Molecular analysis of Fusarium wilt resistance and yield related traits in chickpea (*Cicer arietinum* L.)

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Research work embodied in this thesis was carried out at

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CERTIFICATE

Certified that the work incorporated in the thesis entitled **"Molecular Analysis of Fusarium Wilt Resistance and Yield Related Traits in Chickpea (*Cicer arietinum* L.)"** submitted by **Mr. Manje Gowda SJ** was carried out under my supervision. The material obtained from other sources has been duly acknowledged in the thesis.

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DECLARATION

I hereby declare that the thesis entitled **"Molecular Analysis of Fusarium Wilt Resistance and Yield Related Traits in Chickpea (*Cicer arietinum* L.)"** submitted for the degree of Ph.D. to the **University of Pune** has not been submitted by me for a degree at any other university.

Date: 22nd Jan 2009

Manje Gowda SJ,

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For Indian farming community

CONTENTS

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Manje Gowda

List of abbreviations

°C - degree Celsius
AFLP - amplified fragment length polymorphism
AMMI - Additive Main effects and Multiplicative Interaction
APS - ammonium per sulphate
ASAP - allele specific associated primer
avr - gene for avirulence
BAC - bacterial artificial chromosome
bn, mn - Billion, Million
bp - base pair
cDNA - complementary deoxyribonucleic acid
CIM - Composite Interval Mapping
cm - centimeter
cM - centimorgan
cv - cultivar
DAI - days after infection
DArT - Diversity Array Technology
DH - doubled haploid
DNA - deoxyribonucleic acid
dNTPs - Deoxy ribonucleotide tri phosphate
DTT - dithiothreitol
EDTA – ethylene diamine tetra acetate
E- QTL – Epistatic QTL
EST - expressed sequence tag
FOC - *Fusarium oxysporum* f. sp. *ciceri*
FOC1 - *Fusarium oxysporum* f. sp. *ciceri* race1
g, mg, μg, ng - gram, milligram, microgram, nanogram
GEI – Genotype x Environment interaction
ha - hectare
HCl - hydrochloric acid
IAA - iso-amyl alcohol
IPCA - interaction principal component axes
IM – Interval Mapping
ISSR - inter simple sequence repeat
K_2O - Potassium oxide
Kb, Mb - Kilobase pair, Megabase pair
KCl - Potassium chloride
Kg/ha - Kilogram per hectare
L, mL, μL - liter, milliliter, microliter
LG - linkage group
LOD - Log of the odd (Base 10 logarithm of the likelihood ratio)
M, mM, μM - molar, millimolar, micromolar
MAS - marker assisted selection
$MgCl_2$ - Magnesium chloride
mha - million hectares
MIM – Multiple Interval Mapping

min - minute
mM - millimolar
mmole, mmole - millimole, micromole
M-QTL – Main effective QTL
mRNA - messenger RNA
MT - Metric tonnes
Mt - million tonnes
NaCl - sodium chloride
NaOH - sodium hydroxide
NIL - Near isogenic line
nm - nanometer
PAGE - polyacrylamide gel electrophoresis
PCR - polymerase chain reaction
QTL - quantitative trait loci
R genes - resistance genes
RAPD - random amplified polymorphic DNA
RFLP - restriction fragment length polymorphism
RGA - resistance gene analog
RIL - recombinant inbred line
RNA - Ribonucleic acid
rpm - revolutions per minute
RT-PCR – reverse transcriptase-polymerase chain reaction
SAGE - serial analysis of gene expression
SCAR - Sequence characterized amplified region
SDS - sodium dodecyl sulphate
SNP - single nucleotide polymorphism
SSR - simple sequence repeat
STMS - sequence tagged microsatellite site
TAE - Tris-acetate EDTA
TAM - Tagged-Array Marker
TBE - Tris-borate-EDTA
TE - Tris-EDTA
TEMED - Tetramethylethylenediamine
Tm - Melting temperature
TRIS - Tris-hydroxymethyl aminomethane
U - unit
λ260, λ280 - absorption at 260nm, 280nm
µM – micromolar

List of figures

Chapter 1

Chapter 2

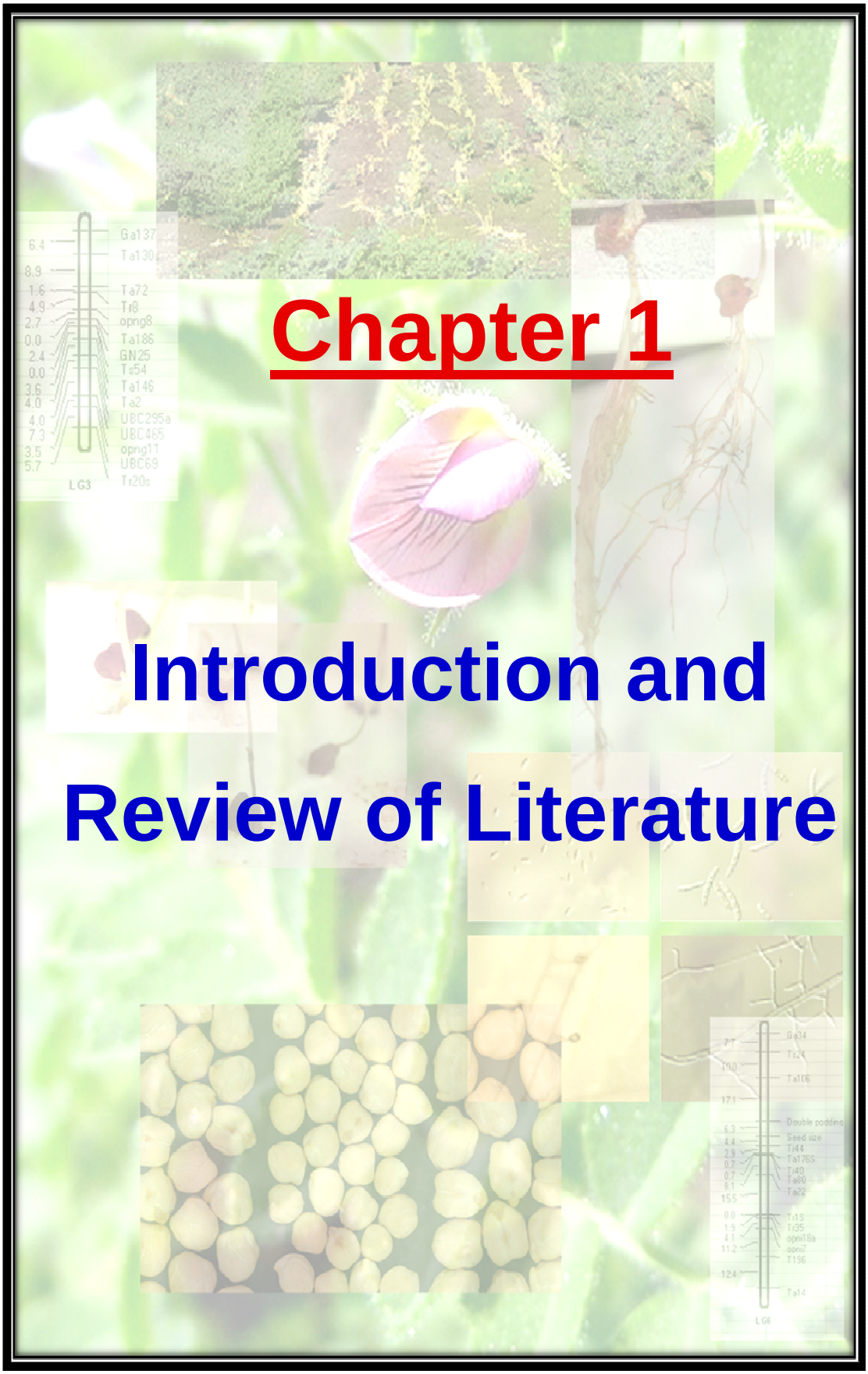
Chapter 3

List of Tables

Chapter 1

Chapter 2

Chapter 3



Chapter 1

Introduction and Review of Literature

Grain legumes are crop plants belonging to the legume family with papilionaceous flowers and pods containing seeds. Legumes fix the atmospheric nitrogen by symbiosis with *Rhizobium*, which provides them organic proteins. Grain legumes are cultivated primarily for their seeds, which are rich in carbohydrate and protein. Legume grains contain 20 to 25% protein by weight, which is double the protein content of wheat and three times that of rice grains. For this reason, pulses are sometimes called "poor man's meat". Hence, cereals, which are deficient in lysine, are commonly consumed along with pulses to form a complete protein diet. While legumes are generally high in protein content, and the digestibility of the proteins is also high, they often are relatively poor in the content of essential amino acid methionine. The rise in import of pulses and their value (Fig 1.1) in India shows the increasing demand and the need for improvement and research in legumes.

1.1 Chickpea: A valuable grain legume

Cultivated chickpea, *Cicer arietinum L.*, is a self-pollinated, diploid ($2n=2x=16$) annual pulse crop with a genome size of 740 Mbp (Arumuganathan and Earle 1991). Globally it is the third most important food legume (Fig 1.2a), grown in over 40 countries representing all the continents. Over 95% of the area, production and consumption of chickpea is in developing countries. During 2007-08, the global annual chickpea production was 9.31 Mt from an area of 11.67 Mha, giving an average productivity of 786 kg/ha (Table 1.1). During the past 20 years, the global chickpea area increased by 7%, yield by 24% and production by 33% (FAOSTAT, 2008). Chickpea has majority of its cultivation in dry areas of the Indian subcontinent (Saxena, 1990) and India is the principal chickpea producing country with a share of 90% in this region. Though, chickpeas are grown and locally consumed, India is also the world's largest importer of chickpeas accounting for about 20% of global imports (Fig 1.1). These figures reflect on the growing demand for chickpea and other pulses as well as the immense strain on crop production and yield. Having a capacity to stand in drought conditions, this crop does not have the requirement of being fed with nitrogen fertilizers. Chickpea through its biological nitrogen fixing (BNF) capability meets 80% of its nitrogen requirement and can fix up to 140 kg N/ha from air. It leaves substantial amount of residual nitrogen behind for subsequent crops and improve soil health, long-term fertility and sustainability of the agro-ecosystems.

Particulars	2001	2002	2003	2004	2005	2006	2007
Area (Million Ha)	9.46	10.39	9.66	10.56	10.36	10.85	11.67
Production (Million Tonnes)							
India	3.86	5.47	4.24	5.72	5.47	5.60	**5.97**
Pakistan	0.40	0.36	0.68	0.61	0.87	0.48	0.84
Turkey	0.54	0.65	0.60	0.62	0.60	0.55	0.52
Australia	0.26	0.13	0.20	0.14	0.12	0.23	0.31
Iran	0.27	0.30	0.29	0.29	0.27	0.31	0.31
Myanmar	0.12	0.21	0.23	0.22	0.23	0.22	0.23
Canada	0.46	0.16	0.07	0.05	0.10	0.16	0.22
Others	1.02	1.01	0.83	0.79	0.87	0.99	0.91
Total	**6.91**	**8.29**	**7.13**	**8.43**	**8.53**	**8.54**	**9.31**
Imports	1.12	0.86	0.92	0.74	0.86		

Table 1.1: Global scenario of chickpea area, production and imports

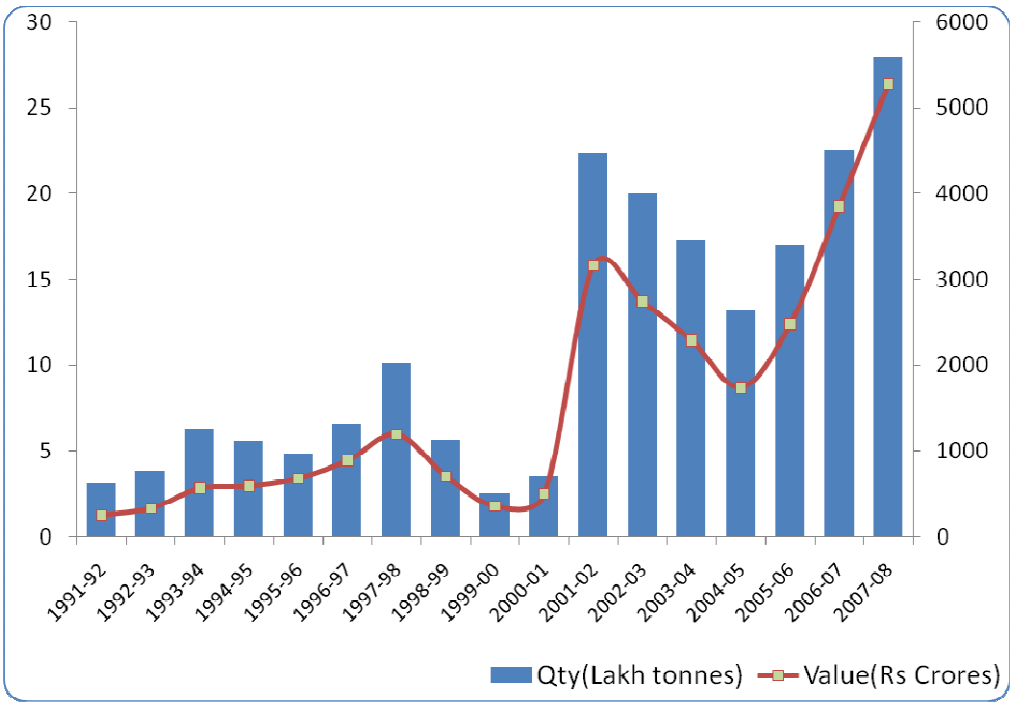


Fig 1.1: Import of total pulses by India (Quantity and Value)

1.1.1 Area, production and productivity

Chickpea, India's most important food legume is currently grown in about 6.7 m ha in India and 11.67 m ha in worldwide (FAOSTAT, 2008). Presently, the most important chickpea producing countries are India (65%), Pakistan (9%), Turkey (6%), Iran (4%), Mexico (3%), Myanmar (3%), Ethiopia (2%), Australia (2%), and Canada (1%) (Fig 1.2) (Millan *et al.*, 2006). During the past 30 years, the chickpea area has remained stagnant, however the production has increased from 6.9 m t (during 2001) to 9.31 m t (during 2007) because of increase in productivity from 614 to 797 kg/ha during this period (Fig 1.3). There was a reduction in the chickpea area in northern India but it was largely compensated by increase in the chickpea area in central and southern India.

1.1.2 Morphology

Chickpea (*Cicer arietinum* L.) has a deep tap root system, which enhances its capacity to withstand drought conditions. It is well adapted to areas having relatively cooler climatic conditions and a low level of rainfall. The aerial portion is profusely branched, erect or spreading, reaching a height of 0.2-1 m, appearing glandular pubescent, olive, dark green or bluish green in color. Leaves are imparipinnate, glandular-pubescent with 3-8 pairs of leaflets with rachis ending in a terminal leaflet. Leaflets are ovate to elliptic, 0.6-2.0 cm long, 0.3-1.4 cm wide; margin serrate, apex acuminate to aristate, base cuneate; stipules 2-5 toothed or absent. The inflorescence consists of solitary flowers, sometimes two per inflorescence and borne on 0.6-3 cm long peduncles, 7-10 mm long calyx; while the bracts are triangular or tripartite; the corolla is 0.8-1.2 cm long and varies from white, pink, purplish (fading to blue), or blue. The staminal column is diadelphous (9-1) with a sessile, inflated and pubescent ovary (Duke, 1981; Cubero, 1987; van der Maesen, 1987). The seeds (1-2 or maximum 3) are contained in a pod, which is rhomboid ellipsoid, inflated and glandular-pubescent. The seed color varies from cream, yellow, brown, black or green.

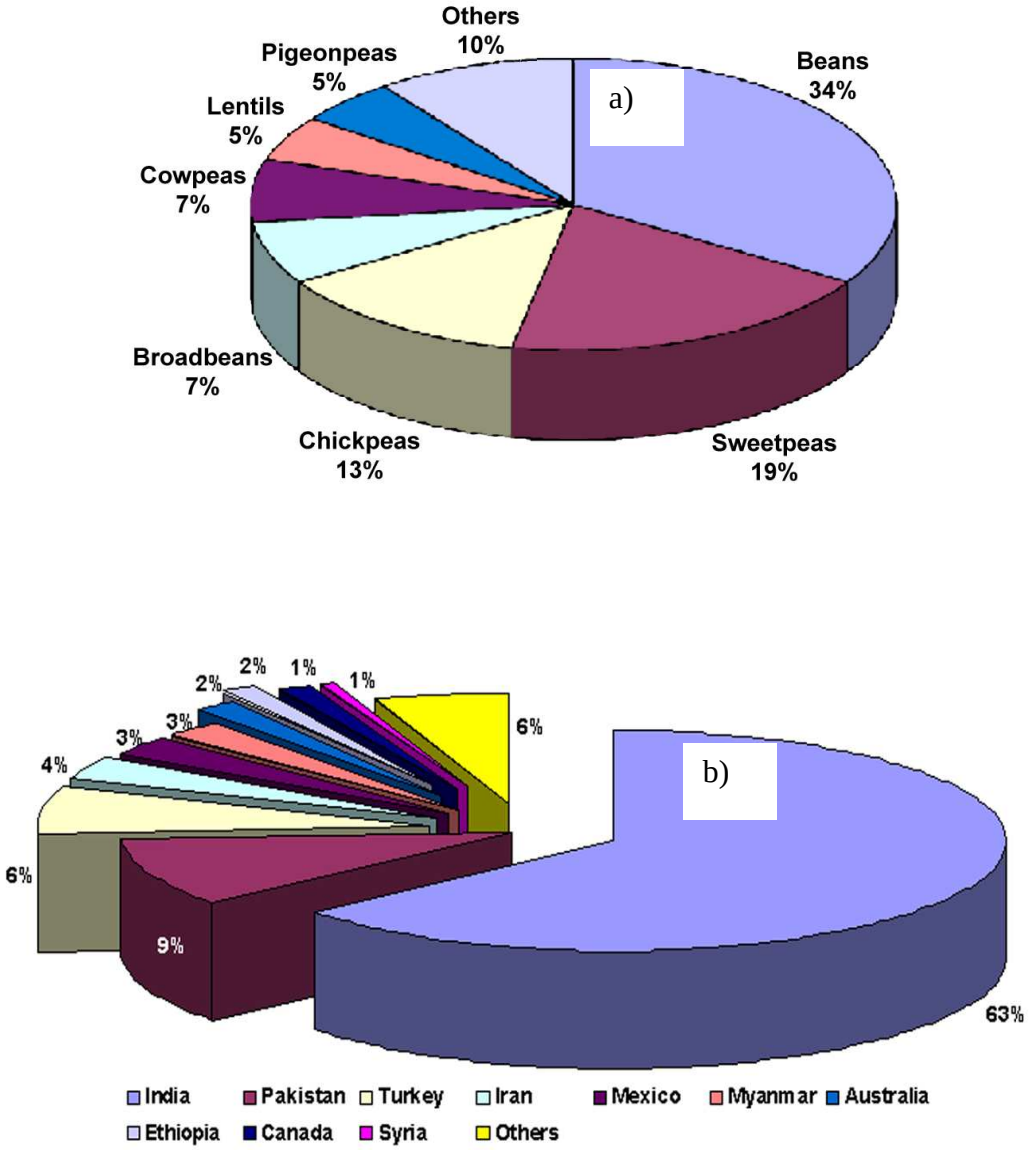


Fig 1.2: Contribution of Indian agriculture to global production of chickpea. a) Global production of chickpea in comparison with other legume crops. b) India is the world's largest producer of chickpeas, contributing to >60% of the total global produce. Source: FAOSTAT Data, 2008 (http://faostat.fao.org).

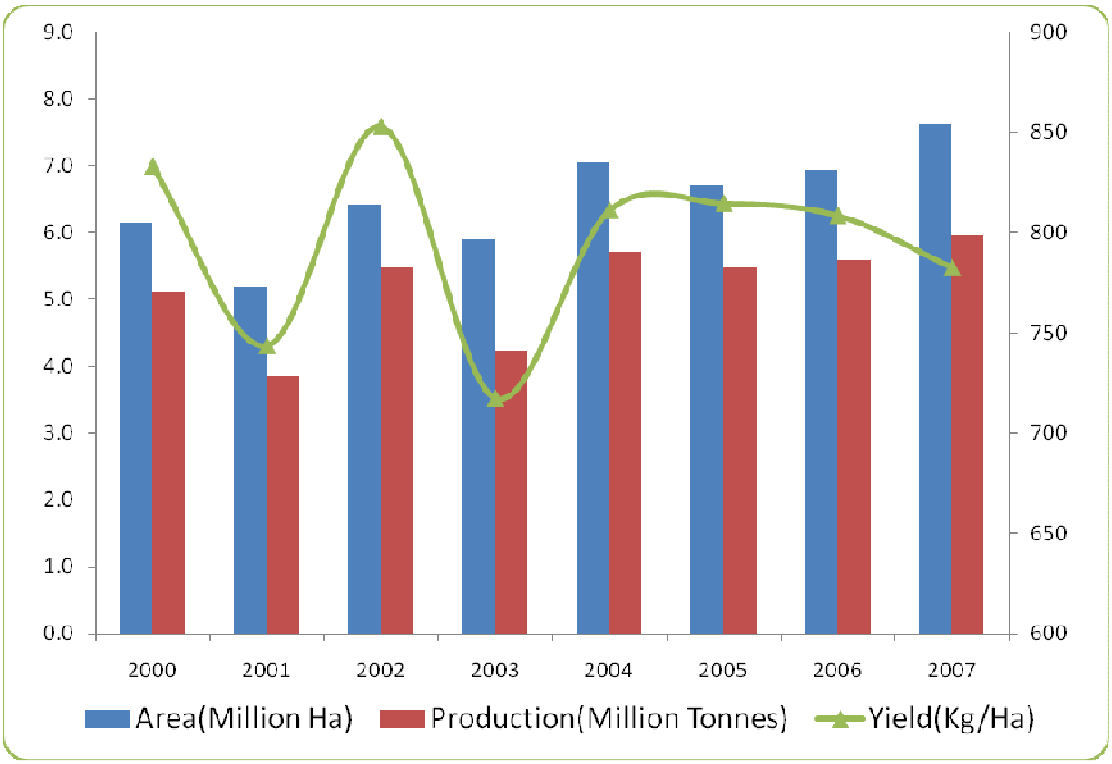


Fig 1.3: Chickpea area, production and productivity of chickpea in India (FAOSTAT, 2008)

Seeds may be rounded to angular with a smooth or wrinkled, or tuberculate seed coat, which is laterally compressed with a median groove around two-thirds of the seed forming a beak at the anterior end; during the cryptocotylar germination cotyledon tips remain in the seed coat in intimate contact with the endosperm (Duke, 1981; Cubero, 1987 van der Maesen, 1987).

1.1.3 Origin and domestication

Chickpea (*Cicer arietinum* L.) is one of the legume crops domesticated in the Old World. Most probably, it has originated in an area of south-eastern Turkey and Syria. It was first grown in Turkey about 7,000 B.C. It is believed to have been domesticated from *C. reticulatum* Ladizinsky, a closely related wild species. Three wild annual *Cicer* species, *C. bijugum*, *C. echinospermum*, and *C. reticulatum*, closely related to chickpea, cohabit with the cultivar in this area. Chickpea is not known to occur in the wild and some of the earlier reports on its mistaken wild status could be due to volunteers or escapes from cultivation. After domestication in the Middle East, the crop spread throughout the Middle East, the Mediterranean region, India, and Ethiopia

(Ladizinsky 1975; van der Maesen 1987). Its introduction in Mexico, Argentina, Chile, Peru, Australia and the US is a recent event (Duke 1981). Chickpea is most widely grown in South Asia and the Mediterranean region (Saxena 1990; Singh and Ocampo 1997; FAOSTAT, 2008). A good knowledge of the various *Cicer* species is essential to enable the scientific community to make efficient use of the genetic resources in chickpea improvement.

1.1.4 Distribution

The *Cicer* species occur from sea level (e.g. *C. arietinum*, *C. montbretii*) to over 5000m (*C. microphyllum*) near glaciers in the Himalayas. *C. arietinum* is found only in cultivation and cannot colonize successfully without human intervention. The wild species (e.g. *C. reticulatum*, *C. bijugum*) occur in weedy habitats (fallow or disturbed habitats, roadsides, cultivated fields of wheat, and other places not touched by man or cattle), mountain slopes among rubble (*e.g. C. pungens*, *C. yamashitae*), and on forest soils, in broad-leaf or pine forests (e.g. *C. montbretii*, *C. floribundum*).

1.1.5 Season

The yield from chickpea is maximum when grown on sandy, loam soils having an appropriate drainage system as this crop is very sensitive to excess water. The production of chickpea or 'chana' is also affected in excessive cold conditions. Chickpea is sown in the months of September to November in India and is considered as a rabi crop. The Desi type chickpea reaches physiological maturity in 95-105 days and Kabuli type in 100-110 days. The crop is harvested when its leaves start drying and shedding and harvesting can be done manually or with the help of a harvester. In India, it is harvested between February and April. This crop is often cultivated as a sole crop but sometimes it is also grown in rotation with other crops such as sorghum, pearl millet, wheat and coriander.

1.1.6. Taxonomy

Chickpea is the only domesticated species under the genus *Cicer*, which was originally classified in the tribe Vicieae of the family Leguminosae and sub family, Papilionoideae. Based on the pollen morphology and vascular anatomy, *Cicer* is now

set aside from the members of Vicieae and is classified in its own monogeneric tribe, Cicereae Alef. The tribe, Cicereae comes closer to the tribe, Trifolieae, which differs from the former in having hypogeal germination, tendrils, stipules free from the petiole, and nonpapillate unicellular hairs.

The genus *Cicer* comprises 43 species and is divided into two subgenera. The subgenus, Pseudononis is characterized by small flowers (normally 5-10 mm), subregular calyx, with hardly gibbous base, with sub linear nearly equal teeth. It comprises two sections, Mono cicer (annuals, with firm erect or horizontal stems branched from the base or at middle) and section, Chamaecicer (annuals or perennials, with thin creeping branched stem, and small flowers). The section, Mono cicer comprising all annual species most important to breeders, is subdivided into three series, arietina (characterized by imparipinnate leaves, with none to small arista), cirrhifera (leaves ending in a tendril, with short arista), and Macro-aristae (leaves imparipinnate, long arista). The subgenus, Viciastrum (perennials, characterized by medium large flowers, calyx strongly gibbous at the base, with unequal teeth) comprises two sections, Polycicer and Acanthocicer.

1.1.6.1 Cytotaxonomy

Chromosome number in *Cicer* species can be generalized as 2n=2x= 16, although varying numbers both for chickpea (2n= 2x= 14, 16, 24, 32) and other wild *Cicer* species (2n=14, 16, 24) have been reported, but could not be confirmed by other workers (http://www.icrisat.org/chickpea/taxonomy). Studies on biosystematic relations between chickpea and its wild relatives following interspecific hybridization have been limited to the 9 annual species, *C. arietinum* (chickpea), *C. reticulatum, C. echinispermum, C. judaicum, C. pinnatifidum, C. bijugum, C. cuneatum, C. chorassanicum*, and *C. yamashitae*. Based on the crossability and morphological similarities, the 9 annual species have been classified into 4 groups: the above first 3 species as group 1, the next 3 species along with *C. yamashitae* as group 2, and the remaining 2 species as two separate groups. Of these, only two species, *C. reticulatum* and *C. echinospermum* produced viable hybrids with chickpea. Gene exchange is normal between chickpea and *C. reticulatum*, while it is restricted due to high sterility in the hybrids involving *C. echinospermum*. In general, based on morphology,

physiology, and genetics, *C. reticulatum* comes closest to the cultigen, making it a possible progenitor of chickpea. However, considering the polymorphic nature of ancestral populations and complex nature of domestication, one cannot rule out the other possibilities, such as *C. reticulatum* and the cultigen sharing a common ancestor or a polyphyletic origin of chickpea.

1.1.6.2 Chemotaxonomy

Based on the electrophoretic study of water-soluble seed protein patterns, a close affinity between chickpea and *C. reticulatum* has been found. Assessment of allelic variation for 23 isozyme loci in 36 accessions representing 8 wild species and 25 accessions of the cultivar, following four genetic groups were recognized: Group one (*C. reticulatum*, *C. arietinum*, and *C. echinospermum*), group two (*C. bijugum*, *C. pinnatifidum*), group three (*C. judaicum*, *C. yamashitae*, *C. chorassanicum*, *C. anatolicum*, *and C. songaricum*; the latter two are perennials) and group four (*C. cuneatum*) (http://www.icrisat.org/chickpea/taxonomy). These groupings showed good agreement with those based on morphological studies, and partial agreement with those obtained from cross ability and cytogenetic studies.

1.1.7 Cultivar types

Two major cultivar types designated as 'desi' (= microsperma) and 'kabuli' (= macrosperma) have emerged under domestication. In addition 'gulabi', pea shaped forms of local importance are also recognized (Moreno and Cubero, 1978). Desi chickpeas are small and angular with rough brown to yellow testas, while kabuli types are relatively large, plump, and with smooth cream-colored testas. Kabuli types are considered relatively more advanced because of their larger seed size and reduced pigmentation achieved through conscious selection. A study at ICRISAT revealed that desi and kabuli types differ in their dietary fiber components of seed, both qualitatively and quantitatively. Kabuli types contain higher amount of dietary fiber, particularly cellulose and hemicellulose.

Kabuli and desi classification also reflects utilization: whereas kabulis are usually utilized as whole grains, desis as whole seeds, de-hulled splits (dhal) or flour. Seeds are ground to flour and used in confectionery. Young shoots or green pods,

shelled for the peas and eaten as a snack or vegetable. Chickpea is also known for its use in herbal medicine and cosmetics. An acrid liquid from the glandular hairs of the plant is collected by spreading a cloth over the crop at night, which absorbs the exudation with the dew. The exudate contains about 94% malic acid and 6% oxalic acid and is used medicinally.

Kabuli × desi crosses are used in many breeding programs to combine genes for cold tolerance, bold seededness, resistance to Ascochyta blight and long vegetative growth more frequently found in kabuli types, while genes for heat and drought tolerance, resistance to Fusarium wilt and early flowering contributed by the desi types (Singh, 1987).

1.1.8 Nutrition

Chickpea is mainly employed for human consumption and also a small proportion forms the part of animal and poultry feed. Chickpea has one of the highest nutritional compositions of any dry edible legume and is not reported to contain any specific major anti-nutritional factors (Williams and Singh, 1987). On an average, chickpea seed contains 22% protein, 64% total carbohydrates, 42% starch, 6% fat, 10% crude fiber, 17% soluble fibers and 3% ash (Fig 1.4). The mineral component is high in phosphorus (340 mg/100 g), calcium (190 mg/100g), magnesium (140 mg/100g), iron (7 mg/100 g) and zinc (3 mg/100 g). Chickpea protein has the highest digestibility when compared to other dry edible legumes. The lipid fraction is high in unsaturated fatty acids, primarily linoleic and oleic acids. They are also a good source of calcium, magnesium, potassium, phosphorus, iron, zinc and manganese (Ibrikci *et al*., 2003). Chickpeas do not contain as high amounts of isoflavones as soybeans do but provide more beneficial carotenoids such as β-carotene than genetically engineered “Golden Rice” (Abbo *et al*., 2005). Thus, chickpea is considered a functional food or nutraceutical (Agharkar, 1991; McIntosh and Topping, 2000; Charles *et al*., 2002). While it is a cheap source of protein and energy in the developing world, it is also an important food to the affluent populations to alleviate major food-related health problems. However, more research is necessary to elucidate and extend the food and nutraceutical benefit of this important food legume through breeding.

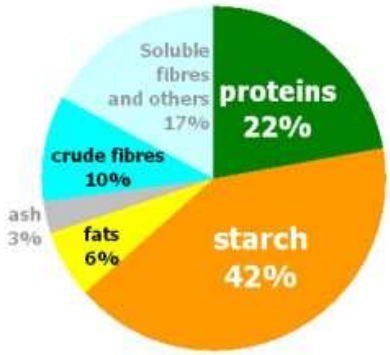


Fig 1.4: Nutrient composition of chickpea seeds

1.1.9 Yield and losses

The potential seed yield of about 5 t/ha has been reported in chickpea. However, the realized seed yield hovers around 850 kg/ha (Fig 1.5) (world average ≈ 0.8 t/ha, FAOSTAT, 2008), which has stagnated over the years. A series of biotic and abiotic stresses reduce the yield and yield stability, leaving room for only marginal improvements. This affects development of widely adapted cultivars and susceptibility to several biotic and abiotic stresses. Generally, the crop produces excessive vegetative growth under high input conditions and is unable to translate the biomass into high seed yields. The major abiotic constraints to productivity include drought, heat, cold and salinity and the key biotic constraints are Ascochyta blight (*Ascochyta rabii*), Fusarium wilt (*Fusarium oxysporum*), Dry root rot (*Rhizoctonia bataticola*), Botrytis grey mould (*Botrytis cinerea*), Collar rot (*Sclerotium rolfsii*), Root-knot nematode (*Meloydogyne incognita* and *M. javanica*), Stunt-virus, Pod borer (*Helicoverpa armigera*), and Cutworm (*Agrotis ipsilon*).

Amongst the causal agents of biotic stresses, about 67 fungi, 3 bacteria, 22 viruses and 80 nematodes have been reported on chickpea (Nene *et al.*, 1996) but only few of these cause economically important diseases (Haware, 1998). There has been an increase in different chickpea pathogens like fungi, bacteria and viruses over a period of past 17 years. The maximum number of pathogens has been reported from India alone with the number rising to 89 pathogens in 1995 from 35 in 1978 (Nene *et al.*, 1996). *Helicoverpa armigera*, which feeds on foliage, flowers and developing seeds, is the most important pest of chickpea, while stunt is the most important and prevalent viral disease in the most chickpea growing regions of the world.

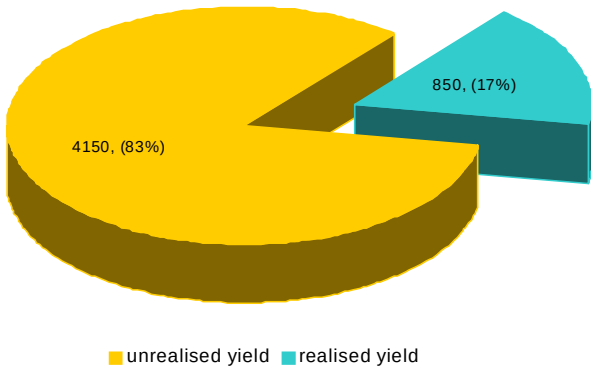


Fig 1.5: The potential seed yield and the realized seed yield (productivity) of chickpea

1.1.10 Diseases

One of the major constraints in realization of full yield potential of chickpea is wilt caused by a Deuteromycetes fungal pathogen *Fusarium oxysporum* Schlechtend.: *Fr.* f. sp. *ciceri* (Padwick) Matuo & K. Sato. The pathogen penetrates the vascular bundles of roots of chickpea plants and stops or reduces water uptake to the foliage. The infected plants ultimately wilt and die. The disease is highly destructive and worldwide in occurrence (Kraft *et al*., 1994). It has been reported from almost all chickpea growing areas of the world including the Indian subcontinent, Iran, Peru, Syria, Ethiopia, Mexico, Spain, Tunisia, Turkey and US (Halila and Strange, 1996). The disease is capable of causing 100% yield loss. Annual yield losses due to wilt have been estimated at 10%–90% (Jimenez-Diaz *et al.*, 1989; Singh and Reddy, 1993). Persistence of the pathogen in soil and its capacity to survive there for years even in the absence of host (Haware *et al*., 1996) renders its control difficult. Soil applications of fungicides are costly and lead to indiscriminate killing of beneficial soil microflora. The disease, to some extent, can be managed by use of biocontrol agents, which provide eco-friendly control of the disease (Hervas *et al.*, 1997, 1998; Landa *et al.*, 2001). Non-pathogenic *Fusarium oxysporum*, *Bacillus* species and *Pseudomonas flourescens* were identified suitable for biocontrol of wilt (Hervas *et al.*, 1997; Landa *et al.*, 2001, 2004). Efficacy of wilt management was improved when biocontrol agents were combined with cultural practices such as sowing dates (Landa *et al.*, 2004). More economic, effective and eco-friendly method of disease management is, however, by race-specific vertical resistance genes of the host, which

are available in the cultigen *C. arietinum* (Jimenez-Diaz *et al.*, 1993; Jalali and Chand 1992; Sharma *et al.*, 2005).

Among the economically important fungal diseases of chickpea are root diseases like Fusarium wilt and root rots caused by a complex of soil borne fungi, foliar diseases like Ascochyta blight and Botrytis gray mould, of which wilt and blight are the most devastating diseases affecting chickpea in tropical and temperate regions, respectively. Especially Ascochyta blight and pod borer, drought and cold are major constraints to yield improvement and adoption of the crop by farmers. Therefore, improving resistance to biotic and tolerance to abiotic stresses as well as a general increase in dry matter are major aims of chickpea breeders around the world.

1.2. Linkage map

1.2.1 Linkage map construction

A linkage map may be thought of as a 'road map' of the chromosomes derived from two different parents (Paterson, 1996). The maps indicate the position and relative genetic distances between markers along chromosomes, which are analogous to signs or landmarks along a highway. The most important use for linkage maps is to identify chromosomal locations containing genes and QTLs associated with traits of interest; such maps may then be referred to as 'QTL' (or 'genetic') maps. 'QTL mapping' is based on the principle that genes and markers segregate via chromosome recombination (called crossing-over) during meiosis (i.e. sexual reproduction), thus allowing their analysis in the progeny (Paterson, 1996). Genes or markers that are close together or tightly-linked will be transmitted together from parent to progeny more frequently than genes or markers that are located further apart. The frequency of recombinant genotypes can be used to calculate recombination fractions, which may be used to infer the genetic distance between markers. By analyzing the segregation of markers, the relative order and distances between markers can be determined, lower the frequency of recombination between two markers, closer they are situated on a chromosome (conversely, higher the frequency of recombination between two markers, further away they are situated on a chromosome). Markers that have a recombination frequency of 50% are described as 'unlinked' and assumed to be located far apart on the same chromosome or on different chromosomes. Mapping

functions are used to convert recombination fractions into map units called centi Morgans (cM). Linkage maps are constructed from the analysis of many segregating markers. Three main steps of linkage map construction are: (1) production of a mapping population; (2) identification of polymorphism and (3) linkage analysis of markers.

1.2.1.1 Mapping populations

The construction of a linkage map requires a segregating plant population (i.e. a population derived from sexual reproduction). The parents selected for the mapping population normally differ for one or more traits of interest. Population sizes used in preliminary genetic mapping studies generally range from 50 to 250 individuals (Mohan *et al*., 1997), however larger populations are required for high-resolution mapping. Several different populations can be utilized for mapping (McCouch and Doerge, 1995; Paterson, 1996). F_2 populations, derived from F1 hybrids, and backcross (BC) populations, derived by crossing the F_1 hybrid to one of the parents, are the simplest types of mapping populations developed for self pollinating species. Their main advantages are that they are easy to construct and require only a short time to produce. Inbreeding from individual F_2 plants allows the construction of recombinant inbred (RI) lines, which consist of a series of homozygous lines, each containing a unique combination of chromosomal segments from the original parents. The length of time needed for producing RI populations is the major disadvantage, because usually six to eight generations are required. Doubled haploid (DH) populations may be produced by regenerating plants by the induction of chromosome doubling from pollen grains, however, the production of DH populations is only possible in species that are amenable to tissue culture (e.g. cereal species such as rice, barley and wheat). The major advantages of RI and DH populations are that they produce homozygous or 'true-breeding' lines that can be multiplied and reproduced without genetic change occurring. This allows for the conduct of replicated trials across different locations and years. Thus, both RI and DH populations represent 'immortal' resources for QTL mapping. Furthermore, seed from individual RI or DH lines may be transferred between various laboratories for further linkage analysis and the addition of markers to existing maps, ensuring that all collaborators examine identical material (Young, 1994; Paterson, 1996).

1.2.1.2 Identification of polymorphism

The second step in the construction of a linkage map is to identify DNA markers that reveal differences between parents (i.e. polymorphic markers). It is critical that sufficient polymorphism exists between parents in order to construct a linkage map (Young, 1994). In many cases, parents that provide adequate polymorphism are selected based on the level of genetic diversity between parents (Anderson *et al*., 1993; Joshi and Nguyen, 1993; Yu and Nguyen, 1994; Collard *et al.*, 2003). Once polymorphic markers have been identified, they must be screened across the entire mapping population, including the parents (and F1 hybrid, if possible). This is known as marker 'genotyping' of the population. Therefore, DNA needs to be extracted from each individual of the mapping population when DNA markers are used. Significant deviations from expected ratios can be analysed using chi-square tests. Generally, markers segregate in a Mendelian fashion although distorted segregation ratios may be encountered (Sayed *et al*., 2002; Xu *et al*., 1997).

1.2.1.3 Linkage analysis of markers

The final step of the construction of a linkage map involves coding data for each DNA marker on each individual of a population and conducting linkage analysis using computer programs. Missing marker data can also be accepted by mapping programs. Although linkage analysis can be performed manually for a few markers, it is not feasible to manually analyze and determine linkages between large numbers of markers that are used to construct maps; computer programs are required for this purpose. Linkage between markers is usually calculated using odds ratios (i.e. the ratio of linkage versus no linkage). This ratio is more conveniently expressed as the logarithm of the ratio, and is called a logarithm of odds (LOD) value or LOD score (Risch, 1992). LOD values of >3 are typically used to construct linkage maps. A LOD value of 3 between two markers indicates that linkage is 1000 times more likely (i.e. 1000:1) than no linkage (null hypothesis). LOD values may be lowered in order to detect a greater level of linkage or to place additional markers within maps constructed at higher LOD values. Commonly used software programs include Mapmaker/ EXP (Lander *et al*., 1987; Lincoln *et al*., 1993) and MapManager QTX (Manly *et al*., 2001), which are freely available from the Internet. JoinMap is another

commonly used program for constructing linkage maps (Stam, 1993). Linked markers are grouped together into 'linkage groups', which represent chromosomal segments or entire chromosomes. Referring to the road map analogy, linkage groups represent roads and markers represent signs or landmarks.

1.2.1.4 Genetic distance and mapping functions

Distance along a linkage map is measured in terms of the frequency of recombination between genetic markers (Paterson, 1996). Mapping functions are required to convert recombination fractions into centiMorgans (cM) because recombination frequency and the frequency of crossing-over are not linearly related (Kearsey and Pooni, 1996; Hartl and Jones, 2001). When map distances are small (<10 cM), the map distance equals the recombination frequency. However, this relationship does not apply for map distances that are greater than 10 cM (Hartl and Jones, 2001). Two commonly used mapping functions are the Kosambi mapping function, which assumes that recombination events influence the occurrence of adjacent recombination events, and the Haldane mapping function, which assumes no interference between crossover events (Kearsey and Pooni, 1996; Hartl and Jones, 2001). It should be noted that distance on a linkage map is not directly related to the physical distance of DNA between genetic markers, but depends on the genome size of the plant species (Paterson, 1996). Furthermore, the relationship between genetic and physical distance varies along a chromosome (Tanksley *et al*., 1992; Young, 1994; Kunzel *et al*., 2000).

1.2.2 Genome mapping in chickpea

Generation of an integrated genetic map of the crop, comprising loci of both economic and scientific importance is a central goal of chickpea genetics. Until recently, the low level of polymorphism in the chickpea genome and the scarcity of co-dominant DNA-based markers were serious constraints to achieving this goal. The advent of sequence tagged microsatellite site (STMS) markers (Huttel *et al*., 1999; Winter *et al*., 1999) provided the opportunity to integrate the different available maps. In recent years, STMS markers were indeed applied for the generation of almost all published genetic maps of chickpea developed employing populations from crosses between *C. arietinum* and *C. reticulatum* (Tekeoglu *et al*., 2002; Benko-Iseppon *et al*., 2003; Rakshit *et al*., 2003; Pfaff and Kahl, 2003; Abbo *et al*., 2005), *C. arietinum* × *C.*

echinospermum (Collard *et al*., 2003) and intra-specific populations (Cho *et al*., 2002; Flandez-Galvez *et al*., 2003; Udupa and Baum, 2003; Cho *et al*., 2004; Cobos *et al*., 2005). Most of the authors compared their maps to the most extended genetic map of chickpea (Winter *et al*., 2000). The model map is based on an interspecific cross between the cultigen and a *C. reticulatum* accession. The emerging body of data now allows to draw three conclusions: (i) STMS markers are indeed elite anchor markers for merging genetic maps in chickpea, (ii) dominant markers are transferable between populations only in rare cases, and their identity needs to be confirmed by either linkages to other markers co-segregating in at least two populations, or sequencing and conversion into e.g. a sequence characterised amplified region (SCAR) marker, (iii) the map of Winter *et al.* (2000) together with its amendments developed on the same population may be employed as a reference map for genetic mapping in chickpea and comparative mapping between chickpea and other legumes, at least until a comprehensive integrated map becomes available.

