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Occurrence, Complete Genome Sequencing, and Development of Diagnostics for Black Pepper Virus F Infecting Black Pepper in India

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ABSTRACT

The occurrence of black pepper virus F (BPVF) was identified for the first time from India, and its complete genome sequence was determined using overlapping fragments obtained through reverse transcription polymerase chain reaction (RT-PCR). The RNA 1 and RNA 2 of the Indian isolate of BPVF (BPVF-IND) contained 6376 and 3340 nucleotides potentially coding for proteins of 230.7 kDa and 114 kDa respectively. Comparison of the RNA 1 sequence of BPVF-IND with that of BPVF from Brazil (BPVF-BR-PA) and China (BPVF- ZYP-1) revealed an identity of 95% and 90%, respectively, while RNA 2 showed an identity of 96% and 90%. The phylogenetic analysis of the Pro-Pol region of RNA 1 and coat protein region of RNA 2 revealed close clustering of all three BPVF isolates well separated from other species of the genus, *Fabavirus*. Diagnostics assays based on the RT-PCR and RT-recombinase polymerase amplification (RT-RPA) were developed for the sensitive detection of the virus that will help in the identification and propagation of virus-free black pepper plants.

1 | Introduction

Black pepper is the most widely used and highly valued spice worldwide and is native to India. Among the black pepper-producing countries worldwide, India is the largest consumer of black pepper and occupies a third position in production (Sabu, Kuruvila, and Manojkumar 2020). Viral disease is the third important disease of this crop causing yield loss up to 82% depending on the severity of the disease (Bhat et al. 2018). So far, the occurrence of viruses, such as *Piper* yellow mottle virus (PYMoV) (genus: *Badnavirus*; family: *Caulimoviridae*) and cucumber mosaic virus (CMV) (genus: *Cucumovirus*; family: *Bromoviridae*), are reported in black pepper from India and other black pepper growing countries, such as Vietnam, Indonesia, Brazil, Malaysia, Philippines, Sri Lanka, Thailand and China (Lockhart et al. 1997; Sarma et al. 2001; De Silva, Jones, and Shaw 2002). Recently, the occurrence of three additional viruses, namely, black pepper virus F (BPVF) (genus: *Fabavirus*; family: *Secoviridae*), black pepper virus E (BPVE) (genus: *Enamovirus*; family: *Solimovridae*) and black pepper virus B (BPVB) (genus: *Badnavirus*; family: *Caulimoviridae*) was reported from China (Ma et al. 2022). Later, BPVF was also reported from Brazil (Kauffmann et al. 2023). With this in view, we performed RT-PCR tests to check the occurrence of BPVF, BPVE, and BPVB in Indian black pepper and other *Piper* species. Our study indicated the occurrence of only BPVF in India while BPVE and BPVB were absent.

Black pepper virus F (Family: *Secoviridae*; subfamily: *Comovirinae*) is a divergent member of the genus *Fabavirus*. The

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RNA 1 of BPVF 1 codes for a polyprotein that is further processed into four mature proteins such as RNA helicase (HEL), viral protein genome-linked protein (VPg), 3C cysteine proteinase (Pro), and RdRp. Similarly, RNA 2 yields three mature proteins namely, movement protein (MP), large coat protein (LCP), and small coat protein (SCP). RNA 1 and RNA 2 are linked to VPg at the 5′ end and have poly A tail at 3′ ends (Fuchs et al. 2022; Ma et al. 2022; Kauffmann et al. 2023). The present paper describes the occurrence and complete genome sequencing of BPVF from Indian black pepper and the development of RT-PCR and RT-recombinase polymerase amplification (RT-RPA) based diagnostics for the quick and sensitive detection of the virus in plants.

2 | Materials and Methods

2.1 | Virus Isolates

Forty-eight plant samples representing 22 black pepper varieties, 10 black pepper germplasm accessions, and 14 *Piper* species maintained at ICAR-Indian Institute of Spices Research (ICAR-IISR), Kozhikode, Kerala, India were used to study the occurrence of BPVF, BPVE and BPVB in India (Table S1). A known BPVF-infected black pepper plant belonging to the variety, Panniyur-7 maintained through vegetative propagation in the greenhouse at the ICAR-Indian Institute of Spices Research, Kozhikode, Kerala, India was used for the complete genome sequencing of the virus. The same plant was used as positive control for the standardisation of RT-PCR and RT-RPA assays along with a known virus-free plant that was used as negative control in the assay. The RT-RPA assay was validated using field samples of black pepper plants with and without symptoms.

2.2 | Primer Designing

Two sets of primers each (AIB 872-873 and AIB 874-875 for BPVF; AIB 876-877 and AIB 878-879 for BPVE, and AIB 880-881 and AIB 882-883 for BPVB) were designed for the detection of BPVF, BPVE, and BPVB based on the multiple sequence alignment of corresponding viruses available in the GenBank (Table 1). For the complete genome sequencing of the BPVF isolate from India, eight primer pairs for RNA 1 and five primer pairs for RNA 2, producing overlapping sequences at the ends were designed based on the pairwise comparison of two BPVF isolates (BR-PA and ZYP-1) available in the GenBank (Table 1). The primers (AIB 874-875) designed based on the large coat protein (LCP) sequence were used for the detection of BPVF through RT-PCR. Similarly, the forward (AIB 895) and reverse (AIB 896) primers for RT-RPA were designed based on the multiple sequence alignments of the coat protein sequence of the BPVF isolates (BR-PA, ZYP-1, and IND), as per the guidelines of the RPA reaction kit manufacturer (TwistDx, United Kingdom) (https://www.twistdx.co.uk). All the designed primers were synthesised at the Eurofins, Bangalore, India.

