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Mango Malformation Disease in Egypt: 1- Isolation and Detection of Viroid-Like RNA and other Associated Organisms

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Mango (*Mangifera indica*) tree is affected by mango malformation disease (MMD). Neither virus like particles, bacteria nor phytoplasma like organisms have been detected in extracts, tissues and thin sections of diseased malformed leaves and inflorescences. While it was found that the malformed tissues contained low molecular weight specific RNA molecules with circular and linear strands as detected by R-PAGE. These molecules were not detected in inflorescences and leaves from healthy trees. These RNA molecules were soluble in LiCl and migrate in 5% PAGE. The mobility of MM-RNA in PAGE is not affected by heat denaturation.

The fungi (*Fusarium moniliforme* and *F. solani*) and mites were also found to be associated with MMD. *F. moniliforme* was isolated from malformed inflorescences of cvs. Hindi, Zebda (75% F), Taimour and Dabsha (100% F). While *F. solani* was only isolated from Hindi and Taimour (25% F). However, the mites were only found to be associated with Hindi and Taimour cvs.

Ultra-thin sections of malformed leaves showed important variations in cytoplasmic membranes and endoplasmic reticulum. Infected cells developed the paramural bodies known as a plasmalemmasomes. These bodies can be correlated with the presence of viroid or viroid-like RNA. Thus, it is suggested that the cause of mango malformation is a viroid like-RNA and that it is a disease of viroid etiology.

Key words: Bacteria, fungi, malformation, mango, mites, phytoplasma, viroid and virus.

Mango malformation is well known in India and has also been confirmed in most mango-growing countries as Pakistan, Bangladesh, Israel, Egypt, Sudan, South Africa, Mexico, Cuba, Brazil, Central America, Australia, USA, and recently, the United Arab Emirates (Kumar *et al.*, 1993). Three distinct types of symptoms described by some investigators are punchy top of seedlings, vegetative malformation and floral malformation. Intermediate stages of these types of symptoms have been observed in nature (Pathak, 1980). Majumder and Sinha (1972) observed that no mango fruit set on malformed panicles in varieties such as *Langara*, *Chausa* and *Dashehri* and they found appreciable fruit set on 'light' type of panicles in variety *Bombay green*.

There are five types of causal agents of mango malformation as suggested by various investigators which may cause some confusion in such syndromes. These agents are:

1. *Fungi:* Varma *et al.* (1971) published the first record of a fungus, *Fusarium moniliforme* associated with malformed mango shoots and inflorescences. Knight and Campbell (1995) and Salazar (1995) showed that most evident points have indicated that the fungus *Fusarium moniliforme* var. *subglutinans* is the causal agent. Kumar and Beniwal (1992) demonstrated that the association of *Fusarium* spp. with malformed tissues was not successful in pathogenicity tests, using *F. moniliforme* var. *subglutinans* as the test fungus.
2. *Phytoplasma:* Kishtah *et al.* (1985) reported that no phytoplasma could be detected in sap or thin sections of mango seedlings or trees showing malformation disease. Culturing an extract of malformed tissue did not change the red colour of the PPLO (Pleuro-pneumonia-like organisms) broth medium. Negative results were obtained when such extracts were checked with conjugated antisera of *Spiroplasma citri* (SC). Examination of thin sections of petals, leaf midribs and fine roots from malformed mango trees revealed that phloem and xylem tissues were apparently free from any pathogen.
3. *Mites*: Hassan (1944) hypothesized the causal organism to be an eriophyid mite, *Aceria (Eriophyes) mangiferae*, as he observed such mites to be associated with both vegetative and floral malformation in mango trees cultivated in Egypt. Similar views were later expressed in India by Narasimhan (1954). Bindera and Bakhetia (1971) who could not establish any correlation between bud-mite population and incidence of malformation. They also found that inoculation of plants with mango bud mites from malformed panicles did not induce malformation and control of bud-mite failed to reduce the extent of malformation.
4. *Viruses:* Bindera and Bakhetia (1971) have successfully transmitted the disease by grafting or budding. On the contrary, Prasad *et al.* (1965) could not transmit the disease by grafting, inarching and budding.
5. *Physiological:* Prasad *et al.* (1965) and Knight and Campbell (1995) noticed little difference in mineral constituents of healthy and diseased tissues. They also added that the disease could not be cured by injecting or spraying diseased trees with solutions of boron, calcium, copper, iron, magnesium and other elements. The involvement of hormonal imbalance was indicated by Singh *et al.* (1991b).

