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Isolation and Characterization of *Fusarium moniliforme* var. *subglutinans* from Malformed Mango

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عزل وتصنيف فطر فيوزاريوم مونيليفوروم (*Fusarium moniliforme* var. *subglutinans*) من المانجو المشوهة

الملخص: تحدث تشوهات المانجو في أغلب المناطق التي تزرع فيها، وهناك تقارير حول التشوهات في الأزهار وفي النمو الخضري. ومن المتفق عليه فإن الفطريات المسببة للمرض هي (*Fusarium moniliforme var. subglutinans*) أو (*Fusarium subflutinanas*) وللتعرف على علاقة فطر (*F. moniliforme*) بمرض التشوه فقد جمعت عينات صحيحة ومصابة من الأنسجة الزهرية و الخضرية لأصناف المانجو المزروعة في مناطق باكستان المختلفة. وعند عزل وزراعة الفطر، كان تلازم الفطر مع المرض متكررا بنسبة بلغت ٩٠ –٩٤%. وكان استرجاع الفطر من النسيج الذي لم تظهر عليه أعراض المرض بنسبة أقل (١٢ – ١٥ %). ولم توجد هناك فروق معنوية تذكر بين الأصناف في مدى قابليتها للإصابة بالمرض، بينما تم التعرف على أنواع بذرية وفصائل مقاومة لمرض تشوه المانجو. تم الفحص المعملي لمعرفة صفات نمو الفطر على أوساط زراعية مختلفة وتحت ظروف درجات حرارة وإضاءة ورقم هيدروجيني (pH) مختلفة. وقد كان نمو خيوط الفطر أفضل على دكستروز البطاطس مقارنة بتسعة أنواع أخرى من الأوساط الزراعية تم اختبارها. كان النمو مثاليا عند الرقم الهيدروجيني المتعادل تحت درجات حرارة تراوحت بين ٢٥ و ٣٠ درجة مئوية. عادة لا تتم مكافحة مرض التشوه بالمبيدات، إلا أنه تم فحص مدى حساسية الفطر تحت ظروف مختبريه لست أنواع من المبيدات على ثلاث تراكيز مختلفة لمعرفة إمكانية استخدام المكافحة الكيميائية.

ABSTRACT: Mango malformation occurs in most mango growing regions of the world. Floral and vegetative malformation have been reported. There is general agreement that the fungal pathogen *Fusarium moniliforme* var. *subglutinans* or *Fusarium subglutinans* is the causal agent. Healthy and malformed samples of both floral and vegetative tissues were collected from different varieties of mango grown in several locations to verify the association of *F. moniliforme* with mango malformation disease in Pakistan. The fungus was isolated and cultured. Frequency of fungal association with the disease ranged between 90- 94%. There was less recovery of fungus from asymptomatic tissue (12-15%). There was no difference among the commercial mango varieties in the level of susceptibility to this disease. However, seedling germplasm and land races showing resistance to mango malformation were identified. The *in vitro* growth characters of the fungus were determined on different culture media, at varying temperatures, light and pH conditions. Mycelial growth on potato dextrose agar was better than nine other media tested. At pH 7.00, the ideal temperature for growth was between 25-30° C. Normally, the malformation is not controlled by fungicide application. The *in vitro* sensitivity of fungus to six fungicides at three concentrations was determined to seek potential means of chemical control.

Mango malformation is a growth disorder seen in both vegetative and inflorescence tissues and occurs in many mango growing regions of the world (Ploetz *et al.*, 1994). Infected trees bear very little or no fruit. Mango malformation has become a major threat to the mango industry of Pakistan where growers have started to remove infected orchards. Floral and vegetative malformation have been characterized (Kumar and Beniwal, 1987). In both cases, irregular bunchy growth develops, which also has been named "bunchy top" (Nirvan, 1953; Schlosser, 1971).

