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Phylogenomic appraisal of morpho-pathogenicity try-out based identified pathogen causing stem and crown rot in *Trifolium alexandrinum* L.

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Egyptian clover (*Trifolium alexandrinum* L.) is a leguminous crop cultivated in subtropical regions of the world as winter fodder. Stem and Crown rot of E. clover caused significant crop losses in Pakistan for the last few years. White mycelia and sclerotia of the fungus appeared on plants. The two-year survey data (2017-2018) of E. clover growing Provinces Punjab and Khyber Pakhtunkhwa revealed that the average disease severity index of Stem and Crown rot was 65.4% (2017) and 67.7% (2018) in Punjab and 60.9% (2017) and 64.8% (2018) in KPK. Putative fungal pathogen from infected plant tissues was isolated and identified using an integrated taxonomic study. Hyphae septate, almost transparent, developed into sclerotia (hard black) on PDA culture media. Internal transcribed spacer (ITS), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 28S rRNA region (LSU), Calmodulin (CAL), and heat shock protein (HSP60) genetic regions were explored and deposited to NCBI with MT995056, MT937071, MT995060, MT954957, and MT978151 GenBank accession numbers respectively. Mophogenomics identified the fungus as *Sclerotinia trifoliorum* Erikss and proved to be a pathogen of Egyptian clover by fulfilling Koch's postulates. The average disease severity index was 44.6% on the cultivar Early (Agaiti), 20.8% on Late (Pachati) in Punjab province and 41.7% on Early (Agaiti) and 19.21% on Late (Pachati) in KPK province.
Keywords: Etiology; egyptian clover; Stem and Crown rot; disease severity.

INTRODUCTION

Several fodder crops are grown in Pakistan; Egyptian clover (*Trifolium alexandrinum* L.) is the king of winter fodder crops by providing three-fourths of winter fodders. It belongs to the clover group known as Egyptian clover worldwide. It is highly favored among livestock farmers due to its long-time (November-May) fodder availability and nutrition. The E. clover cultivated countries are Egypt, India, Pakistan, Australia, Afghanistan, Southern Europe, the USA (California), Turkey, and South Africa (Knight, 1985). Pakistan ranks third based on area cultivation under E. clover (0.71 Million hectares) after India 2 Million hectares and Egypt 1.1 Million hectares (Muhammad *et al*., 2014). It has started to cultivate in Sindh province, Pakistan, in 1904 and afterward in Khyber Pakhtunkhwa (KPK) province in 1924, from where it shifted to Punjab province tracts. In Pakistan, the green fodder yield of E. clover was recorded 90.71-136.07 metric tons/ ha in 2014. Among cultivated areas of Pakistan, Punjab province shares 0.68 million ha under cultivation of E. clover with green fodder production of 21 million tons, while KPK shares 0.017 million ha area with 0.05 million tons' green fodder production (Iqbal and Iqbal, 2014).

Diseases reduce E. clover production; among these, fungal diseases are significant constraints. Recently, in Pakistan, various farmers and research organizations reported the emergence of a new disease (Stem and Crown rot) on E. clover. The disease appeared severely and spread throughout the country. The symptoms were produced initially as; whitish mycelial growth, later on wilting, and ultimately dying of plants in patches of varying size. Some of the research scientists from India reported two Sclerotinia spp. i.e., *S. sclerotiorum and S. trifoliorum*, the disease's cause (Kumar *et al*., 2003; Rathi *et al*., 2007a; Pande *et al*., 2008).

In contrast, Saira *et al*. (2016) reported *S. sclerotiorum* causing Stem and Crown rot in Pakistan. In the present study, comprehensive research was conducted to establish this disease's etiology by adopting conventional and molecular approaches. The most critical step in this research was the determination of disease etiology (Ul Haq and Ijaz 2020).

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Fungi associated with disease-affected plant tissues were collected from various country locations. Initially, the morpho-cultural characteristic described previously to identify isolated fungus was studied; furthermore, the morphological identification was confirmed through molecular characterization, and preliminary, it was reported that *S. trifoliorum* is the cause of Stem and Crown rot in Pakistan (Faraz *et al*., 2021). The symptoms were produced initially as; whitish mycelial growth, wilting, and ultimately dying of plants in patches of varying size. The research was conducted in the Fungal Molecular Biology Laboratory (FMB Lab.), University of Agriculture Faisalabad.

MATERIALS AND METHODS

***Disease assessment under field conditions*:** Surveys of E. clover cultivated Punjab and Khyber Pakhtunkhwa province Pakistan were conducted (Table 1) to document the Stem and Crown rot disease prevalence. Disease severity was recorded using Dixon (1975) disease assessment key.

