MARKER ASSISTED SELECTION AND GENE PYRAMIDING FOR MULTIPLE DISEASE RESISTANCE IN TOMATO

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Abstract: Tomato (*Lycopersicum esculentum*) crop meet substantial damages due to several pathogens causing serious diseases. Previously, the disease was managed using several methods including fungicide application and other cultural practices. The factors including limited availability of commercially acceptable resistant cultivars and negative environmental impact of chemical application have prompted the use of identification and use of genetic resistance to complement other practices in disease management. In the present study, 46 genotypes were screened for resistance to *Fusarium wilt (I1)* and *Tomato yellow leaf curl virus (Ty-2)* to develop fresh market tomato resistant lines. Molecular markers were used to identify resistant gene against both the diseases. Gene pyramiding for disease resistant genes was done by crossing cultivated variety Roma and an advanced line 1008.The present work provided greenhouse, field, molecular marker and laboratory protocols used in the screening and selection process that were applied to segregating populations during generation advance over three years to develop two multiple disease resistant F5 fresh market tomato lines. Resistance of two lines against the abovementioned diseases was confirmed in subsequent evaluations. The identified multiple disease resistant lines were subjected to agro-morphological studies. Analysis of variance was used to analyze the data which was subsequently followed by LSD test to estimate significant variation among the developed lines and their parents. Mean values were highest for number of fruits per plant and fruit width. Other traits like vine length, fruit weight and number of branches per plant also showed statistically high values. Line with superior traits and characters was selected and identified for yield and quality enhancement programs.

Keywords: Solanum lycopersicum, diseases resistance, molecular markers, gene pyramiding

Introduction

Fusarium wilt is a vascular disease of a soil born fungus called *Fusarium oxysporum*. Specialized form of a pathogen called *formae specialis* are capable of infecting a wide range of host including, banana, cabbage, tulip, flax, watermelon, gladioli and tomato (Mamta *et al*., 2013). *Fusarium oxysporum* is divided into three host specific races called *I1*, *I2*, and *I3* (Papoola *et al*., 2013). The pathogen invades the plant by producing hyphae which penetrates down the root epidermis. Entering through root cortex, mycelium advances towards xylem and colonizes there by forming microconidia. The resulting invasion causes brown discoloration of roots with partial or complete rotting. Further progression of infection leads to stunting or withering of the whole plant (Koike *et al*., 2006). Ilyas *et al*., 2016 reported tomato fields in Pakistan are in danger by the invasion of this deadly pathogen causing heavy economical losses. Further studies evaluated that presence of the pathogen is more likely found in warmer areas of Pakistan (Arshad *et al*., 2014). Begomovirus like TYLCV reaches the esophagus by gaining the entry in phloem in the form of virions, finally entering the canal of insect. During the movement, the viorions are capable of releasing certain components and take permanent residence in the hemocoel. Next destination is the entry in salivary system and finally transmitted to plants (Britto *et al*., 2015). Typical symptoms after invasion includes reduction in leaf size, leaf mottling, dropping of flowers and yellowing of leaf edges (Efrat *et al*., 2009). Disease management by using non-judicial and extensive can cause cost production. Using a resistant source is only the safe option to control and manage the disease (Zeeshan *et al*., 2016).

Marker assisted selection offers an opportunity to investigate some of the limiting factors associated with phenotypic selection, providing an efficient selection of a single quantitative or qualitative trait in several crops. Use of molecular markers has increased the efficiency of traditional breeding. But, the marker to be used should meet certain qualifications like, association between QTLs and markers should be closely linked, breeding population should exhibit polymorphism and marker used should be cost effective (Fooland and Panthee, 2011). Selection on the basis of markers allows the classification based on phenotypic evaluation. Breeding inbred lines resistant to multiple diseases is a difficult goal. Managing segregating population to achieve the desired outcome is challenging. The objective of the current study is to use MAS on F5 segregation population to evaluate a line resistant to Fusarium wilt and tylcv.

