

ASSOCIATION OF *LASIODIPLODIA THEOBROMAE* WITH DIFFERENT DECLINE DISORDERS IN MANGO (*MANGIFERA INDICA L.*)

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Abstract

Mango decline has assumed an alarming position due to increasing losses day by day in the orchards of Pakistan. The problem is intensified due to dearth of reliable information and suitable control strategies. The present studies were planned to characterize the isolates of the fungus *Lasiodiplodia theobromae*, test their virulence and evaluate different fungicides to find out effective ones for field application. Ten isolates were identified from 10 mango growing districts of the Punjab province of Pakistan. After inoculation on 10 months old seedlings, the fast growing isolates viz., LT-3, LT-6 and LT-7 resulted in 66.66% mortality and 3.0, 4.9 and 4.5 cm² pathogenicity lesion, respectively. Five fungicides viz., Thiophanate-methyl 70 WP, Carbendazim 50 WP, Precure combi (Thiophanate-methyl + Diethofencarb) 65 WP, Copper oxychloride 50 WP and Captan 50 WP with two doses of concentration, 50 and 100 ppm, were applied *In vitro* by food poison technique. Colony diameter in amended Petri plates was recorded after 2, 4, 6 and 8 days of inoculation. Thiophanate-methyl, Carbendazim and Precure combi showed 100% decrease over control at 50 and 100 ppm doses while Captan and Copper oxychloride exhibited only 26.84 and 7.8 and 35.26 and 20.2% decrease at both the tested doses, respectively. The results of the present studies will be helpful to devise management strategies for the control of mango decline in the Punjab province of Pakistan.

Introduction

Mango (*Mangifera indica L.*) is an important fruit plant of Indo-Pak subcontinent and known to be cultivated since ages. The mango production has increased in non-traditional mango producing areas and includes parts of Asia, West Africa, Australia, South America and Mexico. Mango enjoys prime place in the list of exportable fruits being good source of foreign exchange earning for Pakistan. Although soil and climatic conditions are suitable in Pakistan particularly in the Punjab and Sindh provinces but diseases are some of the significant causes of its low production.

Lasiodiplodia theobromae (Pat.) Griffon & Moube [synonym: *Botryodiplodia theobromae*] is a cosmopolitan and diverse species. As disease agent, pathogen is encountered in its anamorph state, named as *Lasiodiplodia*. This fungus attacks more than 280 species of plants in different parts of the world (Domsch *et al.*, 1980). In Pakistan, it has been reported on more than 50 plant species (Ahmed *et al.*, 1997). It is a common soil-borne saprophyte or wound parasite, distributed throughout the tropics and subtropics associated with different decline syndromes (Domsch *et al.*, 1980). Decline complex is observed in the form of twig blight, tip dieback, gummosis and bark splitting

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(Malik *et al.*, 2005). Drying of tip, discoloration and darkening of bark some distance from the tip are common symptoms. Later, it moves downward involving bigger branches as well. As a result, the leaves are shed followed by exudation of gum from the diseased portions. In severe cases, bark splitting or cracking has also been noticed. These symptoms may be found alone or in combination of two or more symptoms in different mango orchards of the world (Ploetz, 1999; Iqbal *et al.*, 2007). The fungus also attacks the collar region and severity of symptoms turns the plant to decline phase. The injuries caused by insects or physical damages provide avenues for penetration and then further propagation of this fungus in the host plant. The plants affected with these problems are not properly managed and resultantly, the inoculum of this fungus is increasing day by day and becoming very aggressive. When it infects collar or stem portion of the mango plant, healthy looking plant dies within a couple of days. Morphology of the isolates and pathogenicity studies are important in characterization. Morphological features and virulence of *L. theobromae* isolates have been studied previously (Shahbaz *et al.*, 2005) but studies on classification of isolates identified from diversity of field symptoms are imperative. The nature of survival/spread of the pathogen and pre-disposing factors also demand integrated management for this malady.

