MOLECULAR TAGGING AND MAPPING OF RESISTANCE TO LATE LEAF SPOT AND RUST IN GROUNDNUT (*Arachis hypogaea* L.)

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GENETICS AND PLANT BREEDING

By
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CERTIFICATE

This is to certify that the thesis entitled **"MOLECULAR TAGGING AND MAPPING OF RESISTANCE TO LATE LEAF SPOT AND RUST IN GROUNDNUT (*Arachis hypogaea* L.)"** submitted by **Mr. YOGENDRA P. KHEDIKAR,** for the degree of **DOCTOR OF PHILOSOPHY** in **GENETICS AND PLANT BREEDING**, to the University of Agricultural sciences, Dharwad is a record of research work done by him during the period of his study in this university under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

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Affectionately Dedicated to

The Almighty &
Beloved Parents

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Khedikar

YOGENDRA P. KHEDIKAR.

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ABBREVIATIONS

QTL	: Quantitative Trait Loci
LOD	: Log of odd ratio
cM	: centiMorgan
µl	: Microliter
U	: Unit
pM	: Picomoles
mM	: Micromoles
ng	: Nanogram
PAGE	: Polyacrylamide gel electrophoresis
LG	: Linkage group
R^2	: Phenotypic variance
RIL	: Recombinant Inbred Line
SSD	: Single seed descent
LLS	: Late leaf spot
E1	: *Kharif* 2004
E2	: *Kharif* 2005
E3	: *Kharif* 2006
E4	: *Kharif* 2007
S1	: 70 days to score LLS
S2	: 90 days to score LLS
S1	: 70 days to score Rust
S2	: 90 days to score Rust
LLN	: Leaf length
LWD	: Leaf width
PLHT	: Plant height
PBR	: Number of primary branches
PPP	: Pods per plant
YPP	: Yield per plant
YKGH	: Plot yield
HSW	: 100 seed weight
SLNG	: Shelling percentage

Introduction

I. INTRODUCTION

Groundnut (*Arachis hypogaea* L.), also known as peanut, is an important oilseed crop in tropical and subtropical regions of the world. It is a native of South America and grown in six continents but mainly in Asia, America and Africa in over 100 countries with a world production of 35.9 m t from an area of 25.2 m ha (FAO, 2006). China, India and USA are the major producers of the crop. Though, India is a leading producer of the crop but productivity is lower (913 kg/ha) than the USA (2863 kg/ha) and China (2645 kg/ha).

Groundnut seed is an important source of oil (44-50%), dietary protein (25%) and carbohydrate (20%). Groundnut haulms are excellent fodder (Cook and Crosthwaite, 1994) and cake is used for animal feed (Savage and Keenan, 1994). Plant roots left behind after harvest add valuable nutrients to the soil, which is particularly important in the less developed countries where crop is mainly grown under low input condition.

The cultivated tetraploid groundnut ($2n = 4x = 40$) is member of genus *Arachis* and belongs to the family *Leguminosae*, subfamily *Fabaceae*, tribe *Aeschynomeneae*, subtribe *Stylosanthenae* (Krapovickas and Gregory, 1994). Based on the differences in the branching pattern and presence of reproductive node on the main stem, the species has been classified into two subspecies, *hypogaea* and *fastigiata* (Krapovickas and Rigoni, 1960). Further each subspecies has been divided into two

botanical varieties viz. subsp. *hypogaea* into var. *hypogaea* (virginia) and var. *hirsuta* and subsp. *fastigiata* into var. *fastigiata* (valencia), var. *vulgaris* (spanish), var. *peruviana* and var. *aequatoriana* (Karpovickas and Gregory, 1994).

Arachis hypogaea is believed to have originated in South America via hybridization of two diploid wild species (*A. duranensis and A. ipaensis*) followed by rare spontaneous duplication of the chromosomes (Halward *et al.*, 1991; Young *et al.*, 1996). The resultant allotetraploid plant would have had hybrid vigor but reproductively isolated from wild relatives. Therefore, all land races of groundnut are probably derived from one or a few plants and consequently low diversity for traits of agricultural interest exists, and a narrow genetic base of the cultivars, constraining progress of the crop at conventional and molecular level. Paradoxically, the wild diploid *Arachis* species are genetically very diverse and have been selected during evolution by a range of abiotic and biotic stresses, providing a rich source of variation in agronomically important traits; but sterility barriers have hampered the use of wild species in breeding.

The low productivity of the crop in India is ascribed to many biotic and abiotic stresses in the cultivation of the crop. Among the biotic stresses, the two major foliar diseases viz., late leaf spot (*Phaeoisariopsis personata* [(Berk. and Curt.) Deighton] and rust (*Puccinia arachidis* Speg.) are widespread and economically most important. They often occur together and cause yield loss up to 50-70 per cent in the crop (Subrahmaniyam *et al.*, 1984). Besides adversely affecting the pod yield

and its quality, they affect the yield and quality of haulm. Though several effective fungicides are available to control the diseases, development of resistant cultivars is considered the best strategy to surmount additional cost of production and hazardous effect of fungicides on the soil and environment.

Identification of resistant and susceptible lines from the different sources of gene pools is difficult through conventional screening technique because of their co-occurrence and defoliating nature of late leaf spot. High levels of resistance to these diseases has been transferred from wild species to cultigen (Moss *et al.*, 1997; Nigam *et al.*, 1992; Reddy *et al.*, 1996; Reddy *et al.*, 1992; Stalker and Beaute 1993) but the conventional breeding has failed in combining resistance with cultivars having good agronomic traits. Resistant sources often suffer from undesirable traits like low productivity, long duration and poor adaptability besides poor pod and seed traits like thick shell and low shelling percentage. The complex nature of inheritance with recessive genes conferring resistance has hindered the progress of disease resistance breeding.

The advent of molecular markers has given some edge to the resistance breeding. Molecular markers are superior to morphological and protein makers. They are neutral, occur throughout the genome, not influenced by the environment, co-dominant, and monitored in any tissue and stage of the plant and often follow expected Mendelian segregation. Marker-assisted selection (MAS) can offer an effective and efficient breeding tool for detecting, tracking, retaining, combining, and pyramiding

disease resistance genes (Kelly and Miklas, 1998 and 1999). MAS can improve the efficiency of conventional breeding especially in the case of low heritable and recessive traits, where phenotypic selection is difficult, expensive, lack accuracy or precision (Crouch, 2001). Development of disease is mostly erratic and it varies according to season, location and year. Moreover creation of artificial disease epiphytotics is costly and time consuming and also availability of hot spot for a particular disease is one of the paramount factors for screening and MAS can act as an elixir in such circumstances. Identification of resistant or susceptible lines at seedling stage is possible, when MAS is employed. Linkage drag is also one of the serious problems while transferring resistance from unadapted wild and weedy germplasm into elite lines and it can be dissected out through tightly linked markers. It can help in the introgression of resistance from wild relatives and fastest recovery of the recurrent parent genome can be achieved by using foreground and background selection approach. Since resistance to LLS and rust is governed by recessive genes (Nevill, 1982; Kalekar *et al.*, 1984; Knauft, 1987; Paramsivam *et al.*, 1990; Motagi, 2001), MAS can save one generation of selfing to select recessive genes using linked markers. For efficient MAS, one requires germplasm with useful traits, suitable mapping population for the trait of interest, precise screening techniques and efficient marker system, which can detect higher levels of polymorphism.

Microsatellites or simple sequence repeat (SSR) markers are considered as potential markers of choice because they are hyper-variable

and co-dominant (Gupta and Varshney, 2000). They are more polymorphic than other type of markers in groundnut (Hopkins *et al.*, 1999; He *et al.*, 2003; Ferguson *et al.*, 2004; He *et al.*, 2005; Mace *et al.*, 2006; Nimmakayala *et al.*, 2007) and easy availability of SSR markers developed at various laboratories (Hopkins *et al.*, 1999; He *et al.*, 2003; Ferguson *et al.*, 2004; Moretzsohn *et al.*, 2004 and 2005; Mace *et al.*, 2007; Cuc *et al.* (unpublished); Bertioli *et al.* (unpublished); Knapp *et al.* (unpublished) made them important marker system to resolve higher level of polymorphism

Construction of genetic linkage map is an essential step for breeders in order to use molecular breeding strategies for improving biotic and abiotic stress resistance (Azhaguvel *et al.*, 2006) and further identification of potential genomic regions and transfer them into important cultivars. In *Arachis*, attempts have been made to construct linkage map in diploid (Halward *et al.*, 1993; Garcia *et al.*, 1995; Milla, 2003; Garcia *et al.*, 2005) and tetraploid (Burow *et al.*, 2001; Herselman *et al.*, 2004) species using RFLP, RAPD and AFLP markers. But genetic map based on breeder friendly SSR markers would be more useful for marker assisted selection. SSR based genetic linkage maps have been developed only in diploid wild species (Moretzsohn *et al.*, 2005; Gobbi *et al.*, 2006). Developing linkage map in the cultivated tetraploid (AABB) groundnut is urgently required to make progress in marker assisted selection.

In the present investigation, a mapping population (TAG 24 × GPBD 4) comprising 268 RILs in F_8 generation and segregating for LLS and rust resistance has been employed with the following objectives:

1. Phenotyping of the mapping population for resistance to late leaf spot (LLS) and rust besides various agronomic traits.
2. Genotyping of the RIL mapping population with SSR markers
3. Construction of genetic linkage map and
4. Identification of markers or QTL associated with resistance to LLS, rust and agronomic traits contributing to yield.

Review of Literature

II. REVIEW OF LITERATURE

Late leaf spot and rust are the most destructive, widely distributed and economically important foliar diseases of the groundnut causing severe damage to the crop (McDonald *et al.*, 1985; Kokalis Burelle *et al.*, 1997). They are commonly present wherever groundnut is grown but their incidence and severity vary between localities and seasons. Each disease alone is capable of causing substantial yield loss but when they occur together losses are further increased. For instance, rust and late leaf spot together can cause up to 70 per cent yield loss in India (Subrahmanyam *et al.*, 1984). These foliar diseases besides reducing the yield, also have an adverse effect on seed quality and grade characteristics, deteriorate the quality of plant biomass and thus render the fodder unsuitable as animal feed. Moreover, the control of these diseases through application of plant protection measures not only increases the cost of cultivation but also lead to environmental and health hazards. Hence, use of resistant cultivars is the best means of reducing crop losses.

Identification of resistance sources, knowledge of components, mechanism of resistance and the number of loci contributing to resistance are the prerequisites for the success of disease resistance breeding program (Dwivedi *et al.*, 2002). Several sources of resistance to LLS and rust have been reported in *A. hypogaea* (Waliyar *et al.*, 1993a; Anderson *et al.*, 1993; Mehan *et al.*, 1996; Singh *et al.*, 1997). Majority of resistant sources belong to subspecies *fastigiata* var. *fastigiata* and are

land races from South America (Subrahmanyam *et al.*, 1989). They posses a high degree of resistance to rust and moderate levels to LLS but have undesirable pods and seed characters hence were commercially unaccepted. Resistant sources in wild *Arachis* species show immune reaction to rust (Subrahmanyam *et al.*, 1983) and from immune to highly resistant reaction to LLS (Abdou *et al.*, 1974; Subrahmanyam *et al.*, 1985).

The cross compatibility barriers, the linkage of resistance with many undesirable pod and seed characteristics, complex nature of resistance to these diseases, and the long period of time required for developing stable tetraploid interspecific derivatives affected the success of transferring resistance to important diseases like rust and LLS from *Arachis* species to the cultivated groundnut. In spite of these obstacles, a few interspecific derivatives, ICGV 87165, GPNCW 1, GPNCW 2, GPNCW 3, GPNCW 4, ICGV 86699, ICGV 87167 possessing high levels of resistance to foliar diseases have been developed in India and USA (Nigam *et al.*, 1992; Stalker and Beaute 1993; Reddy *et al.*, 1996; Moss *et al.*, 1997) but these lines have not been released for cultivation due to agronomically undesirable traits like late maturity and inferior pod and seed characteristics in comparison with commercially grown cultivars.

Most of the released cultivars are susceptible to late leaf spot and rust diseases. A few cultivars with moderate resistance to these diseases have been released namely ICGV 87160 and ICGV 86590 in India (Reddy *et al.*, 1992), Southern Runner in USA (Gorbet *et al.*, 1987); ICGV-SM 86715 in Mauritius (Moss *et al.*, 1998); Yue You 223 in China

(Liang *et al.*, 1999). The longer duration and low shelling outturn hamper the adaptation of these cultivars by farmers. Progress in resistance breeding is limited because of absence of high levels of resistance in cultivated groundnut and the linkage of resistance with long duration, lower partitioning and with undesirable pod (highly reticulated, constricted, prominently ridged and conspicuously beaked pods with thick shells) and seed (purple or blotched seed color) characteristics (Wynne *et al.*, 1991; Singh *et al.*, 1997).

2.1 COMPONENTS OF RESISTANCE

Recognition of epidemiological components of rate-reducing resistance to foliar diseases of groundnut has provided a major strategy for current breeding efforts (Anderson *et al.*, 1990; Chiteka *et al.*, 1988; Johnson *et al.*, 1986).

Complex nature of resistance to leaf spots is reported (Kornegay *et al.*, 1980; Anderson *et al.*, 1986 and 1993; Green and Wynne, 1987; Iroume and Knauft, 1987; Jogloy *et al.*, 1987), and several components contribute to resistance, including initial infection, lesion size, sporulation, and defoliation (Green and Wynne 1986; Chiteka *et al.*, 1988; Anderson *et al.*, 1993; Waliyar *et al.*, 1993b). Resistance to leaf spot in groundnut has generally been associated with late maturity (Norden *et al.*, 1982; Miller *et al.*, 1990). Resistance to LLS in *A. hypogaea* is characterized by longer latent period, reduced sporulation of pathogen, and less defoliation on host (Nevill, 1981). Sporulation, lesion size, lesion number and latent period are important components of resistance to LLS

and are highly correlated to each other and with percentage of necrotic area on leaf (Chiteka *et al.*, 1988; Anderson *et al.*, 1990). Lesion diameter, defoliation and sporulation from glasshouse study are correlated with field disease score (Subrahmanyam *et al.*, 1982). Nevill (1981) observed longer incubation periods, fewer lesions and lower sporulation rates in the resistant genotypes as compared to susceptible genotypes and also reported high correlation among components of resistance and proposed polygenic systems acting to control the expression of all the components. Walls (1984) found that the latent period, sporulation and lesion diameter measured in the greenhouse as the most effective parameters for estimating field resistance to LLS. Motagi (2001) reported incubation period, lesion size and lesion on main-stem as the important components of resistance having strong association with field disease score, defoliation and remaining green leaf area. Percent defoliation had a highly significant positive association with field disease score (Dwivedi *et al.*, 2002). Resistance in *Arachis* species does involve inefficiency of inoculum to induce lesions (Foster, 1980; Subrahmanyam *et al.*, 1985).

The sources of resistance to rust in *A. hypogaea* exhibit component mechanisms that reduce the rate of disease development. Resistance to rust is attributed to longer incubation period, reduction in latent period, less number of pustule, lesion size, smaller pustule, less ruptured pustules and reduced damage to leaf area (Subrahmanyam *et al.*, 1983; Reddy and Khare, 1988; Mehan *et al.*, 1994). Infection frequency, pustule diameter, percent ruptured pustule, leaf area damage are correlated to

each other and with mean field rust score. The incubation period is negatively correlated with other components. In contrast, the characterized sources of resistance in wild *Arachis* species and their interspecific derivatives have more dramatic effects on the pathogen. In particular, uredosori are observed to be very small on the accessions of wild *Arachis* species and are slightly depressed and do not rupture their uredospores (Subrahmanyam *et al.*, 1983).

2.2 GENETICS OF RESISTANCE

Resistance to LLS is reported as partial type and is similar to "slow rusting" type of resistance. Nevill (1982) proposed that resistance to LLS is controlled by multiple recessive genes. Motagi (2001) reported duplicate recessive genes controlling resistance to LLS and favorable resistance alleles came from inter-specific sources like CS 16 (ICGV 86855). Resistance to LLS has been reported to be determined by two genes (Tiwari *et al.*, 1984) and five-locus recessive genes in the crosses involving cultivated groundnut and wild *Arachis* species (Sharief *et al.*, 1978). Other studies report predominantly additive genetic variance for most of the components of resistance to LLS (Kornegay *et al.*, 1980; Hamid *et al.*, 1981; Anderson *et al.*, 1986; Jogloy *et al.*, 1987).

Resistance to rust in *A. hypogaea* is conferred either by a few recessive genes (Kalekar *et al.*, 1984; Knauft, 1987; Paramsivam *et al.*, 1990) or predominantly controlled by additive, dominance and additive × additive and additive × dominance genetic effects (Reddy *et al.*, 1987; Verman *et al.*, 1991). Motagi (2001) reported resistance to rust conferred

by duplicate complementary recessive genes (9:7), while Singh *et al.*, (1984) concluded that rust resistance in diploid species is partially dominant as compared to the recessive resistance in *A. hypogaea*.

2.3 PROBLEMS IN CONVENTIONAL RESISTANCE BREEDING

Conventional plant breeding have had limited success in enhancing genetic resistance against diseases due to lack of genetic information and complexity of genome. Genetic studies on LLS and rust revealed that resistance is mostly controlled by recessive genes hence necessitating more generation and large population to identify resistant segregants. Further, when the disease occurs together they interfere with each other, leading to difficulties in identification of resistant lines to these diseases. Transfer of resistance to these diseases from land races and wild relatives to cultivated background is difficult due to linkage drag *viz.*, undesirable traits like thick shell, low yield, poor adaptability and long duration are associated with resistance. Under these circumstances, newly emerging biotechnological tools like marker assisted selection can play crucial role in the success of disease resistance breeding.

2.4 NEED FOR MARKER ASSISTED SELECTION IN GROUNDNUT

Groundnut is predominantly an inbreeding crop so the most commonly used breeding methods are pedigree selection, bulk-pedigree selection, and single seed descent. Backcross breeding has not been extensively utilized because most of the economically important traits in the crop have complex inheritance pattern. (Wynne and Gregory, 1981;

Knauft and Wynne, 1995). Marker assisted selection (MAS) can improve the efficiency of conventional plant breeding. Molecular markers are advantageous for traits where conventional phenotypic selection is difficult, expensive, or lacks accuracy and include resistance to pests and diseases as well as tolerance to abiotic stresses, quality parameters and complex agronomic traits with low heritabilities (Crouch, 2001). Some of the traits that justify application of MAS are early and late leaf spot, rust, nematode resistance, leaf minor and *Spodoptera* where low to moderate levels of resistance is available in cultivated species of groundnut but very high levels of resistance is present in wild species and also traits which are associated with seed quality like oleic / linoleic ratio (O/L ratio), and drought tolerance (water use efficiency, partitioning, specific leaf area and total transpiration) which are difficult to measure in large segregating generations and substantially influenced by genotype by environment interactions, may also benefit from markers assisted selection (Dwivedi *et al.*, 2002). The molecular markers can help in pyramiding of important resistance genes through increased adoption of backcross breeding. MAS can also assist in the acceleration of recapitulation of the genome and introgression of important traits from the wild relative sources.

The conventional breeding methods can investigate the genetic control of quantitative traits such as yield and yield contributing traits in a segregating population. (Falconer, 1981; Hallauer and Miranda Fo,1988), although valuable but insufficient to provide information on, chromosomal regions regulating the variation of each trait, the simultaneous effects of

each chromosome region on other traits and genetic basis (pleiotropy and /or linkage) of such associated traits. Some of these constraints can be overcome by using molecular markers which not only allow for the identification of quantitative trait loci (QTL) associated with these traits but also enable us to assess the effects of the same QTL region on other traits (Tanksley, 1993; Prioul *et al.*, 1997) and more importantly on yield (Stuber *et al.*, 1987 and 1999).

2.5 ADVANTAGES OF MAS IN RESISTANCE BREEDING

Molecular markers are useful in disease resistance breeding as they can substitute phenotypic screening in the early phase of breeding program and to identify resistant lines at juvenile stage to save time and cost of screening. It helps in easy identification and transfer of recessive genes and to monitor alien gene introgression, reduces the linkage drag and aids in eliminating undesirable traits in much shorter time frame than those expected through conventional breeding programs. It facilitates map-based cloning of disease resistance genes and pyramiding of genes for multiple disease resistance in a single cultivar, faster recovery of the recurrent parent genome in the backcross breeding programme (Tanksley *et al.*, 1989). It could also reduce the need for phenotypic selection that may be inappropriate in identifying genotypic differences and in selection of rare recombinants between tightly linked resistance genes.

2.6 REQUIREMENT OF MAS

Molecular markers offer great scope for improving the efficiency of conventional plant breeding. The essential requirements for developing

MAS system are (i) availability of germplasm with substantially contrasting phenotypes for the traits of interest, (ii) highly accurate and precise screening techniques for phenotyping mapping population for the trait of interest,(iii) identification of flanking markers closely associated with the loci of interest and the flanking region on either side and (iv) simple robust DNA marker technology to facilitate rapid and cost-effective screening of large population (Paterson *et al.*, 2004).

2.7 MOLECULAR MARKERS STUDIES IN GROUNDNUT

The cultivated groundnut has been analyzed by several marker system including RFLPs, RAPDs, (DAF and SCAR), AFLPs and Microsatellites; variation has been observed among diverse genotypes in approximately 5 per cent of the marker analyzed, but the number is much lower between pairs of *A. hypogaea* lines.

2.7.1 Restriction Fragment Length Polymorphism (RFLPs)

RFLPs represented the first marker system that detected large number of polymorphisms. RFLPs are produced by digesting genomic DNA with restriction endonucleases that recognize specific sites on a DNA sequence and then cleave the DNA strand in or near recognition sites of the sequence. Fragments thus produced can be separated by size on a gel electrophoresis plate. Plants often produced so many fragments that the resulting gel is not interpretable. For complex genomes, a probe is made from cloned DNA that is homologous to a specific DNA sequence in the species being investigated (Botstein, 1980). Radioactivity is used to label

probes and bands are visualized when the unhybridized radioactivity is washed away and then an autoradiograph is produced. RFLPs are robust, reliable and transferable across populations but it is time consuming, laborious, expensive and large amount of DNA is required

In *Arachis*, Kochert *et al.* (1991) observed very low level of RFLP variability among the allotetraploids U.S cultivars and *Arachis monticola*, a wild species. RFLPs revealed very low level of variability in unadapted germplasm lines though considerable morphological and physiological variability existed among the lines (Halward *et al.*, 1991). Paik-Ro *et al.* (1992) assessed Restriction Fragment Length Polymorphisms among accessions within six groundnut species of the *Arachis* section and observed significant amount of variation present among the *Arachis* species and *Arachis monticola* was found to be more closely related to *A. hypogaea* subspecies *hypogaea* than to subspecies *fastigiata*. Kochert *et al.* (1996) observed no variation between *A. hypogaea* and *A. monticola*. RFLPs have been used to analyse the species in the section *Arachis* and cluster that formed (Kochert *et al.*, 1991) corresponded closely with morphological groups (Stalker, 1990). Stalker *et al.* (1995) used RFLPs to study genetic diversity among eighteen accessions of *A. duranensis* Krapov. Abd W.C.Gregory and found large amount of variation in the species. Based on RFLP analysis Kochert *et al.* (1996) concluded that the cultivated groundnut resulted from the cross between *A. duranensis* and *A. ipaensis* Krapov. and W.C.Gregory, and chloroplast analysis indicated that *A. duranensis* was the female progenitor. Gimenes *et al.* (2002) used

RFLPs to study genomic relationship between AA genome, BB genomes and AABB genome species. The lowest genetic variation was detected within accessions of *A. duranensis* (17 accessions), followed by *A. batizocoi* (4 accessions) and *A. cardenasii* (9 plants of accession GKP 10017).

2.7.2 Random Amplified polymorphic DNAs (RAPDs)

The assay developed by Williams *et al.* (1990) which detects nucleotide sequence polymorphisms in DNA by using a single primer of arbitrary nucleotide sequence. RAPDs are quick, simple, inexpensive, multiple loci from a single primer is possible and small amount of DNA is required to carry out this assay but is less popular due to problems such as poor reproducibility and transferability, faint or fuzzy products, and difficulty in scoring bands, which lead to inappropriate inferences.

Halward *et al.* (1992) used primers of arbitrary sequence to study variability in germplasm and reported very little variation in *Arachis* species and also proposed dominant behavior of the markers prevented the differentiation of heterozygotes from homozygotes with certainty, limiting the usefulness of arbitrary primer amplification products as markers in the construction of a genetic linkage map in groundnut. Lanham *et al.* (1992) detected significant amount of variation (81.66%) between *A. hypogaea* and synthetic amphidiploid using RAPD. Hilu and Stalker (1995) observed maximum variation among accessions of *A. cardenasii* and *A. glandulifera* whereas the least amount of variation was observed in *A. hypogaea* and *A. monticola* and based on RAPD assay proposed that *Arachis duranensis* was most closely related to the

domesticated groundnut and is believed to be the donor of the A genome. Bhagwat *et al.* (1997) observed 5.5 per cent polymorphism and were able to detect variation among the different plant height mutants and pod size mutants by using RAPD assay. Bhagwat *et al.* (2001) reported high degree of polymorphism among closely related 14 groundnut genotypes using single RAPD primers.

Subramanian *et al.* (2000) studied RAPD differences among 70 selected genotypes representing variability for several morphological, physiological, and other characters with 48 primers. Of these, seven (14.6%) yielded polymorphic amplification products. Dwivedi *et al.* (2001) assessed genetic diversity among 26 accession using eight RAPDs. The genetic similarity (S_{ij}) ranged from 59.0 to 98.8 per cent, with an average of 86.2 per cent and identified five accessions with diverse profiles for mapping and genetic enhancement studies. Five accessions with diverse DNA profiles were identified for mapping and genetic enhancement in groundnut.

2.7.3 Amplified Fragment Length Polymorphism (AFLPs)

The assay is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. The technique involves three steps, restriction of the DNA and ligation of oligonucleotide adapters, selective amplification of sets of restriction fragments, and gel analysis of the amplified fragments. PCR amplification of restriction fragments is achieved by using the adapter and restriction site sequence as target sites for primer annealing. The selective amplification is achieved by the use of

Arachis species and can also detect high level polymorphism than RAPDs and RFLPs.

Milla *et al.* (2005) used the AFLP technique to determine intra- and interspecific relationships among and within 108 accessions of 26 species of *Arachis* section and revealed that A-genome accessions KG 30029 (*Arachis helodes*) and KSSc 36009 (*Arachis simpsonii*) and B-genome accession KGBSPSc 30076 (*A. ipaensis*) were the most closely related to both *Arachis hypogaea* and *Arachis monticola*. This finding suggests their involvement in the evolution of the tetraploid groundnut species.

2.7.4 Simple Sequence Repeats (SSRs) or Microsatellites

This is an extensively used marker system and detects highest polymorphism in groundnut. Among the different classes of molecular markers, SSR markers are often chosen as the preferred markers for a variety of applications in breeding because of their multi-allelic nature, co-dominant inheritance, relative abundance and extensive genome coverage (Gupta and Varshney, 2000). This method includes DNA polymorphism using specific primers designed from the sequence data of a specific locus. Primers complementary to the flanking regions of the simple sequence repeat loci yield highly polymorphic amplification products (Weber *et al.*, 1989). Polymorphisms appear because of variation in the number of tandem repeats (VNTR loci) in a given repeat motif. This method is technically simple, robust, reliable and transferable between populations. Large amount of time and labour required to

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generate primers and usually it require polyacrylamide gels to resolve bands.

