Bulk segregant analysis identifies SSR markers associated with *Alternaria* blight resistance in linseed (*Linum usitatissimum* L.)

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Abstract

Aim : *Alternaria* blight caused by *Alternaria lini* is one of the major diseases of linseed which severely affects the yield and productivity. Here, we utilized F_2 mapping population derived from a resistant (JRF-4) and a susceptible (Chambal) genotypes of linseed and SSRs to identify the markers associated with *Alternaria* blight resistance using bulk segregant analysis approach.

Methodology : A population consisting of 154 F_2 individuals was developed from the cross between JRF-4 (resistant) and Chambal (susceptible). All 154 F_2 individuals were screened with 100 polymorphic SSRs to identify extreme phenotype. Two bulk of extremes phenotypes (disease resistant and disease susceptible) from F_2 mapping population were used for the bulked segregant analysis. The SSR primers that distinguished the parental lines were used to amplify the DNA from two bulks and banding pattern was observed to identify the SSRs that can differentiate the resistant and susceptible phenotypes bulk for *Alternaria* blight. Markers validation was carried out by amplifying DNA from individual plants of each bulk.

Results : Out of 100, only 10 markers showed polymorphism among the bulks and of which only three markers viz., LUSc 898_3_12, Lu 2472 and Lu 3078 were able to differentiate the disease resistant and susceptible individuals from F_2 population. Further, single marker linear regression approach was used to validate the association of selected polymorphic markers with the disease. The markers LUSc 898_3_12 and Lu 2472 showed significant regression which confirmed their linkage with *Alternaria* blight resistance.

Interpretation : The two markers having significant regression can be used for diseases resistance breeding during marker assisted selection.

Key words: *Alternaria* blight, Bulk segregant analysis, Linseed, SSR marker

Screening of resistant and susceptible parent with SSRs to identify polymorphic SSRs

Resistant and Susceptible parent crossing

Resistant and Susceptible parent crossing

Resistant and Susceptible F_2 individual identification through polymorphic SSRs

SB

(10 susceptible F_2 individual bulk)

RB

(10 resistant F_2 individual bulk)

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Introduction

Flax or linseed (*Linum usitatissimum* L., $2n=2x=30$) is an annual crop belonging to family Linaceae. It is among the oldest fiber and oilseed crops in the world. The use of flax for the production of linen dates back 5000 years. Presently, the total world production of linseed is approximately 2.65 million tonnes, with Canada (34%), the Russian Federation (15%) and China (13%) being the main producers (FAO, 2017). India is the sixth largest producer in the world with contribution to global linseed area and production 13% and 5.5%, respectively (Kaur et al., 2018). It is a highly valuable crop and every part of the plant has a specific economic importance. Flax seeds have 30–45% oil (Diederichsen, 2001) constituted by seed coat and its embryo. Stem provides fibre of good lusture, tensile strength and durability having good blending property. Linseed oil consists of high level of omega-3 fatty acids (~65%) among which alpha-linolenic acid (ALA) is the important omega-3 fatty acid having considerable benefit to humans and animals. ALA has been shown to play beneficial role in heart disease, strokes, and certain types of cancer by interfering with the effects of estrogen. Linseed oil cake is also a good feed for milk cattle and poultries and also as manure (Bibi et al., 2015). Another important compounds extracted from linseed is lignans that offers several potential health benefits. Lignan present in oil has anti-carcinogenic effect (Chauhan et al., 2009). Flax fibre is utilized in making high-grade paper, upholstery tow, insulating materials, rugs, yarn, linen and other textiles.

There are several biotic and abiotic stresses that adversely affect linseed production. Among them, *Alternaria* blight caused by *Alternaria lini* Dey and *A. linicola* Grooves and Skolko, is a major biotic stress limiting crop yield in hot and humid environment (Singh and Singh, 2004a; 2005). The genetic improvement of linseed through conventional breeding approaches has resulted in several high yielding and disease resistant varieties. It has been earlier reported that resistance against *Alternaria* blight in linseed is mainly controlled by additive gene action with the minor role of non-additive gene effect (Tiwari et al., 2012; Singh et al., 2003). However, conventional breeding approaches supplemented with marker assisted selection has laid the foundation for changing the selection criteria solely from selection of phenotypes towards selection of genes. MAS is an efficient, precise and time saving approach of crop improvement.

