Pak. J. Bot., 44(2): 813-820, 2012.

BARK BEETLE, *HYPOCRYPHALUS MANGIFERAE* STEBBING (COLEOPTERA: CURCULIONIDAE: SCOLYTINAE) IS A VECTOR OF MANGO SUDDEN DEATH DISEASE IN PAKISTAN

ASAD MASOOD AND SHAFQAT SAEED

University College of Agriculture, Bahauddin Zakariya University, Multan, Pakistan
**Corresponding authors: E-mail: bumblebeepak@gmail.com*

Abstract

Role of bark beetle *Hypocryphalus mangiferae* (Coleoptera: Curculionidae: Scolytinae) was assessed and proved as a definitive vector in the transmission of Mango sudden death disease (MSDD). Studies were carried out in the southern Punjab, Pakistan. Healthy plants of mango (*Mangifera indica* L.) were enclosed with beetles with and without fungus inoculum by employing a technique "Mesh Enclosure Method" (MEM). Symptoms expression revealed that the wilting was significant in 3 and 6 months but no significant differences in oozing were found and also other symptoms like black streaks and cankers were observed in the plants on which infested beetles were introduced. Re-isolation of fungi after 7.5 months showed higher frequency of *Lasiodiplodia theobromae* compared to *Ceratocystis fimbriata*. In order to confirm the results, both of the fungi were also isolated from adult beetles and beetle's frass. Based on our findings, *H. mangiferae* was found to transmit the infection of Mango Sudden Death Disease in healthy plants which necessitates an integrated approach considering the disease and its vector for the sustainable management of MSDD in orchards.

Introduction

Mango (*Mangifera indica* L., Anacardiaceae) is recognized as the most important and popular fruit crop in the tropical and subtropical areas of the world. In recent years, mango production and quality has been declining due to the occurrence of a variety of abiotic and biotic factors, some leading to the development of sudden death of trees (Masood *et al*., 2009; Masood *et al*., 2011). Abiotic factors include nutrients deficiency, drought, temperature fluctuations, mechanical injuries and improper management practices i.e. plouging and intercropping (Schaffer *et al*., 1988; Ploetz, 2003; Malik *et al*., 2004; Nafees *et al*., 2010; Masood *et al*., 2012). The mango production is also impaired by a number of pests and diseases (Anwar *et al*., 2011). The most common insect pests attacking mango in Pakistan are mango hoppers, *Idioscopus clypealis* (Leth), and *Amritodus atkinsoni* (Leth), midge, *Erosomya indica* (Grover & Prasad), mealybug, *Drosicha stebbingii* (Green), scale, *Aulacaspis tubercularis* (Newstead), fruit flies, *Bactrocera zonata* (Saunders), *B. dorsalis* (Hendel), thrips, *Frankliniella occidentalis* (Pergande) and bark beetle, *Hypocryphalus mangiferae* (Stebbing) (Mohyuddin & Mahmood, 1993). Masood *et al*., (2008) reported four species of Scolytidae (bark beetle) breeding in Mango in Southern Punjab, the most frequent being *H. mangiferae* also attacking healthy mango trees. *H. mangiferae* preferably attacks mango trees stressed by disease, nutrient imbalance, or drought (Wood, 1982; Ploetz *et al*., 1996).

The mango production is hampered by the attack of number of significant diseases (Khalid & Alam, 2002) but Mango sudden death disease (MSDD) is regarded as one of the most serious threats not only in Pakistan but all over the mango growing regions of the world (Kazmi *et al*., 2005). Recently, incidence of this menace was found 20 and more than 60% in Punjab and Sindh Provinces of Pakistan, respectively and 60% in Al Batinah region of Oman (Al Adawi *et al.*, 2006; Saeed *et al.*, 2006). In Brazil, Oman, and Pakistan, fungal species isolated from diseased mango tree were *Ceratocysis fimbriata* Ellis & Halst, *C. omanensis* and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl (Ribeiro, 1980; Al Adawi *et al*., 2006; Saeed *et al*., 2008; Masood *et al*., 2011; Saeed *et al*., 2011). Ploetz *et al.*, (1996) have demonstrated *Cladosporium* spp., *Collectotrichum gloeosporioides*, *Fusarium* spp., *Pestalotiopsis* spp., *Phomopsis* spp., from this disease complex trees in Florida and *C. fimbriata* and *L. theobromae* were reported to produce wilting of many tree species (Kila, 1993) in India, Egypt and Malaysia (Verma *et al*., 1970; Acuna & Waite, 1977; Lim & Khoo, 1985).

