

**RAPD ANALYSIS FOR TAGGING h_2 LOCUS OF WILT
(*Fusarium oxysporum* f.sp. *ciceri*) RESISTANCE IN
CHICKPEA (*Cicer arietinum* L.)**

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*Thesis submitted to the
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in

GENETICS AND PLANT BREEDING

By

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CERTIFICATE

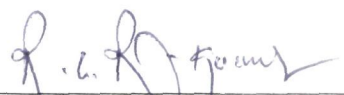
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(Thippeswamy S.)

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Introduction

I. INTRODUCTION

Chickpea (*Cicer arietinum* L.), a diploid species with $2n=16$ chromosomes is the third most important grain legume in the world after dry beans and dry peas. It is a self pollinated crop with natural cross pollination ranging between zero to one per cent (Singh, 1987). Most probably chickpea originated in south eastern Turkey (Ladizinsky, 1975). There are two types of chickpea under cultivation: *desi* (local), characterized by small, angular, coloured seeds and *kabuli* (an allusion to origin in the Afghani capital, Kabul, before it reached India) characterized by large, ram-head shaped, beige coloured seeds. It is believed that, the small seeded *desi* type is the original form of chickpea and that the *kabuli* type developed probably through mutation. The *desi* types account for about 80 to 85 per cent of total world chickpea production (Singh, 1987; Jambunathan *et al.*, 1994).

Chickpea is grown in all the continents except Antarctica. However, its cultivation is more extensive in the semi-arid tropics particularly in the rainfed ecology of the Indian subcontinent, the Mediterranean region, the West Asian and North African region, Eastern Africa and Latin America. Expansion of this crop to new niches, such as Australia in recent years, has been remarkable. This crop is of immense importance in Asia, which grows more than 90 per cent of chickpea mainly in the fragile and rainfed ecosystem. Chickpea is cultivated globally on about 11.3 million hectares adding 8.8 million tonnes of grains annually to the global food basket with an average productivity of 706 kg per ha (Anon., 2001). India has the distinction of being the largest chickpea producer and accounts for over 66 per cent and 69 per cent of the total area and production in the world.

It grows chickpea on about 7.50 million hectares producing 6.10 million tonnes of grains, which represents 33 per cent and 47 per cent of the national pulse acreage and production respectively. In Karnataka, it is an important pulse crop, particularly in the northern districts, grown annually over an area of 2.93 lakh hectares with production of 1.41 lakh tonnes. Its productivity is dismally low with about 3.73 quintals per hectare as compared to national average of about 6.94 quintals per hectare (Anon., 2002).

The annual growth rate of chickpea production and productivity has been very slow at 1.90 and 0.60 per cent respectively. The main reasons for low productivity and unstable yields are biotic and abiotic stresses affecting at various stages of the crop growth. Pulses in general and chickpea in particular are highly susceptible to many of the biotic stresses, which take heavy toll of the total production. Diseases are one of the major constraints of chickpea production. Nearly 115 pathogens with diverse effects have been reported so far, on chickpea from different parts of the world (Nene, 1980), including fungi, bacteria, viruses, mycoplasma like organisms and nematodes. A few of them are devastating (Nene and Reddy, 1987). Fusarium wilt caused by *Fusarium oxysporum* f. sp. *ciceri* and Ascochyta blight caused by *Ascochyta rabiei* are most destructive and widespread. Of these two, Fusarium wilt is a serious problem in Eastern Africa and South Asia in general and India in particular, especially at latitudes ranging from 0-30° (Faris and Gowda, 1990; Haware *et al.*, 1990; Van Rheenen, 1991). It has been estimated that the production loss due to wilt globally is 6,23,000 tonnes which costs 245 million US \$, of which 4,73,000 tonnes is accounted by South Asia alone (Anon., 2000). In

southern states of India including Karnataka, wilt is the only serious disease causing heavy losses. According to an estimate about 10 per cent loss in yield due to wilt is considered to be a regular feature in chickpea growing states of India and may cause more than 90 per cent losses under favourable conditions (Srivastava *et al.*, 1984; Gupta *et al.*, 1986; Jimenez-Diaz *et al.*, 1993; Cortes *et al.*, 2000).

In India, the wilt disease was first observed by Butler in 1918 (Chaturvedi *et al.*, 1997). The fungus is soil borne and survives through chlamydospores on seeds (Haware *et al.*, 1978). Once soil is infected by the pathogen, it can survive in the soil for more than six years even without its host. Lentil, pigeonpea and pea are symptomless carriers of the pathogen (Haware and Nene, 1982). The existence of pathogenic races in populations of *F. oxysporum* f. sp. *ciceri* is now well established. Haware and Nene (1982) were the first to show the occurrence of races of the pathogen, viz., 1, 2, 3 and 4 based on the differential reactions in the artificial inoculation of ten chickpea differentials with different isolates of the pathogen from India. Three additional races were identified (0, 5 and 6) in Spain (Jimenez-Diaz *et al.*, 1989b) and in USA a distinct race that was similar to race 6 was found (Phillips, 1988).

In Karnataka, Annigeri-1 has been the only variety under cultivation since four decades. This variety is susceptible to wilt pathogen. Since this was the only variety being popularly grown in the state, it has lead to the spread of the inoculum in chickpea growing areas of the state. Although, effective fungicides have been identified (Hanounik and Reddy, 1984), they are impractical at field level. Hence, the best strategy to

manage this disease is through use of resistant cultivars. Although, efforts are on to develop high yielding wilt resistant varieties through conventional breeding, the progress has not been very satisfactory. The conventional approach requires screening a large set of segregating populations over several generations under uniform sick plots. This obviously is tedious, cumbersome and time consuming. In view of these, efforts have been made to develop alternate approaches which complement and increase the efficiency of conventional breeding methods.

Identification of biochemical or molecular markers (tags) for resistance genes could lead to quicker assessment of resistance even at the seedling stage and to overcome the need for maintaining virulent isolates of the wilt pathogen in the sick plots. 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 analysed in plant tissue is usually 50 or less. Moreover, isozyme electrophoresis of chickpea has revealed insufficient polymorphism to be useful in finding tags for wilt resistance (Tullu, 1996). A potentially much larger number of markers can be resolved by means of molecular markers. Among which, Restriction Fragment Length Polymorphism (RFLP) technology has been used to establish high density gene maps for an increasing number of crop species (Obrien, 1993). However, only seven RFLPs have been identified and added to the chickpea genetic map (Udupa *et al.*, 1993; Simon and Muehlbauer, 1997).

Random Amplified Polymorphic DNA (RAPD) represents another method for identifying additional genetic variation within the populations

(Welsh and McClelland, 1990; Williams *et al.*, 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 of several orders larger than the total number of morphological or isozyme markers, leading to a much higher level of marker saturation on genetic maps. RAPD analysis is also much faster than RFLP analysis. Despite of the recency of its first use in 1990, RAPDs already have been employed widely for mapping of different genomes (Williams *et al.*, 1990, 1993; Paran *et al.*, 1991; Kiss *et al.*, 1993; Paran and Michelmore, 1993; Torres *et al.*, 1993; Hunt and Page, 1995; Miklas *et al.*, 1996).

Among four prevailing races in India, race 1 is widespread and most destructive race in Central and South India. The genetics of wilt resistance against race 1 in chickpea has been worked out. It has been reported that at least three independent loci designated as H₁, H₂ and H₃ govern resistance to wilt (Upadhyaya *et al.*, 1983a; Singh *et al.*, 1987a and b). Partially recessive allele in homozygous form at either of first two loci and dominant allele at third locus delay wilting. But any two of these alleles together confer complete resistance. Recently Mayer *et al.* (1997) have identified ASAP markers linked to H₁ locus. There is need to tag H₂ locus also, so that the markers for H₁ and H₂ loci together can be used for effective screening of breeding populations against race 1 and pyramiding the resistance genes to achieve complete resistance to wilt. The development of mapping population specifically to tag H₂ locus is another important aspect of this problem. The recombinant inbred lines developed by crossing parental lines polymorphic at H₂ locus of wilt resistance could be the best population to tag H₂ locus.

Keeping these aspects in view, the present investigation was undertaken with the following objectives.

- 1) Development and evaluation of RILs for wilt reaction
- 2) RAPD analysis of parental genotypes
- 3) Association of RAPD markers with wilt reaction
- 4) Genetic variability studies in RILs for seed yield and yield components

Review of Literature

II. REVIEW OF LITERATURE

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *ciceri* is one of the major diseases of chickpea causing significant yield losses. Development of wilt resistant varieties is the cheapest and easy method of management of chickpea wilt as the pathogen is soil borne and chemical control is expensive and impractical (Vidyashekar and Muthamilan, 1995). The development and maintenance of wilt sick plots is a difficult proposal. Therefore, identification of molecular marker for Fusarium wilt resistance genes helps for quicker identification of resistant progeny in the segregating populations (Mayer *et al.*, 1997). Apart from this, a sound knowledge of genetic variability for different yield component traits and their association with seed yield helps the breeder to identify high yielding and wilt resistant genotypes. The available literature on the above aspects is being presented below in the following headings.

2.1 Fusarium wilt of chickpea

2.2 Molecular tagging of disease resistance genes

2.3 Genetic variability and association for yield and yield components

2.1 FUSARIUM WILT OF CHICKPEA

More than 115 pathogens with diverse effects have been reported so far on chickpea from different parts of the world (Nene, 1980). But only a few of them are devastating (Nene and Reddy, 1987). Fusarium wilt caused by *Fusarium oxysporum* Schlecht emd. : Fr. f. sp. *ciceri* (Padwick) Matuo & K. Sato and Ascochyta blight caused by *Ascochyta rabiei* (Pass.)

Lar. are most destructive and widespread. In Eastern Africa, South Asia, India and Mexico, *Fusarium* wilt is more important than *Ascochyta* blight especially at latitudes ranging from zero to 30° (Faris and Gowda, 1990; Haware *et al.*, 1990; Van Rheenen, 1991). *Fusarium* wilt is reported from many countries of Asia, Africa, North and South America and South Europe with yield losses ranging from 10 to 90 per cent (Srivastava *et al.*, 1984; Gupta *et al.*, 1986; Jimenez-Diaz *et al.*, 1993; Cortes *et al.*, 2000). It has been estimated that the loss due to wilt globally is 6,23,000 t however, 4,73,000 t of this is accounted by the South Asia alone (Anon., 2000).

The disease is observed in a highly susceptible cultivar within 25 days after sowing of the crop, which is also referred as early wilt. Early wilting causes more loss than late wilting and the extent of damage by late wilting depends on the stage at which disease develops (Haware and Nene, 1982).

2.1.1 Pathogen

Fusarium wilt of chickpea was first mentioned in 1918 (Nene *et al.*, 1980). Prasad and Padwick (1939) reported that *Fusarium* species they isolated was classified in the section, *Elegans* and subsection, *Orthoceras*. However, Prasad and Padwick (1939) did not name the fungal isolate as a separate species. Comparisons of the isolates with all known species of the sub-section under identical conditions was necessary. The problems were elaborately studied by Padwick (1940) who named the pathogen as *Fusarium orthoceras* var. *ciceri*. Later, the causal agent was reported to be *Fusarium lateritum* f.sp. *ciceri* (Erwin, 1958) based on several isolates

collected in 1954, 1955 and 1956 from wilt affected plants. ICRISAT concluded that the 'wilt complex' should result from a number of distinct diagnosable diseases. 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; Nene *et al.*, 1980; Kraft *et al.*, 1988).

Based on the classification system of Snyder and Hansen (1940), Chattopadhyay and Sen Gupta (1967) renamed *Fusarium orthoceras* var. *ciceri* as *Fusarium oxysporum* f. sp. *ciceri*. The major characteristics of the species *oxysporum* are production of abundant microconidia, macroconidia, presence of chlamydospores and rapid growth on potato dextrose agar (PDA) (Nelson *et al.*, 1983; Booth, 1984).

2.1.2 Ecology and epidemiology

The behaviour of root infecting fungi in soil is complex (Haware *et al.*, 1990). Important factors in the epidemiology of root infecting fungi are inoculum density, inoculum distribution, plant age, host plant resistance, air and soil temperature, soil moisture, soil nutrients and plant density. The fungus, *F. oxysporum* f.sp. *ciceris* is both seed borne and soil borne and persists in soil in the absence of chickpea for more than five years (Nene, 1988; Kraft *et al.*, 1994). The formae speciales of *F. oxysporum ciceris* produce three types of asexual spores *viz.*, microconidia, macroconidia and chlamydospores. Formation of abundant sporodochia on the stems of plants affected by Fusarium wilt could provide a source of inoculum for localized dispersal by splashing rain (Burgess, 1981).

2.1.3 Infection and pathogenesis

A common host symptom of *Fusarium* wilt infection is vascular vessel colonization. In chickpea, penetration occurs mainly through the cotyledons and or to a lesser extent in the zone of elongation and maturation (Jimenez-Diaz *et al.*, 1989a). *Fusarium oxysporum* commonly penetrates root hairs or epidermal cells just behind the root tip or within the zone of elongation and proceeds intercellularly and intracellularly to the primary meristem as has been shown in other crops (Machardy and Beckmann, 1981). After the endodermal and pericycle tissue have been penetrated, the xylem is colonized and the pathogen is distributed upward (Nelson, 1981). Because of proliferation of the pathogen below the perforation plates of the xylem vessel elements, sap flow then is blocked (Mace *et al.*, 1971).

2.1.4 Wilting phenomenon

Wilting caused by *Fusarium oxysporum* f.sp. *ciceri* can be either by mechanical plugging of water ducts (xylem) by mycelium, wilt toxins or hydrolytic enzymes (Green, 1981). According to Green (1981) and Paquin and Waygood (1957), the first chemically defined wilt toxins were lycomarasmin and fusaric acid. Fusaric acid (5-butytylpicdicinic acid) increases transpiration and causes furrowing over the vascular bundles (Lakshminarayanan and Subramanian, 1955). This reaction was demonstrated *in vivo* in tomato (Lakshminarayanan and Subramanian, 1955; Gaumann, 1958) and in pea (Kern, 1972).

Vascular discolouration or browning is a diagnostic symptom in vascular wilt diseases. Dark melanin pigment accumulation in host cells

results from an increase in both phenolic and polyphenoloxidase and other enzyme activity (Green, 1981). The browning originates in xylem parenchyma adjacent to colonized vessels and staining is transferred to the vessels (Pegg, 1981). Vessels could also be blocked by the staining (Corden and Chambers, 1966). Vascular discolouration can occur in infected plants prior to the expression of external symptoms as shown in tomato (Mace *et al.*, 1971). Wilting appears to be host originated resulting from water shortage and hormonal imbalances and the shortage arises in response to the injury of tissues and not as the cause of injury by the pathogen (Beckmann, 1987).

2.1.5 Environment and disease development

Fusarium wilts in general are warm soil diseases and very severe at 28°C or near the optimum temperature for growth of the pathogen in culture (Bruehl, 1987; Bhatti and Kraft, 1992a). Gupta *et al.* (1987) reported that wilt in chickpea was maximum at a temperature of 25°C that severity decreased with an increase or decrease in soil temperature. Bhatti and Kraft (1992a) reported that only mild symptoms (10-15 per cent of foliage with wilting leaves) were observed at 15°C at lower inoculum levels (100-500 propagules/g). They also indicated that chickpea wilt symptoms become prevalent at 25 and 30°C at all inoculum levels (100, 500, 1000 and 5000 propagules/g) and temperature accounted for 57.9 per cent of the variability in wilt severity of chickpea, whereas inoculum density accounted for only 14.10 per cent.

The importance of environment (temperature) was mentioned in other studies (Upadhyaya *et al.*, 1983b; Singh *et al.*, 1987a) as a

contributing factor to the difficulties observed in classifying wilting patterns in segregating chickpea populations.

Suppressive soil is another factor affecting *Fusarium* wilt incidence. Under field conditions the percentage of clay is inversely proportional to the number of *Fusarium* wilt propagules per gram of soil (Nelson, 1981). In general, suppressive soils have more montmorillonite clays compared to non-suppressive soils. These clays were found to stimulate bacterial activity and they might check the growth of *Fusarium* (Baker, 1981).

Eventhough temperature appears to be the most important factor, light and nutrients also affect *Fusarium* wilt development either solely or by interacting with temperature. Increased moisture was associated with increased wilt severity (Bhatti and Kraft, 1992b). Nutrients, especially nitrogen could have a profound effect on wilt development but the effect is not clearly defined in the host pathogen interaction (Beckmann, 1987).

2.1.6 Race pattern of *Fusarium oxysporum* f. sp. *ciceri*

Fusarium oxysporum f.sp. *ciceri* is primarily pathogenic to chickpea but can also attack lentil, pea and pigeonpea without causing visible symptoms (Haware and Nene, 1982). Intraspecific variation in plant pathogenic fungi became apparent from studies of host specificity of such *Fusarium* species as, *F. oxysporum ciceri*, *F. oxysporum lini* and *F. oxysporum pisi* and different pathotypes within one species (*F. oxysporum ciceri* race 1, 2, 3 etc.) (Correll, 1991; Heath, 1991). The strains of the *Fusarium* wilt pathogen have been divided into formae speciales on the basis of virulence to a particular host or group of hosts (Correll, 1991). According to Nene *et al.* (1980), Chauhan (1962) first attempted to study

variability in *Fusarium oxysporum* f.sp. *ciceri*. Later, multilocation trials conducted at ICRISAT provided evidence of differences in reaction to some lines to *Fusarium* wilt pathogens indicating the existence of distinct physiologic races (ICRISAT, 1979).

Seven races designated as 0, 1, 2, 3, 4, 5 and 6 of *F. oxysporum* have been identified. Races 1, 2, 3 and 4 are reported to occur in India (Haware and Nene, 1982), while races 0, 5 and 6 occur in Spain (Jimenez-Diaz *et al.*, 1989b). Phillips (1988) reported the occurrence of race 6 in California based on the disease reaction of ten wilt differential lines described by Haware and Nene (1982). No difference has been established to date between race 6 found in California and race 6 found in Spain (Kraft *et al.*, 1994). The cultivars used to determine the race situations in chickpea and their wilt reaction are shown in Table 1.

Controversies have been reported on the *Fusarium* wilt evaluation using different scoring methods. Haware and Nene (1982) used resistant (R) = 0 - 20 per cent, Medium (M) = 21 - 50 per cent and above 50 per cent as susceptible (S). Jimenez-Diaz *et al.* (1989b) used R= 1 - 33 per cent, M= 34 - 66 per cent and S= 67 - 100 per cent and El-Hadi (1993) used R= 1 - 10 per cent, M= 11 - 33 per cent and S= 33 per cent and above.

Availability of a complementary, reliable and quicker method for identifying races would be of great value in screening and categorizing isolates into races. Earlier studies on serological, electrophoretic and biochemical variability have revealed that the four physiological races *F. oxysporum* f.sp. *ciceri* have a close antigenic relationship and common

Table 1. Reaction of chickpea differential lines to races of *Fusarium oxysporum* f. sp. *ciceri*

Race differentials	India ^a				Spain ^b			California ^c
	Race 1 (Hyderabad)	Race 2 (Kanpur)	Race 3 (Gurdaspur)	Race 4 (Hissar & Jabalpur)	Race 0	Race 5	Race 6	
JG-62	S	S	S	S	R	S	S	S
C-104	S	S	R	S	M	S	M	S/M
JG-74	R	S/M	R	R	R	M	R	R
CPS-1	R	S	S/M	M	R	M	R	R
BG-212	R	S	M	M	R	R	R	R
WK-315	R	R	S/M	R	R	R	R	R
Annigeri-1	S	S	S	S	-	-	-	R
Chaffa	S	S	S/M	S	-	-	-	R
L-550	S	S	S	S	-	-	-	S/M
850-3/27	S	M	M	M	-	-	-	R
Spain^b								
P-2245	S	S	S	S	S	S	S	
12-071/10054	M	R	R	R	S	R	R	
ICCV-2	R	M	M	M	R	S	M	
ICCV-4	R	S	S	S	R	S	M	
PV-24	S	S	S	S	M	S	S	

R=Resistant, S=Susceptible, M=Moderate

^a India, Haware and Nene (1982)

^b Spain, Jimenez-Diaz *et al.* (1989b)

^c California, Phillips (1988)

isozyme patterns (Desai *et al.*, 1992a and b). With the advent of molecular marker technology, DNA marker based studies in *Fusarium oxysporum* f.sp. *ciceri* races have mainly focussed on the use of RAPD-PCR and mitochondrial DNA-restriction fragment length polymorphism approaches (Kelly *et al.*, 1994; Perez-Artes *et al.*, 1995). More recently microsatellite markers also have been used to distinguish four races of *F. oxysporum* f.sp. *ciceri* prevalent in India (Barve *et al.*, 2001).

2.1.7 Sources of resistance to Fusarium wilt of chickpea

In many breeding programs carried out by national and international institutions, development of *Fusarium* wilt resistant chickpea is a major objective. Chickpea breeding lines and accessions can be efficiently screened in the field, greenhouse or in the laboratory. Field screening according to experiences at ICRISAT (Haware *et al.*, 1992), includes the selection of a wilt sick-plot in slightly alkaline vertisols and growing a susceptible cultivar for 2 to 3 seasons during which time the infected plant debris are incorporated into the soil. Artificially and naturally infested soils were used successfully across the world to screen chickpea materials for resistance (Table 2).

Screening in the greenhouse using the pot culture method was standardized by Nene *et al.* (1980). Large earthen pots, 35 cm in diameter are filled with non-autoclaved vertisol followed by the application of the *Fusarium* wilt fungus and autoclaved chickpea stem bits (200 g) in the top 15 cm of vertisol in the pots. The susceptible cultivar, JG-62 is planted to each pot and after wilting the plants are chopped and incorporated in the soil in the pot. The process is repeated and the

Table 2. Sources of resistance to Fusarium wilt in chickpea

Sl. No.	Material screened	Screening method	Resistant source identified	Reference
1	400 lines	Sick plot	42 lines (PPK1, 2, GW-1,3-1, 9, 6, 10, BCP-2,-3, -4 and -5	Dandnaik and Zote, 1988
2	277 lines (2 years)	Sick plot	ICC-184, ICC-1437, ICC-3099, 3328, 3355 and 11322	Karki <i>et al.</i> 1988
3	225 lines (5 years)	Sick plot	ICC 2862, 9023, 9032, 10803, 1558 and 11551	Nene <i>et al.</i> , 1989
4	251 germplasm lines	Sick plot	69 lines (PPK1, PPK2, SSP1, SSP2 and RRAN76)	Shukla <i>et al.</i> , 1989
5	40 germplasm lines	Sick plot	ICC-11323 and ICC-324	Ahmed <i>et al.</i> , 1990
6	211 promising ICRISAT lines	Sick plot	22 lines	Ahmed <i>et al.</i> , 1990
7	79 lines (3 years)	Sick plot	20 lines	Bakr and Ahmed, 1990
8	44 lines	Sick pot	14 lines (ICC 85107 and 12263)	Kaushal and Singh, 1990
9	1283 accessions (3 years)	Sick plot	117 lines (ICC-14532, 14619, 14631, 14735, 15125 and 15233)	Reddy <i>et al.</i> , 1990a
10	Chickpea lines in AICRP trials	Sick plot	Avrodhi, ICC 48 and GCP 6	Reddy <i>et al.</i> , 1990b
11	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
12	39 varieties/lines	Sick plot	GNG 295 and PDG83-31	Kapoor <i>et al.</i> , 1991
13	22 accessions and 48 wild species	Sick plot	10 Wild species	Reddy <i>et al.</i> , 1991
14	13,500 germplasm lines	Sick plot	150 <i>desi</i> and 10 <i>kabuli</i> lines	Haware <i>et al.</i> , 1992
15	188 genotypes	Glasshouse lab. condition	GL1271, GL8319 and ILC-3870	Kapoor and Sugha, 1992
16	Crosses of 6 wilt resistant 74 high yielding lines	Sick plot	58 (BCP-15, BCP-23, BCP-28, BCP-29 and BCP-30)	Zote <i>et al.</i> , 1993
17	51 lines	Sick plot	12 (ICC-12263 and ICCV-10)	Ali <i>et al.</i> , 1994
18	11 wild species	Sick plot	9 wild species	Kaiser <i>et al.</i> , 1994
19	31 ICARDA lines	Sick plot	Flip-85-130C	Iftikhar <i>et al.</i> , 1997
20	60 lines	Sick plot	GCP 9302, 9310 and 9313	Jani <i>et al.</i> , 1999
21	81 lines	Sick plot	10 (JG-22, JG-33, KPG-316 and SAKI-93130)	Yadava <i>et al.</i> , 2000
22	570 lines	Sick plot	GCP9504, H92-71, FG-711, FG-712, FG-703, H 82-2, IFC-97-1 and WCGT 95-50	Gurha <i>et al.</i> , 2002

procedure assures about 90 per cent wilt in susceptible lines. 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). At 3-4 nodal stage seedlings were removed from the perlite, pruned while submerged in the spore suspension and after about 5 min in the spore suspension were replanted into the perlite. Plants were then scored as susceptible or resistant.