***Isolation of fungal isolate(s) from infected tissues of E. Clover*:** Samples comprising infected (symptomatic) and healthy tissues of E. clover plants were processed for fungal isolation associated with E. clover. First, infected plants' Stem and the crown area were cleaved and observed for Sclerotia under a stereomicroscope (EMZ-5TR, Meji Techno, Japan). Afterward, 3-4 mm pieces from the margin of healthy and disease parts were cut and washed under running tap water; air-dried sclerotia (at 25°C) were disinfected with 1% sodium hypochlorite (Daejung, Korea) and consecutive washing with distilled water thrice. These surface disinfected pieces and sclerotia were placed on blotter paper to remove excess water and placed on potato dextrose agar (PDA) containing Petri dish in the laminar flow cabinet (LHS-4AG-FS, ESCO, Singapore). These plates were incubated in the incubator (IFC-110-8, ESCO, Singapore) at 20-25℃ and 12 hours of alternate light and dark. Purification was done using a single hyphal tip technique.

***Taxon morphology*:** Characteristics like texture, the color of fungal colonies, sclerotia size, and shape were studied on a PDA culture medium. Ascospore morphology (size, shape, and ascocarp) were studied by carpogenic germination test of sclerotia. Sclerotia (7 to 20) isolated from Stem and Crown rot of E. clover were placed on autoclaved sand in a glass Petri plate. Petri plates were incubated at 15℃. Apothecia formation, ascospores shapes, and measurements were recorded (Njambere *et al*., 2008).

***Oxalic acid production*:** The Color change (none, slight or intense) due to pH indicator was recorded after 36 and 72 hours of incubation (Njambere *et al*., 2008).

***DNA extraction, PCR analysis, and sequencing*:** The morphologically identified *Sclerotinia* species from diseased tissues were tested at the DNA level for their validated identification.

Table 1. Surveyed districts with locations and GPS coordinates of two provinces of Pakistan for Stem and Crown rot disease assessment and sampling of *E. clover*.

Regions	Districts	Tehsils /Locations	GPS Coordinates
Central Punjab	Faisalabad	Chak Jhumra	31.5629° N, 73.1881° E
		Faisalabad Sadar	31.4504° N, 73.1350° E
		Jaranwala	31.1959° N, 73.2559° E
		Chiniot	31.7292° N, 72.9822° E
		Sammundri	31.0646° N, 72.9520° E
	Okara	Pakpattan	30.2027° N, 73.2311° E
		Okara	30.8138° N, 73.4534° E
		Renala Khurd	30.4817° N, 73.3559° E
	Jhang	Athra Hazari	31. 1100° N, 72. 600° E
		Ahmadpur Sial	30.4031° N, 71.4453° E
		Jhang	31.2780° N, 72.3117° E
		Shorkot	30.3000° N, 72.2400° E
	Toba Tek Singh	Gojra	33.9680° N, 74.6971° E
		Kamalia	30.4359° N, 72.3900° E
		Pirmahal	30.7679° N, 72.4378° E
		Toba Tek Singh	30.9709° N, 72.4826° E
Northern Punjab	Gujranwala	Gujranwala Sadr	32.1877° N, 74.1945° E
		Kamoke	31.9765° N, 74.2220° E
		Hafizabad	32.0712° N, 73.6895° E
		Wazirabad	32.4459° N, 74.0960° E
	Sargodha	Bhalwal	32.8419° N, 74.7659° E
		Bhera	30.2882° N, 72.1253° E
		Sargodha	32.0740° N, 72.6861° E
		Shahpur	31.7874° N, 73.9397° E
		Sillanwali	31.4934° N, 72.3223° E
	Lahore	Kasur	31.1187° N, 74.4632° E
		Nankana Sahib	31.4492° N, 73.7125° E
		Sheikhpura	31.7167° N, 73.9850° E
		Raiwind	31.2456° N, 74.2128° E
	Sialkot	Daska	32.3387° N, 74.3530° E
		Zafarwal	32.3370° N, 74.9033° E
		Narowal	32.1014° N, 74.8800° E
		Sialkot	32.4945° N, 74.5229° E
Southern Punjab	Bahawalpur	Ahmadpur East	29.8370° N, 71.1526° E
		Bahawalpur Saddar	29.4180° N, 71.6706° E
		Hasilpur	29.4244° N, 72.3319° E
	Bahawal-nagar	Bahawalnagar	30.0025° N, 73.2412° E
		Chishtian	29.4800° N, 72.5200° E
		Fort Abbas	29.1137° N, 72.5116° E
		Minchinabad	30.0960° N, 73.3359° E
	Multan	Vehari	29.6000° N, 71.7333° E
		Lodhran	29.3159° N, 71.3759° E
		Khanewal	30.1711° N, 71.5555° E
	Muzaffar-garh	Alipur	29.2347° N, 70.5441° E
		Jatoi	29.5069° N, 70.8536° E
		Kot Addu	30.4685° N, 70.9606° E
		Muzaffargarh	30.0736° N, 71.1805° E
Khyber Pakhtun-khwa	Dera Ismail Khan	D.I. Khan	31.8626° N, 70.9019° E
		Daraban	31.7369° N, 70.3301° E
	Peshawar	Peshawar	34.0151° N, 71.5249° E
	Nowshera	Nowshera	34.0105° N, 71.9876° E
		Pir Sabaq	34.0315° N, 72.0379° E

