



Full Length Article

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),

calmodulin (CAL), chitin synthase (CHS) and beta-tubulin (β TUB) (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 μ L of sterile distilled water (SDW) and



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

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).

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. Woolly thick mycelium was hyaline and septate. Black conidiophores were also observed and conidia were hyaline with size of 15–20 × 2.5–5 μ m, 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

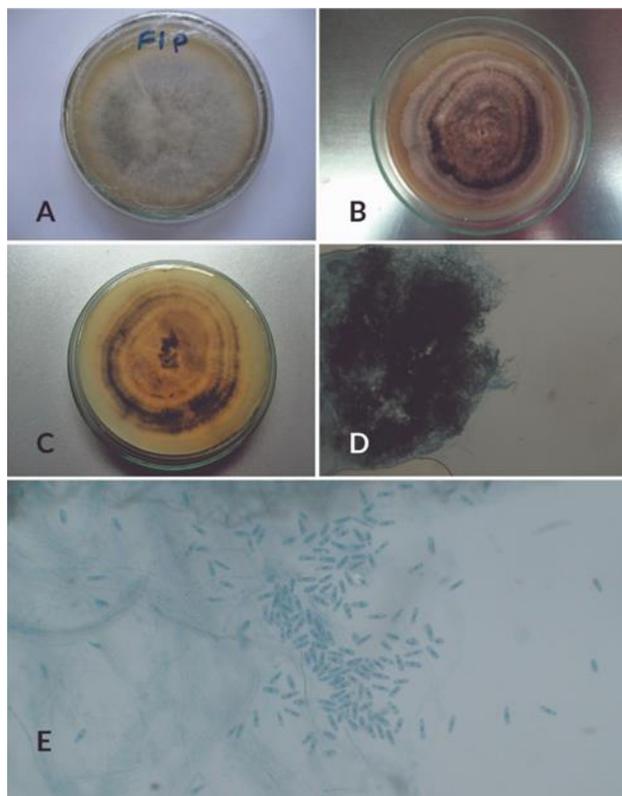


Fig. 2: Morphological characteristics of *Colletotrichum*-Group-a; **A:** Orange grey mycelial growth of representative isolate of the group, **B:** Black colony growth after 10 days, **C:** opposite side of the petri dish showed orange circular growth, **D:** Ruptured conidiophores, **E:** conidia and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

acervuli. Mycelium was hyaline and septate. Conidia were hyaline with size of $12.5\text{--}20 \times 3.5\text{--}5.5 \mu\text{m}$, 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

