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Standardization of artificial screening technique for okra enation leaf curl disease resistance in wild okra (*Abelmoschus moschatus* ssp. *Moschatus*) germplasm

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ABSTRACT

Okra enation leaf curl disease (OELCuD) is a recently emerged whitefly insect vector transmissible viral disease of okra which adversely affects the quality and quantity of okra fruits. Okra enation leaf curl virus (OELCuV) is a main disease causative agent. Two year (*kharif* 2018 and 2019) field experiments were conducted at research farm of ICAR-National Bureau of Plant Genetic Resources, Pusa, New Delhi for field screening of 10 wild okra (*Abelmoschus moschatus* ssp. *moschatus*) accessions, viz. EC360586, EC360794, EC360830, EC360900, EC359730, EC359836, EC359870, EC360351, EC361111 and EC361171. Out of 10 accessions, 4 accessions, viz. EC360794, EC360586, EC360830 and EC361171 showed resistance (R) reaction in both the seasons. Whitefly population was also monitored in both the rainy (*kharif)* season, since, OELCuD is transmitted by whitefly insect vector. Majority (>77%) of okra genotypes were moderately preferred by the whiteflies. To further validate the resistance reaction in these 4 field resistant accessions, artificial screening experiment was conducted using viruliferous whitefly vector and VRO 6 as susceptible check. Prominent okra enation leaf curl symptom appeared after a minimum incubation period of 15 days under controlled conditions. Betasatellite (DNA-β) molecule of OELCuV was amplified (1.3 kb) using PCR for virus detection and these 4 accessions were found free from virus. These 4 promising accessions would serve as resistance source in breeding programmes to develop varieties resistant to OELCuV.

Keywords: *Abelmoschus moschatus* ssp. *moschatus*, Artificial screening, DNA-β, OELCuV, Whitefly

Okra [*Abelmoschus esculentus* (L.) Moench] belongs to Malvaceae family is an important source of carbohydrates, protein, dietary fibre, vitamin C, vitamin K and unsaturated fatty acids. India is considered as the world leader in okra production, producing 6.35 million tonnes per year, which accounts to 66% of the global production (Anonymous 2014). Okra is cultivated all across the India and Andhra Pradesh (18%), West Bengal (14%), Bihar (13%), Gujarat (11%) and Odisha (9%) contribute to majority of the country's production.

Okra yield is reduced economically due to infection of several diseases, of which viral diseases are the main cause of yield losses (Usha 1980). Among these viral diseases, okra enation leaf curl disease (OELCuD) causes severe losses to wild okra (*Abelmoschus esculentus* (L.) *Moench*) in India. Initial symptom of OELCuD comprises small pin-head enations on abaxial leaf surface followed by a warty, rough texture of leaves with later upwards leaves curling. In advanced stage of infection, plants are severely stunted and fruits are deformed with reduced size (Singh 1996). Okra enation leaf curl virus belongs to the genus *Begomovirus* which is a recently emerged viral disease in okra. OELCuV is transmitted by whitefly (*Bemisia tabaci*) (Lazarowitz 1992). Genome of new world begomoviruses comprises DNA-A and DNA-B molecule, each of 2.6–2.8 kb whereas, old world begomovirus genome includes DNA-A molecule associated with Betasatellite (Brown *et al*. 2012). Betasatellites are ss-DNA circular molecule having 1.3 kb size assist in virus replication, insect transmission and movement in plants (Zhou *et al*. 1997, Briddon *et al*. 2003, Jose and Usha 2003, Cui *et al*. 2004, Li *et al*. 2005). Chandran *et al*. (2013) reported association of an alphasatellite with okra enation leaf curl disease.

There is a lack for identified resistant source in cultivable okra against OELCuD. Wild okra germplasm harbours a potential source of resistance against OELCuD (Kumari *et al*. 2021). Hence, certain wild okra (*Abelmoschus moschatus* ssp. *moschatus)* germplasm were evaluated against whitefly

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transmitted OELCuD adopting an innovative reliable artificial screening technique with a view to find out effective resistance trait which can be further utilized in resistant breeding programmes.

