Prevalence, incidence and molecular characterization of *Phomopsis vexans* (*Diaporthe vexans*) causing leaf blight and fruit rot disease of brinjal in Karnataka (India)

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Abstract

The distribution, prevalence and incidence of *Phomopsis vexans* in six major brinjal growing agro-climatic zones of southwest India is reported. *P. vexans* was isolated from diseased leaf and fruit samples from six zones and was studied for its morpho-cultural and molecular characteristics. Eighteen isolates were tested for their pathogenicity on 30-days old brinjal seedlings. The ITS regions of these fungal isolates were used for the molecular identification followed by phylogenetic analysis. The incidence of leaf blight and fruit rot disease was high in northern transition zone (NTZ: 10.6–25.3% and 21–33.3%) followed by southern dry zone (SDZ: 8.3–18% and 22.3–62%) and central dry zone (CDZ: 10–17% and 29–39%). All the isolates exhibited similarities in colony morphology. Variation was observed with regard to number of pycnidia, colony growth and type. Among the 24 isolates, 18 belonged to G-type and the rest could not be ascertained to either colony type. The 18 G-type isolates produced leaf blight and fruit rot symptoms 25–28 and 45–55 days post inoculation, respectively. In the phylogenetic analysis, all the 24 isolates formed a single clade, thus confirming their close genetic relatedness, though they were isolated from different agro-climatic zones of southwest India. Phylogenetic analysis of complete ITS2 sequence showed the presence of two distinct groups based on substitutions and indels observed among the populations where six isolates from NDZ and CDZ formed a distinct group from the rest of the isolates.

Key words – agro-ecological distributions – molecular phylogeny – pathogenicity – *Phomopsis vexans* – *Solanum melongena*

Introduction

Brinjal (*Solanum melongena* L.) is an important vegetable crop cultivated in the tropics and sub-tropics, and grown extensively in China, India, Bangladesh, Pakistan and Philippines. It is also

cultivated in America, Europe and other parts of Asia. It is one of the most important vegetable crops of India (Zeven & Zhukovsky 1975, Rashid 1976, Sekara et al. 2007). It is susceptible to various biotic and abiotic stresses at different stages of growth and development. Fungal pathogens cause several diseases, the most significant being leaf blight and fruit rot (*Phomopsis vexans*), leaf spots (*Alternaria melongenae* and *Cercospora melongenae*), damping-off (*Pythium aphanidermatum*), wilt (*Verticillium dahliae*), and root rot (*Sclerotinia sclerotiorum*) (Shivaprakasam & Soumini Rajgopalan 1974, Iqbal et al. 2003). Fruit rot and leaf blight, caused by *Phomopsis vexans*, is a major disease reducing yield and marketable value of crop by 20−30% (Beura et al. 2008, Pandey 2010).

P. vexans associated with brinjal has been reported from many areas in the warmer parts except for Europe and a few African countries (Harter 1914, Smith et al. 1988). The pathogen is believed to originate from South Asia (Prance & Nesbitt 2005) and reported to also infect some wild *Solanum* species (Datar & Ashtaputre 1988). It is readily transmitted through seeds internally as well as externally (Porter 1943, Vishunavat & Kumar 1993). The sexual stage of the fungus has not been found in nature although Gratz (1942) reported the occurrence of perithecia on potato dextrose agar, and assigned the name *Diaporthe vexans*. Harter (1914) reported this pathogen as host specific on brinjal, but it has been reported on other hosts including *Acacia*, *Capsicum*, *Prunus* and *Sorghum* (Udayanga et al. 2011).

Phomopsis vexans has been reported from Assam, Jammu, Karnataka and Pantnagar (Srinivasa et al. 2005, Thippeswamy et al. 2005, Akhtar & Chaube 2006, Muneeshwar et al. 2011, Das & Sarma 2012, Jayaramaiah et al. 2013). Currently, brinjal is extensively grown in southwest India with the area under cultivation increasing every year. The present study was undertaken to quantify leaf blight and fruit rot disease incidence, detection, variation among isolates and to determine genetic relatedness of *P. vexans* in diverse agro-climatic zones of southwest India.

Materials & Methods

Field survey and data collection

The survey was carried out in southwest India for three years (2011−13) to coincide with crop maturity between pre-flowering to first harvest stage (70−120-days old). Survey area covered approximately 1200−1350 km^2 in each year. Based on the rainfall pattern, distribution, soil types, elevation, topography and type of vegetation, ten agro-climatic zones were classified by the Department of Agriculture, (Government of Karnataka) although this study was restricted to six agro-climatic zones (Table 1).

Sampling and data collection

A total of 303, 333 and 342 fields were assessed during the years 2011, 2012 and 2013, respectively. Disease prevalence and incidence were recorded. Disease prevalence was expressed as a proportion of fields, and calculated as:

Disease prevalence = Number of fields with disease/Total number of fields evaluated ×100.

In each field, 100 plants were assessed in each corner and at the centre (a total of 500 plants/field). Where possible the same fields were sampled during successive surveys but, in the absence of the same fields, adjacent brinjal fields were sampled. Fields were selected along the route separated by about 8−10 km from the survey transect.

The disease incidence was recorded from June to October for three consecutive years; 100 plants were assessed in each corner and at the centre (a total of 500 plants/field) as followed by McKinney (1923) and Hossain et al. (2010). The disease incidence was calculated as:

Disease incidence = (number of infected plants with leaf blight/with fruit rot / total number of plants) × 100.

Isolation, identification and cultural studies

Samples showing leaf blight and fruit rot symptoms were collected during the field survey and were cut into small pieces (~0.5 cm). These were surface sterilized with 2% sodium

hypochlorite (NaOCl) solution for 2−3 min. The samples were then washed with sterile distilled water, blotter dried and placed on potato dextrose agar (PDA, HiMedia Laboratories, Mumbai), and incubated for up to 7 days at room temperature (28±2ºC). Fungal isolates were identified based on micro-morphology and pure cultures were established. A total of 182 isolates of *Phomopsis* were isolated and 24 of these, representing all the study zones, were selected for further study. Variations in colony morphology, colony type and pycnidia, number of pycnidia per plate and average growth of the colony per day was calculated and differences were analyzed (Kanematsu et al. 1999, 2000, Udayanga et al. 2011).

Pathogenicity

Eighteen isolates of *P. vexans* were tested for pathogenicity on *S. melongena* (cv. purple pusa long) by whole plant inoculation under field conditions. Seedlings at six leaf stage were inoculated with conidial suspension of *P. vexans* (1×10^6 conidia/ml) by foliar spray (Dhingra & Sinclair 1995, Mathur & Kongsdal 2003). Plants sprayed with sterile distilled water served as controls. Inoculated plants were covered with a polythene bag for 5 days to maintain high humidity. The experiments were conducted in triplicates of 25 plants for each isolate. Time required for disease expression was recorded from the day of inoculation to the development of symptoms. The disease incidence was calculated as before.

ITS-rDNA sequencing and phylogenetic analysis

The fungal isolates were grown on PDA medium for 8−10 days. Fungal mycelia were harvested (500 mg), freeze dried and ground to a fine powder with liquid nitrogen in a mortar and pestle. Genomic DNA was extracted following the protocol of Zhang et al. (1998) and used for PCR. The internal transcribed spacer (ITS) region of ribosomal DNA containing 18S-ITS1-5.8S-ITS2-28S were amplified by using ITS1 (5'-CGGATCTCTTGGTTCTGGCA-3') and ITS4 (5'-GACGCTCGAACAGGCATGCC-3') primer pair (White et al. 1990). The PCR amplification was carried out in 25 µl reaction mixture containing 1 µl of DNA sample with 2.5 µl of 10 × PCR buffer, 2.5 µl of 2.5 mM MgCl$_2$, 2.0 µl of 2 mM dNTPs, 1.0 µl of each forward and reverse primer (20pM) and 0.2 µl of Taq DNA Polymerase (Sigma Aldrich, USA) and made up to 25 µl with nuclease free water (14.8 µl). The amplification was performed using the cycling program of initial denaturation at 95º C for 3 min; followed by 35 cycles of denaturation at 94º C for 30 s; annealing at 55º C for 30s, and extension at 72º C for 1 min; and a final extension for 10 min at 72º C. The PCR was performed using Advanced Thermus25 Thermo cycler (Peqlab, Germany). The amplified PCR product was run on 1.5% agarose gel along with 1 kb DNA marker. Amplified PCR products were sequenced directly.

