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MOLECULAR CHARACTERIZATION OF *LASIODIPLODIA THEOBROMAE* ASSOCIATED WITH CITRUS GUMMOSIS FROM MAJOR CITRUS GROWING AREAS OF PUNJAB, PAKISTAN

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ABSTRACT

The worldwide extension of cultivation areas of citrus has contributed to the emergence of various diseases. Gummosis is one of the most significant diseases of citrus. The disease is caused by several species of *Botryosphaeriacae,* the most prominent ones are *Lasiodiplodia* spp. The objective of this study was molecular identification and characterization of *Lasiodiplodia theobromae* associated with citrus gummosis from major citrus growing areas of Punjab, Pakistan. This study includes activities like collection of diseased samples, isolation, morphological identification, molecular characterization and pathogenicity of *L. theobromae* on citrus bark tissues. To identify the cause of citrus gummosis disease, symptomatic diseased bark tissues were collected from 30 localities of 5 cities of the province Punjab. *L. theobromae* was isolated from all the infected samples. Preliminary identification was based on morphological and cultural characters of isolated fungi. For molecular studies nucleotide sequences of the ITS region (ITS1-5.8S-ITS2) was used. For pathogenicity analysis all identified isolates of *L. theobromae* inoculated on *Citrus jambhiri* which is commonly used as rootstock in Punjab. Isolates of *L. theobromae* produced typical symptoms of citrus gummosis and significant difference in virulence was found. Our data confirms the association of *L. theobromae* with citrus gummosis in Punjab, Pakistan. No other genus of Botryosphaeriacae except Lasiodiplodia was found to be associated with this disease. This preliminary research work opens new frontiers for the development of sustainable management strategies for the control of gummosis disease in citrus orchards.

Key words: Citrus, Gummosis, *Lasiodiplodia theobromae*, Punjab, rDNA-ITS.

INTRODUCTION

Citrus belong to family *Rutacea*e and sub-family *Aurantiodae* by Davies and Albrigo (1994). Citrus fruit is the most important tree fruit crop in the world with estimated production was 124,246 thousand tons in the year of 2016. Pakistan has favorable environmental conditions for fruit production and citrus holds 1st position in terms of area and production (GOP, 2016). In Pakistan citrus annual production was 1907.4 thousand tons (FAO, 2017). Punjab province contributes a main portion of the total acreage and production of citrus fruit in Pakistan. More than 95% of citrus is being produced in the Punjab (GOP, 2016). Citrus orchards are kept in great reverence however its current status is threatened by a number of problems, in which citrus gummosis caused by multiple species of *Botryosphaeriaceae* which is considered to be important fungal disease of citrus trees (Whiteside *et al*., 1988). It is a significant fungal disease that damages citrus and related genera globally and accounts 10 to 30% of losses in citrus orchards worldwide (Timmer *et al.*, 2000). The disease symptoms are categorized by the development of gum filled pockets within or beneath the bark and the oozing of gum from the cracks in the bark of the trunks and/or limbs. Conspicuous brown streak with hardened gum on the bark turns dark brown and develops longitudinal cracks in the stems. The *Botryosphaeriaceae* consists of a collection of morphologically and phylogenetically diverse fungi with an extensive host range and geographic distribution globally. These fungi infect woody plants including both native trees and economically important crops by Slippers and Wingfield (2007). *Lasiodiplodia theobromae* a member of *Botryosphaeriaceae* family is mainly involved in citrus gummosis development whereas other genera of *Botryosphaeriacea (Diplodia*, *Dothiorella*, *Neofusicoccum*, *Neoscytalidium)* are also reported to be associated with citrus gummosis (Polizzi *et al.,* 2011; Adesemoye *et al*., 2014). In Pakistan no current data is available on citrus gummosis; earlier identifications of *L. theobromae* were based exclusively on morphology. For species identification in genus *Lasiodiplodia,* only morphological technique is virtually impossible. To resolve this difficulty, Internal Transcribed Spacer (ITS) sequences have been extensively used to differentiate among species, specifically cryptic species which appear currently in the genus (Burgess *et al*., 2006; Alves *et al*., 2008). We recently found that citrus gummosis is prevalent in citrus orchards grown in in different localities of Punjab province of Pakistan. We have designed this work with the main objective of authentic

identification and characterization of *L. theobromae* associated with citrus gummosis on morphological and molecular basis, because without proper identification of the pathogen, an effective management strategy cannot be devised. The second objective was to determine their pathogenicity and virulence in disease development in most cultivated citrus rootstock in Punjab.