Attempts have been made to determine a physiological basis for the disorder. Lack of balanced nutrition and disturbed levels of endogenous growth-regulators have been considered. Mites, mycoplasma like organisms and different species of plant parasitic fungi have been reported to occur in the malformed tissues (Kausar, 1959; Sumanwar *et al.*, 1960; Malik and Raza, 1985; Singh and Dhillon, 1993). The etiology of the disorder remains controversial.

ISOLATION AND CHARACTERIZATION OF *FUSARIUM MONILIFORME* VAR *SUBGLUTINANS* FROM MALFORMED MANGO TISSUES

There is general agreement that *Fusarium moniliforme* var. *subglutinans* or *Fusarium subglutinans* is the causal agent (Sharmita and Gupta, 1991). Varma *et al.* (1974) isolated *F. moniliforme* var. *subglutinans* from more than 300 samples of malformed vegetative shoots and inflorescences collected from different parts of India. A similar isolation of *F. moniliforme* var. *subglutinans* has been reported by Ploetz (1994) from Florida.

The isolation and characterization of floral and vegetative fungal isolates of *F. moniliforme* var. *subglutinans* from malformed mango samples taken randomly from mango orchards in Pakistan is reported here. Further knowledge of the prevalence of this fungus in malformed mango tissues and its morphological and physiological characteristics will provide insight into pathogenecity and control of *F. moniliforme* var. *subglutinans* in mango. The *in vitro* response of fungal isolates to fungicides is also reported. Detailed observations were made on field resistance of mango varieties to mango malformation disease. The goal is to develop environmentally safe and economically sound disease control measures as part of an integrated disease management strategy.

Materials And Methods

ISOLATION AND CHARACTERIZATION: Healthy and malformed samples of floral and vegetative shoots were collected from three mango varieties (Langra, Dashaheri, Anwar Retol) grown at several locations in Punjab, Pakistan. All plant samples were pre-sterilized with 0.1% mercuric chloride. Tissue samples were aseptically transferred to PDA in 90-mm Petri plates. Fungus isolations were made by standard techniques (Pathak, 1987). Except for temperature and light studies, all culture plates were wrapped in aluminum foil and incubated at 25°C. The tissue samples were allowed to incubate for 4 days. At that time, growing hyphal tips were transferred to fresh plates for further growth, purification and subsequent characterization.

Growth of the colony (mm/unit time), color and sporulation were described for the isolates. Confirmation of identification was based on characters described by Booth (1971).

Ten culture media (basal medium, corn meal dextrose peptone agar, Richard's agar, Czapeks dox agar, corn meal peptone agar, malt extract agar, malt extract glucose agar, water agar, potato starch agar and potato dextrose agar) were evaluated for their ability to support mycelial growth of *F. moniliforme* var. *subglutinans*. Basal medium was supplemented with a range of carbon sources to test the response of the fungus to different nutritional supplements.

Effect of temperature on fungal growth was studied at increments of 5°C from 10°C to 35°C. In order to study the effect of light on mycelial growth of *F. moniliforme* var. *subglutinans*, PDA plates were incubated under 24 hours continuous light, 16 hours light/ 8 hours dark, 12 hours light/ 12 hours dark, 8 hours light/ 16 hours dark and 24 hours dark. Effect of pH was determined in the range 3 to 10. The pH of the medium was adjusted by the addition of 0.1 N HCl or 1.0 N NaOH solutions. For all temperature, light and pH studies, PDA was used as the culture medium. Observations were made every 24 hours up to 168 hours.

FUNGICIDE SENSITIVITY: The *in vitro* sensitivity of *F. moniliforme* var. *subglutinans* to six fungicides (Benlate, Antracol, Topsin-M, Dithane M-45, Anvil, and Nordox) was determined. Three concentrations of each fungicide (10 mg/kg, 20 mg/kg, 50 mg/kg) were added to culture plates at the time of pouring the PDA medium into plates. After solidification, 5 mm discs of seven-day-old cultures were placed in the centre of test plates and incubated at 25°C. After seven days, data was collected on radial growth of mycelium.

