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RESEARCH ARTICLE

Cultural, morphological and molecular characterization of *Stemphylium vesicarium* isolates causing onion blight

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Abstract
Stemphylium blight caused by *Stemphylium vesicarium* (Wallr.) is one of the important diseases of onion which causes considerable losses in seed as well as bulb crops, particularly in Northern India. Morpho-molecular characterization for variability among 11 isolates of *S. vesicarium* isolated from different onion cultivars of four states namely, Delhi, Punjab, Karnataka and Maharashtra was done. Variable colony growth was observed as either cottony or velvety and pigmentation was recorded as whitish, light to dark grey to brownish with a filiform margin. The mean colony diameter ranged between 44.53 and 71.64 mm. Conidial colour among different isolates varied from light brown to brown with the mean conidial length ranging between 17.96 and 30.99 µm and the breadth between 11.63 and 17.95 µm. The longitudinal septation of conidia ranged from 0 to 4 and transverse septation varies from 0 to 5. Molecular detection by PCR assay using ITS1F/ITS4R primer amplified about ~ 550 bp amplicon, whereas, *β-tubf1* and *β-tubr1* primer amplified ~ 1400 bp amplicons. The study revealed that accurate identification of *S. vesicarium* based on morphological observation supplemented with molecular characterization will be helpful in understanding the variability among isolates of S. *vesicarium* prevalent in a wide range of geographical conditions.

Keywords *Stemphylium vesicarium* · Onion · Cultural characterization · Sequence · Phylogeny

Introduction

Onion (*Allium cepa* L.) is one of the most important vegetable crops cultivated in India. It is an endospermic monocot that belongs to the family Alliaceae. India is the second-largest onion-growing country globally, with 26.64 million tonnes of production (2021-22). The major onion-producing states are Maharashtra, Karnataka, Madhya Pradesh, Gujarat, Bihar, Andhra Pradesh, Rajasthan, Haryana and Telangana. Maharashtra ranks first in onion production with a share of 39%, followed by Madhya Pradesh with 17% in 2020-21. Indian onions are famous for their pungency and are available round the year. There is a lot of demand for Indian onions worldwide, and major importing countries are Bangladesh, Malaysia, Sri Lanka, UAE, Nepal and Indonesia (APEDA, 2022). Onion is used as a spice, vegetable, salad, and condiment for flavouring several medicines and food items in many countries around the world. (Vohra et al. 1973; Hassan and Hussein 2007). It is a rich source of vitamin C, carbohydrates and protein, including minerals like calcium and phosphorus. Besides, it has many chemical compounds with anti-cancer, anti-cholesterol and anti-inflammatory properties (Slimestad et al. 2007). Onion is best known for its insecticidal and fungicidal properties (Mishra et al. 2014).

Onions are affected by many biotic and abiotic stresses during crop production. Among them, fungal diseases such as damping off, Stemphylium blight, downy mildew, basal stem rot and purple blotch are known to cause substantial losses during seed production. Stemphylium blight [*Stemphylium vesicarium* (Wallr.) Simmons] is one of the critical diseases that causes considerable losses in seed as well as bulb crops. The disease is becoming a major concern in recent years, especially in Northern and Eastern India. The initial symptoms appear as small, yellowish-brown to tan, water-soaked lesions at the 3- to 4-leaf stage (Raghavendra

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Rao and Pavgi 1975). As the disease progresses, extensive necrosis of infected leaves develops from the tip. After infection, the *S. vesicarium* produces host-specific toxins associated with necrosis (Singh et al. 2000). During the later stage of disease development, desiccation and premature lodging of onion make it more susceptible to secondary and post-harvest infections. The intensity of disease is greater in seed crops than in bulb crops. Plants with severe infection produce bulbs of small size that are unmarketable or sold at a lower price (Raghavendra Rao and Pavgi 1975).

Cultivating resistant varieties is the most economical and efficient method of plant disease management. Still, they may become susceptible due to the evolution of new virulent pathogen races. Therefore, continuous identification of durable resistance to a range of virulence present in the pathogen population is necessary. To understand the population structure of pathogens, analysis of variability among isolates using morphological, cultural and molecular characterization is essential. Under natural conditions, *S. vesicarium* has been known to express a wide range of variability in the expression of disease symptoms depending upon the environmental conditions and onion genotypes, as reported by various workers (Hosen et al. 2009; Arzanlou et al. 2012; Nisha 2013). With its backdrop, this study was undertaken to measure the variability among different isolates of *S. vesicarium* collected from four Indian states.

