Pak. J. Bot., 49(4): 1605-1613, 2017.

PATHOGENIC CHARACTERIZATION OF *LASIODIPLODIA* CAUSING STEM END ROT OF MANGO AND ITS CONTROL USING BOTANICALS

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Abstract

Two widely cultivated mango fruit varieties White chounsa and Sindhri were collected from two major mango growing areas of Punjab and Sindh Provinces of Pakistan. This study was focused on pathological characterization of predominant postharvest diseases such as stem end rot of mango (*Mangifera indica)* caused by *Lasiodiplodia theobromae*, and evaluation of bio-control activity by different plant extracts. *L. theobromae* aggressiveness of isolates was tested by artificial inoculations under controlled conditions, all isolates proved pathogenic in varying degree of aggressiveness on (Sindhri and White chounsa) with reference to control. Calculated standard error mean varied in lesion area produced by pathogens 6–63cm^2 (Sindhri) and 60-170 cm^2 (White chounsa). Re-isolation of respective fungi verified the Koch's postulates. Plant extract of *Datura stramonium*, *Aloe-vera*, *Eucalyptus camaldulensis*, were used to control the radial growth of *L. theobromae*. Comparative analysis showed *D. Stramonium* and *E. camaldulensis* extracts most efficiently reduced the growth of *Lasiodiplodia* isolates, in comparison to *Aloe-vera* extract, restrict the 15-20% growth. All pathological results and treatments were significant at $p<0.05$ through ANOVA. This study emphasizes the behavior of pathogens which could be helpful in mango breeding to introduce resistance toward *Lasiodiplodia* and referred plants provide the best alternative of chemical fungicides.

Key words: Botanicals, Pathogenicity, Sindhri, White chounsa.

Introduction

According to worldwide statistical analysis, Pakistan stands fourth largest producer of the mango fruit (Minfal, 2009) after India, China, and Thailand. The river belts of Sindh and Chenab are the traditional zones of mango production in Pakistan. Sindhri and white chounsa are the two best and dominant varieties of mango (Ghafoor *et al.*, 2010). The frequency of these verities has prevalence because of their delicious taste and appetizing aroma (Maqbool *et al.*, 2007). Mango is the reliable sources of farm income in southern Punjab and lower Sindh (Arshad, 2008). The yield of mango is unfavorably hampered by the several biotic and abiotic stresses (Shahbaz *et al*., 2009). Postharvest diseases are the principal risk to the mango cultivars. Among them, postharvest losses occur because of infections, by certain physiological disorders, or by the attack of bacteria and fungi (Shivashankar, 2014). Quantitative postharvest losses of mango, amount 8.6 million tons worth US$ 335.2 million per year (Singh *et al*., 2013). In Pakistan, traditional technologies used for postharvest fruit processing, packaging, transportation, handling, storage, and consumption, are responsible for causing 20-40% loss of fruit and vegetables (Tahir *et al.*, 2002), Ultimately inducing the major economic losses. Postharvest diseases prune the natural fruit quality. In most cases, blemished fruit failed to meet the required standard of choice and causes the economic loss in the global markets (Arauz, 2000). During storage mango becomes more susceptible to postharvest diseases, because of physiological changes and senescence which facilitate the growth of pathogens (Prusky, 2009). Correspondingly, Jabbar (2011) described that susceptibility of mango fruit to postharvest diseases increases after harvesting and favored pathogen development. In Pakistan postharvest administration is a noteworthy challenge confronted by the mango industry (Amin *et al.*, 2008). During 2007-2008, about 20% declines were witnessed in export because, in the international market, Pakistan obtained lowest price (per kg) because of the poor quality of fruit. Major fungal pathogens such as *Fusarium* sp., are responsible for the malformation of mango and rotting (Fida & Iram, 2014) Moreover, *Lasiodiplodia theobromae* causal agents of stem end rot and quick decline (Rees, 2012) are the serious menaces to fruit quality and the agrarian economy of Pakistan (Korsten, 1993). In Florida, *Lasiodiplodia theobromae* and *Fusicoccum aesculi* were found causative symptoms linked with the decline on cvs Keit and Tommy Atkins (Ploetz *et al*., 1996). Latter *Lasiodiplodia* species have also been reported from Brazil as relating with mango dieback and stem-end rot (Costa *et al.*, 2010). Postharvest losses aggravate in Pakistan because 99% mango fruit is harvested manually which causes physical damage, sap burns injuries, and bruising. Stem end rot is the predominant fungal disease in Pakistan, requiring systematic study. Previously, considerable work has been done on the morphological identification and characterization of *Lasiodiplodia* species (Shahbaz *et al*., 2005). A more detailed investigation is still needed to understand the relationship between the aggressive behavior of fungal pathogens involved in post–harvest fungal diseases of mango and their control. The goal of present study is to analyze the pathogenic behavior of *L. theobromae* fungal pathogen causing post-harvest (stem end rot) disease of mango on Sindhri and White chounsa varieties of mango, and to fathom the reasons about the post-harvest fungal pathogens and market losses of mangoes and their control by using the different plant extracts.

Material and Methods

Collection of mango samples: Two major varieties (White chounsa and Sindhri) of mango fruit were collected from different districts of Punjab and Sindh (Table 1). The observed fruit size was almost equal for both Sindhri and White chounsa, and the age of mango fruit was 6months. Punjab is the second largest province of Pakistan located at the northwestern edge of the geologic Indian plate; while Sindh is located on the western corner, sharing the border with Iranian plateau in the west. Geographically it is the third largest province of Pakistan.

