

**MOLECULAR EVIDENCE TO *IN VITRO* POLLEN BIOASSAY
FOR WILT RESISTANCE IN CHICKPEA**

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MARCH, 2007

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FOR WILT RESISTANCE IN CHICKPEA**

*Thesis submitted to the
University of Agricultural Sciences, Dharwad
in partial fulfillment of the requirements for the*

Degree of

Doctor of Philosophy

in

GENETICS AND PLANT BREEDING

By

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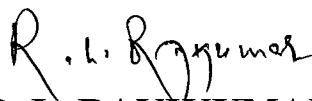
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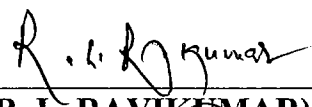
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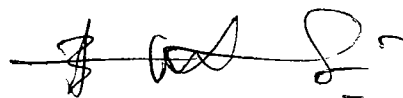
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Affectionately Dedicated
to
Beloved Parents
Smt. Ratnamma , Sri Bhushanam
& Brother Ravi

ACKNOWLEDGEMENT

I wish to express my profound sense of gratitude to **Dr. R. L. Ravikumar**, Associate Professor, Department of Genetics and Plant Breeding, University of Agricultural Sciences, Dharwad and chairman of my Advisory Committee for his guidance, timely advises and encouragement during the course of my research and preparation of the thesis.

I am also grateful to other members of my Advisory Committee, **Dr. P. M. Salimath**, Professor and Head, Department of Genetics and Plant Breeding. **Dr. M. V. C. Gowda**, Professor, Department of Genetics and Plant Breeding, **Dr. S. Lingaraju**, Professor, Department of Plant Pathology and **Dr. B. Fakrudin**, Associate Professor, Department of Plant Biotechnology for providing expert advice in their area of specialization and critical reviewing of the manuscript.

I express my heartfelt thanks to my seniors, juniors, friends and loved ones for their pleasant company, affectionate encouragement and needy inspiration during the entire period of study.

Above all I am very grateful to my father Sri Bhushanam, mother Smt. Ratnamma and brother Ravi whose constant encouragement is the source of inspiration in obtaining the Ph.D.

I won't miss this opportunity to acknowledge the **CSIR** for providing the financial assistance in the form of fellowship during my Ph.D. programme.

DHARWAD
MARCH, 2007


(D. Ratna Babu)

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Introduction

I. INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the only cultivated species within the genus *Cicer*. The crop is self pollinated diploid ($2n=2x=16$) with a relatively small genome (740 Mb). With over 10 million ha under cultivation, chickpea is second only to common bean (*Phaseolus vulgaris*) and third in production among the legumes. It is commercially grown as a cool season crop in a range of climates across the world from Mediterranean to sub-tropical. World production was 7.5 million tonnes in 2003 from an area of 10.3 million hectares (Anonymous, 2004). In recent years, chickpea production has expanded into new regions, including Australia and North America and into marginal land of traditional chickpea production regions in India, Pakistan, Iran, Turkey, Mexico and Ethiopia. The major producers, viz. India, Pakistan and Turkey contribute 65 per cent, 9.5 per cent and 6.7 per cent, respectively to the world harvest (Anonymous, 2005). The current average seed yield of chickpea is only 750 kg/ha (Anonymous, 2004). Despite its broad adaptation, production is restricted by several biotic and abiotic stresses. Among the biotic stresses, *Fusarium* wilt is the major disease limiting the chickpea productivity.

Wilt of chickpea, caused by *Fusarium oxysporum* Schelectendahl, Fries. f.sp. *ciceris* (Padwick) Matuo and Ic. Sato, was first described by Padwick in India (Padwick, 1940) and has since been reported from several other countries (Nene, 1980). It is a serious problem in India, Iran, Pakistan, Nepal, Burma, Spain and Tunisia. In India, it is estimated to cause 10 per cent annual yield loss (Singh and Dahiya, 1973). Likewise an estimated annual loss of 12 million rupees was reported from Pakistan (Sattar *et al.*, 1953). Under favourable conditions it may cause more than 90 per cent yield loss (Srivastava *et al.*, 1984; Gupta *et al.*, 1986; Jimenez-Diaz *et al.*, 1993 and Cortes *et al.*, 2000). The fungus is soil-borne and survives through chlamydospores on seeds (Haware *et al.*, 1978). The pathogen can survive in the soil

for more than five years even without its host. Lentil, pigeonpea and pea were identified as symptomless carriers of the fungus (Haware and Nene, 1982).

There are eight races of *F. oxysporum* f.sp. *ciceris* (race 0, 1A, 1B/C, 2, 3, 4, 5 and 6) which are identified by differential disease reactions on a set of chickpea cultivars (Haware and Nene, 1982; Jimenez-Diaz *et al.*, 1993; Phillips, 1988). Race 1A has been reported in India (Haware and Nene, 1982), California and the Mediterranean region (Jimenez-Diaz *et al.*, 1993). However, despite variation in symptom type, race and geographical distribution, all *F. oxysporum* f. sp. *ciceris* isolates tested so far are in a single vegetative compatibility group (Nogales-Moncada, 1997). Race 1 is widespread in central and peninsular India and race 2 in northern India. Race 3 and 4 are location specific and are prevalent in the Punjab and Haryana states of India (Haware *et al.*, 1992).

Among the four prevailing races in India, race 1 is widespread and most destructive race in central and south India. It has been reported that at least three independent loci govern resistance to race 1 of *Fusarium* in chickpea (Singh *et al.*, 1987a). However, earlier work on *Fusarium* wilt at this centre (Brindha and Ravikumar, 2005) and elsewhere (Upadhyaya *et al.*, 1983a,b) indicated that two major independent loci h_1 and h_2 , effectively govern resistance to race 1 in chickpea. The dominant alleles at both h_1 (H_1) and h_2 (H_2) loci result in early wilting, while recessive at either one ($h_1h_1H_2$ or $H_1h_2h_2$) produce late wilting, whereas recessive at both the loci ($h_1h_1h_2h_2$) result in resistance.

Because of the diverse, widespread, soil borne nature and capacity to survive in soil for many seasons even without host made the pathogen very difficult to handle leading to heavy yield losses in chickpea. All most all the existing varieties including national check A-1 of chickpea are susceptible to wilt, which led to spread of the inoculum in chickpea growing areas of India. Although effective fungicides have been

identified, they are impractical at field level. Hence, the best strategy to manage this disease is through use of resistant cultivars. In spite of availability of resistant sources in germplasm, lack of simple screening technique for selection of resistance is the major limiting factor in the development of resistant varieties. The conventional approach of screening in wilt sick plots, which is being used for the past many years, had its own limitations like difficulty in maintenance of uniform sick plots, presence of other soil borne fungi, laborious and time consuming nature of screening.

Attempts have been made to develop alternate approaches like development of molecular markers and marker based screening/selection for resistance. In this direction ASAP marker CS-27₇₀₀ (Mayer *et al.*, 1997) linked to H_1 locus of wilt susceptibility and RAPD markers OPA-07₁₆₄₄, OPK-09₁₈₀₁ (Thippeswamy *et al.*, 2005) and A07C₄₁₇ (Soregaon *et al.*, 2007) linked to H_2 locus were developed. The marker assisted selection is comparatively quick, robust and possible theoretically for every kind of trait. However, marker assisted selection (MAS) is highly sophisticated and expensive. It is yet to demonstrate its superiority over conventional selection process (Vasil, 1990; Lockhart and Winzeler, 2000).

Alternatively, a simple but effective technique of screening the genotypes based on the performance of their pollen grains is emerging as a viable alternative to sporophytic screening and/or molecular marker assisted selection. Gametophytic selection can be easily combined with conventional breeding methods to obtain higher efficiency (Hormaza and Herrero, 1996). It has been shown time and again that two phases – gametophytic (haploid) and sporophytic phase (diploid) – do share common gene expression profile (Tanksley *et al.*, 1981; Sari-Gorla *et al.*, 1986; Pedersen *et al.*, 1987; Willing *et al.*, 1988; Ottaviano and Sari-Gorla, 1993; and Hormaza and Herrero, 1992). Therefore, the selection at the haploid stage would be relevant for a particular trait (particularly resistance to biotic and abiotic stress tolerance) at haploid

stage would reflect the performance at sporophytic stage of the trait. Accordingly a variety of selection pressures such as temperature (Frova *et al.*, 1986; Sato and Peet, 2005), moisture (Mulcahy *et al.*, 1988; Patil *et al.*, 2004), salinity (Koval, 2000), ozone (Feder, 1986), pathotoxin (Bino *et al.*, 1988; Sharma and Tewari, 1996; Shobha Rani and Ravikumar, 2006), herbicide (Sari-Gorla *et al.*, 1989) were enforced during gametophytic generation. The responses of gametophyte to selection pressure were found very effective in identification and selection of genotypes at sporophytic level. Such results have brought considerable attention to gametophytic selection as a new tool in crop improvement methods.

Despite the encouraging results and promising potential of gametophytic selection of plants with desirable traits, there are only a few reports of its use in conventional breeding (Shobha Rani and Ravikumar, 2006; Clarke *et al.*, 2004). It is yet to receive a wide spread acceptance among plant breeders. There were some instances where gametophytic response failed to show its association with sporophytic response (Ottaviano and Mulcahy, 1989; Landi *et al.*, 1989), especially in case of quantitative traits as many genes with small effects control the phenotype (Pfahler, 1983; Landi *et al.*, 1989). Further most of the earlier investigations on similarities of gametophytic and sporophytic responses were based on only the limited number of genotypes and the results were not validated and confirmed across a range of germplasm lines. Another important reason for non-exploitation of pollen bioassay in plant breeding was lack of genetic evidence. In this direction the present investigation on *in vitro* pollen bioassay for resistance to *Fusarium* wilt of chickpea, aims to track the inheritance of pollen response to pathotoxin as a trait in recombinant inbred lines and progenies of different crosses. Furthermore, the study also makes an attempt to track the association between sporophytic and gametophytic generations for resistance to wilt in chickpea through the use of molecular markers and thereby provide genetic

evidence of *in vitro* pollen bioassay as a screening technique for wilt resistance in chickpea at allelic level.

The preliminary studies at this centre have shown that the fungal toxin, fusaric acid has effect on *in vitro* pollen germination and tube growth (Ravikumar *et al.*, 2002). The present investigation was taken up to develop *in vitro* pollen bioassay as an effective and reliable screening technique to *Fusarium* wilt resistance in chickpea. The specific objectives of the study are

1. Evaluation of selected germplasm and parental lines of chickpea for wilt resistance
2. Validation of identified molecular markers for both H_1 and H_2 loci of wilt resistance in selected genotypes
3. Standardization of *in vitro* pollen bioassay as a screening technique for wilt resistance
4. Association between sporophytic and gametophytic reaction to wilt resistance in recombinant inbred lines
5. Selection of F_5 generation progenies based on *in vitro* pollen bioassay for wilt resistance and its validation in F_6 generation using molecular markers
6. A comparative analysis of selection of genotypes for wilt resistance based on pollen bioassay and molecular markers linked to wilt resistant loci

Review of Literature

II. REVIEW OF LITERATURE

The important aspect in any plant breeding programme is creation of variability and selection. There are two phases *viz.*, diploid and haploid gamete phases in the life cycle of plant which may be available for any kind of selection. However, the only phase extensively used is diploid phase (plant) in spite of several advantages of selection at pollen (haploid) phase over diploid stage. The utility of gamete selection in crop improvement was proposed by Stadler in 1940s as an indirect selection rather than direct utilization of pollen. However, there are not many instances where haploid phase was exploited in plant breeding programmes. Recently Mulcahy (1979) through their pollen competition studies showed that gamete or haploid assay and selection play an important role in sporophytic performance. Followed by that evidence, several workers have shown that there is an overlap in gene expression of gamete and sporophytic phase using biochemical and molecular evidences.

In the present study attempts have been made to show the association between *in vitro* pollen reaction and *Fusarium* wilt reaction of chickpea genotypes. The wilt resistance in chickpea was used as a model system to show the efficiency of pollen bioassay in comparison to molecular marker based selection for selection of wilt resistance in chickpea. The literature available on male gametophytic selection in different plants, *Fusarium* wilt resistance in chickpea and genetic variability studies for seed yield and yield components is reviewed under the following headings.

2.1 Pollen/male gamete bioassay and selection

2.2 Chickpea *Fusarium* wilt resistance: A model system for pollen bioassay

2.3 Genetic variability and association for yield and its components

2.1 POLLEN BIOASSAY AND SELECTION

A general phenomenon in plants is that they have two phases, sporophytic or diploid and gametophytic or haploid, which alternate in the life cycle. However, it was evident that the gametophytic phase is diminishing in terms of both size and length compared to the sporophytic phase (Heslop-Harrison, 1979). In angiosperms this reduction has reached its peak since the male gametophyte (the pollen and pollen tube) consists of just three haploid cells (the vegetative cell and two sperm cells) and the female gametophyte (embryo sac) of six haploid cells (three antipodals, two synergids and the egg cell) and one binucleate cell (the central cell).

For the past many years research in plant breeding has been directed towards the sporophytic phase, whereas the influence of the gametophytic phase was considered as “passive”. There are, however many studies that deal with basic aspects of angiosperm gametophyte from pollen development, structure, pollen germination, tube growth, pollen-pistil interaction increase and fertilization process (Mascarenhas, 1989, 1990). It is only during 1970’s that the possibility of the gametophytic selection in plant breeding was put forward.

Though the method of gamete selection was suggested as early as in 1940’s (Stadler, 1944), much progress was not made in this direction. However in the last two decades, new evidences suggest that the gametophyte exists independently for a period that expresses its own genetic information. When subjected to selection, it can influence the genetic constitution of the resulting sporophytic generation (Mulcahy, 1979). Pollen competition hypothesis (Mulcahy, 1979) was based on the hypothesis that under pollen competition, only the fastest growing pollen grains achieve fertilization leading to vigorous progeny (Ottaviano *et al.*, 1983; Quesoda, 1996). Two main lines of evidence support the hypothesis that selection on the gametophytic phase occurs in nature (Hormaza and Herrero, 1994). One is the overlap in the genetic

expression between the gametophytic and the sporophytic generations, that results in the correlation between both generations in their behaviour against different external factors. The other is the competition among male gametophytes in the pistil that results in differences in the vigour of the next sporophytic generation depending on the intensity of the competition.

2.1.1 Pollen gene expression

The gene expression at pollen grain level was evidenced by expression of proteins and nucleic acids in the gametes.

2.1.1.1 Proteins

One of the first studies pointed out that the activity of alcohol dehydrogenase in maize is expressed in the sporophytic and gametophytic phases (Schwartz and Osterman, 1976). However, the key moment was in 1981 when Tanksley *et al.* (1981) published their work on tomato and concluded that 62 per cent of the isozymes studied were expressed in gametophyte, 58 per cent were expressed in both phases and 3 per cent only in pollen indicating 95 per cent of overlap. The overlap rates seems to be very similar, for example 72 per cent in *Zea mays* (Sari-Gorla *et al.*, 1986), 81 per cent in *Prunus* (Weeden, 1986), 60 per cent in barley (Pedersen *et al.*, 1987) and between 74 and 80 per cent in populus (Rajora and Zsuffa, 1986).

Pollen gene expression was conclusively proved in case of maize alcohol dehydrogenase gene expression (Ottaviano and Mulcahy, 1989) where heterozygous F_1 produced stained (adh^+) and unstained (adh^-) pollen in 1:1 ratio. Similar evidences were found in case of β -galactosidase enzyme activity, small pollen traits, amylase or amylopectin grain content (Ottaviano and Sari-Gorla, 1993) and Rf_3 a fertility restoration factor for MS-S cytoplasm (Laughman and Gabay, 1973) in maize.

2.1.1.2 Nucleic acid overlap

The hybridization between mRNAs synthesized by the gametophyte and cDNAs from the sporophyte was studied in *Tradescantia paludosa* (Mascarenhas *et al.*, 1986) and found about 30,000 different mRNAs in the sporophyte shoot tissue and 20,000 in the gametophyte. Hybridization results have shown 60 per cent overlap. The per cent of overlap may still be increased by studying additional diploid tissue also (Hormaza and Herrero, 1992).

Apart from the above, mRNA analysis of pollen revealed presence of 20,000-24,000 different mRNAs in maize (Willing and Mascarenhas, 1984) and about 65 per cent of pollen mRNA are similar to that of sporophytic stage (Willing *et al.*, 1988). Post meiotic activation and accumulation of different sets of mRNAs at different stages of pollen development suggest the major role of those genes during pollen germination, early tube growth and maturation (Ottaviano and Sari-Gorla, 1993). In *Brassica* (Ruiter *et al.*, 1999) with regard to water-stress-inducible genes (*bopc-15* and *bopc-34*). There was overlap in leaf (diploid) and anther (haploid) tissues. All of these reports indicate pollen gene expression and an overlap occurring to a greater extent in the genetic expression of both sporophytic and gametophytic phases.

2.1.2 Correlated response between sporophytic and gametophytic phases

Three kinds of genes expression can be envisaged according to their expression in the gamete and sporophytic phases (1) genes expressed only in the sporophyte (diploid expression) (2) genes expressed only in the gametophyte (haploid expression) and (3) genes expressed in both gametophyte and sporophyte (haplo-diploid expression). The third group is suitable for gametophytic selection. Genes expressed only in sporophyte can also be used but should be closely linked to

genes expressed in gametophyte (Hormaza and Herrero, 1992). Along with the overlap of genetic expression there is a need of correlation between two phases at phenotypic level. The studies on the possibility of correlated response between the two generations is hinted (Ravikumar and Chikkodi, 1998) and proved by subjecting to selection against different external agents (biotic and abiotic).

2.1.2.1 Biotic factors

Interesting studies in relation to pollen bioassay began in 1973, when it was noticed in *Zea mays* that genotypic resistance to the fungus *Helminthosporium maydis* was related to the pollen response to the toxin produced by the pathogen (Laughman and Gabay, 1973). After a gap of more than a decade, studies were taken up by Hodgkin and McDonald (1986), where they found that pollen from fungus-susceptible *Brassica* plants were also affected by the toxic compounds extracted from a culture of *Alternaria brassicicola*. They concluded that in the F₁, pollen from plants resulted from selected pollen was more resistant to the toxin than pollen from plants descended from non-selected pollen. Bino *et al.* (1988) also found that germination of pollen from different *Lycopersicon* species sensitive to *Alternaria alternaria* was lower on a media with toxin, than that of pollen from resistant plants.

Increased yields were also observed in the lines bred by pollen selection than in the original plants (Meliyan and Balashova, 1994) when pollen was selected against toxin applied stigmatic tissue. In case of *Brassica* – *Alternaria* interaction, destruxin B, a pathotoxin of *Alternaria brassica* could distinguish resistance and susceptibility differences in pollen grain bioassay (Sharma and Tewari, 1996). Further in case of sunflower, similar phenomenon was observed (Ravikumar, 1997 and Ravikumar and Chikkodi, 1998) by using pathotoxin of *Alternaria helianthi*.

Similar experiments have been carried out for different components like ethofumesate (herbicide) resistance in sugar beet (Smith, 1986), chlorsulfuron (herbicide) resistance in maize (Sari-Gorla *et al.*, 1989, Frascoroli and Songstad, 2001), kanamycin (antibiotic) resistance in tomato (Bino *et al.*, 1987) which support the association of two phases. Discriminatory role of fusaric acid, a fungal toxin between *Fusarium* wilt susceptible and resistant plant pollen in case of chickpea was indicated by Ravikumar *et al.* (2002). Earlier such role of fusaric acid was indicated by Wenzel and Foroughi-Wehr (1990) where they identified resistant barley somaclones to *Helminthosporium sativum* using fusaric acid as a selective agent in *in vitro* culture medium.

2.1.2.2 Abiotic factors

2.1.2.2.1 Temperature stress

Early studies in this field were carried out by Zamir *et al.* in 1981, demonstrating the higher competitive ability of pollen from *Lycopersicon hirsutum*, a plant that lives at high altitudes, compared to that of *Lycopersicon esculentum* grown under cold conditions. Further work (Zamir *et al.*, 1982) concluded that this effect was due to haploid genome. Furthermore, they showed that some genes responsible for cold tolerance were located in haploid genome linked to sporophytic isozymes. However, lack of correlation was observed in case of *Lycopersicon* (Maisonneuve and Den Nijs, 1984, Den Nijs *et al.*, 1986), since there were differences in responses to cold temperature between the haploid and diploid phases.

In spite of those discrepancies, there are several studies indicating significant correlation between gametophyte and sporophyte behaviour in relation to temperature

stress in maize (Frova *et al.*, 1986, Lyakh and Soroka, 1993), cotton (Rodriguez-Garay and Barrow, 1988), rice (Yo-Ichiro Sato, 1993), tomato (Fernandez *et al.*, 1995) and *Cucurbita pepo* (Johannsson and Steptenson, 1998). Most recently experiments on tomato (Sato and Peet, 2005) indicated higher magnitude of reduction in pollen release and germination at higher temperatures in temperature-susceptible cultivar compared to temperature-tolerant cultivars.

The tissues of some plant species respond to high temperature by synthesizing special proteins called heat shock proteins (HSPs) which protect the cells against thermic stress. Similar HSPs were observed under stress in mature pollen of *Zea mays* (Frova *et al.*, 1986), *Nicotiana tabacum* and *Lilium longiflorum* (Herpen *et al.*, 1989) and *Sorghum bicolor* (Frova *et al.*, 1991).

2.1.2.2.2 Moisture stress

Being a highly complex trait, screening for moisture stress tolerance, no single parameter was proven to be effective. Hence an alternative technology like pollen assay may serve the purpose. Mulcahy *et al.* (1988) in *Lycopersicon*, showed that male gametophytes produced from moisture stress tolerant varieties are more tolerant than those of non-tolerant varieties. Assessment of *in vitro* pollen performance of a few selected sunflower genotypes under PEG induced stress and the performance at field level of respective sporophytes indicate correlated response between the two stages (Keshavamurthy, 1994).

Morgan (1999) showed that the genes conditioning differences in osmoregulation in wheat leaves, are also expressed in pollen grains and corresponding allelic difference was found in pollen grains. Positive association between seedling and pollen parameters under moisture stress conditions was observed in case of sorghum (Patil *et al.*, 2004).

2.1.2.2.3 Salinity

One of the first pieces of indirect evidence on salinity tolerance was the work by Eiskowitch and Woodell (1975), where they could observe more resistant pollen of coastal plant (*Armeria maritima*) to sea water than those grown away from the sea coast. Later studies on tomato from Sari-Gorla *et al.* (1988) and Sacher *et al.* (1983) showed that the genotypes grown under salt stress produced pollen grains tolerant to salt stress. Koval (2000) indicated the contribution of gametophyte in the inheritance of salt tolerance in barley.

2.1.2.2.4 Metal toxicity

Studies consisted of crosses between copper tolerant and non tolerant clones of *Mimulus guttatus* indicated that pollen from tolerant plants was able to germinate and grow at metal concentration that clearly inhibited the germination and growth of the pollen from non-tolerant plants (Searcy and Mulcahy, 1985a). With the same plant material, further studies (Searcy and Mulcahy, 1985b) were carried out where, flowers from plants watered with a solution that contained copper and from plants watered with a control solution were pollinated with pollen from metal tolerant and non-tolerant plants. The results indicated that, there were significant differences in number of fertilized ovules and viable seeds. Correlation between sporophyte and gametophyte was also found for aluminum tolerance in *Lycopersicon esculentum* (Searcy and Mulcahy, 1990), germination of pollen from aluminum sensitive cultivars is reduced in the presence of the metal but not that of pollen from aluminum resistant cultivars.

Apart from above abiotic factors, other stress factors like ozone resistance of male gametophyte was also studied. In *Nicotiana tabacum* and in *Petunia hybrida*, it

was found (Feder and Sullivan, 1969; Feder, 1986) that pollen germination and pollen tube growth were lower after exposing the male gametophyte to high ozone concentrations *in vitro*, but that the response was variable depending on the origin of the pollen grains: pollen from sporophytes susceptible to ozone was more affected than those from less susceptible sporophytes. Similar correlation in the tolerance to ozone between sporophytes and male gametophytes were obtained by Hormaza *et al.* (1996) in several fruit and nut tree species (Rosaceae).

2.1.3 Pollen selection: An evidence for correlated response

Pollen genotype selection for genes expressed in both the haploid and diploid phases of the plant life cycle can lead to correlated response detectable in the sporophyte.

Results reported in the literature have been produced using two different criteria.

- i) Selection for pollen competitive ability and
- ii) Selection for tolerance to environmental stresses applied during pollen development in the anther or during pollen tube growth in style.

2.1.3.1 Pollen selection for competitive ability

In nature, there may often be more pollen grains on the stigma than are necessary to fertilize all of a flower's ovules, creating an opportunity for competition between pollen. The first account of pollination density affecting both mean and variance was provided by Ter Avanesia (1949, 1978) in *Vigna*, cotton and wheat. The characters affected were those expressing plant vigour and fertility. Similar results are reported for a number of different species: McKenna (1986) indicated the effect of pollen competition on sporophytic vigour in two distylous species of *Turnera*

ulmifolia; Schlichting *et al.* (1987) studied pollen competition and observed offspring variance in *Lotus corniculatus*; Stephenson *et al.* (1986) observed the effect of pollen load size on fruit maturation and sporophyte quality in *Cucurbita pepo*; Gambler and Mulcahy (1996) evidenced pre-anthesis pollen competition and association between pollen size and renner complex in *Oenothera* species; Quesada *et al.* (1996) found the effect of pollen competition on the reproductive performance in F₁ and backcross generation of cucurbit hybrids. Whereas Consuelo and White (1996) suggested that genes determining pollen size in beans have little or no effect on sporophytic traits such as seed size and seed yield.

The methods based on the variation of the distance that competing pollen tubes have to cover in the style was first suggested by Correns (1928) in *Dianthus chinensis*. The most comprehensive results by this approach have been obtained in a study of a maize population (Ottaviano *et al.*, 1982; Ottaviano *et al.*, 1988). It was shown that: (1) a maize population possess a large amount of genetic variability for pollen competitive ability, (2) while pollen germination and tube growth in the early stage are essentially sporophytically controlled, the pollen tube growth rate in later stages shows a large amount of variability controlled by genes expressed in the post-meiotic phases (3) selection acting on this variability effects competitive ability and produces correlated responses in the sporophytic generation for seedling vigour, kernel weight and root growth *in vitro*. The same method adapted to different flower structures has revealed the effect of male gametophytic selection (MGS) on the sporophytic generation in *Dianthus chinensis* (Mulcahy and Mulcahy, 1975) and in *Anchusa officinalis* (McKenna, 1986).

Better quality progeny have been claimed to be produced when pollen tubes are required to grow for longer distance or when heavy loads of pollen are applied to stigma (Mulcahy and Mulcahy, 1987). Snow and Spira (1991) studied the individual difference in per cent pollen germination. They found significant differences in pollen

tube growth rate for four of the six pairs of pollen donors in Hibiscus. They have concluded that in Hibiscus populations, individuals are more likely to differ in pollen growth rates than in pollen germinability.

2.1.3.2 Pollen selection against different stresses

A number of experimental results show that MGS can be effective for improving tolerance to environmental stresses. The first report of this effect was provided by Zamir *et al.* (1982) and Zamir and Vallejos (1983) in tomato for response to low temperature stresses. The material used was F₁ from an interspecific cross between *Lycopersicon hirsutum*, a species adapted to low temperatures and a sensitive species, *L. esculentum*. The response to selection applied to developing microspores and especially to pollen tubes growing in the style was detected by isozyme analysis. It was shown that the progeny from stressed gametophytes received a higher proportion of genome from *L. hirsutum*, the genes controlling the character are localized in two different chromosomes and are expressed in the post-meiotic phase.

Analogous results have been obtained for tolerance to salt. F₁ plants obtained from cross between the cultivated tomato, *Lycopersicon esculentum* (Salt non-tolerant) and *Lycopersicon penelli* (salt tolerant) were grown in a saline environment and in a control environment without salt (Sacher *et al.*, 1983). F₂ plants generated from selfed F₁ plants under saline stress were grown both under saline and non-saline conditions, showing a higher dry weight in both situations. This suggests that fertilization under saline stress increases both tolerance to salt and the mean vigour of the plants. With identical plant material and following the same experimental approach, Sari-Gorla *et al.* (1988) found a preferential segregation in the F₂ in favour of an isozyme marker from the salt-tolerant parent.

Positive results have been obtained in maize exposed to high temperature (Petolino *et al.*, 1990). In this experiment, the flowers were pollinated and placed in

culture media under high and normal temperature regimes during the fertilization. Later, mature plants were developed by *in vitro* culture of the embryos obtained. The results showed that plants derived from the heat-treated flowers were more heat-tolerant than plants derived from normal temperature-treated flowers. The contribution of gametophyte in the inheritance of salt tolerance was proven by Koval (2000). In his study, crosses were made between F_3 and BC_1 hybrids of the tolerant cultivars Rannii 1 and Pirkka with the sensitive cultivar K-30356 and found that the third generation progenies of heterozygous plants grown in saline conditions show elevated salinity tolerance.

In a study conducted by Chikkodi and Ravikumar (2000), two set of seven hybrids of sunflower, one set from selective and the other from non-selective fertilization, were evaluated for reaction to *Alternaria* leaf and stem blight under natural epiphytotic conditions. Selection (using culture filtrate) for resistant pollen on the stigmatic surface resulted in a corresponding increase in progeny resistance. The study demonstrates the transmission of selected trait from the pollen generation to progeny. Further studies (Chikkodi and Ravikumar, 2003) on the same plant material revealed that the selected F_2 populations were positively skewed containing relatively more number of resistant plants than its control. The results indicate that the selected F_2 population had more number of plants with selected traits than the counterparts suggesting that the resistance has been transmitted to F_2 generation.

The effect of pollen selection for herbicide resistance and its persistence up to second sporophytic generation in case of maize was given by Frascaroli and Songted (2001). They performed pollen selection in two genetic backgrounds for chlorsulfuron resistance. Plants of two backcross (BC) lines segregating in 1 (heterozygote, resistant): 1 (homozygote, susceptible) ratio for herbicide resistance were used as pollen donor in the crosses with an unrelated susceptible genotype (tester). The test

cross(TC) progenies so obtained from selective fertilization were screened against the herbicide. This resulted in 89 per cent to 100 per cent resistant plants in the TC progenies derived from heterozygous BC plants, while BC plants homozygous of the susceptible allele gave poor seed set and no resistant plants were found. Further, these resistant TC plants when selfed resulted in expected 3:1 ratio of resistant and susceptible plants indicating the inheritance of herbicide resistant allele through pollen selection.

Using PEG as a selective agent for moisture stress in sorghum, Ravikumar *et al.* (2003) showed that pollen selection had a significant effect on grain yield through its components and developmentally related traits. Clarke *et al.* (2004) clearly demonstrated that chilling tolerant pollen of chickpea 'won the race' to fertilize the ovule at low temperature, using flower colour as a morphological marker. They could also found the improved chilling tolerant progeny which is derived by pollen selection. Walker and associates (2006) could show that glyphosate application to hemizygous soybean plants shortly before the onset of flowering was lethal to male gametes that do not carry the glyphosate tolerance transgene. More recently, Shobha Rani and Ravikumar (2006) indicated that the gametophytic selection can be easily combined with classical plant breeding methods and the populations improved through gametophytic selection appear to be more promising as the pollen selection allowed the selection of rare favourable allelic combinations that would hardly be detected at the sporophytic level.

However, so far only a few investigations have clearly demonstrated the persistence of positive response to gametophytic selection in the succeeding generations. It has been hypothesized that the lack of positive response to gametophytic selection can occur when the traits under selection are quantitative in nature (Ottaviano and Mulcahy, 1989; Landi *et al.*, 1989) or have very little genetic

variability in the gametic pool (Fernandez-Munoz *et al.*, 1995) or are under transient epigenetic effects (Jorgensen, 1993; Jablonka and Lamb, 1998). Especially with regard to quantitative traits, it has been argued that lack of positive selection probably comes from the nature of quantitative traits, as many genes with small effects control the final phenotype (Pfahler, 1983; Landi *et al.*, 1989).

2.2 CHICKPEA *FUSARIUM* WILT RESISTANCE : A MODEL SYSTEM FOR POLLEN BIOASSAY

2.2.1 *Fusarium* wilt of chickpea

Wilt of chickpea (*Cicer arietinum*), caused by *Fusarium oxysporum* Schelectendahl, Fries f.sp. *ciceris* (Padwick) Matuo and Ic. Sato, was first described by Padwick in India (Padwick, 1940) and has since been reported from several other countries (Nene, 1980). It is a serious problem in India, Iran, Pakistan, Nepal, Burma, Spain and Tunisia. It has also been reported from Bangladesh, Ethiopia, Malawi, Mexico, Peru, Syria and the USA. In India, where 85 per cent of the total chickpea crop is grown, *Fusarium* wilt is estimated to cause a 10 per cent annual yield loss (Singh and Dahiya, 1973). Likewise an estimated annual loss of 12 million rupees was reported from Pakistan (Sattar *et al.*, 1953). Under favourable conditions it may cause more than 90 per cent yield losses (Srivastava *et al.*, 1984; Gupta *et al.*, 1986; Jimenez-Diaz *et al.*, 1993 and Cortes *et al.*, 2000).

2.2.2 Symptoms and disease development

The disease can be observed in a highly susceptible cultivar within 25 days after sowing in the field. The characteristic symptoms include sudden drooping of leaves and petioles, internal (Xylem) discoloration, yellowing of leaves, stunting and finally, death of the plant (Westerlund *et al.*, 1974). A common host symptom of *Fusarium* wilt infection is vascular vessel colonization. Wilting can be either by

mechanical plugging of xylem by mycelium, wilt toxin or hydrolytic enzymes (Green, 1981). Further the browning originates in xylem parenchyma adjacent to colonized vessels and staining is transferred to the vessels (Pegg, 1981). Vascular discoloration may occur in infected plants prior to the expression of external symptoms (Mace *et al.*, 1971).

The fungus is soil borne and survives through chlamydospores in seed (Haware *et al.*, 1978) and in dead plant debris in the soil. The fungus can survive in the soil for more than five years. Lentil, pigeonpea and pea were identified as symptomless carriers of the fungus (Haware and Nene, 1982). Optimum temperature for the fungus and for infection is around 25°C (Gupta *et al.*, 1987). The importance of temperature was mentioned in other studies (Upadhaya *et al.*, 1983b and Singh *et al.*, 1987a) as an important contributing factor to the difficulties observed in classifying wilting patterns in segregating chickpea populations. Apart from temperature, other factors like light and nutrients also have a role in disease development. Increased moisture was associated with increased wilt severity (Bhatti and Kraft, 1992).

