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Surveillance and morpho-molecular characterization of *Sclerotium rolfsii* causing root rot of bell pepper in Pothohar Plateau, Pakistan

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Abstract

Sclerotium rolfsii is widely distributed and economically important soil-borne pathogen causing root rot and southern blight in bell pepper. During 2015-16 and 2016-17 cropping season, farmer's fields/greenhouses/low plastic tunnels of bell pepper were surveyed to assess the percent disease incidence in Pothohar Plateau (Rawalpindi, Chakwal, Jhelum, Attock and Islamabad territory) Punjab, Pakistan. The mean disease incidence at seeding stage in greenhouses varied from 0% to 8% with the average 4.1%. In low plastic tunnels at seeding stage the mean incidence was 0% to 15.5% with the average 7.4%. At maturity stage in greenhouses, the mean incidence varied from 0% to 12% with the average 4.5%. However, the mean incidence at maturity stage in open fields varied from 7% to 26.9% with the average 14.4%.Morphological characterization of 50 recovered isolates of *Sclerotium rolfsii*s howed silky-white or white to light cream colonies with submerged, thin flat, medium fluffy and fluffy texture. The sclerotia were round, shiny, cream to brown or dark brown and developed at aerial hyphae or surface. The formation of sclerotia was scattered throughout the plate or formed at peripheral ring of Petri dish. The hyphae were hyaline, thin walled and have infrequent cross walls with clamp connections. Mycelial branching was at right angle and developed hyphae were slightly constricted at the branch origin. The DNA of 5 highly virulent isolates were extracted and amplified using Large Subunit gene (LSU). The sequences share 99-100% similarity with *S. rolfsii* reference isolates and submitted to GenBank (accession numbers MG195620 to MG195624 respectively). *S. rolfsii*was ascertained as the causal agent, based on morphological & molecular evidence, which suggests further investigation for the management of this potential threat to bell pepper production in Pakistan.

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Introduction

Capsicum (chili and bell pepper) contributes a major share among vegetables in Pakistan. *Sclerotium rolfsii* is a soil borne fungal pathogen of bell pepper that associated to cause white mold, southern blight and stem rot. On global perspective, estimated yield loss of 1-60% and 10-20 million dollars have been linked with *S. rolfsii*(Kator *et al.,* 2015).

The disease favors in moist conditions and temperature above 29°C. Affected plants may emerged singly or grouped in circular patches with early symptoms appeared as water-soaked spots on crown part and lower stem at or near the soil line. Diseased foliage turns pale green, with chlorosis and wilting and sudden death. A dense whitish fungal hypha appeared on the crown and lower stem.

The dark brown sclerotia were later produced and serve as inoculums for the next crop (Remesal *et al*., 2010; Xie and Vallad, 2016).

Sclerotium root rot of bell pepper are presently, causing significant losses in Pakistan. Although some work has been conducted on the incidence and management of disease in Pakistan (Jabeen*et al.,* 2014; Javaid and Iqbal, 2014), but these studies carry limited details regarding incidence of disease. Therefore, the aim of this study was to document theincidence of root rot caused by *S. rolfsii*, characterization of isolates employing morpho-molecular tools andidentification of pathogenic behavior of isolates.

Materials and methods

Survey and sample collection

In the present study, farmer's fields/greenhouses/low plastic tunnels of bell pepper were surveyed during 2015-16 and 2016-17 after intensive field's inspection in Pothohar Plateau, which includes Rawalpindi, Chakwal, Jhelum and Attockand Islamabad territory. The sampling was done in ×+ manner for the assessment of incidence and collection of diseased samples. Percent disease incidence was documented following below mentioned formula;

$$\text{Disease Inicidence\%} = \frac{\text{No. of infected plants}}{\text{Total no. of plants observed}} \times 100$$

Isolation and purification

The symptomatic portions of roots were cut into 5 to 10 mm² pieces and tissues were surface sterilized by dipping in sodium hypochlorite (1% NaCLO) for 1-2 mins, dipped thrice in sterile distilled water (SDW) and blotted dry using filter paper. After that the sterilized tissues were plated on Petri dish (90 mm) containing potato dextrose agar (PDA). The Petri plates were incubated in a growth chamber at 25 ± 2°C for 5 days and cultures were purified using hyphal tip method.