In spite of these pathogenic fungi, bark beetle species are expected to be involved as putative vectors (Ribeiro, 1980; Al Adwai *et al.*, 2006; Masood *et al.*, 2008). MSDD is a disease complex in which pathogenic fungi, bark beetle and cultivation method are involved (Malik *et al.*, 2004; Al Adwai *et al*., 2006; Masood *et al*., 2011). Early authors, Westerdijk & Buisman, (1929) supposed that MSDD might be transmitted and spread by various dissemination means i.e., air, water and insects. Recently, Van Wyk *et al*., (2007) reported that MSDD is primarily caused by *C. fimbriata* but typical symptoms of MSDD were also observed in mango plants after inoculation with *Lasiodiplodia theobromae* and the fungus was frequently isolated from declining mango trees in Pakistan (Shahbaz *et al*., 2009; Saeed *et al*., 2011). It is suspected that bark beetles are essential for transmission of both fungus species. Nevertheless, no single species of bark beetle attacking on mango was verified to be an effective vector for any fungal species.

Many fungal pathogens belonging to genus *Ceratocystis* have an intimate association with scolytid beetles which may play very significant role as vectors (Moller & Devay, 1968). Elm bark beetles (*Scolytus scolytus, S. multistriatus*) were identified as effective vectors of *Ophiostoma ulmi and O. neo-ulmi* (Favaro & Battisti, 1993; Battisti *et al*., 1994; Faccoli & Battisti, 1997). Young beetles emerging from diseased trees are mostly contaminated with fungal spores and thus inoculate healthy host trees when feeding on the bark (Fransen & Buisman, 1935; Webber & Gibbs, 1989). Transmission of *Ceratocystis fagacearum* (Brentz) Hunt causing oak wilt in North America was intensively

studied (Gibbs & French, 1980). For this fungus two different types of transmission occur: underground spread via grafted roots and overland spread over longer distances by insect vectors or vehicles. Regarding the second mode infection depends on the presence of wounds in the bark. Several insect species were proved or expected to be efficient vectors including bark boring species like Scolytidae or Cerambycidae (both Coleoptera) as well as sap feeding species like Nitidulidae (Coleoptera), Lepidoptera, Diptera, Hymenoptera or Orthoptera (Craighead & Nelson, 1960). Even wounds are present the whole year, successful infection also depends on season and the age of wounds. Nothing is known so far about insect transmission of the common pathogen, *Lasiodiplodia theobromae*. Wounds seem to be nessessary for successful infection of healthy plants (Shahbaz *et al*., 2009) and thus herbivorous insects might facilitate infection.

However, the interplay of these components in the development of MSDD was not well studied and no work seems to have been done in the transmission of fungal pathogens through mango bark beetle. Therefore, the main objective of the present study was to prove the hypothesis that *H. mangiferae* is able to transmit this malady into healthy trees. We followed the stepwise method for proving and fulfilling Koch's postulates (modified by Leach, 1940): i) the beetle transmits fungal pathogens from diseased to healthy plants in enclosure; ii) the fungus is recovered from healthy plant after inoculation of disease; iii) the bark beetle carry the inoculum of fungus on its body or in frass; iv) the same fungal pathogen is associated with the diseased trees in the field.

Material and Methods

Location and plant material: The experiments were performed at Bahauddin Zakariya University (BZU), Multan, Pakistan (30.263°N, 071.506°E, latitude 100m (ASL). Mango plants (3-4 years old) cv. "Chounsa late" were planted in 45x 60 cm pots containing a porous pot media consisting of gravelly clay-loam, rotted farm yard manure and sterilized wheat and rice straw. Plants were then shifted in an insect proof compartment made up of fine mesh cloth less than 0.5mm mesh. (Leach, 1940). The whole experiment was performed under lathe house conditions. Before shifting in insect proof partition, the potted plants were maintained for at least one month under the lathe house for their better adaptation. To confirm the plants under test as disease free, isolations were made from the main stem by excising 5 mm² pieces with sterilized scalpel by standardized procedure (Al Adawi *et al.*, 2006; Masood *et al*., 2011) The sampling sites were then covered with parafilm after sterilizing with 1% Sodium Hypochlorides (NaOCl).

