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MOLECULAR CHARACTERIZATION OF *BOTRYOSPHAERIACEAE* CAUSING STEM END ROT OF MANGO IN PAKISTAN

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Abstract

In this study, molecular, morphological and pathological characterization of postharvest fungal pathogens associated with stem end rot of mango fruit was done. A systematic survey was conducted in the farmer (uncontrolled practices) and demo blocks (controlled practices) of 6 districts of Punjab and Sindh for the disease assessment of collected 25-30 samples of mangoes. The stem end rot disease was found 100% prevalent in all the locations while disease incidence was high in farmer blocks. DNA was isolated from the stem end rot isolated fungal strains and the amplified products from ITS-5.8S-rDNA were sequenced which showed 96-100% similarity with previously identified sequences in Genbank. As a result of molecular sequencing analysis, two species of Botryosphaeriaceae, *Lasiodiplodia theobromae* and *Botryosphaeria dothidea* were detected. The pathogenicity tests were applied to determine the aggressiveness of postharvest fungal pathogens by plug placement method and found high toxicity on mango twigs and potato tubers but detached mango leaves showed no lesions. Results obtained through this work would be supportive to develop the management policies to reduce the afflictions in orchards caused by mango stem end rot.

Key words: Mango rot, Botryosphaeriaceae, *Botrosphaeria dothidea*, *Lasiodiplodia theobromae,* Pathogencity.

Introduction

Mangifera indica L., commonly known as mango, is a species of flowering plant in the sumac and poison ivy family Anacardiaceae, is a popular, predominant seasonal fruit originated primarily in the tropical and subtropical regions of the world (Srivastava *et al.*,1964). Pakistan produces approximately 8.5% of the world's total production and mostly exports to Iran, Middle East, Japan, Germany and China assembling its important contribution as a significant foreign exchange earning the fruit crop (Rashid *et al*., 2016). In Pakistan, soil and climatic conditions support production of mango in terms of quality and yield but the country is unable to attain the desired results. Numerous issues contribute towards the low production of mango. Unchecked utilization of unhealthful seeds forming infected seedlings, insect assault, mango malformation (vegetative and reproductive) and diseases (anthracnose, quick decline, powdery mildew, sooty mold, stem blight and fruit rot) are the major risks to the industry in the world's top mango producing countries including Pakistan. These afflictions arise primarily attributable to in accessibility of nutrients from soil, ineffective utilization of resources, lack of knowledge concerning the ripening and quality protection practices. Still we are unable to deal with problems occurring owing to unawareness of farmers, lack of phytosanitary measures in orchards and services relating storage and marketing. Our Government ought to take projects to instruct farmers, devise strategies for the expansion of infrastructure, access to markets and provide amenities at low cost. Economically most significant postharvest decay of mango fruit in a number of countries is stem end rot. Stem end rot has been reported from all mango growing areas of the world. The term "Stem End Rot" is used to describe the lesions developing at the pedicle end of fruit after harvest and leading the entire decay of fruit. It is caused by different species of Botryosphaeriaceae including *Botryosphaeria dothidia*, *Neoffusicoccum parvum* and *Lasiodiplodia theobromae*. Members of Botryosphaeriaceae contaminate fruits through endophytic colonization and mechanical injury. In this research first time molecular approaches have been developed for estimation of microbial assortment at specie level in complicated communities. By this research, it would be possible in future by DNA based techniques to reveal fungal variability in ecosystem and propose potential advantages of perceptive and rapid detection. Present position of mango fruits in Punjab and Sindh really convey imperative attention and rapid contribution to comprehend the ecology of these identified fungal pathogens species so, first time this study focused primarily on isolation and characterization of pathogens related with stem end rot disease in Punjab and Sindh, Pakistan by means of morphological, molecular and pathological data.

Materials and Methods

Survey, Sampling and disease assessment: A systematic survey of 6 famous mango growing districts of Punjab (Multan, Muzafar Ghar, Rahim Yar Khan) and Sindh (Tando Allah Yar, Matiyari, MirpurKhas) was carried out for two famous mango varieties, Sindhri and Chounsa (Fig. 1). The disease was assessed on the basis of appearance of symptoms and samples were collected from the farmer (Without improved practices) and demonstrated blocks (with improved practices). Samples were brought in laboratory for evaluation of the disease using the standards scales (Tables 1,2,3) for disease assessment (Akhtar & Alam, 2002) and regular monitoring of the samples was done on the basis of color, firmness and severity of disease for two weeks to determine prevalence, severity and incidence of the disease.

