.* (1984) confirmed that the avirulence in *B. lactucae* to match the specific resistance genes located in lettuce cultivars is controlled as predicted by a gene-for-gene relationship. However, the linkages between loci controlling avirulence were also reported in Bremia-lettuce pathosystem. Norwood *et al.* (1983) suggested linkage between two avirulent loci to R_2 and R_{11} of the lettuce cultivars. In the similar line, the findings of Michelmore *et al.* (1984) indicated the linkage among three loci for avirulence to R_5, R_8, and R_{10} of lettuce cultivars. Later, Norwood and Crute (1984) provided more evidence for these linkage relationships by making crosses involving 12 heterothallic isolates of the pathogen. Much of the evidence was obtained from a cross

in which avirulence segregated simultaneously in the F_1 generation for eight of the 11 specific resistance factors examined.

The gene-for-gene hypothesis was again confirmed in lettuce and *B. lactucae* system by making crosses between heterothallic isolates of the pathogen on differential cultivars of the host (Ilott *et al.*, 1989). The other host-parasite interactions such as Soybean-*Phytophthora megasperma* f.sp. *glycinea*; Rice-*Magnaporrhe grisea*; Potato-*Phytophthora infestans* were also shown to follow gene-for-gene hypothesis pathosystem (Layton and Kuhn, 1988., Silue *et al.*, 1992 and Al-Kherb *et al.*, 1995). However, detailed studies have often shown that the interaction between some genotypes of host and pathogen are more complex (Lawrence *et al*., 1981) and the number of genes involved in pathogenicity always depend on the host and the pathogen interaction (Statler, 1990).

Whisson *et al*. (1994) carried out inheritance and mapping of avirulence gene through molecular marker (RAPD) in F_2 progenies of cross between two different isolates of *Phytophthora sojae*. The segregation of virulence against soybean resistance genes *Rps1a, 3a*, and *5* revealed that the avirulence genes *Avr1a, 3a* and *5* were dominant to virulence and avirulence against these three resistance genes appeared to be conditioned by one locus for *Avr1a* and two independent, complementary dominant loci for both *Avr3a* and *Avr5*. Further, linkage analysis of segregating genetic characters in the F_2 populations showed linkage between avirulence genes Avr3a and Avr5 (10.27 cM, LOD 9.25) Avr3a and RAPD marker OPH4-1 (5.03 cM, LOD 3.25), and Avr5 and OPH4-1 (5.03 cM, LOD 3.25) and suggested the order of genes as Avr3a - OPH4-1 - Avr5.

Tyler *et al*. (1995) studied inheritance and mapping of avirulence factors in the outcrosses of three *Phytophthora sojae* isolates against soybean resistance genes and observed the dominant nature of avirulence in six cases (*Rps1a, Rps1b, Rps1c, Rps1d, Rps1k* and *Rps3a*) and semi-dominant to dominant in four cases (*Rps3b, Rps3c, Rps4* and *Rps6*). The segregation of avirulence among F_2 progenies supported the presence of single dominant avirulence gene (*Avr 1a, Avr 1b* and *Avr 3a*) in the pathogen corresponding to three resistance genes (*Rps1a, Rps1b* and *Rps3a*) in the host. The linkage between four pairs of RFLP markers were detected with the genetic distance ranged from 0 to 11 cM (centimorgan). In addition, RFLP 121 P1 was found to be linked to the avirulence gene (*Avr 1b*) against *Rps1b* at a distance of 11cM.

Sicard *et al*. (2003) examined inheritance of avirulence and linkage between molecular markers and avirulence genes in 97 F_1 progenies derived from a cross between Finnish and Californian isolates (SF5and C82P24) of *Bremia lactucae*. The virulence phenotyping result on 24 differential lettuce cultivars indicated single dominant gene governance of avirulence as per 1:1 (avirulence: virulence) segregation ratio on most of the cultivars, however, digenic controlled avirulence (*Avr11*) was also observed on *Dm11* with 3:1 segregation ratio of avirulence to virulence. The closest linkage between AFLP marker (pCTGC02) and avirulence genes (*Avr7*) was noticed at 1 cM.

A comprehensive genetic linkage map assembled from avirulence phenotypes and closely associated molecular markers will provide a vital starting point for cloning and characterizing of avirulence genes in *S. graminicola*. Cloning and characterizing avirulence genes serves as basic tools for the attempt directed towards revealing the nature and functions of factors in plant pathogens that induce or overcome resistance in the host (May *et al*., 2002). Numerous avirulence genes that have been isolated from bacterial, viral, and fungal plant pathogens encode putative proteins with diverse sequence and structural characteristics (Leach and White, 1996 and De Wit and Joosten, 1999). However, neither avirulence genes nor avirulence gene product has been described in *S. graminicola*. Clear information on avirulence genetics will aid in deciphering the molecular events involved in pathogenesis and in understanding plant-pathogen interactions in general.

2.4 Inheritance of resistance and allelic relationship

After the onset of the hybrid cultivar era for pearl millet, downy mildew resistance has been a major research focus for the scientists of both ICRISAT and the Indian national program involved in improvement of this crop. The durability of disease resistance against single or multiple races/pathotypes in the particular area is almost dependent on the deployment of resistant cultivars carrying different resistance genes to the pathogen. Henceforth, breeding for resistant cultivars requires prior information of the number and nature (dominance or recessive) of resistance genes. This information is somewhat unclear in the previous studies, particularly in relation to dominance (Singh *et al.,* 1993., Hash *et al.,* 1997., Hash *et al.,* 1999 and Hash and Witcombe, 2002).

Appadurai *et al.* (1975) studied inheritance of resistance in 1165 inbred lines of pearl millet under field conditions and suggested that resistance for downy mildew in pearl millet is simply inherited and being determined by one or two major dominant

genes as one set segregated into 3:1 and another set into 15:1 resistance to susceptibility in F_2 generations.

Gill *et al.* (1975) examined 47 F_1 hybrids of resistant and susceptible (RxR, RxS, SxR and SxS) inbred lines under sick plot conditions. The result showed that the combination of RxR and RxS were all resistant, however, the combinations the SxR segregated into 8R:5S indicated the differential reaction of different crosses to the pathogen. The F_{1s} of SxS cross combinations was either resistant or susceptible implied that the inheritance pattern of resistance was rather complex and could involve genic interactions or cytoplasmic-genetic interactions or both.

Later, Gill *et al.* (1978) carried out inheritance study under sick plot conditions with parents, F_1, F_2 and back crosses generations of 10 crosses (RxS) of pearl millet and revealed the presence of two duplicate dominant factors conferring resistance to downy mildew as *P* values showed a good fit for the 15:1 ratio in the F_2 generations of all the ten crosses that was further supported by 3:1 test cross ratio. The gene symbols of *DMlDM2, DM1dm2,* and *dm1DM2* for resistant and dm_1dm_2 for susceptible genotypes were proposed.

Singh *et al.* (1978) crossed 12 parents in diallele fashion (R x R, R x S, S x S) without reciprocal and screened all four generations (parents, F_1 and F_2) using a sick plot screening protocol. The F_{1s} of the crosses between R x R were resistant while S x S were susceptible. However, The F_1 of R x S crosses showed complete susceptible reaction that indicated the dominance of susceptibility over resistance. All the three categories produced resistant to susceptible plants in F_2, but the intensity of resistant plants in R x R group was higher than in the R x S and S x S groups. The plant to plant variation in scoring within the generation indicated the quantitative nature of inheritance for DM inheritance.

Basavaraju *et al.* (1980, 1981) investigated the genetic architecture of resistance to downy mildew in pearl millet with six generations of crosses (parents, F_1, F_2, back crosses) under artificial epiphytotic condition and revealed the additive, dominance and epistatic effects in the inheritance of resistance against DM of pearl millet. The epistatic effects were more prominent followed by dominance and additive effects. Of the epistatic effects, the negative value of dominance x dominance mostly reinforced the resistance. Heterosis was found due to the interaction of additive x additive and dominance x dominance effects. Hence, it was concluded that resistance to downy mildew is due to a series of non-allelic interactions instead of simple inheritance.

The inheritance of resistance to downy mildew in pearl millet was studied by Pethani *et al*. (1980) in line x tester combinations and they suggested the dominant nature of resistance over susceptibility and role of minor genes in resistance to downy mildew. Shinde *et al.* (1984) screened all six generations of crosses under natural condition and revealed the contribution of both additive and non-additive gene effects in the inheritance of downy mildew resistance in pearl millet. Duplicate effects were observed in most of the crosses studied.

Dass et *al.* (1984) and Deswal *et al*. (1998) estimated the gene effect of downy mildew resistance in pearl millet by the analysis of generations means of six populations (P_1, P_2, F_1, F_2, B_1 and B_2) in two pearl millet crosses under two environmental conditions and reported the dominance of resistance over susceptibility. The highly significant χ^2 values for three-parameter model indicated the presence of epistatic gene effects. However, both additive and dominance effects were noticed. Higher magnitude of the latter revealed that dominance effects played greater role in the inheritance and was probably controlled by digenic interactions with complementary gene action. Similar results were reported by Tyagi and Singh (1989) and Kataria *et al.* (1994) and they suggested the complex pattern of inheritance of resistance to downy mildew and indicated simple selection would not be effective for incorporation of resistance to DM.

Deswal and Govila (1994) examined genetics of resistance to downy mildew in six generations (P_1, P_2, F_1, F_2, B_1 and B_2) of the cross R x R, R x S and S x S at two locations (Delhi and Tamil Nadu) under artificial epiphytotic conditions. The negligible disease reactions in the F_2 generations and backcross of R x R cross indicated similar genes for DM resistance (allelic relationship) in genotypes. In the R x S cross, all F_{1s} were resistant that suggested dominance of resistance over susceptibility. Inheritance of downy mildew resistance appeared in digenic ratio, complementary at Delhi (9:7) and duplicate at Villupuram, Tamil Nadu (15:1). However, some modifier gene or genes were also expected to operate.

Singh and Talukdar (1998) studied inheritance of downy mildew resistance in F_1, F_2 and back cross progenies of the cross DMRP 292 (IP18292 derived) x Tift 23DB and found all F_1 plants as resistant that suggested dominant nature of resistance to downy mildew. The segregation of resistant and susceptible plants in the F_2 generation was observed in the ratio 3:1, no segregation in the back cross with resistant parents and 1:1 ratio in the test cross implied resistance to DM in DMRP 292 (IP18292 derived) controlled by a single dominant gene (*Rsg1*). However, they also speculated the

probability of two major genes for resistance in IP 18292 through Quantitative trait loci (QTL) analysis.

Joshi and Ugale (2002) carried out generation mean analysis with 11 basic generations (P_1, P_2, F_1, F_2, B_1, B_2, B_1F_1, B_2F_2, L_1, L_2 and L_3) of three crosses between six diverse line of pearl millet screened under artificial epiphytotic conditions over two environments. The F_2 segregation ratio of cross 1 at environment 1 was 15:1 and 13:3 at environment 2 suggesting the digenic nature of inheritance of resistance. Cross 2 segregated into 229:27 Mendelian ratios entailed tetragenic interactions whereas cross 3 showed trigenic interactions with 55:9 segregation and suggested that resistance is not simply inherited but controlled by several loci. They also reported the non-allellic interactions as duplicate dominant and complementary epistasis. In a few cases where clear Mendelian segregation have been observed, Hash *et al.* (2003) also reported one, two or even three dominant genes for the resistance to downy mildew in pearl millet.

Angarawai *et al*. (2008) determined the number of loci governing the nature of inheritance of DM resistance in five pearl millet Nigerian elite lines using factorial mating scheme of North Carolina Design II through six generations and estimated the involvement of loci ranging from 0.3 to 20.0. The result clearly indicated the inheritance of resistance to DM is a quantitative character in the set of parents studied.

Although in the above studies pearl millet downy mildew resistance was generally found to be dominant over susceptibility and controlled by one or more dominant genes with some modifiers or quantitative nature of resistance, a complete picture of its inheritance is not yet available.

Material and Methods

CHAPTER III

MATERIAL AND METHODS

The present investigation was carried out at International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Telangana, India during 2013-2016.

3.1 Experimental material

3.1.1 General

Common methodologies are described under this heading. The finer details wherever needed are given in the respective heads. The experiments were repeated for confirmation.

3.1.1.1 Sterilization

3.1.1.1.1 Potting

Potting mixture containing alfisol, sand and farmyard manure in a proportion of 3:2:1 (v/v/v) was sterilized in an autoclave at 121°C for 15 minutes under 1.05 kg/cm^2 pressure for 2 h/day on two consecutive days.

3.1.1.1.2 Seed

Seeds of all the host genotypes were surface-sterilized with two per cent sodium hypochlorite (NaOCI) for five minutes, washed thoroughly with sterilized distilled water and dried at room temperature (approximately 25°C) prior to sowing.

3.1.1.1.3 Glassware

Glasswares used in this study were sterilized at 160 °C for 90 minutes in a hot air oven.

3.1.1.2 Collection and maintenance of isolates

Sclerospora graminicola isolates (Table 3.1), surveyed during the field surveys (1992–2014) in major pearl millet growing states of India, were collected from Dryland Cereal Pathology lab, ICRISAT. All the isolates used in the study were maintained on pot-grown seedlings of a highly susceptible genotype 7042S through asexual generations (Table 3.1). Infected plants of each isolate were kept separately in an individual polyacrylic isolation chambers (Plate 3.1) measuring 60cm × 60cm × 90cm

Table 3.1. List of *Sclerospora graminicola* isolates collected from different pearl millet growing states of India

Identity	Location	State	Year of collection	Maintenance host
Sg 543	Aurangabad	Maharashtra	2010	7042 S
Sg 545	Aurangabad	Maharashtra	2011	7042 S
Sg 546	Tanda, Aurangabad	Maharashtra	2010	7042 S
Sg 556	Kothigaon, Banaskantha	Gujarat	2010	7042 S
Sg 557	Lodhnoor, Banaskantha	Gujarat	2010	7042 S
Sg 558	Gagana, Banaskantha	Gujarat	2010	7042 S
Sg 559	Jamdi, Banaskantha	Gujarat	2010	7042 S
Sg 560	SK Nagar, Banaskantha	Gujarat	2010	7042 S
Sg 561	IARI	New Delhi	2010	ICMP 451
Sg 562	Jaipur	Rajasthan	2012	7042 S
Sg 564	Sikar	Rajasthan	2012	7042 S
Sg 566	Sikar	Rajasthan	2012	7042 S
Sg 569	Aligarh	Uttar Pradesh	2012	7042 S
Sg 571	Bulandshasar	Uttar Pradesh	2012	7042 S
Sg 573	Sambhal	Uttar Pradesh	2012	7042 S
Sg 574	Sambal	Uttar Pradesh	2012	7042 S
Sg 575	Baduan	Uttar Pradesh	2012	7042 S
Sg 576	Jodhpur	Rajasthan	2012	7042 S
Sg 578	Kheda	Gujarat	2012	7042 S
Sg 580	Aanand	Gujarat	2012	7042 S
Sg 585	Kheda	Gujarat	2012	7042 S
Sg 586	Jaisalmer	Rajasthan	2013	7042 S
Sg 587	Jaisalmer	Rajasthan	2013	7042 S
Sg 589	Jaisalmer	Rajasthan	2013	7042 S
Sg 590	Jaisalmer	Rajasthan	2013	7042 S
Sg 591	Jaisalmer	Rajasthan	2013	7042 S
Sg 592	Durgapura	Rajasthan	2014	7042 S
Sg 593	Agra	Uttar Pradesh	2014	7042 S
Sg 594	Aligarh	Uttar Pradesh	2014	7042 S
Sg 595	Aligarh	Uttar Pradesh	2014	7042 S
Sg 596	Bulandshasar	Uttar Pradesh	2014	7042 S
Sg 598	Sambhal	Uttar Pradesh	2014	7042 S
Sg 599	Sambhal	Uttar Pradesh	2014	7042 S
Sg 603	Hathras	Uttar Pradesh	2014	7042 S
Sg 604	Hathras	Uttar Pradesh	2014	7042 S
Sg 048	Mysore	Karnataka	1994	852 B
Sg 151	Durgapura	Rajasthan	1997	Nokha Local
Sg 153	Patancheru	Telangana	1997	843 B

Table 3.1(Cont.)

Identity	Location	State	Year of collection	Maintenance host
Sg 597	Sambhal	Uttar Pradesh	2014	7042 S
Sg 601	Baduan	Uttar Pradesh	2014	7042 S
Sg 018	Patancheru	Telangana	1992	7042 S
Sg 139	Jodhpur	Rajasthan	1997	Nokha Local
Sg 409	Patancheru	Telangana	2004	PMB 11571-2
Sg 431	Patancheru	Telangana	2005	7042 S
Sg 510	Baduan	Uttar Pradesh	2008	7042 S
Sg 521	Rewari	Haryana	2009	7042 S
Sg 526	Jodhpur	Rajasthan	2009	7042 S
Sg 529	CAZRI,Jodhpur	Rajasthan	2009	7042 S
Sg 019	Patancheru	Telangana	1992	7042 S
Sg 021	Ahmednagar	Maharashtra	1993	7042 S
Sg 150	Jalna	Maharashtra	1997	834 B
Sg 200	jamnagar	Gujrat	1998	ICMP 451
Sg 212	Durgapura	Rajasthan	1998	ICMP 451
Sg 298	IARI	New Delhi	1999	W 504-1-1
Sg 384	Barmer	Rajasthan	2003	ICMP 451
Sg 445	Banaskantha	Gujrat	2005	Pioneer 7777
Sg 457	Sujnapur, jaipur	Rajasthan	2006	ICMP 451
Sg 492	Iglas	Uttar Pradesh	2007	ICMP 451
Sg 519	Rewari	Haryana	2009	7042 S
Sg 542	Aurangabad	Maharashtra	2010	7042 S

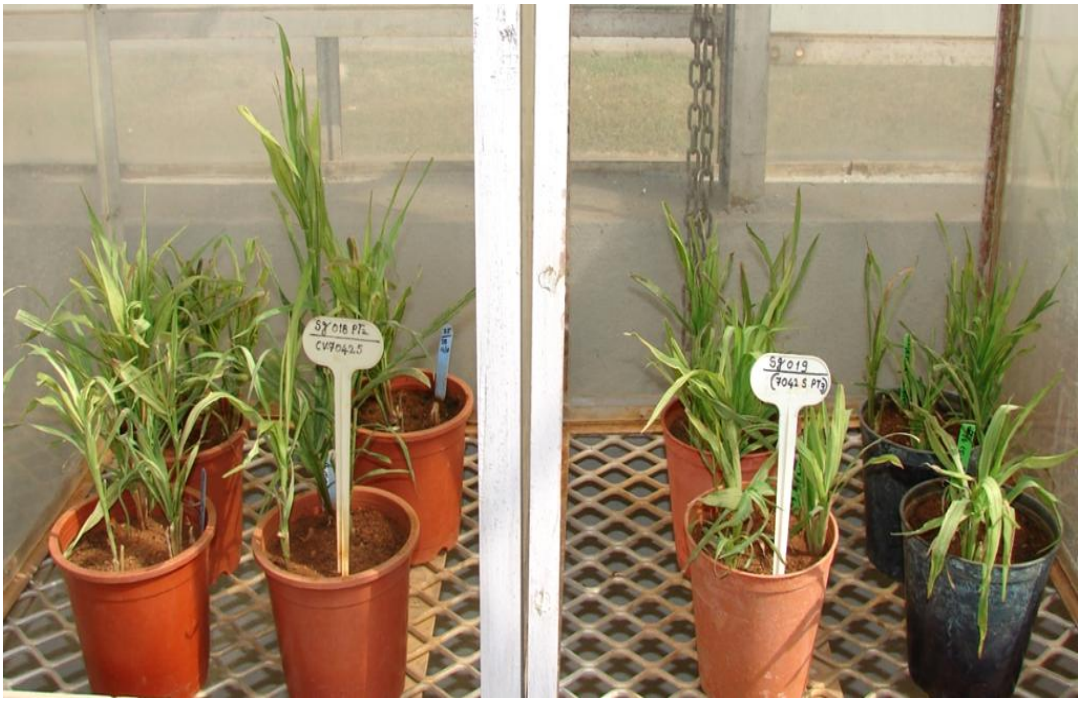


Plate 3.1. A polyacrylic isolation chamber with downy mildew infected pearl millet plants of 7042S inside in a green house at 25±2ºC at ICRISAT, Patancheru

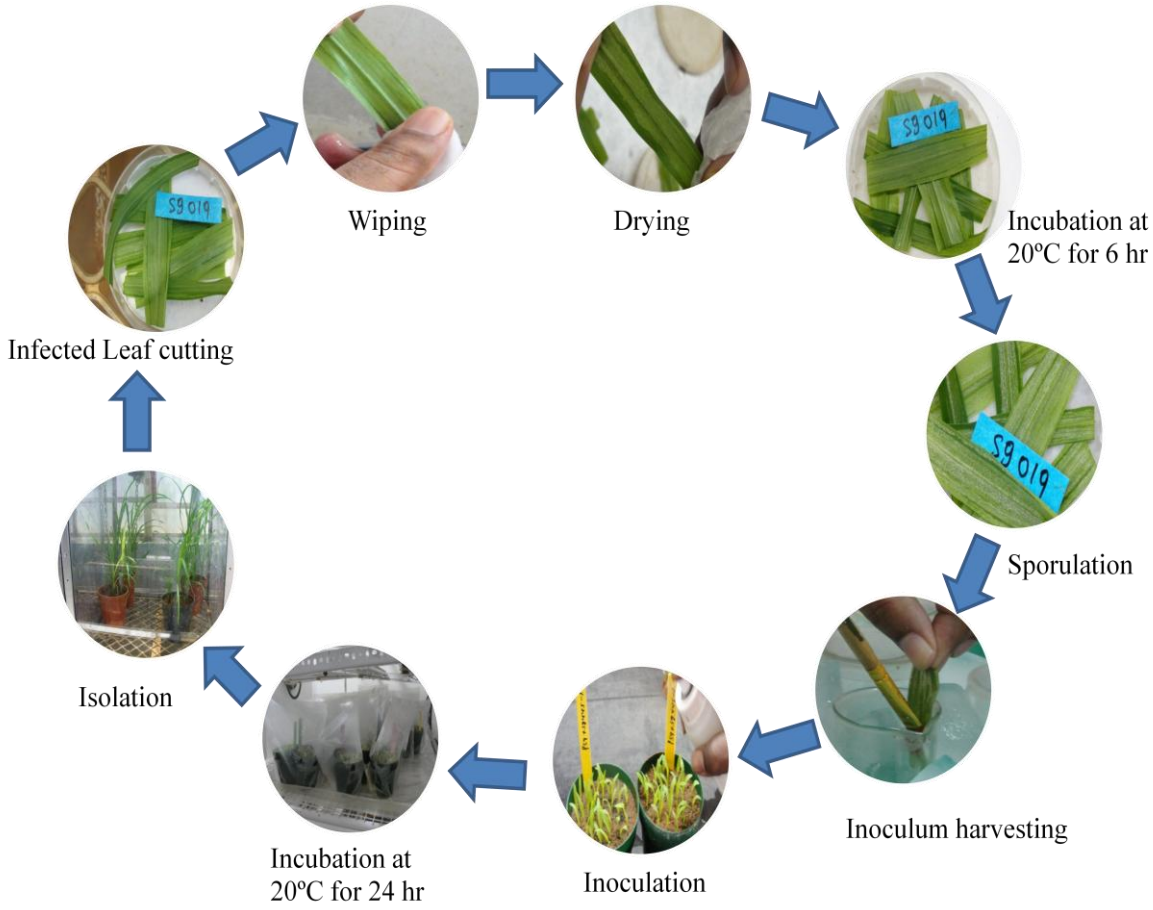


Plate 3.2. Schematic representation of maintenance of isolates of *Sclerospora graminicola* at ICRISAT, Patancheru under glass house conditions

in a greenhouse at 25± 2°C. Each isolate was inoculated onto a fresh set of 7042S seedlings, once a month, with sporangia from the previous generation (Plate 3.2). Plants were kept free from insect pests and other diseases and were adequately fertilized and watered. The sporangial inocula for inoculations were collected from these systemically infected plants.

3.1.1.3 Preparation of inoculum

Infected leaves from individual isolates were collected, excised into pieces and washed in running tap water, using a cotton swab to remove old downy growth from the leaf surface. These leaf pieces were wiped dry with tissue paper and placed with their abaxial surfaces up in plastic-tray humidity chambers lined with moist blotting paper. The humidity chambers were incubated in dark at 20°C for 6 h. The incubator was programmed in such a way that the temperature was reduced to 2°C after 6 h of incubation in order to prevent the release of zoospores from mature sporangia until the sporangial collection next day. Sporangia from sporulating leaves were harvested into ice-cold (4°C) distilled sterilized water, separately for each isolate, using a soft camel hair brush. The sporangial suspension was filtered through a double layered muslin cloth to remove leaf pieces and other particles. The concentration of sporangia was measured using a haemocytometer and adjusted to a desired concentration (1×10^6 ml^{-1}).

3.1.1.4 Inoculation technique

Potted seedlings were spray-inoculated with sporangial suspension at the coleoptile to first-leaf stage using an atomizer in the inoculation chamber and covered immediately with moist polyethylene sheet to provide >95% relative humidity necessary for infection. Inoculated seedlings were incubated in the dark at 20°C for 24 h. The pots were then transferred to greenhouse bench under mist where temperature was maintained at 25±2°C.

3.2. Development and validation of SSRs

3.2.1 Pathogen isolates

Sclerospora graminicola isolates (Table 3.1) collected during the field surveys (1992–2014) in major pearl millet growing states of India, being maintained separately under controlled conditions through asexual progenies either on the same host from

which they were collected or on an universally susceptible genotype 7042S, were used in the present study (Thakur *et al*., 1998b).

3.2.2 DNA extraction

The DNA was extracted by modifying the procedures described by Sastry *et al.* (1995). The modified protocol was as follows: the sporangia of *S. graminicola* from sporulating leaves were harvested into ice-cold ethanol and centrifuged at 4000 rpm in Eppendorf centrifuge 541 7C. The supernatant was decanted and 800 µl of CTAB buffer (0.5 M Tris-HCI, pH 8.0, 0.5 M EDTA, pH 8.0 and 3% CTAB) was added to the pellet. After gentle mixing with pipette the contents were incubated at 65^{o}C for one hour. After cooling the contents to room temperature, an equal volume of chloroform: phenol (1:1) was added to the slurry and mixed gently. The content was centrifuged at 10000 rpm, for 10 minutes. The aqueous phase was removed and an equal volume of chloroform: Isoamyl alcohol (24:1) was added, mixed well and centrifuged for 10 minutes at 10000 rpm. The aqueous phase was removed and DNA was precipitated by adding 0.6 volume of isopropanol. The product was incubated for 15 minutes at -20 ^{o}C and centrifuged at 5000 rpm for five minutes. The supernatant was discarded and pellet was washed twice with 70% ethanol and suspended in 50 µl of T_{50} E_{10} buffer (50mM Tris-HCl, 10 mM EDTA, pH 8.0). The aliquot was treated with 50 µl /ml of RNase and incubated at 37^{o}C for overnight. An equal volume of chloroform: phenol was added to the solution, mixed well by gentle pippeting and centrifuged at 5000 rpm for five minutes. The aqueous phase was transferred to another tube and DNA was precipitated by adding 15 µl of 3 M sodium acetate and 2.5 volume of chilled absolute ethanol. The aliquot was centrifuged at 5000 rpm for five minutes and aqueous phase was removed. The DNA pellet was washed twice with 70% ethanol, dried at room temperature and dissolved in $T_{10}E_1$ (10 mM Tris-HCI, pH 8.0, 1 mM EDTA, pH 8.0). The qualitative and quantitative analysis of DNA was done by gel electrophoresis on 0.8% agarose and by using a NanoDrop spectrophotometer (Thermo SCIENTIFIC NANODROP 8000). The purified DNA was standardized at 12.5 ng/ µl and stored at -20^{0}C.

3.2.3 Mining of SSRs

The assembled scaffolds sequences of *S. graminicola* isolate Sg 445-1 was used for mining of SSRs through Perl script MISA (MIcroSAtellite searching tool; Thiel *et al*., 2003) to screen perfect and compound microsatellites. Criteria to screen microsatellite was set as per Khera *et al.* (2015) i.e mono-nucleotide repeat being repeated 10 or more time; di-nucleotide repeat being repeated 6 or more times; similarly

tri- to hexa-nucleotide repeats being repeated 5 times or more. The maximum size of interruption allowed between two successive microsatellites in compound microsatellites was 100 bp.

