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Screening of Bhut Jolokia (*Capsicum chinense* Jacq.) Germplasm of North East India against Chilli Leaf Curl virus

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ABSTRACT

Bhut Jolokia (Capsicum chinense Jacq.) is a type of chilli extensively grown in North Eastern region of India, predominantly in the states of Assam, Nagaland and Manipur. Bhut Jolokia is recognized as the 7th hottest chilli in the world having a scoville heat unit of 1,041,427. Leaf curl virus disease of chilli is considered as the most serious disease problem prevailing in Bhut Jolokia growing North Eastern region causing serious losses.The disease is characterized by severe upward curling, reduction in leaf size, leaf thickening, vein clearing and stunted plant growth. The severely affected plants are stunted bearing hardly any fruits. PCR detection technique was standardised using two chilli leaf curl virus specific primers at various annealing temperatures using gradient PCR. Primer pairs ChLCVF1-ChLCVR1 and ChLCVF2-ChLCVR2 successfully yielded 550bp and 568bp PCR products at annealing temperature of 45 °C and 48.9 °C, respectively.Thirty different Bhut Jolokia genotypes were collected from various localities of Assam, Nagaland and Manipur based on their fruit morphological characters. These genotypes were screened against ChLCV under field conditions and confirmed by PCR detection using ChLCVF2-ChLCVR2 primer pair at 180 days after transplanting, resulted 4 highly susceptible, 11 susceptible, 14 moderately susceptible and 1 symptomless genotypes. Partial sequencing of three ChLCV isolates showed 99.00 per cent similarity. However, sequence similarity search of these isolates with 20 known ChLCV isolates worldwide recorded 86.00 per cent to 97.00 per cent similarity. Sequence similarity of Bhut Jolokia ChLCV isolates showed 87.00 per cent to 90.00 per cent homology with Indian ChLCV isolates indicating the virus from Jorhat to be distinct strain from Indian isolates, for which the name ChLCV-Bhut Jolokia Jorhat (ChLCV-BJ-JRT) strain is proposed.

Key words: *Bhut Jolokia, PCR, ChLCV (Chilli leaf curl virus), Scoville heat units, Partial sequencing*

INTRODUCTION

'Bhut Jolokia' (*Capsicum chinense* Jacq.) is extensively grown in North Eastern region of India, predominantly in the states of Assam, Nagaland and Manipur. It belongs to the family Solanaceae with chromosome number 2n=24. It is locally know as Bhut Jolokia (Ghost chilli) or Bih Jolokia (poison chilli) in Assam, Naga Jolokia in Nagaland and Oo-morok (tree chilli) in Manipur.

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Molecular analysis with randomly amplified polymorphic DNA (RAPD) markers confirmed that 'Bhut Jolokia' is an inter-specific hybrid, mostly *Capsicum chinense* with some *Capsicum frutescence* genes[1]. A serious constraint in production of Bhut Jolokia is due to virus diseases, especially chilli leaf curl virus. Chilli leaf curl virus, a begomovirus of the family geminiviridae, consists of a large number dicot plant infecting viruses, which are transmitted by white fly, *Bemesia tabaci* of the family Aleyrodidae[10]. Their diseases, symptoms produced might be similar with other cultivable chilli varieties. Viral infection starts at early plant growth stage as leaf curl towards mid rib and gets deformed. Consequently, plants remain stunted. In severe infection stage, flower buds abscise; pollen development obstructs leading to no fruit setting or production of small fruits. Leaf curl disease enhances the infestation of thrips and mites causing severe losses[6]. The north eastern region is known for cultivation of Bhut Jolokia, the farmers of this region are facing a problem with this viral disease infestation. In case of chilli, leaf curl resistant genotypes under open field conditions have been reported (eg. Pusa Jwala and Pant C-1)[12, 8] but there are no reports on screening, especially with Bhut Jolokia genotypes. Hence to identify resistant genotypes against leaf curl virus disease screening was carried out under field conditions. Different disease reactions were recorded based on disease scale and confirmed by PCR assay[2, 7, 9].

