मसूर *(लेन्स कुलिनेरिस* मेडिकस प्रजाति *कुलिनेरिस)* में म्लानि के प्रतिरोधी जीन टैगिंग

"Tagging gene(s) for resistance to wilt in lentil (*Lens culinaris* Medikus subsp. *culinaris*)"

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"Tagging gene(s) for resistance to wilt in lentil (*Lens culinaris* Medikus subsp. *culinaris*)"

By

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This is to certify that the thesis entitled **"Tagging gene(s) for resistance to wilt in lentil *(Lens culinaris* Medikus subsp. *culinaris)*"** submitted to the Faculty of Post Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfillment of requirement of degree of **Doctor of Philosophy in Genetics and Plant Breeding** embodies the result of a bonafide research work carried out by **Mr. Jitendra Kumar Meena** under my guidance and supervision. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that any help or source of information, as has been availed during the course of the investigation, has been duly acknowledged.

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Date: 1/1/19

Place: New Delhi

Jitendra

(Jitendra Kumar Meena)

This research work is dedicated

To

Farmer community

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Date:

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Abbreviations

AFLP	:	Amplified Fragment Length Polymorphism
BSA	:	Bulk Segregant Analysis
cM	:	Centi Morgan
CTAB	:	Cetyl Trimethyl Ammonium Bromide
CSAUAT	:	Chandra Shekhar Azad University of Agriculture and Technology
D.F.	:	Degree of Freedom
dNTPs	:	Deoxy nucleotide Triphosphate
DNA	:	Deoxyribo Nucleic Acid
EDTA	:	Ethyle Diamine Tetra Acetic Acid
EST	:	Expressed Sequence Tag
F.	:	Fusarium
F_1	:	First Filial Generation
F_2	:	Second Filial Generation
FAO	:	Food and Agricultural Organization
GBPUAT	:	Govind Ballabh Pant University of Agriculture and Technology
ICARDA	:	International Center for Agricultural Research in the Dry Areas
IARI	:	Indian Agricultural Research Institute
ISSR	:	Inter Simple Sequence Repeat
MAS	:	Marker Assisted Selection
M.P.	:	Madhya Pradesh
MW	:	Molecular Weight
R	:	Resistance
RB	:	Resistance Bulk
RAPD	:	Random Amplified Polymorphic DNA
RFLP	:	Restriction Fragment Length Polymorphism
PPM	:	Part Per Million
PCR	:	Polymerase Chain Reaction
S	:	Susceptible
SB	:	Susceptible Bulk
SSR	:	Simple Sequence Repeat

CHAPTER 1 *Introduction*

Lentil (*Lens culinaris* Medikus *subsp. culinaris*) is a winter sown pulse crop with genome size (haploid) of 4063 Mbp (Arumuganathan and Earle, 1991). It belongs to the genus *Lens* of *Leguminaseae* family. Historical evidences indicate that lentil was domesticated during 8^{th}-9^{th} millennia B.C. in Eastern Mediterranean region (Zohary, 1972; Zohary and Hopf, 1973; Cubero, 1981). The classification of *Lens* species has been done by many researchers and it has undergone various changes through many taxonomic classifications based on physiological, morphological, cytogenetic and now a day's molecular studies. Ferguson *et al*. (2000) classified genus *Lens* into four species - *Lens culinaris* ssp. *odemensis*; *Lens culinaris* ssp.*culinaris*; *Lens culinaris* ssp. *tomentosus* and *Lens culinaris* ssp. *orientalis*. Using genotyping by sequencing Wong *et al*. (2015) classified lentil species into four gene pools: a) primary gene pool comprising of species *Lens tomentosus*, *Lens culinaris* and *Lens orientalis,* b) secondary gene pool comprising of species *Lens odemensis* and *Lens lamottei* , c) tertiary gene pool comprising of species *Lens ervoides* and d) quaternary gene pool comprising of species *Lens nigricans*.

The lentil is mainly grown in the countries like Canada, China, Turkey, Bangladesh, India, Australia, Nepal, Ethiopia, USA and Syria. During 2014, lentil was grown in 1.89 million hectare area in India and its production was around 1.13 million tons (FAO, 2015). India is important producer and consumer of lentil. The global productivity of lentil is 1000 kg / ha and the productivity in India is around 600 kg / ha. The low productivity in India is due to low genetic variability in cultivated lentils lines, susceptibility to abiotic and biotic stresses and short growing period. In India, lentil is mostly grown in rainfed areas of Eastern India (Chhattisgarh, Bihar, Jharkhand and West Bengal) and Central India (Uttar Pradesh, Madhya Pradesh). Lentil is mainly utilized for food and fodder. Lentil has exceptionally rich source of high quality protein for vegetarians and resource poor people who are not able to meet the expense of diversified diet. It is good source of energy, vitamins (vitamin A, vitamin K, riboflavin, vitamin E) iron, zinc, folate, selenium, carbohydrate, fiber, mineral and antioxidant compounds which makes lentil a highly valuable food crop (Thavarajah *et al*. 2011). Lentil straw is also valuable animal feed. As compared to other cereals, pulses and

oilseeds, it has good cooking quality and it requires minimum precooking processing (Ezzar *et al*., 2012).

Lentil crop exhibits low seedling vigour, low pod setting, flower drop rate is very high, slow leaf area development, low harvest index and poor dry matter accumulation. Several biotic and abiotic stresses affect lentil productivity. Fungus (fusarium wilt, rust and ascochyta blight), bacteria, virus and insects and pests (aphids) cause severe yield losses to lentil crop. Among these, Fusarium wilt is most important widespread disease of lentil causing economic yield losses. This soil borne disease is caused by *Fusarium oxysporum* Schlecht: Fr. f. sp. *lentis* Vasudeva and Srinivasan. The pathogen belongs to the order hypocreales class Ascomycetes (Taylor *et al.,* 2007) and reproduces by asexual spores, macro and micro conidia as well as chlamydospores (Khare 1980 and Beniwal *et al*., 1993).

Fusarium wilt is widely reported from lentil growing countries in South Asia, West Asia and North Africa regions (Erskine *et al*., 1994). Fleischmann (1937) was first to report it from Hungary. Later it was reported from different countries by different workers (Padwick, 1941, Wilson and Brandsberg, 1965, Ujevic *et al*., 1965, Kotava *et al*., 1965, Bayya *et al*., 1998, Bayya *et al*., 1986, Bahl *et al*., 1993, Karki, 1993 and Hulluka and Tadesse, 1994). In India, this disease is reported from Assam, Bihar, Haryana, Punjab, Himachal Pradesh, Madhya Pradesh, Rajasthan, Uttar Pradesh and West Bengal (Agrawal *et al*. 1993; Chaudhary *et al*., 2009 and 2010).

Infection at different crop stages results in variable yield losses (Vasudeva and Srinivasan, 1952; Claudius and Mehrotra, 1973; and Khare *et al.,* 1979) in different environment for different varieties. Infection at seedling stage can result in drooping, drying of leaves and seedling death which leads to complete failure of crop. Whereas, infection at adult stage results sudden drooping of top leaflets, dull green foliage, shrivelling of seeds and wilting of branch or whole plant which reduces the crop yield. Different researchers (Khare *et al*., 1979; and Agrawal *et al*., 1993) have reported wilt incidence in the range of 50-78% from different fields. In Mediterranean country like Syria, Bayaa *et al*. (1986) reported yield loss in the range of 5-72%. Erskine and Bayaa (1996) reported negative correlation between wilt incidence and grain yield, higher the disease lower the yield.

The pathogen is warm-weather pathogen reported in acidic and sandy soils. The disease incidence appears in November and incidence is reduced in December and January reappearing again in February and March (Vasudev and Srinivasan, 1952; Kannaiyan and Nene, 1976). Soil moisture and temperature determine the fungal growth and symptom expression. The maximum disease incidence is reported in sandy loam soil at 25% soil moisture and 7.6 to 8.0 pH. Temperature between 17^0C and 31^0C favours the disease development. The most favourable air and soil temperature for growth of the pathogen is 28°C. Chlamydospore of fungus can survive in soil up to 5 years. For disease resistance breeding efficient screening protocols for evaluation of host plant resistance are essential. Sick plot screening technique for screening against *fusarium oxysporium* f.sp. *lentis* was discussed by Kraft *et al*. (1994) and Bayaa *et al*. (1994). The method is cost effective and can be simulated in normal environmental conditions for wilt development. However, sometimes the screening of genotypes in the wilt sick plot is not very efficient due to effect of drought and other root rot pathogens. Therefore, screening for wilt resistance under controlled conditions is necessary to study the inheritance of resistance and mapping of gene(s) for resistance for characterized isolate of fungus. This eliminates interaction from other soil borne pathogen. Several researchers (Dhingra and Sinclair, 1985; Porta-Puglia *et al*. 1994; and Porta-Puglia and Aragona, 1997) reviewed the strategies for screening and breeding for disease resistance.

The success of disease management depends on the knowledge of disease cycle and epidemiology because host genotype and climate factors influence growth, survival and dissemination of pathogen. Several reports on durable resistance (for soil-borne fungal pathogens) in grain legumes have been published (Lenne and Allen, 1998; Muehlbauer and Kaiser, 1994). The development of wilt resistance varieties is feasible mean for controlling this disease. The knowledge of inheritance pattern of disease resistance is essential for deciding the breeding strategy for development of wilt resistant varieties. Inheritance of resistance to wilt in lentil has been previously reported to be under control the monogenic dominant gene (Komboj *et al*., 1990 and Abbas, 1995). Muehlbauer *et al*. (1989); Tadmor *et al*. (1987); Vaillancourt and Slinkard (1993); and Zamir and Ladizinsky, (1984) used morphological and isozyme markers for genetic linkage studies.

The effectiveness and efficiency of disease resistance breeding programmes has improved due to DNA markers which are good indicators of genetic distances and diversity among accessions because of their selective neutrality. Restriction fragment length polymorphism (RFLP) markers were used by Havey and Muehlbauer (1989) for constructing first genetic linkage in lentil. Later, random amplified polymorphic DNA (polymerase chain reaction (PCR)-based markers) were used to study diversity, taxonomy and phylogeny in *Lens* (Sharma *et al*., 1996; Ford *et al*., 1997; Ferguson *et al*., 2000), to develop linkage maps (Eujayl *et al*., 1997, 1998a; and Rubeena *et al*., 2003), for tagging of gene(s) of interest (Eujayl *et al*., 1998b, 1999; Ford *et al*. 1999; Chowdhury *et al*. 2001; and Tullu *et al*. 2003) and for determination of pathogen population structure (Ford *et al*., 2000). Amplified fragment length polymorphism (AFLP) markers have also been used in lentil for studying genetic diversity (Sharma *et al*. 1996), cultivars identification (Zavodna *et al*., 2000) linkage map construction (Eujayl *et al*., 1998a; Duran Y and De La Vega MP, 2004; Hamwieh *et al*., 2005; and Kahraman *et al*., 2004) and identification of linked markers (Tullu *et al*., 2003). Simple sequence repeat (SSR) are tandem repeats of 2-5 nucleotide DNA core sequences spread throughout the genome. Duran Y and De La Vega MP (2004) and Hamwieh *et al*. (2005) used SSRs to construct linkage maps in lentil.

Conventional mapping requires genotyping of each progeny of mapping population with several molecular markers and this is tedious, time consuming and costly. To overcome this problem two alternative strategies (selective genotyping and bulk segregant analysis) have been proposed. In selective genotyping individuals are genotyped from the two tails of the phenotypic distribution (Navabi *et al*., 2009). In bulk segregant analysis, DNA from groups of individuals with similar phenotype are pooled together. The variation is measured in these pools of segregants and a likely map position is assigned using the established correlation (Michelmore *et al*., 1991). For bulk segregant analysis (BSA) mapping population is developed using diverse parents which are highly variable for the trait(s) to be mapped, are utilized. Initially F_2 populations were developed from interspecific crosses between wild species and cultivars (Havey and Muehlbauer, 1989; Muehlbauer *et al*., 1989; Tahir *et al*., 1993; Vaillancourt and Slinkard, 1993). Such populations exhibited low recombination and small map sizes (Tadmor *et al*., 1987). Reports on gene tagging for resistance to fusarium wilt in chickpea have been published earlier (Mayer *et al*. (1997); Halilia *et*

al. (2009); Hiremath *et al*. (2012) and Patil *et al*. (2014). In pigeon pea Kotreshet *et al*. (2006); Prasanthi *et al*. (2009) and Singh *et al*. (2013) tagged gene(s) for wilt resistance. Only three reports (Komboj *et al*., 1990; Eujayl *et al*., 1998; and Hamwieh *et al*., 2005) are published on inheritance of wilt resistance in lentil and identification of linked molecular marker. Therefore, the present investigations were focussed on the following objectives:

1. To screen the lentil germplasm for Fusarium wilt resistance
2. To study the mode of inheritance of wilt resistance in lentil
3. To tag/map gene(s) for wilt resistance in lentil

CHAPTER 2 *Review of literature*

2.1. Lentil:

Lentil is a winter season crop belonging to Leguminosae family. Seed is the main part of the plant which is consumed as food for preparation of dal. It is grown in wide spread cultivated area of Mediterranean region as well in south west Asia.

2.1.1 Taxonomy:

Moench in 1794 (Westphal, 1974) used name *Lens esculenta*, later on Medikus (1787) validly published *Lens culinaris*. Initially the genus included five species: *L. culinaris, L. nigricans, L. montbretii, L. ervoides and L. orientalis.* Alefeld (1866) classified lentil into eight sub-species. However, Barulina (1930) did not recognise this classification and reported that the cultivated lentil originated from *L. orientalis* and it exhibited the chromosome number (2n=14) and found its similarities with its *L. orientalis* and *nigricans*. They have also categorised the *L. culinaris* into two sub species (microsperma and macrosperma). Morphologically, the microsperma types distinguished from the macrosperma type based on cotyledons colour and pigmentation of flower. Williams *et al*. (1974) classified lentil in family Leguminosae and sub family Papilionaceae. They have also grouped sub species *L. culinaris and L. orientalis under L. culinaris*. Van Oss *et al*. (1997) categorised the genus *Lens* into seven taxa the cultivated lentil *L. culinaris* Medikus subsp. *culinaris*, its wild progenitor *L. culinaris* subsp. *orientalis* (Boiss.) Ponert, *L. odemensis* (Ladz, *L. ervoides* (Brign.) Grande, *L. nigricans* (M. Bieb.) Godr. and two recently recognized species, *L. tomentosus* Ladiz. and *L. lamottei* Czefr. *Lens* was re-classified by Ferguson *et al*. (2000) into seven taxa split into four species *L. culinaris* Medikus subsp. *culinaris*, subsp. *orientalis* (Boiss.) Ponert, subsp. *tomentosus* (Ladiz.) Ferguson *et al*. (2000) subsp. *odemensis* (Ladiz.) Ferguson *et al*. (2000) *L. ervoides* (Brign.) Grande *L. nigerians* (M. Bieb.) Godr. *L. lamottei* Czefr. The classification given by Ferguson *et al*. (2000) is widely accepted by the researchers.

2.1.2 Evolution:

The evolution of lentil has been around Central Asia and it spreads till the near East. The cultivated species *L. culinaris* subsp. *orientalis* have been grown in Turkey, Lebanon, Israel, Uzbekistan, Jordan and Syria (Cubero, 1981). Ladizinsky (1979b)

studied 22 lentil genotypes which comprised of different species of lentil. The seed protein profile of 22 genotypes have been recorded and found that *L. culinaris, L. orientalis and L. nigricans* showed some similarity with each other and *L. ervoides* was found different from the rest of the studied species. *L. nigricans* have been reported to be progenitor of *L. culinaris* on the basis of domestication of lentil in southern Europe (Renfrew, 1973). Except the reports of Renfrew (1973), most of the studies have reported that *L. orientalis* is the progenitor of the cultivated species of lentil (Ladizinsky, 1979a; Barulina, 1930; Zohary, 1972 and Williams *et al*., 1974). According to the report by Singh (2001), *L. orientalis* might have first originated from perennial species and later on became the progenitor of the cultivated species. *L. orientalis* is the presumed progenitor of the domestic of *L. culinaris* and the two species are crossable and produce fully fertile progeny (Muehlbauer *et al*., 2006).