The water culture screening technique (Nene *et al.*, 1980) is similar to that previously described by Roberts and Kraft (1971). The screening procedure involves a single spore culture of *F. oxysporum* f.sp. *ciceri*, multiplication of the inoculum in 100 ml liquid media in 250 ml flasks on a shaker for 10 days at 25-30°C, adjusting the inoculum concentration to 6.5×10^5 or 1×10^6 , inoculation of 14 days old seedlings grown in autoclaved sand and transferring the seedlings to glass tubes containing 20 ml of inoculum. A susceptible cultivar is used as a control and is inoculated along with each set of lines being evaluated. Also, for each test line, an uninoculated seedling is kept as a check.

Resistant sources to Fusarium wilt in chickpea have been identified using different screening techniques. A partial list of identified sources of resistance to Fusarium wilt of chickpea is given in Table 2.

2.1.8 Genetics of Fusarium wilt resistance

The understanding of genetics of a trait is a pre-requisite for developing appropriate breeding strategies. Particularly breeding for

disease resistance requires a thorough understanding of race pattern and genes governing resistance to each race. With the proper documentation of race pattern and genetics of resistance to each race, tremendous success has been achieved for resistance to rust in wheat. Although field, laboratory and greenhouse techniques to screen for resistance to soil borne pathogens like *Fusarium oxysporum* have been developed; there are contradicting reports on genetics of resistance.

Resistance to Fusarium wilt is reportedly conferred by a single gene with incomplete dominance (Ayyar and Iyer, 1936) or by a single recessive gene (Lopez, 1974; Haware *et al.*, 1980; Tiwari, 1981; Kumar and Haware, 1982; Sindhu *et al.*, 1983). The gene symbol (*rfo*) for the recessive gene and (*Rfo*) for its dominant allele was designated by Sindhu *et al.* (1983).

In crosses involving JG-62 (susceptible) and H355 and CPS1 as resistance, the number of susceptible lines were greater than expected and did not fit the expected ratio based on a single gene (Tiwari, 1981; Kumar and Haware, 1982). In another study, the ratio obtained in F₂ population (Phillips, 1983) indicated monogenic recessive inheritance. However, it was not confirmed by segregation in the F₃ and F₄ generations. This suggested the probable involvement of multiple genes.

Intensive race specific inheritance studies of Fusarium wilt resistance in chickpea started only after 1982 when Haware and Nene characterized four races of *Fusarium oxysporum* f.sp. *ciceri*. Three independent genes conferring resistance to Fusarium wilt race 1 have been reported (H₁/h₁, H₂/h₂, H₃/h₃); complete resistance is conferred when one of the H₁/h₁ or H₂/h₂ genes is present as recessive homozygote

and H_3/h_3 as a dominant (Upadhyaya *et al.*, 1983a and b). More recently, three independent genes for race 2 (Gumber *et al.*, 1995; Kumar, 1998), two for race 4 (Tullu *et al.*, 1999), one for race 5 (Tekeoglu *et al.*, 2000) and two for race 0 (Rubio *et al.*, 2003) have been reported.

Upadhyaya *et al.* (1983a) reported that, different chickpea genotypes varied as to the time required before the initial symptoms of Fusarium wilt appeared. In particular, C-104 wilts much later than JG-62, the difference appears to be controlled by a single gene. Upadhyaya *et al.* (1983a) proposed that two genes governed resistance in WR-315 x JG-62 and both must be in the homozygous recessive state for complete resistance. These genes were named as h_1 and h_2 . Further, studies by Upadhyaya *et al.* (1983b) confirmed that the cultivar C-104 appears to differ from WR-315 and CPS-1 by a single locus, which results in delayed wilting when in homozygous recessive form. The same group also suggested that data are consistent with the hypothesis that JG-62 carried the two genes in a homozygous dominant condition ($H_1H_1H_2H_2$), C-104 is homozygous recessive at the second locus ($H_1H_1h_2h_2$) and the resistant parents (WR-315, CPS-1, BG-212 and P-436-2) are homozygous recessive at both loci ($h_1h_1h_2h_2$). They also identified another late wilting genotype K-850.

More complex pattern of inheritance of resistance to race 1 of Fusarium wilt of chickpea was reported by Singh *et al.* (1987a). They found that at least three independent *loci* controlled resistance to race 1, designated H_1 , H_2 and H_3 . Partially recessive alleles (degree of dominance=0.6) in homozygous form at either of the first two loci as found in cultivars K-850 ($h_1h_1H_2H_2h_3h_3$) and C-104 ($H_1H_1h_2h_2h_3h_3$) respectively

and the dominant allele at the third locus in cultivars H-208 ($H_1H_1H_2H_2H_3H_3$) delay wilting but any two of these alleles together confer complete resistance (Table 3). Further Singh *et al.* (1987b) studied the reaction of parents, F_1 and F_2 generations of crosses of chickpea cultivars K-850 with C-104 and JG-62 and F_3 progenies of K-850 x C-104 to race 1 of *Fusarium oxysporum* f.sp. *ciceri*. They obtained a monogenic ratio from the cross K-850 with JG-62 and digenic ratio from the K-850 x C-104 cross. This revealed that K-850 carried a recessive allele for resistance at a locus different and independent of that carried by C-104 and recessive alleles at both *loci* together confer complete resistance.

Singh *et al.* (1991) studied F_1 and F_2 generations of the two crosses H-208 x K-850 and H-208 x C-104. They indicated that cultivar H-208 carried a dominant allele for late wilting to race 1 of *Fusarium oxysporum* f.sp. *ciceri* at a locus different from the two reported earlier. Anupama (2001) reported two recessive genes under homozygous condition for resistance to race 1 of *Fusarium* wilt in a cross between ICCV-2 ($h_1h_1h_2h_2h_3h_3$) and JG-62 ($H_1H_1H_2H_2h_3h_3$). The digenic ratio of 9:6:1 obtained in F_2 generation was confirmed by the F_3 progeny and RIL population. Recently Girase and Deshmukh (2002) also obtained trigenic ratio in two crosses *viz.*, JG-62 x ICC-4958 and JG-62 x Vijay for *Fusarium* wilt resistance.

Race 2 of *Fusarium oxysporum* was reported by Haware and Nene (1982). A good number of reports on the inheritance of resistance to *Fusarium* wilt race 1 (ICRISAT isolate) in chickpea are available. Very little information is available regarding race 2 (Kanpur isolate) and other races of *Fusarium* wilt. Pathak *et al.* (1975) reported that, under field

Table 3. Genetic constitution and wilt reaction of selected chickpea genotypes

Sl. No.	Cultivar	Genetic constitution	Wilt reaction (race 1)	Reference
1.	JG-62	H ₁ H ₁ H ₂ H ₂ h ₃ h ₃	Early wilting	Singh <i>et al.</i> (1987a and b); Anupama (2001)
2.	K-850	h ₁ h ₁ H ₂ H ₂ h ₃ h ₃	Late wilting	Singh <i>et al.</i> (1987a and b)
3.	C-104	H ₁ H ₁ h ₂ h ₂ h ₃ h ₃	Late wilting	Singh <i>et al.</i> (1987a and b); Mayer <i>et al.</i> (1997)
4.	H-208	H ₁ H ₁ H ₂ H ₂ H ₃ H ₃	Late wilting	Singh <i>et al.</i> (1987a); Singh <i>et al.</i> (1991)
5.	WR-315	h ₁ h ₁ h ₂ h ₂ h ₃ h ₃	Resistant	Singh <i>et al.</i> (1987a)
6.	CPS-1	h ₁ h ₁ h ₂ h ₂ h ₃ h ₃	Resistant	Singh <i>et al.</i> (1987a)
7.	P-436-2	h ₁ h ₁ h ₂ h ₂ h ₃ h ₃	Resistant	Singh <i>et al.</i> (1987a)
8.	BG-212	h ₁ h ₁ h ₂ h ₂ h ₃ h ₃	Resistant	Singh <i>et al.</i> (1987a)
9.	JG-74	h ₁ h ₁ h ₂ h ₂ h ₃ h ₃	Resistant	Singh <i>et al.</i> (1987a)
10.	ICCV-2	h ₁ h ₁ h ₂ h ₂ h ₃ h ₃	Resistant	Anupama (2001)

conditions, the resistance to race 2 is controlled by a single recessive gene. Gumber *et al.* (1995) studied the inheritance to Fusarium wilt (race 2) in chickpea in the cross P-165 x C-104. The number of early wilting and late wilting plants fitted well to 13:3 ratio suggesting that two genes governing the resistance to race 2.

Studies of Kumar (1998) with a set of three crosses *viz.*, WR-315 x C-104 (resistant x susceptible), WR-315 x K-850 (resistant x late wilter) and K-850 x GW-517 (late wilter x late wilter) indicated the involvement of the three *loci* (two recessive and one dominant alleles), homozygous recessive forms at the first two *loci* confer resistance and a dominant allele at the third locus complements the other two *loci* to late wilting whereas susceptibility occurred when first two *loci* were in dominant form.

The parents F₁, F₂ and F₃ of three crosses (JG-62 x JG-74, JG-62 x PG-114 and JG-74 x PG-114) were screened, utilizing the Pantnagar isolate (race 3) of *F. oxysporum* f.sp. *ciceri* by Dikshit and Singh (1994). Cross JG-74 x JG-62 (resistant x susceptible) showed monogenic recessive inheritance of resistance. However, PG-114 x JG-62 (resistant x susceptible) showed resistance to wilt under influence of two recessive genes. They suggested the use of bulk population breeding and backcross breeding for the development of resistant varieties.

Inheritance of resistance to race 4 of Fusarium wilt was studied by Tullu *et al.* (1998) using 100 F₅ derived F₇ recombinant inbred lines (RILs) that had been developed from the cross of breeding lines C-104 x WR-315 (late wilter x resistant). They found that the gene for resistance to race 4 segregating in 1:1 ratio as expected for single gene. Further Tullu *et al.*

(1999) performed genetic analysis on the F_1 , F_2 and F_3 families from the cross of JG-62 x Surutato-77. Results indicated that two independent genes controlling resistance to race 4 of *Fusarium* wilt of chickpea. Tekeoglu *et al.* (2000) used race 0 and race 5 to study *Fusarium* wilt resistance in F_6 derived recombinant inbred lines (RILs). A 1 resistant : 1 susceptible segregation ratio was observed for both races, indicating that resistance to each race is controlled by a single gene. They proposed the gene symbols to races 0, 4 and 5 of the pathogen, respectively. More recently a 3:1 resistant to susceptible ratio was observed in the RIL population of CA-2139 x JG-62 cross indicating the involvement two genes for resistance to race 0 of *Fusarium oxysporum* f.sp. *ciceri* of chickpea (Rubio *et al.*, 2003). Presence of either gene is sufficient for resistance.

2.2 MOLECULAR TAGGING OF DISEASE RESISTANCE GENES

Chickpea breeding aims at the improvement of the crop using gene stock already present in cultivated species or wild relatives. Lines displaying the desired traits are crossed, and the offspring tested for the presence of the desired new gene combinations in several rounds of selection from F_2 to F_8 . This, time consuming and costly process can be speeded up considerably by the use of DNA markers. These enable the breeder to characterize breeding lines, construct horizontal resistance (*i.e.*, pyramiding of resistance genes) and detect the presence of recessive genes in the heterozygous state (Winter, 1997). All this makes the development of DNA markers compliment for a successful crop management.

The process of locating genes of interest via linkage to markers is referred as gene tagging (Sharma, 2003). The basic requirements of tagging plant disease resistance genes with molecular markers are the development of a test population and availability of genetic marker. Different test populations have been employed for tagging genes in chickpea which are presented below.

2.2.1 Test populations

2.2.1.1 Segregating populations

Traditional analysis of F_2 population derived from true F_1 hybrid or back cross populations are most commonly used for mapping purposes. The major limitation to these populations is that they are ephemeral because we can not get true breeding seeds by selfing individuals from these populations and as a result, the usefulness of the F_2 and BC populations would be limited to one grow out and under a single environment (Soller and Beckmann, 1990; Young, 1994). The other limitation of using F_2 is the need to score the entire population for each marker and phenotyping for soil borne diseases is not possible where susceptible plants die before flowering or producing seeds.

Bulked segregant analysis (BSA) is an alternative method to overcome problems associated with F_2 population (Michelmore *et al.*, 1991). In this method, polymorphism survey is conducted in the DNA bulks made from resistant and susceptible individual homozygous plants, which differ genetically for the regions containing resistant genes. The resistance genes tightly linked to the markers produce polymorphic band between resistant and susceptible bulks. While, unlinked regions give rise

to monomorphic banding pattern. Further confirmation of the linkage is necessary by screening the population from which the bulk DNA was derived using the markers which show polymorphisms. Michelmore *et al.* (1991) identified three RAPD markers linked to downy mildew resistance genes in lettuce. For the first time, Mayer *et al.* (1997) used BSA to identify RAPD markers linked to the h_1 locus of *Fusarium* wilt (race 1) resistance in chickpea.

2.2.1.2 Homozygous populations : Recombinant inbred lines

Population of recombinant inbred lines (RILs) derived by single seed decent of F_2 plants to the F_6 or F_7 has been proposed as a means of establishing permanent mapping populations (Burr *et al.*, 1988; Burr and Burr, 1989). In developing RILs, it is expected that each line contains a different combination of linkage blocks from the original parents due to several rounds of meiosis and that these differing linkage blocks in individual RILs serve as a basis for linkage analysis.

Thus, RILs represent stable homozygous lines, which can be scored for the parental alleles and to determine recombination frequencies.

Gene maps derived from RILs show a higher resolution of closely linked *loci* as compared to F_2 populations (Burr and Burr, 1989). Mapping with RILs began in plants when Domoney *et al.* (1986) used them to map protein genes in peas. Later, Burr *et al.* (1988) in maize and Tahir (1990) in lentil used RILs to construct linkage groups.

Since several measurements of quantitative traits can be made on the inbred lines at multilocations, they are better suited for QTL mapping

when compared to the restriction of single environments in the case of F_2 populations. Additional mapping data can easily be added to the data set to expand the linkage map. To date, RILs are the most extensively used population for tagging of disease resistance genes in chickpea. RILs obtained through the crossing of different parents have been used for identification of RAPD markers linked with genes for resistance to race 1, 4, 5 and 0 of *Fusarium* wilt (Mayer *et al.*, 1997; Ratnaparkhe *et al.*, 1998a and b; Tullu *et al.*, 1998, 1999; Tekeoglu *et al.*, 2000; Winter *et al.*, 2000; Rubio *et al.*, 2003).

Near isogenic lines (NILs) and doubled haploid lines (DHLs) are the other two homozygous populations. There are no reports of using these populations for tagging of resistance genes in chickpea so far. Rajesh *et al.* (2002) used NILs for identification of an STMS (sequence tagged microsatellite) marker for the double-podding gene in chickpea. One of the limitations of using the NILs is that only one trait can be mapped.

2.2.2 Genetic markers

Any characteristic that is useful to distinguish the plant varieties is known as a marker. These genetic markers are useful in identifying or tagging of economically important traits. Genetic markers are broadly classified into morphological, biochemical and molecular markers.

2.2.2.1 Morphological markers

The morphological markers are usually observable characters, which differ between the selected parents. Characters such as leaf size and type, growth habit, flower colour, seed coat colouration and podding

are some morphological markers. Morphological traits such as leaf and pod type and plant habit exhibit monogenic inheritance and could be good genetic markers (Muehlbauer and Singh, 1987). However, flower colour and number of pods per node segregate independently with the Fusarium wilt resistance in chickpea (Singh *et al.*, 1987a and 1988; Rajesh *et al.*, 2002). On the other hand, morphological markers have major limitations in that they are limited in number, may have undesirable effects on phenotype, show dominance recessive interaction and may not be environmentally neutral (Keim *et al.*, 1990; Dudley, 1993).

2.2.2.2 Biochemical markers

Isozymes are the biochemical markers, which are various forms of the same enzyme differing in the electrophoretic mobility. Markert and Moller (1959) studied isozyme analysis for the first time. The alternate products known as allozymes encode these variant electrophoromorphs. Isozyme markers can be used to assess variability in plant population to develop isozyme fingerprints to identify cultivars and to locate and tag disease resistant genes (Weeden *et al.*, 1988). In peas, the dominant gene controlling resistance to race 1 of *F. oxysporum* FW, is linked to a seed esterase *Est-S* (Hunt and Barnes, 1982). Unfortunately, *Cicer arietinum* exhibits lower polymorphisms at isozyme *loci* (Gaur and Slinkard, 1990). This was also observed from the studies of Oram *et al.* (1987) and Tuwafe *et al.* (1988).

2.2.2.3 Molecular markers

DNA markers are ubiquitous, innumerable, discrete, non-deleterious, inherited by Mendelian laws, unaffected by environment and

free from epistatic interactions (Soller and Beckmann, 1990; Tanksley *et al.*, 1992). A major break through occurred when it was realised that genetic maps could be constructed using pieces of chromosomal DNA as direct markers for segregation pattern of chromosomal segments (Botstein *et al.*, 1980).

A potentially much larger number of markers can be resolved by the means of Restriction Fragment Length Polymorphisms (RFLPs). DNA regions affected by mutational events will have different size distributions of DNA restriction fragments generated by endonuclease digestion compared to regions unaffected by this event. This yields Restriction Fragment Length Polymorphism (RFLP) (Botstein *et al.*, 1980).

RFLP markers have been used to locate the genes for economically important traits such as resistance to downy mildew in lettuce (Michelmore *et al.*, 1994), dwarf mosaic virus in maize (McMullen and Louie, 1989), powdery mildew in barley (Hinze *et al.*, 1991) and leaf blast in rice (Yu *et al.*, 1991). However, only seven RFLPs have been identified and added to the chickpea genetic map (Simon and Muehlbauer, 1997).

Random amplified polymorphic DNA (RAPD) represents another method for identifying additional genetic variation within populations. Two groups, Williams *et al.* (1990) at DuPont company and Welsh and McClelland (1990) at the California Institute of Biological Research independently and simultaneously developed a random amplified polymorphic DNA (RAPD) assay based on the amplification of random DNA segments with single, short, synthetic oligonucleotide primers of arbitrary nucleotide sequence (about 10 bp); the latter group called their

assay Arbitrarily-Primed Polymerase Chain Reaction (AP-PCR). The former described genetic mapping applications while the later focused on genome fingerprinting.

RAPDs have been shown to be useful for DNA fingerprinting (Welsh and McClelland, 1990; Williams *et al.*, 1990; Caetano-Anolles *et al.*, 1991), detection of genetic polymorphism and varietal identification (Hu and Quiros, 1991) and mapping (Williams *et al.*, 1990; Paran and Michelmore, 1993) in different crop plants.

RAPD markers have been used to tag the disease resistance genes such as resistance to downy mildew in lettuce (Michelmore *et al.*, 1991), stem rust resistance gene *Pg3* in oats (Penner *et al.*, 1993) gene for resistance to *Pseudomonas syringe* pv. *tomato* in tomato (Klein-Lankhorst *et al.*, 1991; Martin *et al.*, 1991) and most recently Fusarium wilt resistance genes in chickpea (Mayer *et al.*, 1997; Ratnaparkhe *et al.*, 1998a, Tullu *et al.*, 1998, 1999; Rubio *et al.*, 2003).

AFLP (Amplification Fragment Length Polymorphism), STMS (Sequence Tagged Microsatellite Sites), ISSR (Inter Simple Sequence Repeats), RAMPO (Random Amplified Microsatellite Polymorphism), SCARs (Sequence Characterized Amplified Regions) and CAPS (Cleaved Amplified Polymorphic Sequence) are the list of other useful molecular markers.

2.2.3 Molecular markers in chickpea

A major goal of breeding and marker assisted selection in chickpea is the tagging of genes controlling resistance to the most aggressive fungal

pathogens *viz.*, *Ascochyta* and *Fusarium*. Molecular markers were also used for phylogenetic analysis, diversity and tagging of double podding in chickpea.

2.2.3.1 Disease resistance

Among 115 pathogens that affect chickpea, *Fusarium oxysporum* and *Ascochyta rabiei* which cause wilt and blight respectively are the most destructive and wide spread. Several studies have been conducted to tag resistance to these diseases using molecular markers which have been reviewed here.

2.2.3.1.1 Fusarium wilt

In bulk segregant analysis of selected F₃ plants of a intraspecific cross (C-104 x WR-315), Mayer *et al.* (1997) identified two primers (UBC-170 and CS-27) that produced random amplified polymorphic DNA (RAPD) markers associated with *Fusarium* wilt race 1 resistance. Analysis of the RILs with these primers yielded an estimate of 7 per cent recombination between the two markers and the locus for wilt resistance and 6 per cent recombination between the *loci* corresponding to the two RAPD markers. After cloning and sequencing they designed two allele specific associated primers (ASAPs), CS-27₇₀₀ and UBC-170₅₅₀. CS-27 amplified a fragment linked to the allele for susceptibility to race 1 of *Fusarium* wilt. Whereas UBC 170 produced a single band for both resistant and susceptible genotypes, thus demonstrating locus specificity rather than allele specificity. Further analysis with additional markers placed the two *loci* near one end of linkage group VI on the linkage map of Simon and Muehlbauer (1997).

The gene for resistance to *Fusarium oxysporum* f. sp. *ciceri* race 4 segregated in 1:1 ratio expected for single gene and was located 9 map units from race 4 resistance locus and was on the same side of the resistance gene in the F₅ derived F₇ RILs that had been developed from the cross of breeding lines C-104 x WR-315 (Tullu *et al.*, 1998). In the interspecific cross RILs Ratnaparkhe *et al.* (1998b) identified ISSR marker UBC-855₅₀₀ that was found to be linked in repulsion with the race 4 of *Fusarium* wilt resistance gene at a distance of 5.2 cM. Location of UBC-855₅₇₀ at a distance of 0.6 cM from CS-27₇₀₀ indicated that genes for resistance to *Fusarium* wilt race 1 and 4 are closely linked. This ISSR marker was mapped to the linkage group VI of *Cicer* genome. Tullu *et al.* (1999) further confirmed the linkage of CS-27₇₀₀ with the *Fusarium* wilt pathogen race 4 in F₁, F₂ and F₃ families of JG-62 x Surutato-77 cross.

Linkage analysis in 131 F₆ derived RILs of an interspecific cross indicated that the genes for resistance to race 4 (*foc-4*) and race 5 (*foc-5*) were in same linkage group and were separated by 11.2 centi Morgans (Tekeoglu *et al.*, 2000). In addition CS-27₇₀₀ was located between the two resistance genes and was 7.2 and 4 cM from the genes for resistance to race 4 and 5 respectively. Winter *et al.* (2000) observed 3.7 and 18 cM distances between CS-27 and genes for resistance to races 4 and 5 respectively and mapped to linkage group II. This suggests that linkage group VI of Simon and Muehlbauer (1997) is same as linkage group II of Winter *et al.* (2000).

Recently Rubio *et al.* (2003) identified a RAPD marker OPJ-20₆₀₀ linked with the resistance gene of *Fusarium* wilt race 0 of chickpea. The distance between marker and resistant locus was 6 cM. Till date there are

no markers identified for other three races (race 2, 3 and 6) of *Fusarium* wilt. The identified tags for resistance to different races will be useful in gene introgression.

2.2.3.1.2 Ascochyta blight

Ascochyta blight, a wide spread foliar disease is predominant in WANA (West Asia and North Africa) countries and restricted only by low annual rainfall and elevated temperatures (Singh *et al.*, 1992). The genetics of resistance to Ascochyta blight in chickpea seems to be complex and does not fit a simple gene-for-gene interaction. Moreover, Santra *et al.* (2000) provided evidence that at least three quantitative trait loci (QTLs) control Ascochyta blight resistance in chickpea. Galvez *et al.* (2002) identified seven QTLs conditioning Ascochyta blight resistance.

