TAGGING OF GENE FOR RESISTANCE TO POST FLOWERING STALK ROT IN MAIZE(*Zea mays*) CAUSED BY *Macrophomina phaseolina*

By

P.SUNEETHA.B.Sc. (Ag.)

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CERTIFICATE

MS.SUNEETHA.P has satisfactorily prosecuted the course of research and that the thesis entitled **TAGGING OF GENE FOR RESISTANCE TO POST FLOWERING STALK ROT IN MAIZE (*Zea mays*) CAUSED BY *Macrophomina phaseolina*** submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by her for a degree of any university.

Date:
Place: Hyderabad

(G.ANURADHA)
Chairman of the Advisory Committee

CERTIFICATE

This is to certify that the thesis entitled "**TAGGING OF GENE FOR RESISTANCE TO POST FLOWERING STALK ROT IN MAIZE**(*ZEA MAYS*) **CAUSED BY *Macrophomina phaseolina***" submitted in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE IN AGRICULTURE (AGRICULTURAL BIOTECHNOLOGY**) of the Acharya N.G. Ranga Agricultural University, Hyderabad is a record of the bonafide research work carried out by **Ms. P. SUNEETHA** under my guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee. No part of the thesis has been submitted by the student for any other degree or diploma has been published. The published part has been fully acknowledged. All assistance and help received during the course of the investigation have been duly acknowledged by the author of the thesis.

(G. ANURADHA)
Chairman of the Advisory Committee

Thesis approved by the Student's Advisory Committee.

Chairman: **(Dr.G.ANURADHA)** _____
Principal scientist (Breeding)
Biotechnology Unit
ARI
Rajendranagar,Hyderabad-500 030.

Member: **(Dr.K.MANORAMA)** _____
Professor
Department of Agricultural Biotechnology
College of Agriculture
Rajendranagar, Hyderabad-500 030.

Member: **(Dr. R. SUDHAKAR)** _____
Senior Scientist (Plant Pathology)
SRTC
Rajendranagar, Hyderabad-500 030.

DECLARATION

I, **SUNEETHA.P,** hereby declare that the thesis entitled **TAGGING OF GENE FOR RESISTANCE TO POST FLOWERING STALK ROT IN MAIZE(*Zea mays*) CAUSED BY *Macrophomina phaseolina*** submitted to **Acharya N.G. Ranga Agricultural University** for the degree of **MASTER OF SCIENCE IN AGRICULTURE (AGRICULTURAL BIOTECHNOLOGY)** is a result of original research work done by me. I also declare that the thesis or part thereof has not been published earlier elsewhere in any manner.

Date: **(SUNEETHA.P)**

Place: Hyderabad

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LIST OF ABBREVATIONS

AGE	:	Agarose Gel Electrophoresis
Bp	:	Base pairs
BPPTI	:	Hyderabad Pathology Post Flowering Stalk rot tolerant inbred
BSA	:	Bulk segregant analysis
cM	:	Centi Morgan
CTAB	:	Cetyl trimethyl Ammonium Bromide
DMR	:	Directorate of Maize Research
dNTP	:	Deoxyribo Nucleoside Tri-Phosphate
EDTA	:	Ethylene Diamine Tetra Acetic acid
EtBr	:	Ethidium Bromide
EtOH	:	Ethanol
ISSR	:	Inter Simple Sequence Repeats
M	:	Molar
M.ha	:	Million hectares
MAS	:	Marker Assisted Selection
MB	:	Mega bases
Mg	:	Milli gram
mM	:	milli molar
Ng	:	Nanogram
PCR	:	Polymerase Chain Reaction
PFSR	:	Post Flowering Stalk Rot
Phi	:	Pioneer Hybrid International
RAPD	:	Random Amplified Polymorphic DNA
RFLP	:	Restriction Fragment Length Polymorphism
RILs	:	Recombinant Inbred Lines
rpm	:	Revolutions per minute

SNP’s	:	Single nucleotide Polymorphism
SSR	:	Simple Sequence Repeats
TAE	:	Tris Acetate EDTA
Taq	:	*Thermus aquaticus*
TBE	:	Tris Borate EDTA
TE	:	Tris EDTA
Tris HCl	:	Tris (hydroxyl methyl) amino methane hydro chloride
UV	:	Ultra violet
Λ	:	Absorbance
µg	:	Microgram
µl	:	Microlitre
D/s		Date of sowing

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Author	:	**P.SUNEETHA**
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Department	:	**AGRICULTURAL BIOTECHNOLOGY**
Major Advisor	:	**Dr. G.ANURADHA** **Principal Scientist (Breeding)** **Biotechnology Unit** **Agricultural Research Institute** **Rajendranagar, Hyderabad – 30.**
University	:	**ACHARYA N. G. RANGA AGRICULTURAL UNIVERSITY**
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ABSTRACT

Maize is one of the most important economic cereal crops and an ideal forage crop. It occupies a prominent position in global agriculture. 67% of maize is used for live stock feed and 25% of maize for human consumption, industrial purposes and the balance is used as seed.The productivity of maize is influenced by both biotic and abiotic factors. Maize suffers from about 110 diseases on a global basis.

In India there are four downy mildews, four stalk rots, three foliar diseases, root rots and other diseases affecting kernel and other aerial parts. Disease spectrum varies in different agro climatic zones. More serious diseases are leaf blight, stalk rots, downy mildews and rusts. Stalk rots take a heavy toll, among which stalk rots caused by *Macrophomina phaseolina* (Tassi) Goid and *Fusarium moniliformae* results in 30-40% losses. Post flowering stalk rot (PFSR) is the complex disease caused by three fungi,*viz*., *Cephalosporium acremonium, Macrophomina phaseolina, Fusarium moniliformae* and one bacterium *Erwinia carotovora var zeae*. Out of these, post flowering stalk rot caused by *Macrophomina phaseolina* is the important disease of maize in the state of Andhra Pradesh.

In order to tag the post flowering stalk rot resistant gene maize inbred lines BPPTI-34 (resistant) and BPPTI -66(Susceptible), were crossed to produce F_1. F_1's were selfed as well as back crossed to the susceptible parent to derive F2 and BC_1F_1 populations respectively. Parents P_1 and P_2, F_1 and two mapping populations F_2 and BC_1F_1 were artificially inoculated with the *Macrophomina phaseolina* culture. F_1s inoculated with the culture showed resistant reaction revealing for that resistance for post flowering stalk rot is governed by dominant gene. F_2 population segregated in 3:1 ratio i.e 87 resistant: 27 susceptible and BC_1F_1 population segregated in the ratio of 1:1 i.e 26 resistant: 24 susceptible showing that resistance to post flowering stalk rot is governed by single dominant gene.

A total of 150 microsatellite markers distributed on entire genome were used to screen the parents. Of these, 54 SSR markers from ten chromosomes were found polymorphic in the parents. These fifty four markers were used to screen the bulk DNAs prepared from 10 plants each of resistant and susceptible plants from F_2 and BC_1F_1 populations to find the markers linked to the resistance gene. By bulked segregant analysis (BSA) the marker umc 1269 clearly distinguished resistant and susceptible bulks as that of resistant and susceptible parents indicating that this marker is tightly linked to the gene for resistance to post flowering stalk rot.

CHAPTER-I

INTRODUCTION

Maize is one of the most important economic cereal crops and an ideal forage crop. It occupies a prominent position in global agriculture after wheat and rice. It is being grown primarily as a food grain crop to meet the food demand for humans and animals . In India maize ranks fourth next to rice, wheat and sorghum with an area of 7.73 million hectares with production of 14.71 million tones and productivity of 4073 kg/ha (Centre for monitoring Indian economy 2007). In AP, the importance of maize has increased recent times, due to its requirement to poultry farming . In AP, the area under maize has been increased from 4.0 lakh hectares in 1997-1998 to 7 lakh hectares in 2007-2008 (economic & statistical bulletin A.P 2007-2008)

Among the cereals, maize is rich in starch, protein, oil and sucrose. It is used in many ways such as grains for human consumption, cattle feed, poultry feed. Many of its byproducts are used as raw materials in different industries. It has industrial usage in the form of value added products in recent years. Globally 67%of maize is used for live stock feed, 25% for human consumption, industrial purposes and balance is used as seed.

The productivity of maize is influenced by both biotic and abiotic factors. Diseases and pests are the major constraints. Maize suffers from about 110 diseases on a global basis. In India 29 diseases caused by fungi, bacteria and viruses are noticed on maize. Disease spectrum varies in different agro climatic zones. More serious diseases are leaf blight; stalk rots downy mildews and rusts.

Post flowering stalk rot (PFSR) is the complex disease caused by three fungi,*viz., Cephalosporium acremonium, Macrophomina phaseolina, Fusarium moniliformae* and one bacterium *Erwinia carotovora var zeae*. Out of these, post flowering stalk rot caused by *Macrophomina phaseolina* is common in the state of Andhra Pradesh. This Charcoal rot is prevalent in comparatively drier maize growing areas of India particularly Andhra Pradesh, West Bengal, Bihar, Uttar Pradesh and Delhi where there is scarcity of irrigation especially after flowering stage of the crop growth. Charcoal rot is found to be prevalent particularly in *rabi* season when summer temperatures during post flowering period becomes comparatively high (35 - 45^0C). The pathogen also attack many other hosts, which helps in its perpetuation. Since the fungus is a facultative parasite it is capable of living saprophytically on dead organic tissues particularly on many of its natural hosts producing sclerotial bodies. The fungus survives over winter as a sclerotia in the soil and infects the host at susceptible crop stage through roots and proceed towards stem. The characteristic symptoms of the disease become apparent as the plants approach maturity. The disease generally appears early after flowering. Incidence of this disease at grain filling stage reduces the grain yield by 30%. (Payak&Sharma, 1983) The plant dries from the basal nodes and basal nodes turns into straw colour. Pith is badly disintegrated. The infected stalks may split longitudinally into a mass of fibres. A distinguishing character of the disease is the presence of small black sclerotia in the pith of the affected stalks. Roots are also invaded and show black sclerotia in the disorganized tissue. Horizontal blackening of vascular bundles is seen which hampers the conduction of water and nutrients leading to premature drying. Generally dried ears with broken shanks are seen hanging from the plants. This

disease has gained considerable importance because most of the commercially grown cultivars have shown a high level incidence of post flowering stalk rot.

Widely cultivated improved varieties are genetically uniform and susceptible for the disease. Severity and significance of damage caused by the disease have necessitated the developmental strategies to control and manage the disease, so as to reduce the yield loss. Though use of chemicals like mercuricidal compounds and antibiotics were resorted in early fifties, environmentally safe and stable chemical control at very low concentrations are yet to be developed. However, exploitation of host plant resistance appears to be the best reliable method for management of disease

Conventional breeding for resistance has produced impressive results. However in conventional breeding for selection of resistant plant, creation of disease through artificial inoculation is the basic step along with congenial weather for disease development. With the advent of new techniques it is now possible to select resistant plant without creating any disease epidemics. These new tools needed to reinvigorate the science of plant breeding will come from the rapidly evolving field of biotechnology. Marker-assisted selection (MAS) is one such that can provide an effective and efficient breeding tool for detecting, tracking, retaining, combining, and pyramiding disease resistance genes. Marker assisted selection can potentially increase the efficiency of traditional breeding programs by speeding up the time of varietal release, lowering plant population requirements, and reducing input intensive and time consuming field evaluation. The use of MAS techniques in further improving grain yield under biotic stresses in maize appears very promising.

Identification and characterization of major genes for qualitative resistance and polygenic characters controlling quantitative resistance have contributed to great deal to the success in breeding resistant cultivars. Recently major genes conferring disease resistance in several crop species have been mapped with linked DNA markers, facilitating the MAS for disease resistance in these crops (Melchinger, 1990). Marker assisted selection has been successfully used in selecting resistant crops in the absence of pathogens (Melchinger, 1990). In marker assisted selection , individuals carrying target genes are selected in a segregating population based on tightly linked markers rather than on their phenotypes. Thus, population can be screened at early seedling stage and in various environments. It also helps to overcome interference from interaction between different alleles of a locus or different loci.MAS increases the efficiency and accuracy of selection, especially for traits which are difficult to phenotype.

Requirements for marker assisted breeding include:

1. Availability of diverse germplasm with useful characters.
2. Identification of flanking markers closely linked to gene or QTL.
3. Simple robust polymerase chain reaction (PCR) based marker technology to facilitate rapid cost effective screening of large breeding populations.
4. Highly accurate and precise screening techniques for phenotyping of mapping populations.

Molecular markers , a class of genetic markers are based on differences in the DNA nucleotide sequences of chromosomes of different individuals These differences are referred to as polymorphisms at DNA level, and they arise as a result of insertions, deletions, duplications, and substitutions of nucleotides. Molecular

markers offer many advantages over morphological markers as they are phenotypically neutral occur throughout the genome, neither influenced by environment nor by pleotropic and epistatic interactions and expression is not dependent on plant age. A variety of Marker systems have been developed to visualize these DNA polymorphisms (Paterson, 2000). The most common molecular markers currently in use are restriction fragment length polymorphism (RFLP) , randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats(SSR) and single nucleotide polymorphisms(SNPs). In principle gene mapping involves identification of polymorphic markers between parents and establishing marker-trait association using segregating populations (Michelmore, 1991).

Microsatellite or simple sequence repeat (SSR) markers have been successfully used for genomic mapping, DNA fingerprinting, and marker-assisted selection in many plant species (Powell *et al* 1996)

In the present project, an attempt has been made to tag the gene associated with the post flowering stalk rot resistance using molecular markers. The objectives of the project were as follows.

- To tag the gene for resistance to post flowering stalk rot in maize specifically to *Macrophomina phaseolina*.
- To provide marker for screening of germplasm for resistance to post flowering stalk rot specific to *Macrophomina phaseolina.*

CHAPTER II

REVIEW OF LITERATURE

Maize is one of the most intensively studied crops and hence abundant information is available on genetics of several hereditary traits. Adequate knowledge on impact of damage by diseases, genetics and mechanisms of resistance, factors affecting breeding of disease resistant crops have been generated. Losses due to maize diseases are estimated at 12% in USA while in India it is approximately 13.2% (Payak&Sharma,1983). The following is the literature available on genetic analysis of resistance to post flowering stalk rot (PFSR) disease resistance in *Zea mays*.