Table 1. No of isolates with their locations (Punjab and Sindh).

Mango varieties	White chounsa	Sindhri
No. location	Punjab	Sindh
1	PL1	SL1
2	PL2	SL2
3	PL3	SL3
4	PL4	SL4
5	PL5	SL5
6	PL6	SL6
7	PL7	SL7
8	PL8	SL8
9	PL9	SL9
10	PL10	SL10
11	PL11	SL11
12	PL12	SL12

PL (Punjab *L. theobromae*) and SL (Sindh *L. theobromae*)

Pure culture of *Lasiodiplodia theobromae*: Further studies were conducted in Mycology laboratory of Fatima Jinnah women university, Rawalpindi, Pakistan. Collected mangoes were thoroughly washed with the distilled water and surface sterilized with 70% ethanol and washed again three times with distilled water to avoid inhibition growth of fungus. After seven days, the sample was collected from mango parts, exhibiting the disease symptoms of stem end rot. Mango tissue was excised from the diseased portion with the help of surgical blade and cultured on the general PDA media (Potato extract 4g, Dextrose 20g, Agar15gL^{-1}) plates. Inoculated plates were incubated at 28 ºC for seven days for fungus growth (Fig. 1).

Morphology: All isolates were identified morphologically on the basis of colony (color, shape, texture) and conidia (spore size, shape, Septation) under the compound microscope fitted with the ocular micrometer at 10X (Olympus, Japan).

Molecular analysis: Total genomic DNA of 24 fungal isolates was isolated using modification of phenol extraction method (Reader & Broda, 1985). DNA concentration was estimated to 25ng through lambda DNA standards (Reader & Broda, 1985). Gene 5.8S and two flanking ITS1 and ITS2 internal transcribed spacers were amplified by using the modification of protocol proposed by Mohankumar *et al.*, 2010. The primers sequence used for the amplification were ITS1F (5'- TCC GTA GGT GAA CCT GCG G-3') (Gardes & Bruns, 1993) and ITS4R (5' TCC TCC GCT TAT TGA TAT GC-3') (White *et al*., 1990). The 50 µl reaction mixture was prepared which contained 25ng of template DNA, 20 pmol primers ITS1 & ITS4, 10mM of dNTPs, and 5 µl PCR buffer with NH4 (SO4)2, 5 µl MgCl2 and 1U Taq DNA polymerase (Fermentas) PCR conditions were as follows: initial denaturation at 95°C for 1 min, followed by 30 cycles of denature (95°C), annealing (55°C) and extension (72°C) for 1 min each with final elongation at 72°C for 7 min PCR products were observed at 2% Agarose gel to determine the gene amplification and the amplified bands were compared against 1kb ladder (Fermentas). Amplified PCR product was purified with PCR purification kit (Fermentas).

Phylogeny trees: Final nucleotide sequence of 24 isolates was submitted to GenBank NCBI. The obtained sequences were compared with previously identified sequences in GenBank using the Basic Local Alignment Search Tool (BLAST). Sequences were aligned together with those retrieved from GenBank using Geneious.R.10 (Kearse *et al.*, 2012). Eight isolates were randomly selected, on the basis of highly aggressive, aggressive, moderately aggressive, non-aggressive for construction of phylogeny tree. Tree was constructed in Geneious.R10. Software using Tamura-Nei genetic distance model combination with the neighbor-joining method.

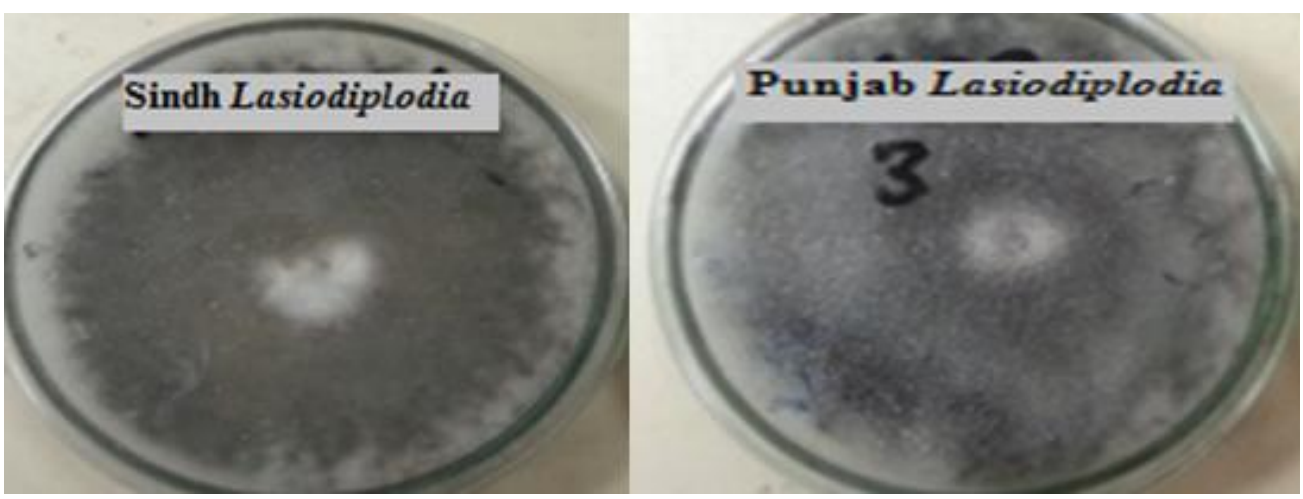

Fig. 1. *Lasiodiplodia theobromae* isolates on PDA agar medium.

