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FIRST REPORT OF *CURVULARIA LUNATA* AND *ALTERNARIA ALTERNATA* CAUSING LEAF BLIGHTS ON TOMATOES IN SINDH, PAKISTAN

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Abstract

The tomato is an economically important crop that grows worldwide. The numerous fungal pathogens attacking tomato crops and causing severe diseases lead to yield loss. A few studies conducted regarding the identification of tomato fungal pathogens in Pakistan. However, no report was found to identify the fungal pathogens of tomato crops grown in Sindh. This study aimed to isolate, identify and characterize the tomato fungal pathogens in District Kamber Shahdadkot. Subsequently, thirty diseased tomato plant samples (leaves & stems) were collected from distinct villages of Taluka Kamber District Kamber Shahdadkot. These samples were collected in polythene bags and marked with symbols like S1, S2 and so on (S=Sample). All samples were brought into Botany Department lab and CBC Shah Abdul Latif University Khairpur. PDA media was prepared, sterilized and poured into petri plates. Prior to the inoculation, pieces from samples were soaked in a 1% solution of sodium hypochlorite (NaOCl) for a minute. The petri plates were inoculated separately and incubated at natural room temperature (26^0C to 28^0C) for arising and growth. Three days later, the inoculation site manifests signs and symptoms of fungal development. Within a week fungus reached at preferable growth and spores were formed. Two slides from each petriplate were prepared i. e. one from the center of the cultures and other from the margins of the cultures. In order to finish this application, slide-sized scotch tape was cut up and immersed on the surface of cultures gently. The off cuts of the scotch tap introduced the fungal materials (mycelia and spores), which were pressed with love to the glass slides and observed. The fungi identification was made by using applications of morphological characteristics like morphology of the cultures, nature, and type of the mycelium, the shape of spores, and the color of the spores. Following these characters two fungal species were identified i. e. *Curvularia lunata* and *Alternaria alternata.*

Keywords: *Solanum lycopersicum*, *Curvularia lunata*, *Alternaria alternata*, Mycelium, hyphae, Potato Dextrose Agar, sodium hypochlorite, autoclave.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is an economical prime vegetable, cultivated in all parts of the world (Joseph *et al*., 2017). Tomato is considered a model crops species, belonging to the Solanaceae family (Zakawa *et al*., 2019). The tomato is second highest-devour vegetable crop next to the potato at all over the world (Garg*et al*., 2020). It is consumed in diverse ways such, as preparing several dishes, like sauces, paste ketchup, salads and drinks (Nugroho *et al*., 2019). The tomato has achieved a good value having varieties of vitamins for example, vitamins A, C, E and antioxidants (Kumar *et al*., 2017), minerals, Ca, Fe, and P (Meena and Bahadur, 2014). In addition the tomato is a solid source of lycopene and carotenoid, the natural antioxidant which appears to be an active compound in the prevention of cancer, cardiovascular risk and slowing down cellular aging (Cheng *et al*., 2017).

The prime cultivating tomatoes in Pakistan are Riogrande, Money maker, and Roma. All these diversified tomatoes have been inaugurated from Europe and America (Khokhar, 2013). All introduced tomato cultivars are growing in every province of Pakistan (Memon, 2013). The annual world total production of tomato is 189.1 million metric tons (FAO, 2022). In Pakistan the total area used for cultivation of tomatoes was around 63 thousand

hectares and approximately 600 thousand tons of tomatoes were in total production. However, during the same period the Sindh province produced around 163 thousand tons of tomatoes using 14.4 thousand hectares (Wahid *et al*., 2017). Every year the number of food industries getting bigger, will also attract the demand for tomato made foods (Tahir *et al.*, 2012). In Pakistan contrast to the rest of the growing countries the tomato crop output is little owing to many fatal diseases created by bacteria, viruses, nematodes and fungi (Raza et *al*., 2016). Fungi at the post-harvest stage cutup the production by around half (Ippolito *et al*., 2005). The main causes of decreased tomato crop health and productivity are lower-quality seeds and abiotic factors including drought and salinity (Zhu *et al*., 2014).

The fungal tomato plant pathogen *Curvularia lunata* was first reported in Pakistan in 2016 (Heba-Alla *et al*., 2021). It was noticed in the survey of tomato crops growing on the subject of University of Punjab, Lahore Pakistan. *Curvularia lunata* causes fruit rot disease in tomato plants which is characterized by watery soaked lesions on tomato fruit which later convert into thin brown to black in colour (Iftikhar *et al*., 2016).

