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Molecular characterization of curvularia leaf blight disease of olive and determination of pathogenicity by a novel disease rating scale

[a] Abida, [a] Muhammad Fahim Abbas *, [a] Tamoor Khan, [a] Sana Batool, [b] Kokab Jabeen, [a] Naimatullah Koondhar, [a] Sana Ullah, [a] Muhammad Arshad, [a] Ali Nawaz, [a] Ahmed Khan

[a] Department of Plant Pathology, Faculty of Agriculture, Lasbela University of Agriculture Water and Marine Sciences, Uthal, Balochistan, Pakistan, [b] Islamabad Center for Research and Innovation, Islamabad, Pakistan.

Authors' Contribution: **Abbas, M.F.** designed research experiments, experitments were executed by **Abida, T. Khan, S. Batool, K. Jabeen, N. Koondhar, S. Ullah & M. Arshad. A. Nawaz & M. Ahmed** collected and analysed the data.

*Corresponding Author's Email Address: fahim.abbas@luawms.edu.pk

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ABSTRACT

During the last decade, olive (*Olea europaea*) production has significantly increased in Pakistan due to oil producing ability and medicine importance. Arbequina, Arbosana, BARI Zaitoon-1 and BARI Zaitoon-2 has recently introduced in Sukhan, Uthal Balochistan. During 2023-24, symptoms of Curvularia leaf blight disease (CLBD) first appeared, with a 97% disease incidence. Subsequently, the infection progressed, eventually covering the entire leaf and olive plant. For the first time, a novel disease rating scale for CLBD of olive was accomplished. White fungal colonies (n=89) were recorded which turned into brownish black. Virulence category of isolates were characterized as moderately (43), slightly (37) and highly (9) virulent with 37.5% disease severity. Under microscope, straight to slightly curved conidia with 3 to 4 septation were recorded in highly virulent isolates and range (n=3) of conidial and conidiophores length and width was observed as 23±2.1 µm to 30±2.4 µm and 10.4±0.8 µm to 12.3 µm ±1.2 µm and 99.6±20.4 µm to 130.6±25.5 µm and 4.2±0.5 µm to 4.8±0.7 µm, respectively. The internal transcribed spacer (ITS) regions were amplified through polymerase chain reaction (PCR). Sequences from highly virulent isolates (n=9) were deposited to NCBI with GenBank Accession numbers PP565050 to PP565058. The nucleotide sequences of local isolates were compared with sequences from all available isolates of different *Curvularia* species (n=12) known to cause blight disease across several host (n=26) species in Pakistan. The obtained ITS sequences as a causal agent of CLBD of olive were exhibiting 98 to 100% and 99 to 100% genetic similarity with previously reported isolates of *C. lunata* (n=5) and *C. geniculate* (n= 4). According to our knowledge, it is first comprehensive study of CLBD of olive caused by *C. lunata* and *C. geniculate* in Balochistan as well as in Pakistan. This emerging disease has serious threat to olive production in the province as well as in the country and precaution measures are required to manage this distractive disease.

Keywords: Cultural identification, first report, *Curvularia lunata*, *Curvularia geniculate*, genetic similarity.

INTRODUCTION: In Pakistan, an annual expenditure of 38 billion rupees is allocated to meet the demand for edible oil. During last five years, olive *(Olea europaea* L.) is recognized as a significant fruit crop due to its dual production of fruit and oil, making it a pivotal contributor to the country's economy (Galán *et al.*, 2005). The optimal production of olives has been recorded in regions lying between 30 degrees to 50 degrees north and south latitudes, characterized by long summers, cold winters, and low humidity. Pakistan possesses highly suitable climatic conditions and ecological zones conducive to the commercial cultivation of olives. Over the past five years, olive cultivation has seen significant growth across various regions in Pakistan with 45 million olive trees in the Western hills of Baluchistan, Punjab, Sindh, Azad Kashmir, Khyber Pakhtunkhwa (KPK), Swat, Chitral, and Dir (Khaliq *et al.*, 2020). The existence of such huge quantity of trees indicates that the agro-climatic conditions of these areas are conducive for cultivation of olive in the country. The increase in olive cultivation will play a vital role in the agricultural landscape and economy of these regions.