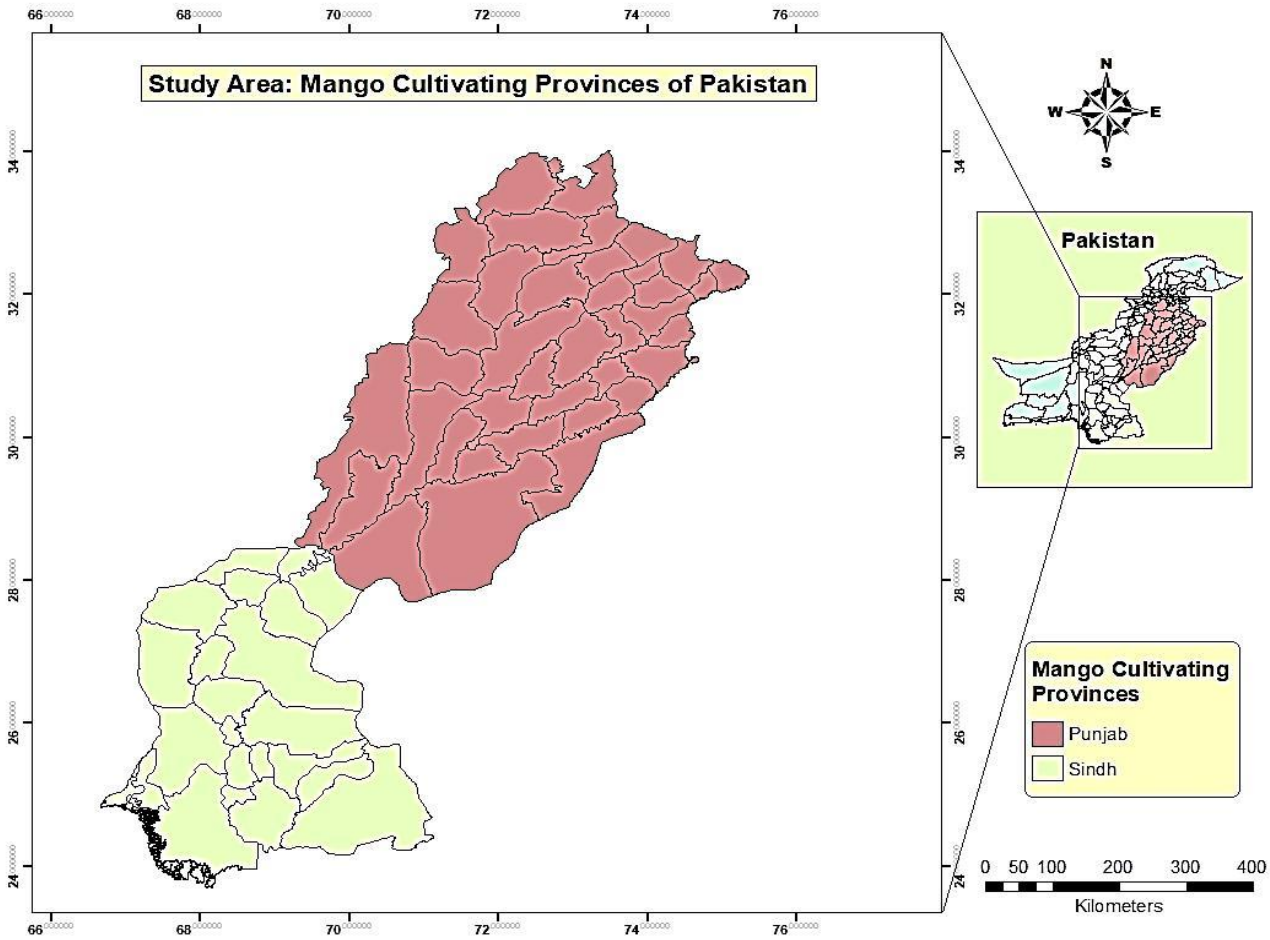


Fig. 1. Study area map showing Mango producing Provinces of Pakistan.

Table 1. Grading scale for fruit color (Hassan, 2006).

Grading	Color
1	Green
2	Beaker
3	Yellow 25%
4	Yellow 50%
5	Yellow 75%
6	Yellow 100%

Table 2. Grading scale for fruit firmness (Hassan, 2006).

Grading	Firmness
1	Hard
2	Sprung
3	B/W Sprung and eating ripe
4	Eating ripe
5	Over ripe

Table 3. Scale for assessment of disease severity (Hafiz *et al*., 2018).

Grading scale	Affected area of fruit (%)	Severity
0	0	No disease
1	1-5	Trace of disease
2	6-25	Mild disease
3	26-50	Moderate disease
4	51-75	Severe disease
5	76-100	Very severe disease

Pathogen isolation, preservation and identification: The pathogen was isolated from fruit samples by tissue segment method on general potato dextrose agar and specific malt extract agar medium at 28°C (Rangaswami, 1958). Morphological studies were accomplished by determining macroscopic and microscopic characteristics of each isolate. Cultures were characterized on the basis of colony morphology and conidial characteristics (Sutton, 1980). Appearance, color, shape, texture and growth rate of isolates was determined on Potato Dextrose Agar medium after 7 days of incubation at 28°C (Rayner, 1970). Fruiting structure and conidial characteristics (shape, color of hyphae, septation, fruiting bodies, pigmentation and other visible features) were examined under compound microscope (Razak, 1999).

Molecular characterization: Genomic DNA was isolated by Phenol extraction method (Alves *et al*., 2004). For this purpose, 50mg of mycelium was crumbed with sterilized blade from surface of 1 week old fungal culture. Mycelium was squeezed with glass rod suspended in 1000µl of DNA extraction buffer. After homogenization, 500µl of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added in mycelium and crushed for few seconds. Mixture was transferred to 1.5ml eppendorff tube and centrifuged for 10min at 10,000rpm. After that, supernatant was separated circumspectly from pellet and added 500µl of isopropanol and 50µl of sodium acetate (3M, pH4.8) in pellet and mixed smoothly with hands until the emergence of a thread

in mixture and centrifuged subsequently. Then pellet was separated and rinsed with 500µl of 70% EtOH followed by centrifugation. Pellet was detached from wash buffer and samples were put on tissue paper for air drying. After complete drying of two hours, samples were suspended in 200µl of TE buffer and incubated at 35°C for 30min. Isolated DNA was purified by adding 1µL of RNAse (100µl/m) to each sample. Traces of DNA that still remained were then vortexed for complete suspension. A part of internal transcribed spacer region of 5.8S ribosomal DNA operon was amplified with the primer pair: ITS1-F (5' CTTGGTCATTTAGAGGAAGTAA 3') and ITS4-R (5' TCCTCCGCTTATTGATATGC 3') (White *et al*., 1990). PCR Thermo cycler was used for amplification reactions (Arzanlou *et al*., 2012). PCR products were run on 2% agarose and the amplified bands were compared with 1kb ladder (Fermentas). The 5.8S rDNA gene fragments of fungal isolates were analyzed electrophoretically to test the amplification of DNA obtained from the fungal isolates (Burgess *et al*., 2005). PCR products were purified with Silica Bead Gel Extraction Kit (Thermo scientific) by following the instructions of manufacturers with minor modifications. Sequencing of ITS strands of amplified products was done by the service provided by Macrogen, Korea. Obtained nucleotide sequences were read and edited manually by using DNA Dragon software. Sequences were compared with previously identified sequences in Genbank using the Basic Local Alignment Search Tool (BLAST). Sequences were aligned and gaps were eliminated by using statistical software MEGA (version 5) and a phylogenetic tree was built using neighbor-joining method in MEGA 5. to determine the phylogenetic relationship among the isolates. Transitions and transversions in equal ratio were included and the support of internal nodes of tree was assessed by bootstrap method with 1000 replicates.

Pathogenicity tests: Pathogenicity experiments were carried out in the laboratory by artificial inoculations of pathogens by mycelial plug method (Szczech *et al*., 2008) on three different hosts to determine the aggressiveness of isolates. For this purpose, fresh leaves and twigs from healthy mango tree (Host-pathogen interaction) and young recently harvested potato tubers (Non-host-pathogen interaction) were used. Samples in uniform size were selected for this study. Samples were surface sterilized by dipping in 70% ethanol for 5 min then washed for three times with changing of distilled water and air dried at room temperature for 24 hours. After complete drying, samples were inoculated with mycelial plug of 1 week old culture by using plug placement method. After 2 weeks of incubation, samples were cut in longitudinal section from the point of inoculation and necrotic lesions were recorded longitudinally and vertically (Lin *et al*., 2002; Choiseul *et al*., 2006; Peters *et al*., 2008 and Azad & Shamsi, 2011). Experiments were conducted by using three replicates for each isolate and diseased area was determined by the equation (Area = πlw). At the end of trials, the causal agents in the infected parts were re-isolated on PDA medium as depicted in the isolation of pathogens. Colonies were sub cultured and characters of recovered pathogens were compared with original isolates to confirm Koch's postulates (Fig. 2).

