FINE MAPPING OF QTL-*QSlb.pau-3.04* FOR SOUTHERN LEAF BLIGHT RESISTANCE IN MAIZE (*Zea mays* L.)

Dissertation

Submitted to the Punjab Agricultural University
In partial fulfillment of the requirements
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DOCTOR OF PHILOSOPHY
In
BIOTECHNOLOGY
(Minor Subject: Plant Breeding & Genetics)

By

Kirandeep Kaur
(L-2013-A-48-D)

School of Agricultural Biotechnology
College of Agriculture

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CERTIFICATE-I

This is to certify that the dissertation entitled, "**FINE MAPPING OF QTL-*QSlb.pau*-3.04 FOR SOUTHERN LEAF BLIGHT RESISTANCE IN MAIZE (*Zea mays* L.)"** submitted for the degree of Ph.D., in the subject of **Biotechnology** (Minor subject: **Plant Breeding & Genetics**) of the Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Kirandeep Kaur** (**L-2013-A-48-D**) under my supervision and that no part of this dissertation has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

Major Advisor
(Dr. Yogesh Vikal)
Sr. Geneticist
School of Agricultural
Biotechnology
Punjab Agricultural University
Ludhiana-141004

CERTIFICATE II

This is to certify that the dissertation entitled, "**FINE MAPPING OF QTL-*QSlb.pau*-3.04 FOR SOUTHERN LEAF BLIGHT RESISTANCE IN MAIZE (*Zea mays* L.)"** submitted by **Kirandeep Kaur (L-2013-A-48-D)** to the Punjab Agricultural University, Ludhiana, in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**, in the subject of **Biotechnology** (Minor subject: **Plant Breeding & Genetics**) has been approved by the Student's Advisory Committee along with External Examiner after an oral examination on the same.

(Dr. YogeshVikal)
Major Advisor

(Dr. Sujay Rakshit)
External Examiner
Director,
Indian Institute of Maize Research
PAU Campus, Ludhiana,
Punjab-141004, INDIA

(Dr. G. S. Mangat)
Head of Department

(Dr. Gurinder Kaur Sangha)
Dean, Postgraduate Studies

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ABSTRACT

Southern leaf blight (SLB) caused by *Cochliobolus heterostrophus* (Drechsler), is a serious disease throughout the world where maize is grown under warm and humid conditions, leading to enormous yield losses. Exploiting genes and quantitative trait loci (QTLs) related to SLB is helpful for improving fungal resistance. In earlier study at Punjab Agricultural University, Ludhiana a set of 325 $F_{2:3}$ families and F_4 progenies derived from the cross of CM139 as the resistant (female) parent and CM140 as the susceptible (male) parent were phenotyped for resistance to SLB under field conditions. A total of 172 polymorphic SSR markers were genotyped on F_2 population.Three probable QTL viz. *qSLB2.1*, *qSLB3.1*, *qSLB3.2* were detected for SLB resistance in bins of 2.05-2.08, 3.04 and 3.06-3.09. The marker interval *phi099-umc1729* spanning *qSLB3.1* was considered as major putative QTL. In present study, the QTL *qSLB3.1* spanning region was saturated with more SSR markers on 298 RIL population of the same cross. The detected QTL region (13.7 cM) was fine mapped to 1.2 cM region flanked with marker *MSSR1-MSSR20* explaining phenotypic variance of 15.1 per cent at log-likelihood of 6.9. The 1.2 cM region corresponds to 269.3 Kb and comprises only six candidate genes. The candidate gene based markers were designed, to study the differential expression patterns of candidate genes through quantitative real-time PCR (qRT-PCR). Two of the candidate genes *viz. GRMZM2GO88371* and *GRMZM5G862219* showed 3.48 and 6.40 fold higher expression in CM140 at 48h and 72h respectively. *GRMZM2GO88371* and *GRMZM5G862219* have their biological function in lipid metabolism and β oxidation respectively that is involved in the defense pathway and may be one of the potential candidate genes conferring resistance against *C. heterostrophus*. The SLB QTL linked flanking markers were employed for mobilization of *qSLB3.1* QTL into the background of CM140 through marker assisted backcross breeding (MABB). The F_1s of cross (CM139 X CM140) were backcrossed to recurrent parent to generate BC_1F_1 population. A total of 64 plants out of 420 were selected on the basis of foreground selection. Recombinant selection for the carrier chromosome was done to identify single and double recombinants. The plants having maximum recurrent parent recovery for carrier chromosome were selected and backcrossed with CM140 to generate BC_2F_1 generation. The data generated from this study can serve as valuable genomic resource for maize breeding programmes. It will enable the researcher to multi-thronged and focused approaches for sustainable development of new genotypes by pyramiding it with other desirable genes using MABB.

Keywords: Southern Leaf Blight, Mapping, QTL, Candidate genes, Marker assisted selection

Signature of the major advisor **Signature of the student**

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ਕੋਕੀਲੋਬੋਲਸ ਹੀਟੀਰਿਊਸਟੋਫਸ ਦੁਆਰਾ ਹੋਣ ਵਾਲੇ ਦੱਖਣੀ ਝੁਲਸ ਰੋਗ ਸੰਸਾਰ ਭਰ ਦੇ ਗਰਮ ਅਤੇ ਨਮੀ ਵਾਲੇ ਖੇਤ ਵਿੱਚ ਉਗਾਈ ਜਾਣ ਵਾਲੀ ਮੱਕੀ ਦਾ ਮੁੱਖ ਰੋਗ ਹੈ। ਜਿਸ ਨਾਲ ਝਾੜ ਬੁਰੀ ਤਰ੍ਹਾਂ ਨਾਲ ਘੱਟਦਾ ਹੈ। SLB ਨਾਲ ਸੰਬੰਧਿਤ ਜੀਨ ਅਤੇ QTLs ਨੂੰ ਇਸ ਉੱਲੀ ਦਾ ਟਾਕਰਾ ਕਰਨ ਲਈ ਵਰਤਿਆ ਜਾ ਸਕਦਾ ਹੈ। ਪੰਜਾਬ ਐਗਰੀਕਲਚਰਲ ਯੂਨੀਵਰਸਿਟੀ, ਲੁਧਿਆਣਾ ਵਿੱਚ ਹੋਏ ਪਹਿਲਾ ਅਧਿਐਨ ਵਿੱਚ ਪ੍ਰਤੀਰੋਧੀ ਮਾਦਾ ਜੰਮਹੇਤੂ CM139 ਅਤੇ ਸੰਵੇਦਨਸ਼ੀਲ ਨਰ CM140 ਦੇ ਕਰਾਸ ਤੋਂ ਪ੍ਰਾਪਤ $F_{2:3}$ ਅਤੇ F_4 ਜੰਨਸੰਖਿਆ ਉੱਪਰ 172 ਪੌਲੀਮੋਰਫਿਕ SSR ਮਾਰਕਰ ਜੀਨੋਟਾਇਪਡ ਕੀਤੇ ਗਏ। ਤਿੰਨ *QTL, qSLB2.1, qSLB3.1, qSLB3.2* ਬਿਨ *2.05-2.08, 3.04* & *3.06-3.09* ਵਿੱਚ SLB ਪ੍ਰਤੀਰੋਧੀ ਪਾਏ ਗਏ। ਮਾਰਕਰ ਅੰਤਰਾਲ *phi099-umc1729 qSLB3.1* ਨੂੰ ਮੁੱਖ QTL ਮੰਨਿਆ ਗਿਆ। ਮੌਜੂਦਾ ਅਧਿਐਨ ਵਿੱਚ ਉਸੇ ਹੀ ਕਰਾਸ ਦੀ 298 RIL ਜੰਨਸੰਖਿਆ ਉੱਪਰ SSR ਮਾਰਕਰ ਨਾਲ ਸਪੈਨਿੰਗ ਖੇਤਰ ਵੱਧ ਤ੍ਰਿਪਤ ਸੀ। ਪਹਿਚਾਣਿਆ ਗਿਆ QTL ਖੇਤਰ (13.7 cM) 1.2 cM ਖੇਤਰ ਉੱਪਰ ਫਾਇਨ ਮੈਪਡ ਕੀਤਾ ਗਿਆ। ਜਿਸ ਵਿੱਚ *MSSR1-MSSR20* ਮਾਰਕਰ ਲੋਗ ਲੀਤਾ 6.9 ਉੱਪਰ 15.1% ਅਕ੍ਰਿਤੀ ਵੇਰੀਏਂਸ ਵਰਣਨ ਕਰਦੇ ਹਨ। 1.2 cM ਖੇਤਰ 269.3kb ਦਾ ਸੀ ਅਤੇ ਸਿਰਫ 6 ਉਮੀਦਵਾਰ ਜੀਨ ਦਾ ਸੀ। ਉਮੀਦਵਾਰ ਜੀਨ ਆਧਾਰਿਤ ਮਾਰਕਰ ਪਰਿਮਾਣਾਤਮਕ ਅਸਲ ਸਮਾਂ PCR (qRT-PCR) ਦੇ ਰਾਹੀਂ ਉਮੀਦਵਾਰ ਜੀਨ ਉਕਤੀ ਦੇ ਪੈਟਰਨ ਨੂੰ ਅਧਿਐਨ ਕਰਨ ਲਈ ਡਿਜ਼ਾਇਨ ਕੀਤਾ ਗਿਆ। ਦੋ ਉਮੀਦਵਾਰ ਜੀਨ *GRMZM2G088371* ਅਤੇ *GRMZM5G862219, CM140* ਉੱਪਰ ਕ੍ਰਮਵਾਰ 48 ਅਤੇ 72 ਘੰਟਿਆਂ ਵਿੱਚ 3.48 ਅਤੇ 6.40 ਸੁਣਾ ਵੱਧ ਉਕਤੀ ਕੀਤੀ। *GRMZM2G088371* ਦੇ ਅਤੇ *GRMZM5G862219* ਦੀ ਲਿਪਿਡ ਮੈਟਾਬੋਲਿਜ਼ਮ ਵਿੱਚ ਬਾਇਓਲਾਜੀਕਲ ਫੰਕਸ਼ਨ ਅਤੇ ਰੱਖਿਆ ਪਾਥਵੇਅ ਵਿੱਚ β ਆਕਸੀਕਰਣ ਵਿੱਚ ਭੂਮਿਕਾ ਸੀ ਅਤੇ ਇਹ *ਕੋਕੀਲੋਬੋਲਸ ਹੀਟੀਰਿਊਸਟੋਫਸ* ਦੇ ਵਿਰੁੱਧ ਯਕੀਨੀ ਪ੍ਰਤੀਰੋਧਤਾ ਲਈ ਇਕ ਸੰਭਾਵੀ ਉਮੀਦਵਾਰ ਹੋ ਸਕਦਾ ਹੈ। SLB QTL ਨਾਲ ਸੰਬੰਧਿਤ ਮਾਰਕਰਾਂ ਨੂੰ *qSLB3.1* ਦੀ ਗੋਲਬੰਦੀ ਲਈ ਲਗਾਇਆ ਗਿਆ। 420 ਵਿੱਚੋਂ 64 ਪੌਦਿਆਂ ਨੂੰ ਫੋਰਗਰਾਊਂਡ ਚੋਣ ਦੇ ਆਧਾਰ ਤੇ ਚੁਣਿਆ ਗਿਆ। ਆਪਣੇ ਵਾਹਕ ਕ੍ਰੋਮੋਸੋਮ ਲਈ ਰੀਕੰਬੀਨੇਂਟ ਚੋਣ ਇਕੱਲੇ ਅਤੇ ਦੋਹਰੇ ਰੀਕੰਬੀਨੇਂਟ ਦੀ ਪਹਿਚਾਣ ਕਰਨ ਲਈ ਕੀਤੀ ਗਈ। ਜਿੰਨ੍ਹਾਂ ਪੌਦਿਆਂ ਵਿੱਚ ਵਾਹਕ ਕ੍ਰੋਮੋਸੋਮ ਲਈ ਸਭ ਤੋਂ ਵੱਧ ਰੀਕਰੰਗ ਹੰਮਹੇਤੂ ਰਿਕਵਰੀ ਹੋਈ । ਉਹਨਾਂ ਨੂੰ ਚੁਣ ਲਿਆ ਗਿਆ ਅਤੇ ਦੋਹਰੇ ਰੀਕੰਬੀਨੇਂਟ ਦੀ ਪਹਿਚਾਣ ਕਰਨ ਲਈ ਕੀਤੀ ਗਈ। ਜਿਨ੍ਹਾਂ ਪੌਦਿਆਂ ਵਿੱਚ ਵਾਹਕ ਕ੍ਰੋਮੋਸੋਮ ਲਈ ਸਭ ਤੋਂ ਵੱਧ ਰੀਕਰੰਗ ਜੰਮਹੇਤੂ ਰਿਕਵਰੀ ਹੋਈ ਉਹਨਾਂ ਨੂੰ ਚੁਣ ਲਿਆ ਗਿਆ ਅਤੇ CM140 ਨਾਲ ਬੈਕਕਰਾਸ ਕੀਤਾ ਗਿਆ ਤਾਂ ਕਿ BC_2F_1 ਤਿਆਰ ਕੀਤਾ ਜਾ ਸਕੇ। ਇਸ ਅਧਿਐਨ ਤੋਂ ਪੈਦਾ ਹੋਇਆ ਡਾਟਾ ਮੱਕੀ ਦੇ ਬਰੀਡਿੰਗ ਪ੍ਰੋਗਰਾਮ ਲਈ ਇੱਕ ਮੁੱਲਵਾਨ ਜੀਨੋਮਿਕ ਸ੍ਰੋਤ ਦੇ ਤੌਰ ਤੇ ਕੰਮ ਆ ਸਕਦਾ ਹੈ । ਇਹ ਖੋਜਕਾਰਾਂ ਨੂੰ ਇਸ ਨੂੰ ਹੋਰ ਇੱਛਤ ਜੀਨਾ ਨਾਲ ਪਿਰਾਮਿਡ ਕਰਕੇ MABB ਰਾਹੀਂ ਹੰਡਣਸਾਰ ਮੱਕੀ ਦੇ ਨਵੇਂ ਜੀਨੋਟਾਇਪ ਤਿਆਰ ਕਰਨ ਲਈ ਕੰਮ ਆ ਸਕਦੀ ਹੈ।

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CHAPTER I

INTRODUCTION

Maize (*Zea mays* L.) is one of the most versatile emerging cereal crops belonging to family Poaceae. Globally maize is known as queen of cereals due to its highest genetic yield potential among the cereals. It is the third most important crop for food, feed and basic raw material for various industries after wheat and rice. It is virtually grown in every country in the world with a total production of 26.26 million tons (FAOSTAT 2017). The productivity in India was 3.12 tons/ha, with production of 28.72 million tons from 9.22 million haduring 2017 (FAOSTAT 2017). The projected demand for maize production by 2050 is around 121 million tons in India (Amarasinghe *et al* 2008). Raju *et al* (2010) reported a growth rate of maize area of three per cent per annum in the early years of 21st century. However scope for area expansion is limited so it becomes imperative to explore geographical regions as well as germplasm having high yield potential under biotic and abiotic stresses.

Maize is affected by various biotic and abiotic stresses which cause enormous yield loss during commercial cultivation. About 61 diseases have been reported in maize by Payak and Sharma (1985). These include blights, stalk rot, downy mildew, leaf spot, etc. Maydis leaf blight caused by the fungus C*ochliobolus heterostrophus* (Drechs.) Drechs. [Anamorph = *Bipolarismaydis* (Nisikado) Shoemaker; synonym =*Helminthosporium maydis* Nisikado] is a serious foliar disease distributed widely in maize-producing areas throughout the world where it is grown under warm and humid conditions (White 1999). It is also known as southern leaf blight (SLB) because of its being widespread in the Southern United States. It causes significant qualitative and quantitative losses in parts of India, Africa, Western Europe and Southern United States.

Three races of *C. heterostrophus* known as O, T and C have been reported (Smith *et al*1970; Wei *et al* 1988). It was not considered an important pathogen until 1970 when *C. heterostrophus* race T became prevalent in the U.S. Corn Belt. Race T was highly pathogenic on Texas male-sterile cytoplasm (cms-T), causing a major disease epidemic in 1970 and 1971 (Ullstrup 1972). Since that time, cms-T has been eliminated from elite germplasm and effective polygenic resistance has been introduced. Race C shows high pathogenicity to cultivar with Charrua male-sterile cytoplasm (Wei *et al* 1988). Race O is most prevalent in tropical and sub-tropical areas. It infects the broad range of maize genotype despite the type of cytoplasm. The current predominant form of *C. heterostrophus* is race O (Lennon *et al* 2017) and causes yield losses up to 40 per cent (Fisher *et al* 1976; Byrnes *et al* 1989). This disease can reduce the grain yield up to extent of 41per cent in susceptible cultivars (Sharma and Rai 2000).

Severity and symptoms of the disease depend on the host germplasm and the race of the pathogen (Ali *et al* 2011a). Race O causes small lesions and becomes diamond shape and

rectangular as they mature and vary from 2 to 6 mm wide and 3 to 22mm long. These lesions are restricted to leaf veins (Ali *et al* 2011b). Race T attacks on leaves, husks as well as ears and produces tan with yellow-green or chlorotic halos. Later on, lesions develop to dark brown borders. The shape of lesions is elliptical or spindle and range from 6 to 12 mm wide by 6 to 27 mm long (Ullstrup 1972). Race O and T can be discriminated by pathogenicity test of cms-T plants and physiological /morphological characters on culture media (Leonard 1977; Warren *et al* 1977). Lesions caused by Race C are necrotic 5 mm long and tend to cause wilt (Wei *et al* 1988).

Southern leaf blight occurs in various states of India, mostly in Maharashtra, Gujarat, Jammu and Kashmir, Rajasthan, Meghalaya, Andhra Pradesh, Bihar, Uttar Pradesh, Punjab, Haryana and Delhi. The first report was given by Munjal and Kapoor (1960) for the presence of SLB in India. Mitra in 1931 has first time reported SLB in Punjab on maize and is responsible for maximum damage. At Punjab Agricultural University, Ludhiana, blighting of leaves was observed in maize lines with texas male sterile cytoplasm (Khehra *et al* 1976). Therefore, it is of concern for breeders to develop SLB resistant hybrids. The pathogen can reside overwinter in infected maize tissues on the soil surface or seed, but not in debris buried at 5-20 cm (Ullstrup 1972). Management of crop debris between growing seasons can help to reduce the initial amount of inoculum. However, residue management may not be compatible with conservation farming techniques. Fungicides could be used to provide adequate protection from SLB in the absence of host resistance. Mancozeb, Thiram and Carboxin are the chemicals that have been reported to be effective against SLB (Schenck and Stelter 1974; Kommedahl and Lang 1973). Indofil M 45 at the concentration of 0.2 per cent is considered to be the most effective in managing SLB. Fungicidal control is warranted only in high value crops like hybrid seed production plots or commercially viable crops like QPM, sweet corn, popcorn and baby corn etc. Tilling fields at the end of the season is very helpful because it will break down the infected plant residue left from diseased plants, reducing chances of spores germinating next season. Crop rotation is also recommended to reduce chances of further infection when economically viable. Enhancing host resistance is the most economical and environment user-friendly approach for SLB management.

It has been observed that disease resistance in maize is complex in nature. Qualitative disease resistance is generally controlled by one or a few genes, while quantitative disease resistance is controlled by polygenes. Resistance controlled by multiple genes is more effective and durable, but it is more difficult to manipulate the resistance of this type due to the unknown effects of the environment and the barriers to combine many loci into one genotype. However, such improvement and use of such sources in regular breeding programme is a long-term approach. Therefore, quantitative trait loci (QTL) mapping is a highly effective approach for studying genetically complex forms of plant disease resistance and can provide

useful information for breeding programs, since it allows the estimation of genomic locations and genetic effects of chromosomal regions related to the expression of quantitative traits. Most of the studies have been undertaken in genetic mapping of QTL to assist the development of varieties with improved host resistance (Stuber *et al* 1987; Paterson 1998). Many qualitative plant resistance genes have been cloned and their downstream pathways have been characterized (Hammond-Kosack and Parker 2003; Rathjen and Moffett 2003) but little is known about the molecular genetic basis or mechanism of action of quantitative plant disease resistance genes. Numerous studies have been published on the mapping of disease resistance quantitative trait loci (QTL) to particular genomic regions (Bubeck *et al* 1993; Dingerdissen *et al* 1996; Clements *et al* 2000; Brown *et al* 2001).

The genetic resistance to SLB is either additive or recessive in effect and can be quantitative also (Balint-Kurti *et al* 2008; Burnette and White 1985; Holley and Goodman 1989; Lim and Hooker 1976; Scott and Futrell 1975; Zwonitzer *et al* 2010; Kump *et al* 2011). The monogenic recessive resistance to race O of *C. heterostrophus* was first identified in a Nigerian maize inbred, and the gene was named as *rhm1* (Smith and Hooker 1973). Subsequently, *rhm1* was transferred to many maize inbred lines as the main resistance source to SLB (Cai *et al* 2003).Two linked recessive genes (*rhm1* and *rhm2*) have been identified on chromosome 6 (Zaitlin *et al* 1993; Chang and Peterson 1995).Previous studies have been published on SLB QTL mapping for field resistance from populations of different crosses in maize (Bubeck 1991; Jiang *et al* 1999; Carson *et al* 2004; Balint-Kurti *et al* 2006; Balint-Kurti *et al* 2008; Lenon *et al* 2017), locating different QTL number and position or effect from different populations. This clearly indicates that genetics of this disease is complex and it varies with background effect. Thus, it is required to identify QTL specific to agro-climatic region, so that QTL could be exploited in maize breeding programme to develop resistant inbred lines.

At Punjab Agricultural University an inbred line CM139, resistant and CM140, susceptible to SLB has been identified. Preliminary phenotyping for SLB has been done on $F_{2:3}$ families of cross CM139 x CM140. The disease reaction data showed normal distribution, indicating that resistance for SLB is under polygene control (Chauhan 2013). QTL mapping using 72 SSR markers on 92 $F_{2:3}$ families' detected three putative QTL viz. *qSLB2.1*, *qSLB3.1* and *qSLB3.2* for SLB resistance in bins 2.05-2.08, 3.04 and 3.06-3.09 respectively. The marker interval *phi099-umc1729* spanning *qSLB3.1*in bin 3.04 showed highest QTL peak explaining phenotypic variance of 46.3 per cent at log-likelihood of 12.41 and was considered as major QTL. The QTL *qSLB3.1* found in bin 3.04 was also reported earlier and suggested that bin 3.04 is a 'hotspot' region for SLB resistance (McMullen and Simcox 1995; Wisser *et al* 2006). Bin 3.04 had also been detected as a 'hotspot' for viral resistance. Similarly, Jiang *et al* (1999) identified a QTL for SLB in the vicinity of bin 3.04 using tropical germplasm. SLB

resistance QTL has been identified in bin 3.04 both at juvenile stage (Balint- Kurti and Carson 2006) and at adult plant stage (Carson *et al* 2004). Lennon *et al*(2017) also identified four significant QTL associated with SLB in bins 2.04, 3.04, 3.05, and 8.05.Therefore, the genomic region spanning QTL,*qSLB3.1*detected by Chauhan (2013) needs further exploration with more markers to identify candidate genes linked with SLB resistance QTL. The linked markers will be used to follow marker assisted breeding for SLB resistance.

Keeping in view the epidemics of SLB in Punjab and importance of the QTL region identified in hotspot bin 3.04, the present investigation was undertaken with following objectives:

1. Phenotypic evaluation of RIL population against southern leaf blight.
2. Saturating flanking region on both sides of SLB QTL-*QSlb.pau-3.04* with more SSR markers based on maize genome sequence.
3. Identification and expression analysis of candidate genes.

CHAPTER II

REVIEW OF LITERATURE

Maize (*Zea mays* L.) an important food, feed and fodder crop ranks next to rice and wheat with respect to area and grain production. In India, maize is grown in a range of agro ecological zones. India is the fifth largest producer of maize in the world. On global basis, out of 112 diseases recorded on maize, 35 have been reported in India. Primary diseases include seed rots and seedling blights, root and stalk rots, foliar diseases and ear rots (Paliwal 2000).The reduction in maize yield is affected by many fungal, bacterial and viral diseases. One of the fungal diseases that affect maize yield severely is southern leaf blight (SLB). Southern leaf blight caused by the fungus *Cochliobolus heterostrophus* (Drechs.) Drechs. [Anamorph = *Bipolaris maydis* (Nisikado) Shoemaker; synonym = *Helminthosporium maydis Nisikado*] is a serious foliar disease of maize distributed widely in maize-producing areas throughout the world.

The causal agent of this disease has three races: race O, race T (Hooker *et al* 1970; Ullstrup 1970; Hooker 1972) and race C (Wei *et al* 1988). Among these races, race O has major effect on maize fields of Punjab, which causes reduction in large proportion of yield. Several models of genes that cause resistance to race O of this disease have been proposed. These models are: model with multiple genes (Pate and Harvey 1954), two linked recessive gene model (Craig and Fajemisin 1969) and model including two independent recessive genes with complementary effects (Thompson and Bergquist 1984). Considerable research has been undertaken in genetic mapping of quantitative trait loci (QTL) to assist the development of varieties with improved host resistance. Genetic mapping can detect QTL in discrete regions of plant genomes (Stuber *et al* 1987). Single-gene resistance is more easily mapped and utilized, as compared to polygenic resistance. Hypothetically, resistance controlled by multiple genes is more effective and durable, but resistance of SLB type is also more difficult to manipulate due to the unknown effects of the environment and the barriers to combine many loci into one genotype. The prospect for maize improvement is a challenge as the resistance to this disease is complex in nature. Considering the complexity of SLB resistance and the utility of QTL mapping for identification of genomic regions involved in resistance and its further exploitation in breeding programs, the literature was thus reviewed under following headings:

2.1 Historical background

SLB (no race designation) was first discovered in the United States in 1923 (Robert 1953). It killed the green tissue of the leaves, effectively reduced the photosynthetic source area of the plant. With the exception of isolated outbreaks, prior to the 1970 epidemic, SLB was not considered as major pest of corn in the United States (Carson 2016). The first warning sign of a problem was reported in the Philippines by Mecado and Lantican (1961)

with later confimations by Aala (1964) and Villareal and Lantican (1964, 1965). However, these observations were during below average rainfall seasons and differences between cms-T and normal cytoplasm for SLB were not readily apparent.

In the central Corn Belt states of Illinois, Indiana, and Iowa in 1969, an unidentified disease was infecting the leaves and ears of plants in several production fields. Examinations by Ullstrup (1972), of a seed field in southern Iowa showed that *H. maydis* was involved in the rotting of the ears. Hybrids without cms-T showed no ear or stalk infections and very few lesions on the leaves. Scheifele *et al* (1970) reported the same disease in corn growing in Southern Illinois. An estimate of the total loss of corn from the 1970 epidemic in North America was 254 million hectoliters of grain (Tatum 1971).

Since 1971, cms-T has not been a factor in corn production. Later research revealed that the mitochondrial gene *T-urf13* responsible for inducing male sterility in cms-T corn was also responsible for its susceptibility to SLB race T (Forde *et al* 1978; Dewey *et al* 1986, 1987; Wise *et al* 1987). Levings (1990) showed that there was an interaction between URF-13, the protein coded for by *T-urf13*, and the fungal toxins of SLB race T accounted for the specific susceptibility of cms-T genotypes to fungal attack.

Race T of SLB is more destructive to host plants than race O because of its tendency to form lesions on the leaf sheaves, ear husk, developing grain on the ear, as well as leaf blades (Carson 2016). Both races though can predispose the host plants to secondary infections of various stalk rotting diseases increases harvest losses further due to stalk lodging and dropped ears. Race T's ability to infect developing grain can cause substantial losses at harvest by direct destruction of the kernels. SLB race C was reported in 1971 from a Brazilian cultivar Charrua. It has been used extensively in China as destructive to susceptible hybrids as SLB race-T was to cms-T hybrids during the epidemic of 1970-1971 in North America. Wei *et al* (1988) stated that a major epidemic of SLB race-C similar to that in North America could occur in China or elsewhere if the majority of hybrids being grown are of cms-C genotype.

2.1.2 Occurrence and distribution

In the 1970s SLB caused by *Helminthosporium maydis* T. resulted in catastrophic losses in maize crops in the USA and UK (Ullstrup1972, Carson 1998, Manamgoda *et al* 2012). Later, a similar fungus was described on maize in New Jersey, USA and was named as *Heliminthosporium inconspicum* (Tarr 1962). These two species were generally identical as discussed by *Drechseler* (1923). Drechsler (1925) isolated a *Helminthosporium* sp. from maize leaves and found it to be distinct from *Helminthosporium turcicum* in conidial morphology. Later on, he identified this fungus as *Ophiobolus heterostrophus*. Nisikado and Miyake (1926) identified the conidial stage of this fungus as *H. maydis*. Nisikado (1928) divided *Helminthosporium* into two subgenera: *Cylindro-helminthosporium* and *Eu-*

heliminthosporium. Hilum morphology can also be used to differentiate *Bipolaris* and *Drechslera*. In *Drechslera* a flat scar exists within the lowest part of the basal cell, whereas in *Bipolaris* it is inconspicuous or very slightly protuberant (Lutrell 1964,; Alcorn 1988). The sexual morphs of *Drechslera* have been linked to *Pyrenophora* whereas the sexual morphs of *Bipolaris* were regarded as *Cochilobolus* (Drechsler 1934). The name *Heliminthosporium* based on *Heliminthosporium maydis* as the type species should be conserved for the species in *Cylindro-heliminthosporium* and *Eu-heliminthosporium* in accordance with the provision in Article 14 of the International Code of Botanical Nomenclature or this provision might later be abandoned (Lanjouw 1961).

Drechsler (1934) assigned the name *Cochliobolus heterostrophus* to the sexual stage of *H.maydis.* Shoemaker (1959) classified *H. maydis* under the genus *Bipolaris* and renamed it as *Bipolaris maydis.* Conidial stage of *H. maydis* was isolated from sorghum seeds in Punjab (Mitra 1931). Ullstrup (1941) reported physiological variability in *H. maydis* isolates from the United States and further Ullstrup (1954) identified more isolates of *H. maydis* with cultural and pathological differences. Singh and Singh (1963) reported this fungus on maize from Uttar Pradesh. The interest on this line of work was revived with the outbreak of a severe epiphytotic of *H. maydis* in the United States when Hooker *et al* (1970) reported race T from this region. Origin of SLB is not very clear. Race T was isolated from herbarium specimens collected in 1955 from the United States (Nelson *et al*1970). Leonard (1971) believed that race T was introduced into the United States or, alternatively, it originated as a result of mutation. One of the reports discussed that race T of this fungus had been the predominant member of the pathogen population in the United States since several years. Subsequently, this pathotype was reported from other parts of the world also (Craig 1971; Terry 1973; Danquah *et al* 1975).

In India, *H. maydis* was first reported by Munjal and Kapoor (1960) from Maldah in West Bengal Later on, Nath *et al* (1973) isolated race T of *H. maydis* from sorghum seeds in India. Khehra *et al* (1976), Bains *et al* (1977-78) and Sharma *et al* (1978) reported the presence of *H. maydis* isolates closely resembling race T in different characters from Punjab. Misra (1979) collected *H. maydis* variants from different parts of India with varied morphological and pathological characters. Race C has been found in China and is specific to cms-C cytoplasm maize and race O infects all types of maize that do not carry resistance genes, regardless of the type of cytoplasm (Smith 1975). In addition to maize, *D. maydis* is also known to infect sorghum and teosinte.

Sharma *et al* (1978) reported the outbreak of *H. maydis* from Ludhiana, Rajasthan, and now became a serious disease particularly in Jammu and Kashmir, Himachal Pradesh, Sikkim, Meghalaya, Punjab, Haryana, Rajasthan, Delhi, UP, Bihar, MP, Gujarat, Maharashtra, Andhra Pradesh, Karnataka and Tamil Nadu. The disease is prevalent in warm

humid temperature ranging from 20-30 ˚C during cropping period (Singh and Srivastava 2012). The *C. hterostrophus* injures or kills the leaf tissues and thereby reduces the area of chlorophyll which involved in photosynthesis. If considerable leaf area is killed, then vigour and yields are reduced drastically. If much of the green area is killed starch formation is restricted and the kernels become chaffy (Goudar *et al* 2019). The blighted leaves are not suitable for fodder because of the lowered nutrition value.

The disease is currently known worldwide, widespread in the tropics and subtropics or being more important in regions with warm (20˚-32˚ C) and damp climate (Ellis and Holliday 1971; Singh *et al* 1979; Forutan and Rahimian 1990; White 1999; Martyniyuk 2003; Anjos *et al* 2004; Shah *et al* 2007; Martinez *et al* 2010).

