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Original Research Paper

A comprehensive survey on the occurrence of *Polerovirus* (*Solemoviridae*) causing yellowing disease on cucurbitaceous crops in Tamil Nadu, India

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ABSTRACT

A roving survey were conducted in and around Coimbatore, Dindigul, and Tiruchirappalli districts of Tamil Nadu in which commercially field grown bitter gourd, ridge gourd, pumpkin, sponge gourd, snake gourd and bottle gourd were observed with the virus symptoms *viz*., yellowing with interveinal chlorosis, downward rolling and mottling of leaves with stunted growth of plants. Sampling of symptomatic leaves from infected fields revealed an average yellowing disease incidence of 54.42% among the different cucurbitaceous crops. Molecular assay through reverse transcription-PCR (RT-PCR) using universal and specific primer pairs of *Polerovirus* was performed with the collected symptomatic leaf samples showed the association of polerovirus. In addition, samples tested for serological assay through dot immune binding assay (DIBA) using polyclonal antibody of *Cucurbit aphid-borne yellows virus* (CABYV) were also found positive. The study results revealed 81% and 100% of symptomatic samples were detected with Poleroviruses through RT-PCR and DIBA assays, respectively. The amplified DNA fragments of RNA dependent RNA polymerase (RdRp) gene and coat protein (CP) gene of *Polerovirus* associated with bitter gourd crop were cloned using pGEM-T vector and sequenced in both the orientations. Nucleotide sequencing analysis of RdRp and CP gene confirms the association of *Cucurbit aphid-borne yellows virus* (CABYV), a *Polerovirus* belonging to the family *Solemoviridae* sharing an identity of 96.34% and 100%, respectively. The phylogenetic analysis of RdRp genomic region of CABYV showed a single cluster with various isolates of Asian continent, whereas, CP genomic region exhibit minimal variation within the amplified region, grouping into single cluster with different isolates of Asian, North America, Europe, and South America. These results confirmed the alarming nature of *Polerovirus* occurrence, causing yellowing disease on cucurbitaceous crop in Tamil Nadu.

Keywords: Cucurbit crops, polerovirus, RT-PCR, serological assay

INTRODUCTION

Cucurbitaceous crops are economically important in India, where they are widely cultivated and consumed as vegetables. Worldwide, cucurbits are consumed in various forms, including mature fruits, whole immature fruits, and seeds. These plants, which are typically climbing herbs, thrive in tropical and subtropical regions. Cucurbit cultivation faces significant challenges due to viral infections and more than 70 different viruses reported to affect cucurbitaceous crops globally (Lecoq & Katis, 2014). A study conducted in India has documented a variety of viral infection in cucurbits, characterized by symptoms such as yellowing with interveinal chlorosis, downward curling, mottling of leaves and stunting. These symptoms are results from infections of *Polerovirus* (F: *Solemoviridae*), *Crinivirus* (F: *Closteroviridae*), *Zucchini yellow mosaic virus* (F: *Potyviridae*), and *Begomovirus* (F: *Geminiviridae*) (Nagendran et al., 2015 & 2017; Kumari et al., 2021 & 2022; Krishnan et al., 2022b).

The genus Poleroviruses have single-stranded RNA genomes with a linear structure and they are restricted to the phloem. They are exclusively transmitted by different aphid species through a circulative and non-propagative manner (Mayo et al., 2000). The yellowing symptom of the disease is marked by phloem necrosis, streaking, yellowing, leaf rolling and thickening, as well as stunting. Worldwide, eight species of *Polerovirus* have been identified to infect

broad range of cucurbit crops (Knierim et al., 2014; Kidanemariam et al., 2019; Kumar et al., 2022). *Polerovirus* infections displaying symptoms have been reported to cause fruit yield reductions ranging from 10% to 100%, while, leaving fruit quality un-affected (Relevante et al., 2012; Desbiez, 2019). In India, the emergence of yellowing symptoms on bitter gourd caused by *Polerovirus* was first documented with the identification of cucurbit aphid-borne yellows virus (CABYV) (Suveditha et al., 2017). Later, Krishnan et al. (2022a) characterized the first complete genome of the CABYV isolates infecting squash and watermelon in India. In the present study, a comprehensive survey was conducted to document the incidence of *Polerovirus* associated with the yellowing disease in different cucurbit crops cultivated in Tamil Nadu through molecular and serological assays.