In Pakistan, morpho-anatomical characterization of Azerbaijan (Azerbaiijan), BARI Zaitoon-I, BARI-2, Earlik I, Earlik-II, Erlik (Israel), Frantoio, FS-17 (Italy), Gemlik (Turkey), Hamdi (Tunisia), HP Olive, Manzanilla (Spain), Mariana, Nabali (Palestine), Naqvi, Nocellara, QR Olive, Sorani and Souri (Lebanon) were recorded (Ahmad *et al.*, 2023) and genetic characterization of Sohawa-Selection, Bulkasar-Selection, Chugtai-Selection, Bari-Zatoon, Arbequina, Arbosana, Manzanilla, Coratina, Frontaio, Pendolino, Ottobratica, Gemlik and Koroneiki) with 63 SSR markers were confirmed (Iqbal *et al.*, 2021). Olea-Berberis-Punica, Olea-Olea-Dodonaea, OleaOlea-Zanthoxylum and Olea-Ficus-Ricinus were recorded by cluster analysis and Detrendent correspondence analysis (DCA) technique from Azad Jammu and Kashmir. From Balochistan, the morphological characterization of Koroneiki, Biancolilla, Carola, Kaisy and Sorani were studied from Lorelai (Palwasha *et al.*, 2022). In Pakistan, the production of olive is low as compared to other olive producing country of the world due to several biotic and abiotic factors. All olive growing areas of the country have ideal temperature and humidity for the growth and development of phytopathogenic fungi that are affecting olive production. The information about phytopathogens of olive is limited in the country and *Colletotrichum acutatum* (Nawaz *et al.*, 2022) and *Alternaria alternata* (Alam and Munis, 2019) were only confirmed as the causal organism of olive. The information about other pathogens of olive is still unknown. In Pakistan, morphological characterization and molecular identification were used to confirm *Fusarium* rot (Mehmood *et al.*, 2017), root rot (Qamar *et al.*, 2019) and crown rot (Qamar *et al.*, 2019) on strawberry and mucor rot (Abbas *et al.*, 2020), *Fusarium* fruit (Abbas *et al.*, 2017), anthracnose (Naz *et al.*, 2017), *Diplodia* rot (Abbas and Naz, 2018) and *Neopestalotiopsis* fruit rot (Abbas *et al.*, 2022) on loquat in Pakistan. The *Curvularia* genus has more than 40 species with wide host range and caused significant yield loess in fruits, crops and vegetables (AbdElfatah *et al.*, 2021). The cultural characterizations (colony morphology) and morphological identification (curved or lunate shaped conidia, septae number and colony morphology) were used for the confirmation of this pathogen (Santos *et al.*, 2018). These characteristics overlap within several species which leads false identification. New molecular approaches such as molecular identification through polymerase chain reaction (PCR) and nucleotide sequences were further adopted for the reliable identification of phytopathogenic fungi (Nawaz *et al.*, 2022).

OBJECTIVES: The objectives of the current study were to assess the incidence of CLBD of olive at Sukhan and confirmation through cultural characterization and morphological identification. Additionally, the study aimed to develop a comprehensive disease rating scale for CLBD of olive and to conduct sequence analysis to further understand the genetic characteristics of the pathogen.

MATERIAL AND METHODS: **Survey and sample collection:** Four olive verities, Arbequina, Arbosana, BARI Zaitoon-1 and BARI Zaitoon-2 and were introduced on 150 acres at Sukhan (25.5820° N, 66.3343° E), Uthal, Balochistan into 6 different plots. In a single plot, 3364 olive plants were sown with 18-foot row to row and plant to plant distance. During the recent year, a comprehensive survey was conducted from individual olive variety and 100 olive plants were selected randomly from three to five-years-old which were catagrized with noveal rating scale (table 1). The information about variety, infected, healthy and missing olive plants were recorded (table 2) and infected samples were shifted into sterile plastic bags.

Disease grade	Description	Infection (%)
0	No symptoms on leave	0
1	One CLBD spot	1-10
3	Few CLBD symptoms on few leave	11-26
5	Few CLBD symptoms on many leave	27-50
7	Many large CLBD symptoms	51-75
9	Mostly large CLBD symptoms	76-100

Table 1: Novel rating scale for CLB disease categories infecting olive.

Incidence and novel severity scale of CLBD: The infected leaves from CLBD were classified on the basis of symptoms (figure 1) and incidence along with severity (table 2) was calculated.

$$\text{Disease } incidence\ (\%) = \frac{number\ of\ infected\ olive\ plants}{Total\ olive\ plants} X\ 100$$

$$\text{Disease } severity\ \ (\%) = \frac{sum\ of\ all\ ratings}{Total\ ratings\ X\ maximum\ disease\ grade} X\ 100$$