Materials and methods

Collection of infected samples

The plants of different onion cultivars showing typical Stemphylium blight symptoms were collected from Delhi (cvs. Punjab Naroya, Pusa Sowmya, Pusa Red and Pusa Riddhi), Punjab (cvs PRO 6 and L28), Karnataka (cvs., Rajoli Local, Double Red and Bhima Red) and Maharashtra (cvs. Nasik Red and Pune Fursungi) states and were used for pathogen isolation (Table 1).

Table 1 Collection of different onion cultivars showing Stemphylium blight symptoms

Sl. No.	Isolates name	Cultivars Name	Place of collection
1	SV-1	Punjab Naroya	IARI, New Delhi
2	SV-2	Pusa Sowmya	IARI, New Delhi
3	SV-3	Pusa Red	IARI, New Delhi
4	SV-4	Pusa Riddhi	IARI, New Delhi
5	SV-5	Pro 6	Ludhiana, Punjab
6	SV-6	L28	Ludhiana, Punjab
7	SV-7	Rajoli Local	Raichur, Karnataka
8	SV-8	Double Red	Bidar, Karnataka
9	SV-9	Bhima Red	Dharwad, Karnataka
10	SV-10	Nasik Red	Pune, Maharashtra
11	SV-11	Pune Fursungi	Nasik, Maharashtra

Isolation of pathogen

The pathogens from the diseased plants were isolated through the tissue bit transfer method. Briefly, the leaf area of diseased parts, and some healthy portions, were cut into small bits with a sharp sterilized blade. These bits were surface sterilized with 2% NaOCl, followed by 3–4 washings using sterilized distilled water. The surface dried bits were transferred to sterilized 11 cm Petri plates containing potato dextrose agar (PDA) medium and incubated for seven days at 25 ± 1 °C. To maintain the identity of individual isolates, they were designated with an isolate name as SV-1 to SV11.

Purification and maintenance of the pathogen isolates

Pure culture of the *S. vesicarium* was obtained by using a single spore isolation technique. The single spores located under the microscope were selected individually and transferred on sterilized Petri plates. The peripherally developed mycelia were subsequently picked up aseptically for subculturing. The isolates were multiplied as required and were preserved in a refrigerator at a low temperature (4 ± 1 °C).

Pathogenicity test

The potted onion plants were inoculated with different isolates of *S. vesicarium* to know their pathogenicity, as per Basallote-Ureba et al. (1999). The isolates were cultured on PDA, and the sporulating cultures plates were flooded with 10 mL sterile double distilled water. The colony surface was gently scraped using a spatula, followed by cotton gauze filtration to remove mycelial fragments, and the conidial concentration was determined using a hemocytometer. Depending on the number of conidia present, the suspension concentration was adjusted to 4×10^4 conidia per mL and trypan blue solution was added to the suspension to differentiate dead conidial cells from live cells. The conidial suspension of different isolates was inoculated to the eight-week-old onion plants of varitiety Punjab Naroya by spraying with a glass atomizer. The inoculated plants were covered with moist polythene bags and incubated under day/night temperatures of 22–26 °C/ 18–20 °C for four days. Later the plants were moved to a greenhouse to observe the disease development up to three weeks after inoculation.

Cultural variability

All eleven isolates were multiplied on PDA, and 5.0 cm mycelial discs from ten days old culture were transferred aseptically to new Petri plates containing PDA and incubated

for seven days at 25 ± 1 °C. Three replications of each isolate were maintained in the CRD design. The cultural characteristics of isolates, viz. colony colour, type and diameter were recorded at seven days and sporulation at ten days after inoculation. For sporulation studies, a 10 mm mycelial disc was homogenized in 3 mL of sterilized distilled water, and the number of spores was counted using a hemocytometer.

Morphological variability

All eleven isolates from 10-d old cultures grown over a PDA medium were observed using a compound microscope for variation in morphological characteristics. The morphological characters, viz. length and width of conidia (µm), colony growth (mm) on 7th day, conidial septation, colony colour (using RHS colour chart, 5th edition), colony surface texture, colony margin shape and growth pattern were measured using Magplus software. Ten recordings per replication were made for the purpose.

Molecular detection by PCR assays

DNA extraction, PCR amplification, and sequencing

Each fungal isolate was grown in 40 mL of potato dextrose broth in 100 mL Erlenmeyer flasks using an orbital shaker (150 rpm) at 25°C for 10d. Mycelial mat from freshly grown cultures was harvested by filtration using filter paper and dried with sterile blotting paper, and used immediately for DNA extraction or stored at -80ºC for further use. Isolation of DNA from eleven isolates was done using the CTAB method and stored at -20°C. Sequences of the internal transcribed spacer regions (ITS1F and ITS4R) of the nuclear ribosomal DNA (rDNA) were amplified using forward primer ITS1(5′-TCCGTAGGTGAACCTGC GC-3′) and reverse primer ITS4 (5'TCCTCCGCTTATTGA TATGC-3') (White et al. 1990). Sequences of the β-tubulin region were amplified using forward primer *β-tubf1* (5′CAGCTCGAGCGTATGAACGTCT-3′) and reverse primer *β-tubr1* (5′TGTACCAATGCAAGAAAGCCTT-3′) (McKay et al. 1999).