All experimental treatments were replicated four times. Data were analysed in a completely randomised design and means were separated by LSD/Tukey's test (SAS, 1988; Steel and Torrie, 1980).

DISEASE INCIDENCE AND GENETIC RESISTANCE: Twenty-three mango orchards from different locations of Punjab were inspected to assess the incidence and response of various mango cultivars to mango malformation during 1996. Six mango varieties (Langra, Chausa, Anwar Retol, Dashehari, Fajri and Sindri) were selected for intensive observations and inspected during flowering and fruit setting period. Data were collected from 100 randomly selected inflorescences from five plants of each variety. Disease intensity was recorded following the scale of Kumar and Beniwal (1987) and varieties were rated as resistant, moderately resistant, tolerant, moderately tolerant and susceptible.

Results and Discussion

CULTURE AND PHYSIOLOGY: The data summarising the association as percent recovery of *F. moniliforme* var. *subglutinans* with malformation is presented in Table 1. There are clear differences between asymptomatic and malformed tissues. Maximum isolations were obtained from the pedicel or peduncle tissues of malformed

TABLE 1

***Association and Percent Recovery of F. moniliforme* var. *subglutinans* from healthy (H) and malformed (M) parts of different varieties of mango.**

Plant Part		Number of Isolations	% Recovery of Fungus from cv. Langra	% Recovery of Fungus cv. Dushehari	% Recovery of Fungus from cv. Anwar Retol
1. Panicle	H	340	13.82^{c}	12.33^{c}	12.59^{dc}
	M	340	92.65^{a}	91.41^{a}	90.88^{a}
2. Shoot Behind Panicle					
a) Shoot-Panicle Joint	H	105	0.00	0.00	0.00
	M	105	33.33^{b}	35.24^{b}	33.33b
b) 5 cm Below Joint	H	105	0.00	0.00	0.00
	M	105	25.70^{c}	26.67^{c}	25.71^{c}
c) 10 cm Below Joint	H	105	0.00	0.00	0.00
	M	105	18.09^{d}	16.89^{d}	16.19^{d}
d) 15 cm Below Joint	H	105	0.00	0.00	0.00
	M	105	14.29^{dc}	13.33^{e}	13.33^{e}
e) 20 cm Below Joint	H	105	0.00	0.00	0.00
	M	105	9.52^{f}	8.57^{f}	8.57^{f}
f) 25 cm Below Joint	H	105	0.00	0.00	0.00
	M	105	2.86^{h}	1.90^{h}	1.90^{h}
g) 30 cm Below Joint	H	105	0.00	0.00	0.00
	M	105	2.86^{h}	1.90h	1.90^{h}
3. Twig Supporting Shoot Behind Panicle					
a) Twig-Shoot Joint	H	75	0.00	0.00	0.00
	M	75	0.00	0.00	0.00
b) 25 cm Below Joint	H	75	0.00	0.00	0.00
	M	75	0.00	0.00	0.00
c) 50 cm Below Joint	H	75	0.00	0.00	0.00
	M	75	0.00	0.00	0.00
d) 75 cm Below Joint	H	75	0.00	0.00	0.00
	M	75	0.00	0.00	0.00
e) 100 cm Below Joint	H	75	0.00	0.00	0.00
	M	75	0.00	0.00	0.00
f) 125 cm Below Joint	H	75	0.00	0.00	0.00
	M	75	0.00	0.00	0.00

Mean/percentages were separated by Tukey's test. Values of different superscripts are significantly different ($p<0.05$)

panicles or inflorescence of three mango varieties. Percent recovery of fungus was 93, 94 and 91 from malformed samples of Langra, Dashehari and Anwar Retol, respectively. Isolation of *F. moniliforme* var. *subglutinans* from asymptomatic panicle tissues ranged between 12-15%. Isolations from other tissues along the stem of malformed panicles below the panicles were reduced linearly. There was no recovery of fungus beyond the panicle/twig-shoot joint.