Alternaria alternata is another fungal species that causes leaf blights, leaf spots (Meena, *et al*., 2016) and cankers on tomato plants. The infections caused by *Alternaria alternata* on tomato plants start from the tips and margins of mature leaves in the form of black or brown spots and then transfer to the stem, spread and kill the plant in good moisture environment (Akhtar *et al*., 2004).

Alternaria solani is a notorious fungal pathogen that develops the early blight disease in tomato crops (Fry, 2008; Kumar and Srivastava, 2013). Early blight disease appears on tomato plant during seedling as well as at the young age (Camlica and Tozlu, 2019). At the seedling stage *Alternaria solani* infect the stem just above the soil line. The symptoms that appear on the plant body are, brown spots on leaves up to half of the inch in diameter, concentric rings called bull's eye, lesions on fruits and stem rots of the crop (Pandey *et al*., 2003). In Pakistan *Alternaria solani* was first observed in 2011 with respect to the Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan escorted by disease prevalence of 10–100% and yield dropping from 20 to 80% (Akhtar *et al*., 2011). Considering the lack of tomato recognition fungal pathogens in Sindh, this study tackled the main objectives to isolate, morphological identification and characterize the tomato fungal pathogens in District Kamber Shahdadkot Sindh.

MATERIALS AND METHODS

Samples collection: The tomato crops were visited regularly from the beginning of the growing season until the fruits started to form in Taluka Kamber District Kamber Shahdadkot. The plant parts infected by fungi were collected in polythene bags and taken to the laboratory in the Department of Botany, Shah Abdul Latif University Khairpur for further process.

Isolation of fungal pathogens: To isolate and culture the fungi present in infected parts of tomato plant i.e leaves and stems, the Potato Dextrose Agar (PDA) media was prepared using 40gm of PDA powder, 15 gm of agar, 20 gm of dextrose, and 1000 ml distilled water (Rijal, 2015). Required laboratory apparatus and PDA media were autoclaved at 120°C for 25 minutes. After sterilization, 35 mg/liter of Streptomycin was put into the media to inhibit the genesis of colonies of bacteria (Yong, 2015). Sterilized media was poured into petri plates. Pieces of 5mm were cut from each sample with sterilized blade/ scissor for inoculation. Prior to the inoculation, pieces from samples were scrubbed with 1% suspension of sodium hypochlorite (NaOCl) for a minute (Akhtar *et al*., 2011). After inoculation total petri plates were set in the laboratory providing room temperature for the best growth and development of fungal colonies (Kumar *et al.*, 2017). Checked and observed the culture each day without lifting the covers of petri plates. Growth of fungi appeared in radial form in petri plates after two or three days at the point of inoculation. Within a week, fungal mycelia reached desirable growth, and spores were formed.

Morphological identification of fungal pathogens: For morphological identification of candidate fungi, confocal microscope was used at Center for Biodiversity and Conservation, Shah Abdul Latif University, Khairpur, respectively. To complete the application two slides were prepared from all petri plates. For the first petri plate fungal material was taken from the middle of the plate while other slide was prepared from the margins of the plate. The material was taken from the plates by dipping scotch tape onto the surface of the media. The pieces of scotch tape having fungal mycelia and spores were softly pressed on plane glass slides and observed using distinct power objectives of a confocal microscope. Pictures of fungal spores and mycelia were taken by a charge-coupled device camera. References including Kibemo, 2017; Zheng *et al*., 2015; Alex *et al*., 2013; Ramjegathesh and Ebenezar, 2012 were followed for morphological identification of candidate fungal pathogens.