Cultures of bark beetle and fungi: During mid July, 2007, bark beetle infested logs were cut from diseased mango trees situated at "Faraz mango farm" near Khanewal Road, Multan (30.270ºN, 71.250ºE at GPS position) and transferred into cages in the lab of BZU. Cast acrylic rearing cages (45x60x45 cm) fitted with a black mesh cloth (0.5 mm gauze) on its top side and a transparent collecting jar with a light source at its bottom were used. The culture of bark beetles was maintained by placing infested logs (40-50 cm long, 30-35 cm in diameter) in rearing cages on fortnightly basis (Masood *et al*., 2009). Rearing of consecutive generations in a lab stock, a separate culture was set up on logs from healthy trees for two generations at 25 ± 3°C and 60 ± 5% relative humidity, to obtain pathogen free stock of *H. mangiferae.*

Isolation of fungal pathogens *Ceratocysitis fimbriata* and *Lasiodiplodia theobromae* was performed on plates of potato dextrose agar amended with streptomycin (SPDA) as the standard medium of isolation (Masood *et al*., 2011). Small bark pieces (5mm^2) were dissected from diseased stem portion and inoculated on media plates that were incubated at 25 ± 2°C for 8-10 days. Once ascomata had been developed, ascospores were transferred into new SPDA plates for further isolation. After 7-10 days, the fungal isolates were microscopically identified on the basis of culture morphology, spores and conidia of the specific fungi (Upadhyay, 1981; Masood *et al*., 2011). Isolates of *C. fimbriata* and *L. theobromae* have been deposited at Plant Pathology and Mycology Department, Punjab University, Lahore, Pakistan (Accession number FCBP 1012).

Pathogenecity through bark beetle: Infection tests were conducted using completely randomized design of five treatments and five individual plants as replicates in the lathe house from mid of April to November, 2008. In first treatment (T_1), fungi from the lab culture (*C. fimbriata* and *L. theobromae*) were inoculated into healthy mango plants by making two slanting T-cuts alternatively into the main stem with sharp knife. A piece of each fungal colony (5 mm) was placed in one of each incision and covered with parafilm. In second treatment (T_2) beetles collected from diseased mango trees in field were applied to the mango plants. In order to verify fungal inoculum on the beetles, isolations of fungi were made (*C. fimbriata* and *L. theobromae*). In third treatment (T_3) spore free beetles from the lab culture were used. In the fourth treatment (T_4) spore free beetles were allowed to crawl on fungal infected media (*C. fimbriata* and *L. theobromae*) for 30 minutes prior to enclosure. In the fifth treatment (T_5), healthy plants were used to serve as control. In all treatments Mesh Enclosure Method (MEM) was used for application of beetles. A cloth of fine mesh was fixed around the lower part of main stem of individual healthy mango plants to prevent escape of beetles (dimension 15×30 cm, Fig. 1). In each cage per plant, 20 randomly selected beetles were released according to the treatments. All the treatment plants were placed in insect-proof fine mesh screens (in lathe house) to prevent contamination across treatments by emerging beetles.

Success of infection was estimated by the occurrence of MSDD symptoms. For measuring plant wilting, we counted the number of wilted leaves out of total leaves in each observation and calculated the wilting percentage. Mortality was calculated as frequency of dead plants out of five and number of oozing was counted on the caged part of each plant. Appearance of wilting, mortality and oozing on plants were assessed every fortnight between April to November 2008. Length of cankers (cm) and length of black streaks (cm) were observed on dissected plants at the end of experiment. To describe differences of infection, symptoms on plants were analyzed in three specific periods i.e. three months post infection (pi), six months pi and at the end of experiment 7.5 months pi. At the end of the experiment all plants were dissected for measuring internal symptoms.

