Open Access

Phytopathogenomics and Disease Control*., *2025, 4(1), 29-37.
ISSN (Online):2957-5842; ISSN (Print):2957-5834
DOI: https://doi.org/10.22194/Pdc/4.1058
https://societyfia.org/journal/PDC

Society for Innovative Agriculture

Etiological and Molecular Characterization of *Sclerotinia sclerotiorum* Associated with Stem and Crown Rot in Alfalfa

Honey Arooj[1*], Ahmad Nisar[1], Sidra Gill [3,4] and Nabeeha Aslam Khan[1]

[1]Department of Plant Pathology, University of Agriculture, 38040 Faisalabad, Pakistan:, [3]Department of Botany, Faculty of Chemical and Biological Sciences, The Islamia University of Bahawalpur,Pakistan, Current address; [4]Centre de Recerca en Agrigenòmica-CRAG 08193 Spain

*Corresponding author's e-mail: honeyarooj00@gmail.com

Alfalfa (*Medicago sativa*), a member of the family Fabaceae**,** is one of the oldest and most important forage crops in the world, commonly referred to as the "Queen of Forages." Despite its high nutritional value and agronomic significance, alfalfa is susceptible to a wide range of diseases caused by fungi, bacteria, and viruses. Among these, stem and crown rot**,** caused by *Sclerotinia sclerotiorum*, represents a serious threat to alfalfa production. This disease significantly contributes to yield losses, with a substantial portion of the crop potentially lost each year. Due to its wide host range, *S. sclerotiorum* has emerged as a global problem, impacting various economically important crops and resulting in considerable annual losses. Keeping in view high yield losses due to this disease, this research has been mainly deals withEtiological and Molecular Characterization of *Sclerotinia sclerotiorum* associated with Stem and Crown Rot in Alfalfa. There is also a need to study and understanding of the conditions for *S. sclerotiorum* inoculum production for managing and controlling the stem and crown rot disease caused by *S. sclerotiorum.* During survey maximum disease incidence was recorded. Morphological characterization of the pathogen was based on the size of sclerotia and morphology of the ascospore. *S. sclerotiorum* were characterized on the basis of morphological as well as molecular technique (using ITS primers-ITS1/ITS4). The sequences were submitted to Fungal Molecular Biology Laboratory Culture Collection University of Agriculture Faisalabad (FMB CC UAF) given accession number FMB 0123. Single parsimonious phylogenetic tree was constructed in which queried sequences present in separate clade indicated that all queried sequences are phylogenetically diverse from other sequences present in separate clade indicated that all queried sequence is phylogenetically diverse from other sequences present in comparison and has maximum similarity with *S.sclerotiorum*.

Keywords: Alfalfa, Fabaceae, *Sclerotinia sclerotiorum*, etiological, molecular characterization, crown rot, parsimonious phylogenetic tree and phylogenetically diverse.

INTRODUCTION

Alfalfa, originated from South- western Asia with Iran as the regional center, and is known to be cultivated as the oldest forage crop (Goplen *et al*., 1987). The Spanish and Portuguese conquistadors first brought Alfalfa to the Americas. And then it grew in Peru and Mexico due to meeting its ideal conditions for growth there and then it is spread in "Chile, Argentina, eventually to Uruguay, in 1775 (Bolton *et al*., 1975). Alfalfa in Montana, Iowa, Missouri and Ohio was grown to some extent, in the late 1800s. While the atmosphere of Southwestern states was very suitable for Spanish Chilean supplies of alfalfa and in the northern and eastern states, the weather of winter hardiness required for productive development of alfalfa was missing (Bolton *et al*., 1975). However, colonists of New England introduced alfalfa as "Lucerne" to their new homeland. Taxonomically, alfalfa belongs to the Plantae kingdom and sub-kingdom is Tracheobionta, and its class is Magnoliopsida and its Sub-class is Rosidae and belonging to order Fabales and the family is Fabaceae (pea family) and Magnoliopsida is its class and the genus is *Medicago* and specie name is *sativa*. Alfalfa is also known as luzerna, blalusem, chilean clover, jatt, kabayonca, medick, yonja, luzerna herbal buffalo, mielga, mu su, sai pi li ka, root, purple medick, luzerne, murasaki-umagoyashi, sinimailanen, and Lucerne (Wiersema & Leon,

Arooj, H., A. Nisar, Gill,S., & N.A. Khan. (2025). Etiological and Molecular Characterization of *Sclerotinia sclerotiorum* Associated with Stem and Crown Rot in Alfalfa. *Phytopathogenomics and Disease Control*, *4*,29-37.
[Received 15Dec 2024; Accepted 21 May 2025; Published 23 Jun 2025]