3.2.4 Designing of primers

The predicted SSRs were subjected to primer design using primer3 standalone software (http://bioinfo.ut.ee/primer3/). Here, a novel scheme to reduce the search space was introduced as follows - Instead of searching the entire genome space for designing primers for flanking region of SSRs, only the flanking 200 bp regions of the SSRs on either side was extracted using custom scripts. This was used as input for primer3 to drastically reduce the search space and time. The pseudo-coordinates of the primers designed were then replaced with the original genome coordinates using custom scripts. Primer3 was run with default parameters as: 20-80 G/C %, annealing temperature between 57-63^{0}C, amplicon size 200-450 bp, and primer length 18-27 bp. Out of 2702 primers, 106 were selected on the basis of the following criteria: (1) more than 3 bases of GC rich (GCC, CGG, CCG, CCC, CGG, GCG, GGG, GGC) at the 3$^{'}$ end was eliminated; (2) 3$^{'}$ end containing either G or C was selected; (3) poly AAAAA, TTTTT, GGGGG, CCCCC were also removed. The primers were custom synthesized by Eurofins Genomics Pvt Ltd (Bangalore, India).

3.2.5 PCR amplification and gel electrophoresis

Standard PCR reactions were carried out in a volume of 20 µl, containing 2 µl of 10X buffer (plus 1.5mM Mg^{2+}), 1 µl of forward primer (1.4 pmol/µl), 1 µl of reverse primer (7.1 pmol/µl), 2 µl of 0.2 mM dNTP, 2.5 units of DNA polymerase (KAPA BIOSYSTEMS) and 1 µl of 12.5 ng of genomic DNA template. DNA amplification was accomplished in a PCR system (Thermal cycler: BIO-RAD T100^{TM}) with an initial denaturation at 94 °C for 1 min, followed by 40 cycles each of 1 min at 94 °C, 30 s at 62 °C, and 1 min at 72 °C, followed by a 10 min final extension at 72 °C. Amplification was primarily tested for four isolates *viz*., Sg 019, Sg 200-1, Sg 445 and Sg 457. Each PCR products were run on 1.2% agarose gel at 90 V for a quality check. Subsequently, the amplification was carried out using DNA of 12 diverse isolates (Sg 019, Sg 021, Sg 150, Sg 200-1, Sg 212, Sg 298, Sg 384, Sg 445, Sg 457, Sg 492, Sg 519 and Sg 542-1) to test the polymorphism. Further, the polymorphic primers were screened on 60 pathotypes for diversity analysis. PCR products were separated on 3% MetaPhor^{TM} Agarose (Lonza, USA).

3.2.6 Cross-genus transferability

The primer pairs developed for *Sclerospora graminicola* were tested for cross-genus transferability on the *Pernosclerospora sorghi* and *Phytophthora infestans* isolates using the identical PCR setup as described earlier for *S. graminicola*.

3.3 Establishment of F_1 progenies from a cross between avirulent and virulent isolates of *S. graminicola*

3.3.1 Selection of self sterile isolate based on oospore formation

A total 52 isolates of *Sclerospora graminicola* collected from different pearl millet growing areas of India were collected form Cereal Pathology Lab, ICRISAT, India and maintained on susceptible cultivar 7042S in the isolation chambers as described in 3.1.1.2. To identify self sterile (heterothallic) isolates, infected leaves of each isolate were allowed to mature for the formation of oospores. Since, isolates were in isolation chambers, oospore formation could take place only in homothallic isolates having both the mating types. Necrotic leaf pieces collected from two month old seedlings infected by each isolate were dried under shade in brown paper bags and stored at room temperature (25±2°C) until observation. Leaf pieces (1 cm^2) were surface sterilized with NaOCl (2%) and washed thoroughly with sterilized distilled water. These leaf pieces were cleared by incubating them at 40ºC in NaOH (5%) for 12 to 16 h. Cleared leaf pieces were rinsed in distilled water and observed under a microscope using a 10× objective for the presence of oospores. Isolates which did not show oospore formation were selected as self sterile isolates for further studies.

3.3.2 Evaluation of virulent/avirulent reactions of self sterile isolate

To identify virulent and avirulent isolates with different mating types, out of 52, 33 self sterile, heterothallic isolates, including reference mating type isolates Sg 018 (*Mat-1*) and Sg 019 (*Mat-2*), being maintained at ICRISAT were tested for virulent/avirulent reactions on P 7-4, P 310-17, 700651, 7042R, IP 18292, IP 18293 and 852B and two known downy mildew (DM) susceptible lines - ICMP 451 and 7042S. Sporangial inocula of the 33 isolates were raised on seedlings of a highly susceptible genotype 7042S in isolation chambers in the greenhouse. Sporangial suspensions for all the isolates were prepared described in 3.1.1.3 and were adjusted to concentration 1×10^6 ml^{-1}. Pot-grown seedlings of the pearl millet lines P 7-4, P 310-17, 700651, 7042R, IP 18292, IP 18293 and 852B and two known DM susceptible lines - ICMP 451 and 7042S were spray-inoculated at coleoptile stage using an atomizer. The inoculated

seedlings were incubated at 20ºC with >90% RH for 24 h, and then transferred to greenhouse benches at 25±2°C and >95% RH for disease development for the next 2 weeks as detailed in 3.2.1.4. DM incidence was recorded 14 days after inoculation as per cent infected plants. The genotypes with ≤10% incidence were considered resistant and those with >50% disease incidence as susceptible to a particular isolate (based on reaction type Sg 018 and Sg 019 were selected as avirulent and Sg 445 was selected as virulent isolates for crossing to generate segregating populations.

3.3.3. Confirmation of mating type of test isolate (Sg 445-1)

Reference isolates of *S. graminicola* of different mating types Sg 018 (*Mat*-1) and Sg 019 (*Mat*-2) and test isolates Sg 445-1 (single zoospore selection from Sg 445) were maintained on 7042S. To detect mating type of the test isolate, Sg 445-1 was crossed with both the reference mating type isolates (Sg 018 × Sg 445-1), and (Sg 019 × Sg 445-1). Sporangial inoculum of each isolate (1×10^6 sporangia ml^{-1}) was prepared separately in ice-cold distilled sterile water. Sporangial suspensions of Sg 018 and Sg 445-1, and Sg 019 and Sg 445-1 was mixed in equal proportion (1:1) and spray inoculated on the highly susceptible pearl millet line 7042S separately. The inoculated seedlings were incubated and transferred to isolation chambers as described in 3.1.1.4. The infected seedlings were maintained in the isolation chambers and allowed to mature. The necrotic tissues from these infected seedlings (>2 month old) were observed for oospore formation as described in 3.3.2.

3.3.5 Establishment of F_1 progenies form oospores generated from Sg 019×Sg 445-1 crosses

As the cross between Sg 019 and Sg 445-1 resulted in oospores production (absent in Sg 018 × Sg 445-1), plants inoculated with Sg 019 × Sg 445-1 were used to generate segregating populations. For this purpose, the matured infected leaf samples from the plants inoculated with cross Sg 019 × Sg 445-1 were collected, dried in shade, ground and strained to make fine powder containing F_1 oospores. This leaf powder was again checked for the presence of oospores Sterilized potting mixture (soil, sand, and farmyard manure in a ratio of 3:2:2 by volume) was prepared as described in 3.2.1.1 and infested with oospore inoculum as described by Pushpavathi *et al*. (2006b). The pots (15-cm diameter) containing infested mixture were sown with a susceptible genotype 7042S (25 seed per pot). Each pot was covered with a polythene bag and incubated at 40 °C for 3-4 days for rapid seed germination. Pots were transferred to polyacrylic

isolation chambers in a greenhouse at 25 ± 2 °C to avoid cross contamination from other isolates. Pots were watered adequately everyday and observed regularly for DM symptoms on the seedlings. As the first infected seedling in a pot was noticed, it was removed from the pot and was transplanted in another pots containing sterilized soil and shifted to isolation chambers.Since infected seedlings occurred infrequently and rarely, each infected seedling was assumed to have infection from a single oospore. Sporangia from each infected seedling were maintained separately on 7042S as an individual F_1-progeny in isolation polyacrylic chambers measuring 60 cm × 45 cm × 45 cm in a greenhouse at 25± 2°C (Plate 3.3).

3.4 Inheritance and molecular mapping of avirulence genes in *S. graminicola*

3.4.1 Phenotyping of mapping populations

3.4.1.1 Selection of differential lines

To know the differential reaction of F_1 progenies, ten pearl millet lines *viz.*, P 7-4, J 2480, ICMB 07111, ICMB 09999, ICMB 12444, 852 B, ICMB 01333, IP 18292, ICML 22 and 81 B P-6 (Table 3.2) were screened against the parents of F_1 progenies i.e Sg 019 and Sg 445-1. The lines showing differential reactions to Sg 019 and Sg 445-1 were selected for further screening against F_1 progenies.

3.4.1.2 Evaluation of F_1 progenies

Sporangial inocula of the 121 F_1 progenies and parents Sg 445-1 and Sg 019 were raised on seedlings of a highly susceptible genotype 7042S in isolation chambers in the greenhouse as detailed in 3.1.1.2. Sporangial inocula from all progenies and parents were prepared and spore concentration was adjusted to 1×10^6 ml^{-1} as described in 3.1.1.3. Pot-grown seedlings of all five selected differential lines *viz.*, 852 B, ICMB 01333, IP 18292, ICML 22 and 81 B P-6 and a susceptible check 7042S were spray-inoculated at coleoptile stage using an atomizer as described in 3.1.1.4. The inoculated seedlings were incubated at 20ºC temperature with >90% RH for 24 h, and then transferred to greenhouse benches at 25±2°C and >95% RH for disease development for the next 2 weeks. DM incidence was recorded 14 days after inoculation as per cent infected plants. Based on disease incidence (DI), F_1-progenies were classified into two phenotypic groups as: virulence reaction type (≥10.0% DI) and avirulence reaction type (≤10.0% DI) and the data obtained were used to study inheritance of avirulence in *S. graminicola* (Plate 3.4).

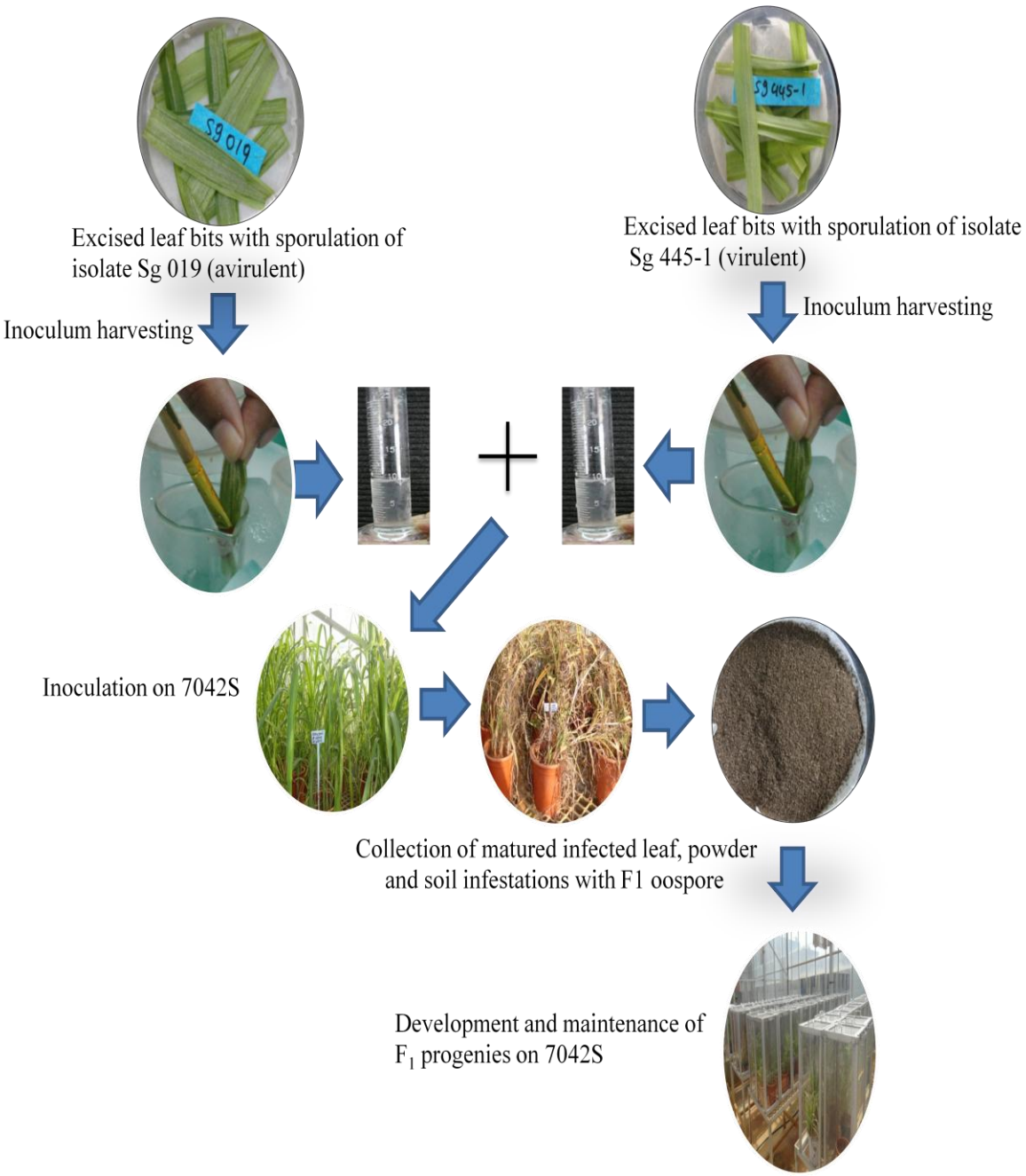


Plate 3.3. Schematic representation of development of F_1 progenies from cross of Sg 019 x Sg 445-1

Table 3.2. List of pearl millet differential lines used for phenotyping of F_1 progenies

Line	Derived from/Pedigree	Origin	References	Sg 019	Sg 445-1
P 7-4	IP 6118	Nigeria	Thakur *et al*. (1997)	A	*
J 2480	-	India	Chaudhary *et al*. (2012)	V	V
ICML 22 [a]	IP 2696	Chad	Singh *et al*. (1994)	A	V
81 B P-6[b]	Tift 23 D2B1	ICRISAT	Anand Kumar *et al*. (1984)	A	V
852 B [c]	MC 103 × Serere 17B-12-2)-1	India	Thakur *et al*. (2004)	A	V
ICMB 01333	HHV-S1-64-3-2-3-2-1	ICRISAT	Thakur *et al*. (2009)	A	V
ICMB 07111	(ICMB 96111× 4038-4-4-B)-2-2-5-4	ICRISAT	Rai *et al*. (2014)	A	A
ICMB 09999	(81 BX 4025-3-3-B)-8-1-B	ICRISAT	Rai *et al*. (2014)	A	A
ICMB 12444	(ICMB 95444× ICMB 93333)-24-2-B-4-B-B-B	ICRISAT	Rai *et al*. (2014)	A	*
IP 18292[d]	D2WS.GL. Yellow	ICRISAT	Thakur *et al*. (2004)	A	V
7042S	IP 2696	India	Thakur *et al*. (2004)	V	V

*Intermediate reaction, - unknown [a] ICML 22 (7042 R), developed through pure line selection from IP 2696 from chad. [b] downy mildew resistant selection from gamma radiation treated Tift 23 D2B1. [c]852B = (MC 103XSerere 17B-12-2)-1, MC 103 (Maiwa Composite) from Nigeria and Serere 17B-12-2 from Uganda. [d]IP 18292 (D2WS GL Yellow) Dwarf White Sheath Glossy Yellow line derived from a multiple cross involving dwarfing gene d2d2 from ifton (USA), the White Sheath trait gene wsws from IP 7626 (India), and the glossy trait gene gl1gl1 from IP 8275 (India).

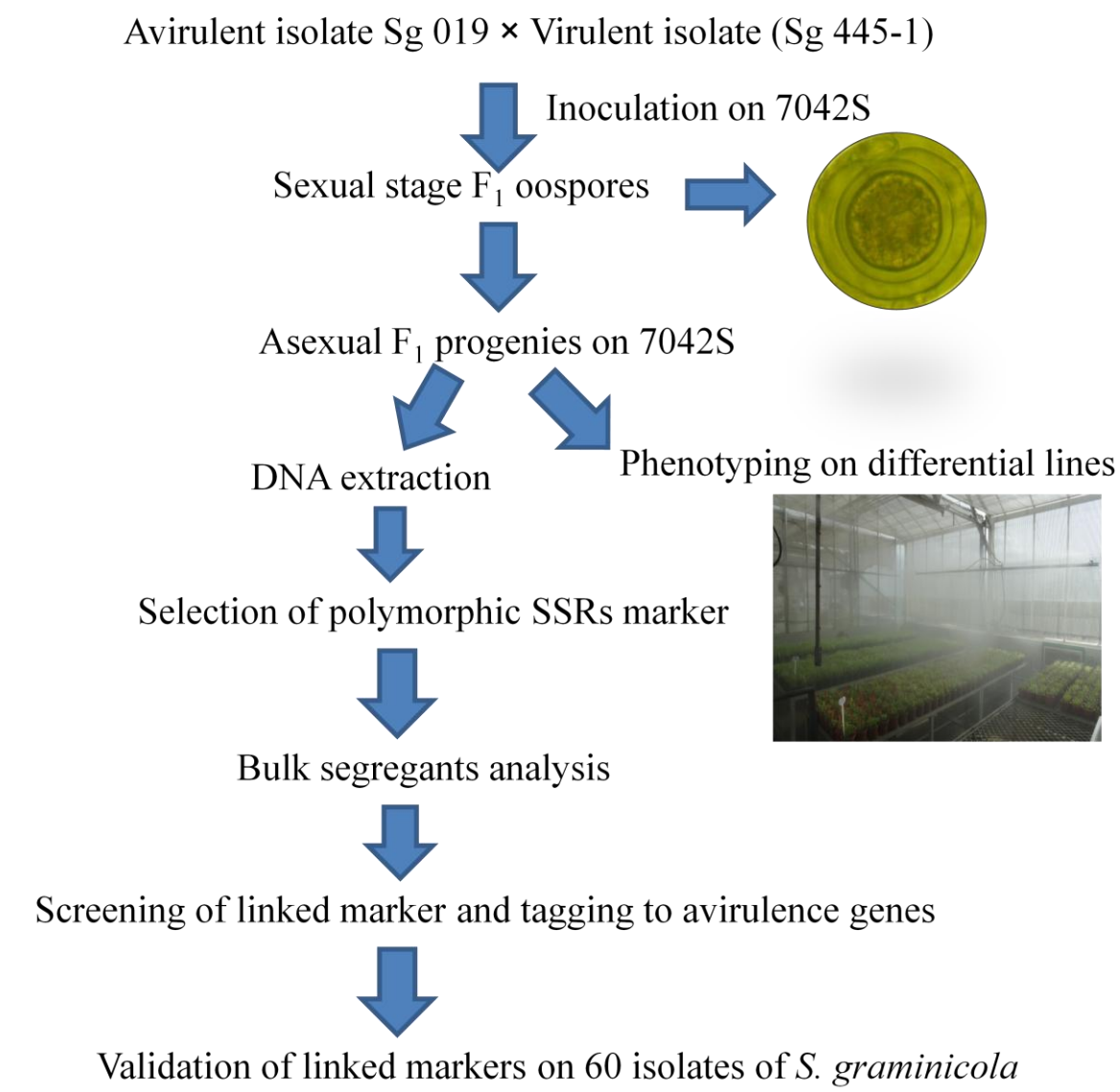


Plate 3.4. Schematic representation of inheritance of avirulence and gene tagging on F_1 progenies from cross of Sg 019 x Sg 445-1

3.4.2 Identification of markers linked to avirulence gene (*Avr*)

3.5.2.1 DNA extraction

The genomic DNA of all 121 F_1-progenies and Sg 019 and Sg 445-1 were extracted in pure form as described in 3.2.2. The qualitative and quantitative analysis of DNA was done by gel electrophoresis on 0.8% agarose and by using a NanoDrop spectrophotometer (Thermo SCIENTIFIC NANODROP 8000). The purified DNA was standardized at 12.5 ng/ul and stored at -20^0C.

3.4.2.2 Selection of polymorphic marker between parents Sg 019 and Sg 445-1

A total of 106 primers were screened against avirulent parent (Sg 019) and virulent parent (Sg 445-1) and based on results of gel electrophoresis in 3.2.5, 18 polymorphic SSRs markers between Sg 019 and Sg 445-1 (Sg_SSR 01, Sg_SSR 07, Sg_SSR 09, Sg_SSR 13, Sg_SSR 38, Sg_SSR 45, Sg_SSR 46, Sg_SSR 47, Sg_SSR 49, Sg_SSR 65, Sg_SSR 68, Sg_SSR 92, Sg_SSR 93, Sg_SSR 95, Sg_SSR 98, Sg_SSR 99, Sg_SSR 102 and Sg_SSR 103) were selected for bulk segregants analysis (Table 3.3).

3.4.2.3 Bulk segregants analysis (BSA)

BSA was carried out by modifying the protocol of Michelmore *et al*. (1991). Four groups were formulated based on result of phenotyping of F_{1s} on four differential lines (852 B, ICMB 01333, IP 18292 and ICML 22); Bulk 1 (P 2, P 3, P 4, P 5, P 6, P 7, P 8, P 9, P 13, P 14, P 15, P 19, P 21 and P 30), avirulent on all differential lines; Bulk 2 (P18, P 20, P 60 and P 111) virulent on all differential lines; Bulk 3 (P 51, P81, P83, P 84, P 86, P 88, P89 and P90) virulent on 852 B, ICMB 01333 and ICML 22 but not on IP 18292; Bulk 4 (P 26, P 27, P 28, P 29, P 31, P 32, P 33, P 34, P 35, P 36, P 37, P 38, P 41, P 44, and P 47) virulent on ICMB 01333, ICML 22 and IP 18292 but not on 852 B. Aliquots (12.5 ng/µl) of each isolates in a particular group were pooled together in equal quantity to make four bulks and then screened with selected 18 polymorphic SSRs.

3.4.2.4 PCR amplification and gel electrophoresis

PCR reactions and gel electrophoresis were performed as described in 3.2.5.

Table 3.3. List of polymorphic SSRs markers between Sg 019 and Sg 445-1

Marker	Repeats	FORWARD PRIMER1 (5'-3')	REVERSE PRIMER
Sg_SSR 01	$(TAT)_7$	TCCGCGTACCCTGATTTAGC	CGGAAGCGTTTGGTCATACG
Sg_SSR 07	$(TAA)_{25}$	TTAGCGGAAGGAGAACCACG	TTCGTCTCCCGGGTAAAAGC
Sg_SSR 09	$(TTG)_5$	GGACATAACCCTCCCAGTGC	GAGAGAGGGAACTACGCACG
Sg_SSR 13	$(TCC)_6$	TCGATTTCATCCCCGCTAGC	AGTGAAGCTATCGTGCTCCG
Sg_SSR 38	$(TAC)_8$	TTCGATCAACCTCCAGCACC	TGCGTCCTTACCGCTAAAGG
Sg_SSR 45	$(TCT)_9$	TTTGGCGTTTTCGTGTTGGG	CATCCATCTCTCTCGCCTCC
Sg_SSR 46	$(GAA)_5$	CCTACCCCTGGAAAAGCTGG	GATACGATGCTCCACGGAGG
Sg_SSR 47	$(GAA)_5$	TCGGAAGCTGTATTCGACCG	CACGCTCCAGATCGGTATCC
Sg_SSR 49	$(TGC)_5$	AAACGTGGCTGGGTATCTGG	TGACCCTCGATTTGTGGACG
Sg_SSR 65	$(TTC)_6$	CCTTCTGTGATAGGCCGTCC	CTATCCAGGTCCGGATTGGC
Sg_SSR 68	$(TCT)_5$	CTCTTTGTTGCAACCACGGG	CCAGCAGCTGATAGGGATGG
Sg_SSR 92	$(ATAGT)_6$	AAGTCCCCACCATGTCGAAC	CTCCTCGACCTCGTATTCGC
Sg_SSR 93	$(TATAG)_{13}$	CGACCTAGTTGAATGCAGCG	GCGACGGTCATGAGCAAATC
Sg_SSR 95	$(ATAGT)_6$	AAGTCCCCACCATGTCGAAC	CTCCTCGACCTCGTATTCGC
Sg_SSR 98	$(TTGTTA)_5$	TCGACGGCAGTATGTTACGG	TCTTCATCGCCCAGTTCGTG
Sg_SSR 99	$(ATAGTA)_7$	GCTTCACGCTTGTTGCTGAC	CGCAAGCCTACTACCACCTC
Sg_SSR 102	$(TAG)_8(TA)_6$	ACCTGACATTCCAGCCGATG	GCCTGTAAATGGTGTAGAAGCC
Sg_SSR 103	$(TAC)_5at(CTA)_{11}$	ACTGAGCCAAGTTACCGACG	AAGGAAGCTACGGCCAAGTC

3.4.2.5 Validation of marker

In order to determine the diagnostic value of avirulence linked microsatellite marker for the purpose of marker assisted selection, a set of 60 isolates of *S. graminicola*, collected from different pearl millet growing regions of India, were raised on seedlings of a highly susceptible genotype 7042S in isolation chambers in the greenhouse. Sporangia from sporulating leaves of each isolate were harvested in ice-cold distilled sterile water and spore concentration was adjusted to 1×10^6 sporangia ml^{-1}. Pot-grown seedlings of IP 18292 and a susceptible check 7042S were spray-inoculated at coleoptile stage using an atomizer as discussed in 3.4.1.2. The inoculated seedlings were incubated at 20ºC temperature and >90% RH for 24 h, and then transferred to greenhouse benches at 25±2°C and >95% RH for disease development for the next 2 weeks. DM incidence was recorded 14 days after inoculation as per cent infected plants. Based on disease incidence (DI), isolates were classified into two phenotypic groups as: virulent reaction type (≥10.0% DI) and avirulence reaction type (≤10.0% DI). To verify marker trait association, the 60 isolates of *S. graminicola* were screened with linked marker Sg_SSR 49. Based on the allele fragment sizes of parent Sg 019 (avirulent) and Sg 445-1 (virulent) in 3.4.2.4, the genotyping data of 60 isolates were scored as: Allele 1 (130 bp) and Allele 2 (120 bp). The amplified fragment size of 130 bp (Allele 1) was considered associated with the presence of avirulent gene that was shown avirulence reaction on IP 18292.

3.5 Inheritance of DM resistance in pearl millet genotypes and allelic relationships among the resistance genes

3.5.1 Selection of pearl millet genotypes and pathogen isolates

Seeds of pearl millet genotypes were taken from genetic stocks maintained at Cereal Pathology Lab, ICRISAT, Patancheru. To select resistant and susceptible lines for the inheritance of DM resistance in pearl millet genotypes and allelic relationships among resistance genes, pearl millet lines were screened with three isolates of *S. graminicola* (Table 3.4). Based on disease incidence (DI) (Table 3.4), 834 B, IP 18294-P1 (selection from IP 18294) and IP 18298-P1 (selection from IP 18298) were selected as resistant (≤10.0% DI) and 81B as susceptible parents (≥85.0% DI) to generate crosses for inheritance and allelism study. To facilitate the selection of single plant derivatives with complete resistance and complete susceptibility, the process of selfing and single plant propagation was done to reveal such hidden variability. In order that

the gene(s) conferring residual variability for resistance to DM from the susceptible parent does not interfere with the segregation pattern of resistance genes from the resistant parent, and vice versa, creation of homozygosity in both parents was considered absolutely necessary. Therefore, selected lines were selfed for three consecutive generations to obtain true pure inbred lines. Since, pearl millet is a protogynous species with a large number of small florets on its inflorescences, chances of self pollination of some florets, in the absence of emasculation for crossing, do exist. Uses of morphological markers were important factors in ensuring 100% crossing in this study. Characteristics of selected lines are summarized in Table 3.5.

The use of a single-spore culture is highly recommended for inheritance and allelism studies. To conduct the inheritance and allelism study, three single zoosporic *S. graminicola* isolates *viz*., Sg 200-1, Sg 526-1 and Sg 542-1 were collected from Cereal Pathology Lab, ICRISAT Patancheru. Selection of pathogen isolates were done based on the disease reactions data as: virulence reaction type (≥10.0% DI) on 81B and avirulence reaction type (≤10.0% DI) on 834 B, IP 18294-P1 and IP 18298-P1 (Table 3.4). The isolates *viz.,* Sg 200-1, Sg 526-1 and Sg 542-1 selected for inheritance and allelism study were maintained in isolation chambers as described in 3.1.1.2.

