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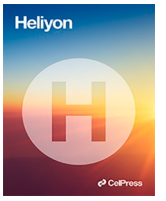

Research article

Morpho-cultural and molecular variability of *Stemphylium vesicarium* causing Stemphylium leaf blight in tropical onions

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ABSTRACT

The cultural, morphological, and molecular variations among 22 isolates of *Stemphylium vesicarium* (Wallr.) E. Simmons, collected from different locations, was carried out. Significant variations were observed among the *Stemphylium* isolates regarding colony diameter, mean radial growth rate and sporulation on PDA media. The colonies of *S. vesicarium* isolates exhibited diverse mycelial growth characteristics, including velvety, cottony, or fluffy textures and a range of colours from whitish to dark grey, olivaceous with a greenish tinge or brownish. The margins of the colonies were observed to be filiform, displaying a filamentous appearance, with entire and undulate shapes and a whitish colouration. The conidiophores of *Stemphylium* isolates displayed a wide range of dimensions, with average length ranging from 36.81 μm to 66.44 μm and average breadth from 3.05 μm to 6.96 μm. Similarly, the conidia exhibited variations in size, colour, shape, average length (23.31 μm–43.18 μm) and average breadth (12.84 μm–23.13 μm). The conidia were mainly light brown or brown and displayed ovoid, oblong, or ovoid-to-oblong shapes. The number and presence of transverse and longitudinal septa also varied among the isolates. Fifteen RAPD primers generated 192 banding patterns. SV4, OPL5, and SV5 were identified as the most polymorphic primers, while OPA3, OPF10, OPN7, OPS7, and OPS10 produced the fewest polymorphic bands. The average polymorphic information content (PIC) value was 0.37, with OPA5 and OPC8 showing the highest PIC values. Cluster analysis based on genetic similarity revealed five distinct clusters, but no clear correlation between isolates and their collection sites was observed. In the phylogenetic analysis, based on Internal transcribed spacer (ITS) region and Glycerol-3-Phosphate Dehydrogenase 1(*gpd1)* gene sequences, 20 isolates obtained from diseased onion leaves formed a distinct cluster and exhibited sequence similarity with ex-type sequence of *Stemphylium vesicarium*. Additionally, two isolates from diseased garlic samples showed similarity with ex-type sequence of *Stemphylium eturmiunum*. This is the first-time report of *S. eturmiunum* on Indian garlic under field conditions.

1. Introduction

Onion (*Allium cepa* L.), belonging to the *Amaryllidaceae* family, holds significant importance worldwide due to its culinary,

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Table 1
Details of *Stemphylium* isolates collected from different locations in India.

Isolate	Host	Location	State	Gen bank accession number (ITS)	Gen bank accession number (*gpd*)	Coordinates
SV-01	Onion	Masit, Alwar	Rajasthan	OP473945	OR490766	28°04′38.9″N 76°48′22.1″E
SV-02	Onion	Bhubaneswar	Orissa	OP473946	OR490767	20°17′34.3″N 85°50′30.6″E
SV-03	Onion	Pantnagar	Uttarakhand	OP473947	OR490768	29°01′20.3″N 79°29′01.3″E
SV-04	Onion	Rohtak	Haryana	OQ346312	OR490769	28°55′25.1″N 76°35′26.3″E
SV-05	Onion	Mandsaur	Madhya Pradesh	OP473948	OR490770	24°00′44.0″N 75°21′14.2″E
SV-06	Onion	Bengaluru	Karnataka	OP473949	OR490771	13°08′29.5″N, 77°30′04.8″E
SV-07	Onion	Jobner	Rajasthan	OP473950	OR490772	26°57′54.8″N 75°22′36.5″E
SV-08	Onion	Tripura	Tripura	OP473951	OR490773	23°54′23.5″N 91°18′47.4″E
SV-09	Onion	Jammu	J&K	OP473952	OR490774	32°39′26.2″N 74°47′55.2″E
SV-10	Onion	Nasik	Maharashtra	OP473953	OR490775	28°36′32.3″N 77°06′08.1″E
SV-11	Onion	Shikohpur (1)	Haryana	OP473954	OR490776	28°22′19.1″N 76°59′21.3″E
SV-12	Onion	Junagadh	Gujarat	OP473955	OR490777	21°50′10.4″N, 70°43′32.8″E
SV-13	Onion	Shikohpur (2)	Haryana	OP473956	OR490778	28°22′17.3″N 76°59′20.6″E
SV-14	Garlic	New Delhi	Delhi	OP473957	OR268890	28°38′43.0″N, 77°09′11.3″E
SV-15	Onion	Mathura	Uttar Pradesh	OP473958	OR490779	27°36′25.8″N 77°35′44.5″E
SV-16	Onion	Bathinda	Punjab	OP473959	OR490780	30°12′47.8″N 74°54′22.2″E
SV-17	Onion	Ludhiana	Punjab	OP473960	OR490781	30°53′59.7″N 75°47′34.5″E
SV-18	Onion	Palampur	Himachal Pradesh	OP473961	OR490782	32°05′54.8″N 76°32′33.9″E
SV-19	Garlic	Bilaspur	Himachal Pradesh	OP473962	OR268891	31°22′16.4″N 76°38′37.8″E
SV-20	Onion	Rajgurunagar	Maharashtra	OP473963	OR490783	18°50′35.4″N 73°53′00.5″E
SV-21	Onion	Hisar	Haryana	OP473964	OR490784	29°09′04.1″N 75°42′44.9″E
SV-22	Onion	New Delhi	Delhi	OP473965	OR490785	28°38′43.0″N, 77°09′11.3″E

nutritional, and medicinal value. It occupies an important agricultural area of approximately 5.77 million hectares worldwide, and its global production is estimated at approximately 106.59 million metric tons [1]. India has become the largest onion producer, producing 26.64 million metric tons from an area of 1.62 million hectares [1]. Despite being one of the largest onion producers in the world, India needs to catch up compared to other nations such as South Korea, the Netherlands, and the USA in terms of productivity. Onion is generally grown under open field conditions and is susceptible to various fungal diseases that can infect during cultivation, transportation, and storage. Several fungal pathogens are associated with different foliar diseases of onion [2–5].

Among fungal diseases, Stemphylium leaf blight (SLB) has resurfaced as a significant limiting factor in *Allium* spp. cultivation, especially in winter crops [6–8]. The genus Stemphylium, belonging to the group of filamentous ascomycetes, encompasses various endophytic, saprophytic, and plant pathogenic species [9–11]. Among all the Stemphylium species, *S. vesicarium* is found to be mainly associated with leaf blight of onion [8,12–14]. SLB is characterized by the appearance of small yellow-tan, water-soaked lesions, which rapidly progress into elongated, spindle-shaped, or ovate spots, sometimes with pinkish margins [8,14]. *Stemphylium vesicarium* causes severe damage, particularly to the leaves and seed stalk of onion plants, leading to substantial losses in the onion seed crop. It severely affects the leaves and seed-stalk of onion and cause losses of up to 80–85 % in seed crop [7,15]. It also reduces the quality and quantity of both bulbs and seeds [16].