1.2.3 Genetic to physical mapping

One of the logical spin-offs of a genetic map, the construction of a complete physical map of a genome, still represents a challenge for chickpea genomics. However, a physical map is fundamental to any progress towards a more complete understanding of the structure, composition and function of the genome. This cannot be achieved by mere recombination mapping. More so, the isolation of genes of agronomic importance (e.g. genes encoding receptor kinases, proteins of signal transmission, transcription factors, regulatory proteins or small regulatory RNAs, or enzymes of defense pathways) inevitably necessitates a physical map.

In essence, the era of physical mapping in chickpea is beginning now. It will, and has to be succeeded by an era of DNA sequence analysis. Moreover, the first steps towards this goal have already been made: at least four bacterial artificial chromosome (BAC) libraries are available, but under-used, and a cytogenetic map of the chickpea chromosomes is close to completion (Millan *et al*., 2006). One of the BAC libraries has been described in detail (Rajesh *et al*., 2004). A second one, derived from the fusarium-resistant chickpea cultivar (ICC 4958) was established in a binary vector V41 with 5× coverage of the genome. The library has been spotted onto

high-density nylon filters (close to 14,000 clones/filter) and used for hybridization experiments. These experiments clearly proved, that some markers, which were located on the integrated genetic map (Winter *et al*., 2000; Benko-Iseppon *et al*., 2003), and later on sequenced, are either low-copy (e.g. the thaumatin [PRP5]-encoding gene), middle-repetitive (e.g. the gene encoding *N*-hydroxycinnamoyl-benzoyl transferase, a protein catalysing a particular step in the phytoalexin synthesis pathway), or highly repetitive (marker CS27, a *Ty*3-gypsy-like LTR retrotransposable element CaRep; Staginnus *et al*., 1999, 2001). In addition, a series of 141 resistance gene analogues (RGAs) have been identified in this BAC library. Clustering of the various R-genes was neither observed in the BACs nor suggested by genetic mapping of RGAs (Huttel *et al*., 2002). Lichtenzveig *et al*. (2005) also constructed a BAC and a BIBAC library for chickpea; the two libraries contain a total of 38,016 clones and are equivalent to ca. 7.0x genomes of chickpea. Thus, the available BAC libraries could be employed for the generation of a physical map and as potential resources for whole genome sequencing, which should be a future perspective in chickpea genomics.

An alternative route to physical mapping has already started in collaboration between the laboratory of J. Dolezel (Olomouc, Czech Republic) and the University of Frankfurt with the aim to bridge the gap between the recombination-based genetic map and the chromosome-based map. The chromosomes were isolated from root tip cells synchronized for their mitosis, separated by fluorescent cell sorting and identified by their size. As a proof of principle, the localization of 5S-rDNA on chromosomes 2 (B) and 7 (G), that had already been shown by fluorescent *in situ* hybridizations (Gortner *et al*., 1998; Staginnus *et al*., 1999) was confirmed. Moreover, the smallest LG 8, identified by the STMS GAA46, corresponds to the smallest chromosome 8 (H). None of the other chromosome fractions contains the sequence of this marker (Vl´acilov´a *et al*., 2002). Exploiting this technology, linkage group (LG) 1 has already been identified as chromosome F (or G), LG 2 as chromosome F (or G), LG 3 as chromosome C (or D), LG 4 as chromosome B, LG 5 as chromosome C (or D), LG 6 as chromosome E, LG 7 as chromosome A, and LG 8 as chromosome H, respectively. At present, the separation is brought to perfection, and packages of at least 10 different linkage-group-specific markers address the precise identification of linkage group–chromosome relationships. The resulting map then will allow the

identification of the most interesting chromosomes carrying a particular trait (or gene), opening an avenue for the isolation and characterization of the underlying sequence, its transcription and regulation, and mechanism of action of the encoded protein. These features are badly needed for an understanding of basic plant properties for example, yield, resistances towards abiotic and biotic stresses, growth and development and seed quality.

1.3 Fusarium wilt

1.3.1 Pathogen - *Fusarium oxysporum* f. sp. *ciceri*

Classification and identification schemes for *Fusarium* are traditionally based exclusively on a morphological species concept derived from cultural characteristics, shared morphological trait of the anamorph, host range, and to a lesser extent, teleomorph micromorphology (Booth, 1971). The systematics of *Fusarium* remains controversial and confusing (Gams and Nirenberg, 1989), due to the conflicting morphological species concepts employed in taxonomic treatments of this genus (Booth, 1971; Gerlach and Nirenberg, 1982; Nelson *et al.*, 1983). Gerlach and Nirenberg's system (1982) is the most differentiated, including 73 species and 26 varieties; while 44 species and 7 varieties have been recognized by Booth (1971) and, 30 species by Nelson *et al.* (1983). On the other hand, in more recent times molecular systematics based on discrete DNA sequence data offers an objective phylogenetically based system of classification for *Fusarium* and its teleomorphs (Bruns *et al.*, 1991). Previous investigations employing cladistic analysis of DNA sequences from multiple unlinked loci in *Fusarium* species have revealed the utility of gene phylogenies inferred from mitochondrial small subunit (mtSSU) rDNA, nuclear 28S rDNA, β-tubulin gene and nuclear translation elongation factor 1α (O'Donnell *et al.*, 1998; Baayen *et al.*, 2000), however, nuclear rDNA ITS gene tree was found to be composed of non-orthologous sequences (O'Donnell and Cijelnik, 1997).

1.3.1.1 Habitat and host range

Fusarium is a large cosmopolitan genus of pleoanamorphic hyphomycetes whose members are responsible for a wide range of plant diseases (Farr *et al.*, 1989), mycotoxicoses and mycotic infections of humans and other animals (Nelson *et al.*, 1994). The species *Fusarium oxysporum* is well represented among the soil borne

fungi, in every type of soil, all over the world (Burgess *et al.*, 1981) and is considered to be a normal constituent of the rhizosphere of plants (Appel and Gordon, 1994). Some strains of *Fusarium oxysporum* are pathogenic to different plant species; they operate by penetrating into the roots and causing either root rots or tracheomycosis by invasion of the vascular system, causing wilt and sudden death disease leading to severe economic damages to many crop species. Typically, the vascular wilt causing *Fusarium oxysporum* species invade only living root tissues, tend to be specialized or host specific, and suppressed by saprophytes (Hillocks, 2001). Depending on the plant species and plant cultivars infected, *Fusarium oxysporum* is classified into more than 120 *forma speciales* (Armstrong and Armstrong, 1981) and further subdivisions into races are often made based on their virulence to a set of differential host cultivars (Cornell, 1991). However, the genetic basis of host specificity (*forma speciales*) and cultivar specificity (pathogen races) of *F. oxysporum* is not fully understood (Baayen *et al*., 2000). The presently accepted classification for the Fusarium wilt pathogen *Fusarium oxysporum* f. sp. *ciceri* is: Form-class: Fungi Imperfecti, Form-order: Moniliales, Form-family: Tuberculariaceae, Form-genus: *Fusarium*, Form-species: *oxysporum*, forma specialis: *ciceri*. *Fusarium oxysporum* f.sp. *ciceri* is reported from most of the chickpea growing areas.

1.3.1.2 Life cycle

Insight into the life cycle of wilt pathogens is important to understand their survival, causation of disease in a spatial framework and interactions at the host-parasite interface leading to disease resistance or susceptibility. Beckman and Roberts (1995) have addressed these topics and proposed a model explaining the interactions between vascular wilt causing pathogens and their host plants, wherein, the pathogens have distinct saprophytic and parasitic phases in their life cycles. The life cycle of soil-borne, wilt causing fungi including their saprophytic and parasitic growth and successive phases of colonization and pathogenesis is represented in Figure 1.6. There are three distinct phases in the pathogen life cycle: **i)** Determinative phase, **ii)** Expressive phase and **iii)** Saprophytic phase. In the determinative phase the extent of colonization of the host vascular system is determined, while in the expressive phase mainly disease symptoms are developed, and the saprophytic phase is characterized by the survival of the pathogen by formation of long-lived resting structures. During

disease congenial conditions, the pathogen, after invasion of the root tissue, acquires significant cortical colonization, then it enters the second phase of vascular invasion and spreads along with the transpiration pull. The spread and colonization of the xylem vessels by the pathogen plugs the conducting vessels leading to disruption of water uptake by the plants and thus causes wilting in the susceptible plants.

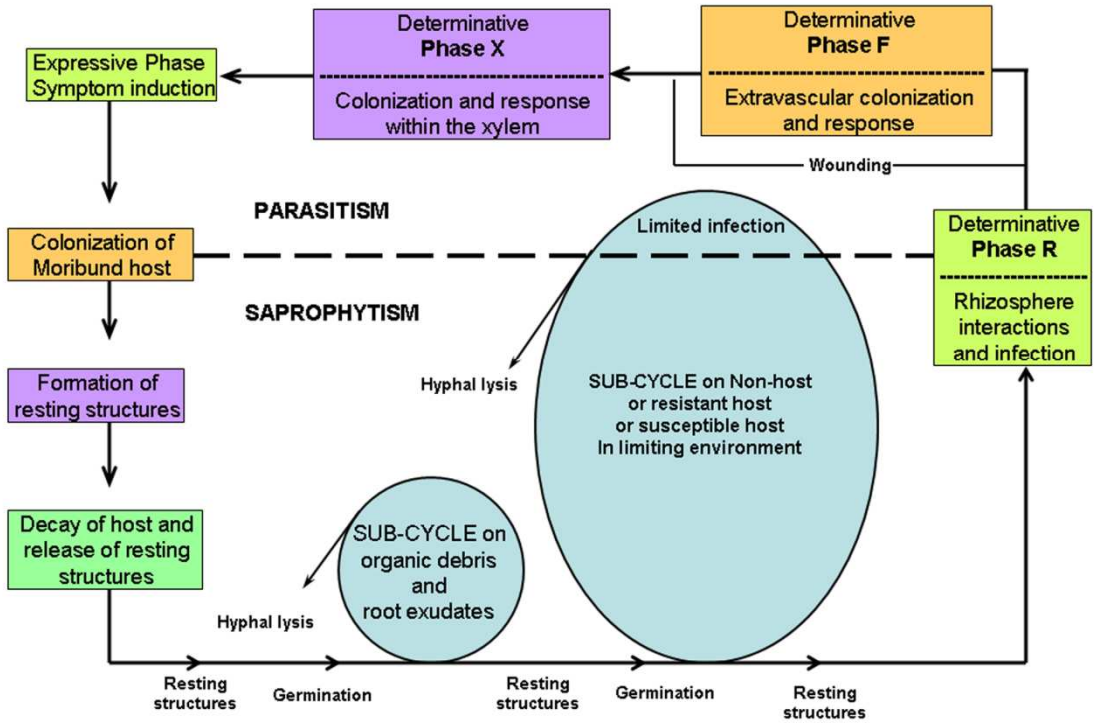


Fig 1.6: Schematic representation of the life cycle of wilt causing soil borne fungi, depicting saprophytic and parasitic growth and successive phases of colonization and pathogenesis (Beckman and Roberts, 1995)

1.3.1.3 Physiological specialization in Fusarium

Haware and Nene (1982) reported existence of four physiological races (1, 2, 3 and 4) of *F. oxysporum* f. sp. *ciceri* in India using 10 chickpea lines as differentials. Two additional races (0 and 5) were later identified from Spain and Tunisia (Halila and Strange 1996) whereas another (race 6) was reported from California, USA (Phillips 1988). Race 1 was subsequently divided into two races named as race 1A (from India) and race 1B/C (from Spain) based on variation in reaction on differential host lines (Trapero-Casas and Jimenez-Diaz 1985; Jimenez Diaz *et al*., 1993). Race 1B/C was also found in USA (California), Syria, Turkey and Tunisia. Thus, a total of eight

physiological races of the pathogen have been reported worldwide. The races 0 and 6 were later also reported in India (Rahman *et al*., 1998). The geographical distribution of races shows regional specificity for their occurrence in different regions of the world. Among the eight races, 0, 1B/C, 5 and 6 are primarily found in the Mediterranean region and the USA (Phillips 1988; Jimenez Diaz *et al.*, 1989, 1993; Halila and Strange 1996; Jimenez-Gasco *et al.*, 2001), whereas races 1A, 2, 3 and 4 are restricted to the Indian subcontinent (Haware and Nene 1982).

Apart from region-specificities, the eight races can also be divided into two groups based on symptomatology of infected plants i.e., yellowing syndrome and wilting syndrome (Trapero-Casas and Jimenez-Diaz 1985). Of the eight races, six (1A, 2, 3, 4, 5 and 6) cause wilting syndrome and are economically more important than races 0 and 1B/C that cause yellowing syndrome (Haware and Nene 1982; Jimenez-Diaz *et al.*, 1993; Kelly *et al.*, 1994). Plants infected with races causing wilting syndrome wilt within three to four weeks of inoculation with no visible yellowing of leaves. On the other hand, infection with races 0 and 1B/C leads to progressive foliar yellowing of plant leaves coupled with vascular discoloration. The wilting of infected plants eventually starts six to seven weeks after inoculation. Wilting and yellowing symptoms have been so far considered race-specific; however, evidence is emerging to indicate that both types of symptoms can be caused by a single race. Race 0, which is considered to cause yellowing syndrome, led to the wilting of plants of *C. reticulatum* (PI489777) within 30 days of inoculation with no evident foliar yellowing (Tekeoglu *et al.*, 2000).

Despite the occurrence of several races, overall genetic makeup of the fungus all over the world is narrow. All *F. oxysporum* f. sp. *ciceri* isolates were found to belong to a single vegetative compatibility group (Nogales-Moncada, 1997). DNA fingerprinting of races with repetitive sequences also suggested monophyletic lineage (Jimenez-Gasco *et al.*, 2004). Despite this, geographically isolated populations of the fungus displayed genetic and pathological diversity. The Iranian isolates comprised at least three vegetative compatibility groups (VCGs) (Zamani *et al.*, 2004), whereas the four Indian races were phylogenetically distinct from each other (Barve *et al.*, 2001; Chakrabarti *et al.*, 2001; Sivaramakrishnan *et al.*, 2002). Indian populations of pathogen were also genetically as well as pathologically distinct from those in other

countries as is evident from DNA fingerprinting studies (Barve *et al.*, 2001) and confinement of races 1A, 2, 3 and 4 (wilting pathotypes) to the India and 0 and 1B/C (yellowing pathotypes) to the Mediterranean region and California. Thus, at least two different populations of the pathogen exist worldwide, one native to India and another to other parts of the world. Unlike *F. oxysporum* f. sp. *malvacearum*, which evolved from two different populations, the populations of *F. oxysporum* f. sp. *ciceri* have evolved from a common ancestor or a single individual (Jimenez-Gasco *et al.*, 2002). The propagules of *F. oxysporum* f. sp. *ciceri* from the founder population then disseminated to different geographical areas possibly through seed where these diverged independently to races by stepwise acquisition of virulence (Jimenez-Gasco *et al.*, 2004). The evolution of geographically distinct virulence appears to be correlated to cultivation of chickpea germplasm lines in these regions. Resistance to wilt occurs mostly in 'desi' genotypes (Haware *et al.*, 1980). Interestingly, races 1A, 2, 3 and 4, which inhabit India are also the most virulent ones, whereas those from the Mediterranean region or the USA are less virulent (Haware and Nene 1982; Jimenez-Diaz *et al.*, 1993; Halila and Strange 1996). Evidently, there exists a correlation between evolution to races and cultivation of chickpea lines.

Race identification based on differentials is time consuming and can be erroneous if temperature is not conducive for wilt development. Alternatively, DNA-based diagnostics assays, which are fast, do not need screening of differential lines and are not influenced by environment, are being developed for the pathogen and its races (Kelly *et al.*, 1994; Jimenez-Gasco *et al.*, 2001; Jimenez-Gasco and Jimenez-Diaz, 2003). Random amplified polymorphic DNA (RAPD) markers have been used successfully to detect the pathogen in soil (Gracia-Pedrajas *et al.*, 1999) and distinguish between yellowing and wilting pathotypes either from isolated cultures (Kelly *et al.*, 1994) or from infected chickpea plants without fungal isolation (Kelly *et al.*, 1998). The technique was further refined to develop RAPD-based detection system for races 0, 1B/C, 5 and 6 (Jimenez-Gasco *et al.*, 2001). RAPD markers are less robust and the results may sometimes be ambiguous. To facilitate precise identification of races 0, 1A, 5 and 6, more robust markers called as sequence characterized amplified regions (SCARs) have also been developed (Jimenez-Gasco and Jimenez-Diaz, 2003). The utility of these assays to replace the traditional method based on host reaction for identification of the pathogen and its races is still to be

confirmed. However, these assays might need further refinements before these could be used routinely by the pathologists or breeders.

1.3.2 The disease: Fusarium wilt

Wilt in chickpea was first reported by Butler in 1918. McKerral (1923) who considered the disease to be soil borne and the putative causal organism *Fusarium* spp. was isolated from the soil samples analyzed. An association of *Fusarium* spp. and *Macrophomina phaseolina* (Tassi) Goid, with wilted plants was reported by Dastur (1935). However, the latter could not prove pathogenicity of the isolated *Fusarium* spp. and concluded that wilt was due to abiotic factors (Dastur, 1935).

1.3.2.1 Disease management

Fusarium wilt, caused by *Fusarium oxysporum* f.sp. *ciceri* (FOC), is a major constraint to chickpea production worldwide (Jalali and Chand, 1992). Annual chickpea yield losses due to Fusarium wilt vary from 10-15% (Trapero-Casas and Jimenez-Diaz, 1985; Jalali and Chand, 1992), and at times under specific conditions is capable of completely destroying the crop (Halila and Strange, 1996).

1.3.2.2 Cultural practices

Chickpea wilt has been reported to increase with higher levels of soil inoculum. Occurrence of wilt disease, its severity and disease progression is directly proportional to the density of the pathogen population. Presence of high levels of FOC propagules leads to 100% wilting much earlier than lower initial levels of FOC propagules (Bhatti and Kraft, 1992). Thus, it may be possible to early forecast the severity of diseases induced by soil-borne pathogens by assessing the initial pathogen population (Fry, 1982).

Avoidance of planting in heavily infested fields is advised to minimize the effects of wilt disease; however, availability of land is a limiting factor in Indian conditions. Moreover, as the pathogen can survive in soil for longer periods (Haware *et al.*, 1996) crop rotation, is not an effective practice for reducing wilt incidence. On the other hand, cultural practices like deep ploughing during summer and removal of host debris from the field can considerably reduce inoculum levels. Solarization

(covering the soil with transparent polythene for 6-8 weeks during summer months) is known to effectively control the chickpea wilt (Chauhan *et al.*, 1988). However, it is not a practical option in India as the poor farmer is already strapped for resources. Control of seed transmission of wilt can be achieved by using disease free seed, obtained from plants grown in disease free areas. The seed-borne inoculum can also be controlled by seed dressing with fungicides like Benlate-T (benomyl 30% + thiram 30%) at 0.25% rate (Haware *et al.*, 1978).

1.3.2.3 Biocontrol

The most effective and practical way to manage wilt is to use resistant cultivars (Jimenez-Diaz *et al*., 1991; Jalali and Chand, 1992; Kraft *et al*., 1994; Jimenez-Gasco, *et al*., 2004). However, occurrence of pathogenic races in FOC curtails the effectiveness of host resistance. Cultivation of varieties possessing resistance to specific races of the pathogen prevalent in a region or locality is the most economical disease management strategy (Jalali and Chand 1992). The use of biological control using either bacterial or fungal antagonists may enhance the effectiveness of resistant cultivars for management of Fusarium wilt in chickpea. Biological control by non-host *F. oxysporum* isolates (Ogawa and Komada, 1985; Paulitz *et al*., 1987; Mandeel and Baker, 1991; Alabouvette *et al*., 1993; Hervas *et al*., 1995; Larkin *et al*., 1996; Fuchs *et al*., 1997; Hervás *et al*., 1997) and incompatible races of the same *forma specialis* (Biles and Martyn, 1989; Martyn *et al*., 1991; Hervas *et al*., 1995) is seen as a promising strategy for management of Fusarium wilt diseases. Hervas *et al.* (1995) showed that prior inoculation of germinated chickpea seeds with either incompatible FOC races or non-host *F. oxysporum* isolates can suppress Fusarium wilt caused by the highly virulent FOC race 5. Further studies (Hervas *et al*., 1997; 1998) supported the potential of the non-host *F. oxysporum* isolate Fo90105 as a biocontrol agent against Fusarium wilt of chickpea.

Various mechanisms are involved in the biological control of Fusarium wilt by non-host *F. oxysporum* isolates, these include saprophytic competition for nutrients; parasitic competition for infection sites; and enhanced resistance due to rapid induction of defense responses within the host (Schneider, 1984; Alabouvette, 1986; Matta, 1989; Mandeel and Baker, 1991; Fuchs *et al*., 1997). These mechanisms may

function in parallel and not necessarily exclusive of one another, and several other mechanisms are speculated to be responsible for disease suppression by many biocontrol agents (Mandeel and Baker, 1991). Certain plant defense responses, namely phytoalexin synthesis and accumulation of chitinase and β-1,3-glucanase activities, may be involved in the non-host resistance of chickpea against non-host *F. oxysporum* isolates (Armero *et al*., 1993; Cabello, 1994; Armero, 1996). Stevenson *et al.* (1997) concluded that chickpea phytoalexins (the pterocarpans maackiain and medicarpin) are fundamental components of the resistance mechanism of this plant to Fusarium wilt.

1.3.3 Host responses to pathogen

1.3.3.1 Resistance mechanisms

Several different kinds of resistance mechanisms are exhibited by the plants, which are more or less regulated via different genetic frameworks. Additionally, there are several different definitions of the forms of resistance, which have been changed over a period. The four categories i) escape, ii) tolerance, iii) resistance and iv) immunity, described by Chahal and Gosal (2002) are fairly descriptive of the various mechanisms that influence the occurrence and severity of disease from a crop yield perspective.

1.3.3.2 Escape

The mechanism relies on avoidance of contact with the disease causal agent. Abscission of diseased leaves or growth and flowering early in the season are examples of escape mechanisms. The escape strategy can also be utilized to some extent by agronomical practice, like early or late planting and the use of fertilizers (Barbetti *et al.*, 1975; Chahal and Gosal, 2002). Deployment of early maturing varieties is one of the regular practices in several crops.

1.3.3.3 Tolerance

Here although the plant may show some visible disease symptoms, it does not suffer any adverse effects from infection, while the pathogen also is able to reproduce. A variant of tolerance is recovery, where a diseased plant is restored to healthy status by

various in planta mechanisms. Examples include the woody plants, which form new xylem tissue around Verticillium-infected tissues (Hiemstra, 1998).

1.3.3.4 Resistance

Resistance is a hereditary capability to limit pathogen growth. Resistance does not necessarily imply complete abolishment of pathogen activity. The common distinction of different forms of resistance is the vertical and horizontal resistance (Parlevliet and Zadoks, 1977; Vanderplank, 1984), effective against different pathogens, depending on their life style and reproductive strategies (McDonald and Linde, 2002). In vertical resistance, the plant has the ability to completely block the pathogen growth, the determinant of virulence of the pathogen. Vertical resistance is further sub-divided into race-specific resistance, where the resistance is active against some genotypes (races) of the pathogen, but not all races; while race non-specific resistance is the ability to block all known isolates of a pathogen, but where some plant genotypes show susceptible phenotype (Hammond-Kosack and Parker, 2003). Vertical resistance can be due to the presence of a resistance (R) gene according to the gene-for-gene resistance model (Flor, 1947) where the plant R gene recognizes a pathogen avirulence (Avr) gene, leading to a rapid response and resistance.

Horizontal resistance is often inherited as a quantitative trait. This type of resistance can be governed by multiple factors, and is in some cases referred to as 'basal resistance' (Hammond-Kosack and Parker, 2003), which can be confusing since induced resistance due to recognition of non-specific pathogen components like chitin or flagellin often is referred to as 'basal resistance' (de Torres *et al.*, 2006). The horizontal ("basal") resistances can also be governed through non-induced components like physical characteristics of the plant, toxin resistance and its chemical composition (i.e. the chemical structure of its antimicrobial secondary metabolites, like glucosinolates, phytoalexins, oxylipins etc.). Horizontal resistance does not breakdown like gene-for-gene type resistance, but may erode over time.

1.3.3.5 Immunity or non-host resistance

As all pathogens are not able to attack all plants, the events where all interactions between all genotypes of a pathogen and all genotypes of a plant are incompatible

(= no disease develops) are designated as immunity or non-host resistance. There have been many hypotheses about the mechanisms of non-host resistance – i) the pathogen fails to recognize the plant as a potential host, ii) the plant contains multiple "R genes" or "R genes" targeting indispensable structures of the pathogen, which makes it virtually impossible for the pathogen to break the induced resistance of the plant (Hammond-Kosack and Parker, 2003; Holub and Cooper, 2004), iii) the pathogen lacks the appropriate virulence factors and is thus unable to overcome the basal resistances of the non-host (Holub and Cooper, 2004).

1.3.4 Defense responses

Active defense responses are being elucidated in various plants, which include calcium and ion fluxes, increase of reactive oxygen species (ROS) during the oxidative burst (Lamb and Dixon 1997) and hypersensitive cell death (HR) (Greenberg, 1997). The expression of transcription factors and protein kinases, as well as elevation in cytosolic calcium, is integral to the signalling of these defenses (Grant and Mansfield, 1999). The expression of various defense genes also leads to the production of antimicrobial compounds such as pathogenesis-related (PR) proteins (Van Loon and Van Strien 1999) and phenylpropanoids (Dixon *et al.*, 2002).

Numerous defense responses vary in their timing, ranging from rapid responses, such as HR and callose depositions, followed by induced defenses like the salicilic acid (SA)- or methyl jasmonate (MeJ)-induced antimicrobial peptides. One of the rapid responses against the pathogen is deposition of callose that work as a barrier against pathogens that try to penetrate the cell and limits nutrient leakage from the cell, thus being efficient against both necrotrophs and biotrophs (Flors *et al.*, 2005). However, callose deposition is reported to negatively influence SA accumulation, which leads to the counter-intuitive result that loss of callose synthase can result in enhanced resistance against some biotrophic pathogens (Vogel and Somerville, 2000). Other modulations of the physical barriers against the pathogen are also known, such as lignification and thickening of the cell wall.

A long lasting resistance is then achieved by the plant, such as systemic acquired resistance (SAR), which in essence keeps the plant on alert to defend itself from future attacks (Grant and Lamb, 2006). Grafting studies have shown that SAR

requires SA locally. The mobile signal still remains elusive, but is dependent on a lipid transfer protein (Maldolando *et al.*, 2002). Another induced resistance requires ET, JA and (cytosolic) NPR1 is referred to as induced systemic resistance (ISR), a long lasting response triggered by non-pathogenic rhizobacteria, which is not associated to elevated levels of pathogenesis-related (PR) proteins (Pieterse *et al.*, 2001). ISR is, in many respects, to be regarded as a priming of defenses (Verhagen *et al.*, 2004), similar to BABA (β-amino-butyric acid)-induced resistance (BABA-IR). BABA-IR is, however, dependent on the SAR or an ABA-dependent signalling, depending on pathogen (Ton and Mauch-Mani, 2004).

1.3.4.1 Chickpea defense responses to Fusarium

The host in response to pathogen invasion, presents defenses, mainly at two levels (**i**) Structural: in the vascular tissue, where the upward movement of the pathogen is arrested by compartmentalization of the pathogen through the formation of callose, gelgum and tyloses, which are mainly the derivatives of celluloses and hemi-celluloses and progressive suberinization and lignin deposition (**ii**) Biochemical: the endodermis and xylem parenchyma, where the invading pathogen is restricted by infusion of phenolic compounds, and by hydrolytic enzymes like chitinases and glucanases. Fungal elicitors are known to induce the production of phenyl ammonia lyase (PAL) and peroxidase, which are involved in the synthesis and depolymerization of lignin precursors. The rapid increase and higher levels of PAL and peroxidases activity was found in resistant cultivars as compared to the susceptible cultivars (Aguilar *et al.*, 2000). Phenolics may function as either phytoalexins or be incorporated into structural barriers such as phenol-conjugated, lignified or suberised cell walls of appositions (Aist, 1983). Phytoalexins have been implicated as fundamental components of chickpea resistance mechanism to Fusarium wilt (Stevenson *et al.*, 1997).

1.4 Genetics of chickpea wilt resistance

Genetics of Fusarium resistance is complex, since at least for resistance to race 1, a minimum of two out of three detected resistance genes are required (van Rheenen, 1992). Several studies employing inter- and intra-specific recombinant inbred line (RIL) populations have demonstrated the organization of resistance genes for

Fusarium wilt races 1, 3, 4 and 5 (*foc1*, *foc3*, *foc4* and *foc5*; Mayer *et al.*, 1997; Ratnaparkhe *et al.*, 1998a; Tullu *et al.*, 1998; Winter *et al.*, 2000; Sharma *et al.*, 2004) in two adjacent resistance gene clusters on linkage group (LG) 2 flanked by STMS markers GA16 and TA96 (*foc1–foc4* cluster) and TA96 and TA27 (*foc3–foc5* cluster), respectively (Fig 1.7). Apart from the resistance genes per se, other sequences coding for proteins putatively involved in the chickpea's defense reaction were localized in close vicinity to the Fusarium resistance gene clusters, like the sequence of one of the markers tightly linked to the *foc4* and *foc5* loci is similar to a PR-5 thaumatin-like protein gene and another is homologous to the gene for anthranilate N-hydroxy cinnamoyl-benzoyltransferase, a regulator of the phytoalexin pathway, both important components of the plant's defense against pathogens. Huttel *et al.* (2002) isolated a series of RGAs from both *C. arietinum* and *C. reticulatum* using two degenerate primer pairs targeting sequences in the NBS domain. A total of 48 different RGAs were grouped into 9 different sequence classes, and were members of the Toll-interleukin receptor (TIR)-NBS-LRR and coiled coil (CC)-NBS-LRR groups. Thirty of these RGAs were mapped on the reference genetic map of chickpea (Winter *et al.*, 2000), where they could be located on principally five linkage groups, some of them as clusters on LGs 2 and 5, respectively (Fig 1.7). While, Flandez-Galvez *et al.* (2003) mapped 12 RGA markers that clustered on three LGs.

It is usually accepted that the difference in resistant and susceptible cultivars lies in the speed with which they can activate the defense mechanisms and accumulate substances like callose to restrict the growth and spread of the pathogen. However, there is still a debate about the role of fungal toxins in vascular wilt diseases. *Fusarium oxysporum* is known to produce the toxin 'fusaric acid' in culture filtrates, but most of the disease symptoms are postulated to be caused by the plant response to infection. Early studies on genetics of wilt resistance were restricted to race 1 where it was shown to be inherited by a recessive gene (Ayyar and Iyer 1936; Kumar and Haware 1982; Sindhu *et al.*, 1983). With the identification of phenomenon of late wilting in some genotypes susceptible to race 1 (Upadhyaya *et al.*, 1983), the focus was shifted to genetics of late wilting. The late wilting was found to be a monogenic trait and was controlled by three independent genes named as h_1, h_2 and H_3, each of which delayed onset of disease symptoms (Singh *et al.*, 1987a, b). Combination of any of the two late wilting genes (h_1 h_1 or h_2 h_2 or h_1 H_3 or h_2 H_3) was required for

complete resistance to race 1. (Upadhyaya *et al.*, 1983; Singh *et al.*, 1987a, b) (Table 1.2). The race 1 of FOC used in these studies was from India, hence, the race 1 described here should be considered as race 1A.

Similar to race 1A, resistance to race 2 was initially found to be conferred by a single recessive gene (Pathak *et al.*, 1975), however, later studies revealed involvement of two (Gumber *et al.*, 1995) or three genes (Kumar 1998). The phenomenon of late wilting was also reported after inoculation with race 2 (Gumber *et al.*, 1995). Of the three genes, *a* or *b* in homozygous recessive form or C in dominant form conferred late wilting (Kumar, 1998). Complete resistance was expressed when both *aa* and *bb* were present. Interestingly, the third gene whether it is homozygous recessive or homozygous dominant or heterozygous, did not influence the expression of complete resistance by other two genes or imparted any role in complete resistance. The F_3 data of Kumar (1998) and that of F_2 of Gumber *et al.* (1995) also did not fit well to the three and two gene theories, respectively. This possibly points towards the involvement of fewer/more genes than three in race 2 resistance. Using the F_2 and RILs derived from the same parents that were used by Kumar (1998) to show involvement of three genes, Sharma *et al.* (2005) demonstrated that resistance to race 2 was governed by a single recessive gene. Differences in results between the two studies can be attributed to the evaluation techniques used.

Genetics of resistance to other races of the pathogen is comparatively less studied. The resistance to race 3 was found to be monogenic (Sharma *et al.*, 2004, 2005), however, its dominant or recessive nature is unknown as a RIL population was used. Resistance to race 4 was monogenic recessive in some lines (Tullu *et al.*, 1998; Sharma *et al.*, 2005) whereas it was digenic recessive in Surutato-77 (Tullu *et al.*, 1999). Similar to races 1 and 2, the phenomenon of late wilting was also detected for race 4.

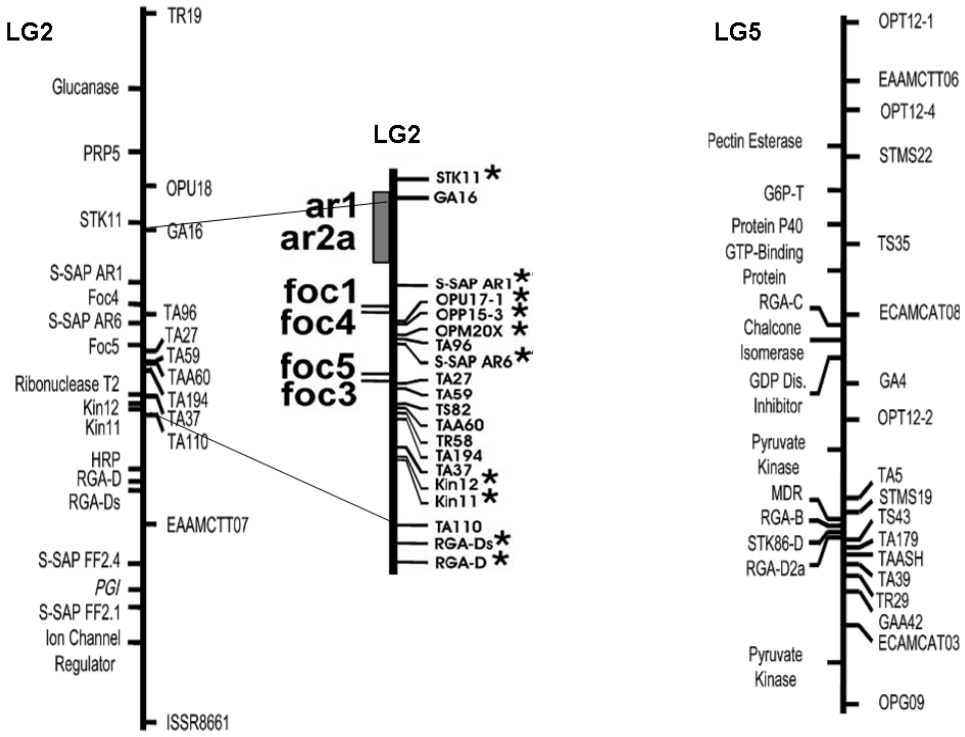


Fig 1.7: LG2 and LG5 from the integrated genetic map of chickpea generated by Millan *et al.* (2006) with data from Winter *et al.* (2000), Huttel *et al.* (2002) and Pfaff and Kahl (2003). Markers on the left of the vertical bar are derived from genes and those on the right are STMS or dominant framework markers. Only a few markers necessary for demonstrating the context within the linkage groups are shown. Detailed map of LG2 is in the centre depicting the vicinity of the Fusarium resistance gene clusters including Fusarium resistance genes and QTL for Ascochyta blight resistance (ar1, ar2a, indicated by the shaded box) on the left side of the vertical bar. Loci marked with an asterisk are potentially involved in pathogenesis, either encoding RGAs or pathogenesis-related proteins. (Millan *et al*., 2006).

Table 1.2: The genetic constitution and Fusarium wilt to race 1 reactions of chickpea cultivars

Cultivar	ICC #	Genetic constitution	Wilt reaction
JG-62	4951	$H_1\ H_1\ H_2\ H_2\ h_3\ h_3$	Early-wilting
K 850	5003	$h_1\ h_1\ H_2\ H_2\ h_3\ h_3$	Late-wilting
C 104	4928	$H_1\ H_1\ h_2\ h_2\ h_3\ h_3$	Late-wilting
H 208	4954	$H_1\ H_1\ H_2\ H_2\ H_3\ H_3$	Early-wilting
WR 315	8933	$h_1\ h_1\ h_2\ h_2\ h_3\ h_3$	Resistant
CPS 1	10130	$h_1\ h_1\ h_2\ h_2\ h_3\ h_3$	Resistant
P 436-2	554	$h_1\ h_1\ h_2\ h_2\ h_3\ h_3$	Resistant
BG 212	11088	$h_1\ h_1\ h_2\ h_2\ h_3\ h_3$	Resistant
JG-74	6098	$h_1\ h_1\ h_2\ h_2\ h_3\ h_3$	Resistant

(http://www.icrisat.org/ChickPea/Pedigree/Chickpeaintro.htm accessed on 29-11-08)

There are only a couple of studies on the inheritance of resistance to race 5, which showed it to be governed by a single gene (Tekeoglu *et al.*, 2000; Sharma *et al.*, 2005). However, it is yet to be ascertained whether the resistance gene(s) in two lines are the same or different. Resistance to race 0 was found to be monogenic (Tekeoglu *et al.*, 2000) as well as digenic, which may be either dominant or recessive (Rubio *et al.*, 2003). The genes conferring resistance to different races and their effect on wilting have been presented in Table 1.3.

1.4.1 Slow wilting

Apart from vertical form of resistance, slow wilting resistance in chickpea after inoculation with *F. oxysporum* f. sp. *ciceri* has also been observed (Sharma *et al.*, 2005). Slow wilting is a race-specific phenomenon and differs from late wilting in three aspects: latent period, disease progress rate, and final disease severity. In comparison to slow wilting, late wilting refers to susceptible lines showing a prolonged latent period. Late wilting lines eventually show 100% wilt. The phenomenon of slow wilting in chickpea is similar to that of slow mildewing and slow rusting in crops such as pea and wheat.

Table 1.3: Genetics of resistance to different races of the chickpea wilt pathogen *Fusarium oxysproum* f. sp. *Ciceri* (Sharma and Muehlbauer, 2007)

Fusarium race	Name of the resistance gene	Effect of resistance gene on wilting	Reference
0	*foc-0* $_1$ /*Foc-0* $_1$,	Complete resistance[b]	Rubio *et al.* (2003)
	foc-0 $_2$ /*Foc-0* $_2$ [a]		
1A	*h* $_1$ (syn *foc-1*),	Late wilting	Singh *et al.* (1987)
	h $_2$		
	H $_3$		
1B/C	-		
2	*foc-2* [c]	Late wilting	Sharma *et al.* (2005)
3	*foc-3*/*Foc-3* [a]	Complete resistance	Sharma *et al.* (2004, 2005)
4	*foc-4*	Complete resistance	Tullu *et al.* (1998), Sharma *et al.* (2005)
	Two recessive genes	Complete resistance	Tullu *et al.* (1999)
5	(*foc-5*/*Foc-5*)[a]	Complete resistance	Tekeoglu *et al.* (2000), Sharma *et al.* (2005)

[a]Dominant/ recessive nature not known
[b]Effect of individual genes in resistance not known
[c]Kumar (1998) found it to be governed by three genes, *a*, *b* and *C*. Each of the three genes led to late wilting whereas the first two genes conferred complete resistance

(–), Genetics of resistance not known

The genetics of slow wilting resistance in chickpea have not been determined, however, it might involve host genes other than vertical resistance ones. These genes appear to be minor ones, which additively slow the development of wilt as is evident from identification of slow wilting lines from cross of resistant and susceptible parents (Sharma and Muehlbauer, 2007).

1.4.2 Molecular markers linked to Fusarium wilt resistance genes

The first wilt resistance gene to be tagged in chickpea was H_1 (syn. *foc-1*, Mayer *et al*., 1997). The gene was located 7.0 cM from RAPD markers CS-27_{700} and UBC-170_{550} and an Allele Specific Associated Primer (ASAP) marker. Subsequently, markers linked closely to *foc-1* (Sharma *et al.*, 2004; Sharma and Muehlbauer, 2005), *foc-0* (Rubio *et al.*, 2003; Cobos *et al.*, 2005), *foc-2* (Sharma and Muehlbauer 2005), *foc-3* (Sharma *et al.*, 2004), *foc-4* (Ratnaparkhe *et al.*, 1998a, b; Tullu *et al.*, 1998, 1999; Tekeoglu *et al.*, 2000; Winter *et al.*, 2000; Benko-Iseppon *et al.*, 2003), the second resistance gene for *foc4* (Tullu *et al.*, 1999) and *foc5* (Ratnaparkhe *et al.*, 1998b; Tekeoglu *et al.*, 2000; Winter *et al.*, 2000; Benko-Iseppon *et al.*, 2003; Sharma and Muehlbauer 2005) were identified. Comparison of different studies (Tekeoglu *et al.*,, 2000; Ratnaparkhe *et al.*,, 1998a, b; Winter *et al.*, 2000; Huttel *et al.*, 2002; Benko-Iseppon *et al.*, 2003; Pfaff and Kahl 2003; Sharma *et al.*, 2004), indicated that four genes (*foc-1, foc-3, foc-4* and *foc-5*) should be in the same linkage group. Based on marker data of Benko-Iseppon *et al.* (2003), Huttel *et al.* (2002) and other studies, Millan *et al.* (2006) also proposed linkage of *foc-1, foc-3, foc-4* and *foc-5*. Conclusive evidence on clustering of five resistance genes (*foc-1, foc-2, foc-3, foc-4* and *foc-5*) was presented later by Sharma and Muehlbauer (2005) who mapped the genes using an intra-specific RIL population derived from the cross of WR-315 and C-104.

Screening of the progeny plants carrying wilt resistance gene(s) can be facilitated with marker assisted selection (MAS). Chickpea breeders are aiming to exploit MAS for resistance breeding. Efficacy of MAS, however, depends upon closeness of the marker to the gene. Marker density in the LG 2 is still low to facilitate MAS for wilt resistance genes and their positional cloning. There is a need to saturate the chromosomal region harboring wilt resistance genes with more markers to achieve these objectives. Sources of resistance to wilt are available within the

cultigens. MAS can be exploited effectively by using polymorphic markers for *C. arietinum* populations rather than for interspecific ones. Such markers will also be useful for map based cloning as the differences in genetic and physical distances among markers would be minimum (Winter *et al.*, 2000; Benko-Iseppon *et al.*, 2003). With the advent of STMS markers and availability of sources of resistance to all races in *C. arietinum*, it is now possible to map genes using intra-specific populations.

1.5. Mapping of quantitative traits using molecular markers

Most of the economically important traits of crop plants are controlled by QTLs and their expression is often influenced by the environment in which the plants are grown. The heritability of these traits is low and selection by conventional plant breeding method may delay the process or lead to failure in crop improvement. The estimation of the traits such as agronomic and yield related traits needs large sample size, technical facilities and labour. However, these estimates cannot be reliable if the heritability of the trait under study is governed by QTLs. Marker assisted selection (MAS) is widely adopted to transfer the QTLs and theoretically, it is more effective than phenotypic selection when correlation between the marker genotype scores and the phenotypic values is greater than the square root of heritability of the trait (Dudley 1993). Tagging of DNA markers with the phenotypic traits needs a comprehensive study, which is discussed below.