3.5.2 Generation of progenies segregating for DM resistance

Staggered sowings of lines were carried out to get synchronization in flowering time. The susceptible plants of 81B were recovered by spraying Ridomil MZ 72 WP (2g/l) and used as susceptible parent. For inheritance study, three F_{1s} (81B × 834B, 81B × IP 18294-P1, 81B × IP 18298-P1) were generated by crossing susceptible line as female, with each three resistant lines as male. To carry out allelism study, three F_{1s} (834B × IP 18294-P1, 834B × IP 18298-P1 and IP 18294-P1 × IP 18298-P1) were produced by crossing resistant lines with each other in diallele fashion without reciprocal crosses during March- June 2014. All crosses were made in the greenhouse. Specific characteristics related to seed (color and size) of the parents (Table 3.5) were used to assure the hybrid nature of F_{1s}. In the subsequent post-rainy season during November- February 2014, each F_1 (8-10 panicles) was selfed using parchment paper bags for the production of F_2 seeds. To develop BC_1P_1, back cross progenies with susceptible parent, [(81B × (81B × 834B), 81B × (81B × IP 18294-P1) and 81B × (81B × IP 18298-P1)] and BC_1P_2, back cross progenies with resistant parent, [(834B × (81B × 834B), IP 18294-P1 × (81B × IP 18294-P1) and IP 18298-P1 × (81B × IP 18298-P1)],

single head pollen of each F_1 was used to pollinate the corresponding susceptible and resistant parents, respectively (Plate 3.5 and Plate 3.6).

3.5.3 Inoculum preparation

To inoculate all parents, F_{1s}, BC_1P_{1s}, BC_1P_{2s} and F_2 populations, all three isolates (Sg 200-1, Sg 526-1 and Sg 542-1) were raised on seedlings of a highly susceptible genotype 7042S in isolation chambers in the greenhouse. The systemically infected leaves were collected, excised into pieces, washed under running tap water to remove old sporangia, wiped dry with tissue paper, placed with their abaxial surfaces up in humidity chambers lined with moist blotting paper and were incubated in dark to induce sporulation at 20°C for 6 h. Sporangia from sporulating leaves of each isolate were harvested, separately in ice-cold distilled sterile water and sporangial concentration was adjusted to 1×10^6 ml^{-1} as discussed in 3.1.1.3.

3.5.4 Sowing, inoculation and disease evaluation for inheritance study

The experiment was carried out in glasshouse in two replications in Complete Randomized Design (CRD). In each replication, for inheritance study, three pots of the parents and F_{1s}, five pots each of both BC_1P_{1s} and BC_1P_{2s} and 15 pots of F_{2s} were planted for each cross. Whereas, for allelism study, three pots of the parents and F_{1s} and 15 pots of F_{2s} were planted for each cross. Seeds were sown in 15-cm diameter pots (30-35 seeds/pot) filled with sterilized soil-sand-FYM mix (2:1:1) and placed in a greenhouse bay maintained at 30±1ºC. The 48 hours pot-grown seedlings of all parents, six F_{1s} (three S × R and three R × R), three BC_1P_{1s}, three BC_1P_{2s} and six F_2 (three S x R and three R x R) populations were spray-inoculated as mentioned in 3.1.1.4 with an aqueous sporangial suspension (ca. 1×10^6 ml^{-1}) of all three isolates (Sg 200-1, Sg 526-1 and Sg 542-1) of *S. graminicola* using an atomizer and covered immediately with moist polyethylene sheet to provide >95% relative humidity. Inoculated seedlings were incubated in the dark at 20°C for 24 h and then transferred onto greenhouse bench at 25±2°C. Observations of individual seedlings were recorded 14 days after inoculation as diseased or healthy as reported by Sharma *et al*. (2015).

Table 3.4. Disease reaction of pearl millet lines to different isolates of *S. graminicola*

Entry	Identity	Sg 200-1*	Sg 526-1*	Sg 542-1*
1	834 B	R	R	R
2	IP18294-P1	R	R	R
3	IP18298-P1	R	R	R
4	81B	S	S	S

*R= resistant; S= susceptible.

Table 3.5. Description of the Pearl millet genotypes used for inheritance and allelic study

Genotype	Origin	Pedigree /Derived from	Special character				
834 B	ICRISAT	ICMB 4	Presence of long bristles on spikelet	Purple glumes, bold seed, loose spikelet	Matures early	Medium height	Round, and Dark gray seed
IP18294-P1	Mali	P 5307	Zebra stripes	Semi compact to compact spikelet	Medium	Tall	Elliptical, Brown
IP18298-P1	Burkina Faso	CVP 610	Glossy leaves	Semi compact to compact spikelet	Medium	Medium	Globular, Grey
81B	ICRISAT	ICMB 1	Bushy at the seedling stage	Compact spikelet	Matures late	Dwarf	Obovate-globular, light gray

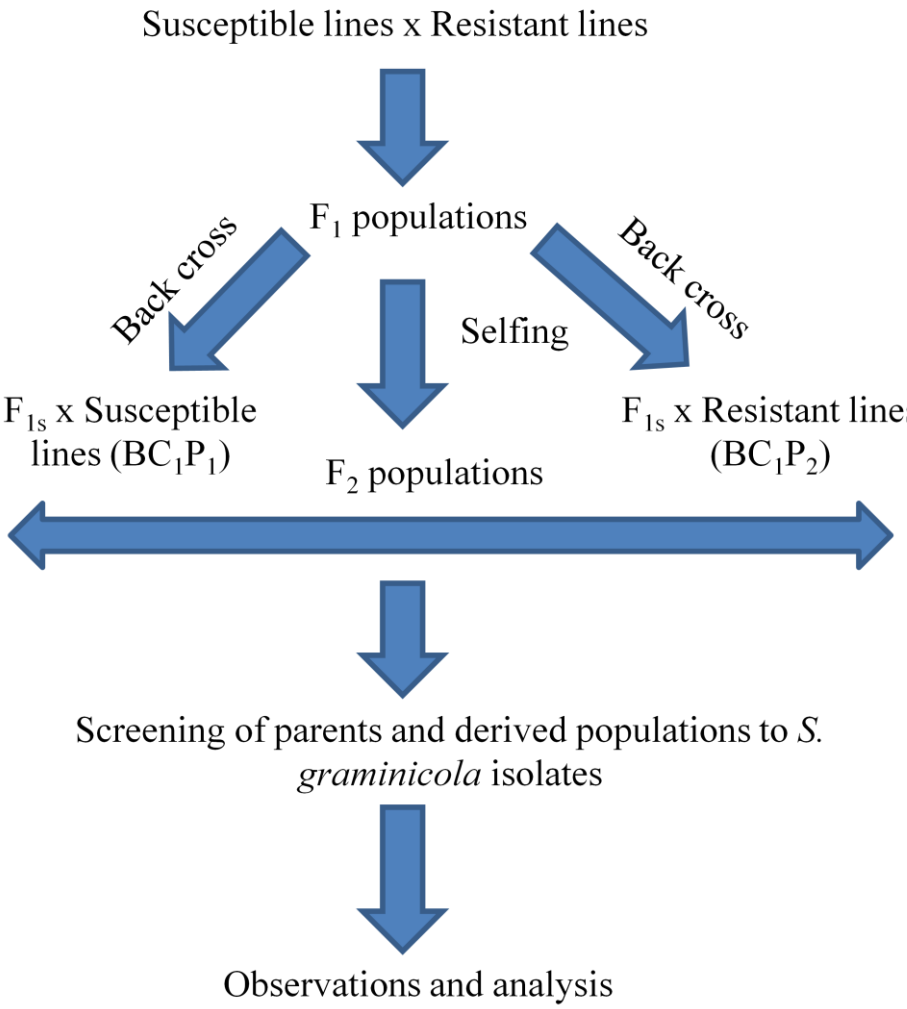


Plate 3.5. Schematic representation of inheritance of resistance in Pearl millet genotypes to *S. graminicola* isolates

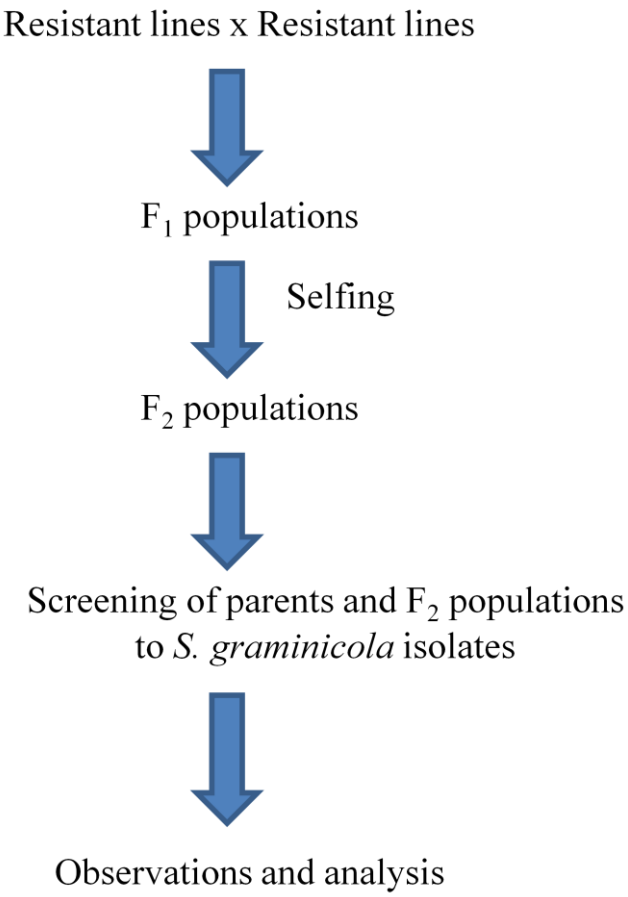


Plate 3.6. Schematic representation of allelism study between resistance genotypes of pearl millet to *S. graminicola* isolates

3.5.5 Statistical analysis

3.5.5.1 Data analysis of SSRs

Gel photographs were used to score the all visible and unambiguously scorable fragments amplified by SSR primers. The fragment sizes for all markers were used for the basic statistics using PowerMarker version 3.25 (http://www.powermarker.net) (Liu and Muse, 2005) including the polymorphic information content (PIC), allelic richness as determined by total number of the detected alleles and number of alleles per locus, gene diversity and average heterozygosity (%) and analysis of molecular variance (AMOVA). To identify the genetic structure of *S. graminicola* populations and assign individuals to populations, the software STRUCTURE version 2.3.3 (Pritchard *et al*., 2000) was used. Optimal numbers of groups (K) were derived through STRUCTURE run with K varying from 1 to 20 and three runs for each K value. To determine the true value of K, ad hoc statistic ΔK was followed (Evanno *et al.,* 2005). Parameters were set to 1,00,000 burn-in periods and 2,00,000 Markov Chain Monte Carlo (MCMC) replications after burn-in with an admixture and allele frequencies correlated model.

The Neighbor-Joining method proposed by Saitou and Nei (1987) was used for genetic diversity. The tree was constructed based on the simple matching dissimilarity matrix of SSR markers genotyped across the *S. graminicola* populations as implemented in DARwin 6.0.12 programme (http://darwin.cirad.fr/darwin).

3.5.5.1.1 Polymorphic Information Content (PIC):

A closely related diversity measure is the polymorphism information content (PIC) (Anderson *et al*., 1993). PIC of SSRs was calculated as follows:

$$\widehat{PIC_1} = 1 - \sum_{u=1}^{k} \tilde{p}_{lu}^2 - \sum_{u=1}^{k-1} \sum_{v=u+1}^{k} 2\tilde{p}_{lu}^2 \tilde{p}_{lv}^2$$

P_{lu} is the population frequency of u^{th} allele at l^{th} locus and P_{lv} is the population frequency of v^{th} allele at l^{th} locus in the set of genotypes investigated

3.5.5.1.2 Gene diversity:

Gene diversity often referred to as expected heterozygosity, is defined as the probability that two randomly chosen alleles from the population are different. An unbiased estimator of gene diversity at the l^{th} locus is as follows:

$$\widehat{D_l} = (1 - \sum_{u=1}^{k} \tilde{p}_{lu}^2)/(1 - \frac{1+f}{n})$$

3.5.5.1.3 Heterozygosity:

Heterozygosity is simply the proportion of heterozygous individuals in the population. At a single locus and it was estimated as follows:

$$\widehat{H}_l = 1 - \sum_{u=1}^{k} \tilde{P}_{luv}$$

3.5.5.1.4 Allele frequencies:

The sample allele frequencies are calculated as $\tilde{p}_u = n_u/(2n)$, with the variance estimated as follows:

$$var(\tilde{p}_u) \widehat{=} \frac{1}{2n}(\tilde{p}_u + \tilde{P}_{uv} - 2\tilde{p}_u^2)$$

where, $\widehat{=}$ means "estimated by".

The sample genotype frequencies $\tilde{P}_{uv}$ are calculated as n_{uv}/n. Both the $\tilde{p}_u$s and $\tilde{P}_{uv}$ s are unbiased maximum likelihood estimates (MLEs) of the population frequencies. Confidence intervals for allele and genotype frequencies are formed by resampling individuals from the data set.

3.5.5.1.5 Unweighted neighbor-joining tree:

The Neighbor-Joining method proposed by Saitou and Nei (1987) was used for assessing genetic diversity. The tree was constructed based on the simple matching dissimilarity matrix of SSR markers genotyped across the *S. graminicola* populations as implemented in DARwin 6.0.12 programme (http://darwin.cirad.fr/darwin).

3.5.5.2 Inheritance and tagging of avirulence

Chi square values were calculated to compare observed ratios of avirulent versus virulent phenotypes. Genotyping data were assembled in a unique input file to elaborate the genetic linkage among markers. MAPMAKER/EXP 3.0 (Lincoln and Lander, 1987) was used to build the genetic linkage map. Linkage was considered significant if the logarithm of odds (lod) score was 3.0 and maximum genetic distance of <50 centimorgan (cM). For consideration of the positions of SSR loci relative to the target

locus, linkage distances were calculated as two-point data. Marker trait association analysis was carried out using PlabQTL software v1.2 (Utz and Melchinger, 1996) and the results were obtained as the estimates for adjusted R^2 value and the F statistic for each marker. The significance of R^2 value explains whether the marker is linked to a locus for a trait under the study. F value <0.05 indicates significance of adjusted R^2 value and shows significant association of the marker with trait.

3.5.5.3 Inheritance and allelism study

The observed ratios of resistant to susceptible plants in the segregating populations in greenhouse were compared to theoretical ratios using chi-square test after pooling of plants from all the replications. The chi-square test was applied to test the segregation ratio of the phenotypic classes by using the program GENES (Cruz, 2001). Ten phenotypic ratios: 1:0 (alleles in the same locus); 3:1 (Single dominant gene); 15:1 (two independent dominant genes); 9:7 (two complementary dominant genes); 13:3 (two epistatic genes, one dominant and one recessive); 63:1 (three independent dominant genes); 57:7 (one dominant and two complementary genes); 27:37 (three complementary dominant genes); 61:3 (two dominant and one recessive genes); 49:15 (one dominant and two recessive genes) were tested.

Results and discussion

CHAPTER IV
RESULTS AND DISCUSSION

Information regarding the isolates of *Sclerospora graminicola* and its co-evolutionary potential in terms of increased threats from a rapidly changing population adapted to the newly developed resistant varieties is useful for disease-management strategies. Therefore, there should be greater emphasis on studying the biology of the pathogen and interaction with its host. The impetus for fundamental molecular research on *S. graminicola* has been repressed in the past by lack of good molecular tools, the lack of DNA sequence information, and in particular lack of a route for functional analysis of genes. However, work in the last few years on *Phytophthora*, *Bremia* and *Plasmopara* (Lees *et al*., 2006., May *et al*., 2002 and Sicard *et al*., 2003) in the diagnosis and determination of mating type, studying genetic structure and population genetics, inheritance and mapping of avirulence genes through molecular markers has significantly altered these aspects of research and it is now possible to examine the relationship between host and pathogen at molecular level. To increase the durability of resistance, attempts have been made to identify several sources of resistance against one or multiple pathotypes (Hash *et al*. 2006 and Sharma *et al*., 2015). However, it would be nice to know the mode of inheritance of DM resistance and the relationship or independence between genes for resistance while breeding for durable resistance.

Results of various experiments conducted on aforementioned objectives are presented and discussed experiment-wise under following headings.

5. Development of Simple Sequence Repeat (SSR) markers for diversity analysis of *Sclerospora graminicola*
6. Development of segregating population from a cross between avirulent and virulent isolates of *S. graminicola*
7. Inheritance and molecular mapping of avirulence genes in *S. graminicola*
8. Inheritance of downy mildew resistance in pearl millet genotypes and allelic relationships among resistance genes

4.1 Development of Simple Sequence Repeat (SSR) markers for diversity analysis of *S. graminicola*

4.1.1 Distribution of SSRs

The assembled scaffolds were screened for the presence of SSRs in the *S. graminicola* isolate Sg 445 genome using perl script MISA. In total 7453 SSRs were identified from 3912 SSR containing sequences of which 235 (3.3%) SSRs were of compound type and remaining 7218 (96.7%) were of perfect type (Table 4.1). The mining of SSRs in genome sequence of *S. graminicola* reflected ~27 % of sequences with perfect SSRs, that represented higher frequencies than earlier report of Garnica *et al*. (2006) and Schena *et al*. (2008) across *Phytophthora* species. Nevertheless, these differences might reflect the different criteria used to select the SSRs. The sequences containing more than one SSR were 1554 (10.73%). The overall relative abundance and density of SSRs in the assembled scaffolds were 34.57 /Mb and 459.67 bp/Mb, respectively (Table 4.2). The relative abundance of SSRs in fungi is found to be in between 9-1453 SSRs/Mbp depending on the size and microsatellite availability (Murat *et al*., 2011). Henceforth, oomycetes with complex and longer genome could be speculated to follow the similar line as fungi. The maximum number of SSRs was found in scaffold 639 of size 106597 bp.

4.1.2 Validation of markers

Out of 2702 primers, 106 were selected (Appendix A.) on the basis of the following criteria: (1) more than 3 bases of GC rich (GCC, CGG, CCG, CCC, CGG, GCG, GGG, GGC) at the 3' end was eliminated; (2) 3' end containing either G or C was selected; (3) poly AAAAA, TTTTT, GGGGG, CCCCC were also removed. All 106 SSRs markers were screened on four *S. graminicola* isolates. Primers showed good amplification. To identify polymorphic markers for diversity analysis all primers were tested on 12 diverse *S. graminicola* isolates. This study revealed 39 polymorphic primers. However, six primers (Sg_SSR 62, Sg_SSR 66, Sg_SSR 70, Sg_SSR 74, Sg_SSR 86 and Sg_SSR 30) were not consistent with the results and thus removed from further study. Nine primer sets (Sg_SSR 01, Sg_SSR 07, Sg_SSR 13, Sg_SSR 38, Sg_SSR 45, Sg_SSR 46, Sg_SSR 47, Sg_SSR 65 and Sg_SSR 102) showed polymorphisms between only two isolates (Sg 019 and Sg 445-1), whereas Sg_SSR 99 and Sg_SSR 4 generated three to four bands, therefore not selected for genetic diversity

Table 4.1. Summary of microsatellite search in genome of *S. graminicola*

Total number of sequences examined	14481
Total size of examined sequences (bp)	215561828
Total number of identified SSRs	7453
Number of SSR containing sequences	3912
Number of sequences containing more than 1 SSR	1554
Number of SSRs present in compound formation	235

Table 4.2. Distribution, frequency, relative abundance and density of classified repeat types

Repeats	Total No	Total bp	Abundance	Density	SSR %
A/T	2157	22697	10.01	105.3	28.9
C/G	2095	22246	9.72	103.2	28.1
Total	**4252**	**44943**	**19.725199**	**208.49**	**57**
CA/AC/GT/TG	253	3342	1.17	15.5	3.4
GA/AG/CT/TC	358	5416	1.66	25.1	4.8
AT/AT	643	8938	2.98	41.5	8.6
CG/CG	1	12	0.00	0.1	0.0
Total	**1255**	**17708**	**5.8219**	**82.148**	**16.8**
AAC/GTT/ACA/TGT/CAA/TTG	146	2526	0.68	11.7	2.0
AAG/CTT/AGA/TCT/GAA/TTC	776	13263	3.60	61.5	10.4
AAT/ATT/ATA/TAT/TAA/TTA	267	5283	1.24	24.5	3.6
ACC/GGT/CAC/GTG/CCA/TGG	28	456	0.13	2.1	0.4
ACG/CGT/CGA/GAC/TCG/GTC	99	1596	0.46	7.4	1.3
ACT/AGT/CTA/TAG/TAC/GTA	158	3243	0.73	15.0	2.1
AGC/GCA/TGC/GCT/CTG/CAG	114	1839	0.53	8.5	1.5
AGG/CCT/GGA/TCC/GAG/CTC	86	1440	0.40	6.7	1.2
ATC/GAT/TCA/TGA/CAT/ATG	139	2421	0.64	11.2	1.9
Total	**1813**	**32067**	**8.410**	**148.76**	**24.4**
AAAC/AACA/ACAA/CAAA/GTTT/TGTT/TTGT/TTTG	1	20	0.00	0.1	0.0
AAAG/CTTT/GAAA/TTTC/AAGA/AGAA/TCTT/TTCT	8	180	0.04	0.8	0.1
AAAT/AATA/ATAA/ATTT/TAAA/TATT/TTAT/TTTA	10	236	0.05	1.1	0.1
AACC/ACCA/CAAC/CCAA/CGTT/GTTG/TGGT/TTGG	1	20	0.00	0.1	0.0
AACT/ACTA/CTAA/TAAC/ATTG/GATT/TGAT/TTGA	3	68	0.01	0.3	0.0
AAGC/AGCA/GCAA/CAAG/TTCG/TCGT/CGTT/GTCC	2	40	0.01	0.2	0.0
AAGG/AGGA/CCTT/CTTC/GAAG/GGAA/TCCT/TTCC	6	120	0.03	0.6	0.1
AAGT/AGTA/GTAA/TAAG/TTCA/TCAT/CATT/ATTC	9	260	0.04	1.2	0.1
AATC/ATCA/TCAA/CAAT/TTAG/TAGT/AGTT/GTTA	3	68	0.01	0.3	0.0
AATG/ATGA/TGAA/GAAT/TACT/TTAC/ACTT/CTTA	0	0	0.00	0.0	0.0
AATT/ATTA/TAAT/TTAA	8	180	0.04	0.8	0.1
ACAG/AGAC/CAGA/CTGT/GACA/GTCT/TCTG/TGTC	10	304	0.05	1.4	0.1
ACAT/ATAC/ATGT/CATA/GTAT/TACA/TATG/TGTA	2	328	0.01	1.5	0.0
ACCT/TGGA/CCTA/GGAT/CTAC/GATG/TACC/ATGG	2	100	0.01	0.5	0.0
ACGG/CGGA/GGAC/GACC/TGCC/GCCT/CCTG/CTGC	1	28	0.00	0.1	0.0
ACGT/ATGC/CATG/CGTA/GCAT/GTAC/TACG/TGCA	2	44	0.01	0.2	0.0
ACTC/TGAG/AGTG/TCAC/CACT/GTGA/CTCA/GAGT	6	136	0.03	0.6	0.1
ACTG/TGAC/CTGA/GACT/CAGT/TCAG/GTCA/AGTC	3	60	0.01	0.3	0.0
AGAT/TCTA/ATAG/TATC/ATCT/TAGA/CTAT/GATA	12	476	0.06	2.2	0.2
ATCC/GGTA/GTAG/AGGT/GATG/TCCA/CCAT/CATC	2	48	0.01	0.2	0.0
Total	**91**	**2716**	**0.4221**	**12.6**	**0.9**

Table 4.2. (Cont.)

Repeats	Total No	Total bp	Abundance	Density	SSR %
AAAAG/CTTTT	1	25	0.00	0.1	0.0
AAATT/AATTT	1	25	0.00	0.1	0.0
AACGT/ACGTT	1	25	0.00	0.1	0.0
AATAC/ATTGT	6	200	0.03	0.9	0.1
AATAG/ATTCT	3	135	0.01	0.6	0.0
AATAT/ATATT	4	105	0.02	0.5	0.1
ACAGT/ACTGT	2	50	0.01	0.2	0.0
ACTAT/AGTAT	12	570	0.06	2.6	0.2
ACTCT/AGAGT	1	145	0.00	0.7	0.0
ATATC/ATATG	1	25	0.00	0.1	0.0
Total	**32**	**1305**	**0.1484**	**6.0539**	**0.4**
AAAAAG/CTTTTT	1	36	0.00	0.2	0.0
AAAGAC/CTTTGT	1	30	0.00	0.1	0.0
AACAAT/ATTGTT	1	30	0.00	0.1	0.0
AACCCT/AGGGTT	1	48	0.00	0.2	0.0
AAGAGC/CTCTTG	2	60	0.01	0.3	0.0
AAGCAG/CTGCTT	1	30	0.00	0.1	0.0
AATAGT/ACTATT	1	42	0.00	0.2	0.0
ACGATG/ATCGTC	1	30	0.00	0.1	0.0
AGCTCC/AGCTGG	1	42	0.00	0.2	0.0
Total	**10**	**348**	**0.0463**	**1.6144**	**0**
Sum Total	**7443**	**99087**	**34.57**	**459.7**	**100.0**

analysis. The remaining 22 polymorphic markers were used to study genetic diversity among 60 *S. graminicola* isolates (Table 4.3).

The number of alleles per locus (N_A) varied from 2 to 4 among the markers (Table 4.3). A total of 57 alleles with an average of 2.59 alleles per locus were detected, that appears to be in low number as compared to 3 to 4 alleles with an average of 3.2 in *Peronospora tabacina* (Trigiano *et al*., 2012); 2 to 9 alleles with an average of 4.3 in *Pseudoperonospora cubensis* (Kanetis *et al*., 2009); 2 to 8 alleles with a mean of 5.2 in *P. sorghi* (Perumal *et al*., 2008) and unusually high (43) alleles with an average of 7.0 in *P. viticola* (Gobbin *et al*., 2003). The frequency of major alleles (M_{AF}) per locus was 0.474 to 0.940, with an average of 0.721. In addition, the observed heterozygosity (H_O) values were 0.0000-0.7667 with an average of 0.2667, and expected heterozygosity (H_E) values were 0.1244-0.6296 with an average of 0.3945. The polymorphic information content (PIC) values ranged between 0.1167 and 0.6012, with an average of 0.3301. Similar observations were reported for H_E, H_O and PIC value by Gobbin *et al*. (2003) among isolates of *Plasmopara viticola*. The PCR product sizes ranged from 110-320 bp. The estimated frequency of null alleles showed probabilities ranging around zero for most of SSR loci. However, the negative values for null allele frequency imply observed heterozygote genotypes in excess of statistical expectations. The probabilities of identity (PI) ranged from 0.211 to 0.786, with an average of 0.4672 for the entire isolates over 22 loci and PI (sib) ranged from 0.519592 to 1.27875, with an average of 0.8429.

Among 22 markers, Sg_SSR 85 was the most informative SSR marker because of its high polymorphic information content (PIC), low probability of identity and high values of expected and observed heterozygosity (Table 4.3). The presence of a null allele in an appreciable frequency was observed wherever the observed heterozygosity was markedly less than the expected heterozygosity. Similar results have been documented by Gobbin *et al*. (2003) on *Plasmopara viticola*.