The polymerase chain reaction was performed in 100µL reaction mixture containing 4.0µL of template DNA, 40µL Taq polymerase mixture, 52 µL Nuclease free water and 2.0 µL forward and reverse primer (each) for both ITS and β-tubulin genes. PCR conditions include: initial denaturation at 95 °C for 5.0 min, 39 cycles of denaturation at 95 °C for 45 s, annealing at 56 °C for 1.0 min and extension at 72 °C for 1.0 min followed by a final elongation for 10.0 min at 72 °C. After the PCR reaction, 2.0µL of loading dye was added to the 25.0µL of amplification products and loaded into a 1.5% horizontal agarose gel in TAE buffer pre-stained with ethidium bromide (1.0 µg/mL) along with a 1.5 kb ladder marker. Electrophoresis was carried out at 110v for 1.0 h, and the gel was visualized under UV illumination. The resultant PCR products of both ITS and beta-tubulin were sent to Barcode Biosciences for further sequencing.

'The obtained sequences of ITS and beta-tubulin gene were blasted in the GenBank database of NCBI to as follows The ITS and β sequences were compared by using Blast analysis (http://www.ncbi.nlm.nih.gov). The generated and downloaded sequences were edited and aligned using MEGA ver.8. Phylogenetic analysis was performed using the NJ method with a bootstrap of 1000 replicates (Tamura et al. 2007). The phylogenetic tree was generated based on ITS and B-tubulingene sequence, the representative isolates were analyzed with the reference isolates of *S. vesicarium*, *A. porri* and *S. solani* for ITS sequence and *S. vesicarium*, *A. porri*, *A. alternata* and *S. solani* for B-tubulinsequences to out-group during cluster analysis.

Results

Cultural variability

Significant variations were recorded among different isolates of *S. vesicarium* for various cultural characteristics like colour, type, margin and diameter of the colony and also for its sporulation. *S. vesicarium* isolates when grown on the PDA media showed distinct variations with respect to colony characters (Fig. 1). Colonies observed for mycelial growth were either cottony or velvety with colours ranging from whitish, light to dark grey to brownish in colour. Colony margins were mostly filiform, with a whitish colour (Table 1; Fig. 1). Conidial colour among different isolates varied from light brown to brown (Table 2).

Morphological variability

From the data presented in (Table 3), significant variation was noticed among different isolates for colony diameter after seven days of incubation. The colony diameter indicated the growth ability of the fungal mycelium. The isolate Sv-5, with a mean colony diameter of 71.64 mm, was the fastest-growing, while the Sv-11 isolate, with a 44.53 mm diameter, was the slowest. Further variations were recorded among different isolates for various morphological characteristics such as conidial shape and septation. Pathogen isolates observed for sporulation showed significant variation, with the mean ranging from 51.54 to 59.27 conidia mm^{-2} (Table 2). The maximum (30.99 µm) and minimum (17.96 µm) mean conidial length were observed in isolate Sv-5 and Sv-3, respectively. While maximum (17.95 µm)

