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Identification of viruses Naturally infecting Patchouli (*Pogostemon cablin* (Blanco) Benth.) in India

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ABSTRACT: Patchouli (*Pogostemon cablin*) is considered one of the most important aromatic medicinal plants. A number of viruses are known to attack patchouli plant, causing mosaic symptoms and significantly reduce the quality and quantity of patchouli production. To prevent crop loss or minimise crop damage, accurate viral detection and identification are required before developing virus control methods. However only a handful of information is available in India for their identification. This study aims to identify and characterise the viruses associated with mosaic disease of patchouli using RT-PCR. Infected plant samples were collected from different patchouli cultivated states in India such as Assam, Karnataka and Palampur. RT-PCR employing pair of degenerated primers, specific to both potyvirus and fabavirus, yielded DNA fragments representing *Peanut stripe virus* and *Broad bean wilt virus 2*, respectively. Furthermore, RT-PCR using the designed primers specific to 3' end of PStV yielded amplicon that represent nuclear inclusion b protein (partial), coat protein (CP, complete) and 3' UTR. RT-PCR with the designed primers specific to BBWV2 produced an amplicon that encompases the large and small CP (partial). The PCR amplicons were subjected to nucleotide sequencing. The Phylogenetic analysis clustered Indian PStV isolates together with those from Taiwan, Vietnam and Thailand in a single clade. According to phylogenetic analysis of BBWV2, Indian isolates were clustered with Chinese and German isolates. Thus, RT-PCR assay developed here can act as a diagnostic tool for detection and epidemiological investigation of PStV and BBWV2.

Keywords: *Broad bean wilt virus 2*, *Peanut stripe virus*, Patchouli, Phylogenetic, RT-PCR.

INTRODUCTION

Pogostemon cablin (Blanco Benth.), also known as patchouli, belongs to the family *Lamiaceae*, a member of mint family. It is an aromatic perrenial herb having great commercial value due to the presence of oil glands. Though, the whole plant is known to contribute to oil extraction, oil glands present on leaves account for the major part of extraction (Singh *et al.*, 2009). Patchouli is found to be a native of the Philippines and is well cultivated in many South Asian countries, such as Indonesia, India, Malaysia, China, Singapore as well as West Africa and Vietnam (Maheshwari *et al*., 1993). The oil can be obtained from its shade dried leaves by the process of steam distillation and has strong sweet, herbaceous aromatic and spicy fragrance. Patchouli's unique fragrant properties makes for a huge business potential in the global market, since it is considered to be better than most essential oil yielding plants (Varshney. 2000). Apart from its aromatic properties, patchouli is also regarded as a medicinal plant and has roots among ancient Chinese and Indian traditional medicinal practices. Studies showed that the primary components of patchouli oil, patchouli alcohol (PA) and pogostone, had great therapeutic value (Swamy *et al*., 2015). Moreover, it also exhibits several biological properties like being antimicrobial, antifungal and anti-oxidant, inflammatory, platelet, thrombotic, depressant, mutagenic (Swamy *et al*., 2015). Due to its fixative properties the oil is used as a base material in perfumery and soap industries (Hasegawa *et al*., 1992). Patchouli cultivation is seriously threatened by the presence of various pest and pathogens that can be caused by fungi, viruses and nematodes (Sreedevi *et al*., 2009). Viruses pose a greater threat than others and cause significant decline in the quality and the quantity of patchouli production. Several viruses have been reported that naturally infect patchouli plants and are fully or partially characterised. These viruses have been characterized and sequenced as *Patchouli mildmosaic virus* (PatMMV) (Natsuaki *et al*., 1994) and *Peanut stripe virus* (PStV) [Singh *et al*., 2009]. Some viruses infecting patchouli plants are published as disease notes or characterised at the preliminary level; e.g., potexvirus *Patchouli virus X* (PatVX) (Meissner *et al*., 2002), *Patchouli mottle virus* (PaMoV) (Natsuaki *et al*., 1994), *Pepper ringspot virus* (PRV), Patchouli mosaic virus (PaMV) (Gama. 1979), *Tobacco necrosis virus* (TNV) - a presumed Rhabdovirus (Kitajima *et al*.,1979) and yellow mosaic of Patchouli (Sastry *et al*., 1981; Zaim *et al*., 2013**).** Patchouli plants that are infected by the viruses, either partially or fully characterized, generally belong to family *Potyviridae* and *Secoviridae*. According to current ICTV criteria, the family *Potyviridae* consists of 12 genera on the basis of host range, genomic features, and phylogeny*: Arepavirus,*

Bevemovirus, Brambyvirus, Celavirus, Ipomovirus, Macluravirus, Poacevirus, Potyvirus, Roy movirus, Rymovirus, Tritimovirus and three unassigned species. Among the family *Potyviridae*, *Potyvirus* is the largest genus infecting more than 230 hosts (Inoue-Nagata *et al*., 2022; Wylie *et al*., 2017). The virions range from 680-900 nm in length and are transmitted by aphids or by mechanical means. The genome is linear, single stranded positive sense monopartite RNA of about 8500-12000 nucleotides with a poly (A) tail at 3' end and presumably a genome-inked protein (VPg) at its 5' end. The genome is translated into single polyprotein and later it is processed into 10 functional proteins by virus encoded proteases (Inoue-Nagata *et al*., 2022).

The viruses belonging to the genus *Fabavirus* [family *Secoviridae*, species *Broad bean wilt virus 2* (BBWV2)]**,** are transmitted in a non-persistent manner by several aphid species (Kobayashi *et al*., 1999). The fabavirus virions have a nucleocapsid structure and it shows icosahedral symmetry and have an approx. diameter of about 30nm. The genome comprises of two single stranded positive-sense genomic RNA molecules (RNA1 and RNA2) and their 3' termini are polyadenylated and encapsidated separately (Lisa *et al*., 1996). A polyprotein of RNA1 is processed proteolytically into 5 functional proteins that are involved in genome replication and expression viz., protease cofactor (Co-Pro), helicase (Hel), protease (Pro), polymerase (Pol) and genome linked viral protein (VPg). A polyprotein of RNA 2 is also processed further into movement protein (MP), large coat protein (LCP) and small coat protein (SCP) (Kwak *et al*., 2013)