2.1.3 Gene pool:

L. culinaris subs. *orientalis* comes under primary gene pool (Ladizinsky and Alder, 1976). *L. culinaris* subs. *orientalis* is fully cross compatible with cultivated lentil (Robertson and Erskine, 1997). The secondary gene pool comprised of *L. nigricans*, it can be crossed with the cultivated lentil but the seed set is considerably lower than *L. culinaris* subsp. *culinaris* × *L. culinaris* subsp. *orientalis* (Muehlbauer and Slinkard, 1981).

2.2 Pathogen: lentil fuasrium wilt:

Genus *Fusarium* was proposed by Link (1809) for species with fusiform, nonseptate spores borne on stroma and was based on *Fusarium roseum* (Booth, 1971). Synder and Hansen (1940) reported that the genus *Fusarium* belongs to the class fungi imperfecti and includes many species and many forms within species. They placed all the species in the section of Elegans under *F. oxysporum.* However, the fungus was renamed by Chattopadhyay and Sengupta (1967) to *F. oxysporum* Schlecht. ex Fr. f. sp. *lentis* Vasudeva and Srinivasan. *Fusarium* is a cosmopolitan genus of filamentous ascomycete fungi. This genus comprised of many plant pathogens (toxin-producing), and these are very important for utilisation in agriculture. Eight strains of *Fusarium* were reported by Khare *et al.* (1975) and Kannaiyan and Nene (1978) were reported seven strains.

The study of Indian races exhibited no variation in virulence that plays a major role in breaking the resistance of genotypes of lentil. *F. oxysporum* have been known

to limit the productivity and yield of crops by causing vascular wilt disease (Nelson *et al*., 1983). The majority of the vascular wilt isolates are specific strains. These specific strains that infect small number of host's species and can be differentiated from each by utilizing sub specific species (Summer *et al*., 2003). Belabid *et al*. (2004) performed experiments on 32 Algerian isolates of *Fusarium* and studied virulence and vegetative compatibility. They have grouped them as a single race. The response of these isolates with the susceptible lines is very aggressive. Forty three cultural and morphological groups were reported by (Chaudhary, 2008) by studying 333 isolates from various states and regions of India. These isolates were classified in three different groups on the grounds of their disease reactions against seven different differentials of lentil. In the same line, genetic variability studies against twenty four isolates revealed two sub-populations with little genetic variations. These populations were selected and collected from north eastern Indo-Gangetic plains zone of India and molecular analysis was performed by 40 RAPD and 12 SSR primer pairs (Datta *et al*., 2009).

2.2.1 Life cycle and host range:

The life cycle of the *Fusarium* wilts has been studied and it was found that crops like lentil, chickpea and lupin have similar life cycle, the only difference between the life cycles of these crops are the hosts which they infect. Once the host roots are infected by the pathogen, it crosses the cortex of the plant and then reaches to the xylem tissues. As it reaches the xylem, it spreads quickly and reaches to vascular system becoming systemic in the host tissues and subsequently infects the seed/grain. The pathogen sits in the xylem vessels and produces micro conidia by branching of mycelium. The micro conidia detach from the mycelium and are carried in upward direction in the vascular system until movement of the micro conidia stopped, at which point they germinate and the mycelium penetrates the wall of the adjacent vessel. The pits form the way for movement of these conidia between the vessels. The fungus then enters in all tissues of the plant and reaches to the surface where it profusely sporulates. The water economy of infected plants are severely compromised by blockage of vessels which subsequently results in closure of stomata, wilting of leaves, death of leaves and these symptoms ultimately becomes the cause of death of the whole plant. The fungus survives as chlamydospores and mycelium in seed and soil, roots and stem tissues buried in the soil and on infected crop residues for more than 6 years (Singh *et al*., 2007). Chlamydospores are formed in terminal intercalary, old cultures (smooth or rough walled), and may also be formed alone/in pairs/ in chains. Micro conidia are borne on

short and simple conidiophores, which arise laterally on the hyphae. They occur in many shapes i.e. straight, curved oval, cylindrical. While in case of macro conidia, it is borne on the branches of conidiophores. Macro conidia are fusoid, thin walled and pointed at both ends having one to six septate. It measures around 3.5 - 4.5 × 25 – 65 µm. The spores of the pathogen are dispersed through water, wind or movement of soil or plant debris. The movement through seed provides a route for spreading the infection at long distances and for transmission of the disease into new areas.

2.2.2 Symptoms of wilt disease:

Wilt mainly refers to loss of turgidity due to non-availability of water to the plant. It may be partial wilting where the plant recovers in the later stage or complete wilting leading to death of the plant. The lentil wilt disease is of two types namely, seedling wilt and adult plant wilt and appears in patches in the field at both the stages. Vasudeva and Srinivasan (1952) have studied the wilt in crops and reported that in broadcast crop, the disease will appear in isolated patches, more or less circular in outline and the disease appears to progress along the lines when sown in rows. The symptoms of the wilt are wilting of the top leaves of plants, stunting, shrinking and curling of the leaves from the lower part of the plants, this curling of the leaves symptom moving upward to the stems of the infected plant and later causes yellowing and death of the plant. Khare (1980) reported symptoms of the effect of wilt on root which includes reduction in growth with brownish discolouration (prominent) and proliferation of secondary roots above the tap root injury.

Vasudeva and Srinivasan (1952) observed that curling of leaves began from lower end and extended upwards, drooping of the crown was followed by death of the plant whereas root system was poorly developed and discoloured brown. Khare (1980) also reported that the seedling infection included seed rot and sudden drooping. Dubey and Singh (2004) found that wilt can affect at any of the growth stages of the crop and there was no external rotting of roots and black discolouration involving of xylem and pith.

2.2.3 Occurrence:

Lentil wilt caused by *F. oxysporum* f.sp. *lentis* is an important disease reported from all the lentil growing regions worldwide except Australia (Beniwal *et al*., 1993; Tosi and Cappelli, 2001). Vasudeva and Srinivasan (1952) reported the attack of

Fusarium wilt in 1949 in Delhi which destroyed the crop by 67%. In India wilt is caused by *F. oxysporum* f.sp. *lentis* (*Fol*) is a most important disease in Bihar, Madhya Pradesh, West Bengal and Uttar Pradesh and in some other areas, where lentil is grown (Agrawal *et al*., 1991). Predominance of *fusarium* diseases on pea was found in Jabalpur district of India which was confirmed by pathogenicity tests involving pathogen spores and metabolites and *F. oxysporum* showed severe pathogenicity on pea varieties grown in that region (Sharma *et al*., 2011).

2.3. Screening for resistance to vascular wilt:

A. Lentil:

Khare and Sharma (1970) screened 10 lentil lines against *Fusarium* wilt. L-9-12, B-25, NP-11 and T-36 exhibited the low incidence of wilt (1.82, 2.75, 3.12, and 3.55 respectively) under field conditions. Khare *et al*. (1973) found out those five lines of lentil viz., J- 52, 733, 769, 774 and 795 resistant to *F. oxysporum* f.sp. *lentis.* Kannaiyan and Nene (1976) evaluated the 158 lines against *F. oxysporum* f.sp. *lentis* and reported that thirty two lines were found resistant. Pandya *et al*. (1980) studied the resistance of Pant-406 line of lentil against seven races proposed by Kannaiyan (1974), and found it immune to race 5, resistant against races 3 and 6 and semi-resistant against race 4. Omar *et al*. (1988) evaluated 12 lines of lentil against wilt and root rot diseases caused by *Rhizoctonia solani, Fusarium solani, F. oxysporum, F. moniliforme, Gliocladium roseum, Verticillium* spp. and *Pythium butleri* .They reported lines H5, H6 and H81 as resistant and lines F29, K270 and F300 as semi-resistant whereas the line ILL16370 was susceptible. Erskine and Bayaa (1990) screened 162 germplasm lines under greenhouse conditions. Twenty nine germplasm lines were reported as resistant. Bhat *et al*. (2003) screened thirteen Indian lentil genotypes. The results of the study classified the genotypes into different groups, immune, highly resistant, moderately resistant and moderately susceptible. No genotypes were found immune to the disease. SKL-16 was found to be highly resistant and exhibited highest yield. SKL-1, SKL-29 and SKL-9 were moderately resistant and SKL-12, SKL-8, SKL-13, SKL-3 and SKL-15 were moderately susceptible to the disease. Bayaa *et al*. (1997) evaluated core collection comprising of 577 germplasm from thirty three countries for wilt resistance in North Syria. They analysed variation among selected genotypes on the temporal pattern of wilting. Among study of different countries genotypes belonging to Egypt, Romania, India, Chile and Iran were found to be most resistant accessions.

Pradhanang *et al*. (1993) reported that screening for resistance to *fusarium* wilt among 90 lines of lentil under natural and controlled conditions, showed that all of them were susceptible including the line Simal which is widely cultivated in the North India. Tzvetelina *et al.* (2006) evaluated 32 lentil genotypes belonging to different geographical locations for their reaction to *F. oxysporum* f.sp. *lentis* in greenhouse conditions. Genotypes 91-001, 91-028 and 98-001 were found susceptible with approximately 45 and 50 % of total wilted plant. Mohammadi *et al*. (2011) screened 55 developed lines against the collected isolates under wilt sick plot and controlled condition. The data of the greenhouse and field data has led to the identification of three resistant genotypes namely FLIP2007-42 L, 81S15 and FLIP2009-18 L resistant under both the conditions.

Pouralibaba *et al*. (2015) evaluated 196 lentil landraces under wilt sick plot and in green house under controlled condition. Only twelve accessions were identified as resistant while remaining was found susceptible. Fatima *et al*. (2015) screened 28 lentil accessions against *fusarium* wilt and reported 16 accessions as highly susceptible, 7 as susceptible, 3 as moderately resistant whereas remaining two expressed resistance reaction. Joshi (2006) reported that lentil varieties i.e. Bari Masur-4, Shital, Simal, Khajura-1, Simrik and Maheswor Bharati possessed field resistance to vascular wilt at Khumaltar, Nepal. Gharti *et al*. (2011) observed that genotypes ILL 7715 and ILL 9993 were resistant and ILL 7164, ILL 590, PL 406 and F 2003-49L revealed moderate resistance to wilt / root rot complex in Nepalgunj condition. Singh *et al*. (2017) has done screening of F_5 mapping population generated by crossing ILL10829× ILWL30 against *fusarium* wilt in the wilt sick plot. Mapping population of ILL10829 × ILWL30 manifested resistant reaction for 15 recombinant inbred lines (RILs) against *Fusarium* wilt. Yadav *et al*. (2017) screened 185 lentil genotypes for wilt and reported resistance in 15 genotypes (Arun, Sagun, M-Bharati, RL- 13, DPL- 62, ILL8191, ILL1672, RL-85, ILL6811, RL-77, ILL6468, ILL6260, ILL9996, ILL7164, ILL6024 and RL-21).Twenty three genotypes (ILL8187, ILL9949, L 39-S-66, ILL8132, ILL7980, LN-0135, ILL1920, FLIP05-24L(ILL10045), RL-51, LN-0111, FLIP2009, ILL9932, ILL7157, ILL6025, RL-44, ILL602, RL6, ILL3490, LN0137, ILL2526 and ILL6256 expressed moderate resistance. Sixty seven genotypes showed moderate susceptibility and remaining genotypes were highly susceptible.

B. Chickpea:

Haware *et al*. (1992) screened over 13,500 accessions of chickpea germplasm for resistance to race 1 of *F. oxysporum* f.sp. *ciceri*. One hundred and sixty genotypes were found to be resistant. These included 10 Kabuli types. Halila and Strange (1996) screened 1915 'Kabuli' accessions screened for resistance to (*F. oxysporum* f sp. *ciceri* race 0 and reported resistance in 110 accessions.

Iqbal *et al*. (2010) screened 145 genotypes against of lentil wilt under artificial disease condition. These genotypes were collected from various sources. The incidence of disease was recorded at two stages 1) at the growth of seedling and 2) at reproductive stages. Significant variation was recorded at both the stages. At seedling stage genotypes C-44, ILC 182, FLIP98-227C, FLIP98-54C, KC-89, 90395, C-235, CM2000, FLIP98-38C, FLIP97-129C, FLIP97-172C, FLIP98-107C, ILC7374, FLIP98-230C and FLIP98-231C were found resistant, sixty five genotypes were found to be tolerant and remaining sixty six genotype were found to have susceptible reaction. In reproductive stage, no genotype were found to be resistant to the wilt, genotypes FLIP98-231C, 90395, C-235, FLIP98-38C, C-44, ILC7374E101×PB91, FLIP98-54C, FLIP98-107C, FLIP98-226C, FLIP98-230C and FLIP98-227C showed tolerance to wilt and remaining 133 genotypes exhibited susceptibility. Kumar *et al*. (2015) screened one hundred one genotypes of chickpea against to *fusarium* wilt disease caused by *F. oxysporum* f.sp. *ciceri* .The disease incidence observation were recorded at seedling and reproductive stage. The results showed that 57 lines were resistant, 28 were tolerant while 16 were susceptible to the wilt disease at seedling stage. On the contrary, 31 genotypes were resistant, 26 were tolerant and 44 were susceptible at reproductive stage. As compared to seedling stage, invariably the disease incidence at physiological maturity stage increased in all the genotypes. On an average basis 56.44% disease resistance was recorded at early stage and 30.69% at reproductive stage, whereas 15.84% disease incidence was observed at seedling stage and 43.56% at reproductive stage. The results depicts that reproductive stage is more sensitive to the wilt in comparison to seedling stage.

C. Pigeonpea:

Anjaneya reddy and Saifulla (2005) evaluated seven promising pigeonpea genotypes and twelve host differentials for host plant resistance against *F. udum* under

wilt sick plot conditions. Among the seven promising genotypes screened, C-11 was moderately resistant while all other genotypes viz., ICPL-87119, ICPL-93001 and ICPL-96061 were resistant with the wilt incidence of less than 10 per cent. While, the twelve host differentials screened to identify different isolates, seven differentials viz., ICP8859, ICP8863, ICP 9145, ICP 9174, C 11, BDN 1 and BDN 2 were under resistant group, ICP 8858 fell under moderately resistant group and five differential viz., ICP 8862. LRG 30, ICP 2376, ICP 26 and TTB 7 were under susceptible group. Kathiria (2015) evaluated eight pigeonpea genotypes (ICP 8863, ICPL 84060, BSMR 853, AGT 2, GT 101, T 15-15, AVPP 1 and LRG 41) in pot and water culture screening using artificial inoculation. Both screening techniques identified genotype ICP 8863 as resistant against *F. udum*. Bhaskar (2016) studied 55 pigeonpea genotypes along with check to identify the sources of resistance to *fusarium*. Out of 55 entries, only two entries (ICPL-87119 and IPAC-68) were resistant to wilt disease. Mishra *et al*. (2016) screened thirty promising lines of pigeonpea in the sick field as well as under greenhouse conditions for resistance against *fusarium* wilt. Out of thirty lines, 18 genotypes showed resistant reaction, 7 were moderately resistant and 5 were susceptible under sick plot. On the contrary, in case of greenhouse screening 17, 7 and 6 genotypes showed resistant, moderately resistant and susceptible respectively.

2.4. Wilt resistance breeding:

Reliable information of genetics and inheritance of genes or traits play a pivotal role in transfer of *fusarium* wilt resistance genes from donors to recipient parents. Gene tagging and molecular mapping of molecular markers linked to the wilt requires firstly information about the inheritance pattern of wilt resistance.