2.2.3 Pathotypes and race patterns

Variation in symptom types (pathotypes) and pathogenic races have been reported to correlate geographical regions and to polymorphism in molecular markers. Two pathotypes, designated yellowing and wilting, have been differentiated by pathogenicity tests (Trapero-casas and Jimenez-Diaz, 1985). The yellowing pathotype includes progressive foliar yellowing with vascular discoloration, followed by plant death within 40 days of inoculation. The wilting pathotype induces severe chlorosis and flaccidity combined with vascular discolouration, followed by plant death within 20 days of inoculation. These two pathotypes can be distinguished unambiguously by

RAPD marker, they formed two distinct groups in cluster analysis (Kelly *et al.*, 1994), suggesting that they may not be closely related.

In addition to variation in symptom type, there are eight races of *F. oxysporum* f.sp. *ciceris* (race 0, 1A, 1B/C, 2, 3, 4, 5 and 6) which are identified by differential disease reactions on a set of chickpea cultivars (Haware and Nene, 1982, Jimenez-Diaz *et al.*, 1993; Phillips, 1998). Reaction of differentials to different races was reported by Jimenez-Gasco *et al.* (2004). As with pathotypes, some of these races can be distinguished by RAPD markers (Jimenez-Gasco *et al.*, 2001). In susceptible chickpea cultivars, race 0 and 1B/C induce the yellowing syndrome (yellowing pathotype) whereas race 1A, 2, 3, 4, 5 and 6 induce the wilting syndrome (wilting pathotype) (Trapero-casas and Jimenez-Diaz, 1985; Jimenez-Diaz *et al.*, 1993). These races also have distinct geographical distribution. Race 2, 3 and 4 have only been described from India (Haware and Nene, 1982), whereas race 0, 1B/C, 5 and 6 are found mainly in Mediterranean region, as well as in the USA (California) (Jimenez-Diaz *et al.*, 1993; Halila and Strange, 1996). Race 1A has been reported in India (Haware and Nene, 1982), California and the Mediterranean region (Jimenez-Diaz *et al.*, 1993). However, despite variation in symptom type, race and geographical distribution, all *F. oxysporum* f.sp. *ciceris* isolates tested so far are in a single vegetative compatibility group (Nogales-Moncada, 1997). In India also, the races are geographically distinct. Race 1 is widespread in central and peninsular India and race 2 in northern India. Race 3 and 4 are location specific and are prevalent in the Punjab and Haryana states of India (Haware *et al.*, 1992). Though there is lot of phenotypic and geographic diversity was observed in *F. oxysporum* f.sp. *ciceris*, it was proved that the evolutionary origin is stepwise (Jimenez-Gasco *et al.*, 2004) and monophyletic in nature (Jimenez-Gasco *et al.*, 2002; Jimenez-Gasco *et al.*, 2004).

2.2.4 Sources of resistance

Effective screening for disease resistance requires accurate simulation of natural environmental conditions where plants are exposed to the inoculum. Optimum inoculation and incubation conditions should be established so that susceptible and resistant genotypes can be easily differentiated. Screening can be performed directly in the field, in the greenhouse or in the laboratory. Evaluation of breeding material can be accomplished easily and directly in the field with little expense by the use of naturally or artificially infested fields or plots. The main advantage of sick plots is that they allow simultaneous screening of a large amount of genetic material under environmental conditions similar to those of cultivated plants (Infantino *et al.*, 2006).

Several methods have been described for development of wilt sick plots (WSPs) (Nene *et al.*, 1981). WSPs should be established based on the presence of the disease as indicated by visual symptoms and reisolation of the causal fungus. An average concentration of about 3,000 propagules/g soil (ppg) induced 100 per cent incidence of wilt in the susceptible line ICC 4951 in a WSP in Pakistan (Ali *et al.*, 1994).

Similarly 3,283 ppg of race 1 of *FOC* killed the line JG-62 and affected the late wilting line K-850 but did not affect the resistant line WR-315 (Kraft *et al.*, 1994). While at the lower inoculum levels (483 ppg), 100 per cent wilt incidence was observed only on JG-62. At Beja, Tunisia, a WSP infested with race 0 of *FOC* presented a level of inoculum equivalent to 1795 cf/g of soil and resulted in a uniform wilting reaction of the susceptible check ILC-482 across the plot (Halila and Strange, 1997).

Evaluation of germplasm in controlled environments is an important tool in many breeding programmes focused on disease resistance. Screening in the

greenhouse or growth chamber allows breeding material to be challenged with well characterized isolates without interaction with other phytopathogenic organisms throughout the year. Screening chickpea in controlled conditions is usually done in pots as described by Nene and Haware (1980) and Jimenez-Gasco *et al.* (2001). Tekeoglu *et al.* (2000) reported a technique for evaluating chickpea for resistance to *FOC*. chickpea seeds are immersed in metalaxyl to control seed-borne oomycetes and incubated in a germination chamber at 22°C and 100 per cent relative humidity for 3 days. Seedlings are transferred to plastic flats containing sterile, coarse perlite and placed in a greenhouse maintained at 21-26°C until plants reach the third to fourth node stage. Seedlings are then removed from the perlite and approximately one-third of the root system pruned. The remainder of the seedling's roots are submerged in spore suspension for 5 min prepared from potato dextrose broth (10^6 spores ml⁻¹) and replanted in the perlite. Scoring should be done every 2 days by counting the percentage of dead plants for 6-8 weeks.

Typical field conditions in the pots can be simulated by subirrigating the pots, so that the surface soil remain dry (Phillips, 1988). Ratnaparkhe *et al.* (1998a) used plastic trays filled with coarse perlite for screening of RILs in greenhouse (21-26°C). Disease scoring scales used to phenotype resistance and susceptibility for race identification have varied considerably among different studies (Table 2.1) (Sharma *et al.*, 2005). Resistant sources to *Fusarium* wilt in chickpea that have been identified using different screening techniques are listed in Table 2.2.

2.2.5 Genetics of *Fusarium* wilt resistance

To develop any breeding strategy, understanding the genetics of that particular trait is very essential. Similarly breeding for *Fusarium* wilt resistance, a thorough understanding of the genetics is desirable. In spite of established screening techniques, there were contradicting reports on the genetics and inheritance of wilt

Table 2.1. Disease scoring scales used in different studies to phenotype chickpea differential lines for resistance to races of *Fusarium oxysporum* f.sp. *ciceris*

Reference	Disease scoring scale (% wilt incidence)		
	Resistant	Moderately susceptible	Susceptible
Haware and Nene (1982)	0-20	21-50	>50
Phillips (1988)	0-20	21-50	>50
Jimenez-Diaz <i>et al.</i> (1989)	0-33	34-66	67-100
El-Hadi (1993)	1-10	11-50 ^a	51-100
Tullu (1996)	1-10	11-90 ^b	91-100

^a Disease reaction: medium

^b Disease reaction: intermediate

Table 2.2 Sources of resistance to Fusarium wilt in chickpea

Sl. No.	Material screened	Screening method	Resistant source identified	Reference
1	40 germplasm lines	Sick plot	ICC-11323 and ICC-324	Ahmed <i>et al.</i> (1990)
2	211 promising ICRISAT lines	Sick plot	22 lines	Ahmed <i>et al.</i> (1990)
3	79 lines (3 years)	Sick plot	20 lines	Bakr and Ahmed (1990)
4	44 lines	Sick plot	14 lines (ICC-85107 and ICC-12263)	Kaushal and Singh (1990)
5	1283 accessions (3 years)	Sick plot	117 lines (ICC-14532, 14619, 14631, 14735, 15125 and 15233)	Reddy <i>et al.</i> (1990a)
6	Chickpea lines in AICRP trials	Sick plot	Avrodhi, ICC-48 and GCP-6	Reddy <i>et al.</i> (1990b)
7	1904 lines	Sick plot	FLIP 84-43C, FLIP-85-20C, FLIP-85-29C, FLIP-85-30C, LIC-127, 219, 237, 267 and 513	Jimenez-Diaz <i>et al.</i> (1991)
8	39 varieties/lines	Sick plot	GNG-295 and PDG 83-31	Kapoor <i>et al.</i> (1991)
9	22 accessions and 48 wild species	Sick plot	10 wild species	Reddy <i>et al.</i> (1991)
10	188 genotypes	Glasshouse lab, condition	GL-1271, GL-8319 and ILC-3870	Haware <i>et al.</i> (1992)
11	Crosses of 6 wilt resistant 74 high yielding lines	Sick plot	58 (BCP-15, GCP-23, BCP-28, BCP-29 and BCP-30)	Zote <i>et al.</i> (1993)
12	51 lines	Sick plot	12 (ICC-12263 and ICCV-10)	Ali <i>et al.</i> (1994)
13	11 wild species	Sick plot	9 wild species	Kaiser <i>et al.</i> (1994)
14	300 diverse lines	Sick plot	ICCV-89402, ICCV-90902, ICCV-90254, HG-209, H88-2, RSG-180, GF-888426	Gupta (1995)

Table 2.2 Contd.....

Sl. No.	Material screened	Screening method	Resistant source identified	Reference
15	5 kabuli chickpeas	Sick plot	ICC-6045	Tadesse (1995)
16	31 ICARDA lines	Sick plot	FLIP-85-130C	Ifrikhar <i>et al.</i> (1997)
17	60 lines	Sick plot	GCP-9302, 9310 and 9313	Jani <i>et al.</i> (1999)
18	81 lines	Sick plot	10 (JG-22, JG-33, KPG-316 and SAKI-93130)	Yadava <i>et al.</i> (2000)
19	570 lines	Sick plot	GCP-9504, H92-71, FG-711, FG-712, FG-703, H 82-2, IFC-97-1 and WCGT 95-50	Gurha <i>et al.</i> (2002)
20	PDG 4, PDG 3, JG-2	Sick plot	PDG-4	Singh <i>et al.</i> (2002)
21	40 advanced lines from ICARDA	Sick plot	FLIP 90-131C, FLIP 96-152C, FLIP 96153C, FLIP 96-155C, FLIP-158C, ICCV-95503	Khan <i>et al.</i> (2002)
22	Gujarat gram 1 + checks (15 locations)	Sick plot	Gujarat Gram 1	Pithia <i>et al.</i> (2003)
23	PBG 5, PBG 1 (12 seasons)	Sick plot	PBG-5	Sandhu <i>et al.</i> (2004)
24	L 550, JGK 1, BG 1003, KAK 2 (2 seasons, 13 location in 1 st and 9 locations in 2 nd season)	Sick plot	JGK-1	Gaur <i>et al.</i> (2004)
25	ICCV-2, L-550, Vihar, KAK-2, BG-1003, JG-62 (5 seasons)	Sick plot	Vihar	Jamadagni <i>et al.</i> (2005)
26	15 high yielding genotypes	Sick plots	H 92-67, H 00-256, H 97-93, H 00-216, H 01-07, H 01-08, H 01-9, H 01-10, H 01-67, H 01-74, H 01-79	Waldia <i>et al.</i> (2005)

resistance in chickpea. The genetic studies on *Fusarium* wilt resistance were started way back in mid 1930s. The earliest report on genetics of *Fusarium* wilt was given by Ayyar and Iyer (1936), indicating single gene with incomplete dominance. The role of single recessive gene was reported by Lopez (1974); Haware *et al.* (1980); Tiwari (1981); Kumar and Haware (1982) and Sindhu *et al.* (1983). Identification of different pathotypes and races further complicated the understanding of inheritance pattern which resulted in many reports with contradicting results. All the reports to date are reviewed race-wise in the Table 2.3

2.2.6 Molecular studies on *Fusarium* wilt of chickpea

Identification of molecular markers for resistance genes could lead to a quicker assessment of susceptibility, allow screening of seeds or seedling and reduce the need for maintaining virulent isolates of the wilt pathogen. Isozyme electrophoresis can provide relatively quick and easy access to useful and reliable molecular markers; however the total number of isozymes that can be readily analyzed in plant tissue is usually 50 or less. A potentially much larger number of markers can be resolved by means of restriction fragment length polymorphism (RFLPs). RFLP technology has been used to establish high density gene maps for an increasing number of crop species (O'Brien, 1993). However, only seven RFLPs have been identified and added to the chickpea gene map (Simon and Muehlbauer, 1997).

Random amplified polymorphic DNA represents another method for identifying additional genetic variation within populations (Williams *et al.*, 1990; Welsh and McClelland, 1990). There is virtually no limit to the number of primers that can be designed for use in RAPD reactions. Consequently, the number of potential RAPD markers can be several orders of magnitude larger than the total number of morphological or isozyme markers, leading to a much higher level of marker saturation on genetic maps.

Table 2.3 Studies on inheritance of wilt resistance

Race	No. of genes controlling resistance/Nature of inheritance	Genotypes used	References
Race 0	Single gene	131 RILs of cross ICC-4958 (<i>C. arietinum</i>) x P1489777 (<i>C. reticulatum</i>)	Tekeoglu <i>et al.</i> (2000)
	Single gene	RILs of cross CA-2156 x JG-62	Rubio <i>et al.</i> (2003)
	Two genes (either of the two was sufficient to confer resistance)	RILs of cross CA-2139 x J4-62	Rubio <i>et al.</i> (2003)
Race 1A	Two recessive genes	JG-62, C-104, H-208, K-850	Upadhyaya <i>et al.</i> (1983a)
	Two recessive genes	JG-62, C-104, H-208, K-850	Upadhyaya <i>et al.</i> (1983b)
	Three independent loci	K-850, C-104, H-208	Singh <i>et al.</i> (1987a)
	K-850 and C-104 carry different recessive loci	K-850, C-104, JG-62	Singh <i>et al.</i> (1987b)
	H-208 carry dominant allele for late wilting	H-208, K-850, C-101	Singh <i>et al.</i> (1991)
	Two recessive genes under homozygous condition	ICCV-2, JG-62	Anupama (2001)
	Four recessive genes	Vijay, ICC-4958, JG-62	Girase and Deshmukh (2002)
	Monogenic nature of resistance in WR-315	100 RILs and F ₂ population of WR-315 and C-104	Sharma <i>et al.</i> (2005)
	Two recessive genes	RILs of cross JG-62 x WR-315	Brindha and Ravikumar(2005)

Table 2.3 Contd.....

Race	No. of genes controlling resistance/nature of inheritance	Genotypes used	References
Race 2	Two genes for resistance Three loci (two recessive and one dominant allele) Single recessive gene	P-165, C-104 WR-315, C-104, K-850, GW-5/7 100 RILs and F ₂ population of cross WR-315 x C-104	Gumber <i>et al.</i> (1995) Kumar (1998) Sharma <i>et al.</i> (2005)
Race 3	Two recessive genes Monogenic nature Monogenic resistance in WR-315	JG-62, J4-74, PG-114 100 RILs of WR-315 x C-104 100 RILs and F ₂ pop of cross WR-315 x C-104	Dikshit and Singh (1994) Sharma <i>et al.</i> (2004) Sharma <i>et al.</i> (2005)
Race 4	Recessive monogenic nature in WR-315 Diagenic nature in Surutato -77 Monogenic nature of resistance in WR-315	100 RILs of C-104 x WR-315 JG-62, Surutato-77 100 RILs and F ₂ population of cross WR-315 x C-104	Tullu <i>et al.</i> (1998) Tullu <i>et al.</i> (1999) Sharma <i>et al.</i> (2005)
Race 5	Single gene Monogenic resistance	131 RILs of cross ICC-4958 (<i>C. arietnum</i>) x PI489777 (<i>C. reticulatum</i>) 100 RILs of cross WR-315 x C-104	Tekeoglu <i>et al.</i> (2000) Sharma <i>et al.</i> (2005)

For the first time, two RAPD primers (UBC-170 and CS-27) associated with *Fusarium* wilt race 1 resistance were identified (Mayer *et al.*, 1997) using bulk segregant analysis of selected F₃ plants of cross C-104 x WR-315 and they could design two allele specific associated primers (ASAPs), CS-27₇₀₀ and UBC-170₅₅₀ out of which CS-27₇₀₀ amplified a fragment linked to the allele for susceptibility to race 1 (allele specific) whereas UBC-170₅₅₀ produced a single band in both resistant and susceptible genotypes indicating locus specificity rather than allele specificity. Recombination between two marker loci was 6 per cent and both loci were located on same side of the resistance locus and were placed on one end of the linkage group VI on the linkage map developed by Simon and Muehlbauer (1997).

Further studies (Tullu *et al.*, 1998) revealed that the RAPD marker previously shown to amplify fragments linked to race 1 resistance also amplified fragment associated with race 4 resistance. The RAPD loci CS-27₇₀₀, UBC-170₅₅₀ and the gene for resistance to race 4 segregated in 1:1 ratios expected for single genes. Both RAPD markers were located 9 map units from the race 4 resistance locus were on the same side of the resistance gene. Their results also indicated that the genes for resistance to race 1 and 4 are 5 map units apart. Tullu *et al.* (1999) further confirmed the linkage of CS-27₇₀₀ with race 4 resistant gene by using F₁, F₂ and F₃ families of JG-62 x Surutato-77 cross.

In the interspecific cross RILs, Ratnaparkhe *et al.* (1998b) identified ISSR marker UBC-855₅₇₀ that was found to be linked in repulsion with the race 4 resistance gene at a distance of 5.2 cM. Location of UBC-855₅₇₀ at a distance of 0.6 cM from CS-27₇₀₀ indicate that genes for resistance to race 1 and 4 are closely linked.

Presence of race 4 and 5 resistance genes on the same linkage group at a distance of 11.2 cM was revealed (Tekeoglu *et al.*, 2000) in the linkage analysis of 131 F₅ derived RILs of an interspecific cross. In addition, CS-27₇₀₀ was located

between these two loci at a distance of 7.2 and 4 cM from resistance loci of race 4 and 5 respectively. The same CS-27₇₀₀ is also known to be linked to the genes for resistance to race 4 and 5 at a distance of 3.7 and 18 cM respectively on linkage group 2 (Winter *et al.*, 2000) suggesting that linkage group VI of Simon and Muehlbauer (1997) is same as linkage group of Winter *et al.* (2000). Validation of CS-27₇₀₀ in different commonly used parental lines (Ravikumar *et al.*, 2003) and in F₅ progenies of cross JG-62 x WR-315 (Brindha and Ravikumar, 2005) also proved its strong association to the wilt resistance locus for race 1.

RAPD marker OPJ-20₆₀₀ was identified (Rubio *et al.*, 2003) to be linked with the resistance gene of race 0 and the distance established between the marker (OPJ-20₆₀₀) and resistance locus (for race 0) was 6 cM.

Recently resistance gene (*foc-3*) for race 3 was identified and mapped on linkage group 2 (Sharma *et al.*, 2004) and was indicated to be linked with other two resistance loci for race 1 and 4 with a map distance of 9.8 cM and 8.7 cM respectively, whereas the latter two loci are closely linked at 1.1 cM distance. They could demonstrate the presence of two clusters of wilt resistance genes on linkage group 2. One that contains loci linked to resistance of race 1 and 4 and other comprises the loci linked to race 3 and 5.

The RAPD markers linked to second gene of resistance are being identified (Thippeswamy *et al.*, 2005; Soregaon *et al.*, 2007). The tagging of different resistance loci would facilitate in pyramiding of wilt resistance genes to chickpea.

2.2.7 Pollen germination and selection in chickpea

In vitro pollen germination is a very convenient and effective technique to study many basic and applied aspects of pollen biology (Heslop-Harrison, 1987, Kristen and Kappler, 1990; Shivanna and Rangaswamy, 1992). The composition of

the medium used for pollen germination varies from species to species. The medium formulated by Brewbaker and Kwack (1963) has been used for pollen of a number of species including chickpea (Srinivasan *et al.*, 1997).

For screening chickpea genotypes tolerant to cold Shivanna *et al.* (1997) used Brewbaker and Kwack (BK) medium, could observe limited pollen tube growth (<200 μm) and around 80 per cent pollen germination but with lot of inconsistency. Polyethylene glycol (PEG) has been shown to improve markedly *in vitro* pollen germination and/or pollen tube growth in many species (Zhang and Croes, 1982; Read *et al.*, 1993; Shivanna and Sawhney, 1995).

The BK medium was improved by testing different effects of PEG (Shivanna *et al.*, 1997) and could obtain best response for chickpea pollen in a medium containing 2.5 per cent sucrose and 10 per cent PEG. Thus the improved medium contained sucrose 2.5 % + PEG 10 % + boric acid 100 mg L⁻¹ + calcium nitrate 300 mg L⁻¹ + magnesium sulphate 200 mg L⁻¹ + potassium nitrate 100 mg L⁻¹. The medium with above composition along with different concentration of fusaric acid was successfully used to differentiate resistant and susceptible genotypes of chickpea against *Fusarium* wilt race 1 (Ravikumar *et al.*, 2002).

2.3 GENETIC VARIABILITY AND CHARACTER ASSOCIATION

The information on the magnitude of variability for different quantitative and qualitative traits along with their heritability and relationship with yield in any crop species play a vital role in formulating efficient breeding programme.

2.3.1 Variability, heritability and genetic advance

In a highly self-pollinated crop like chickpea, the breeding strategy for varietal development generally involves selection of potential genotypes from the existing germplasm, utilizing them in the hybridization programme and isolation of superior

genotypes in the segregating population. This necessitates a thorough knowledge of genetic variability, heritability and the genetic advance that can be achieved through selection. The available information on variability, heritability and genetic advance is summarized in Tables 2.4, 2.5 and 2.6, respectively.

2.3.2 Association between yield and its components

Grain yield is a function of several component traits. The genetic correlations between yield and its component traits are helpful in assessing the consequences of selecting for one or more traits. The information on genetic correlations between yield and its component characters is summarized in Table 2.7.

2.3.3 Path coefficient analysis

Yield and its component traits sharing linear correlations may present a confusing picture due to the possible interrelationship between the components themselves. Path analysis helps to further resolve the correlation coefficients indicating the direct and indirect effects of various component characters on seed yield. The technique of path analysis was developed by Wright (1921) and is still considered as a valuable tool in detecting the real merit of characters contributing to yield.

An analysis of 50 F_2 progenies from each of two crosses (C 214 x SH 214 and C 214 x H 432) (Jatasra *et al.*, 1978) revealed a greater direct effect of pod number as well as branches per plant on seed yield. They also observed high negative effects of plant height and days to maturity on seed yield. Similarly study on F_1 and F_2 of 45 crosses involving 15 parental varieties (Katiyar, 1979) indicated that seeds per pod and number of primary branches per plant exhibited the greatest positive direct effects on yield in F_1 and F_2 , respectively.

Table 2.4 Summary of review on variability for various traits in chickpea

Sl. No.	Character		Material studied	Reference
1	Plant height at maturity	High	330 lines (205 desi + 125 kabuli types) 28 genotypes 96 cultivars 10 exotic genotypes 16 cultivars	Kumar <i>et al.</i> (1981) Dasgupta (1992) Bhatia <i>et al.</i> (1993) Wahid and Ahmed (1999) Sial <i>et al.</i> (2003)
		Low	40 diverse genotypes	Arora (1991)
2.	Number of primary branches per plant	High	330 lines (205 desi + 125 kabuli types) 40 local and introduced cultivars 28 genotypes 96 cultivars 70 genotypes 2 crosses (P-9623 x T39-1 and RS-11 x T39-1)	Kumar <i>et al.</i> (1981) Mandal and Bahl (1983) Dasgupta (1992) Bhatia <i>et al.</i> (1993) Chavan <i>et al.</i> (1994) Vijayalakshmi <i>et al.</i> (2000)
		Moderate	40 diverse genotypes	Arora (1991)
3.	Days to 50% flowering	High	P ₁ , P ₂ , F ₁ and F ₃ generation of 2 crosses (JG-315 x L550 and TLM x L550) 40 genotypes obtained by single, double and multiple crosses	Agrawal (1985) Rao and Kumar (2000)
		Moderate	132 lines	Rang <i>et al.</i> (1980)
		Low	330 lines (250 desi + 125 kabuli types) 140 varieties	Kumar <i>et al.</i> (1981) Islam <i>et al.</i> (1984)
4	100 seed weight	High	P ₁ , P ₂ , F ₂ and F ₃ generations of 2 crosses (JG-315 x L-550 and TLM x L-550) 42 diverse genotypes 66 genotypes 70 genotypes 40 diverse genotypes 96 cultivars 44 varieties 50 genotypes 40 cultivars	Agrawal (1985) Jivani and Yadavendra (1988) Singh and Singh (1989) Sharma <i>et al.</i> (1990) Arora (1991) Bhatia <i>et al.</i> (1993) Rao <i>et al.</i> (1994) Kumar <i>et al.</i> (1999) Nimbalkar (2000)

Table 2.4 Contd.....

Sl. No.	Character		Material studied	Reference
			2 crosses (P-9623 x T 39-1 and RS-11 x T 39-1)	Vijayalakshmi <i>et al.</i> (2000)
			40 diverse genotypes	Arora and Jeena (2001)
			36 diverse genotypes	Jeena and Arora (2001)
			136 genotypes	Hegde <i>et al.</i> (2002)
			33 diverse genotypes	Yadav (2003)
			14 genotypes	Burli <i>et al.</i> (2004)
5	Number of pods per plant	High	132 lines	Rang <i>et al.</i> (1980)
			330 lines (205 desi + 125 kabuli type)	Kumar <i>et al.</i> (1981)
			125 germplasm lines and 20 promising cultivars	Islam and Begum (1985)
			42 diverse genotypes	Jivani and Yadavendra (1988)
			66 genotypes	Singh and Singh (1989)
			40 diverse genotype	Arora (1991)
			96 cultivars	Bhatia <i>et al.</i> (1993)
			70 genotypes	Chavan <i>et al.</i> (1994)
			44 varieties	Rao <i>et al.</i> (1994)
			40 lines derived via multiple inter varietal crosses	Rao (1996)
			100 genotypes	Tripathi (1998)
			50 genotypes	Kumar <i>et al.</i> (1999)
			2 crosses (P-9623 x T 39-1 and RS-11 x T 39-1)	Vijayalakshmi <i>et al.</i> (2000)
			26 diverse genotypes	Kumar <i>et al.</i> (2001)
			362 accessions	Naghavi and Jahansouz (2005)
			24 gulabi genotypes	Patel and Babbar (2005)
		Moderate	28 genotypes	Dasgupta <i>et al.</i> (1992)
		Low	140 varieties	Islam <i>et al.</i> (1984)
6.	Seed yield per plant	High	132 lines	Rang <i>et al.</i> (1980)
			330 lines (250 desi + 125 kabuli types)	Kumar <i>et al.</i> (1981)
			40 local and introduced cultivars	Mandal and Bahl (1983)
			125 germplasm lines and 20 promising cultivars	Islam and Begum (1985)
			66 genotypes	Singh and Singh (1989)
			40 diverse genotypes	Arora (1991)
			44 varieties	Rao <i>et al.</i> (1994)
			40 lines derived via multiple inter varietal crosses	Rao (1996)
			30 F ₄ progenies	Wanjari <i>et al.</i> (1996)

Table 2.4 Contd.....

Sl. No.	Character		Material studied	Reference
			100 genotypes	Triptahi (1998)
			50 genotypes	Kumar <i>et al.</i> (1999)
			10 exotic genotypes	Wahid and Ahmed (1999)
			40 genotypes obtained by single double and multiple crosses	Rao and Kumar (2000)
			36 diverse genotypes	Jeena and Arora (2001)
			136 genotypes	Hegde <i>et al.</i> (2002)
			30 genotypes	Sable <i>et al.</i> (2002)
			33 diverse genotypes	Yadav <i>et al.</i> (2003)
			14 genotypes	Burli <i>et al.</i> (2004)
			F ₃ progenies and biparental population	Singh (2004)
			362 accessions	Naghavi and Jahansour (2005)
			24 kabuli genotypes	Patel and Babbar (2005)
			12 lines	Obaidullah <i>et al.</i> (2006)
		Moderate	2 crosses (P-9623xT39-1 and RS-11xT39-1)	Vijayalakshmi <i>et al.</i> (2000)

Table 2.5 Summary of review on heritability for various traits in chickpea

Sl. No.	Character		Material studied	Reference
1	Plant height at maturity	High	70 genotypes 18 varieties 44 varieties 36 genotypes 34 genotypes 14 genotypes 18 elite genotypes and 2 cultivars	Sharma <i>et al.</i> (1990) Misra (1991) Rao <i>et al.</i> (1994) Arshad <i>et al.</i> (2002) Singh <i>et al.</i> (2002) Burli <i>et al.</i> (2004) Saleem <i>et al.</i> (2005)
		Moderate	58 genotypes 10 exotic genotypes 16 cultivars	Sandhu <i>et al.</i> (1988) Wahid and Ahmed (1999) Sial <i>et al.</i> (2003)
2	Number of primary branches	High	32 genotypes Biparental population 16 cultivars	Khorgade <i>et al.</i> (1985) Kampli <i>et al.</i> (2002) Sial <i>et al.</i> (2003)
		Moderate	2 cross (P-9623xT39-1 and RS-11 x T39-11) 34 genotypes	Vijayalakshmi <i>et al.</i> (2000) Singh <i>et al.</i> (2002)
		Low	36 genotypes	Arshad <i>et al.</i> (2002)
3	Days to 50% flowering	High	45 varieties 18 varieties 4 crosses 8 cultivars and their 21 F ₁ s 28 F ₁ hybrids 17 genotypes 34 genotypes 16 cultivars 24 advanced lines 14 genotypes 18 varieties	Govil and Kumar (1989) Misra (1991) Panchabhai <i>et al.</i> (1992) Jahagirdar <i>et al.</i> (1994) Jahagirdar <i>et al.</i> (1996) Kumar and Krishna (1998) Singh <i>et al.</i> (2002) Sial <i>et al.</i> (2003) Arshad <i>et al.</i> (2004) Burli <i>et al.</i> (2004) Misra (1991)
4	100-seed weight	High	70 genotypes 18 varieties 8 cultivars and their 21 F ₁ hybrids 44 varieties 28 F ₁ hybrid 34 varieties 30 F ₄ progenies	Sharma <i>et al.</i> (1990) Misra (1991) Jahagirdar <i>et al.</i> (1994) Rao <i>et al.</i> (1994) Jahagirdar <i>et al.</i> (1996) Mathur and Mathur (1996) Wanjari <i>et al.</i> (1996)

Table 2.5 Contd.....

Sl. No.	Character		Material studied	Reference
			17 genotypes 50 genotypes 40 cultivars 40 diverse genotypes 36 diverse genotypes 34 genotypes 30 diverse genotypes 16 cultivars 24 advanced lines 14 genotypes 30 genotypes 80 diverse genotypes 18 elite genotypes and 2 cultivars	Kumar and Krishna (1998) Kumar <i>et al.</i> (1999) Nimbalkar (2000) Arora and Jeena (2001) Jeena and Arora (2001) Singh <i>et al.</i> (2002) Sable <i>et al.</i> (2003) Sail <i>et al.</i> (2003) Arshad <i>et al.</i> (2004) Burli <i>et al.</i> (2004) Kaur <i>et al.</i> (2004) Jeena <i>et al.</i> (2005) Saleem <i>et al.</i> (2005)
		Moderate	2 crosses (P-9623 x T 39-1 and RS-11 x T 39-1)	Vijayalakshmi <i>et al.</i> (2000)
5.	Number of pods per plant	High	18 varieties 70 genotypes 8 cultivars and their 21 F ₁ hybrids 28 F ₁ hybrids 50 genotypes 6 intervarietal crosses 26 diverse genotypes 34 genotypes 80 diverse genotypes 18 elite genotypes and 2 cultivars	Misra (1991) Chavan <i>et al.</i> (1994) Jahagirdar <i>et al.</i> (1994) Jahagirdar <i>et al.</i> (1996) Kumar <i>et al.</i> (1999) Singh <i>et al.</i> (2000) Kumar <i>et al.</i> (2001) Singh <i>et al.</i> (2002) Jeena <i>et al.</i> (2005) Saleem <i>et al.</i> (2005)
		Moderate	10 exotic genotypes 16 cultivars	Wahid and Ahmed (1999) Sial <i>et al.</i> (2003)
		Low	36 genotypes	Arshad <i>et al.</i> (2002)
6	Seed yield per plant	High	70 genotypes 28 F ₁ hybrids 34 varieties 50 genotypes 34 genotypes 30 diverse genotypes 4 cross populations	Chavan <i>et al.</i> (1994) Jahagirdar <i>et al.</i> (1996) Mathur and Mathur (1996) Kumar <i>et al.</i> (1999) Singh <i>et al.</i> (2002) Sable <i>et al.</i> (2003) Deshmukh <i>et al.</i> (2004)
			80 diverse genotypes	Jeena <i>et al.</i> (2005)

Table 2.5 Contd.....

Sl. No.	Character		Material studied	Reference
			18 elite genotypes and 2 cultivars	Saleem <i>et al.</i> (2005)
		Moderate	30 F ₄ progenies 17 genotypes 10 exotic genotypes	Wanjari <i>et al.</i> (1996) Kumar and Krishna (1998) Wahid and Ahmed (1999)
		Low	44 varieties 36 genotypes 16 cultivars	Rao <i>et al.</i> (1994) Arshad <i>et al.</i> (2002) Sial <i>et al.</i> (2003)

Table 2.6 Summary of review on genetic advance for various traits in chickpea

Sl. No.	Character		Material studied	Reference
1	Plant height at maturity	High	16 cultivars 44 varieties 36 genotypes	Dumbre <i>et al.</i> (1984) Rao <i>et al.</i> (1994) Arshad <i>et al.</i> (2002)
		Moderate	18 elite genotypes and 2 cultivars	Saleem <i>et al.</i> (2005)
		Low	34 genotypes	Singh <i>et al.</i> (2002)
2	Number of primary branches	High	32 genotypes 18 varieties 40 diverse genotypes Biparnetal population	Khorgade <i>et al.</i> (1985) Misra (1991) Arora and Jeena (2001) Kampli <i>et al.</i> (2002)
		Moderate	2 crosses (P-9623 x T 39-1 and RS-11 x T 39-1)	Vijayalakshmi <i>et al.</i> (2000)
		Low	36 genotypes 34 genotypes	Arshad <i>et al.</i> (2002) Singh <i>et al.</i> (2002)
3	Days to 50 per cent flowering	High	32 genotypes 45 varieties 8 cultivars and their F ₁ hybrids 28 F ₁ hybrids 14 genotypes	Khorgade <i>et al.</i> (1985) Govil and Kumar (1989) Jahagirdar <i>et al.</i> (1994) Jahagirdar <i>et al.</i> (1996) Burli <i>et al.</i> (2004)
		Low	34 genotypes 24 advanced lines	Singh <i>et al.</i> (2002) Arshad <i>et al.</i> (2004)
4	100-seed weight	High	8 cultivars and their 21 F ₁ hybrids 44 varieties 28 F ₁ hybrids 34 varieties 50 genotypes 40 cultivars 40 diverse genotypes 36 diverse genotypes 34 genotypes 30 genotypes 16 cultivars 14 genotypes 80 diverse genotypes	Jahagirdar <i>et al.</i> (1994) Rao <i>et al.</i> (1994) Jahagirdar <i>et al.</i> (1996) Mathur and Mathur (1996) Kumar <i>et al.</i> (1999) Nimbalkar (2000) Arora and Jeena (2001) Jeena and Arora (2001) Singh <i>et al.</i> (2002) Sable <i>et al.</i> (2003) Sial <i>et al.</i> (2003) Burli <i>et al.</i> (2004) Jeena <i>et al.</i> (2005)
		Moderate	18 varieties	Misra (1991)

Table 2.6 Contd.....