Sharmita and Gupta (1991) inoculated mango seedlings with *F. moniliforme* (*Gibberella fujikuroi*) var. *glutinans* isolate 19A (infected by virus-like particles (VLP) or 7B (VLP-free) in pots. Symptoms appeared 8 months after inoculation. Malformation developed on plants inoculated with isolate 19A but not on plants inoculated with isolate 7B.

Finally, one hundred years ago since it was first recognized such syndromes, the problem of mango malformation is still un-solved (Kumar *et al.*, 1993). Thus, this work aimed to continue investigation on such problem, trying to detect and identify the causal agents based on the biological, morphological and molecular properties.

Materials and Methods

Agents associated with malformed tissues:

Samples of naturally malformed inflorescences of mango were collected from mango orchards at Qualubia Governorate. Samples were used for isolation and detection fungi, bacteria, mites, phytoplasma, virus and viroid.

Fungi were isolated on PDA according to the technique described by Hawker (1950). The isolated fungi were identified using the description of Barnett and Hunter (1987). Bacteria were isolated on nutrient agar medium using the method described by Abdel-Sayed (1984). Another portions of malformed tissues were used for investigation of mites using the method described by Munger and Gilmore (1963).

Detection of phytoplasma was done by using Dienes' stain (consisting of 2.5g methylene blue, 1.25g azore 11, 10.0g maltose and 0.25g sodium carbonate, and 100 ml distilled water). The stain was filtered through filter paper (Whatman No.1) and serial dilutions (0.5-1.0% v/v) were made in distilled water (Hayflick, 1965).

Transverse hand sections of healthy and diseased leaf blade were cut into distilled water using a single edge razor. The sections were transferred to Dienes' stain (Hayflick, 1965) for 10 min. The sections were examined by light microscope.

Detection of the virus:

Samples of malformed tissues were used for detection of viruses as follows: negative staining of clarified infected sap was examined by electron microscopy (Uyeda *et al.*, 1975).

Extraction of total nucleic acids:

Nucleic acids were isolated from frozen healthy and infected mango tissues as described by Morris and Smith (1977). Briefly, 1-5g of plant tissue were extracted in 8ml of extraction buffer consisting of 0.1M glycine-NaOH, pH 9.0, 50mM NaCl, 1mM (Na_2EDTA), 2% SDS and 1% sodium lauryl sarcosine. The extract was mixed with 8ml of Tris-HCl buffered phenol pH 7.6, containing 0.17% 8-hydroxyquinoline and 0.1% 2-mercaptoethanol then stirred for 15 min. after adding 8ml of chloroform. Samples were centrifuged at 6000 rpm for 15 min., the aqueous phase was recovered and the nucleic acids were precipitated by adding 2.5 volume of absolute ethanol and 0.10 volume of 3M sodium acetate pH 5.2, then kept overnight at –20°C. The nucleic acids were centrifuged at 10000 rpm for 30 min. The pellet was dissolved in 1ml of TE-buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) then reprecipitated with ethanol and sodium acetate as described above. The pellets were again dissolved in TE-buffer and the nucleic acid concentration was determined spectrophotometrically.

Analysis of nucleic acids extracts:

Return-PAGE was carried out as described by Singh *et al.* (1988). A sample of 6µl of purified nucleic acid was mixed with 2 µl of the dyes xylene cyanol F.F and bromophenol blue, then subjected to each well of gel (6%), first mixture of

electrophoresis was carried out at room temperature with high salt buffer (89 mM tris, 89 mM Boric acid, 2.5 mM EDTA) for 2.5 hr. The second mixture of electrophoresis was carried out with low salt buffer (1/8 the cover of high salt buffer) at 70°C in water bath and electrophoresed for 20 hr. Gels were stained with silver nitrate.

Tissue processing and electron microscopy:

Ultra-thin sections were prepared as employed by Abdel-Ghaffar (1994) by cutting five different of healthy and malformed mango leaves into 1x1 pieces followed by pre-fixing in 2.5% glutaraldehyde and 2% paraformaldehyde for 4 hr, then post fixing in 1% osmium tetraoxide for 1.30 hr and dehydrating in ascending grade series of ethanol. The specimens were treated with propylene oxide and then embedded in super plastic mixture in embedding capsules. The sections were cut with a diamond knife and the grids containing sections were stained with 5% uranyl acetate then with lead citrate and examined with a Zeiss 10C transmission electron microscope at National Research Centre, Dokki, Egypt.