MATERIALS AND METHODS

Screening of Bhut Jolokia germplasm

Morphologically different 30 Bhut Jolokia accessions were collected based on *Capsicum* descriptors[4] from north eastern states *viz.*, Assam, Nagaland and Manipur (Table 1). Healthy fruits were selected and seeds were extracted. The seeds were washed with clean water and were soaked in potassium nitrate (0.3%) overnight and shade dried. The seeds sown in plastic trays in nursery and at 6-8 leaves stage the seedlings were transplanted in the main field (241.5 m^2). Each accession had 12 plants and maintained a spacing of 75 cm x 75 cm and followed standard agronomic practices. No pesticides were applied during cropping period. These accessions were designated as BJ001 to BJ030 and screened against chilli leaf curl virus under open field conditions during 2013-15 at Jorhat, Assam.

Data collection

Scoring for incidence and ChLCV disease severity on the plants was done at 60, 120 and 180 days after transplanting based on the disease scale [2, 7, 9] with slight modifications (Table 2).

Viral DNA detection: DNA extraction

The leaf samples were collected from accessions to confirm the phenotypic screening. DNA extraction was carried out by following suitable method [3] with some modifications.

Primer for PCR amplification

Two primer pairs especially targeting ChLCV were used for amplification of PCR products. The primer pair ChLCV1 Forward 5'AGAATTATGTCCAAGCGACCA3' and Reverse 5'AAGCGTTGGGGATACACAAA3' [11] was used for amplification. ChLCV2 specific primer pair Forward 5'TCCCTTCCTCCAAATTGTTG3' and Reverse 5'TTGTTTTTGCCTGGTTTTCC3' which was designed using primer3 software from the known chilli leaf curl virus isolates derived from National Centre for Biotechnology Institute (NCBI).

Optimisation of PCR annealing temperature

Optimisation of annealing temperature for primer ChLCV2 was be done by using gradient PCR. The gradient PCR experiment was performed at temperatures between 46 °C-50 °C *viz.*, 46 °C, 47.1 °C, 48.3 °C, 48.9 °C and 50 °C with a reaction mixture of 25 µl.

PCR amplification of ChLCV DNA

PCR was performed in 25 µl volume using ChLCV specific primer pair. The reaction mixture composition was 2.5 µl 10X PCR buffer (with 17.5 mM $MgCl_2$), 2.0 µl of 2mM dNTPs, 2.0 µl of 10 pmol/µl forward and reverses primers, 1.8 µl Taq DNA polymerase (1U/ µl), 2.0 µl template (50ng/ µl), 12.7 µl of

nuclease free water. For primer pair, ChLCVF-ChLCVR DNA amplification parameters were 40 cycles of denaturation for 1 min at 94 ^{o}C, 48.9 ^{o}C for 1 min, 72 ^{o}C for 2 min and final extension at 72 ^{o}C for 10 min. Amplification products were maintained at 4 ^{o}C prior to gel electrophoresis.

Gel electrophoresis

The PCR products were analysed in 1.2 per cent agarose gel electrophoresis in 1X TBE buffer containing 0.5 µg/ml of ethidium bromide. One µl of 6X gel loading dye were mixed with 2 µl of each DNA sample and loaded in the well. One µl of 100 base pair DNA ladder was loaded in one well as molecular weight standards. The electrophoretic gel was run at 30 mAmp in BIO-RAD electrophoretic apparatus till the dye has migrated one- third of the distance in the gel. Migrated DNA was visualized using a UV transilluminator and captured the gel images using the geldoc (Alpha Innotech, USA). Banding patterns were then observed and compared between individuals showing viral presence and absence.

Partial Sequencing

PCR fragment obtained from three infected ChLCV samples of Jorhat were sequenced at Bioserve Biotechnology (I) Pvt. Ltd, Hyderabad. Sequenced products were assembled using Bio-edit software (www.mbio.ncsu.edu/bioedit/bioedit) and compared with known ChLCV isolates using bio-informatic tool (www.ncbi.nlm.nih.gov/BLAST). These sequences were aligned in a global multiple sequence alignment programme, multalin (www.multalin.toulouse.inra,fr/multalin/) and phylogenic analysis was conducted by using MEGA6 neighbor-joining method.

RESULTS

Screening for ChLCV resistance

Disease incidence

The percentage of ChLCV incidence at 60, 120 and 180 days after transplanting are given in Table 3. At 60 days after transplanting (DAT) only BJ013 accession have shown ChLCV symptoms whereas other accessions were symptomless. At 120 DAT more than 50 per cent accessions were ChLCV infected. At 180 DAT all the accessions were infected except BJ001 remained symptomless.