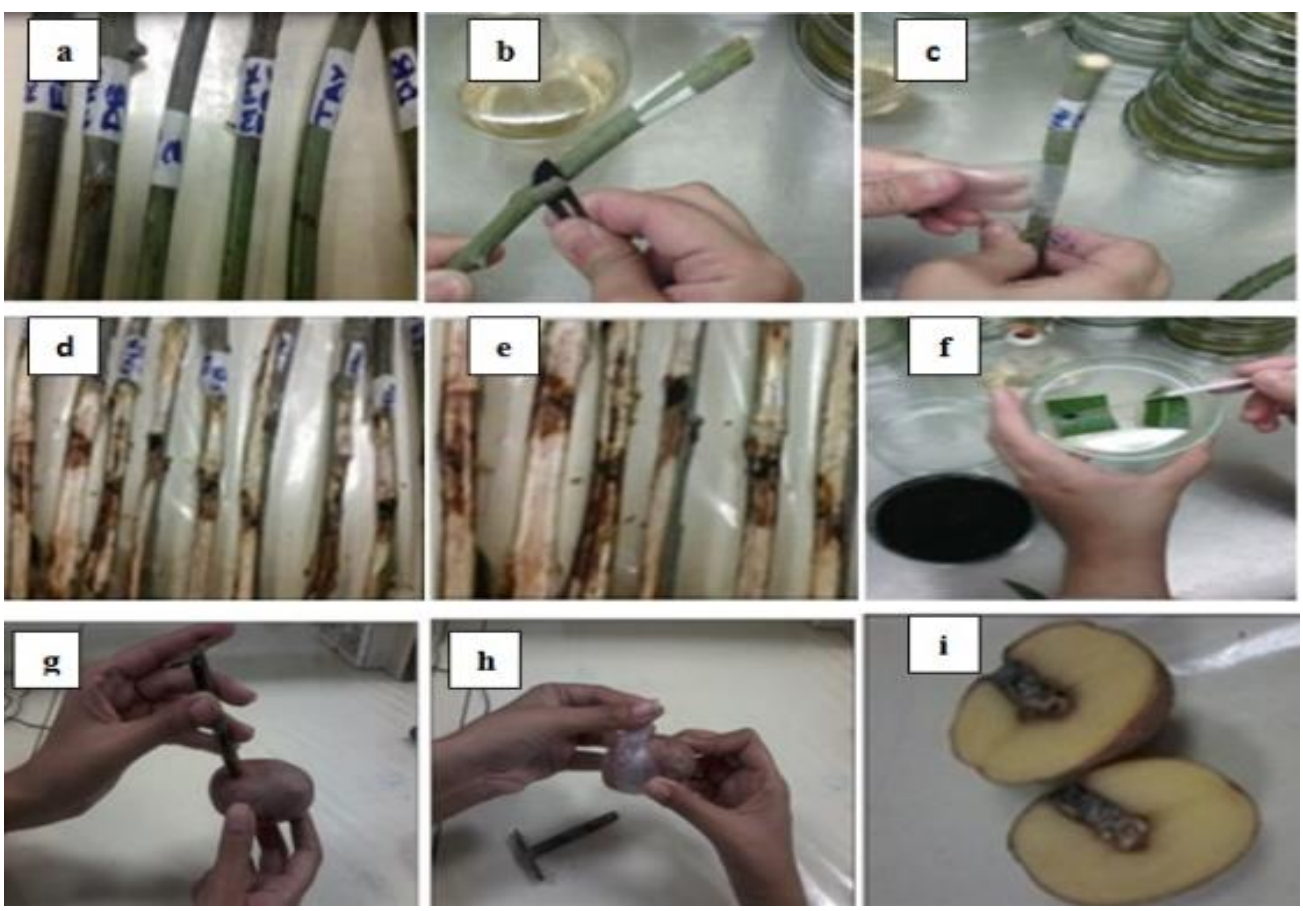


Fig. 2. (a-c) Inoculation of pathogens on detached mango twigs. (d-e) Development of disease lesions on inoculated twigs. (f) Inoculation of pathogens on detached mango leaves. (g-h) Inoculation of pathogens on potato tubers. (i) Development of disease symptom.

Results and Discussion

Disease assessment and fungal identification: Mango fruit samples from all locations showed visible symptoms of stem end rot disease and disease was found 100% prevalent in all surveyed orchards of Punjab and Sindh. Disease incidence was recorded high in farmer blocks as compared to demo blocks. Disease severity was high in Multan district followed by Tando Allah Yar and Matiyari and it was negligible in Rahim Yar Khan (overall disease incidence and severity was less in demo block or farmer blocks). Overall, disease prevalence, incidence and severity were highest in Multan (Table 4). Disease symptoms appeared as brown to black staining on skin of the fruit. Lesions were observed on fruit samples emerging from stem and extending down along the fruit causing softening of fruit skin and watery pulp. Symptoms were more severe internally than externally. Investigations on mango postharvest fungal pathogens revealed the occurrence of typical symptoms of mango fruit stem end rot in Punjab and Sindh which was attributed to two species of *Botryosphaeriaceae* reported in present investigation. Disease emerged as brown to black discoloration on fruit. In previous investigations, similar disease signs were reported to be originated by various species of *Botryosphaeriaceae* worldwide (Ramos *et al.*, 1991).

Colony color of *Lasiodiplodia theobromae* was observed as white at initial stage of growth, darken from the centre which turned to olivaceous grey, reverse dark grey after 3-4 days and finally black after one week. The texture was fluffy and cottony. On maturity, small, dark brown and shiny black pycnidia were observed on culture plates. Conidial walls were thick and ellipsoidal in shape. *Botryosphaeria dothidea* mycelium was fluffy initially white, turned green grey to dark grey and grey-blue pigmentation were observed within few days on reverse side of Petri plate. Aerial hyphae cumulative and forming dark grey to black pycnidia which were mostly solitary or sometimes aggregated, globose covered with mycelium. Conidia were hyaline, single celled, spindle shaped, ellipsoid, thin walled, without septum and showed slight variations in size. Conidia were slightly tapered at apex and wider at center (Fig. 3abc and Table 5).

Table 4 Prevalence, incidence and severity in farmer and demo blocks of mango in Punjab and Sindh.

Origin	District	Farmer block			Demo block		
		Prevalence	*Incidence (%)	**Severity (1-5) scale	Prevalence	*Incidence (%)	**Severity (1-5) scale
Punjab	Multan	100	73.23	1-5	100	88.73	1-5
	MuzaffarGarh		45	1-2		25	1-2
	Rahim Yar Khan		33.33	1		6.66	0
Sindh	Matiyari	100	43.33	1-4	100	36.66	1-3
	MirpurKhas		15	1-2		13.33	1
	Tando Allah Yar		80	1-5		41.66	1-4

Disease assessment

*Prevalence of the diseased fields in which the disease symptoms were observed to the total number of fields visited in each location.

*Disease incidence was assessed by counting the number of apparently diseased mangoes per site with respect to overall mangoes.