MATERIALS AND METHODS

Sample collection: In 2015-2016, samples of the infected bark of citrus gummosis were collected from 30 orchards of major citrus growing areas of Punjab province that included Sargodha, Bhalwal, Kot Momin, Toba Tek Singh and Faisalabad. Coordinates of each diseased plant from which samples originated were recorded by hand held GPS (Garmin Montana 680T, USA). The diseased samples were collected from trees of 02 to 25 years old, including 'Kinnow mandarin' (*C. reticulata* Blanco), sweet oranges (*C. sinensis*), grapefruits (*C. paradisi*), acid limes (*C. aurantifolia*) and lemon (*C. limon*). Sample trees were grown on the rough lemon (*C. jambhiri*) rootstock. After collection, the diseased samples were placed in polyethylene bags with supporting data such as samples numbers, variety of host, locations. Samples were tightly sealed to maintain their humidity and brought to Molecular Plant Pathology laboratory, University of Agriculture, Faisalabad, Pakistan.

Fungal Isolation and Morphological identification: Diseased samples were immediately processed for surface sterilization with 70% ethanol for 1 min to eliminate epiphytic contaminations and after washing with dH_2O, dried on filter paper. Around 2-4 mm bark pieces were cut from the edge of the lesions and were placed on Potato dextrose agar (PDA) supplemented with Streptomycin. When the fungal hyphae were grown out from the diseased bark, a few of them were picked with sterile needle and cultured separately on the PDA medium. The isolated fungus was examined under stereoscope (SWIFT Instruments, USA) for colony appearance including its texture mainly, color, shape of hyphae and spores were observed under microscope (Micros, Austria). To study the colony morphology, sporulation of the pure cultures was done by transferring a mycelial plug to 2% water agar (20g /L dH_2O) mixed with sterile host material (citrus), branch sections (Mehl *et al*., 2011) and leaves (Inderbitzin *et al*., 2010). Plates were placed under continuous ultraviolet light (Pavlic *et al*., 2004). Identification of *Lasiodiplodia* genus was based on the description given by Phillips *et al*. (2013).

Molecular characterization: Fungal DNA was extracted by using a slightly modified CTAB method as outlined by Moller *et al.* (1992) and quantified by Nanodrop spectrophotometer (Epoch 2 BioTek, USA) at 260 nm in ng/µl. For molecular characterization of associated pathogen ITS region of rDNA was amplified by using the primers ITS-1F (TCCGTAGGTGAACCTGCGG) and ITS-4R (TCCTCCGCTTATTGATATGC) as described by White *et al.* (1990). PCR amplification conditions were optimized and final amplifications carried out in total 25 µl reaction. Each reaction contained 12.5 µl of Dream Taq Green PCR Master Mix (Thermo Scientific, USA), 1 µl of each forward and reverse primer, 1 µl of fungal DNA template and 9.5 µl of nuclease free water. Amplifications were done in a thermocycler (Bio-Rad T-100) programmed at 94ºC for 2 min; followed by 35 cycles at 94ºC for 30 s, 55ºC annealing for 30s, and 72ºC for 1 min; a final extension for 10 min at 72ºC. The amplified PCR products of ITS gene region were analyzed by agarose gel electrophoresis (1.0% (w/v) (Nanopac-300, UK). 0.5X Tris Borate-acid EDTA (TBE buffer) was used as running buffer and stained by ethidium bromide (100µg/ml) (Fisher Biotech, Australia). Electrophoresis was completed at 90 V for 40 min. The size of PCR products of ITS region was compared to O'gene ruler^{TM} 1kb DNA ladder (Thermo Scientific, USA). Purification of PCR products were done by using the PCR Purification Kit (Favor Prep^{TM}, Favorgen, Taiwan) according to manufacturer's instruction. The complete nucleotide sequences of PCR products were obtained commercially from MCLAB California, USA by using dideoxynucleotide chain termination (Sanger *et al*., 1977). A preliminary information of the sequences of our isolates were obtained by comparison with the sequences deposited already in NCBI database (National Centre for Biotechnology Information) by doing BLAST searches (Basic Local Alignment Search Tool) and the sequences were deposited into GenBank (https://www.ncbi.nlm.nih.gov/) by Bankit method.

Phylogenetic analysis: Phylogenetic analysis of ITS region was performed with the isolates of *L. theobromae* associated with citrus gummosis disease and aligned to the reference sequences (Obtained from NCBI, DNA data bank) (Table 2**)**. Sequence alignments were performed by using MUSCLE (Multiple Sequence Comparison by Log-Expectation) method. All sequence gaps at the start and end were removed to improve the alignments before phylogenetic analysis. Tree was drawn to a scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method by Nei and Kumar (2000) and in the units of the number of base differences per site. The evolutionary history was inferred using the Neighbor-Joining method by Saitou and Nei (1987). Molecular Evolutionary Genetics Analysis software MEGA7 was used for evolutionary analysis (Kumar *et al.,* 2016). The phylogenetic tree was rooted with *Guignardia philoprina*.