ChLCV disease severity under field conditions and PCR assay

All the accessions have shown different disease severity as shown (Fig. 1). Based on these disease reactions the accessions were sorted as mentioned (Table 4).

PCR detection and amplification of ChLCV DNA

The primer pair ChLCVF2-ChLCVR2 were used in detection of ChLCV in the Bhut Jolokia accessions and was successfully yielded at 568 base pair as shown (Fig. 2). The accessions have shown positive for PCR amplification (Table 4).

Phylogenetic tree analysis of ChLCV isolates

PCR fragment obtained from the infected three ChLCV samples of Jorhat were sequenced at Bioserve Biotechnology (I) Pvt. Ltd, Hyderabad. Sequenced products were assembled using Bio-edit software (www.mbio.ncsu.edu/bioedit/bioedit). The results recorded a 490 base pair, 491 base pair and 491 base pair sequence for the three ChLCV isolates of Jorhat. The three sequences of ChLCV *viz.*, ChLCV1, ChLCV2 and ChLCV3 were shown 99.00 per cent homology between three ChLCV isolates. The multiple alignment of Jorhat isolates with other isolates shown the sequence similarity ranging between 86.00 to 97.00 per cent. The comparison of ChLCV- Bhut Jolokia Jorhat isolates with ChLCV Indian isolates showed sequence similarity ranging from 87.00 per cent to 90.00 per cent indicating the virus from Jorhat to be distinct strain from Indian isolates, for which the name ChLCV- Bhut Jolokia strain (ChLCV-BJ-JRT) is proposed.

Table 1: List of Bhut Jolokia accessions

Accession No.	Village	District	State
BJ001	Alengmora	Jorhat	Assam
BJ002	Alengmora	Jorhat	Assam
BJ003	Namdeuri	Jorhat	Assam
BJ004	Hatigarh	Jorhat	Assam
BJ005	Alengmora	Jorhat	Assam
BJ006	Silonijan	Golaghat	Assam
BJ007	Silonijan	Golaghat	Assam
BJ008	Silonijan	Golaghat	Assam
BJ009	Senapati	Senapati	Manipur
BJ010	Dikoi	Dimapur	Nagaland
BJ011	Dikoi	Dimapur	Nagaland
BJ012	Sukori	Dimapur	Nagaland
BJ013	Senjum	Dimapur	Nagaland
BJ014	Tipomia	Jorhat	Assam
BJ015	Borbhula	Jorhat	Assam
BJ016	Dhekiajuli	Jorhat	Assam
BJ017	Harupathar	Golaghat	Assam
BJ018	Borpathar	Golaghat	Assam
BJ019	Ungma	Mokukchung	Nagaland
BJ020	Longjung	Mokukchung	Nagaland
BJ021	Longjung	Mokukchung	Nagaland
BJ022	Jorhat	Jorhat	Assam
BJ023	Jorhat	Jorhat	Assam
BJ024	Jorhat	Jorhat	Assam
BJ025	Dibrugarh	Dibrugarh	Assam
BJ026	Khuwang	Dibrugarh	Assam
BJ027	Dolonikher	Dibrugarh	Assam
BJ028	AAU-1	Jorhat	Assam
BJ029	AAU-2	Jorhat	Assam
BJ030	AAU-3	Jorhat	Assam

Table 2: Disease scale based on symptom severity

Symptoms	Symptom severity grade	Disease reaction
No symptom	0	Symptomless
0-5% curling and clearing of upper leaves	1	Highly resistant (HR)
6-25% curling, clearing of leaves and swelling of veins	2	Resistant (R)
26-50% curling, puckering of leaves and swelling of veins	3	Moderately resistant (MR)
51-75% leaf curling and stunted plant growth	4	Moderately susceptible (MS)
>75% curling and deformed small leaves, stunted plant with small and no or small fruits	5	Susceptible (S)
100% curling deformed small leaves, stunted plant growth without plant growth	6	Highly susceptible (HS)