MATERIALS AND METHODS

Collection of plant materials

A roving survey was conducted during the major cropping season of 2023-24 in Coimbatore, Dindigul, and Tiruchirappalli districts of Tamil Nadu, India, focusing on cucurbitaceous crops such as bitter gourd, ridge gourd, pumpkin, sponge gourd, snake gourd, and bottle gourd. The survey aimed to assess the per cent disease incidence (PDI) and occurrence of poleroviruses in these crops during different growing seasons. Leaf samples showing yellowing virus symptoms were collected and evaluated. The disease incidence was calculated based on the proportion of symptomatic plants observed in relation to the total number of plants in each field (Table 1). In total, 69 symptomatic leaf samples were collected along with

Table 1 : Detection of *Polerovirus* causing yellowing disease in cucurbits samples

Surveyed location	Crop	Disease incidence (%)	No. of samples	Number of samples positive *Polerovirus* primer		DIBA
				Genus specific primer	CABYV specific primers (CABYVUp/ CABYVDo)	
Coimbatore						
Orchard, TNAU	Bittergourd	92	4	4	3	4
	Pumpkin	60	2	2	2	2
	Sponge gourd	34	3	2	2	3
Kuppepalayam	Bitter gourd	78	9	8	6	9
	Ridge gourd	65	8	6	3	8
	Snake gourd	45	3	3	1	3
	Bottle gourd	34	4	3	3	4
Thappattai	Bitter gourd	60	8	7	5	8
Kilavan Pudur	Ridge gourd	75	7	7	4	7
Gomangal ampudur	Bitter gourd	80	6	4	4	6
Total			54	46	33	54
Dindigul						
Mangarai	Bitter gourd	48	6	3	3	6
Dindigul	Ridge gourd	42	4	3	2	4
Total			10	6	5	10
Tiruchirappalli						
Allithurai	Ridge gourd	25	3	2	2	3
	Sponge gourd	24	2	2	2	2
Total			5	4	4	5
Grand total			**69**	**56**	**42**	**69**

Table 2 : Primer details used for the detection of poleroviruses associated with cucurbit yellowing disease

Primer ID	Sequence (52 -32)	Target gene/region[a]	Amplicon size (bp)	Reference
Pol-Gen-Up2	GATGARGGTCGYTACCG	RdRp	600	Lotos et al. (2014)
Pol-Gen-Down2	ACCTCGACTTTRAARCC			
CABYVUp	GTCCGAAACCGCCTGACGC	CP	294	Lotos et al. (2014)
CABYVDo	TCGAGGTTCGAGCAAGCTG			

[a]RdRp: RNA dependent RNA polymerase; CP: coat protein

apparently healthy samples from various farmer holdings. These samples were then brought to the Plant Virology Laboratory and were stored at -80^0 until further analysis.

RT-PCR based detection of *Polerovirus*

A quantity of 100 mg infected leaf samples was used to extract total RNA using TRI reagent (Sigma Aldrich, USA) as per the manufacturer's instruction. Two-step RT-PCR assay was performed for the molecular detection of Poleroviruses species associated with the yellowing disease using previously reported primer pairs (Table 2). In the first step, extracted the total RNA to synthesis cDNA using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. In the second step, PCR was performed with the cDNA as template using the *Polerovirus* universal and CABYV specific primer pairs. After PCR assay, the PCR products were electro phorizedin 1.2% agarose gels stained with ethidium bromide and documented with UV transilluminator (UVITEC Cambridge, UK).

