BIODIVERSITAS
Volume 22, Number 2, February 2021
Pages: 615-623

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d220213

Genetic analysis and pathogenic characterization of *Alternaria tenuissima* induced fruit rot of bitter gourd

SEHRISH IFTIKHAR[1], WAHEED ANWAR[1], ADNAN AKHTER[1,♥], SAJID ALI[1], HAFIZ AZHAR ALI KHAN[1], MUHAMMAD KHURSHID[2], MUHAMMAD SALEEM HAIDER[1]

[1]Institute of Agricultural Sciences, University of Punjab. Quaid-e-Azam Campus, P.O. Box. 54590, Lahore, Pakistan.
Tel.: +92-42-26023186, Fax.: +92-42-99231846, ♥email: adnanakhter.iags@pu.edu.pk
[2]Institute of Biochemistry and Biotechnology, University of the Punjab. Quaid-e-Azam Campus, P.O. Box. 54590, Lahore, Pakistan

Manuscript received: 29 November 2020. Revision accepted: 9 January 2021.

Abstract. *Iftikhar S, Anwar W, Akhter A, Ali S, Khan HAA, Khurshid M, Haider MS. 2021. Genetic analysis and pathogenic characterization of Alternaria tenuissima induced fruit rot of bitter gourd. Biodiversitas 22: 615-623.* Bitter gourd (*Momordica charantia* Linn.), belongs to *Cucurbitaceae* family, is widely cultivated in areas with warm climate. In 2017, fruits of bitter gourd-bearing rot symptoms were observed in the Punjab province of Pakistan. The disease-causing fungal isolate was collected from the diseased fruits on potato dextrose agar (PDA). Microscopic examination revealed short conidiophores arose singly, measuring 79.8-158.5 µm long and 3.94-7.89 µm thick. The size of conidia varied from 25.7 to 46.45 µm and 8.55-14.39 µm in length and width respectively, which were characteristics of *Alternaria* spp. To confirm the identity and molecular characterization of the isolate, the internal transcribed spacer (*ITS*) region, translation elongation factor 1 alpha (*TEF1-α*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and RNA polymerase II large subunit 2 (*RPB2*) genes were amplified. The sequence analysis of amplicons and phylogenetic studies specified the homology of isolated *Alternaria* spp. with the previously reported *A. tenuissima* in GeneBank. The pathogenicity tests conducted on the fruits of bitter gourd confirmed the disease development with typical *Alternaria* induced rot symptoms, thus satisfied Koch's postulate. To our knowledge, this is the first record of *A. tenuissima* causing fruit rot of bitter gourd in Pakistan.

Keywords: *Alternaria* rot, emerging disease, microscopic examination, *Momordica charantia*, phylogenetics, phytopathogenic

Abbreviations: PDA: potato dextrose agar; ITS: internal transcribed spacer; region, TEF1-α: translation elongation factor 1 alpha, GAPDH: glyceraldehyde 3-phosphate dehydrogenase; RPB2: RNA polymerase II large subunit 2

INTRODUCTION

Bitter gourd (*Momordica charantia* L.), also known as "bitter melon", is an important vegetable crop in the tropics, which is mainly cultivated in Asia and Africa (Nagarani et al. 2014; Xu et al. 2015). It is used as a vegetable and has been used in various Asian traditional medicines(Polito et al. 2016a). It is a tropical vine of the *Cucurbitaceae* family. It is an annual plant of climbing nature and the fruit is rich in vitamins, carbohydrates, phosphorus, minerals, essential oils, and sterols (Joseph et al. 2013; Dandawate et al. 2016; Saad et al. 2017). The fruit of bitter gourd varies in shape and size. Bitter gourd has economic values and has been used against cancer (Li et al. 2012), diabetes and immunodeficiency virus (HIV) (Paul and Raychaudhuri 2010), has cytotoxic activity (Zhang et al. 2014), the healing activity of gastric ulcers (Gürdal and Kültür 2013). Bitter gourd can stimulate digestion as most of the bitter-tasting foods.

Alternaria spp. are known as major plant pathogens causing leaf spots and blights on a wide variety of host plants (Nowicki et al. 2012; Bowers et al. 2013). Other members of this genus are saprophytic and colonize the dead or decaying plant material. The colonies are usually black or grey in color and produce club-shaped spores. The symptomatic dark-colored spores cause new infections in other places. The airborne spores are ubiquitous and can be found in the soil and water. *Alternaria* isolates secret toxins and cell wall degrading enzymes, which help in the acquisition of nutrients and decomposition of plant cell walls (Choi et al. 2013; Taheri 2010).

Proper classification of an organism is only possible after its correct identification. Precise identification of phytopathogens has a significant role in disease control and management (Sales and Pashazadeh 2020). It helps in quarantine strategy (Aksoy et al. 2017). The traditional way of species identification was based on morphological characteristics like spore shape and colony appearance but it becomes difficult for closely related species and requires a taxonomic expert (Manamgoda et al. 2012). Molecular characterization has been possible since the advent of PCR and advance sequencing techniques (Marakli 2018). Sequencing of unique DNA segments can resolve closely related species quickly in a precise manner (Lakshman et al. 2016; Gherbawy et al. 2018). These unique segments act as DNA barcodes and are flanked by conserved regions that can be amplified with universal primers. These include *ITS* (internal transcribed spacer) region of rDNA, *TEF-1-α* (translation elongation factor 1 alpha), *GAPDH*, *RPB2*, *EF1a*, etc. *ITS* is most efficient for fungal identification up to species level (Seena et al. 2010; Schoch et al. 2012; Al-Nadabi et al. 2018). The ITS regions have been broadly

used in molecular phylogeny and taxonomical studies as they can be readily amplified using small quantities of DNA (Schoch et al. 2012; Elfar et al. 2018). It includes small and large ribosomal subunits sequences (*ITS1*, *ITS2*) on DNA and gives a good resolution at family, genus, or species level hence proved to be an ideal (Asemaninejad et al. 2016; Řezáčová et al. 2016). The translation elongation factor 1 alpha (*TEF-1-α*) loci, involved in translation processes, is highly conserved. The *TEF-1α* gene is used to illustrate the genetic diversity, examine the intra- and inter-specific variation, and analyze the phylogenetic diversity among eukaryotic organisms (Klopfenstein et al. 2017; Luo et al. 2017). The objectives of this study were to isolate fruit rot causing pathogen from the bitter gourd, followed by molecular and phylogenetic characterization of the disease-causing isolate and, confirmation of the pathogenic ability of the collected isolate to establish Koch's postulates. To the best of our knowledge, this is the first study highlighting the spread of *Alternaria* rot to new geographic locations as well as an emerging threat to bitter gourd production in Pakistan.

