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ORIGINAL ARTICLE

Genetic diversity, mating type and pathogenicity of two *Phytophthora* species infecting black pepper in India

Fathimath Zumaila[1,2] · A. Jeevalatha[1] · C. N. Biju[1]

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Abstract

Phytophthora capsici and *P. tropicalis* are the two species of *Phytophthora* associated with foot rot disease of black pepper in India. High genetic diversity amongst the *Phytophthora* species contributes to its wide host range and variability in the virulence pattern. In the present study, genetic diversity of *Phytophthora* species infecting black pepper was analysed using RAMS (Random Amplified Microsatellites) and REP (Repetitive Extragenic Palindromic)-PCR fingerprinting. Forty-eight isolates, 24 each of *P. capsici* and *P. tropicalis* collected from major black pepper growing states, such as Karnataka, Kerala, Tamil Nadu and Goa, were used in the study. The analyses revealed a total of 160 loci of which 150 (93.75%) were polymorphic. UPGMA cluster and PCoA analysis based on combined RAMS and REP-PCR data clearly grouped the *P. capsici* and *P. tropicalis* isolates into two clusters which were further divided into four sub-clusters viz., I & II (*P. capsici*) and III & IV (*P. tropicalis*). The study clearly indicated that all the isolates were genetically unique and the entire population was heterogeneous. REP-PCR primers showed more polymorphic loci than RAMS primers. Further, sixteen isolates were selected for morphological and infectivity analyses under in vitro conditions. The isolates exhibited varied colony morphology, sporangial shapes and belonged to A1 mating type. Under in vitro conditions, all the sixteen black pepper *Phytophthora* isolates could infect nutmeg, tomato, chilli, pumpkin, and cucumber and few of the isolates could infect cardamom. None of the isolates could infect coconut, areca nut and vanilla.

Keywords *P. capsici* · *P. tropicalis* · *Piper nigrum* · RAMS · REP-PCR

Introduction

Black pepper is one of the spice crops with paramount role in the global market and is cultivated across many countries. India being the fifth largest producer accounts for about 66,000 metric tonnes as of 2019 data (FAO 2023). Major producers of black pepper in India are Karnataka, Kerala and Tamil Nadu. In Karnataka, black pepper was cultivated in 211,497 ha with an annual production of 36,000 tonnes followed by Kerala 82,124 ha with 22,000 tonnes and Tamil Nadu 6,576 ha with 1750 tonnes in 2020–21 (Spices Board 2023). According to surveys, the cultivation has been reduced to one-third due to the deleterious disease foot rot (Hema et al. 2007) and it is one amongst the major problems in black pepper production. Being a highly valued commercial crop and its immense trade value in the market, its cultivation is very important and its production constraints are a matter of concern.

Phytophthora is detrimental to its host under epiphytotic conditions and it infects majority of the horticultural crops with economic importance. It is an oomycete soil-borne pathogen which reproduces both sexually and asexually that favours easy propagation and persistence in a population under field conditions. Although sexual reproduction has a major role in genetic recombination, inter-specific hybridization (Donahoo and Lamour 2008), hyphal anastomosis (Stephenson et al. 1974), parasexual recombination (Gu et al. 2000) and migration add to the genetic diversity within the

✉ A. Jeevalatha
A.Jeevalatha@icar.gov.in

Fathimath Zumaila
fathimathzumaila@gmail.com

C. N. Biju
bijucn123@rediffmail.com

[1] Division of Crop Protection, ICAR-Indian Institute of Spices Research, Kozhikode 673012, Kerala, India

[2] Department of Botany, University of Calicut, Malappuram 673635, Kerala, India

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population. The genetic diversity ultimately alters the virulence pattern and rewards the pathogen with better adaptability to adverse environmental variations. The selection pressure and the mutations within the population often lead to the prevalence of fungicide-resistant isolates (Pereira et al. 2020). Unravelling the genetic makeup at the molecular level helps in better understanding of the pathogen diversity and to design a successful management strategy (Burdon 1993).

Genetic diversity studies give an insight on the genetic composition that exists within a population. Variability often directs towards better adaptability of the individuals in the changing environmental conditions. Various approaches reported to analyse the genetic diversity of a population include isozyme analysis (El-Esawi et al. 2017), AFLP (Markert et al. 2010), RAPD (Fu et al 2003), SNP marker (Jara-Arancio et al. 2022), mtDNA analysis (Wang et al. 2009), RAMS and REP-PCR analyses (Truong et al. 2010). The present study employed the benefits of RAMS and REP-PCR as these have proved to be ideal candidates to delineate the genetic diversity due to its precise reproducibility. RAMS (random amplified microsatellites) technique was developed by Zietkiewicz et al. (1994) and it is successfully used to study genetic diversity in fungi (Hantula et al. 1996). RAMS analysis combines the SSR and RAPD techniques to overcome the drawback of each of these techniques and to increase specificity of the analysis.

Short intergenic repeated sequence was initially identified in *Escherichia coli* (Shirzad-Aski et al. 2016) and *Salmonella typhimurium* which had highly conserved inverted repeats, such as REP (Repetitive Extragenic Palindromic) and ERIC (Enterobacterial Repetitive Intergenic Consensus) sequence fingerprinting. Since the agarose gel analysis of these PCR products produced unique fingerprints for closely related strains, it can be used to study the genetic diversity between them (de Bruijn 1992). Box elements are the combinations of three subunits, such as boxA, boxB and boxC, which are 59, 45 and 50 nucleotides long, respectively (Bilung et al. 2018; Mishra et al. 2015). Box elements have a role in the regulation of transcription (Borba et al. 2020). Arbitrarily primed PCR fingerprint can be obtained faster, and no prior knowledge on the sequence information is required (Welsh et al. 1991). REP, ERIC, BOX and M13 were used successfully to study the genetic diversity in *Phytophthora* species (Masanto et al. 2019; Truong et al. 2012).

In India, two species of *Phytophthora* viz., *P. capsici* and *P. tropicalis* are associated with the foot rot disease of black pepper (Bhai et al. 2022; Jeevalatha et al. 2021). Recently, Bhai et al. (2022) studied the diversity amongst the isolates of these two species infecting black pepper through ITS rDNA sequencing, ITS-RFLP, SSCP and MLST analyses. In the present study, we aimed to explore the genetic diversity of *P. capsici* and *P. tropicalis* infecting black pepper in India using RAMS and REP-PCR fingerprinting besides identifying potentiality of these isolates to infect other crops.

Materials and methods

Phytophthora cultures and species identification

One hundred and fifty three *Phytophthora* isolates of black pepper collected from major black pepper growing areas of Kerala, Karnataka, Tamil Nadu and Goa during the period 1997 to 2021 were used in species identification. These cultures were obtained from National Repository of *Phytophthora* at ICAR—Indian Institute of Spices Research, Kozhikode, Kerala, India. The isolates were cultured in carrot agar medium (Brasier 1967) and maintained at 25 ± 1 °C. Ypt1 gene-based species-specific primers (Jeevalatha et al. 2021) were used in a PCR assay to identify these isolates at species level. The result of species identification was compared with earlier reports of Bhai et al. (2022) who used ITS rDNA sequencing and ITS-RFLP for identification of *Phytophthora* species infecting black pepper. Forty-eight isolates, 24 isolates each of *P. capsici* and *P. tropicalis* listed in Table 1 were selected based on the location (Fig. 1) and year of collection for the genetic diversity analysis.

DNA extraction

Agar plugs from 3-day-old cultures were inoculated to Ribeiro's broth (Erwin and Ribeiro 1996) and actively growing mycelia were harvested on the 4th day, and blot-dried on sterile filter paper. DNA extraction from *Phytophthora* mycelia was carried out according to Sheji et al. (2009) with slight modifications (Jeevalatha et al. 2021).

