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Full Length Article

Molecular Characterization Molecular Characterization of Fungal Pathogens Associated with Citrus Withertip/Dieback from Major Citrus Growing Areas of Punjab, Pakistan

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Abstract

Citrus withertip/dieback is an emerging threat to the citrus production which results into yield losses including death of the plants. Characteristic symptoms are leaf chlorosis, drooping of the leaves, dieback spreading towards main stem and black pustules on the dead twigs. The current study was designed for molecular characterization of casual organism associated with citrus withertip/dieback. Samples were collected from four main citrus growing district (Sargodha, Kot Momin, Bhalwal and Faisalabad) of Punjab, Pakistan. It was found that different species of *Colletotrichum* were associated with citrus withertip/dieback on morphological basis. Pathogenicity test were performed following Koch's postulates to confirm the association of fungal species with citrus dieback. To characterize the pathogens, Whole genome of seven Pakistani isolates were sequenced, submitted to NCBI and have given accession numbers. Multi-gene phylogenetic and Average Nucleotide identity (ANI) analysis revealed that two different species of *Colletotrichum*; *C. gloeosporioides* and *C. siamense* were responsible for Citrus withertip/dieback in Punjab, Pakistan. © 2020 Friends Science Publishers

Keywords: *Colletotrichum*; Citrus; Punjab; Dieback; Average Nucleotide identity

Introduction

Pakistan is in top 15 *Citrus* producing countries and area covered by *Citrus* is 206569 ha producing 1907.4 thousand tonnes, 98% of which is produced by Punjab province only (FAO 2017). Area in Punjab with *Citrus* orchards is 183210 ha with 2135.895 thousand tones production (GOP 2016–2017). The most widely grown and used variety is kinnow (*Citrus reticulata*) which covers the 86% of the total area in Pakistan (Altaf 2006). Important districts for citrus cultivation are Sargodha, Sahiwal, Multan, Jhang, Lahore, Gujranwala, Mianwali and Sialkot.

Citrus withertip/dieback is serious damage causing disease in Pakistan. Symptoms are characterized by yellowing of the leaves and drooping of leaves occurs as wilting progressed leading to death of the plants. Symptoms start from the tip of the twigs and extend towards the main stem causing dieback (Al-Sadi *et al*. 2014). Black pustules were clearly observed on dead twigs and in severe cases silvery appearance was found on twigs. Citrus withertip was first time reported in Mexico by Benyahia *et al*. (2003) caused by *Colletotrichum gloeosporioides*. In Ghana, *C. gloeosporioides* was found to be associated with Cassava stem tip dieback disease (Moses *et al*. 1996). Eucalyptus dieback was also caused by *C. gloeosporioides* in South Africa (Smith *et al*. 1998). In Oman, *Fusarium solani*, *Lasiodiplodia theobromae*, *Neoscytalidium dimidiatum* and *L. hormozganensis* were discovered to be associated with *Citrus* on lime seedlings (Al-Sadi *et al*. 2014).

Lately, molecular techniques based on DNA have been developed for the identification and characterization of fungal species. In past, there are many studies in which phylogeny is inferred using internal transcribed spacer (ITS) region of rDNA to distinguish the complex fungal species (Cen *et al*. 1994; Moses *et al*. 1996; Smith *et al*. 1998) but using ITS sequences alone are skeptical to identify and characterize the closely related complex fungal species (Weir *et al*. 2012; Hassan *et al*. 2018). While the technology progressed, different methods have been adapted all over the world for identification and diversification of the species among the genera (Liu *et al*. 2016; Rodriguez-Galvez *et al*. 2017). ITS sequences alone cannot distinguish the *Colletotrichum* species. For *C. gloeosporioides* complex, six protein coding genes are used as secondary barcodes other than ITS region; actin (ACT), glutamine synthetase (GS), glyceraldehyde 3-phosphate dehydrogenase (GAPDH),

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calmodulin (CAL), chitin synthase (CHS) and beta-tubulin (βTUB) (Weir *et al*. 2012; Hassan *et al*. 2018).

Although, citrus withertip/dieback is an emerging disease, it has been neglected from many years. Despite the importance of this disease no cultural and molecular studies have been performed to identify the causal agent(s). It has become a necessity to fill this gap to understand the etiology of this disease which will enable us to adopt better management practices. Current study was designed to properly investigate the causal agent of citrus withertip/dieback in the orchards of Punjab, Pakistan and to become a milestone for future studies.