A comprehensive understanding of pathogen population structure and how variation arises within the populations is a prerequisite to devising any management strategies. Variability studies are crucial for documenting changes within populations and individuals, particularly in terms of morphological, cultural, and pathogenic characteristics. In field conditions, *S. vesicarium* has exhibited significant variability in the expression of disease symptoms. This variability is influenced by factors such as the onion cultivars and environmental conditions [14,17,18]. Standard sets of differential hosts, as proposed by Ref. [19], are commonly used to detect and assess variability in pathogen populations. In situations where standard sets of differential hosts are not available for specific host-pathogen systems, alternative methods are employed to detect variability in pathogen populations [20]. The traditional approach involves the utilization of morphological, cultural and chemical markers. Such markers are, however, very much influenced by the cultivation medium, prolonged subculturing, and environmental conditions [21]. Molecular methods, such as AFLP, ITS-RFLP, RAPD, URP-PCR, and other markers, complement cultural and morphology-based methods for assessing pathogen variability [22,23]. DNA's Internal Transcribed Spacer (ITS) region is extensively utilized for systematic molecular studies, primarily at the species level [23]. Its high variability enables differentiation within species, enhancing our understanding of fungal diversity and relationships.

Currently, not a single commercial onion variety resistant to SLB disease is available in the market worldwide. The disease is commonly managed through fungicides with a single-site mode of action. However, this practice can contribute to the development of resistance among pathogen populations. Reports have indicated decreased sensitivity or reduced effectiveness of widely used fungicides in managing SLB [8]. Understanding pathogen variability across different regions of onion cultivation is vital for any resistant variety developmental programs. Very few reports are available on variability studies of *Stemphylium,* the causal pathogen of SLB in onions. Therefore, this experiment aimed to isolate, characterize and compare the isolates of Stemphylium recovered from diseased

Table 2
Colony characteristics of *Stemphylium vesicarium* isolates.

Isolate	Colony type	Colour	Colony elevation	Margin	Radial diameter (mm) on 7th day	Radial growth rate (mm/day)	Sporulation (number of conidia/plate)
Sv-01	Cottony, non-appressed	Whitish light grey centre	Flat	Filliform	50.56^{h}	3.61^{h}	1.46×10^{5bc}
Sv-02	Cottony, non-appressed with concentric zones	Whitish light grey centre	Raised	Filliform	59.93^{ef}	4.28^{ef}	1.20×10^{5bc}
Sv-03	Velvety, non-appressed	Olivaceous green with light grey centre	Flat	Filliform	56.91^{fg}	4.06^{fg}	1.50×10^{5bc}
Sv-04	Cottony, non-appressed	Light grey to whitish	Raised	Filliform	65.90^{bc}	4.70^{bc}	1.30×10^{5bc}
Sv-05	Cottony, non-appressed	Whitish	Flat	Filliform	68.17^{ab}	4.87^{ab}	1.13×10^{5c}
Sv-06	Cottony, non-appressed	Light grey to whitish	Raised	Entire	58.58^{efg}	4.18^{efg}	1.06×10^{5c}
Sv-07	Cottony, non-appressed	Light grey with whitish centre	Raised	Filliform	51.25^{h}	3.66^{h}	1.40×10^{5bc}
Sv-08	Cottony, non-appressed	Whitish	Flat	Undulate	59.38^{ef}	4.24^{ef}	1.37×10^{5bc}
Sv-09	Velvety, non-appressed centre with concentric rings	Whitish light grey centre	Flat	Undulate	61.43^{de}	4.39^{de}	1.70×10^{5b}
Sv-10	Cottony, non-appressed	Whitish	Raised	Filliform	70.13^{a}	5.01^{a}	1.53×10^{5bc}
Sv-11	Velvety, non-appressed	Whitish light grey centre	Flat	Undulate	64.03^{cd}	4.57^{cd}	1.66×10^{5b}
Sv-12	Cottony, non-appressed	Light grey with whitish centre	Flat	Filliform	58.66^{efg}	4.19^{efg}	1.30×10^{5bc}
Sv-13	Velvety, appressed centre with concentric rings	Olivaceous green with light grey centre	Raised	Filliform	61.68^{de}	4.40^{de}	1.60×10^{5bc}
Sv-14	Cottony, non-appressed	Whitish light grey centre	Flat	Filliform	59.62^{ef}	4.26^{ef}	2.43×10^{5a}
Sv-15	Velvety, appressed centre with concentric rings	Whitish light grey centre	Raised	Undulate	55.68g	3.97^{g}	1.06×10^{5c}
Sv-16	Cottony, non-appressed centre	Whitish light grey centre	Flat	Entire	71.00^{a}	5.07^{a}	1.33×10^{5bc}
Sv-17	Cottony, non-appressed centre	Concentric zones of dark and light grey	Flat	Filliform	71.37^{a}	5.09^{a}	1.53×10^{5bc}
Sv-18	Cottony, non-appressed centre	Light grey with whitish centre	Flat	Entire	61.71^{de}	4.40^{de}	1.36×10^{5bc}
Sv-19	Cottony, non-appressed centre	Concentric whitish zones	Raised	Filliform	60.64^{de}	4.33^{de}	2.26×10^{5a}
Sv-20	Cottony, non-appressed centre	Whitish	Raised	Entire	68.47^{ab}	4.89^{ab}	1.43×10^{5bc}
Sv-21	Cottony, non-appressed centre	Whitish	Raised	Entire	63.56^{cd}	4.54^{cd}	1.36×10^{5bc}
Sv-22	Cottony, non-appressed centre	Dark grey with whitish centre	Flat	Entire	55.40^{g}	3.95^{g}	1.73×105^{b}
C.V.$_{0.05}$					2.05	2.04	18.61

The values with the same letter do not differ significantly as per the Tukey test ($P < 005$).

samples collected from various onion-growing regions of India based on cultural, morphological, and molecular methods.

2. Material and methods

2.1. Collection, isolation, and maintenance

Twenty symptomatic leaf samples of onion and two from garlic were collected from India's geographically distinct central onion-growing regions from 2021 to 2023 (Table 1). Twenty-two fungal isolates were isolated and further used in this study. The symptomatic leaves were washed with running tap water and gently rubbed. Then, the samples from infected area were cut into small pieces of size (0.5–1.0 cm) [24]. The cut pieces were then placed on a glass slide to prevent direct contact between the samples and the sterilized moist blotter paper inside the Petri plates. The plates were then covered with a lid and incubated at 25 °C for 48–72 h (Fig-S1). A portion of the infected plant samples was examined under a light microscope to check for the sporulation of *Stemphylium vesicarium*. A sterilized dissecting needle was dipped into an agar medium to coat the tip with a thin layer of agar. The needle tip was then touched to the sample surface on the stage of a stereo microscope to collect a few *Stemphylium vesicarium* conidia. These conidia were transferred onto a 2 % PDA medium in a Petri dish and spread with an inoculation loop. The plates were incubated at 25 °C for 48 h. Under a microscope, a single germinated conidium was marked by making a circle around it with a needle. The agar block containing the germinated conidium was then cut out and transferred to another PDA plate for mycelial growth. After 7–10 days, the

Fig. 1. Cultural variability of 22 *Stemphylium* isolates causing Stemphylium leaf blight of onion and garlic.

obtained single-spore pure cultures of fungal isolates were identified using morphological and molecular methods and maintained at a temperature of 4 °C in the refrigerator.