2.3 | Preparation of Template

A crude extract prepared by grinding 50 mg of the leaf sample in 500 μL of 0.5 N NaOH, followed by diluting the ground extract in 0.1 M tris buffer (1:10) was used as a template for the detection of BPVB through PCR (Greeshma, Bhat, and Jeevalatha 2023). Total RNA isolated from the leaf sample using the TRIzol method (Siju, Madhubala, and Bhat 2007) was used as the template in RT-PCR for the detection of BPVF and BPVE and for determining the complete genome sequence of BPVF. Crude extract was prepared as described in Suzuki et al. (2016) in a laminar airflow cabinet by grinding 10 mg of the leaf tissue in 500 μL of 100 mM Tris–HCl (pH 8.0), followed by dilution to 10^{-2} in the same buffer was used as a template for the detection of BPVF by RT-RPA. Both the RNA templates were denatured by heating at 80°C for 10 min followed by rapid cooling in ice for 10 min before use in RT-PCR.

2.4 | PCR, RT-PCR, and Sequencing

The PCR reaction for the detection of BPVB was carried out using 12.5 μL of 2x EmeraldAmp GT Master Mix (Takara Bio Inc., Japan), 10 pM each of forward and reverse primers, 1 μL of template, and 9.5 μL of nuclease-free water in a total reaction volume of 25 μL. The RT-PCR reaction mix for the detection of BPVF and BPVE was comprised of 1x *Taq* buffer with 1.5 mM $MgCl_2$, 10 mM dithiothreitol, 100 μM dNTP mix, 10 pM each of forward and reverse primers, 10 U of RNase inhibitor, 100 U of RevertAid reverse transcriptase, 3 U of *Taq* polymerase, 1 μL template and RNase free water to make a total reaction volume of 50 μL. The temperature conditions used were an initial denaturation temperature of 94°C for 3 min (for BPVB) or 42°C for 45 min for cDNA synthesis (for BPVF and BPVE) followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 48°C–60°C (depending on Tm of the primer pair, see Table 1) for 30 s and extension at 72°C for 1.0–1.5 min (depending on the size of the expected product), and a final extension at 72°C for 10 min. The amplified products were subjected to 1% agarose gel electrophoresis and visualised through the ChemiDoc Imaging System (Biorad Laboratories, USA). RT-PCR products were eluted from the gel, purified, and cloned into pTZ57R/T vector (Thermo Scientific, Lithuania) and sequenced from both directions using the facility at the Eurofins Pvt. Ltd., Bangalore, India.

2.5 | Sequence Analysis

The identity of the sequences was confirmed through blastn and was assembled manually based on the consensus sequences at the overlapping regions. The open reading frames (ORFs) in the assembled sequence were predicted using the NCBI ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder) and translated into an amino acid acid sequence using the Expasy Translate tool (SIB) (https://web.expasy.org/translate/). The complete genome sequence of the two BPVF isolates and other fabaviruses used for the analysis were retrieved from the GenBank (Table S2). The putative cleavage sites within the polyprotein were identified based on comparison with the cleavage sites of BPVF isolates and other fabaviruses. A multiple sequence alignment was performed using Clustal Omega (https://www.ebi.ac.uk/jdispatcher/) and phylogenetic trees were constructed using the maximum-likelihood method in MEGA 11 with a bootstrap analysis of 1000 replicates using the Jones-Taylor-Thornton (JTT) model for the Pro-Pol amino acid (aa) sequence of RNA 1 and the coat protein (CP) amino acid sequence of RNA 2.

TABLE 1 | Details of primers used for the amplification and detection of the black pepper virus F (BPVF), black pepper virus E (BPVE), black pepper virus B (BPVB) through RT-PCR and RT-RPA.

Virus	Primer	Sequence (5′ … 3′)	Nucleotide position (bp) with reference to ON568199 and ON568200	Product size (bp)	Assay
BPVF	AIB 872 (FP)	TGGTATTTGCCGCAACACAG	5132–5988 (RNA 1)	857	RT-PCR
	AIB 873 (RP)	GGCCACTACCCATACGATAG			
	AIB 874 (FP)	TGGGTCTCACAACAAAGGCG	1494–2474 (RNA 2)	981	
	AIB 875 (RP)	CAGCTGGATACACAGAAACAG			
	AIB 926 (FP)	TGGTTAAGAAAATTTCCCAATCTG	1–301 (RNA 1)	301	
	AIB 917 (RP)	ACATATCTCCTCACCCCATC			
	AIB 897 (FP)	CAACACCTAGATTTCGCATCGG	118–1250 (RNA 1)	1133	
	AIB 898 (RP)	CAACATCTGTCACCCATTG			
	AIB 899 (FP)	AACGAGCTTTTGGTCCTAG	1155–2379 (RNA 1)	1225	
	AIB 900 (RP)	CTCACAGCATATGGGGTCGA			
	AIB 901 (FP)	CTGTCTACGATGATTTGGGTGC	2289–3280 (RNA 1)	992	
	AIB 916 (RP)	GTACAACTCGCAGACCTTCTC			
	AIB 903 (FP)	ACAACTATGCGAGAGAAGGTC	3248–4458 (RNA 1)	1211	
	AIB 904 (RP)	GGTCCTGATTCCTGTGCATACT			
	AIB 905 (FP)	CCTAAAGAATGGCATCTTGGC	4316–5462 (RNA 1)	1147	
	AIB 906 (RP)	GAGTCTTATCCACACCATCAG			
	AIB 907 (FP)	CAGTGCACCCATCAATAACCC	5364–6256 (RNA 1)	893	
	AIB 908 (RP)	GGCTTTTGTAGCTTGCATTGTG			
	AIB 920 (FP)	TGATAGTGGTAAGCGTCTGCG	6044–6342 (RNA 1)	299	
	AIB 927 (FP)	TAAAAGAAATAGTAAAGATTTCTCTTTATTCATC	1–370 (RNA 2)	520	
	AIB 922 (RP)	CTTCAGCTGAGGGCAACATAC			
	AIB 909 (FP)	CAGGAGCGTGGTATGCTATAAC	170–1307 (RNA 2)	1138	
	AIB 910 (RP)	CCGCTCTCTTTCCTAAGGT			
	AIB 911 (FP)	CCCTGATATTGTGATACCTTTG	1164–2379 (RNA 2)	1216	
	AIB 912 (RP)	GTCTGAGAAAGGGTTCCACTG			
	AIB 913 (FP)	ACTGTTACTGGGGCACAATC	2276–3291 (RNA 2)	1016	
	AIB 914 (RP)	GCAACAAGAAATGCAGCAAGG			
	AIB 924 (FP)	TTATGAACACGAAGTGGCAC	2973–3334 (RNA 2)	362	
	Oligo dT anchor primer	GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV			
	AIB 895 (FP)	ACATGCCACCAAACATCTTCAATGAGCTAG	1709–1912 (RNA 2)	204	RT-RPA
	AIB 896 (RP)	GCATCTCATTGTCAGGTGCCTGCAACCATTCAG			
BPVE	AIB 876 (FP)	TCGGACAACTCTTCAACGGG	2424–3382 (ORF 2)	959	RT-PCR
	AIB 877 (RP)	AGTGAGTGCGTTCCTGACTG			
	AIB 878 (FP)	AACACGGAAACGATCCAACG	4034–4593 (ORF 3)	560	
	AIB 879 (RP)	TACGTGTCACCAACCGGAAG			