4.1.3 Diversity analysis

4.1.3.1 Dendrogram analysis

Dendrogram for 60 *S. graminicola* test isolates was developed with 22 SSR markers data by using genetic distance and the dissimilarity matrix through neighbor-joining (NJ) method. Cluster analysis showed random distributions of all isolates into five major clusters (Fig. 4.1). Cluster I contained 12 isolates from four states (Rajasthan-5, Uttar Pradesh-4, Telangana-2 and Maharashtra-1), Cluster II comprised

Table 4.3. Number of allele (N_A), total possible number of genotypes (tG), allele Size Range (ASR), allele Frequencies (AF), expected (He) and observed heterozygosity (Ho), estimated frequency of null alleles (Fn), probability of identity (PI), probability of identity of sibling (PI_{sib}), and Polymorphic information content (PIC) of 22 *S. graminicola* SSR loci on 60 isolates

Locus No.	Marker	N_A	tG	ASR	AF				H_E	H_O	Fn	PI	PIsib	PIC
1	Sg_SSR 66	2	3	110-120	0.633	0.367			0.464	0.000	0.317	0.395	0.706	0.357
2	Sg_SSR 95	3	6	285-305	0.658	0.267	0.075		0.490	0.533	-0.029	0.328	0.683	0.423
3	Sg_SSR 94	3	6	170-185	0.407	0.525	0.068		0.554	0.102	0.291	0.294	0.598	0.479
4	Sg_SSR 92	2	3	290-300	0.708	0.292			0.413	0.517	-0.073	0.430	0.780	0.328
5	Sg_SSR 35	4	10	270-300	0.025	0.675	0.292	0.008	0.459	0.600	-0.097	0.371	0.721	0.380
6	Sg_SSR 9	2	3	160-170	0.322	0.678			0.437	0.000	0.304	0.413	0.746	0.366
7	Sg_SSR 3	2	3	150-160	0.661	0.339			0.448	0.000	0.309	0.405	0.729	0.372
8	Sg_SSR 6	3	6	160-180	0.742	0.233	0.025		0.395	0.067	0.235	0.427	0.812	0.334
9	Sg_SSR 103	4	10	200-230	0.025	0.825	0.108	0.042	0.305	0.300	0.004	0.502	0.955	0.286
10	Sg_SSR 99	2	3	290-310	0.758	0.242			0.367	0.483	-0.085	0.468	0.851	0.299
11	Sg_SSR 34	3	6	240-260	0.034	0.940	0.026		0.115	0.121	-0.005	0.786	1.279	0.167
12	Sg_SSR 16	3	6	190-210	0.931	0.060	0.009		0.129	0.138	-0.008	0.764	1.252	0.178
13	Sg_SSR 14	2	3	230-250	0.133	0.867			0.231	0.200	0.025	0.618	1.071	0.204
14	Sg_SSR 49	2	3	120-130	0.433	0.567			0.491	0.000	0.329	0.380	0.669	0.371
15	Sg_SSR 78	2	3	170-180	0.933	0.067			0.124	0.133	-0.008	0.774	1.261	0.117
16	Sg_SSR 52	4	10	270-300	0.017	0.867	0.067	0.050	0.242	0.250	-0.007	0.586	1.058	0.231
17	Sg_SSR 55	2	3	310-320	0.617	0.383			0.473	0.767	-0.200	0.390	0.694	0.361
18	Sg_SSR 65	2	3	120-130	0.636	0.364			0.463	0.627	-0.112	0.395	0.708	0.380
19	Sg_SSR 96	3	6	280-300	0.042	0.822	0.136		0.304	0.017	0.220	0.512	0.954	0.301
20	Sg_SSR 98	2	3	210-220	0.720	0.280			0.403	0.559	-0.111	0.438	0.796	0.346
21	Sg_SSR 22	2	3	270-280	0.373	0.627			0.468	0.000	0.319	0.393	0.701	0.382
22	Sg_SSR 85	3	6	160-180	0.474	0.325	0.202		0.630	0.509	0.074	0.211	0.520	0.601
		57							**0.382**	**0.269**	**0.077**	**0.467**	**0.843**	**0.330**

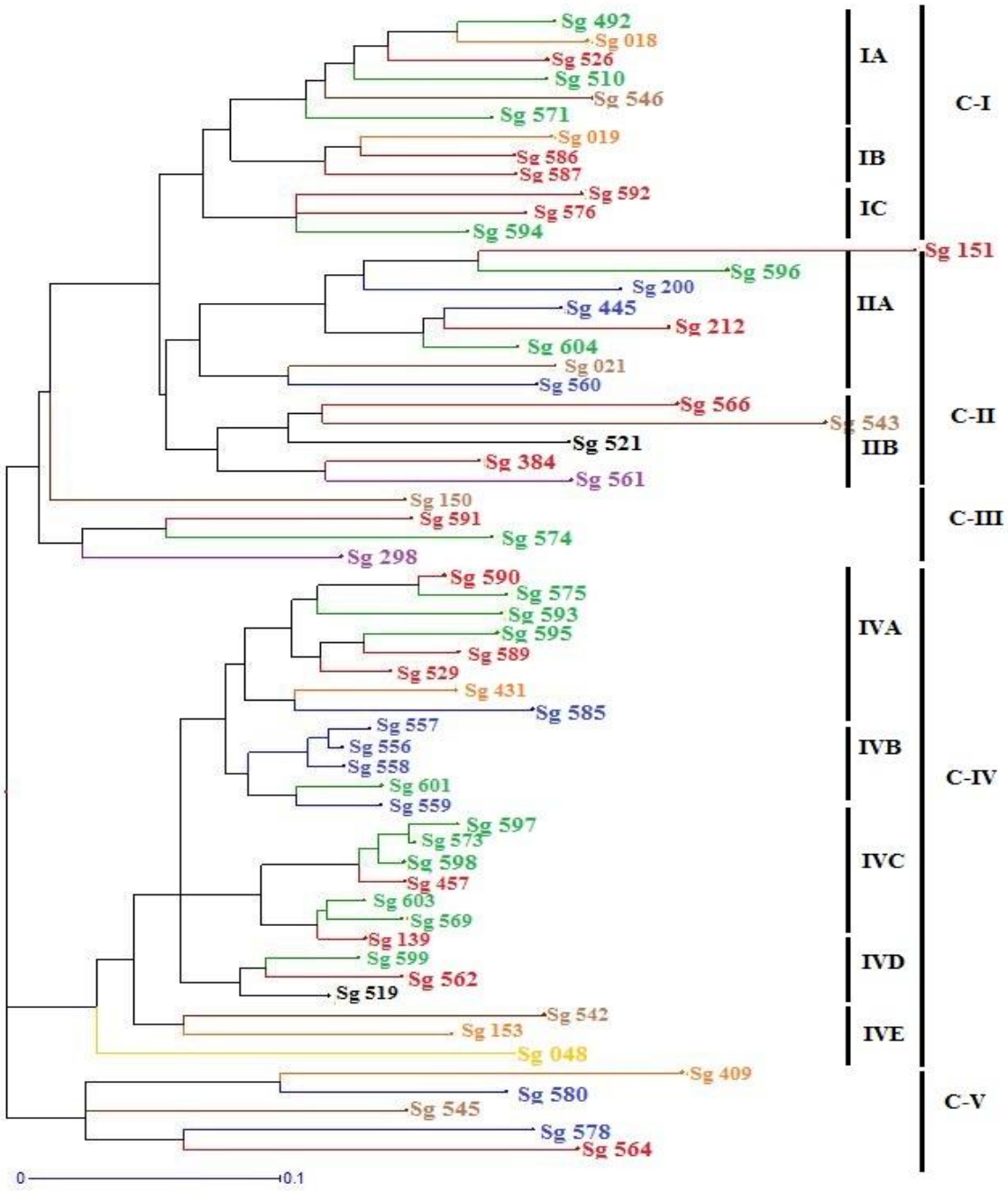


Fig 4.1. Neighbor-joining (N-J) tree showing the relationships between 60 *S. graminicola* isolates; color of genotypes represents the region specificity as Red- Rajasthan, Blue-Gujarat, Green- Uttar Pradesh, Orange- Telangana, Brown- Maharashtra, Purple- New Delhi, Light yellow- Karnataka and Black- Haryana

13 isolates from six states (Rajasthan-4, Uttar Pradesh-2, Gujarat-3, Maharashtra-2, New Delhi-1 and Haryana-1), Cluster III represented three isolates from three states (Rajasthan-1, Uttar Pradesh-1 and New Delhi-1), Cluster IV constituted 25 isolates from six states (Uttar Pradesh-10, Rajasthan-6, Gujarat-5, Telangana-2, Haryana-1 and Maharashtra-1), whereas Cluster V grouped five isolates from four states (Gujarat-2, Rajasthan-1, Telangana-1 and Maharashtra-1). Large variation was observed in the isolates and these groups were further divided into two sub-groups. Cluster I was sub-grouped into cluster IA with six, cluster IB and cluster IC with three isolates each. Similarly, cluster II was sub-grouped into two sub-clusters; cluster IIA and cluster IIB with eight and five isolates, respectively. Cluster IV being the largest group, contained eight isolates in IVA, five in IVB, seven in IVC, three in IVD and two in IVE. Sg 150 and Sg 048 appeared separately in the dendrogram and were not assigned to any group. Cluster III and cluster V was not divided into any further sub-cluster. *S. graminicola* isolates labeled with different colors to represent different regions/states got clustered in all the major clusters and even in the sub clusters. However, region specific grouping was noticed for Rajasthan isolates in Clade I, Gujarat isolates and Uttar Pradesh isolates in Clade IV. Isolates grouped into Clade II, Clade III and Clade V showed no sign of region specificity (Appendix B). These results are in agreement with the report of Sharma *et al*. (2010).

4.1.3.2. Analysis of molecular variance (AMOVA)

Analysis of molecular variance (AMOVA) revealed 56.21% of the variance over 22 SSRs loci due to the differences among the isolates within states, 35.28 % due to within isolates and 8.51% was due to differences among the states (Table 4.4). The little contribution of variation among states towards the total variance could be explained by random distribution of isolates across the cluster grouping. However, this variation between states might be because of clustering of some isolates (Sg 556, Sg 557 and Sg 558) from Gujarat and (Sg 575, Sg 593 and Sg 595) from Uttar Pradesh in the region-specific groups. The adaptation of some isolates to specific host genotype in a particular region is well reported by Thakur *et al*. (2004). The maximum genetic variation towards the total variance detected within states could be due to recombination occurring within the field population. The variation contributed by within isolates towards the total variance could be attributed

to the presence of large amount of heterozygosity in the pathogen that might be expected due to out-crossing nature of *S. graminicola*. The existence of different types of sexual compatibility types and sexual mechanisms like secondary homothallism and heterothallism may probably permits out-crossing within *S. graminicola*. This result is very much supported by the findings of Singru *et al*. (2003) and Sharma *et al*. (2010).

4.1.3.3. Population structure

Genetic analysis of 60 *S. graminicola* isolates using a evanno-based approach suggested the presence of genetically distinct population structure with variable log likelihood values *L*(*K*) at different runs (*K*= 2–19). Therefore, inference of exact value of *K* was not straight forward with the distribution of *L*(*K*) (Fig. 4.2, Appendix C, D, E and F). True number of populations (*K*) is often identified using the maximal value of *L*(*K*). However, in most of the simulations, *L*(*K*) at larger *K*s showed either plateau or slight increase in the graph (Fig. 4.2, Table 4.5), although a plateau started developing at *K*=5. The slight increase in *L*(*K*) value were noticed between *K* = 6 and *K* = 7. The model choice criterion to detect the most probable value of *K* was Δ*K*. The highest value of Δ*K* =192.86 was found at *K* = 2 (Table 4.5, Fig. 4.2), which indicated that the entire population can be grouped into two subgroups *viz.*, SG1 and SG 2 (Fig. 4.3a). Based on the membership fractions, isolates with the probability of ≥ 80 % were assigned to corresponding subgroups while others categorized as admixture (Fig. 4.3a). SG1 consisted of 30 isolates from seven different states, of which, nine belongs to Rajasthan, six to Uttar Pradesh, five to Gujarat and remaining ten from four different states. SG2 contained 24 isolates from six different states with maximum 10 isolates from Uttar Pradesh followed by Rajasthan (6) Gujarat (5), Telangana (1), Haryana (1) and Maharashtra (1). The remaining six isolates were retained to be admixture between three groups. However, the ad hoc static value of Δ*K* at *K*=5, Fig 4.3b, suggested partitioning of all isolates into five different groups (SG 3, SG 4, SG 5, SG 6 and SG 7). The partitioning divided 30 isolates of SG 1 at *K*=2 to three groups, 11 to SG 3, 9 to SG 4 and 6 to SG 5 (Fig 4.3b). While, 24 isolates of SG 2 classified into two groups SG 6 and SG 7 with 12 and 10 isolates, respectively (Fig 4.3b). The remaining four isolates of SG 1 and 2 isolates of SG 2 grouped in the admixture category (Fig 4.3b). The results at *K*=5 were well supported by cluster analysis (Table 4.5) where SG 1 represented by cluster I, SG 3 by cluster II, SG 2 and maximum isolates of SG 4 by cluster IV and SG 5 by cluster V. Out of four, three isolates of cluster III were grouped into admixture category and one

into SG 4. Two isolates of SG 5 were noticed to mix with cluster II. Besides this, the slight increase of ΔK at K=7 indicated the possibilities of further differentiation of isolates. Further classification of isolates at K=7 suggested similar grouping of 27 isolates as been done at K=5 and cluster analysis (Fig 4.3c). The remaining 33 isolates were categorized as admixture. However, Fig. 3d suggested that there could be more differentiation of *S. graminicola* populations.

Interestingly, some isolates of Gujarat (Sg 556, Sg 557 and Sg 558) and Uttar Pradesh (Sg 575, Sg 593 and Sg 595) were clustered together throughout the grouping (Cluster IV and SG 2) that suggested regional specificity for some isolates due to restricted gene flow. These results were in agreement with those of Sharma *et al*. (2010). In addition, another three isolates (Sg 212, Sg 445 and Sg 604) belonging to three different states, Rajasthan, Gujarat and Uttar Pradesh respectively, were clustered together across the grouping (Cluster II and SG 3) that might be due to the operation of similar natural selection force in these regions.

Table 4.4. Summary of analysis of molecular variance (AMOVA) over 22 SSRs loci in *Sclerospora graminicola* isolates

Source of variation	Sum Of Square	Percentage of variation
Among States	84.91	8.51
Within States	560.74	56.21
Within Isolates	352.00	35.28
Total	997.65	100

Overall Fst or θ = amount of inbreeding-like effects within isolates = 0.011

Overall Fis or f = amount of inbreeding-like effects within states = 0.296

Overall Fit or F = amount of inbreeding-like effects among states = 0.303.

Table 4.5. Summary of population structure in *Sclerospora graminicola* isolates

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	3	-1607.73	0.47	NA	NA	NA
2	3	-1433.23	0.55	174.5	106.2	192.83
3	3	-1364.93	1.63	68.3	36.1	22.18
4	3	-1332.70	5.47	32.2	25.7	4.69
5	3	-1326.13	52.11	6.6	6.4	0.12
6	3	-1325.97	13.20	0.2	7.4	0.56
7	3	-1333.17	12.81	-7.2	53.8	4.20
8	3	-1394.20	20.14	-61.0	21.3	1.06
9	3	-1433.97	14.30	-39.8	1.1	0.07
10	3	-1472.67	29.34	-38.7	24.9	0.85
11	3	-1486.43	40.25	-13.8	19.9	0.50
12	3	-1520.13	6.51	-33.7	14.6	2.24
13	3	-1539.27	36.14	-19.1	33.7	0.93
14	3	-1524.70	45.31	14.6	45.8	1.01
15	3	-1555.90	32.31	-31.2	27.7	0.86
16	3	-1559.37	13.26	-3.5	2.3	0.17
17	3	-1560.57	41.60	-1.2	16.9	0.41
18	3	-1544.87	37.49	15.7	979.8	26.13
19	3	-2509.00	1628.65	-964.1	1914.3	1.18
20	3	-1558.80	16.79	950.2	NA	NA

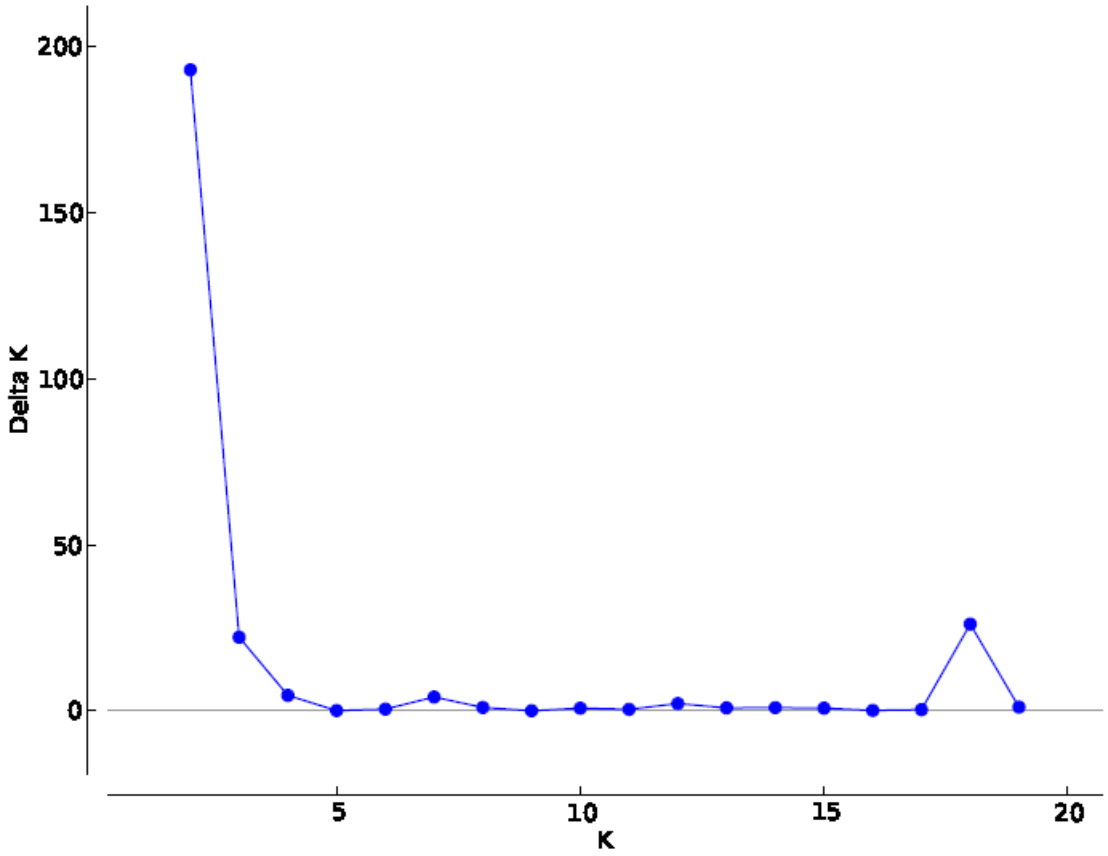


Fig 4.2. Mean of the absolute values of $L''(K)$ averaged over 20 runs divided by the standard deviation of $L(K)$

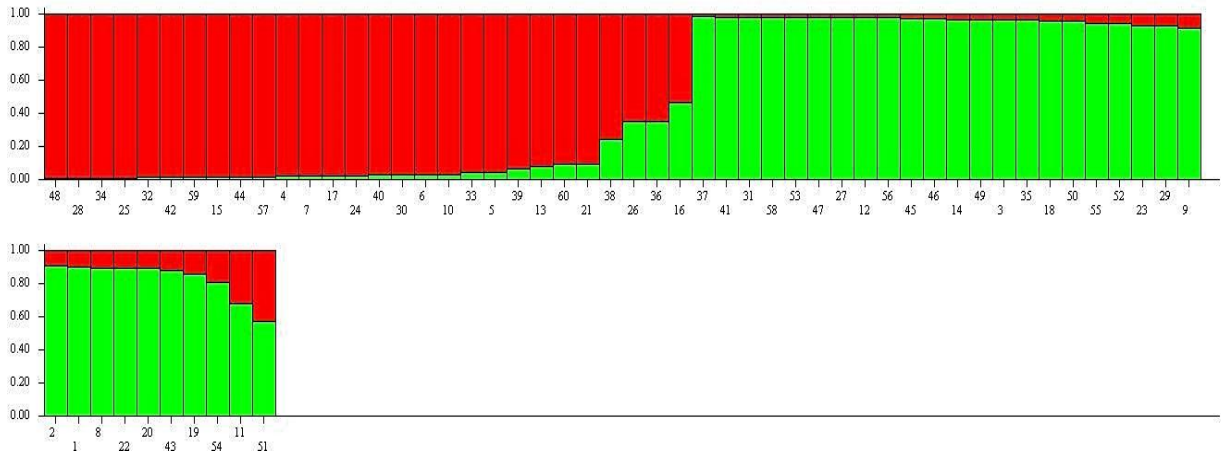


Fig 4.3a. Population structure of 60 *S. graminicola* isolates at *K=2* based on 22 SSR loci using STRUCTURE (Pritchard *et al*., 2000). Different colors represent sub-isolates (or groups)

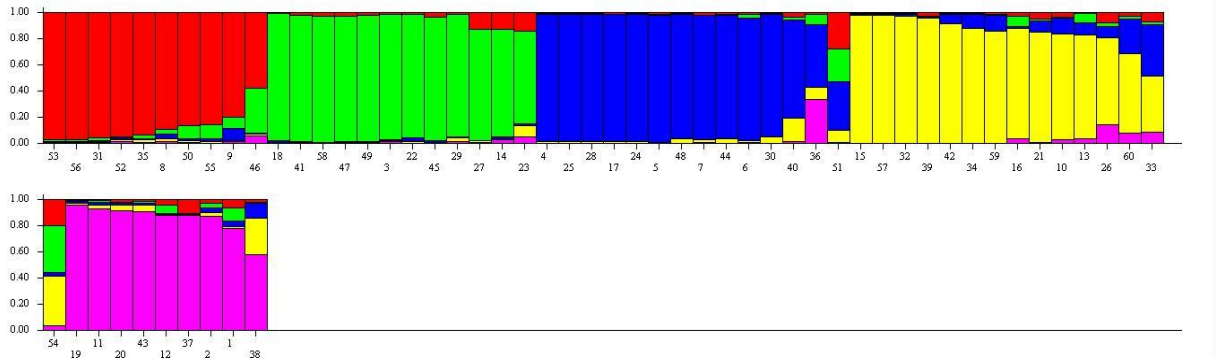


Fig 4.3b. Population structure of 60 *S. graminicola* isolates at *K=5* based on 22 SSR loci using STRUCTURE (Pritchard *et al*., 2000). Different colors represent sub-isolates (or groups)

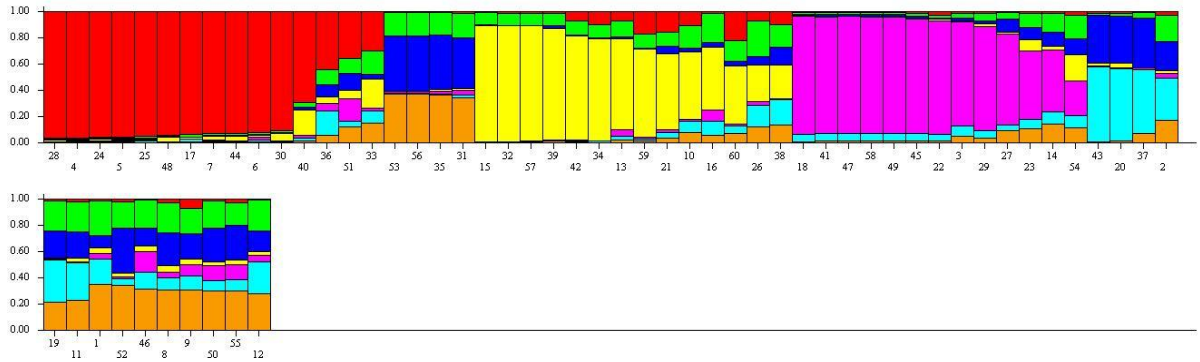


Fig 4.3c. Population structure of 60 *S. graminicola* isolates at *K=7* based on 22 SSR loci using STRUCTURE (Pritchard *et al*., 2000). Different colors represent sub-isolates (or groups)

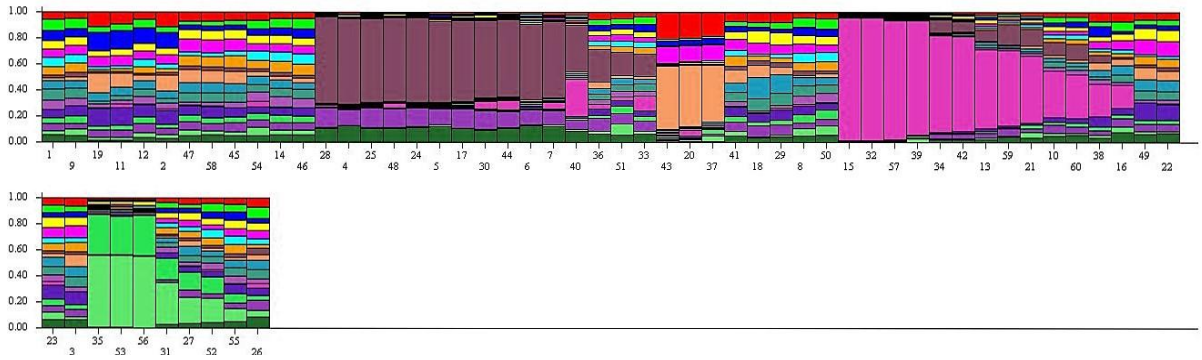


Fig 4.3d. Population structure of 60 *S. graminicola* isolates at *K=18* based on 22 SSR loci using STRUCTURE (Pritchard *et al*., 2000). Different colors represent sub-isolates (or groups)

4.1.4 Cross-genus amplification

Microsatellite primers developed for a distinct species have been shown to be useful for other closely related species (Davis and Strobeck, 1998). Dutech *et al*. (2007) reported 34% cross-species transferability of microsatellite primers in fungi. In the present study, primer pairs developed from *S. graminicola* amplified the DNA fragments of related oomycetes genera *Pernosclerospora sorghi* and *Phytophthora infestans*. Out of 106 primers, 72 showed amplification in *P. sorghi*, whereas 19 of 36 primers showed amplification in *P. infestans* (Table 4.6), although they were not examined further. Similar cross genus transferability of *P. sorghi* microsatellite loci to other species of *Peronosclerospora* as well as to *Peronospora* and *Sclerospora* species were reported by Perumal *et al*. (2008). In contrast, limited cross amplification was demonstrated with *Peronospora tabacina* loci in three other *Peronospora* species and in a single species of *Plasmopara* (Trigiano *et al*., 2012). The paucity of transferability was reported in *P. viticola*, in which only 34% of SSR could work across members of the same genera (Dutech *et al*., 2007).

In-silico development of SSRs and its validation to assess the genetic variation of *S. graminicola* is a significant step towards understanding the biological functions and evolutionary processes. The information gathered from population genetics studies may improve models of disease epidemics and forecasting, enhance the evaluation of risks to established plant cultivars, or assist in targeting control measures. For instance, incorporating knowledge of a pathogen's population structure into breeding for disease resistance may provide insight into the potential long-term and global effectiveness of resistance breeding lines. The alternative approach, which relies on using individual strains, is fraught with a lack of knowledge on the representativeness of such strains, and the possibility of a sudden upswing in strains capable of overcoming such resistance. Furthermore, knowledge of the means and magnitude of pathogenic migration is also critical in the formulation of regulations governing the international trade in plant germplasm.

Table 4.6. Amplification details of *S. graminicola* primers to other oomycetes

Genus	Total Primer used	Amplification	No Amplification	Percentage
Sclerospora	106	106	0	100.0
Pernosclerospora	106	72	34	67.9
Phytophthora	36	19	17	52.7

Table 4.7. Observation on oospore formation in the *Sclerospora graminicola* isolates

S.N.	Isolate No.	Oospore formation		S.N.	Isolate No.		
		No Oospore	Oospores			No Oospore	Oospores
1	Sg 018	√		28	Sg 532	√	
2	Sg.019	√		29	Sg 533	√	
3	Sg 021		√	30	Sg.535	√	
4	Sg 048	√		31	Sg 540	√	
5	Sg 139	√		32	Sg 541		√
6	Sg 150	√		33	Sg 542	√	
7	Sg 151	√		34	Sg 543	√	
8	Sg 153		√	35	Sg 544	√	
9	Sg 200	√		36	Sg 545		√
10	Sg 212	√		37	Sg 546		√
11	Sg 298	√		38	Sg 547	√	
12	Sg 334		√	39	Sg 548		√
13	Sg 384	√		40	Sg 549	√	
14	Sg 409		√	41	Sg 550		√
15	Sg 431	√		42	Sg 551		√
16	Sg 445	√		43	Sg 552	√	
17	Sg 457	√		44	Sg 553	√	
18	Sg 492	√		45	Sg 554	√	
19	Sg 510		√	46	Sg 555	√	
20	Sg 519		√	47	Sg 556	√	
21	Sg.520		√	48	Sg 557	√	
22	Sg 521		√	49	Sg 558		√
23	Sg 526	√		50	Sg 559		√
24	Sg 528	√		51	Sg 560		√
25	Sg 529	√		52	Sg 561		√
26	Sg 530	√					
27	Sg 531		√				

4.2 Development of segregating population from a cross between avirulent and virulent isolates of *S. graminicola.*

4.2.1 Selection of self sterile isolates of *S. graminicola*

A total 52 isolates of *Sclerospora graminicola* collected from different pearl millet growing areas in India were used to identify self sterile (heterothallic) isolates. As isolates were in isolation chambers, oospore formation could take place only in homothallic isolates having both the mating types. The necrotic leaf pieces (1 cm^2) collected from at least one month old seedlings infected by each isolate were observed separately under microscope and out of 52 isolates, 33 were found to be self-sterile (no oospore) i.e heterothallic in nature, whereas oospores formation was observed in the remaining 19 isolates (Table 4.7).