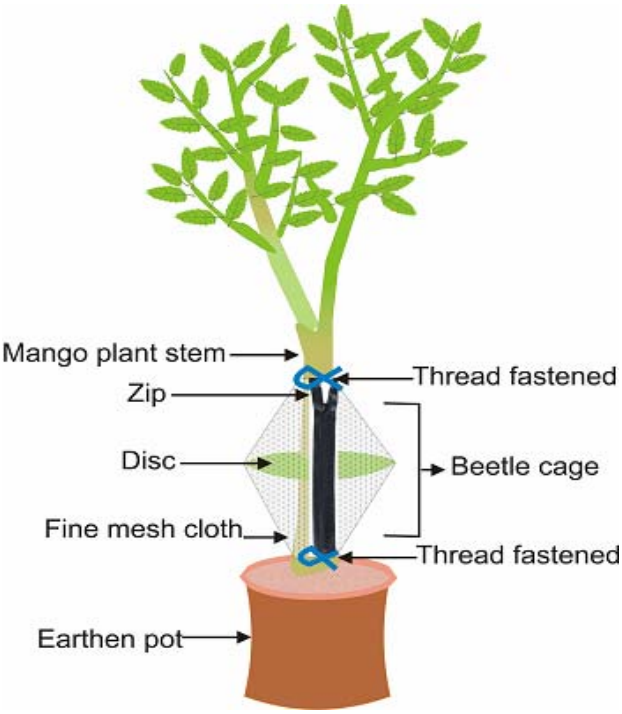

Fig. 1. Mesh Enclosure Method (MEM) for transmission of disease through beetle.

Isolation from frass and beetles: To confirm fungal pathogens in frass and beetles, a total of 320 beetles and their frass were collected periodically from infested plants and put in sterilized plastic tubes. The beetles were immersed in 1% NaOCl for three minutes followed by four serial washing with sterilized distilled water and then blotted dry on sterile filter paper. Beetles were slightly crashed and then aseptically placed onto potato dextrose agar (PDA). Small quantities of frass (<0.015g) emitted out from beetle's holes, were inoculated on PDA. All plates were incubated at 25 ± 2°C for 7-10 days and growth of fungus observed thereafter (Masood *et al*., 2011).

Re-isolation frequencies of fungi from test plants: For re-isolation of fungi from treated mango plants, the stem portion was removed from the point of inoculation and made small pieces (0.5mm) of wood stripes (5-15 with average 10 per plant). Each strip was placed on PDA and the frequency of isolation was calculated (Masood *et al*., 2011). For statistical comparison reisolation frequencies were calculated (no. of isolates with the fungi/ total no. of isolates × 100) (Jankowiak & Bilanski, 2007).

Data analysis: The data of disease symptoms (wilting, mortality, oozing, cankers, and black streaks) and isolation frequencies of fungi were subjected to statistical analysis using one way ANOVA. In a second step means were compared pairwise using least significant difference (LSD) test. The data was analyzed using computer software Anon., (2008).

Results

Pathogenecity through *H. mangiferae*: The number of wilted leaves and subsequent mortality of plants as well as MSDD symptoms increased in all treatments from first to second and third observation. There were also significant differences in disease symptoms i.e., black streaks, cankers and oozing among the 5 treatments at all 3 observation (Table 1). After 7.5 months, in treatments with fungal inoculum (T_1, T_2, T_4), significant higher amount of leaves showed wilting compared to treatment with spore free beetles and control (Table 1). Likewise, mortality decreased significantly from inoculated fungi, beetles of diseased trees and beetles with spores to spore free beetles and control. At the end of the experiment 100% mortality occurred in artificially inoculated plants whereas control showed 20% mortality (Table 1). At the end of the experiment treated plants also showed conspicuous disease specific symptoms, i.e. black streaks and cankers in cambial tissue as well as oozing, except of control plants. There was statistically significant difference in length of black streaks and canker ($F=3.4$, $p=0.029$ and $F=15.1$, $p<0.0001$, respectively). Streak length and canker length increased from disease inoculated (T_1) to treatments with spore free beetles (T_3), beetles with spores (T_4) and beetles collected from diseased trees (T_2) (Table 1). Analysis of variance indicated significant differences among treatments with beetles of diseased trees (T_2) and spore free beetles (T_3) in comparison with control (T_5) (Table 1). Maximum oozing did only occur in plants treated with beetles. Significant differences were indicated between control (T_5) and both treatments with beetles of diseased trees (T_2) and spore free beetles (T_3) (Table 1).

Re-Isolation of fungi from treated plants: Re-isolation revealed both species of fungi i.e., *Ceratocystis fimbriata* and *Lasiodiplodia theobromae* in all treated plants except control (Table 2). Significant difference in re-isolation frequency of both fungus species was observed in the first (July) study only but not in the second (November). Re-isolation frequencies for *L. theobromae* were much higher compared to *C. fimbriata.* Astonishingly, in the treatment with spore free beetles high infestation was also recorded (Table 2).