Anon (1998) at there are about sixteen diseases causing severe setback in the maize production. In India there are four downy mildews, four stalk rots, three foliar diseases, root rots and other diseases affecting kernel and other aerial parts. Stalk rots take a heavy toll, among which stalk rots caused by *Macrophomina phaseolina* and *Fusarium moniliformae* results in 30-40% losses.

In India, Raju and Lal, 1976 listed nine fungi and three bacteria while Payak and Sharma, 1983 have reported only eight fungi and three bacteria as causal agents of stalk rots of maize.

Post Flowering Stalk Rot

Maize production is severely limited due to attack of a complex disease commonly called post flowering stalk rot (PFSR)(Sharma *et al.*,1993).Stalk rot is a serious and wide spread disease in maize that reduces both yeild and quality (Chiang and Wilcoxson,1961;Stack, 1999; Sobek and Munkvold1,1999; Jim,1999 and Yang *et al.*,2005) The disease is caused by several species of pathogen that occur singly or

as a part of a complex (Smeltzer,1958; Stack ,1999 and Yang 2002). Different pathogens have been reported to be associated with the maize stalk rot in different countries or regions (Byrne *et al.*,1995; Jim,1999 and Thon *et al.*,2000). Complex of three fungi viz *Cephalosporium acremonium, Macrophomina phaseolina, Fusarium moniliformae* and one bacterium *Erwinia caratovora var Zeae* causes the disease (Shankarlingam,2003 and Harlapur *et al.*, 2002). However,YasminAhmad *et al.*,2000 reported that *Macrophomina phaseolina, Fusarium moniliforme [Gibberella fujikuroi]* were the two causal organisms of stalk rot.

The term stalk rot is often used to include stalk breakage, stalk lodging, premature death of plant and occasionally root lodging. Severity of stalk rots is greatly influenced by temperature, rainfall, soil drainage, soil type and available nutrients. The rabi maize, that is being popularised in the eastern parts of the country may suffer from charcoal rot disease caused by *Macrophomina phaseolina* (Tassi) Goid.)], if the pathogen gets timely entry into the host. *In vitro* studies show that the spread of the pathogen within the maize stalk is influenced by high temperature, the optimum being 38°C (Kaiser *et al.*, 1979). Mechanical injuries and insect damage also tend to increase the severity of stalk rots. Dodd, 1980 reported that the high yielding varieties with good plant structure have succeeded in acquiring more phytosynthates from stalk. Because of this, balance between source and sink is being impaired thus predisposing the stalks to the infection by these pathogens. There after, rot weakens the stalk and account for lodging. Stalk rot occurs in a plant in which the amount of carbohydrate available to roots is insufficient to maintain viability of root cells. Resistanceto soil microorganisims surrounding roots dependent upon energy production of the phenols and the other anti microbial agents.

Good control over these diseases can be obtained through cultural management of soil and water through cultural practises, and by avoiding or decreasing green bug damage by applying insecticides or growing resistant cultivars (Doupnik,1984 and Evangelista and Tangonan, 1991). However, this approach is difficult to consider, because most of the area planted is rainfed and drought-prone. Positive correlation was recorded between disease resistance, crude protein and sugar content (Gill *et al.,* 2005). ShankarLingam and Venkatesh ,2005 developed twenty one agronomically desirable inbred lines resistant to post flowering stalk rot with disease rating of 2-5 on a 1-9 scale 1-immune and 9-susceptible

Macrophomina phaseolina. is a polyphagus fungus that infects numerous host-plants (maize, sunflower, pepper,) and causes large yield losses (Nagy and Fischl, 2002). The fungus causes charcoal rot which can lead to severe losses especially under arid conditions (Netzahualcoyotl *et al.,* 2001). Holiday and Punithalingam, 1970 for the first time described the fungi *Macrophomina phaseolina*. its transmission, geographical distribution and hosts. They aiso reported that plant debris is the main transmitting agent for this disease. Also scleortia and pycnidia occur on the seed surface and under the seed coat. The pathogen also attack many other hosts, which helps in its perpetuation. Since the fungus is a facultative parasite it is capable of living saprophytically on dead organic tissues, particularly many of its natural hosts producing sclerotial bodies. Sclerotia are probably the main source of infection which also occurs through conidia. Sclerotia showed no loss in viability after 8 months and the fungus was recovered from stubble after 24-42 weeks. (Holliday and Punithalingam,1970). On the basis of sclerotial morphology, two groups of isolates could be formed, the one with oblong shape

having irregular edges and the other being round with regular edges (Meenashekhar *et al.*,2006). During winter the fungus remains as a sclerotia in the soil and infects the host at susceptible crop stage through roots and proceed towards stem. On the basis of disease expression, out of seven *Macrophomina phaseolina* isolates from different agro-ecological zones of India, the Hyderabad isolate was observed to be most virulent by giving maximum disease (8.8), while the Coimbatore isolate was found to be the least virulent as it exhibited a highest rating of 5.2 in the susceptible inbred CM 120 on a scale of 1-9. The noteworthy observation of the study was that sclerotial size and number had positive correlation with virulence (Meenashekhar *et al.*, 2006).

To explore the genetic differentiation among *Macrophomina* isolates from each host, these isolates were examined by restriction fragment length polymorphism and random amplified polymorphic DNA (RAPD) analysis by Su *et al.*, 2001. No variations were observed among isolates in restriction patterns of DNA fragments amplified by polymerase chain reaction covering the internal transcribed spacer region, 5.8S rRNA and part of 25S rRNA, suggesting that *M. phaseolina* constitutes a single species More colonization of maize roots occurred when maize was grown in soil containing maize isolates compared with isolates from other hosts. Amplification of the ITS region with AD primers the entire ITS< sub>1</ sub>, rDNA and ITS2 region was amplified successfully for all the isolates studied. A single band of 750 bp was uniformly amplified in all the isolates and no polymorphism was visualized (Meena shekhar *et al.*, 2006). Sankar and Sharma,2001 isolated *Trichoderma viride* isolate for controlling charcoal rot. Seeds of 1kg treated with 12g of this isolate produced plants with maximum shoot length, dry matter, test weight and yield

Symptoms of damage

Charcoal rot incidence is more under dry warm conditions 28-35^0 C and moisture stress (Uppal *et al.,*1936). Desai *et al.,* 1982 surveyed in Karnataka and reported that the stalk rot due to *Macrophomina phaseolina* and *Fusarium moniliformae* is more in *rabi.* The characteristic symptoms of the disease become apparent as the plants approach maturity. The disease generally appears early after flowering. Plants affected by *M. phaseolina* show evidence of pre-mature ripening. The lower internodes become straw colored. The pith becomes badly disintegrated. The most frequent symptoms are a dry or wet, dark rot of the lower stem. In maize and sorghum this usually occurs near maturity, the cortex is destroyed, lodging may take place. A distinguishing character of the disease is the presence of the numerous small black sclerotia on the vascular fibers of the affected stalks. Small black sclerotia are found on the of the affected stalks(Holiday and Punithlingam ,1970).The infected stalks may split longitudinally into a mass of fibres. The longitudinal cutting of affected stalks reveals horizontal blackening of vascular bundles that hampers conduction of water and nutrients leading to death of the plant in dry conditions. Roots are also invaded and show black sclerotia in the disorganised tissue. In severe cases the ear formation is hampered. Generally the dried ears with broken shanks are seen hanging from the plants

Macrophomina phaeolina predominates and sclerotia are seen on disintegrated pith on longitudinal cutting of shredded stems. Under well fertilized and normal irrigated conditions *Cephalosporium acremonium* predominates and it is characterized by typical blackening of lower internodes. Towards the later crop growth stages *Fusarium* and *Erwinia d*ominates. *Erwinia* causes soft rot of the basal

internodes and a sweet fermenting odor emanates. The rotting may extend to the shank and ears.

Lawrence *et al. (*1987) reported that occludation of stem protoxylem vessel elements collapsing of intracellular material around vascular bundles and penetration of fungal hyphae penetrated throughout the cells. They also reported the following losses.

Loss of yield due to premature plant death, so normal grain filling is stopped. Total grain weight on stalk rot affected plants is less than the weight of grains on healthy plants. Another component of yield loss is that plants with stalk rot may lodge and can not be harvested with mechanical equipment. Harvesting is slowed if stalk rot is severe and losses also occur due to time loss during harvest. Losses also occur with ear rot as a result of the ear on lodged plant coming in contact with soil. This results in reduced grain quality and potential dockage when the grain is marketed. Yield loss estimates are difficult to obtain because losses due to stalk rots occur in many ways.

In USA, an average of 7.15% yield loss has been reported from stalk rot on maize which amounts to a loss of $500,000,000 on overall crop (Christensen and wilicoxson,1966). Cook, 1973 reported a mean reduction of 18.7% in cob weight and 11.2%in 1000 grain weight in plants infected with stalk rot. Mukesh Kumar *et al.*, 1998 observed a direct correlation between disease sevearity and varietal susceptibility. They observed that variety expressed different degrees of the yield loss and the loss was maximum in Kiran followed by Navajyoti and Deccan 107.

Inoculum Preparation

Shanmugam and Govindaswamy, 1973 studied the physiological aspects of *Macrophomina phaseolina* causing groundnut root rot and found that *Macrophomina phaseolina* grew best on Richards medium at pH 5.0. Waser *et al.*,1990 isolated *Macrophomina phaseolina* from soyabean and reported that the pathogen grew best on PD agar and PD juice at 35^0C

For all post flowering stalk rot pathogens, tooth pick method of inoculation is the most desirable for large scale field inoculation because inoculation procedure can be performed rapidly and inoculum preparation is easy. In this method only five to ten seconds are required for inoculating each plant. Sprague (1954) concluded that stalk rots developed when inoculation was made at silking than at a later stage and maximum disease severity occurred when the plants were inoculated at pollination. Raju and Lal 1976 reported that the maize plants upto flowering were less susceptible, became most susceptible during flowering and then susceptibility decreased as the plants mature. The most appropriate plant stage for inoculation is between tasseling and pollination through insertion of tooth pick into the injury created in the second internode with a insertion needle. Splitting the stalk open and observing the rot is the most reliable method of determining the amount and extent of stalk rot. Inoculated plants were cut at the third internode after physiological maturity and vertical stalk sections of each hybrid were used for disease assessment using a disease severity index based on the discoloration of the pith tissue (Zamani, 2006).

Christenson and Wilcoxson,1960 stated that the hybrids and inbreds can be inoculated on the same date that differ as much as ten days in the time of pollination

as the exact timing of inoculation is not critical. Siva Reddy, 1999 reported that the resistance to post flowering stalk rot screened can be easily screened in disease sick plots. However, for effective disease reaction artificial inoculation using tooth pick method is necessary for effective disease reaction.

Disease sick plot development is criteria in success of breeding for resistance to PFSR. Screening reinforced with artificial inoculation using tooth pick method is effective in supplementing the disease sick plot techniques of screening against PFSR. The methodology followed is suitable for screening against a multi pathogen disease complex (Shankar lingam and Venkatesh, 2005).

The score may be done in different ways including a numerical scale (Young,1943; Reece,1949;Andrew,1954 and Kochler,1960). One of the most common method is to estimate the percentage of tissue decayed in an internode. Whenever the rot extends beyond the internode inoculated, the total rating scale is equal to the sum of the disease rating of the internodes infected.

Rind puncture was the most desirable method of evaluating stalk quality because it is simple to use and non destructive to the plants (Anderson and White, 1994). Khan and Paliwal1979 used 0-5, White and Renfro, 1972 used 1-10. These two scales are generally in use for the stalk rots screening. Payak and Sharma 1983 suggested 1-9 index scale.1-immune reaction, 9-susceptible reaction, 5-resistant reaction which is now the most widely used method.

Genetics of Resistance

During last sixty years enormous research was carried out on maize stalk rot disease. However, few reports are concerned with genetics and molecular analysis of

this disease (Pe *et al.,* 1993). However, Koehler,1953 stated that inheritance of resistance to any of the rot is largely independent of inherent resistance to other rots.

The most promising and cheapest way to control stalk rot disease might be obtained by means of producing resistant lines (Stackman and Harar 1957). Ulstrup, 1966 stated that utilization of genetic resistance is likely to be more effective strategy to control the disease. The resistance to PFSR disease complex was found to be genetically controlled and most of the studies have indicated a polygenic nature of inheritance of resistance mechanisim (Payak and Sharma 1979). Singh (1979) found only one or two dominant genes to determine resistance to stalk rots. Such an uncomplicated pattern will allow resistance genes to be incorporated into any susceptible background by backcrossing accompanied by artificial inoculation and detection of resistant plants. Transfer of resistance is not expected to pose any problem for developing improved population or hybrids.

There was a genetic response for selection of resistant materials to post flowering stalk rot. Response of recurrent selection against stalk rots reported by Devey and Russell,1983. Breeding for resistance to stalk rots was greatly complicated by the relatively large number of fungi that can cause stalk rots and by the effects of various environmental factors that predispose plants to stalk rot (White,1999).

Bramel Cox *et al.,*1988 reported that the inheritance of resistance to *Fusarium moniliforme* and *M. phaseolina* is controlled by a multiple-locus complex with distinct heterotic patterns based on artificial inoculation techniques. Resistance appeared to be dependent on the environment. Tenkouano *et al.,* 1992 studied the genetics of nonsenescence and charcoal rot resistance in sorghum. They reported

that nonsenescence was regulated by dominant and recessive epistatic interactions between two nonsenescence-inducing loci and a third locus with modifying effects. Similar such information was obtained for charcoal rot resistance. However, the authors concluded that "nonsenescence alone can not account for, and should not be used as the sole breeding criterion for, resistance to charcoal rot in sorghum".