1999). The pod is coiled and has a diameter of 6 to 8 mm. The production of seeds is up to 220,000 per lb and is of 1 to 2mm in length and width is 1 to 2 mm and thickness is 1.2 mm and of kidney-shaped and much smaller than the size and length of the pods (Teuber & Brick, 1988). Alfalfa, *Medicago sativa L*, is considered as the largest forage crop of the world (Putnam *et al*., 2010), with an 8.3 million metric tons global hay market in 2017 (ITC, 2018). Alfalfa is known to be an important crop due to its usage in the production of dairy and meat and that's why it is known to be a high-quality forage due to its high protein content (Ball *et al*., 2001). Alfalfa has also the ability to fix nitrogen by symbiosis of rhizobia and the production of ample biomass yield and more significantly it has the ability to tolerate drought conditions (Singer *et al*., 2018). Alfalfa contains high levels of protein in leaves and it can also fulfill the nitrogen requirements (Heichel, 1983). Alfalfa is known to be a deep-rooted crop so that's why it has the efficiency of nutrient uptake and improved usage of water efficiency as well as excellent qualities of Nitrogen fixation in sustainable agriculture (Kulkarni *et al.*, 2018). Alfalfa stems grow dense xylem tissue (wood) and *have* cell walls composed of hemicellulose, lignin, cellulose, and pectin (Theander & Westerlund, 1993).Since leaves of alfalfa are known to be the machinery of photosynthesis activity and therefore the leaves of alfalfa have very high concentration of enzymes and very thin cell wall which facilitate gaseous exchange and absorption of light. The composition of stem of alfalfa is shown in Table 1. Stems and leaves of alfalfa have low concentrations of starch and simple sugars (Raguse & Smith, 1966), whereas, roots of alfalfa store 150 to 350gkg^{-1} DM of starch and has approximately 20g kg^{-1} DM of lipid content (Dhont *et al*., 2002).

Table 1. Composition of Immature (Bud Stage) and Mature (Full Flower) Alfalfa Stem Material.

Component	Immature bud stage	Mature full flower
	Dry Matter	
Lignin	156	173
Pectin	128	120
Soluble carbohydrates	57	50
Starch	5	3
Lipid	8	9
Hemicellulose	103	125
Cellulose	273	303
Ash	80	56

Pakistan possesses a diverse livestock population. It not only provides food security by providing, meat, milk and self-employment to both men and women, but also serve a major role in alleviating scarcity of employment among smallholder farmers. Thus, it has an almost 54.5 percent of share in agricultural GDP and near about 11.5 percent of share in national GDP. According to the economic survey of Pakistan, the livestock population consists of approximately 163.5 million heads, including pigs, cattle, asses, goats, horses, mules, camels, and buffaloes. As the population of livestock is increasing by 4.3 percent per year; consequently, their feeding requirements are also increasing. For better and nutritious development of the population of livestock a complete supply of well-developed forage is required. So, forage crops play a significant and principal role in fulfilling the feeding requirements of livestock. But the major issue is the limited production and scarcity of forage crop (Azam & Shafique, 2017).This exasperating situation stems from a nearly 2 percent reduction in forage availability per decade, marked by two periods of shortageone in November-January (in winter months) and the other one is in May-June (in summer seasons). Currently, forage is cultivated over 10.4 percent covering an area of 22.66 million hectares. The area was contribution of the provinces towards forage cultivation is 83.66 percent by Punjab province and 4.5 percent share is by NWFP and Sindh is contributing near about 11 percent and on the other hand, 1.47 percent share is by Baluchistan province. According to Agric. Statistics survey, Pakistan is harvesting annually forage of 51.9 million tons at an area of 2.4 million hectares and approximately 22.6 tons fodder is harvested per hectare in the country (Azam & Shafique, 2017).

In July 1982, Stem and Crown Rot Disease of Alfalfa caused by a species named Selerotinia was observed in several broadcast alfalfa stands used for seed production in the Touchet-Gardinia region near Walla Walla, southeast Washington. Alfalfa corona and stem rot, caused by *S. sclerotiorum*is known to occur in cool, humid European and North American regions (Adams & Ayers, 1979). Yet, with its dry and hot desert climate, alfalfa fields in eastern Washington were not expected to occur. Nevertheless, in April, May, and June of 1982, the dense plant canopy combined with the cool and wet conditions provided near-optimum conditions for a significant occurrence of this disease activity. Several fields in this development area displayed substantial browning and dieback caused by Sclerotinia during June and July. There areno *Sclerotinia*-resistant alfalfa cultivars and the only methods of control are cultural practices.

Sclerotinia sclerotiorum is known to be a serious threat towards a number of crops including alfalfa and many other forage legumes growing in the countries of temperate climates. This is known to be a serious because it causing devasting losses such as thinning of alfalfa stands and also by lowering alfalfa yields by causing Stem and Crown Disease of Alfalfa (Elgin *et al*., 1988). This disease can damage alfalfa plants of all ages but it can cause severe attack during the seedling stage in the temperate cool climate with humid conditions (Kanbe *et al*., 1997). Damage typically becomes evident in the spring after vegetation resumes. In susceptible

