ISOZYMES AND BIO-CONTROL ANALYSIS OF FUSRIAUM SPP. FROM POST-HARVEST DISEASED MANGO OF PAKISTAN

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Abstract- Electrophoretic studies of Isozymes and whole cell protein using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out to evaluate genetic diversity and intra specific variations among *Fusarium solani* and *F.oxysporum* causative fungal pathogens of mango rotting in Pakistan. In present study 47 isolates were studied for morphological variability and in vitro bio-efficiency of plants against *Fusarium* spp. Among them 37 isolates randomly selected for molecular analysis. Results revealed morphological characteristics of isolates were similar to each other. 24 phenotypes obtained by using four isozymes i.e. Lactate dehydrogenase (LDH), Malate dehydrogenase (MDH), Glucose-6-phosphate dehydrogenase (G6PDH), β-glucosidase (β-glu). Among studied isolates MDH exhibit lowest activity. Isozymes banding pattern obtained by SDS-PAGE was used to compute Jaccards similarity coefficients which ranged 0.25 to 1.0 indicating genetic diversity of the isolates. Cluster analysis with UPGMA showed isolates belonged to two main groups. Genetic distance observed from D=0.15 to D=1.0. Among 37 isolates 90% belongs to *Fusarium solani*. Three plant species *Azadirachta indica, Moras alba, Citrus lemon.L* were used as Anti-Fungal Botanicals (AFB) to control the growth of *Fusarium* spp. Comparative analysis of all three AFBs showed Moras alba most effective against *F.solani* and *F.oxysporum*. Anova showed that all genotypes and treatments were significantly different from each other at 5% probability level. In conclusion, cluster analysis of protein by SDS-PAGE, and electrophoretic detection of isozymes banding patterns were useful tools for differentiating Genetic diversity among *Fusarium* spp. and AFBs are as preferred commonly used fungicides because of their environment friendly nature. This understanding can aid in devising ways of managing this potentially pathogenic fungus.

Index Terms— *Fusarium solani, F.oxysporum*, Isozymes, Post-Harvest Mango Rotting, Plant extract bio-control

1 INTRODUCTION

Mango production unfavorably hampered by the several biotic stresses in Pakistan. Punjab and Sindh mango production constitute major proportion of country agrarian economy. But their market value degraded by attack of *F.solani* and *F.oxysporum* caused mango rotting.

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It's grown on 1987.38 thousands hectare area and produces 1846.0 thousand tons of mangos (Govt. of Pakistan, 2010). Pakistan exports 7-10% of its total production valued at around US$20 million per year [1]. Post-harvest diseases destroy 10-30% of the total yield of crops while this figure can be 100% if the conditions are suitable for disease development [8]. Technologies used for post-harvest processing, packaging, transportation, handling, storage and consumption in Pakistan are traditional which caused 20-40 percent loss of fruit and vegetables During 2012 and 2013 mango diseased samples were collected from demonstrated (improved cultural practices e.g, harvesting, desapping, drying, grading etc) and farmer (without culture practices) blocks of Sindh

and Punjab mango orchards. Mango sample assessed for post-harvest diseases incidence and associated fungal pathogens. Mango rotting, Stem end rot, anthracnose and side rots are the predominant fungal diseases in Pakistan, requiring systematic study. The fungus *Fusarium* spp. induces rotting, wilting, terminated or wrinkled fruits and leaves. The isozymes study of *Fusarium* spp. defines genetic diversity and the polymorphism in isolates. In present study four different isozymes were studied in *F.solani* and *F.oxysporum* to analyze the intraspecific genetic diversity within the isolates and fungus growth reduction by using the environment friendly botanicals. Genetic distances among strains have been evaluated through analyses of isozymes and molecular markers [13]. Three different anti-fungal plant species (*Azadirachta indica, Citrus lemon.L, Moras Alba*) were used to control the growth of *Fusarium*. The goal of present study is to understand reasons about the post-harvest fungal pathogens and market losses of mangoes, and the objectives were (i) Genetic characterization of post-harvest diseases caused by fungi through SDS-PAGE (ii) Relationship of pathogenic fungi on the basis of their molecular characterization (iii) Evaluate the plant extract to control *Fusarium* growth