RAMS and REP-PCR analyses

The details of primers used for RAMS and REP-PCR analyses are listed in Table 2. PCR amplification was carried out using four RAMS primers in 20 µl reaction volume containing 10 µl of Emerald Amp GT PCR Master Mix (2X Premix; Takara Bio Inc, Shiga, Japan), 0.8 µM of primer and 20 ng of DNA. Thermal cycling was performed in Gradient Thermal Cycler IG-96GEP (iGene Labserve, New Delhi, India) following the thermal cycle conditions given in Table 2 (Hantula et al. 1996). The amplicons were run in 2.5% agarose gel in TAE buffer at 80 V for 3 h and visualised under UV trans-illuminator. PCR amplification using REP-PCR markers was carried out in 25 µl reaction volume containing, 12.5 µl of Emerald Amp GT PCR Master Mix (2X Premix; Takara Bio Inc., Shiga, Japan), 0.4 µM primer

Table 1 Details of *Phytophthora* isolates included in the present study

S. No.	Name of species	Isolate	Isolation source	Place of collection	State/UT
1	*Phytophthora capsici*	98–81	Black pepper leaf	Kalpakancherry, Malappuram	Kerala
2		98–164	Black pepper leaf	Kargode, Sirsi, Uttara Kannada	Karnataka
3		00–42	Black pepper	Sirsi, Uttara Kannada	Karnataka
4		01–04	Black pepper stem	Adivaram, Kozhikode	Kerala
5		02–20	Rhizosphere soil	Silent Valley, Palakkad	Kerala
6		05–06	Black pepper spike	Peruvannamuzhi, Kozhikode	Kerala
7		05–14	Black pepper leaf	Appangala, Kodagu	Karnataka
8		05–19	Rhizosphere soil	Chettalli, Kodagu	Karnataka
9		06–12	Black pepper leaf	Irikkur, Kannur	Kerala
10		07–03	Black pepper leaf	Chelavoor, Kozhikode	Kerala
11		07–06	Black pepper leaf	Wayanad	Kerala
12		08–01	Black pepper root	Peruvannamuzhi, Kozhikode	Kerala
13		10–02	Black pepper leaf	Bommanahally, Bangalore	Karnataka
14		11–13	Black pepper leaf	Kargunda, Kodagu	Karnataka
15		11–16	Black pepper leaf	Cherambane, Kodagu	Karnataka
16		13–17	Rhizosphere soil	Meppadi, Wayanad	Kerala
17		13–21	Black pepper leaf	Adimali, Idukki	Kerala
18		13–41	Black pepper leaf	Madikai, Kasaragod	Kerala
19		13–42	Black pepper leaf	Madikai, Kasaragod	Kerala
20		13–48	Black pepper root	Goa	Goa
21		18–12	Black pepper leaf	Idukki	Kerala
22		20–05	Black pepper leaf	Panniyur, Kannur	Kerala
23		20–06	Black pepper leaf	Ambalavayal, Wayanad	Kerala
24		21–01	Black pepper leaf	Panniyur, Kannur	Kerala
25	*Phytophthora tropicalis*	97–19	Black pepper stem	Kottakkal, Malappuram	Kerala
26		97–55	Black pepper root	Valparai, Coimbatore	Tamil Nadu
27		98–65	Black pepper leaf	Valnoor, Kodagu	Karnataka
28		98–93	Black pepper leaf	Adyanadka, Dakshin Kannad	Karnataka
29		98–175	Black pepper root	Isloor, Sirsi, Uttara Kannada	Karnataka
30		99–136	Black pepper leaf	Silver Cloud, Wayanad	Kerala
31		00–38	Black pepper	Sidapur, Kodagu	Karnataka
32		01–20	Black pepper	Silent Valley, Palakkad	Kerala
33		03–07	Black pepper leaf	Adivaram, Kozhikode	Kerala
34		05–17	Black pepper leaf	Chettalli, Kodagu	Karnataka
35		06–11	Black pepper berries	Edayannur, Iritti, Kannur	Kerala
36		06–17	Black pepper leaf	Rasi Estate, Yercaud, Salem	Tamil Nadu
37		08–07	Black pepper stem	Koorachundu, Kozhikode	Kerala
38		09–01	Black pepper stem	Peruvannamuzhi, kozhikode	Kerala
39		09–18	Rhizosphere soil	Pamathatt, Kasaragod	Kerala
40		09–25	Rhizosphere soil	Kanjirapuzha, Palakkad	Kerala
41		11–20	Black pepper leaf	Sunnapulikote, Kodagu	Karnataka
42		11–29	Rhizosphere soil	Wayanad	Kerala
43		11–30	Rhizosphere soil	Wayanad	Kerala
44		13–23	Rhizosphere soil	Nedumkandam, Idukki	Kerala
45		13–33	Rhizosphere soil	Kumali, Idukki	Kerala
46		13–45	Black pepper leaf	Pilicode, Kasaragod	Kerala
47		13–53	Black pepper root	Goa	Goa
48		16–06	Black pepper leaf	Appangala	Karnataka

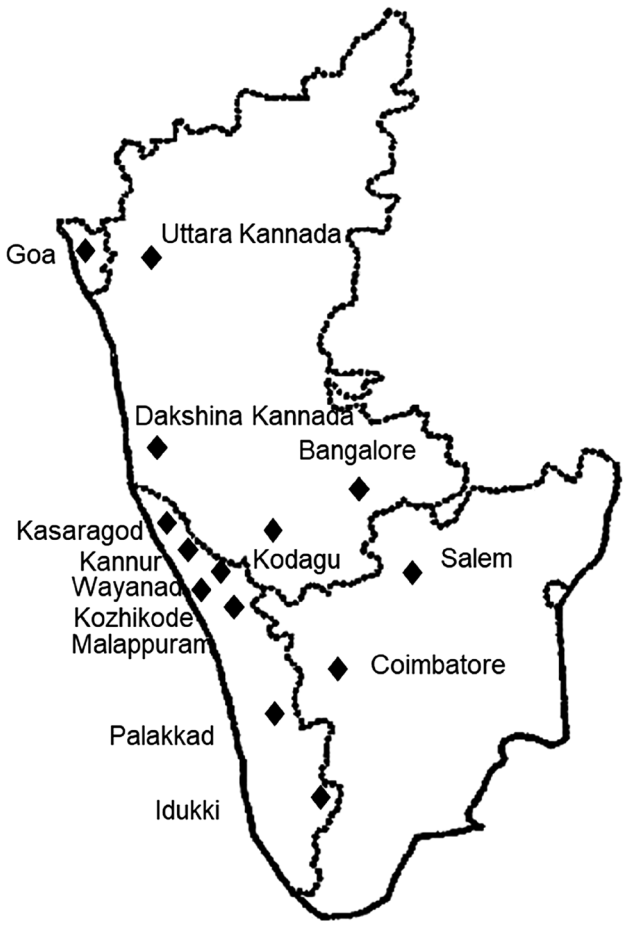

Fig. 1 Map of South India showing the location of collection of the isolates across four states: Kerala, Karnataka, Tamil Nadu and Goa

and 20 ng DNA template. Thermal cycling was performed using Gradient Thermal Cycler IG-96GEP (iGene Labserve, New Delhi, India) following the temperature profile given in Table 2. The amplicons were run in 1.5% agarose gel in TAE buffer at 80 V for 3 h and visualised under UV trans-illuminator.

Genetic diversity analysis

RAMS and REP-PCR generated bands were scored as either present (1) or absent (0). Strong and reproducible bands were scored and used in the analysis. Genetic diversity analysis was performed for RAMS data alone, REP-PCR data alone as well as combining the scoring patterns of both RAMS and REP-PCR data. Unweighted Pair-Group Method using Arithmetic Averages (UPGMA) clustering was performed using DICE similarity coefficient in NTSYSpc Version 2.10e (Rohlf 2000). Genetic diversity indices were calculated using POPGENE version 1.32 (Yeh et al. 1997). The binary data obtained from RAMS, REP-PCR and combined RAMS & REP-PCR analyses were subjected to principal coordinate analysis (PCoA) using GenAlEx 6.5 software with default parameter settings (Peakall and Smouse 2006, 2012).

Colony morphology and sporangia characters

Sixteen isolates representing different sub-clusters of UPGMA dendrogram constructed based on combined RAMS and REP-PCR data were selected for further study. The pure cultures of the isolates were grown on carrot agar media at 25 ± 1 °C. The colony morphology patterns were photographed on the fifth day after inoculation. The sporangial characters were studied using the 3-day-old cultures. Five mm discs of actively growing cultures were placed in a Petri plate containing sterile distilled water and incubated at 25 ± 1 °C under illumination for 48 h. The sporangial production was checked and photographed using Leica DM 5000 B microscope (Leica Mikrosystems Vertrich GmbH, Germany).

Mating type determination

Determination of mating type of *Phytophthora* isolates was carried out on 10% clarified V8 juice agar (Li et al. 2017). The isolates were paired with known A1 isolate (ATCC-MYA- 2338) and A2 isolate (ATCC-MYA-4034) of *P. capsici* obtained from American Type Culture Collection, Manassas, USA (www.atcc.org) and incubated at 25 ± 1 °C in dark for up to 10 days. The plates were observed for the presence of oospores at the junction of the two colonies using Leica DM 5000 B microscope (Leica Mikrosystems Vertrich GmbH, Germany). The pairing between the same isolates served as control. All the pairing experiments were performed in triplicates.