2.1.3 Symptoms

The long, dry and sunny periods during the growing season are unfavourable, whereas rainy season with high humidity and warm temperature favours the development of SLB disease. Symptoms and severity of SLB depends on the pathogen race and host germplasm. Race O initially produced small and diamond-shaped lesions which elongate as they mature. Final lesions were tan coloured, rectangular (2-6 x 3-22 mm) in shape and restricted by leaf veins (Ullstrup 1972). Lesions of SLB caused by race T were oval and slightly larger (6-12 x 6-27 mm) than those caused by race O. Lesions were usually characterized by dark and brown borders. Race T caused lesions on all above ground parts of the plant (including stems, sheaths and ears) and also caused ear rots. Seedlings from race T infected seeds were wilted and died within 3 to 4 weeks (Ullstrup 1972). Peet and Maschtti (1972) reported that rainfall, relative humidity and temperature were critical factors in the spread of *D. maydis*. Under severe disease pressure, usually when infection occurred prior to silking, lesions tends to coalesce and results in blighting of the entire leaf. In such circumstances, plants were predisposed to lodging.The SLB is generally a late season diseaseas disease development increases after anthesis (White 1999).

2.1.4 Damage

Gregory *et al* (1978) observed that several factors such as susceptible varieties, crop growth stage and planting time contributed to high disease intensities and ultimate yield losses. The extent and severity of this disease varied from season to season. Infection initiated late in the season, led to considerable losses in grain yield of maize. SLB disease causes significant yield losses in cultivars developed from subtropical or temperate germplasm from 9.7 % to 11.7 % depend upon the weather conditions (Bera and Giri 1979; Harlapur *et al* 2000; Sharma and Sharma 2006; Kumar and Saxena 2007).Seedlings grown from kernels infected with the pathogen might die within four weeks following planting. Increased application of nitrogen fertilizer and increased crop density were associated with increased disease severity.

SLB, predominantly caused by race O, is still a significant problem in the southern Atlantic coast area of the United States and parts of India, Africa and Western Europe. It can cause grain yield losses of 40 per cent or more (Ullstrup 1972; Fisher *et al* 1976; Gregory *et al* 1979; Byrnes *et al* 1989). Zwonitzer *et al* (2009) also came forward with reports of 15-46 per cent losses due to SLB. The damage caused by SLB resulted in loss of photosynthetic leaf area due to foliar lesions which reduced photosynthate production for grain filling. Further damage was caused by lodging, which occurred when plants divert sugars from the stalks for grain filling during severe disease pressure. SLB had been potentially damaging the crop and caused significant losses in warm and humid environments (Thompson and Bergquist 1984).

Ali *et al* (2011b) revealed that damage was most critical when infection occurs prior to silking and also if weather conditions persist favorable for disease development during the reproductive growth stages. If cultivating varieties with the Texas source of male sterility, infection by Race T pathotypes can lead to extensive stalk and ear rots. Singh and Srivastava (2012) reported that SLB disease causes the significant yield losses in cultivars developed from subtropical or temperate germplasm ranging from 9.7 per cent to 11.7 per cent depending upon the weather conditions. It is a widespread disease in India with the potential to cause 30-40 per cent yield losses in hot and humid tropical and subtropical regions (Kumar *et al* 2009).In many instances disease resulted in reduction in the value and quality of the grain and might increase harvesting costs due to lodging of plants when affected.

2.1.5 Management strategies

The cultural methods could prevent the spread of all races of *D. maydis* by managing crop debris between growing seasons, as *D. maydis* overwintered in the leaf and sheath debris. It had been observed that burying residues by plowing had reduced the occurrence of SLB as opposed to minimal tillage, which could leave residue on soil surface. Another form of cultural control used to limit SLB was crop rotation with non-host crops (Sumner and Littrell 1973).Several fungicides had been used to control SLB but it was difficult to suppress seed and soil borne pathogen. The fungicides must be applied to plants immediately at the initial stage of SLB infection. Depending on the environmental conditions, re-applications of fungicides might be necessary during the growing season.

Stoner *et al* (1954) demonstrated that *D. maydis* was controlled by timely application of Nabam and Zinc sulphate together or Zineb alone. Miller (1972) observed that Zineb and Maneb provided an effective protection against *D. maydis* under the green house conditions whereas 1.7 Kg/ha of Manozeb in 23.4 or 28.0 liters of water/ha was found to be effective as observed by Schenk and Stelter (1974). Yield from plants with effective disease control were consistently better than non sprayed plants. Effectiveness of foliar spray of tilt for management of SLB was recommended by Kumar *et al* (2009). According to them integrated SLB management strategies involving biological, cultural and chemical methods were

appropriate. They also reported that this disease was adequately managed by spraying garlic and neem leaf extract @ 5per cent in field. Afterwards, they found that soil application of *Trichoderma* was more effective than foliar spray. Soil application of *T. viride* resulted in minimum disease intensity of SLB followed by *T. harzianum*. More recently, a fungicide namely Indofil M45 (200 gm/100 l of H_2O) had been under usage as recommended by Mohan (1980).

The best practice for management of this disease is breeding for host resistance. Both single gene and polygene resistance sources had been discovered. In some resistant hybrids flecking had been found, but was only a reaction to resistance and would not cause loss of economic significance. During the last three decades, considerable research efforts had been directed towards the quest for stable resistant sources and their subsequent utilization in the development of high yielding resistant cultivars. Misra and Singh (1971) reported only 4 varieties of resistant maize during 2 seasons in Bihar on the basis of environment impact on resistance mechanism against *D. maydis* in maize. On the other hand, Jha *et al* (1977) observed that none of the 19 varieties of maize were completely immuned to *D. maydis* under natural conditions whereas Ganga-2, Ganga-4 and Ganga-5 were only relatively resistant.

Sharma and Payak (1990) identified two inbred lines namely, CM 104 and CM 105 which in a span of 14 years had been able to sustain resistance to race O at a high level with the resistance rating not exceeding 2.0 (in a scale of 1-highly resistant to 5-highly susceptible). In the last three decades of research in the All India Coordinated Maize Improvement Project, though several high yielding hybrids and composites were developed but had either lower or at par level of resistance to this disease as compared with CM 104/CM 105.

Fifty one maize genotypes were analyzed by Rai *et al* (2009) against *D. maydis*. They recorded significant variability for disease severity under artificial conditions of full season maturity group. The per cent disease severity ranged from 1.5 to 4.5 at leaf stage. Out of 51, 26 genotypes were rated as resistant, 8 moderately resistant, 13 moderately susceptible, 2 susceptible and 2 highly susceptible against this disease. Kaur *et al* (2010) screened twenty maize inbred lines against SLB under artificial epiphytotic conditions during monsoon 2005 and 2006 and reported one genotype as resistant, nine as moderately resistant and eleven as susceptible. The resistant genotype derived from the cross between LET DR99 and ENT 49-2.

Singh *et al* (2014) evaluated 119 maize genotypes to identify the new sources of resistance to *D.maydis* under artificial epiphytotic condition at three locations *i.e* Almora, Nagenahalli and Varanasi for consecutive 12 years (1999-2011). On the basis of combined data, it was inferred that 41 were resistant, 41 showed moderately resistant reaction while 24 and 13 were categorized as susceptible and highly susceptible respectively. Among these, ten lines *viz.* V53, V 178, V 190, V 336, V 340, V 341, V 345, V 348, CM 104 and CM 145

showed high level of resistance, whereas five inbreds *viz*. V 49, CM 126, CM 127, CM 202 and CM 212 showed high level of susceptibility across the environment. Mubeen *et al* (2015) accessed 12 maize genotypes : NC-2703 (hybrid), NC-2003 (hybrid), SP-3 (inbred line), NCML-73 (inbred line), NRL-6 (inbred line), NRL-4 (inbred line), Soan-3 (variety), Rakaposhi (variety), Margala (variety), EV-1097 (variety), Local-Y (variety), Local-W (variety) against SLB both under laboratory and field conditions. The SP-3 and NCML-73 inbreds were found highly resistant whereas Local-W as moderately resistant and other genotypes were least resistant in *in vitro* analysis. Under field screening, Margala, NRL-4, EV-1097 showed maximum resistance while SP-3, NCML-73, NC-2703, NRL- 6 and Local-Y maize genotypes showed moderate resistance. NC-2003, Rakaposhi and Soan-3 showed least resistance during field evaluation. It indicates that scrutinizing of germplasm for resistance is the pre-requisite to study its genetics and utilization in breeding programs.

Lai *et al* (2016) recommended that SLB can be well controlled by rhizobacteria-mediated induced systemic resistance in combination with reduced but appropriate application of dithiocarbamate fungicides just before a heavy infection period. An appropriate use of rhizobacteria can enhance plant growth and help plants overcome negative effects caused by dithiocarbamates. They developed a strategy that when corn plants were drench-treated with *Bacillus cereus* C1L. Frequency of spraying dithiocarbamate fungicides could be decreased. The treatment of *B. cereus* C1L was able to protect maize from southern leaf blight while residues of dithiocarbamates on leaf surface were too low to provide sufficient protection.

2.1.6 Genetics of SLB resistance

Plant pathologists and breeders recognized two general types of resistance: qualitative and quantitative. Qualitative resistance conferring a high level of resistance was usually race-specific and based on single dominant or recessive genes. In contrast, quantitative resistance in plants was usually partial and race-nonspecific in phenotype; oligogenic or polygenic in inheritance and was conditioned by additive or partially dominant genes. It was easier to work with qualitative resistance in crop genetic studies and in breeding but quantitative resistance was often the more useful in an agronomic context, due to its generally higher durability and broader specificity (Lindhout 2002; Parlevliet 2002).

After 1970 epiphytotic, several researchers began extensive investigation into the nature of resistance to *D. maydis*. Lim and Hooker (1972) studied some quantitative sources of SLB resistance available in maize germplasm by using different combinations of normal and cms-T cytoplasms in conjunction with race O and/or race T of the pathogen. Their research demonstrated that resistance genes were partially dominant and that no cytoplasmic-genomic interaction was associated with resistance.

Smith and Hooker (1973) determined that resistance from the Nigerian source was controlled by a single recessive gene, designated as *rhm* gene, conferring a high level of

resistance to the pathogen up to anthesis, at which time resistance was lost. The *rhm1* gene had been of limited use in commercial maize hybrids in the United States due to its recessive inheritance and its ineffectiveness in providing effective control after anthesis.

Lim (1975) and Lim and Hooker (1976) showed that additive gene action was much greater than non-additive effects, but neither study used an experimental design nor genetic model that would allow estimation of heritability, numbers of effective factors or the relative importance of additive, dominance and epistatic gene action. Another type of resistance had been observed in some Nigerian inbred lines in which the reaction to infection was small, circular and chlorotic lesion supporting little sporulation.

Thompson and Bergquist (1984) emphasized on mature plant resistance, with disease ratings taken at several weeks after flowering. They also concluded that resistance in inbred NC250 to *D. maydis* race O was controlled by relatively few loci having additive genetic effects and this type of resistance might be incorporated into susceptible inbreds by backcrossing.

Burnette and White (1985) derived 12 families from crosses of nine resistant and three susceptible inbreds. When the Hayman and Mather model was fit to the data, highly significant additive genetic effects were evident in all families with 49.0 to 96.9 per cent of the total variation. Estimates of broad-sense heritability ranged from 29.5 to 69 per cent and the estimated numbers of effective factors ranged from 2.2 to 14.6 per cent. Although virulence to these nuclear genes had not been observed in nature. Kolmer and Leonard (1986) were able to increase the aggressiveness of *D. maydis* race O to a partially resistant maize inbred line through recurrent selection. Their selection increased not only the level of general aggressiveness of *D. maydis* to all maize inbreds tested, but also specific virulence to the partially resistant inbred line on which the recurrent selection was practiced. They speculated that the reason (s) of such selection had not been observed in nature may be due to rare occurrence of sexual reproduction in *D. maydis*, the low heritability of virulence, and the constantly changing genetic makeup of maize hybrids being grown.

Holley and Goodman (1989) documented several sources of resistance to SLB in tropical maize. They selected three highly resistant inbred lines (X105A, H5 and X306B) to detect new sources for SLB resistance. These inbreds were intercrossed (X105A x H5; X306B x H5 and X105A x X306B) and were also top crossed to a known resistant (NC250) and susceptible (B73) inbred. They presented a preliminary data on their mode of inheritance. Resistant lines from these crosses had shown resistance equivalent to the level of resistance available from NC250. When the tropical inbreds were crossed to B73, the inbreds from crosses of X105A x H5 and X105A x X306B showed additive type of gene action whereas X306B x H5 cross showed recessive type of gene action. It was also concluded that, some

minor modifying genes that combine with resistance genes of NC250 in positive epistatic manner to induce SLB resistance in these lines.

Zaitlin *et al* (1993) derived 102 F_3 families from the cross of $H95_{rhm}$ × B73 to study the linkage of *rhm* gene to RFLP marker loci. The results indicated that *rhm* was tightly linked to two RFLP marker loci (*umc85* and *p144*) that were mapped to the short arm of chromosome 6 (bin 6.00).Chang and Peterson (1995) proposed that there were two linked recessive genes (*rhm1 and rhm2*) controlling resistance to SLB. It was also postulated that one of the two genes was a copy of the other created by duplication. The dominant status at either locus makes a functional product and thus abolished resistance.

Carson *et al* (2004) identified that the additive effects of QTLs associated with SLB on chromosomes 1, 4 and 10. Balint-Kurti and Carson (2006) detected QTLs for SLB in bins 1.06, 1.08/09, 2.09, 3.04, 3.06, 5.07, 6.00, 7.02 and 10.06/07. They found no epistatic interactions between any of these QTLs. Balint-Kurti *et al* (2008) detected an epistatic interaction between the two QTLs in bin 3.04, with a partial phenotypic variation (R^2) of 2.2 and an additive effect of 0.23. Nageri *et al* (2011) detected additive mode of inheritance for SLB in three maize recombinant inbred populations derived from the crosses of B73 x CML254, CML254 x B97 and B97 x Ki14. These populations were assessed in two environments: Clayton, NC, in the summer and Homestead, FL, in the winter.

Kump *et al* (2011) postulated that small additive effects were responsible for SLB resistancein the maize nested association mapping (NAM) population. No significant epistatic interactions were observed between them. Bian *et al* (2014) analysed the genetic architecture of resistance to SLB in the NAM population. It was observed that resistance was associated with more than 30 loci with small additive effects by using joint linkage mapping and genome-wide association study. Thirty-three QTLs were identified with small additive effects across 25 NAM families.

Yang *et al* (2017) detected a quantitative trait locus, $qMdr_{9.02}$, present on chromosome 9 which was associated with resistance to three important foliar maize diseases *viz.* southern leaf blight, gray leaf spot and northern leaf blight. Through fine-mapping, association analysis, expression analysis, insertional mutagenesis and transgenic validation, it was demonstrated that *ZmCCoAOMT2*, gene within $qMdr_{9.02}$, encodes a caffeoyl-CoA O-methyltransferase was associated with the phenylpropanoid pathway and lignin production, conferring quantitative resistance to both southern leaf blight and gray leaf spot.

2.2 Molecular studies in maize

The maize genome is one of the most extensively analyzed among the plant genomes. It had been used in development and evaluation of an array of molecular markers for various purposes in genetics and breeding. Besides the well demonstrated utility of markers in genotype differentiation and analysis of genetic diversity in maize germplasm, application of

DNA based markers was also of considerable significance for mapping and marker assisted selection (MAS) for resistance to major biotic/abiotic stresses affecting production and productivity. Judicious integration of conventional and molecular approaches in maize breeding programmes was vital for efficient utilization of genetic resources, and improving the production and post-harvest characteristics of maize germplasm (Prasanna and Hoisington 2003).

2.2.1 Detection of genetic polymorphism

Various types of DNA polymorphisms that occur in or near genic sequences can be used as genetic markers. These include restriction fragment length polymorphisms (RFLPs), single strand conformational polymorphisms (SSCPs), simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs) (Liu and Corder 2004). SSRs have proven to be highly polymorphic and useful as genetic markers in many plant species including maize (Senior and Heun 1993), *Arabidopsis* (Depeiges *et al* 1995), soybean (Akkaya *et al* 1995; Rongwen *et al* 1995), sweet potato (Jarret and Bowen 1994) and wheat (Plaschke *et al* 1995; Röder *et al* 1995).

A survey of 859 primer pair sequences of SSR markers was used by Sibov *et al* (2003) to characterize the level of polymorphism between two elite inbred lines, L-08-05F and L-14-4B. About 54 per cent of the markers gave clearly scorable amplification products, 13 per cent did not amplify and 33 per cent could not be scored on agarose gels. Out of total markers, 213 markers were found to be polymorphic.

Song *et al* (2004) screened 439 SSR markers between the two parents, high oil line (By 804) and a normal line (B73), and 158 markers were found to be polymorphic with 36 per cent of polymorphism. Zhang *et al* (2006) analyzed 778 pairs of SSR markers to detect the polymorphisms between parents, R15 and Ye478 and 159 SSR markers were found to be polymorphic i.e. 20.44 per cent level of polymorphism was observed.

Zhang *et al* (2008) detected polymorphism between elite high-oil inbred (By804), selected from BHO and a normal line (B73) by using 543 SSR markers. Out of 543 markers, 183 markers were found to be polymorphic with 33.7 per cent of polymorphism. The frequency of homozygous markers ranged from 13.76 to 34.48 per cent, with an average of 23.78 per cent. The frequency of homozygous markers from B73 varied between 12.31 to 33.79 per cent, with an average of 23.59 per cent. The frequency of heterozygous markers from the parents ranged from 42.86 to 65.52 per cent, with an average of 52.63 per cent.

Shi *et al* (2010) analyzed 500 SSR markers to identify QTLs for plant height in a new dwarf germplasm of maize. To map the dwarfing gene in Ai 2003 (a dwarf inbred line of maize), a segregation population containing 255 $F_{2:3}$ families was constructed by crossing Ai 2003 to Ji 257 (inbred line with normal plant height). 158 markers out of 500 showed polymorphism between Ai 2003 and Ji 257. Zwonitzer *et al* (2010) screened B73 and Ki14

with ≈200 SSR markers and 1536 SNP markers to identify polymorphic markers. A total of 765 SNPs and 106 SSR markers were found to be polymorphic.

Polymorphism analysis between B73 and *parviglumis* using 713 SSR makers revealed 220 (30.9 percent) polymorphic. The polymorphic markers were then applied to the F_2 population analysis. A total of 320 polymorphic markers were identified after testing 873 SSR markers on B73 and *diploperennis*, and the polymorphism was 36.6 per cent. These markers were used to analyze the backcross (BC) population. In this study, B73, *parviglumis* and *diploperennis* were different subspecies of the genus *Zea.* The SSR analysis showed that the polymorphic ratio between B73 and *diploperennis* was higher than that between B73 and *parviglumis.* The result implied that the sequence divergence between B73 and *parviglumis* was less than that of B73 and *diploperennis* (Wang *et al* 2012).

Xu *et al* (2013) used whole-genome sequences from 16 maize inbreds and 1 wild relative to determine SSR abundance and to develop a set of high-density polymorphic SSR markers. They identified 264 658 SSRs across these 17 genomes. Then they compared SSR sequences and e-polymerase chain reaction analysis and created new database, including 111 887 SSRs, that could be develop as polymorphic markers *in silico*. Among these markers they found that 58.00, 26.09, 7.20, 3.00, 3.93, and 1.78 per cent of them had mono, di-, tri-, tetra-, penta-, and hexa-nucleotide motifs, respectively and the polymorphic information content for 35 573 polymorphic SSRs out of 111 887 loci varied from 0.05 to 0.83.

2.2.2 Construction of molecular linkage maps

Marker-dense genetic maps contribute greatly to the understanding of evolutionary processes, enable marker assisted selection and mapping of agronomic traits and facilitate many aspects of crop improvement. Instead of this, high-resolution genetic maps are essential for positional cloning of genes and for providing the genetic framework for physical map construction. Significant progress in plant genome mapping had been made by using specialized mapping populations and molecular markers suitable for genome-wide, high-throughput mapping.

Genetic mapping in maize was first carried out using morphological markers generating a genetic map consisting of 62 morphological trait loci (Emerson *et al* 1935). The first generation of molecular marker maps in maize was constructed using restriction fragment length polymorphisms (RFLPs) (Coe *et al* 1987, Burr *et al* 1988).

Gardiner *et al* (1993) constructed linkage map using RFLP markers on F_3 population of cross TX303 xC0159. A total of 215 markers consisting of 159 genomic clones, 16 isozymes and 35 cloned genes of defined function had been assigned to 10 chromosomes. Genetic mapping with DNA probes and PCR-based markers had identified many loci in the maize genome (Davis *et al* 1999). These maps were later saturated with SSRs and other types of PCR based markers (Lee *et al* 2002). Sharopova *et al* (2002) developed 1051 novel SSR

markers for maize from microsatellite-enriched libraries and by identification of microsatellite containing sequences in public and private databases. Three mapping populations were used to derive map positions for 978 of these markers. The main mapping population was the intermated B73 xMo17 (IBM) population.

Sibov *et al* (2003) generated genetic map with 117 markers of 1634 cM in length with an average interval of 14 cM between adjacent markers. Song *et al* (2004) used 155 SSR markers to construct a genetic linkage map of 1759.1 cM length with an average interval of 11.65 cM. Briggs *et al* (2007) genotyped the BC_1 plants for 294 polymorphic markers, including 270 SNPs, three indels, and 21 SSRs for construction of a genetic linkage map. The molecular markers were assembled into a linkage map of 1474.9 cM. The average distance between adjacent markers was 5.4 cM.

Zhang *et al* (2008) derived a mapping population of 298 $F_{2:3}$ family lines containing Beijing high-oil (BHO) maize germplasm to map QTLs for high oil, starch and protein. The F_2 individuals were genotyped with 183 SSR markers and constructed a genetic linkage map, which spanned 1605.7 cM. The genetic distance between adjacent markers ranged from 0.2 to 33.3 cM, with the average interval of 8.77 cM. The distance between adjacent markers in the map was lower than 20 cM for 92.49 per cent of markers. Shi *et al* (2010) constructed a genetic linkage map by using 114 SSR markers covering the entire maize genome. The total map length was 2852.1 cM with an average distance of 27.4 cM.

A linkage map was generated by Zwonitzer *et al* (2010) using 871 molecular markers (SSR and SNP markers) covering a total map length of 1697.3 cM. The average distance between mapped markers was 1.9 cM. The markers *dupssr14* and *PHM14046.9* on chromosome 8 (bin 8.08) were inverted in the linkage map constructed in this study compared with the marker order reported in the intermated IBM reference map (IBM2 2005 Neighbors map). Ding *et al* (2011) developed 225 $F_{2:3}$ families to construct the genetic map consisting of 180 SSR markers distributed on 10 chromosomes. The map spanned a total length of 1987.7 cM with an average distance of 11.0 cM between markers.

Jiang *et al* (2011) developed the genetic linkage map for QTLs mapping of coleorhizae length (CL) in maize using 255 SSR markers, and it covered 10 maize chromosomes. The total length of maize genome reached 2399 cM. The genetic distance between adjacent markers ranged from 0 to 45.9 cM, where the average distance was 9.4 cM and 14.51 per cent of the intervals were greater than 20 cM. The number of markers per linkage group varied from 11 to 43. A linkage genetic map was constructed using a F_2 population derived from a cross between a elite maize inbred, B73, and its progenitor, teosinte (*Z. mays* ssp. *mexicana*), using 205 SSR markers and one morphological marker. The polymorphic markers were clustered into 10 groups, covering 10 chromosomes of maize x teosinte, with a total length of 1947.8 cM and an average interval of 9.4 cM (Zhou et al 2011).

Wang *et al* (2012) constructed a genetic linkage map of B73 x*parviglumis* with 220 polymorphic SSR markers, which included linkage groups (10 chromosomes) with 172 SSR markers and total length of map was 2210.8 cM. The longest linkage group was 327.3 cM and the shortest was 76.1 cM. The average genetic distance between two neighbouring loci was 12.8 cM and the distribution of markers on the chromosomes was relatively even, without crowding in any region. The 320 polymorphic markers were used for analyzing the BC population of cross B73 x*diploperennis*. In the segregation analysis among the individual plants, 258 markers were mapped into 10 linkage groups, which had a total map length of 1357.7 cM with an average genetic distance between neighbouring markers of 5.3 cM.

Molecular linkage maps had also been constructed among maize × teosinte crosses, including *Z. mays* ssp × *mexicana* (Doebley and Stec 1991), *Z. mays* ssp × *parviglumis* (Doebley and Stec 1993, Lauter and Doebley 2002, Briggs *et al* 2007), *Z. mays* ssp × *huehuetenangensis* (Mano *et al* 2005), *Z. luxurians* (Omori and Mano 2007, Mano *et al* 2008). All these maps had different map length coverage depending upon the diversification of two species.

The construction of high-density linkage maps appears more feasible since the advent of next-generation sequencing (NGS), which eases SNP discovery and high-throughput genotyping of large population. Khanal *et al* (2015) compared the linkage maps and precision of QTL detection in an intermated recombinant inbred line (IRIL) population and a selfed recombinant inbred line (RIL) population. Both, IRIL and RIL, populations were developed from inbred lines CG60 and CG102. The populations were grown in two environments to evaluate traits, and inbred lines from each population were genotyped with SSR and SNP markers for linkage map construction and QTL identification. Both RIL and IRIL populations was simulated to compare the precision of QTL detection. In the empirical study, the linkage map was longer in RIL as compared with IRIL, and the average QTL support interval was reduced by 1.37-fold in the IRIL population compared with the RIL population.

A high-density genetic map was constructed using a genotyping by sequencing (GBS) strategy with 4183 bin markers (100-Kb intervals with no recombination events). The total genetic distance covered by the linkage map was 1545.65 cM and the average distance between adjacent markers was 0.37 cM with a physical distance of about 0.51 Mb. It has been demonstrated that there was a relatively high degree of collinearity between the genetic map and the B73 reference genome (Zhou *et al* 2016).

Su *et al* (2017) also used GBS for large-scale SNP discovery and simultaneous genotyping of all F_2 individuals from a cross between two varieties of maize (SG-5 and SG-7) that are in clear contrast in yield and related traits. The SNP genotypic data was utilized to construct an intra-specific genetic linkage map consisting of 3,305 bins on 10 linkage groups spanning 2,236.66 cM at an average distance of 0.68 cM between consecutive markers.

Rameker *et al* (2018) generated the linkage map consisted of 259 *Mu*-anchored fragments, 34 SCARs, and 614 SSR markers, distributed over the ten maize chromosomes. From the linkage analysis, it was observed that three SNP loci associated with kernel starch synthesis genes (*sh2*, *su1*, *wx1*) linked to either *Mu*-TD loci or SSR markers, which may be useful for maize breeding programs. In addition, QTL analysis was done to determine the chromosomal location of traits related to grain yield and kernel quality.

Linkage mapping, mainly using RFLPs and SSRs, had been carried out in maize by numerous laboratories since the 1980s and till to-date with high density SNP markers. A large amount of data is now available in the Maize GDB database (Anonymous 2019) for selection of markers and their utilization in breeding programs.

2.2.3. Mapping of SLB resistance gene(s)/QTL

Maize, a cross-pollinated species with a comparatively small genome, is amenable to gene investigation, offering researchers the opportunity to study the location and behavior of factors underlying quantitative genetic variation. A number of quantitative traits have been extensively investigated using conventional biometrical approaches. The development of mapping techniques in the last few decades had opened novel avenues for the identification of chromosomal regions harboring QTLs controlling important traits.

In maize, the majority of disease resistance deployed in elite varieties in the field was quantitative in nature. Cloning genes conditioning quantitative resistance was much more challenging because of their modest phenotypic effects. Paterson *et al* (1988) reported the first use of a complete RFLP linkage map to resolve quantitative traits into discrete Mendelian factors, in an interspecific backcross of tomato. Six QTLs controlling fruit mass, four QTLs for the concentration of soluble solids and five QTLs for fruit pH were mapped. The chromosomal segments associated with quantitative resistance were also identified with the aid of molecular markers (Tanksley 1993; Kearsey and Farquhar 1998).

Linkage analysis using $F_{2:3}$ population, revealed that the RFLP marker, *umc85* was closely linked to SLB resistance locus *rhm* (Zaitlin *et al* 1993). Similarly they also found that RFLP marker, *agrP144*, was tightly linked to *rhm* at a distance of 0.5 cM and *rhm* had been mapped to bin 6.00. Moon *et al* (1999) identified a major QTL for SLB resistance in a set of recombinant inbred lines (RILs), but the technique did not allow mapping that QTL to a specific chromosomal location. Jiang *et al* (1999) identified a single QTL for resistance to SLB on chromosome 3 in a cross between an inbred line derived from a tropical lowland population (S_8 from CIMMYT Population 21) and one derived from a tropical highland population (S_5 from CIMMYT Pool 1).

Cai *et al* (2003) used a combination of the amplified fragment length polymorphism (AFLP) technique and bulked segregant analysis (BSA) to identify molecular markers linked to the *rhm* gene for resistance to SLB in a large F_2 population. One co-dominant AFLP

marker, *p7m36*, was mapped to a position 1.0cM from *rhm* gene on opposite side of the marker *agrP144* and also developed an STS (sequence-tagged site) marker linked to *rhm* gene from *p7m36* marker.

Carson *et al* (2004) derived a random set of RILs ($F_{2:7}$) from the cross of the inbred lines Mo17 (resistant) and B73 (susceptible) and were evaluated for resistance to SLB. The RILs were genotyped for 234 SSR, RFLP and isozyme markers. When means of disease ratings over years were fitted to models, a total of 11 QTLs were found to condition resistance to SLB, depending upon which disease ratings were used in the analyses. When the area under disease progress curve (AUDPC) data were combined and analyzed over environments, seven QTLs, on chromosomes 1, 2, 3, 4, 7 and 10 were found to come from the resistant parent Mo17. An additional QTL for resistance on chromosome 1 came from the susceptible parent B73. The eight identified QTLs accounted for 46 per cent of the phenotypic variance for resistance to SLB. QTL x environment interactions often were highly significant but, with one exception, where differences were attributed in the magnitude of QTLs effects between years and not due to changes in direction of effects. QTLs on the long arm of chromosome 1 and chromosomes 2 and 3 showed consistent large effects, and accounted for most of the phenotypic variance. No significant additive x additive epistatic effects were detected. These data supported earlier reports of the polygenic inheritance of resistance to SLB of maize.

Gao *et al* (2005) also identified that the resistance to SLB was under the control of a single recessive allele, *rhm*, by analysed pairs of isogenic lines, F_2 population consisting of a total of 687 individuals, and 120 F_3 families. The disease ratings were also studied together with the segregation scores of a single copy DNA probe, *agrP144*. Among 687 F_2 plants, 161 plants were rated as resistant and 526 as susceptible, fitting into 1:3 ratio. Among the 120 F_3 families, there were 32 susceptible, 58 segregating and 30 resistant rows, fitting the expected 1:2:1 ratio for a single gene model. Using *agrP144* as a marker, 99.5 per cent RFLP allelic segregation scores corresponded to the disease ratings obtained from field data, indicating that the marker *agrP144* is tightly linked to the *rhm* locus. Similarly, the AFLP marker *p7m36* also is closely linked to the locus but on the opposite side of the marker *agrP144*.

Balint - Kurti and Carson (2006) derived a set of 192 maize RILs from a cross between the inbred lines Mo17 and B73 and evaluated at 3 week old seedlings in the greenhouse for resistance to SLB. Six significant QTLs were identified for disease resistance, located on chromosomes 1, 2, 3, 6, 7 and 8. Results were compared with a previous study that had used the same RIL population and pathogen isolate, but had examined resistance in mature rather than juvenile plants. There was a very weak but significant correlation between the overall resistance phenotypes of the RILs scored at mature and juvenile plants. Two QTLs were found in similar positions on chromosomes 1 and 3 at both growth stages. Other QTLs were specific to one growth stage or the other. Twenty-three of these RILs, together with the

parental lines, were inoculated in the greenhouse with four *D. maydis* isolates. Results indicated that the quantitative resistance observed was largely isolate non-specific.

