Journal of Animal & Plant Sciences, 34(6): 2024, Page: 1582-1593
ISSN (print): 1018-7081; ISSN (online): 2309-8694
https://doi.org/10.36899/JAPS.2024.6.0834

MOLECULAR CONFIRMATION OF ToLCNDV RESISTANCE IN CUCUMBER THROUGH AGROINOCULATION AND FIELD SCREENING

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ABSTRACT

Viral diseases, especially ToLCNDV causes serious threat to cucumber cultivation in India and many other countries of the world. Screenings for resistance to ToLCNDV in cucumber were elucidated in the current work for the first time under natural epiphytotic (RBD with three replications) as well by artificial screening (CRD with three replications) through agro-inoculation. Specific DNA-A primers were used in PCR amplification as it is a bipartite virus. Among the four cucumber genotypes studied, mean percent disease index and incidence ranged from 0.00 to 92.00% and 0.00 to 93.3% in 2023 and 2024 respectively. Under natural epiphytotic conditions, the genotype DC 70 was found to be free from ToLCNDV infection while DC 773 recorded 75.00 PDI and 87.50% disease incidence and DC 769 with 42.85 PDI and 71.42% disease incidence in 2023 while DC 70 and P-85 were found to be free from ToLCNDV infection and DC 773 recorded 65.71 PDI and 85.00% disease incidence and DC 769 with 40.00 PDI and 75.00% disease incidence in 2024.In artificial screening, the genotype DC 70 was found to be free from ToLCNDV infection followed by P-85 with 22.91 PDI and 46.60% disease incidence (inoculated)and 9.20 PDI and 6.66% disease incidence (control)while DC 773 recorded 92.00 PDI and 93.3%disease incidence (inoculated) and 22.40 PDI and 40.00%disease incidence(control). Out of the four genotypes screened, DC 70 showed a durable immune reaction and the resistant source identified is a good candidate for theresistant breeding programmes for ToLCNDV in cucumber.

Key words: Cucumber, ToLCNDV, Natural epiphytotic conditions, Artificial screening, Agro-inoculation, PCR, Resistant and Susceptible.

Published first online November 05, 2024 Published final December 22, 2024

INTRODUCTION

Cucumber (*Cucumis sativus* L.) being native to India, is a widely consumed fresh market vegetable that is used in many different dishes. Being a highly cross-pollinated crop, it is monoecious in nature and prefers warm, sunny weather for optimal growth and development. Viral diseases, especially ToLCNDV causes greater threat to cucumber cultivation in India and other countries of world. Further the globalization and climatic variations speed up their transmission.

The major infectious agents associated with cucurbits include Zucchini yellow mosaic virus (ZYMV), Cucumber mosaic virus (CMV), Watermelon bud necrosis virus (WBNV) and Tomato leaf curl New Delhi virus (ToLCNDV). Among them, the largest general member of the Geminiviridae family, the Begomovirus, is posing a serious threat to cucurbitaceous crops in India. The virus is transmitted by whiteflies (*Bemisia tabaci*) and is the cause of the tomato leaf curl New Delhi virus (ToLCNDV). Since 20th century, the begomovirus in cucurbits became a significant problem in India, resulting in 50% loss in northern states of India (Tiwari *et al*., 2012). ToLCNDV was first identified in India during 1995 as a variation of the Tomato yellow leaf curl virus (TYLCV) by (Srivastava *et al*., 1995). Depending on the nature of genome present, this virus is bipartite contains two 2.6–2.7 kb circular single-stranded DNA strands, known as DNA-A and DNA-B, which are encapsulated in geminate particles and necessary for fundamental viral operations (Fortes *et al*., 2016). In general, beta satellite and monopartite begomovirus are linked however, a recent study also shown that beta satellite and bipartite begomovirus are also associated (Venkataravanappa *et al.,* 2019). It is also known that beta satellite plays significant influence on the host range of the associated begomovirus.

On the other hand, ToLCNDV has been rapidly expanding its host range and reaching new geographic areas in recent years (Malathi *et al.,* 2017). However, a recent report shows that it also infects tomato (Prabhandakavi *et al.* 2018), chayotes and cucurbits (Patil *et al.* 2017) in the southern region of the country.ToLCNDV has caused substantial harm to a variety of crops, particularly cucurbits, such as melons. In central Spain, its impact on open-field melon production has been particularly severe with reported losses reaching as high as 20% (Saez *et al.* 2017).It has also been linked to immense economic losses, especially reduced yield and cracked, unmarketable fruits in Spain and other key cucurbit production regions (Siskos *et al*., 2022). Diseaseincidence embrace greater significance results in diminished fruit yield.The use of genotypes with different backgrounds, virus isolates, the timing of inoculation and screening techniques used to determine the resistance. In this study, four cucumber genotypes were studied under natural epiphytotic conditions and challenge inoculation(agro-inoculation) for ToLCNDV and also for the molecular confirmation of the resistance in the genotypes.

MATERIALS AND METHODS

Plant materials: Cucumber genotypes *viz.*, DC 70, DC 773, DC 769 and P-85developed from Indian Agricultural Research Institute, NewDelhi **(Table 1)** were used as plant materials for this experiment. Samples of infected plants exhibiting different ToLCNDV symptoms such as mosaic, yellowing, upward curling, puckering, and stunting were randomly collected and brought to the laboratory for molecular analysis. Disease index and incidence were computed and reported as percentages.