Materials and Methods

Samples collection

In 2015–2016, samples were obtained from orchards of four major citrus growing areas of Punjab province (Sargodha, Kot Momin, Bhalwal and Faisalabad) from plants showing characteristic symptoms of withertip/dieback (Fig. 1). Samples were obtained from two orchards of each district. The diseased samples were collected from trees of cultivar Kinnow (*C. reticulata*). The diseased samples were placed in paper bags with labels, were brought to Molecular Plant Pathology laboratory, University of Agriculture, Faisalabad (UAF), Pakistan and placed in refrigerator at 4°C for further use.

Morphological identification and pathogenicity analysis

Diseased portion from the edges of lesions were cut into 2–3 mm size pieces and used for isolation of pathogen. Samples were disinfected with 70% ethanol for one minute and with distilled water again. These samples were placed aseptically onto Potato Dextrose Agar (PDA) media and incubated at 30–35°C in N-BIOTEK incubator (NB-205LF) for 6 days and observe daily for growth. Sub culturing was done from mycelium on PDA in the test tubes (30 mL cap.) to get pure culture and then stored in refrigerator at 4°C. Different morphological characters; colony colour and growth, conidia size and fruiting bodies were observed.

Pathogenicity trials were done by using Koch's postulates in green house. One-year old kinnow plants were transferred in pots (9″ (diameter) x 8.5″ (depth)) filled using aseptic soil. For preparation of spore suspension, the protocol of Mello *et al*. (2004) was followed. The counting of spores was done with hemocytometer and 10^4/mL spore suspension was inoculated on healthy plants by spraying. After the appearance of symptoms, re-isolation was done following the Koch's postulates.

Whole genome sequencing and phylogenetic analysis

Fungal DNA was extracted by modified CTAB method (Moller *et al*. 1992) from pure fungal culture. Pellet were dissolved in 25 μL of sterile distilled water (SDW) and stored at -20°C. Extracted DNA was used for the preparation of sequencing libraries at the Cook's Lab, University of California, Davis, USA, following Qiagen® QIAseq FX ®DNA Library Kit protocol. Illumina HiSeq 4000 sequencing was performed at the Genome Center, University of California, Davis, USA. Assembly of genomes was done by using SPAdes 3.9.0 pipeline (Bankevich *et al*. 2012). Assemblies were produced using 'careful' mode in SPAdes to avoid miss-pairing of contigs by scaffolding. Phylogenetic relationship was determined by ML method of MEGA 7.0 with 1000 replicate of six genes (ACT, CAL, CHS1, GAPDH, GS, TUB2) along with ITS to identify the species of *Colletotrichum*. Average nucleotide identity (ANI) was measured by using Pyani (Pritchard *et al*. 2016).

Fig. 1: Trees showing characteristics symptoms of withertip/dieback: **A**; progressed dieback no fruit formation, **B**; onset of dieback, **C**; Leaf chlorosis

Results

Morphological characters

After isolation and purification, two different groups of fungi were identified belonging to *C. gloeosporioides* complex. One group resembles with *C. gloeosporioides*; Grey-orange color colony growth with concentric rings was observed on the opposite side of the petri dish. Wooly thick mycelium was hyaline and septate. Black conidiophores were also observed and conidia were hyaline with size of 15–20 × 2.5–5 μm, cylindrical with obtuse base and rounded apex (Fig. 2). Other groups members resemble with *C. siamense*; White colored colony growth observed which later turn into grey color with scattered light orange