Fabaviruses and Potyviruses pose severe threat in the cultivation of patchouli and can amount to significant losses in its production (Noveriza *et al*., 2012). Since patchouli is maintained only through vegetative propagation, the survival of viruses becomes easy in the virus- infected plants. Hence, virus detection in the early growth stage and selection of healthy mother plants are the pre-requisite to control their further spread. The symptoms are generally mild to severe mosaic with thickened or stunted leaf growth. To stop the loss or to minimize the damage of the crop, suitable disease management strategies are implemented. But an accurate virus detection and identification is a requisite to devise virus management strategies. It is a challenging task as different viruses often express similar symptoms. So, for the identification of the viruses different molecular techniques have been developed that are also rapid, sensitive and cost effective in nature. Virus identification methods that are most commonly used include: enzyme-linked immunosorbent assay (ELISA) and reverse transcription polymerase chain reaction (RT-PCR). ELISA is the most commonly used diagnostic tool. However, producing highly specific antisera against viruses is difficult because of multiple infection and for the detection of large numbers of samples. Consequently, RT-PCR was used to identify various plant viruses because to its great sensitivity and specificity (Irfan and Abhishek 2023). RT-PCR technique using universal primers has been applied for identification of genera potyvirus and fabavirus (Gibbs and Mackenzie 1997; Zheng *et al*., 2010; Ferrer *et al*., 2007). RT-PCR were used to detect potyvirus and fabavirus from patchouli samples. Since very little study have been attempted for the identification of viruses associated with patchouli plants in India, the present investigation was carried out to identify and characterise viruses on the basis of CP gene sequence. This RT-PCR assay described here may be used as a diagnostic tool for PStV and BBWV2 detection and epidemiological study.

MATERIALS AND METHODS

Collection of patchouli leaf samples. Patchouli plants exhibiting typical virus like symptoms such as mild to severe mosaic, thickened or stunted leaf growth were collected from different patchouli cultivation areas in India from Assam, Karnataka and Palampur (Fig. 1). The leaf samples were stored at -80°C deep freezer for further experimental work.

Total RNA Extraction and cDNA Synthesis. 100 mg of infected patchouli leaves showing mosaic symptoms and healthy leaves were subjected to total RNA extraction using a combination of CTAB and TRIzol (GeNei™) method as described by Jordon *et al.* (2015). The extracted RNA was used as a template for cDNA synthesis (reverse transcription) and polymerase chain reaction (PCR). First strand cDNA synthesis was done in 20µl reaction mixture using 2 µl (1 µg) of total RNA as template, 1µl (0.5 µM) of oligo d(T) primer, 2µl of dNTP mix (5mM each), 1 µl of RT enhancer, 4 µl of 5 x RT buffer, 1µl of reverse transcriptase enzyme (Thermos fisher scientific, US) and 9 µl of nuclease free water to make a final volume of 20 µl . The reaction mixture was then incubated at 42 °C for 90 minutes and terminated at 95°C for 10 minutes to yield cDNA.

RT-PCR based detection of potyvirus/fabavirus. Potyvirus and fabavirus were directly detected from the patchouli plants using a set of degenerate primer pairs named CPUP/CP9502 (Van Der Vlugt *et al*., 1999) and fab5'R1F/ fab5'R1R (Ferrer *et al*., 2007), respectively in RT-PCR. The potyvirus specific primer (CPUP/ CP9502) amplify partial coat protein gene and 3'UTR of the potyviruses. The fabavirus specific primers (Fab5'R1F/ Fab5'R1R) yield an amplicon corresponding to the 5'NTR of RNA1 segment.

PCR was carried out in an automated thermocycler (BIO RAD, T100 thermal cycler). RT-PCR involving both degenerate primers specific to each group was carried out in a 20µl reaction mixture containing 2 µl of above synthesized cDNA, 10 µl of 2X Gene Taq Green PCR mastermix (Puregene, Genetix), 0.5µl (10pmol) of each of upstream and downstream primers (each) and 9µl of Milli Q water to make a final volume of 20 µl. Thermocycler programme for potyvirus amplification included 35 cycles each at 94°C for 5 min, 94°C for 30sec, 50°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min whereas the fabavirus amplification included 94°C for 5min, 94°C for 30 sec, 55°C for 30sec, 72°C for 1min with final step at 72°C

for 10min. The amplified products were then electrophoresed on 1% agarose gel prepared in 1X TAE (Tris-Acetic acid- EDTA) buffer which contained ethidium bromide. After electrophoresis, the amplified PCR-products were visualized under UV light in a gel documentation system (BIO RAD, Gel Doc™ XR+, USA).

Designing of primers for specific detection of PstV and BBWV2. Nucleotide sequence containing the complete coat protein gene of PStV isolates were retrieved from the GenBank (accession no- AF063222, AY968604, DQ925418, AF200624, AF200623, Y11774, U34972, U05771, AJ132156, AF073380). The primers were designed manually from the conserved regions of the CP gene after aligning using ClustalW Multiple Alignment Bioed it Version 7.2.5. The sequence of the primers designed for specific detection of PStV used in this study are listed in Table 1. Gradient PCR was done to optimize the primer pair with different annealing temperature. The 20 µl reaction mixture contained 2 µl of above synthesized cDNA, 10 µl of 2X Gene Taq Green PCR mastermix (Puregene, Genetix), 0.5µl (10pmol) of each of forward and reverse primers and 7 µl of nuclease free water to make a final volume of 20 µl. The optimized thermocycling steps includes 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 40 seconds., annealing at 57°C for 30seconds, elongation at 72°C for 1 min and final extension step at 72°C for 5 minutes. The products were analysed on 1.0 % agarose gel stained with EtBr and visualized under UV light.

Fig. 1. Patchouli (*Pogostemon cablin*) leaves showing typical mosaic symptoms.

Table 1: Primers sequence used in the PCR assay for detection of PStV and BBWV2.

Primer Name	Sequence (5'→3')	References
CPUP: CP9502:	TGAGGATCCTGGTGYATHGARAAYGG GCGGATCCTTTTTTTTTTTTTTTT	Van Der Vlugt *et al.* (1999)
PstV for: PstV rev:	GGCTATCCTGAATTGCTCCAAGA TGAGGACATGCAGAGTGATT	This study
Fab5'R1F: Fab5'R1R:	AAATATTAAAACAAACAGCTTTCGTT TTCAAAGCTCGTGCCATNTYATTKGC	Ferrer *et al.* (2007)
BBWV2 for: BBWV2 rev:	CTGGCTTTCAAGCAAGGG ACTTCTTGCTCCCACGCAC	This study

For BBWV2 detection, the nucleotide sequences of RNA 2 segment (containing complete LCP and SCP gene of BBWV2 isolates) were downloaded from GenBank database. Using ClustalW Multiple Alignment Bioedit Version 7.2.5, these sequences were aligned and primers were designed manually from the conserved part of the CP gene (Table 1). The designed primer encompasses the partial LCP as well as SCP genes. The annealing temperature was optimized using gradient PCR. The PCR was carried out in 20 µl reaction volume comprising 2 µl of cDNA, 10 µl of 2X Gene Taq Green PCR mastermix (Puregene, Genetix), 1µl (10pmol) of each of forward and reverse primers and 7 µl of nuclease free water. The optimized thermocycling profile includes 94°C for 5 minutes, 94°C for 30 seconds, 53°C for 30 seconds, 72°C for 1minutes with final step at 72°C for 10minutes.