2.2.3.2 Phylogenetic analysis

RAPD markers were used to distinguish between nine different *Cicer* taxa representing the cultivated chickpea and eight other related annual wild species (Ahmad, 1999). A total of 115 reproducibly scorable RAPD markers were generated. All except 14 were polymorphic and these were utilized to deduce genetic relationships among the annual *Cicer* species. Four distinct clusters were observed and represented *C. arietinum*, *C. reticulatum* and *C. echinospermum* in first cluster followed by *C. chorassanicum* and *C. yamashitae* in second cluster, while *C. pinnatifidum*, *C. judiacum* and *C. bijugum* formed the third cluster. *Cicer cunneatum* did not cluster with any of the species and was most distantly placed from the cultivated species.

Phylogenetic analysis of microsatellite polymorphism [(TAA)_n] showed no relationship between accession and geographic origin, which is compatible with the recent expansion of this crop throughout the world (Udupa *et al.*, 1999). A phylogenetic tree based on the conservation of 90 microsatellite flanking sequences revealed that chickpea and the other members of its crossability group were more closely related to the perennial *C. anatolicum* than to other annual species of the genus (Choumane *et al.*, 2000). More recently ISSR markers have been used to study phylogenetic relation between cultivated, annual and perennial wild species of *Cicer* (Iruela *et al.*, 2002; Rajesh *et al.*, 2003). These studies indicated the possibility of gene flow between *Cicer reticulatum* and *Cicer echinospermum*.

2.2.3.3 Genetic diversity

Analysis of genetic diversity in chickpea using RFLP technique has shown little molecular diversity (Udupa *et al.*, 1993). Alternatively, RAPD markers are frequently used for fingerprinting due to simplicity of this technique (Winter and Kahl, 1995). Banerjee *et al.* (1998) used RAMPO analysis to study genetic diversity in five chickpea accessions. The combinations of RAPD analysis and probing with microsatellite repeats (TG)₁₀, (GATA)₄ and (CAC)₅ have revealed polymorphic and genotype specific unique DNA banding patterns.

In India, Sant *et al.* (1999) used RAPD and microsatellites to study genetic diversity in 29 elite Indian chickpea genotypes. Among the various microsatellites (AAC)₅, (ACT)₅, (AAG)₅ and (GATA)₄ were able to differentiate all 29 chickpea cultivars.

2.2.3.4 Double podding

A gene that confers double podding with the symbol “S” is considered important for breeding higher yielding cultivars in chickpea. Out of 400 RAPD, 100 STMS and 100 ISSR markers, one STMS marker, TA-80 was polymorphic and is placed 4.84 cM away from double podding gene *S* (Rajesh *et al.*, 2002).

2.3 GENETIC VARIABILITY AND CHARACTER ASSOCIATION

A thorough understanding of the genetic variation for different traits, their heritability and relationship with yield is important for any crop improvement programme to be successful.

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, actual genetic variation, heritable and the genetic advance that can be achieved through selection.

The magnitude of variability, heritability and genetic advance differed depending on the material used. However, the majority of workers have reported high variability, heritability and genetic advance for most of the characters studied. The information on variability, heritability and genetic advance is summarized in Table 4.

Table 4. Summary of review on variability, heritability and genetic advance for various traits in chickpea

Character		Reference		
		Variability	h^2 (bs)	GA
Plant height at harvest	High	Chandra (1968), Bharadwaj and Singh (1980), Kumar <i>et al.</i> (1981), Singh <i>et al.</i> (1990), Sandhu <i>et al.</i> (1991), Dasgupta <i>et al.</i> (1992), Bhatia <i>et al.</i> (1993), Rao <i>et al.</i> (1994), Naseem <i>et al.</i> (1995), Singh <i>et al.</i> (1995)	Chandra (1968), Sharma <i>et al.</i> (1990), Mishra (1991), Chavan <i>et al.</i> (1994), Mishra <i>et al.</i> (1994), Rao <i>et al.</i> (1994), Patil (1996)	Mandal and Bahl (1983), Dumbre <i>et al.</i> (1984), Agarwal (1986), Rao <i>et al.</i> (1994), Patil (1996)
	Moderate	Arora (1991), Shiraiwa and Hashikawa (1995), Patil (1996)	Chand <i>et al.</i> (1975), Esser (1976), Mandal (1977), Rastogi and Singh (1977), Sharma <i>et al.</i> (1989), Dasgupta <i>et al.</i> (1992)	Chavan <i>et al.</i> (1994), Gaded (1996)
	Low	Pushpa <i>et al.</i> (1993), Mishra <i>et al.</i> (1994)	-	-
Number of primary branches at harvest	High	Kumar <i>et al.</i> (1981), Mandal and Bahl (1983), Singh <i>et al.</i> (1990), Singh and Rao (1991), Dasgupta <i>et al.</i> (1992), Chavan <i>et al.</i> (1994), Patil (1996)	Mishra (1991), Chavan <i>et al.</i> (1994)	Mishra (1991), Chavan <i>et al.</i> (1994), Rao <i>et al.</i> (1994), Patil (1996)
	Moderate	Arora (1991)	Patil (1996)	-
	Low	-	Singh and Rao (1991)	-

Table 4. Contd.....

Character	References		
	Variability	$h^2(bs)$	GA
100 seed weight	Filippetti (1990), Salimath and Patil (1990), Sharma <i>et al.</i> (1990), Singh <i>et al.</i> (1990), Arora (1991), Kumar <i>et al.</i> (1991), Singh and Rao (1991), Bhatia <i>et al.</i> (1993), Maynez <i>et al.</i> (1993), Bhoyte <i>et al.</i> (1994), Rao <i>et al.</i> (1994), Shiraiwa and Hashikawa (1995), Patil (1996)	Salimath and Bahl (1985), Salimath and Patil (1990), Sharma <i>et al.</i> (1990), Kumar <i>et al.</i> (1991), Sandhu <i>et al.</i> (1991), Singh and Rao (1991), Dasagupta <i>et al.</i> (1992), Chavan <i>et al.</i> (1994), Jahagirdar <i>et al.</i> (1994), Mishra <i>et al.</i> (1994), Rao <i>et al.</i> (1994), Kumar and Singh (1995), Patil (1996)	Sharma <i>et al.</i> (1990), Kumar <i>et al.</i> (1991), Jahagirdar <i>et al.</i> (1994), Rao <i>et al.</i> (1994), Kumar and Singh (1995), Patil (1996)
	-	Chand <i>et al.</i> (1975), Rastogi and Singh (1977)	Agarwal (1986), Mishra (1991)
	-	-	Arora (1991), Mishra (1991), Wanjari <i>et al.</i> (1996)
Days to 50% flowering	Agarwal (1985)	Agarwal (1985), Jahagirdar <i>et al.</i> (1994)	Agarwal (1985), Jahagirdar <i>et al.</i> (1994)
	Dasagupta <i>et al.</i> (1992), Rao <i>et al.</i> (1994)	Mishra (1991), Jahagirdar <i>et al.</i> (1994)	-

Table 4. Contd.....

Character		Variability	References		GA
Number of pods per plant	High	Filippetti (1990), Arora (1991), Kumar <i>et al.</i> (1991), Sandhu <i>et al.</i> (1991), Dasagupta <i>et al.</i> (1992), Bhatia <i>et al.</i> (1993), Chavan <i>et al.</i> (1994), Jahagirdar <i>et al.</i> (1994), Mishra <i>et al.</i> (1994), Rao <i>et al.</i> (1994), Shiraiwa and Hashikawa (1995), Patil (1996)	Mishra (1991), Kumar <i>et al.</i> (1991), Dasagupta <i>et al.</i> (1992), Chavan <i>et al.</i> (1994), Jahagirdar <i>et al.</i> (1994), Mishra <i>et al.</i> (1994), Rao <i>et al.</i> (1994), Shiraiwa and Hashikawa (1995), Patil (1996)		Kumar <i>et al.</i> (1991), Mishra (1991), Chavan <i>et al.</i> (1994), Jahagirdar <i>et al.</i> (1994), Mishra <i>et al.</i> (1994), Rao <i>et al.</i> (1994), Patil (1996)
	Moderate	Dasagupta <i>et al.</i> (1992), Rao <i>et al.</i> (1994)	Mandal (1977), Salimath and Bahl (1985)		-
Seed yield per plant	Low	Patil and Phadnis (1977), Islam <i>et al.</i> (1984), Singh <i>et al.</i> (1990)	Chandra (1968), Rao <i>et al.</i> (1994)		-
	High	Filippetti (1990), Patil (1990), Arora (1991), Sandhu <i>et al.</i> (1991), Singh and Rao (1991), Dasagupta <i>et al.</i> (1992), Bhatia <i>et al.</i> (1993), Jirali <i>et al.</i> (1994), Rao <i>et al.</i> (1994), Srivastava and Jain (1994), Naseem <i>et al.</i> (1995), Shiraiwa and Hashikawa (1995), Singh <i>et al.</i> (1995), Wanjari <i>et al.</i> (1996)	Mishra <i>et al.</i> (1988), Sandhu <i>et al.</i> (1989), Singh and Rao (1991), Chavan <i>et al.</i> (1994), Jahagirdar <i>et al.</i> (1994), Patil (1996)		Mishra <i>et al.</i> (1988), Dasgupta <i>et al.</i> (1992), Chavan <i>et al.</i> (1994), Jahagirdar <i>et al.</i> (1994), Rao <i>et al.</i> (1994), Patil (1996)
	Moderate	Singh <i>et al.</i> (1990)	Mishra (1991), Wanjari <i>et al.</i> (1996)		Mandal and Bahl (1983), Mishra (1991)
	Low	-	Salimath and Patil (1990), Rao <i>et al.</i> (1994), Wanjari <i>et al.</i> (1996)		Wanjari <i>et al.</i> (1996)

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 5.

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.

Bahl *et al.* (1976) observed high direct effect of 100 seed weight on seed yield. While, Singh *et al.* (1976) reported that the pods per plant had the largest positive direct effect on grain yield and is the major factor contributing to the grain yield. They also reported that, the number of branches per plant and the number of seeds per pod in combination with the pods per plant were also important for improving yield. Katiyar *et al.* (1977) and Jatasra *et al.* (1978) observed 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.

Maximum direct effect of pods per plant on seed yield was observed by Tomar *et al.* (1982) as compared to other characters. However, 100

Table 5. Summary of review on association pattern of yield component traits with seed yield in chickpea

Character	Correlation	Reference
Plant height at harvest	Positive	Yadav (1990), Mishra (1991), Shinde and Saraf (1991), Roshan <i>et al.</i> (1993), Bhambota <i>et al.</i> (1994), Naseem <i>et al.</i> (1995), Rana <i>et al.</i> (1995), Rao (1998)
	Negative	Govil (1980), Salimath and Patil (1990)
Number of primary branches at harvest	Positive	Geletu <i>et al.</i> (1995), Rana <i>et al.</i> (1995), Singh <i>et al.</i> (1995), Patil (1996), Rao (1998)
	Negative	Singh <i>et al.</i> (1995), Patil (1996)
Number of pods per plant	Positive	Filippetti (1990), Yadav (1990), Kharat <i>et al.</i> (1991), Mishra (1991), Sandhu <i>et al.</i> (1991), Shinde and Saraf (1991), Dasagupta <i>et al.</i> (1992), Bhatia <i>et al.</i> (1993), Roshan <i>et al.</i> (1993), Bhambota <i>et al.</i> (1994), Bhoyte <i>et al.</i> (1994), Chavan <i>et al.</i> (1994), Mishra <i>et al.</i> (1994), Rao <i>et al.</i> (1994), Naseem <i>et al.</i> (1995), Singh <i>et al.</i> (1995), Patil (1996), Rao (1998), Berger and Turner (2000)
100 seed weight	Positive	Salimath and Bahl (1986), Mishra (1991), Shinde and Saraf (1991), Dasagupta <i>et al.</i> (1992), Bhatia <i>et al.</i> (1993), Bhoyte <i>et al.</i> (1994), Chavan <i>et al.</i> (1994), Jirali <i>et al.</i> (1994), Mishra <i>et al.</i> (1994), Naseem <i>et al.</i> (1995), Singh <i>et al.</i> (1995), Patil (1996), Rao (1998)
	Negative	Chand <i>et al.</i> (1975), Rastogi and Singh (1977), Sindhu and Prasad (1987), Filippetti (1990), Roshan <i>et al.</i> (1993)
Days to 50% flowering	Positive	Paliwal <i>et al.</i> (1987), Mishra (1991), Chavan <i>et al.</i> (1994)

seed weight and number of primary branches were the other components exerting a substantial direct effect on seed yield per plant. They also observed that, indirect effect of these traits were also positive as compared to direct effect of other traits. Singh and Paroda (1986) also found that, pods per plant had the largest positive direct effect on grain yield.

Seeds per plant and 100 seed weight showed maximum direct and positive contribution towards seed yield (Ram *et al.*, 1980; Reddy and Rao, 1988; Gupta *et al.*, 1989). A similar high positive direct effect of seeds per plant followed by 100 seed weight, indicating that these characters are most promising for increasing yield potential in breeding programme was reported by Yadav (1990).

Jivani and Yadavendra (1988) suggested that branches per plant, pods per plant and seed weight should be given prime importance in indirect selection for increased yield owing to their greater direct effects on yield. Sharma and Maloo (1988) showed that pod number was the character to have greatest influence on seed yield followed by primary branches. Bhambota *et al.* (1994) reported that pods per plant and plant height had considerable positive direct effect on seed yield. Further, the number of branches had a negative direct effect on yield but a positive indirect effect via pods per plant. Sarvaliya and Goyal (1994) found high direct effect of pod number per plant and 100 seed weight on seed yield. Chavan *et al.* (1994) concluded that branches per plant, pods per plant should be used as selection criteria for yield improvement. Singh *et al.* (1995) found high direct effect of biomass per plant followed by 100 seed weight on seed yield.

Bhattacharya *et al.* (1995) concluded that harvest index and days to 50 per cent flowering influence seed yield greatly under moisture stress. Arora and Jeena (1999) in a study of path analysis in 43 genotypes indicated that plant height, pods per plant were important characters in selection for seed yield. Rao and Kumar (2000) found that days to 50 per cent flowering, duration of reproductive phase had positive direct effect on yield while plant height, days to maturity, 100 seed weight had negative direct effect.

Study of path analysis in 34 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 50 per cent flowering had negative direct effect (Singh *et al.*, 2001). The indirect effects of characters *viz.*, number of branches, 100 seed weight were also much appreciable indicating that these characters played an important role in determining yield. In short, the review given here reveals pod number per plant as the most important character in selection for yield owing to its high direct correlation with seed yield.

Material and Methods

III. MATERIAL AND METHODS

The present study was undertaken to identify RAPD markers associated with H₂ locus governing resistance to race 1 of *Fusarium oxysporum* f. sp. *ciceri* in chickpea. The details of the experiments conducted in the present study are given under the following heads.

1. Development and evaluation of RILs for wilt reaction
2. RAPD analysis of parental genotypes
3. Association of RAPD markers with wilt reaction
4. Genetic variability studies in RILs for seed yield and yield components

The material and methods employed in these experiments are given below.

3.1 EXPERIMENTAL MATERIAL

Two parental genotypes K-850, a late wilter (h₁h₁ H₂H₂ h₃h₃) and WR-315, a wilt resistant (h₁h₁ h₂h₂ h₃h₃) differing in respect of H₂ locus were selected for this study.

3.2 EXPERIMENTAL SITE

All the experiments except field screening of RILs for wilt reaction were conducted at the department of Genetics and Plant Breeding, College of Agriculture, Dharwad, Karnataka. Dharwad is located at a latitude of 15° 31' N and longitude of 75° 07' E at an altitude of 678 m above msl and the soil type was black. The weather parameters prevailed at College

of Agriculture, UAS, Dharwad, Karnataka during evaluation of RILs for morphological traits (2002 *Rabi*) are presented in Table 6.

Field screening of RILs for wilt reaction was carried out in wilt sick plots at International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru near Hyderabad, situated at an altitude of 545m above msl and at a latitude of 17° 32' N and longitude of 78° 16' E.

3.3 EXPERIMENTAL DETAILS

3.3.1 Development and evaluation of RILs for wilt reaction

3.3.1.1 Development of RILs

A set of recombinant inbred lines (RILs) derived from the cross K-850 X WR-315 was used as a test population for tagging of H₂ locus. The RILs were produced at the College of Agriculture, Dharwad, by following single seed descent method (with a seed from each segregating plant contributing to SSD bulk from F₂ onwards). The generations of the cross were rapidly advanced with no intentional selection utilizing all the three seasons in a year (Plate 1).

The late wilting genotype K-850 was crossed manually to resistant genotype WR-315 to produce hybrid seeds. All the F₁ plants were space planted and true F₁s were identified and harvested. One Seed from each F₂ plant was harvested to generate SSD bulk population. The same procedure was continued to advance the bulk through generations up to F₅. In all the generations the nurseries were protected from the infestation of wilt, rot, pod borer and other pests and diseases. The seed from each of 178 plants harvested separately in F₅ to produce 178 F₆ recombinant

Table 6. Mean monthly meteorological data during 2002 at Main Agricultural Research Station, University of Agricultural Sciences, Dharwad

Month	Relative humidity (%)	Maximum temperature (°C)	Minimum temperature (°C)	Rainfall (mm) in 2002	Average rainfall (1950-1994)
January	55	30.1	14.5	0.0	0.0
February	50	31.9	17.9	62.7	0.0
March	49	36.0	20.0	0.0	7.0
April	53	37.2	21.7	67.0	50.0
May	62	34.9	23.0	57.9	90.0
June	80	29.6	21.9	60.5	112.0
July	79	28.4	21.3	15.0	158.0
August	82	26.6	20.5	48.1	102.0
September	73	29.8	20.0	6.6	105.0
October	67	30.7	20.4	103.6	137.0
November	62	30.5	17.1	7.0	34.0
December	47	30.2	14.4	0.0	6.0
Annual	63	31.3	19.4	428.4	801.0



K - 850

X



WR-315

F₁

***Rabi* 2000**



F₂

Summer 2001



F₃

***Kharif* 2001**



F₄

***Rabi* 2001**



F₅

***Kharif* 2002**



F₆

***Rabi* 2002**

Plate1: Schematic representation of development of Recombinant Inbred Lines (RILs) in chickpea

inbred lines (RILs), which were utilized for tagging of H₂ locus governing fusarium wilt resistance.

3.3.1.2 Evaluation of RILs for wilt reaction

The RILs and parental genotypes along with early wilting check JG-62 were evaluated in wilt sick plot at ICRISAT, Patancheru, Hyderabad. The wilt sick plot maintained at ICRISAT has been considered as standard for evaluation of chickpea wilt (Haware *et al.*, 1992). All 178 F₆ RILs were planted on 20th October 2002 in single rows of 4 m length and parents were sown in three rows of same row length in wilt sick plot on conserved moisture. The susceptible early wilting genotype JG-62 was sown after every three rows as an early wilting check and also source of inoculum. After every 20 rows resistant cultivar WR-315 was planted. All the plants of JG-62 in all the rows showed wilting within 20 days indicating early wilting and also uniform distribution of inoculum of *Fusarium oxysporum* throughout the experimental block. None of the RILs showed early wilting.

The following observations were recorded in wilt sick plot evaluation.

- i) Germination and number of plants per row
- ii) Number of wilted plants

The first observation on late wilting was recorded on 37th day and second on 62nd day (Haware *et al.*, 1992). During observation, dead plants were uprooted and examined. Only those showing characteristic typical vascular wilt were recorded as death due to pathogen.

Based on the number of cumulative dead plants due to wilt infestation in each RIL, per cent wilt was calculated. RILs showing zero to thirty per cent wilted plants were considered as resistant, 31 to 49 per cent as moderate (segregating) and more than or equal to 50 per cent as susceptible (Haware and Nene, 1982; Phillips, 1988; Jimenez-Diaz *et al.*, 1989b). RILs which showed resistant or susceptible (Late wilting) reaction were considered as homozygous lines and taken for further analysis (Plate 2).

3.3.1.3 Confirmation in pot culture

The results obtained on wilt reaction of RILs in wilt sick plot were further confirmed in pot culture experiments under controlled green house conditions at the department of Genetics and Plant Breeding, UAS, Dharwad, by randomly selecting forty four recombinant inbred lines. The pathogen *Fusarium oxysporum* race 1 was obtained from ICRISAT, Hyderabad which is the most prevalent race in India. The fungus culture derived from a single spore, was maintained on Potato Dextrose Agar (PDA) in petriplates. The pathogen was mass multiplied on sand-maize meal mixture (Abeygunawarlana and Wood, 1957) in a ratio of 95:5 w/w and watered to 20 per cent by volume. Each of the 500 ml conical flasks were filled with 200 g of the mixture and sterilized. A mycelial disc of five mm diameter cut by sterile cork borer from the margin of an actively growing culture was transferred aseptically to each flask. The flasks were incubated at $27 \pm 1^{\circ}$ C for 20 days by shaking periodically in order to permit uniform growth.

One hundred grams of the mass multiplied inoculum was thoroughly mixed with three kg of an autoclaved pot mixture for each pot



K-850 (Late wilting)



Recombinant inbred lines



Recombinant inbred lines



Recombinant inbred lines

Plate 2: Testing for wilt reaction of parental genotypes and RILs of chickpea in wilt sick plot (ICRISAT)

of 30 cm diameter. The susceptible cultivar JG-62 was grown in the pots (5 to 10 seeds) and allowed to wilt. After the plants had wilted, the plant debris were incorporated in to the pot mixture. Three seeds were sown in each pot and five pots per genotype were used for each of the 44 selected RILs, parents and check. After germination only one seedling was retained and maintained by supplying micronutrient solution (Hoagland and Arnon, 1950) every week. First observation was recorded after JG-62 started wilting and continued until the end of the experiment. None of the RILs showed early wilting along with JG-62. The RILs with all dead plants were considered as susceptible while the RILs with the healthy plants were taken as resistant and compared with field reaction (Plate 3).

3.3.2 RAPD ANALYSIS OF PARENTAL GENOTYPES

The two parents K-850 (late wilter) and WR-315 (resistant) were used to identify primers showing polymorphism. The markers, which exhibited polymorphism between the parents, were used for bulk segregant analysis.

3.3.2.1 DNA extraction

The DNA was extracted from the parental genotypes by following CTAB extraction method (Saghai-Maroo *et al.*, 1994) with few modifications.

1. One to two grams of fresh young leaf from shoot apex was harvested. The sample was crushed in liquid nitrogen in a precooled pestle and mortar to make fine powder.



JG-62 (Early wilting)



K-850 (Late wilting)



WR-315 (Resistant)



**Testing for wilt reaction of RILs in
pot culture**

**Plate 3: Testing for wilt reaction of parental genotypes and RILs of
chickpea in pot culture**

2. The powder was transferred into a sterile polypropylene tube containing 15 ml extraction buffer (2-3% w/v CTAB, 1.4 M NaCl, 20mM EDTA, 100 mM Tris-HCl, pH 8, 0.03% β mercapto ethanol).
3. The samples were incubated at 60°C for 30 minutes by mixing gently after every 15 minutes.
4. 15 ml of chloroform-isoamyl alcohol mixture (24:1 v/v) was added to the tube containing the sample and extraction buffer.
5. The solution was shaken gently for 10 minutes at room temperature.
6. The solution was centrifuged for 10 minutes at room temperature at 6000 rpm.
7. Step four was repeated if the upper layer did not look clear.
8. Final aqueous phase was transferred to a new sterile tube and DNA precipitated with 0.7 volume of cold iso-propanol. DNA was hooked out but if DNA precipitate was not seen, the solution was centrifuged for 15 minutes at 6000 rpm.
9. The supernatant was removed and the pellet was washed once in 2ml of 70 per cent ethanol and then air-dried for 10 to 15 minutes.
10. DNA was suspended in 500 to 700 μ l high salt TE (10 mM Tris HCl, 1 mM EDTA, 2 M NaCl, pH 8) in sterile eppendorf tube and kept for 45 minutes at room temperature.
11. When DNA was fully suspended, 5 μ l of RNase (10 mg/ml) was added and incubated at 37°C for one hour or at room temperature overnight.

12. An equal volume of cold phenol : chloroform : iso-amylalcohol (25:24:1 v/v/v) was added and mixed well (The molecular grade phenol buffered to pH 8 was used). The solution was centrifuged for five minutes at 14,000 rpm.
13. Top layer was pipetted in to another eppendorf tube and an equal volume of chloroform : iso-amylalcohol (24:1 v/v) solution was added and centrifuged for five minutes at 14,000 rpm.
14. The top layer was pipetted in to new eppendorf tube and 0.5 volume of 7.5 mM ammonium acetate solution and two volumes of absolute ethanol was added, mixed thoroughly and centrifuged at 14,000 rpm for 5 minutes.
15. DNA pellet was removed and washed twice with 250 μ l of 70% ethanol. The supernatant solution was dispensed and the pellet was fan dried for 10 minutes.
16. The DNA was dissolved in appropriate volume (150-250 μ l) of TE buffer (10 mM Tris, 1mM EDTA pH 8).