Hopkins *et al.* (1999) captured 26 microsatellites from the groundnut genomic DNA library and observed 23% polymorphism in collection of 22 groundnut DNAs, representing both cultivated and wild species. Raina *et al.* (2001) used twenty-one RAPD and 29 ISSR primers to assess genetic variation and interrelationships among subspecies and botanical varieties of cultivated groundnut and phylogenetic relationships among cultivated groundnut and wild species of the genus *Arachis*. Both random and ISSR primers revealed 42.7 and 54.4 per cent polymorphism, respectively This study strongly support the view that *Arachis monticola* ($2n = 4x = 40$) and *A. hypogaea* are very closely related, and indicate that *A. villosa* and *A. ipaensis* are the diploid wild progenitors of the tetraploid species.

He *et al.* (2003) isolated 56 different microsatellites by using SSR enrichment procedure and observed 33.9 per cent of polymorphism among th· genotypes suggesting higher level of DNA polymorphism by these markers than other DNA markers in cultivated groundnut. Moretzsohn *et al.* (2004) screened 67 TTG SSR markers to study polymorphism of seven accessions and observed only 4.4 per cent polymorphism in cultivated groundnut. Ferguson *et al.* (2004) generated 110 sequence tagged microsattelites sites (STMS) markers for the cultivated groundnut and in there study 81 per cent of $(ATT)_n$ and 70.8 per cent of $(GA)_n$ showed polymorphism in groundnut. Krishna *et al.* (2004)

has shown molecular diversity using microsatellite markers in the cultivated Valencia groundnut (subspp. *fastigiata*) and results indicated that considerable genetic variations was present among the analyzed genotypes. He *et al.* (2005) have developed 130 simple sequence repeat (SSR) markers in groundnut and observed 29.23 per cent polymorphism among 24 groundnut accessions. Eight SSR markers were found useful to classify botanical varieties. Mace *et al.* (2006) screened 23 SSR markers across 22 groundnut genotypes with varying levels of resistance to rust and late leaf spot and showed 52 per cent polymorphism with PIC value ≥0.5.

Bravo *et al.* (2006) evaluated the transferability of microsatellite primers and the assay of genetic variability between and within the germplasm of some species of the *Arachis* section and reported 78 per cent were found to be polymorphic. All loci had transferability to all the species analyzed. Upadhyaya *et al.* (2007) studied genetic diversity in composite collection containing 916 accessions with 21 SSR markers and revealed considerable variation among the accessions (0.819 PIC value; 490 alleles) A total of 101, 50, 11.group-specific unique alleles in wild *Arachis*, *fastigiata* and *hypogaea*, respectively were identified. Clustering of different genotypes into *fastigiata*, *hypogaea* and wild spp was observed and based on common origin, some of the accessions from *fastigiata* grouped with *hypogaea*. Kottapalli *et al.* (2007) used 73 microsattelite markers to genotype 72 accessions from the US groundnut minicore.

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Moderate levels genetic variation was found and the genetic distance values (D) ranged from 0.88 to 0.254.

Nimmakayala *et al.* (2007) used 96 SSR primers to screen 30 species representing A, B and D genomes of *Arachis* with various ploidy levels (18 diploid, 9 tetraploid and one aneuploid) along with two cultivated groundnut varieties. Of these, 50 (52.08 %) were found to be polymorphic. Tang *et al.* (2007) assessed the genetic variation from the four sets of 24 accessions each from the four botanical varieties of the cultivated groundnut using 34 microsatellites. Among these accessions, 10-16 pairs of microsattelites primers showed polymorphism. Barkley *et al.* (2007) studied diversity and phylogenetic relationship among groundnut species by using 31 microsatellites with attached M13 tail, which consists of all but one of the 112 accession from the minicore. A total of 477 alleles were detected in this data set with an average of 15.4 alleles per locus. The mean Polymorphism Information Content (PIC) score was 0.687. Gimenes *et al.* (2007) isolated thirteen microsatellite loci and characterized 16 accessions of *A. hypogaea*. The level of variation found in *A. hypogaea* using microsatellites was higher than with other markers. Cross transferability of the markers was also high and found same repeated sequence in almost all the wild species as in *A. hypogaea* after sequencing of amplified fragments.

The studied markers systems in groundnut revealed very low level of molecular polymorphism compared to other crop species (Stalker and Mozingo, 2001). Singh *et al.* (1998) proposed several reasons for the low

level of polymorphism in groundnut at the DNA sequence even in the presence of significant level of morphological variation *viz.*, limited use of variability present in the germplasm, use of limited number of enzymes and primers and lack of use of advanced methodologies to trap molecular polymorphism in groundnut. Hence, a need to explore new methodologies such as single nucleotide polymorphism (SNP) with greater power to reveal polymorphism is emphasized (Paterson *et al.*, 2004). It is an up coming markers system which hold promise to detect high level of polymorphism due to their high frequency in the genome and their frequent linkage to genes.

2.7.5 Genetic Mapping in Groundnut

Construction of genetic linkage map is necessary to apply marker assisted selection tool in crop improvement programme but it was a hard task for groundnut researchers because of its low level of genetic polymorphism due to single event of polyploidization but recently explosion of robust molecular marker methods revealed significant amount of polymorphism in the crop.

Halward *et al.* (1993) constructed first genetic map in groundnut by using a cross between two diploid species *A. stenosperma* and *A cardenasii*. RFLP markers were used from genomic as well as cDNA libraries of groundnut *A. hypogaea* cv.GK7. Partial genomic library was constructed by pstI digestion of genomic DNA and cloning the 1-2 Kb fragments. The cDNA libraries were made from shoot and root tissue. The F_2 population (87 individuals) was analysed at seven restriction sites

(BamHI, DraI, EcoRI, HaeIII, HindIII and RsaI). Out of the 100 genomic and 300 cDNA probes used in the study 15 and 190 respectively, gave polymorphic profile between the parents. Of the 205 probes that showed polymorphism, 132 were analyzed for segregation since the rest revealed complex patterns and hence could not be mapped. Of the 132, 117 segregating loci were distributed on 11 linkage groups. A map distance of 1400 cM was covered with a 20 cM resolution. This map covers 80 per cent of the groundnut genome (Table 1).

Garcia *et al.* (1995) constructed a linkage map having one tetraploid parent and the other being the diploid species *A. cardenasii*. Seventy three RFLP probes and 70 RAPD markers were screened against 46 introgression lines from cross between *A. hypogaea* ($2n = 4x = 40$) and *A. cardenasii* Krapovickas and W.C.Gregory ($2n = 2x = 20$) for the introgression of *A. cardenasii* chromosome segments. A total of 34 cDNA RFLP probes and 45 RAPD primers identified introgressed chromosomal segments in one or more lines. The introgression segments covered 10 out of the 11 linkage groups, smallest of which were RFLP markers and the largest had 3-4 adjacent markers at a distance of 30-40 cM. Garcia *et al.* (2005) also used a backcross population *A. stenosperma* × (*A. stenosperma* × *A. cardenasii*) and 39 shared RFLPs to place 167 RAPD loci onto the RFLP map. The RAPDs covered a total genetic length of 800 cM and mapped onto 11 markers groups.

Herselman *et al.* (2004) used 60 $F_{2:3}$ lines derived from two *A. hypogaea* (ICG1 2991 and ICGV-SM 93541) genotypes. A total of 308

Table 1: Different mapping population used and details of construction of genetic linkage map in groundnut

Sl. No	Mapping Population	Features of Genetic Map	Genome Coverage	Genome	Reference
1	F_2, interspecific ; *A. stenosperma* × *A. cardenassi*	11 linkage groups with 117 RFLP Loci	1063 cM	AA	Halward *et al.*, 1993
2	Backcross population : A.hypogaea × {*A. batizocoi* × (*A. cardenasii* × *A. diogoi*)}	23 linkage groups with 370 RFLP loci.	2210 cM	AABB	Burow *et al.*, 2001
3	F_2 population: *A. kuhlmanni* × *A. diogoi*	12 linkage groups with 102 AFLPs	1068.1 cM	AA	Milla, 2003
4	$F_{2:3}$population : ICG 12991(Spanish) × ICGVSM 93541(Land race)	5 linkage groups with 12 AFLP marker loci	139.4 cM	AABB	Herselman *et al.*, 2004
5	F_2 Population : *A. duranensis* × *A. stenosperma*	11 linkage group with 204 microsattelite loci	1230.89 cM	AA	Moretzsohn *et al.*, 2005
6	Backcross population : *A. stenosperma* × (*A. stenosperma* × *A. cardenassi*)	11 linkage groups with 167 RAPD loci	800 cM	AA	Garcia *et al.*,2005
7	F_2 Population: *A. ipaensis* × *A. magna*	11 linkage group with 94 microsattelite loci	754.8 cM	BB	Gobbi *et al.*, 2006

Courtesy: Dr. R. K. Varshney, Senior Scientist, ICRISAT, Patancheru-502 324

AFLP primers and 144 primer combinations were used to identify markers associated with aphid resistance and identified 20 putative markers. Of which, 12 are mapped on 5 linkage groups covering a map distance of 139.4 cM This study represents first report on the identification of molecular markers linked to aphid resistance to groundnut rosette disease (GRD) and the construction of first partial genetic linkage map of the cultivated groundnut.

Burow *et al.* (2001) constructed the first molecular map representing the entire tetraploid genome of the groundnut. To introduce variability from diploid wild species into tetraploid cultivated *Arachis hypogaea* a synthetic amphidiploid TxAG-6 (*A. batizocoi*K9484 × (*A. carenasii* GKP10017 × *A. diogoi*GKP10602)^{4x}) was used as a donor parent to generate backcross population of 78 progenies. Three hundred seventy RFLP loci were mapped onto 23 linkage groups using a BC1 mapping population. A total of 917 bands were observed, for an average of 4.1 bands per probe. A mean of 1.68 loci per probe were mapped. The total length of tetraploid map was spanning 2210 cM, which was slightly greater than twice the length of (1063 cM) of the diploid map (Garcia *et al.*, 1995). The tetraploid map developed based on an inter-specific cross is useful in terms of locating specific genes of interest in the inter-specific cross and also provides valuable information about genome organization and genome evolution.

Milla (2003) constructed a genetic linkage map for an F_2 population of *A. kuhlmannii* × *A. diogoi*. The map consisted of 102 AFLP markers grouped into 12 linkage groups and spanning 1068.1 cM.

Moretzsohn *et al.* (2005) as a first step towards the introgression of resistance genes into cultivated groundnut, a linkage map based on microsatellite markers was constructed, using F_2 population obtained from a cross between two diploid wild species with AA genome (*A. duranensis* and *A. stenosperma*). A total of 271 new microsatellite markers were developed from SSR enriched genomic libraries, EST and data mining, sequence available at Genbank and another 162 published groundnut microsatellite markers were screened against both the progenitors. Two hundred and four of these (47.1%) polymorphic were screened against 93 F_2s. The resulting linkage map consists of 11 linkage group covering 1,230.89 cM of the total map distance, with an average distance of 7.24 cM between markers. This is the first microsatellite based map published for *Arachis* and the first map based on sequences that are publicly available.

Gobbi *et al.* (2006) constructed a B genome map. They have used 93 F_2s derived from a cross between *A. ipaensis* (KG30076) and *A. magna* (KG30097), both diploid species with B Genome. 94 polymorphic markers were screened which spanned 11 linkage groups with a total distance of 754.8 cM. Size of the linkage groups ranged from 5.6 cM to 130.7 cM.

The above mapping studies in groundnut revealed lack of comprehensive molecular genetic map based on a mapping population derived from the cross of two cultivated (4*x*) groundnut varieties/cultivars. This may be ascribed to two main reasons *viz.*, non availability of the mapping population with diverse genetic background that segregate for

agronomic traits, and unavailability of adequate and appropriate genomics tools to detect existing genetic variation in primary gene pool (Varshney *et al.*, 2006). Mapping populations derived from wild species show considerable amount of polymorphism but dissipates in the successive generations. Hence, there is an exigency to explore new marker assay like SNPs rather than targeting wild species based material, which can track down the molecular variation in groundnut.

2.7.6 DNA markers associated with resistance

Molecular markers do play very important role in the introgression breeding. It makes the selection process easy, effective and offers a mechanism to eliminate undesirable traits associated with hybridizing diverse genotypes. Several attempts have been made to transfer desirable genes from wild relatives in to the cultivated background with the aid of molecular markers.

2.7.6.1 Rust Resistance

Varma *et al.* (2005) studied variation among parents and identified microsatellite markers associated with rust resistance in groundnut. The parents, F_1, F_2 $BC_1P_1F_1$ and $BC_1P_2F_1$ of two crosses (ICGV 99003 × TMV2; ICGV 99005 × TMV2) were evaluated for resistance to rust using 25 SSRs. Of these, Seven primer pairs detected polymorphic variation among ICGV 99003 × TMV 2 and eight primer pairs between ICGV 99005 × TMV 2 and none of the microsatellite primer pairs showed intra accession variability among parents. The highly resistant and susceptible F_2 plants were

selected to form bulks and analyzed using BSA to identify markers linked with resistance to rust. BSA did not provide the expected results so later on all the resistant and susceptible plants were analyzed for marker – trait association along with the parents. They have reported association of rust resistance with two microsatellite alleles *namely* $3A01_{275}$ and $3A01_{387}$ in the cross ICGV 99003 × TMV 2 and the $5D5_{270}$, $5D5_{295}$, $5D5_{335}$ in cross ICGV 99005 × TMV2. The susceptibility to rust is associated with markers $3A01_{293}$ and $3A01_{412}$ in the cross ICGV 99003 × TMV2 and the markers $5D5_{260}$, $5D5_{288}$,and $5D5_{312}$ in the cross ICGV 99005 × TMV2.

Mondal *et al.* (2007) used 117 F_2 lines segregating for rust derived from resistant parent VG 9514 and susceptible parent TAG 24 and tagged RAPD marker $J17_{1300}$ by using modified Bulk Segregant Analysis (BSA) that was tightly linked to rust having 18.5 cM away from resistance gene. Resistant and susceptible F_2 bulks (10 each) were prepared based on scoring data. Parental screening was done on 160 RAPD primers and polymorphic primers were tested on resistant and susceptible bulks along with two parents. The primers which showed polymorphism in parents and bulks were used to screen all 117F_2 plants and the same primers were used to screen a set of 11 resistant and eight susceptible breeding lines/ cultivars. Of these, 11 primers detected reproducible polymorphism between the parents. Among the 11 polymorphic primers, one primer (J7) generated polymorphic DNA fragments, $J7_{1350}$ and $J7_{1300}$ between the resistant and susceptible bulks. Based on linkage analysis results confirmed that $J7_{1300}$ was in repulsion phase and $J7_{1350}$ in coupling

phase. To test the magnitude of association with rust resistance, simple regression analysis was carried out. The results showed that $J7_{1300}$ and $J7_{1350}$ individually explained 9.4 per cent and 27.9 per cent of phenotypic variance, respectively.

2.7.6.2 Other diseases

Stalker and Mozingo (2001) reported association of RAPD markers with a gene conferring resistance to *Cercosporidium arachidicola* sporulation, lesion diameter, defoliation and overall rating. A marker was also associated with resistance to southern corn rootworm damage. In addition, they associated markers with *Cylindrocladium* black rot resistance and sporulation of *C. arachidicola* in a cross between cultivar NC 7 and PI 109839, which represent the first report of molecular markers being associated with resistance genes in an *A. hypogaea* × *A. hypogaea* cross.

Milla (2003) used AFLP markers to establish marker-trait association for tomato spotted wilt virus (TSWV) resistance in groundnut. One hundred seventy nine individuals derived from F_2 population of *A. kuhlmannii* Krapov. and W. C. Gregory × *A. diogoi with* total of 13*Eco*RI/*Mse*I primer combinations were used to tag makers associated with TSWV. The study identified five closely linked markers to TSWV resistance. All the five markers were located on the same linkage group (LG V) within a distance of 62.7 cM and among them, four markers originated from *A. diogoi*.

Herselman *et al.* (2004) identified and mapped AFLP markers linked to aphid resistance using F_2 population segregating for aphid resistance in combination with AFLP and Bulk Segregant Analysis (BSA). BSA was performed on 20 F_2s (10 homozygous aphid resistant and 10 homozygous aphid susceptible) from 139 F_3 lines. The single recessive gene for aphid resistance was mapped between markers MI-TTG/M-GAA1 and P-TCA/ M-ACT1 on linkage group 1. These markers showed the best associations with aphid resistance and explained 76.1 per cent and 31.2 per cent phenotypic variation and mapped 22.8 cM apart. The gene mapped 3.9 cM from marker MI-TTG/M-GAA1 and 18.9 cM from marker P-TCA/M-ACT1. This is consistent with a single recessive gene for aphid resistance (Van der Merwe, 2001). These markers may be useful in MAS and would serve to select against the aphid-resistant allele of parent ICG 12991.

Lei *et al.* (2006) reported a SCAR marker AFs-412 converted from AFLP marker E45/M53-440 which was closely linked with resistance to *Aspergillus flavus* infection from the twenty genotypes of groundnut.

Garcia *et al.* (2006) used 59 RAPD markers including 25 (17 mapped plus eight unmapped *A. cardenasii*-specific markers) and 34 (7 mapped plus 27 unmapped *A. batizocoi*-specific markers) to evaluate progenies of four interspecific crosses at different selfing and backcross generations.

Material and Methods

Table 2: Mean performance of parents for components of resistance to late leaf spot and rust and some physiological traits days

Parents	LLS			Rust				DM (days)	HI (%)	PC (%)
	IP	LP_{50}	FDS	IP	LP_{50}	FDS	RGLA			
TAG 24	4.6	16.5	7.6	5.2	17.5	5.0	18.3	96	68.4	63.8
GPBD 4	10.3	39.0	4.0	16.7	39.0	2.8	63.3	110	53.3	63.1

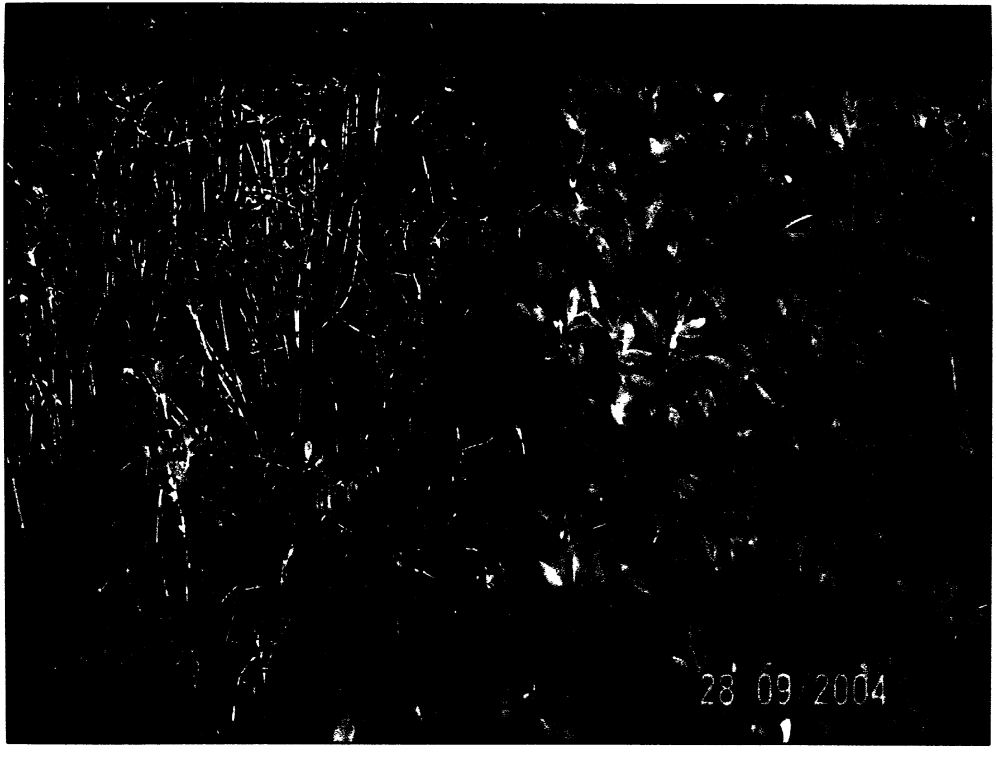
IP - Incubation Period
DM - Days to Maturity
LP_{50} - Latent Period
HI - Harvest Index
FDS - Field Disease Score
PC - Partitioning Coefficient
RGLA - Remaining Green Leaf Area

Source : Motagi, 2001

Table 3: Yield loss (%) due to rust and LLS under early and late sown conditions

Parents	Pod Yield		Kernel Yield		Oil Yield		Fodder Yield	
	Early	Late	Early	Late	Early	Late	Early	Late
TAG 24	32.6	35.4	27.4	39.5	38.1	50.2	36.0	37.2
GPBD 4	13.1	10.9	15.8	17.3	14.8	21.6	10.6	20.9

Source : Motagi, 2001



TAG 24 GPBD 4

Plate 1: Response of TAG 24 and GPBD 4 to foliar diseases

Station, Raichur, Karnataka. CS 16 is a Virginia bunch interspecific-derivative (*A. hypogaea* × *A. cardenasii*, ICGV 86855) resistant to foliar diseases developed at ICRISAT, Patancheru, India.

3.2 DEVELOPMENT OF MAPPING POPULATION

The mapping population was developed at the University of Agricultural Sciences, Dharwad from the cross TAG 24 × GPBD 4. F_1s were selfed to produce F_2s and advanced through Single Seed Descent (SSD) till F_6 generation. Each F_6 line epitomizes the individual F_2 plant from which it is derived (Plate 2).

3.3 PHENOTYPING OF MAPPING POPULATION FOR LATE LEAF SPOT AND RUST

Phenotyping of RILs was carried out at Dharwad, a known hotspot for foliar diseases, during the rainy seasons 2004(E1), 2005(E2) and 2006(E3) for LLS and 2007(E4) for rust under artificial epiphytotics.

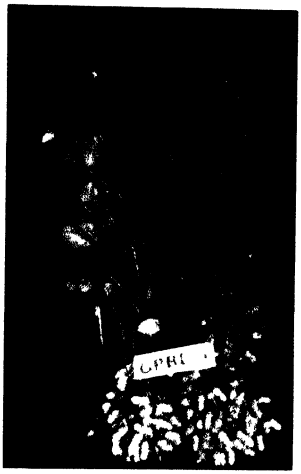
3.3.1 Production of LLS and Rust Inoculums

The inoculums were produced and maintained separately on TMV-2 for LLS and mutant 28-2 (resistant to LLS) for rust. The LLS conidia and rust urediniospore were isolated by soaking and rubbing of infected leaves in water for 30 minutes and used for inoculation on test material separately.

The 268 RILs were sown in Randomized Block Design (RBD) with two replications. Test materials were treated with seed protectant before sowing. Ten seed of each RIL were planted in 1 m rows with 30 cm and

P_1

X



P_2

F_1

⊗

F_2

Followed Single Seed Descent (SSD) method after F_2 generation

Recombinant inbred lines segregating for LLS and rust in F_6 generation

Legend

X Crossed with

⊗ Self pollination

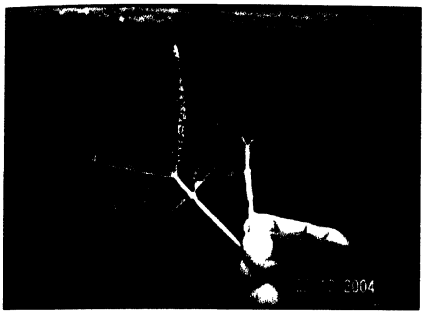
Plate 2 : Development of TAG 24 x GPBD 4 mapping population

10 cm inter and intra-row spacing, respectively. The two parents *viz.*, TAG 24 and GPBD 4 were sown as controls after every 50 rows. All the necessary agronomic practices were followed to raise a healthy crop.

Artificial disease epiphytotics were created in separate screening experiments for the two diseases using "spreader row technique". TMV 2 and mutant 28-2 (resistant to LLS but susceptible to rust) were used as spreader rows for LLS and rust, respectively. Spreader rows were planted at every 10^{th} row as well as border around the field to maintain the effective inoculum load. Thirty five days after sowing, plants were inoculated uniformly in the evening with LLS / rust for a week. The inoculum containing 20,000 conidia / urediniospore per ml water and mixed with Tween 80 (0.2 ml/ 1,000 ml of water) as a mild surfactant and atomized on the plants using knapsack sprayer. The weather conditions favored good development of diseases (Appendix I-IV). High humidity was maintained by irrigating the field in the night by sprinkler or furrow irrigation. Additional inoculum was provided by placing pots containing diseased plants at every 50 rows (Plate 3a, 3b and 3c). The non-targeted disease *i.e.* rust / LLS in LLS and rust experiments were controlled by spraying fungicide carbendazim (bavistin) 1 g litre^{-1} and tridemorph (calixin) 1 ml litre^{-1}, respectively.

3.3.2 Disease Scoring

Disease scoring was done at 70 days (Stage 1) and 90 days (Stage 2) for LLS and 70 days (Stage 1), 80 days (Stage 2) and 90 days (Stage 3) for



Late leaf spot

Rust

Plate 3a: Disease symptoms of late leaf spot and rust

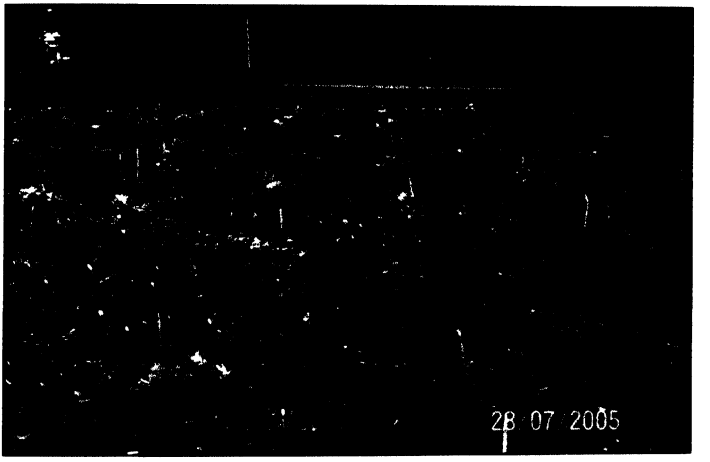


Plate 3b: Creation of artificial disease epiphytotics for LLS and rust using spreader row technique

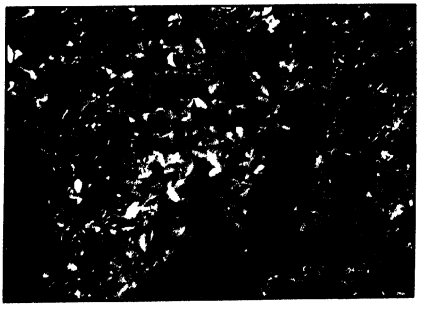


Plate 3c: RILs segregating for LLS and rust

rust by using modified 9 point scale (Subbarao *et al.*, 1990) (Table 4a and 4b; Fig 1a and 1b).

3.4 AGRONOMIC TRAITS

The agronomic traits which are contributing for yield were studied in *kharif* 2007 (E4) at Patancheru location. The 268 RILs were sown in Augmented plot design. Fourty seeds of each line were planted in 4 m row with 10 cm between plant and 60 cm between the rows spacing. The Parents TAG 24 and GPBD 4 were replicated after every 9th row. All the agronomic practices and control measures of important foliar diseases were followed to have proper growth of the crop. Observations were recorded on 9 traits *viz.*, leaf length (LLN), leaf width (LWD), plant height (PLHT), primary branches (PBR), pods per plant (PPP), yield per plant (YPP), plot yield (YKGH), 100 seed weight (HSW) and shelling percentage (SLNG).