Cloning, Sequencing and Phylogenetic analysis. In order to confirm the identity, PCR amplicon were cloned usingp JET 1.2 cloning kit as per the instructions given by the manufacturer (Thermo Fisher Scientific, US). The cloned vectors were then transformed into *E. coli* DH5α cells. Two positive clones from independent PCR products were sequenced for each isolates using an automated sequencer company (Superworth Bio, New Delhi, India) employing pJET1.2 universal primers following the Sanger's dideoxy chain termination method (Sanger *et al.*, 1977).

The consensus sequences were constructed for each sequences (PStV and BBWV2) using Bioedit software Version 7.2.5 and compared to the sequences registered in the GenBank by the Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/BLAST/) to know their homology among themselves and with others sequences of PStV and BBWV2 available at GenBank. Multiple sequence Alignment (MSA) of nucleotide and amino acid sequences were generated for each virus isolates obtained in this study and those retrieved from GenBank. MSA was performed by Clustal W Multiple Alignment available in Bioedit Version 7.2.5 (Hall, 1999).

For phylogenetic studies, Molecular Evolutionary Genetic Analysis [(MEGA version 11.0; (Tamura *et al.*, 2021)], was used and tree were generated by neighbour-joining (NJ) method.

RESULTS AND DISCUSSION

In the current study, two viruses namely PStV and BBWV2 that naturally infect patchouli plants were identified on the basis of sequences from their CP genes. Since viruses adversely affect the quality and quantity of patchouli production, their identification at the early stage is a must to devise a virus control

strategy. The common symptoms noticed during the investigation were plants with typical mosaic symptoms of pale green or yellow patches, and depending on the severity malformation of leaves and stunting of plants. These symptoms were similar to previous reports of a viral infection caused by potyvirus and fabavirus (Noveriza *et al*., 2012; Singh *et al*., 2009; Zaim *et al*., 2013). Both new and old plantations were affected by the disease, and its severity ranged from 57 to 73% (Noveriza *et al*., 2012). RT-PCR was performed to discover viruses that naturally infect patchouli plants, using total RNA extracted from patchouli leaf samples. RT-PCR of total RNA from patchouli leaf samples, showed positive amplification of potyvirus. The degenerate primers (CPUP and CP9502) amplified a DNA fragment of 800bp covering partial coat protein (CP) and 3' UTR, thus indicating the tentative presence of potyvirus (Fig. 2).

To know the complete information of the virus species, primers specific to PStV (PstV for and PstVrev) were designed. The primer pair (PstVfor& rev) further amplified a DNA fragment of 1300bp representing polymerase gene, complete CP gene and 3' UTR (Fig. 3). The amplicons were cloned, sequenced and submitted to GenBank database with the accession numbers ON152317, ON152319-ON152322, ON186489. The nucleotide analysis of sequences under research revealed PStV homology.

A comparison of nucleotide and protein sequence between Indian PStV (ON152317, ON152319-ON152322, ON186489) isolates, with other isolates retrieved from GenBank showed 89-96% and 93-97% similarities respectively (Table 2). Nucleotide sequence analysis of all the obtained PStV sequences, revealed a long open reading frame with a terminating codon TAA at position 1086. Each sequence comprised of 225bp of nuclear inclusion b protein (polymerase), 861bp of complete coat protein gene, followed by a 3'UTR of 216bp (ON152317, ON186489), 210bp (ON152322, ON152320, ON152321) and 209bp (ON152319).

Comparison of amino acid sequence alignment of Indian PStV isolates (under study) with other isolates retrieved from GenBank (Table 2) showed a putative cleavage site (EVXXQ/S) and coat protein of identical size- 288 amino acids. Three different protease cleavage site for the production of CP gene inpotyvirus are Q/S, Q/G and Q/A and the putative cleavage site Q/S was found in the PStV sequence (Fig. 4). The N-terminal regions of coat proteins among Indian PStV isolates and that of Vietnam (DQ925418), Thailand (AF200623, AF200624) and Taiwan (AF063222, AY968604) were found to be highly conserved, both in sequence (94-96%) and in length (52aa). Moreover these isolates also showed conserved core and C-terminal regions. The CP of each isolates are characterised by the presence of conserved DAG motif near the N-terminal region which is essential for aphid transmissibility and characteristic of most of the potyviruses (Atreya *et al*., 1991; Harrison *et al*., 1988). Beside this, the 6 conserved motifs owned by potyvirus were also found in the amino acid sequence of the PStV coat protein gene of this study. These motifs include EN/DTERH, QMKAAA, YAFDFYE, MVWCIE/DNG, WV/TMMDGD/E/N and P/R/AYMPRYG (Fig. 4). These sites are likewise conserved in the potyvirus family (Zheng *et al*., 2008). Coat protein gene was subjected to nucleotide sequence determination in the absence of full genome sequence of the viruses and serve as the basis for the classification (Adams *et al*., 2005; Inoue-Nagata *et al*., 2022). The sequence homology (94-96% at nt and 95-97% at aa) found between CP gene of PStV isolates of this study with the previously reported isolates suggested that they were the strain of same potyvirus. (Khan *et al*., 1993, 1990; Shukla & Ward 1988, 1989). This was further supported by the findings that their 3'UTR shared 90-95% identity with the published PStV sequences in GenBank, which was within the cut off range of identifying the strain of same virus (Frenkel *et al*., 1989). Based on these criteria, nucleotide/protein sequences of CP and 3'UTR under study, suggest that the virus isolates belong to genus *Potyvirus* of strain PStV. The occurrence of PStV (Singh *et al*., 2009) and PaYMV (Zaim *et al*., 2013) in patchouli has been reported previously from India. But these studies provide information on the partial coat protein gene. Our study however focuses on the amplification of complete coat protein gene along with 3'UTR and partial polymerase gene of PStV.

