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Molecular Mapping of a Gene Conferring Fusarium Wilt Resistance in Lentil (*Lens culinaris* Medikus subsp. *culinaris)* using Bulked-segregant Analysis

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ABSTRACT

Background: Vascular wilt caused by *Fusarium oxysporum* f.sp. *lentis* Vasu. and Srini. is a serious disease of lentil (*Lens culinaris* Medikus), causes severe yield losses worldwide. For effective disease resistance breeding the inheritance and mapping of wilt resistance gene (s) is necessary. Therefore, the present investigation was focused on study the mode of inheritance and tag/map gene (s) for fusarium wilt resistance in lentil.

Methods: Bulked segregant analysis (BSA) approach was used to identify markers that were tightly linked to *Fusarium* wilt resistance gene. The inheritance and mapping of wilt-resistance gene (s) in lentil was investigated in F_2 and $F_{2:3}$ populations derived from L9-12×ILL10965 cross, whereas L9-12 and ILL10965 were susceptible and resistant parents, respectively.

Result: More than two hundreds SSRs markers were surveyed for the parental polymorphism, of which twenty nine were found polymorphic. These polymorphic SSRs were used for the bulked-segregant analysis (BSA) using both parents and its respective resistant and susceptible bulks, and three SSRs *viz*. PBALC233, PBALC1409 and PBALC203 could distinguish the respective 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, PBLAC233 was also found present on the same linkage group at a distance of 10.2 cM from PBLAC1409.

Key words: *Fusarium oxysporum*, *Lens culinaris*, Linkage, Mapping.

INTRODUCTION

Lentil (*Lens culinaris* Medikus subsp. *culinaris*) is a diploid (2n=2x=14 chromosomes), self-pollinating, annual, cool-season grain legume crop, having haploid genome of 4,063 Mbp size (Arumuganathan and Earle 1991). From Mediterranean region, lentils has been spread to various parts of the world and ultimately evolved into six geographical groups (Cubero, 1981). Although, lentil is cultivated nearly in 52 countries, major share is occupied by countries like India, Canada, Turkey, Bangladesh, Iran, China, Nepal and Syria. Currently, the global area under lentil cultivation is about 6.10 m ha, producing 6.33 m tons of grains with an average production of 1,038 kg/ha (FAOSTAT, 2018). In India, lentil is cultivated in about 2.21 m ha with production and productivity of 1.62 m tons and 731 kg/ha, respectively (FAOSTAT, 2018).

Lentil grains are immensely valued for its richness in protein (22-35%), minerals (K, P, Fe, Zn, Se) and vitamins (A, K, E, folate, thiamin, β carotene, riboflavin, niacin, pantothenic acid and pyridoxine) for human nutrition (Sarker *et al.,* 2018; Kiran *et al*., 2021). Lentil productivity is constrained by various biotic and abiotic factors of which vascular-wilt (*Fusarium oxysporum* Shlecht. Emend. Snyder and Hansen f. sp. *lentis* Vasudeva and Srinivasan) is the most important yield limiting factor in Sub-Saharan Africa, South Asia, West Asia and North Africa (WANA), causing severe economic yield losses (Erskine *et al*., 2011).

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It is a soil-borne, host-specific fungus infecting only cultivated lentil (*Lens culinaris* spp. *culinaris*) and wild vetch (*Vicia montbretii*). It prefers warm and dry conditions and mostly infects the crop during reproductive crop growth stage. Its symptoms include drooping and wilting of top leaflets resembling water-deficit stress, stunting of plants, shedding of leaflets and ultimate plant death. Although, it survives on the debris of infested plants, seed transmission has also been reported (Erskine *et al*., 1990). In India, Fusarium wilt is the main reason which limits the production

of lentil in majority of the lentil growing states including Uttar Pradesh, Madhya Pradesh, Himachal Pradesh, Bihar, West Bengal, Assam, Rajasthan, Haryana and Punjab (Chaudhary *et al*., 2009).

The genetics of wilt-resistance in lentil is essential to understand the number of gene(s) controlling the trait in different background for its effective deployment in the breeding programme. However, limited reports are available on the genetics of lentil wilt resistance. Based on test of allelism, Kamboj *et al*. (1990) reported five dominant genes governing the lentil wilt resistance, of which two showed duplicate gene action and complimentary gene action in different genetic backgrounds. Further, Abbas (1995) and Eujayl *et al*. (1998) reported dominant single gene inheritance for wilt resistance.