Morphological characterization

Colony characters *viz.* colony diameter, color, reverse color, texture, topography, margin of colony, hyphal characteristics and resting structures (sclerotia) were visually studied. The microscopic characters *viz.* hyphal dimensions were noted. The mean and standard deviation data was analyzed statistically using SPSS statistical software 16.020.

Pathogenicity test

The inoculum of *S. rolfsii*was colonized on wheat grains for 14 days. Three days prior to transplantation 10-12 seeds mixed in the upper 2 cm fumigated soil layer. Additional three bell pepper seedlings per isolate were included without inoculation (10-12 healthy wheat seeds), served as control. The control and treated seedlings were maintained in a growth chamber for 15 days at 22-30°C day temperature, 20-22°C night temperature and 60-70% moisture.

The disease severity index for *Sclerotium*root rot was evaluated according to the Le *et al.* (2012) disease rating scale. The virulence of the isolates was further evaluated according to Disease severity index (DSI).

$$\text{DSI (\%)} = \frac{\text{Sum of ll disease ratings}}{\text{Total no. of rating} \times \text{Maximum disease grade}} \times 100$$

Molecular characterization

Five highly virulent isolates confirmed by pathogenicity test were subjected to molecular

characterization. DNA of pure fungal cultures was extracted (Raeder and Broda, 1985). Large subunit gene (LSU) was amplified using LROR (5'-ACCCGCTGAACTTAAGC-3') and LR5 (5'-TCCTGAGGGAAACTTCG-3') primers (Vilgalys and Hester, 1990). The amplified product of PCR was purified using ExoSAP-IT (Affymetrix, California) and sequenced in both directions by Genscript Inc. (Piscataway, New Jersey) with the same PCR primers. Sequences of the partial forward and reverse regions were aligned with MUSCLE(Edgar, 2004). The representative isolates of present study as well as reference sequences from GenBank were selected for phylogenetic analysis based on high similarity index. The phylogenetic tree was generated using Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993).

Results and discussion

Symptomatology

The initial symptoms of *Sclerotium* root rot infection is poor plant growth at the top and wilting of the leaves. Water-soaked lesions appeared on root and lower stem part near the soil line. White cottony growth covered the infected roots surface (Fig.1).

The roots became rotted and plant eventually died. Numerous mustard seed like sclerotia were often produced on stem and root surface and the surrounding soil.

Table 1. Morphological and disease severity index (DSI) of *S. rolfsii* isolates.