Materials and methods

Plant material comprised a variety Roma and a line 1008. Roma carries a resistant gene against *Ty-2* but shows no resistance against *I1*. On the other hand, 1008 is resistant for *I1* and susceptible for *Ty-2* (Akbar *et al*., 2016). A cross was made between two lines and 96 F2 plants were produced. Individual F2 plants were grown for DNAisolation and selection. From F2 onwards, PCR based molecular screening for both *I1* and *Ty-2* gene was done. F2 plants with screened resistant genes were allowed to grow and set fruit for seed collection. Seeds from F3 families were again sown to get F4 progenies. Process of molecular screening and selection continued till F5 where lines with both genes in homozygous condition were selected and grown for further morphological studies. Six morphological traits were observed in the developed lines which included, vine length, fruit width, fruit length, fruit weight, number of branches per plant and number of fruits per plant. ANOVA followed by LSD test was used to analyze genetic variation

between the traits. Statistix 8.1 software was used in the current study.

DNA isolation and marker analysis

Fresh young leaves from 96 F2 plants were collected for DNA extraction. Leaves were crushed using liquid nitrogen to make a fine powder. Squashing needles were used for fine crushing. Each sample was transferred to labeled 1.5ml ependorf tube. 2XCTAB was added in each tube, incubated for 60min at 65˚C. Following incubation 25:24:1 of phenol, chloroform and isoamyl alcohol was added. After placing the tubes for half hour at room temperature, tubes were centrifuged for 15min at 8000rpm. Clear supernatant of 400µl was extracted and transferred to new tubes, following addition of 400µl 2 propanol in each tube. Next step was incubation for 3 hours at -20˚C. DNA pellet was formed after centrifuging the tubes at 8000rpm for 15 minutes. Pellet was washed with ethanol (70%). Tubes were dried by inverting them for several hours. 50µl of TE buffer was added in each tube. DNA sample was checked on 1% agarose gel stained with ethidium bromide through electrophoresis. Tubes were stored at 4˚C (Ali *et al*., 2016).

Two PCR based molecular markers were used to screen the genotypes and lines. Marker TOM-144 (forward primer CTGTTTACTTCAAGAAGGCTG, reverse primer ACTTTAACTTTATTATTGCGACG) was used for *I1* amplification. While P1-16 (forward primer CACACATATCCTCTATCCTATTAGCTG , reverse primer CGGAGCTGAATTGTATAAACACG) was used for *Ty-2* amplification. Amplification reactions was carried out in 16 ul reaction volumes containing 1µl genomic DNA (20 – 50 ng/µl), 0.5µl each of forward and reverse primers (10 µM / µl), 1.2µl of dNTPs (25 mM each) , 0.4 µl of Taq DNA Polymerase (2 units, Enzynomix), 1X Taq Buffer and 1.6µl $MgCl_2$ (2.5 mM).

PCR amplification was carried out in DNA Thermal Cycler (Applied Bio System) set at an initial denaturation of 5 min at 94°C; 32 cycles of 94°C for 45 sec, 52°C for 45 sec, and 72°C for 45 sec. One additional cycle of 7 min at 72°C was used for final extension. Amplification product was subjected to 2% gel electrophoresis. 100bp DNA ladder was used for *I1* gene while Ikb DNA ladder was used for *Ty-2* product size calculation. Data was scored on the basis of presence and absence of gene.

Results and discussion Parmer e*t al*., 2013 recommended a set of SSR marker TOM-144 for the identification of resistant genes in tomato genotypes. Marker provided two sharp bands of 199+299bp for resistant lines while a single band of 199bp for susceptible line (Figure 1).TOM-144 is linked to *I1* gene that confers resistance to fusarium wilt in tomato. Among the 46 selected tomato genotypes 17 showed the presence of *I*1 gene, while the remaining genotypes were lacking this gene. The genotypes that possessed *I*1 gene include 1008, 017868, 1311,017878, 017859, 1173, 017869, 0101, Sashaltai, 017890, 017909, 017865, 017872, 017870, 1002, kht-5 and 017887 (Table 1).

Similarly, Yang *et al*., 2014 developed a sequence characterized amplified region (SCAR) marker P1-16 which is tightly linked to *Ty-2* gene providing resistance against tomato yellow leaf curl virus. Banding pattern of 800bp shows the genotype is susceptible while 900bp band confers resistance to tylcv (Figure 2). 12 genotypes were found resistant for TYLCV which included1003,Roma,Sashaltai,017882,017883,KHT5,Zarnita,017887,Subarctic,1219 ,1004 and Bushbeef (Table 1).