Molecular markers have been widely used in different crops for tracking loci and genomic regions for various agronomic and disease resistant traits (Phillips and Vasil, 1994; Gupta and Varshney, 2004). Among all the molecular markers, SSR is most widely used and the most powerful tool for variety identification and has much potential in genetic and breeding studies (Wang et al., 2007). SSR markers can also be utilized in QTL identification for selecting certain traits, such as disease resistance that is essential for any breeding program. These microsatellite markers are utilized in genomic assisted breeding for selecting ELS resistant genotypes in groundnut (Zongo et al., 2017). Blast resistance gene in rice (Kumbhar et al., 2013) and rust resistance

gene in pea (Upadhyay et al., 2016) have also been identified through SSR markers. Bulk segregant analysis (BSA) is a selective genotyping strategy for selecting two pooled DNA samples of individuals from a segregating population derived from a single cross. It is less time-consuming method for identifying potential markers linked to any trait (Michelmore et al., 1991). Bulk sample analysis can be used for any population with significant phenotypic difference for the target trait among individuals (Zou et al., 2016). One strength of this analysis is that it does not require a linkage map construction and could be utilized where insufficient polymorphism exists for map (Quarrie et al., 1999). An additional advantage is that this approach relies on dramatic reduction in the number of marker assay when compared to building a genetic map for the purpose of identifying markers associated with a trait (Wenzl et al., 2007). Thus, the present study was undertaken to identify molecular markers associated with *Alternaria* blight disease resistance in linseed through BSA in early segregating generation.

Materials and Methods

Mapping population and SSR markers: The F_2 mapping population comprises a total of 154 plants derived from cross of JRF-4 and Chambal genotypes of linseed (Fig. 2 A and B). The JRF-4 is a fiber type and *Alternaria* blight resistant genotype while Chambal is oil type and *Alternaria* blight susceptible genotype (Singh et al., 2008). All the 154 F_2 plants along with parental lines were grown in field at C.S.A. University of Agriculture and Technology, Kanpur during the crop year 2014-2015. The SSR markers for the present investigation was selected from the previous report (Cloutier et al., 2012) and also developed from genome sequence of linseed. Out of total 100 SSRs used, 50 were selected from published linkage map (Cloutier et al., 2012) and 50 from genome sequence data of linseed.

Inheritance for *Alternaria* blight in F_2 population : The morphological screening of F_2 plants was carried out for *Alternaria* blight response along with their parents at experimental field of CSIR-National Botanical Research Institute, Lucknow, India. This disease attacks all the aerial parts of the plant, however, on leaves, the disease appears as small, dark brown spots which results in their twisting and drying. In severe infection, the plant may die. In the present study, *Alternaria* blight screening was done at flowering (Stage 1) and harvesting (Stage 2) stage using the 0 to 5 scale (Tiwari et al., 2012) (Supplementary Table 1) based on percent damaged area of leaf. For genetic study, scale 0-3 was collectively categorized as resistant (R) whereas, scale 4-5 were taken as susceptible (S) plants.

DNA extraction and SSR analysis : The genomic DNA was extracted using DNeasy plant mini kit (Qiagen, Valencia) as per manufacturer's protocol. The quality of DNA was assessed by electrophoresis in 0.8% agarose gel and the concentration was determined with flurometer (Qubit, Invitrogen). Finally, DNA was normalized to $10 \text{ ng } \mu\text{l}^{-1}$ for PCR amplification. PCR amplification was carried out in $10 \text{ } \mu\text{l}$ reaction volume containing 10 ng of

genomic DNA, 1× PCR master mix (FermentasInc, USA), 1.0 µl (5 pmol µl⁻¹) each of forward and reverse primers in Veriti PCR machine (Applied Biosystems). The amplification was carried out with an initial denaturation of 5 min at 95°C, followed by 35 cycles of denaturation for 30 sec at 94 °C, annealing for 45 sec at 52-58 °C- (primer specific), extension for 30 sec at 72 °C and final extension for 15 min at 72 °C. The amplified PCR products were analyzed using 3.0 % agarose gel at constant power of 150V for 2 hrs.

Bulk segregant analysis: Two bulk of extreme phenotypes (disease resistant and disease susceptible) from F₂ mapping population were used for the bulked segregant analysis following Michelmore *et al.* (1991). Equal quantities of DNA were bulked from 10 susceptible plants to prepare susceptible bulk (SB). Likewise, resistant bulk (RB) was prepared by pooling equal amount of DNA from 10 resistant plants. The SSR primers that distinguished the parental lines were used to amplify the DNA from two bulks and banding pattern was observed to identify the SSRs that can differentiate the resistant and susceptible phenotypes bulk. Marker validation was carried out by amplifying DNA from individual plants of each bulk.

