3 Biotech (2023) 13:178
https://doi.org/10.1007/s13205-023-03606-y

ORIGINAL ARTICLE

Identification and characterization of a novel begomovirus, *Withania leaf curl virus* associated with leaf curl disease of *Withania somnifera*

Asifa Khan[1] · Sana Tabanda Saeed[1] · Soumya Sinha[1] · Sujata Singh Yadav[1] · Abdul Samad[1]

Received: 9 December 2022 / Accepted: 3 May 2023 / Published online: 12 May 2023

Abstract

Begomovirus is the largest genus of the family Geminiviridae with wide host range and responsible for a considerable amount of economic damage to many important crops globally. *Withania somnifera* (Indian ginseng) is an important medicinal plant with high demand in pharmaceutical industries worldwide. During the routine survey in 2019, typical characteristic viral symptoms such as severe leaf curling, downward rolling of the leaves, vein clearing, and poor growth of Withania plants with 17–20% disease incidence were observed in Lucknow, India. Typical symptoms, abundant presence of whiteflies, PCR and RCA based detection indicated the amplification of ~ 2.7 kb and suspected the causal pathogen to be a begomovirus, associated with a betasatellite (~ 1.3 kb). Transmission electron microscopy revealed the presence of twinned particles of ~ 18–20 nm in diameter. Full genome sequencing (2758 bp) of the virus and its analysis showed only 88% sequence identity with the begomovirus sequences present in the database. Hence, based on the nomenclature guidelines we concluded that the virus associated with the present disease of *W. somnifera* is a novel begomovirus and its name is proposed as *Withania leaf curl virus*.

Keywords Begomovirus · *Withania somnifera* · Phylogenetic analysis · *Bemisia tabaci*

Introduction

Withania somnifera (Syn: ashwagandha, suranjan, punir, winter cherry, Indian ginseng) is an evergreen, xerophytic, woody, short, perineal shrub that belongs to the family Solanaceae. It is naturally found in drier regions ranging from the Mediterranean through tropical Africa through South Africa and from the Canary and Cape Verde Islands to the Middle East and Arabia, Afghanistan, Baluchistan, Pakistan, Sri Lanka, China, Nepal and India. In India, it is extensively distributed in Uttar Pradesh, Punjab, Maharashtra, West Bengal, Gujarat and Rajasthan (Kulkarni and Dhir 2008). This plant is a rich source of some important secondary metabolites like, steroids, alkaloids, flavonoids, phenolics, saponins, and glycosides, etc., which are widely used in traditional medicine (Saleem et al. 2020). It has broad array of pharmacological properties, such as an immunomodulatory, anti-oxidant, anti-inflammatory, antimicrobial, antistress, antitumor, neuroprotective, cardioprotective, antihypertensive, and antidiabetic activities (John 2014). In the last 2 decades, an active component of Withaferin A (WFA) has shown tremendous cytotoxic activity suggesting its potential as an anti-carcinogenic agent in the treatment of several cancers and Alzheimer's disease (Dutta et al. 2019). The chemical profile and pharmacological activities of *W. somnifera* have been extensively reported. Several clinical studies have reported its safety for use in humans (Saggam et al. 2021). Its herbal formulations are available in the market, named as, ashwagandharishta, and stresswin.

Begomovirus is a genus of plant viruses belongs to the family Geminiviridae. It has exhibited its potential prevalence from ornamental, vegetable, agricultural to medicinal and aromatic plants (MAPs) (Brown et al. 2015). The transmission of begomovirus is carried out generally by whiteflies (Basak 2016). Its genome consists of closed, single-stranded, circular DNA. On the basis of presence of one or two genomic components, they are divided into monopartite

Asifa Khan and Sana Tabanda Saeed contributed equally to this work.