3.3.2.2 DNA quantity and quality estimation

The concentration of DNA was assessed by a spectrophotometer and also by gel electrophoresis using 0.8 per cent agarose gel with known concentrations of uncut DNA.

To test the quality of DNA, samples were run on 0.8 per cent agarose gel in 1X TAE buffer and stained with ethidium bromide and checked for contamination by RNA (which usually runs ahead) and the

DNA was evaluated by comparing it with a standard undigested DNA sample

3.3.2.3 Polymerase Chain Reaction

Requirements

Template DNA

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

Primers

Commercial kits 'A', 'B', 'E', 'F', 'K' and 'AG' of random decamer DNA primers were obtained from Operon Technologies Inc. Alamedas, USA and two specific primers CS-27 and UBC-170 (Mayer *et al.*, 1997) were also synthesized from the same company and 12C gene family primer was got synthesized from M/S Bangalore Genei Pvt. Ltd., Bangalore, India are given in Table 7. A total of 123 primers were used for PCR amplification. The working concentrations of random (5 pM per μ l) and specific (2 pM/ μ l) (Mayer *et al.*, 1997) primers were prepared using autoclaved sterile distilled water.

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.

Table 7. Primers used for tagging of wilt resistance gene in chickpea (*Cicer arietinum* L.)

Sl. No.	Primer	Sequence (5' to 3')	GC %
01	OPK-01	CATTCGAGCC	60
02	OPK-02	GTCTCCGCAA	60
03	OPK-03	CCAGCTTAGG	70
04	OPK-04	CCGCCCAAAC	70
05	OPK-05	TCTGTGCGAGG	60
06	OPK-06	CACCTTTCCC	60
07	OPK-07	AGCGAGCAAG	60
08	OPK-08	GAACACTGGG	60
09	OPK-09	CCCTACCGAC	70
10	OPK-10	GTGCAACGTG	60
11	OPK-11	AATGCCCCAG	60
12	OPK-12	TGGCCCTCAC	70
13	OPK-13	GGTTGTACCC	60
14	OPK-14	CCCGCTACAC	70
15	OPK-15	CTCCTGCCAA	60
16	OPK-16	GAGCGTCGAA	60
17	OPK-17	CCCAGCTGTG	70
18	OPK-18	CCTAGTCGAG	60
19	OPK-19	CACAGGCGGA	70
20	OPK-20	GTGTCGCGAG	70
21	OPA-01	CAGGCCCTTC	70
22	OPA-02	TGCCGAGCTG	70
23	OPA-03	AGTCAGCCAC	60
24	OPA-04	AATCGGGCTG	60
25	OPA-05	AGGGGTCTTG	60
26	OPA-06	GGTCCCTGAC	70
27	OPA-07	GAAACGGGTG	60
28	OPA-08	GTGACGTAGG	60
29	OPA-09	GGGTAACGCC	70
30	OPA-10	GTGATCGCAG	60
31	OPA-11	CAATCGCCGT	60
32	OPA-12	TCGGCGATAG	60
33	OPA-13	CAGCACCCAC	70
34	OPA-14	TCTGTGCTGG	60
35	OPA-15	TTCCGAACCC	60
36	OPA-16	AGCCAGCGAA	60
37	OPA-17	GACCGCTTGT	60
38	OPA-18	AGGTGACCGT	60
39	OPA-19	CAAACGTGCGG	60
40	OPA-20	GTTGCGATCC	60
41	OPB-01	GTTTCGCTCC	60
42	OPB-02	TGATCCCTGG	60
43	OPB-03	CATCCCCCTG	70

Table 7. Contd.....

Sl. No.	Primer	Sequence (5' to 3')	GC %
44	OPB-04	GGACTGGAGT	60
45	OPB-05	TGCGCCCTTC	70
46	OPB-06	TGCTCTGCCC	70
47	OPB-07	GGTGACGCAG	70
48	OPB-08	GTCCACACGG	70
49	OPB-09	TGGGGGACTC	70
50	OPB-10	CTGCTGGGAC	70
51	OPB-11	GTAGACCCGT	60
52	OPB-12	CCTTGACGCA	60
53	OPB-13	TTCCCCCGCT	70
54	OPB-14	TCCGCTCTGG	70
55	OPB-15	GGAGGGTGTT	60
56	OPB-16	TTTGCCCGGA	60
57	OPB-17	AGGGAACGAG	60
58	OPB-18	CCACAGCAGT	60
59	OPB-19	ACCCCCGAAG	70
60	OPB-20	GGACCCTTAC	60
61	OPE-01	CCCAAGGTCC	70
62	OPE-02	GGTGCGGGAA	70
63	OPE-03	CCAGATGCAC	60
64	OPE-04	GTGACATGCC	60
65	OPE-05	TCAGGGAGGT	60
66	OPE-06	AAGACCCCTC	60
67	OPE-07	AGATGCAGCC	60
68	OPE-08	TCACCACGGT	60
69	OPE-09	CTTCACCCGA	60
70	OPE-10	CACCAGGTGA	60
71	OPE-11	GAGTCTCAGG	60
72	OPE-12	TTATCGCCCC	60
73	OPE-13	CCCGATTCCG	70
74	OPE-14	TGCGGCTGAG	70
75	OPE-15	ACGCACAACC	60
76	OPE-16	GGTGACTGTG	60
77	OPE-17	CTACTGCCGT	60
78	OPE-18	GGACTGCAGA	60
79	OPE-19	ACGGCGTATG	60
80	OPE-20	AACGGTGACC	60
81	OPAG-01	CTACGGCTTC	60
82	OPAG-02	CTGAGGTCCT	60
83	OPAG-03	TGCGGGAGTG	70
84	OPAG-04	GGAGCGTACT	60
85	OPAG-05	CCCACTAGAC	60
86	OPAG-06	GGTGGCCAAG	70
87	OPAG-07	CACAGACCTG	60

Table 7. Contd.....

Sl. No.	Primer	Sequence (5' to 3')	GC %
88	OPAG-08	AAGAGCCCTC	60
89	OPAG-09	CCGAGGGGTT	70
90	OPAG-10	ACTGCCCCGAC	70
91	OPAG-11	TTACGGTGGG	60
92	OPAG-12	CTCCCAGGGT	70
93	OPAG-13	GGCTTGGCGA	70
94	OPAG-14	CTCTCGGCGA	70
95	OPAG-15	CCCACACGCA	70
96	OPAG-16	CCTGCGACAG	70
97	OPAG-17	AGCGGAAGTG	60
98	OPAG-18	GTGGGCATAC	60
99	OPAG-19	AGCCTCGGTT	60
100	OPAG-20	TGCGCTCCTC	70
101	OPF-01	ACGGATCCTG	60
102	OPF-02	GAGGATCCCT	60
103	OPF-03	CCTGATCACC	60
104	OPF-04	GGTGATCAGG	60
105	OPF-05	CCGAATTCCC	60
106	OPF-06	GGGAATTCGG	60
107	OPF-07	CCGATATCCC	60
108	OPF-08	GGGATATCGG	60
109	OPF-09	CCAAGCTTCC	60
110	OPF-10	GGAAGCTTGG	60
111	OPF-11	TTGGTACCCC	60
112	OPF-12	ACGGTACCAG	60
113	OPF-13	GGCTGCAGAA	60
114	OPF-14	TGCTGCAGGT	60
115	OPF-15	CCAGTACTCC	60
116	OPF-16	GGAGTACTGG	60
117	OPF-17	AACCCGGGAA	60
118	OPF-18	TTCCCGGGTT	60
119	OPF-19	CCTCTAGACC	60
120	OPF-20	GGTCTAGAGG	60
121	CS-27 R	AGTGGTCGCGATGGGGCCATGGTG	66.66
	CS-27 F	AGCTGGTCGCGGGTCAGAGGAAGA	62.50
122	UBC-170 R	ATCTCTCCTGCATCACAAG	47.36
	UBC-170 F	ATCTCTCCTGTGTGTGTG	50.00
123	I2 C GENE FAMILY SENSE	TGGAGAGTTCCCTACACTTGAG	50.00
	I2 C GENE FAMILY ANTI-SENSE	TTCTCTTCAAGGTAGTTGGCAG	45.45

Thermocycler

Primus 96^{plus} supplied by MWG AG Biotech, Auzinger strasse TA, Ebersberg, Germany was used for cyclic amplification of DNA.

3.3.2.4 Amplification reaction mixture

Amplification reaction mixture was prepared in 0.2 ml thin walled flat cap PCR tubes containing following components. The total volume of each reaction mixture was 25 μ l.

Sl. No.	Components	Amount in μ l	
		RAPD	Specific primer
1	10X buffer with 15 mM Mg Cl ₂	2.5	2.5
2.	Mg Cl ₂ (25 mM)	1.0	--
3.	dNTP mix (2.5 mM each)	1.0	1.0
4.	Primer (5 pM/ μ l)	1.0	--
	Forward primer (2 pM/ μ l)	--	1.0
	Reverse primer (2 pM/ μ l)	--	1.0
5.	Template (10 ng/ μ l)	3.0	3.0
6	Sterile distilled water	16.16	16.16
7	Taq DNA polymerase (3U/ μ l)	0.34	0.34

3.3.2.5 PCR amplification

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

Sl. No.	Step	Temperature (° C)	Duration (min.)	Cycles
1.	Denaturation	95	5	1
2.	Denaturation	94	1	45
3.	Annealing	36	1	
4.	Extension	72	2	
5.	Final extension	72	8	1

For specific primers, the amplification was performed as suggested by Mayer *et al.* (1997).

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

The above RAPD profile was performed in the absence of amplification at this (62°C) annealing temperature.

3.3.2.6 Separation of amplified products by agarose gel electrophoresis

20 µl of the amplified products from each tube along with 2 µl of loading dye were separated on 1.1 per cent agarose gel (for specific primers it was 2 per cent gel) using 1X TAE buffer of pH 8.0 along with Lambda DNA *Hind III* digest and ΦX174 DNA *Hae III* digest as DNA molecular weight marker. The gels were photographed using gel documentation system (Uvi tec, Cambridge, England).

The primer which produced specific bands in any one of the parents were considered as polymorphic and were selected for further analysis of RILs.

3.3.3 ASSOCIATION OF RAPD MARKERS WITH WILT REACTION

For this study, the DNA from all 164 RILs was isolated by following the method as described in 3.3.2.1

3.3.3.1 Bulk Segregation Analysis

Ten primers out of 123 oligonucleotide primers showed parental polymorphism. In order to select the putative primers linked to wilt resistance or susceptibility, from among 164 RILs, ten homozygous lines each showing susceptibility and resistance were selected for BSA. Equal quantity of DNA from ten susceptible and resistant RILs was mixed separately to form susceptible and resistant bulks. The DNA from these bulks was used as template in PCR amplification using primers which showed parental polymorphism. The primers which showed polymorphic amplification in both parents and bulks were selected. Three primers showed polymorphism and were selected for the analysis of individual RILs. These primers were further used for amplification of all the 164 RILs as described in 3.3.2.5.

3.3.3.2 Statistical analysis

The two parental genotypes differ only for H₂ locus of wilt reaction which make the genotype susceptible (late wilting) or resistant (No wilting). The RILs were also classified in to resistant or susceptible. It is expected to show 1:1 (resistant : susceptible) segregation in the RILs. The single gene segregation of wilt reaction in RILs was tested using chi square test for confirmation of involvement of single gene. The same test was used to know the random distribution of markers. Linkage between the marker and wilt reaction was identified by testing wilt reaction and marker for 1:1:1:1 ratio in RILs. The linkage of markers and wilt reaction locus was calculated using map maker version 3.0 software. In this, loci were first divided into linkage groups at a LOD score of three by two point

analysis using the 'group' command. Marker order in linkage group was determined using the 'try' command of the program. Marker orders obtained were confirmed by multipoint analysis applying the 'ripple' function. Final map distances were calculated by applying the 'Kosambi' function provided by the program.

3.3.3.3 Verification of RAPD markers with wilt susceptibility in commonly used parental lines of chickpea

Six chickpea genotypes viz., BG-256, ICCV-10, A-1, WR-315, JG-62 and K-850 which are commonly used as donor parents in breeding programmes in India were selected for this study. The seeds of the selected genotypes were sown in both wilt sick plot and pots as described in 3.3.1.2 and 3.3.1.3 to know their reaction to wilt. The DNA was isolated by following the modified CTAB method and was used in PCR amplification with two RAPD markers (OPK-09 and OPA-07). The PCR products were separated on 1.1 per cent agarose gels, stained with ethidium bromide and scored for presence or absence of the markers.

3.3.2 GENETIC VARIABILITY STUDIES IN RILs FOR SEED YIELD AND YIELD COMPONENTS

A total of 167 entries involving 164 RILs, two parents (K-850 and WR-315) and a national check, A-1 were tested in the Botanical garden of College of Agriculture, Dharwad, during the month of October, 2002. The experiment was laid out in Randomized Block Design (RBD) with two replications. Each entry was represented by a plot of two rows of one 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 conditions with protective irrigations by following all the recommended agronomic practices including insecticidal sprays for good crop growth.

3.3.4.1 Collection of data

The following observations were recorded on each of the five competitive plants selected at random per genotype per replication.

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 percent 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 (g):** The weight in grams (g) of a counted sample of 100 seeds from each plant was recorded. In case where the seed number was less than 100, it was calculated from the weight of available number of seeds and converted to 100 seed weight.
6. **Seed yield per plant:** The weight of seeds in grams (g) from each plant was recorded.

3.3.4.2 Statistical methods

The mean of data collected for six characters on 167 genotypes was subjected for following statistical analysis.

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	M ₁	-
Genotypes (g)	g-1	M ₂	$\sigma_e^2 + r\sigma_g^2$
Error	(r-1) (g-1)	M ₃	σ_e^2
Total	(rg-1)	M ₁ + M ₂ + M ₃	

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,

$$\text{Genotypic variance } (\sigma_g^2) = \frac{\text{MSS (genotypes)} - \text{MSS (error)}}{\text{Number of replications}} = \frac{M_2 - M_3}{r}$$

$$\text{Phenotypic variance } (\sigma_p^2) = \sigma_g^2 + \text{MSS error} = \frac{M_2 - M_3}{r} + M_3$$

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)

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

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).

$$r_p = \frac{\text{Cov } X_p Y_p}{\sqrt{X_p^2 \times Y_p^2}}$$

Where,

r_p = Phenotypic correlation

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

X_p^2 and Y_p^2 = 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.

$$r_g = \frac{\text{Cov } X_g Y_g}{\sqrt{X_g^2 \times Y_g^2}}$$

Where,

r_g = Genotypic correlation

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

X_g^2 and Y_g^2 = 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 for RILs 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 towards achieving the objectives are presented under following headings.

4.1 Development and evaluation of RILs for wilt reaction

4.2 RAPD analysis of parental genotypes

4.3 Association of RAPD markers with wilt reaction

4.4 Genetic variability studies in RILs for seed yield and yield components

4.1. DEVELOPMENT AND EVALUATION OF RILs FOR WILT REACTION

The population of recombinant inbred lines (RILs) derived by single seed descent method represent stable homozygous lines, which can be scored for the parental alleles and to determine recombination frequencies. RILs serve as ideal mapping populations for the tagging of important traits. Two genotypes which are polymorphic for H₂ locus of wilt resistance were used in the present study.

The late wilting genotype K-850 with genetic constitution of $h_1h_1H_2H_2h_3h_3$ was crossed with wilt resistant genotype WR-315 ($h_1h_1h_2h_2h_3h_3$). From each F₂ plant of this cross, a seed was taken to constitute the SSD bulk and advanced to the next generation. This procedure was continued up to F₅ and F₅ to F₆ individual plant progenies were taken to get more seeds for evaluation. Thus, a total of 178 F₆ recombinant inbred lines were developed which were utilized for tagging of H₂ locus governing Fusarium wilt resistance.

One hundred seventy eight F₆ RILs along with parents were tested for resistance to race1 of fusarium wilt in wilt sick plot at ICRISAT during *rabi* 2002. The difference at H₂ locus causes late wilting in chickpea. As expected none of the RILs showed early wilting. The observations on late wilting were recorded at 37th day and 62nd day. Based on cumulative wilting percentage, the recombinant inbred lines were grouped into three groups viz., susceptible (late wilting), resistant and moderate (segregating) (Table 8). The late wilting of the RILs was not uniform. It ranged from 50 to 100 per cent. However, the genotypes showing more than 50 per cent were taken under one group as late wilting.

Out of 178 RILs, 84 lines were resistant to race1 of Fusarium wilt (Table 8), while eighty lines were susceptible (late wilters) like K-850. The 14 RILs showed moderate (segregating) for late wilting reaction and thus were not considered for further analysis. For confirmation, forty four RILs along with parental lines were tested under pot culture. The results obtained support the reaction of genotypes observed under wilt sick plot in 41 RILs. Only three genotypes showed differential reaction compared to field reaction (Table 9). The data on field reaction of RILs for wilt was subjected to chi-square test to know the goodness of fit to 1:1 ratio (Susceptible: Resistance) (Table 10). The calculated χ^2 value (0.097) was non-significant at both 5 and 1 percent level.

4.2 RAPD ANALYSIS OF PARENTAL GENOTYPES

The DNA isolated from two parents (K-850 and WR-315) was used as template in PCR reaction. The parental polymorphism was studied

Table 8. Reaction of chickpea RILs to Fusarium wilt race 1 in wilt sick plot at ICRISAT

Sl. No.	Genotype	Cumulative wilt (%)	Wilt reaction
1.	6-01	50	S
2.	6-02	71	S
3.	6-03	16	R
4.	6-04	29	R
5.	6-05	15	R
6.	6-06	74	S
7.	6-07	7	R
8.	6-08	56	S
9.	6-09	57	S
10.	6-10	21	R
11.	6-11	41	M
12.	6-12	50	S
13.	6-13	0	R
14.	6-14	23	R
15.	6-15	3	R
16.	6-16	74	S
17.	6-17	5	R
18.	6-18	21	R
19.	6-19	11	R
20.	6-20	12	R
21.	6-21	70	S
22.	6-22	38	M
23.	6-23	34	M
24.	6-24	59	S
25.	6-25	71	S
26.	6-26	0	R
27.	6-27	35	M
28.	6-28	0	R
29.	6-29	0	R
30.	6-30	24	R
31.	6-31	0	R
32.	6-32	0	R
33.	6-33	66	S
34.	6-34	50	S
35.	6-35	53	S
36.	6-36	13	R
37.	6-37	52	S
38.	6-38	26	R
39.	6-39	29	R
40.	6-40	82	S
41.	6-41	0	R
42.	6-42	82	S
43.	6-43	78	S
44.	6-44	0	R
45.	6-45	40	M
46.	6-46	60	S

Table 8. Contd.....

Sl. No.	Genotype	Cumulative wilt (%)	Wilt reaction
47.	6-47	89	S
48.	6-48	71	S
49.	6-49	41	M
50.	6-50	52	S
51.	6-51	0	R
52.	6-52	83	S
53.	6-53	68	S
54.	6-54	74	S
55.	6-55	70	S
56.	6-56	3	R
57.	6-57	100	S
58.	6-58	9	R
59.	6-59	16	R
60.	6-60	50	S
61.	6-61	6	R
62.	6-62	0	R
63.	6-63	2	R
64.	6-64	27	R
65.	6-65	0	R
66.	6-66	0	R
67.	6-67	56	S
68.	6-68	57	S
69.	6-69	71	S
70.	6-70	23	R
71.	6-71	68	S
72.	6-72	88	S
73.	6-73	0	R
74.	6-74	0	R
75.	6-75	10	R
76.	6-76	51	S
77.	6-77	50	S
78.	6-78	13	R
79.	6-79	57	S
80.	6-80	78	S
81.	6-81	74	S
82.	6-82	55	S
83.	6-83	75	S
84.	6-84	0	R
85.	6-85	75	S
86.	6-86	29	R
87.	6-87	37	M
88.	6-88	26	R
89.	6-89	91	S
90.	6-90	14	R
91.	6-91	63	S
92.	6-92	79	S
93.	6-93	58	S

Table 8. Contd.....

Sl. No.	Genotype	Cumulative wilt (%)	Wilt reaction
94.	6-94	65	S
95.	6-95	0	R
96.	6-96	22	R
97.	6-97	87	S
98.	6-98	54	S
99.	6-99	3	R
100.	6-100	51	S
101.	6-101	23	R
102.	6-102	52	S
103.	6-103	59	S
104.	6-104	70	S
105.	6-105	26	R
106.	6-106	0	R
107.	6-107	5	R
108.	6-108	52	S
109.	6-109	50	S
110.	6-110	52	S
111.	6-111	39	M
112.	6-112	52	S
113.	6-113	60	S
114.	6-114	52	S
115.	6-115	0	R
116.	6-116	26	R
117.	6-117	83	S
118.	6-118	78	S
119.	6-119	13	R
120.	6-120	52	S
121.	6-121	19	R
122.	6-122	50	S
123.	6-123	3	R
124.	6-124	0	R
125.	6-125	5	R
126.	6-126	69	S
127.	6-127	28	R
128.	6-128	35	M
129.	6-129	8	R
130.	6-130	0	R
131.	6-131	3	R
132.	6-132	20	R
133.	6-133	36	M
134.	6-134	29	R
135.	6-135	51	S
136.	6-136	16	R
137.	6-137	0	R
138.	6-138	3	R
139.	6-139	0	R
140.	6-140	8	R

Table 8. Contd.....

Sl. No.	Genotype	Cumulative wilt (%)	Wilt reaction
141.	6-141	60	S
142.	6-142	0	R
143.	6-143	52	S
144.	6-144	12	R
145.	6-145	75	S
146.	6-146	59	S
147.	6-147	55	S
148.	6-148	0	R
149.	6-149	0	R
150.	6-150	38	M
151.	6-151	73	S
152.	6-152	39	M
153.	6-153	51	S
154.	6-154	38	M
155.	6-155	0	R
156.	6-156	61	S
157.	6-157	36	M
158.	6-158	67	S
159.	6-159	81	S
160.	6-160	70	S
161.	6-161	9	R
162.	6-162	81	S
163.	6-163	65	S
164.	6-164	0	R
165.	6-165	0	R
166.	6-166	0	R
167.	6-167	60	S
168.	6-168	12	R
169.	6-169	0	R
170.	6-170	56	S
171.	6-171	7	R
172.	6-172	0	R
173.	6-173	0	R
174.	6-174	17	R
175.	6-175	22	R
176.	6-176	79	S
177.	6-177	0	R
178.	6-178	51	S
179.	WR-315	0	R
180.	K-850	50	S

R : Resistant
M : Moderate
S : Susceptible

Table 9. Reaction of selected chickpea RILs to race 1 of Fusarium wilt in wilt sick pots

Sl. No.	Genotype	Wilt reaction	Sl. No.	Genotype	Wilt reaction
1	6-02	S	23	6-81	S
2	6-13	R	24	6-83	S
3	6-15	R	25	6-89	S
4	6-16	S	26	6-105	R
5	6-19	R	27	6-106	R
6	6-24	S	28	6-117	S
7	6-26	R	29	6-123	R
8	6-28	R	30	6-124	R
9	6-29	R	31	6-137	R
10	6-32	R	32	6-145	S
11	6-41	R*	33	6-151	S
12	6-44	R	34	6-155	R
13	6-47	S	35	6-159	S
14	6-48	S	36	6-162	S
15	6-52	S	37	6-164	R
16	6-56	R	38	6-166	R
17	6-60	S	39	6-167	S*
18	6-68	S*	40	6-169	R
19	6-69	S	41	6-172	R
20	6-70	R	42	6-173	R
21	6-72	S	43	6-176	S
22	6-73	R	44	6-177	R

*genotypes differing in their reaction in pot culture experiment

R : Resistant

S : Susceptible

Table 10. Single locus goodness of fit for 1:1 ratio of RILs reaction to race 1 of Fusarium wilt in chickpea

Locus	Susceptible	Resistance	χ^2	Probability
Race 1	80	84	0.097	0.755

using 123 oligonucleotide primers. All the primers produced scorable amplified products except four viz., OPK-01, OPK-03, OPK-20 and OPE-04 (Table 11). Initially 23 primers produced polymorphic amplified products between the parents. After continuous repetition only 10 primers (OPA-07, OPB-16, OPE-12, OPF-15, OPF-17, OPF-18, OPF-19, OPK-09, OPK-13 and OPAG-16) produced consistent polymorphism between the parents (Table 11).