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Sample	Condition	Variety	Sample No	Condition	Variety
OL1	Missing	Arbequina	OL51	Disease	Arbosana
OL2	Missing	Arbequina	OL52	Disease	Arbosana
OL3	Disease	Arbequina	OL53	Disease	Arbosana
OL4	Disease	Arbequina	OL54	Disease	BARI Zaitoon-1
OL5	Disease	Arbequina	OL55	Disease	BARI Zaitoon-1
OL6	Disease	Arbequina	OL56	Healthy	BARI Zaitoon-1
OL7	Disease	Arbequina	OL57	Healthy	BARI Zaitoon-1
OL8	Disease	Arbequina	OL58	Disease	BARI Zaitoon-1
OL9	Disease	Arbequina	OL59	Disease	BARI Zaitoon-1
OL10	Disease	Arbequina	OL60	Disease	BARI Zaitoon-1
OL11	Disease	Arbequina	OL61	Disease	BARI Zaitoon-1
OL12	Missing	Arbequina	OL62	Disease	BARI Zaitoon-1
OL13	Missing	Arbequina	OL63	Disease	BARI Zaitoon-1
OL14	Missing	Arbequina	OL64	Disease	BARI Zaitoon-1
OL15	Disease	Arbequina	OL65	Disease	BARI Zaitoon-1
OL16	Disease	Arbequina	OL66	Disease	BARI Zaitoon-1
OL17	Disease	Arbequina	OL67	Disease	BARI Zaitoon-1
OL18	Disease	Arbequina	OL68	Disease	BARI Zaitoon-1
OL19	Missing	Arbequina	OL69	Disease	BARI Zaitoon-1
OL20	Disease	Arbequina	OL70	Disease	BARI Zaitoon-1
OL21	Missing	Arbequina	OL71	Disease	BARI Zaitoon-1
OL22	Disease	Arbequina	OL72	Disease	BARI Zaitoon-1
OL23	Disease	Arbequina	OL73	Disease	BARI Zaitoon-1
OL24	Disease	Arbequina	OL74	Disease	BARI Zaitoon-1
OL25	Disease	Arbequina	OL75	Disease	BARI Zaitoon-1
OL26	Disease	Arbequina	OL76	Disease	BARI Zaitoon-1
OL27	Disease	Arbequina	OL77	Disease	BARI Zaitoon-2
OL28	Disease	Arbequina	OL78	Disease	BARI Zaitoon-2
OL29	Disease	Arbequina	OL79	Disease	BARI Zaitoon-2
OL30	Disease	Arbequina	OL80	Disease	BARI Zaitoon-2
OL31	Disease	Arbequina	OL81	Disease	BARI Zaitoon-2
OL32	Disease	Arbequina	OL82	Disease	BARI Zaitoon-2
OL33	Disease	Arbequina	OL83	Disease	BARI Zaitoon-2
OL34	Disease	Arbosana	OL84	Disease	BARI Zaitoon-2
OL35	Disease	Arbosana	OL85	Disease	BARI Zaitoon-2
OL36	Disease	Arbosana	OL86	Disease	BARI Zaitoon-2
OL37	Disease	Arbosana	OL87	Disease	BARI Zaitoon-2
OL38	Missing	Arbosana	OL88	Disease	BARI Zaitoon-2
OL39	Disease	Arbosana	OL89	Disease	BARI Zaitoon-2
OL40	Disease	Arbosana	OL90	Disease	BARI Zaitoon-2
OL41	Disease	Arbosana	OL91	Disease	BARI Zaitoon-2
OL42	Missing	Arbosana	OL92	Disease	BARI Zaitoon-2
OL43	Disease	Arbosana	OL93	Disease	BARI Zaitoon-2
OL44	Disease	Arbosana	OL94	Disease	BARI Zaitoon-2
OL45	Disease	Arbosana	OL95	Disease	BARI Zaitoon-2
OL46	Disease	Arbosana	OL96	Disease	BARI Zaitoon-2
OL47	Disease	Arbosana	OL97	Disease	BARI Zaitoon-2
OL48	Disease	Arbosana	OL98	Disease	BARI Zaitoon-2
OL49	Disease	Arbosana	OL99	Disease	BARI Zaitoon-2
OL50	Disease	Arbosana	OL100	Disease	BARI Zaitoon-2

Table 2: Sample collection from four olive varieties growing at Sukhan, Uthal, Balochistan.

incidence along with severity (table 2) was calculated.

Cultural characterization and morphological identification: The tissue (5cm) from individual infected leaf sample was sterilization with sodium hypocloride (1%) for 3 min. and dipped thrice into sterile distilled water (SDW). The tissues were dried on the double layer of sterile filter papers and shifted to petri plates comprising of sterile Czapek Dox Agar (CZDA) (Abbas and Naz, 2018). The isolation procedure was performed in sterile conditions and petri plates were incubated at 25 ± 2°C with 70% relative humidity. For the purpose of the pure culture, active mycelial plug (3 mm) was shifted to new petri plates comprising of sterile CZDA media. After 5 days, color and texture of the fungal colony was observed. The mycelium and spores were observed under the microscope and spore size, shape, and color was recorded.

Pathogenicity test: In the pathogenicity test, asymptomatic, healthy olive leaves of all growing varices were plucked from Sukhan orchard. The leaves were surface sterilized and a spore suspension (1 × 106 spore/mL) was applied to each leaf (Abbas *et al.*, 2016). The negative control with SDW was included in the pathogenicity test. The leaves were observed after 7 days and isolates were categorized as highly, moderately and slightly virulent. The pathogen was re-isolated and cultural characterization and morphological identification was compared with infected culture.

Molecular identification: The genomic DNA from highly virulent isolates (n=9) of CLBD was extracted through phenol chloroform, isoamyl alcohol extraction methods and confirmed on 2% (w/v) agarose gel (White, 1990). The ITS regions were amplified through PCR with ITS-1 (5′ TCC GTA GGT GAA CCT GCG G 3′) and ITS-4 (5′ TCC TCC GCT TTA TTG ATA TG 3′) primers. The PCR reactions mixture (PRM) was consisting of the PCR reaction buffer, $MgCl_2$, the mix dNTPs, ITS-1/ITS-4 primers, *Taq DNA polymerase* and individual DNA from each highly virulent isolate.