2.2. Cultural and morphological characterization of isolates

For macroscopic descriptions, isolates were cultured on potato dextrose agar medium in Petri plates. Mycelial discs (5 mm) from 7-day-old cultures were transferred to the centre of new potato dextrose agar plates and incubated at 25±1°C for 7 days, with three replications maintained for each isolate in a completely randomized design. After 7 days of growth, cultural characteristics such as colony type, colony colour, type of margins, colony elevation, and colony diameter were recorded. The diameter of the fungal colony formed was measured daily using a digital calliper, and the radial growth rate was calculated using the formula described by Ref. [25], where the radial growth rate (mm/day) is equal to RGR max (D max/2) divided by the number of days. To study the sporulation, isolates were cultured on potato dextrose agar plates for 14 days and placed under cool fluorescent light with a light/dark cycle of16/8 h. A loopful of cultures were dispersed in 5 ml of distilled water using an inoculation loop, and the resulting spore suspension was used to count the number of spores using a hemocytometer. A compound microscope was used with a Progres 2.7 version mounted camera (Jenoptik make, USA) to analyze each isolate's shape, size, and septation of the conidiophore and conidia. Measurements were taken with the help of the software MagVision and 30 recordings per replication were taken to ensure accuracy. Isolates were classified as

Table 3
Variability in the conidial size, colour and shape of *Stemphylium vesicarium* isolates.

Isolates	Conidiophore size and septation			Conidial size		Conidial septation		Colour	Shape
	Conidiophore Length	Conidiophore Breadth	Septation (No.)	Conidial Length	Conidial Breadth	Longitudinal septation	Transverse septation		
	Mean (μm)	Mean (μm)	Mean	Mean (μm)	Mean (μm)	Mean	Mean		
Sv-01	55.93^{fgh}	4.99^{bcd}	3.24^{c}	33.71^{gh}	12.84^{g}	4.25^{a}	3.12^{a}	Brown	Oblong
Sv-02	66.44^{a}	5.00^{bcd}	2.42^{e}	27.33^{ij}	18.43^{cd}	2.15^{de}	2.00^{bc}	Light Brown	Ovoid
Sv-03	56.42^{efgh}	4.31^{def}	2.28^{e}	36.21^{def}	21.16^{b}	1.52^{hi}	2.00^{bc}	Light Brown	Oblong
Sv-04	57.71^{defg}	6.96^{a}	3.30^{c}	43.18^{a}	21.55^{b}	1.90^{efgh}	2.39^{b}	Light Brown	Oblong
Sv-05	48.31^{i}	3.05^{h}	3.33^{c}	39.30^{bc}	21.42^{b}	1.35^{i}	2.62^{ab}	Brown	Oblong
Sv-06	61.24^{bcd}	4.55^{cd}	2.14^{e}	23.66^{k}	17.30^{def}	1.95^{efg}	2.05^{bc}	Brown	Oblong
Sv-07	60.04^{cdef}	4.21^{defg}	2.32^{e}	34.08^{fgh}	13.07^{g}	3.74^{b}	2.57^{ab}	Brown	Ovoid to Oblong
Sv-08	56.41^{efgh}	3.33^{fgh}	3.92^{b}	35.43^{efg}	17.96^{de}	1.55^{hi}	2.30^{bc}	Light Brown	Ovoid to Oblong
Sv-09	60.70^{cde}	4.16^{defg}	2.93^{cd}	34.30^{efgh}	21.78^{ab}	2.53^{c}	2.365^{bc}	Light Brown	Oblong
Sv-10	47.72^{i}	3.32^{fgh}	2.19^{e}	33.70^{gh}	18.50^{cd}	2.00^{efg}	2.31^{bc}	Brown	Ovoid to Oblong
Sv-11	57.97^{defg}	5.51^{b}	2.98^{cd}	40.78^{b}	21.88^{ab}	2.15^{de}	2.18^{bc}	Light Brown	Ovoid to Oblong
Sv-12	60.72^{cde}	4.11^{defgh}	2.26^{e}	29.16^{i}	16.69^{ef}	1.70^{ghi}	2.04^{bc}	Brown	Oblong
Sv-13	53.78^{gh}	6.46^{a}	2.67^{de}	40.74^{b}	21.19^{b}	2.10^{ef}	2.12^{bc}	Light Brown	Ovoid to Oblong
Sv-14	63.33^{abc}	3.12^{h}	4.60^{a}	41.38^{ab}	22.51^{ab}	2.63^{c}	2.31^{bc}	Light Brown	Oblong
Sv-15	65.14^{ab}	4.44^{cde}	2.30^{e}	26.46^{j}	15.86^{f}	1.90^{efgh}	1.65^{c}	Light Brown	Oblong
Sv-16	53.33^{h}	4.31^{def}	3.14^{cd}	32.57^{h}	19.59^{c}	1.75^{fghi}	2.12^{bc}	Brown	Ovoid to Oblong
Sv-17	39.72^{kl}	3.45^{efgh}	3.06^{cd}	32.25^{h}	21.22^{b}	1.88^{efgh}	2.30^{bc}	Light Brown	Ovoid to Oblong
Sv-18	45.82^{ij}	4.23^{defg}	2.33^{e}	23.31^{k}	16.71^{ef}	2.06^{efg}	2.43^{b}	Light Brown	Ovoid
Sv-19	42.99^{jk}	3.27^{gh}	4.63^{a}	25.91^{jk}	16.96^{def}	2.13^{ef}	2.26^{bc}	Light Brown	Ovoid
Sv-20	48.40^{i}	5.31^{bc}	3.24^{c}	36.47^{de}	17.98^{de}	1.89^{efgh}	2.15^{bc}	Light Brown	Oblong
Sv-21	36.8^{1l}	4.09^{defgh}	2.31^{e}	37.98^{cd}	21.52^{b}	1.55^{hi}	2.02^{bc}	Brown	Oblong
Sv-22	52.44^{h}	5.52^{b}	3.14cd	40.96^{ab}	23.13^{a}	2.50^{cd}	2.20^{bc}	Brown	Oblong
C.V.$_{0.05}$	2.86	7.77	1.41	2.56	2.95	7.72	12.98		

The values with the same letter do not differ significantly as per the Tukey test ($P < 005$).

