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Phytoplasma Associated with Mango Malformation Disease in Egypt

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Scanning electron microscopy (SEM) of different mango samples showing typical symptoms of malformation disease was conducted. The investigations revealed the presence of phytoplasma units in the cortical cells as well as parenchymatous cells of vascular tissues of rachises and petioles of mixed inflorescences (phyllody symptoms) of both Hindy and Taymor mango cultivars.

Different populations of phytoplasma units were observed in the investigated sections. The diameter of such units ranged between 0.6-0.8 µm. Budding and binary fission of phytoplasma were also observed as a developmental stages of its growth.

In addition, a histological study was conducted to fetch how far the different tissues were affected. In general, tannic sacs of all studied parts were increased in their frequency and density due to infection. The resin ducts of the infected samples of both leaf petiole and rachis showed bigger diameter. Phloem obliteration was clearly observed, while the cambium and its cambial derivatives were disorganized.

Keywords: Malformation, mango, phyllody, phytoplasma and SEM.

Mango malformation is a very destructive disease of an international importance. It occurs in most mango growing countries worldwide (Kishta *et al.*, 1985; Burhan, 1991; Kumar *et al.*, 1993 and Freeman *et al.*, 1999). Both vegetative and floral malformations were found on the affected trees.

Most of the previous researches attributed the disease to fungi (Kuti Babu and Raw, 1998; Kumar and Ram, 1999 and Freeman *et al.*, 1999). In many cases the pathogenicity of the isolated fungi was not proved, so our attention was spotted on how to throw light on the role of phytoplasma(s) on this disease.

Phytoplasma (formerly known as mycoplasma like organisms) are non-cultivable prokaryotes of the class Mollicutes, as they resisted all attempts to culture them *in vitro* in cell free media (Chang *et al.*, 2004 and El-Banna *et al.*, 2007). Phytoplasma(s) are unicellular wall-less units bounded by membrane and appear in sieve elements as rounded or ovoid units ranging in diameter from 400 to 900 nm (Guo *et al.*, 1996; Siddique *et al.*, 1998 and El-Banna *et al.*, 2007).

In the four decades since their discovery, and based on evidence provided by electron microscopy or transmission experiments, phytoplasma have been implicated as the etiological agent of several hundreds of plant diseases worldwide

(Davis and Sinclair, 1998). Phytoplasma(s) are known to cause diseases to woody trees and other plants, *i.e.* Dodonia (Borth *et al.*, 1995), pear and apple (Schneider and Jibb, 1997), eucalyptus (Marcone *et al.*, 1996b)) and hazelnut (Jamantiene *et al.*, 2000).The previous diseases are characterized by phyllody, witches-broom, proliferation, stunting, dieback and decline resembling or identical to symptoms observed on malformed mango trees.

Early in 1979, La and Chang described typical symptoms of phyllody of the inflorescences of Jujube trees, beside twigs heavily covered and crowded with abnormally small leaves. Mycoplasma like organisms were detected in the vascular bundles of such leaves and inflorescences. Since that date, many publications were announced concerning the association of phytoplasma with phyllody and virescence symptoms.

Recently, in 2004 Chang *et al.* indicated that Aster yellows phytoplasma was responsible for the disease characterized by symptoms of phyllody and virescence of the flower spikes of the ornamental plants, poker statice and lace. The phytoplasma units were detected in sieve elements of midribs taken from leafy parts formed instead of floral parts (phyllody) by EM.

Phytoplasma diseases are often accompanied by anatomical changes culminating in necrosis and disorganization of phloem tissues. Early, in 1977 Esau described the anatomical alteration in phloem tissues of spinach affected with Aster yellows disease. The alteration included phloem degeneration, involving sieve tubes necrosis and abnormal cell proliferation. In Italian grapevine infected with flavescence dorée (FD) disease caused by phytoplasma, sieve elements and the associated companion cells became necrotic (Credi, 1994). He also added that abnormal proliferation of sieve tubes was also observed. Others pointed out that *Vicia faba* plants infected by phytoplasma of the same disease (FD) showed high titer of phytoplasma units without collapse of the sieve elements, while shoot tissues demonstrated low titer and sieve elements degradation (Lherminier *et al.*, 1994).