Pathogenicity test and statistical analysis: Pathogenicity test was conducted by inoculation of stem of 1-year old Rough lemon plants. We used rough lemon (*Citrus jambhiri*) for pathogenicity assays because rough lemon is the most usual root stock used in Punjab, Pakistan due to its adaptability to the local conditions. For this purpose, surface sterilization of stems was done with 70% ethanol for 1 min and a cut made on the stem with a disinfected blade. Mycelia plugs from 3 to 5 days old cultures of each fungus growing on PDA were inoculated on the freshly wounded surfaces. Each isolate was inoculated in triplicate (n=3) and negative controls (n= 3) inoculated with no colonized agar plugs. A portion of moistened cotton was placed on inoculated surface of the stem and subsequently covered with Parafilm to prevent it from desiccation. Plants were placed under controlled conditions (25 ± 5°C and 12-h dark and light cycle) in a greenhouse. After 1 week, the Parafilm and cotton was removed. Gummosis lesions were observed on all inoculated plants. The length of each Gummosis lesion was recorded. To complete the Koch's Postulates Re-isolation of inoculated pathogen was done from inoculated diseased tissues. To check the variability in virulence between all inoculated isolates of the *L. theobromae,* statistical analysis was done. Pathogenicity data was analyzed in CRD under factorial arrangement in Statistix 8.1 (Analytical software, 2003). The probability level was checked with $\alpha \leq 0.05$ using LSD test to measure the differences in the size of gummosis lesion length induced by pathogen associated with citrus gummosis.

RESULTS

Fungal isolation and Morphological characterization: A particular number of samples collected from symptomatic citrus plants produced characteristic *Botryosphaeriaceae* which were initially identified as white colonies, fluffy mycelium with profuse dense straight aerial hyphae and 15 days later greenish or gray mycelium turned into black color. Microscopic examination revealed that hypha of Botryosphaeriaceae was broad, brown, with many septa and granular cytoplasm without conidia. To identify the genera, sporulation of conidia was done on 2% water agar medium. The isolates had common features such as the fast spreading, immersed branched, septate hyaline mycelium. Pycnidial black colored fruiting bodies were produced. Initially conidia were hyaline, unicellular and ellipsoidal with granular contents. On maturity conidia were changed into bi-celled, thick walled dark brown in color with longitudinal striations (Fig. 2). All isolates characters were consistent with genus *Lasiodiplodia* (Phillips *et al*., 2013). No other genera of *Botryosphaeriaceae* apart from *Lasiodiplodia* were isolated from infected samples.

Fig. 1. Typical symptoms of gummosis disease on *C. reticulata* Blanco. A: Dark brown to light brown diseased wood. B: Affected bark darker than the surrounding healthy tissues. C-D: Cracked infected bark.

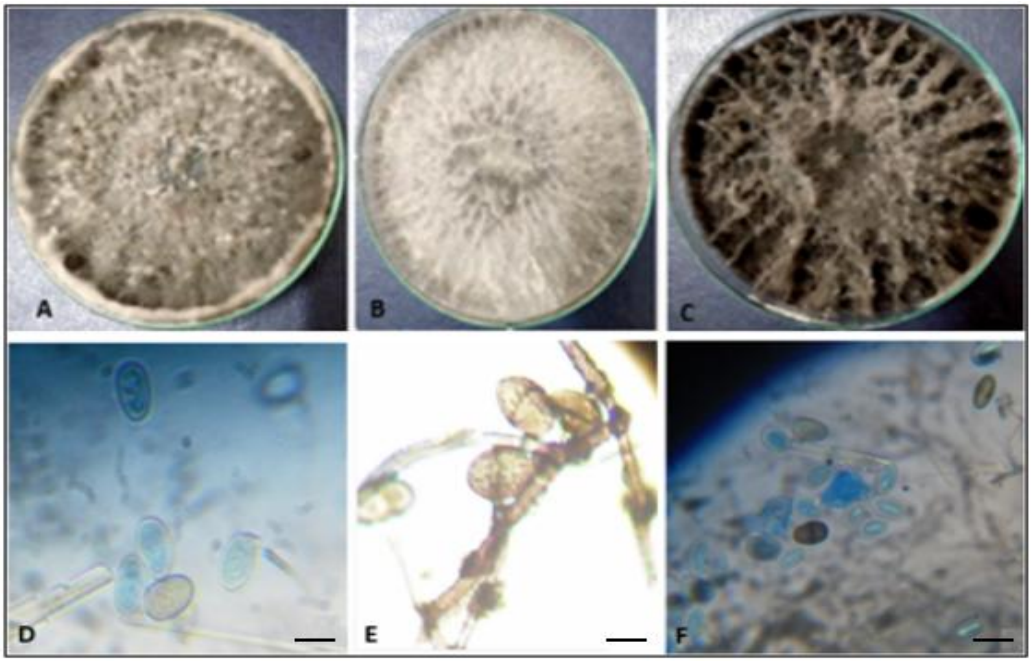

Fig. 2. Isolates of *Botryosphaeriaceae* showing fluffy grayish to black mycelia having profuse dense straight aerial hypha (A-C). Conidial morphology of *Lasiodiplodia* isolates (D-E). D: Immature whitish Conidia with thin walls. E: Mature conidia (dark brown) with uniseptate and longitudinally striate in thick walls. F: Immature and mature conidia exist together. Scale bars: D-F= 10 µm.

