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PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF BACTERIAL WILT RESISTANCE IN BRINJAL

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ABSTRACT
Nine genotypes of brinjal (Swarna Sree, Swarna Shobha, Swarna Mani, Swarna Pratibha, Swarna Shyamali, HAB-381, HAB-792, HAB-900 and HAB-901) were characterized for resistance to bacterial wilt. Among them only HAB-901 was found wilt resistant (81.3% plant survival). RAPD rs 724 gene sequence was used to design six SSR primers for assessing genetic variation and relationship among the nine genotypes. Clustering analysis showed two major clusters in which the HAB-381 was grouped separately while the remaining eight genotypes formed cluster 2. HAB-900 and HAB-792 were also found wilt resistant in previous studies. Hence, it can be concluded that HAB-901, HAB-900 and HAB-792 may be having related wilt resistance genes, but the resistance may have broken down in HAB-900 and HAB-792 due to heavy nematode infection.

INTRODUCTION

Brinjal or eggplant (*Solanum melongena* L. 2n = 24) is an important solanaceous crop grown throughout the world (Khan and Singh, 2014). Brinjal cultivation in India is estimated to cover about 7.60% vegetable area (area of 0.71 million hectares) with a contribution of 8.3% (13.56 million tonnes) to total vegetable production (NHB, 2015). Major brinjal producing states are West Bengal, Orissa, Bihar, Gujarat, Maharashtra, Jharkhand, Karnataka, Uttar Pradesh and Andhra Pradesh. Although vast area is under its cultivation, production is limited by many biotic stresses especially in eastern plateau. And among them bacterial wilt incited by *Ralstonia solanacearum* Yubuuchi is a serious disease, which limits eggplant production from 4.24 to 86.14 per cent (Sabita *et al,* 2000). This disease is widely distributed in tropical, subtropical and some warm temperate regions of the world with a host range of 44 plant families (Ji *et al.*, 2005; Hayward, 1991).

India being the centre of origin of brinjal, abundant genetic diversity is available, offering tremendous scope for its improvement. For enriching the genetic diversity germplasm need to be constantly evaluated and characterized which can be further utilized for pre breeding and various crop improvement programme. Moreover, yield being a complex character is influenced by a number of component characters and they are polygenically inherited. Hence the present investigation was taken to characterize brinjal germplasm along with the established varieties for their wilt resistance and yield traits which will be utilized in development of high yielding wilt resistant varieties.

Moreover, many of the commercial varieties are susceptible to bacterial wilt (Gopinath and Madalgeri, 1986) and there is no other effective control strategy to manage bacterial wilt (Wang *et al.*, 2000). The only effective means of bacterial wilt control is to screen and identify resistant sources. Until recently, only few resistant cultivars of brinjal have been developed. Inheritance of bacterial wilt resistance in brinjal is still unclear. Moreover the wilt resistance shows breakdown due to nematode susceptibility of the already established wilt resistant varieties. Deberdta *et al*. (1999) showed infection of tomato roots by root-knot nematodes reduced genetic resistance to bacterial wilt. Hussain and Bora (2009) found that the increased wilt incidence at higher inoculum level of the nematode and bacteria and low wilt incidence at lower inoculum levels of nematode with bacteria.

Conventional breeding being laborious, time-consuming and dependent on environmental conditions, usage of molecular markers is an efficient alternative. Modern molecular techniques help to identify the specific molecular marker for the resistant genes which act as versatile tools and have found their own position in various fields like taxonomy, physiology, embryology, genetic engineering, *etc.* (Joshi *et al*., 1999). PCR based molecular markers were used for characterization of resistance in wheat leaf rust (Vasistha *et al*., 2014), mungbean yellow mosaic (Kalaria *et al*., 2014) and alternaria blight of mustard (Visalakshi *et al*., 2013). Molecular markers also accelerate selection and eliminate the effects of environmental variation during selection (Malyshev and Kartel, 1997). Hence in the present study deals with sequence specific primers (SSRs)

for molecular characterization of wilt resistance.

MATERIALS AND METHODS

Selection of plant Material

Popularly cultivated released varieties of brinjal from ICAR-RCER, RC Ranchi, Jharkhand (Swarna Sree, Swarna Shobha, Swarna Mani, Swarna Pratibha, Swarna Shyamali) and wilt resistant advanced breeding lines (HAB-381, HAB-792, HAB-900, HAB-901) were chosen for characterization of resistance to bacterial wilt. The trials were conducted during main season of 2011-12 and 2012-13 at Experimental Farm of ICAR RCER Research Centre, Ranchi (23.35º N and 85.33º E at 629m altitude). Total annual rainfall was 1430mm with 1100 mm during June to September and the average maximum and minimum temperatures 37°C and 40°C respectively.