RAPD analysis of parental lines with 123 oligonucleotide primers yielded 875 and 882 amplified products in susceptible parent K-850 and resistant parent WR-315 respectively. On an average 7.10 and 7.20 fragments per primer were produced in K-850 and WR-315 respectively. The primer OPE-14 produced as high as 21 fragments in both susceptible and resistant parents while OPA-06 produced only one fragment. The 120 random primers used are from six kits of Operon technologies viz., OPA, OPB, OPE, OPF, OPK and OPAG, each kit containing 20 primers. In kit A the highest mean number of products (12) were produced by OPA-13 in both the parents, while OPA-06 produced only one amplified product in both the parents. OPB-08 and OPB-18 produced maximum number of amplified products (10) in kit B, while OPB-02 and OPB-20 produced minimum of three scorable bands in both susceptible and resistant parents.

In kit E, OPE-14 produced as many as 21 scorable bands, whereas OPE-13 and OPE-17 produced only two bands in both the parents. In kit F maximum number of bands per primer was 11 with primer OPF-05 and minimum was three with OPF-12 and OPF-13 in both susceptible and

Table 11. Primers producing polymorphic markers among the resistant and susceptible parents of chickpea

Primer	Banding pattern					
	K-850			WR-315		
	Major bands	Minor bands	Total	Major bands	Minor bands	Total
A01	8	1	9	8	1	9
A02	7	3	10	7	3	10
A03	5	5	10	5	5	10
A04	7	3	10	7	3	10
A05	6	1	7	6	1	7
A06	1	0	1	1	0	1
A07	4	2	6	3	2	5
A08	4	1	5	4	1	5
A09	8	2	10	8	2	10
A10	6	5	11	6	5	11
A11	5	1	6	5	1	6
A12	1	0	1	1	0	1
A13	10	2	12	10	2	12
A14	3	0	3	3	0	3
A15	4	2	6	4	2	6
A16	4	1	5	4	1	5
A17	9	0	9	9	0	9
A18	6	4	10	6	4	10
A19	4	2	6	4	2	6
A20	7	1	8	7	1	8
Total	109	36	145	108	36	144
Mean	5.45	1.8	7.3	5.4	1.8	7.2
B01	2	2	4	2	2	4
B02	3	0	3	3	0	3
B03	4	0	4	4	0	4
B04	5	0	5	5	0	5
B05	7	1	8	7	1	8
B06	3	6	9	3	6	9
B07	8	0	8	8	0	8
B08	3	7	10	3	7	10
B09	4	5	9	4	5	9
B10	4	5	9	4	5	9
B11	2	2	4	2	2	4
B12	6	1	7	6	1	7
B13	4	2	6	4	2	6
B14	2	4	6	2	4	6
B15	2	2	4	2	2	4
B16	2	5	7	3	4	7
B17	3	3	6	3	3	6
B18	6	4	10	6	4	10
B19	4	0	4	4	0	4
B20	2	1	3	2	1	3
Total	76	50	126	77	49	126
Mean	3.8	2.5	6.3	3.85	2.45	6.3

Table 11. Contd.....

Primer	Banding pattern					
	K-850			WR-315		
	Major bands	Minor bands	Total	Major bands	Minor bands	Total
E01	3	0	3	3	0	3
E02	3	7	10	3	7	10
E03	9	4	13	9	4	13
E04	0	0	0	0	0	0
E05	5	2	7	5	2	7
E06	6	2	8	6	2	8
E07	11	0	11	11	0	11
E08	3	0	3	3	0	3
E09	5	5	10	5	5	10
E10	0	4	4	0	4	4
E11	6	8	14	6	8	14
E12	6	0	6	7	0	7
E13	2	0	2	2	0	2
E14	10	11	21	10	11	21
E15	4	4	8	4	4	8
E16	3	8	11	3	8	11
E17	2	0	2	2	0	2
E18	6	6	12	6	6	12
E19	6	3	9	6	3	9
E20	8	5	13	8	5	13
Total	98	69	167	99	69	168
Mean	4.9	3.45	8.4	4.95	3.45	8.4
F01	6	1	7	6	1	7
F02	4	4	8	4	4	8
F03	6	2	8	6	2	8
F04	2	5	7	2	5	7
F05	8	3	11	8	3	11
F06	3	2	5	3	2	5
F07	4	2	6	4	2	6
F08	7	2	9	7	2	9
F09	5	5	10	5	5	10
F10	6	0	6	6	0	6
F11	4	1	5	4	1	5
F12	2	1	3	2	1	3
F13	2	5	7	2	5	7
F14	3	0	3	3	0	3
F15	1	5	6	4	4	8
F16	4	5	9	4	5	9
F17	6	0	6	7	0	7
F18	4	0	4	8	0	8
F19	7	0	7	5	0	5
F20	3	4	7	3	4	7
Total	87	47	134	93	46	139
Mean	4.35	2.35	6.7	4.65	2.3	7
K01	0	0	0	0	0	0
K02	4	1	5	4	1	5
K03	0	0	0	0	0	0

Table 11. Contd.....

Primer	Banding pattern					
	K-850			WR-315		
	Major bands	Minor bands	Total	Major bands	Minor bands	Total
K04	9	2	11	9	2	11
K05	4	0	4	4	0	4
K06	4	3	7	4	3	7
K07	4	4	8	4	4	8
K08	4	3	7	4	3	7
K09	7	1	8	6	1	7
K10	4	5	9	4	5	9
K11	4	0	4	4	0	4
K12	4	5	9	4	5	9
K13	4	4	8	4	3	7
K14	4	3	7	4	3	7
K15	2	6	8	2	6	8
K16	6	0	6	6	0	6
K17	5	2	7	5	2	7
K18	3	2	5	3	2	5
K19	11	0	11	11	0	11
K20	0	0	0	0	0	0
Total	83	41	124	82	40	122
Mean	4.15	2.05	6.2	4.1	2	6.1
AG01	4	3	7	4	3	7
AG02	12	1	13	12	1	13
AG03	6	2	8	6	2	8
AG04	5	3	8	5	3	8
AG05	3	4	7	3	4	7
AG06	5	3	8	5	3	8
AG07	8	5	13	8	5	13
AG08	6	10	16	6	10	16
AG09	6	0	6	6	0	6
AG10	11	4	15	11	4	15
AG11	5	4	9	5	4	9
AG12	5	0	5	5	0	5
AG13	5	1	6	5	1	6
AG14	3	0	3	3	0	3
AG15	10	1	11	10	1	11
AG16	4	0	4	8	0	8
AG17	7	0	7	7	0	7
AG18	4	0	4	4	0	4
AG19	1	2	3	1	2	3
AG20	5	0	5	5	0	5
Total	115	43	158	119	43	162
Mean	5.75	2.15	7.9	5.95	2.15	8.1
CS27	4	2	6	4	2	6
UBC170	3	7	10	3	7	10
12 C Gene family primer	2	3	5	2	3	5
Grand Total			875			882
Average No. of bands per primer			7.1			7.2

resistant parents. OPK-04 and OPK-19 produced 11 amplified products which was higher in kit K, while the minimum was four in OPK-05. The primers OPAG-08 yielded the highest of 16 scorable amplified products in both parents while, OPAG-14 and OPAG-19 produced only three in kit AG.

Among six kits, each with 20 primers, kit E primers produced maximum number of bands (335) in both the parents followed by kit AG (320), Kit A (289), kit F (273) and kit B (252) while minimum number of bands were produced in kit K (246). The average number of bands per primer in susceptible and resistant parent was highest in kit E (8.4; 8.4), followed by AG (7.9; 8.1), kit A (7.3; 7.2), F (6.7; 7.0) and B (6.3; 6.3) and it was least in kit K (6.2; 6.1).

The primer OPA-07 was the only primer which showed one specific amplified scorable band in susceptible parent and that was absent in resistant parent in kit A and in kit B, the polymorphic primer was OPB-16. Both kit E and kit AG also had only one polymorphic primer each viz., OPE-12 (Plate 4) and OPAG-16 respectively. In kit F as many as four primers produced polymorphic bands between the parents (OPF-15, OPF-17, OPF-18 and OPF-19) (Plate 4). In kit K, two primers (OPK-09 and OPK-13) showed an extra amplified scorable product in susceptible parent that was absent in resistant parent.

4.3 ASSOCIATION OF RAPD MARKERS WITH WILT REACTION

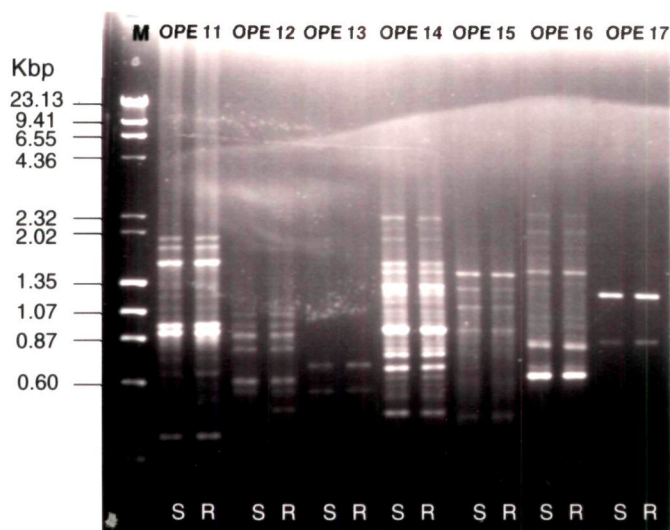
4.3.1 Bulk Segregation Analysis

Bulked segregant analysis was used in this study to identify putative RAPD markers linked to resistance or susceptibility. An equal



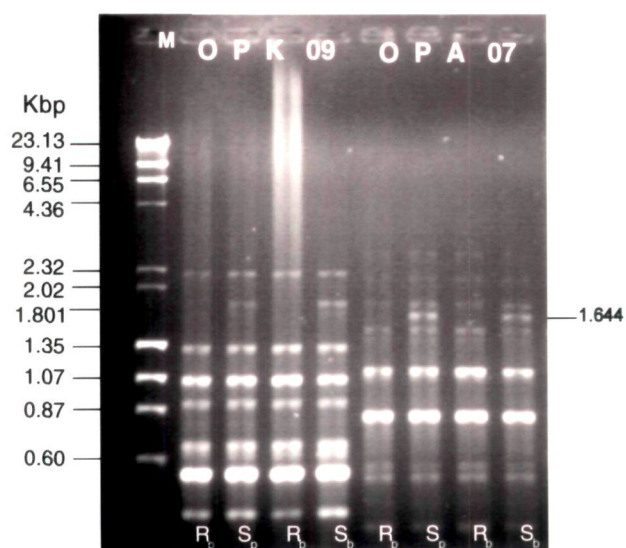
Parental polymorphism in Chickpea amplified with F kit primers

(M: DNA marker ladder, S: Susceptible parent, R: Resistant parent)



Parental polymorphism in Chickpea amplified with E kit primers

(M: DNA marker ladder, S: Susceptible parent, R: Resistant parent)



RAPD markers detecting polymorphism between wilt resistant and susceptible parental lines and RIL bulks in Chickpea

(M: DNA marker ladder, Sp: Susceptible parent, Rp: Resistant parent, Rb: Resistance bulk, Sb: Susceptible bulk)

volume of standardized DNA (ng/ μ l) from ten homozygous susceptible and ten homozygous resistant RILs were pooled separately to make resistant (R) and susceptible (S) bulk. Out of ten primers, which showed polymorphism between the parents, only three primers amplified polymorphic product in parents and bulks. The primer OPK-09 and OPK-13 of kit K and OPA-07 of kit A produced an extra band in susceptible parent and susceptible bulk (Plate 4).

4.3.2 Segregation of RAPD markers

The primers which produced polymorphic DNA fragment in resistant and susceptible parents and bulks were considered as putatively linked with wilt reaction. These primers were used to screen the DNA of all the individual RIL's to study the association between wilt reaction and RAPD marker. Three primers (OPK-09, OPK-13 and OPA-07) were used for DNA amplification of 164 RILs and scored for presence and absence of specific polymorphic band. Among three primers OPK-09 had 70 percent of GC content which produced 1.801 Kbp polymorphic band in susceptible parent K-850 and susceptible bulk. Whereas OPK-13 and OPA-07 had only 60 percent GC in their sequence and produced 1.254 Kbp and 1.644 Kbp polymorphic amplified product respectively in susceptible parent and bulk (Table 12).

RAPD marker (1.801 Kbp), which was amplified with OPK-09 was produced in 87 RILs and was absent in 77 RILs out of 164 RILs studied (Plate 5). The primer OPK-13 produced 1.254 Kbp RAPD marker in 93 recombinant inbred lines and was absent in 71 lines. Among 164 RILs, 91

Table 12. Primer sequence, GC per cent and approximate size of the polymorphic amplified product of three primers tagged with Fusarium wilt reaction in bulk segregant analysis (BSA)

Primer	Sequence (5' - 3')	GC per cent	Approximate size of polymorphic amplified product (Kbp)
OPK-09	CCCTACCGAC	70	1.801
OPK-13	GGTTGTACCC	60	1.254
OPA-07	GAAACGGGTG	60	1.644

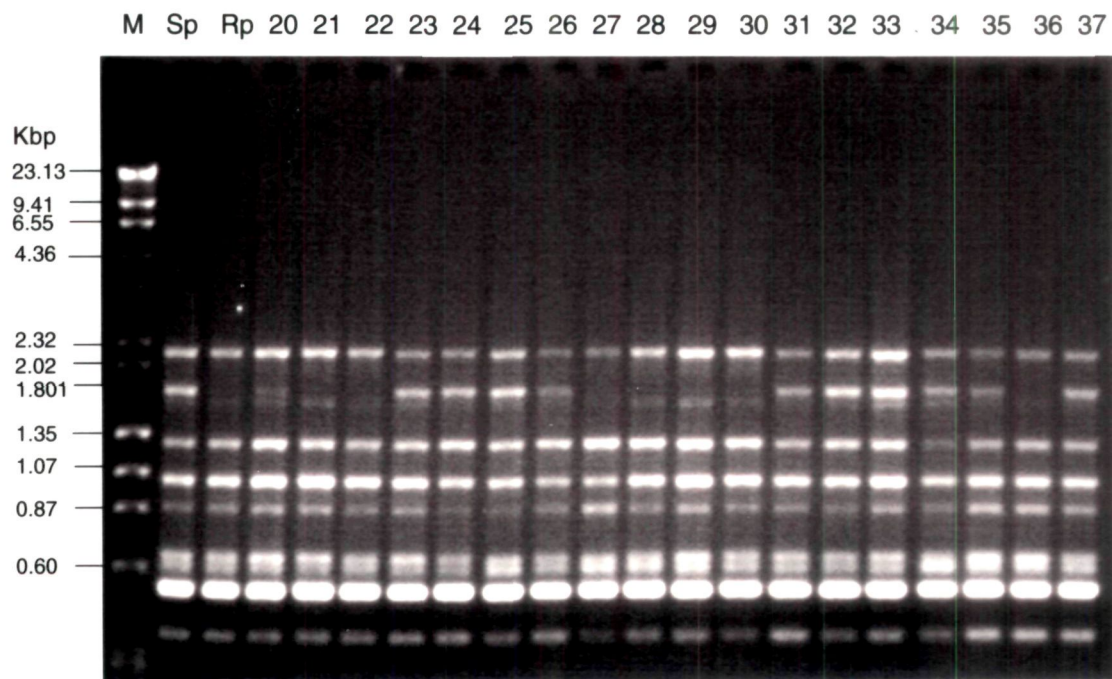
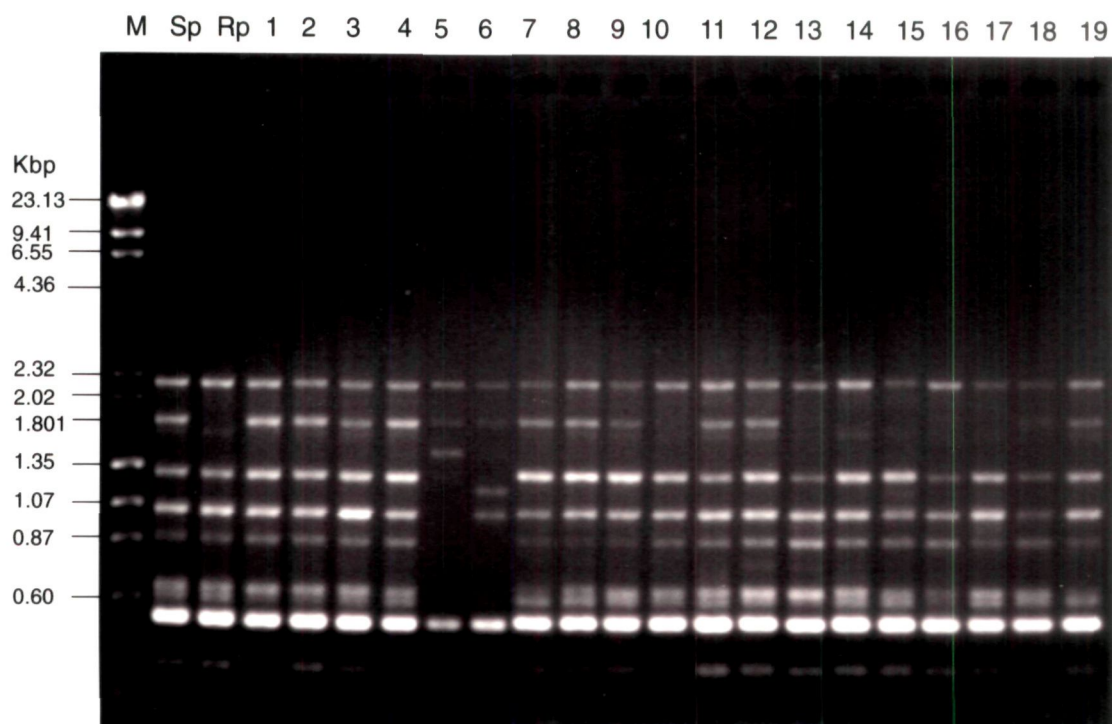


Plate 5: PCR amplification for RAPD marker (OPK - 09) in RILs of Chickpea

(M: DNA marker ladder, Sp: Susceptible parent, Rp: Resistant parent, Lane 1 to 37 are RILs)

were identified with 1.644 Kbp amplification product of OPA-07 (Plate 6) while it was absent in remaining 73 RILs (Table 13).

The score of presence and absence of the bands in a number of recombinant inbred lines was subjected for chi-square analysis to test their single locus goodness of fit for 1:1 ratio. The OPK-09 (1.801 Kbp) had χ^2 value of 0.609, while OPK-13 (1.254 Kbp) and OPA-07 (1.644 Kbp) recorded χ^2 values of 2.951 and 1.970 respectively, which were non significant.

4.3.3 Linkage analysis and determination of distance between the RAPD markers and wilt reaction locus

The RILs were classified into four categories based on presence and absence of specific amplified products and wilt reaction. Out of 80 susceptible RILs, OPK-09 (1.801 Kbp) DNA fragment was present in 72 RILs while it was absent in eight. Similarly out of 84 resistant lines the marker was present in 15 resistant lines. The RAPD marker OPK-13₁₂₅₄ was present in 47 susceptible and 46 resistant lines, while it was absent in 33 susceptible and 38 resistant lines. Similarly, OPA-07 primer produced a polymorphic RAPD marker OPA-07₁₆₄₄ in 71 susceptible and 21 resistant lines while it was absent in nine susceptible and 63 resistant lines.

4.3.4 Joint segregation analysis of RAPD markers and wilt reaction

Joint segregation pattern of wilt reaction of RILs and three RAPD markers was analysed for two loci to test for the goodness of fit for 1:1:1:1 ratio and the results are presented in Table 14.

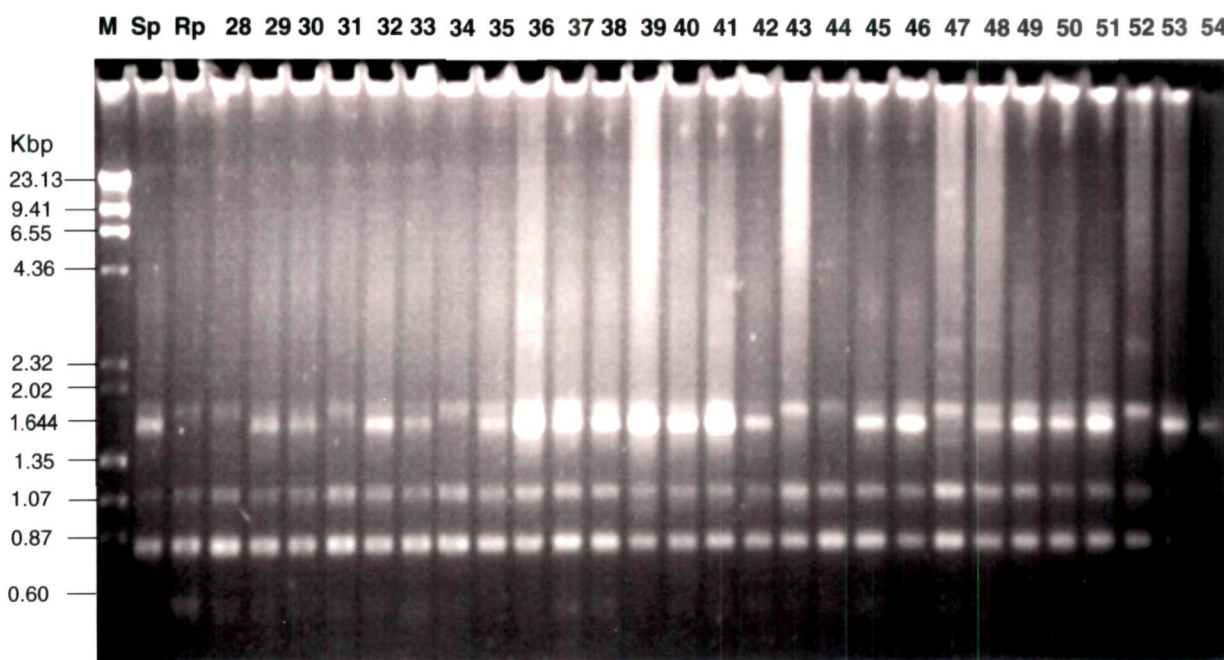
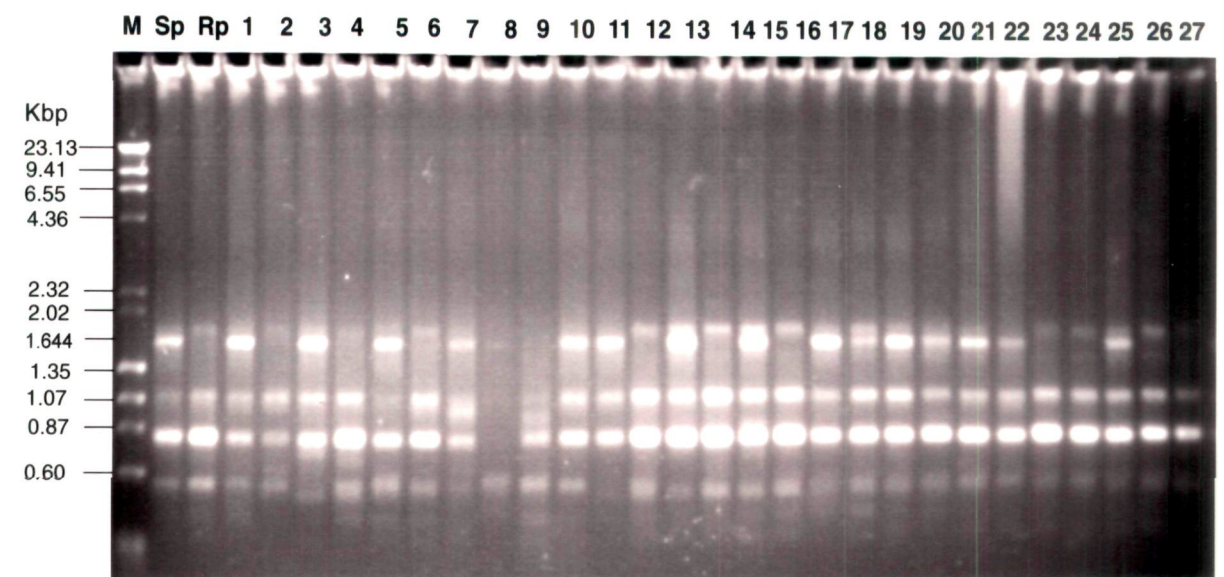


Plate 6: PCR amplification for RAPD marker (OPA- 07) in RILs of Chickpea

(M: DNA marker ladder, Sp: Susceptible parent, Rp: Resistant parent, Lane 1 to 54 are RILs)

Table 13. Single locus goodness of fit for 1:1 ratio (Presence : Absence) of RAPD markers associated with wilt susceptibility in chickpea

Locus	+	-	χ^2	Probability
OPK-09	87	77	0.609	0.435
OPK-13	93	71	2.951	0.085
OPA-07	91	73	1.970	0.160

+ - Presence of polymorphic amplified product

- - Absence of polymorphic amplified product

Table 14. Two loci goodness of fit for 1:1:1:1 ratio for three RAPD markers and reaction to Fusarium wilt in chickpea

Locus	Susceptible		Resistance		χ^2	Probability
	+	-	+	-		
OPK-09	72	8	15	69	85.80**	0.0
OPK-13	47	33	46	38	3.268	0.352
OPA-07	71	9	21	63	68.49**	0.0

+ - Presence of polymorphic amplified product

- - Absence of polymorphic amplified product

** Significant at 1 % level

The segregation pattern of RILs were analysed for independent assortment of two genes. Four classes of genotypes were expected and the chi-square analysis for goodness of fit for 1:1:1:1 segregation for OPK-09 and wilt reaction, indicated a significant chi-square value (85.80) as the 1.801 Kbp amplified product of OPK-09 was absent in eight susceptible RILs and was present in 15 resistant RILs.