3.4.1 Leaf length (mm): Measured on the third leaf, apical leaflet of the main stem when fully expanded.

3.4.2 Leaf width (mm): Measured on the third leaf fully expanded apical leaflet on the main stem at its widest point.

3.4.3 Plant height (cm): Measured from cotyledonary axil average of 5 randomly selected representative plants.

3.4.4 Number of primary branches: Number of primary branches (n+1).

3.4.5 Pods per plant: Total number of pods per plant.

ıa. Modified 9-point scale used for field screening groundnut genotypes for resistance to rust disease

ıcore	Description	Disease severity (%) ψ
1.	No disease	0
2	Pustules sparsely distributed, largely on lower leaves	1-5
3	Many pustules on lower leaves, necrosis evident, very few pustules on middle leaves	6-10
4	Number of pustules on lower and middle leaves, severe necrosis on lower leaves	11 20
	Severe necrosis of lower and middle leaves, pustules may be present on top leaves but less severe	21-30
6	Extensive damage to lower leaves, middle leaves, necrotic with dense distribution of pustules, pustules on top leaves	31-40
7	Severe damage of lower and middle leaves, pustules densely distributed on top leaves	41 60
8	100 per cent damage to lower and middle leaves, pustules on top leaves, which are severely	61-80
9.	Almost all leaves withered, bare stems seen	81-100

Percentage leaf area damaged by rust

ıle 4b: Modified 9-point scale used for screening groundnut genotypes for resistance to late leaf spot disease

Disease score	Description	Disease severity (%) ψ
1.	No disease	0
2	Lesions present largely on lower leaves; no defoliation	1 5
3	Lesions present largely on lower leaves, very few on middle leaves; defoliation of some leaf lets evident on lower leaves	6-10
4	Lesions present on lower and middle leaves but severe on lower leaves; defoliation of some leaf lets evident on lower leaves	11-20
5	Lesions present on lower and middle leaves, over 50 % of defoliation of lower leaves	21 30
6	Severe; eosins on lower and middle leaves; lesions present but less severe on top leaves; extensive defoliation of lower leaves; some defoliation on middle leaves	31-40
7	Lesions on all leaves but less severe on top leaves, defoliation of all lower and middle leaves	41-60
8	Defoliation of all lower and middle leaves; severe lesions on top leaves evident.	61-80
9	Almost all leaves defoliated, leaving bare stem; some leaflets my remain, but show severe leaf spot	81 100

ψ Percentage leaf area damaged by late leaf spot (Subbarao *et al.* 1990)

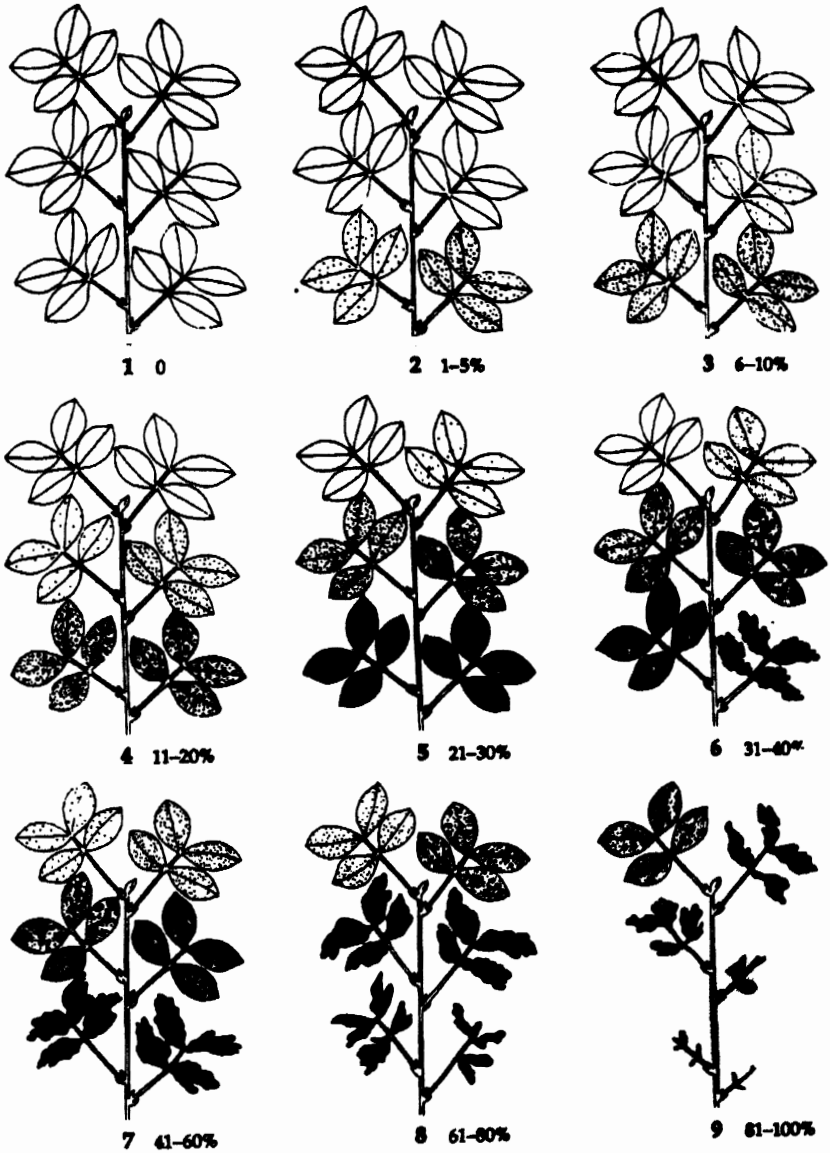


Fig. 1a: The modified 9-point scale for field evaluation of rust of groundnut

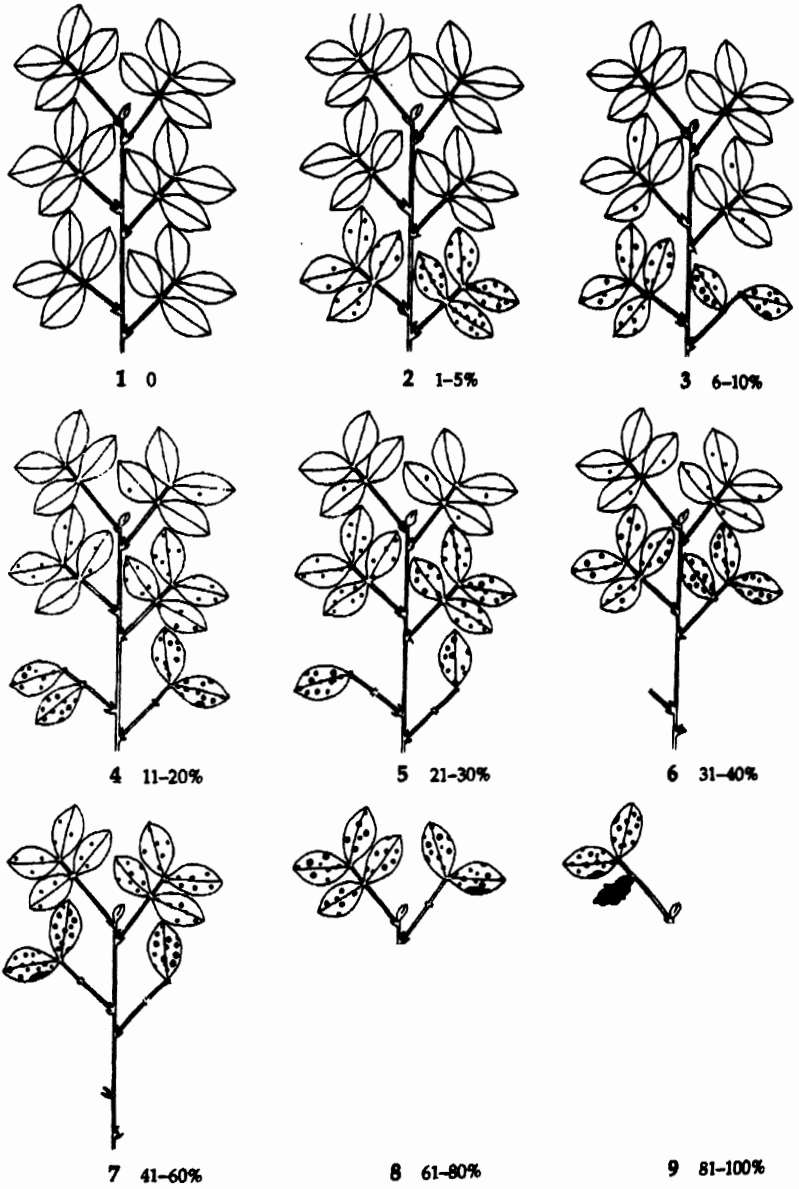


Fig. 1b: The modified 9-point scale for field evaluation of late leaf spot of groundnut

3.4.6 Yield per plant (g): Total weight of dried and cleaned pods obtained from single plant.

3.4.7 Plot yield (Kg/ha): Total weight of dried and cleaned pods obtained from net plot was used to calculate the plot yield per hectare.

3.4.8 100 seed weight (g): The weight of 100 kernels was taken as test weight or 100 seed weight.

3.4.9 Shelling percentage: Shelling percentage was calculated as seed mass over pod mass in per cent

3.5 GENOTYPING OF MAPPING POPULATION

3.5.1 DNA isolation of Parents and Recombinant Inbred lines (RILs)

Young tissues of parents and RILs were collected from two week old plants grown in greenhouse and SIGMA® Genelute Plant Genomic DNA Kit was employed to isolate DNA as per the following procedure.

Crush the plant tissue in liquid nitrogen.

- Take 100 mg of ground leaf tissue in collection tube and add 400 µl of lysis solution.
- Mix it thoroughly by vertexing and keep it in water bath for incubation at 65°C for 10 min.
- Add 130 µl of precipitation solution and invert to mix.
- Chill on ice for 5 min and centrifuge at 12,000 rpm for 5 min to pellet the debris.

- Transfer the supernatant to blue filtration column and spin it for 1 min at 12,000 rpm.
- Add 700 µl binding solution to filtrate and mix it thoroughly by inverting.
- Transfer 700 µl of mixture to binding column and centrifuge for 1 min at 12,000 rpm.
- Repeat the step with remainder of the mixture and then transfer column to new collection tube.
- Add 500 µl of wash solution to column, centrifuge at 12,000 rpm for 1 min and transfer the column to new collection tube.
- Repeat the step twice to remove all the contaminants.
- Transfer column to new collection tube and add 60 µl of elution solution (pre-warmed to 65^{0}C) to columns.
- Spin at 12,000 rpm for 1 min.
- After 5 min, repeat the step to elute out the remaining DNA from the binding column.

3.5.2 Quantification of DNA

DNA quality was checked and quantified on 0.8% agarose gel with known concentration of uncut lambda DNA standard.

3.6 ANALYSIS OF PARENTS AND RILS USING SSR MARKERS

Initially the parents, TAG 24 and GPBD 4 were screened for polymorphism by using 1089 available SSR markers (Hopkins *et al.*, 1999;

He *et al.*, 2003; Ferguson *et al.*, 2004; Moretzsohn *et al.*, 2004 and 2005; Mace *et al.*, 2007; Cuc *et al.*(unpublished); Bertioli *et al.* (unpublished); Knapp *et al.* (unpublished). Of these, 908 produced scorable bands and 67 primers (6.15%) were found polymorphic (Appendix V, Table 5 and Plate 4a), which were then employed for genotyping the mapping population (Plate 4b).

3.6.1 PCR Amplification

Polymerase Chain Reactions (PCR) were performed by using a Touch - Down PCR profile (Table 6) and an amplification protocol (Table 7) appropriate for each pair of primers. DNA amplification was performed in 5 µl reaction mixture using Gene Amp® PCR system 9700. The reaction mixture contained 5 ng/µl template DNA, 10 pM / µl SSR primers pair (Forward and Reverse), 25 mM Mgcl2 (Bioline), 2 mM DNTP's, 10X PCR buffer (Bioline), and 5U/µl Taq DNA polymerase (Bioline) for unlabeled primers and for labeled primers 5 ng/µl template DNA, 10 pM/µl SSR primers pair (Forward-labelled and Reverse), 25 mM $Mgcl_2$ (Qiagen), 2 mM DNTP's, 10X PCR buffer (Qiagen) and 5U/µl Taq DNA polymerase (Qiagen).

3.6.2 Electrophoresis

Before loading PCR Products in the sequencing gel, amplification was checked on 1.4 per cent agarose gel. For the separation of DNA fragments, non-denaturing polyacrylamide gel electrophoresis (PAGE) and capillary electrophoresis were used.

Table 5: Parental polymorphism with SSRs in TAG 24 x GPBD 4 mapping population

Source	No. of primers screened	No. of Polymorphic markers in TAG24 x GPBD4	Percent polymorphism
Hopkins *et al.* (1999)	26	3	11.53
He *et al.* (2003)	158	9	5.69
Ferguson *et al.* (2004)	226	15	6.63
Moretzsohn *et al.* (2004, 2005)	338 (67 + 271)	25	7.39
Mace *et al.* (2006)	79	0	0
Cuc *et al.* (unpublished)	170	8	4.70
Bertioli *et al.* (unpublished)	46	0	0
Knapp *et al.* (unpublished)	46	7	15.21
Total	**1089**	**67**	**6.15**

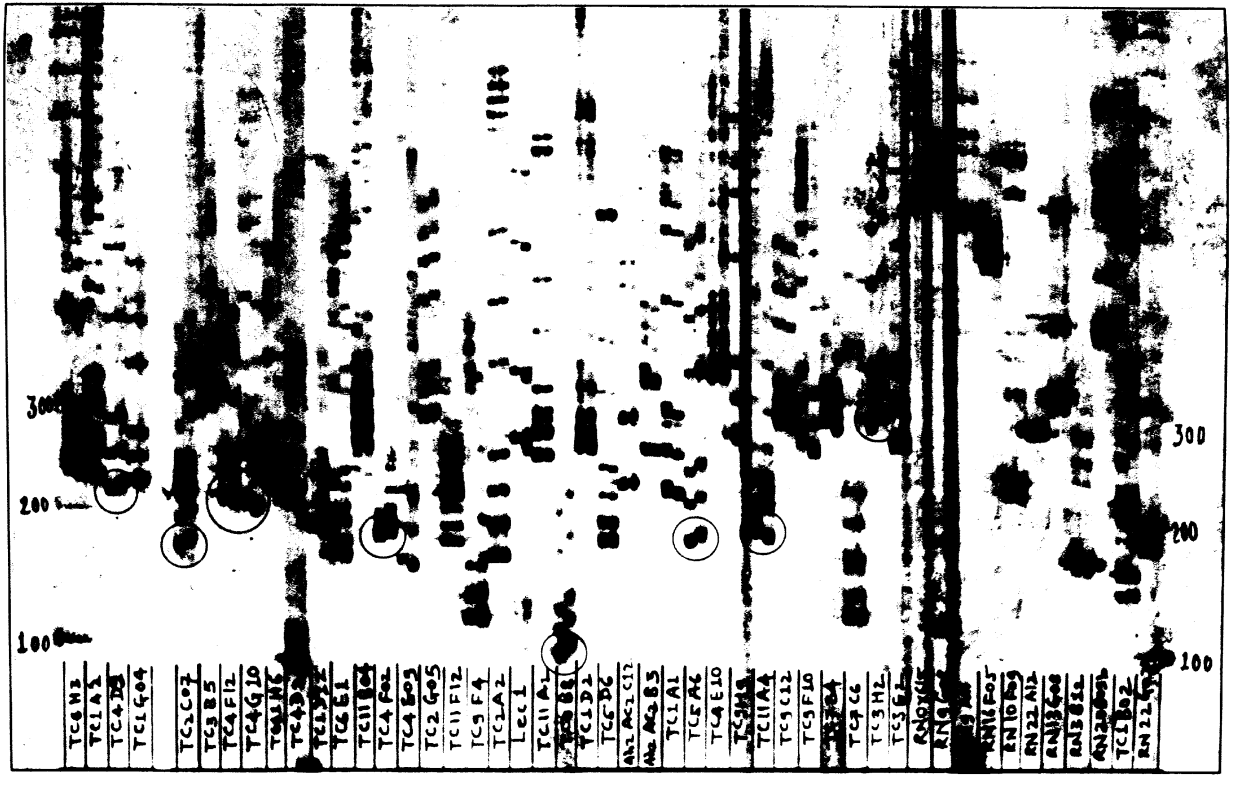


Plate 4a: Screening of parents using SSR markers on PAGE

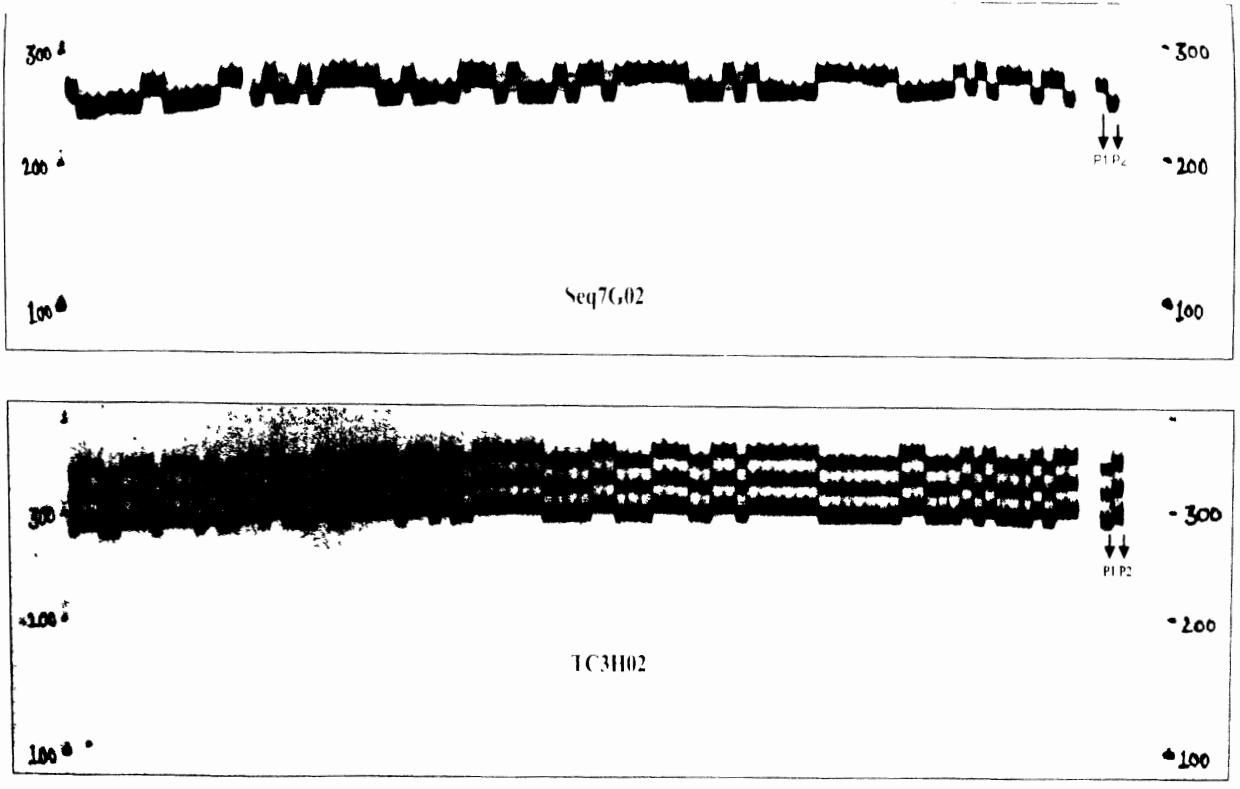


Plate 4b: Genotyping of RILs with SSR markers on PAGE

Table 6: Touch- Down PCR profiles for the targeted microsatellite loci

Sl. No.	Steps	Temperatures (°C)			Time	Cycles
		55-45	60-55	65-60		
1	Initial denaturation	95	95	95	3 min	
2	Denaturation	94	94	94	20 sec	5 cycles
3	Annealing	65	60	55	20 sec	
4	Primer extension	72	72	72	30 sec	
5	Denaturation	94	94	94	20 sec	30 cycles
6	Annealing	59	56	48	20 sec	
7	Primer extension	72	72	72	30 sec	
8	Final extension	72	72	72	20 min	
9	Store at	4	4	4		

Table 7: PCR reactions for the targeted microsatellites loci

Components	Concentration^{-1} µl	Protocol No		
		1	2	3
		PCR Reaction (5 µl)	PCR Reaction (5 µl)	PCR Reaction (5 µl)
Primers (F + R)	10 pM	0.25	0.25	0.5
Mg^{2+} (Bioline)	25 mM	0.5	0.5	0.5
DNTP's	2 mM	0.5	0.5	0.5
Buffer (Bioline)	10X	0.5	0.5	0.5
Taq polymerase(Bioline)	5U	0.03	0.04	0.03
Double distilled water	-	2.23	1.21	1.47
Template	5 ng	1	2	1.5

Protocol No. 1- Universal Protocol
Protocol No. 2 was exclusively used for GM series microsatellite primers.
Protocol No. 3 -for TC1A1, TC4D9, TC4E09 primers

Contd.....

Table 7 contd.....

Components	Concentration^{-1} µl	Protocol No		
		4	5	6
		PCR Reaction (5 µl)	PCR Reaction (5 µl)	PCR Reaction (5 µl)
Primers (F + R)	10 pM	0.1	0.15	0.1
Mg^{2+} (Bioline)	25 mM	1.0	0.75	0.5
DNTP's	2 mM	0.5	0.5	0.25
Buffer (Bioline)	10X	0.5	0.5	0.4
Taq polymerase(Bioline)	0.5U	0.4	1.0	0.5
Double distilled water	-	0.5	1.10	2.25
Template	5 ng	2	1	1

Protocol No. 4- Seq1967 and PM183
Protocol No. 5 - XIP121, Seq18A5 (b) and PM434
Protocol No. 6- XIP176

Contd.....

Table 7 contd.....

Components	Concentration [1] µl	Protocol No.			
		7	8	9	10
		PCR Reaction (5 µl)	PCR Reaction (5 µl)	PCR Reaction (5 µl)	PCR Reaction (5 µl)
Primers (F + R)	10 pM	0 13	0.15	0.05	0.2
Mg^{2+} (Qiagen)	25 mM	0.4	0.4	0.4	0.3
DNTP's	2 mM	0 38	0.38	0.38	0.5
Buffer (Qiagen)	10X	0.5	0.5	0.5	0.5
Taq polymerase(Qiagen)	0 5U	0 04	0.04	0 04	0.06
Double distilled water		2.6	2 5	2 6	2 4
Template	5 ng	1	1	1	1

Protocol No 7 Seq17E03 TC11A02 TC9F10
Protocol No 8 Seq5D05 TC4F12 TC11A04
Protocol No 9 Seq2D12B TC6H03
Protocol No 10 Seq8D09

Non-Denaturing Polyacrylamide Gel Electrophoresis (PAGE)

After PCR amplification, 1ul of orange dye was added to 5 µl reaction mixture. Then 2 µl of this reaction mixture was loaded by using Hamilton syringe on each lane of 96-track of 6% non-denaturing PAGE and as the base pair marker, 100bp DNA ladder was loaded on both the corners of the gel. Recipe for 6% gel consisted of 52.5 ml of distilled water, 7.5 ml of 10X TBE, 15 µl of acrylamide-methylbisacrylamide 29:1 (V/V), 100µl of TEMED and 450 µl of ammonium per sulphate (APS). Electrophoresis was run at 900 volts for 2 hours in 0.5X TBE running buffer, using BIORAD sequencing gel unit.

PCR products were visualized by using silver staining protocol (Kolodny, 1984). Initially, the gel was rinsed with distilled water for 5 min with gentle shaking followed by soaking in 0.1% CTAB for 20 min (1.5 g in 1.5 litre of water) then kept in 0.3% liquid ammonia for 15 min (19.5 ml of 25% liquid ammonia solution in 1.5 litre of water) and later placed in 0.1% silver nitrate solution (1.5 g of silver nitrate + 6 ml of 1M NaOH in 1.5 litre of water and then titrated with ammonia solution till it became colorless) followed by rinsing in water for 1 min. After this gel was kept for developing in solution (22.5 g sodium carbonate and 400 µl of formaldehyde in 1.5 ml of water) till bands became conspicuous. The gel was kept in water for 5 min to stop the staining reaction and fixed in 3% glycerol.

After staining the gel, bands were scored as A, B, H, and O. Where, A represents homozygosity for the allele from female parent (TAG 24), B indicates homozygosity for the allele from male parent (GPBD 4) and H denotes heterozygote *i.e.* presence of A and B alleles and O represents off type (neither A nor B allele). A subjective score was given to each marker from 1 to 4 denoting the fragment amplification quality, where 1 indicates single and strong band, 2 represents single and weak band, 3 denotes multiple and strong bands and 4 indicates multiple and weak bands.

b) Capillary Electrophoresis (ABI 3100 Genetic Analyzer)

Amplified products of 10 primer pairs were separated by using capillary electrophoresis. Total volume of 15 µl contains 1 µl PCR products of FAM, VIC and 1.5 µl of NED and PET were mixed separately to a mix of 7 µl of formamide, 0.2 µl of Liz-500 size standard and 2.8 µl of double distilled water (adjusted as per dye and number of primers used for multiplexing). Then the samples were kept for denaturation for 5 min at 94°C and chilled on ice for 5 min. Before placing plates containing samples were centrifuged at 900 rpm for 1 minute and kept in ABI 3100 genetic analyzer. The "G5" dye set, "Genescan POP4" run module and GS500Liz analysis module were employed and the fragments were separated in 36cm capillary array. After completion of the run, the A and B peak patterns were sized using Genescan and scored by using Genotyper software (Plate 4c).