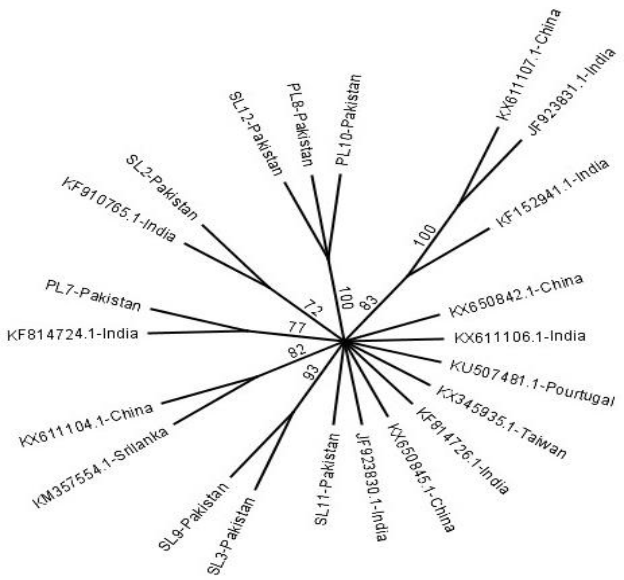

Fig. 2. Un-rooted Phylogenetic tree showing the relationship of ITS-sequence of *Lesiodiplodia* with the similar public sequences, genetic distances were calculated using the Tamura-Nei model, the phylogenetic tree was constructed using the neighbor-joining method.

Correlation between aggressiveness and genetics: The other tree (Fig. 3) was constructed between the isolates of Sindhri and White chounsa isolates in Geneious. R10. software using Tamura-Nei genetic distance model combination with the neighbor-joining method. Bootstrap support values were evaluated using 50 bootstrap replicates.

Pathogenicity tests on detached mango fruit: Pathogenicity tests were carried out on healthy mature mango fruits of the same age and size to determine the aggressive and non-aggressive behavior of fungal pathogens. Punjab (White chounsa) and Sindh (Sindhri) were chosen for disease assessment. Mango fruits were surface sterilized with 1% NaCl solution for 10-15minutes, washed with distilled water and allowed to dry. After that fruits were inoculated by 5mm fungal agar plug of seven days old culture. The inoculated portion wrapped with parafilm. In comparison to diseased mango fruit, the control mango samples were non-colonized by agar plug only and experiment were conducted in complete randomized design (CRD) plan, where three biological replicates (three mango fruits) were used for each isolate. The inoculated fruit covered with the polythene bag and incubated with the cotton plug (wet) to maintain the humidity. The experiment was conducted at room temperature. After 24h, the inoculum was removed. The appearance of the lesion was observed for 3–10 days after inoculation (Lelliott & Stead, 1987) and diameter was measured in two dimensions on each mango fruit, both horizontally and vertically to the stem (Sakalidis *et al*., 2011). The lesion area was calculated by using the formula:

Area of oval = πlw: π =3.14, l=length, w=width. Disease reaction was scored on the 0–5 point scale using a modification of the disease scoring scale (Corkidi *et al.*, 2006) (Table 2).

Table 2. Disease severity scale.

No	Affected area	Disease severity
1.	0%	No disease
2.	1-5%	Trace
3.	6-25%	Mild
4.	26-50%	Moderate
5.	51-71%	Severe
6.	76-100%	Very severe

Re-isolation of the fungi from inoculated mango fruit was done in order to confirm the Koch's postulates (Mahasuk *et al*., 2009; Pakdeevaraporn *et al*., 2005)

Source of plant leaves: Fresh leaves of *D. stramonium*, *Aloe vera*, and *E. camaldulensis* collected from Rawalpindi (north-west) and Multan (southeast). Leaves were sterilized with 70% ethanol and shed dried.

Preparation of plant extracts, and fungal growth inhibition by bioassay: Dried leaves were crushed with the help of mortar and pestle to a fine powder (10 g) each plant powder was added in 100 ml distilled water. The solution was boiled for 10 minutes to remove the secondary pathogens (fungi, bacteria) and centrifuged at 10000g for 5 minutes. The supernatant was used for the antifungal activity. Ten ml of each plant extract (supernatant) was added in 90 ml of PDA media. In a case of *Aloe-vera*, the 10ml fresh gel was added directly in 90 ml PDA media and this media was centrifuged at 10000g for 5min. Media plates were allowed to solidify and inoculated with 5mm fungal disk of seven-day-old culture. Inoculated plates were grown at 28ºC for 10 days (Table 3).

Bio-efficiency of plant extracts against *Lasiodiplodia*: The antifungal efficiency of plants was observed on agar plate by using the formula (Vincent, 1947).

$$\text{Percentage of inhibition: } [\% = \frac{C - T}{C} \times 100]$$

C = Growth in absence of Plant extract
T = Growth of fungus in the presence of plant extract

Table 3. Plants description used for antifungal activity.