Toman and White (1993) inoculated their F_1,F_2 and BC_1F_1 maize populations with a conidial suspension of *Colletotrichum graminicola* and concluded that a single dominant gene could be best explain the obtained data. Cheng and Song (1999) based on their field survey data reported that in maize, resistance to stalk rot caused by *F.graminearum* was controlled by a single gene. Yang *et al.,* 2005 reported that the resistance to stalk rot in maize was controlled by one dominant gene when inoculated with a single purified pathogen, but seemed to be controlled by multiple genes when inoculated with mixed pathogens.

Marker assisted selection (MAS)

This refers to selection of plants carrying genomic regions that are involved in the expression of traits of interest through molecular markers. With the development and availability of array of molecular markers and dense molecular genetic maps in crop plants, MAS has become possible for traits both governed by major genes as well as minor genes or QTL's. MAS improve the efficiency of conventional plant breeding procedures through precise transfer of genomic regions of interest (foreground selection) and accelerating the recovery of recurrent parent genome (background selection). Currently, MAS for simply inherited traits is more widely employed than MAS for polygenic traits (Babu *et al.,* 2004). Molecular

markers offer great scope for mapping and tagging of agriculturally important genes which form the basis for marker assisted selection in crop plants.

There are three types of markers:

1) Morphological markers: these visually characterize phenotypic traits or characters, 2) Biochemical markers: these include allelic variants of enzymes called isozymes, and 3) DNA(or molecular) markers: these reveals sites of variation in DNA.

Among these three types of markers, morphological and biochemical markers are limited, often unstable and are influenced by environmental conditions. DNA markers on the other hand are unlimited in number, stable and will not be influenced by environmental conditions.

DNA markers

DNA markers have enormous potential to improve the efficiency and precision of conventional plant breeding via marker-assisted selection (MAS). The large number of quantitative trait loci (QTLs) mapping studies for diverse crop species have provided an abundance of DNA marker–trait associations. DNA-based markers possess the potential to be used as markers for desirable characters that are difficult or time-consuming for conventional breeding methods. (Alexandrova,1999) The DNA markers have been used to study both monogenic as well as quantitative disease resistance.

Sharma (1993) reported that besides conventional methods of pathogen detection and breeding resistant cultivars, recent developments in molecular biology techniques, particularly the advent of various DNA markers, have greatly influenced plant protection methods. DNA markers linked to specific resistance gene can be

used in marker-assisted selection for resistance breeding, gene pyramiding and map-based cloning of the resistance genes. However, it takes over months to determine the resistance of breeding materials by inoculation assays. DNA markers, which can be identified within short period of time, should therefore be a powerful alternative for disease resistance screening (Onozaki *et al.*,2003).

Techniques which are particularly promising in assisting selection for agronomically important traits involve the use of molecular markers such as random-amplified polymorphic DNAs (RAPDs), restriction fragment length polymorphisms (RFLPs), microsatellites (SSRs)and PCR-based DNA-markers such as sequence characterized amplified regions (SCARs) and amplified fragment length polymorphisms (AFLPs) (Alexandrova,1999).

Polymerase Chain Reaction (PCR) based markers

Since the advent of PCR technology (Mullis and Faloona, 1997), a variety of PCR methods have been developed for various molecular biological applications. In particular, several PCR based markers were developed, which immensely simplified the DNA finger printing (genotyping) work for MAS breeding.

Simple sequence repeats (SSR) or microsatellites

Simple sequence Repeat (SSR) markers also known as micro satellites, are tandemly repeated motifs of 1-6 nucleotides found in all the prokaryotic and eukaryotic genome (Zane *et al.*, 2002). According to Pupko and Gvaur, 1999 any number of tandem repeats of a certain nucleotide combination may be regarded as a microsatellite. These repeats are found in both coding and noncoding regions (Hancock 1995) and are usually characterized by a high degree polymorphism (Zane *et al.*, 2002). Microsatellite loci are inherently unstable with high mutation rates, a

phenomenon that was reported to be caused by DNA polymerase slippage and/or unequal recombination (Li *et al.,* 2002). Due to their high mutability, SSRs play a significant role as molecular markers.

Microsatellites offer several advantages compared to other molecular markers.

They are highly reproducible, highly polymorphic, PCR based and readily portable with in a species Edward *et al.,* 1996. In a recent study comparing RAPD's and AFLPS for the genetic analysis of yeast, Gallego *et al.,* 2005 reported that SSR analysis gave highest level of information content. Similar results were reported earlier in Soya bean (Powell *et al.,* 1996). Microsatellites have also attracted scientific attention because they have been shown to be part of or linked to some genes of agronomic interest Yu *et al.,* 2000. All these positive attributes coupled with multi allelic mature, co-dominant transmission, relative abundance, extensive genome coverage and reqirement of only a small amount of template DNA have contributed to the extraordinary increase of interest in SSR's in many organisims (Zane *et al.,* 2002)

Inter simple sequence repeats

The inter simple sequence repeats (ISSR-PCR) (Zietkiewiez *et al.,.,* 1994) is another method which relies on two primers for PCR. It involves the amplification of regions between adjacent , inversely oriented microsatellites using a simple sequence repeats containing primer and can be undertaken for any species that contains sufficient number and distribution of SSR motifs and has the advantage that genomic sequence data is not required. This technique amplifies large number of

DNA fragments per reaction, representing multiple loci across the genome, is an ideal method for finger printing varieties.

New generation DNA markers

Single nucleotide polymorphism (SNPs) are the most common type of polymorphisms in the genome. They are considered to be useful markers for genome mapping and finger printing because of their high number, co-dominant inheritance, stability and simplicity compared to co dominant microsatellite markers (Rafalski, 2002 & Nasu *et al.*,2002). A large number of PCR based SNP detection techniques are available . Development and application of SNP markers is gaining momentum especially after the release of genome sequence information.

Earlier resistance to corn stalk rots was considered to be controlled by multiple genes (Stack, 1999). Pe *et al*. (1993) worked on maize stalk rots by inoculating an F2 population with a conidial suspension of *Fusarium graminearum*. and analyzed them with 95 RFLP markers and ten cloned RAPD markers. Results indicated that the resistance to *F.graminearum* was controlled by quantitative trait loci. Four quantitative trait loci were found on different chromosome regions. However, later Chen and Song, 1999 found that the resistance to any specific pathogen was controlled by one dominant gene in all of the tested inbred lines of maize.

Philip *et al*. (2009) reported that few resistance (R) genes and quantitative trait loci provide control to one or more races of the pathogen. To better understand monogenic resistance and improve breeding efficiency, tagging and mapping of a gene in host differential cultivar provides resistance to a range of races.

Yang *et al.*, 2004 reported that gene for resistance to maize stalk rot(*Rfg*1) caused by *Fusarium graminearum* is present on chromosome 6 between SSR marker mmc0241 and RFLP marker bnl3.03 with a genetic distance of 3.0cM and 2.0cM.

Genetics of resistance to stalk rot caused by *Pythium inflatum* in maize was studied by Yang *et al.*, 2005. They concluded that resistance is governed by a dominant gene. They also mapped the gene for resistance using molecular markers (RFLP, SSR, RAPD). They mapped the gene Rpil on chromosome 4, in between the SSR markers bnlg 1937 and agri 286 at 1.6 cM away from the former and 4.1 cM distant from the latter.

Molecular mapping of genomic regions harboring QTLs for stalk rot resistance in sorghum was carried out by Reddy *et al.*, 2008. A population of recombinant inbred lines (RILs) from F9 generation derived from IS22380 susceptible) × E36-1 (resistant), along with parents were phenotyped in sick plots at two locations (Dharwad and Bijapur, Karnataka, India). A total of 85 polymorphic marker loci (62 nuclear and 4 genic SSRs, 19 RAPDs) was available for the construction of genetic map, spanning 650.3 cM in all the ten linkage groups. Analysis with QTL Cartographer (2.5b), adopting composite interval mapping method (LOD > 2.0) at both locations, revealed 5 QTLs at Dharwad and 4 QTLs at Bijapur locations for the component traits of charcoal rot disease resistance. QTLs for number of internodes crossed, length of infection and per cent lodging accounted for 31.83, 10.76 and 18.90 per cent at Dharwad location and 14.87, 10.47 and 26.44 per cent phenotypic variability at Bijapur location, respectively. The QTLs for number of internodes crossed by the rot, length of infection and percent lodging were

common across two locations. These QTLs, consistent over environments for the component traits, are likely to assist in marker-assisted selection (MAS) for charcoal rot resistance in sorghum.

Philip *et al*. (2009) tagged and mapped the (*Pse-1*) gene in host differential cultivar of common Bean UI-3 that provides resistance to races 1, 5, 7, and 9 of *Psp*. Cosegregation for resistance to races 1, 5, 7, and 9, in a recombinant inbred population, Canadian Wonder ×UI-3 (CU), confirmed the effect of *Pse-1* against multiple races of the pathogen. Bulked-segregant analysis in the CU population identified six random amplified polymorphic DNA (RAPD) markers tightly linked (0–3.3 cM) to *Pse-1*. Three of the RAPDs completely linked with *Pse-1* in the population were converted to sequence characterized amplified region (SCAR) markers.

Pereira, 2003 reported that quantitative trait loci (QTL) mapping has received particular attention for studying disease resistance which refines the information about the inheritance of disease resistance. So, a breeding programme that includes QTL mapping procedures is better oriented, maximizing the chances in favor to overcome a disease via host-plant resistance. The colinearity and genome synteny involving the domesticated grasses facilitate the study and the utilization of QTL mapping related to disease resistance. Combination of traditional approaches with QTL mapping in disease resistance could result in more rapid and precise population improvement.

Bulk segregant analysis

Usual method to locate and compare loci regulating a major QTL requires a segregating population of plants each one genotyped with a molecular marker.

However, plants from such population can also be grouped according to the phenotypic expression and tested for the allelic frequency differences in the population bulks (Quarrie *et al.,* 1999).

The method of bulk segregant analysis (BSA) was initially proposed by Michelmore *et al.,* 1991 in their studies on downy mildew resistance in lettuce. It involves comparing two pooled DNA samples of individuals from a segregating population originating from a single cross. Within each pool, or bulk, the individuals are identical for the trait or gene of interest but vary for all other genes. Two pools contrasting for a trait (e.g., resistant and susceptible to a particular disease) are analyzed to identify markers that distinguish them. Markers that are polymorphic between the pools will be genetically linked to loci determining the trait used to construct the pools will be genetically linked to the loci determining the trait used to construct the pools. BSA has two immediate applications in developing genetic maps. Detailed genetic maps for many species are being developed by analyzing the segregation of randomly selected molecular markers in single populations. As a genetic map approaches saturation, the continued mapping of polymorphisms detected by arbitrarily selected markers becomes progressively less efficient. Bulked segregant analysis provides a method to focus on regions of interest or areas sparsely populated with markers. Also, bulked segregant analysis is a method of rapidly locating genes that do not segregate in populations initially used to generate the genetic map (Michelmore *et al.*,1991)

The second variant of bulk segregant an only in the analysis applies when using the pools of genetically diverse individuals like varietal mixtures or composite populations that differ from the first only in the number of alleles likely to be present

at any marker locus. Number of markers have been identified corresponding to different agronomic traits. In such cases, the genotypes have to be analysed individually as well as in bulk to measure the frequency with which a particular alleles occur (Quarrie *et al.*,1999).

CHAPTER III

MATERIAL AND METHODS

To tag the gene(s) for resistance to post flowering stalk rot, mapping population was developed using maize inbred lines developed at Maize Research Centre ANGRAU.

Materials

The inbred lines BPPTI-34 resistant to the post flowering stalk rot and BPPTI-66 susceptible to the post flowering stalk rot were procured from Maize Research Centre, ANGRAU.

Inoculum

Macrophomina phaseolina culture prepared on Honey peptone medium was used to inoculate the two mapping populations, parents P_1, P_2, F_1, F_2, BC_1F_1 for creating artificial disease conditions (Fig 1)

Simple sequence repeats (SSRs)

Maize SSRs selected randomly overall the genome were used for screening parents, F_1, F_2, and BC_1F_1 Mapping populations. One hundred and fifty maize SSRs from maize data base (Sigma) were used for screening (table 1)

Development of mapping population

Mapping population for tagging the gene(s) conferring resistance to the disease was developed from the cross between the susceptible parent BPPTI-66 and the resistant variety BPPTI-34 and Crosses were effected during *Rabi* 2008 at the Maize Research Centre, Rajendranagar. The F_1s were selfed as well as backcrossed with the susceptible parent to produce F_2 and BC_1F_1 respectively during *Kharif* 2009.

Thus the mapping population comprised of F_2, BC_1F1 generations of the cross between BPPTI-34 and BPPTI-66. The mapping populations *viz.*,F_2, BC_1F_1 (backcross with BPPTI-66) along with the parents and F_1 were screened for post flowering stalk rot at Maize Research Centre, ARI, Rajendranagar, Hyderabad during early *Rabi* 2009(D/s 2-9-2009). Three rows of the susceptible check were raised all around the experimental plot to provide the disease inoculum facilitating screening. To make the disease screening still more affective, the two mapping populations, F_2, BC_1F_1 (backcross with BPPTI-66), parents P_1, P_2 and F_1 were challenged artificially by treating with the inoculum prepared from the diseased susceptible check.

Phenotyping for post flowering stalk rot resistant

The two populations (F_2 and BC_1F_1) along with the parents and F_1 were evaluated for post flowering stalk rot resistance under field conditions using the disease screening methodology.

Phenotypic data analysis

Test of significance among F_2 and backcross generations was done by chi-square method. χ^2 test was applied for testing the deviation of the observed segregation from theoretical segregation. Chi-square was calculated using the formula.

$$\chi^2 = \frac{\Sigma(O-E)^2}{E}$$

Where,

O = Observed frequency

E = Expected frequency

$\sum$ = Summation of the data

If the calculated values of χ^2 is significant at 5 per cent level of significance, is said to be poor and one or more observed frequencies are not in accordance with the hypotheses assumed and *vice versa*. So, it is also known as goodness of fit. The degree of freedom (df) in χ^2 test is (n-1). Where n = number of classes.