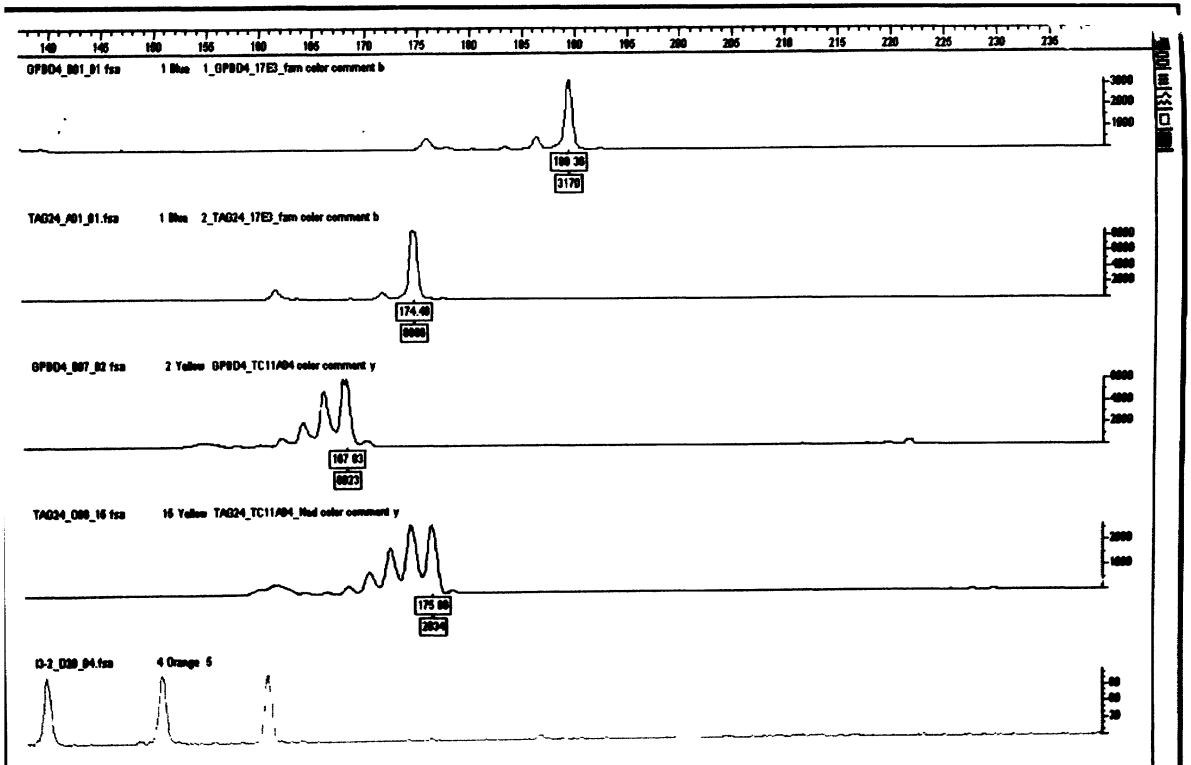


Plate 4c: Screening of parents using capillary electrophoresis (ABI 3100 Genetic Analyzer)

3.7 STATISTICAL DATA ANALYSIS

3.7.1 Phenotypic data analysis

3.7.1.1 Analysis of Variance (ANOVA)

The analysis of variance at different stages of disease scoring in different environments was performed to test the significance of differences between genotypes and pooled analysis of the data to assess the contribution of different sources to the total variation by following Panse and Sukhatme (1961).

ANOVA for single environment

Source of variation	Degrees of Freedom	Mean Sum of Square	Expected Value of M.S.S.
Replication (r)	r-1	M1	
Genotypes (g)	g-1	M2	$\sigma^2_e + r\sigma^2_g$
Error	(r-1)(g-1)	M3	σ^2_e
Total	(rg-1)	M1 + M2 + M3	

3.7.1.2 Estimation of genetic parameters

In order to assess and quantify the genetic variability among the genotypes, different parameters were estimated as given below:

3.7.1.3 Estimation of variance components

Phenotypic and genotypic variances were estimated using the following formulae.

Phenotypic and genotypic variances were estimated using the following formula.

Genotypic variance (σ_g^2) $= \dfrac{\text{MSS (genotypes)} - \text{MSS (error)}}{\text{No. of replications}} = \dfrac{M_2 - M_3}{r}$

Phenotypic variance (σ_p^2) = σ_g^2 + MSS error = $\dfrac{M_2 - M_3}{r} + M_3$

Where,

σ^2_p = Phenotypic variance

σ^2_g = Genotypic variance

$\sigma 2_e$ = Environmental variance

3.7.1.4 Coefficient of Variability

Both genotypic and phenotypic coefficients of variability were estimated as per the method suggested by Burton and Devane (1953).

a) Genotypic Coefficient of Variation (GCV)

$$GCV = \frac{\sigma_g}{\bar{X}} \times 100$$

Where,

σ^2_g = Genotypic variance

$\bar{X}$ = Mean of the characters

b) Phenotypic Coefficient of Variation (PCV)

$$PCV = \frac{\sqrt{\sigma_p^2}}{} \times 100$$

Where,

σ^2_p = Phenotypic variance

$\overline{X}$ = Mean of the characters

The GCV and PCV values were classified as described by Sivasubramanian and Menon (1973).

GCV and PCV values	Classification
0-10	Low
10-20	Medium
20 and above	High

3.7.1.5 Heritability (h^2 (b.s.))

Heritability in broad sense was computed as the ratio of genetic variance to the total phenotypic variance as suggested by Hanson *et al.* (1956) and expressed as percentage.

$$h^2 = \frac{\sigma_g^2}{\sigma_p^2} \times 100$$

Where,

σ^2_g = Genotypic variance

σ^2_p = Phenotypic variance

Heritability (broad sense) estimates were categorized into high, moderate and low by Robinson *et al.* (1966).

Heritability (%)	Classification
5-10	Low
10-30	Medium
30-60	High
>60	Very high

3.7.1.6 Genetic advance (GA)

Genetic advance was estimated by using the formula given by Johnson *et al.* (1955).

$$GA = h^2 \times k \times \sigma_p$$

Where,

h^2 = heritability estimate

σ^2_{ph} = Phenotypic standard deviation

K = Selection differential at 5% is equal to 2.06 of selection (Lush, 1949)

3.7.1.7 Genetic advance as percent of mean (GAM)

$$GAM = \frac{GA}{\bar{X}} \times 100$$

Where,

$\bar{X}$ = Grand mean of the trait

GA = Genetic advance

The Genetic Advance as percent of Mean (GAM) was categorized by Johnson *et al.*, 1955)

GAM (%)	Classification
0-10	Low
10-20	Medium
20 and above	High

3.7.1.8 Standard Error (S.E.m)

$$S.E.m = \frac{\sqrt{(N - 1)}}{N} \frac{(Error\ MS)}{r}$$

Where,

N = Number of Individuals

Error MS = Error mean sum of square

r = Number of replications

3.7.1.9 Coefficient of Variation (C.V.)

$$CV = \frac{\sqrt{Error\ MS}}{GM} \times 100$$

Where,

Error MS = Error mean sum of square

GM = Grand mean

3.7.2 Correlation coefficient (r)

Correlation coefficient (r) among the different stages of LLS, rust and among different agronomic traits was estimated by using software GenStat (9.1th edition).

3.8 GENOTYPING DATA ANALYSIS

3.8.1 Single Marker Analysis (SMA)

Single Marker Analysis was performed to tag and confirm potential SSR markers linked to the trait based on phenotypic and genotypic data pertaining to the 268 RILs, which is based on simple linear regression method (Haley and Knott, 1992).

3.8.1.1 Linkage Analysis

Sixty seven polymorphic markers were used for genotyping 268 RILs. Chi-square (χ^2) test was performed on the genotypic data to test the null hypothesis of expected 1:1 Mendelian segregation on all the scored markers. Of these, 20 markers showed segregation distortion (SD). Due to less number of polymorphic markers, even the distorted markers were also used for linkage map construction and QTL analysis.

The linkage analysis was performed using MAPMAKER/ EXP V 3.0 (Lander *et al.*, 1987; Lincoln *et al.*, 1992). A minimum LOD score of 3.0 and maximum recombination fraction (θ) of 0.5 were set as threshold values for linkage group determination.

Twenty one linkage groups were defined with the "Make Chromosome" command and a set of markers were used as anchors. The most likely marker order within each linkage group was estimated by using three point analyses ("three point" command). Marker orders were confirmed by comparing the Log-likelihood of the possible orders using

multipoint analysis ("compare" command) and by permuting all the adjacent triple orders ("ripple" command).

In the second step, LOD score was set to 3.0 in order to include new markers in the linkage groups. The "try" command was used to determine the exact position of the new marker orders. The new marker orders were again confirmed with the "first order", "compare" and or "ripple" commands.

Recombination fraction was converted into map distances in centiMorgans (cM) using Haldane mapping function (Haldane, 1919). The intermarker distances calculated from mapmaker were used to construct linkage map by using MAPCHART version 2.2 (Voorrips, 2006). Out of 67 markers, 59 markers could land on 13 linkage groups, which spanned 909.4 cM of the groundnut genome and eight markers remained ungrouped. The previously mapped markers were used to designate and orientate linkage groups.

3.8.1.2 QTL Analysis

To map significant chromosomal regions (QTL) controlling resistance to LLS and rust, the combined phenotypic and genotypic data were subjected to PLABQTL version 1.1w (Utz and Melchinger, 1996).

The means, predicted means [derived using best- linear- unbiased-predictions (BLUP) of means adjusted for replicate using REML], square root and $\log_{10}$ transformed means of the RILs were used for QTL mapping at different environments. To determine QTL across the environments

means across environments and their transformed values were used. QTL analysis was performed using the method of Composite Interval Mapping (CIM) (Zeng, 1994; Jansen and Stam, 1994) as in PLABQTL version 1.1 w (Utz and Melchinger, 1996).

Composite interval mapping (CIM) combines the approaches of Interval Mapping (IM) and Single Marker Analysis (SMA) in multiple regression frameworks (Haley and Knott, 1992). Cofactors are identified using stepwise regression with an F to enter and F to delete threshold value of 3.5 in PLABQTL. Once the model containing cofactors is built, the entire genome is rescanned using interval mapping.

The presence of putative QTL in an interval was tested by using a critical value for LOD threshold as determined by PLABQTL using the Bonferroni chi-square approximation (Zeng, 1994) corresponding to genome wise type - I error of 0.25. As the mapping population comprised of RILs, the additive model "AA" was used for analysis in which additive × additive epistatic effects were included. The point at which the LOD score had the maximum value in the interval was taken as the estimated QTL position. The coefficient of determination also known as coefficient of variance (R^2) explained by the QTL was used as a measure of the magnitude of association and it is estimated as the square of the partial correlation coefficient. Estimates of the additive effect of each detected QTL, the total LOD score, the total proportion of phenotypic variance explained by all the detected QTL were obtained by fitting a multiple linear regression model that simultaneously included all the detected QTL for the

traits in question. The LOD score was calculated from the F- value for the multiple regressions (Haley and Knott, 1992) as

$$LOD = n/n\ (1+ p*F/DFres)*0.2171$$

Where,

p = Number of parameter fitted (Haley and Knott, 1992)

$$F\ ratio = SSR\ (full) - SSR\ (red)/pMSE(full)$$

Where,

SSR (full) = Sum of square for regression with full model, *i.e.* with QTL and cofactors

SSR (red) = Sum of square for regression with reduced model *i.e.* without the QTL

MSE (full) = SSE/DEF= residual mean square (full model)

pMSE = Number of estimated QTL effects

Dfres = Number of degrees of freedom for residual sum of square in multiple regression;

The percentage of phenotypic variance (R^2) explained by a QTL was estimated. This is based on the partial correlation of putative QTL with observed variable, adjusted for cofactors (Kendall and Stuart, 1961). In the simultaneous fit, the cofactors are ignored and only the putative QTL initially detected and their estimated position were used in multiple

regressions to obtain the final estimates of the additive effects and the percentage of phenotypic variation for the particular trait that could be explained by the QTL. The additive effect was calculated as half the differences between genotypic values of two homozygotes (Falconer, 1989):

$$\text{Additive effect} = (\text{Parent } P_2 - \text{Parent } P_1)/2.$$

The QTL analysis across the environments was done by the same methodology using means across the environments.

3.8.1.3 QTL - Analysis of Variance (ANOVA)

Analysis of QTL × Environment interactions

The ANOVA was carried out by using PLABQTL version 1.1w (Utz and Melchinger, 1996) in the following way:

Source of Variation	DF	E (MS)
Environment	E -1	
Genotypes	G -1	
QTL	Q	VC + f1 VCqe + E VCd + f2 VCq
Residuals	G -1-Q	VC + E VCd
Genot × Env	(G -1) (E – 1)	
QTL × Env	Q (E-1)	VC + f1 VCqe
Res × Env	(G – 1-Q) (E - 1)	VC

Where,

Q = Number of detected QTL effects (additive, dominance)

E = Number of environments

G = Number of genotypes

VCq = Genetic variance explained by the QTL effects

VCd = Unexplained residual genetic variance (deviation)

VCqe = Variance component QTL × Env. interactions

VCde = Variance component Res. × Env. interactions

VC = VCe /R + VCde, with R being the number of replications in a single environment and VCe the pooled plot error.

The ANOVA table for QTL, especially the variance component VC from the column denoted VComp, are calculated in the following manner, where expectations of Mean Squares (MS) were taken analogously to Knapp (1994) and Bliss (1967).

VC (Genotypes) = [MS (Genotypes) – MS (Genotype × Env.)] / E

Where

E = Number of macro environments (approximative result if Genotype × Environments are unbalanced)

VCq = VC (Genotypes) - VCd

Experimental Results

IV. EXPERIMENTAL RESULTS

With a view to identify putative QTL associated with resistance to late leaf spot (LLS) and rust besides agronomic traits, initially phenotyping of 268 recombinant inbred lines (RILs) for the traits of interest was carried out in four environments *viz.*, *kharif* 2004 (E1), *kharif* 2005 (E2), *kharif* 2006 (E3) for LLS and *kharif* 2007 (E4) for rust at UAS, Dharwad and for agronomic traits at ICRISAT, Patancheru. Disease score was recorded as per modified 9 point scale (Subbarao *et al.*, 1990) at 70 days (stage 1) and 90 days (stage 2) after sowing for LLS and 70 days (stage 1), 80 days (stage 2) and 90 days (stage 3) for rust.

Genotyping of RILs was carried out using available microsatellite markers in groundnut. A total of 1089 markers were screened against TAG 24 (susceptible) and GPBD 4 (resistant) parents. Of these, 908 (83.37%) could amplify and 67 markers were found to be polymorphic (6.15%), which were then used for genotyping the RILs.

The phenotyping and genotyping data derived from 268 RILs were used for linkage map construction and for identifying markers and QTL associated with different traits. The results obtained in the investigation are presented here under.

4.1 PHENOTYPING

4.1.1 Analysis of variance (ANOVA)

The analysis of variance for individual environments for LLS and rust diseases and pooled analysis across environments and different

stages for LLS were performed on the field data on RILs. The variances due to genotypes were significant at stage 1 and stage 2 in all the three environments for LLS and at all the three scoring stages for rust (Table 8 and 9). Analysis of variance for 12 agronomic traits revealed significance among the genotypes for all the traits except days to maturity, pods per plant and yield per plant (Table 10).

Pooled analysis of variance for LLS showed highly significant differences among the genotypes and between the stages and environments (Table 11). Among the interactions, stages × environment and genotype × environment revealed significant differences. The differences were non-significant for genotype × stage and environment × genotype × stage. Per cent contribution of sources to variation revealed maximum contribution by stages (30.77%), genotypes (30.46%) and environment (9.15%) indicating predominant contribution by stage and genotype than environment. Among the interactions, genotype × environment contributed 9.23 per cent followed by stage × environment (5.42%).

4.1.2 Genetic components of variation

The genetic coefficient of variation (GCV) and phenotypic coefficient of variation (PCV) estimates for LLS were high at stage 1 compared to stage 2 indicating higher magnitude of variation at the initial stages. High PCV and moderate to high levels of GCV revealed substantial variation for both LLS and rust at different stages and environments (Table 12). Among the agronomic traits moderate to high level of GCV and

Table 8: ANOVA for late leaf spot in TAG 24 × GPBD 4 mapping population

Source of variation	D.F.	Mean Sum of Square					
		Kharif 2004 (E1)		*Kharif* 2005 (E2)		*Kharif* 2006 (E3)	
		S1	S2	S1	S2	S1	S2
Replication	1	2.03	3.29	0.00	0.06	11.54	24.67
Genotype	267	2.97**	3.55**	3.33**	3.51**	1.73**	3.37**
Error	267	1.03	1.49	0.53	0.33	0.50	0.72

Significant at P ≤ 0.01

Table 9: ANOVA for rust in TAG 24 × GPBD 4 mapping population

Source of variation	D.F.	Mean Sum of Square		
		S1	S2	S3
Replication	1	11.06	0.28	0.19
Genotype	267	1.11**	1.35**	1.86*
Error	267	0.50	0.41	0.66

** Significant at P ≤ 0.01

S1 - 70 days to score LLS S2 - 90 days to score LLS

S1 - 70 days to score rust S2 - 80 days to score rust S3 - 90 days to score rust

Table 10: ANOVA for agronomic traits in TAG 24 × GPBD 4 mapping population

Source of variation	D.F.	Mean Sum of Square								
		LLN	LWD	PLHT	PBR	PPP	YPP	YKGH	HSW	SLNG
Block	14	59 99	11 07	44 26	0.36	80 87	33.02	486521	45.04	105 21
Genotype	265	19 42*	4 21*	26 16**	0 13**	43 77	19 86	327792*	41 12*	59.91**
Error	15	13 45	3 83	8 10	0 03	57 08	22 54	185813	18.21	15 60

** Significant at P 0 01 * Significant at P 0 05

LLN Leaf length (mm) LWD Leaf width (mm) PLHT - Plant height (cm)

PBR - No of primary branches PPP Pods per plant YPP - Yield per plant (g)

YKGH - Plot yield (kg/ha) HSW 100 seed weight (g) SLNG - Shelling percentage

Table 11: Pooled Analysis of variance for late leaf spot in TAG 24 × GPBD 4 mapping population

Source of variation	D.F.	Mean sum of Square	F value	Percent contribution of Variation
Environment	2	603.09**	668.45	9.15
Replication × Environment	3	6.98**	7.73	0.16
Genotype	267	15.03**	16.66	30.46
Genotype × Environment	534	2.28*	2.52	9.24
Stages	1	4052.27**	4491.47	30.77
Stages × Environment	2	357.36**	396.09	5.42
Genotype × Stages	267	0.73**	0.91	1.49
Environment × Genotype × Stages	534	0.57**	0.64	2.34
Error	1605	0.902		10.93

** Significant at P ≤ 0.01, * Significant at P ≤ 0.05

Table 12: Estimates of variance components, broad sense heritability and genetic advance for LLS and rust in TAG 24 × GPBD 4 population

Environments / Traits	Scoring stage	GCV (%)	PCV (%)	$h^2b.s$ (%)	GAM (%)
LLS					
E1	S1	21.00	31.00	48.50	31.05
	S2	14.60	22.84	40.87	18.70
E2	S1	21.99	25.82	73.53	38.04
	S2	19.76	21.71	82.81	36.52
E3	S1	24.76	33.55	55.40	37.88
	S2	18.71	23.56	64.70	30.49
Pooled		49.57	52.63	88.62	95.37
Rust					
E4	S1	16.33	26.54	37.88	20.22
	S2	15.79	21.61	53.40	23.39
	S3	14.39	20.86	47.61	20.15

GCV - Genetic coefficient of Variation, PCV-Phenotypic coefficient of Variation, h-b.s- heritability in Broadsense
GAM - Genetic Advance as percent of Mean
E1 - *Kharif* 2004 E2 - *Kharif* 2005 E3 - *Kharif* 2006 E4 - *Kharif* 2007
S1 - 70 days to score LLS S2 - 90 days to score LLS S1 - 70 days to score rust S2 - 80 days to score rust
S3 - 90 days to score rust

PCV was noticed for all the traits except days to maturity, pods per plant and yield per plant . GCV was non estimable in these traits due to high error variance (Table 13).

In general, heritability was moderate to high for both the diseases but very high heritability was observed in E2 at both the stages for LLS. Among the agronomic traits, heritability was very high for plant height, pods per plant and shelling percentage and moderate to high for other traits.

In general, high genetic advance (>20) was observed for both the diseases at different stages and environments. High genetic advance was evident for plant height and yield and low to moderate for other agronomic traits.

Higher estimates of components of variation for LLS and rust, plant height, primary branches, shelling percentage, 100 seed weight and yield per plant and plot yield revealed highly heritable nature of variation in these traits.

4.1.3 Correlation

Correlation coefficients were employed to assess consistency and stability of the disease scores across stages and environments. Highly significant and positive correlations were observed between stages in each of the environment for both the disease and the correlations were high even across environments for LLS revealing consistency between stages and across environments (Table 14).

Table 13: Estimates of variance components, broad sense heritability and genetic advance for agronomi traits in TAG 24 × GPBD 4 population

Traits	GCV (%)	PCV (%)	$h^2b.s$ (%)	GAM (%)
LLN	4.72	8.51	30.74	5.25
LWD	2.55	8.49	9.02	1.57
PLHT	17.21	20.71	69.03	29.41
PBR	7.47	8.52	76.92	13.32
PPP	NE	33.24	NE	NE
YPP	NE	31.87	NE	NE
YKGH	28.73	43.66	43.33	38.68
HSW	12.46	16.70	55.71	18.91
SLNG	10.43	12.13	73.96	18.24

NE : Non estimable

GCV – Genetic coefficient of Variation, PCV-Phenotypic coefficient of Variation, h2b.s -heritability in Broadsense
GAM – Genetic Advance as percent of Mean
LLN – Leaf length (mm) LWD – Leaf width (mm) PLHT – Plant height (cm)
PBR – No. of primary branches PPP – Pods per plant YPP – Yield per plant (g)
YKGH – Plot yield (kg/ha) HSW – 100 seed weight (g) SLNG – Shelling percentage

Table 14: Correlation matrix for late leaf spot and rust over stages and across seasons in TAG 24 × GPBD 4 mapping population

		LLS						Rust		
	Scoring stage (days)	**S1E1**	**S1E2**	**S1E3**	**S2E1**	**S2E2**	**S2E3**	**S1E4**	**S2E4**	**S3E4**
Late leaf spot	S1E1	1								
	S1E2	0.59**	1							
	S1E3	0.53**	0.56**	1						
	S2E1	0.76**	0.56**	0.44**	1					
	S2E2	0.69**	0.80**	0.60**	0.66**	1				
	S2E3	0.47**	0.58**	0.74**	0.45**	0.65**	1			
Rust	S1E4	0.11	0.13	0.22	0.13	0.15	0.10	1		
	S2E4	0.15	0.11	0.23	0.15	0.13	0.09	0.87**	1	
	S3E4	0.16	0.12	0.20	0.19	0.16	0.14	0.76**	0.86**	1

** Significant at P ≤ 0.01, * Significant at P ≤ 0.05

E1 - *Kharif* 2004 E2 - *Kharif* 2005 E3 - *Kharif* 2006 E4 - *Kharif* 2007

S1 - 70 days to score LLS S2 - 90 days to score LLS S1 - 70 days to score rust S2 - 80 days to score rust

S3 - 90 days to score rust

In general positive correlations were observed between all the morphological and agronomic traits (Table 15). The association was very strong among the different morphological traits *viz.*, leaf length, leaf width, plant height and primary branches. Primary branches exhibited strong correlation with some yield contributing traits *viz.*, pods per plant, yield per plant, 100 seed weight and shelling percentage, while leaf length and leaf width had higher correlations with 100 seed weight and shelling percentage. The association was very strong among the yield traits *viz.*, pods per plant, yield per plant and plot yield and between yield per plant, plot yield and 100 seed weight and 100 seed weight with shelling percentage .

4.1.4 Frequency distribution of RILs

The variation existed in the RILs for LLS and rust is represented graphically using frequency distribution of means in three screening environments for LLS and one environment for rust (Fig. 2 and 3).

The disease scores were plotted on X-axis against genotype frequency on Y - axis with equal class intervals. The resulting histogram showed near normal curves for both the diseases with slightly skewed towards susceptibility for LLS in later stages. In general the distribution of RILs was within the parental limits for both the diseases with only few RILs exhibiting slightly more susceptible reaction than the susceptible parent TAG 24. All the agronomic traits showed normal distribution and the transgressive segregants were observed in both the directions (Fig. 4).

Table 15: Correlation matrix for agronomic traits in TAG 24 × GPBD 4 mapping population

	LLN	LWD	PLHT	PBR	PPP	YPP	YKGH	HSW	SLNG
LLN	1.00								
LWD	0.87**	1.00							
PLHT	0.58**	0.45**	1.00						
PBR	0.67**	0.66**	0.49**	1.00					
PPP	0.24**	0.28**	0.20**	0.47**	1.00				
YPP	0.30**	0.31**	0.18**	0.44**	0.84**	1.00			
YKGH	0.25**	0.22**	0.21**	0.27**	0.58**	0.58**	1.00		
HSW	0.55**	0.51**	0.32**	0.45**	0.27**	0.39**	0.55**	1.00	
SLNG	0.61**	0.63**	0.34**	0.58**	0.29**	0.30**	0.25**	0.57**	1.00

** Significant at P ≤ 0.01, * Significant at P ≤ 0.05

LLN - Leaf length (mm)
LWD - Leaf width (mm)
PLHT - Plant height (cm)
PBR - No. of primary branches
PPP - Pods per plant
YPP - Yield per plant (g)
YKGH - Plot yield (kg/ha)
HSW - 100 seed weight (g)
SLNG - Shelling percentage

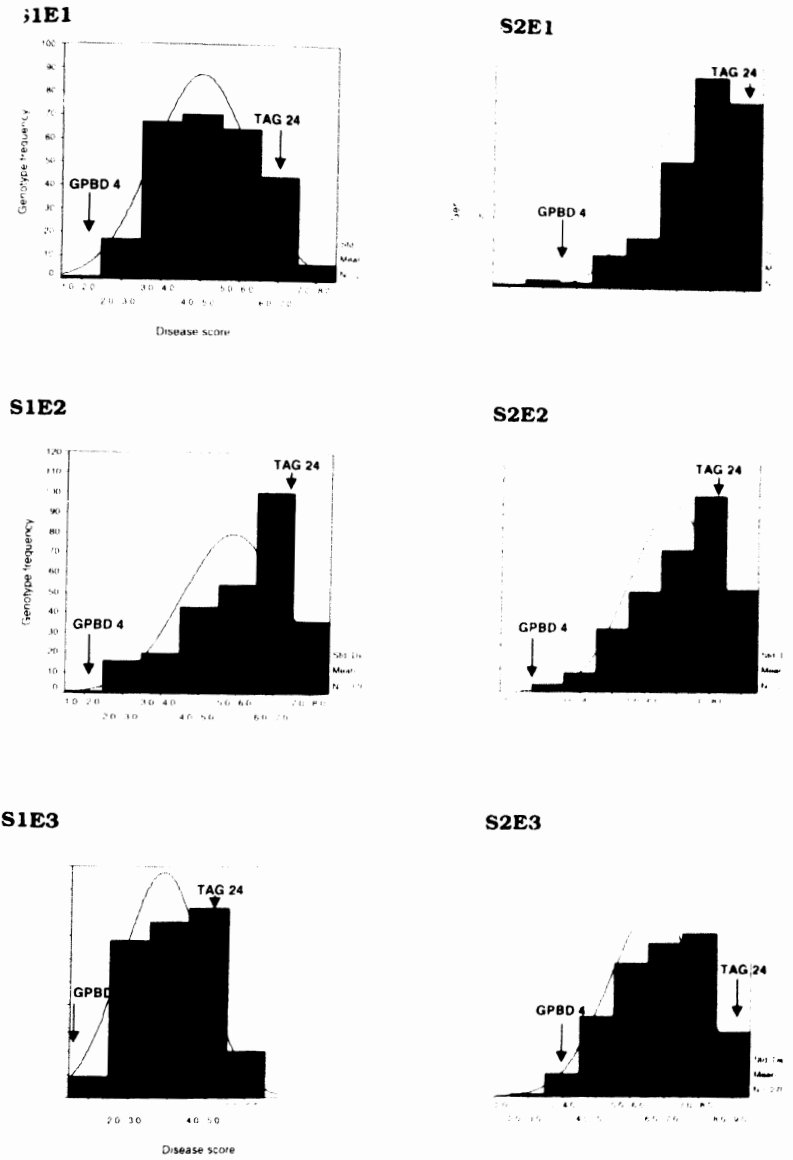


Fig. 2: Frequency distribution of RILs for LLS in TAG 24 × GPBD 4 mapping population

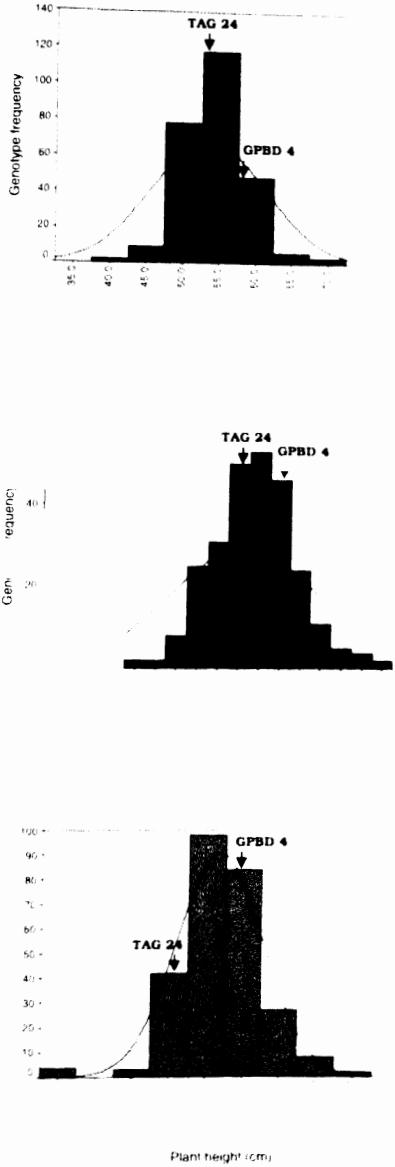
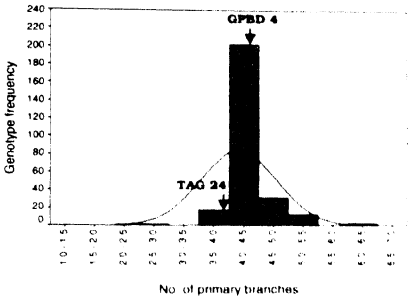
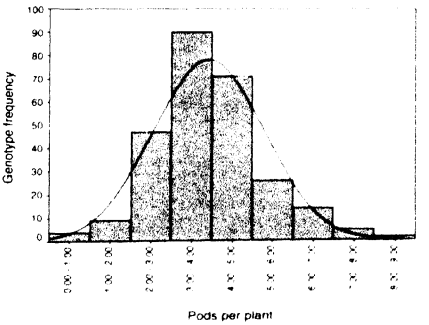


Fig. 4: Frequency distribution of RILs for agronomic traits in TAG 24 × GBPD 4 mapping population

Fig. 4 Contd.....