Multiple sequence alignment and phylogenetic analysis

The ITS-rDNA sequences of all the 24 *Phomopsis* isolates obtained in this survey and additionally *P. vexans* sequences retrieved from GenBank database were used for multiple sequence alignment and for further phylogenetic analysis. The sequences were aligned using ClustalW2 (Larkin et al. 2007) online program (www.ebi.ac.uk) and alignments were visually inspected. Phylogenetic tree was constructed using Neighbour-Joining (NJ) method in MEGA6.0 (Tamura et al. 2011). The Kimura-two-parameter model was used to generate the NJ tree. For all analyses, 1000 bootstrap replicates were performed to evaluate the node to support the generated tree. In another phylogenetic analysis, only the ITS2 sequence was extracted by subjecting to ITS2 online database (Eddy 1998, Koetschan et al. 2010) and these sequences were analyzed by multiple sequence alignment, and a phylogenetic tree was constructed by N-J method as described above.

Statistical analysis

The three-year disease incidence data and pathogenicity experiments data were subjected to statistical analysis and expressed as mean incidence along with standard error. Values were subjected to arcsine transformation and analysis of variance (ANOVA) using SPSS v. 16.0 (SPSS Inc., Chicago, IL, USA). Significant effects were determined by magnitude of F values ($P<0.05$). Means were separated by Tukey's HSD (honestly significant difference) test.

Results

Disease prevalence and incidence of leaf blight and fruit rot

The prevalence of leaf blight and fruit rot was maximum in SDZ (80−90%) followed by EDZ (70−85%), CDZ (66−80%) and STZ (80−83%) agro-climatic zones (Table 1). The lowest disease prevalence was observed in NDZ (55−53%) followed by NTZ (42−60%) and NET zone, NED (0−20%) (Fig. 1). The leaf blight incidence ranged between 8−25% in all the three years. The maximum leaf blight incidence was observed in NTZ (Savanur, 25.35%) and lowest incidence was recorded in STZ (H.D. Kote, 8.6%), SDZ (Chamarajanagar, 8.3%; Pandavapura, 8.6%; Malavalli, 12%) and EDZ (Doddaballapur, 8.6%). Fruit rot incidence ranged from 15−62% in all the study zones. The maximum incidence of fruit rot was recorded in SDZ (Malavalli, 62%) and the minimum was in CDZ (Sira, 15%).

Morphological characterization of fungal isolates

Leaf blight symptoms developed as small necrotic areas on the leaves with appearance of pycnidia at later stages of disease development. The infected fruit also showed the appearance of necrotic lesions on its surface with concentric rings at the beginning followed by the appearance of dark pycnidia later. Pycnidia were dark, pyriform, ostiolate, immersed and erumpent and prominently visible with the disease development (Fig. 2).

The number of pycnidia varied in each isolate. Conidia were single celled, biguttulate, and 4.6−7.4 × 1.2−2.0 μm. The shape and size of the pycnidia varied among isolates. Based on micro-morphological and cultural features, the fungal pathogen was identified as *P. vexans*. The cultural characteristics of 24 isolates of *P. vexans* are presented in Table 2.

The fungal colonies were whitish in colour with a wavy margin (Fig. 3). Pycnidia were submerged and produced all over the surface except in six isolates (Fig. 4). Out of 24 isolates, 18 isolates belonged to G-type colony and the rest could not be assigned to either of G or W type colony as they did not produce pycnidia.

Pathogenicity

Among 24 isolates of *P. vexans*, 18 isolates (except isolates MK5, 8, 13, 14, 17 and 20) were pathogenic to brinjal and significant differences in disease incidence upon challenge inoculation were observed among the six isolates (MK3, 10, 16, 19, 22 and 24). The leaf blight symptoms were produced 20−25 days post inoculation and fruit rot symptoms after 45−55 days. The results of pathogenicity test and disease incidence of leaf blight disease are presented in Fig. 5. Four isolates (MK10, 16, 19 and 22) produced disease incidence of >80%, 10 isolates (MK1, 2, 7, 9, 11, 12, 15, 18, 21 and 23) an incidence of 60−70%, and two isolates (PVMK3 and 24) 50−55% disease incidence.
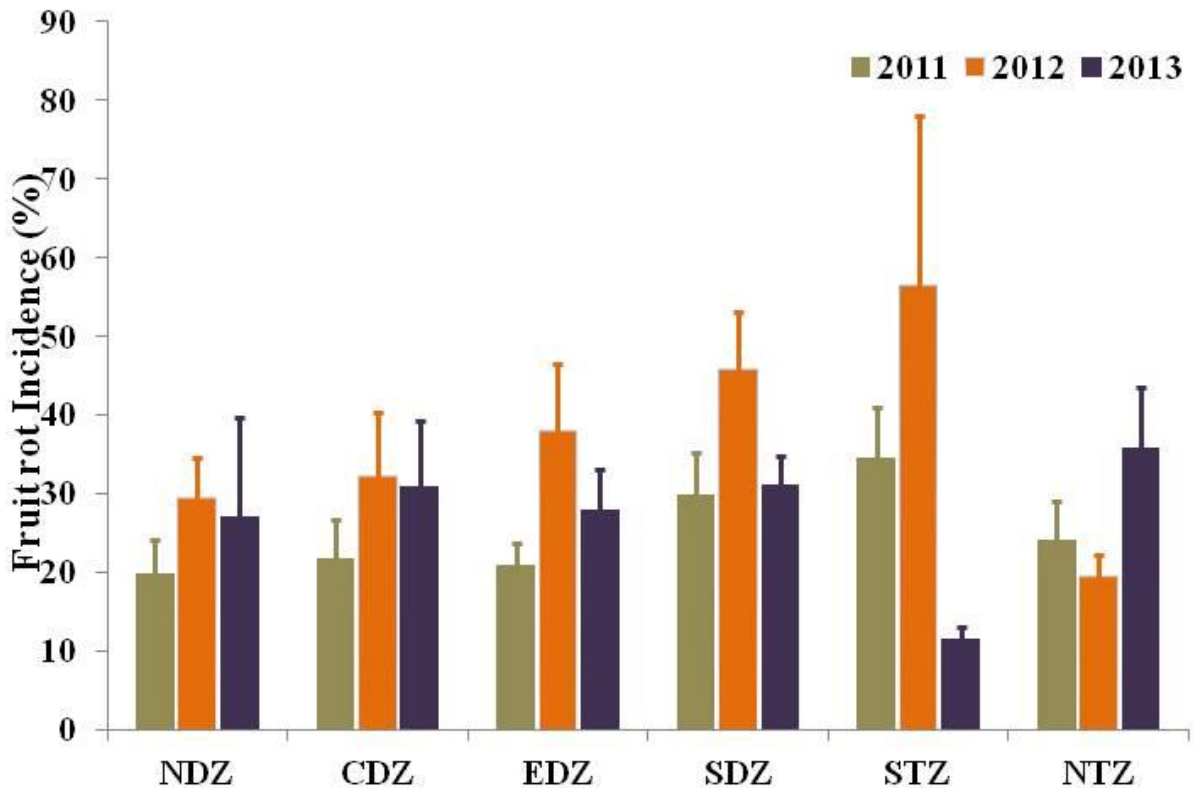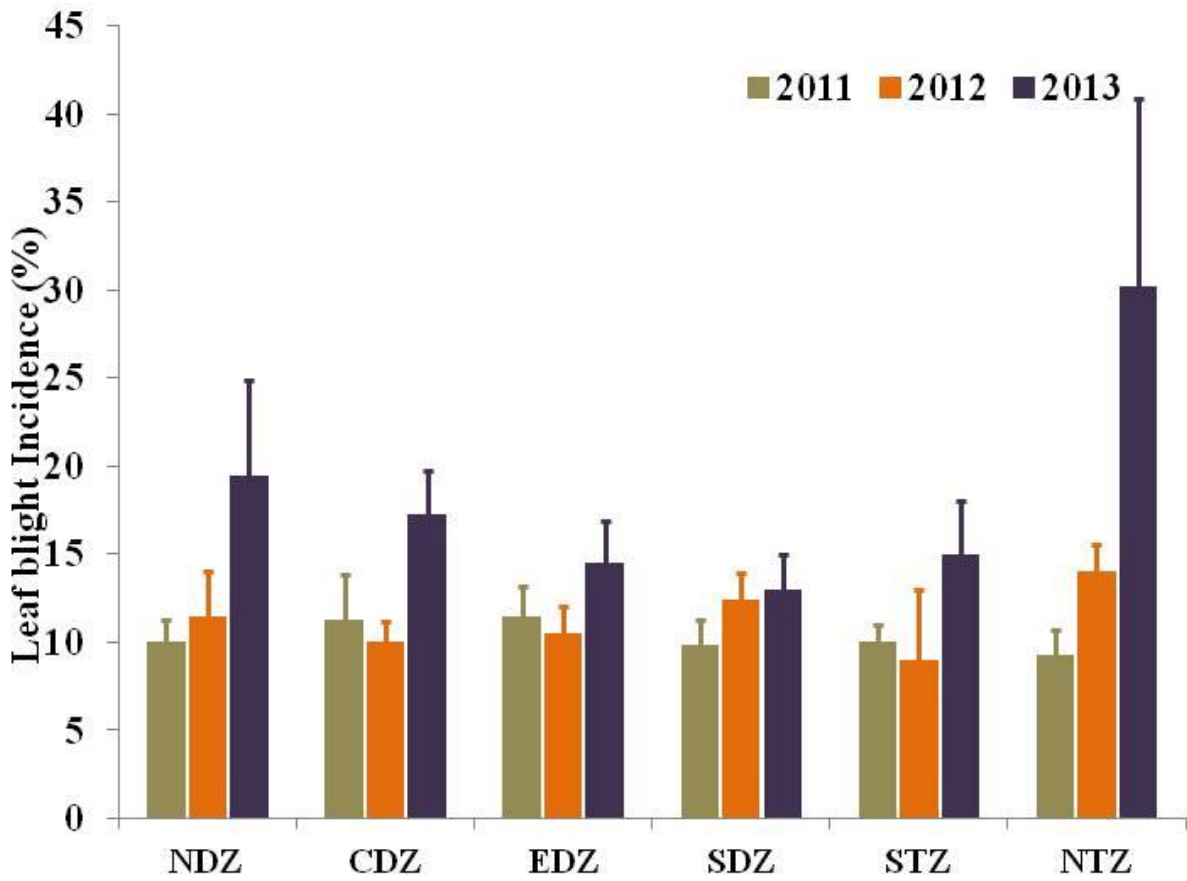
Fig. 1 – Leaf blight and fruit rot incidence recorded from different agro-climatic zones of Karnataka during 2011–13.