Our results do not agree well with those of Ploetz (1994), who reported that all malformed floral panicles were infected and about 50% of the non-malformed panicles were infected by the fungus. Ploetz (1994) also observed that an average of 84.5% of the small pedicel and peduncle tissue pieces of malformed panicles were infected and fungus was detected in 2.2% of branches supporting malformed panicles, which is close to our estimates. Our data are in agreement with the findings of Crooks and Rijkenberg, (1985) who have reported a highly significant correlation between isolation of *F. moniliforme* var. *subglutinans* and malformation of infected tissue but a lack of correlation between isolation of fungus from healthy tissues.

Out of ten culture media tested, potato dextrose agar resulted in the best growth (Table 2) of *F. moniliforme* var. *subglutinans* i.e. 48.61 mm averaged 168 hours after inoculation, whereas potato starch agar was found to be the least effective for mycelial growth, producing only 22.11mm averaged after 168 hours. The morphological features of the fungus seen on various

TABLE 2

Effect of culture media and incubation period on mycellial growth (diameter in mm) of F. moniliforme* var. *subglutinans.

Culture Media/ Hours Incubated	24	48	72	96	120	144	168	Mean
Basal Medium	7.00	19.00	31.50	41.25	54.50	64.25	72.75	41.46^{d}
Corn meal dextrose peptone agar	7.00	17.50	29.50	38.25	42.00	48.75	53.25	33.75^{g}
Richard's agar	7.50	17.00	29.75	44.00	51.00	64.00	78.50	41.68^{d}
Czapek's dox agar	6.75	15.50	27.25	35.25	41.25	53.75	64.50	34.89^{f}
Corn meal peptone agar	8.75	17.75	27.50	37.00	45.50	57.50	64.75	36.96^{e}
Malt extract agar	7.75	20.00	29.75	46.50	53.50	67.25	77.50	43.18^{c}
Malt extract glucose agar	6.50	25.50	36.00	47.00	57.75	67.25	73.00	44.71^{b}
water agar	7.50	18.25	22.25	26.50	37.00	45.50	51.00	29.71^{h}
Potato starch agar	9.75	13.00	15.75	18.50	28.75	32.00	37.00	22.11^{i}
Potato dextrose agar	8.50	24.00	35.00	50.50	60.50	73.75	88.00	48.61^{a}
Mean	7.70^{g}	18.75^{f}	28.43^{e}	38.48^{d}	47.18^{c}	57.40^{b}	66.03^{a}	----

Means with different superscripts are significantly different ($p<0.05$)

TABLE 3

Culture characteristics of isolated fungal colonies of F. moniliforme* var. *subglutinans.

Character	Color of Medium	Color of Mycelium Outside	Color of Mycelium Inside	Color of substrate	Mycelial Growth	Macrospore Production	Microspore Production	Chlamydospore Production
Culture Medium								
Basal Media	Transparent	White	Light Orange	Light Orange	Excellent	Good	Excellent	Absent
Corn Dextrose Peptone Agar	Cream	White	Light Pink	Pink	Good	Poor	Good	Absent
Richard's Agar	Transparent	White	Creamy	Light Orange	Good	Poor	Excellent	Absent
Czapeks Dox Agar	Transparent	White	Creamy	Light Orange	Good	Poor	Excellent	Absent
Corn Meal Peptone Agar	Creamy	White	Creamy	Yellow (Concentric Rings)	Good	Good	Excellent	Absent
Malt Extract Agar	Creamy	White	White	Violet	Scanty	Scanty	Scanty	Absent
Malt Extract glucose Agar	Creamy	White	White	Purple	Scanty	Scanty	Scanty	Absent
Water Agar	Transparent	No Growth	No Growth	Transparent	No Growth	Scanty	Scanty	Absent
Potato Starch Agar	Creamy	White	White	Creamy	Poor	Poor	Good	Absent
Potato Dextrose Agar	Creamy	Light Orange	Light Orange	Orange	Excelent	Poor	Excellent	Absent