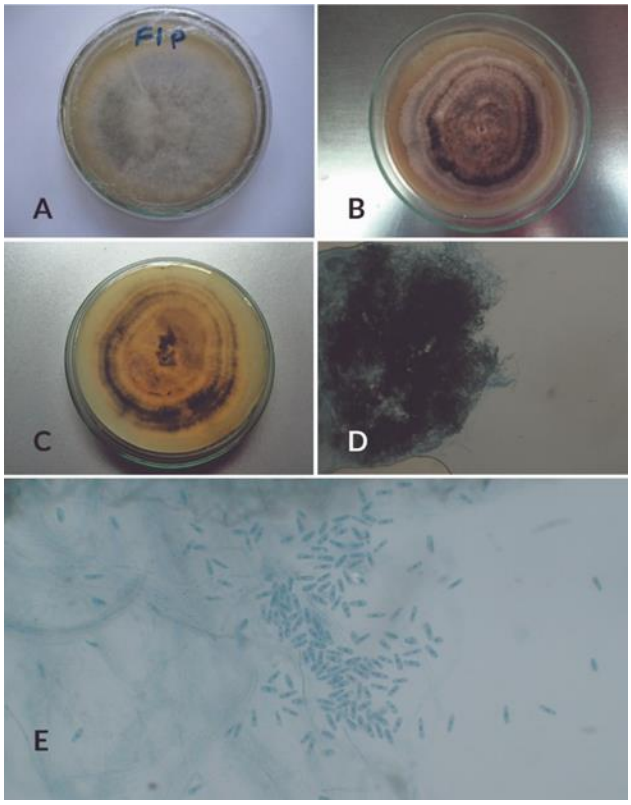

Fig. 2: Morphological characteristics of *Colletotrichum*-Group-a; **A**: Orange grey mycelial growth of representative isolate of the group, **B**: Black colony growth after 10 days, **C**: opposite side of the petri dish showed orange circular growth, **D**: Ruptured conidiophores, **E**: conidia and mycelium under microscope at 20X

acervuli. Mycelium was hyaline and septate. Conidia were hyaline with size of 12.5–20 × 3.5–5.5 μm, cylindrical to fusiform in shape and aseptate (Fig. 3).

Pathogenicity assay

Pathogenicity trial on one-year old Kinnow plants confirmed that causal agents of the citrus withertip/dieback are *C. siamense* and *C. gloeosporioides*. Onset of disease was recorded after 10 days of inoculation from tips of the plants following leaf chlorosis, defoliation and dieback. Plants died after four weeks of inoculation. Characteristic symptoms were recorded during the pathogenicity trial (Fig. 4). Re-isolation of the fungi was performed successfully to confirm the Koch's postulates from disease seedlings.

Molecular identification and phylogenetic analyses

Genome size of *C. siamense* strains were ~56 Mbp and *C. gloeosporioides* were of 56–61 Mbp. The assembled genomes were deposited to NCBI and accession number were assigned to respective genomes (Table 1). Six genes (ACT, CAL, CHS1, GAPDH, GS, TUB2) along

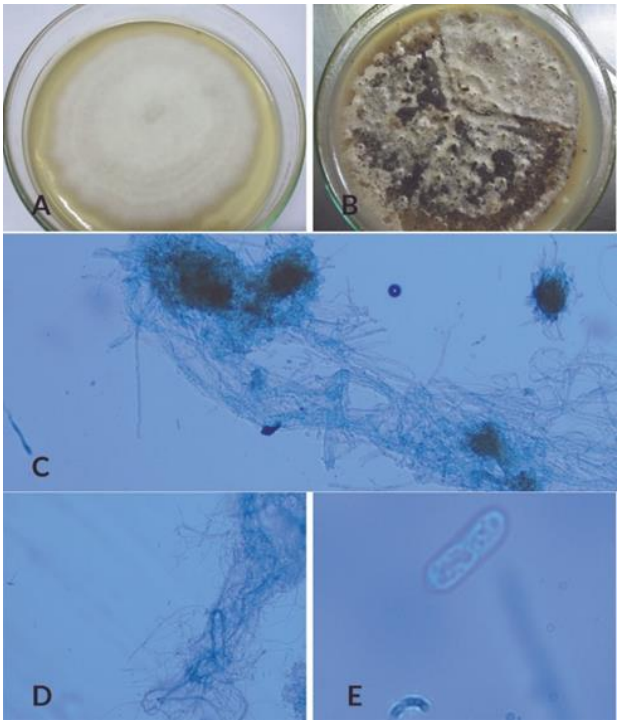

Fig. 3: Morphological characteristics of *Colletotrichum*-Group-b; **A**: White mycelial growth of representative isolate of the group, **B**: Colony growth after 14 days showed scattered orange and black conidiophore, **C**: fruiting bodies along with mycelium, **D**: conidia and mycelium under microscope (20X), **E**: At 40X showing granular structure of conidia