Statistical analyses : The analysis of variance at different stages for *Alternaria* blight through phenotypic disease scoring of F₂ and parents was done using the WINDOSTAT software (www.windostat.org). Phenotypic coefficient of variance (PCV), genotypic coefficient of variance (GCV) and heritability in broad sense (h²) were computed for all stages of disease scoring.

Single marker analysis (SMA) through linear regression analysis was performed using F₂ genotypic data of 10 polymorphic markers and F₂₃ (141 individuals) phenotypic data of

Alternaria blight disease score to identify potential SSR markers associated *Alternaria* blight resistance. The coefficient of determination (R²) was used as a measure of magnitude of association.

Results and Discussion

F₂ individuals were scored from 0 to 5 on the basis of percent disease infection. Out of 154 F₂ individuals, 115 were resistant and 39 were found susceptible. This disease scoring data was used to prepare frequency distribution graph (Supplementary Fig. 1) and also to perform chi-square to test the goodness of fit. Chi-square value revealed high probability for a good fit (P>0.99) towards 3:1 ratio, which implies towards governance of disease resistance by one dominant gene. Previously, Chauhan *et al.* (2013) also reported that one dominant gene is responsible for *Alternaria* blight resistance in linseed. Singh *et al.* (2003) observed that *Alternaria* blight in linseed is mainly attributed to additive gene action.

Analysis of variance showed significant differences between F₂ mapping population for *Alternaria* blight indicating towards considerable genetic variability among them. The parent JRF-4 showed mean disease score of 0.66 for *Alternaria* blight which confirms it as disease resistant genotype as compare to other parent, i.e., Chambal (4.33). The estimates of coefficient of variation, GCV (103.27) and PCV (104.56) for parents were very high along with high broad sense heritability. High estimates of GCV and PCV suggested substantial variation among the lines of population. High heritability in broad sense indicated high selection intensity of *Alternaria* blight. The mean disease score of F₂ population was 2.79 showing moderate disease resistance

Supplementary Table 1: Disease score scale adopted in F₂ population for *Alternaria* blight in linseed

Scale	Disease intensity	Disease reaction
0	0%	No visible disease damage
1	1-10%	Few lesions mostly on older leaves
2	11-25%	Numerous lesions on older leaves
3	26-50%	Abundant lesions, some defoliations and leaf damage
4	51-75%	Abundant lesions, moderate leaf damage and defoliation
5	76-100%	Severe leaf damage and defoliation

Table 1: Estimates of Mean, GCV, PCV and h² for *Alternaria* blight disease of parents along with F₂ mapping population (JRF-4 X Chambal) in linseed

	Parents		F ₂ (JRF-4 x Chambal)
	JRF-4	Chambal	
Mean	0.66	4.33	2.79
GCV		103.27	39.56
PCV		104.56	41.64
h ² b.s		97.56	90.26

Supplementary Table 2 : Phenotypic disease scoring of F₂ population for *Alternaria blight* in linseed at different stages

Line	Stage 1	Stage 2	Line	Stage 1	Stage 2	Line	Stage 1	Stage 2
JRF-4	0	1	40	2	1	82	2	2
Chambal	5	5	41	4	2	83	3	4
1	2	1	42	4	2	84	2	2
2	2	1	43	4	2	85	2	2
3	2	3	44	4	2	86	2	1
4	2	1	45	3	1	87	3	4
5	2	1	46	0	1	88	2	2
6	0	1	47	3	3	89	2	2
7	3	1	48	4	2	90	2	2
8	5	5	49	5	2	91	2	2
9	1	3	50	1	1	92	3	3
10	4	-	51	2	1	93	3	4
11	4	5	52	2	1	94	3	3
12	2	5	53	3	1	95	2	3
13	4	5	54	5	1	96	1	3
14	5	-	55	2	1	97	1	3
15	4	4	56	2	1	98	2	3
16	0	1	57	2	1	99	1	3
17	3	1	58	2	1	100	1	1
18	2	1	59	3	3	101	3	3
19	1	1	60	3	2	102	3	3
20	2	1	61	3	1	103	3	-
21	2	1	62	4	2	104	3	5
22	3	3	63	3	1	105	1	2
23	4	4	64	2	2	106	5	-
24	3	4	65	2	1	107	4	4
25	2	2	66	5	1	108	3	5
26	2	3	67	4	2	109	2	-
27	2	3	68	3	2	110	4	-
28	4	5	69	3	1	111	2	3
29	3	3	70	1	2	112	2	2
30	0	1	71	2	2	113	3	4
31	1	3	72	3	4	114	2	2
32	5	5	73	2	2	115	3	-
33	2	3	74	3	2	116	1	2
34	3	3	75	0	2	117	3	4
35	2	-	76	4	4	118	3	4
36	1	1	77	3	5	119	1	2
37	3	4	78	3	4	120	3	5
38	4	5	79	2	2	121	2	-
39	1	1	80	4	4	122	3	4
40	2	4	81	2	3	123	1	-
124	3	3	135	3	3	146	3	4
125	2	2	136	3	4	147	2	2
126	3	3	137	1	1	148	1	1
127	5	5	138	3	-	150	3	3
128	1	-	139	3	4	151	3	3
129	3	4	140	3	4	152	2	2
130	2	2	141	1	2	153	3	-
131	1	2	142	3	3	154	3	-
132	3	4	143	2	2			
133	3	3	144	3	3			
134	2	2	145	3	3			