A. Lentil:

Komboj *et al*. (1990) studied the segregation pattern for wilt reaction in F_2, BC (P_1), BC (P_2) and F_3 generations in field and glasshouse conditions indicated that resistance to *Fusarium* wilt is under the control of two dominant duplicate genes in Pant L 234. In JL 446 and LP 286, two independent dominant genes have been reported with complementary gene effects. They have also reported dominant gene in genotypes JL 446 and LP 286. These lines were found susceptible to the wilt. Eujayl *et al*. (1998) reported single dominant gene for wilt resistance in lentil. With the help of RAPD markers linkage map for the disease have been developed and OPK-15 900 was found

to be linked with the trait at distance of 10.8 cM on linkage group 6. Hamwieh *et al*. (2005) reported one SSR and AFLP marker flanking the *Fusarium* wilt resistant gene by 8.0 and 3.5 cM respectively. Datta *et al*. (2011) collected different isolates from various agro-climatic zones in India. Around 100 isolate of *F oxysporum* f.sp. *lentis* causing agent of vascular wilt in lentil. Among them 15 isolates of *F. oxysporum* f.sp. *lentis* were selected for molecular diversity by three molecular markers. Twenty RAPD markers produced around 105 reproducible bands, out of which 81 bands were reported to have polymorphic loci and 24 were found to be monomorphic.

B. Chickpea:

The wilt is reported by Ayyar and Iyer (1936) in chickpea, this presumes to be one of the first reports about *fusarium* wilt resistant gene in chickpea. The resistance to *fusarium* wilt in chickpea have been reported to be governed by a single gene with incomplete dominance. Haware *et al*. (1980) reported single recessive gene for resistance to *fusarium* wilt. Haware and Nene (1982) identified the second race of *F. oxysporum* and they have also discovered the phenomenon of late wilting after inoculation with race 2. Upadhyaya *et al*. (1983a) evaluated chickpea genotypes for the variation in time of wilting in response to race 1 of Pathogen *F. oxysporium*. Genotype C 104 was identified as resistant genotype and JG 62 as susceptible. Single genes have been reported for time difference of wilting with early wilting partially dominant to late wilting. This study revealed identification of two genes h1 and h2 genes, involved in the inheritance of resistance in chickpea to race 1. Upadhyaya *et al*. (1983b) conducted experiments for identifying genes for wilt in chickpea. They have studied mapping population derived from cross JG 62 and C 104. Genotype JG 62 was early-wilting and C 104 was late-wilting. The result reported segregation of two genes and exhibited the requirement of both the genes in homozygous recessive state for complete resistance. Singh *et al*. (1987a) reported three genes H1 H2 H3 to control resistance to race 1 of pathogen. Partial recessive allele at first two loci and dominant allele at the third locus causes delay in wilting. But any of the mentioned allele when work together imparts complete resistance. Singh *et al*. (1987b) screened F_1 and F_2 generations of crosses of chickpeas derived from K 850 with C 104 along with parents (P_1, P_2) and F_3 progenies of crosses obtained by crossing K850 × C104 to race 1 of *F. oxysporum* f.sp. *ciceri*. Monogenic ratio was derived from the cross K 850 with JG62. The study of the cross between the K 850 × C 104 cross showed digenic ratio. The result indicated that K850

carried a recessive allele at different locus than C 104. Recessive alleles identified at both the locus together confer complete resistance to wilt. Gumber *et al*. (1995) studied inheritance pattern of *Fusarium* wilt (race 2) by hybridizing genotypes P165 and C104 in chickpea. They reported 13:3 ratio, which shows occurrence of two resistance genes against race 2 of the pathogen. Two independent genes were identified by Tullu *et al*. (1999) to genes control the resistance of race 4 of *fusarium* wilt in chickpea. The study was performed on F_1, F_2 and F_3 families from the cross derived from JG62 × Surutato-77. Tekeoglu *et al*. (2000) focussed on two races of the pathogen (race 5 and race 0) for studying *fusarium* wilt resistance in recombinant inbred lines.

Sharma *et al*. (2004) have also reported the genetics of *fusarium* wilt resistance in chickpea genotype WR315. For identifying the different races of the pathogen (*F. oxysporum* f.sp. *ciceri*) a set of differentials were used under study. Recombinant inbred lines (RILs) with population size of 100 derived from the cross WR 315 (resistant) and C 104 (susceptible) was used to study the genetics of resistance to different races 1A, 2, 3, 4, and 5 of *F. oxysporum* f.sp. *ciceris* and a population of 26 F_2 plants from a cross between the same two parents was used to study inheritance of resistance to race 2. Segregations of the recombinant inbred line for resistance to each of the 5 races propose that single genes in WR 315 govern resistance to each of the races of the pathogen. Ratio of 1:3 depicts resistant to susceptible ratio in the F_2 population. This shown that resistance to WR 315 to race 2 was governed by a single recessive gene. Sharma *et al*. (2004 and 2005) also reported monogenic inheritance of resistance to race 3.

C. Pigeonpea:

In pigeon pea inheritance mechanism of resistance to *fusarium* wilt is not fully understood by researchers and whatever information is available, it shows variability. Pal (1934) reported that resistance to wilt was controlled by many factors. Shaw (1936) reported two complementary genes for resistance to wilt. According to the research done so far resistance to *Fusarium* wilt has been shown to be governed by single dominant gene (Pawar and Mayee, 1986; Singh *et al*., 1998; and Karimi *et al*., 2010), single recessive gene (Jain and Reddy, 1995) two complementary genes (Parmita *et al*. 2005), two genes (Okiror, 2002), major genes (Parmita *et al*., 2005). Ajay *et al*. (2013) studied mode of inheritance in F_2 and F_3 generations derived from crosses BRG-1 × ICP-8863 (cross 1) and TTB-7 × ICP-8863 (cross 2). Cross 1 exhibited 9 (susceptible): 7 (resistant) ration and cross 2 exhibited 13 (susceptible): 3 (resistant) ration in

segregation generations respectively. The histogram showed normal distribution, which is skewed towards susceptibility. This specifies that susceptibility was dominant over resistance and is governed by two or more genes. Chaithanya *et al*. (2011) assessed F_2 progenies of crosses LRG-41 × ICPL-8863 and TRG-22×ICPL-87119 for *Fusarium* wilt resistance. The utilization of SCAR14f/r marker that shown that the inheritance was governed by a single dominant gene. The resistant plants demonstrated amplification of band of 937 bp and this amplified band is absent in susceptible plants. The genotypic data segregates in the ratio of 3 resistant: 1 susceptible. The ratio 3:1 confirms single dominant gene for wilt resistance in pigeon pea

Karimi *et al*. (2010) assessed pigeonpea genotypes to study the mode of inheritance of *fusarium* wilt resistance for identifying different genes governing resistance to wilt. F_1, F_2 and backcross populations were developed by crossing genotypes ICEAP 00554, ICEAP 00557 (resistant) and KAT 60/8, ICP 7035 (susceptible accessions). The evaluation against *Fusarium* wilt resistance was done in the Parents, F_1, F_2 and backcrosses (BC_1F_1 and BC_2F_1) populations. Recessive gene was detected from cross ICP 7035 × KAT 60/8. F_2 populations derived from ICEAP 00554 × KAT 60/8, ICEAP 00557 × ICP 7035, ICEAP 00554 × ICP 7035, ICEAP 00557 × KAT 60/8, crosses exhibited a 3:1 ratio which indicated that resistance to *Fusarium* wilt was under the control of major gene. Patil *et al*. (2013) assessed the inheritance pattern of resistance against two variants of *Fusarium* wilt by using highly resistant (ICP8863) and highly susceptible (Type 7) genotypes. Homogeneity and hybrid testing was conducted by using with molecular markers (PPMC-1, OPP-17, PPMC-2, CCB-10 and PPMC-3) for development of mapping populations. Phenotyping of mapping population was carried out against the studied two variants in the pots inoculated with 5% of inoculum (w/w) under greenhouse conditions. The result revealed existence of two recessive genes against variant 2 in ICP8863 and single recessive gene against variant 1. Singh *et al*. (2013) performed genetic diversity analysis by using SSR markers for identifying number of genes governing the trait. Thirty six elite cultivated pigeon pea accessions was studied for their variable level of resistance to *fusarium* wilt. Twenty-four polymorphic SSRs were screened which amplified a total of 59 alleles with high PIC value of 0.52. Singh *et al*. (2016) studied genotypes BDN-2001-9, BWR-133, IPA-234 and BDN-2004-1 for inheritance of resistance to *fusarium* wilt disease (casual organism *F. udum*) in pigeon pea. Based on the study one dominant gene was

reported in BDN-2004-1 and BDN-2001-9, two dominant complimentary genes in resistance source IPA-234 and two duplicate dominant genes in BWR-133. Further, six SSRs (ASSR-366, ASSR-363, ASSR-148, ASSR-1, ASSR- 229 and ASSR-23) were reported to be associated with *fusarium* wilt resistance.

2.5 Molecular marker and gene tagging/mapping:

2.5.1 Genetic markers:

Generally, genetic markers are classified into three major class viz. (1) Morphological markers which reveal morphological variation of traits or characters based phenotypic analysis (2) Biochemical markers are the allelic variants of enzymes and (3) DNA markers reveal sites of variation in DNA (Winter and Kahl, 1995; Jones *et al.,* 1997 b). Morphological markers represent the phenotypic characters like flower colour, plant height, days to flowering and maturity etc. These can be visualized and recorded in the field.

Isozyme markers are Differences in enzymes detected by electrophoresis using specific staining represent the Isozyme marker. Both these marker are limited in number and are influenced by plant stage and environment (Winter and Kahl, 1995). Inspite of these limitations morphological and biochemical markers have been widely utilized by the plant breeders (Weeden *et al.*, 1994; and Eagles *et al.*, 2001). Molecular markers also known as genetic markers are DNA tags which are known to have difference in DNA sequences between two or more individuals. Molecular markers occur at specific site in plant genome. Molecular markers do not influence expression of any trait and are in close proximity to trait and inheriting together with trait under consideration. Paterson (1996) reported that molecular markers arise from DNA mutations [substitution (point mutations), rearrangements (insertions or deletions) or errors in replication of tandem repeated DNA]. Molecular markers do improve efficiency of selection in breeding programs, saving time and providing opportunity for pyramiding of two or more genes in a single genetic background.

2.5.2. Molecular or DNA markers:

Initially in lentil Restriction Fragment Length Polymorphism (RFLP), AFLP (Amplified fragment length polymorphism) and Random amplified polymorphic DNA (RAPD) markers were used to study genetic variability and phylogeny within and among different *Lens* species (Havey and Muehlbauer, 1989; Aboelwafa *et al*., 1995;

Sharma *et al*., 1995; Ahmad and McNeil, 1996; Sharma *et al*., 1996; Ford *et al*., 1997) and trait mapping (Eujayl *et al*., 1998b; Tullu *et al*., 2003; Duran *et al*., 2004; Kahraman *et al*., 2004; and Hamwieh *et al*., 2005). In the recent years molecular markers are the preferential tool for diversity analysis in lentil. Although, RAPD loci were much quicker to be identified, but being a dominant marker for one in every two primers detected a polymorphism and is not gene specific, hence it is less preferred one compared to other advanced PCR based markers. Among various PCR based molecular markers, Simple Sequence Repeats (SSRs) have become marker of choice in many crops because it offers many advantages such as locus specificity, co dominance nature, multi-allelic due to high mutation rate, high reproducibility, relative abundance and it provide the facility of whole genome scan (Powell *et al*., 1996; and Varshney *et al*., 2009). It is very powerful in differentiating closely related individuals in many plant species. Microsatellites or simple sequence repeats (SSRs) are small tandem repeats of DNA, usually 2–5 bp in length, that occur in most eukaryotic genomes. Roder *et al*. (1998), Winter *et al*. (1999) and Cho *et al*. (2000) used SSR markers for genome mapping and tagging various traits of agronomic importance. SSRs are random and frequently distributed in eukaryotic genome (Tautz, 1989). Polymorphism based on repeat units in genome is reflected by SSR (Jacob *et al.* 1991; Litt and Luty, 1989; and Weber and May, 1989). The composition and number of SSRs is variable. Molecular markers application is reliable tool for understanding of phylogeny and diversity of *fusarium* spp. However, till date numerous studied have done on *fusarium* wilt in molecular breeding. (O'Donnell, 2000).

2.6. Gene Tagging for wilt resistance using bulked segregant analysis (BSA) approach:

A. Lentil:

Due to the presence of different races/path types of *fusarium* spp. and uneven concentration of inoculum accurate phenotyping becomes hurdle for transferring wilt resistance gene to locally adapted cultivars is difficult. It also encountered the problem of linkage drag. Use of molecular markers paves the wave in reducing the linkage drag and thereby efficiently tagging gene for resistance to wilt. There is slow development of molecular markers in lentil due to their low polymorphism in chickpea genome (Kazan and Muehlbauer, 1991; Labdi *et al*., 1996; Ahmad and Slinkard, 1992; Mayer *et al*., 1997). Earlier reports for using biochemical marker (isozymes) such as RFLP

and RAPD in mapping of disease resistance gene were not so successful (Kazan and Muehlbauer, 1991; Kazan *et al*., 1993; and Mayer *et al*., 1997). But as the research progressed first gene (H1) for wilt resistance have been tagged in chickpea by Mayer *et al*. (1997) (syn. *foc-1*). Further molecular markers closely linked to wilt resistance have been identified by several researchers as *foc-01* (Rubio *et al*., 2003; and Cobos *et al*., 2005), *foc-1* (Sharma *et al*., 2004b; and Sharma and Muehlbauer, 2005), *foc-2* (Sharma and Muehlbauer, 2005), *foc-3* (Sharma *et al*., 2004b; and Sharma and Muehlbauer, 2005), *foc-4* (Tullu *et al*., 1998; Tullu *et al*., 1999; Winter *et al*., 2000; Tekeoglu *et al*., 2000; Benko-Iseppon *et al*., 2003; Sharma *et al*., 2004b; Sharma and Muehlbauer, 2005) and *foc*-5 (Ratnaparkhe *et al*. 1998b; Tekeoglu *et al*., 2000; Winter *et al*., 2000; Benko- Iseppon *et al*., 2003; Sharma and Muehlbauer, 2005). Eujayl *et al*. (1998) developed linkage map by using RAPD markers and identified marker OPK-15900 linked with Fusarium wilt resistance gene (*Fw*) at a distance of 10.8 cM. They have also established its linkage with markers in coupling (OP-B17800 and OP-D15500) and repulsion phase (OP-C04650). The linked markers have been converted into locus-specific sequence characterized amplified region (SCAR) markers for marker-assisted selection. The linkage map has been made by using 86 recombinant inbred lines encompassing eighty nine RAPDs and 79 AFLPs along with six co dominant RFLP markers. They have also identified molecular markers linked to the single dominant gene by using recombinant inbred lines conditioning *fusarium* vascular wilt resistance (*Fw*). Hamwieh *et al*. (2005) identified two molecular markers one SSR marker and one AFLP markers linked to Fusarium wilt at distance of 8.0 and 3.5 cM. Molecular markers have been very useful in establishing hybridity of F_1 plants to developing mapping populations without any segregation distortion (Solanki *et al*., 2010).