Isolation of fungi from adult bark beetle and its frass: *C. fimbriata* and *L. theobromae* were detected in beetles and in their frass of all beetle containing treatments. Frequency of isolation from beetles as well as its frass was found statistically significant among treatments (Table 3). The highest isolation frequencies of *C. fimbriata* were obtained from beetles and its frass that were collected from plants inoculated with beetles from infested tree and beetle crawled on fungal media, respectively (Table 3). Highest frequencies of *L. theobromae* on beetles were recorded from the beetles crawled on media but isolation frequency of this fungus was significantly higher in treatments with spore free beetles (Table 3).

Table 1. Assessment of host symptoms appeared on mango plants cv. Chounsa late during April to end November, 2008 (Mean ± SE).

Treatment plants*	Holes (number)	After 3 months		After 6 Months		After 7 ½ months		After dissection of plants		
		Wilting (%)	Mortality (out of five)	Wilting (%)	Mortality (out of five)	Wilting (%)	Mortality (out of five)	Black streak (cm)	Canker (cm)	Oozing (Number)
Inoculated fungi (T1)	-	28 ± 3.4a	0.2 ±.2a	51 ± 4a	0.6 ±.25a	89 ± 5.1a	1.0 ± 0b	11 ± 3.3ab	4.5 ± 0.45ab	1.2 ± 0.58ab
Beetle of disease tree(T2)	41.2 ± 6.1b	12 ± 5.8bc	0a	36 ± 8.3ab	0.4 ±.2a	85 ± 6.7a	0.8 ± 0.2b	15 ± 4.3a	4.8 ± 0.63a	7.2 ± 1a
Beetles with spores (T3)	66.2 ± 11.4ab	20 ± 5.2ab	0a	44 ± 5.3a	0.4 ± .2a	94 ± 4a	0.8 ± 0.18b	9.9 ± 3.7ab	4.0 ± 0.63ab	6.6 ± 1.9ab
Spore free beetles (T4)	82.8 ± 17.1a	7.0 ± 3c	0a	24 ± 6.4bc	0.2 ± 0.06a	56 ± 6.2b	0.6 ± 0.07ab	5.7 ± 2.4bc	3.2 ± 0.45b	7 ± 2.1a
Plants without beetles (T5)	-	5.0 ± 3 c	0a	8 ± 4.1c	0.0 a	16 ± 2.9c	0.2 a	0.0c	0.0 c	0.0 b
ANOVA results F;df; P	2.86; 2; 0.096	4.96; 4; 0.006	1; 4; 0.43	8.5; 4; 0.0001	0.349; 4; 1.18	39.7; 4; <0.0001	2.5; 4; 0.071	3.4; 4; 0.029	15.1; 4; <0.0001	2.4; 4; 0.081

Treatments sharing the common letters are not statistically significant. T1= artificially inoculated fungi (*C. fimbriata* and *L. theobromae*) into healthy pod plants without beetles. T2= introduced beetles on plants which were collected directly from infested mango trees. T3= introduced spore free beetles into healthy pod plants (Beetles reared on healthy logs in laboratory). T4= beetles reared on healthy logs were crawled on fungal media (*C. fimbriata* and *L. theobromae*) and then inoculated on healthy plants. T5= healthy plants were used as control without beetles

Table 2. Re-isolation frequency (%) of *Ceratocystis fimbriata* and *Lasiodiplodia theobromae* during July –November, 2008.

Treatment plants	Number of isolates from plants		First isolation, July 2008		Second isolation, Nov. 2008	
	July 2008	Nov. 2008	*Ceratocystis fimbriata*	*Lasiodiplodia theobromae*	*Ceratocystis fimbriata*	*Lasiodiplodia theobromae*
Inoculated fungi (T1)	73	40	0.8 ± 0.8b	71.4 ± 13 a	18 ± 15.6 a	40 ± 17b
Beetles of diseased tree(T2)	47	45	3.3 ± 2ab	32.6 ± 12bc	4.0 ± 4.0a	34 ± 13.6ab
Beetles with spores (T3)	52	55	5.6 ± 2.5 a	56.65 ± 12.7ab	16.0 ± 4.5a	44.7 ± 16.5 b
Spore free beetles (T4)	66	45	0.00 b	47.3 ± 13.7ab	1.33 ± 1.3a	26 ± 11.7ab
Plants without beetles (T5)	69	30	0.000 b	0.000 c	0.0 a	0.00 a
ANOVA results F;df;P	-	-	2.7; 4; 0.059	5.6; 4; 0.003	1.27; 4; 0.312	1.74; 4; 0.179

For re-isolation of fungi, the stem portion was removed from the point of inoculation and made small pieces (0.5mm) of wood stripes (5-15 with average 10 per plant). Each strip was placed on PDA and the frequency of isolation was calculated.