4.2.2 Selection of isolates with differential virulent/avirulent reactions and identification of mating type of test isolate

All self sterile, heterothallic isolates including reference mating type isolates Sg 018 (*Mat-1/*A_1) and Sg 019 (*Mat-2/*A_2) were tested for virulent/avirulent reactions on seven host differentials (P 7-4, P 310-17, 700651, 7042R, IP 18292, IP 18293 and 852B) and two known downy mildew (DM) susceptible lines - ICMP 451 and 7042S. The screening results identified Sg 445-1 as the most virulent isolate and the two reference mating type isolates Sg 018 and Sg 019 as avirulent; hence they were selected for crossing (Table 4.8). To detect mating type of the test isolate, Sg 445-1 was crossed with both the reference mating type isolates (Sg 018×Sg 445-1 and Sg 019×Sg 445-1). Oospore production was observed in the cross Sg 019 × Sg 445-1. However, no oospore formation was observed in Sg 018 × Sg 445-1. This indicated *Mat-1/*A_1 mating types of Sg 445-1. Thus, two parents Sg 019 (avirulent) and Sg 445-1 (virulent) of different mating types were selected for generation of segregating population for avirulent/virulence loci.

Table 4.8. Disease reaction of avirulent and virulent isolates on differential lines

Isolate	Mating type	Downy mildew incidence (%)								
		700651	7042R	7042S	852B	ICMP451	IP18292	IP18293	P 310-17	P7-4
Sg 018	*Mat 1*	4	47	97	0	94	0	4	0	8
Sg 019	*Mat 2*	0	38	95	0	91	0	0	0	3
Sg 445	-	53	75	100	100	100	80	46	63	86

Table 4.9. Segregation for avirulence (A) and virulence (V) in F_1 progenies of *S. graminicola* on five differential lines of pearl millet

Differential lines	Observed No.*	Expected ratio	Expected No.*	χ^2	*P value*
IP18292	96 A : 25 V	3:1	90.5 A : 30.5 V	1.36	0.25
852B	91 A : 30 V	3:1	90.5 A : 30.5 V	0.01	0.91
ICMB 01333	53 A : 68 V	1:1	60.5 A : 60.5 V	1.86	0.17
ICML 22	53 A : 68 V	1:1	60.5 A : 60.5 V	1.86	0.17
81 B-P6	0 A : 121 V	-	-	-	-

*A- avirulent; V- virulent

Table 4.10. Proposed avirulence genotypes of Sg 019 and Sg 445-1 on differential lines

Isolates	Avirulent genes			
	IP18292	852B	ICMB 01333	ICML 22
Sg 019	AaBb	AaBb	Aa	Aa
Sg 445-1	aabb	aabb	aa	aa

4.2.3 Establishment of F_1 progenies

The 121 F_1 progenies were established separately from F_1 oospores developed from Sg 019 × Sg 445-1 and maintained on 7042 S as an individual F_1-progeny in isolation polyacrylic chambers measuring 60 cm × 45 cm × 45 cm in a greenhouse at 25± 2°C. The possibility of biased sampling for a/virulence was not apparent due to segregation in clear Mendelian ratios to several resistance factors. In order to decipher the dominance of avirulence over virulence, number of genes associated with the inheritance of avirulence on different lines and involvement of any possible inhibitory or modifying factor responsible inheritance of avirulence, parents and F_1 progenies were screened on five differential lines (852 B, ICMB 01333, IP 18292, ICML 22 and 81 B P-6) along with susceptible check 7042 S. Both parents and all F_1 progenies were virulent on susceptible check 7042 S that implied no loss of virulence during the recombination.

4.3 Inheritance and molecular mapping of avirulence genes in *S. graminicola*

4.3.1 Selection of differential lines

To know the differential reactions of F_1 progenies, ten lines *viz.,* P 7-4, J 2480, ICMB 07111, ICMB 09999, ICMB 12444, 852 B, ICMB 01333, IP 18292, ICML 22 and 81 B P-6 were screened against the parents of F_1 progenies i.e Sg 019 and Sg 445-1 and based on screening result (refer Table 3.2 in Material and Methods), five lines (ICML 22, 852 B, ICMB 01333, 81B-P6 and IP 18292) exhibiting resistant reaction (<10% DI) to Sg 019 and susceptible reaction (>60% DI) to Sg 445-1 were selected to test the reaction for F_1 progenies.

4.3.2. Reaction of F_1 progenies on IP 18292 and 852 B

Sporangial inocula (1×10^6 ml^{-1}) of all 121 F_1 progenies and parents Sg 445 and Sg 019 were tested for their reaction as virulent or avirulent on 48 hours pot-grown seedlings (coleoptile stage) of all five selected differential lines *viz.,* 852 B, ICMB 01333, IP 18292, ICML 22 and 81 B P-6 along with a susceptible check 7042 S. Of the 121 F_1 progenies, 96 were found avirulent (A) and 25 virulent (V) on IP 18292. The segregation on 852 B was observed as 91 avirulent and 30 virulent F_1 progenies. This indicated that segregations of 121 F_1 progenies follows expected ratio of 3A:1V, the test cross ratio for two dominant genes (Table 4.9, Appendix G). Segregation of avirulence in F_1 generations, in 3:1 ratio,

implied the heterozygous nature of avirulence gene and involvement of two dominant genes in heterozygous state for avirulence in Sg 019. This result was largely in agreement with the other studies of segregation of avirulence in oomycetes (Norwood *et al.*, 1983., and Sicard *et al*. 2003). Michelmore *et al*. (1984) observed segregation of avirulence in F_1 progenies of the cross CG1 (virulent isolate of *Bremia lactucae*) x $IMF_1$7c (avirulent isolate of *B. lactucae*) towards R_{11} factor in the lettuce cultivar Capitan and reported the governance of avirulence by two independent dominant genes in the $IMF_1$7c isolate of *B. lactucae*. The presence of heterozygosity in *S. graminicola* is due to out-crossing nature of the pathogen that leads to random mating between two dissimilar thalli of opposite mating types. However, Pushpavathi *et al*. (2006c) observed segregations of a/virulence in F_2 generation after crossing the two homozygous parents for avirulence (AA) and virulence (aa) in *S. graminicola* and reported the governance of a/virulence by one gene on resistant factor of IP 18292. The probable genotypes for avirulence in Sg 019 and virulence in Sg 445-1 towards IP 18292 and 852B might be AaBb and aabb respectively (Table 4.10).

4.3.2.2 Segregation of F_1 progenies on ICMB 01333, ICML 22 and 81 B-P6

Segregation of avirulence in F_1 progenies on ICMB 01333 and ICML 22 was different from IP 18292 and 852B. The reaction of F_1s on ICMB 01333 and ICML 22 had a good fit to the segregation ratio of 1A:1V as 52 avirulent and 68 virulent progenies (Table 4.9). The results suggested the monogenic dominant nature of avirulence gene in Sg 019 towards the resistance gene of ICMB 01333 and ICML 22**.** Pushpavathi *et al*. (2006c) reported monogenic nature of governance of avirulence in the avirulent isolate (110-9) of *S. graminicola* to a corresponding resistance gene in IP 18292.

The differential segregation of avirulence gene(s) in Sg 019, monogenic towards ICMB 01333 and ICML 22 and digenic towards IP 18292 and 852B, might be due to dissimilar resistance genes between ICMB 01333/ICML 22 and IP 18292/852B. However, it needs to be verified through allelism study by making crosses between them. Norwood and Crute (1984) earlier reported similar segregation of avirulence in F_1 progenies of CS9 (virulent isolate of *B. lactucae*) x W5 (avirulent isolate of *B. lactucae*) in the ratio of 1A:1V (one dominant gene) towards resistant genes R_6 (cv. Sabine) and R_7 (cv. Mesa 659) whereas 3A:1V (two dominant gene) towards R_{11} (cv. Capitan) in lettuce cultivars. Similar findings were revealed by Sicard *et al*. (2003) in 97 F_1 progenies of a cross between avirulent and

virulent isolates of *B. lactucae* towards two different resistant genes R_3 as monogenic and R_{11} as digenic.

No segregation for avirulence was observed on 81 B-P6 to all 120 F_1 progenies suggesting complex nature of inheritance of avirulence. The complexity in inheritance of avirulence were reported earlier by Norwood and Crute (1984) in SF3 isolate of *Bremia lactucae* against R_1 factors (cv. Blondine) and postulated the presence of heterozygosity for inhibitory loci (AaIi). Contrastingly, Hulbert *et al*. (1988) and Ilott *et al*. (1989) speculated the occurrence of segregation distortion or genome instability or hyperploidy in the *Bremia* isolate that lead to loss of avirulence. Ilott *et al*. (1989) found complete loss of avirulence in the 17 progenies of the cross between CS9 (virulent) and CG1 (avirulent) against cv. Valmaine of Lettuce and confirmed the presence of homozygous inhibitory loci in CS9 as reported earlier by Norwood and Crute (1984). The complete loss of avirulence in F_1 progenies on 81B-P6 should be further verified by making crosses between another homozygous avirulent isolates with Sg 445-1. Therefore, the probable genotypes for avirulence in parents Sg 019 and virulence in Sg 445-1 might be Aa and aa, respectively on ICMB 01333 and ICML 22 (Table 4.10).

4.3.3 Bulk segregants analysis

The bulked segregants analysis (BSA) developed by Michelmore *et al*. (1991) in plant has been widely used by various workers for identifying genetic markers linked to genes of interest in the other eukaryotic genomes. BSA has proven to be a useful tool for identifying specific genes in fungi and oomycetes (Dioh *et al*., 2000 and Beattie *et al*., 2007). In the present investigation, out of 106 SSR primer pairs evaluated for polymorphism between two parents Sg 019 and Sg 445-1, 18 (Sg_SSR 01, Sg_SSR 07, Sg_SSR 09, Sg_SSR 13, Sg_SSR 38, Sg_SSR 45, Sg_SSR 46, Sg_SSR 47, Sg_SSR 49, Sg_SSR 65, Sg_SSR 68, Sg_SSR 92, Sg_SSR 93, Sg_SSR 95, Sg_SSR 98, Sg_SSR 99, Sg_SSR 102 and Sg_SSR 103) were identified as polymorphic. Among 18 primers, only three primers Sg_SSR 49, Sg_SSR 68 and Sg_SSR 99 revealed polymorphisms between two parents and four bulks. Thus, these three markers were used to genotype all F_1s to identify markers linked to Avr gene(s) (Fig. 4.4).

4.3.4 Genetic linkage among markers and Marker-trait association

Genotypic data of F_1 progenies generated using three SSR markers (Sg_SSR 49, Sg_SSR 68 and Sg_SSR 99) were analyzed through MAPMAKER/EXP 3.0 (Lincoln and Landers, 1987) (Fig. 4.5). Two markers Sg_SSR 49 and Sg_SSR 68 were found to be linked with a LOD value of 22.5 and genetic distance of 7.4 cM while third marker Sg_SSR 99 was unlinked (Table 4.11). Similar study of Tyler *et al*. (1995) revealed genetic linkage among four pairs of RFLP markers (LOD score of >3.0) and one group of three linked RFLP markers with the genetic distance ranged from 0 to 11cM. The association of markers with avirulence trait was performed using PlabQTL software with genotypic data of the putative linked microsatellite markers generated from the F_1 progenies as well as phenotypic data for avirulence/virulence of F_1 progenies on four differential lines. The genetic distance between marker Sg_SSR 49 and the avirulence gene in Sg 019 on IP 18292 and ICMB 01333 was found to be 0 cM (Table 4.12, Fig. 4.6). However, second gene governing avirulence in Sg 019 on IP 18292 and 852 B could not be mapped. The LOD value for marker Sg_SSR 49 and avirulence on three differential lines ICML 22, IP 18292 and ICMB 01333 varied from 3.38 to 7.43 and 9.66 to 9.79 for Sg_SSR 68 on ICMB 01333 and IP 18292 (Table 4.12). The data were further subjected to linear model regression analysis and significance of R^2 was identified using F value comparison. The significant R^2 values higher than 10 for the trait of avirulence on three host differentials *viz*., IP 18292, ICMB 01333 and ICML 22 with Sg_SSR 49 and two host differentials IP 18292 and ICMB 01333 with Sg_SSR 68 indicated the linkage of these markers with avirulence loci in Sg 019 (Table 4.12). In the same way, genetic linkage was reported among RAPD markers and avirulence gene by Whisson *et al*. (1994); RFLP marker and avirulence trait by Tyler *et al*. (1995) in *P. sojae* and AFLP marker with avirulence trait by Sicard *et al*. (2003) in *B. lactucae*. Genetic linkage map could not be developed in the present study due to paucity of polymorphic markers.

Fig 4.4. SSR markers detecting polymorphisms between parents avirulent (A, Sg 019), virulent (V, Sg 445-1) and four bulks assembled from phenotyping of 120 F_1 progenies of *S. graminicola*

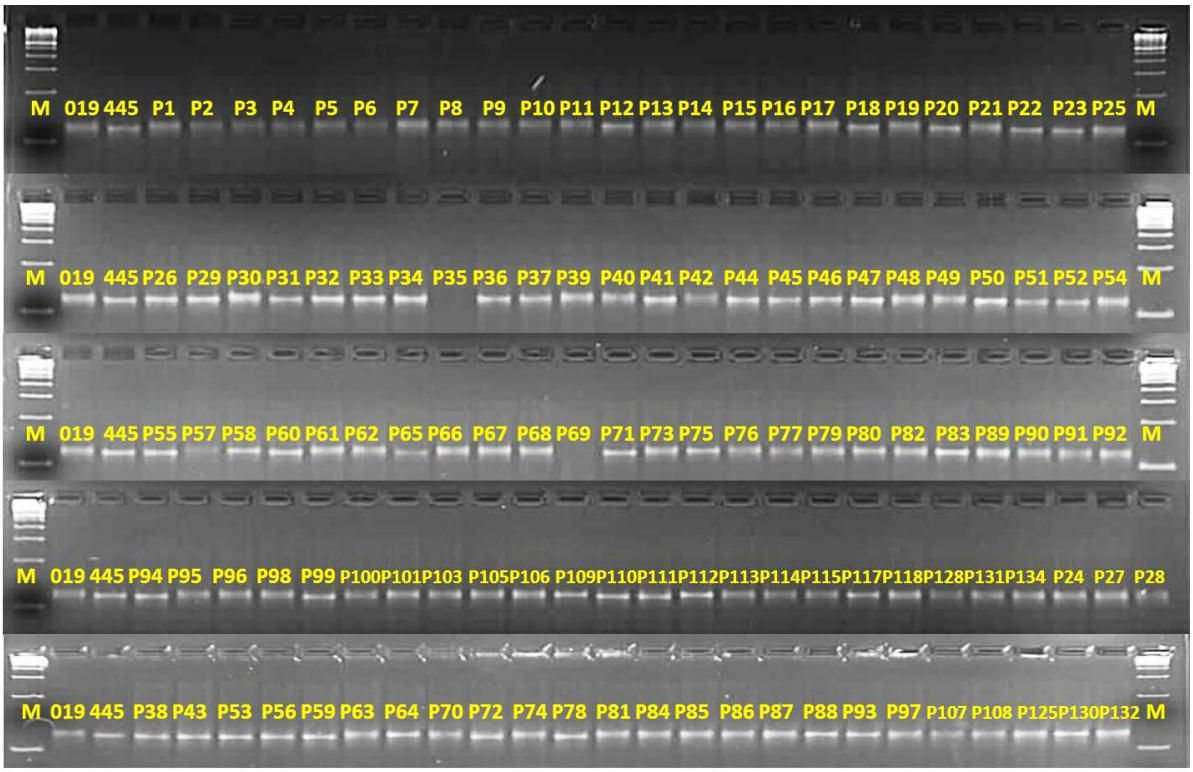


Fig 4.5. Genotyping with marker Sg_SSR 49 linked to avirulence gene in 121 F_1 progenies of *S. graminicola* derived from Sg 019 × Sg445-1. M= 100 bp ladder

Table 4.11. Segregation of linked makers in F_1 progenies of *Sclerospora graminicola*

Marker	Repeat Motif/ region	Primers	Marker analysis		Dist. (cM)	Lod score
			Allele 1[a]	Allele 2[b]		
Sg_SSR 49	$(TGC)_5$ (scaffold 992_size46649)	AAACGTGGCTGGGTATCTGG TGACCCTCGATTTGTGGACG	77	42	7.4	22.5
Sg_SSR 68	$(TCT)_5$ (scaffold 3537_size13791)	CTCTTTGTTGCAACCACGGG CCAGCAGCTGATAGGGATGG	80	39		
Sg_SSR 99	$(ATAGTA)_7$ (scaffold 3540_size13786)	GCTTCACGCTTGTTGCTGAC CGCAAGCCTACTACCACCTC	81	38	Unlinked	

[a] Allele 1- 130 bp (avirulent fragment) [b] Allele 2- 120 bp (virulent fragment)

Table 4.12. Marker trait association in the F_1 progenies of *Sclerospora graminicola*

Trait	Marker	Lines	*R^2 (%)	*R^2adj. (%)	LOD value	Distance (cM)
Avirulence	Sg_SSR 68	IP 18292	31.3±7.0	30.7	9.79	7.4
	Sg_SSR 49	IP 18292	23.5±6.8	22.2	6.96	0
	Sg_SSR 68	ICMB 01333	31.0±7.0	30.4	9.66	7.4
	Sg_SSR 49	ICMB 01333	24.8±6.8	23.5	7.43	0
	Sg_SSR 49	ICML 22	12.2±5.6	11.4	3.38	-

*R^2 values are significant at $p<0.05$ and $p<0.01$ level of significance.

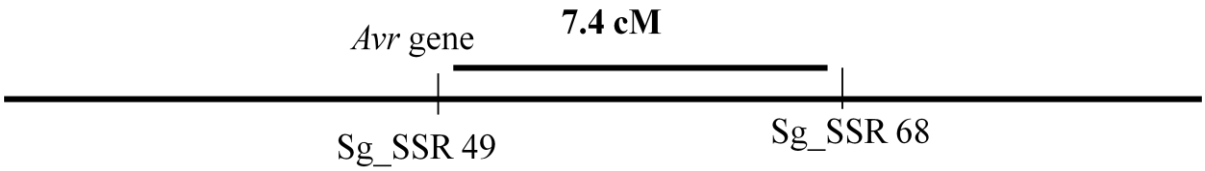


Fig 4.6. Proposed tagging of putative marker Sg_SSR 49 to avirulent gene in *S. graminicola* isolate Sg 019

A comprehensive genetic linkage map assembled from avirulence phenotypes and closely associated molecular markers will provide a vital starting point for cloning and characterizing of avirulence genes in *S. graminicola*. Cloning and characterizing avirulence genes serve as basic tools for the attempt directed towards revealing the nature and functions of factors in plant pathogens that induce or overcome resistance in the host (May *et al*., 2002). Numerous avirulence genes that have been isolated from bacterial, viral, and fungal plant pathogens encode putative proteins with diverse sequence and structural characteristics (Leach and White, 1996 and De Wit and Joosten, 1999). However, neither avirulence genes nor avirulence gene product has been described in *S. graminicola*. Clear information on avirulence genetics will aid in deciphering the molecular events involved in pathogenesis and in understanding plant- pathogen interactions in general.

4.3.5 Validation of marker

In order to determine the diagnostic value of avirulence linked microsatellite marker for the purpose of marker assisted selection, a set of 60 isolates of *S. graminicola*, collected from different pearl millet growing regions of India, were phenotyped on pearl millet lines IP 18292 and genotyped by linked SSR marker Sg_SSR 49. Isolates expected to carry avirulence gene showed high level of avirulence to the cultivar IP 18292 (Table 4.13, Fig 4.7). Genotyping with avirulence linked marker Sg_SSR 49 amplified fragment size of 130 bp (Allele 1) and was associated with the presence of avirulent gene in 44 isolates that were shown avirulent reaction on IP 18292 (Table 4.13).

4.4 Inheritance of downy mildew resistance in pearl millet genotypes and allelic relationships among resistance genes

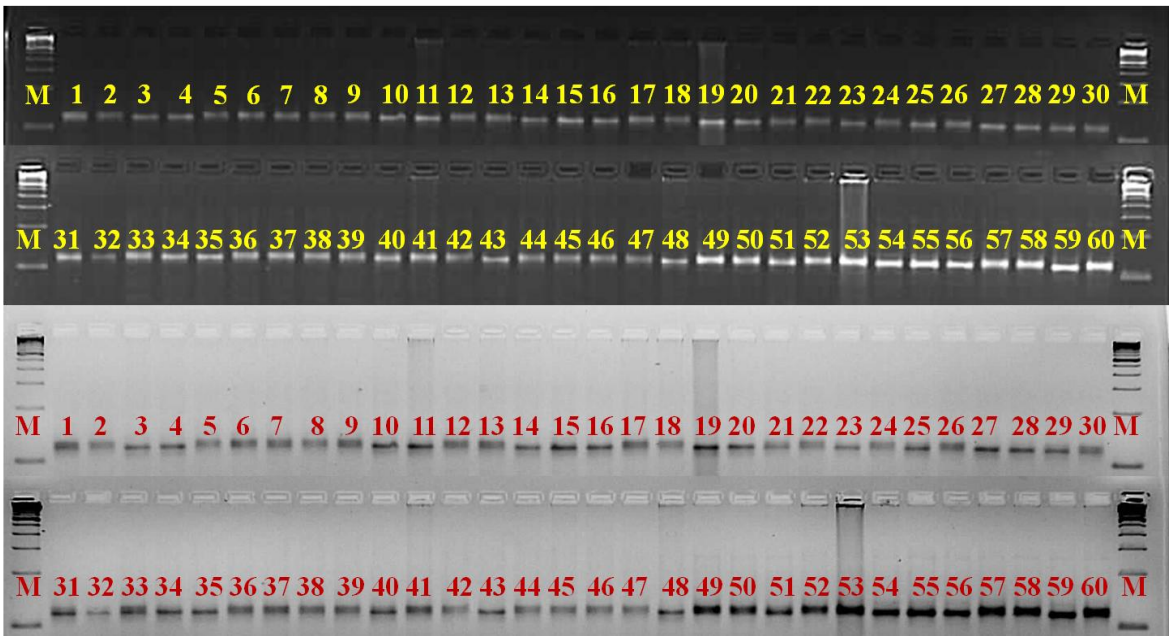
Pearl millet is a diploid crop and parent materials selected for this study was homozygous inbred lines developed after selfing for number of generations. Maternal effect for downy mildew resistance/ susceptibility have been reported to be absent in most of the studies (Aanand kumar *et al*., 1983., Yadav *et al*., 1993 and Yadav, 1994 and Yadav, 1996). Resistance or susceptibility in pearl millet-downy mildew pathosystem is measured in absolute relative terms of percentage. Hence, complete susceptibility or resistance in pearl millet is a rare phenomenon as host and pathogen are highly out-crossable in nature. Therefore in this study, pearl millet lines with ≥ 90% DI and ≤ 10% DI were selected as susceptible and resistant source, respectively. The presence of residual variability for

resistance to downy mildew in apparently susceptible genotypes has been documented by Singh *et al*. (1988). Similarly, variability for susceptibility in apparently resistant genotypes has also been reported by Singh and Talukdar (1988). To facilitate the selection of single plant derivatives with complete resistance and complete susceptibility, the process of selfing and single plant selection was followed to reveal such hidden variability. To ascertain that the gene(s) conferring residual variability for resistance to downy mildew from the susceptible parent does not interfere with the segregation pattern of resistance genes from the resistant parent, and vice versa, creation of homozygosity in both parents was considered absolute necessary. Therefore, selected lines were selfed for three consecutive generations to obtain true pure inbreed lines. Since, pearl millet is a protogynous species with a large number of small florets on its inflorescences, chances of self pollination of some florets, in the absence of emasculation for crossing, do exist. Therefore, the resistant plants in F_1 generations were selected to generate F_2 and back cross generations.

Table 4.13. Disease reactions of 60 *S. graminicola* isolates on pearl millet genotype IP 18292 and genotyping of all isolates with Sg_SSR 49 marker

S.No	Isolates	Reaction	Fragment size (bp)*	S.No	Isolates	Reaction	Fragment size (bp)*
1	Sg 543	Avirulent	130	31	Sg 200	Avirulent	130
2	Sg 560	Avirulent	130	32	Sg 212	Avirulent	130
3	Sg 561	Avirulent	130	33	Sg 384	Virulent	120
4	Sg 569	Avirulent	130	34	Sg 445	Virulent	120
5	Sg 573	Virulent	120	35	Sg 492	Avirulent	130
6	Sg 575	Avirulent	130	36	Sg 519	Virulent	120
7	Sg 578	Virulent	120	37	Sg 542	Avirulent	130
8	Sg 580	Virulent	120	38	Sg 598	Virulent	120
9	Sg 587	Virulent	120	39	Sg 566	Avirulent	130
10	Sg 590	Virulent	120	40	Sg 576	Avirulent	130
11	Sg 591	Avirulent	130	41	Sg 586	Avirulent	130
12	Sg 592	Virulent	120	42	Sg 545	Avirulent	130
13	Sg 593	Virulent	120	43	Sg 526	Avirulent	130
14	Sg 595	Avirulent	130	44	Sg 457	Avirulent	130
15	Sg 596	Avirulent	130	45	Sg 557	Virulent	130
16	Sg 599	Avirulent	130	46	Sg 558	Virulent	130
17	Sg 603	Virulent	120	47	Sg 559	Virulent	130
18	Sg 604	Virulent	120	48	Sg 562	Avirulent	120
19	Sg 048	Avirulent	130	49	Sg 564	Avirulent	120
20	Sg 151	Avirulent	130	50	Sg 571	Avirulent	120
21	Sg 153	Avirulent	130	51	Sg 574	Avirulent	120
22	Sg 597	Avirulent	130	52	Sg 585	Virulent	130
23	Sg 018	Avirulent	130	53	Sg 589	Virulent	130
24	Sg 139	Avirulent	130	54	Sg 594	Avirulent	120
25	Sg 409	Virulent	120	55	Sg 601	Avirulent	120
26	Sg 431	Avirulent	130	56	Sg 521	Virulent	130
27	Sg 510	Avirulent	130	57	Sg 150	Avirulent	120
28	Sg 529	Virulent	120	58	Sg 298	Avirulent	120
29	Sg 019	Avirulent	130	59	Sg 546	Avirulent	120
30	Sg 021	Avirulent	130	60	Sg 556	Avirulent	120

*****fragment size 130bp-Allele1-avirulent; 120bp-Allele2-virulent reaction



M- Marker, **1.** Sg 543 **2**. Sg 545 **3**. Sg 546 **4.** Sg 556 **5**. Sg 557 **6**.Sg 558 **7**. Sg 559 **8**. Sg 560 **9**. Sg 561 **10**. Sg 562 **11**. Sg 564 **12**. Sg 566 **13**. Sg 569 **14**. Sg 571 **15**. Sg 573 **16**. Sg 574 **17**. Sg 575 **18**. Sg 576 **19**. Sg 578 **20**. Sg 580 **21**. Sg 585 **22**. Sg 586 **23**. Sg 587 **24**. Sg 589 **25**. Sg 590 **26**.Sg 591 **27**. Sg 592 **28**. Sg 593 **29**. Sg 594 **30**. Sg 595 **31**. Sg 596 **32**. Sg 598 **33**. Sg 599 **34**. Sg 603 **35**. Sg 604 **36**. Sg 048 **37**. Sg 151 **38**. Sg 153 **39**. Sg 597 **40**. Sg 601 **41**. Sg 018 **42**. Sg 139 **43**. Sg 409 **44**. Sg 431 **45**. Sg 510 **46**. Sg 521 **47**. Sg 526-1 **48**. Sg 529 **49**. Sg 019 **50**. Sg 021 **51**. Sg 150 **52**. Sg 200-1 **53**. Sg 212 **54**. Sg 298 **55**. Sg 384 **56**. Sg 445 **57**. Sg 457 **58**. Sg 492 **59**. Sg 519 **60**. Sg 542-1

Fig 4.7. Genotyping of Sg_SSR 49 marker on 60 *S. graminicola* isolates

4.4.1 Inheritance of resistant gene in pearl millet lines to *S. graminicola* isolates

The inheritance study results of all the parents and the different generations (F_1, F_2, BC_1P_1 and BC_1P_2) from each cross to three isolates of *S. graminicola* (Sg 200-1, Sg 526-1 and Sg 542-1) are summarized in Table 4.14, 4.15 and 4.16. The Susceptible parent 81B was severely infected, showing >90% of disease incidence (DI), while resistant parents 834 B, IP 18294-P1 and IP 18298-P1 expressed <10% DI to all the three isolates.

4.4.1.1 81B × 834B to Sg 200-1

The results of inheritance study are summarized in Table 4.14. A total of 109 plants of F_1, 941 plants of F_2, 304 plants of BC_1P_1 and 383 plants of BC_1P_2 were screened to Sg 200-1. Almost all plant of F_{1s} and BC_1P_2 was observed as resistant. In F_2 generation, resistant and susceptible plants were found in 619 and 222 in number, respectively. Out of 304 plants of BC_1P_1, 160 plants were found as resistant whereas 149 plants displayed susceptible reactions.