**Disease severity on mango fruit was assessed by a five point rating scale.

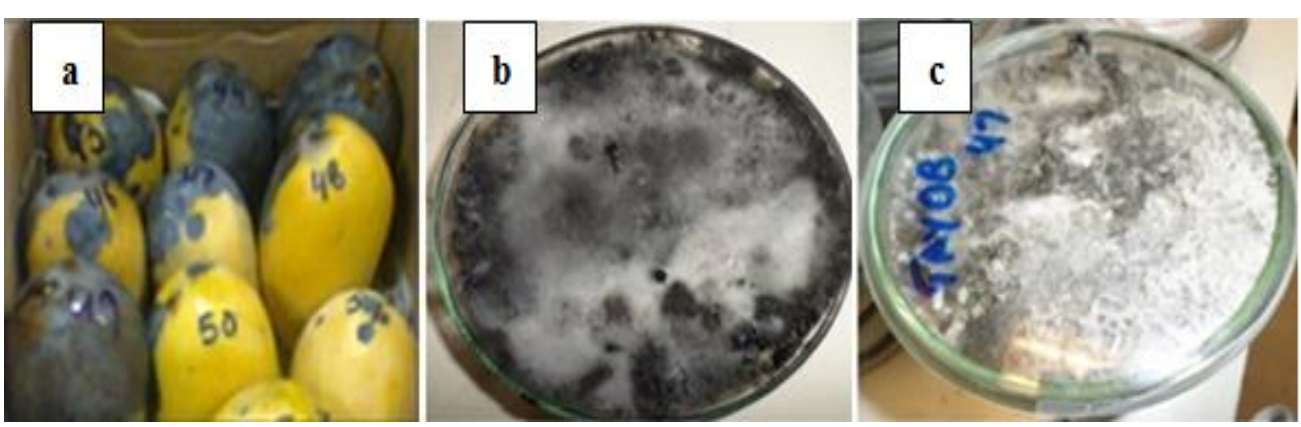


Fig. 3. (abc) Samples showing symptoms of stem end rot. (b) Morphology of L. theobromae on PDA medium. (c) Morphology of B. dothidea on PDA medium.

Table 5. Conidial dimensions of Botryosphaeriaceae species examined in this study.

Identity	Conidial size (µm)	L/W	Paraphyses
Lasiodiplodia theobromae	17-35 × 13-15	1.3	septate
Botryosphaeria dothidea	19-30 × 4-6	4.7	aseptate

It was assessed in the study that fraction of infected fruits was higher because of fruit surface injuries during transport, handling and packaging (Paull, 2001) therefore, high disease incidence might be owing to the physiological position of fruits. It was suggested that these fungi may take place as endophytes in mango stem tissues and take over the stem end of fruit during maturation, in this manner causing stem end rot (Johnson, 2008). Disease was found very severe in district Multan. Climatic conditions in Multan are favorable for mango production but the focal point of farmers might be production only and attention is not given towards the quality of fruit and disease management practices. Lack of management in orchards as well as excessive use of nitrogen fertilizers in Punjab could be the major causes of development of fungal diseases in mango orchards. Cultural and conidial characteristics are the most useful aspects for the isolation, identification and discrimination of pathogens. Isolates used in this study were assessed with respect to their growth rates on artificially synthesized medium. All isolates showed fast growth rates of 90mm within one week of incubation at 28°C.These results were according to preceding temperature studies accomplished with Botryosphaeriaceae isolates from California in which *L. theobromae* isolates attained maximum growth rates at 28°C. Conidial color, shape and presence of septa are major characteristics for identification of *B. dothidea* and *L. theobromae*. *B. dothidea* can be differentiated from other species by producing white to dark brown colonies, hyaline, holoblastic and subcylindrical conidiogenous cells and fusiform, unicellular, hyaline and aseptate conidia, rarely forming a septum. The utility of these morphological distinctions is in agreement with previous literature (Urbez-Torres *et al.*, 2006).

Molecular characterization: High quality DNA was achieved and ITS-5.8S rDNA-ITS region of isolates was amplified. PCR products having single amplification fragments were in the range of 580-600bp (Fig. 4abcd). Nucleotide sequences of ITS region of 5.8S rDNA were submitted in Genbank and accession numbers for isolated species were obtained (Table 6). Blast searches in Genbank identified the isolates as *Lasiodiplodia theobromae* and *Botryosphaeria dothidea*. High similarities (96-100%) were examined for both species (Fig. 5). The evolutionary history was inferred using the neighbor-joining method. The analysis involved 47 nucleotide sequences. All positions containing gaps and missing data were eliminated. To get insight into the phylogenetic relationship between isolates of species, a detailed molecular phylogeny approach was applied. Two main clades were obvious in the phylogenetic tree. Phylogenetic reconstructions distinguished ITS sequences into two further subclades based on evolutionary relationships among the isolates of *L. theobromae* and *B. dothidea*. DNA sequencing analysis verified the morphological identification of *B. dothidea* and *L. theobromae*. The rDNA-ITS sequencing marker is used as a commanding tool in identifications and determination of fungus variations (White *et al*., 1990).These ITS markers are extensively used to determine the relationships within fungal species in addition to detection and identification of fungi up to specie level (Nilsson *et al*., 2008).

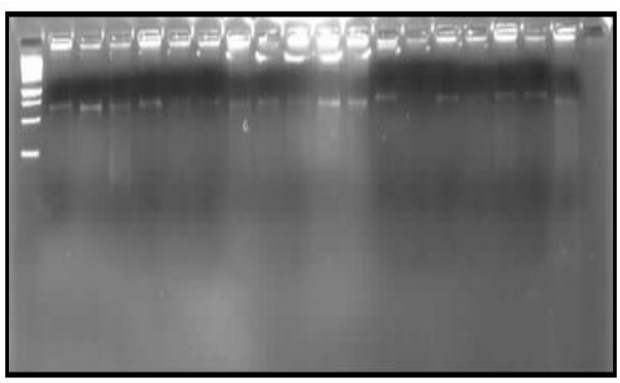


Fig. 4a. Purified PCR products.

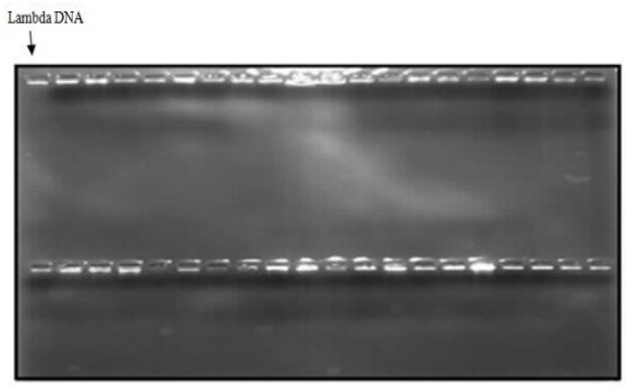


Fig. 4b. Quantified DNA.

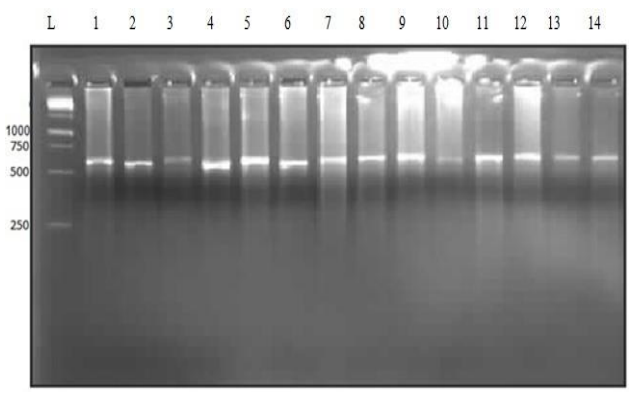


Fig. 4c. ITS amplification of DNA generating fragments ranging in 580-600bp.