Pathogenicity tests: The Tomato (*Solanum lycopersicum* L.) variety Roma was used for the pathogenicity test. The in vivo experiment was conducted on tomato crop at the village Bahram buthi Taluka Kamber District Kamber Shahdadkot Sindh. For this work fresh young healthy leaves and stems of three-month-old tomato plants were used (Kibemo, 2017). The leaves and stems of selected plants were cleansed with tape water to do away with dust particles & other materials. Washed leaves and stems were surface decontaminated in 1% of sodium hypochlorite solution by submerging them for one minute and then were left to be dry (Kohler *et al*.,

2018). The stems of selected plants for pathogenicity test were bored at two regions i. e above the soil line and mid of the stem length with the depth of 0.5 cm by sterilized cork borer. The six-day-old culture of fungal isolates washed with sterilized distilled water and was inoculated into the holes and packed using scotch tape to control the entrance of foreign contaminates (Firdous *et al*., 2009). The same method was applied for leaves inoculation but here sterilized needle was used to make little holes on surface of selected leaves to inoculate the piece of fungal mycelium with few spores. The inoculated victims were left to get results and were observed daily. After seven days the symptoms of the disease appeared at the point of inoculation on leaves and stems (Shah *et al*., 2009).

RESULTS

Morphological identification of fungal pathogens: Observed, recorded and labeled the following important morphological features of the various fungi from different petri plates. Morphology of cultures mycelium type and its nature, color, figure and arrangement of spores, were taken as diagnostic characteristics for the identification of candidate fungal isolates (Kibemo, 2017; Zheng *et al*., 2015; Alex *et al*., 2013; Ramjegathesh and Ebenezar, 2012).

Curvularia lunata

Symptomatology of candidate fungal isolates (*Curvularia lunata*) on tomato leaves: Tomato leaf samples were collected from different tomato crops of Taluka Kamber and marked with symbols like S3, S5, S8, S11, S16 and S20 (Figure 1). Among these infected tomato leaves the sample S3 shows the symptoms of irregular-sized spots, total discoloration and necrosis. The leaf samples S5, S16 and S20 show similar symptoms. The infection starts with blighting of leaf tips and margins towards the middle of the lamina. Due to the infection tissue damage and yellow colour appeared. The S8 leaf sample is damaged completely, with no living tissue with chlorophyll. The tomato leaf having the symbol of S11 is also completely damaged by pathogens; various-sized spots are showing on entire the surface of the lamina.

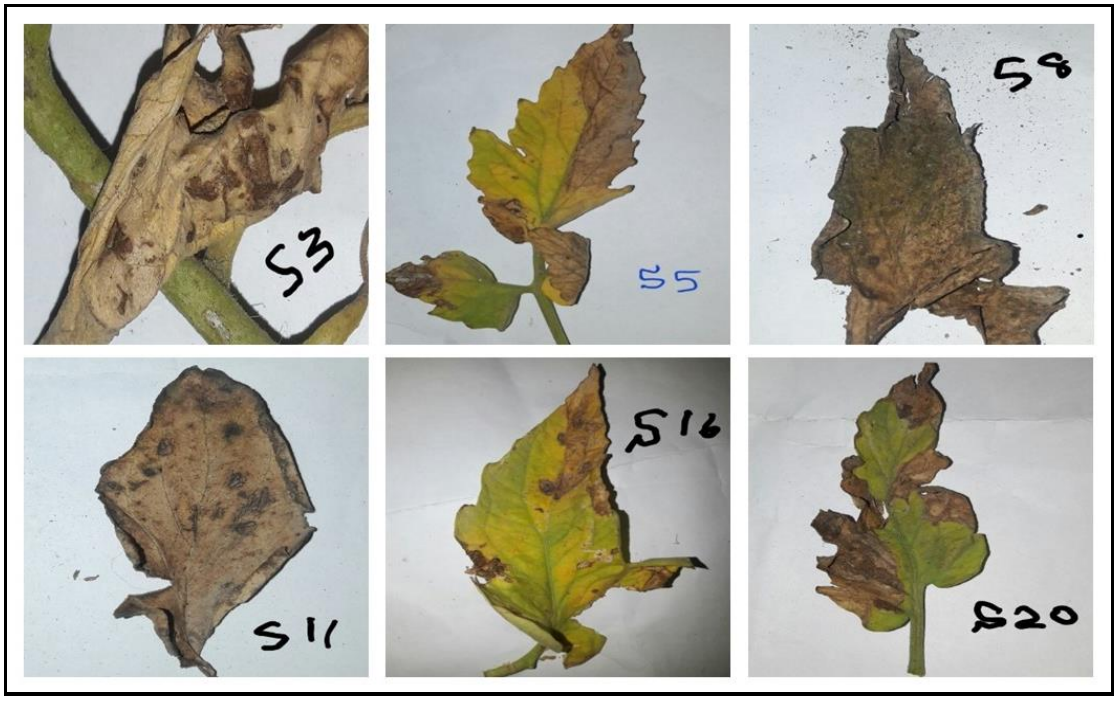

Figure. 1 Leaf sample S3 shows irregular-sized spots with discoloration and necrosis. S8 and S11are completely blighted with no living tissue. Sample S5, S16 and S20 show the start of infection from the margins towards the midrib and yellow colour of leaves.