Screening of cucumber genotypes against ToLCNDV under natural epiphytotic conditions: The research was conducted at Department of Vegetable Science, Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore. Field screening of four cucumber genotypes was conducted against ToLCNDV under natural epiphytotic condition as Coimbatore is the hotspot for the diseases of cucurbits due to it's climatic conditions (average temperature of 27-35^{0}C in March to June) during the cropping seasons. During the period the natural ToLCNDV vector population pressure was increased due to elevated normal temperature under natural epiphytotic conditions and except for the use of insecticides, all farm practices of cucumber cultivation were carried out in order to raise healthy crop. Further to increase the vector population pressure, one row of ToLCNDV susceptible genotype DC 773 was planted along with every genotype in the experimental field. A randomized block design with three replications was used to set up the experiment. Seeds of the four genotypes were sown at a spacing of 1.5m x 1.5m.The first season sowing was effected on 16.03.2023 and the second season on 17.03.2024. Weather data for both the seasons is provided in **Annexure I.**

Disease scoring procedure: Each plant was given a score on 0–5 rating scale **(Table 2)** based on the area of the leaf covered by symptoms, in accordance with the rating methodology as reported by Islam *et al.* (2011). Symptom evaluation was done by visual scoring of ToLCNDV symptoms. Disease assessment was carried out for four genotypes *viz.,* DC 70, DC 773, DC 769 and P-85 evaluating their susceptibility to ToLCNDV disease. Per cent disease index was graded using a modified scale ranging from 0 to 5, as suggested by Islam *et al*. (2011) presented in Table 2. Percent disease index of ToLCNDV as per the formula defined by McKinney 1923, which assisted comparison among genotypes.

$$\text{Per cent disease index (PDI)} = \frac{\text{Sum of all numerical ratings}}{\text{Total number of plants observed}} \text{x} \frac{100}{\text{Maximum disease grade}}$$

Depending on the percent disease index values, the genotypes were distinguished into various classes *viz.* ,immune (PDI% = 0), resistant (PDI% = 1-10), moderately resistant (PDI% =11-25), moderately susceptible (PDI% = 26-50), susceptible (PDI% = 51-75) and highly susceptible (PDI% = 76-100).

Percent disease incidence: Cucumber genotypes were screened under both natural epiphytotic conditions and artificial screening to identify the resistant genotype for exploiting in further breeding programme. Per cent disease incidence was calculated by the formula given by Tiwari *et al.* (2012).

$$\text{Disease incidence (\%)} = \frac{\text{Total number of plants infected with ToLCNDV}}{\text{Total number of plants observed}} \text{x100}$$

Artificial screening through Agroinoculation: The ToLCNDV agro-infectious construct was obtained from Department of Plant Pathology, TNAU, Coimbatore .*Agrobacterium tumefaciens* containing recombinant plasmids of DNA A, DNA B and β satellite of ToLCNDV grown in LB broth with an optical density (OD) of 1.0 at 600nm. A total of sixty seeds of the four genotypes were sown in plastic pots of 6 x 6 cm size and maintained in the controlled environment under completely randomized design with three replications. To improve seed germination seeds were submerged in sterile water for 48 hours. Ten days old seedlings were inoculated with liquid culture using the pinpricking technique. For artificial virus inoculation, germinated seed hypocotyls were pinpricked under laminar air flow chamber. The following day the pinpricked seeds were planted in pots which contained a mixture of autoclaved red soil and FYM (3:1) after being submerged in agroinoculation buffer for 14 hours at 37˚C in the dark.

Following the protocol described by Vignesh *et al.* (2023), a total of 60 plants at the two leaves stage (10 days old) of cucumber seedlings of both resistant and susceptible genotypes were agroinfiltrated with ToLCNDV infectious clone. Stem inoculation around the developing nodal region of the stem used 30-gauge needle containing 20 µl of an *Agrobacterium* culture. Thereafter both resistant and susceptible genotypes of agroinoculated and control plants were grown in an insect-proof growth chamber with a photoperiod of 16 h/8 h (day and night), temperature 28±2°C and 60% relative humidity (**Fig.1**). All plants were observed regularly and leaf samples from each genotype were collected from terminal part of both agroinoculated and control plants. Cucumber leaf samples were preserved at -80˚C for future experimental analysis.

DNA isolation and PCR mediated amplification: A specific degenerative primer GKNDV DNA-A was used to detect the presence of coat protein gene of ToLCNDV in all the agroinfiltrated leaf samples (Vignesh *et al.,* 2023). The assay of ToLCNDV infection was assured by PCR amplification of leaf samples. The presence of DNA-A component was examined in both symptomatic and asymptomatic leaf samples of agroinoculated and control plants. In order to confirm the suspected virus PCR was carried out by utilizing a pair of primers developed from coat protein gene area of a well characterized ToLCNDV DNA-A. The coat protein gene of the ToLCNDV DNA-A genome was found using a specific degenerative primer, GKNDV DNA. For molecular validation of the presence or absence of ToLCNDV genome, PCR amplification was performed on all plant samples using the GKNDV DNA-A-F and GKNDV DNA-A-R primer sets that are unique to the ToLCNDV DNA-A.