Inoculum Preparation

Disease sick plot development is crucial in successes of breeding for disease resistance to post flowering stalk rot. Screening reinforced with artificial inoculation using tooth pick method is effective in supplementing the disease sick plot technique of screening against post flowering stalk rot. The methodology followed is suitable for screening against a multiple pathogen disease complex (Shankar Lingam and Venkatesh, 2005).

Artificial inoculation was done with the tooth picks on which the disease causal organisms were grown in the laboratory. For this purpose, infected maize stems with post flowering stalk rot were collected, cut into small bits and surface sterilized with 0.1% mercuric chloride for one minute followed by washing with sterile distilled water. Finally a single bit was aseptically transferred to culture tubes containing the sterile PDA medium and incubated for 10 days to get the stock culture of pathogen.

100 ml (peptone 1g, honey 5ml and distilled water 94 ml) sterilized (20 minutes) and cooled honey peptone medium was poured under aseptic condition into a sterilized, wide mouthed bottle screw cap, containing tooth picks. Then from stock culture, two loops of mycelial suspension were seeded in bottle containing tooth

picks under aseptic conditions. Then bottles were incubated at 35^0c for 7 days. The tooth picks covered with mycelia of the fungus were then used for field inoculation.

Disease screening methodology

The experimental material was screened for post flowering stalk rot resistance under field conditions using infector rows of the susceptible check *viz.*, CM600 during *Rabi,* 2008 at Maize Research Centre, ANGRAU, Rajendranagar, Hyderabad. Two mapping populations (F_2 and BC_1F_1) was sown in two rows of 2 m length with 30 x 20 cm spacing. The crop was raised adopting the recommended package of practices. Three rows of the susceptible check were raised all around the experimental plot to provide the disease inoculum facilitating screening of the entries under field conditions. To make the disease screening still more affective, the entries were challenged artificially by treating with the inoculum of mycelial spores prepared from the diseased susceptible check. The most appropriate plant stage for inoculation is between tasseling and pollination, through insertion of tooth pick into the site of injury created in the second internode with an insertion needle (Fig2,3). Classification for the reaction for the pathogen was done on an individual plant basis, splitting the stalk open and observing the rot is the most reliable method of determining the amount and extent of stalk rot and the 1-9 index scale, suggested by Payak and Sharma (1983) was followed for scoring disease.(table 2)

Level of resistance/susceptibility of the entries to the disease was determined by Percentage Disease Index (PDI)

$$PDI = \frac{\text{Number of plants infected}}{\text{Total number of plants analyzed}} \times 100$$

On the basis of the PDI, the entries were grouped into the following three categories. (table 3)

Study of parental polymorphism

DNA extraction protocol

DNA was extracted from leaf tissue of one month old plants using MaCouch *et al.* (1988) method described as under:

Steps followed

- Fresh leaves of maize were collected, freezed in liquid nitrogen and stored at -80^0 C until use. 0.5 g of leaves were ground using mortar and pestle in liquid nitrogen until finely ground. Frozen ground leaf tissue was transferred to 15 ml poly propylene centrifuge tubes.
- 5 ml of 60^0 C extraction buffer and 50 mg PVP/0.5 g leaf tissue were added. The contents were mixed by inversion and incubated in an waterbath maintained at 60^0 C (with shaking) for 45 min.
- The tubes were removed from water bath and cooled to room temperature for 5 min.
- 6 ml of chloroform: isoamyl alcohol (24:1) was added and mixed by inversion to form an emulsion.
- After mixing thoroughly centrifugation at 3000 rpm for 20 min was done in a table top centrifuge at room temperature.

- Top aqueous solution was transferred to new 15 ml centrifuge tubes using wide-bore pipette tip. Chloroform-isoamyl alcohol extraction step was repeated to remove cloudiness (PVP) in aqueous phase.
- Two volumes of cold (-20^0 C) isopropanol was added and mixed by inversion and placed in freezer (-20^0 C) for 10 min to accentuate precipitation. The solution was left at 4-6^0 C to precipitate overnight.
- Centrifugation was done at 3000 rpm for 6 min.
- Supernatant was poured off and pellet was washed with cold (0-4^0 C) 70% v/v ethanol. The pellet was dried at 37^0 C in an oven or vacuum until dry (~1 hour).
- The pellet was dissolved in 300 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) overnight at 4-6^0 C and transferred to 1.5 ml Eppendorf tubes.
- 3 µl RNase A (10 mg/ml) was added and incubated in 37^0 C water bath for approximately 1 hr. 3 µl proteinase K (1mg/ml) was also added and incubated at 37^0 C for 20 min.
- 150 µl of phenol and 150 µl of chloroform was added to each Eppendorf tube.
- Vortexing was done for a brief period and centrifuged in microfuge at 14,000 rpm for 15 min. Upper layer was collected in new 1.5 ml tube. 50 µl TE was added to phenol phase.
- The samples were left overnight in freezer. Samples were centrifuged at 14,000 rpm for 15 min. Later the samples were drained and washed with 70% v/v ethanol. Ethanol was removed.
- Tubes were vacuum-dried. 100-200 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) was added and allowed time for complete resuspension.

3.3.3 Estimation of quality and quantity of DNA

Estimation of quantity and quality of the DNA was done based on spectrophotometric measurement of UV absorbance at 260 nm since DNA has maximum absorbance at 260 nm. Absorbance corresponds approximately 50 µg/ ml of double stranded DNA, 40 µg/ ml of single stranded DNA/RNA and 20 µg/ ml of oligonucleotide.

Procedure

- An aliquot of 10 µl of the DNA sample was diluted in TE buffer in a ratio of 1:1000 in a microcuvette, mixed well and absorbance was determined at 260, 280 and 320 nm against TE buffer blank. The DNA concentration was calculated using the following formula

Amount of DNA (µg/ ml) = Dilution factor x Standard (50 µg/ ml) x absorbance at 260 nm

The ratio of absorbance 260 to OD 280 provided information on the purity of the DNA

1) If the ratio is 1.8 to 2.0 the sample of DNA is relatively pure
2) If the ratio is < 1.8 the sample of the DNA is either contaminated with phenol or protein
3) If the ratio is > 2.0 the sample of DNA is contaminated with RNA.

PCR amplification

PCR amplification was performed in a 10 µl volume of PCR reaction mix containing the following.

1. Taq buffer (10X) with Mg Cl_2 1 µl (1X)

2. dNTP mix (2.5 mM each) 1mM (1.0 µl)

3. Taq DNA polymerase (3U/µl) 1 U (0.2 µl)(Bangalore Genei)

4. Forward primer 0.2 µM (0.4 µl)

5. Reverse primer0.2 µM (0.4 µl)

6. Genomic DNA (50 ng/µl) 2.0 µl

7. Sterile distilled water – 5.0 µl

PCR amplification was carried out on thermal cycler (Eppendorf, or Applied Biosystems, USA). Thermal cycling conditions for PCR amplification are as follows.

Initial denaturation at 94^0C for 5 min

Denaturation at 94^0C for 1 min,

Primer annealing at □ 55 for 30 sec, } 35 cycles

Extension at 72^0C for 45 sec,

This was followed by final extension of 72^0C for 8 min.

The amplified products were checked on ethidium bromide stained agarose gels (3 %) and polymorphic primers were noted. A primer was considered polymorphic, if it amplified a band in one parent and absence of same band in the other parent

Agarose gel electrophoresis (AGE)

PCR samples were mixed with bromo-phenol blue (0.25% bromo-phenol blue and 40% (w/v) sucrose dissolved in water) and run on a 3% agarose gel (Sigma/ Bangalore Genei) containing ethidium bromide (10 mg/ml) along with the marker 50 bp DNA ladder (Bangalore Genei,) at 5.3 V/cm (Bio-Rad PowerPac 300)

for 2 hr in 1.0 X Tris-Acetic acid-EDTA (TAE) buffer (242 g Tris base, 57.1 ml Acetic acid, 100ml 0.5 M EDTA mixed and made up the volume to 1 litre with double distilled water and pH adjusted to 8.5). The resolved PCR bands were documented using Bio-Rad Molecular Imager Gel Doc XR System.

Parental polymorphism and screening of mapping population

A set of 150 microsatellites or simple sequence repeats (SSR) primers were used to screen the parents, BPPTI-66 and BPPTI-34 for identification of polymorphic SSRs. Of these markers, 54 SSRs that showed polymorphism between the parents were used for screening the susceptible and resistant bulks through bulked segregant analysis. Consistency of the bands was checked by repeating the reaction twice and the reproducible bands were scored in all the samples for each of the primers separately. As the SSR marker is the co dominant marker bands were present in both resistant and susceptible parents.

Bulked Segregant Analysis

Following parental polymorphism using appropriate SSR primers, closely linked markers to resistance were identified by bulked segregant analysis (BSA). BSA is a method used for rapidly identifying markers linked to any specific gene or genomic region. BSA was initially proposed for screening qualitative traits known to express variation at a single locus of large effect (Hill *et al.* 1998; Michelmore *et al.* 1991 ; Lynch and Walsh, 1997.). Each pool or bulk contains individuals that are identical for a particular trait or genomic region but arbitrary at all unlinked regions. The two bulks are genetically dissimilar at the selected region but seemingly heterozygous at all other regions. The essence of this procedure is creation of a bulk sample of DNA for analysis by pooling DNA from individuals with similar

phenotypes (resistant/susceptible). One bulk contained DNA from ten plants that were resistant to post flowering stalk rot and the second bulk contained DNA from ten plants that were susceptible to the disease from F_2 & BC_1F_1 populations. The markers identified to be polymorphic between the parents were used to screen the susceptible and resistant bulks.

Table 1 : List of 150 SSR Markers used in parental polymorphism studies

S. No	Marker	Chro mo-some	Forward primer sequence	Reverse primer sequence
1	umc1354	1	GATCAGCCCGTTCAGCAAGTT	GAGTGGAGGCGGAGGATCTG
2	umc1566	1	ATCTCGTCTACCTAACCCACCCTC	CAGGTGAAGAATCTGGTGAGGTC
3	bnlg2204	1	AGGCGACTTAGCTGCAGAAG	CGACTTTCGGTTTGGAAAAG
4	umc1126	1	CAACAGGGTGAACCCTCTGTACTT	AATATGGTGTTGTGATTTGCATCG
5	umc1812	1	TACAAGGAAGGCAAGTTCATCCTC	ATGCAGGTGACATTCATCATCATC
6	umc 1082	1	CCGACCATGCATAAGGTCTAGG	GCCTGCATAGAGAGGTGGTATGAT
7	umc1353	1	AGACAGGATCATCGAAAACACACA	ACCTCAGCCTCCTCGTCAACTACT
8	umc1071	1	AGGAAGACACGAGAGACACCGTAG	GTGGTTGTCGAGTTCGTCGTATT
9	umc1160	1	CGTTTGATATGATGTGGAGATTCG	AAGCTTGTGAATGTTCTGGATGTC
10	umc1177	1	CGTGTACCGCTCCTCTATAGTCGT	AAGTGGCCGAATTCATCCTTTATT
11	umc1222	1	CTCAGAACAGAAGCCATCAAAAGC	CGTCTTCGTGAGAGACATCCTGT
12	umc1269	1	TATATTAGAGGCACCTCCCTCCGT	AGCTGCTTCAGCGACTTTGG
13	umc1363	1	TGTTTAAGTGTTGGCAGAAAGCAA	TCTCCCTCCCCTGTACATGAATTA
14	umc2012	1	CTTGCATTGAACGACGACCTG	CGTACGCTTGCAATGCTTCTCT
15	umc1661	1	ACGAGACTCCCTCCTCTCCTCTC	GGAGTAAACTGTTGAAAGGCCCAT
16	umc1622	2	CTGGATGAGGAGGAAGAATACGAG	CCTCGGATTTTCCAAAACATTTCT
17	umc1419	2	CTCATCACAACTAGCGCCACTCTA	ATAGTGCAGAGGTCATCGTGGC
18	umc1776	2	AAGGCTCGTGGCATACCTGTAGT	GCTGTACGTACGGGTGCAATG
19	umc1026	2	TCGTCGTCTCCAATCATACGTG	GCTACACGATACCATGGCGTTT
20	umc1736	2	CCATCCACCACTAGAAAGAGAGGA	TTAATCGATCGAGAGGTGCTTTTC
21	umc2214	2	ACCCCCTGATTCTCTCTTACGTTT	CTGGATGAGGAGGAAGAATACGAG
22	mmc0231	2	GAGCGACTGCGAGACGG	AGATCGCGCCACCGCTC
23	bnlg2248	2	CCACCACATCCGTTACATCA	ACTTTGACACCGGCGAATAC
24	bnlg1064	2	CTGGTCCGAGATGATGGC	TCCATTTCTGCATCTGCAAC
25	umc1185	2	AGTAAAAGAGGCAAGGACTACGGC	GCGGCGATATATACGAGGTTGT
26	umc1542	2	TAAAGCTATGATGGCACTTGCAGA	CATATTTGCCTTTGCCCTTTTGTA
27	umc1265	2	GCCTAGTCGCCTACCCTACCAAT	TGTGTTCTTGATTGGGTGAGACAT
28	umc1756	2	ATCTCAGGTACTCTGCCTACGGG	AACAGAGGGTAGCTTGTGGCCT
29	umc2184	2	CTTGGCCTACTCCAAGTTCTCG	AGTAGAGCAGCACCATCCCG
30	bnlg1017	2	ATTGGAAGGATCTGCGTGAC	CAGCTGGTGGACTGCATCTA
31	umc2118	3	CGTCTCCGTCTGCAGTCACTATTA	TATGGTCCTCGGAGTTTGTTTGTT
32	umc1780	3	CTGTCCCCAGGTTGCTGTAGTAGT	CATGATGTACCCGCAACAAATG
33	umc2276	3	CTAGGTAGCCAGCTAGGTACGGGT	AGTGGAGCTTCTTCATCCTACCG