No.	Botanical name	Local name	Family	Part used	Medicinal use
1.	*Datura stramonium*	Datura	Solanaceae	Leaves	Antimicrobial
2.	*Aloe vera*	*Aloe-vera*	Asphodelaceae	Pulp	Antimicrobial
3.	*Eucalyptus camaldulensis*	Safeda	Myrtaceae	Leaves	Antimicrobial

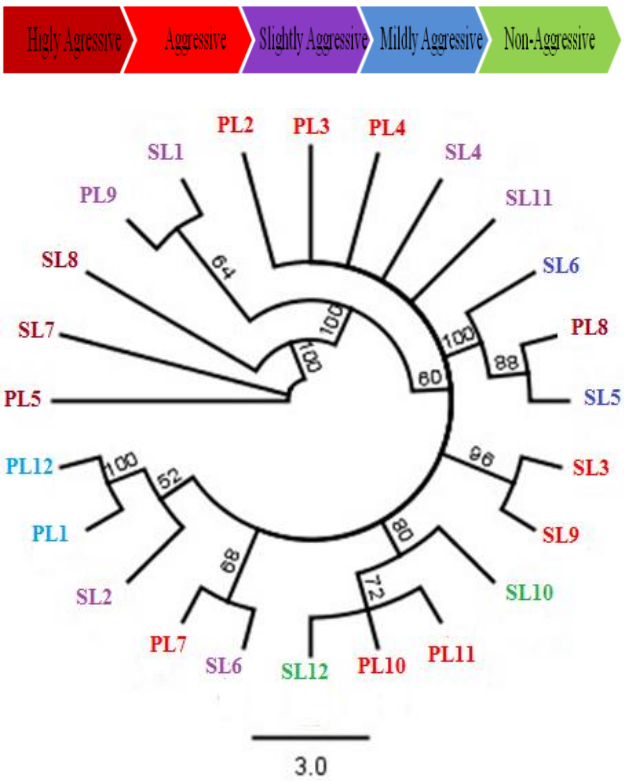

Fig. 3. Correlation between the aggressiveness and molecular phylogeny, within the isolates of *Lasiodiplodia* (Sindh and Punjab). Genetic distances were calculated using the Tamura-Nei model, the phylogenetic tree was constructed using the neighbor-joining method.

Statistical analysis: Data recorded for various characteristics were analyzed, with completely randomized design (CRD) a two-factor factorial analysis of variance (ANOVA) technique, using statistix 8.1. For significant F value, the least significant difference (LSD) was used for mean comparison at 0.05% level (Sakalidis *et al.,* 2011). Clustering was done through Minitab 17 statistical software for grouping and characterized as aggressive, slightly aggressive, moderately aggressive, highly aggressive, and non-aggressive. Phylogenetic tree was prepared by using the Geneious (Kearse *et al.,* 2012) software.

Results and Discussion

Morphology: *L. theobromae* isolates were morphologically characterized on PDA media. The maximum growth was observed at 30˚C temperature and pH~6. These findings are similar to (Jacobs & Rehner, 1998); indicating the observed temperatures ranging 25 - 30°C are the most favorable for the majority of *Botryodiplodia* sp., that cause decline and die-back of mango (Table 4).

Similar results consisting limited morphological differences in isolates of *L. theobromae* have already been reported by Al-Adawi *et al*. (2003) and also supported by Punithalingam (1980) who reported that size of conidia on maturity ranged usually 20–30 x 10-15 µm. Correspondence with limited differences is in agreement with (Arshad, 2008) where observed septate conidia were 20.3 – 23.3 x 10.3 - 12.8 µm.

Table 4. Morphological characterizations of *L. theobromae* on PDA media.

No.	Morphology	Characteristics	Growth time
1.	Colony color	Grey	Initial
		Black	After 10 days
2.	Pycnidia color	Shiny black	
3.	Pycnidia shape	Oblong , Globose	
4.	Conidia	Aseptate	Initial
		1-septa	After 6 days
5.	Conidia length	18-25µm	After 10 days
6.	Conidia width	11-15µm	After 10 days
7.	Spores wall	Thin	Initial
		Thick	After 10 days
8.	Spores color	Pale brown	Initial
		Dark brown	After 10 days

Molecular analysis: Internal Transcribed Spacer (ITS1 and ITS4) regions amplified approximately 600 to 650bp. Bootstrap support values were evaluated using 100 bootstrap replicates. The combined dataset contained 1,065 characters with identical sites 661 (62.6%) having pairwise identity (95.4%) (Fig. 2).

The minimum genetic identity (83.87%) and maximum (100%) was observed among all the isolates. As representing in the tree, all the eight isolates showed (100%) similarity to *Lasiodiplodia* (AC-JF923830.1-India).

Correlation between aggressiveness and genetics: Tree is devided into two groups, one containing sister taxa of highly aggressive isolates on sindhri and White chounsa (PL5 and SL7) while other group further divided into two clades with one outer taxa containing Highly aggressive isolates of Sindhri (SL8). The clade having sister texa of slightly aggressive isolates (PL9 and PL11) and other major clade containg all the isoltes. The color alphabates indicateing the scale of aggressiveness from highly arrgessive to non-aggressive as represents in the scale above the tree. PL is stands for Punjab *Lesiodiplodia* and SL is representing Sindh *Lesiopiplodia* (Fig. 3).