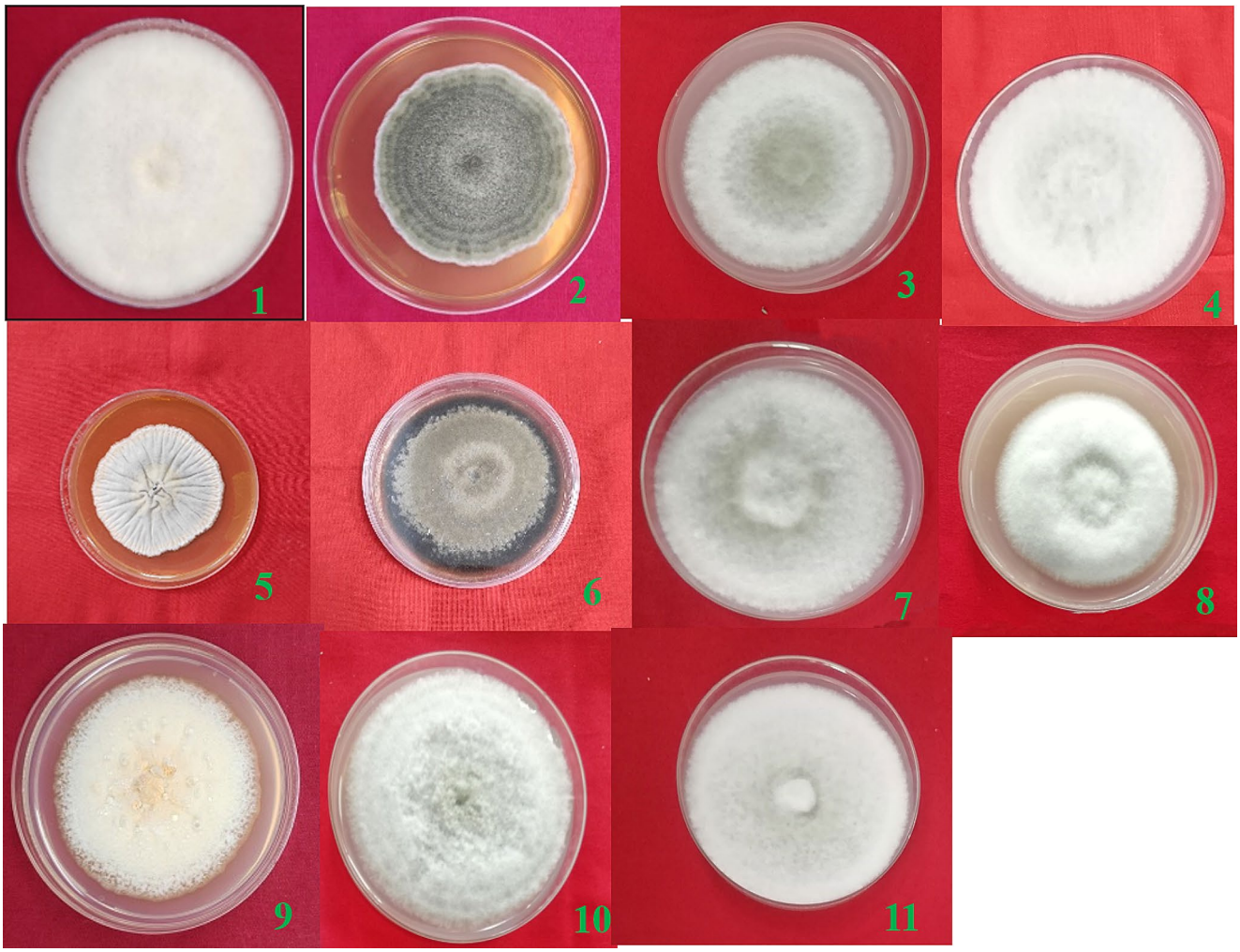

Fig. 1 Cultural variability of different *Stemphylium vesicarium* isolates **No 1–11: Isolate code = SV 1- SV 11 (SV–*Stemphylium vesicarium*)**

and minimum (11.63 µm) conidial width were observed in isolate Sv-7 and Sv-11, respectively. The septation in conidia, both longitudinal and transverse varied significantly among different isolates. Longitudinal septation varied from 0 to 4 and Transverse from 0 to 6.

Molecular detection by PCR assays

PCR assays were carried out using ITS1F/ITS4R and *β-tubf1* and *β-tubr1* primer pairs. About ~ 550 base pairs amplicons were amplified in PCR assay with the ITS1F/ITS4R primer pairs (Fig. 2) and around ~ 1400 base pairs were consistently obtained in the *β-tubf1* and *β-tubr1* pair from the eleven isolates of *S.vesicarium* collected from four states (Fig. 3).

Sequence analysis

A BLASTn sequence identity search of GenBank database and pairwise comparison of ~ 550 bp of ITS gene sequences of eleven *S. vesicarium* isolates (Acc. No. OP521668, OP521669, OP521670, OP521671, OP521672, OP521673, OP521674, OP521675, OP521676, OP521677, and OP521678) in onion revealed 99.45–99.82% sequence identity with earlier reported *S. vesicarium* strain in mango (Acc. No. MH879836), *Populus cathayan* Rehd (Acc. No. KT192286), Asparagus (Acc. No. MH628103) and in onion (Acc. No. MN596829). Further, sequence comparison of ~ 1400 bp amplicons of β-tubulin gene of 11 onion *S. vesicarium* (Acc. No. OP832359, OP832360, OP832361, OP832362, OP832363, OP832364, OP832365, OP832366,