Phenotypic characterization for wilt

Nine genotypes of brinjal in two replications were evaluated for wilt resistance in bacterial wilt sick plots (48.67 X10^6cfu/g) (Rahman *et al*., 2011) during rainy season and artificially inoculated with bacterial suspension in the third leaf axil from the top (Rashmi *et al*., 2012). Observations on percent plant survival were recorded at 90 days after transplanting. Scoring was done on the basis of percentage of survival *viz*., ≥80% as resistant and <80% as susceptible (Sinha *et al*., 1988).

Phenotypic characterization for yield and yield components

Data on fruit yield per plant (Kg), no. of fruits per plant, plant height (cm), no. of branches/plant, fruit weight (gm), fruit length (cm), fruit breadth (cm) and days to 50% flowering were collected from selected genotypes from each replication. Means of observations were subjected to statistical analysis.

DNA extraction from brinjal leaves

Nine different varieties of brinjal (*Solanum melongena*) leaves were surface sterilized with 0.1% of $HgCl_2$ and used for the isolation of genomic DNA using CTAB method (Doyle and Doyle 1987; Doyle and Doyle, 1990) followed by RNase treatment (Healey *et al*., 2014). Quantification of genomic DNAs was determined by NanoDrop 8000 spectrophotometer. 80 nanogram of isolated DNA was checked at 0.8% agarose gel was subjected to PCR amplification.

PCR amplification of wilt resistance genes

a) Primer designing

The wilt resistance rapd rs 724 (Cao *et al*., 2001; http://www.ncbi.nlm.nih.gov/nuccore/ EU547499.1) gene sequence selected and six SSR primers were designed using BLAST PRIMER software from the NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). Wilt resistance SSR primer sequence is listed below (Table 1).

b) PCR *amplification of wilt resistant gene in different brinjal varieties:*

PCR was carried out in a final reaction volume of 25 µl using ABI Veriti Thermal Cycler (Xcelris Labs Ltd. Ahmedabad, India). Composition of reaction mixtures for PCR is given in Table 2. PCR was performed at 94ºC for 4 min; 40 cycles of 15 s at 94ºC, 30 s at 52-56ºC, and 45 s at 72ºC (as per protocol followed by Xcelris Labs Ltd (Ahmedabad, India) using a ABI Veriti Thermal Cycler. To confirm the targeted PCR amplification, 5 µl of PCR product from each tube was mixed with 1 µl of 6X gel loading dye and electrophoresed on 1.5 % Agarose gel containing Ethidium bromide at constant 5V/cm for 30 min in 1 X TAE buffer. The amplified product was visualized under UV light and documented by gel documentation system (Biorad, USA).

Sequencing of identified genes and clustering

Established marker of wilt resistance rs 724 gene (http://www.ncbi.nlm.nih.gov/nuccore/ EU547499.1) was selected for the identification of wilt resistant genes in different brinjal varieties. After the PCR amplification of the selected marker genes, obtained PCR products were sequenced from the next generation sequencing. Sequencing was performed on Illumina MiSeq Sequencing System (Illumina Inc.). Furthermore, the amplified DNA sequence of the same markers of different brinjal varieties were aligned to generate the cluster pattern (Xiong, 2006) for wilt resistance through the CLC genomics workbench beta 9 (QIAGEN, USA).

RESULTS AND DISCUSSION

Phenotypic characterization of brinjal germplasm against bacterial wilt resistance

Among the nine genotypes screened for wilt resistance, only HAB-901 was found resistant (81.3% plant survival) (Figure

Table 1: Six SSR primers designed from rs 724 (wilt resistance) gene

Primers designed from rs 724 Name of primer	Forward/Reverse Primer	Sequence (5'->3')	Product length (bp)
SSR-1	Forward primer	GACCCTTCCAGCACGATCAG	197
	Reverse primer	TGGGGTGGCTTCCATTTTCA	
SSR-2	Forward primer	ACGAGCATACTCAGAGAGCC	147
	Reverse primer	GACATTCCGTTGACACTTGGC	
SSR-3	Forward primer	CCCTTCCAGCACGATCAGTTA	196
	Reverse primer	CTGGGGTGGCTTCCATTTTC	
SSR-4	Forward primer	AACAGGACAGGCCAAGTGTC	90
	Reverse primer	TTCCTTGTTCTGGCTCAAGTGT	
SSR-5	Forward primer	CAGGACAGGCCAAGTGTCAA	87
	Reverse primer	TCCTTGTTCTGGCTCAAGTG	
SSR-6	Forward primer	GCCCAAAAGATGACAAGCAT	188
	Reverse primer	TCCTTGTTCTGGCTCAAGTG	