Table 3: Percentage of ChLCV disease incidence in the Bhut Jolokia germplasm

Accession No.	60 DAT	120 DAT	180 DAT
BJ001	0	0	0
BJ002	0	33.33	41.66
BJ003	0	8.33	50.00
BJ004	0	16.66	58.33
BJ005	0	8.33	33.33
BJ006	0	18.18	33.33
BJ007	0	33.33	75.00
BJ008	0	50.00	100.00
BJ009	0	16.66	41.66
BJ010	0	16.66	66.66
BJ011	0	16.66	58.33
BJ012	0	33.33	58.33
BJ013	25.00	41.66	100.00
BJ014	0	25.00	66.66
BJ015	0	25.00	100.00
BJ016	0	8.33	16.66
BJ017	0	16.66	33.33
BJ018	0	33.33	50.00
BJ019	0	8.33	41.66
BJ020	0	16.66	41.66
BJ021	0	25.00	58.33
BJ022	0	25.00	50.00
BJ023	0	25.00	66.66
BJ024	0	11.11	75.00
BJ025	0	25.00	41.66
BJ026	0	16.66	33.33
BJ027	0	16.66	66.66
BJ028	0	41.66	50.00
BJ029	0	41.66	75.00
BJ030	0	50.00	100.00

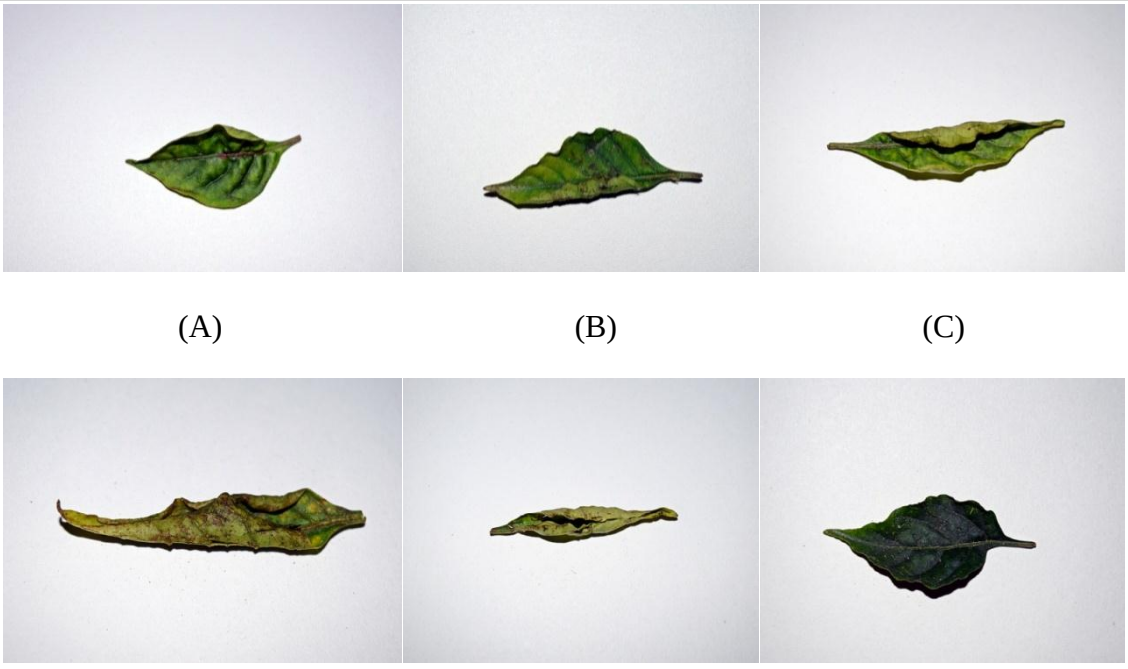
DAT = Days after transplanting

Table 4: Disease severity in Bhut Jolokia germplasm under field conditions and PCR assay

Accession No.	0	1	2	3	4	5	6	PCR
BJ001	0							-
BJ002					4			+
BJ003					4			+
BJ004						5		+
BJ005					4			+
BJ006					4			+
BJ007						5		+
BJ008							6	+
BJ009					4			+
BJ010						5		+
BJ011						5		+
BJ012						5		+
BJ013							6	+
BJ014						5		+
BJ015							6	+
BJ016					4			+
BJ017					4			+
BJ018					4			+
BJ019					4			+
BJ020					4			+
BJ021						5		+
BJ022					4			+
BJ023						5		+
BJ024						5		+
BJ025					4			+
BJ026					4			+
BJ027						5		+
BJ028					4			+
BJ029						5		+
BJ030							6	+

Table 5: Assignment of genotypes to specific disease severity based on screening against ChLCV disease