Transmission trials:

Hundred of healthy mango seedlings were used for studying under greenhouse condition of pathogen transmission by grafting.

Results

1- Agents associated with malformed tissues:

Samples of mango inflorescences and leaves showing malformation symptoms (Fig. 1) were subjected to detect suspected causal agents.

Data in Table (1) show that isolation trials have resulted in two fungal isolates identified as *Fusarium moniliforme* and *Fusarium solani*. Results of isolation using PDA plates showed that *F. moniliforme* was isolated with 100% frequency from infected samples of Taimour and Dabsha cultivars and 75% frequency from infected Hindi and Zebda samples. On the contrary, *F. solani* was isolated from Hindi and Taimour with 25% frequency. In the same time, *F. solani* was not associated with malformed tissues of Zebda and Dabsha cultivars.

The results indicate also that mites were found to be associated with inflorescences of Hindi and Taimour only. In the same time it was not found to be associated with Zebda and Dabsha cvs (Table 1).

No virus like particles were observed in both negatively stained clarified sap and in ultra thin section from diseased mango leaves or inflorescences by electron microscopic examination (Fig. 5).

No phytoplasma-like bodies could be detected by light microscope in phloem tissue of petiole, blade and leaf midrib of hand thin sections of diseased mango leaves, stained with Dienes' stain (Fig. 2).

Table 1. Isolated agents from malformed mango tissues

Isolated organism	Variety							
	Hindi		Zebda		Taimour		Dabsha	
	P *	F%	P	F%	P	F%	P	F%
Fungi								
F. moniliforme	+	75	+	75	+	100	+	100
Fusarium solani	+	25	-	-	+	25	-	-
Bacteria	-	-	-	-	-	-	-	-
Mites	+		-		+		-	
Phytoplasma	not detected							
Virus	not detected							
Viroid	Viroid-like RNA							

* P= Persistence and F= Frequency.

Fig. 1. Mango inflorescences and leaves showing malformation symptoms.

Isolation of viroid-like RNA associated with the mango malformation:

Sections taken from malformed mango trees, which were grafted, on healthy mango seedling, and showing typical malformation symptoms developed 9-12 months latter as the natural infection. The results indicated that twenty seedlings from 50 grafted-seedlings developed symptoms of malformation. When the previously malformed tissues were tested early for the presence of expected fungi, bacteria, phytoplasma, virus and mites, it was found free from all these agents. In addition, examining the crude sap on plate agar by electron microscope and the hand sections by Dienes' stain revealed negative results. On the other hand, *Fusarium moniliforme* and a mite appeared as a secondary infection on mango var. Dabsha only.

RNA species detected in malformed mango trees:

The total nucleic acids extracted from healthy and malformed inflorescences and leaves were fractionated by 5% PAGE. The specific RNA was detected in nucleic acid preparations from malformed inflorescences and leaves (Fig. 3). It was not found in nucleic acid prepared from healthy inflorescences and leaves (Fig. 4). This RNA which associated with mango malformation samples migrated as a sharp band in 5% gel. Another RNA species was detected in nucleic acid preparations from healthy and malformed inflorescences and leaves. A MM-RNA appeared to be present in high concentration in leaves of malformed inflorescences, being readily detected by PAGE of nucleic acid extracts as little as 1.0 g fresh leaf tissues. It is clear that malformation symptoms on mango trees are localized in the inflorescences and nearest leaves of them, thus, we decided to investigate the possibility that the causal agent was confined to these lesion sites.

Electrophoretic analysis of nucleic acids preparations from naturally malformed inflorescences, leaves and grafted seedlings which induced similar malformation symptoms, with return PAGE, showed the existence of a nucleic acid with the structural properties of viroid-like RNA (Fig. 4).

The cytopathic differences were observed in MMD tissues. It was the presence of many cytoplasmic vesicles formed by invagination of the plasmalemma and or the tonoplast (Fig. 5). These paramural bodies had different sizes, containing multiple membrane-enclosed chambers [watch spring like multilayered membrane body (WSL)] and multi-vesicular bodies [plasmalemmasomes (PLS)] which can be correlated with the presence of viroid or viroid-like RNA. As well as the onset of gross-symptom were not found in healthy tissues.