Fig. 4: Pathogenicity Trail on one-year old *Citrus* plants; **A**: Onset of disease; **B**: Plant died after four weeks post-inoculation

with ITS were extracted, concatenated and used to identify and to inferred the phylogeny of different *Colletotrichum* species presented in Table 2. The inferred phylogeny revealed two *Colletotrichum* species. COLG-31 and COLG-95 had affinity to *C. gloeosporioides*_IMI-356878 with a bootstrap value of 100%. While COLG-34, COLG-38, COLG-44, COLG-50 and COLG-90 grouped with different *C. siamense* isolates, also with strong bootstrap support

Table 1: Pakistani strains isolated from different district of Punjab

Strains	No. of scaffolds	Longest fragment	Shortest fragment	Genome size	Rate of N	Rate of GC	Scaffold N50	No. of sequences >=3 kb	Accession Number
COLG-31	4142	149620	128	56492417	1.07E-05	0.52263813	25160	3084	VNWS00000000
COLG-34	17516	100753	128	61427343	4.23E-06	0.52996204	16081	4046	WEZJ00000000
COLG-38	8793	261654	128	59174470	3.89E-06	0.53030821	38314	2165	WEZK00000000
COLG-44	6278	115451	128	58427301	1.49E-05	0.52538398	16594	4337	WEZL00000000
COLG-50	6784	80160	128	56927278	8.78E-07	0.52498492	16038	4281	WEZM00000000
COLG-90	5456	279223	128	57669018	1.73E-06	0.52938951	48402	1875	WEZN00000000
COLG-95	5237	373335	60	56028021	1.03E-05	0.52332074	20160	3635	WEZO00000000

Table 2: The list of fungal genomes used for comparative analysis

Isolates Used in the analysis	Strain name	Gene Bank Assembly Accession No.	Host	Origin
C. fructicola	1104-7	GCA_002314275.1	Apple	China
C. fructicola	15060	GCA_002887685.1	Mango	China
C. fructicola	Nara gc5	GCA_000319635.1	*Fragaria x ananassa*	Japan
C. gloeosporoides	030206	GCA_002189585.1	Apple	China
C. gloeosporoides	cg-14	GCA_000446055.1	Avocado cv. Fuente	Israel
C. gloeosporoides	cg01	GCA_003666125.1	*Huperzia serrata*	China
C. gloeosporoides	ES026	GCA_003568745.1	*H. serrata*	China
C. gloeosporoides	TYU	GCA_002901105.1	*Taxus cuspidata*	South Korea
C. incanum	MAFF238704	GCA_001625285.1	*Raphanus sativus* L.	Japan
C. incanum	MAFF238712	GCA_001855235.1	*R. sativus* var. *longipinnatus*	Japan
C. graminicola	M1001	GCA_000149035.1	N/A	N/A
C. graminicola	M5	GCA_001951205.1	*Zea mays*	Brazil
C. sublineola	CgSl1	GCA_001951195.1	*Sorghum bicolor*	USA
C. sublineola	TX430BB	GCA_000696135.1	*S. bicolor*	USA
C. higginsianum	IMI 349063	GCA_001672515.2	*Brassica rapa* subspp. *chinensis*	Trinidad & Tobago
C. coccodes	NJ-RT1	GCA_002249775.1	pepper fruit	USA
C. coccodes	RP180a	GCA_002249805.1	pepper fruit	USA
C. tofieldiae	0861	GCA_001625265.1	*Arabidopsis thaliana*	Spain
C. tofieldiae	CBS 127615	GCA_001618715.1	*Agapanthus* spp.	Portugal
C. tofieldiae	CBS 168.49	GCA_001618705.1	*Lupinus polyphyllus*	Germany
C. tofieldiae	CBS 495.85	GCA_001618725.1	*Tofieldia calyculata*	Switzerland
C. tofieldiae	CBS 130851	GCA_001618735.1	*Semele androgyna*	Germany
C. fioriniae	HC89	GCA_002930455.1	Apple	USA
C. fioriniae	PJ7	GCA_000582985.1	*Fragaria x ananassa*	New Zealand
C. fioriniae	HC91	GCA_002930425.1	N/A	N/A
C. acutatum	C71	GCA_001662755.1	N/A	N/A
C. acutatum	1	GCA_001593745.1	*Capsicum annuum*	Korea
C. nymphaeae	SA-01	GCA_001563115.1	N/A	N/A
C. lindemuthianum	89 A2 2-3	GCA_001693025.2	*Phaseolus vulgaris*	USA
C. lindemuthianum	83.501	GCA_001693015.2	*P. vulgaris*	USA
C. salicis	CBS 607.94	GCA_001563125.1	*Salix* spp.	Netherlands
C. spinosum	CBS 515.97	GCA_004366825.1	N/A	N/A
C. simmondsii	CBS122122	GCA_001563135.1	Papaya	Australia
C. tanaceti	BRIP57314	GCA_005350895.1	*Tanacetum cinerariifolium*	Australia
C. sansevieriae	Sa-1-2	GCA_002749775.1	N/A	Japan
Colletotrichum spp.	JS-367	GCA_003122705.1	Mulberry	South Korea
C. gloeosporioides	SMCG1	GCA_003243855.1	Chinese fir	China
C. chlorophyti	NTL11	GCA_001937105.1	N/A	N/A
C. lentis	CT-30	GCA_003386485.1	*Lens culinaris* sspp. *culinaris*	Canada
C. orbiculare	104-T	GCA_000350065.2	Cucumber	N/A
C. sidae	CBS 518.97	GCA_004367935.1	N/A	N/A
C. truncatum	MTCC 3414	GCA_002632455.2	*Capsicum annuum*	India
C. trifolii	543-2	GCA_004367215.1	N/A	N/A
C. falcatum	Cf671	GCA_001484525.1	Sugarcane	India
C. musae	GM20	GCA_002814275.1	N/A	N/A
C. higginsianum	MAFF305635	GCA_004920355.1	N/A	N/A
C. graminicola	M5	GCA_001951205.1	Maize	Brazil
Colletotrichum spp.	PG-2018a	GCA_006783085.1	Perilla	N/A