Infectivity analysis

Eight isolates each from *P. capsici* and *P. tropicalis* species representing different sub-clusters were selected for infectivity analysis. Infectivity of *P. capsici* and *P. tropicalis* isolates was tested on black pepper and also on other crops, such as arecanut, tomato, pumpkin, vanilla, nutmeg, coconut, chilli, cucumber and cardamom, using leaf /fruit inoculation method under in vitro conditions. Either the leaf or the fruit was inoculated with 5 mm disc obtained from 3-day-old actively growing *Phytophthora* cultures and incubated in moist chamber at 25 ± 1 °C. The experiment was performed in triplicates. The symptom development was monitored for up to 5 days. The length and the width of the lesion were measured, and the area of lesion at 48 h after inoculation was computed (da Silva et al. 2012) using the following formula:

Table 2 Primer sequences and cycle conditions of RAMS and REP-PCR

Primer	Sequence	Temperature cycle	References
RAMS			
CCA	5'DD$(CCA)_5$	95 °C 10 min 95 °C 30 s 64 °C 45 s 72 °C 2 min 72 °C 7 min- 35 cycles	Hantula et al. (1996)
GT	5'YHY$(GT)_5$G	95 °C 10 min 95 °C 30 s 53 °C 45 s 72 °C 2 min 72 °C 7 min- 35 cycles	Hantula et al. (1996)
ACA	5'BDB$(ACA)_5$	95 °C 10 min 95 °C 30 s 49 °C 45 s 72 °C 2 min 72 °C 7 min- 35 cycles	Hantula et al. (1996)
CGA	5'DHB$(CGA)_5$	95 °C 10 min 95 °C 30 s 60 °C 45 s 72 °C 2 min 72 °C 7 min- 35 cycles	Hantula et al. (1996)
REP-PCR			
BOX1A	5′-CTACGGCAAGGCGACGCTGACG-3′	95 °C 7 min, 94 °C 1 min, 53 °C 1 min, 65 °C 8 min, 65 °C 16 min- 30 cycles	Versalovic et al. (1991)
ERIC1R	5′-ATGTAAGCTCCTGGGGATTCAC-3′		
ERIC2	5′-AAGTAAGTGACTGGGGTGAGCG-3′	95 °C 7 min 94 °C 1 min 52 °C 1 min 65 °C 8 min 65 °C 16 min- 30 cycles	Versalovic et al. (1991)
REP1R-I	5′-IIIICGICGICATCIGGC-3′		
REP2-I	5′-ICGICTTATCIGGCCTAC-3′	94 °C 7 min 94 °C 1 min 40 °C 1 min 65 °C 8 min 65 °C 16 min- 35 cycles	Versalovic et al. (1993)
M13	5′-GAGGGTGGCGGTTCT-3′	93 °C 3 min 93 °C 1 min 55 °C 1 min 72 °C 1 min 72 °C 10 min- 45 cycles	Zaccardelli et al. (2008)

$$S = \pi (L \times W)/4$$

where S is the surface area of lesion, L is the length of lesion and W is the width of lesion. ANOVA was performed with R studio R version 4.2.1 (R Core Team 2022).

Results

Species identification

PCR analysis of 153 *Phytophthora* isolates using species-specific primers showed that 71 isolates belonged to *P. capsici* and 82 isolates to *P. tropicalis.* Of the 92 isolates collected from Kerala across seven districts, 49 isolates were *P. capsici* and 43 isolates were *P. tropicalis*. Out of 52 isolates collected from 4 districts of Karnataka, 20 isolates were *P. capsici* and 32 isolates were *P. tropicalis.* All the three isolates collected from Tamil Nadu were of *P. tropicalis* and out of 6 isolates from Goa, 2 were *P. capsici* and 4 were *P. tropicalis.* The results of ypt1 gene-based species-specific PCR assay were similar to the results obtained using ITS rDNA sequencing and ITS-RFLP assay to differentiate *P. capsici* and *P. tropicalis* isolates of black pepper (Bhai et al. 2022).

RAMS and REP-PCR analyses

RAMS analysis of 48 isolates including *P. capsici* and *P. tropicalis* revealed 71 loci, of which Kerala isolates accounted for about 88.73% polymorphism, Karnataka for 83.1%, Tamil Nadu for 11.27% and Goa for 49.3%. The combined data for all states showed 90.14% of polymorphic loci. A total of 89 loci were revealed through REP-PCR analysis and Kerala isolates accounted for 96.63% of polymorphic loci, Karnataka for 78.65%, Tamil Nadu for 8.9% and Goa for 41.57%. Combined results showed that 96.63% of the loci were polymorphic. The combined RAMS and REP-PCR data revealed 160 loci and 93.75% were polymorphic in isolates from all states. Shannon diversity index and Nei's gene diversity of Kerala population were 0.4414 and 0.2892, respectively and those for Karnataka isolates were 0.4100 and 0.2717, respectively (Table 3). Agarose gel images of RAMS and REP-PCR analyses are given in Supplementary Fig. 1.

The diversity parameters for each primer were also computed individually (supplementary table1) and it was found that amongst RAMS primers, greater polymorphism was observed for GT (100%) and ACA primer revealed lowest polymorphism (80.95%). Whereas in case of REP-PCR primers, loci amplified by BOX1, M13 and Rep-1R & Rep-2I primers were of 100% polymorphic with 28, 21 and 23 loci, respectively. ERIC1R & ERIC2 primers showed 82.35% polymorphic loci.

UPGMA cluster analysis based on combined RAMS & REP-PCR data clearly grouped *P. capsici* and *P. tropicalis* isolates into two clusters which was further divided into four sub-clusters I, II, III & IV (Fig. 2). *P. capsici* isolates were grouped in sub-clusters I and II, whereas *P. tropicalis* spp. were grouped in sub-clusters III and IV. The isolates from each state were distributed randomly in the population group and hence we could not correlate the cluster generated with

Table 3 Genetic variations and polymorphic features of 24 isolates each of *P. capsici* and *P. tropicalis* infecting black pepper

Marker	No. of isolates	Total no. loci	No. of polymorphic loci (% of total)	Nei gene diversity (h)	Shannon diversity index (I)	Population group*
RAMS						
Combined (all states)	48	71	64 (90.14)	0.3204	0.4751	I, II, III, IV
Kerala	30	71	63 (88.73)	0.3128	0.4654	I, II, III, IV
Karnataka	14	71	59 (83.10)	0.3124	0.4605	I, III, IV
Tamil Nadu	2	71	8 (11.27)	0.0467	0.0681	III
Goa	2	71	35 (49.30)	0.2042	0.2981	I, IV
REP-PCR						
Combined (all states)	48	89	86 (96.63)	0.2660	0.4158	I, II, III, IV
Kerala	30	89	86 (96.63)	0.2704	0.4223	I, II, III, IV
Karnataka	14	89	70 (78.65)	0.2392	0.3698	I, III, IV
Tamil Nadu	2	89	8 (8.99)	0.0372	0.0544	III
Goa	2	89	37 (41.57)	0.1722	0.2514	I, IV
RAMS + REP − PCR						
Combined (all states)	48	160	150 (93.75)	0.2901	0.4421	I, II, III, IV
Kerala	30	160	149 (93.12)	0.2892	0.4414	I, II, III, IV
Karnataka	14	160	129 (80.62)	0.2717	0.4100	I, III, IV
Tamil Nadu	2	160	16 (10.00)	0.0414	0.0605	III
Goa	2	160	72 (45.00)	0.1864	0.2721	I, IV

*Population group I and II—*P. capsici*, III and IV—*P. tropicalis*

the geographical origin of the isolates. The isolates which were grouped closer showed up to 97% similarity, which indicated that the isolates were unique and the population was found to be heterogeneous.

REP-PCR analysis could alone differentiate *P. capsici* and *P. tropicalis* and group them into separate clusters (Supplementary Fig. 2a) as observed in dendrogram based on combined RAMS and REP-PCR data. But, in RAMS dendrogram, slight variation was observed. Sub-cluster II of *P. capsici* (with three isolates) was found to form major cluster with sub-clusters III and IV of *P. tropicalis* isolates (Supplementary Fig. 2b). However, four sub-clusters were maintained in RAMS dendrogram also.