cultivars, dead plants become evident shortly after vegetation resumes. Later in the spring, plants can tolerate the pathogen, exhibiting slowed disease development symptoms such as damaged crowns and a reduced number of wilting plants (Pratt & Rowe, 1995). Lucerne varieties vary greatly in tolerance to Stem and Crown Rot disease as compared to other crop plants (Khan, 2002; Pratt & Rowe, 1998; Pratt, 1996).
The accessibility of molecular methods has made our understanding towards *S. sclerotiorum*infection strategies and pathogenic growth considerably advanced.*S. sclerotiorum*'s capability to infect numerous plant species and tissuesand can survive and infect crops during different environmental conditions because the pathogen produce sclerotia that can remain viable in the soil for many yearsand therefore it can infect a wide range of plants for a number of years. On the other hand, sclerotia are playing a major role in disease cycles by germinating ascospores from apothecia for local colonization, triggering the disease cycle through either vegetative or sexual reproduction.(Bardin & Huang, 2001; Bolton *et al*., 2005).
The main aims of this research were to Identified *Seclerotinia sclerotiorum* on a Morphological, cultural and Genetic basis.

MATERIALS AND METHODS

***Acquisition of pathogen*:** Isolate of *Seclerotinia sclerotiorum* acquired from Fungal Molecular Biology Culture Collection (FMB-CC), University of Agriculture Faisalabad having accession number.

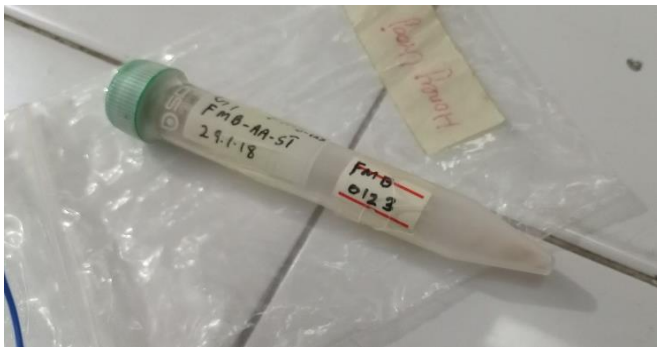


Figure 1. Isolate of *Seclerotinia sclerotiorum* acquired from FMB-CC.

For the isolation of *S. sclerotiorum*, Potato dextrose agar (PDA), a common media was prepared by following these steps; Ingredients for one-liter media; Potato (250g), Agar Agar(20g), Glucose (20g) and 1 litre Water. Peeled 250 g potatoes were taken and boiled for 20 mint in 500ml of water to get the potato starch, and 20 g of dextrose was added in 500ml of water in a flask. In another flask 20 g agar agar was dissolved in 300ml of flask water. Then both flask contents were collectively combined to make 1liter volume, and then autoclaved for 20 minutes at ±121 ° C at 15 psi. After that the media under aseptic condition was poured into the petri dishes in the laminar air flow chamber. 20ml of media was poured into each petri plate, and left for solidification. After that, the petri dishes were wrapped and will be processed for further procedure.
***Revival of Culture*:** The Isolate of *Seclerotinia sclerotiorum* acquired from FMB-CC-UAF laboratory are placed on the PDA containing petri plates with the help of sterilized forceps under laminar flow. Then wrapped them carefully and incubate them at 20°C with PH 5.0 for 7 days. Plates were observed on daily basis.

Figure 2. Isolation of fungus *S. sclerotiorum*

To avoid the contamination during handling, after 8 days when pathogen grows on media plates and the pathogen also develop their sclerotia with mycelium, a single sclerotium has been taken by using sterilized needle from the petri plates and transferred it into other petri plates. The whole procedure was completed in a laminar air flow chamber. For the growth of the fungus the plates were incubated at ±25°C in the incubator. The isolated fungus was later on observed microscopically on the basis of morphology of the fungus size, shape, color of conidia etc. for pathogen identification.
***Morpho-culture characterization of the isolate*:** For the identification of the pathogen, morphological features of isolated fungal pathogens such as mycelial pattern, sclerotial characters, conidia, conidiophore, distinctive character of apothecia, and ascospores examined. Single hyphal tip of fungal isolate were transferred to new PDA plates and incubated for a week. Colony characteristics and sclerotial fungal morphology have been examined with naked eye. Permanent slides from the colony were prepared and analyzed to observe the fungal structures at various magnifications under a light microscope.
Morphological features of the teleomorphic phases of isolate was examined under mmicroscope.The sclerotia are firstly

surface sterilized and then placed in the sterilized petri plates having 9cm diameter and filled with sterilized wet sand and then these plates were incubated at ±4°C for 5-6 weeks. The Petri plates containing germinating sclerotia were then placed for examination under incubator having fluorescent radiation of 260 µmol / m2 / s and illuminated for 3-4 days at ±20°C (Huang *et al*., 2005). Morphological features like ascospores, apothecia and sclerotiawere examined under the microscope (40X and 100X).