(Continues)

TABLE 1 | (Continued)

Virus	Primer	Sequence (5′ … 3′)	Nucleotide position (bp) with reference to ON568199 and ON568200	Product size (bp)	Assay
BPVB	AIB 880 (FP)	ATTCGAGTAACCCAGCCAGC	1836–2780 (ORF 3)	945	
	AIB 881 (RP)	TAAGGCCACTTCTTCTCGCC			
	AIB 882 (FP)	ACAGACAGCAGTCGGAACAG	1131–1624 (ORF 2)	495	
	AIB 883 (RP)	TGTACTCGCCGATAGTCACG			

FIGURE 1 | Symptoms observed on black pepper virus F (BPVF) infected plants.

2.6 | Standardisation and Optimization of RT-RPA for the Detection of BPVF

To set the RT-RPA reaction, the supplied freeze-dried reaction pellet of the Twist Amp basic kit was dissolved in 29.5 μL of rehydration buffer, 2.4 μL each of forward and reverse primer (480 nM) (Table 1), 100 U of RevertAid reverse transcriptase (0.5 μL), 10 U of RNase inhibitor (0.25 μL) and 7.45 μL nuclease-free water. The resultant mixture was aliquoted equally (8.5 μL each) into five PCR tubes, and a 1 μL template (crude extract) was added to the tubes. For initiating the RT-RPA reaction, 0.5 μL of 280 mM magnesium acetate solution (14 mM) was added and the tube was incubated for 40 min at 39°C with moderate mixing and spinning briefly after 4 min. The RT-RPA reaction was stopped by incubating the tube at 65°C for 10 min. Initially, to check the specificity of designed primers, three RT-RPA reactions were set up using positive control, negative control, and water control (without a template). RT-RPA products were run on a 2% agarose gel and visualised under the ChemiDoc Imaging System. Reaction conditions were optimised using different concentrations of magnesium acetate (12–20 mM), temperature (37°C–41°C), and different duration of incubation time (10–40 min).

2.7 | Determination of Sensitivity and Specificity of the Assay

To determine the detection limit of RPA and PCR, 10 ng of the recombinant plasmid harbouring BPVF insert was 10-fold serially diluted from 10 ng to 0.001 fg (10^{0} to 10^{-10}) with nuclease-free water, and each dilution was subjected to RPA and PCR. The limit of detection of BPVF was also determined by subjecting each of the 10-fold serially diluted crude extract (10^{0} to10^{-10}) prepared in 0.1 M tris buffer (pH 8) to RT-RPA. The specificity of the assay was determined by including the crude extracts from black pepper plants infected with PYMoV and CMV.

2.8 | Validation of RT-RPA for the Detection of BPVF

For validating the optimised RT-RPA assay, the crude extract isolated from field samples of black pepper was tested by RT-RPA assay along with RT-PCR using total RNA isolated by the TRIzol method as a template.

3 | Results

3.1 | Occurrence of BPVF

Out of 48 plant samples tested, only three samples collected from Panniyur 2, Panniyur 3, and Panniyur 7 varieties of black pepper were positive for BPVF. The symptoms in BPVF-positive plants varied from apparently healthy to mild chlorotic specs and yellow mottles (Figure 1). None of the tested samples showed the presence of BPVE and BPVB. Both primers based on RdRp (AIB 872-873) and LCP (AIB 874-875) regions of BPVF produced bands at the expected size (Figure 2). The identity of amplicons were confirmed through sequencing that showed an identity of 92% and 97% with RdRp, and 97% and 99% with LCP region of BPVF isolates from China and Brazil, respectively.