The attempts to test fungicides against the fungus *L. theobromae* under laboratory conditions are reported previously (Ahmad *et al.*, 1995). Shelar *et al.*, (1997) examined *In vitro* efficacy of seven fungicides viz., Aureofungin, Carbendazim, Captan, Benomyl, Mancozeb, Copper oxychloride and Thiophanate-methyl against *L. theobromae* using solid and liquid Richard's media. The results demonstrated that Benomyl (0.1%), Captan (0.2%), Carbendazim (0.1%), Mancozeb (0.25%) and Thiophanate-methyl (0.1%) were highly effective against the fungus in both solid and liquid media. Banik *et al.*, (1998) observed that Carbendazim @ 0.04% completely inhibited the growth of *L. theobromae* followed by Thiophanate-methyl @ 0.045%. Inclusion of some new fungicides along with previously tested ones may ensure selection of effective fungicides. The objectives of the present studies were to determine the infection levels of *L. theobromae* associated with different decline disorders in Pakistan, characterize isolates of ecological proximity or distant origins, test their virulence and determine minimum inhibitory concentration (MIC) values of different fungicides to find out the most suitable ones for field applications. The results of the present work will be helpful to manage the disease under field conditions in future.

Materials and Methods

Samples collection: Samples showing typical symptoms of twig blight, dieback, gummosis, bark splitting and collar rot were taken from 10 mango growing districts of the Punjab province of Pakistan (Table 1). The plants were noted with the vigilant observation and categorized in their respective disorder according to the characteristic symptoms of each disorder. Three samples of a particular disorder were collected from each of four orchards visited in each district. A total of 48 samples of each and 240 of five disorders were obtained from different locations. The scraped diseased pieces along with some healthy portion were kept in polythene bags and brought to the laboratory immediately after excision. Base information regarding symptom category was recorded on each bag as described previously (Iqbal *et al.*, 2007).

Table 1. Prefixion and origin of *L. theobromae* isolates obtained from 10 different locations of Pakistan.

Isolate	Location/District	Varietal host	Status/Origin	Field symptoms
LT-1	Multan	Malda	Local	Twig blight
LT-2	Sahiwal	Dusehri	Local	Twig blight
LT-3	Lodhran	Sensation	Exotic	Tip die back
LT-4	Bahawalpur	Chaunsa	Local	Tip die back
LT-5	Pakpattan	Dusehri	Local	Gummosis
LT-6	Faisalabad	Langra	Local	Gummosis
LT-7	Khanewal	Dusehri	Local	Bark splitting
LT-8	Rahim Yar Khan	Chaunsa	Local	Bark splitting
LT-9	Jhang	Saroli	Local	Collar rot
LT-10	Vehari	Sindhri	Local	Collar rot

Fungal isolation and characterization: Ten tissue pieces, 5 mm long, excised from each sample were surface disinfested for 10 sec., in 70% ethanol and for 2 min., in 1% NaOCl solution. The tissues were rinsed twice in sterilized deionized water, dried on sterile blotting papers and placed into glass Petri plates containing Potato dextrose agar (PDA) medium (Pathak, 1987; Ploetz & Gregory, 1993; Akhtar, 2000). The plates were incubated at 25°C with a photoperiod of 12 hours. After 72 hours, growth of hyphal tips was observed. The distinct fungal growth colonized on bits was purified and identified (Booth, 1971; Sutton, 1980). The single spored isolates were prefixed from LT-1 to LT-10. The origins and disease phenotypes associated with the isolates are indicated in Table 1. The purified cultures were lyophilized and duplicate copies stored at 10°C for future use. Lyophilized cultures were revitalized and single conidium isolates transferred to glass Petri dishes (Ø 90 mm) containing PDA, and Mango leaf agar (MLA) composed of 2% oxide agar, 20 g ground mango leaves and 1000 ml distilled H₂O. Two perpendicular measurements were taken of the colony diameter daily until the mycelia of the fastest growing isolates had covered the plates. Final observations were recorded 6 days after incubation. Average colony diameter of each isolate was calculated from the six readings per isolate. Morphological and cultural features were studied after 12 days growth on PDA using a light microscope (Olympus, Japan).