Figure 3. Revival culture of fungus *S. sclerotiorum*

Molecular identification

***Extraction of DNA*:** As defined by Plattner *et al*. (2009), complete genomic DNA was extracted. The fungal spores were inoculated on the potato dextrose broth (100ml) and placed on 180rpm at 28 ° C in the shaking incubator for 72 hours. The culture was then poured into a 50ml falcon tube and centrifuged at 13000rpm for 25-30min. (Note: Set water bath at 65 ° C, centrifuge at 4 ° C before work starts). Take pallet into pestle mortal after centrifuging discarded supernatant Ground with sterilized CTAB solution and preheated sand. After grinding, pass the grinded sample into a 15 ml falcon tube and invert the falcon every 10 minutes after incubation at 65 ° C in a water bath for 30 mins. And then equal volume of chloroform isoamyl alcohol is added into each falcon tube after 30 minutes, shake well and centrifugate at 4 ° C at 900rpm for 15 minutes. Take supernatant and add chloroform isoamyl alcohol to the same amount of chloroform isoamyl alcohol again centrifuged in the same amount. After centrifugation, the supernatant was moved into a new sterilized falcon tube and 2 propanol was added similarly chilled, centrifuged at 4 ° C @ 900 rpm for 10 minutes. After centrifugation a pellet was formed. Then the supernatant was discarded and the pellet was washed for 2-3 minutes with 70 % ethanol. The falcon was placed inverted position on tissue paper after discarding the supernatant until it became dried. The pellet was then dissolved in 25 µL R40. A Nano drop spectrophotometer has been used to measure DNA.

Table 2. Reagents and Quantity used in the Extraction of DNA.

Reagents	Quantity
CTAB	20.0 g
100Mm tris HCL	25.0 ml
NaCl	81.9 g
20 Mm EDTA	40.0 ml

***PCR Conditions for the amplification of ITS region of fungal DNA*:** Reaction volume of PCR was 25 micro liters. It contained d3H_2O, 10X Taq buffer, $MgCl_2$, dNTPS, Taq DNA polymerase, ITS primers (ITS1/ITS4) and template DNA. Polymerase chain reaction (PCR) was conducted with forward primer ITS-1 (5′-TCC GTA GGT GAACCT GCG G-3′) and reverse primer ITS-4 (5′-TCC TCC GCT TAT TGATAT GC-3′) (White *et al.,* 1990) to amplify rDNA-ITS regions of the fungal isolate PSHB1. PCR was done according to the described method by Hayakawa *et al.,* (2006).

***Agarose gel electrophoresis*:** For PCR product analysis, electrophoresis (using 1 percent agarose gel) was used. Agarose gel was prepared by dissolving 01 gram of agarose in a 100ml 0.5X TAE buffer, then adding 2.5µl of ethidium bromide to the staining agent, pouring it into a gel tray containing a comb, then loading it into gel wells after solidification of the gel samples and supplying the voltage to run. The macromolecules were segregated as agarose gel on the basis of the mass to charge ratio. Gel visualization was achieved using the gel documentation system (GDS) and the gel images were saved.

Table 3. Reagents for TAE buffer 50X 500ml.

Ingredient	Quantity
Sodium EDTA	9.3g/40ml
Tris base	121g/350ml
Glacial acetic acid	28.55ml

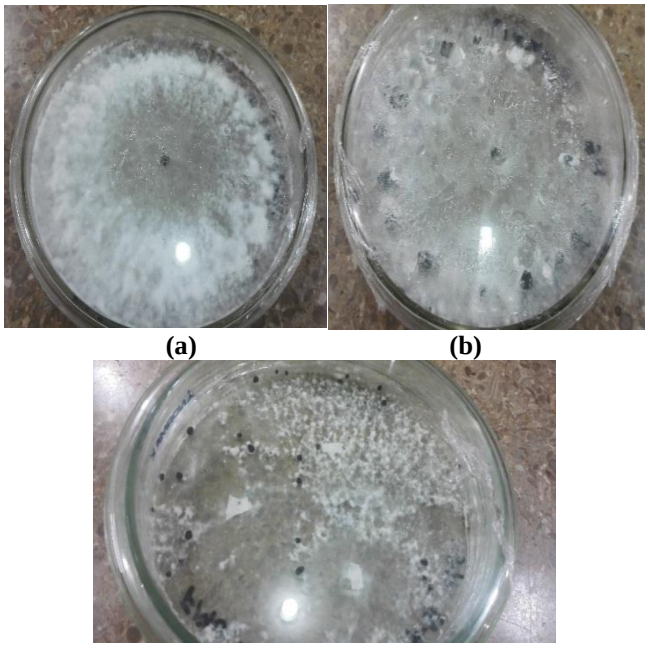
***Purification of PCR product*:** Purification of PCR product was done by using PCR purification kit. Protocol followed as given by manufacturer of purification kit: Transfer the PCR product in Eppendorf, Added 5 volume favor prep PCR cleaning (FAPC) buffer in Eppendorf having PCR sample, Place FAPC column into collection tube, Vortex the mixture of FAPC column and PCR product and transfer into FAPC column (*incubate the elution buffer at 55°), Centrifuged the mixture @ 9000 for 30 sec, Discard flow through and centrifuged @ 13000 rpm for 3min, Shift the column in elution tube, Add 40µl elution buffer in the center of membrane of FAPC column then wait for 1 minute, Take flow through with pipette, again poured into column and centrifuge for 1 minutes @ 13000 rpm and Stored at -20°C.