MATERIALS AND METHODS

Study area and sample collection

A field survey was conducted in Punjab, Pakistan, during 2017. The areas included Kasur and Lahore, districts of Punjab, Pakistan (Figure 1). A total of 10 farms were selected for sampling from each district. The infected fruits were sampled and analyzed for the isolation and identification of phytopathogenic fungi. Infected fruit samples were excised and packed in polyethylene bags. All the bags were labeled and kept at 4 °C in the laboratory at University of the Punjab, Lahore, Pakistan.

Procedures

Isolation and maintenance of fungi

Bitter gourd tissue was cut into small pieces (0.5 cm) and surface sterilized with sodium hypochlorite (NaOCl; 1%) for 1 min after lesion excision, followed by dipping in 70% ethanol for 30 seconds (Wei et al. 2020). The pieces were washed with autoclaved dH_2O and embedded onto PDA media (Difco) aseptically. The plates were kept at 25 °C with a 16 h photoperiod for three to four days to allow hyphal growth. The pathogens were purified by subculturing the growing hyphal tips out of the tissue sample to a new PDA plate. The spores in the purified culture plate were collected from 7-14 days old culture plates by adding a small volume (3 mL) of sterile water and rubbed the surface gently with Drigalski spatula. The spore suspension was collected and 50 µL from suspension was spread to a new PDA plate to get single spore culture. The plate was observed using a stereoscopic microscope (Labomed®) to examine the germinating spores. A single spore was picked up with a needle tip to a new PDA plate. PDA slants of the stock culture were preserved at 4°C.

Morphological characterization of fungi

A single spore colony on a PDA plate was allowed to grow up to seven days at 25 ±1 °C to examine its morphological characteristics. The shape, color, growth pattern and margins of the colony were observed. Detailed mycelial characteristics were observed including septation and texture with the help of microscope (Labomed®) at 40× and 100× resolution. Reproductive structures and spore formation were investigated. The presence of elongated secondary conidiophores, spore size, shape, and color were investigated.

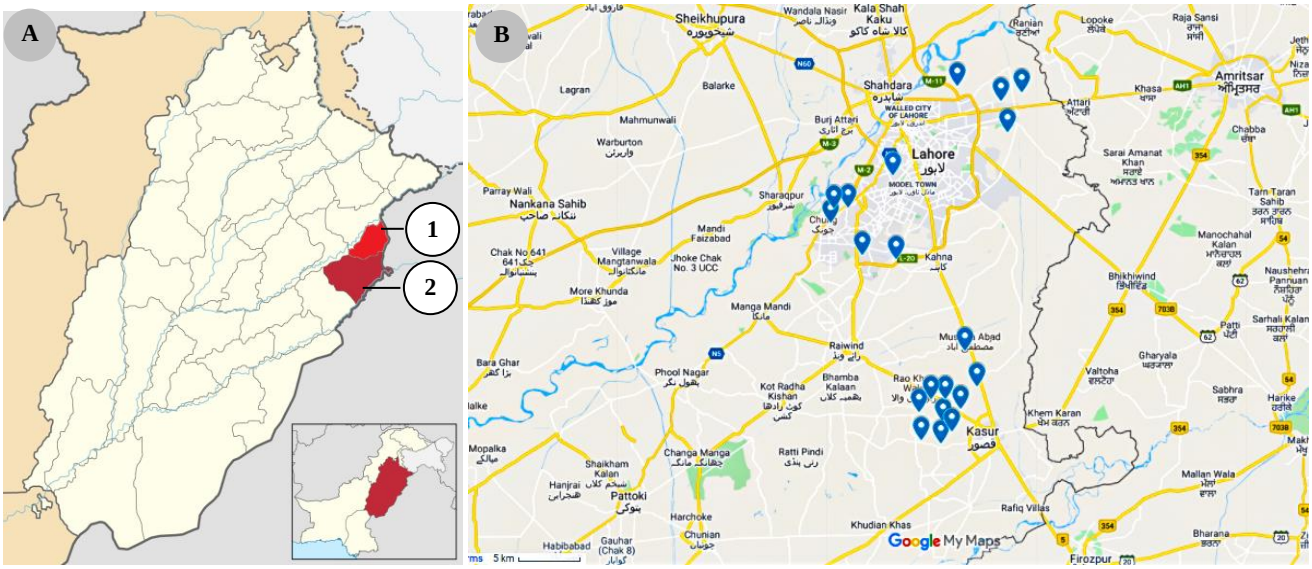

Figure 1. A. Location of Lahore (1) and Kasur (2) districts, in Punjab Province, Pakistan; B. Indicating the sampling site

Molecular characterization of Alternaria tenuissima

Different molecular markers including *TEF1-α*, *GAPDH*, *RPB2* and *ITS* region, were used to characterize the pathogen species. Total genomic DNA of the fungi was isolated using a modified CTAB method described by Wang et al. (2015). The fungi were inoculated in potato dextrose broth with shaking at 150 rpm for 5 days. The media was prepared as mentioned previously except for agar. After 5 days, the mycelium was filtered with Whatman filter paper no.1. The filtered mycelial mass was ground in liquid nitrogen with pestle and mortar. β-mercaptoethanol (200 µL) was added in already prepared 20 mL of pre-warmed (65°C) CTAB buffer and 700 µL of this buffer was added into powered mycelium followed by incubation in the water bath at 65 °C for 30 min. Then 700 µL of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 13000 rpm for 10 min. Then 500 µL of isopropanol was added to supernatant and mixed gently. The tubes were incubated at -20°C for 2 h. After incubation, the tubes were centrifuged at 13000 rpm for 5 min and the pellet was collected. Afterward, the pellet was washed with 70% ethanol and air-dried. The dried pellet was suspended in 100 µL of 5% T.E buffer. Agarose gel (1%) was used to check the DNA quality by separation at 100 V for 30 min at 3 V cm-1. Agarose gel (1%) was prepared by adding 1 gram agarose (Sigma-Aldrich) in 100 ml TAE buffer and heated for 3 min. Ethidium bromide (3 µL) was added to the gel before electrophoresis to a final concentration of 0.5 µg mL-1 before solidification. The gel was exposed to UV light and the picture was taken with a gel documentation system. DNA ladder of 1Kb was used as a marker.