Cloning, sequence analysis and phylogeny

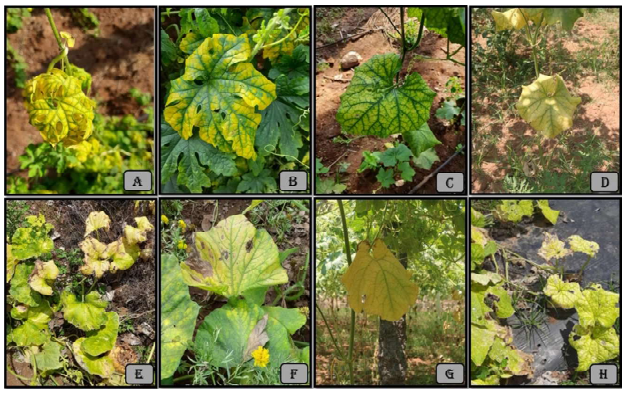
The amplified DNA fragments of RdRp and CP gene from a representative sample of bitter gourd were separated under agarose gel electrophoresis and purified the gel using QuickGel extraction kit (Qiagen, Germany) and were cloned into pGEM-T easy vector system (Promega, Madison, Wisconsin, USA) and sequenced in both the directions with M/s Biokart Private Limited, Bangalore. Further, Basic local alignment search tool (BLASTn) was used to analyse the nucleotide sequences for identification of virus associated at species level. The resultant sequences of RdRp and CP was performed using the Molecular evolutionary genetics analysis (MEGA 11) software, which is integrated with the MUSCLE algorithm (Kumar et al., 2018). For phylogenetic tree construction, homologous sequences were retrieved from Genbank and incorporated. Further, estimation of phylogenetic relationship of virus species was done through the neighbour joining (NJ) method with the 1000 bootstrap replications.

Serological detection of *Polerovirus* through dot immuno binding assay (DIBA)

According to the protocol outlined by Dijkstra et al. (1998) and Ali et al. (2012), the infected leaf samples were homogenized with an extraction buffer [TBS (0.02M Tris 2.42 g; 0.5 M NaCl 29.24 g; distilled water 1 L; pH 7.5) + 50 mM DIECA] at a 1:10 w/v ratio. Clarified sap from leaf samples were blotted onto a PVDF Transfer Membrane (Immobilon® PSQ, Millipore). The membrane was then blocked and incubated with primary and secondary antibodies specific for CABYV (DSMZ, Germany). Finally, the membrane was fixed as per the protocol. The color development was assessed by visually inspecting the membrane with the naked eye.

RESULTS AND DISCUSSION

During 2023-2024, six cucurbitaceous crops such as bitter gourd, ridge gourd, pumpkin, sponge gourd, snake gourd and bottle gourd crops showing yellowing symptoms with interveinal chlorosis leaving the veins green, downward rolling, chlorotic patches, mottling along with the presence of aphid and stunted plant growth were observed (Fig.1). The highest disease incidence of yellowing was observed in bitter gourd (92%), while, the lowest incidence was recorded in sponge gourd (24%) (Table 1). A total number of 69 symptomatic leaf samples were collected from four different locations in Coimbatore, two locations in Dindigul and one location in Tiruchirappalli districts of Tamil Nadu.