growth media are presented in (Table 3). The data shown here are comparable to the characters described by Booth (1971) for this species of *Fusarium*. On PDA plates, the fungus produced an easily identifiable purple colour in the substratum. Microscopic examination revealed that the fungus produced microconidia in chains, which were smaller in size, but abundant compared to macroconidia. The fusiform, smooth and 0-1 septate microconidia in chains measured 6.5-10.4 µm x 2.6 µm. The macroconidia were fusoid, thin walled with curved apical cells and pedicellate basal cell, measuring 16.9-19.5 µm x 2.6-3.9 µm. The chlamydospores were absent from the cultures. Our data confirmed the identity of *F. moniliforme* var. *subglutinans* and its association reported with mango malformation disease (Crooks and Rijkenberg, 1985; Kumar and Beniwal, 1987; Ploetz and Gregory, 1993; Ploetz, 1994).

Twelve carbon sources were tested as a substitute for dextrose/glucose. The quantity of substitute was adjusted to carbon equivalent of 5g of dextrose. The substitutes and their net effect on mycelium growth and sporulation are presented in (Table 4). Glucose remained the best substitute for mycellial growth followed by inositol, L-asparagine, and starch. Nicotinic Acid failed to substitute for dextrose. Chattopadhya and Nandi (1981) reported pectin better than glucose/dextrose as a source of carbon for the growth of *F. moniliforme* var. *subglutinans*. Pectin was not included in our investigation and its ability to

TABLE 4

Effect of carbon sources on growth of fungal mycelium and macrospore production.

Carbon Source	Growth (mm/unit time)	Number of Macropores
Sucrose	37.71[e]	8.50[c]
Fructose	34.46[d]	8.00[c]
Mannitol	34.17[d]	11.75a[b]
Starch	38.89[b]	5.25[d]
Glycine	23.50[h]	7.00[cd]
Tyrosine	25.64[g]	11.00[b]
L-Aspargine	29.68[f]	5.50[d]
Inositol	36.75[c]	13.50[a]
Glutamine	32.82[e]	6.75[cd]
Adenine	25.75[g]	13.50[a]
Nicotinic Acid	4.57[i]	13.25[a]
Glucose	41.43[a]	11.00b

Mean are average values after 168 from inoculation. Means with different superscripts are significantly different ($P<0.05$)

TABLE 5

***Effect of three concentrations of six fungicides on in vitro growth (diameter in mm/unit time) of Fusarium moniliforme* var. *subglutinans*.**

Concentration	10 mg/kg	20 mg/kg	50 mg/kg	Mean	Control
Fungicide					
Benlate	0.00	0.00	0.00	0.00[d]	89.50
Topsin-M	0.00	0.00	0.00	0.00[d]	89.50
Dithane M-45	39.00	7.50	0.00	15.50[b]	89.50
Antracol	34.50	12.50	0.00	15.66[b]	89.50
Anvil	15.00	10.00	7.50	10.83[c]	89.50
Nordeaux	45.50	34.00	27.00	35.50[a]	89.50
Mean	23.33[a]	10.66[b]	5.75[c]	—	—

Means with different superscripts are significantly different ($P<0.05$)

support the growth of present isolate of fungus could not be ascertained. There were significant differences among carbon sources to induce sporulation/macroconidia production, which were not dependent on abundance of mycellial growth. The data on physiological behaviour of the fungus at different temperature, light, and pH were statistically analysed and the variances (F-value) were found to be highly significant. Means are presented in Figure 1 for temperature, 2 for light and 3 for pH of the temperatures tested. Maximum growth of *F. moniliforme* var. *subglutinans* was observed at 30 ± 2°C followed by growth at 25 ± 2°C. Fungus was unable to grow at 5 ± 2°C but produced a little mycelial growth at 10 ± 2°C (Figure 1). Mycelial growth of the fungus varied slightly with change in the duration of light and continued as the incubation period prolonged. Continuous light and 12h-light/12h dark were most suitable exposures for mycelial growth (Figure 2). Maximum mycelial growth of fungus was observed at pH 7 and 8, whereas at pH 3 fungus was unable to grow. The fungus responded positively to the alkaline conditions (Figure 3).