Virulence of *L. theobromae* isolates: The isolates of *L. theobromae* were tested for their ability to cause symptoms of decline on mango seedlings. Inoculum was prepared by transfer of lyophilized cultures to PDA and incubation under cool-white fluorescent light for 12 days at 25°C to stimulate sporulation. Healthy uninfected 10 months old seedlings were selected for the experiment. Artificial inoculations were done by cutting a small flap on the basal portion of the stem and inserting a 5 mm agar piece containing viable culture of the fungus (*L. theobromae*) as described by Chauhan (1994). Inoculated sites were covered with parafilm (Iwaki, Japan) to maintain a saturated environment for 2 days. Three seedlings were inoculated with each isolate. Control seedlings were inoculated with sterile PDA. Seedlings were arranged in a completely randomized block design in a growth room. Temperature in the growth room was set at 25°C with 12 hours photoperiod using cool-white fluorescent light. After 30 days, lesion development was measured distal to the point of inoculation. Re-isolations were made from diseased tissue to confirm pathogenicity of the respective isolates.

Evaluation of fungicides: The *In vitro* sensitivity of the fungus *L. theobromae* to five fungicides viz., Thiophanate-methyl 70 WP, Carbendazim 50 WP, Precure combi 65 WP, Copper oxychloride 50 WP and Captan 50 WP was tested by food poison technique (Borum & Sinclair, 1968). Two minimum inhibitory concentrations (MICs; 50 and 100 ppm) of each fungicide were added to PDA medium at the time of pouring into Pyrex glass Petri plates (Ø 90 mm). After solidification, 5 mm discs of 7 days old culture of *L. theobromae* were placed in the centre of test plates and arranged in completely randomized design with three replications. The plates were incubated at 25 °C with 12 hours light and dark cycling. Data on mean colony growth (mm) were recorded after 2, 4, 6 and 8 days of inoculation.

Statistical analysis: Data obtained from the experiments on tissue infection (%), colony growth, mortality (%) after inoculation and pathogenicity lesions were subjected to analysis of variance technique. Treatment means were compared by employing least significance difference (LSD) test at 5% probability using MSTATC statistical computer package (Michigan State University, East Lancing, MI).

Results

Fungal isolation and characterization: The fungus *L. theobromae* was found associated with all the 5 disorders viz., twig blight, tip die back, gummosis, bark splitting and collar rot with mean infection of 54.62, 46.75, 12.00, 14.13 and 56.25%, respectively (Table 2). Maximum recovery of all the disorders (42.47%) was noted in Multan district followed by Faisalabad (38.76%). Least infection (31.33%) was recorded in Pakpattan. Maximum infection by twig blight (61.0%), tip die back (58.67%), gummosis (17.0%) and bark splitting (19.0%) was recorded in Multan district while collar rot showed maximum infection (63.33%) in Faisalabad.

All the fungal isolates originating from cultivars of different mango growing districts exhibited morphological features typical of the species *L. theobromae*. Cultures were initially white to smoke grey, with fluffy, aerial mycelium on PDA. Colonies soon became gray or black and fast spreading with immersed, superficial and branched septate mycelia. Black colour of mycelia on obverse and reverse sides of Petri plates was quite visible (Table 3). The upper surface gradually developed prominent fruiting bodies. Shiny black pycnidia were produced on the surface. Conidia were initially hyaline, unicellular and subovoid to ellipsoid, with a granular content. Mature conidia were bicelled, cinnamon to dark brown, thick walled, ellipsoidal. Concisely, this study identified black coloured mycelia with few medium grey, not grouped, subglobose pycnidia, fawn coloured and ellipsoid conidia as main morphological and physical features of most of the isolates of *L. theobromae*. These distinctive characteristics were retained when single spores were subcultured. In case of linear growth of the fungus, there was significant variation among isolates for cultural characters. After 6 days incubation, based on cultural variation the isolates were classified into three groups viz., fast, intermediate and slow growing. The isolates LT-3, LT-6 and LT-7 represented significantly fast growing isolates, LT-2, LT-8 and LT-9 intermediate and LT-1, LT-4, LT-5 and LT-10 slow growing (Table 3). Fast growing isolates secured mean linear growth of 79-80, intermediate 70-74 and slow growing 47-64 mm.