2. MATERIAL AND METHODS

2.1 Microorganism, Maintenance and Media Preparation

Fresh samples of mango were collected from demonstrated (DB) and farmer blocks (FB) of Sindh and Punjab. In Sindh, samples were collected from Mir pur khas (MPK), Matiari (KNA) and Tandu Allah Yar (TAY) while in Punjab collected from Multan (MU), Muzaffar ghar (MG), Rahim Yar Khan (RYK). Punjab (White Chounsa) and Sindh (Sindhri) were chosen for the disease assessment. Mango fruit was surface sterilized with ethyl alcohol. when rotting disease appeared on the mango fruits, diseased muscle cut and cultured at 280C on V8 media composed of (v8 juice 200ml, distilled water 800ml, $CaCO_3$ 3g, agar 20g)

2.2 Morphology Analysis

Morphological studies were conducted by picking a small fungal plug using a sterile needle and transferred to a clean glass slide. Small culture segments were obtained from four positions of the culture plate, two at right angle to each other, one from very close to the inoculation point and another midpoint of radius. Macroscopic characterization studied for colony color and growth on V8 and PDA media at 280C incubation. Microscopic characterization was studied on the basis of septum formation in the hyphae, mycelia color, presence of micro-spores, and absence of chlamydospores.

2.3 Enzyme Production

The V8 broth media was prepared and autoclaved, a small plug of fungus added and culture was incubated at 280C in shaker incubator for 4 to 6 days at 60rpm for the production of enzyme. After 6 days the broth medium was filtered and mycelium mat was collected from media.

2.4 Lyophilization and Extraction

Mycelia mat was kept at -180C in the refrigerator for 24 hours. Chilled mat crushed with the help of mortar and pestle until it become fine lyophilized powder [4]. Urea based protein extraction buffer was used.

2.5 Electrophoresis and Enzyme Staining

Isozymes analysis was done on SDS-PAGE by using four isozymes (Lactate dehydrogenase, Malate dehydrogenase, Glucose-6-phosphate dehydrogenase, and β-glucosidase). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) used to check purity of enzyme under denaturation conditions, using 12.5% separating and 4.5% stacking gel. The glycine based running buffer was used and prepared by adding Glycine: 14.4g; Tris-base: 3.0g; SDS: 1.25g; Distilled water: 1000ml. After electrophoresis, the gel was stained with coomessie brilliant blue. Molecular mass of standers (Lactate dehydrogenase: 35 kDa; Glucose-6-phosphate dehydrogenase: 52 kDa; Malate dehydrogenase: 35 kDa; β-glucosidase: 92 - 102 kDa. The gel was prepared by [11] protocol. After electrophoresis, the gel was incubated for 2h at room temperature, on shaking, in staining solution prepared by adding the methanol :400ml; acetic acid :60ml; Coomassie brilliant blue :2.25g; Distilled water: 500ml. After staining the gel washed by distilled water and de-stained in de-staining solution prepared by methanol: 50ml; acetic acid: 200ml; Distilled water: 1000ml for 3 hours [6]

Table 1 Enzymes used for determination of isozymes polymorphism in *Fusarium* spp.

Isozymes	Acronym	E.C Number	Buffer system
Lactate Dehydrogenase	LDH	1.1.1.27	Sodium Phosphate Buffer
Malate Dehydrogenase	MDH	1.1.1.37	Potassium Phosphate Buffer
Glucose-6-Phosphate Dehydrogenase	G6PDH	1.1.1.49	Glycine buffer
B-glucosidase	B-glu	3.2.1.20	Acetate buffer

2.6 Plant Bio-control

Three plants (*Azadirachta indica, Lemon citrus L. and Moras alba*) selected on the basis of their antifungal activities. Plant leaves were washed and shed dried for 24 hours. 250 grams dried leaves from plant were taken and 250ml distilled water was added and crushed with mortar and pestle to obtain their extract [2]. Crushed leaves were filtered by fine muslin cloth and the filtrate was collected. Filtrate was subjected to 3000rpm for 5min and supernatant was decanted. Distilled water (50ml) was added into (50 ml) plant extract on basis of random dilution method [14].

2.7 Agar Dilution Method

Plant extracts was tested for their efficiency against the pathogens by using agar dilution method [16]. Different concentrations of plant extracts (25% and 10%) were obtained by amending V8 media. The amended medium was dispensed into sterile Petri plates and allowed to solidify. Teramycine (100 µg/100ml) were added to prevent the growth of bacteria.