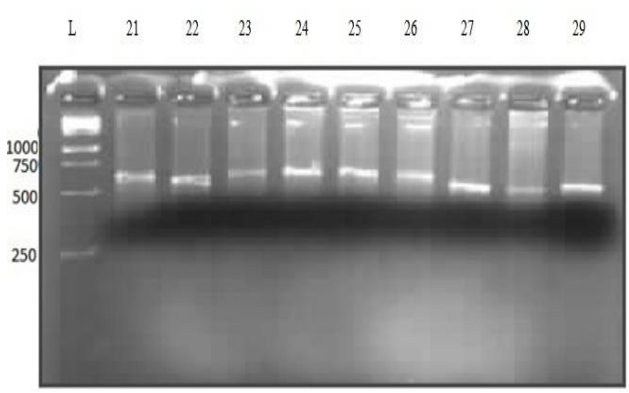


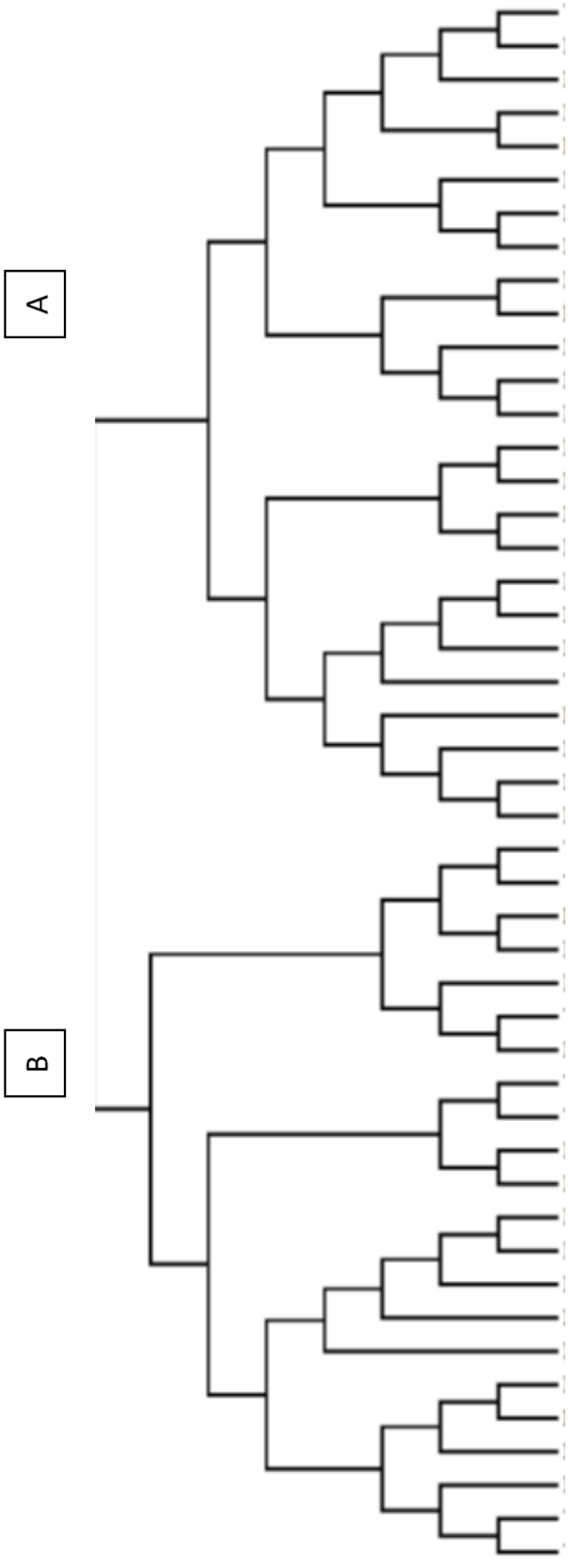
Fig. 4d. DNA amplification with ITS primers generating 580-600bp fragments.

Table 6. Fungal isolates of mango retrieved from GenBank used for phylogenetic analysis.

Isolate code	Identity	Source	Host	Location	Accession number	Similar species	Similarity (%)
mlt-1	*L. theobromae*	Fruit	*M. indica* White Chounsa	Punjab, Pakistan	AB908429	*L. theobromae* HM466959	99
mlt-2	*L. theobromae*	Fruit	*M. indica* White Chounsa	Punjab, Pakistan	AB908435	*L. theobromae* GU066603	97
mlt-3	*L. theobromae*	Fruit	*M. indica* White Chounsa	Punjab, Pakistan	AB908428	*L. theobromae* KC895526	98
mlt-7	*L. theobromae*	Fruit	*M. indica* White Chounsa	Punjab, Pakistan	AB908436	*L. theobromae* HM346874	95
mlt-10	*B. dothidea*	Fruit	*M. indica* White Chounsa	Punjab, Pakistan	AB908438	*B. dothidea* KF293850	99
mlt-11	*L. theobromae*	Fruit	*M. indica* White Chounsa	Punjab, Pakistan	AB908432	*L. theobromae* KC960898	99
mlt-70	*B. dothidea*	Fruit	*M. indica* White Chounsa	Punjab, Pakistan	AB906439	*B. dothidea* KF293944	99
mlt-71	*L. theobromae*	Fruit	*M. indica* White Chounsa	Punjab, Pakistan	AB906441	*L. theobromae* FJ150695	99
mlt-72	*B. dothidea*	Fruit	*M. indica* White Chounsa	Punjab, Pakistan	AB906440	*B. dothidea* KF516940	100
mlt-73	*L. theobromae*	Fruit	*M. indica* White Chounsa	Punjab, Pakistan	AB906442	*L. theobromae* GQ502452	99
mg-3	*L. theobromae*	Fruit	*M. indica* White Chounsa	Punjab, Pakistan	AB906430	*L. theobromae* JX464083	100
mg-4	*L. theobromae*	Fruit	*M. indica* White Chounsa	Punjab, Pakistan	AB906431	*L. theobromae* HM466960	100
mg-8	*L. theobromae*	Fruit	*M. indica* White Chounsa	Punjab, Pakistan	AB908437	*L. theobromae* GU066603	98
mg-12	*L. theobromae*	Fruit	*M. indica* White Chounsa	Punjab, Pakistan	AB908431	*L. theobromae* KC484812	100
mg-30	*L. theobromae*	Fruit	*M. indica* White Chounsa	Punjab, Pakistan	AB906428	*L. theobromae* JX464096	100
mg-31	*L. theobromae*	Fruit	*M. indica* White Chounsa	Punjab, Pakistan	AB906429	*L. theobromae* GQ469929	100
mg-54	*L. theobromae*	Fruit	*M. indica* White Chounsa	Punjab, Pakistan	AB906432	*L. theobromae* GU066603	99
ryk-1	*L. theobromae*	Fruit	*M. indica* White Chounsa	Punjab, Pakistan	AB908425	*L. theobromae* JX464083	99
ryk-2	*L. theobromae*	Fruit	*M. indica* White Chounsa	Punjab, Pakistan	AB908426	*L. theobromae* FJ150695	99
ryk-3	*B. dothidea*	Fruit	*M. indica* White Chounsa	Punjab, Pakistan	AB908427	*B.dothidea* KF293953	100
ryk-4	*B. dothidea*	Fruit	*M. indica* White Chounsa	Punjab, Pakistan	AB908433	*B. dothidea* KC197787	100
ryk-5	*B. dothidea*	Fruit	*M. indica* White Chounsa	Punjab, Pakistan	AB908434	*B. dothidea* JX096631	98
mat-6	*L. theobromae*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906419	*L. theobromae* FJ150695	99
mat-7	*L. theobromae*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906420	*L. theobromae* KF294005	99

Table 6. (Cont'd.).