The above data on physiological behaviour of *F. moniliforme* var. *subglutinans* may not be unique but certainly an appropriate attempt to describe its isolates from Pakistan. One can expect variation in physiological behavior of strains or isolates gathered from different sources. The knowledge of physiological aspects is necessary to work towards confirming the Koch's postulates for mango malformation.

FUNGICIDE SENSITIVITY: The data on *in vitro* sensitivity of the fungus to six fungicides at three doses are presented in Table 5. The fungus responded differentially to various chemicals and doses. Two fungicides, Beneate and Topspin-M, inhibited fungal growth at 10 mg/kg. Dithane M-45 and Antracol were effective in complete inhibition of fungal growth at a much higher concentration of 50mg/kg. The remaining two fungicides, Anvil and Nordeaux, failed to completely inhibit the fungal growth. There was an incomplete inhibition of growth by Anvil at all concentrations. It is known from numerous studies reported in the literature, that the aerial-tree sprays of fungicides do not control the spread of mango malformation (Ploetz *et al.*, 1994). Our *in vitro* sensitivity data has clearly demonstrated the fungicide sensitivity of the *F. moniliforme* var. *subglutinans*.

There is a need for finding better application methods to determine chemical control of mango malformation disease. Darvas (1987) has reported efficacy of trunk injection of fungicides to control mango malformation, which deserves further investigation in the light of our data. For finding chemical control, an application method with better penetration and/or an appropriate time of application will be necessary to achieve the desired results. Equally important is the doze of fungicide and additives applied through spray methods. The investigators may not have reached the effective concentration along with other parameters of foliar application. The lack of *in vitro* inhibition by two fungicides in the present case could be a consequence of an ineffective doze as well. The inability of fungicides to control mango malformation disorder also calls for further investigations into its cause(s) other than *F. moniliforme* var. *subglutinans*.

DISEASE INCIDENCE AND GENETIC RESISTANCE STUDIES: Detailed responses of mango varieties to mango malformation were noted on a data sheet prepared according to the methods of Kumar and

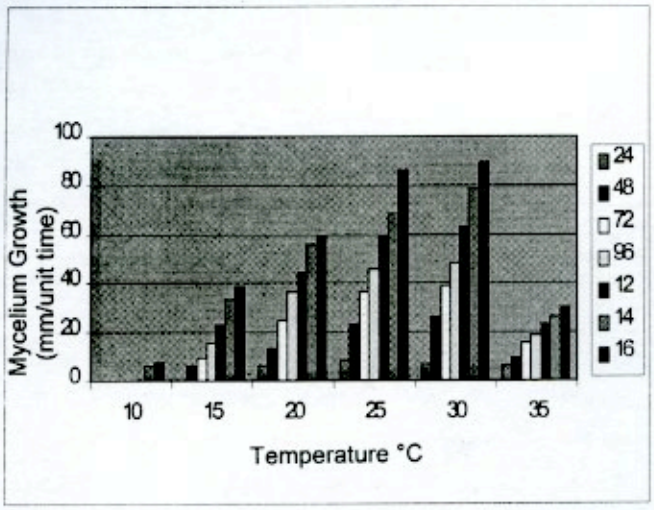

Figure 1. Effect of temperature and time on mycelium growth measured from 24 hours to 168 hours of fungal culture.