Sample No	Isolate Name	Virulent Category	Sample No	Isolate Name	Pathogenicity test
OL-3	CUR-1	Moderately	OL-54	CUR-45	Slightly
OL-4	CUR-2	Moderately	OL-55	CUR-46	Moderately
OL-5	CUR-3	Moderately	OL-58	CUR-47	Moderately
OL-6	CUR-4	Slightly	**OL-59**	**CUR-48**	**Highly**
OL-7	CUR-5	Slightly	OL-60	CUR-49	Slightly
OL-8	CUR-6	Moderately	OL-61	CUR-50	Slightly
OL-9	CUR-7	Moderately	OL-62	CUR-51	Moderately
OL-10	CUR-8	Slightly	OL-63	CUR-52	Moderately
OL-11	CUR-9	Moderately	OL-64	CUR-53	Slightly
OL-15	CUR-10	Moderately	OL-65	CUR-54	Moderately
OL-16	CUR-11	Moderately	OL-66	CUR-55	Slightly
OL-17	**CUR-12**	**Highly**	OL-67	CUR-56	Moderately
OL-18	CUR-13	Slightly	OL-68	CUR-57	Moderately
OL-20	CUR-14	Moderately	OL-69	CUR-58	Slightly
OL-22	CUR-15	Slightly	OL-70	CUR-59	Slightly
OL-23	CUR-16	Moderately	OL-71	CUR-60	Slightly
OL-24	CUR-17	Slightly	**OL-72**	**CUR-61**	**Highly**
OL-25	CUR-18	Moderately	OL-73	CUR-62	Slightly
OL-26	CUR-19	Slightly	OL-74	CUR-63	Moderately
OL-27	CUR-20	Slightly	OL-75	CUR-64	Moderately
OL-28	CUR-21	Moderately	OL-76	CUR-65	Slightly
OL-29	**CUR-22**	**Highly**	OL-77	CUR-66	Moderately
OL-30	CUR-23	Slightly	OL-78	CUR-67	Slightly
OL-31	CUR-24	Moderately	OL-79	CUR-68	Slightly
OL-32	CUR-25	Slightly	OL-80	CUR-69	Moderately
OL-33	CUR-26	Slightly	**OL-81**	**CUR-70**	**Highly**
OL-34	CUR-27	Moderately	OL-82	CUR-71	Moderately
OL-35	CUR-28	Slightly	OL-83	CUR-72	Slightly
OL-36	CUR-29	Slightly	OL-84	CUR-73	Moderately
OL-37	CUR-30	Slightly	OL-85	CUR-74	Slightly
OL-39	**CUR-31**	**Highly**	OL-86	CUR-75	Moderately
OL-40	CUR-32	Slightly	OL-87	CUR-76	Moderately
OL-41	CUR-33	Slightly	OL-88	CUR-77	Moderately
OL-43	CUR-34	Moderately	OL-90	CUR-78	Moderately
OL-44	CUR-35	Moderately	**OL-89**	**CUR-79**	**Highly**
OL-45	CUR-36	Slightly	OL-91	CUR-80	Moderately
OL-46	CUR-37	Moderately	OL-92	CUR-81	Moderately
OL-47	CUR-38	Moderately	OL-93	CUR-82	Moderately
OL-48	**CUR-39**	**Highly**	OL-94	CUR-83	Slightly
OL-49	CUR-40	Moderately	OL-95	CUR-84	Slightly
OL-50	CUR-41	Moderately	OL-96	CUR-85	Moderately
OL-51	**CUR-42**	**Highly**	OL-97	CUR-86	Slightly
OL-52	CUR-43	Moderately	OL-98	CUR-87	Slightly
OL-53	CUR-44	Slightly	OL-99	CUR-88	Moderately
			OL-100	CUR-89	Slightly

Table 3: Renaming of samples and virulence category of CLB disease of olive.

A negative control (without DNA) was always carried out. The PRM was initial heated (95 ºC) for 5 min. and 35 cycles of denature (95 ºC), annealing (56 ºC) and extension (56 ºC) was performed at 1 min. The final extension (72ºC) was applied for 7 min. The PCR amplified products (PAP) were analyzed in 1% agarose gel and size of the fragment was confirmed with 1 kb DNA ladder.

Sequencing and sequence analysis: The PAP from individual isolate was purified with the standard protocol of Thermo Scientific GeneJET PCR Purification Kit (#K0701) and sequenced in both directions. The obtained sequences were checked with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and final sequences were submitted to National Center for Biotechnological Information (NCBI) (https://www.ncbi.nlm.nih.gov/) to obtain the GenBank accession numbers. The Basic Local Alignment Standard Tool (BLAST) was used to compute genetic homology between sequenced and previously reported isolates. Previously reported ITS sequences of CLBD were downloaded from NCBI and compared with the sequence of local isolates. Molecular Evolutionary for Genetic Analysis (MEGA) version 7 were used to compute percentage genetic homology of local isolates with reference isolates.

RESULTS: Prevalence, incidence and CLBD severity: The CLBD was recorded at olive orchard of Sukhan, Balochistan and all four growing varieties (Arbequina, Arbosana, BARI Zaitoon-1 and BARI Zaitoon-2) were found to be susceptible (table 2). During the early stage of infection, irregular brown spots were appeared which combined at later stage of infection and covered the entire leaf and olive plant (figure 1). The incidence and severity (figure 2a) of CLBD was recorded as 97% and 37.5%, respectively.