Isolate code	Identity	Source	Host	Location	Accession number	Similar species	Similarity (%)
mat-12	*B. dothidea*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906425	*B. dothidea* EU162756	99
mat-13	*B.dothidea*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906426	*B. dothidea* GQ421485	99
mat-14	*L. theobromae*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB908430	*L. theobromae* KC960898	100
mat-20	*L. theobromae*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906421	*L. theobromae* JX464065	95
mat-25	*L. theobromae*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906422	*L.theobromae* JX868690	99
mat-26	*L. theobromae*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906423	*L. theobromae* KC484820	99
mat-32	*B. dothidea*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906427	*B. dothidea* KC556959	98
mat-56	*L. theobromae*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906424	*L. theobromae* KF466496	95
mpk-6	*L. theobromae*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906434	*L. theobromae* JX868680	99
mpk-7	*L. theobromae*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906435	*L. theobromae* JX139602	100
mpk-48	*L. theobromae*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906433	*L. theobromae* FJ904843	100
mpk-49	*L. theobromae*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906436	*L. theobromae* HM346877	99
mpk-54	*L. theobromae*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906437	*L. theobromae* JQ031157	100
mpk-55	*L. theobromae*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906438	*L. theobromae* KC895526	100
tay-14	*B. dothidea*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906443	*B. dothidea* JX139033	100
tay-32	*B. dothidea*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906444	*B. dothidea* KC960887	98
tay-42	*B. dothidea*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906445	*B. dothidea* KF293913	99
tay-47	*L. theobromae*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906446	*L. theobromae* HM346878	100
tay-48	*L. theobromae*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906447	*L. theobromae* JX464083	100
tay-52	*L. theobromae*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906448	*L. theobromae* KC662361	98
tay-53	*L. theobromae*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906450	*L. theobromae* KF516938	99
tay-54	*L. theobromae*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906449	*L. theobromae* HM46695	99
tay-56	*L. theobromae*	Fruit	*M. indica* Sindhri	Sindh, Pakistan	AB906451	*L. theobromae* JX139033	100



Location	Isolate#	Identified fungi
A1	Tando Allah Yar 52	*Lasiodiplodia theomae*
	Multan 3	*Lasiodiplodia theomae*
	Matiyari 26	*Lasiodiplodia theomae*
	Mirpur Khas 7	*Lasiodiplodia theomae*
	Rahim Yar Khan 2	*Lasiodiplodia theomae*
	Mirpur Khas 55	*Lasiodiplodia theomae*
	Matiyari 56	*Lasiodiplodia theomae*
	Mirpur Khas 48	*Lasiodiplodia theomae*
	Matiyari 13	*Botryosphaeria dothidea*
	Rahim Yar Khan 5	*Botryosphaeria dothidea*
	Muzaffar Garh 12	*Lasiodiplodia theomae*
	Multan 73	*Lasiodiplodia theomae*
	Multan 7	*Lasiodiplodia theomae*
	Matiyari 12	*Botryosphaeria dothidea*
	Matiyari 32	*Botryosphaeria dothidea*
	Muzaffar Garh 4	*Lasiodiplodia theomae*
	Mirpur Khas 49	*Lasiodiplodia theomae*
	Matiyari 6	*Lasiodiplodia theomae*
	Muzaffar Garh 30	*Lasiodiplodia theomae*
A2	Muzaffar Garh 3	*Lasiodiplodia theomae*
	Tando Allah Yar 53	*Lasiodiplodia theomae*
	Rahim Yar Khan 4	*Botryosphaeria dothidea*
	Muzaffar Garh 54	*Lasiodiplodia theomae*
	Matiyari 20	*Lasiodiplodia theomae*
	Mirpur Khas 6	*Lasiodiplodia theomae*
	Tando Allah Yar 47	*Lasiodiplodia theomae*
	Tando Allah Yar 48	*Lasiodiplodia theomae*
	Rahim Yar Khan 1	*Lasiodiplodia theomae*
	Matiyari 14	*Lasiodiplodia theomae*
	Matiyari 7	*Lasiodiplodia theomae*
B1	Tando Allah Yar 32	*Botryosphaeria dothidea*
	Multan 2	*Lasiodiplodia theomae*
	Tando Allah Yar 54	*Lasiodiplodia theomae*
	Tando Allah Yar 56	*Lasiodiplodia theomae*
	Multan 11	*Lasiodiplodia theomae*
	Multan 10	*Botryosphaeria dothidea*
	Tando Allah Yar 54	*Lasiodiplodia theomae*
	Multan 71	*Lasiodiplodia theomae*
	Muzaffar Garh 8	*Lasiodiplodia theomae*
	Multan 70	*Botryosphaeria dothidea*
	Matiyari 25	*Lasiodiplodia theomae*
	Multan 72	*Botryosphaeria dothidea*
	Rahim Yar Khan 3	*Botryosphaeria dothidea*
	Muzaffar Garh 31	*Lasiodiplodia theomae*
B2	Multan 1	*Lasiodiplodia theomae*
	Tando Allah Yar 14	*Botryosphaeria dothidea*
	Tando Allah Yar 42	*Botryosphaeria dothidea*

Fig. 5. A neighbor-joining phylogenetic tree obtained from ITS region of 5.8S-rDNA sequencing data.

Assessment of aggressive behavior of *Botryosphaeria dothidea* and *Lasiodiplodia theobromae by* pathogenicity assays

Same length together with identity in the nucleotide sequence of ITS-5.8S rDNA-ITS2 fragments of isolates signified the rDNA-ITS region of isolates was highly conserved. Several investigations have revealed that using a single gene region is not sufficient to demarcate the cryptic species (Slippers *et al*., 2004). However to determine species boundaries, at least two gene regions are required. This approach divulged the presence of mysterious species in various genera of family Botryosphaeriaceae (Pavlic *et al.*, 2004).

Detached mango twigs: Infection signs became visible as brown colored scratches at the point of inoculation which were spread out upward and downward from the point of inoculation. Brown streaks were examined in woods while no symptom of disease was monitored in twigs of control experiment (Fig. 2abcdef). All tested pathogens were proved pathogenic on detached twigs. Isolates showed variability in aggressive behavior. Clustering was done by XLstat and isolates were divided into four groups based on dissimilar pathogenic behavior (Fig. 6, Table 7). Mean lesion length on detached twigs affected by pathogens ranging between 1.333-4.1cm and the width on detached twigs ranged between 0.467-1cm. Largest lesion area covered by pathogens was calculated for isolate number 3 (12.456cm²) and 6 (11.544c²m) and these were collected from Multan (Punjab) while isolate 32, 34 and 35 showed negligible disease symptoms and lesion area of these isolates was 2.649cm², 3.363cm² and 3.133cm² respectively. These isolated were assembled from Matiyari and Mirpur Khas (Sindh).

Detached mango leaves: After 2 weeks of incubation, wilting and drying was observed in inoculated leaves but no visible signs of stem end rot were appeared on leaf parts inoculated with pathogenic culture plug.