Table 1 Summary of agro-ecological zones and prevalence of leaf blight and fruit rot disease of brinjal during 2011, 2012 and 2013 in major brinjal growing regions of Karnataka.

Agro-Ecological Zones	Mean annual precipitation (mm)	Regions covered in each zone	Prevalence of Phomopsis leaf blight and fruit rot disease (%)		
			2011	2012	2013
Northern Dry Zone (NDZ)	465–786	Bhagalkot	(4/8)* 50.0	(5/11) 45.4	(7/12) 58.3
		Gadag	(8/15) 53.3	(5/10) 50.0	(8/16) 50.0
		Mundaragi	(8/14) 57.14	(6/10) 60.0	(7/13) 53.84
		Gokak	(5/9) 55.5	(8/12) 75.0	(5/9) 55.5
		Mean±SE	**(25/46) 54.34±1.54**	**(24/43) 55.81±6.55**	**(27/50) 54±1.73**
Central Dry Zone (CDZ)	454–718	Hosadurga	(6/9) 66.6	(7/10) 70.0	(6/11) 54.54
		Arasikere	(10/12) 83.3	(8/14) 57.14	(6/12) 50.0
		Koratagere	(7/9) 77.7	(8/10) 80.0	(7/9) 77.7
		Sira	(7/8) 87.5	(10/12) 83.3	(7/13) 53.84
		Mean±SE	**(30/38) 78.94±4.52**	**(33/46) 71.73±5.88**	**(26/45) 57.77±6.3**
Eastern Dry Zone (EDZ)	679–889	Gubbi	(8/9) 88.8	(10/13) 76.92	(7/9) 77.7
		Anekal	(5/9) 55.5	(10/12) 83.3	(8/10) 80.0
		Doddaballapur	(7/9) 77.7	(7/10) 70.0	(7/10) 70.0
		Bhagepalli	(8/11) 72.72	(6/9) 66.6	(8/9) 88.8
		Mulbagal	(7/9) 77.77	(11/15) 73.3	(10/12) 83.33
		Gouribidnur	(8/10) 80.0	(9/14) 64.28	(7/14) 50.0
		Mean±SE	**(43/57) 75.43±4.52**	**(53/73) 72.60±2.85**	**(47/64) 73.43±5.6**
Southern Dry Zone (SDZ)	671–889	K.R. Nagar	(8/10) 80.0	(10/10) 100	(9/10) 90.0
		Mysore	(10/11) 90.9	(10/12) 83.33	(7/9) 77.7
		Nanjangud	(7/10) 70.0	(8/9) 88.8	(8//11) 72.72
		Nagamangala	(9/11) 81.81	(13/15) 86.6	(8/10) 80.0
		Pandavapura	(10/12) 83.33	(12/15) 80.9	(9/10) 90.0
		Mandya	(7/12) 58.33	(10/13) 76.92	(11/13) 84.61
		Malavalli	(9/14) 64.28	(6/9) 66.6	(13/19) 68.42
		Chamarajanagar	(8/13) 61.53	(4/8) 50.0	(9/13) 69.23
		Yalandur	(12/15) 80.9	(8/10) 80.0	(10/11) 90.90
		Mean±SE	**(80/98) 81.63±3.77**	**(81/91) 89.01±4.73**	**(94/106) 88.67±7.1**
Southern Transition Zone (STZ)	612–1054	H.D. Kote	(10/12) 83.33	(12/13) 92.3	(12/14) 87.71
		Hunsur	(14/15) 93.33	(15/18) 83.3	(11/14) 78.57
		Mean±SE	**(24/27) 88.88±1.66**	**(27/31) 87.09±4.5**	**(23/28) 82.14±4.57**
Northern Transition Zone (NTZ)	619–1303	Belgaum	(5/9) 55.5	(7/12) 58.33	(5/10) 50.0
		Shiggoan	(7/12) 58.33	(6/14) 42.85	(9/16) 56.25
		Savanur	(5/9) 55.5	(6/10) 60.0	(6/12) 50.0
		Dharwad	(6/10) 60.0	(8/13) 61.53	(7/11) 63.63
		Mean±SE	**(23/40) 57.5±1.11**	**(27/49) 55.10±4.32**	**(27/49) 55.10±3.24**
		Overall Mean	**(225/306) 73.52**	**(245/333) 73.57**	**(244/342) 71.34**