The RAPD primer OPK-13 produced 1.254 Kbp product in 46 resistant and 47 susceptible RILs and it was absent in 33 susceptible and resistant RILs. The chi-square value for two loci goodness of fit for 1:1:1:1 ratio for the RAPD markers and reaction to race1 of Fusarium wilt was 3.268 which was non-significant indicating the absence of linkage between OPK-13 (1.254 Kbp) and wilt reaction. The 1.644 Kbp amplified product of OPA-07 was absent in nine susceptible RILs and present in 21 resistant RILs. Chi-square value for goodness of fit for 1:1:1:1 ratio for OPA-07 and wilt reaction was 68.49 which was significant.

The χ^2 test indicated that two RAPD markers OPA-07 and OPK-09 were linked to susceptibility allele. The segregation patterns of RAPD markers and wilt resistance among RILs were analysed using MAPMAKER version 3.0. The distance between disease reaction locus and OPK-09₁₈₀₁ was 8.20 cM and of that with OPA-07₁₆₄₄ was 12.90 cM with lod scores of 20.44 and 13.57 respectively. The distance between OPA-07₁₆₄₄ and OPK 09₁₈₀₁ was 19.56 cM. Utilizing these values a representative linkage map of wilt reaction locus using RAPD markers was developed and it is presented in Plate 7.

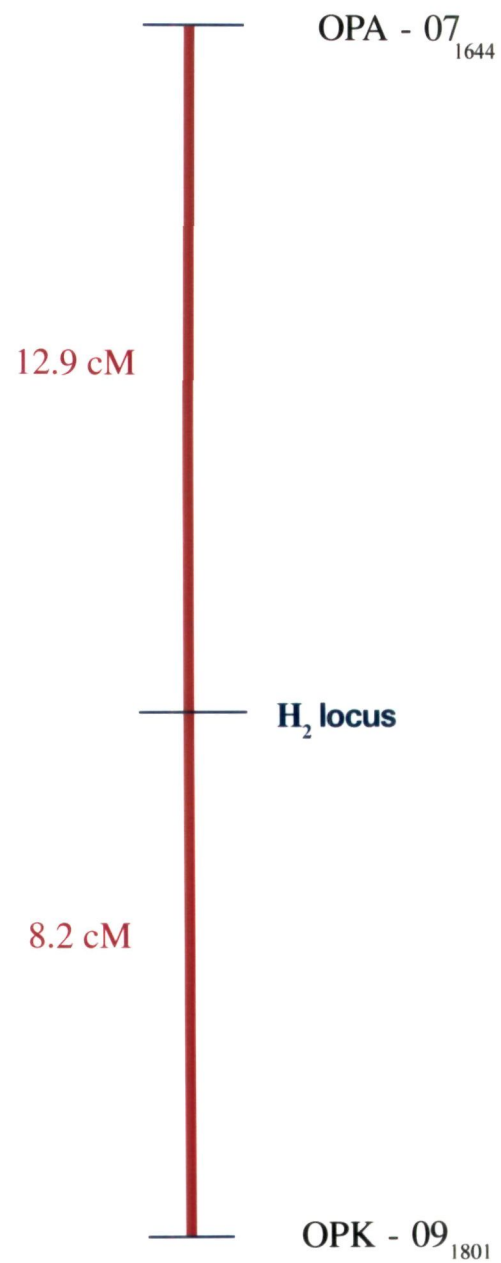


Plate 7: Linkage map of Fusarium wilt resistance (H₂ locus) in Chickpea

4.3.4 Verification of RAPD markers with wilt susceptibility in commonly used parental lines of chickpea

Six chickpea genotypes *viz.*, BG-256 (bold seeded with high seed yield), ICCV-10 (resistant to wilt), A-1 (National check), WR-315 (wilt resistant check), JG-62 (early wilt susceptible check) and K-850 (late wilter, good combiner, nodulator and bold seeded) which are commonly used as donor parents in breeding programmes in India, were surveyed with the two markers (OPK-09 and OPA-07) associated with late wilting of chickpea.

Initial wilt symptoms were observed within 17 days in JG-62 and all the plants were completely wilted within 25 days in this genotype. The time of wilting differed taking more than 30 days in four genotypes *viz.*, K-850, BG-256, A-1 and ICCV-10 suggesting late wilting. The resistant genotype WR-315 did not show wilting till maturity. Out of eight genotypes, two late wilters *viz.*, BG-256 and K-850 showed amplification of OPK-09₁₈₀₁ and it was absent in two late wilters (ICCV-10 and A-1), early wilter (JG-62) and resistant (WR-315) lines. The other RAPD marker OPA-07₁₆₄₄ linked to susceptibility was present in all the four late wilters and the early wilter JG-62. It was absent in resistant genotype WR-315. (Table 15).

4.4 GENETIC VARIABILITY STUDIES IN RILs FOR SEED YIELD AND YIELD COMPONENTS

4.4.1 Variability, heritability and genetic advance

The mean values obtained for various characters included in the present study in respect of all the recombinant inbred lines are presented

Table 15. Reaction of commonly used chickpea parental lines to *Fusarium oxysporum* f.sp. *ciceri* race 1 and the associated RAPD markers

Genotype	Wilt reaction	Markers	
		OPK-09 ₁₈₀₁	OPA-07 ₁₆₄₄
BG-256	Late wilting	+	+
JG-62	Early wilting	-	+
WR-315	Resistant	-	-
ICCV-10	Late wilting	-	+
K-850	Late wilting	+	+
A-1	Late wilting	-	+

in Appendix I. Analysis of variance for six characters included in the present study are presented in the Table 16. It is evident from the Table 16, genotypic mean sum of squares were highly significant for all the characters studied except plant height. The mean, range, genotypic and phenotypic coefficients of variability, heritability estimates and the genetic advance predicted as per cent of mean in respect of six characters are given in Table 17.

4.4.1.1 Plant height

The mean values for plant height ranged from 22.17 to 44.66 cm with overall mean plant height of 35.78 cm, revealing wide range among the RILs. Among the RILs, the line 6-10 (22.17 cm) was the shortest and 6-79 (44.66 cm) was the tallest.

The coefficients of variation at phenotypic and genotypic levels were moderate (11.87 and 10.07 respectively). The heritability estimate was high (72.0) and genetic advance as per cent of mean (17.60) was moderate for this attribute.

4.4.1.2 Number of primary branches

The range was very narrow for the number of primary branches. The mean values ranged from two to four with a mean of 2.91. The RILs 6-47 and 6-115 showed lowest (2.00) number of primary branches whereas 6-34 had the highest number of four primary branches per plant.

The coefficient of variation was found to be moderate for this character both at phenotypic (12.63) and genotypic (11.58) levels. High

Table 16. Analysis of variance for different characters in recombinant inbred lines of chickpea

Source	d.f.	MSS for the characters					
		Plant height (cm)	Number of primary branches	Days to 50% flowering	Number of pods per plant	Test weight (g)	Seed yield per plant (g)
Genotype	166	31.01*	0.25**	41.08**	390.89**	37.46**	23.93**
Error	166	5.03	0.02	0.38	12.96	4.30	1.98

*, ** Significant at 5 and 1% level respectively.

Table 17. The mean, range, phenotypic and genotypic coefficient of variability, heritability (H) and genetic advance over mean (GAM) of different characters in recombinant inbred lines of chickpea

Sl. No.	Characters	Mean	Range	PCV	GCV	H	GAM
1.	Plant height (cm)	35.78	22.17 - 44.66	11.87	10.07	72.0	17.60
2.	Number of primary branches	2.91	2.0 - 4.0	12.63	11.58	84.0	21.99
3.	Days to 50% flowering	50.14	37.00 - 64.00	9.08	9.00	98.2	18.36
4.	Number of pods per plant	46.36	16.17 - 98.00	30.64	29.66	93.7	59.15
5.	Test weight (g)	24.46	15.50 - 32.35	18.68	16.65	79.4	30.54
6.	Seed yield per plant (g)	11.82	3.52 - 25.15	30.44	28.01	84.7	53.13

heritability estimate (84.00) and genetic advance as per cent of mean (21.99) were observed for this trait.

4.4.1.3 Days to 50 per cent flowering

Number of days taken for 50 per cent flowering ranged between 37 (6-41) to 64 (6-178) days with overall mean of 50.14 days. Low phenotypic coefficient of variation (9.08) and genotypic coefficient of variation (9.00) were observed for this trait. The RILs exhibited high heritability estimate (98.2) coupled with moderate genetic advance (18.36) for this trait.

4.4.1.4 Number of pods per plant

The pod number showed a very wide range from 16.17 in RIL 6-26 to 98.00 in RIL 6-37, with an overall mean number of pods per plant in the present material was 46.36.

The coefficient of variability at phenotypic (30.64) and genotypic (29.66) levels was highest among the characters studied. The highest heritability estimate (93.7) with high genetic advance (59.15) were registered for this trait.

4.4.1.5 Test weight (g)

The observed mean values for this trait varied from 15.50 to 32.35 g with a mean of 24.46 g impinging upon the moderate variability present in the RILs. The highest test weight was recorded in RIL 6-64 (32.35 g) and the lowest was in RIL 6-170 (15.50 g).

The coefficient of variation in terms of both phenotypic and genotypic values were moderate i.e., 18.68 and 16.65 respectively. Heritability estimate was high (79.4) with high amount of genetic advance over mean (30.54).

4.4.1.6 Seed yield per plant (g)

A wide variability for seed yield with a range of 2.52 g to 25.15 g with a mean seed yield of 11.82 g was observed among RILs. The RIL, 6-33 recorded the highest seed yield and 6-26 recorded the lowest.

The genotypic coefficient of variation was 28.01 and phenotypic coefficient of variation recorded was 30.44. High heritability estimate of 84.7 per cent with high genetic advance of 53.13 per cent was observed for this trait.

4.4.2 Character association studies

The genotypic and phenotypic correlation coefficients were determined to know the nature of relationship existing between seed yield and their component characters as well as the association among component characters themselves.

The degree of association of different quantitative characters with seed yield and also among themselves at genotypic and phenotypic levels are given in Table 18 and 19 respectively.

Seed yield per plant had highly significant positive association with number of pods per plant (0.762; 0.735), plant height (0.536; 0.463), test

Table 18. Genotypic correlations for different characters in recombinant inbred lines of chickpea

Characters	Number of primary branches	Days to 50% flowering	Number of pods per plant	Test weight (g)	Seed yield per plant (g)
Plant height (cm)	0.197*	-0.035	0.254**	0.647**	0.536**
Number of primary branches		0.045	0.399**	0.030	0.287**
Days to 50% flowering			0.013	0.127	0.025
Number of pods per plant				-0.130	0.762**
Test weight (g)					0.415**

*, ** Significant at 5 and 1% level respectively.

Table 19. Phenotypic correlations for different characters in recombinant inbred lines of chickpea

Characters	Number of primary branches	Days to 50% flowering	Number of pods per plant	Test weight (g)	Seed yield per plant (g)
Plant height (cm)	0.158*	-0.025	0.237**	0.532**	0.463**
Number of primary branches		0.036	0.368**	0.043	0.248**
Days to 50% flowering			0.014	0.116	0.026
Number of pods per plant				-0.105	0.735**
Test weight (g)					0.410**

*, ** Significant at 5 and 1% level respectively.

weight (0.415; 0.410) and number of primary branches per plant (0.287; 0.248) at both genotypic and phenotypic levels. Significant positive association of plant height with number of pods per plant (0.254; 0.237), test weight (0.647; 0.532) and number of primary branches (0.197; 0.158) were observed at both genotypic and phenotypic level. Among the other traits significant genotypic and phenotypic correlations were recorded between number of primary branches and number of pods per plant (0.399; 0.368).

4.4.3 Path coefficient analysis

The path coefficient analysis at genotypic (Table 20) and phenotypic (Table 21) levels was worked out for seed yield per plant by taking component traits into consideration and the results are presented below.

The number of pods per plant (0.873; 0.809) and test weight (0.564; 0.493) recorded the highest direct positive effects, both at phenotypic and genotypic levels. Plant height had negative direct effect (-0.040) on seed yield at genotypic level while it was positive at phenotypic level (0.020). However, the values were low and negligible. The number of primary branches (-0.067; -0.072) per plant and days to 50% flowering (-0.057; -0.040) recorded low negative direct effects at both genotypic and phenotypic level. The highest indirect effect was recorded by test weight on seed yield via plant height at genotypic level, while it was by number of pods per plant via number of primary branches at phenotypic level (0.298).

Table 20. Genotypic path coefficient analysis of different characters on seed yield in recombinant inbred lines of chickpea

Character	Plant height (cm)	Number of primary branches	Days to 50% flowering	Number of pods per plant	Test weight (g)	Correlation with seed yield (g)
Plant height (cm)	-0.040	-0.013	0.002	0.222	0.365	0.536**
Number of primary branches	-0.008	-0.067	-0.003	0.348	0.017	0.287**
Days to 50% flowering	0.001	-0.003	-0.057	0.012	0.072	0.025
Number of pods per plant	-0.010	-0.027	-0.001	0.873	-0.074	0.762**
Test weight (g)	-0.026	-0.002	-0.007	-0.114	0.564	0.415**

Diagonal values indicate direct effect and other values indicate indirect effect.

** Significant at 1% level.

Table 21. Phenotypic path coefficient analysis of different characters on seed yield in recombinant inbred lines of chickpea

Character	Plant height (cm)	Number of primary branches	Days to 50% flowering	Number of pods per plant	Test weight (g)	Correlation with seed yield (g)
Plant height (cm)	0.020	-0.011	0.001	0.192	0.262	0.463**
Number of primary branches	0.003	-0.072	-0.001	0.298	0.021	0.248**
Days to 50% flowering	0.000	-0.003	-0.040	0.011	0.057	0.026
Number of pods per plant	0.005	-0.027	-0.001	0.809	-0.052	0.735**
Test weight (g)	0.010	-0.003	-0.005	-0.085	0.493	0.410**

Diagonal values indicate direct effect and other values indicate indirect effect.

** Significant at 1% level.

4.4.4 Identification of superior RILs

RILs which are significantly superior over national check A-1 and superior over high yielding parent K-850 are presented in Table 22 and 23 respectively. Among 164 RILs 29 recorded significantly higher yield compared to check A-1. Among the 29 lines, fourteen lines were resistant to wilt. Test weight, which is important contributor of seed yield was significantly higher compared to A-1 in all the RILs except three RILs. Twenty four lines had high yield than better parent K-850 and six recombinant inbred lines (6-28, 6-33, 6-37, 6-38, 6-83 and 6-131) among them recorded significantly higher yield per plant. Three recombinant inbred lines *viz.*, 6-28, 6-38 and 6-131 recorded significantly higher yield over both K-850 and A-1 and resistant to Fusarium wilt.

Table 22. Significantly superior genotypes over national check A-1

Sl. No.	Genotypes	Plant height (cm)	No. of primary branches	Days to 50% flowering	No. of pods Per plant	Test weight (g)	Seed yield per plant (g)	Wilt Reaction
1.	6-4	37.33	3.0	50.50	50.00	28.09	17.05	R
2.	6-6	34.67	3.2	61.00	45.66	29.16	15.10	S
3.	6-9	32.00	2.7	59.00	43.33	25.26	15.52	S
4.	6-25	36.83	2.8	44.50	65.17	25.22	17.03	S
5.	6-28	41.50	2.7	51.00	60.83	30.71	21.63	R
6.	6-30	37.33	3.0	50.00	70.16	20.07	16.80	R
7.	6-33	42.33	2.8	45.00	85.33	30.10	25.15	S
8.	6-37	37.33	3.6	47.00	98.00	24.45	22.47	S
9.	6-38	39.00	3.0	64.00	85.67	22.70	23.32	R
10.	6-40	37.33	3.3	46.00	62.33	23.82	15.20	S
11.	6-41	38.00	2.7	37.00	72.33	17.08	14.95	R
12.	6-42	40.33	3.2	51.00	57.33	30.09	16.25	S
13.	6-46	38.67	2.6	48.00	44.50	30.03	14.90	S
14.	6-67	42.33	3.0	49.50	51.83	27.63	15.23	S
15.	6-83	38.33	3.7	49.50	67.00	32.04	20.23	S
16.	6-85	44.00	3.3	48.50	65.00	32.26	18.32	S
17.	6-86	37.67	3.3	47.00	61.67	26.98	16.57	R
18.	6-94	39.67	2.8	47.50	61.17	26.05	15.92	S
19.	6-106	40.00	3.2	48.00	79.33	23.34	18.57	R
20.	6-108	39.33	3.2	53.50	59.83	30.35	17.28	S
21.	6-118	40.00	2.7	58.00	54.66	29.28	15.35	S
22.	6-123	40.00	3.5	47.00	56.67	28.79	15.98	R
23.	6-127	39.17	3.3	49.50	67.17	30.75	17.20	R
24.	6-130	39.33	3.0	48.00	51.83	32.01	18.67	R
25.	6-131	38.33	2.8	45.50	60.67	28.48	19.12	R
26.	6-132	40.16	3.0	47.50	61.17	22.20	16.55	R
27.	6-156	40.50	3.2	56.00	56.16	30.96	17.37	S
28.	6-165	40.33	3.2	49.50	55.83	28.73	15.20	R
29.	6-168	39.50	3.5	54.00	52.50	29.88	15.32	R
30.	K-850	43.50	3.5	58.00	57.66	28.86	15.23	S
31.	WR-315	36.50	2.8	46.00	38.66	17.02	07.21	R
32.	A-1	37.33	2.3	44.00	45.66	19.34	11.86	S
R : Resistant; S : Susceptible					CD	4.08	3.78	

Table 23. Superior genotypes over better parent K-850

Sl. No.	Genotypes	Plant height (cm)	No. of primary branches	Days to 50% Flowering	No. of pods per plant	Test weight (g)	Seed yield per plant (g)	Wilt reaction
1	6-4	37.33	3.0	50.50	50.00	28.09	17.05	R
2	6-9	32.00	2.7	59.00	43.33	25.26	15.52	S
3	6-25	36.83	2.8	44.50	65.17	25.22	17.03	S
4	6-28	41.50	2.7	51.00	60.83	30.71	21.63	R
5	6-30	37.33	3.0	50.00	70.16	20.07	16.80	R
6	6-33	42.33	2.8	45.00	85.33	30.10	25.15	S
7	6-37	37.33	3.6	47.00	98.00	24.45	22.47	S
8	6-38	39.00	3.0	64.00	85.67	22.70	23.32	R
9	6-42	40.33	3.2	51.00	57.33	30.09	16.25	S
10	6-67	42.33	3.0	49.50	51.83	27.63	15.23	S
11	6-83	38.33	3.7	49.50	67.00	32.04	20.23	S
12	6-85	44.00	3.3	48.50	65.00	32.26	18.32	S
13	6-86	37.67	3.3	47.00	61.67	26.98	16.57	R
14	6-94	39.67	2.8	47.50	61.17	26.05	15.92	S
15	6-106	40.00	3.2	48.00	79.33	23.34	18.57	R
16	6-108	39.33	3.2	53.50	59.83	30.35	17.28	S
17	6-118	40.00	2.7	58.00	54.66	29.28	15.35	S
18	6-123	40.00	3.5	47.00	56.67	28.79	15.98	R
19	6-127	39.17	3.3	49.50	67.17	30.75	17.20	R
20	6-130	39.33	3.0	48.00	51.83	32.01	18.67	R
21	6-131	38.33	2.8	45.50	60.67	28.48	19.12	R
22	6-132	40.16	3.0	47.50	61.17	22.20	16.55	R
23	6-156	40.50	3.2	56.00	56.16	30.96	17.37	S
24	6-168	39.50	3.5	54.00	52.50	29.88	15.32	R
25	K-850	43.50	3.5	58	57.66	28.86	15.23	S
26	WR-315	36.50	2.8	46	38.66	17.02	07.21	R
27	A-1	37.33	2.3	44	45.66	19.34	11.86	S
					CD	4.08	3.78	

R : Resistant ; S : Susceptible

Discussion

V. DISCUSSION

Despite considerable international investment in chickpea breeding, the annual growth rate (0.60 per cent) of productivity of the crop has not improved significantly. The annual growth rate of chickpea production is also very slow at 1.90 per cent in the last few decades. One of the main constraints for increasing yield in West Asia and North African countries which are the main producers of chickpea, is the susceptibility of the plant to foliar disease, Ascochyta blight (Saxena and Singh, 1987; Singh *et al.*, 1992; Kaiser, 1997) and in the Indian subcontinent to the vascular disease, Fusarium wilt caused by *Fusarium oxysporum* f.sp. *ciceri* (Podwick) Matuo sato (Srivastava *et al.*, 1984; Jimenez-Diaz *et al.*, 1993; Ratnaparkhe *et al.*, 1998b). Consequently, chickpea breeding in India aims at high yielding cultivars that combine long lasting resistance against Fusarium wilt with tolerance to drought.

Despite considerable international efforts to breed such lines conventionally, cultivars with long lasting resistance against the soil borne diseases are still lacking. The major problem encountered by breeders is the divergence of the pathogen and development and maintenance of uniform wilt sick plots. Screening of segregating populations in wilt sick plots, although is considered most efficient means of identifying wilt resistant segregants (Jimenez-Diaz *et al.*, 1993), plants grown in sick plots take several weeks to exhibit symptoms. The other problem of selection of resistant plants in the segregating population is the complexity of resistance which is controlled by three genes (Nene and Sheila, 1992; Singh *et al.*, 1992). This necessitates the exploration of more efficient selection and breeding strategies than are currently employed.

In recent years, the use of molecular markers has accelerated plant breeding in a number of areas including disease resistance. The identification of molecular markers or tags for *Fusarium* wilt resistance genes could lead to quicker assessment of resistance even at seedling stage and to overcome the need for maintaining virulent isolates of the wilt pathogen in the sick plots. Inheritance of resistance to race 1 of *Fusarium* wilt has been well established (Upadhyaya *et al.*, 1983a and b; Singh *et al.*, 1987a), which indicates that it is controlled by three genes H_1 , H_2 and H_3 and recessiveness at the first two loci together confer complete resistance. Two markers, CS-27₇₀₀ and UBC-170₅₅₀ have been identified previously that amplified fragments linked to *Fusarium* wilt race 1 resistance (Tullu, 1996; Mayer *et al.*, 1997). The primer pair CS27F/CS27R amplified a fragment linked to the H_1 allele of susceptibility to race 1 of *Fusarium* wilt and thus constitute Allele Specific Associated Primer (ASAP), whereas UBC-170F/UBC-170R produced a single band for both resistant and susceptible genotypes, thus demonstrating locus specificity. The classical Mendelian analysis of wilt resistance has shown that two major recessive genes h_1 and h_2 at different loci are mainly responsible for resistance to wilt in chickpea. Therefore selecting for the presence of both h_1 and h_2 , the breeders will be assured of selecting for major loci that govern resistance to wilt. Therefore markers for both the loci will greatly increase the speed and efficiency of conventional breeding. In this direction identification of DNA markers for H_2 locus offer the potential to detect resistance to wilt at seedling stage in the absence of wilt sick plots.

