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*Budget Department*
*היחידה לתקציבים*

October 16, 2007

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Dear Mr./Ms.

Re: Project TA-MOU-02-M21-030

Please find enclosed a copy of the Final Report for the above mentioned grant.
Covering period December 1ST, 2007 to September 30th, 2007

Sincerely,

R. Lerer

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# FINAL REPORT

**Covering period**: December 1st, 2002 to September 30th, 2007

**Submitted to the U.S. Agency for International Development; Bureau for Global Programs, Field Support and Research; Center for Economic Growth and Agricultural Development**

**TITLE OF PROJECT**: Epidemiology and control of mango malformation disease

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## 3. Executive Summary

Mango malformation was first reported in Israel in 1968, Egypt in 1934, and the United States (Florida) in 1972. This disease is one of the most important constraints to production, since fruit are not produced on affected panicles. Although the etiology of mango malformation has been confused for many years, there is unequivocal evidence that the fungus, *Fusarium mangiferae*, is the causal agent of disease. The epidemiology of malformation is poorly understood; however, it has been reported that the disease may be spread with the assistance of the mango bud mite, *Aceria mangiferae*. Since there is an exchange of propagation material between these and other countries where the disease is present, such as India, *F. mangiferae* could be moved in infected tissue between countries.

Viability of spores and inoculum of *F. mangiferae* in infected panicles declined very rapidly in soil. In Israel, in infected trees, the pathogen was not detected in seed, seed coat or flesh, whereas under certain field conditions in Egypt, infections were apparently seedborne. In Egypt and in Israel, utilization of molecular markers determined that inoculum of the pathogen is airborne with infections in seedlings descending from top to lower stem sections and roots. Minor infections of roots may occur, but the pathogen did not infect plants systemically. The mango bud mite can facilitate transfer of spores within trees to the apical bud, which is a major infection site. However, wind is the main mode of spore dispersal. When sprayed at the appropriate time, the fungicide prochloraz effectively reduced the development of malformed panicles. A reliable molecular method for detection of the pathogen was developed. Molecular techniques determined that a uniform population exists in Israel compared to diverse populations in Egypt, the US and elsewhere. It appears that certain cultivars, some growing in Egypt, are more resistant than others to mango malformation disease.

One of the primary aims of this research was to assure that disease-free propagation material is available to the local, small farmers in Egypt. Based on project results, a pamphlet that contained vital information on how to maintain and cultivate disease-free seedlings was produced and distributed to over 3,000 such farmers.

By virtue of the USAID-MERC funds, the Egyptian laboratory was fully equipped to conduct state-of-the-art molecular detection and population genetic studies. Training to this effect was completed in Egypt.

Joint Israeli-Egyptian work that stemmed from the research project was presented at an international conference where it received wide recognition. An audience of more than 350 researchers, extension specialists and farmers were exposed to the scientific results that were published in a proceedings article.

A peer-reviewed article co-authored by the Israeli PI and co-workers, and Egyptian PI and co-workers was published in the reputable scientific journal *Plant Pathology*, further accrediting the scientific work conducted in the project.

The research teams from Israel, Egypt and the US met at least five times within the lifetime of the project to plan, discuss, and conduct their mutual research. A concerted effort to deal with the mango malformation problem on a regional basis, with the expertise of the U.S. collaborator, promoted a very fruitful interaction. This will lead to continued efforts to solve the problem beyond the duration of this research project; future work is planned.

## 4. Research Objectives

Although research on mango malformation disease, caused by the fungal pathogen *Fusarium mangiferae*, has been conducted since the late 1800s, little progress has been made to understand the epidemiology of the disease. Furthermore, cultural, mechanical and chemical attempts to control the disease had been unsuccessful.

**The main goals of the research were:**

(1) Determine source of inoculum, survival and epidemiology of disease.

(2) Evaluate chemical control methods, pathogen interaction with mites and screening of susceptible/resistant mango varieties to the disease.

(3) Investigate the population biology and genetic diversity of the pathogen.