Molecular identification and phylogenetic analysis: The amplicon of ITS region from all the population DNAs yielded a single band with an approximately 600-650 bp in size (Fig. 4), which was sequenced. An initial comparison of isolates sequences was done to the sequences which already submitted in NCBI by using BLAST search. The percentage of similarity between isolates sequences and available sequence in databank ranging from 98–100%. BLAST confirms the isolates sequences belong to *Lasiodiplodia* spp. For Phylogenetic analysis of ITS region of *Lasiodiplodia* spp. MUSCLE alignment was made between 10 sequenced isolates of *Lasiodiplodia* spp. and previously identified reference sequences of *Lasiodiplodia* spp. available in the databases. The reference sequences which were used are *L. gilanensis*, *L. margaritacea*, *L. gonubiensis*, *L. crassispora, L. viticola, L. missouriana, L. exigua, L. pseudotheobromae* and *L. theobromae* mentioned in Table 2. The result of phylogenetic analysis of *Lasiodiplodia* spp. sequences showed that 10 isolates of *Lasiodiplodia* spp. sequences (with Genbank accession numbers MH260011, MH260012, MH260013, MH260014, MH260015, MH260016, MH260017, MH260021, MH260019, MH260020 given in Table 1) clustered with reference sequences of *L. theobromae* originated from India (MG051345, MG661769), Sri Lanka (KU296922), Iran (KF466495, KF535908), Malaysia (KP998517) and China (KU877346, KY393057) and are indistinguishable. The result showed that isolates from Punjab, Pakistan are closely related with different isolates of *L. theobromae* of neighboring countries (Fig. 5).

Table 1. Isolates of *Lasiodiplodia theobromae* recovered from citrus gummosis samples in this study.

*No.	Isolates	Area	Coordinates	Host	Species	Genbank Accessions No. ITS of rDNA
1	MZA009	Sargodha	32°2'40.800"N72°45'4.833"E	*C. reticulata* Blanco	*L. theobromae*	MH260011
2	MZA013	Sargodha	32° 6'35.16"N 72°48'5.58"E	*C. sinensis*	*L. theobromae*	MH260012
3	MZA027	Bhalwal	32°17'2.807"N72°56'25.99"E	*C. sinensis*	*L. theobromae*	MH260013
4	MZA048	Kot Momin	32°12'36.52"N73°1'36.37"E	*C. reticulata* Blanco	*L. theobromae*	MH260014
5	MZA052	Kot Momin	32°11'38.22"N 73°0'40.56"E	*C. limon*	*L. theobromae*	MH260015

6	MZA070	Faisalabad	31°25'54.10"N73°3'38.31"E	*C. reticulata* Blanco	*L. theobromae*	MH260016
7	MZA075	Faisalabad	31°17'13.84"N73° 9'18.01"E	*C. reticulata* Blanco	*L. theobromae*	MH260017
8	MZA081	Faisalabad	31°1'18.102"N73°5'23.782"E	*C. paradisi*	*L. theobromae*	MH260021
9	MZA102	Toba Tek Singh	30°53'11.03"N72°31'56.26"E	*C. sinensis*	*L. theobromae*	MH260019
10	MZA107	Toba Tek Singh	31°4'41.150"N72°25'26.13"E	*C. reticulata* Blanco	*L. theobromae*	MH260020

*No. represents amplified PCR products of each isolates.

Fig. 3. Map of *L. theobromae* isolates originated from major citrus growing areas of Punjab, Pakistan.