4.4.1.2 81B × 834B to Sg 526-1

The results of inheritance study are shown in Table 4.15. A total of 108 plants of F_1, 850 plants of F_2, 383 plants of BC_1P_1 and 427 plants of BC_1P_2 were screened to Sg 526-1. Almost all plant of F_{1s} and BC_1P_2 showed as resistant reactions. In F_2 generation, resistant and susceptible plants were found in 651 and 199 in number, respectively. Out of 383 plants of BC_1P_1, 196 plants were found as resistant whereas 187 plants exhibited susceptible reactions.

4.4.1.3 81B × 834B to Sg 542-1

The results of inheritance study are detailed in Table 4.16. A total of 109 plants of F_1, 905 plants of F_2, 335 plants of BC_1P_1 and 385 plants of BC_1P_2 were screened to Sg 542-1. Almost all plant of F_{1s} and BC_1P_2 exhibited resistant reaction. In F_2 generation, resistant and susceptible plants were found in 677 and 228 in number, respectively. Out of 335 plants of BC_1P_1, 176 plants were found as resistant whereas 159 plants showed susceptible reactions.

4.4.1.4 81B × IP 18294-1 to Sg 200-1

The results of inheritance study are summarized in Table 4.14. A total of 92 plants of F_1, 692 plants of F_2, 302 plants of BC_1P_1 and 274 plants of BC_1P_2 were screened to Sg 200-1. Almost all plant of F_{1s} and BC_1P_2 was observed as resistant. In F_2 generation, resistant and susceptible plants were found in 533 and 159 in number, respectively. Out of 302 plants of BC_1P_1, 155 plants were found as resistant whereas 147 plants exhibited susceptible reactions.

4.4.1.5 81B × IP 18294-1 to Sg 526-1

The results of inheritance study are described in Table 4.15. A total of 122 plants of F_1, 657 plants of F_2, 409 plants of BC_1P_1 and 385 plants of BC_1P_2 were screened to Sg 526-1. Almost all plant of F_{1s} and BC_1P_2 exhibited resistant reaction to the pathogen isolate. In F_2 generation, resistant and susceptible plants were found in 506 and 151 in number, respectively. Out of 409 plants of BC_1P_1, 218 plants were found as resistant whereas 199 plants showed susceptible reactions.

4.4.1.6 81B × IP 18294-1 to Sg 542-1

The results of inheritance study are summarized in Table 4.16. A total of 114 plants of F_1, 730 plants of F_2, 323 plants of BC_1P_1 and 306 plants of BC_1P_2 were screened to Sg 542-1. Almost all plant of F_{1s} and BC_1P_2 showed resistant reaction to the pathogen. In F_2 generation, resistant and susceptible plants were found in 558 and 172 in number, respectively. Out of 323 plants of BC_1P_1, 175 plants were found as resistant whereas 148 plants showed susceptible reactions.

4.4.1.7 81B × IP 18298-1 to Sg 200-1

The results of inheritance study are summarized in Table 4.14. A total of 150 plants of F_1, 765 plants of F_2, 322 plants of BC_1P_1 and 322 plants of BC_1P_2 were screened to Sg 200-1. Almost all plant of F_{1s} and BC_1P_2 was observed as resistant. In F_2 generation, resistant and susceptible plants were found in 715 and 50 in number, respectively. Out of 322 plants of BC_1P_1, 241 plants were found as resistant whereas 81 plants exhibited susceptible reactions.

4.4.1.7 81B × IP 18298-1 to Sg 526-1

The results of inheritance study are described in Table 4.15. A total of 140 plants of F_1, 787 plants of F_2, 422 plants of BC_1P_1 and 374 plants of BC_1P_2 were screened to Sg 526-1. Almost all plant of F_{1s} and BC_1P_2 exhibited resistant reaction to the pathogen isolate. In F_2 generation, resistant and susceptible plants were found in 735 and 52 in number, respectively. Out of 422 plants of BC_1P_1, 311 plants were found as resistant whereas 111 plants showed susceptible reactions.

4.4.1.7 81B × IP 18298-1 to Sg 542-1

The results of inheritance study are summarized in Table 4.16. A total of 122 plants of F_1, 859 plants of F_2, 369 plants of BC_1P_1 and 288 plants of BC_1P_2 were screened to Sg 542-1. Almost all plant of F_{1s} and BC_1P_2 showed resistant reaction to the pathogen. In F_2 generation, resistant and susceptible plants were found in 823 and 36 in number, respectively. Out of 369 plants of BC_1P_1, 281 plants were found as resistant whereas 88 plants showed susceptible reactions.

Overall the F_1 generations of the crosses, 81B × 834B, 81B × IP 18294-P1, and 81B × IP 18298-P1, exhibited almost resistance (R) reaction to each isolate. The almost all resistant plants in F_1 generations of the three crosses (81B × 834B, 81B × IP 18294-P1 and 81B × IP 18298-P1) to each isolate indicating that the resistance in these lines is governed by dominant gene(s). The F_2 generations of each of three S × R crosses (81B × 834B, 81B × IP 18294-P1, 81B × IP 18298-P1) and corresponding BC_1P_1 generations 81B × (81B × 834B), 81B × (81B × IP 18294-P1) and 81B × (81B × IP 18298-P1) showed clear cut segregations to all the three isolates for resistant and susceptible plants. The resistance and susceptible plants in F_2 generations of the cross 81B × 834B and 81B × IP 18294-P1 showed a good fit to the segregation ratio of 3R:1S to all three isolates, suggesting dominant monogenic control of DM resistance in 834B and IP 18294-P1. The corresponding $BC_1P_{1s,}$ 81B × (81B × 834B) and 81B × (81B × IP 18294-P1), had good fit to 1R:1S ratio expected for monogenic inheritance to each isolate. However, the F_2 populations from the cross 81B × IP 18298-P1 exhibited a good fit to the segregation ratio of 15R:1S to all three isolates indicating the involvement of two dominant genes for resistance in IP 18298-P1. The corresponding $BC_1P_{1,}$ 81B × (81B × IP 18298-P1), showed 3R:1S ratio anticipated for digenic inheritance to each isolates. Whereas, all plants of three

BC_1P_2s, 834B × (81B × 834B), IP 18294-P1 × (81B × IP 18294-P1) and IP 18298-P1 × (81B × IP 18298-P1) expressed resistant reaction to each isolate.

4.4.1.8 Allelism tests

4.4.1.8.1 834B × IP 18294-1 to Sg 200-1

The results of allelism study are summarized in Table 4.17. A total of 715 plants of F_2 populations were screened to Sg 200-1. Out of 715 plants, 666 showed resistant reaction whereas 49 plants exhibited susceptible reactions to the pathogen isolate Sg 200-1.

4.4.1.8.2 834B × IP 18294-1 to Sg 526-1

The results of allelism study are depicted in Table 4.18. A total of 690 plants of F_2 generations were screened to *S. graminicola* isolate Sg 526-1. Out of 690, 640 plants expressed resistant reaction whereas 50 plants exhibited susceptible reactions to the pathogen.

4.4.1.8.3 834B × IP 18294-1 to Sg 542-1

The results of allelism study are detailed in Table 4.19. A total of 383 plants of F_2 populations were screened to *S. graminicola* isolate Sg 542-1. Out of 383 plants, 356 exhibited resistant reaction whereas 27 plants showed susceptible reactions to the pathogen.

4.4.1.8.4 834B × IP 18298-1 to Sg 200-1

The results of allelism study are summarized in Table 4.17. A total of 715 plants of F_2 generations were screened to Sg 200-1. All plants showed resistant reactions to the pathogen isolate.

4.4.1.8.5 834B × IP 18298-1 to Sg 526-1

The results of allelism study are described in Table 4.18. A total of 866 plants of F_2 populations were screened to Sg 526-1. No segregations were observed to this pathogen isolate.

4.4.1.8.6 834B × IP 18298-1 to Sg 542-1

The results of allelism study are detailed in Table 4.19. A total of 913 plants of F_2 generations were screened to Sg 542-1. All plants exhibited resistant reactions to the pathogen isolate.

4.4.1.8.7 IP 18284-1 × IP 18298-1 to Sg 200-1

The results of allelism study are summarized in Table 4.17. A total of 717 plants of F_2 generations were screened to Sg 200-1 and all plants showed resistant reactions to the pathogen isolate.

4.4.1.8.8 IP 18284-1 × IP 18298-1 to Sg 526-1

The results of allelism study are described in Table 4.18. A total of 687 plants of F_2 populations were screened to Sg 526-1. No segregations were observed to the pathogen isolate.

4.4.1.8.8 IP 18284-1 × IP 18298-1 to Sg 542-1

The results of allelism study are summarized in Table 4.19. A total of 729 plants of F_2 generations were screened to Sg 542-1 and all plants showed resistant reactions to the pathogen isolate.

Allelism tests were conducted by crossing all three resistant parents with each other (834B, IP 18294-P1 and IP 18298-P1) to know whether the same gene (s) or different genes governs resistance in these genotypes to the test isolates. The results are summarized in Table 4.17, 4.18 and 4.19. The F_1 generations of the crosses, 834B × IP 18294-P1, 834B × IP 18298-P1 and IP 18294-P1 × IP 18298-P1, displayed almost resistance reaction to each isolate (data not shown). Out of three crosses (834B × IP 18294-P1, 834B × IP 18298-P1 and IP 18294-P1 × IP 18298-P1), 834B × IP 18294-P1 showed a good fit to the segregation ratio of 15R:1S to all the three isolates suggesting the involvement of two independent dominant resistance genes. This concluded that the resistance gene in 834B is different to the resistance gene present in IP 18294-P1. The lack of segregation in the F_2 populations of the cross 834B × IP 18298-P1 and IP 18294-P1 × IP 18298-P1 to all three isolates indicated allelic relationship between resistance gene of IP 18298-P1 with both 834 B and IP 18294-P1. These results further confirm the presence of two dominant resistance

genes in IP 18298-P1 to all three isolates of *S. graminicola*. Out of these two resistant genes, one gene is present in 834B and the second gene is present in IP 18294-P1.

The simple mode of inheritance of resistance determined by single gene is perhaps the most common (Mendelian) type in host-pathogen interactions. However, it is also possible for genetic studies to reveal two or more genes for resistance to the same pathogen in one host. Single (Appadurai *et al*., 1975 and Singh and Talukdar, 1998), two or more dominant genes acting independently (Gill *et al*., 1978 and Joshi and Ugale, 2002) have been found in one line of pearl millet to control the resistance to downy mildew. Resistance was also found to be governed by recessive gene (Singh *et al*., 1980 and Pethani *et al*. 1980) and multiple genes with epistatic effect (Deswal and Govilla, 1994). In the present study, resistance in 834 B and IP 18294-P1 to all three isolates was observed to be governed by single dominant gene. Two dominant genes appeared to control resistance in IP 18298-P1 to all three isolates. Differential digenic control of resistance to different pathotypes of *S. graminicola* was earlier observed by Deswal and Govilla (1994). In contrast, the findings of Joshi and Ugale (2002) explained the combinations of dominant and recessive genes as tri- and tetragenic interactions in pearl millet to the downy mildew pathogen.

Deswal and Govilla (1994) observed no segregation in F_2 generations of a cross of two resistant cultivars of pearl millet and suggested same gene for resistance to downy mildew in both the cultivars. The allelic relationship between resistance genes to three isolates of *S. graminicola* in this study suggested that 834 B and IP 18294-P1 contain one non-allelic dominant gene each designated as *RSg2* and *RSg3* respectively (Table 4.20). The designation of *Rsg1* has been attributed to the resistance gene identified in IP 18292 in the previous work of Singh and Talukdar (1998). Two dominant resistance genes in IP 18298-P1 to all three isolates in this study were symbolized as *RSg2* and *RSg3* (Table 4.20). The high resistance level of IP 18298 in this study is well supported by the findings of Singh (1995) where IP 18298 were found resistant to several field populations of *S. graminicola* in pearl millet-DM field nurseries in India and Western Africa. The multiple-pathotypes (10) resistance of IP 18298 in the study of Thakur *et al*. (2009) also affirmed the high level of resistance in IP 18298 than IP 18294. Nevertheless, there could be possibilities of having more number of DM-resistant genes in these lines that would be revealed by involving more number of resistant lines and more number of diverse *S.*

graminicola isolates. The lines used in this study, IP 18294-P1 and IP 18298-P1 and 834B have origin from three different countries, Mali and Burkina Faso and ICRISAT (India), respectively. This geographical diversity of lines could be representing diversity for resistance genes as well (Caicedo, 2008). Therefore, use of resistant sources of diverse origin in the breeding programs can help to diversify and broaden the genetic base for DM resistance in pearl millet to cope up with the evolving virulences of the pathogen.

The results obtained here have important implications in breeding programs which aim to deploy pathotype-specific DM resistant genes or pyramid different genes conferring resistance to different pathotypes of *S. graminicola* into the elite cultivars. According to Thakur *et al*. (2008), pyramiding of genes is a strategy to develop varieties with durable resistance. The accumulation of resistance genes with major effects delays the appearance of new races of the pathogen. The basis for this stability of resistance is the decrease in pathogen fitness when a number of virulence genes are necessary to overcome the resistance of the host (Van Der Plank, 1984). Therefore, a potential strategy in order to maintain disease resistance for a long period of time would be the introgression of several resistance genes in a single variety. The data obtained in the present work allow the breeder to choose a number of sources of resistance for pyramiding, in order to put together the best possible combination of genes in new cultivars. These varieties expressing durable resistance would be, therefore, resistant to a large number of pathotypes of the pathogen over a long period of time.

Table 4.14. Segregation analysis for resistance in the pearl millet lines and in the populations derived from crosses between them to *S. graminicola* isolates Sg 200-1

Cross	Generations	Number of Plants[a]		Expected ratio[b]	X^2	*P* val[c]	R factors
		R	S				
81B × 834B	81B	24	199	0:1			1D
	834B	193	11	1:0			
	F_1	106	3	1:0			
	F_2	619	222	3:1	0.87	0.35	
	BC_1P_1	160	149	1:1	0.39	0.53	
	BC_1P_2	369	14	1:0			
81B × IP 18294-P1	81B	24	199	0:1			1D
	IP 18294-P1	201	5	1:0			
	F_1	91	1	1:0			
	F_2	533	159	13:3	1.51	0.22	
	BC_1P_1	155	147	1:1	0.21	0.64	
	BC_1P_2	265	9	1:0			
81B × IP 18298-P1	81B	24	199	0:1			2D
	IP 18298-P1	218	0	1:0			
	F_1	147	3	1:0			
	F_2	715	50	15:1	0.1	0.74	
	BC_1P_1	241	81	3:1	0.00	0.95	
	BC_1P_2	320	2	1:0			

[a]R = resistant; S = susceptible. [b] all possible phenotypic ratio were analyzed and best explained one was shown in the table. [C]*P* = probability.

Table 4.15. Segregation analysis for resistance in the pearl millet lines and in the populations derived from crosses between them to *S. graminicola* isolates Sg 526-1

Cross	Generations	Number of Plants[a]		Expected ratio[b]	X^2	*P* val[c]	R factors
		R	S				
81B × 834B	81B	20	202	0:1			1D
	834B	204	2	1:0			
	F_1	100	8	1:0			
	F_2	651	199	3:1	1.14	0.28	
	BC_1P_1	196	187	1:1	0.21	0.64	
	BC_1P_2	416	11	1:0			
81B × IP 18294-P1	81B	20	202	0:1			1D
	IP 18294-P1	272	24	1:0			
	F_1	120	2	1:0			
	F_2	506	151	3:1	1.42	0.23	
	BC_1P_1	218	191	1:1	1.78	0.18	
	BC_1P_2	357	28	1:0			
81B × IP 18298-P1	81B	20	202	0:1			2D
	IP 18298-P1	161	0	1:0			
	F_1	140	0	1:0			
	F_2	735	52	15:1	0.17	0.67	
	BC_1P_1	311	111	3:1	0.38	0.53	
	BC_1P_2	371	3	1:0			

[a]R = resistant; S = susceptible. [b] all possible phenotypic ratio were analyzed and best explained one was shown in the table. [C]*P* = probability.

Table 4.16. Segregation analysis for resistance in the pearl millet lines and in the isolates derived from crosses between them to *S. graminicola* isolates Sg 542-1

Cross	Generations	Number of Plants[a]		Expected ratio[b]	X^2	*P* val[c]	R factors
		R	S				
81B × 834B	81B	23	205	0:1			1D
	834B	201	1	1:0			
	F_1	106	3	1:0			
	F_2	677	228	3:1	0.02	0.89	
	BC_1P_1	176	159	1:1	0.86	0.35	
	BC_1P_2	366	19	1:0			
81B × IP 18294-P1	81B	23	205	0:1			1D
	IP 18294-P1	193	7	1:0			
	F_1	107	7	1:0			
	F_2	558	172	3:1	0.80	0.37	
	BC_1P_1	175	148	1:1	2.257	0.133	
	BC_1P_2	296	10	1:0			
81B × IP 18298-P1	81B	23	205	0:1			2D
	IP 18298-P1	198	0	1:0			
	F_1	122	0	1:0			
	F_2	823	36	15:1	0.48	0.49	
	BC_1P_1	281	88	3:1	0.26	0.61	
	BC_1P_2	287	1	1:0			

[a]R **=** resistant; S **=** susceptible. [b] all possible phenotypic ratio were analyzed and best explained one was shown in the table. [C]*P* **=** probability.

Table 4.17. Allelism test for genetic characterization of the resistance to *S. graminicola* isolates Sg 200-1

S.No	F_2 populations[a] (P_1x P_2)	Number of F_2 Plants[b]		Expt. ratio[c]	X^2	*P val*[d]	R factors	
		R	S				P_1	P_2
1	834B × IP18294-P1	666	49	15:1	0.444	0.505	1D	1D
2	834B × IP18298-P1	715	0	-	-	-	-	-
3	IP18294-P1 × IP18298-P1	717	0	-	-	-	-	-

[a]All crosses come from resistant × resistant parents. [b]R = resistant; S = susceptible. [c]all possible phenotypic ratio were analyzed and best explained one was shown in the table. [d]*P* = probability. [e] P_1-Parent 1; P_2-Parent 2

Table 4.18. Allelism test for genetic characterization of the resistance to *S. graminicola* isolates Sg 526-1

S.No	F_2 populations[a] (P_1x P_2)	Number of F_2 Plants[b]		Expt. ratio[c]	X^2	*P val*[d]	R factors	
		R	S				P_1	P_2
1	834B × IP18294-P1	640	50	15:1	1.169	0.279	1D	1D
2	834B × IP18298-P1	866	0	-	-	-	-	-
3	IP18294-P1 × IP18298-P1	687	0	-	-	-	-	-

[a]All crosses come from resistant × resistant parents. [b]R = resistant; S = susceptible. [c]all possible phenotypic ratio were analyzed and best explained one was shown in the table. [d]*P* = probability. [e] P_1-Parent 1; P_2-Parent 2

Table 4.19. Allelism test for genetic characterization of the resistance to *S. graminicola* isolates Sg 542-1

S.No	F_2 populations[a] (P_1x P_2)	Number of F_2 Plants[b]		Expt. ratio[c]	X^2	*P val*[d]	R factors	
		R	S				P_1	P_2
1	834B × IP18294-P1	356	27	15:1	0.420	0.517	1D	1D
2	834B × IP18298-P1	913	0	-	-	-	-	-
3	IP18294-P1 × IP18298-P1	729	0	-	-	-	-	-

[a]All crosses come from resistant × resistant parents. [b]R = resistant; S = susceptible. [c]all possible phenotypic ratio were analyzed and best explained one was shown in the table. [d]*P* = probability. [e] P_1-Parent 1; P_2-Parent 2

Table 4.20. Proposed dominant resistant genes in pearl millet lines that confer resistance to downy mildew against *S. graminicola*

Resistant source	Genes (allele)
834B	*RSg2*
IP 18294-P1	*RSg3*
IP 18298-P1	*RSg2*
	RSg3

Summary and conclusions

CHAPTER V
SUMMARY AND CONLUSIONS

In the present investigation, studies pertaining to development of simple sequence repeat markers and its validation, inheritance and molecular mapping of avirulence and inheritance and allelic study of resistance genes in pearl millet to the isolates of *S. graminicola* were carried out. All the experiments were conducted at ICRISAT, Patancheru, Telangana, India. The results obtained are summarized below.

A total of 14481 sequences spanning 215561828 bp were screened to find simple sequence repeats (SSRs) using a microsatellite search tool, MISA. From 3912 SSR containing sequences, in total of 7453 SSRs were identified, of which 235 (3.3%) SSRs were of compound type and remaining 7218 (96.7%) were of perfect type. The overall relative abundance and density of SSRs in whole genome were 34.57/Mb and 459.67bp/Mb respectively.

A total of 2702 Simple sequence repeat primers were developed through *in-silico* method. Out of 2702 markers, 106 were custom synthesized and screened for diversity analysis with 60 isolates of *Sclerospora graminicola*. The gel electrophoresis result showed 22 scorable polymorphic markers. Allele frequencies i.e 57 alleles with an average of 2.59 alleles per locus were detected. The frequency of major alleles (M_{AF}) per locus was 0.47-0.94, with an average of 0.72. In addition, the H_O values were 0.00- 0.76 with an average of 0.26, and H_E values were 0.12- 0.62 with an average of 0.39. The polymorphic index content (PIC) values were 0.11- 0.60, with an average of 0.33. The PCR product sizes were ranged between 110-320 bp. Dendrogram developed through neighbour joining (NJ) method across 60 *S. graminicola* populations revealed five major clusters. Analysis of molecular variance (AMOVA) revealed 56.21% of the variance over 22 SSRs loci due to the differences among the isolates within states, 35.28% due to within individual isolates and 8.51% was due to differences among the states. Genetic analysis of 60 *S. graminicola* isolates at high delta K value using an Evanno-based approach provided evidence for the presence of two genetically distinct population structures with six admixtures. However, unstable graph of delta K verses K, suggested that there could be more differentiation of *S.*

graminicola populations. Besides, *S. graminicola* primer pairs also amplified the fragments from other oomycetes *P. sorghi* (67.9%) and *Phytophthora* species (52.7%).

Two distinct mating types Sg 019 (*Mat 2/A_2*) and Sg 445-1/Sg 018 (*Mat 1/A_1*) were selected for generation of segregating population. From the cross between Sg 019 and Sg 445-1, 121 F_1 progenies were established separately from oospores on the susceptible line 7042S. In order to decipher the nature of genes responsible for inheritance of avirulence, Parents and 121 F_1 progenies were screened on five differential lines (IP 18292, 852B, ICMB 01333, ICML 22 and 81B-P6) along with susceptible check 7042 S. Avirulence in Sg 019 was segregated in F_1 progenies on IP 18292 and 852 B in the ratio of 3:1 (avirulence: virulence) that implied the heterozygous nature of avirulence gene and involvement of two loci for avirulence in Sg 019. The probable genotypes for avirulence and virulence in Sg 019 and Sg 445-1 designated as AaBb and aabb respectively. Segregation of F_1 progenies on ICMB 01333 and ICML 22 appeared in the ratio of 1:1 indicated the monogenic control of avirulent gene in Sg 019. The complete loss of avirulence in F_1 progenies against 81B-P6 suggested complex inheritance. Therefore, the probable genotypes for avirulence and virulence in parents Sg 019 and Sg 445-1 might be Aa and aa, respectively on ICMB 01333 and ICML 22.

In the modified bulk segregants analysis (BSA), only three primers Sg_SSR 49, Sg_SSR 68 and Sg_SSR 99 revealed polymorphisms between two parents and four bulks. Genetic linkage among three markers revealed two markers Sg_SSR 49 and Sg_SSR 68 to be linked with a LOD value of 22.5 and genetic distance of 7.4 cM. The association of markers with avirulence trait showed tight linkage (0 cM) of Sg_SSR 49 to the avirulence trait on IP 18292 and ICMB 01333 with LOD value of 6.96 and 7.43, respectively. Linkage of Sg_SSR 68 with avirulence trait was observed at 7.4 cM distance with LOD value of 9.66 to 9.79 for on ICMB 01333 and IP 18292. The significant R^2 values higher than 10 for the trait of avirulence on three host differentials *viz*., IP 18292, ICMB 01333 and ICML 22 with Sg_SSR 49 and two host differentials IP 18292, ICMB 01333 with Sg_SSR 68 indicated the linkage of these markers with avirulence loci in Sg 019. In order to determine the diagnostic value of avirulence linked microsatellite marker, a set of 60 isolates of *S. graminicola* were phenotyped on pearl millet line IP 18292 and genotyped by linked SSR marker Sg_SSR 49. Isolates expected to carry avirulence gene showed high level of avirulence to the cultivar IP 18292. Genotyping with avirulence linked marker Sg_SSR 49

amplified fragment size of 130 bp (Allele 1) and was associated with the presence of avirulent gene in 44 isolates that were shown avirulent reaction on IP 18292.

In order to decipher the inheritance and allelism of resistant genes, three resistant 834 B, IP18294 and IP18298 and one susceptible line 81 B were selected. Crosses (Three S × R and three R × R) were generated in diallel fashion without reciprocal crosses in the greenhouse. F_{1s} (8-10 panicles) were selfed for the production of F_2 seeds and back crosses $BC_1F_1P_1$ (S x F_{1s}) and $BC_1F_1P_2$ (R x F_{1s}) were generated. Parents and derived populations were screened with three selected isolates of *S. graminicola* Sg 200, Sg 526 and Sg 542. The almost all resistant plants in F_1 generations of the three crosses (81B x 834B, 81B x IP 18294 and 81B x IP 18298) to all the isolates indicated that the resistance in these lines is governed by dominant gene(s). The resistance and susceptible plants in F_2 generations of the cross 81B x 834B and 81B x IP 18294 showed a good fit to the segregation ratio of 3R:1S to all three isolates, suggesting dominant monogenic control of DM resistance in 834B and IP 18294. The corresponding $BC_1P_{1s,}$ 81B x (81B x 834B) and 81B x (81B x IP 18294), had good fit to 1R:1S ratio expected for monogenic inheritance to each isolate. However, the F_2 populations from the cross 81B x IP 18298 exhibited a good fit to the segregation ratio of 15R:1S to all three isolates indicating the involvement of two dominant genes for resistance in IP 18298. The corresponding $BC_1P_{1,}$ 81B x (81B x IP 18298), showed 3R:1S ratio anticipated for digenic inheritance to the isolates.

Allelism tests were conducted by crossing all three resistant parents 834B, IP 18294 and IP 18298 with each other to know whether the same gene (s) or different genes governs resistance in these genotypes to the test isolates. Out of three crosses (834B x IP 18294, 834B x IP 18298 and IP 18294 x IP 18298), 834B x IP 18294 showed a good fit to the segregation ratio of 15R:1S to all the three isolates suggesting the involvement of two independent dominant resistance genes. This concluded that the resistance gene in 834B is different from the resistance gene present in IP18294. The lack of segregations in the F_2 populations of the cross 834B x IP18298 and IP18294 x IP18298 to all the three isolates indicated allelic relationship between resistance gene of IP 18298 with both 834 B and IP 18294. Therefore, allelic relationship between resistance genes to three isolates of *S. graminicola* in this study suggested that 834 B and IP 18294 contain one non-allelic dominant gene designated as *RSg2* and *RSg3*, respectively. Two dominant resistance genes in IP 18298 to all the three isolates in this study were symbolized as *RSg2* and *RSg3*.

Future strategies

- Study on the relative contributions and rates of mutation, recombination, natural selection, gene flow, genetic drift, migration and maintenance of variation in populations (relative importance of asexual vs. sexual reproduction) will improve our understanding towards the pathogen populations and its evolutionary potential in terms of increased threats from a rapidly changing populations.
- Studies should be conducted on sexual compatibility types of *S. graminicola* which is one of prime source of development of variation in natural populations.
- Microsatellites markers are suitable tools to answer some unsolved problems like: (1) quantitative contribution of oospore-initiated infection into an epidemic; (2) period of the epidemic where oospore derived infections are possible and probable; (3) number of secondary infections arise from a single primary infection and (4) how far the sporangia can migrate from the source of inoculum. These questions could be solved by the developed SSRs markers in our study.
- Similar digenic segregation of avirulence of Sg 019 on IP 18292 and 852 B indicated two common resistant factors in both the cultivars. In the same way, resistant factors of ICML 22 and ICMB 01333 showed similar behavior with Sg 019. It needs to be verified by inheritance and allelic study.
- Complete inhibition of avirulence loci of Sg 019 on 81B-P6 and partial modification of avirulence on ICML 22 and ICMB 01333 by inhibitory factors of Sg 445-1 have to be affirmed by developing more progenies between Sg 445-1 and other isolates of *S. graminicola*.
- Recessive resistant factors in IP 18294 and IP 18298 should be ascertained and designated by making more crosses with other susceptible lines against differential isolates of *S. graminicola*.