✉ Abdul Samad
samad_cimap@yahoo.co.in

[1] Plant Pathology Department, CSIR-Central Institute of Medicinal and Aromatic Plants, P.O. CIMAP, Lucknow 226015, India

(DNA-A) or bipartite (DNA-A and DNA-B) begomoviruses (Fauquet et al. 2008). DNA-A virion-sense strand encodes two open reading frames (ORFs) (AV1 and AV2) and four in the complementary sense (AC1, AC2, AC3 and AC4) and five ORFs (AC1, AC2, AC3, AC4 and AC5) in New World and Old World begomoviruses, respectively. DNA-B encodes two ORFs, BV1 and BC1 in virion-sense and complementary-sense strands, respectively. Both the genomic components (DNA-A and DNAB) share 200 nt common region (CR), which is present within the intergenic region, where replication origin is present. Begomoviruses are often found to be associated with satellite DNA molecules such as betasatellites, alphasatellites and/or deltasatellites (Lozano et al. 2016). Recent reports have shown the growing incidences and threatening effect of begomoviruses infection on medicinal and aromatic plants (Saeed and Samad 2017).

Withania somnifera has been often reported to be infected by fungal diseases such as *Alternaria dianthicola*, *Pseudocercospora fuligena*, *Choanephora cucurbitarum*, etc. (Maiti et al. 2007; Saroj et al. 2012, 2014). The carmine red spider mite (*Tetranychus urticae*) and mealybug species *Phenacoccus solenopsis* are the most prevalent pests found on this plant in India (Sharma et al. 2012; Sharma and Pati 2013). However, very few reports of begomoviruses infection on *Withania somnifera* have been found so far. Tobacco leaf curl virus (Phatak and Raychaudhuri 1967), Eggplant mottled dwarf virus (Al-Musa and Lockhart 2008) and Jatropha mosaic India virus (Baghel et al. 2012) have been reported to be infecting *W. somnifera*. Earlier reports were based on partial sequence and incomplete characterisation of the associated viruses and symptoms were quite different in contrast to present disease.

Typical symptoms like downward leaf curling, rolling, leaf deformation, vein clearing, and infestation of whiteflies were observed for the first time on the leaves of *W. somnifera* in the experimental fields of CSIR-Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India. The identification and molecular characterization of the pathogen responsible for the disease associated with *W. somnifera* is described in the present study.

Materials and methods

Sample collection

During the survey, in October–November, 2019, *W. somnifera* leaves exhibiting characteristic viral symptoms were collected from the experimental field of CSIR-CIMAP, Lucknow, India. Ten symptomatic and three asymptomatic (healthy) samples were taken from different corners of the fields for downstream analysis.

Sap inoculation

Mechanical sap inoculation on healthy *W. somnifera, Nicotiana benthamiana, N. glutinosa and Chenopodium amaranticolor* seedlings were done using pre dusted carborundum leaves with the sap obtained by naturally infected leaf tissue ground in the presence of chilled phosphate buffer in an autoclaved pestle and mortar. Just after inoculation, leaves were gently washed with distilled water. Plants inoculated with phosphate buffer only served as mock control. All the plants were kept under glasshouse at 25 ± 2 °C for the development of symptoms.

Transmission electron microscopy

Healthy and infected leaf samples were processed for the electron microscopy as described by Garg et al. (2001), and preparations were negatively stained in 2% w/v aqueous uranyl acetate. The grids were dried and screened under a JEOL-100S transmission electron microscope at 80 kV.

Whitefly transmission

For transmission studies, healthy, starved indigenous B-biotype whiteflies (taken from CSIR-NBRI, Lucknow) were used for acquisition feeding (24 h) on infected *W. somnifera* leaves and then transferred to healthy plants (10/plant) for an overnight inoculation feeding. After inoculation feeding, whiteflies were killed by spraying mild (0.01%) insecticide/acaricide (OMITE-Propagite 57% EC). The plants were kept under glasshouse for further observations.