Morphology of cultures/ Nature of growth: The colonies of fungi got appeared on the PDA media surface with white to dark brown mycelial growth (Iftikhar *et al*., 2016) but changed into grayish-black when reached desirable growth (Alex *et al*., 2013). The lower surface of each colony was showing a blackish colour without any pigment diffusion in the medium. The upper regions of the colonies were velvet to flocculus (Figure 2)**.**

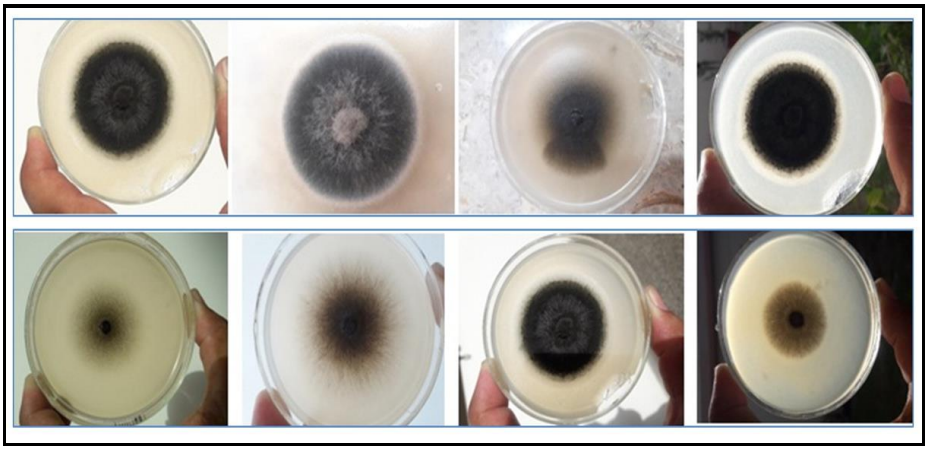

Figure. 2 Morphology of fungal cultures on PDA media in different petri plates,

microscopic examination for morphological identification of *Curvularia lunata:* On the nature basis the mycelia were hyaline, septate, branched, and conidia were broadly ellipsoidal or clavate, brownish in colour, smooth-walled, septate, mostly contained 4 cells, slightly curved and produced in sympodial orders (Alex *et al*., 2013). The middle cells of conidia were broad, thick and darker than sub-terminal cells (Kamaluddeen *et al*., 2013). Subsequently, the following entire characteristics of S3, S5, S8, S11, S16 and S20 samples were identified as *Curvularia lunata* (Figure 3,4,5,6,7, and 8).

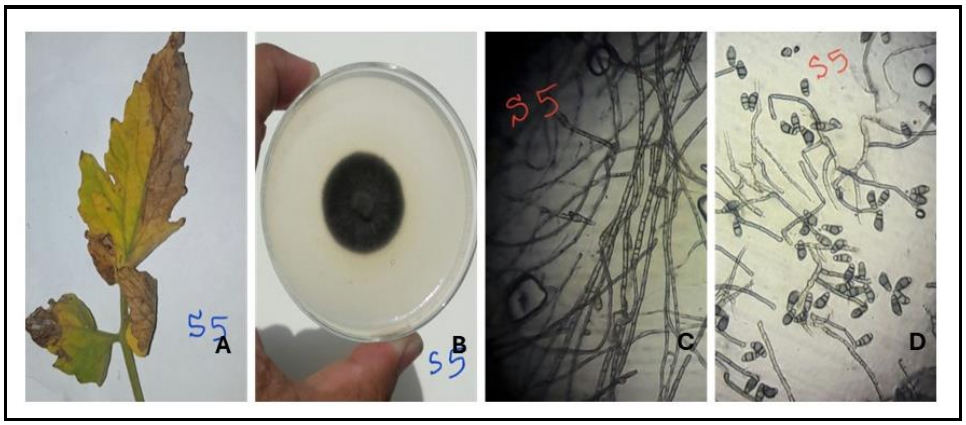

Figure. 3. ***Curvularia lunata*** **(sample S5).** A. Tomato leaf sample blighted by *Curvularia lunata* from tips and margins and chlorosis, B and C. Fungal culture in PDA media with light gray from the center while black towards the margins, D. Fungal mycelium branched and septate with dark colour, conidia on tip of conidiophores septate with three to four cells.