MATERIALS AND METHODS

Field screening of wild okra germplasm: A total of 10 accessions of wild okra, viz. EC360586, EC360794, EC360830, EC360900, EC359730, EC359836, EC359870, EC360351, EC361111 and EC361171 along with 4 check, viz. Arka Anamica, VRO 6, Pusa Sawani and Prabhani Kranti were sown at the research farm of ICAR-National Bureau of Plant Genetic Resources, Pusa, New Delhi in randomized block design (RBD) during rainy (*kharif*) season 2018 and 2019 with one row for each accession with plant to plant spacing 30 cm × 30 cm and row to row spacing 75 cm × 75 cm.

Per cent disease index (PDI) was computed on 10 plants of each accession. Observations were noted thrice at an interval of 25 days during vegetative crop growth stage. Cupping of leaves and petiole bending in plant were considered as the characteristic symptom of OELCuD. The scale 0–4 as suggested by Cao *et al*. (2009) was used for evaluation of resistance/susceptible reaction of the genotypes to OELCuD and also for computation of per cent disease index (PDI) with slight modification.

1. Resistant (R) PDI ≤ 10%,
2. Moderately resistant (MR) PDI 10.1 to 20.0%
3. Moderately susceptible (MS) PDI 20.1 to 40.0%
4. Highly susceptible (HS) PDI ≥40.1%

Number of plants infected in each entry was monitored and PDI was computed as:

$$\text{Per cent Disease Index} = \frac{\text{Sum of all ratings}}{\text{Highest grade} \times \text{Total number of plants examined}} \times 100$$

Computation of whiteflies population during kharif *season:* Whiteflies (*B. tabaci*) serve as insect vector for OELCuV and subsequent disease development. *B. tabaci* populations were observed on 3 leaves/plant, each from lower, middle and upper canopy of plants. Observations were noted thrice at 25 days interval during vegetative crop growth phase from 3 randomly identified symptomatic plants of each wild okra accession.

Further, the mean and critical difference (CD) values were calculated to know the whitefly preferences to wild accessions and were graded into four groups: (i) negligible preference (genotypes with values < Mean-CD), (ii) Moderate preference (genotypes with values between Mean-CD and <Mean), (iii) High preference (genotypes with values between and equal to Mean and Mean+CD) and (iv) Very high preference (genotypes with values > Mean+CD) (Manoharan *et al*. 1982).

Virus source and artificial screening of wild okra: Symptomatic leaf samples from 3 randomly selected okra plant were collected from ICAR-National Bureau of Plant Genetic Resources, New farm area, New Delhi (Fig 1).

Fig 1 Okra plant showing okra enation leaf curl disease symptom on susceptible okra cultivar in field.

Duplicate leaf samples were stored at -80°C. Five wild okra accessions, viz. EC360794, EC360586, EC360830, EC361171 and EC361148 along with VRO 6 (susceptible check) were sown in ICAR-National Bureau of Plant Genetic Resources, glasshouse during *kharif* 2020 and 2021 to carryout artificial screening experiment.

Insect transmission: Three treatments were used for the artificial inoculation of viruliferous whiteflies on okra germplasm. For each treatment 5 replicates with 5 pots as well as uninoculated control were kept. In first treatment (T_1) 5 viruliferous whiteflies were used while in second treatment (T_2) and third treatment (T_3) employed 10 as well as 15 viruliferous whiteflies respectively for inoculation to 20 days old healthy okra seedlings (Table 1).