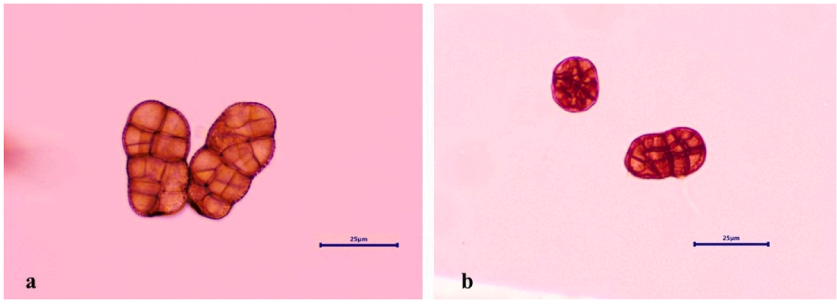

Fig. 2. Morphological characteristics of *Stemphylium* spp. conidia (a) *Stemphylium vesicarium* (b) *Stemphylium eturmiunum*. Scale bars = 25 μm.

Stemphylium spp. based on Simmons [12,26] descriptions. The experiment was laid out in a completely randomized design. The collected data were subjected to one-way analysis of variance (ANOVA). Subsequent comparisons of the means were performed using Tukey's test at a significance level of $p \leq 0.05$.

2.3. Genomic DNA isolation

Mycelial bit of 10 days old *Stemphylium* culture of all the isolates was transferred to Potato dextrose broth (PDB) in aseptic condition under the laminar airflow and incubated at 25 ± 1 °C. Mycelial mat of fungus was harvested after 15–18 days and later was stored at −20 °C for further use. Genomic DNA was extracted from all the isolates of *Stemphylium* using CTAB method [27]. The dried mycelium was ground to a fine powder in liquid nitrogen using a pre-chilled pestle and mortar. The fine powder was transferred to a 1.5 ml centrifuge tube, and 700 μl of extraction buffer (100 mM Tris HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA pH 8.0, 2 % CTAB, 1 % PVP and 0.5 % β-mercaptoethanol) was added to the tube. The mixture was incubated in a water bath at 60 °C for 1 h. To each tube, an equal volume (700 μl) of chilled chloroform: isoamyl alcohol (24:1) was added. The contents were mixed thoroughly, and tubes were spun at 10,000 rpm for 15 min in a cooling centrifuge at 4 °C. The aqueous phase was then transferred to a new tube, and an equal volume of pre-chilled isopropanol was added and kept at −20 °C overnight. The mixture was left overnight at 4 °C or for 1 h at −20°C. Centrifuged the mixture at 10,000 rpm for 10 min at room temperature and discarded the supernatant. Added 500 μl of 70 % ethanol and centrifuged the mixture at 10,000 rpm at 4 °C. Discarded the supernatant and dried the pellet until the ethanol evaporated completely. Finally, 70 μl of nuclease-free water was added to the pellet and stored at −20 °C. The concentration of DNA was measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

2.4. Molecular characterization using RAPD marker, ITS region and gpd1gene

Twenty-five RAPD markers were used to screen three randomly selected isolates for polymorphism. The primers showing consistent polymorphism among isolates were selected for further RAPD profiling of all the twenty-two *Stemphylium* isolates. Similarly, all the isolates were characterized by amplifying and sequencing the Internal transcribed spacer (ITS) region and the Glycerol-3-Phosphate Dehydrogenase 1 (*gpd*1) gene. The amplification of the ITS region was performed using ITS1 and ITS4 primers (5′-TCCGTAGGT-GAACCTGCGG-3′ and 5′-TCCTCCGCTTATTGATATGC-3′) [28]. The amplification of the *gpd1* gene was carried out using *gpd1* f and *gpd1* r primers (5′-CAACGGCTTCGGTCGCATTG-3′ and 5′-GCCAAGCAGTTGGTTGTGC-3) [29]. The annealing temperature and base sequence of the primers used for screening are presented in supplementary table (Table S1).

2.5. PCR amplification and visualization

Amplification using polymerase chain reaction (PCR) [30] was performed in 0.2 ml PCR tubes in an Eppendorf Pro S thermal cycler using 50–60 ng of genomic DNA for RAPD analysis of each isolate in a final volume of 25 μl per reaction. The stock and final concentration of different components used in PCR reaction/mixture for RAPD is given in Table S2.The reaction mixture in PCR tubes was given a short spin or vortexed in a microcentrifuge (Thermo Fisher Scientific) and placed in 96 well thermal cyclers programmed for initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, at annealing temperature (Table S1) for 1 min, 72 °C for 2 min and a final extension at 72 °C for 5 min 25 μl of the amplified product and 5 μl of the 6X loading dye were added to make the final concentration of the loading buffer in reaction samples 1X (Table S2). The PCR product was resolved on 1.2 per cent agarose gel. The gel was prepared in 0.5X TAE buffer. Ethidium bromide was added at a concentration of 0.5 μg μl^{-1}. The gel was run at 5V/cm under UV light and photographed using the Alfa Imager gel documentation system (Alfa Imager EC, Protein Simple, USA). All reactions were performed twice, and a one kb DNA ladder (GeneDirex, Inc.) was used as a size marker for DNA fragments.

2.6. Data generation using RAPD primers

The banding pattern obtained from agarose gel electrophoresis was digitalized to a binary matrix (0 and 1) for the absence and presence of RAPD bands. The data of all the primers were combined. The Numerical Taxonomy System of Multivariate Statistical

Table 4
Polymorphism survey in 22 *Stemphylium vesicarium* isolates based on RAPD genotyping.

Sl. no.	Marker	Polymorphic Bands	Monomorphic Bands	PIC	EMR	Discriminating power	Resolving power
1	OPA1	7	1	0.43	2.10	0.52	2.44
2	OPA3	5	1	0.37	2.23	0.80	3.55
3	OPA5	8	0	0.44	2.00	0.56	1.66
4	OPA10	6	0	0.34	2.50	0.42	1.65
5	OPC8	6	1	0.44	2.75	0.52	2.55
6	OPF10	5	0	0.37	2.86	0.67	1.36
7	OPF14	6	1	0.36	3.68	0.63	2.82
8	OPG8	6	1	0.37	2.45	0.83	1.82
9	OPG16	7	1	0.36	2.77	0.84	3.00
10	OPL5	13	0	0.37	7.14	0.70	8.82
11	OPN7	5	0	0.29	3.86	0.40	2.27
12	OPS7	5	0	0.37	2.77	0.69	3.18
13	OPS10	11	1	0.36	4.36	0.84	2.73
14	SV4	14	0	0.37	5.82	0.83	6.00
15	SV5	5	1	0.35	3.27	0.57	3.09
	Total	109	8	0.37	3.37	0.65	3.13

Programme (NTSYSpc) software package 2.10e [31] was used to obtain a similarity matrix based on Jaccard's similarity coefficient through the SIMQUAL route. A dendrogram was then constructed based on the unweighted pair group method of arithmetic average (UPGMA) within the SAHN programme of NTSYSpc software [32]. Two key measures of the genetic marker's quality or informativeness are heterozygosity (*H*) and polymorphic information content (PIC). In order to streamline the molecular studies, we utilized an online tool (https://irscope.shinyapps.io/iMEC/) for calculating heterozygosity (*H*), discriminating power (*D*), resolving power (*R*) and PIC values [33].