2.8 Inoculation

5mm uniform disk from seven days old cultures of *Fusarium* spp. were inoculated on petri plates containing 10 ml V8 medium. Both the control (without plant extract) and bio control plates were kept in incubator at 28oC. Fungal growth was measured after 3rd, 5th and 7th day. Three replicates were prepared for each sample. Percentage inhibition of bio-control growth calculated in comparison to control growth using given formula [17]. I = C-T/ C ×100 (C = Total radial growth in control, T = Total radial growth in treatment) MIC The minimum inhibition concentration was taken from the results of the fungi static activity. The lowest bio-control extract and chemical concentrations with highest inhibition percentage were taken as MIC [7].

2.9 Statistical Analysis

Data recorded for bio-control characteristics were analyzed with analysis of variance (ANOVA) technique using statistix 8.1 software [12]. For significant F value, LSD was used for mean comparison at 0.05 % level. All the computational work calculated by using NYSYSpc.

3. RESULTS AND DISCUSSION

3.1 Morphology Analysis

Macroscopic Characterization: Cottony white, fluffy, off white or pale yellow colonies were appeared on the v8 media plate after 7 days of incubation. The growth rate of all isolates was measured on 7th and 15th days of incubation. The isolates of different location showed the different growth rates. Fungus strain isolated from the diseased mango samples of Multan and Tandu Allha Yar variety showed maximum growth diameter (7cm) on v8 medium. Isolates from Rahim Yar Khan and Mir Pur Khas showed medium growth (5cm) while isolates from Muzaffar Ghar and Matiari variety of mango showed lowest growth (<5cm) these results similar to [5]. Microscopic Characterization of all isolates analyzed under the microscope at 40x. *F.solani* and *F.oxysporum* contain abundant micro-conidia and few macro-conidia with 0 to1 septa, and chlamydospores were absent. Kidney shape spores were observed [9]. Microscopic Characterization of all isolates analyzed under the microscope at 40x. *F.solani* and *F.oxysporum* contain abundant micro-conidia and few macro-conidia having 0 to1 septa, and chlamydospores were absent. Kidney shape spores were observed comparable with the investigation of Kumar (199).

3.2 SDS-PAGE Analysis

Polymorphism was observed on the basis of four isozymes. On the SDS-PAGE 24 different phenotypes were appeared. Protein profile of different isolates showed common bands size of 26 to 66 kDa was

present among all 37 isolates. The MPKDB-3 (Mir Pur Khas demonstrated block) showed different banding pattern [3].

3.3 Isozymes Analysis

The LDH banding pattern showed minimum similarity 16% with MPKDB-3 and maximum similarity 83% with isolate KNADB-43 (Matiari demonstrated block). While G6PDH pattern showed minimum similarity at 14% with MPKDB-3 and maximum 83% with TAYFB-18 (Tandu Allah Yar farmer block). The β-glucosidase showed minimum similarity at 10% with MUDB-61(Multan demonstrated block), and 83% with KNADB-25. MDH activity was less prominent in all isolates.

MDH minimum similarity 0% with MUDB-3 and maximum similarity 40% with MGDB-7 (Muzaffar Ghar demonstrated block), TAYFB-13(Rahim Yar Khan farmer block), and RYKDB-61(Rahim Yar Khan demonstrated block). MPKDB-3 exhibit very less homogeneity with LDH and G6PDH banding pattern.

3.4 Cluster Analysis

There were two major cluster observed in dendrogram i.e. A and B. cluster A had only one isolate MPKDB-3 while the rest were in cluster B. Similarly cluster B was further divided in to two cluster B-1 and B-2. Cluster A and B had coefficient of similarity of 0.25 while cluster B-1 and B-2 were having similarity of 0.29.

Figure 1 β-Glu, LDH, G69DH, MDH isozymes in comparison with *Fusarium* isolates on SDS-PAGE
Column 1: Molecular Marker, Column 2: B-glucisidase, Column 3: Lactate dehydrogenase,
Column 4: Glucose-6-phosphate dehydrogenase, Column 5: Malate dehydrogenase