Four weeks after inoculation, approximately 100 mg of fresh apical leaves from four genotypes were harvested. Total genomic DNA was extracted from leaves exhibiting severe, mild and symptomless samples. Total DNA from leaf tissues were extracted by CTAB DNA extraction method as described by Doyle and Doyle (1990). 20 µL of nuclease free water, 0.5 µL of primer forward (10 pM), 0.5 µL of primer reverse (10 pM), and 3.5 µL of 1 × AccuPower® PCR Mastermix (Bioneer)were used to produce the mixture for the PCR reaction. The PCR reaction setup was 94^0C for 4 mins, 30 cycles of denaturation for 30 sec at 94^0C annealing for 30 sec at 58^0Cand extension for 1 min at 72^0CC (DNA A) reaction was finished with a final extension for 20 min at 72^0C. To determine the amplicon size, the PCR products were resolved on a 1% agarose gel and stained with ethidium bromide.

Sequence of ToLCNDV DNA- A specific primers used: Forward primer (GKNDV DNA-A-F): 5'CGCAGGTTGTGGTTGAACTG 3' (20 nt)

Reverse primer (GKNDV DNA-A-R): 5' GCAAAACAATGTGGGCTCGT 3'(20 nt)

Statistical analysis: Results from the natural and artificial screening experiments were expressed as mean. Before statistical analysis per cent disease index and incidence values were converted to arcsine transformation.Then data were subjected to analysis of variance (Two way ANOVA) for RBD and (One way ANOVA) for CRD followed by DMRT was performed. In SPSS software version 16, to perform a Univariate analysis using the general linear model (GLM) was followed and presented in table (Gomez and Gomez, 1984). The significance level was based on the probability of $P \leq 0.05$.

RESULTS AND DISCUSSION

Screening of cucumber genotypes against ToLCNDV under natural epiphytotic conditions: The morphological characteristics of four genotypes were statistically analyzed. Except DC 70, all genotypes showed expression of ToLCNDV incidence on 45 days after sowing. Based on the observed percent disease index (PDI), the screened genotypes showed significant differences in response to ToLCNDV infection and disease incidence ranged from immune to susceptible reactions, and there was a slight variation in the sowing seasons. Under natural field conditions, the ToLCNDV disease index varied among genotypes from 0% to 75.00% (Table 3). Results showed that genotype DC 773 recorded significantly highest disease index (75.00%) and incidence (87.50%) with the lowest yield of 1.06 kg/plant proved to be susceptible followed by DC-769 with the disease index of 42.85% and 71.42%disease incidence with the yield of 1.32 kg/plant (Table 4),categorized as moderately susceptible. In 2023, the temperature ranged from 32°C to 37°C and the RH being72-92% during the cropping period. In 2024, the temperature ranged between 32°C-39°C and the RH being 72-92% which is very conducive for ToLCNDV disease inoculum as well for the rapid vector multiplication. Hence, represent the natural epiphytotic condition. Singh *et al*. (2024) also observed that disease incidence in commercially cultivated sponge gourd varieties varied from 76.50% to 96.10% were proved to be susceptible to ToLCNDV under natural screening in the same weather conditions. P 85 showed 8.57 PDI and 28.57%disease incidence with a yield of 1.69 kg/plant proved to be resistant to ToLCNDV. DC 70 was found to be totally free from ToLCNDV symptoms with a yield of 2.01 kg/plant (Table 4) determined as immune to ToLCNDV during both the seasons (March -June, 2023 and 2024).However, during 2024significantly highest disease index was recorded in DC 773 (65.71%) and disease incidence (85.00%) with the lowest yield of 1.10 kg/plant proved to

be susceptible followed by DC 769 with 40.00%and 75.00% respectively(Table 3) with yield of 1.47 kg/plant(Table 5) expressed as moderately susceptible. DC 70 and P 85 were found to be free from ToLCNDV symptoms with a yield of 2.19 and 1.89 kg/plant and proved resistant to ToLCNDV. Likewise, field survey conducted in various bitter gourd cultivation areas of Coimbatore revealed symptoms such as mosaic, slight leaf curl and blistering with disease incidence value fluctuated from 65-80% Vignesh *et al*. (2023) reported that mean disease incidence in ash gourd field survey was about 75% in the same season.

From this study it is manifested that the genotype DC 70 of cucumber expressed stable immune reaction against ToLCNDV under natural epiphytotic conditions during both the growing seasons whereas P 85 was found to be immune during 2024.According to Pandey *et al*. (2022) all the Arya lines of *Cucumis melo* under study scored zero for disease scoring, designating the immunity to ToLCNDV. Moreover, the ability of DC 70 genotype capable to grow profusely and produce high yield in both the seasons as well as showed immune response when challenged by an infectious ToLCNDV virus clone under glasshouse and most likely by a natural viral inoculum in an open field may offer an efficient way to induce a ToLCNDV immune response while using less insecticides. Mostly occurrence of various types of virus (both mono- and bipartite) and inherently mixed infections, variations in whitefly populations as well by rainfall could have contributed the variation in the disease incidence in genotypes of cucumber (Maruthi *et al*., 2005)

Artificial screening against ToLCNDV by agroinoculation: ToLCNDV is a serious disease in cucumber causing 80-100% yield loss depending upon the stage of infection. High temperature and RH is conducive for the disease incidence due to active vector population. Screening of viral diseases pose serious problem in disease resistance research. And this is the first report on ToLCNDV screening in cucumber through infectious clone by agroinoculation which was developed by Vignesh *et al*. (2023). Globally vegetable crop production is seriously threatened by Tomato leaf curl New Delhi virus (ToLCNDV). Presently, it is common in India and has also been occurred in countries like Pakistan, Bangladesh, and Thailand. ToLCNDV is becoming a serious threat to the production of cucurbit crops due to its host range extension, which is being supported by its evolution and potential for a different mechanism of transmission (Juarez *et al*., 2019). In epidemic situation, the crops infected with ToLCNDV disease can cause 100% yield loss. Therefore, identifying ToLCNDV resistant sources in other species of cucumber is crucial to mitigate the effects of this virus. As a result, intensive screening of a wide range of genetic resources is required to discover resistant sources for use in cucumber breeding programmes. An artificial screening study was carried out with four distinct genotypes of cucumber, to verify whether ToLCNDV was truly due to pathogenicity on the above genotypes. Yamamoto *et al.* (2021) observed more severe symptoms of ToLCNDV-[ES-Alm-Cuc-16] in agroinoculated cucurbit plants, especially in melon and zucchini compared to field-grown plants probably due to inoculation during the cotyledon stage prior to the occurrence of true leaves.