Table 2 Colony characteristics of different isolates of *Stemphylium vesicarium*

S. No.	Isolates	Colony characteristics			
		Colour	Growth Type	Margin	Conidial Colour
1	SV-1	Whitish	Cottony	Filiform, whitish	Brown
2	SV-2	Greyish	Cottony	Entire, whitish	Light brown
3	SV-3	Whitish to light grey center	Cottony	Filiform, whitish	Light brown
4	SV-4	Whitish	Cottony	Filiform, whitish	Light brown
5	SV-5	Whitish to light grey	Velvety	Filiform, whitish	Light brown
6	SV-6	blackish with dark grey center	Velvety	Filiform, blackish	Brown
7	SV-7	Whitish	Velvety	Entire, whitish	Light brown
8	SV-8	Whitish with light grey center	Velvety	Filiform, whitish	Light brown
9	SV-9	whitish	Velvety	Filiform, whitish	Brown
10	SV-10	Whitish	Velvety	Filiform, whitish	Light brown
11	SV-11	Whitish	Cottony	Filiform, whitish	Brown

OP832367, OP832368 and OP832369) shared 98.80–100% sequence similarity with the earlier reported *S. vesicarium* in *Limonium cordatum* (Acc. No. MT881940), *Limoniastrum monopetalum* (Acc. No. MT671893) and in *Lobularia maritima* (Acc. No. MT671903) (Table 4).

Phylogenetic analysis

A phylogenetic tree was constructed using partial ITS and β-tubulin gene sequences of all the representative isolates of *S. vesicarium* (Acc. No. OP521668, OP521669, OP521670, OP521671, OP521672, OP521673, OP521674, OP521675, OP521676, OP521677 and OP521678 and (Acc. No. OP832359, OP832360, OP832361, OP832362, OP832363, OP832364, OP832365, OP832366, OP832367, OP832368 and OP832369. During cluster analysis, it was observed that the eleven representative isolates of *S. vesicarium* were clustering among themselves and also with the earlier reported reference isolates of *S. vesicarium* isolates. However, representative isolates of *S. vesicarium* were clearly out-grouped from the reference isolates of *A. porri* and *S. solani* for ITS sequence and of *A. porri*, *A. alternata* and *S. solani* for B-tubulinsequences (Figs. 4 and 5). Thus, the pathogen was identified as *S. vesicarium* based on molecular characteristics. Hence, the results of the present molecular study confirmed the association of *S. vesicarium* with eleven onion isolates from states viz., Karnataka, Maharashtra, Delhi and Punjab.

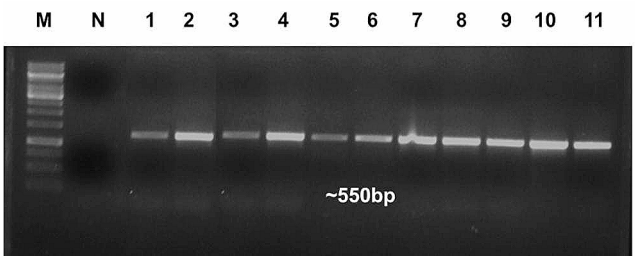

Fig. 2 Gel pictures showing the amplicon of ITS region of eleven isolates M- Molecular marker (100 bp), N–Distilled water, (1–11)-Isolates of *S. vesicarium*

Pathogenic variability

All eleven isolates exhibited the ability to induce disease symptoms, albeit with variations in key parameters such as incubation period and lesion characteristics (including number, size, and colour). The incubation period ranged

Table 3 Conidial characteristics of different isolates of *Stemphylium vesicarium*

S. No	Isolates	Colony characteristics		Conidial characteristics			
		Diameter (mm^2)	Density (conidia/mm^2)	Length (µm)	Width (µm)	Transverse septation	Longitudinal septation
1	SV-1	55.83	55.42	23.30	12.55	1–3	0–3
2	SV-2	52.67	59.27	18.97	14.62	0–4	0–4
3	SV-3	47.55	57.49	17.96	11.24	1–3	1–4
4	SV-4	63.14	54.05	28.86	15.06	0–4	0–4
5	SV-5	71.64	53.79	30.99	14.71	1–4	0–4
6	SV-6	53.01	53.83	27.80	17.10	1–6	0–3
7	SV-7	51.16	54.39	26.16	17.95	2–4	0–2
8	SV-8	48.59	54.23	29.16	16.31	2–5	1–3
9	SV-9	50.49	54.27	21.56	13.26	1–4	0–3
110	SV-10	54.15	52.41	26.04	13.32	0–3	0–2
11	SV-11	44.53	51.54	21.23	11.63	1–3	0–1
	C.D	2.550	0.010	0.310	0.009		