Table 3. Isolation frequency of *Ceratocystis fambriata* and *Lasiodiplodia theobromae* isolated from adult beetles and its frass from April-November, 2008.

Beetle treated plants	Number of beetle released	Number of isolates (beetles)	Number of isolates (frass)	Isolation from beetle		Isolation from beetle's frass	
				Ceratocystis fimbriata	*Lasiodiplodia theobromae*	*Ceratocystis fimbriata*	*Lasiodiplodia theobromae*
Beetles of disease tree (T2)	20	95	50	2.80 ± 0.54a	4.6 ± 0.58b	0.92 ± 0.36ab	1.8 ± 0.54a
Beetles with spores (T3)	20	110	50	1.04 ± 0.31b	19.3 ± 2.82a	1.8 ± 0.54b	2.2 ± 0.40a
Spore free beetles (T4)	20	115	55	0.60 ± 0.36b	2.09 ± 0.72b	0.36 ± 0.13a	5.24 ± 1.4b
ANOVA results F;df; P				8.3 ;2; <0.005	29.67; 2; <0.0001	3.92; 2; 0.049	4.49; 2; 0.035

A total of 320 beetles (20 per plant) were collected and small quantities of frass (<0.015g) emitted out from beetle‘s holes, were inoculated on PDA. Each beetle and small quantity of frass was placed individually on PDA and isolation frequencies were calculated (no. of isolates with the fungal growth/ total no. of isolates x 100).

Discussion

The ability of an arthropod to act as a vector can be tested by using the 4 principles given by Leach (1940) which are also compatible with Koch's postulates (Whetzel, 1918). Testing the postulates implies a bioassay forcing the putative arthropod vector to colonize test plants. Mesh Enclosure Method (MEM) was successfully used in our attempt to test fungus transmission by bark beetle to young mango plants (Fig. 1). In contrast to gel capsule method originally devised by Graham (1967) and adopted by Fraedrick *et al*., (2008) we achieved higher colonization rates with MEM and an increase of beetle population by the factor 2 to 6 (Table 1). In our preliminary experiments confined area of capsule (3.81cm²) for beetle activity seemed to be a limiting factor which resulted in accumulation of frass and moisture and induced ultimately high mortality of beetles. Graham (1967) and *Fraedick et al*., (2008) reported also high mortality of beetles within the capsule and suggested to replace the dead with live beetles while attachment of capsule with the stem. That is why we used MEM technique for fungal transmission through beetles. But, the main disadvantage of MEM was that the distribution of galleries within the cage could not be predicted or manipulated due to large area for beetle activity. Moreover, due to the long experimental period individual beetles were able to produce more than one entrance hole.

From all the treated plants, the same two fungus species, *C. fimbriata* and *L. theobromae* were re-isolated which were also involved in MSDD in Oman, Brazil and Pakistan also (Ribeiro, 1980; Al Adawi *et al*., 2006; Masood *et al*., 2011; Saeed *et al*., 2011). The *C. fimbriata* was obtained in significantly higher frequency in the plants treated with crawled beetles on fungal media as compared to artificially inoculated plants, plants treated with spore free beetles and control in the first re-isolation. In all treatments re-isolation frequency increased to second isolation but there was no significant difference between treatments due to high variability of values.

Re-isolation frequency of *L. theobromae* was much higher compared to the former species *C. fimbriata* but frequencies declined from first to second re-isolation. The difference in development of the two fungus species might be attributed to different temperature or to direct competition. Low re-isolation frequencies of *C. fimbriata* in July corresponded to average temperature higher than 34°C in Multan, whereas the optimum temperature for the growth of *Ceratosystis* sp., is 23-27°C and is checked above 35°C (Upadhyay, 1981; Yaun & Mohammad, 2002; Van Wyk *et al*., 2004) and *Lasiodiplodia* sp., show maximum growth at temperature $\geq$ 35°C and slow down at $\leq$25°C (Damm, 2007). In November average temperature was about 23°C in that region, favoring growth of *C. fimbriata* but was far less as compared to *L. theobromae* and *vice versa*. It was shown by Shahbaz *et al*., (2009) that moist conditions are necessary for establishment of *L. theobromae* resulting in higher damages in spring and autumn. This requirement might be overcome by transmission by *H. mangiferae* as vector which transports inoculums directly into cambial layer of host tree. Further, it might be possible that competition between fungi species induced decline of *L. theobromae* from first to second re-isolation. Growth of virulent *C. fimbriata* might have suppressed the growth of less virulent *L. theobromae* (Al Adawi *et al*., 2006).