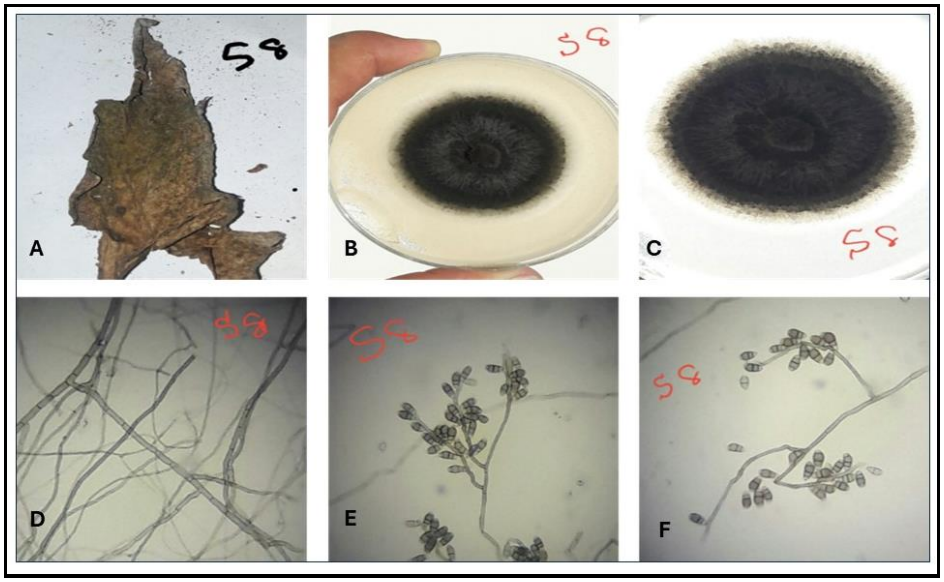

Figure. 4 ***Curvularia lunata*** **(sample S8).** A. Tomato leaf sample totally blighted by *Curvularia lunata* no chlorophyll and living tissues are seen, B and C. Fungal culture on PDA media with black colour, D. Fungal mycelium branched and septate with grey colour, E and F. Conidia on tip of Conidiophores septate with three to four cells.

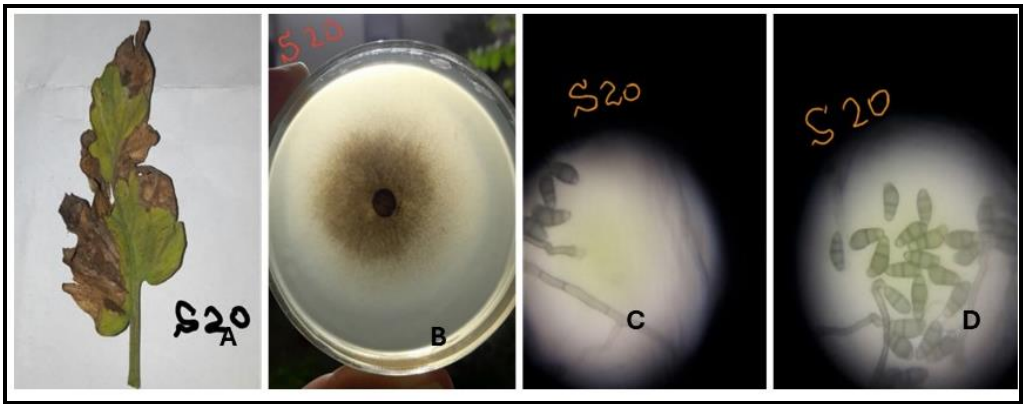

Figure 5 ***Curvularia lunata*** **(sample 20)** A.Tomato leaf sample by *Curvularia lunata*, infection started from the leaf margins and tips and grew towards the midrib resulting chlorosis, B. Radial growth of fungal culture with dark brown color on PDA media, C. Fungal mycelium is showing branched and septate hypha with few conidia, D. Fungal spores/conidia slightly curved, septate with thickened and darker middle cells.