S. No	Marker	Chro mo-some	Forward primer sequence	Reverse primer sequence
34	umc2048	3	GCTGAAGTCCCAACCACCAC	TTGACATGTTCTACCATCTCACCAA
35	umc2256	3	GGTCCTAGTCGTTAATTCTTTTAGCG	GGTCAAGGACTCTTCTTCCTCCTT
36	umc2377	3	CCTTCAAACCAAATGTACAGCAGC	CTCCTCAACGACAGCGTGTACC
37	umc1886	3	GTTTGACAGCACAAGTGCAAGAAA	GAGGTGGACATTGGACAACACC
38	bnlg1647	3	CGTCGTCTGTGGACGTACTG	AGAAGCTCACAAGCCTGCTC
39	umc2259	3	GGCTCGACTTCGAGGACACC	GAGGAGGAGAGGGACAGGGAAG
40	umc1968	3	CTTCCCCTCCGCTACTGCTC	GTACTTGGTGTTGTCGCTCTTCTTC
41	umc1773	3	GGATCACACTATCGAGTCAGCGAT	CAAGGTAGCGTCGTCCTCCTC
42	umc1501	3	CCACATTTGGCTGAATTTGTTGTA	CTTGTTGGCTAGAAATTTGCCTTG
43	umc2265	3	AAGACGGTCCCGAAGAAAGC	CTGGACGTGGACTCAGACACC
44	umc1539	3	GAGTCCAGGCAGCACGCTAGT	GAGCAGCACACGAGGACCAG
45	bnlg2241	3	GTGCACACTCTCTTGCATCG	TAGTCAGCATCTGCCGTGTC
46	umc1008	4	TCTAGCTTGTGGTGGTGGTTGA	ACATGAGCACAAAGACTGACGC
47	umc2279	4	TGTCTCCTCCCAGGTCGTAGTGT	CAGAAGAGTAACCACACTGAACACACA
48	umc1902	4	CCTCATCTCTCATGGGATGGATA	TTCAGCATGACATATCATACAGTAGCA
49	bnlg2291	4	CCTCTCGATGTTCTGAAGCC	GTCATAACCTTGCCTCCCAA
50	bnlg1890	4	ACCGGAACAGACGAGCTCTA	GTCCTGCAAAGCAACCTAGC
51	umc1682	4	AGCAAGCAAGCAAGTCACTGAGTA	AGCAAGCAAGCAAGTCACTGAGTA
52	bnlg1126	4	GAGATCGAAGGTCATGGCAC	GAGATCGAAGGTCATGGCAC
53	umc2309	4	CATCTCCTACCAGCTCACCCC	CATCTCCTACCAGCTCACCCC
54	umc1490	4	GCCCTAGCTTGCTAATTAACTAACA	GCCCTAGCTTGCTAATTAACTAACA
55	umc1964	4	CTTCTCACTGTCGCAGAACAAGAG	CTTCTCACTGTCGCAGAACAAGAG
56	umc1702	4	ACGAGGCTCTTCCGAGTTCC	ACGAGGCTCTTCCGAGTTCC
57	umc2027	4	CAAATATCTTCGCAGCTCCAAATC	CAAATATCTTCGCAGCTCCAAATC
58	dupssr	4	TTCTTTAACTATTGGAAGCCCA	TTCTTTAACTATTGGAAGCCCA
59	bnlg1444	4	GCATGGATGGAGAAAGAGGA	GCATGGATGGAGAAAGAGGA
60	umc1109	4	GCAACACAGGACCAAATCATCTCT	GCAACACAGGACCAAATCATCTCT
61	umc2291	5	CTCGACGAGTTCAAGCGCTAC	AACTTCTCCTGGCGAGCATCT
62	bnlg1879	5	TGCTCTCACAAGATGGTGGA	CCACAGGATAAAATCGGCTG
63	umc1349	5	ACGACCAGTGCTTCGCTCAC	AGTTTCCATCGTATGATGTCGAGG
64	bnlg389	5	GGTCACCCTCCCTTTGCAG	ATTGCCTACACAGTTTGATTGG
65	umc1153	5	CAGCATCTATAGCTTGCTTGCATT	TGGGTTTTGTTTGTTTGTTTGTTG
66	umc1097	5	CTCGTCAACGTCAACCCAAGTAAG	CTGTTAGATGTGCGACAACAGAGC
67	umc1325	5	GGAGGTCATGCGTGTAAATAGGTC	ATATTGTACAGGAGCAGCTGGGAC
68	bnlg565	5	TAAGAACGACGAACGGTAACTG	GCTCACTGCACGCCAACAC

S. No	Marker	Chro mo-some	Forward primer sequence	Reverse primer sequence
69	mmc0351	5	GCAGTGCATGTATCTGATCTAC	AGGCTCTCTTGATCCTTCA
70	umc1429	5	GGGCCCTGTTAATCCTCATCTG	TCCTCCTTTCTCTCATGTTTCTCG
71	umc1221	5	GCAACAGCAACTGGCAACAG	AAACAGGCACAAAGCATGGATAG
72	umc1155	5	TCTTTTATTGTGCCCGTTGAGATT	CCTGAGGGTGATTTGTCTGTCTCT
73	umc1537	5	CATGAATCACACTTGGATGTGGTC	AGAAGCTGTCCTCGTTCAAGCTC
74	bnlg118	5	CTTCCAGCCGCAACCCTC	CCAACAACGCGGACGTGA
75	umc1375	5	AGTTCGACTTCGACCCGGAC	GTCAGGCTTCTTCTCGACACACC
76	umc1002	6	AGCTAGCTATACACCGCCAGG	TCAGTTTGGAACAGGGAAAAGTA
77	umc2309	6	CTGTGTTTTGTGTATTAGCGCCAG	GTCGAAATTCCTGACACAAAAAGG
78	umc1257	6	CAACGGAAGTGGCTGTAGAGTTTT	ACAGAGCATGTCAGGTATTTGCAG
79	umc1314	6	ACAAACTAAACTGCATGTACCCCC	CACAAATGATGTGGTGGCAATATC
80	umc1520	6	AGCAAATATATGAGCAATTAAGAACAGG	GTGTCGCCACCTATAATTTGATGA
81	bnlg1136	6	TAACCGGATGAGCATCTTCC	CATCAGCTTCAACGAGTTCG
82	umc2324	6	GATCCTCTGTCGCCAAACACTAAG	AGATGGTGACGATGAGTGATGAAC
83	umc1753	6	AAGATCTTGCTCCGTTTCCTCTCT	TTCAGATGCAAATCTCTTTTCGCT
84	umc2006	6	AGTCCATCACCATCCCTGGC	GCAGAACTATTGTCAGTTAACCTTGCAT
85	umc1250	6	GAGGCAAGAGCTAGGTCTCGATAG	CTGCTGCTTTTGGTGTTGTCTCT
86	bnlg1174	6	CGCATTCCAAGAACAATGAA	TTCGATTGGTGGGAAGATTC
87	umc1379	6	AAGTCGTAGTCAGCGAGGGCTT	GAACCACAGCTATGCTCGCC
88	umc2065	6	CAAGGTTCGGTCCTTCTTCTCC	GACACCTCGTCGTCGGTCAC
89	bnlg1372	6	AACTTTTGGCATTGCACTGG	CGTAAGTGCACACGGCATTA
90	umc2322	6	CTGCAGGTCCCTGAGCCTCT	AACAAGTGGCGACAGACAAAGATT
91	umc2177	7	ACCATGCATGTCTCACGTCACT	GGGTACGTGCTGTGGAGGAC
92	umc1241	7	TGAAGCAAGTCACTGGTAAGAGCA	TGACACACCCATACTTCCAACAAG
93	umc1401	7	CTCTGGTCCATCCTCATCGACT	TCTCTTGATCACATATCGATCCCA
94	mmc0411	7	CGATGCAAGAGTGTCAAGTA	ACTCCCTAGTGCAAAAATCA
95	phi091	7	ATCTTGCTTCCATAAGATGCACTGCTCT	CTCAGCTTCGGTTCCTACACAGT
96	umc1543	7	TTCCTCTACCAGCTGCCCGT	GTGAGCAGAAGCTTGAGGCG
97	umc2333	7	GAATTGGATGTTATTCGGATCGTC	TGCCTCCTTTTTCTGATCTACACC
98	phi116	7	GCATACGGCCATGGATGGGA	TCCCTGCCGGGACTCCTG
99	umc1672	7	CTTTGTTCCAGATCCATCTTTTCG	CCTAGACCCCACTCATGTGGTAAC
100	umc1159	7	TTCCCATGTTCATTTCAGGTTCTT	TCATGGGTTTTGAGGCTGTATTTT
101	umc2325	7	CCTAGGAACTCTGATGGCTATGGA	CTACGATATCCACCTCTACCACCG
102	umc1879	7	TTTCTGATTAGCTAGAAGCGCGAG	GATAAAACAGTACTGGGCCGATTG
103	bnlg1666	7	GCTGGTAGCTTTCAGATGGC	TGTCCCTCCTCCAGTTTCAC

S. No	Marker	Chro mo-some	Forward primer sequence	Reverse primer sequence
104	bnlg2259	7	ACCATTGATTTCATGGTATTGG	GCGGATAATGACATTGGGTC
105	umc1154	7	CCACCACAAGACAAGACAAGAATG	CCTGATCGATCTCATCGTCGT
106	umc1786	8	ACCGTGACTTCCTCCTCATAACTG	CATTTTTCGCATTTAGGAAATCCA
107	umc2042	8	GCAGTCTCTCCACTACCAGAGCAT	AACAGAGGAGTACGAGGAGGAGC
108	umc1913	8	AAACAACTATCCATGTGGCTGACC	CGTTCAGTACAATTTGGCTCAGTG
109	bnlg2046	8	TTGGTGAAACGGTGAAATGA	CTGGTGAGCTTCACCCTCTC
110	bnlg1823	8	TGTGACTCCATACCGCACAT	CTCATCATGTTGTACATGGCG
111	umc1663	8	GCTTGCACTAGCTTTAGCTCCATC	CGGGATCAGTCGTTACAAACATAG
112	bnlg1131	8	TTAGTTGGGTAAACGTGCAC	GCATCAGGGGGTAGTTGAGA
113	umc1075	8	GAGAGATGACAGACACATCCTTGG	ACATTTATGATACCGGGAGTTGGA
114	bnlg1194	8	GCGTTATTAAGGCAAGCTGC	ACGTGAAGCAGAGGATCCAT
115	umc1974	8	ACAAGGAGACCCTCCTCAGCTAGT	GTAAGCTGTGGCCATACTACCACC
116	umc2146	8	GTCTCCGTCCACCTCCTGTG	GTCATGGGAATGTGCTGGATG
117	umc1984	8	CTCTGGCCTCTGATACCAGTTGAT	CATCCTCCTGCAGCTGTTAACTC
118	umc2154	8	GCTAGTAGTAGTTCCAACGAAGCAACA	GTCACCATCTCCAGGTGCAAGT
119	umc1846	8	ATTATTGGTCACAGGCCCTACCTT	TTAGGCCCTCGTCTTGTAGACTTG
120	bnlg1031	8	AATCGGTGAGGCTTCACAAC	ATGCCTACCTACCACCATGC
121	umc1957	9	CATGATCGCCGGGATTAATACTAC	GTCCAAGGACGACGATTACGAC
122	umc1370	9	GGGAGCACACACAGTAGTACTCGAT	AGAGGCTCTCCTCCTTCAAGCTC
123	umc1366	9	GTCACTCGTCCGCATCGTCT	CCTAACTCTGCAAAGACTGCATGA
124	umc1137	9	ATCAGTCACTCTTCTGCCTCCACT	GGCTGGATAATGTTGTAGCTGGTC
125	umc1982	9	TTCATCTTCCTAGTCTCGTCTCCG	AATCGTACTTGGAGGAGGCGTT
126	umc1867	9	TGGTCTTCTTCGCCGCATTAT	ATAAGCTCGTTGATCTCCTCCTCC
127	bnlg1583	9	ATCAAGCTTATCGAGAGAGAGAGAG	CGACGGTGGAAAGACTGC
128	umc2336	9	ATCTCACCGCACGTAACTGAGACT	CCTATGCTCTTGCTCTTCCTGGTA
129	bnlg1372	9	AGCGGTGCTCAAATAGGAG	CGCCGGCTTCCCTCAC
130	phi022	9	TGCGCACCAGCGACTGACC	GCGGGCGACGCTTCCAAAC
131	umc2337	9	CTAACGTACACACATCTTGGCTGG	GAGTACCTCCGCCCACTCATC
132	umc1231	9	CTGTAGGGCTGAGAAAAGAGAGGG	CGACAACTTAGGAGAACCATGGAG
133	umc1494	9	CCACAGCAGCTCAGCTCGTC	GACGACCGCTACTTCTTCAGCA
134	umc1794	9	AGAGTAGATGCCGTGTAAGCAACC	CGACCGGGGTAGTGTAGTGATAAG
135	umc1310	9	AACTCCGAGATCTACGACAACAGC	GAGGAAGAGTTGGCCAGGATG
136	umc1380	10	CTGCTGATGTCTGGAAGAACCCT	AGCATCATGCCAGCAGGTTTT
137	phi041	10	TTGGCTCCCAGCGCCGCAAA	GATCCAGAGCGATTTGACGGCA
138	bnlg1547	10	TTGGATCAACTTACCCAGGC	ACATGCGTGCTACCCATACA