Tree was constructed in Geneious.R10.software using Tamura-Nei genetic distance model combining with the neighbor-joining method. Bootstrap support values were evaluated using 50 bootstrap replicates. The combined dataset contained 909 characters with identical sites 534 (63.3%) having pairwise identity (93.6%). The minimum genetic identity (78.38%) was found between the PL3 and PL12 while maximum genetic identity (100%) was observed among all the isolates.

Pathological Characterization of *Lasiodiplodia theobromae* isolates: The morphologically similar fungal isolates showed variable pathogenic (Highly aggressive to Slightly-aggressive) responses towards mango cultivar (Sindhri and White chounsa) under controlled conditions. *L. theobromae* was proved to be pathogenic through artificial inoculations method on detached mango fruit (Fig. 4). Positive results for postharvest symptoms were obtained through artificial inoculations. This finding is in line with the work of Palejwala *et al*. (1987) and Kumar *et al*. (1993) both of these groups provided similar results on different fruits and plants and established the fact that wounding is

required for disease to rots and this is in line with previous studies from all over the world (Ismail *et al*., 2012). The comparison of lesion mean produced by postharvest fungal pathogens showed that lesions were significantly larger than control (agar plug only) at $p<0.05$. The fungus that was inoculated successfully re-isolated from infected fruit supporting Koch's postulates.

The pathogenicity trials were carried out in triplicates (three fruit). Mean lesion length of different isolates was ranged 1.9 cm (min) to 7.2 cm (max) on White chounsa variety and 0.8cm (min) to 3.3cm (max) on Sindhri variety. The appearance of lesions produced in stem end rot disease by *L. theobromae*, was brownish black and started from stem (collar) region and spread linearly along the fruit resulting in the softening of skin and pulp became watery which could be punctured with the finger. Lesion size (area) observed, was variable 60-170 cm^2 (White chounsa) and 6–63 cm^2 (Sindhri) (Fig. 5).

Present study reveals the extensive association of fungal isolates with mango losses after harvesting. The reason behind considering Sindhri and White chounsa for pathogenicity trails was that these two cultivars of Pakistan are regarded as the exporter commodity. Bar graph indicating the diseased area on Sindhri ranged from 6 to 63 cm^2 showed that Sindhri cultivar is more resistant (Figs. 5 & 6) to rots and this is in line with previous studies from all over the world (Malik *et al.*, 2005). This resistance to rot could be due fact that Sindhri variety is hard in texture while White chounsa cultivar has soft texture and prone to rots.

The variability in the pathogenic behavior of isolates of same species is due to evolution among isolates. Similar results were obtained by Shah *et al*. (2010); where these authors collected and studied thirteen isolates of *Lasiodiplodia theobromae* in terms of their morphological and the pathological characterization isolated from the pear fruit grown in the Punjab. The pathogenicity trials were carried out on fruit. Pathogenicity results demonstrated that isolates of *L. theobromae* were the most pathogenic towards mango.

Fig. 4. Mango fruits showing symptoms of stem end rot disease.

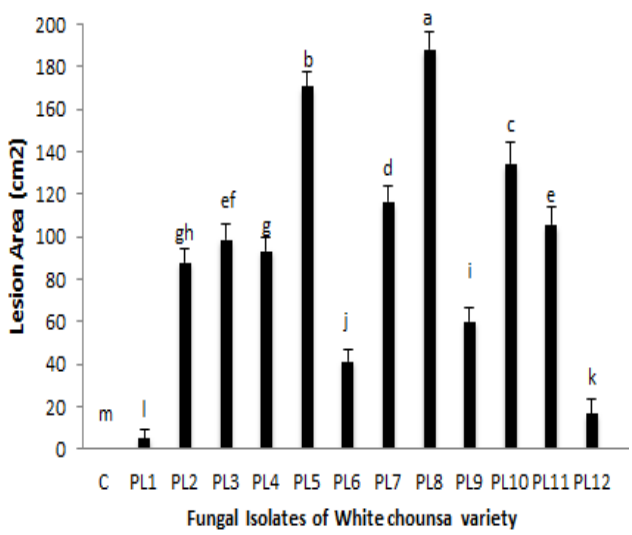

Fig. 5. Mean lesion area (cm^2) on White chounsa mango caused by *L. theobromae* isolates. A bar above column represents standard error of the mean and lesion area which are significantly different at $p<0.05$ have different lettering. PL represents the Punjab *Lasiodiplodia* isolates and C representing the control

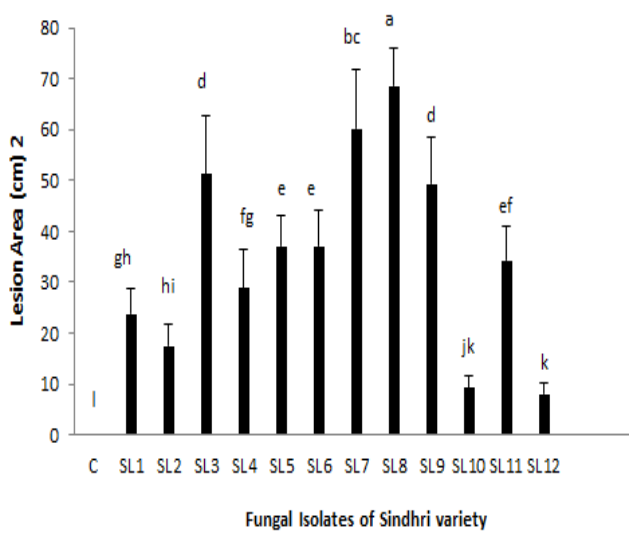

Fig. 6. Mean lesion area (cm^2) on Sindhri mango affected by *L. theobromae* isolates. A bar above column represents standard error of the mean and lesion area significantly different at $p<0.05$ have different lettering. SL represents the Sindh *Lasiodiplodia* isolates and C representing the control