GPBD 4
TAG 24
Genotype frequency
No. of primary branches


Genotype frequency
Pods per plant

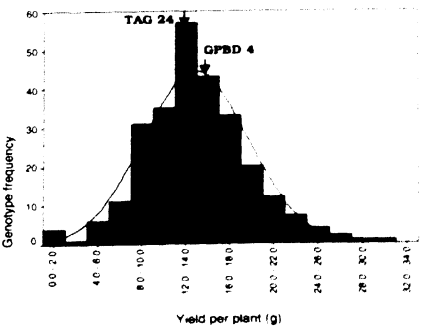
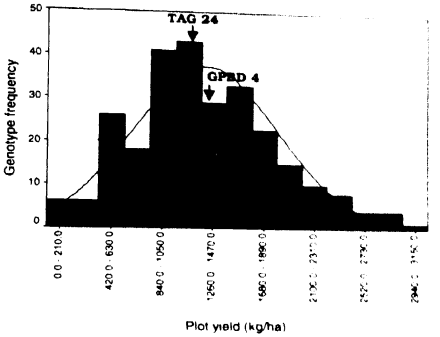
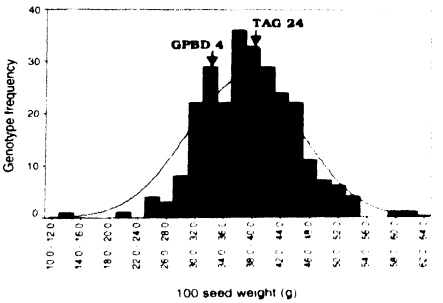
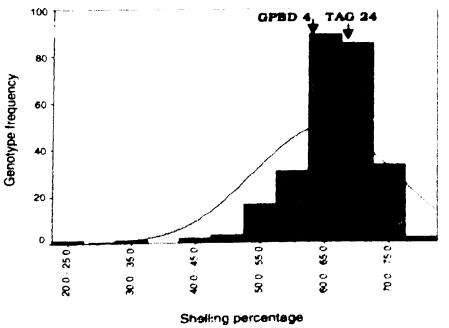

TAG 24
GPBD 4
Genotype frequency
Yield per plant (g)

Fig. 4 Contd.....


TAG 24
GPBD 4
Genotype frequency
Plot yield (kg/ha)


TAG 24
GPBD 4
Genotype frequency
100 seed weight (g)


GPBD 4, TAG 24
Genotype frequency
Shelling percentage

4.1.5 Mean performance of parents

The mean disease score of parents is presented in Table 15. GPBD 4 has shown consistently lower disease incidence than TAG 24 at all the scoring stages and environments for both the diseases. The parents TAG 24 and GPBD 4 exhibited differential performance for some agronomic traits *viz.*, plant height (19.78 cm and 28.06 cm), shelling percentage (66.89 and 60.25), pods per plant (14.99 and 25.73) and 100 seed weight (40.33 g and 33.21 g), while it was comparable for other traits.

4.1.6 Mean and Range of RILs

The disease incidence increased with stages for both LLS and rust in different environments. The ranges of RILs for LLS and rust were comparable and within the limits of the range of values exhibited by the parents. When examined with mid-parental values the mean values for RILs were slightly inclined towards susceptible parent in the case of LLS while for rust it was towards the resistant parent (Table 16). RILs exhibited much wider range of variation as compared to parental differences for all the agronomic traits (Table 17).

4.2 SINGLE MARKER ANALYSIS (SMA)

Detection of putative markers associated with LLS and rust was carried out by single marker analysis (SMA), which used simple linear regression method (Haley and Knott, 1992) to find out the significant marker - trait association. The phenotypic and genotypic data pertaining to 268 RILs was utilized to the tag putative markers.

Table 16: Mean and Range of parents and RILs for late leaf spot and rust

Environments / Traits	Scoring stage	Mean		Range		Mid parent value	RILs	Mean ± SE
		TAG 24	GPBD 4	TAG24	GPBD4			
LLS								
E1	S1	6.75	1.75	6.5 - 7.0	1.5 - 2.0	4.25	1.5 - 7.0	4.49 ± 0.71
	S2	9.00	3.00	9.0 - 9.0	2.0 - 4.0	6.00	1.5 – 9.0	6.95 ± 0.86
E2	S1	7.00	1.75	7.0 - 7.0	1.5 – 2.0	4.37	2.0 - 7.0	5.38 ± 0.51
	S2	7.75	2.00	7.5 - 8.0	2.0 - 2.0	4.87	2.5 - 8.5	6.38 ± 0.40
E3	S1	4.75	1.00	4.5 - 5.0	1.0 - 1.0	2.87	1.0 - 5.5	3.14 ± 0.49
	S2	8.50	3.50	8.0 - 9.0	3.5 - 3.5	6.00	2.5 - 9.0	6.14 ± 0.45
Rust								
	S1	5.00	2.25	4.5 - 5.5	2.0 - 2.5	3.62	2.0 - 6.0	3.38 ± 0.49
E4	S2	5.50	3.35	5.0 - 6.0	3.0 - 3.5	4.42	2.0 - 6.0	4.34 ± 0.45
	S3	7.00	3.50	7.0 - 7.0	3.5 - 3.5	5.25	3.0 - 8.0	5.38 ± 0.57

E1 - *Kharif* 2004
S1 - 70 days to score LLS
S1 - 70 days to score rust

E2 - *Kharif* 2005
S2 - 90 days to score LLS
S2 - 80 days to score rust

E3 - *Kharif* 2006
S3 - 90 days to score rust

E4 - *Kharif* 2007

Table 17: Mean and Range of parents and RILs for agronomic traits

Environments / Traits	Mean		Range		Mid parent value	RILs	Mean ± SE
	TAG 24	GPBD 4	TAG24	GPBD4			
LLN	51.27	55.22	45.2 - 57.0	50.6 - 63.4	53.24	37.6 - 68.8	50.93 ± 3.66
LWD	24.33	25.8	20.0 - 27.6	22.8 - 29.6	25.06	15.8 - 30.4	23.77 ± 1.95
PLHT	19.78	28.06	14.4 - 22.8	21.2 - 32.6	23.92	12.60 - 44.0	24.32 ± 0.18
PBR	4.14	4.31	3.8 - 5.2	4.0 - 5.2	4.22	2.60 - 6.40	4.16 ± 2.35
PPP	14.99	25.73	9.8 - 20.8	14.0 - 53.0	20.36	1.40 - 8.32	3.92 ± 7.5
YPP	13.25	14.09	7.36 - 16.98	7.0 - 30.32	13.67	3.94 - 31.0	13.85 ± 4.7
YKGH	1119.44	1264.43	242.65 - 1499.89	510.0 - 2729.79	1191.93	96.09 - 3128.74	1296.14 ± 430.19
HSW	40.33	33.21	34.6 - 46.5	27.7 - 41.5	36.77	13.61 - 60.5	37.83 ± 4.25
SLNG	66.89	60.25	56.6 - 73.0	51.6 - 64.3	63.57	24.30 - 75.41	62.37 ± 3.23

LLN - Leaf length (mm)
LWD - Leaf width (mm)
PLHT - Plant height (cm)
PBR - No. of primary branches
PPP - Pods per plant
YPP - Yield per plant (g)
YKGH - Plot yield (kg/ha)
HSW - 100 seed weight (g)
SLNG - Shelling percentage

4.2.1 Putative markers associated with LLS

Single marker analysis revealed 28 (E1), 35 (E2) and 23 (E3) markers associated with LLS across stages in different environments (Table 18). Six markers *viz.*, PM436 (1.49 - 4.29%), Lec1 (1.75 - 4.89%), TC2G05 (3.03 - 4.42%), gi-1107 (1.73 - 4.02%), PM179 (1.48 - 5.78%) and TC1A01 (1.44 - 3.51%) were found to be consistent across seasons.

4.2.2 Putative markers associated with rust

Single marker analysis with 67 markers revealed 11 markers associated with rust (Table 19). Among them, four markers *viz.*, XIP103, TC9H09, TC4G10 and GM624 were found at all the three scoring stages. The maximum phenotypic variation was contributed by XIP103 (33.8 - 40.6%) followed by TC9H09 (2.59 - 4.6%), TC4G10 (2.36 - 3.4%) and GM624 (1.99 - 3.5%).

4.2.3 Putative markers associated with agronomic traits

Putative markers associated with 9 agronomic traits and their respective phenotypic variations are listed in Table 20.

4.2.3.1 Leaf length and Leaf width

SMA revealed 8 and 15 single markers associated with leaf length and leaf width, respectively. Among them five markers *viz.*, TC1A01, Seq7G02, PM137, GM670 and XIP108 were found to be common for both the traits. The marker TC1A01 (3.53%) contributed maximum phenotypic variation in leaf length and TC4F12 (8.72%) in leaf width.

Table 18: Putative markers associated with late leaf spot in TAG 24 × GPBD 4 population

Environment 2004 (E1)				Environment 2005 (E2)				Environment 2006 (E3)			
S1		S2		S1		S2		S1		S2	
Markers	R^2_{adj} (%)	Marker	R^2_{adj} (%)	Markers	R^2_{adj} (%)	Markers	R^2_{adj} (%)	Markers	R^2_{adj} (%)	Markers	R^2_{adj} (%)
PM436	4.67**	Seq7G02	5.14**	PM179	5.78**	PM179	5.23**	Seq17E3	5.26**	Seq17E3	4.63**
TC6H03	3.6**	Lec1	4.89**	Seq13E06	4.21**	XIP 524	3.72**	TC2G05	3.35**	TC2G05	3.59**
TC9F10	3.53**	TC2G05	4.42**	GM633	3.44**	Seq13E06	3.57**	TC9F10	2.48**	PM179	2.73**
Lec1	3.12**	PM436	4.29**	XIP524	3.16**	gi 1107	3.35**	TC6H03	2.46**	XIP 524	2.21*
Seq7G02	2.95**	gi 1107	4.02**	gi 1107	2.9**	TC5A07	3.35**	TC1A01	2.41**	TC1A01	2.09*
TC2G05	2.85**	TC9F10	3.86**	Lec1	2.81**	GM 633	3.05**	Seq2E06	1.89*	XIP176	1.99*
XIP107c	2.51**	TC6H03	3.61**	Seq11C08	2.53**	TC2G05	3.03**	GM 660	1.41*	Lec1	1.75*
gi 1107	2.27*	TC1A01	3.51**	TC5A07	2.34**	Lec1	2.76**	TC9H09	1.2*	gi 1107	1.73*
Seq13E06	1.9*	GM 660	2.12*	Seq5D5	2.32**	Seq17E3	2.46*	XIP 176	1.2*	PM436	1.49*
TC2C07	1.49*	TC2C07	1.78*	PM436	2.2*	PM377	2.32*			PM377	1.47*
PM179	1.48*	TC9H09	1.68*	Seq17C09	2.2*	PM436	2.31*			XIP 103	1.43*
TC1A01	1.44*	Seq17E3	1.61*	Seq17E3	1.52*	Seq11C08	2.12*			TC3H02	1.4*
GM660	1.35*	Seq17C09	1.31*	TC2G05	1.46*	TC3H02	1.55*			Seq11C08	1.38*
		TC4G10	1.26*	PM377	1.12*	TC1A01	1.52*			TC11A04	1.36*
		AH4-101	1.23*			TC11A04	1.37*				
						Ah4-04	1.34*				
						PM 137	1.3*				
						TC4D09	1.27*				
						TC4F02	1.26*				
						TC1A02	1.19*				
						Seq5D5	1.18*				

** Significant at P ≤ 0.01, * Significant at P ≤ 0.05
S1 – 70 days to score LLS S2 – 90 days to score LLS

Table 19: Putative markers associated with rust in TAG 24 × GPBD 4 population

Environment 2007 (E4)					
S1		**S2**		**S3**	
Markers	**R^2_{adj} (%)**	**Markers**	**R^2_{adj} (%)**	**Markers**	**R^2_{adj} (%)**
XIP103	36.59**	XIP103	40.58**	XIP103	33.8**
TC9H09	4.58**	TC9H09	2.59**	TC9H09	4.14**
TC4G10	3.93**	TC4G10	2.36**	TC4G10	3.52**
GM624	2.68**	Seq2E06	1.99*	GM624	3.5**
Seq2E06	2.11*	GM624	1.88*	XIP407c	2.07*
XIP395	1.94*	XIP407c	1.39*	Seq13E06	1.36*
TC3E05	1.53*	PM50	1.31*	GM699	1.26*
		Seq13E06	1.28*		

** Significant at P ≤ 0.01, * Significant at P ≤ 0.05
S1 = 70 days to score rust S2 = 80 days to score rust S3 = 90 days to score rust

Table 20: Putative markers associated with agronomic traits in TAG 24 × GPBD 4 population

Agronomic traits							
LLN		LWD		PLHT		PBR	
Markers	R^2_{adj} (%)	Markers	R^2_{adj} (%)	Markers	R^2_{adj} (%)	Markers	R^2_{adj} (%)
TC1A01	3.53**	TC4F12	8.72**	GM633	8.56**	Seq5D5	3.84**
Seq7G02	2.77**	GM699	3.95**	XIP176	6.82**	GM660	3.13*
PM137	2.7**	GM670	3.43**	PM179	4.79**	TC2G05	2.44*
GM670	2.55**	Seq2B10	3.09**	Seq11C08	4.23**	AH4-101	2.02*
Seq13E06	2.16*	TC1A01	3.02**	TC3E05	3.17*	TC9H09	1.94*
XIP407c	1.76*	PM137	2.82**	TC11A04	2.65*	TC9F10	1.8*
Seq5D5	1.42*	XIP272	2.34**	XIP103	2.57*	TC9H03	1.79*
XIP108	1.19*	XIP108	1.95*	TC1A02	2.16*	GM 624	1.52*
		PM50	1.79*	TC5A07	1.78*		
		PM183	1.78*	Seq19D09	1.6*		
		TC11A04	1.77*	TC7H11	1.42*		
		TC1A02	1.73*	GM 670	1.37*		
		GM692	1.55*	PM 50	1.26*		
		PM179	1.54*	Seq5D5	1.23*		
		Seq7G02	1.53*	PM 137	1.1*		

** Significant at P ≤ 0.01, * Significant at P ≤ 0.05

LLN - Leaf length (mm) LWD - Leaf width (mm) PLHT - Plant height (cm) PBR - No. of primary branches

Table 20 contd.....

Agronomic traits									
PPP		**YPP**		**HSW**		**YKGH**		**SLNG**	
Markers	**R^2_{adj} (%)**	**Markers**	**R^2_{adj} (%)**	**Markers**	**R^2_{adj} (%)**	**Markers**	**R^2_{adj} (%)**	**Markers**	**R^2_{adj} (%)**
Seq5D5	10.23**	Seq5D5	6.85**	XIP 103	4.90**	XIP103	9.98**	GM633	5.35**
XIP103	8.18**	XIP103	6.11**	GM670	3.79**	Seq5D5	7.64**	TC9F04	4.96**
PM179	4.42**	TC1B02	1.59*	GM699	3.06**	TC1B02	2.23*	TC1B02	4.94**
TC1B02	3.78**	TC9F10	1.43*	GM633	1.91*	Ah4-101	1.59*	XIP524	3.91*
TC9F10	2.94**	TC6H03	1.24*	XIP108	1.48*	Ah4-04	1.53*	PM179	3.29*
GM633	2.93**	TC2G05	1.23*	PM137	1.44*			Seq11C08	2.62*
TC6H03	2.58**	GM687	1.23*	Ah4-101	1.35*			TC3E05	1.34*
TC2G05	2.47**			Seq2D12B	1.27*			AH4-101	1.32*
GM660	2.21**			XIP121	1.17*			TC6H03	1.25*
AH4-101	1.85*							TC9F10	1.25*
TC9H09	1.62*								
XIP395	1.42*								

** Significant at P ≤ 0.01, * Significant at P ≤ 0.05
PPP - Pods per plant YPP - Yield per plant (g) YKGH - Plot yield (kg/ha)
HSW - 100 seed weight (g) SLNG - Shelling percentage

4.2.3.2 Plant height

SMA for plant height revealed a total of 15 single markers linked to the trait with R^2 ranging from 1.1 - 8.56 per cent. The highest phenotypic variation was observed in GM633 (8.56%) followed by XIP176 (6.82%), PM179 (4.79%) and Seq11C08 (4.23%).

4.2.3.3 Primary branches

Eight single markers were associated with primary branches and contribution ranged from 1.52 - 3.82 per cent. Seq5D5 (3.84%) showed maximum phenotypic variation followed by GM660 (3.13%), TC2G05 (2.44%) and AH4-101 (2.02%).

4.2.3.4 Pods per plant and yield per plant

Twelve single markers in pods per plant and 7 markers in yield per plant were found to be associated and among them, 6 markers *viz.*, Seq5D5, XIP103, TC1B02, TC9F10, TC6H03, and TC2G05 were common between these traits. The markers Seq5D5 (10.23%, 6.85%) and XIP103 (8.18%, 6.11%) contributed high phenotypic variation in pods per plant and yield per plant, respectively.

4.2.3.5 100 seed weight and plot yield

A total of 9 and 5 single markers were tagged with 100 seed weight and plot yield, respectively and two markers XIP103 and Ah4-101 were common between the traits. The markers XIP103 (4.90%), GM670 (3.79%) and GM699 (3.06%) in 100 seed weight and XIP103 (9.98%) and Seq5D5 (7.64%) in yield contributed maximum phenotypic variation.

4.2.3.6 Shelling percentage

A total of 10 single markers were found linked with shelling percentage with contribution ranging from 1.25 - 5.35 per cent. The highest phenotypic variation was recorded in GM633 (5.30%) followed by TC9F04 (4.96%), TC1B02 (4.94%) and XIP524 (3.91%).

4.3 LINKAGE MAP CONSTRUCTION

Since, there is no comprehensive linkage map developed in the cultivated groundnut, linkage map construction was one of the major objectives of the present investigation. The linkage map was constructed using software MAPMAKER/EXP V 3.0 (Lander *et al.*, 1987; Lincoln *et al.*, 1992) multipoint analysis with minimum LOD score of 3.0 and maximum recombination fraction (θ) of 0.5 were set as threshold for linkage group determination. Out of 1089 microsatellite markers screened, only 67 were found to be polymorphic between parents of the TAG 24 × GPBD 4 mapping population. The chi-square (χ^2) test was conducted to test the Mendelian segregation ratio (expected 1:1) and 20 markers showed segregation distortion (SD). But due to paucity of polymorphic markers, all the 67 markers were used for linkage map construction. A total of 59 markers were mapped on 13 linkage groups (LGs) spanning 909.4 cM and 8 markers remained ungrouped. The number of markers mapped per linkage group ranged from two (LG 3, LG 11, LG 12 and LG 13) to ten (LG 4 and LG 5). The lengths of linkage groups were ranging from 6.00 cM (LG 12) to 226 cM (LG 4) with an average distance of 15.25 cM (Table 21). The linkage map constructed based on TAG 24 × GPBD 4 was used for

Table 21: Genetic markers assigned to linkage groups

Linkage group (LGs)	Number of Microsatellites	Likelihood ratio (LOD)	Length (cM)	Average distance (cM)
LG 1	7	-597.00	173.70	24.81
LG 2	3	-411.96	86.60	28.86
LG 3	2	-189.89	15.60	7.80
LG 4	8 (+ 2 synthetic markers)	-1039.99	226.90	28.36
LG 5	9 (+1 synthetic markers)	-798.00	76.90	8.54
LG 6	5 (+1 synthetic markers)	-661.69	63.90	12.78
LG 7	5	-474.43	66.20	13.24
LG 8	3	-391.49	74.10	24.70
LG 9	3(+1 synthetic markers)	-290.20	16.50	5.50
LG 10	3	-336.33	28.70	9.56
LG 11	2	-216.93	19.00	9.50
LG 12	2	-182.54	6.00	3.00
LG 13	2	-267.49	70.90	35.45

identification and mapping of QTL for resistance to LLS and rust besides agronomic traits (Fig. 5).

4.4 QTL MAPPING

The foremost step towards QTL mapping is to have linkage map with good coverage of markers. The partial linkage map developed from the cross TAG 24 × GPBD 4 was used for QTL analysis. QTL analysis was done by using software PLABQTL version 1.1w (Utz and Melchinger, 1996). The RIL means for LLS and rust and best linear unbiased predictors (BLUPs) for agronomic traits were used for the analysis. In order to take care of distribution abnormalities, if any, square root transformed means (SQ) and $\log_{10}$ transformed (LOG) means in LLS were also utilized for the identification QTL. Frequency distribution of rust scoring data showed normal distribution so data was not transformed. Two environment data *viz.*, E1 and E2 yielded eight QTL and E3 did not reveal any QTL and hence data pertaining to the first two environments was utilized to assess Q × E interaction.

4.4.1 QTL for LLS

4.4.1.1 Environment 1

Stage 1

One QTL (*Seq9E08 - XIP407c*) at stage 1 was identified, which was mapped on LG 13 with a LOD of 3.09. This QTL explained 2.7 per cent of phenotypic variation with 0.14 additive effect and the favorable allele for

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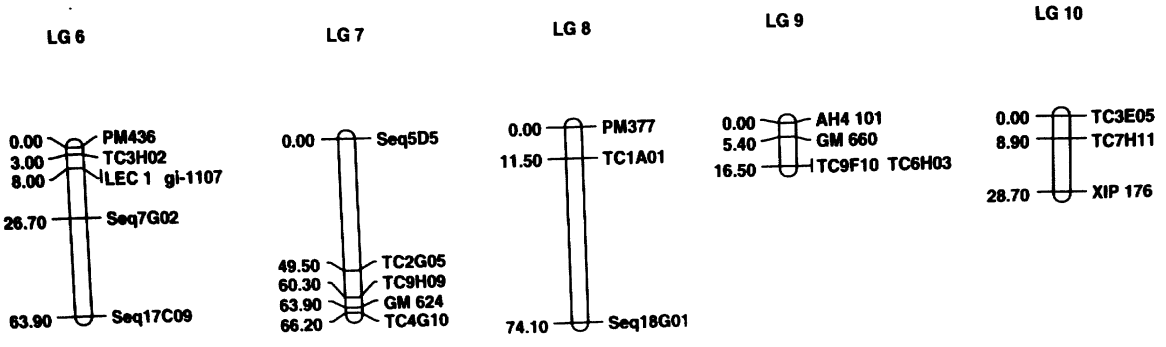
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3.00
8.00
26.70
63.90
PM436
TC3H02
LEC 1 gi-1107
Seq7G02
Seq17C09
LG 7
0.00
49.50
60.30
63.90
66.20
Seq5D5
TC2G05
TC9H09
GM 624
TC4G10
LG 8
0.00
11.50
74.10
PM377
TC1A01
Seq18G01
LG 9
0.00
5.40
16.50
AH4 101
GM 660
TC9F10 TC6H03
LG 10
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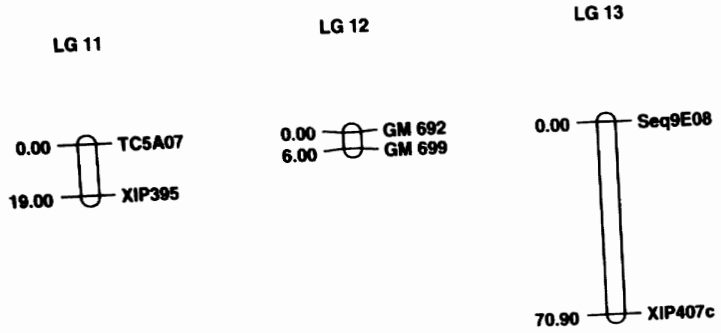
8.90
28.70
TC3E05
TC7H11
XIP 176

Contd.....


LG 11
0.00
TC5A07
19.00
XIP395
LG 12
0.00
GM 692
6.00
GM 699
LG 13
0.00
Seq9E08
70.90
XIP407c

this locus has contributed by the susceptible parent, TAG 24 (Table 22, Fig. 6 and 7)

Stage 2

A total of three QTL flanked by the markers *Lxc1 - Seq7G02*, *TC2G05 - TC9H09* and *GM660 - TC9F10* with LOD score 2.84, 2.51 and 3.01, respectively were identified. The first QTL (*Lxc1 - Seq7G02*) positioned on LG 6 expressed 5.7 per cent of the phenotypic variation with 0.22 additive effects. The second QTL (*TC2G05 - TC9H09*) located on LG 7 accounted for 4.7 per cent of phenotypic variation with an additive effect of 0.17 and the third QTL (*GM660 - TC9F10*) mapped on LG 9 explained 3.6 per cent of phenotypic variation with 0.14 additive effect. The favorable alleles in all the QTL were contributed by the resistant parent GPBD 4 and they together explained 14.0 per cent of the total phenotypic variation.