2.7. Sequencing alignment and phylogenetic analysis

For phylogenetic analysis, the nucleotide sequences were aligned using Muscle - Multiple Sequence Alignment, a multiple sequence alignment program [34]. The aligned sequences were deposited in the GenBank database. MEGA X version software was used to perform phylogenetic-based analysis [35]. The maximum parsimony (MP) methods of phylogenetic inference were used for construction of the phylogenetic trees. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm. The stability of the tree was evaluated using 1000 bootstrap replications. The edited ITS and *gpd1* sequences were compared with other available *Stemphylium* spp. sequences available in the GenBank.

3. Results and discussion

3.1. Cultural and morphological variability

The traditional taxonomic system of fungal classification relies on morphological characteristics to identify and classify species and races [36]. In all examined sections of symptomatic onion and garlic leaves, a consistently whitish-brown to grey coloured fungal colony was isolated. Significant variation was observed among the isolates regarding cultural characteristics such as colony type, colour, elevation, margin, diameter, and sporulation (Table 2). Two types of colonies, either cottony or velvety, were observed. Furthermore, variations within cottony-type colonies were also evident in the appressed and non-appressed centers. Isolates were observed with marked variation for their colony colour, which ranged from whitish, light to dark grey and olivaceous with greenish tinge colour (Fig. 1). Regarding colony elevation, two types were observed either colony were raised or flat. The margins of colonies varied from filiform and entire to undulated types. The results on colony diameter and mean radial growth rate on PDA media after an incubation period of 7 days showed significant variations among the pathogen isolates. The highest colony diameter on PDA media was recorded in isolate Sv-17 (71.37 mm), followed by isolate Sv-16 (71.00 mm), Sv-10 (70.13 mm) and Sv-20 (68.47 mm). In comparison, the lowest colony diameter was recorded for isolates *viz.*, Sv-01 (50.56 mm), preceded by Sv-07 (51.25 mm) and was statistically at par with each other. The fastest mean radial growth rate (mm/day) was observed in isolate Sv-17 (5.09 mm/day), followed by Sv-16 (5.07 mm/day) and Sv-10 (5.01 mm/day). However, isolates Sv-17, Sv-16 and Sv-10 were statistically at par with each other. The least mean radial growth rate (mm/day) was recorded in isolate Sv-01 (3.61 mm/day), preceded by Sv-07 (3.66 mm/day). Both Sv-01 and Sv-07 were statistically at par with each other. For sporulation, most of the isolates did not show any significant variation. In PDA media, 14 days after incubation, the highest sporulation was observed in isolate Sv-14 (2.43×10^5conidia/plate), whereas isolates Sv-06 and Sv-15 recorded least spore count (1.06×10^5 conidia/plate). Microscopic image analysis was done for the morphological characterization and precise measurements of the conidia and conidiophores. Morphological variability was observed among *Stemphylium* isolates for size, septation, shape, and colour of conidiophores and conidia (Table 3). The average maximum and minimum length of conidiophores was observed in isolate Sv-02 (66.44 μm) and Sv-21 (36.81 μm), respectively. The maximum breadth of conidiophore was recorded in isolate Sv-04 (6.96 μm), whereas the minimum was recorded in isolate Sv-05 (3.05 μm). Seven of the twenty-two isolates were brown, while fifteen were observed with light brown-coloured conidiophores. Morphological data related to conidial

Table 5
Jaccard's similarity coefficient of 22 *Stemphylium vesicarium* isolates based on RAPD data analysis.

	Sv-01	Sv-02	Sv-03	Sv-04	Sv-05	Sv-06	Sv-07	Sv-08	Sv-09	Sv-10	Sv-11	Sv-12	Sv-13	Sv-14	Sv-15	Sv-16	Sv-17	Sv-18	Sv-19	Sv-20	Sv-21
Sv-02	0.70																				
Sv-03	0.77	0.71																			
Sv-04	0.62	0.73	0.67																		
Sv-05	0.60	0.69	0.67	0.63																	
Sv-06	0.62	0.65	0.62	0.64	0.69																
Sv-07	0.48	0.52	0.61	0.59	0.62	0.46															
Sv-08	0.69	0.74	0.71	0.76	0.68	0.68	0.58														
Sv-09	0.71	0.70	0.77	0.64	0.63	0.59	0.54	0.74													
Sv-10	0.53	0.68	0.68	0.69	0.73	0.70	0.59	0.74	0.69												
Sv-11	0.59	0.54	0.63	0.61	0.65	0.64	0.58	0.66	0.60	0.62											
Sv-12	0.60	0.68	0.64	0.64	0.65	0.70	0.54	0.77	0.68	0.78	0.67										
Sv-13	0.53	0.62	0.56	0.61	0.64	0.57	0.67	0.59	0.59	0.61	0.60	0.57									
Sv-14	0.51	0.50	0.62	0.58	0.55	0.50	0.61	0.57	0.61	0.47	0.61	0.56	0.57								
Sv-15	0.61	0.62	0.59	0.65	0.75	0.66	0.50	0.73	0.62	0.70	0.71	0.65	0.55	0.53							
Sv-16	0.72	0.71	0.77	0.73	0.62	0.71	0.59	0.78	0.78	0.71	0.66	0.74	0.58	0.56	0.61						
Sv-17	0.72	0.75	0.76	0.66	0.68	0.66	0.58	0.69	0.82	0.67	0.53	0.66	0.67	0.58	0.61	0.77					
Sv-18	0.64	0.61	0.71	0.68	0.60	0.59	0.60	0.70	0.66	0.61	0.67	0.65	0.63	0.63	0.62	0.69	0.66				
Sv-19	0.42	0.50	0.47	0.52	0.56	0.52	0.54	0.54	0.57	0.56	0.60	0.61	0.65	0.54	0.58	0.49	0.53	0.65			
Sv-20	0.68	0.73	0.74	0.70	0.65	0.64	0.55	0.75	0.78	0.67	0.63	0.75	0.56	0.58	0.66	0.83	0.76	0.73	0.58		
Sv-21	0.67	0.69	0.69	0.76	0.71	0.65	0.58	0.72	0.70	0.69	0.66	0.69	0.61	0.55	0.73	0.75	0.69	0.70	0.59	0.82	
Sv-22	0.65	0.65	0.75	0.70	0.64	0.61	0.63	0.68	0.80	0.67	0.65	0.67	0.66	0.56	0.60	0.81	0.74	0.67	0.60	0.77	0.77