*Average of 10 observations

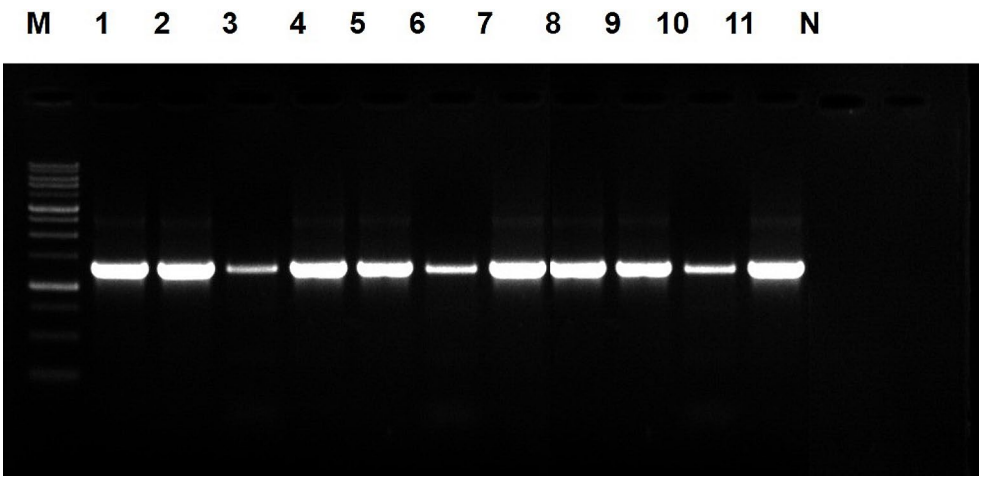

Fig. 3 Gel pictures showing the amplicon of β-tubulin region of eleven isolates M- Molecular marker (1 kb), N–Distilled water, (1–11)-Isolates of *S. vesicarium*

Table 4 Accession number given by National Center for Biotechnology Information (NCBI)

Isolates	ITS	BETA TUBULIN
SV-1	OP521668	OP832359
SV-2	OP521669	OP832360
SV-3	OP521670	OP832361
SV-4	OP521671	OP832362
SV-5	OP521672	OP832363
SV-6	OP521673	OP832364
SV-7	OP521674	OP832365
SV-8	OP521675	OP832366
SV-9	OP521676	OP832367
SV-10	OP521677	OP832368
SV-11	OP521678	OP832369

from 7 to 10 days across different isolates, marking the onset of symptoms. Notably, six isolates displayed whitish lesions, while one isolates exhibited a yellowish hue (Table 5). Conversely, only four isolates displayed brown-colored lesions. Based on lesion size, the isolates were categorized into four distinct groups: Group I (< 1.50 mm), Group II (1.50–2.00 mm), Group III (2.01–2.50 mm), and Group IV (> 2.50 mm). Group I, encompassing 18% of the isolates (Sv-07, Sv-08), were classified as mildly virulent. Meanwhile, Group II, comprising 18.18% of isolates (Sv-09, Sv-17), exhibited a moderate level of virulence. Group III, consisting of 27.27% of isolates (Sv-05, Sv-06, Sv-10), were categorized as virulent, and Group IV, comprising 36.36% of isolates (Sv-01, Sv-02, Sv-03, Sv-04), were deemed highly virulent. These findings align with previous research, as reported by Llorente et al. (2012), which also identified four distinct virulence groups in *S. vesicarium* isolates based on lesion lengths induced by artificial inoculations. Similar observations of variation in incubation period, lesion color, and lesion size were documented in 79 *S. vesicarium* isolates during artificial inoculation by Basallote-Ureba et al. (1999) and 36 *S. vesicarium* isolates by Hassan et al. (2020).

Discussion

Limited published information is available in India with respect to morphological and molecular characterization of *S. vesicarium* causing Stemphylium blight of Onion. Identification of pathogen isolates based on morphological characters alone may give ambiguous results about the representative pathogen. However, combined morphological and molecular analysis helps in unambiguous identification of different isolates of pathogens. In our studies, diseased samples from four states were analyzed for both morphological and molecular characterization. Variation was observed in the cultural characteristics among different isolates of *S. vesicarium*. It was observed that considerable variability was present in the natural population of S. *vesicarium*. Several workers have also reported variability in cultural characteristics among different *S. vesicarium* isolates (Pei et al. 2011; Arzanlou et al. 2012; Nisha 2013; Hassan et al. 2020). Similarly, a significant difference was observed for morphological studies and our findings were comparable with that of many workers who observed wide morphological variability in *S. vesicarium* (Hassan et al. 2006; Pei et al. 2011; Arzanlou et al. 2012; Mc Kenzie 2013; Poursafar et al. 2016; Woudenberg et al. 2017; Hassan et al. 2020).