Because methods for *in vitro* cultivation of phytoplasmas are not available yet, the lack of efficient techniques for their diagnosis, their differentiation and detection is difficult. The techniques based on symptomatology and electron microscopy supply useful basic information (Borth *et al.*, 1995; Guo *et al a.*, 1996; Marcone *et al.*, 1996a and Siddique *et al.*, 1998). El-Banna and El-Deeb (2001) pointed out that phytoplasma was detected for the first time in Egypt in sections prepared from malformed mango inflorescences using scanning electron microscopy (SEM).

In the present work, samples of small leaflets formed on mixed inflorescences, their petioles and rachises formed on floral mango malformations were investigated using SEM to check the presence of phytoplasma. In addition, a histological study was conducted to fetch how far the different tissues were affected.

Materials and Methods

Disease symptoms:

Mango (*Mangifera indica* L) trees (8-10 years), cvs Hindy and Taymor grown in El-Fayoum and El-Menufya (Nobariya district) governorates were labeled during March and April 2002. Most of the inspected trees were severely infected with mango malformation disease. Samples were collected from both healthy and malformed parts for detecting the presence of phytoplasma as well as its effect on different tissues using both light and electron microscopy.

Internal investigation of naturally infected inflorescences:

A- Light microscopy:

Specimens for microscopy were taken from the leaf petioles and rachises of the mixed inflorescences. Samples were killed and fixed in FAA (5 ml formalin + 5 ml glacial acetic acid + 90 ml ethyl alcohol 70 %) solution for 24 h then dehydrated to a final concentration of n-butanol and then embedded in paraffin wax. Thereafter, microtome sections were made and stained with safranin – light green combination according to Sander (1993).

B- Scanning electron microscopy (SEM):

Samples of floral and vegetative malformed leaf blade, leaf petiole of mixed inflorescences and rachises of floral malformations were cut, fixed in 2.5% glutaraldehyde for 24 h at 4°C. Samples, then post-fixed in 1% osmium tetroxide for 1 h at room temperature (Harley and Fergusen, 1990). The specimens were then dehydrated with ascending concentrations of acetone, critical point dried, and finally sputter coated with gold. The examination, measurements and photographing were done through a Jeol Scanning Electron Microscope (JSM-T 330 A) equipped with image recording and processing system (Sem Afore).

Phytoplasma concentration was estimated in the different tissues of infected leaf petiole and rachis. The titer of phytoplasma was determined in multiple sections of the inspected phloem tissues of the aforementioned parts of mango trees according to Guo, *et al* (1996).If the maximum number of phytoplasma unit per phloem element is less than five in any section, the titer was considered to be low. If the maximum number was more than 50 the titer was considered to be high. A titer between these two extremes was considered moderate.

Results and Discussion

Symptoms:

According to field observations, both Hindy and Taymor mango trees at any age were highly susceptible to infection by mango malformation. The inflorescences were heavily covered with abnormal small leaves (Fig. 1), giving which is known as phyllody symptoms (mixed inflorescences). On the other hand, vegetative malformations (proliferations) were observed on twigs, giving them a bushy appearance. These observations are confirmed by the findings of Khadhair *et al.* (1998) who stated that phytoplasma taxa, can be compared for many characters including the propensity to induce particular symptoms such as phyllody,