Phylogenetic tree with appreciable bootstrap values (1000) was constructed using the nucleotide sequences to determine relationship between PstV isolates (present study) with the previously reported PStV isolates using *Azuki bean mosaic virus* as an outgroup. Dendrogram generated showed two different groups, Group I and Group II (Fig. 5). Group I includes all the isolates from India; Assam (ON152317 & ON186489), Karnataka (ON152322 & ON152320), Palampur (ON152321 & ON152319), Vietnam (DQ925418), Thailand (AF200624 & AF200623) and Taiwan (AF063222 & AY68604), clustered in a single clade signifying their close resemblance. Group II includes isolates from China (KF439722) and USA (U34972 & U05771).

Similarly, fabavirus, BBWV2 has been detected based on RT-PCR technique in patchouli.RT-PCR using fabavirus group specific degenerate primer pairs(Fab5'R1F/ Fab5'R1R) amplified DNA fragments of expected size (~ 400bp) which were cloned and sequenced (Fig. 6). Furthemore, specific primers (BBWV2for & BBWV2rev) designed against CP gene sequences of BBWV2 also amplified a DNA fragment of ~ 1kb (Fig. 7). Amplicons were subjected to nucleotide sequence determination and phylogenetic analysis and were submitted to GenBank with accession numbers ON602251and ON638916-ON638920. Patchouli was found to be infected with fabavirus, PatMMV and first characterised from Japan by Ikegami *et al.* (1998) and co-worker. However, PatMMV considered an isolate of BBWV at serological level having similar particle morphology.

Table 2: Percent homology of nucleotide sequence (below the diagonal line) and amino acid sequences (above the diagonal line) of Indian PStV isolates (this study) with those previously reported in GenBank.

	ON152317 (This study)	ON186489 (This study)	ON152322 (This study)	ON152320 (This study)	ON152321 (This study)	ON152319 (This study)	AF063222	DQ925418	AY968604	AF200624	AF200623	U34972	KF439722	U05771	AB012663
ON152317 (This study)		98	98	98	99	98	96	96	96	95	95	94	95	95	93
ON186489 (This study)	99		98	98	98	98	96	95	96	94	94	94	94	94	93
ON152322 (This study)	98	98		100	98	98	96	95	96	94	94	94	94	94	93
ON152320 (This study)	98	98	100		98	98	96	95	96	94	94	94	94	94	93
ON152321 (This study)	98	98	99	99		99	96	96	97	95	95	94	95	95	94
ON152319 (This study)	98	98	99	99	100		97	96	97	95	95	94	95	95	94
AF063222	94	94	95	95	95	96		97	100	96	96	96	96	96	94
DQ925418	94	94	95	95	96	96	96		97	98	98	96	96	96	94
AY968604	94	94	95	95	95	96	99	95		96	96	96	96	96	94
AF200624	94	94	94	94	95	95	95	97	95		100	96	96	96	93
AF200623	94	94	94	94	95	95	95	97	95	100		96	96	96	93
U34972	93	93	93	93	94	94	94	94	95	94	94		100	100	94
KF439722	92	93	93	93	93	94	94	94	95	94	94	99		100	94
U05771	92	93	93	93	93	94	94	94	95	94	94	100	99		94
AB012663	89	89	89	89	90	90	90	90	90	90	90	89	89	89	

Table 3: Percent homology of nucleotide sequence (below the diagonal line) and amino acid sequences (above the diagonal line) of Indian BBWV2 isolates (this study) with those previously reported in GenBank.

	ON602251 (This study)	ON638916 (This study)	ON638917 (This study)	ON638918 (This study)	ON638919 (This study)	ON63920 (This study)	OK05854	MW55659	AF104335	HQ283389	AB013616	AB0320403	AF225954	AB018698	AJ132844	JX183226	KT988974
ON602251 (This study)		100	97	97	97	97	97	97	96	95	97	96	90	95	96	97	61
ON638916 (This study)	100		98	97	97	97	97	97	96	95	97	96	91	95	96	97	62
ON638917 (This study)	97	97		97	97	97	97	97	96	95	97	96	91	95	96	97	62
ON638918 (This study)	97	97	99		97	96	97	97	95	95	96	95	90	95	96	96	62
ON638919 (This study)	97	97	97	97		100	97	97	96	96	97	96	91	95	96	97	62
ON63920 (This study)	96	97	97	97	100		97	97	96	96	97	96	91	95	96	97	62
OK05854	91	91	92	92	91	91		99	98	97	99	98	92	97	98	99	62
MW55659	91	91	91	91	91	91	93		98	98	99	98	92	97	98	99	62
AF104335	89	89	89	90	90	90	90	91		99	98	97	91	96	97	97	62
HQ283389	89	89	89	90	90	89	90	91	96		97	96	91	96	96	97	62
AB013616	87	87	87	87	87	87	86	86	87	86		99	93	98	98	99	62
AB0320403	85	85	86	86	85	85	86	85	86	85	91		92	97	97	98	62
AF225954	79	79	79	79	80	80	81	80	81	81	81	80		92	93	93	57
AB018698	80	80	80	80	80	80	81	80	82	82	82	81	89		97	97	62
AJ132844	84	84	84	84	84	84	85	85	84	83	85	84	79	81		98	62
JX183226	83	83	84	84	84	84	86	85	84	84	85	85	80	80	90		62
KT988974	62	62	62	61	62	62	62	62	61	61	62	62	56	56	60	61	

After sequencing the amplified DNA fragment (1032bp), BLASTn and BLASTp analysis showed highest homology with BBWV*2*. Comparison of nucleotide and deduced amino acid sequence between Indian isolates (ON602251and ON638916-ON638920) with earlier reported isolates of BBWV2 showed that the nucleotide percentage similarities ranged between 79 to 91% and protein similarities ranged between 92 to 97% (Table 3). BBWV2 isolates under study revealed highest sequence identity with isolates from China (MW55659) and Germany (OK05854) (91% at nucleotide and 97% at amino acid). The nucleotide sequence analysis of CP gene of BBWV2 (under study) showed partial LCP (596bp) and SCP (435bp). Alignment of amino acid sequences (344 residue) of all the Indian BBWV2 isolates with previously described isolates from GenBank showed putative cleavage site Q/S or Q/A between LCP and SCP (Fig. 8).