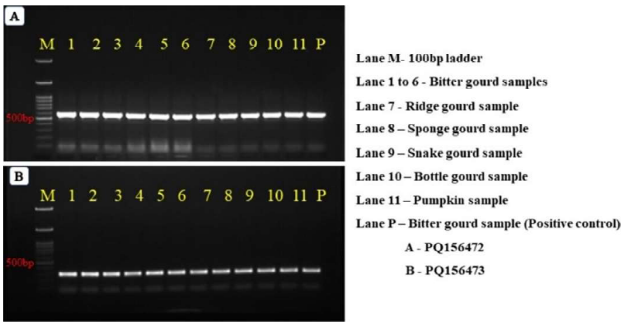


Leaves showing A & B; yellowing with downward rolling and interveinal chlorosis of bitter gourd; C: interveinal chlorosis with downward rolling of ridge gourd; D: leathery with interveinal chlorosis in snake gourd; E: yellowing in pumpkin; F: interveinal chlorosis in bottle gourd; G: yellowing in sponge gourd; and H: yellowing with leathery leaves of ridge gourd

Fig. 1 : Symptoms variation of yellowing disease associated with cucurbitaceous crops of Tamil Nadu

In this study, yellowing disease caused by the genus *Polerovirus* was identified through molecular and serological assays. In recent years, the report on the rapid spread of yellowing disease in cucurbitaceous crops had played a significant role in declining the production leading to huge economic losses. The global spread of yellowing disease may be attributed to the widespread presence of aphid vectors, which facilitate the disease's transmission across different regions. Previously, occurrence and molecular characterization of poleroviruses such as CABYV, MABYV and LABYV were reported in water melon, cucumber, bitter gourd, squash, teasel gourd, musk melon, ivy gourd, snake gourd, satputia, ridge gourd, bottle gourd, ash gourd and sponge gourd from India (Krishnan et al. 2022a & 2022b; Tripathi et al. 2023; Nagendran et al., 2023; Verma et al., 2023).

Out of 69 isolates with yellowing symptoms, 56 samples (81.11%) have produced the expected amplicon of ~600 bp in RT-PCR assay with the Pol-Gen-Up2/Pol-Gen-Down2 primer pair corresponding to RdRp gene of poleroviruses. Whereas only 42 samples (60.86%) have successfully yielded desired amplicon of ~300 bp with CABYVUp/CABYVDo primer pair, which targets the coat protein region of CABYV (Fig. 2). Similarly, Nagendran et al., (2023) has characterized CABYV and LABYV isolates infecting cucurbits in Uttar Pradesh state (India) withPol-Gen-Up2/Pol-Gen-Down2. Further, serological detection through DIBA was performed using polyclonal antibody of CABYV in 1:1000 dilution. A clarified sap of bitter gourd, ridge gourd, pumpkin, sponge gourd, snake gourd and bottle gourd crops were blotted on the membrane. DIBA assay reacted positive in all the symptomatic samples with the polyclonal CABYV antibody, resulting in a violet colour at the membrane spots, whereas no color change was observed in the apparently healthy samples of the same crops (Fig. 3).



A: RT-PCR amplification of *Polerovirus* infected different cucurbit crop samples using universal primer pair (Pol-Gen-Up2/Pol-Gen-Down2); and B: PCR amplification using CABYV specific primer pair (CABYVUp/CABYVDo)

Fig. 2 : Detection of *Polerovirus* causing yellowing disease in cucurbitaceous crops

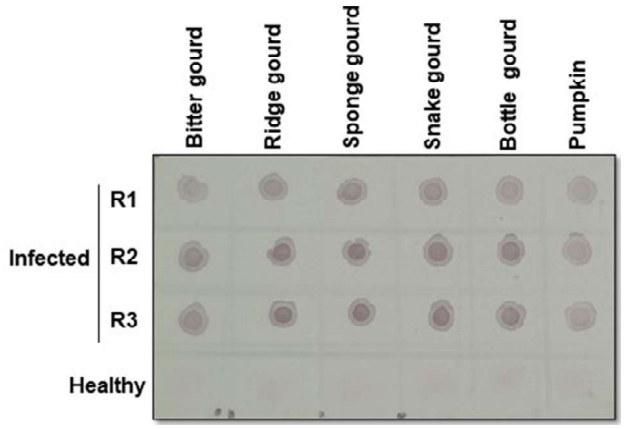


Fig. 3 : Dot immunobinding assay with leaf samples of cucurbitaceous crops

Despite the high conservation of the nucleotide region in the P2 gene, which encodes the RNA-dependent RNA polymerase in the *Polerovirus* genus, the reduced detection efficiency observed between the primer pairs. Lack of primer specificity, phloem-limited nature of the *Polerovirus* and low titer of virus in the infected tissues might have compromised the effectiveness of

