

Full Length Research Paper

Consistent association of fungus *Fusarium mangiferae* Britz with mango malformation disease in Pakistan

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Mango malformation disease (MMD) deforms the natural shape of panicles and shoots. The disease incitant is of great concern due to its complexity and mode of infection. Recently, a new species *Fusarium mangiferae* Britz was confirmed as the etiological agent of MMD in African and Asian clade. There was a need to confirm the fungus in other Asian countries. We investigated the association of *F. mangiferae* with malformed branches of five exotic and five indigenous cultivars of *Mangifera indica* L. in Pakistan. *F. mangiferae* proved to be the dominant fungus hosting majority of the malformed tissues. Among the indigenous cultivars, maximum tissue infection of 96.66% was found in cultivar Anwar Rataul and minimum was found in cultivar Late Chaunsa (48.33%). In exotic ones, maximum and minimum infections of 97.33 and 70.67% were noted in the cultivars Sensation and Pop, respectively. Light and transmission electron microscopy proved helpful in investigating the morphological matrix and ultrastructure of the propagules of fungus *F. mangiferae*.

Key words: *Mangifera indica*, microconidium, Pakistan, tissue assay, transmission electron microscopy.

INTRODUCTION

Mango (*Mangifera indica* L.) occupies a prominent position amongst fruit crops due to its specific nature, growth pattern and diversity. In Pakistan, mango is cultivated on an area of 0.1515 m ha with annual production of 1.674 m tones (Anonymous, 2005). It is affected by various animate and inanimate diseases. Among animate ones, malformation is the most important malady causing colossal losses every year. Overall yield losses may be as high as 90%. In severe cases, the loss may be almost total (Ploetz, 1999; Summanwar, 1967). Britz et al. (2002) identified a new species (*Fusarium mangiferae*) that is associated with mango malformation disease (MMD) in the mango orchards of South Africa. Later, presence of the same species was also confirmed in Egypt and Israel

(Ploetz et al., 2002; Freeman et al., 2004). In the world, three additional taxa have also been found to be associated with MMD: *Fusarium sterilihyphosum* from South Africa and Brazil and *Fusarium* sp. nov. and *Fusarium proliferatum* from Malaysia (Marasas et al., 2006). In *F. mangiferae*, microconidial chains and chlamydospores are not formed rather, it produces polyphialides. This species could also be differentiated morphologically from two other species that are associated with MMD (Nirenberg and O'Donnell, 1998; Britz et al., 2002). Recently, Otero-Colina et al. (2010) reported nine phylogenetically distinct *Fusarium* spp. within the *Gibberella fujikuroi* species complex including one species within the African clade (*Fusarium pseudocircinatum*), two species within the Asian clade (*F. mangiferae* and *F. proliferatum*) and at least six species within the American clade (*F. sterilihyphosum* and five undescribed *Fusarium* spp.). They described a new species as *Fusarium mexicanum*.

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Table 1. Recovery of *F. mangiferae* from malformed tissues of five local cultivars obtained from five mango growing areas of the Punjab province of Pakistan.

Area	Percent recovery from each cultivar					Mean of district
	Aman Dusehri	Malda	Late Chaunsa	Fajri	Anwar Rataul	
Shujabad	91.66 ^{bc}	75.00 ^{gh}	83.33 ^{de}	78.33 ^{ef}	100.00 ^a	85.66 ^a
Vehari	100.00 ^a	65.00 ^j	41.66 ^{mn}	71.66 ^{ghi}	100.00 ^a	75.66 ^b
Layyah	100.00 ^a	76.66 ^{fg}	38.33 ^{no}	68.33 ^{ij}	95.0 ^{ab}	75.66 ^b
Rahim Yar Khan	83.33 ^{de}	58.33 ^k	33.33 ^o	70.00 ^{hij}	88.33 ^{cd}	66.66 ^c
Faisalabad	66.66 ^{ij}	50.00 ^l	45.00 ^{lm}	43.33 ^{mn}	100.00 ^a	60.99 ^d
Mean of cultivars	88.33 ^b	64.99 ^c	48.33 ^d	66.33 ^c	96.66 ^a	

*Means sharing similar letter(s) do not differ significantly ($p = 0.05$) by LSD test

Pathogenicity tests have been reported to be completed successfully in Mexico with local strains of *Fusarium* sp. different from *F. mangiferae* and *Fusarium oxysporum*. *F. mexicanum* has been reported as a novel etiological agent (Rodríguez-Alvarado et al., 2008; Otero-Colina et al., 2010).