Amplification of ITS1, TEF1-α, GAPDH, RPB2 gene regions

The concentration of isolated genomic DNA was measured with NanoDropTM (Thermo Fisher Scientific). The DNA was diluted to 50 ng μL^{-1} using the formula (M1V1=M2V2). The *ITS* was amplified with the primer pairs ITS1 and ITS4 (White et al. 1990), the *TEF1-α* with EF1-728F and EF1-986R (Carbone and Kohn 1999), the *GAPDH* with gpd1 and gpd2 (Berbee et al. 1999), and *RPB2* with RPB2-5F2 (Sung et al. 2007) and fRPB2-7cR (Liu et al. 1999). The amplification was performed according to the procedure of Woudenberg et al. (2014). MyCyclerTM Thermal Cycler (Bio-Rad Laboratories Inc.) was used to perform PCRs. A total volume of 12.5 µL was used for PCR reactions. The genomic DNA (1 µL; <250ng), dNTPs (40 µM of each), primer (0.2 µM of each), $MgCl_2$ (1.5 µM), 0.25 Unit GoTaq® Flexi DNA polymerase (Promega) and GoTaq® Flexi buffer (2 µL) (Promega, Madison, WI, USA) was used in a reaction mixture. In the GAPDH and ITS reaction mixture 1 µM MgCl2 was used, while in the *RPB2* and *TEF1-α* PCR mixtures, 2 µL genomic DNA was used. In the case of RPB2 mixture, 0.5 U GoTaq® Flexi DNA polymerase was used.

PCR conditions for *ITS* and *GAPDH* consisted of an initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C, 48°C and 72°C for for 30, 30 and 90 s, respectively. The *TEF1-α* was amplified with 40 cycles, denaturation at 94°C for 30 s and annealing temperature of 59°C for 30 s. The final extension was performed for 7 min at 72°C. For the amplification of partial RPB2 gene, 30 cycles were performed with denaturation at 94°C, annealing at 60°C and extension at 72°C for 45, 45 and 120 s, respectively. The PCR products were run on agarose gel (1%). The gel image was taken with a gel documentation system. DNA ladder of 1Kb was used as a marker. The amplified PCR products were processed for sequencing from 1st BASE Malaysia (Sanger et al. 1977). All the generated sequences were deposited in the GenBank.

Phylogenetic analysis

Homologous sequences of *ITS1*, *TEF1- α*, *GAPDH*, and *RPB2* loci of similar fungal species were taken from NCBI GenBank using the Basic Local Alignment Search Tool (BLAST) (2017). The alignment of consensus sequences and phylogenetic analysis was performed using the neighbor-joining technique with the help of MEGA6 software version 6.0 (Tamura et al. 2013).

Pathogenicity test

Pathogenicity tests were performed on the fruits of bitter gourd by inoculating a drop spore suspension (10 µl containing 5×10^5 spores mL^{-1}). The inoculated fruits were wrapped in a plastic bag and incubated at 26°C. Fruits were arranged randomly into four replicates and each replicate contains five fruits. The symptoms were evaluated 5 days post-inoculation (dpi) by visually estimating the percentage of fruit area covered with pathogen-related symptoms. Koch's postulates were confirmed by re-isolating the pathogen from artificially inoculated fruits and comparing the resultant cultures with the original cultures.

RESULTS AND DISCUSSION

Isolation and identification of *Alternaria tenuissima*

The fungal species associated with affected bitter gourd fruits were isolated from the samples collected during the survey and identified as *A. tenuissima* on the basis of cultural and genetic characters. A total of 6 isolates were obtained. The symptoms observed were brown-colored circular necrotic spots that became sunken. At later stages, the spots enlarged in size and developed an irregular shape which coalesced, and gradually covered the entire surface of the fruit (Figure 2).

Morphological characterization of *Alternaria tenuissima*

Morphological characterization of representative isolate of *A. tenuissima* isolate was done by observing the colony growth on PDA according to Simmons (2007). Subsequently, macro and micro characteristics, including vegetative and reproductive structures were also studied.

Macroscopic characters

The fungus colonies produced, after incubation at 26°C for 5-6 days with a 12-h photoperiod on PDA, were regular and flat, with a rough texture. The outer edge of the colony was olive-black, with a dark black center (Figure 3). The

topography of the mycelium was spreading, hairy, and velvety (Figure 3.A).

Microscopic characters

The conidiophores were short, arising singly and flexuous pale. The conidiophores measured 79.8-158.5 µm long and 3.94-7.89 µm thick. The size of spore varied from 25.7 to 46.45 µm long and 8.55-14.39 µm wide. Morphology was typical of *Alternaria tenuissima*. The spores were borne singly with 1-4 transverse septa (Figure 3.B). The spores were oval to oblong and occasionally branched. Based on morphological characteristics of the isolates, especially, colony morphology, and microscopic observations of spores, the isolates were identified as *A. tenuissima* (Figure 3.C).

Molecular characterization of *Alternaria tenuissima*

To confirm the molecular identity of *A. tenuissima*, the *ITS1*, *GAPDH*, *TEF1-α*, and *RPB2* regions were amplified followed by sequencing. The accession numbers for all the amplified sequences of isolate are given in Table 1. The sequences were submitted to GenBank: LT670913, LT707522, LT707523, and LT707524 for ITS, GAPDH, RPB2, and *TEF1-α* . The ITS1 region consisted of 547 bp, TEF1-α of 284 bp, GAPDH of 608bp, and RPB2 of 931bp. There was 99-100% percentage similarity among our fungal isolates and available sequences in NCBI database based on BLAST analysis of nucleotide sequences.