Alternaria alternata

Three prepared slides loaded with fungi (Mycelia and spores) from the petri plates having the symbols of S6, S15and S17 (S= Sample) were observed. The fungi on these three slides were identified as *Alternaria alternata*. To confirm the identification method of Woundenberg *et al*., 2013; Lawrence *et al*., 2013 was applied.

Tomato leaf symptoms infected by *Alternaria alternata:* A total three tomato leaf samples i. e S6, S15, and S17 infected by *Alternaria alternata* were found which show similar sign and symptoms. The infection starts from the lower leaves with the development of yellow colour of leaf and brown coloured markings. Symptoms arise from the tips and margins of leaves which move towards the central areas resulting blighted leaves (Akhtar *et al*., 2004).

Morphology of cultures/ Nature of growth: The fungal cultures in petri plates inoculated with leaf samples S6, S15 and S17 appeared with dark olivaceous brown to dark blackish brown on the surface of PDA media (Khodaei and Arzanlou, 2013).

Microscopic observations of *Alternaria alternata:* Microscopic examination of fungi loaded in scotch tape revealed septate hyphae, straight or curved primary conidiophores which give rise to conidia in long chains (Akhtar *et al*., 2004). The conidia were light brown in colour having tapered apices mostly formed in chains but sometimes singly with muriform shape (Ramjegathesh and Ebenezar, 2012). Conidia were long ellipsoid, obclavate, moderate in size, divided into septums, straight conidia, and dark brown to olivaceous green and smooth as suggested by (Khodaei and Arzanlou, 2013). Sometimes secondary conidiophores may be formed with one or a few conidiogenous lociapically or laterally (Figure 5). These observations and scrutinizes are consistent with the morphology of *Alternaria alternata* (Kpotzo *et al*., 2011).

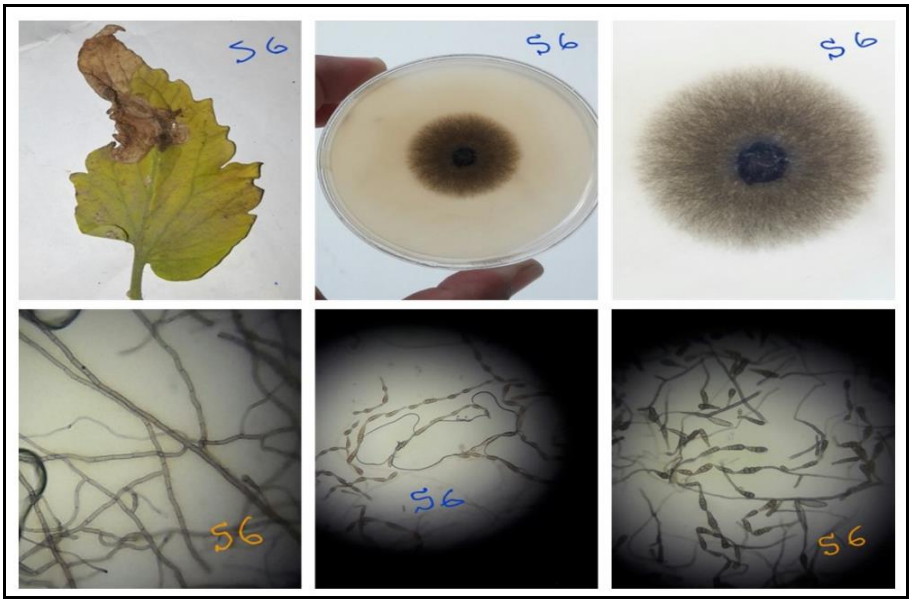

Figure 6 ***Alternaria alternata*** **(Sample 6).** A. Tomato leaf sample infected by *Alternaria alternata* showing the chlorosis of leaf and brown colored lesion, infection started from the tips or margins of lamina, B and C. Fungal culture appeared in radial growth with olivaceous brown color on media, D. Fungal mycelium is branched, and septate, E and F. Long chain of conidia with tapered apices and olivaceous green.