Similarly coefficient of similarity between cluster B-2.1 and B-2.2 was 0.33 while cluster B-2.1.1, B-2.1.2 and cluster B-2.2.1; B-2.2.2 had similarity 0.38. Two main groups at the similarity level of 100% in un- weighted pair group method with arithmetic average (UPGMA) analysis. Since the enzymes levels and isozymes patterns are known to vary, depending on various environmental factors [15]. MPKFB-35 (Mir Pur Khas farmer block) was 100% similar with MUFB-50 (Multan farmer block). Isolate MPKFB-48 100% similar with MGDB17. Unambiguous interpretation requires genetic crosses to determine the mode of inheritance of these markers. The polymorphic isozymes system could serve as an indicator of genetic variability in *Fusarium* isolates and in identifying and characterizing these isolates. A comparison made in present work revealed that morphological characters only characterized *Fusarium* spp. at species level and did not display their genetic relationships, whilst isozymes revealed great genetic variations among individual isolates and distinguished those from each other. These results were consistent with previous study [10]. In this study, attempts have been made to obtain a better resolution for all tested isozymes, including culture conditions, incubation time and temperature,

extracting buffer systems, concentrations of the polyacrylamide and electrophoretic time. Isolate TAYDB-25 showed 100%similarity with isolates MGFB-45 (Muzaffar Ghar farmer block). Isolate KNADB-24 and KNADB-3 100% similar with MGFB-18.

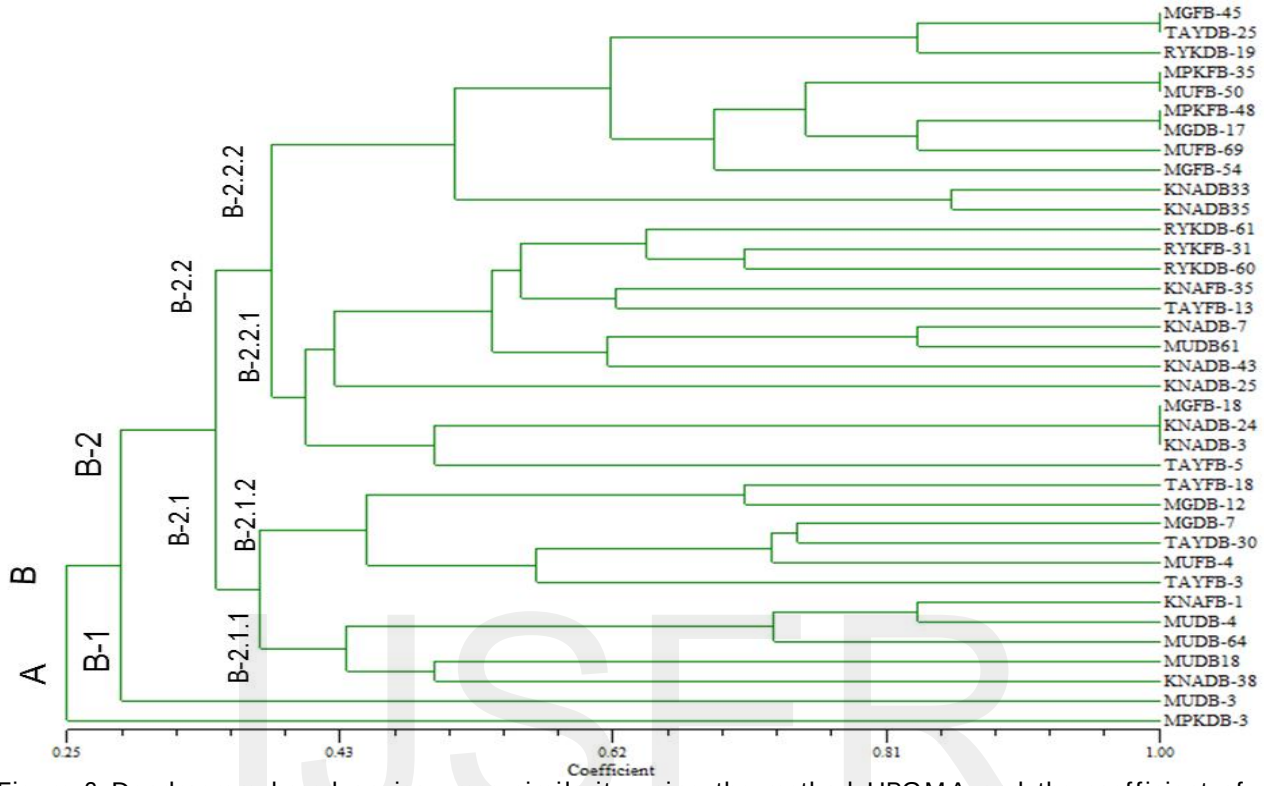

Figure 2 Dendrogram based on isozymes similarity using the method UPGMA and the coefficient of Jaccards (NYSYS-pc)
(TAYFB-13, KNADB-7, KNAFB-1: Belongs to *Fusarium oxysporum*)