***Sequencing*:** Purified products were filled into sequencing tubes after purification of PCR products, and ITS1 primers were added and then shipped for sequencing. Sequences collected were trimmed using software and matched. Using compatible sequences to create phylogenetic tree. In order to access how many necessary sequences are connected to each other, the phylogenetic tree was created. Using BLAST tool and literature, the trimmed sequences were queried against NCBI database. Trimmed sequences were sent to Genbank to retrieve and coordinate ITS sequences of known isolates from the NCBI database.

***Preservation*:** In the Fungal Molecular Biology Lab, the dry Filter paper technique and PDA slants will be used for the preservation of isolates in Fungal Molecular Biology Culture Collection (FMB-CC), University of Agriculture Faisalabad as well as in the Bank for First Fungal Culture, Institute of Agricultural Sciences, University of Punjab. The isolated cultures were used in Punjab, Pakistan to further experiment with the etiology and management of stem and crown rot disease in alfalfa (*Madicago sativa*).

RESULTS

***Revival of Culture*:** *Seclerotinia sclerotiorum* acquired from Fungal Molecular Biology Culture Collection (FMB-CC), University of Agriculture Faisalabad having accession number FMB 0123.



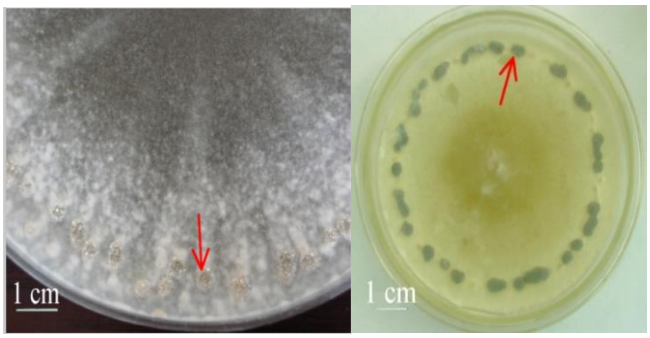
(a) **(b)**

(c)

Figure 4 (a,b,c). Pure culture of isolates of *S. sclerotiorum*

The culture was then grown on the growth medium for further process of morphological, molecular identification and also for pathogenicity test. Thus, to carry out this process culture was passed to the PDA media afterward. In light and dark times, plates were held at an optimal temperature of 25°C for 12hrs. Identification was based on morphology.

***Morphological identification of the characters of S. sclerotiorum*:** Morphological features of the teleomorphic phases of isolate was examined under microscope. Sclerotia are firstly surface sterilized and then placed in the sterilized petri plates having 9cm diameter and filled with sterilized wet sand and then these plates were incubated at ±4°C for 5-6 weeks. The Petri plates containing germinating sclerotia were then placed for examination under incubator having fluorescent radiation of 260 µmol / m2 / s and illuminated for 3-4 days at ±20°C (Huang *et al*., 2005). Morphological features like ascospores, apothecia and sclerotia were examined under the microscope (40X and 100X).



(a) **(b)**

Figure 5(a,b). Morphological identification of the Fungus

Pathogen isolates were also made from infected stem pieces and also from lesion surface and from sclerotial places that supplied creamy white colored mycelial colonies on the PDA medium. The hyphae were hyaline in color, 4 -4.5 microns in diameter, branched septate, and multinucleate. On PDA medium, the pathogen produced sparse and creamy white mycelial colonies that later turned fluffy and light brown in color.

The entire medium of the petri plates took four days for the full germination of mycelial colonies. After the full germination of mycelium on the petri plates, the initiation of sclerotia started in ring or scattered form on the sclerotia after 72 hours or with 6-7 days. Actually, the sclerotia during the growing ager is of silvery white in color and as the time proceed it turned into dark brownish and then with the passage of time into dark blackish in color on the fungal culture. They were of circular to oval shaped and also of cylindrical shape and ranged in size from 4.0 to 8.7 x 3.0 to 5.5 mm (with the average size of 4.77 x 3.73 mm). Approximately 38-39 sclerotia were produced per plate with the average fresh and dry weight of 10 sclerotia was 0.215 g and 0.103g respectively.

Table 4. Cultural and morphological characteristics of *Sclerotinia sclerotiorum.*

Cultural & morphological parameter*	Description
Hyphae color	Hyaline
Diameter	4 -4.5 µm
Colony color	Creamy white to light brown
Colony growth rate (cm/day)	(1.2) 24h, (4.3) 48h, (6.8) 72h, (9.0) 96h
Type of growth	Sparse, fluffy
Initiation of sclerotia	formation 72 h after incubation
Color of sclerotia	Whitish to blackish
Shape of sclerotia	Spherical to oval or cylindrical
Average size of sclerotia	4.68 x 3.73 mm
Average number of sclerotia per plate	(90 mm) 38.7
Position of sclerotia in Petri plate	Appeared in form of ring at the periphery or scattered in the center of the plate

*Mean of four replications

***Molecular identification*:** PCR products were analyzed through gel electrophoresis. Wells were loaded with purified PCR products of FMB respectively. Last well was loaded with 1KB ladder. Discrete bands of 550bp are shown in figure:

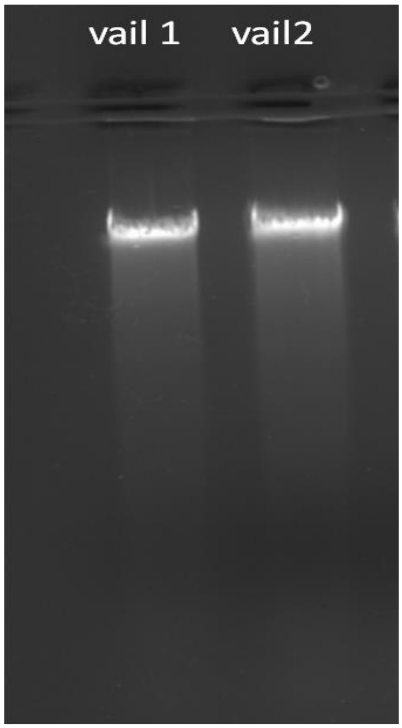


Figure 6. Gel picture of DNA extracted from isolates.

Sequences obtained were trimmed by Chromas software and in silico characterization was done. Then homology search tool BLAST (Basic Local Alignment Search Tool) was used to queried the trimmed sequences against Genbank database. All queried sequences got similarities with sequences of *S. sclerotium*. It confirmed the results got through morphological identification of fungal isolates. Trimmed sequences were deposited to Genbank having accession number FMB 0123. Then on homology basis, retrieved the ITS (internal transcribe spacer) sequences of *S. sclerotium* from NCBI (National Center for Biotechnology Information) database and alignment of all these sequences long with our submitted sequences (accession number FMB 0123) was done by using Clustal Omega Software. MEGA6.06 was used to construct phylogenetic tree of aligned sequences with maximum parsimony method.

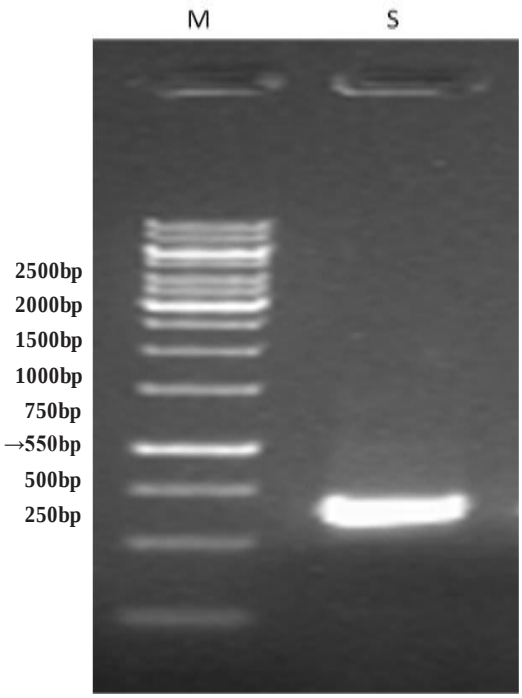


Figure 7. PCR Products of fungal isolates.
M = 1KB marker
S = Purified PCR product (Sample)

***Culture Preservation and Submission to FMB-CC-UAF culture collection*:** The isolate of S. sclerotium was preserved as Dry Filter Paper Technique. The preserved fungal culture was deposited in Fungal Molecular Biology Laboratory Culture Collection University of Agriculture Faisalabad (FMB-CC-UAF). The submitted isolates were given unique accession number by this culture collection which are available online on the website of the world data center for microorganisms (WDCM). The detail of the submitted isolate is given in Table 5.

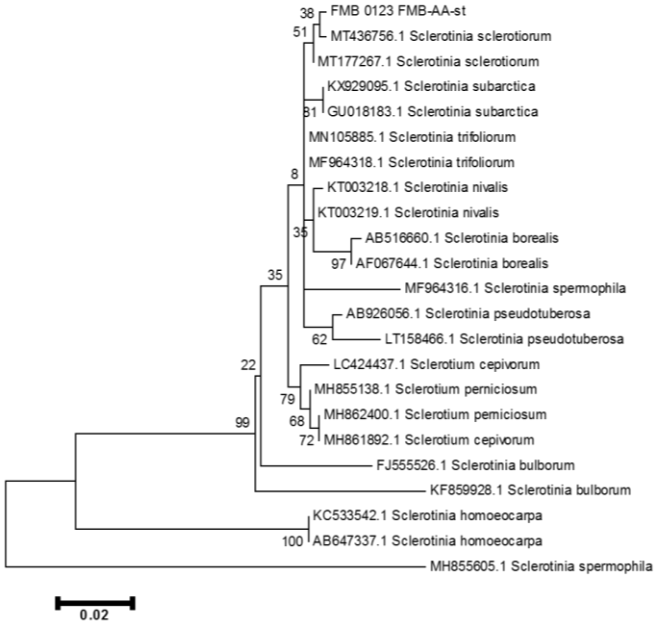


Figure 8. Single parsimonious phylogenetic tree was constructed in which queried sequences present in separate clade indicated that all queried sequences are phylogenetically diverse from other sequences present in separate clade indicated that all queried sequence is phylogenetically diverse from other sequences present in comparison and has maximum similarity with *S. sclerotiorum*.