Sr. No	Isolate	Colony			Margins	Texture	Hyphal Diameter (µm)	Sclerotium				DSI (%)
		Diameter	Color (Front)	Color (Reverse)				Color	Formation	Location	Shape	
1	DGADY01	8.7±0.1	Silky-white	White	Irregular	Fluffy	5.3±0.5	Dark brown	Peripheral	Surface	Round	73.33
2	DGADY02	8.9±0.1	White to light cream	White	Irregular	Fluffy	5.1±0.6	Dark brown	Scattered	Surface	Round	80.00
3	DGADY03	8.4±0.1	Silky-white	White	Irregular	Thin flat	5.2±0.2	Dark brown	Scattered	Surface	Round	80.00
4	DGADY04	7.2±0.1	Silky-white	White	Irregular	Submerged	5.2±0.6	Dark brown	Scattered	Surface	Round	73.33
5	DGADY05	8.3±0.1	Silky-white	White	Irregular	Fluffy	5.4±0.2	Dark brown	Peripheral	Aerial & surface	Round	100.00
6	DGADY06	8.7±0.1	Silky-white	White	Irregular	Thin flat	5±0.4	Cream to brown	Peripheral	Surface	Round	80.00
7	DGADY07	8.3±0.1	Silky-white	White	Irregular	Thin flat	5.1±0.6	Dark brown	Scattered	Surface	Round	80.00
8	DGADY08	8.6±0.1	Silky-white	White	Irregular	Fluffy	5.1±0.3	Dark brown	Peripheral	Surface	Round	86.67
9	DGADY09	8.9±0.1	Silky-white	White	Irregular	Fluffy	4.8±0.3	Dark brown	Peripheral	Aerial & surface	Round	100.00
10	DGADY10	9.0±0	White to light cream	White to light cream	Irregular	Fluffy	5.4±0.2	Dark brown	Peripheral	Surface	Round	80.00
11	DGADY11	9.0±0	Silky-white	White	Irregular	Medium fluffy	5.2±0.4	Cream to brown	Scattered	Aerial & surface	Round	100.00
12	DGADY12	9.0±0	Silky-white	White	Irregular	Medium fluffy	5±0.2	Dark brown	Scattered	Surface	Round	80.00
13	DGADY13	8.4±0.1	White to light cream	White	Irregular	Medium fluffy	5.2±0.8	Dark brown	Scattered	Aerial & surface	Round	73.33
14	DGADY14	9.0±0	White to light cream	White to light cream	Irregular	Thin flat	5.2±0.4	Dark brown	Peripheral	Surface	Round	100.00
15	DGADY15	9.0±0	White to light cream	White to light cream	Irregular	Medium fluffy	5.3±0.3	Dark brown	Peripheral	Surface	Round	100.00
16	DGADY16	8.7±0.1	Silky-white	White	Irregular	Fluffy	5.1±0.5	Dark brown	Peripheral	Aerial & surface	Round	80.00
17	DGADY17	8.3±0.1	White to light cream	White	Irregular	Fluffy	5±0.3	Dark brown	Scattered	Surface	Round	66.67
18	DGADY18	9.0±0	White to light cream	White	Irregular	Fluffy	5.2±0.8	Dark brown	Scattered	Surface	Round	80.00
19	DGADY19	8.2±0.1	White to light cream	White	Irregular	Medium fluffy	5.2±0.4	Cream to brown	Peripheral	Surface	Round	86.67
20	DGADY20	7.3±0.1	Silky-white	White	Irregular	Thin flat	5±0.3	Dark brown	Peripheral	Surface	Round	80.00
21	DGADY21	8.7±0.1	Silky-white	White	Irregular	Submerged	5.1±0.5	Dark brown	Peripheral	Surface	Round	73.33
22	DGADY22	9.0±0	Silky-white	White	Irregular	Fluffy	5.5±0.4	Cream to brown	Peripheral	Aerial & surface	Round	80.00