Artificial screening was done to confirm field screening results on four selected genotypes. Cucumber genotypes showed immune to highly susceptible reactions under natural field study during both the seasons were selected for artificial screening to confirm the resistance of the genotypes. Hence, employing artificial screening systems allows for disease evaluation trials regardless of weather conditions that might be any period of time in the season (Kaur *et al.,* 2021).In this study, four genotypes of cucumber were successfully agroinoculated with ToLCNDV, three genotypes displayed ToLCNDV symptoms, and one genotype did not express any symptoms at all (Table 2). Differential symptom reactions against ToLCNDV was expressed though same concentration of inoculum was agroinoculated into each genotype during the agroinoculation process. In order to evaluate their potential for use in new region, south Indian resistant/tolerant tomato cultivars were artificially screened against the Bangladesh ToLCV. This was done because conventional breeding for ToLCNVD resistance is a slow and challenging procedure (Hanson *et al*., 2000).

Susceptible genotypes initially showed symptoms with light yellowing of the leaves. Later, the puckering of apical leaves, curling, and yellowing with mosaic symptoms were noticed. Stunting and leaf curling were noted during severe stages of infection. Similar pattern of symptoms such as leaf curling, mosaic yellowing, and stunting were reported as serious pathogenicity to pumpkin, zucchini and melon as shown for other Mediterranean or Asian isolates and suggested that ToLCNDV had increased pathogenicity (Vo *et al.*,2022). Symptoms, percent disease index, and percent disease incidence have also been provided in Table 6 &7.

The artificial screening results showed that both control and inoculated DC 70 plants on 28 dpi to be symptomless immune reaction suggested that genotype could resist ToLCNDV incidence than other genotypes. Symptomless immune reaction of DC 70 might be due to genetic variability of the crop species which is crucial for the resistant genotype. Furthermore, different genetic background of genotypes may be the cause for various symptom expression of virus spread and infection. Infected plants expressed a typical disease symptom at 28 dpi. Similarly, Islam *et al.* (2011) identified ToLCNDV

resistance in sponge gourd lines, DSG-6 and DSG-7 through sterilized virion inoculation in insect-proof net houses while Kaur *et al.* (2021) confirmed potential resistance to ToLCNDV in *L. cylindrica* and *L. acutangula* germplasm. The disease index % were significantly higher in DC 773 inoculated plants which expressed the highest PDI (92.00%) (Table 7) and highly susceptible reactions such as severe mosaic, yellowing, curling of leaves, and stunted plants. DC 773 control plants expressed the moderately susceptible reactions such as mosaic, yellowing and curling on a fewer leaves with PDI of 29.87%(Table 7). These symptoms were similar to those depicted for ToLCNDV infection on melon (Trisciuzzi *et al.,* 2018). Based on the study, disease transmission qualities implied on changing behaviors and types of disease-causing capacity as well as the potential for wider host ranges and disease dissemination. These findings are consistent with earlier reports from Yamamoto *et al. (*2021) on cucumber, melon and zucchini and Mei *et al.* (2023) on *Cucumis melo.*

In DC 769 inoculated plants PDI (52.50%) (Table 7) with susceptible reactions *viz.*, mosaic of young leaves, yellowing, distortion of leaves were observed while 18.75% PDIin DC 769 control plants caused moderately susceptible reactions such as young leaf mosaic and mild yellowing of leaves (Table 7).Results of this study also implied the susceptible reaction of cucumber plants, which is concurrent with the report by Ruiz *et al*. (2017).DC 85 inoculated plants had22.91% PDI(Table 7) with moderately resistant symptoms *viz.*, mosaic of young leaves while DC 85 control plants showed resistant reactions *viz.*, mild mosaic of young leaveswith9.20%PDI. The reasons for the variations in per cent disease incidence and index of ToLCNDV might be due to difference in the inoculum source, vector population, climatic conditions and geographic location. These findings were concurrent with earlier research on pumpkin plants studied by Dhillon *et al.* (2021).

Among the four screened genotypes of inoculated and control plants percent disease incidence ranged from 0.00 to 93.30% (Table 7). DC 773 had significantly highest percentage of disease incidence, followed by DC 769 (73.00 and 20.00%) and P-85 (46.60 and 6.66%) (Table 6). Similarly, ridge gourd varieties such as RHRG-2, SVRGH-54, VRGH-2 and VRGH-336 were identified as highly susceptible to ToLCNDV *via* inoculation observed by Sohrab *et al,* (2013). In contrast, DC 70 had the lowest or no score at 28 dpi (0.00 & 0.00%) (Table 7). High temperature encouraged the proliferation of whiteflies might be the cause for high disease incidence percentage in the field during both the seasons. The corresponding trend in *Luffa actangula* per cent disease incidence was reported by Parrella *et al*.(2017) in pumpkin plants. Moreover, investigation of seed transmission of ToLCNDV is crucial for potential quarantine measures to prevent virus spread into new geographical areas.