Molecular characterization

Total genomic DNA was isolated from symptomatic and asymptomatic leaves of *W. somnifera* using CTAB method as described by Khan et al. (2007). PCR was performed in 25 µl PCR mixture containing 2.5 µl 10X buffer, 0.8 µl of each primer (10 µM), 1 µl of 10 mM dNTPs, 0.8 µl of DNA templates (30 ng), 18.8 µl of Milli Q and 0.3 µl of *Taq* DNA polymerase (3 U/µl) using begomovirus CP-specific primers (Hallan et al. 1998) for initial screening of begomovirus infection in the respective samples. Amplified PCR products were processed for the whole genome amplification. Two sets of overlapping primers K1F/K1R and K2F/K2R (Kumar et al. 2011) and universal primers for the detection of DNA-B and alphasatellite components (Rojas et al. 1993) were used. Primer pair β01/β02 was used to detect the presence of betasatellite (Briddon et al. 2002). Amplified PCR

products were cloned and sequenced in CSIR-CIMAP using ABA sequencer 3730 (Applied Biosystems).

Rolling Circle Amplification (RCA) was performed using (RCA, Templiphi GE Healthcare, USA) to amplify the full-length genome of the begomoviruses (Inoue-Nagata et al. 2004). The RCA product was digested independently with various restriction enzymes (*Bam*HI, *Hinc*II, *Sal*I, *Kpn*I, *Pst*I, and *Xba*I) to linearize full length genomic DNA. The restricted fragments were resolved on a 1% agarose gel.

Sequences were analysed using BLASTn and deposited to Genbank database. Phylogenetic analysis was carried out with representative sequences of begomoviruses available in the NCBI database using the Neighbour Joining method in MEGA 7 software with 1000 bootstrap value (Kumar et al. 1994).

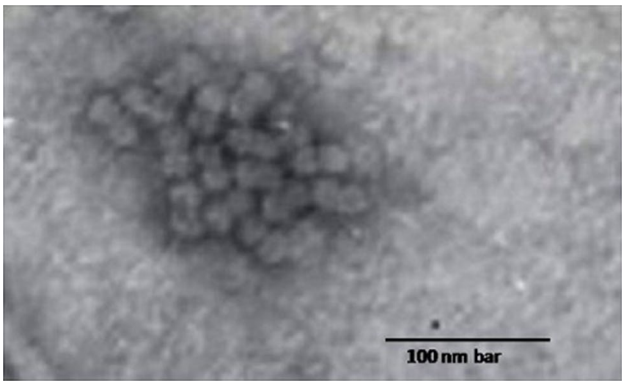

Fig. 2 TEM image showing twinned icosahedral virus particles (~ 18–20 nm in diameter) negatively stained with uranyl acetate in infected samples (Bar-100 nm)

Results and discussion

During the survey, infected plants showed typical severe downward leaf curling, rolling, vein clearing, wilting, leaf deformation and poor growth (Fig. 1). Based on the symptoms and heavy infestation of whiteflies, infection of begomoviruses was suspected. Mechanically inoculated *W. somnifera* and other plants did not express any visible local or systemic symptoms on the leaves and all were also found negative in PCR tests. Disease incidence was estimated about 17–20 per cent on the basis of visual plant population.

Partially purified virus preparations from the infected samples were observed under TEM; 10–15 windows were screened and revealed the presence of scattered twinned icosahedral particles of ~ 18–20 nm in diameter (Fig. 2).

B. tabaci-based transmission was found positive in five of the eight *Withania* plants, and developed symptoms similar to those observed on the naturally infected plants in the fields.

PCR amplicon of ~ 771 bp was obtained using coat protein specific primers in eight out of ten symptomatic samples, whereas no amplification was obtained in asymptomatic samples. Samples from which CP gene was amplified were used for further detection of genomic and satellite components of begomoviruses. Genomic DNA-A fragment was amplified in two parts of ~ 1200 bp and ~ 1700 bp via PCR (Fig. 3) and find out the actual length of the DNA segment by removing excess bases. However, presences of DNA-B and alphasatellite components were not detected by PCR. Betasatellite of 1376 bp was detected using primers β01/β02.