Principal coordinate analysis

PCoA analysis was carried out based on the distance matrix with data standardisation for 71, 89 and 160 loci generated by RAMS, REP-PCR and combined RAMS & REP-PCR, respectively. In case of combined RAMS & REP-PCR, the first three coordinates explained 84.51% cumulative variation. The topology obtained by PCoA analysis was similar to UPGMA dendrogram. The scatter plot divided the 48 isolates into four groups i.e. clusters I & II comprising *P. capsici* isolates (indicated by blue circle) and clusters III & IV comprising *P. tropicalis* isolates (indicated by green circle) (Fig. 3). PCoA analysis using RAMS data alone explained 76.40% cumulative variation in first three coordinates. The topology obtained by PCoA analysis was slightly different than UPGMA dendrogram. *P. tropicalis* isolates were found in two separate clusters as observed in UPGMA dendrogram. However, in case of *P. capsici* isolates, the isolates were not clustered as observed in UPGMA dendrogram (Supplementary Fig. 3a). PCoA analysis using REP-PCR data alone explained 87.58% cumulative variation in first three coordinates. The topology obtained by PCoA analysis based on REP-PCR data was similar to UPGMA dendrogram (Supplementary Fig. 3b).

Colony morphology and sporangia characters

Colony morphology studies of sixteen isolates (8 isolates each of *P. capsici* and *P. tropicalis*) showed that the isolates exhibited different growth patterns like modified chrysanthemum (PC98-81, PC05-06, PC07-03 and PT98-93), stellate with concentric rings (PC01-04), cottony with concentric rings (PC02-20), modified stellate (PC06-12, PC18-12, PT03-07, PT09-01 and PT11-29), stellate (PC20-05), modified petaloid (PT06-17) and stellate with cottony mycelium (PT13-23 and PT13-53) (Fig. 4). The sporangia produced by the isolates were papillate and exhibited a variety of shapes like naviculate, globose, elongated, elliptical, pyriform, limoniform, ovoid-obpyriform, ovoid

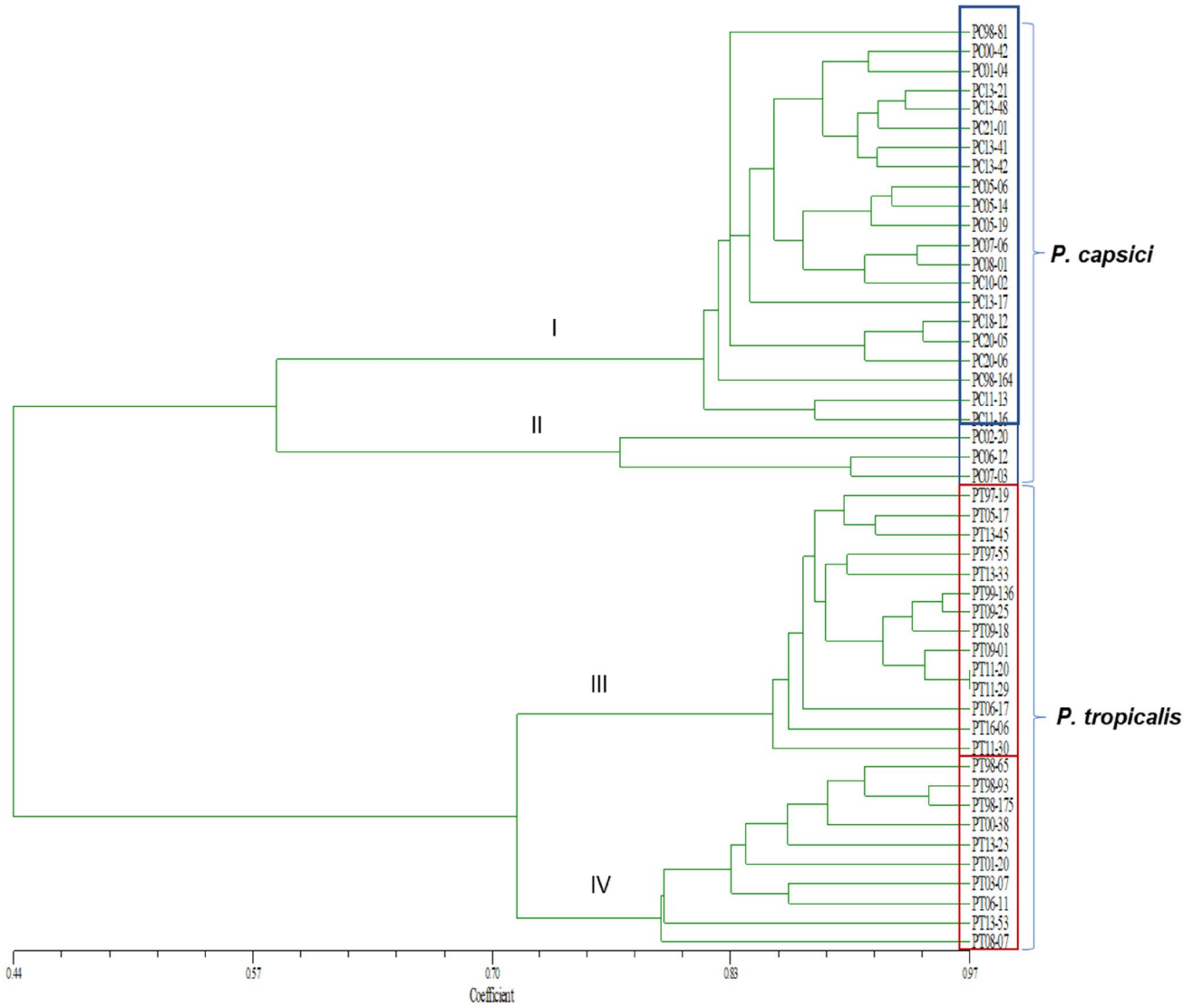

Fig. 2 UPGMA dendrogram of 48 *Phytophthora* isolates (24 *P. capsici* and 24 *P. tropicalis* isolates) based on combined RAMS and REP-PCR data

and obpyriform (Fig. 5). There was no correlation between culture morphology, sporangia shape and clustering of isolates.

Mating type determination

Mating type of *Phytophthora* isolates was determined using pairing test. Oospores were observed in all the isolates when paired with ATCC-MYA-4034 (A2 mating type) and not with ATCC-MYA-2338 (A1 mating type) which confirmed that all the 48 isolates belonged to A1 mating type.

Infectivity analysis

In infectivity analysis, all the 16 *Phytophthora* isolates from black pepper could infect nutmeg, cucumber, tomato and pumpkin. But only few isolates viz., PC06-12, PC20-05, PT06-17 and PT13-23 could infect cardamom (Fig. 6). Upon inoculation in black pepper, the isolates produced blackish lesions with fimbriate margin. In case of nutmeg, a dark brown-coloured lesion was observed which covered the entire leaf surface at later stage. The symptoms on cucumber appeared as water-soaked lesion, and white cottony mycelial growth was observed at a later stage. A light brownish lesion was observed on tomato leaves and in pumpkin, and water-soaked lesion with sunken spot was observed. Brown-coloured lesion with a yellow