(Fig. 5). Another method of pairwise comparison was used to determine the specie relationship using Average nucleotide identity (ANI) of 95%. ANI_{95} also supported the phylogenetic analysis dividing the isolates into two different groups. COLG-31 and COLG-95 grouped with *C. gloeosporioides* with high ANI value (98%) along with high pairwise genome alignment coverage (92–96%), supporting strong relationship with *C. gloeosporioides*. Strains COLG-

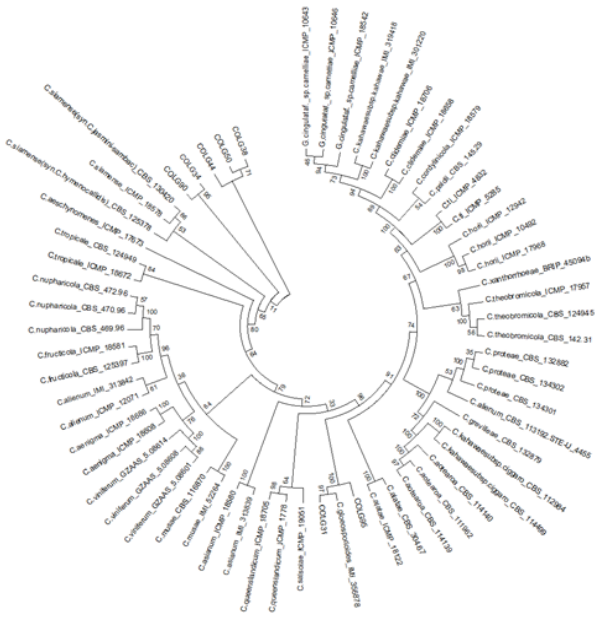

Fig. 5: Phylogenetic analysis using ITS, ACT, CAL, CHS1, GAPDH, GS, TUB2 genes of Pakistani isolates with Publicly available strains from NCBI by ML method