Note: Values in parenthesis (n/N) indicates 'n-number of fields with disease' and N-total number of fields evaluated in each study site for a particular year.
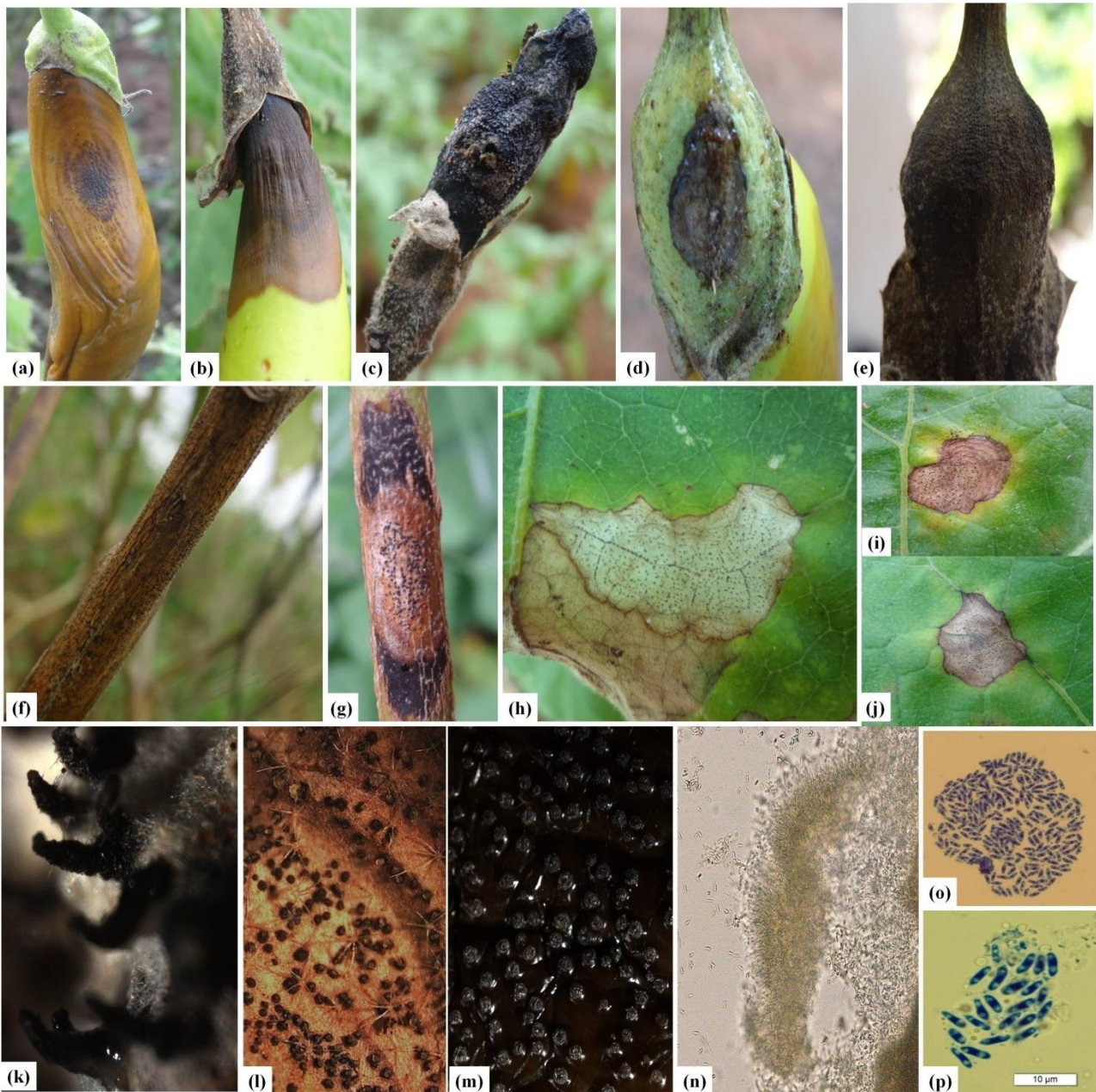
Fig. 2 – Disease symptoms caused by *Phomopsis vexans*. (a, b) fruit rot of brinjal, (c) mummified fruit, (d, e) blight on calyx, (f, g) stem blight on severely infected plants, (h–j) leaf blight, (k) cirri of *P. vexans*, pycnidia on affected leaf and fruit, (n) pycnidia (100 μm), (o, p) alpha conidia.

Table 2 Variations in colony morphology, type, diameter, development and number of pycnidia of 24 selected *Phomopsis vexans* isolates.

Isolate No.	Origin	Appearance of colony	Colony type[b]	Colony colour		Colony diameter cm[c]	Development of pycnidia[d]	No. of pycnidia[e]
				Top view	Reverse view			
PV_MK1 (8)[a]	K.R. Nagar	Floccose	G	White with pale brown stripes	Grey	7.15 (10.21)	18–20	90–120
PV_MK2 (8)	Mysore	Floccose	G	White	Grey	8.8 (12.57)	12–15	>500
PV_MK3 (8)	Nanjangud	Floccose	G	White, with wavy margin	Grey	7.9 (11.28)	18–20	90–140
PV_MK4 (8)	Nagamangala	Wavy	G	White, with wavy margin	Grey	8.5 (12.14)	10–12	>500
PV_MK5 (8)	Mulbagal	Floccose	-	White with pale brown margin	Brownish	6.9 (9.85)	0	-
PV_MK6 (8)	H.D. Kote	Velvety	G	White	Grey	8.4 (11.58)	20–25	10–15
PV_MK7 (8)	Hunsur	Floccose	G	White	Grey	8.9 (12.71)	15–20	50–80
PV_MK8 (7)	Belgaum	Velvety	-	Pale brown with white	Grey	8.8 (12.14)	0	-
PV_MK9 (8)	Shiggoan	Wavy	G	Mixture of brown and white	Grey	9.0 (12.85)	18–20	120–150
PV_MK10 (6)	Pandavapura	Floccose	G	Pale brown with whitish margin	Grey	5.2 (7.42)	18–20	50–60
PV_MK11 (8)	Bhagalkot	Velvety	G	White	Grey	9.0 (12.85)	10–12	200–220
PV_MK12 (8)	Gadag	Floccose	G	White	Grey	8.5 (12.14)	15–20	>500
PV_MK13 (6)	Mundaragi	Floccose	-	Pale brown with white	Brownish	7.1 (10.14)		-
PV_MK14 (8)	Gokak	Velvety	-	White	Grey	6.2 (8.85)	0	-
PV_MK15 (8)	Hosadurga	Wavy	G	White, with wavy margin	Grey	9.0 (12.64)	10–12	>500
PV_MK16 (8)	Arasikere	Wavy	G	White, with wavy margin	Grey	9.0 (12.85)	10–12	>500
PV_MK17 (8)	Koratagere	Velvety	-	White	Grey	8.5 (12.14)	0	-
PV_MK18 (8)	Mandya	Wavy	G	White, with wavy margin	Grey	9.0 (12.42)	10–12	>500
PV_MK19 (8)	Malavalli	Wavy	G	White, with wavy margin	Grey	9.0 (11.25)	10–12	>500
PV_MK20 (8)	Chamarajanagar	Velvety	-	White	Grey	8.2 (11.71)	0	-
PV_MK21 (7)	Yalandur	Wavy	G	White, with wavy margin	Grey	9.0 (12.85)	18–22	>500
PV_MK22 (6)	Savanur	Wavy	G	White, with wavy margin	Grey	8.9 (11.12)	10–12	>500
PV_MK23 (6)	Dharwad	Floccose	G	White	Grey	9.0 (11.25)	10–15	300–350
PV_MK24 (8)	Gouribidnur	Floccose	G	White	Grey	8.4 (10.93)	15–18	300–350

Note: a-value in parenthesis indicates total number of fungal isolates established from each locality; b-colony types were distinguished based on Kanematsu et al. (1999, 2000) and Udayanga et al. (2011); c-values in parenthesis are the average colony growth in mm/day; d-number of days required to express pycnidial locules; e-number of pycnidia per plate.

Table 3 *Phomopsis vexans* ITS sequences used in the construction of phylogenetic tree and complete elucidation of ITS2 sequence for phylogenetic analysis.