Roma is resistant to tylcv and line 1008 is resistant to fusarium wilt (Table 1). A cross was made between them to develop a hybrid which was allowed to self fertilize to produce F2 generation. 96 F2 plants were selected and screened for both *I1* and *Ty-2* genes following similar strategy of molecular screening (Table 2). 51 plants were resistant for *I1* gene while 40 were resistant for *Ty-2* gene. On the other hand 45 lines were absent with resistant gene *I1* while 56 lines were susceptible for *Ty-2* gene. Line L5P2 bearing both the genes were selected and allowed to self fertilize to develop F3 lines. Total of 41 plants were selected for molecular screening against two resistant genes (table 2). Out of 41 F3 lines, 28 were resistant for *I1* and 25 were resistant *Ty-2* gene. While 13 were susceptible for *I1* gene and 16 were susceptible for *Ty-2* gene. Line L3P4 was screened with both the genes which was selected and allowed to grow F4 progeny. Total of 20 F4 plants were produced at Hazara Agricultural Research Station, Abbottabad. Two lines L1P4 and L1P9 were screened with both the genes. These lines were again allowed to grow and produce F5 progeny. 20 F5 lines were selected for molecular screening (Table 2). Two lines L2P4 and L2P2 were screened with both resistant genes which were found in homozygous condition. These two lines developed were subjected to morphological studies to evaluate the genetic variability among the developed lines and there successive parents.

Morphological traits under study included vine length, fruit width, fruit weight, fruit length, number of branches per plant and number of fruits per plant. Present study suggested positive values for fruit size, fruit width, fruit length, number of branches per plant and number of fruits per plant. These characters are attributed towards high yield in tomato crop, therefore they should be considered as primary yield components necessary for high yield in tomato. Similar studies were conducted by Livia *et al*., 2012 who evaluated the correlation between the yield and fruit quality characteristics in tomato. Traits like fruit weight, fruit width, number of fruits per plant can be used as selection parameters for developing elite lines through heterosis breeding (Muhammad *et al*., 2013). Certain similarities and differences were investigated in the current study and the related study done by earlier scientists, which may be due to different breeding material and different environmental conditions. Lines developed were high yielding and agro-morphological studies revealed that quality traits are well maintained in the plants. Field screening during generation advance was done under natural disease pressure. Effective and inexpensive methods were used to enable efficient selection of a genotype carrying different combination of genes for disease resistance.

Breeding for disease resistance is always challenging due to certain factors like, multiple races and species of pathogens and lack of an easy method for molecular screening. The strategy alternating Marker assisted selection with filed selection offers an opportunity for horticultural performance and selection of resistance. MAS is reported with high efficiencies as compared to phenotypic selection where marker tightly linked to a specific gene is identified using molecular markers (Wencai and Francis, 2005). Pyramiding a breeding line against multiple disease resistance offers potential strategy to reduce the impact of different diseases invading tomato crop. Pyramided lines developed during the present study could be beneficial for sustainable tomato production in the areas prone to different pathogens effecting tomato crop annually

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Table 1. Molecular screening of selected germplasm for *I1* and *Ty-2* resistant genes

S.No.	Genotypes	*I1*	*Ty-2*	S.No.	Genotypes	*I1*	*Ty-2*
1	017863	_	_	24	017872	+	_
2	Coldera	_	_	25	017870	+	_
3	1008	+	_	26	017903	_	_
4	017868	+	_	27	Nepoli	_	_
5	1311	+	_	28	1002	+	_
6	017878	+	_	29	017882	_	+
7	1003	_	+	30	017883	_	+
8	Nagina	_	_	31	Naqeeb	_	_
9	9601	_	_	32	Anahi	_	_
10	Roma	_	+	33	017906	_	_
11	1315	_	_	34	Kht-5	+	+
12	017856	_	_	35	1314	_	_
13	017859	+	_	36	0201	_	_
14	1173	+	_	37	Zhezha	_	_
15	017869	+	_	38	017904	_	_
16	0101	+	_	39	Zarnita	_	+
17	017862	_	_	40	Longkeeper	_	_
18	Sashaltai	+	_	41	017887	+	+
19	1004	_	+	42	Bushbeef	_	+
20	017890	+	_	43	Subarctic	_	+
21	017909	+	_	44	Riogrande	_	_
22	1315	+	_	45	017902	_	_
23	017871	_	_	46	1219	_	+