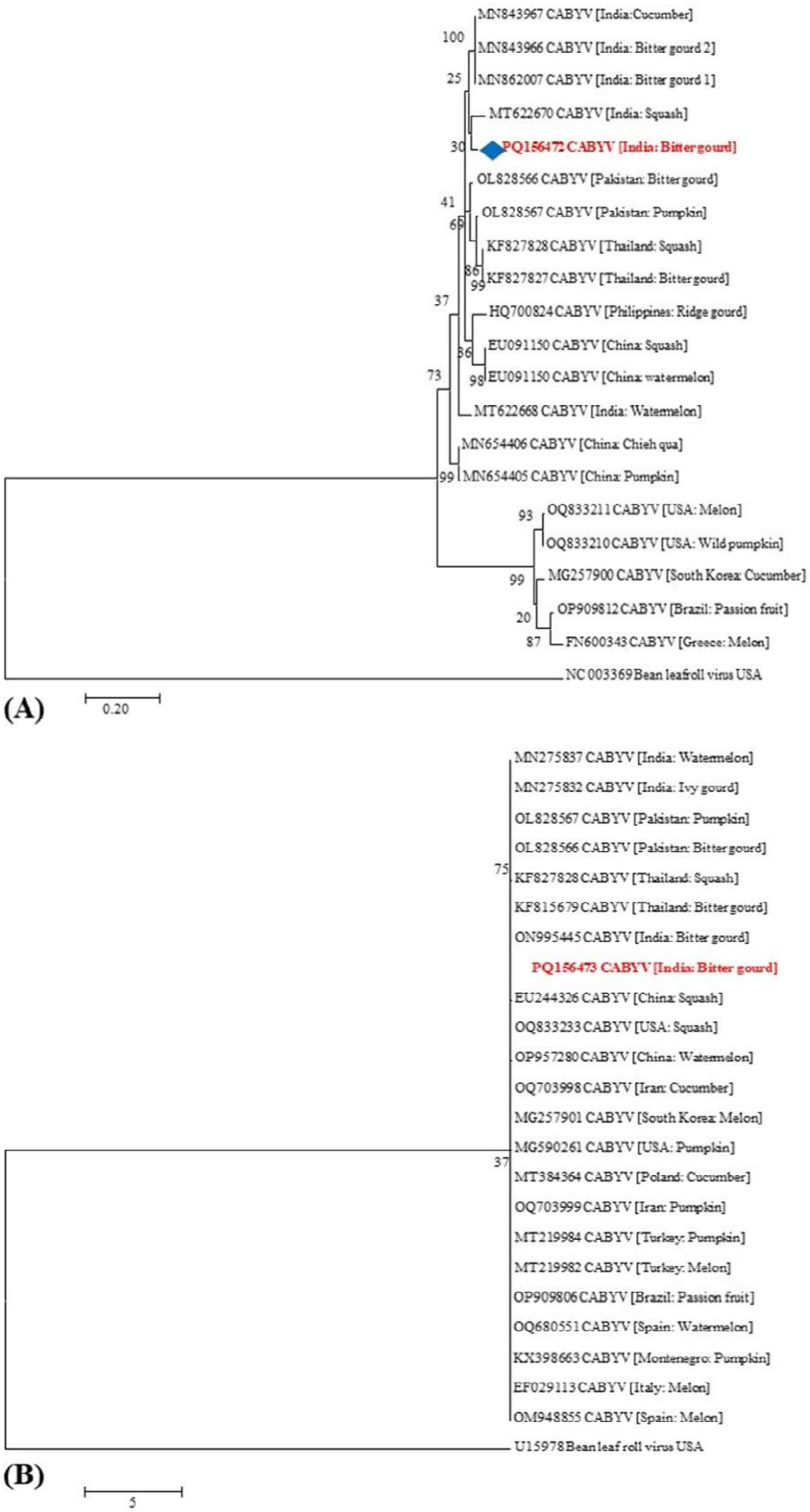


Fig 4 : Phylogenetic relatedness of polerovirus genomic region, RdRp (A) and CP (B) associated with yellowing disease of bittergourdin India with the previously reported isolates

these molecular assays (Tourrette et al., 2021; Nagendran et al., 2023; Kumari et al., 2021). Overall, the serological assay detected *Polerovirus* among 100% samples, whereas, RT-PCR assay detected among 81.11% of samples.

As a representative of all the samples, the RdRp and CP fragments of bitter gourd sample were cloned into pGEM-T vector and sequenced. In sequence analysis, nucleotide sequence of RdRp of bitter gourd (PQ156472) showed highest identity of 96.34% with previously reported CABYV isolate (squash) from Uttar Pradesh, India (MT622670). Similarly, the nucleotide sequence of CP region of bitter gourd (PQ156473) showed the highest identity of cent per cent with previously reported CABYV isolate (water melon) from Karnataka, India (MN275837). The phylogenetic analysis was performed using homologous sequence of CABYV corresponding to RdRp and CP genomic regions with the respective sequences were retrieved from NCBI database. The phylogenetic tree of RdRp genomic region of CABYV showed less genetic distances among the different isolates of Asian continent and was grouped under single cluster, whereas isolate from other countries of North America, East Asia, South America, and Europe continents were formed separate cluster. Similarly, the CP sequences grouped with all other isolates of CABYV with less genetic distance among different isolates of Asian, North America, Europe, and South America and were grouped under single cluster (Fig 4). Results from these molecular and serological assays confirms the association of *Polerovirus* causing yellowing disease with cucurbitaceous crops in Tamil Nadu.