Efforts to commercially control the wilt using chemical and biological means have not succeeded much due to its high cost and complexity of incorporation into the soil during crop growth. Hence, development and deployment of resistant cultivars is most effective, economical and environmentally friendly way of managing wilt in lentil (Bayaa *et al*. 1995). Further, field based screening methods for the identification of donors is tedious and sometimes gives inconsistent results. Thus, more precise and efficient strategy like identification of closely linked DNA markers to the resistance gene can provide effective tool for tagging, mapping and pyramiding of resistant gene in desirable agronomic background. Based on resistance and susceptible reaction in lentil cultivars eight races of fusarium wilt pathogen identified by Hiremani and Dubey (2018) and Chaithra *et al*., 2019 also isolated twelve *Fusarium oxysporum* f. sp. *ciceri* isolates from chickpea cultivars that could be help in race specific wilt resistance lentil variety.

Marker assisted breeding research has been taken up for various biotic stresses in lentil such as ascochyta blight (Gupta *et al*. 2012) and for fusarium wilt in chickpea (Pratap *et al*. 2017). Even for Fusarium wilt resistance, Eujayl *et al*. (1998) has reported a linked RAPD marker $OPK15_{900}$ at 10.8 cM distance, whereas Hamwieh *et al*. (2005) mapped the gene on LG 6 which was found flanked by SSR59-2B and P17m30710 (AFLP) at a distance of 8.0 cM and 3.5 cM respectively. The present investigation was aimed to identify the SSR marker linked with Fusarium wilt resistance gene in lentil using bulked-segregant analysis.

MATERIALS AND METHODS

Plant material and development of mapping population

The genotypes, L9-12 and ILL10965 which was earlier identified by our group as susceptible and resistant to the Fusarium wilt respectively, are used for the development of F_2 mapping population. These resistant and susceptible genotypes are identified from a set of 93 diverse genotypes which were screened against fusarium wilt in well-established sick-plot at Rafi Ahmad Kidwai (RAK) College, Sehore, India (23°12′ N, 77°05 E, 502 m AMSL) and also under controlled conditions.

Hybridization was performed during *rabi* 2014-15 at ICAR-Indian Agricultural Research Institute, New Delhi, India (28°63''24' N, 77°15''14' E, 218 m AMSL). A total of 11 putative F_1s were raised as offseason nursery at IARI, RS Wellington during summer 2015. Then, four true hybrids were identified using polymorphic SSR markers and harvested individually to produce F_2 populations during *rabi* 2015-16. One of the F_2 population having 120 plants was raised in the farm of IARI, New Delhi and were harvested individually to get the $F_{2:3}$ populations.

Phenotyping of parents and mapping population

To work out the genetics of the disease resistance, the parents, their $F_{1's}$ and $F_{2:3}$ families were phenotyped against Fusarium-wilt in a well-established wilt-sick plot at RAK College of Agriculture (Sehore), which is also the hot-spot for *Fusarium* wilt in India, using infector row technique. The parents and 120 individuals of $F_{2:3}$ population were raised in individual rows during *rabi* 2015-16 and *rabi* 2016-17 respectively under sick-plot conditions as described by Eujayl *et al*. (1998). The planting of $F_{2:3}$ and parental lines were done at a row-to-row and plant-to-plant distance of 5.0 cm and 25.0 cm respectively, with the row-length of 4.0 meter.

DNA extraction and PCR analysis

Genomic DNA extraction was done using 2.0 g leaf tissue of 21 days old seedlings with CTAB method. Twenty µl PCR reaction mixture constituted of 10× buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$ and 0.01% gelatin), 200 µM dNTPs, 0.5 µM forward and reverse primers, 1U Taq DNA polymerase (Sigma-Aldrich, USA) and nearly 40 ng DNA. The amplification was performed in a thermal cycler (Applied Biosystems, Singapore). The PCR amplification protocol comprised of denaturation at 94°C for 4 min followed by 30 cycles of 94°C for 1.0 min, annealing at 59 to 62°C for 30 seconds, extension at 72°C for 1 min and final extension at 72°C for 10 min. Electrophoretic separation of amplified products were performed in 3.0% Metaphor agarose gel (Lonza, USA) in TBE buffer at 100 V for nearly 3 h and stained using ethidium bromide. Gel documentation system (Syngene) was used to record the gel photograph using CCD camera. DNA ladder 50bp (MBI, Fermentas, Lithuania) was used as marker and more than two hundreds SSR primer pairs were used to study the parental polymorphism.