Isolates The dendrogram results showed that the Matiari and Muzzafar ghar block have very much similarity. Morphology of fungal spores was similar to *F.solani* and *F.oxysporum* on the basis of mat color, microscopic spore characterization. Isozymes analysis by SDS-PAGE was simple and sophisticated technique. It has been prove successful in supporting the taxonomical analysis and provided the Genetic similarity 25 to 100% and genetic distance D= 0.15 to D=1.0 between showed among *Fusarium* isolates on the basis of four isozymes. 24 electrophoretic phenotypes (Polymorphism) were observed.

3.5 Plant Bio-control MIC

Results showed that the effective concentration of *Moras alba* (White Mulberry) was 10% (v/v). *Citrus lemon L.* (Lemon) and *Azadirachta indica* (Neem) was effective at 25 % concentration (25 ml plant leaves extract in 100 ml media). Growth diameter obtained on bio-control plate compared with the growth obtained on control plate Comparison reveals that *Moras alba* shows the best results, while the *Citrus lemon L.* was least effective, and *Azadirachta indica* showed acceptable results. The table 1 showed the percentage of inhibition by using AFB plant extract in farmer and demonstrated blocks of Punjab and Sindh orchids of Pakistan.

3.6 Average Growth Control by Plant Extracts

In Punjab the maximum average growth mean on control treatment was 4.0720 cm. *Moras alba* treatment retarded the growth of all genotypes of fungus on average growth of 0.5cm was observed followed by *Azadirachta indica* with 2.2756cm and *Citrus lemon L.* with 3.853cm. Isolates MG-DB 12 and MG-DB 19 were found

most susceptible for all three treatments followed by isolates MG-D B21, MG-DB 7 and MG-FB5 4. While in Sindh maximum average growth mean on control treatment was 4.9 cm.

Table 1 Location vise Inhibitory effects of plant leaf extract on in vitro growth of *Fusarium* spp.

			Azadirachta indica (25%)		*Citrus lemon.L* (25%)		*Moras* alba (10%)	
Province	Locations	Mango Variety	Farmer Block	Demonstrated Block	Farmer Block	Demonstrated Block	Farmer Block	Demonstrated Block
Punjab	Rahim Yar Khan	White chunsa	44%	52%	0%,	2%	90%	90%
Punjab	Multan	White chunsa	40%	55%	4%	10%	90%	90%
Punjab	Muzzafar Ghar	White chunsa	53%	16%	21%	10%	90%	90%
Sindh	Tandu Allah Yar	Sindhri	24%	16%	14%	5%	90%	90%
Sindh	Matiari	Sindhri	0%	3%	2%	7%	90%	90%
Sindh	Mir Pur Khas	Sindhri	7%	13%	1%	10%	90%	90%

The *Moras alba* treatment retarded the growth of all genotypes of fungus on average growth of 0.5cm was observed, followed by *Azadirachta indica* with 1.5cm and *Citrus lemon L*. with 1.7cm. Isolate KNA-DB 60 was found most susceptible for all three treatments followed by isolate KNA-FB 3, KNA-FB 35 and TAY-DB 30. Genotype mean is the mean of all treatments showed the overall results of plant extracts against *Fusarium* isolates of Sindh.

4. CONCLUSION

It is necessary to monitor the post-harvest diseases of mango from time to time. More surveys and detailed investigations about the pathogenic behavior and genetic variability of fungal pathogens are required for the determination of potential threat to Pakistani mango industry. Genetic distance is a measure of the genetic divergence between species or between populations within a species. Data obtained show polymorphism of mango pathogens providing evidence of variability among isolates. Significant differences in the isozyme pattern between isolates of cluster A and cluster B indicated, however, that the two groups of isolates were of different ancestry. Most of the isolates get similarity levels of > 50%, thus, conclude, that the host plant exerts the primary selection pressure, and *Fusarium* spp.