Fig. 1 **a** Healthy plants of *W. somnifera* and **b** infected plants of *W. somnifera* exhibiting vein clearing, severe leaf curling and rolling symptoms in the field condition

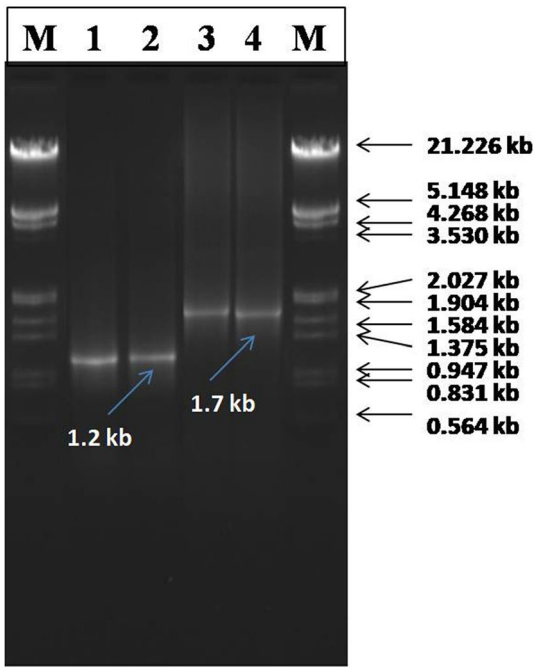

Fig. 3 PCR amplification of the full genome of DNA-A using primers K1F/K1R and K2F/K2R generating two overlapping fragments of ~ 1.2 kb and ~ 1.7 kb, respectively, from infected *W. somnifera* samples. M- λ *Eco*RI/ *Hind* III DNA marker, Lanes 1 and 2—PCR products of ~ 1.2 kb and Lanes 3 and 4—PCR products of ~ 1.7 kb

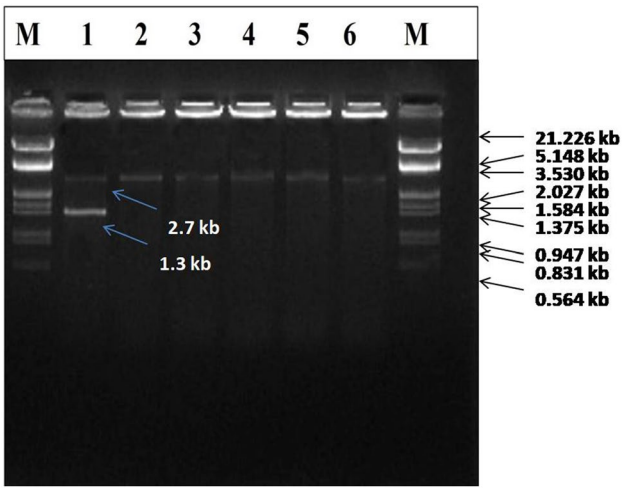

Fig. 4 Restriction profile of RCA product (generated from DNA isolated from infected *W. somnifera* samples), digested with different restriction enzymes. M- λ *Eco*RI/ *Hind* III DNA marker, Lane 1—*Hinc*II, Lane 2—*Bam*HI, Lane 3—*Kpn*I, Lane 4—*Pst*I, Lane 5—*Sal*I and Lane 6—*Xba*I

The presence of a begomovirus and associated betasatellite was also validated by rolling circle amplification. The RCA products restricted with *Hinc*II showed the presence of two fragments of 2.7 kb and 1.3 kb corresponding to DNA-A and betasatellite of begomovirus while *Bam*HI, *Kpn*I, *Pst*I, *Sal*I and *Xba*I generated 2.7 kb fragment only, suggesting these enzymes have single restriction site in DNA A and no restriction site in betasatellite (Fig. 4).