23	DGADY23	9.0±0	Silky-white	White	Irregular	Fluffy	5.3±0.7	Dark brown	Peripheral	Aerial & surface	Round	80.00
24	DGADY24	8.9±0.1	Silky-white	White	Irregular	Submerged	5.2±0.2	Dark brown	Scattered	Surface	Round	80.00
25	DGADY25	9.0±0	Silky-white	White	Irregular	Medium fluffy	4.9±0.4	Dark brown	Peripheral	Surface	Round	73.33
26	DGADY26	8.2±0.1	Silky-white	White	Irregular	Fluffy	5.4±0.2	Dark brown	Scattered	Surface	Round	80.00
27	DGADY27	8.6±0.1	Silky-white	White	Irregular	Fluffy	5.3±0.7	Dark brown	Peripheral	Aerial & surface	Round	80.00
28	DGADY28	9.0±0	White to light cream	White	Irregular	Submerged	5.5±0.5	Dark brown	Scattered	Surface	Round	66.67
29	DGADY29	7.9±0.1	White to light cream	White to light cream	Irregular	Fluffy	5.2±0.4	Dark brown	Peripheral	Surface	Round	80.00
30	DGADY30	9.0±0	Silky-white	White	Irregular	Fluffy	5.4±0.5	Dark brown	Peripheral	Surface	Round	46.67
31	DGADY31	7.9±0.1	Silky-white	White	Irregular	Fluffy	5±0.2	Dark brown	Peripheral	Aerial & surface	Round	80.00
32	DGADY32	9.0±0	Silky-white	White	Irregular	Thin flat	4.9±0.4	Cream to brown	Peripheral	Surface	Round	80.00
33	DGADY33	9.0±0	Silky-white	White	Irregular	Thin flat	5.3±0.7	Dark brown	Peripheral	Surface	Round	86.67
34	DGADY34	8.6±0.1	Silky-white	White	Irregular	Submerged	5.1±0.2	Dark brown	Scattered	Surface	Round	80.00
35	DGADY35	8.4±0.1	Silky-white to light cream	White	Irregular	Medium fluffy	5.4±0.3	Cream to brown	Scattered	Aerial & surface	Round	46.67
36	DGADY36	8.6±0.1	Silky-white	White	Irregular	Medium fluffy	5.3±0.8	Dark brown	Peripheral	Surface	Round	73.33
37	DGADY37	9.0±0	White to light cream	White	Irregular	Medium fluffy	5.5±0.4	Dark brown	Peripheral	Surface	Round	80.00
38	DGADY38	8.2±0.1	White to light cream	White	Irregular	Medium fluffy	5.4±0.7	Dark brown	Peripheral	Surface	Round	80.00
39	DGADY39	8.2±0.1	White to light cream	White	Irregular	Medium fluffy	5±0.5	Dark brown	Peripheral	Aerial & surface	Round	73.33
40	DGADY40	9.0±0	Silky-white	White	Irregular	Submerged	5.3±0.8	Dark brown	Peripheral	Surface	Round	73.33
41	DGADY41	7.4±0.1	White to light cream	White to light cream	Irregular	Thin flat	5.3±0.7	Dark brown	Peripheral	Surface	Round	80.00
42	DGADY42	9.0±0	Silky-white	White	Irregular	Fluffy	5.3±0.4	Cream to brown	Scattered	Aerial & surface	Round	86.67
43	DGADY43	8.5±0.1	White to light cream	White	Irregular	Fluffy	5.3±0.7	Cream to brown	Scattered	Surface	Round	46.67
44	DGADY44	9.0±0	Silky-white	White	Irregular	Submerged	5.7±0.4	Dark brown	Peripheral	Surface	Round	80.00
45	DGADY45	8.5±0.1	Silky-white	White	Irregular	Thin flat	5.3±0.3	Dark brown	Peripheral	Surface	Round	40.00
46	DGADY46	9.0±0	Silky-white	White	Irregular	Medium fluffy	5.3±0.7	Dark brown	Peripheral	Aerial & surface	Round	80.00
47	DGADY47	9.0±0	Silky-white	White	Irregular	Fluffy	5.3±0.3	Dark brown	Scattered	Aerial & surface	Round	86.67
48	DGADY48	8.6±0.1	White to light cream	White	Irregular	Fluffy	5.3±0.7	Dark brown	Peripheral	Aerial & surface	Round	80.00
49	DGADY49	9.0±0	Silky-white	White	Irregular	Medium fluffy	5.1±0.5	Dark brown	Peripheral	Surface	Round	80.00
50	DGADY50	9.0±0	Silky-white	White	Irregular	Thin flat	5.2±0.5	Dark brown	Peripheral	Surface	Round	73.33

Survey

At seedling stage in greenhouses, the maximum *Sclerotium* root rot incidencewas recorded in Gujar Khan (8%), followed by Chakwal (7.5%) and Rawalpindi (5%). Conversely, no disease was observed in Islamabad and Taxila. In low plastic tunnels, the highest *Sclerotium* root rot mean incidence was in Chakwal (15.5%), followed by KallarKahar (15%) and Rawalpindi (6.8%). The lowest (4.7%) incidence was in tehsil Gujar Khan. Conversely, *Sclerotium* root rot was not observed in Taxila and ChoaSaidan Shah. At maturity stage in greenhouses, the highest mean incidence was recorded at Gujar Khan (12%) followed by Chakwal (7.5%) and Rawalpindi (6.3%). Conversely, root rot caused by *Sclerotium* was not observed in Islamabad and Taxila. In open fields at maturity stage, the highest *Sclerotium* root rot mean incidence was recorded in Chakwal (26.9%), followed by Jhelum (22.5%), Gujar Khan (17.7%) and Attock (14.5%).