A RIL population developed from a cross between the maize lines NC300 (resistant) and B104 (susceptible) was evaluated by Balint-Kurti *et al* (2006) for resistance to SLB disease and for days to anthesis (DTA) in four environments. Entry mean and average genetic correlations between disease ratings in different environments were high (0.78 to 0.89 and 0.9) and the overall entry mean heritability for SLB resistance was 0.89. When weighted mean disease ratings were fitted to a model using multiple interval mapping, seven potential QTLs were identified, the two strongest being on chromosomes 3 (bin 3.04) and 9 (bin 9.03-9.04). These QTLs explained a combined 80 per cent of the phenotypic variation for SLB resistance. Some time-point-specific SLB resistance QTLs were also identified. There was no significant correlation between disease resistance and DTA. Six putative QTLs for DTA were identified, none of which coincided with any SLB resistance QTL.

Balint-Kurti *et al*(2007) generated intermated B73 x Mo17 (IBM) population, an advanced intercross RIL derived from a cross between the maize lines B73 (susceptible) and Mo17 (resistant) and was evaluated in four environments for resistance to SLB disease caused by *D. maydis* (race O). Two environments were artificially inoculated, while two were not inoculated and consequently had substantially lower disease pressure. They identified four common QTLs for SLB resistance in all environments, two in bin 3.04 and one each in bins 1.10 and 8.02/3. They found no significant correlation between disease resistance and DTA. A direct comparison was made between SLB QTL detected in two populations, independently derived from the same parental cross: the IBM advanced intercross population and a conventional RIL population. Several QTLs for SLB resistance were detected in both populations, with the IBM providing between 5 and, in one case, 50 times greater mapping resolution.

Balint-Kurti *et al* (2008) identified loci contributing to SLB resistance in two RIL populations derived from crosses between maize inbred lines H99 and B73 to produce HB population, and B73 and B52 to develop BB population. QTLs for SLB resistance were mapped in bins 3.04 (two), 6.01 and 8.05 in the HB population and on bin 2.07 in the BB population.

Zwonitzer *et al* (2009) characterized the loci responsible for the increased SLB resistance of NC292 and NC330. Genomic regions that differentiated NC292 and NC330 from B73 and which contributed to NC292 and NC330's enhanced SLB resistance were identified. Ten NC250P-derived introgressions were identified in both the NC292 and NC330 genomes of which eight were shared between genomes. Disease resistance QTL (dQTLs) were mapped in two $F_{2:3}$ populations derived from lines very closely related to the original parents of NC292 and NC330 (B73*rhm1*x NC250A and NC250A x B73). Nine SLB dQTLs

were mapped in the combined populations using combined SLB disease data over all locations. Of these, four dQTLs precisely co-localized with NC250P introgressions in bins 2.05-2.06, 3.03, 6.01 and 9.02 and three were identified near NC250P introgressions in bins 1.09, 5.05-5.06 and 10.03.

Later on, Zwonitzer *et al* (2010) derived maize RIL population from a cross between lines Ki14 and B73, and evaluated the evidence for the presence genes or loci conferring multiple disease resistance (MDR). Each disease was scored in multiple separate trials. Highly significant correlations between the resistances and the three diseases were found. The highest correlation was identified between SLB and gray leaf spot (GLS) resistance (r = 0.62). Nine, eight, and six QTLs were identified for SLB, GLS, and northern leaf blight (NLB) resistance, respectively. QTLs for all three diseases co-localized in bin 1.06, while QTLs co-localizing for two of the three diseases were identified in bins 1.08-1.09, 2.02/2.03, 3.04/3.05, 8.05, and 10.05.

Nageri *et al* (2011) identified QTLs for resistance to SLB in three maize recombinant inbred populations assessed in two environments: Clayton, NC, in the summer and Homestead, FL, in the winter. The three populations were derived from the crosses of B73 x CML254, CML254 x B97, and B97 x Ki14. Each of these populations was derived from a cross between a temperate maize line (B73 or B97) and a tropical maize line (Ki14 or CML254). QTLs were identified by separate analysis of each population and by joint connected and disconnected analyses of all the populations. The most significant QTLs identified were on chromosomes 3, 8, 9 and 10.

Pengfei *et al* (2011) developed 330 $F_{2:3}$ families from a T14 (resistant) x T4 (susceptible) cross and identified QTLs associated with SLB in sweet corn. Seven QTLs out of 18 QTLs were identified at the seedling stage on chromosomes 3, 4, 6 and 9, and the phenotypic variance explained by each QTL ranged from 4.02 to 35.4 per cent. The 11 other QTLs were identified at the milk stage on chromosomes 3, 4, 6, 8, 9 and 10 and accounted for 3.50 to 13.65 per cent of the phenotypic variance. Two QTLs were detected in both years; these were located on chromosome 3 and 4 in the marker intervals *umc2275* to *umc2008* and *umc2287* to *bnlg2162*, respectively. One QTL, located on chromosome 3 between *umc2275* and *umc2008*, was detected at both developmental stages.

Belcher *et al* (2012) derived near isogenic lines (NILs) by crossing NC292 x B73 and NC330 x B73. The goals of this study were to determine the effects of each introgression on resistance to SLB and to two other foliar fungal diseases of maize, NLB and GLS. This was achieved by generating and testing a set of NILs carry single or combinations of just two or three introgressions in a B73 background. Introgressions 3B at bin 3.03-3.04, 6A at bin 6.01 and 9B at bin 9.02-9.03 conferred significant levels of SLB resistance in the field.

Introgression 6A was the only introgression that had a significant effect on juvenile plant resistance to SLB and introgressions 6A and 9B conferred resistance to multiple diseases.

Zhao *et al* (2012) reported that *rhm1* was a major recessive disease resistance locus for SLB. To further narrow down its genetic position, F_2 population and BC_1F_1 population derived from the cross between resistant ($H95_{rhm}$) and susceptible parents (H95) of maize were constructed. Using newly developed markers, *rhm1* was initially delimited within an interval of 2.5 Mb, and then finally mapped to 8.56 kb interval between InDel marker *IDP96-503* and SSR marker *A194149-1*. Three polymorphic markers *IDP961-504*, *IDP B2-3* and *A194149-2* were shown to be co-segregated with the *rhm1* locus. Sequence analysis of the 8.56 kb DNA fragment revealed that it contained only one putative gene with a predicted amino acid sequence identical to *lysine histidine transporter 1* (*LHT1*). Comparative sequence analysis indicated that the *LHT1* in $H95_{rhm}$ harboured a 354 bp insertion in its third exon as compared with that of susceptible alleles in B73, H95 and Mo17. The 354 bp insertion resulted in a truncation of the predicted protein of candidate resistance allele (*LHT1*-$H95_{rhm}$). They suggested *LHT1* as the candidate gene for *rhm1* against SLB.

Zhao *et al*(2013) fine mapped a major gene *RppP25* that confers resistance to southern rust in inbred line P25 and did candidate gene analysis of *RppP25* from the near-isogenic line F939, which harbors *RppP25* in the genetic background of the susceptible inbred line F349. The inheritance of resistance to southern rust was investigated in the BC_1F_1 and BC_3F_1 populations, which were derived from a cross between F939 and F349 (as the recurrent parent). The 1:1 segregation ratio of resistance to susceptible plants in these two populations indicated that the resistance is controlled by a single dominant gene. Ten markers, including three simple sequence repeat (SSR) markers and seven insertion/deletion (InDel) markers, were developed in the *RppP25* region. *RppP25* was delimited to an interval between P091 and M271, with an estimated length of 40 kb based on the physical map of B73. In this region, a candidate gene was identified that was predicted to encode a putative nucleotide-binding site leucine-rich repeat (NBS-LRR) protein.

Lennon *et al* (2017) aims to identify teosinte alleles that, when present in a temperate maize background, confer a significant level of resistance to SLB. By using ten populations of BC_4S_2near isogenic lines (NILs), four significant QTL associated with SLB in bins 2.04, 3.04, 3.05, and 8.05 were identified. And in $F_{2:3}$populationssingle marker analysis was done and validated allelic substitution effects predicted from the original NIL population analysis. An allele at the QTL in bin 2.04 was shown to confer resistance to both SLB and a second maize foliar disease, GLS.

Li*et al* (2018) studied with the nested association mapping (NAM) population, the marker densities of 1106 to 7386 SNPs for linkage mapping, and from 1.6 to 28.5 million markers for association mapping were used. They identified 49 SLB and 48 NLB resistance-

related unique QTLs in linkage mapping, and multiple loci in association mapping with candidate genes involved in known plant disease-resistance pathways. Furthermore, independent natural populations with 282 diversified inbred lines were sequenced for four selected candidate genes based on their biological functions. Three of them demonstrated significant associations with disease resistance.

2.3 Identification of candidate genes and their expression analysis

Candidate genes are generally the genes with known biological function directly or indirectly regulating the developmental processes of the investigated traits, which could be confirmed by evaluating the effects of the causative gene variants in an association analysis (Zhu and Zhao 2007). In general, significant components of QTLs in a chromosomal region affecting genetic variation of investigated traits are causative genes, so the ultimate pinpoint location of a QTL, with number of genes assembled in about ~20cM confidence interval, to a specific polymorphic gene is inevitably involved in candidate gene analysis.

Użarowska *et al* (2009) validated the candidate genes putatively associated with resistance to (potyviruses sugarcane mosaic virus) SCMV and maize dwarf mosaic virus MDMV in maize by qRT-PCR, coding for metallothionein-like protein, S-adenosylmethionine synthetase, germin - like protein or 26S ribosomal RNA.

Poland *et al* (2011) evaluated a 5,000 nested association mapping population for resistance to northern leaf blight, a maize disease of global economic importance. Twenty-nine quantitative trait loci were identified, and most had multiple alleles. With genome-wide nested association mapping, using 1.6 million SNPs, multiple candidate genes related to plant defense, including receptor-like kinase genes similar to those involved in basal defense were identified.

Kump *et al* (2011) also used nested association mapping population for evaluation against SLB resistance. With joint-linkage analysis 32 QTLs were identified with predominantly small, additive effects on SLB resistance. Later on, genome-wide association tests of maize HapMap SNPs were conducted by imputing founder SNP genotypes onto the NAM RILs. SNPs both within and outside of QTL intervals were associated with variation for SLB resistance. Many of these SNPs were within or near sequences homologous to genes previously shown to be involved in plant disease resistance.

Galli *et al* (2013) studied softwares to identify the most stably expressed candidate reference genes in samples from seven stages of grain development and from eight landrace varieties in maize. The results of the analysis performed using geNorm indicated that tubulin (*TUB*) and actin (*ACT*) were the most suitable reference genes among all experimental conditions, while glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) showed the least stability. The expression of phytoene synthase gene (*PSY1*), the first enzyme in the carotenoid biosynthetic pathway, was overestimated when the least stable candidate gene

(*GAPDH*) was used as the internal control instead of the most stable gene pair (*ACT* + *TUB*), thus highlighting the importance of validating reference genes before conducting a RT-qPCR experiment to obtain accurate results. Thus they explained the stability of genes for using them as reference genes to normalize RT-qPCR data from maize landraces during multiple stages of grain development.

Xu *et al* (2014) identified total 524 nsSNPs that were associated with 271 candidate genes involved in plant hormone regulation, carbohydrate and sugar metabolism, signaling molecules regulation, redox reaction and acclimation of photosynthesis under drought stress in maize. Most of the nsSNPs identified were clustered in bin 1.07 region that harbored six previously reported QTL with relatively high phenotypic variation explained for drought tolerance.

Gowda *et al* (2015) gained insights into the genetic architecture underlying the resistance to maize lethal necrosis disease (MLND) by genome-wide association study (GWAS) and genomic selection. They used two association mapping (AM) panels comprising a total of 615 diverse tropical/subtropical maize inbred lines. All the lines were evaluated against MLND under artificial inoculation. Both the panels were genotyped using genotyping-by-sequencing. GWAS revealed 24 SNPs that were significantly associated ($P < 3 \times 10^{-5}$) with MLND resistance. These SNPs are located within or adjacent to 20 putative candidate genes that are associated with plant disease resistance.

Yang *et al* (2017) identified quantitative trait locus, $qMdr_{9.02}$, associated with resistance to three important foliar maize diseases-southern leaf blight, gray leaf spot and northern leaf blight. The putative candidate gene*ZmCCoAOMT2*, which encodes a caffeoyl-CoA *O*-methyltransferase associated with the phenylpropanoid pathway and lignin production, within $qMdr_{9.02}$ conferred quantitative resistance to both southern leaf blight and gray leaf spot.

Chen *et al* (2018) with comparative genomics study using *Arabidopsis* abiotic stress-responsive *MYB* protein sequences they identified 46 *ZmMYB* genes which were involved in abiotic stress responses of *Zea mays*. An expression pattern analysis of the 46 *ZmMYB* genes under abiotic stress treatments was used to identify 22 *MYB* genes that were induced by one or more of the stress treatments. *ZmMYB30* was highly upregulated under the four stress treatments. The ectopic expression of *ZmMYB30* in transgenic *Arabidopsis* plants promoted salt-stress tolerance and also increased the expression of a number of abiotic stress-related genes, allowing the plants to overcome adverse conditions.

Li *et al* (2018) studied with the nested association mapping (NAM) population, the marker densities of 1106 to 7386 SNPs for linkage mapping, and from 1.6 to 28.5 million markers for association mapping were used. They identified 49 SLB and 48 NLB resistance-related unique QTLs in linkage mapping, and multiple loci in association mapping with

candidate genes involved in known plant disease-resistance pathways. The four major candidate genes selected based on their biological functions. Three of them demonstrated significant associations with disease resistance. The first gene *GRMZM2G441903* encodes a protein with an AN1-like zinc finger domain, that functions in DNA binding and zinc ion binding, whose homologous genes encode proteins that act as important regulators of the stress response. The second candidate gene, *GRMZM2G463580*, a representative of the LRR-type genes, represents a direct hit of two SLB-associated markers and encodes a member of the LRR protein kinase family. The third candidate gene, *GRMZM2G383122*, represented a direct hit of an NLB-associated marker (with a BPP of 0.81) and encodes BCL-2-associated athanogene 3 (BAG3). The homologue of this gene in *Arabidopsis* encodes a BAG protein, which regulates apoptotic-like processes ranging from pathogen attack and abiotic stress to plant development. The last gene, *GRMZM2G099363* (*ZmCCoAOMT2*), was identified based on four associations with SLB (with the highest BPP of 0.78) in the environments of both the US and China. Its homologue in *Arabidopsis* encodes an S-adenosyl-L-methionine-dependent methyltransferase superfamily protein (CCoAOMT1), which has been implicated in O-methyltransferase activity.

2.4 Marker assisted selection

Backcrossing is a plant breeding method most commonly used to incorporate one or a few genes into an adapted or elite variety. In most cases, the parent used for backcrossing has a large number of desirable attributes but is deficient in only a few characteristics (Allard 1999). The method was first described in 1922 and was widely used between the 1930s and 1960s (Stoskopf *et al* 1993). Mapping and tagging of agriculturally important genes have been greatly accelerated by an array of molecular markers in crop plants. The use of DNA markers in backcrossing greatly increases the efficiency of selection. The practical application of molecular markers in crop improvement is in marker assisted selection (MAS), that is of great importance as it helps in improving the efficiency of plant breeding through specific transfer of genomic regions of interest (foreground selection) and accelerating the recovery of the recurrent parent genome (background selection). MAS have been more extensively employed for simply inherited traits.

Frisch *et al* (2001) performed marker assisted selection to accelerate recovery of the recurrent parent genome (RPG) in backcross program. Their objective was to compare various selection strategies and breeding plans for the simultaneous introgression of two genes with respect to the RPG recovered and the number of marker data points (MDP) required. Investigation involves computer simulations with a published genetic map in maize consisting of 80 markers and assuming selection for dominant target genes on the basis of phenotypic evaluation. It was concluded that in breeding programs with three backcross generations, the least MDP were required when (i) applying selection strategies consisting of

three or four selection steps on the basis of presence of the target genes and selection indices calculated from the marker genotype, (ii) increasing the population size from early to advanced generations, and (iii) merging the target genes in an early generation. These principles can be used for optimizing the design of marker-assisted backcross programs for the simultaneous introgression of two genes.

An STS marker converted from AFLP tightly linked marker to the *rhm* gene for SLB is useful in MAS or for map based cloning of this gene (Cai *et al* 2003). Flint Garcia *et al (*2003) compared phenotypic selection and MAS for stalk strength and 2- generation of European corn borer (2-ECB) resistance and demonstrated that MAS could be effective tool to select for stalk strength and 2- ECB resistance.

Yang *et al* (2004) used marker assisted selection in rice for β-carotene. Transformants with β-carotene, produced in the background of "Nagdong", a japonica rice cultivar. This transformed was used for introgressing the carotenoid locus into the elite cultivar "Ilpum". A total of 220 SSR markers were surveyed and 38% of them were found to be polymorphic between the "Ilpum" as the recurrent parent and "Nagdong" transformant as a donor parent. Backcross progenies generated were genotyped by transgene (*PAC4-2*) specific PCR-based marker. A total of 240 BC_3F_2 plants were produced which were derived from thirteen BC_3F_1 plants containing the caroteniod gene. To develop near isogenic line, additional experiments performed for background selection using SSR markers. Traits of agronomic importance such as heading date, panicle number and culm length were also evaluated.

Babu *et al* (2005) converted normal maize inbred line, V25 into QPM version using two generation marker assisted backcross breeding. They employed *phi057* and *umc1066* for foreground selection of *opaque2* (*o2*) gene. Manna et al (2005) used three SSR markers (*phi057*, *phi112* and *umc1066*) to improve efficiency of QPM breeding in Uganda. They used six normal and seven QPM lines and lines were evaluated for polymorphism in *o2* locus using three SSR markers. They got polymorphism using *phi057* and *phi112*. They used *phi057* to monitor introgression of the *o2* allele from CML176 to 136R.

Benchimol *et al* (2005) reported marker assisted backcross selection in maize. They have chosen SSR markers to simulate a target allele and three criteria (02, 04 and 06 markers per chromosome) were tested to evaluate the most efficient parameters for performing marker assisted backcross (MAB) selection. They used 53 polymorphic SSRs to genotype 186 BC_1 maize plants produced by crossing the inbred maize lines L-08-05 (donor parent) and L-14-4B (recurrent parent). In the second backcross (BC_2) generation 180 plants was produced and were screened with markers which were not recovered from the first backcross (BC_1) generation. A total of 480 plants were evaluated in the third backcross (BC_3) generation from which 48 plants were selected for parental genotype recovery. Recurrent genotype recovery

averages in three backcross generations were compatible with those expected in BC_4 or BC_5, indicating genetic gain due to the marker-assisted backcrossing. The target marker *phi037*was efficiently transferred. Six markers per chromosome showed a high level of precision for parental estimation at different levels of maize genome saturation and donor alleles were not present in the selected recovered pure lines. Phenotypically, the plants chosen based on this criterion (06 markers per chromosome) were closer to the recurrent parent than any other selected by other criteria (02 or 04 markers per chromosome). They concluded that six microsatellites per chromosome is a more efficient parameter than 02 and 04 markers per chromosome for deriving a marker assisted backcross (MAB) experiment in three backcross generations.

Babu *et al* (2005) reported two generation marker aided backcrossing for rapid conversion of normal maize lines to quality protein maize (QPM). They used 72 SSR markers on 74 individuals heterozygous for *opaque2* gene in BC_2F_1 population. Out of 74 plants 27% had 75-80% recurrent parent genome recovery, 24% had 80-85% and 1.35% was upper and lower extremes of recurrent parent genome recovery. 14 plants showed more than average recurrent parent genome recovery (87.5%). Highest recovery of recurrent parent genome was 95.75%.

Viicher *et al* (2006) investigated the marker assisted introgression in backcross breeding program. They investigated the efficiency of marker assisted introgression in backcross populations derived from inbred lines by simulation. Background genotypes were simulated assuming that the genetic model of many genes of small effects in coupling phase explains the observed breed difference and variance in backcross populations. They observed that the markers are efficient in introgression backcross programs for simultaneously introgressing an allele and selecting for the desired genomic background. They used a marker spacing of 10-20 cM which gave an advantage of one to two backcross generations selection relative to random or phenotypic selection. They concluded that for relatively precisely mapped quantitative trait loci, flanking markers or marker haplotypes should cover 10 cM -20 cM around the estimated position of the gene, to ensure that the allele frequency does not decline in later backcross generations.

Jompuk *et al* (2006) used three populations, i.e., Pop61C1, Pop62C6 and Pop65C6 from the International Maize and Wheat Improvement Center (CIMMYT) for screening with two *o2* specific markers (*phi057* and *phi112*) for the selection of S0-plants having QPM gene. 24 out of 40 Pop61C1 plants (60%), 34 out of 35 Pop62C6 plants (97%) and 24 out of 30 Pop65C6 plants (80%) *o2* positive plants using *phi057* marker. While using *phi112* marker they identified 34 (85%), 35 (100%) and 30 (100%) *o2* plants from the respective populations were identified. It has been further implicated that *phi112* was a dominant marker so it only indicates the difference between QPM inbreds and normal maize whereas *phi057* is a co-

dominant marker and it identify the heterozygote of maize plants and therefore can identify the contamination of non-QPM presented in CIMMYT population. So, *phi057* is more feasible than *phi112*to follow MAS for *opaque2*gene and to identify QPM line in the short period of time.

Kil *et al* (2008) used background selection of near isogenic lines (NILs) for bacterial leaf pustule resistance gene in soybean. Five near isogenic lines from separate backcrosses (BCs) of BLP-susceptible Hwangkeumkong x BLP-resistant SS2-2 (HS) and BLP-susceptible Taekwangkong x SS2-2 (TS) were surveyed for foreground and background selection based on the four-stage selection strategy. First, 15 BC individuals were selected through foreground selection using the SSR markers *Satt486* and *Satt372* flanking the *rxp* gene. Among them, 11 BC plants showed the BLP-resistant response. The HS and TS lines chosen in foreground selection were again screened by background selection using 118 and 90 SSR markers across all chromosomes, respectively. Eventually, five individuals showing greater than 90% recurrent parent genome content were selected in both HS and TS lines. These NILs will be a unique biological material to characterize the *rxp* gene.

Septiningsih *et al* (2009) followed marker assisted backcrossing strategy to transfer the tolerant *Sub1* allele into the mega varieties. Two breeding lines IR49830 and IR40931 were used as donors. The mega varieties Samba Mahsuri and CR1009 from India, IR64 from the Philippines (IRRI), Thadokkham 1 (TDK1) from Laos, and BR11 from Bangladesh were used as recipient parents. Population sizes ranged from 343 to 697 plants for the BC_1 generation and 69 to 320 plants at the BC_2 generation. The fully converted *Sub1* lines were selected at the BC_2F_2 or BC_3F_2 generation. Recombinants were initially identified using two markers tightly flanking the *Sub1* region. Populations of 2240 BC_1F_2 plants for IR64/IR40931, and 2137 BC_2F_2 and 279 BC_3F_1 plants for BR11/IR40931 were screened with flanking markers to identify recombination events between *Sub1*A and *Sub1*C. One plant with the desired genotype was successfully selected in the BC_2F_2 generation. The tolerant version of IR64 was developed following a similar approach. Among the 440 BC_1F_1 and 188 BC_2F_1 plants that were genotyped, single plant was selected. Out of 162 BC_2F_2 progeny from the selected plant, single plant was selected based on its possession of the *Sub1* locus and maximum recipient genome, Due to the small size of the TDK1/IR40931 population from the earlier backcrosses and segregation bias of several markers toward the tolerant parent there were no ideal *Sub1* plants selected out of the BC_2F_2 population. Screening of larger BC_3F_1 and BC_3F_2 populations subsequently facilitated the identification of several BC_3F_1 plants with a small *Sub1* introgression. One of the selected BC_3F_1 plants was self-pollinated and among the 190 BC_3F_2 progenies, single plant was identified with the desired small *Sub1* introgression.

Zhang *et al* (2010) used marker assisted selection for *o2* introgression lines with *o16*gene. They used QCL3021 line as donor parent. QCL3021 line carries *o16* gene. Taixi 19

having *o2* gene was used as recipient parent. They used *umc1066* SSR marker for foreground selection for *o2* gene and *umc1141* for *o16* gene in every backcross and inbred generations.

Kumar (2011) reported the marker assisted backcrossing scheme in rice using microsatellite markers. They used 72 polymorphic SSR markers for genotyping 123 BC_1 rice plants developed from a cross between two rice cultivars IR64 (recurrent parent) and Tetep (Donor parent with *Pi-kh* resistance gene). The BC_2 generation produced 144 plants and screened with markers not amplified in the BC_1 generation. 400 plants were produced in the BC_3 generation and 48 plants were selected for recurrent parent genotype recovery. The target marker (*RM206*) was transferred efficiently. The recurrent parent genotype recovery averages in three backcrosses indicates that marker-assisted backcrossing produced a genetic gain. Backcross transfer at genotype level showed high accuracy when six markers per chromosomes were used even in a saturated rice genome. Morpho-agronomically, the plants in SC3 criteria (six markers per chromosome) were closer to the recurrent parent than in any other criteria. Thus, it was concluded that six markers per chromosome can be considered as the best option to perform marker-assisted background selection in three generations.

A total of 116 SSR markers for background selection were employed by Iftekharuddaula *et al* (2011) in backcrosses between a *SUB1* donor IR40931-33-1-3-2 and BR11. BR11-*Sub1* identified in a BC_2F_2 plant, possessed BR11 type SSR alleles on all fragments analyzed except the *SUB1* QTL. Yield, yield-component parameters and grain physico-chemical properties showed successful recovery of the BR11 traits in BR11-*Sub1*, with yield potential ranging from 5.2 to 5.6 t/ha, not significantly different from the recurrent parent mega variety BR11.

Kostadinioc *et al* (2016) developed QPM versions of two commercial ZP inbreds through MAS with *opaque2* specific molecular markers, while maintaining their good agronomic performances and combining abilities. Donor line was a tropical QPM line CML 144. After two backcross and three selfing generations, six near isogenic lines (NILs) with 93% recovery of the recurrent parent genome were created from one cross. Average increments of 30% in tryptophan content and 36% in quality index were obtained, as well as kernels with less than 25% opaque endosperm. Grain yield was increased by 11-31% and combining abilities of the improved lines were on a par with the original line.

Recently, QPM version of three popular commercial hybrids viz., HM-4, HM-8 and HM-9 has been developed using MAS (Hossain *et al* 2018).Gene-based SSR markers (*umc1066* and *phi057*) were deployed for introgression of *o2* allele. Background selection using genome-based SSRs helped in recovering (>96%) of recurrent parent genome. The newly developed quality protein maize (QPM) inbreds showed modified kernels (25–50% opaqueness) coupled with high degree of phenotypic resemblance to the respective recipient lines, including grain yield. In addition, endosperm protein quality showed increased lysine

and tryptophan in the inbreds to the range of 52-95% and 47-118%, respectively.

Goswami*et al* (2019) examined that HKI1128 as parental lines of three popular hybrids in India was converted to QPM. The HKI1128Q was further stacked with β-carotene gene. Severe segregation distortion for *crtRB1* was observed in BC_1F_1, BC_2F_1 and BC_2F_2. Background selection by 100 SSRs revealed mean recovery of 91.07% recurrent parent genome varying from 88.78% to 93.88%. Across years, introgressed progenies possessed higher mean β-carotene (BC: 9.22 µg/g), β-cryptoxanthin (BCX: 3.05 µg/g) and provitamin A (proA: 10.75 µg/g) compared to HKI1128Q (BC: 2.26 µg/g, BCX: 2.26 µg/g and proA: 3.38 µg/g). High concentration of essential amino acids, viz. lysine (mean: 0.303%) and tryptophan (0.080%) in endosperm, was also retained. Multi-year evaluation showed that introgressed progenies possessed similar grain yield (1,759=1,879 kg/ha) with HKI1128Q (1,778 kg/ha). Introgressed progenies with higher lysine, tryptophan and proA hold immense potential as donors and parents in developing biofortified hybrids.

CHAPTER III

MATERIAL AND METHODS

The present experiments were conducted using the field and laboratory facilities at the School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana, during the years 2015, 2016 and 2017.

3.1 Plant materials

The experimental material consisted of two parental inbred lines viz. CM139 and CM140, and their derived 298 RIL population (F_8, F_9, F_{10}). The CM139 is resistant to southern leaf blight (SLB) having moderate height, light green silk, open tassel, light green leaves with wavy leaf blade, orange flint bold and round kernels whereas, CM140 is susceptible to SLB having moderate height, light pink silk, lack open tassel, semi erect and dark green leaves, dull to normal orange flint kernels. The pedigree information of these two parental lines is given in Table 3.1. These two parental lines belong to different heterotic pools of a hybrid, Parkash which is grown widely in Northern region of Punjab.

Table 3.1: Pedigree of the parental lines:

Inbred line	Pedigree	Heterotic pool
CM139 (female)	(Tarun×MS_1 yellow)-63-1-1-	Semi-exotic
CM140 (male)	J617-61-1-1-1-1-1-1-1-1	Indegenous

The RILs are advanced by single seed descent method. A total of 298 RILs were raised during *kharif* seasons of 2015, 2016 and 2017 in alpha lattice design having 30 blocks in each two replications. Each RIL was planted in one row of three metre (15 plants). The plants were sown with a plant-to-plant and row-to-row distance of 20 cm and 60 cm respectively. Standard agronomical practices were followed for raising the crop.

3.2 Screening of RIL population with *Drechslera maydis* isolate

3.2.1 Culturing of *D. maydis* and its maintenance

The most virulent isolate of *D. maydis*, *Dm1* was procured from Maize Section of Department of Plant Breeding and Genetics and used for inoculations. The *Dm1* isolate was cultured and maintained on potato dextrose agar (PDA) medium (Fig. 3.1A). The medium was prepared by dissolving 24.0 gm of potato dextrose broth (HIMEDIA) with 20.0 gm agar per liter of the medium. The pH of the medium was maintained to 6.2. The total volume was made to 1000 ml by adding distilled water. The media was dispensed in glass tubes, autoclaved at 15 psi for 20 min and after autoclaving placed in a slant at room temperature until media solidified. The 5 mm bits from actively growing cultures were placed on the media and cultures were then incubated for 5 days at 25 ± 1°C. The cultures were stored at 4°C for further days.

3.2.2 Inoculum preparation

The mass inoculation of SLB was performed by culturing *Dm1* on sorghum (*Sorghum bicolor* L.) seeds following the method of Lim (1975). The sorghum seed flasks were prepared by soaking sorghum seeds overnight in water, draining off the excess water, filling the flasks with the moistened seed, and then autoclaving for 1 h at 15 psi. The flasks were inoculated with 5 mm bits taken from the surface of 2-week-old cultures of *Dm1*using sterile inoculation loop under aseptic conditions. The inoculated flasks containing sorghum seeds incubated at 26°C for 2-3weeks for the proper growth of the fungus till all the sorghum seeds turned black in color (Fig. 3.1B).

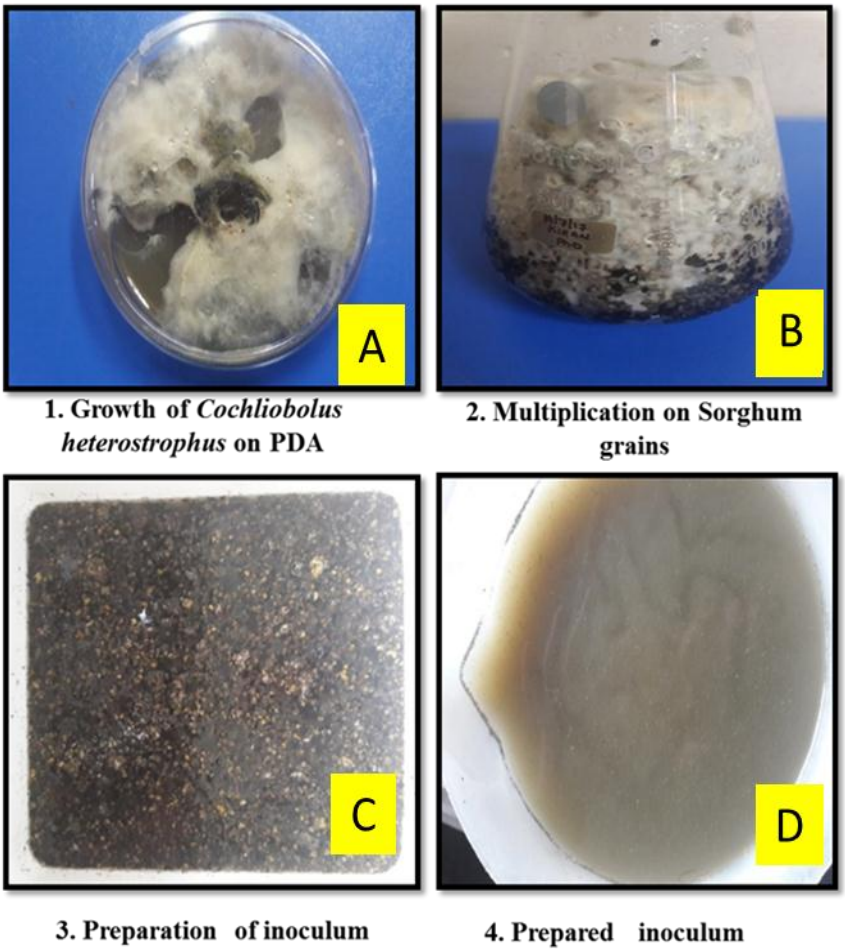

Fig 3.1: Preparation of inoculum of most virulent isolate *Dm1* of *Cochliobolus heterostrophus* by culturing on PDA medium and its multiplication on sorghum seeds

3.2.3 Field inoculations

Experimental materials were inoculated at the seven to nine leaf growth stage (V_{7-9}, Fig. 3.2) by placing 20 to 30 grains of infected sorghum seeds with isolate *Dm1*(Fig. 3.1C) in the whorls of each plant of each $F_{8,}$ $F_{9}$and F_{10} RILs (Fig. 3.2A, B & C). The remaining sorghum seeds were meshed by adding 15 gm of carboxy methyl cellulose and dissolved in 5 litres warm water for foliar spray (Fig. 3.1D). For the spray, inoculum was prepared with

concentration of 2 x 10^6 spores/ml (for different concentration of spore, suspentions were prepared using haemocytometer). This was then used as a spray over the field for proper disease development (Fig. 3.2D). After inoculation, water was sprayed twice to provide free moisture to initiate fungal growth.