B. Chickpea:

The first wilt resistance gene to be tagged in chickpea was H1 with CS 27F/CS 27R (ASAP) (Mayer *et al*., 1997), which amplifies a fragment of 700 bp linked to the allele for susceptibility**.** Mayer *et al*. (1997) have conducted experiments to link molecular markers linked with wilt resistance and susceptible locus (UBC- 170550 and CS-27700) respectively. Seven percent recombination was identified between markers UBC-170 and CS-27. Two allele specific primers have been developed by cloning the sequence. CS-27 amplified a fragment linked to the allele for susceptibility to race 1

(H1 locus) of *Fusarium* wilt and primer UBC-170 showed a single fragment (resistant and susceptible) genotypes. Ratnaparkhe *et al*. (1998) identified ISSR marker associated with *fusarium* wilt resistance against race 4. UBC- 855500 marker was found to be linked with *fusarium* wilt resistance gene in repulsion at a distance of 5.2 cM. This marker co-segregated with RAPD primer, CS-27700, which was earlier shown to be linked with race 1 of *Fusarium* wilt resistance gene and was mapped to LG 6 of the *Cicer* genome. The study exhibited that the markers linked with race 1 and race 4 were closely linked. The marker UBC-855500 is located at 0.6 cM from CS-27700 and is present on the same side of the wilt resistance gene. Tullu *et al*. (1998) studied inheritance of wilt resistance against race 4 and reported RAPD marker against race 4. Kumar (1998) performed experiments to study the inheritance of race 2 of pathogen resistance to *fusarium* wilt in chickpea in crosses 'WR315' × 'K850' (resistant × tolerant) and 'K850' × 'GW5/7' (tolerant × tolerant) and 'WR315' × 'Cl04' (resistant × susceptible) to evaluate the number of genes governing the trait of interest and study the complementation and to investigate resistant segregants which are responsible in a cross between two tolerant genotypes. Two recessive and one dominant genes have been reported to be control wilt resistance trait in chickpea by tests of F_2 and F_3 generations of these crosses under controlled conditions at ICRISAT, Patancheru, India,

Winter *et al*. (2000) developed a comprehensive reference map using 130 $F_{6:7}$ RIL's from the inter-specific cross between cultivated chickpea line ICC-4958 × *C. reticulatum* (PI-489777) and localized resistance genes for fusarium wilt races 4. Benko-Iseppon *et al*. (2003) developed DNA amplification fingerprinting (DAF) markers linked to resistance loci. SCAR loci derived from DAF markers closely linked to the FOC-4 resistance locus 2.0 cM in chickpea was observed between marker R-2609-1 and the race 4 resistance locus. Seven other markers flanked this locus were in a range from 4.1 to 9.0 cM. These are the most closely linked markers available for this locus up to date. Kumar *et al*. (2003) validated CS-27700 (ASAP) primer with wilt susceptibility in commonly used parental lines of chickpea. Brinda and Kumar (2005) showed the independent segregation of DNA marker linked to H1 (CS27700) and H2 (A07C417) by using RIL's of cross JG62 (H1H1H2H2 susceptible early winter) × WR315 (h1h1h2h2 resistant).

Soregaon *et al*. (2007) identified molecular marker linked to H2 locus of *fusarium* wilt resistance gene for Race 1. They have used RIL population derived from

cross between K850 (h1h1H2H2, susceptible late winter) × WR315 (h1h1h2h2, resistant) segregating for H2 locus alone. The primer A07C417 amplified an extra fragment of 417 bp in susceptible parent and co-segregated in susceptible bulk. This marker show monogenic segregation ratio of 1:1 in the RIL's.

Gowda *et al*. (2009) mapped the FOC1, FOC2 and FOC3 genes with previously unreported SSR markers that closely flank the genes. They also validated the markers using 16 diverse chickpea genotypes. After linkage analysis, 19 markers showed association with one or more wilt resistance genes. All these markers were located on LG2. Further they carried out validation of markers viz. TA110, TA96, H1B06 and TA194 which were determined to be tightly linked with FOC1, FOC2 and FOC3 were carried out. They reported thirteen genotypes resistant to FOC1 and all of these were determined to carry the allele associated with resistance for the marker TA110. Among the three FOC1 susceptible genotypes, JG62 carried the susceptibility associated TA110 allele. Overall, TA110 correctly identified 14 of the 16 genotypes as either resistant or susceptible to either of the FOC races.

Halila *et al*. (2009) tagged and mapped a second gene conferring resistance to the chickpea wilt pathogen, *F. oxysporum* f.sp. *ciceris* race 0, to linkage group 2 (LG2) of the chickpea genetic map. Two genes controlled resistance against race 0 remaining unmapped while, segregate independently; one present in accession JG62 (Foc01 / foc01) and mapping to LG5 and the second present in accession CA2139 (Foc02 / foc02). The resistance to race 0 of wilt pathogen confer complete by both the gene but separately. Sequence tagged microsatellite sites (STMS) markers sited on LG2 were strongly associated with *Foc02 / foc02* revealed by genotypic analysis using these ten resistant lines paired with ten susceptible RILs, selected in the same population.

Mahmood *et al*. (2011) investigated the inheritance of wilt resistance of chickpea genotypes under field conditions. Inheritance of resistance to *F. oxysporum* was studied in a set of crosses among six resistant CM-98, Aug-786, Bittal-98, Balksar-2000, Wanhar-2000, Punjab-2000 and one disease susceptible parent viz., AUG-424. F_1, F_2 and F_3 generations indicated that resistance was conferred by a single recessive allele at the same locus in the six resistant parents. The resistance was successfully transferred from resistant parents to susceptible parent by hybridization and pedigree and bulk selection. Ali M and Gupta. S. (2012) were validated SSR markers linked to resistance among Pakistani germplasm to races of *F. oxysporium* f.sp. *ciceris* (FOC).

They have analysed markers in Pakistani germplasm, induced mutants and local lines. Most of the (SSR) markers showed good correlation with phenotypic evaluation of genotypes of different races of *F. oxysporium* f.sp. *ciceris* (FOC).

Padaliya *et al*. (2013) carried out marker assisted characterization of six chickpea genotypes differing for *fusarium* wilt reaction using seven molecular markers reported by earlier workers linked to disease resistant/susceptibility. In this study, four different markers (namely, CS-27, UBC-170, CS-27A and UBC-825) linked to susceptibility and three microsatellite based markers (TA-59, TA-96 and TR-19) linked to resistance allele were validated. It was observed that two Random Amplified Polymorphic DNA (RAPD) markers, CS-27 and UBC-170 and one sequenced characterized amplified region (SCAR) CS- 27A700 gave amplification of 700, 550 and 700 bp, respectively in susceptible genotype only as reported by earlier worker. The inter simple sequence repeat (ISSR) marker UBC-825 produced amplification of 1200 bp in susceptible genotypes (JG-62 and GG 4) and intermediate genotype (Chaffa). Three sequence tagged microsatellites site (STMS) primers (TA-59, TA-96 and TR-19) produced specific allele in wilt resistant genotypes. The PCR amplification of TA-59 primer generated two alleles, out of which the allele of 258 bp was present only in resistance genotypes. The alleles of 265 bp amplified by primer TA- 96 was present only in resistance genotypes and absent in other genotypes. The marker TR-19 amplified allele of 227 bp in resistant genotypes. Further, the sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS PAGE) analysis of seed storage protein showed a difference in protein profile among studied genotypes but none of polypeptide fragment was specific to wilt resistance or susceptibility.

Patil *et al*. (2014) constructed molecular map of chickpea using F_9:F_{10} recombinant inbred lines from an intraspecific cross between *fusarium* wilt susceptible (JG 62) and resistant (WR 315) genotypes. A total of 23 markers with LOD scores of > 3.0 were mapped on the recombinant inbred lines (RILs). Twenty sequence tagged microsatellites (STMSs) and three amplified fragment length polymorphisms (AFLPs) covered 300.2 cM in five linkage groups at an average inter-marker distance of 13 cM. Early and late wilting due to *fusarium* infection was recorded in RILs at 30 and 60 DAS, respectively. There was a significant variation among RILs for wilt resistance for both early and late wilting. QTLs associated with early (30 days after sowing (DAS) and late (60 DAS) wilting are located on LG II. The flanking markers for these QTLs were the

same as those of previous reports. Five STMS markers located on LG II of reference map (interspecific) were mapped on LG II of the present map (intraspecific) with minor changes in the order of markers indicating the conservation of these genomic regions across the *Cicer* species.

Masuria *et al*. (2017) validated different markers viz., CS-27, UBC-170, CS-27A, OP-U17-1, UBC-825, TA-59, TA-96 and TR-19 previously reported to be linked to wilt disease either susceptibility or resistance in chickpea. Among susceptibility linked markers CS-27700 and UBC-8251200 validated for all susceptible genotypes, whereas CS-27A700 was not validated only in susceptible genotypes, GG-4 while OP-U17-11014 was not validated in GG-4. The resistance linked markers UBC-170500 was validated in all resistant genotypes. The markers TA-59258 and TA-96265 did not give specific amplicon in resistance genotypes GG-1, GG-2 and Annigeri, GG-1 respectively, while TR-19227 did not amplify specific product in resistance genotypes Annigeri, GG-1 and GG-2. Out of eight markers studied, three markers CS-27700, UBC-8251200 and UBC-170500 were validated in fifteen diverse chickpea genotypes and found to be consistence for marker assisted characterization.

C. Pigeon pea:

Kotresh *et al*. (2006) used bulk segregant analysis with 39 RAPD primers which led to identification of two markers (OPM03704 and OPAC11500) that were associated with *Fusarium* wilt susceptibility allele in a pigeon pea F_2 population derived from GS1×ICPL87119. Prasanthi *et al*. (2009) used bulk segregant analysis to identify molecular markers linked to a major resistance gene using the F_2 population of two crosses LRG-41 × ICPL-87119 and ICPL-7035 × ICPL-8863. Random primers primer OPG 08950 was found to produce a consistent marker which differentiated resistant from susceptible parent and bulk. An identified random amplified polymorphic DNA marker OPG08 linked to *Fusarium* wilt resistance in pigeon pea was cloned and sequenced. Kumar (2010) identified two QTLs linked to *fusarium* wilt resistance. The first QTL was spans over 40.0 cM in LG-1 and flanked by the SSR markers CcM0444 and CcM0494. The second QTL was spans over 30.0 cM in LG-4 and flanked by CcM1027 and CcM0995. Both the QTLs have explained only 6% of phenotypic variation. Saxena (2010a) used 30 SSR markers to assess the DNA polymorphism in a set of 32 pigeon pea genotypes. As a result, five parental combinations were identified

for developing genetically diverse mapping populations suitable for the development tightly linked markers for *fusarium* wilt and sterility mosaic disease resistance.

2.7. Linkage mapping:

2.7.1 Construction of linkage maps:

Mapping population is essentially required for construction of a linkage map. Parents exhibiting diversity for one or more trait (s) are selected for development of mapping population. The plant population size of 50 to 250 individuals has been used by breeders for initial genetic mapping (Mohan *et al*., 1997). High-resolution mapping requires larger population size. Several different mapping populations (F_2, $F_{2:3}$, BC, DH's, RIL's, CSSL's etc.) are used for gene(s) / QTL's mapping in different field crops. (McCouch and Doerge, 1995; Paterson, 1996). In self-pollinated crops F_2 populations back cross populations are used commonly. Recombinant inbred lines (RIL's) are derived by inbreeding of individual F_2plants.Several softwares are available for genetic linkage mapping like LINKAGE (Suiter *et al*., 1983), MAPMAKER/EXP (Lander *et al*., 1987), GMENDEL (Echt *et al.,* 1992), Join Map (Stam, 1993 and 1995), and Map Manager QTX (Manly *et al*., 2001). Among which the JoinMap (Stam, 1993 and 1995) most widely used and a commercial program, while all others are available at the internet freely.

2.7.2 Linkage maps in lentil:

Plant breeders and Geneticists can use linkage maps of agricultural crops for improving tagging of genes like disease resistance and by their association with genetic markers, and in QTL mapping. The initial genetic map of lentil was constructed by Zamir and Ladizinsky, 1984; and Tadmor *et al*., 1987 utilizing sing morphological and isozyme markers. Havey and Muehlbauer (1989) were constructed linkage map in lentil which covering small portion of genome of this crop. Subsequently RAPD, AFLP, RFLP, ISSR and resistant gene analogs were used to construct different linkage maps using different populations. Eujayl *et al*. (1998a) constructed linkage map using 86 recombinant inbred lines (RILs) with 89 RAPD and 79 AFLP primers. Rubeena *et al*. (2003) reported linkage map based on 100 RAPDs, 11 ISSRs (inter-simple sequence repeats) and three resistance gene analog (RGA) markers. Duran Y and De La Vega MP (2004) used 62 RAPDs, 29 ISSRs 65 AFLPs and four morphological and one microsatellite marker for constructing linkage map in lentil. Hamweih *et al*. (2009)

identified 14 new microsatellite markers for lentil from genomic library of cultivated lentil ILL5588. They utilized these SSRs for diversity analysis of lentil core collection developed by ICARDA. Tanyolac *et al*. (2010) constructed molecular linkage using RIL population (Precoz × WA8949041) of 94 plants and 166 markers. The developed linkage map possessed 11 linkage groups spanning 1396.3 cM and average map distance was 8.4cM. The studied RAPD and ISSR markers were evenly spread across genome 391 AFLP markers generated were clustered in linkage group 1.

Gupta *et al*. (2012) reported that the construction of linkage map from interspecific population (L 830 × ILWL 77) of 114 F_2 plants. RAPD revealed higher polymorphism in comparison to ISSR. Eleven linkage groups were reported from this study covering 3843.4 cM. Average marker distance was 19.3 cM .The most recently Kaur *et al*. (2014) identified SNP markers from EST database. Used intraspecific mapping population Cassab × ILL2024 segregating for boron tolerance to identify genomic region associated with Boron tolerance in lentil.

CHAPTER 3 *Material and Methods*

3.1 Lentil wilt screening:

3.1.1 Materials:

In present study the experimental material comprised of 93 lentil genotypes representing lentil varieties and advanced lines and exotic germplasm lines of Mediterranean origin from ICARDA.