In order to know the phylogenetic relationship with other BBWV2, the CP nucleotide sequence (partial) of Indian isolates (ON602251 and ON638916-ON638920) were compared with the sequence of other BBWV2 isolates along with BBWV1 isolates as outgroup. Based on phylogenetic analysis RNA2 genome of BBWV2 isolates is divided into two groups, namely Group I and Group II (Fig. 9). Group II includes isolates from Japan (AB013616, AB032403 & AB018698), Singapore (AF225954), China (AJ132844), and Korea (JX183226). While Group I is further divided into two subgroup, Ia and Ib. Subgroup Ia comprises all the Indian isolates [Assam (ON602251 & ON638916), Karnataka (ON638917 & ON638918), and Palampur (ON638919 & ON638920)] along with isolates from Germany (OK058514) and China (MW556592). The subgroup Ib includes isolates from China (HQ283389) and Korea (AF104335).

To the best of our knowledge, this is the first report of BBWV2 infection in patchouli plant from India, showing mixed infection with PStV, a *Potyvirus*. Earlier report suggested that the infection of BBWV2 increased abruptly with co-infection with *Cucumber mosaic virus* (CMV), Pepper mottle virus, Pepper mild mottle virus and/or Potato virus Y (Kwak *et al*., 2013).

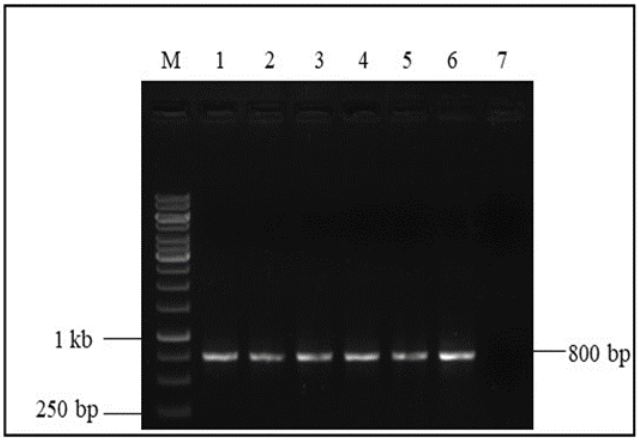

Fig. 2. RT-PCR amplification of potyvirus gene from virus suspected patchouli leaves using degenerate primers (CPUP & CP9502). M=1kb marker; lane 1 & 2= leaf samples from Assam; lane 3 & 4= leaf samples from Karnataka; lane 5 & 6= leaf samples from Palampur; lane 7= negative control (RNA from healthy samples)

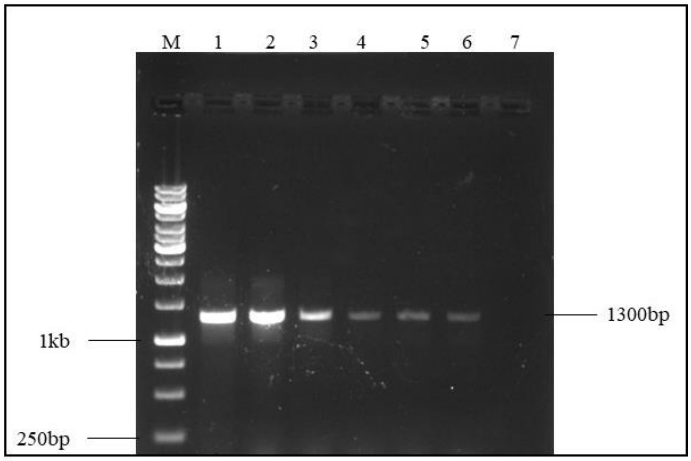

Fig. 3. RT-PCR of RNA from patchouli samples using PStV specific primers (designed in this study, PstV for &PstV rev) on 1% agarose gel showing amplicon size of about 1300bp. M=1kb marker; lane 1 & 2= leaf samples from Assam; lane 3 & 4= leaf samples from Karnataka; lane 5 & 6= leaf samples from Palampur; lane 7= negative control (RNA from healthy samples).