Bulked-segregant analysis (BSA)

BSA, as proposed by Michelmore *et al*. (1991) was performed to tag the Fusarium wilt resistance gene in lentil. The resistant and susceptible bulks were constituted from the 10 F_2 homozygous individuals each, showing extreme phenotype for the wilt disease and having 30 ng/µL DNA. The polymorphic SSR makers identified between resistant and susceptible parents were used for studying polymorphism among the resistant and susceptible bulks.

The amplification of allele in resistant parent and resistant bulk or susceptible parent and susceptible bulk was the basis of association of marker with gene controlling wilt resistance. The polymorphic primers identified between resistant and susceptible bulks are used to genotype all the F_2 plants of mapping population.

Segregation and linkage analysis

The individuals of $F_{2:3}$ families which were derived from the F_2 population were screened for wilt resistance in the wilt sick plot at RAK College, Sehore (Madhya Pradesh, India). The segregating populations were classified in two distinct classes as resistant and susceptible. Chi square test (Gomez and Gomez 1984) for a fixed ratio hypothesis was used to analyze the data (tested at 5% level of significance). MapMaker ver. 3.0 (Lander *et al*. 1987) was used to determine linkage between resistant gene and markers, and linkage map was constructed using Kosambi mapping function at LOD 3.0 (Kosambi 1944).

RESULTS AND DISCUSSION

Inheritance of wilt resistance

The key approach of controlling wilt disease in lentil is by resistance breeding which has resulted in the identification of resistant accessions in both wild and cultivated lentil (Erskine *et al*., 1994). These resistant lines have been extensively used by the breeders and famers throughout the world (Bayaa *et al*. 1997). Although, a number of resistant sources to Fusarium wilt including released varieties are known but only few reports about the inheritance of this deadly disease is published (Kamboj *et al*. 1990; Abbas 1995).

In this study, the genetics of inheritance of wilt resistance gene in lentil was studied in F2 population which was derived from the cross, L9-12 × ILL10965 after the selfing of F_1 plants. The parent L9-12 exhibited high susceptibility to wilt with the score of >50.0% wilting while, ILL10965 exhibited moderate resistant reaction with the score of <2.0-10% wilting. Chi-square test for the studied F_2 population confirmed the segregation ratio of 3:1, meaning that the wilt resistance in lentil was under the control of monogenic dominant gene (Table 1). The 120 $F_{2:3}$ progeny-rows were 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). The results were in conformity with the previous reports of Eujayl *et al.* (1998) and Hamwieh *et al.* (2005) in which single dominant gene control of rust resistance have been reported. On the similar note, Abbas (1995) also reported single dominant gene control of wilt resistance in the crosses studied at ICARDA, Syria; while Kamboj *et al*. (1990), reported five independently segregating genes controlling wilt resistance in Indian germplasms.

Mapping of wilt resistance gene in ILL10965

This study was conducted to tag/map the gene(s) controlling resistance to Fusarium wilt in lentil using SSR markers. For tagging/mapping wilt resistance gene F2 population was used and its homozygosity for wilt resistance gene 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 segregant analysis. For the parental polymorphism survey, a total of 212 SSRs markers have been used of which 29 SSRs exhibited polymorphism between parental lines L9-12 and ILL10965. These markers were then used 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 (Table 2). These three bulk discriminating primers were then used to screen the entire F_2 population consisting of 120 individuals. The amplification profile of parents and individuals of resistant and susceptible bulks of F_2 population of wilt is presented in Fig 1. The data with respect to segregation of individual marker locus are presented in Table 3. All the three markers which differentiated the bulk, showed goodness of fit with the expected 1:2:1 ratio. Similar segregation of markers was also observed by Dikshit *et al*. (2016) in lentil and Chaithanya *et al.* (2011) in pigeonpea.

The data generated was analyzed using Map Maker_*ver*.3.0. The resistance gene was found flanked by SSR markers, PBALC203 and PBALC1409 at distance of

Table 1: Segregation for wilt disease reaction in F_2 population.

Cross (F_2)	Wilt disease reaction in F_2		Expected ratio	χ^{2*}	df	P-Value
	Resistant	Susceptible				
L9-12 × ILL10965	92	28	3:1	0.17	1	0.680

Where, df: Degree of freedom; *Value for significance at P= 0.05.