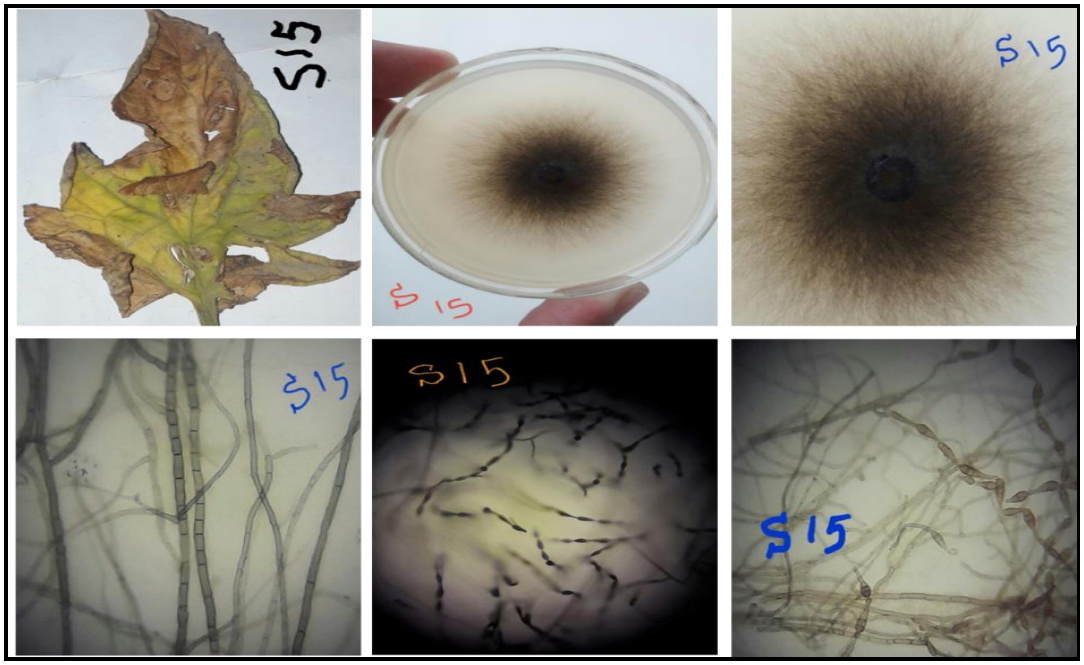

Figure 7. ***Alternaria alternata*** **(sample 15).** A. Tomato leaf sample blighted from tips and margins resulting necrosis and chlorosis, B and C. Fungal culture in dark brown color with tufting growth of hyphae, D. Fungal mycelium is septate, branched and dark in color, E and F. Fungal spores/conidia in long chains.

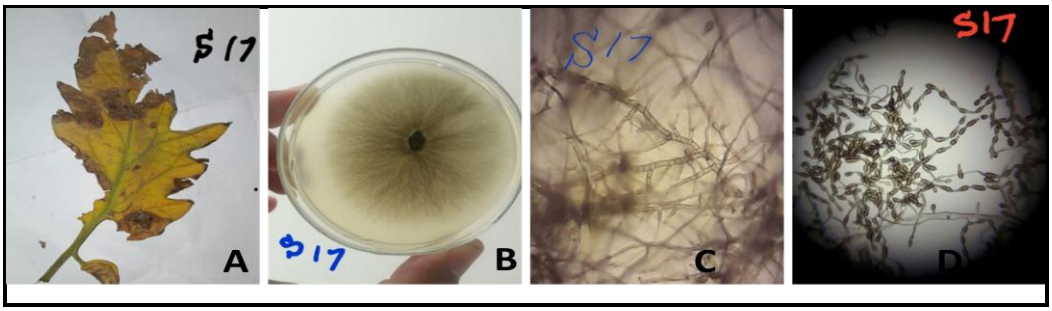

Figure 8 ***Alternaria alternata*** **(Sample 17).** A. Blighted leaf from tip and margins leading chlorosis, B. fungal culture with tufting hyphae and olivaceous colour, C. Fungal mycelium branched and septate light brown in colour, D. Fungal spores/conidia in long chain with tapered apices.

DISCUSSIONS

The tomato plant (Solanum lycopersicum L.) is a financially rewarding, essential, and model crop of the Solanaceae family (Saand *et al*., 2015; Saleem *et al*., 2013). This crop has been attacked by numerous disease-causing agents like; insects, nematodes, bacteria, fungi, viruses and viroids which lead to yield loss and heavy economic damage (Arie *et al*., 2007). Although the tomato crop is infected with enormous number of fungal pathogens described earlier. Moreover, this study identified the two fungal species i.e. *Curvularia lunata* and *Alternaria alternata*. The morphological and cultural characteristics features for each isolate were authenticated by following previously suggested protocols, methods and recommendations.