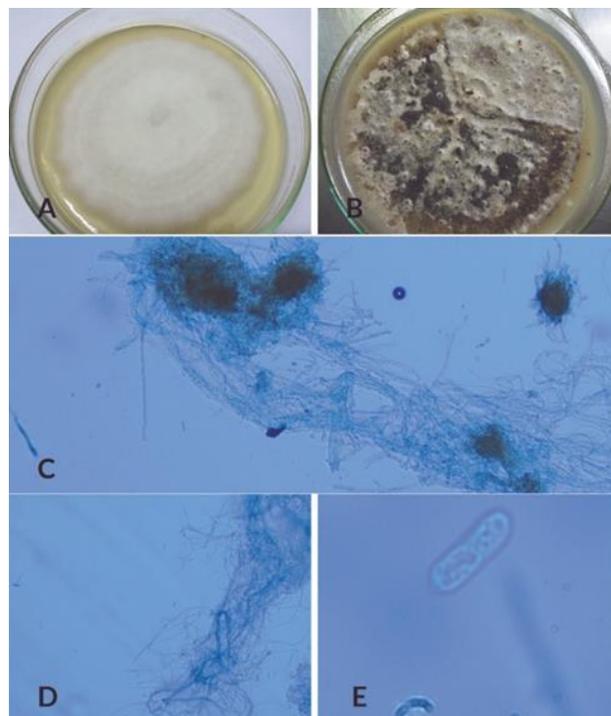


Fig. 3: Morphological characteristics of *Colletotrichum*-Group-b; **A:** White mycelial growth of representative isolate of the group, **B:** Colony growth after 14 days showed scattered orange and black conidiophore, **C:** fruiting bodies along with mycelium, **D:** conidia and mycelium under microscope (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 infer 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
<i>C. fructicola</i>	I104-7	GCA_002314275.1	Apple	China
<i>C. fructicola</i>	15060	GCA_002887685.1	Mango	China
<i>C. fructicola</i>	Nara gc5	GCA_000319635.1	<i>Fragaria x ananassa</i>	Japan
<i>C. gloeosporioides</i>	030206	GCA_002189585.1	Apple	China
<i>C. gloeosporioides</i>	cg-14	GCA_000446055.1	Avocado cv. Fuente	Israel
<i>C. gloeosporioides</i>	cg01	GCA_003666125.1	<i>Huperzia serrata</i>	China
<i>C. gloeosporioides</i>	ES026	GCA_003568745.1	<i>H. serrata</i>	China
<i>C. gloeosporioides</i>	TYU	GCA_002901105.1	<i>Taxus cuspidata</i>	South Korea
<i>C. incanum</i>	MAFF238704	GCA_001625285.1	<i>Raphanus sativus</i> L.	Japan
<i>C. incanum</i>	MAFF238712	GCA_001855235.1	<i>R. sativus</i> var. <i>longipinnatus</i>	Japan
<i>C. graminicola</i>	M1001	GCA_000149035.1	N/A	N/A
<i>C. graminicola</i>	M5	GCA_001951205.1	<i>Zea mays</i>	Brazil
<i>C. sublineola</i>	CgS11	GCA_001951195.1	<i>Sorghum bicolor</i>	USA
<i>C. sublineola</i>	TX430BB	GCA_000696135.1	<i>S. bicolor</i>	USA
<i>C. higginsianum</i>	IMI 349063	GCA_001672515.2	<i>Brassica rapa</i> subsp. <i>chinensis</i>	Trinidad & Tobago
<i>C. coccodes</i>	NJ-RT1	GCA_002249775.1	pepper fruit	USA
<i>C. coccodes</i>	RP180a	GCA_002249805.1	pepper fruit	USA
<i>C. tofieldiae</i>	0861	GCA_001625265.1	<i>Arabidopsis thaliana</i>	Spain
<i>C. tofieldiae</i>	CBS 127615	GCA_001618715.1	<i>Agapanthus</i> spp.	Portugal
<i>C. tofieldiae</i>	CBS 168.49	GCA_001618705.1	<i>Lupinus polyphyllus</i>	Germany
<i>C. tofieldiae</i>	CBS 495.85	GCA_001618725.1	<i>Tofieldia calyculata</i>	Switzerland
<i>C. tofieldiae</i>	CBS 130851	GCA_001618735.1	<i>Semele androgyna</i>	Germany
<i>C. fioriniae</i>	HC89	GCA_002930455.1	Apple	USA
<i>C. fioriniae</i>	PJ7	GCA_000582985.1	<i>Fragaria x ananassa</i>	New Zealand
<i>C. fioriniae</i>	HC91	GCA_002930425.1	N/A	N/A
<i>C. acutatum</i>	C71	GCA_001662755.1	N/A	N/A
<i>C. acutatum</i>	1	GCA_001593745.1	<i>Capsicum annuum</i>	Korea
<i>C. nymphaeae</i>	SA-01	GCA_001563115.1	N/A	N/A
<i>C. lindemuthianum</i>	89 A2 2-3	GCA_001693025.2	<i>Phaseolus vulgaris</i>	USA
<i>C. lindemuthianum</i>	83.501	GCA_001693015.2	<i>P. vulgaris</i>	USA
<i>C. salicis</i>	CBS 607.94	GCA_001563125.1	<i>Salix</i> spp.	Netherlands
<i>C. spinosum</i>	CBS 515.97	GCA_004366825.1	N/A	N/A
<i>C. simmondsii</i>	CBS122122	GCA_001563135.1	Papaya	Australia
<i>C. tanacetii</i>	BRIP57314	GCA_005350895.1	<i>Tanacetum cinerariifolium</i>	Australia
<i>C. sansevieriae</i>	Sa-1-2	GCA_002749775.1	N/A	Japan
<i>Colletotrichum</i> spp.	JS-367	GCA_003122705.1	Mulberry	South Korea
<i>C. gloeosporioides</i>	SMCG1	GCA_003243855.1	Chinese fir	China
<i>C. chlorophyti</i>	NTL11	GCA_001937105.1	N/A	N/A
<i>C. lentis</i>	CT-30	GCA_003386485.1	<i>Lens culinaris</i> ssp. <i>culinaris</i>	Canada
<i>C. orbiculare</i>	104-T	GCA_000350065.2	Cucumber	N/A
<i>C. sidae</i>	CBS 518.97	GCA_004367935.1	N/A	N/A
<i>C. truncatum</i>	MTCC 3414	GCA_002632455.2	<i>Capsicum annuum</i>	India
<i>C. trifolii</i>	543-2	GCA_004367215.1	N/A	N/A
<i>C. falcatum</i>	Cf671	GCA_001484525.1	Sugarcane	India
<i>C. musae</i>	GM20	GCA_002814275.1	N/A	N/A
<i>C. higginsianum</i>	MAFF305635	GCA_004920355.1	N/A	N/A
<i>C. graminicola</i>	M5	GCA_001951205.1	Maize	Brazil
<i>Colletotrichum</i> 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%. ANI₉₅ 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-

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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