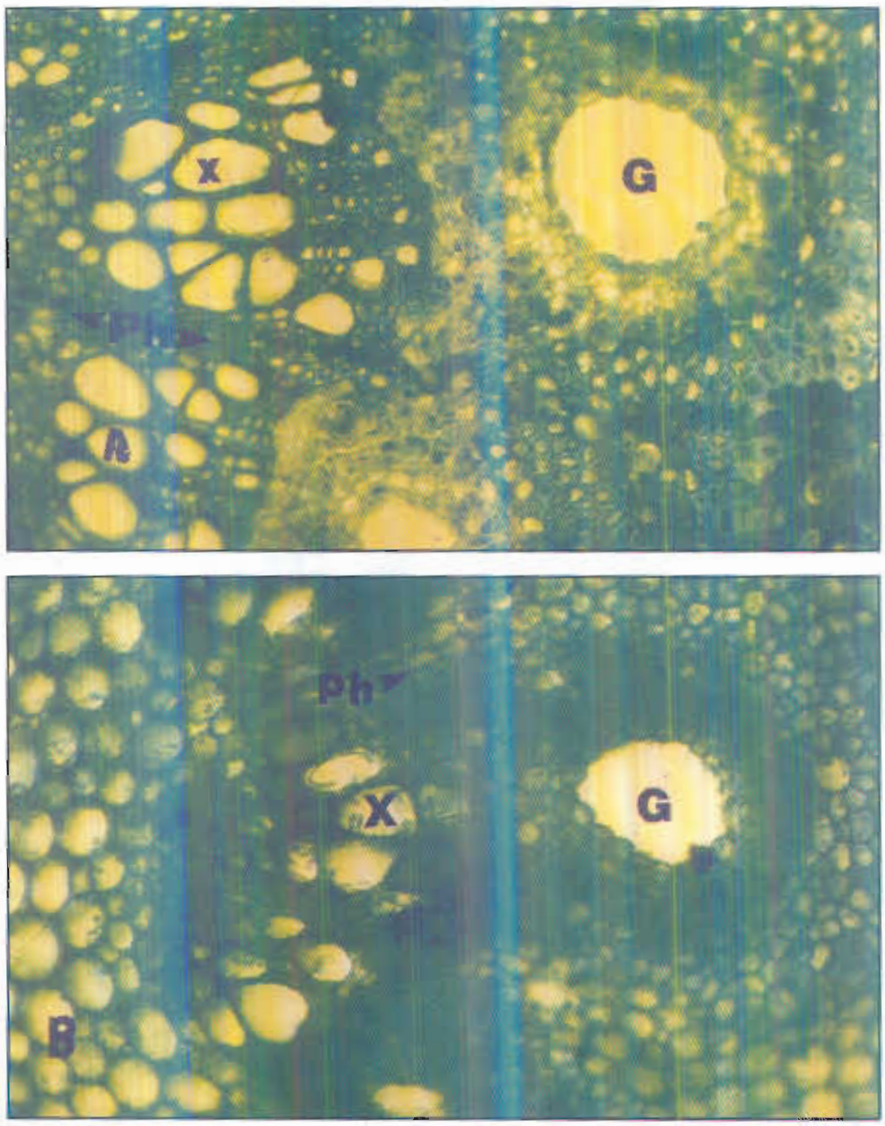

Fig. 2. Light micrographs of transverse sections of mango malformed leaves treated with Dienes' stain. Hand cross sections of leaf blade (A) Healthy (B) Diseased, xylem and phloem remain unstained. Whereas: G= gland, Ph= phloem and X= xylem.