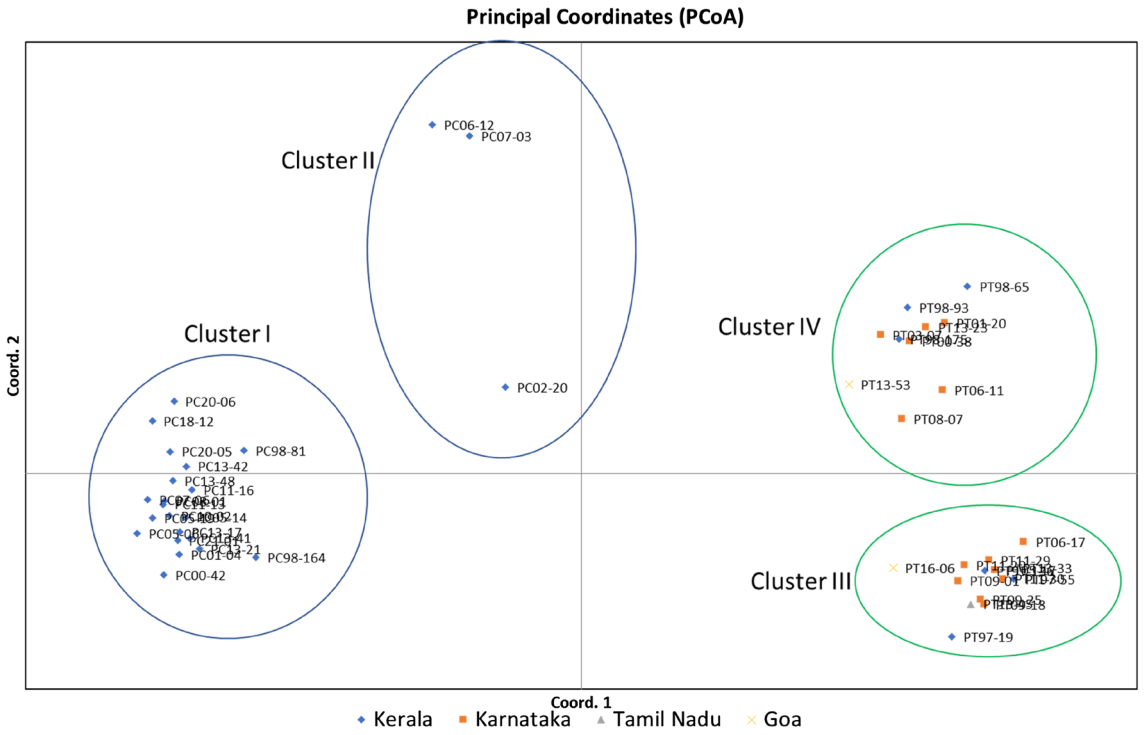

Fig. 3 Principal coordinate analysis (PCoA) plot based on combined RAMS and REP-PCR data for four *Phytophthora* populations (Kerala, Karnataka, Tamil Nadu and Goa) showing separation of the population into four clusters (*P. capsici*—Cluster I & II; *P. tropicalis*—Cluster III & IV)

margin was observed on cardamom leaves. In black pepper, the isolate 97–55 was highly virulent as it showed larger lesion area (10.54 cm^2) followed by 06–12 (8.82 cm^2) and 98–81 was least virulent (0.45 cm^2). The isolates 13–23, 13–53 and 01–04 displayed a comparable pattern of virulence in nutmeg (10.71 cm^2, 10.46 cm^2 and 10.33 cm^2 respectively), whereas 18–12 was the least virulent (0.77 cm^2). The lesion area ranged from 0.41 cm^2 to 2.58 cm^2 in tomato and there was no significant variation in virulence pattern between the isolates. In chilli, the isolate 13–23 (14.52 cm^2) showed high virulence which was on a par with the isolates, 06–17 (14.01 cm^2), 07–03 (13.86 cm^2) and 03–07 (13.73 cm^2). The least virulent isolates were 09–01 (0.79 cm^2) and 98–81 (0.64 cm^2). In case of pumpkin, isolate 13–53 (9.94 cm^2) was highly virulent followed by 06–17 (6.75 cm^2), and 97–55 (0.28 cm^2) was the least virulent isolate. Not all the isolates could infect cardamom. The isolates, 06–17, 20–05, 13–23 and 05–06 only could infect cardamom with a lesion of 0.21–0.77 cm^2 (Table 4).

When the isolates were considered, 06–12 and 97–55 were highly virulent in black pepper and chilli but less virulent in the rest of the crops. The isolates, 20–05 and 05–06 were moderately virulent in black pepper and chilli and less virulent in nutmeg and pumpkin. 02–20, 06–17 and 11–29 were moderately virulent in both black pepper and nutmeg, but 02–20 was the least virulent in the rest of the crops, whereas 06–17 and 11–29 were highly virulent in chilli. The isolates, 07–03, 18–12, 09–01, 13–23 and 13–53 were moderately virulent in black pepper, but 07–03, 13–23 and 13–53 were highly virulent in both chilli and nutmeg, whereas 13–53 was highly virulent in pumpkin. Isolates 98–81, 01–04, 98–93 and 03–07 were less virulent in black pepper, but 01–04 was highly virulent in chilli and nutmeg, and 98–93 was moderate. 98–81 was moderately virulent in pumpkin. None of the isolates could infect crops such as coconut, arecanut and vanilla (Fig. 6). The virulence patterns amongst the isolates were also random and no correlation was observed with the UPGMA cluster.

Discussion

Phytophthora foot rot disease has been reported in many countries like Vietnam (Truong et al. 2008), Malaysia (Farhana et al. 2013), Ethiopia (Jibat et al. 2021), where *P. capsici*, *P. tropicalis and P. nicotianae,* (Roy et al.

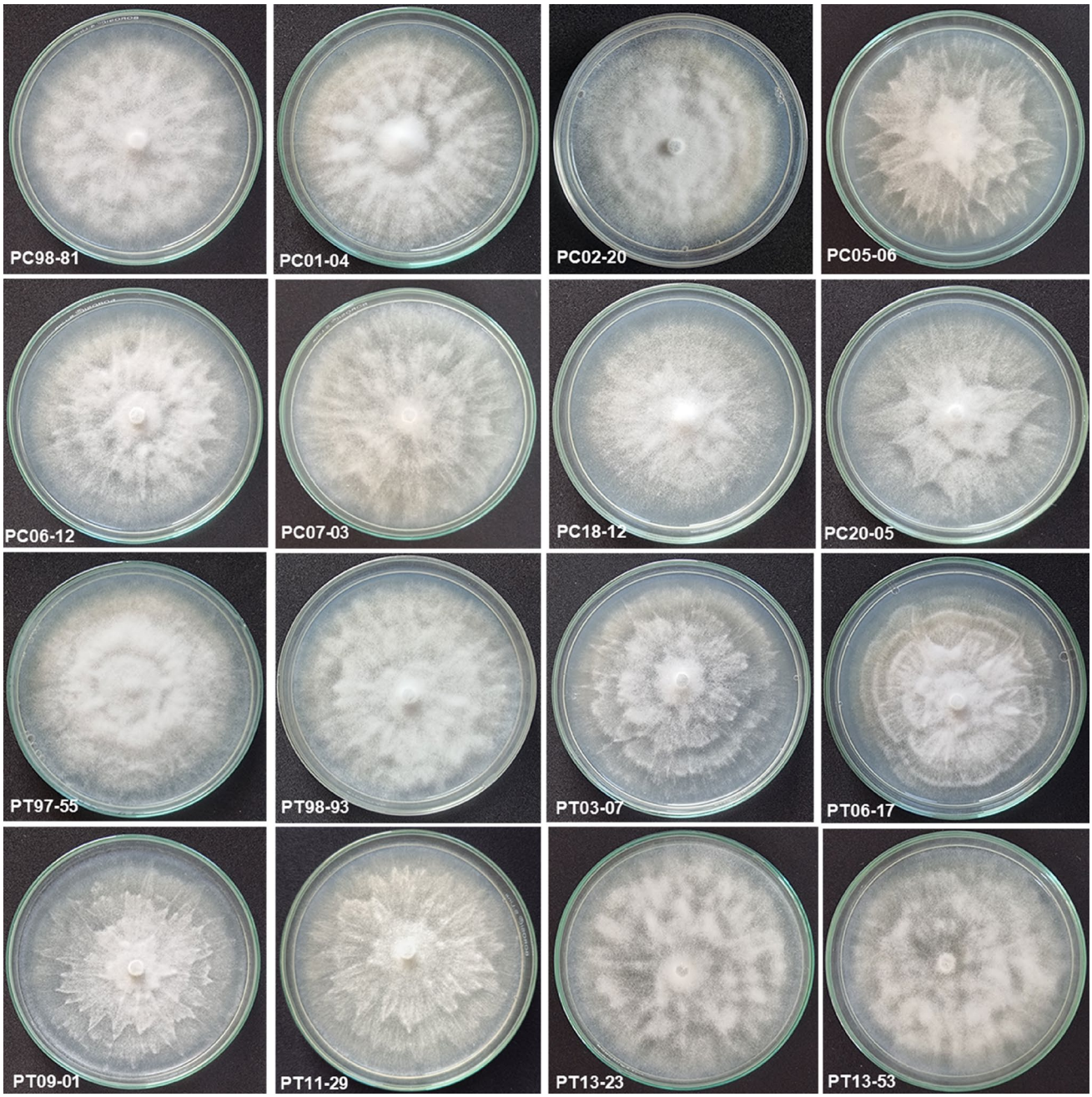

Fig. 4 Colony morphology of selected *Phytophthora* isolates: PC98-81 Modified chrysanthemum; PC01-04 Stellate with concentric rings; PC02-20 Cottony with concentric rings; PC05-06 Modified chrysanthemum; PC06-12 Modified stellate; PC07-03 Modified chrysanthemum; PC18-12 Modified stellate; PC20-05 Stellate; PT97-55 No pattern; PT98-93 Modified chrysanthemum; PT03-07 Modified stellate; PT06-17 Modified petaloid; PT09-01 Modified stellate; PT11-29 Modified stellate; PT13-23 Stellate with cottony mycelia; PT13-53 Stellate with cottony mycelia

2009) are the causal organisms. In India, the role of two species of *Phytophthora* has already been elucidated with black pepper foot rot disease. It is also equally important to discern the coexistence of these species within a population. The major black pepper producers in India include Karnataka, Kerala and Tamil Nadu and hence, the present study mainly focussed on the isolates collected from these states. The present study constitutes the first report on genetic diversity of *Phytophthora* species infecting black pepper in India unravelled through RAMS and REP-PCR analyses.