Isolate	Source	Origin	GB Acc. No.[a]	Year	ITS2 length	Reference
PV_MK-01	Seed	Karnataka	KJ002759.1	2013	162	This study
PV_MK-02	Seed	Karnataka	KJ002760.1	2012	162	This study
PV_MK-03	Fruit	Karnataka	KJ002770.1	2011	162	This study
PV_MK-04	Fruit	Karnataka	KJ002771.1	2012	162	This study
PV_MK-05	Fruit	Karnataka	KJ002772.1	2012	162	This study
PV_MK-06	Fruit	Karnataka	KJ002773.1	2011	162	This study
PV_MK-07	Fruit	Karnataka	KJ002774.1	2013	162	This study
PV_MK-08	Leaf	Karnataka	KJ002775.1	2011	162	This study
PV_MK-09	Leaf	Karnataka	KJ002776.1	2011	162	This study
PV_MK-10	Leaf	Karnataka	KJ002777.1	2012	162	This study
PV_MK-11	Seed	Karnataka	KJ002778.1	2012	162	This study
PV_MK-12	Leaf	Karnataka	KJ002779.1	2012	161	This study
PV_MK-13	Leaf	Karnataka	KJ002780.1	2011	161	This study
PV_MK-14	Leaf	Karnataka	KJ002781.1	2013	163	This study
PV_MK-15	Seed	Karnataka	KJ002782.1	2011	163	This study
PV_MK-16	Seed	Karnataka	KJ002783.1	2011	163	This study
PV_MK-17	Leaf	Karnataka	KJ002784.1	2013	163	This study
PV_MK-18	Fruit	Karnataka	KJ002785.1	2012	-	This study
PV_MK-19	Fruit	Karnataka	KJ002786.1	2012	-	This study
PV_MK-20	Seed	Karnataka	KJ002787.1	2013	162	This study
PV_MK-21	Leaf	Karnataka	KJ002788.1	2013	162	This study
PV_MK-22	Seed	Karnataka	KJ002789.1	2013	162	This study
PV_MK-23	Leaf	Karnataka	KJ002790.1	2012	162	This study
PV_MK-24	Fruit	Karnataka	KJ002791.1	2013	162	This study
VUS1	Leaf	Karnataka	KJ002767.1	2014	162	GenBank
VUS9	Leaf	Karnataka	KJ002766.1	2014	162	GenBank
PV1	-	Karnataka	GU373628.1	-	-	GenBank
PV2	-	Karnataka	GU373629.1	-	-	GenBank
PV3	-	Karnataka	GU373630.1	-	-	GenBank
PV4	-	Karnataka	GU373631.1	-	-	GenBank
PV5	-	Karnataka	GU373632.1	-	-	GenBank
PV6	-	Karnataka	GU373633.1	-	-	GenBank
MAFF 712047	-	Japan	AB105171.1	2009	-	GenBank
CBS 127.14	-	Netherlands	KC343229.1	2013	162	GenBank
BhSPv	-	West Bengal	AB934366.1	2014	162	GenBank
BSPv	-	West Bengal	AB934367.1	2014	162	GenBank
KbFPv	-	West Bengal	AB934368.1	2014	162	GenBank
TnSPv	-	West Bengal	AB934369.1	2014	162	GenBank
Gui01	-	China	DQ855274.1	2006	-	GenBank
Pv6	-	Karnataka	KF994970.1	2014	162	GenBank
Pv5	-	Karnataka	KF994969.1	2014	-	GenBank
Pv4	-	Karnataka	KF994968.1	2014	162	GenBank
Pv3	-	Karnataka	KF994967.1	2014	-	GenBank
Pv1	-	Karnataka	KF994965.1	2014	-	GenBank