Table 2: Composition of PCR Master Mix

PCR components	Quantity/volume
2X PCR Master Mix	12.5 μl
Primers (10pmol/μl)	1.0 μl
DNA Templates	80 ng
Nuclease Free water	To make up final vol. 25 μl
Total Volume	25 μl

Table 3: Performance of brinjal germplasm against bacterial wilt under artificial wilt sick plot conditions

Name of genotype	Survival % at 90 DAT
Swarna Sree	28.1
Swarna Sobha	9.38
Swarna Mani	0
HAB-381	62.5
HAB-792	71.9
HAB-900	62.5
HAB-901	81.3
Swarna Shyamali	34.4
Swarna Pratibha	28.1

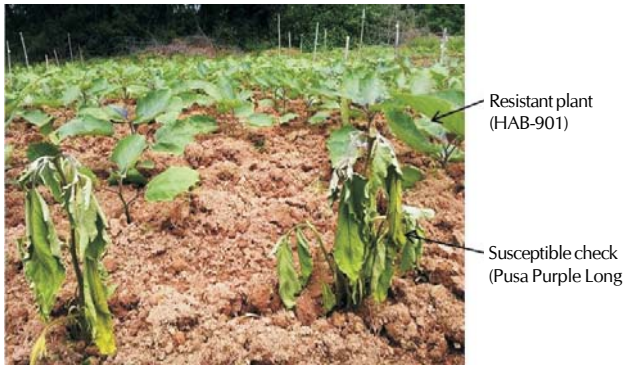

Figure 1: Wilt susceptible (Pusa Purple Long) and resistant (HAB-901) plant of brinjal

1). Although Swarna Shyamali (CH-249) and Swarna Pratibha (CH-309) are wilt resistant varieties as reported by Sharma *et al*. (1995), they were susceptible to wilt; reason may be due to break down of resistance (Table 3). Deberdta *et al*. (1999) reported that high temperatures reduce the expression of resistance. And also genetic resistance to *R. solanacearum* is often diminished as a result of nematode infection due to synergistic effect (Siddiqui *et al*., 2013). Hence it can be concluded that nematodes provide wounds through which the bacteria may enter and also release metabolites useful for bacterial growth. The resistance reactions and mechanism of resistance are very location and environment specific and it is genetically determined (Mew and Ho, 1977; Gopalakrishnan *et al*., 2005; Ajjappalavara *et al*., 2008; Bainsla *et al*., 2016).

Phenotypic characterization of brinjal germplasm for yield component

In addition to yield, different yield components were also characterized in all the nine genotypes. Highest yield was recorded in Swarna Sree (1.58 Kg/ Plant), highest number of fruit per plant in HAB-381 (23.53), maximum fruit weight (239.63 gm) and earliest flowering (32.33 days) in Swarna Shyamali (Table 4). Due importance should be given to fruit breadth, fruit length, plant height and percent plant survival

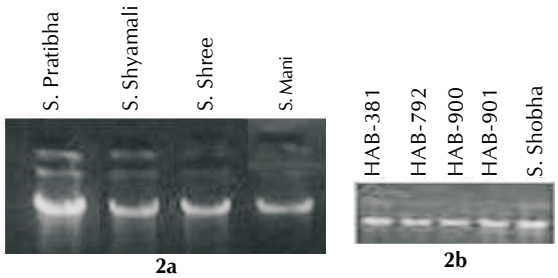

Figure 2: DNA gel electrophoresis of nine different brinjal varieties. 2a S. Pratibha, S. Shyamli, S. Shree, S. Mani and 2b HAB-381, HAB-901, HAB-900, HAB -792 and S. Shobha

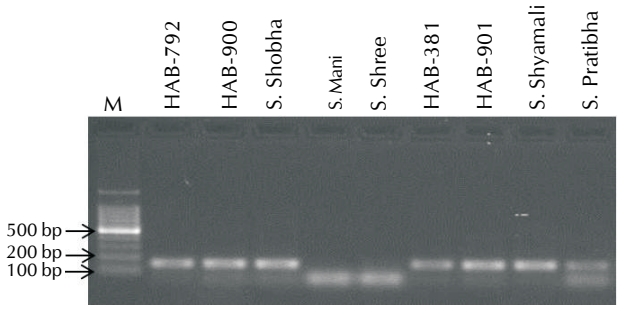

Figure 3 : PCR amplicon generated through SSR-2 marker (M: 1kbp DNA ladder) Characterization using 6 SSR markers (RAPD rs724)