It was evident from the present study that there existed considerable variations amongst both the species. Size of the sample often influenced the genetic diversity

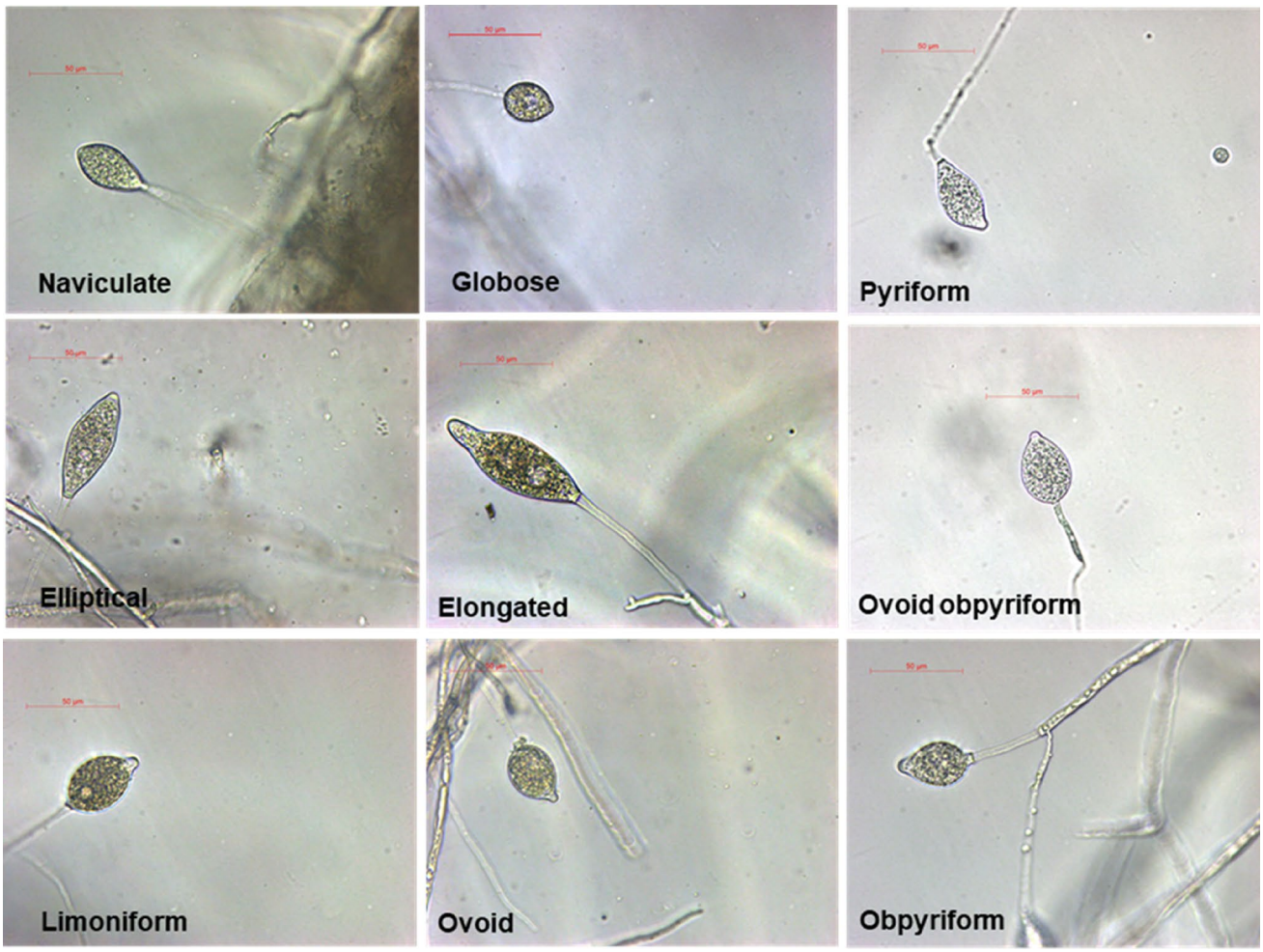

Fig. 5 Microscopic view of sporangia of *Phytophthora* isolates

estimation. Based on the availability of the cultures from the national repository, a greater number of isolates were used from Kerala followed by Karnataka i.e. nearly half the quantum. The diversity estimates were greater for Kerala isolates, but when considering the sample size, the Karnataka population was found to be more diverse. The samples collected from Tamil Nadu and Goa were considerably less and hence not sufficient to describe the diversity of these populations.

Through this study, we have identified that the black pepper infecting *Phytophthora* spp. population in India is highly diverse and the two species of *Phytophthora* could be clearly delineated using combined RAMS and REP-PCR data. REP-PCR analysis could alone differentiate *P. capsici* and *P. tropicalis* and group them into separate clusters as observed in dendrogram based on combined RAMS and REP-PCR data. But, in RAMS dendrogram, slight variation was observed. Similarly, both the markers generated comparable number of polymorphic loci. However, those identified by REP-PCR primers is slightly higher. The topology obtained by PCoA analysis was similar to UPGMA dendrogram, and this indicates the robustness of application of combined RAMS and REP-PCR data or REP-PCR data alone in differentiating the two-sister species infecting black pepper. Several reports are available on the use of REP-PCR fingerprinting in genetic diversity analysis of *Phytophthora* species. REP-PCR fingerprints detected variations in *P. infestans* populations and were highly reproducible (Bouws and Finckh 2007). Recently, 81 isolates of *P. palmivora* in Indonesia and Japan were studied using REP-PCR primers, and the clustering of the isolates was similar to microsatellite markers (Masanto et al. 2019).

All the isolates analysed in this study were unique and the population was found to be heterogeneous. There was no correlation between UPGMA clustering and geographical origin of the isolates. A study from Vietnam on *P. capsici* infecting black pepper revealed that the *P. capsici* population in Vietnam is clonal and also no correlation was observed with the geographical origin of the isolates (Truong et al. 2010). The genetic diversity study of *P. capsici* infecting