Note: a-Accession numbers are provided for the whole sequence of 18S-ITS1-5.8S-ITS2-28S region from which complete ITS2 sequences were retrieved using ITS2 online database; b-No complete ITS2 sequences found when subjected to ITS2 online database for annotation to retrieve complete ITS2 sequence. This is due to unidentifiable end sequences of LSU.
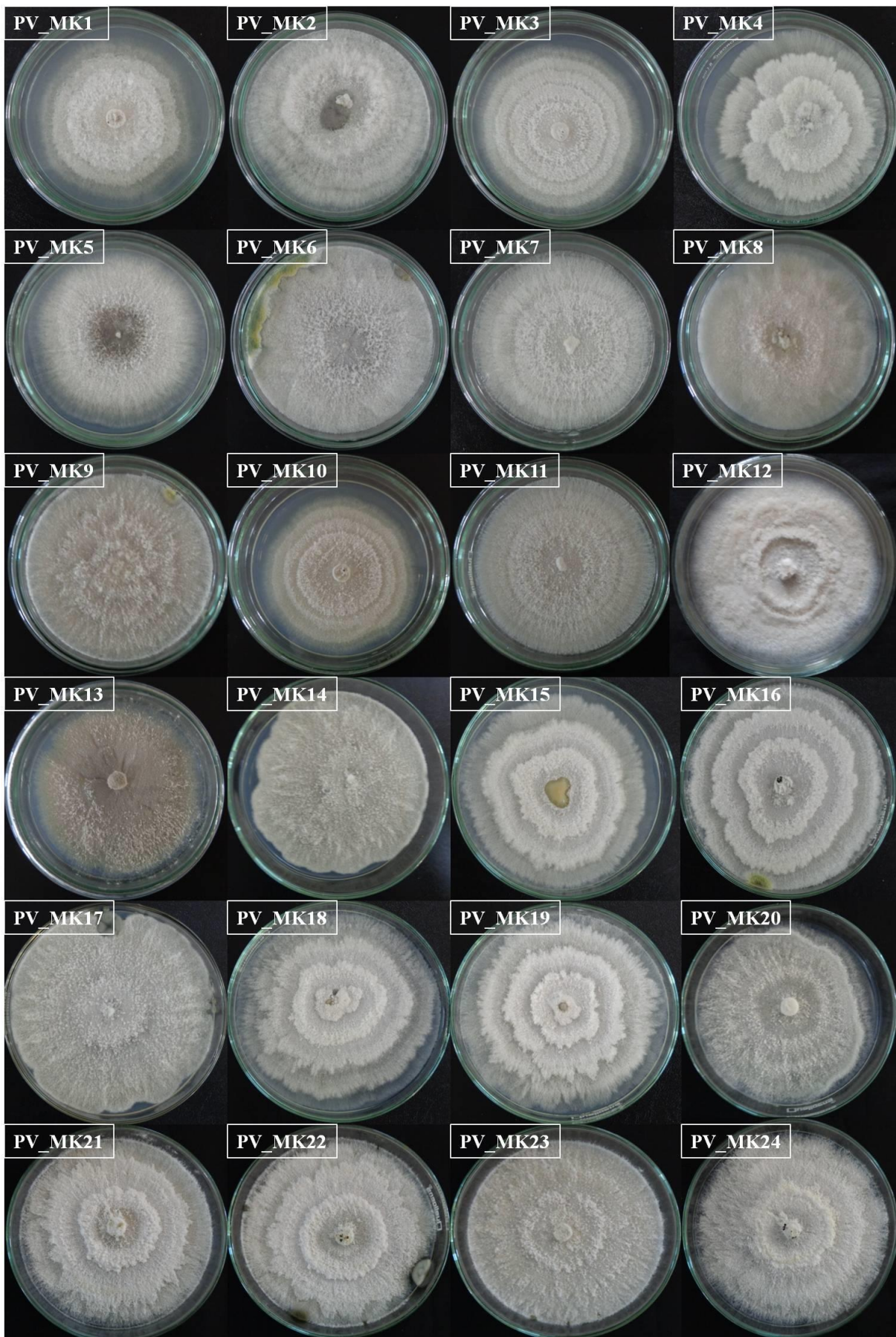
Fig. 3 – Colony morphology of *Phomopsis vexans* isolates on potato dextrose agar medium after 15 days incubation at room temperature (top view).
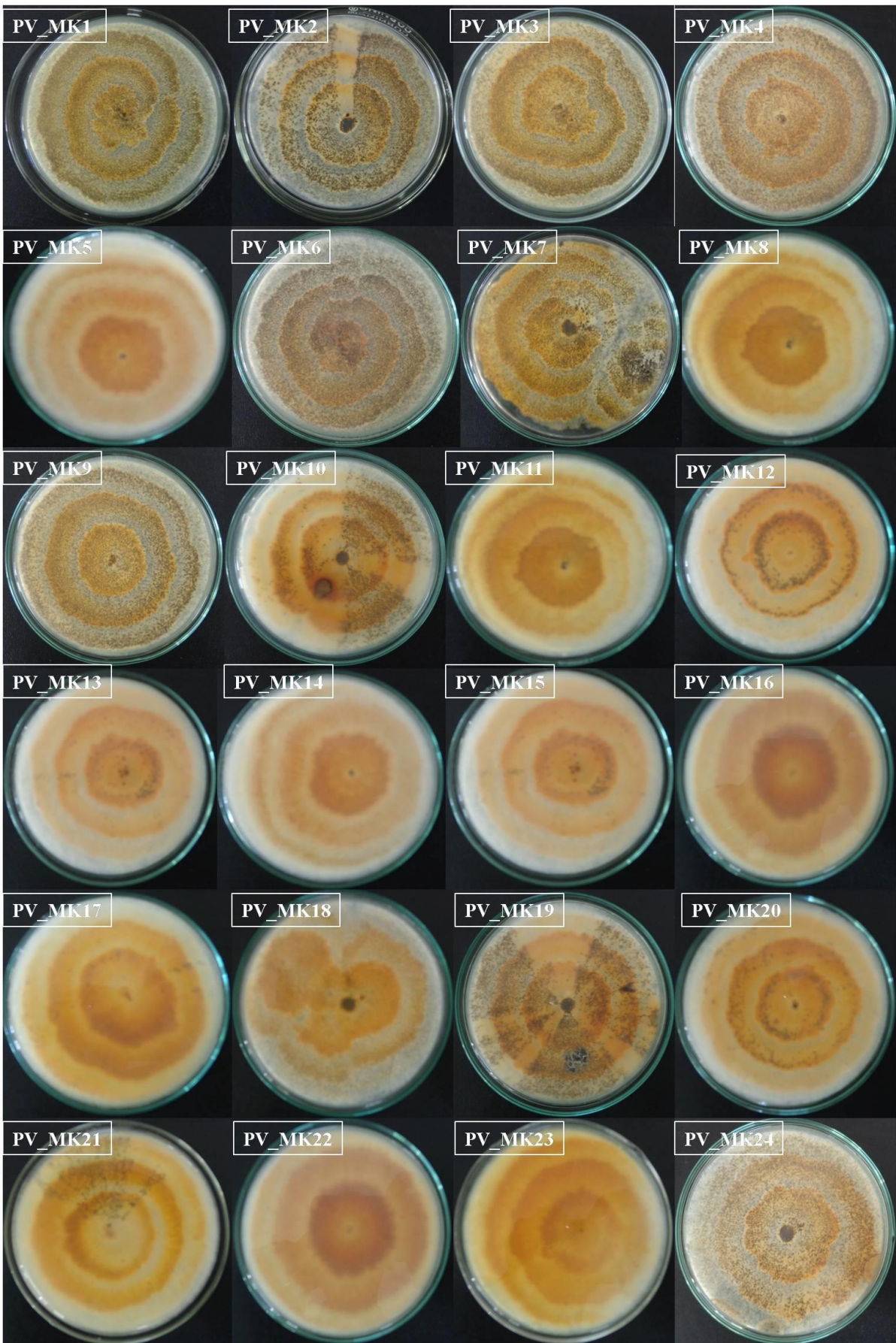
Fig. 4 – Colony morphology of *Phomopsis vexans* isolates on potato dextrose agar medium after 15 days incubation at room temperature (reverse).
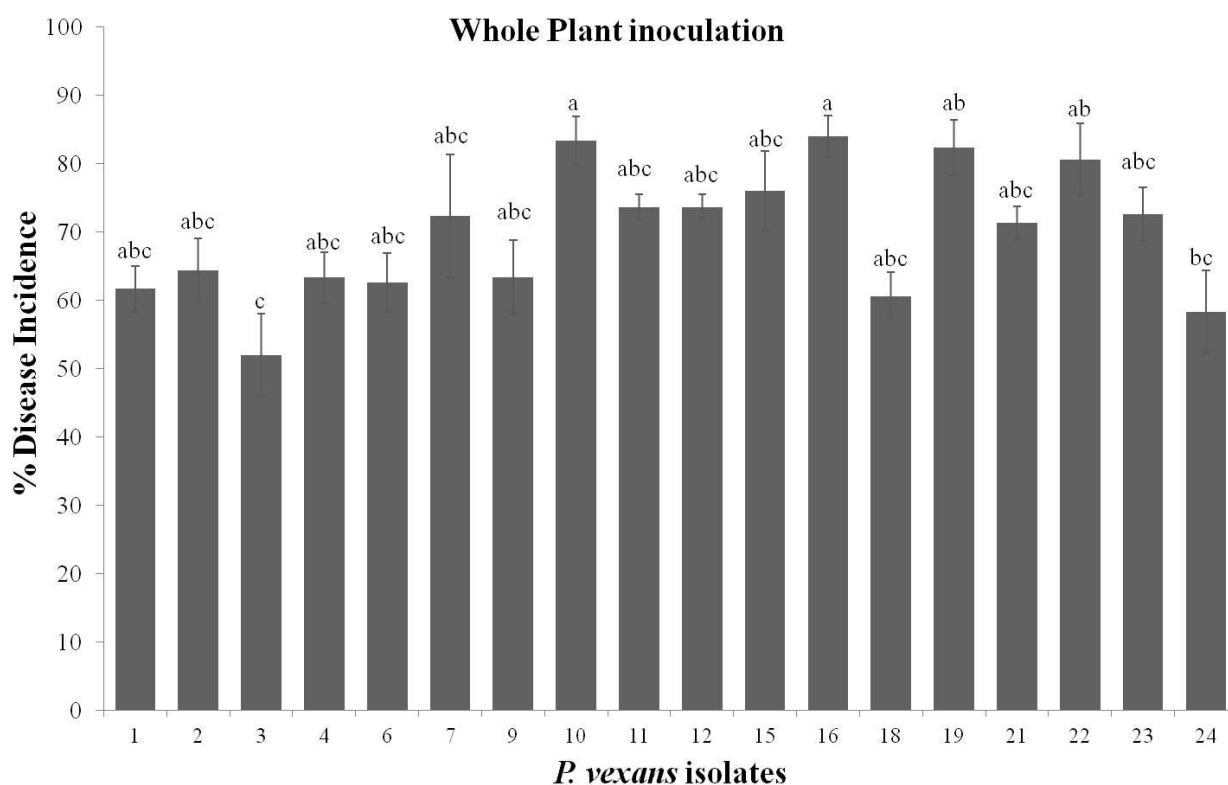
Fig. 5 – Per cent incidence of leaf blight symptoms caused by different isolates of *Phomopsis vexans* by whole plant inoculation. Means annotated by the same letter are not significantly different according to Tukey's HSD test (P<0.05).

Molecular identification and phylogenetic analysis

The PCR amplification using ITS1 and ITS4 universal primers yielded an expected 550–570 bp amplicon in all the 24 isolates. The sequences obtained were submitted to GenBank - KJ002759-KJ002760 (02), KJ002770-KJ002791 (22). The ITS sequences obtained in the present study and the ITS sequences retrieved from GenBank database were used for the construction of phylogenetic trees and are presented in Table 3.

All the 24 isolates formed a major clade with three distinct sub-clades (Fig. 6). The phylogenetic trees (analysis) showed that 16 isolates (PV-MK1 to MK11 and MK18, 19, 22 and 24) clearly shared a single sub-clade 1, six isolates (PV-MK12 to17) formed another sub-clade 2 and two isolates (PV-MK20 and MK21) formed sub-clade 3.