Table. 2 Reaction of Resistance in F2-F5 generations against two genes

S.No.	**Total No. of plants**	**Total No. resistant plants (*I1*)**	**Total No. of susecptibe plants (*I1*)**	**Total No. resistant plants (*Ty-2*)**	**Total No. of Susceptible plants (*Ty-2*)**
F2 progeny	96	51	45	40	56
F3 progeny	41	28	13	25	16
F4 progeny	20	18	02	14	06
F5 progeny	20	18	02	14	06

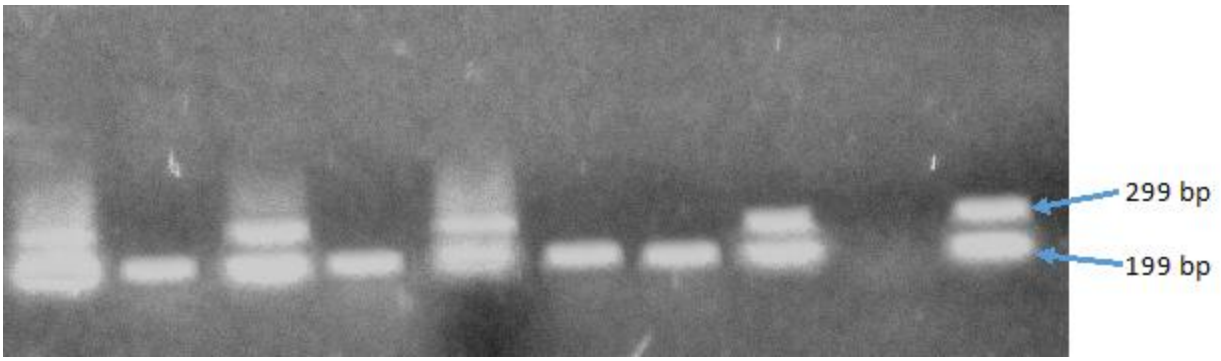

Figure 1. PCR analysis of tomato genotypes for the presence of *I1* gene using SSR marker TOM-144. Genotypes having both the bands of 199+299bp are resistant to Fusarium wilt.

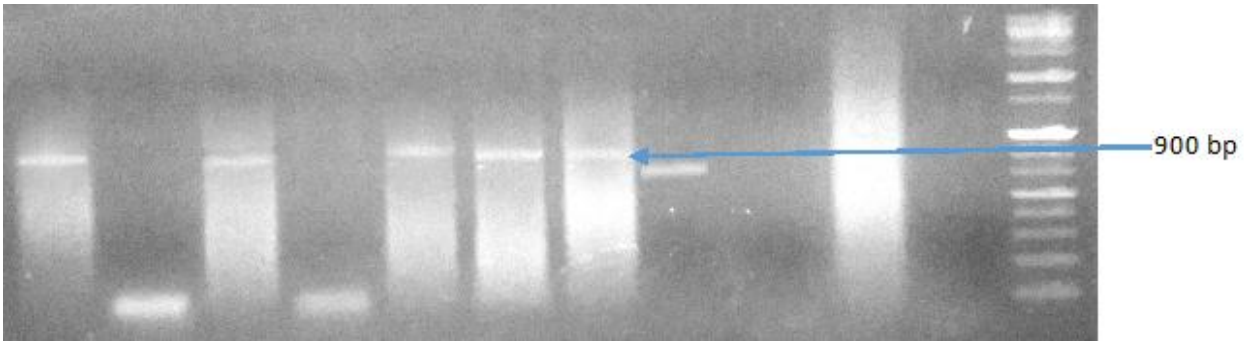

Figure 2. PCR analysis of tomato genotypes for the presence of Ty-2 gene using SCAR marker P1-16. Genotypes having 900bp fragments are resistant to tomato yellow leaf curl virus.

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