Sl. No.	Character		Material studied	Reference
			30 desi genotypes	Kaur <i>et al.</i> (2004)
		Low	24 advanced lines 18 elite genotypes and 2 cultivars	Arshad <i>et al.</i> (2004) Saleem <i>et al.</i> (2005)
5	Number of pod per plant	High	70 genotypes 8 cultivars and their F ₁ hybrids 44 varieties 28 F ₁ hybrids 100 genotypes 50 genotypes 6 inter varietal crosses 26 diverse genotypes 36 genotype 16 cultivars 80 genotypes	Chavan <i>et al.</i> (1994) Jahagirdar <i>et al.</i> (1994) Rao <i>et al.</i> (1994) Jahagirdar <i>et al.</i> (1996) Tripathi (1998) Kumar <i>et al.</i> (1999) Singh <i>et al.</i> (2000) Kumar <i>et al.</i> (2001) Arshad <i>et al.</i> (2002) Sial <i>et al.</i> (2003) Jeena <i>et al.</i> (2005)
		Moderate	36 genotypes	Singh <i>et al.</i> (2002)
		Low	18 elite genotypes and 2 cultivars	Saleem <i>et al.</i> (2005)
6	Seed yield per plant	High	70 genotypes 44 varieties 28 F ₁ hybrids 34 varieties 100 genotypes 50 genotypes 30 diverse genotypes 16 cultivars 80 diverse genotypes 24 kabuli genotypes	Chavan <i>et al.</i> (1994) Rao <i>et al.</i> (1994) Jahagirdar <i>et al.</i> (1996) Mathur and Mathur (1996) Tripathi (1998) Kumar <i>et al.</i> (1999) Sable <i>et al.</i> (2003) Sial <i>et al.</i> (2003) Jeena <i>et al.</i> (2005) Patel and Babbar (2005)
		Moderate	18 diverse varieties 2 crosses (P-9623 x T 39-1 and RS-11 x T 39-1) 34 genotypes	Misra (1991) Vijayalakshmi <i>et al.</i> (2000) Singh <i>et al.</i> (2002)
		Low	35 genotypes 18 elite genotypes and 2 cultivars	Arshad <i>et al.</i> (2002) Saleem <i>et al.</i> (2005)

Table 2.7 Summary of review on association pattern of yield component traits with seed yield in chickpea

Sl. No.	Character		Material studied	Reference
1	Plant height at maturity	Positive	80 diverse genotypes 24 genotypes 28 elite strains 14 cultivars 17 kabuli genotype 80 diverse genotypes 12 genotypes	Arora <i>et al.</i> (2003) Arshad <i>et al.</i> (2004) Brar <i>et al.</i> (2004) Ciftci <i>et al.</i> (2004) Toker and Cagragan (2004) Jeena <i>et al.</i> (2005) Obaidullah <i>et al.</i> (2006)
		Negative	61 lines 50 genotypes 24 genotypes	Islam <i>et al.</i> (1982) Kumar <i>et al.</i> (1999) Kumar <i>et al.</i> (2002)
2	Number of primary branches per plant	Positive	50 F ₂ progenies of 2 crosses (C-214 x SH-214 and C-214 x H 432) 2 crosses (P-9632 x T 39-1 RS-11 x T 39-1) 80 diverse genotypes 28 elite strains 14 cultivars 219 genotypes 17 kabuli genotypes 12 genotypes	Jatasra <i>et al.</i> (1978) Vijayalakshmi <i>et al.</i> (2000) Arora <i>et al.</i> (2003) Brar <i>et al.</i> (2004) Ciftci <i>et al.</i> (2004) Qureshi <i>et al.</i> (2004) Toker and Cagrgan (2004) Obaidullah <i>et al.</i> (2006)
		Negative	30 genotypes 30 cultivars	Yadav and Sharma (1998) Yadav <i>et al.</i> (2001)
3	Days to 50% flowering	Positive	50 F ₂ progenies of two cross (C-214 x SH-214 and C-214 x H 432) 70 genotypes 20 elite genotypes	Jatasra <i>et al.</i> (1978) Chavan <i>et al.</i> (1994) Saleem <i>et al.</i> (2002)
		Negative	30 genotypes 30 cultivars 46 diverse genotypes 16 genotypes	Yadav and Sharma (1998) Yadav <i>et al.</i> (2001) Bhaduoria <i>et al.</i> (2003) Sial <i>et al.</i> (2003)
4	100-seed weight	Positive	50 genotypes 20 elite genotypes 80 diverse genotypes 24 genotypes 80 diverse genotypes 12 genotypes	Kumar <i>et al.</i> (1999) Saleem <i>et al.</i> (2002) Arora <i>et al.</i> (2003) Arshad <i>et al.</i> (2004) Jeena <i>et al.</i> (2005) Obaidallah <i>et al.</i> (2006)
		Negative	28 F ₁ hybrids 30 genotypes 16 genotypes 17 kabuli genotypes	Bakhsh <i>et al.</i> (1998) Yadav and Sharma (1998) Sial <i>et al.</i> (2003) Toker and Cagragan (2004)

Table 2.7 Contd.....

Sl. No.	Character		Material studied	Reference
5	Number of pods per plant	Positive	50 F ₂ progenies of 2 crosses (C-214 x SH-214 and C-214 x H-432) 28 F ₁ hybrids 2 crosses (P-9623 x T 39-1 and RS-11 x T 39-1) 31 genotypes 20 elite genotypes 80 diverse genotypes 16 genotypes 33 divergent genotypes 24 genotypes 28 elite strains 14 cultivars 219 genotypes 17 kabuli genotypes 80 diverse genotypes 30 genotypes 12 genotypes	Jatasra <i>et al.</i> (1978) Bakhsh <i>et al.</i> (1998) Vijayalakshmi <i>et al.</i> (2000) Narayana and Reddy (2002) Saleem <i>et al.</i> (2002) Arora <i>et al.</i> (2003) Sial <i>et al.</i> (2003) Yadav <i>et al.</i> (2003) Arshad <i>et al.</i> (2004) Brar <i>et al.</i> (2004) Ciftci <i>et al.</i> (2004) Qureshi <i>et al.</i> (2004) Toker and Cagragan (2004) Jeena <i>et al.</i> (2005) Rao and Rao (2005) Obaidallah <i>et al.</i> (2006)

Path analysis of data on seed yield and six related traits in 36 genotypes (Paliwal *et al.*, 1987) revealed that 100-seed weight had the highest positive direct effect on yield, followed by pods per plant, seeds per pod and days to maturity. Study on 80 diverse genotypes (Salimath and Bahl, 1986) indicated that branches per plant, pods per plant and 100-seed weight had positive direct effects on seed yield.

Study on path analysis in 30 diverse populations of chickpea (Singh *et al.*, 1999) revealed that number of pods per plant and 100-seed weight had maximum direct effect on yield. However, primary and secondary branches showed indirect effect upon yield through number of pods per plant. Similarly a study conducted on 40 chickpea genotypes by Kheder and Maloo (1999) indicated that the pods per plant had the highest direct effect on seed yield, followed by seeds per pod, 100-seed weight and primary branches per plant.

Path analysis carried out in 40 genetically diverse genotypes of chickpea (Singh and Singh, 2002) revealed that pods, test weight and secondary branches had negative direct effect but it had high positive indirect effect via pods per plant on seed yield. Similarly, Kumar *et al.* (1999) studied 50 chickpea genotypes and observed that number of pods per plant and 100-seed weight showed negative direct effects on yield, but showed indirect effects via biological yield per plant and harvest index.

The study on 34 chickpea genotypes revealed that total biological yield had highest direct effect on grain yield followed by number of pods, days to maturity while, 100-seed weight, number of primary branches, days to flowering had negative direct effect (Singh *et al.*, 2001).

Path analysis carried out in 57 chickpea genotypes (Pratap *et al.*, 2002) revealed that biological yield, number of pods per plant and harvest index have positive direct effect on grain yield. High direct effects of number of pods per plant,

100-seed weight, number of seeds per pod and harvest index on seed yield was reported in study on 31 chickpea genotypes (Narayana and Reddy, 2002). Similarly analysis on 14 chickpea cultivars revealed strong direct effects of the biological yield, harvest index and number of seeds per plant on the seed yield (Ciftci *et al.*, 2004).

Kumar *et al.* (2003) conducted path analysis on 26 genetically diverse chickpea genotypes and indicated that harvest index exhibited the highest significant positive association with seed yield, followed by pods per plant, biological yield per plant and secondary branches per plant. Similarly a study on eighty diverse genotypes of chickpea (Arora *et al.*, 2003) revealed that biological yield per plant had the highest positive direct effect on seed yield per plant, followed by harvest index, pods per plant, 100-seed weight and seeds per pod.

Path coefficient analysis on 30 divergent chickpea genotypes revealed that pods per plant, number of seeds per pod, 100-seed weight and biological yield and moderate to high positive direct effect on seed yield per plant indicating that these characters may be given due weightage while making selection for seed yield improvement (Yadav *et al.*, 2003). While Rao and Rao (2005) indicated that total dry matter per plant had the highest direct effect on grain yield, followed by harvest index and high indirect effects through secondary branches per plant, primary branches per plant and plant height in a study conducted on 30 chickpea genotypes. The studies indicate that pod number per plant as the most important character in selection for yield owing to its high direct effect on seed yield.

Material and Methods

III. MATERIAL AND METHODS

The present investigation was undertaken to develop pollen bioassay as a laboratory method to evaluate chickpea genotypes for wilt resistance in particular and as a technique in general. Further, the efficiency of the pollen bioassay was compared to that of molecular markers in identification of wilt resistant genotypes in chickpea. The selected diverse genotypes, recombinant inbred lines (RILs) and selected progenies of different crosses were tested for molecular markers linked to *Fusarium* wilt resistance in chickpea and for their response to pollen bioassay in the laboratory. A comparative analysis of pollen bioassay and molecular marker assisted selection was made to test the usefulness of pollen bioassay as a technique.

The chickpea wilt caused by race 1 of *Fusarium oxysporum* f.sp. *ciceris* is controlled by three independent loci and partially recessive alleles in homozygous form at either of the first two loci and the dominant allele at the third locus delay wilting, but any two of these alleles together confer complete resistance (Upadhyaya *et al.*, 1983a, b; Singh *et al.*, 1987b; Brindha and Ravikumar, 2005). With this background, the genotypes for the present study were carefully selected. The pollen bioassay and molecular marker analysis was carried out in three different sets of genotypes.

- a. Diverse genotypes of chickpea – representing genetic diversity.
- b. Recombinant inbred lines (RILs) of a cross between early wilting JG-62 ($H_1H_1H_2H_2$) and resistant line WR-315 ($h_1h_1h_2h_2$) segregating for both H_1 and H_2 loci and
- c. F_5 generation progenies of cross between late wilting BG-256 ($h_1h_1H_2H_2$) and resistant line WR-315 ($h_1h_1h_2h_2$) segregating for only one H_2 locus.

The following experiments were conducted in the present study.

- 3.1 Evaluation of selected chickpea genotypes for wilt resistance using *in vitro* pollen bioassay, molecular markers and *in vivo* screening
- 3.2 Combined analysis of pollen bioassay and molecular markers in recombinant inbred lines (RILs) of chickpea.
- 3.3 Selection of F₅ progenies of chickpea based on pollen bioassay and their validation with molecular markers in F₆ generation.
- 3.4 Evaluation of chickpea genotypes, RILs and F₅ generation progenies for yield and yield components

3.1 EVALUATION OF SELECTED GENOTYPES FOR WILT RESISTANCE USING *IN VITRO* POLLEN BIOASSAY, MOLECULAR MARKERS AND *IN VIVO* SCREENING

Sixteen genotypes were selected for this experiment which included eight ICC accessions obtained from International Crop Research Institute for Semi-Arid Tropics (ICRISAT) and another eight varieties and established lines which are being used in different breeding programmes at UAS, Dharwad. The wilt reaction of selected sixteen genotypes is presented below. In case of ICC accessions, the late wilting types were not differentiated and grouped under wilt resistant.

Genotypes	Wilt reaction	Genotypes	Wilt reaction
Varieties/ local cultivars		ICC accessions	
JG-62	Early wilting	ICC-12236	Wilt resistant
WR-315	Wilt resistance	ICC-12237	Wilt resistant
K-850	Late wilting	ICC-96030	Wilt resistant
BG-256	Late wilting	ICC-12234	Wilt resistant
Karikadli	Early wilting	ICC-12249	Wilt resistant
ICCV-2	Wilt resistant	ICC-12429	Wilt resistant
A-1	Late wilting	ICC-96029	Wilt resistant
ICC-4958	Late wilting	ICC-12252	Wilt resistant

3.1.1 *In vitro* pollen bioassay of selected genotypes for wilt resistance

The *in vitro* pollen germination medium suggested by Shivanna *et al.* (1997) with slight modification was used for the present study. The composition of the pollen germination medium used in the present study is given below.

Composition	Concentration
Sucrose	2.5%
Polyethylene glycol	10%
Boric acid	100 mg L ⁻¹
Ca(NO ₃) ₂	200 mg L ⁻¹
MgSO ₄	200 mg L ⁻¹
KNO ₃	100 mg L ⁻¹

Bioassay consisted of two aspects *i.e.* pollen germination and pollen tube growth.

3.1.1.2 *In vitro* pollen germination

The selected genotypes were grown in the field following standard agronomic practices. Each genotype was grown in two rows of 3 meter length. At flowering, the flowers which were about to dehisce were collected in morning hours (8 to 9 am) in petriplates. The pollen grains were collected in cavity slides after dehiscence and incubated in humid chamber (petriplate with moist filter paper below and top) at 80-90 per cent relative humidity for 20 minutes at room temperature.

The sitting drop method in cavity slide was used for pollen germination. Hundred micro litre (µl) of the above mentioned media was kept in each cavity and incubated pollen grains were sprinkled on to the media uniformly. Then the cavity slides were incubated in humid chamber (petriplate with moist filter paper below and top) at 80-90 per cent RH at room temperature for 45 minutes. The number of pollen grains germinated (pollen grains with length at least equal to their diameter) and non-germinated were counted in each field using projection microscope (SPICON, SP-

585/SP-585 A) and expressed as per cent pollen germination. In each field on the projection microscope (SPICON, SP-585/SP-585 A) five germinated pollen grains were randomly selected and the tube length was measured on the screen of the projection microscope which was marked in specific units (1 unit=0.05 mm). The above mentioned pollen germination medium (3.1.1.1) gave very high pollen germination and tube growth in all the selected genotypes. Hence, the above mentioned medium was used for all the studies.

3.1.1.3 Standardization of *in vitro* Pollen Germination Medium (PGM) for pollen bioassay

Fusaric acid (FA) is one of the toxins produced by the chickpea wilt causing pathogen *Fusarium oxysporum* f.sp. *ciceris*. The commercially available fusaric acid was obtained from Sigma (cat F-6513) and used in the pollen germination medium (PGM) for pollen bioassay. For standardization of PGM for pollen bioassay the PGM with different concentrations of fusaric acid, viz. 0 (control), 25, 50, 75, 100 and 150 μg of FA/ml of PGM was prepared. The pre-conditioned pollen of susceptible check JG-62 and wilt resistant genotype WR-315 were tested for their pollen germination and pollen tube growth as mentioned in 3.1.1.2 in all the six concentrations of FA. The increased concentration of fusaric acid in the PGM did not greatly affect the pollen germination of all the genotypes. However, the pollen tube growth was significantly reduced with increase in FA concentration in both the genotypes. The inhibition of tube growth was not uniform in both the genotypes. The results are presented below.

Fusaric acid concentration ($\mu\text{g ml}^{-1}$ of PGM)	Pollen tube length	
	JG-62 (mean μm)	WR-315 (mean μm)
0 (control)	241.13 $\pm$ 1.99	362.63 $\pm$ 1.33
25	228.93 $\pm$ 1.53	349.08 $\pm$ 1.08
50	156.63 $\pm$ 1.45	306.13 $\pm$ 1.85
75	95.25 $\pm$ 1.98	252.50 $\pm$ 1.55
100	87.13 $\pm$ 1.52	194.13 $\pm$ 1.21
150	63.13 $\pm$ 0.61	161.35 $\pm$ 1.12

At 150 µg of FA/ml of PGM, both the genotypes showed more than 50 per cent reduction in tube growth with respect to control. While at 25 µg of FA/ml, there was not much difference with respect to control in both the genotypes. Based on the above results of pollen tube growth, three concentrations, viz. 50, 75 and 100 µg of FA/ml of PGM along with control were selected for all the experiments to screen the genotypes.

3.1.1.4 Performance of selected genotypes under *in vitro* pollen bioassay

The selected sixteen genotypes were grown in the field. At flowering, the pollen grains of all the genotypes were tested for pollen germination and tube growth at selected toxin concentrations of 0, 50, 75 and 100 µg/ml of PGM. Four replications for each concentration with two cavities per replication were used for all the genotypes and five randomly chosen fields per cavity were scored for pollen germination and tube length as mentioned in 3.1.1.2.

3.1.1.5 Determination of pollen parameters

(a) Pollen germination (per cent of control)

The values of pollen germination at each toxin level were converted into per cent of control for each genotype using the following formula.

$$\text{Per cent of control (pollen germination)} = \frac{\text{Per cent germination at a given toxin level}}{\text{Per cent germination at control}} \times 100$$

(b) Pollen tube length (per cent of control)

The values of pollen tube length (µm) at each toxin level were converted into per cent of control for each genotype using the following formula.

$$\text{Per cent of control (pollen tube growth)} = \frac{\left(\frac{\text{Tube length in control}}{\text{Tube length at a given toxin level}} \right)}{\text{Tube length in control}} \times 100$$

(c) Toxin required to inhibit 50 per cent pollen tube growth

The per cent control values were used to calculate the concentration of toxin required to inhibit 50 per cent pollen growth (LD_{50} values) by using Probit analysis. The LD_{50} values were determined for each genotype and replication separately. Greater the LD_{50} values, greater is the tolerance of pollen grains vice-versa. The LD_{50} values for the pollen tube growth inhibition with four replications were analyzed following Completely Randomized Design. The analysis of variance is presented below.

Source of variation	Degrees of freedom	Mean sum of squares
Genotypes (g)	(g-1)	MSSg
Error	(gr-g)	EMSS
Total	(gr-1)	

Where,

r = number of replications (4)

g = number of genotypes (16)

S.Em was computed using mean sum of squares due to error from Completely Randomized Design-analysis (CRD).

$$\text{S.Em. (genotypes)} = \sqrt{\frac{\text{error MSS}}{r}}$$

Factorial analysis

Data on pollen tube growth of sixteen genotypes at four toxin concentrations were analyzed using Two Factorial Completely Randomized Design. First factor was assigned with genotypes and the second was with toxin concentrations. The structure of analysis is as follows.

Source of variation	Degrees of freedom	Mean sum of squares
Factor A (Genotypes)	(a-1)	MSSa
Factor B (toxin levels)	(b-1)	MSSb
Interaction (AxB)	(a-1) (b-1)	MSSi
Error	ab(r-1)	EMSS
Total	(abr-1)	

Where,

a = number of genotypes (16)

b = number of toxin levels (4)

r = number of replications (4)

S.Em estimates were computed using mean sum of squares due to error factor from Two Factorial Completely Randomized Design analysis.

$$\text{S.Em. (genotypes)} = \sqrt{\frac{\text{error MSS}}{r \times b}}$$

$$\text{S.Em. (toxin levels)} = \sqrt{\frac{\text{error MSS}}{r \times a}}$$

$$\text{S.Em. (interactions)} = \sqrt{\frac{\text{error MSS}}{r}}$$

3.1.2 Analysis of selected genotypes for molecular markers linked to wilt resistance loci

Two genes H_1 and H_2 are important for *Fusarium* wilt resistance. Molecular markers linked to H_1 and H_2 loci have been identified (Mayer *et al.*, 1997;

Thippeswamy *et al.*, 2005; Soregaon *et al.*, 2007). The ASAP marker CS-27₇₀₀ developed by Mayer *et al.* (1997) linked to susceptibility at H_1 locus and three RAPD markers OPA-07₁₆₄₄, OPK-09₁₈₀₁ (Thippeswamy *et al.*, 2005) and A07C₄₁₇ (Soregaon *et al.*, 2007) linked to susceptibility at H_2 locus were used in the present study.

3.1.2.1 DNA extraction

The selected sixteen genotypes were grown in small plastic pots separately and DNA was extracted from the growing bud of each genotype by following rapid method of isolation (Edwards *et al.*, 1991) with some modifications. The procedure of DNA extraction is presented below.

1. Tender growing bud of young seedlings was harvested and crushed in 400 μ l of extraction buffer (200 mM Tris-HCl, 250 mM NaCl, 25 mM EDTA and 0.5 per cent SDS maintained at 8 pH) using 1.5 ml centrifuge tube and micro pestle.
2. The crushed sample was centrifuged at 9000 rpm for 4 minutes at 4°C.
3. The supernatant was transferred to fresh tube and equal volume of chloroform: isoamylalcohol mixture (24:1 v/v) was added and shaken vigorously and centrifuged at 9000 rpm for 7 minutes at 4°C.
4. The supernatant was carefully transferred to fresh tube and equal volume (400 μ l) of pre-chilled isopropanol was added and mixed gently.
5. The above solution was centrifuged at 9000 rpm for 7 minutes to pellet the DNA.
6. The supernatant was discarded and the pellet was washed with 70 per cent ethanol and then air-dried for 15-20 minutes.
7. The DNA pellet was suspended in 100 μ l of T₁₀E₁ (10 mM Tris-HCl, 1 mM EDTA maintained at pH 8) in sterile centrifuge tube and kept for 1 hr at room temperature.

The quality and concentration of extracted DNA was assessed by gel electrophoresis using 0.8 per cent agarose.

3.1.2.2 Polymerase chain reaction

Template DNA

Purified genomic DNA extracts (30 ng) of the parents were used as template DNA.

Primers

- a. CS-27₇₀₀ allele specific associated primers (ASAP) were custom synthesized at Sigma, Bangalore.
- b. Three RAPD primers OPA-07, OPK-09 (Thippeswamy *et al.*, 2005) and A07C₄₁₇ (Soregaon *et al.*, 2007) associated with h_2 locus of wilt resistance were used for validation.

Primer sequences

CS-27 (F)	5'AGCTGGTCGCGGGTCAGAGGAAGA3'
(R)	5'AGTGGTCGCGATGGGGCCATGGTG3'
OPA-07	5'GAAACGGGTG3'
OPK-09	5'GGGTAACGCC3'
A07C	5'GAAACGGGTGC3'

Taq DNA polymerase

Taq DNA polymerase (3 units per μ l) and 10X Taq buffer were obtained from M/S Bangalore Genei Pvt. Ltd., Bangalore.

dNTPs

Individual dNTPs such as dATP, dGTP, dCTP and dTTP were obtained from M/S Bangalore Genei, Pvt., Ltd., Bangalore.

Thermal cycler

Eppendorf Master cycler gradient® was used for cyclic amplification of DNA.

PCR reaction mix

The PCR reaction mixture consisted of 1 µl of 30 ng template DNA, 1 µl (5ng/µl) of primer, 1 µl of 0.1 mM dNTP mix, 0.34 µl of Taq DNA polymerase, 2.5 µl of 10x Taq assay buffer in a final volume of 25 µl.

3.1.2.3 PCR amplification

For ASAP

The PCR amplification for ASAP primer was done by following the method proposed by Mayer *et al.* (1997).

Sl. No.	Step	Temperature (° C)	Duration (min.)	Cycles
1.	Denaturation	95	5	1
2.	Denaturation	94	20 sec.	40
3.	Annealing	62	1	
4.	Extension	72	1	
5.	Final extension	72	8	1

For RAPD

The PCR amplification for RAPD analysis was performed according to Williams *et al.* (1990) with certain modifications.

Sl. No.	Step	Temperature (° C)	Duration (min.)	Cycles
1.	Denaturation	95	5	1
2.	Denaturation	94	1	40
3.	Annealing	36	1	
4.	Extension	72	2	
5.	Final extension	72	8	1

3.1.2.4 Separation of amplified products by agarose gel electrophoresis

The amplified products from each tube along with 2 µl of loading dye were separated on 1.2 per cent and 2 per cent gels for RAPD and ASAP amplified products respectively. 1x TAE buffer of pH 8.0 was used as electrolyte and λ DNA (*Eco*RI + *Hind* III double digest) and 100 bp ladder were used as molecular weight markers for RAPD and ASAP amplified products respectively. The gels were photographed using gel documentation system (Uvitech, Cambridge, England).

3.1.2.5 Scoring of the bands linked to h_1 and h_2 loci

The scoring of the bands linked to different loci of wilt susceptibility was done based on the presence or absence of the specific bands amplified by the respective primers. The details of the bands linked to resistant loci produced by different primers are presented below.

Marker	Locus	Size of specific band (bp)
CS-27 ₇₀₀	h_1	700
OPA-07 ₁₆₄₄	h_2	1644
OPK-09 ₁₈₀₁	h_2	1801
A07C ₄₁₇	h_2	417

3.1.3 *In vivo* screening of selected genotypes for wilt resistance

The 16 selected genotypes were studied for wilt resistance at seedling stage by following the procedure developed recently by Ravikumar and Ratna Babu (2007). The selected genotypes were studied for wilt reaction at '0' and 15 ppm fusaric acid under *in vivo* condition in glass vials.

The seeds of chickpea genotypes were surface sterilized and incubated in water for 48 hours. The uniformly germinating seeds were planted in plastic cups (8 cm diameter) filled with sterilized peat moss and allowed to grow for seven more

days. The nine days old seedlings of chickpea were used to study their response to varied concentrations of phytotoxin. Fusaric acid (Sigma F6513), a well known phytotoxin produced by several pathogenic strains of *Fusarium oxysporum*, the causal organism of wilt in a great variety of plants (Kern, 1972). On the 9th day the seedlings were uprooted from cups and the roots were cleaned very gently under sterile water, dried for a few minutes and then placed in 50 ml vials containing 15 ml solutions of '0' and 15 ppm fusaric acid (FA). Glass beads were used to keep the seedlings in an upright position. The vials were covered with aluminum foil to protect the roots from light and were incubated at ambient temperature in a glass house for 15 days. Ten seedlings for '0' and 15 ppm of fusaric acid were used as replicates and the experiment was repeated two times at different periods of time.

The number of days for wilting at 15 ppm FA was counted and the genotypes wilted on 6th and 9th day were classified as early wilters and late wilters, respectively while those remained healthy for 12 days were grouped as resistant.

3.1.4 Comparative analysis of selected genotypes for pollen reaction, molecular markers and *in vivo* seedling screening

The rank correlation (Spearman, 1904) between the toxin concentrations for 50 per cent inhibition of pollen tube growth and number of days taken for wilting of genotypes during *in vivo* screening was determined.

The genotypes were classified into resistant or susceptible based on seedling wilt reaction to fusaric acid. The genotypes were also classified as resistant and susceptible based on pollen bioassay. Similarly, the genotypes were classified based on molecular markers. The classification of genotypes based on pollen bioassay, seedling reaction and molecular markers was compared to determine the efficiency of pollen bioassay as a technique to select the chickpea genotypes for wilt resistance.

3.2 COMBINED ANALYSIS OF POLLEN BIOASSAY AND MOLECULAR MARKERS IN RECOMBINANT INBRED LINES

3.2.1 Selection of material

The plant material used for this experiment included RILs of a cross between highly susceptible early wilting genotype JG-62 containing susceptibility linked molecular markers at both H_1 and H_2 and resistant WR-315. JG-62 is an early wilting genotype containing susceptibility linked molecular markers at both H_1 and H_2 . JG-62 is designated as $H_1H_1H_2H_2$ while WR-315 is designated as $h_1h_1h_2h_2$ with reference to wilt. The RILs of the cross JG-62 x WR-315 (F_5 generation) were available at this department (Brindha and Ravikumar, 2005) and obtained for this study. A set of 139 RILs were used for the study. The lines were grown in the Botanical Garden, Agricultural College, Dharwad during 2004 *rabi* and advanced to F_7 generation using single seed descent method.

3.2.2 Molecular marker analysis of RILs

The two molecular markers CS-27₇₀₀ (Mayer *et al.*, 1997) and A07C₄₁₇ (Soregaon *et al.*, 2007) which were validated for *Fusarium* wilt in selected genotypes were used to analyze the 139 RILs. All the RILs were sown in plastic pots separately and DNA was isolated as described in 3.1.2.1. The DNA extracted from the 139 RILs and their parents was screened for ASAP markers CS-27₇₀₀ (Mayer *et al.*, 1997) and RAPD marker A07C₄₁₇ (Soregaon *et al.*, 2007) separately. The polymerase chain reaction and scoring of amplified specific bands were performed as mentioned in 3.1.2.2 and 3.1.2.5 respectively.

Statistical analysis

a) Goodness of fit for ASAP marker CS-27₇₀₀ and RAPD marker A07C₄₁₇

Single locus goodness of fit for 1:1 segregation of presence and absence of ASAP marker CS-27₇₀₀ and RAPD marker A07C₄₁₇ was carried out separately using chi-square test (Snedecor and Cochran, 1956b).

b) Goodness of fit for 1:1:1:1 segregation of wilt reaction

Two loci of resistance H_1 and H_2 segregate independently. It is expected that the two linked markers also segregate independently (Brindha and Ravikumar, 2005). Therefore, inheritance of two markers together was tested against digenic goodness of fit for 1:1:1:1 ratio using chi-square test (Snedecor and Cochran, 1956b).

3.2.3 Pollen bioassay of selected RILs

Fifty randomly selected RILs were grown in the field during *rabi* 2005. Each entry was grown in two rows of 2 meter length. All these selected RILs were sown 3 times in the field at an interval of 15 days for staggered flowering. It was ensured that flowers of all the 50 RILs were available for a longer duration in order to perform pollen bioassay in the laboratory. At flowering, the pollen grains were collected in cavity slides after dehiscence and incubated at 80-90 per cent RH for 20 minutes at room temperature. The pre-conditioned pollen was subjected to pollen bioassay at four selected concentrations of toxin (0, 50, 75 and 100 μg FA/ml PGM) as mentioned in 3.1.1.4. Four replications for each concentration with two cavities per replication were prepared for all the genotypes. In five randomly chosen fields per cavity the pollen tube length was measured as given in 3.1.1.2. The observed pollen tube length were converted to per cent control and the toxin concentration required for 50 per cent pollen tube growth inhibition (LD_{50} values) for each genotype and replication were determined as mentioned earlier (3.1.1.5). The LD_{50} values with four replications were analyzed following Completely Randomized Block Design and the genotypes were compared. Susceptible and resistance groups were formed based on the relative concentration of toxin required for 50 per cent inhibition of pollen tube growth.

The data on pollen tube length (μm) of each genotype at all the four toxin levels was subjected to Two Factorial Completely Randomized Block design for

testing the variability among the RILs (1st factor), for the toxin levels (2nd factor) and the interaction effect as given in 3.1.1.5. The S.E.m. of genotypes, toxin levels and interaction effects were used to compare the critical differences of all the treatment combinations (3.1.1.5).

3.2.4 Analysis of molecular markers and pollen bioassay in RILs

a) The 50 RILs were ranked based on the pollen bioassay using the toxin concentrations required to inhibit the 50 per cent tube growth (LD₅₀ values).

b) Based on the molecular markers analysis using the markers linked to h_1 locus (CS-27₇₀₀) and h_2 locus (A07C₄₁₇), the RILs were grouped into 3 groups viz., early wilting (amplification for both linked markers), late wilting (amplification for any one of the linked marker) and resistant (absence of both the markers).

c) The mean LD₅₀ values of the above three groups were compared using unpaired t-test (Snedecor and Cochran, 1956a).

3.3 SELECTION OF PROGENIES BASED ON POLLEN BIOASSAY AND THEIR VALIDATION WITH MOLECULAR MARKERS

The F₅ progenies of a cross between late wilting BG-256 and resistant WR 315 were used for this experiment. BG-256 is a late wilting genotype having susceptibility linked molecular marker at H_2 locus while WR 315 is resistant with no susceptibility linked marker. Eighty F₅ progenies of this cross (BG-256 x WR-315) were available at this department. All the eighty F₅ lines were grown in the Botanical Garden, College of Agriculture, Dharwad during *rabi* 2005. Twenty two F₅ progenies were selected randomly and pollen bioassay was performed using four different levels of toxin with four replications per toxin level as described earlier (3.1.1.4). The observations on pollen tube growth were recorded as mentioned in 3.1.1.2. The LD₅₀ values were determined for each of the selected progenies replication wise and CRD

analysis was performed to compare the progenies as given in 3.1.1.5. Susceptible and resistant groups were formed based on the relative LD₅₀ values.

Data on pollen tube growth of 22 F₅ progenies with four treatment effects were analyzed for variability using Two Factorial Completely Randomized Block Design. First factor was assigned with F₅ progenies, while second with the toxin levels. The structure of analysis followed and S.E.m calculation was done as given in 3.1.1.5.

The F₆ generation of the selected 22 progenies were grown in pots and DNA was isolated. The DNA of these 22 lines was analyzed for presence or absence of DNA markers linked to *H₁* and *H₂* loci as given earlier (3.1.2). Based on the molecular marker data the 22 F₆ progenies were grouped into two groups: late wilting and resistant lines.