(4) Develop a reliable molecular diagnostic method for detection of the pathogen in infected mango tissue.

Significant gaps exist in what is known about the etiology, epidemiology and control of mango malformation. These deficiencies directly and indirectly impact the management of this disease and were, thus, a major focus of this project.

Until 1966 the etiology of disease was in dispute. With the completion of Koch's postulates it became clear that malfomation is caused by a fungus; however, confusion still existed regarding the identity of the species of *Fusarium* and whether other species were involved. The use of modern technology in the form of molecular tools and new fungicides with novel mechanisms of control were used to manage and curtail the spread of the pathogen in nurseries and on grafting scions, and expand cultivation and increase yields of mango in the region.

Mango malformation was first reported in Israel in 1968, in Egypt in 1934, and in the United States (Florida) in 1972 (Goldman, 1975; Ibrahim et al., 1975; Malo & McMillan, 1972; Pinkas and Gazit, 1992; Ploetz, 2000; Ploetz, 1994). In Egypt and in Israel, it is found in all producing regions and is the most important constraint to production in both countries (Campbell, 1982; Crane, 1997; Ibrahim et al., 1975; Knight, 1995; Manners, 1996; Pinkas and Gazit, 1992). Malformation affects seedlings and grafted plants in the nursery, and vegetative and floral apices in the field. Since fruit are not produced on affected panicles, malformation has a very serious effect on fruit production. Mangoes are grown in several Middle Eastern countries, but are most important in Egypt where it has been stated that "Mangoes are an important national crop in Egypt and a major item within the National Food Basket" (Anonymous, 1997).

The most recent monetary figures that are available for Egypt are from 1998 (Ploetz et al. 2002). During that year, 215,657 tons of mangoes were produced that had a value of more than US$150 million. Malformed panicles do not produce fruit. Thus, the proportion of panicles that are affected by malformation could be used to estimate the amount of fruit that would have been produced in the disease's absence. On this basis, fruit worth an estimated US$14.6 million was lost due to malformation only in governorates that were surveyed in 1998 (Ploetz et al. 2002).

In Israel, the south and central mango cultivation regions are heavily affected by the disease. These areas contain 20% of the total production. Until 1996 the disease had not been recorded in the northern mango cultivation region, which contains 80% of the total production. However, since 2000 many malformation

outbreaks have been recorded in the north and caused extreme concern. Estimated loss due to the disease was US$4.3 million in 2002.

In both countries, extensive mango plantings have been established over the past 5 years. Since malformation affects most of these areas, economic losses could be significantly higher than that recorded in the past.

Although the etiology of mango malformation has been confused for many years, there is unequivocal evidence that the fungus, *Fusarium mangiferae*, is the causal agent (Ploetz & Prakash, 1997; Freeman et al., 1999). Koch's postulates have been confirmed for *F. mangiferae* in Egypt, Florida, Israel, and South Africa. Another species of *Fusarium*, *F. oxysporum*, has been reported to also cause malformation in Egypt (El Khoreiby, 1997; Abdel-Sattar, personal communication). Little is known about the genetic diversity and population biology of *F. mangiferae* isolates causing malformation. To this end, various studies used vegetative compatibility groups (VCG) of the fungus (Leslie, 1995; Ploetz 1994; Ploetz et al., 1999; Shaw et al. 1993). Other studies applied RAPD and AFLP for the detection of genetic variation of this fungus (Zheng and Ploetz 2002; Freeman, Newman, U. Lavi, unpublished). In recent years, additional *Fusarium* species such as *F. sterilityphosum*, *Fusarium* sp. *nov.* and *F. proliferatum* were implicated in malformation, although their causal roles have not been reported (Marasas et al., 2006).

**Epidemiology**

Malformation is spread by grafting, and infected nursery stock is a common means by which the disease is moved to new areas. In contrast, within-tree and tree-to-tree spread in nurseries and orchards is poorly understood. Most reports indicate that the disease moves slowly in affected orchards (references in Kumar & Beniwal, 1992). Spores of *F. mangiferae* are probable infective propagules since they are the only spores that are produced by the fungus and form profusely on dead malformed tissues.