Figure 2. A-C. Symptoms of fruit rot of bitter gourd observed in the field

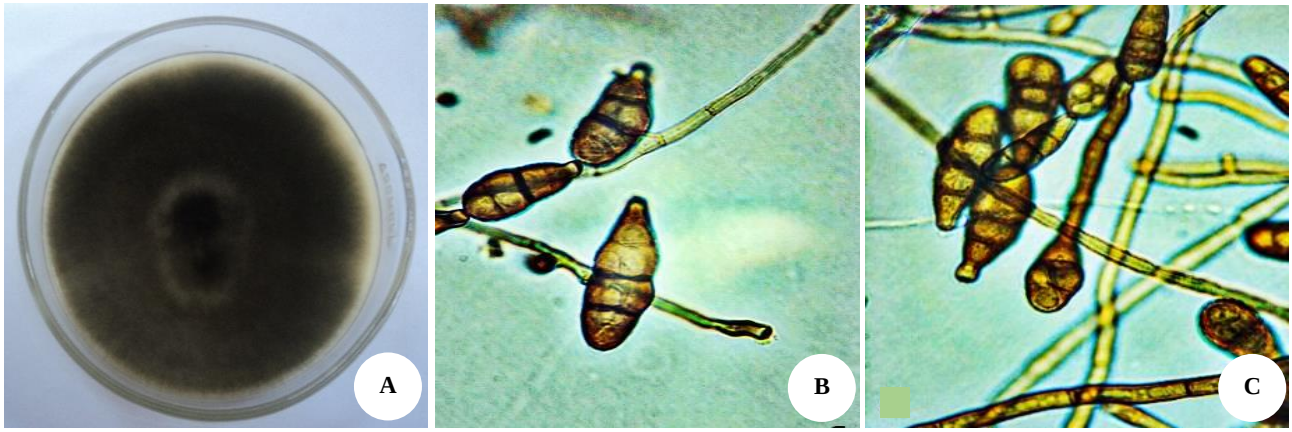

Figure 3. A. Mycelium development of *Alternaria tenuissima* on PDA, B-C. Spores of *A. tenuissima*

Table 1. Details of GenBank accession numbers of *ITS1*, *TEF1-α*, *GAPDH*, and *RPB2* genes of *Alternaria tenuissima*

Region	GenBank No.
8S rRNA gene, *ITS1*	LT670913
Partial gene for translation elongation factor 1 alpha (*TEF1-α*)	LT707524
Partial gene for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)	LT707522
Partial gene for RNA polymerase II large subunit 2 (*RPB2*)	LT707523

Phylogenetic analysis of *Alternaria tenuissima*

Genetic relationship between *A. tenuissima* isolates was observed by the neighbor-joining tree assembled on the basis of the available *ITS1*, *GAPDH*, *TEF1-α*, and *RPB2* gene sequences of *A. tenuissima* isolate and those available in the database. The neighbor-joining tree revealed that on the basis of *ITS1* gene sequence, *A. tenuissima* isolate (LT670913) showed maximum homology with *A. tenuissima* isolates KX139157, JX205160, JX867218, and HQ647307. On the basis of the translation elongation factor 1 alpha (*TEF1-α*) gene sequences in NCBI database and our fungal isolate of *A. tenuissima*(LT707524) made a separate clade and showed maximum homology with LC136865, LC136863, LC136862, and LC136864 *A. tenuissima* isolates (Figure 4). Based on the RNA polymerase II large subunit 2 (*RPB2*) gene sequences, *A. tenuissima* isolate**s** **(**LT707523) showed maximum similarity with LC134328, LC134327, LC134326 and LC134326 *A. tenuissima* isolates. In the neighbor-joining tree, on the basis of available partial glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene sequences, *A. tenuissima* isolates (LT707522) showed maximum similarity with *A. tenuissima* isolates making a clade, which includes LC134316, LC134319, LC134315, and LC134318. A comparative analysis of *ITS1*, *GAPDH*, *TEF1-α*, and *RPB2* gene sequences showed that our fungal strains are very close to *A. tenuissima* isolates.

Pathogenicity test

Alternaria tenuissima inoculation produced initial symptoms 5 dpi on bitter gourd fruit. The symptoms observed were brown-colored circular and necrotic spots that became sunken. At later stages, the spots were enlarged in size and had irregular shapes, which coalesced and gradually covered the entire surface of the fruit (Figure 5). The re-isolation of fungal isolates from infected fruit yielded typical cultures of *A. tenuissima*, thus satisfied Koch's postulates. *A. tenuissima* was re-isolated from the inoculated fruits; control fruits inoculated with distilled water showed no symptoms. Based on morphological characters, molecular identification and pathogenicity test, the fungus was identified as *A. tenuissima*.