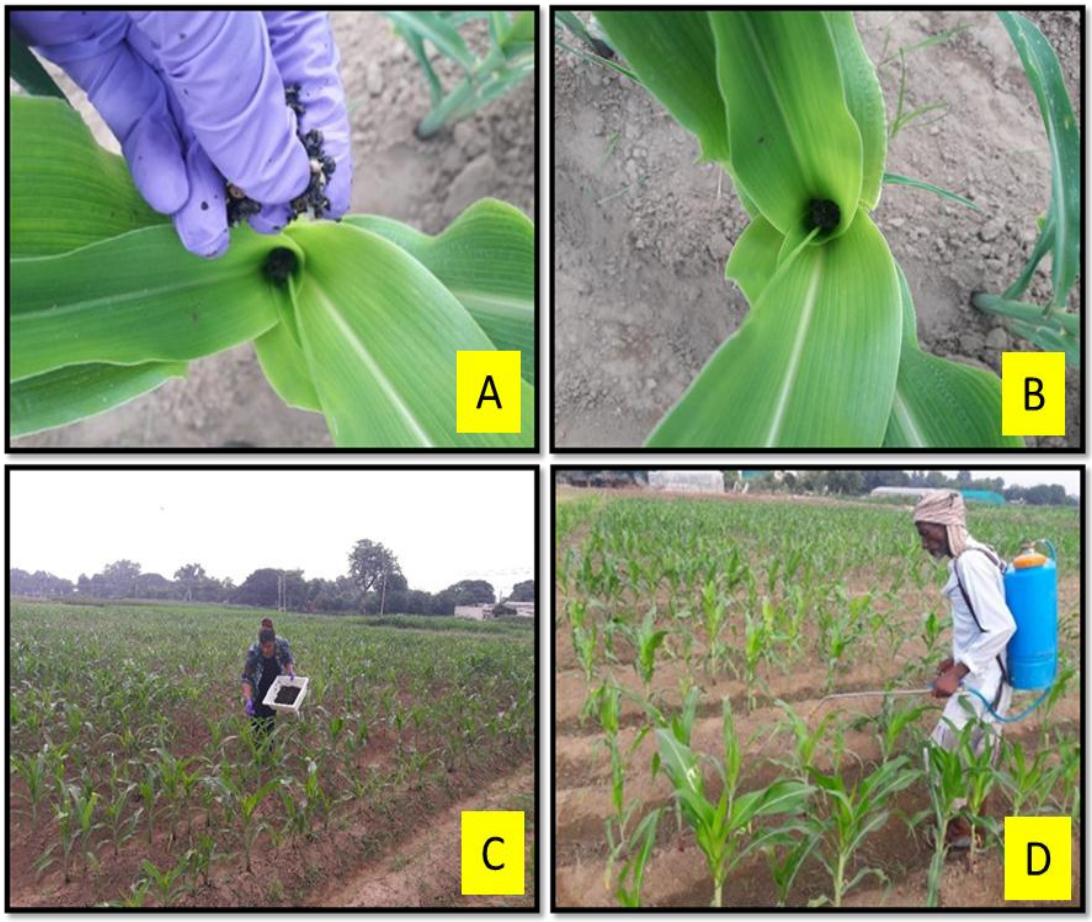

Fig 3.2: Field inoculation of RILs along-with parents; with infected sorghum seeds in whorls of each plant of each RIL (A, B, C). Spraying of inoculum over the plants (D).

3.2.4 Scoring of disease reaction

The disease reaction of SLB was recorded on 10 plants in each row after 15, 30 and 45 days of inoculations following 1-9 scale of Miller *et al* (1970). Phenotypes with a rating of 1 or 2 had very little or no necrotic lesion development, whereas phenotypes with ratings of 6-9 had large necrotic lesions. The disease reaction of each individual in each RIL population was scored, and the average within each line was calculated and used for the data analysis. The symptoms and reaction used for scoring of SLB is given in Table 3.2.

Table 3.2: Scale used for scoring SLB infection and reaction

Scale used	Infection	Reaction
1.0-2.0	Very slight to slight infection, one or two to few scattered lesions on lower leaves	Immune to highly resistant
3.0	Light infection, moderate number of lesions on lower leaves only	Resistant
4.0-5.0	Moderate infection, abundant lesions are on lower leaves, few on middle leaves	Moderately resistant
6.0-7.0	Heavy infection, lesions are on lower and middle leaves and extending to upper leaves	Moderately susceptible
8.0-9.0	Very heavy infection, lesions abundant on almost all leaves, plants prematurely dry and killed by the disease	Susceptible

3.2.5 Statistical analysis

Disease severity was calculated from disease score data using formula given by Miller *et al* (1972):

$$\text{Per cent disease severity} = \frac{\text{Sum of class rating}}{\text{Total number of plants} \times \text{Maximum rating}} \times 100$$

Area under disease progress curve (AUDPC) was calculated from three data points *i.e.* the initial, middle and final disease severity data as suggested by Shaner and Finney (1977) using formula:

$$\text{AUDPC} = \sum_{i=1}^{n=1} [(X_{i+1}+X_i)]\,[t_{i+1}-t_i]\,2$$

Where,

X_i = the proportion of host tissue damaged at the ith day

t_i= the time in days after appearance of the disease at the ith day

n = the total number of observations.

The unit for sample data is %

The unit for *t* is time period

The unit of the AUDPC is %-development stage unit

3.3 Genotyping of RIL population

3.3.1 Genomic DNA isolation and estimation of quantity and quality of DNA

Genomic DNA from 298 RILs along-with both the parents (CM139 and CM140) were isolated using the CTAB (cetryl trimethyl ammonium bromide) method as modified by Saghai-Maroof *et al* (1984). Young leaves were harvested from each plant (one leaf from each plant) of each RIL (10 plants per RIL) and were placed in white butter paper bag marked

with appropriate number and stored in -80ºC deep freezer (Thermo) used. The leaves were ground to a fine powder using pre-chilled sterilized pestle and mortar in liquid nitrogen so as to make the leaves brittle and to reduce the DNase activity and were transferred immediately to a 50 ml falcon tubes. After all the samples were ground, 15 ml of 2X CTAB extraction buffer was added to the powdered samples. The composition of extraction buffer is given in Table 3.3.

The leaf powder was suspended in the buffer by inverting the tubes gently. The homogenate was incubated at 65ºC for 45-60 min with occasional mixing in a water bath. After incubation, 15 ml of chloroform: isoamyl alcohol (24:1) was added to the homogenate and tubes were placed on a rotary shaker for 45 min for the uniform and constant mixing of the contents, till it made a dark green emulsion of the organic phase. The tubes were then centrifuged at 10,000 rpm for 10 min at room temperature in a fixed angle centrifuge.

After centrifugation, the supernatant (aqueous phase) was transferred into another 50 ml oakridge tube using a large bore tip. 5 µl of heat-treated RNase (10 mg/ml) was then added to a final concentration of 10 µg/ml to each sample and incubated at 37ºC in water bath for half an hour. After the RNase treatment, about 0.8 volume of chilled isopropanol was added and the tubes were gently inverted several times. Then the tubes were placed at -20°C for 30 minutes. The DNA formed a white cotton thread-like precipitate and good quality DNA floated at the top. Tubes were then centrifuged at 10,000 rpm for 10 min. After discarding the supernatant, washings with 70 per cent ethanol were given and DNA was air dried by simply inverting the tubes. The DNA was finally dissolved in appropriate quantity of 1X TE buffer (Tris-EDTA buffer- 10 mM Tris HCl, 1 mM EDTA, pH 8.0).

Quantity and quality of DNA was checked by agarose gel electrophoresis. For agarose gel electrophoresis, 0.8 gm of agarose was dissolved in 100 ml of 0.5X TBE electrophoresis buffer (45 mM Tris base, 45 mM Boric acid and 1mM EDTA). The mixture was heated till the agarose dissolved completely i.e. when the solution became transparent. It was cooled down to 55-60°C with constant stirring and 5 µl of ethidium bromide (10 mg/ml) was added to final concentration of 0.5 µg/µl buffer

Table 3.3: Composition of 2X CTAB extraction buffer

Components	Final concentration
CTAB	2.0 percent
Tris HCl	100 mM
NaCl	1.4 M
EDTA	20 mM
2- Mercaptoethanol*	0.2 percent

*****Mercaptoethanol was added just before use

The agarose solution was then poured into already prepared gel mould with combs and was left for 30-40 min for solidification. DNA samples were prepared by adding 1X loading dye (6X loading dye consists of 0.25 per cent w/v bromophenol blue, 0.4 per cent w/v sucrose or 30 per cent Glycerol in sterile water) to the DNA such that the final concentration of loading dye was 1X. When the gel solidified, it was transferred to the electrophoresis tank and the DNA samples were loaded into wells with the help of micropipette. Along with the DNA samples, DNA of known concentration of variable ranges (100 ng/µl, 200 ng/µl, 400 ng/µl, 500 ng/µl and 1000 ng/µl) was also loaded. After loading, the gel was run for about 30 mins-1 hours at a constant voltage of 5V/cm. The gel was then visualized under UV transilluminator. Using photo gel documentation system, the DNA samples were photographed. The intensity of fluorescence of each sample was compared with that of the known DNA concentration and then DNA concentration of each sample was ascertained. The quality of DNA sample was judged based on whether the DNA formed a single high molecular weight band (good quality) or a smear (degraded/ poor quality).

3.3.2 Selection of SSR primers

For polymorphism survey, SSR (simple sequence repeat) markers were selected, spanning region *qSLB*3.1 of chromosome 3 covering 3.02 to 3.07 bins. A total of 101 primers for chromosome 3 were screened for parental polymorphism. The polymorphic markers were then analyzed on 298 RIL population. These simple sequence repeat (SSR) primers were chosen from the Maize Genetics and Genomics Database (MaizeGDB) (http://www.maizegdb.org/) and the map used was IBM 2008 Neighbors Frame 3.

3.3.3 In *silico* identification of new SSR markers:

3.3.3.1 Identification of SSR sequences

The MISA microsatellite finder tool (Thiel *et al* 2003) was used for designing the new SSR markers in nucleotide sequence download from the MaizeGBD database. MISA was run on a standard Linux server, several helper scripts and programs were used. Initially the FASTA file was downloaded having the nucleotide sequence spanning the QTL region from marker *umc1030* to *umc2000*. The maizeGDB database was used for fetching out the region. Five main files were used to run the MISA:

- misa.in
- misa.pl
- p3_in.pl
- p3out.pl

The command lines that were used for mining the SSRs is as follows:

1. Perl misa.pl maize.fasta.
2. cpsequence.fasta.misaBackup_sequence.fasta

3. perl p3_in.pl maize.fasta.misa
4. primer3_core<maize.fasta.p3in >maize.fasta.p3_out
5. grep “sequence_ID=” sequence.fasta.p3_out
6. Perlp3_out.pl sequence.fasta.p3_out sequence.fasta.misa
7. Upon its successful termination, the result files were saved.

3.3.3.2 Designing SSR primers

After identification of SSR sequences within specified region, the SSR primers were designed from the flanking sequence using pearl primer software with the criteria of excluding primers containing more than 3 repeats or 4 runs, bisulphite PCR excluded repeats per/run. Exclude percentage GC content and only consider primers with 40-60 per cent GC content. A total of 22 SSR markers were designed and synthesized from IDT Technologies, USA. (Appendix I)

3.3.4 PCR amplification and agarose gel electrophoresis

In vitro amplification using polymerase chain reaction (PCR) was performed in a 96 well and 384 well microtiter plate in Eppendorf and Applied Biosystems master cyclers. The PCR mix for each 10 µl reaction volume contained the following components: 100 ng template DNA, 2X PCR master mix buffer, 0.37 µM each of forward and reverse primers. Components used in PCR reaction and their stock and final concentration are given in Table 3.4. The PCR plate was covered with a mat and placed in a 96 and 384 well thermal cycler. The PCR amplification involved the following thermal profile: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50-65°C (depending on the primer) for 1.5 min and extension at 72°C for 1.5 min.

Table 3.4: Concentration of the different components used in the PCR reaction

Component	Stock concentration	Quantity	Final concentration
Sterile water	-	1.5µl	-
Template DNA	20 ng/µl	1.5 µl	100 ng
Master mix buffer	2X	4.0 µl	1X
Forward Primer	5 µM	1.5 µl	0.375 µM
Reverse Primer	5 µM	1.5 µl	0.375 µM
Total		20 µl	-

This was followed by a final extension step at 72°C for 7 min. The product was kept on hold at 4°C after the PCR. A negative control (without template DNA) was included in each plate during every amplification reaction.

After amplification, electrophoresis of the amplified PCR product was done by loading it in 3 per cent agarose gel was used to separate approx..40 -50 bp, prepared in 0.5X

TBE buffer. For preparing 3 per cent agarose gel, 15 gm agarose was dissolved in 500 ml of 0.5X TBE electrophoresis buffer. The mixture was heated till the agarose dissolved completely and the mixture became transparent. It was cooled to about 55-60°C with constant stirring and 5 µl of ethidium bromide (10 mg/ml) was added to a final concentration of 0.5µg/µl. The agarose solution was poured into an already prepared gel mould with combs and left for about 30 min for solidification. From the total product, 10 µl of each sample was loaded in 3 per cent agarose gel. The PCR products were resolved by running gel at 5V/cm for 3-5 hours. The gels were then visualized under gel documentation system and photographed.

3.3.5 Gel Preparation for PAGE

We move from agarose to PAGE for the difference of 5 bp to 6 bp. For preparation of polyacryamide gel (6.00 %) ingredients used are given in Table 3.5 and procedure for preparation of gel was followed as under:

a) Preparation of 40.0 per cent Acrylamide-bis acrylamide solution

Separately weighed 38.0 gm Acrylamide + 2.0 gm Bis-acrylamide (Amresco, USA) was dissolved in 50 ml double distil water and final volume was made to 100 ml.

b) Preparation of 10 X TBE

For preparation of 1 litre solution: Separately weighed 108gm Tris-base (890 mM) + 55gm boric acid (890 mM) + 7.44 gm EDTA were dissolved in 700 ml of double-distilled water using magnetic stirrer. After this pH was adjusted at 8.0 and final volume was made to 1 liter, then the solution was autoclaved.

c) Setting of PAGE assembly

The gasket was placed on rectangular plate and spacers were aligned carefully on the both sides of rectangular plate. After this, the notched plate was placed carefully on the above set plate and clips were fixed on the plate and assembled apparatus.

d) Pouring of gel solution in PAGE assembly

All the ingredients mentioned in Table 3.5 were mixed, but APS and TEMED were added at the last, just before pouring of gel solution in PAGE glass plates. The 100 well comb was fixed immediately after pouring the gel solution. The gel was allowed to solidify for 50-60 min.

e) Placing of gel in PAGE assembly

The comb and gasket were removed after gel solidification and placed the glass plates in PAGE assembly (CBS, Scientific). 0.5 X TBE buffer was added in the lower and upper chamber to dip the electrodes and gel.

f) Staining and loading of the gel

Seventy five µl of ethidium bromide (Amresco, USA) was added in lower chamber for staining. The gel was pre-run for 2 hours at 300 V before loading the samples. After that

10 µl PCR product of each sample was loaded on 6.0 per cent polyacrylamide gel. PCR products were resolved by running the gel at 300 V for 3-6 hours depending on the size of the bands. The gels were visualized under UV light and photographed using gel documentation system at regular intervals.

Table 3.5: Composition of various chemical compounds used for preparation of PAGE

Component	Concentration	Quantity
Acrylamide-bis acrylamide solution	40.00 %	22.5 ml
TBE	10.00 X	7.50 ml
Ammonium persulphate (APS)	0.07 % (w/v)	0.105 gm
TEMED (Amresco, USA)	0.08% (w/v)	120 µl
Double-distil water	-	99.88 ml
Total volume	6.0 %	150

3.3.6 Scoring of SSR markers data

The major objective is to properly score and identify the correct amplicon in the gel. So, each gel was scored thrice to avoid any scoring error. SSR markers are co-dominant manner and the individual bands were scored as 'A' for the resistant parent type (CM139) band, 'B' for susceptible parent type (CM140) band and 'M' for the missing data.

The segregation of the marker locus in 298 RILs was checked for the deviation from the expected Mendelian ratio by standard Chi square test (χ^2) on the basis of banding pattern through MapDisto software (Lorieux 2007).

Following equation was used to calculate the chi-square values:

$$\chi^{2(n-1)}d.f = \sum (O-E)^2 / E$$

Where,

N = Number of genotypic classes

D.F. = Degrees of Freedom

$\sum$ = Summation

O = Observed number of bands in each class

E = Expected number of bands in each class

3.3.7 Linkage map construction using Mapdisto software

Mapdisto version 2.0 (Lorieux 2007) was used to develop the linkage map that gave representation in the form of a graphic to mark the position of genes within a linkage group with a threshold value of LOD score of 3.0 and recombination fraction of 0.3. Kosambi mapping function was used for the construction of linkage map in Mapdisto. The order of markers on linkage group was assigned from 'Auto-order' command. The final order of markers on each linkage group was reconfirmed using 'Ripple' command.

3.3.8 QTL analysis

Composite interval mapping (Lander and Botstein 1989) was performed using Windows QTL Cartographer 2.5 (Wang *et al* 2007) to analyze QTL position in RILs. In composite interval mapping, QTL were scanned at a window size of 10.0 cM. The threshold values were based on the likelihood of odds ratio (LOD) scores from 1,000 permutations of the original at a significance level of P = 0.01 (Churchill and Doerge 1994). The LOD threshold used in the study was averaged from the threshold value calculated for each trait. For the marker locus closest to the QTL peak, the additive effect and the proportion of phenotypic variance explained by the QTL (R^2) were obtained using the Windows QTL Cartographer.

3.4 Identification of candidate gene spanning in the QTL region

After fine mapping of the SLB QTL the genomic region bracketed by the flanking markers *MSSR1* and *MSSR20* was fetched out. Putative genes in the B73 genomic sequences have been predicted by using the Maize Genetics and Genomic Database *i.e* Maize GDB (https://www.maizegdb.org/). The evidence-based gene prediction was conducted by searching the landmark region between the flanking markers using the data source maize B73 RefGen_v4. The filtered existing predicted genes were surveyed, spanning the fine-mapped QTL interval on the database in the tracks. The potential identities of the predicted coding sequences were subsequently determined by using Blast2GO, mapman and pfam bioinformatics platform for high-quality functional annotation.

3.5 Relative expression analysis of candidate genes

3.5.1. Collection of leaf samples

The leaf samples were collected from each line i.e. CM139 and CM140 from both control (without infection) and infected plants with *Dm1* isolate at intervals of 6h, 12h, 18h, 24h, 48h, 60h and 72h with three biological replicates. Leaf tissue samples were gently cleaned with tissue paper and cotton to remove the adhered soil. Immediately plant tissue was frozen in liquid nitrogen and kept at -80°C until RNA isolation.

3.5.2 RNA isolation

To prepare diethylpyrocarbonate (DEPC) water, 1 ml of DEPC was added into 1 litre autoclaved distilled water and allowed it to stand for overnight (12h) at 37˚C. The microtubes, tips, pestle-mortars, glassware were fully immersed in DEPC water overnight at 37˚C. After overnight treatment the tips and microtubes were filled in tip boxes and jam jars respectively. The tip boxes and jam jars were kept in hot air oven at 80˚C for overnight baking, which resulted in complete drying of moisture. Pestle-mortars were wrapped with foil after drying. Thereafter, all the material was autoclaved at 121˚C for 20 min. The total RNA was isolated using TRIZol reagent with following steps:

1. The frozen tissue was transfer into pre-chilled mortar and then crushed into small particles until it becomes powdery with liquid nitrogen.
2. Transferred the homogenized sample into 1.5 ml tube and added 1 ml of TRIZol reagent per 30-40 mg of tissue.
3. Kept the tube at room temperature for 5 min.
4. Centrifuged tube at 12000Xg for 5 min at 4°C.
5. Collected supernatant and transferred it to new 2 ml centrifuge tube without disturbing the pellet.
6. To the above solution, added 0.2 ml of chloroform, caped the tube and mixed for 15 seconds until milky.
7. Kept the solution at room temperature for 5 min.
8. Centrifuged at 12000Xg for 15 min at 4°C. The solution was separated into three layers, the top layer had RNA.
9. Carefully transfered the top layer into new 1.5 ml tube without disturbing or touching middle layer.
10. Added 1ml of isopropanol and mixed gently. Kept the mixture at room temperature for 10 min.
11. Centrifuged at 12000X g for 10 min at 4°C to precipitate RNA.
12. Carefully removed the supernatant.
13. Added 0.5 ml of 75% cold ethanol, gently taped 2-3 times with finger and centrifuged at 7500X g for 5 min at 4°C and discarded the supernatant.
14. Dried the pellet by leaving the tube open for 5-8 min.
15. Resuspended the RNA pellet in RNase-free water (not more than 20-25 µl).
16. Kept the tube on ice for 5-7 min to dissolve the pellet.
17. Stored the samples at -80°C and later on proceeded to downstream application.

3.5.3 Assessment of RNA quality and quantity

3.5.3.1 Agarose gel electrophoresis

RNA quality was checked by formaldehyde agarose gel electrophoresis. The gel electrophoresis tank, combs, mould, measuring cylinder were cleaned properly with 0.5 per cent SDS followed by ethanol. To prepare denaturing gel, 1.2 gm of agarose was dissolved in 100 ml of 1X MOPS buffer (prepared in 0.1 per cent DEPC water). The composition of 10X MOPS buffer (pH 7.0) is given in Table 3.6. This mixture was heated until agarose dissolved completely. Then, it was cooled to 60°C with constant stirring and 5 µl of ethidium bromide and 1.78 ml of formaldehyde was added. After that, agarose solution was poured into gel casting tray and was left for 40-50 min. for solidification at room temperature. RNA sample was prepared by mixing 5 µl of 50 per cent glycerol and 5 µl of RNA. When the gel solidified, it was transferred to the electrophoresis tank and the RNA samples were loaded

into wells with the help of micropipette. After loading, the gel was run for about 3-4h at a constant voltage of 5V/cm. The gel was then visualized under UV transilluminator. Quality of RNA was judged on the basis of whether the RNA separated into three distinct bands (5s,18s and 28s) on gel with middle band thicker as compared to other two; upper and lower band (good quality) or formed a smear (degraded).

Table 3.6: Composition of 10X MOPS Buffer

Components	Composition
MOPS (200 mM)	41.9 g
Sodium Acetate (50 mM)	8.2 g
EDTA (10 mM)	3.72 g
Double distilled water	To make 1 litre volume

3.5.3.2 Nanodrop spectrophotometer

RNA quality was also checked by Thermo scientific NanoDrop™1000 spectrophotometer. In this 1 µl sample was pipetted onto the end of a fiber optic cable (the receiving fibre). A second fiber optic cable (the source fibre) was then brought into contact with the liquid sample causing the liquid to bridge the gap between the fibre optic ends. The gap was controlled to both 1 mm and 0.2 mm paths. A pulsed xenon flash lamp provided the light source and a spectrometer utilizing a linear CCD array was used to analyze the light after passing through the sample. The instrument was controlled by PC based software, and the data was logged in an archieve file on the PC.

3.5.4 cDNA synthesis

Reverse transcription of total RNA to cDNA was carried out using Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit according to the manufactures' instruction using total RNA as template for each sample as follows:

1. All components of cDNA synthesis kit were thawed on ice and mixed properly.
2. The following reagents were added into a sterile, nuclease-free tube on ice:
    I. Template RNA- 2 µl
    II. Oligo $(dT)_{18}$ primer- 1 µl
    III. Water, nuclease-free- 7 µl
3. This mixture was incubated at 65°C for 5 min. and then cooled immediately on ice. It's highly optional when the template is GC-rich or contains secondary structures.
4. To the reaction mixture 5X Reaction buffer (4 µl), Ribolock RNase inhibitor (1 µl), 200 U Revert AidMuLV RT (1µl) and 10mM dNTP Mix (2 µl) was added. The volume of the reaction mixture was made to 20 µl by adding RNase free water. The components of the mixture were mixed gently and centrifuged.

5. The reaction mixture was then incubated at 42°C for 60 min.
6. Terminated the reaction by heating at 70°C for 5 min.
7. Briefly centrifuged.
8. The cDNA product was then stored at -20°C.

3.4.4 Confirmation of cDNA synthesis using 26S rRNA primers

cDNA synthesis was confirmed using 26S rRNA primers (Forward primer 5´CACAATGATAGGAGGAGCCGAC3´ and Reverse primer 5´CAAGGGAACGGGC TTGGCAGAATC3´) (Singh *et al* 2004). *In vitro* amplification using polymerase chain reaction (PCR) was performed in a 96 well microtiter plate and strips in Eppendorf and Applied Biosystems master cyclers. The PCR mix for each 20 µl reaction volume contained the following components: 100 ng template DNA, 1X Master mix 4 µl and 0.25 µM each of forward and reverse primers. Components used in PCR reaction and their stock and final concentration are given in Table 3.7. A negative control (without template DNA) was included in each amplification reaction. The PCR plate was covered with a mat and placed in a 96 well thermal cycler. The PCR amplification profile for 26S rRNA is given in Table 3.8.

Table 3.7: Concentration of the different components used in the PCR reaction

Component	Stock concentration	Quantity	Final concentration
Sterile water	-	9.3 µl/8.5µl	-
Template DNA	40 ng/µl	2.5 µl	100 ng
PCR buffer	10X	4.0 µl	1X
Forward Primer	5 µM	1.0 µl	0.25 µM
Reverse Primer	5 µM	1.0 µl	0.25 µM
Total		20 µl	-

Table 3.8: PCR amplification profile for 26S rRNA

Steps		Temperature (°C)	Time
I	Initial denaturation	94°C	5 min.
II	Denaturation	94°C	1 min.
III	Primer annealing	55°C	1 min.
IV	Extension	72°C	1 min. (Repeat II to IV steps for 30cycles)
V	Final extension	72°C	7 min.
VI	Storage	4°C	∞

3.5.6 Gel electrophoresis

After amplification, 3 µl of 6X loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water) was added to each of the amplified product and mixed thoroughly. Electrophoresis of the amplified cDNA was done by loading it in 1.5 per cent agarose gel prepared in 0.5X TBE buffer (45mM Tris base, 45mM Boric acid, 1mM EDTA, pH 8.0). For preparing 1.5 per cent agarose gel, 2.25g agarose was dissolved in 150 ml of 0.5X TBE electrophoresis buffer. The mixture was heated till the agarose dissolved completely and the mixture became transparent. It was cooled to about 55-60ºC with constant stirring and 5 µl of ethidium bromide (10 mg/ml) was added to a final concentration of 0.5 µg/µl. The agarose solution was poured into an already prepared gel mould with combs and left for about 30 min. for solidification. From the total product, 10 µl of each sample was loaded in 1.5 per cent agarose gel. The PCR products were resolved by running gel at 5V/cm for 3-5 hours. The gels were then visualized under gel documentation system and photographed.

3.5.7 Testing of RT primers amplification

The cDNA was used to check the amplification of candidate gene specific primers with normal PCR. The PCR mix for each 20 µl reaction volume contained the following components: 120 ng template DNA, 1X PCR Master mix reaction buffer and 0.25 µM each of forward and reverse primers. Components used in PCR reaction and their stock and final concentration are enlisted in Table 3.9. The PCR amplification involved the following thermal profile: initial denaturation at 95°C for 3 min., followed by 5 cycles of denaturation at 95°C for 30 sec., annealing at 60°C for 30 sec. and extension at 72°C for 1 min. This was followed by 35 cycles of denaturation at 95°C for 20 sec., annealing at 60°C for 20 sec. and extension at 72°C for 30 sec. The final extension step at 72°C was given for 7 min. The product was kept on hold at 4°C after the PCR (Table 3.10). A negative control (without template DNA) was included in amplification reaction.

The amplification was checked by preparing 4 per cent agarose gel in 0.5X TBE electrophoresis buffer. The mixture was heated till the solution became clear. It was cooled down to 60°C with constant stirring. Ethidium bromide was added to a final concentration of 5µg/ml. Then it was put into gel casting tray and left for 10-30 min. for solidification. The amplified products were loaded into well after mixing with 6X loading dye. Along with samples, DNA ladder (GeneRuler™50bp DNA Ladder) with known size of fragments was also loaded in first well. After loading, the gel was subjected to electrophoresis at 130 - 150 V for an hour. The gel was visualized under UV light and photographed using photo gel documentation system. The size of DNA fragment was checked by comparing with that of standard marker.

Table 3.9: Concentration of the different components used in the PCR reaction

Component	Stock concentration	Quantity	Final concentration
Sterile water	-	8.8 µl/8.0µl	-
Template DNA	40 ng/µl	3.0 µl	120 ng
Master mix buffer	10X	4.0 µl	1X
Forward Primer	5 µM	1.0 µl	0.25 µM
Reverse Primer	5 µM	1.0 µl	0.25 µM
Total		20 µl	-

Table 3.10: PCR amplification conditions for gene RT primers

Steps		Temperature (°C)	Time
I	Initial denaturation	95°C	3 min.
II	Denaturation	95°C	30 sec.
III	Primer annealing	60°C	30 sec.
IV	Extension	72°C	1min. (Repeat II to IV steps for 5cycles)
V	Denaturation	95°C	20 sec.
VI	Primer annealing	60°C	20 sec.
VII	Extension	72°C	30 sec. (Repeat V to VII steps for 35cycles)
VII	Final extension	72°C	7 min.
VIII	Storage	4°C	∞

3.6 qRT-PCR analysis

3.6.1 qRT-PCR amplification

Candidate genes expression was studied by qRT-PCR using ROSHE qRT-PCR machine. qRT-PCR was performed in triplicates using cDNA as a template. 18S gene was used as a reference gene for relative quantification of target genes. The PCR reaction mix (20 µl) was prepared by adding 80 ng of cDNA template, 0.25 µM of forward and reverse primers, 10 µl of 2X Takara SYBR® Premix Ex TaqTM II and 6 µl nuclease free water (Table 3.11). PCR amplification conditions were used as follows (Table 3.12).

Table 3.11: Concentration of the different components used in the PCR reaction

Component	Stock concentration	Quantity	Final concentration
Sterile water	-	6.0µl	-
cDNA	40 ng/µl	2.0 µl	80 ng
Forward Primer	5 µM	1.0 µl	0.25 µM
Reverse Primer	5 µM	1.0 µl	0.25 µM
SYBR	2X	10 µl	1X
Total		20 µl	-

Table 3.12: PCR amplification profile for qRT-PCR

Steps		Temperature (°C)	Time
I	Initial denaturation	94°C	3 min.
II	Denaturation	94°C	10 sec.
III	Primer annealing	60°C	30 sec.
IV	Extension	72°C	30 sec. (Repeat II to IV steps for 40 cycles)
V	Melt curve	55°C	30 sec.