Sequences of DNA-A (2758 bp) and betasatellite (1347 bp) were deposited to NCBI Genbank database with the accession numbers OP617239 and OP617240, respectively. The genome organization is found to be of Old World monopartite begomoviruses consist of six ORF's, two on virion sense strand and four on complementary strand, interspaced by an IR (Intergenic region). The viral sense strand has ORF AV1 (encoding CP) which is partially overlapped by the small ORF AV2 (Pre- CP), while the complementary sense strand contains ORFs AC1 (Rep), AC2 (TrAP), AC3 (REn), and AC4. BLASTn analysis of DNA-A has shown maximum similarity to *Cherry tomato leaf curl virus* (LN906594) with 88% sequence identity; suggesting it to be a novel begomovirus. Based on the nomenclature guidelines for *Begomovirus* (Brown et al. 2015), we concluded the present virus to be a novel begomovirus for which we proposed the name *Withania leaf curl virus*. Phylogenetic analysis revealed that since *Withania leaf curl virus* (Acc.no OP617239) is a new virus, its DNA-A sequence formed a separate clade and it shared a common ancestor with *Cherry tomato leaf curl virus* (LN906594) (Fig. 5). Betasatellite sequence showed 98% identity with *Ageratum yellow vein betasatellite* (KF364485) reported from Lucknow, India and shared the same clade of the phylogenetic tree (Fig. 6).

The infection of *W. somnifera* by Tobacco leaf curl virus (Phatak and Raychaudhuri 1967), Eggplant mottled dwarf virus (Al-Musa and Lockhart 2008) and Jatropha mosaic India virus (Baghel et al. 2012) are the few reports with partial sequences and that have not been fully characterised are available in the literature. However, this is the first natural occurrence of a novel begomovirus, *Withania leaf curl virus* along with *Ageratum yellow vein betasatellite* associated with severe leaf curling, rolling and vein clearing on *W. somnifera*. It is one of important medicinal herb of versatile use and mostly people preferred to keep in the house, garden and also cultivated in the commercial fields. The present and earlier reported viruses are spreading in nature by vectors and causing significant yield reduction; therefore, their management strategies should be implemented using available findings to protect other economically important crops including medicinal and aromatic plant.