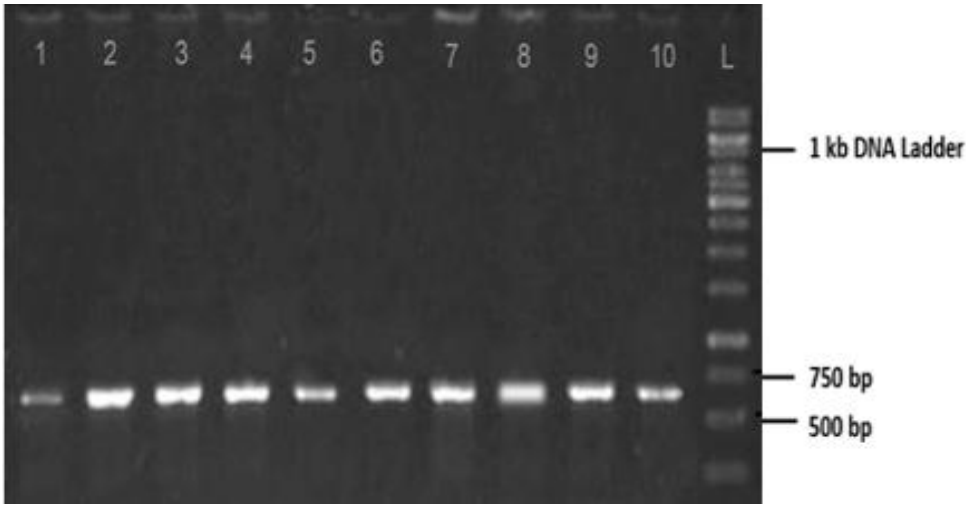

Fig. 4. PCR products of ITS region of rDNA of *L. theobromae* isolates. The size of each band ranges between 600-650bp by comparison to 1kb Ladder. Detail of each PCR No. (1-10) is mentioned in Table 1.

Table 2. Description of the reference *Lasiodiplodia* species and their sequences obtained from GenBank.

Isolate No.*	Species	Host	Locality	Collector	ITS Accession No.
MAR35	*L. gilanensis*	*Syzygium cordatum*	South Africa-2017	J.W.M. Mehl	KY052997
PED34	*L. gilanensis*	*Syzygium cordatum*	South Africa - 2017	J.W.M. Mehl	KY053013
MAR34	*L. gilanensis*	*Syzygium cordatum*	South Africa - 2017	J.W.M. Mehl	KY052996
CBS138289	*L. margaritacea*	*Combretum elaeagnoides*	South Africa - 2016	L.A. Shuttleworth	KP872320
CBS138290	*L. margaritacea*	*Combretum elaeagnoides*	South Africa - 2016	L.A. Shuttleworth	KP872321
CBS138291	*L. margaritacea*	*Combretum elaeagnoides*	South Africa - 2016	L.A. Shuttleworth	KP872322
CBS115812	*L. gonubiensis*	*Syzygium cordatum*	South Africa - 2014	B. Slippers	KF766191
CMM4468	*L. gonubiensis*	*Anacardium humile*	Brazil-2015	M.S.B. Netto	KT325571
CBS115812	*L. gonubiensis*	*Syzygium cordatum*	Portugal-2007	A. Alves	DQ458892
WAC12533	*L. crassispora*	*Santalum album*	Australia-2008	T.I. Burgess	DQ103550
CMM0283	*L. crassispora*	*Vitis vinifera*	Brazil-2014	K.C. Correia	KJ450853
CBS110492	*L. crassispora*	Unknown	Portugal-2007	A. Alves	EF622086
CMM3872	*L. subglobosa*	*Jatropha curcas*	USA-2014	A.R. Machado	KF234558
CMM3872	*L. subglobosa*	*Jatropha curcas*	USA-2017	A.R. Machado	NR147350
CMW41372	*L. viticola*	*Vitis vinifera*	South Africa - 2015	L.A. Shuttleworth	KP872342
UCD2553AR	*L. viticola*	*Vitis vinifera*	USA 2012	J.R. Urbez-Torres	HQ288227
UCD2199MO	*L. missouriana*	*Vitis vinifera*	USA -2012	J.R. Urbez-Torres	HQ288226
CBS128311	*L. missouriana*	*Vitis vinifera*	USA -2017	J.R.Urbez-Torres	NR145222
BL185	*L. exigua*	*Vitis vinifera*	Italy-2015	B.T. Linaldeddu	KJ638319
BL186	*L. exigua*	*Vitis vinifera*	Italy-2014	B.T. Linaldeddu	KJ638320
CBS137785	*L. exigua*	*Melaleuca uncinata*	Italy-2014	B.T. Linaldeddu	NR147353
CBS116459	*L.pseudotheobromae*	*Gmelina arborea*	Portugal-2008	A. Alves	EF622077
CERC2314	*L.pseudotheobromae*	*Juglans regia*	China-2015	G.Q. Li	KR261714
CERC3475	*L.pseudotheobromae*	*Eucalyptus urophylla*	China-2017	G.Q. Li	KX278027
UVQISO3	*L. theobromae*	*Grevillea robusta*	Sri Lanka-2017	N. Mahadevan	KU296922
ZM13582	*L. theobromae*	*Firmiana simplex*	China-2016	M. Zhang	KU877346
KER-U-LTAPL1	*L. theobromae*	*Prunus domestica*	Iran-2013	H. Mohammadi	KF466495
KER-U-LTGG2	*L. theobromae*	*Prunus domestica*	Iran-2013	N. Soltaninejad	KF535908
AKB4T5	*L. theobromae*	*Acacia mangium*	Malaysia-2017	A. Abdul Latiff	KP998517
HBZHB014	*L. theobromae*	*Ziziphus jujuba*	china-2016	R. Zang	KY393057
H1	*L. theobromae*	Unknown	India-2017	T. Arunprasath	MG661769
MKSvv2	*L. theobromae*	*Vitis vinifera*	India. 2017	S. Mahadeva kumar	MG051345
CBS447.68	*G. philoprina*	*Taxus baccata*	Portugal-2009	A.J.L. Phillips	FJ824768