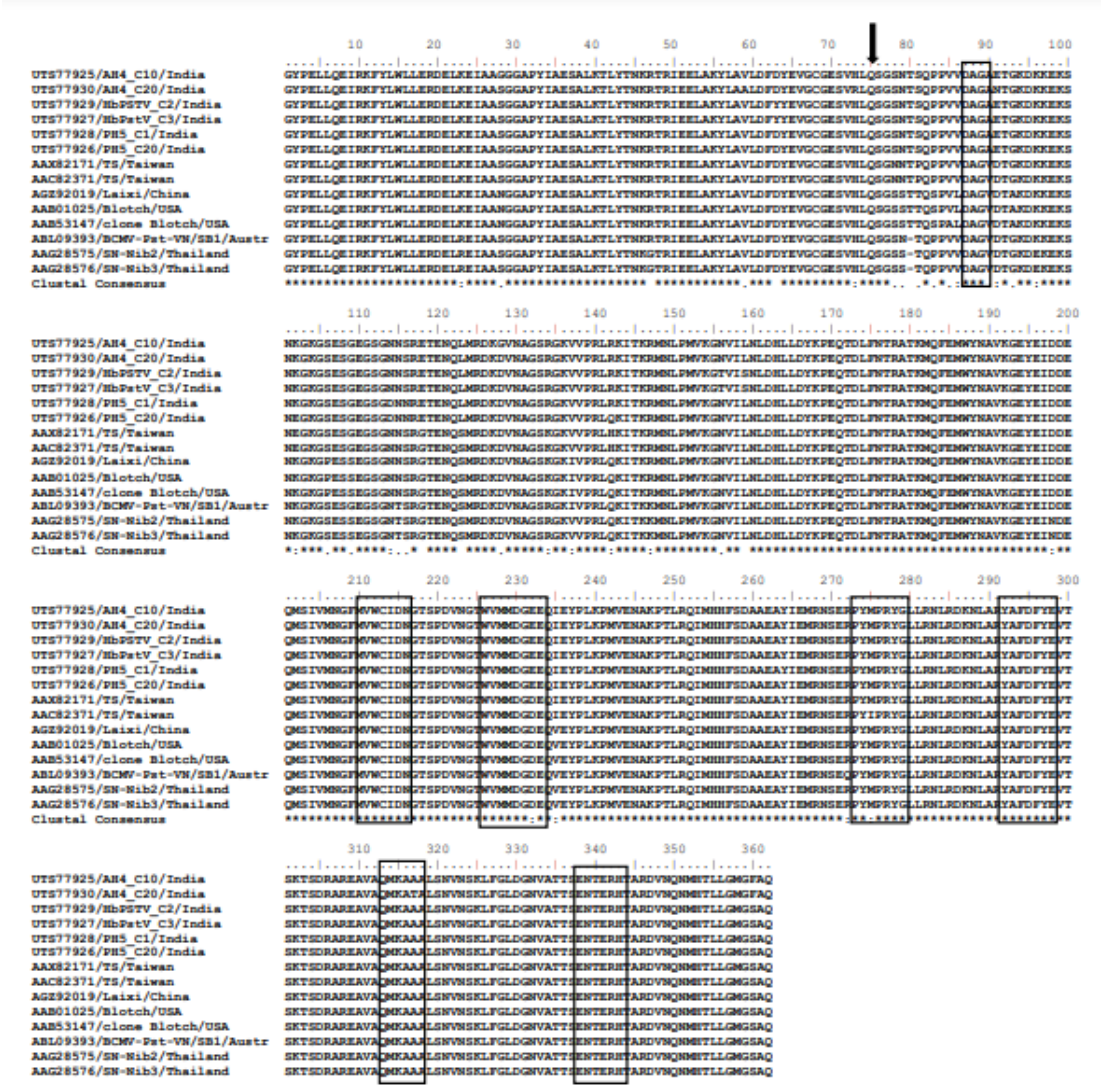

Fig. 4. Comparison of the amino acid sequences of Indian PStV isolates (under study, Accession no.UTS77925-UTS77930) with isolates retrieved from GenBank. Identical amino acids are indicated by asterisk, gaps are indicated by dashes, putative cleavage sites is indicated by vertical arrow and conserved sites in CP potyvirus are indicated in box.

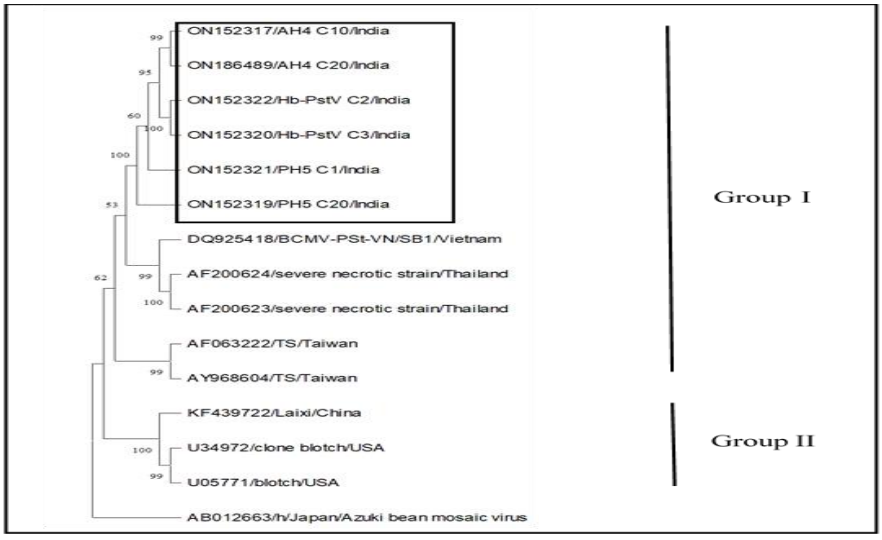

Fig. 5. Phylogenetic tree based on the CP gene nucleotide sequences to show the relationship of Indian isolates (under study, Accession no. ON152317, ON152319-ON152322, ON186489) with other isolates of PStV obtained from GenBank. Dendograms were constructed in MEGA using neighbour joining method with Azuki bean mosaic virus as an outgroup (Tamura *et al*., 2021).

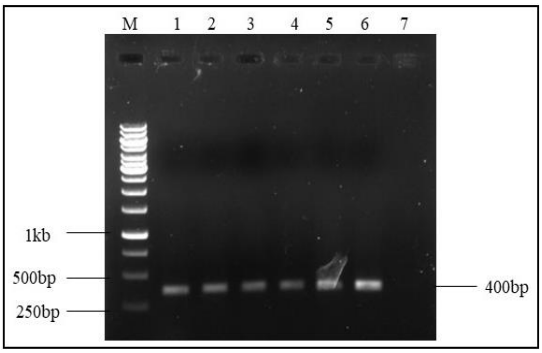

Fig. 6. RT-PCR amplification of fabavirus gene from patchouli samples with degenerate primers (Fab5'R1F & Fab5'R1R) on 1% agarose gel M=1kb marker; lane 1 & 2= leaf samples from Assam; lane 3 & 4= leaf samples from Karnataka; lane 5 & 6= leaf samples from Palampur; lane 7= negative control (RNA from healthy samples).

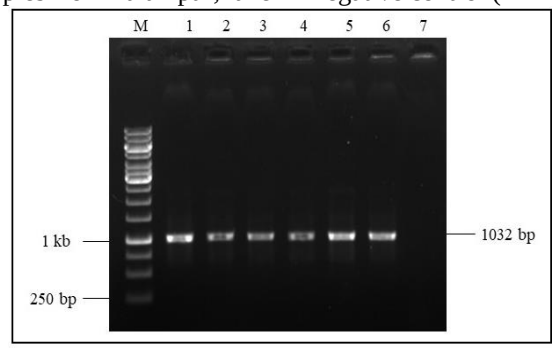

Fig. 7. RT-PCR amplification of BBWV2 gene from patchouli samples using specific primers (designed in this study, BBWV2 for & BBWV2 rev) on 1% agarose gel M=1kb marker; lane 1 & 2= leaf samples from Assam; lane 3 & 4= leaf samples from Karnataka; lane 5 & 6= leaf samples from Palampur; lane 7= negative control (RNA from healthy samples.