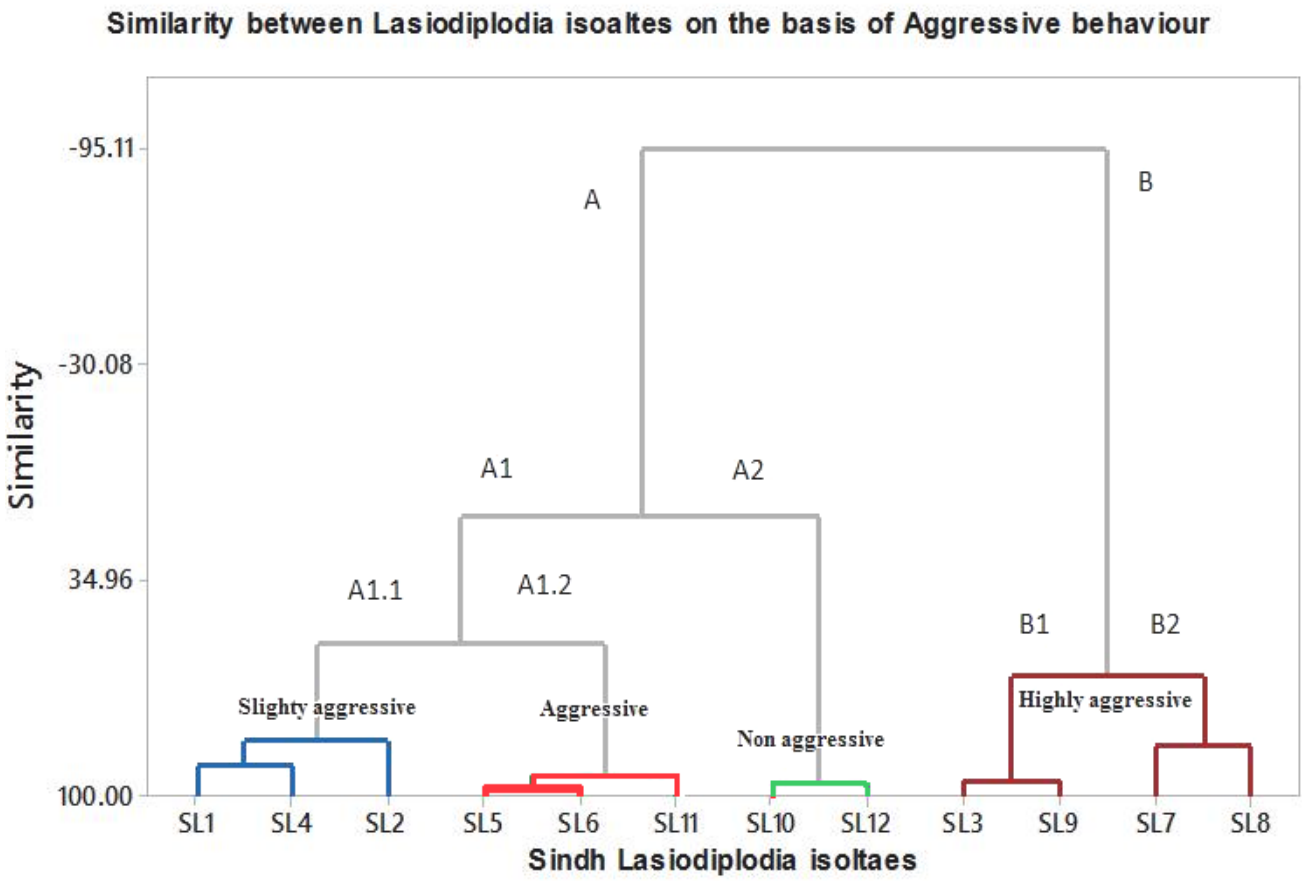

Fig. 7. Dendrogram clustering showing the aggressive behavior similarity of *L. theobromae* isolates on White chounsa variety.