The lowest (7%) disease incidence was recorded in Islamabad. The highest disease incidence at maturity stage in open fields might be attributed to the

favorable temperature and humidity for disease development.

Fig. 1. White cottony growth of *Sclerotium* root rot covered the infected roots surface.

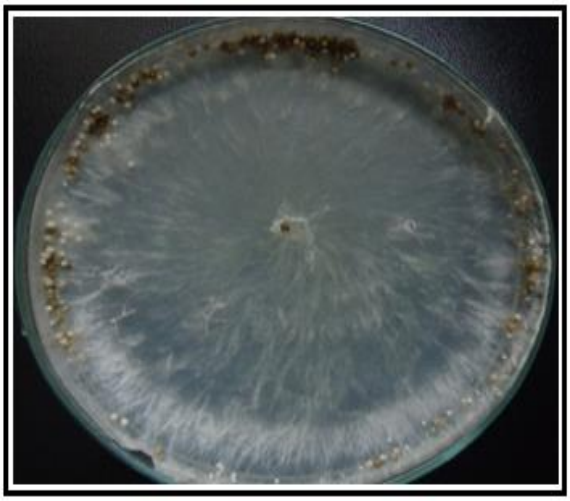

Fig. 2. White to light cream, thin-flat colony of *S. rolfsii.*

The temperature above 29˚C and high humidity are the conducive environmental conditions for the *sclerotium* root rot disease development (Kator*et al.,* 2015). Ideal temperature for mycelial growth ranges between 25 to 35°C, and the optimal temperature for sclerotia formation ranges from 27°C to 35°C (Punja, 1985). Mycelial growth is little or none at 10 or 40°C, but sclerotia can survive at temperature as low as -10°C. Sclerotia germinate well at 25-35% relative humidity (Edmunds *et al.*, 2003). The minimum mean incidence of *Sclerotium* root rot at seedling stage in greenhouses and low plastic tunnels may be due to the fact thatat the time of survey the environmental conditions were not favorable for the pathogen development.

Cultural characteristics of *Sclerotium rolfsii*

The whole Petri plate was rapidly covered with mycelium in 3 days and aerial hyphae also cover the lid of the petri plate. Pure cultures of *S. rolfsii* produced silky-white (Fig. 2) or white to light cream colonies (Fig. 3).

The colonies showed distinct texture i.e. submerged (7 isolates), thin flat (10 isolates), medium fluffy (15 isolates) and fluffy texture (20 isolates) (Table 1).

The sclerotia were round, shiny in appearance and mature sclerotia having the mustard seed like appearance. The sclerotial diameter ranged from 0.5-2.0 mm. The sclerotia produced by eight isolateswere cream to brown. Whereas, the rest of forty-two isolates developed dark brown sclerotia.

Fig. 3. Silky-white, fluffy colony of *S. rolfsii* on PDA medium.

The thirty-five isolates (70%) developed sclerotia on the surface of colony, and fifteen isolates (30%) had sclerotia at both aerial hyphae and surface.

The formation of sclerotia by seventeen isolates (34%) were scattered throughout the plate (Fig. 4). The rest of thirty-three isolates (66%) formed sclerotia at peripheral ring of Petri dish (Fig. 5) as shown in Table 1.