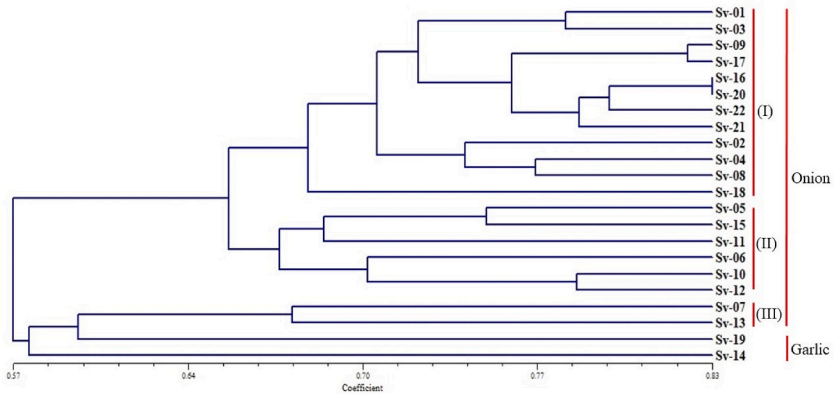

Fig. 3. Phenogram of RAPD profile of 22 isolates of *Stemphylium* constructed using NTSYSpc. Three groups (I ± III) were resolved by UPGMA analysis.

variability exhibited a significant variation in length, breadth, mean conidial septations and colour among the isolates. The morphological characteristics of mature conidia for *S. vesicarium* (Fig. 2a) and *S. eturmiunum* (Fig. 2b) are presented, respectively. The average maximum (43.18 μm) and minimum (23.31 μm) length were recorded in isolate Sv-04 and Sv-18, respectively. Similarly, the maximum mean conidial breadth was observed in isolate Sv-22 (23.13 μm), whereas the minimum mean value for conidial breadth was recorded in isolate Sv-01 (12.84 μm). The highest mean value for longitudinal septation was recorded as Sv-01 (4.25), followed by Sv-07 (3.74) and Sv-14 (2.63), while the lowest was observed in isolate Sv-05 (1.35). The maximum mean value for transverse septation was recorded in isolate Sv-12 (3.12), while the minimum value was observed in isolate Sv-15 (1.65). Brown-coloured conidium was observed in nine isolates, whereas light brown-coloured conidium was found in thirteen isolates. The shape of the conidium varied from ovoid to oblong. Oblong-shaped conidium was observed in twelve isolates, whereas three isolates were recorded with ovoid. Seven isolates out of twenty-two were found to be having ovoid to oblong shaped conidium. These findings align with a study conducted by Refs. [11,14] where the colour of conidiophores in various *Stemphylium* spp. ranged from hyaline to light brown. Other researchers [17,37] have also reported variations in conidiophore colour among *S. vesicarium* isolates, ranging from light brown to brown. Similar findings were reported by Ref. [18] in a study involving 24 isolates of *S. vesicarium*, where the length of conidia varied from 14.6 μm to 30.6 μm, and the breadth ranged from 4.7 μm to 15.7 μm. The isolates in the present study exhibited conidia that were ovoid, oblong, or ovoid to oblong in shape. The conidia displayed various shades of brown, including deep brown, light brown, or medium brown [14]. also observed variations in conidial length and breadth among 36 isolates of *S. vesicarium*, where the conidia were ovoid to oblong in shape and ranged from 15.80 to 39.75 × 22.04–7.26 μm [17]. reported that the conidia of *S. vesicarium* were 20–24 × 12–15 μm in length and breadth, respectively, and exhibited an oblong to broadly ovoid shape, often with 1–3 transverse and 1–4 longitudinal or oblique septa, sometimes constricted at one or more of the septa [38]. found 3–5 transverse septa and 2–4 longitudinal or oblique septa in *S. luffae* whereas [39] documented brown-coloured conidia in *S. botryosum* and *S. solani*.

3.2. Genetic variability based on RAPD markers

Molecular markers are valuable tools for studying the evolutionary adaptations of pathogenic fungi, including their ability to assimilate resistant genotypes, adapt to diverse habitats, and respond to fungicides. Also, molecular marker techniques have contributed to the analysis of variability in organisms where stable morphological markers are lacking, allowing for a deeper understanding of their genetic diversity and population dynamics [20].In this study, 15 RAPD primers were selected for fingerprinting of 22 *Stemphylium* isolates. DNA fingerprinting of *Stemphylium* isolates collected from various geographical regions showed significant polymorphism within the isolates. They produced distinct banding patterns ranging in size from approximately 250 to 2500 bp (Fig.S2 a-c). The RAPD primers produced 192 consistently amplified banding patterns from 22 isolates (Table 4). The primer SV4 produced 14 polymorphic banding patterns, followed by OPL5 and SV5, which amplified 13 and 11 polymorphic banding patterns, respectively.PIC can be defined as the probability that the marker genotype of a given offspring will allow the deduction, in the absence of crossing over, of which of the two marker alleles of the affected parents it received [33]. RAPD primers, OPA5 and OPC8 were recorded with the highest PIC value of 0.44, followed by OPA1, having a PIC value of 0.43, with a mean value of 0.37. Effective multiplex ratio is the product of the fraction of polymorphic loci for an individual assay. In other words, the number of loci polymorphic in the germplasm set of interest is analyzed per experiment fraction of polymorphic loci. The effective multiplex ratio was observed to be highest in OPL5 (7.14), whereas the least was recorded for primer OPA5. Discriminating power is the probability that two randomly chosen individuals exhibit different banding patterns and are thus distinguishable from one another. The primer OPG16 and OPS10 were recorded the highest discriminating power (D) of 0.84, indicating their usefulness in discriminating isolates, while the least was observed for primer OPN7 (0.40). Resolving power is based on the distribution of alleles within the sampled genotypes and strongly correlates with the ability to distinguish between analyzed samples. The division of samples into two groups is based on the presence or absence of a band, ideally present in one part of the samples while absent from the other. Resolving power (R) was observed highest for primer OPL5