3.2 | Complete Genome Sequencing and Analysis

The complete genome sequence of RNA 1 and RNA 2 of BPVF from India (BPVF-IND) determined in the present study was

deposited in the NCBI database with accession no: PQ014407 and PQ014408. The complete nucleotide sequence of the BPVF-IND isolate comprised RNA 1 with 6376 nt (GC content 42%), and RNA 2 with 3340 nt (GC content 43%) (Figure 3a,b; Figure S1a,b). RNA 1 and RNA 2 of BPVF-IND contain a single ORF potentially encoding a protein of 230.7 kDa (2049 aa) and 114 kDa (1019 aa) respectively. RNA 1 has 145 nt in the 5′untranslated region (UTR) and 81 nt in the 3′ UTR, while RNA 2 has 147 nt in the 5′-UTR and 133 nt in the 3′-UTR. The four cleavage sites in RNA 1 (AQGQ/F, ATAQ/S, AEGQ/A, ANAQ/C) predicted based on comparison with the cleavage sites of BPVF isolates (BR-PA and ZYP-1) and other fabaviruses yielded five cleavage products, such as an unknown protein (nt 146-1642), helicase (nt 1643-3448), VPg (nt 3449-3532), protease (nt 3533-4177) and RdRp (nt 4178-6295) (Figure 3a,b) (Table S3). Similarly, the two cleavage sites predicted in RNA 2 (AEGQ/A, AEGQ/M) yielded three cleavage products, namely, movement protein (nt 148-1407), large coat protein (nt 1408-2619), and small coat protein (nt 2620-3207) (Table S3). A comparison of the cleavage sites of BPVF with those of known fabaviruses revealed that the cleavage sites are common in all the three BPVF isolates, but differ from the other fabaviruses (Table S4). As reported for other fabaviruses, BPVF also contained conserved sequence motifs (CAGCUUUC) in the 5′-UTR at 57–64 nt position in RNA 1 and 99–106 nt position in RNA 2. Among the different proteins of RNA 1 of BPVF-IND, VPg shared the most identity (31%–71% at aa level) with the other fabaviruses, while unknown protein shared the least identity (19%–30% at aa level) (Table S5). Among the proteins of RNA 2, the identity shared is almost similar (20%–28% at the aa level) with other fabaviruses (Table S6).

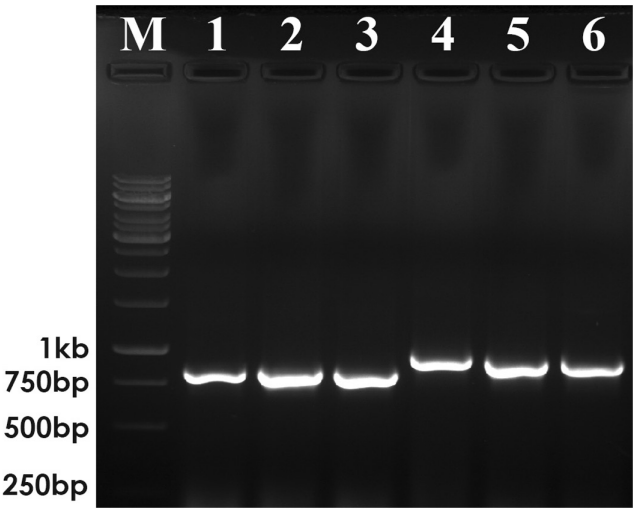

FIGURE 2 | Detection of black pepper virus F (BPVF) by reverse transcription-polymerase chain reaction (RT-PCR) in black pepper. Lane M: Molecular weight marker; Lanes 1–3 and 4–6 are loaded with RT-PCR products obtained with primers AIB 872-873 and AIB 874-875 in Panniyur 2, Panniyur 3 and Panniyur 7 varieties of black pepper, respectively. The details of the primers are provided in the Table 1.

The ICTV species demarcation criteria for the members of the family *Secoviridae* are amino acid sequence identity of < 80% for Pro-Pol and < 75% for CP (Fuchs et al. 2022). In the present study, the RNA 1 nucleotide and polyprotein sequences of BPVF-IND shared an identity of 95% and 99% with BR-PA, and 90% and 98% with ZYP-1 isolates of BPVF, respectively, while the identity with other fabaviruses ranged from 45% to 48% and 34% to 39% (Table S5). In RNA 2, the nucleotide and polyprotein sequence shared an identity of 96% and 99% with BR-PA and 90% and 97% with ZYP-1 isolates of BPVF. Whereas, it shared an identity of only 37%–41% in nucleotide and 22%–27% in amino acid sequences with other fabaviruses (Table S6). Thus, based on the ICTV guidelines the present isolate is a strain of BPVF. Between the two known strains of BPVF, the BPVF-IND is closer to the strain BR-PA than to the ZYP-1 strain. Phylogenetic tree analysis using amino acid sequences of Pro-Pol of RNA 1 and CP of RNA 2 of BPVF isolates and 10 other fabaviruses showed close clustering of all three BPVF isolates, which was consistent with the results of percent identity analysis (Figure 4a,b). Among different species, grapevine fabavirus (GFabV) is the closest species to BPVF.

3.3 | Development of Diagnostic Assays for BPVF

RT-PCR and RT-RPA assays performed using total RNA isolated by TRIzol method as template were able to detect BPVF in