The presence of shiny and gummy material on sclerotia surface is owing to the production of extracellular polysaccharides. The filamentous fungi is promising producer of β D-glucan as the extracellular matrix and hyphal cell wall contain more than 75% polysaccharides (Fliegeret *al.,* 2003).Sclerotia were small, round and tan to dark brown or black. Variability in mycelial growth rate, cultural morphology, sclerotialcolour, size and formation were observed in *S. rolfsii* by many researchers (Akramet *al.,* 2008; Almeida *et al.,* 2001; Okereke and Wokocha, 2007).

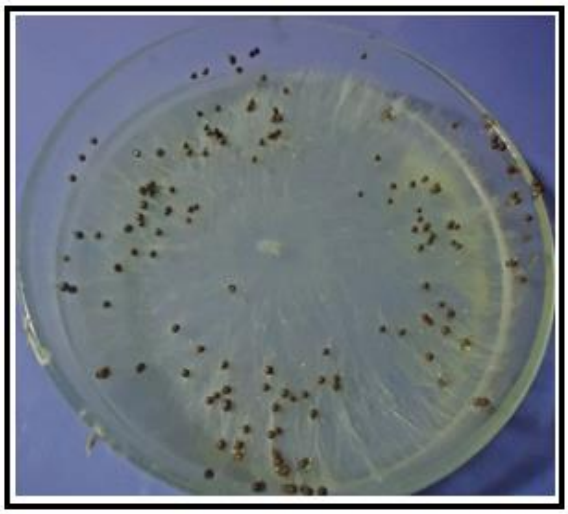

Fig. 4. Sclerotia scattered on whole Petri dish.

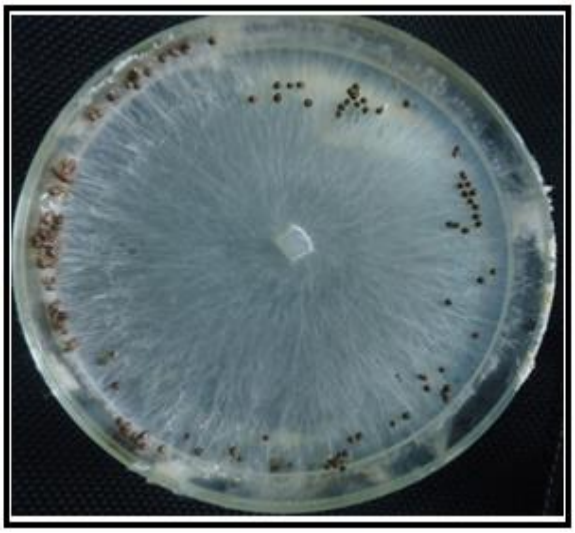

Fig. 5. Sclerotia scattered on periphery of Petri dish.

Hyphal characteristics

The fan-shaped mycelial expanse was observed growing outward. The hyphae were hyaline, thin walled and have infrequent cross walls.

All the isolates produced clamp connections. Feeding branches arise from the main branch. Mycelial branching was at right angles and developed hyphae that are slightly constricted at the branch origin, often a septum near the origin.

Fig. 6. Root rot caused by *S. rolfsii*; Poor top growth and wilting of the leaves.

The average hyphal width ranged from 4.8 µm to 5.7 µm. Maximum hyphal width (5.7 µm) was observed in isolate DGADY44 while minimum (4.8 µm) was in isolate DGADY09 (Table 1).

Pathogenicity test

The symptoms developed by *Sclerotiumrolfsii* on artificial inoculated bell pepper seedlings after 10 days include poor top growth and wilting of the leaves

(Fig. 6). Water-soaked lesions appeared on roots and lower stem part. White cottony growth covered the infected root surface. The roots become rotted and plant eventually died. Notably, 4 (8%) isolates viz. DGADY05, DGADY09, DGADY14 and DGADY15 exhibited highly virulent response with 100% DSI. In contrast, 42 (84%) isolates were moderately virulent and 4(8%) exhibited low virulence (Table 1). The fungi re-isolated from artificially inoculated roots were identical in morphology to the original isolates on PDA and fulfilling the Koch's postulates.