The distribution of *F. mangiferae* in affected trees suggests that vegetative and floral buds are the primary sites of infection and that systemic colonization of older, subtending tissues does not occur. Freeman et al. (1999) transformed isolates of *F. mangiferae* from mango with the GUS reporter gene (β-glucoronidase), and used them to artificially inoculate mango. Their results verified that bud and flower tissues of the host are primary infection sites, and that wounds provide points of entry for the pathogen.

Root infection by *F. mangiferae* has been reported to cause malformation, either at the root collar or in the canopy (Abdel-Sattar, 1973; Kumar & Beniwal, 1992). Unfortunately, plants in these studies were not examined prior to planting in infested soil to determine whether they were pathogen-free and, once plants were in an experiment, no precautions were taken to protect them from other sources of inoculum. Thus, it is not clear whether the root system is actually an infection court. Likewise, it is not clear whether the pathogen survives in infected flowers that have dispersed on the ground throughout the growing season or in soil. Determining the factors that influence infection and symptom development may shed light on the natural spread of the pathogen, as well as assist the development of reliable control methods.

Spread on a small scale is most clearly demonstrated in nurseries (Prakash & Srivastava, 1987). Since seed do not appear to harbor the fungus (Saeed & Schlosser, 1972; Youssef et al., 2007), seedlings should be disease-free. However, the incidence of malformation on seedlings is often high in nurseries and conflicting evidence is apparent in Egypt (Sattar, personal communication). Since seedling nurseries are

often located in affected orchards in Egypt (Ploetz et al. 2002), it is logical to assume that seedlings are infected due to their close proximity to affected trees. Exactly how this is accomplished is not known.

The mango bud mite, *Aceria mangiferae*, is often observed in high numbers on malformed trees. Although *A. mangiferae* does not cause malformation, it may be an important component of the malformation disease cycle. *A. mangiferae* feeds on epidermal cells in floral and vegetative buds of mango, and has been shown to carry *F. mangiferae* on its body (Abdel-Sattar, 1973; Manicom, 1989). Thus, contaminated mites might facilitate infection via their feeding activities (Crookes & Rijkenberg, 1985). Once relationships among *A. mangiferae*, *F. mangiferae* and mango are fully elucidated, they should provide useful insights into the epidemiology and control of malformation.

**Management and control**

Several approaches have been used to manage malformation, but most have been ineffective. The best way to avoid problems with the disease is to establish new plantings with pathogen-free nursery stock. Scion material should never be taken from an affected orchard, and any affected plants that are observed in the nursery should be removed and burned immediately. Nurseries should also not be established in orchards, especially when they are affected by malformation.

Once the disease is found in an orchard, control is possible, but time-consuming. In these cases, cultural management has been most effective (Manicom, 1989; Narisimhan, 1959; Singh et al., 1974). Affected terminals and the subtending three nodes are cut from trees, removed from the field and burned. Unfortunately, pruning to manage malformation is not practical for all producers, some of whom are unable or unwilling to devote the effort that is required to ensure that this approach succeeds. In addition, it may be difficult or impossible to impose this treatment on large trees such as those prevalent in Egypt.

A diverse array of pesticides, hormones and growth regulators has been tested for the control of malformation. These measures have been, at best, marginally effective. Singh et al. (1994) tested sulphates of cobalt, cadmium and nickel for malformation control in India. It is unlikely, though, that these compounds could be used safely on this food crop due to their human toxicity. Darvas (1987) reduced the percentage of malformed inflorescences by about one-half (from 96% to 48%) by injecting 'Keitt' trees with the fungicide fosetyl-Al. This reduction was significant ($P < 0.05$), but the increase in fruit yield, 46 to 95 kg of fruit per tree, was not. Results with other fungicidal compounds have been generally less effective (e.g., Chakrabarti & Ghosal, 1989; Diekman et al., 1982). In general, the protected, internal location of the pathogen in affected trees makes control of this disease a difficult proposition. Protectant fungicides, no matter how toxic to the fungus, would be effective only in reducing the spread of the disease, and effective systemic fungicides are limited.