The nucleotide length of ITS2 region varied from 161–163 bp in all the sequences except for 14 isolates of *P. vexans* from GenBank database due to unidentifiable LSU end sequences (Table 3). These ITS2 complete sequences were selected and analyzed phylogenetically. The tree clearly showed two groups (Fig. 8) where 18 isolates (MK1 to MK11 and MK18 to 24) formed a major group 1 and six isolates (PV-MK12 to 17) formed a smaller group 2 (Fig. 7). The variations could be attributed to variations observed in the form of indels and substitutions of nucleotides at various positions among the isolates of *P. vexans*. MSA analysis of complete ITS2 sequences indels were observed in four isolates (MK14 to MK17) at position 151 (G) and 152 (A) and deletions were observed among the six isolates (MK12 to MK17) where T is deleted at position 164 in all six isolates which formed the group 2 in ITS2 phylogenetic tree (Fig. 8). Substitutions were observed at two positions: at nucleotide position 145 in which C was substituted by G (Pv6_KF994970) and at position 149 where substitution of G in place of A occurred in *P. vexans* isolate MK12 to MK17 (KJ002779-KJ002784). These six isolates with variation were collected from NDZ and CDZ.
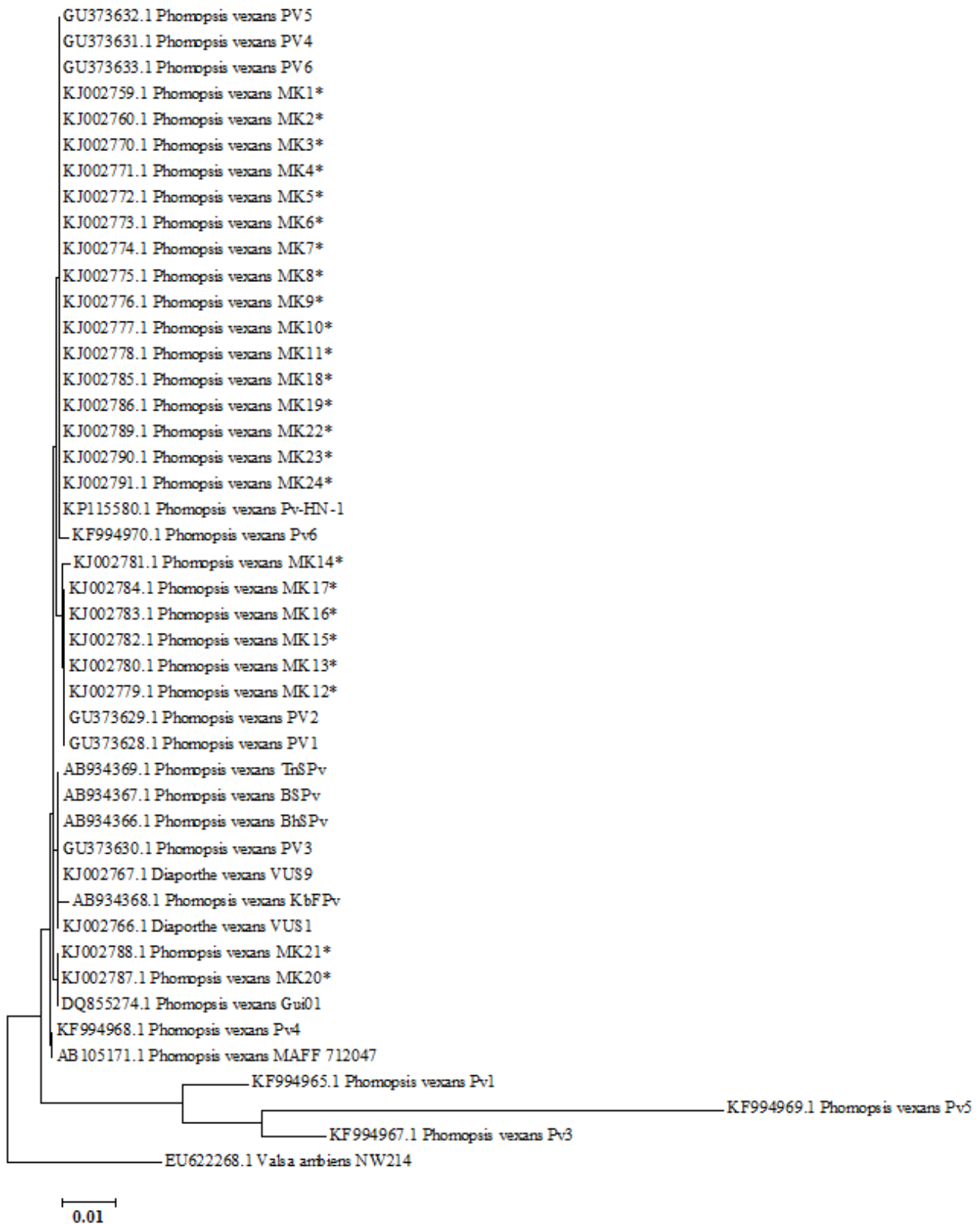
Fig. 6 – Phylogenetic tree constructed by Neighbour-Joining method using ITS regions of *Phomopsis vexans* isolates from Karnataka. Tamura-Nei Substitution model and nearest neighbor-interchange search options with 1000 bootstrap replicates were used.
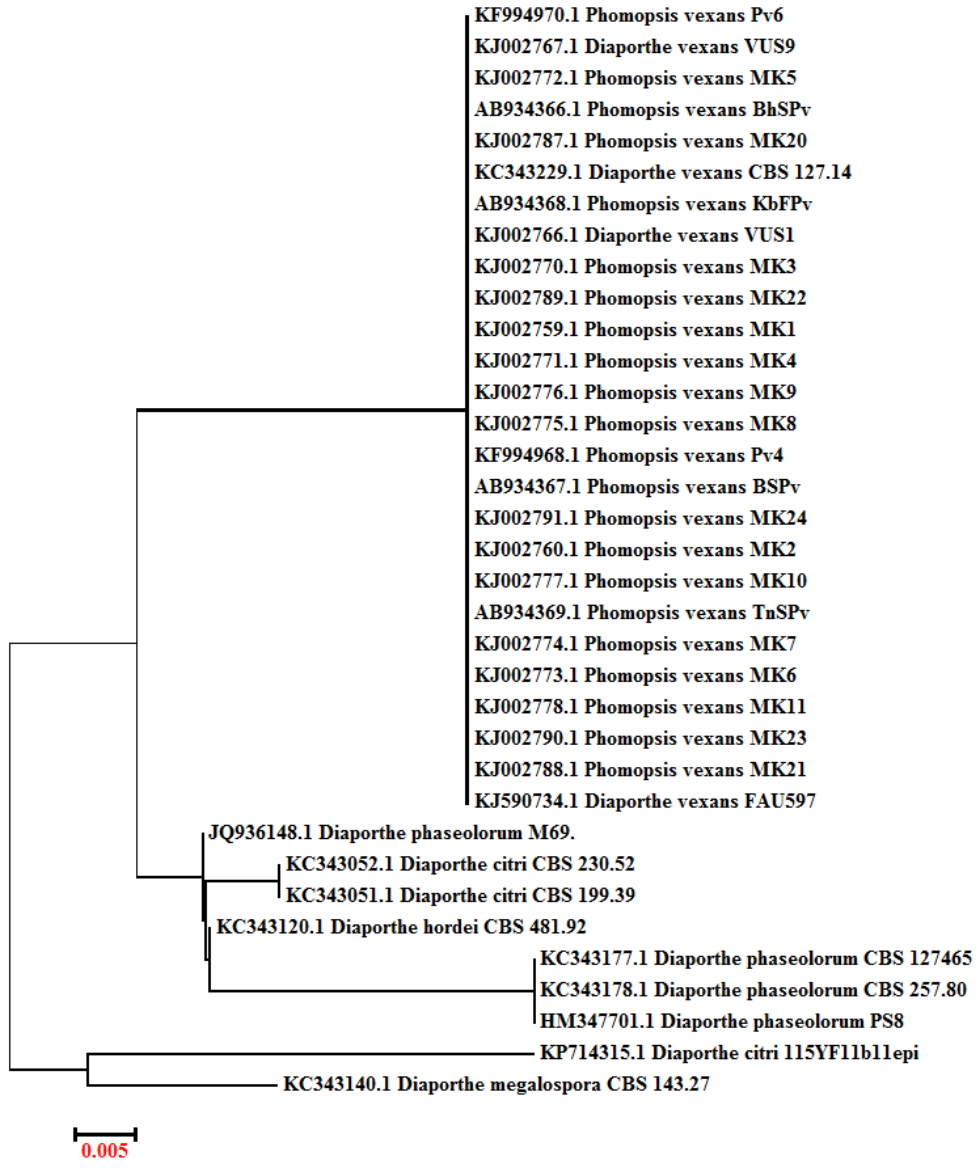
Fig. 7 – Phylogenetic tree constructed by Neighbour-Joining method using complete ITS2 sequences of *Phomopsis vexans* isolates. Tamura-Nei Substitution model and nearest neighbor-interchange search options with 1000 bootstrap replicates were used.