1.5.1 Methodology of QTL mapping in plants using DNA markers

1.5.1.1 Microsatellite markers

Simple sequence length polymorphism (SSLP markers), also known as simple sequence repeats (SSRs), or microsatellites, consist of tandemly repeated di-, tri- or tetra-nucleotide motifs and are a common feature of most eukaryotic genomes. The number of repeats is highly variable because slipped strand mis-pairing causes frequent gain or loss of repeat units. With high level of allelic diversity, microsatellites are valuable as molecular markers, particularly for studies of closely related individuals. PCR-based markers are designed to amplify fragments that contain a microsatellite using primers complementary to unique sequences surrounding the repeat motif (Weber and May, 1989). Differences in the number of

tandem repeats are readily assayed by measuring the molecular weight of the resulting PCR fragments. As the differences may be as small as two base pairs, the fragments are separated by electrophoresis on polyacrylamide gels or using capillary DNA sequencers that provide sufficient resolution. Without prior sequence knowledge, microsatellites can be discovered by screening libraries of clones. Clones containing the repeat motif must be sequenced to find unique sites for primer design flanking the repeats. Microsatellite marker development from pre-existing sequence is far more direct. Good reviews of microsatellite marker development include those of McCouch *et al.* (1997) and Zane *et al.* (2002). Microsatellites discovered in non-coding sequence often have a higher rate of polymorphism than microsatellites discovered in genes. However, in some species such as spruce (*Picea* spp.) with highly repetitive genomes, SSR markers developed from gene sequences have fewer instances of null alleles, i.e. failure of PCR amplification (Rungus *et al.*, 2004). Microsatellite markers have several advantages. They are co-dominant; the heterozygous state can be discerned from the homozygous state. The markers are easily automated using fluorescent primers on an automated sequencer and it is possible to multiplex (combine) several markers with non-overlapping size ranges on a single electrophoresis run. The results are highly reproducible, and markers are easily shared among researchers simply by distributing primer sequences. Although SSRs are abundant in most eukaryotic genomes, their genomic distribution may vary. Uneven distributions of microsatellites limit their usefulness in some species.

1.5.1.2 QTL mapping approaches

Genetic maps act as the first step towards understanding the genetics of individual crop plants. Genetic maps based on molecular marker technologies are now available for all major cereal species, including wheat (Snape *et al.*, 2006). At present, genetic maps are widely used to locate genes of interest so that the maps can be fully annotated with the locations of genes governing quality, agronomic performance, disease resistance, adaptability, or any other trait. This helps in direct manipulation of the desired trait by MAS. There are three approaches to map the QTLs; a) Single marker analysis, b) Interval mapping (IM) and c) Composite interval mapping (CIM)

(a) Single marker analysis: The simplest approach to identify QTLs, with data on an experimental cross is to perform analysis of variance (ANOVA) at each of the marker loci (Soller *et al.*, 1976). At each genetic marker, the population is split into two groups, according to their genotypes at the marker and compares the two group phenotypes means by a t-test. It is accompanied with a LOD score, defined as the (base 10) log-likelihood ratio comparing the hypotheses (Broman, 2002). Marker loci with large LOD scores are indicated to be linked to a QTL.

a) The phenotypes in the two groups are normally distributed with distance means but a common variance and

b) The phenotypes for all individuals follow a common normal distribution, independent of genotype.

The above approach has following weaknesses- i) if a QTL is not located at a marker, its effect will be attenuated as a result of recombination between the marker and the QTL; ii) At each genetic marker, we must discard individuals whose genotypes are missing; iii) When the markers are widely spaced a QTL may be quite far from all markers, and so the power for QTL detection will decrease; iv) This approach considers only one locus at a time (Single QTL model); in the presence of several QTLs, this approach fails.

(b) Interval mapping (IM): To overcome the disadvantages of single QTL model, multiple QTL model was proposed to give greater power for QTL detection, better separation of linked QTLs and to allow the examination of interactions among the QTLs. Lander and Botstein (1989) developed interval mapping, which overcomes the first three weaknesses of ANOVA at marker loci described above. This method, which continues to be the most popular approach for QTL mapping, makes use of a genetic map using linked markers and like ANOVA assumes the presence of single QTL. Each location in the genome is positioned, one at a time, as the location of the putative QTL.

Given the marker genotype data (and assuming no crossover interference), one can calculate the probability that an individual has genotype HH (or HL) at a putative QTL. These QTL probabilities depend only on the genotypes at the flanking markers. In interval mapping one assumes that given QTL genotypes, the phenotype follows a normal distribution with mean μH or μL according to whether the QTL genotype is

HH or HL, respectively and a common standard deviation. With the given genotypes at the markers flanking the QTL, the conditional QTL genotype probabilities, the marker genotype data, as mixing proportions, QTLs can be detected. For the QTL at each position in the genome (or in practice, at steps of 0.05 cM), three parameters are calculated μH, μL and and also a LOD score; the (base 10) log-likelihood ratio, by comparing the hypothesis that there is a single QTL at the given location with the hypothesis that there is no QTL anywhere in the genome. The LOD score, as a function of chromosome position, forms a profile log-likelihood. The genomic region, which has large LOD score, indicates the genomic interval, which harbors the QTL. Churchill and Doerge, (1994) suggested permutation test to generate genome wide threshold LOD, using the assumptions that there are no QTLs (ie. the phenotypes are simple normally distributed; independent of the marker data).

The advantages of interval mapping are two fold - i) it makes more complete use of the marker genotype data (making proper allowance for the missing data) and ii) it considers positions between markers as putative locations for a QTL effects. In case of dense genetic markers and relatively complete marker genotype data, interval mapping provides little advantage over ANOVA. The disadvantage being similar to ANOVA, which assumes single QTL model and it is not ideal in the presence of multiple especially linked QTLs.

(c) Composite interval mapping (CIM): Methods that make use of multiple QTL models can provide increased sensitivity, resolve linked QTLs and allow the examination of interactions between QTLs. The simplest multiple-QTL method is multiple regressions, the obvious extension of ANOVA at the marker loci. Cowen (1989) appears to be the first to have recommended the use of multiple regressions in this context. Jansen and Zeng independently developed a method which attempts to reduce the multi-dimensional search for identifying multiple QTLs to a one–dimensional search (Jansen 1993; Jansen and Stam, 1994; Zeng, 1994). This is actually a hybrid of interval mapping and multiple regression on marker genotypes. One includes other markers (on the same chromosomes and on different chromosome) as repressors while performing interval mapping, in an effort to control for the effects of QTLs in other intervals, so that there will be greater power for QTL detection and also the effects of background QTLs will be precisely estimated. Zeng (1994) referred

to this approach as composite interval mapping (CIM). Similar to interval mapping, LOD threshold is calculated by whole genome scanning.

(d) Multiple interval mapping (MIM): MIM uses multiple marker intervals simultaneously to construct multiple putative QTL in the model for QTL mapping. Multiple–interval mapping is much like CIM, but the additional repressors are not required to reside at the marker loci. Therefore, when compared with the current methods such as IM and CIM, MIM tends to be more powerful and precise in detecting QTLs (Kao *et al.*, 1999). To detect a QTL using the MIM model, model selection procedures are considered because all possible subset selection is not feasible. There are at least three basic model selection techniques, forward, backward, and stepwise selections, for exploring the relationship between the independent and dependent variables (Kleinbaum *et al.*, 1988; Miller, 1990). As MIM uses multiple QTLs, the computation burden is heavy when compared with the one-QTL model (CIM and IM). MIM has the potentiality to be more powerful and more precise in QTL mapping by directly conditioning putative QTL and incorporating possible epistasis in the model. Thus, more genetic variation can be controlled in the model. With the estimates of QTL parameters, other composite genetic parameters, such as the genetic variance components and heritabilities can also be estimated. Based on the MIM results, genotypic values of individuals can also be estimated to allow desired genotypes to be selected in MAS under various requirements (e.g. cost, efficiency, and trait correlations) (Kao *et al.*, 1999).

1.5.2 Marker assisted selection (MAS)

Molecular markers are powerful tools that can be used for MAS and also as landmarks for map-based cloning of genes. Molecular markers associated with QTLs have been reported for many important traits. Once linkage between a QTL and molecular marker is determined, the QTL can be transferred into other genetic backgrounds using MAS. Molecular markers are increasingly being used to tag genes or QTLs of agronomic importance, offering the possibility of their use in MAS for chickpea breeding (Sharma and Muehlbauer, 2007). In addition to their use in MAS, molecular markers have been used to isolate genes via map-based cloning (Stein and Graner, 2004). The potential value of genetic markers, linkage groups and their

association with agronomic traits has been known for more than 80 years. The usefulness of MAS was recognized as early as 1923 when Sax demonstrated in beans an association between seed size and seed coat pigmentation. The concept of selection based on genotype rather than phenotype created strong interest among plant breeders (Tanksley *et al.*, 1989; Paterson *et al.*, 1995). The molecular-marker based (RFLP) map in plants was first demonstrated in tomato and consisted of 57 loci (Bernatzky and Tanksley, 1986). Since then, maps have been constructed for nearly all crop plants (Philips and Vasil, 2001), allowing in principle, the application of MAS in plant breeding as originally proposed by Sax (1923) and Thoday (1961). The rationale relies on the discovery of phenotype/genotype associations between genome regions (as assayed by molecular markers) and traits in segregating populations (such as F_2, RIL, DH, BC, etc.). These are derived by analysis of segregation of simply inherited traits and by QTL analysis for complex traits (Lee, 1995). The identification of markers tightly linked to target genes/QTLs and their conversion, if necessary, to a PCR platform has made MAS feasible in some plant breeding programmes (Langridge and Chalmers, 1998). MAS can increase the efficiency and accuracy of selection, especially for traits that are difficult to phenotype or are recessive (Varshney *et al.*, 2006).

Markers selected for use in MAS should be reliable and easily shared among researchers. Co-dominant markers are preferred to avoid the need for progeny testing. Sometimes less desirable markers for MAS such as RAPDs, ISSRs and AFLPs are useful for finding markers linked to the desired allele. Once such a marker is found, it is possible to extract and sequence the corresponding band. This sequence can be used to develop co-dominant markers such as cleaved amplified polymorphic sequences (CAPS) (Konieczny and Ausubel, 1993) or to sequence characterized amplified regions (SCARs) (Paran and Michelmore, 1993). SCAR and CAPS markers are co-dominant and simplify the screening of large number of individuals. When a genetic map exists, markers can be positioned on the map and other linked markers can be substituted. The additional markers are useful for high resolution mapping to find markers more closely linked to the desired allele or ultimately for positional cloning of the underlying gene. Following are some of the major components of MAS aimed at enhancing the efficiency of plant breeding:

(1) Accelerating the selection of small number of traits that are difficult to follow due to complex inheritance or strong environmental influence.
(2) Selection for traits of substantial economic importance, in cases where the biological assays are unreliable and/or not cost-effective.
(3) Accumulating disease-resistance genes by gene pyramiding. Once an effective resistance gene is present in a breeding line, it is difficult to select for additional resistance genes due to epistatic effects.

Using molecular markers, additional resistance genes can be accumulated into elite lines while maintaining pre-existing resistance genes.

1.5.3 Chickpea molecular breeding

The chickpea cultigen contains high morphological variation, but narrow overall genetic variation, from which many desirable traits may have been excluded through selection (Abbo *et al*., 2003). For the desirable but missing traits from advanced breeding programs, such as durable resistance/tolerance to the many major biotic and abiotic stresses, breeders have begun to source germplasm more widely, from landraces and closely related species. To speed up the process of recombining 'wild' genes into elite genotypes, molecular tools have been integrated with classical breeding approaches. This has included the generation of molecular markers linked to the genes conditioning desirable traits, for efficient pyramiding of the traits. Molecular markers associated with quantitative trait loci (QTL) for resistance to biotic stresses and some morphological traits have been located on both intra-specific and interspecific linkage maps and, importantly, chickpea genotypes tolerant to most major biotic and abiotic stresses have been identified (Millan *et al*., 2006). The use of resistant or tolerant cultivars is considered to be the most efficient and effective means of controlling major stresses. However, a major problem for disease-resistant cultivars is that the resistance is incomplete and/or breaks down against new virulent races of pathogens that arise from mutation and genetic recombination.

Wild *Cicer* species have also been identified as sources for resistance to some stresses (Singh *et al*., 1981; Collard *et al*., 2003; Croser *et al*., 2003) and, although interspecific crosses between wild species and *C. arietinum* have only been successful for *Cicer reticulatum* and *Cicer echinospermum* (Singh and Ocampo 1997; Collard *et*

al., 2003), there still exists much potential for transferring resistance genes from wild *Cicer* species into cultivated chickpea. Detailed information regarding the number, nature and diversity of genes controlling resistance/tolerance to biotic and abiotic stresses is essential for successful breeding programs. However, problems in dissecting polygenic traits and accurately measuring the underlying physiological mechanisms controlling tolerance to abiotic stresses make this difficult. As a result, molecular genetic studies have not provided a consistent picture of the genetic basis for biotic stress resistance, especially for resistance to Ascochyta blight (Millan *et al*., 2006). The narrow genetic variation in cultivated chickpea has limited the generation of informative molecular markers, while QTL for certain stresses differ with developmental stage, bioassay environmental conditions, the genotypes/fungal isolates used, and classifications for resistance and susceptibility. For example, numerous genetic mechanisms controlling Ascochyta blight resistance have been proposed, including single/multiple genes of dominant/recessive nature with modifiers and additive effects, as well as single/multiple QTL. The use of recombinant inbred line (RIL) populations was identified as a strategy to enable resistance studies to be performed on near homozygous individuals with temporal and spatial replication (Tekeoglu *et al*., 2000). Recent achievements have been made using RIL populations to study Ascochyta blight and Fusarium wilt resistance (Cobos *et al*., 2006; Iruela *et al*., 2007). An important QTL for Ascochyta blight resistance was identified on linkage group 2, which appears to cluster with a major gene for resistance to Fusarium wilt.

1.5.4 Functional genomics

Specific genes involved in resistance to biotic and abiotic stresses in chickpea have not been characterized using the genetics approach, but an enhanced understanding of the chickpea stress response at the genomic level may enable this. Plant stress responses are complex and diverse, and every gene involved, from recognition to signaling to direct involvement, forms part of a coordinated response network. Until recently, the genes and pathways of gene activation controlling effective stress resistance in chickpea remained unknown. Several approaches, including differential screening of cDNA libraries (Ichinose *et al*., 2000) and the placement of resistance gene analogues onto existing linkage maps (Rajesh *et al*., 2002a), have identified

candidate genes that are involved in ascochyta blight resistance. Functional genomics provides opportunities for illuminating the mechanisms of chickpea resistance/tolerance to major biotic and abiotic stresses, possibly providing information concerning the molecular pathway(s) used by the plant, as well as the function of the candidate genes involved. Functional genomics incorporates several parallel approaches and tools, such as EST generation, transcript profiling, transgenics and reverse/forward genetics, for high throughput studies of gene function. Ultimately the goal is to link the genome to the phenome, but understanding of the functional roles of genes is very limited compared with the knowledge of sequence information. Thus, a major challenge is to analyze and interpret the large-scale gene sequence data being produced to discover and understand the functional roles of underlying genes. Functional genomics has become widely useful for studying the stress responses of plants, such as tomato (Gibly *et al*., 2004), rice (Fujiwara *et al*., 2004), maize (Baldwin, 1998), cassava (Lopez *et al*., 2005), soybean (Moy *et al*., 2004) and *Arabidopsis thaliana* (Huitema *et al*., 2003).

1.6 Future trends for genomics-assisted breeding

Chickpea and other grain legumes have been 'orphaned' with regard to investment in molecular research compared with cereals and horticultural crops of high economic value. This scenario is slowly changing with efforts from organisations such as the European Union (EU), who have implemented a Grain Legumes Integrated Project (GLIP) to facilitate coordinated research in grain legumes. Recently, a GLIP dissemination event held in Madrid (Spain) unveiled current and future research interests (http://www.grainlegumes.com), which are focused on the importance of chickpea alongside major grain crops like field pea and the model legume *Medicago truncatula*. The main aim of the GLIP is to understand the interrelationships of the multiple signalling systems that control stress-adaptive responses in legumes. To dissect the mechanisms of abiotic stress tolerance in legumes, gene expression patterns and metabolomic changes induced by various abiotic stresses in field pea, chickpea and *M. truncatula* will be analysed using various genomic approaches. This is coupled with detailed genetic mapping of crosses between salinity tolerant and sensitive varieties in chickpea and *M. truncatula*. The approach was implemented to help evaluate control mechanisms exerted by QTL on gene expression patterns and to

identify regulators of gene expression and metabolic adaptation. The proposed outcomes of this project are: (1) identification of candidate genes induced by salinity, drought or cold stress in *M. truncatula*, field pea and chickpea, (2) generation of SSH cDNA libraries of field pea, chickpea and *M. truncatula* exposed to drought and salinity stress conditions, (3) identification of molecular markers associated with QTL linked to abiotic stress tolerances in *M. truncatula* and chickpea, (4) fine mapping of *M. truncatula* and chickpea QTL for salinity tolerance, and (5) generation of a 'LeguStressChip' to serve as a diagnostic tool to screen legume germplasm for stress tolerance (http://www.grainlegumes.com). The GLIP is also using the genomics approach to develop tools for transferring the information gained from model plants (including *M. truncatula*, *Lotus japonicas* and *Arabidopsis thaliana*) to grain legume crops, such as chickpea, field pea, faba bean, alfalfa and clover. Such a large scale coordinated research project will greatly accelerate our understanding of stress tolerance in chickpea and other legumes and will boost the technology transfer from model crops to cultivated species.

Chickpea gene expression studies carried out to date using microarrays and SuperSAGE has identified candidate chickpea genes for resistance/tolerance to major biotic and abiotic stresses. Combining larger-scale gene expression profiling (e.g. SuperSAGE and/or increased EST generation) with the use of near isogenic germplasm contrasting only for the trait of interest will greatly enhance the identification of genes directly involved in resistance/tolerance to key biotic and abiotic stresses. Recently a series of powerful functional genomics tools for model legumes and chickpea have emerged that will shape the future of research in this field. For chickpea, a relatively dense integrated genetic map with most linkage groups related to chromosomes was developed (Vlacilova *et al.*, 2002). Together with the existence of several bacterial artificial chromosome (BAC) libraries, this will greatly facilitate map based gene/QTL cloning, genome sequencing and physical map construction. In fact, positional cloning of Ascochyta blight resistance genes from QTL1 is currently in progress. In addition, colinear mapping, making use of cross-species synteny, has enabled the recent placement of the same QTL from chickpea from different genetic backgrounds on the *M. truncatula* genome (Bian *et al.*, 2007). With the advent of efficient chickpea transformation protocols (Senthil *et al.*, 2004), important clones from the binary BAC library (Lichtenzveig *et al.*, 2005) may be

readily used in high-throughput transgenic studies. Also of great importance is the development of powerful and high throughput array-based genotyping tools, such as Diversity Array Technology (DArT) and Tagged-Array Marker (TAM), which are beginning to be applied to legumes and have the capacity to enhance chickpea genomics.

1.7 Objectives of the thesis work

Although India is the major producer of chickpea in the world, still it fails to meet the domestic demand. We are importing the chickpea from other countries mainly due to less productivity. To meet the growing demand it is essential to increase the chickpea productivity which can be achieved by reducing the loss caused by biotic and abiotic stresses and developing the agronomically superior and high yielding varieties. In view of improving the chickpea productivity, research work on mapping of Fusarium wilt and yield related traits was initiated at Plant Molecular Biology group of National Chemical Laboratory, in collaboration with various other chickpea breeding research organizations. The importance of the chickpea crop in Indian perspective and the need for improvement of chickpea yield by direct or indirect methods necessitated the work, which was carried out keeping in mind the following objectives

1) Construction of chickpea framework linkage map using JG62 x Vijay RIL population.
2) Tagging of Fusarium wilt resistance genes (*foc1*, *foc2* and *foc3*).
3) QTL analysis of yield and yield related traits viz; Plant height, Plant spread, Branches per plant, Days to 50% flowering, Days to maturity, Pods per plant, Seed weight and Yield per plant segregating in the JG62 x Vijay population.

1.7.1 Organization of the thesis

I have organized my thesis in the following order:

Chapter 1: Introduction and Review of Literature

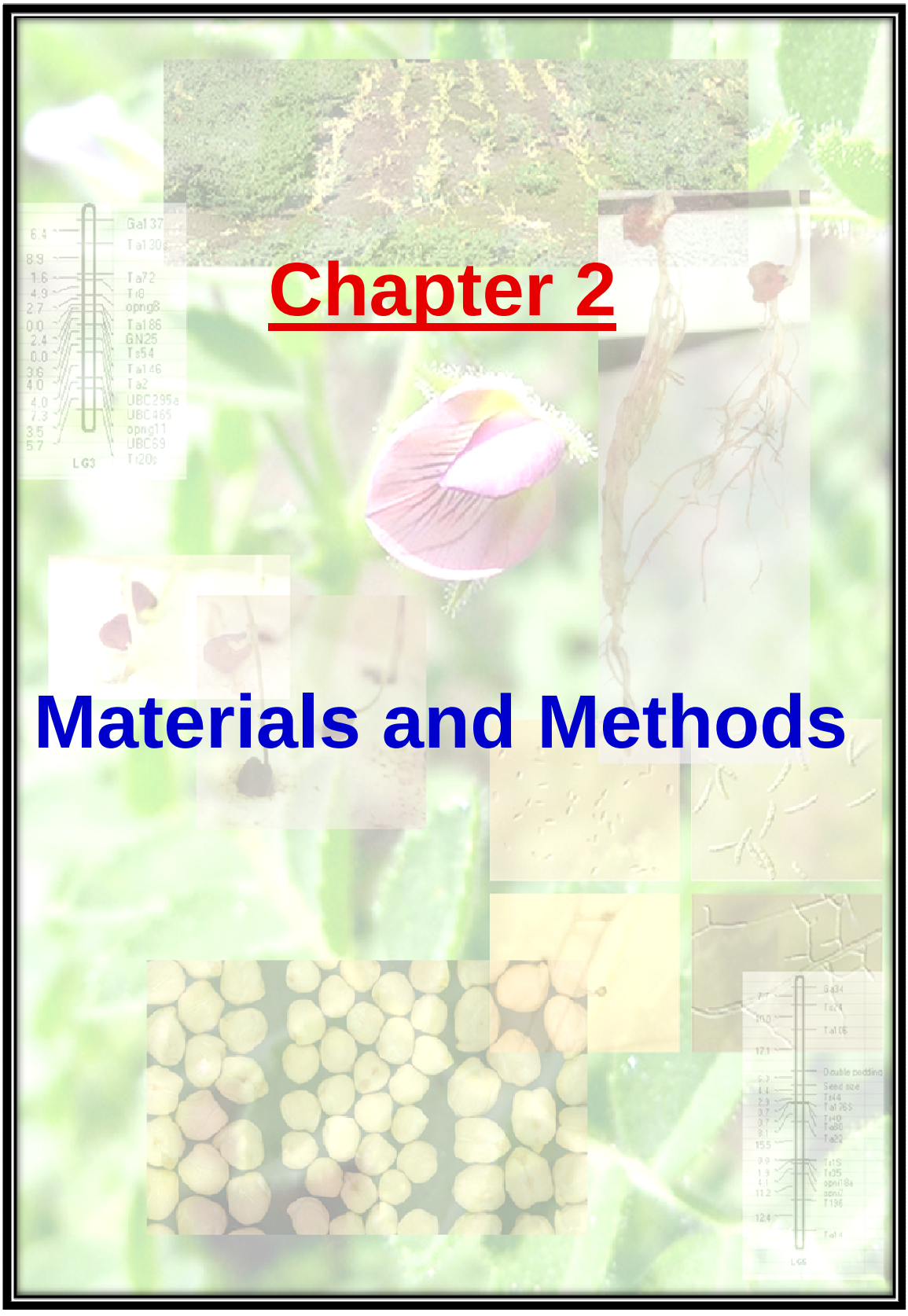
Chapter 2: Materials and Methods

Chapter 3: Results

Chapter 4: Discussion

Chapter 5: Thesis Summary and Future Directions

Bibliography



Chapter 2

Materials and Methods

2.1 Plant material

Based on the morphological and genetic diversity, two popular varieties (Vijay and JG-62) of chickpea available at Pulses Improvement Project (PIP), Mahatma Phule Krishi Vidyapeeth (MPKV), Rahuri were selected for crossing program. These two genotypes are different for many agro-economically important traits which are listed in Table 2.1.

Table 2.1: Main features of the parental genotypes

Parents	**Pedigree**	**Origin**	**Special features**
JG-62	Selection from germplasm	Jabalpur (Madhya Pradesh)	Highly susceptible to wilt, twin podded, early maturing, medium sized seeds
Vijay	P-1270 x Annigeri	Rahuri (Maharashtra)	Wilt resistant, drought tolerant, high yielding, wider adaptability with high pod number

2.1.1 Methods

To raise different self and backcross generations within a stipulated period, in addition to the regular rabi season, off season nurseries were grown at PIP, Mahatma Phule Krishi Vidyapeeth, Rahuri. Chickpea is a self pollinated crop in which anthesis takes place one or two days before opening of flower. The healthy buds which are likely to open within day or two were emasculated in the morning and pollinated on same day between 10 to 11 a.m. (Khan and Akhtar, 1934). JG62 x Vijay cross was effected and enough crossed seeds were harvested. The schematic details of various generations, raised during regular and off season at Rahuri are given in Table 2.2.

Table 2.2: Schematic details of different generations (P_1 = JG62 and P_2 = Vijay)

Place	Season	Cross/self	Stage of seed
P.G.I. Farm	Rabi (Oct to Jan)	P_1 x P_2	F_1
P.G.I. Farm	Early rabi (Sept to Dec)	Self of P_1 x P_2	F_2
Pulse Improvement Project, MPKV, Rahuri	Late rabi (Dec to Mar)	Self of F_2 Self of F_3 to F_9	F_3 F_4 to F_9

2.1.2 Conduct of experiments

A uniform piece of land was selected for experiment. It was ploughed, harrowed; stubbles of the previous crop were collected and brought to fine tilth. The experiment was conducted in randomized block design with two replications during rabi season. Sowing was done in rows of 3m length and 30cm apart, accommodating 20 plants at 15cm distance in a row. Fully developed single plumpy seeds were dibbled at 15cm distance in each row. Fertilizer dose was applied at uniform rate of 25kg N and 50kg P_2O_5/ha at the time of sowing. The operations like thinning, weeding, hoeing, irrigation and plant protection were carried out regularly as per need and stage of the crop. The crop growth was uniform and satisfactory. The experimental plots were surrounded by non experimental border rows of variety PG12, in order to avoid border effect.

The recombinant inbred line (RIL) population comprising 197 lines was developed at Pulse Research Station, MPKV Rahuri, India by single seed descent method from F_2 generation onwards, bulked plant-wise at F_9 generation. For the molecular analysis, ninty three randomly selected RILs were used. The parents JG62 and Vijay along with the RILs were grown at two different locations; Rahuri (for three years 2003, 2004 and 2005) and Dharwad (for two years, 2006 and 2007). The phenotypic data were collected in the successive years from all the RILs in a row to avoid biased selection. The RIL population was used to identify QTLs for various agronomic and yield related traits using different protocols as detailed below.

2.2 Screening for Fusarium wilt resistance

2.2.1 Pot culture

One hundred F_9 recombinant inbred lines (RILs) from a cross between two *Cicer arietinum* cultivars, JG62 and Vijay, were used to map the fusarium wilt resistance genes. JG62 is an early wilting genotype highly susceptible to Foc races 1, 2 and 3, while Vijay is resistant to them. The RIL population was grown in experimental field of Pulses Research Station, MPKV, Rahuri, India. The lines were tested for their reaction to wilt in sick pots under controlled conditions. Single spore isolates of *Fusarium oxysporum* f.sp. *ciceri* races 1, 2 and 3 were obtained from ICRISAT, Patancheru, India and maintained on fresh potato dextrose agar (PDA). The inoculums were further cultured with sterile corn-meal-sand mixture (CMS) in conical flasks and incubated for 21 days at room temperature. The infested CMS mixture was mixed thoroughly with autoclaved soil mixture (clay loam, sand, FYM; 1:1:1 v/v) at (1:12 w/w) in pots (Brinda and Ravikumar, 2005). Seeds of the susceptible cultivar JG62 were surface disinfected with 70% alcohol and grown in all the pots and the plants were allowed to wilt. Only the pots in which JG62 was completely wilted within 25 days of sowing were used for the experiment. Ten similar pots without pathogen inoculums were used as control to grow JG62 and none of these plants developed disease symptoms. Seeds of the parents and the RIL population were surface disinfected with 70% alcohol and sown in the sick pots to study their wilt reaction. The experiment was conducted with two replications and ten plants per RIL in each replication. The numbers of wilted and healthy plants in each RIL were noted at weekly intervals starting from three weeks after sowing and the data were recorded up to 9^{th} week after sowing. The RILs were evaluated separately for Foc races 1, 2 and 3. Reaction of RILs was based on wilt incidence, where the line with more than 80% wilting was treated as susceptible and that with less than 20% wilting as resistant.

2.2.2 Hydroponic culture

Cicer arietinum seeds of cultivars Vijay (R), JG-62 (S) and 100 RILs were obtained from the Mahatma Phule Krishi Vidyapeeth (MPKV), Rahuri, Maharashtra, India. For germination, seeds were wrapped in wet sterile muslin cloth and stored at room

temperature (24-26^{o}C) in dark for 3-4 days till sprouting. While the seeds sprouted the trays and floats were made ready. The Styrofoam sheets were cut to a size that they fitted into trays, and holes were punched into the Styrofoam sheets using a cork borer in a square lattice so as to accommodate around 20 genotypes. Then the sprouted seeds were transferred onto Styrofoam floats placing each sprouted seed into the holes punched earlier, and these floats were placed in the glass trays containing water and growth media and kept in controlled conditions at 22^{o}C and 60% relative humidity under white light and normal day conditions (14 h light/10 h dark). Seedlings were grown hydroponically under sterile conditions on floats in sterile water containing macro- and micro- nutrients (half strength Hoagland's nutrient medium, (Hoagland and Arnon 1950)).

Plants were seven days old at the time of pathogen infection. Freshly prepared spore suspension (10 ml of 1x10^{6}spores/ml) of *Fusarium oxysporum* f.sp. *ciceri*, was added to the sterile hydroponic trays. After two days the water in the trays was mixed with a sterile glass rod to ensure uniform spread of the fungus. A few seeds of JG-62 (S) were sown in each tray as an indicator of infection. Seedlings grown in similar trays with no pathogen served as an uninfected plant control. Data were recorded from 20 days after infection till 60 days in five days interval.

2.2.3 Validation

Fourteen *C. arietinum* genotypes (ICC4958, WR315, K850, Vihar, Vishal, PG94255, PG94091, PG94262, PG96006, PG5, PG97030, PG12, PG110 and Virat) in addition to Vijay and JG62, were collected from central India and evaluated for their reaction to Foc races 1, 2 and 3 using the controlled pot culture technique as detailed before. Genomic DNA of these lines was amplified with the markers linked to the respective disease resistance genes and association of the phenotypes with the marker genotypes was determined.

2.3 Evaluation of agronomic and yield traits

The experiment was conducted in randomized block design with two replications during winter seasons of 2003-04, 2004-05, 2005-06, 2006-2007 and 2007-2008. The two parental genotypes, Vijay and JG62, also served as checks and were sown after

every 10 rows of the RILs. For recording observations, ten competitive plants were selected randomly. Data about nine agronomic and yield traits were recorded.

2.3.1 Plant height (Pht)

Plant height was obtained by measuring height of the most tip of the plant from ground level at maturity and expressed in cm.

2.3.2 Plant spread (Psp)

Maximum horizontal spread of the plant was recorded at maturity

2.3.3 Branches per plant (Brp)

Total number of branches per plant was recorded, which included both primary and secondary branches.

2.3.4 Days to 50% flowering (Dfl_{50})

The numbers of days required from the date of sowing to the 50% of plants were flowering.

2.3.5 Days to maturity (Dmt)

Number of days required from the date of sowing to complete maturity of the population in the plot was recorded.

2.3.6 Pods per plant (Pdp)

The total number of pods from each observational plant was counted at the time of harvesting.

2.3.7 Seeds per pod (*spp*)

The seeds per pod were recorded as number of seeds present within the pod (one or two) from each observational plant.

2.3.8 Double podding (*Sfl*)

This was a qualitative trait, genotypes having twin pods or two pods from single node were scored as double podded plants and others as single podded plants.

2.3.9 Seed weight (Swt)

Seed weight is the measure of the weight of the 100 seeds expressed in grams. 100 seeds from observational plants were weighed in electronic weighing balance.

2.3.10 Yield per plant (Yld)

The weight of seeds obtained from each observational plant was recorded in gram.

2.4 Chemicals, enzymes and oligonucleotides

The *Taq*-DNA polymerase was procured from Bangalore Genei (India) and used with the 10 X buffer supplied, unless otherwise stated. Oligonucleotides were custom synthesized from Sigma Genosys (India). Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) primers were from University of British Columbia (Canada). Laboratory reagents were obtained from the following companies: Sigma Genosys (India), Qualigens (India), Bangalore Genei (India), GE Health care (USA), Promega (USA) and Cambrex Bioproducts (USA).

2.5 DNA extraction and quantification

2.5.1 Methodology

The parents (Vijay and JG62) and all recombinant inbred lines were grown in pots in controlled condition at National Chemical Laboratory, Pune. The genomic DNA was extracted by using 20 days old seedling leaves by modified Sarkosyl method (Doyle and Doyle, 1987) outlined below.

- Leaves of 3-week old plants were harvested, immediately transferred into plastic vials and stored in liquid nitrogen.

- One or two grams of leaf sample was submerged in liquid nitrogen and then ground to fine powder and quickly transferred to a tube containing 7.5ml of ice-cold extraction buffer (0.35M sorbitol, 0.1M Tris, 5mM EDTA, pH 7.5).
- The tube was briefly shaken and 7.5ml nuclei lysis buffer (2M NaCl, 0.2M Tris, 50mM EDTA, 2% CTAB, pH 7.5) was then quickly added.
- This was followed by addition of 3ml of 5% Sarkosyl solution.
- Sample sets were incubated at 65°C in the water bath for 20 minutes and allowed to cool it to room temperature.
- 18ml of chloroform/isoamyl alcohol (24:1) was added to each tube, shook well and centrifuged at 10,000 rpm for 15 minutes.
- Aqueous layer was removed and centrifuged again with 15ml chloroform/ isoamyl alcohol mixture at 10,000 rpm for 10 minutes.
- The aqueous layer was transferred to a new tube and equal volume of chilled isopropanol was added to it.
- DNA spool was removed out and washed with 70% ethanol, then centrifuged at 8,000rpm for 5 min.
- Dried DNA pellet was suspended in 500µl TE buffer (10mM Tris and 1mM EDTA, pH 8.0).
- RNAse treatment was given and kept at 37°C for 90 minutes.
- Chloroform/isoamyl alcohol (24:1) was added and kept for 5 minutes. Centrifuged the sample at 10,000 rpm for 10 minutes.
- The aqueous phase was mixed with equal volume of chilled isopropanol for precipitation. Centrifuged at 10,000 rpm for 10 minutes.
- Washed the DNA pellet with 70% ethanol and centrifuged at 10,000rpm for 10 minutes.
- Dissolved the DNA in Tris-EDTA (TE) buffer and stored at -20°C until further use.

2.5.2 DNA quantification

Extracted genomic DNA (1µl) was loaded on a 0.8% agarose gel in 0.5 X TAE (Tris-acetate EDTA) buffer, which contains ethidium bromide. The DNA concentration of the sample was estimated by visual comparison of the band with known dilutions of bacteriophage DNA (50 ng, 100 ng, 200 ng, 500 ng etc). Purity and concentration of the extracted DNA for each sample was also checked spectrophotometrically at 230nm, 260nm, 280nm and 320nm. The DNA quality was determined by calculating the ratio A260/A280 nm and it was ensured that the ratio ranged between 1.7 and 2.0. The A260/A230 ratio denoted the contamination of DNA with organic compounds, the DNA quality was best if the ratio was greater than 1.5. The absorption at 320 nm, was recorded to ensure that the DNA solution was without any turbidity.

2.6 PCR amplification using various DNA primers

2.6.1 RAPD analysis

RAPD assays were performed by using 800 random 10-mer oligonucleotide primers obtained from the University of British Columbia (UBC), Canada. Amplification reaction was carried out in 25 µl volumes containing 10 ng of genomic DNA, 1.5mM $MgCl_{2,}$ 50 mM KCl, 0.1 mM dNTPs, 5pmoles primer and 0.6U Taq DNA polymerase (Bangalore Genei Pvt. Ltd., India). All RAPD-PCR amplifications were performed in PTC-200 thermocycler (MJ Research, USA). The thermal cycling protocol as described by Winter *et al.* (2000) was followed.

Initial denaturation: 94 °C for 5 min	
5 cycles:	94 °C for 60 s
	37 °C for 45 s
	72 °C for 90s
35 cycles	94 °C for 5s
	40 °C for 20s
	72 °C for 90s
Extension	72 °C for 5 min

2.6.2 ISSR analysis

A set of one hundred ISSR primers (UBC 801-900) were used for the analysis using DNAs of both the parents. The primers, which gave clear and reproducible polymorphic patterns, were used for further analysis. A 25 µl reaction mixture consisted of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl2, 0.1mM dNTPS, 0.4 mM spermidine, 0.2 mM of primer, 1 unit of *Taq* DNA polymerase and 20 ng of genomic DNA. All ISSR-PCR amplifications were performed in PTC-200 thermocycler (MJ Research, USA). The thermal cycling protocol as described by Ratnaparkhe *et al.* (1998a) was followed.

Initial denaturation:	94 °C for 5 min
40 cycles:	94 °C for 60 s
	55 °C for 45 s
	72 °C for 2 min
Extension:	72 °C for 5 min

2.6.3 SSR analysis

The SSR analysis was carried out by using 510 chickpea SSR markers. Among these markers, 22 primers were reported by Huttel *et al*. (1999), 180 primers by Winter *et al.* (1999), 95 primers by Sethy *et al.* (2003, 2006a, b), 200 primers by Lichtenzveig *et al.* (2005) and 13 primers by Choudhary *et al*. (2006)). Fifteen *Medicago truncatula* SSRs (Eujayl *et al.*, 2004) were also used to check cross-species utility of the primers. The polymerase chain reaction (PCR) with chickpea-STMS primer pairs was performed as described by Huttel *et al.* (1999) and Winter *et al.* (1999), with some modifications. The PCR was carried out with a PTC-200 thermocycler (MJ Research, Inc., USA) in 15 µl reaction volume. In order to increase the screening efficiency of markers, microsatellites with compatible annealing temperatures of primer pairs and no overlapping size of amplification products were multiplexed in the PCR. The total reaction volume, primer concentration and amount of DNA sample were optimized for each microsatellite combination as recommended by Winter *et al.* (2000).

Initial denaturation:	94 °C for 5 min
40 cycles:	94 °C for 60 s
	55 °C for 45 s
(Annealing temperature depending on T_m of primers)	
	72 °C for 2 min
Extension:	72 °C for 5 min

2.7 Resolution of PCR products using various methods

2.7.1 Agarose gel electrophoresis

The amplified products were resolved on 2% agarose gels in 0.5 X TAE buffer, visualized and further gel documented with Image Master VDS gel documentation system (Amersham Pharmacia Biotech, Denver, USA) under UV light. Agarose was dissolved in 0.5X TAE buffer by slow, circular motion and the mixture was boiled in microwave oven for 3 min. Proper care was taken to avoid over boiling/ frothing of agarose. The agarose solution was cooled to 40-50°C and poured on gel casting trays fitted with 24 well combs. About 4 µl of Bromophenol blue loading dye was added to 25 µl amplified PCR product and was loaded on the gel. The gel electrophoresis was carried at 100V, 50 mA for 45 min to 90 min and stained with 200 ml ethidium bromide staining solution with 5 µl of ethidium bromide stock (10 mg/ ml) for 10-15 min, with slow circular motion. Following staining, the gels were destained with plain ultrapure water gently for 5 min. The stained gels were visualized on gel documentation system (Amersham Pharmacia Biotech, USA) and digital images were stored in tiff format. All SSR- amplified products were resolved initially on 3% metaphor-agarose gels in 0.5 X TBE buffer, visualized and gel documented.

2.7.2 Polyacrylamide gel electrophoresis

The SSR primer products unable to resolve on Metaphor gels were resolved on 0.4 mm polyacrylamide using the sequencing gel unit from Life Technologies, USA. The Bind plate-larger glass plate (33.3 x 41.9 cm) was treated with 4 ml of methacryloxypropyl-trimethoxysilane (Plus one Bind- Silane, Amersham Pharmacia Biotech), in 1 ml of acidic ethanol (0.5% glacial acetic acid in 95% ethanol) to

covalently attach the gel onto the glass plate. The plates were dried for 5 min and the excess silane was removed using a paper tissue moistened with 95% ethanol. The Repel plate- smaller glass plate (33.3 x 39.4 cm) was treated with 1 ml of a 2% solution of dimethyldichlorosilane in octamethyl cyclo-octasilane (Plus one Repel-Silane ES, Amersham Pharmacia Biotech) for complete release of gel from this plate. The plates were dried for 5 min and excess silane was removed with a tissue paper moistened in distilled water. The gel solution was prepared by mixing 50 ml of the urea: acrylamide solution in TBE with 200 µl of freshly prepared 10% ammonium per sulfate and 50 µL of TEMED (Tetramethylethylenediamine). The gel solution was poured into the assembled gel plates (0.4 mm thickness) using a pointed beaker. The gel was allowed to polymerize for 60 min.

2.7.2.1 Prerun

The sequencing gel was run at 60 W (42 mA; 1500 V) for 60 min or until the gel temperature reaches 55 °C in 1 X TBE (Tris-Borate EDTA) buffer. The samples were denatured for 5 min at 94°C in the thermocycler and immediately placed on ice. About 8 µl was loaded to the gel as quickly as possible and the electrophoresis was performed at 60 W for 80/100 min at 50-55 $^{\circ}C$.

2.7.2.2 Silver staining

The gel bound to binding plates was removed from repel plate and fixed with fixer solution (200 ml of crude alcohol, 10 ml of acetic acid in 1790 ml of double distilled water) for 20 min in gentle circular motion. The gel plate was drained and silver stained with staining solution (4 gm of silver nitrate in 2 liter of double distilled water) for 30 min. After staining, the plate was drained free of staining solution and developed using developer solution (30gm of NaOH, 5 ml formaldehyde in 2 liter of double distilled water) for 5-10 min. After the appearance of sharp and dark bands, the gel was treated with stop solution (10 ml of acetic acid in 1790 ml of doubled distilled water) to end the staining process. The gel was completely washed with ultrapure water and further dried for gel documentation.

2.8 Construction of framework map

2.8.1 Scoring of marker data

The genotype of each sample in case of RAPD and ISSR analysis was scored as presence or absence of amplified DNA locus. In case of SSR primers, scoring was done based on the size variation of the alleles in the parents. The RILs with a genotype of Vijay were given score 'a' and that of JG62 were given 'b'.

2.8.2 Linkage group construction

The χ^2 test was used to assess goodness-of-fit to the expected 1:1 segregation ratio for each marker. All markers including those with distorted segregation were used for linkage analysis performed using JoinMap Ver. 3.0 (van Ooijen and Voorrips 2001). This software calculates the multipoint map distance estimates for a recombinant-inbred population and converts to estimates of gametic recombination *R* by inverse application of the mapping function (Kosambi 1944). These estimates are transformed via the function $r = 2R/ (1 + 2R)$ (Haldane and Waddington, 1931) to estimate expected recombination 'r' after selfing to homozygosity.

The markers were classified into linkage groups (LGs) using the minimum LOD threshold of 3.0 and maximum recombination fraction of 0.4 for the JoinMap program. If the recombination value of any two loci were more than 0.4, then they were declared as separate linkage groups. The choice of a stringent LOD threshold of 3.0 for ordering of markers suggests comparison with other genome maps (Nelson *et al*., 2006). Comparison of the present map with the interspecific map developed by Winter *et al.* (2000) was performed using MapChart Ver. 2.2 (Voorrips 2002). In the comparison, the LGs of the present map were designated with Arabic numerals; whereas the LGs of the map of Winter *et al.* (2000) were designated with Roman numerals. The genetic map was finally drawn using the computer software Mapchart Ver. 2.1 (Voorips, 2002).

2.8.3 Statistical analysis for wilt resistance

Disease reaction of each RIL to different races of the pathogen and each marker data were analyzed by chi-square to determine goodness of fit to the expected segregation

ratio of one resistant: one susceptible. Data generated by different markers were recorded in a binary fashion. Linkage between the markers and resistance genes was established using JoinMap ver. 3.0 (van Ooijen and Voorrips, 2001). The map was constructed at LOD 3.0 with Kosambi (1944) mapping function.