Random Amplified Polymorphic DNA (RAPD) markers, are being used extensively as markers of various traits in different crops. The RAPD

approach can expedite gene tagging. This is a flexible application of PCR, can also serve as a step towards greater characterization of DNA polymorphisms via tools such as ASAPs. RAPD analysis is effective in linkage analysis when used with designated crosses and F₂ derived RILs. After the pioneering work of Williams *et al.* (1990), the RAPD technique has become one of the essential tools for molecular breeding. One of the prominent achievements made by this technique is identification of numerous DNA markers linked to resistant genes in different crops such as resistance to downy mildew in lettuce (Michelmore *et al.*, 1991), stem rust resistance gene *Pg3* in oats (Penner *et al.*, 1993), gene for resistance to *Pseudomonas syringe* pv. *tomato* in tomato (Klein-Lankhorst *et al.*, 1991; Martin *et al.*, 1991) and most recently Fusarium wilt resistance genes in chickpea (Mayer *et al.*, 1997; Ratnaparkhe *et al.*, 1998a; Tullu *et al.*, 1998, 1999; Rubio *et al.*, 2003). It was precisely with this objective the present work was taken up and results of various experiments conducted during the course of investigation are discussed below.

5.1 DEVELOPMENT AND EVALUATION OF RILs FOR WILT REACTION

The parental genotypes for development of RILs were carefully chosen. The female parent K-850 ($h_1h_1H_2H_2h_3h_3$) and male parent WR-315 ($h_1h_1h_2h_2h_3h_3$) differed only for H_2 locus. The female K-850 with dominant allele at H_2 locus and recessive allele at H_1 locus is defined as a late wilter, while the male parent WR-315 with recessive alleles at all the three loci is considered as resistant. The wilt reaction of the parental lines was confirmed in sick plots of ICRISAT and also in pot culture experiments. Several previous studies have also shown similar reactions of chosen parents (Singh *et al.*, 1987a and b; Singh *et al.*, 1991). The

genetic analysis of wilt resistance in *Fusarium oxysporum* race 1 involving parents, F₁, F₂ and F₃ generations of crosses of chickpea cultivars K-850 with C-104 and JG-62 indicated a monogenic ratio from the cross K-850 with JG-62 and digenic ratio from the K-850 x C-104 cross. This revealed that K-850 carried a recessive allele for resistance ($h_1h_1H_2H_2h_3h_3$) at a locus different and independent of that carried by C-104 ($H_1H_1h_2h_2h_3h_3$) and recessive alleles at both loci together confer complete resistance (Singh *et al.*, 1987a). The recessiveness at all the three loci ($h_1h_1h_2h_2h_3h_3$) which makes WR-315 a resistant genotype was also well established (Tullu 1996; Mayer *et al.*, 1997).

After confirming the phenotype of K-850 and WR-315, they were crossed to develop the F₁ hybrid. The F₁ was advanced to F₂ and from F₂ onwards the material was handled following single seed descent (SSD) method up to F₆ generation and thus obtained 178 F₆ recombinant inbred lines (RILs). The RILs were increased to provide sufficient seed for evaluation of wilt resistance in wilt sick plots at ICRISAT, pot culture experiments at UAS, Dharwad and evaluation of many agronomic traits in replicated field experiments. The advantage of using RILs is that, contrary to the often used F₂ populations, dominant and codominant markers has a similar information content which allows the integration of rapidly applied RAPD into a framework of codominant markers like STMSs.

These RILs were screened for their reaction to race 1 of *Fusarium oxysporum* f.sp. *ciceri* in wilt sick plots at ICRISAT. The wilt sick plot at ICRISAT was uniformly infested with *Fusarium oxysporum* f. sp. *ciceri* race 1, which is evident from the death of highly susceptible check JG-62 in all the rows within 20 days. The check JG-62 was planted after every three

rows throughout the plot and in all the rows, the death of JG-62 was uniform across the plot. Similarly, the parental genotype K-850 showed late wilting (after 32 days) and WR-315 no wilting in all the rows throughout the plot. These results clearly indicate the uniform distribution of optimum inoculum throughout the plot to differentiate early, late and no wilting genotypes (Haware *et al.*, 1992). The phenotyping of RILs was carried out in such uniform sick plot and the reaction of RILs was recorded. After screening of RILs in wilt sick plot, a few lines were randomly selected and their reaction to wilt was confirmed in wilt sick pots at Department of GPB, UAS, Dharwad. Out of 44 RILs and parental lines evaluated in pots, 41 RILs showed similar reaction to wilt, thus confirmed the results of wilt sick plots at ICRISAT.

Out of 178 RILs, 84 were resistant to race 1 of *Fusarium* wilt and eighty lines were susceptible (late wilters) like K-850, whereas fourteen lines showed segregation. In susceptible (late wilters) RILs, the degree of susceptibility and time of wilting varied. Such variation could be due to single replication testing. Apart from this several QTLs with modifier effects are presumed to influence the degree of wilting (Vidya Gupta, 2004). The segregation in RILs could be due to the fact that they are from a plant that was heterozygous at the wilt resistance locus and continuing segregation within the RILs in the succeeding generations (Mayer *et al.*, 1997; Tekeoglu *et al.*, 2000). The data on resistance and susceptibility (late wilting) of RILs closely fit a 1:1 segregation ratio suggesting a single locus segregation of late wilting and no wilting in the cross of K-850 x WR-315, thus supporting the contention that the cultivars differ at the H_2 locus (Singh *et al.*, 1987b). Therefore, it is confirmed that the genotype K-

850 has two recessive alleles at H₁ and H₃ loci and dominant allele at H₂ locus. The proposed genotype of K-850 *i.e.*, h₁h₁H₂H₂h₃h₃ is validated in this study. Similarly the other parent WR-315 is resistant to wilt pathogen race 1 and proposed to contain recessive alleles at all the three loci (h₁h₁h₂h₂h₃h₃). The genes for resistance to different races of *Fusarium* wilt segregated in 1:1 ratio which is expected for a single gene as also evident from earlier studies (Mayer *et al.*, 1997; Ratnaparkhe *et al.*, 1998a; Tullu *et al.*, 1998, 1999; Tekeoglu *et al.*, 2000).

5.2 RAPD ANALYSIS OF PARENTAL GENOTYPES

Identification of markers or tags for wilt resistance in chickpea could lead to quicker assessment of resistance. Isozyme electrophoresis can provide relatively quick and easy access to useful and reliable markers. However, the total number of isozymes that can be readily analysed in plant tissue is usually fifty or less. Moreover, isozyme electrophoresis of chickpea has revealed insufficient polymorphism to be useful in finding tags for wilt resistance (Oram *et al.*, 1987; Tuwafe *et al.*, 1988; Gaur and Slinkard, 1990). A potentially much large number of markers can be resolved by the means of Restriction Fragment Length Polymorphisms (RFLPs). However, only limited RFLPs have been identified and added to the chickpea genetic map (Simon and Muehlbauer, 1997). The RILs of the interspecific cross were used to develop a genetic map of chickpea (Santra *et al.*, 2000). However the validity of these maps in the intraspecific crosses has not yet been proved. In the present study RAPD markers were used in an intraspecific cross to tag H₂ locus. Random amplified polymorphic DNA (RAPD) represents another method for identifying additional genetic variation within populations (Welsh and

McClelland, 1990; Williams *et al.*, 1990). There is virtually no limit to the number of primers that can be designed for use in RAPD reactions of magnitude larger than the total number of morphological or isozyme markers, leading to a much higher level of marker saturation on genetic map.

Differences between parental genotype and the markers to differentiate the parents are the two important criteria in gene tagging. Parental polymorphism is the basic requirement for tagging of any gene. In the present study, 123 oligonucleotide primers were used to identify the DNA marker that show difference between the late wilting (K-850) and resistant parents (WR-315). All the primers produced scorable amplified products except four *viz.*, OPK-01, OPK-03, OPK-20 and OPE-04. These primers may have special requirements for amplification in terms of PCR reagents or temperature profile since all the reaction parameters were identical for all the primers, differences in the clarity of the banding patterns are likely due to the specific requirements of a primer. Weeden *et al.* (1992) also reported variation in the efficiency of primer amplification. RAPD analysis of parental lines with 123 oligonucleotide primers yielded 875 and 882 amplified products in susceptible (K-850) and resistant (WR-315) parents respectively. Initially 23 primers produced polymorphic amplified products between the parents. After continuous repetition only ten primers produced consistent polymorphism between the parents. These primers produced reproducible banding profile during replicate amplifications. Such amplifications in chickpea using RAPD primers has been reported by several workers (Mayer *et al.*, 1997; Rubio *et al.*, 2003).

In general, the polymorphism either for isozyme marker or DNA marker is less in chickpea (Oram *et al.*, 1987; Tuwafe *et al.*, 1988; Gaur and Slinkard, 1990; Rubio *et al.*, 2003). However, the degree of polymorphism detected in parental lines in this study was comparatively higher than previously reported. The probability of finding polymorphism and a marker within a specified distance of a target gene depends on genome size, the number of primers screened and the sequence divergence in the region scanning the target gene. In the present study 1.60 per cent of PCR products involving 123 primers were different between parents. This is not surprising considering that the parental lines were within the cultivated species. However ten out of 123 primers (8.13 per cent) tested produced polymorphic bands.

Interestingly allele specific associated primers CS-27₇₀₀ and UBC-170₅₅₀ which were earlier identified as tags for wilt reaction (Mayer *et al.*, 1997) did not produce polymorphism between K-850 and WR-315. This might be due to the fact that Mayer *et al.* (1997) used C-104 and WR-315 as parents which were differing at H₁ locus and not at H₂ locus. The CS-27₇₀₀ was linked to susceptibility at H₁ locus and both the parental lines K-850 and WR-315 carry recessive resistant allele at H₁ locus and hence were not polymorphic. However, UBC-170₅₅₀ which was produced in both resistant and susceptible parents WR-315 and C-104 respectively (Mayer *et al.*, 1997) thus locus specific were not validated in this cross as well as in other crosses studied elsewhere (Vidya Gupta, 2004).

The homologous primer (12c gene family primer) that was related to resistance to race 2 of *Fusarium oxysporum* f.sp. *lycopersici* in tomato (Simons *et al.*, 1998) did not show polymorphism in chickpea parental

lines. The heterologous primers were specific to *12c* gene of tomato conferring resistance to race 2 of *Fusarium oxysporum* f.sp. *lycopersici*. The *12c* locus in tomato is a multigenic family. The DNA markers selected in one plant species may not be applicable in other (Sejji Murayama *et al.*, 1999).

5.3 IDENTIFICATION OF RAPD MARKERS LINKED TO WILT REACTION

5.3.1 Bulk Segregant Analysis (BSA)

Bulk Segregant Analysis (BSA) was used in this study to identify putative RAPD markers linked to resistance or susceptibility. Bulk segregant analysis involves screening for differences between two pooled DNA samples derived from a segregating population that originated from a single cross. Each pool or bulk contains individuals selected to have identical genotypes for a particular genome region (target locus or region) but random genotypes at loci unlinked to the selected region (Michelmore *et al.*, 1991). Therefore, the two resultant bulked DNA samples differ genetically only in the selected region and are seemingly heterozygous and monomorphic for all other regions. Bulk segregant analysis improved the efficiency of primer screening.

An equal volume of standardized DNA (ng/ μ l) from ten homozygous susceptible and ten homozygous resistant RILs were pooled to make susceptible (S) and resistant (R) bulks respectively. Out of ten primers which showed parental polymorphism, seven did not cosegregate closely with resistant and susceptible bulks. Only three primers amplified polymorphic fragment that were apparently linked to susceptibility. The

primer OPK-09 and OPK-13 of Kit K and OPA-07 of Kit A produced an extra band in susceptible parent and susceptible bulk also. Thus these three RAPD markers were apparently linked to late wilting (H_2 locus). Michelmore *et al.* (1991) identified three RAPD markers linked to downy mildew resistance gene in lettuce using BSA. In chickpea, Mayer *et al.* (1997) used BSA of F_3 plants of an intra specific cross (C-104 x WR-315) that produced random amplified polymorphic DNA (RAPD) markers associated with resistance to race 1 of Fusarium wilt. More recently Chantret *et al.* (2000) and Panday *et al.* (2002) used BSA to identify QTLs for powdery mildew resistance in wheat and anthracnose disease resistance in sorghum, respectively. The confirmation of the linkage of three RAPD markers with late wilting locus is necessary by screening individual progenies/lines of the mapping population. The putative markers identified during BSA were further confirmed in segregating test populations for net blotch in barley (Molnar *et al.*, 2000) and for anthracnose disease resistance in sorghum (Panday *et al.*, 2002).

5.3.2 Segregation of RAPD markers

Three primers (OPK-09, OPK-13 and OPA-07) which showed polymorphism in parental lines and BSA were used for amplification of 164 RILs. The progenies were scored for presence or absence of specific polymorphic band produced by each of the primer in parents and BSA. The 1.801 Kbp RAPD product of the primer OPK-09 was present in 87 lines and absent in 77 RILs. The χ^2 analysis of goodness fit for 1:1 ratio was non significant for this RAPD marker (OPK-09₁₈₀₁) in the RILs. The results indicate that the RAPD marker OPK-09₁₈₀₁ segregates as alleles at a single locus. Similarly, the RAPD markers, OPK-13₁₂₅₄ and OPA-07₁₆₄₄

also showed 1:1 segregation in RILs. The marker OPK-13₁₂₅₄ was present in 93 and absent in 71 while the other marker OPA-07₁₆₄₄ amplified in 91 lines and failed to amplify in 73 lines. Both the markers segregated as alleles at a single locus independently. The RAPD loci studied showed virtually complete agreement with expectations of Mendelian segregation, which is necessary for linkage analysis. The Mendelian inheritance of the RAPD markers indicated that most lines are relatively unbiased and show low heterozygosity. The lines would thus appear to constitute a suitable population for the analysis of h₂ locus of wilt resistance.

5.3.3 Linkage analysis and determination of distance between the RAPD markers and wilt reaction

In this study the wilt reaction and three RAPD markers clearly showed expected Mendelian segregation of 1:1 ratio independently. To know the linkage between RAPD markers and late wilting loci, RILs were classified into four categories based on presence and absence of specific amplified products and their reaction of wilt to examine for the independent assortment of two genes. The chi-square analysis for goodness of fit for 1:1:1:1 segregation of OPK-09₁₈₀₁ and wilt reaction indicated a significant χ^2 value (85.80) suggesting that the susceptibility and RAPD marker are linked. Similarly the χ^2 value (68.49) was significant for OPA-07₁₆₄₄ and wilt susceptibility indicating the linkage. However χ^2 value (3.268) was non significant for OPK-13₁₂₅₄ and wilt reaction indicating that OPK-13₁₂₅₄ and wilt reaction assort independently and there is no linkage between them in the RILs studied. Similar kind of results were recorded by Liu *et al.* (2002) who in their study found that out of nine primers showing polymorphisms between the powdery mildew

resistance and susceptible DNA pools of wheat, none was found to be linked to resistance gene when tested on individual progenies.

The exact distance between the RAPD markers and late wilting locus was calculated using map maker programme. The distance between late wilting locus (h_2) and OPK-09₁₈₀₁ was 8.90 cM and that with OPA-07₁₆₄₄ was 12.90 cM. The RAPD markers were found distributed on both the sides of the H_2 locus. These are the first RAPD markers linked to H_2 locus. However, it may be emphasized here that closer markers on both the sides are needed to start map based cloning. Both the markers were found in repulsion phase with resistance. In several lines resistance was associated with presence of RAPD markers. The presence of fragments amplified by OPK-09₁₈₀₁ in 15 and OPA-07₁₆₄₄ in 21 resistant RILs indicated that the marker is present in both coupling and repulsion phases with H_2 locus. Haley *et al.* (1993) also observed the absence of marker linked to susceptibility in the backcross progenies and RILs.

From this, DNA markers closely linked to both H_1 and H_2 loci of *Fusarium* wilt susceptibility will be available. By selecting for the presence of these two markers, breeder will be assured of identifying segregants carrying major loci that governs susceptibility to *Fusarium oxysporum* race 1. Inversely, the segregants without these DNA markers are the ones that carry resistant genes at two major loci. Therefore, these markers will greatly increase the speed and efficiency of conventional breeding for wilt resistance by reducing the need for subjective disease screening. These markers also offer the potential to detect resistance to wilt prior to seed germination.

The genetic studies on wilt resistance indicated that the recessive alleles at both H_1 and H_2 in homozygous condition confer complete resistance. Susceptibility linked ASAP marker for H_1 locus of resistance to *Fusarium oxysporum* race 1 (Mayer *et al.*, 1997) and DNA markers linked to resistance to races 4, 5 and 0 have been reported in chickpea (Ratnaparkhe *et al.*, 1998a and b; Tullu *et al.*, 1998, 1999; Tekeoglu *et al.*, 2000; Winter *et al.*, 2000; Rubio *et al.*, 2003). In the present study RAPD markers linked to h_2 locus of wilt resistance to race 1 of *Fusarium oxysporum* have been identified. Thus both these markers can be utilized for early selection of completely resistant genotypes in breeding programmes. These markers will also be helpful in pyramiding of wilt resistant genes to achieve broad based resistance leading to wide adaptability of genotypes.

Due to dominant nature of RAPD markers, it is not possible to differentiate between homozygous and heterozygous plants for linked allele. However, the individuals of a resistant phenotype is considered as homozygous for the recessive alleles. In this study, both the DNA markers linked to dominant susceptibility alleles of h_2 locus as that of CS-27₇₀₀ linked to H_1 locus (Mayer *et al.*, 1997). Therefore, the potential exists for a breeder to pick individuals that are not only homozygous for susceptibility but also heterozygous for susceptibility in the segregating generations. All the susceptible plants (both homozygous and heterozygous) can be easily eliminated in the early generation itself at a very early stage. Therefore, these markers will be very useful in the identification of homozygous and completely resistant plants through the process of elimination of both homozygous and heterozygous susceptible plants.

5.3.4 Verification of RAPD markers with wilt susceptibility in commonly used parental lines of chickpea

Six commonly used parental lines were tested for their reaction to race 1 of *Fusarium* wilt. Four among six were late wilting (BG-256, NCV-10, K-850 and A-1), one was early wilting (JG-62) and WR-315 was resistant. The RAPD marker OPA-07₁₆₄₄ was present in all five susceptible parental lines validating the marker in these lines. Similarly, Ravikumar *et al.* (2003) validated the susceptibility marker CS-27₇₀₀ in commonly used parental lines. Another marker OPK-09₁₈₀₁ was present in two late wilting genotypes K-850 and BG-256 and absent in the other two, indicating that the marker might be present in both coupling or repulsion phases with the wilt susceptible H₂ locus in chickpea germplasm. Though similar observations were made by Mayer *et al.* (1997) and Tullu *et al.* (1998), these results need to be further confirmed in many germplasm lines as the genotypes used here were very few among the array of chickpea germplasm and they did not represent the available range of germplasm lines and cultivars. Presence of both the RAPD markers in late wilting genotype BG-256, clearly indicate that the genotype of BG-256 as h₁h₁H₂H₂h₃h₃ similar to that of K-850.

5.4 GENETIC VARIABILITY STUDIES IN RILs FOR SEED YIELD AND YIELD COMPONENTS

Historically, yield improvement in any crop can be traced back to exploitation of new source of germplasm or traits that harness genetic variability. Progress in breeding programme depends upon 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 genomes interspecific crosses were preferred to maximise 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 to further map the RAPD marker linked to H₂ locus.

An understanding of the nature of association of yield component characters like plant height, number of primary branches, days to 50 per cent flowering and test weight with seed yield is expected to provide new dimension in selection strategy. The results of genetic variability studies of present investigation in RILs of K-850 (late wilter) x WR-315 (resistant) cross for seed yield and yield components are discussed below.

5.4.1 Variability studies

The analysis of variance indicated highly significant variation for number of primary branches, days to 50 per cent flowering, number of pods per plant, test weight and seed yield per plant and significant variation for plant height. Such high variability has been observed in RILs of JG 62 x ICCV-2 cross (Anupama, 2001), in 30 RILs of two crosses

(Chander *et al.*, 2001) and in forty different genotypes (Lokendrakumar *et al.*, 1999). 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 of variation observed was wider for number of pods per plant, days to 50 per cent flowering, test weight, seed yield per plant and number of primary branches per plant. While narrow range was observed for plant height at harvest, this might be due to less difference in the plant height of parents K-850 and WR-315, which were differing at H₂ locus of *Fusarium oxysporum* f. sp. *ciceri* race 1 resistance.

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 cross, high variability was observed for number of pods per plant and seed yield per plant followed by moderate variability for test weight, number of primary branches and plant height. The results are in conformity with the reports of Yaman *et al.* (1997) in the genetic resource material collected from Argean region for pod number and seed yield per plant, Upadhyaya *et al.* (2002) in chickpea core collection consisting of 1956 accessions for test weight, Arora (1991) for number of primary branches and Patil (1996) in *desi* x *desi*, *desi* x *kabuli* and *kabuli* x *kabuli* crosses of chickpea for plant height. Least variability was observed for days to 50 per cent flowering contradicting to the

earlier reports who observed moderate variability for days to 50 per cent flowering (Dasgupta *et al.*, 1992; Rao *et al.*, 1994).

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 the present study, among which highest was for days to 50 per cent flowering, number of pods per plant followed by seed yield per plant, number of primary branches and least was for test weight and plant height. High estimates of heritability along with high variation was observed for number of pods per plant and seed yield per plant. Similar results were also reported by Mishra *et al.* (1994) in their evaluation of thirty two genetically diverse genotypes of chickpea. Chavan *et al.* (1994) observed high heritability for days to 50 per cent flowering and number of primary branches per plant in seventy genotypes of chickpea collected from all over India.

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 percentage of mean was recorded for number of pods per plant, seed yield, test weight and number of primary branches. These results are in accordance with the findings of Patil (1996) where he studied inheritance of yield and yield components in *desi* x *desi*, *desi* x *kabuli* and *kabuli* x *kabuli* crosses of chickpea. Moderate genetic advance as expressed in percentage of mean was observed for days to 50 per cent flowering and

plant height. Similar results were also reported by Arora and Jeena (2000). 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, number of pods per plant, test weight and number of primary branches had high heritability estimates coupled with high genetic advance indicating the substantial contribution of additive genetic variance in the expression of these characters. An overall observations of the findings of heritability estimates and genetic advance, it could be concluded that the selection criteria based on seed yield per plant, number of pods per plant, test weight and number of primary branches will be useful for the improvement of seed yield in chickpea crosses.

5.4.2 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 about an improvement in the other. But if they are negatively associated improvement in one character may adversely effect 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 important characters without sacrificing the gain in the other.

In the present investigation, five yield components were investigated and their relationship with yield as well as among themselves were examined using correlation analysis. A critical look at genotypic and phenotypic correlation tables, in general indicates that the genotypic correlation coefficients were slightly higher than their respective phenotypic correlation coefficients. It indicated the environmental component had influence on association of the characters. Similar were the findings of Jivani and Yadavendra (1988).

It was noticed that seed yield was positively correlated with all the traits studied both at phenotypic and genotypic levels. Similar results were obtained by Arora and Jeena (1999) in their association analysis for yield and other quantitative traits in forty three chickpea genotypes. However, based on the magnitude of correlation values number of pods per plant, plant height, test weight and number of primary branches were regarded as very closely related to yield. Many reports indicate the association of number of pods per plant with seed yield (Naseem *et al.*, 1995; Singh *et al.*, 1995; Patil, 1996; Rao, 1998). Therefore they can be considered as principle yield determining factors. These traits are easily visible and can be used as selection criteria to score large number of plants in a breeding programme.

Association among important yield components indicated that number of pods per plant had significant and positive association with plant height and number of primary branches. These results are in agreement with earlier findings of Lokendrakumar *et al.* (1999). Highly significant association was observed between test weight and plant height. However, correlation of test weight with number of pods was negative.

Similar results were reported by Singh *et al.* (2001). Such a situation calls for breaking of negative correlation so that better gains in yield can be realised by selection of both the traits *i.e.*, test weight and number of pods. Among other associations plant height had positive association with number of primary branches, which in turn was associated with pods per plant which was a major yield component. Therefore from the present study it is clear that pods per plant, plant height, test weight and number of primary branches were the major yield components in chickpea.

5.4.3 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 (path coefficients) (Yadava *et al.*, 2002).

In the present study, path analysis was used to workout the direct and indirect effects of five characters on seed yield. These 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 four traits which had significant positive correlation with seed yield only number of pods per plant and test weight had high positive direct effects on seed yield. Similar results were also obtained by Arora and Jeena (1999) for number of pods per plant and for test weight by Reddy and Rao (1988) and Yadava *et al.* (2002).