These fungal isolates were cultured on YPSU medium (yeast extract 4g+Succrose 15g+K2Hpo4 1g+MgSo4 5g and pH maintained at 6.5) and incubated at 20ºC and 180 rpm in a shaker incubator (I 4000, IRMECO, Germany) to get fresh mycelia (Yelton *et al*., 1984). The genomic DNA was extracted using GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA), following the protocol described by the manufacturer. The extracted DNA sample was diluted 50 times in d_3H_2O and stored at 4°C prior to their use as templates in Polymerase chain reactions. The PCR analysis was conducted to amplify the taxonomically informative DNA regions. The DNA barcode's internal transcribed spacer region (ITS), large subunit ribosomal RNA (LSU), calmodulin (CAL), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and heat shock protein 60 (HSP60) were explored for characterization using primer pairs given in Table 2. VeritiTM 96 wells thermal cycler (Applied Biosystems) was used for PCR analysis. The PCR conditions were optimized for amplifying each investigated locus using gradient PCR. The PCR thermal program for generated amplicons of each locus was comprised of initial denaturation at 95°C for 3 minutes followed by the loop of 40 cycles (denaturation at 95°C for 45 sec, annealing for 30 sec, and extension at 72°C, for 40 sec), and a final extension at 72°C, for 8 minutes. The PCR products were stored at 4°C for downstream applications. PCR products were separated on high-resolution agarose gel (0.8%) (ACTGene, USA), as shown in Appendix II. The gel visualization was carried out under UV-transilluminator (Trans Lum ™ SOLO, BIOTOP) and pictured in the gel documentation system (GDS) of Bio-Rad. The required amplicons were excised from the agarose gel for DNA elution using FavorPrep Gel Extraction Kit (FAVORGEN, BIOTECH CORP, Taiwan) and then cloned in TA cloning vector, pTZ57R/T (InsTAclone ™ PCR cloning kit) for sequencing (Eurofins Genomics DNA sequencing services). The sequencing was conducted bidirectionally using the same primers used in PCR analysis. The trimming of generated sequences of investigated loci was performed using BioEdit v.7.2.6.1 to get high-quality (HQ) DNA sequences. The HQ DNA sequences were searched for homology using the homology search tool BLASTn and were then deposited in GenBank to get accession numbers.

***Molecular species delimitation*:** The reference sequences were extracted from the NCBI database (National Center for Biotechnology) against each locus to supplement each locus, generating sequences for generating alignments using the MAFFT program (multiple sequence alignment program). The alignment of all investigated loci was viewed and concatenated through Geneious software version v. 4.8.5. Finally, the phylogenetic analysis was performed on concatenated datasets using PUAP* for phylogram construction.

***Pathogenicity test*:** Morphogenomiclly identified fungal isolates associated with infected tissues of Stem and Crown rot were used to confirm pathogenicity test following Koch's postulates on the cultivar Early (Agaiti), Late (Pachati), Anmol, Lyallpur late, Super late Faisalabad cultivars of E. clover.

***Fungal Inoculum preparation*:** Large-scale sclerotia medium (54g cornmeal+3.5g vermiculate+37.5mL solution of 1% casamino acids+0.97g yeast extract+38mL distilled water pH of 5.9) was prepared in canning jars. After three months of incubation, sclerotia were harvested by shaking the jar to break up the sclerotia mass; distilled water (300-400mL) was added to the jar, shaken, and removed the water. (Nelson *et al*., 1988).

***Crop establishment*:** The healthy pure seed of cultivars mentioned above was collected from Ayyub agricultural research institute (AARI) Faisalabad, Pakistan. The seed was sown by adopting the recommended agronomic practices in 12 inches' earthen pots, trays (3×5 feet) at FMB net house, research area Department of plant pathology, and UAF.

***Inoculation of fungi to E. clover plants*:** Sclerotia of *Sclerotinia* spp. were mixed in the pot soil and trays before establishing the crop. Soil without sclerotia served as control. Stem inoculation was done by putting an agar plug, taken from an actively growing culture, on ten one-month-old healthy Egyptian clover plants. In comparison, control plants were exposed to a plain agar plug.

***Disease assessment*:** After inoculation, symptom development was observed daily visually. Re-isolation of *sclerotinia* spp. from disease samples was done.