4.4.1.2 Environment 2

Stage 1

Two QTL at marker interval *XIP524 - TC4D09* (LG 1) and *GM633 - PM179* (LG 4) were detected with 3.3 and 3.2 LOD scores, respectively. The QTL (*XIP524 - TC4D09*) explained 2.9 per cent phenotypic variation with 0.22 additive effect and the second QTL (*GM633 - PM179*) explained 6.2 per cent of phenotypic variation having an additive effect of 0.29. The favorable alleles for these QTL came from the resistant parent GPBD 4 and together accounted for 9.1 per cent of phenotypic variation.

Table 22: QTLs associated with resistance to late leaf spot at different stages and environments in TAG 24 x GPBD 4 population

Environment	Scoring stage	LG	Marker interval	Position (cM)	LOD	R^2 (%)	Additive effect
E1	S1	13	*Seq9E08 - XIP407c*	70	3.09	2.7	0.14
	S2	6	*Lec1 Seq7G02*	20	2 84	5.7	-0 22
		7	*TC2G05 TC9H09*	50	2 51	4.7	-0.17
		9	*GM660 - TC9F10*	16	3.01	3.6	-0 14
E2	S1	1	*XIP524 TC4D09*	150	3.31	2.9	-0.22
		4	*GM633 PM179*	4	3 2	6 2	-0 29
	S2	1	*XIP524 TC4D09*	150	2 56	3 8	-0 26
		4	*GM633 PM179*	4	2 77	5 8	0 29
E3	S1						
	S2						

LG- Linkage group cM -centiMorgan R- Phenotypic variance LOD- Logarithm of odds ratio
and + sign indicates the direction of favorable alleles - indicate GPBD 4 and + indicate TAG 24
E1 - *Kharif* 2004 E2 - *Kharif* 2005 E3 - *Kharif* 2006
S1 - 70 days to score LLS S2 - 90 days to score LLS

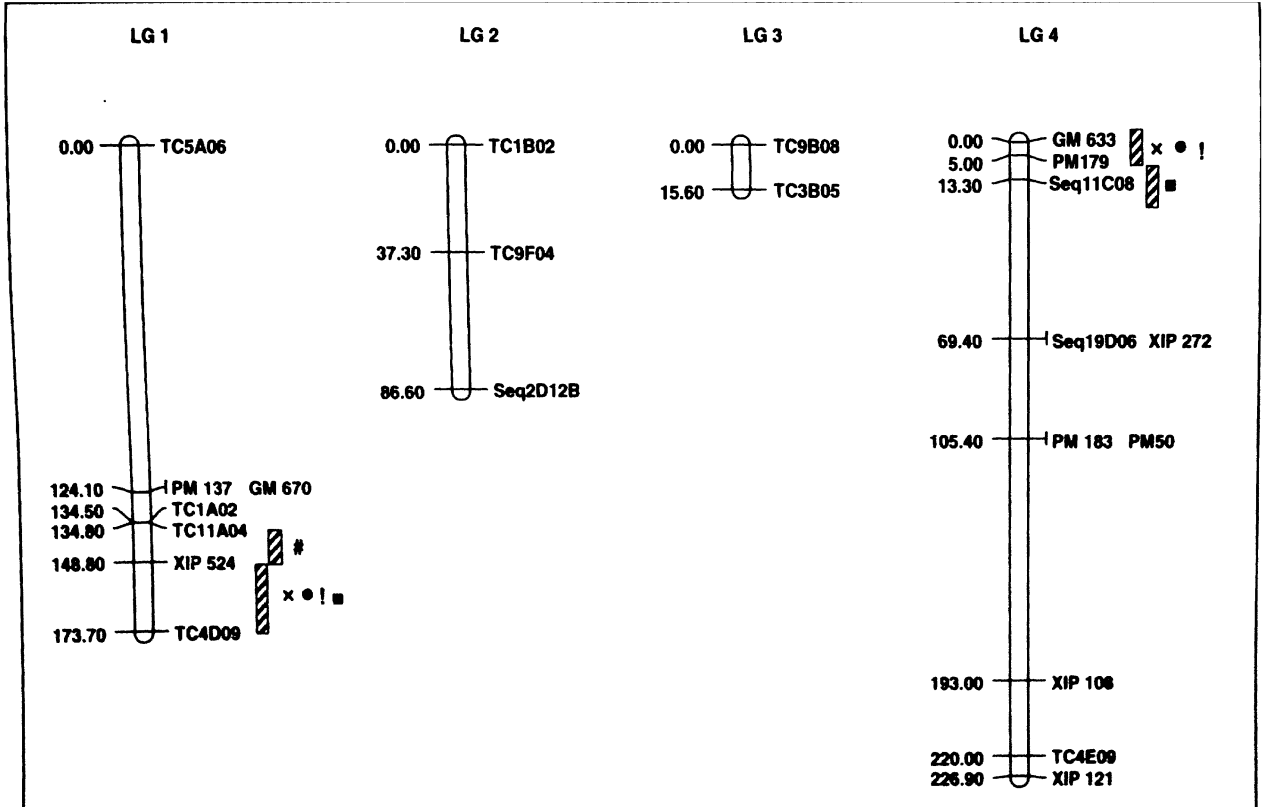


Fig. 6: Genetic linkage map showing resistance to LLS and rust QTLs in TAG 24 x GPBD 4 mapping population

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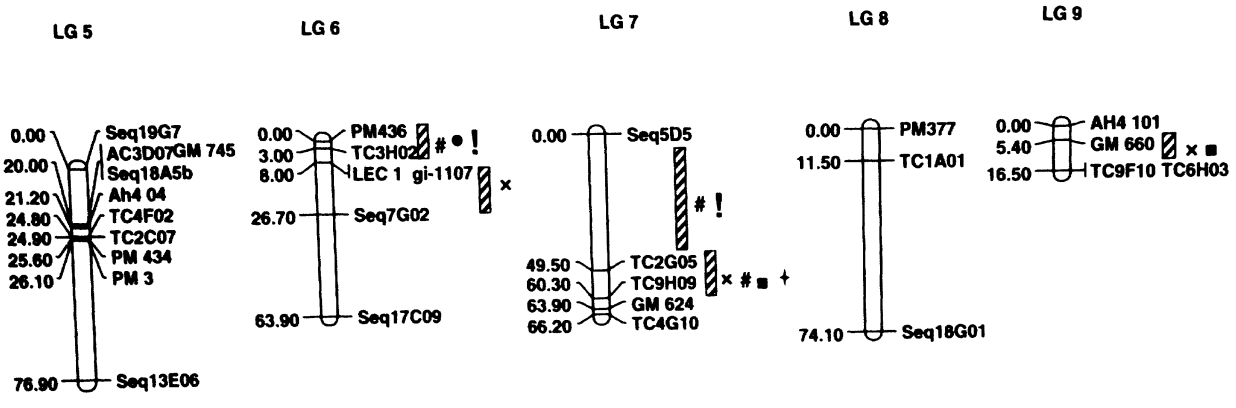
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LG 6
LG 7
LG 8
LG 9
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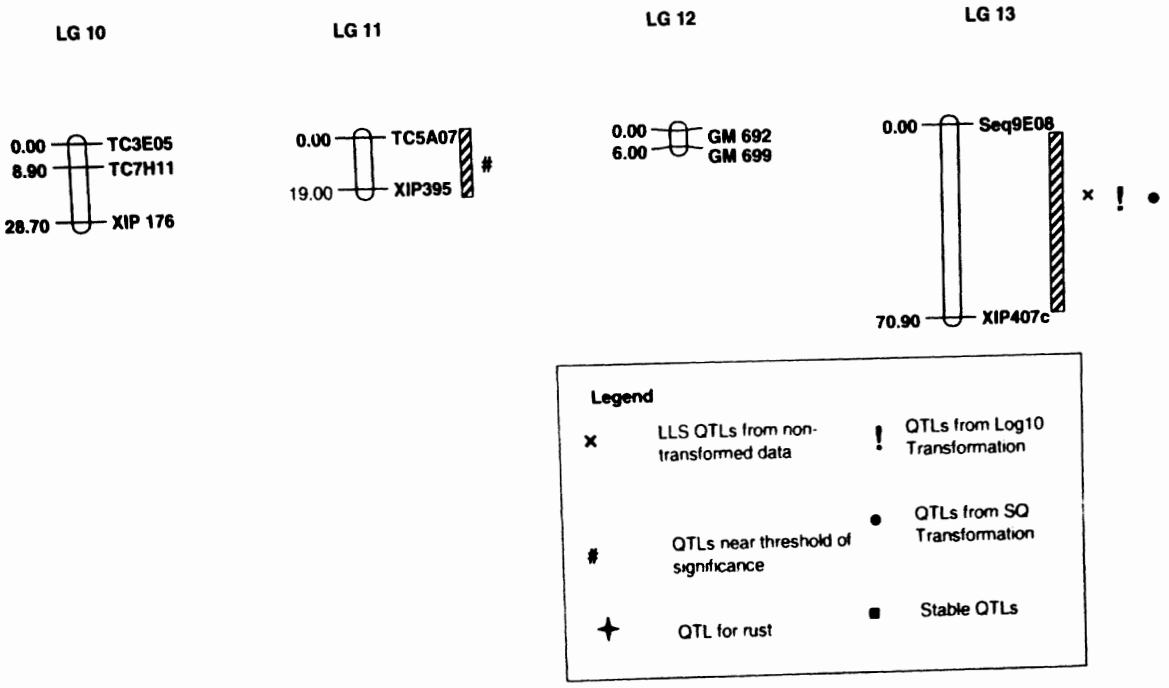
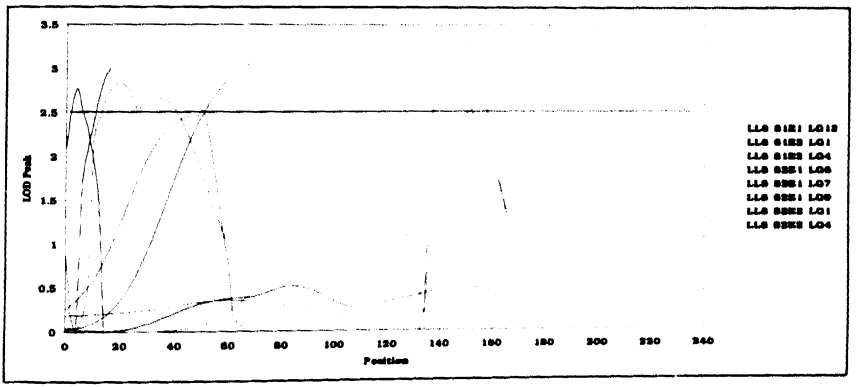
20.00
21.20
24.80
24.90
25.60
26.10
76.90
Seq19G7
AC3D07GM 745
Seq18A5b
Ah4 04
TC4F02
TC2C07
PM 434
PM 3
Seq13E06
0.00
3.00
8.00
26.70
63.90
PM436
TC3H02
LEC 1 gi-1107
Seq7G02
Seq17C09
0.00
49.50
60.30
63.90
66.20
Seq5D5
TC2G05
TC9H09
GM 624
TC4G10
0.00
11.50
74.10
PM377
TC1A01
Seq18G01
0.00
5.40
16.50
AH4 101
GM 660
TC9F10 TC6H03

Contd.....


LG 10
0.00
TC3E05
8.90
TC7H11
28.70
XIP 176
LG 11
0.00
TC5A07
#
19.00
XIP395
LG 12
0.00
GM 692
6.00
GM 699
LG 13
0.00
Seq9E08
× ! •
70.90
XIP407c
Legend
× LLS QTLs from non-transformed data
! QTLs from Log10 Transformation
QTLs near threshold of significance
• QTLs from SQ Transformation
✦ QTL for rust
■ Stable QTLs



S1- Stage 1 S2- Stage 2 E1- Environment 1 E2- Environment 2
LG- Linkage Group.

Fig. 7: QTL LOD Peak for LLS at different stages and environments

Stage 2

The same two QTL detected at stage 1 co mapped at stage 2 with change in magnitude of phenotypic variation and effect but without any change in the direction. These QTL accounted for 3.8 per cent and 5.8 per cent of phenotypic variation with 0.26 and 0.29 additive effects, respectively. Total contribution of these QTL to phenotypic variation was 9.6 per cent.

4.4.1.3 Environment 3

The data from environment 3 did not yield any QTL at different stages.

4.4.2 QTL near threshold of significance (LOD 1.85 - 2.47)

4.4.2.1 Environment 1

Two QTL (*PM436 - TC3H02* and *GM660 - TC9F10*) were detected and they were mapped on LG 6 and LG 9 and explained together 7.8 per cent (4.3 and 3.5 per cent phenotypic variation, respectively) variation at stage 1 (Table 23).

4.4.2.2 Environment 2

At stage 2, the two QTL (*TC2G05 - TC9H09*) on LG 7 and (*TC5A07 -XIP395*) on LG 11 were identified and contributed 3.4 and 4.0 per cent phenotypic variation, respectively.

4.4.2.3 Environment 3

The QTL flanked by the markers *TC11A07 - XIP524* and *Seq5D5 - TC2G05* mapped on LG 1 and LG 7 and accounted for 1.4 and 3.6 per cent phenotypic variation at stage 2.

Table 23: QTLs associated with late leaf spot at near threshold of significance

Environment	Scoring stage	LG	Marker interval	Position (cM)	LOD	R^2 (%)	Additive effect
E1	S1	6	*PM436 TC3H02*	0	2.26	4.3	-0.16
		9	*GM660 TC9F10*	16	2.38	3.5	0.15
E2	S2	7	*TC2G05 TC9H09*	50	1.85	3.4	-0.20
		11	*TC5A07 XIP395*	0	1.86	4.0	0.22
E3	S2	1	*TC11A04 XIP524*	144	2.47	1.4	0.30
		7	*Seq5D5 TC2G05*	48	2.45	3.6	0.22

E1 *Khanti* 2004 E2 *Khanti* 2005 E3 *Khanti* 2006
S1 70 days to score LLS S2 90 days to score LLS

The favorable alleles that had increasing additive effects for all these QTL were from the resistant parent GPBD 4.

4.4.3 QTL × Environment interaction

Analysis of variation for QTL × environment revealed significant differences between the genotype, environment and genotype × environment at both the stages and QTL × environment at stage 1 (Table 24a and 24b).

Two significant QTL flanked by the markers *XIP524 - TC4D09* (LG 1) and *PM179 - Seq11C08* (LG 4) were detected at stage 1 and QTL explained 2.1, 4.6 per cent of phenotypic variation with 0.18, 0.25 additive effects, respectively. The resistant parent GPBD 4 contributed the favorable allele for these QTL. A total 6.7 per cent phenotypic variation for the trait has been accounted by these QTL (Table 25, Fig. 6 and 8)

At stage 2, two stable QTL between the markers *TC2G05 - TC9H09* (LG 7) and *GM660 - TC9F10* (LG 9) were observed with LOD score 3.02 and 2.65, respectively. Individually they explained 5.3 per cent and 2.9 per cent of phenotypic variation with 0.28 and 0.21 additive effects, respectively and together their contribution was 8.2 per cent with favorable alleles coming from GPBD 4.

4.4.4 Effect of transformation on the identification of QTL

4.4.4.1 Environment 1

The data transformation has not affected the appearance of QTL (*Seq9E08 - XIP407c*) at stage1 while at stage 2, the QTL (*LEC 1 - Seq7G02*)

Table 24a: Analysis of variance pertaining to G × E analysis at stage 1 for late leaf spot

Source of variation	DF	Mean sum of Square	F value
Environment	1		
Genotypes	266	2.57**	3.71
QTL	2	21.92*	4.40
Residuals	264	2.43**	3.58
Genotype × Environment	266	0.69	
QTL × Environment	2	2.70*	3.99
Residual × Environment	264	0.67	

** Significant at P < 0.01, * Significant at P < 0.05

Table 24b: Analysis of variance pertaining to G × E analysis at stage 2 for late leaf spot

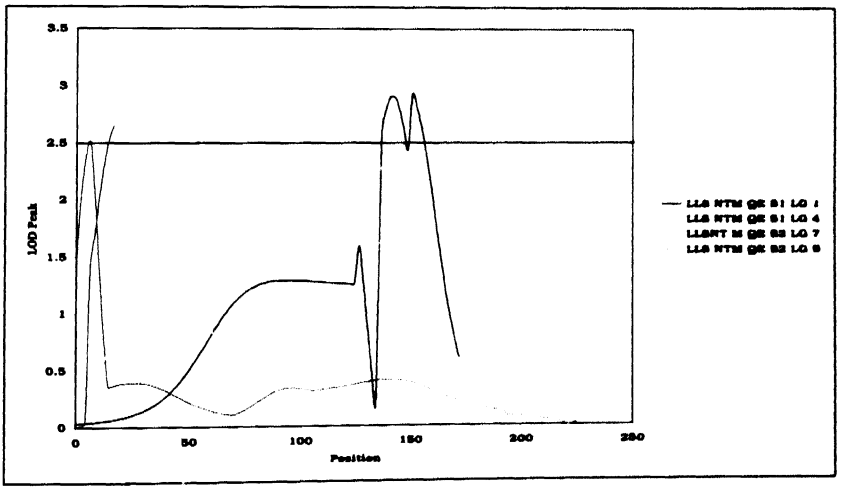
Source of variation	DF	Mean sum of Square	F value
Environment	1		
Genotypes	266	2.86**	4.30
QTL	2	30.94**	7.65
Residuals	264	2.65**	4.02
Genotype × Environment	266	0.66	
QTL × Environment	264	0.65	
Residual × Environment	2	1.47	2.24

** Significant at P ≤ 0.01, * Significant at P ≤ 0.05

Table 25: QTLs associated with late leaf spot across environments

Scoring stage	LG	Marker interval	Position (cM)	LOD	R^2 (%)	Additive effect
S1	1	*XIP 524 - TC4D09*	150	2.92	2.1	-0.185
	4	*PM 179 - SeqHC08*	6	2.51	4.6	-0.254
S2	7	*TC2G05 - TC9H09*	50	3.02	5.3	-0.286
	9	*GM 660 - TC9F10*	16	2.65	2.9	-0.203

S1 - 70 days to score LLS S2 - 90 days to score LLS



NTM – Non transformed means QE- QTL × Environment

Fig. 8: QTL LOD Peak for LLS in across environment

and (*TC2G05 - TC9H09*) disappeared upon data transformation. At stage 2 the QTL (*GM660 - TC9F10*) was retained in square root transformation but displaced by (*Seq5D5 - TC2G05*) in $\log_{10}$ transformation. The new QTL positioned on LG 7, explained 5.4 per cent phenotypic variation with favorable allele contributed from GPBD 4 (Table 26a and 26b, Fig. 6, 9 and 10).

4.4.4.2 Environment 2

The data transformation did not affect the QTL identification at the both stages.

4.4.4.3 Environment 3

Though no QTL were detected from original data, transformation resulted in the detection of a new QTL (*TC3H02 - Lec1*) at stage 1. The QTL was mapped on LG 6 and explained 3.5 to 3.7 per cent of phenotypic variation with favorable alleles coming from GPBD 4 (Table 26a and 26b).

The QTL, Seq9E08 - *XIP407c* (stage 1) in and *GM660 TC9F10* (stage 2) in E1 and *XIP524 - TC4D09 and GM633 - PM179* (stage 1 and 2) in E2 were detected from transformed as well as non-transformed data (Table 27).

4.4.4.4 QTL across the environments

At stage 1, the QTL (*PM179 - Seq11C08*) was detected in all the data analyses while the QTL (*XIP524 - TC4D09*) was retained in log 10 transformation but replaced by (*TC11A04 - XIP524*) in square root transformation and it was mapped on LG 1 with 1.0 per cent contribution

Table 26a: QTL associated with late leaf spot based on square root transformation

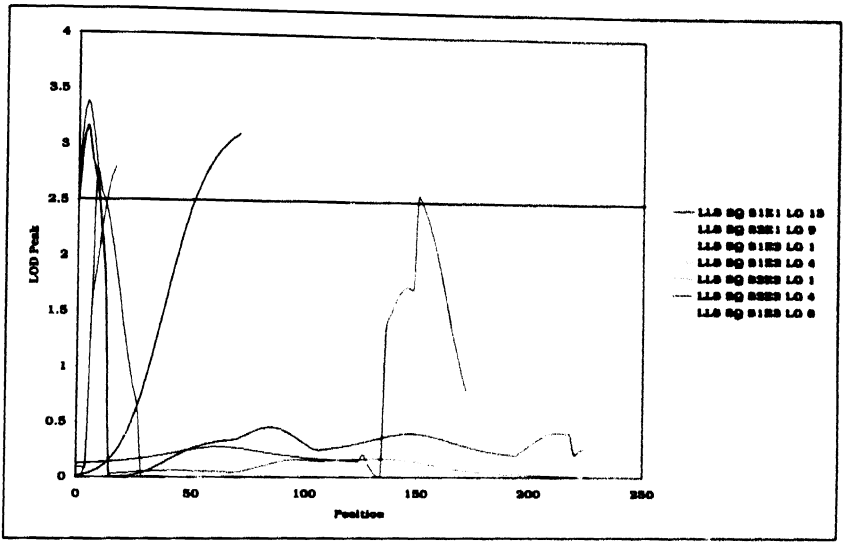
Environment	Scoring stage	LG	Marker interval	Position (cM)	LOD	R^2 (%)	Additive effect
E1	S1	13	*Seq9E08 XIP407c*	70	3.11	2.6	0.051
	S2	9	*GM660 TC9F10*	16	2.80	3.7	-0.056
E2	S1	1	*XIP524 TC4D09*	150	3.36	3.1	-0.062
		4	*GM633 PM179*	4	3.35	6.0	-0.079
	S2	1	*XIP524 TC4D09*	150	2.66	3.9	0.062
		4	*GM633 PM179*	4	3.06	6.0	-0.070
E3	S1	6	*TC3H02 Lec1*	8	2.76	3.7	0.052
	S2						

E1 *Kharif* 2004 E2 *Kharif* 2005 E3 *Kharif* 2006
S1 70 days to score LLS S2 90 days to score LLS

Table 26b: QTL associated with late leaf spot based on Log_{10} transformation

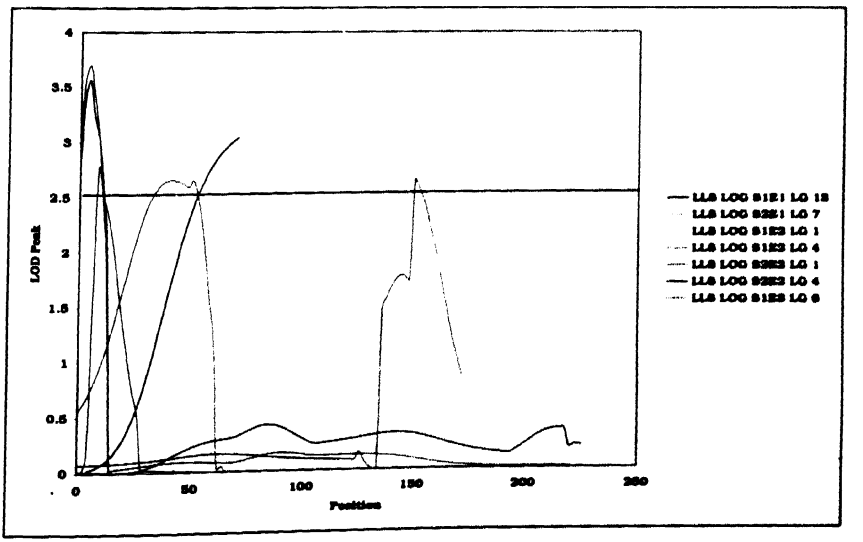
Environment	Scoring stage	LG	Marker interval	Position (cM)	LOD	R^2 (%)	Additive effect
E1	S1	13	Seq9E08 - *XIP407c*	70	3.04	2.4	0.021
	S2	7	Seq5D5 - TC2G05	40	2.65	5.4	-0.037
E2	S1	1	*XIP524* - *TC4D09*	150	2.93	3.1	-0.026
		4	*GM633* - *PM179*	4	3.70	6.4	-0.035
	S2	1	*XIP524* - *TC4D09*	150	2.63	3.8	-0.023
		4	*GM633* - *PM179*	4	3.56	6.8	-0.028
E3	S1	6	*TC3H02* - *Lec1*	8	2.76	3.5	-0.025
	S2	-	-	-	-	-	-

E1 - *Kharif* 2004 E2 - *Kharif* 2005 E3 - *Kharif* 2006
S1 - 70 days to score LLS S2 - 90 days to score LLS



SQ- Square root transformation

Fig. 9: QTL LOD Peak for LLS using square root transformed means



LOG- Log_{10} transformation

Fig. 10: QTL LOD Peak for LLS using Log_{10} transformed means

Table 27: Common QTL associated with late leaf spot in non-transformed and transformed data sets

			Non – transformed		Transformed			
					Square root		Log10	
Scoring stage/ Environment	Marker interval	LG	R^2 (%)	Additive effect	R^2 (%)	Additive effect	R^2 (%)	Additive effect
E1S1	Seq9E08 - *NIP407c*	13	2.7	0.14	2.6	0.051	2.4	0.021
E1S2	GM660 - *TC9E10*	9	3.6	-0.14	3.7	-0.056	-	-
E2S1	*NIP524* - *TC4D09*	1	2.9	-0.22	3.1	-0.062	3.1	-0.026
	GM633 - *PM179*	4	6.2	0.29	6.0	-0.079	6.4	-0.035
E2S2	*NIP524* - *TC4D09*	1	3.8	-0.26	3.9	-0.062	3.8	-0.023
	GM633 - *PM179*	4	5.8	-0.29	6.0	-0.070	6.8	-0.028

E1 - *Kharif* 2004 E2 - *Kharif* 2005 E3 - *Kharif* 2006
S1 - 70 days to score LLS S2 - 90 days to score LLS

to the phenotypic variation. Likewise at stage 2 the QTL (*TC2G05 - TC9H09*) was identified in all the analyses and the QTL (*GM660 - TC9F10*) have disappeared upon transformation (Table 28a and 28b, Fig. 6, 11 and 12)

4.4.5 QTL for Rust

The QTL flanked by the markers *TC2G05 - TC9H09* was detected in stage 1 and 3 but not in stage 2. The QTL positioned on LG 6 accounted for 4.6 and 4.7 per cent to the phenotypic variation, respectively. The additive effects were 0.17 and 0.22 at two stages with favorable allele coming from the resistant parent, GPBD 4 (Table 29, Fig. 7 and 13).

4.4.6 QTL for agronomic traits

The results are presented in Table 30, Fig.14 and 15.

4.4.6.1 Leaf length and leaf width

A single QTL for leaf length was co-mapped for leaf width flanked by the markers *TC1A01 - Seq18G01* and accounted for 4.7 per cent and 3.7 per cent of phenotypic variation.

4.4.6.2 Plant height

A total of three QTL were identified for plant height between the marker interval *TC11A04 - XIP524, GM63 - PM179 and TC7H11 - XIP176*. These QTL explained a total of 18.8 per cent phenotypic variation and were contributed by TAG 24.