The selection based on pollen bioassay was validated by comparing the mean values of LD₅₀ toxin levels of two groups (late wilting and resistant formed based on molecular markers) using unpaired t-test (Snedecor and Cochran, 1956a).

3.4 EVALUATION OF SELECTED GENOTYPES, RILs AND F₅ GENERATION PROGENIES FOR YIELD AND YIELD COMPONENTS

The fifteen genotypes (Trial-1), 125 RILs (Trial-2) and 80 F₅ generation progenies (Trial-3) were evaluated in separate field trials in the Botanical Garden of College of Agriculture, Dharwad, during *rabi* 2005. Each trial was laid out separately in Randomized Complete Block Design (RCBD) with three replications. All the package of practices were same for all the three trials. Each entry was represented by a single row of two meter length per replication with a spacing of 30 cm between rows and 10 cm between plants within a row.

The experiment was conducted under residual soil moisture with protective irrigations by following all the recommended agronomic practices including pesticidal sprays for good crop growth.

3.4.1 Collection of data

Five plants per replication per genotype were randomly selected in all the trials. The following observations were recorded on each of the five selected competitive plants.

1. **Plant height at harvest:** The plant height was measured in centimeters (cm) from ground level to the tip of main stem at harvest.
2. **Number of primary branches:** The total number of branches arising directly from the main stem were counted at the time of harvest.
3. **Days to 50 per cent flowering:** The number of days from sowing to the opening of flowers in 50 per cent of plants in each genotype was recorded.
4. **Number of pods per plant:** Total number of pods per plant were counted at the time of harvest.
5. **Test weight:** The weight in grams (g) of a counted sample of 100 seeds from each replicatio was recorded.
6. **Seed yield per plant:** The weight of seeds in grams (g) from each plant was recorded.

3.4.2 Statistical methods

The mean of data collected for six characters was subjected for following statistical analysis. For each trial statistical analysis was carried separately.

1. Analysis of variance (ANOVA)

The analysis of variance for different characters was carried out using the mean data in order to partition variability due to different sources by following Panse and Sukhatme (1961).

Source of variation	d.f.	M.S.S.	Expected values of M.S.S.
Replication (r)	r-1	MSSr	-
Genotypes (g)	g-1	MSSg	$\sigma_e^2 + r\sigma_g^2$
Error	(r-1)(g-1)	EMSS	σ_e^2
Total	(rg-1)		

Where,

g = number of genotypes

r = number of replications

2. Estimation of genetic parameters

In order to assess and quantify the genetic variability among the genotypes for the characters under study, the following parameters were estimated as given below:

i) Estimation of variance components

Phenotypic and genotypic variances were estimated using the following formula,

$$\text{Genotypic variance } (\sigma_g^2) = \frac{\text{MSSg} - \text{EMSS}}{r}$$

$$\text{Phenotypic variance } (\sigma_p^2) = \sigma_g^2 + \text{EMSS} = \frac{\text{MSSg} - \text{EMSS}}{r} + \text{EMSS}$$

ii) Coefficient of variability

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

a) Genotypic coefficient of variability (GCV)

$$\text{GCV} = \frac{\sqrt{\sigma_g^2}}{\bar{X}} \times 100$$

b) Phenotypic coefficient of variability (PCV)

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

Where,

σ_g^2 = Genotypic variance

σ_p^2 = Phenotypic variance

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

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

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

iii) Heritability

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

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

Where,

σ_g^2 = Genotypic variance

σ_p^2 = Phenotypic variance

Heritability estimates were classified into low, moderate and high by following Hanson *et al.* (1956).

Heritability	Classification
0 to 30	Low
30 to 60	Medium
60 and above	High

iv) Genetic advance (GA)

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

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

Where,

h^2 = Heritability estimate

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

σ_p = Phenotypic standard deviation

v) Genetic advance as per cent of mean (GAM)

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

Where,

$\bar{X}$ = General mean of the character

Classification of GAM is as follows (Johnson *et al.*, 1955)

GAM	Classification
0 to 10	Low
10 to 20	Medium
20 and above	High

vi) Correlation analysis

The correlation coefficients were worked out to determine the degree of association of a character with yield and also among the yield components.

Phenotypic correlations were computed by using the formula given by Weber and Moorthy (1952).

$$r_p = \frac{\text{Cov}(X_p Y_p)}{\sqrt{V(X)_p \cdot V(Y)_p}}$$

Where,

r_p = Phenotypic correlation

$\text{Cov } X_p Y_p$ = Phenotypic covariance between the characters 'x' and 'y'

$V(X)_p$ and $V(Y)_p$ = Phenotypic variance of the characters 'x' and 'y' respectively

The genotypic correlation coefficients were also calculated as phenotypic correlation coefficients using the formula given below.

$$r_g = \frac{\text{Cov } X_g Y_g}{\sqrt{V(X)_g \cdot V(Y)_g}}$$

Where,

r_g = Genotypic correlation

$\text{Cov } X_g Y_g$ = Genotypic covariance between the characters 'x' and 'y'

$V(X)_g$ and $V(Y)_g$ = Genotypic variance of the characters 'x' and 'y' respectively

Both genotypic and phenotypic correlation coefficients were compared against table 'r' values given in Fisher and Yates (1963) table at (n-2) degrees of freedom at the probability levels of 0.05 and 0.01 to test their significance.

vii) Path coefficient analysis

Path coefficient analysis was carried out by using the correlation coefficients to know the direct and indirect effects of the six component characters on yield as suggested by Wright (1921) and illustrated by Dewey and Lu (1959).

Path coefficients were obtained by solving the simultaneous equations which express the basic relationship between correlations and path coefficients. The equations were as follows

$$r_{1,y} = P_{1,y} + r_{1,2}P_{2,y} + r_{1,3}P_{3,y} + \dots + r_{1,7}P_{7,y}$$

$$r_{2,y} = P_{2,y} + r_{2,1}P_{1,y} + r_{2,3}P_{3,y} + \dots + r_{2,7}P_{7,y}$$

$$r_{3,y} = P_{3,y} + r_{3,1}P_{1,y} + r_{3,2}P_{2,y} + \dots + r_{3,7}P_{7,y}$$

...

...

$$r_{6,y} = P_{6,y} + r_{6,2}P_{1,y} + r_{6,2}P_{2,y} + \dots + r_{6,6}P_{6,y}$$

where, $r_{1,y}$ to $r_{6,y}$ denote the correlation coefficients between independent characters 1 to 6 and dependent character 'y', $r_{1,2}$ to $r_{5,6}$ denote the correlation coefficients between all possible combinations of independent characters. $P_{1,y}$ to $P_{6,y}$ denote the effects of characters 1 to 6 on character y.

Experimental Results

IV. EXPERIMENTAL RESULTS

The results of various experiments conducted in the present study are presented under following headings.

- 4.1 Evaluation of selected chickpea genotypes for wilt resistance using *in vitro* pollen bioassay, molecular markers and *in vivo* seedling screening
- 4.2 Combined analysis of pollen bioassay and molecular markers in recombinant inbred lines (RILs) of chickpea
- 4.3 Selection of F₅ progenies of chickpea based on pollen bioassay and their validation with molecular markers
- 4.4 Evaluation of chickpea genotypes, RILs and F₅ generation progenies for yield and yield components

4.1 EVALUATION OF SELECTED CHICKPEA GENOTYPES FOR WILT RESISTANCE USING *IN VITRO* POLLEN BIOASSAY AND MOLECULAR MARKERS AND *IN VIVO* SEEDLING SCREENING

4.1.1 Performance of selected genotypes under *in vitro* pollen bioassay

The sixteen selected genotypes were tested for *in vitro* pollen germination and pollen tube growth at different levels of fusaric acid (fungal toxin) under laboratory conditions.

Pollen germination

The analysis of variances (Table 4.1) indicated that there was a significant variation for pollen germination, among the selected genotypes, toxin levels and their interaction.

The results of *in vitro* pollen germination of selected genotypes at different toxin levels are presented in Table 4.2. The results indicate that there was more than

Table 4.1. ANOVA for *in vitro* pollen germination in chickpea genotypes at different toxin levels

Source	df	MS	F
Genotypes	15	231.17**	33.67
Toxin levels	3	663.48**	96.64
Interaction	45	35.01**	5.10
Error	192	6.87	

*,** significant at 5% and 1% respectively

Table 4.2. Effect of toxin on *in vitro* pollen germination in chickpea genotypes

Sl. No	Genotype	Germination (%)						
		T1 (control)	T2 (50µg/ml)	T3 (75µg/ml)	T4 (100µg/ml)	T1-T2	T1-T3	T1-T4
1	JG-62	92.05 (100)	90.56 (98.37)	79.81 (86.70)	74.08 (80.47)	1.50 (1.63)	12.24** (13.30)	17.98** (19.53)
2	WR-315	94.39 (100)	93.57 (99.13)	87.92 (93.14)	87.2 (92.38)	0.82 (0.87)	6.47** (6.86)	7.19** (7.62)
3	K-850	91.14 (100)	91.10 (99.96)	88.14 (96.72)	82.65 (90.69)	0.04 (0.04)	2.99 (3.28)	8.49** (9.31)
4	BG-256	88.58 (100)	97.04 (109.6)	84.93 (95.88)	80.11 (90.44)	-8.46** (-9.55)	3.65 (4.12)	8.47** (9.56)
5	Karikadle	84.85 (100)	87.14 (102.7)	78.94 (93.04)	76.87 (90.60)	-2.29 (-2.70)	5.91** (6.96)	7.98** (9.40)
6	ICCV-2	76.45 (100)	77.68 (101.6)	78.54 (102.7)	76.66 (100.3)	-1.24 (-1.62)	-2.10 (-2.74)	-0.21 (-0.28)
7	A-1	82.46 (100)	88.23 (106.9)	79.47 (96.37)	77.52 (94.01)	-5.76** (-6.99)	2.99 (3.63)	4.94* (5.99)
8	ICC-12236	82.22 (100)	82.05 (99.80)	82.05 (99.79)	80.83 (98.31)	0.16 (0.20)	0.17 (0.21)	1.39 (1.69)
9	ICC-12237	86.84 (100)	85.96 (99.00)	87.48 (100.7)	86.51 (99.63)	0.87 (1.00)	-0.64 (-0.74)	0.32 (0.37)
10	ICC-96030	88.58 (100)	87.81 (99.13)	85.07 (96.04)	80.02 (90.34)	0.77 (0.87)	3.51 (3.96)	8.56** (9.66)
11	ICC-12434	80.54 (100)	81.69 (101.4)	77.47 (96.19)	78.93 (97.99)	-1.15 (-1.42)	3.07 (3.81)	1.62 (2.01)
12	ICC-12249	86.52 (100)	84.78 (97.99)	79.61 (92.01)	81.58 (94.29)	1.74 (2.01)	6.91** (7.99)	4.94* (5.71)
13	ICC-4958	87.24 (100)	85.02 (97.45)	78.94 (90.48)	79.95 (91.64)	2.22 (2.55)	8.31** (9.52)	7.30** (8.36)
14	ICC-12429	90.32 (100)	85.89 (95.10)	83.00 (91.90)	78.78 (87.23)	4.43* (4.90)	7.32** (8.10)	11.54** (12.77)
15	ICC-96029	92.39 (100)	87.37 (94.57)	89.89 (97.29)	80.99 (87.66)	5.02** (5.43)	2.51 (2.71)	11.40** (12.34)
16	ICC-12252	80.35 (100)	77.29 (96.19)	76.24 (94.88)	77.47 (96.42)	3.06* (3.81)	4.11* (5.12)	2.88* (3.58)
	Treatment mean	86.56	86.45	82.34	80.01			

Per cent of control values are represented in parenthesis

*,** significant at 5% and 1% respectively

S.Em (interactions) = 1.310

75 per cent pollen germination in all the genotypes irrespective of the toxin levels. However, there was a gradual reduction in mean pollen germination with increase in the toxin concentration. The mean pollen germination in control was 86.56 per cent and it was 86.45, 82.34 and 80.01 per cent at 50, 75 and 100 μg of FA per ml of PGM respectively.

Although there was a reduction in mean pollen germination values with increase in toxin concentration, the reduction was not uniform across the genotypes. At 50 μg of FA per ml of PGM, significant reduction of pollen germination with reference to control was observed only in three genotypes, viz. ICC-96029 (5.02%), ICC-12429 (4.43%) and ICC-12252 (3.06%). At the same toxin level BG-256, A-1, Karikadle, ICCV-2 and ICC-12434 recorded increased pollen germination than the control. At 75 μg of FA six genotypes recorded significant reduction in pollen germination. The highest reduction was observed in JG-62 (12.24%) followed by ICC-4958 (8.31%), while ICCV-2 and ICC-12237 did not shown any reduction. At 100 μg of FA, the highest reduction was observed in JG-62 (17.98%) followed by ICC-12429 (11.54%) and ICC-96029 (11.40%) while ICCV-2 did not show any reduction in pollen germination.

Pollen tube growth

The analysis of variance (Table 4.3) indicated that there was highly significant variation for mean pollen tube growth among the selected genotypes, toxin levels and their interaction.

The results of *in vitro* pollen tube growth of selected sixteen genotypes at different toxin levels are presented in Table 4.4.

The basal pollen germination medium (control) comprising of sucrose (2.5%), polyethylene glycol (10%), boric acid (100 mg L⁻¹), CaNO₃ (200 mg L⁻¹), MgSO₄

Table 4.3 ANOVA for *in vitro* pollen tube growth in chickpea genotypes at different toxin levels

Source	df	MS	F
Genotypes	15	70433.31**	3498.43
Toxin levels	3	448365.30**	22270.38
Interaction	45	3002.72**	149.15
Error	192	20.13	

*, ** significant at 5% and 1% respectively

Table 4.4. Effect of toxin on *in vitro* pollen tube growth in chickpea genotypes

Sl. No	Genotype	Pollen tube length (μ m)						
		T1 (control)	T2 (50 μ g/ml)	T3 (75 μ g/ml)	T4 (100 μ g/ml)	T1-T2	T1-T3	T1-T4
1	JG-62	241.13 (100)	155.13 (64.33)	95.25 (39.50)	87.13 (36.13)	86.00** (35.67)	145.88** (60.50)	154.00** (63.87)
2	WR-315	360.13 (100)	306.13 (85.01)	255.00 (70.81)	194.13 (53.90)	54.00** (14.99)	105.13** (29.19)	166.00** (46.10)
3	K-850	368.25 (100)	268.00 (72.78)	189.63 (51.49)	163.88 (44.50)	100.25** (27.22)	178.63** (48.51)	204.38** (55.50)
4	BG-256	318.00 (100)	219.88 (69.14)	181.38 (57.04)	135.38 (42.57)	98.13** (30.86)	136.63** (42.96)	182.63** (57.43)
5	Karikadle	295.00 (100)	196.25 (66.53)	125.38 (42.50)	91.88 (31.14)	98.75** (33.47)	169.63** (57.50)	203.13** (68.86)
6	ICCV-2	417.13 (100)	343.50 (82.35)	275.00 (65.93)	171.25 (41.05)	73.63** (17.65)	142.13** (34.07)	245.88** (58.95)
7	A-1	316.25 (100)	246.13 (77.83)	197.88 (62.57)	141.25 (44.66)	70.13** (22.17)	118.38** (37.43)	175.00** (55.34)
8	ICC-12236	272.13 (100)	182.75 (67.16)	146.63 (53.88)	77.88 (28.62)	89.38** (32.84)	125.50** (46.12)	194.25** (71.38)
9	ICC-12237	436.00 (100)	351.63 (80.65)	246.50 (56.54)	151.63 (34.78)	84.38** (19.35)	189.50** (43.46)	284.38** (65.22)
10	ICC-96030	459.25 (100)	400.88 (87.29)	269.38 (58.66)	200.38 (43.63)	58.38** (12.71)	189.88** (41.34)	258.88** (56.37)
11	ICC-12434	250.88 (100)	150.63 (60.04)	109.38 (43.60)	98.25 (39.16)	100.25** (39.96)	141.50** (56.40)	152.63** (60.84)
12	ICC-12249	353.88 (100)	342.50 (96.79)	292.38 (82.62)	236.00 (66.69)	11.38** (03.21)	61.50** (17.38)	117.88** (33.31)
13	ICC-4958	430.50 (100)	356.75 (82.87)	259.63 (60.31)	181.13 (42.07)	73.75** (17.13)	170.88** (39.69)	249.38** (57.93)
14	ICC-12429	243.75 (100)	143.13 (58.72)	105.38 (43.23)	90.75 (37.23)	100.63** (41.28)	138.38** (56.77)	153.00** (62.77)
15	ICC-96029	317.63 (100)	312.50 (98.39)	212.00 (66.75)	112.00 (35.26)	5.13 (01.61)	105.63** (33.25)	205.63** (64.74)
16	ICC-12252	256.88 (100)	160.00 (62.29)	137.25 (53.43)	101.13 (39.37)	96.88** (37.71)	119.63** (46.57)	155.75** (60.63)
	Treatment mean	333.55	258.48	193.63	139.63			

Per cent of control values are represented in parenthesis

*, ** significant at 5% and 1% respectively

S.Em (interactions) = 2.243

(200 mg L⁻¹) and KNO₃ (100 mg L⁻¹) has given the highest pollen tube growth in all the genotypes. At control the highest pollen tube growth was observed in the genotype ICC-96030 (459.25 µm) followed by ICC-12237 (436 µm), while the lowest was in JG-62 (241.13 µm).

The basal pollen germination medium was supplemented with varying levels of toxin ranging from 50 µg to 100 µg FA per ml of an interval of 25 µg/ml. The genotype ICC-96030 recorded the highest pollen tube growth (400.88 µm) at 50 µg of FA while, ICC-12249 recorded the highest at both 75 (292.38 µm) and 100 µg (236.0 µm). The least pollen tube growth was recorded in JG-62 at both 50 (155.13 µm) and 75 µg (95.25 µm) and ICC-12236 (77.88 µm) at 100 µg of FA.

The increase in toxin concentration significantly reduced the pollen tube growth in all the genotypes (Table 4.4). However, the degree of reduction varied among the genotypes. The highest reduction of pollen tube growth with respect to control at 50, 75 and 100 µg/ml was recorded in ICC-12429 (100.63 µm), ICC-96030 (189.88 µm) and ICC-12429 (100.63 µm) respectively. At 50 µg/ml the least reduction was recorded in ICC-96029 (5.13 µm), while ICC-12249 recorded least reduction at both 75 (61.50 µm) and 100 µg/ml (117.88 µm). Effect of different concentrations of fusaric acid on *in vitro* pollen tube growth of early wilting, late wilting and resistant genotypes are presented in Plate 1.

The reduction in pollen tube growth was converted to per cent of control values (value in parenthesis of Table 4.4). The highest reduction with respect to control at 50 µg of FA was observed in ICC-12429 (41.28%) while least was recorded in ICC-96029 (1.61%). Similarly highest reduction at 75 µg of FA was observed in JG-62 (60.50%) followed by Karikadle (57.50%) and ICC-12429 (56.77%), while the least was in ICC-12249 (17.38%) followed by WR-315 (29.19%). The reduction levels at 100 µg of FA with respect to control were as follows: The highest reduction



Control

50 µg/ml

75 µg/ml

100 µg/ml

Early wilter (JG-62)



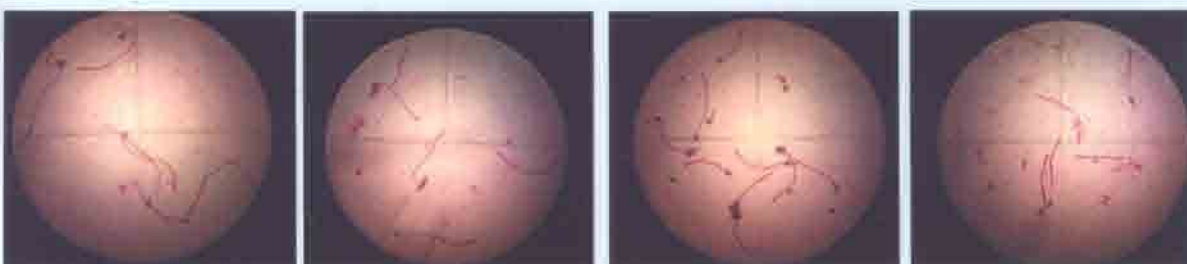
Control

50 µg/ml

75 µg/ml

100 µg/ml

Late wilter (K-850)



Control

50 µg/ml

75 µg/ml

100 µg/ml

Resistant (WR-315)

Plate 1. Effect of different concentration of fusaric acid on *in vitro* pollen tube growth of early wilter (JG-62), late wilter (K-850) and resistant (WR-315) genotypes of chickpea

was observed in ICC-12236 (71.38%) followed by Karikadle (68.86%) and ICC-12237 (65.22%), while the least reduction was recorded in ICC-12249 (33.31%) followed by WR-315 (46.10%).

In general there was reduction of pollen tube length in all the genotypes but the degree of reduction varied among the genotypes. The variation in degree of pollen tube growth inhibition was quantified by calculating the toxin concentration required to inhibit 50 per cent pollen tube growth (LD_{50} values). The analysis of variance of LD_{50} values of selected sixteen genotypes (Table 4.5) indicated that there was a significant variation among the genotypes for LD_{50} values.

The highest toxin concentration required for inhibition of 50 per cent pollen tube growth was observed in ICC-12249 (122.4 μg) followed by WR-315 (109.1 μg), while the least was recorded in ICC-12429 (64.3 μg) followed by JG-62 (66.53 μg) (Table 4.6).

4.1.2 *In vivo* screening of selected genotypes for wilt resistance

The sixteen selected genotypes were studied for wilt reaction at '0' and 15 ppm FA. The number of days taken for appearance of wilting symptoms and death of the seedlings for each genotype are presented in Table 4.7. Four genotypes Karikadle, ICC-12434, JG-62 and ICC-12429 took 6 days for wilting at 15 ppm FA and were grouped as early wilters, while ten genotypes, viz. A-1, ICCV-2, ICC-96030, ICC-4958, ICC-96029, BG-256, K-850, ICC-12237, ICC-12252 and ICC-12236 took 9 days and were grouped as late wilters. The remaining two genotypes (ICC-12249 and WR-315) survived for 12 days and only on 13th day wilting symptoms were observed. These two genotypes were grouped as resistant. All the genotypes remained healthy upto 15 days at '0' ppm FA (control) when the experiment was terminated. Wilting symptoms in early wilting, late wilting and resistant genotypes on 6th, 9th, and 12th day are presented in Plate 2.

Table 4.5 ANOVA for LD₅₀ of toxin for inhibition of pollen tube growth in chickpea genotypes

Source	df	MS	F
Genotypes	15	999.27**	271.119
Error	48	3.69	

*, ** significant at 5% and 1% respectively

Table 4.6 Variation in toxin levels for 50 per cent pollen tube growth inhibition in chickpea genotypes

Sl. No.	Genotype	Fusaric acid(μ g/ml)
1	ICC-12249	122.4 A
2	WR-315	109.1 B
3	A-1	92.41 C
4	ICCV-2	90.39 CD
5	ICC-96030	88.98 CD
6	ICC-4958	88.33 CD
7	ICC-96029	87.89 CD
8	BG-256	85.21 DE
9	K-850	84.03 DE
10	ICC-12237	81.15 EF
11	ICC-12252	76.34 FG
12	ICC-12236	72.04 GH
13	Karikadle	67.68 HI
14	ICC-12434	66.71 HI
15	JG-62	66.53 HI
16	ICC-12429	64.30 I

Table 4.7 Seedling reaction of chickpea genotypes to root feeding of fusaric acid

Sl. No.	Genotype	Days to wilting		Grouping
		0 ppm	15 ppm	
1	ICC-12249	15	13	R
2	WR-315	15	13	
3	A-1	15	9	LW
4	ICCV-2	15	9	
5	ICC-96030	15	9	
6	ICC-4958	15	9	
7	ICC-96029	15	9	
8	BG-256	15	9	
9	K-850	15	9	
10	ICC-12237	15	9	
11	ICC-12252	15	9	
12	ICC-12236	15	9	
13	Karikadle	15	6	EW
14	ICC-12434	15	6	
15	JG-62	15	6	
16	ICC-12429	15	6	

R = Resistant, LW = Late wilting, EW = Early wilting



Plate 2. Seedling reaction of early wilting (JG-62), late wilting (K-850) and resistant (WR-315) genotypes of chickpea to root feeding of fusaric acid (15 ppm)

4.1.3 Analysis of selected genotypes for molecular markers linked to wilt resistance loci

The genomic DNA of selected sixteen genotypes were screened for the identified molecular markers linked to *Fusarium* wilt resistance loci H_1 and H_2 .

The ASAP marker CS-27₇₀₀ developed by Mayer *et al.* (1997) linked to susceptibility at H_1 locus and three RAPD markers OPA-07₁₆₄₄, OPK-09₁₈₀₁ (Thippeswamy *et al.*, 2005) and A07C₄₁₇ (Soregaon *et al.*, 2007) linked to susceptibility at H_2 locus were used in the present study. The markers : CS-27₇₀₀, OPA-07₁₆₄₄, OPK-09₁₈₀₁ and A07C₄₁₇ amplified specific fragments of size 700, 1644, 1801 and 417 bp respectively. The sixteen selected genotypes were studied for the presence or absence of the specific bands amplified by the respective primers and the results are presented in Table 4.8.

Five genotypes *viz.*, JG-62, Karikadle, ICC-12236, ICC-12434 and ICC-12429 showed amplification of CS-27₇₀₀ on the other hand 10, 2 and 13 genotypes had specific amplification for OPA-07₁₆₄₄, OPK-09₁₈₀₁ and A07C₄₁₇ markers respectively. Only one genotype WR-315 did not show amplification for any of the markers. It was also observed that the markers OPK-09₁₈₀₁ and OPA-07₁₆₄₄ were not validated in different genetic backgrounds. For example OPK-09₁₈₀₁ was absent in JG-62 and Karikadle which are early wilting and OPA-07₁₆₄₄ was absent in A-1 and BG-256 which are late wilting and reported to be having dominant allele at H_2 locus (Ravikumar *et al.*, 2003). Therefore, these two markers (OPK-09₁₈₀₁ and OPA-07₁₆₄₄) were not considered for the analysis. Only the other two markers, CS-27₇₀₀ linked to H_1 locus of susceptibility and A07C₄₁₇ linked to H_2 locus of susceptibility were considered. Out of sixteen genotypes studied four genotypes *viz.*, Karikadle, ICC-12434, JG-62 and ICC-12429 amplified for both markers, ten genotypes *viz.*, A-1, ICCV-2, ICC-96030, ICC-4958, ICC-96029, BG-256, K-850, ICC-12237, ICC-12252

Table 4.8 Reaction of selected genotypes to identified molecular markers linked to H_1 and H_2 loci of *Fusarium* wilt resistance in chickpea

Sl. No.	Genotype	Markers			
		CS-27 ₇₀₀	OPA-07 ₁₆₄₄	OPK-09 ₁₈₀₁	A07C ₄₁₇
1	JG-62	+	+	-	+
2	WR-315	-	-	-	-
3	K-850	-	+	+	+
4	BG-256	-	-	-	+
5	Karikadle	+	+	-	+
6	ICCV-2	-	-	-	+
7	A-1	-	-	-	+
8	ICC-12236	+	+	-	-
9	ICC-12237	-	+	-	+
10	ICC-96030	-	+	-	+
11	ICC-12434	+	-	-	+
12	ICC-12249	-	+	-	-
13	ICC-4958	-	+	+	+
14	ICC-12429	+	+	-	+
15	ICC-96029	-	+	-	+
16	ICC-12252	-	-	-	+

and ICC-12236 amplified for anyone of the markers. While, two genotypes (ICC-12249 and WR-315) did not show any marker (Plate 3).

4.1.4 Comparative analysis of selected genotypes for pollen bioassay and *in vivo* seedling screening

The genotypes were classified based on pollen bioassay and *in vivo* seedling screening (Table 4.9). The two genotypes which recorded high LD₅₀ values, viz. ICC-12249 (122.4 µg) and WR-315 (109.1 µg) remained healthy upto 12th day of root feeding with 15 ppm FA. The four genotypes which recorded the low LD₅₀ values for pollen tube growth inhibition, viz. ICC-12429 (64.3 µg), JG-62 (66.53 µg), ICC-12434 (66.71 µg) and Karikadle (67.68 µg) took only 6 days for showing wilting symptoms under *in vivo* seedling screening. The other ten genotypes recorded intermediate LD₅₀ values and took 9 days for appearance of wilting symptoms and death (Table 4.9).

The results of Spearman's rank correlation (Table 4.10) indicate that there was a highly significant positive correlation (0.805) between *in vivo* seedling screening and *in vitro* pollen bioassay in terms of days to wilting of seedling and LD₅₀ requirement for pollen tube growth inhibition, respectively.

4.1.5 Comparative analysis of selected genotypes for pollen bioassay and molecular markers

The genotypes were grouped into three based on the molecular markers : the genotypes having both markers (supposed to be early wilting), any one (supposed to be late wilting) none (supposed to be resistant). Four genotypes viz., Karikadle, ICC-12434, JG-62 and ICC-12429 showed positive for both the markers and grouped as early wilting, while two genotypes ICC-12249, WR-315 did not have any marker and grouped as resistant. The remaining ten genotypes amplified for any one of the two makers and were grouped as late wilting.

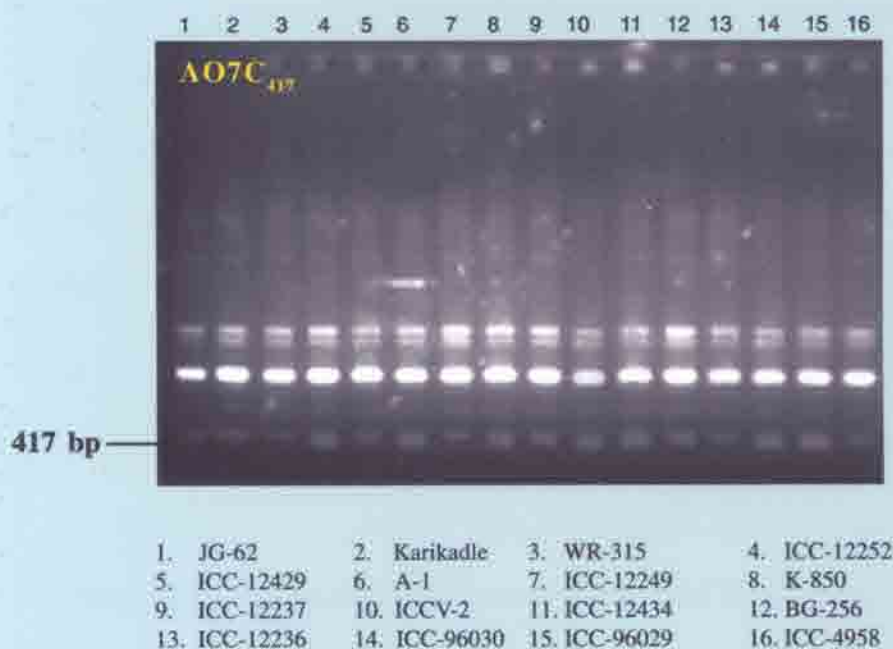
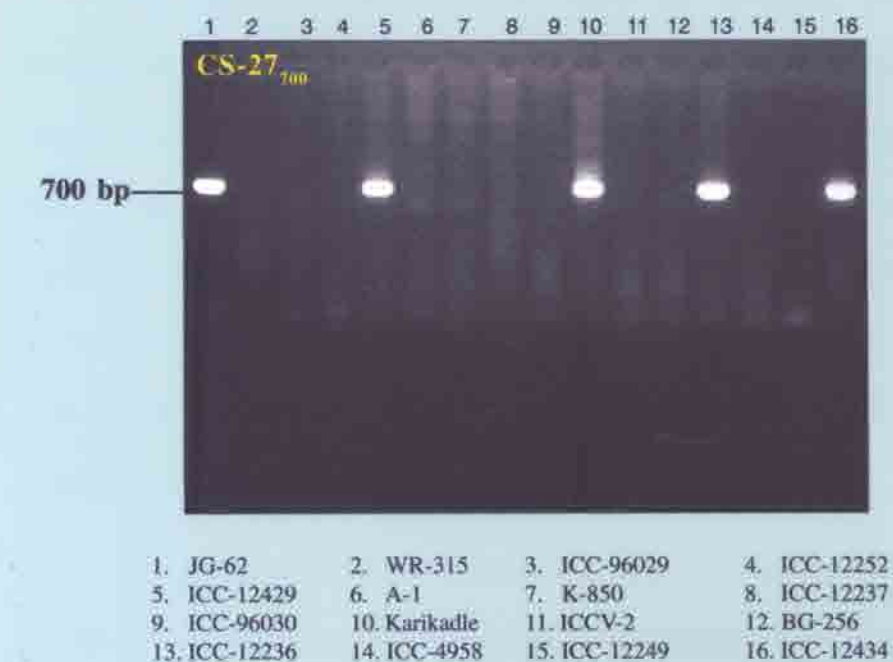


Plate 3. Amplification of DNA markers linked to H_1 (CS-27₇₀₀) and H_2 (AO7C₄₁₇) loci of wilt resistance in chickpea genotypes

The genotypes were classified based on molecular markers and pollen bioassay and compared (Table 4.11). The two genotypes recorded maximum LD₅₀ values *viz.*, ICC-12249 (122.4 µg) and WR-315 (109.1 µg) did not have any DNA markers *viz.*, CS-27₇₀₀ and A07C₄₁₇. In case of four genotypes which recorded the least LD₅₀ values for pollen tube growth inhibition *viz.*, ICC-12429 (64.3 µg), JG-62 (66.53 µg), ICC-12434 (66.71 µg) and Karikadle (67.68 µg) showed the presence of both the markers. The other ten genotypes recorded intermediate LD₅₀ values showed positive for any one of the two markers (Table 4.11).

The mean LD₅₀ values of toxin for early wilting, late wilting and resistant groups were calculated and compared using t-test (Table 4.9). The mean LD₅₀ values of genotypes showing presence of both the markers was 66.41 µg, the mean LD₅₀ value of genotypes showing presence of any one of the markers was 84.67 µg, while the mean LD₅₀ value of genotypes showing absence of both the markers was 115.75 µg. The results of unpaired t-test (Table 4.12) indicate that there was significant difference between groups of genotypes showing presence of both markers, genotypes showing presence of any one of the linked markers and those lacking both the markers.

4.1.6 Comparative analysis of wilt reaction of selected genotypes based on *in vivo* seedling reaction, pollen bioassay and molecular markers

The genotypes grouped separately based on molecular markers, pollen bioassay and *in vivo* seedling reaction were compared (Table 4.13).