***Response of E. clover cultivars against Stem and crown rot in Pakistan*:** While surveying the E. clover cultivated area of

Table 2. List of primers with their genetic regions, primer name, annealing temperature, amplicon and refrences followed for molecular analysis of fungal samples of E. clover diseased tissues.

Genetic regions	Primers names	Annealing temperature	Amplicon size (bp)	References
Internal Transcribed Spacer (*ITS*)	ITS1-F/ITS4	55°C	~684	White *et al*., 1990
The Large Subunit of Ribosomal RNA gene (*LSU*)	LROR/LR7	54°C	~1300	Rehner and Samuels, 1994
Calmodulin (*cmdA*)	CALDF1/CALDR2	61°C	~500	Andrew and Kohn, 2009
Glyceraldehyde 3-Phosphate Dehydrogenase (*gapdh*)	G3PDHF/ G3PDHR	64°C	~ 870	Staats *et al*., 2005
Heat Shock Protein (HSP60)	HSP60F/HSP60R	55°C	~1028	Staats *et al*., 2005

Punjab and Khyber Pakhtunkhwa Provinces of Pakistan, disease severity index was recorded using the reference key described by Dixon (1975).

RESULTS

***Disease Severity Assessment*:** During the 2017 and 2018 surveys of Punjab and Khyber Pakhtunkhwa provinces (KPK), sixteen districts cultivating E. clover were surveyed to record Stem and Crown rot severity. During 2017 among three (Northern, Central, and Southern) regions of Punjab province, the highest disease severity index of Stem and Crown rot was in Sargodha, Northern Punjab (83.3%), Okara, Central Punjab (74.3%), and Muzaffargarh, Southern Punjab (55.3%) and 2018, the highest disease severity index was in Sargodha, Northern Punjab (86%), Okara, Central Punjab (75%), and Muzaffargarh, Southern Punjab (59.3%). The two-year survey data revealed that the average disease severity index from Punjab province was 65.4% and 67.7%. The data recorded from KPK showed the highest disease severity index in the Darban district, 66.3% (2017) and 67.7% (2018), with the average disease severity index 60.9% and 64.8% (2017 & 2018) (Figure 1).

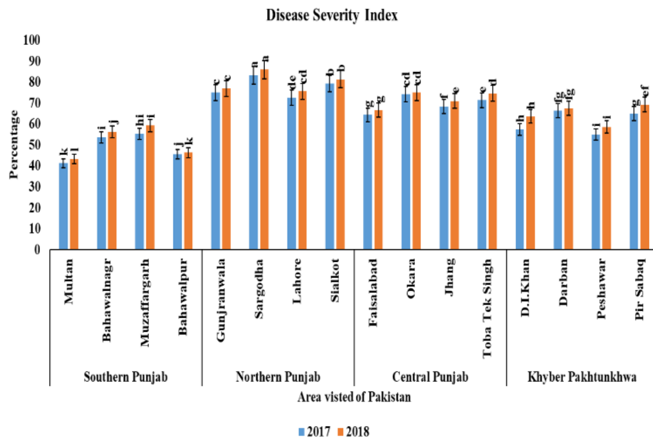

Figure 1. Disease severity indexing of Stem and Crown rot on E. Clover recorded at twelve locations of three regions (southern Punjab, Northern Punjab and central Punjab) of Punjab province and four locations of Khyber Pakhtunkhwa province, Pakistan

***Taxon Morphology*:** White mycelia of the fungus appeared on diseased plants (Figure 2), which later turned to sclerotia (hard black) 2-7×2-10 mm (average 4.3×5.8 mm) as temperature raises to 25ºC, present inside or attached to the outer surface of the stem. After five to seven days of incubation, white mycelial growth with no conidial formation, hyaline, and septate hyphae appeared, which developed into hard black sclerotia of various irregular patterns (4×10 mm) on PDA medium. Ascospores were hyaline, biguttulate, uniseriate, ellipsoid, large (13.2-17.8 x 6.8-8.9 µm) or small (10.6-13.4 ×5.8-6.8 µm). In addition, one to several greyish septa and darker stipe apothecia were raised from sclerotia Figure 3 (Njambere *et al*., 2008). All the isolates did not produce oxalic acid on a semi-selective medium.