Molecular confirmation of agroinoculated plants by PCR amplification: The PCR analysis demonstrated that ToLCNDV infection was the cause of the symptoms displayed on several genotypes employed in artificial screening. At 28 dpi Cucumber genotypes inoculated with ToLCNDV expressed typical symptoms compared with control plants. In this study, only ToLCNDV inoculum was used as positive control. Using a set of GKNDV DNA-A primers, DNA collected from both symptomatic and non-symptomatic leaf samples were subjected to PCR for a preliminary screening of ToLCNDV infection. The coat protein gene of ToLCNDV DNA-A was amplified using a primer pair of GKNDV DNA-A (40 nt).According to PCR results, the samples resulted positive for GKNDV DNA-A primer displayed amplification of DNA-A band at an amplicon size of 630 bp was seen in symptomatic leaves of DC 773, 769 and P-85genotypesconfirmed the presence of ToLCNDV infection transmitted by *Agrobacterium* mediated agroinoculation(Fig1a&1b).Likewise, molecular detection for employing begomovirus specific deng primers and coat protein gene primers revealed the ToLCNDV presence in bitter gourd samples reported by Kiran *et al*.(2021) and similar tendency on amplicon size of 630 bp in agroinoculated cucurbits crops for ToLCNDV coat protein DNA-A gene through conventional PCR was previously reported by Vignesh *et al.*(2023) in bitter gourd. However, no such amplicon was found in symptomless leaf samples of DC 70 as confirmed through PCR assay.

In PCR investigation, samples showing positive using degenerate primers indicated begomovirus infection. Similarly, Saha *et al*. (2014) confirmed begomovirus associated with tomato leaf curl disease in tomato samples from the Brahmaputra valley of Assam. By employing PCR amplification, the viral genome was recognized in all symptomatic plants infected with ToLCNDV. Out of 36 leaf samples of four genotypes including both inoculated and control leaf samples were subjected to preliminary detection in PCR using GKNDV DNA-A primer which amplified for DNA-A. PCR analysis confirmed that24 samples amplified CP gene of ToLCNDVDNA-A(**Table 1**).

Table: 1 Sources and performance of cucumber genotypes used in the current study..

Genotype	Source	Bitterness	Fruit Colour	Salient Features
DC-70	IARI, New Delhi	+	light green	Monoecious, resistant to leaf curl disease and Downy Mildew, fruits 13-16 cm long, black spine
DC-773	IARI, New Delhi	+	dark green	Monoecious, fruits 40-43 cm long, white spine
DC 769	IARI, New Delhi	++	Darkgreen	Monoecious, fruits 35-40 cm long, black spine
P-85	IARI, New Delhi	+	Light green	Monoecious, fruits 25-30 cm long, black spine

Table: 2 Disease reaction scale (gradings) in cucumber for ToLCNDV disease

Severity grade	Symptom	Rating scale	Severity range	Response value
0	No symptoms	Immune (I)	0%	0
1	Mild mosaic of young leaves covering < 10% of the surface	Resistant (R)	1-10 %	0.25
2	Mosaic of young leaves covering < 25% of the surface	Moderately Resistant (MR)	11-25 %	0.5
3	Mosaic of young leaves covering <50% of the surface, blistering and puckering of leaves	Moderately Susceptible (MS)	26-50 %	0.75
4	Severe mosaic of young leaves covering < 75% of the surface, distortion of leaves	Susceptible (S)	51-75%	1
5	Severe mosaic of young leaves covering > 75% of the surface, distortion of leaves and stunting of the plants	Highly Susceptible (HS)	76-100%	1

The coefficient of infection was calculated by multiplying % disease by the 'response value' assigned to each severity grade. Thus, the coefficient of infections combined the percentage of infection and its severity.

Table: 3 Response of the different genotypes used in field screening of ToLCNDV under epiphytotic conditions (March-June 2023 and 2024).

Genotypes	Season	Percent Disease Index (PDI)	Percent Disease Incidence	Coefficient of infection	Disease reaction	Symptoms observed
DC-70	2023	0.00 (0.00^{d})	0.00 (0.00^{d})	0.00	Immune (I)	No symptoms
	2024	0.00 (0.00^{c})	0.00 (0.00^{c})	0.00	Immune (I)	No symptoms
DC-773	2023	75.00 (60.00^{a})	87.50 (69.30^{a})	75.00	Susceptible (S)	Severe mosaic, yellowing, curling of leaves and stunting of the plants
	2024	65.71 (54.16^{a})	85.00 (67.21^{a})	65.71	Susceptible (S)	Severe mosaic, yellowing, curling of leaves and stunting of the plants
DC-769	2023	42.85 (40.89^{b})	71.42 (57.68^{b})	32.13	Moderately Susceptible (MS)	Mosaic of young leaves and slight yellowing of leaves
	2024	40.00 (39.23^{b})	75.00 (60.00^{b})	30.00	Moderately Susceptible (MS)	Mosaic of young leaves and slight yellowing of leaves
P-85	2023	8.57 (17.02^{c})	28.57 (32.31^{c})	2.14	Resistant(R)	Mild mosaic of young leaves
	2024	0.00 (0.00^{c})	0.00 (0.00^{c})	0.00	Immune (I)	No symptoms
SE (d)	2023	1.888	2.596	-	-	-
	2024	1.594	5.656	-	-	-
CD (P≤0.05)	2023	4.113	2.229	-	-	-
	2024	3.473	4.85	-	-	-