2.9 Statistical analysis and QTL mapping

2.9.1 The analysis of variance (ANOVA)

The phenotypic data analysis was performed using IRRISTAT for Windows Ver. 5.0 (IRRI, 2005) using 'Cross site analysis module' (Fig 2.1). The analysis of variance (ANOVA) revealed significant differences among the parental genotypes for all the traits evaluated. The GxE interaction (GEI) of RILs with the environments was deciphered by using AMMI (Additive Main effects and Multiplicative Interaction) model with IRRISTAT (IRRI, 2005) software through "Cross site analysis module". Five year's data at two sites were treated as five environments in the analysis. The sum of squares was first partitioned into genotype, environment, and GEI, then, the sum of squares for GEI term was further partitioned by principal components analysis using the AMMI model (Crossa *et al.*,1990; Gauch, 1992) using the formula

$$Y_{ij} = \mu + g_i + e_j + {}^{h}\sum_{n=1} \lambda_n \alpha_{ni} \gamma_{nj} + R_{ij}$$

where Y_{ij} is the value of the i^{th} genotype in the j^{th} environment, μ is the grand mean, g_i is the mean of the i^{th} genotype minus grand mean, e_j is the mean of the j^{th} environment minus the grand mean, λ_n is the singular value for the principal component analysis axis n, α_{ni} and γ_{nj} are the principal component scores for principal component analysis axis n of the i^{th} genotype and j^{th} environment, respectively and R_{ij} is the residual. Broad sense heritability (H^2) was estimated as: genotypic variance/phenotypic variance (σ_g^2/σ_p^2 X 100). Correlation coefficients among the seven traits were calculated by using Qgene (Nelson, 1998).

2.9.2 QTL mapping

The QTLs were identified by single locus QTL analysis through CIM using Windows QTL Cartographer Ver. 2.5 (Basten *et al*., 1994; Wang *et al*., 2004) (Fig 2.2). For each trait the analysis was carried out using data from individual environment. The

threshold LOD scores for detection of QTLs were calculated based on 1000 permutations (Doerge and Churchill, 1996). The Model 6 of the CIM was used with forward regression and backward elimination module of QTL Cartographer for scanning intervals of 2 cM between the markers and putative QTLs with a window size of 10 cM. Five markers were used as the background control for forward-backward stepwise regression. The position, genetic effects and percentage of phenotypic variation of the QTLs were estimated at the significant LOD peak in the region under consideration. But the flanking markers with LOD value above threshold were also considered and used for indicating the base of QTL peaks. Multiple trait analysis involving MCIM was conducted using the module JZmap QTL available in Windows QTL Cartographer with an objective to detect the pleiotropic QTLs. The confidence intervals were obtained by marking positions ± 1 LOD from the peaks. QTLs in the adjacent intervals and / or with overlapping confidence intervals were treated as a single QTL. Two locus analysis was conducted using QTLNetwork Ver. 2.0 (http://ibi.zju.edu.cn/software/qtlnetwork), where P=0.05 was used as the threshold for detecting putative M-QTLs or E-QTLs. QTL Network analysis reveals the graphic presentation of the genetic architecture with QTL and epistasis (Fig 2.3).

The QTLs identified using CIM for various traits were grouped; linkage group wise and QTL plots were drawn using QTL Mapchart (Voorrips 2002). The QTLs were designated as *QX.ncl-Y*, where X denotes the phenotypic trait abbreviation and Y represents the chromosome on which the quantitative trait locus was located. Each QTL was defined on one line, along the linkage group map with the extent of QTL intervals and peak of the QTL. Different styles and colors of lines were used to represent various QTLs of different traits.

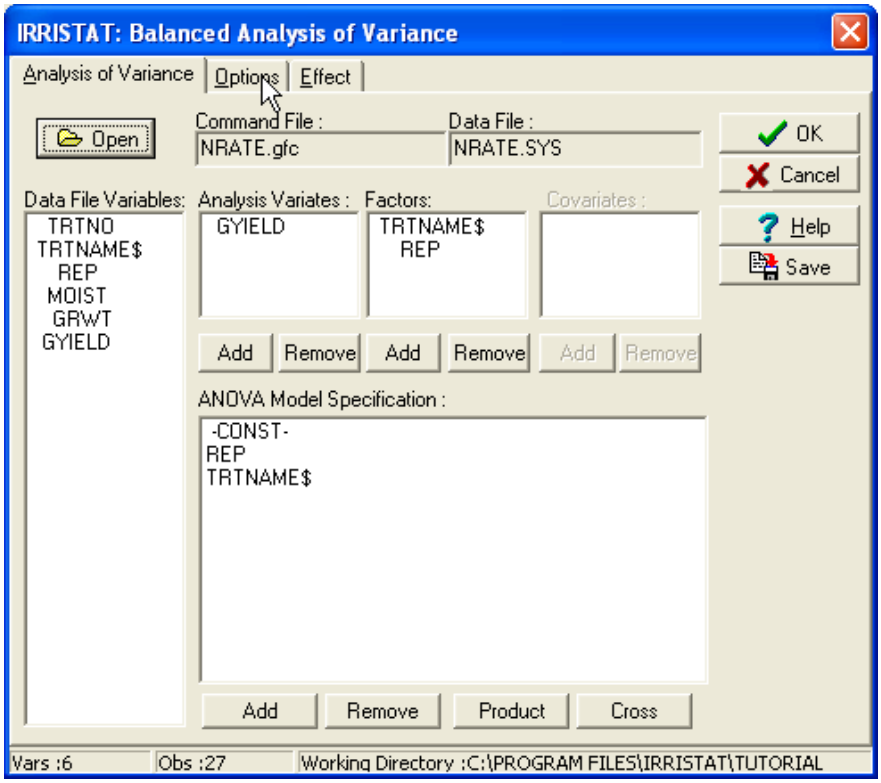


Fig 2.1: Analysis of variance by IRRISTAT software ver. 5.0

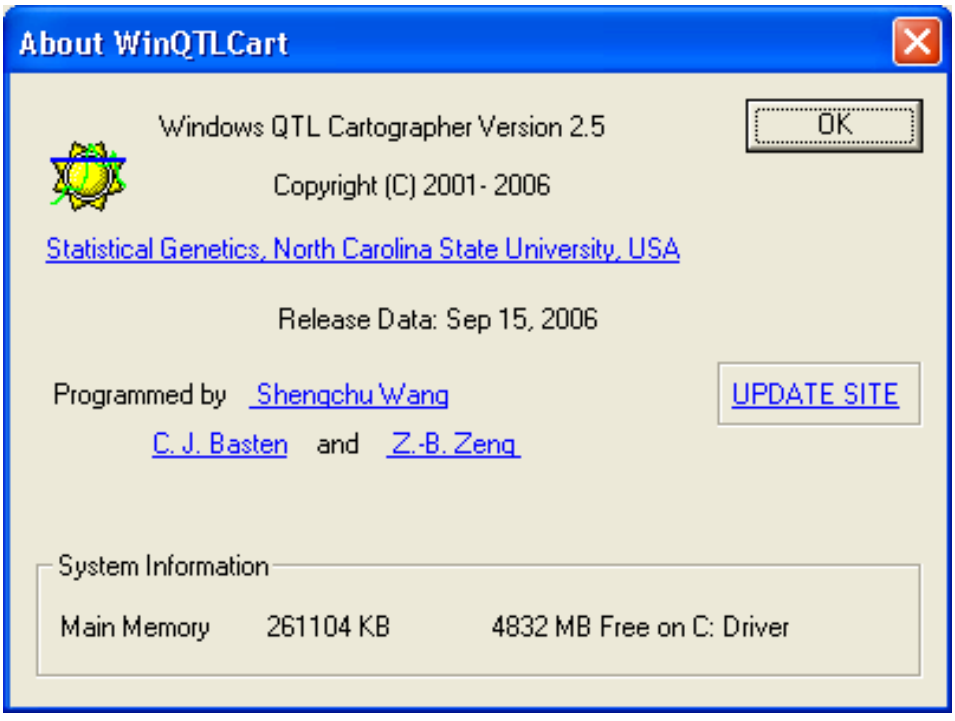


Fig 2.2: Windows QTL cartographer for CIM QTL

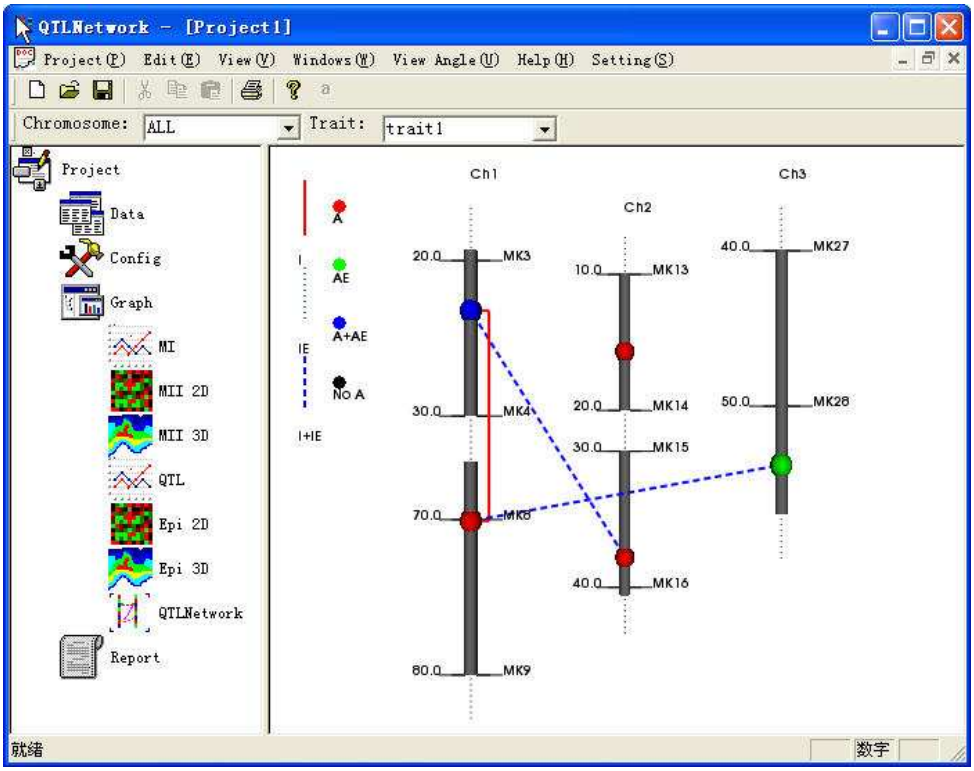
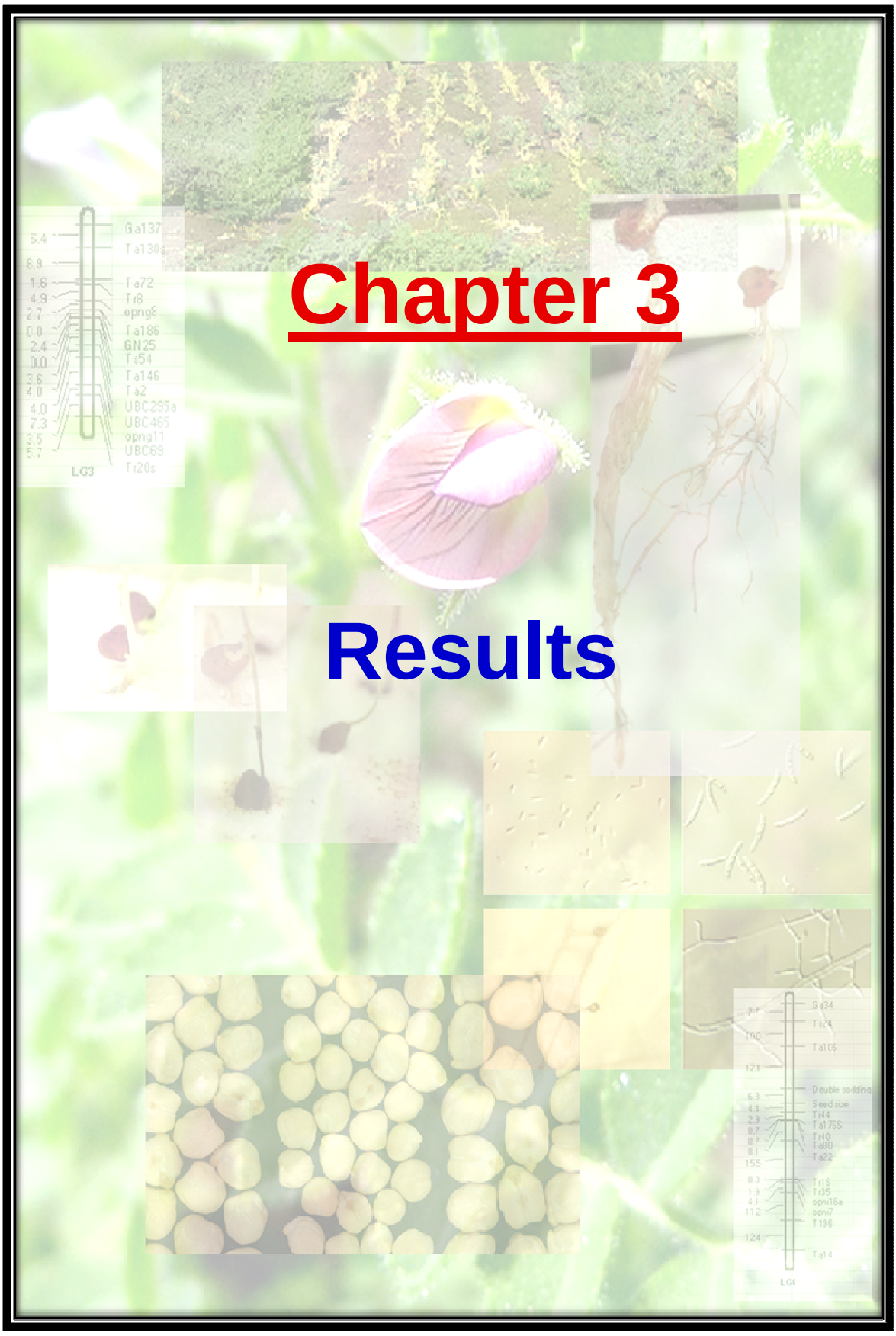


Fig 2.3: Graphic presentation of the genetic architecture with QTL and epistasis by using QTL Network.



Chapter 3

Results

The Recombinant Inbred Line (RIL) population of JG62 x Vijay, comprising ninety-three lines was grown under five environments (three consecutive years; 2003-05 at Rahuri and two years 2006-07 at Dharwad), in randomized block design with two replications. The parental survey and linkage groups were constructed as per the protocol discussed in the previous chapter of Materials and Methods. The QTL analysis was performed for yield and yield related traits and the results are given below.

3.1 Construction of framework map

3.1.1 Parental analysis

A total of 1520 PCR based markers comprising 800 RAPD, 100 ISSR primers, 504 SSRs, 100 chickpea EST-SSRs, 15 Medicago SSRs and one allele specific associated primer (ASAP) were used for parental screening. The details of the primers used for population screening are presented in Table 3.1. A representative gel picture for the chickpea SSR markers is presented in Fig 3.1. The marker data as defined in the previous chapter were converted into allele scores and analysed by using JoinMap ver. 3.0 (Van Ooijen and Voorrips, 2001).

3.1.2 Construction of linkage map

Out of 1,520 primers screened between the parents of JV (JG62 × Vijay) population, only 171 (11.30%) primers revealed clear and consistent polymorphism generating 175 reproducible and segregating markers for linkage analysis. Fifteen Medicago SSR primers were used as an alternative resource to increase the marker density of chickpea intra-specific map. Though these primers gave amplification, they were monomorphic with the parents. Similarly all the 100 EST-SSR primers also gave monomorphic banding pattern with the parents. Among the other primers used; RAPDs and ISSRs were least polymorphic and varying in their reproducibility (Table 3.1).

The linkage analysis revealed eight linkage groups with 135 markers (120 SSRs, 9 RAPDs, 1 ASAP, three fusarium wilt resistance genes (*foc1*, *foc2* and *foc3*) and two yield related qualitative traits (double podding (*Sfl*) and seeds per pod (*spp*)) (Fig 3.2).

Table 3.1 Primers used for parental analysis

Primers	**Source**	**No. of primers**	**Polymorphism**	**% of Polymorphism**
RAPD	*UBC	800	10	1.25
ISSR	*UBC	100	3	3.00
Chickpea SSRs				
	Huttel *et al.*, 1999	22	6	27.28
	Winter *et al.*, 1999	174	72	41.38
	Sethy *et al.*, 2003, 2006a, 2006b	95	26	27.37
	Lichtenzveig *et al.*, 2005	200	50	25.00
	Choudhury *et al.*, 2006	13	3	23.10
Chickpea EST-SSRs	Jayashree *et al.*, 2005	100	0	0.00
ASAP	Mayer *et al*., 1997	1	1	100
Medicago SSRs	Eujayl *et al.*, 2004	15	0	0.00
Total		**1520**	**171**	**11.30**

* UBC = University of British Columbia, Canada

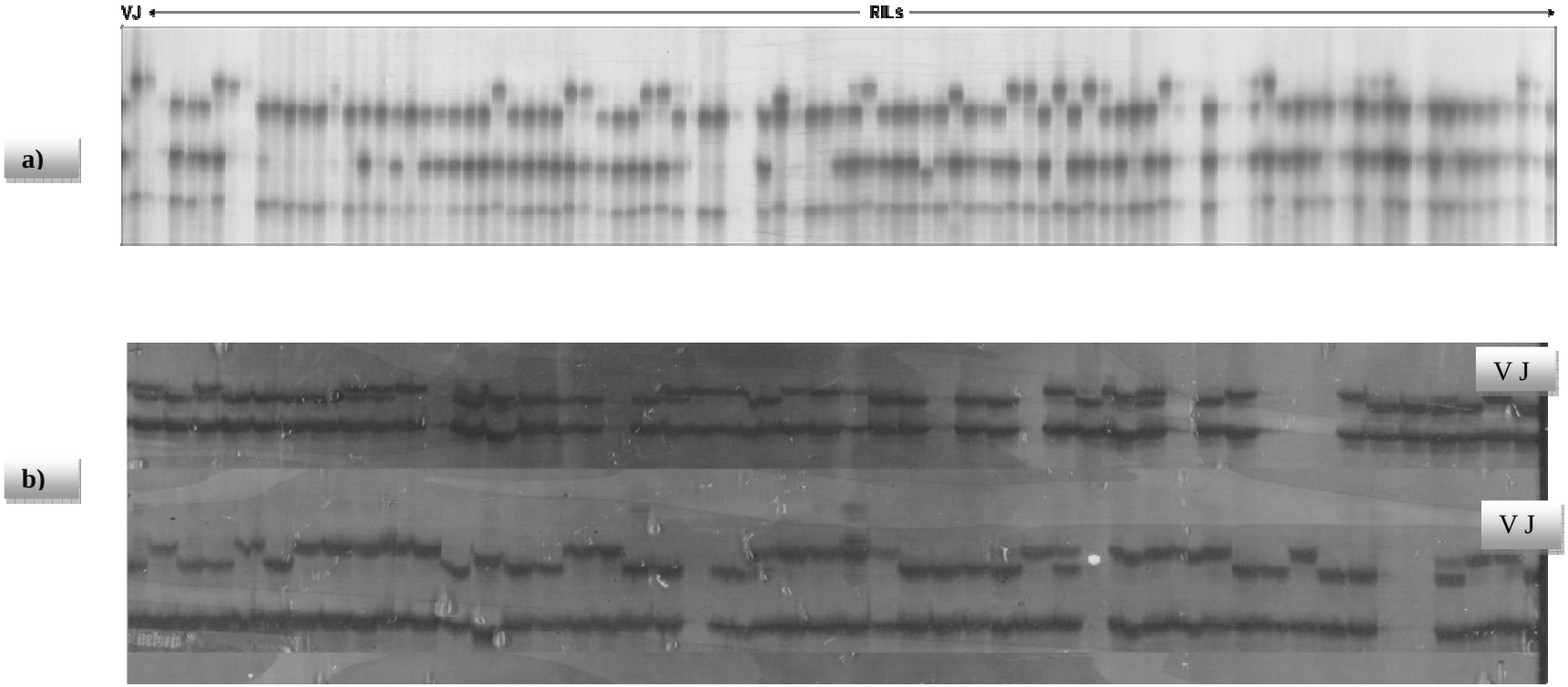


Fig 3.1: Representative segregation pattern of chickpea SSR [a) TA96, b) H1B09] profiles for F_9RILs.

This map covered 568.6 cM with an average marker density of 4.21 cM. Forty markers comprising one RAPD, three ISSRs and thirty six chickpea SSRs were unlinked. The main features of the intra-specific map were presented in Table 3.2 and 3.3.

LG-2 was the longest linkage group with 21 markers and spanned 102.1 cM with an average marker density of 4.85 cM (Fig 3.2). LG-1 was the densest linkage group with a marker density of 2.14 cM and had 37 markers spanning 77.3 cM. This group corresponded to LGs III and LG V of the interspecific map of Winter *et al.* (2000). The LG-2 corresponded mainly to LG-I and LG IV of previously published reference map. The LG-3 had 28 markers spanning 89.5 cM and shared many markers from LG-II of interspecific map developed by Winter *et al.* (2000). LG-4 had seven markers distributed with average marker interval of 8cM. LG-5 spanned 58.9 cM having 25 markers with marker density of 4.21cM and corresponded to LG-VII of the interspecific map. The *Sfl* gene was mapped on LG6 and was flanked by two new STMS markers TA80s and TA106s. This group is corresponds to LG VI of Winter *et al.* (2000). The LG7 and LG8 comprised all the newly developed STMS markers. In LG 8 RAPDs were more in number than STMS markers. These LGs lacked common markers and could not be compared with the LGs of Winter *et al.* (2000) map. Inversions were observed with respect to marker orders in all the linkage groups between the present and the interspecific map of Winter *et al.* (2000).

The correlation between number of markers on each LG and length of the respective LG gave an indication of distribution of markers over the linkage groups. This correlation coefficient was 0.58 ($P < 0.001$) for the intra-specific map, which indicates less random distribution of markers among the LGs. Of the 135 markers mapped in this population, 40 markers did not segregate according to the expected Mendelian ratio ($P < 0.001$). All the marker types used in the present study exhibited different levels of skewness; however, SSRs were the most distorted markers.

Table 3.2: The main features of chickpea intra-specific map

Linkage groups	**8**
Total No. of Markers	**175**
Linked / Mapped Markers	**135**
Distorted Markers	**40**
Total Length	**568.6 cM**
Av. Marker Density	**4.21 cM**

Table 3.3: The main features of individual linkage groups of chickpea intra-specific map

Linkage group	Total Markers	Total length (cM)	Marker density (cM)
1	37	77.3	2.14
2	21	102.1	4.85
3	28	89.5	3.20
4	7	56.1	8.00
5	14	58.9	4.21
6	17	89.6	5.27
7	5	36.7	7.34
8	6	58.4	9.73
Total	**135**	**568.6**	**4.21**

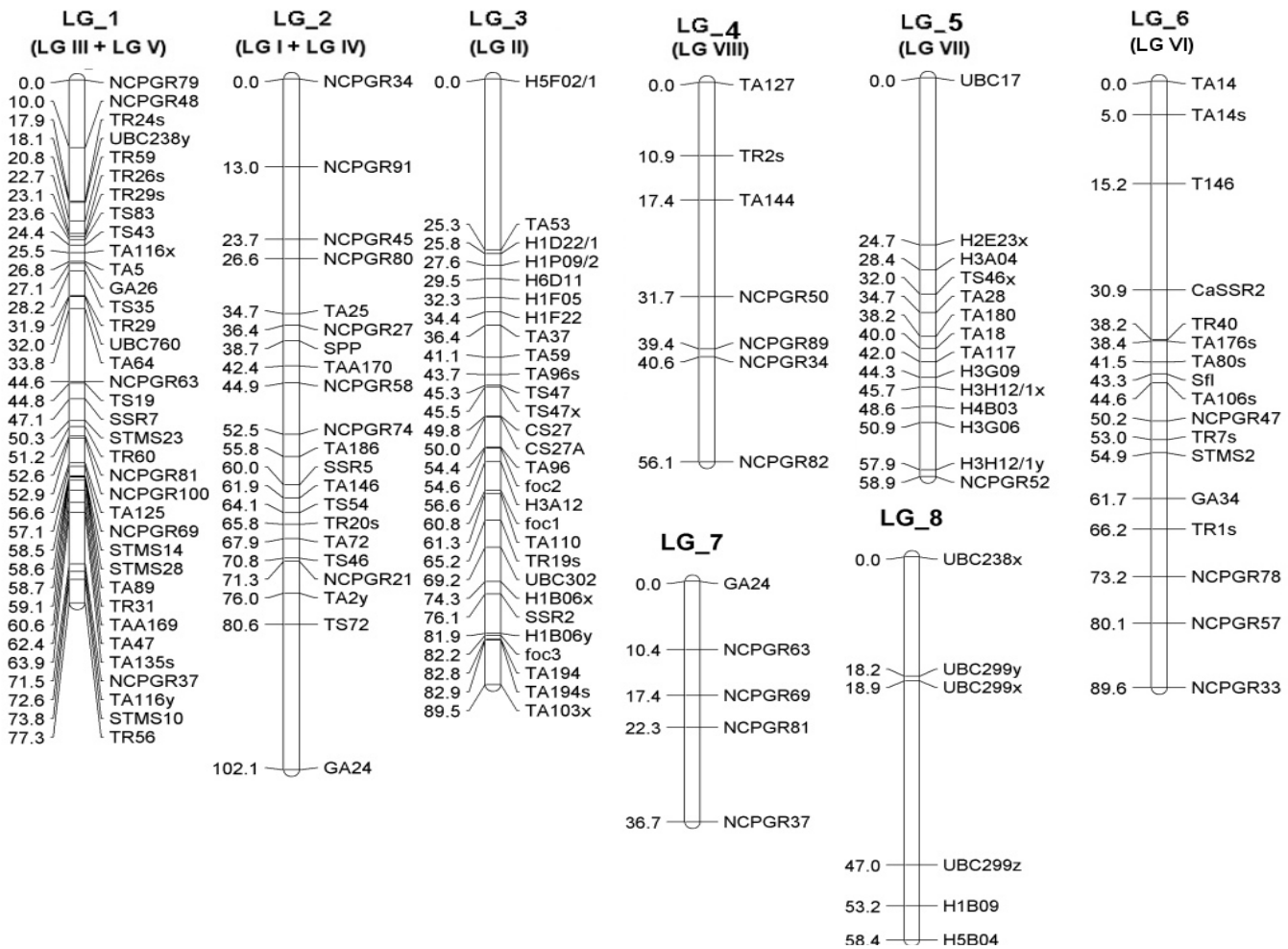


Fig 3.2: Chickpea intra-specific map. Corresponding LGs of Winter *et al*. (2000) reference map have been indicated in *Roman numerals* in parentheses

3.2 Fusarium wilt

3.2.1 Genetics of wilt resistance in chickpea

In hydroponic culture plants inoculated with FOC1 were observed for disease symptoms at different time intervals. The JG-62 (Susceptible) seedlings which were inoculated with Foc cultures started developing a distinct yellow coloration at 10 days after inoculation as compared to the uninfected healthy seedlings. At 25-30 days after infection, the JG-62 (S) plants showed complete wilting while the Vijay (Resistance) plants along with uninfected JG-62 (S) showed normal healthy growth (Fig 3.3). It was observed that the total root length was similar in susceptible and resistant cultivars in the uninoculated controls when observed after 20 days, which became markedly smaller and weaker in susceptible cultivar, after inoculation with FOC at the same time. However, in the resistant cultivar inoculation with FOC increased lateral root branching, which were longer and more in number. Such long lateral root branches were not observed in the susceptible inoculated plants, in which the whole root system appeared dark brown and dead (Fig 3.3).

Reactions of the chickpea lines for Foc races 1, 2 and 3 were assessed following the independent inoculations with respective isolates of Foc in pot culture experiments. Disease screening allowed unambiguous classification of resistant and susceptible phenotypes. Among the 100 RILs, 55 RILs were resistant and 45 were susceptible to Foc1, whereas for Foc2, 49 were resistant and 51 were susceptible (Table 3.4). The RILs also segregated in 1:1 ratio for resistance and susceptibility to Foc3, indicating that resistance to each race was monogenic in this population. The susceptible parent, JG62, completely wilted in 25 days after sowing, whereas Vijay was resistant and did not develop any wilting symptoms till maturity for all the three races. The susceptible RILs took 25-32 days for complete wilting. The chi-square analysis of disease reaction data of the RILs indicated a good fit to the 1:1 segregation ratio expected for single genes conferring resistance to each of the three Foc races. These chickpea genes were earlier designated as *foc1*, *foc2* and *foc3* for resistance to Foc races 1, 2 and 3, respectively (Tekeoglu *et al.*, 2000).

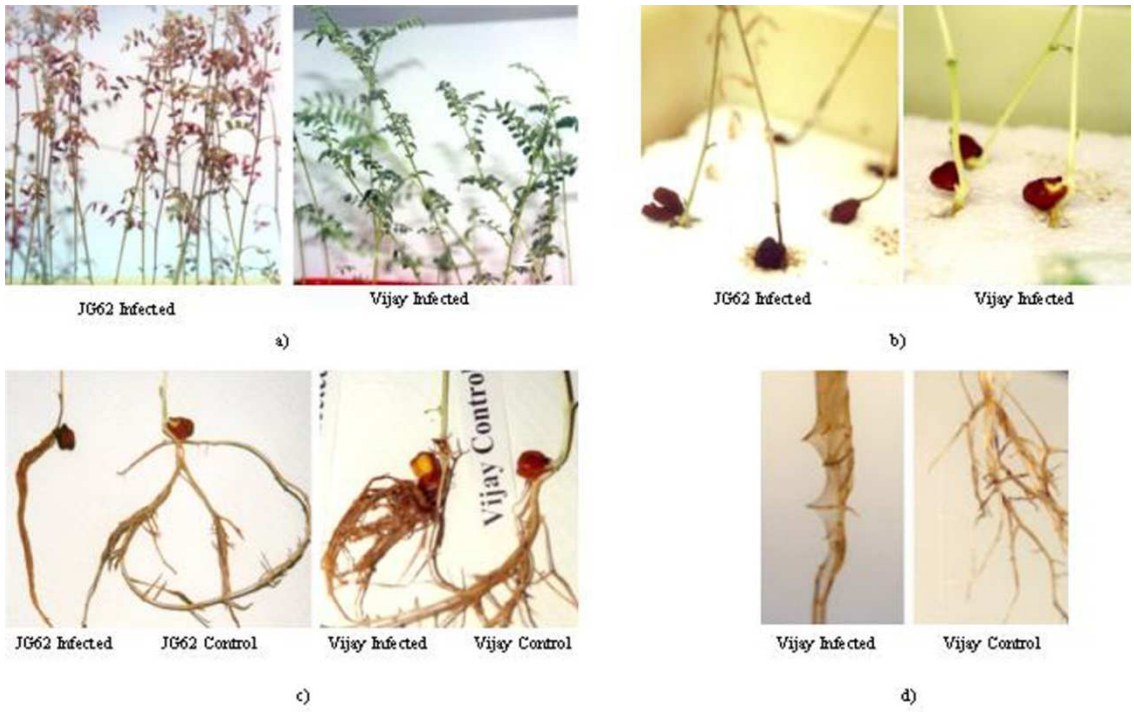


Fig 3.3: a) Chickpea seedlings hydroponically growing in growth chamber; b) JG-62 seedling showing wilting symptoms after infection with FOC1 while Vijay seedlings are healthy after infection; c) Root morphology of JG-62 and Vijay after infection; d) Difference between infected roots of Vijay covered with fungal mycelial mass and non-infected roots without any fungal mycelia.

3.2.2 Genetic mapping of Foc resistance genes

After parental screening, 175 polymorphic markers were selected for screening the full population. In linkage analysis, 19 markers showed association with wilt resistance genes. All these markers were located on LG II of the reference map of chickpea (Winter *et al.*, 2000), as determined using the STMS markers. In this study, new STMS markers closely linked to the resistance genes for Foc races 1, 2 and 3 were identified. The locus order and genetic distances among the genes *foc1*, *foc2*, *foc3* and linked markers are illustrated in Fig 3.4. Two STMS markers, TA110 and H3A12 flanked *foc1* at 2.1 and 3.9 cM, respectively. Race 2 resistance gene (*foc2)* was tagged with two new STMS markers, TA96 and H3A12 at a distance of 0.2 cM and 2.7 cM, respectively, whereas; *foc3* gene was flanked with TA194 and H1B06y at 0.7 and 0.2 cM, respectively.

3.2.3 Validation of the markers

The genomic DNA of sixteen varieties was extracted. The markers TA110, TA96, H1B06y and TA194 (Table 3.5) which were tightly linked with *foc1*, *foc2* and *foc3* were validated with these varieties. Thirteen genotypes were resistant to Foc1 and all of these amplified the allele associated with resistance for the marker TA110 (Fig 3.5). Of the three race1 susceptible varieties, JG62 amplified the TA110 allele associated with susceptibility; K850 and PG5 amplified the allele associated with resistance. Similarly for TA96, ten of the thirteen Foc2 resistant genotypes amplified the allele associated with resistance. For two Foc2 susceptible genotypes, JG62 and PG5, the TA96 allele associated with susceptibility was amplified. However, for K850 the resistant allele of TA96 was amplified. On the contrary, Vishal and PG12, which appeared Foc2 resistant, amplified the susceptibility allele associated with TA96. For Foc3 Ta194 marker amplified resistance type allele in fourteen genotypes and susceptible alleles in two genotypes. H1B06y showed resistance alleles in eleven genotypes with susceptible allele in five genotypes (Table 3.5).

Table 3.4: Disease reaction of RILs to races 1, 2 and 3 of *Fusarium oxysporum* f.sp. *ciceri* (Foc) and frequencies of marker genotypes

Gene/ Marker	Resistant^	Susceptible	χ2 (1:1)	Marker/ Marker	Resistant	Susceptible	χ2 (1:1)
foc1	55	45	1.00	H1B06y	57	43	2.00
foc2	49	51	0.02	H1F05	56	36	4.30*
foc3	53	47	0.40	H1F22	56	37	3.85*
TA103x	43	57	2.00	H1P09/2	48	43	0.36
TA110	52	48	0.20	H6D11	54	38	2.77
H3A12	56	44	1.40	TS47	53	40	1.81
TA59	53	40	1.81	UBC302	45	48	0.11
TA96	57	43	2.00	TA37	50	43	0.53
TA96s	49	44	0.28	TA144	53	32	5.55*
TR19s	50	41	0.96	CS27A	44	49	0.28
H1B06x	52	48	0.20	TA194	59	41	3.20*

[a] Resistant and susceptible refer to the reaction of the RILs to Foc races 1, 2 or 3

* Significant at P = 0.05

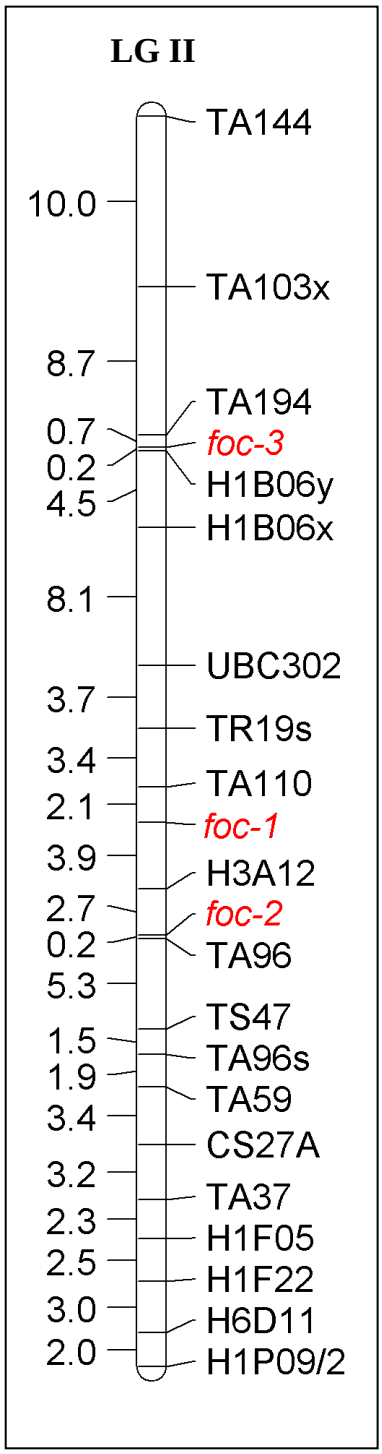


Fig 3.4: Mapping of *foc1*, *foc2* and *foc3* genes conferring resistance to races 1, 2 and 3 of *Fusarium oxysporum* f.sp. *ciceri*. Marker and gene names are shown on the right and estimated map distances between them are shown in the left.

Table 3.5: Disease reaction of different chickpea genotypes to Foc races 1, 2 and 3 and marker genotypes[a]

Sr No.	Genotypes	Foc1	Ta110	Foc2	Ta96	Foc3	H1B06y	Ta194
1	Vijay	R	R	R	R	R	R	R
2	JG62	S	S	S	S	S	S	S
3	ICC4958	R	R	R	R	R	R	R
4	WR315	R	R	R	R	R	R	R
5	K850	S	R	S	R	S	S	R
6	Vihar	R	R	R	R	R	R	R
7	Vishal	R	R	R	S	R	R	R
8	PG94255	R	R	R	R	R	R	S
9	PG94091	R	R	R	R	R	R	R
10	PG94262	R	R	R	R	R	R	R
11	PG96006	R	R	R	-	R	R	R
12	PG5	S	R	S	S	S	R	R
13	PG97030	R	R	R	R	R	S	R
14	PG12	R	R	R	S	R	S	R
15	PG110	R	R	R	R	R	R	R
16	Virat	R	R	R	R	R	S	R

[a] For Foc1 and Foc3: R – Resistant, S – Susceptible; For markers: R – presence of resistance associated allele, S – presence of susceptibility associated allele, - not amplified

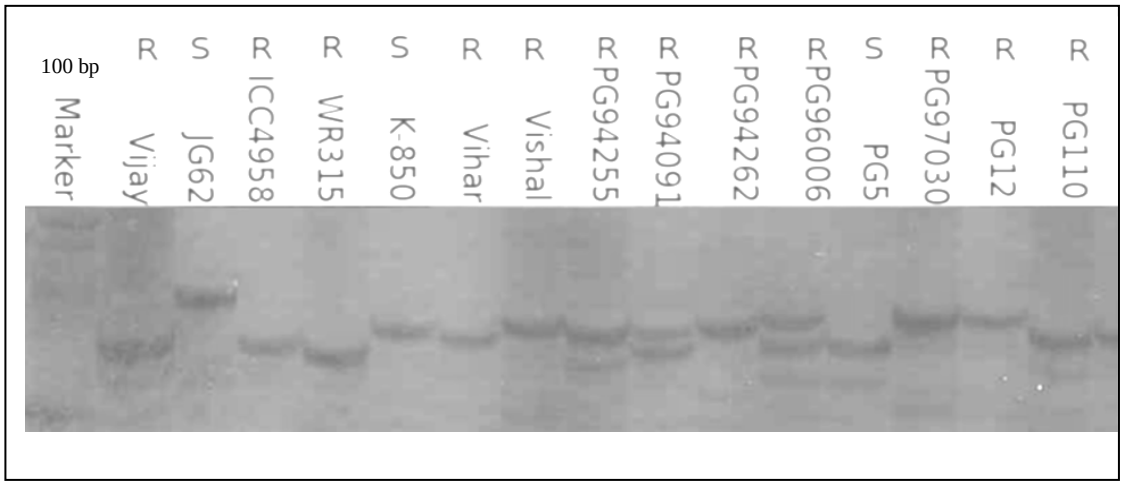


Fig 3.5: Amplification of genomic DNA from different chickpea varieties with STMS primer TA110. Names of the lines are at the top. The phenotypic reactions of chickpea lines to Foc1 is given on the top (R: Resistant, S: Susceptible)

Interestingly, the same thirteen genotypes, which were resistant to Foc1and Foc2, also exhibited resistance to Foc3. Ten of these thirteen genotypes amplified the allele associated with resistance for the marker H3B06y. In contrast, three Foc3 resistant genotypes amplified the H3B06y allele associated with susceptibility (along with two Foc3 susceptible genotypes; JG62 and K850). PG5, which appeared susceptible to Foc3, amplified the H3B06y allele associated with resistance. In case of TA194, twelve Foc3 resistant genotypes amplified the resistance allele. While on one hand, a Foc3 resistant genotype, PG94255, amplified the susceptibility associated TA194 allele; PG5, which was Foc3 susceptible, amplified the resistance associated TA194 allele. Overall, TA110 correctly identified 14 of the 16 genotypes as either resistant or susceptible to Foc1 race while TA96 correctly identified 12 of the 16 genotypes as either resistant or susceptible to Foc2. H1B06y identified 12 out of 16 while TA194 identified 13 out of 16 genotypes as either resistant or susceptible for Foc3.

3.3 Analysis of qualitative yield traits

3.3.1 Double podding (*Sfl*)

Parent JG62 is a double podding variety in which a single node will give rise to two pods as shown in Fig 3.6. Sixteen markers showed association with double podding gene and mapped on LG6. Two SSR markers TA80s and TA106s showed close segregation with *sfl* in the F_9 population and flanked the double podding (*Sfl*) gene at 3.1 and 1.2 cM, respectively (Fig 3.6).

3.3.2 Seeds per pod (*spp*)

Many chickpea varieties have only one seed per pod while a few varieties have two seeds per pod. This two seeds per pod trait is genetically inherited and controlled by single recessive gene. Parent Vijay showed the two seeds per pod character while JG62 had single seed per pod. Data were collected from each RIL in the field and analysed for their cosegregation with the mapped molecular markers. *spp* gene was tagged with two flanking STMS markers NCPGR27 and TA170 at 2.3 and 3.7cM, respectively on LG2 (Fig 3.7).

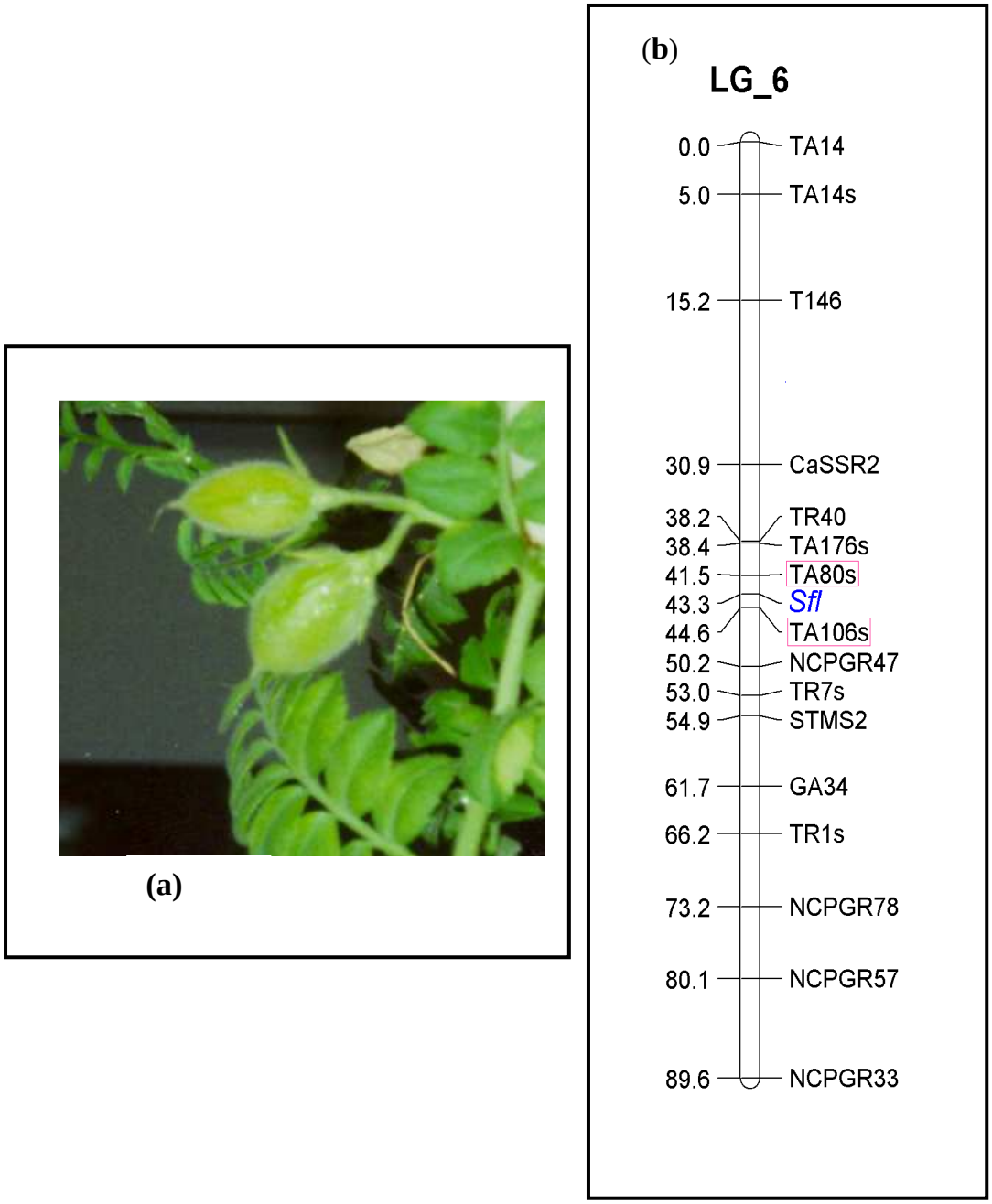


Fig 3.6: (a) Double pods in chickpea and (b) Mapping of double podding gene (*Sfl*).