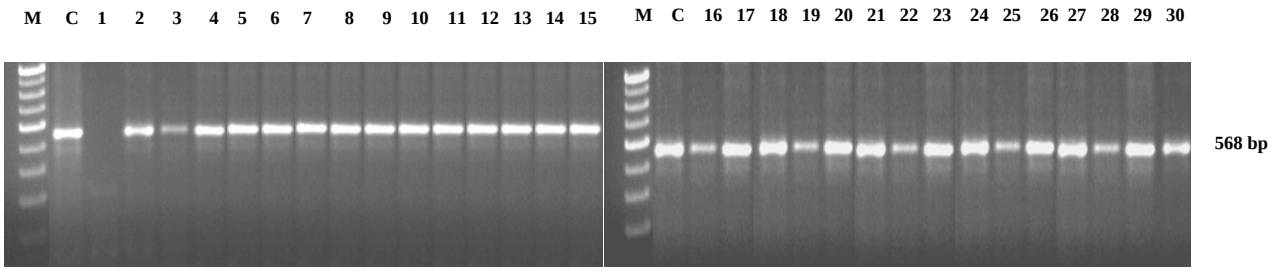
Disease severity	Genotypes
Symptomless (SL)	BJ001
Highly resistant (HR)	-
Resistant (R)	-
Moderately resistant (MR)	-
Moderately susceptible (MS)	BJ002, BJ003, BJ005, BJ006, BJ009, BJ016, BJ017, BJ018, BJ019, BJ020, BJ022, BJ025, BJ026, BJ028
Susceptible (S)	BJ004, BJ007, BJ010, BJ011, BJ012,BJ014, BJ021, BJ023, BJ024, BJ027, BJ029
Highly susceptible (HS)	BJ008, BJ013, BJ015, BJ030



(A) (B) (C)

(D) (E) (F)

Fig. 1: Disease severity based on percentage of leaf curling A. 0-5 per cent curling, B. 6-25 per cent curling, C. 26-50 per cent curling, D. 51-75 per cent curling, E. 76-100 per cent curling, F. No ChLCV disease symptoms



(G) (H)

Fig. 2: (G)(H). ChLCV amplified product on agarose gel using ChLCV specific primer. M : 100bp DNA ladder, C : Positive ChLCV sample, Lane 1-30 : ChLCV infected samples from 30 genotypes

DISCUSSION

Phenotypic screening of 30 Bhut Jolokia genotypes at 180 days after transplanting against chilli leaf curl virus disease (ChLCV) was done, based on the symptoms by following a disease scale [2, 7, 9]. The phenotypic observations were further confirmed by PCR analysis. The ChLCV specific primer was used for detection and PCR products yielded at 568 base pair. Results revealed that 4 highly susceptible, 11 susceptible, 14 moderately susceptible and 1 symptomless genotypes (Table 5). Similarly, Indian Institute of Vegetable Research, Varanasi has screened for ChLCV in a total of 321 genotypes representing 4 *Capsicum* spp. incidence was recorded at 60, 120 and 180 days after transplanting on 20 plants of each genotype with symptom severity on a 0-5 scale and found symptom-less (7), highly resistant (27), resistant (14), moderately resistant (53), moderately susceptible (125), susceptible (76) and highly susceptible (19) categories [7]. The other report showed three symptomless genotypes against pepper leaf curl virus under field and glass house conditions along with PCR assay[6]. In this research the screening of Bhut Jolokia germplasm against ChLCV

revealed one symptomless genotype *viz.*, BJ001 under field condition and confirmed by PCR assay.

The earlier report proposed a new ChLCV Oman strain (ChLCV-OM) which is distinct from ChLCV Pakistan strain (ChLCV-PK) based on their sequence homology ranging between 88.00 per cent to 91.10 per cent[5]. This supports the proposed nomenclature of Bhut Jolokia ChLCV Jorhat strains (ChLCV-BJ-JRT1, ChLCV-BJ-JRT2, ChLCV-BJ-JRT3) based on sequence homology ranging from 87.00 per cent to 90.00 per cent with other known ChLCV Indian isolates. However, the maximum nucleotide identity of 97.00 per cent was shared with Oman isolates (GenBank accession numbers JN604500, JN604494, JN604491. KF229720, JN604490, HF968755).

CONCLUSION

This is the first report on screening Bhut Jolokia germplasm against a distinct Indian chilli leaf curl virus (ChLCV-BJ-JRT) disease. However further confirmation is required under green house or net house condition. The information generated on specific disease reaction of ChLCV against each accession will be useful to select appropriate genotype for developing integrated pest management.

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