About ~ 550 bp and ~ 1400 amplicons were observed during PCR assay using ITS1F or ITS4R and *β-tubf1* and *β-tubr1* primer pairs. Phylogenetic analysis of representative isolates of *S.vesicarium* were clearly out grouped

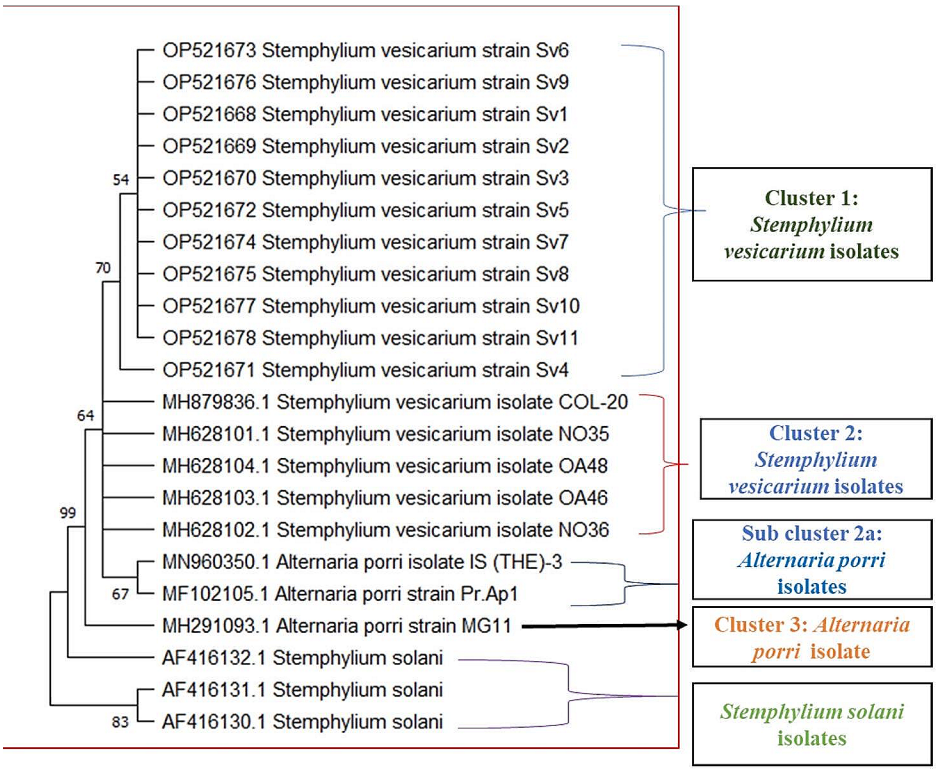

Fig. 4 Phylogenetic tree obtained from an analysis of the ITS sequences from *Stemphylium vesicarium*

from other pathogens such as *A. porri* and *S. solani* for ITS sequence and of *A. porri*, *A. alternata* and *S. solani* for β-tubulinsequences. In earlier studies, Chaisrisook et al. (1995) analyzed the genomic similarity of geographically diverse *Stemphylium* species which were isolated from the alfalfa using RAPD markers and observed DNA polymorphisms among 28 isolates from five morphology based taxonomic species of Stemphylium and one isolate each of *Pithomyces chartarum* and *P. atro-olivaceus*. Wang et al. (2010) explained species of *Stemphylium* based on molecular characterization using ITS and gpd genes and phylogenetic analyses and reported that *S. variabilis* and *S. phaseolina* are two distinct phylogenetic species. Al-amiri et al. (2016), while working on leaf spot causing fungi *S. lycopersici* in tomato based on the sequence analysis of a combined dataset of the internal transcribed spacer and glyceraldehyde-3-phosphate dehydrogenase regions and observed the presence of a very low level of genetic differentiation between populations of *S. lycopersici.* This work on *S. vesicarium* is novel as it deals with the study of molecular characterization using ITS and β-tubulin in *S. vesicarium* causing *Stemphylium* blight of onion. This study revealed the presence of variability in the natural population among different isolates for pathogenic, morphological and cultural traits. Molecular characterization further helps in the accurate identification of isolates. Therefore, understanding variability studies show some light with respect to the Stemphylium blight pathogen and will help a researcher in planning to carry out research work in this area.