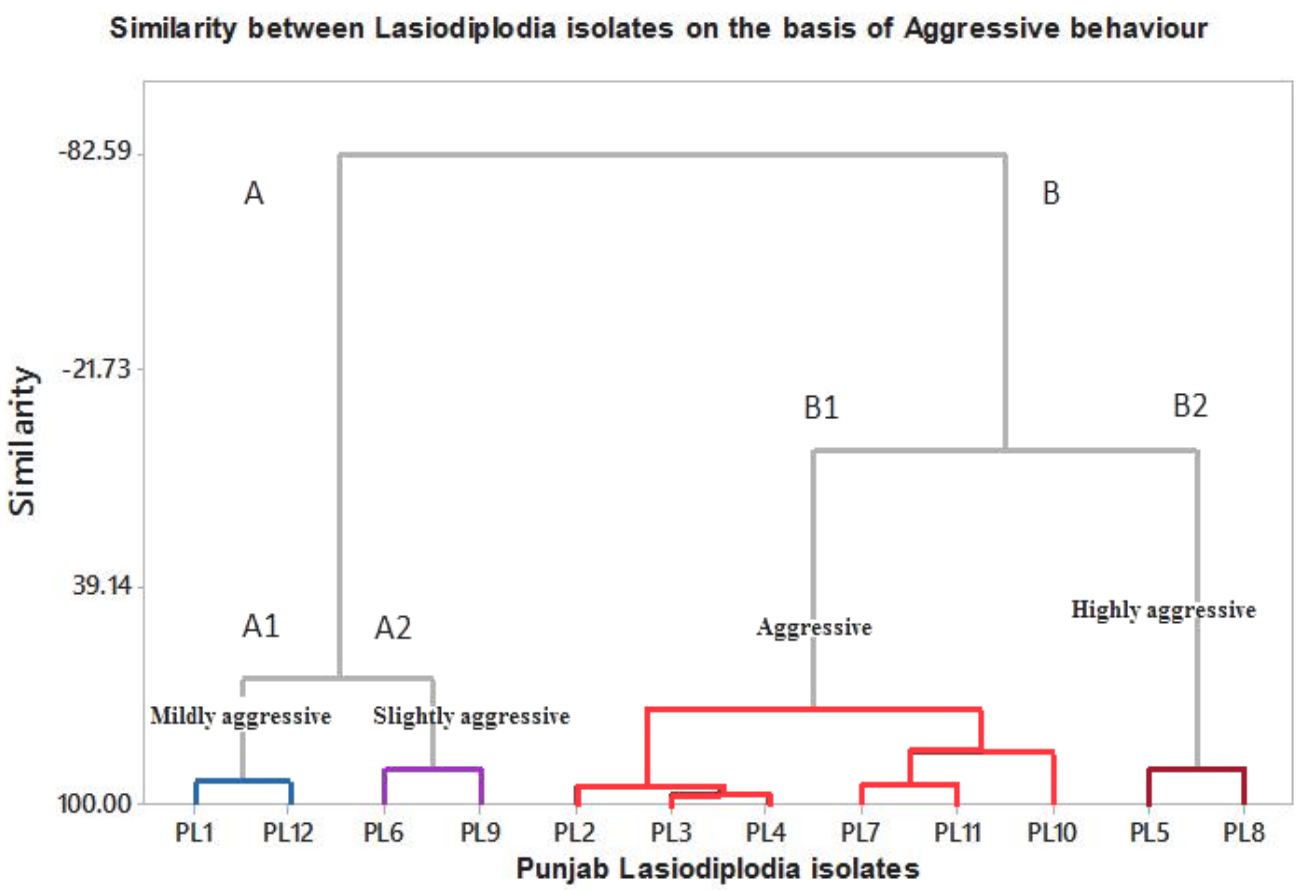

Fig. 8. Dendrogram clustering showing the aggressive behavior similarity of *L. theobromae* isolates on Sindhri variety.

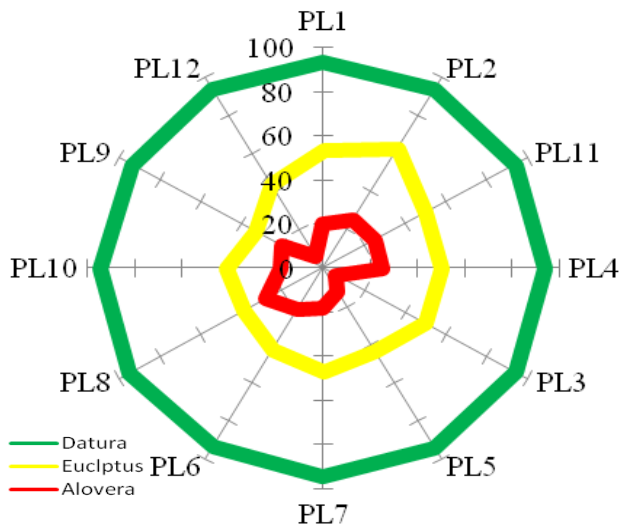

Fig. 9. Radar Chart indicates the reduction in radial growth by using the plant extract against *L.theobromae* isolates collected from White chounsa (Punjab mango variety). The values indicate the growth diameter (mm).

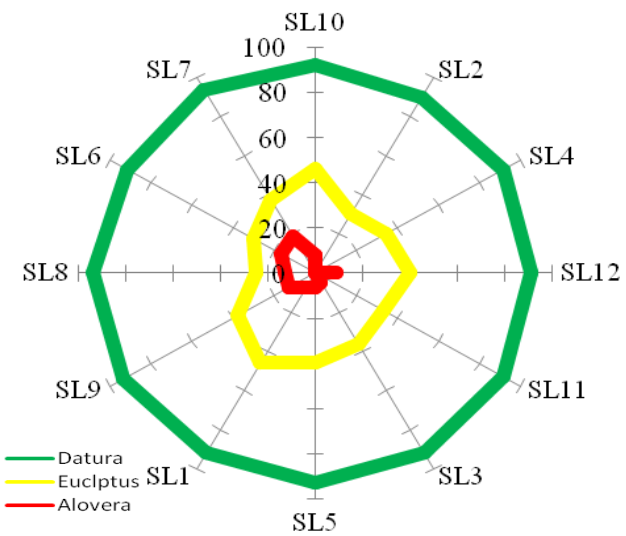

Fig. 10. Radar Chart indicates the variability in a reduction of radial growth by using the plant extract against *L.theobromae* isolates collected from Sindhri (Sindh mango variety). The values indicate the growth diameter (mm).