Table 5. The detail of the submitted isolate with accession number and Isolate code.

Isolate Name	FMB accession no.	Isolate code
Sclerotinia sclerotium	FMB 0123	MW012390.1

DISCUSSION

In present study, the purpose of this research, is to evaluate the *S. sclerotiorum* isolates in alfalfa through molecular and morphological approaches. The isolate analyzed was identified as *S. Sclerotiorum* has shown considerable genotypic and morphological variability through ITS-rDNA sequence analysis. The identity of the pathogen was confirmed on the basis of morphological characterization, that is ascospore morphology and sclerotia size. Differentiation of species among plant pathogenic species was confirmed as *S. Sclerotiorum*by morphological characterization. Saharan & Mehta (2008), indicate that *S. Sclerotiorum*, compared to other species, has the ability to produce large-sized sclerotia and monomorphic ascospores.In our research it is found out that sclerotial bodies are of 4.77 x 3.75 mm large, and of irregular shape with dark brown to blackish in color are produced. This has confirmed the identity of S. sclerotiorum. Sclerotiahas been subjected to carpogenic germination in order to study ascospore morphology. It was reported by Sanogo & Puppala (2007) that S. sclerotiorum produced beige to tanned colored apothetical discs. After 45 daysSclerotia produce stipe in our research. The black stipe initials were melanized, increased in length and cup shaped after fifteen days of incubation; and apothecium of ochraceous in color was produced from the stipe of sclerotia were produced. The apothecium varied in size from 1.0 to 1.6cm. The ITS region comprising 18S rRNA, ITS1, 5.8S rRNA, ITS 2 and 28S rRNA can be regarded as a fungi-identification environmental barcode (Bellemain *et al*., 2010). It has been reported that the 18S-28S rRNA gene has approximately 600 bp (Wang *et al*., 2008). In our study, the PCR product generated an amplicon length of ~550 bp and the same was sequenced by the Sanger dideoxy sequencing method at Excelris genomics, Ahmedabad. The sequence was analyzed with BLAST in NCBI after sequencing. The 18S-28S rRNA gene nucleotide sequence acquired from the fungus study had a 99 percent match with *S. sclerotiorum.* This molecular confirmation complements and substantiates the morphological observations. Moreover, the detection of significant genotypic and morphological variability among isolates suggests the potential for regional adaptation or microevolution within *S. sclerotiorum* populations. Such variability has been previously reported and may influence virulence, host range, and resistance to fungicides. The integration of morphological and molecular approaches not only improved the accuracy of pathogen identification but also highlighted the importance of comprehensive diagnostics in disease management. Morphological assessments provided rapid and cost-effective screening, while molecular tools offered precise, confirmatory evidence. Together, these methods serve as a robust platform for future work in pathogen surveillance, resistance breeding, and epidemiological studies. Given the pathogen's broad host range, survival through sclerotia in soil, and the production of long-lasting reproductive structures like apothecia and ascospores, management of *S. sclerotiorum* requires a multidisciplinary approach. Accurate identification is the cornerstone of such efforts, enabling timely deployment of resistant cultivars, crop rotation strategies, and effective chemical or biological control measures.

***Conclusion*:** The observed morphological and cultural features closely aligned with typical characteristics of *S. sclerotiorum*. Molecular techniques, especially ITS sequencing, provided robust evidence confirming pathogen identity. The study confirms *S. sclerotiorum* as the causative agent of stem and crown rot in alfalfa through morpho-cultural and molecular characterization. This combined methodology enhances diagnostic reliability and underpins future control strategies and these findings provide valuable

insights for developing early detection tools and integrated management strategies against the disease.

CRediT author statement: Honey Arooj designed the study, conducted experiments, analyzed data, and drafted the manuscript. Ahmad Nisar supported sample collection, pathogen isolation, and initial data compilation. Sidra Gill assisted with molecular characterization and laboratory analysis. Nabeeha Aslam Khan contributed to experimental design and data interpretation, and reviewed the manuscript.

Acknowledgement**:** I acknowledge the work instructions provided by Imran ulhaq and whole team of authors for their support.

Ethical statement**:** This article does not contain any studies with human participants or animal performed by any of the authors.

Availability of data and material**:** We declare that the submitted manuscript is our work, which has not been published before and is not currently being considered for publication elsewhere.

Consent to participate**:** Honey Aroojand Ahmad Nisar conceived the idea and prepared manuscript.

Consent for publication**:** All authors are giving the consent to publish this research article in Phytopathogenomics and Disease Control.

SDGs addressed**:** Zero Hunger; Responsible Consumption and Production; Climate Action

Policy referred**:** National Agricultural Policies, International Policies

Publisher's note: All claims stated in this article are exclusively those of the authors and do not necessarily represent those of their affiliated organizations or those of the publisher, the editors, and the reviewers. Any product that may be evaluated/assessed in this article or claimed by its manufacturer is not guaranteed or endorsed by the publisher/editors.