S. No	Marker	Chro mo-some	Forward primer sequence	Reverse primer sequence
139	umc1678	10	GTAGAGATCGATTCGCTAACCTGC	AGTTGTTCCGTTCCGTCCTTATC
140	umc1196	10	CGTGCTACTACTGCTACAAAGCGA	AGTCGTTCGTGTCTTCCGAAACT
141	umc1645	10	CATCGTCATCAGTTCTTCCATGAG	GTGATTGACAGAGGACCATCAGAA
142	umc1038	10	CGTCACACTCCTCTGCCACTT	GAGGATTCAGAACTCGACTCGG
143	umc2053	10	ATCTCTCCCTCGCTCTCCTTCTC	AGCAGCAGGTTGGTCGAATG
144	umc2034	10	TATCTCCTCCGATCCTAACACCCT	GCTCATACGGAGGGTCAGCTAAG
145	umc1345	10	ACCGCAGCAGCAGAGAAGAG	GAACATCTGGGTCACCTCTGTCAT
146	umc2348	10	AGTCAGACCGACGCACTCACTAA	TAACATCATCATCAGCGACGATTT
147	bnlg1074	10	CATGCTAATAGCCTACCGGG	TTTCCCCCTGATTCGTTATG
148	umc1477	10	GAAGCCGCAGAACATCCTCTT	AAGCTGTACACGTCGCCTTCC
149	bnlg2190	10	TCCTCCTTCATCCCCTTCTT	CCCAGTATCATTGCCCAATC
150	umc2021	10	AAACTCAAGCTCGGAATGTACTGC	CGATACTGATCTACTTCACGCTGG

Table 2 : Disease rating Scale of Post flowering stalk rot caused by *Macrophomina phaseolina* in maize

SCORE	SYMPTOM
1	25%of the inoculated internode discoloured.
2	26-50%o of the inoculated internode discoloured
3	51-75%of the inoculated internode discoloured
4	76-100% of the inoculated internode discoloured.
5.	Discolouration of less than 50% of adjacent internodes
6.	Discolouration of more than 50% of adjacent internodes
7.	Discolouration of more than three internodes
8.	Discolouration of more than four internodes
9.	Discolouration of more than five internodes and plants prematurely killed

Table 3 : **Classification of the entries based on Per cent Disease Index (PDI)**

PDI (per cent)		Disease reaction
1	→	Immune (I)
< 5	→	Resistant(R)
9	→	Susceptible (S)

Fig.1: Inoculum of *Macrophomina phaseolina* used in inoculation

Fig.2: Preparation for insertion of toothpick coated with inoculum

Fig.3:Insertion of tooth pick coated with inoculum

CHAPTER IV

RESULTS

The present investigation was carried out with two objectives molecular tagging of gene(s) for post flowering stalk rot resistance and providing marker for post flowering stalk rot resistance in maize. The results of the study are presented hereunder.

Development of mapping population

Maize inbred lines BPPTI-34 and BPPTI-66 were selected as resistant and susceptible parents respectively for crossing**,** and crosses were carried out during *rabi* 2008. The F_1 of the cross was simultaneously selfed and backcrossed to susceptible parent to raise F_2 and backcross populations ($BC_1F_{1)}$ respectively during *kharif* 2009. F_2 and backcross populations ($BC_1F_{1)}$ along with parents and F_1 were raised during early *rabi* 2009, (D/s2-9-09). (Fig, 4)

Phenotyping of the mapping population

The two populations F_2 and BC_1 F_1 along with the parents and F_1's were evaluated for post flowering stalk rot resistance under field conditions using the standard disease screening methodology as detailed under material and methods. The F_2 population consisted of 114 plants and while BC_1F_1 population consisted 50 plants.

Maize inbred line BPPTI-34 was selected as a resistant parent for crossing has shown the disease score of 2.2 according to the Payak and Sharma scale (1983) and BPPTI-66 which was taken as susceptible parent has shown the disease score of 5.6.

F_1 was artificially inoculated with one of the post flowering stock rot disease causing organism *Macrophomina phaseolina.* All the F_1 plants showed resistance reaction to post flowering stalk rot . By following the standard disease screening the disease score was given as 2 as the 26-50% of the inoculated internode discoloured. F_1s were selfed to produce F_2 mapping population and crossed to susceptible parent (BPPTI-66) to produce BC_1F_1 population. (Fig 5,6)

F_2 seed was sown during *rabi* 2009. F_2 population (114) was screened for disease resistance through artificial inoculation. Of these 114 plants 87 showed resistant reaction and 27 showed susceptible reaction. Of 87 plants 26-50% of the inoculated internode was discolored and the disease score was given as 2 and in remaining 27 plants discoloration of more than 50% of adjacent internodes and the disease score was given 6 according to the standard disease scoring method. The F_2 population when screened for disease segregated into a ratio of is 3:1 i.e 87 resistant : 27 susceptible plants

A total of 50 BC_1F_1 plants produced by crossing F_1 with the susceptible parent BPPTI-66 were sown in *rabi* 2009 along with the F_2 population. A disease score of 2 was observed on 26 plants and a disease score of 6 was observed on 24 plants proving that BC_1F_1 plants segregated in 1:1 ratio (table 4).(fig,7)

Inheritance of post flowering stalk rot tolerance

A field trail was conducted to study the inheritance of post flowering stalk rot resistance in maize utilizing the parents BPPTI-66 and BPPTI-34. F_1 and two populations *viz.*, F_2, BC_1F_1 during early *rabi* 2009. The female parent BPPTI-66 was susceptible to post flowering stalk rot whereas, male parent BPPTI-34 was resistant to post flowering stalk rot. The F_1 individuals of this cross shown resistant reaction

with the disease score 2. In the F_2 population among 114 plants, 87 were resistant and 27 were susceptible. Chi-square tests were performed to determine the goodness of fit to a ratio of 3:1. Results indicated that the ratio of resistant plants to susceptible plants was fitted to the ratio of 3:1. That results pointed out that a dominant gene controlled the reaistance to pathogen *Macrophomina phaseolina* and the resistance was from the resistant parent BPPTI-34.

In order to confirm the conclusion obtained from F_2 population the BC_1F_1 population obtained from the cross F_1×BPPTI-66 was tested with this pathogen. There was 26 resistant plants and 24 susceptible plants in 50 BC_1F_1 individuals. After examining by □2 test, the goodness of fit was always 1:1 in the BC_1F_1 generation (table 5).

Tagging of post flowering stalk rot resistant gene employing SSR markers

Parental polymorphism

One hundred and fifty maize SSR (simple sequence repeats) primers (Sigma) which were randomly distributed all over the ten chromosomes were screened between the susceptible parent (BPPTI-66) and the resistant parent (BPPTI-34). Of these, 54 markers showed polymorphism i.e 36 % (table 6).

Bulked Segregant Analysis (BSA)

DNA from ten resistant and ten susceptible plants from F_2 and BC_1F_1 populations were used to made resistant and susceptible bulks respectively. Polymorphic SSR markers between two bulks were identified by BSA, which provides a rapid, technically simple alternative for identifying markers linked to specific genes (traits). The 54 markers identified to be polymorphic between parents

were used for bulked segregant analysis of the resistance and susceptible bulks from F_2 and BC_1F_1 populations. Out of 54 markers, marker *viz.*, umc-1269 showed polymorphism between susceptible and resistance bulks as that of resistant and susceptible parents.

The marker umc-1269 amplified a fragment of about 150 bp length in the resistant parent and the resistant bulks and a fragment of 130 bp length in the susceptible parent and susceptible bulks (fig:8,9).

Table 4 : Disease score of Parents, F_1, F_2 and BC_1F_1

Mapping population	Disease reaction	Number of plants	Disease score
BPPTI-34	RESISTANT	20	2.2
BPPTI-66	SUSCEPTIBLE	20	5.6
F_1	RESISTANT	80	2
F_2	RESISTANT	87	2
F_2	SUSCEPTIBLE	27	6
BC_1F_1	RESISTANT	26	2
BC_1F_1	SUSCEPTIBLE	24	6

Table 5 : **Results of $\chi 2$ test to the F_2 and BC_1F_1 population**

Mapping population	Year	Total	Resistant	Susceptible	R/S ratio	$\chi 2$	p
F_2	2009	114	87	27	3.18/1	0.111	>0.05
BC_1F_1	2009	50	26	24	1.04/1	0.08	>0.05

Table 6: List of Polymorphic SSR markers

S. No	Marker	Chr.	Forward primer sequence	Reverse primer sequence
1	**bnlg2204**	1	AGGCGACTTAGCTGCAGAAG	CGACTTTCGGTTTGGAAAAG
2	**umc1812**	1	TACAAGGAAGGCAAGTTCATCCTC	ATGCAGGTGACATTCATCATCATC
3	**umc1071**	1	AGGAAGACACGAGAGACACCGTAG	GTGGTTGTCGAGTTCGTCGTATT
4	**umc1177**	1	CGTGTACCGCTCCTCTATAGTCGT	AAGTGGCCGAATTCATCCTTTATT
5	**umc1269**	1	TATATTAGAGGCACCTCCCTCCGT	AGCTGCTTCAGCGACTTTGG
6	**umc1363**	1	TGTTTAAGTGTTGGCAGAAAGCAA	TCTCCCTCCCCTGTACATGAATTA
7	**umc1661**	1	ACGAGACTCCCTCCTCTCCTCTC	GGAGTAAACTGTTGAAAGGCCCAT
8	**umc1622**	2	ACTTTGACACCGGCGAATAC	CCTCGGATTTTCCAAAACATTTCT
9	**umc1419**	2	CTCATCACAACTAGCGCCACTCTA	ATAGTGCAGAGGTCATCGTGGC
10	**umc1776**	2	AAGGCTCGTGGCATACCTGTAGT	GCTGTACGTACGGGTGCAATG
11	**umc2214**	2	ACCCCCTGATTCTCTCTTACGTTT	CTGGATGAGGAGGAAGAATACGAG
12	**bnlg2248**	2	CCACCACATCCGTTACATCA	ACTTTGACACCGGCGAATAC
13	**umc1542**	2	TAAAGCTATGATGGCACTTGCAGA	CATATTTGCCTTTGCCCTTTTGTA
14	**umc1756**	2	ATCTCAGGTACTCTGCCTACGGG	AACAGAGGGTAGCTTGTGGCCT
15	**umc2118**	3	CGTCTCCGTCTGCAGTCACTATTA	TATGGTCCTCGGAGTTTGTTTGTT
16	**umc2276**	3	CTAGGTAGCCAGCTAGGTACGGGT	AGTGGAGCTTCTTCATCCTACCG
17	**umc2377**	3	CCTTCAAACCAAATGTACAGCAGC	CTCCTCAACGACAGCGTGTACC
18	**bnlg1647**	3	CGTCGTCTGTGGACGTACTG	AGAAGCTCACAAGCCTGCTC
19	**umc1773**	3	GGATCACACTATCGAGTCAGCGAT	CAAGGTAGCGTCGTCCTCCTC
20	**umc2265**	3	AAGACGGTCCCGAAGAAAGC	CTGGACGTGGACTCAGACACC
21	**umc1008**	4	TCTAGCTTGTGGTGGTGGTTGA	ACATGAGCACAAAGACTGACGC
22	**bnlg2291**	4	CCTCTCGATGTTCTGAAGCC	GTCATAACCTTGCCTCCCAA
23	**umc1682**	4	AGCAAGCAAGCAAGTCACTGAGTA	AGCAAGCAAGCAAGTCACTGAGTA
24	**umc1490**	4	GCCCTAGCTTGCTAATTAACTAACA	GCCCTAGCTTGCTAATTAACTAACA
25	**umc2027**	4	CAAATATCTTCGCAGCTCCAAATC	CAAATATCTTCGCAGCTCCAAATC
26	**umc1109**	4	GCAACACAGGACCAAATCATCTCT	GCAACACAGGACCAAATCATCTCT
27	**bnlg389**	5	GGTCACCCTCCCTTTGCAG	ATTGCCTACACAGTTTGATTGG
28	**umc1097**	5	CTCGTCAACGTCAACCCAAGTAAG	CTGTTAGATGTGCGACAACAGAGC
29	**umc1221**	5	GCAACAGCAACTGGCAACAG	AAACAGGCACAAAGCATGGATAG
30	**umc1002**	6	AGCTAGCTATACACCGCCAGG	TCAGTTTGGAACAGGGAAAAGTA
31	**umc1314**	6	ACAAACTAAACTGCATGTACCCCC	CACAAATGATGTGGTGGCAATATC

S. No	Marker	Chr.	Forward primer sequence	Reverse primer sequence
32	**bnlg1136**	6	TAACCGGATGAGCATCTTCC	CATCAGCTTCAACGAGTTCG
33	**umc2006**	6	AGTCCATCACCATCCCTGGC	GCAGAACTATTGTCAGTTAACCTTGCAT
34	**umc1379**	6	AAGTCGTAGTCAGCGAGGGCTT	GAACCACAGCTATGCTCGCC
35	**umc2322**	6	CTGCAGGTCCCTGAGCCTCT	AACAAGTGGCGACAGACAAAGATT
36	**mmc0411**	7	CGATGCAAGAGTGTCAAGTA	ACTCCCTAGTGCAAAAATCA
37	**phi116**	7	GCATACGGCCATGGATGGGA	TCCCTGCCGGGACTCCTG
38	**umc1159**	7	TTCCCATGTTCATTTCAGGTTCTT	TCATGGGTTTTGAGGCTGTATTTT
39	**bnlg2259**	7	ACCATTGATTTCATGGTATTGG	GCGGATAATGACATTGGGTC
40	**umc2042**	8	GCAGTCTCTCCACTACCAGAGCAT	AACAGAGGAGTACGAGGAGGAGC
41	**bnlg1823**	8	TGTGACTCCATACCGCACAT	CTCATCATGTTGTACATGGCG
42	**umc1075**	8	GAGAGATGACAGACACATCCTTGG	ACATTTATGATACCGGGAGTTGGA
43	**umc2146**	8	GTCTCCGTCCACCTCCTGTG	GTCATGGGAATGTGCTGGATG
44	**umc1846**	8	ATTATTGGTCACAGGCCCTACCTT	TTAGGCCCTCGTCTTGTAGACTTG
45	**umc1370**	9	GGGAGCACACACAGTAGTACTCGAT	AGAGGCTCTCCTCCTTCAAGCTC
46	**umc1137**	9	ATCAGTCACTCTTCTGCCTCCACT	GGCTGGATAATGTTGTAGCTGGTC
47	**umc2336**	9	ATCTCACCGCACGTAACTGAGACT	CCTATGCTCTTGCTCTTCCTGGTA
48	**umc1231**	9	CTGTAGGGCTGAGAAAAGAGAGGG	CGACAACTTAGGAGAACCATGGAG
49	**umc1310**	9	AACTCCGAGATCTACGACAACAGC	GAGGAAGAGTTGGCCAGGATG
50	**umc1380**	10	CTGCTGATGTCTGGAAGAACCCT	AGCATCATGCCAGCAGGTTTT
51	**umc1645**	10	CATCGTCATCAGTTCTTCCATGAG	GTGATTGACAGAGGACCATCAGAA
52	**umc2034**	10	TATCTCCTCCGATCCTAACACCCT	GCTCATACGGAGGGTCAGCTAAG
53	**bnlg1074**	10	CATGCTAATAGCCTACCGGG	TTTCCCCCTGATTCGTTATG
54	**umc2021**	10	AAACTCAAGCTCGGAATGTACTGC	CGATACTGATCTACTTCACGCTGG