Genetic resistance has been reported among different mango cultivars, but little consensus exists on which perform the best (Ploetz, 2000). Prakash & Srivistava (1987) indicated that "There is great variation in the susceptibility of existing varieties." However, controlled inoculations with reference cultures were not conducted to make these evaluations (Ploetz, 2000). Reports on cultivar resistance have been based on observations which are made in nonreplicated test orchards. Thus, cultivars listed as "resistant" may have come from healthy nursery stock or may have escaped infection once planted in the field. Alternatively, the variable response of different cultivars in different locations may have been caused by different, undefined pathotypes of *Fusarium* spp. Therefore, research on resistance to malformation and pathogenic variation in *Fusarium* spp. causing malformation is required.

## 5. Methods and Results

### a. Epidemiology

(i) Timing of sporulation and spores dispersal:
The time of release of spores of the fungal pathogen, *Fusarium mangiferae*, was determined by sampling infected panicles of different ages (young immature, partially mature, mature with open flowers) three times a month from the beginning of flowering (April) till the end of flowering (July) (Table 1).

Table 1. Spore quantification[a] from *F. mangiferae*-infected mango inflorescences according to phenological characteristics and chronology[b]

| | April | May | June |
|---|---|---|---|
| Bud break | 1,417[a] b[c] | 26,833 a | - |
| Mid stage | 1,928 c | 14,888 b | 24,055 a |
| Mature and malformed | 2,389 c | 77,550 b | 196,100 a |

[a] Spores (spores/ml) were quantified after agitating 1 g infected panicles, of different ages sampled at various periods, and diluted serially onto a *Fusarium*-selective medium. In control healthy panicles from healthy trees, no pathogen spores were detected.
[b] Flowers at bud break (inflorescences of 1 to 3 cm long), mid stage of flowering (inflorescences of 5 to 8 cm long) and mature malformed flowers (inflorescences of 10 to 15 cm long) were sampled at three periods: April, May and June. Six replicate inflorescences of 1 g weight each per age and period were agitated for 30 min in a rotary shaker at 120 rpm.
[c] Spore quantification per phenological stage at different periods followed by a common letter are not significantly different ($P<0.05$) according to Duncan's Multiple Range test.

**As can be seen, the time of release of spores show that in time and with maturity, inoculum density increases. The peak of inoculum production was found in mature malformed panicles later on in the season.**

(ii) Infection of bark, fruit and seed.

Bark:
Since the pathogen may survive on external plant tissue, pieces of bark were sampled from different infected orchards in different governorates in Egypt (Nubaria, Kalubia, Giza and Fayoum regions) during July, August, September, October and November, the period when infected panicles have and are dispersing inoculum. Tissues were cut from the chosen parts, washed thoroughly in tap water, the surface sterilized followed by successive washings in sterile distilled water and dried on sterilized filter paper. Stock of Petri-dishes containing PDA were plated with surface sterilized pieces of mango barks. Pure culture of each isolate was obtained (Table 2).

Table 2. Percentage of fungi isolated from mango barks from different localities in Ismailia, Sharkia, Nubaria, Kalubia, Giza and Fayoum Governorates from July to November 2003.