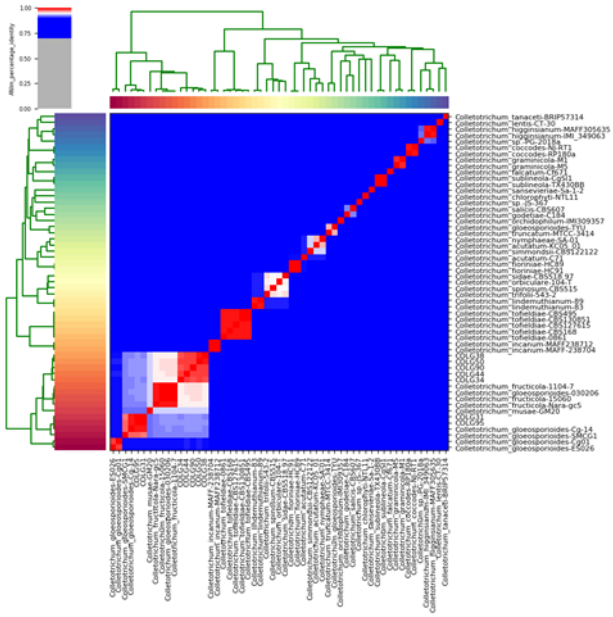

Fig. 6: Average Nucleotide identity (ANI) Analysis all the genomes available on NCBI along with Pakistani isolates dividing into two distinguish groups; *C. siamense* and *C. gloeosporioides*

34, COLG-38, COLG-44, COLG-50 and COLG-90 formed individual group with 99% ANI and 92–94% genome alignment coverage (Fig. 6). Phylogenetic analysis revealed close affinity with *C. siamense*, however no genome for *C. siamense* was available in public databases to compare with before June 2019.

Discussion

Citrus withertip/dieback has been prevailing in the Pakistan from decades; however, the pathogens associated with this disease have never been identified and characterized on molecular basis. For this purpose, the study was designed to identify and characterize the pathogens associated with citrus withertip/dieback which ultimately will help in better understanding of the disease and its management. In 2015, samples were collected from different orchards of Punjab; dieback symptoms were observed resulting considerable yield losses following the complete plant death. The recent discovery of several new species of fungi associated with tropical plants led us to speculate that more than one fungus is associated with dieback of *Citrus* in Pakistan. *In vitro* morphological characters are important for distinguishing among *Colletotrichum* species (Hu *et al*. 2015) and on this basis the isolated fungi were further divided into 2 sub-groups. The identified isolates of *C. gloeosporioides* and *C. siamense* showed distinct colony morphologies, with small variations in appressoria, conidia sizes and shape, which were similar to morphological characters of *C. gloeosporioides* (Kimaru *et al*. 2018) *and C. siamense* (Cristóbal-Martínez *et al*. 2017) (Fig. 2 and 3). All of the tested *Colletotrichum* isolates showed similar symptoms and degree of virulence when tested for Koch's postulates. For molecular characterization of isolated pathogenic fungi, high quality draft genomes were produced to check the gene contents of both species and to understand the host-pathogen interaction. Since ITS region is not enough to identify the *Colletotrichum* species (Weir *et al*. 2012), so six different genes along with ITS (ACT, CAL, CHS1, GAPDH, GS, TUB2) was used to identify different species in given isolates. However, in previous research only *C. gloeosporioides* and *C. theobromicola* were reported to cause dieback (Smith *et al*. 1998; Singh *et al*. 2015; Hawk *et al*. 2018). *C. siamense* is first time reported to cause dieback on citrus along with *C. gloeosporioides*. ANI_{95} has been used widely to differentiate the isolates on specie level (Goris *et al*. 2007; Richter and Rosselló-Móra 2009). Average nucleotide identity (ANI) analysis and phylogenetics analysis clearly distinct the isolates belonging to two different species within the Musae clade. Until now, *F. solani*, *L. hormozganensis*, *N. dimidiatum* and *L. theobromae* is found to be associated with Citrus Dieback (Ferrari *et al*. 1996; Al-Sadi *et al*. 2014). But present studies revealed that *C. gloeosporioides* and *C. siamense* for citrus dieback/withertip in Punjab, Pakistan. These findings led to the better understanding of the disease and can help for further analysis to establish management strategies for Citrus withertip/ dieback. In Future, these isolates may play a fundamental part in refining our understanding to the extent of cryptic species diversity in *Colletotrichum* complexes.

Conclusion

We identified two species from *C. gloeosporioides* complex that caused dieback on citrus, based on morphological, pathogenesis, and molecular analyses. The identification of a new *Colletotrichum* spp. causing dieback on citrus redirects the importance of further research on *Colletotrichum* taxonomy to alleviate the risk to the citrus fruit industry not only in Pakistan but throughout the world.

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