Table 28a: QTLs associated with late leaf spot across environments based on square root transformation

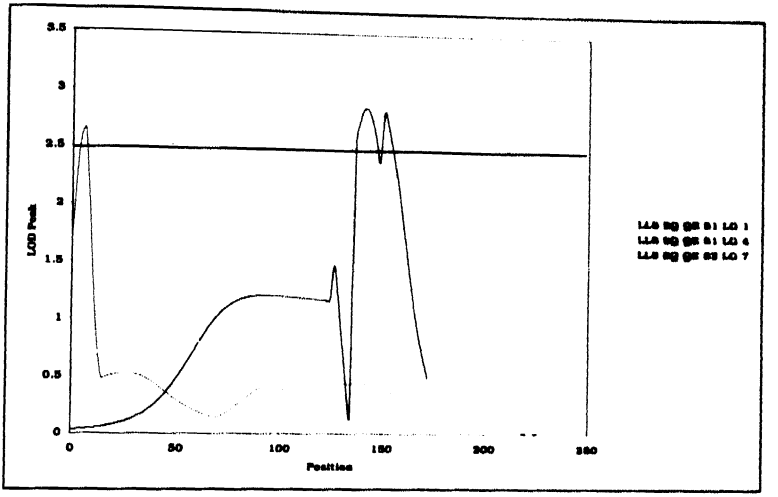
Scoring stage	LG	Marker interval	Position (cM)	LOD	R^2 (%)	Additive effect
S1	1	TC11A04 - XIP524	140	2.86	1.0	-0.031
	4	PM179 - ScqTC08	6	2.64	4.8	-0.063
S2	7	TC2G05 - TC9H09	50	3.05	5.5	0.063

S1 – 70 days to score LLS S2 – 90 days to score LLS

Table 28b: QTLs associated with late leaf spot across environments based on log_{10} transformation

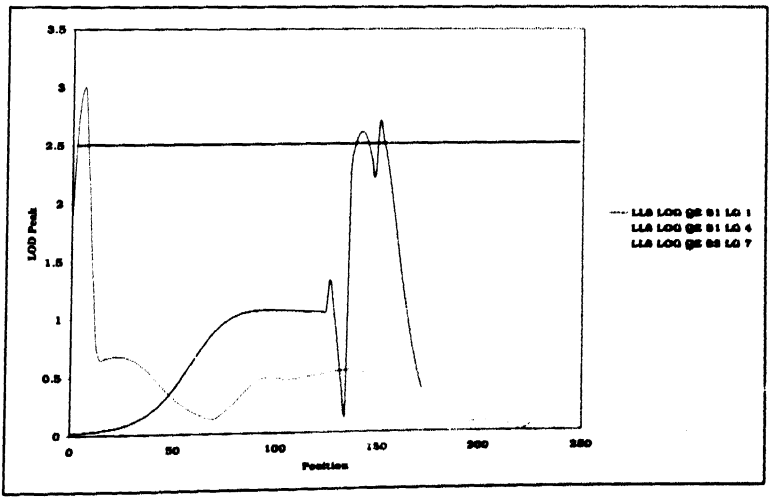
Scoring stage	LG	Marker interval	Position (cM)	LOD	R^2 (%)	Additive effect
S1	1	*XIP524 - TC2G05*	150	2.69	2.2	-0.020
	4	*PM 179 - Seq1IC08*	6	2.99	5.2	-0.028
S2	7	*TC2G05 - TC9H09*	50	2.57	5.2	-0.023

S1 - 70 days to score LLS S2 - 90 days to score LLS



SQ – Square root transformed means QE- QTL x Environment

Fig. 11: QTL LOD Peak for LLS in across environment using square root transformed means



LOG – Log_{10} transformed means, QE- QTL x Environment

Fig. 12: QTL LOD Peak for LLS in across environment using Log_{10} transformed means

Table 29: QTLs associated with resistance to rust at different stages

Scoring stage	LG	Marker interval	Position (cM)	LOD	R^2 (%)	Additive effect
S1	7	TC2G05 - TC9H09	60	2.53	4.6	-0.165
S2	-	-	-	-	-	-
S3	7	TC2G05 - TC9H09	60	2.55	4.7	-0.223

S1 - 70 days to score rust, S2 - 80 days to score rust, S3 - 90 days to score rust

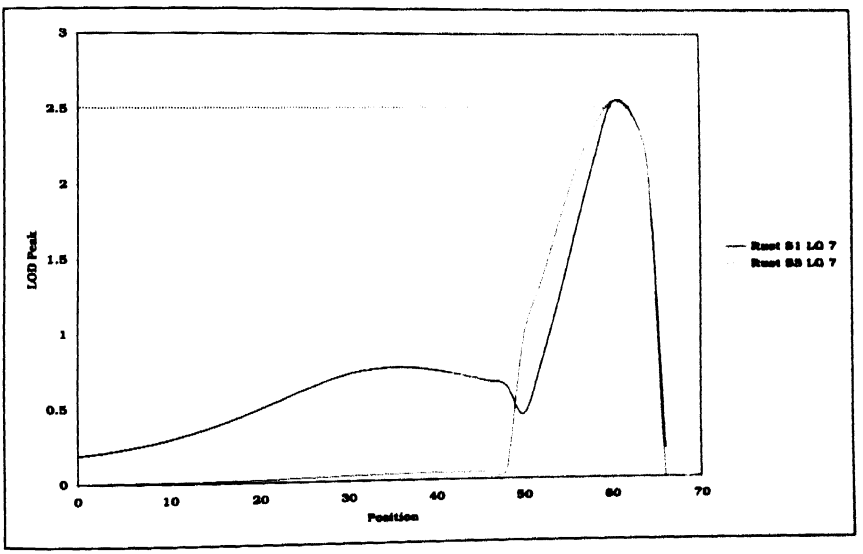


Fig. 13: QTL LOD Peak for rust at different stages

Table 30: QTLs associated with agronomic traits

Trait	LG	Marker interval	Position (cM)	LOD	R^2 (%)	Additive effect
LLN	8	*TC1A01 - Seq18G01*	32	2.71	4.7	0.448
LWD	8	*TC1A01 - Seq18G01*	30	2.70	3.7	0.156
PLHT	1	*TC11A04 - XIP524*	136	3.71	3.2	0.610
	4	*GM633 - PM179*	0	7.09	9.0	1.013
	10	*TC7H11 - XIP176*	28	5.04	6.6	0.882
Cumulative					18.8	
PBR	7	*Seq5D5 - TC2G05*	22	2.50	5.7	0.075
PPP	2	*TC1B02 - TC9F04*	0	2.86	4.3	-0.869
	7	*Seq5D5 - TC2G05*	8	7.69	11.3	1.841
Cumulative					15.6	
YPP	7	*Seq5D5 - TC2G05*	8	4.46	7.5	0.054
YKGH	7	*Seq5D5 - TC2G05*	0	5.70	8.0	31.613
HSW	-	-	-	-	-	-
SLNG	2	*TC1B02 - TC9F04*	22	3.87	5.7	2.168
	4	*GM633 - PM179*	0	2.71	5.1	-1.373
Cumulative					10.8	

LLN - Leaf length (mm)
PBR - No. of primary branches
YKGH - Plot yield (kg/ha)
LWD - Leaf width (mm)
PPP - Pods per plant
HSW - 100 seed weight (g)
PLHT - Plant height (cm)
YPP - Yield per plant (g)
SLNG - Shelling percentage

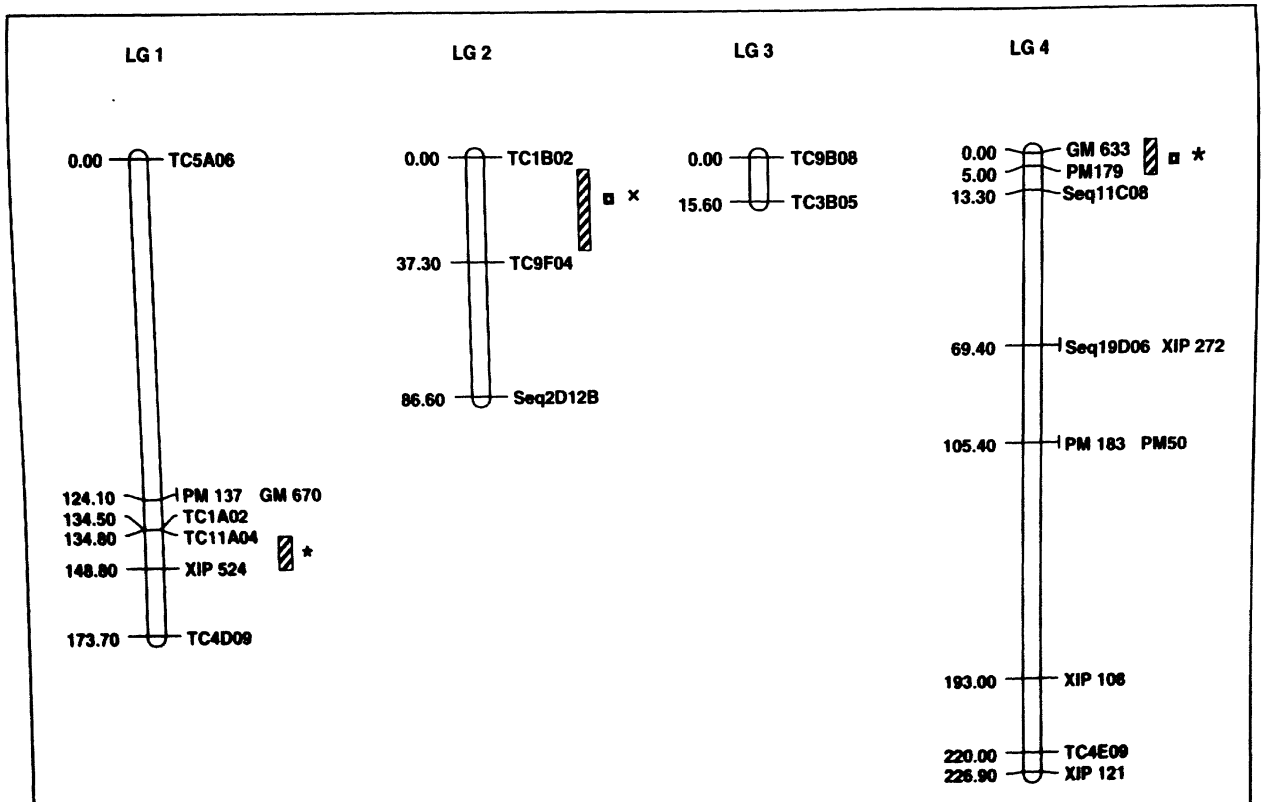
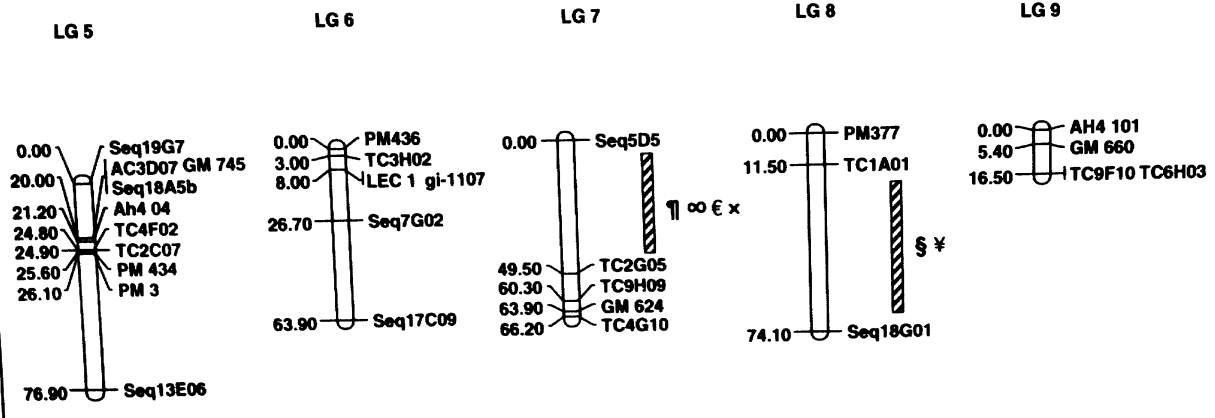
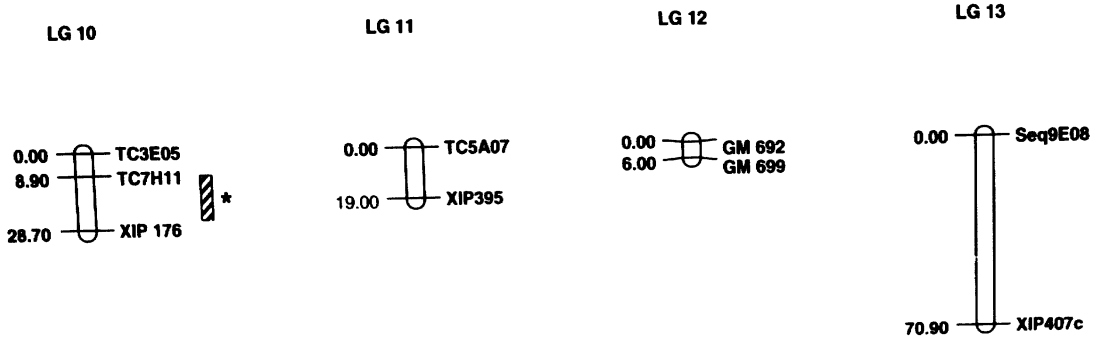


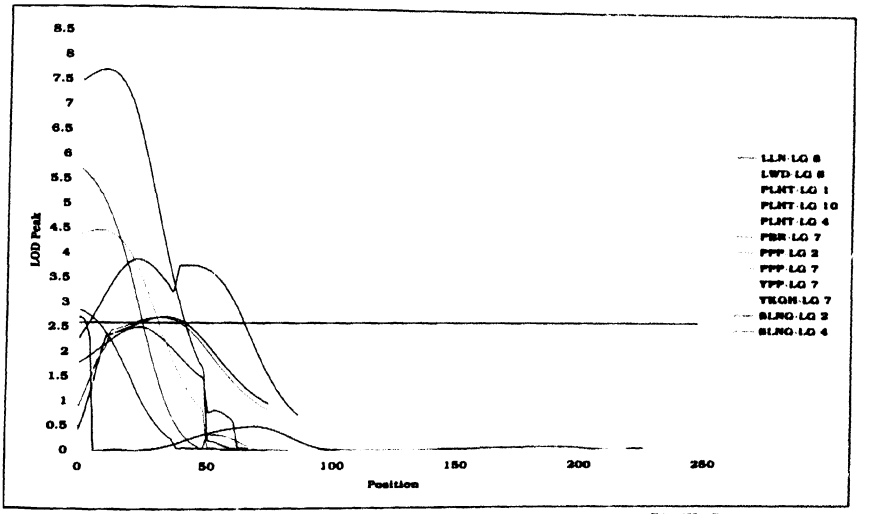
Fig. 14: Genetic linkage map showing QTLs for agronomic traits in TAG 24 x GPBD 4 mapping population

Contd.....


LG 5
0.00
20.00
21.20
24.80
24.90
25.60
26.10
76.90
Seq19G7
AC3D07 GM 745
Seq18A5b
Ah4 04
TC4F02
TC2C07
PM 434
PM 3
Seq13E06
LG 6
0.00
3.00
8.00
26.70
63.90
PM436
TC3H02
LEC 1 gi-1107
Seq7G02
Seq17C09
LG 7
0.00
49.50
60.30
63.90
66.20
Seq5D5
¶ ∞ € x
TC2G05
TC9H09
GM 624
TC4G10
LG 8
0.00
11.50
74.10
PM377
TC1A01
§ ¥
Seq18G01
LG 9
0.00
5.40
16.50
AH4 101
GM 660
TC9F10 TC6H03

Contd.....


LG 10
0.00
8.90
28.70
TC3E05
TC7H11
XIP 176
*
LG 11
0.00
19.00
TC5A07
XIP395
LG 12
0.00
6.00
GM 692
GM 699
LG 13
0.00
70.90
Seq9E08
XIP407c
Legend
§ LLN
¥ LWD
• PLHT
¶ PBR
× PPP
€ YPP
▪ YKGH
■ SLNG



LLN- Leaf Length LWD- Leaf Width PLHT- Plant Height

PBR- No. of Primary Branches PPP- Pods Per Plant YPP- Yield Per Plant
YKGH- Yield per Hectare SLNG- Shelling Percent

Fig. 15: QTL LOD Peak for agronomic traits

4.4.6.3 Primary branches

One QTL was detected for primary branches flanked by the markers *Seq5D5 - TC2G05* with 5.7 per cent of phenotypic variation and the favorable allele came from the parent TAG 24.

4.4.6.4 Pods per plant

Two QTL for pods per plant between the markers *TC1B02 - TC9F04* and *Seq5D5 - TC2G05* explained 15.6 per cent phenotypic variation. The favorable alleles contributed by both the parents, TAG 24 and GPBD 4 (*Seq5D5 - TC2G05* and *TC1B02 - TC9F04*).

4.4.6.5 Yield per plant and plot yield

The QTL flanked by the markers *Seq5D5 - TC2G05* was co mapped for yield per plant and plot yield and contributed 7.5 per cent and 8.0 per cent to the phenotypic variation with the favorable allele coming from TAG 24.

4.4.6.6 Shelling percentage

Two QTL between the marker interval *TC1B02 - TC9F04* and *GM633* - PM179 were identified for shelling percentage and they contributed 10.8 per cent to the phenotypic variation. Both the parents TAG 24 (*TC1B02 - TC9F04*) and GPBD 4 (*GM633 - PM179*) contributed the favorable alleles.

Discussion

V. DISCUSSION

Groundnut (*Arachis hypogaea* L.) is one of the principal oilseed crops of the world. The LLS and rust are the major foliar diseases and they often occur together leading to 50 - 70 per cent yield loss in the crop. Development of cultivars with resistance to foliar diseases is the best strategy to diminish cost of cultivation, soil and environment pollution. Majority of the wild species harbor resistance to these diseases but the introgression is thwarted due to cross compatibility barrier and linkage drag. Hence limited success has been achieved in groundnut resistance breeding.

The advent of biotechnological tools like molecular markers has revolutionized the conventional resistance breeding in gaining the better success through MAS. The major constraints in resistance breeding are surmounted by new emerging marker technology by providing easiest way of introgression, selection and pyramiding favorable genes in breeder's perspective. Identification of resistant breeding material to the major foliar diseases is one of challenging objectives for the groundnut breeders. Because of simultaneous occurrence of LLS and rust and dominating and defoliating nature of LLS makes the selection for rust resistance very difficult. Mapping of resistance genes is important in these diseases because resistance is quantitative in nature and governed by recessive genes.

The development of genetic linkage map will greatly expedite the ability of breeders to tag and follow the introgression of specific chromosome segments linked to desirable traits from wild species into breeding lines of cultivated groundnut and also without the availability of a genetic map, it is difficult to utilize molecular markers or to combine molecular and conventional genetic techniques in groundnut improvement programs (Halward *et al.*, 1993). Since, there is no comprehensive genetic map in the tetraploid cultivated groundnut (Varshney *et al.*, 2007) the present investigation emphasizes linkage map construction based on SSR markers, tagging of putative markers associated with resistance and identification of QTL contributing to resistance in TAG 24 × GPBD 4 population. The mapping population was derived from the parents TAG 24 and GPBD 4, as they differ for resistance to LLS and rust, growth habit, harvest index, oil quality besides yield components.

RILs consist of a series of homozygous lines, each containing a unique combination of chromosomal segments from the original parents. RILs are the eternal resource of QTL mapping studies because they produce true breeding lines that can be reproduced and multiplied without change occurring. Therefore, multi-location, multi-environment data can be generated. Furthermore, seed from individual RI lines may be transferred between different laboratories for further linkage analysis and the addition of markers to existing maps. The length of time needed for producing RI populations is the major disadvantage, because usually six to eight generations are required.

The mapping population consisting of 268 RILs exhibited significant variation for resistance to LLS and rust. The magnitude of variation was moderate to high as revealed by phenotypic coefficient of variation and with high to very high heritability, the population revealed substantially high heritable variation. High positive correlation between disease scores at different stages and across environments revealed consistency and stability in the disease development. The near normal to normal distribution revealed quantitative nature of resistance. The distribution of RILs for both the diseases was within the parental limits but in the case of LLS few lines exhibiting more susceptibility than susceptible parent revealing the contribution of favorable alleles from one of the parents. The influence of G × E interaction on disease ontogeny, necessitate screening of this population in multiple environments and locations. Non significant correlation between LLS and rust indicated independent inheritance of the diseases.

As for the agronomic traits are concerned, very high magnitude of variation coupled with high heritability for plant height and yield traits *viz.*, plot yield, 100 seed weight, and shelling percentage revealed considerable heritable variation in the population. Mostly normal distribution was observed for agronomic traits showing their quantitative nature of inheritance. Association analysis revealed strong correlation among morphological (leaf length, leaf width, plant height and primary branches) and yield traits (pods per plant, yield per plant, plot yield, hundred seed weight and shelling percentage) and also between some

morphological and yield traits revealing genetic association due to linkage and / or pleiotropy. Transgressive segregants for all the agronomic traits were observed indicating the contribution of favorable alleles by both the parents.

Twenty six RILs have been identified based on resistance to LLS, rust and among them 21 RILs having desirable productive features. These lines can be further utilized in breeding for resistance to foliar diseases. (Appendix VI).

The limited DNA-level of variability observed in the cultivated groundnut by RFLP (Halward *et al.*, 1991) and RAPD (Kochert *et al.*, 1991) is mainly ascribed to its origin by single event of polyploidization and further isolation from wild relatives (Halward *et al.*, 1991; Young *et al.*, 1996). Simple sequence repeats (SSRs) are the markers of choice because they are ubiquitous throughout the genome, multi allelic, co dominant and breeder friendly (Gupta and Varshney, 2000). Infact, several hundred SSR markers are currently available in groundnut (Hopkins *et al.*, 1999; He *et al.*, 2003; Ferguson *et al.*, 2004; Moretzsohn *et al.*, 2004 and 2005; Mace *et al.*, 2007; Cuc *et al.* unpublished; Bertioli *al.* unpublished; Knapp *et al.* unpublished). High level of polymorphism in SSRs over RFLP and RAPD was observed by Hopkins *et al.*, 1999 (23%), He *et al.*, 2003 (33.9%), Ferguson *et al.*, 2004 (70.8- 81%), He *et al.*, 2005 (29.23%), Mace *et al.*, 2006 (52%) and Nimmakayala *et al.*, 2007(52.08%). Very low polymorphism (6.15%) observed between the parents TAG 24 and GPBD 4 revealed their narrow genetic base and it was comparable to 4.4 per cent

reported by Moretzsohn *et al.* (2004) among 7 accessions of *Arachis hypogaea*. Hence, while developing mapping populations for the traits of interest, screening of different genotypes or germplasm using molecular markers and the combination of genotypes which gives higher polymorphism could be a better approach (Anderson *et al.*, 1993; Mace *et al.*, 2006).

A total of 20 markers (29.85%) out of 67 markers showed segregation distortion which is relatively less compared to Burow *et al.*, 2001(68%) and Moretzsohn *et al.*, 2005 (51%). This could be due to more similarity and less genetic diversity between the parents in the present investigation as compared to use of wild species and synthetic parents leading to sterility in those studies. Reduced distortion could also be due to large size of the mapping population (268 RILs) employed in the present study as compared to earlier studies. Segregation distortion affects the estimation of map distances and the order of markers when many distorted markers are present. In other words segregation distortion affects the construction of linkage map and thus QTL analysis.

Mere identification of markers based on resistant or susceptible germplasm lines will not have any practical utility in the breeding programme but tagging of the markers with the traits of interest and assessing their contribution towards phenotypic variation will substantiate the utility of markers. In the present investigation, a total of 34 markers were found to be associated with LLS by single marker analysis (SMA) and the phenotypic contribution ranged from 1.12 to 5.78

per cent. Six markers *viz.*, PM436 (1.49 - 4.29%), Lec1 (1.75 - 4.89%), TC2G05 (3.03 - 4.42%), gi-1107 (1.73 - 4.02%), PM179 (1.48 - 5.78%) and TC1A01 (1.44 - 3.51%) were prevalent in different seasons (Table 31). Though this is the first report of markers associated with LLS, the proportion of phenotypic variance explained by the individual markers is quite less (<5%) demanding a need to explore more markers to track prominent ones with greater contribution.

A total of 11 markers were associated with rust and their phenotypic variation ranged from 1.26 to 40.58 per cent. Among them, 4 markers were found to be common across stages of scoring and XIP103 contributing substantially (33.8 - 40.6%) to the total variation and it smacks the very tightly linked association with rust (Table 31). Further validation of this marker in outside the original mapping population would strengthen the reliable association of this marker with rust paving the way for application in MAS.

Varma *et al.* (2005) screened 25 SSR markers in two mapping populations (ICGV 99003 × TMV 2 and ICGV 99005 × TMV 2) and identified 5 markers associated with rust resistance. Mace *et al.*, 2006 used 23 SSR markers in 22 genotypes and identified 12 markers associated with resistance to LLS and rust. In the present investigation, all these markers were employed but none were found to be associated with LLS or rust indicating genotypes specific association.

As many as 45 markers were found to be associated with 9 different agronomic traits and their contribution for total phenotypic variation

Table 31: Prominent markers associated with LLS, rust and agronomic traits

Trait	Marker	R^2 (%)
LLS	PM436	1.49 - 4.29
	Lec1	1.75 - 4.89
	TC2G05	3.03 - 4.42
	gi-1107	1.73 - 4.02
	PM179	1.48 - 5.78
	TC1A01	1.44 - 3.51
Rust	XIP103	33.8 - 40.6
Agronomic traits		
PBR,PPP,YPP,YKGH	Seq5D5	6.85 - 10.23
PPP,YPP,HSW,YKGH	XIP103	4.90 - 9.98
PLHT,SLNG	GM 633	5.35 - 8.56
PLHT,SLNG	PM179	3.29 - 8.56
LLN,LWD	GM670	2.55 - 3.43
LWD	TC4F12	8.72
PLHT	XIP176	6.82

LLN - Leaf length (mm)
PBR - No. of primary branches
YKGH - Plot yield (kg/ha)
LWD - Leaf width (mm)
PPP - Pods per plant
HSW - 100 seed weight (g)
PLHT - Plant height (cm)
YPP - Yield per plant (g)
SLNG - Shelling percentage

ranged from 1.1 to 10.23 per cent. Among them, seven markers viz., Seq5D5 (primary branches, pods per plant, yield per plant, plot yield) XIP103 (pods per plant, yield per plant, 100 seed weight, and plot yield) GM633 and PM179 (plant height and shelling percentage), GM670 (leaf length and leaf width), TC4F12 (leaf width) and XIP176 (plant height) were found to contribute significantly (>5%) to phenotypic variation (Table 31). The significant contribution to phenotypic variation and concurrent association of some of the markers with several agronomic traits indicate the potential for the molecular approach to dissect the traits in the mapping population. Concomitant association of XIP103 with rust and important agronomic traits demands molecular dissection of this locus for further use in MAS.

SMA is the simplest tool and test the significance of phenotypic groups based on ANOVA or regression models. It is preliminary and least informative and does not reveal the location and effects of detected QTL precisely. Hence the mapping of the markers on the linkage map to find out their distance from the gene and effects is pertinent.

There were only two earlier reports on the construction of genetic linkage maps based on SSRs in diploid species of groundnut (Moretzsohn *et al.*, 2005; Gobbi *et al.*, 2006) and the present study constitutes the first attempt on the development of SSR based tetraploid linkage map. The map consists of 59 markers mapped on 13 linkage groups and spanning 909.40 cM with an average distance of 3.0 to 35.45 cM. The estimated map distance in diploid species is 1424.70cM (Moretzsohn

et al., 2005) and 2849.40 cM in tetraploid and hence the map coverage is 32 per cent (909.40 cM) and with only 13 out of 20 possible LGs, it can be considered mostly a partial map. The map coverage is much lower than Moretzsohn *et al.*, 2005(86.4%) and Gobbi *et al.*, 2006 (52.97%) but the diploid maps are of less significance to genetic improvement of cultivated groundnut. When compared with other tetraploid maps, it is far superior to AFLP map by Herselman *et al.*, 2004 (139.4 cM; 5 LGs) but less dense than RFLP map by Burow *et al.*, 2001 (2210 cM; 23 LGs).