Potato tubers: Visible disease symptoms as brown lesions initiating from the point of inoculation spreading longitudinally and vertically on vascular tissues were observed. Pathogens showed variations in lesion development. Tubers in control remained unsymptomatic during experimentation (Fig. 2ghi). Clustering analysis was conducted and isolates were segregated into four groups depending on the level of aggressiveness (Fig. 7, Table 8).

Table 7. Grouping of isolates based on aggressive behavior on detached mango twigs.

Aggressive (A1) (6.576 – 8.926 cm²)	Highly aggressive (A2) (10.075 – 12.456 cm²)	Moderately aggressive (B1) (5.070 – 6.179 cm²)	Slightly aggressive (B2) (2.649 – 4.682 cm²)
Botryosphaeria dothidea (isolate 5, 7, 9, 21, 22)	*Botryosphaeria dothidea* (isolate 26)	*Botryosphaeria dothidea* (isolate 20, 25, 31, 41, 42)	*Botryosphaeria dothidea* (isolate 39, 40)
Lasiodiplodia theobromae (isolate 1, 4, 8, 10, 15, 16, 17, 19, 23, 24, 27, 28, 33, 36, 38, 45)	*Lasiodiplodia theobromae* (isolate 2, 3, 6, 12, 14)	*Lasiodiplodia theobromae* (isolate 11, 18, 29, 44, 46, 47)	*Lasiodiplodia theobromae* (isolate13, 30, 32, 34, 35, 37, 43)

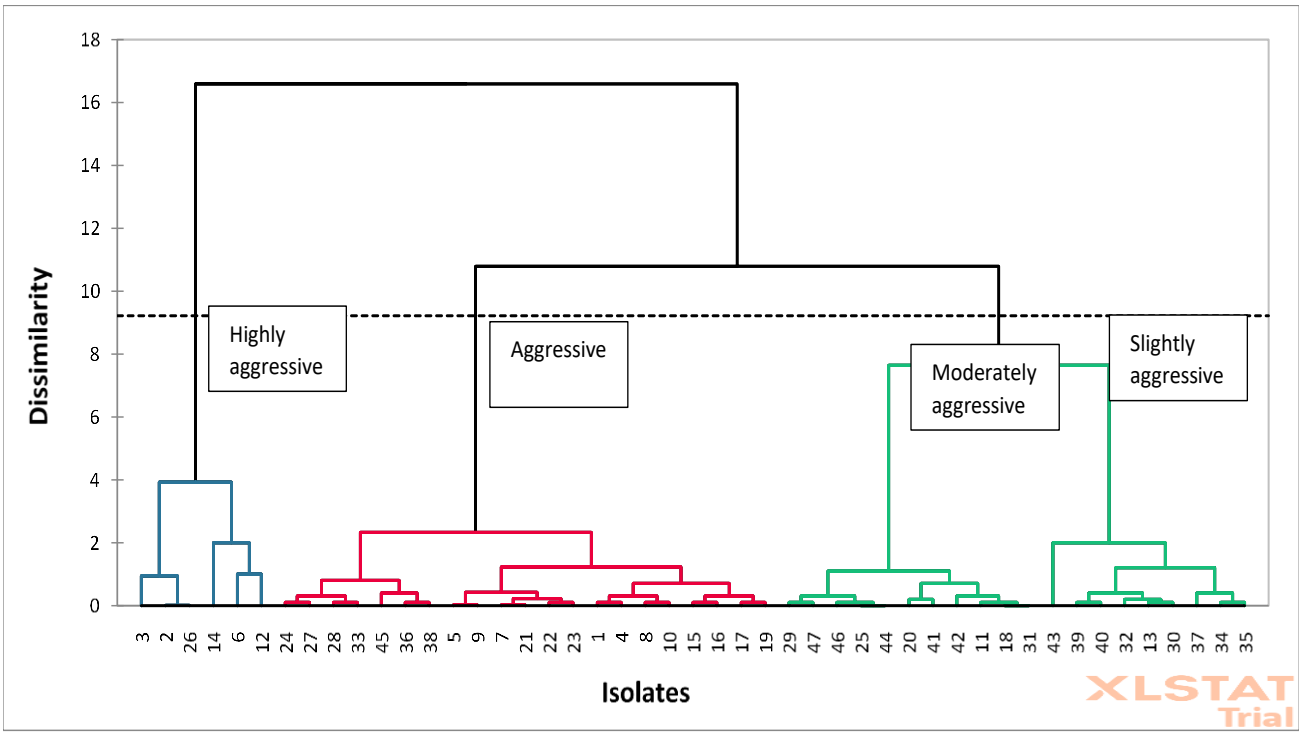


Fig. 6. Dendrogram showing clustering based on dissimilarity in aggressiveness of pathogens on detached mango twigs.

Table 8. Grouping of isolates based on aggressive behavior on potato tubers.

Aggressive (A1) (13.952 – 16.924 cm²)	Highly aggressive(A2) (18.495 - 19.582 cm²)	Moderately aggressive (B1) (9.598 – 12.893cm²)	Slightly aggressive (B2) (5.757 – 8.938cm²)
Botryosphaeria dothidea (isolate 9, 20, 21, 22, 39, 40)	*Botryosphaeria dothidea* (0)	*Botryosphaeria dothidea* (isolate 5, 7,31)	*Botryosphaeria dothidea* (isolate 25, 41)
Lasiodiplodia theobromae (isolate 1, 2, 6, 8, 14, 15, 19, 24, 37)	*Lasiodiplodia theobromae* (isolate 3,13,16)	*Lasiodiplodia theobromae* (isolate 10, 12, 17, 18, 23, 27, 29, 30, 32, 33, 34, 45, 47)	*Lasiodiplodia theobromae* (isolate 11, 28, 35, 36, 38,42, 43, 44, 46)