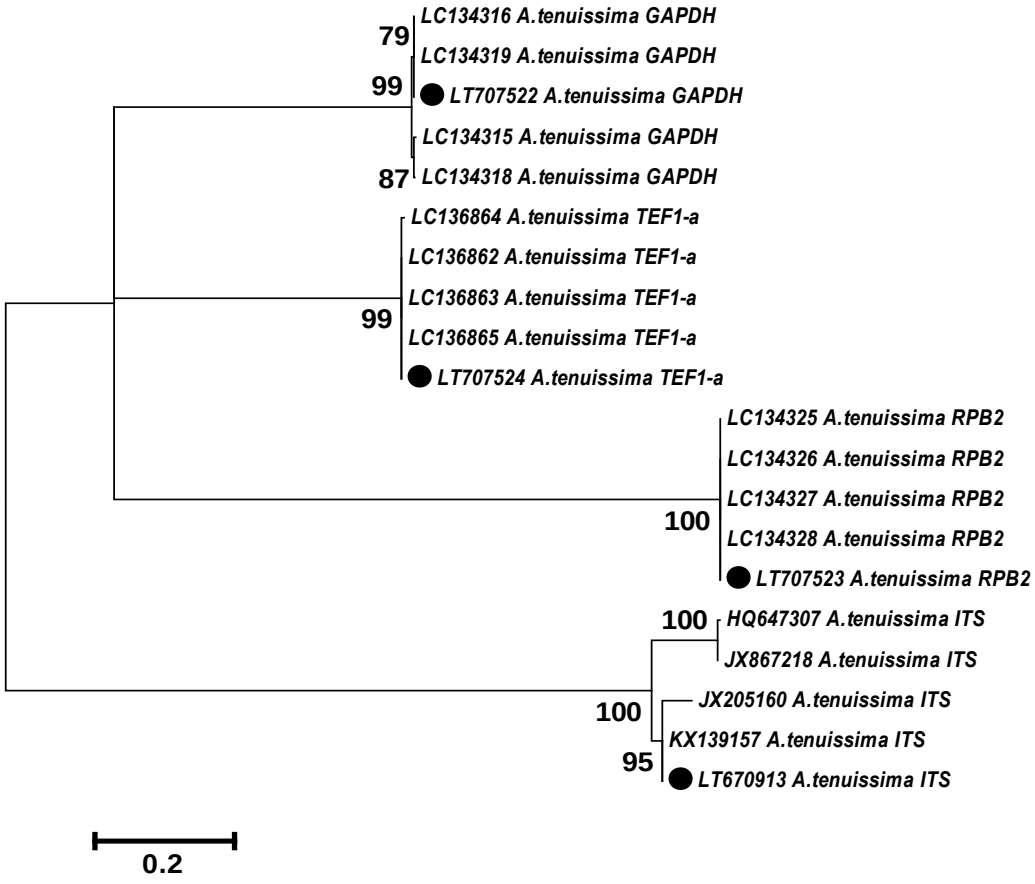

Figure 4. Unrooted phylogenetic tree representing the relationships among *Alternaria tenuissima* species. The *ITS1*, *GAPDH*, *TEF1-α*, and *RPB2* gene sequences of the isolates from this study were aligned with reference sequences of *Alternaria* spp. retrieved from GenBank using ClustalW© program. The phylogenetic tree was constructed by MEGA6 software version 6.0 (Tamura et al. 2013)

Figure 5. *Alternaria tenuissima* isolates symptoms development at 5 dpi. All the fruits were inoculated with one droplet (10 µL) of spore suspension of *A. tenuissima*. Arrow sign pointing towards the symptoms of *A. tenuissima*

Discussion

The family Cucurbitaceae includes vegetables with high nutritional value and are economically very important. However, due to various diseases, quality and yield have been reported by various farmers. *Alternaria* spp. are among the pathogens that cause serious damage to the crops including sorghum, wheat, broad beans and etc. Others include *A. tenuissima* and *A. cucumerina* on cucurbits (Neeraj and Verma 2010; Ziedan et al. 2018; Ma et al. 2020). In this study, *Alternaria tenuissima* was initially identified by analyzing morphological characters and further confirmed by molecular characterization as well as evaluated by the pathogenicity trials. The clear differentiation between the large-spored *Alternaria* spp. cannot be made on the basis of cultural and morphological characteristics as there are comparatively limited morphological features with narrow differences, which results in overlapping and incorrect identification of the isolates (Anees et al. 2010). The characteristics like colony color, shape and texture are also influenced by cultural conditions (Diguta et al. 2011). The limitations presented by the morphological characterization can be compensated by using the molecular techniques, which appear to be more precise and rapid (Buckingham 2011; Barley and Thomson 2016; Morin et al. 2019; Haridas et al. 2020).

The genus *Alternaria* was first recognized by Nees and Berkeley (Nees 1817; Berkeley 1836) as a pathogenic fungus causing diseases on various host plants belonging to the *Brassicaceae* family as *Macrosporium brassicae* Berk. Later, the fungus was renamed *Alternaria brassicae* (Berk.) (Saccardo 1886). Afterward, this genus was extensively studied by Neergaard specifically based on parasitism, economic significance and taxonomy (Neergaard 1945). Afterward Joly, (1964) described the morphological variations of *Alternaria* species by adopting a simplified key for characterization to confirm the identity of common species. Ellis (1976) reported the main characteristics of several *Alternaria* species as in "Dematiaceous Hyphomycetes" and "More Dematiaceous Hyphomycetes". Lawrence et al. (2013), divided the genus *Alternaria* into eight taxonomic groups, while later 19 additional phylogenetically distant clades were proposed by Woudenberg et al. (2013, 2014), Grum-Grzhimaylo et al. (2016), and Lawrence et al. (2016). Multiple *Alternaria spp.* namely *alternata, tenuissima, arborescens, brassicae, malorum*, and *infectoria* are among the most common species of *Alternaria* genus (Gabriel et al. 2016; Escrivá et al. 2017).

Recent diagnosis techniques e.g., PCR is more reliable for pathogen detection (Khan et al. 2018). The ribosomal genes and their ITS regions have been extensively used for distinguishing and naming of the species (Yang et al. 2020), along with the study of genetic diversity (Ghosh et al. 2019; Turzhanova et al. 2020), phylogenetic relationship (Ozkilinc et al. 2018; Liu et al. 2019; Tao et al. 2019), and taxonomic studies (Mahmoudi et al. 2017; Poursafar et al. 2018). In fungi, ITS region is used in species identification and phylogenetic studies of closely related species (Mmbaga et al. 2011). Since there is a higher degree of variation in ITS gene sequence, it has been extensively applied in molecular taxonomy to discriminate at species levels or sometimes even within species (Al Ghafri et al. 2019). The present results are parallel to those from India by Mukerji and Bhasin (1986), as they reported that the cause of fruit rot of *M. charantia* was *Alternaria tenuissima* (Kunze. ex Pers.). *Alternaria alternata* (Fr.) Keisler, *A. tenuissima* Kunze ex Pers has been reported in Pakistan by Sultana and Gaffar (2007) as seed inhabited pathogen of *M. charantia*. Various *Alternaria* species were found to be associated with various angiosperm families but *A. alternata* usually inflicts members of Cucurbitaceae. Besides, *A. tenuissima* and *A. cucumerina* have recently been reported to cause pathogenic infections on cucurbitaceous plants (Alka et al. 2020). In addition, *Alternaria* species were also previously reported to cause disease on other plants including potato, tomato and apple (Bessadat et al. 2014; Harteveld et al. 2014; Tymon et al. 2016).

In the present study, *TEF1-α*, *ITS1*, *GAPDH* and *RPB2* genes were amplified and sequenced for the confirmation of the identity of the isolates at the species level. Genetic relationship between *A. tenuissima* isolate was observed by

the neighbor-joining tree assembled on the basis of the available *ITS1*, *GAPDH*, *TEF1-α*, and *RPB2* gene sequences. A comparative analysis of gene sequences of *ITS1*, *GAPDH*, *TEF1-α*, and *RPB2* showed that our fungal isolates are very close to *A. tenuissima* isolates. According to our findings, it is the only study confirming the association of *Alternaria tenuissima* causing bitter gourd fruit rot. The effective post-harvest control measures can be designed against *Alternaria tenuissima* of *M. charantia* using the findings of current research work.