The two genotypes ICC-12249 and WR-315 which showed the absence for both the linked markers (supposed to be resistant) recorded high levels of LD₅₀ toxin for pollen tube growth inhibition and also remained healthy for 12 days under *in vivo* seedling screening at 15 ppm FA. Four genotypes Karikadle, ICC-12434, JG-62 and

Table 4.9 Comparison of pollen bioassay and *in vivo* seedling reaction of chickpea genotypes

Sl. No.	Genotype	LD ₅₀ Fusaric acid(μ g/ml)		Days to wilting at 15 ppm FA	Grouping
1	ICC-12249	122.4	A	13	R
2	WR-315	109.1	B	13	
3	A-1	92.41	C	9	LW
4	ICCV-2	90.39	CD	9	
5	ICC-96030	88.98	CD	9	
6	ICC-4958	88.33	CD	9	
7	ICC-96029	87.89	CD	9	
8	BG-256	85.21	DE	9	
9	K-850	84.03	DE	9	
10	ICC-12237	81.15	EF	9	
11	ICC-12252	76.34	FG	9	
12	ICC-12236	72.04	GH	9	
13	Karikadle	67.68	HI	6	EW
14	ICC-12434	66.71	HI	6	
15	JG-62	66.53	HI	6	
16	ICC-12429	64.30	I	6	

R = Resistant, LW = Late wilting, EW = Early wilting

Table 4.10 Rank correlation between pollen bioassay and *in vivo* seedling reaction

	LD ₅₀ Fusaric acid(μ g/ml)	Days to wilting
LD ₅₀ Fusaric acid(μ g/ml)	1	
Days to wilting	0.805**	1

** Significant at 1%

Table 4.11 Comparison of LD₅₀ of toxin for inhibition of pollen tube growth and molecular marker reaction in chickpea genotypes

Sl. No.	Genotype	Fusaric acid (µg/ml)	Markers		Grouping
			CS-27 ₇₀₀	A07C ₄₁₇	
1	ICC-12249	122.4 A	-	-	R
2	WR-315	109.1 B	-	-	
3	A-1	92.41 C	-	+	LW
4	ICCV-2	90.39 CD	-	+	
5	ICC-96030	88.98 CD	-	+	
6	ICC-4958	88.33 CD	-	+	
7	ICC-96029	87.89 CD	-	+	
8	BG-256	85.21 DE	-	+	
9	K-850	84.03 DE	-	+	
10	ICC-12237	81.15 EF	-	+	
11	ICC-12252	76.34 FG	-	+	
12	ICC-12236	72.04 GH	+	-	
13	Karikadle	67.68 HI	+	+	EW
14	ICC-12434	66.71 HI	+	+	
15	IG-62	66.53 HI	+	+	
16	ICC-12429	64.30 I	+	+	

R = Resistant, LW = Late wilting, EW = Early wilting

Table 4.12 Variation in mean toxin requirement for 50 per cent pollen tube growth inhibition in different resistant groups of chickpea genotypes

Marker reaction CS-27 ₇₀₀ ; A07C ₄₁₇	No. of Genotypes	Mean toxin (LD ₅₀)
Both positive	4	66.41 ± 0.71 ^a
Either of the one positive	10	84.67 ± 2.05 ^b
Both negative	2	115.75 ± 6.65 ^c

Values with the same superscript do not differ

Table 4.13 Comparison of molecular markers, seedling screening and pollen bioassay of selected chickpea genotypes for wilt reaction

Sl. No.	Genotype	Markers		Days to wilting at 15 ppm FA	LD ₅₀ Fusaric acid(μ g/ml)	Grouping
		CS-27 ₇₀₀	A07C ₄₁₇			
1	ICC-12249	-	-	13	122.4	R
2	WR-315	-	-	13	109.1	
3	A-1	-	+	9	92.41	LW
4	ICCV-2	-	+	9	90.39	
5	ICC-96030	-	+	9	88.98	
6	ICC-4958	-	+	9	88.33	
7	ICC-96029	-	+	9	87.89	
8	BG-256	-	+	9	85.21	
9	K-850	-	+	9	84.03	
10	ICC-12237	-	+	9	81.15	
11	ICC-12252	-	+	9	76.34	
12	ICC-12236	+	-	9	72.04	
13	Karikadle	+	+	6	67.68	EW
14	ICC-12434	+	+	6	66.71	
15	JG-62	+	+	6	66.53	
16	ICC-12429	+	+	6	64.30	

R = Resistant, LW = Late wilting, EW = Early wilting

ICC-12429 which showed the presence for both linked markers (supposed to be early wilting) recorded least LD₅₀ values ranging from 64.30 to 67.68 for pollen tube growth inhibition and took only 6 days for wilting and death of seedlings during *in vivo* root feeding of seedlings. The remaining 10 genotypes showed the presence of any one linked marker (supposed to be late wilting) recorded moderate LD₅₀ values ranging from 72.04 to 92.41 for pollen tube growth inhibition and took 9 days for wilting and death of seedlings during *in vivo* root feeding with 15 ppm FA.

4.2 COMBINED ANALYSIS OF POLLEN BIOASSAY AND MOLECULAR MARKERS IN RECOMBINANT INBRED LINES OF CHICKPEA

4.2.1 Molecular marker analysis of RILs

The two molecular markers CS-27₇₀₀ (Mayer *et al.*, 1997) and A07C₄₁₇ (Soregaon *et al.*, 2007) which were found to be linked to H_1 and H_2 loci of susceptibility in different genetic backgrounds were used for the present study. A total of 139 RILs were studied for presence or absence of the DNA markers. Out of 139 RILs studied, 61 RILs showed the presence of ASAP marker CS-27₇₀₀ (linked to h_1 locus) and 78 RILs showed the absence of this marker. Similarly 79 RILs showed the presence of RAPD marker A07C₄₁₇ (linked to h_2 locus) and 60 RILs showed absence of the marker (Appendix I). The chi-square analysis (Table 4.14) for single locus goodness of fit for 1:1 ratio for the individual markers CS-27₇₀₀ and A07C₄₁₇ separately indicated non-significant chi-square values of 2.079 and 2.597 respectively. The 139 RILs segregated in expected 1:1 ratio for both the markers.

The RILs were classified into four categories based on the presence or absence of both the markers together. Both the markers were present in 34 RILs and both the markers were absent in 33 RILs. The marker, CS-27₇₀₀ alone was present in 27 RILs and the marker, A07C₄₁₇ alone was present in 45 RILs (Table 4.15). The segregation

Table 4.14 Monogenic inheritance of DNA markers linked to H₁ and H₂ loci in chickpea RILs of JG 62 x WR 315

Marker	+ve	-ve	χ^2 (1:1)
CS-27 ₇₀₀	61	78	2.079
A07C ₄₁₇	79	60	2.597

Table 4.15 Digenic inheritance of DNA markers linked to H₁ and H₂ loci in chickpea RILs of JG 62 x WR 315

Marker CS-27 ₇₀₀ ; A07C ₄₁₇	Number of RILs	χ^2 (1:1:1:1)
+ve +ve	34	4.855
+ve -ve	27	
-ve +ve	45	
-ve -ve	33	

pattern of RILs was analyzed for digenic inheritance ratio. The chi-square analysis for two loci (digenic) segregation ratio of 1:1:1:1 (Table 4.15) indicate that there is a goodness of fit for the expected 1:1:1:1 ratio.

4.2.2 Pollen bioassay of selected RILs

Fifty randomly selected RILs of cross JG-62 x WR-315 were studied for *in vitro* pollen tube growth under varied toxin concentrations. The analysis of variance (Table 4.16) indicated that there was significant variation for mean pollen tube length among the RILs, the different toxin levels and interaction effects as well.

The results of *in vitro* pollen tube growth of 50 RILs at different toxin levels are presented in Table 4.17.

The basal pollen germination medium (control) has given the highest pollen tube growth in all the 50 RILs. At control the highest pollen tube growth was observed in RIL No. 20 (472 μm) and the lowest was observed in RIL No. 33 (197.5 μm). The RILs 4 (391.01 μm), 83 (313.00 μm) and 59 (264.63 μm) recorded the highest pollen tube growth at 50, 75 and 100 μg of FA/ml of PGM respectively. While RILs 32 (151.50 μm), 5 (91.00 μm) and 33 (62.50 μm) recorded the least pollen tube growth at 50, 75 and 100 μg of FA respectively.

The increase in toxin concentration has significantly reduced the pollen tube growth in all the 50 RILs (Table 4.17). The highest reduction of pollen tube growth with respect to control in 50, 75 and 100 $\mu\text{g}/\text{ml}$ was recorded in RIL number 49. While, RILs 34 (35.63 μm) 99 (80.00 μm) and 65 (110.88 μm) recorded the least reduction at 50, 75 and 100 $\mu\text{g}/\text{ml}$ of PGM respectively.

The pollen tube growth values at different toxin levels were converted to per cent of control (values in parenthesis of Table 4.17). The values indicate that the highest reduction in pollen tube growth with respect to control at 50 (45.56%) and 75

Table 4.16 ANOVA for *in vitro* pollen tube growth in chickpea recombinant inbred lines at different toxin levels

Source	df	MS	F
Genotypes	49	62565.19**	3377.19
Toxin levels	3	1532525.46**	82723.70
Interaction	147	2881.14**	155.52
Error	600	18.53	

*, ** significant at 5% and 1% respectively

Table 4.17 Effect of toxin on *in vitro* pollen tube growth in chickpea recombinant inbred lines

Sl. No	RIL No.	Pollen tube length (µm)						
		T1 (control)	T2 (50µg/ml)	T3 (75µg/ml)	T4 (100µg/ml)	T1-T2	T1-T3	T1-T4
1	2	284.50 (100)	207.25 (72.85)	160.50 (56.41)	88.63 (31.15)	77.25** (27.15)	124.00** (43.59)	195.88** (68.85)
2	3	331.00 (100)	245.75 (74.24)	142.50 (43.05)	105.25 (31.80)	85.25** (25.76)	188.50** (56.95)	225.75** (68.20)
3	4	470.75 (100)	391.00 (83.06)	285.50 (60.65)	209.88 (44.58)	79.75** (16.94)	185.25** (39.35)	260.88** (55.42)
4	5	239.25 (100)	155.00 (64.79)	91.00 (38.04)	85.25 (35.63)	84.25** (35.21)	148.25** (61.96)	154.00** (64.37)
5	6	439.50 (100)	347.25 (79.01)	254.25 (57.85)	151.00 (34.36)	92.25** (20.99)	185.25** (42.15)	288.50** (65.64)
6	7	368.50 (100)	266.25 (72.25)	194.00 (52.65)	113.50 (30.80)	102.25** (27.75)	174.50** (47.35)	255.00** (69.20)
7	10	295.00 (100)	213.50 (72.37)	183.50 (62.20)	160.75 (54.49)	81.50** (27.63)	111.50** (37.80)	134.25** (45.51)
8	14	409.25 (100)	260.50 (63.65)	189.50 (46.30)	162.25 (39.65)	148.75** (36.35)	219.75** (53.70)	247.00** (60.35)
9	16	413.50 (100)	327.00 (79.08)	246.00 (59.49)	151.00 (36.52)	86.50** (20.92)	167.50** (40.51)	262.50** (63.48)
10	20	472.00 (100)	347.25 (73.57)	280.50 (59.43)	250.25 (53.02)	124.75** (26.43)	191.50** (40.57)	221.75** (46.98)
11	24	363.75 (100)	287.25 (78.97)	207.50 (57.04)	144.25 (39.66)	76.50** (21.03)	156.25** (42.96)	219.50** (60.34)
12	26	368.00 (100)	251.00 (68.21)	177.63 (48.27)	97.50 (26.49)	117.00** (31.79)	190.38** (51.73)	270.50** (73.51)
13	27	414.38 (100)	344.25 (83.08)	299.75 (72.34)	241.13 (58.19)	70.13** (16.92)	114.63** (27.66)	173.25** (41.81)
14	29	470.75 (100)	391.00 (83.06)	285.50 (60.65)	203.88 (43.31)	79.75** (16.94)	185.25** (39.35)	266.88** (56.69)
15	30	314.50 (100)	228.25 (72.58)	182.75 (58.11)	132.00 (41.97)	86.25** (27.42)	131.75** (41.89)	182.50** (58.03)
16	32	215.50 (100)	151.50 (70.30)	116.75 (54.18)	62.50 (29.00)	64.00** (29.70)	98.75** (45.82)	153.00** (71.00)
17	33	197.50 (100)	146.00 (73.92)	93.75 (47.47)	62.50 (31.65)	51.50** (26.08)	103.75** (52.53)	135.00** (68.35)
18	34	320.00 (100)	284.38 (88.87)	222.13 (69.41)	167.50 (52.34)	35.63** (11.13)	97.88** (30.59)	152.50** (47.66)
19	35	253.00 (100)	197.00 (77.87)	129.00 (50.99)	99.00 (39.13)	56.00** (22.13)	124.00** (49.01)	154.00** (60.87)
20	36	348.38 (100)	279.63 (80.27)	245.75 (70.54)	189.38 (54.36)	68.75** (19.73)	102.63** (29.46)	159.00** (45.64)
21	37	328.75 (100)	231.50 (70.42)	184.00 (55.97)	101.50 (30.87)	97.25** (29.58)	144.75** (44.03)	227.25** (69.13)
22	38	449.63 (100)	350.00 (77.84)	288.13 (64.08)	248.38 (55.24)	99.63** (22.16)	161.50** (35.92)	201.25** (44.76)
23	39	285.38 (100)	210.00 (73.59)	162.00 (56.77)	88.63 (31.06)	75.38** (26.41)	123.38** (43.23)	196.75** (68.94)
24	40	463.63 (100)	345.38 (74.49)	279.75 (60.34)	167.25 (36.07)	118.25** (25.51)	183.88** (39.66)	296.38** (63.93)
25	43	346.75 (100)	244.25 (70.44)	235.13 (67.81)	165.88 (47.84)	102.50** (29.56)	111.63** (32.19)	180.88** (52.16)

Table 4.17. Contd.....

Sl. No	RIL No.	Pollen tube length (μm)						
		T1 (control)	T2 (50 $\mu\text{g/ml}$)	T3 (75 $\mu\text{g/ml}$)	T4 (100 $\mu\text{g/ml}$)	T1-T2	T1-T3	T1-T4
26	46	286.63 (100)	173.50 (60.53)	115.00 (40.12)	98.00 (34.19)	113.13** (39.47)	171.63** (59.88)	188.63** (65.81)
27	47	464.50 (100)	315.00 (67.81)	215.00 (46.29)	177.25 (38.16)	149.50** (32.19)	249.50** (53.71)	287.25** (61.84)
28	49	439.50 (100)	239.25 (54.44)	144.00 (32.76)	100.00 (22.75)	200.25** (45.56)	295.50** (67.24)	339.50** (77.25)
29	50	364.88 (100)	273.00 (74.82)	230.50 (63.17)	203.00 (55.64)	91.88** (25.18)	134.38** (36.83)	161.88** (44.36)
30	52	288.50 (100)	221.50 (76.78)	190.50 (66.03)	161.63 (56.02)	67.00** (23.22)	98.00** (33.97)	126.88** (43.98)
31	54	414.50 (100)	352.13 (84.95)	240.88 (58.11)	178.75 (43.12)	62.38** (15.05)	173.63** (41.89)	235.75** (56.88)
32	55	389.75 (100)	330.00 (84.67)	218.25 (56.00)	126.50 (32.46)	59.75** (15.33)	171.50** (44.00)	263.25** (67.54)
33	56	325.50 (100)	186.50 (57.30)	131.50 (40.40)	105.50 (32.41)	139.00** (42.70)	194.00** (59.60)	220.00** (67.59)
34	58	289.50 (100)	219.75 (75.91)	175.75 (60.71)	63.50 (21.93)	69.75** (24.09)	113.75** (39.29)	226.00** (78.07)
35	59	441.13 (100)	372.88 (84.53)	305.88 (69.34)	264.63 (59.99)	68.25** (15.47)	135.25** (30.66)	176.50** (40.01)
36	60	414.25 (100)	329.75 (79.60)	286.50 (69.16)	226.50 (54.68)	84.50** (20.40)	127.75** (30.84)	187.75** (45.32)
37	64	238.25 (100)	184.38 (77.39)	153.63 (64.48)	125.38 (52.62)	53.88** (22.61)	84.63** (35.52)	112.88** (47.38)
38	65	240.63 (100)	188.25 (78.23)	148.13 (61.56)	129.75 (53.92)	52.38** (21.77)	92.50** (38.44)	110.88** (46.08)
39	66	393.13 (100)	327.75 (83.37)	270.13 (68.71)	227.13 (57.77)	65.38** (16.63)	123.00** (31.29)	166.00** (42.23)
40	67	290.25 (100)	218.75 (75.37)	177.00 (60.98)	111.25 (38.33)	71.50** (24.63)	113.25** (39.02)	179.00** (61.67)
41	68	262.75 (100)	202.00 (76.88)	160.50 (61.08)	114.00 (43.39)	60.75** (23.12)	102.25** (38.92)	148.75** (56.61)
42	70	313.25 (100)	232.50 (74.22)	184.75 (58.98)	122.75 (39.19)	80.75** (25.78)	128.50** (41.02)	190.50** (60.81)
43	73	307.00 (100)	252.50 (82.25)	195.00 (63.52)	100.00 (32.57)	54.50** (17.75)	112.00** (36.48)	207.00** (67.43)
44	77	292.25 (100)	214.50 (73.40)	169.25 (57.91)	94.00 (32.16)	77.75** (26.60)	123.00** (42.09)	198.25** (67.84)
45	78	472.00 (100)	347.25 (73.57)	280.50 (59.43)	248.25 (52.60)	124.75** (26.43)	191.50** (40.57)	223.75** (47.40)
46	83	465.00 (100)	380.50 (81.83)	313.00 (67.31)	164.25 (35.32)	84.50** (18.17)	152.00** (32.69)	300.75** (64.68)
47	86	362.25 (100)	271.25 (74.88)	217.75 (60.11)	133.25 (36.78)	91.00** (25.12)	144.50** (39.89)	229.00** (63.22)
48	93	398.88 (100)	339.13 (85.02)	262.38 (65.78)	210.00 (52.65)	59.75** (14.98)	136.50** (34.22)	188.88** (47.35)
49	96	285.00 (100)	205.50 (72.11)	160.00 (56.14)	90.50 (31.75)	79.50** (27.89)	125.00** (43.86)	194.50** (68.25)
50	99	238.00 (100)	181.75 (76.37)	158.00 (66.39)	72.50 (30.46)	56.25** (23.63)	80.00** (33.61)	165.50** (69.54)
	Treatment mean	345.23	261.12	202.44	143.73			

Per cent of control values are represented in parenthesis

*, ** significant at 5% and 1% respectively

S.E.m (interactions) = 2.165

µg of FA (67.24%) was observed in RIL number 49. While at 100 µg it was in RIL number 58 (78.07%). The RILs 34 (11.13%), 27 (27.66%) and 59 (40.01%) recorded least per cent of reduction at 50, 75 and 100 µg of FA respectively.

The toxin concentration required to inhibit 50 per cent pollen tube growth (LD_{50} value) was calculated for all the RILs for comparison. The analysis of variance of LD_{50} values (Table 4.18) indicated that there was a significant variation among the RILs for LD_{50} values. The highest LD_{50} value was observed in RIL number 27 (127.10 µg) followed by RIL number 59 (123.40 µg) while least was recorded in RIL number 49 (54.04 µg) followed by 56 (59.98 µg) (Table 4.19).

4.2.3 Comparative analysis of molecular markers and pollen bioassay in RILs

Based on molecular marker analysis, the selected 50 RILs were grouped into early wilting (presence of both the linked markers), late wilting (presence of any one of the linked markers) and resistant (absence of both the markers). Ten RILs amplified for both the markers and grouped as early wilting, 27 RILs showed only one of the two markers and were grouped as late wilting, while in 13 RILs the two markers were absent and were grouped as resistant.

The groups classified based on molecular markers and pollen bioassay were compared (Table 4.19). The LD_{50} values of the group of RILs showing absence of both the markers ranged from 105.90 µg to 127.10 µg. The LD_{50} values of the group showing presence of any one of the markers ranged from 73.65 to 105.80 µg/ml. For the group of RILs showing presence for both the markers, the LD_{50} values ranged from 54.04 µg to 73.52 µg. The RIL numbers 52, 34, 55 and 37 deviated from their respective groups (Table 4.19). For example the RIL 52 had only one marker and grouped under late wilting but with respect to LD_{50} values it was placed under resistant group. Similarly RIL 37 having single marker was grouped under early wilting.

Table 4.18 ANOVA for LD₅₀ of toxin for inhibition of pollen tube growth in recombinant inbred lines

Source	df	MS	F
Genotypes	49	1397.82**	103.01
Error	150	13.54	

*, ** significant at 5% and 1% respectively

Table 4.19 Comparison of LD₅₀ of toxin for inhibition of pollen tube growth and molecular marker reaction in selected RILs of chickpea

Sl. No.	RIL No.	Fusaric acid(µg/ml)		Markers		Grouping
				CS-27 ₇₀₀	A07C ₄₁₇	
1	27	127.10	A	-	-	R
2	59	123.40	AB	-	-	
3	52	121.00	AB	-	+ D	
4	50	119.70	ABC	-	-	
5	66	118.70	ABCD	-	-	
6	10	117.70	ABCDE	-	-	
7	60	115.80	ABCDE	-	-	
8	36	115.40	ABCDE	-	-	
9	38	114.40	BCDEF	-	-	
10	64	108.20	CDEFG	-	-	
11	65	107.10	DEFG	-	-	
12	20	106.70	DEFG	-	-	
13	43	105.90	EFG	-	-	
14	78	105.80	EFG	-	+	LW
15	93	103.20	FG	-	+	
16	34	102.90	FG	-	- D	
17	29	101.30	G	-	+	
18	4	90.73	H	-	+	
19	68	89.84	H	+	-	
20	54	88.20	HI	-	+	
21	83	86.44	HIJ	+	-	
22	30	86.09	HIJK	+	-	
23	67	84.70	HIJKL	+	-	
24	24	84.31	HIJKL	-	+	
25	70	84.09	HIJKL	-	+	
26	73	83.11	HIJKL	+	-	
27	16	83.03	HIJKL	+	-	
28	86	82.86	HIJKL	-	+	
29	40	82.35	HIJKL	-	+	
30	99	81.86	HIJKLM	-	+	
31	6	81.08	HIJKLM	+	-	
32	55	80.67	HIJKLM	+	+ D	
33	35	80.62	HIJKLM	-	+	
34	77	78.23	HIJKLMN	-	+	
35	39	77.12	IJKLMN	-	+	
36	2	76.76	IJKLMN	+	-	
37	96	76.70	IJKLMN	-	+	
38	58	75.11	JKLMNO	+	-	
39	7	74.67	JKLMNO	+	-	
40	32	73.65	JKLMNO	-	+	
41	47	73.52	KLMNO	+	+	EW
42	33	73.52	KLMNO	+	-	
43	37	72.88	LMNO	-	+ D	
44	3	71.94	LMNO	+	+	
45	14	71.92	LMNO	+	+	
46	26	69.26	MNOP	+	+	
47	5	66.00	NOP	+	+	
48	46	63.20	OPQ	+	+	
49	56	59.98	PQ	+	+	
50	49	54.04	Q	+	+	

D = Deviation from the group, R = Resistant, LW = Late wilting, EW = Early wilting

The mean LD₅₀ values of toxin for early wilting, late wilting and resistant groups were calculated and compared using t-test (Table 4.20). The mean LD₅₀ values of RILs showing presence of both the markers was 68.41 µg, the mean LD₅₀ value of RILs showing presence of CS-27₇₀₀ alone was 82.08 µg, the mean LD₅₀ value of RILs showing presence of OAP-07C₄₁₇ was 87.34, while the mean LD₅₀ value of RILs showing absence of both the markers was 114.08 µg. The results of unpaired t-test (Table 4.20) indicate that there was significant difference between groups of RILs showing presence of both markers, RILs showing presence of any one of the linked markers and RILs lacking both the markers. There was no significant difference between mean values of group of RILs showing presence of marker CS-27₇₀₀ and the group showing presence of marker linked to H₂ locus.

4.3 SELECTION OF F₅ PROGENIES OF CHICKPEA BASED ON POLLEN BIOASSAY AND THEIR VALIDATION WITH MOLECULAR MARKERS

Twenty two F₅ progenies of cross between BG-256 (*h₁h₁H₂H₂*) and WR-315 (*h₁h₁h₂h₂*) were randomly selected from 80 available progenies and pollen bioassay was performed as mentioned earlier.

The analysis of variance (Table 4.21) indicated that there was significant variation for *in vitro* pollen tube growth for both the factors (22 progenies and 4 toxin levels) and their progeny x toxin levels interaction.

The results of pollen bioassay of 22 F₅ progenies (Table 4.22) indicated that the highest levels of pollen tube growth were recorded at basal pollen germination media (control) in all the progenies. The addition of toxin reduced the pollen tube growth in all the progenies. The mean pollen tube length values in control, 50, 75 and 100 µg of FA per ml of PGM were 378.66 µm, 302.92 µm, 240.190 µm and 183.67 µm respectively.

Table 4.20 Variation in mean toxin requirement for 50 per cent pollen tube growth inhibition in different resistant groups of chickpea RILs

Marker reaction CS-27 ₇₀₀ ; A07C ₄₁₇	No. of RILs	Mean toxin (LD ₅₀)	
+ve +ve	10	68.41 ± 2.45 ^a	
+ve -ve	10	82.08 ± 1.62 ^b	84.71 ^b
-ve +ve	17	87.34 ± 3.19 ^b	
-ve -ve	13	114.08 ± 2.06 ^c	

Values with the same superscript do not differ

Table 4.21 ANOVA for pollen tube growth in selected F_5 progenies of chickpea at different toxin levels

Source	df	MS	F
Genotypes	21	30514.70**	2454.42
Toxin levels	3	618086.03**	49715.23
Interaction	63	2053.85**	165.20
Error	264	12.43	

*, ** significant at 5% and 1% respectively

Table 4.22 Effect of toxin on *in vitro* pollen tube growth in selected F_5 progenies of chickpea

Sl. No	Progeny No.	Pollen tube length (μ m)						
		T1 (control)	T2 (50 μ g/ml)	T3 (75 μ g/ml)	T4 (100 μ g/ml)	T1-T2	T1-T3	T1-T4
1	9	309.50 (100.00)	266.00 (85.95)	213.00 (68.82)	144.00 (46.53)	43.50** (14.05)	96.50** (31.18)	165.50** (53.47)
2	12	439.00 (100.00)	312.25 (71.13)	294.63 (67.11)	243.13 (55.38)	126.75** (28.87)	144.38** (32.89)	195.88** (44.62)
3	13	374.00 (100.00)	318.75 (85.23)	248.25 (66.38)	203.75 (54.48)	55.25** (14.77)	125.75** (33.62)	170.25** (45.52)
4	15	423.63 (100.00)	309.00 (72.94)	243.88 (57.57)	221.50 (52.29)	114.63** (27.06)	179.75** (42.43)	202.13** (47.71)
5	18	342.25 (100.00)	286.75 (83.78)	227.25 (66.40)	161.00 (47.04)	55.50** (16.22)	115.00** (33.60)	181.25** (52.96)
6	19	314.75 (100.00)	218.50 (69.42)	181.00 (57.51)	146.25 (46.47)	96.25** (30.58)	133.75** (42.49)	168.50** (53.53)
7	20	398.88 (100.00)	339.13 (85.02)	262.38 (65.78)	210.00 (52.65)	59.75** (14.98)	136.50** (34.22)	188.88** (47.35)
8	25	346.75 (100.00)	244.25 (70.44)	235.13 (67.81)	190.13 (54.83)	102.50** (29.56)	111.63** (32.19)	156.63** (45.17)
9	27	349.00 (100.00)	290.50 (83.24)	229.25 (65.69)	176.88 (50.68)	58.50** (16.76)	119.75** (34.31)	172.13** (49.32)
10	29	446.50 (100.00)	398.63 (89.28)	335.00 (75.03)	200.00 (44.79)	47.88** (10.72)	111.50** (24.97)	246.50** (55.21)
11	30	355.00 (100.00)	305.75 (86.13)	253.75 (71.48)	197.13 (55.53)	49.25** (13.87)	101.25** (28.52)	157.88** (44.47)
12	32	311.50 (100.00)	241.00 (77.37)	202.00 (64.85)	144.50 (46.39)	70.50** (22.63)	109.50** (35.15)	167.00** (53.61)
13	33	370.63 (100.00)	281.38 (75.92)	239.00 (64.49)	202.88 (54.74)	89.25** (24.08)	131.63** (35.51)	167.75** (45.26)
14	45	375.00 (100.00)	318.75 (85.00)	221.50 (59.07)	184.25 (49.13)	56.25** (15.00)	153.50** (40.93)	190.75** (50.87)
15	66	410.50 (100.00)	266.25 (64.86)	213.75 (52.07)	161.75 (39.40)	144.25** (35.14)	196.75** (47.93)	248.75** (60.60)
16	69	316.50 (100.00)	312.63 (98.78)	214.50 (67.77)	113.50 (35.86)	3.88 (1.22)	102.00** (32.23)	203.00** (64.14)
17	73	410.50 (100.00)	347.75 (84.71)	256.75 (62.55)	211.00 (51.40)	62.75** (15.29)	153.75** (37.45)	199.50** (48.60)
18	83	261.25 (100.00)	193.00 (73.88)	159.50 (61.05)	95.00 (36.36)	68.25** (26.12)	101.75** (38.95)	166.25** (63.64)
19	108	406.00 (100.00)	291.00 (71.67)	222.00 (54.68)	155.50 (38.30)	115.00** (28.33)	184.00** (45.32)	250.50** (61.70)
20	109	471.50 (100.00)	363.25 (77.04)	299.63 (63.55)	200.75 (42.58)	108.25** (22.96)	171.88** (36.45)	270.75** (57.42)
21	114	462.00 (100.00)	398.13 (86.17)	270.13 (58.47)	246.63 (53.38)	63.88** (13.83)	191.88** (41.53)	215.38** (46.62)
22	120	436.00 (100.00)	361.63 (82.94)	262.00 (60.09)	231.25 (53.04)	74.38** (17.06)	174.00** (39.91)	204.75** (46.96)
Treatment mean		378.66	302.92	240.19	183.67			

Per cent of control values are represented in parenthesis

*, ** significant at 5% and 1% respectively

S.Em (interactions) = 1.763

The pollen tube growth of progenies with reference to control was calculated. There was significant reduction at all the toxin levels in all the progenies except in case of progeny number 69 (3.88 μm) at 50 $\mu\text{g/ml}$. The progeny number 66 recorded highest reduction at 50 (144.25 μm) and 75 (196.75 μm) μg of FA with reference to control. The least reduction at 50, 75 and 100 μg of FA compared to control were recorded in progeny numbers 69 (3.88 μm), 9 (96.50 μm) and 25 (156.63 μm) respectively (Table 4.22).

The critical concentration of toxin required for inhibition of 50 per cent of pollen tube growth (LD_{50} value) was calculated for all the 22 progenies. The analysis of variance (Table 4.23) indicated that there was significant variation among the progenies with respect to LD_{50} values. The LD_{50} values of 22 progenies were compared for their critical differences and ranked accordingly (Table 4.24).

The F_6 generation of above selected 22 progenies were subjected to molecular marker analysis using the linked marker to H_2 locus (A07C₄₁₇). The progenies segregate only for H_2 locus. The results of molecular marker analysis were presented in Table 4.24. Out of 22 progenies, 11 showed the presence of the marker (A07C₄₁₇) linked to H_2 locus and 11 progenies did not produce the amplicon linked to H_2 locus. Based on the presence or absence of the molecular marker linked to h_2 locus (A07C₄₁₇) the 22 progenies were classified into late wilting (presence of A07C₄₁₇) and resistant (absence of A07C₄₁₇) groups.

The mean LD_{50} values of toxin for late wilting and resistant groups were calculated and compared using t-test (Table 4.25). The LD_{50} values of the group of progenies showing presence of A07C₄₁₇ ranged from 76.53 μg to 98.97 μg while the group lacking the marker ranged from 99.34 μg to 137.50 μg . The progeny numbers 12, 45 and 109 deviated from their respective groups. For example progeny number 12 showing presence of A07C₄₁₇ was grouped under late wilting but with respect to

Table 4.23 ANOVA for LD₅₀ of toxin for inhibition of pollen tube growth in F₅ progenies of chickpea

Source	df	MS	F
Genotypes	21	928.76**	77.25
Error	66	12.02	
Total	87		

*,** significant at 5% and 1% respectively

Table 4.24 Comparison of LD₅₀ values and molecular marker reaction in segregating progenies of chickpea

Sl. No.	Progeny No.	Fusaric acid(µg/ml)	Markers		Grouping
			CS-27 ₇₀₀	A07C ₄₁₇	
1	25	137.50 A	-	-	R
2	12	137.40 A	-	+ D	
3	33	115.80 B	-	-	
4	30	112.00 BC	-	-	
5	13	106.80 BCD	-	-	
6	20	103.20 CDE	-	-	
7	15	102.80 CDE	-	-	
8	27	101.20 CDEF	-	-	
9	120	100.80 CDEF	-	-	
10	73	99.34 DEFG	-	-	
11	114	98.97 DEFG	-	+	LW
12	29	97.34 DEFG	-	+	
13	9	96.81 DEFG	-	+	
14	18	96.56 DEFG	-	+	
15	32	96.29 DEFG	-	+	
16	45	94.28 EFG	-	- D	
17	19	91.51 EFGH	-	+	
18	109	90.59 FGHI	-	- D	
19	69	88.47 GHI	-	+	
20	83	82.81 HIJ	-	+	
21	108	80.13 IJ	-	+	
22	66	76.53 J	-	+	

D = Deviation from the group

R = Resistant, LW = Late wilting

Table 4.25 Variation in mean toxin requirement for 50 per cent pollen tube growth inhibition in resistant groups of chickpea progenies

Marker reaction CS-27 ₇₀₀ ; A07C ₄₁₇	No. of F ₆ progenies	Mean toxin (LD ₅₀) concentrations
-ve -ve	11	105.84 ^a
-ve +ve	11	94.80 ^b

Values with the same superscript do not differ

LD₅₀ values it was placed under resistant group. Similarly progenies 45 and 109 lacking the marker were grouped under late wilting. The mean LD₅₀ values of progenies showing absence of the marker was 105.84 µg, while the mean LD₅₀ value of progenies showing presence of A07C₄₁₇ marker was 94.80 µg. The results of unpaired t-test indicate that there was significant difference between those two groups.