Figure 2. Whitish mycelial mat of the *S. trifoliorum* covering Stem and Crown rot disease afferected E. clover plants under field conditions

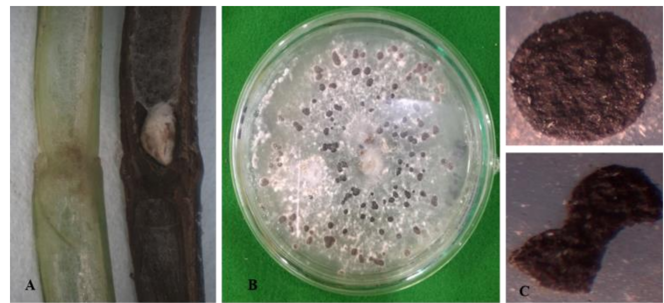

Figure 3. Pictorial comparison of asymptomatic and symptomatic stems of E. clover (A) white mycelial growth with no conidial formation, hyaline septate hyphae which developed into hard black sclerotia of various irregular patterns on PDA medium (B) hard black sclerotia of various irregular patterns (4×10 mm) (C)

***Molecular Species delimitation*:** The most parsimonious phylogenetic tree was constructed using PAUP* v. 4.0a161 software. The generated sequences of documented taxonomically informative loci were supplemented with available sequences for the alignment analysis (Ul Haq et al., 2021). The Multiple Alignment using Fast Fourier Transform (MAFFT) web-based tool aligned each locus's dataset. The aligned datasets were concatenated, viewed in Geneious software, and saved in nexus format. The saved file was run as an input file in PAUP software. The most parsimonious tree was constructed using the bootstrap method in full heuristic search with 1000 bootstrap replicates with random stepwise addition. Tree-bisection-reconstruction (TBR) was in branch

Table 3. List of Sclerotinia species and FMB isolates their respective host, culture accessions, Genbank accession numbers and primers used for the construction of the phylogenetic tree.

Species/ isolate	Host	Culture accession number	GenBank Accession Numbers				
			ITS	GAPDH	CAL	HSP60	LSU
FMB-SFB-TA	*T. alexandrinum*	FMB 0090	MT995056	MT937071	MT954957	MT978151	MT995060
Sclerotinia trifoliorum	Turfgrass	CBS 171.24	MF964318	KF878375	MF964267	KF871408	MH866296
S. *sclerotiorum*	Forest	CBS 499.50	MH856725	KF878370	KF871381	KF871403	MH868246
S. *pseudotuberosa*	Forest	CBS 331.35	MH855694	KF878368	KF871379	KF871401	MH867209
S. *bulborum*	Forest	CBS 297.31	MH855218	KF878363	KF871374	KF871396	MH866668
S. *pseudotuberosa*	*Quercus robur*	CBS 655.78	AY526234	KF878369	KF871380	KF871402	-
S. *pseudotuberosa*	*Quercus* sp.	CBS 312.37	KF859931	KF878366	KF871377	KF871399	-
S. *pseudotuberosa*	*Quercus robur*	CBS 327.75	AY526231	KF878367	KF871378	KF871400	-
S. *minor*	*Lactuca sativa*	CBS 339.39	KF859929	KF878364	KF871375	KF871397	-

T. alexandrinum = Trifolium alexandrinum

swapping algorithm mode with reconnection limit, 8, and branches were collapsed when maximum branch length was zero. Gaps were treated as a new state (5th base), with the 'MulTrees' option in effect. All characters were of unord type and had equal weight. For parsimony analysis, different metrics, including tree length (TL), homoplasmy index, consistency index (CI), rescaled consistency index (RC), and retention index (RI), were calculated. The majority rule (50%) consensus tree was based on 465 parsimony-informative characters (PIC), with tree length 958. The calculated matrices of this analysis were 0.7725 rescaled consistency index (RC) and 0.8895 retention index (RI); however, the total consistency index (CI) was 0.8685; whereas the CI value after excluding uninformative characters was 0.8122.

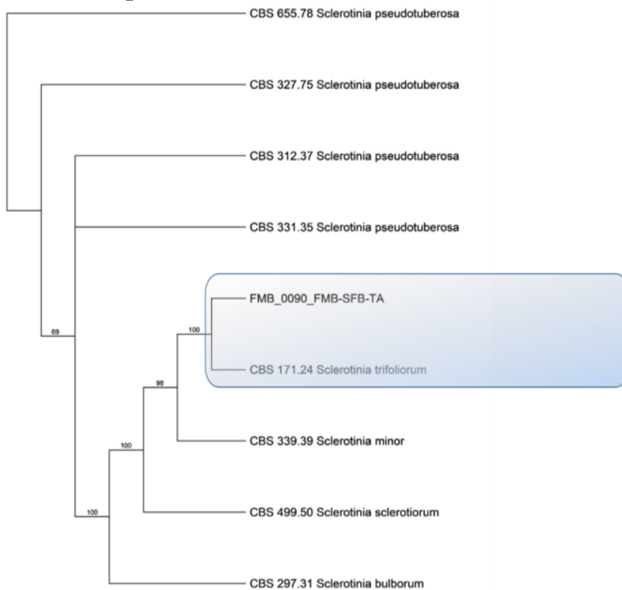

Figure 4. The Most parsimonious phylogenetic tree of *S. trifoliorum* using the combined dataset (ITS, LSU, CAL, GAPDH, and HSP60) with PAUP* v. 4.0a161 software

Similarly, the total homoplasmy index (HI) was 0.1315; however, after excluding uninformative characters, the HI value was 0.1878. The maximum parsimonious tree generated using a concatenated dataset showed the place of our fungal isolates Table 3, FMB-SFB-TA (FMB 0090), close to the strain of Sclerotinia trifoliorum, CBS 171.24 with 100% bootstrap support (Figure 4). Hence, the polyphasic approach for identifying and classifying fungal isolate, FMB-SFB-TA (FMB 0090), sampled from Stem and Crown rot affected E. clover, showed *Sclerotinia trifoliorum*.