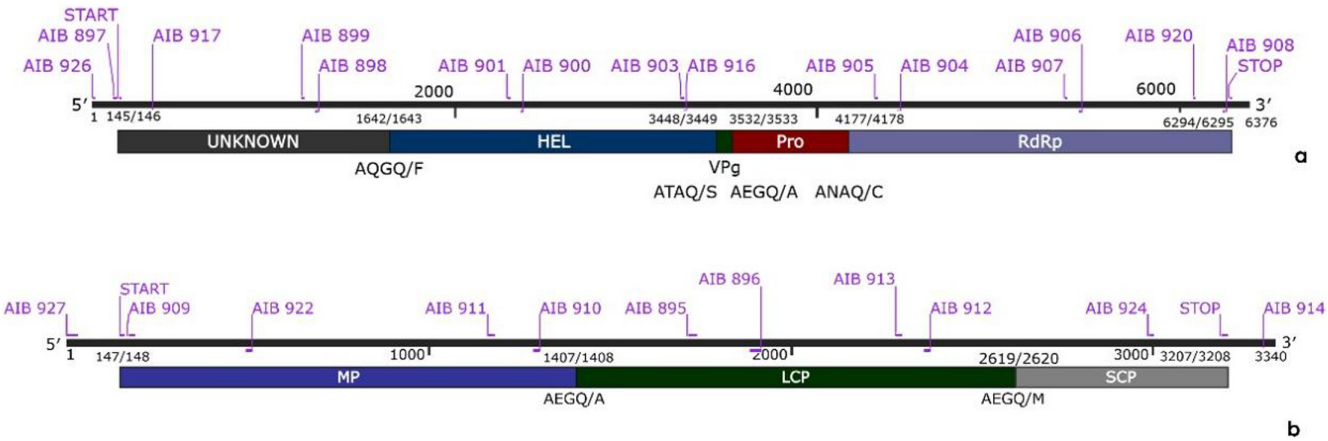

FIGURE 3 | Genome organisation of RNA 1 (a) and RNA 2 (b) of an Indian isolate of black pepper virus F (BPVF-IND). The position of mature proteins within the polyprotein are indicated by boxes, and the corresponding predicted cleavage sites are indicated below the boxes. The position of primers used for the amplification are shown and the details of the primers used are provided in the Table 1.

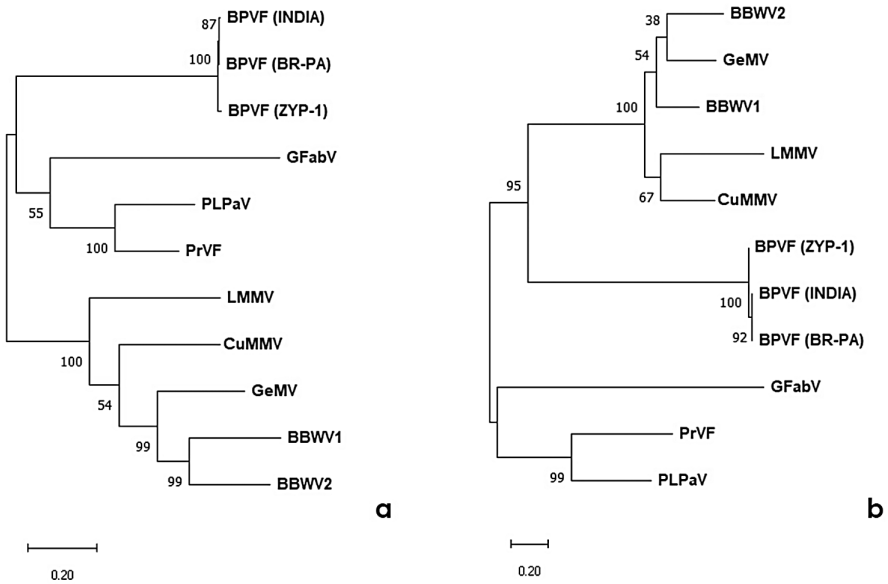

FIGURE 4 | Phylogenetic tree based on the amino acid sequence of (a) Pro-Pol and (b) CP of an Indian isolate of black pepper virus F (BPVF-IND) with other fabaviruses using MEGA 11 by the maximum-likelihood method with Jones-Taylor-Thornton (JTT) models with 1000 bootstrap replicates.

infected sample and, there was no amplification in healthy and water controls (Figure S2a,c). Similar results were observed in RT-RPA, when crude extract was used as a template. However, no amplification was observed when the crude extract was subjected to RT-PCR (Figure S2b,d). A magnesium acetate concentration of 14 mM, temperature of 40°C, and an incubation time of 20 min were found optimum for the detection of BPVF through RT-RPA (Figure S3).

3.4 | Determination of Detection Limit and Specificity of the Assay

The detection limit of BPVF in the RPA assay was 0.01 fg (10^{-9}) using recombinant plasmid DNA as a template (Figure 5a,b) while it was 100 fg (10^{-5}) in the PCR assay (Figure 5c) indicating that RPA is 10^4 times more sensitive than PCR. The detection limit of RT-RPA assay was 10^{-5} when crude extract was used as a template (Figure 5d). RT-RPA performed using crude extracts from PYMoV and CMV-infected black pepper plant did not show amplification indicating that the primers are specific for the detection of BPVF (Figure 5e).

3.5 | Validation of RT-RPA Assay

Validation of RT-RPA assay was carried out using crude extract from field samples of different varieties of black pepper. Thirty-four samples including nine plants with symptoms such as chlorotic specs and yellow mottle, and 25 asymptomatic plants were used in the assay. Of these, 17 plants including all nine symptomatic and eight asymptomatic plants tested positive for BPVF in the RT-RPA assay (Figure 6a,b). As crude extract as a template did not work in RT-PCR, the total RNA isolated from all the 34 plants by the TRIZol method when subjected to RT-PCR also showed similar results (Figure 6c,d).

4 | Discussion

Viral disease is one of the major threats to the productivity of black pepper in all black pepper-producing countries including India. Being perennial and vegetatively propagated plants, the viruses spread easily through planting materials (Bhat et al. 2018). Until now, only PYMoV and CMV were known to be associated with black pepper. Recently based on the next-generation sequencing approach, the occurrence of three additional viruses, namely, BPVF, BPVE, and BPVB, and their complete genome sequence was reported from China (Ma et al. 2022). Subsequently, the occurrence and complete genome sequence of a BPVF isolate from Brazil was also reported based on high throughput sequencing (Kauffmann et al. 2023). India is one of the largest black pepper-producing countries in the world. Given this, the present study was undertaken to check for the occurrence of these three new viruses in Indian black pepper. Out of 48 samples tested that included released varieties of black pepper, germplasm accessions, and different *Piper* species collected and maintained at the ICAR-Indian Institute of Spices Research, Kozhikode, India, only samples from three varieties of black pepper showed the occurrence of BPVF and none of the samples were positive for BPVE and BPVB. The symptoms of BPVF-infected plants varied from asymptomatic to mild chlorotic specs and yellow mottles. None of the other *Piper* spp. (except *P. nigrum*) tested positive for all three viruses. A high percent incidence of BPVF was reported in China (84%) and Brazil (66.66%), while only 6% of the tested population was infected with BPVF in India. To understand the variability between the Indian isolate of BPVF and the other two known isolates, we determined the complete genome sequence of RNA 1 and RNA 2 of the BPVF-IND. Both RNA 1 and RNA 2 of BPVF-IND encode a single large polyprotein that is further cleaved to yield five and three mature proteins respectively by virus-encoded proteases. Percent identity analysis based on the