Five wild okra accessions, viz. EC360794, EC360586, EC360830, EC361171 and EC361148 along with VRO 6 (susceptible check) were used to carry out artificial screening experiment under controlled conditions. The minimum number of whitefly (*Bemisia tabaci*) adults required for transmission of OELCuV was determined by allowing adult whiteflies (0, 5, 10, 15 in number) for 24 h Acquisition Access Period (AAP) on symptomatic okra plant and then transferred whiteflies to 20 days old healthy okra seedlings for 24 h Inoculation Access Period (IAP). After 24 h of IAP whiteflies were killed using 0.01% imidacloprid spray to ensure 24 h of IAP. For each accession five plants were tested in each cage.

Table 1 Effect of treatment with different number of viruliferous whiteflies under artificial conditions on okra germplasm

Treatment		Number of plant infected/ Plant inoculated	Days taken to appear symptom
EC361148	Inoculation with 5 viruliferous whiteflies	2/5	20–25
EC361148	Inoculation with 10 viruliferous whiteflies	4/5	20–25
EC361148	Inoculation with 15 viruliferous whiteflies	5/5	20–25
EC361148	Control	0/5	NA (not appeared)
EC361171	Inoculation with 5 viruliferous whiteflies	0/5	NA
EC361171	Inoculation with 10 viruliferous whiteflies	0/5	NA
EC361171	Inoculation with 15 viruliferous whiteflies	0/5	NA
EC361171	Control	0/5	NA
EC360794	Inoculation with 5 viruliferous whiteflies	0/5	NA
EC360794	Inoculation with 10 viruliferous whiteflies	0/5	NA
EC360794	Inoculation with 15 viruliferous whiteflies	0/5	NA
EC360794	Control	0/5	NA
EC360586	Inoculation with 5 viruliferous whiteflies	0/5	NA
EC360586	Inoculation with 10 viruliferous whiteflies	0/5	NA
EC360586	Inoculation with 15 viruliferous whiteflies	0/5	NA
EC360586	Control	0/5	NA
EC360830	Inoculation with 5 viruliferous whiteflies	0/5	NA
EC360830	Inoculation with 10 viruliferous whiteflies	0/5	NA
EC360830	Inoculation with 15 viruliferous whiteflies	0/5	NA
EC360830	Control	0/5	NA
VRO 6	Inoculation with 5 viruliferous whiteflies	4/5	15–20
VRO 6	Inoculation with 10 viruliferous whiteflies	5/5	15–20
VRO 6	Inoculation with 15 viruliferous whiteflies	5/5	15–20
VRO 6	Control	0/5	NA

After imidacloprid spray, artificially designed insect-proof cages made up of Acrylic sheet 5 nm size: 24"×15"×10" provision with sliding shutter used to maintain the okra plants and symptoms were monitored regularly (Fig 2). In all cases the transmission rates (Number of infected plants divided by number of inoculated plants) were estimated.

Molecular identification of OELCuV: Total nucleic acid was extracted from each viruliferous whitefly inoculated wild okra accession along with VRO 6 (susceptible check) using cetyltrimethyl ammonium bromide method (Doyle and Doyle 1990). OELCuV complete betasatellite molecule was amplified using Beta01F5'-GGTACCACTACGCTACGCAGCAGCC3'/ Beta02R3'CACATGGGGACCCTCCCATCCATGG-5' universal primer pair (Briddon *et al*. 2002). PCR reactions