A collection of Brazilian isolate showed a new phylogenetic lineage in the *G. fujikuroi* species complex as a sister clade to *F. sterilihyphosum* was formed. Mango malformation disease in Brazil is now considered to be caused by a distinct phylogenetic lineage of *Fusarium*, and to a lesser extent by *F. sterilihyphosum*. The new phylogenetic lineage together with *F. mangiferae* and *F. sterilihyphosum*, are the only known taxa of *Fusarium* that are proven to be capable of causing mango malformation (Lima et al., 2009).

The prime objectives of this study were to confirm the presence of dominant species associated with malformed tissues of diverse origins and study its ultrastructure using transmission electron microscope (TEM).

MATERIALS AND METHODS

Sampling

The studies to determine the fungal association were conducted during the flowering cycle (March to April) under Pakistan conditions. Five indigenous cultivars viz. Aman Dusehri, Malda, Late Chaunsa, Fajri and Anwar Rataul and five exotic viz. Sensation, Keitt, Mommy-k, Pop and Alphanso were used in this study. Sample collection for indigenous cultivars was done in five mango growing areas of the Punjab province (Table 1). Six locations were selected in each area to collect the samples of indigenous cultivars. Each location contributed five panicles along with 6 to 8 cm shoot portion representing one cultivar each. Thirty (30) samples of each exotic cultivar were obtained from Mango Research Station, Shujabad (Multan), Pakistan because most of the exotic cultivars are grown there. Immediately after clipping, the samples were brought to the laboratory in an ice box to avoid heating during transit (Iqbal et al., 2003).

Microscopic assay

The experiment was arranged in completely randomized design

(CRD) with 3 replications. Ten tissues of 5 mm size were cut from peduncles and panicle-shoot juncture of each sample. After disinfestation with 1% NaOCl solution for 2 min, the tissues were placed in glass Petri plates containing potato dextrose agar (PDA) medium using standard isolation protocol (Ploetz and Gregory, 1993). The plates were incubated at 25°C with alternating cycling of light and darkness. The colonies of *F. mangiferae* were purified on carnation leaf agar (CLA) medium and the identification was done on the basis of typical macro and microconidia (Nelson et al., 1983; Britz et al., 2002).

Ultrastructural assay

The culture blocks derived from malformed tissues of cultivar Sensation were processed for transmission electron microscopy starting from addition of glutaraldehyde (5%), followed by post fixation with 0.2% osmium tetra oxide and treatment with 5% aqueous uranyl acetate for 16 to 18 h (Hameed, 2003). Dehydration was done for 30 min each in 30, 50 and 70% ethanol. Exchange of alcohol was done with 100% acetone and then infiltration was done in 1:1 acetone/spur resin and for 24 to 72 h in spur resin alone. Polymerized resin blocks were used for cutting 120 to 200 nm thick ultrathin sections (Roberts, 2002). Finally, sections were double stained with uranyl acetate and lead citrate as described previously (Anjum, 2001) and examined under a transmission electron microscope (JEOL JEM-1010, Japan) operating at 80 kV.

Data pertaining to recovery of *F. mangiferae* having two way interaction of district x cultivars were subjected to analysis of variance. Mean separations were determined at $P = 0.05$ by LSD test.