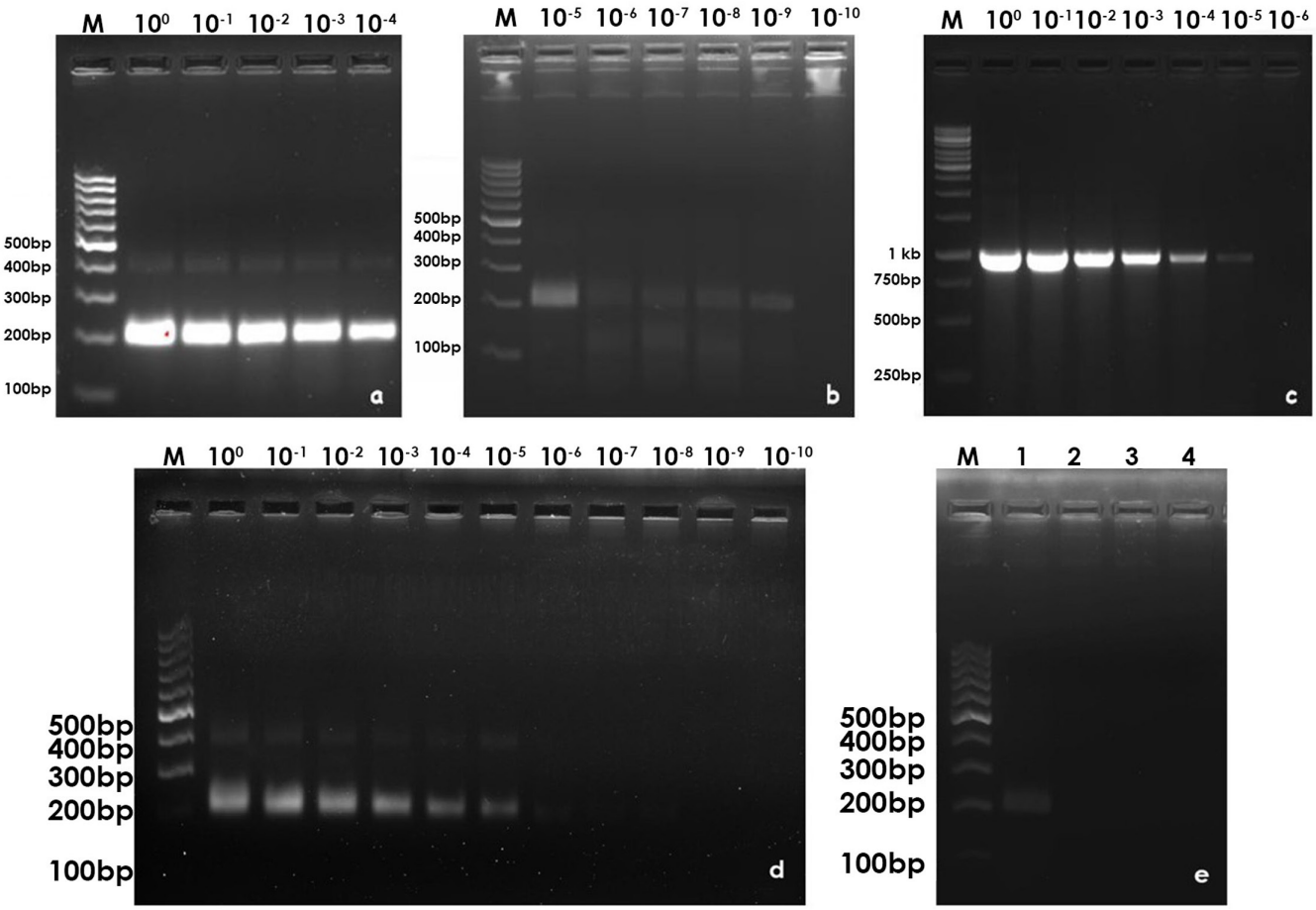

FIGURE 5 | Comparison of the sensitivity of recombinase polymerase amplification (RPA) (a, b) and polymerase chain reaction (PCR) (c) assays for detection of black pepper virus F (BPVF) using recombinant plasmid DNA harbouring BPVF insert. Sensitivity of RT-RPA assay using crude extract as a template (d). Lanes 10^0 to 10^{-10} show different dilutions of the recombinant plasmid DNA/crude extract. Specificity evaluation of RT-RPA for detection of BPVF (e). Lane 1: Known BPVF-infected sample, Lane 2: PYMoV-infected sample, Lane 3: CMV-infected sample, Lane 4: Healthy control. Lane M: Molecular weight markers.

complete RNA 1 and RNA 2 sequence of BPVF-IND revealed a very high identity both at the nucleotide and amino acid level with the other two isolates of BPVF. Among the two isolates of BPVF, BPVF-IND shared maximum identity with BR-PA isolate of BPVF. The low identity shared by BPVF-IND with other fabaviruses confirms that BPVF is a divergent member of the genus *Fabavirus*. In the phylogenetic tree constructed based on Pro-Pol and CP, all three BPVF isolates were clustered together indicating their close relationship.