3.6.1.2 Statistical analysis

The relative expression of target genes were normalized to reference gene expression for each set of sample and results were analyzed by ΔΔCt method. According to this method:

- ΔCt = Ct mean (target gene) - Ct mean (reference gene)
- ΔΔ Ct =ΔCt (target sample) - ΔCt (control sample)
- $2^{-\Delta\Delta CT}$ represents fold change in gene expression in stress condition in relative to control conditions (Relative gene expression)

3.7 Transfer of *qSLB3.1* using marker assisted selection

3.7.1 Generation of BC_1F_1 population

Crosses were attempted between CM139 X CM140 in *kharif* 2017 to develop F_1 hybrid. The F_1s were raised in spring 2018 and were backcrossed with CM140 to generate BC_1F_1 population. The BC_1F_1 population was grown in *kharif* 2018 using the standard agronomic practices. The crossing scheme for generation of BC_1F_1 progeniesis illustrated in Fig 3.3.

3.7.2 Screening of BC_1F_1 population against SLB

BC_1F_1 population plants were inoculated at the seven to nine leaf growth stage (V_{7-9}, Fig. 3.2A) by placing 20 to 30 grains of infected sorghum seeds with isolate *Dm1* in the whorls of each plant. The disease reaction of SLB was recorded on each 420 plants after 15, 30 and 45 days of inoculations following 1-9 scale of Miller *et al* (1970) as given in Table 3.2. Same procedure was followed as mentioned above for inoculations and scoring disease.

3.7.3 Foreground selection of *qSLB.pau_3.1*in BC_1F_1

BC_1F_1 population was analyzed for *qSLB3.1* linked flanking SSR markers, *MSSR1* and *MSSR20* along with QTL peak marker *MSSR9* of chromosome 3. Heterozygous plants for all the three markers were selected for background selection of carrier chromosome.

3.7.4 Recombinant selection for the carrier chromosome

This method was used to reduce the introgressed segment (linkage drag) that were transferred from the donor parent. The markers flanking to QTL linked markers were surveyed on selected plants. After identification of individuals using foreground markers, single and double recombinant individuals carrying the recurrent parent alleles were selected. The selected plants with minimum linkage drag were further backcrossed to generate BC_2F_1 population.

The banding pattern generated by the 13 SSR markers employed for recombinant selection of recurrent parent genome in BC_1F_1 population was scored in a co-dominant manner. The individual bands were scored as ‘A’ for the recurrent parent type band and ‘B’ for the presence of both the parent allele i.e. heterozygous. GGT (Graphical Genotyping) requires input in the form of GGT data files (or a spreadsheet, see further on). GGT data files were derived from two sources of data: A locus file, containing marker names and raw marker scores and a (linkage) map file, specifying marker positions on a linkage map. The locus file was a plain text file. It contains data on marker alleles using the MapDisto type of coding. The map file was also a plain text file consisting of two columns, markers and their map positions. A GGT data file was constructed by merging a locus and a map file using the option ‘Build GGT-file’. Common input data consist of a matrix of marker scores with markers arranged in rows and genotypes arranged in columns. GGT 2.0 was used to visualize data of markers with known map positions on a genetic map represented graphically by estimated lengths of genomic compositions as coloured chromosome bar segments.

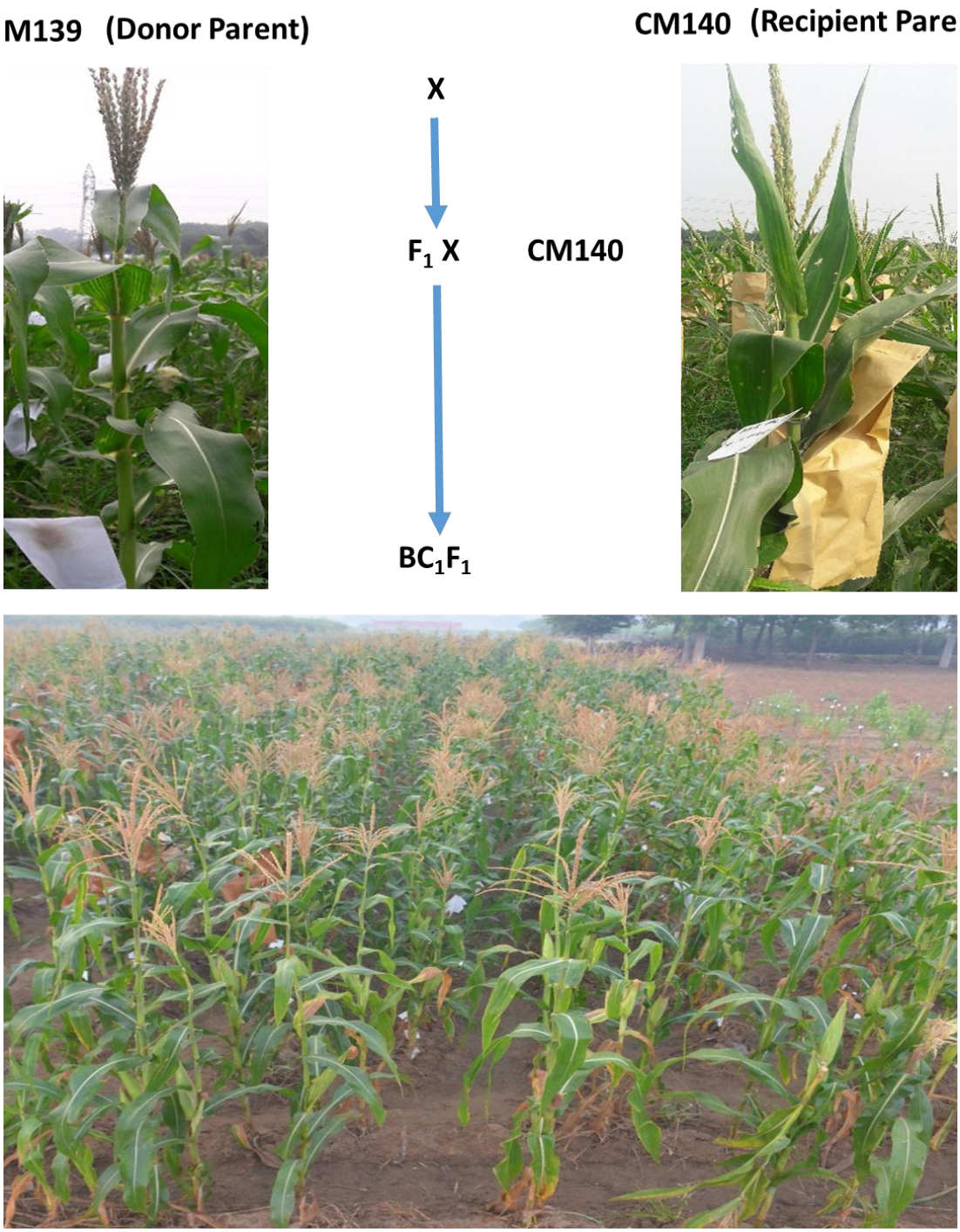

Fig. 3.3: Crossing scheme for generation of backcross population from the cross of CM139 X CM140

CHAPTER IV

RESULTS AND DISCUSSION

The present investigation was carried out using the mapping population from the cross between CM139 as the female parent (resistant to SLB) and CM140 as the male parent (susceptible to SLB) and their derived 298 RIL population to identify the QTL conferring resistance to southern leaf blight.

The present investigation was carried out as five different experiments:

Experiment I was aimed for phenotypic evaluation of RILs for southern leaf blight (SLB) resistance using 1-9 scale. Experiment II was undertaken for genotyping the RIL population using already available SSR markers on maize genome database and new SSR markers between the QTL regions were designed for fine mapping. Experiment III was aimed to identify candidate gene spanning in the QTL region and Experiment IV was undertaken to study the relative expression analysis of the candidate genes in chromosome 3. Experiment V was carried out to transfer the SLB resistance QTL in the background of CM140 inbred through marker assisted backcross breeding. The results obtained during the present investigation have been discussed under following heading:

4.1 Phenotypic evaluation of RILs

4.1.1 SLB resistance

4.2 Genotyping of RILs using SSR markers

4.2.1 Parental Polymorphism survey

4.2.2 Genotyping of mapping population

4.3 Molecular mapping

4.3.1 Construction of linkage map

4.3.2 Linkage analysis

4.3.2.1 Mapping of SLB resistance

4.3.2.2 Fine Mapping of putative QTL for SLB resistance

4.4 Identification of candidate genes

4.5 qRT-PCR

4.5.1 RNA isolationand cDNA synthesis

4.5.2 Relative expression analysis of candidate genes

4.6 Transfer of SLB resistance QTL through MAS

4.6.1 Evaluation of BC_1F_1 plants for SLB resistance

4.6.2 Foreground selection in BC_1F_1 generation

4.6.3 Recombinant Selection

4.7 Implication for pyramiding of SLB resistance QTL

4.1 Phenotypic evaluation of RILs

4.1.1 SLB resistance

Disease reaction data of RILs was taken after 15, 30 and 45 days of inoculation using 1-9 scale (Miller *et al* 1970) during *kharif* seasons of 2015, 2016 and 2017 (Fig. 4.1). The field view of RIL population of cross CM139 x CM140 is presented in Fig. 4.2. The SLB disease reaction exhibited by parental lines is shown in Fig. 4.3. .The range of disease severity among RIL population after 15, 30 and 45 days of inoculation fall within range of 5.11-59.4%, 24.4-83.3%, 34.4-100% respectively for year 2015. During *kharif* season 2016 the range was 7.2-33.8%, 13.3-76.8% and 27.27- 99.7% after 15, 30 and 45 days of inoculation respectively. Likewise, it varied between 3.4-57.5%, 23.14-76.2% and 35.6-99.7% after 15, 30 and 45 days of inoculation respectively for the year 2017 (Table 4.1).

Table 4.1: Mean and range of southern leaf blight severity and AUDPC between parents (CM139, CM140) and among 298 RILs derived from the cross of CM139 and CM140 during *kharif* seasons (2015-2017)

Days of incubation	*Kharif* 2015		*Kharif* 2016		*Kharif* 2017		Parental Mean (2015-2016)	
	Mean disease severity (%)							
	RILs		RILs		RILs		CM139	CM140
	Mean	Range	Mean	Range	Mean	Range	Mean	Mean
15 days	32.2	5.1 - 59.4	20.5	7.2 - 33.8	30.4	3.4-57.5	2.8	9.0
30 days	53.8	24.4 - 83.3	45.0	13.3 – 76.8	49.6	23.1-76.2	27.2	63.4
45 days	68.7	34.4 - 100	63.4	27.2 – 99.7	67.6	35.6-99.7	33.9	89.1
AUDPC	1006.6	535.3 - 1478	823.4	570.0 - 1076.8	961.4	700.0-1222.8	488.6	1216.6

The mean disease severity of RIL population after 15, 30 and 45 days of inoculations was 27.81%, 49.52% and 66.56%, whereas CM139 had 2.86%, 27.23% and 33.9% and CM140 had 9.03%, 63.4% and 89.16%, respectively over the years 2015-2017. The area under disease progress curve (AUDPC) ranged from 535.34-1478, 570.0-1076.82, and 700.0-1222.8 in consecutive years 2015, 2016 and 2017 respectively, among the RILs. The AUDPC for CM139 was 453.6, 536.15 and 476.05 whereas for CM140 was 1201.95, 1248.65 and 1199.45 for the years 2015, 2016 and 2017 respectively. SLB severity data showed a normal distribution after 30 days of inoculations demonstrating the characteristics of a quantitative trait but was little skewed towards susceptibility after 45 days of inoculations. The frequency distribution of RILs at different days of severity is presented in Figs. 4.4, 4.5 and 4.6. SLB severity data showed a normal distribution after 30 days of inoculations demonstrating the

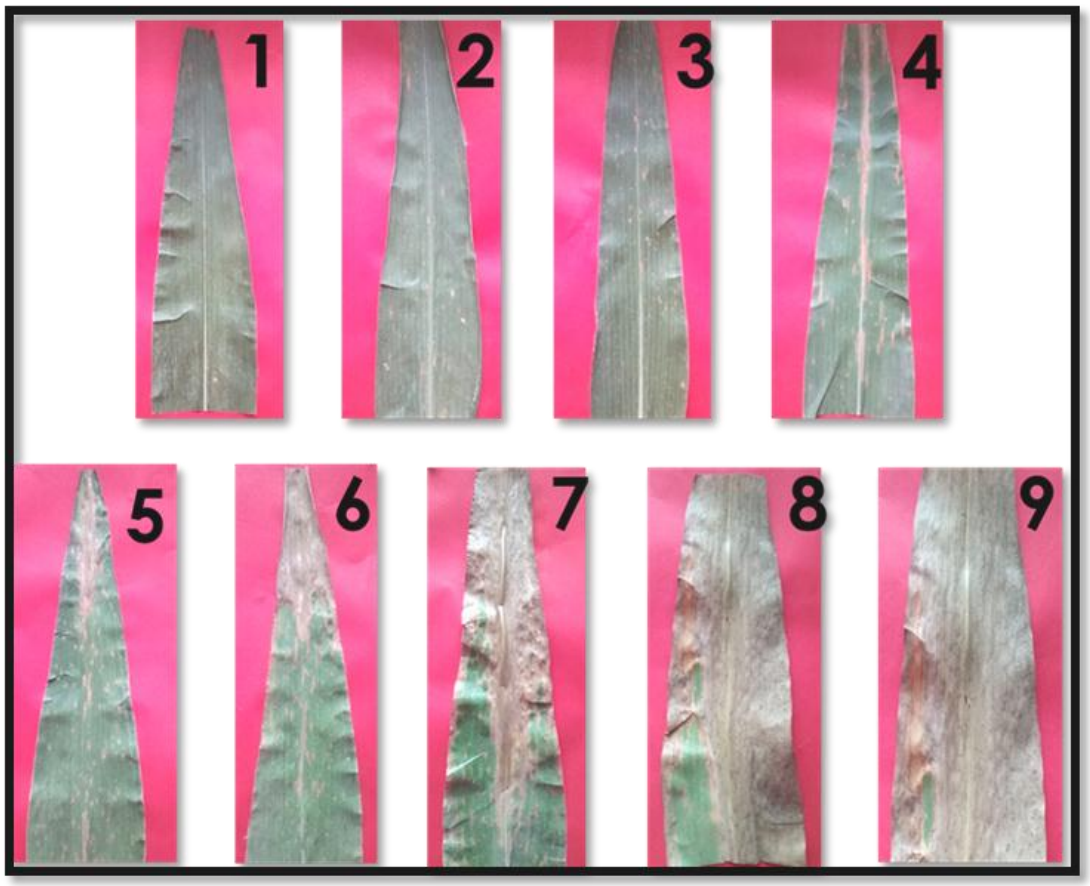

Fig.4.1: Southern leaf blight disease rating scale from 1 to 9 on infected maize leaves

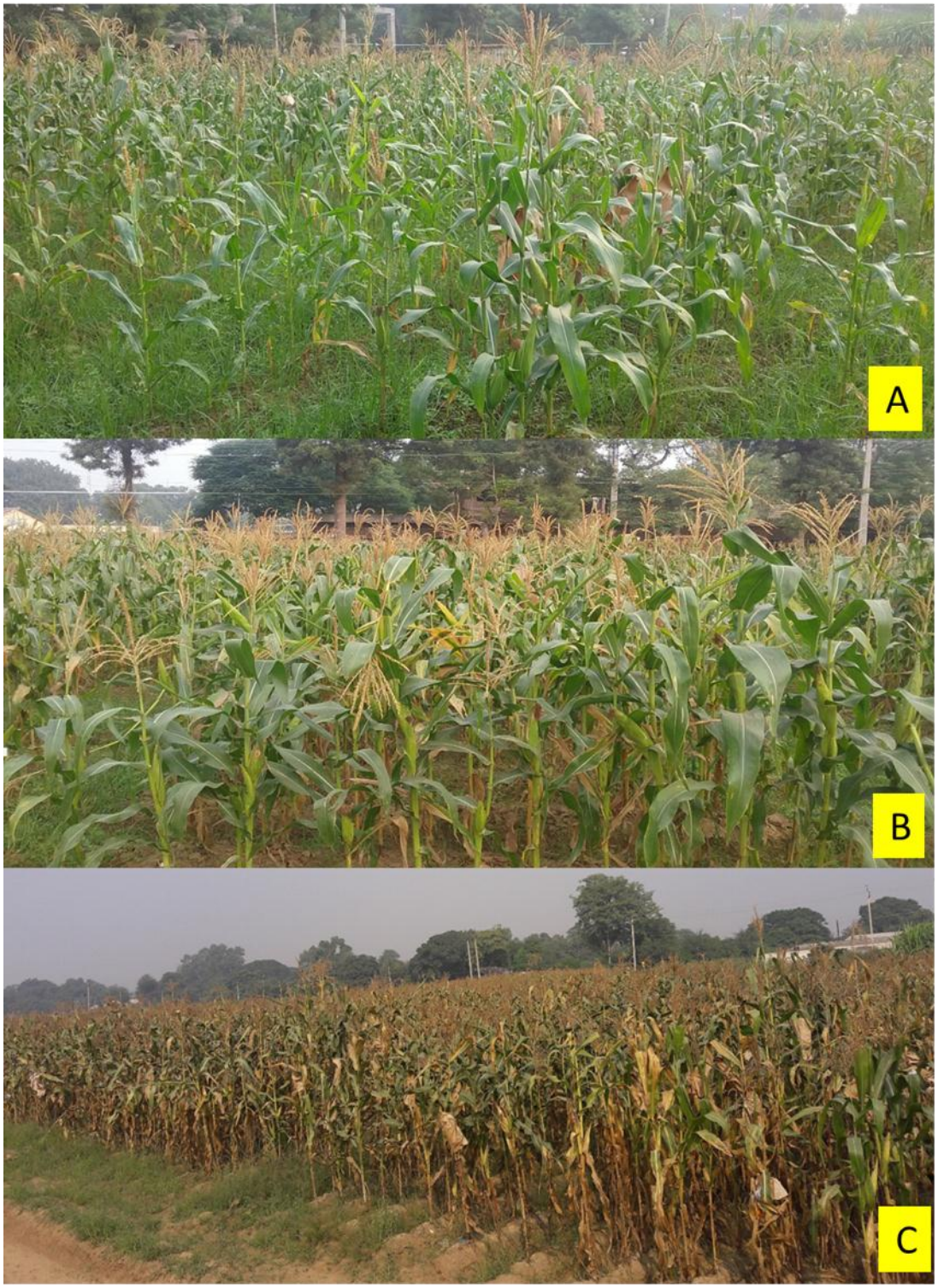

Fig. 4.2: Field view of RIL population (F_8, F_9 and F_{10}) derived from cross of CM139 (R)x CM140 (S) during *kharif season* years 2015 (A), 2016 (B) and 2017 (C)

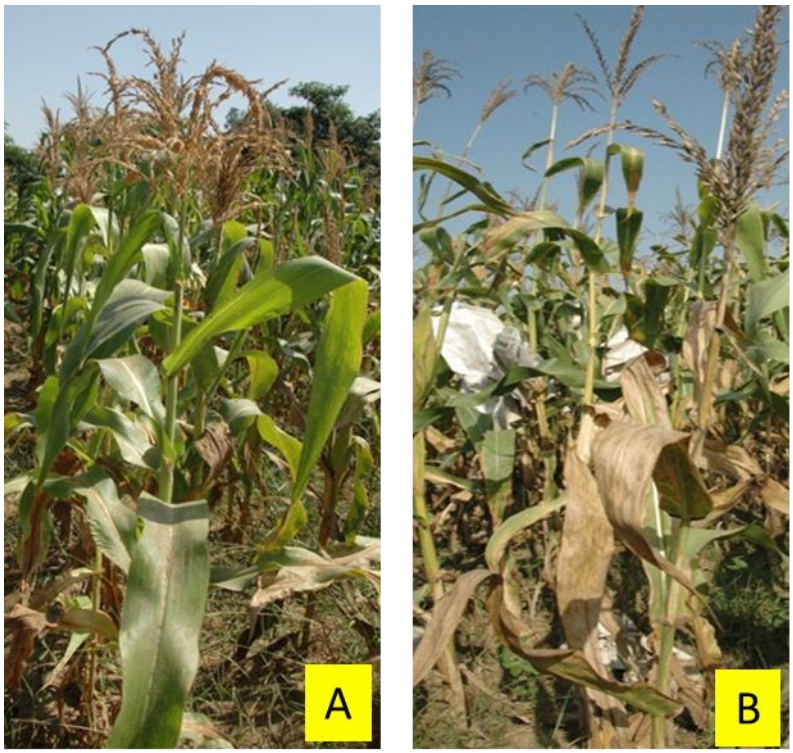

Fig. 4.3: Disease reaction of southern leaf blight on CM 139, resistant parent (A) and CM140, susceptible parent (B)

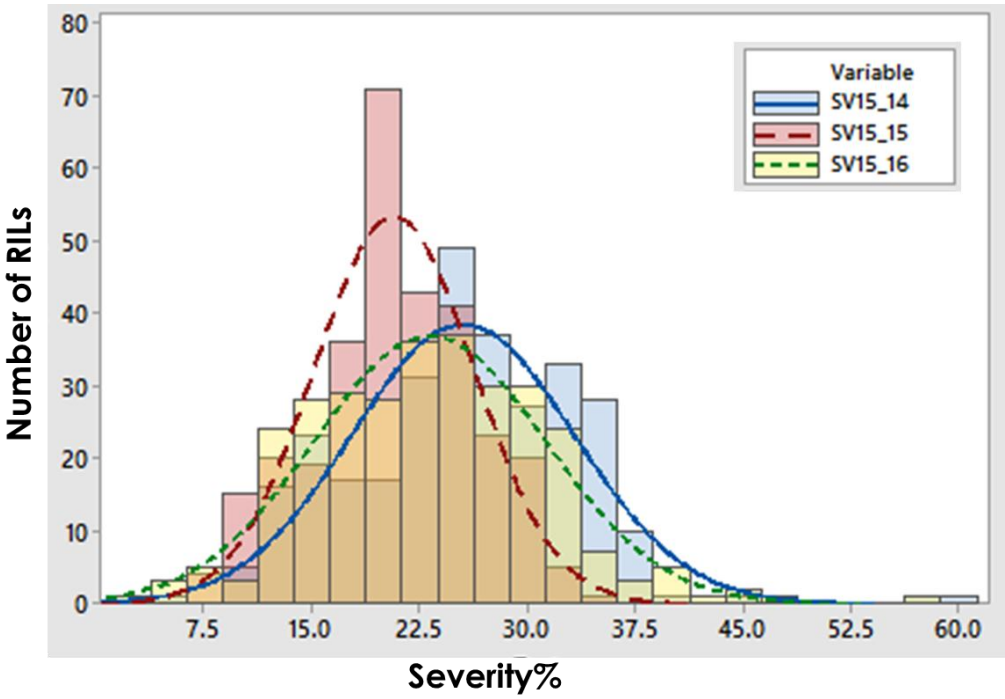

Fig. 4.4: Frequency distribution of disease severity at 15 days after inoculation during *kharif* seasons (2015-2017)

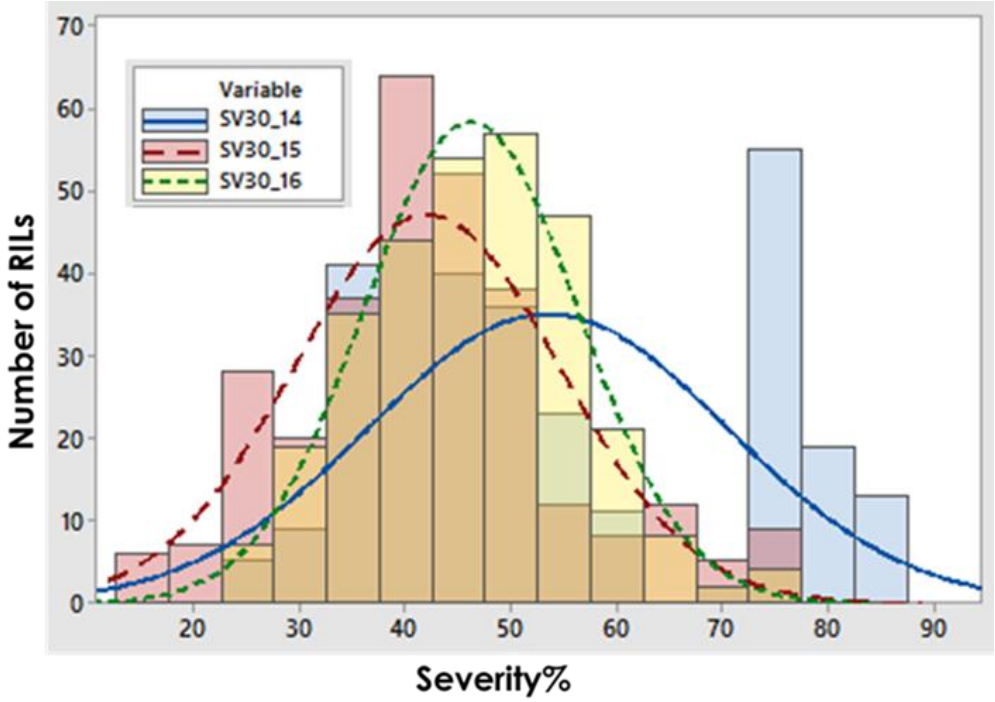

Fig. 4.5: Frequency distribution of disease severity at 30 days after inoculation during *kharif* seasons (2015-2017)

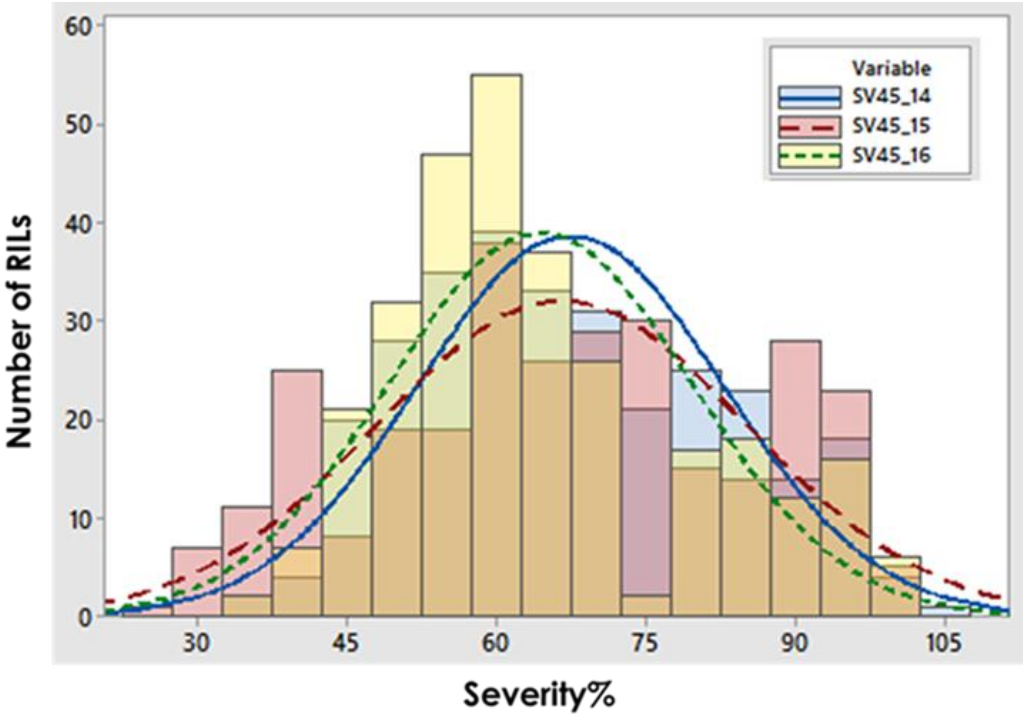

Fig. 4.6: Frequency distribution of disease severity at 45 days after inoculation during *kharif* seasons (2015-2017)

characteristics of a quantitative trait but was little skewed towards susceptibility after 45 days of inoculations.

The pooled analysis was also done to analyze the variation in increment of disease reaction after 15, 30 and 45 days of inoculation at Ludhiana during 2015-2017. ANOVA was performed to test for statistical significance. Environment, genotype, and the genotype-environment interactions were significant sources of variation in the combined analysis, while the effects of blocks within replication, replication and block-genotype interaction were non significant. Environment, genotype, and the genotype-environment interactions were significant sources of variation in the combined analysis, while the effects of blocks within replication and replications.

The significant difference in disease development between genotypes was observed with coefficient of variance (CV) of 14.67, 8.28, 5.34 and 4.98 at severity 15, 30, 45 and AUDPC respectively for 300 RILs population at 5% level of significance (Table 4.2). The high level of variation associated with environment may reflect that development of diseases varied over years. Similarly significant genotype and genotype- environment interactions were observed in other studies (Klump *et al* 2011, Balint Kurti *et al* 2007).

Table 4.2: Pooled analysis of variance of area under disease progress curve and disease severity at 15, 30 and 45 days after inoculation

Source	F-value				
	DF	**Severity 15**	**Severity 30**	**Severity 45**	**AUDPC**
Year	**2**	45.55****	122.61****	24.97****	117.04****
Rep	**1**	0.04^{ns}	0^{ns}	1.44^{ns}	0.43^{ns}
Block	**29**	0.91^{ns}	0.96^{ns}	0.79^{ns}	0.61^{ns}
TRT	**299**	14.91****	57.17****	103.21****	128.73****
Block*TRT	**839**	1.12^{ns}	0.91^{ns}	1.06^{ns}	0.84^{ns}
Year *TRT	**105**	4.96****	4.08****	6.55****	6.39****
CV		**14.67**	**8.28**	**5.34**	**4.98**

ns= non significant; **** highly significant

Again the pooled analysis was done to find Pearson correlation between disease severity at 15, 30 and 45 days and area under disease progress curve (Table 4.3). The highest significant positive correlation was observed between AUDPC and severity 45 (0.977) while AUDPC and severity 30 had 0.976 but correlation between severity 15 and severity 30 was0.062 whereas, between severity 15 and severity 45 was 0.598. Cooper *et al* (2018) observed Pearson's correlation coefficients for Goss's wilt, NLB (northern leaf blight), GLS (gray leaf spot), and SLB in the IBM population and found that Goss's wilt was significantly correlated with NLB, but not with GLS and SLB.

Table 4.3: Phenotypic correlation coefficient between southern leaf blight severity at 15, 30 and 45 days and area under disease progress curve (AUDPC)

Source	Correlation matrix (Pearson)			
	Severity 15 days	**Severity 30 days**	**Severity 45 days**	**AUDPC**
Severity 15 days	1	0.62****	0.51****	0.64****
Severity 30 days		1	0.94****	0.98****
Severity 30 days			1	0.98****
AUDPC				1

Sowing of F_8 RILs was done on 20th June 2015, whereas sowing of F_9 RILs was done on 27th June, 2016 and during these two years, the temperature was 31ºC and 32.0ºC while on 5th July 2017, it was 34.0ºC. During 2015, relative humidity was 57.0 per cent, 60 per cent in year 2016 and it was much higher upto 64 per cent in 2017. The temperature during inoculation and data recording varied from 34.0ºC-39.0ºC, 31.0ºC-36.0ºC and 33.0ºC-38.0ºC during 2015, 2016 and 2017, respectively. Similarly, relative humidity after 15 days of inoculation was 61 per cent, 68 per cent and 70 per cent for the years 2015, 2016 and 2017 respectively. At the time of data recording after 45 days of inoculation, humidity during 2015, 2016 and 2017 was 96.0, 60, and 82 per cent, respectively. These observations revealed that the favourable conditions like high humidity and more rainfall were very conducive for SLB development, whereas very hot and dry weather conditions resulted in slow disease development. Peet and Maschtti (1972) reported rainfall, relative humidity and temperature as critical factors in the spread of D. *maydis*. Same effects of rain and humidity were observed by Carson *et al* (2004) during the years 1995 and 1996. Dai *et al* (2018) indicated that geographic location and temperature did have an effect on sensitivity of *C. heterostrophus*. Similar results were obtained in present investigation indicating the effects of humidity and temperature on disease development over the years.

There was concern that disease ratings might be affected by variations between lines in time to maturity (Balint-Kurti *et al* 2008). The high level of variation associated with environment likely reflected the fact that disease was assessed in relatively diverse environments at Ludhiana. There was also increase in the disease development after 45 days of inoculation as compared to after 30 days of inoculation. Similar results were observed by Zwonitzer *et al* (2010) reported the significant difference between the

disease development when disease scoring was done in northern Ohio and in central North Carolina. Increase in disease development after anthesis was also reported by Pengfei *et al* (2011). Similar observations were made by Yong-xiang *et al* (2018) that necrotrophic leaf diseases like SLB, tend to occur before anthesis and the symptoms become much more severe after anthesis. These results clearly indicated that breeding for disease resistance require thorough evaluation of lines, populations in diverse regions and rating at different interval of times to have broad and durable resistance over the environments.