Stage 1= At flowering; Stage 2= At harvesting; (-)= Died

Table 2 : SSR ID, primer sequence, amplicon size and R^2 value of 10 polymorphic SSRs used in the present study

SSR ID	Primer sequence (5'-3')	Amplicon size (bp)	R^2 value
Lu 2472	F: TAAATGTTTCCCGCCAAAC R: TTTGGAAATGGGAAGTGAGG	250	70.5
LuC 8166563_1	F: CCGACCCATCTGGAATTATG R: TCAATATTGTGCATGGGATTT	130	50.3
LuC_8167177_1	F: CCGACCAGTTCATGGAGATT R: CGCAAACCCAACTGTCTTT	153	23.2
Lu 3078	F: AGGGGGAAGACACAGATGAG R: CTTAAGGCTGTAAGAAGGTCAAT	211	19.6
LuC_8252549_1	F: CCAGTAGGCCGTACGTCAAT R: GATTGGCATTGAGGAGGA	127	14.3
LUSc 898_3_12	F: GGAAGAGAGGAAATGGGGAG R: TCTTCCTCATCCAACGACC	144	11.4
LUSc_540_2_1	F: CTGCTAACC GCATAGCCTTC R: AACCACAGCCAGAAATCCAG	151	11.2
LUSc_464_2_24F	F: GTAACGGAACGGAACAGAA R: CAACCACTTTGGGCTTCATT	156	8.2
Lu2103F	F: GCAGTCGTTGCACTTCATGT R: AGTGGTGAAGGAGCAGCAAG	233	6.3
Lu2929F	F: TCTTCCTTTACCGTGAAAAACG R: CCTTGCATCGAGAAGTTTCC	230	2.1

(Table 1). The GCV (39.56) and PCV (41.64) estimates observed very high but PCV was slightly higher than GCV. Relatively small difference between GCV and PCV estimates showed less effect of environment on the incidence of *Alternaria* blight. High GCV and PCV coupled with high heritability in broad sense suggests significant role of additive gene action in the inheritance of this trait. Zongo *et al.* (2017) also suggested high GCV and PCV along with high heritability in broad sense against ELS disease for F_2 population in groundnut.

The parental lines used to develop mapping population (JRF-4 x Chambal) were surveyed with 100 SSRs to identify polymorphic markers and ~10% polymorphism was found among the parental lines used. A similar degree of marker polymorphism was also reported in ground nut (Shobha *et al.*, 2012). The SSR ID, primer sequences and amplicon size of identified 10 polymorphic markers is given (Table 2). Wu *et al.*, (2016) also suggested that polymorphic microsatellites are useful for genetic linkage map construction, germplasm classification, gene identification, QTL mapping and marker assisted breeding in linseed. However, Govindaraj *et al.* (2005) reported that irrespective of level of polymorphism, the success of linked marker for a particular trait was assessed on the basis of their screening over segregating generations.

SSR markers linked to *Alternaria* blight: In linseed, *Alternaria* blight is a major disease causing severe damage to the crop. In order to fasten genetic improvement for developing resistant line of linseed identification of molecular markers is an important step

towards marker assisted breeding. Bulk segregant analysis (BSA) approach proposed by Michelmore *et al.* (1991) is a rapid method to identify markers linked with specific traits. BSA reduces the number of marker assays as compared to other approaches of genotyping for the purpose of identifying marker-trait association (Wenzl *et al.*, 2007). Spielmeier *et al.* (1998) developed AFLP based linkage map of linseed using double haploid population, and identified two QTLs associated with *Fusarium* wilt. Since then no other reports are available on molecular markers related to any disease in linseed. In this study, an effort was made to identify SSR markers associated with *Alternaria* blight resistance using BSA approach. Based on disease incidence in F_2 mapping population, 10 extreme susceptible and 10 extreme resistant plants were selected and DNA from these was pooled separately to prepare susceptible (SB) and resistant bulk (RB), respectively.