4.4 EVALUATION OF SELECTED GENOTYPES, RILs AND F₅ GENERATION PROGENIES FOR YIELD AND YIELD COMPONENTS

4.4.1 Genetic variability and genetic components of variation

The fifteen selected genotypes, 125 RILs and 80 F₅ generation segregating progenies were studied for variability and genetic components of variation separately. The results are presented below experiment wise.

4.4.1.1 Analysis of variance

The three sets of plant material viz., 15 selected genotypes, 125 RILs and 80 F₅ generation progenies were evaluated for yield and yield components. The analysis of variance (Table 4.26) revealed that there was significant difference among treatments within each set of plant material for all the six quantitative traits viz., days to 50 per cent flowering, plant height, number of primary branches, number of pods per plant, test weight and seed yield per plant.

4.4.1.2 Genetic variability

The mean values of all the six quantitative characters studied of selected genotypes, RILs and segregating progenies were presented in Appendix II, III and IV, respectively. The mean, range, phenotypic coefficient of variation (PCV), genotypic coefficient of variation (GCV), heritability and genetic advance as per cent of mean

Table 4.26 Analysis of variance for six characters of selected genotypes, RILs and segregating progenies of chickpea

Source of variation	df	Days to 50% flowering	Plant height	Number of primary branches	Number of pods per plant	Test weight	Seed yield per plant
Selected genotypes	14	370.80**	19.63*	0.81**	253.66**	16.89**	22.85**
Recombinant inbred lines	124	14.26**	28.78**	2.19**	823.08**	9.74**	88.87**
F ₅ progenies	79	14.65**	31.89**	2.09**	791.01**	39.31**	83.01**

*, ** = Significant at 5% and 1% respectively

(GAM) were calculated for the six quantitative traits of all the three sets of plant material separately and presented character wise in Table 4.27.

4.4.1.2.1 Days to 50 per cent flowering

The mean number of days taken for flowering in selected genotypes, RILs and segregating progenies were 46.49, 47.33 and 47.33 days respectively. The trait ranged from 23.33 (ICC-96029) to 56.67 (WR-315) days in selected genotypes, from 43.00 (RIL-19) to 51.00 (RILs-20 and 74) days in RILs and from 43.00 (progeny-96) to 49.67 (progeny-20) days in the segregating progenies. The phenotypic and genotypic coefficients of variance were high in selected genotypes (25.56; 23.05) and were low in both RILs (5.35; 4.18) and segregating progenies (5.44; 4.23). High heritability estimates were observed in all the three sets *viz.*, genotypes (81.30%), RILs (61.10%) and F_5 progenies (60.50%). The GAM values were high (42.80) for genotypes, on the other hand the recombinant inbred lines (6.74) and F_5 progenies (6.78) recorded low GAM values. Although the mean values did not differ much for 3 sets of plant material, the range, PCV, GCV, heritability and GAM values were more for genotypes compared to RILs and F_5 progenies.

4.4.1.2.2 Plant height

The selected genotypes, RILs and F_5 progenies recorded a mean plant height of 37.14, 37.12 and 36.95 cm respectively. The plant height ranged from 31.30 (ICC-96029) to 41.93 (ICC-12429) cm in selected genotypes, from 29.13 (RIL-146) to 46.78 (RIL-48) cm in RILs and from 30.45 (Progeny-26) to 46.78 (Progeny-58) cm in F_5 progenies. The trait recorded low estimates of PCV and GCV in all the three experiments: it was 9.50 and 5.10 respectively in genotypes, 9.32 and 7.81 respectively in RILs and 9.64 and 8.38 respectively in F_5 progenies. The trait recorded low heritability values in selected genotypes (28.80%) and high in both RILs

Table 4.27 The mean, range, phenotypic and genotypic coefficients of variation, heritability and genetic advance as per cent of mean of different characters in selected genotypes, RILs and segregating progenies of chickpea

Sl. No	Character	Material studied	Mean	Range	PCV	GCV	H (%)	GAM
1	Days to 50% flowering	A	46.49	23.33 - 56.67	25.56	23.05	81.30	42.80
		B	47.33	43.00 - 51.00	5.35	4.18	61.10	06.74
		C	47.33	43.00 - 49.67	5.44	4.23	60.50	06.78
2	Plant height (cm)	A	37.14	31.30 - 41.93	9.50	5.10	28.80	05.63
		B	37.12	29.13 - 46.78	9.32	7.81	70.30	13.49
		C	36.95	30.45 - 46.78	9.64	8.38	75.60	15.02
3	Number of primary branches	A	5.10	4.00 - 5.80	11.62	9.31	64.30	15.29
		B	4.72	2.80 - 7.57	19.26	17.50	82.60	32.83
		C	4.86	3.33 - 7.11	18.03	16.72	86.10	31.89
4	Number of pods per plant	A	60.52	34.71 - 69.00	16.82	14.32	72.20	25.10
		B	55.70	15.53 - 97.77	29.89	29.66	98.50	60.62
		C	46.43	16.56 - 84.44	34.99	34.96	99.80	71.96
5	Test weight(g)	A	15.53	11.53 - 18.35	15.91	14.96	88.40	28.98
		B	14.21	09.42 - 19.37	13.75	12.11	77.60	21.95
		C	17.65	09.73 - 26.17	20.62	20.45	98.30	41.76
6	Seed yield per plant(g)	A	10.44	2.87 - 14.07	27.20	26.04	91.60	51.34
		B	12.81	2.59 - 24.61	35.44	32.36	83.40	60.83
		C	10.45	2.71 - 27.31	55.42	47.58	73.70	83.35

A = 15 selected genotypes, B = 125 RILs, C = 80 F₅ progenies

(70.30%) and F₅ progenies (75.60%). The GAM estimate was low in genotypes (5.63) and was moderate in both RILs (13.49) and segregating progenies (15.02). The plant height exhibited high heritability but moderate genetic advance as per cent of mean in RILs and F₅ progenies, while it was low heritability and GAM in selected progenies.

4.4.1.2.3 Number of primary branches

The highest mean number of primary branches recorded in selected genotypes (5.10), followed by F₅ progenies (4.86) and RILs (4.72). The primary branches ranged from 4.00 (ICC-96029) to 5.80 (ICC-4958) in selected genotypes, while it was from 2.80 (RIL-97) to 7.57 (RIL-70) and 3.33 (Progeny-30) to 7.17 (Progeny-95) in F₅ progenies. Moderate phenotypic (11.62, 19.26 and 18.03 for genotypes, RILs and F₅ progenies respectively) and genotypic (9.31, 17.50 and 16.72 for genotypes, RILs and F₅ progenies respectively) coefficients of variation were recorded for all the three sets of plant material except for low GCA values in case of selected genotypes. High heritability (64.30, 82.60 and 86.10 for genotypes, RILs and F₅ progenies) coupled with moderate to high GAM (15.29, 32.83 and 31.89 for genotype, RILs and F₅ progenies respectively) values were recorded for this trait in all the three sets of genotypes studies.

4.4.1.2.4 Number of pods per plant

The selected genotypes, RILs and F₅ progenies recorded a mean number of pods per plant of 60.52, 55.70 and 46.43 respectively. The F₅ progenies recorded the least mean number of pods per plant. The number of pods ranged from 34.71 (ICC-96029) to 69.00 (ICC-12249) in selected genotypes, from 15.53 (RIL-97) to 97.77 (RIL-70) in RILs and from 16.56 (Progeny-30) to 84.44 (Progeny-95) in F₅ progenies. The PCV and GCV were moderate in selected genotypes (16.82; 14.32) and were high in both RILs (29.89; 29.66) and F₅ progenies (34.99; 34.96). High heritability

coupled with high GAM were observed in all the three sets *viz.*, genotypes (72.20%; 25.10 respectively) RILs (98.50%; 60.62 respectively) and segregating progenies (99.80%; 71.96 respectively).

4.4.1.2.5 Test weight

The highest mean test weight was observed in F₅ progenies (17.65 g) followed by genotypes (15.53 g) while the least was observed in RILs (14.21 g). Among genotypes the highest test weight was recorded in ICC-12429 (18.35 g) while the least was observed in ICC-96029 (11.53 g). Among RILs the highest was selected in RIL-70 (19.37 g) while the least was in RIL-97 (9.44). Among the F₅ progenies highest and least were recorded in Progeny-95 (26.17 g) and Progeny-30 (9.73 g) respectively. The PCV and GCV were moderate in selected genotypes (15.91; 14.96) and RILs (13.75; 12.11) and recorded high in F₅ progenies (20.62; 20.45). High heritability coupled with high GAM was recorded in all the three sets *viz.*, selected genotypes (88.40%; 29.98), RILs (77.60%; 21.95) and segregating progenies (98.30%; 41.76).

4.4.1.2.6 Seed yield per plant

The mean seed yields per plant recorded were 10.44, 12.81 and 10.45 g in genotypes, RILs and F₅ progenies respectively. The trait ranged from 2.87 (ICC-96029) to 14.07 (ICC-12429) grams in genotypes, from 2.59 (RIL-97) to 24.61 (RIL-90) grams in RILs and from 2.71 (Progeny-30) to 27.31 (Progeny-95) grams in segregating progenies. High phenotypic and genotypic coefficients of variation were recorded in all three sets *viz.*, genotypes (27.20; 26.04), RILs (35.44; 32.36) and F₅ progenies (55.42; 47.58). Similarly all three sets: selected genotypes (91.60%; 51.34), RILs (83.40%; 60.83) and F₅ progenies (73.70%; 83.35) recorded high heritability and GAM.

RILs and F_5 progenies, which are significantly superior over national check A-1 are presented in Table 4.28 and 4.29 respectively. Out of 125 RILs studied 36 recorded significantly higher yield compared to national check A-1. Among these 36 RILs, 24 RILs recorded significantly high number of pods, 8 RILs recorded significantly high number of pods and primary branches per plant while, 7 RILs recorded significantly high number of pods, primary branches and test weight compared to national check A-1, which are important contributing characters to seed yield. Among 80 F_5 progenies studied 9 recorded significantly higher seed yield per plant compared to national check A-1. All these 9 progenies also recorded significantly high number of pods, test weight and number of primary branches. While only ICC-12429 out of 15 selected genotypes recorded significantly higher yield per plant compared to national check A-1.

4.4.2 Character association

The phenotypic association between the seed yield and yield components *viz.*, days to 50 per cent flowering, plant height, number of primary branches, number of pods per plant, test weight and seed yield per plant in 15 selected genotypes, 125 RILs and 80 F_5 progenies were calculated separately and the results are presented character wise in Table 4.30.

The trend of character associations in three different sets of plant material *viz.*, selected genotypes, recombinant inbred lines and F_5 progenies were similar. The number of primary branches, number of pods per plant and test weight showed significant positive association with seed yield and among themselves in all the three sets of plant material studies. Plant height also recorded positive association with seed yield per plant in all the three experimental material. However, the value was significant only in selected genotypes. Association of plant height with number of

Table 4.28 Significantly superior chickpea RILs of JG-62 x WR-315 over national check A-1

Sl No.	RIL No.	Days to 50% flowering	Plant height (cm)	Number of primary branches	Number of pods per plant	Test weight (g)	Seed yield per plant (g)
1	16	48.33	35.11	5.27	65.70	15.37	17.97
2	23	47.67	35.78	5.27	66.33	15.47	18.01
3	28	47.00	31.33	5.57	77.30	16.17	19.76
4	34	47.33	32.45	5.67	81.73	16.67	20.18
5	36	49.00	38.56	5.07	62.93	14.90	16.23
6	39	48.00	36.11	5.40	68.30	15.80	18.50
7	40	47.67	34.45	5.73	82.27	16.70	20.18
8	47	45.00	46.78	5.37	68.80	15.67	18.47
9	48	47.67	36.67	5.63	77.40	16.47	19.97
10	50	49.00	36.11	5.50	76.03	16.07	19.68
11	55	47.67	36.33	5.50	73.30	16.00	18.91
12	56	50.00	41.00	5.67	78.20	16.90	20.02
13	58	43.67	38.33	5.23	65.80	15.20	17.17
14	63	48.00	38.11	5.37	67.53	15.50	18.31
15	64	49.67	38.44	7.00	93.70	18.17	22.04
16	70	45.00	36.11	7.57	97.77	19.37	24.26
17	82	46.67	41.56	5.07	64.80	14.90	16.31
18	87	50.00	31.89	5.07	63.13	14.90	16.00
19	90	48.00	39.33	7.53	95.00	19.10	24.61
20	91	44.33	37.78	5.73	83.27	17.10	20.54
21	94	49.67	39.33	5.87	90.27	17.90	20.93
22	95	49.00	38.11	7.33	93.73	18.90	23.10
23	98	49.00	40.44	6.90	93.47	18.07	21.88
24	100	48.33	37.89	6.07	90.33	18.00	21.78
25	108	48.33	37.91	6.87	91.60	18.00	21.47
26	115	47.67	40.77	5.50	71.83	15.97	18.80
27	116	48.00	40.23	5.40	68.80	15.80	18.50
28	118	49.00	39.07	5.13	65.20	15.07	16.72
29	119	41.67	39.40	5.27	65.47	15.30	17.80
30	122	46.00	39.90	5.33	66.33	15.47	18.24
31	125	47.33	41.50	5.77	87.40	17.20	20.97
32	132	47.00	39.67	5.27	65.60	15.37	17.80
33	137	48.67	40.53	5.47	70.47	15.90	18.64
34	138	49.00	39.07	5.20	65.30	15.10	16.77
35	139	45.00	39.03	5.13	64.80	15.00	16.53
36	141	50.00	39.07	5.20	65.13	15.17	17.04
37	150	44.67	40.40	5.43	68.87	15.87	18.63
38	A-1	55.33	38.07	5.21	63.89	16.47	12.16
CD				0.61	3.33	1.49	3.80

Table 4.29 Significantly superior chickpea F₅ progenies of BG-256 x WR-315 over national check A-1

Sl No.	Progeny No.	Days to 50% flowering	Plant height (cm)	Number of primary branches	Number of pods per plant	Test weight (g)	Seed yield per plant (g)
1	1	43.33	38.78	6.33	73.44	23.01	21.55
2	35	44.67	37.89	6.22	72.27	22.84	21.03
3	59	47.67	36.67	6.92	83.60	26.10	25.06
4	64	44.33	40.00	6.56	78.49	25.99	24.11
5	81	46.00	38.11	6.56	78.42	24.13	23.89
6	83	48.00	38.11	6.22	71.98	22.67	19.44
7	95	45.00	36.11	7.11	84.44	26.17	27.31
8	106	45.67	35.56	5.89	69.08	22.23	17.89
9	109	45.67	40.44	6.00	70.53	22.32	17.97
10	A-1	55.33	38.07	5.21	63.89	16.47	12.16
CD				0.53	1.12	0.76	1.91

Table 4.30 Phenotypic correlation for different characters in selected genotypes, RILs and segregating progenies of chickpea

	Material studied	Plant height	Number of primary branches	Number of pods per plant	Test weight	Seed yield per plant
Days to 50% flowering	A	-0.198	-0.177	-0.170	-0.240	-0.166
	B	-0.102	0.090	0.112	0.092	0.107
	C	-0.107	-0.010	0.005	0.005	-0.062
Plant height	A		0.582*	0.562*	0.626*	0.650**
	B		0.168	0.184*	0.163	0.173
	C		0.138	0.160	0.157	0.145
Number of primary branches	A			0.708**	0.762**	0.835**
	B			0.916**	0.854**	0.850**
	C			0.948**	0.925**	0.920**
Number of pods per plant	A				0.789**	0.855**
	B				0.909**	0.918**
	C				0.972**	0.956**
Test weight	A					0.863**
	B					0.843**
	C					0.925**

*, ** = Significant at 5% and 1% respectively
A = 15 selected genotypes, B = 125 RILs, C = 80 F₅ progenies

branches, pods and test weight was positive, but significant with all the 3 traits only in selected genotypes.

Days to 50 per cent flowering recorded negative association with seed yield in genotypes and F_5 progenies while it was positive in RILs. It also associated positively with number of pods per plant and test weight in RILs and F_5 progenies while associated negatively with those two characters in selected genotypes. Days to flowering also recorded negative association with plant height and number of primary branches in all three sets except in RILs for number of primary branches. None of the quantitative traits associated significantly with days to 50 per cent flowering.

4.4.3 Path coefficient analysis

The path coefficient analysis was computed to find out relative contribution of five different quantitative traits towards seed yield. The phenotypic path coefficient analysis for 15 selected genotypes, 125 RILs and 80 F_5 progenies was carried out separately (Table 4.31) and the results are described hereunder.

The path analysis of different sets of plant material indicate that the highest direct contribution to the seed yield was recorded by number of pods per plant followed by number of primary branches in all the three sets of plant material studied. While, the contribution of test weight towards seed yield was high in selected genotypes but not in RILs and segregating progenies. Though the direct contribution of test weight was less, it recorded highest indirect contribution through number of pods per plant towards seed yield per plant in all the three sets of plant material resulting in high correlation.

The traits days to flowering and plant height did not show any significant direct effects for seed yield in any of the three sets of experimental material studied. There was no direct contribution as well in case of days to flowering with any of the

Table 4.31 Phenotypic path coefficient analysis of different characters on seed yield in selected genotypes, RILs and segregating progenies of chickpea

	Material studied	Days to 50% flowering	Plant height	Number of primary branches	Number of pods per plant	Test weight	Correlated seed yield per plant
Days to 50% flowering	A	0.039	-0.018	-0.054	-0.060	-0.073	-0.166
	B	0.006	-0.001	0.005	0.093	0.004	0.107
	C	-0.066	0.001	-0.001	0.004	0.000	-0.062
Plant height	A	-0.008	0.090	0.179	0.199	0.189	0.650*
	B	-0.001	0.006	0.008	0.153	0.006	0.173
	C	0.007	-0.013	0.017	0.148	-0.014	0.145
Number of primary branches	A	-0.007	0.052	0.308	0.250	0.231	0.835**
	B	0.001	0.002	0.050	0.764	0.033	0.850**
	C	0.001	-0.002	0.125	0.878	-0.081	0.920**
Number of pods per plant	A	-0.007	0.050	0.218	0.354	0.239	0.855**
	B	0.002	0.001	0.046	0.835	0.035	0.918**
	C	0.000	-0.002	0.119	0.926	-0.085	0.956**
Test weight	A	-0.009	0.056	0.235	0.279	0.303	0.863**
	B	0.001	0.002	0.043	0.759	0.039	0.843**
	C	0.000	-0.002	0.116	0.900	-0.088	0.925**

Residual values (A = 0.1272, B = 0.1557, C = 0.0786)

*, ** = Significant at 5% and 1% respectively,

A = 15 selected genotypes, B = 125 RILs, C = 80 F₅ progenies

Diagonal values indicate direct effects

traits studied. Plant height contributed indirectly through pods per plant and number of primaries in all the three sets of plant material. While the same trait recorded positive indirect contribution through pods per plant, test weight and number of primaries in selected genotypes resulting in significant positive correlation with seed yield per plant.

Number of primary branches contributed indirectly via pods per plant in all three sets of plant material and through pods per plant and test weight in selected genotypes to seed yield resulting in highly significant correlation. Similarly number of pods per plant contributed indirectly through primary branches in all three sets and it was through both primary branches and test weight in selected genotypes resulting in high correlation with seed yield per plant.

_____ *Discussion*

V. DISCUSSION

Chickpea (*Cicer arietinum* L.) is commercially grown as a cool season crop in a range of climates across the world from Mediterranean to sub-tropical. World chickpea production was 7.5 million tonnes in 2003 from an area of 10.3 million hectares (Anonymous, 2004). In recent years, chickpea production has expanded into new regions, including Australia and North America and into marginal land of traditional chickpea production regions in India, Pakistan, Iran, Turkey, Mexico and Ethiopia. The major producers India, Pakistan and Turkey contribute 65 per cent, 9.5 per cent and 6.7 per cent respectively, to the world harvest (Anonymous, 2005). Despite its broad adaptation, production is restricted by the environmental conditions under which the crop is grown. Among the biotic stresses, *Fusarium* wilt is reported to be in widespread and cause heavy losses upto 90 per cent under favorable conditions (Srivastava *et al.*, 1984; Gupta *et al.*, 1986; Jimenez-Diaz *et al.*, 1993 and Cortes *et al.*, 2000).

Many traditional chickpea cultivars are susceptible to soil-borne diseases and sources of resistance (mainly *desi* cultivars) were included in breeding programmes (Singh, 1987; Kaiser *et al.*, 1994). However, development of resistant cultivars is hindered by the pathogenic variability of the fungus and difficulty in development of wilt sick plots. Screening of segregating populations in wilt sick plots, although is considered most efficient means of identifying wilt resistant segregants, plants grown in sick plots take several weeks to show symptoms. The other problems are difficulty in maintaining the uniform wilt sick plots and elimination of other soil borne pathogens like *Sclerotium* and *Rhizoctonia* which also infect chickpea. Because of non-uniformity of wilt sick plots, there is every chance of escape of susceptible genotypes which in turn questions the reliability of identified resistant sources and selections in segregating population. Similarly presence of other soil borne pathogens

and their interactions influence the expressions of resistance. The identification and selection of resistant segregants are further complicated due to complexity of resistance which is controlled by 2-3 or more genes (Singh *et al.*, 1987a,b; Girase and Deshmukh, 2002). In this context there is an urgent need to develop alternate screening technique to select wilt resistant genotypes. In this direction, attempts were made to develop DNA based markers, which would be useful in the selection of resistant genotypes.

Marker assisted selection (MAS) became possible for wilt resistance with the identification of molecular markers for H_1 (Mayer *et al.*, 1997) and H_2 loci (Thippeswamy *et al.*, 2005 and Soregaon *et al.*, 2007). However, marker assisted selection has some inherent disadvantages. It is technology intensive, requires sophisticated laboratory and many a times their application is restricted to limited number of related genotypes or RILs in which they were identified. For example, the DNA marker linked to H_2 locus of *Fusarium* wilt susceptibility identified in the cross K-850 x WR-315 was not validated in the progenies of another cross JG-62 x WR-315 (Brindha, 2003). Other limitations like dominant nature of molecular markers identified which are linked to H_1 and H_2 loci of wilt resistance of chickpea, made it difficult to differentiate heterozygotes from dominant homozygotes. Alternatively a simple but effective technique of screening the genotypes based on the performance of their pollen grains is emerging to be a strong alternative to conventional selection methods and marker assisted selection (MAS).

The technique of pollen selection is proving to be a promising tool which can be easily combined with conventional breeding methods to increase their efficiency. Interest in male gametophytic selection (MGS) as a tool was identified in early 1980's following the realization that there is high proportion of overlap of gene expression between the gametophytic and sporophytic stage (Hormaza and Herrero 1992;

Hormaza *et al.*, 1996). The overlapping relationship between gametophyte and sporophyte was first shown by Tanksley *et al.* (1981). In their study on seven dimer-enzymes in tomato, it was determined that 18 out of 30 i.e. 60 per cent of the structural genes which are expressed in sporophyte of tomato are also expressed in the pollen. In addition, they have also observed that about 95 per cent of the genes which are expressed in the pollen are also expressed in the sporophyte. The haploid-diploid gene expression implies that selection exerted at the gametophytic level can also lead to detectable changes in allelic frequencies at the sporophytic level (Clegg *et al.*, 1978; Ravikumar *et al.*, 2007). Accordingly, a variety of selection pressures such as temperature (Zamir *et al.*, 1982; Clarke *et al.*, 2004), pathotoxin (Laughnan and Gabay, 1973; Shobha Rani and Ravikumar, 2006), herbicide (Sari-Gorla *et al.* 1989), metal (Searcy and Mulcahy, 1985a,b) and water stress (Ravikumar *et al.*, 2003) were enforced during gametophytic generation and were found very effective in increasing the frequency of resistant individuals in progeny (Hormaza and Herrero, 1996; Ravikumar and Patil, 2002). Further, investigations have clearly demonstrated the persistence of positive response to gametophytic selection in the succeeding generations also. For example, Frascaroli and Songstad (2001) following their pollen selection experiments showed a consistent and marked improvement in maize for chlorosulfuron resistance at the end of second sporophytic generations. Similarly, Chikkodi and Ravikumar (2003) have shown that the persistence of effect of gamete selection in F₃ generations.

The pollen based technique has two important applications: (i) pollen bioassay as a technique to screen the genotypes for the selected trait and (ii) pollen selection or gamete selection to channelise the genetic variability to increase the frequency of desirable types in the progeny. In this direction, attempts were made in the present study to develop a simple *in vitro* pollen bioassay as an alternate technique to field testing and MAS in identification of wilt resistant genotypes. Further, a comparative

analysis of *in vitro* pollen bioassay and MAS was made for selection of wilt resistance in chickpea. The results of the experiments conducted in the present investigation are discussed below under the following headings.

5.1 Pollen bioassay as an *in vitro* screening technique for wilt resistance

5.2 Study on molecular markers linked to wilt resistance loci in chickpea

5.3 Evaluation of diverse chickpea genotypes for wilt resistance, *in vitro* pollen bioassay and molecular markers

5.4 *In vitro* pollen bioassay and molecular markers analysis of selected RILs for wilt resistance

5.5 Selection of chickpea progenies for resistance to wilt using pollen bioassay and their validation with molecular markers

5.6 Evaluation of chickpea genotypes, RILs and F₅ progenies for yield and yield components

5.1 POLLEN BIOASSAY AS AN *IN VITRO* SCREENING TECHNIQUE FOR WILT RESISTANCE

The pollen bioassay can be effectively carried out under *in vitro* conditions in the laboratory using pollen germination medium by incorporating selective agent to the pollen germination medium (Laughnan and Gabay, 1973).

The *in vitro* pollen germination medium suggested by Shivanna *et al.* (1997) with minor modifications was used for the present study as a basal pollen germination medium. The PGM used in this study comprised sucrose (2.5%), polyethylene glycol (10%), boric acid (100 mg L⁻¹), CaNO₃ (200 mg L⁻¹), MgSO₄ (200 mg L⁻¹) and KNO₃ (100 mg L⁻¹). This medium has given more than 75 per cent pollen germination in all the selected genotypes. Ravikumar *et al.* (2002) have also observed such high levels

of germination and good tube growth irrespective of the genotypes of chickpea used. It has been reported that addition of stress factors such as temperature, herbicide, pathotoxin, etc. (Zamir *et al.*, 1982; Sari-Gorla *et al.*, 1989; Ravikumar, 1997) inhibit pollen germination and tube growth in different crop plants. The pathogen stress was created by addition of pathotoxin in the form of culture filtrate (Hodgkin and McDonald, 1986; Ravikumar, 1997), partially purified toxin (Ravikumar and Chikkodi, 1998) and purified toxin (Bino *et al.*, 1988).

In the present study, it was observed that partially purified pathogen culture filtrate inhibited chickpea pollen germination and tube growth. However the results were not consistent and a lot of variation was observed in repeat experiments. It could be due to variation in production of toxin by the pathogen. The quantity of toxin extracted is the function of initial inoculum, the amount of substrate (PDB) used for multiplication, growing conditions, multiplication rate of pathogen, etc. The pollen bioassay is a very sensitive technique; the amount of pathotoxin produced in culture filtrate is a very critical factor. Therefore in the absence of proper quantification of toxins produced in culture filtrate, the effect of culture filtrate on pollen is not reliable. Alternatively, commercially available fusaric acid (Sigma cat # 6513) was used as a stress factor. Fusaric acid (FA) is one of the fungal toxins produced by *Fusarium oxysporum* (Gaumann, 1957). It was reported that the virulence of *Fusarium oxysporum* was related to the amount of fusaric acid produced by the pathogen in potato (Venter and Styen, 1998). The addition of FA to *in vitro* pollen germination medium inhibited chickpea pollen germination and tube growth and the inhibition was not uniform across genotypes (Ravikumar *et al.*, 2002). The study also indicated that the effect of FA was more on the pollen grains produced by the susceptible genotypes compared to resistant genotypes. Different concentrations of selective agent (fusaric acid) was incorporated for pollen screening of diverse genotypes, RILs and

segregating progenies of chickpea in this study. The effect of different stress factors on *in vitro* pollen germination and tube growth has been reported in different crop plants (Mulcahy, 1971; Laughnan and Gabay, 1973; Mulcahy and Mulcahy, 1975; Maisonneuve and Den-Nijs, 1984 and Hodgkin and McDonald, 1986). Hence, fusaric acid, one of the pathotoxins produced by *Fusarium oxysporum* f.sp.*ciceris* was used for *in vitro* pollen bioassay of chickpea genotypes.

5.2 MOLECULAR MARKERS LINKED TO WILT RESISTANCE LOCI IN CHICKPEA

The genetic constitution and inheritance pattern of genes conferring resistance to race 1 have been reported. Using F_1 s and F_2 plants and F_3 families of different crosses, three independently functioning resistance loci designated as H_1 , H_2 and H_3 ; and breeding lines carrying each of these genes have been identified (Upadhaya *et al.*, 1983a, b; Singh *et al.*, 1987a). Singh *et al.* (1987a) suggested the genetic constitution for C-104, a line susceptible to race 1 (late wilting), to be $H_1H_1h_2h_2h_3h_3$, the homozygous recessive gene at the second locus confers late wilting. WR-315 ($h_1h_1h_2h_2h_3h_3$), a resistant genotype, differs from C-104 only at the first locus. However, it was observed in another study that the dominant alleles at both H_1 and H_2 loci cause early wilting and dominance at any one locus (either H_1 or H_2) lead to late wilting while homozygous recessive at H_1 and H_2 confer complete resistance (Brindha and Ravikumar, 2005). Molecular markers linked to H_1 and H_2 loci of susceptibility have been identified. The ASAP marker CS-27₇₀₀ developed by Mayer *et al.* (1997) linked to susceptibility at H_1 locus and three RAPD markers OPA-07₁₆₄₄, OPK-09₁₈₀₁ (Thippeswamy *et al.*, 2005) and A07C₄₁₇ (Soregaon *et al.*, 2007) linked to H_2 locus were used in the present analysis. The ASAP marker CS-27₇₀₀ linked to H_1 locus has been validated in different genetic backgrounds and found useful (Ravikumar *et al.*, 2003; Brindha and Ravikumar, 2005).

Sixteen genotypes showing differential reactions were selected for this study. Among the 16 genotypes 8 genotypes viz., JG-62, WR-315, ICCV-2, A-1, BG-256, K-850, ICC-4958 and Karikadle are established lines of which JG-62 and Karikadle are early wilters, BG-256, K-850, ICCV-2 and A-1 are late wilters and WR-315 is resistant (Ravikumar *et al.*, 2003). While, the other 8 genotypes were ICC accessions which were not clearly categorized for their wilt resistance.

The ASAP marker linked to H_1 and three RAPD markers supposed to be linked to H_2 were tested in sixteen selected genotypes for their validation before employing them for further use in this study. The markers OPK-09₁₈₀₁ and OPA-07₁₆₄₄ were putatively linked to H_2 locus of wilt susceptibility in the RILs of K-850 x WR-315 (Thippeswamy *et al.*, 2005). However, these two markers were not validated in the 16 genotypes studied. The early wilters Karikadle and JG-62 are expected to have both the linked markers. However, OPK-09₁₈₀₁ was absent in JG-62 which was one of the parental lines used for the development of RILs for the study. Hence, DNA marker OPK-09₁₈₀₁ was considered as non-reliable marker for further use in this study. The deviation could be due to recombination between H_2 locus and marker. Similarly another RAPD marker OPK-07₁₆₄₄ was absent in late wilter A-1 and BG-256 which were reported to be having dominant allele at H_2 locus (Ravikumar *et al.*, 2003). Therefore, the RAPD marker OPA-07₁₆₄₄ was also excluded from this study. These two markers were also found not reliable in earlier studies (Brindha, 2003). The RAPD markers are not highly reliable and in majority of the cases, their usage was restricted to the populations or genotypes in which they were identified (Collard *et al.*, 2005). Such deviations in RAPD markers have been observed in other studies also (Miklas *et al.*, 1996). Among the other two markers CS-27₇₀₀ is an allele specific marker linked to H_1 locus and was found to be working in different genetic backgrounds and genotypes (Brindha and Ravikumar, 2005). In the present study also

it was positive for all the early wilting genotypes and hence was used in all the experiments of this study.

Recently another marker A07C₄₁₇ has been identified in our laboratory (Soregaon *et al.*, 2007). It was also tested in different genotypes and found to be linked to H_2 locus in all the genotypes. The marker A07C₄₁₇ was identified using the recombinant inbred lines of cross K-850 ($h_1h_1H_2H_2$) x WR-315 ($h_1h_1h_2h_2$) and found linked to H_2 locus of wilt susceptibility. All the four susceptible genotypes, viz. JG-62, Karikadle, ICC-12434 and ICC-12429 showed amplification for this marker indicating its consistency in different genotypes. Similarly highly resistant genotypes WR-315 and ICC-12249 did not show amplification for this marker. Further to validate this marker, it was tested in 139 RILs of cross JG-62 ($H_1H_1H_2H_2$) x WR-315 ($h_1h_1h_2h_2$). The chi-square analysis revealed that the marker segregated in an expected 1:1 ratio. These results confirm that the marker is linked to H_2 locus of wilt susceptibility across genotypes. Hence, it was considered as an useful marker for the present study. Similarly the marker CS-27₇₀₀ linked to H_1 locus also segregated in expected 1:1 ratio in the RILs of the cross JG-62 x WR-315. Further, the digenic segregation analysis of both the linked markers CS-27₇₀₀ and OPA-07₄₁₇ in RILs of cross JG-62 x WR-315 showed expected digenic 1:1:1:1 ratio. These results establish conclusively the association of A07C₄₁₇ to H_2 locus of *Fusarium* wilt susceptibility. Based on above results, markers CS-27₇₀₀ (linked to H_1 locus) and A07C₄₁₇ (linked to H_2 locus) were employed in all the experiments of the present investigation.