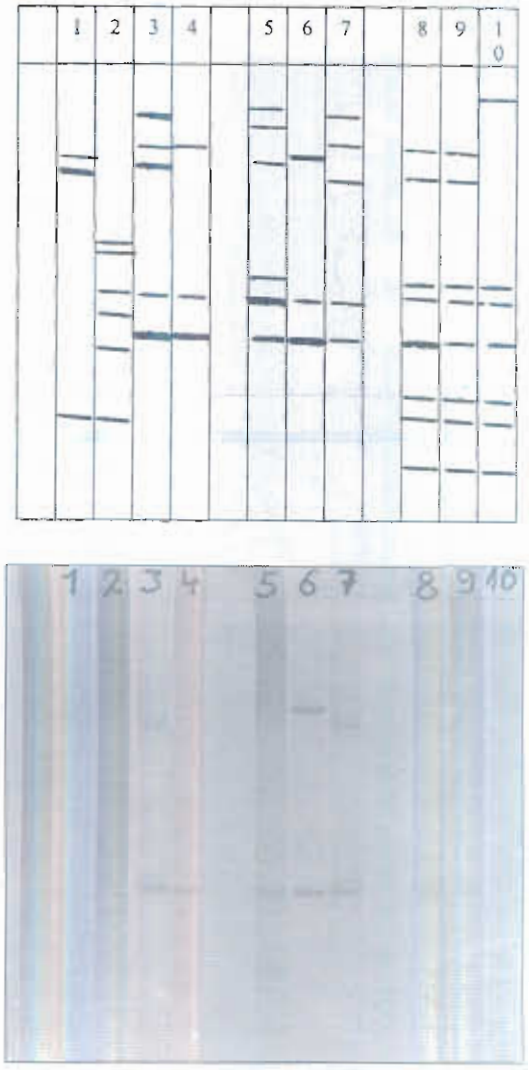

Fig. 3. Polyacrylamide gel electrophoretic analysis of total nucleic acid extracted from malformed inflorescences of different mango varieties.

1- Healthy inflorescences. 2- Healthy leaves.
3- Malformed grafted seedling. 4- Malformed seedling.
5- Malformed leaves of Dabsha. 6- Malformed leaves of Zebda.
7- Malformed leaves of Taimour. 8- Malformed inflorescences of Dabsha.
9- Malformed inflorescences of Zebda. 10- Malformed inflorescences of Taimour.

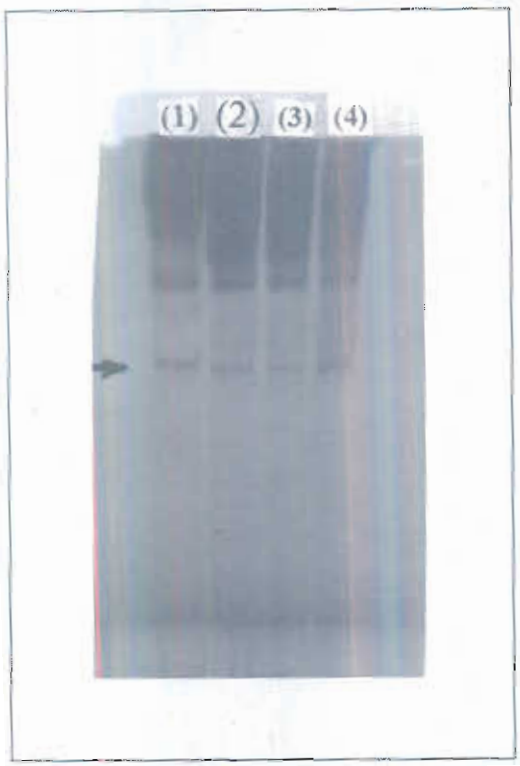

Fig. 4. Return polyacrylamide gel electrophoretic analysis of nucleic acid extracted from mango.

1- Malformed leaves of Dabsha. 2- Malformed leaves of Zebda.
3- Malformed leaves of Taimour. 4- Malformed leaves of Hindi.