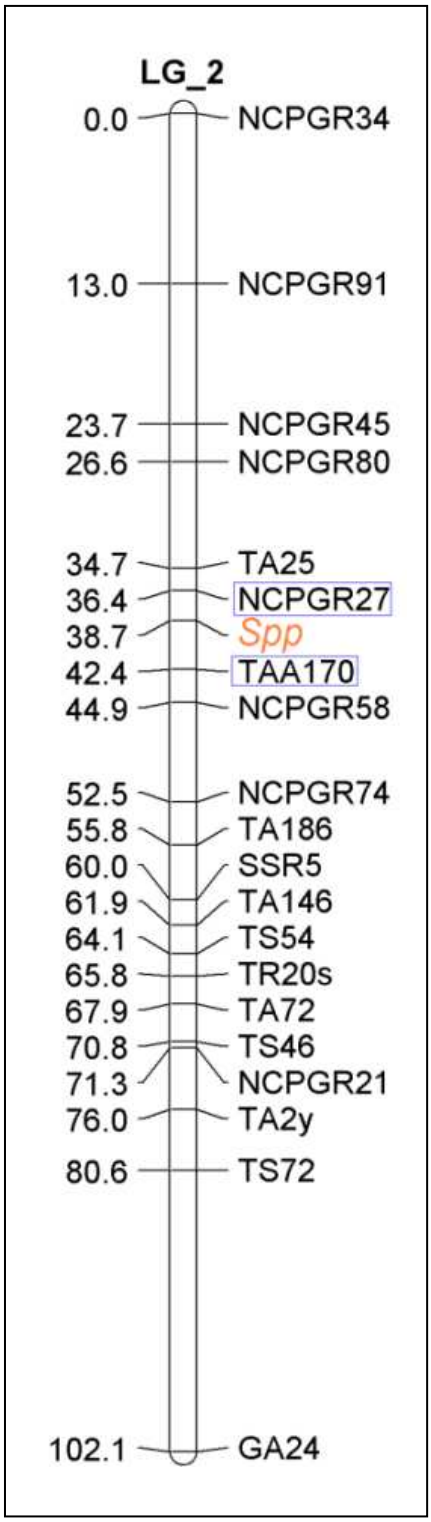


Fig 3.7: Mapping of number of seeds per pod (*SPP*) on LG 2 (LG III of Winter *et al*., 2000).

3.4 Analysis of quantitative yield traits

3.4.1 Phenotypic characteristics of F_9 RIL population

The parents JG62, Vijay and their RIL progeny were analysed for various quantitative traits contributing to yield and the yield related traits at two different locations (Rahuri and Dharwad) as detailed in the previous chapter of Materials and Methods. Both the parents showed a statistically significant difference for all the traits across different environments. Table 3.6 depicts the range of eight traits, namely Plant height (Pht), Plant spread (Psp), Branches per plant (Brp), Pods per plant (Pdp), Days to 50% flowering (Dfl_{50}), Days to maturity (Dmt), Yield per plant (Yld), and 100 Seed weight (Swt) in the parents and the RILs. The phenotypic distribution for each trait in the population is shown graphically in Fig 3.8 to 3.11.

The parents JG62 and Vijay showed a highly significant difference between them for Psp (24.7 to 33.8) and Pdp (26 to 52), compared to other traits like Pht, Brp, Yld, Swt, Dfl and Dmt (Table 3.6). Although difference between the parents were smaller in other traits, they were significant in the RIL population (Table 3.6). For Pht and Brp, the difference between the parents was less while range in population was found to be wide. All the traits showed good fit to normal distribution (Fig 3.5 to 3.8). Transgressive segregants were observed in all the environments and comparisons between the best parent and the best RIL showed a significant difference for all the traits. Continuous phenotypic variation and transgressive segregation for all the traits observed in the RIL population revealed the quantitative inheritance of these traits.

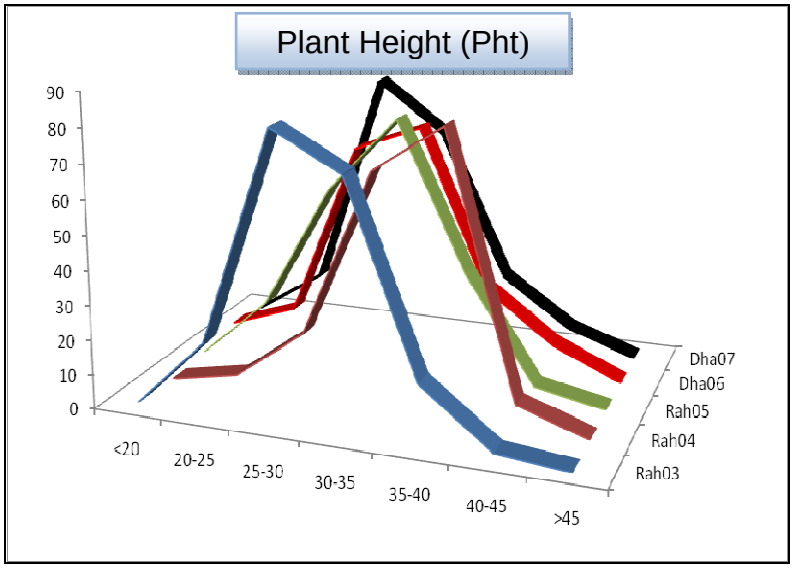
3.4.2 Correlation among yield and yield related traits

Simple correlation coefficients for three years, among the traits were calculated using Qgene ver. 2 (Nelson, 1998) software and are presented in Table 3.7. (Since only two environment data were available for Days to 50% flowering, it was not included in the correlation studies.) In 2003 Pht showed positive and significant correlation with only Yld and Swt, but in 2004 it was significantly correlated with all the traits. Yld was significantly correlated with Pht, Psp, Brp, Pdp and Swt in all the three years. Swt was significantly and positively correlated with Pht and Yld in all the three environments. The highest positive correlation was observed between Yld and Pdp (0.866), followed by Psp and Brp (0.777) as well as Pdp and Brp (0.693). Yield showed significant positive correlation with Pht, Brp, Pdp and Swt, while it was also significantly and positively correlated with Psp. Dmt was significantly correlated only with Swt.

Table 3.6: Parental values and population distribution parameters of the quantitative traits

Traits	Parental lines		population		Broad sense heritability
	JG62*	Vijay*	Mean*	Range	
Plant height (cm) (Pht)	43.0±4.2	40.2±4.5	38.9±4.7	28.0 - 49.0	77
Plant spread (cm) (Psp)	24.7±7.3	33.8±2.5	35.6±7.4	18.8 - 52.0	36
Number of branches per plant (Brp)	10.3±4.4	12.8±1.9	17.9±6.5	7.3 - 37.0	62
Number of pods per plant (Pdp)	26.0±5.6	52.0±4.6	58.0±30.4	7.0 - 175.0	64
Yield per plant (g) (Yld)	7.2±2.8	10.2±1.5	10.6±9.7	1.4 - 86.7	52
100-seed weight (g) (Swt)	15.6±0.8	18.2±0.8	16.5±3.6	10.4 - 30.9	84
Days to 50% flowering (Dfl)	42.5±2.4	44.3±3.1	45.3±4.6	42.3-54.6	81
Days to maturity (Dmt)	100.0±4.5	112.0±3.0	108.3±5.0	100 - 123	63

*: The values are mean ± S.D.



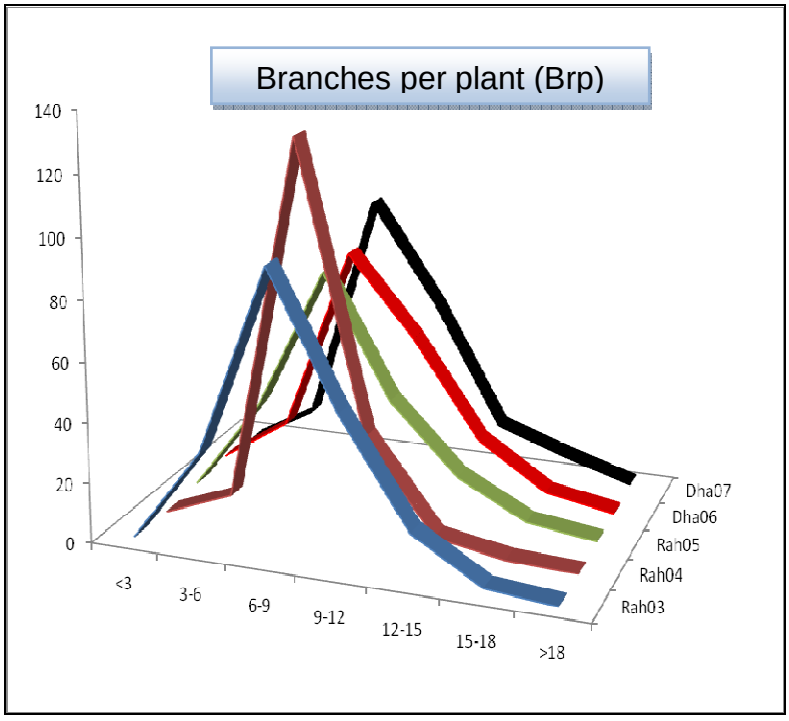
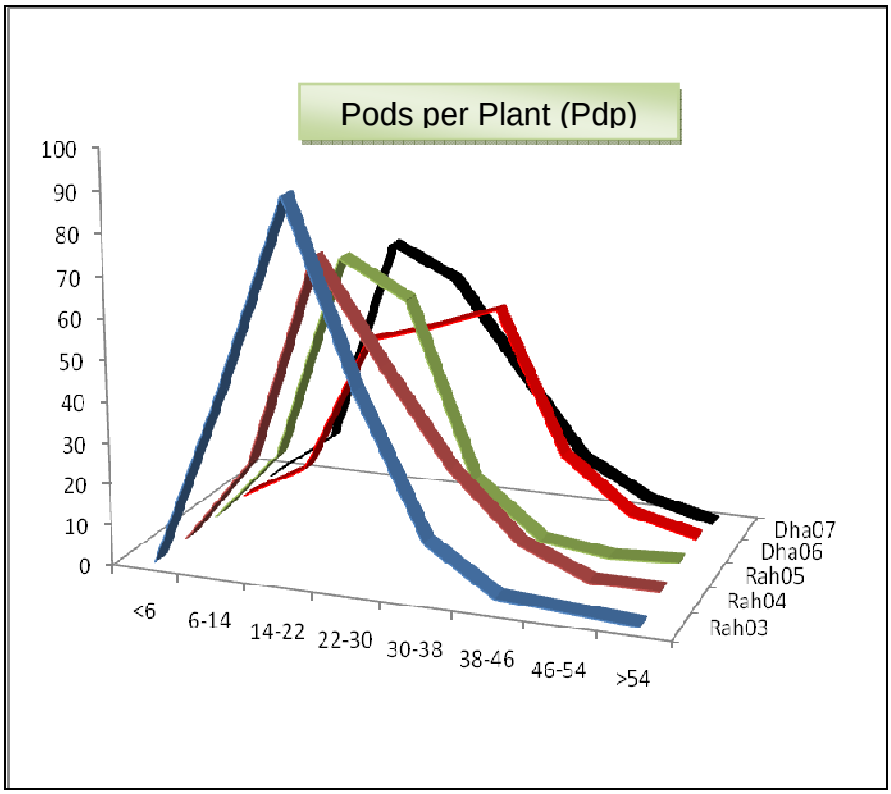


Fig 3.8: Pht and Brp frequency distribution in the JG62 x Vijay population



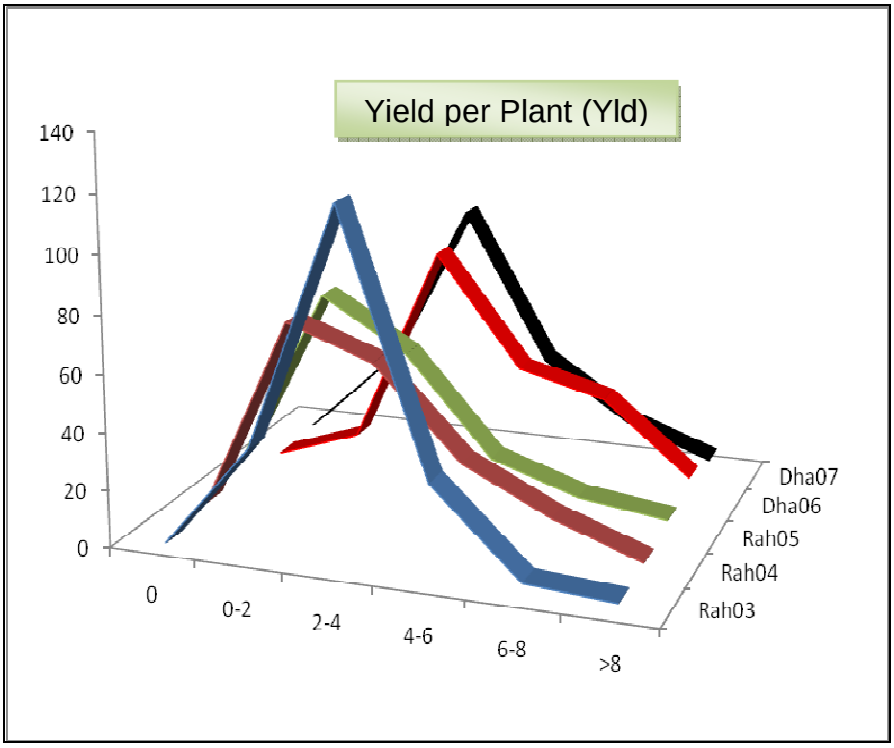
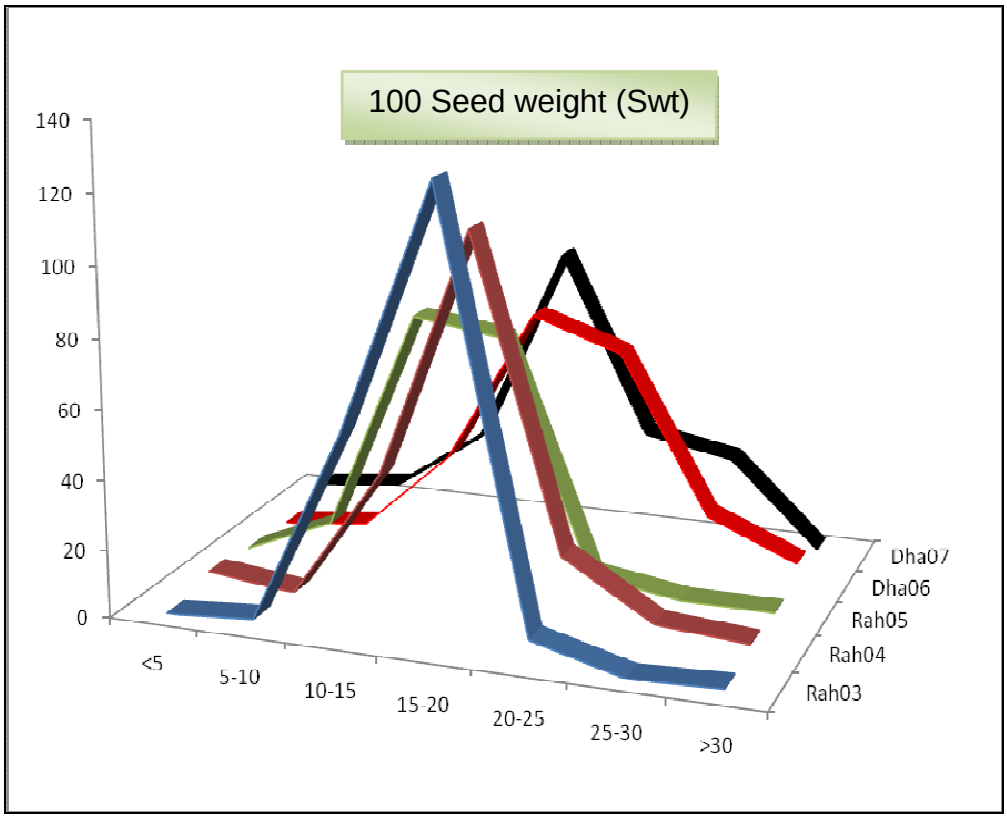


Fig 3.9: Pdp and Yld frequency distribution in the JG62 x Vijay population



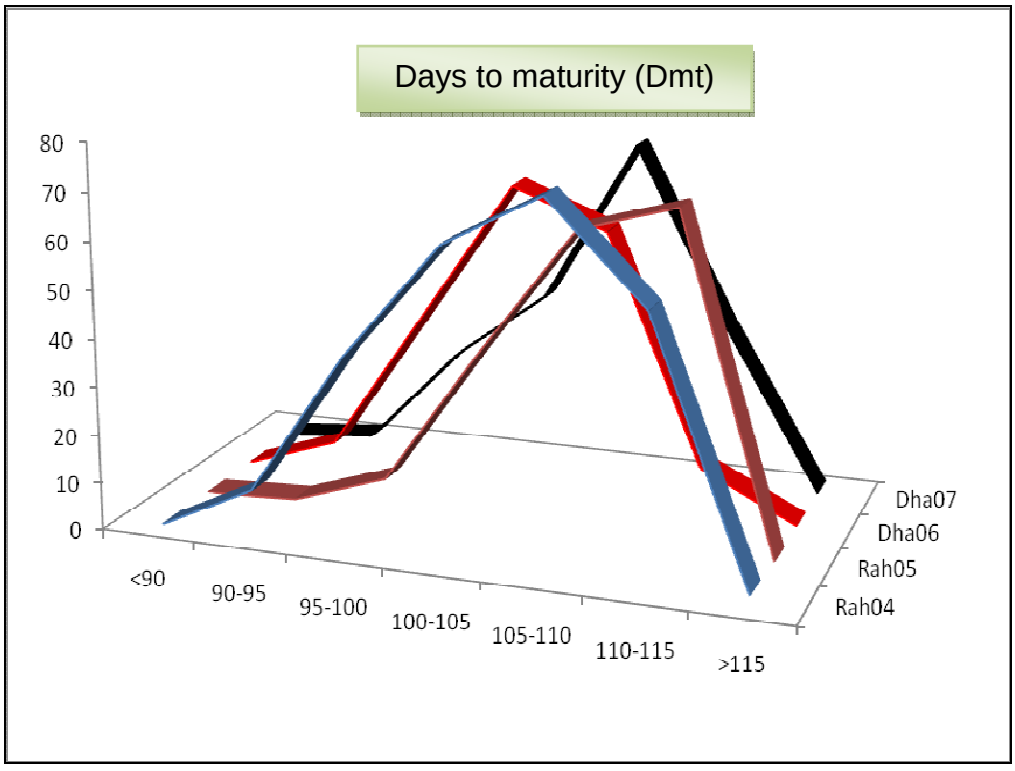
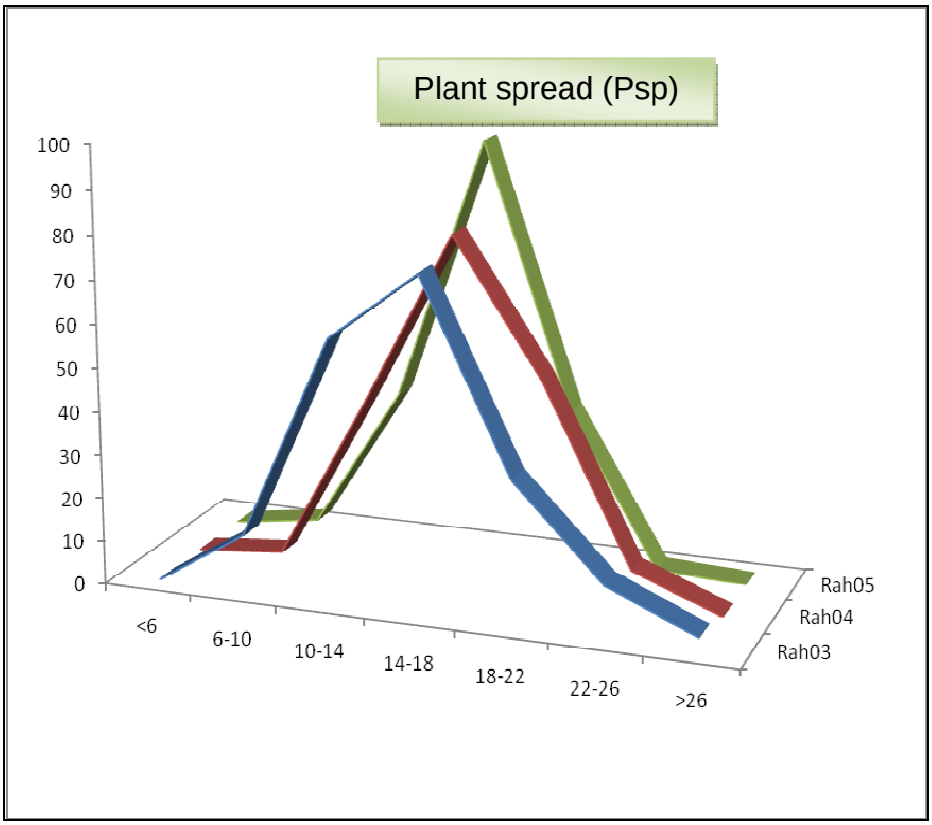


Fig 3.10: Swt and Dmt frequency distribution in the JG62 x Vijay population



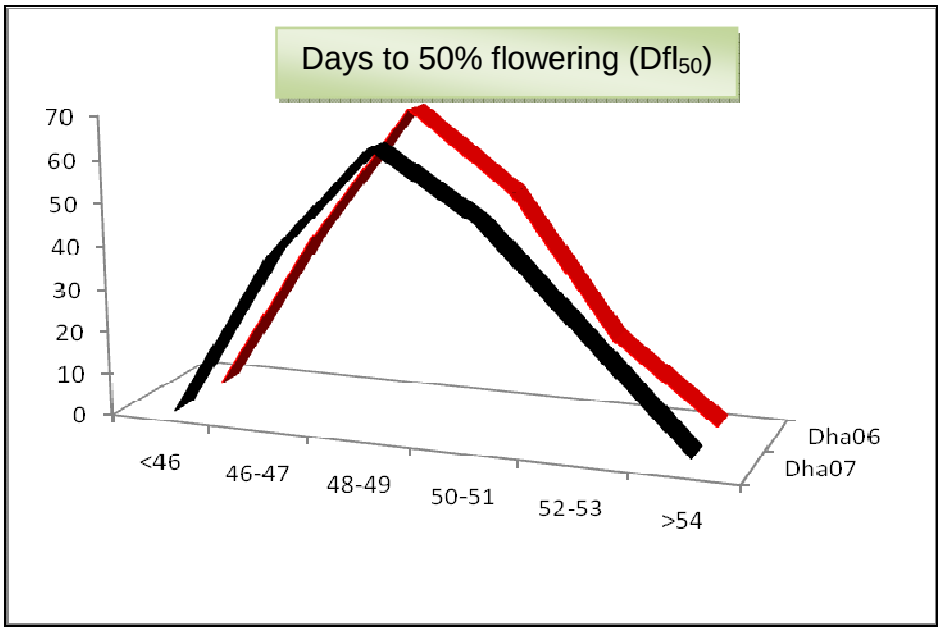


Fig 3.11: Psp and Dfl_{50} frequency distribution in the JG62 x Vijay population (* For Psp data for 3 years from Rahuri for Dfl_{50} two years from Dharwad)

3.4.3 AMMI (Additive main effect and multiplicative interaction)

The analysis of variance (ANOVA) for Pht, Psp, Brp, Pdp, Dfl_{50}, Dmt, Yld and Swt with AMMI model is presented in Tables 3.8 and 3.9. Contribution to the sum of squares due to Genotype, Genotype x Environment Interaction (GEI) and Environment were calculated as percentage of total sum of squares (Tarakanovos and Ruzgas, 2006). For all the traits AMMI model (Tables 3.8 and 3.9) deciphered the GEI into 4 principal components, the first interaction principal component axes (IPCA 1) and the second component (IPCA II) score accounted for a large portion of the sum of squares with GEI for all the traits except for Brp. Psp and Dfl_{50}.

3.4.3.1 Plant height

The AMMI analysis of variance of Pht tested in five environments showed that 58% of the total sum of squares (TSS) was attributable to genotype x environmental effect, 26% to genotypic effect, and only 16% to environment effect (Table 3.8). Among the 58% of GEI contributed, 42% was controlled by first two principal components. The AMMI2 biplot (Fig 3.12) explained 72% of the GE interaction. Environments C (Rah05), D (Dha06) and E (Dha 07) had longer vectors (high GEI) and were further from the centre of the biplot. Environment B (Rah04) had shorter vector (less GEI), while environment A (Rah03) showed a length close to zero and hence had no or least GE interaction.

3.4.3.2 Plant spread

Environment played a major role in plant spread where 66.5% of total phenotypic variation was controlled by environmental factors. Genotype x environment interaction contributed 21% and genotype effect was very less with 12% of total variation (Table 3.9). The AMMI2 biplot analysis (Fig 3.12) for Psp showed that all the three environments were highly diverse. First two principal components only explained 100% of total GE interactions.

Table 3.7: Simple correlations among the yield and yield related traits

Trait	Pht03	Psp03	Brp03	Pdp03	Yld03	Swt03	Pht04	Psp04	Brp04	Pdp04	Yld04
Psp03	-0.039										
Brp03	0.165	0.247*									
Pdp03	0.104	0.312*	0.515*								
Yld03	0.274*	0.217*	0.469*	0.718*							
Swt03	0.197*	0.045	0.076	-0.101	0.303*						
Pht04	0.021	0.068	0.007	-0.053	0.03	0.171					
Psp04	-0.031	-0.035	-0.067	-0.075	-0.009	0.042	0.451*				
Brp04	0.018	0.017	0.067	0.124	0.198*	0.081	0.363*	0.777*			
Pdp04	-0.2	-0.057	-0.084	-0.028	-0.074	0.092	0.578*	0.668*	0.503*		
Yld04	-0.096	-0.114	-0.027	0.069	0.009	0.085	0.515*	0.523*	0.395*	0.866*	
Swt04	0.056	0.113	0.156	0.166	0.227*	0.245*	0.212*	0.182	0.112	0.12	0.228*
Pht05	0.208*	-0.036	0.13	0.283*	0.215*	-0.112	-0.118	-0.12	-0.098	-0.066	-0.004
Psp05	0.045	0.185	0.042	-0.02	-0.046	-0.023	0.048	0.075	0.107	0.164	0.163
Brp05	0.129	0.146	0.081	0.042	0.083	0.141	0.189	0.228*	0.248*	0.168	0.1
Pdp05	0.006	0.14	0.21	0.192*	0.074	-0.115	0.125	0.093	0.166	0.133	0.054
Yld05	0.266*	0.088	0.045	0.064	0.182	0.137	0.158	-0.012	0.051	-0.039	-0.047
Swt05	0.117	0.001	0.042	0.174	0.272*	0.107	-0.004	-0.152	-0.071	-0.164	-0.143
Dmt04	0.307*	0.136	0.179	0.026	0.206*	0.136	0.118	-0.016	-0.085	-0.107	-0.03
Dmt05	-0.06	-0.188	-0.159	-0.04	-0.025	0.082	-0.097	-0.159	-0.112	-0.107	-0.071

*Significant at P < 0.05 (environment 03=2003; 04=2004; 05=2005)

Table 3.7: Simple correlations among the yield and yield related traits (Contd……)

Trait	Swt04	Pht05	Psp05	Brp05	Pdp05	Yld05	Swt05	Dmt04
Psp03								
Brp03								
Pdp03								
Yld03								
Swt03								
Pht04								
Psp04								
Brp04								
Pdp04								
Yld04								
Swt04								
Pht05	0.076							
Psp05	0.12	0.004						
Brp05	0.098	0.084	0.274*					
Pdp05	-0.01	0.197*	0.294*	0.692*				
Yld05	0.329*	0.203*	0.034	0.546*	0.326*			
Swt05	0.054	0.262*	-0.128	0.212*	0.227*	0.408*		
Dmt04	0.173	-0.023	0.229*	0.024	0.015	-0.011	-0.181	
Dmt05	0.048	0.204*	-0.177	0.056	0.149	0.104	0.302*	-0.078

*Significant at P < 0.05 (environment 03=2003; 04=2004; 05=2005)

3.4.3.3 Branches per plant

Using ANOVA the Brp sum of squares was partitioned into genotype, environment and GE interaction. Using principal component analysis the GE interaction was further partitioned. The results of AMMI analysis (Table 3.8) revealed that only 26.6% of total variability was justified by the GE interaction, where as major 65% by the environment and 8% by the genotypic effect. The AMMI2 biplot analysis (Fig 3.13) for Brp showed that environments B and C had longer vector (showed high GEI) but environment D had very short vector (low GEI). One RIL (line No. 32) had the longest vector for environment B, thus displaying specific adaptation for this environment. The AMMI2 biplot explained 88% of the total GE interactions.

3.4.3.4 Days to maturity

AMMI analysis for Dmt showed that GE interaction influenced 45% of total variation followed by environmental effect with 38% and least 16% explained by genotypic effect (Table 3.8). The AMMI2 biplot showed that first two principal components explained 99% of the total GE interactions (Fig 3.13). Environment B (Rah04), C (Rah05) and E (Rah07) were showed high GE interactions. There were six recombinant inbred lines (2, 6, 7, 13, 25 and 32) which showed specific adaptability to environment D *i.e.* for the year 2006 Dharwad. All the RILs were clustered very close to the center.

3.4.3.5 Pods per plant

Analysis of variance for Pdp showed that GE interaction had major influence which explained 52% of the total phenotypic variation. 27.8% was governed by environmental effect and 20% was due to genotypic effect (Table 3.9). AMMI biplot analysis showed (Fig 3.14) that environment B (Rah04) and C (Rah05) were involved in high GE interactions but environment A (Rah03) showed the least interactions. Environments D (Dha06) and E (Dha07), although showed interactions there was less variation or similar interactions with genotypes between them.

Table 3.8: Analysis of variance for Pht, Brp, Dmt and Pdp

Pht

Source	*df*	*S.S.*	*M.S.*	*F*	*% explained*
G	92	6773.46	73.6246		25.9
E	4	4207.23	1051.81		16.1
GxE	368	15168.9	41.2199		**58**
IPCA 1	95	7869.75	82.8394	3.1***	30.1
IPCA 2	93	3064.16	32.948	1.4*	11.7
IPCA 3	91	2827.87	31.0755	1.9***	-
IPCA 4	89	1407.14	15.8105		-
TOTAL	464	26149.6	56.35		

Brp

Source	*df*	*S.S.*	*M.S.*	*F*	*% explained*
G	92	1399.48	15.2117		8.3
E	4	10932.3	2733.07		**65.12**
GxE	368	4453.05	12.1007		26.6
IPCA 1	95	3180.27	33.4765	7.18***	19
IPCA 2	93	736.685	7.92134	2.66***	4.4
IPCA 3	91	483.579	5.31406	9.006***	-
IPCA 4	89	52.5126	0.590029	********	-
TOTAL	464	16784.8	36.17		

Dmt

Source	*df*	*S.S.*	*M.S.*	*F*	*% explained*
G	92	14489.1	157.49		16.4
E	3	33841.8	11280.6		38.4
GxE	276	39854	144.398		**45.2**
IPCA 1	94	37473.9	398.658	30.484	42.5
IPCA 2	92	2301.06	25.0115	28.474	2.6
IPCA 3	90	79.0547	0.878385	********	0.9
TOTAL	371	88184.8	36.17		

Pdp

Source	*df*	*S.S.*	*M.S.*	*F*	*% explained*
G	92	43511.9	472.956		20.1
E	4	60136.1	15034		27.8
GxE	368	112337	305.263		**52.1**
IPCA 1	95	74348.9	782.62	5.62***	34.4
IPCA 2	93	22737.4	244.488	2.88***	10.5
IPCA 3	91	12190	133.956	3.89***	5.6
IPCA 4	89	3060.45	34.3871	********	
TOTAL	464	215985	465.4		

The AMMI components were denoted as IPCA1, IPCA2, IPCA3 and IPCA4 ANOVA was calculated from the values of RILs across all environments, significance of AMMI components were indicated with asterisk symbol (*)***P<0.001, *P<0.05

Table 3.9: Analysis of variance for Swt, Yld, Psp and Dfl_{50}

	Swt				
Source	***df***	***S.S.***	***M.S.***	***F***	***% explained***
G	92	2510.91	27.2925		35.9
E	4	188.744	47.1859		2.7
GxE	368	4299.41	11.6832		**61.4**
IPCA 1	95	2007.77	21.1345	2.51***	28.7
IPCA 2	93	1031	11.086	1.58*	14.7
IPCA 3	91	758.971	8.34034	1.48*	10.8
IPCA 4	89	501.667	5.63671	********	
TOTAL	464	6999.1	15.1		

	Yld				
Source	***df***	***S.S.***	***M.S.***	***F***	***% explained***
G	92	2650.92	28.8144		15.6
E	4	5197.47	1299.37		30.4
GxE	368	9197.43	24.993		**54**
IPCA 1	95	6603.4	69.5094	5.62***	38.7
IPCA 2	93	1244.88	13.3858	2.88***	7.3
IPCA 3	91	878.569	9.65461	3.89***	5.1
IPCA 4	89	470.585	5.28747	********	
TOTAL	464	17045.8	36.73		

	Psp				
Source	***df***	***S.S.***	***M.S.***	***F***	***% explained***
G	92	3282.26	35.67		11.95
E	2	18274.5	9137.25		**66.57**
GxE	184	5892.26	32.02		21.46
IPCA 1	93	4077.99	43.84	2.19***	14.85
IPCA 2	91	1814.27	19.93	1.73***	6.6
TOTAL	278	27449	98.73		

	Dfl_{50}				
Source	***df***	***S.S.***	***M.S.***	***F***	***% explained***
G	92	45717.7	233.254		**76.3**
E	1	1498.97	1498.97		2.5
GxE	93	12719.9	64.8975		21
IPCA 1	93	12719.9	64.8975	2.5***	20.9
TOTAL	186	59936	152.5		

The AMMI components were denoted as IPCA1, IPCA2, IPCA3 and IPCA4 ANOVA was calculated from the values of RILs across all environments, significance of AMMI components were indicated with asterisk symbol (*). ***P<0.001, *P<0.05

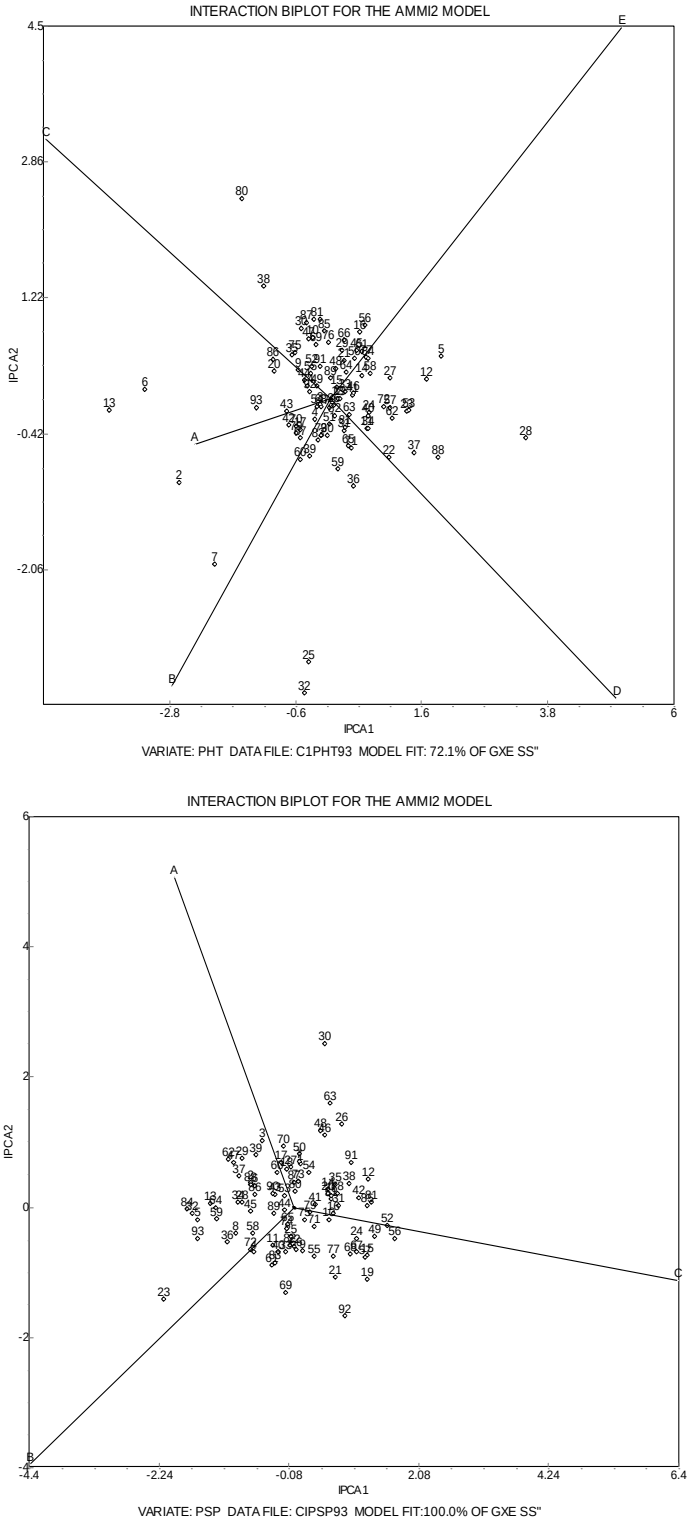


Fig 3.12: Biplot analysis of the GE interaction for the AMMI2 model for Pht and Psp (A, B, C, D and E are Environments: A= Rah03, B= Rah04, C= Rah05, D= Dha06 and E= Dha07. Genotypes: 1 to 93)

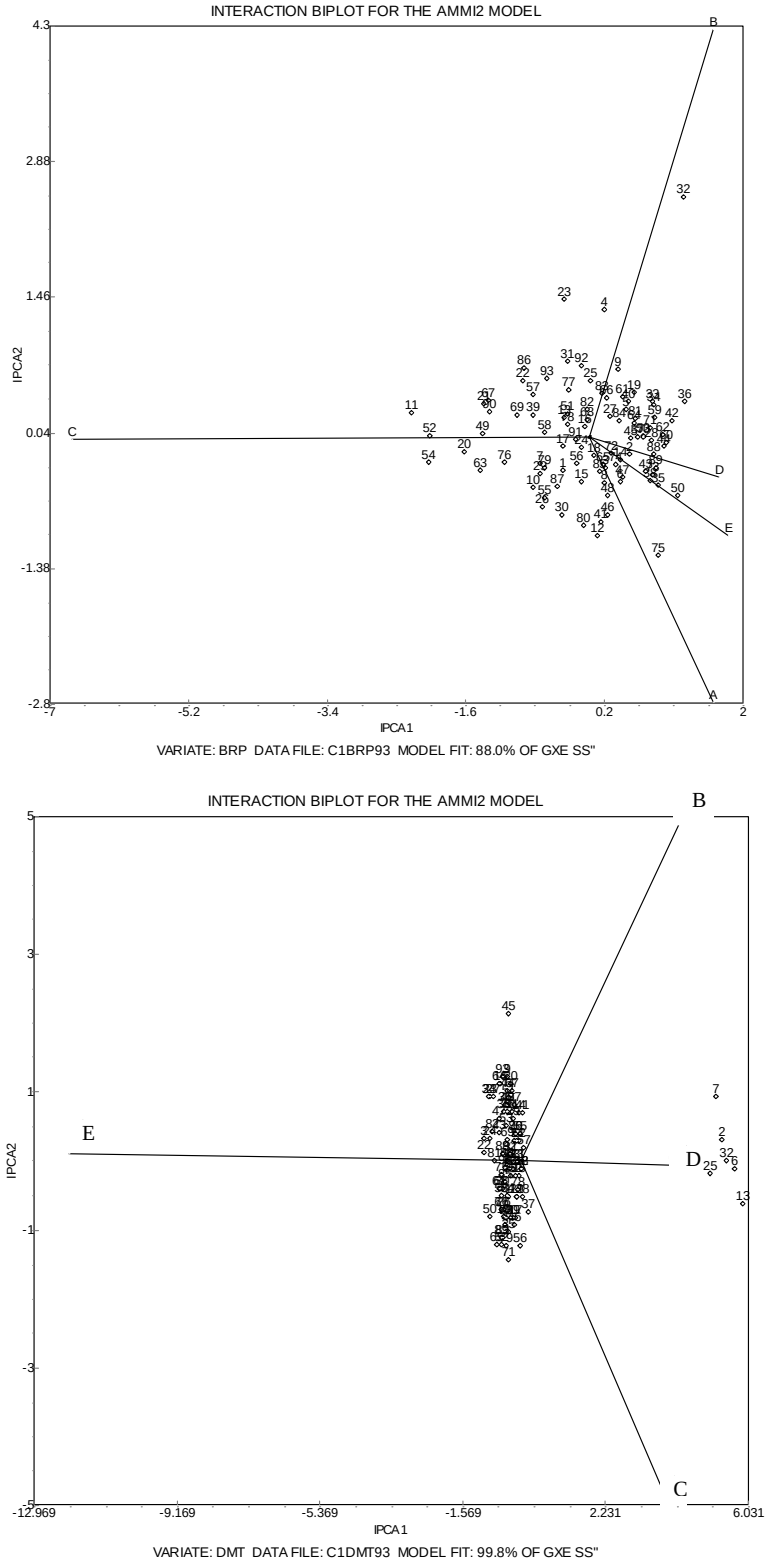


Fig 3.13: Biplot analysis of the GE interaction for the AMMI2 model for Brp and Dmt (A, B, C, D and E are Environments: A= Rah03, B= Rah04, C= Rah05, D= Dha06 and E= Dha07. Genotypes: 1 to 93)

3.4.3.6 Seed weight

Environment played the least role for 100 seed weight with control of 2.7% of total variation. GE interaction governed 65% of total variation and genotypic effect was also up to 36% of total variation (Table 3.9). Biplot analysis (Fig 3.14) showed that environments B, C and E were involved in high GE interaction and environments A and D were involved in the least GE interactions. First two PCs explained up to 70% of GE interactions.

3.4.3.7 Days to 50% flowering

Effect of genotype was more on days to 50% flowering, which explained 76% of total variation followed by GEI (21%) and environment (2.5%) (Table 3.9). Biplot analysis showed that all the genotypes were clustered into two groups (Fig 3.15). It was observed that most of the genotypes and environments were dispersed around the biplot.

3.4.3.8 Yield per plant

The most of the phenotypic variation for yield was explained by GE interaction (54%) and environment (30%) (Table 3.9). AMMI biplot (Fig 3.15) analysis showed the first two components explaining 85% of total interaction. Environments A and E showed the least interaction with genotypes where as remaining three environments showed high GE interactions (Fig 3.15).