The significant achievement of the present study is the authentication of the pathogenic behavior of respective isolates by successful re-isolation of fungus from fruit. Isolates of Punjab were proved more aggressive towards fruit than the isolates from Sindh. As the dendrogram indicates the occurrence of two clusters A-(non-aggressive) containing 4 isolates and B-(aggressive) contain 8 isolates (Fig. 8). Cluster A is further grouped into A1-(non-aggressive) and A2-(slightly aggressive). Figure 8 also indicates almost 66% isolates are observed aggressive on the White chounsa variety. While dendrogram of Sindhri variety (Fig. 7) represent the cluster A is divided further into A2-(non-aggressive) and A1-(slightly aggressive to aggressive). Cluster B, contains all aggressive isolates. About 66% isolates are non-aggressive on Sindhri variety, completely contradicted to White chounsa containing 66% aggressive isolates. This is in clear agreement with pathogenic studies conducted on *L. theobromae* isolates of mango by (Khanzada *et al*., 2004; Sakalidis *et al*., 2011); both groups found that the dominant symptoms were developed apparently in stem of the inoculated plant in comparison to root.

Aggressiveness is due to the reason that in Punjab, orchards are relatively smaller and closer to each other while in Sindh the situation is almost reverse (Meer *et al*., 2013). Another important fact for more aggressive behavior of Punjab isolates is excessive use of nitrogen fertilizers, which also facilitate the attack of pathogens; Arshad *et al.* (2007) argued that the different sources of carbon and nitrogen influenced the growth of *L. theobromae*. Maximum growth was observed with sucrose and potassium nitrate. Another study of Shelar *et al.* (1997) also succeeded in developing the maximum growth of *L. theobromae* with sucrose and potassium nitrate. The environmental conditions play crucial role in the disease development and pathogens spread as the weather is hot and humid (54% to 95%) in Pakistan because environmental factors, harvesting techniques, packaging, transportation, and marketing all factors are in the favor of pathogens attack and destroy the fruit quality.

Effect of plant extracts on radial growth of fungus: The present study tested the antifungal activity of three different plant extracts. All the isolates (Aggressive and non-aggressive) were used in the experiment. All plants showed antifungal efficiency at varying degree in methodology. Significant results were obtained in previous study conducted by Sharma *et al.* (2013) in which, *D. stramonium* leaf extract showed greater fungicidal activity against *R. stolonifera.* A similar finding was also observed by Soni *et al.* (2012) and confirmed antifungal activity of a concoction brewed from *D. stramonium* against *F.mangiferae*. *Datura* plants contain tropane alkaloids such as hyoscyamine, scopolamine, and atropine in all parts, but phytochemicals are rich in leaves. The leaves of the plants have already been reported to contain high phytoconstituents than any other parts of the plant (Malik *et al.*, 2015).

E. camaldulensis showed 40-50% reduction (growth mean value of control and *E. camaldulensis*) in fungal radial growth; these finding were in excellent agreement with work of Bashir & Tahira (2012) and explained that the leaf extract of *E. camaldulensis* was the most efficient antifungal against *Fusarium* sp.

The least effective plant extract is *Aloe-vera*, which controls the 10-20% fungal growth (Figs. 9-10). Ezeibekwe *et al.*, 2009 investigation showed that *Aloe-vera* gel in concentrations 25, 50 and 100% proved ineffective against the *Fusarium sp.* and *Botryodiplodia theobromae*. Phytochemicals present in *Aloe vera* are tannins carbohydrates, terpenoids, alkaloids, and flavonoids (Nidiry, 2011). Overall plant antifungal efficiency was batter on the isolates of White chounsa as compare to isolates of Sindhri. The reason is Sindhri variety of mango contains natural resistance to disease. These results are in line with early observations of Fida & Iram (2014) who brilliantly identified that antifungal activity of different plant extract against *Fusarium* sp. which was efficient on White chounsa than Sindhri. Biocontrol is prophylactic way to control the plant pathogens (Akthar *et al.*, 2017).

Conclusion

Poor management, culture practices causing major loss of mango. A detailed investigation of the pathogenic behavior of fungal pathogens is needed for the identification of the potential threat to mango industry of Pakistan. This study will be helpful in mango breeding, to introduce resistance towards *Lasiodiplodia theobromae.* The lack of tolerance to these fungal isolates is a major factor of concern worldwide and in the development of transgenic plants possessing the tolerance for species of *Lasiodiplodia.* Punjab variety is less resistant to diseases because of its texture as well as environmental and chemical factors; further work on variety maintenance could be helpful to induce the resistance. Moreover, the analysis provides the appropriate disease control strategies for suppression of mango postharvest fungal diseases i-e control use of fungicides, development of botanical disease controls (*Datura Stramonium*) and use of appropriate techniques for harvesting, storage, and distribution.

Acknowledgment

This research was carried out as a part of grant supported by Government of Australia through ACIAR under Pakistan-Australia Agricultural Sector Linkages Program (ASLP). The financial support is greatly appreciated. A special gratitude to *Dr. Alice Nagata*, for reviewing this article.

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(Received for publication 19 April 2016)