Cultural characterization and pathogenicity test: During the initial stage of the fungal growth, white colonies were observed on CZPK dox agar medium and turned into black at later stage of growth (figure 2b) due to presence of spores in the culture. On the bases of cultural confirmation, a total number of 87 isolates were confirmed and renamed from OL1 to OL100 to CUR-1 to CUR-89 (table 2).

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The CLBD was recorded on the artificially inoculated leaf but no growth was observed on negative control (figure 2d) and maximum 43 isolates were recorded as moderately, 37 were slightly and 9 were highly virulent (table 3). Re-isolating the pathogen from a leaf that had been artificially infected, it was compared to the original fungal culture. The similar cultural and morphological characterization were recorded from the artificially inoculated leaf.

Figure 1: Curvularia leaf blight disease symptoms of olive at Sukhan, Balochistan.

Figure 2: Novel disease rating scale (a), cultural characterization (b), morphological identification (c) and pathogenicity test confirmation (d) of CLBD of olive at Sukhan, Balochistan.

Morphological identification: Highly virulent isolates (CUR-12, CUR-22, CUR-31, CUR-39, CUR-42, CUR-48, CUR-61, CUR-70 and CUR-79) were subjected to morphological confirmation (table 4).

Isolate	PCR confirmation	Specie Name	GBAN	GS (%)
CUR-12	Amplified	*Curvularia lunata*	PP565050	98%
CUR-22	Amplified	*C. lunata*	PP565051	99%
CUR-31	Amplified	*C. lunata*	PP565052	98%
CUR-39	Amplified	*C. lunata*	PP565053	99%
CUR-42	Amplified	*C. lunata*	PP565054	100%
CUR-48	Amplified	*C. geniculate*	PP565055	99%
CUR-61	Amplified	*C. geniculate*	PP565056	99%
CUR-70	Amplified	*C. geniculate*	PP565057	100%
CUR-79	Amplified	*C. geniculate*	PP565058	100%
Negative control	No amplification	-	-	-

Table 4: The PCR confirmation of ITS regions and GenBank Accession Number from highly virulent isolates of CLBD disease. GBAN = GenBank Accession Number, GS = Genetic similarity. PC.

Under the microscope, straight to slightly curved conidia with 3 to 4 septation were recorded (figure 2c). Conidial length and breadth ranges were noted as 23±2.1 µm to 30±2.4 µm and 10.4±0.8 µm to 12.3 µm ±1.2 µm, respectively (table 4). The range of conidiophores length and width were recorded as 99.6±20.4 µm to 130.6±25.5 µm and 4.2±0.5 µm to 4.8±0.7 µm, respectively.

Molecular identification: Highly virulent isolates were further confirmed through PCR by observing a 650 bp amplified ITS fragment when compared with 1KB genomic DNA ladder and no amplification was observed in the negative control (table 5).

Sequencing and sequence analysis: The sequences from highly virulent isolates of CLBD were submitted to NCBI and provided GenBank accession numbers (table 5). The available ITS sequences of *C. aeria*, *C. australiensis*, *C. buchloes*, *C. crepinii*, *C. geniculate*, *C. hawaiiensis*, *C. inaequalis*, *C. lunata*, *C. protuberans*, *C. spicifera* and *C. tuberculate* causing blight disease of *Chamaedorea seifrizii*, *Chenopodium quinoa*, *Citrus reticulate*, *Cucurbita pepo*, *Curcuma longa*, *Dalbergia sissoo*, *Dypsis lutescen;s*, *Eriobotrya japonica*, *Ficus religiosa*, *Fragaria ananassa*, *Gerbera jamesonii*, *Jasminum grandiflorum*, *Medicago sativa*, *Musa paradisiaca* L., *Oryza sativa*, *Diospyros kaki*, *Prunus persica* L., *Rosa Rubiginosa*, *Saccharum officinarum*, *Shorea robusta*, *Solanum melongen*, *S. nigrum*, *Trifolium alexandrinum* and *Zea mays* L. were downloaded from NCBI (table 6).

To determine the evolutionary history, the Maximum Likelihood method which is predicated on the Kimura 2-parameter model was employed and displayed in figure 3. The sequences were exhibiting 98 to 100% and 99 to 100% genetic similarity with previously reported isolates of *C. lunata* and *C. geniculate* (table 5).

DISCUSSION: The cultivation of olive trees is crucial for Balochistan, providing a stable agricultural foundation for its populace alongside various other benefits. The species from the genus *Curvularia* are distributed worldwide and caused several diseases including necrotic spots, leaves blights and fruits rots and is responsible for significant yield losses (AbdElfatah *et al.*, 2021).

Table 3: Renaming of samples and virulence category of CLB disease of olive.