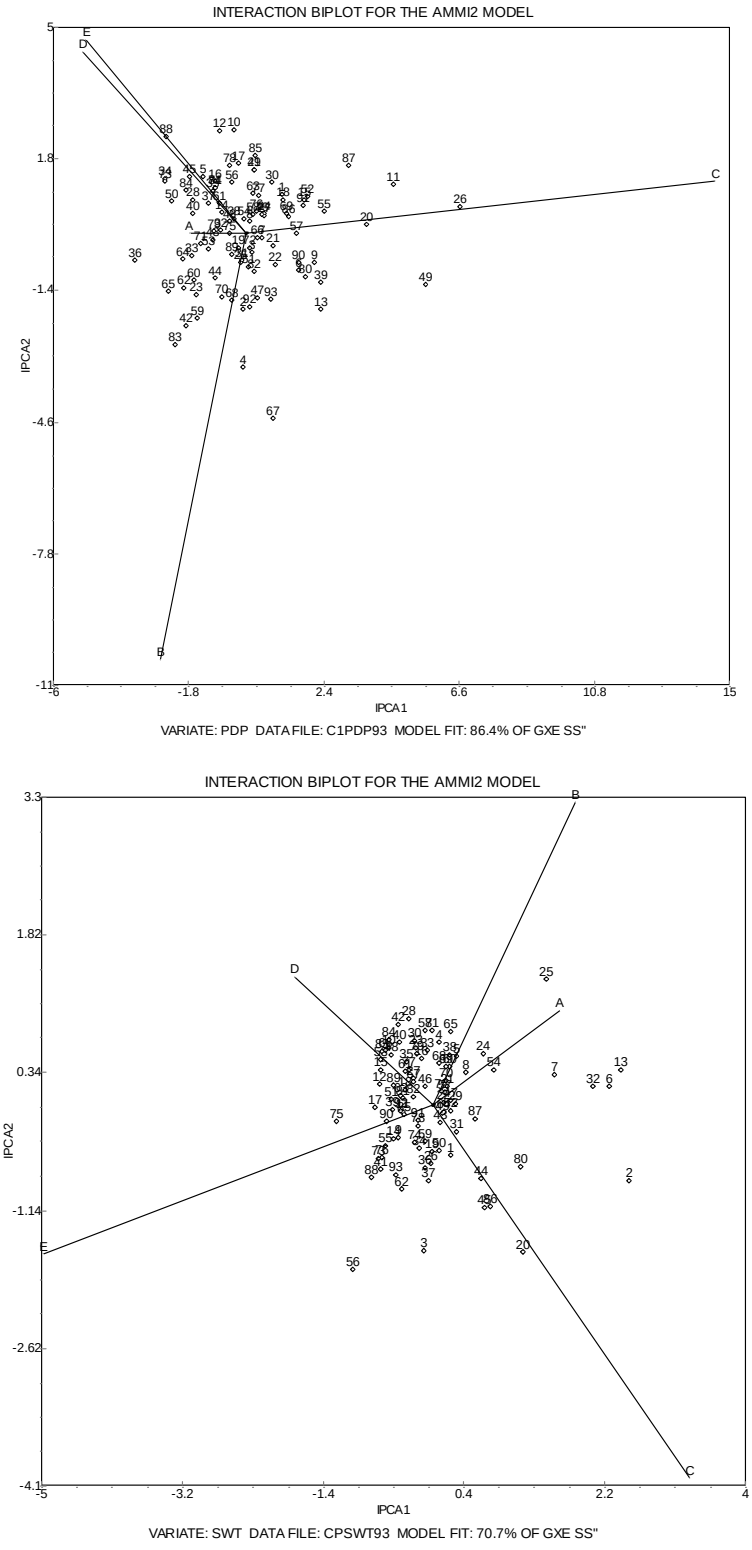


Fig 3.14: Biplot analysis of the GE interaction for the AMMI2 model for Pdp and Swt (A, B, C, D and E are Environments: A= Rah03, B= Rah04, C= Rah05, D= Dha06 and E= Dha07. Genotypes: 1 to 93)

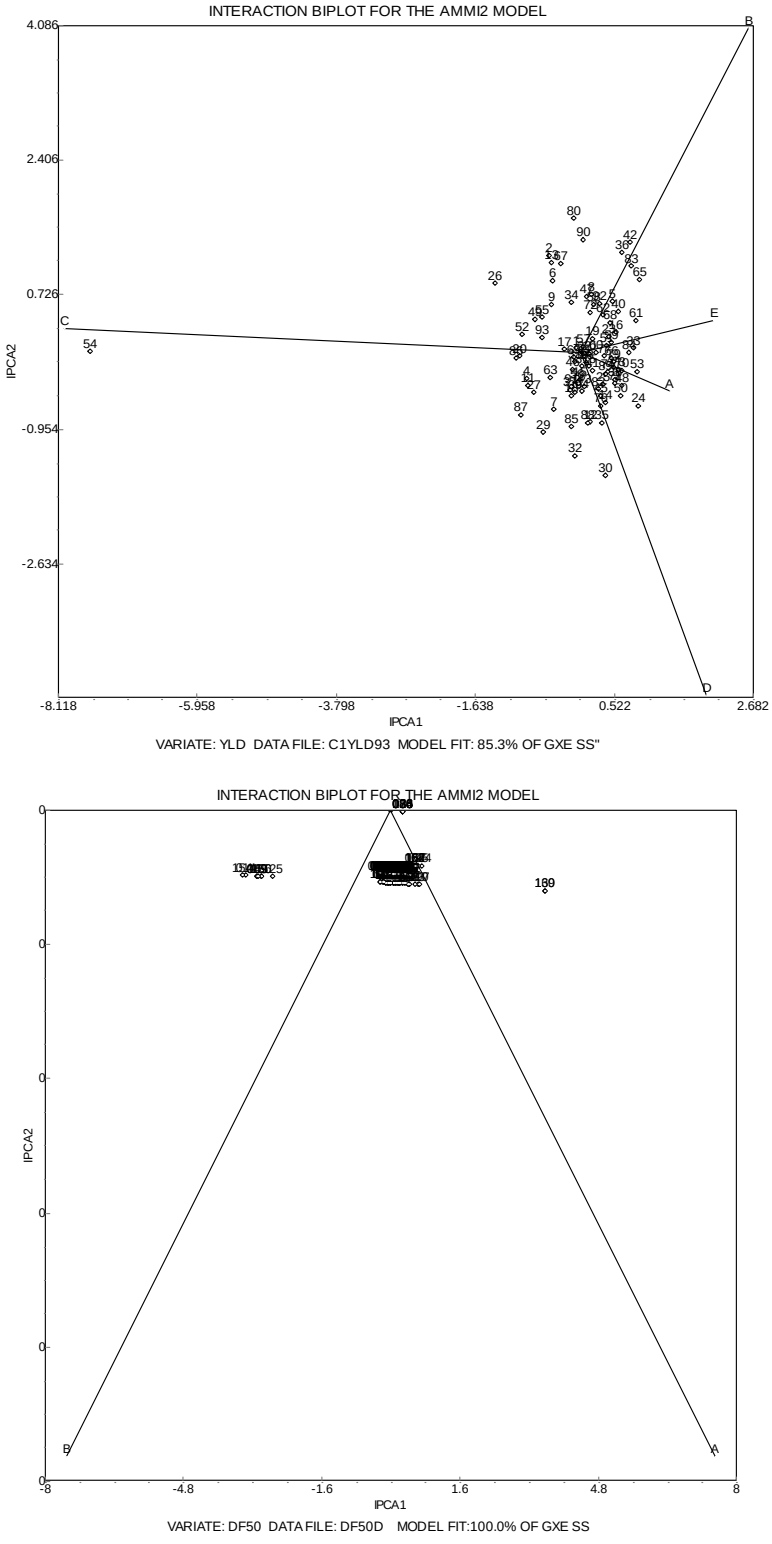


Fig 3.15: Biplot analysis of the GE interaction for the AMMI2 model for Yld and Dfl_{50} (A, B, C, D and E are Environments: A= Rah03, B= Rah04, C= Rah05, D= Dha06 and E= Dha07. Genotypes: 1 to 93) (Dfl_{50} - (A and B are Environments: A= Dha06 and B= Dha07).

3.5 Single locus QTLs analysis [Composite Interval Mapping (CIM)]

In the RIL population 80 significant QTLs (LOD ≥ 3.0) (Table 3.10) were identified for the eight yield and yield related traits. The positions and effects of significant QTLs are summarized in Table 3.11 to 3.18 and the QTLs were mapped on their respective LGs (Fig 3.16 and 3.17). The number of significant QTLs for individual traits ranged from three (Psp) to eighteen (Pdp). A total of 18 significant QTLs were detected for Pdp followed by 14 QTLs for Brp, while only three QTLs were detected for Psp. The marker NCPGR80 was associated with QTLs for seven traits *viz*. Pht, Psp, Brp, Pdp, Dmt, Dfl_{50} and Yld, while another marker TA64 was associated with QTLs for four traits *viz.* Pht, Swt, Dfl_{50} and Dmt. The highest phenotypic variation of 43.2% was explained by *QSwt.ncl-1.2*, followed by *QDmt.ncl-2.2* (36%). Most of the detected QTLs were environment specific and only 10 of the 80 QTLs were stable which expressed in more than one environment. This was particularly evident in case of Pdp QTLs, where 17 of the 18 Pdp QTLs were environment specific, on the contrary five of the fourteen Brp QTLs expressed in more than one environment. Among the QTLs identified, LG1 was associated with most number of the QTLs (33), wherein these QTLs were clustered in three groups (Fig 3.16). LG2 also had at least one QTL for each trait. Brp and Pdp QTLs were distributed across six LGs, except LGs 6 and 7, while the three Psp QTLs were mapped on the LGs 2, 4 and 5, respectively (Fig 3.16 and 3.17). Graphical representation of the clustering of these QTLs is represented in Figures 3.18 and 3.19 for LG1 and LG2, respectively.

3.5.1 QTLs for Pht

About 12 significant QTLs were identified for Pht which are dispersed on five linkage groups (Table 3.11). One common QTL (*QPht.ncl-1.3)* was detected in two locations, Rahuri and Dharwad. The contribution of the phenotypic variation ranged from 7.5% to 23.9%. Vijay contributed for Pht through 6 QTLs and JG62, for the remaining 6 QTLs (Table 3.10). Majority of QTL were on LG 2 (4 QTLs) followed by LG 1 (3QTLs) and LG 3 and LG5 (2 QTLs each) (Fig 3.16 and 3.17).

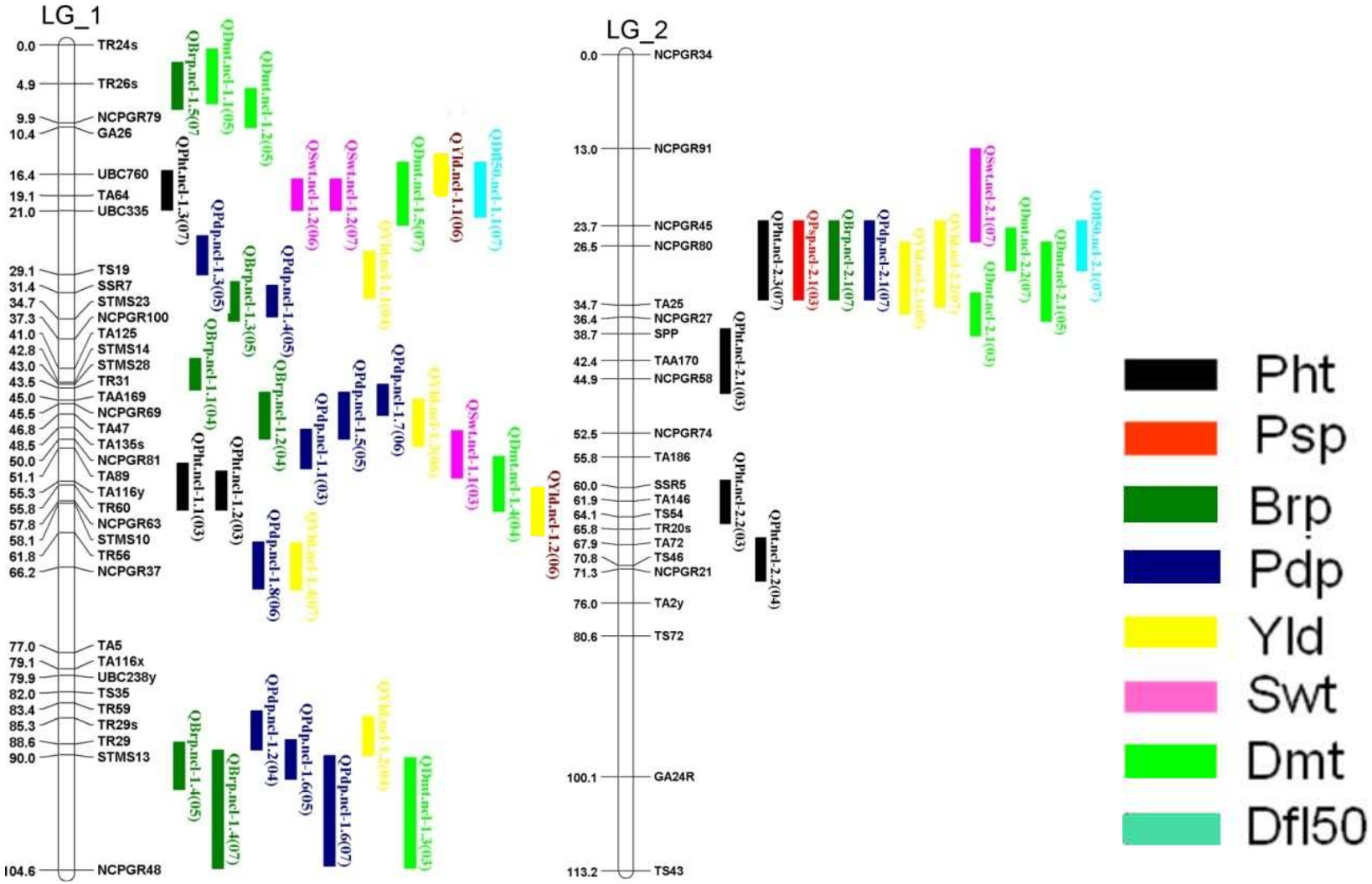


Fig 3.16: The linkage map (LG1 and LG2) showing QTLs for eight quantitative traits detected in the JV (JG62 × Vijay) mapping population. The QTLs were designated using the letter 'Q' followed by the trait name abbreviations, ncl (Name of the organization), the linkage group number and the QTL number for the trait.

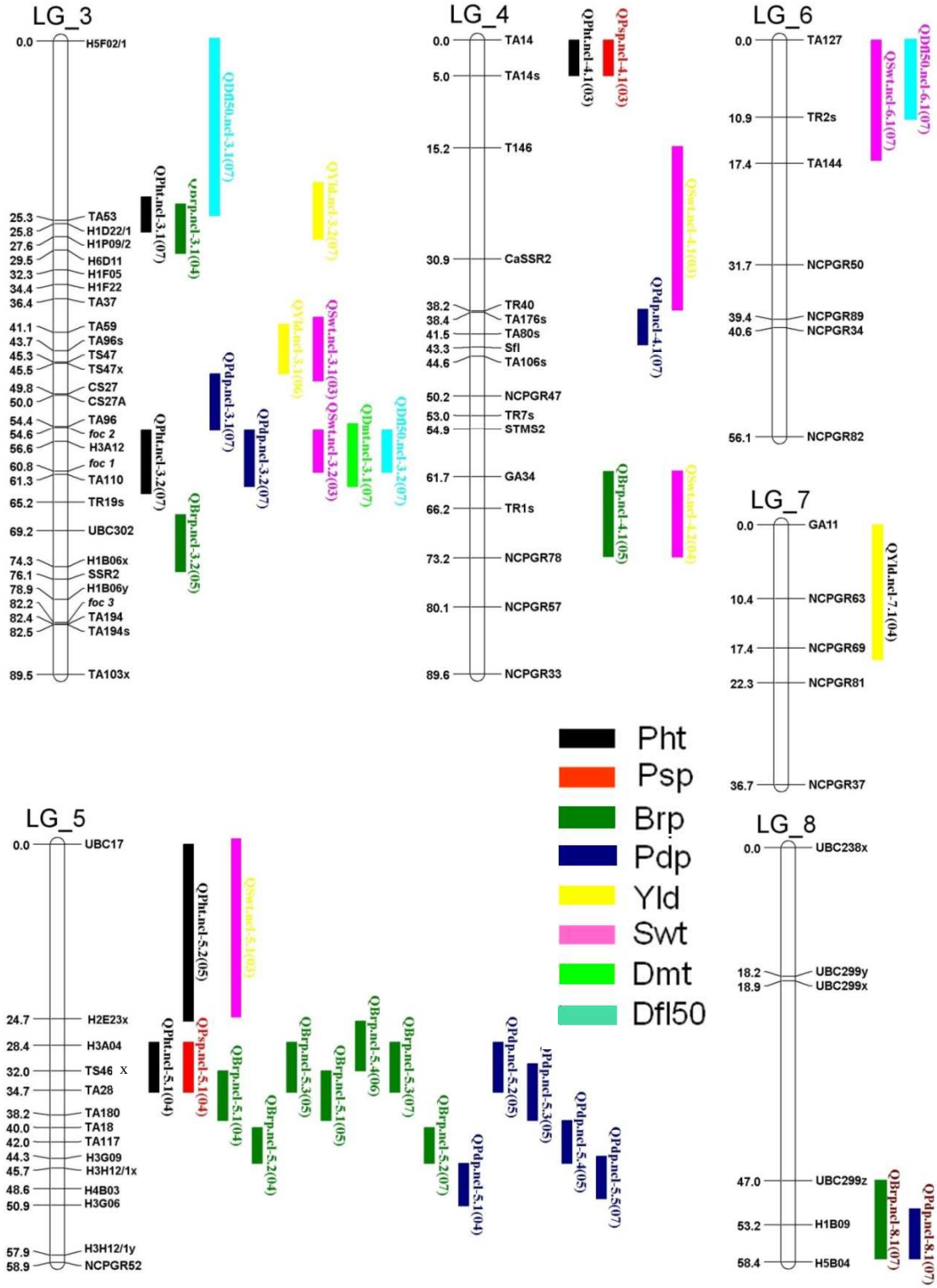


Fig 3.17: The linkage map (LG3 to LG8) showing QTLs for eight quantitative traits detected in the JV population. The QTLs were designated using the letter 'Q' followed by the trait name abbreviations, ncl (Name of the organization), the linkage group number and the QTL number for the trait.

Table 3.10: The number of significant QTLs identified and the contribution of each parent towards trait enhancing alleles of the QTLs

		Population		
			No. of trait enhancing alleles contributed by each parent	
Sl No.	**Trait**	**No. of QTLs**	**JG62**	**Vijay**
1	Pht	12	6	6
2	Psp	3	2	1
3	Brp	14	9	5
4	Pdp	18	9	9
5	Yld	10	3	7
6	Swt	9	7	2
7	Dmt	9	7	2
8	Dfl_{50}	5	3	2
	Total	**80**	**46**	**34**

3.5.2 QTLs for Psp

Only three significant QTLs were identified on three different LGs for plant spread (Table 3.12). All these three QTLs appeared to be pleiotropic with Pht QTLs. The contribution of the phenotypic variation ranged from 12 to 21%. Two QTLs were influenced by alleles of JG 62 and one QTL was influenced by alleles of parent Vijay (Table 3.10).

3.5.3 QTLs for Brp

For Brp, 14 QTL were identified on six LGs with most of them mapped on LG1 (5QTLs) followed by LG 5 (4 QTLs) (Table 3.13). The LGs 2, 4 and 8 each had one QTL controlling the number of branches. *QBrp.ncl-2.1* explained 27.83% of total phenotypic variation with Vijay allele is influencing to increase the number of branches. *QBrp.ncl-1.4* explains 22.6% and *QBrp.ncl-5.3* explains 16.78% of total variation, contributed by poor parent, suggesting the importance of alleles from JG62. Among the 14 QTLs, nine were influenced by alleles of JG 62 and five QTLs were governed by alleles of parent Vijay. Five QTLs (*QBrp.ncl-1.2*, *QBrp.ncl-1.3*, *QBrp.ncl-5.1*, *QBrp.ncl-5.2* and *QBrp.ncl-5.3*) expressed in more than one environment. Nine QTLs showed pleiotropic effect with one or another trait.

3.5.4 QTLs for Pdp

Eighteen QTLs were identified for Pdp (Table 3.14), which were distributed in six LGs, except LG6 and LG7. *QPdp.ncl-1.6* was consistent and expressed in more than one environment. Total phenotypic variation explained by individual QTLs ranged from 7.5 to 32.2%. Nine QTLs each were influenced by both the parental alleles.

3.5.5 QTLs for Dfl_{50}

Total five QTLs were identified on four LGs and all of them were environmental specific (Table 3.15). Three QTLs were influenced by JG62 alleles and two QTLs by Vijay alleles. Total phenotypic variation explained by these QTLs ranged from 8.1% (*$QDfl_{50}$.ncl-6.1*) to 35% (*$QDfl_{50}$.ncl-2.1*).

3.5.6 QTLs for Dmt

Eight significant QTLs were mapped on three LGs (LG1, 2 and 3) (Table 3.16; Fig 3.16 and 3.17). One QTL was (*QDmt.ncl-2.1*) stable across environments. Seven QTLs were influenced by alleles of JG 62 and two QTLs by Vijay alleles (Table 3.10). QTL, *QDmt.ncl-2.2*, explained 36% of total variation followed by *QDmt.ncl-1.3* (21.6%) and *QDmt.ncl-1.5* (20.3%).

3.5.7 QTLs for Swt

A total of nine QTLs were identified for Swt on 6 LGs, with majority mapped on LG1, LG3 and LG4 (Table 3.17). Among these nine QTLs, seven had negative additive effect, suggesting the contribution of alleles from inferior parent JG62. One major and stable QTL was identified and mapped on LG1. This QTL (*QSwt.ncl-1.2*) explained 43.44% of total phenotypic variation and expressed in more than one environment. Two QTLs on LG2 (*QSwt.ncl-2.1*) and LG6 (*QSwt.ncl-6.1*) contributed 22.17 and 21.54% phenotypic variation, respectively.

3.5.8 QTLs for Yld

Ten QTLs were identified for plant yield which were distributed on three different linkage groups (Table 3.18), LG1 alone carrying six QTLs followed by two QTLs each on LG2 and LG3. All the QTLs were environment specific. *QYld.ncl-2.2* explained 32% of total variation followed by *QYld.ncl-1.6* with 21%. Seven QTLs were influenced by alleles of parent Vijay while three QTLs were governed by alleles of JG62. The parent Vijay exhibited higher phenotypic values than JG62 for all the traits except Pht. Six of the twelve Pht QTLs, Vijay alleles decreased plant height in the population while the JG62 allele increased the trait value. Similarly for Psp, Pdp, Dfl_{50}, Dmt, Yld and Swt QTLs, the alleles from Vijay positively influenced the phenotypic values (Table 3.10).

Table 3.11: Results of composite interval mapping for Pht

LG	Marker	Position	LOD	QTL name	A	PVE (%)
1	NCPGR63	57.8	3.33	*QPht.ncl-1.1*	-1.35	10.58
2	TAA170	44.4	3.75	*QPht.ncl-2.1*	-2.05	***18.88***
2	TA146	61.9	3.41	*QPht.ncl-2.2*	1.45	9.63
4	TA14	2.0	3.39	*QPht.ncl-4.1*	1.31	10.88
1	TA89	55.1	2.93	*QPht.ncl-1.2*	-1.48	7.57
2	TS46y	70.8	3.85	*QPht.ncl-2.2*	2.14	12.93
5	TS46x	32.0	2.98	*QPht.ncl-5.1*	-1.8	10.89
5	UBC17	0.0	4.86	*QPht.ncl-5.2*	1.99	16.26
1	**TA64**	**19.1**	**6.83**	***QPht.ncl-1.3***	**-7.28**	***23.94***
2	NCPGR80	26.5	3.28	*QPht.ncl-2.3*	5.39	13.51
3	H1D22/1	25.8	3.19	*QPht.ncl-3.1*	-4.59	8.64
3	*foc1*	60.8	4.53	*QPht.ncl-3.2*	5.66	13.08

(Markers underlined: QTLs contributing for more than one trait; QTLs in bold letters expressed in more than one environment, Italisized in PVE – heighest variability contribution)

Table 3.12: Results of composite interval mapping for Psp

LG	Marker	Position	LOD	QTL name	A	PVE (%)
2	NCPGR80	28.5	3.00	*QPsp.ncl-2.1*	1.8	16.79
4	TA14	0.0	3.12	*QPsp.ncl-4.2*	-1.55	11.79
5	TS46x	34.0	4.49	*QPsp.ncl-5.1*	-2.65	***21.25***

(Markers underlined: QTLs contributing for more than one trait; bold and Italisized in PVE – heighest variability contribution)

Table 3.13: Results of composite interval mapping for Brp

LG	Marker	Position	LOD	QTL name	A	PVE (%)
1	STMS28	42.9	3.37	*QBrp.ncl-1.1*	1.36	11.19
1	**TA47**	**46.8**	**2.95**	***QBrp.ncl-1.2***	**1.08**	**8.64**
3	H1P09/2	27.6	3.17	*QBrp.ncl-3.1*	-1.3	8.72
5	**TA28**	**34.7**	**3.34**	***QBrp.ncl-5.1***	**-1.03**	**8.93**
5	**TA117**	**42**	**2.59**	***QBrp.ncl-5.2***	**-0.9**	**6.92**
1	SSR7	33.4	3.21	*QBrp.ncl-1.3*	-2.85	12.96
1	**STMS13**	**92**	**6.14**	***QBrp.ncl-1.4***	**-3.25**	***22.66***
3	UBC302	71.2	2.90	*QBrp.ncl-3.2*	-2.33	11.33
4	TR1s	66.2	2.72	*QBrp.ncl-4.1*	1.83	7.49
5	**TS46x**	**32**	**4.59**	***QBrp.ncl-5.3***	**-2.89**	**16.78**
5	H3A04	28.4	4.16	*QBrp.ncl-5.4*	0.11	11.19
1	TR26s	5	4.78	*QBrp.ncl-1.5*	-0.7	9.96
2	NCPGR80	26.5	9.25	*QBrp.ncl-2.1*	1.28	***27.83***
8	H1B09	53.2	4.81	*QBrp.ncl-8.1*	-0.68	10.8

(Markers underlined: QTLs contributing for more than one trait; QTLs in bold letters expressed in more than one environment, Italisized in PVE – heighest variability contribution)

Table 3.14: Results of composite interval mapping for Pdp

LG	Marker	Position	LOD	QTL name	A	PVE (%)
1	TA89	51.1	2.97	*QPdp.ncl-1.1*	-2.38	10.41
1	TR29	88.6	2.91	*QPdp.ncl-1.2*	6.03	9.82
5	H4B03	48.6	3.44	*QPdp.ncl-5.1*	8.71	12.42
1	UBC335	27.0	2.61	*QPdp.ncl-1.3*	-12.6	14.67
1	SSR7	33.4	2.90	*QPdp.ncl-1.4*	-11.9	9.94
1	TA47	46.8	3.93	*QPdp.ncl-1.5*	12.55	11.65
1	**STMS13**	**90.0**	**2.81**	***QPdp.ncl-1.6***	**-9.13**	**7.69**
5	TS46x	32.0	7.15	*QPdp.ncl-5.2*	-18	***27.51***
5	TA28	36.7	5.65	*QPdp.ncl-5.3*	-16.1	21.45
5	TA117	42.0	3.68	*QPdp.ncl-5.4*	-12.2	11.83
1	NCPGR69	45.5	5.12	*QPdp.ncl-1.7*	0.18	17.88
1	TR56	65.8	3.59	*QPdp.ncl-1.8*	0.17	16.62
2	NCPGR80	26.5	5.26	*QPdp.ncl-2.1*	6.27	13.11
3	CS27A	50.0	4.75	*QPdp.ncl-3.1*	7.12	18.62
3	H3A12	58.6	5.10	*QPdp.ncl-3.2*	10.87	***32.19***
4	TA80s	41.5	3.36	*QPdp.ncl-4.1*	6.36	7.56
8	NCPGR81	51.0	2.96	*QPdp.ncl-8.1*	-5.96	8.31
5	H3H12/1x	45.7	4.47	*QPdp.ncl-5.5*	-4.95	11.14

(Markers underlined: QTLs contributing for more than one trait; QTLs in bold letters expressed in more than one environment, bold and Italisized in PVE – heighest variability contribution)

Table 3.15: Results of composite interval mapping for Dfl_{50}

LG	Marker	Position	LOD	QTL name	A	PVE (%)
1	TA64	19.1	7.00	*QDfl$_{50}$.ncl-1.1*	-15.45	***21.84***
2	NCPGR80	26.5	8.08	*QDfl$_{50}$.ncl-2.1*	23.51	***35.30***
3	H5F02/1	0.0	2.90	*QDfl$_{50}$.ncl-3.1*	14.84	8.12
3	*foc1*	60.8	4.07	*QDfl$_{50}$.ncl-3.2*	15.16	13.17
6	TA127	0.0	2.99	*QDfl$_{50}$.ncl-6.1*	-24.4	8.15

(Markers underlined: QTLs contributing for more than one trait; bold and Italisized in PVE – heighest variability contribution)

Table 3.16: Results of composite interval mapping for Dmt

LG	Marker	Position	LOD	QTL name	A	PVE (%)
1	TR24s	0.0	4.68	*QDmt.ncl-1.1*	-1.95	13.89
1	TR26s	7.0	2.86	*QDmt.ncl-1.2*	-2.04	15.78
1	STMS13	100	5.28	*QDmt.ncl-1.3*	-1.82	***21.62***
2	**TA25**	**34.5**	**3.21**	***QDmt.ncl-2.1***	**-1.54**	**9.56**
1	TR60	55.9	3.81	*QDmt.ncl-1.4*	-2.04	13.48
1	TA64	19.1	6.08	*QDmt.ncl-1.5*	-8.57	***20.28***
2	NCPGR80	26.5	7.50	*QDmt.ncl-2.2*	12.22	***36.01***
3	*Foc1*	60.8	4.18	*QDmt.ncl-3.1*	-5.22	15.72

(Markers underlined: QTLs contributing for more than one trait; QTLs in bold letters: expressed in more than one environment, bold and Italisized in PVE – heighest variability contribution)

Table 3.17: Results of composite interval mapping for Swt

LG	Marker	Position	LOD	QTL name	A	PVE (%)
1	TA89	51.1	2.94	*QSwt.ncl-1.1*	0.72	8.39
3	TA59	43.1	4.93	*QSwt.ncl-3.1*	1.04	15.10
3	H3A12	56.6	3.53	*QSwt.ncl-3.2*	-0.9	10.25
4	CaSSR2	30.9	4.21	*QSwt.ncl-4.2*	-1.04	13.72
5	UBC17	0.0	3.13	*QSwt.ncl-5.1*	-0.71	9.00
4	TR1s	66.2	3.03	*QSwt.ncl-4.2*	-1.36	9.34
1	**TA64**	**19.1**	**7.96**	***QSwt.ncl-1.2***	**-5.79**	***43.44***
2	NCPGR45	23.7	5.03	*QSwt.ncl-2.1*	-4.2	***22.17***
6	TR2s	10.9	4.99	*QSwt.ncl-6.1*	-3.55	***21.54***

(Markers underlined: QTLs contributing for more than one trait; QTLs in bold letters expressed in more than one environment, bold and Italisized in PVE – heighest variability contribution)

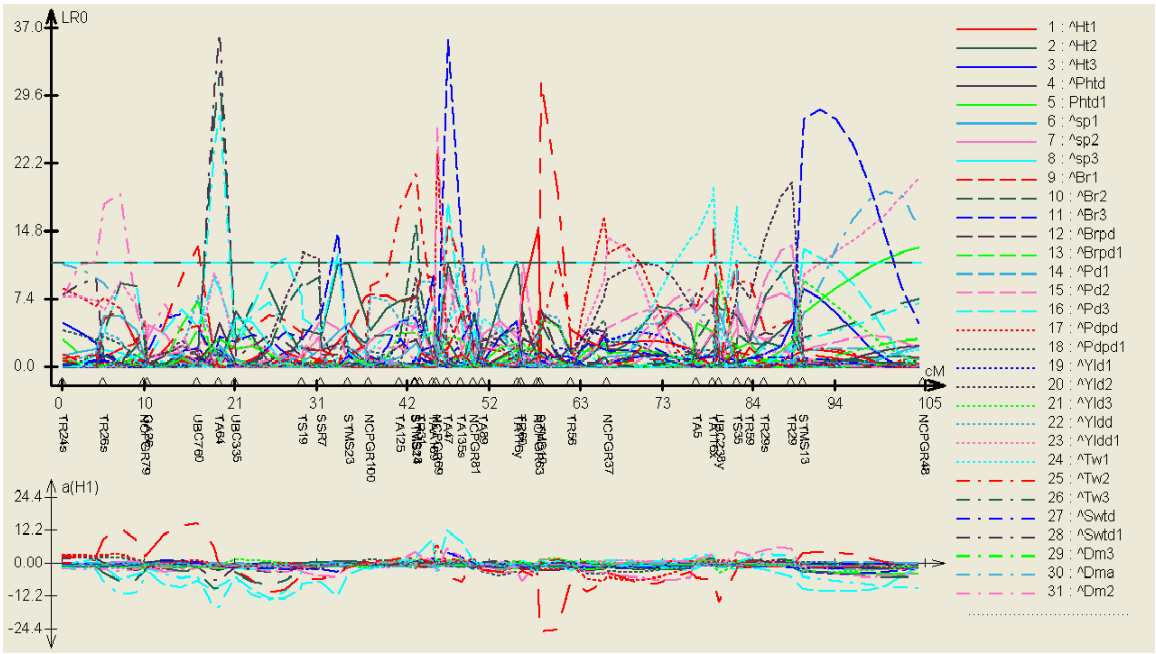


Fig 3.18: QTL clusters mapped on LG1 governing various yield traits

Table 3.18: Results of composite interval mapping for Yld

LG	Marker	Position	LOD	QTL name	A	PVE (%)
1	TS19	29.1	2.95	*QYld.ncl-1.1*	-1.66	9.12
1	TR29	88.6	4.39	*QYld.ncl-1.2*	1.79	15.22
2	TA25	34.5	2.73	*QYld.ncl-2.1*	-3.4	10.81
1	TA135s	48.5	2.93	*QYld.ncl-1.3*	-1.18	8.61
3	TA59	43.1	2.82	*QYld.ncl-3.1*	1.51	9.78
1	NCPGR37	66.3	3.10	*QYld.ncl-1.4*	0.14	13.85
2	NCPGR80	30.5	5.03	*QYld.ncl-2.2*	0.33	***32.98***
3	H5F02/1	24.0	3.07	*QYld.ncl-3.2*	0.14	14.24
1	UBC760	16.4	2.87	*QYld.ncl-1.5*	7.31	9.51
1	STMS10	58.1	6.85	*QYld.ncl-1.6*	-4.94	***21.99***

(Markers underlined: QTLs contributing for more than one trait; QTLs in bold letters expressed in more than one environment)

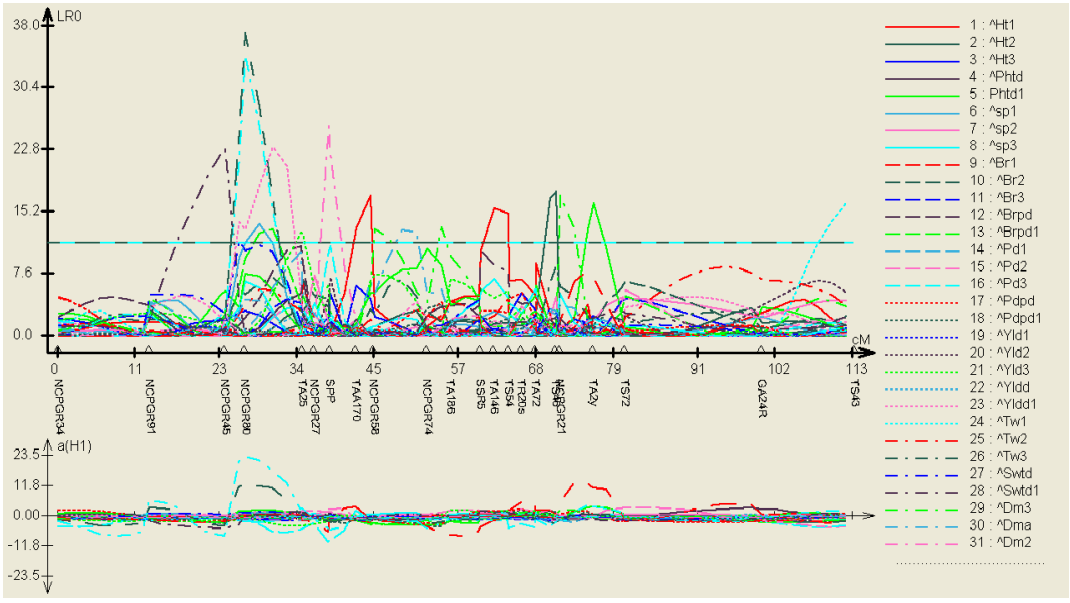


Fig 3.19: QTL clusters mapped on LG2 governing various yield traits

3.6 Multiple Composite Interval Mapping (MCIM)

Single-locus multiple-trait composite interval mapping was also conducted using JV population. All the QTLs detected through single locus MCIM were also observed in joint MCIM. In the JV population, at least one QTL for each trait was detected using single locus MCIM and 37 QTLs were detected using joint MCIM (Fig 3.20). Among these, three QTLs were pleiotropic (Table 3.19).

Table 3.19: Pleiotropic QTLs based on MCIM analysis

Traits	LG	Marker interval	Position
Pht+Psp+Brp+Pdp+Dmt+Yld	LG2	NCPGR80-TA25	26.5-35.0
Brp+Pdp+Yld	LG1	TR29-STMS13	85.6-89.1
Pht+Pdp+Swt+Dfl_{50}	LG3	H3A12-TA110	56.0-61.3

3.7 Two Locus analysis

QTL interactions were studied by using QTL Network software. QTLs are mainly divided into main effective QTLs and epistatic QTLs (Fig 3.21) based on their expression or interactins. Main effective QTLs have their own genotypic effect and sometimes they show interaction with environment also. Epistatic QTLs are usually involved in QTL x QTL interactions as well as QTL x QTL x Environment interactions (Fig 3.21). Two-locus QTL analysis was performed and the results are summarized in Tables 3.20 and 3.21. Six traits (Pht, Psp, Brp, Pdp, Dmt and Swt) showed QE and QQE interactions. In this population, epistatic interactions were detected only for two traits Psp and Brp. Three (*QPht.ncl-3.2*, *QSwt.ncl-3.2*, *QDmt.ncl-2.1*) of the five M-QTLs were also identified through single locus CIM analysis either in the same and/or adjacent marker intervals. Three M-QTLs (*QPsp.ncl-4.1*, *QBrp.ncl-3.1 and QPdp.ncl-4.1*) exhibited QE interactions. Remaining three QTLs were main effect QTLs which had their own individual effect without any interaction with the environment. The epistatic analysis revealed six QQE interactions involving eleven QTLs in the RIL population (Table 3.21).

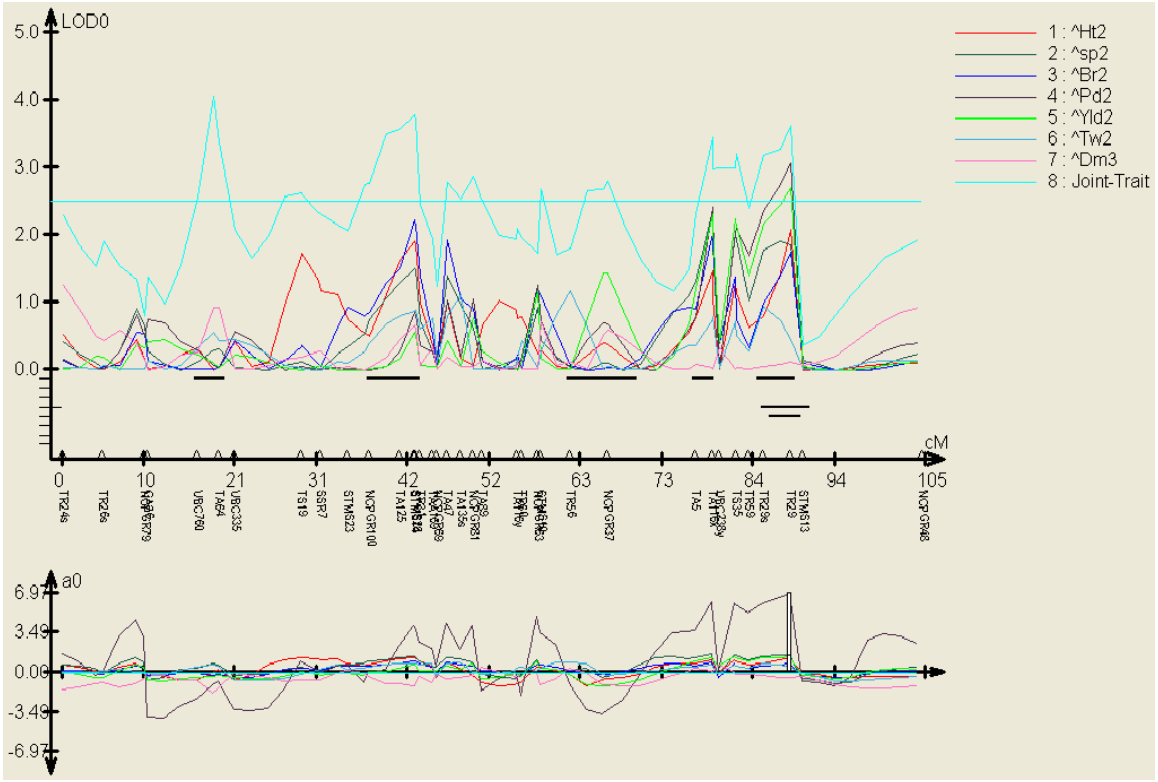


Fig 3.20: QTL clusters mapped for yield and seed weight traits based on MCIM

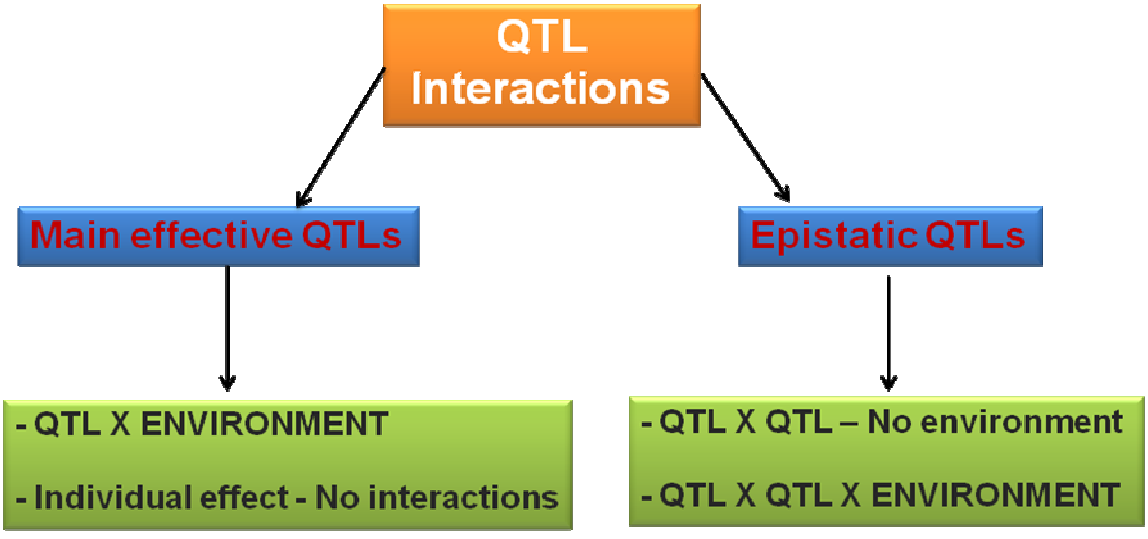


Fig 3.21: Outline of the general QTL interactions

Among these epistatic QTLs, four (*QBrp.ncl-2.1*, *QBrp.ncl-5.1*, *QBrp.ncl-4.1* and *QBrp.ncl-5.2*) were also detected in single locus CIM analysis. Definition of the graphic meta system for genetic architecture presentation based on QTL Network analysis has been described in Table 3.22. One main effective QTL was identified for plant height (Fig 3.22). This QTL (*QPht.ncl-3.2*) is independent of environmental influence and even not interacting with any other QTLs. Interestingly this QTL is associated with wilt resistance gene (*foc1*) and influenced by JG-62 alleles. The same QTL is also detected through CIM analysis upto LOD 5.6. Based on two locus analysis three QTLs for plant spread were identified (Fig 3.23). One is main effective QTL (*QPsp.ncl-4.1*) which is having its own individual effect as well as it is interacting with environment (Rah05) (Table 3.20). Two other QTLs identified are on LG3 (*QPsp.ncl-3.2* and *QPsp.ncl-3.3*) were not having their own individual effect but when they interact each other they will act as a main effective QTLs (Table 3.21). These epistatic QTLs interacted with environment (Rah05) by exhibiting Q x Q x E interactions (Table 3.21). One main effective QTL was identified (*QPdp.ncl-4.1*) for Pods per plant on LG4. This QTL is showed additive as well as additive and environment interactions. It interacted in both Rahuri (Rah05) and Dharwad (Dha07) environments. One QTL each were detected for Days to maturity and seed weight, which are showed only main individual or additive effect but not showed any interaction with environments.