Figures in the parenthesis are transformed values

In a column, means with similar letter (s) is/are not significantly different by DMRT at $P \leq 0.05$

Table: 4 Morphological parameters (March -June 2023)

Geno types	Fruit length (cm)	Fruit girth (cm)	Fruit weight (g)	No. of fruits /plant	Crop duration	Days forfirst harvest	Yield per plant (kg)
DC-70	19.10 (25.91^{d})	12.89 (21.04^{a})	210.12 (14.51^{a})	8.30 (16.74^{b})	93.00 (74.66^{a})	40.20 39.35^{c})	2.01 (8.15^{a})
DC-773	29.10 (32.65^{b})	11.80 (20.09^{b})	211.56 (14.56^{a})	6.20 (14.42^{d})	78.70 (62.51^{c})	50.50 (45.29^{a})	1.06 (5.91^{d})
DC 769	37.00 (37.46^{a})	12.01 (20.28^{b})	219.83 (14.84^{a})	7.12 (15.48^{c})	76.73 (61.16^{c})	47.42 (43.52^{b})	1.32 (6.6^{c})
P-85	21.80 (27.83^{c})	9.80 (18.24^{c})	175.83 (13.28^{b})	9.40 (17.85^{a})	89.00 (70.63^{b})	48.00 (43.85^{b})	1.69 (7.47^{b})
Mean	26.75	11.63	204.34	7.76	84.36	46.53	1.52
SE(d)	1.06	0.52	9.20	0.28	3.78	2.01	0.07
CD (P≤0.05)	2.29	1.12	19.96	0.61	8.19	4.36	0.15

Figures in the parenthesis are transformed values
In a column, means with similar letter (s) is/are not significantly different by DMRT at P ≤ 0.05

Table: 5 Morphological parameters (March - June 2024)

Geno types	Fruit length (cm)	Fruit girth (cm)	Fruit weight (g)	No. of fruits /plant	Crop duration	Days forfirst harvest	Yield per plant (kg)
DC 70	20.10 (26.64^{c})	15.10 (22.87^{a})	210.98 (14.54^{b})	10.00 (18.43^{b})	95.00 (77.08^{a})	45.87 (42.63^{b})	2.19 (8.51^{a})
DC 773	36.23 (37.01^{b})	11.99 (20.26^{b})	221.89 (14.91^{b})	6.50 (14.77^{c})	73.77 (59.19^{c})	51.57 (45.9^{a})	1.10 (6.02^{d})
DC 769	39.9 (39.17^{a})	12.8 (20.96^{b})	237.45 (15.43^{a})	6.20 (14.42^{c})	81.30 (64.38^{b})	49.66 (44.81^{a})	1.47 (6.96^{c})
P-85	20.07 (26.62^{c})	15.27 (23.00^{a})	177.17 (13.33^{c})	10.67 (19.07^{a})	94.00 (75.82^{a})	49.67 (44.81^{a})	1.89 (7.90^{b})
Mean	29.08	13.79	211.87	8.34	86.02	49.19	1.66
SE(d)	1.11	0.64	8.10	0.39	3.82	2.25	0.06
CD (P≤0.05)	2.40	1.39	17.58	0.85	8.28	4.80	0.13

Figures in the parenthesis are transformed values
In a column, means with similar letter (s) is/are not significantly different by DMRT at P ≤ 0.05

Table: 6 Reaction of genotypes for ToLCNDV under artificially inoculated condition

Genotypes	Inoculum	Time taken for symptom expression	Number of infected plants/agroinocuated plants	Per cent Disease Incidence	PCR of ToLCNDV DNA-A
DC-70 (V)	DNA-A+B+β satellite	28 dpi	0/15	0.00 (0.00^{g})	-
DC-70 (C)	-	38 DAS	0/15	0.00 (0.00^{g})	-
DC-773 (V)	DNA-A+B+β satellite	28 dpi	14/15	93.30 (17.79^{a})	+
DC-773(C)	-	38 DAS	5/15	40.00 (11.54^{d})	+
DC 769 (V)	DNA-A+B+β satellite	28 dpi	11/15	73.00 (15.68^{b})	+
DC 769 (C)	-	38 DAS	3/15	20.00 (8.13^{e})	+
P-85 (V)	DNA-A+B+β satellite	28 dpi	7/15	46.60 (12.47^{c})	+
P-85 (C)	-	38 DAS	1/15	6.66 (4.68^{f})	+
SE(d)	-	-	-	1.227	-
CD (P≤0.05)	-	-	-	2.602	-

Figures in the parenthesis are transformed values
In a column, means with similar letter (s) is/are not significantly different by DMRT at P ≤ 0.05
dpi- days post inoculation DAS- Days after sowing

Table: 7 Response of cucumber genotypes against ToLCNDV under artificial screening by agroinoculation.