Fig.4: F_2 population of cross BPPTI-66×BPPTI-34

Fig.5: Plant with symptoms of Post flowering stalk rot

Fig.6: Symptoms of post flowering stalk rot in split open stalk

Fig.7: Overall field view of population after disease development

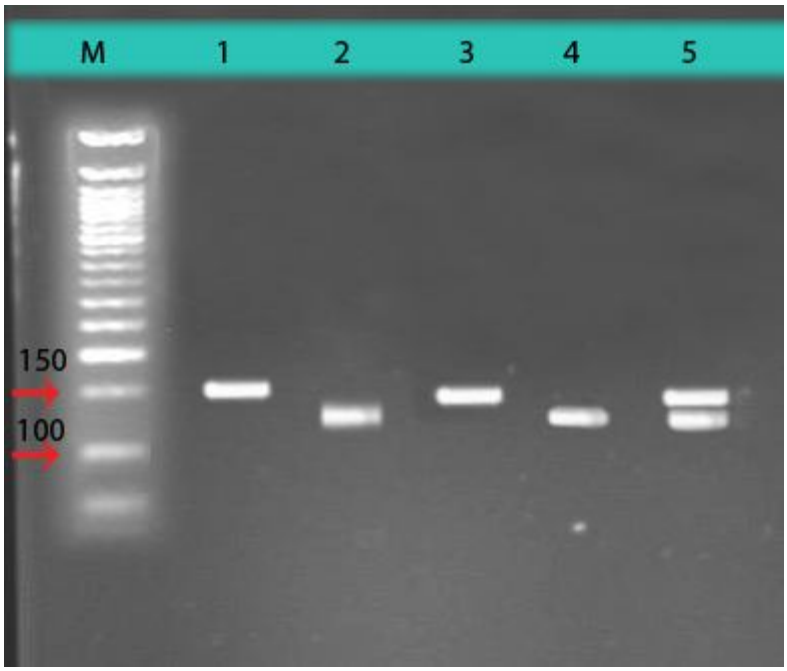


Fig8: Amplification pattern of the tagged marker umc1269

M- 50 bp DNA Marker 1-BPPTI -34 (resistant parent)

2-BPPTI-66 (susceptible parent)

3-Resistant Bulk 4-Susceptible Bulk 5- F_1

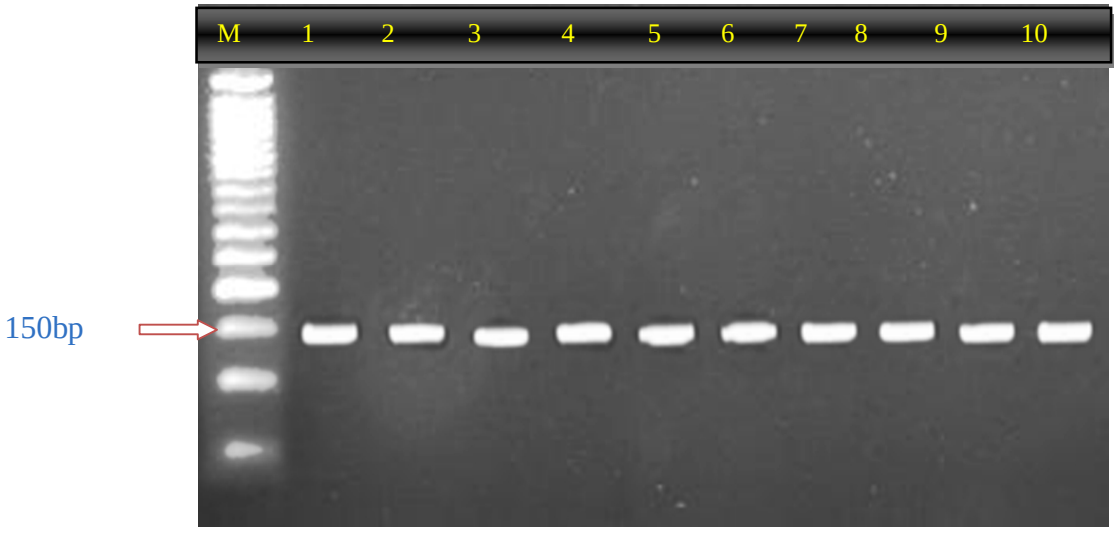


Fig. 9: Amplification pattern of resistant plants used for bulking

Lanes 1-10: F_2 resistant plants used for bulking M- 50 bp DNA Marker

CHAPTER V

DISCUSSION

Maize is one of the most important crops in world agricultural economy. In maize breeding, the main objective is the development of cultivars with good seed quality, composition and high stable yields which are hampered by factors such as environment, disease and insect pests, and lodging.

Maize suffers from about 110 diseases on a global basis of which 29 diseases are known in India caused by fungi, bacteria and viruses. Inherently low yield potential apart, biotic stresses constitute the major yield destabilizing factors. Of which, leaf blight, stalk rots, downy mildews and rusts among diseases contribute significantly to yield losses. There are about 16 diseases causing severe setback in the maize production. In India there are four downy mildews, four stalk rots, three foliar diseases, root rots and other diseases affecting kernel and other aerial parts. Stalk rots take a heavy toll, among which stalk rots caused by *Macrophomina phaseolina* (Tassi) Goid and *Fusarium moniliformae* are causing 30-40% losses (Anon ,1998). In India, Raju and Lal,1976 listed nine fungi and three bacterium while Payak and Sharma, 1983 have reported only eight fungi and three bacteria as causal agents of stalk rots of maize. But the post flowering stalk rot is caused by three fungi viz *Cephalosporium acremonium, Macrophomina phaseolina, Fusarium moniliformae* and one bacterium *Erwinia caratovora var Zeae* (Shankarlingam,2003 and Harlapur *et al.,* 2002).

Post flowering stalk rot caused by *Macrophomina phaseolina* commonly known as “charcoal rot” disease is influenced by high temperature, the optimum

being 38°C and low soil moisture conditions. A distinguishing character of the disease is the presence of the numerous small black sclerotia on the vascular fibers of the affected stalks. Small black sclerotia are found on the affected stalks (Holiday and Punithlingam 2005).The infected stalks when split longitudinally a mass of fibers is observed. The longitudinal cutting of affected stalks reveals horizontal blackening of vascular bundles that hampers conduction of water and nutrients leading to death of the plant in dry conditions. As this disease occurs after flowering i.e at grain filling stage, economic losses are more. There has been mean reduction of 18.7% in cob weight and 11.2%in 1000 grain weight in plants infected with stalk rot (Cook, 1973).There are number of methods available to control this disease. However, exploitation of host plant resistance appears to be the reliable economic method of disease management. But, in conventional breeding for selection of resistant plant, creation of disease through artificial inoculation is the basic step which also requires congenial weather for disease development, also large populations have to be screened which is time consuming and labour oriented. These situations call for expeditious development and deployment of alternative approaches to identify resistant sources quite at early stage of development without waiting till it's late reproductive stage at which the disease appears. In such situations molecular marker technology is of great help offering reliable solutions.

Keeping the foregoing in view the present investigation was undertaken with the objective of identifying molecular markers linked to the gene for resistance to post flowering stalk rot (PFSR)

In the present study, BPPTI-66 which was reported as susceptible variety and while BPPTI-34 as a resistant variety as per the scale given by Payak and

Sharma,1983 were selected as parents for developing mapping populations for tagging of post flowering stalk rot resistance genes. ingam and Venkatesh, 2005 developed 21 agronomically desirable inbred lines resistant to PFSR with disease rating of 2-5 on a 1-9 scale (1=immune 9= susceptible). In the present study, parents P_1, P_2, first filial generation (F_1), second filial generation (F_2), and backcross generation (BC_1F_1) were evaluated for post flowering stalk rot under field conditions using the standard disease screening methodology. Phenotypic evaluation of the mapping populations (F_2, BC_1F_1) along with the parents for post flowering stalk rot resistance reveals that there are significant differences in the individual plants for their reaction against post flowering stalk rot.

In the present investigation, inheritance of post flowering stalk rot resistance was studied in the segregating generations (F_2, BC_1F_1) of the cross between BPPTI-66 and BPPTI-34. The female parent BPPTI-66 was highly susceptible to post flowering stalk rot whereas, the corresponding male parent BPPTI-34 was resistant to post flowering stalk rot. The resultant F_1 was resistant indicating resistance appears to be a dominant character. F_1 was selfed as well as backcrossed to the susceptible parent to obtain F_2 and BC_1F_1 generations respectively. In the F_2 and BC_1F_1 populations ,segregation ratios of 3 : 1 and 1:1 (resistant: susceptible) respectively were observed indicating that the resistance to *Macrophomina phaseolina* is governed by a single dominant gene and the resistance was from the resistant parent BPPTI-34 which is in agreement with the finding by Yang *et al.*, (2005). Yang *et al.*, (2005) reported that the resistance to maize stalk rot appeared to be controlled by one dominant gene when inoculated with a single purified pathogen, but seemed to be controlled by multiple genes when inoculated with mixed

pathogens. Toman and White (1993) inoculated their F_1, F_2 and BC_1F_1 maize populations with a conidial suspension of *Colletotrichum graminicola* and concluded that a single dominant gene could best explain the obtained data. Hence, the present finding that the resistance to post flowering stalk rot caused by *M.phaseolina* is controlled by a single dominant gene is in line with the earlier findings as the disease was the result of inoculation by a single pathogen i.e *Macrophomina phaseolina*. Earlier Cheng and Song (1999) reported similar finding in maize i.e resistance to stalk rot caused by *F.graminearum* was a single gene controlling trait.

In the recent past, molecular marker technology has greatly facilitated the identification and mapping of genes for precise manipulation of agronomic traits in crop plants. DNA from 10 resistant plants and 10 susceptible plants each from both populations (F_2 and BC_1F_1) were collected and used in bulk segregant analysis. (Michelmore *et al.*,1991).

Out of 150 SSR markers screened, 54 SSR's were detected to be polymorphic between the parents amounting to a polymorphism percentage of 36.5 %. (fig 10) The polymorphic markers were used to screen bulks collected from 10 resistant plants and ten susceptible plants following Bulked segregant analysis (Michelmore *et al.*, 1991).

In the present study the marker umc1269 on chromosome 1 clearly expressed polymorphism between the susceptible and resistant bulks as well as in parents. Yang *et al., (*2004) carried out genetic analysis and molecular mapping of resistant gene to post flowering stalk rot caused by *Fusarium graminearum* through BSA from F_2 segregating population and concluded that the resistant gene *Rfg1* was tagged and mapped to chromosome 6 using molecular markers between two linked SSR marker

mmc0241 and RFLP marker bnl3.03 with a genetic distance of 3.ocM and 2.0cM respectively .

Yang *et al.* 2005 concluded that stalk rot resistance is governed by single dominant gene. They also mapped the gene for resistance to stalk rot caused by *Pythium inflatum* using molecular markers (RFLP, SSR &RAPD) by performing BSA from resistant and susceptible bulks of F_2 They mapped the gene *Rpi*l on chromosome 4, in between the SSR markers bnlg 1937 and agri 277 at 1.6 cM away from the former and 4.1 cM distant from the latter.

From literature it is understood that umc 1269 is present near to the gene that produce Glutathione S-transferase (GST) an enzyme reported for chemical defense in plants (fig 11) (Kathleen 1988). Investigation is necessary to confirm the candidate gene for (PFSR). When we searched the maize genome database for candidate genes near the tightly linked marker umc 1269 for post flowering stalk rot (PFSR), few genes related to disease resistance were observed *viz.,* gst12 (glutathione S-transferase12), prc3 (proteasome component3),smt2 (sterol methyl transferase2) of which, Glutathione-S-transferase enzyme reported to be involved in plant defense mechanism.(Kathleen, 1988)

More markers on the chromosome1 near the umc 1269 are to be used to map the gene for post flowering stalk rot. In addition, advanced mapping populations like recombinant inbred lines or backcross populations needs to be developed to exactly pinpoint the gene(s) under lying post flowering stalk rot. Also, maize genome sequencing information can be used to further identify the candidate gene(s) for post flowering stalk rot. After identification the post flowering stalk rot gene can be transferred to the popular inbred lines to develop post flowering stalk rot resistant hybrid.