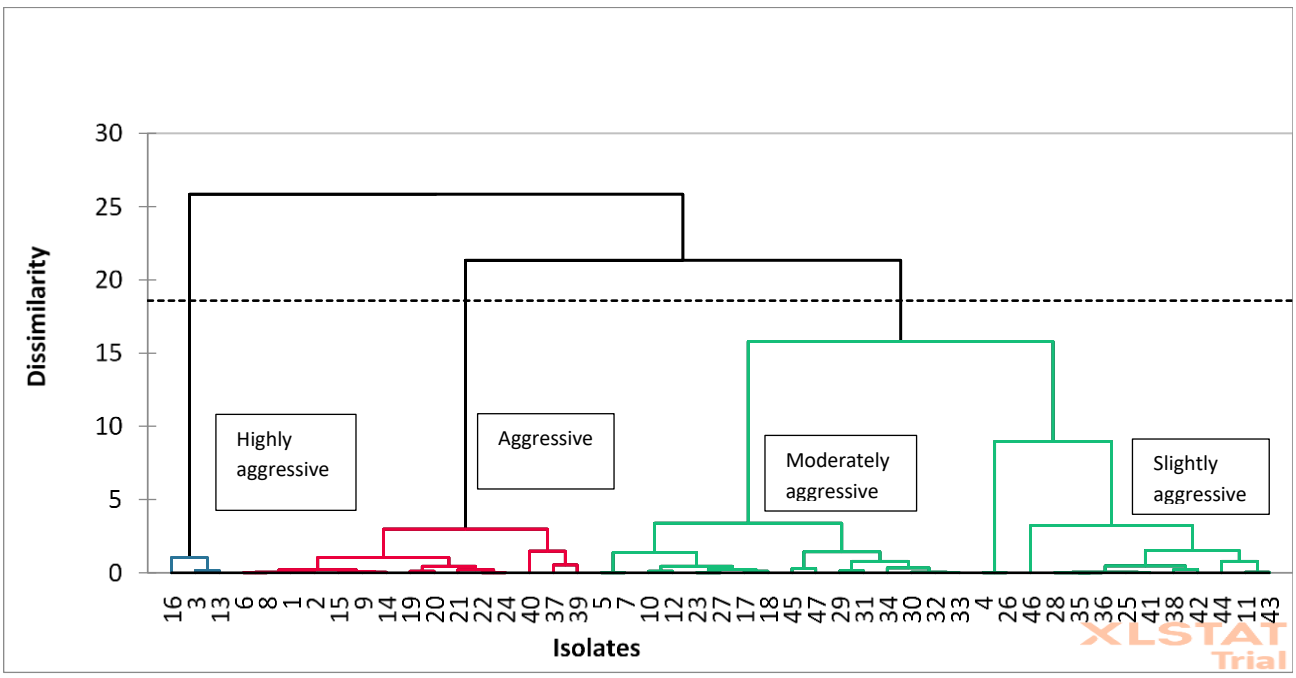


Fig. 7. Dendrogram showing clustering based on dissimilarity in aggressiveness of pathogens on potato tubers.

Table 9. Analysis of variance for length, width and area (lesion) of isolated pathogens on detached mango twigs.

Source	DF	SS	MS	F	P
Length (lesion) of isolated pathogens on detached twigs					
Replicate	2	0.0095	0.00475	0.01	0.9904
Error	138	67.9123	0.49212		
Total	140	67.92			
Grand mean			2.9780		
CV			23.56		
Width (lesion) of isolated pathogens on detached twigs					
Replicate	2	0.04142	0.02071	1.08	0.3429
Error	138	2.64936	0.01920		
Total	140	2.69			
Grand mean			0.8376		
CV	16.54				
Area(lesion) of isolated pathogens on detached twigs					
Replicate	2	4.198	2.09900	0.41	0.6624
Error	138	701.121	5.08059		
Total	140	705.319			
Grand mean			7.8395		
CV			28.75		

Mean lesion length was in range of 1.7-3.467cm and width ranging in 0.9-2.2cm was recorded on potato tubers. Largest lesions on potato tubers were established by isolate number 3, 13 and 16. Lesion area produced by these isolates was 19.582 cm², 18.557 cm² and 18.495 cm² respectively and they were isolated from district Multan and Muzaffar Garh. Isolate 25 and 43 (5.757 cm² and 5.758 cm²) showed slight lesions on potato tubers. They were isolated from Matiyari and Tando Allah Yar. Tables 9 & 10 showed the results of ANOVA for the length, width and area (lesion) of isolated pathogens on detached mango twigs and potato tubers. The P values at 95% confidence limit showed that isolates were not significantly different. Isolates from Punjab proved more pathogenic on detached mango twigs and potato tubers compared to isolates collected from Sindh.

A comparison was made among the aggressiveness of all isolates on detached twigs, leaves and potato tubers. Pathogens showed most severe symptoms on potato tubers followed by detached twigs but detached leaved remained asymptomatic in the whole trial of pathogencity (Fig. 8). Koch's postulates were confirmed by reisolation of pathogens successfully from artificially infected tissues and similar results were obtained in pure cultures. The upward and downward development inside the dynamic tissues indicates the distinguished endophytic nature of these pathogens (Ploetz, 2004). All investigated isolates of *B. dothidea* and *L. theobromae* confirmed that they were proficient to spread by the internal tissues inoculation points causing brown to black discoloration. Similar results were achieved by Khanzada *et al.*, (2004) who demonstrated that inoculations of mango fruit with *Botryosphaeria* species marked an assortment of external and internal indications such as necrosis and discoloration of vascular tissues. The most severe results of aggressiveness on potato tubers might be due to the fact that potato is a source of carbohydrates and sucrose which encouraged the maximum mycelial growth as well as pycnidial production. In a previous research *Botryodiplodia theobromae* was found most aggressive and virulent to potato tubers (Salami & Popoola, 2007).

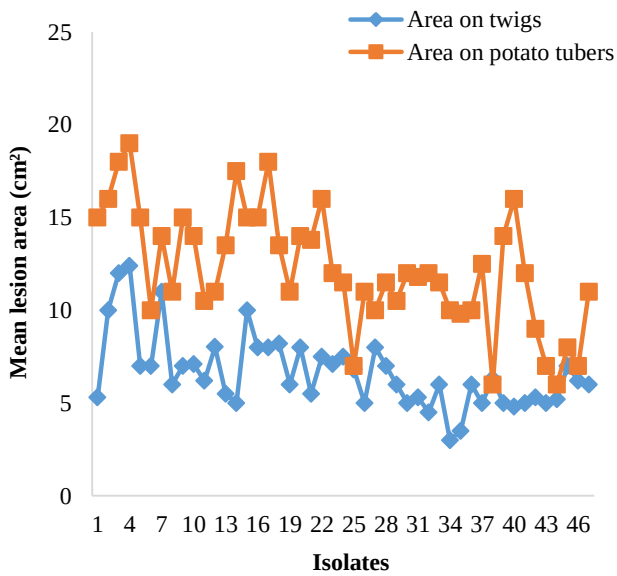


Fig. 8. Comparison of mean lesion area (cm²) covered by pathogens on potato tubers and detached twigs of mango.

Table 10. Analysis of variance for length, width and area (lesion) of isolated pathogens on potato tubers.

Source	DF	SS	MS	F	P
Length (lesion) of isolated pathogens on potato tubers					
Replicate	2	0.0435	0.02177	0.11	0.8998
Error	138	28.4230	0.20596		
Total	140	28.4665			
Grand mean			2.4716		
CV			18.36		
Width(lesion) of isolated pathogens on potato tubers					
Replicate	2	0.00440	0.00220	0.04	0.9625
Error	138	7.92596	0.05743		
Total	140	7.93035			
Grand mean			1.5461		
CV			15.50		
Area (lesion) of isolated pathogens on potato tubers					
Replicate	2	0.97	0.4849	0.04	0.9640
Error	138	1822.22	13.2045		
Total	**140**	1823.19			
Grand mean			12.207		
CV			29.77		

Conclusion

It was concluded that stem end rot is prevalent in Punjab and Sindh and morphological and molecular studies revealed that *Lasiodiplodia theobromae* and *Botryosphaeriadothidea* were the main cause of mango stem end rot. According to pathogencity results, fungal pathogens of districts in Punjab were more aggressive than pathogens of Sindh. Isolated pathogens were proved more aggressive on potato tubers compared to detached twigs but detached were not proved good host for pathological analysis of *Lasiodiplodia theobromae* and *Botryosphaeria dothidea*. ITS sequencing analyses are proficient to determine genetic variability among the isolates, providing information on genetic variations of these species as a source for future studies planning the management of disease.

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