5.3 EVALUATION OF DIVERSE CHICKPEA GENOTYPES FOR WILT RESISTANCE, *IN VITRO* POLLEN BIOASSAY AND MOLECULAR MARKERS

5.3.1 *In vivo* seedling assay for wilt resistance

The *in vivo* seedlings assay demonstrated that by root feeding fusaric acid (FA) it is possible to differentiate resistant and susceptible genotypes in chickpea at

seedling stage. Fusaric acid causes wilt early in susceptible genotypes compared to resistant genotypes. The necrotic lesions and death was early in early wilters compared to late wilters and resistant genotypes. The wilting and death of the seedlings occurred in all the replicated seedlings on the 6th day in early wilters and on 9th day in all the seedlings of late wilting genotypes. None of the seedlings of resistant genotypes showed wilting symptoms before 12 days. A positive correlation between virulence of *Fusarium oxysporum* and production of fusaric acid has been reported in potato (Venter and Styen, 1998). Fusaric acid has been used in the past to screen seedlings of different crops for resistance to plant diseases (Chatani *et al.*, 1996). The role of fusaric acid in screening chickpea for identification of resistance to *Fusarium* wilt was reported by Ravikumar and Ratna Babu (2007). The present study show further evidence for *in vivo* screening of chickpea genotypes for wilt resistance through root feeding of fusaric acid. From the *in vivo* seedling assay it was observed that ICC-12249 and WR-315 were resistant, Karikadle, ICC-12434, JG-62 and ICC-12429 were susceptible early wilters and A-1, ICCV-2, ICC-96030, ICC-4958, ICC-96029, BG-256, K-850, ICC-12237, ICC-12252 and ICC-12236 were susceptible late wilters among the 16 genotypes tested.

5.3.2 Pollen bioassay of selected genotypes

The *in vitro* pollen germination medium (PGM) suggested by Shivanna *et al.* (1997) with slight modification was used for the present study. The pathotoxin (fusaric acid) as stress factor was incorporated at different concentrations to PGM. The effect of toxin concentration on pollen germination and tube growth of different genotypes was used as a measure of resistance.

Pollen germination

The pollen germination per cent was highest in basal pollen germination medium with zero toxin (fusaric acid) in all the genotypes. In general, the incorporation of different levels of toxin to the basal medium reduced the mean pollen

grain germination of the genotypes. For example, the mean germination of all the genotypes was 86.56 per cent in the control while it was 86.45, 82.34 and 80.01 per cent in PGM with 50, 75 and 100 μg FA/ml of PGM. But, the reduction was not uniform across the genotypes. In genotypes, viz. BG-256, A-1, Karikadle, ICCV-2 and ICC-12434 there was no reduction in germination per cent with addition of toxin up to 50 μg /ml of PGM. Infact, there was a slight increase in germination at that concentration (50 μg FA). None of the genotypes studied recorded less than 75 per cent germination irrespective of toxin concentrations. There was no definite trend in the effect of toxin on pollen germination in selected genotypes. Therefore, the toxin effect on *in-vitro* pollen germination was not considered as a sensitive parameter for pollen bioassay in the other experiments of this study. On the contrary Bino *et al.* (1988) observed significant effect of pathotoxin on pollen germination of tomato genotypes. Further, the effect was more in susceptible lines compared to resistant lines. They suggested that *in vitro* pollen germination could be used to differentiate resistant and susceptible genotypes in tomato. Similarly, Sato and Peet (2005) also observed higher reduction in pollen germination in susceptible genotypes of tomato compared to resistant genotypes under temperature stress.

Pollen tube growth

A number of studies have demonstrated positive correlation between pollen competition ability in terms of pollen tube growth rate with several sporophytic traits (Mulcahy, 1971; 1974; 1979; Ottaviano *et al.*, 1980 and Snow, 1986). This parameter has been extensively used in pollen bioassay studies as well (Ravikumar *et al.*, 2002 and Golyshikina and Gigadlo, 2000). In the present study, pollen tube growth was measured as a responsive indicator for tolerance of the pollen grains of different chickpea genotypes to the pathotoxin.

The pollen tube growth was highest in basal pollen germination medium with '0' toxin in all the selected genotypes. The incorporation of toxin in the basal PGM steadily reduced the mean pollen tube growth in the genotypes. The reduction in pollen tube growth with addition of toxin was observed in all the genotypes tested. The factorial analysis indicated that there was significant effect of toxin level on mean pollen tube growth of the selected genotypes. The reduction of pollen tube growth was significant at all the three toxin levels (50, 75 and 100 µg of FA/ml PGM) in all the genotypes in comparison with control. This significant reduction in pollen tube growth with increase in toxin levels in the media, suggests the inhibitory effect of toxin on the pollen tube growth. Further the degree of reduction in pollen tube growth was different in different genotypes. The toxin level and genotype interaction was significant. Such interactions were also observed in earlier studies also. For example, in sunflower against *Alternaria helianthi* (Shobha Rani and Ravikumar, 2006). The results clearly indicate that the pollen grains of different genotypes respond differently to toxin.

The variation in degree of tube growth inhibition was quantified by calculating the toxin concentration required to inhibit 50 per cent pollen tube growth (LD₅₀ values). The analysis of variance of LD₅₀ values of selected 16 genotypes indicated that there was a significant variation among the genotypes for toxin requirement to inhibit 50 per cent tube growth. These values ranged from 64.30 µg to 122.40 µg. The highest LD₅₀ value was observed in ICC-12249 (122.40 µg) followed by WR-315 (109.10 µg). Both the genotypes are highly resistant to wilt. Similarly, the highly susceptible early wilters ICC-12429 (64.30 µg), JG-62 (66.53 µg), ICC-12434 (66.71 µg) and Karikadle (67.68 µg) recorded very low values of LD₅₀. On the other hand the late wilters K-850 (84.03 µg), BG-256 (85.21 µg), ICC-96029 (87.89 µg), ICC-4958 (88.33 µg), ICC-96029 (88.98 µg), ICCV-2 (90.39 µg) and A-1 (92.41 µg) recorded

moderate values. These results indicate that the genotypes which are resistant at sporophytic level also require high level of toxin in the PGM to inhibit *in vitro* pollen tube growth and vice versa. The LD₅₀ values for *in vitro* pollen tube growth inhibition can be taken as a measure of resistance of the genotypes at sporophytic level against *Fusarium* wilt.

The effect of pathotoxin/stress factor on *in vitro* pollen tube growth is also reported in different crop plants, in chickpea (Ravikumar *et al.*, 2002); in cherry (Golyshikina and Gigadlo, 2000); and in sunflower (Ravikumar *et al.*, 1997). This tolerance of pollen grains to pathotoxin could be due to the expression of genes responsible for resistance to wilt at the gametophytic level.

The *in vivo* root feeding studies conducted on the above selected 16 genotypes at seedling stage using pathotoxin (fusaric acid) showed significant differences among genotypes for days to wilting. Based on the number of days for wilting, the genotypes were classified as resistant, late wilting and early wilting genotypes. The genotypes identified as resistant based on *in vivo* root feeding also required the highest toxin levels for inhibition of 50 per cent pollen tube growth. Similarly, the susceptible genotypes recorded the lowest toxin requirement for 50 per cent inhibition of pollen tube growth. The late wilters recorded moderate LD₅₀ values ranging from 72.04 µg to 92.41 µg. The mean LD₅₀ toxin values of these groups were determined. The resistant group required highest toxin concentration (116.13 µg) compared to late (84.68 µg) and early wilting groups (66.31 µg). These group means were compared and found to be significantly different from each other. There was significant positive correlation between sporophytic and gametophytic tolerance to fusaric acid as evidenced from *in vivo* seedling reaction to the toxin. Thus providing a conclusive evidence that pollen bioassay as a technique can be used to identify and differentiate not only resistant and susceptible genotypes but also it can clearly differentiate late wilting and resistant

genotypes which is the major limiting factor in established conventional screening technique using wilt sick plots and sick pots.

Many *in vitro* pollen bioassay studies which proved successful in *Zea mays* (Laughnan and Gabay, 1973), in tomato (Bino *et al.*, 1988), in *Brassica* (Hodgkin and McDonald, 1986; Sharma and Tewari, 1996), in sunflower (Ravikumar *et al.*, 1997), in cherry (Golysheikina and Gigadlo, 2000), in chickpea (Ravikumar *et al.*, 2002) and in tomato (Sato and Peet, 2005) were based on only a limited number of genotypes. The present investigation involved a large number of genotypes and hence the results are highly reliable. Further, the study also showed that this technique can be used not only to differentiate resistant and susceptible genotypes, but also early and late wilters among susceptible genotypes, which is difficult even under well maintained sick plots.

5.3.3 Molecular marker analysis of selected genotypes for wilt resistance

The two molecular markers *viz.*, CS-27₇₀₀ (linked to H_1 loci of *Fusarium* wilt) and A07C₄₁₇ (linked to H_2 locus of *Fusarium* wilt) were used for this study. The markers were used to analyse the 16 selected genotypes for the presence and/or absence of H_1 and/or H_2 alleles in each of the selected genotypes. The presence of both markers suggests that the genotype contain dominant alleles at both H_1 and H_2 loci and is early wilting. The presence of any one marker is late wilting and none is resistant. Out of 16 genotypes, four were positive for both the markers, two were negative for both the markers and 10 were positive for any one of the marker of which CS-27₇₀₀ alone was present in one genotype while A07C₄₁₇ alone was present in nine genotypes. Both the markers were present in Karkadle, ICC-12434, JG-62 and ICC-12429. These genotypes showed susceptible early wilting reaction in *in vivo* root feeding experiment also. Both the markers were absent in genotypes WR-315 and ICC-12249 of which WR-315 was reported to be highly resistant in different studies

(Upadhayaya *et al.*, 1983a,b). The ICC-12249 also showed resistant reaction along with WR-315 in the root feeding experiment. The ASAP marker CS-27₇₀₀ alone was present in ICC-12236 while A07C₄₁₇ alone was present in A-1, ICCV-2, ICC-96030, ICC-4958, ICC-96029, BG-256, K-850, ICC-12237, ICC-12252 and ICC-12236 and were grouped as late wilting. All these genotypes showed late wilting reaction in root feeding studies.

The classification of genotypes into late wilting, early wilting and resistant based on molecular markers was in total agreement with classification of genotypes based on their response to *in vivo* root feeding of fungal toxin. The molecular markers are very efficient in identifying the wilt resistance in chickpea and highly reliable across different genetic backgrounds. In majority of the studies the molecular markers are genotype specific (Collard *et al.*, 2005). However, these two markers are very useful and hence applied to classify genotypes in other experiments of this study. It is interesting to note that molecular markers are useful in identifying late wilters also among susceptible genotypes. Hitherto, such differences among susceptible genotypes was not frequently reported in literature.

5.3.4 Comparative analysis based on pollen bioassay and molecular markers for resistance in selected genotypes

Sixteen genotypes were analysed for their wilt resistance and susceptibility using three different techniques (a) *in vivo* seedling evaluation by root feeding the pathotoxin (fusaric acid) (b) Molecular marker analysis using DNA markers CS-27₇₀₀ and A07C₄₁₇ linked to H_1 and H_2 loci of wilt resistance respectively and (c) *in vitro* pollen bioassay. Attempts were also made to assess the efficiency of pollen bioassay as a technique to identify resistance in chickpea with comparison to other two techniques.

Based on the root feeding experiments, the genotypes were classified into resistant, early wilting and late wilting genotypes depending on the days taken for appearance of wilt symptoms on the seedlings during root feeding of fusaric acid. Genotypes ICC-12429, JG-62, ICC-12434 and Karikadle wilted very early and classified as early wilters while A-1, ICCV-2, ICC-96030, ICC-4958, ICC-96029, BG-256, K-850, ICC-12237, ICC-12252 and ICC-12236 wilted three days later compared to early wilter and were grouped as late wilters while the other two (ICC-12249 and WR-315) genotypes did not show any symptoms even after another three days of root feeding and hence grouped as resistant.

Molecular marker analysis of the 16 genotypes facilitated to classify them into resistant, late wilting and susceptible groups based on the presence and/or absence of CS-27₇₀₀ and/or A07C₄₁₇. Out of 16 genotypes two (WR-315, ICC-12249) were resistant while four genotypes (karikadle, ICC-12434, JG-62 and ICC-124299) were susceptible. The remaining ten genotypes (A-1, ICCV-2, ICC-96030, ICC-4958, ICC-96029, BG-256, K-850, ICC-12237, ICC-12252 and ICC-12236) were late wilters. The genotypes which were found resistant in root feeding were also resistant based on molecular markers. Similar is the case with late and early wilters. The results clearly suggest that the markers used are robust and applicable in a wide range of genotypes.

The genotypes which were classified as resistant based on their sporophytic response to root feeding also required higher toxin in *in vitro* pollen bioassay for 50 per cent inhibition of pollen tube growth. The mean LD₅₀ toxin required for resistant genotypes was 116.13 µg. Similarly, the early wilters required less toxin (66.31 µg) for 50 per cent inhibition in pollen bioassay. The late wilters recorded moderate (84.68 µg) values for toxin under *in vitro* pollen bioassay. The study clearly showed that there is significant positive relationship between sporophytic and gametophytic response to pathotoxin. Therefore, the *in vitro* pollen bioassay can be effectively used

to differentiate the genotype in terms of their level of resistance. It is equally efficient as that of testing genotypes in wilt sick plots.

Comparative studies of grouping of genotypes done based on pollen bioassay and molecular markers indicate that the genotypes of these two sets of groups were same. This indicates that the pollen bioassay is as effective as molecular marker based screening. The results clearly indicate that the pollen response to toxin under *in vitro* conditions is due to resistant alleles and not due to any epigenetic or adaptive effects. This is a direct genetic evidence for the *in vitro* pollen bioassay as technique for screening for chickpea genotypes for wilt resistance. The pollen bioassay is cost effective, robust and can be used for traits lacking specific DNA markers such as tolerance to high temperature (Frova *et al.*, 1986; Sato and Peet, 2005), low moisture (Mulcahy *et al.*, 1988; Morgan, 1999; Patil *et al.*, 2004), salinity (Koval, 2000) and ozone (Feder and Sullivan, 1969; Feder, 1986).

5.4 IN VITRO POLLEN BIOASSAY AND MOLECULAR MARKERS ANALYSIS OF SELECTED RILs FOR WILT RESISTANCE

Out of available 139 F₇ generation RILs of cross JG-62 x WR-315, 50 RILs were randomly selected for conducting *in vitro* pollen bioassay studies.

5.4.1 *In vitro* pollen bioassay of selected RILs

Since the pollen germination per cent as a parameter did not show consistent results in response to pathotoxin under *in vitro* conditions for selected genotypes, only pollen tube growth was considered in this experiment.

Like in selected genotypes, the pollen tube growth was highest in the basal pollen germination medium with '0' toxin in all the 50 RILs studied. The incorporation of toxin to basal PGM led to gradual reduction in pollen tube growth in all the RILs as in different genotypes tested. The factorial analysis indicated that there

was significant variation for mean pollen tube growth among the 50 RILs, toxin levels and their interactions. The reduction of mean pollen tube growth was significant in all the three toxin treatments (50, 75 and 100 $\mu\text{g/ml}$). The similar significant reduction in pollen tube growth with increase in toxin level was observed in selected genotypes also suggesting the inhibitory role of toxin on the pollen tube growth. The genotype x toxin interaction was significant, indicating that the effect of toxin on pollen tube growth inhibition was not uniform across RILs.

The degree of reduction in pollen tube growth varied among the RILs. The degree of reduction in individual RILs were quantified by computing the toxin required to inhibit 50 per cent pollen tube growth (LD_{50} value) for each of the RILs separately. The analysis of variance of LD_{50} values of 50 RILs indicated that there was a significant variation among the RILs for the LD_{50} values ranging from 54.04 μg to 127.10 μg .

The variation in the requirement of toxin for inhibition of 50 per cent tube growth indicate that different RILs respond differently to toxin stress. The RILs 27, 59, 52, 50, 66, 10, 60, 36, 38, 64, 65 and 20 required more than 106 $\mu\text{g/ml}$ for 50 per cent inhibition. On the other hand, RILs 47, 33, 37, 3, 14, 26, 5, 46, 56 and 49 required less than 73.5 $\mu\text{g/ml}$ toxin for 50 per cent inhibition. The results indicate that the RILs segregate for pollen tolerance to pathotoxin. The RILs are derived from hybridization of susceptible (JG-62) x resistant (WR-315) parents. They differ for both H_1 and H_2 loci of wilt susceptibility and hence segregate for both loci resulting in early wilters, late wilters and resistant (Brindha and Ravikumar, 2005). The similar segregation has been observed for pollen tolerance to pathotoxin. Therefore, the pollen tolerance, which indirectly indicate the sporophytic tolerance segregate like any other trait and inherited. It is under genetic control like sporophytic resistance or tolerance.

5.4.2 Molecular marker analysis of selected RILs for wilt resistance

The 50 RILs which were used for pollen bioassay were also used for molecular marker analysis. The RILs were analysed for presence of the two established DNA markers, CS-27₇₀₀ and A07C₄₁₇ linked to H_1 and H_2 loci of wilt susceptibility respectively.

Among the 50 RILs, CS-27₇₀₀ (linked to H_1 locus) alone was present in 10 RILs, A07C₄₁₇ (linked to H_2 locus) alone was present in 17 RILs, both the markers were present in 10 RILs and none in 13 RILs. From the earlier experiments it was established that genotypes showing presence for both markers can be grouped as early wilters, with any one of them as late wilters and none as resistant. Considering the above, among the 50 RILs studied, 13 were resistant, 10 were susceptible while remaining 27 (10 having CS-27₇₀₀ and 17 having A07C₄₁₇) were late wilters. Further the segregation of the two markers in these 50 RILs also fit into the expected 1:1:1:1 ratio.

5.4.3 Comparative analysis based on pollen bioassay and molecular marker for resistance in 50 selected RILs

Based on molecular marker analysis the selected 50 RILs were grouped into resistant (absence of both the linked markers), late wilting (presence of any one of the linked markers) and susceptible early wilting (presence of both the markers) groups. The mean toxin LD₅₀ values for each group of RILs were determined. The RILs of resistant group required highest toxin concentration (114.08 µg) compared to late wilters (84.71 µg) and early wilters (68.41 µg) for 50 per cent inhibition of pollen tube growth. The mean LD₅₀ value of resistant group was significantly different from late wilters and the mean LD₅₀ values of late wilters was significantly different from early wilting RILs.

As expected, all the resistant RILs had higher LD₅₀ toxin values (required more toxin) compared to late wilting RILs with an exception of only one line. Excluding the deviated line the range of LD₅₀ values for resistant group was from 105.90 to 127.10 µg. In case of late wilting RILs, the LD₅₀ toxin values were lower than resistant RILs and more than that of susceptible RILs with an exception of only two lines. The range of LD₅₀ values for late wilting group was 73.65 to 105.60 µg excluding the deviated lines. Similarly, the susceptible RILs recorded least LD₅₀ values compared to late wilters and resistant RILs with an exception of one line. Excluding the deviated line the range of LD₅₀ value of this group was 54.04 to 73.52 µg. There was correlation between results of classification of RILs based on molecular markers and *in vitro* pollen bioassay.

These results indicate that even in the RILs which are segregating for resistance, the pollen bioassay can be used to identify resistant segregants. Although 50 RILs were studied, the deviations from expectations were not many - one in resistant group, two in late wilting group and one in susceptible group. Those deviations could be due to the recombinations between the allele and the tagged marker also. There is a need to test the RILs for their wilt resistance to confirm such recombination in the progenies. Attempts are being made to phenotype all the RILs in wilt sick plot at ICRISAT.

The molecular marker analysis clearly provided the genetic evidence for the *in vitro* pollen bioassay in terms of its response to the selective agent fusaric acid and wilt resistance. The presence of marker indicates the presence of allele linked to wilt susceptibility, leading to susceptibility of sporophyte. The pollen grains also showed susceptibility when both the alleles were present. Similarly, in absence of markers, the sporophyte was resistant and pollen grains also showed resistance for *in vitro* pollen bioassay. Molecular markers can be used to identify late wilters and early wilters

among susceptible genotypes. Presence of any one marker indicates presence of susceptible allele at H_1 or H_2 locus only making the plant late wilting. Interestingly, the late wilting and early wilting segregants can be easily identified by using *in vitro* pollen bioassay also. The results clearly indicate that the resistance in the pollen grains depends on the presence of genes responsible for sporophytic resistance. The same set of genes may be responsible for sporophytic and gametophytic resistance. Although it was hypothesized that the same genes are implicated for resistance in sporophyte and gametophyte (Sari-Gorla *et al.*, 1989), this study is the first experimental evidence to prove the hypothesis.

5.5 SELECTION OF CHICKPEA PROGENIES BASED ON POLLEN BIOASSAY AND THEIR VALIDATION WITH MOLECULAR MARKERS

This experiment was conducted to test the efficiency of pollen bioassay in selection of resistant progenies in a segregating population. For this study 22 F_5 progenies of cross BG-256 ($h_1h_1H_2H_2$) x WR-315 ($h_1h_1h_2h_2$) were randomly selected from available 80 progenies and were subjected to pollen bioassay for resistance.

As observed in earlier experiments, the addition of toxin (FA) inhibited pollen tube growth in all the selected F_5 progenies. The reduction was not uniform in the selected progenies. The factorial analysis revealed significant effect of toxin on pollen tube growth. There was a significant toxin x progeny interaction suggesting the effect of toxin was not uniform across progenies. The reduction in tube growth was not similar in all the progenies. The toxin required for inhibition of 50 per cent of pollen tube growth of all the progenies was determined. The analysis of variance indicated that there is significant variation among the progenies for LD_{50} toxin values which mean that there is variability for the resistance (based on pollen bioassay) in the progenies. These progenies were obtained from the cross between late wilter and

resistant genotypes. They segregate for late wilting and resistance. It was observed that progenies also segregate for pollen tolerance for toxin stress. Further the range of LD₅₀ values among the progenies indicated that the progenies segregate for late wilting and resistance. The LD₅₀ values ranged from 76.53 µg to 137.50 µg. Similar range was observed for late wilters and resistant genotypes in earlier experiments involving 16 genotypes and RILs. The results further confirm that the toxin range observed to differentiate; susceptible early wilting, late wilting and resistant genotypes can be generalized to identify resistant genotypes in any genetic background. The 22 progenies were classified as resistant and late wilting based on LD₅₀ values. Among 22 progenies 8 to 10 progenies recorded higher LD₅₀ values can be considered as resistant lines and remaining as susceptible.

All the 22 F₅ progenies were advanced to F₆ generation. The resistance or susceptibility of the progenies was studied using linked molecular markers. The progenies segregate for *H*₂ locus of wilt resistance. Consequently the progenies segregate only for A07C₄₁₇ linked to *H*₂ locus. Based on the presence or absence of this marker the progenies were classified into two groups; late wilting and resistant. Out of 22 progenies tested, 11 were positive for the marker and another 11 were showed the absence of the marker, hence resistant. The F₅ progenies which were considered as resistant based on *in vitro* pollen bioassay were resistant in F₆ progenies also. The selection of progenies also indicated that these 11 genotypes required high toxin for inhibition of pollen tube growth.

The mean toxin LD₅₀ values of these two groups, resistant and late wilting, differed. The mean LD₅₀ values of the resistant progenies was 105.84 µg while it was 94.80 µg for the late wilting groups, and were significantly different from each other. Within group analysis of resistant and late wilting

progenies showed a few exceptions. The progeny numbers 45 and 109 which belong to resistant group based on molecular markers showed late wilting based on pollen bioassay. Similarly, progeny number 12 which belong to late wilting group based on marker showed resistant reaction ~~based on pollen bioassay~~. Except for these progenies, 10 F₆ progenies which were considered as resistant based on molecular markers were also resistant based on pollen bioassay in F₅ generation. These results indicate that the resistance identified based on pollen bioassay is inherited to next generation, supporting that the pollen response to toxin stress is determined by the alleles is inherited from the sporophyte.

All the three experiments involving different genetic material, viz. diverse genotypes, recombinant inbred lines and F₅ progenies showed that the toxin level for inhibition of 50 per cent of pollen tube growth in resistant (>105 µg), late wilting (85 - 95 µg) and susceptible genotypes (~ 65 µg) were consistent, although these experiments were conducted in different years. The technique is validated not only in different genetic backgrounds, it was also effective over time and space.

In all the three experiments the *in vitro* pollen bioassay was found effective in the selection of resistant genotypes. Pollen bioassay was able to differentiate resistant, late wilting and susceptible genotypes which is very difficult even in sick plots. The results were consistent on repetitions. The results also indicate that the selection for resistance using pollen bioassay is as effective as that of molecular markers. The technique is as efficient as MAS. The study also evidenced that the selection of resistance based on pollen bioassay is genetically inherited.

Although practical benefit of pollen selection was demonstrated in several crop plants (Hormaza and Herrero, 1996; Chikkodi and Ravikumar, 2000; Clarke *et al.*, 2004; Dominguez *et al.*, 2005), there was reluctance among plant breeders for acceptance to apply in plant improvement programmes. The main reason was, most of

the earlier results were based on limited number of genotypes and there was no genetic evidence for pollen based selection. The present study has unequivocally proved the utility of pollen bioassay in identifying resistance across different genetic backgrounds. It has also provided genetic evidence for pollen based selection. Now there is a clear evidence to show that the pollen bioassay has emerged as an effective laboratory technique for screening and selecting the resistant genotypes of chickpea against *Fusarium* wilt. The pollen bioassay has obvious advantages - speed and economy of testing; pollen from a single plant can be used to study the tolerance of the sporophyte against a wide range of stress factors; operates at haploidy without interference with dominance; successful for traits even when their genetic control is not known; does not require expensive equipment or sophisticated technology and can be effectively integrated to improve the efficiency of conventional breeding methods.

The present study involving linked DNA markers to resistant loci, three different sets of diverse genotypes and experiments performed over a period of time clearly show that the selection of genotypes for resistance to *Fusarium* wilt, based on pollen bioassay is as reliable as molecular markers assisted selection and/or sporophytic selection if not more.

5.6 EVALUATION OF SELECTED GENOTYPES, RILs AND F₅ PROGENIES FOR YIELD AND YIELD COMPONENTS

Progress in any breeding programme depends on the extent of variability present and the amount of variability recombined during hybridization which becomes available for selection. High genetic variability in mapping population is very important for DNA marker analysis and mapping of genes for different traits. Genetic variation within cultivated chickpea is minimal (Oram *et al.*, 1987; Kazan *et al.*, 1993) and has prompted several researchers to use wide hybridization for linkage analysis (Winter *et al.*, 2000). Also, in other crops with more variable genome,

interspecific crosses were preferred to maximize polymorphism in linkage analysis. In chickpea, interspecific cross was used to map several genes and most comprehensive map of chickpea genome is available (Winter *et al.*, 2000). An understanding of the level of genetic variability available in the RIL population will be useful in mapping different loci linked to important traits like wilt resistance.

An understanding of the nature of association of yield component characters like, days to 50 per cent flowering, plant height, number of primary branches and test weight with seed yield is expected to provide new dimension in selection strategy. Such association will be useful to understand association of different QTLs for desirable and undesirable traits. The results of present investigation on genetic variability studies in three different sets of plant material, viz. 15 selected genotypes, 125 RILs of cross JG-62 x WR-315 and 80 F₃ segregating progenies of cross BG-256 x WR-315 for seed yield and yield components are discussed below.

5.6.1 Variability studies

The analysis of variance indicated highly significant variation for days to flowering, plant height, number of pods per plant, test weight and seed yield per plant and significant variation for number of primary branches per plant in all the three sets of plant material studied. Such a high variability has been observed in RILs of cross JG-62 x ICCV-2 (Anupama, 2001) and in 330 lines including 205 *desi* and 125 *kabuli* types (Kumar *et al.*, 1981). The availability of wide variability for morphological characters is encouraging from the point of research efforts aimed not only at improving yield by amelioration of variability of morphological characters but also from the point of mapping of useful genes.

One of the ways in which the variability is assessed is through a simple approach of examining the range of variation. The range observed was wider for plant

height, number of primary branches number of pods per plant, test weight and seed yield per plant in RILs of cross JG-62 x WR-315 and F_5 progenies of BG-256 x WR-315 cross compared to the 15 selected genotypes. While, days to flowering recorded high amount of variation in case of selected genotypes compared to RILs and F_5 progenies. Overall, there is high amount of variation among the RILs and F_5 progenies compared to the selected genotypes which could be due to diversity among parental genotypes.

The extent of variability as measured by GCV and PCV also give information regarding the relative amount of variation in different characters. In the present study, high variability was observed for days to 50 per cent flowering in selected genotypes while RILs and F_5 progenies recorded low levels of variation. The results of high variability are in conformity with the reports of Agrawal (1985) in P_1 , P_2 , F_1 and F_2 generations of two different crosses (JG-315 x L550 and TLM x L550) while results of low coefficients of variation were in agreement with the findings of Islam *et al.* (1984) in 140 different varieties. Low coefficients of variation for plant height and moderate variation for number of primary branches were observed in all the three sets of plant material which are in agreement with the results of Arora (1991) in 40 diverse genotypes for plant height and number of primary branches. Number of pods per plant recorded moderate variability in selected genotypes while it was high in both RILs and F_5 progenies. Similar observations of moderate and high variability were noticed in evaluation studies of 28 genotypes (Dasgupta *et al.*, 1992) and in 362 accessions (Naghavi and Jahansouz, 2005), respectively. Moderate to high coefficients of variation for test weight and high variation for seed yield per plant were recorded in all the three sets of plant material whose results were in accordance with the findings of Vijayalakshmi *et al.* (2000) in two different crosses viz., P 9623 x T 39-1 and RS 11 x T 39-1 for test weight and of Singh (2004) in F_3 progenies and biparental population.

The coefficient of variation indicates only the extent of variability existing for various traits, but does not give any information about the heritable portion of it, as the estimates of heritability. High estimates of heritability was observed for all the traits in all the three sets of plant material except for plant height in selected genotypes which recorded low heritability estimate. High estimates of heritability along with high level variation was observed for number of pods per plant and seed yield per plant in RILs and segregating progenies. Similar results were observed by Chavan *et al.* (1994) in their evaluation of 70 genotypes and by Kumar *et al.* (2001) in 26 diverse genotypes.

Heritability indicates only the effectiveness with which selection of genotypes can be based on their phenotypic performance but fail to indicate the amount of progress expected from selection. Therefore, heritability estimates appear to be more meaningful when accompanied by estimates of genetic advance. The high genetic advance as expressed in per cent of mean was recorded by number of primary branches, number of pods per plant, test weight and seed yield per plant in all the three kinds of plant material except in case of number of primary branches in selected genotypes. These results are in accordance with the findings of Arora and Jeena (2001) in 40 diverse genotypes for number of primary branches and test weight and with the results of Jahagirdar *et al.* (1996) in 28 F_1 hybrids for number of pods per plant and seed yield per plant. The traits, days to flowering and plant height recorded low and medium GAM values respectively in both RILs and F_3 progenies while, selected genotypes recorded high GAM for days to flowering and low for plant height. A relative comparison of heritability estimates and expected genetic advance will give an idea about the nature of gene action governing a particular character. The comparison of these two estimates made in this study revealed that seed yield per plant and number of pods per plant had high heritability estimates coupled with high

genetic advance in all the three sets of plant material except in selected genotypes for number of pods per plant. This indicates the substantial contribution of additive genetic variance in the expression of these characters. From this, it can be inferred that the selection criteria based on seed yield per plant and number of pods per plant will be useful for the improvement of seed yield in chickpea crosses.

The high yielding genotypes, RILs and F_5 progenies which were significantly superior over national check A-1 were identified. Among 125 RILs, 37 recorded significantly higher yield compared to national check. Out of these 37 significantly high yielding RILs, 24 recorded significantly high number of pods and primary branches per plant while 7 RILs recorded significantly high number of pods, primary branches per plant and test weight, compared to national check indicating the contribution of respective characters to these RILs towards the higher yields. Among the 80 F_5 progenies studied, 9 recorded significantly higher seed yield per plant compared to A-1 and all these 9 recorded significantly high number of pods, primary branches per plant and test weight than national check indicating the contribution of these characters for their higher yields. The contribution of these characters towards yield were also indicated by Yadav *et al.* (2003) for number of pods per plant and test weight and by Singh *et al.* (2001) for number of primary branches. Among the 15 selected genotypes studied, only one genotype was found superior over check in terms of yield. The superior RILs and segregating progenies could be useful for commercial cultivation in Karnataka. These lines are to be tested in large scale and multilocation trials before releasing them for cultivation.

5.6.2 Correlation analysis

Seed yield is a complex character and is multiplicative and product of many factors called yield components. To make effective selection for higher yields, a thorough understanding of yield contributing characters, interrelationship among

themselves and with yield is necessary. Selection for one component may bring about a simultaneous change in the other. If the association is positive, improvement in one character will simultaneously bring an improvement in the other. But, if they are negatively associated, improvement in one character may adversely affect the other and the net economic gain may be towards the negative side. The correlation is a powerful tool to study the character association and is therefore, very useful to facilitate selection for improvement of characters without scarifying the gain in the other.

In the present investigation, five yield components were studied for their relationship with yield as well as among themselves using correlation analysis individually in 15 selected genotypes, 125 RILs and 80 F₅ segregating progenies.

It was noticed that the seed yield was positively correlated with all the traits studied except for days to 50 per cent flowering in all the three sets of plant material. Similar results were obtained by Obaidullah *et al.* (2006) and Jeena *et al.* (2005) in their association analysis for yield and other quantitative traits in twelve and eight diverse chickpea genotypes respectively. However, based on the magnitude of correlation values, number of primary branches, number of pods per plant and test weight were regarded as very closely related traits to yield. Many reports indicated the association of seed yield with number of primary branches and number of pods per plant (Obaidullah *et al.*, 2006; Toker and Cagrgan, 2004; Qureshi *et al.*, 2004; Ciftci *et al.*, 2004; Brar *et al.*, 2004; Arora *et al.*, 2003 and Vijayalakshmi *et al.*, 2000). Therefore, they can be considered as principal yield determining factors. These traits are easily visible and can be used as selection criteria to score large number of segregating lines, RILs and diverse genotypes in any breeding programme. Days to 50 per cent flowering recorded negative association with seed yield in selected genotypes and segregating progenies while it was associated positively in case of RILs. Positive

association of this trait with seed yield was earlier indicated in the correlation studies of 20 elite genotypes (Saleem *et al.*, 2002), while negative association was observed in a study involving 46 diverse genotypes of chickpea (Bhaduoria *et al.*, 2003).

Association among important yield components indicated that number of pods per plant had significant and positive association with number of primary branches while test weight had significant positive association with both number of primary branches and number of pods per plant in all the three sets of plant material studied. Among the associations, plant height in case of selected genotypes recorded significant positive association with all other characters including seed yield per plant. Therefore, from the present study it was clear that pods per plant, number of primary branches and test weight were the major yield components in chickpea.