Finally, morphological and cultural characters of *Curvularia* species appeared on media with dark brown having septate conidia along with basal scar (Kamaluddeen *et al*., 2013). The conidia are curved or straight with thickened middle cells (Madrid *et al*., 2014). The *Curvularia lunata* old colonies are usually found in gray to blackish and clavate-shaped conidia having dark brown color with four septa (Alex *et al*., 2013). Following those suggested characteristics features were observed in *Curvularia lunata* tomato fungal isolates of this study having several features including; branched septate mycelium with light/dark grey or brown in color, conidia septate three to four-celled, middle cells were thick and darker than sub terminal cells. Thus, S3, S5, S8, S11, S16 and S20 samples were identified as *Curvularia lunata* (Figures 3,4,5,6,7 and 8) (Kamaluddeen *et al*., 2013; Alex *et al*., 2013).

Our study revealed the first-ever report of tomato fungal pathogens identification in Sindh. The identification of *Alternaria alternata* and its taxonomy is commonly based on morphological and cultural criteria (Hong *et al*., 2005). The protocols and recommendations suggested by Simmons (2007) are authentic tools to identify *Alternaria alternata* on the basis of morphological features including; sporulation

pattern, conidia morphology, and primary and secondary conidiophores. The small-spored species such as *Alternaria tenuissima*, *Alternaria arborescens* and *Alternaria infectoria* are complex to determine from *Alternaria alternata* by morphology bases (Taralova *et al*., 2011). Nevertheless, *Alternaria infectoria* shows discrete secondary conidiophores and *Alternaria tenuissima* depicts the unbranched conidia in chains, this could distinguish these two from *Alternaria alternata* on the basis of morphological characters (Simmons, 2007).

Moreover, small spored fungi could produce the toxins which involved in pathogenicity whereas; *Alternaria infectoria* cannot (Rotem, 1994). Although molecular studies also revealed the distinction between small and large-spored species of the *Alternaria* genus (Andrew *et al*., 2009; Peever *et al*., 2004). The first or second conidia in every chain often slightly longer, however, 2ndary chains were septate or without septums and the tip of conidia are unbranched with brown in color are diagnostic characteristic feature to identify the *Alternaria alternata* plant fungal pathogen (Khodaei and Arzanlou, 2013). Subsequently, isolates (S6, S15, S17) appeared in their cultural and morphological features including; culture dark or olivaceous brown color, mycelium septate and branched, long chains of conidia with tapered apices identified as *Alternaria alternata* species (Figure 9,10 and 11) (Akhtar *et al*., 2004; Simmons, 2007; (Ramjegathesh and Ebenezar, 2012; Khodaei and Arzanlou, 2013).

CONCLUSIONS

In conclusion, this study recognized two fungal species, Alternaria alternata and Curvularia lunata, as big hazards to tomato crops in District Kamber Shahdadkot, Sindh. The identification was basically based on the morphology and cultural features of the fungi. The Curvularia lunata was characterized by septate branched mycelia, with dark brown to grayish-black color, and conidia having four cells with brown color and a little curved shape. On the other hand, Alternaria alternata display dark olive brown to blackish brown with septate mycelia, straight or curved, with conidia set in long chains possessing tapering apices, septate, and ranging in color from light brown, dark brown to olivaceous green, and smooth. These findings support the significance of authentic identification of fungal pathogens for effective disease control plan in tomato cultivation. Understanding the specified characteristics and conduct of these pathogens can assist in action targeted control measurements, such as fungicides applications, crop rotation, or disease resistant cultivar preference. However, the presences of these pathogens focus-on the need for uninterrupted monitoring and inspection to mitigate the risk of crop yield losses due to fungal diseases. Furthermore, this study subsidize to the existing knowledge of fungal species affecting tomato plants in the area, providing valuable perceptions for more advanced research and development of eco-friendly agricultural applications. This research assign the preliminary work for future studies aimed at elucidating the genetic diversity, process of pathogenicity, and ecological interactions of these fungi within the agro ecosystem. Finally, such endeavors are critical for ensuring food security and livelihoods in tomato-producing areas like District Kamber Shahdadkot, Sindh.

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