Table 3.1: The list of materials used in the study along with its source

S. No.	Genotypes	Source	S. No.	Genotypes	Source
1	L4721	IARI, New Delhi	48	IPL222	IIPR, Kanpur
2	L4712	IARI, New Delhi	49	IPL227	IIPR, Kanpur
3	L4717	IARI, New Delhi	50	IPL335	IIPR, Kanpur
4	L4076	IARI, New Delhi	51	KLB14-12	CSAUAT, Kanpur
5	L4715	IARI, New Delhi	52	IPL331	IIPR, Kanpur
6	L4590	IARI, New Delhi	53	RKL1003-21C	ARS, Kota
7	L4716	IARI, New Delhi	54	IPL81	IIPR, Kanpur
8	L4718	IARI, New Delhi	55	PL194	GBPUAT,Pantnagar
9	L4719	IARI, New Delhi	56	VL524	Almora
10	L4147	IARI, New Delhi	57	RVL13-5	Sehore,M.P.
11	L4720	IARI, New Delhi	58	RKL14-26	ARS, Kota
12	L4714	IARI, New Delhi	59	RVL13-7	Sehore,M.P.
13	L4713	IARI, New Delhi	60	VL148	Almora
14	L4709	IARI, New Delhi	61	VL525	Almora
15	L4710	IARI, New Delhi	62	DKL37	Dhaula kaun
16	L4593	IARI, New Delhi	63	RLG195	RARI, Durgapura
17	L4711	IARI, New Delhi	64	IPL315	IIPR, Kanpur
18	L4592	IARI, New Delhi	65	PL-165	GBPUAT, Pantnagar
19	L4708	IARI, New Delhi	66	RKL24C-59	ARS, Kota
20	L 9-12	IARI, New Delhi	67	DPL62	IIPR, Kanpur
21	L1373	IARI, New Delhi	68	IPL329	IIPR, Kanpur
22	L4739	IARI, New Delhi	69	IPL220	IIPR, Kanpur
23	L4737	IARI, New Delhi	70	KLS14-1	CSAU, Kanpur
24	L4730	IARI, New Delhi	71	IPL576	IIPR, Kanpur

25	L4726	IARI, New Delhi	72	NDL14-22	Faizabad
26	L4727	IARI, New Delhi	73	LL1374	PAU, Ludhiana
27	L4117	IARI, New Delhi	74	IPL406	IIPR, Kanpur
28	LL1320	PAU, Ludhiana	75	RKL12-11E-119	ARS ,Kota
29	LL1316	PAU, Ludhiana	76	IPL228	IIPR, Kanpur
30	L1318	IARI, New Delhi	77	PL191	GBPUAT,Pantnagar
31	IG 69549	ICARDA, Aleppo, Syria	78	IPL321	IIPR, Kanpur
32	IG 70238	ICARDA, Aleppo, Syria	79	NDL14-21	Faizabad,U.P.
33	IG 71487	ICARDA, Aleppo, Syria	80	IPL325	IIPR, Kanpur
34	ILL 10916	ICARDA, Aleppo, Syria	81	RVL11-6	Sehore, Bhopal
35	ILL 10921	ICARDA, Aleppo, Syria	82	RKL603-1	ARS, Kota
36	ILL 10965	ICARDA, Aleppo, Syria	83	VL149	Almora
37	PL6-9	GBPUAT, Pantnagar	84	DKL14-20	Dhaulkuan
38	DPL15	IIPR, Kanpur	85	IPL316	IIPR, Kanpur
39	SLC101	RARS, Sahillongani	86	PL172	GBPUAT,Pantnagar
40	PL178	GBPUAT, Pantnagar	87	KLS218	CSAUAT, Kanpur
41	IPL332	IIPR, Kanpur	88	IPL533	IIPR, Kanpur
42	HUL57	Varanasi	89	RLG192	RARI,Durgapura
43	PL175	GBPUAT, Pantnagar	90	IPL330	IIPR, Kanpur
44	PL157	GBPUAT, Pantnagar	91	RL3-5	IGKV, Raipur
45	KLS13-3	CSAUAT, Kanpur	92	PL192	GBPUAT,Pantnagar
46	KLB13-6	CSAUAT, Kanpur	93	Sehore 74-3	JNKVV, Jabalpur
47	IPL334	CSAUAT, Kanpur			

3.1.2 Methodology:

3.1.2.1 Wilt sick plot screening /Field screening:

The methodology for developing and maintaining wilt sick plots was suggested by Bayaa and Erskine (1990), Bayaa *et al*. (1995 and 1997) and Eujayl *et al*. (1998). For screening of *Fusarium* wilt resistant genotypes wilt sick plot screening is most common and widely used method. Screening in wilt sick plot involves planting of material to be screened along with susceptible checks. Based on observations of disease symptoms the plants are scored based on rating scale. The experimental material for the present study comprised of 93 lentil genotypes. Susceptible cultivar 'Sehore 74-3' was grown between every two rows of test material. Observations on wilt incidence were recorded at fortnightly interval after appearance of the disease symptoms. The genotypes on the basis of wilt incidence recorded were categorized into different categories *i.e.* highly susceptible, susceptible, moderate susceptible, moderate resistant and resistant. In wilt sick plot large no. of germplasm lines can be screened in cost effective manner.

Figure 3.1. Screening of lentil genotypes in wilt sick plot at Sehore, Madhya Pradesh.

3.1.2.2 Screening under controlled conditions/Greenhouse screening:

The results of field screening can be confirmed by glass house screening. A set of lentil genotypes comprising 93 was evaluated under greenhouse conditions. (Bayaa *et al*., 1994, Khare *et al*., 1993; and Kraft *et al*., 1994) have reviewed glass house screening techniques. Subsequently, genotypes were compared and confirmed the reaction against *F. oxysporium.* In this method, fungus can be multiplied on Potato dextrose agar, lentil extract dextrose, and Richards's solution. In pots pathogen is mixed in soil and planted with susceptible check. Lentil seeds of test entries as well as susceptible check, were sterilized by dipping in 2.0 percent sodium hypochorite for 2 minutes, the sterilized seeds were rinsed in sterile water for 3-4 times in order to wash off sodium hypochorite. Surface sterilized seeds were sown in plastic pots having 2/3 volume of sterilized sand/soil. All the pots were irrigated regularly for normal germination under greenhouse conditions. Inoculum of pure culture of *F. oxysporium,* isolated from naturally wilt infected lentil plants was used for inoculum preparation. Single spore culture of *F. oxysporium* was multiplied on 100 g of 9:1 sand: lentil meal medium for 15 days at 28-30^{0}C. Two hundred grams of these inoculums was mixed well with 2kg autoclaved soil and placed in one 15 cm plastic pots. Lentil seeds were sown in plastic pots filled with sterilized potting mixture soil, sand and FYM 1:1:1 proportion. Five seeds of each test genotype were planted in each pot. A highly susceptible check 'Sehore 74-3' was used as control during screening.

Fig 3.2: Screening of lentil genotypes against fusarium wilt under glass house

The plants sown in pots were observed for the development of wilt periodically and the final wilt incidence was recorded at sixty days after sowing. Glass house screening is required to avoid interference from other soil borne pathogens and confirm the results of wilt sick plot. Screening for resistance to lentil wilt, in general, must take into account two factors: the varied timing of symptom expression among genotypes and the uneven and patchy distribution of the disease in the field.

The appearance of disease symptoms, the percentage of dead plants was recorded following the method proposed by Bayaa and Erskine (1990).

Calculation of Disease Incidence

$$\text{Disease } \textit{incidence} \text{ (percent)} = \frac{\text{Total number of plants examined}}{\text{No. of plants infected}} \times 100$$

1. Scoring for wilt resistance reaction

The following disease scale was adopted for evaluating the genotypes against wilt disease incidence.

Rating Scale	Wilt incidence (per cent)	Reaction
1	1% or less plants wilted	Resistant
3	2-10% plants wilted	Moderately Resistant
5	11-20% plants wilted	Moderately Susceptible
7	21-50% plants wilted	Susceptible
9	Above 50% plants wilted	Highly Susceptible

3.2 Inheritance of wilt resistance in Lentil:

3.2.1 Methodology:

3.2.1.1 Crossing programme:

For crossing programme the two susceptible (L 9-12 and Sehore 74-3) and two resistance genotypes (ILL10921 and ILL10965) are used as parent .The parents were selected based on *Fusarium* wilt reaction and crosses were made among selected parents during rabi 2014-15 at the research farm station of IARI, New Delhi. The parental genotypes were sown in five rows plot with 2.5m row length, 25 cm row to row spacing and maintaining 5cm plant to plant distance. The standard packages of agronomic practices were followed to raise the crop. The details of the parentage and their crosses are presented in table 3.2. To verify the maternal effects reciprocal crosses also were made.

A. Resistant × Susceptible
   1. ILL10921 × L9-12
   2. ILL10965 × L9-12
   3. ILL10965 × Sehore 74-3

B. Susceptible × Resistant (Reciprocal)
   1. L9-12 × ILL10921
   2. L9-12 × ILL10965
   3. Sehore 74-3 × ILL10965

3.2.1.2 Raising F_1 generation:

The F_1 seeds collected separately from the each cross in rabi season 2014-15. Subsequently, these seeds were sown in off season at Wellington, regional station, IARI, Tamil Nadu in summer 2015. The plants were space planted in layout of 5 × 25 cm plant to row distance to produce sufficient number of F_2 seeds with parents. It help in screening of crossing plants and remove of selfed plants. The recommended package practices were followed .The seeds of raised materials were collected separately at maturity.

3.2.1.3. Screening of parents, $F_{1'S}$, $F_{2'S}$ and $F_{2:3}$ families:

The parents and F_1,s were screened at Sehore, M.P. and greenhouse condition at IARI New Delhi. In *rabi* 2015-16, the F_2 seeds were grown at Sehore, M.P. in hot spot for *Fusarium* wilt and recorded observation on segregation population .The infector row planted as a check after each 10 rows which help in spreading of the secondary inoculum. The raised plants materials were tagged and numbered to maintain proper identity of plant population before observations recorded. $F_{2:3}$ plant population was phenotyped at Sehore, M.P. during rabi 2015-16.

3.2.2 Statistical analysis:

3.2.2.1 F_2 segregation for wilt reaction:

The Chi-square test to determine goodness-of-fit was used to compare the actual genetic ratio with those calculated for Mendelian segregation.

$$\text{Chi-square } (\chi^2) = \Sigma \{(O-E)^2/E\}$$

Where, O= Observed number of individuals E= Expected number of individuals

Joint segregation test was performed to find out the segregation of two genes (wilt resistance and the microsatellite marker). Analysis for detection of linkage between the segregating genes were carried out following Mather (1951) by setting the distribution frequencies in a two way contingent table. Chi-square was first calculated for deviation due to segregation of resistance gene and the putatively linked microsatellite marker individually and then for deviation due to joint segregation of resistance gene and the microsatellite marker. Linkage was detected by subtracting the deviation due to individual gene segregation from the compound deviation due to combined segregation of genes.

3.3 Identification of molecular marker linked with the wilt resistance gene(s):

3.3.1 Parental polymorphism survey:

Four parents L 9-12, Sehore74-3, ILL10921 and ILL10965 were used for parental polymorphism survey using more than 300 SSR primers. This includes two susceptible (L 9-12 and Sehore 74-3) and two resistance (ILL10921 and ILL10965) parents.

3.3.2 Mapping population:

F_2 plants population derived from cross L9-12 × ILL10965 comprising of 120 individuals were used for the mapping of wilt resistance gene (s) using bulked segregant analysis (BSA) approach of Michelmore *et al.* (1991). For this parents, their respective bulks and individual plants were used for identification of molecular marker linked with the rust resistance gene(s). F_2 mapping population plants were randomly selected and tagged at early stage. Leaf samples from these plants were taken for DNA isolation. These plants were also characterised with respect to their wilt reaction at later stage.

3.3.3 Methodology:

3.3.3.1 Bulked segregant analysis (BSA) approach:

In most species, bulked segregant analysis is the classical way to find genetic markers of disease resistance genes. In context of lentil, there are only a few examples of disease resistance genes that have been tagged using BSA. In lentil, this method has been used to identify markers that are tightly linked to genes for resistance to *Fusarium* vascular wilt and Ascochyta blight (Chowdhury *et al*. 2001; Eujayl *et al*., 1998b; and Ford *et al*., 1999). Bulk Segregant Analysis (BSA) is method for rapid identification of markers in a genomic region linked to phenotypic traits controlled by major gene(s). It was first described by Michelmore *et al*. (1991). BSA partitions a population from a single cross into two pools, or bulks, according to a single trait, so that each bulk contains individuals corresponding to a particular phenotype or specific section of a phenotypic range. The method uses marker measurements of pooled genomic DNA samples from each bulk to measure correlation between marker and phenotype and thereby designate a probable location for the gene based on that correlation. This method relies on the availability of bulked DNA samples collected from individuals that segregate for the two extreme divergent phenotypes within a single population. In context of present study the parental polymorphism survey was carried out between L9-

12 and ILL10965 by using 302 SSR markers. Equal quantity of DNA from 10 resistant and 10 susceptible plants of F_2 mapping population were bulked separately to constitute resistant and susceptible bulk, respectively. To identify putative markers linked to *Fusarium* wilt resistance in F_2 mapping population, these resistant and susceptible bulks along with parents were tested in BSA using polymorphic markers for the two parents. The markers distinguishing two bulks and parents were considered as putatively linked to *Fusarium* wilt resistance gene in ILL 10965. Such putative markers identified in BSA were used for genotyping of whole F_2 population.

3.3.3.2. Genomic DNA extraction and purification:

From 21-day old seedlings, the genomic DNA was extracted using cetyl trimethyl ammonium bromide (CTAB) method. Leaf tissue (5 g) was powdered in liquid nitrogen by using a pre chilled motor and pestle. The powdered leaf tissue was transferred to polypropylene tube containing 15 ml DNA extraction buffer (50 mM TrisHCl, 150 mM NaCl, 100 mM EDTA, 10 % CTAB and 0.002 cm3 of β-mercapto ethanol). The tubes were incubated at 65º C for 30 min. Two third volume of chloroform: isoamyl alcohol (24:1, v/v) was added to this sample and mixed. The sample was then centrifuged at 10,000 rpm for 10 min at room temperature for separation of organic and aqueous phase. Organic phase was discarded and aqueous phase was collected in another tube and 0.6 v/v chilled isopropanol was added. The sample was kept for 15 - 25 min at -20 ºC and centrifuged at 10,000 rpm at 4 ºC for 12 min. DNA pellet was rinsed and washed with 70 % ethanol for 10 - 15 min. The pellet was dried at room temperature for 12 hours and then dissolved in TE buffer (pH 8.0). DNA was purified by RNAse treatment as described by Murray and Thompson (1980).

3.3.3.3 DNA Quantification:

Quantification of isolated DNA can be done either by spectrophotometric measurements or by agarose gel electrophoresis. In the present study, quantification was done by agarose gel electrophoresis of the isolated DNA samples along with known quantity of uncut λ lambda DNA.

3.3.3.4 Preparation of gel:

0.8 percent gel was made by mixing 0.8 g of agarose in 100 ml of 1 × TBE buffer. The solution was melted in a microwave oven, 5 µl of ethidium bromide was added to the metaphor solution after cooling it to 40°C and poured into a gel casting

tray set with combs. Once the gel polymerized, combs were removed and gel kept immersed in 1 × TBE in the buffer tank for loading the samples.

3.4.2.2 Estimation of DNA quantity:

Two µl of each DNA sample was taken along with 1 µl of 10× loading dye (MBI, Ferments) and the final volume was made up to 10 µl using sterile distilled water in separate 0.5 ml Eppendorf. Also four concentrations of lambda uncut DNA solutions 25, 50, 100 and 200 ng were prepared with loading dye and made up with sterile water to 10 µl. The whole 10 µl of the standards (λ DNA) and samples were loaded in the wells and resolved at a constant volume of 50 V for 1 hour. The approximate amount of DNA was estimated by making comparison of each of the samples with the known concentrations of uncut λ DNA loaded side by side. The DNA samples were diluted with TE buffer, according to their individual concentrations to make the final concentration of the DNA as 15 ng / µl.

3.4.3 Primer selection:

A set of 260 EST SSRs (Kaur *et al*., 2011 and Jain *et al*., 2013) and 60 genomic SSRs (Hamwieh *et al*., 2005 and 2009, Saha *et al*., 2010) were used for studying the polymorphism of 96 genotype of lentil. Seventy three SSR primers exhibiting polymorphism in the studied lentil genotypes were utilized in the present study. Because of the monomorphic nature or their nonspecific amplification the remaining EST-SSRs and genomic SSRs were not considered for the study.

3.4.4 Polymerase chain reaction:

The PCR amplifications were carried out in 20 µl reaction mix which comprised of 40 ng of template DNA, 10 picomole of forward and reverse primer, 0.2 milli molar of dNTPs (Bangalore Genei), 1x PCR buffer and 1 unit Taq DNA polymerase (Bangalore Genei). The products after amplification with microsatellite markers were resolved using a 3% metaphor gel. A 50 bp DNA ladder was used for detection of the size PCR products. DNA samples were electrophoresed for 4 hours at a voltage of 100 V in 1X TBE buffer and gel was photographed using a CCD camera attached to the gel documentation system (Syngene). The PCR cycling conditions for the SSR primer were along the following lines: primary denaturation at 94°C for 3 min followed by 30 cycles with 30 sec at 94°C, 30 sec at 54 – 60°C (depending upon the primer), 1 min at 72°C with final extension at 72°C for 5 min.