Genotypes	Percent Disease Index	Coefficient of infection	Disease reaction	Symptoms
DC-70 (V)	0.00 (0.00[g])	0.00	Immune (I)	No visible symptoms and normal growth
DC-70 (C)	0.00 (0.00 [g])	0.00	Immune (I)	No visible symptoms and normal growth
DC-773 (V)	92.00 (73.57[a])	92.00	Highly Susceptible (HS)	Severe mosaic, yellowing, curling of leaves and stunting of the plants
DC-773(C)	29.87 (33.13[c])	22.40	Moderately Susceptible (MS)	Mosaic of young leaves, curling and puckering of leaves
DC 769 (V)	52.50 (46.43[b])	52.50	Susceptible (S)	Mosaic of young leaves, yellowing, distortion of leaves
DC 769 (C)	18.75 (25.66[e])	9.38	Moderately Susceptible (MS)	Mosaic of young leaves and slight yellowing of leaves
P-85 (V)	22.91 (28.60[d])	11.45	Moderately Resistant (MR)	Mosaic of young leaves
P-85 (C)	9.20 (17.66[f])	2.30	Resistant (R)	Mild mosaic of young leaves
SE(d)	1.19	-	-	-
CD (P≤0.05)	2.53	-	-	-

Figures in the parenthesis are transformed values
In a column, means with similar letter (s) is/are not significantly different by DMRT at P ≤ 0.05
dpi- days post inoculation DAS- Days after sowing

Fig:1. PCR amplification results of ToLCNDVDNA-Afrom agroinoculated cucumber plants usinguniversal primer pair of GKNDV DNA-A.

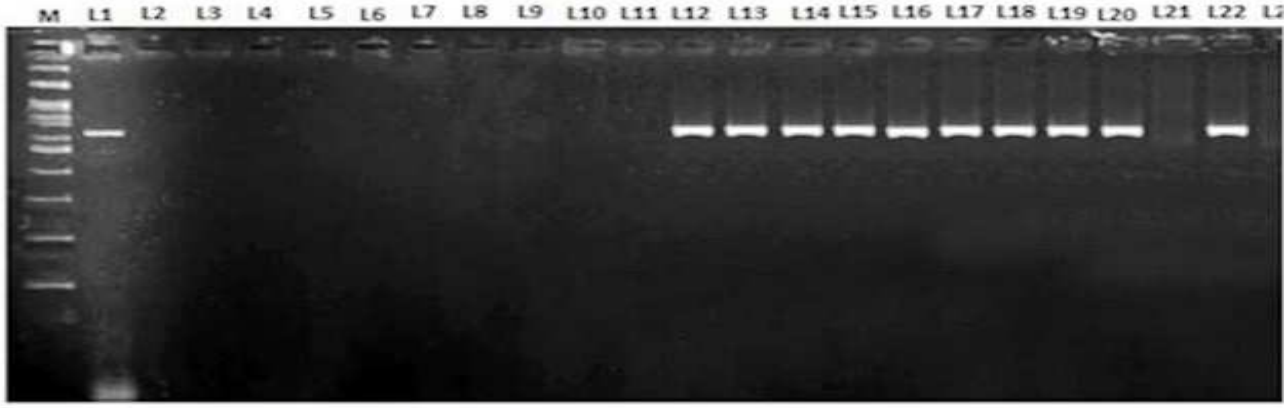


Fig:1 a. Lane M: 1kb ladder;L1: Positive control;L2: DC 70 control; L3-L11: DC 70 inoculated; L12: DC 769 control; L13-L19: DC 769 inoculated; L20: P-85 control; L21-L24:P-85 inoculated.