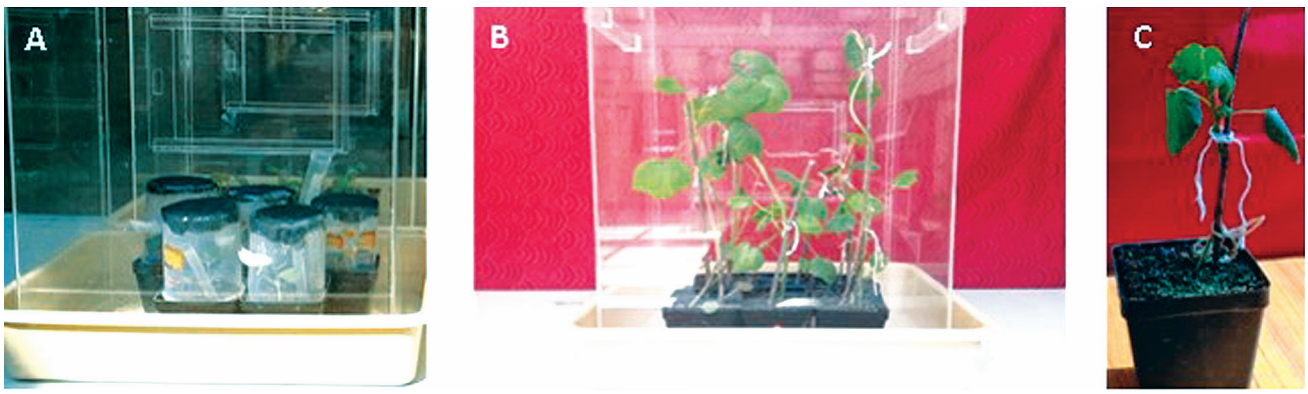

Fig 2 Artificial screening of wild okra against okra enation leaf curl virus using whitefly vector A, Cage inoculation of viruliferous whitefly along with susceptible check; B, Resistant response to tested wild okra accession; C, Okra enation leaf curl disease symptom development in the susceptible check.

were carried out in a DNA Engine (Peltier thermal cycler) machine.

Total volume of PCR reaction prepared was 25 µl (100 pmol DNA template, 1.5U Taq DNA Polymerase, 25 mM $MgCl_2$, 2.5 mM dNTPs, 25 pmol of each primer and nuclease free water). Total number of amplification cycles used were 35 with initial denaturation at 94°C for 4 min and final extension at 72°C for 10 min. The cycling conditions were denaturation at 94°C for 45s, annealing at 59°C for 50s, and extension at 72°C for 90s. Amplified PCR products were electrophoresed (1 h at 80 volts) on 1% agarose gel and seen on a Gel documentation system. Desired size of 1.3 kb amplified products corresponding to the OELCuV of okra leaf was obtained. PCR product of 1.3 kb was sequenced and sequence similarity was analysed using BLAST.

RESULTS AND DISCUSSION

Disease occurrence in germplasm: Field screening was conducted on the basis of disease symptoms and scoring was done for OELCuD in wild okra accessions at New Delhi location during the main cropping seasons of *kharif* 2018 and 2019. Okra genotypes showed varied OELCuD symptoms. Majority (>72%) of wild okra accession exhibited typical top leaves curled symptom during two years of field screening.

During the first year of field screening (*kharif* 2018) average per cent disease index values were 16.14 while during the second year (*kharif* 2019) average PDI values were 13.11 which clearly indicated that disease progress was higher during the first year. Out of 10 accessions, 4 lines, viz. EC360794, EC360586, EC360830 and EC361171 showed resistant (R) reaction during *kharif* 2018 and 2019.

Response to whiteflies Bemisia tabaci: During *kharif* 2018, the mean population of whiteflies (*B. tabaci*) per leaf was 0.502 whereas during *kharif* 2019, the mean population of whiteflies per leaf was 0.433. During both the seasons, genotypes were grouped based on whitefly population into either moderate or high preference. Among the checks only VRO 6 in *kharif* 2018 and 2019 showed very high preference whereas rest showed high preference of whiteflies. Further, promising okra genotypes namely EC360586, EC360794, EC360830 and EC361171 showed moderate preference of whitefly population in both the years of field screening.

Standardization of screening technique: The artificial screening technique was perfectly standardized and used to screen 5 wild okra accessions and 1 susceptible check (VRO 6) with initial lower population of whitefly and without any chance for escape under artificial conditions for consecutive two years. Out of 3 inoculation treatment tested (using 5, 10 and 15 viruliferous whiteflies) only inoculation with 15 viruliferous whiteflies were found to be efficient to develop complete okra enation leaf curl symptom in wild okra (EC361148) tested plant after 20–25 days of inoculation where as for susceptible check (VRO 6) 10 viruliferous whiteflies were sufficient to produce okra enation leaf curl symptom after 15–20 days of inoculation (Table 1).