Composition of reaction mixture for PCR:

Reagents	**Stock concentrations**	**Volume (µl)**	**Final concentration**
Sterile dd H_2o	-	9.8	-
PCR buffer	10 ×	2.0	1 ×
dNTP mix	2mM	1.0	250 µM
Primer forward	40 ng/ µl	1.0	40 ng/µl
Primer reverse	40 ng/ µl	1.0	40 ng/µl
Taq polymerase	3 unit/ µl	0.3	0.33 units
DNA template	10.0 ng/ µl	5.0	40 ng
Total	-	20	-

CHAPTER 4 *Results*

This study was undertaken to assess the resistance in lentil germplasm collected from various places against wilt pathogen, understand its inheritance and locate the wilt resistance gene by mapping using microsatellite markers.

4.1. Screening of lentil germplasm for fusarium wilt resistance:

4.1.1. Reactions of promising lines of lentil against *Fusarium* wilt under field conditions:

Ninety three lentil genotypes were screened against *fusarium* wilt in sick plot. The result classified the studied lentil genotypes in four groups; moderately resistant, moderately susceptible, susceptible and highly susceptible reaction. Genotypes L4709, L4710, L4712, L4713, L4714, L4716, L4717, L4718, L4719, L4720, LL1374, IG 69549, IG 70238, IG 71487, ILL 10916, ILL 10921, ILL10965, IPL334, PL175 and DPL15 expressed moderate resistance reaction, genotypes L4592, L4593, L4711, L4715,IPL321, IPL332, IPL576, PL178, PL192 and HUL57 exhibited moderate susceptibility, genotypes L1318, L1373, L4076, L4117,L4590, L4708, , L4721, L4726, L4727, L4737, L4739, LL1320, IPL220, IPL222, IPL227, IPL228, IPL315, IPL325, IPL335, IPL406, IPL533, PL157, PL172, PL191, PL194, RKL24C-59, RKL603-1, RKL14-26, RLG195, VL524, NDL14-21, NDL14-22, KLB13-6, DKL14-20 and DPL62 exhibited susceptibility while genotypes L 9-12, L4147, L4730, LL1316, Sehore 74-3, IPL81, IPL316, IPL329, IPL330, IPL331, PL6-9, PL-165, KLS14-1, KLS13-3, KLS218, RLG192, RL3-5, KLB14-12, RKL1003-21C, RKL12-11E-119,VL149, VL148, VL525, RVL11-6, RVL13-5, RVL13-7, DKL37 and SLC101expressed high susceptibility reaction against the *fusarium* wilt. Bhat *et al.* (2003) and De *et al.* (2003) also screened lentil germplasm for *fusarium* wilt resistance and reported similar findings.

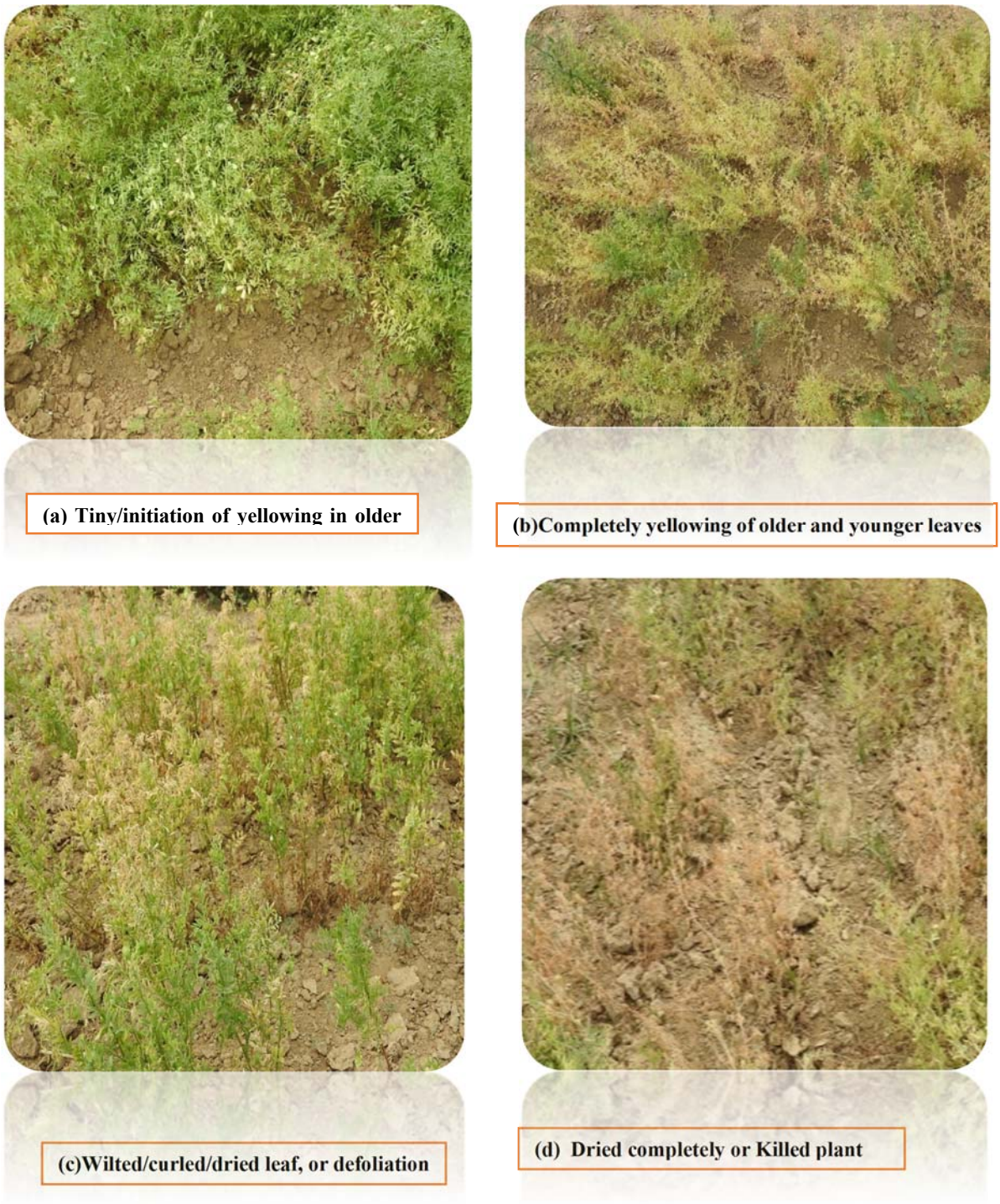

Fig. 4.1 Wilt symptoms in lentil genotypes recorded during field screening

Table 4.1 Reactions of lentil genotypes against *Fusarium* wilt in sick plot

Reactions	Genotypes
Resistant (≤1 %)	-
Moderately resistant (2-10%)	L4709, L4710, L4712, L4713, L4714, L4716, L4717, L4718, L4719, L4720, LL1374, IG 69549, IG 70238, IG 71487, ILL 10916, ILL 10921, ILL10965, IPL334, PL175, DPL15
Moderately susceptible (11-20%)	L4592, L4593, L4711, L4715,IPL321, IPL332, IPL576, PL178, PL192, HUL57
Susceptible (21-50%)	L1318, L1373, L4076, L4117, L4590, L4708, L4718, L4721, L4726, L4727, L4737, L4739, LL1320, IPL220, IPL222, IPL227, IPL228, IPL315, IPL325, IPL335, IPL406, IPL533, PL157, PL172, PL191, PL194, RKL24C-59, RKL603-1, RKL14-26, RLG195, VL524, NDL14-21, NDL14-22, KLB13-6, DKL14-20, DPL62
Highly susceptible (>50%)	L 9-12, L4147, L4730, LL1316, Sehore 74-3, IPL81, IPL316, IPL329, IPL330, IPL331, PL6-9, PL-165, KLS14-1, KLS13-3, KLS218, RLG192, RL3-5, KLB14-12, RKL1003-21C, RKL12-11E-119,VL149, VL148, VL525, RVL11-6, RVL13-5, RVL13-7, DKL37, SLC101

4.1.2. Reactions of lentil genotypes against *Fusarium* wilt under greenhouse condition:

Screening of genotypes under greenhouse condition exhibited moderate resistance in genotypes L4709, L4710, L4713, L4714, L4716, L4718, IG 69549, IG 70238, IG 71487, ILL 10916, ILL 10921 and ILL 10965. Moderately susceptible genotypes were L4708, HUL57, PL175, PL178, PL192, IPL321, IPL332, IPL576, IPL334, DPL15, L4719, L4720, LL1374, and L4593. While, L1318, L1373, L4076, L4117, L4590, L4592, L4711, L4712, L4715, L4717, L4721, L4726, L4727, L4737, L4739, LL1320, IPL220, IPL222, IPL227, IPL228, IPL315, IPL325, IPL335, IPL406, IPL533, DPL62, DKL14-20, RKL14-26, RKL24C-59, RKL603-1, RLG195, KLB13-6, VL524, PL157, PL172, PL191, PL194, NDL14-21 and NDL14-22 expressed susceptible reaction, genotypes L 9-12, LL1316, L4147, L4730, Sehore 74-3, RLG192, IPL81, IPL316, IPL329 IPL330, IPL331, RL3-5, PL-165, PL6-9, KLS13-3, KLS14-1, KLS218, KLB14-12 ,RKL1003-21C, RKL12-11E-119, RVL11-6, RVL13-5, RVL13-7, VL148**,** VL149, VL525, DKL37 and SLC101 showed high susceptibility against the *fusarium* wilt. Pande *et al*. (2007) also screened lentil genotypes for wilt resistance under controlled conditions and reported similar findings.

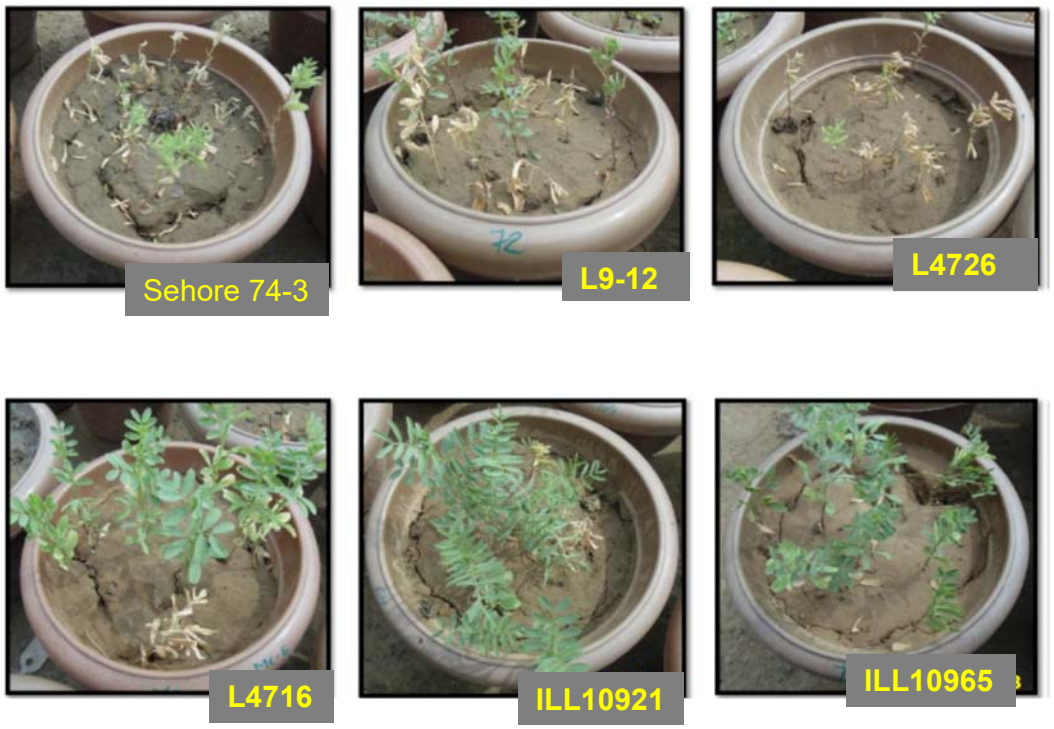

Fig. 4.2 Different degree of susceptibility and resistance in lentil genotypes under greenhouse condition:

Table 4.2 Reactions of lentil genotypes against *Fusarium* wilt in controlled conditions:

Reactions	Genotypes
Resistant (≤1 %)	-
Moderately resistant (2-10%)	L4709, L4710, L4713, L4714, L4716, L4718, , IG 69549, IG 70238, IG 71487, ILL 10916, ILL 10921, ILL 10965
Moderately susceptible (11-20%)	L4708,HUL57,PL175,PL178,PL192,IPL321, IPL332, IPL576, IPL334, DPL15, L4719, L4720, LL1374 , L4593
Susceptible (21-50%)	L1318, L1373, L4076, L4117, L4590, L4592, L4711, L4712, L4715, L4717, L4721, L4726, L4727, L4737, L4739, LL1320, IPL220, IPL222, IPL227, IPL228, IPL315, IPL325, IPL335, IPL406, IPL533, DPL62, DKL14-20, RKL14-26, RKL24C-59, RKL603-1, RLG195, KLB13-6, VL524, PL157, PL172, PL191, PL194, NDL14-21, NDL14-22
Highly susceptible (>50%)	L 9-12, LL1316, L4147, L4730, Sehore74-3, RLG192, IPL81, IPL316, IPL329 IPL330, IPL331, RL3-5, PL-165, PL6-9, KLS13-3, KLS14-1, KLS218, KLB14-12 ,RKL1003-21C,RKL12-11E-119, RVL11-6, RVL13-5, RVL13-7, VL148, VL149 ,VL525, DKL37, SLC101

4.2. Inheritance of wilt resistance:

The entire set of genotypes was screened in wilt sick plot and under controlled conditions. Based on results of screening, two indigenous lentil cultivar L9-12 and Sehore 74-3 were identified as susceptible and two genotypes ILL10921 and ILL10965 as moderate resistant. L9-12 and Sehore 74-3 exhibited high susceptibility to wilt with the score of >50.0% wilting while, ILL10965 and ILL10921 exhibited resistant reaction with the score of 2.0% -10 % wilting. In this study, the genetics of inheritance of wilt resistance gene in lentil was studied in a F_2 population which was derived from the crosses, L9-12×ILL10965, ILL10965× Sehore74-3, ILL10921 × L9-12, Sehore 74-3 × ILL10965, L9-12×ILL10921 and ILL10965×L9-12 after the selfing of F_1 plants.

4.2.1. Observations on F_1, F_2 and $F_{2:3}$ families:

All the plants of F_1 populations were screened for their wilt reaction. Individual plants of the different F_2 populations were observed for disease reaction and classified as either susceptible (S) or resistant (R). Presence or absence of the marker traits in each plant was also recorded. The number of plants in different crosses analysed under present investigation was above 100, in the F_2 population. F_2 ratios of most of the crosses were further confirmed by analysing the $F_{2:3}$ families for wilt reaction.

4.2.2 Mode of inheritance of wilt resistance:

The crosses (Resistant × Susceptible and Susceptible × Resistant) were made to the study inheritance pattern of wilt resistance in lentil. Total six crosses were made for studying the mode of inheritance of wilt resistance in lentil (Table 4.3). The F_1 of direct (resistant × susceptible) and reciprocal (susceptible × resistant) crosses were found to be resistant. This indicated dominance of resistance over susceptibility without any maternal effect. The F_2 population of all the six crosses individually segregated in a good ratio of 3R: 1 S. The F_2 analysis for goodness of fit to the ratio of 3R: 1S showed that the segregation in all the crosses were in agreement with the expectation, with high degree of confidence (P = 0.228-0.913). This confirmed the monogenic dominance of wilt resistance in lentil in the material studied. To substantiate the conclusion of fitness of the observed data to 3:1 ratio, heterogeneity for 3:1 ratio was calculated, which too was found to be non-significant (P = 0.849). This demonstrated the homogeneity in different crosses for fitness to 3: 1 ratio. A total of 679 plants in F_2 generation over all the 6 crosses were classified into 497 resistant and 182 susceptible classes and pooled

$\chi^2$was calculated which was 1.17. This again was found to be non-significant for 1 d.f. at 5% level (P=0.228).