Isolation frequency from beetles was low for both fungus species reaching up to 2.8% for *C.fimbriata* and 19.3% for *L. theobromae*. Salle *et al*., (2005) reported high variability of isolation frequency of several fungi from *Ips typographus* between years and stands. Most common *Ophiostoma piceaperdum* was isolated on average from 20.5% to 37.2% of beetles in different years whereas isolation frequency of *Ceratocystis polonica* ranged from 0% to 4.2% in same years. Regarding storage of inoculum *H. mangiferae* is able to carry it on the body as well as in alimentary tract. This can be concluded from different isolation frequencies of both fungus species from beetles and feces and wood dust produced by beetles i.e., frass (Table 3). However, virulence of frass was conspicuously lower compared to whole beetles, although surface of specimens was washed with 1% NaOCl prior to isolation. This situation is similar to European Spruce bark beetle, *I. typographus* L., whose culticular transmission of blue-stain fungus, *Ophiostoma polonicum* Siemasko (Syn. *Ceratocystis polonicum*) was demonstrated at higher frequencies than transmission in the digestive tract which is also possible (Furniss *et al.,* 1990). Several bark beetle species have developed mycangia in different location on body which carry inoculum during migration (Six, 2003; Harrington, 2005) i.e., adult female of *Xyleborus glabratus* (Coleoptera: Scolytidae) has mycangia near mandible while *Xyleborus* sp., have similar structures at the base of elytra and intersegmental sacs between pronotum or mesonotom (Fraedrich *et al.*, 2008). However, the presence of mycangia was not yet proved in *H. mangiferae.*

Based on Leach's postulates, we confirmed the transmission of *Ceratocystis fimbriata* and *Lasiodiplodia theobromae* by the bark beetle, *H. mangiferae* and thus proved its function for spread of MSDD. Therefore, spread MSDD seems to follow similar pathways as Dutch elm desease in Central Europe, caused by *Ophiostoma ulmi* transmitted by *Scolytus* species (Coleoptera, Scolytidae) (Lanier & Peacock, 1981; Brasier, 1987) or oak wilt in North America, caused by *Ceratocystis fagacearum* (Brentz) Hunt and transmitted by a variety of sap feeding insects (Gibbs & French, 1980). Masood *et al*., (2011) also demonstrated the symptoms of MSDD and reported the attack of bark beetle on diseased trees. In our experiment cankers, black streaks and oozing occurred on all tested plants (except control) that can be related to one or the other factor (beetle or fungus) involved in the disease complex. Cankers and black streaks are more evident on plants infected by fungi independent of the presence of bark beetles. On the other hand, oozing was more abundant on plants attacked by bark beetle compared to artificial fungus inoculation and control. However, wilting as the most serious symptom finally resulting in plants death appeared to depend on the presence of fungi (Table 1).

Although we proved *H. mangiferae* as a vector of *C. fimbriata* and *L. theobromae*, several other ways of transmission seem to be possible: (i) Frass emitted from

galleries might be the source of inoculums for spread over large distances either through air or water during surface irrigation. The spores of *C. fimbriata* f. *platani* contained in frass excavated by beetle are disseminated through water which is an important dispersal factor in France, Switzerland and Italy (Grosclaude *et al.*, 1991; Kile, 1993). Malik *et al*., (2004) suggested that the diseased tree is the main reservoir for the development of bark beetle population and serve as breeding place for the bark beetle and pathogens. This has been demonstrated in other bark beetle-pathogen systems like Dutch elm disease and oak wilt vectored by bark beetles (Gibbs & French, 1980, Webber & Gibbs, 1989). (ii) Gibbs & French (1980) reported the importance of grafted roots for transmission of *C. fagacearum* between adjacent oak trees. Even in plantations of mango fruit trees, the distance between trees is reduced for economic reasons favoring direct contact of roots and probability of spontaneous root grafting may be possible. Ploetz *et al*., (1996) also suggested that water stress and mechanical injuries to roots may predispose the mango plants to the infection of mango sudden death and attraction of beetles. (iii) Nitidulid beetles (Coleoptera, Nitidulidae) and other sap feeding insects are able to imbibe inoculum exuded from wounds of diseased trees or inoculum might adhere to the mouthparts of feeding arthropods. This inoculum of oak disease (*C. fagacearum*) might be transferred to healthy oak trees (Gibbs & French, 1980). In MSDD it may be possible that inoculum is oozed out by sap flow which is one of the typical symptoms. This source on diseased trees is accessible for any sap feeding arthropod and thus, transmission to a health plants may occur. Anyway, for successful infection of healthy trees the presence of wounds in the bark seems to be essential. These might be caused by insect vectors itself i.e., if the vector is a saproxylic species or by mechanical injuries while pruning or accidentally by machines for cultivation (Malik *et al*., 2004). The same wounds are also susceptible to inoculum carried by wind or water.