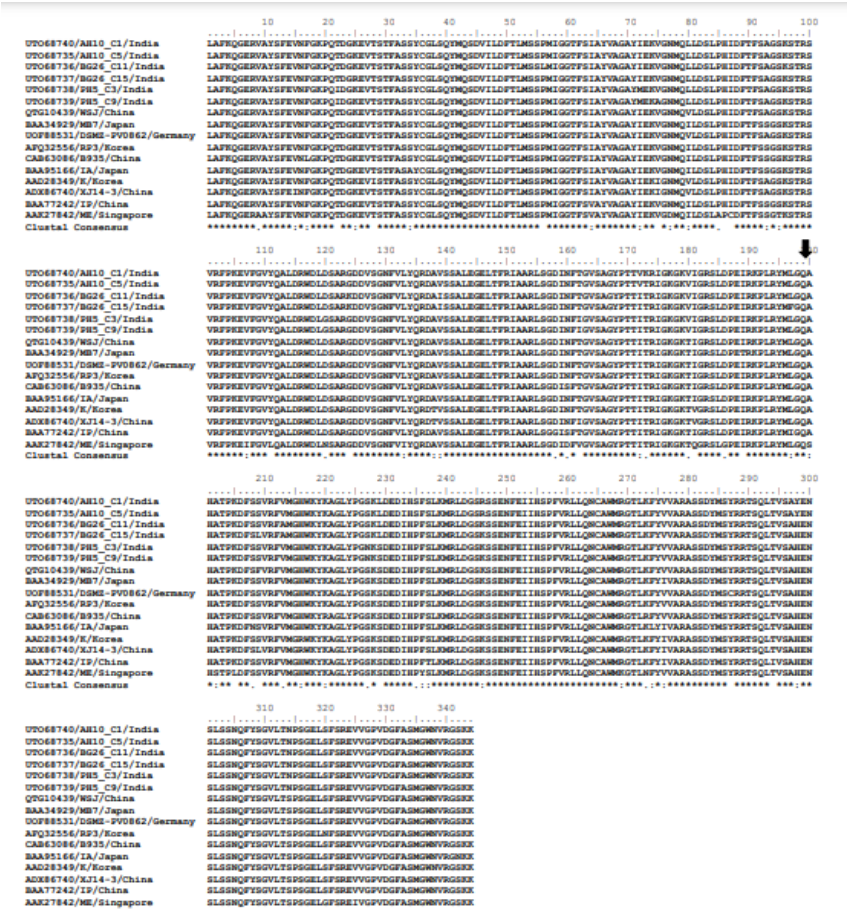

Fig. 8. Comparison of the amino acid sequences of BBWV2 Indian isolates (under study, Accession no. UTO68735-UTO68740) with the isolates obtained from GenBank. Identical amino acids are indicated by asterisk, putative cleavage site between LCP and SCP is indicated by vertical arrow.

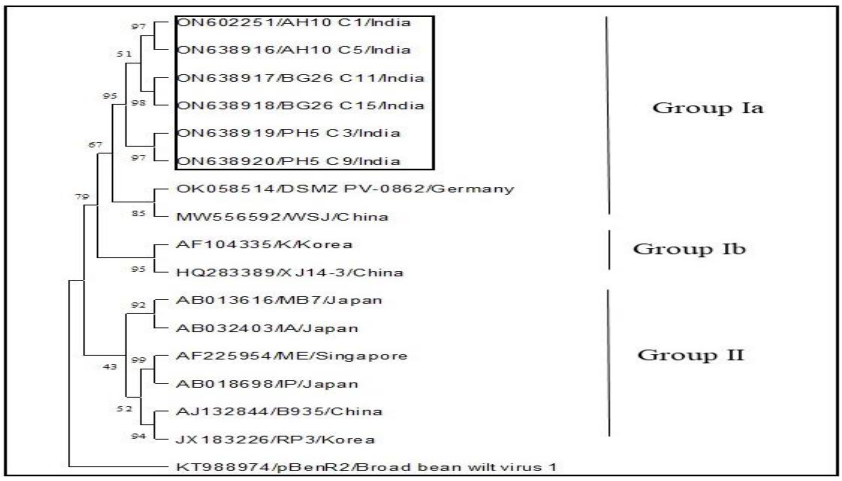

Fig. 9. Phylogenetic tree derived from the nucleotide sequences to show the relationship of Indian isolates (under study, Accession numbers ON602251, ON638916-ON638920) with previously reported BBWV2 isolates downloaded from GenBank. Dendograms were constructed in MEGA using neighbour joining method with Broad bean wilt virus 1 as an outgroup (Tamura *et al*., 2021).

CONCLUSIONS

PStV (potyvirus) and BBWV2 (fabavirus) have been identified as the viruses associated with the mosaic disease of patchouli plants in India based on the highest nucleotide identities of CP gene sequence and their phylogenetic relationship. Phylogenetic analysis of PStV isolates under study showed close resemblance with Taiwan, Vietnam and Thailand. The phylogenetic analysis of BBWV2 showed its close resemblance with China and Germany isolates. Consequently, the present study reveals the virus diversity, subgrouping, evolutionary history within the patchouli viruses in India.

FUTURE SCOPE

The results presented here would provide a comparative analysis of PStV and BBWV2 viral isolates at the nucleic acid level. This would help in the development of methods for a more accurate diagnosis, understanding of their mode of infection, and eventually the establishment of virus-resistant strains.

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Conflict of Interest. None.

REFERENCES

Adams, M. J., Antoniw, J. F. & Fauquet, C. M. (2005). Molecular criteria for genus and species discrimination within the family Potyviridae. *Archives of virology*, *150*(3), 459-479.

Atreya, P. L., Atreya, C. D. & Pirone, T. P. (1991). Amino acid substitutions in the coat protein result in loss of insect transmissibility of a plant virus. *Proceedings of the National Academy of Sciences*, *88*(17), 7887-7891.

Ferrer, R. M., Luis-Arteaga, M., Guerri, J., Moreno, P. & Rubio, L. (2007). Detection and identification of species of the genus Fabavirus by RT–PCR with a single pair of primers. *Journal of virological methods*, *144*(1-2), 156-160.

Frenkel, M. J., Ward, C. W. & Shukla, D. D. (1989). The use of 3′ non-coding nucleotide sequences in the taxonomy of potyviruses: application to watermelon mosaic virus 2 and soybean mosaic virus-N. *Journal of General Virology*, *70*(10), 2775-2783.

Gama, M. I. C. S. (1979). Estudossobre um complexo de virus infectandopatchuli (*Pogostemon Patchouli* Pellet.) e obstensao de plantassadiasporcultura de meristema. *Brasilia: Tese de Mestrado*, *72*.

Gibbs, A. & Mackenzie, A. (1997). A primer pair for amplifying part of the genome of all potyvirids by RT-PCR. *Journal of Virological methods*, *63*(1-2), 9-16.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In *Nucleic acids symposium series* (Vol. 41, No. 41, pp. 95-98). [London]: Information Retrieval Ltd., c1979-c2000..

Harrison, B. D. & Robinson, D. J. (1988). Molecular variation in vector-borne plant viruses: epidemiological significance. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences*, *321*(1207), 447-462.

Hasegawa, Y., Tajima, K., Toi, N. & Sugimura, Y. (1992). An additional constituent occurring in the oil from a patchouli cultivar. *Flavour and fragrance journal*, *7*(6), 333-335.

Inoue-Nagata, A. K., Jordan, R., Kreuze, J., Li, F., López-Moya, J. J., Mäkinen, K. & Wylie, S. J. (2022). ICTV Virus Taxonomy Profile: Potyviridae 2022. *Journal of General Virology*, *103*(5), 001738.