It was found that 15 viruliferous whiteflies per plant efficiently transmit OELCuV in 20 days old wild okra seedlings and the whiteflies were subjected to 24 h AAP and 24 h IAP (Table 2). Wild okra accession EC361148 and VRO 6 susceptible check developed a conspicuous OELCuD symptom whereas 4 wild okra accessions, viz. EC360794, EC360586, EC360830 and EC361171 found resistant against OELCuD (Fig 2).

Table 2 Transmission efficiency of okra enation leaf curl virus (OELCuV) by whitefly vector using 24 h of Acquisition Access Period (AAP) and Inoculation Access Period (IAP)

Number of whiteflies used (I)	Plants infected (R)/ Plants inoculated (N)	Transmission rate (T=R/N)
0	0/5	0.00
5	2/5	0.40
10	4/5	0.80
15	5/5	1.00

Detection of OELCuV: PCR amplification of OELCuV betasatellite (DNA-β) molecule using universal primer pair (Beta01F/ Beta02R) amplified at 1.3 kb for wild okra accession EC361148. Four wild okra accessions, viz. EC360794, EC360586, EC360830 and EC361171 were found free from virus, where no virus amplification was observed. DNA-β amplification was noticed in EC361148 and VRO 6 susceptible check. PCR product of 1.3 kb has been sequenced and found 98.47–99.18% sequence similarity with okra enation leaf curl virus.

The whitefly transmission characteristics for OELCuV are similar as that of circulative, non-propagative mode of geminiviruses transmission (Gray and Banerjee 1999). Efficiency of virus transmission depends on the sex of whiteflies. Generally female whiteflies transmit viruses more efficiently than male (Muniyappa *et al*. 2000, Czosnek *et al*. 2001). Our findings showed 15 viruliferous whiteflies are sufficient for effective virus transmission which also correlates with the finding of Venkataravanappa *et al*. (2015). Pasupathi *et al*. (2021) showed the effect of the age of okra plants for varying whitefly resistance responses on the transmission rate of okra enation leaf curl virus by its vector whitefly (*Bemisia tabaci*). They found that the efficiency of transmission of OELCuV was highest when 7 days old seedlings were inoculated and the transmission had decreased when the age of seedlings increased. Understanding the resistance mechanisms of the okra accessions and interactions between plant viruses and their insect host can pave the way for novel approaches to protect plant from virus infection.

Lack of resistance for OELCuD in cultivated species of okra has forced the breeders to look out for wild species which are regarded as stable and reliable sources of resistance to OELCuD (Singh *et al.* 2007). A number of wild species, viz. *A. angulosus, A. crinitus*, *A. ficulneus, A. manihot*, *A. pungens*, *A. tetraphyllus* and *A. tuberculatus* were observed to be free from OELCuV (Singh *et al.* 2009). In our experiment it was consistently observed that *Abelmoschus moschatus* ssp. *moschatus* have high level of

resistance against OELCuD.

To curtail yield loss, use of synthetic pesticide spray against whitefly vector is commonly followed by farmers but it is costly and has adverse impact on user and non-target species through persistence in food and environment. Thus, attention is given to develop resistant variety which is environmentally safe and long lasting in nature (Eigenbrode and Trumble 1994, Nataraja *et al*. 2013). In the present study we did field screening work and found 4 accessions, viz. EC360794, EC360586, EC360830 and EC361171 resistant to OELCuD in both the *kharif* season 2018 and 2019. These 4 wild okra accessions selected for further study at artificial inoculation condition to validate the consistency of resistance against OELCuD and found resistant in both field as well as artificial conditions.

These 4 promising accessions found resistant would serve as resistance source in breeding programmes to develop okra varieties resistant to OELCuV.

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