Though highest numbers of markers (1089 SSRs) were screened in the present study but limited polymorphism (67 SSRs) remained the biggest constraint in the construction of a good skeletal / framework map. The SSRs employed were mostly of genomic origin and hence use of genic SSRs may yield better results (Varshney *et al.*, 2005). Alternatively use of a larger number of highly polymorphic markers like SNPs (single nucleotide polymorphisms) and DArTs (Diversity Array Technologies) could be utilized in the development of framework map which could be later enriched with co-dominant SSRs (Paterson *et al.*, 2004).

The present investigation is the pioneering attempt to identify QTL associated with LLS, rust and yield related traits and it was carried out by using phenotypic and genotypic segregation data based on 268 RILs. QTL analysis revealed 11 QTL associated with resistance to LLS each contributing 1.4 to 6.2 per cent of the phenotypic variation (Table 32). But they were minor and screen specific. Minor QTL are prone for inconsistency, environmental conditions vary according to season and low

heritability of the traits can be probable reason for appearance of screen specific QTL. Inconsistencies have been found in other host-pathogen system where many QTL of small and medium effect were segregating (Ender and Kelly, 2005) so that screen specific QTL were likely to occur.

Since resistance to rust and LLS is complex with several components of resistance (Kornegay *et al.*, 1980; Subrahmanyam *et al.*,1982 and1983; Anderson *et al.*, 1986 and 1993; Green and Wynne, 1986 and 1987; Iroume and Knauft, 1987; Jogloy *et al.*, 1987; Chiteka *et al.*, 1988; Reddy and Khare, 1988; Anderson *et al.*, 1993; Waliyar *et al.*, 1993b; Mehan *et al.*, 1994; Motagi, 2001; Dwivedi *et al.*, 2002), the power of QTL detection could be increased by phenotyping the mapping population for the components of resistance like incubation period, latent period, lesion size, lesion on main stem for LLS and rust and also number of pustule, pustule diameter for rust.

Classical genetic analyses indicated multiple recessive genes governing resistance in LLS (Sharief *et al.*, 1978; Nevill, 1982) but as few recessive genes for rust resistance (Kalekar *et al.*, 1984; Knauft, 1987; Paramsivam *et al.*, 1990). In the present investigation, many single markers (34) with less contribution (1.12 - 5.78%) of phenotypic variation and 10 QTL with small effects (1.4 - 6.2%) were associated with LLS giving an indication that resistance to LLS is possibly conferred by many loci. Paradoxically, in the case of rust few single markers (11) with one marker (XIP103) contributing as much as 40.58 per cent phenotypic variation and

one single QTL (4.5 - 4.6%) indicated the possibility of few oligogenes controlling the resistance.

Data analysis across environments revealed four stable QTL for LLS and the phenotypic variation ranged from 2.1 to 5.3 per cent. Since these QTL were also present in some of the individual analysis they could be considered as potential regions carrying disease resistance genes and can be targeted for MAS. Among them, the QTL between the markers *XIP524 - TC4D09 and PM179 - Seq11C08* were specific to stage 1 while *TC2G05 - TC9H09 and GM660 - TC9F10* to stage 2 (Table 32). The stage specific QTL could be influencing different components of resistance operating at different stages of host-pathogen interaction. In the case of rust only one QTL between the markers *TC2G05 - TC9H09* (4.6 - 4.7%) was detected and it was also associated with LLS (Table 29). The favorable alleles for all the QTL have come from the resistant parent GPBD 4 except Seq9E08 - *XIP407c* for which resistance allele was contributed from the susceptible parent TAG 24.

Data transformation is known to affect QTL detection in the absence of normality. In the present investigation Log_{10} and square root transformation were attempted on the LLS data because it showed deviation from normal distribution. The transformation resulted in the loss of three QTL and detection of one new QTL in three different environments indicating its relevance in QTL detection.

Many of the single markers and QTL associated with LLS were found to be independent of rust thus revealing largely independent nature

Table 32: QTL associated with LLS at different stages and environments

Marker interval	S1		S2		QTL across environment			
					S1		S2	
	R^2 (%)	Additive effect	R^2 (%)	Additive effect	R^2 (%)	Additive effect	R^2 (%)	Additive effect
Seq9E08 - XIP407c	2.7[¥]	0.14						
Lec 1 - Seq7G02			5.7[¥]	-0.22				
TC2G05 - TC9H09			4.7[¶]	-0.17			5.3	-0.29
			3.4[¥]	-0.20				
GM660 - TC9F10	3.5[¥]	-0.15	3.6[¶]	-0.14			2.9	-0.20
XIP524 - TC4D09	2.9[¥]	0.22	3.8[¥]	0.26	2.1	0.19		
GM633 - PM179	6.2[¥]	-0.29	5.8[¥]	0.29				
PM436 - TC3H02	4.3[¶]	-0.16						
TC5A07 - XIP395			4.0[¥]	-0.22				
TC11A04 - XIP524			1.4[×]	-0.30				
Seq5D5 - TC2G05			3.6[×]	-0.22				
PM179 - Seq11C08					4.6	-0.25		

¥ - Environment 1, ¶ - Environment 2, × - Environment 3

of LLS. However, 4 of the 11 markers and the one QTL associated with rust were also found influencing LLS indicating existence of some genomic regions influencing both the diseases.

Six QTL (*TC11A04 - XIP524, GM633- PM179, TC7H11 - XIP176, Seq5D5- TC2G05, TC1B02 - TC9F04* and *TC1A01 - Seq18G01)* were identified for 9 agronomic traits with phenotypic variation ranging from 3.2 - 11.3 per cent. Among them, the QTL flanked by the markers *Seq5D5 - TC2G05* was commonly observed in pods per plant, yield per plant, plot yield and primary branches. The QTL (*GM633- PM179)* was associated with plant height, shelling percentage and also LLS resistance thus, revealing their pleiotropic nature and the phenotypic correlations obtained for theses traits support the QTL results. The favorable alleles for all the agronomic traits were contributed by TAG 24 except for a QTL each for pods per plant (*TC1B02 - TC9F04)* and shelling percentage (*GM633 - PM179*) by GPBD 4. Based on contribution to the phenotypic variation two QTL between the markers *Seq5D5- TC2G05* (7.5 - 11.3%) and *GM633 - PM179* (5.1 - 9.0%) can be considered as major QTL (Table 33). Since the phenotypic assessment of agronomic traits is based on only one evaluation, the stability of the QTL should be ascertained by evaluating the mapping population over seasons and locations.

The present linkage map consisted of 13 linkage groups, of them 10 carried QTL associated with different traits (Fig 16). Among them, LG 1, LG 4, LG 6, LG 7, LG 9, LG 11 and LG 13 carried QTL for LLS and LG 7 for rust. Six linkage groups viz., LG 1, LG 2, LG 4, LG7, LG 8 and LG 10

Table 33: QTL associated with agronomic traits

Marker interval	LLN		LWD		PLHT		PBR		PPP		YPP		YKGH		HSW		SLNG	
	R^2	Add. Effect	R^2	Add. Effect	R^2	Add. Effect	R^2	Add. Effect	R^2	Add. Effect	R^2	Add. Effect	R^2	Add. Effect	R^2	Add. Effect	R^2	Add. Effect
TC1A01 - Seq18G01	4.7	0.45	3.7	0.16													5.7	2.17
TC11A04 XIP524					3.2	0.61												
GM633 PM179					9.0	1.01											5.1	1.37
TC7H11 XIP176					6.6	0.88												
Seq505 TC2G05							5.7	0.08	11.3	1.84	7.5	0.05	8.0	31.61				
TC1B02 TC9F04									4.3	4.87								

LLN - Leaf length (mm)
LWD - Leaf width (mm)
PLHT - Plant height (cm)
PBR - No. of primary branches
PPP - Pods per plant
YPP - Yield per plant (g)
YKGH - Plot yield (kg/ha)
HSW - 100 seed weight (g)
SLNG - Shelling percentage

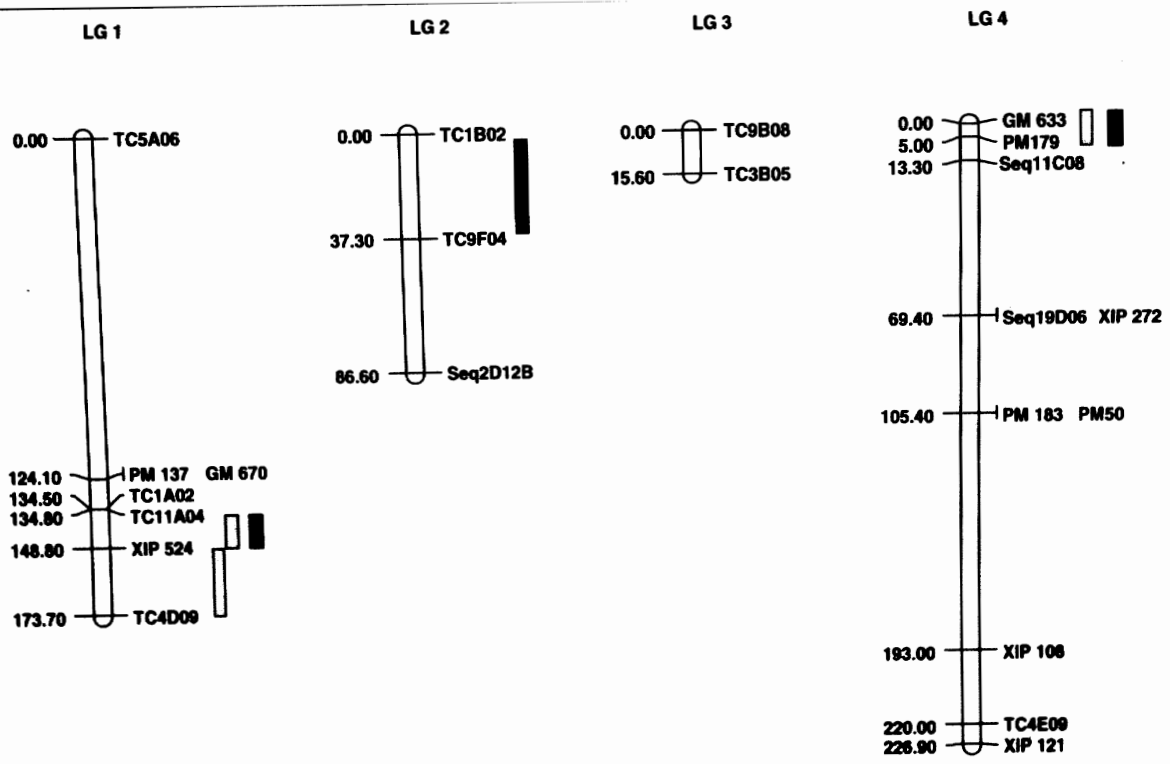
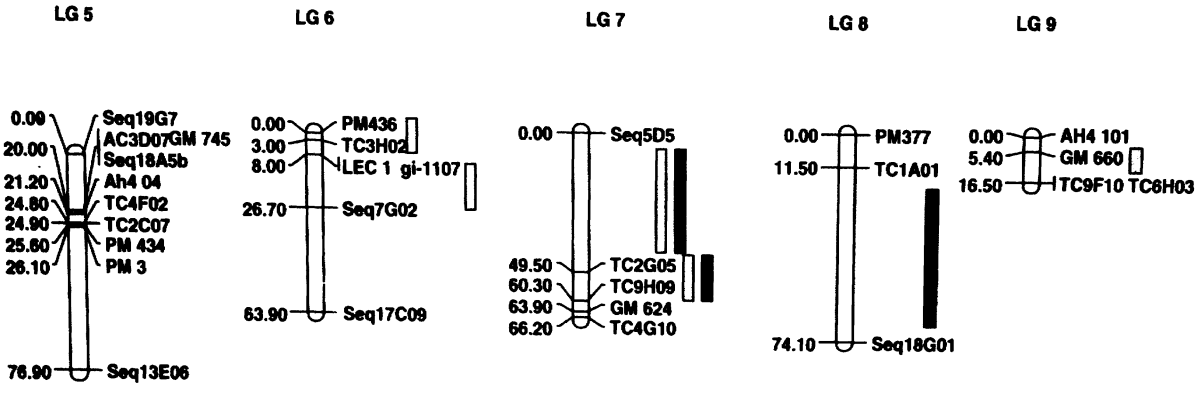
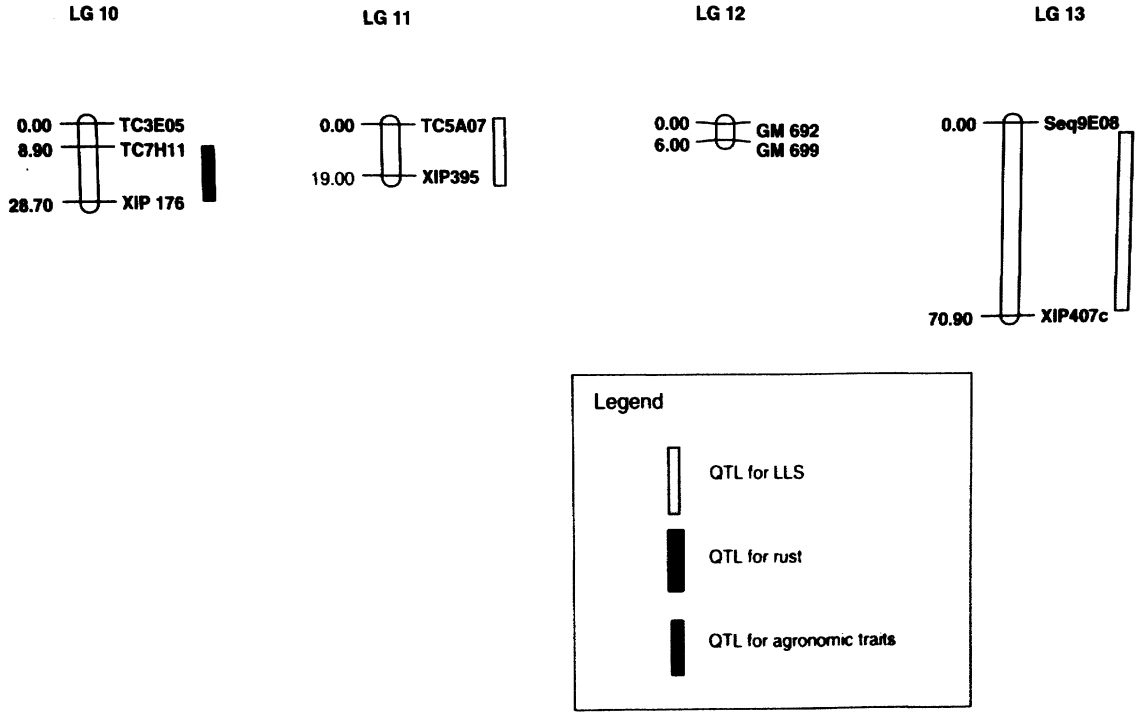


Fig. 16: Genetic linkage map showing QTL for LLS. rust and agronomic traits in TAG 24 x GPBD 4 mapping population

Contd.....


LG 5
0.00
20.00
21.20
24.80
24.90
25.60
26.10
76.90
Seq19G7
AC3D07GM 745
Seq18A5b
Ah4 04
TC4F02
TC2C07
PM 434
PM 3
Seq13E06
LG 6
0.00
3.00
8.00
26.70
63.90
PM436
TC3H02
LEC 1 gi-1107
Seq7G02
Seq17C09
LG 7
0.00
49.50
60.30
63.90
66.20
Seq5D5
TC2G05
TC9H09
GM 624
TC4G10
LG 8
0.00
11.50
74.10
PM377
TC1A01
Seq18G01
LG 9
0.00
5.40
16.50
AH4 101
GM 660
TC9F10 TC6H03

Contd.....


LG 10
0.00
TC3E05
8.90
TC7H11
28.70
XIP 176
LG 11
0.00
TC5A07
19.00
XIP395
LG 12
0.00
GM 692
6.00
GM 699
LG 13
0.00
Seq9E08
70.90
XIP407c
Legend
QTL for LLS
QTL for rust
QTL for agronomic traits

harbored QTL for agronomic traits. Three linkage groups *viz.*, LG 1, LG 4 and LG 7 revealing QTL for LLS, rust and agronomic traits were found to carry important genomic regions.

The results of SMA were found in accordance with QTL mapping in certain cases. The marker TC2G05 and the QTL (*TC2G05 - TC9H09*) were found to be stable across the stages and environment in LLS. In the case of agronomic traits, TC1A01 marker and the QTL (*TC1A01 - Seq18G01*) were found to be associated with leaf length and leaf width. The markers GM633, TC11A04 and TC7H11 were found to be common between SMA and QTL mapping for plant height. The SMA identified marker Seq5D5 was found associated with primary branches, pods per plant, yield per plant and plot yield and the marker TC1B02 for shelling and pods per plant, the results of QTL analysis also indicated the role of QTL flanked by these markers. In the present investigation, mostly the small effects QTL were obtained in LLS and rust but failed to detect single major QTL and the minor QTL are highly environment dependent (Ender and Kelly, 2005). A prominent marker (XIP103) associated with rust resistance detected in single marker analysis remained ungrouped. Non-detection of major QTL could be due to partial linkage map, large marker interval or less number of polymorphic markers due to narrow genetic base of the population (Ender and Kelly, 2005). Hence, there is an exigency to saturate the map with other types of markers *viz.*, AFLPs, SNPs and DArT using the same population or develop a new mapping population derived from the genetically diverse parents.

Conclusion

As there was no SSR based molecular maps in the cultivated groundnut due to lack of suitable mapping population and marker system to resolve polymorphism. But recently, the availability of SSR marker from various labs and mapping population has made success in the construction of first SSR based genetic linkage map in tetraploid cultivated groundnut.

Identification of late leaf spot and rust resistant lines is difficult due to their co-occurrence and defoliating nature of LLS. Hence to surpass these obstacles, it is necessary to have an alternative approach like tagging of resistance genes using molecular markers. The markers identified in the present study (XIP103) can be directly used for MAS as well as QTL detected for LLS and rust need to be validated and further transfer them into elite susceptible lines.

Summary

VI. SUMMARY

The mapping population comprising 268 RILs derived from the cross between susceptible parent TAG 24 and resistant parent GPBD 4. The population segregating for late leaf spot and rust was utilized in phenotyping for resistance and productivity traits and genotyping with SSRs for constructing linkage map and identification of markers and QTL associated with the traits.

Phenotyping of mapping population was carried out at UAS, Dharwad for three seasons *viz.*, *Kharif* 2004, *Kharif* 2005 *and Kharif* 2006 for LLS at 70 days and 90 days and *Kharif* 2007 for rust at three stages, 70 days, 80 days and 90 days. Artificial disease epiphytotics were created using spreader row technique and diseases were scored by using modified 9 point scale (Subbarao *et al.*, 1990). Nine agronomic traits *viz.*, leaf length, leaf width, plant height, number of primary branches, pods per plant, yield per plant, plot yield, 100 seed weight and shelling percentage were studied at ICRISAT, Patancheru in *Kharif* 2007.

Analysis of variance for all these traits revealed significant differences among genotypes. Pooled analysis of variance revealed the predominant contribution by stages, genotypes and environments and among the interactions, genotype × environment followed by stages × environment were significant.

- Components of variance, PCV and GCV revealed moderate to high magnitude of variation for resistance to both the diseases and all the agronomic traits except days to maturity, pods per plant and yield per plant.

- High to very high heritability estimates in LLS, high in rust and moderate to high in agronomic traits revealed considerable amount of heritable variation existing among the lines. Low to high genetic advance showed the expected progress possible when phenotypic selection is operated for different traits.

- Highly positive correlation observed between the different stages for LLS and rust and in different environments for LLS showed consistency in disease reaction of RILs between stages and across environments but it was non-significant between LLS and rust indicating their independent nature of inheritance.

- Frequency distribution of LLS showed near normal distribution at stage 1 and skewed towards susceptible parent at stage 2 in different environments, while it was almost normal distribution for rust at all the stages. The distribution of RILs for diseases was mostly within the parental limits with a few lines exhibiting more susceptibility than susceptible parents revealing the contributions of favorable alleles from the resistant parent GPBD 4.

- Agronomic traits showed normal distribution and strong correlation among morphological and yield traits and also between some morphological and yield traits revealing genetic association due to linkage and / or pleiotropy. The transgressive segregants in both the

directions indicated the contribution of favorable alleles from both the parents.

- Parental polymorphism was assessed using 1089 SSR markers from different sources and out of them 67 markers was found to be polymorphic between the parents, which were later utilized for genotyping the 268 RILs.

- Single marker analysis (SMA) revealed 34 markers associated with LLS with phenotypic contribution ranging from 1.12 to 5.78 per cent in different environment. Among them, PM436, Lec1, TC2G05, gi-1107, PM179 and TC1A01 were found to be consistent across seasons with significant contribution to the phenotypic variation.

- Eleven markers were found to be associated with rust contributing 1.26 to 40.58 per cent to phenotypic variation. XIP103 contributed as much as 33.8 to 40.6 per cent of variation at different stages.

- Detection of large number of markers with small effects in LLS compared to rust may indicate the possibility of many loci contributing to resistance to LLS and few oligogenes for rust.

- SMA for agronomic traits identified 15 (plant height), 8 (primary branches), 10 (shelling percentage), 8 (leaf length), 15 (leaf width), 12 (pods per plant), 7 (yield per plant), 9 (100 seed weight) and 5 (plot yield) markers associated with different traits with 1.1 to 10.23 per cent contribution to phenotypic variation.

- Seven markers viz., Seq5D5 (primary branches, pods per plant, yield per plant, plot yield) XIP103 (pods per plant, yield per plant, 100 seed weight and plot yield) GM633 and PM179 (plant height and shelling percentage), GM670 (leaf length and leaf width), TC4F12 (leaf width) and XIP176 (plant height) were found to contribute significantly (>5%) to phenotypic variation.

- Partial linkage map was constructed using segregation data derived from 67 markers by MAPMAKER/EXP V 3.0. A total of 59 markers were mapped on 13 different linkage groups spanning 909.40 cM covering approximately 32 per cent of the groundnut genome.

- QTL analysis revealed 10 QTL associated with resistance to LLS each contributing 1.4 to 6.2 per cent of the phenotypic variation in different environments. Among them, only two QTL (*TC2G05 - TC9H09* and *Seq5D5 - TC2G05)* appeared in two environments (E1 and E2) and others (*XIP524 - TC4D09, GM633 - PM179, GM660 - TC9F10, Seq9E08 - XIP407c, Lec1 - Seq7G02, PM436 - TC3H02, TC5A07 - XIP395* and *TC11A04 - XIP524*) were detected in one of the three environments revealing inconsistency in QTL detection over environments.

- Data analysis across environments revealed four stable QTL (*XIP524 - TC4D09, PM179 - Seq11C08* and *TC2G05 - TC9H09, GM660 - TC9F10)* for LLS and the phenotypic variation ranged from 2.1 to 5.3 per cent. These QTL are stage specific, which may determine different components of resistance operating at different stages.

- Log_{10} and square root transformation of the data has resulted in the loss of three QTL (*Lec1 - Seq7G02, TC2G05 - TC9H09* and *GM660 - TC9F10*) and appearance of one new QTL (*TC3H02 - Lec1*) indicating its relevance.

- In the case of rust only one QTL between the markers *TC2G05 - TC9H09* (4.6 - 4.7%) was detected and it was also associated with LLS.

- The favorable alleles for all the QTL have come from the resistant parent GPBD 4 except *Seq9E08 - XIP407c* in LLS which was contributed from the susceptible parent TAG 24.

- Many of the markers and QTL associated with LLS did not affect rust revealing largely independent nature of LLS. However, 4 of the 11 markers and the QTL associated with rust were also found influencing LLS indicating existence of some genomic regions influencing both the diseases.

- Six QTL (*TC11A04 - XIP524, GM633- PM179, TC7H11 - XIP176, Seq5D5- TC2G05, TC1B02 - TC9F04* and *TC1A01 - Seq18G01*) were identified for 9 agronomic traits with phenotypic variation ranging from 3.2 - 11.3 per cent. Among them, the QTL flanked by the markers *Seq5D5 - TC2G05* was commonly observed in pods per plant, yield per plant, plot yield and primary branches. The QTL (*GM633 - PM179*) was associated with plant height, shelling percentage and also LLS resistance thus, revealing their pleiotropic

nature. The favorable alleles for all the agronomic traits were contributed by TAG 24 except for a QTL each for pods per plant and shelling percentage by GPBD 4.

- As far as resistance is concerned GPBD 4 harbors favorable alleles and TAG 24 for yield related traits.
- Mostly minor QTL have been detected for LLS and rust indicates further molecular dissection of these loci. Four QTL for morphological and yield related traits contributed 7.5 – 11.3% could be directly use for marker assisted breeding.
- Only XIP 103 has contributed significantly towards phenotypic variance, but it has to be validated and associations with resistance or susceptibility need to confirm and further it could be use in marker assisted selection.
- Twenty six RILs having resistance to LLS, rust and 21 lines with desirable agronomic features have been selected and later these lines can be used in resistance breeding.
- The study indicated a need for further saturation of genetic map using AFLPs, SNPs or DArT markers for improved detection of QTL affecting late leaf spot and rust resistance besides agronomic traits for which substantial variation exists in the mapping population.

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Appendices

Appendix I: Weather data of year 2004

Parameters	June	July	August	September	October	November
T_{Max} °C	28.8	27.2	27.0	28.6	30.1	30.2
T_{Min} °C	21.5	21.0	20.3	19.9	18.4	15.9
RH (%)	80	79	83	77	65	52
Rainy Days	5	3	12	11	3	-
RF (mm)	43.8	24.8	160.7	222.1	64.6	0.6

Appendix II: Weather data of year 2005

Parameters	June	July	August	September	October	November
T_{Max} °C	30.9	27.4	27.1	27.5	29.6	29.4
T_{Min} °C	21.5	21.0	20.4	20.3	19.1	14.9
RH (%)	76	83	81	85	70	51
Rainy Days	10	19	15	14	9	1
RF (mm)	151.0	290.2	138.8	194.5	89.4	38.0

T_{Max} - Temperature maximum, T_{Min} - Temperature minimum, RH - Relative humidity, RF - Rainfall

Appendix III: Weather data of year 2006

Parameters	June	July	August	September	October	November
T_{Max} °C	29.5	26.6	26.3	29.2	30.0	29.2
T_{Min} °C	20.6	20.4	19.6	19.2	19.1	18.1
RH (%)	78	87	85	77	67	70
Rainy Days	14	18	17	10	3	-
RF (mm)	212.4	176.1	115.2	91.4	38.6	55.4

Appendix IV: Weather data of year 2007

Parameters	June	July	August	September	October	November
T_{Max} °C	29.7	27.0	27.1	27.2	29.7	29.5
T_{Min} °C	21.3	21.1	20.5	20.3	19.4	15.1
RH (%)	80	85	85	83	68	53
Rainy Days	10	14	12	11	3	2
RF (mm)	220.1	211.2	176.0	180.8	74.8	54.0

T_{Max} - Temperature maximum. T_{Min} - Temperature minimum. RH - Relative humidity. RF - Rainfall