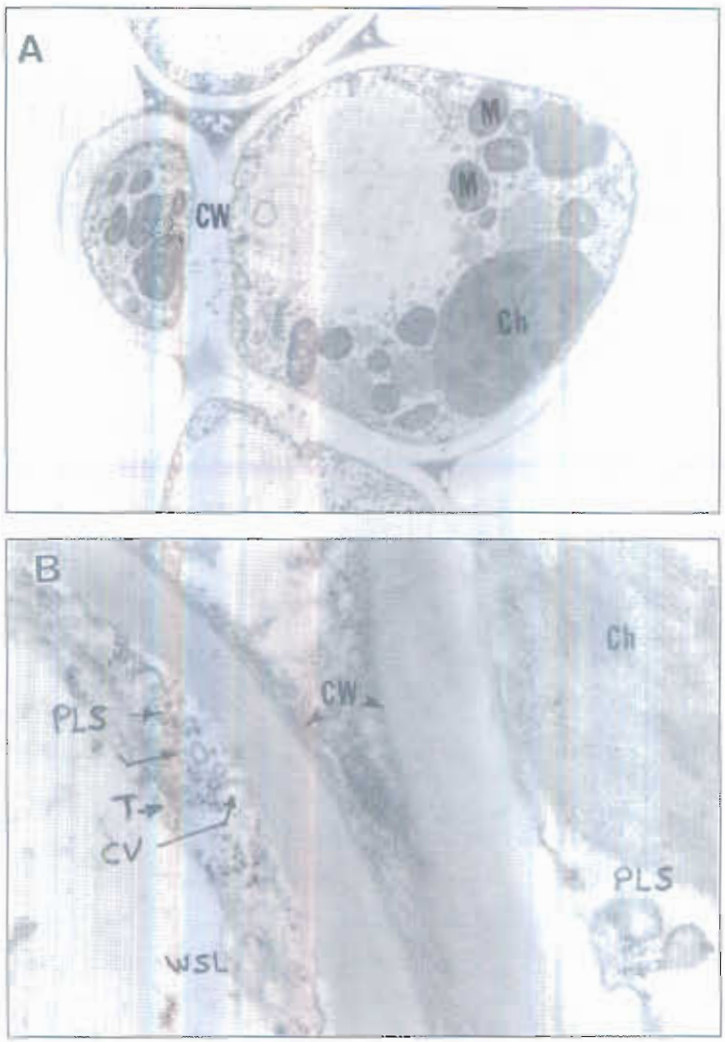

Fig. 5. Transmission electron micrograph of malformed mango leaves.

A. The normal cell of healthy leaves. (6000X).

B. The infected cells of diseased leaves, plasmalemmasome formed by invagination of plasma membrane into vacuole, Mitochondria close degenerate may have vacuoles and change of size. Reduction of chloroplasts (6000 X). Multiple vesculated paramural bodies or plasmalemmasomes formed by tonoplast or plasma membranes. It's close to degenerate and change of size and shape (10000 X).

Whereas: Ch= chloroplast, CW= cell wall, M= mitochondria, CV= cytoplasmic vacuoles, PL= plasma membrane, PLS= plasmalemmasomes, T= tonoplast, WSL= watch spring like.

Discussion

Malformation of inflorescences and leaves of early natural infection on mango trees and seedlings (showed erect growth, in particular the production of small leaves with various forms of epinasty and an abnormal unevenness of surface) were found free from bacteria, phytoplasma like organism and virus but were associated with fungi and sometimes with mites. These samples were used as a source for isolation and detection of viroid-like RNA. On the other hand, fungi were found on malformed inflorescences and leaves, as a secondary infection. In this way, Kumar and Beniwal (1992) mentioned that association of *Fusarium* species with malformed tissues was not successfully demonstrated in pathogenicity tests, using *F. moniliforme* var. *subglutinans* as the test fungus. In addition, the mites were found in association with cvs. Hindi and Taimour but not found on cvs. Zebda and Dabsha. This result confirmed that mites are not the causal agent and agreed with Bindera and Bakhetia (1971) who found that inoculation of plants with mango bud mites from malformed panicles did not induce malformation and control of bud-mite failed to reduce the extent of malformation.

Ultra-thin sections from malformed leaves or inflorescences did not reveal any pathogens. No virus or virus-like particles were detected in extracts or ultra thin sections by electron microscopy. This evidence, together with that of Beniwal and Bhatnagar (1975) and Kishtah *et al.* (1985) suggest that the causal agent is not a virus. Too over growth, sterile flowers and bunchy top symptoms of mango malformation is a feature of many phytoplasma diseases, (Schlösser, 1970). However, this study as well as other studies did not reveal the phytoplasma or phytoplasma like bodies in the phloem or xylem tissues of hand thin sections (Beniwal and Bhatnagar, 1975 and Kishtah *et al.*, 1985). Sharmita and Gupta (1991) found a mycovirus (ds-RNA) of *Fusarium moniliforme* var. *glutinans* as an agent of mango shoot malformation.