H. mangiferae was formerly reported as an indigenous scolytid breeding in mango trees (Mohyuddin & Mahmood, 1993). While, the association of fungi and the bark beetle has been reported just recently in Pakistan and Oman (Al Adawi *et al.*, 2006; Masood *et al.*, 2008; Masood *et al.*, 2011) Therefore, it seems to be possible that MSDD and its rapid spread throughout the region during the last decade is the result of the new coincidence of an indigenous vector and an introduced pathogen. In a similar way the spread of *O. ulmi* originating from East Asia over Central Europe occurred, resulting in dieback of indigenous elm species during the last century (Webber & Gibbs, 1989).

In spite of pathogen and vector, different predisposing abiotic factors can be contributed to development of MSDD (Schaffer *et al.*, 1988; Ploetz *et al.*, 1996). The mango trees are increasingly vulnerable to the infection due to improper irrigation, root injuries either by termites or ploughing and lack of phytosanitary measures in the orchards (Tsopelas & Angelopoulos. 2004; Malik *et al*., 2004). Therefore, it is highly important to develop integrated management systems for mango production to minimize the risk of and the damage through MSDD. It should involve orchard management practices like controlled irrigation, light hoeing, balance use of nutrients, avoid tillage and intercropping as well as removal and elimination of infected trees or their branches without leaving inoculum or beetles in the orchard as a phytosanitary measure (Malik *et al*., 2004; Masood *et al*., 2011; Masood *et al*., 2012). Direct control measures against pathogen and vector are use of fungicides and insecticides as curative measures as soon as initial wilting signs appear (Malik *et al.*, 2004; Akem, 2006; Masood *et al*., 2011[a]) and control of the vector *H. mangiferae* (Fokunang *et al.*, 2000). A monitoring system should be developed to recognize the activity of the bark beetle which can be alleviated by using trap tree logs followed by its destruction (Saeed *et al.*, 2010). Chemical means like use of pyrethroid and new chemistry insecticides i.e., deltamethrin and bifenthrin, and emamectin benzoate and imidaclopred are effective against the attack of mango bark beetle and olive bark beetle (Lozeno *et al.*, 2001; Masood *et al*., 2011[a]) which should be regarded as the last possible alternative. In this way, loss in mango production might be reduced to economically justifiable level.

This study has proved that the bark beetle, *H. mangiferae* can be incriminated as a definitive vector of mango sudden death disease in Pakistan and thus, as a key factor in spread of the disease. The biology of transmission, although not completely understood due to involvement of different factors indicates that integrated management systems are requested. The dispersal potential of bark beetle within and between the orchards is inevitable for future prospective as well as investigation of other ways of transmission. Different means of disease dissemination and cultivation methods should be addressed in interactive ways in the study of MSDD with the intention of integrated disease management.

Acknowledgements

This work was supported by Higher Education Commission (HEC) batch-III as Indigenous scholarship grant and by Min. FAL National Agriculture Research Centre (NARC) Project:" Etiology and management of sudden death phenomenon in Mango" and "Australia-Pakistan, Agriculture Sector Linkages Program" (ASLP) Mango Projects. The financial assistance from these quarters is highly appreciated. Many thanks for their help in identification of bark beetle to Carlos Flechtmann, *Museum of Entomology Ilha Solteira, São Paulo, Brazil* and Dr. Thomas Harrington**,** *Dept. of Plant Pathology, Iowa State University- USA* for identification of fungi. I also extend my gratitude to Dr. Axel Gruppe, *Institute of Animal Ecology, Technische Universität München, Germany* for his positive comments.

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(Received for publication 17 June 2010)