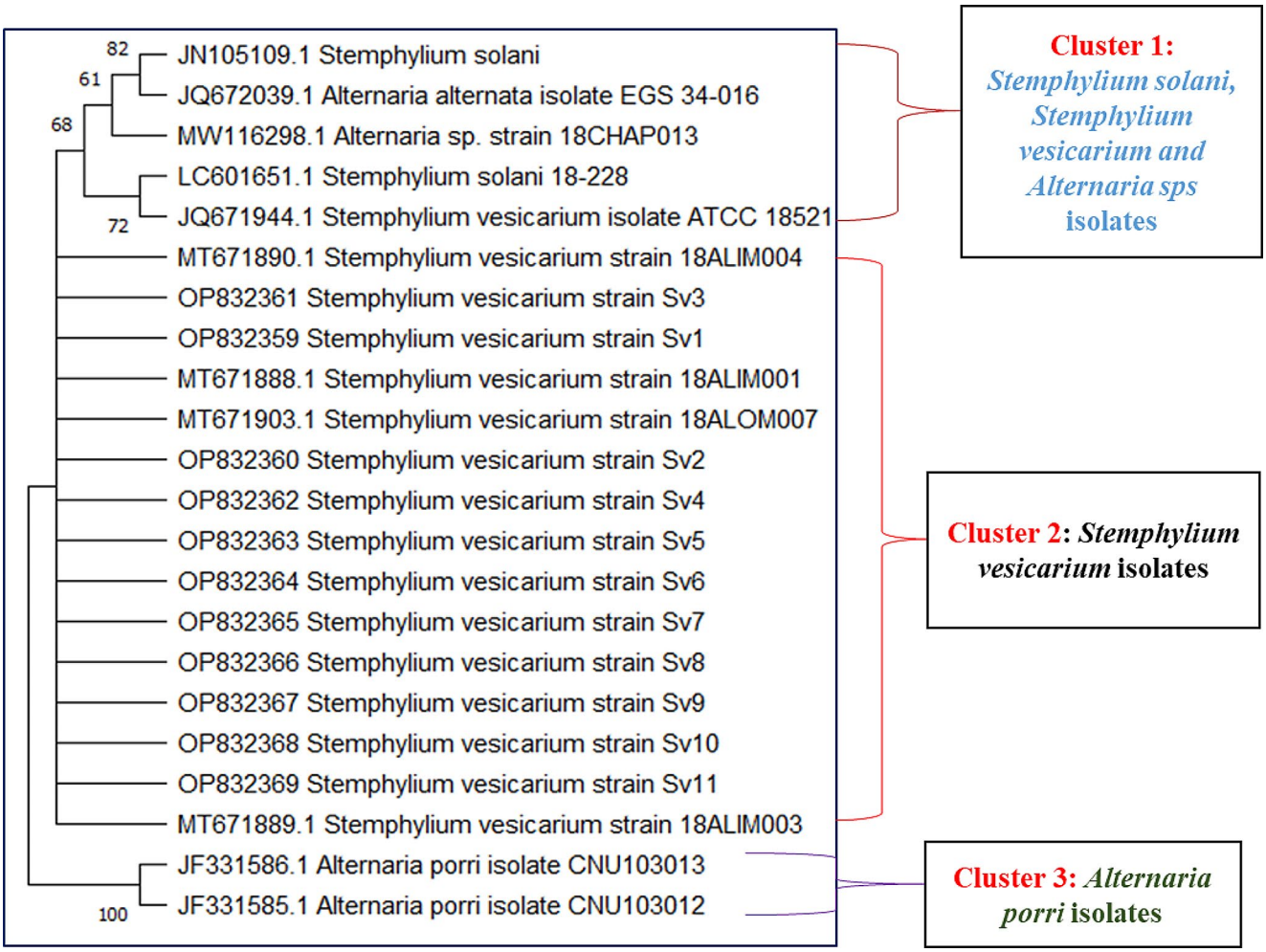

Fig. 5 Phylogenetic tree obtained from an analysis of the β-tubulinsequences from *Stemphylium vesicarium*

Table 5 Pathogenic variability in *Stemphylium vesicarium* isolates

Isolates name	Incubation period	Size of lesion		Colour
		Range	Mean	
SV-1	07	02-3.5	2.47	White
SV-2	07	2.5-4.0	2.69	White
SV-3	07	02–03	2.88	White
SV-4	07	02–03	2.64	White
SV-5	10	02–03	2.12	White
SV-6	10	02–03	2.20	White
SV-7	08	01–02	1.30	Yellow
SV-8	09	01–02	1.13	Brown
SV-9	09	01–03	1.72	Brown
SV-10	10	02–03	2.10	Brown
SV-11	09	01–03	1.59	Brown

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Author contributions Conceptualization of research (AK, JA, DV, SJ); Designing of the experiments (AK, JA, DV, NP); Contribution of experimental materials (AK, JKR, JA); Execution of field/lab experiments and data collection (NP, AK, JA, GPM, SJ); Analysis of data and interpretation (NP, SJ, AK, JA, GPM); Preparation of the manuscript (NP, AK, SJ, JA, GPM).

Declarations

Conflict of Interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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