Table 2. Recovery of fungus *L. theobromae* from mango tissues affected by different decline disorders.

Disorder	Sample source	Tissue infection (%)				Mean infection (%)
		Multan	Sahiwal	Pakpattan	Faisalabad	
Tip die back	Twigs	61.00 ab*	54.00 e	48.66 f	54.99 de	54.62 b
Gummosis	Twigs/branches/stem	58.67 bc	42.00 g	38.00 h	48.33 f	46.75 c
Bark splitting	Limbs/stem	17.00 ij	10.67 k	8.00 l	12.33 k	12.00 e
Collar rot	Bark	19.00 i	15.67 j	7.00 l	14.83 j	14.13 d
	Mean	42.47 a	34.47 c	31.33 d	38.76 b	

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

Table 4. Induction of symptoms on 10 months old seedlings after inoculation with *Lasiodiplodia theobromae* isolates.

Isolate	Mortality (%)	Pathogenicity lesion (cm ²)
LT-1	0.00 c*	1.00 d
LT-2	33.33 b	2.50 bc
LT-3	66.66 a	3.00 b
LT-4	0.00 c	0.00 e
LT-5	0.00 c	0.00 e
LT-6	66.66 a	4.90 a
LT-7	66.66 a	4.50 a
LT-8	33.33 b	2.30 c
LT-9	33.33 b	1.90 c
LT-10	0.00 c	0.00 e
Control	0.00 c	0.00 e

*Mean percentages in each column sharing similar letter(s) do not differ significantly (p=0.05) by LSD test.

Virulence of *L. theobromae* isolates: After 15 days of inoculation, initial symptoms like browning and blackening of the collar region were observed on test seedlings. The affected portion gradually turned dried and dead extending on upper and lower sides of the inoculation sites and leading to mortality of the seedlings in severe symptom manifestation. Infected area was measured after one month on all the inoculated seedlings. The fast growing isolates viz., LT-3, LT-6 and LT-7 showed 66.66% mortality and 3.0, 4.9 and 4.5 cm² pathogenicity lesion, respectively (Table 4). Intermediate growing isolates viz., LT-2, LT-8 and LT-9 showed 33.33% mortality and 2.5, 2.3 and 1.9 cm² pathogenicity lesion, respectively. Slow growing isolates were unable to kill the seedlings however isolate LT-1 produced only 1.0 cm² lesion on the inoculated seedling. Re-isolations from the affected parts confirmed the identification of inoculated isolates. Control plants did not produce disease signs and remained quite healthy till the termination of the experiment.

Evaluation of fungicides: Thiophanate-methyl, Carbendazim and Precure combi proved to be the best fungicides completely suppressing the growth of *L. theobromae* at both the tested MICs (Table 5). Copper oxychloride exhibited the least efficacy. Thiophanate-methyl, Carbendazim and Precure combi showed 100% decrease over control at both the concentrations while Captan and Copper oxychloride exhibited only 26.84 and 7.8 and 35.26 and 20.2% decrease at 50 and 100 ppm, respectively (Table 5). MIC of 100 ppm gave better efficacy as compared to 50 ppm after all the days of study. It showed 78.20, 78.42, 75.88 and 63.20% decrease over control after 2, 4, 6 and 8 days of study, respectively (Fig. 1).