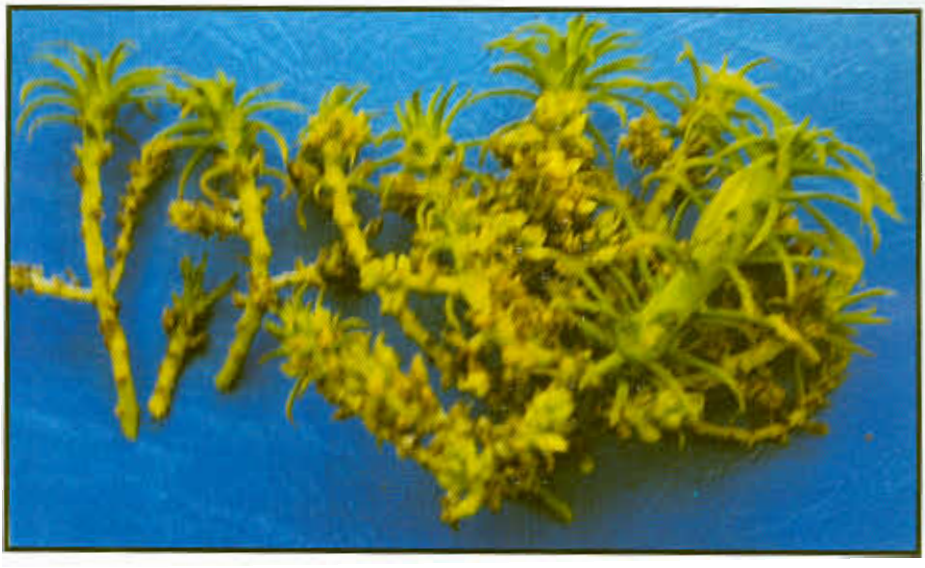

Fig. 1. The mixed inflorescences (phyllody symptoms) on Hindy mango trees grown in Nobariya plantation.

proliferation, witches-broom or hyperplasia. Sinclair and Griffiths (2000) pointed out that phytoplasma diseases are characterized by phyllody symptoms which are attributed to the virescence agents responsible for these characteristic symptoms. This was also indicated by Pracros *et al.* (2006) who indicated the responsibility of phytoplasma on symptoms of flower malformations including uprighted inflorescences, virescence, phyllody and big bud. They indicated that phytoplasma multiplication in the phloem sieve tubes, results in deregulation of floral meristem gene expression.

Internal investigation of naturally infected inflorescences:
A - Light microscopy:

The microscopic investigations of both cross and longitudinal sections of healthy and malformed samples (leaf blade, leaf petiole and stem), showed an increase in the phloem area due to malformation. The increased area of phloem contained less functioning sieve elements. A higher rate of obliteration was observed in the phloem sieve elements and their companion cells as well. The malformed materials revealed also an increase in the diameter of resin ducts (Fig. 2). Meantime, the area of xylem tissue was also increased, but with less vessels number and less diameter (Fig. 3).

This reduction of active phloem sieve elements and xylem vessels indicated an impairment of translocation. It could be also suggested that the high rate of phloem obliteration lead to a very low auxin transport which consequently produces the small leaves (malformation syndrome).

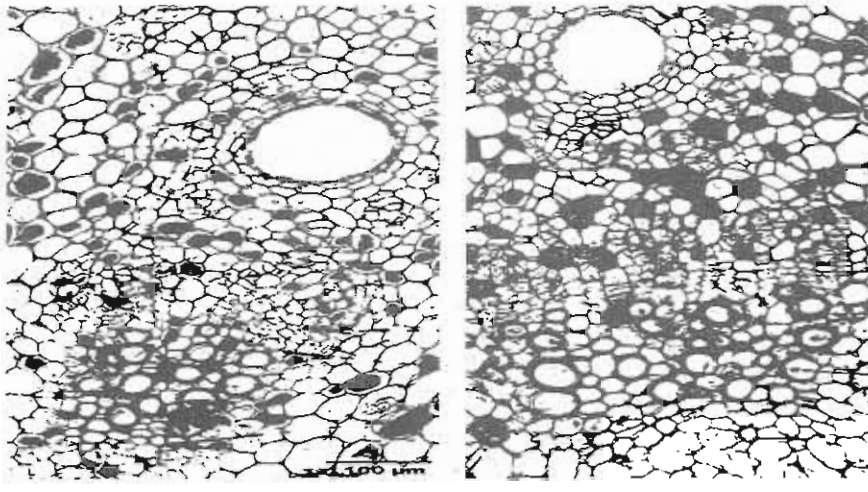

Fig. 2. Light micrographs for cross-sections of the vascular bundles of the leaf petioles of both healthy (A), and malformed mango plants (B).