Morphological and molecular analyses revealed that the isolated fungus was typical of *Alternaria tenuissima*. Pathogenicity tests further confirmed Koch's postulate. Here we confirm that *A. tenuissima* is the cause of fruit rot of bitter gourd in Pakistan, while its potential extended speciation needs further investigation. Future studies need to be designed to understand the ecology, epidemiology and diversity of *Alternaria tenuissima* among other cultivated crops and vegetables. Moreover, the efforts should be directed to contain the further spread of pathogen by characterizing the pathogenicity genes and mode of infection, and to identify suitable resistance genes in crop plants to be exploited in the breeding programs.

ACKNOWLEDGEMENTS

The authors are grateful to the University of Punjab, Lahore for providing the funds to complete the study.

REFERENCES

Aksoy HM, Kaya Y, Ozturk M, Secgin Z, Onder H, Okumus A. 2017. *Pseudomonas putida*-induced response in phenolic profile of tomato seedlings (*Solanum lycopersicum* L.) infected by *Clavibacter michiganensis* subsp. *michiganensis*. Biol Control 105: 6-12. DOI: 10.1016/j.biocontrol.2016.11.001

Al Ghafri AB, Maharachchikumbura SS, Hyde KD, Al-Saady NA, Al-Sadi AM. 2019. A new section and a new species of *Alternaria* encountered from Oman. Phytotaxa 12: 279-89.

Alka K, Vandana K, Udit N. 2020. Symptomatology and etiology of *Alternaria* diseases of cucurbits in India. HortFlora Res Spectrum 9: 9-14.

Al-Nadabi HH, Maharachchikumbura SS, Agrama H, Al-Azri M, Nasehi A, Al-Sadi AM. 2018. Molecular characterization and pathogenicity of *Alternaria* species on wheat and date palms in Oman. Eur J Plant Pathol 15: 577-88. DOI: 10.1007/s10658-018-1550-4.

Anees M, Tronsmo A, Edel-Hermann V, Hjeljord LG, Héraud C, Steinberg C. 2010. Characterization of field isolates of *Trichoderma* antagonistic against *Rhizoctonia solani*. Fun Biol 114: 691-701. DOI: 10.1016/j.funbio.2010.05.007.

Asemaninejad A, Weerasuriya N, Gloor GB, Lindo Z, Thorn RG. 2016. New primers for discovering fungal diversity using nuclear large ribosomal DNA. PloS one 8: e0159043. DOI: 10.1371/journal.pone.0159043.

Barley AJ, Thomson RC. 2016. Assessing the performance of DNA barcoding using posterior predictive simulations. Mol Eco 25 (9): 1944-57. DOI: 10.1111/mec.13590.

Berbee M, Pirseyedi M, Hubbard S. 1999. *Cochliobolus* phylogenetics and the origin of known, highly virulent pathogens, inferred from ITS and glyceraldehyde-3-phosphate dehydrogenase gene sequences. Mycologia 91: 964-977. DOI: 10.1080/00275514.1999.12061106.

Berkeley MJ. 1836. Fungi. In: Smith JE (ed.) The English Flora. Academic Press. London.

Bessadat N, Benichou S, Kihal M, Henni DE. 2014. Aggressiveness and morphological variability of small spore *Alternaria* species isolated from Algeria. J Exp Biol Agric Sci 2: 265-278.

Bowers RM, Clements N, Emerson JB, Wiedinmyer C, Hannigan MP, Fierer N. 2013. Seasonal variability in bacterial and fungal diversity of the near-surface atmosphere. Env Sci Tech 47: 12097-106.

Buckingham L. 2011. Molecular diagnostics: Fundamentals, Methods and Clinical Applications, 2nd ed. F.A. Davis Company, Philadelphia.

Carbone I, Kohn LM. 1999. A method for designing primer sets for speciation studies in filamentous Ascomycetes. Mycologia 1: 553-556.

Dandawate PR, Subramaniam D, Padhye SB, Anant S. 2016. Bitter melon: A panacea for inflammation and cancer. Chinese J Nat Med 14: 81-100. DOI: 10.1016/S1875-5364(16)60002-X.

Diguta C, Vincent B, Guilloux-Benatier M, Alexandre H, Rousseaux S. 2011. PCR ITS-RFLP: A useful method for identifying filamentous fungi isolates on grapes. Food Microbiol 28: 1145-1154. DOI: 10.1016/j.fm.2011.03.006.

Elfar K, Zoffoli JP, Latorre BA. 2018. Identification and characterization of *Alternaria* species associated with moldy core of apple in Chile. Plant Dis 102: 2158-69. DOI: 10.1094/PDIS-02-18-0282-RE.

Ellis MB. 1976. More Dematiaceous Hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey.

Escrivá L, Oueslati S, Font G, Manyes L. 2017. *Alternaria* mycotoxins in food and feed: An overview. J Food Qual. DOI: 10.1155/2017/1569748.

Gabriel MF, Postigo I, Tomaz CT, Martínez J. 2016. *Alternaria alternata* allergens: Markers of exposure, phylogeny and risk of fungi-induced respiratory allergy. J Environ Intl 89: 71-80. DOI: 10.1016/j.envint.2016.01.003.

Gherbawy Y, Hussein MA, Runge F, Spring O. 2018. Molecular characterization of *Alternaria alternata* population isolated from Upper Egyptian tomato fruits. J Phytopath 166: 709-21. DOI: 10.1111/jph.12752.

Ghosh S, Mazumder M, Mondal B, Mukherjee A, De A, Bose R, Das S, Bhattacharyya S, Basu D. 2019. Morphological and SSR marker-based genetic diversity analysis of Indian mustard (*Brassica juncea* L.) differing in *Alternaria brassicicola* tolerance. Euphytica 215: 206. DOI: 10.1007/s10681-019-2523-1.

Grum-Grzhimaylo AA, Georgieva ML, Bondarenko SA, Debets AJM, Bilanenko EN. 2016. On the diversity of fungi from soda soils. Fungal Divers 76: 27-74. DOI: 10.1007/s13225-015-0320-2.

Gürdal B, Kültür Ş. 2013. An ethnobotanical study of medicinal plants in Marmaris (Muğla, Turkey). J Ethnopharmacology 146: 113-26. DOI: 10.1016/j.jep.2012.12.012.

Haridas S, Albert R, Binder M, Bloem J, LaButti K, Salamov A, Andreopoulos B, Baker SE, Barry K, Bills G, Bluhm BH. 2020. 101 Dothideomycetes genomes: A test case for predicting lifestyles and emergence of pathogens. Stud Mycol 96: 141-53. DOI: 10.1016/j.simyco.2020.01.003.