***Kochs Postulates Testing Using Selected Fungal Pathogens*:** Symptoms, appearance and disease development were assessed by visual observation every week. Initially, water-soaked lesions appeared on stem bleaching and wilting led to crown rot. Next, whitish mycelia appeared on the Stem and Crown area of diseased plants. Later on, mycelia turned into black hard sclerotia in adverse conditions inside or outside the Stem of diseased plants and ultimately the plant's death. Finally, the disease appeared in patches of severity (Figure 5). Re-isolation of fungi was done from Stem and Crown rot infected plants. Only *S. trifoliorum* was isolated from artificially inculcated diseased plants. The pathogenicity test results confirmed that *S. trifoliorum* produced symptoms on the tested cultivars. Furthermore, the pathogen produced the symptoms with varying intensities in given conditions on all the tested cultivars, proving them susceptible to the disease.

***The response of E. Clover cultivars against Stem and Crown rot in Pakistan*:** While surveying, only two cultivars, Early (Agaiti) and Late (Pachati) of E. Clover were extensively grown by growers in Punjab and KPK. The surveyed data revealed that the average disease severity index from Punjab province was 44.6% and 20.8% on Early (Agaiti) and Late (Pachati) during 2017. The highest disease severity index (59.16%) of Stem and Crown rot was recorded from Sargodha (Northern Punjab region), (56.67%) from Toba Tek Singh (Central Punjab region), and (36.17%) from Muzaffargarh (Southern Punjab region) on Early (Agaiti) cultivar of E.

clover. While the highest disease severity index (25.34%) of Stem and Crown rot was recorded from Lahore (Northern Punjab), (20.67%), from Faisalabad (Central Punjab), and (19.34%) from Bahawalnagar (Southern Punjab) on Late (Pachati) cultivar of E. clover.

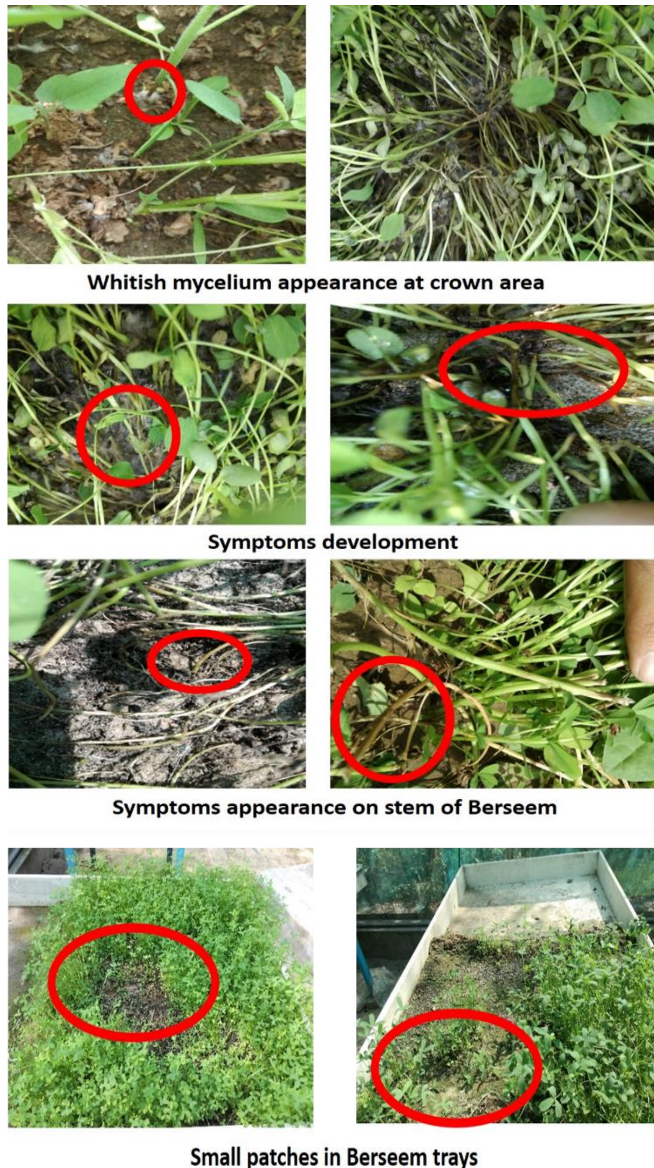

Figure 5. **Pathogenicity *confirmation* following the Koch's postulates, sign and symptoms intiation, disease development and complete rotting of E. clover**