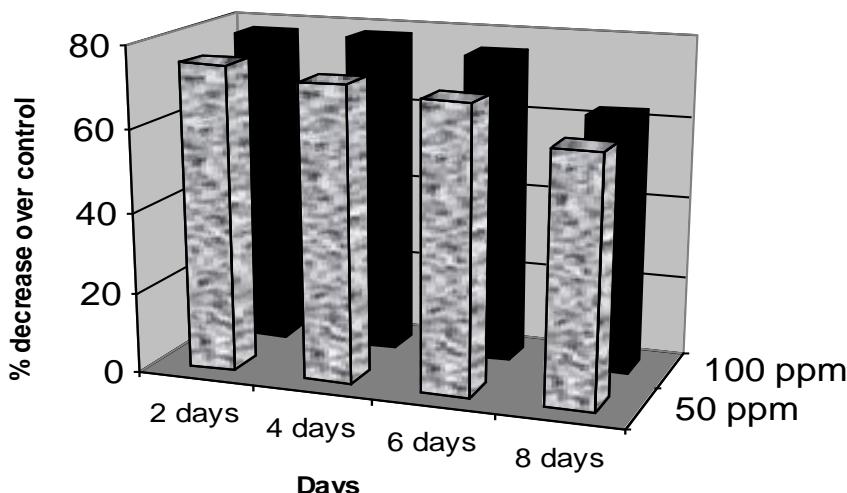


Fig. 1. Effect of 50 and 100 ppm concentrations of five fungicides on inhibiting colony diameter of fungus *L. theobromae* after four study periods.

Discussion

Gonzalez *et al.*, (1999) found the fungus (*L. theobromae*) associated with decline of mango trees. All the decline disorders observed during different stages of the present study were almost the same as observed by Ploetz *et al.*, (1997). Jadeja (2000) and Mahmood *et al.*, (2002) also reported maximum infection of *L. theobromae* in mangoes affected by decline symptoms. Narasimhudu & Reddy (1992) isolated *L. theobromae* from mango trees affected by gummosis and successfully proved its pathogenicity. In the same way, Rodriguez & Mathos (1988) also confirmed dieback, floral necrosis and gummosis caused by *L. theobromae* on 10-year-old trees of mango in Peru.

Amongst five disorders, collar rot proved to be the severe one with 56.25% infection in the mango orchards of four districts. Twig blight, tip dieback and gummosis were previously common symptom manifestations in mango orchards. Collar rot symptoms have started inflicting mango orchards few years back (Malik *et al.*, 2005). It has emerged as a new threat of extinction to mango orchards. Rotted bark portion ever shows frequent association of *L. theobromae*. All the decline disorders are found in the mango orchards alone or in combination with each other. Gummosis is found combined with other disorders like dieback, collar rot and wilting. This indicates that all these mango disorders might have originated by the same inoculum. On the other hand, gummosis might be the characteristic symptom of severe dieback, wilting and bark splitting/cracking (Rios-Castano & Reuther, 1967-68).

L. theobromae requires moist conditions for initial establishment and symptom development. This is why the disease is more severe in February-March and August-September (due to favourable temperature and high humidity). Once fungus establishes, it may survive even in dry months as observed in some mango orchards of Pakistan. Multan is the major mango growing area of the Punjab province. Maximum infection of all the decline disorders except collar rot was observed in this district.

Fast growing isolates viz., LT-3, LT-6 and LT-7 caused 3.0, 4.9 and 4.5 cm² pathogenicity lesion on inoculated mango seedlings. Higher mortality (66.66%) was also recorded in the seedlings infected by the same isolates. This shows the fast colonizing

nature of the isolates. The slow growing isolates LT-4, LT-5 and LT-10 could not produce pathogenicity lesions or mortality and proved to be non-pathogenic.