In the current study, we provided the first report of *C. lunata* and *C. geniculate* pathogens causing leaf blight disease of olive in Balochistan province of Pakistan. The symptom development is an initial step for CLBD diagnosis and irregular leaf spots were recorded at Sukhan, Balochistan (figure 1). The blight symptoms were found to be similar on all observed olive plants and similar symptoms were associated with CLBD of olive orchards growing at El-Kawamel and El-Kawther of the experimental Farm located at Faculty of Agriculture, Sohag University, Sohag, Egypt (Mohamed *et al.*, 2021). The host range of the CLBD is widely studied and it has been identified as the causative agent of tomato fruit and leaf blight in Pakistan (Iftikhar *et al.*, 2016) and Egypt (AbdElfatah *et al.*, 2021), bell pepper (Menaria, 2011) and rice (Kamaluddeen *et al.*, 2013) in India, maize in China (Hou *et al.*, 2013) and Cocoa in Mexico (Cuervo-Parra *et al.*, 2012). The prevalence of CLBD of olive was confirmed from Balochistan Province of Pakistan and prevalence within specific geographical region has significant value as it important step to initiate the precaution measures. The *Curvularia* sp. is associated with fruit rot of tomato in Pakistan (Iftikhar *et al.*, 2016) and Uthal is famous for the production of tomato in Balochistan. The olives were recently introduced in Sukhan and this disease might be transferred from tomato to olive plants.

The species in the genus *Curvularia* were confirmed on cultural characteristics and conidia morphology and number of septae (Santos *et al.*, 2018). To ensure the traditional confirmation of CLBD, cultural characterizations were further adopted. The genus *Curvularia* is from Deuteromycotina and comprised of more than forty species which were confirmed on cultural characteristics (Chung and Tsukiboshi, 2005). Mohamed *et al.* (2021) observed white to light gray fungal colonies of CLBD associated with olive and later these colonies were turned into dark black in color.

Lunate and curved shaped dark brown spores were recorded in the local isolates which confirmed *Curvularia* sp. as the causal organism

	Conidium				Conidiphore		
Isolate	ML (µm) ± SD	MW (µm) ± SD	Septation	Shape	ML (µm) ± SD	MW (µm) ± SD	Septation
CUR-12	23 ± 2.1	10.4 ± 0.8	3-4	SSC	99.6 ± 20.4	4.2 ± 0.5	3-10
CUR-22	24 ± 2.0	10.5 ± 0.9	3-4	SSC	105.2 ± 24.1	4.5 ± 0.6	3-10
CUR-31	24 ± 2.2	10.7 ± 0.7	3-4	SSC	112.3 ± 22.2	4.4 ± 0.5	3-10
CUR-39	23 ± 2.2	10.2 ± 1.1	3-4	SSC	96.6 ± 17.1	4.3 ± 0.4	3-10
CUR-42	30 ± 2.4	12.3 ± 1.2	3-4	SSC	130.6 ± 25.5	4.8 ± 0.7	4-12
CUR-48	25 ± 2.2	11.3 ± 1.0	3-4	SSC	102.4 ± 22.4	4.7 ± 0.6	3-10
CUR-61	28 ± 2.1	10.9 ± 1.1	3-4	SSC	107.6 ± 22.3	4.5 ± 0.4	3-10
CUR-70	29 ± 2.2	10.6 ± 1.2	3-4	SSC	110.5 ± 23.2	4.3 ± 0.3	3-10
CUR-79	27 ± 2.3	10.4 ± 0.7	3-4	SSC	114.2 ± 22.1	4.3 ± 0.3	3-10

Table 5: Morphological identification of CLB disease infecting olive at Sukhan, Balochistan.
ML = mean length, MW= mean width, SD = standard deviation, SSC = Straight to slightly curved.

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(Ariyawansa *et al.*, 2015). The morphological confirmation was more reliable as compared to cultural identification or symptomological confirmation because it provided accurate diagnosis (Iftikhar *et al.*, 2016).