Plant height and number of primary branches had significant positive correlation with seed yield but they had negative direct effect on seed yield. This is mainly due to their positive indirect contribution through number of pods per plant and test weight. Thus for selection of high yielding genotypes in chickpea, number of pods per plant and test weight should be used as primary selection criteria.

5.4.4 Identification of superior RILs

The high yielding RILs which were significantly superior over national check A-1 and better performing than better parent K-850 were identified. Among 164 RILs, 29 recorded significantly higher yield compared to A-1. Out of these 29, 24 had higher seed yield than better parent K-850. Except five lines all the 29 superior RILs had significantly higher test weight than A-1. Six recombinant inbred lines (6-28, 6-33, 6-37, 6-38, 6-83 and 6-131) among them recorded significantly higher yield per plant. Three recombinant inbred lines *viz.*, 6-28, 6-38 and 6-131 recorded significantly higher yield over both K-850 and A-1 and resistant to Fusarium wilt.

The high yielding capacity of these 29 recombinant inbred lines was mainly due to higher number of pods, high test weight and higher plant height. Among the high yielding RILs, 14 recorded resistance against race 1 of Fusarium wilt. A-1 is the most popular variety in Karnataka with more than 80 per cent area. However, A-1 is susceptible to wilt and a large area is affected by wilt. Therefore, these superior RILs with high yield over A-1 and resistance to wilt 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. Among the superior RILs, several resistant lines with desirable test weight and other yield components were observed which could serve as sources of resistance along with desirable yield components.

5.5 FUTURE LINE OF WORK

- 1) The RAPD markers identified in the present study are based on only one cross. The marker has to be tested across a large number of genotypes and crosses to validate the marker
- 2) After verification of marker attempts may be made to develop SCAR (Sequence Characterized Amplified Region) marker for this locus
- 3) High yielding and wilt resistant RILs are to be tested in large scale and multilocation trials to confirm their performance
- 4) The markers identified for h_1 and h_2 loci may be utilised for gene pyramiding

Summary

VI. SUMMARY

India is the largest producer of chickpea accounting for 69 per cent of world's total production. Despite intensive research efforts in crop improvement, the annual growth rate of productivity is low (0.60 per cent). One of the main constraints for increasing yield is the susceptibility of presently grown cultivars to many biotic and abiotic stresses. Among biotic factors, Fusarium wilt caused by *Fusarium oxysporum* f.sp. *ciceri* is reported to be important in many countries of Asia, Africa, North and South America with yield losses ranging from 10-90 per cent.

Consequently, chickpea breeding in India aims at high yielding cultivars that combine long lasting resistance against Fusarium wilt. However, the major problem encountered for the development of high yielding wilt resistant genotypes is the divergence of the pathogen and development and maintenance of uniform wilt sick plots. The 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 maintenance of uniform sick plots is very difficult, this necessitates the exploration of more efficient selection and breeding strategies than that are currently employed. The identification of molecular markers for Fusarium wilt resistance could lead to quicker assessment of resistance even at seedling stage thereby overcome the need for maintaining virulent isolates of the wilt pathogen in the sick plots.

Inheritance of resistance to race 1 (most prevalent and virulent race in India) of Fusarium wilt is well established, three genes H₁, H₂ and H₃

and recessiveness at the first two loci (H_1 and H_2) together confer complete resistance. Two specific primers, CS-27₇₀₀ and UBC-170₅₅₀ have been identified previously that are linked to H_1 locus of *Fusarium* wilt resistance. Identification of DNA markers for h_2 locus offer the potential to detect resistance to wilt at seedling stage in the absence of wilt sick plots, which will greatly increase the speed and efficiency of conventional breeding. In the present study attempts were made to identify DNA markers linked to h_2 locus of *Fusarium* wilt race 1 resistance by utilizing the RILs developed by crossing a late wilter (K-850) with a resistant (WR-315) genotype in chickpea. The salient findings of the investigations are presented below.

1. The parental genotypes for development of RILs were carefully chosen. The female K-850 ($h_1h_1H_2H_2h_3h_3$) with dominant allele at h_2 locus and recessive allele H_1 locus is defined as late wilter, while the male parent WR-315 ($h_1h_1h_2h_2h_3h_3$) with recessive alleles at all the three loci is considered as resistant. The wilt reaction of the parental lines were confirmed in sick plots at ICRISAT and also in wilt sick pots at UAS, Dharwad.
2. The susceptible K-850 was crossed to resistant WR-315 to develop the F_1 . The F_1 was advanced to F_2 and from F_2 to F_6 , the generations were advanced by following single seed descent method, thus obtained 178 F_6 recombinant inbred lines (RILs).
3. The RILs were screened for their reaction to race 1 of *Fusarium oxysporum* f.sp. *ciceri* in uniformly infested wilt sick plot at ICRISAT. The uniform infection was evident by the wilting of susceptible check JG-62 within 20 days. The check was planted after every three rows

throughout the experimental block. Among the 178 RILs tested for wilt reaction in wilt sick plots, 84 were resistant, 80 were late wilting and 14 showed segregation. The segregating were eliminated from further analysis.

4. Wilt reaction of RILs was further confirmed in wilt sick pots at Department of Genetics and Plant Breeding, University of Agricultural Sciences, Dharwad. Forty one out of forty four randomly selected RILs showed similar reaction as that of wilt sick plot.
5. The data on resistant (84 RILs) and late wilting (80 RILs) of RILs closely fit a 1:1 (susceptible : resistant) segregation ratio suggesting a single locus segregation of late wilting in the cross of K-850 x WR-315, thus supporting the contention that parents differ at the h_2 locus.
6. 123 oligonucleotide primers were used to identify the polymorphic DNA marker between the parents. All the primers except four produced scorable amplified products yielding 875 and 882 amplified products in susceptible (K-850) and resistant (WR-315) parents respectively. After replicated amplifications only ten primers produced polymorphism between the parents.
7. Allele specific associated primers CS-27₇₀₀ and UBC-170₅₅₀ which were earlier identified as tags for H_1 locus of wilt resistance (Mayer *et al.*, 1997) did not produce polymorphism between K-850 and WR-315 as they did not differ at H_1 locus. The homologous primer (12 C gene family primer) that was related to resistance to race 2 of *Fusarium oxysporum* f.sp. *lycopersici* in tomato (Simons *et al.*, 1998) also did not show polymorphism.

8. Bulk segregant analysis was carried out by bulking the DNA of ten homologous susceptible and resistant RILs. Three primers (OPK-09, OPK-13 and OPA-07) out of ten, produced extra amplified products in susceptible parent and susceptible bulk. Thus these three RAPD markers were apparently linked to late wilting (h_2) locus. The remaining primers did not show polymorphism in BSA.
9. The DNA of 164 RILs was used to amplify with three putatively linked RAPD markers. The marker OPK-09₁₈₀₁ was present in 87 lines, OPK-13₁₂₅₄ in 93 and OPA-07₁₆₄₄ in 91 RILs out of 164 RILs. All the three primers showed a segregation ratio of 1:1 (presence : absence) in the RILs confirming single locus segregation for each of these three markers.
10. The chi-square analysis for goodness of fit for 1:1:1:1 segregation of OPK-09₁₈₀₁ and wilt reaction indicated a significant χ^2 value suggesting that the susceptibility and RAPD marker were linked. Similarly OPA-07₁₆₄₄ was also linked to late wilting locus.
11. The exact distance between the RAPD markers and late wilting locus was calculated using Map Maker programme. The distance between late wilting locus (H_2) and OPK-09 was 8.90 cM and that with OPA-07 was 12.90 cM. It appears that the markers were shown to be present on either side of the gene.
12. For validation, the two primers were tested on 6 genotypes/parental lines. Among two primers, OPA-07₁₆₄₄ amplified in all the wilt susceptible parental lines while OPK-09₁₈₀₁ was present in two late wilting parental lines (BG-256 and K-850) and absent in two susceptible genotypes.

13. Analysis of variance for all the traits under study indicated significant variation among the RILs for all the traits. Very high variability was observed for number of pods per plant and seed yield per plant followed by moderate variability for test weight, number of primary branches and plant height. This abundant variability reflects on the possible chances of utilizing these immortal RILs for mapping of valuable genes.
14. Seed yield per plant, number of pods per plant, test weight and number of primary branches had 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.
15. In general it was observed that the genotypic correlation coefficients were higher than the phenotypic correlation coefficients. Number of pods per plant, plant height, test weight and number of primary branches were positively associated with seed yield, among these only two traits (pod number and test weight) had high positive direct effects on seed yield. For selection of high yielding chickpea genotypes, number of pods per plant and test weight should be used as primary selection criteria.
16. Among the RILs, 29 recorded significantly higher yield than national check A-1. Fourteen of these recorded resistance against race 1 of Fusarium wilt. Three recombinant inbred lines viz., 6-28, 6-38 and 6-131 recorded significantly higher yield over both K-850 and A-1 and resistant to Fusarium wilt.

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Appendix

Appendix I. Mean values of RILs for six quantitative traits in chickpea

Sl. No.	Genotypes	Plant height (cm)	No. of primary branches	Days to 50% flowering	No. of pods Per plant	Test weight (g)	Seed yield per plant (g)
1.	6-1	36.83	2.7	48.50	34.50	27.42	10.35
2.	6-2	30.83	2.5	45.50	24.16	25.61	07.80
3.	6-3	33.00	2.3	46.50	41.83	23.40	13.44
4.	6-4	37.33	3.0	50.50	50.00	28.09	17.05
5.	6-5	30.17	3.3	49.50	45.33	19.21	09.70
6.	6-6	34.67	3.2	61.00	45.66	29.16	15.10
7.	6-7	35.50	2.8	60.00	51.00	25.93	13.65
8.	6-8	31.00	3.0	60.00	35.17	27.92	07.70
9.	6-9	32.00	2.7	59.00	43.33	25.26	15.52
10.	6-10	22.17	2.5	58.00	29.67	23.61	09.78
11.	6-12	32.50	2.8	47.50	50.33	22.70	12.48
12.	6-13	31.16	3.1	42.00	43.67	15.93	09.02
13.	6-14	34.66	2.5	54.00	32.66	26.14	11.47
14.	6-15	33.33	3.3	50.00	37.33	27.63	10.72
15.	6-16	33.83	3.0	49.00	39.83	26.30	13.69
16.	6-17	37.00	2.7	50.00	43.17	24.73	12.20
17.	6-18	35.67	3.0	49.00	35.50	29.31	11.68
18.	6-19	32.50	2.5	45.00	38.67	20.85	10.48
19.	6-20	41.17	3.1	48.50	45.33	25.29	14.08
20.	6-21	42.00	3.0	48.50	68.50	20.97	13.53
21.	6-24	33.33	2.6	44.00	53.83	21.39	13.70
22.	6-25	36.83	2.8	44.50	65.17	25.22	17.03
23.	6-26	28.67	2.6	47.50	16.17	16.29	03.52
24.	6-28	41.50	2.7	51.00	60.83	30.71	21.63
25.	6-29	36.00	2.2	51.00	41.33	23.03	12.15
26.	6-30	37.33	3.0	50.00	70.16	20.07	16.80
27.	6-31	31.67	3.0	47.50	60.83	17.46	13.18
28.	6-32	31.83	3.0	51.00	45.17	17.07	12.30
29.	6-33	42.33	2.8	45.00	85.33	30.10	25.15
30.	6-34	31.00	4.0	54.00	59.17	21.19	13.30
31.	6-35	39.00	3.3	51.00	86.83	19.52	13.75
32.	6-36	31.00	2.7	48.00	75.17	17.72	11.93
33.	6-37	37.33	3.6	47.00	98.00	24.45	22.47
34.	6-38	39.00	3.0	64.00	85.67	22.70	23.32
35.	6-39	31.50	2.7	62.50	35.33	24.33	09.44
36.	6-40	37.33	3.3	46.00	62.33	23.82	15.20
37.	6-41	38.00	2.7	37.00	72.33	17.08	14.95
38.	6-42	40.33	3.2	51.00	57.33	30.09	16.25
39.	6-43	38.33	3.6	51.50	67.66	22.80	13.60
40.	6-44	32.33	3.0	59.00	56.50	20.87	08.17
41.	6-46	38.67	2.6	48.00	44.50	30.03	14.90
42.	6-47	32.00	2.0	47.00	35.66	19.54	09.55
43.	6-48	39.83	2.5	52.00	35.67	26.67	11.70

Contd.....

Sl. No.	Genotypes	Plant height (cm)	No. of primary branches	Days to 50% flowering	No. of pods Per plant	Test weight (g)	Seed yield per plant (g)
44.	6-50	39.00	2.7	56.00	46.50	24.50	12.60
45.	6-51	31.83	2.6	50.00	24.33	25.89	06.22
46.	6-52	34.50	2.5	51.00	32.67	30.02	10.28
47.	6-53	31.66	2.7	52.00	38.33	20.38	07.58
48.	6-54	38.83	2.8	51.00	29.00	31.24	11.50
49.	6-55	35.00	3.0	47.00	37.66	29.46	11.92
50.	6-56	34.67	2.6	53.00	38.67	26.43	11.43
51.	6-57	30.92	3.2	51.50	37.66	24.21	10.22
52.	6-58	39.16	2.8	47.00	40.83	31.54	13.48
53.	6-59	32.83	2.8	47.00	31.50	18.11	08.12
54.	6-60	35.50	3.2	45.50	45.00	25.09	11.92
55.	6-61	31.83	2.3	41.50	43.83	16.94	09.73
56.	6-62	34.17	3.5	57.00	40.83	23.15	10.13
57.	6-63	34.16	3.3	47.00	24.83	23.09	08.45
58.	6-64	37.50	2.8	48.50	35.16	32.35	12.42
59.	6-65	35.83	2.8	47.00	24.33	28.33	08.88
60.	6-66	34.33	3.0	48.00	25.33	26.90	07.83
61.	6-67	42.33	3.0	49.50	51.83	27.63	15.23
62.	6-68	31.33	2.5	45.00	31.67	19.43	07.20
63.	6-69	36.66	2.7	47.00	35.00	23.54	08.52
64.	6-70	33.83	2.8	51.00	38.00	18.63	09.30
65.	6-71	37.33	3.2	48.50	43.67	25.89	11.88
66.	6-72	37.33	3.0	51.50	40.83	29.67	13.35
67.	6-73	31.17	2.5	54.00	32.33	18.37	05.73
68.	6-74	35.16	3.5	48.50	26.83	21.91	07.95
69.	6-75	32.00	3.1	47.00	54.67	18.31	09.87
70.	6-76	42.83	2.8	49.00	41.16	30.39	13.00
71.	6-77	43.50	3.2	49.00	45.67	23.12	11.35
72.	6-78	41.00	3.0	48.00	32.50	26.20	10.30
73.	6-79	44.66	3.6	49.50	44.00	25.88	09.77
74.	6-80	39.83	3.0	47.00	46.50	28.77	12.53
75.	6-81	41.66	3.0	49.00	42.66	30.31	11.77
76.	6-82	29.16	3.1	51.00	67.33	22.68	13.80
77.	6-83	38.33	3.7	49.50	67.00	32.04	20.23
78.	6-84	34.67	3.2	46.00	56.67	19.83	11.77
79.	6-85	44.00	3.3	48.50	65.00	32.26	18.32
80.	6-86	37.67	3.3	47.00	61.67	26.98	16.57
81.	6-88	33.17	3.3	48.50	57.83	19.06	11.05
82.	6-89	37.50	3.0	48.50	46.33	27.09	11.13
83.	6-90	29.33	2.6	48.00	41.50	16.38	07.93
84.	6-91	38.00	2.6	49.00	53.16	25.54	11.20
85.	6-92	36.67	3.0	50.50	44.66	27.64	12.78
86.	6-93	36.30	2.5	51.00	28.33	27.85	07.48
87.	6-94	39.67	2.8	47.50	61.17	26.05	15.92

Contd.....

Sl. No.	Genotypes	Plant height (cm)	No. of primary branches	Days to 50% flowering	No. of pods Per plant	Test weight (g)	Seed yield per plant (g)
88.	6-95	33.83	3.2	60.00	59.66	18.34	10.48
89.	6-96	31.83	2.6	58.00	63.83	18.12	13.18
90.	6-97	36.33	2.8	53.00	43.66	29.27	13.93
91.	6-98	39.83	2.3	47.00	29.00	25.06	07.55
92.	6-99	34.50	2.7	50.50	39.50	25.70	09.90
93.	6-100	34.33	2.8	48.50	36.33	30.30	10.32
94.	6-101	27.33	3.0	48.00	45.00	18.32	09.07
95.	6-102	37.66	3.1	50.00	50.33	27.04	13.73
96.	6-103	44.17	3.0	48.50	42.83	28.73	12.08
97.	6-104	32.66	3.3	47.50	44.50	19.63	09.98
98.	6-105	30.66	3.0	47.00	46.17	20.11	08.87
99.	6-106	40.00	3.2	48.00	79.33	23.34	18.57
100.	6-107	34.00	3.0	56.50	46.00	20.42	10.03
101.	6-108	39.33	3.2	53.50	59.83	30.35	17.28
102.	6-109	37.83	2.8	56.50	43.17	27.89	11.95
103.	6-110	39.17	3.0	48.00	46.50	25.46	10.80
104.	6-112	40.00	3.0	47.50	44.67	24.73	11.18
105.	6-113	38.33	2.3	47.50	43.33	25.40	10.02
106.	6-114	30.33	3.6	47.00	62.17	20.09	11.28
107.	6-115	31.50	2.0	62.00	50.50	21.71	10.08
108.	6-116	33.00	3.0	57.00	55.83	21.27	12.45
109.	6-117	33.50	2.7	49.50	55.33	27.23	13.45
110.	6-118	40.00	2.7	58.00	54.66	29.28	15.35
111.	6-119	42.66	3.0	53.00	41.17	28.23	10.37
112.	6-120	32.33	2.8	48.50	44.66	25.26	11.25
113.	6-121	34.00	2.6	58.00	47.67	25.46	11.20
114.	6-122	41.33	3.0	56.50	73.33	21.67	12.33
115.	6-123	40.00	3.5	47.00	56.67	28.79	15.98
116.	6-124	31.66	3.3	47.00	56.50	15.70	09.30
117.	6-125	33.17	3.3	49.50	73.67	18.18	13.42
118.	6-126	39.50	3.0	46.00	48.13	27.16	12.40
119.	6-127	39.17	3.3	49.50	67.17	30.75	17.20
120.	6-129	38.17	3.5	48.50	47.50	29.23	12.70
121.	6-130	39.33	3.0	48.00	51.83	32.01	18.67
122.	6-131	38.33	2.8	45.50	60.67	28.48	19.12
123.	6-132	40.16	3.0	47.50	61.17	22.20	16.55
124.	6-134	30.83	3.2	50.00	51.00	25.39	11.67
125.	6-135	41.00	3.8	52.00	68.67	21.33	12.78
126.	6-136	32.00	2.5	49.50	36.83	23.93	10.27
127.	6-137	36.33	2.5	47.50	33.50	23.25	07.28
128.	6-138	35.83	2.7	47.50	27.00	23.79	06.72
129.	6-139	38.33	3.3	55.00	46.50	23.15	10.46
130.	6-140	35.00	2.3	56.00	29.83	24.56	08.77
131.	6-141	36.67	2.8	50.00	31.00	31.24	09.45

Contd.....

Sl. No.	Genotypes	Plant height (cm)	No. of primary branches	Days to 50% flowering	No. of pods Per plant	Test weight (g)	Seed yield per plant (g)
132.	6-142	36.50	3.0	46.50	25.33	22.27	06.43
133.	6-143	35.17	3.0	54.00	50.66	19.23	10.40
134.	6-144	34.17	3.0	55.00	36.50	20.64	08.83
135.	6-145	33.00	3.0	47.00	41.67	26.21	11.78
136.	6-146	36.50	3.0	48.00	36.66	26.22	12.15
137.	6-147	33.33	2.7	52.00	26.83	19.59	06.17
138.	6-148	30.83	2.8	47.00	49.16	24.97	12.55
139.	6-149	32.50	2.3	49.50	23.33	28.28	07.04
140.	6-151	40.00	2.7	51.00	37.50	30.79	12.69
141.	6-153	40.66	3.0	50.50	38.33	28.56	11.77
142.	6-155	35.00	3.3	58.00	48.83	21.11	09.55
143.	6-156	40.50	3.2	56.00	56.16	30.96	17.37
144.	6-158	31.33	2.3	46.50	44.33	18.95	10.30
145.	6-159	38.50	3.2	58.00	35.33	29.44	10.02
146.	6-160	39.50	2.7	48.50	37.00	30.56	12.79
147.	6-161	36.00	3.6	51.50	41.33	21.19	09.00
148.	6-162	34.33	2.3	45.00	39.83	22.53	10.00
149.	6-163	30.67	3.5	61.50	47.16	19.74	09.05
150.	6-164	31.33	3.0	46.50	37.00	18.00	07.45
151.	6-165	40.33	3.2	49.50	55.83	28.73	15.20
152.	6-166	44.00	2.8	56.50	32.33	28.09	12.18
153.	6-167	36.00	2.8	54.00	23.83	28.97	07.97
154.	6-168	39.50	3.5	54.00	52.50	29.88	15.32
155.	6-169	39.33	2.6	47.00	44.83	20.87	10.72
156.	6-170	28.50	3.0	46.50	42.00	15.50	06.70
157.	6-171	34.50	3.2	48.50	46.00	26.12	11.04
158.	6-172	37.50	3.3	48.50	44.50	27.66	13.04
159.	6-173	35.00	3.3	47.00	42.33	22.90	10.52
160.	6-174	35.00	3.7	48.00	45.16	23.41	10.05
161.	6-175	31.00	3.0	49.00	35.83	20.19	10.12
162.	6-176	36.00	3.3	46.50	43.33	22.72	10.45
163.	6-177	36.50	2.8	48.00	35.67	26.54	10.28
164.	6-178	31.00	2.6	37.00	49.17	16.59	09.10
165.	K-850	43.50	3.5	58.00	57.66	28.86	15.23
166.	WR-315	36.50	2.8	46.00	38.66	17.02	07.21
167.	A-1	37.33	2.3	44.00	45.66	19.34	11.86

**RAPD ANALYSIS FOR TAGGING h_2 LOCUS OF WILT
(*Fusarium oxysporum* f.sp. *ciceri*) RESISTANCE IN
CHICKPEA (*Cicer arietinum* L.)**

THIPPESWAMY S.

2004

Dr. R. L. RAVIKUMAR
MAJOR ADVISOR

ABSTRACT

The major constraint for increasing yield in chickpea is the susceptibility of presently grown cultivars to *Fusarium* wilt. Screening of large segregating populations and development and maintenance of uniform wilt sick plots are the major problems encountered for the development of high yielding and wilt resistant genotypes. Identification of molecular markers for resistance will solve such problems and increase the efficiency of conventional breeding. The present investigation was carried out at College of Agriculture, Dharwad to identify RAPD markers linked to h_2 locus of *Fusarium* wilt race 1 resistance. The RILs developed by crossing a late wilter, K-850 ($h_1h_1H_2H_2h_3h_3$) with a resistant genotype WR-315 ($h_1h_1h_2h_2h_3h_3$) were screened for their reaction to race 1 of *Fusarium oxysporum* f.sp. *ciceri* in wilt sick plot at ICRISAT, Hyderabad and was further confirmed in wilt sick pots in the glass house. The RILs showed a 1:1 segregation ratio for wilting (susceptible) and no wilting (resistant) suggesting single locus segregation of late wilting.

Ten out of 123 oligonucleotide primers produced polymorphism between the parents. In bulk segregant analysis three primers (OPK-09, OPK-13 and OPA-07), produced an extra amplified product in susceptible parent and susceptible bulk. The linkage analysis suggested two RAPD markers (OPK-09₁₈₀₁ and OPA-07₁₆₀₄) are linked to h_2 locus of susceptibility. The distance between h_2 locus and OPK-09₁₈₀₁ was 8.2 cM and that with OPA-07₁₆₀₄ was 12.90 cM on either side of the gene.

Analysis of variance among the RILs indicated high to moderate variability for seed yield, number of pods, test weight and number of primary branches. High heritability estimates coupled with high genetic advance indicated the importance of additive genetic variance in the expression of those characters. Number of pods and test weight had high positive direct effects on yield and these should be used as primary selection criteria. Among the RILs, 29 recorded significantly higher yield than national check A-1. Out of which 14 were resistant to race 1 of *Fusarium* wilt.