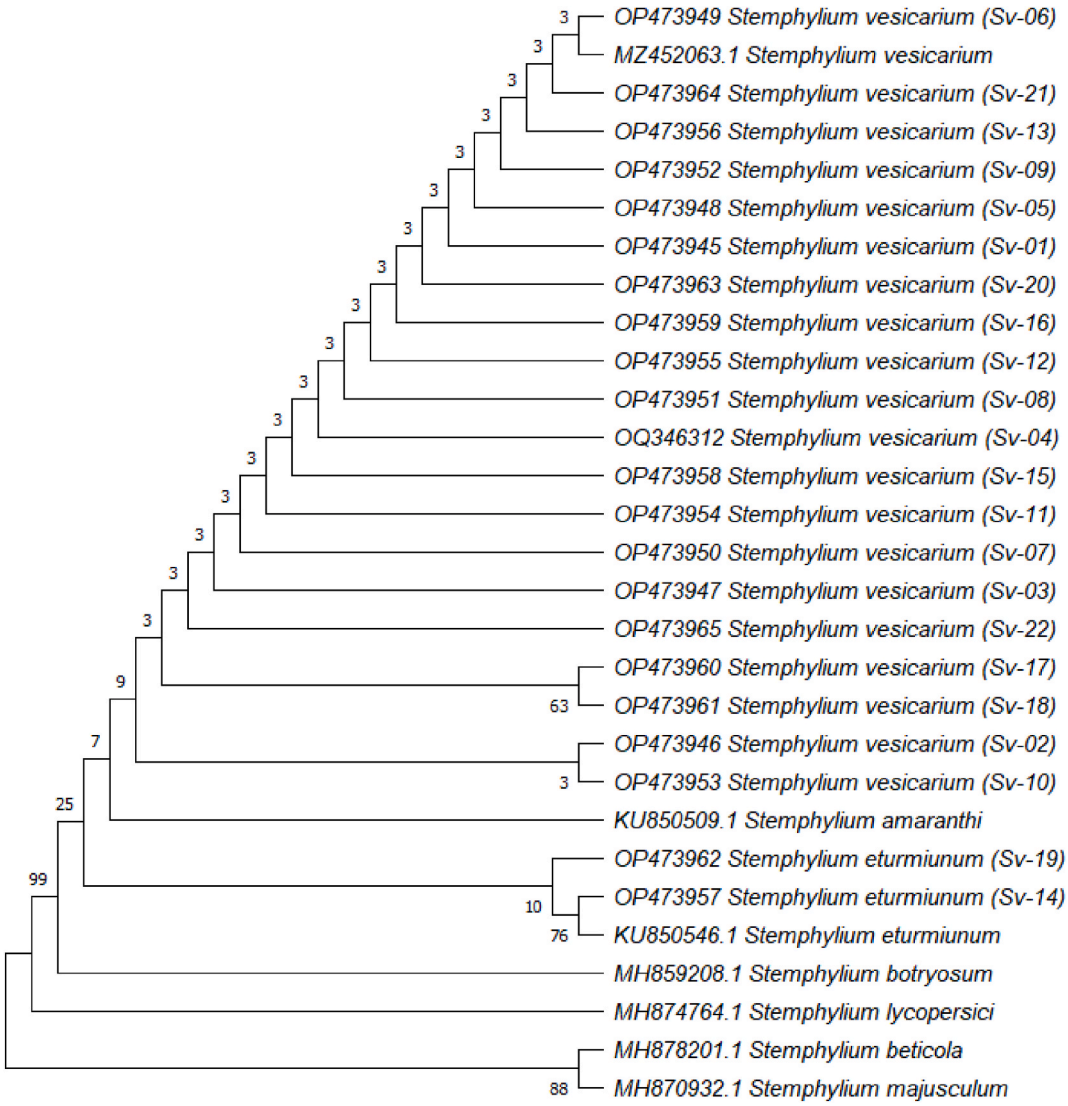

Fig. 4a. Phylogenetic relationship among Stemphylium isolates based on ITS region. The evolutionary history was inferred using the Maximum Parsimony method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

(8.82), whereas the least was recorded for primer OPF10 (1.36) with an average value of 3.13.

Jaccard's pair-wise similarity coefficient values were estimated among *Stemphylium* isolates based on RAPD data. The genetic similarity of the isolates varied from 0.42 to 0.83 (Table 5). The minimum average genetic similarity of 0.43 was found between Sv-01 and Sv-19, while a maximum similarity of 0.83 was observed between Sv-16 and Sv-20, indicating that these two isolates have the maximum resemblance among themselves compared to all other isolates. Cluster analysis was conducted on the taxonomic distance matrix with the Unweighted Pair Group Method based Arithmetic Average (UPGMA) and dendrogram generated (Fig. 3). Isolates were grouped into 3 clusters in the dendrogram generated with the RAPD markers at the genetic similarity of 0.57. In Cluster I, 12 isolates (Sv-01, Sv-02, Sv-03, Sv-04, Sv-08, Sv-09, Sv-16, Sv-17, Sv-18, Sv-20, Sv-21 and Sv-22) were grouped. Cluster II consist of 6 isolates (Sv-5, Sv-6, Sv-10, Sv-11, Sv-12, and Sv-15) whereas cluster III consist of 2 isolates Sv-07 and Sv-13. The isolates in cluster I showed approx. 65 % similarity with cluster II. All isolates grouped under Cluster I, II and III were obtained from symptomatic onion leaf samples. Two isolates, Sv-14 and Sv-19, obtained from garlic showed approximately 57 % genetic similarity, with most isolates obtained from onion leaves. The RAPD primers used in this study demonstrated a high level of polymorphism among different isolates of *Stemphylium*, indicating that RAPD markers are suitable for studying the genetic diversity of this pathogen. This suggests that the pathogen has evolved the capacity to adjust to the various environmental conditions it encounters during the cultivation of allium crops, both on a broader scale and in specific microenvironments. The utilization of RAPD primers has unveiled genetic differences among the *Stemphylium* isolates, as indicated by the extent of polymorphism observed. Similar findings have been reported by other