RESULTS

Morphological characteristics

The colonies starting from single-conidium displayed a prominent purple pigmentation on the reverse side of the glass Petri dishes (Pyrex) after 14 days. Sclerotia or chlamydospores like propagules were found to be absent in all the cultures under study. Three septate macroconidia were always present and their apical cells were often curved (Figure 1A). Microconidia were abundant and were mostly fusiform, oval to elliptical, with 0 to 1 septate and formed on polyphialides in false heads. Obovoid microconidia specific for *F. mangiferae* were also identified (Figure 1B).

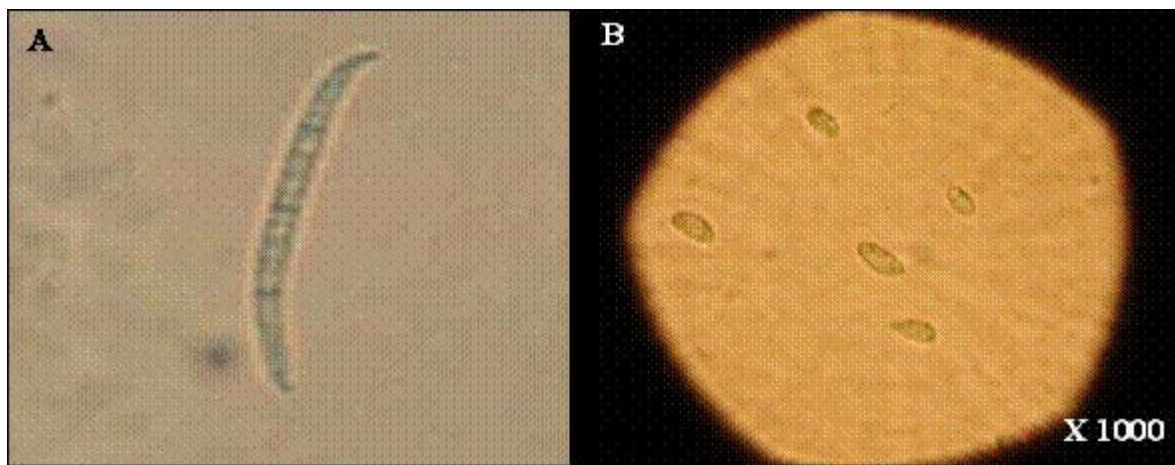


Figure 1. Macro and microconidia of *F. mangiferae*. A: 3-septate (four celled) typical macroconidium; B: obovoid microconidia.

Table 2. Recovery of *F. mangiferae* from malformed tissues of five exotic cultivars grown in the Punjab province of Pakistan.

Cultivar	Origin	Tissue assay		
		Examined	Infected	Infection (%)
Sensation	Exotic	300	292	97.33 ^a
Keitt	Exotic	300	281	93.67 ^a
MomY-K	Exotic	300	289	96.33 ^a
Pop	Exotic	300	212	70.67 ^b
Alphanso	Exotic	300	280	93.33 ^a

*Means sharing similar letter(s) do not differ significantly ($p = 0.05$) by LSD test.

Indigenous cultivars

Consistent isolations were made from malformed parts of all the selected locations of different areas of Punjab. Fungus *F. mangiferae* was found to be widely distributed in symptomatic tissues of mango. Cv. Aman Dusehri exhibited maximum recovery (100%) of *F. mangiferae* in Vehari and Layyah, and Anwar Rataul exhibited maximum recovery (100%) of *F. mangiferae* in Shujabad, Vehari and Faisalabad. Anwar Rataul showed maximum tissue infection of 96.66%, but Late Chaunsa had minimum infection percentage of 48.33 among the 5 tested cultivars. Aman Dusehri, Fajri and Malda had 88.33, 66.33 and 64.99% infection, respectively (Table 1). Amongst the areas, the maximum tissue infection of 85.66% was recorded in Shujabad, while the least infection (60.99%) was found in Faisalabad. There was considerable and statistically significant variation in infection levels among the cultivars and mango growing areas.