REFERENCES

Adams, P.B. (1979). Ecology of *Sclerotinia* species. *Phytopathology*, *69*, 896-899.

Azam, A., (2017). Agriculture in Pakistan and its Impact on Economy. A Review. *International Journal of Advanced Science and Technology*, *103*, 47-60.

Ball, D.M., (2001). Understanding Forage quality. *Turkish Journal of Field Crops 16*, 15-22.

Bardin, S.D., (2001). Research on biology and control of *Sclerotinia* diseases in Canada. *Canadian Journal of Plant Pathology, 23*, 88-98.

Bellemain, E., (2010). ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. *BMC microbiology*, *10*, 1-9.

Bolton, J. L., (1975). Alfalfa Science and Technology. *EE. UU. 1*, 7-8.

Bolton, M.D., (2005). *Sclerotinia sclerotiorum* (Lib.) de Bary: Biology and molecular traits of a cosmopolitan pathogen. *Molecular Plant Pathology*, 1-16.

Dhont, C., (2002). Alfalfa Root Carbohydrates and Regrowth Potential in Response to Fall Harvests. *Crop Science*, *42*, 754-765.

Elgin J. E., (1988). Breeding for yield and quality. *Alfalfa and Alfalfa Improvement 29*, 809-825.

Goplen, B.P, Baenziger, L.D., Bailey, A.T.H., Gross, M.R., & Waddington (1987). Morphological, Physiological, and Genetic Responses to Salt Stress in Alfalfa: *Agronomy Journal*, *10*, 577.

Heichel, G.H., (1983). Alfalfa. *In Crop—Water Relations* (I. D. Teare and M. M. Peet, Ed.) p. 127- 155. New York, NY: John Wiley and Sons, Inc.

ITC (International Trade Center).2018. http://www.intracen.org/itc/market-info-tools/trade-statistics/Google Scholar

Kanbe, M., Fujimoto, Y., Mizukami, S., Inami & Fukaya (1997). Increase of resistance of alfalfa to *Sclertotinia* crown and stem rot through recurrent selection. *Breeding Science*, *47*, 347-351.

Khan, U.Q., (2002). Evolving resistant varieties to Sclerotinia root and crown rot in alfalfa. *Asian Journal of Plant Sciences*, *5*, 596-8.

Kulkarni, K., Tayade, S., Asekova, J., Tae Song, J., Grover Shannon & Lee. (2018). Harnessing the potential of forage legumes, alfalfa, soybean, and cowpea for sustainable agriculture and global food security. *Frontiers in Plant Science, 9*, 1314.

Plattner, A., Kim, J., Reid, G., Hausner, Y. W., Lim, Y., Yamaoka & Breuil. (2009). Resolving taxonomic and phylogenetic incongruence within species Ceratocystiopsisminuta. *Mycologia*, *101*, 878-887.

Pratt, R.G., (1996). Screening for resistance *Sclerotinia trifoliorum* in alfalfa by inoculation of excited leaf tissue. *Phytopathology*, *86*, 923928.

Pratt, R.G., & Rowe (1995). Comparative pathogenicity of isolates of *Sclerotinia trifoliorum* and *S. sclerotiorum* on alfalfa. *Plant Disease, 79*, 474-477.

Pratt, R.G. & Rowe (1998). Evaluation of simplified leaf inoculation procedures for identification of quantitative resistance to *Sclerotinia trifoliorum* in alfalfa seedlings. *Plant Disease*, *82*, 1161-1164.

Putnam, D., Ruselle, S., Orloff, J., Kuhn, J., Fitzhugh, L., Godfrey & Kiess (2010) Fiber length and pulping characteristics of switchgrass, alfalfa stems, hybrid poplar and willow biomasses. *Bioresource Technology*, *101*, 215-221.

Raguse, C.A., & Smith .(1966). Some Nonstructural Carbohydrates in Forage Legume Herbage. *Journal of Agricultural and Food Chemistry 14*, 423-426.

Saharan, G.S., & Mehta .(2008). Sclerotinia diseases of crop plants: biology, ecology and disease management. *Springer Science & Business Media*.

Sanogo, S., & Puppala .(2007). Characterization of a darkly pigmented mycelial isolate of *Sclerotinia sclerotiorum* on Valencia peanut in New Mexico. *Plant Disease 91*, 1077-1082.

Singer, S.D., (2018). Molecular improvement of alfalfa for enhanced productivity and adaptability in a changing environment. *Plant, Cell Environment 41*, 1955-1971.

Teuber, L. R., (1988). Morphology and anatomy. 1988. Alfalfa and alfalfa improvement. Madison, WI: American society of agronomy.

Theander, O. (1993). Quantitative analysis of cell wall components. *Forage cell wall structure and digestibility*, *1*, 83-104.

Wang, J., Wang, R., Li, Y., Li, G., Tian, L., Goodman, W., Fan, J., Zhang, J., Li, J., & Guo .(2008). The diploid genome sequence of an Asian individual. *Nature 456*, 60-65.

Wiersema, J. H., (1999). World Economic Plants: A Standard Reference. CRC Press, New York, USA.