Management of viral diseases in black pepper plants depends strongly on the detection and elimination of virus-infected plants. The asymptomatic occurrence of the virus in black pepper necessitates the requirement for a specific, sensitive, and affordable diagnostic assay for the production of virus-free plants for propagation (Bhat et al. 2018). RT-PCR requires a well-equipped laboratory for its performance and is time consuming. A rapid isothermal amplification assay, such as RPA, offers a perfect alternative as it can be performed at minimal laboratory conditions (Daher et al. 2016). In the present study, we have developed both RT-PCR and RT-RPA for the detection of BPVF infection. RT-RPA assay developed for BPVF detection in the present study is a promising technique owing to its compatibility for detection using crude extract and shorter incubation time (20 min). No cross reactions were produced against black pepper samples infected with other viruses (PYMoV and CMV) confirming the specificity of the assay. RT-RPA using crude extract as a template could detect the virus up to 10^{-5} dilutions. Furthermore, the assay could detect the virus up to 0.01 fg and was 10^4 times more sensitive than PCR when recombinant plasmid was used as a template. A similar higher sensitivity of RPA was reported for the detection of barley yellow dwarf virus (Kim, Kim, and Jeong 2020), chilli veinal mottle virus (Jiao et al. 2020), citrus concave-gum associated virus (Liu et al. 2021), cucumber green mottle mosaic virus (Jiao et al. 2019) and large cardamom chirke virus (Malavika, Bhat, and Greeshma 2024). RT-RPA was also successful in the detection of BPVF even in asymptomatic plants using crude extract as a template. RT-PCR failed to detect BPVF when the crude extract was used as a template which might be due to the presence of contaminants or PCR inhibitors in the extract. Due to the ease of template preparation, minimal incubation time, and higher sensitivity, the RT-RPA assay developed in the present study is suitable for the identification of BPVF-free black pepper plants in the field, thus reducing the spread of the virus.

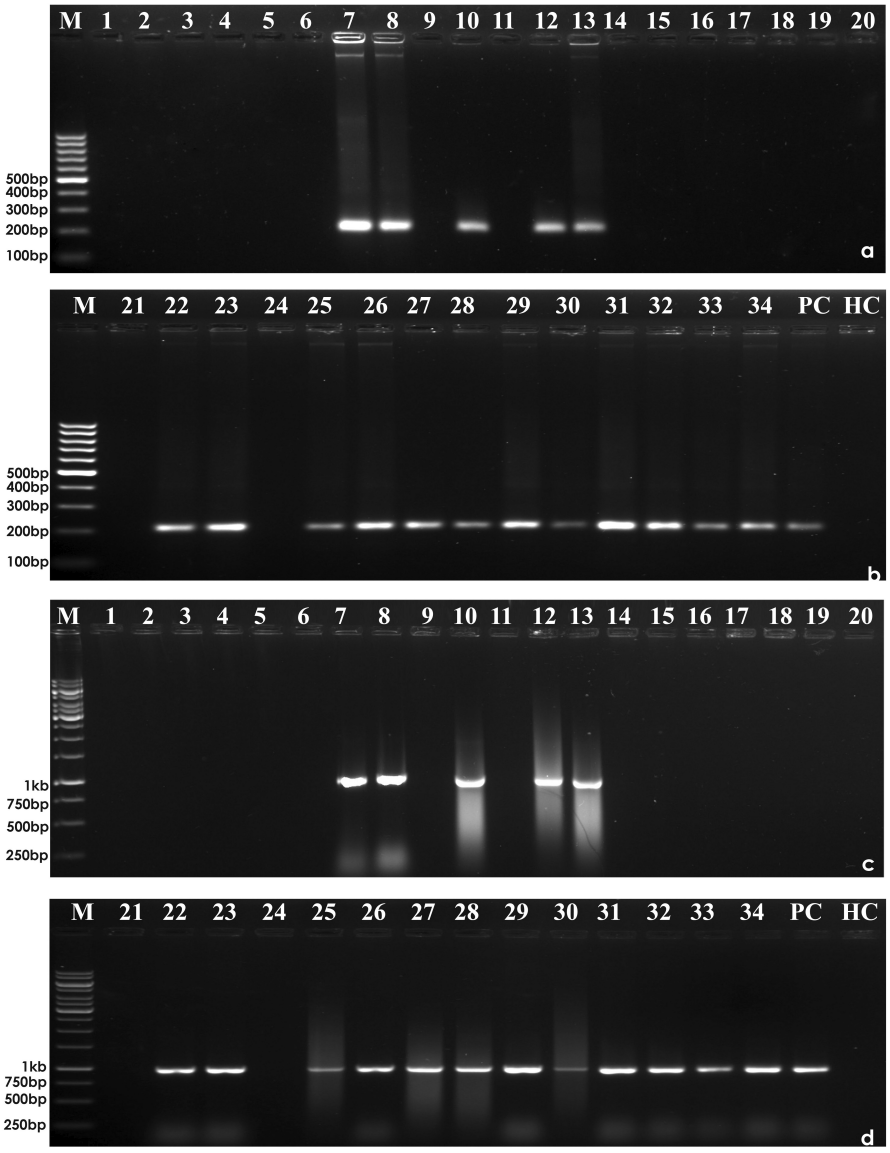

FIGURE 6 | Validation of reverse transcription-recombinase polymerase amplification (RT-RPA) (a, b) and reverse transcription-polymerase chain reaction (RT-PCR) (c, d) for the detection of BPVF in field samples of black pepper. Lanes 1–10 show field samples of Panniyur 2, lanes 11–20 show Panniyur 3 samples and lanes 21–34 show samples of Panniyur 7. Lane PC shows positive control and HC indicates healthy control. Lane M shows molecular weight markers.

Author Contributions

A.I. Bhat conceptualised, designed experiments, and finalised the manuscript; P. Malavika and M. Greeshma performed all experiments and wrote the manuscript; all authors have read and approved the final manuscript.

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Ethics Statement

The present research did not involve any experimentation on humans or animals.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in GenBank at https://www.ncbi.nlm.nih.gov/nucleotide?cmd=search, reference number PQ014407, PQ014408.