Exotic cultivars

F. mangiferae showed significant infection levels in exotic

cultivars. The examination of malformed parts of different exotic cultivars revealed the dominance of fungus *F. mangiferae*. Tissue assay exhibited maximum infection of 97.33% in Sensation colonizing 292 tissues out of 300. This was followed by Mommy-k, Keitt and Alphanso with 96.33, 93.67 and 93.33% infection, respectively. However, all these four cultivars were statistically alike. The least infection of 70.67% was shown by Pop which is still much higher (Table 2). No exotic cultivar was found to be free of infection. Infection frequency in tissues depicts the extensive and massive colonization by the fungus *F. mangiferae*.

Ultrastructural assay

Ultrastructure of the fungal propagules revealed interesting morphological matrix and features. The *in vitro* grown fungal culture obtained from malformed parts of cultivar Sensation showed longitudinal and transverse sections of hyphae when viewed under TEM. The longitudinal section of hypha was septate (Figure 2A). Oval to fusiform, single and bicelled, and microconidia, which are characteristic features of *F. mangiferae* were also observed with similar

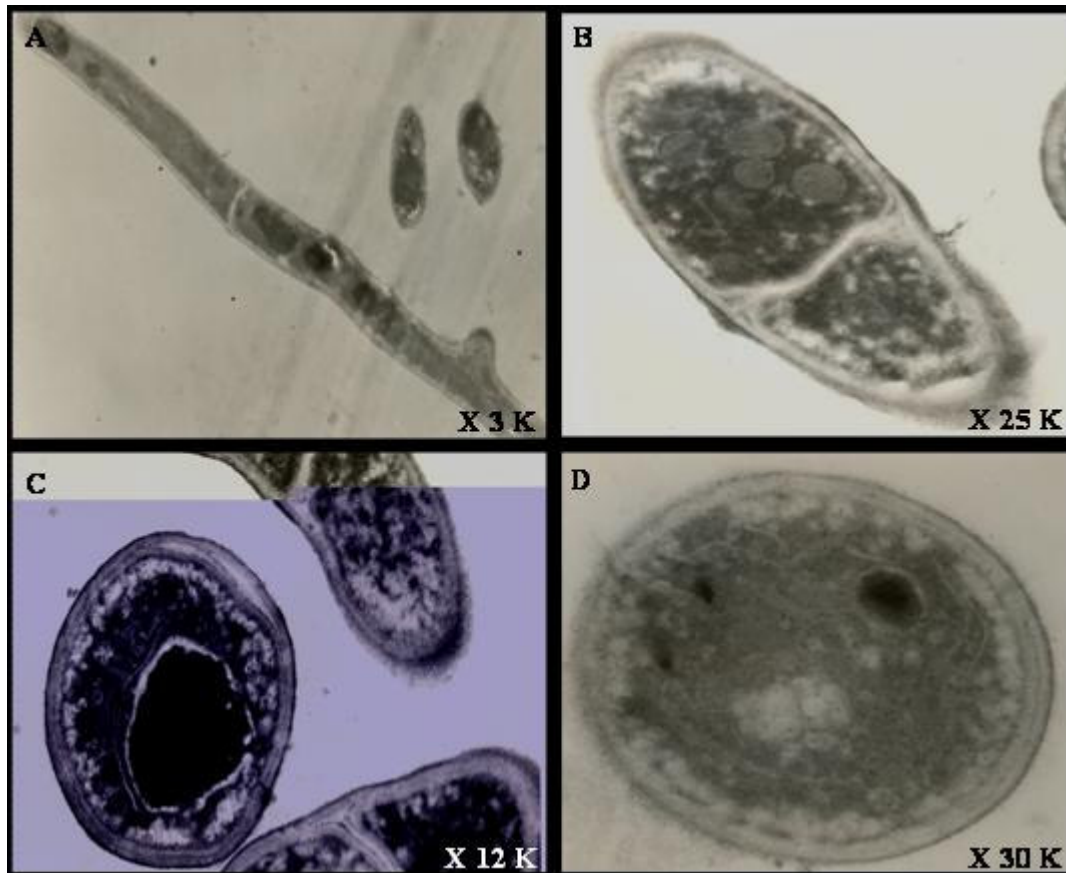


Figure 2. Ultrastructure of fungal propagules obtained from pure culture of fungus *F. mangiferae*. A: Longitudinal septate hypha; B: septate microconidium; C: cross section of hypha; D: magnified cross section of fungal hypha.