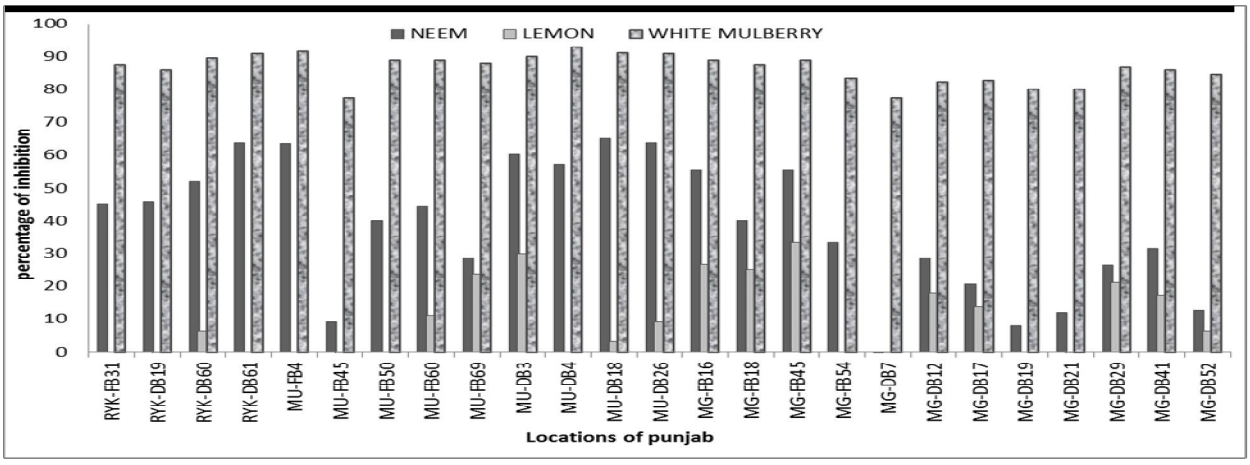

Figure 3 Comparative results of Neem, Lemon, white mulberry to control radial growth of *Fusarium* isolates of Punjab orchids.

undergo a significant saprotrophic phase in its life history. Two plant species *Azadirachta indica* and *Moras alba* were successfully controlled the growth of *Fusarium* spp. and helpful in reduction of post-harvest disease. But the Citrus lemon was not satisfactory to control the growth of *Fusarium* spp.

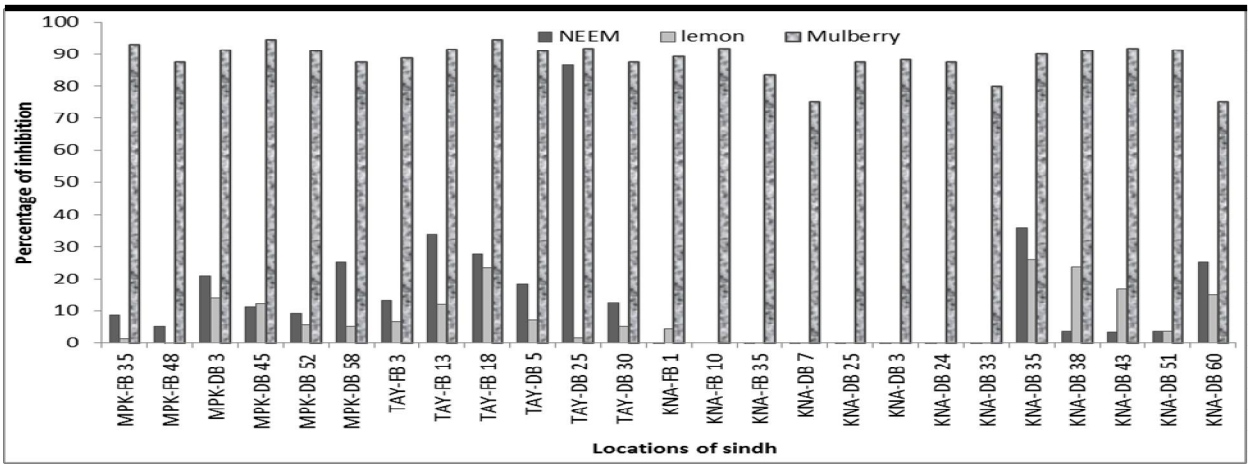

Figure 4 Comparative results of Neem, Lemon, white mulberry to control radial growth of *Fusarium* isolates of Sindh orchids.

The reported plant species should be used on other fungal species to control their growth. Other species of *Moras* should also be applied to check the antifungal activity.

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