No lesions developed in the healthy control plants inoculated with sterile distilled water.

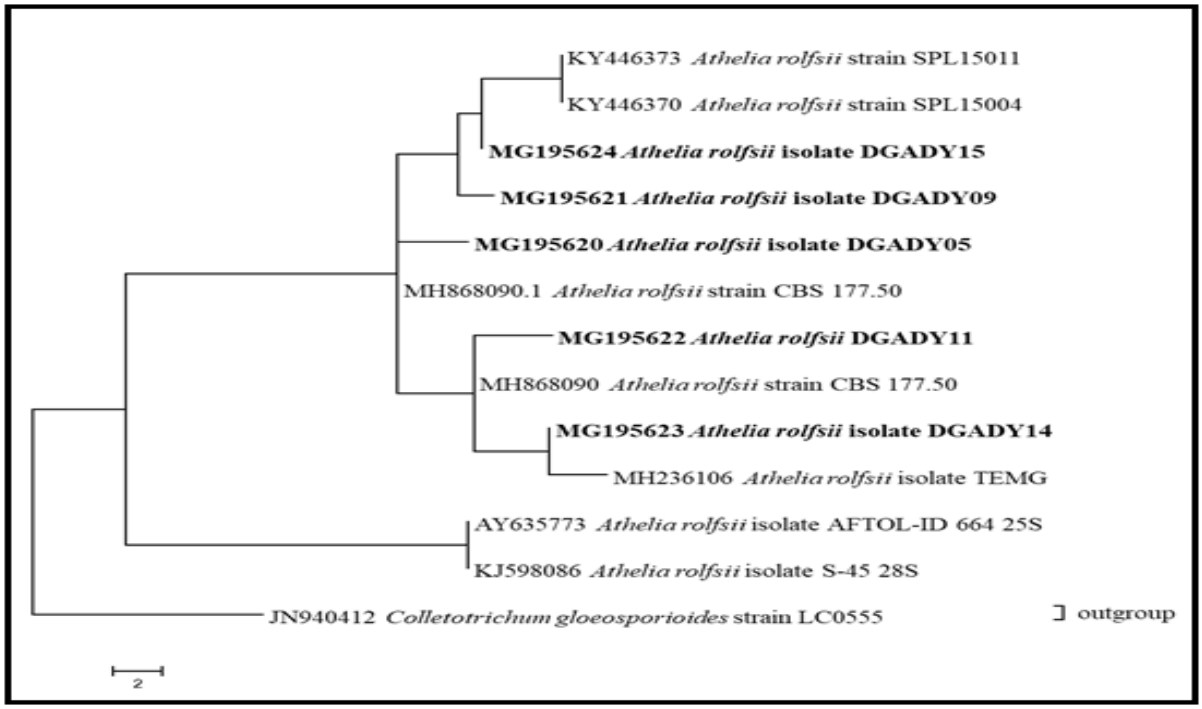

Fig. 7. Molecular phylogeny of *S. rolfsii* (Teleomorph: *Athelia rolfsii*), generated from aMaximum Likelihood method analysis.

Molecular characterization

The isolates of S. rolfsii (Teleomorph: *Athelia rolfsii*) shared 99-100% genetic homology with the sequences reported from different regions in GenBank. A phylogram was generated using the partial sequences of LSU gene (Fig. 7). The reference sequences of S. rolfsiiwere obtained from GenBank with C. gloeosporioides (strain LC0555) as an outgroup. A total of 629 sites in the final dataset were processed.

The isolates of present study were grouped into five different clades and each aligned with S. rolfsii isolates reported from different regions. Morphological characteristics based on cultural and microscopic morphology in this study was in agreement with phylogenies obtained from molecular data.

Conclusion

The present study reports S. rolfsii causing root rot of bell pepper in Pothohar Plateau, Punjab, Pakistan based on morpho-molecular characterization. Pathogenicity test further identifies the pathogenic variability of isolates.

The detailed studies are needed to explore the management practices to minimize the damage and future losses caused by this disease.

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