*CMW = Culture Collection Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.
CMM: Culture Collection of Phytopathogenic Fungi, Universidade Federal Rural de Pernambuco, Recife, Brazil.
CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands.
UCD = University of California, Davis, USA.
CERC= Culture Collection of China Eucalypta Research Center, Chinese Academy of Forestry, ZhanJiang, GuangDong, China.
WAC= Department of Agriculture Western Australia Plant Pathogen Collection, Perth, Western Australia.
CBS447.68 *Guignardia philoprina* used as outgroup.

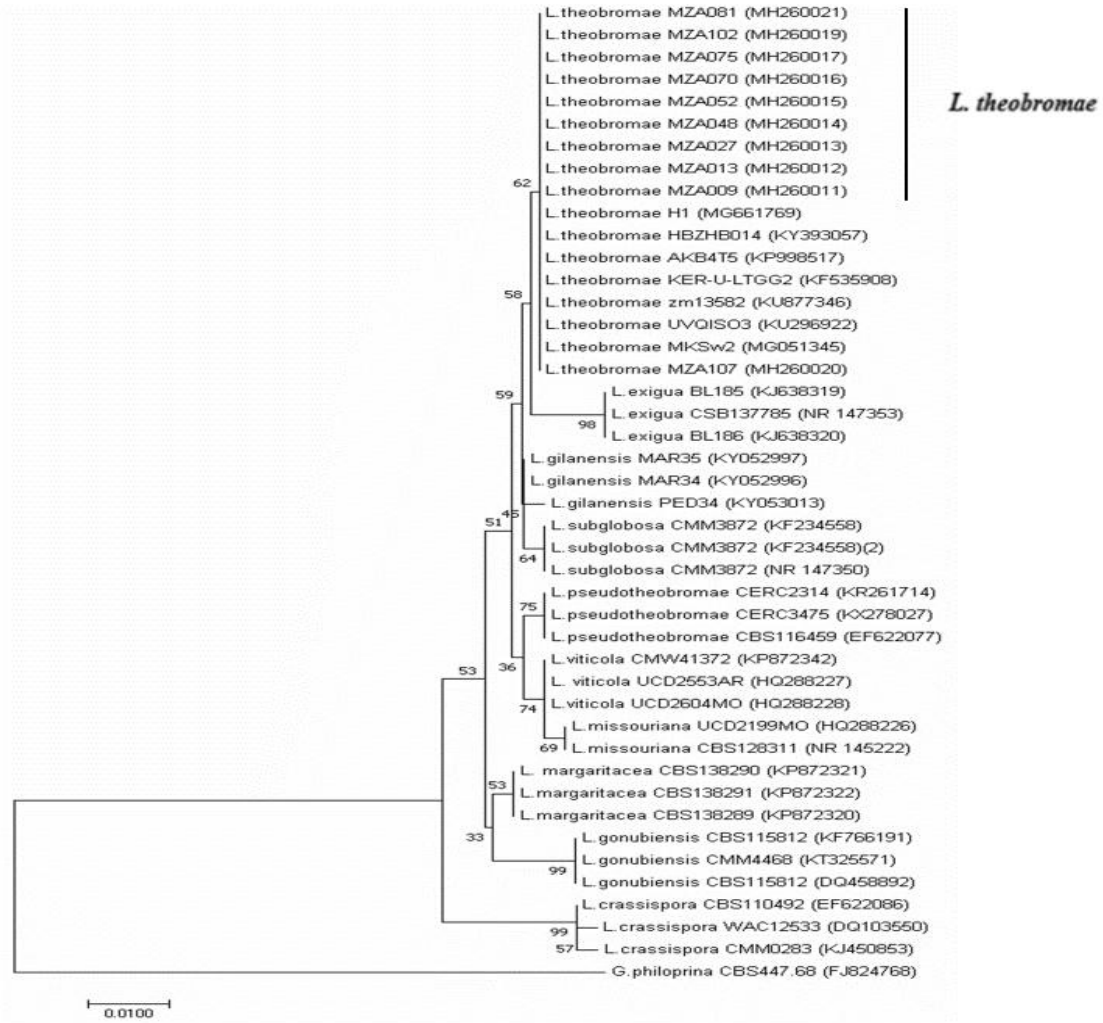

Fig. 5. Neighbor joining phylogenetic tree based upon MUSCLE alignment of ITS region rDNA nucleotides of *Lasiodiplodia theobromae* associated with citrus gummosis and reference sequences of *Lasiodiplodia* spp. available in the database. All isolates of *L. theobromae* originated from major citrus growing areas of Punjab are closely related with respect to their reference sequences originated from neighboring countries. Numbers at the nodes are percentage bootstrap confidence values (1000 replicates). The tree is rooted with *G. philoprina*. The optimal tree with the sum of branch length = 0.1095 is shown. The analysis contained 44 nucleotide sequences with 391 positions in the final dataset. Phylogenetic analysis was conducted in MEGA7.