Harteveld DOC, Akinsanmi OA, Drenth A. 2014. Pathogenic variation of *Alternaria* species associated with leaf blotch and fruit spot of apple in Australia. Eur J Plant Pathol 139: 789-799. DOI: 10.1007/s10658-014-0433-6.

Joly P. 1964. Le Genre *Alternaria*. Paul Lechevalier, Paris.

Joseph B, Jini D. 2013. Antidiabetic effects of *Momordica charantia* (bitter melon) and its medicinal potency. Asian Pacific J Tropical Dis 1: 93-102. DOI: 10.1016/S2222-1808(13)60052-3.

Khan M, Wang R, Li B, Liu P, Weng Q, Chen Q. 2018. Comparative evaluation of the LAMP assay and PCR-based assays for the rapid detection of *Alternaria solani*. Front Microbiol 9: 1-11. DOI: 10.3389/fmicb.2018.02089.

Klopfenstein NB, Stewart JE, Ota Y, Hanna JW, Richardson BA, Ross-Davis AL, Elías-Román RD, Korhonen K, Keča N, Iturritxa E, Alvarado-Rosales D. 2017. Insights into the phylogeny of northern hemisphere *Armillaria*: Neighbor-net and Bayesian analyses of translation elongation factor 1-α gene sequences. Mycologia 109: 75-91. DOI: 10.1080/00275514.2017.1286572.

Lakshman DK, Jambhulkar PP, Singh V, Sharma P, Mitra A. 2016. Molecular identification, genetic diversity, population genetics and genomics of *Rhizoctonia solani*. In: Chowdappa P, Sharma P, Singh D, Misra AK (eds.). Perspectives of Plant Pathology in Genomic Era. Today & Tomorrow's Printers and Publishers. New Delhi.

Lawrence DP, Gannibal PhB, Peever TL, Pryor BM. 2013. The sections of *Alternaria*: Formalizing species-group concepts. Mycologia 105: 530-546. DOI: 10.1080/00275514.2017.1286572.

Lawrence DP, Rotondo F, GannibalPhB. 2016. Biodiversity and taxonomy of the pleomorphic genus *Alternaria*. Mycol Progress 15: 3. DOI: 10.1007/s11557-015-1144-x.

Li CJ, Tsang SF, Tsai CH, Tsai HY, Chyuan JH, Hsu HY. 2012. *Momordica charantia* extract induces apoptosis in human cancer cells through caspase-and mitochondria-dependent pathways. Evidence-Based Comp Altern Med. DOI: 10.1155/2012/261971.

Liu HF, Liao J, Chen XY, Liu QK, Yu ZH, Deng JX. 2019. A novel species and a new record of *Alternaria* isolated from two *Solanaceae* plants in China. Myc Prog 18: 1005-12. DOI: 10.1007/s11557-019-01504-3.

Liu YJ, Whelen S, Hall BD. 1999. Phylogenetic relationships among ascomycetes: Evidence from an RNA polymerse II subunit. Mol. Biol Evol 16: 1799-1808. DOI: 10.1093/oxfordjournals.molbev.a026092.

Luo Y, Hou L, Förster H, Pryor B, Adaskaveg JE. 2017. Identification of *Alternaria* species causing heart rot of pomegranates in California. Plant Dis 21: 421-427. DOI: 10.1094/PDIS-08-16-1176-RE.

Ma G, Bao S, Zhao J, Sui Y, Wu X. 2020. Morphological and molecular characterization of *Alternaria* Species causing leaf blight on watermelon in China. Plant Dis. DOI: 10.1094/PDIS-01-20-0130-RE.

Mahmoudi R, Shams-Bakhsh M, Safaie N. 2017. Phylogenetic analysis of *Alternaria raphani*, *A. arborescens* and *A. brassicicola* from canola in Iran. Biosci Biotechnol Res Commun 1: 105-11. DOI: 10.1111/ppa.12955.

Manamgoda DS, Cai L, McKenzie EH, Crous PW, Madrid H, Chukeatirote E, Shivas RG, Tan YP, Hyde KD. 2012. A phylogenetic and taxonomic re-evaluation of the *Bipolaris-Cochliobolus-Curvularia* complex. Fungal Div 56 131-144. DOI: 10.1007/s13225-012-0189-2.

Marakli S. 2018. A Brief Review of Molecular Markers to Analyse Medically Important Plants. Intl J Life Sci Biotechnol 1: 29-36.

Mmbaga MT, Shi A, Kim MS. 2011. Identification of *Alternaria alternata* as a causal agent for leaf blight in Syringa species. Plant Path J 27: 120-127. DOI: 10.5423/PPJ.2011.27.2.120.

Morin E, Miyauchi S, San Clemente H, Chen EC, Pelin A, de la Providencia I, Ndikumana S, Beaudet D, Hainaut M, Drula E, Kuo A. 2019. Comparative genomics of *Rhizophagus irregularis*, *R. cerebriforme*, *R. diaphanus* and *Gigaspora rosea* highlights specific genetic features in Glomeromycotina. New Phytol 222 (3): 1584-1598. DOI: 10.1111/nph.15687

Mukerji KG, Bhasin J. 1986. Plant diseases of India. A sourcebook. Tata Mc. Graw Hill Publishing Ltd., New Delhi.

Nagarani G, Abirami A, Siddhuraju P. 2014. Food prospects and nutraceutical attributes of *Momordica* species: A potential tropical bioresources-A review. Food Sci Human Wellness 3: 117-26. DOI: 10.1016/j.fshw.2014.07.001.

National Center for Biotechnology Information, 2017. BLAST: Basic Local Alignment Search Tool.

Neeraj N, Verma S. 2010. *Alternaria* diseases of vegetable crops and new approaches for its control. Asian J Exp Biol Sci 1: 681-692.

Neergaard P. 1945. Danish species of *Alternaria* and *Stemphylium* taxonomy, parasitism, economical significance. Oxford University Press, London.

Nees Von, Esenbeck GG. 1817. System der Plize Urid Schwamme. Stahel, Wurzburg.

Nowicki M, Nowakowska M, Niezgoda A, Kozik E. 2012. *Alternaria* black spot of crucifers: Symptoms, importance of disease, and perspectives of resistance breeding. Veg Crops Res Bull 76: 5-19.

Ozkilinc H, Rotondo F, Pryor BM, Peever TL. 2018. Contrasting species boundaries between sections *Alternaria* and *Porri* of the genus *Alternaria*. Plant Pathol 67: 303-314.