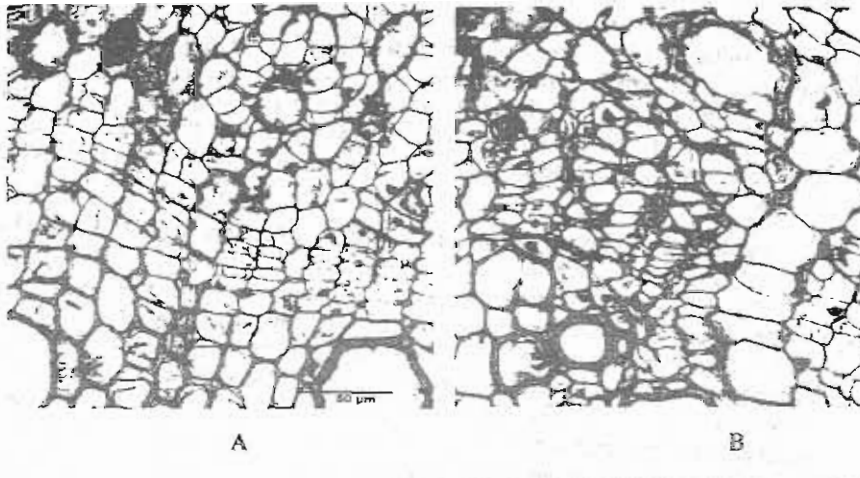

Fig. 3. Light micrographs illustrating the cambium layer and its cambial derivatives of the rachis of both healthy (A), and malformed (B) mango inflorescences.

The microscopic investigations of the vascular bundles showed also a disorganized cambium, which consequently produced irregular cambial derivatives. The cambial zone showed larger disorganized area (Fig. 3). Raafat *et al.* (1995), mentioned also that the vascular tissues of the malformed mango stem showed clear reduction in the functioning sieve elements accompanied with a higher rate of sieve element obliteration. Symptoms caused by phytoplasma indicate disorders of the phloem function which seem to be a unifying symptom for massive phytoplasma infection and is histopathologically indicated (Christensen *et al.*, 2005). Pracros *et al.* (2006) described typical flower malformations including uprighted inflorescences, virescence, phyllody and big bud as a result of infection of periwinkle plants by the stolbur phytoplasma. They also mentioned that phytoplasma causes disorders of floral development suggesting that the genetic regulation pathway of flower development is impaired.

B- Scanning electron microscopy (SEM):

The different samples, of leaf blades and leaf petioles were investigated using SEM. Phytoplasma units were detected in the phloem tissue as well as in the parenchymatous tissues between xylem and phloem. Phytoplasma units were observed almost in all investigated sections as rounded bodies identical in their shape, with a diameter ranged from 0.6-0.8 µm (Figs. 4, 5 & 6). The observed phytoplasma units are identical to those visualized by Guo *et al.* (1996), Andersen *et al.* (1998), Jamantiene *et al.* (2000) and EL-Banna *et al.* (2007). The phytoplasma units were attached with the inner surface of the cell plasma membrane (Fig. 6). The adhesion is a functional aspect as the phytoplasma uses the sterols of the cell membrane for their growth and division (Christensen *et al.*, 2005). On the other hand, budded units were also detected (Fig. 4B) indicating their identity as phytoplasma as many reports pointed out that reproduction of phytoplasma is partially through budding (Marcone *et al.*, 1996b and Khadhair *et al.*, 1998 and Christensen *et al.*, 2005). Tymon *et al.* (1998) stated that phytoplasma units were detected in young inflorescences of coconut trees suffering from coconut yellowing disease.