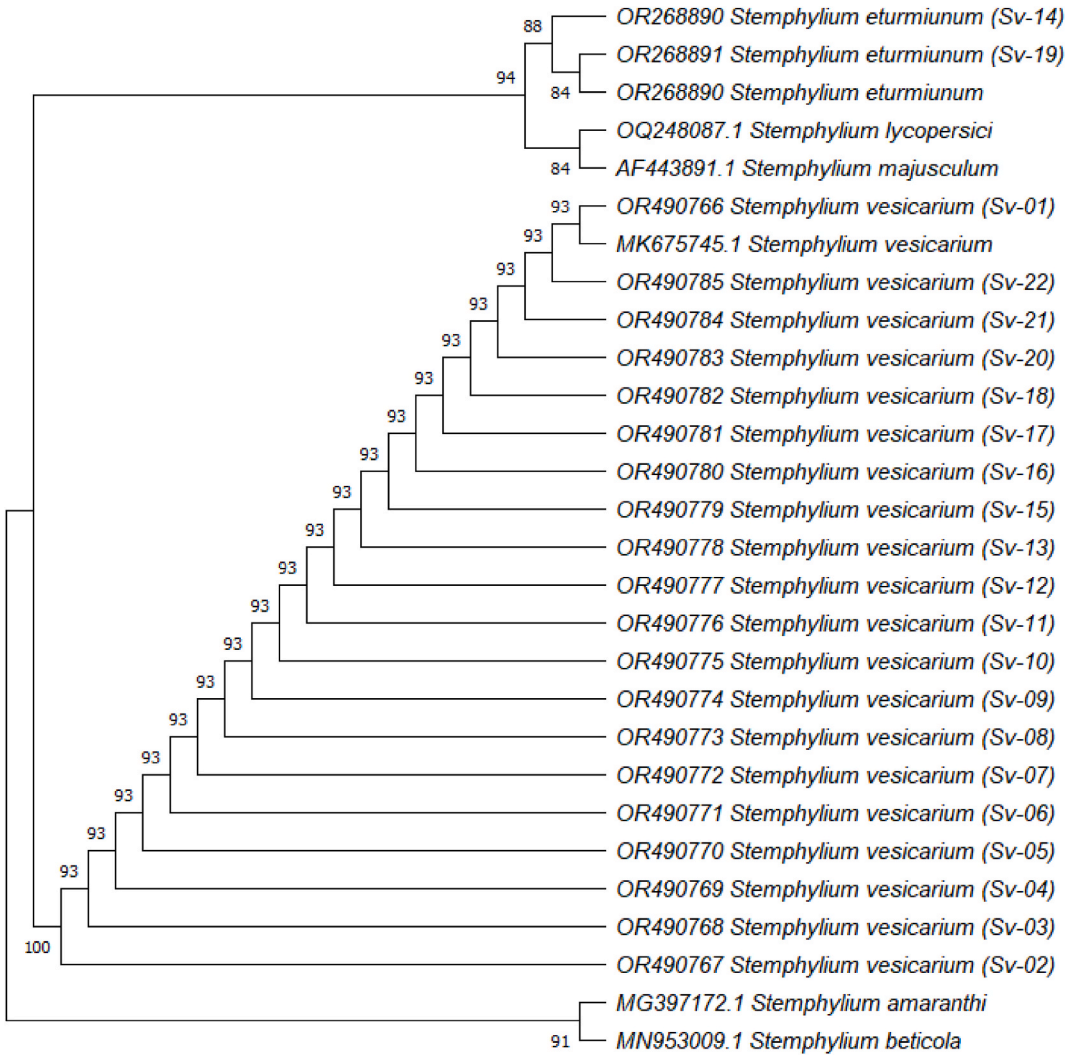

Fig. 4b. Phylogenetic relationship among Stemphylium isolates based on *gpd* region. The evolutionary history was inferred using the Maximum Parsimony method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

researchers who have also found RAPD markers to be effective in studying genetic diversity in *S. vesicarium* and other phytopathogenic fungi [40,41]. A relationship between the 22 *Stemphylium* isolates and their respective collection sites was not established based on RAPD analysis. These findings align with previous studies that have reported similar observations [41,42].

3.3. Molecular characterization of Stemphylium isolates based on ITS region and gpd1 gene

Accurate identification is crucial for formulating effective, sustainable field management strategies. Traditional diagnostic methods are based on a connection between host identification and the morphology of associated pathogens. However, in order to overcome challenging identification issues in fungal pathogens, genomic rDNA regions have been examined [43]. The genetic studies, involving the ITS and *gpd1* regions along with an analysis of morphological features, successfully facilitated the differentiation of species within a complex polymorphism. The morphological identification was confirmed through molecular characterization using Internal transcribed spacer (ITS) and Glyceraldehyde-3-phosphate dehydrogenase 1 (*gpd1*) gene sequences. PCR-based amplification of all the isolates with both forward and reverse primers of ITS (ITS1 and ITS4) and *gpd1* (*gpd1* f and *gpd1* r) gene were carried out. Amplified products were separated and sequenced. The sequence length was 560 bp in the case of ITS, and 590 bp for *gpd1*. The ITS region of fungal DNA plays a crucial role in molecular systematics, allowing differentiation not only between species but also within a species. The *gpd1* fragment proved to be more useful in establishing well-supported relationships among the species and isolates of *Stemphylium* due to its greater resolving power [44]. These findings confirmed the identity of all the isolates under investigation. Consequently, the molecular ITS and *gpd1* region did not reveal significant variation among the *Stemphylium* isolates. Rarely, variations can be observed

among individual rDNA repeats, particularly within the ITS and *gpd1* regions [36]. The amplified products (Fig. S3 a-b) were sent to sequencing, and after receiving the sequencing result, sequences were analyzed for their quality through Bio-edit software. The sequences from the amplified product were blasted with the NCBI GenBank database (webpage: http://blast.ncbi.nlm.nih.gov) and compared with the reference sequences available in the NCBI GenBank database. Similarity between 99 and 100 per cent was obtained (Table S3).

3.4. Phylogenetic analysis

The sequences of all 22 isolates were compared with five *Stemphylium* spp. (*S. vesicarium*, *S. eturmiunum*, *S. majusculum*, *S. lycopersici* and *S. beticola*) whose sequences were retrieved from the NCBI database. The phylogenetic tree was constructed based on ITS and *gpd1*regions sequences of 22 isolates (Fig. 4a and b). Parsimony analysis of the ITS found the most parsimonious tree with length = 212, consistency index of 1.0000, the retention index of 1.0000. Similarly, for *gpd1* gene the most parsimonious tree with length = 200, consistency index of 0.9672, and retention index of 0.9936 was found. Species of *Stemphylium* formed a well-supported clade in parsimony analyses of the ITS region and *gpd1* gene with bootstrap values of 100 %. Twenty isolates, obtained from onion leaves, were grouped along with *Stemphylium vesicarium* retrieved sequence for both ITS and *gpd1* regions. Isolates, Sv-14 and Sv-19, which were obtained from garlic leaves grouped with *Stemphylium eturmiunum*. The phylogenetic analysis of ITS and *gpd1* sequences conducted in this study reaffirmed previous findings regarding the taxonomy of Stemphylium [11].Geographical variation in the causal agent of leaf blight of garlic disease is evident, with *Stemphylium vesicarium* reported in Spain and Turkey, while *S. solani* is the predominant species in China. Association of *Stemphylium vesicarium* with leaf blight of onion is well established in India whereas in garlic it was considered to be the same pathogen earlier. The other three *Stemphylium* spp.of which DNA sequences were retrieved from the GenBank, were grouped separately, and none of our isolates were grouped along with them. In the phylogenetic study, despite having different geographical origins, the sequences of *S. vesicarium* were grouped [45]. This suggests that there is genetic similarity or shared evolutionary history among these sequences of isolates that overrides the influence of geographic location.

4. Conclusion

Significant variations for cultural characteristics viz., colony type, colour, elevation, margin, diameter, and sporulation were observed. Similarly, morphological variability for size, septation, shape, and colour of conidiophores and conidia was documented. Molecular variability using 15 RAPD primers exhibited significant polymorphism among the isolates. But the clustering was independent of geographical locations. DNA barcoding using ITS and *gpd1* were able to identify the Stemphylium isolates and *gpd1* proved to be more useful in establishing well-supported relationships. Further, it was recorded that *S. eturmiunum* is the causal agent of SLB in garlic which has not yet been reported under Indian field conditions. Based on our studies, we can conclude that there is significant variation among the Stemphylium spp. isolated. The minor limitation in this study is that emphasis has been on a single disease with a single host. The broader goal should be to understand the overall diversity of Stemphylium spp. in India, studying isolates from additional hosts or environments. Also, work on determining the virulence of these isolates needs to be studied. Our studies are a stepping stone for in depth work towards development of SLB resistant onions.

CRediT authorship contribution statement

Rahul Chandel: Writing – original draft, Investigation, Formal analysis. **Deeba Kamil:** Writing – review & editing, Conceptualization. **Amrender Kumar:** Software, Formal analysis. **Yashpal Taak:** Writing – review & editing, Visualization. **Anil Khar:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e39107.

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