The 10 polymorphic SSRs identified among the parental lines (JRF-4 x Chambal) were used to survey two bulk of DNA that could differentiate both the bulks. Out of 10 polymorphic SSRs, three SSR marker viz., LUSc 898_3_12, Lu 2472 and Lu 3078 distinguished the resistant bulk and susceptible bulk (Fig. 1 a,b,c). The markers LUSc 898_3_12, Lu 2472 and Lu 3078 were further validated on 10 resistant and 10 susceptible extreme individuals of F_2 population. All the three SSR markers were able to differentiate resistant and susceptible plants at individual level also. Therefore, the three SSRs, i.e., LUSc 898_3_12, Lu 2472 and Lu 3078 distinguishing between extreme individuals along with their bulk revealed their high association with disease resistance loci as compared to other SSRs.

Fig. 1a : Bulk segregant analysis for *Alternaria* blight using SSR marker LUSc 898_3_12, **RI** : Resistant Individual, **SI** : Susceptible Individual, **RB** : Resistant Bulk, **SB** : Susceptible Bulk, **RP** : Resistant Parent (JRF-4), **SP** : Susceptible parent (Chambal), **M**: 100 bp Ladder.

Fig. 1b : Bulk segregant analysis for *Alternaria* blight using SSR marker Lu 2472; **RI** : Resistant Individual, **SI** : Susceptible Individual, **RB** : Resistant Bulk, **SB** : Susceptible Bulk, **RP** : Resistant Parent (JRF-4), **SP** : Susceptible parent (Chambal), **M**: 100 bp Ladder.

Fig. 1c : Bulk segregant analysis for *Alternaria* blight using SSR marker Lu 3078; **RI** : Resistant Individual, **SI** : Susceptible Individual, **RB** : Resistant Bulk, **SB** : Susceptible Bulk, **RP** : Resistant Parent (JRF-4), **SP** : Susceptible parent (Chambal), **M**: 100 bp Ladder.

Fig. 2 (A-B) : Infection of *Alternaria* blight in Linseed on (A) Leaves and (B) Capsules.

Further validation of association of SSR markers with *Alternaria* blight resistance was carried out through single marker analysis. The 10 polymorphic SSRs were further subjected to genotyping of all the 154 F_2 plants and marker data scored. A single marker analysis was carried out through regression analysis by using marker data of 3 SSRs on 154 F_2 plants and their respective phenotype. The regression values (R^2) for 10 SSRs were ranged from 2% to 70% (Table 2). The markers Lu 2472 and LUSc 898_3_12 had higher R^2 value (70.5 and 50.3 %) as compare to others which confirms their association with *Alternaria* blight resistance in linseed. However, the marker Lu 3078 showed relatively low regression value ($R^2=19.6\%$) which indicates its poor association towards disease resistance. The BSA approach have been widely used to identify the linked markers which could improve particular trait during marker assisted breeding programme especially for disease resistance. Shoba et al. (2012) reported three SSRs for late leaf spot disease viz., PM 375162, pPGPseq5D5220 and PM

384 but single marker analysis validated only PM384 its association with the disease. Uma et al. (2016) reported three SSR markers (VuUGM02, VuUGM08 and VuUGM19) for their association with rust resistance in cowpea through BSA and also significant regression for linkage between VuUGM02 and rust resistance gene. Medini et al. (2014) identified three AFLP markers for septoria leaf blotch in Tunisian old durum wheat and binary logistic regression analysis on the diversity panel showed that two out of three AFLP markers were significantly associated with the disease resistance. Shen et al. (2003) reported that bulk segregant analysis and QTL mapping based on SSR markers revealed one chromosome regions each at 3B, 2D and 6B chromosome were responsible for fusarium head blight in wheat.

The markers identified in this study, i.e., LUSc 898_3_12 and Lu 2472 are of high value for marker assisted breeding of linseed to develop *Alternaria* blight resistance lines. However, to be more precise, further application of more

stringent markers such as SNP can be deployed over a wide range of population to get more closer association of marker with *Alternaria* blight in linseed.

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