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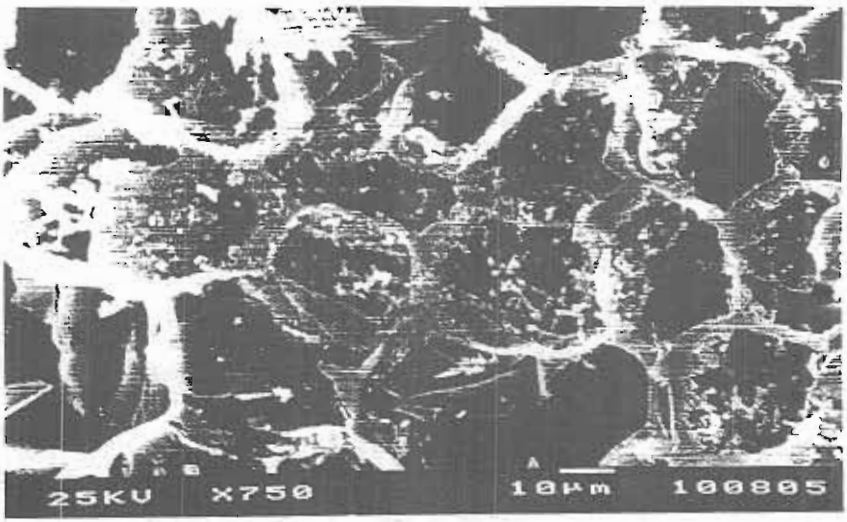
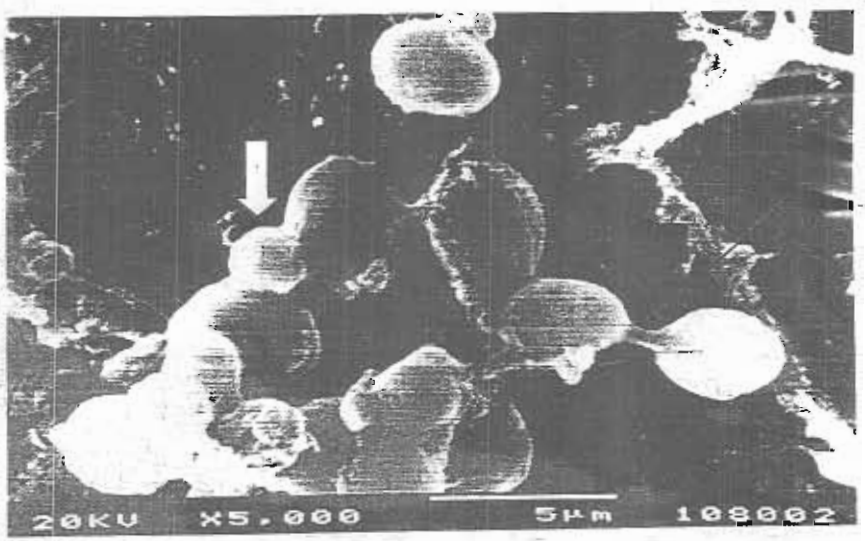


A



B

Fig. 4. Scanning electron micrographs showing the corrosive presence (A), of the phytoplasma units in the cortical cells of the malformed inflorescences. (B), few units in higher magnification as budding (thick arrow) and binary fission (thin arrow) are obvious.

Fig. 5. Scanning electron micrographs showing the occurrence of phytoplasma (arrow) in xylem parenchymatous cells (longitudinal sections).

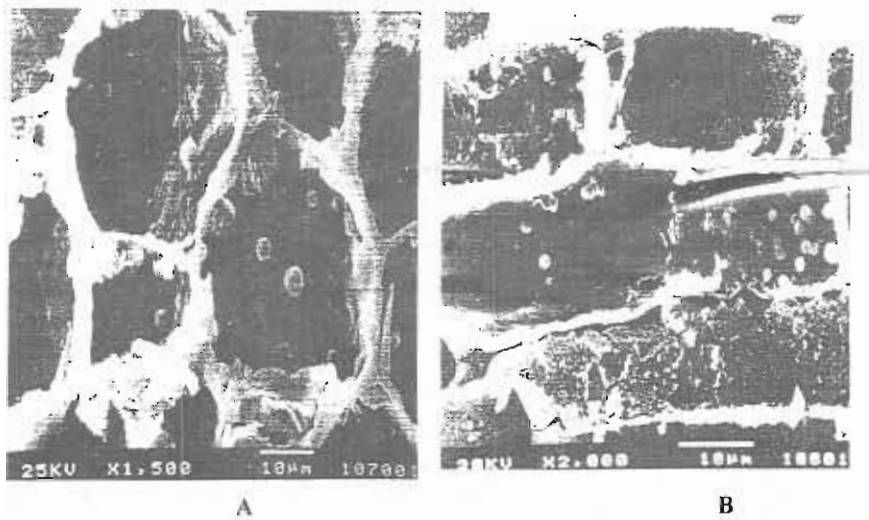


Fig. 6. Scanning electron micrographs presenting the occurrence of the phytoplasma in the phloem parenchymatous cells of leaf base formed on mixed inflorescence.