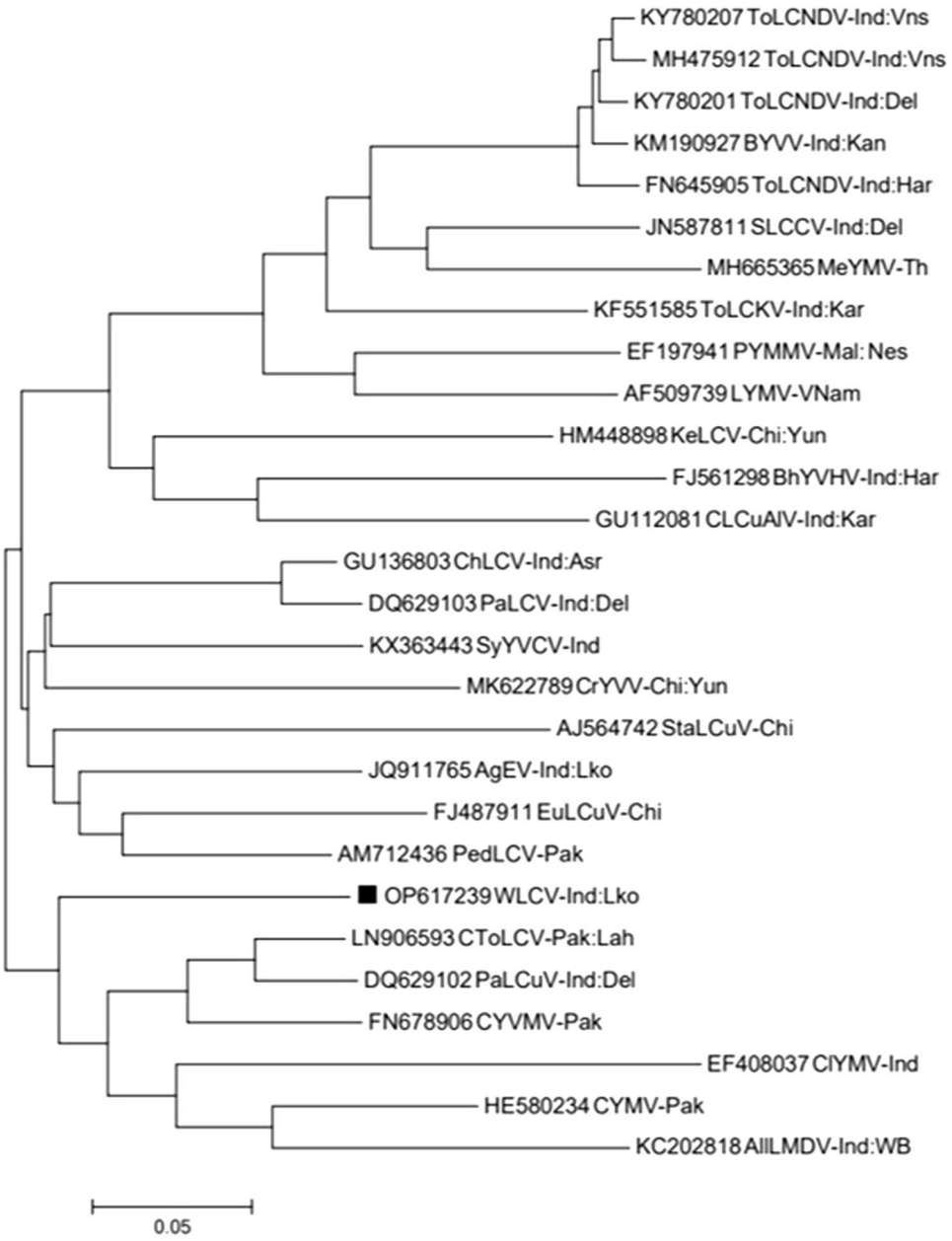

Fig. 5 Phylogenetic relationship based on nucleotide sequence of DNA-A of begomovirus isolate (OP617239) infecting *W. somnifera* with different begomoviruses using MEGA 7.0 neighbour joining tree method with 1000 bootstrap value. *Withania leaf curl virus* (Acc. no OP617239) forms a separate clade and it shares a common ancestor with *Cherry tomato leaf curl virus* (LN906594)