4.2Genotyping of RILs using SSR markers

4.2.1 Parental polymorphism survey

A total of 101 SSR markers spanning the chromosome 3 of maize genome were analyzed on CM139 and CM140 inbred lines for parental polymorphism and a detailed list of SSR markers surveyed is enlisted in Table 4.4. The SSR markers surveyed, had covered different bin regions of chromosome 3. Out of 101 SSR markers, 66 SSR markers were monomorphic and 35 were polymorphic showing overall 36.63 per cent of polymorphism. Further, for a fine map new 22 SSR markers spanning the region between the identical QTL of maize genome were again analyzed on CM139 and CM140 inbred lines for parental polymorphism. Out of 22 SSR markers, 10 SSR markers were monomorphic and 12 were polymorphic showing overall 54.5 per cent of polymorphism (Table 4.5).

Table 4.4: List of SSR markers surveyed for parental polymorphism

Sr. No.	Marker	Bin no.	Status of marker	Sr. No.	Marker	Bin no.	Status of marker
1	*phi453121*	3.0	P	22	*umc1717*	3.04	M
2	*umc2118*	3.0	P	23	*umc1655*	3.04	M
3	*umc1793*	3.0	P	24	*umc1835*	3.04	M
4	*umc1970*	3.01	M	25	*phi374118*	3.04	M
5	*bnlg1144*	3.02	P	26	*umc1750*	3.04	M
6	*umc1458*	3.02	P	27	*umc2263*	3.04	M
7	*umc1886*	3.02	P	28	*umc1900*	3.04	M
8	*bnlg1647*	3.02	P	29	*umc1920*	3.04	M
9	*bnlg1523*	3.02	P	30	*umc1527*	3.04	P
15	*bnlg1447*	3.03	M	31	*umc1030*	3.04	P
16	*umc2258*	3.03	P	32	*umc1772*	3.04	M
17	*umc1729*	3.04	P	33	*umc1608*	3.04	M
18	*umc1425*	3.04	P	34	*umc1965*	3.04	M
19	*umc2158*	3.04	M	35	*umc1351*	3.04	M
20	*umc1495*	3.04	M	36	*umc1721*	3.04	M
21	*umc2033*	3.04	M	37	*umc2262*	3.04	P

Sr. No.	Marker	Bin no.	Status of marker
38	*umc2000*	3.04	P
39	*phi036*	3.04	P
40	*phi099*	3.04	P
41	*umc1012*	3.04	P
42	*umc1167*	3.05	M
43	*umc1501*	3.05	M
44	*pdupssr5*	3.05	M
45	*umc1347*	3.05	M
46	*bnlg602*	3.05	M
47	*bnlg1399*	3.05	M
48	*mmc0022*	3.05	M
49	*umc1907*	3.05	M
50	*umc1839*	3.05	M
51	*umc1616*	3.05	M
52	*umc1300*	3.05	M
53	*umc2127*	3.05	P
54	*umc2155*	3.05	P
55	*umc1539*	3.05-3.06	M
56	*umc1742*	3.04	M
57	*umc2260*	3.04	P
58	*bnlg1638*	3.04	P
59	*umc1504*	3.04	M
60	*umc1223*	3.04	M
61	*bnlg1957*	3.04	M
62	*umc1419*	3.04	M
63	*umc1527*	3.04	P
64	*bnlg1638*	3.04	P
65	*bnlg1019*	3.04	P
66	*bnlg2117*	3.04	P
67	*umc2260*	3.04	P
68	*umc1392*	3.04	M
69	*umc1968*	3.04	M
70	*umc1683*	3.04	M
71	*umc1773*	3.04	M
72	*phi243966*	3.04	M

Sr. No.	Marker	Bin no.	Status of marker
73	*bnlg1113*	3.04	M
74	*mmc0132*	3.04	M
75	*phi029*	3.04	M
76	*bnlg2136*	3.04	M
77	*umc1968*	3.04	M
78	*umc1087*	3.04	M
79	*nc030*	3.04	M
80	*bnlg2047*	3.04	M
81	*umc2264*	3.04	M
82	*umc2024*	3.04	M
83	*umc1380*	3.05	M
84	*bnlg1022*	3.05	M
85	*umc1874*	3.05	M
86	*phi073*	3.05	M
87	*bnlg1456*	3.05	P
88	*umc2020*	3.05	M
89	*bnlg1245*	3.05	M
90	*umc1174*	3.05	P
91	*umc1600*	3.05	M
92	*umc1628*	3.05	M
93	*bnlg197*	3.06	P
94	*umc2174*	3.08	P
95	*mmc0001*	3.09	M
96	*dupssr33*	3.09	M
97	*umc1010*	3.09	M
98	*bnlg1754*	3.09	P
99	*umc1641*	3.09	P
100	*umc1594*	3.09	P
96	*umc1639*	3.09	P
97	*umc1578*	3.09	P
98	*umc2008*	3.09	P
99	*phi047*	3.09	M
100	*umc1813*	3.09	M
101	*umc2048*	3.10	P

Table 4.5: List of new SSR markers surveyed for parental polymorphism:

Sr. No.	Marker	Status of Marker	Sr. No.	Marker	Status of Marker
1	*MSSR1*	P	12	*MSSR12*	P
2	*MSSR2*	P	13	*MSSR13*	M
3	*MSSR3*	P	14	*MSSR14*	M
4	*MSSR4*	P	15	*MSSR15*	M
5	*MSSR5*	M	16	*MSSR16*	M
6	*MSSR6*	P	17	*MSSR17*	M
7	*MSSR7*	M	18	*MSSR18*	P
8	*MSSR8*	P	19	*MSSR19*	M
9	*MSSR9*	P	20	*MSSR20*	P
10	*MSSR10*	P	21	*MSSR21*	M
11	*MSSR11*	P	22	*MSSR22*	M

Various studies indicated that level of polymorphism in any experimental material depends on the markers employed as well as the genetic architecture of the lines used for the study. Jiang *et al* (1999) used 150 SSR markers to detect polymorphism that showed 57.33 per cent of polymorphism in *Zea mays*. Sibov *et al* (2003) surveyed 859 SSR markers and 213 markers were found to be polymorphic between two elite inbred lines, L-08-05F and L-14-4B. The observed level of polymorphism was 45.9 per cent. Song *et al* (2004) analyzed 439 SSR markers to detect polymorphism between high oil line (By 804) and a normal line (B73) and 158 markers were found to be polymorphic that showed 36 per cent of polymorphism. Shi *et al* (2010) screened 500 SSR markers and 158 markers showed polymorphism with 31.6 per cent of polymorphism between Ai 2003 (dwarf line) and Ji 257 (inbred line with normal plant height).

Polymorphism analysis done by Wang *et al* (2012) with 713 SSR makers, showed 220 (30.9 percent) polymorphic markers for cross between B73 and *parviglumis*. For another cross (B73 x *diploperennis*) a total of 320 polymorphic markers were identified from 873 tested SSR markers and the polymorphism was 36.6 per cent. Kumari *et al* (2018) did the genetic diversity analysis in maize using 22 SSR markers. From those 22 SSR primers, 9 primers were polymorphic and displayed clear size differences and a total of 19 polymorphic alleles were generated through the 9 polymorphic primers with an average polymorphism information content and genetic diversity value of 0.297 and 0.373, respectively. They found *phi064* and *phi053* as the best marker for identification of genotypes as revealed by PIC values (0.367). These studies showed that range of polymorphism between the parents using SSR markers varied from 30.9 per cent to 57.33 per cent.

In the present study, the level of polymorphism with available SSR markers from maize genome database revealed 36.63 per cent polymorphism while newly synthesized markers showed 54.5 per cent level of polymorphism. Similar range of polymorphism per cent was observed in other studies as discussed above. It clearly indicates that the level of polymorphism in any experimental material is attributed to how diverse the lines are from each other and also on the selection of marker employed for the study.

4.2.2 Genotyping of mapping population

A total of 35 polymorphic SSR markers were used to genotype 298 RILs. All the 35 markers which showed polymorphism between both parents also distinguished RIL population clearly. While selecting the markers, care was taken to represent all the bins, especially the bin harbouring the putative QTLs for southern leaf blight (*qSLB.pau_3.04*) reported from analysing $F_{2:3}$ population (Chauhan 2013). Later on, the 12 more newly synthesised polymorphic SSR markers were genotyped on the same set of RILs. The genotyping of RIL population using SSR markers is shown in Fig. 4.7.

Chi square (χ^2) analysis of markers was done to study whether the segregation of markers follow the Mendelian ratio (1:1) or distorted ratio. A total of 47 markers were studied for their chi square analysis and out of these polymorphic markers 39 of the markers showed Mendelian segregation ratio of 1:1 (Table 4.6). Significant deviation from expected segregation was observed for 8 markers. Out of eight markers, two markers showed segregation distortion towards parent CM139 and six markers distorted towards CM140.

Segregation distortion was first reported in maize by Mangelsdorf and Jones (1926) and subsequently reported by Burnham (1936); Rhoades (1942); Longley (1945); Helentjaris *et al* (1986); Wendel *et al* (1987) and Gardiner *et al* (1993). This could occur in pollen (Cameron and Moav 1957; Loegering and Sears 1963; Endo 1982; Finch *et al* 1984; Tsujimoto and Tsunewaki 1985), in megaspores or in both (Rick 1966). The reasons for this distortion might be resulted from a selection process during gametogenesis, fertilization or germination (Lyttle 1991).

Liu *et al* (1996) suggested that increased segregation distortion was expected solely because of increased recombination. Increased recombination lengthened the map, thereby increasing the number of independent tests represented by markers across the genome. If segregation distortion was caused by segregation distortion loci (SDL), all markers in the vicinity of the SDL would be affected (Vogl and Xu 2000). Whereas Chunhui *et al* (2016) used high-density markers to identify segregation distortion regions (SDRs) and suggested that the regions with high recombination rate and high gene density usually tended to have little segregation distortion. It indicates that segregation distortion depends on the coverage of markers along the chromosome and recombination rate between the markers. Also some genomic regions are hot spots for recombination while regions near to telomere and centromere tends to have low recombination rate.

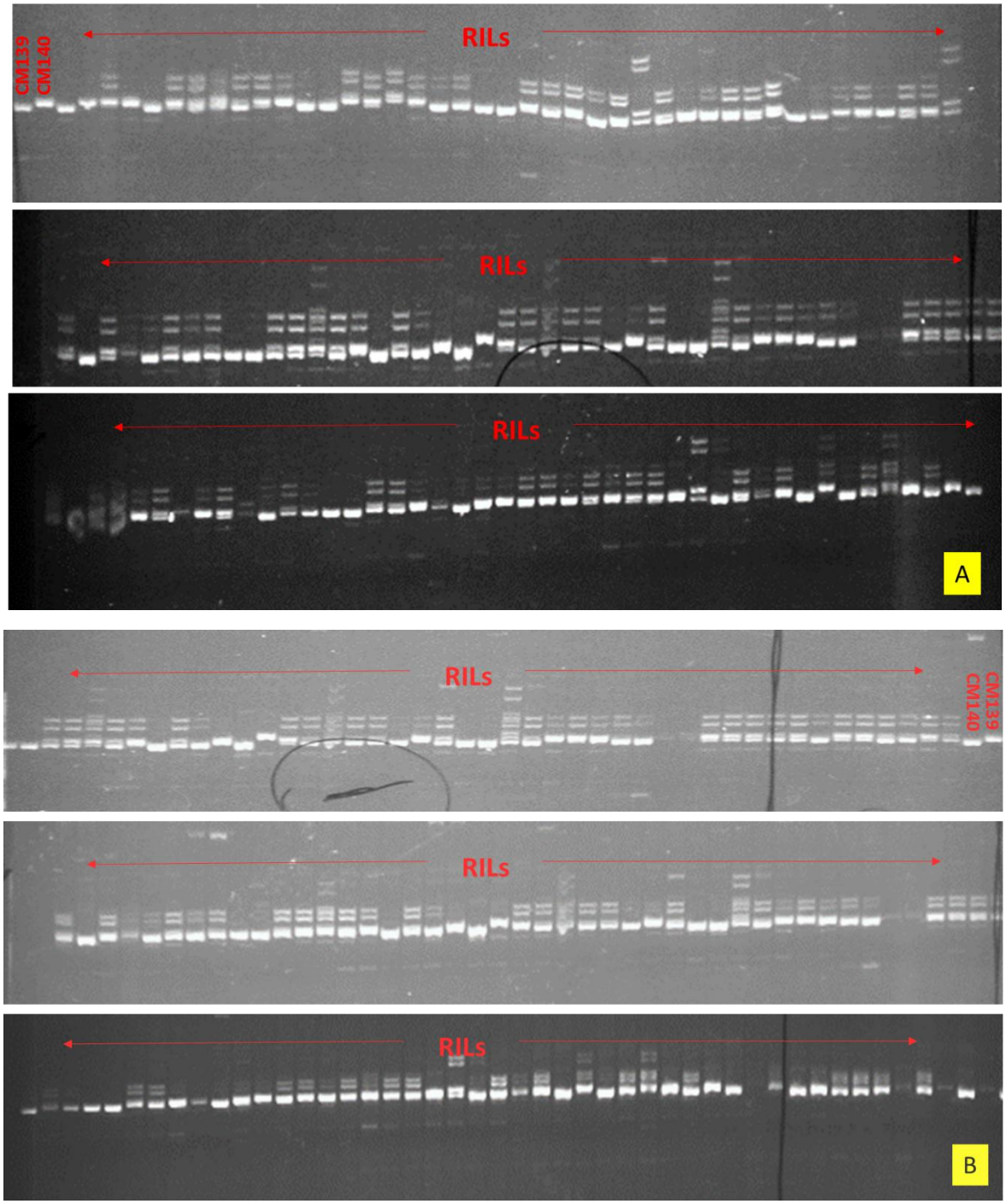

Fig. 4.7: Genotyping of RILs derived from the cross of CM139 (R) and CM140 (S) with SSR markers *viz.MSSR*9 (A) and *MSSR*20 (B) resolved on polyacrylamide gel

Table 4.6: Chi square (χ^2) analysis of SSR markers showing Mendelian or distorted segregation

S. No.	Marker	$\chi^2_{1:1}$	*P*	Status	S. No.	Marker	$\chi^2_{1:1}$	*P*	Status
1	*umc2048*	8.00	0.00468	**	24	*phi099*	3.20	0.07364	ns
2	*umc1594*	0.31	0.57851	ns	25	*umc1012*	1.57	0.21000	ns
3	*umc1639*	3.37	0.06646	ns	26	*phi036*	0.34	0.55841	ns
4	*umc1641*	0.50	0.47950	ns	27	*umc2258*	4.63	0.03144	*
5	*umc1578*	14.52	0.00014	***	28	*bnlg1647*	0.02	0.89463	ns
6	*umc1754*	0.20	0.65472	ns	29	*umc1886*	9.92	0.00163	**
7	*umc2008*	2.25	0.13333	ns	30	*bnlg1523*	2.18	0.13965	ns
8	*umc2174*	0.86	0.35238	ns	31	*bnlg1144*	9.93	0.00163	**
9	*bnlg197*	0.31	0.57851	ns	32	*umc1458*	2.65	0.10364	ns
10	*bnlg1456*	2.60	0.10686	ns	33	*umc1793*	9.93	0.00163	**
11	*umc2127*	0.05	0.82726	ns	34	*phi453121*	15.91	0.00007	****
12	*umc2155*	1.08	0.29870	ns	35	*umc2118*	15.21	0.00010	****
13	*umc1174*	2.32	0.12813	ns	36	*MSSR1*	0.02	0.89463	ns
14	*umc1425*	0.01	0.91042	ns	37	*MSSR2*	0.00	1.00000	ns
15	*umc2260*	2.78	0.09558	ns	38	*MSSR3*	2.18	0.13965	ns
16	*umc2262*	0.12	0.73244	ns	39	*MSSR4*	0.02	0.90129	ns
17	*umc2117*	0.02	0.90129	ns	40	*MSSR6*	2.65	0.10364	ns
18	*umc1030*	1.00	0.31731	ns	41	*MSSR8*	1.00	0.31731	ns
19	*umc1729*	0.00	1.00000	ns	42	*MSSR10*	3.19	0.07415	ns
20	*umc2000*	0.13	0.71798	ns	43	*MSSR11*	2.85	0.09148	ns
21	*bnlg1019*	0.21	0.64636	ns	44	*MSSR12*	0.55	0.46018	ns
22	*bnlg1638*	3.57	0.05878	ns	45	*MSSR18*	0.02	0.89974	ns
23	*umc1527*	0.00	1.00000	ns	46	*MSSR20*	0.89	0.34523	ns
					47	*MSSR9*	2.65	0.10364	ns

4.3 Molecular mapping

4.3.1 Construction of linkage map

The genotypic data of 298 RILs with 35 SSR markers were analyzed using MAPDISTO software to construct a genetic linkage map by using the LOD score of 3.0 and recombination fraction of 0.3. The all SSR markers were grouped together into one linkage group for chromosome 3. The linkage map generated using 35 SSR markers is presented in Fig. 4.8A. It had genomic coverage of 410.1 cM and maximum distance between the two markers was found to be 23.2 cM between the marker interval of *phi453121* and *umc2118*. The minimum distance of 4.2 cM was identified between marker interval of *umc2048* and *umc1594* on chromosome 3. Distribution of SSR markers on chromosome 3 was in the same order of bins and map positions as in linkage map generated by IBM2 2008 Neighbors Frame 3 (http://www.maizegdb.org).

Several maps for different populations had been constructed with varied map length and marker intervals. Sibov *et al* (2003) constructed a genetic map with total length of 1634 cM and average interval between adjacent markers was 14 cM. A total map length of 1759.1 cM with an average interval of 11.65 cM was ascertained by Song *et al* (2004), whereas Shi *et al* (2010) reported total map length of 2852.1 cM with an average distance of 27.4 cM. Similarly, Zwonitzer *et al* (2010) developed a linkage map covering a total length of 1697.3 cM with 1.9 cM of average distance between markers. The average distance between the markers was 6.5 cM as reported by Pengfei *et al* (2011) whereas Jiang *et al* (2011) accounted the average genetic distance of 9.4 cM.

The total map length of 1987.7 cM with an average distance of 11.0 cM between markers was given by Ding *et al* (2011), while Wang *et al* (2012) constructed linkage map of total length 2210.8 cM and the average genetic distance between two neighboring loci was 12.8 cM. Therefore, the average distance between the SSR markers on a linkage group varied from 1.9 cM to 27.4 cM and in this study the average distance between the markers was 4.6 cM. There were a few gaps of more than 10 cM on the map, that region is likely to be densed by genotyping with more SSR markers. Su *et al* (2017) used the SNP genotype data and constructed high density intra-specific genetic linkage map of maize which consists of 3,305 bins on 10 linkage groups spanning 2,236.66 cM at an average distance of 0.68 cM between consecutive markers.

Chauhan (2013) developed linkage map using $F_{2:3}$ families of cross CM139 x CM140. The total map length was 345.1 cM and the length of chromosome 3 was 66.4 cM. The chromosome 3 covered nine markers *phi453121*, *phi104127*, *bnlg 1144*, *phi099*, *umc1729*, *umc2000*, *bnlg1019*, *bnlg197* and *bnlg1754*. In the present study the marker density had been increased and the map length extended to 410.1 cM using RILs of the same cross. It was clear from these studies that as we go on increasing the marker density on map, the average

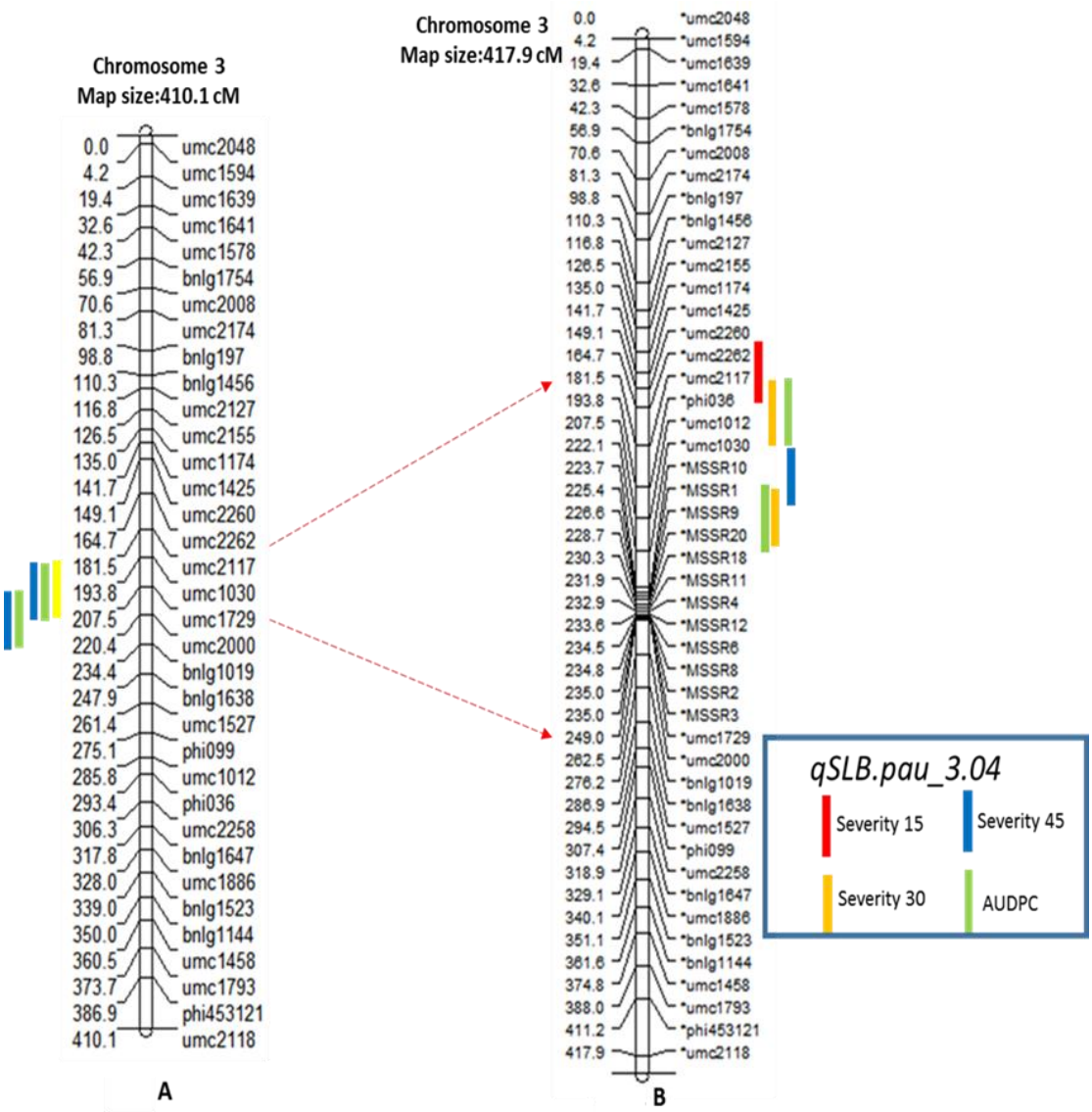

Fig. 4.8: Linkage map of chromosome 3 based on 35 SSR markers (A). Saturated linkage map with more SSR markers spanning *qSLB.pau_3.04* QTL for fine mapping (B). Both maps showing chromosomal location of significant QTL for southern leaf blight resistance in maize

distances between the markers decreases. So, saturation of markers is required to get the more precise map.

4.3.2 Linkage analysis

4.3.2.1 Mapping of SLB resistance

Both genotypic data of 35 SSR markers and phenotypic SLB severity data at different disease ratings along with AUDPC over the years (2015-2017) was subjected to QTL mapping for SLB resistance. The QTLs for above mentioned traits were mapped on chromosome 3. The disease severity data after 15, 30 days and 45 days along with area under disease progress curve, depicted two QTL positions on chromosome 3. At severity 30 single QTL designated as *qSLB.pau2_3.04*with flanking markers *umc2117-umc1729* was detected, whereas at severity 45 two QTL were observed viz. *qSLB.pau2_3.04* and *qSLB.pau*3_3.04 with flanking markers *umc2117-umc1729* and *umc1030-umc2000* respectively. Both these QTL were co-localized with area under disease progress curve and designated as *qaudpc.pau2_3.04* and *qaudpc.pau3_3.04* (Fig 4.9). The total phenotypic variance of 19.89 per cent was explained by AUDPC QTL whereas 19.74 per cent variance was explained by QTL detected at severity 45 (Table.4.7). The flanking markers *umc2117-umc1729* spanning 13.7 cM region on reference genome B73RefGen_v4 had physical distance of 395.7 kbp which was reduced from 768 kbp having flanking markers as *phi 099-umc1729*as reported by Chauhan (2013). It has been observed that QTL region was narrowed down with marker *umc1729* which was same as detected by Chauhan (2013) as flanking and new markers *umc1030* and *umc 2117* was found closer to QTL.

Table 4.7 Marker intervals showing the association of putative QTL with SLB resistance analyzed by composite interval mapping over the years (2015-2017)

Trait	Peak marker	Flanking Marker	Lod Score	Additive Effect	Phenotypic variance (%)
Severity 30 (*qSLB.pau*2_3.04)	*umc1030*	*umc2117-umc1729*	4.00	5.90	8.60
Severity 45 (*qSLB.pau*2_3.04)	*umc1030*	*umc2117-umc1729*	5.30	7.00	11.00
(*qSLB.pau*3_3.04)	*umc1729*	*umc1030-umc2000*	4.30	6.23	8.70
AUDPC *qaudpc.pau2_3.04*	*umc1030*	*umc2117-umc1729*	6.09	123.35	12.30
(*qaudpc.pau3_3.0*)	*umc1729*	*umc1030-umc2000*	3.77	86.90	7.60

The present study revealed that genetics of SLB resistance is in additive manner and AUDPC QTL had maximum additive effect of 123.35. Previous studies also showed that the genetics of SLB resistance could be additive or recessive in effect (Scott and Futrell 1975; Lim and Hooker 1976; Thompson and Bergquist 1984; Burnette and White 1985; Holley and Goodman 1989; Carson *et al* 2004; Balint-Kurti *et al* 2008; Kump *et al* 2011; Zwonitzer *et al* 2010; Nageri*et al* 2011). In maize, many reports were present on QTL identification for SLB disease resistance (Bubeck *et al* 1993; Jiang *et al* 1999; Carson *et al* 2004; Balint-Kurti and Carson 2006; Balint-Kurti *et al* 2007, 2008; Zwonitzer *et al* 2010; Pengfei *et al* 2011; Belcher 2012; Ali *et al* 2013). Our results are reliable with most of the earlier studies of the inheritance of SLB resistance in maize. A comparison of results of all published SLB resistance QTL suggested that bin 3.04 was a 'hotspot' region for SLB resistance. Bin 3.04 had also been detected as a 'hotspot' for viral resistance (McMullen and Simcox 1995; Wisser *et al* 2006). Jiang *et al* (1999) identified a QTL for SLB in the vicinity of bin 3.04 using tropical germplasm. Bin 3.04 was of particular interest as it was identified as an SLB resistance QTL in both juvenile (Balint-Kurti and Carson 2006) and mature plants (Carson *et al* 2004).

4.3.2.2 Fine mapping of putative QTL for SLB resistance

Further the fine mapping of putative QTL in bin 3.04 (*qSLB.pau3_3.04*) was done by designing new SSR markers through MISA software between the region spanning the QTL linked with flanking markers *umc2117*and *umc1729*. The 12 new SSR markers along with 35 SSR markers were analysed and linkage map was constructed with genomic coverage of 417.9 cM. The minimum distance between the markers *MSSR8* and *MSSR2* was 0 cM and maximum distance was 17.8 cM between markers *umc2262* and *umc2117* (Fig 4.8B).

Combined analysis over the years was done with phenotypic and genotypic data and four QTL associated with SLB resistance were detected. The QTL for SLB severity at 15 days was mapped between the marker intervals of *umc2262-phi036* and was designated as *qSLB.15pau*_3.04. The QTL for SLB severity at 15 days was neither detected by Chauhan (2013) nor in the present study as discussed above. This infers that saturation of genomic regions by markers increases the probability of detection of QTL which otherwise go undetected with low genomic coverage. So, marker density should be increased while mapping complex quantitative traits. Two QTL were associated with SLB severity after 30 days of inoculation and 22.5 per cent total phenotypic variance was depicted (Table 4.8). Both QTL were designated as *qSLB.pau*2_3.04 and *qSLB.pau*3_3.04. These two QTL were also co-localized with AUDPC whereas SLB severity at 45 days a QTL was found between marker intervals of *MSSR10-MSSR9* and designated as *qSLB.pau*4_3.04 explaining phenotying variance of 11 per cent among the RILs. The major QTL which spanned region between *MSSR1* and *MSSR20* showed the maximum phenotypic variance of 15.1 per cent with peak

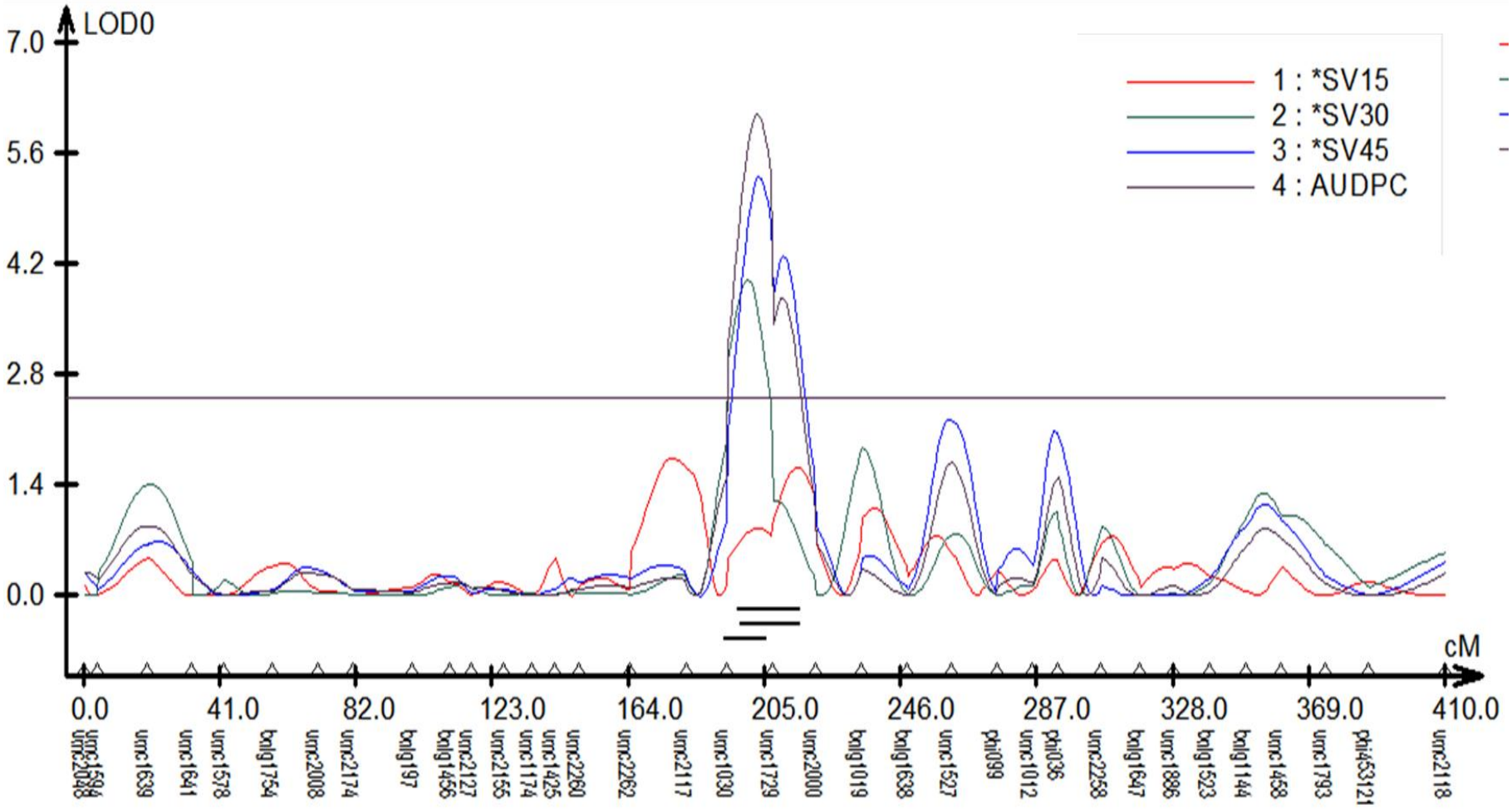

Fig. 4.9: QTL likelihood plot of chromosome 3 showing putative QTL for SLB resistance between flanking markers*umc2117-umc1729* and*umc1030* and *umc2000* using RILs derived from cross of CM139 (R) and CM140 (S) over the years (2015-2017)

marker as *MSSR9* (Fig. 4.10). All the QTL for severity at different ratings (after 15, 30 and 45 days of inoculation) were present together one by one with slight overlapping between them inferring 43.8 per cent of total phenotypic variance which is in agreement with earlier study that 46.3 per cent of phenotypic variance explained by Chauhan (2013) between the marker interval of *phi099-umc1729.*

Table 4.8 List of putative QTL associated with SLB resistance detected after fine mapping over the years (2015-2017)

Trait	Peak marker	Flanking Marker	Lod Score	Additive Effect	Phenotypic variance (%)
Severity 15 (*qSLB.15pau*_3.04)	*umc2117*	*umc2262-phi036*	2.50	6.00	10.30
Severity 30 (*qSLB.pau*2_3.04) (*qSLB.pau*3_3.04)	*umc1012*	*phi036-umc1030*	5.05	4.50	7.40
	MSSR9	*MSSR1-MSSR20*	6.97	6.60	15.10
Severity 45 (*qSLB.pau*4_3.04)	*MSSR1*	*MSSR10-MSSR9*	6.15	7.02	11.00
AUDPC (*qaudpc*15)	*umc1012*	*phi036- umc1030*	4.11	69.53	6.13
	MSSR9	*MSSR1-MSSR20*	6.40	106.90	13.93

It could be concluded that QTL window has been significantly reduced to an average distance of 1.2 cM from the earlier distance of 13.7cM. We had found that QTLs for SLB resistance were splittted into four QTLs within the same bin 3.04 which was earlier detected. As single QTL using $F_{2:3}$ generation (Chauhan 2013). Thus, fine mapping enabled us to detect QTL for each rating which together could be exploited both for juvenile and adult plant resistance. Hence it is justified that bin 3.04 is the hot spot region for SLB resistance. Further this region could be narrowed down by candidate gene mapping.