The present investigation provides evidence that malformed inflorescence is probably induced by a viroid, viroid-like RNA or satellite RNA according to the following:

1- As mentioned by Bindera and Bakhetia (1971) that mango malformation was transmitted by grafting to mango seedlings and emphasized by Burhan (1991) who believed that the disease was introduced into the United Arab Emirates with imported seedlings and agreed with Kumar (1992) who showed that grafting as the most common method of mango propagation, plays a decisive role in the spread of the disease, because diseased scions are impossible to distinguish on a tree in an off year.

2- Obtained results showed that total nucleic acid preparations subjected to polyacrylamide gel electrophoresis revealed low molecular weight RNA band which is discernible in extracts from malformed inflorescences and leaves, but not in extracts of healthy ones. These results were obtained by using R-PAGE (Schumacher *et al.*, 1980, and Singh *et al.*, 1991a). In the R-PAGE procedure, viroid or viroid-like RNA is obtained by two independent electrophoresis runs.

During the first run with "high salt" buffer RNA (linear form) move from top towards the bottom of the gel. Second run (return) is conducted with reversed current polarity, RNA (circular form) migrate or return from the bottom towards the top of the gel. High temperature was found to be critical for successful separation of these viroid or viroid-like RNA especially, during the second run (Schumacher *et al.*, 1980 and Singh *et al.*, 1991a). On the other hand, denatured RNA contained circular molecular and other properties of structure of viroid-like RNA closely resembled that of a viroid (Riesner *et al.*, 1979 and Sanger *et al.*, 1979). The low molecular weight RNA and the fact that no conventional virus particles were found in malformed inflorescences, suggest that malformed mango may have viroid or viroid-like RNA etiology.

The present investigation showed that the main cytopathic changes were the presence of large numbers of paramural bodies. Paramural bodies of the type reported herein had been also detected in citrus exocortis viroid CEVd, potato spindle tuber viroid (PSTVd) and cucumber pale fruit viroid (CPFVd) infected but not in healthy leaves (Semancik and Vanderwoude, 1976; Hari, 1980; Wahn, 1980; El-Dougdoug *et al.*, 1993 and El-Dougdoug *et al.*, 1998).

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مرض تشوه المانجو في مصر:
١- عزل وتشخيص RNA مشابه للفيرويد والكائنات المصاحبة الأخرى

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** قسم النبات الزراعي - كلية الزراعة بمشتهر - جامعة الزقازيق/فرع بنها - مصر.

تصاب المانجو بمرض تشوه الشماريخ الزهرية، وبفحص الشماريخ الزهرية المشوهة لم يكتشف جزيئات الفيروس أو خلايا بكتيرية أو مشابهات الفيتوبلازما في المستخلصات النباتية وفى القطاعات الرقيقة للأوراق والشماريخ المشوهة بينما وجدت تحتوي علي أنواع خاصة من الحمض النووي الريبوزي (RNA) ذات بناء دائري وخيطي وأوزان جزيئية منخفضة كما هو واضح بإستخدام التفريد الكهربى لرقائق R-PAGE مما يدفع الى الزعم بأن مرض تشوه المانجو ربما يكون ناتجا عن إصابة فيرويد او مشابهات الفيرويد حيث لا يوجد هذا الحمض النووي في أوراق وشماريخ الأشجار السليمة.

وقد وجد فطري فيوزاريوم مونيليفورم وفيوزاريوم سولاني والأكاروسات مصاحبة للنورات المشوهة حيث تم عزل فطر فيوزاريوم مونيليفورم من النورات المشوهة لأصناف المانجو هندي وزبدية بنسبة ٧٥% والتيمور والدبشة بنسبة ١٠٠% بينما عزل فطر فيوزاريوم سولاني من أصناف الهندي والتيمور بنسبة ٢٥% فقط. كما وجد الأكاروس مصاحبا فقط للشماريخ المشوهة لصنفي الهندي والتيمور. وقد أظهرت القطاعات فائقة الرقة في السمك للأوراق المشوهة اختلافات هامة في الجدر السيتوبلازمية والشبكة الإندوبلازمية حيث أظهرت الخلايا المصابة تراكيب خاصة من نوع (Paramural bodies) تعرف باسم (plasmalemmasomes). هذه الأجسام كانت مختلفة في الحجم والشكل والتراكيب الداخلية ومرتبطة بوجود الفيرويد أو مشابهات الفيرويد. وعلي ذلك فنحن نعتبر أن مسبب تشوه المانجو هو مشابهات الفيرويد.