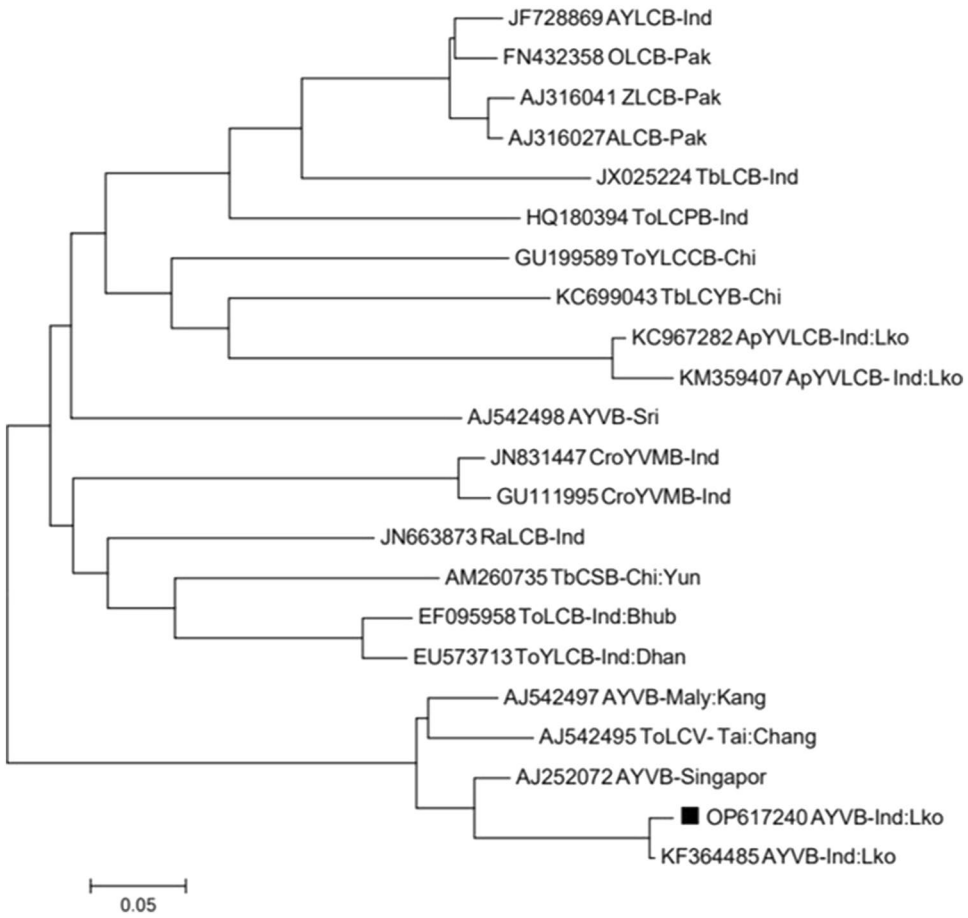

Fig. 6 Phylogenetic relationship based on nucleotide sequence of betasatellite DNA of begomovirus isolate (OP617240) infecting *W. somnifera* with different begomoviruses using MEGA 7.0 neighbour joining tree method with 1000 bootstrap value. Betasatellite sequence has maximum identity with *Ageratum yellow vein betasatellite* (KF364485) reported from Lucknow, India, and shares the same clade in the phylogenetic tree

Acknowledgements Authors are thankful to the Director, CSIR-CIMAP, Lucknow, for the necessary facilities, and DST-New Delhi for INSPIRE-SRF to Soumya Sinha.

Author contributions STS and AK did molecular experiments and data analysis. SS and SSY helped in computational analysis and manuscript preparation. AS overall monitored the experiments and compilation of manuscript.

Declarations

Conflict of interest There are no conflicts of interest to declare.

Human and animal studies This article does not contain any research involving human or animal participants.

Accession numbers Withania leaf curl virus, DNA-A: OP617239 (GenBank). Ageratum yellow vein betasatellite: OP617240 (GenBank).

References

Al-Musa AM, Lockhart B (2008) Occurrence of Eggplant mottled dwarf virus in Jordan. J Phytopathol 128:283–287

Baghel G, Naqvi QA, Snehi SK, Khan MS, Raj SK (2012) Molecular identification of three isolates of Jatropha mosaic India virus associated with mosaic disease of *Withania somnifera* in India. Arch Phytopathol 17:2114–2119

Basak J (2016) Tomato yellow leaf curl virus: a serious threat to tomato plants world-wide. J Plant Pathol Microbiol 7:346

Briddon RW, Bull S, Mansoor S, Amin I, Markham PG (2002) Universal primers for the PCR-mediated amplification of DNA β: a molecule associated with some monopartite begomoviruses. Mol Biotechnol 20:315–318

Brown JK, Zerbini FM, Navas-Castillo J, Moriones E, Ramos Sobrinho R, Silva JCF, Fiallo-Olivé E, Briddon RW, Hernández-Zepeda C, Idris A, Malathi VG, Martin DP, Rivera Bustamante R, Ueda S, Varsani A (2015) Revision of Begomovirus taxonomy based on pairwise sequence comparisons. Arch Virol 160:1593–1619

Dutta R, Khalil R, Green R, Mohapatra SS, Mohapatra S (2019) *Withania somnifera* (Ashwagandha) and withaferin A: potential in integrative oncology. Int J Mol Sci 21:5310

Fauquet CM, Briddon RW, Brown JK, Moriones E, Stanley J, Zerbini FM, Zhou X (2008) Geminivirus strain demarcation and nomenclature. Arch Virol 153:83–821