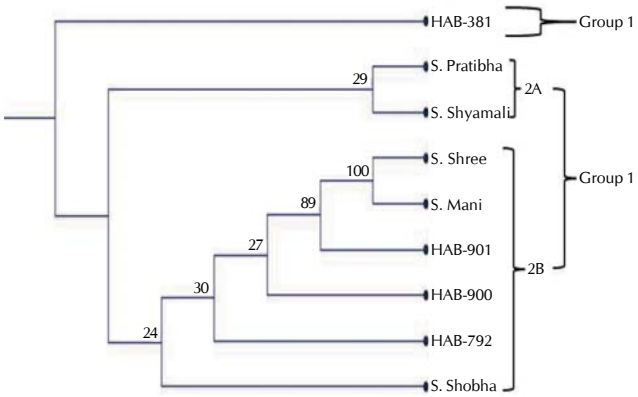

Figure 4: Dendrogram showing the genetic variation between brinjal genotypes for wilt resistance

against wilt for development of high yielding wilt resistant cultivars of brinjal (Singh *et al*., 2014). Plant height and fruit length as important selection criteria were reported by many researchers (Singh and Kumar, 2004; Sharma and Swaroop, 2000; Pathania *et al*., 2005; Patel and Sarnaik, 2004).

DNA extraction of plant material

DNA was isolated from the selected plant material of different brinjal varieties and quantity of the DNA was checked in gel electrophoresis (Figure 2).

DNA amplification through the SSR marker

Band length between 100 to 200 bp was obtained in HAB-792, HAB-900, Swarna Shobha, HAB-381, HAB-901 and Swarna Shymali; less than 100 bp band obtained in Swarna

Table 4: Mean performance of brinjal genotypes for yield and some of its components

Genotypes	Yield/ plant(kg)	No. of fruits/plant	Plant height(cm)	No. of branches /plant	Fruit weight(gm)	Fruit length(cm)	Fruit breadth(cm)	Days to 50% flowering
Swarna Sree	1.58	11.2	87.4	2.63	222.17	9.87	8.25	46.33
Swarna Shobha	1.33	10.6	92.6	3	169.97	11.1	7.17	46
Swarna Mani	0.72	3.8	120.5	2.93	227.33	9.29	8.79	58.33
HAB-381	1.15	23.53	84	2.8	57.67	11.9	3.91	37
HAB-792	1.09	33	84.6	2.6	58.57	12.9	3.41	49.33
HAB-900	1	23.07	76.53	2.53	79.9	8.19	5.24	46
HAB-901	0.93	12.07	80.2	3.47	76.33	8.41	5.13	40.33
Swarna Shyamali	1.46	8.2	89	2.67	239.63	8.35	8.96	32.33
Swarna Pratibha	1.42	13.6	85	3.27	120.73	15.1	4.83	47.33
CV	40.35	41.84	10.87	18.75	14.80	6.09	5.82	7.98
C. D at 1%	N/A	11.29	N/A	N/A	49.17	1.23	0.72	N/A

Shree, Swarna Mani and Swarna Pratibha (Figure 3).

Based on the sequencing data obtained from amplification using six SSR primers, dendrogram was constructed (Figure 4). Two major clusters were formed through the construct in which HAB-381 grouped separately while the remaining eight genotypes formed cluster 2. Swarna Pratibha and Swarna Shyamali formed a single subcluster (2A) in cluster 2 and the remaining six genotypes (Swarna Sree, Swarna Mani, HAB-901, HAB-900, HAb-792 and Swarna Shobha) formed another sub cluster (2B) in cluster 2 with sequential clustering habit. Similarly, segregation of eight brinjal varieties into two main clusters based on RAPD analysis was reported by Sharmin *et al*., 2011. SSR analysis of five brinjal genotypes generated two clusters by Khorsheduzzama *et al*., 2008.

Although HAB-901 was found phenotypically resistant, it did not form a separate cluster with regard to wilt resistance. In previous studies, HAB-900 and HAB-792 were found to be resistant (Unpublished data). Hence, it can be concluded that HAB-901, HAB-900 and HAB-792 may be having related wilt resistance genes, but the resistance may have broken down in these two genotypes due to heavy nematode infection. Nirmaladevi and Tikoo (1992) reported that the wilt resistant hybrids became susceptible to wilt when the parents had partially dominant/recessive source of resistance to wilt and/ or nematode. Similarly, Perez *et al*. (2006) concluded that there are big differences between Mi-gene-resistant tomato cultivars as far as nematode host status, and that some should be considered tolerant rather than resistant. Different workers have reported inheritance pattern for bacterial wilt resistance ranging from monogenic dominant/recessive to inhibitory type in different cross combinations (Ajjappalavara *et al*., 2005;

Chaudhary, 2000; Bainsla *et al*., 2016). Thus the future prospective should be for nematode characterization and interaction with bacterial wilt in the genotypes HAB-901, HAB-900 and HAB-792 which can be successfully used in brinjal improvement programme.

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