Binary fission was also detected (Fig. 4B) as a method of vegetative reproduction for phytoplasma. Musetti and Favali (2003) detected phytoplasma budding units in the phloem tissues of *Catharanthus roseus* leaf petioles infected by apple proliferation phytoplasma. Regarding the titer of phytoplasma units, it was obvious from the inspected sections that it varied from low (Fig. 6A) to heavy (Fig. 6B) according to the statement applied by Guo *et al.* (1996).

The titer (No) of phytoplasma units was estimated. Data in Table (1) generally show that concentration of phytoplasma units was higher (75 units/ cell) in phloem parenchyma than in the xylem (32 units/ cell). The mean diameter of phytoplasma units was high; being 0.88 µm in xylem parenchyma cells compared with 0.83 µm in phloem parenchyma cells. The calculated mean diameter of phytoplasma units was in the range of almost all the published data about this point (Guo *et al.*, 1996; Andersen *et al.*, 1998; Jamantiene *et al.*, 2000 and Chang *et al.*, 2004).

Our results suggest for the first time the association of phytoplasma with the disease known as mango malformation, and according to these findings we can say that phytoplasma(s) might play a role in this syndrome. This conclusion was confirmed by that no fungal structures or other plant pathogens were observed in all the inspected sections. Further advanced studies concerning the molecular biology based methods using specific primers adopted for detection of phytoplasma will be carried out on infected mango trees to confirm the responsibility of the detected phytoplasma for this serious disease.

Table 1. Concentration and mean diameter of phytoplasma units detected in two different infected tissues

Tissue	Phloem Parenchyma			Xylem parenchyma		
	High	Moderate	low	High	Moderate	low
Phytoplasma titer (No)	75	35.3	5.75	32	15	5.4
Diameter (µm)*	0.820	0.873	0.79	0.934	0.782	0.928
Mean diameter		0.83			0.88	

* Diameters of 200 phytoplasma units were measured using a recording and processing image system (Sem Afore) equipped to an SEM.

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ارتباط الفيتوبلازما بمرض تشوه المانجو

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اجري فحص بالمجهر الالكتروني الماسح لقطاعات مختلفه من اجزاء اشجار المانجو المظهره لأعراض نموذجيه لمرض التشوه في المانجو . و قد اظهر الفحص وجود وحدات الفيتوبلازما في خلايا القشرة و كذلك في الخلايا البرانشيميه لانسجة الحزم الوعائيه لمحاور النوره و أعناق الأوراق الصغيرة المتكونه بدل الازهار علي النورات المختلطة (اوراق و أزهار) وPhyllody المعروفه باسم (تورق الازهار) و ذلك في عينات المانجو من الاصناف هندي و تيمور. و قد سجلت تعدادات مختلفه من وحدات الفيتوبلازما في الانسجة التي تم فحصها و قد تراوح قطرها بين ٠,٦-٠,٨ ميكروميتر. كما لوحظت وحدات متبرعمه و اخري في حالة انقسام ثنائي كمراحل من تطور الفيتوبلازما.

كما أجريت دراسه هستولوجيه لمعرفة كيف تأثرت الأنسجه المختلفه بالأصابه و عموما وجد أن الخلايا التاينيه في جميع الانسجه التي تم فحصها قد زادت في العدد أو الكثافه نتيجه للأصابه . وقد أظهرت القنوات الراتنجيه زياده في القطر في انسجة محاور الازهار و قواعد الأوراق كما لوحظ تهشم في أنسجة اللحاء كما لوحظ أيضا عدم انتظام في الكامبيوم و مشتقاته.