The highest disease severity index of Stem and Crown rot in Khyber Pakhtunkhwa was recorded in Pir Sabaq district (44.67%) and Darban district (23.17%) on Early (Agaiti) and Late (Pachati), respectively. The average disease severity index from Khyber Pakhtunkhwa province was 41.7% and 19.21% on Early (Agaiti) and Late (Pachati) (Figure 6). The surveyed data revealed that the average disease severity index from Punjab province was 46.48% and 21.31% on Early (Agaiti) and Late (Pachati) during 2018. Similarly, in the year 2018, the highest (61.33%) disease severity index of Stem and Crown rot was recorded from Sargodha, Northern Punjab (58.33%) from Okara, Central Punjab, and (39.67%) from Muzaffargarh, Southern Punjab on Early (Agaiti) cultivar of E. clover. While the highest (27.67%) disease severity index of Stem and Crown rot was recorded from Gujranwala, Northern Punjab (19.5%) from Faisalabad, Central Punjab, and (20.67%) from Bahawalnagar, Southern Punjab on Late (Pachati) cultivar of E. clover. The highest disease severity index of Stem and Crown rot in KPK was recorded from Pir Sabaq district (46.16%) and Darban district (25.33%) on Early (Agaiti) and Late (Pachati), respectively. The average disease severity index from KPK province was 42.8% and 22% on Early (Agaiti) and Late (Pachati) (Figure 7).

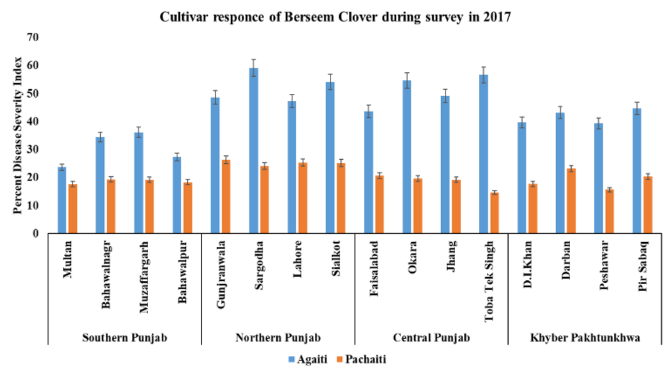

Figure 6. **Response of Agaiti and Pachaiti cultivar of E. Clover against Stem and Crown rot disease at twelve locations of three regions (southern Punjab, Northern Punjab and *central* Punjab) of Punjab province and four locations of Khyber Pakhtunkhwa province, Pakistan during the survey in 2017**

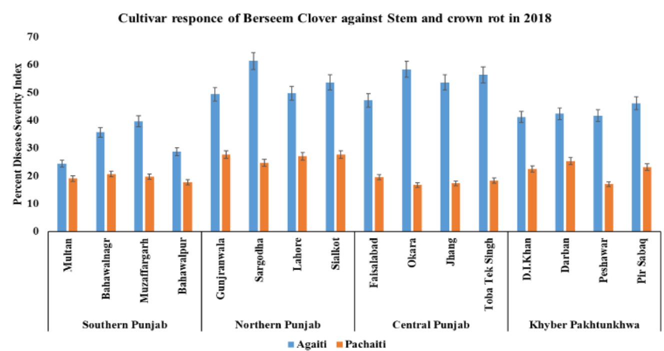

Figure 7. **Response of Agaiti and Pachaiti cultivar of E. Clover against Stem and Crown rot disease at twelve locations of *three* regions (southern Punjab, Northern Punjab and central Punjab) of Punjab province and four locations of Khyber Pakhtunkhwa province, Pakistan**

DISCUSSION

While surveying various selected locations of two provinces of Pakistan (Punjab and KPK), the data recorded had made it possible to index the disease severity of Stem and Crown rot of E. Clover and provide reliable information on disease prevalence in the country. As a result, Saira *et al*. (2016) claimed in their first-ever report that 17 % of crop losses in E. clover are due to Stem and Crown rot disease caused by *S. sclerotiorum* in Pakistan.

The etiology determination of Stem and Crown rot of Besrseem clover was the first significant challenge in this research study. However, two species of Sclerotinia were reported causing this disease previously. Therefore, various fungal pathogens were isolated from the infected plant parts. Literature consulted depicts *S. trifoliorum* and *S. sclerotiorum* as stem rot disease-causing agents in E. clover from India (Pande *et al*., 2008). However, Saira *et al*. (2016) contradicted the previous findings and claimed only *S. sclerotiorum* as a causal agent for E. clover stem rot from Pakistan.