Table 4.3 Segregating pattern for wilt resistance between different resistance (R) × susceptible (S) crosses of lentil

S. No.	Crosses	F_1 phenotype	No of F_2 plants			Expected ratio	χ2	P value
A	Resistance × Susceptible		Total	R	S			
1.	ILL10965 × L9-12	R	116	85	31	3:1	0.184	0.668
2.	ILL10965 × Sehore 74-3	R	112	80	32	3:1	0.762	0.383
3.	ILL10921 × L9-12	R	114	86	28	3:1	0.012	0.913
B	Susceptible × Resistance							
4.	Sehore74-3 × ILL10965	R	125	90	35	3:1	0.60	0.438
5.	L9-12 × ILL10965	R	120	92	28	3:1	0.17	0.680
6.	L9-12 × ILL10921	R	92	68	24	3:1	1.449	0.228
	Total (6df)						3.177	-
	Pooled over 6 crosses		679	497	182		1.179	0.228
	Heterogentity (5 df)						1.998	0.849

These all observations confirmed the monogenic dominance of wilt resistance over susceptibility in lentil. For confirmation of F_2 data, F_2 derived F_3 ($F_{2:3}$) families in three crosses were analysed for wilt reaction. It was observed that the $F_{2:3}$ family data of all the three crosses fitted well in the ratio of 1 (Breeds True for resistance): 2 (Breeds True for segregating): 1 (susceptible) with non-significant χ^2 values (Table 4.4). It confirmed that wilt resistance is controlled by a single dominant gene in these crosses. The results are in agreement with earlier reports of Eujayl *et al*. (1998) and Hamwieh *et al*. (2005).

Table. 4.4. F_2 segregation pattern for wilt resistance in $F_{2:3}$ families of four crosses in lentil:

Crosses	No of plants in each family	No of families	Breeds true for resistance	Segregating families	Breeds true for susceptible	Expected ratio	$\chi 2$	P value
L9-12 × ILL10965	25-30	30	8	14	8	1:2:1	1.20	0.54
ILL10965 × Sehore 74-3	25-30	30	7	14	9	1:2:1	0.40	0.818
L9-12 × ILL10921	25-30	30	8	15	7	1:2:1	1.79	0.409

4.3. Tagging wilt resistance gene:

The study was conducted to map the gene (s) controlling resistance to *Fusarium* wilt in lentil using SSR markers. F_2 population was used for tagging genes for wilt resistance and its homozygosity was determined by screening $F_{2:3}$ progeny rows against the wilt disease in the well-established sick-plot at Sehore (India).

Ten plants each of the non-segregating resistant and susceptible plant progeny rows were used for development of resistant and susceptible bulks for the bulk segregants analysis. Similar approach for tagging and mapping gene(s) for rust resistance in lentil was reported by (Mishra *et al*., 2003 and Dikshit *et al*., 2016). For the parental polymorphism survey, a total of 302 SSR markers have been used of which 80 SSRs exhibited polymorphism between parental lines L9-12 and ILL10965. These polymorphic SSR markers were utilized to study the polymorphism between the resistant and susceptible bulks of F_2 population using BSA. The BSA identified three SSRs namely PBALC233, PBALC1409 and PBALC203 which discriminated the two extreme bulks viz resistant and susceptible bulks too (Table 4.5). Using the identified three bulk discriminating primers were then used to screen the entire F_2 population consisting of 120 individuals plants. The data with respect to segregation of individual marker locus are presented in (Table 4.6). All the three markers which differentiated the bulk, showed good fit with the expected 1:2:1 ratio. Similar segregation of markers

was also recorded by Mishra *et al*. (2003) and Dikshit *et al.* (2016) in lentil for rust resistance. The data generated was analyzed using MapMaker_*ver*.3.0. The resistance gene *Fw* was found flanked by SSR markers, PBALC203 and PBALC1409 at distance of 8.2 cM and 9.4 cM respectively .The map of wilt resistant locus with linked SSR markers is presented as Figure 4.5.

Table 4.5. Lists of polymorphic SSRs segregating with putative wilt resistance gene.

S. No.	Primer	Forward sequence (5'-3')	Reverse sequence (5'-3')	Tm (°C)
1	PBALC233	AGTTGAAGACGGTGCAAA	CGAGAATGATGACCTTTAAGA	56
2	PBALC1409	GGGTCATTGTTATTTAGTTGC	CTTTTGGGTACTACTCCCATT	56
3	PBALC203	CATAGTCAACACTTGGTCGTT	GTCCACAATGAAACTCATCAC	56

Table 4.6. Segregation of SSR markers in F_2 population (L9-12×ILL10965).

Marker	Marker classes			Total plants	Chi square	P Value
	MM	Mm	mm			
PBALC 233	34	55	31	120	0.982	0.682
PBALC 1409	34	53	33	120	0.653	0.721
PBALC 203	33	56	32	120	1.649	0.438

The amplification profile of parents and individuals of resistant and susceptible bulks of F_2 plant population of wilt is presented in Figure 4.3.

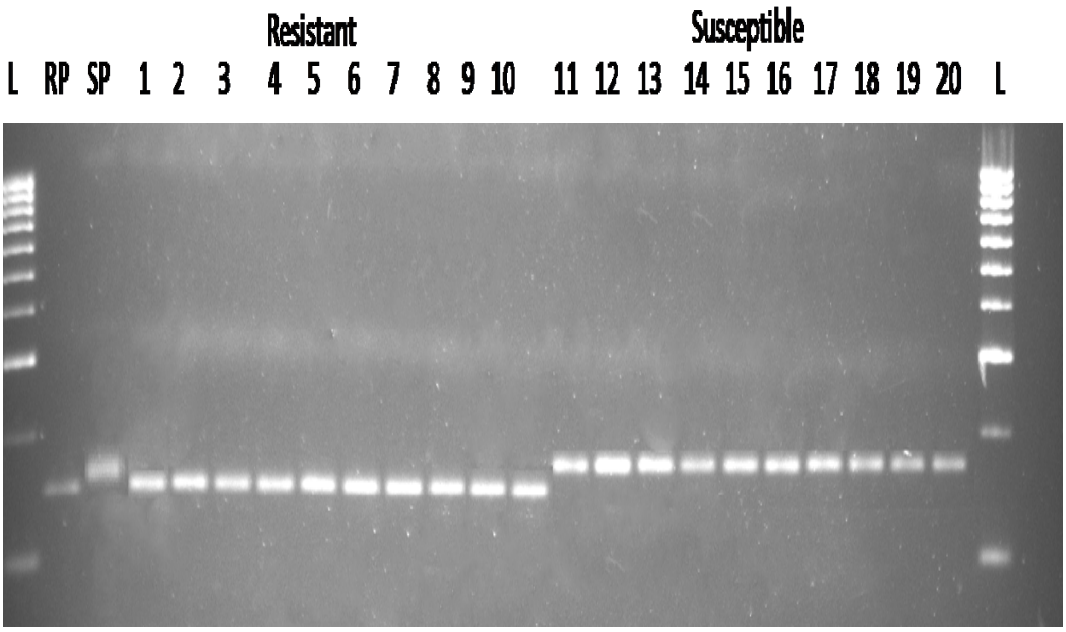

Fig 4.3 Amplification profile of SSR marker PBALC 203 in parents and the selected individuals utilized for making two extreme bulks for wilt expression, from F_2 mapping population. Where, RP: Resistant parent (ILL 10965), SP: Susceptible parent (L 9-12), L= 100 bp ladder

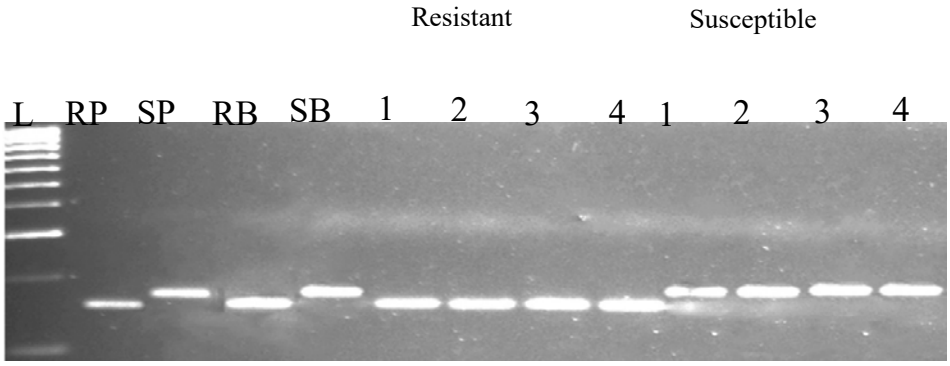

Fig 4.4 Amplification profile of SSR marker PBALC 203 in parents, Resistance bulk (RB), Susceptible Bulk (SB) and the selected individuals utilized for making two extreme bulks for wilt expression, from F_2 mapping population. Here, RP: Resistant parent (ILL 10965), SP: Susceptible parent (L 9-12), L= 100 bp ladder

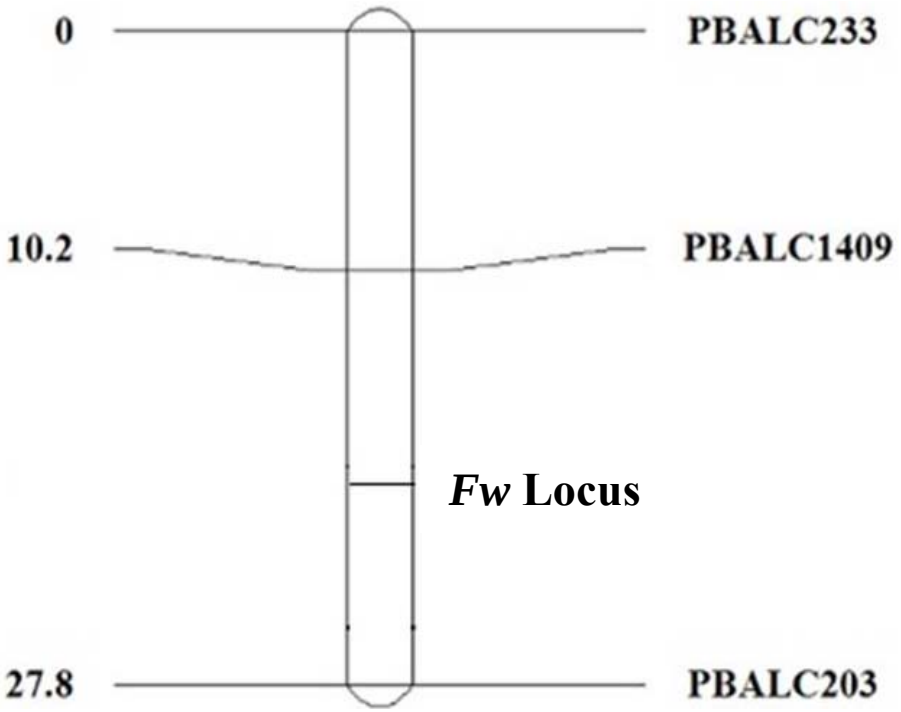

Fig. 4.5 Linkage-map of wilt resistant locus (*Fw*) with linked SSR markers

CHAPTER 5 *Discussion*

The lentil is protein rich legume crop cultivated in wide range of agro-climatic conditions. It occupies special position in human diet all over world due to its protein richness and easy digestibility. Lentil is orphan crop grown in rainfed areas across the world. Lentil production is affected by several abiotic (drought, heat, cold etc.) and biotic stresses (rust and wilt etc.). Among biotic stresses fusarium wilt caused by *F. oxysporium* f.sp *lentil* cause severe loss in lentil yield. Under epidemic conditions it can cause more than 50 percent losses or even 100 percent loss at seedling stage (Khare *et al*., 1979; Agrawal *et al*., 1993).

Fusarium wilt can be controlled by various means like physical, cultural and chemical but no single approach was observed as an effective approach in controlling this disease. Non availability of resistant varieties for cultivation in Central India is cause of concern and there is an urgent need for development of wilt resistant bold seeded variety. The development of a resistance variety via gene transfer is the possible solution for this problem but it requires knowledge on mode of inheritance of resistance gene(s).

The present research was carried out on screening of wilt resistance germplasm lines to study the inheritance of wilt resistance and molecular tagging of wilt resistance gene (s) in lentil. These aspects of the study have a direct impact on the development of new wilt resistant varieties in lentil. For this an attempt has been made by analysing the parents, F_1, F_2 and $F_{2:3}$ families for wilt reaction under natural epiphytotic conditions. For gene tagging, SSR markers (SSR's) were used. The aspect-wise discussion of results of the present investigation are detailed below

5.1. Screening for wilt resistance:

Successful screening for disease resistance is primarily depends on availability and accessibility of diverse germplasm collections and also on accuracy of the screening methods utilized in the study (Infantino *et al*., 2006). It is also very important for identifying susceptible and resistance genotypes and identification of suitable resistance parents for use in hybridization programme. Sick plot screening helps in screening against soil borne pathogens. It is most common method for screening large number of genetic material at low cost. In present study the wilt sick plot screening

was carried out following the methods suggested by Bayaa and Erskine (1990), Bayaa *et al*. (1995 and 1997), and Eujayl *et al*. (1998). The 93 genotypes were screened in wilt sick plot. Susceptible cultivar 'Sehore 74-3' was planted between every two rows as infector row for further spread of pathogen inoculum which helps in effective screening. Observations on wilt incidence were recorded at fortnightly interval just after appearance of the disease. But in case of wilt sick plot some other epidemic factors like drought, problematic soils are also contribute to disease incidence and sometimes escape occurs. Hence, controlled or glasshouse screening techniques was used for further validation of results obtained from field screening. Several germplasm lines exhibited reaction ranging from resistance to susceptible reaction against fusarium wilt in both conditions and were categorised in different group based on disease incidence viz., highly susceptible, susceptible, moderate susceptible, moderate resistant and resistant. The fusarium wilt screening helps in identification of donors for wilt resistance. . In this study six genotypes IG 69549, IG70238, IG71487, ILL 10916, ILL10921 and ILL10965 were identified as resistant through both the disease screening method. Similar approaches for screening wilt resistance have been reported for *F. oxysporum* f.sp. *medicaginis* (Weimer) Snyd. & Hans in *Medicago truncatula* Gaertn. (Rispail and Rubiales, 2014) and *F. oxysporum* Schlent.f.sp. *pisi* (JC Hall) Snyd. and Hans in pea (Bani *et al*., 2012).

5.2. Inheritance of wilt resistance:

In this study, the genetics of inheritance of wilt resistance gene in lentil was studied in an F_2 plant population (L9-12 × ILL10965). The parent L9-12 exhibited high susceptibility to wilt with the score of >50.00 percent wilting while, ILL10965 exhibited highly resistant reaction with the score of 2.0-10 percent wilting. Chi-square test for the studied F_2 populations confirmed the segregation ratio of 3:1, meaning that the wilt resistance in lentil was under the control of monogenic dominant gene (Table 4.3). The 120 $F_{2:3}$ progeny-rows expressed as 34 non-segregating wilt-resistant plant progeny row, 58 heterozygote segregating for wilt resistance/susceptibility and 28 non-segregating susceptible plant progeny-rows in 1:2:1 ratio (χ^2= 0.50; P-value is 0.779) confirming the results of F_2 generation. Brinda and Ravikumar (2005) have reported resistance to *Fusarium oxysporum* f.sp. *ciceri* race 1 (Foc1) to be governed by one or two genes. Singh *et al*. (1987) have reported three genes for resistance to the disease.