Zhao *et al* (2012) fine mapped the *rhm1* recessive disease resistance locus for SLB. It was narrow down using F_2 population and BC_1F_1 population derived from the cross between resistant (H95rhm) and susceptible parents (H95). *rhm1* was initially delimited within an interval of 2.5 Mb, and then finally mapped to a 8.56 Kb interval between InDel marker IDP961-503 and SSR marker A194149-1.

4.4 Identification of candidate genes

With the advancement of molecular markers technology and availability of high density linkage maps and whole genome sequence data for several crop species, QTL mapping and gene identification is now routinely followed to predict putative candidate genes

for a given trait. Candidate gene approach is becoming an extensive method for characterizing QTLs.

On the basis of physical position of *MSSR1* and *MSSR20*, the target QTL region was spanned between 269.3 kbp, according to annotation present on maizeGDB, maize genetics and genomics database. (https://www.maizegdb.org/data_ center/ssr?id=255977). In between these two SSRs, a total of 6 genes were predicted (Fig. 4.11). The potential identities of the predicted coding sequences were subsequently determined by using Blast2GO, mapman and pfam bioinformatics platform for high-quality functional annotation. The predicted genes with their putative functions were enlisted in the Table.4.8. Among these six predicted genes, two encode unknown protein, rest of the genes encode for functional protein. *Fasciclin-like arabinogalactan protein 7* is involved in the onset of secondary wall cellulose synthesis and affecting the integrity of cell wall matrix, thus participate in disease resistance (MacMillan*et al* 2010) whereas *serine carboxy peptidase K10B2.2* helps to regulates the intracellular turnover of proteins and required for acylation of antimicrobial terpenes, thus provide protection against soil borne pathogens (Mugford *et al* 2010). The other two genes *viz. GRMZM2GO88371* and *GRMZM5G862219* have their function role inreceptor mediated signal carriers and lipid metabolism & β oxidation respectively. Similar procedure was followed by Guan *et al* (2016) to narrow down the *ygl-1* (leaf colour gene) locus to a region of about 48 kb using 2930 and 2247 individuals of F_2 and F_3 mapping populations, respectively. Within this region three candidate genes were present.

Yang *et al* (2017) distinguished quantitative tait locus, *qMdr9.02*, related with protection from three significant foliar maize diseases-southern leaf blight, gray leaf spot and northern leaf blight.This QTL confers alleles for multiple disease resistance (MDR) that are valuable in crop improvement The putative candidate gene *ZmCCoAOMT2*, which encodes a *caffeoyl-CoA O-methyl transferase* associated with the phenyl propanoid pathway and lignin production, within $qMdr_{9.02}$ conferred quantitative resistance to both southern leaf blight and gray leaf spot. Chen *et al* (2018) using comparative genomics in *Arabidopsis* characterized abiotic stress-responsive *MYB* protein. They identified 46 *ZmMYB* genes which were involved in abiotic stress responses of *Zea mays*. An expression pattern analysis of the 46 *ZmMYB* genes under abiotic stress treatments was used to identify 22 *MYB* genes that were induced by one or more of the stress treatments. *ZmMYB30* was highly upregulated under the four stress treatments. The ectopic expression of *ZmMYB30* in transgenic *Arabidopsis* plants promoted salt-stress tolerance and also increased the expression of a number of abiotic stress-related genes, allowing the plants to overcome adverse conditions.

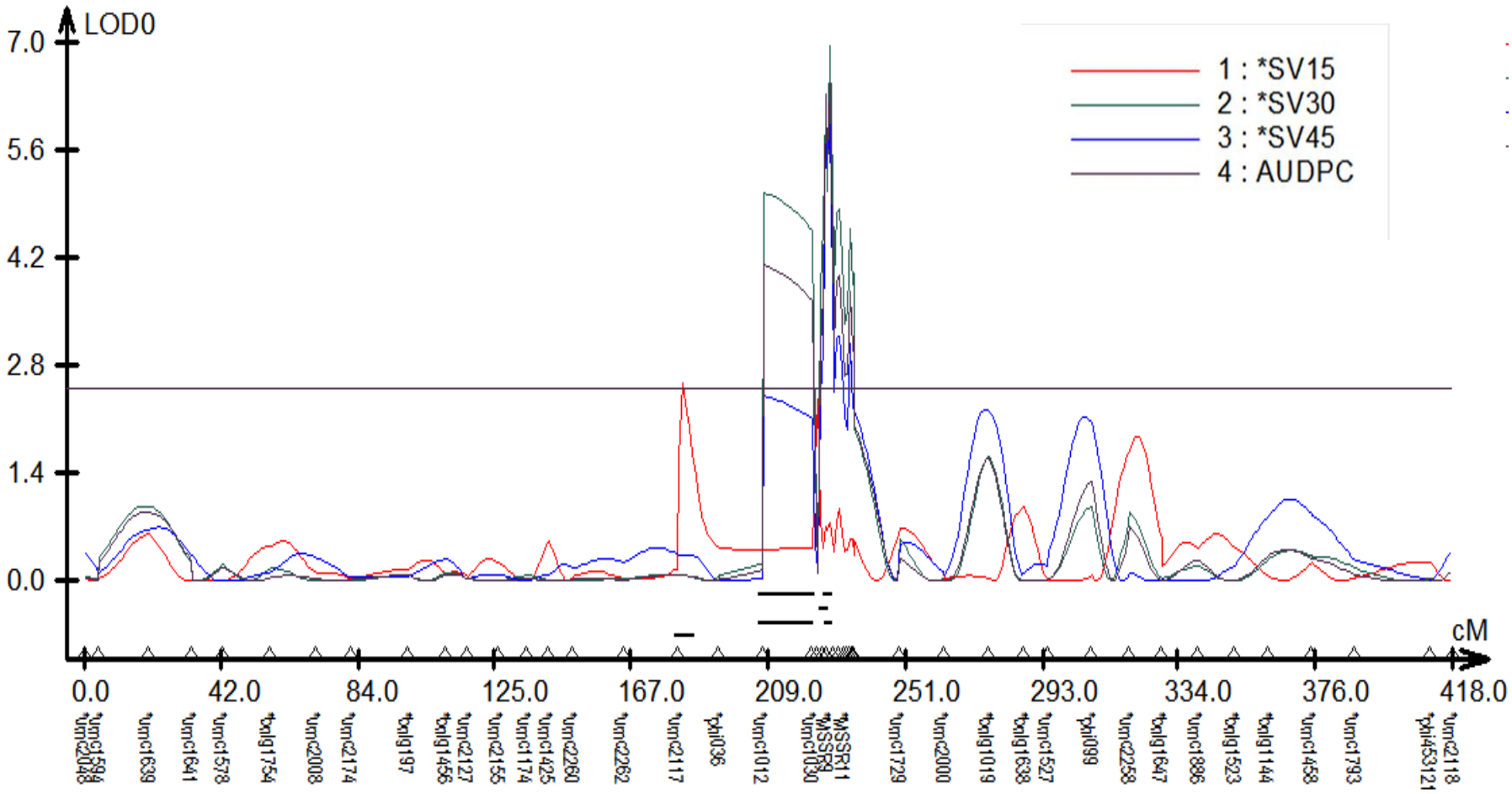

Fig. 4.10: QTL likelihood plot of fine mapped chromosome 3 showing putative QTL for SLB resistance between flanking markers *MSSR*1 and *MSSR*20 using RILs derived from cross of CM139 (R) and CM140 (S) over the years (2015-2017)

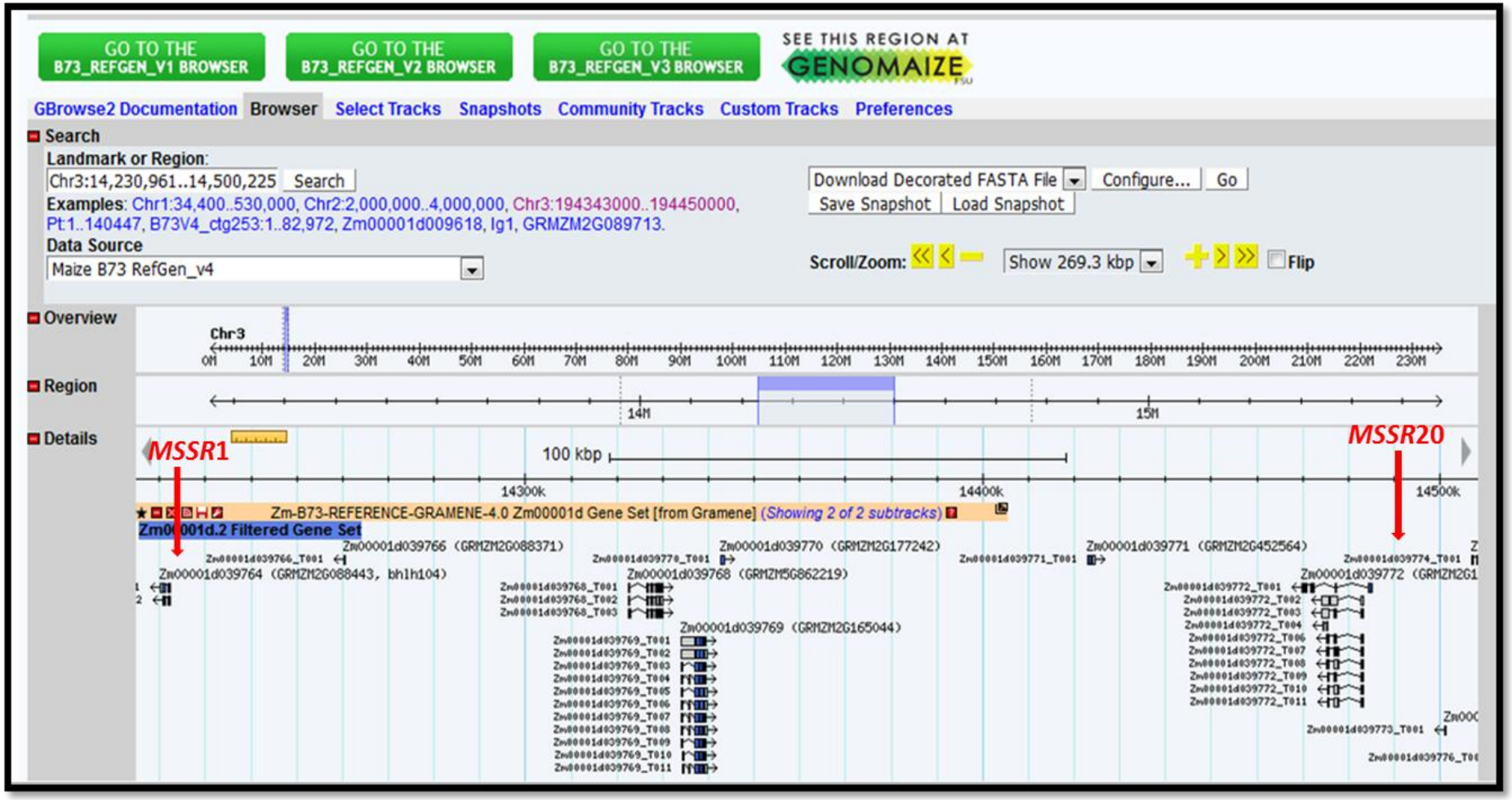

Fig. 4.11: The physical map of 269.7 Kbp genomic region spanning QTL *qSLB.pau_3.04* between flanking markers, *MSSR*1-*MSSR*20 and presence of potential candidate genes in it based on maize B73_REFGEN_V4

Table 4.9: List of candidate genes with their putative functions

S. No.	Gene id	Description	Functional role
1.	*Zm00001d039770 (GRMZM2G177242)*	*fasciclin-like arabinogalactan protein 7*	Involved in onset of secondary wall cellulose synthesis and affecting the integrity of cell wall matrix, thus participate in disease resistance (MacMillan*et al* 2010).
2.	*Zm00001d039771 (GRMZM2G452564)*	unidentified	-
3.	*Zm00001d039772 (GRMZM2G133718)*	*serine carboxypeptidase K10B2.2*	Regulates the intracellular turnover of protiens and required for acylation of antimicrobial terpenes, thus provide protection against soil borne pathogens (Mugford *et al* 2010).
4.	*Zm00001d039766 (GRMZM2G088371)*	*Rapid Alkalinization Factor (RALF)*	Play a critical role in the regulation of plant defense and development (Gupta *et al* 2010).
5.	*Zm00001d039768 (GRMZM5G862219)*	*Acyl CoA dehydrogenase 1 and lipid metabolism*	Involved in initiating β oxidation pathway which ultimately regulates jasmonic acid pathway and thus play role in plant defense (Graham and Eastmond 2002).
6.	*Zm00001d039769 (GRMZM2G165044)*	unidentified	-

4.5 qRT-PCR analysis

4.5.1 RNA isolation and cDNA synthesis

Total RNA was isolated from two parental lines (CM139 and CM140) with TRI-zol reagent (Life technologies) according to manufacturer's instructions at different time intervals of 12h, 24h, 48h and 72h from both control and diseased stressed plants in three biological replicates. Three distinct bands were visible on 1.2 per cent denaturing formaldehyde agarose gel. The first band represented 28S rRNA, second band represented 18S rRNA and third band represented 5S rRNA. Good quality of rRNA was obtained without degradation as no smear was observed and all the three bands were intact (Fig. 4.12)

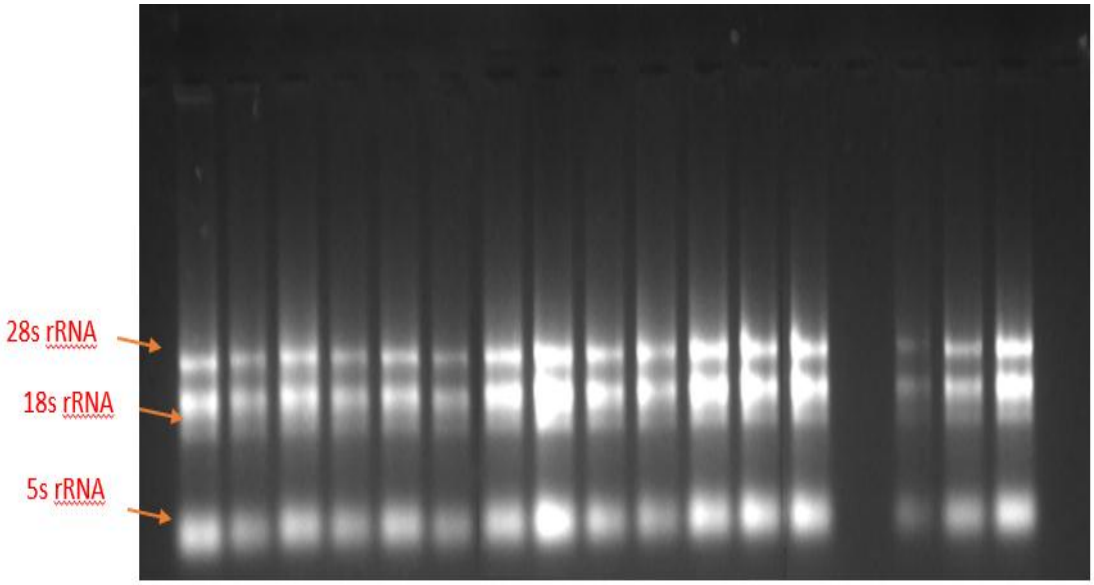

Fig. 4.12: RNA banding pattern on formaldehyde agrose gel

These reagents facilitated isolation of a variety of RNA species of large or small molecular size (Ahmann *et al* 2008). For example, RNA isolated from rat liver, showed discrete bands of high molecular weight RNA between 7 kb and 15 kb in size (composed of mRNA's and hnRNA's), two predominant ribosomal RNA bands at ~5 kb (28S), ~2 kb (18S), and low molecular weight RNA between 0.1 and 0.3 kb (tRNA, 5S).

The cDNA was synthesized with PrimeScript First Strand cDNA Synthesis kit **(**Takara Clontech) and the synthesis of cDNA was confirmed by amplifying it with 26S rRNA primers. The amplified cDNA product was resolved on 1.5 per cent agarose gel and an expected band of 534 bp was obtained (Fig. 4.13). Singh *et al* (2004) designed primers for 26S rRNA gene which was expected to yield a band of 534 bp and it was confirmed by sequencing. It was shown that the expression of 26S rRNA was independent of developmental stages and external stimuli in a range of plant species. Hence, it was proposed to be a suitable internal control in quantitative/semi quantitative expression studies in the plant system.

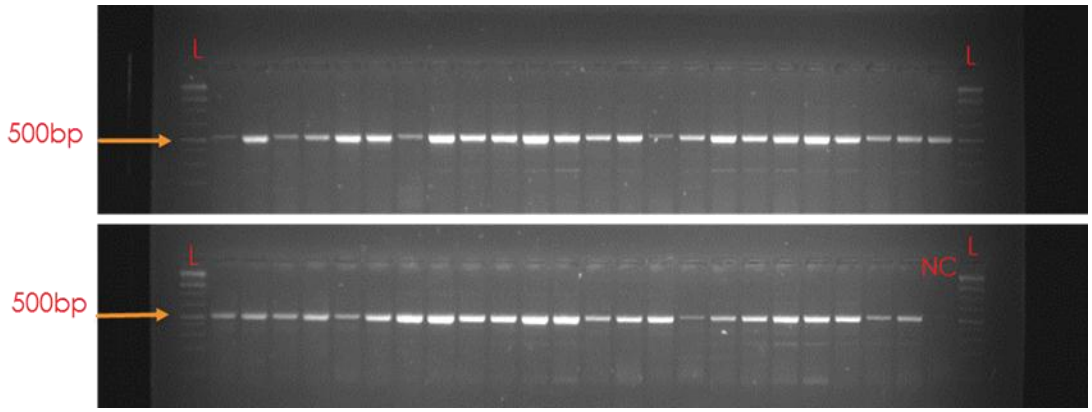

Fig 4.13 Amplification of cDNA with universal primer 26S rRNA L=1Kb DNA Ladder, NC= Negative Control

The cDNA of maize leaf samples at different time intervals was used to check the amplification of candidate gene specific primers with normal PCR. A total of 20 Real Time primers were designed using perl primer, two primers for each gene was designed and only single primers from*Zm00001d039766* and *Zm00001d0397719* genes (Appendix II). All real-time PCR primers must generate a single amplicon of the correct size on agarose gels. Alternatively, a default melting program could be run on real-time PCR at the end of the cycling program. The dissociation curves (first derivatives of the melting curves) should contain a single peak with no shoulders, and also the agarose gels of the amplified product should reveal single bands corresponding to the predicted amplicon length. Sharpe *et al* (2008) designed primers generating amplicons of 75-150 bp in lengthand validated for favorable melting temperatures to ensure a lack of intra-molecular folding. Each primer set was qualitatively examined and optimized with endpoint qRT- PCR. These optimal conditions were then utilized in q (RT)-PCR for each primer pair to verify the amplification of single amplicons via melt curves. The non-amplification at different loci might be due to the lack of primer binding of that primer at the given temperature (60°C). The optimization with the annealing temperature was not done because the recommended annealing temperature for internal control primers is 60°C and the qRT-PCR was performed using same temperature profiles with all primers, otherwise for each primer calibration at each step is required for qRT-PCR. Condori *et al* (2011) showed that most of the amplicons gave a single band of the expected size with selected reference genes. In present study for each genes, melting curve analysis was also performed and a single peak was observed. It clearly indicated that primers must be validated for their amplicon size and number of amplicons generated before proceeding for qRT-PCR.

4.5.2 Relative expression analysis of candidate genes

All four genes *GRMZM2G177242, GRMZM2G452564, GRMZM2G133718 and GRMZM2G165044* showed very litte to no fold change expression at 12h, 24h, 48h and 72h. So, these genes were not considered for any relative expression for resistance to SLB. Two of the genes, *GRMZM2GO88371* and *GRMZM5G862219* showed the relative expression in qRT-PCR. The *GRMZM5G862219* gene involved in lipid metabolism and β oxidation showed maximum expression at 48h and 72h in CM140 whereas at 24h in CM139 inbred (Fig. 4.14A). It indicates that expression of that particular gene in susceptible plants is expressed at lateral stage when fungal pathogen is at the end of their incubation period. By exploiting the lipid metabolism of the respective plant host, fungal pathogens facilitate their virulence and pathogenic development. Studies and evidences reveal that fungi mostly uses the endogeneous lipid metabolism enzymes and endogenous oxylipins to successfully colonize the host, reproduce and synthesize toxins. Lipids play important functions in

membrane trafficking, exo and endocytosis, signal transduction photosynthesis, cytoskeletal rearrangements, in eukaryotes (Wang 2004; Funk 2001; Shea and Del Poeta, 2006). A distinct group of lipids, polyunsaturated fatty acids (PUFA), are enzymatically or non-enzymatically oxygenated to produce oxylipins. These oxylipins have their diverse signaling properties in plants from which jasmonic acid (JA) plays essential roles in defense responses against pathogens (Kachroo and Kachroo 2009; Shea and Del Poeta 2006). For pathogenic fungi the main strategy may be involved is to secrete lipase and lipoxygenase (LOX) inside the host cells which modulate host lipid metabolism by cleaving off the free fatty acids and oxidizing them to produce oxylipins. This possibility has been clearly discussed in case of *F. graminearum* of maize (Voigt *et al* 2005), that depicting of an extracellular lipase *FGL1* resulted in complete inability of these pathogens to colonize in maize ears. This suggests that lipid metabolizing enzyme are involved in the processes of pathogenesis. Thus in a nut shell we can infer that during pathogen attack polyunsaturated fatty acids get oxygenised to produce the oxylipins which directly hinders the growth of the pathogens in plants.

Catalyzes of the first step of peroxizomal fatty acid and betaoxidation during early and post-germinative growth in oilseed species is carried by the *Acyl CoA oxidase 4* which encodes a short chain of *acyl CoA*. Many studies showed that β -oxidation in plants with germinating seeds, mainly have their role in the breakdown of the storage lipids. (Graham and Eastmond 2002). However beside from the role in breakdown of lipids many of the increasing evidences have revealed and indicated that plants tissues which are non fatty in nature also depend on β-oxidation for several other processes, including the synthesis of indole-acetic acid and JA (Graham and Eastmond 2002; Zolman and Bartel 2004). JAs are synthesized by the so called octadecanoid pathway that involves enzymes located in two different subcellular compartments (Vick and Zimmerman 1984; Schaller 2001; Wasternack and Hause 2002). In maize, JA-deficient *opr7opr8* mutants are not capable of surviving under field conditions due to “damping-off” disease (Yan *et al* 2012). In that study, the major pathogen responsible for “damping-off” in the diseased roots of *opr7opr8* was identified to be *Pythium aristosporum*.

Supporting the genetic evidence that maize requires JA for immunity against *P. aristosporum*, exogenous application of JA as a soil drench could rescue normal survival of the plants. Another study with *opr7opr8* double mutants recommended that the use of sterile soil to exclude the plants from soil-borne pathogens is required. JA is also required for immunity against another common opportunistic soil-borne fungus, *F. verticillioides* (Christensen *et al* 2014).

It was very interesting to note that the gene *GRMZM2GO88371* showed maximum fold change expression at 12h and 24h in resistant line CM139 indicating induction of genes

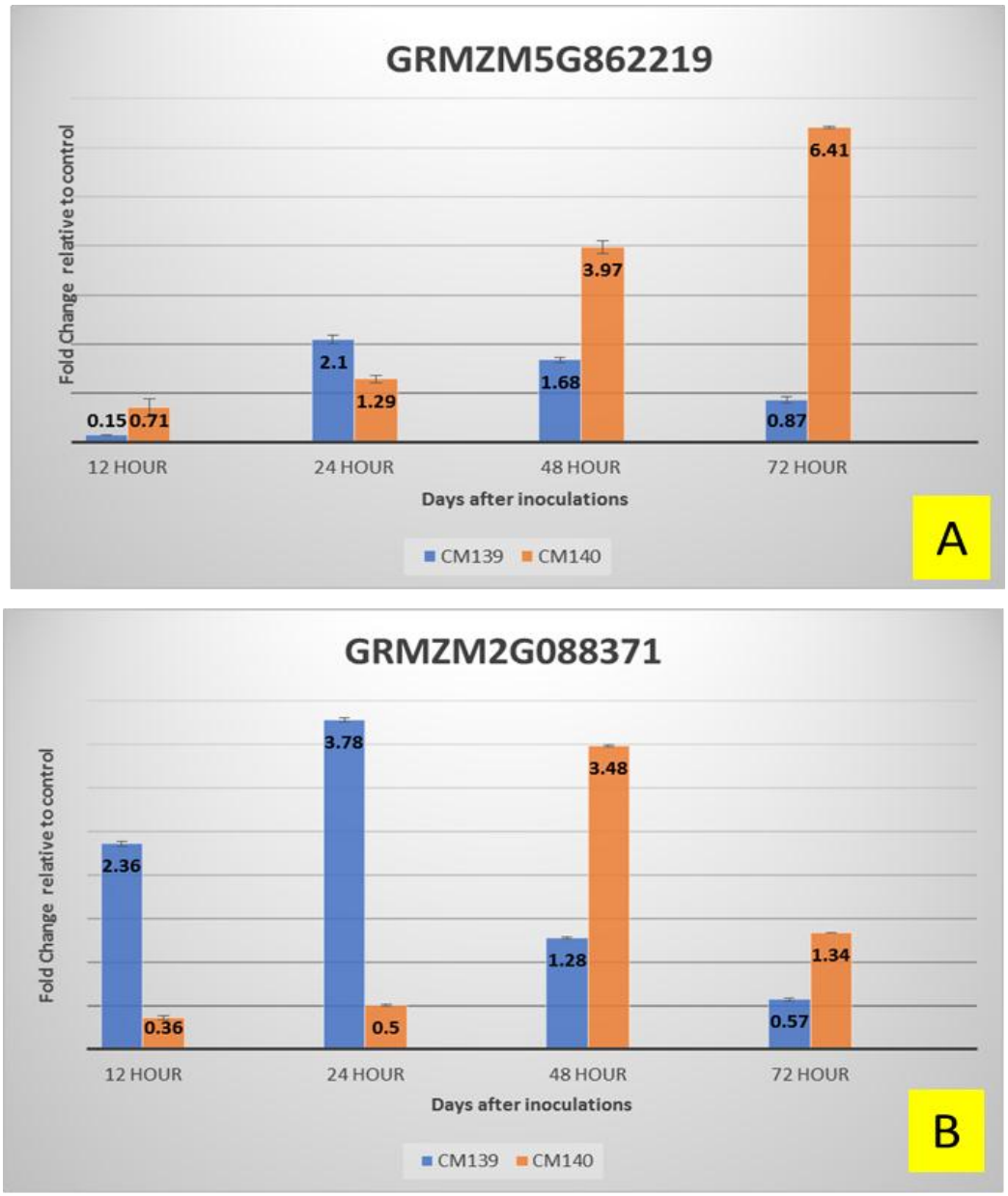

Fig. 4.14: Fold change in expression level of candidate genes *GRMZM5G862219* (A) and *GRMZM2G088371* (B) in CM139 (R) and CM140 (S) inbred lines at different time intervals

at early stages in resistant lines as compared to the susceptible line CM140 which showed maximum expression at 48h (Fig. 4.14B). This gene synthesize a protein (RALF) which function as receptor mediated signal carriers and may involved in the regulation of plant defense and development. *Rapid alkalization factor* (*RALF*) proteins potentially comprise important signaling components which may have a key role in plant biology. The *RALF* gene family contains large number of genes in several plant species, however, only a few *RALF* genes have been characterized to date. Gupta *et al* (2010) through their experiments involving chickpea (*Cicer arietinum*) showed that an attack by the plant pathogenic fungus *Fusarium oxysporum* f. sp. *Ciceri* (Race 1) induced the expression of *RALF* in resistant plants, while *RALF* expression was much lower in susceptible chickpea cultivars. Using the chickpea-*F. Oxysporumplant-pathogen* system, the same group also showed that *RALF* are produced in response to wounding and stress and proposed that a *RALF* induces increase in pH which is detrimental to the fungus. They also suggested that *RALF* peptides may serve as decoy or guardee to plant R proteins thereby reducing the inoculum titer and avoiding a direct interaction between pathogen effector molecules and the plant R protein (Gupta *et al* 2010). A comprehensive and detailed role for *RALF* in cell expansion was established by Haruta *et al* (2014). They showed that *RALF* suppress the elongation of the *Arabidopsis* root primary cells by activating *FERONIA* receptors causing phosphorylation of cell membrane H+ ATP2 at Ser899. *FERONIA* are shown to be receptors of secreted *RALF* peptides through direct binding experiments. The function of *FER* is not only restricted to plant cell growth regulation, but is also important for plant responses to pathogen invasion. The fer mutants have been shown to be less susceptible to powdery mildew *Golovinomyces* (syn. *Erysiphe*) *orontii* and *Pseudomonas syringae*pv. Tomato DC3000, although the mechanism is unknown (Keinath *et al* 2010; Kessler *et al* 2010). Interestingly, a recent study uncovered a surprising connection between pathogenic microbes and their host plantsdeciphering that the pathogenic fungus *Fusarium oxysporum* can secrete *RALF1*-like peptides that can recruit *FER* to switch on the downstream signaling events in their host plants (Masachis *et al* 2016; Thynne *et al* 2016).

4.6 Transfer of SLB resistance QTL through MAS

4.6.1 Evaluation of BC_1F_1 plants for SLB resistance

4.6.2 Foreground selection in BC_1F_1 generation

Backcrossing is the most frequently used breeding approach for incorporation of one or more desirable genes into which is an agronomically good, but lacks one or more important characteristics, into recurring parent (Singh *et al* 2015). The enormous prospects of DNA marker technology has made various research groups and institutes involved in crop

improvement to shift their focus on developing of these markers and their further employment for marker aided selection in plant breeding (Collard *et al* 2005). Disposition of genetic markers in plant species, hastened mapping and tagging of agriculturally significant genes (Babu *et al* 2004). Associations between markers and traits were first reported in maize by Stuber and Moll 1972 using isozymes. Effectiveness of the selection was significantly increased with the use of DNA markers. The molecular markers are now routinely employed marker aided selection breeding programs. Tanksley (1989) suggested indirect selection of desired QTL/gene based on associated marker genotype. This approach was referred to foreground selection. It is used to select traits in the seedling stage, which allows identification of the best plants for backcrossing. The number of individuals that needed to be maintained can be reduced by eliminating progeny that do not carry the desirable allele at the seedling stage. It helps in saving space, time, labour and other resources. Melchinger (1990) effectively exploited marker assisted foreground selection for introgression of disease resistance genes. MAS is quite useful in enhancing the efficiency of selection of target gene and background recovery of the recurrent parent (Bouchez *et al* 2002). Through marker aided foreground selection we can also screen population for traits that are recessive in nature, which is difficult to identify using conventional breeding approach (Akhtar *et al* 2010).