One main effective QTL (*QBrp.ncl-3.1*) and nine epistatic QTLs were detected for branches per plant (Tables 3.20, 3.21; Fig 3.24). The main effective QTL showed both additive and additive x environment interactions. Nine QTLs were epistatic for Brp, as shown in the Fig 3.24, all these QTLs were not having their own additive effect but when they interacts each other and contribute for phenotype (Fig 3.24). Among these nine QTLs, three were mapped on LG2 (Fig 3.24). Both epistatic main effect and epistatic x environment interaction effect was observed between *QBrp.ncl-2.1* and *QBrp.ncl-5.1; QBrp.ncl-2.2* and *QBrp.ncl-4.1*; *QBrp.ncl-2.3* and *QBrp.ncl-4.1* QTLs (Fig 3.24 and Table 3.21). Among these three interactions, for first two QTLs (*QBrp.ncl-2.1* and *QBrp.ncl-5.1*) QQ interaction was influenced by JG62 alleles where as for QQE interaction was influenced by Vijay alleles (Table 3.21). Two QTLs; *QBrp.ncl-3.1*and *QBrp.ncl-4.2* were interacted each other as well as with one environment (Rah05) but in this case alleles of JG62 were influenced for

both QQ and QQE interactions. *QBrp.ncl-5.2* and *QBrp.ncl-8.1* were interacted each other and also with environments (Rah03 and Rah05). JG62 alleles influenced both QQ and QQE interactions except in one environment (Rah03) where Vijay alleles were contributed. Single QTL, *Brp.ncl-4.1* is interacting with two QTLs (*QBrp.ncl-2.2* and *QBrp.ncl-2.3*) for same trait (Brp) showed the pleiotropism during interaction.

Table 3.20: QTLs with main effects and environment interactions for yield traits by two-locus analysis

Marker interval	QTL	LG (position)	A	AE
foc1-TA110	*QPht.ncl-3.2*	LG3 (58.6-61.3)	1.92	-
GA34-TR1s	*QPsp.ncl-4.1*	LG4 (56-64.7)	1.98	2.7 [AE (III)]
CS27A-TA96	*QBrp.ncl-3.1*	LG3 (50-53)	0.8	1.19 (AEII)], 2.68 [AE(III)]
STMS2-GA34	*QPdp.ncl-4.1*	LG4 (54.9-64.6)	2.9	9.5 [AE(III)], -4 [AE(V)]
H3A12-*foc1*	*QSwt.ncl-3.2*	LG3 (58.6-60.8)	0.9	-
NCPGR80-TA25	*QDmt.ncl-2.1*	LG2 (30.5)	1.88	-

* A: additive effect; AE(I), AE(II), AE(III), AE(IV) and AE(V): QTL × environment interaction effects for environments I, II, III, IV and V, respectively

Table 3.21: QTL interactions involving (Q × Q or Q × Q × E) for yield traits by two-locus analysis

Marker interval	QTL (position)	LG	Marker Interval	QTL (position)	LG	AA*	AAE*
Plant spread							
TR19s-UBC302	*QPsp.ncl-3.2 (68.2)*	LG3	H1B06x-*foc3*	*QPsp.ncl-3.3* (80.9*)*	LG3	2.04	5.6 [AAE(III)]
Number of branches per plant							
NCPGR45-NCPGR80	*QBrp.ncl-2.1(25.7)*	LG2	TA28-TA180	*QBrp.ncl5.1(37.7)*	LG5	-0.72	2.4 (AAE III)
spp-TAA170	*QBrp.ncl-2.2(41.7)*	LG2	GA34-TR1s	*QBrp.ncl-4.1(63.6)*	LG4	0.54	3.14 (AAE III)
NCPGR74-TA186	*QBrp.ncl-2.3(53.5)*	LG2	GA34-TR1s	*QBrp.ncl-4.1(63.6)*	LG4	0.62	0.85 (AAE III)
H3A12-*foc1*	*QBrp.ncl-3.1(56.6)*	LG3	TA80s-*Sfl*	*QBrp.ncl-4.2(41.5)*	LG4	-0.36	0.76 (AAE II), -1.7 (AAE III)
H3A04-TS46	*QBrp.ncl-5.2(31.4)*	LG5	UBC299y-UBC299x	*QBrp.ncl-8.1(18.2)*	LG8	-0.67	0.7 (AAE1), -1.8 (AAE III)

* AA: additive effect; AAE(I), AAE(II), AAE(III), AAE(IV) and AAE(V): epistasis associated with environments I, II, III, IV and V, respectively

Table 3.22: Definition of the graphic meta system for genetic architecture presentation based on QTL Network analysis

Graphic meta system	Line (Epistasis)	Circle (Shape)
Red	___ with only epistatic main effect (I)	with only additive effect (A)
Green	. . . . . with only epistasis ×environment interaction effect (IE)	with only additive × environment interaction effect (AE)
Blue	---------- with both I and IE	with both A and AE
Dark	Not available	with no additive related effect

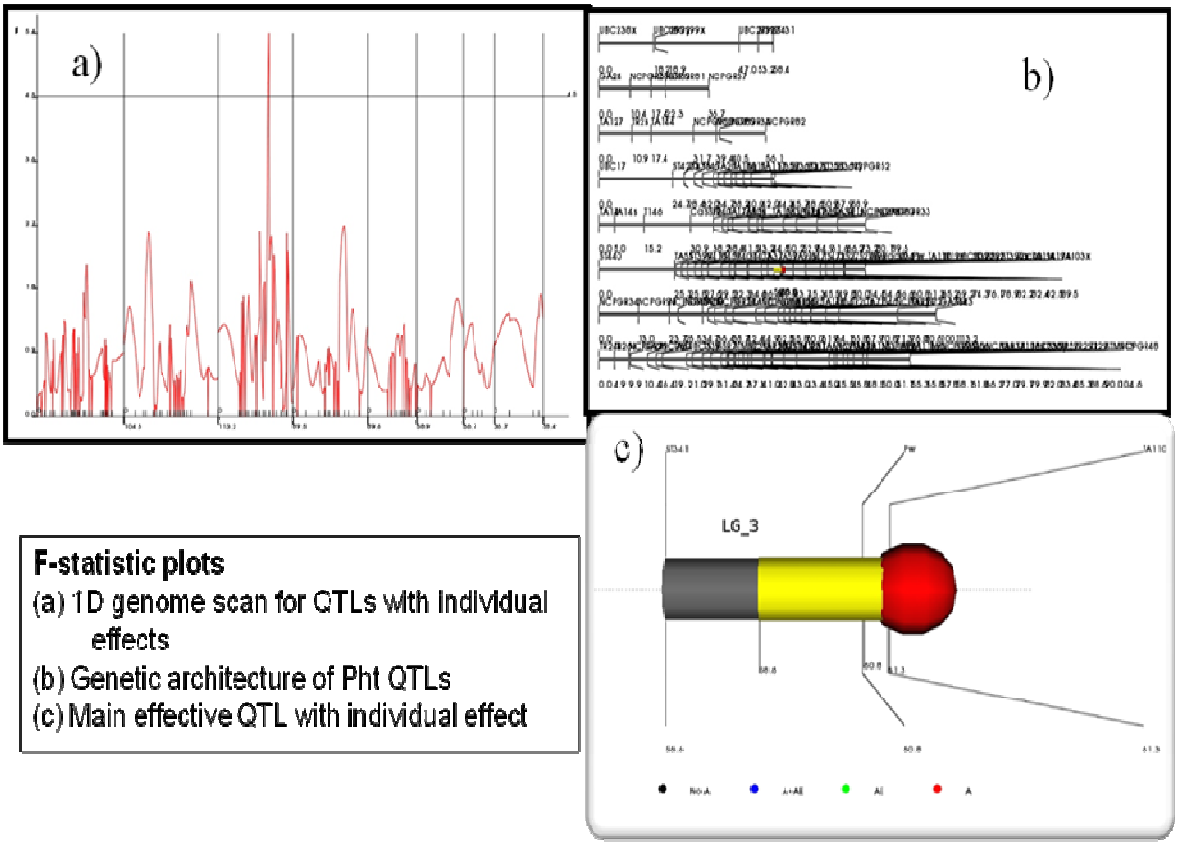


Fig 3.22: QTL x QTL interaction observed in QTL Network analysis for Pht on LG3

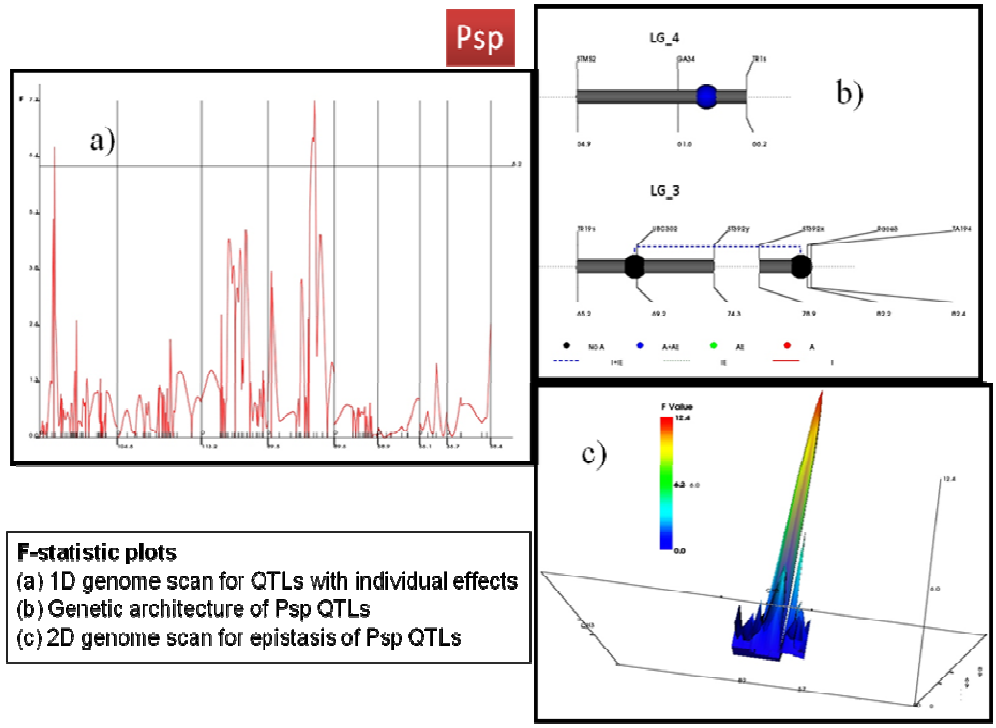


Fig 3.23: QE and QQE interactions observed in QTL Network analysis for Psp

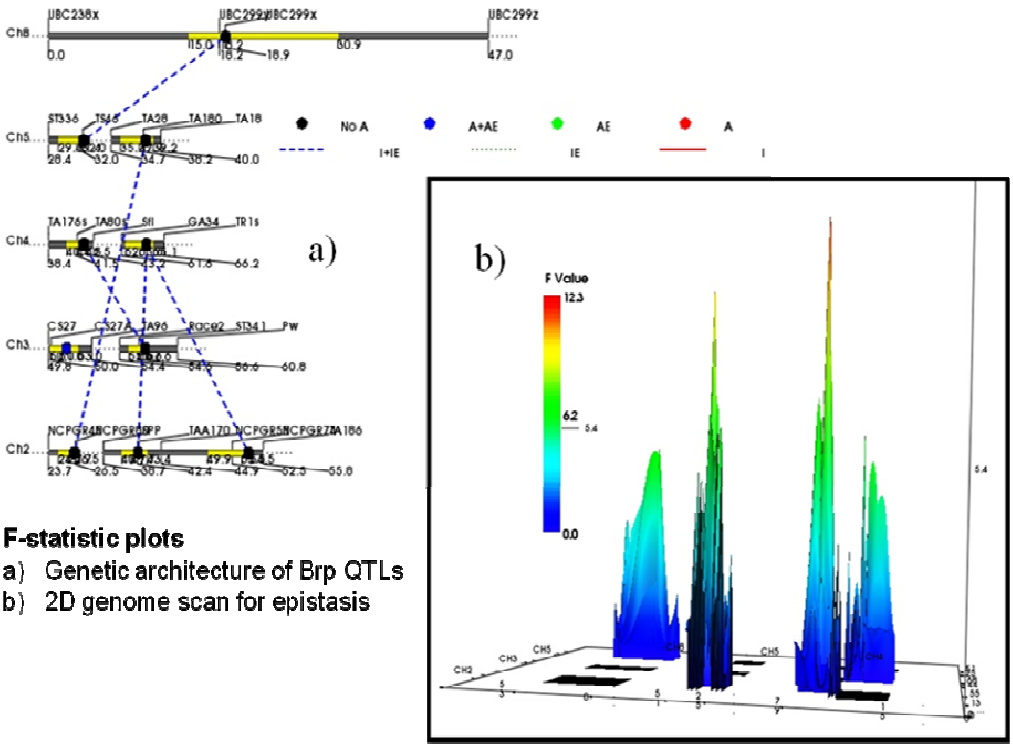


Fig 3.24: QE and QQE interactions observed in QTL Network analysis for Brp

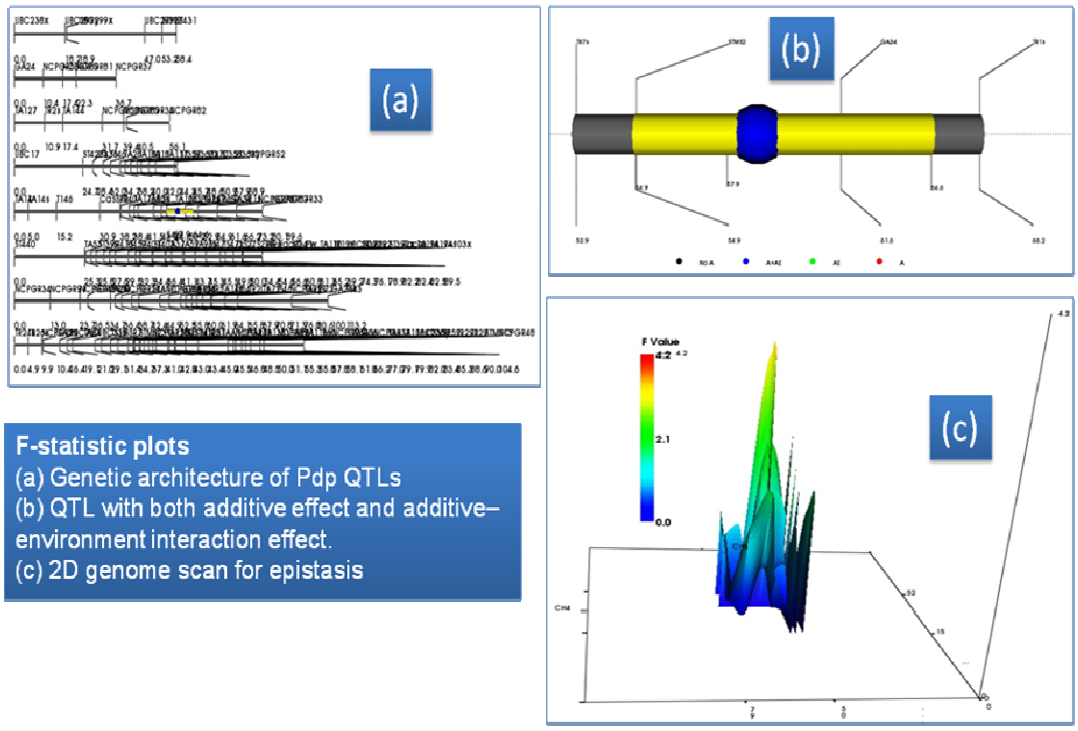


Fig 3.25: QE interactions observed in QTL Network analysis for Pdp

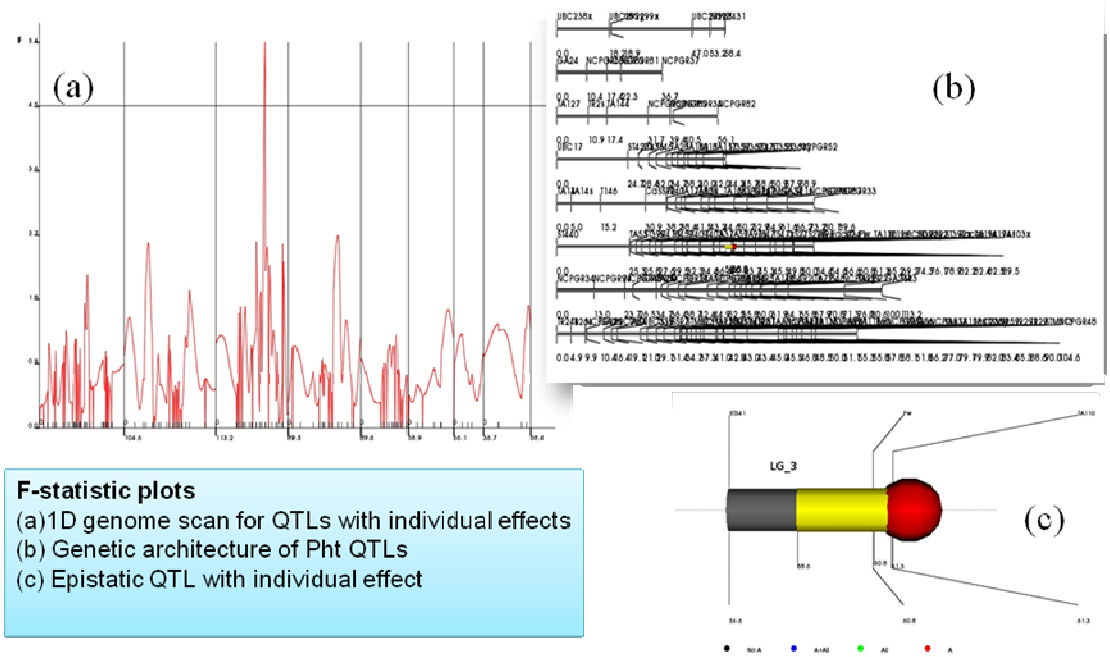
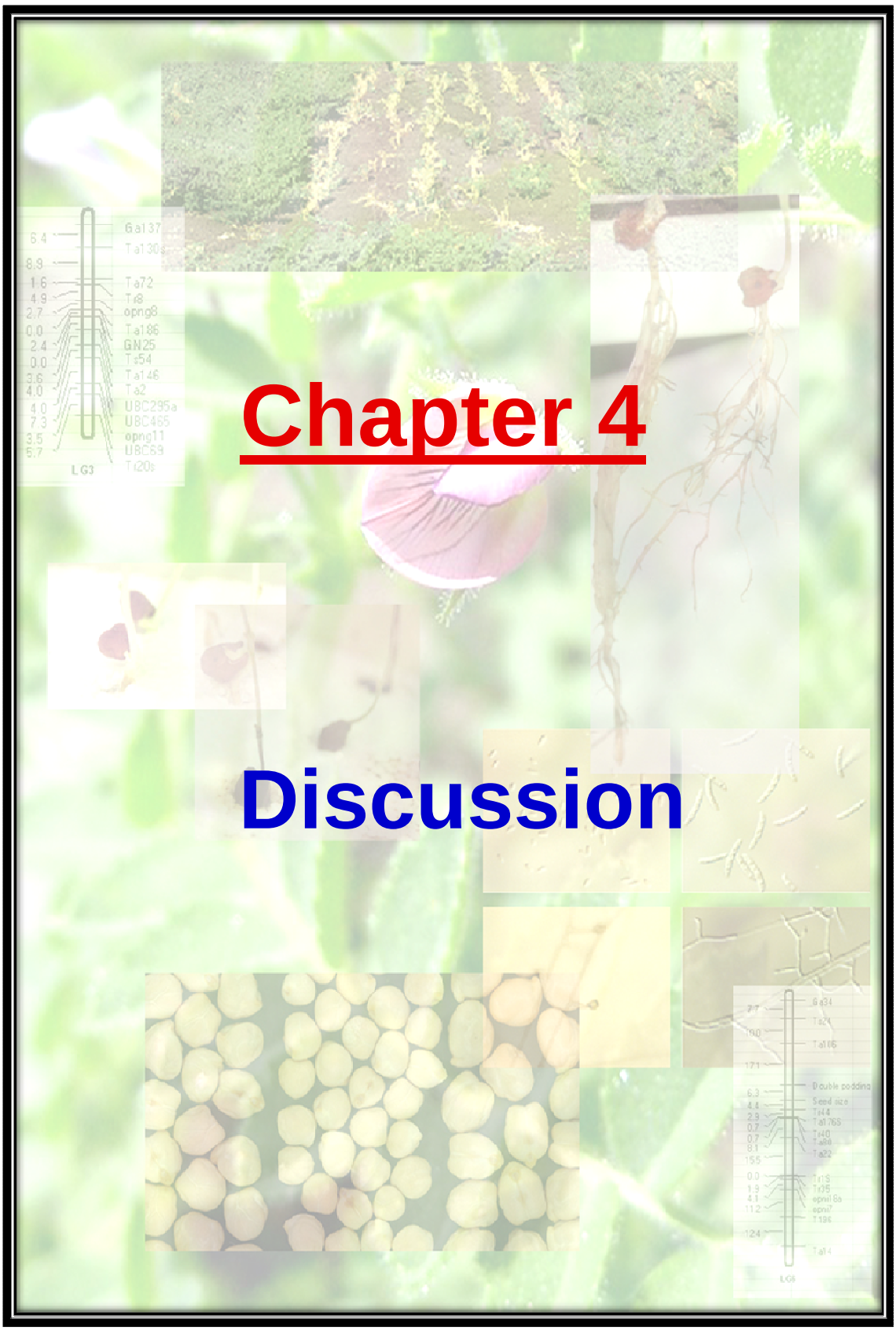


Fig 3.26: Main effective QTL observed in QTL Network analysis for Swt



Chapter 4

Discussion

During the past decade many advances have been accomplished in the construction of linkage maps for crop plants using various molecular marker tools such as RFLP, RAPD, ISSR, SSR, AFLP and SNP (Subudhi and Nguyen, 2004). These maps play an important role in the genetic analysis of agronomic and yield traits including QTL analysis, dissecting QTLs into individual components and map-based gene cloning. However, the availability of linkage maps using intervarietal cross with QTL positions of economic traits is limited in the chickpea. Construction of linkage maps based on codominant SSR markers helps in detecting good polymorphism, validation in other populations and easy comparison with existing maps as compared to maps with many dominant markers such as RAPD, ISSR and AFLP. Furthermore, intra-specific maps with codominant markers are usually considered to be suitable and preferred for MAS against desirable traits located on specific chromosomal region (Torada *et al*., 2006). A framework map using an intra-specific cross has been constructed in order to identify genes for fusarium wilt resistance, double podding, seeds per pod and QTLs for yield related traits in chickpea in the presented study.

Generation of an integrated genetic map of the crop, comprising loci of both economic and scientific importance is a central goal of chickpea genetics. Initially, the low level of polymorphism in the chickpea genome and the scarcity of co-dominant DNA-based markers were serious constraints to achieving this goal. The advent of sequence tagged microsatellite sites (STMS) markers (Huttel *et al*., 1999; Winter *et al*., 1999), however, provided the opportunity to integrate the different available maps. In recent years, STMS markers were indeed applied for the generation of almost all published genetic maps of chickpea developed employing populations from crosses between *C. arietinum* and *C. reticulatum* (Tekeoglu *et al*., 2002; Benko-Iseppon *et al*., 2003; Pfaff and Kahl, 2003; Rakshit *et al*., 2003; Abbo *et al*., 2005), *C. arietinum* × *C. echinospermum* (Collard *et al*., 2003) and intra-specific populations (Cho *et al*., 2002, 2004; Flandez-Galvez *et al*., 2003; Udupa and Baum, 2003; Cobos *et al*., 2005). Most of the previously published maps were compared with most extended genetic map of chickpea (Winter *et al*., 2000). However this map, which currently comprises more than 470 markers, was based on an interspecific cross between the *C. arietinum* and a *C. reticulatum* accession. Collard *et al*. (2003) could not detect similarities between the order of RAPD and ISSR markers in their map as compared to previous studies. Nevertheless, most genomic regions harboring genes

for important traits are not yet sufficiently saturated with co-dominant markers to apply MAS in chickpea breeding programs. Genetic mapping mostly focused on tagging agronomically relevant genes such as ascochyta blight resistance (Tekeoglu *et al.*, 2002; Udupa and Baum, 2003; Collard *et al.*, 2003; Flandez-Galvez *et al.*, 2003; Cho *et al.*, 2004) and fusarium resistance genes (Benko-Iseppon *et al.*, 2003; Sharma *et al.*, 2004), and yield-influencing characters such as double podding and other morphological characters (Cho *et al.*, 2002; Rajesh *et al.*, 2002b).

4.1 Features of the framework map

In the present study an intra-specific linkage map of the chickpea genome was established using an F_9 RIL population. We screened all the available chickpea SSR markers (published till 2008) to construct the intra-specific linkage map. Although far from marker saturation, the map comprised eight linkage groups of the genome, upon which anchor markers were distributed at an informative marker density. These linkage groups may have corresponded to the chromosome number of chickpea (*C. arietinum*, 2n = 16) however, more markers would have to be mapped to make the correlation between linkage groups and chromosomes certain. The linkage map was predominantly constructed using chickpea-STMS markers. Because of the availability of genome-wide anchor markers and stringent linkage criteria (r = 40 cM), linkage groups were established at LOD-score of 3.0. Nonetheless, a strict LOD threshold of 4.0 was set as a multipoint criteria parameter when markers were ordered in each linkage group by multipoint analysis. Similar way in potato, two backcross-linkage maps were constructed at a LOD-score of 2.0 using tomato RFLP markers based on homoeology of the potato and tomato genomes (Bonierbale *et al*., 1988). Whereas in mung bean and cowpea, the best orders of markers were determined at LOD > 2.0 (Menancio-Hautea *et al.*, 1993), although LOD thresholds were set at 2.5 and 3.0 during the preceding two-point and three-point analyses, respectively.

The intra-specific linkage map consisted of 135 (predominantly chickpea STMS) markers, which covered 568.5 cM at an average marker density of 4.21 cM. Relative to the estimated physical size of the chickpea genome (750 Mbp; Arumuganathan and Earle, 1991), 1cM distance in the map is approximately 1.32 Mbp (1,320 Kbp). This marker density is almost twice as sparse as the 750 Kbp/cM

high-density map of tomato (Tanksley *et al*., 1992). This means that another 66 molecular markers may be evenly added into the linkage map to approximate the high-density linkage map of the tomato genome (Flandez-Galvez *et al.*, 2003).

The parental genotypes used in this study were 30.2% polymorphic at 510 microsatellite sites. Huttel *et al*. (1999) and Flandez-Galvez *et al.* (2003) also observed 41% polymorphism using STMS markers among chickpea accessions. A higher level of polymorphism/genetic diversity (48% to 94%) was detected using microsatellite markers in studies that compared a larger number of chickpea cultivars (Weising *et al.*, 1992; Sharma *et al*., 1995; Huttel *et al*., 1999; Sant *et al*., 1999; Winter *et al*., 1999), thereby increasing the informativeness of each marker. The level of DNA polymorphism within chickpea is quite low for high density linkage mapping in the *C. arietinum* genome. In the chickpea intra-specific map, a genetic distance of 1 cM to 1.32 Mbp requires at least 284 evenly distributed markers to resolve a marker density of 2 cM, which is required for marker-assisted pyramiding of genes (Winter, 1997). Consequently, at least 1000 microsatellite sequences should be screened in chickpea to generate similar number of markers. However, there have been only 510 microsatellite loci characterized so far in chickpea (Radhika *et al.*, 2007). Efforts should be directed to characterize more microsatellite loci that are distributed across the whole genome.

The segregation distortion observed in this population (29.6%) was comparable to that reported by Reiter *et al*. (1992) in Arabidopsis and Xu *et al*. (1997) in rice. Most of the distorted loci in this population were skewed in favor of the parent Vijay. This might be due to accumulation of distorted alleles in the population with progressive cycles of selfing undergone in the development of the RILs (Flandez-Galvez *et al*., 2003). In tomato, Paran *et al*. (1995) reported a significant increase in the number of loci that deviated from the expected Mendelian inheritance from F_2 to F_7. They accounted this increase to the cumulative effect of selection against the alleles of one of the parents during propagation of the RILs.

The highly significant correlation (0.58, $P < 0.001$) observed between the lengths of the LGs and the number of markers in the respective LGs, indicated random distribution of the markers in the map. However, non-uniform distribution of markers was observed in some linkage groups (LG8 and LG5) (Fig 3.1). This might

be due to non-random sampling of the genome by the primers used, by uneven distribution of recombination along the length of the LGs (Tanksley *et al*., 1992), or by clustering of some markers due to their preferential targeting of particular genomic regions (Castiglioni *et al*., 1999).

The chickpea intra-specific linkage map developed in this study will serve as a core map in the mapping and tagging of genes for disease resistance, particularly fusarium wilt resistance. As the map becomes saturated with markers, more complex traits known to limit the production potential of chickpea could be dissected and utilized more effectively in national and international breeding programs. This map can be used to integrate with earlier developed or newly developing intra-specific maps to increase the marker density. Finally, the use of chickpea-STMS markers as anchor markers has provided a molecular insight of the genetic evolution of chickpea, which is a logical starting point towards intra-genera comparative mapping in *cicer*.

4.2 Fusarium wilt

Evaluation of chickpea lines for resistance to *Fusarium oxysporum* f.sp. *ciceri* in the fields can be erroneous, especially over different years, because of non uniformity in pathogen infection in field. Few methods were standardized to reproduce chickpea wilt under controlled conditions (Tullu, 1996; Sharma *et al*., 2004, Sharma and Muehlbauer, 2007; Ravi Kumar and Patil, 2004), which can be useful for unbiased evaluation of resistance as well as for studies on inheritance of resistance, as it ensures that all the tested plants are inoculated simultaneously at the same stage with constant inoculums load and are grown under similar environmental conditions. The technique can also be useful to resolve the ambiguity in genetics of resistance to different pathogen races (Pathak *et al*., 1975; Gumber *et al*., 1995; Kumar, 1998; Sharma *et al*., 2005). As the phenotypic expression of wilt resistance gene/s can be effectively studied under controlled conditions, we evaluated F_9 RIL population was evaluated for resistance to Foc races 1, 2 and 3 separately in pot culture experiments.

The segregation of STMS markers in the present study was nearly in complete agreement with the expectations of Mendelian segregation and indicated that most of the lines were relatively unbiased and showed low heterozygosity. Eighteen SSR markers and one RAPD marker were linked to the resistance genes *foc1*, *foc2* and

foc3. The identified SSR markers have clear advantages over other markers because of their allele specific amplification, easier scoring and more reliability. The results demonstrated high efficiency of these markers in predicting desired genotypes, alleviating the time-consuming process involved in a classical breeding program. Resistance to Foc races 1, 2 and 3 was earlier reported to be governed by three, two and one gene, respectively (Sharma *et al*., 2004 and 2005). However, in our population, monogenic inheritance for resistance to all the three Foc races was observed. Similar monogenic inheritance was reported in the population of the cross WR315 x C-104 for *foc1* (Mayer *et al*., 1997) and *foc3* (Sharma *et al*., 2004).

Mayer *et al.* (1997) first reported the marker CS27 which was linked to *foc1* at 7.0 cM and later this marker was converted into an allele specific associated marker (CS27A). However, in our study, CS27A was mapped at 19.0 cM from *foc1*. This increase in the distance between *foc1* and CS27A might be due to integration of new markers between these loci and / or the use of different mapping population. Similarly, Sharma *et al.* (2004) mapped *foc3* with two markers TA96 and TA194. However, in the present study, *foc3* was mapped closer to TA194 at 0.7 cM, but away from TA96. Presence of six new molecular markers between TA96 and *foc3* or different population used for mapping might have contributed to the increased distance between the gene and TA96. Race 3 resistance gene was mapped with two new STMS markers H1B06y and TA194 at 0.2 and 0.7 cM, respectively. Similarly, *foc2* gene was tightly flanked by two STMS markers Ta96 and H3A12, at a distance of 0.2 and 2.7 cM, respectively.

In the previous studies, around seven to eight markers were mapped on the same LG (LG II of Winter *et al*., 2000) (CS27A, Ta27, Ta59, Ta96, Ta110, Ta194, Tr19) (Winter *et al*., 2000; Sharma *et al*., 2004; Cobos *et al*., 2005; Lichtenzveig *et al*., 2005). In the present analysis, we increased the marker density on LG3 (LG II of Winter *et al*., 2000) by adding nine new microsatellite markers (Ta110, Ta103, H1B06, H3A12, H1F05, H1F22, H1P09/2 and H6D11) and an RAPD marker (UBC302). The results obtained by earlier studies (Tullu *et al*., 1998; Tekeoglu *et al*., 2000; Winter *et al*., 2000) indicated two clusters of fusarium wilt resistance genes, one that contained *foc1* and *foc4* and the other comprising *foc3* and *foc5* (Fig 1.7). In our results, these two clusters appeared to be combined as *foc1* and *foc2* are present in

the same cluster, which might be due to higher number of markers mapped in the present study compared to previous studies or due to some inversions observed in the map. These markers can be used in marker assisted selection for *foc1*, *foc2* and *foc3* either independently or in combination. Addition of few more markers in this linkage group will increase the chance of success for positional cloning of these *foc* genes.

The results presented here and those by Udupa and Baum (2003) contributed to the emerging picture of a hot spot for resistance against two diseases (Fusarium wilt and Ascochyta blight) on LG2 of chickpea map (Winter *et al*., 2000) as this LG also harbors QTLs for resistance against pathotypes I and II of *Ascochyta rabiei* (Cho *et al*., 2004). Apart from resistance genes, other genes involved in pathogen defense are also located on the same LG. The region containing Foc gene cluster also harbors sequences with high homology to pathogenesis-related genes such as a Thaumatin-like protein (PrP 5) or the gene coding for N-hydoxycinnamoyl-benzoyltransferase that catalyses one of the first steps in the production of phytoalexins (Benko-Iseppon *et al*., 2003). All these studies conclude that LG2 is important for resistance genes of various diseases in chickpea. Hence, saturating this LG with more STMS markers will pave the way for MAS and positional cloning of these disease resistance genes.

The use of closely linked markers to the Foc resistance genes, developed in this study, could facilitate introgression of these genes from Foc resistant cultivars carrying individual genes into commercially competitive chickpea varieties which are lacking Fusarium resistance. In addition, using the previously published markers for *foc4* and *foc5* (Tullu *et al*., 1998; Tekeoglu *et al*., 2000), they can enable introgression of all the five race specific resistance genes into a single chickpea variety, thus greatly enhancing the spread and durability of wilt resistance. Moreover, anchoring genomic areas of interest with STMS markers has been a very profitable strategy allowing saturation of the genomic region surrounding the Foc resistance genes on LG2. Marker density around these loci in LG2 is promising for further targeted selection of resistant genes. However, we failed to detect markers linked to *foc4* in our population, which clearly demands few more polymorphic and co-dominant markers such as STMS to bring all the resistance genes in a single cultivar and to achieve comprehensive resistance against Fusarium wilt of chickpea.

4.3 Double podding and seeds per pod

In chickpea, various parameters such as seed mass, seed yield, seed size, etc., contribute to yield (Rao *et al*., 1994). Double podding is also one such primary trait for yield improvement. This trait is conferred by a single recessive gene that has been assigned the gene symbol "*s*" or "*sfl*" (Khan and Akhtar, 1934; Ahmad, 1964; Patil, 1966; D'Cruz and Tendulkar, 1970; Singh and Van Rheenan, 1989, 1994). Chickpea usually develops single flower only (hence also a single pod) per node. But one cultivar JG62 (used in present analysis) produces two flowers per node (Rubio *et al.*, 1998). The double podding (*Sfl)* gene was first tagged by Rajesh *et al.* (2002b) and Cho *et al.* (2002) with the marker TA80 at 4.84 cM. In the present study, the gene has been tagged with two new flanking STMS markers TA80s and TA106s at 3.1 and 1.2 cM, respectively. There have been some reports in the past indicating the positive effect of double-podding on chickpea crop yields (Sheldrake *et al*., 1978; Singh and Van Rheenan, 1989, 1994). On the other hand, Knights (1987) reported that it had no effect on yield in diverse genetic backgrounds. The *Sfl* gene has a positive yield stabilizing effect and it is independent of seed size (Rubio *et al*., 2004). It reportedly increases seed yield by 10–18% under moisture-limiting conditions (Sheldrake *et al.*, 1978; Kumar *et al*., 2000). Constitution of the different backgrounds is necessary as it plays a role in the expressivity of the "*s*" allele (Kumar and van Rheenen, 2000). In order to study the effects of double-podding on yield, it was suggested that true isogenic lines should be developed and evaluated in diverse environments (Knights, 1987). Rubio *et al*. (1998) developed NILs for the double-podding trait and tested it at five locations over 2 years to study the role of this trait in yield. The results revealed that the double-podded NILs had more yield stability when compared to single podded lines. Similarly, in another study by Kumar *et al.* (2000), this trait showed stability for the seed yield though it had unstable penetrance and variable expressivity. These results indicated that the double-podded trait conferred more yield stability than the single-podded trait. Therefore, a tightly linked marker to this gene can be utilized to exploit the agronomic importance of this trait.

In chickpea, normally pods contain single seed. However, in some of the cultivars like 'Vijay' two seeds per pod were observed and also it is inherited as

controlled by single recessive gene. The *Spp* trait was tagged by two flanking STMS markers NCPGR27 and TA170 at 2.3 and 3.7cM, respectively (Fig 3.7).

4.4 Quantitative traits

Knowledge of the inheritance of quantitative characters is a basic requirement to identify and integrate interesting genes in linkage maps and to utilize these maps for MAS of these characters to accelerate the development of new cultivars. The knowledge of relative contribution of genetic components and environmental effects in controlling the variation for different quantitative traits is essential for crop improvement (Kumar and Rao, 1996). This information allows geneticists and breeders to employ improved strategies to develop more efficient selection methods and genetic populations (Nyquist, 1991). In chickpea, genetics of resistance to ascochyta blight (Singh and Reddy, 1983; Tewari and Pandey, 1986; Dey and Singh, 1993; Tekeoglu *et al*., 2000), fusarium wilt (Muehlbauer and Singh, 1987; Gumber *et al*., 1995; Kumar, 1998; Tullu *et al*., 1998; Tekeoglu *et al*., 2000; Rubio *et al.*, 2003), chilling tolerance at flowering (Clarke and Siddique, 2003), and flowering time (Or *et al*., 1999) have been extensively analysed.

In the present study we analysed one intra-specific F_9 RIL population for eight yield and yield related traits *viz*. plant height (Pht), plant spread (Psp), number of branches per plant (Brp), number of pods per plant (Pdp), yield per plant (Yld), 100-seed weight (Swt) days to flowering (Dfl_{50}) and days to maturity (Dmt). Although many studies have been performed on several traits of chickpea, this is one of the first reports about the association of molecular markers with the traits such as plant height, plant spread, and number of branches per plant in chickpea.

4.4.1 Phenotyping in multiple environments

Growing genotypes under well-adapted conditions with strong phenotypic expression can lead to over estimation of the genetic component and it could be avoided by including contrasting environments and seasons in which observations are made (Moralejo *et al*., 2004). In accordance, the experimental material consisting of RIL population developed with the cross JG62 x Vijay was grown in five years. Variation in environmental conditions of these years included variation in sowing, rainfall,

average temperature etc. leading to phenotype estimations at different environments. Measurable characters contributing to yield were further considered for precise quantification of the phenotypic traits, which is a basic requirement in any QTL analysis.

4.4.2 Normal distribution

The population means for Pht, Psp, Brp, Dfl_{50}, Dmt, Pdp, Swt and Yld traits in all the environments posed a normal distribution (Fig 3.8 to 3.11), without skewing towards either of the parents, suggesting least epistatic effects between the QTLs (Blanco *et al.*, 2006). The RIL population developed for the present study showed both positive and negative transgressive segregants, suggesting the possibility of finding positive alleles in the poor parent while negative alleles in the parent with better yield traits.

4.4.3 Correlation and heritability

Grain yield and related traits of chickpea (*Cicer arietinum* L.) are quantitative in nature, affected by many genetic factors as well as environment fluctuations (Muehlbauer and Singh, 1987). In chickpea breeding programs, selection is based on yield and yield related characters. Determination of correlation coefficients between yield and yield criteria is important to select favorable plant types for effective chickpea breeding. Although direct selection for the grain yield could be misleading, indirect selection via yield related characters with high heritability might be more effective than the direct selection for yield (Toker, 1998). Traditionally, correlation, regression and path-coefficient analyses have been used in determining character interrelationships and yield criteria for indirect selection (Bahl *et al*., 1976; Singh *et al.*, 1990; Toker and Cagirgan, 2003).

Correlations between the specific traits were analysed (Table 3.7). Pht was significantly and positively correlated with Yld and Swt in the first year (2003), where as correlation was positively significant with Psp, Brp, Pdp, Yld and Swt in 2004. Yield was positively and significantly correlated with Pht, Psp, Brp, Dmt, Pdp and Swt. Days to maturity showed negative correlation with all the traits in 2003 and 2004 but was significantly positively correlated with Pht and Swt in 2005 (Table 3.7).

Generally, Yld, Swt and Pdp are accepted as the most important characters in all the traits studied due to its close relationship with grain yield. The higher the pod numbers the higher grain yield. Furthermore, the number of pods and seed weight were found as one of the most important selection criteria in order to contribute to grain yield because of the fact that they had the significant positive direct effect (Singh *et al*., 1990). Pods per plant should be considered together with branches per plant. Apart from the other selection criteria, the grain weight should solely be evaluated to select large grained genotypes. Similarly, selection criteria in cereals were also evaluated (Walton, 1972; Lee and Kaltsikes, 1973; Godschalk and Timothy, 1988; Cagirgan and Yildirim, 1990). Singh *et al.* (1990) and Toker and Cagirgan (2003) reported that breeding materials should previously be screened and selected for important biotic and abiotic stress factors in the target environment prior to selection for grain yield. Traditional selection procedures will be shortened by these applications.

The broad-sense heritability estimates for the seven traits ranged from 36% (Psp) to 84% (Swt). These values agree with those reported earlier in chickpea (Eser 1976; Muehlbauer and Singh, 1987; Singh, 1987; Abbo *et al.*, 2005). Eser (1976) observed 13% narrow-sense heritability for single plant yield, 25% for number of pods per plant, 30% for plant height and 78% for seed weight in chickpea. She also concluded that seed weight was the least influenced by the environment. Singh (1987) studied chickpea pure lines and estimated the heritability values for several traits *viz*. seed yield, plant height, number of pods per plant, days to flowering, days to maturity and 100-seed weight to range from 49% to 91%. Abbo *et al*. (2005) and Cobos *et al*. (2007) also reported high heritability values for seed weight (71%) and seed size (90%). In the present study we also observed high heritability (84%) for seed weight (Swt).

4.4.4 Analysis of variance

Using ANOVA the yield sum of squares was partitioned into genotype, environment and Genotype x Environment interaction. Using principal component analysis the GE interaction was further partitioned. The results of combined analysis of variance (Table 3.8 and 3.9) showed significant differences for environments and the genotype

× environment interaction, indicating the effect of the environment in the GE interaction, and as the GE interaction was significant, it was possible to proceed further and calculate phenotypic stability (Farshadfar and Sutka, 2003). The results of AMMI analysis (Table 3.8 and 3.9) revealed that 58% of total variability was justified by the GE interaction for Pht, 16% by the environment and 26% by the genotype. Except Psp, Brp and Dfl_{50}, for all other traits GE interaction had influenced largely for total variation, indicating the importance of interactions than the individual (environmental or genotypic) influence for these quantitative traits. Many researchers reported the importance of GE interactions in quantitative traits (Yan, 2002).