Isolate Name	Specie Name	Host	GBAN
FCBP1515	*Curvularia aeria*	*Ficus religiosa*	KT283679
FCBP1503	*C. aeria*	*Jasminum grandiflorum*	KT265688
PAK23	*C. australiensis*	*Eriobotrya japonica*	KT154009
FMB 0177	*C. buchloes*	*Medicago sativa*	MK871667
FCBP1509	*C. crepinii*	*Gerbera jamesonii*	KT283673
PAK13	*C. geniculata*	*E. japonica*	KR259522
PAK22	*C. geniculata*	*E. japonica*	KT154008
CRLB2	*C. geniculata*	*Zea mays L.*	MN366108
CUR-48	*C. geniculata*	*Olea europaea*	PP565055
CUR-61	*C. geniculata*	*O. europaea*	PP565056
CUR-70	*C. geniculata*	*O. europaea*	PP565057
CUR-79	*C. geniculata*	*O. europaea*	PP565058
US-1	*C. hawaiiensis*	*Cucurbita pepo*	MN053866
BLS-4	*C. hawaiiensis*	*Oryza sativa*	MK418956
NAS-C035	*C. inaequalis*	*Fragaria ananassa*	MF461014
NAS-C034	*C. inaequalis*	*F. ananassa*	MF461013
NAS-C028	*C. inaequalis*	*F. ananassa*	MF461012
NAS-C015	*C. inaequalis*	*F. ananassa*	MF461011
NAS-C07	*C. inaequalis*	*F. ananassa*	MF461010
FMB 0006	*C. lunata*	*Chamaedorea seifrizii*	MF448224
FMB 0200	*C. lunata*	*C. seifrizii*	MN971669
7	*C. lunata*	*Chenopodium quinoa*	MZ351412
BOT17	*C. lunata*	*Citrus reticulate*	MH645045
OZ-25	*C. lunata*	*Curcuma longa*	MW015787
H200904	*C. lunata*	*Dalbergia sissoo*	MW048851
FMB-G-DL	*C. lunata*	*Dypsis lutescens*	MG461573
PAK12	*C. lunata*	*E. japonica*	KR259521
PAK11	*C. lunata*	*E. japonica*	KR259520
PAK10	*C. lunata*	*E. japonica*	KR259519
PAK 32	*C. lunata*	*E. japonica*	KU052579
PAK 31	*C. lunata*	*E. japonica*	KU052578
PAK34	*C. lunata*	*E. japonica*	KT280007
PAK35	*C. lunata*	*E. japonica*	KT280008
CUR99	*C. lunata*	*E. japonica*	MN897748
CUR80	*C. lunata*	*E. japonica*	MN897747
CUR75	*C. lunata*	*E. japonica*	MN897746
CUR71	*C. lunata*	*E. japonica*	MN897745
CUR69	*C. lunata*	*E. japonica*	MN897744
CUR65	*C. lunata*	*E. japonica*	MN897743
CUR63	*C. lunata*	*E. japonica*	MN897742
CUR58	*C. lunata*	*E. japonica*	MN897741
CUR53	*C. lunata*	*E. japonica*	MN897740
CUR49	*C. lunata*	*E. japonica*	MN897739
CUR45	*C. lunata*	*E. japonica*	MN897738
CUR33	*C. lunata*	*E. japonica*	MN897737
CUR29	*C. lunata*	*E. japonica*	MN897736
CUR23	*C. lunata*	*E. japonica*	MN897735
CUR19	*C. lunata*	*E. japonica*	MN897734
CUR15	*C. lunata*	*E. japonica*	MN897733
CUR11	*C. lunata*	*E. japonica*	MN897732
CUR7	*C. lunata*	*E. japonica*	MN897731
Cuy22	*C. lunata*	*E. japonica*	KR425588
PF-0601	*C. lunata*	*Musa paradisiaca Linn*	MN722422
CL-37	*C. lunata*	*O. sativa*	ON767111
CL-32	*C. lunata*	*O. sativa*	ON767108.
CL-17	*C. lunata*	*O. sativa*	ON764855
R1	*C. lunata*	*O. sativa*	KF644377
R2	*C. lunata*	*O. sativa*	KF644376
R6	*C. lunata*	*O. sativa*	MH830326
R1	*C. lunata*	*O. sativa*	KF613576
R1R	*C. lunata*	*O. sativa*	KP940576
Bot-06	*C. lunata*	*Diospyros kaki*	OR131279
Cur	*C. lunata*	*Rosa Rubiginosa*	MG837719
CR	*C. lunata*	*Saccharum officinarum*	MN636871
FCBP1508	*C. lunata*	*Shorea robusta*	KT283672
BCIIAGS	*C. lunata*	*Solanum melongen*	KU379554
BCL2IAGS	*C. lunata*	*S. melongen*	KU836756
SL1	*C. lunata*	*S. nigrum*	OP265394
FMB 0201	*C. lunata*	*Trifolium alexandrinum*	MN971670
FMB 0078	*C. lunata*	*T. alexandrinum*	MF611756
PDL20	*C. lunata*	*Z. mays L.*	PP049245
PDL 19	*C. lunata*	*Z. mays L.*	PP033972
PDL 19	*C. lunata*	*Z. mays L.*	PP033958
CUR-12	*C. lunata*	*O. europaea*	PP565050
CUR-22	*C. lunata*	*O. europaea*	PP565051
CUR-31	*C. lunata*	*O. europaea*	PP565052
CUR-39	*C. lunata*	*O. europaea*	PP565053
CUR-42	*C. lunata*	*O. europaea*	PP565054
LC13496	*C. protuberans*	*S. officinarum*	MN215694
AVCS-1	*C. spicifera*	*Aloe perfoliata*	OP365099
PAK177	*C. spicifera*	*E. japonica*	MH204211
FMB-ALF1-MS	*C. spicifera*	*M. sativa*	MG388297
US-1	*C. spicifera*	*O. sativa*	MN153760
R8	*C. spicifera*	*O. sativa*	KF644378
P17	*C. spicifera*	*Prunus persica L.*	ON720268
BCsIAGS	*C. spicifera*	*S. melongena*	KU379555
FMB 0005	*C. tuberculata*	*Archontophoenix alexandrae*	MF448225

Table 6: GenBank accession number of curvularia blight disease available at NCBI.

The spores are reproductive structure of CLBD and diversity among the shape, size and color in the spores were confirmed in different species.