Kumari et al. (2021) predicted a severity of 50-89% of *Polerovirus* incidence with the emerging yellowing disease in cucurbitaceous crop through the risk map generated using ArcGIS tool in the region Varanasi and Mirzapur district of Uttar Pradesh, India. The severity of polerovirus infections can be exacerbated by viruliferous aphids, which transmit the virus persistently, can travel up to 1000 km through the wind (Zeyen et al., 1987). The absence of reliable detection methods is hindering the characterization of various Indian isolates of poleroviruses. Developing robust serological and molecular detection techniques is essential, as they would enable virus detection at the field level and facilitate early identification of the virus. Future efforts will focus on examining genome diversity, developing sensitive molecular and serological detection methods, investigating potential virus reservoirs, and devising management strategies that address both the virus and its vectors. Additionally, work on developing resistant or tolerant plant lines by assessing and selecting from wild varieties should also be focused.

CONCLUSION

Yellowing disease associated with cucurbitaceous crops *viz*., bitter gourd, ridge gourd, pumpkin, sponge gourd, snake gourd and bottle gourd in Coimbatore, Dindigul and Tiruchirappalli districts of Tamil Nadu were identified as strain of CABYV. However, to gain a more comprehensive understanding of the species, strains, and genomic variations of *Polerovirus*, further surveys across various agro-climatic zones in Tamil Nadu are necessary. A detailed investigation into the genomic diversity of poleroviruses will aid in developing effective diagnostic tools for these poleroviruses affecting cucurbitaceous crops in India. Future, research should focus on geographical distribution, transmission pattern of virus, host range and divergence of *Polerovirus* species as well as different symptom expression and their economic impact. This understanding will drive the developing of eco-friendly, effective, and robust management strategies for poleroviruses infecting cucurbitaceous crops.

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