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The pattern of “Literature cited” presented above is in accordance with the guidelines for the Thesis presentation for Professor Jayashankar Telangana State Agricultural University, Hyderabad.

*Original article not found.

Appendices

Appendix A. List of primers used in this study

S.No.	SSR	Forward Primer1 (5'-3')	Tm(°C)	Reverse Primer	Tm(°C)
1	(TAT)7	TCCGCGTACCCTGATTTAGC	59.90	CGGAAGCGTTTGGTCATACG	59.63
2	(TTC)5	CGGACGAGATGGAGAAGACG	59.97	GAGATCAGCGGGATAGTGGC	60.04
3	(TTC)5	GGTAGGGATCGACTCAACGC	60.25	CATCATCTGGGACCCGTAGC	59.97
4	(TAC)6	GTCTTCGATCACCCTCCAGC	60.18	ATAAGCAGGACGTTCGCAGC	61.08
5	(GAA)5	TCCAACTCGCAGGGATATGC	59.89	CTAAAGCGGCATTTGGAGGC	59.90
6	(GTT)7	CCTGAGGTTTTGAGCTGTGC	59.41	CAACCTGCTACACGTGTACG	58.94
7	(TAA)25	TTAGCGGAAGGAGAACCACG	59.75	TTCGTCTCCCGGGTAAAAGC	60.04
8	(GCA)5	ATTTACGCGTCAGCAACAGC	59.83	GTTGCTGAGCCTGCATATGC	59.97
9	(TTG)5	GGACATAACCCTCCCAGTGC	60.11	GAGAGAGGGAACTACGCACG	59.90
10	(TTC)6	AGATCCTGCACCAACTAGCG	59.82	TTTCGCAATGGGTTGATGCG	60.11
11	(GCT)5	TTCTTTGTTTGTGGCCTGCG	59.90	CTCATGAGCGATGCCAAAGC	59.97
12	(GCT)5	TTCTTTGTTTGTGGCCTGCG	59.90	CTCATGAGCGATGCCAAAGC	59.97
13	(TCC)6	TCGATTTCATCCCCGCTAGC	59.97	AGTGAAGCTATCGTGCTCCG	59.90
14	(GAG)5	TGGTCACCCGTTACCTTTCG	59.97	CGCGCGGTCTAAGATTTTCG	60.04
15	(TCT)5	TGGGAGACAACCACTGTTCG	59.89	CACCCATCCATCTCTCTCGC	59.97
16	(CTA)6	AGGGCTATGTGCGTGATAGC	59.97	CGCTATCGTACTGGACACCC	59.97
17	(GAA)5	GGACCAGGTCACCATTCAGG	60.04	CGGGACCCTTTAATCCACCC	60.11
18	(GAA)5	GGACCAGACCACCATTCAGG	60.04	ACTCGAAGGTGGTAAGCACG	60.04
19	(TAA)5	GCCTGTTTGTCAGCTGAACG	60.04	CGACAGCGAAGATCCCTACC	59.97
20	(TTC)5	CCTTATAGGCACGCTCCAGG	59.97	CACCATCCAGATCACCCACC	60.11
21	(ATA)5	TACGCATGCATCTGAGTCCC	59.90	TCTATGACGACTTCGCACCG	59.90
22	(TTC)10	GCCTTCCTTGTCCATCTCCC	60.11	GTTTGTCGCGTCATACTGCC	59.91
23	(TTC)5	CACGCTCCAGATCGGTATCC	60.04	AAGGAAGAAGCGGAAGTCGG	60.04
24	(TTA)5	TACGAATGGTCACCCTGAGC	59.47	CAATTTGAGGCCGCTCATGG	59.90
25	(GAA)5	GGACCAGGTCACCATTCAGG	60.04	CGGGACCCTTTAATCCACCC	60.11
26	(TGT)5	TTTGAAACACGCATGGCTGG	59.97	TAGCAGCCTATCAGCAACCG	59.90

Appendix A. (Cont.)

S.No.	SSR	Forward Primer1 (5'-3')	Tm(ºC)	Reverse Primer	Tm(ºC)
27	(TTA)5	GCATTATGACGCGGATTCGG	59.84	CGTTCAGCATCTACGCATGC	60.04
28	(GCA)5	GGAGAGTTGGACATCGACCC	59.82	ATCAAGAGCACGACCTTGGG	60.04
29	(TCT)5	TTCGTCGTCGTCTACTTCGC	60.18	GGAATTAGAGGACGCCGAGG	59.97
30	(TTA)7	ACGAATGGTCACTCTGAGCC	59.75	TGCGAATTTAAGAGCTGAACGG	59.59
31	(ACG)5	ACGTCCTTCCAAACGAGTCC	59.97	GAAATGGAGTGGTTGGCAGC	59.76
32	(TCA)5	CAGCTGAGGAGTCACTGTGG	60.04	GTGGCACGATTCTCGAATGC	59.97
33	(TCA)5	CAGCTGAGGAGTCACTGTGG	60.04	GTGGCACGATTCTCGAATGC	59.97
34	(GAT)10	TGCCGACTACAGATTCACCG	59.83	GACATTCGCTTGCTTGGTGG	60.11
35	(ACA)6	ATCTAAACCCGCGTCAGACC	59.83	TCCGGGCGTACAGTAATTCG	59.90
36	(CTT)6	TGCTGCCTACTTTCCTGACG	60.04	ATACGAAGCAGCATCGAGGG	59.97
37	(TTA)5	GACAGTATTTGCGGCCATGC	60.25	ACCTGCTCTGCACTCTTTCC	59.96
38	(TAC)8	TTCGATCAACCTCCAGCACC	60.04	TGCGTCCTTACCGCTAAAGG	60.11
39	(CTT)6	CTTCCTCGTCGACCAACTCC	60.11	TTGTACTTCCGATCGGTGGC	60.11
40	(TTC)5	TTCAAGACCCCATCCGTTCG	60.04	CAGTTCAGGACCAGACCACC	59.97
41	(TTA)6	ACAAGACCGAGCAAGTCAGG	59.97	GACGGTGGCTGGATATCTGG	59.97
42	(GAA)6	GGACCAAGTCACCATCCAGG	60.04	ACGGGACCTTTCAATCCACC	59.96
43	(GAT)5	CCGTTTGGTTGGCACTAACG	60.04	ATCATCGCAAACAAGCAGCC	59.83
44	(AGA)5	AGTGCCCTCACATGATAGCG	59.89	AACCCATACGGCTTGACTGG	60.04
45	(TCT)9	TTTGGCGTTTTCGTGTTGGG	60.18	CATCCATCTCTCTCGCCTCC	59.40
46	(GAA)5	CCTACCCCTGGAAAAGCTGG	60.03	GATACGATGCTCCACGGAGG	60.04
47	(GAA)5	TCGGAAGCTGTATTCGACCG	59.90	CACGCTCCAGATCGGTATCC	60.04
48	(TTC)5	CATCGGAGTCCTTAGGTGGC	59.90	CTAGGCACTCGAGAACGACC	59.90
49	(TGC)5	AAACGTGGCTGGGTATCTGG	60.04	TGACCCTCGATTTGTGGACG	60.04
50	(TTC)5	CCTTCTGTGATAGGCCGTCC	59.90	GGACCAAGTCACCATCCAGG	60.04
51	(GTT)5	GTGACAGTGTAGTCCGGACG	60.11	CGGGATGAGTATCGATCAGAGG	59.58
52	(ACA)12	TCTAAACCCGCGTAAGACCG	59.83	CCGAGCATATACCACGACCC	60.04

Appendix A. (Cont.)

S.No.	SSR	Forward Primer1 (5'-3')	Tm(ºC)	Reverse Primer	Tm(ºC)
53	(GTC)5	TTCTGCGCGTTGTTTCTTGG	59.97	CGAATGGTGGCACTTGTTGG	60.04
54	(AGT)8	AAAGTGTCCCACTGGATCCG	59.68	ACATCCAAGATGCCTGAGCC	60.11
55	(CAT)6	TGTGCGTGCATTTCAATGGG	60.04	CATGTTGGTGCATAGCTCGC	59.97
56	(TTA)6	ACTGTTACCGCATTGGGAGG	60.04	TGTGTGCTCCTTCGTGTACC	59.97
57	(TCT)7	AAAGTTGGCTGGGAGGATGG	59.96	TCCTTGCCCGGAGAAATTCC	60.03
58	(TAC)5	ACGAAAGGAATCGCGTAGGC	60.81	GGACGTGATGAGGGAGTTGG	60.11
59	(TTC)5	ACTCGAAGGTGGTAAGCACG	60.04	GGACCAGACCACCATTCAGG	60.04
60	(AGT)7	CGGTGGAGTTACTCGATCGG	59.97	GGTATGGATGATGGGCCTGG	59.96
61	(CTT)6	TGCTGCCTACTTTCCTGACG	60.04	ATACGAAGCAGCATCGAGGG	59.97
62	(CTT)6	CTTCCTCGTCGACCAACTCC	60.11	TTGTACTTCCGATCGGTGGC	60.11
63	(GAG)5	CCGATCAAGTCTCGAGCTCC	59.97	TCGATCTTCTTGCACCCAGG	59.75
64	(CAC)5	CAACAGACGGACCCTTACCC	60.04	AACACGAACAACTTGCTCGC	59.97
65	(TTC)6	CCTTCTGTGATAGGCCGTCC	59.90	CTATCCAGGTCCGGATTGGC	59.97
66	(TGC)5	AAACGTGGCTGGGTATCTGG	60.04	TGACCCTCGATTTGTGGACG	60.04
67	(TGA)8	TGATGATGAAGCCGCTACCC	59.89	AACGTGCTTCATTGTCAGCG	59.76
68	(TCT)5	CTCTTTGTTGCAACCACGGG	59.97	CCAGCAGCTGATAGGGATGG	59.96
69	(ACT)7	GGTATGGATGATGGGCCTGG	59.96	CGGTGGAGTTACTCGATCGG	59.97
70	(GAA)5	CGAGGACGACGAAGAAGAGG	59.90	ACGCTGTAGGAAAGGAGTGC	60.04
71	(GTC)5	GTGCTAGCACGGGTAGATCC	59.97	GTGACATTCGCTGACACTGC	59.84
72	(GAA)5	CACCTACCCCTCAAAGCTGG	60.04	CCAGGTCGGTGTCTGTAACC	60.04
73	(GAG)5	CCGATCAAGTCTCGAGCTCC	59.97	TCGATCTTCTTGCACCCAGG	59.75
74	(ATG)6	CATGTTGGTGCATAGCTCGC	59.97	TGTGCGTGCATTTCAATGGG	60.04
75	(TCC)5	AATACGTAGCCCATCGACGG	59.69	GGACCCACACAACAACATGC	59.97
76	(GAA)5	GCTGGATATGACCGGAGTGG	59.97	ACTGTCATGTGTGGTCCACC	59.89
77	(TTC)6	TTGTAGTCTGGCATACGCGG	60.18	AGATCACGACTCGCTTGAGC	60.18
78	(TTC)5	CCTTCTGTGATAGGCCGTCC	59.90	GGACCAAGTCACCATCCAGG	60.04

Appendix A. (Cont.)

S.No.	SSR	Forward Primer1 (5'-3')	Tm(°C)	Reverse Primer	Tm(°C)
79	(CTT)5	AATCAGGCGATCGGTCTTGG	60.18	CAAGCGTGTGTTGTGGACG	60.01
80	(ATC)10	GACATTCGCTTGCTTGGTGG	60.11	TGCCGACTACAGATTCACCG	59.83
81	(GAA)6	GGACCAGACCACCATTCAGG	60.04	CCAGGTCGGTGTCTGTAACC	60.04
82	(GCT)6	GTCAACGTTTGTGGGGAAGC	59.97	AAGCAGCTGTCCATCAGACC	60.04
83	(AAG)7	TTTGCGCGGATTCAGAAAGC	60.11	ATCATTCGCTTCTCCCGTCC	59.90
84	(TCT)6	GTACCCTCCTTCACACAGCC	60.04	GAAATTCCGATGCCAGCTGC	60.25
85	(CTTC)5	AAGCTTTCCCCATCCAGCTC	60.03	ATTTTCACCCGCGAAATGGC	60.11
86	(ATAA)5	TAGCCCATGTTTGCCATTGG	58.81	GCATTAAAGCGCAGAAACCG	58.74
87	(GAGT)5	CGTCAAATCGCGGTCATGTC	59.98	TTCGCCAGGACTAACGACAC	60.04
88	(AAGG)5	ATTTTCACCCGCGAAATGGC	60.11	AAGCTTTCCCCATCCAGCTC	60.03
89	(GAGT)5	CGTCAAATCGCGGTCATGTC	59.98	TTCGCCAGGACTAACGACAC	60.04
90	(TTAT)5	GCATTAAAGCGCAGAAACCG	58.74	TAGCCCATGTTTGCCATTGG	58.81
91	(GATG)5	AGCATGAGTGATGGAGTGGC	60.11	CGCCAGTGATGACTTGCAAG	59.83
92	(ATAGT)6	AAGTCCCCACCATGTCGAAC	59.96	CTCCTCGACCTCGTATTCGC	60.04
93	(TATAG)13	CGACCTAGTTGAATGCAGCG	59.35	GCGACGGTCATGAGCAAATC	59.97
94	(TATTG)9	TGCAGTGCACGAAGAGAGAG	60.04	GTTACTTTCGGCGTGCGATC	59.98
95	(ATAGT)6	AAGTCCCCACCATGTCGAAC	59.96	CTCCTCGACCTCGTATTCGC	60.04
96	(ATACA)5	GAGGTGGACAGAACAGAGGC	60.04	AGCAAATCTCGGACTCGGTG	60.11
97	(TCATCG)5	GACAAGGCAGCACACACTTG	59.97	TGGAAATTGACCCGGATCCG	60.11
98	(TTGTTA)5	TCGACGGCAGTATGTTACGG	59.90	TCTTCATCGCCCAGTTCGTG	60.39
99	(ATAGTA)7	GCTTCACGCTTGTTGCTGAC	60.66	CGCAAGCCTACTACCACCTC	60.18
100	(AAG)5(ACG)5	GAAACTCTGCGACGTGCTTC	59.84	AAGTGTGACACCGTCTCGTC	59.97
101	(TAC)5(TAG)7	GGTTGAACGTGAACGCTGTC	60.04	CCGATGTGATGCGCAATGAG	60.04
102	(TAG)8(TA)6	ACCTGACATTCCAGCCGATG	60.11	GCCTGTAAATGGTGTAGAAGCC	59.32
103	(TAC)5at(CTA)11	ACTGAGCCAAGTTACCGACG	60.04	AAGGAAGCTACGGCCAAGTC	60.04

Appendix A. (Cont.)

S.No.	SSR	Forward Primer1 (5'-3')	Tm(º C)	Reverse Primer	Tm(º C)
104	(AAG)5(ACG)5	GAAACTCTGCGACGTGCTTC	59.84	AAGTGTGACACCGTCTCGTC	59.97
105	(AT)9t(TA)7	TTGGTTCCTCAGACGTCACG	59.97	ACTTACCTTCTTGTAGACACGTG	58.13
106	(AAG)5(ACG)5	GAAACTCTGCGACGTGCTTC	59.84	AAGTGTGACACCGTCTCGTC	59.97

Appendix B. Clustering of all the tested isolates of *S. graminicola* based on dendrogram and population structure

ID#	Location	State	Year	Cluster analysis	Structure (K=2)	Structure (K=5)	Structure (K=7)	Structure (K=18)
Sg 492	Iglas	Uttar Pradesh	2007	IA	SG 1	SG 1	AD	AD
Sg 018	Patancheru	Telangana	1992		SG 1	SG 1	AD	AD
Sg 526	Jodhpur	Rajasthan	2009		SG 1	SG 1	AD	AD
Sg 510	Baduan	Uttar Pradesh	2008		SG 1	SG 1	AD	AD
Sg 546	Aurangabad	Maharashtra	2010		SG 1	SG 1	AD	AD
Sg 571	Bulandsharh	Uttar Pradesh	2012		SG 1	SG 1	AD	AD
Sg 019	Patancheru	Telangana	1992	IB	SG 1	SG 1	AD	AD
Sg 586	Jaisalmer	Rajasthan	2013		SG 1	SG 1	AD	AD
Sg 587	Jaisalmer	Rajasthan	2013		SG 1	AD	AD	AD
Sg 592	Durgapura	Rajasthan	2014	IC	SG 1	SG 1	AD	AD
Sg 576	Jodhpur	Rajasthan	2012		SG 1	SG 1	AD	AD
Sg 594	Aligarh	Uttar Pradesh	2014		SG 1	SG 1	AD	AD
Sg 151	Durgapura	Rajasthan	1997	IIA	SG 1	SG 5	SG 5	AD
Sg 596	Bulandsharh	Uttar Pradesh	2014		SG 1	SG 3	AD	AD
Sg 200	jamnagar	Gujrat	1998		SG 1	SG 3	AD	AD
Sg 445	Banaskantha	Gujrat	2005		SG 1	SG 3	SG 3	SG 3
Sg 212	Durgapura	Rajasthan	1998		SG 1	SG 3	SG 3	SG 3
Sg 604	Hathras	Uttar Pradesh	2014		SG 1	SG 3	SG 3	SG 3
Sg 021	Ahmednagar	Maharashtra	1993		SG 1	SG 3	AD	AD
Sg 560	Banaskantha	Gujrat	2010		SG 1	SG 3	AD	AD
Sg 566	Sikar	Rajasthan	2012	IIB	SG 1	SG 5	AD	AD
Sg 543	Aurangabad	Maharashtra	2010		SG 1	AD	AD	AD
Sg 521	Rewari	Haryana	2009		SG 1	AD	AD	AD
Sg 384	Barmer	Rajasthan	2003		SG 1	SG 3	AD	AD
Sg 561	IARI	New Delhi	2010		SG 1	SG 3	AD	AD

Appendix B. (Cont.)

ID#	Location	State	Year	Cluster analysis	Structure (K=2)	Structure (K=5)	Structure (K=7)	Structure (K=18)
Sg 150	Jalna	Maharashtra	1997		AD	AD	AD	AD
Sg 591	Jaisalmer	Rajasthan	2013	**III**	AD	AD	AD	AD
Sg 574	Sambal	Uttar Pradesh	2012		AD	SG 4	AD	AD
Sg 298	IARI	New Delhi	1999		SG 1	AD	AD	AD
Sg 590	Jaisalmer	Rajasthan	2013	**IVA**	SG 2	SG 2	SG 2	AD
Sg 575	Baduan	Uttar Pradesh	2012		SG 2	SG 2	SG 2	AD
Sg 593	Agra	Uttar Pradesh	2014		SG 2	SG 2	SG 2	AD
Sg 595	Aligarh	Uttar Pradesh	2014		SG 2	SG 2	SG 2	AD
Sg 589	Jaisalmer	Rajasthan	2013		SG 2	SG 2	SG 2	AD
Sg 529	CAZRI,Jodhpur	Rajasthan	2009		SG 2	SG 2	SG 2	AD
Sg 431	Patancheru	Telangana	2005		SG 2	SG 2	SG 2	AD
Sg 585	Kheda	Gujrat	2012		SG 2	SG 4	AD	AD
Sg 556	Kothigaon, Banaskantha	Gujrat	2010	**IVB**	SG 2	SG 2	SG 2	AD
Sg 557	Lodhnoor, Banaskantha	Gujrat	2010		SG 2	SG 2	SG 2	AD
Sg 558	Banaskantha	Gujrat	2010		SG 2	SG 2	SG 2	AD
Sg 601	Baduan	Uttar Pradesh	2014		SG 2	SG 2	AD	AD
Sg 559	Banaskantha	Gujrat	2010		SG 2	SG 2	SG 2	AD
Sg 597	Sambhal	Uttar Pradesh	2014	**IVC**	SG 2	SG 4	SG 4	SG 4
Sg 573	Sambal	Uttar Pradesh	2012		SG 2	SG 4	SG 4	SG 4
Sg 598	Sambhal	Uttar Pradesh	2014		SG 2	SG 4	SG 4	SG 4
Sg 457	Jaipur	Rajasthan	2006		SG 2	SG 4	SG 4	SG 4
Sg 603	Hathras	Uttar Pradesh	2014		SG 2	SG 4	SG 4	AD
Sg 569	Aligarh	Uttar Pradesh	2012		SG 2	SG 4	SG 4	AD
Sg 139	Jodhpur	Rajasthan	1997		SG 2	SG 4	SG 4	AD

Appendix B. (Cont.)

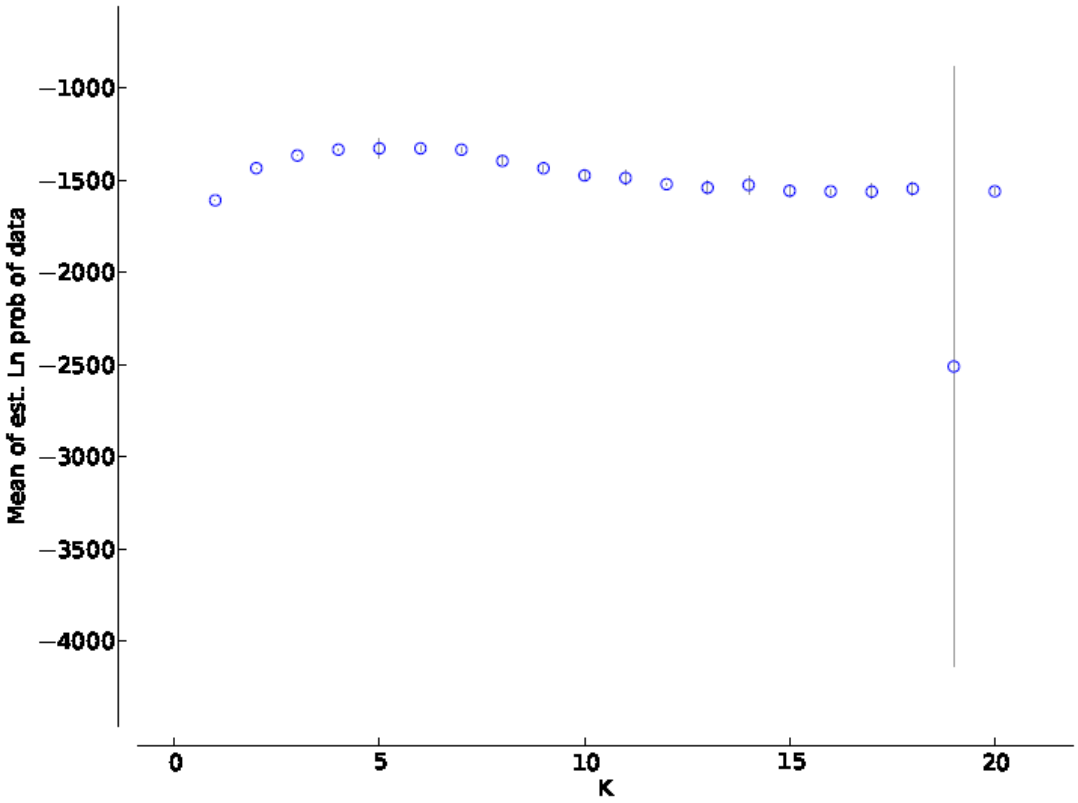
ID#	Location	State	Year	Cluster analysis	Structure (K=2)	Structure (K=5)	Structure (K=7)	Structure (K=18)
Sg 599	Sambhal	Uttar Pradesh	2014		SG 2	AD	AD	AD
Sg 562	Jaipur	Rajasthan	2012	**IVD**	SG 2	SG 4	AD	AD
Sg 519	Rewari	Haryana	2009		SG 2	SG 4	AD	AD
Sg 542	Aurangabad	Maharashtra	2010	**IVE**	SG 2	AD	AD	AD
Sg 153	Patancheru	Telangana	1997		AD	AD	AD	AD
Sg 048	Mysore	Karnataka	1994		AD	AD	AD	AD
Sg 409	Patancheru	Telangana	2004		SG 1	SG 5	SG 5	AD
Sg 580	Aanand	Gujrat	2012		SG 1	SG 5	SG 5	AD
Sg 545	Aurangabad	Maharashtra	2011	**V**	SG 1	SG 5	SG 5	AD
Sg 578	Kheda	Gujrat	2012		SG 1	SG 5	SG 5	AD
Sg 564	Sikar	Rajasthan	2012		AD	SG 5	SG 5	AD

Appendix C. Estimated log probability of data, mean value of Ln likelihood and variance of Ln likelihood of 60 *S. graminicola* isolates produced by structure Harvester core version vA.2

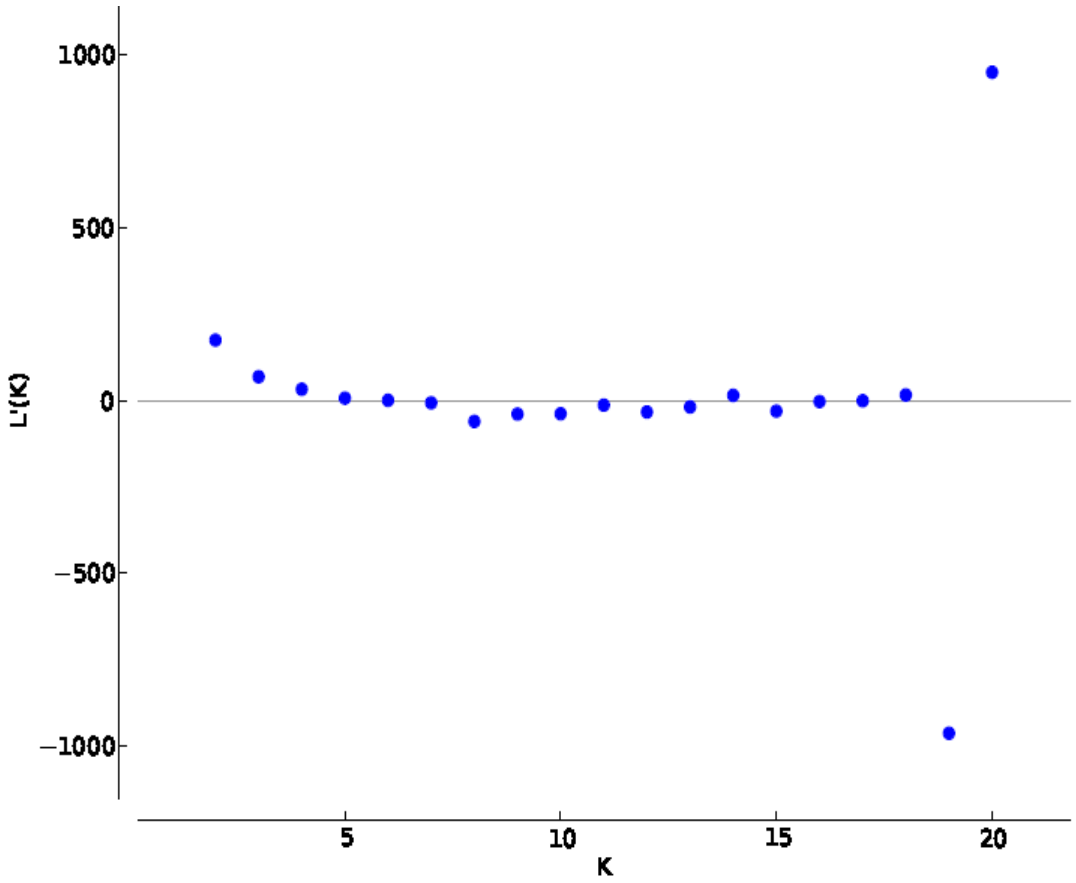
# File name Run #	K	Est. Ln prob. of data	Mean value of Ln likelihood	Variance of Ln likelihood
SSR_1_f1	1	-1608.1	-1599.3	17.6
SSR_3_f3	1	-1607.9	-1599.2	17.5
SSR_2_f2	1	-1607.2	-1598.9	16.5
SSR_5_f5	2	-1433.5	-1403.9	59.1
SSR_4_f4	2	-1433.6	-1404.4	58.4
SSR_6_f6	2	-1432.6	-1404	57.3
SSR_7_f7	3	-1364.6	-1320.8	87.6
SSR_9_f9	3	-1363.5	-1320.3	86.4
SSR_8_f8	3	-1366.7	-1321.1	91.2
SSR_11_f11	4	-1336.2	-1273.5	125.3
SSR_12_f12	4	-1335.5	-1273.6	123.8
SSR_10_f10	4	-1326.4	-1273.4	106.1
SSR_14_f14	5	-1296.5	-1226.9	139.2
SSR_15_f15	5	-1386.3	-1236.6	299.5
SSR_13_f13	5	-1295.6	-1226.6	138
SSR_16_f16	6	-1315.2	-1203.8	222.7
SSR_18_f18	6	-1322	-1205.3	233.5
SSR_17_f17	6	-1340.7	-1216.7	248
SSR_20_f20	7	-1318.8	-1184.3	269.1
SSR_21_f21	7	-1337.3	-1188.9	296.8
SSR_19_f19	7	-1343.4	-1188.2	310.4
SSR_22_f22	8	-1416	-1198.7	434.7
SSR_23_f23	8	-1390.3	-1176.7	427.3
SSR_24_f24	8	-1376.3	-1188	376.8
SSR_27_f27	9	-1435.9	-1173.6	524.7
SSR_25_f25	9	-1447.2	-1171.1	552.1
SSR_26_f26	9	-1418.8	-1160.1	517.2
SSR_28_f28	10	-1439.8	-1171.6	536.4
SSR_30_f30	10	-1496.2	-1164.4	663.7
SSR_29_f29	10	-1482	-1163.7	636.6
SSR_33_f33	11	-1494.1	-1168.3	651.5
SSR_31_f31	11	-1522.3	-1164.8	715
SSR_32_f32	11	-1442.9	-1157.2	571.4
SSR_36_f36	12	-1519.1	-1166.6	705

Appendix C. (Cont.)