Peer Review

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/jph.13418.

References

Bhat, A. I., C. N. Biju, V. Srinivasan, S. J. Ankegowda, and K. S. Krishnamurthy. 2018. "Current Status of Viral Diseases Affecting Black

Pepper and Cardamom." *Journal of Spices and Aromatic Crops* 27, no. 1: 1–16. https://doi.org/10.25081/josac.2018.v27.i1.1009.

Daher, R. K., G. Stewart, M. Boissinot, and M. G. Bergeron. 2016. "Recombinase Polymerase Amplification for Diagnostic Applications." *Clinical Chemistry* 62, no. 7: 947–958. https://doi.org/10.1373/clinchem.2015.245829.

De Silva, D. P. P., P. Jones, and M. W. Shaw. 2002. "Identification and Transmission of *Piper Yellow Mottle Virus* and *Cucumber Mosaic Virus* Infecting Black Pepper (*Piper nigrum*) in Sri Lanka." *Plant Pathology* 51, no. 5: 537–545. https://doi.org/10.1046/j.0032-0862.2002.00757.x.

Fuchs, M., J. M. Hily, K. Petrzik, et al. 2022. "ICTV Virus Taxonomy Profile: *Secoviridae* 2022." *Journal of General Virology* 103, no. 12: 001807. https://doi.org/10.1099/jgv.0.001807.

Greeshma, M., A. I. Bhat, and A. Jeevalatha. 2023. "Rapid Onsite Detection of Piper Yellow Mottle Virus Infecting Black Pepper by Recombinase Polymerase Amplification-Lateral Flow Assay (RPA-LFA)." *Journal of Virological Methods* 315: 114695. https://doi.org/10.1016/j.jviromet.2023.114695.

Jiao, Y., J. Jiang, Y. Wu, and Z. Xia. 2019. "Rapid Detection of Cucumber Green Mottle Mosaic Virus in Watermelon Through a Recombinase Polymerase Amplification Assay." *Journal of Virological Methods* 270: 146–149. https://doi.org/10.1016/j.jviromet.2019.05.008.

Jiao, Y., C. Xu, J. Li, et al. 2020. "Characterization and a RT-RPA Assay for Rapid Detection of Chilli Veinal Mottle Virus (ChiVMV) in Tobacco." *Virology Journal* 17: 1–9. https://doi.org/10.1186/s12985-020-01299-w.

Kauffmann, C. M., A. J. Boari, J. M. Silva, R. Blawid, E. W. Kitajima, and T. Nagata. 2023. "Detection, Characterization, and Occurrence of Black Pepper Virus F in Black Pepper Plants in Brazil." *Tropical Plant Pathology* 48, no. 3: 347–351. https://doi.org/10.1007/s40858-023-00569-6.

Kim, N. K., S. M. Kim, and R. D. Jeong. 2020. "Reverse Transcription Recombinase Polymerase Amplification Assay for Rapid and Sensitive Detection of Barley Yellow Dwarf Virus in Oat." *Plant Pathology Journal* 36, no. 5: 497–502. https://doi.org/10.5423/PPJ.NT.08.2020.0148.

Liu, Z., Z. Dong, B. Zhan, and S. Li. 2021. "Characterization of an Isolate of Citrus Concave Gum-Associated Virus From Apples in China and Development of an RT-RPA Assay for the Rapid Detection of the Virus." *Plants* 10, no. 11: 2239. https://doi.org/10.3390/plants10112239.

Lockhart, B. E. L., K. Kiratiya-Angul, P. Jones, et al. 1997. "Identification of *Piper Yellow Mottle Virus*, a Mealybug-Transmitted Badnavirus Infecting *Piper* spp. in Southeast Asia." *European Journal of Plant Pathology* 103: 303–311. https://doi.org/10.1023/A:1008699414536.

Ma, Y., F. Xing, H. Che, S. Gao, Y. Lin, and S. Li. 2022. "The Virome of *Piper nigrum*: Identification, Genomic Characterization, Prevalence, and Transmission of Three New Viruses of Black Pepper in China." *Plant Disease* 106, no. 8: 2082–2089. https://doi.org/10.1094/PDIS-12-21-2692-RE.

Malavika, P., A. I. Bhat, and M. Greeshma. 2024. "Development of Reverse Transcriptase-Recombinase Polymerase Amplification (RT-RPA) Assay for Rapid Detection of Large Cardamom Chirke Virus." *VirusDisease* 1-8: 302–309. https://doi.org/10.1007/s13337-024-00861-2.

Sabu, S. S., A. Kuruvila, and K. Manojkumar. 2020. "Status of Production and Export of Indian Black Pepper." *Indian Journal of Arecanut, Spices and Medicinal Plants* 22, no. 4: 9–20.

Sarma, Y. R., G. Kiranmai, P. Sreenivasulu, et al. 2001. "Partial Characterization and Identification of a Virus Associated With Stunt Disease of Black Pepper (*Piper nigrum*) in South India." *Current Science* 20: 459–462.

Siju, S., R. Madhubala, and A. I. Bhat. 2007. "Sodium Sulphite Enhances RNA Isolation and Sensitivity of *Cucumber Mosaic Virus* Detection by RT-PCR in Black Pepper." *Journal of Virological Methods* 141, no. 1: 107–110. https://doi.org/10.1016/j.jviromet.2006.11.032.

Suzuki, R., S. Fukuta, Y. Matsumoto, et al. 2016. "Development of Reverse Transcription Loop-Mediated Isothermal Amplification Assay as a Simple Detection Method of *Chrysanthemum Stem Necrosis Virus* in Chrysanthemum and Tomato." *Journal of Virological Methods* 236: 29–34. https://doi.org/10.1016/j.jviromet.2016.07.005.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.