Pathogenicity test and statistical Analysis: On inoculation of all isolates of *L. theobromae* produced characteristic symptoms like orange-brown gummy exudation and cankered necrotic lesions on the bark of stem after 35 days. The size of each lesion increased gradually with the passage of time (Fig. 6). Inoculated all isolates of *L. theobromae* were re-isolated from the diseased plants to complete Koch's postulates. Non-inoculated stem remained asymptomatic; no pathogen was recovered from control plants. It was confirmed *L. theobromae* is one of the main causing agents of citrus gummosis in major citrus areas of Punjab. The lesion length of the necrotic gummosis varied significantly among isolates (Table 3). Among different isolates of *L. theobromae*, minimum gummosis lesion size was 2.94 cm produced after 35 days of post inoculation and maximum gummosis lesion size was calculated 8.27 cm after 56 days. In all inoculated *L. theobromae* isolates, MZA081 showed maximum virulence 7.06 cm and isolate MZA027 showed minimum virulence 3.30 cm. In the interactive effect between Days and Isolates of *L. theobromae*, isolate MZA081 showed maximum gummosis lesion length 10.13 cm at day 56 and isolate MZA027 showed minimum lesion length 1.4 cm at 35 days (Fig.7).

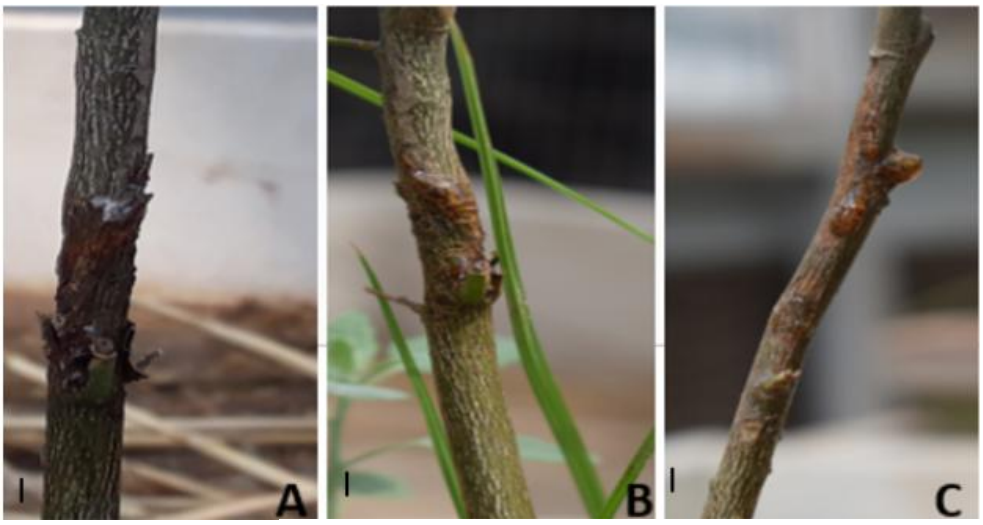

Fig. 6. Typical disease symptoms with orange brown gummy exudation lesions on inoculated bark tissues. Gummosis lesions produced by inoculation of *L. theobromae* isolates (A-C). Scale bars: A-C = 1cm.

Table 3. Lesion lengths of different isolates of *L. theobromae* at different days.

Days (D)	Lesion (cm)
35	2.94 D
42	4.63 C
49	6.43 B
56	8.27 A
Isolates (I)	
MZA013	5.77 D
MZA009	6.73 C
MZA070	4.90 E
MZA027	3.30 G
MZA048	6.92 B
MZA052	3.38 G
MZA081	7.06 A
MZA102	6.94 AB
MZA075	6.94 AB
MZA107	3.68 F
LSD ($p \leq 0.05$)	
D	0.083
I	0.131
D×I	0.262

Means in a column followed by the same letter are not significantly different by LSD test ($P \leq 0.05$ level).