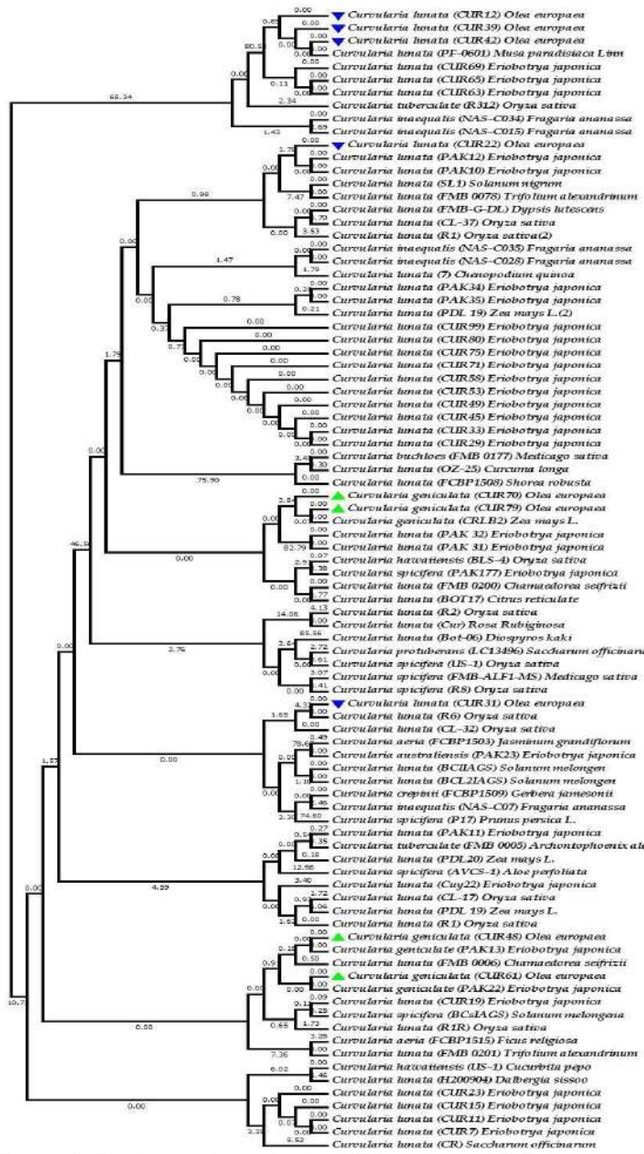

Figure 3: Phylogenetic analysis of ITS regions of CLB disease of olive caused by *C. lunata* ( ) and *C. geniculate* ( ) with previously reported sequences from Pakistan.

The branched hype of CLBD associated with olive were recorded as septate branched hype with 3-6 µm in length, unbranched, erect conidiophores with smooth conidia ranging from 21-31 × 8.5-12.0 µm with 3-septate (Mohamed *et al.*, 2021). On the basis of morphological characterization of conidia, Curvularia was further divided into Maculans (four cells condita), Geniculata (five cell conidia) and Lunata (four-celled, more or less curved spores) (Madrid *et al.*, 2014). In the highly virulent isolates (n=9), the morphological identification confirmed *C. lunata* (n=5) and *C. geniculate* (n= 4). The conidia characteristics alone does not provide accurate identification but distinguish these three types (Aira *et al.*, 2013). For reliable confirmation, new molecular approaches were further adopted. The lengths and sequences of rDNA repeats' ITS sections vary, and they are thought to develop quickly (Faeth and Sullivan, 2003). The ITS regions can be amplified more easily thanks to universal PCR primers, which are made from highly conserved sections that border the ITS. They also have a high copy number of rDNA repeats (up to 30,000 per cell) and are relatively modest (600–700 bp). From highly virulent isolates, ITS regions were amplified through PCR. The evolutionary history of CLBD with Maximum Likelihood method which was predicated on the Kimura 2-parameter model provide reliable information (Kimura, 1980). Using ITS sequence analysis, the 72 isolates were grouped into 19 genera, mostly comprising 2 Basidiomycota and 17 *Ascomycota* genera (Sullivan and Faeth, 2004). Furthermore, Osono and Masuya (2012) considered seasonal, climatic, and tree species variations when examining the species composition and diversity of endophytic fungus in the leaves of eleven distinct *Betulaceae* species. As such, the ITS area is an intriguing topic for evolutionary, phylogenetic, and biogeographic research (Faeth, 2009). The emergence of CLBD in Balochistan as well as in Pakistan possesses

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a future threat to olive production in the country.

CONCLUSION: In the current study, a novel disease rating scale for CLBD of olive was developed for the first time through a comprehensive approach integrating symptom development. Cultural characterization, morphological identification and nucleotide evidences ensuring precise confirmation of CLBD in olives in Pakistan. Our results confirmed first evidence of CLBD of olive at Balochistan and Pakistan.

CONFLICT OF INTEREST: The authors declare that they have no conflict of interest that affects the publication of this article.

LIFE SCIENCE REPORTING: In current research article no life science threat was reported.

ETHICAL RESPONSIBILITY: This manuscript is original research, and it is not submitted in whole or in parts to another journal for publication.

INFORMED CONSENT: The author(s) have reviewed the whole manuscript and approved the final version of the manuscript before submission.

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