Fig. 8 – Multiple sequence alignment showing the substitutions and indels observed among all the isolates of *Phomopsis vexans* used.

Discussion

India is the largest producer of brinjal next to China with an area of 722,000 hectares under cultivation with annual production of 13,444 million tonnes. South west India comprising Karnataka, Telangana, Maharashtra, Tamil Nadu and Kerala covers an area of 52,410 hectares under brinjal cultivation with an annual production of 1.114 million tonnes. The occurrence of leaf blight and fruit rot disease caused by *Phomopsis vexans* along with damping-off in nurseries and tips over blight during early stage of crop development and stem blight (Singh 1992, Udayanga et al. 2011) causes severe damage to production and yield. However, most of the studies in India and other major brinjal growing areas in Asian countries lack quantitative data on disease incidence and severity. The survey covered most of the brinjal growing regions in south west India. The disease prevailed in all zones surveyed with varying degrees of incidence.

The incidence of leaf blight disease was lower than fruit rot incidence. As shown in the data, certain zones though with lower leaf blight incidence, have higher incidence of fruit rot up to 62% (Malavalli: SDZ) followed by 48% (K.R. Nagar: SDZ) and 41% (Bhagepalli and Mulbagal: EDZ). It could be due to the fact that inoculum buildup has taken place during flowering and sufficient inoculum would be available to cause fruit infection later in the season. Leaf blight incidence could be attributed to the inoculum present in soil or seeds and spread by cultural practices, which in turn are responsible for the appearance of damping-off and seedling blight during early phase of its development followed by the appearance of leaf blight and fruit rot disease when plants attain flowering (Singh 1992). The leaf blight disease affects rate of photosynthesis and hence crop productivity is affected. In many zones, the incidence of fruit rot was very high and it was responsible for severe crop losses. The cultural practices including sprinkling irrigation, adapted during cultivation in recent years, might be responsible for the splash dispersal of soil and fruit-borne conidia and must have caused new infections on the fruits resulting in severe fruit rot incidence. The study revealed that use of hybrid seeds was less compared to the seeds collected by farmers, especially in SDZ, STZ and EDZ. The reduced incidence of *Phomopsis* blight was observed when hybrid seeds were sown. The appearance of leaf blight disease on brinjal was high during humid conditions. As the incidence and severity of plant disease are greatly influenced by the weather (Seem 1984), prevailing high relative humidity, moisture and optimum temperature might have helped the survival and spread of the pathogen that in turn resulted in higher incidence and severity of leaf blight and fruit rot disease.

Most of the information available for identification of *P. vexans* was only on morpho-cultural studies. The overlap in conidial size among species and some of the morphological characters vary with cultural conditions (Udayanga et al. 2011). It is no longer possible to delimit *P. vexans* based on morphological characters alone. Variability of *P. vexans* isolates noted by Akhtar & Choube (2006) indicated that significant and substantial differences in radial growth, colony and zonation exist among the isolates. Based on the colour of the colony Kanematsu et al. (1999, 2000) recognized two morphological groups viz., W and G type, where G-type colony is characterized by a few white to grey aerial hyphae, abundant small pycnidial stroma with irregular pycnidial locules and W-type colony was recognized based on aerial hyphae, scattered relatively large stromata, irregular pycnidial locules with alpha and beta conidia. In the present study, variation in colony morphology and pycnidial development among isolates from different agro-climatic zones and a slight variation was seen with respect to the colony diameter and number of pycnidia developed. Of the 24 isolates studied in detail, 18 isolates representing all six zones belonged to G-type. The rest could not be classified due to lack of sporulation and they were identified to species level by ITS sequence analysis. Morpho-cultural and ITS sequence analysis shared close genetic relatedness among different isolates of *P. vexans* represented by wide geographical areas. Cross inoculation studies conducted by Harter (1914) on various solanaceous crops found that *P. vexans* was host specific as it failed to produce any characteristic disease symptoms on tested crops. Both cultural and morphological studies on *P. vexans* conducted by Islam et al. (2010a, b) showed the occurrence of physiological races based on colony variation and the degree of virulence on different cultivars of brinjal.

Recent studies used ITS sequences along with morphology based characters to identify species of *Phomopsis*. Rehner & Uecker (1994) analyzed ITS1 and ITS2 sequence data for 43 strains of *Phomopsis* from a wide range of hosts and reported variation in ITS sequence data, which may lead to the formation of several cryptic species. Though there are limited numbers of sequences available in GenBank database for *P. vexans*, the complete ITS sequence analyses performed in the present study shared 99% similarity among isolates and further ITS2 sequence phylogenetic analysis also showed two major groups with respect to the changes occurred in complete ITS2 sequence.

The ability of fungal species to cause disease and degree of necrosis has been used to differentiate various pathogenic species. Kanematsu et al. (1999) emphasized the need for comparative studies of pathogenic *Phomopsis* species using morphology and molecular data. In the present study, all the isolates (except for PV_MK5, 8, 13, 14, 17 and 20) were pathogenic on brinjal cv. purple pusa long and high virulence (>80% of DI) was observed among four isolates from SDZ (2), CDZ (1) and NTZ (1). There is a need for screening of brinjal cultivars for durable resistance to *Phomopsis* blight and fruit rot disease. The pathogenic nature of fungal isolates may vary from region to region and isolation, identification of virulent and avirulent isolates may help in understanding the host-pathogen interaction. Work on breeding for disease resistance to *Phomopsis* leaf blight has been carried out with some success in India (Kalda et al. 1976, Datar & Ashtaputre 1988, Pandey et al. 2002) and *Solanum* species have been identified as sources of resistance (Sherf & MacNab 1986, Datar & Ashtaputre 1988) to *P. vexans* on brinjal. However, the fungal pathogen infects all varieties of brinjal irrespective of environmental conditions and agro-climatic zones. Pandey et al. (2002) found no brinjal varieties were resistant to *P. vexans* leaf blight, stem blight and fruit rot diseases. Seeds are the main source of *P. vexans* infection and may serve as substrate for pathogen survival for the next season (Islam & Pan 1992, Pan et al. 1995). The pathogen exists in seed coat and the cotyledons cause various degrees of seed discoloration. *P. vexans* is distributed across Karnataka and causes disease and affects productivity irrespective of the cultivars planted.

This is the first comprehensive study on the quantification of disease incidence of *Phomopsis* leaf blight and fruit rot disease on brinjal from Karnataka (India). Results of the present study demonstrated that prevalence and incidence of the disease was higher in southern dry zone, central dry zone followed by northern dry zone and higher incidence of fruit rot justifies its impact on decrease of yield. Two distinct groups of the pathogen were identified based on ITS2-complete sequence data.

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