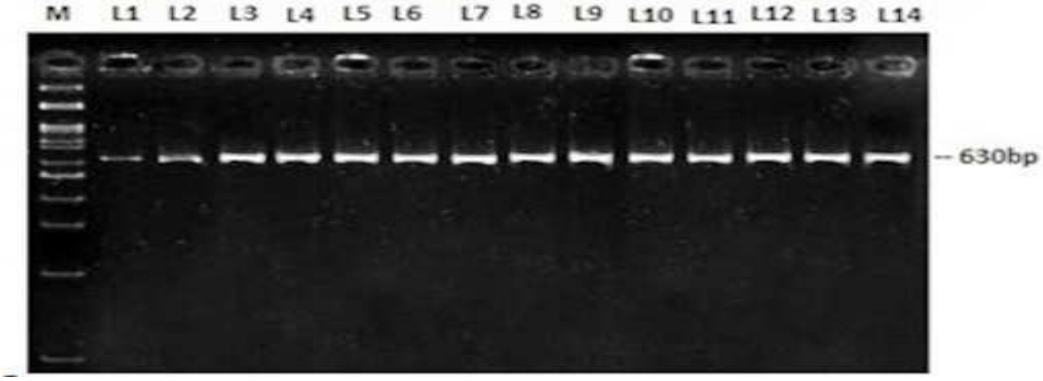
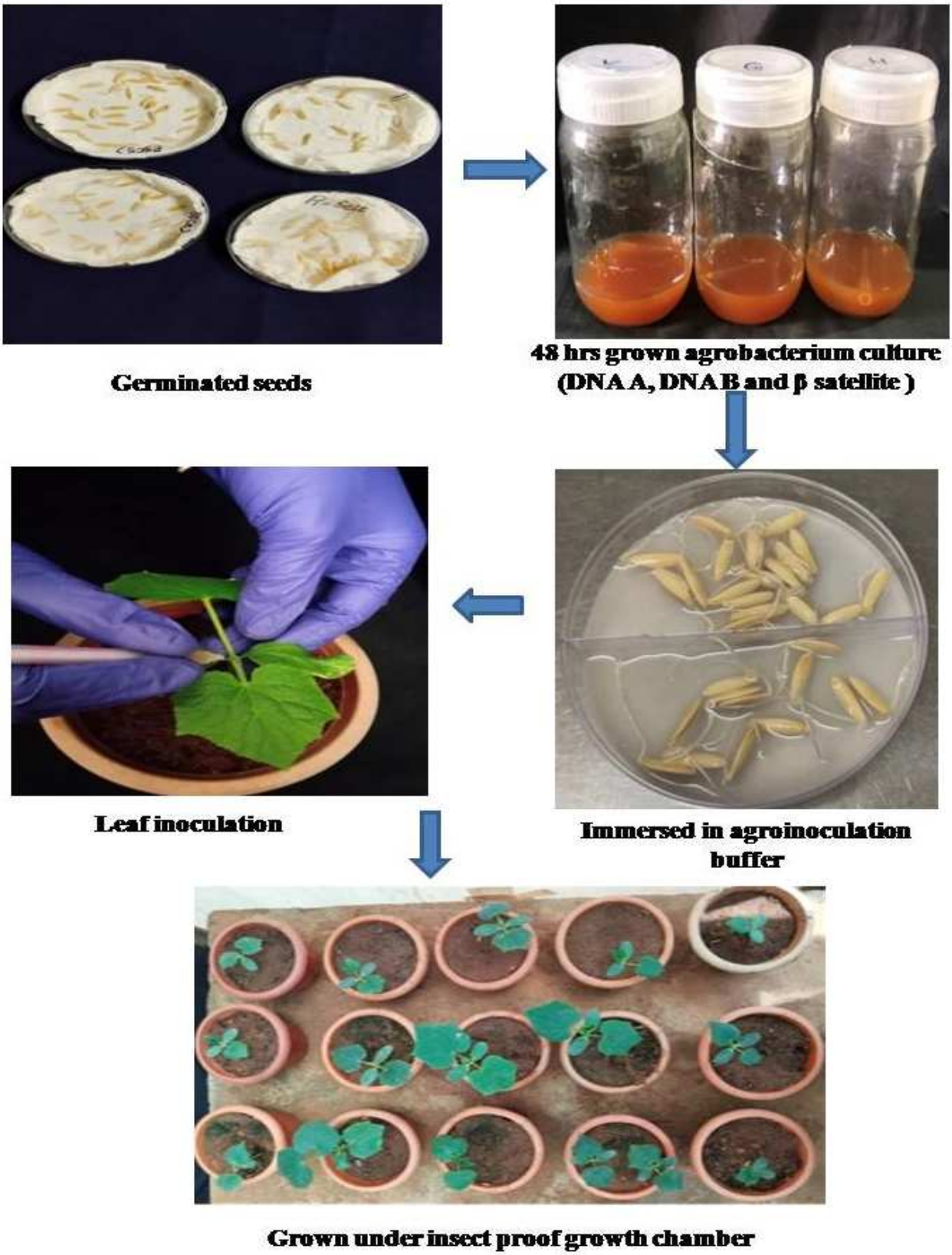


Fig 1: ToLCNDV agroinfectious clone on cucumber



Conclusion: Out of tested genotype DC 70 was found to be free from any visible symptoms of ToLCNDV under both field and artificial screening by agro-inoculation under controlled environmental conditions. The genotype of cucumber DC 70 has proved to be resistant to ToLCNDV. ToLCNDV is the major production constraint experienced by growers in many countries and commercial cultivars resistant to this virus are currently unavailable. DC 70 act as a good source of ToLCNDV resistance that can be introgrossed in to other cucumber cultivars. It may act as a possible source of ToLCNDV resistance that other cucumbers may inherit. The identified resistant genotype will serve as good candidate in breeding program for developing ToLCNDV resistant cultivars in cucumber.

Acknowledgment &Funding: would like to give my deepest thanks to National Agricultural Science Fund (NASF), Indian Council of Agricultural Research, KAB-I, Pusa Campus, New Delhi-110012 for funding to carry out this research

Author contribution: E. N.,- Conducting research work and article writing, V. R., - Chairmen, Seed collection and article correction, G. K., - Advisory committee member & Guide the ToLCNDV Screening, T. K. B., - Research mentor, M. K., - Advisory committee member& Crop Production guidance, D. R.,- Statistical analysis. All co-authors reviewed the final version and approved the manuscript before submission.

Conflicts of interest: The authors declare that there is no conflict of interest.

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ANNEXURE I

Weather data for the period from March to May 2023

Week	Temperature maximum (°C)	Temperature minimum (°C)	Relative Humidity (%)	Rainy Days
12	35.2	24.0	74	0
13	36.3	23.9	72	0
14	38.2	25.2	76	0
15	37.1	25.9	77	0
16	36.9	25.9	80	0
17	38.6	25.8	77	0
18	38.9	26.8	74	0
19	37.0	25.2	80	1
20	34.0	23.6	92	5
21	29.8	23.7	92	3
22	33.6	24.6	89	0
23	33.0	24.3	84	0
24	32.0	24.5	77	0
25	33	24.2	83	0
26	30	23.8	84	2
27	32.4	23.9	88	0

Weather data for the period from March to June 2024

Week	Temperature maximum (°C)	Temperature minimum (°C)	Relative Humidity (%)	Rainy Days
11	33.9	22.9	81	0
12	34.9	22.4	80	0
13	35.1	23.5	83	2
14	35	24.2	82	0
15	36	23.7	74	0
16	37	23.8	78	0
17	35.2	24.6	85	2
18	32.6	23.7	92	4
19	33.5	23.9	90	5
20	35.5	24.9	85	2
21	34.6	24.3	85	1
22	35.4	24.4	87	2
23	34.9	24.5	83	0
24	34.1	24.1	81	0
25	33.2	23.9	90	2
26	32.2	23.3	77	0