| Governorate | % of *Fusarium* spp isolated from July to November, 2003 | | | | | Mean |
|---|---|---|---|---|---|---|
| | July | August | September | October | November | |
| Ismailia | | | | | | |
| El-Dabia | 0 | 0 | 4 | 5 | 2 | 2.2 |
| Abo-Khalifa | 0 | 2 | 3 | 2 | 1 | 1.6 |
| Sharkia | | | | | | |
| El-Sowwah | 2 | 0 | 1 | 3 | 0 | 1.2 |
| El-Sanagra | 1 | 3 | 4 | 2 | 3 | 2.6 |
| Nubaria | 0 | 1 | 2 | 3 | 2 | 1.6 |
| Kalubia | | | | | | |
| El-Kanater | 1 | 2 | 3 | 3 | 2 | 2.2 |
| Tokh | 2 | 3 | 2 | 2 | 1 | 2.0 |
| Giza | | | | | | |
| Kerdasa | 0 | 1 | 2 | 2 | 1 | 1.2 |
| El-Mansoria | 1 | 2 | 2 | 3 | 1 | 1.8 |
| Abo-Rawash | 1 | 1 | 1 | 3 | 2 | 1.6 |
| Nekla | 0 | 2 | 2 | 2 | 0 | 1.2 |
| Kafr | 1 | 1 | 2 | 1 | 0 | 1.0 |
| Hakeem | 2 | 1 | 2 | 3 | 1 | 1.8 |
| Fayoum | | | | | | |
| Senoures | 0 | 0 | 1 | 2 | 0 | 0.6 |
| Sanhour | 0 | 0 | 2 | 2 | 0 | 0.8 |
| Mean | 0.73 | 1.26 | 2.2 | 2.5 | 1.06 | |

Accompanying fungi: Species of *Lasiodipoldia*, *Cladosporium*, *Alternaria*, *Pestalotia*, *Nigrospora*, *Epicoccim*, *Helminthosporium* and *Aspergillus*.

Data presented in Table 2 indicated that isolation from mango barks yielded several fungi i.e., *Fusarium* spp. *Lasiodipoldia* sp., *Cladosporium* sp., *Alternaria* spp., *Pestalotia* spp, *Nigrospora* sp., *Epicoccim* sp., *Helminthosporium* sp. and *Aspergillus* sp. It is also clear that the mean percentage of *Fusarium* spp. compared to other isolated fungi reached 0.73 and 1.26 during July and August, respectively, and increased to 2.2 and 2.5 during September and October, respectively, but decreased to 1.06 during November. *Fusarium* spp. were detected in most inspected mango barks.

**Due to the very low incidence of detection on the bark and unclear identity of these isolates, it does not appear that bark can be considered as an inoculum source for the pathogen, especially when comparing to the massive amounts of spores that are released from affected panicles.**

Fruit and seed:

Forty fruitlets and 40 flowers, 20 healthy and 20 diseased, respectively, were collected from trees (cv. Keitt, Volcani Center, Israel) to determine pathogen presence. The pathogen was quantified from fruit originating from healthy and diseased trees. Half of each sample was surface sterilized and all were plated on a *Fusarium*-selective medium. All the fruit were sliced in half and the seeds (including seed coat) were removed. Each seed, seed coat and flesh sample was cut into five random pieces before plating. Half the seeds, seed coats and flesh (1 cm pieces) from both healthy and diseased trees were placed directly of the *Fusarium*-selective medium, while the other half was surface sterilized before plating. Samples of the fungal culture growing from the different tissues were verified as *F. mangiferae* by the

polymerase chain reaction (see below). The experiments were conducted twice in two consecutive growing seasons of 2002 and 2003 and once again in 2004. Similar results were observed for all experiments therefore one representative experiment is presented (Table 3).

All flowers and fruitlets from diseased panicles were 100% infected, whether surface disinfested or not, whereas *F. mangiferae* was not detected in comparable flowers and fruitlets from healthy trees (data not shown). In 2- and 3-month-old fruit from diseased trees, viable spores were quantified from the fruit's surface, with a greater concentration on younger fruit surface compared to that on mature fruit, whereas the pathogen was not detected on fruit from healthy trees (Table 3). No *Fusaria* were detected from healthy fruits. Only *F. mangiferae* colonies, verified by specific-PCR, were isolated from fruit from the infested orchard.

Table 3. Detection of *Fusarium mangiferae* colony forming units (CFU) on mango fruits from diseased and healthy trees, 2 and 3 months after fruit set in Israel

| | Fruit age (months) | Average weight (g) | CFU/g fruit |
|---|---|---|---|
| Diseased | 2 | 320 | 61.5 ± 2.8 |
| Diseased | 3 | 460 | 27.4 ± 4.5 |
| Healthy | 2 | 340 | 0 |
| Healthy | 3 | 490 | 0 |

[a]Twenty-five fruit from diseased and healthy trees of each age were washed and the supernatant was centrifuged and plated on *Fusarium*-selective medium for CFU enumeration.