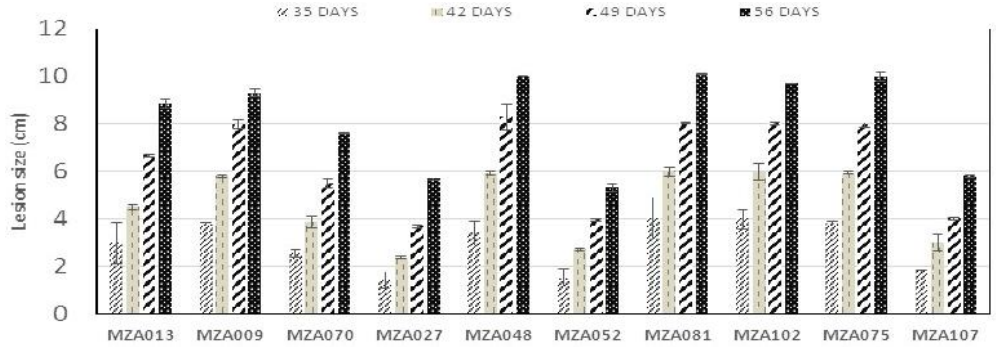

Fig. 7. Expression of gummosis lesion in response to *L. theobromae.* *L. theobromae* produced gummosis lesion length (cm) after 5th weeks of inoculation. Data was recorded for consecutive four weeks after appearance of gummosis. The data included three biological replicates. The bar shows standard deviation for the mean.

DISCUSSION

Preliminary studies have been conducted in past to investigate the citrus gummosis disease in Punjab, Pakistan. Due to dearth of correct identification of the causal organism, management strategies cannot be developed. A comprehensive investigation is required to study the association of pathogens with this disease. This study reveals the molecular characterization, diversity and pathogenicity of causal organism associated with citrus gummosis disease. For this purpose**,** diseased samples of gummosis were collected from different citrus host varieties. During collection of diseased samples exudation of gum from the affected bark on citrus trees was observed. It was noticed that affected bark tissue was darker than the adjacent healthy tissues. When affected diseased bark tissue removed, it looked dark brown to light brown. These all disease symptoms were consistent in major citrus growing areas. *Botryosphaeriaceae* spp. were isolated from diseased tissue and initially identified by morphological characteristics upon culturing the pathogens on potato dextrose media. After sporulation of conidia, it was confirmed that *Lasiodiplodia* genus of *Botryosphaeriaceae* is associated with disease. No other genera of *Botryosphaeriaceae* were found except *Lasiodiplodia*, in the region of Punjab. There is possibility that other genera of *Botryosphaeriaceae* present in this region but *Lasiodiplodia* is clearly dominant. It may also be speculated that *Lasiodiplodia* is well environmentally adapted to the conditions of region. In this study preliminary macro and micro morphological characters of species were used. But these features confuse the identification of species in the genus of *Lasiodiplodia*. Morphological characters can often be confusing due to hybridization, cryptic speciation and convergent evolution. Therefore, more advance and reliable techniques can help for accurate identification of pathogens. To resolve the issues associated with morphological identification, isolated pathogens were identified on molecular level. DNA sequence data are vital in solving taxonomic questions, redefining species boundaries and the correct naming of species. For molecular characterization of associated pathogens, ITS region of rDNA was targeted. Analysis of ITS region of *Lasiodiplodia* spp. revealed that *L. theobromae* are associated with citrus gummosis in Punjab. No diversity was observed within all identified isolates of *L. theobromae*. Moreover, phylogenetic analysis of *L. theobromae* originated from major citrus areas of Punjab showed close resemblance with *L. theobromae* reported from neighboring geographical countries of Pakistan. To figure out the role of *L. theobromae* in disease development, all isolates were inoculated on Rough lemon (*C. jambhiri*). *L. theobromae* was able to spread through the internal tissues above and below points of inoculation resulting in gum formation. The external and internal symptoms disclosed the capacity of *L. theobromae* to cause disease and to spread rapidly through the vascular tissues even if the host is not exposed to stress. A significant difference was also observed in virulence of *L. theobromae* isolates. Our findings are in accordance with Guajardo *et al*. (2018), who reported *L. theobromae* causing gummosis in *C. limon* in Chile. *L. theobromae* also found to be associated with gummosis disease in *C. aurantium*, *C. aurantifolia*, and *C. sinensis* in Mexico (Picos-Muñoz *et al*., 2014). Assuah *et al*. (1999) conducted pathogenicity tests and confirmed that *L. theobromae* cause citrus gummosis in Ghana. Ferrari *et al.* (1996) stated die back and gummosis induced by *L. theobromae* on three citrus tree species (sweet orange, lemon and pummelos). Cedeno and Palacios (1992) reported that *L. theobromae* as the cause of lesions and gummosis on branches of lemon and orange. Sonada and Pelosi (1990) mentioned that *L. theobromae* associated with Rio Grande gummosis lesions in Grapefruit. To our knowledge, this is the first molecular study on citrus gummosis from major citrus growing areas of Punjab, Pakistan and based on the results of present study *L. theobromae* found to be associated with this disease. Citrus is an economically important fruit crop plant of Pakistan, therefore knowledge about the prevalence and geographical distribution of *L. theobromae* will facilitate the development of sustainable control measures for limiting the economic loss in citrus orchards.

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