In the present study the SLB QTL '*qSLB.pau3_3.04'* was transferred into the background of CM140 using marker assisted selection. A total of 420 BC_1F_1 plants were raised by backcrossing F_1 with CM140 during *kharif* 2018. Flanking maker *MSSR1-MSSR20* and the peak marker *MSSR9* enabled the direct selection of resistant plants for southern leaf blight. A total 420 BC_1F_1 plants were analysed and 198 plants were found to be positive for all the three markers. A total of 64 BC_1F_1 plants, having both the alleles, were finally selected based on phenotypic data.

. Similar procedure was followed by Muthusamy *et al* (2014).Seven different crosses between VQL1 and HP 465-43, V-335 and HP 465-35, HKI 1105 and HP 467-6, HKI 323 and HP 467-4, and HKI 161 and HP 467-13 were attempted to generate backcross population. Selection for target allele was done in BC_1F_1, BC_2F_1 populations using a gene specific marker for *crtRB1*allele. The positive plants were again subjected with *phi057* marker for selection for *opaque2* gene. Background selections of the selected heterozygote plants were done using SSRs in BC_1F_1 and BC_2F_1 progenies.

Liu *et al* (2015) subjected two different population *viz.* CML-161 (line1) and CML-171 (line2) for selection of favorable allele *crtRB1-5'TE-2* and *crtRB1-3'TE-1*. 290 plants from line1 and 218 plants from line2 carried desirable allele out of 597 and 462 plants respectively. In the BC_2F_1 generation, 779 plants of CML161 and 1055 plant of the CML-171

were subjected to foreground selection for the two target favourable alleles. A total of 410 individuals had the favourable alleles in the CML -161 population and 497 individuals had the favorable alleles in CML-171 population. Chi square tests showed that segregation ratios for the favourable allele combination in CML-161 and CML-171 population were in a good fit to the Mendelian 1:1 ratio as expected for one gene-pair inheritance (P>0.05). Similar results were observed in our study for each marker segregating into Mendelian 1:1 raio.

4.6.3 Recombinant selection

Selecting individuals homozygous for recurrent parent allele at marker flanking the target allele is referred to as recombinant selection. The purpose of recombinant selection is to reduce the size of the donor chromosome segment containing the target locus (i.e. size of the introgression). This is important because the rate of decrease of this donor fragment is slower than for unlinked regions and many undesirable genes that negatively affect crop performance may be linked to the target gene from the donor parent—this is referred to as 'linkage drag' (Hospital 2005). Flanking markers are used to select rare individuals that are the result of recombination near the target gene, thus minimizing the effects of linkage drag (Ibitoye *et al* 2010). Hospital (2005) computed the mean and variance of the length of the intact donor segment around the target gene, when background selection is applied on two markers flanking the gene, one on each side in any BC generation. It was concluded that selection for distant markers over several successive backcross generations cannot provide a better reduction of linkage drag than using close markers. Using very close markers is the only way to reduce linkage drag substantially. Babu *et al* (2005) analyzed two flanking markers *bnlg*1200 which lies 3.8cM from gene and *bnlg* 2160 which lies at 4.4 cM distance from the gene for selection of either single or double recombinants

The flanking markers on either side of SLB QTL linked markers were analysed for the selection of single or double recombinants. The markers were selected on the basis of map generated in RIL population. A total of 64 BC_1F_1 plants heterozygous for both the QTL linked markers were surveyed with the SSR markers *viz.umc1012, umc1030, MSSR10, MSSR18, MSSR11, MSSR4, MSSR12, MSSR6, MSSR8, MSSR2, MSSR3, umc1729, umc2000* (Fig.-4.15, Fig.-4.16 and Fig.-4.17) These plants with maximum background recovery for the carrier chromosome were seventeen, these plants were selected for further backcrossing to generate BC_2F_1 population (Table 4.9). The background recovery with respect to carrier chromosome varied from 46.7 per cent to 90 per cent (Appendix III). Out of 17 BC_1F_1 plants 15 plants were single recombinants on either side of the SLB QTL linked markers, whereas two of the plants were double recombinants with respect to immediate markers.

Table 4.10: Percentage background recovery for the carrier chromosome 3 in BC_1F_1 plants derived from the cross of CM139 x CM140

Plant No.	A%	H%
43	82.8	17.2
152	76.2	23.8
208	75.4	24.6
213	75.8	24.2
227	85.8	14.2
233	86.6	13.4
234	85.5	14.3
235	84.3	15.7
237	82.7	14.5
241	77.8	22.2
249	75.4	24.6
304	84.3	15.7
357	87.6	12.4
367	90.6	9.4
385	86.4	13.6
393	87.6	12.4
416	87.6	12.4

Septiningsih *et al* (2009) followed marker assisted backcrossing strategy to transfer the tolerant *Sub1* allele into the mega varieties. Two breeding lines IR49830 and IR40931 were used as donors. The mega varieties Samba Mahsuri and CR1009 from India, IR64 from the Philippines (IRRI), Thadokkham 1 (TDK1) from Laos, and BR11 from Bangladesh were used as recipient parents. Population sizes ranged from 343 to 697 plants for the BC_1 generation and 69 to 320 plants at the BC_2F_1 generation. The fully converted *Sub1* lines were selected at the BC_2F_2 or BC_3F_2 generation. Recombinants were initially identified using two markers tightly flanking the *Sub1* region. Populations of 2240 BC_1F_2 plants for IR64/IR40931, and 2137 BC_2F_2 and 279 BC_3F_1 plants for BR11/IR40931 were screened with flanking markers to identify recombination events between *Sub1A* and *Sub1C*. One plant with the desired genotype was successfully selected in the BC_2F_2 generation. The tolerant version of IR64 was developed following a similar approach. Among the 440 BC_1F_1 and 188 BC_2F_1 plants that were genotyped, single plant was selected. Out of 162 BC_2F_2 progeny from the selected plant, single plant was selected based on its possession of the *Sub1* locus and

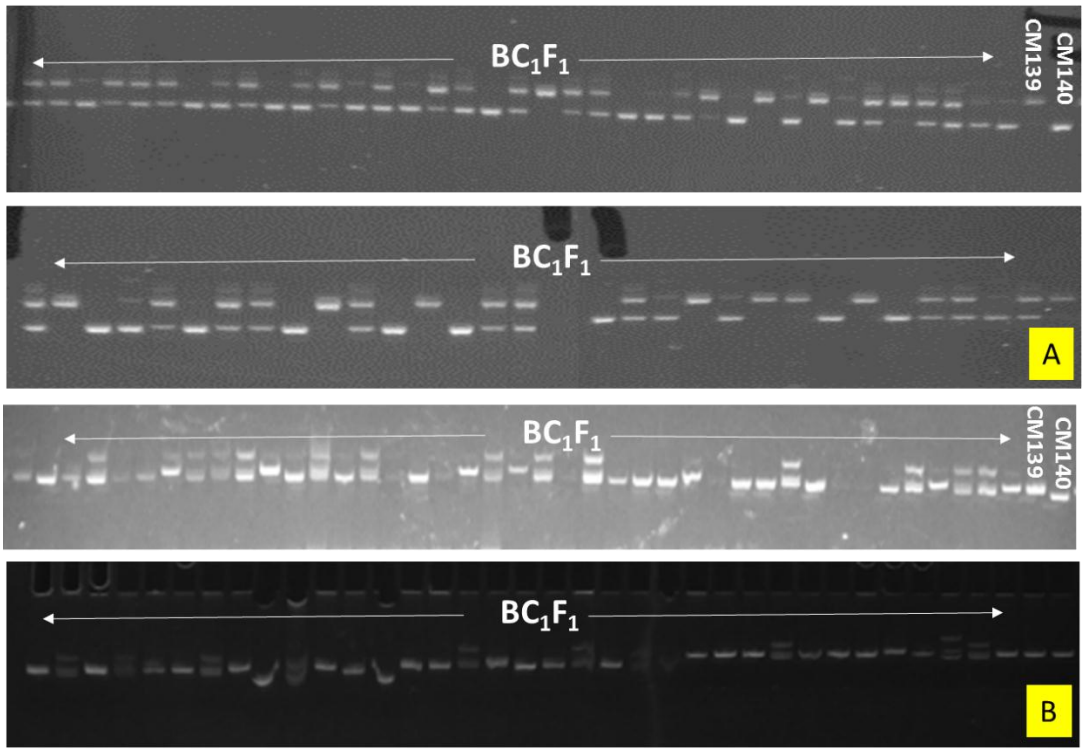

Fig. 4.15: Genotyping of BC_1F_1 derived from the cross of CM139 (R) and CM140 (S) with SSR markers viz. *umc1030* (A) and *MSSR18* (B) resolved on polyacrylamide gel

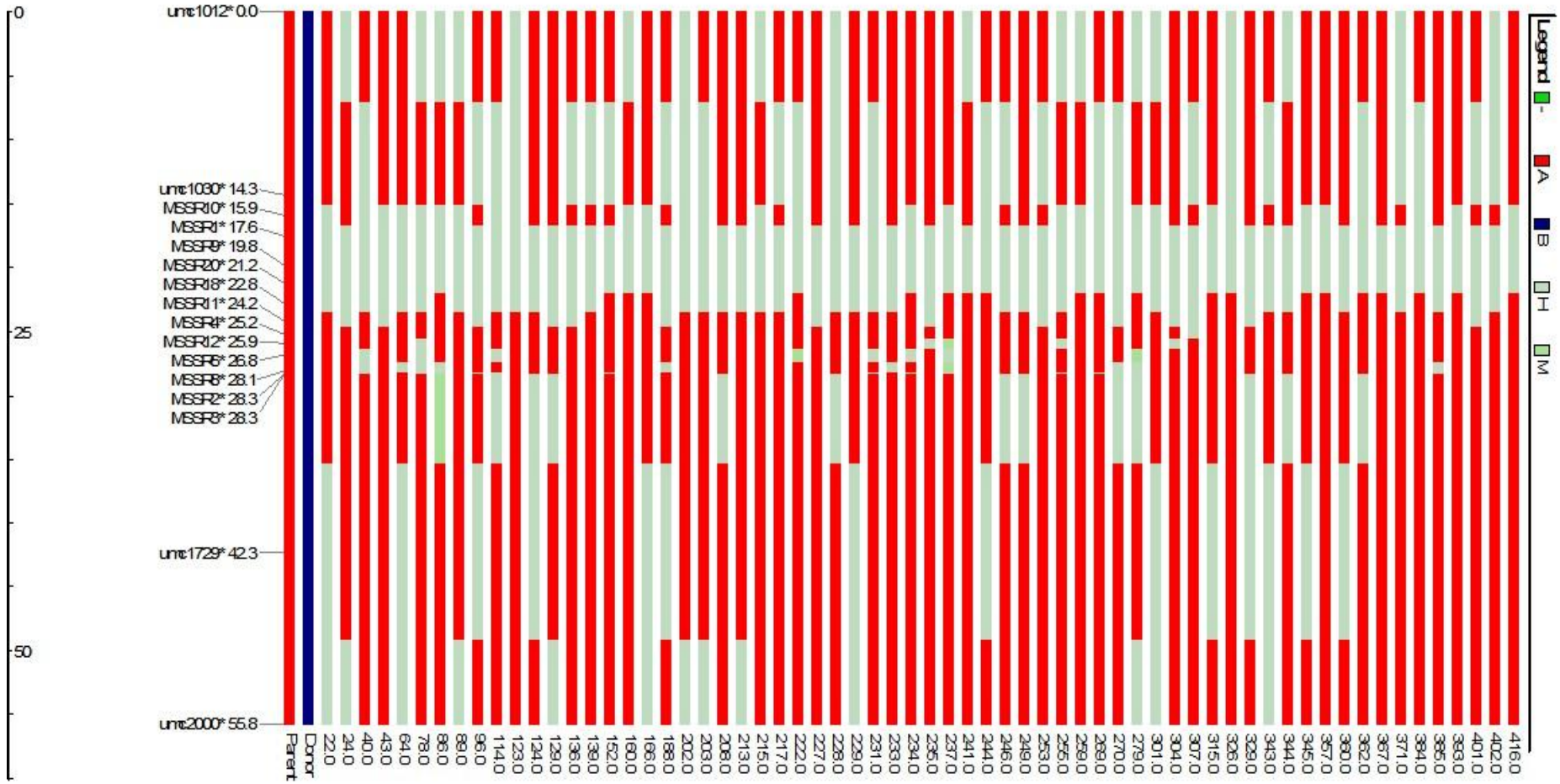

Fig. 4.16: Graphical representation of sixty-four BC_1F_1 plants (positive for QTL linked markers) showing recurrent parent genome recovery for the carrier chromosome 3

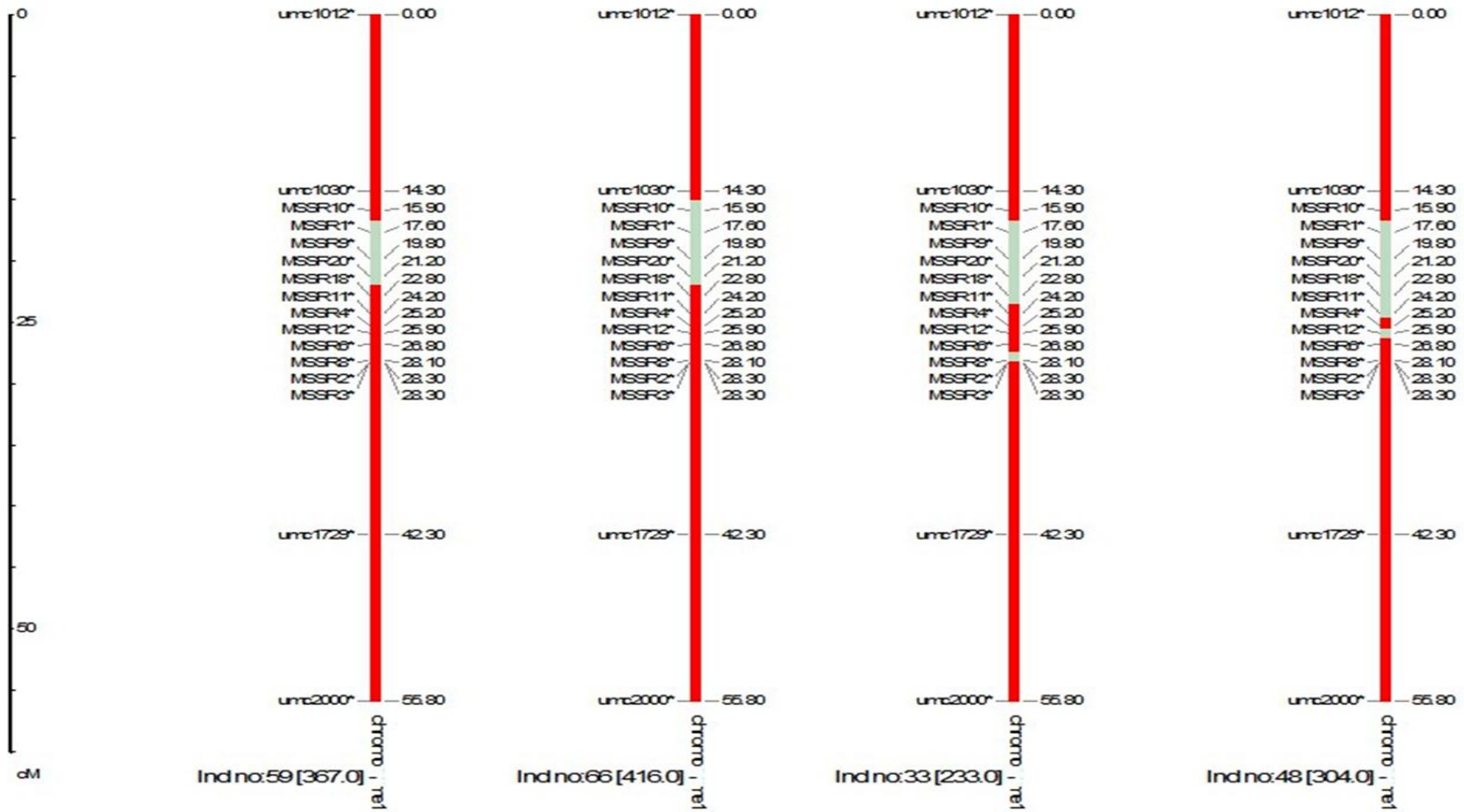

Fig. 4.17: Percentage background recovery for the carrier chromosome 3 in selected BC_1F_1 plants derived from the cross CM139 X CM140

maximum recipient genome. Screening of larger BC_3F_1 and BC_3F_2 populations subsequently facilitated the identification of several BC_3F_1 plants with a small *Sub1* introgression. One of the selected BC_3F_1 plant was self-pollinated and among the 190 BC_3F_2 progenies, single plant was identified with the desired small *Sub1* introgression. This shows how the markers are efficient in breeding strategy for reduction of linkage drag and selection of the appropriate genotype.

In the present study we used backcrosses for introgression of SLB QTL in CM140 maize inbred line. We selected 13 markers covering bin location 3.04 for carrier chromosome recovery. According to Visscher *et al* (1996) with the increment in quality of markers from 3 to 11 gives substantial increase in rate or recurrent parent genome recovery. Hossain *et al* (2018) introgressed recessive *opaque2* (*o*2) allele that enhances endosperm lysine and tryptophan using marker-assisted backcross breeding into three normal inbred lines (*HKI323, HKI1105* and *HKI1128*). Background selection was done using genome-based SSRs helped in recovering of more than >96% of recurrent parent genome. The newly developed quality protein maize (QPM) inbreds showed modified kernels (25-50% opaqueness) coupled with high degree of phenotypic resemblance to the respective recipient lines, including grain yield. Muthusamy *et al* (2014) also used 200 genome wide SSR markers for the background selection. The recovery ranged from 70.3% to 88.4% in BC_1F_1 population. The per cent recovery varied from 86.4 to 93.7 per cent in BC_2F_1 generation.

In conclusion, the main accomplishments of this thesis are that we identified a series of polymorphic markers, and fine mapped the SLB resistance locus to a 269.3 kbp region on the short arm of maize chromosome 3 using RILs. Based on our fine mapping results, we propose that the *Zm00001d039768* and *Zm00001d039766*genes are the potential candidate genes for SLB resistance. The SLB QTL linked markers identified in this study could directly be employed in maize breeding programs.

4.7 Implication for pyramiding of SLB resistance QTL

The prerequisite for pyramiding of two or more genes in a single inbred line for making the lines durable to resistance is the identification of a tightly linked DNA marker. In our study we have identified two SSR markers (*MSSR1*and *MSSR20*) tightly linked to the QTL for southern leaf blight with *MSSR9* as a QTL peak marker and also demonstrated the utility of these markers using BC_1F_1 progeny to incorporate this QTL into inbred line as practice for marker assisted selection (MAS). This QTL could be integrated with another SLB resistance QTL identified from different RIL population (LM5 x CM140) on chromosome 8 and 9 (Kaur *et al* 2010). The identified SLB resistance QTL could also be mobilized into the background of sweet corn, QPM lines as the hybrids of speciality corn are hightly susceptible to southern leaf blight in Northern India. As compared with improving the materials gene-by-gene, pyramiding through MAS using any lines either from cross or a backcross scheme can

be used to improve other maize materials in a cost effective and in shorter time period. Thus gene pyramiding could be successfully applied in maize breeding program for developing new cultivars or inbred lines with favorable genes for enhanced resistance to southern leaf blight from different source lines (CM139 and LM5). At SAB, we have initiated pyramiding of these QTLs in CM140 background.

CHAPTER V

SUMMARY

Maize (Zea *mays* L.) is the third most important cereal crop in Asia. It is a major cereal crop for both livestock feed and human nutrition, worldwide. Biotic stresses have emerged as the major cause of yield instability across diverse crop growing regions of maize. Among biotic stresses, important foliar diseases that have an impact on maize production are southern leaf blight (SLB), gray leaf spot (GLS) and northern leaf blight (NLB). SLB caused by *Cochliobolus heterostrophus* (Drechs.) Drechs. (anamorph = *Bipolaris maydis* (Nisikado) Shoemaker; synonym = *Helminthosporium maydis* Nisikado), is a serious foliar disease of maize throughout the world where maize is grown under warm and humid with the potential to cause 30-40% yield losses. Integrated SLB management strategies include crop rotation, fungicidal seed treatments, tillage, cultural practices and foliar spray that results in disease reduction. But due to the potential harmful effects of fungicide residues on human health and the environment, the inbuilt host resistance is the most cost-effective, eco-friendly and practical criteria for management of this disease. Disease resistance in maize is complex and most identified genetic resistance to SLB is quantitative and can be additive or recessive in effect. Therefore, quantitative trait loci (QTL) mapping and marker assisted selection is a highly effective approach for studying genetically complex forms of plant disease resistance and can provide useful information for breeding programs, since it allows the estimation of genomic locations and genetic effects of chromosomal regions related to the expression of quantitative traits.

The present investigation was undertaken using 298 RILs over the years 2015-2017 from the cross between CM139 as the resistant (female) parent and CM140 as the susceptible (male) parent. The mean disease severity of RIL population after 15, 30 and 45 days of inoculations was 27.81, 49.52 and 66.56, whereas CM139 had 2.86, 27.23 and 33.9 and CM140 had 9.03, 63.4 and 89.16, respectively over the years 2015-2017. The area under disease progress curve (AUDPC) ranged from 535.34-1478, 570.0-1076.82, and 700.0-1222.8 in consecutive years 2015, 2016 and 2017 respectively, among the RILs. The AUDPC for CM139 was 453.6, 536.15 and 476.05 whereas for CM140 was 1201.95, 1248.65 and 1199.45 for the years 2015, 2016 and 2017 respectively. SLB severity data showed a normal distribution after 30 days of inoculations demonstrating the characteristics of a quantitative trait but was little skewed towards susceptibility after 45 days of inoculations. Environment, genotype, and the genotype-environment interactions were significant sources of variation in the combined analysis, while the effects of blocks within replication and replications were non-significant. Significant positive correlation was observed both between AUDPC & severity 45 (0.977) and AUDPC & severity 30 (0.976).

A total of 35 polymorphic SSR markers were genotyped on 298 RILs. The linkage map generated for chromosome 3 had genomic coverage of 410.1 cM. The QTL analysis depicted two QTL positions on chromosome 3 with flanking markers *umc2117-umc1729* and *umc1030-umc2000* explaining maximum phenotypic variance of 12.30 and 11.04 per cent with LOD score 6.9 and 5.3 respectively. The flanking markers *umc2117-umc1729* spanning 13.7 cM region on reference genome B73RefGen_v4 had physical distance of 395.7 kbp which was reduced from 768 kbp.

Further the fine mapping of putative QTL in bin 3.04 was done by designing new SSR markers in between the region spanning the QTL between two flanking markers *umc2117- umc1729* through MISA software. The genotyping data generated from newly designed 12 polymorphic SSR markers along with 35 SSR markers was analysed and saturated linkage map was constructed with genomic coverage of 417.9 cM. The QTL analysis fine mapped the *qSLB.pau_3.04* between the flanking markers *MSSR1* and *MSSR20* at LOD score of 6.97 with phenotypic variance of 15.1 per cent. The QTL window has been significantly reduced to an average distance of 1.2 cM from the earlier distance of 13.7 cM. On the basis of physical position of *MSSR*1 and *MSSR*20, the QTL region spanned between 269.3 Kbp. A total of 6 candidate genes were predicted between these two SSRs.

The expression analysis of these candidates genes were studied between two CM139 and CM140 at different points of infection with the *Dm1* isolate along with non-infected (control) plants. Two of the candidate genes *viz.GRMZM2GO88371* and *GRMZM5G862219* showed 3.48 and 6.40 fold higher expression in CM140 at 48h and 72h respectively. *GRMZM2GO88371* and *GRMZM5G862219* have their biological function in receptor binding signaling lipid metabolism & β oxidation respectively. Both of the genes are involved in the defense pathway and may be one of the potential candidate genes conferring resistance against *C. heterostrophus.*

The linked QTL markers were employed in marker assisted selection for transfer of *qSLB.pau_3.04* in the background of CM140 inbred. A total of 420 BC_1F_1 population was developed from the cross of CM139 x CM140. Foreground selection was done using flanking maker *MSSR1* and *MSSR20* and the peak marker *MSSR9.* A total of 64 plants heterozygous for the three markers were identified. A total of 13 markers on either side of SLB QTL linked markers were analysed for the selection of single or double recombinants. The background recovery for the carrier chromosome was ranged between 46.7 to 90.6 per cent. The plants having maximum recurrent recovery between 85 to 90 per cent were selected for further backcrossing to generate BC_2F_1 population. The markers identified in this study could be successfully used in the marker assisted maize breeding programs.

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APPENDIX I

List of newly designed SSR markers

Oligo Name	Forward sequence(5'-3')	Reverse sequence(5'-3')
M_SSR-1_F	CGCCAAACTGAAGCTGACAG	AGCTAACACGTCCAGAAGGC
M_SSR-2_F	CCCTCTCTCAAACGGTCACC	CAAAGCCTCTCCAAGTCCCA
M_SSR-3_F	ACCACGGCTTCCCCTAGTTA	AGGTGTTGTGACCATGGCTT
M_SSR-4_F	AATCTGCCTGAGCCAGAACC	TGCCTCGCATATCTGAGGTG
M_SSR-5_F	CGCAGCCAATCTTCAGCATC	GCCATCGGTCAGACTCTAGC
M_SSR-6_F	ACGGAAACCTAGCCCCTTTT	GGTTCAACCGCCCCTTTT
M_SSR-7_F	AAATATCTGGCTCGGCTCGG	AAGTTAGAAGTTGGGACGATGA
M_SSR-8_F	TACCAGAGAGCCGCTACCTT	TGGCATTCCCTCGAACAGTC
M_SSR-9_F	GACTAGCAGGGTCCCAACAG	CATTGCAGCGCCAGATGATC
M_SSR-10_F	CCCGACAGGAGGACTACAGA	TATTTACACGGCTGCCCTGG
M_SSR-11_F	GCTGTGACGTGATAACCTGTG	GGCTACGGTACACCATGTGAG
M_SSR-12_F	GTAGTCGTTGTCGTCGTTGTC	AGGAATCTTTCGTCAATGAGC
M_SSR-13_F	ACTTCCACTTGTTGAACTTGC	GGTTTGTTATCCTGGCTGCG
M_SSR-14_F	GAAACGGGAAACTACTCCAC	ACTACTGATGTGCGTCTCTG
M_SSR-15_F	CAATCATCTGCATCTAGTGGA	GAGATACGCCAAGAAAGGAGG
M_SSR-16_F	GACCTCGCCATGAAGATTGC	CCTTGCTCATCACCTTTGAC
M_SSR-17_F	ATTGGAGACCCTACAAATACG	AATGTTGTAAACTCACCTGCG
M_SSR-18_F	CTTCGGACTCCTCCCAAAGG	GCAATCAATAGCCTACCGTTTCTG
M_SSR-19_F	CATCTGGACCCTCTCCTTGC	AAACCCGGCTTGTAGTTGTG
M_SSR-20_F	GCGTAGATATACCTGATTGGG	ATATAGATGGACGGTGTAGATTGG
M_SSR-21_F	CATAGCCTCCGCCTGTAGTG	TAACCAGTAGAACATCATGCTCAG
M_SSR-22_F	TTATCTCTTGATCAGTGGTGTAGC	CTATGGGTTGGTTTGAGTTGC

APPENDIX II

List of Real Time polymerase chain reaction primers

Oligo Name	Forward sequence(5'-3')	Reverse sequence(5'-3')
RT_39772.1	GCTCTCCTTGAATCCCTACG	ACCAAAGTTACCTGTCTTGTTGTC
RT_39772.2	ATCTACCGTGAACTCATCTCG	CATCCACCAACCTCTTCGTC
RT_39768.1	CGCTTCGGATTATTACCAGTC	CACCCATAACCCTTTATTGTACCA
RT_39768.2	ACAATAAAGGGTTATGGGTGC	TCACTTCCATAGTTTGGCTCTG
RT_39769.1	TTTGCCTGGAGGACTACAGAG	ATCTATTTACACGGCTGCCCT
RT_39769.2	CTCTACCATCAGGTGCACCA	CGCATGGATGTAGATGTTGCT
RT_39770.1	GAGTTCCCACTCAACGTCAC	CCTTGTTGACCTGGTAGACG
RT_39770.2	CAGTTCAACACCTTCATCCG	GTGACGTTGAGTGGGAACTC
RT_397719	TTCTGACCATTTGTTCCTCTTGC	GTCCAACATCTATTTCATTGTCGC
RT_39766	TGGTTCTGTGCCTCCTGTTAGC	TGTAGTCGTTGGCGGGTTTCC
RT_39772.1	GCTCTCCTTGAATCCCTACG	ACCAAAGTTACCTGTCTTGTTGTC
RT_39772.2	ATCTACCGTGAACTCATCTCG	CATCCACCAACCTCTTCGTC
RT_39768.1	CGCTTCGGATTATTACCAGTC	CACCCATAACCCTTTATTGTACCA
RT_39768.2	ACAATAAAGGGTTATGGGTGC	TCACTTCCATAGTTTGGCTCTG
RT_39769.1	TTTGCCTGGAGGACTACAGAG	ATCTATTTACACGGCTGCCCT

APPENDIX III

Percentage background recovery for the carrier chromosome 3 in BC_1F_1 plants derived from the cross of CM139 x CM140

Plant No.	A%	H%
22	48.2	51.8
24	60.8	39.2
40	67.2	32.8
43	82.8	17.2
64	46.9	53.1
78	67.2	32.8
86	60.8	26.7
89	60	40
96	46.7	53.3
114	56	44
123	57.9	42.1
124	50.7	49.3
129	61.1	38.9
136	71.5	28.5
139	73.7	26.3
152	76.2	23.8
160	74.8	25.2
166	50.9	49.1
188	45.5	54.5
202	45.8	54.2
203	58.6	41.4
208	75.4	24.6
213	75.8	24.2
215	72.1	27.9
217	73.7	26.3
222	71.4	26.6
227	85.8	14.2
228	45.3	54.7
229	51.2	48.8
231	68.5	31.5
233	86.6	13.4
234	85.5	14.3
235	84.3	15.7
237	82.7	14.5
241	77.8	22.2

Plant No.	A%	H%
244	48.7	51.3
246	61.1	38.9
249	75.4	24.6
253	71.5	28.5
255	70.5	29.5
259	74.8	25.2
269	73.2	26.8
270	54.5	45.5
279	46.7	51.3
301	35.4	64.6
304	84.3	15.7
307	70	30
315	63	37
326	60.6	39.4
329	48.6	51.4
343	36.9	63.1
344	62.5	37.5
345	63	37
357	87.6	12.4
360	63.3	36.7
362	60.8	39.2
367	90.6	9.4
371	60.8	39.2
384	73.4	26.6
385	86.4	13.6
393	87.6	12.4
401	71.5	28.5
402	60.8	39.2
416	87.6	12.4

VITA

Name of the student	**:**	Kirandeep Kaur
Father's name	**:**	S. Karamjit Singh
Mother's name	**:**	Smt. Narinder Kaur
Nationality	**:**	Indian
Date of birth	**:**	28th August, 1990
Permanent home address	**:**	H.No. 589-D, Type II, Rail Coach Factory, Kapurthala, Punjab
EDUCATIONAL QUALIFICATIOIN		
Bachelor degree	**:**	B.Sc. Biotechnology
University and year of award	**:**	Guru Nanak Dev University, Amritsar 2011
%age of marks	**:**	69.0%
Master's degree	**:**	M.Sc. Biotechnology
University and year of award	**:**	Eternal University, Baru Sahib 2013
OCPA	**:**	7.89/10.00
Title of Master's Thesis	**:**	Molecular characterization of puroindoline proteins in wheat and wheat relatives
Ph.D	**:**	Ph. D. (Biotechnology)
University and year	**:**	Punjab Agricultural University, Ludhiana, Punjab, 2019
OCPA	**:**	7.23/10.00
Title of the Dissertation	**:**	Fine mapping of QTL-*QSlb.pau*-3.04 for southern leaf blight resistance in maize (*Zea mays* L.)
Awards/Distinctions/Fellowships	**:**	-