The fruit from diseased and healthy trees were sectioned to determine presence of the pathogen in seed and on seed coats. The pathogen was not detected within the seed or on seed coats of fruit from diseased trees or from healthy trees. Likewise, the pathogen was not detected in the flesh of any of the tested diseased fruit or from healthy ones (data not shown).

**From the data in Table 3, the pathogen may be present on the fruit exterior in Israel but is not seedborne or found within fruit.**

In Egypt, apparent healthy mango fruit of 'Company' (susceptible), 'Misk' (susceptible) and 'Jolic' (resistant) were collected from orchards in EL- Sharkia Governorate. Longitudinal sectioning was carried out to determine the internal rot of the embryo tissues. Percentage of discoloration in embryo tissues was recorded (Table 4).

Table 4. Fungi associated with internal rot of embryo tissues of apparent healthy mango fruits of three cultivars differing in their susceptibility to malformation.

| Cultivar | Reaction to malformation | Associated fungi | % of embryo rot |
|---|---|---|---|
| Company | Highly susceptible | *Fusarium* spp.<br>*Alternaria* spp. | 45% |
| Misk | Susceptible | *Lasiodiplodia* spp.<br>*Fusarium* spp. | 25% |
| Golic | Resistant | Not detected | 0% |

It appears that the highly susceptible 'Company' showed 45% embryo rot followed by 'Misk', which showed 25% infection. The *Fusarium* spp. isolates were verified as *F. mangiferae* according to specific PCR amplification. No embryo infection was observed in 'Golic.'

**The results in Egypt contrast with those in Israel, where no internal infection of fruit and seeds was determined. In future research this subject will need to be clarified.**

(iii) Isolation of the pathogen from different growth tissues

In each of the fifteen branches selected, the causal agent was isolated and identified but without sequential consistency. The causal agent was detected in all infected branches, regardless of age, but the branch sections closer to the infected panicles were more heavily infected (Table 5). Molecular identification by PCR of 122 samples of *Fusarium* cultures, growing from sections plated on *Fusarium*-selective medium, verified that in 93% of the cases *F. mangiferae* was detected.

Table 5. Infection (%) of branch sections of various ages cut from a heavily infected orchard

| Age (years) | Infection (%)[a] |
|---|---|
| 0 (malformed panicles) | 100.0 |
| 1 | 37.3 |
| 2 | 21.3 |
| 3 | 12.0 |
| 4 | 18.7 |
| 5 | 9.3 |
| 6 | 10.7 |
| 7 | 16.0 |
| LSD[b] | 8.7 |

[a]Average % infection of segmented samples according to age, consisting of five replicate pieces, per age, per tree (total fifteen), plated on a *Fusarium*-selective medium after surface disinfestation.
[b]Least significant difference (LSD), Tukey-Kramer multiple comparison test ($P$ 0.05).

**The pathogen remained viable in various parts of the trees for up to 7 years, indicating that it survives for long periods in woody portions of the tree where lateral buds were present. Similar results were obtained from experiments conducted in Egypt (data not shown).**