4.4.5 AMMI biplot analysis

Genotype-by-environment interaction (GEI) is the differential response of genotypes evaluated under different environmental conditions. It is a complex phenomenon as it involves environmental (agro-ecological, climatic and agronomic) conditions and all physiological and genetic factors that determine the plant growth and development. There are many statistical methods for assessing, studying and interpreting GEIs (Flores *et al*., 1998; Hussein *et al*., 2000; Sabaghnia *et al.*, 2006). Some methods are based on linear regression of a genotype means on environmental index, e.g., Finlay and Wilkonson (1963) and Eberhart and Russell (1966). Nonparametric stability statistics, requiring no statistical assumptions, have been proposed by Hüehn (1990a, b) and Kang (1988). Many of the nonparametric methods have recently been compared by Sabaghnia *et al.* (2006). Three newer methods, which help identify important characteristics of GEI are the Additive Main effects and Multiplicative Interactions (AMMI), which was popularized by Gauch and Zobel (1997), Pattern Analysis (PA), which was developed and updated by Watson *et al.* (1996), and GGE Biplot Analysis, which was developed by Yan (2001) and thoroughly documented by Yan and Kang (2003).

Genotype-by-environment interaction data obtained from multi-environment trials (METs) across a wide range of environments can be investigated by pattern analysis to identify genotypes with similar responses across environments, and to identify those environments that discriminate among genotypes in a similar manner (Cooper and Delacy, 1994; Alagarswamy and Chandra, 1998; Delacy *et al.*, 2000).

Pattern analysis is based on the joint complementary use of cluster (CA) and principal component analysis (PCA) to study different aspects of response patterns of genotypes. Since there is an exponential increase in a number of pair-wise comparisons with an increase in a number of environments, inspection of individual comparisons becomes impractical. To overcome this problem, the use of pattern analysis has been proposed (Cooper and Delacy, 1994). Inspection of two-way response plots from environmental and genotypic clusters or the biplots from PCA provides an alternative and complementary way of examining the relationships among genotypes and environments (Cooper and Delacy 1994). In particular, a biplot represents a versatile graphical approach for analyzing METs (Yan, 2001; Yan and Kang, 2003).

Previous studies on predictive assessment revealed that AMMI with only two interaction principal component axes was the best predictive model (Zobel *et al.*, 1988). The contribution of IPCA1 to the GE interaction was greater than that of IPCA2 and IPCA3 for all the traits. Similar results were found in barley (Monica *et al*., 2008). It was observed that most of the genotypes and environments were dispersed around the biplot for all the traits. Most of the RILs were clustered in center indicating all these RILs are stable genotypes and all the environments are very much diverse (Fig 3.12 to 3.15). Among the yield traits analysed Pht, Dmt, Pdp, Swt and Yld shared high GxE interactions. In the previous study on barley, RILs showed moderate GE interactions compare to landraces (Monica *et al.*, 2008) for yield. Genotypes of annual crops evaluated for grain yield on a multi-locational, multi-year basis frequently show GE interactions that complicate selection or recommendation of individual lines (Mohammadi *et al.*, 2007).

Coping with genotype-year (GY) and genotype-location-year (GLY) interaction effects is possible only by selection for yield stability across environments defined as location year combinations (Annicchiarico, 1997). There are two strategies for developing genotypes with low GE interactions. The first is sub-division or stratification of a heterogeneous area into smaller, more homogeneous sub-regions, with breeding programs aimed at developing genotypes for specific sub-regions. The second strategy for reducing GE interaction involves selecting genotypes with better stability across a wide range of environments in order to better predict behavior

(Eberhart and Russell 1966; Tai, 1971). Various methods use GE interaction to facilitate genotype characterization, and as a selection index together with the mean yield of the genotypes. Accordingly, genotypes with minimal variance for yield across environments are considered stable. This idea of stability may be considered as a biological or static concept of stability (Becker and Leon 1988). This concept of stability is not acceptable to most breeders and agronomists, who prefer genotypes with high mean yields and the potential to respond to agronomic inputs or better environmental conditions (Becker, 1981). The high yield performance of released cultivars is one of the most important targets of breeders; therefore, they prefer a dynamic concept of stability (Becker and Leon, 1988).

According to Huehn (1990a, b) nonparametric procedures are easy to use and interpret and additions or deletions of one or few genotypes minimally affect the variation of results. We can even use nonparametric methods for balanced data with normal distributions because they are relatively simple. Stability estimates from nonparametric models based on the ranked classifications of genotypes in a given set of environments do not require previous assumptions and are a good alternative for parametric measurements (Nassar and Huehn 1987; Huehn and Nassar 1989). The interaction concepts of the classifications are strongly related to those required by breeders, i.e. determination of whether the best genotype in one environment is also the best in other environments, and they can define static and dynamic concepts of stability. The results thus show the potential usefulness of AMMI model to identify the genotypes having wider adoptability or specific adaptability which can be used as a genetic resource for breeding.

4.5 Composite interval mapping analysis

The basic principle of using genetic markers to study quantitative trait loci (QTL) is well established (Lander and Botstein, 1989; Carbonell *et al.*, 1992; Haley and Knott 1992; Jansen 1993; Zeng 1993, 1994). Sax (1923) first used pattern and pigment markers in beans to analyze genes affecting seed size by investigating the segregation ratio of F_2 progeny of different crosses. Thoday (1961) proposed the idea of using two markers to bracket a region for detecting QTL. The basic idea of Sax and Thoday for detecting the association of a QTL with a marker rests on the comparisons of trait

means of different marker (chromosomal segment) classes. These methods, such as *t* - test and simple and multiple regressions, directly analyze markers. In recent years, the advent of fine-scale molecular genetic marker maps for various organisms by molecular biology techniques has greatly facilitated the systematic mapping and analysis of individual QTL. Lander and Botstein (1989) proposed a much-improved method, named interval mapping (IM), for QTL mapping. It has been shown that IM has more power and requires fewer progeny than the methods for direct analysis of markers (Lander and Botstein 1989; Haley and Knott 1992; Zeng 1994). Haley and Knott (1992) proposed a regression version of interval mapping to approximate IM. Although Haley and Knott's method could save time in computation and produce similar results to those obtained by IM, the estimate of the residual variance is biased, and the power of QTL detection can be affected (Xu, 1995).

The approach of IM considers one QTL at a time in the model for QTL mapping. Therefore, IM can bias identification and estimation of QTL when multiple QTL are located in the same linkage group (Lander and Botstein 1989; Haley and Knott 1992; Zeng, 1994). To deal with multiple QTL problems, Jansen (1993) and Zeng (1993, 1994) independently proposed the idea of combining IM with multiple regression analysis in mapping. Zeng named this combination "composite interval mapping" (CIM). The approach of CIM is that, when testing for the putative QTL in an interval, one uses other markers as covariates to control for other QTL and to reduce the residual variance such that the test can be improved. The model of CIM includes one QTL and markers. Hoeschele and Vanranden (1993a, b), Satagopan *et al.* (1996), and Sillanpaa and Arjas (1998) used a Bayesian approach in estimation and to identify QTL. Doerge and Churchill (1996) used permutation tests for QTL detection. Mapping for QTL controlling binary trait and ordinal categorical traits is presented by Hackett and Weller (1995).

In deciphering the yield and yield related QTLs we used a RIL population from JG62 x Vijay cross, which could be considered as a smaller population for QTL mapping of such complex traits. However, Price (2006) postulated that QTL positions identified using small populations were nearly same as that of large mapping population. The QTL analysis for important traits in chickpea such as for fusarium wilt (*foc0*, 80 lines) (Cobos *et al.*, 2005), 100 seed weight, days to flowering and

number of seeds per plant (76 lines and 80 lines) (Cho *et al.*, 2002; Cobos *et al.*, 2007) were reported earlier. In wheat frost tolerance (cbf3, 74 lines) (Vaguifalvi *et al.*, 2003), wheat grain protein (Gpc, 74 lines) (Distelfeld *et al.*, 2004) and barley photo period response (Ppd H_1, 94 lines) (Turner *et al.*, 2005), using population less than 100 individuals, accurately predicted the underlying genes, governing these traits. However, it has been reported that the sampling affects the confidence interval and maximum LOD may not be found at true QTL position (Darvasi *et al.*, 1993). The QTL identification carried out in the present study using ninty-three RILs can be considered predictive for further studies.

4.5.1 QTL mapping for various traits under study

There were twelve QTLs mapped for Pht which were distributed on five linkage groups. One QTL (*QPht.ncl-2.3)* showed pleotropism with QTLs for traits Psp, Brp, Dfl_{50}, Pdp and yield with NCPGR 80 as an indicative marker. This result was supported by the correlation observed between these traits. Pht was significantly and positively correlated with Psp, Brp, Dfl_{50}, Pdp and Yld (Table 3.7). QTL '*QPsp.ncl-2.1'* was clustered with QTLs for Brp, Pdp and Yld in LG2. In LG5 also, clustering of QTLs between Psp, Brp and Pdp traits was observed. These results were clearly supported by correlation observed between these traits which were positive and significant. There were five Brp QTLs (*QBrp.ncl-1.2*, *QBrp.ncl-1.4*, *QBrp.ncl-5.1*, *QBrp.ncl-5.2* and *QBrp.ncl-5.3*) which were stable and expressed in more than one environment. The correlation and clustering observed between Brp and other traits was similar. Pods per plant had direct influence to improve the grain yield. Many QTLs were identified for Pdp with even some of them contributing up to 32% of total phenotypic variance. Except one QTL (*QPdp.ncl-1.6*) all other QTLs were expressed in single environment.

In genetic terms, yield is the end result of many different genes acting throughout the life of the plant plus the effect of the environment and environment x genotype interactions, consequently low hereditability is expected, as this study has shown. Environment had major influence on this trait. Correlations between yield and seed weight showed positive values (0.3 and 0.41) (Table 3.7), similar as the results obtained in the other field experiments, where correlations between seed weight and

yield were positive. These results might explain the similar outcome reported by different authors in relation to correlations between yield and yield components (Mandal and Bahl, 1980; Muehlbauer and Singh, 1987; Singh *et al.*, 1990; Kharrat *et al.*, 1991; Kumar and Arora, 1991; Kumar and Bahl, 1992; Maynez *et al.*, 1993; Cobos *et al.*, 2007). The QTL for seed size (*QSwt.ncl-2.1*), Pdp (*QPdp.ncl-2.1*) and another two QTLs for yield (*QYld.ncl-2.1* and *QYld.ncl-2.2*) in the same genomic region in LG2 might explain the correlation between these three traits (Fig 3.16 and Fig 3.17). These results agree with those reported by Cho *et al.* (2002), who found a QTL for seed size and seed number per plant (an important yield component) and Cobos *et al*, (2007) for Yld and Swt in same LG. Similar type of clustering of QTLs was observed in LG1 and LG3 also for these three traits.

In our map, no QTLs for yield or seed size were identified in same genomic region of LG6 where the double-pod gene is present. Similar results were obtained by Cho *et al*. (2002) and Cobos *et al* (2007). Studies performed in different locations and years have shown that this gene has no effect on increased yield and/or decreased seed size (Rubio *et al.*, 1998, 2004). However, the link between QTL for Yld and Dfl_{50} in LG2 might partly explain the positive correlation found between yield and days to flowering. This result agrees with Rubio *et al.* (2004), who performed a multi location / year assay and found a significant and positive effect of earliness on yield in chickpea.

Seed weight is an important aim in chickpea breeding programs because larger seed sizes fetch higher market prices (Upadhyaya *et al.*, 2006). Thus, the significant correlation between seed size and yield seems to be good for obtaining large seed cultivars with high yields. In this study, the QTLs for seed size were closely linked or pleiotropic with QTLs for yield on LG1 and LG3. One major QTL for Swt (*QSwt.ncl-1.2*) was expressed in both Rahuri as well as in Dharwad location with around 43% of the total phenotypic variation (Fig 3.16 and Table 3.17). This QTL was also clustered with one yield QTL (*QYld.ncl-1.1*) which is also supported by significant correlation observed between them. More attempts must be made in the future to saturate this major QTL region (*QSwt.ncl-1.2*) in the LG1 of chickpea map, using robust markers such as STMS (Winter *et al.*, 2000; Tekeoglu *et al.*, 2002; Cho *et al.*, 2004). The

detection of these new QTLs for seed size would help to overcome the selection problem for larger seeds in chickpea.

QTL for Swt on LG2 (*QSwt.ncl-2.1*) in this study might be the same QTL for seed size reported by Cho *et al.* (2002), Abbo *et al.* (2005) and Cobos *et al*. (2007) (Fig 3.16). Abbo *et al*, (2005) located their QTL flanked by markers GA02 and STMS11 (23 cM apart); these markers were not polymorphic in our map. New NCPGR markers were added to same LG but these markers were not used in previously published articles. The QTL for seed size reported by Cho *et al.* (2002) using an intra-specific population was located in a broad genomic region of LG4. In this study, a more precise location was obtained for QTL (*QSwt.ncl-2.1*), flanked by two new SSR markers (11 cM apart). On chickpea maps published before now, this genomic region was poorly saturated and necessitating the addition of more robust markers in this interesting region.

Days to flowering is considered to be an important adaptive trait because crops have to grow in different thermal and photoperiod regimes (Khanna-Chopra and Sinha, 1987). Mediterranean chickpea seem to have evolved towards high day-length sensitivity, while on the Indian subcontinent and in East Africa, they have evolved towards short photoperiods (Roberts *et al.*, 1985; Kumar and Abbo, 2001). The RIL population used in this study was derived from a cross between two Desi Indian chickpea cultivars. Segregation can be clearly observed in the data distribution of the RILs growing under short-day conditions, with significant differences between the parental lines. Both temperature and photoperiod have been reported to affect days to flowering in chickpea (Roberts *et al.*, 1985; Kumar and Abbo, 2001). Transgressive segregation in these assays may be the result of new genetic combinations related with photothermal response. In fact, two major genes (*ppd* and *efl-1*) controlling early flowering have been reported in chickpea (Or *et al.*, 1999; Kumar and van Rheenen, 2000) and complementary gene actions seem to be evident in crosses between early chickpea genotypes (Kumar *et al.*, 1985; Kumar and Rao, 1996). In this study, highly significant QTLs for days to flowering were detected in LG1, 2, 3 and 4. Cho *et al.* (2002) reported one significant QTL for days to flowering in LG3 by using RILs from a cross between an extra-early parent (ICCV 2) and the same genotype used in our RIL as an early parent (JG62). The QTL (*QDfl$_{50}$.ncl-6.1*) might be the same QTL

which was reported by Cho *et al*. (2002). In both the studies an STMS marker Ta127 is a common marker.

In conclusion, LG1 and LG2 could be considered as interesting genomic region for yield traits in chickpea. LG1 had 33 QTLs representing at least one QTL for each trait. Two QTLs for resistance to ascochyta blight have been reported in the same LG (LG2 of this study) (Santra *et al.*, 2000; Tekeoglu *et al.*, 2002; Collard *et al.*, 2003; Flandez-Galvez *et al.*, 2003; Rakshit *et al.*, 2003; Udupa and Baum, 2003; Cho *et al.*, 2004; Iruela *et al.*, 2006) that might be flanking the QTLs detected in this study for Pht, Psp, Brp, Swt and yield. Furthermore, genes for seeds per pod (*spp*) have been located in this linkage group. Clusters of yield traits have been reported in several species such as rice (Mei *et al.*, 2003; Thomson *et al.*, 2003), common bean (Tar'an *et al.*, 2002) and pea (Timmerman-Vaughan *et al.*, 2005). This fact might explain the early association found between resistance to ascochyta blight and late flowering/small seed size (Singh and Reddy, 1993). In terms of breeding for these traits, large segregant populations would have to be used to obtain early flowering, large seeds and resistance to wilt genotypes. MAS would enable simultaneous selection to be performed for all the three traits at an early stage. STMS marker NCPGR80 used in this study was closely linked to QTLs for Pht, Psp, Pdp, Swt and Yld might be useful in the selection of these traits.

In the present study, only 10 QTL of the total 80 QTL were detected in more than one environment, indicating that individual QTL seem to be more sensitive to the environment. This was in agreement with the results reported by Paterson *et al*. (1995) and Cho *et al*. (2004). However, QTL for different traits showed different stabilities. A substantial proportion of QTL for Pht, Brp, Pdp and Swt was active across environments, although the QTL for Psp, Yld and Dmt changed greatly across different trials. Therefore, the present study tends to support the general conclusion made by Tanksley (1992), i.e. a substantial proportion of QTL affecting a trait can be identified under different environments (especially QTL having major effects).

4.6 MCIM analysis

As many of these yield traits were correlated, MCIM and joint MCIM analysis were performed to detect pleiotropic QTLs. Although many QTLs were detected in CIM,

most of them were not detected in the MCIM analysis. This suggests more stringent or higher level of confidence in MCIM than in CIM for detecting pleiotropic QTLs. Additionally, this also suggests that pleiotropy might be one of the possible causes of the correlations observed among the traits. Similar results were observed for yield traits in wheat (Kumar *et al*., 2007).

The coincidence of QTLs for correlated traits with co-directional genetic effects is compatible with two hypotheses. The alleles with similar directions of effects might be in a coupling phase at a number of adjacent loci and such linked complexes might have been generated and maintained by selection prior to or after domestication. Alternatively, allelic variation at a single locus may control pleiotropic variation for a number of characters. Earlier studies have provided evidence for co-localization of QTLs for morphological traits in *Arabidopsis thaliana* (Perez-Perez *et al.*, 2002) and clover (Cogan *et al.*, 2006). Similar studies have been performed for reproductive developmental traits in Arabidopsis, sunflower and clover (Ungerer *et al.*, 2002; Bert *et al.*, 2003; Cogan *et al.*, 2006). The presence of multiple genes or QTLs controlling related traits, as observed in these studies, might be resolved by fine mapping.

4.7 QTL interaction studies

In its broadest sense, epistasis implies that the effect of a particular genotype on the phenotype depends on the genetic background. In its simplest form, this refers to an interaction between a pair of loci, in which the phenotypic effect of one locus depends on the genotype at the second locus. More generally, the effect of one locus might depend on the genotype at several or many loci. In the case of quantitative traits, epistasis describes the general situation in which the phenotype of a given genotype cannot be predicted by the sum of its component single-locus effects (Phillips, 1998). Extensive work on the control of qualitative genetic variation has highlighted the biological importance of epistasis at a ‘locus-by-locus’ level. On the basis of this work, several classic genotype–phenotype patterns that are caused by epistasis such as comb type in chickens, coat colour in various animals, and kernel colour in wheat have been characterized (Carlborg and Haley, 2004).

Epistatic QTL-mapping methods are more flexible than those for individual QTLs as they simultaneously consider the mean effects of multi-locus genotypes on the phenotype. The use of the methodology poses more technical challenges and demands more from the data than individual QTL mapping. For these reasons, epistatic QTL mapping is not yet a standard tool in complex trait studies. Epistasis between pairs of QTLs in which both or one QTL have detectable individual effects has been reported (Li *et al*., 1997), but the extent to which epistasis controls variation in quantitative traits has been poorly explored. There are several methods for mapping epistatic QTLs in experimental populations (Sen and Churchill, 2001) some of the most recent methods are based on simultaneous scans and randomization tests that detect QTLs that do not have individual effects (Carlborg and Anderson, 2002). Such approaches have led to the identification of many, statistically reliable, novel epistatic QTLs.

A majority of the previous reports on QTL analysis in chickpea did not perform any interaction (QE, QQ and QQE) studies. QTL analysis with a provision for detecting these interactions would generally avoid the biased estimate of main effect QTLs and increase the success rate in marker assisted selection (MAS) (Kumar *et al.*, 2007). The two locus analysis performed in this study revealed that the QTLs involved in QE interactions were mainly M-QTLs; while the QTLs involved in QQ / QQE interactions were the QTLs, which had no main effects. This suggests that many of these QTLs have no main effects and exercise their effects through interactions with other QTLs, which are either main effect QTLs or epistatic QTLs. Among the 6 M-QTLs, three were involved in QE interactions, which were detected in up to two environments. Some of the QTLs were stable across the environments and were not involved in QE interactions. Of the 12 E-QTLs detected, all QTLs were involved in either QQ or QQE interactions. In addition to these QQ interactions, there was one instance (for Brp) where the same QTL (*QBrp.ncl-4.1*) was involved in two epistatic interactions. These interactions appear to be pleiotropic interacting with two different QTLs (Table 3.21).

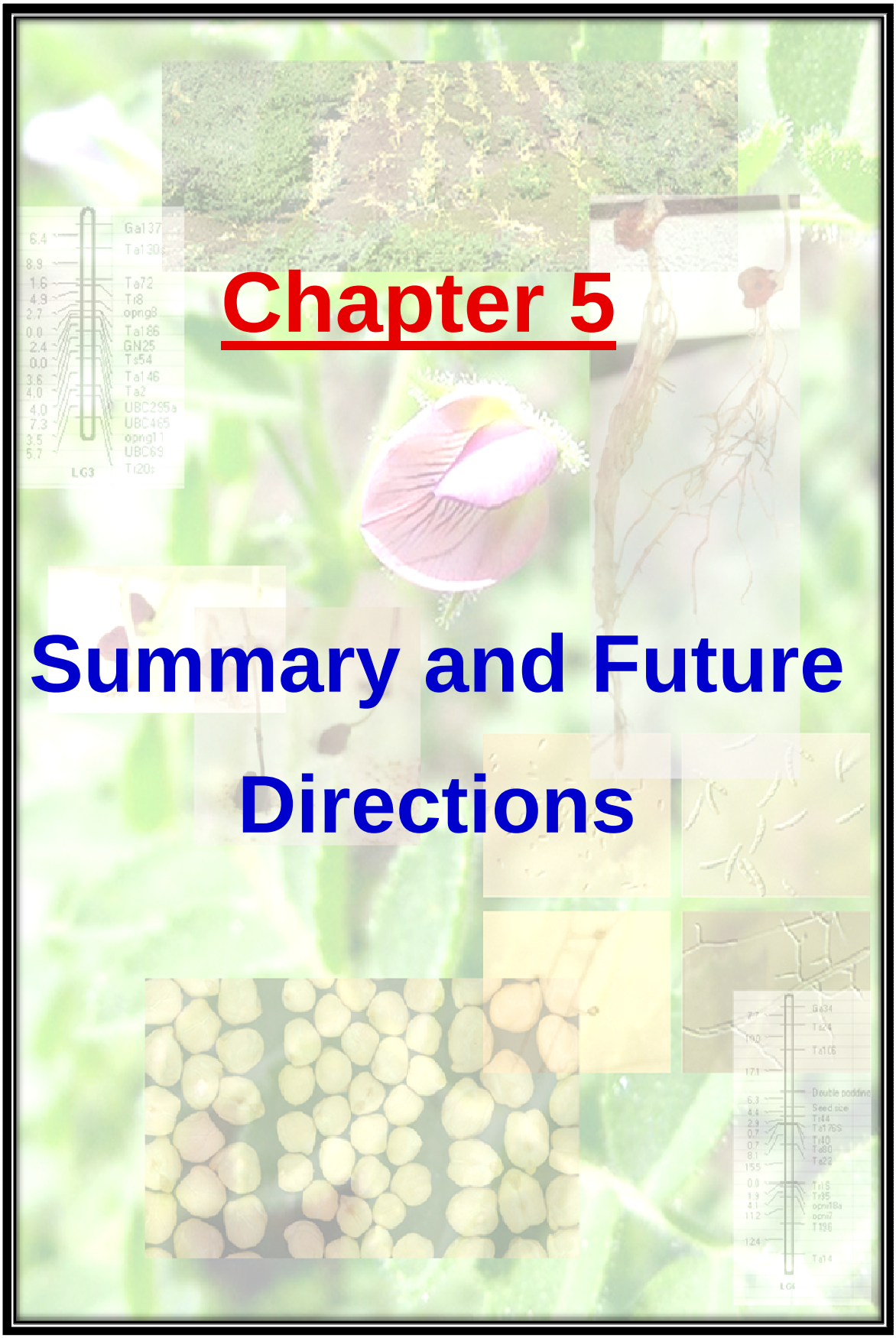
The results of the present study reconfirm that the genetics of yield and yield components is highly complex and the component traits are governed by a large number of major and minor QTLs. Further, these QTLs may have only main effects

and/or they may be involved in epistatic (QQ) or environmental (QE, QQE) interactions. The magnitudes and directions of the additive effects of individual QTLs may also vary due to genetic background of different genotypes and due to epistasis-by-environment interactions. The use of RIL population and performing interaction analyses in the present study permitted detection of many QTLs, some of which were stable across environments.

4.8 Future strategy

The salient challenge of applied genetics and functional genomics is the identification of genes underlying a trait of interest so that they can be exploited in crop improvement programmes (Rensink and Buell, 2005). Modern quantitative genetics is useful for investigating specific properties of individual genes contributing to quantitative traits, through QTL mapping (Paterson, 1995), but classical quantitative genetics describes the aggregate behavior of suites of genes influencing a trait. Though functional genomics will help in identifying the candidate gene responsible for any QTL, epistatic interaction of genes due to genome plasticity makes it possible to produce various phenotypes from little genetic variation (Morgante and Salamini, 2003).

Marker-assisted selection in plant breeding is a promising application of biotechnology. Many valuable crop traits are quantitative in nature, exhibiting substantial environmental variance. Most quantitative traits are influenced by multiple quantitative trait loci (QTLs), which may have different effects within any given environment. The presence and importance of genotype-by-environment interaction (GE) has long been recognized in the testing and recommendation of plant varieties, and no sensible producer would grow a plant variety based on information from a single environment. Information on performance relative to other varieties within a target environment is essential and, since target environments are somewhat unpredictable, knowledge of the nature of GE is also important. The same reasoning must be applied to the discovery and deployment of QTLs. QTL identification must be based on phenotypic data from multiple environments that are representative of a range of target environments, and QTL-by-environment patterns must be investigated before the usefulness of a QTL can be determined.



Chapter 5

Summary and Future Directions

Chickpea is the third most important pulse crop in the world behind dry bean (*Phaseolus vulgaris* L.) and field pea (*Pisum sativum* L.), and India is the largest producer and consumer where as Australia is the largest exporter (FAOSTAT, 2008). Despite a proposed yield potential of 6 metric tons/ha (Singh 1987), actual yields have remained low compared with other pulses (world average ~0.8 metric tons/ha; FAOSTAT 2008), mainly because of biotic and abiotic stresses that reduce yield and yield stability. The necrotrophic foliar fungal disease Ascochyta blight (*Ascochyta rabiei* (Pass.) Labrousse) and the soil-borne necrotrophic fungal disease Fusarium wilt (*Fusarium oxysporum* f. sp. *ciceris*) are considered the most serious biotic stresses. Other minor diseases of chickpea are more geographically localized and include pod borer (*Helicoverpa armigera*) in Australia and India. To accelerate molecular breeding efforts for the discovery and introgression of stress tolerance genes into cultivated chickpea, molecular and functional genomics approaches are rapidly growing. Recently, a series of genetic tools for chickpea have become available that have allowed high-powered functional genomics studies to proceed, including a dense genetic map, large insert genome libraries, expressed sequence tag libraries, microarrays, serial analysis of gene expression, transgenics and reverse genetics.

The chickpea cultigen contains high morphological variation, but narrow overall genetic variation, from which many desirable or economically important traits may have been excluded through selection (Abbo *et al*., 2003). For the desirable but missing traits from advanced breeding programs, such as durable resistance/tolerance to the many major biotic and abiotic stresses, breeders have begun to source germplasm more widely, from landraces and closely related species. To speed up the process of recombining 'wild' genes into elite genotypes, molecular tools have been integrated with classical breeding approaches. This has included the generation of molecular markers linked to the genes conditioning desirable traits, for efficient pyramiding of the traits. Molecular markers associated with QTLs for resistance to biotic stresses and some morphological traits have been located on both intra-specific and interspecific linkage maps and, importantly, chickpea genotypes tolerant to most major biotic and abiotic stresses have been identified (Millan *et al*., 2006). The use of resistant or high yielding cultivars is considered to be the most efficient and effective means of controlling major stresses or increasing the productivity.

5.1 Summary

In the present work an intra-specific map of chickpea has been developed which is used as a platform to locate the genes for fusarium wilt resistance genes (*foc1*, *foc2* and *foc3*) and to identify the genomic regions controlling the QTLs for yield and related traits.

5.1.1 Construction of framework linkage map

For linkage analysis ninety-three RILs were randomly selected from F_9 population. DNA was extracted from individual lines according to the method described by Doyle and Doyle (1987) and Simon and Muehlbauer (1997) with some modifications. The primers used in the present study included 800 RAPDs (UBC1-800), 100 ISSRs (UBC801-900), 24 RGAs, 1 ASAP (CS27) and 504 chickpea SSRs and 100 EST-SSR primers. Optimal PCR conditions were established for each primer type. All the PCR products were run on 2% agarose gel for RAPD and ISSR products. SSR products were resolved on either metaphor gel or in PAGE gels depending on their allelic size difference. All the marker loci were scored at least twice to minimize interpretation errors. The genotypic data for RIL population was generated using 156 SSRs, three ISSR, ten RAPDs and one ASAP marker. For linkage analysis, JOINMAP ver 3.0 (van Ooijen and Voorrips 2001) was used. Analysis was done by using 175 polymorphic markers and constructed the framework map. The recombination frequency (0.4) and LOD value (3.0) were used as threshold limits for linkage group construction.

The linkage analysis revealed eight linkage groups with 135 markers (120 SSRs, 9 RAPDs, 1 ASAP, three fusarium wilt resistance genes (*foc1*, *foc2* and *foc3*) and two yield related qualitative traits (double podding and seeds per pod) (Fig 3.2). This map covered 568.6 cM with an average marker density of 4.21 cM. Forty markers comprising one RAPD, three ISSRs and thirty six chickpea SSRs were unlinked. Linkage groups were assigned to previously reported LG numbers when the groups had two or more SSR loci that had been assigned to a particular chickpea chromosome in previously published skeletal chickpea map which is being used as a reference map (Winter *et al.*, 2000).

LG3 was the longest linkage group with 21 markers and spanned 102.1 cM with an average marker density of 4.85 cM (Fig 1). LG1 was the densest linkage group with a marker density of 2.14 cM and had 37 markers spanning 77.3 cM. The LG2 had 28 markers spanning 89.5 cM and shared many markers from LGII of interspecific map developed by Winter *et al* (2000). The *Sfl* (double podding) gene was also mapped on LG6 and was flanked by two STMS markers TA80s and TA106s. LG5 spanned 58.9 cM with 25 markers with marker density of 4.21cM. The LG7 and LG8 comprised all the newly developed STMS markers. In LG8 RAPDs are more than STMS markers. These LGs lacked common markers and could not be compared with the LGs of Winter *et al.* (2000) map. Inversions were observed with respect to marker orders in all linkage groups between the present and the interspecific map of Winter *et al.* (2000).

The correlation between number of markers on each LG and length of the respective LG gave an indication of distribution of markers over the linkage groups. These correlation coefficient was 0.58 ($P < 0.001$) for the intra-specific map, which indicated less random distribution of markers among the LGs. Of the 135 markers mapped in this population, 40 markers did not segregate according to the expected Mendelian ratio ($P < 0.001$). Different marker types exhibited different levels of skewness; however, SSRs were the most distorted.

5.1.2 Molecular mapping of fusarium wilt resistance genes

Fusarium wilt is a widespread and serious chickpea disease caused by the soil borne fungus *Fusarium oxysporum* f.sp. *Ciceri* (Foc). Breeding for fusarium wilt resistance is challenging due to variability of races and lack of high throughput screening techniques. Here we studied three fusarium wilt races (1, 2 and 3) in controlled conditions to identify DNA markers linked to the resistance genes for the pathogen races. Recombinant inbred lines developed from the cross of JG62 (susceptible) x Vijay (resistant) screened separately for their disease reaction to Foc races 1, 2 and 3.

Reactions of the chickpea lines for Foc races 1, 2 and 3 were assessed following the independent inoculations with respective isolates of Foc in pot culture experiments. Disease screening allowed unambiguous classification of resistant and susceptible phenotypes. Among the 100 RILs, 55 RILs were resistant and 45 were

susceptible to Foc1, whereas for Foc2, 49 were resistant and 51 were susceptible (Table 3.4). The RILs also segregated in 1:1 ratio for resistance and susceptibility to Foc3, indicating that resistance to each race was monogenic in this population. The chi-square analysis of disease reaction data of the RILs indicated a good fit to the 1:1 segregation ratio expected for single genes conferring resistance to each of the three Foc races.

All the three Foc genes were mapped with new closely flanked markers on LG2. The STMS marker H3A12 was mapped on one side of the *foc1* locus at 3.9 cM, whereas TA110 was mapped on the opposite side at a distance of 2.1 cM. The *foc3* gene was mapped 0.2 cM from H1B06y and marker, TA194, at 0.7 cM, flanked the gene on the other side. The TA96 and H3A12 markers were flanked the *foc2* locus at 0.2 and 2.7 cM, respectively. These markers were also tested for their usefulness in other breeding lines for validation indifferent genetic background. The identification of markers linked to wilt resistance genes will be useful to understand their evolution, mechanisms of resistance and their exploitation in wilt resistance breeding and its management.

5.1.3 QTL analysis of yield and yield related traits

Eight yield and yield traits from RIL population were evaluated. The data were recorded from five environments [Rahuri (I: 2003, II: 2004, III: 2005) and Dharwad (IV: 2006 and V: 2007)]. The parental genotypes used to develop the mapping populations were distinct for all the traits. These traits showed good fit to the normal distribution in the population. Transgressive segregants were observed in all the environments. Comparisons between the best parent and the best RIL showed a significant difference for all the traits. The population mean was higher than the better parent for Psp, Brp, Pdp and Yld. The highest positive correlation was observed between Yld and Pdp (0.866), followed by Psp and Brp (0.777) as well as Pdp and Brp (0.693). Yield showed significant positive correlation with Pht, Brp, Pdp and Swt.

Eighty significant QTLs (LOD $\geq$ 3.0) were identified for the yield related traits. QTLs were mapped on the respective linkage groups. The number of significant QTLs for individual traits ranged from three (Psp) to eighteen (Pdp). A total of 18 significant QTLs were detected for the Pdp followed by 14 QTLs for Brp, while only

three QTLs were detected for Psp. The marker NCPGR80 was associated with QTLs for seven traits *viz*. Pht,Psp, Brp, Pdp, Dmt, Dfl_{50} and Yld, while TA64 was associated with QTLs for four traits *viz.* Pht, Swt, Dfl_{50} and Dmt. The highest phenotypic variation of 43.2% was explained by *QSwt.ncl-1.2*, followed by *QDmt.ncl-2.2* (36%). Most of the detected QTLs were environment specific and only 10 of the 80 QTLs expressed in more than one environment. This was particularly evident in case of Pdp QTLs, where 17 of the 18 Pdp QTLs were environment specific, on the contrary five of the fourteen Brp QTLs expressed in more than one environment. Among the QTLs identified, LG1 was associated with most of the QTLs (33), wherein the QTLs were clustered in three groups (Fig 3.12). LG2 also had at least one QTL for each trait. Brp and Pdp QTLs were distributed across six LGs, except LGs 6 and 7, while the three Psp QTLs were mapped on the LGs 2, 4 and 5.

The parent Vijay exhibited higher phenotypic values than JG62 for six of the seven traits except Pht. Among six of the twelve Pht QTLs, Vijay alleles decreased plant height in the population while the JG62 allele increased the trait value. Similarly for Psp, Pdp, Yld and Swt QTLs, the alleles from Vijay positively influenced the phenotypic values. On the contrary, JG62 alleles influenced seven of the nine Dmt QTLs and reduced the days to maturity.

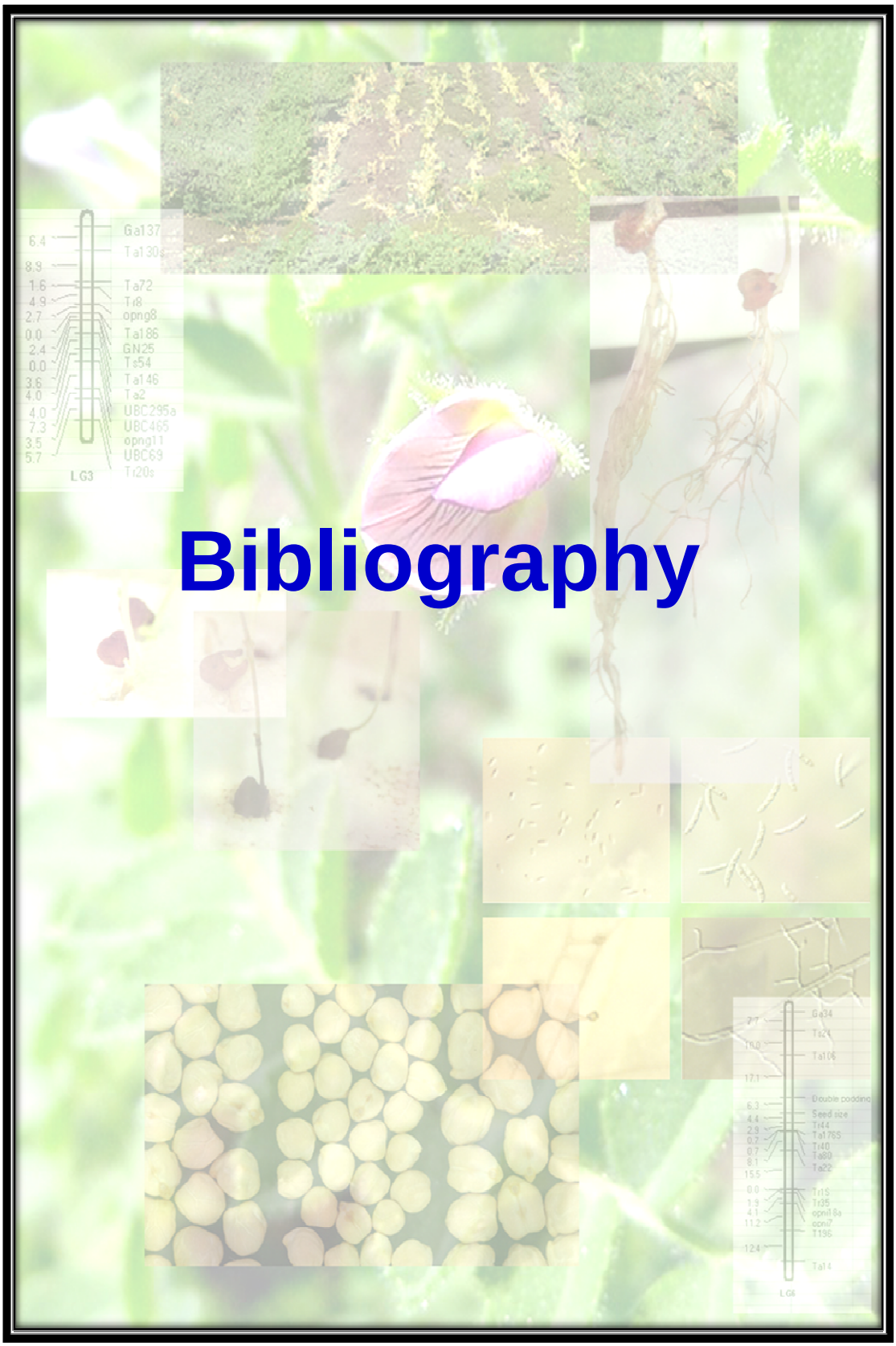
QTL interactions were studied by using QTL Network software. QTLs are mainly divided into main effective and epistatic QTLs. Main effective QTLs have their own genotypic effect and some time they would show interaction with environment. Epistatic QTLs are usually involved in QTL x QTL interactions as well as QTL x QTL x Environment interactions. Six traits showed QTL (Pht, Psp, Brp, Pdp, Dmt and Swt) QE and QQE interactions. Two of the five M-QTLs were also identified through single locus CIM analysis either in the same and/or adjacent marker intervals. Three M-QTLs (*QPsp.ncl-4.1, QBrp.ncl-3.2 and QPdp.ncl-4.1*) exhibited QE interactions. Remaining three QTLs were main effect QTLs which have their own individual effect without any interaction with environment. The epistatic analysis revealed six QQE interactions involving twelve QTLs.

5.2 Future directions

A major aim of chickpea breeding is the development of cultivars with adequate resistance/ tolerance to yield-reducing stresses. Based on this thesis work we propose

few future works that have to be done to improve the chickpea productivity. The future directions are as follows

- Integration of intra-specific maps to increase the marker density by using available chickpea markers and maps.
- Development of more STMS and EST markers for chickpea
- Increase the marker density around the mapped Fusarium wilt resistance genes (*foc1, foc2 and foc3*)
- Identification of *foc4* and *foc5* genes in the same population or pyramiding into the same population or in single popular variety.
- Study the interactions of chickpea with different races of *Fusarium oxysporum* sp. Ciceris to understand the mechanism of pathogen infection and host response.
- Positional cloning of FOC resistance genes
- Validation of identified major QTLs for yield related traits in different environments with using larger population size.
- Fine mapping to dissect the chromosomal regions governing the yield related traits.
- Study of interactions between QTLs and differentiate between main effective and epistatic QTLs.
- Mapping of yield and yield related traits by using association mapping approach and compare the results of linkage mapping and association mapping.
- To establish collaborative efforts with Chickpea Genomics Consortium (www.icgc.wsu.edu) that could allow for high-powered studies on ideal germplasm lines; thus, fast-tracking the overall development of resistant/tolerant cultivars.
- Creating the database and bioinformatics resource capable of integrating and mining chickpea structural and functional genomics data.
- Development of many new and powerful techniques, such as expression QTL mapping and whole-genome sequencing.
- The use of information from studies of model legumes for comparative genomics this promises to enhance breeding efforts in chickpea.



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TECHNICAL SKILLS

- Experience in molecular genetics field such as DNA sequencing, PCR, RAPD, ISSR, SSR, cDNA-AFLP, RNA isolation, plasmid isolation and general biochemical techniques
- Breeding techniques in self- and cross-pollinated crops
- Familiar with computer programs for linkage analysis/mapping like Mapmaker, Join Map, QTL Cartographer, QTL network etc...
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EDUCATION

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- B.Sc. (Agriculture) from University of Agriculture Sciences, GKVK, Bangalore, India (2001)

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AWARDS

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PUBLICATIONS

1. Radhika P, **Gowda M**, Kadoo NY, Mhase LB, Jamadagni BM, Chandra S, Sainani MN, Gupta VS (2007) Development of an integrated intraspecific map of chickpea (*Cicer arietinum* L.) using two recombinant inbred line populations. *Theoretical and Applied Genetics*, **115**(2): 209-216.
2. **Gowda M**, Radhika P, Kadoo NY, Mhase LB, Jamadagni BM, Chandra S, Sainani MN, Gupta VS (2009) Mapping of yield and related QTLs in chickpea (*Cicer arietinum* L.) using two recombinant inbred line populations. (Communicated to *Theoretical and Applied Genetics*.)
3. **Gowda M**, Radhika P, Kadoo NY, Mhase LB, Jamadagni BM, Chandra S, Sainani MN, Gupta VS (2009) Mapping of wilt resistant genes (*foc1,2* and *3*) in chickpea (*Cicer arietinum* L.). (Communicated to *Molecular Breeding*)
4. **Gowda M**, Radhika P, Kadoo NY, Mhase LB, Jamadagni BM, Chandra S, Sainani MN, Gupta VS (2009) Mapping of stable QTLs for yield and related traits in chickpea (*Cicer arietinum* L.). (Manuscript under preparation)
5. **Gowda M**, Randhawa GJ, Phirke PK, Bisht IS, Dhillon BS (2009) Diversity analysis of coloured rice germplasm using morphological and molecular markers (RAPD and SSRs) (under review in Annals of Applied Biology)

Review Articles

1. Gupta VS, **Gowda M**, Kadoo NY, Radhika P and Sainani MN (2005) Quantitative Trait Loci (QTL) Mapping and Marker Assisted Selection (MAS) in Food Legumes. International Food Legumes Research Conference -IV, held at IARI, New Delhi from Oct 18-22, 2005

Posters Presented

1. **Gowda M**, Radhika P, Kadoo NY, Jamdagni BM, Mhase LB and Gupta, VS (2005) Mapping of agro-economically important traits in chickpea (*Cicer arietinum* L.). Poster presented in International Food Legumes Research Conference -IV, held at Indian Agricultural Research Institute, New Delhi, India, from Oct 18-22, 2005.
2. **Gowda M**, Radhika P, Kadoo NY, Jamdagni BM, Mhase LB and Gupta, VS (2005) Mapping of QTLs for yield traits in chickpea (*Cicer arietinum* L.). Poster presented in International conference on New Horizons in Biotechnology (NHBT), held at NIIST, Trivendrum, Kerala, India, from Nov 26-29, 2007.
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