This study proves the major causative relationship of fungus *L. theobromae* with mango decline disorders in the Punjab province of Pakistan. Ragab *et al.*, (1971) described *L. theobromae* as an aggressive and vigorous pathogen causing various types of disease symptoms like tip dieback and twig blight. Similarly, Mahmood *et al.*, (2002) reported the extent of this fungus from roots, stem and branches of declining plants. They inoculated the mango plants with *Phytophthora spp.*, *Fusarium oxysporum* and *L. theobromae* but symptoms were manifested only in seedlings inoculated with *L. theobromae*. They recorded a mortality of 86.7% in mango seedlings which confirms the present results. The present studies have a good support from the latest world literature. The symptoms produced in the present work are almost similar to as described by Ploetz & Prakash (1997). The association of *L. theobromae* with the gummosis, rotting and girdling of mango stem has already been reported. Some other fungi like *F. oxysporum*, *F. solani*, *Dothiorella sp.* and *Phytopathora spp.* have also been identified by some earlier workers. *F. oxysporum*, *F. solani* and *Phytophthora spp.* are ubiquitous soil organisms in Pakistan. The frequent and common fungus associated with bark portion is *L. theobromae* (Mahmood *et al.*, 2002). No doubt, conducive environmental conditions, contribute a lot to predispose mango trees to decline, as this is also the requirement for establishment of *L. theobromae* infection.

Fungicidal evaluation is corroborated by the findings of the previous workers. Banik *et al.*, (1998) screened different fungicides against *L. theobromae* in the laboratory conditions. Carbendazim and Thiophanate-methyl completely inhibited the growth of the fungus at 400 and 450 ppm doses, respectively. Both these fungicides belong to the benzimidazole group and members of this group show good efficacy against *L. theobromae* even at low doses as reflected in the present study. Shelar *et al.*, (1997) evaluated *In vitro* efficacy of seven fungicides against *L. theobromae*, the causal agent of dieback disease of mango. Benomyl (0.1%), Captan (0.2%), Carbendazim (0.1%), Mancozeb (0.25%) and Thiophanate-methyl (0.1%) were highly effective against *L. theobromae* in both solid and liquid media. Mahmood & Gill (2002) examined *In vitro* effect of Topsin-M (Thiophanate-methyl), Benlate (Benomyl), Daconil (Chlorothalonil) and Cuprocuffaro (Copper oxychloride) at the concentrations of 10, 20, 50 and 100 ppm on the mycelial growth of *L. theobromae*. Topsin-M and Benlate showed good efficacy at 20 and 100 ppm doses. Cuprocuffaro was the least effective fungicide against the fungus at all the tested concentrations. In the present study, Thiophanate-methyl (Topsin-M) and two new fungicides Carbendazim (Crest) and Precure combi proved equally effective, completely inhibiting the growth of fungus at 50 and 100 ppm concentrations.

MIC of 100 ppm gave better efficacy as compared to 50 ppm after all the days of study. This shows that fungicidal efficacy was decreased when the dose was reduced as observed in the present study especially in case of Copper oxychloride and Captan (Table 5). In case of Captan, colony diameter of 2.3 mm after 2 days increased to 75.8 mm after 8 days of study. Similarly, colony growth in case of Copper oxychloride was increased from 5.2 mm after 2 days to 80.0 mm after 8 days resulting in complete loss of efficacy. Generally there was decrease in colony growth with the increase in concentration of fungicides. Thiophanate-methyl, Carbendazim and Precure combi retained their efficacy at both tested concentrations even after 8 days. However, the use of fungicides in the laboratory and field depends on their *In vitro* efficacy at minimal, economically acceptable dosages and their efficient and rapid transport to the infection site.

Conclusion

It is inferred from this study that different mango decline disorders prevail as independent or in combination with one another in mango orchards of Pakistan. Amongst other associated fungi, *L. theobromae* is the most common and frequent isolated fungus from all the diseased samples of these disorders. In *In vitro* studies, the fungicides Thiophanate-methyl 70 WP, Carbendazim 50 WP and Precure combi 65 WP were found more effective by completely suppressing the growth of *L. theobromae* even at low dose of 50 ppm as compared to other fungicides tested. The results of the present studies will be helpful to devise management strategies for the control of mango decline under field conditions. Future efforts will be needed to study the major co-factors for facilitating the promising disease incitation by the fungus, *L. theobromae*.

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