Garg ID, Khurana SMP, Kumar S, Lakra BS (2001) Association of geminivirus with potato apical leaf curl in India and its immuno-electron microscopic detection. J Indian Potato Assoc 28:227–232

Hallan V, Saxena S, Singh BP (1998) Ageratum, Croton and Malvestrum harbour geminiviruses: evidence through PCR amplifcation. World J Microbiol Biotechnol 14:931–932

Inoue-Nagata AK, Albuquerque LC, Rocha WB, Nagata T (2004) A simple method for cloning the complete begomovirus genome using the bacteriophage phi 29 DNA polymerase. J Virol Methods 116:209–211

John J (2014) Therapeutic potential of *Withania somnifera*: a report on phyto–pharmacological properties. Int J Pharma Sci Res 5:2131–2148

Khan S, Qureshi MI, Alam T, Abdin MZ (2007) Protocol for isolation of genomic DNA from dry and fresh roots of medicinal plants suitable for RAPD and restriction digestion. Afr J Biotechnol 3:175

Kulkarni SK, Dhir A (2008) *Withania somnifera*: an Indian ginseng. Prog Neuropsychopharmacol Biol Psychiatry 32:1093–1105

Kumar S, Tamura K, Nei M (1994) MEGA: molecular evolutionary genetics analysis software for microcomputers. Bioinformatics 2:189–191

Kumar A, Snehi SKR, Kumar SK, Khan JA (2011) Association of Cotton leaf curl burewala virus and its satellite molecules with leaf distortion symptoms of cotton in India. New Dis Rep 24:18

Lozano G, Trenado HP, Fiallo-Olivé E, Chirinos D, Geraud-Pouey F, Briddon RW, Navas-Castillo J (2016) Characterization of non-coding DNA satellites associated with sweepoviruses (genus Begomovirus, Geminiviridae)–definition of a distinct class of begomovirus-associated satellites. Front Microbiol 7:162

Maiti CK, Sen S, Paul AK, Acharya K (2007) First report of *Alternaria dianthicola* causing leaf blight on *Withania somnifera* from India. Plant Dis 4:467

Phatak HC, Raychaudhuri SP (1967) *Withania somnifera*: an additional host of tobacco leaf curl virus [new record]. Sci Culture 33:234–235

Rojas MR, Gilbertson RL, Russell DR, Maxwell DP (1993) Use of degenerate primers in the polymerase chain reaction to detect whitefly-transmitted geminiviruses. Plant Dis 77:340–347

Saeed ST, Samad A (2017) Emerging threats of begomoviruses to the cultivation of medicinal and aromatic crops and their management strategies. Virus Dis 1:1–7

Saggam A, Limgaokar K, Borse S, Chavan-Gautam P, Dixit S, Tillu G, Patwardhan B (2021) *Withania somnifera* (L.) Dunal: opportunity for clinical repurposing in COVID-19 management. Front Pharmacol 12:623795

Saleem S, Muhammad G, Hussain MA, Altaf M, Bukhari SN (2020) *Withania somnifera* L.: Insights into the phytochemical profile, therapeutic potential, clinical trials, and future prospective. Iran J Basic Med Sci 12:1501

Saroj A, Kumar A, Qamar N, Alam M, Singh HN, Khaliq A (2012) First report of wet rot of *Withania somnifera* caused by *Choanephora cucurbitarum* in India. Plant Dis 2:293

Saroj A, Kumar A, Srivastava AK, Khaliq A, Absar N, Alam M, Samad A (2014) New report of black leaf spot mold (*Pseudocercospora fuligena*) on *Withania somnifera* from India. Plant Dis 9:1275

Sharma A, Pati PK (2013) First record of Ashwagandha as a new host to the invasive mealybug (*Phenacoccus solenopsis* Tinsley) in India. Entomol 1:59–62

Sharma A, Pati PK, Liu TX (2012) First record of the carmine spider mite, *Tetranychus urticae*, infesting *Withania somnifera* in India. J Insect Sci 12:50