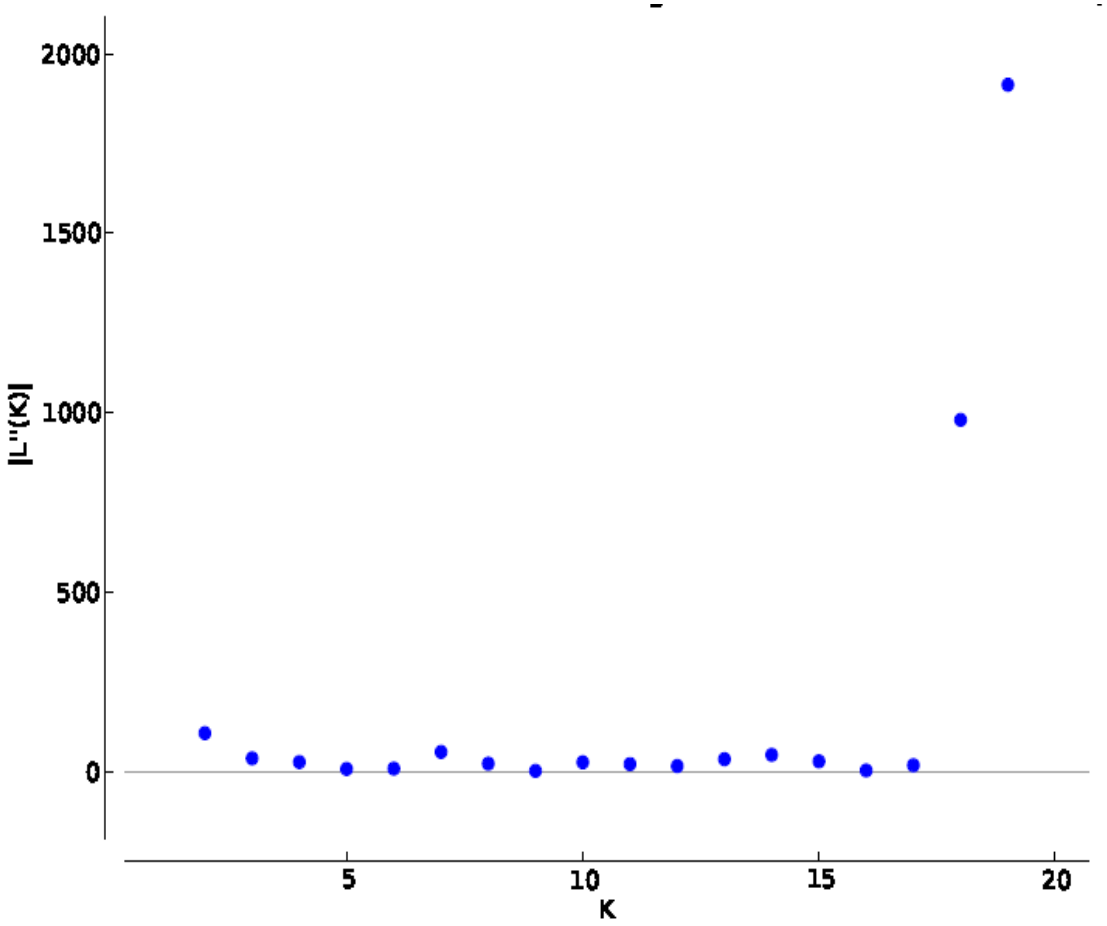
# File name Run #	K	Est. Ln prob. of data	Mean value of Ln likelihood	Variance of Ln likelihood
SSR_34_f34	12	-1527.1	-1168.3	717.6
SSR_35_f35	12	-1514.2	-1164.9	698.6
SSR_38_f38	13	-1562.1	-1176.1	772
SSR_37_f37	13	-1497.6	-1171.1	652.9
SSR_39_f39	13	-1558.1	-1177.6	760.9
SSR_41_f41	14	-1556.4	-1178.3	756.3
SSR_40_f40	14	-1544.9	-1178.7	732.3
SSR_42_f42	14	-1472.8	-1170.3	605.1
SSR_43_f43	15	-1519.9	-1183.8	672.1
SSR_44_f44	15	-1582.4	-1191.3	748.2
SSR_47_f47	16	-1572.3	-1183.7	777.3
SSR_48_f48	16	-1545.8	-1188.6	714.4
SSR_46_f46	16	-1560	-1197.3	725.4
SSR_51_f51	17	-1593.1	-1190.8	804.5
SSR_49_f49	17	-1574.9	-1189.9	769.8
SSR_50_f50	17	-1513.7	-1186.3	654.9
SSR_52_f52	18	-1586	-1204.5	763
SSR_53_f53	18	-1536	-1197.2	677.6
SSR_54_f54	18	-1512.6	-1194.3	636.6
SSR_57_f57	19	-4389.5	-1226.4	6326.3
SSR_55_f55	19	-1551.8	-1194.6	714.4
SSR_56_f56	19	-1585.7	-1197.9	775.6
SSR_60_f60	20	-1577.6	-1207.5	740.3
SSR_58_f58	20	-1553.5	-1204	698.8
SSR_59_f59	20	-1545.3	-1199	692.7



Appendix D. Mean likelihood L(K) (± SD) over 20 runs for each *K* value.



Appendix E. Mean difference between successive likelihood values of *K*



Appendix F. Absolute value of the difference between successive values of $L'(K)$ corresponds to the second order rate of change of $L(K)$ with respect to K

Appendix G. Segregation of F_1 progenies for avirulence on five differential cultivars.

Population	852B			ICMB01333			ICML22			81BP6			IP 18292		
	R-I	R-II	Mean	R-I	R-II	Mean	R-I	R-II	Mean	R-I	R-II	Mean	R-I	R-II	Mean
1	9.3	17.3	**13.3**	3.3	6.9	**5.1**	23.8	41.2	**32.5**	79.4	75.3	**77.3**	2.9	0.0	**1.4**
2	0.0	0.0	**0.0**	0.0	1.9	**1.0**	0.0	5.0	**2.5**	71.2	56.2	**63.7**	0.0	1.5	**0.8**
3	0.0	0.0	**0.0**	8.1	1.4	**4.7**	8.2	12.4	**10.3**	82.9	89.5	**86.2**	7.5	2.5	**5.0**
4	0.0	0.0	**0.0**	3.0	0.0	**1.5**	15.8	19.9	**17.9**	46.6	49.5	**48.0**	1.3	0.0	**0.6**
5	0.0	0.0	**0.0**	8.5	3.5	**6.0**	4.2	1.4	**2.8**	64.1	78.4	**71.2**	12.9	0.0	**6.5**
6	100.0	100.0	**100.0**	10.1	0.0	**5.1**	11.3	2.7	**7.0**	98.7	95.9	**97.3**	0.0	0.0	**0.0**
7	17.0	5.1	**11.1**	11.3	14.6	**13.0**	14.3	18.5	**16.4**	66.8	79.6	**73.2**	0.0	6.5	**3.3**
8	14.3	0.0	**7.1**	14.3	0.0	**7.2**	8.5	5.8	**7.2**	75.6	86.5	**81.0**	3.8	0.0	**1.9**
9	0.0	1.3	**0.6**	1.4	4.5	**2.9**	0.0	0.0	**0.0**	80.6	93.1	**86.8**	0.0	0.0	**0.0**
10	53.3	81.1	**67.2**	4.3	0.0	**2.2**	7.0	1.4	**4.2**	42.0	38.4	**40.2**	5.4	5.3	**5.4**
11	97.5	97.1	**97.3**	6.7	3.7	**5.2**	40.3	32.8	**36.6**	40.8	22.7	**31.8**	8.1	5.2	**6.7**
12	93.5	100.0	**96.7**	13.9	0.0	**7.0**	35.2	57.1	**46.2**	52.7	57.5	**55.1**	2.6	8.5	**5.6**
13	0.0	0.0	**0.0**	1.8	9.2	**5.5**	19.3	19.3	**19.3**	96.5	96.7	**96.6**	1.4	8.2	**4.8**
14	0.0	0.0	**0.0**	17.7	1.7	**9.7**	14.1	0.0	**7.1**	97.4	93.3	**95.4**	1.6	2.7	**2.1**
15	8.9	0.0	**4.4**	2.0	6.6	**4.3**	7.2	1.8	**4.5**	44.1	59.2	**51.7**	0.0	6.8	**3.4**
16	1.1	1.0	**1.0**	15.7	8.6	**12.1**	5.9	6.0	**6.0**	86.2	94.2	**90.2**	0.0	0.0	**0.0**
17	10.5	26.8	**18.6**	70.6	51.0	**60.8**	62.5	56.6	**59.6**	61.8	71.4	**66.6**	7.3	0.0	**3.7**
18	75.7	58.1	**66.9**	87.3	48.6	**68.0**	87.9	61.2	**74.5**	95.2	89.7	**92.5**	100.0	89.6	**94.8**
19	2.4	1.3	**1.8**	5.0	0.0	**2.5**	4.0	1.4	**2.7**	87.7	98.6	**93.1**	3.0	0.0	**1.5**
20	100.0	98.6	**99.3**	100.0	94.4	**97.2**	84.9	93.8	**89.3**	98.8	97.7	**98.2**	98.8	98.6	**98.7**
21	1.4	0.0	**0.7**	4.3	0.0	**2.1**	0.0	1.4	**0.7**	77.3	87.8	**82.5**	7.9	0.0	**4.0**
22	100.0	100.0	**100.0**	0.0	0.0	**0.0**	100.0	66.1	**83.0**	85.0	79.5	**82.2**	5.8	16.6	**11.2**
23	56.9	43.6	**50.3**	16.3	20.4	**18.3**	6.0	16.9	**11.5**	28.8	26.4	**27.6**	10.4	6.9	**8.6**
24	3.9	2.7	**3.3**	100.0	100.0	**100.0**	77.2	65.6	**71.4**	100.0	100.0	**100.0**	7.0	4.5	**5.7**

Appendix G. (Cont.)

Population	852B			ICMB01333			ICML22			81BP6			IP 18292		
	R-I	R-II	Mean	R-I	R-II	Mean	R-I	R-II	Mean	R-I	R-II	Mean	R-I	R-II	Mean
25	1.3	0.0	**0.6**	3.4	14.5	**9.0**	21.1	6.4	**13.8**	45.6	82.8	**64.2**	100.0	98.9	**99.4**
26	1.3	0.0	**0.6**	70.8	100.0	**85.4**	44.7	46.4	**45.6**	93.8	100.0	**96.9**	100.0	100.0	**100.0**
27	3.4	0.0	**1.7**	100.0	97.7	**98.9**	50.6	62.9	**56.8**	100.0	100.0	**100.0**	100.0	100.0	**100.0**
28	0.0	0.0	**0.0**	100.0	97.6	**98.8**	67.5	57.5	**62.5**	100.0	100.0	**100.0**	100.0	100.0	**100.0**
29	0.0	0.0	**0.0**	94.4	100.0	**97.2**	58.7	76.6	**67.7**	91.5	100.0	**95.8**	100.0	100.0	**100.0**
30	0.0	0.0	**0.0**	21.0	0.0	**10.5**	22.8	16.8	**19.8**	52.4	28.3	**40.4**	5.4	7.2	**6.3**
31	1.4	0.0	**0.7**	82.0	100.0	**91.0**	38.8	58.6	**48.7**	95.0	100.0	**97.5**	100.0	97.6	**98.8**
32	0.0	1.6	**0.8**	100.0	92.6	**96.3**	54.7	49.2	**51.9**	100.0	100.0	**100.0**	100.0	100.0	**100.0**
33	0.0	0.0	**0.0**	100.0	93.3	**96.7**	43.8	62.9	**53.3**	100.0	100.0	**100.0**	97.1	100.0	**98.6**
34	0.0	0.0	**0.0**	100.0	100.0	**100.0**	87.6	63.1	**75.4**	100.0	100.0	**100.0**	100.0	100.0	**100.0**
35	0.0	0.0	**0.0**	100.0	98.4	**99.2**	55.5	46.4	**51.0**	100.0	98.4	**99.2**	53.5	3.4	**28.5**
36	0.0	0.0	**0.0**	88.7	100.0	**94.3**	36.4	79.1	**57.7**	98.5	100.0	**99.3**	100.0	100.0	**100.0**
37	0.0	0.0	**0.0**	100.0	98.0	**99.0**	55.8	43.3	**49.6**	100.0	98.6	**99.3**	100.0	100.0	**100.0**
38	0.0	1.3	**0.6**	100.0	100.0	**100.0**	72.0	46.7	**59.3**	100.0	100.0	**100.0**	100.0	100.0	**100.0**
39	0.0	1.4	**0.7**	14.3	2.1	**8.2**	0.0	0.0	**0.0**	52.6	57.1	**54.8**	0.0	1.2	**0.6**
40	0.0	1.6	**0.8**	98.1	91.0	**94.6**	100.0	95.7	**97.9**	95.9	96.4	**96.1**	0.0	1.3	**0.6**
41	1.4	0.0	**0.7**	100.0	96.3	**98.1**	36.8	50.0	**43.4**	100.0	100.0	**100.0**	98.7	98.7	**98.7**
42	0.0	0.0	**0.0**	100.0	100.0	**100.0**	72.6	57.4	**65.0**	100.0	98.6	**99.3**	0.0	1.3	**0.6**
43	0.0	0.0	**0.0**	87.8	100.0	**93.9**	100.0	100.0	**100.0**	100.0	100.0	**100.0**	16.0	3.6	**9.8**
44	4.4	1.4	**2.9**	96.9	100.0	**98.4**	100.0	94.4	**97.2**	100.0	98.6	**99.3**	100.0	98.8	**99.4**
45	0.0	0.0	**0.0**	94.4	88.7	**91.6**	100.0	94.4	**97.2**	97.3	100.0	**98.6**	1.4	0.0	**0.7**
46	0.0	0.0	**0.0**	100.0	97.7	**98.9**	47.6	41.3	**44.5**	100.0	98.3	**99.1**	4.1	0.0	**2.1**
47	0.0	1.4	**0.7**	100.0	97.7	**98.9**	74.6	55.2	**64.9**	100.0	100.0	**100.0**	100.0	100.0	**100.0**
48	35.9	0.0	**17.9**	20.9	2.4	**11.6**	37.6	0.0	**18.8**	81.5	50.6	**66.0**	0.0	0.0	**0.0**

Appendix G. (Cont.)

Population	852B			ICMB01333			ICML22			81BP6			IP 18292		
	R-I	R-II	Mean	R-I	R-II	Mean	R-I	R-II	Mean	R-I	R-II	Mean	R-I	R-II	Mean
49	0.0	0.0	**0.0**	24.9	31.8	**28.4**	0.0	0.0	**0.0**	97.3	96.2	**96.7**	0.0	0.0	**0.0**
50	0.0	30.9	**15.5**	1.9	8.8	**5.3**	7.5	20.6	**14.1**	61.0	49.2	**55.1**	2.6	0.0	**1.3**
51	12.1	63.5	**37.8**	53.6	34.0	**43.8**	26.4	87.0	**56.7**	49.9	56.2	**53.0**	13.9	2.5	**8.2**
52	3.0	4.2	**3.6**	7.4	7.4	**7.4**	8.7	10.2	**9.4**	54.8	63.1	**58.9**	3.5	4.3	**3.9**
53	0.0	0.0	**0.0**	27.3	41.0	**34.2**	0.0	0.0	**0.0**	76.8	72.4	**74.6**	1.3	0.0	**0.7**
54	31.4	0.0	**15.7**	13.8	3.6	**8.7**	20.6	0.0	**10.3**	64.5	44.2	**54.4**	0.0	0.0	**0.0**
55	1.5	0.0	**0.7**	2.4	0.0	**1.2**	10.7	8.3	**9.5**	71.6	75.6	**73.6**	0.0	0.0	**0.0**
56	1.9	0.0	**1.0**	0.0	3.9	**2.0**	19.6	9.5	**14.5**	41.9	62.6	**52.3**	0.0	5.7	**2.8**
57	0.0	0.0	**0.0**	17.4	14.3	**15.9**	14.0	9.4	**11.7**	38.1	40.1	**39.1**	0.0	0.0	**0.0**
58	11.6	0.0	**5.8**	1.8	12.5	**7.1**	5.8	10.5	**8.1**	55.3	74.8	**65.1**	0.0	0.0	**0.0**
59	1.4	0.0	**0.7**	10.9	3.9	**7.4**	0.0	0.0	**0.0**	41.2	38.2	**39.7**	0.0	0.0	**0.0**
60	100.0	97.6	**98.8**	97.6	84.2	**90.9**	68.8	85.5	**77.2**	91.0	90.6	**90.8**	94.8	95.9	**95.4**
61	7.4	6.5	**7.0**	17.8	16.0	**16.9**	46.7	59.3	**53.0**	67.6	54.9	**61.3**	0.0	0.0	**0.0**
62	0.0	5.8	**2.9**	5.9	0.0	**3.0**	3.7	6.0	**4.8**	45.7	40.8	**43.2**	0.0	0.0	**0.0**
63	2.9	7.4	**5.2**	40.8	89.7	**65.2**	3.1	18.6	**10.9**	80.8	100.0	**90.4**	0.0	1.5	**0.7**
64	1.3	0.0	**0.6**	0.0	5.7	**2.9**	6.0	15.0	**10.5**	68.1	88.9	**78.5**	0.0	4.2	**2.1**
65	100.0	98.5	**99.3**	86.5	95.8	**91.1**	0.0	0.0	**0.0**	96.8	98.7	**97.8**	1.5	4.6	**3.0**
66	1.4	1.4	**1.4**	4.2	27.7	**16.0**	0.0	16.5	**8.3**	92.7	98.6	**95.7**	0.0	0.0	**0.0**
67	0.0	0.0	**0.0**	71.7	77.2	**74.4**	0.0	1.6	**0.8**	74.0	79.8	**76.9**	0.0	2.7	**1.4**
68	9.5	2.9	**6.2**	2.1	26.6	**14.3**	6.1	20.3	**13.2**	94.7	87.0	**90.8**	7.6	1.2	**4.4**
69	0.0	0.0	**0.0**	9.5	18.8	**14.2**	3.7	9.4	**6.5**	77.0	89.6	**83.3**	4.4	2.2	**3.3**
70	2.9	1.4	**2.1**	88.3	71.0	**79.7**	4.2	1.3	**2.7**	79.4	86.1	**82.8**	1.7	0.0	**0.8**
71	0.0	0.0	**0.0**	15.7	3.7	**9.7**	0.0	0.0	**0.0**	66.8	66.7	**66.7**	0.0	1.2	**0.6**
72	11.0	2.6	**6.8**	11.8	9.7	**10.7**	6.9	9.4	**8.2**	73.4	74.6	**74.0**	0.0	0.0	**0.0**

Appendix G. (Cont.)

Population	852B			ICMB01333			ICML22			81BP6			IP 18292		
	R-I	R-II	Mean	R-I	R-II	Mean	R-I	R-II	Mean	R-I	R-II	Mean	R-I	R-II	Mean
73	100.0	100.0	**100.0**	20.5	17.1	**18.8**	11.8	12.9	**12.4**	100.0	93.9	**96.9**	9.0	4.1	**6.6**
74	0.0	0.0	**0.0**	16.8	17.7	**17.3**	15.0	16.2	**15.6**	75.0	76.7	**75.9**	0.0	0.0	**0.0**
75	0.0	0.0	**0.0**	5.3	11.0	**8.2**	12.1	12.1	**12.1**	98.8	97.3	**98.0**	1.3	1.4	**1.4**
76	8.1	4.0	**6.0**	47.0	48.1	**47.5**	18.0	16.6	**17.3**	50.8	55.1	**52.9**	0.0	0.0	**0.0**
77	0.0	1.1	**0.5**	10.8	5.9	**8.4**	4.9	0.0	**2.5**	62.0	87.8	**74.9**	6.2	0.0	**3.1**
78	0.0	1.2	**0.6**	98.3	95.2	**96.7**	11.3	8.9	**10.1**	98.3	96.0	**97.2**	0.0	0.0	**0.0**
79	6.8	4.3	**5.6**	42.4	47.3	**44.9**	13.8	11.3	**12.5**	97.0	97.7	**97.3**	0.0	0.0	**0.0**
80	1.5	0.0	**0.7**	8.5	7.7	**8.1**	15.6	7.9	**11.8**	78.2	72.4	**75.3**	0.0	0.0	**0.0**
81	85.5	91.9	**88.7**	77.8	73.6	**75.7**	93.4	94.7	**94.1**	94.2	96.3	**95.3**	8.3	6.3	**7.3**
82	80.9	82.8	**81.8**	15.0	16.3	**15.7**	95.8	94.4	**95.1**	76.6	77.2	**76.9**	8.9	11.4	**10.1**
83	78.9	77.4	**78.2**	47.5	40.6	**44.0**	97.2	95.7	**96.5**	51.2	80.8	**66.0**	10.4	14.2	**12.3**
84	94.3	95.0	**94.6**	46.2	46.0	**46.1**	96.1	94.7	**95.4**	97.6	87.7	**92.6**	8.8	3.3	**6.1**
85	95.0	95.4	**95.2**	21.6	20.2	**20.9**	94.9	96.5	**95.7**	98.5	92.0	**95.2**	15.7	15.2	**15.4**
86	93.8	98.1	**95.9**	39.6	34.7	**37.2**	94.5	98.3	**96.4**	88.6	92.5	**90.5**	13.6	15.2	**14.4**
87	0.0	0.0	**0.0**	12.2	13.6	**12.9**	0.0	6.3	**3.2**	73.7	75.6	**74.6**	0.0	0.0	**0.0**
88	98.7	97.0	**97.8**	43.3	39.0	**41.1**	96.4	96.6	**96.5**	96.6	95.6	**96.1**	1.3	6.2	**3.8**
89	56.8	94.0	**75.4**	68.3	70.8	**69.6**	92.9	94.3	**93.6**	90.0	89.3	**89.6**	4.9	2.4	**3.6**
90	22.8	3.3	**13.1**	21.7	14.7	**18.2**	70.0	61.7	**65.9**	87.2	78.6	**82.9**	0.0	0.0	**0.0**
91	0.0	0.0	**0.0**	8.4	8.0	**8.2**	5.6	4.7	**5.1**	90.2	91.1	**90.7**	4.7	5.7	**5.2**
92	0.0	2.2	**1.1**	0.0	14.2	**7.1**	0.0	13.8	**6.9**	68.5	78.9	**73.7**	0.0	0.0	**0.0**
93	0.0	0.0	**0.0**	15.5	13.3	**14.4**	4.8	5.0	**4.9**	82.3	81.5	**81.9**	2.6	0.0	**1.3**
94	0.0	0.0	**0.0**	94.7	98.5	**96.6**	29.4	25.4	**27.4**	95.6	91.4	**93.5**	0.0	2.6	**1.3**
95	0.0	0.0	**0.0**	0.0	1.8	**0.9**	7.6	7.4	**7.5**	73.8	28.9	**51.4**	7.3	0.0	**3.6**
96	0.0	0.0	**0.0**	12.2	29.1	**20.7**	5.6	4.0	**4.8**	52.6	52.3	**52.4**	9.9	0.0	**4.9**

Appendix G. (Cont.)

Population	852B			ICMB01333			ICML22			81BP6			IP 18292		
	R-I	R-II	Mean	R-I	R-II	Mean	R-I	R-II	Mean	R-I	R-II	Mean	R-I	R-II	Mean
97	0.0	0.0	**0.0**	6.4	12.7	**9.6**	0.0	9.0	**4.5**	52.7	29.8	**41.3**	0.0	0.0	**0.0**
98	0.0	0.0	**0.0**	13.3	13.9	**13.6**	9.7	9.8	**9.7**	67.5	67.5	**67.5**	0.0	0.0	**0.0**
99	100.0	100.0	**100.0**	92.6	100.0	**96.3**	57.9	47.4	**52.6**	100.0	96.7	**98.3**	0.0	0.0	**0.0**
100	0.0	0.0	**0.0**	34.5	37.7	**36.1**	7.3	9.2	**8.3**	86.7	89.8	**88.2**	3.1	0.0	**1.6**
101	0.0	0.0	**0.0**	5.6	6.4	**6.0**	12.0	4.9	**8.5**	54.3	53.2	**53.7**	1.6	0.0	**0.8**
103	2.7	8.0	**5.3**	37.7	29.6	**33.6**	12.5	17.9	**15.2**	76.0	92.3	**84.2**	1.3	0.0	**0.6**
106	0.0	8.8	**4.4**	0.0	1.5	**0.7**	4.9	11.1	**8.0**	85.8	91.5	**88.6**	0.0	0.0	**0.0**
107	0.0	4.1	**2.1**	9.7	4.2	**6.9**	4.9	5.9	**5.4**	67.2	88.3	**77.7**	0.0	2.4	**1.2**
108	0.0	3.9	**2.0**	100.0	100.0	**100.0**	78.7	95.4	**87.1**	100.0	98.5	**99.2**	100	97.6	**98.8**
109	0.0	5.6	**2.8**	1.9	0.0	**0.9**	7.7	5.3	**6.5**	72.5	64.3	**68.4**	6.6	1.1	**3.9**
110	0.0	0.0	**0.0**	1.9	3.5	**2.7**	11.9	17.2	**14.6**	78.6	89.6	**84.1**	11.3	4.0	**7.6**
111	67.9	98.6	**83.2**	91.9	96.6	**94.2**	86.2	89.7	**87.9**	92.8	100.0	**96.4**	86.1	100.0	**93.1**
112	0.0	0.0	**0.0**	1.9	7.7	**4.8**	0.0	1.8	**0.9**	80.7	81.8	**81.3**	0.0	1.3	**0.7**
113	0.0	12.6	**6.3**	15.5	10.0	**12.7**	3.2	11.1	**7.2**	68.4	88.3	**78.3**	1.3	2.4	**1.9**
114	0.0	2.9	**1.5**	1.8	4.2	**3.0**	4.8	4.6	**4.7**	56.7	100.0	**78.4**	11.2	6.0	**8.6**
115	0.0	4.4	**2.2**	4.3	0.0	**2.1**	1.7	1.7	**1.7**	74.0	51.6	**62.8**	0.0	1.5	**0.8**
117	3.7	7.1	**5.4**	0.0	0.0	**0.0**	3.0	2.7	**2.8**	71.4	60.6	**66.0**	8.6	1.2	**4.9**
118	0.0	0.0	**0.0**	2.1	0.0	**1.0**	1.9	5.4	**3.6**	94.2	97.1	**95.6**	0.0	1.2	**0.6**
125	0.0	4.2	**2.1**	10.8	0.0	**5.4**	9.0	4.0	**6.5**	86.1	96.3	**91.2**	0.0	1.1	**0.5**
128	0.0	7.7	**3.8**	2.1	1.6	**1.8**	4.8	22.1	**13.4**	71.5	95.8	**83.7**	10.1	4.1	**7.1**
130	0.0	0.0	**0.0**	0.0	13.5	**6.8**	5.4	8.1	**6.7**	87.1	92.1	**89.6**	1.4	2.4	**1.9**
131	0.0	0.0	**0.0**	2.1	0.0	**1.0**	9.5	9.1	**9.3**	76.7	79.9	**78.3**	2.8	2.3	**2.6**
132	28.9	1.3	**15.1**	5.7	1.7	**3.7**	2.5	3.3	**2.9**	82.0	53.3	**67.7**	0.0	1.3	**0.6**
134	80.6	45.2	**62.9**	0.0	9.7	**4.9**	2.5	9.9	**6.2**	53.1	97.6	**75.4**	0.0	0.0	**0.0**