morphology as under light microscope but they differed in magnification and level of resolution (Figure 2A and B). Transverse sections of fungal hyphae and microconidia were quite evident showing fine ultrastructural details and precision of TEM protocol (Figure 2B, C and D). These proved to be the most discernible indicators amongst the fungal propagules. These results are consistent with the findings of Iqbal et al. (2010).

DISCUSSION

The genus *Fusarium* shows variation in natural occurrence due to its typical genetic characteristics. The cultures initiated from single conidia in these studies provided optimum conditions of temperature and light. The single-spored subsets were studied for various characteristics. The cultures retained the same pigmentation and features even after frequent culturing. This shows the uniformity of growing conditions. Pigmentation specific to species was ever conspicuous on PDA. Mutations were minimized by using the single spore or hyphal tip techniques and by storing the culture on media with low

carbohydrate concentration (Nelson et al., 1983; Iqbal et al., 2006). Morphology of macro and microconidia and conidiogenesis are the significant features used in the description of *Fusarium* spp. The morphological and cultural features in this study were in conformity with the previous illustrations and exhibited similar characteristics described for this species (Britz et al., 2002). The diseased sections yielded typical and abundant macroconidia of the fungus on artificial medium. Similarly, the clarity of ultrastructure shows discernible matrix and fungal propagules like septate hypha, microconidia and transverse sections of hyphae. Sometimes, variation may occur due to the intrinsic biological variability of the fungus and its microenvironment or gradual alteration of hyphal structures during specimen preparation. Use of specific fixatives increased preservation of specific cell components. Glutaraldehyde fixed biological membranes and cross-linked proteins by turning cells into interlocking structure and osmium helped to stabilize protein cross-links (Cole, 1986). The important achievement of this study was identification of species *F. mangiferae* in malformed tissues of diverse origins in Pakistan. This is a significant report on vast comparative assay of the fungus

in symptomatic tissues of local and exotic cultivars. Since inception of malformation, confusing opinions regarding etiology were introduced which were later refuted in the latest world literature. Fungal nature of the disease was first established by Summanwar et al. (1966). Some misleading citations in the literature still ascribed the cause to some other fungi like *F. oxysporum* but role of these fungi has already been negated (Ploetz, 1994; Srivastava, 1998). Its causal role was not obviously verified (Ploetz and Prakash, 1997). A high percentage of sample's infection and frequency of *F. mangiferae* in malformed tissues of all the tested local and exotic cultivars in this study confirms the possible role of this fungus in causing malformation of mango. Recovery of *F. mangiferae* differed from 48.33 to 96.66% in indigenous cultivars, while in exotic cultivars; it ranged from 70.67 to 97.33%. This is corroborated by the findings of Freeman et al. (2004) who confirmed the frequency of *F. mangiferae* in malformed mango parts.

F. mangiferae has also been isolated from healthy trees grown in the vicinity of diseased ones. Fungus is accumulated in the healthy tissues and after achieving maximum infection, symptoms are manifested. *F. mangiferae* has been observed to be frequent in panicle-shoot juncture tissues. The panicles emerging from preinfected shoots do appear malformed. Some workers reported the association of *F. oxysporum* but its pathogenic role in causation of malformation has already been negated. This is also in agreement with the conclusions of Ploetz and Prakash (1997). Light microscopic studies and ultrastructural assay provide a useful insight to glean into morphology and frequency of the fungus. On the basis of these observations, it could be inferred that *F. mangiferae* host and infects the mango tissues ultimately, causing malformation symptoms in mango orchards. The studies have provided new avenues to devise inoculum specific control strategies to eliminate the disease from mango orchards.

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