Ikegami, M., Kawashima, H., Natsuaki, T. & Sugimura, N. (1998). Complete nucleotide sequence of the genome organization of RNAff2 of patchouli mild mosaic virus, a new favavirus. *Archives of virology*, *143*(12), 2431-2434.

Irfan Khan and Abhishek Sharma (2023). Molecular characterization and Phylogenetic Analysis of Viruses Associated with Garlic and Onion in Punjab, India. *Biological Forum – An International Journal*, *15*(2), 285-290.

Jordon Thaden, I. E., Chanderbali, A. S., Gitzendanner, M. A. & Soltis, D. E. (2015). Modified CTAB and TRIzol protocols improve RNA extraction from chemically

complex Embryophyta. *Applications in plant sciences*, *3*(5), 1400105.

Khan, J. A., Lohuis, H., Goldbach, R. W. & Dijkstra, J. (1990). Distinction of strains of bean common mosaic virus and blackeye cowpea mosaic virus using antibodies to N and C or N terminal peptide domains of coat proteins. *Annals of Applied Biology*, *117*(3), 583-593.

Khan, J. A., Lohuis, D., Goldbach, R. & Dijkstra, J. (1993). Sequence data to settle the taxonomic position of bean common mosaic virus and blackeye cowpea mosaic virus isolates. *Journal of General Virology*, *74*(10), 2243-2249.

Kitajima, E. W. & Costa, A. S. (1979). Rhabdovirus-like particles in tissues of five different plant species. *Fitopatologia Brasileira*.

Kobayashi, Y. O., Nakano, M., Kashiwazaki, S., Naito, T., Mikoshiba, Y., Shiota, A. & Honda, Y. (1999). Sequence analysis of RNA-2 of different isolates of broad bean wilt virus confirms the existence of two distinct species. *Archives of virology*, *144*(7), 1429-1438.

Kwak, H. R., Kim, M. K., Nam, M., Kim, J. S., Kim, K. H., Cha, B. & Choi, H. S. (2013). Genetic compositions of Broad bean wilt virus 2 infecting red pepper in Korea. *The plant pathology journal*, *29*(3), 274.

Lisa, V. & Boccardo, G. (1996). Fabaviruses: Broad bean wilt and allied viruses. *The plant viruses: polyhedral virions and bipartite RNA genomes*, 229-250.

Maheshwari, M. L., Vasantha Kumar, T., Sharma, N. & Chandel, K. P. S. (1993). Patchouli-an Indian perspective. *Indian perfumer*, *37*, 9-9.

Meissner Filho, P. E., Resende, R. D. O., Lima, M. I. & Kitajima, E. W. (2002). Patchouli virus X, a new potexvirus from *Pogostemon cablin*. *Ann. Appl. Biol*, *141*(3), 267-274.

Noveriza, R., Suastika, G., Hidayat, S. H. & Kartosuwondo, U. (2012). Potyvirus associated with mosaic disease on patchouli (*Pogostemon cablin* (Blanco) Benth.) plants in Indonesia. *Journal of ISSAAS (International Society for Southeast Asian Agricultural Sciences)*, *18*(1), 131-146.

Natsuaki, K. T., Tomaru, K., Ushiku, S., Ichikawa, Y., Sugimura, Y., Natsuaki, T. & Teranaka, M. (1994). Characterization of two viruses isolated from patchouli in Japan. *Plant Disease*, *78*(11), 1094-1094.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the national academy of sciences*, *74*(12), 5463-5467.

Sastry, K. S. & Vasanthakumar, T. (1981). Yellow mosaic of patchouli (*Pogostemon patchouli*) in India. *Current Science*.

Shukla, D. D. & Ward, C. W. (1988). Amino acid sequence homology of coat proteins as a basis for identification and classification of the potyvirus group. *Journal of General Virology*, *69*(11), 2703-2710.

Shukla, D. D. & Ward, C. W. (1989). Identification and classification of potyviruses on the basis of coat protein sequence data and serology. *Archives of Virology*, *106*(3), 171-200.

Singh, M. K., Chandel, V., Hallan, V., Ram, R. & Zaidi, A. A. (2009). Occurrence of Peanut stripe virus on patchouli and raising of virus-free patchouli plants by meristem tip culture. *Journal of Plant Diseases and Protection*, *116*(1), 2-6.

Sreedevi, S. C., Hegde, Y. R. & Prashanthi, S. K. (2009). Survey for fungal diseases of patchouli in Karnataka. *Biomed*, *4*(2), 109-112.

Swamy, M. K. & Sinniah, U. R. (2015). A comprehensive review on the phytochemical constituents and pharmacological activities of *Pogostemon cablin* Benth.: an aromatic medicinal plant of industrial importance. *Molecules*, *20*(5), 8521-8547.

Tamura, K., Stecher, G. & Kumar, S. (2021). MEGA11: molecular evolutionary genetics analysis version 11. *Molecular biology and evolution*, *38*(7), 3022-3027.

Varshney, S. C. (2000). Vision 2005: Essential oil industry of India. *Indian Perfumer*, *44*(3), 101-118.

Van der Vlugt, R. A. A., Steffens, P., Cuperus, C., Barg, E., Lesemann, D. E., Bos, L. & Vetten, H. J. (1999). Further evidence that shallot yellow stripe virus (SYSV) is a distinct potyvirus and reidentification of Welsh onion yellow stripe virus as a SYSV strain. *Phytopathology*, *89*(2), 148-155.

Wylie, S. J., Adams, M., Chalam, C., Kreuze, J., López-Moya, J. J., Ohshima, K. & Consortium, I. R. (2017). ICTV virus taxonomy profile: Potyviridae. *The Journal of general virology*, *98*(3), 352.

Zaim, M., Ali, A., Joseph, J. & Khan, F. (2013). Serological and molecular studies of a novel virus isolate causing yellow mosaic of patchouli [*Pogostemon cablin* (Blanco) Benth]. *Plos one*, *8*(12), e83790.

Zheng, L., Rodoni, B. C., Gibbs, M. J. & Gibbs, A. J. (2010). A novel pair of universal primers for the detection of potyviruses. *Plant pathology*, *59*(2), 211-220.

Zheng, L., Wayper, P. J., Gibbs, A. J., Fourment, M., Rodoni, B. C. & Gibbs, M. J. (2008). Accumulating variation at conserved sites in potyvirus genomes is driven by species discovery and affects degenerate primer design. *PLoS One*, *3*(2), e1586.

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