5.3. Gene tagging for wilt resistance in lentil:

The information about the genetics of fusarium wilt disease resistance is of immense use in lentil breeding programme intending to develop wilt resistant varieties. There is still need to do more intensive studies on the inheritance of Fusarium wilt resistance involving various diverse resistance and susceptibility sources in lentil.

The study was conducted to map the gene (s) controlling resistance to Fusarium wilt in lentil using SSR markers. F_2 population was used for tagging genes for wilt resistance and its homozygosity was determined by screening $F_{2:3}$ progeny rows against the wilt disease in the well-established sick-plot at Sehore (India). In the present study, bulk segregation analysis has been performed and three SSRs have been identified, which differentiates between resistance and susceptible bulk. These polymorphic SSR markers were utilized to study the polymorphism between the resistant and susceptible bulks of F_2 population using BSA. The BSA identified three SSRs namely PBALC233, PBALC1409 and PBALC203 which discriminated the two extreme bulks viz resistant and susceptible bulks. Bulk segregation analysis is a very useful approach for identifying association between markers and traits. This can be utilized in crops where the less information is available regarding their inheritance of the traits. Many researchers have used this method for dissecting genetics and to identify loci linked to the trait of interest. By applying BSA method SSR markers were identified linked to rust in lentil (Dikshit *et al*., 2016), wilt in chickpea (Gowda *et al*., 2009), water stress in wheat (Altinkut and Gozukirmizi, 2003) drought stress in rice (Venuprasad *et al*., 2009). PBALC203 and PBALC1409 were linked to the resistance genes *Fw*. The resistance gene *Fw* was found flanked by SSR markers, PBALC203 and PBALC1409 at distance of 8.2 cM and 9.4 cM respectively. The results exhibited high efficiency of these SSR markers in identifying genotypes resistant to wilt in lentil, this will subsequently alleviate the time-consuming process involved in breeding programs. Similar approach for tagging and mapping gene(s) for rust resistance in lentil was reported by (Mishra *et al*., 2003 and Dikshit *et al*., 2016). Hamwieh *et al*. (2005) studied the fusarium vascular wilt resistance in lentil. They have identified the Fusarium wilt resistance gene between the markers SSR59-2B and AFLP marker p17m30710 at distances of 8.0 cM and 3.5 cM, respectively.

The linked SSR's markers identified in this study is expected to aid fusarium wilt resistance breeding in lentil and wilt resistance can be incorporated in short span

of time. Further, it will also serve as a replacement for wilt phenotyping; which will help in the precise phenotyping either at very early stage or even in offseason nursery, thus making it cost-effective to grow more generation per annum. However, validation of these markers across populations and against a set of known resistant germplasm is required before employing this marker in wilt-resistance breeding programme. The lentil genome is now completely sequenced which will help in locating these linked markers on the chromosome. Also, the sequences around the gene can be used for designing additional primers for fine mapping of the gene to identify tightly linked markers for an efficient marker aided selection. This eventually can be used for map based cloning of the wilt-resistant gene to understand the molecular and biochemical basis of wilt-resistance in other biological processes. In chickpea resistance genes for wilt resistance have been mapped and tagged in various mapping populations (Tullu *et al*. 1998; Gowda *et al*. 2009 and Tekeoglu *et al*. 2000).

CHAPTER 6 *Summary and Conclusion*

Fusarium wilt is one of the major diseases of lentil crop caused by the fungus *Fusarium oxysporium.* In lentil more than 50 percent yield loss was reported due to *fusarium* wilt in India (Khare *et al.,* 1979; Agrawal *et al.,* 1993). It impedes the production of lentil crop, which contribute in reducing the pulse production of country. To stabilize the production of lentil fusarium wilt resistant varieties of lentil are required. Gene transfer is an effective way to develop disease resistance varieties which requires information on mode of inheritance of gene(s). The present investigations focussed on the following objectives:

1. To screen the lentil germplasm for *Fusarium* wilt resistance
2. To study the mode of inheritance of wilt resistance in lentil
3. To tag/map gene(s) for wilt resistance in lentil.

In present study released lentil varieties and advanced lines developed at different lentil breeding centres of India and exotic germplasm lines of Mediterranean origin from ICARDA were used for *fusarium* wilt screening. To study the mode of inheritance of *fusarium* wilt resistance two different types of crosses viz., Resistance × Susceptible (direct cross) and Susceptible × Resistance (reciprocal cross) were made at research farm of IARI, New Delhi. The F_1 seeds collected separately from the each cross and were sown in off season at Wellington, regional station, IARI, Tamil Nadu. The F_2 seeds were harvest separately from individual plants of F_2 mapping population (L9-12 × ILL10965). These F_2 seeds were grown at Sehore, M.P in hot spot for *Fusarium* wilt to raise the $F_{2:3}$ population and observation were recorded of segregation population for phenotypic study, while, molecular analysis was performed in the Pulse Lab, Division of Genetics, IARI, New Delhi.

The salient findings are presented below:

1. Ninety three genotypes were screened in wilt sick plot using infector row technique. Genotypes L4709, L4710, L4712, L4713, L4714, L4716, L4717, L4718, L4719, L4720, LL1374, IG 69549, IG 70238, IG 71487, ILL 10916, ILL 10921, ILL10965, IPL334, PL175 and DPL15 expressed the moderate resistance reaction and remaining 73 genotypes express moderate susceptible to high susceptible reaction in wilt sick plot.

2. Under the green house or artificial condition any genotypes did not show resistance reaction while twelve genotypes L4709, L4710, L4713, L4714, L4716, L4718, IG 69549, IG 70238, IG 71487, ILL 10916, ILL 10921 and ILL 10965 expressed moderate resistance reaction. While, genotypes L4708, HUL57, PL175, PL178, PL192, IPL321, IPL332, IPL576, IPL334, DPL15, L4719, L4720, LL1374 and L4593 showed moderate susceptibility and remaining genotypes expressed susceptibility to high susceptibility under greenhouse condition.

3. The F_2 segregation of studied six crosses (ILL10965 × L9-12, ILL10965 × Sehore74-3, ILL10921 × L9-12, L9-12 × ILL10965, Sehore74-3 × ILL10956 and L9-12 × ILL10921) exhibited 3:1 ratio of resistance and susceptible plants. The studies of crosses revealed that wilt resistance in lentil is dominant over susceptibility without any maternal effect and controlled by a single dominant gene. For confirmation of F_2 data observation, $F_{2:3}$ families in three crosses (L9-12 × ILL10965, L9-12 × ILL10921 and ILL10965 × Sehore74-3) were evaluated for wilt reaction at Sehore, M.P. The plants of all three crosses exhibited 1:2:1 ratio for resistance, segregating and susceptible reaction respectively. It further confirmed that wilt resistance is controlled by a single dominant gene in these crosses. The gene symbol *Fw* is proposed for this trait.

4. Parents namely ILL 10965 and L 9-12 were used for parental polymorphism survey. A set of 302 SSR primers were used for molecular study in which 80 were found polymorphic between the parents ILL 10965 and L 9-12 and only three markers (PBLAC233, PBALC203 and PBALC1409) exhibited polymorphism between the parents and as well as resistant and susceptible bulks. Linkage analysis revealed the resistance gene *Fw* flanked by SSR markers, PBALC203 and PBALC1409 at distance of 8.2 cM and 9.4 cM respectively. Further, PBLAC233 was also found present on the same linkage group at a distance of 10.2 cM from PBLAC1409.

The resistant genotypes identified in this study IG 69549, IG70238, IG71487, ILL 10916, ILL10921 and IIL10965 can be used as donor in future lentil wilt resistance breeding. Identified molecular markers (PBALC 233, PBALC1409 and PBALC 203) linked to wilt resistant loci in lentil after validation can be used for transfer of the wilt resistance gene into agronomically superior but wilt susceptible cultivars.

ABSTRACT

Fusarium wilt caused by *Fusarium oxysporum* f.sp. *lentis* Vasu. and Srini. is a serious disease of lentil (*Lens culinaris* Medikus), causing severe yield losses worldwide and it is a major disease in Central India. The disease is soil borne causing huge losses and development of wilt resistant varieties is most effective means of controlling this disease. Highly resistant sources of wilt in lentil have not been reported from the Indian lentil breeding programme. Ninety three lentil accessions including twelve varieties, six ICARDA germplasm lines and seventy five advanced breeding lines were evaluated in field and controlled conditions against wilt. Among which six genotypes IG 69549, IG70238, IG71487, ILL10916, ILL10921 and ILL10965 were identified as resistant in wilt sick plot as well as greenhouse condition. The germplasm L9-12 as wilt susceptible parent and ILL10965 as wilt resistance parent were used to develop mapping population. The inheritance of wilt-resistance gene in lentil was investigated in F_2 mapping population (L9-12 × ILL10965) exhibited 3:1 ratio of resistance and susceptible plants and $F_{2:3}$ mapping population plants exhibited 1:2:1 ratio for resistance, segregating and susceptible reaction respectively. It confirmed that wilt resistance in lentil is dominant over susceptibility without any maternal effect and controlled by a single dominant gene. Both wilt resistance gene inheritance and marker segregation was confirmed by Chi-square test, which fitted well the 3:1 ratio. Three hundred two SSR markers were surveyed for the parental polymorphism, in which three SSRs viz. PBALC233, PBALC1409 and PBALC203 were found polymorphic between parents as well as resistance and susceptible bulks. Linkage analysis showed two SSR markers, PBALC203 and PBALC1409 flanking the wilt resistance gene at 8.2 cM and 9.4 cM distance, respectively. Further, PBALC233 was also found present on the same linkage group at a distance of 10.2 cM from PBALC1409.

Keywords : Fusarium wilt, Gene, Lentil, Mapping Population and Markers

सारांश

फुजेरियम ओक्सीस्पोरम फॉर्म प्रजाति लेटिस वासु एवं श्रीनी द्वारा उत्पन्न म्लानि रोग मसूर) लेन्स कुलिनेरिस मेडिकस (की एक गंभीर बीमारी विश्वभर में उपज का भयंकर नुकसान करती है और यह मध्य भारत में बड़ी बीमारी है। भूमि से उत्पन्न यह बीमारी भयंकर नुकसान करती है और म्लानि प्रतिरोधी किस्मो का विकास इस बीमारी को नियंत्रित करने का सबसे प्रभावी माध्यम है। मसूर में म्लानि के अत्यधिक प्रतिरोधी स्रोतों को भारतीय मसूर प्रजनन कार्यक्रम से रिपोर्ट नहीं किया गया है। बारह किस्मो, छः आसीएआरडीए जननद्रव्य वंशक्रम एवं ७५ उन्नत प्रजनन किस्मो सहित ९३ मसूर अभिप्राप्ति का कृषि और नियंत्रित स्थितियों में म्लानि के खिलाफ मूल्यांकन किया गया। इनमे से आईजी६९५४९, ७०२३८, ७१४८७, आइएलएस १९१६,१०९२१ और आइएलएस१०९६५, म्लानि विरक्त क्षेत्र और हरित गृह की स्थिति में म्लानि के प्रतिरोधी के रूप में पहचाने गए थे। जननद्रव्य ९-१२ का उपयोग म्लानि संवेदनशील एवं आरएलएस१०९६५ का उपयोग म्लानि प्रतिरोध पैतृक चित्रण आबादी के विकास में किया गया। मसूर में प्रतिरोधी जीन की विरासत की जांच एफ२ में ३:१ अनुपात का प्रदर्शन प्रतिरोध और संवेदनशील पौधों और एफ्२:३ चित्रण आबादी के पौधों १:२:१ अनुपात का प्रदर्शन क्रमश सत्य प्रतिरोधी नस्ल, सत्य विसंयोजित नस्ल और सत्य संवेदनशील के लिए किया। इसने पुष्टि की कि मसूर में म्लानि प्रतिरोध का प्रभाव संवेदनशीलता पर बिना महत्व के था। और एक प्रभाबी वंशाणु द्वारा नियंत्रित था। दोनों, म्लानि प्रतिरोधकता की वंशानुगति एवं चिन्हक विसंयोजन का पुष्टि काई-स्क्वायर परीक्षण से की गई। जोकि ३:१ अनुपात में अच्छी तरह से समाहित हुई। पैतृक बहुरूपता के लिए ३०२ एसएसआर चिन्हको का सर्वेक्षण किया गया। जिनमे से ३ एसएसआर पीसीएलसी २३३, पीसीएलसी१४०९ एवं पीसीएलसी २०३ पैतृक एवं प्रतिरोधी तथा संवेदनशील पुंज के बीच बहुरूपी पाए गए। सहलग्नता विश्लेशण ने दर्शाया कि दो एसएसआर चिन्हक पीसीएलसी २०३ एवं पीसीएलसी १४०९ म्लानि प्रतिरोधक वंशाणु को क्रमशः ८.२ सीएम व ९.४ सीएम दूरी पर घेरे हुए है। आगे पीसीएलसी २३३ भी इसी सहलग्नता समूह पर पीसीएलसी १४०९ से १०.२ सीएम की दूरी पर स्थित है।

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Appendix-I

Appendix I. Preparation of chemicals, reagents, buffers and solutions

The various solutions and reagents used during the present study were prepared as described below:

(a) 1M Tris-HCl (pH 8.0): 121.1 g of Tris base was dissolved in 800 ml of double distilled water and pH was adjusted to 8.0 with HCl. The volume is adjusted to 1 liter with double distilled water and the prepared solution was sterilized by autoclaving.

(b) 0.5 M EDTA (pH 8.0): 186.1 g of EDTA disodium salt was added to 800 ml of distilled water, stirred continuously on a magnetic stirrer. The pH was adjusted to 8.0 by adding the NaOH pellets. The final volume was made up to 1 liter and sterilized by autoclaving.

(d) 5 M NaCl: 490.7 g of sodium chloride (NaCl) was dissolved in 700 ml of distilled water and the final volume was raised to 1 liter with distilled water.

(e) DNA extraction buffer: DNA extraction buffer was prepared by adding 10 ml of 1 M Tris-HCl, 12.4 ml of 5 M NaCl and 10 ml of 0.5 M EDTA, 1.2 g of SDS and 200 µl of β mercaptoethanol. The final volume was raised to 100 ml by adding distilled water.

(f) 50 × Tris-acetate-EDTA (TAE) buffer

Tris base: 242 g

0.5M EDTA (pH 8.0): 100 ml

Glacial acetic acid: 57.1 ml

The volume was made up to 1 liter and was used at a concentration of 1×

Appendix-II

Appendix II. List of consumables/Equipments

Consumables/Equipments	Description	Source
Markers	1kb DNA ladder 100 bp DNA ladder 50 bp ladder	MBI, Fermentas, Vilnius, Lithuania)
Rnase A	Bovine pancreas RnaseA	Sigma-Aldrich, Spruce Street, St. Louis, USA
Commonly used chemicals	Boric Acids, CTAB, tris, EDTA, DEPC, Sodium hydroxide, HCl, Glacial Acetic acid, β-mercaptoetanol, Sodium Chloride, Iso-amylalcohol, Phenol, Chloroform, Glycerol, Sodium acetate.	Sigma-Aldrich, Spruce Street, St. Louis, USA
Plasticwares	Sealing mats, PCR tubes and PCR plates, Micro tips, Micro centrifuge tubes, Falcon tubes (50ml), Falcon tubes (15ml)	Axygen Scientific Pvt. Ltd. Union City, California, USA
Glasswares	Reagent bottles	Scott Duraan, Mainz, Germany
	Measuring cylinders, Funnel, Volumetric flask and Beaker	Tarsons Products Pvt. Ltd. Kolkata, India
Equipments	VeritiTM thermal cycler	Applied Biosystems, Life Technologies, Singapore
	Electrophoresis apparatus	Bio-Rad laboratories. Washington D.C. USA
	Weighing Balance	YMC Co Ltd. Ishikawa, japan Life technologies
	Multi Wave Microwave	LG Electronics, Noida, India