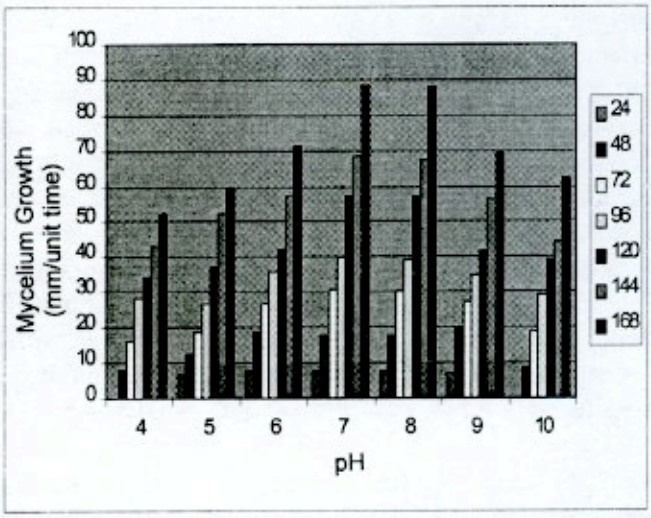

Figure 2. Effect of light on mycelium growth measured from 24 hours to 168 hours of fungal culture.

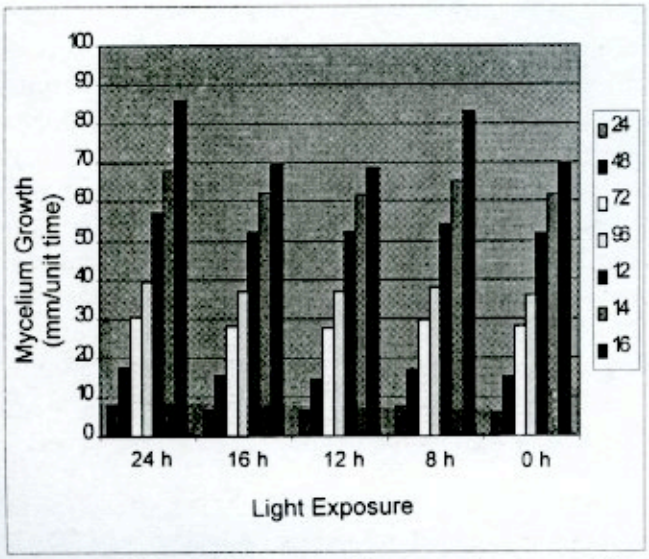

Figure 3. Effect of pH on mycellium growth measured from 24 hours to 168 hours of fungal culture

Beniwal (1987). There were no significant differences among the commercial mango varieties in succeptibility to this disease (data not presented here). The incidence of disease during the 1996 year was universal in all selected orchards and on all six varieties under investigation. The intensity of malformation ranged between 22% and 83% of all inflorescences tagged and observed for developing malformation symptoms in all orchards under investigation. During the course of this survey, we identified land races and seedling germplasm showing resistance to mango malformation (Khan, 1996). None of the cultivars under observation was found to be resistant. These results are consistent with the findings of Prakash and Raoof (1987). They observed the incidence of mango malformation in 121 cultivars during 1978 and 1979. None of them was resistant. Singh (1984) reported the existence of genetic diversity in the genus *Mangifera*. Our observations of resistant land races support his findings. As reported by Sharma and Majumdar (1989), genetic resistance to mango malformation is a dominantly inherited character. Thus, there is a strong potential to successfully select and breed mango malformation resistant varieties that should be exploited.

Conclusions

The fungus species *Fusarium moniliforme* var. *subglutinans* was found to be consistently associated with the mango malformation disease in Pakistan. Fungal isolates from Pakistan have morphological and physiological characteristics of the species as described by Booth (1971). *In vitro* sensitivity tests of the fungus to different doses of fungicides indicate a possibility of developing a safe chemical control by stem injection as reported by Darvas (1987). The commercial varieties of mango are generally susceptible to the disease. However, genetic resistance does exist in the land races and undescribed germplasm of mango. Thus, it is possible to develop programs for selecting and breeding mango malformation resistant varieties, the most suitable disease management strategy.

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