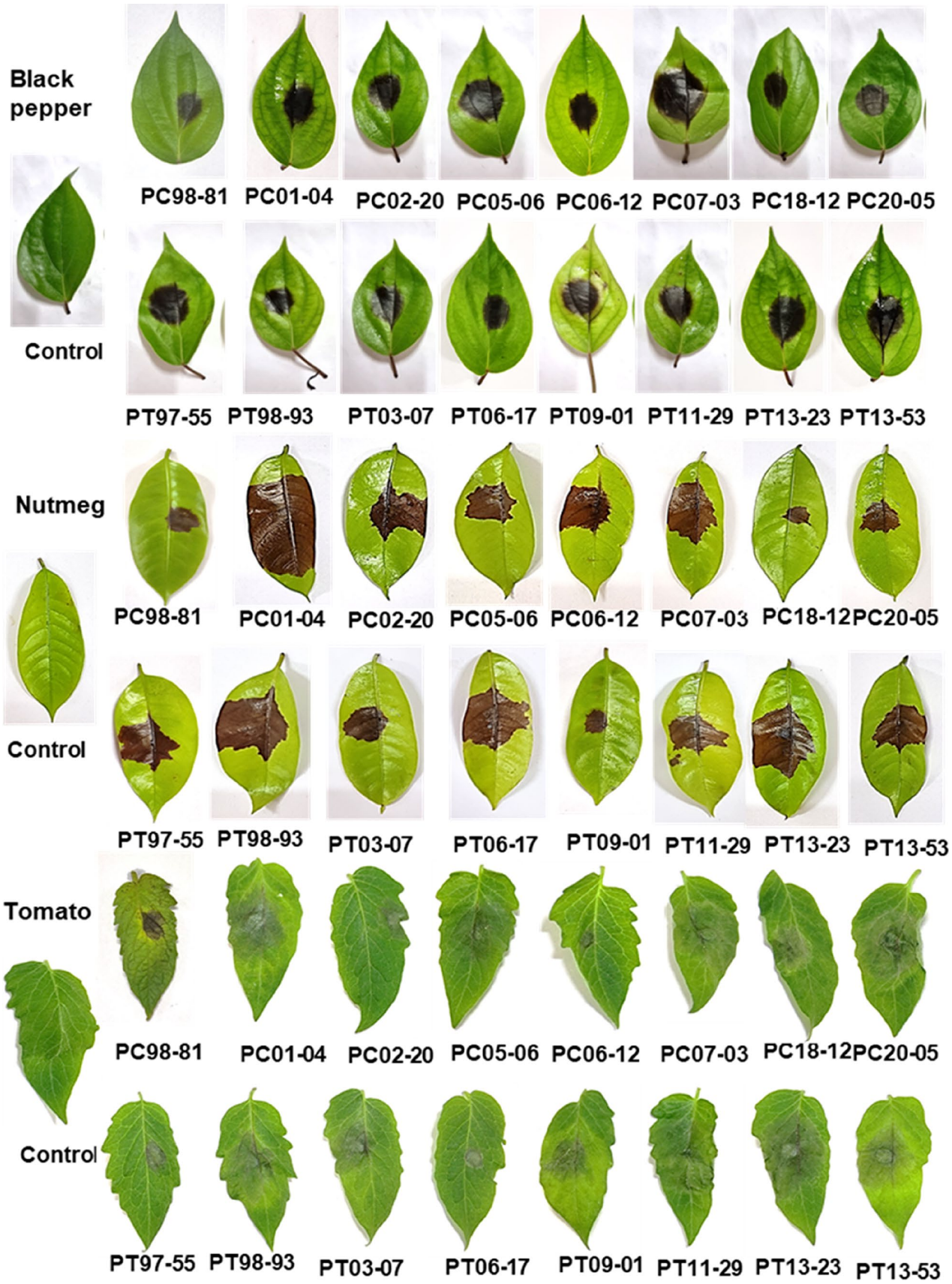

Fig. 6 Infectivity analysis of black pepper *Phytophthora* isolates on various crops (Symptoms photographed at 48 h after inoculation)

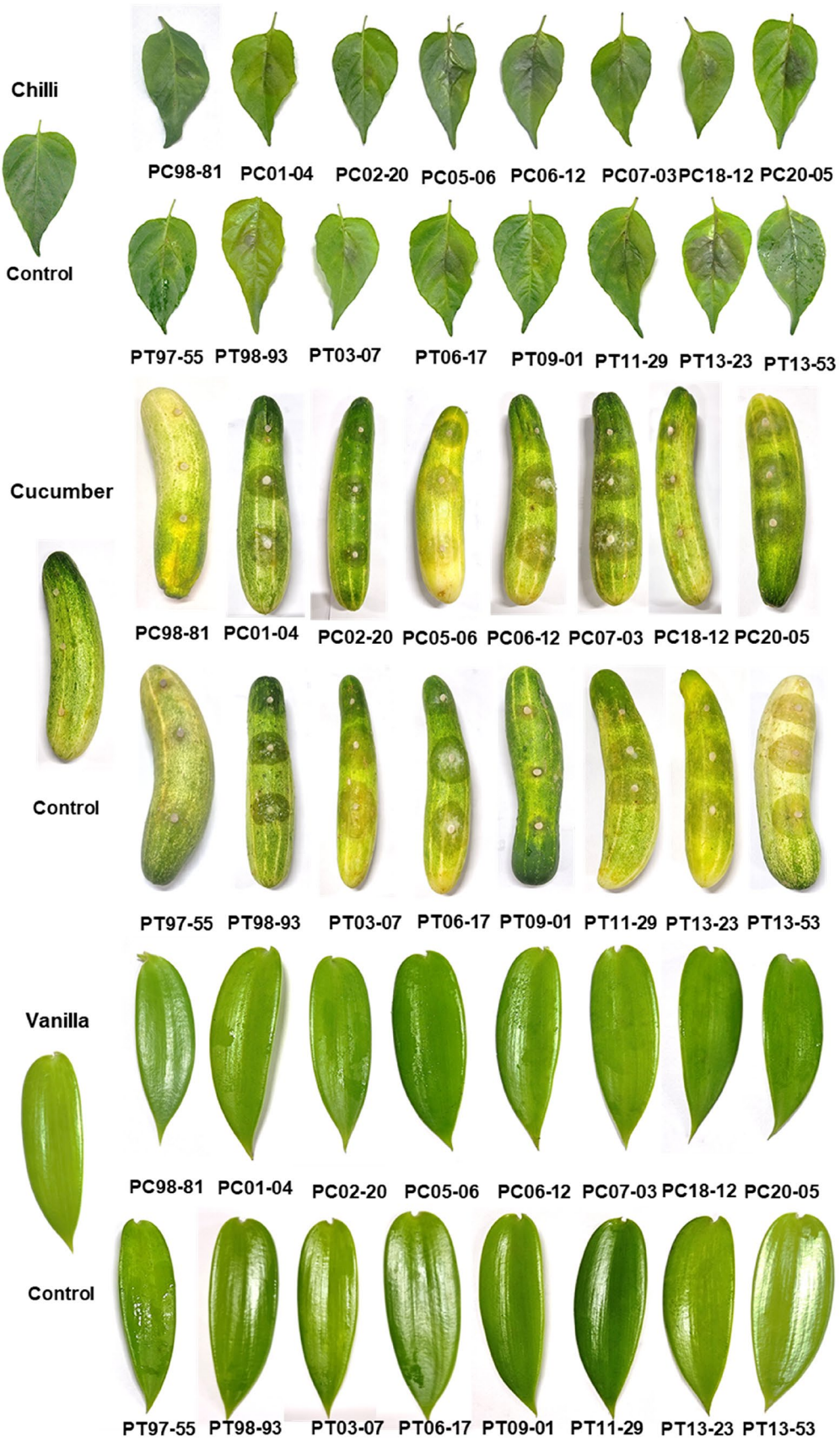

Fig. 6 (continued)

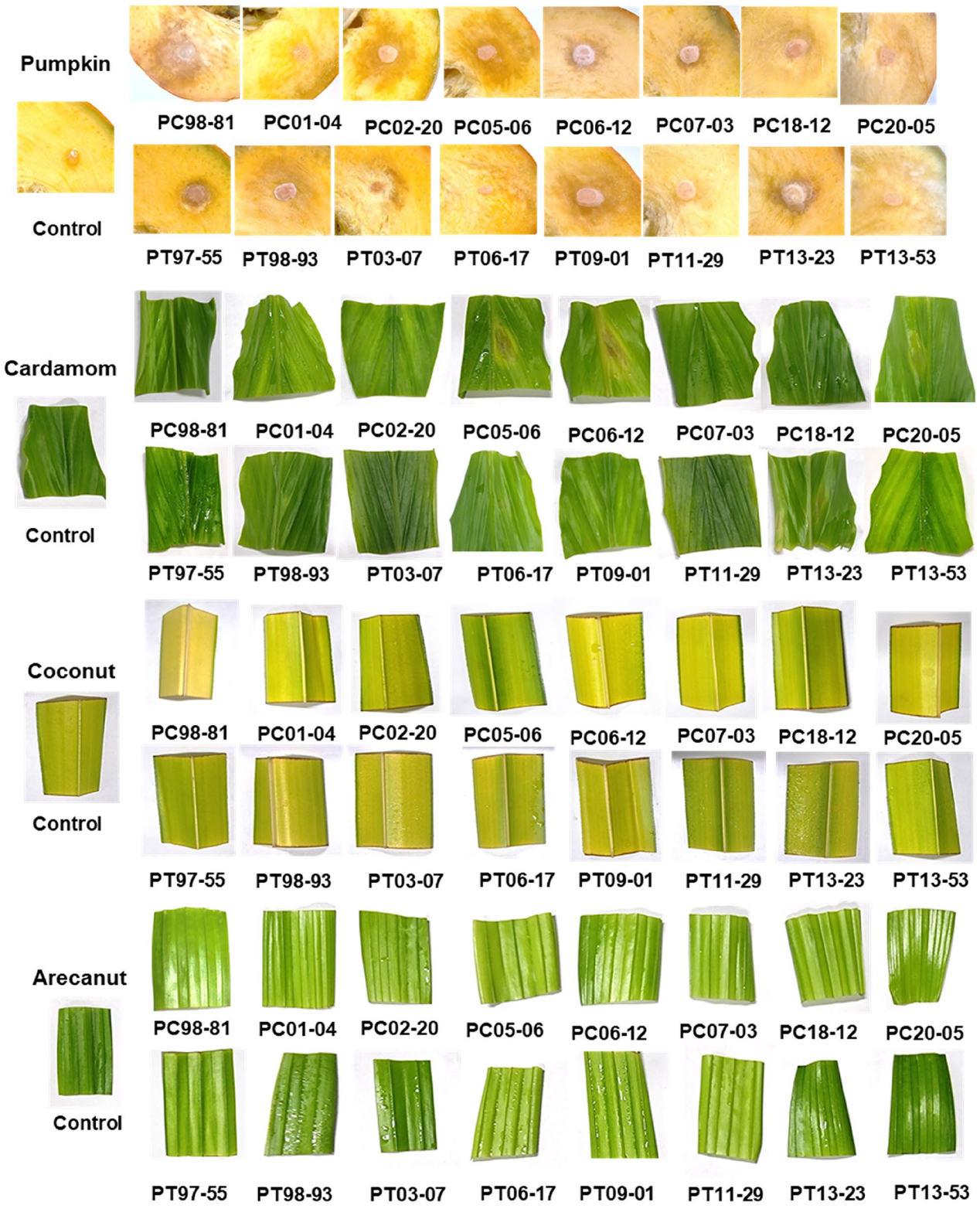

Fig. 6 (continued)