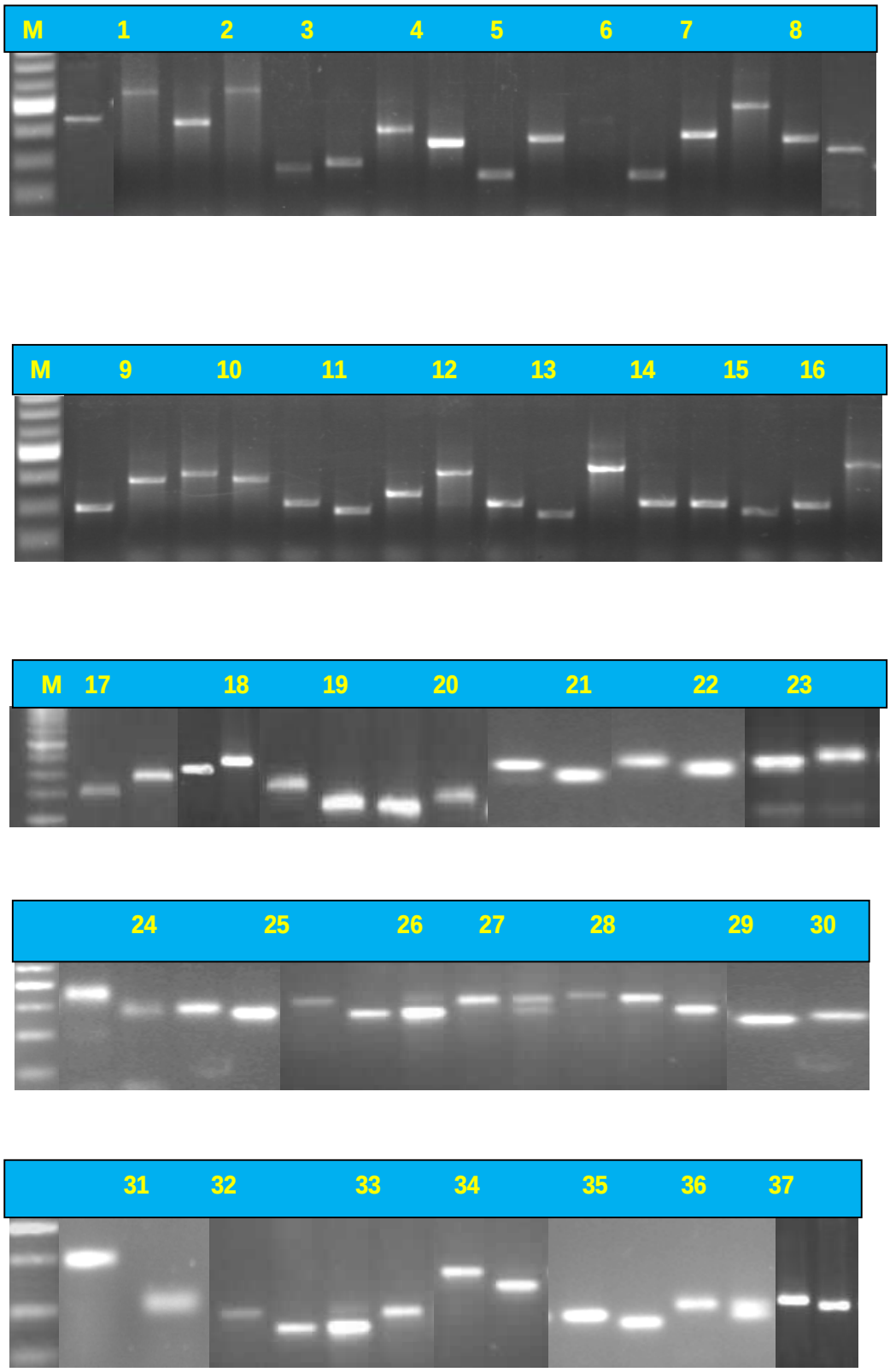
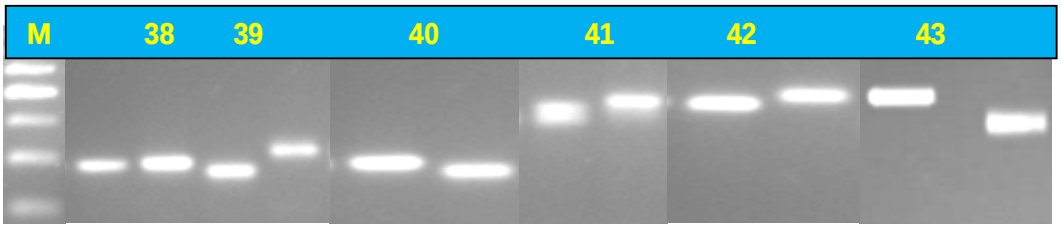
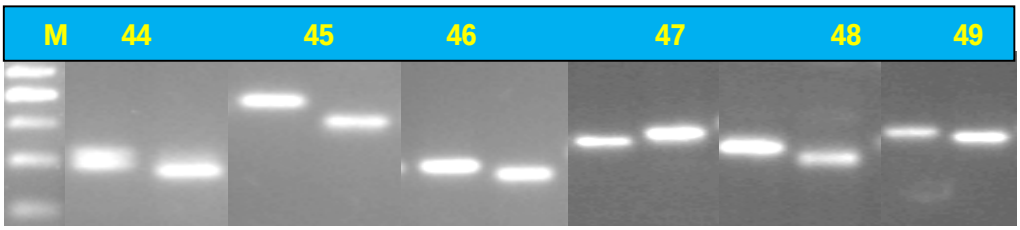


Fig.10: Parental polymorphism between BPPTI-66 (susceptible parent)and BPPTI-34(resistant parent) with maize specific SSR's





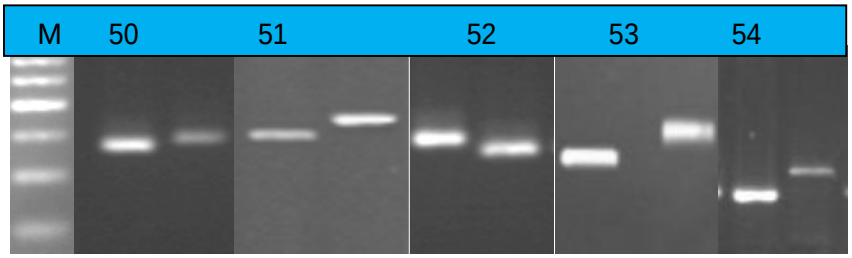


Fig.10 Parental polymorphism between BPPTI-66 (susceptible parent)and BPPTI-34(resistant parent) with maize specific SSR's

1.bnlg2204 2. umc1812 3.umc1071 4.umc1314 5. umc1363 6. umc1661 7. umc1622 8.umc1419 9.umc 1776 10.umc2214 11.bnlg2248 12.umc1542 13.umc1756 14. umc2118 15.umc2276 16.umc2377 17.bnlg1647 18.umc1773 19. umc2265 20.umc1008 21.bnlg2291 22.umc1682 23.umc1490 24.umc2027 25.umc1109 26.bnlg389 27.umc1097 28.umc1221 29.umc1002 30.umc1002 31.umc1177 32.umc1269 33.bnlg1136 34.umc2006 35.umc1379 36.umc2322 37.mmc0411 38.phi116 39.umc1159 40.bnlg2259 41.bnlg1823 42.umc1075 43.umc2146 44.umc1846 45.umc1370 46.umc1137 47.umc2336 48.umc1231 49.umc1310 50.umc1380 51.umc1645 52.umc2304 53.bnlg1074 54.umc2021

Fig.10: Position of umc1269in maize genome(Maize database)

CHAPTER VI

SUMMARY

Significant improvement of the yield in any crop is possible either through development of high yielding varieties/ hybrids or through breeding for tolerant/ resistant varieties/hybrids by the way of cutting down yield losses due to biotic and abiotic stress. In maize, the yield loss due to post flowering stalk rot (PFSR) is to an extent of 30-40%. Though this disease can be managed by chemical methods, breeding resistant varieties is the most economic and environmentally safe option. Breeding for resistance requires a very basic step of screening for pest and disease damage. For this, situation is to be created for development of pest/disease that to on waiting for the proper stage of pest/ disease attack. At this situation, the application of molecular markers play an important role in identifying pest/disease resistant cultivar through already available designed marker (MAS). This technique has already been employedin crop improvement for pest/disease resistance *viz.*, breeding for BLB resistance in rice, charcoal rot resistance in sorghum and downy mildew resistance in bajra. In the present study, an attempt has been made to identify a marker that is closely linked to the gene for resistance to the PFSR caused by *Macrophomina phaseolina* employing bulked segregant analysis. Maize line BPPTI-34 resistant to the PFSR and a susceptible line BPPTI-66 were used to develop mapping populations F_2 and BC_1F_1 . P_1, P_2, F_1, F_2 and BC_1F_1 generations were inoculated with the *Macrophomina phaseolina* culture. The following are the salient findings from this study.

All F_1 Plants shown resistant reaction with the disease score of 2 indicating hat the resistance is governed by single dominant gene.

- The F_2 and BC_1F_1 populations derived from the cross BPPTI-66×BPPTI-34 shown Mendelian segregation ratio of 3:1 and 1:1 respectively for disease reaction (resistance: susceptible). This confirms that the resistance is controlled by a single dominant gene.
- A total of 150 SSR markers were used to screen parents for polymorphism which were selected randomly from all over the genome. Out of these , 54 SSR markers were found polymorphic between two parents with a polymorphic percentage of 36. Out of these 54 polymorphic primers the SSR primer umc 1269 on chromosome 1 clearly distinguished between resistant and susceptible bulks as that of resistant and susceptible parents.
- The marker umc 1269 is tightly linked to the gene that produces glutathione-S-transferase enzyme which is responsible for plant defense.

The study broughtout the fact that the resistance to PFSR of maize caused by *Macrophomina phaseolina* is a single dominant gene controlled trait. The gene for resistance to PFSR is located on chromosome 1 of maize as SSR primer umc 1269 on chromosome 1expressed cosegregation with disease reaction.

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***Originals are not seen**

The literature cited as per the guidelines of ANGRAU

APPENDIX I

LIST OF CHEMICALS USED

1. Ethanol
2. Agarose
3. Bromophenol blue dye (sigma)
4. Chloroform (Qualigens)
5. CTAB
6. dNTP's (Deoxy nucleoside triphosphate) (Genei)
7. EDTA (Ethylene diamine Tetra Acetic Acid) (Himedia)
8. Ethidium bromide (10 mg/ml) (Sigma)
9. Icecold isopropanol
10. Isoamyl alcohol
11. Lamda Hind III marker
12. NAOH pellets
13. Phenol
14. RNase A
15. Sterile distilled water
16. Sucrose
17. Taq polymerase
18. Tris base
19. 50 bp DNA Marker

APPENDIX II

BUFFERS AND STOCK SOLUTIONS

CTAB buffer 100ml

2.0 g CTAB (Hexadecetyl trimethyl- ammonium bromide)

10.0 ml M Tris pH 8.0

4.0 ml 0.5 M EDTA pH 8.0 (Ethylenediamine tetraacetic acid Di-sodium salt)

28.0 ml 5 M Nacl

40.0 ml H_20

1g pvp 40 (Polyvinyl pyrrolidone- vinyl pyrrolidine homopolymer MW 40,000)

Adjust all to pH 8.0 with HCL and make up to 100 ml with H_2O

0.5 M Tris Buffer (pH 8.0)

Dissolved 60.55 g of Tris base in 400 ml of distilled water. Adjust pH to 8.0 by adding HCL. Adjust the volume to 500 ml with H20. Sterilize using an autoclave.

1M EDTA (Ethylenediamine tetra acetic acid)

Dissolve 186.1 grams of EDTA, free acid in about 200 ml of distilled water. Adjust the pH to 8.0 with NAOH and make up the vol to 500 ml with distilled water. Sterlized by autoclaving.

Phenol: Chloroform: Isoamyl alcohol (25:24:1)

Equal parts of equilibrated phenol and chloroform: isoamyl alcohol (24:1) were mixed and stored at room temperature.

TAE buffer (Tris/acetate/EDTA) 50X stock solution

242 g Tris base
57.1 ml Glacial acetic acid
100 ml 0.5 M EDTA (pH 8.0)

Adjust the pH to 8.3 with acetic acid and make up the volume to 1 lit with distilled water. Sterilize by autoclaving.

TE buffer (pH 8.0)

10 mM Tris HCL

1 mM EDTA.

Mix 2 ml of 0.5 M Tris-Cl pH 8.0 with 0.2 ml of 0.5 EDTA, make up the vol to 100 ml with sterile distilled water.

6X Gel loading buffer

0.25% (W/V) Bromo phenol blue

40% (W/V) sucrose in water

RNase preparation

RNase buffer

A.1M Tris (pH 7.5)

B. 5M NaCl

Take 0.5ml of 1M Tris (final concentration 10mM) and 75 µl of 5M NaCl (final concentration 15mM) and make up the volume to 50 ml. Weigh 25 mg of ribonuclease A into a tube and add RNase buffer to a final volume of 5 ml (so final concentration 5mg/ml). keep the tube in a boiling water bath for 10 min, cool and make aliquots of 1 ml in 1.5 ml Eppendorf tubes and store at -20^0c.

PCR components

The PCR components were mixed as given below to give a final reaction volume of 10 µl per sample. For every 5 samples, one extra master mix was made in order to avoid the loss due to pipetting error.

Master Mix (35 reactions)

Components	**Stock concentration**	**Quantity required for 1 reaction (µl)**	**Quantity required for 35 reactions (µl)**
Sterile distilled water	-	5	175
PCR buffer	10 x	1.0	35
dNTP's	2.5 mM	1.0	35
Primer F	2.5pmoles	0.4	14
Primer R	2.5pmoles	0.4	14
Taq DNA Polymerase	1 U / µl	0.2	7
		8.0 µl	280.0 µl

Template DNA – 2.0 µl (20ng/µl)

APPENDIX-III

MATERIALS / EQUIPMENTS USED

1. Micropipettes and tips
2. Microcentrifuge
3. Thermal cycler (PCR machine)
4. PCR strips
5. Gel casting unit, gel trays
6. Power supply unit
7. Microwave oven
8. Electronic balance
9. Gel documentation system including UV trans-illuminator(Geneflash; SYNGENE)
10. Agarose gel electrophoresis system (CBS Scientific).