5.6.3 Path coefficient analysis

Yield being a complex character, is composed of several components some of which affect yield directly while others contribute towards it indirectly. Correlation studies provide an opportunity to study the magnitude and direction of association of yield with its components and also among various components. To accumulate optimum combination of yield contributing characters in a single genotype, it is essential to know the amplification of interrelationship of various characters with standard partial correlation or regression.

In the present study, path analysis was used to work out the direct and indirect effects of five different characters on seed yield in 15 selected genotypes, 125 RILs and 80 segregating F_5 progenies. The traits which not only have high positive correlation but also high direct effect are expected to be useful in selection programmes. Considering this fact, among the three traits which had significant positive correlation with yield, only number of primary branches and number of pods

per plant had high positive direct effects on seed yield in all the three sets of plant material. While test weight had positive direct effect on seed yield only in selected genotypes and RILs but recorded negative direct effect in case of F_5 progenies. Plant height incase of selected genotypes also recorded positive direct effect on seed yield. The positive direct effects of plant height observed in this study are in agreement with results of Katiyar (1979) for number of primary branches, Kumar *et al.* (2003) for number of pods per plant and Paliwal *et al.* (1987) for test weight. While negative direct effects were also reported for test weight on seed yield by Singh *et al.* (2001) in 34 diverse chickpea genotypes. Although test weight recorded negative direct effect, its positive correlation with seed yield was due to its high indirect effect via number of pods per plant. The path coefficient analysis revealed that there is more or less similar trend in all the three sets of plant material, viz. selected genotypes, RILs and F_5 progenies with respect to direct and indirect effects of different traits towards seed yield. It is clear that for selection of high yielding genotypes in chickpea, number of pods per plant, number of primary branches and test weight should be used as primary selection criteria.

Future line of work

1. The pollen bioassay standardized in present investigation should be integrated in the conventional breeding programmes for development of resistant varieties.
2. The validation of pollen bioassay using molecular markers may be supported by screening RILs and different progenies in wilt sick plots.
3. The promising progenies identified in RILs and F_6 progenies can be used in different breeding programmes to develop high yielding genotypes with wilt resistance.

Summary

VI. SUMMARY

Chickpea (*Cicer arietinum*) is an economically important crop of India. Several biotic and abiotic factors limit the chickpea production in the country. The productivity of 810 kg/ha in the country is one of the lowest in the world. The wilt caused by *Fusarium oxysporum* f.sp. *ciceris* is one of the major limiting factors for production. It is difficult to successfully control this disease as it is soil borne. Development of high yielding resistant chickpea varieties appears to be the most viable strategy to overcome this problem. Several features of pathosystem, as well as the development and maintenance of uniform wilt sick plots for selection of resistance hinder the development of resistant cultivars. Consequently, more efficient methods for accelerating the development of resistant cultivars are required. Screening of segregating populations in wilt sick plots is considered the most efficient means of identifying wilt resistant segregates but it is laborious, time consuming, costly and is affected by inoculum concentration and environmental conditions. Alternatively attempts are being made to identify molecular markers for resistance genes to reduce the need for maintaining virulent isolates of the wilt pathogen. However, molecular marker assisted selection has certain disadvantages (Collard *et al.*, 2005). In this context there is an urgent need to develop alternate screening technique to identify wilt resistant genotypes in order to improve the overall productivity of the chickpea. In the present study, attempts were made to develop pollen bioassay as a simple but effective screening technique at laboratory level against *Fusarium* wilt. The efficiency of this technique in the selection of resistance was also compared to that of selection based on DNA markers linked to resistant loci.

The plant material for the present study was carefully selected which include three different sets, viz. (i) 16 diverse genotypes (ii) RILs of cross between early wilter JG-62 and resistant genotype WR-315 and (iii) F₅ progenies of cross BG-256

(late wilter) x WR-315 (resistant line). Four DNA markers were used in the present investigation which included one ASAP marker CS-27₇₀₀ linked to H_1 locus of wilt susceptibility and three putative RAPD markers (OPA-07₁₆₄₄, OPK-09₁₈₀₁ and A07C₄₁₇) linked to H_2 locus. The pollen bioassay was performed using the basal pollen germination medium suggested by Shivanna *et al.* (1997). The pollen germination and tube growth were considered as sensitive pollen parameters to measure resistance. The fusaric acid one of the pathotoxins produced by the pathogen *Fusarium oxysporum* f.sp. *ciceris* was used. The salient findings of the investigation are presented below.

1. Sixteen genotypes were evaluated for wilt reaction. Among 16 genotypes 4 were early wilters, 10 were late wilters and 2 were resistant.
2. Among the DNA markers linked to H_1 locus (CS-27₇₀₀) and H_2 locus (OPA-07₁₆₄₄, OPK-09₁₈₀₁ and A07C₄₁₇), two were not validated in different genetic backgrounds. Two markers CS-27₇₀₀ and A07C₄₁₇ were selected for further analysis.
3. The resistant genotypes WR-315 and ICC-12249 did not have any markers linked to susceptible loci, while the late wilters A-1, ICCV-2, ICC-96030, ICC-4958, ICC-96029, BG-256, K-850, ICC-12237, ICC-12252 and ICC-12236 were positive for either CS-27₇₀₀ linked to H_1 or A07C₄₁₇ linked to H_2 locus of susceptibility.
4. These two markers were tested separately in the 139 RILs of cross JG-62 ($H_1H_1H_2H_2$) x WR-315 ($h_1h_1h_2h_2$). Both of these markers showed expected 1:1 segregation ratios individually. Further, digenic analysis revealed that, both the markers were present in 34 RILs, CS-27₇₀₀ alone was present in 27 RILs, A07C₄₁₇ alone was present in 45 RILs while, both were absent in 33 RILs which

showed expected digenic 1:1:1:1 ratio. The two markers viz., A07C₄₁₇ and CS-27₇₀₀ were considered for further analysis as they are validated in the experimental material.

5. The sixteen selected genotypes were tested for *in vitro* pollen germination and pollen tube growth at different levels of toxin stress (0, 50, 75 and 100 µg of fusaric acid/ml of PGM). There was significant reduction in pollen tube growth in all the genotypes with the addition of toxin indicating the effect of FA on the tube growth. However, the degree of reduction varied among the genotypes, which was quantified by computing the toxin requirements for inhibition of 50 per cent tube growth (LD₅₀ value). Based on requirement of toxin concentration to inhibit 50 per cent tube growth the genotypes were grouped into resistant, late wilting and susceptible.
6. The genotypes classified based on molecular markers and pollen bioassay were compared. The genotypes showing absence of both the markers (resistant group) recorded mean LD₅₀ value of 115.75 µg, the genotypes showing the presence of any one of the linked markers (late wilters) recorded mean LD₅₀ toxin of 84.67 µg. While, genotypes showing presence of both the markers (early wilters) recorded mean LD₅₀ value of 66.41 µg. It was evident that the toxin concentration required for inhibition of pollen tube growth is more for resistant group (115.75 µg) compared to late wilters (84.67 µg) and early wilters (66.41 µg). Further, the mean LD₅₀ values of these three groups were significantly different from each other indicating the effectiveness of FA as a selective agent for *in vitro* screening of the chickpea genotypes for wilt resistance.
7. Fifty randomly selected RILs of cross JG-62 x WR-315 were studied for *in vitro* pollen tube growth under varied toxin concentrations. The increase in toxin

concentration has significantly reduced the pollen tube growth in all the 50 RILs.

The toxin concentration required to inhibit 50 per cent pollen tube growth (LD_{50} value) was determined for the RILs. The RILs showed segregation for LD_{50} toxin requirement. Based on the LD_{50} values the 50 RILs were classified into highly resistant (with high LD_{50} values), moderately resistant (with moderate LD_{50} values) and susceptible (with low LD_{50} values) groups.

8. The DNA markers CS-27₇₀₀ (linked to H_1 locus) and A07C₄₁₇ (linked to H_2 locus) were used to analyse the 50 RILs. Ten RILs amplified the specific bands for both the markers and grouped as early wilters, 27 RILs showed the presence of any one of the linked marker and were grouped as late wilters, while 13 RILs did not show any amplification of both the markers and were grouped as resistant.
9. The RILs classified based on molecular markers and pollen bioassay were compared. The RILs showing absence of both markers (resistant group) recorded mean LD_{50} toxin of 114.08 μ g, the RILs showing presence of any one of the two linked markers (late wilting group) recorded mean LD_{50} toxin of 85.39 μ g, while, RILs showing presence of both the markers (susceptible group) recorded 68.41 μ g of LD_{50} toxin requirement. Only four RILs (RIL Nos. 52, 34, 55 and 37) deviated from their respective groups. The mean LD_{50} values of the three groups were significantly different from each other.
10. The comparative study of pollen bioassay and molecular marker analysis produced similar results in both the experiments – selected genotypes and RILs. The resistant genotypes/RILs based on molecular markers required very high toxin concentration for 50 per cent inhibition of pollen tube growth as evident by mean LD_{50} values. Similarly late wilters required moderate toxin concentration while the early wilter genotypes/RILs required very low toxin concentration. These results unequivocally prove that the *in vitro* pollen bioassay using fusaric

acid as stress factor is an effective simple technique to identify and select resistant genotypes.

11. The pollen reaction of genotypes to toxin stress also showed faithful segregation in the RILs similar to resistant alleles. The pollen bioassay as a trait is inherited and showed normal segregation like molecular markers. These results further confirm that pollen bioassay is an effective, highly reliable technique for screening genotypes and germplasm of chickpea for resistance to *Fusarium* wilt.
12. The efficiency of pollen bioassay in selecting resistant segregants was studied. Twenty-two F_5 progenies of cross BG-256 ($h_1h_1H_2H_2$) x WR-315 ($h_1h_1h_2h_2$) were selected based on the performance of their pollen grains for toxin stress. The progenies showed significant variation for LD_{50} values of toxin. Based on LD_{50} values the progenies were classified as late wilters and resistant progenies.
13. The F_6 generation of the selected 22 progenies were grown and analyzed for their resistance using linked molecular markers. Out of 22 progenies, 11 were positive for the A07C₄₁₇ and the remaining 11 did not produce amplicon linked to H_2 locus. Based on the presence or absence of the marker (A07C₄₁₇) progenies were classified into late wilting and resistant.
14. In general, the F_5 progenies selected as resistant based on *in vitro* pollen bioassay produced F_6 progenies which are resistant. Similarly, the susceptible progenies produced susceptible off springs. However, three progenies numbered 12, 45 and 109 deviated from their respective groups. The resistant progenies had significantly higher mean LD_{50} toxin (105.84 μ g) compared to susceptible late wilters (94.84 μ g).
15. The results involving germplasm lines, RILs and F_5 progenies clearly indicate that pollen bioassay is an efficient technique for selection of resistance. The

efficiency of selection for resistance is on par with molecular markers if not more.

16. It is interesting to observe that the LD₅₀ toxin requirement for the resistant genotypes is more than 105 µg/ml of PGM, for late wilters it is from 84.00 to 95.00 µg/ml PGM and for susceptible genotypes it was around 65 µg/ml of PGM. There was consistency in these values. Hence, we can generalize and recommend the toxin concentrations for selection of early wilters, late wilters and resistant genotypes in chickpea.
17. The genetic variability studies of all the three sets of plant material viz., 15 selected genotypes, 125 RILs and 80 F₅ progenies revealed significant variation in each group for all the traits studied viz., days to 50 per cent flowering, plant height, number of primary branches, number of pods per plant, test weight and seed yield per plant.
18. In all the 3 sets of experimental material the seed yield per plant, number of pods per plant and 100-seed weight and high heritability estimates coupled with high genetic advance indicating the substantial contribution of additive genetic variance in the expression of these characters. High genetic gain from selection may be expected for these traits.
19. Out of 125 RILs studied, 36 recorded significantly higher yield compared to national check A-1. Among these 36 RILs, 24 recorded significantly high number of pods, 8 recorded significantly high number of pods and primary branches per plant while, 7 RILs (numbered 64, 70, 90, 95, 98, 100 and 108) recorded significantly high number of pods, primary branches and test weight compared to national check A-1.
20. Among the 80 F₅ progenies studied 9 recorded significantly high seed yield per plant compared to A-1. All these 9 progenies (numbered 1, 35, 59, 64, 81, 83, 95,

106 and 109) also recorded significantly high number of pods, primary branches and test weight, which are important contributing characters to seed yield.

21. The traits, number of primary branches, number of pods per plant and 100-seed weight were positively associated with seed yield. Among these traits, number of primary branches and number of pods per plant had high positive direct effect on seed yield per plant in all the three sets of plant material. For selection of high yielding chickpea genotypes, number of pods, primary branches per plant and test weight can be used as primary selection criteria.

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Appendices

APPENDIX I

**Reactions of chickpea RILs of cross JG-62 x WR-315 for CS-27₇₀₀ (linked to H1 locus)
and A07C₄₁₇ (linked to H₂ locus) markers**

Sl No.	RIL no	CS-27 ₇₀₀	A07C ₄₁₇	Sl No.	RIL no	CS-27 ₇₀₀	A07C ₄₁₇	Sl No.	RIL no	CS-27 ₇₀₀	A07C ₄₁₇
1	1	+	+	54	57	+	-	107	113	+	-
2	2	+	-	55	58	+	-	108	114	-	+
3	3	+	+	56	59	-	-	109	115	+	-
4	4	-	+	57	60	-	-	110	116	-	+
5	5	+	+	58	61	-	-	111	117	+	-
6	6	+	-	59	62	-	-	112	118	+	+
7	7	+	-	60	63	+	+	113	119	+	+
8	8	+	+	61	64	-	-	114	120	-	+
9	9	-	+	62	65	-	-	115	121	-	+
10	10	-	-	63	66	-	-	116	122	-	-
11	12	-	+	64	67	+	-	117	123	-	-
12	13	-	+	65	68	+	-	118	124	+	-
13	14	+	+	66	69	-	-	119	125	+	+
14	15	+	+	67	70	-	+	120	126	+	-
15	16	+	-	68	71	-	-	121	127	-	+
16	17	-	+	69	72	+	+	122	128	-	+
17	19	+	+	70	73	+	-	123	129	+	-
18	20	-	-	71	74	-	-	124	130	+	+
19	21	+	+	72	75	-	+	125	132	+	+
20	22	+	-	73	76	+	-	126	133	+	-
21	23	+	-	74	77	-	+	127	135	-	+
22	24	-	+	75	78	-	+	128	136	-	+
23	25	-	-	76	80	+	+	129	137	+	+
24	26	+	+	77	81	+	+	130	138	-	+
25	27	-	-	78	82	-	-	131	139	+	-
26	28	-	-	79	83	+	-	132	140	-	-
27	29	-	+	80	84	-	-	133	141	+	-
28	30	+	-	81	85	+	-	134	142	-	+
29	31	+	-	82	86	-	+	135	145	-	+
30	32	-	+	83	87	+	+	136	146	-	+
31	33	+	+	84	88	-	+	137	148	-	+
32	34	-	-	85	90	+	+	138	149	-	+
33	35	-	+	86	91	+	+	139	150	-	-
34	36	-	-	87	92	+	+				
35	37	-	+	88	93	-	+				
36	38	-	-	89	94	-	-				
37	39	-	+	90	95	+	-				
38	40	-	+	91	96	-	+				
39	42	-	-	92	97	-	-				
40	43	-	-	93	98	+	+				
41	44	-	-	94	99	-	+				
42	45	+	+	95	100	-	+				
43	46	+	+	96	101	+	+				
44	47	+	+	97	102	-	-				
45	48	-	+	98	103	-	+				
46	49	+	+	99	104	-	-				
47	50	-	-	100	105	+	+				
48	51	-	-	101	106	-	+				
49	52	-	+	102	107	-	+				
50	53	-	+	103	108	+	+				
51	54	-	-	104	109	+	-				
52	55	+	+	105	110	-	+				
53	56	+	+	106	112	-	+				

APPENDIX II

Mean values of selected genotypes for six quantitative traits in chickpea

Sl No.	Genotype	Days to 50% flowering	Plant height (cm)	Number of primary branches	Number of pods per plant	Test weight (g)	Seed yield per plant (g)
1	JG-62	48.67	38.87	5.05	64.94	17.77	12.13
2	WR-315	56.67	35.37	4.90	57.83	13.67	09.26
3	K-850	55.67	35.67	4.93	58.80	14.27	10.09
4	BG-256	43.00	36.63	4.93	62.43	14.30	10.11
5	Karikadle	52.67	38.93	5.60	67.73	17.89	12.23
6	ICCV-2	51.00	34.60	4.43	46.42	11.93	07.05
7	A-1	55.33	38.07	5.21	63.89	16.47	12.16
8	ICC-12236	54.67	37.40	4.93	63.50	15.83	10.38
9	ICC-12237	54.33	34.77	4.87	56.14	12.70	08.94
10	ICC-96030	24.67	38.13	5.20	64.00	17.57	12.06
11	ICC-12249	45.67	39.00	5.63	67.27	17.77	12.48
12	ICC-4958	47.33	39.43	5.80	68.20	18.02	12.97
13	ICC-12429	53.33	41.93	6.01	69.00	18.35	14.07
14	ICC-96029	23.33	31.30	4.00	34.71	11.53	02.87
15	ICC-12252	41.00	36.93	4.93	62.87	14.83	10.35
Mean		46.49	37.14	5.10	60.52	15.53	10.44
CV		11.05	08.02	6.94	08.82	05.43	07.88

APPENDIX III

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Mean values of RILs for six quantitative traits in chickpea

Sl No.	RIL No.	Days to 50% flowering	Plant height (cm)	Number of primary branches	Number of pods per plant	Test weight (g)	Seed yield per plant (g)
1	1	43.33	38.78	4.27	45.93	13.37	09.63
2	2	49.67	39.33	4.53	51.17	13.77	11.48
3	3	49.00	38.11	3.93	38.13	12.67	06.57
4	4	48.00	38.78	4.13	44.40	13.17	09.06
5	5	45.00	38.55	4.07	43.23	13.00	08.71
6	6	49.00	40.44	4.27	45.93	13.37	09.51
7	7	48.33	37.89	3.73	35.50	11.87	05.01
8	8	48.67	32.22	4.13	44.27	13.17	08.93
9	10	47.67	41.67	3.87	37.87	12.47	05.86
10	12	44.00	40.44	4.47	47.50	13.50	10.27
11	14	49.00	32.89	4.93	62.30	14.70	15.72
12	15	48.00	37.33	4.50	50.97	13.67	11.27
13	16	48.33	35.11	5.27	65.70	15.37	17.97
14	17	49.67	35.78	3.47	33.07	11.17	03.71
15	19	43.00	37.78	4.60	53.47	13.90	12.23
16	20	51.00	31.22	4.80	58.53	14.40	14.20
17	21	47.67	30.45	4.67	55.73	14.17	12.97
18	22	45.67	31.78	3.87	37.13	12.16	05.67
19	23	47.67	35.78	5.27	66.33	15.47	18.01
20	24	48.00	33.22	4.80	59.10	14.40	14.69
21	25	48.00	36.78	3.10	25.47	10.48	03.04
22	26	49.00	35.11	4.87	59.67	14.47	15.18
23	27	41.67	36.78	3.93	37.93	12.57	06.44
24	28	47.00	31.33	5.57	77.30	16.17	19.76
25	29	44.67	37.89	4.47	48.53	13.60	10.97
26	31	46.00	34.67	3.07	19.43	10.40	02.82
27	32	44.00	32.44	4.07	43.80	13.10	08.91
28	33	48.00	38.89	4.67	54.23	14.07	12.64
29	34	47.33	32.45	5.67	81.73	16.67	20.18
30	35	44.00	39.78	4.40	46.90	13.40	10.26
31	36	49.00	38.56	5.07	62.93	14.90	16.23
32	38	49.33	37.78	5.00	62.70	14.77	15.88
33	39	48.00	36.11	5.40	68.30	15.80	18.50
34	40	47.67	34.45	5.73	82.27	16.70	20.18
35	42	47.00	38.44	4.70	56.22	14.20	13.57
36	43	49.00	35.55	4.73	57.33	14.30	13.76
37	44	50.00	33.45	4.67	55.89	14.20	13.42
38	45	48.67	32.78	4.33	46.90	13.40	09.82
39	46	49.00	34.55	3.93	43.60	12.67	06.51
40	47	45.00	46.78	5.37	68.80	15.67	18.47
41	48	47.67	36.67	5.63	77.40	16.47	19.97
42	49	50.00	36.78	4.93	60.40	14.70	15.54
43	50	49.00	36.11	5.50	76.03	16.07	19.68
44	51	47.00	45.67	4.53	51.47	13.77	11.53
45	52	44.33	40.00	4.50	49.53	13.60	11.23
46	53	50.33	39.55	3.97	38.63	12.67	06.78
47	55	47.67	36.33	5.50	73.30	16.00	18.91
48	56	50.00	41.00	5.67	78.20	16.90	20.02
49	57	44.00	39.11	4.73	57.67	14.40	14.33
50	58	43.67	38.33	5.23	65.80	15.20	17.17

Appendix III. Contd.....

Sl No.	RIL No.	Days to 50% flowering	Plant height (cm)	Number of primary branches	Number of pods per plant	Test weight (g)	Seed yield per plant (g)
51	59	47.00	35.78	4.93	60.40	14.67	15.52
52	60	46.67	37.00	4.00	40.40	12.90	07.04
53	61	47.33	37.22	4.90	60.40	14.57	15.41
54	62	46.00	38.11	4.13	43.67	13.10	08.78
55	63	48.00	38.11	5.37	67.53	15.50	18.31
56	64	49.67	38.44	7.00	93.70	18.17	22.04
57	65	52.00	36.33	4.30	46.67	13.37	09.74
58	66	47.00	37.78	4.13	44.77	13.50	09.11
59	68	50.00	41.67	4.53	51.10	13.70	11.39
60	69	49.00	32.00	4.80	59.13	14.40	14.80
61	70	45.00	36.11	7.57	97.77	19.37	24.26
62	71	43.00	34.44	4.87	59.63	14.47	14.94
63	72	47.33	42.00	4.73	57.13	14.40	13.94
64	73	48.00	34.33	4.00	39.60	12.73	06.84
65	74	51.00	34.56	4.27	45.73	13.37	09.33
66	75	44.00	33.89	3.60	35.10	11.80	04.28
67	76	47.00	34.00	4.07	41.10	12.90	07.72
68	77	45.67	35.56	4.47	48.67	13.60	11.10
69	78	44.00	36.44	4.27	45.50	13.27	09.30
70	80	48.00	33.89	4.67	55.93	14.17	13.00
71	81	45.67	40.44	4.47	47.93	13.50	10.40
72	82	46.67	41.56	5.07	64.80	14.90	16.31
73	83	50.00	41.00	4.47	49.20	13.60	11.12
74	84	45.00	35.78	3.63	35.23	11.87	05.00
75	85	47.00	36.11	4.87	60.33	14.50	15.37
76	86	48.00	43.22	3.50	34.97	11.47	03.93
77	87	50.00	31.89	5.07	63.13	14.90	16.00
78	88	49.00	39.44	3.57	35.07	11.57	04.11
79	90	48.00	39.33	7.53	95.00	19.10	24.61
80	91	44.33	37.78	5.73	83.27	17.10	20.54
81	92	43.33	38.78	3.47	34.20	11.37	03.87
82	94	49.67	39.33	5.87	90.27	17.90	20.93
83	95	49.00	38.11	7.33	93.73	18.90	23.10
84	96	48.00	38.78	4.73	57.24	14.30	13.71
85	97	45.00	38.55	2.80	15.53	9.42	02.59
86	98	49.00	40.44	6.90	93.47	18.07	21.88
87	100	48.33	37.89	6.07	90.33	18.00	21.78
88	101	48.67	32.22	3.73	35.93	12.01	05.57
89	102	47.67	41.67	5.00	62.73	14.87	15.93
90	104	44.00	40.44	4.53	51.73	13.87	11.68
91	105	49.00	32.89	4.67	55.73	14.10	12.90
92	107	48.00	37.33	4.07	41.93	12.97	08.14
93	108	48.33	37.91	6.87	91.60	18.00	21.47
94	109	49.67	35.07	4.07	40.47	12.90	07.44
95	110	43.00	35.37	4.20	44.80	13.20	09.23
96	112	51.00	35.40	4.20	45.47	13.23	09.27
97	113	47.67	36.67	4.63	53.57	13.97	12.23
98	114	45.67	36.57	4.57	53.47	13.90	12.01
99	115	47.67	40.77	5.50	71.83	15.97	18.80
100	116	48.00	40.23	5.40	68.80	15.80	18.50
101	117	48.00	37.00	4.67	54.40	14.07	12.67
102	118	49.00	39.07	5.13	65.20	15.07	16.72
103	119	41.67	39.40	5.27	65.47	15.30	17.80

Appendix III. Contd.....

Sl No.	RIL No.	Days to 50% flowering	Plant height (cm)	Number of primary branches	Number of pods per plant	Test weight (g)	Seed yield per plant (g)
104	120	47.00	33.93	3.93	37.90	12.60	06.40
105	121	44.67	36.43	4.53	51.73	13.77	11.63
106	122	46.00	39.90	5.33	66.33	15.47	18.24
107	123	44.00	36.47	4.57	51.93	13.90	11.97
108	124	48.00	32.43	3.73	35.87	11.65	05.20
109	125	47.33	41.50	5.77	87.40	17.20	20.97
110	126	44.00	36.77	4.67	53.80	14.00	12.52
111	127	49.00	36.87	4.67	54.30	14.07	12.60
112	128	49.33	35.80	4.40	47.20	13.47	10.11
113	129	48.00	32.93	3.87	36.67	12.37	05.63
114	130	47.67	37.80	4.80	58.93	14.40	14.67
115	132	47.00	39.67	5.27	65.60	15.37	17.80
116	133	49.00	38.27	4.93	60.40	14.70	15.61
117	136	50.00	37.87	4.83	60.20	14.40	14.83
118	137	48.67	40.53	5.47	70.47	15.90	18.64
119	138	49.00	39.07	5.20	65.30	15.10	16.77
120	139	45.00	39.03	5.13	64.80	15.00	16.53
121	140	47.67	36.13	4.50	49.27	13.60	11.18
122	141	50.00	39.07	5.20	65.13	15.17	17.04
123	142	49.00	32.80	3.80	36.40	12.16	05.57
124	146	47.00	29.13	3.37	28.97	11.72	03.53
125	150	44.67	40.40	5.43	68.87	15.87	18.63
Mean		47.34	37.11	4.72	55.70	14.21	12.81
CV		03.34	05.08	8.05	03.70	06.50	14.44

Mean values of F₆ progenies for six quantitative traits in chickpea

Sl No.	Progeny No.	Days to 50% flowering	Plant height (cm)	Number of primary branches	Number of pods per plant	Test weight(g)	Seed yield per plant(g)
1	1	43.33	38.78	6.33	73.44	23.01	21.55
2	3	49.33	39.33	5.00	48.48	18.95	10.16
3	4	49.00	38.11	4.33	38.97	16.33	05.97
4	6	48.00	38.78	4.56	42.71	17.73	07.18
5	9	45.00	38.55	4.78	45.05	18.39	08.03
6	10	49.00	40.44	4.22	32.42	14.03	04.97
7	11	48.33	37.89	4.56	40.42	17.42	06.95
8	12	48.67	32.22	4.56	41.12	17.64	07.10
9	13	47.67	41.67	4.00	30.96	13.13	03.82
10	14	44.00	40.44	3.44	19.53	11.98	02.90
11	15	49.00	32.89	4.00	29.77	13.04	03.72
12	18	48.00	37.33	4.67	43.53	17.91	07.33
13	19	48.33	35.11	4.00	31.08	13.18	04.43
14	20	49.67	35.78	3.56	20.09	12.06	02.93
15	23	43.33	37.78	4.15	31.34	13.60	04.54
16	25	51.00	31.22	5.56	60.61	19.83	13.52
17	26	47.67	30.45	3.89	25.63	12.53	03.58
18	27	45.67	31.78	3.78	21.00	12.28	03.38
19	28	47.67	35.78	3.44	16.89	11.97	02.77
20	29	48.00	33.22	3.56	20.73	12.09	03.27
21	30	48.00	36.78	3.33	16.56	9.73	02.71
22	31	49.00	35.11	4.45	39.91	16.46	06.10
23	32	41.67	36.78	4.22	33.06	14.27	05.16
24	33	47.00	31.33	4.56	40.94	17.52	06.99
25	35	44.67	37.89	6.22	72.27	22.84	21.03
26	36	46.00	34.67	3.78	21.87	12.50	03.54
27	38	44.00	32.44	4.89	46.41	18.74	08.31
28	39	48.00	38.89	4.89	45.89	18.65	08.03
29	40	47.33	32.45	4.33	38.76	16.25	05.76
30	41	44.00	39.78	3.89	26.98	12.74	03.65
31	42	49.00	38.56	5.00	48.38	18.90	10.00
32	44	49.33	37.78	5.78	65.48	21.58	13.94
33	45	48.00	36.11	4.33	37.83	15.52	05.72
34	47	47.67	34.45	4.22	36.18	14.41	05.22
35	49	47.00	38.44	5.50	59.53	19.61	12.92
36	51	49.00	35.55	5.55	59.92	19.82	13.16
37	54	50.00	33.45	5.67	60.86	20.57	13.58
38	55	48.67	32.78	4.89	47.42	18.78	08.54
39	56	49.00	34.55	5.78	65.44	21.33	13.76

Appendix IV. Contd.....

Sl No.	Progeny No.	Days to 50% flowering	Plant height (cm)	Number of primary branches	Number of pods per plant	Test weight(g)	Seed yield per plant(g)
40	58	45.00	46.78	5.33	55.16	19.09	11.35
41	59	47.67	36.67	6.92	83.60	26.10	25.06
42	60	50.00	36.78	4.78	44.63	18.33	07.95
43	61	49.00	36.11	4.22	34.13	14.32	05.16
44	62	47.00	45.67	5.00	47.45	18.80	08.56
45	64	44.33	40.00	6.56	78.49	25.99	24.11
46	66	50.33	39.55	4.33	37.38	14.79	05.48
47	69	47.67	36.33	4.66	42.85	17.77	07.26
48	71	50.00	41.00	5.22	54.30	19.08	11.29
49	72	44.00	39.11	4.44	39.19	16.38	06.00
50	73	43.67	38.33	5.00	47.55	18.84	09.06
51	75	47.00	35.78	5.67	63.54	21.15	13.59
52	79	46.67	37.00	4.44	39.37	16.43	06.09
53	80	47.33	37.22	5.11	53.49	19.05	11.24
54	81	46.00	38.11	6.56	78.42	24.13	23.89
55	83	48.00	38.11	6.22	71.98	22.67	19.44
56	85	49.33	38.44	5.11	53.17	19.02	11.10
57	88	52.00	36.33	5.33	56.87	19.37	12.56
58	90	47.00	37.78	4.18	31.83	13.64	04.54
59	91	50.00	41.67	5.07	48.52	18.97	10.74
60	92	49.00	32.00	5.11	53.28	19.05	11.15
61	95	45.00	36.11	7.11	84.44	26.17	27.31
62	96	43.00	34.44	4.00	29.76	12.92	03.65
63	97	47.33	42.00	4.45	40.16	17.32	06.22
64	98	48.00	34.33	5.11	51.89	18.99	10.85
65	99	51.00	34.56	5.44	57.96	19.41	12.56
66	103	44.00	33.89	4.78	44.54	17.92	07.87
67	105	47.00	34.00	4.67	42.98	17.90	07.28
68	106	45.67	35.56	5.89	69.08	22.23	17.89
69	107	44.00	36.44	5.44	59.20	19.58	12.90
70	108	48.00	33.89	3.67	20.85	12.14	03.31
71	109	45.67	40.44	6.00	70.53	22.32	17.97
72	110	46.67	41.56	4.78	44.04	17.91	07.51
73	111	50.00	41.00	5.56	59.64	19.70	12.98
74	113	45.00	35.78	5.44	58.87	19.47	12.66
75	114	47.00	36.11	4.33	36.46	14.48	05.37
76	117	48.00	43.22	5.33	55.29	19.37	12.15
77	119	50.00	31.89	4.89	46.27	18.74	08.04
78	120	49.00	39.44	4.33	37.83	14.99	05.48
79	121	48.00	39.33	5.78	67.44	22.19	14.00
80	123	44.33	37.78	4.78	44.67	18.35	07.95
Mean		47.33	36.95	4.86	46.43	17.65	10.45
CV		03.42	04.77	6.73	09.49	02.66	06.30

MOLECULAR EVIDENCE TO *IN VITRO* POLLEN BIOASSAY FOR WILT RESISTANCE IN CHICKPEA

D. RATNA BABU

2007

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ABSTRACT

Fusarium wilt caused by *Fusarium oxysporum* is the major biotic factor limiting chickpea productivity in India. Despite the availability of stable resistance sources, development of resistant varieties is hindered due to lack of effective screening techniques. Conventional screening in sick plots is tedious and time consuming. Alternatively *in vitro* pollen bioassay is emerging as an effective screening technique.

The pollen grains of sixteen diverse genotypes were tested for *in vitro* pollen tube growth by adding different concentrations of fusaric acid to pollen germination medium. The toxin inhibit pollen tube growth and genotypes showed significant variation for the concentration of toxin to inhibit 50 per cent pollen tube growth.

The genotypes were phenotyped for wilt reaction using the *in vivo* seedling reaction to fusaric acid. The genotypes were also tested for their wilt reaction using established molecular markers. The genotypes which were categorized as wilt resistant using molecular markers and *in vivo* seedling reaction were also resistant under *in vitro* pollen bioassay requiring higher toxin concentration for pollen tube inhibition. Further, 50 recombinant inbred lines of cross JG-62 ($H_1H_1H_2H_2$) x WR-315 ($h_1h_1h_2h_2$) were analyzed for their wilt reaction using *in vitro* pollen bioassay. RILs showed significant variation for the toxin concentration to inhibit pollen growth. The RILs were classified into resistant, susceptible late wilters and susceptible early wilters using molecular markers. The resistant RILs required significantly higher toxin concentration for pollen tube growth inhibition, followed by late wilters and early wilters.

The efficiency of pollen bioassay in selecting resistant segregating was studied in F_5 generation of BG-256 x WR-315. The resistant F_5 progenies selected based on pollen bioassay produced resistant F_6 progenies.

The present study involving DNA markers linked to wilt resistance loci, three different sets of genetic material and experiments performed over a period of time clearly showed that the selection of genotypes for *Fusarium* wilt resistance based on pollen bioassay is as reliable as marker assisted selection and/or sporophytic selection of not more.