Table 4 ANOVA results showing the lesion area inflicted by *Phytophthora* isolates on various crops

Isolates	Black pepper	Nutmeg	Tomato	Chilli	Pumpkin	Cardamom
98–81	0.45^{e}	1.84^{gh}	1.21^{a}	0.64^{h}	5.75^{abc}	0.00^{d}
01–04	3.61^{cde}	10.33^{a}	1.56^{a}	9.09^{cd}	2.93^{bcd}	0.00^{d}
02–20	5.21^{bcd}	4.87^{cdefg}	0.66^{a}	4.63^{efg}	1.78^{bcd}	0.00^{d}
05–06	6.14^{bcd}	2.90^{efgh}	1.05^{a}	5.83^{ef}	2.76^{bcd}	0.21^{c}
06–12	8.82^{ab}	6.19^{bcde}	0.41^{a}	11.05^{bc}	2.02^{bcd}	0.00^{d}
07–03	6.07^{bcd}	9.06^{ab}	2.58^{a}	13.86^{ab}	2.31^{bcd}	0.00^{d}
18–12	4.84^{bcde}	0.77^{h}	1.53^{a}	2.02^{gh}	1.16^{cd}	0.00^{d}
20–05	4.06^{cde}	3.59^{defgh}	2.46^{a}	5.95^{ef}	1.89^{bcd}	0.37^{b}
97–55	10.54^{a}	2.17^{fgh}	0.66^{a}	3.77^{fg}	0.28^{d}	0.00^{d}
98–93	2.75^{de}	6.50^{bcd}	2.19^{a}	7.28^{de}	1.63^{bcd}	0.00^{d}
03–07	3.37^{cde}	3.02^{efgh}	0.90^{a}	13.73^{ab}	1.36^{cd}	0.00^{d}
06–17	5.03^{bcd}	7.63^{abc}	1.05^{a}	14.01^{ab}	6.75^{ab}	0.77^{a}
09–01	6.36^{abcd}	2.49^{fgh}	2.13^{a}	0.7 9h	2.05^{bcd}	0.00^{d}
11–29	5.29^{bcd}	5.39^{cdef}	1.83^{a}	10.26^{c}	2.04^{bcd}	0.00^{d}
13–23	4.66^{bcde}	10.71^{a}	1.88^{a}	14.52^{a}	2.28^{bcd}	0.22^{c}
13–53	7.46^{abc}	10.46^{a}	1.72^{a}	10.44^{c}	9.94^{a}	0.00^{d}
CV	44.78	32.04	126.73	20.79	89.19	91.13
t-value	2.03	2.03	2.03	2.03	2.03	2.04
LSD	3.94	2.92	3.13	2.76	4.35	0.15

No infection in coconut, arecanut and vanilla. Values followed by the same superscript letters in each column are not significantly different from each other as detected by DMRT ($p<0.05$)

Capsicum annuum in Bhutan (Rai et al. 2020) could identify polymorphism amongst the isolates and the RAMS clustering corresponded to the geographical origin of the isolates. REP-PCR analysis of *P. palmivora* from Indonesia and Japan showed similar results *i.e.* REP-PCR analysis clustered the isolates based on the geographical region rather than host plant or mating type (Masanto et al. 2019). RAMS analysis of *P. colocasia* from India could identify highly diverse population and the UPGMA cluster did not correspond to geography or phenotype of the isolates (Nath et al. 2016).

All the 48 isolates used in the present study were of A1 mating type. So far, all the *Phytophthora* isolates infecting black pepper in India were identified as A1 mating type (unpublished data). However, the chance of sexual reproduction amongst the South Indian populations of *P. capsici* and *P. tropicalis* cannot be ruled out completely. A2 mating type isolates were reported in *P. capsici* infecting other crops such as cocoa in southern India (Chowdappa and Chandramohan 1997). In addition, there is also a higher possibility of inter-specific hybridization with A2 mating type of other species such as *P. palmivora* which is predominant in Kerala and Karnataka (Chowdappa and Chandramohan 1997). The other attributions for the unique genotypes of the isolates in the present study could be mutations and loss of heterozygosity due to mitotic recombination (Goodwin 1997; Truong et al. 2010; Li and Liu 2021).

Colony morphology revealed that the isolates exhibited variety of growth pattern irrespective of species difference, modified chrysanthemum being the most observed pattern. Aragaki and Uchida (2001) reported that *P. capsici* spp. bear broad sporangia with length-to-breadth ratio less than 1.8 and round sporangial base, whereas the sporangia of *P. tropicalis* was narrow with breadth ratio greater than 1.8 and tapered sporangial base. As per their study, *P. tropicalis* spp. produce chlamydospores which is dissimilar to *P. capsici*, In the present study, elongated sporangia with tapered base and broad sporangia with round base were observed in *P. capsici* and *P. tropicalis*, respectively. Production of chlamydospores was observed only in *P. tropicalis* isolates. All the isolates of *P.capsici* and *P. tropicalis* infecting black pepper in India analysed in this study were of A1 mating type.

P. capsici is a volatile pathogen which leaves an egregious effect on its host, and has a broad spectrum of host range including the members of *Cucurbitaceae* and *Solanaceae* (Meitz et al. 2010), whereas *P. tropicalis* is a pathogen of ornamentals (Hong et al. 2006) and fruit trees (Brumat et al. 2022). Amongst the crops selected for the infectivity analysis, *P. capsici* is reported to infect crops like tomato (Syed-Ab-Rahman et al. 2019), cucumber (Mansfeld et al. 2020), chilli (Majid et al. 2016) and pumpkin (Mohammad and Jose 2018). *P. tropicalis* could infect seedlings of tomato (Orlikowski et al. 2006) and cucumber (Hong et al. 2008) in vitro. The present study examined the ability of black pepper *Phytophthora* isolates to infect other crops including

plantation, spices and vegetables under in vitro conditions and variability in virulence pattern. Both *P. capsici* and *P. tropicalis* were capable of infecting other crops evidenced with the development of symptoms on nutmeg, tomato, cucumber, chilli, pumpkin and cardamom and however, none of the isolates could infect coconut, areca nut and vanilla. It was also observed that there exists a great variability amongst the isolates in exhibiting aggressiveness of the symptom, *i.e.* the most aggressive isolate on one crop was not aggressive on the other. Neither the most virulent nor the least virulent isolates fall into any specific cluster.

Conclusion

Combined data of RAMS and REP-PCR analyses could clearly differentiate the two species of *Phytophthora* infecting black pepper and group them into distinct clusters. Amongst the two methods, REP-PCR analysis is better, clearly differentiated *P. capsici* & *P. tropicalis* and produced more polymorphic loci than RAMS analysis. All the isolates were genetically unique and the population was diverse irrespective of their place of origin. They showed varied colony morphology, sporangial shapes and belonged to A1 mating type. Infectivity analysis revealed that the black pepper isolates could infect crops, such as tomato, cucumber, chilli, pumpkin, nutmeg and few of the isolates, could infect cardamom under in vitro conditions.

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Author contributions FZ—Implementation of the research, analyses and interpretation of results, draft manuscript preparation, AJ—Conceptualization, experimental design, analysis and interpretation of results, manuscript editing, CNB—Analyses and interpretation of results, manuscript editing. All authors reviewed the results and approved the final version of the manuscript.

Data availability All data generated in this study are available within the paper and also as supplementary information.

Declarations

Conflict of interest The authors declare that they have no conflict of interest in the publication.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the author.

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