Paul A, Raychaudhuri SS. 2010. Medicinal uses and molecular identification of two *Momordica charantia* varieties: a review. Electronic J Bio 6: 43-51.

Polito L, Bortolotti M, Maiello S, Battelli MG, Bolognesi A. 2016. Plants producing ribosome-inactivating proteins in traditional medicine. Molecules 21: 1560. DOI: 10.3390/molecules21111560.

Poursafar A, Ghosta Y, Orina AS, Gannibal PB, Javan-Nikkhah M, Lawrence DP. 2018. Taxonomic study on *Alternaria* sections Infectoriae and Pseudoalternaria associated with black (sooty) head mold of wheat and barley in Iran. Mycol Progr 17: 343-356. DOI: 10.1007/s11557-017-1358-1.

Řezáčová V, Gryndler M, Bukovská P, Šmilauer P, Jansa J. 2016. Molecular community analysis of arbuscular mycorrhizal fungi-contributions of PCR primer and host plant selectivity to the detected community profiles. Pedobiologia 59 (4): 179-87. DOI: 10.1016/j.pedobi.2016.04.002.

Saad DY, Soliman MM, Baiomy AA, Yassin MH, El-Sawy HB. 2017. Effects of Karela (Bitter Melon; *Momordica charantia*) on genes of lipids and carbohydrates metabolism in experimental hypercholesterolemia: biochemical, molecular and histopathological study. BMC Compl Altern Med 17 (1): 319. DOI: 10.1186/s12906-017-1833-x.

Saccardo PA. 1886. Hyphomyceteae. In: Sylloge fungorumomninumhucusquecognitorum. The University of Illinois Library, Pavia, Italy.

Sales AJ, Pashazadeh M. 2020. Study of chemical composition and antimicrobial properties of rosemary (*Rosmarinus officinalis*) essential oil on *Staphylococcus aureus* and *Escherichia coli* in vitro. Intl J Life Sci Biotechnol 3: 62-69.

Sanger F, Nicklen S, Coulson AR. 1977. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463-5467. DOI: 10.1073/pnas.74.12.5463

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W. 2012. Fungal Barcoding Consortium. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc Nat Acad Sci USA 17: 6241-6.

Seena S, Pascoal C, Marvanová L, Cássio F. 2010. DNA barcoding of fungi: A case study using ITS sequences for identifying aquatic hyphomycete species. Fungal Divers 1: 77-87. DOI: 10.1007/s13225-010-0056-y.

Simmons EG. 2007. *Alternaria*, An Identification Manual. CBS Biodiversity Series. CBS Fungal Biodiversity Centre ,Utrecht.

Sultana N, Ghaffar A. 2007. Seed borne fungi associated with bitter gourd (*Momordica charantia*). Pak J Bot 39: 2121-2125.

Sung GH, Sung JM, Hywel-Jones, NL, Spatafora JW. 2007. A multi-gene phylogeny of Clavicipitaceae (Ascomycota, Fungi): Identification of localized incongruence using a combinational bootstrap approach. Mol Phyl Evol 44: 1204-1223. DOI: 10.1016/j.ympev.2007.03.011

Taheri-Ardestani S, Sharif-Nabi B, Zare R, Abbassi-Moghadam A. 2010. New *Alternaria* species associated with potato leaf spot in various potato growing regions of Iran. Iran J Plant Pathol 45: 83-86.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30 (12): 2725-2729. DOI: 10.1093/molbev/mst197.

Tao YQ, Jia GG, Aung SL, Wu QL, Lu HX, Deng JX. 2019. Multigene phylogeny and morphology of *Alternaria* reveal a novel species and a new record in China. Phytotaxa 397: 169-176.

Turzhanova A, Khapilina ON, Tumenbayeva A, Shevtsov V, Raiser O, Kalendar R. 2020. Genetic diversity of *Alternaria* species associated with black point in wheat grains. PeerJ. 8: e9097. DOI: 10.7717/peerj.9097.

Tymon LS, Cummings TF, Johnson DA. 2016. Pathogenicity and aggressiveness of three *Alternaria* spp. on potato foliage in the U.S. North-west. Plant Dis 100: 797-800.

Wang YB, Yu H, Dai YD, Wu CK, Zeng WB, Yuan F, Liang ZQ. 2015. *Polycephalomyces agaricus*, a new hyperparasite of *Ophiocordyceps* sp. infecting melolonthid larvae in southwestern China. Mycol Prog 1: 70.

Wei J, Ma W, Liu X, Xu J, Zhang N, Shao W, Chen R, Xu J, Yu G. 2020. First report of leaf spot on *Sorghum bicolor* caused by *Alternaria tenuissima* in China. Plant Dis 104: 2729.

White TJ, Bruns T, Lee SJWT, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols: A Guide to Methods and Applications 18: 315-322. Academic Press, New York.

Woudenberg JHC, Groenewald JZ, Binder M, Crous PW. 2013. *Alternaria* redefined. Stud Mycol 75: 171-212.

Woudenberg JHC, Truter M, Groenewald JZ, Crous PW. 2014. Large-spored *Alternaria* pathogens in section Porri disentangled. Stud Mycol 79: 1-47.

Xu X, Shan B, Liao CH, Xie JH, Wen PW, Shi JY. 2015. Anti-diabetic properties of *Momordica charantia* L. polysaccharide in alloxan-induced diabetic mice. Intl J Bio Macromol 81: 538-543. DOI: 10.1016/j.ijbiomac.2015.08.049

Yang Y, Wang XL, Ji JJ, Chen ZQ, Qu ZY, Liu Y, Zhang YW. 2020. First report of purple spot on dandelion in China caused by *Alternaria tenuissima*. Plant Dis. DOI: 10.1094/PDIS-05-20-1037-PDN.

Zhang LJ, Liaw CC, Hsiao PC, Huang HC, Lin MJ, Lin ZH, Hsu FL, Kuo YH. 2014. Cucurbitane-type glycosides from the fruits of *Momordica*

charantia and their hypoglycaemic and cytotoxic activities. J Fun Foods 6: 564-574. DOI: 10.1016/j.jff.2013.11.025

Ziedan ES, Khattab AE, Sahab AF. 2018. New fungi causing postharvest spoilage of cucumber fruits and their molecular characterization in Egypt. J Plant Protec Res 362-371.