Table 2: Lists of polymorphic SSRs segregating with putative wilt resistance gene.

Primer	Forward sequence (5'-3')	Reverse sequence (5'-3')	Tm (°C)
PBALC233	AGT TGA AGA CGG TGC AAA	CGA GAA TGA TGA CCT TTA AGA	56
PBALC1409	GGG TCA TTG TTA TTT AGT TGC	CTT TTG GGT ACT ACT CCC ATT	56
PBALC203	CAT AGT CAA CAC TTG GTC GTT	GTC CAC AAT GAA ACT CAT CAC	56

Table 3: 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

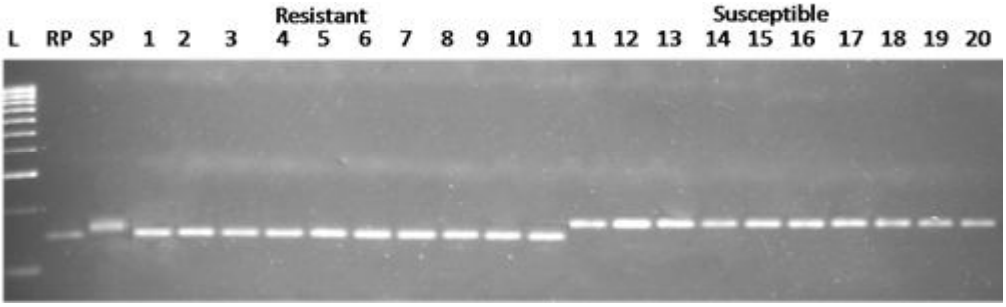

Fig 1: Amplification profile of SSR marker PBALC 233 in parents and the selected individuals utilized for making two extreme bulks for wilt expression, from F_2 mapping population. Where, RP: Resistant parent (ILL 10965), SP: Susceptible parent (L 9-12), L: 100bp ladder

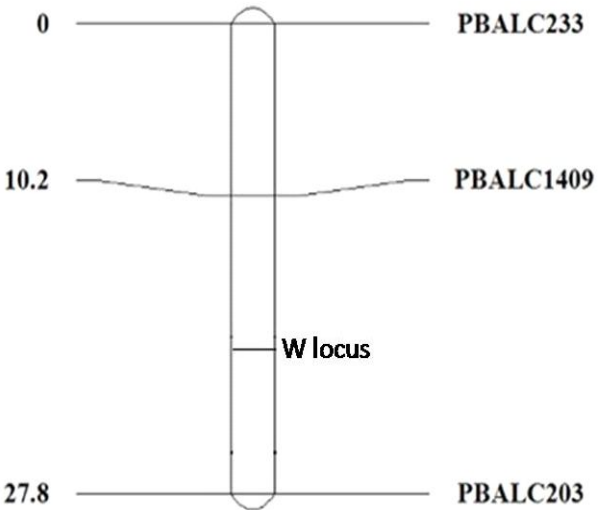

Fig 2: Linkage-map of wilt resistant locus with linked SSR markers.

8.2 cM and 9.4 cM respectively. The map of wilt resistant locus with linked SSR markers is presented as Figure 2. Eujayl *et al*. (1998) reported a RAPD marker (OPK-15_{900}) linked with the *Fw* locus at a distance of 10.8 cM on LG6. Similarly, Halila *et al*. (2009) has been mapped gene conferring fusarium wilt resistance for *Fusarium oxysporum* f. sp *ciceris* race 0, to linkage group 2 (LG2) of the chickpea genetic map.

Further, Hamwieh *et al*. (2005) also localized the Fusarium wilt resistance gene on LG 6 which was found flanked by SSR59-2B and p17m30710 (AFLP marker) at a distance of 8.0 cM and 3.5 cM, respectively. In recent times, a number of relatively dense linkage maps have been reported (Gupta *et al*., 2012; Saha *et al*., 2013; Kaur *et al*., 2014) which can assist in the execution of more precise marker assisted breeding for wilt disease resistance in lentil.

CONCLUSION

Linkage analysis revealed that the resistance gene *Fw* was 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. 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. The information about the genetics of Fusarium wilt disease resistance is of immense use in lentil breeding programme intended to develop wilt resistant varieties. The linked SSRs identified in this study is expected to aid Fusarium wilt resistance breeding in lentil by incorporating gene(s) in a short span of time.

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