Ekins *et al*. (2005) described that growth rate, size and shape of sclerotia, and ascospore dimorphism are the most common and reliable morphological characteristics used for Sclerotinia species identification and differentiation. In this research study, fungal isolates associated with Stem and Crown rot disease affected plant, and un-germinated and untreated seed batches of E. Clover were characterized using morpho-cultural and molecular parameters; Koch's postulates were completed with each fungus to establish the disease etiology. Initially, the fungal isolates' cultural characteristics examinations from diseased plant tissues revealed these isolates as Sclerotinia sp. Findings of Vleugels *et al*. (2013) validate our results. As far as the morpho-cultural characters are concerned, the growth rate of Sclerotinia species varied with a temperature change. *S. trifoliorum* mycelial growth rate is slower as the temperature rises to 25°C, but the fastest growth is found at 5°C when compared with *S. sclerotiorum*, which generally grows faster at high temperature (Tariq *et al*., 1985). This study proved that Sclerotinia isolates produced small and large ascospores showing the dimorphism ascospores characteristics. Historically, Bretag and Mebalds (1987) described the dimorphism in ascospores as a characteristic difference between *S. trifoliorum* and *S. sclerotiorum*. Furthermore, it is also an established fact that *S. sclerotiorum* produces oxalic acid on culture media and plants (Godoy *et al*.,1990), and *S. trifoliorum* does not produce oxalic acid on semi selective medium (Njambere *et al*., 2008). Li *et al.* (2008) attempted to correlate oxalic acid production and *S. trifoliorum* on red clover. They found this Sclerotinia sp. produced no oxalic acid. We also did not observe the oxalic acid production on semi selective medium, while the airborne ascospores of Sclerotinia species produced a change in color on semi selective medium. Morphological characters like ascospores production and the shape of apothecia differentiate the *S. trifoliorum* from *S. sclerotium* (Uhm and Fujii, 1983). The literature on previous research supported the morpho-cultural characterization (as explained in the Results section) done in this research study and proved the fungal isolates (confirmed as a pathogen of Stem and Crown rot), *S. trifoliorum.*

Many researchers have adopted molecular markers to identify and differentiate Sclerotinia species like random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) (Ekins *et al*., 2005), and sequence and phylogenetic analysis of ITS region along with morphological parameters (Mahadevakumar *et al*., 2016). Gargouri *et al*. (2017) claimed that ITS-based sequence analysis showed *S. trifoliorum* had similarities with *S. sclerotiorum*; that is why only ITS marker is not enough to study the genetic diversity Sclerotinia species. Furthermore, no reference isolates of species-specific primers are available on Sclerotinia species to explore the genetic diversity (Baturo-Ciesniewska *et al*., 2017). Manjunatha *et al*. (2019) reported based on morphological and molecular characterization (using only ITS), *S. sclerotiorum* and *S. trifoliorum* cause of Stem and Crown rot on E. clover in India. Similar studies have also been conducted by Saira *et al*. (2016), who identified *S. sclerotiorum* exploring ITS and β- tubulin gene regions and claimed this fungus cause Stem and Crown rot on E. Clover in Pakistan. It is reported that *S. trifoliorum* has "I" introns at DNA sequences on ITS1 and ITS5 primers. Only two base pairs are different in ITS regions of *S. sclerotiorum* and *trifoliorum*. The presence of "I" introns and dimorphism in the ascospores of *S. trifoliorum* differentiated it from *S. sclerotiorum* (Njambere *et al*., 2008). Critically reviewing and analyzing the previous research findings regarding reliable pathogen identification, we went for validation of our morpho-cultural-based identification of S. *trifoliorum* through molecular characterization. This study did not rely only on the ITS; instead, we used five genetic loci (ITS, LSU, HSP60, CAL, and G3PDH), confirming the *S. trifoliorum*. The pathogenicity test was performed with the representative culture of *S. trifoliorum*, following Koch's postulates, and confirmed this fungus as the Stem and Crown rot pathogen, thus proving the etiology of the disease. Recently Baturo-Ciesniewska *et al*. (2017) also reported the *S. trifoliorum* pathogen on Kura clover.

***Conclusion*:** This study revealed that *S. trifoliorum* is the actual cause of emerging disease Stem and Crown rot of Egyptian clover. The disease is prevailing in all the E. clover cultivation regions of Pakistan. Furthermore, results also showed that no cultivar is resistant to the disease.

***Conflict of interest*:** All authors declare that they have no conflict of interests.

***Authors' Contribution Statements*:** Imarn ul haq and Siddra ijaz planned the research, designed the experiment and analyzed the data, Anjum faraz conducted the experiments, recorded the data and prepared the draft. Shahbaz talib sahi and imran khan helped in the editing of the manuscript.

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