(iv) Transfection via grafting

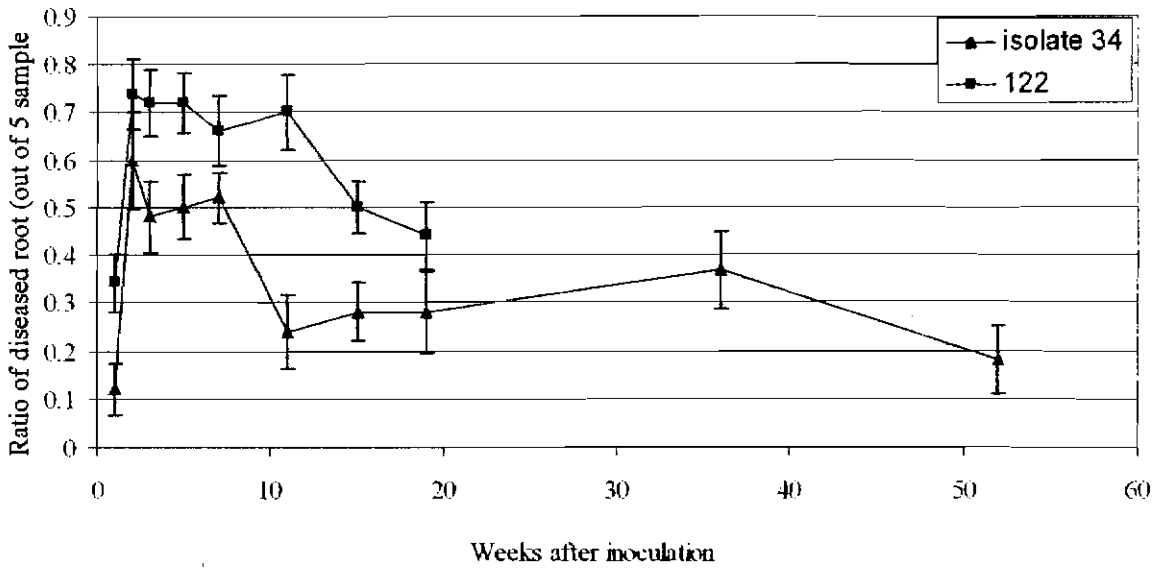
In all of 15 saplings that were used in the graft experiment, the pathogen was isolated above the graft union but not in consistent sequence. In the region below the graft the fungus was found in only 4.3% of the segments, from three out of 15 saplings. Molecular tests by PCR verified identification of the pathogen.

**According to the grafting experiments, movement of the pathogen was mostly acropetal, but isolation of the pathogen in areas below the graft was also detected in a small percentage indicating possible basipetal growth from an infected scion into the rootstock. Similar results were obtained from experiments conducted in Egypt (data not shown).**

(v) Root infections and survival of the pathogen in soil and panicles

Root infections and survival of *F. mangiferae* in seedling roots
Seedlings were inoculated with 100 ml of a final concentration of $10^5$ spores/g soil (isolates 122 and 34). Five plants were inoculated with each isolate and maintained at 25°C for 1 year. Periodically, 5 roots from each plant were sampled and plated to determine percent infection (Fig. 1).

Figure 1




**An immediate increase in colonization was detected in the inoculated roots but in time a gradual decline was observed. In no case did symptoms develop aboveground.**

Survival of *F. mangiferae* in soil under various conditions
Natural soil (92% sand, 8% clay, pH 7.0) was collected from a nearby mango orchard in the Volcani Center. Spore water suspensions of two isolates of *F. mangiferae* were mixed with the soil to a final concentration of $10^5$ spores/g soil (22% moisture content). Infested soil was placed into three replicate flasks, and 1 g soil was sampled every 1 or 2 weeks and plated in serial dilutions to determine surviving colony-forming units (CFU). Nine replicate plates, three per flask representing bulked samples, were assessed per isolate at each period. Survival experiments with isolates 506/2 and 135A, conducted in the winter and summer months, respectively, at 14°C and 33°C average respective soil temperatures, were performed outdoors and compared to controlled conditions at 25°C.

All survival experiments were conducted at least three times with similar results and one of these experiments is presented in Table 6. During the summer months under outdoor conditions, a very rapid decline of isolates 506/2 and 135A was recorded compared with that under winter and controlled conditions (Table 6). During the summer months, isolates 506/2 and 135A declined to 50% of their original levels after 1.6 weeks, as compared to 7.9 and 18.4, and 14.7 and 15.4 weeks, for the respective isolates under respective controlled and winter conditions (Table 6).

Table 6. Survival, expressed in number of weeks needed to recover 50% of the original population of culture-produced spores of isolates 506/2 and 135A of *Fusarium mangiferae* in field soil, under controlled, summer and winter outdoor conditions[a]

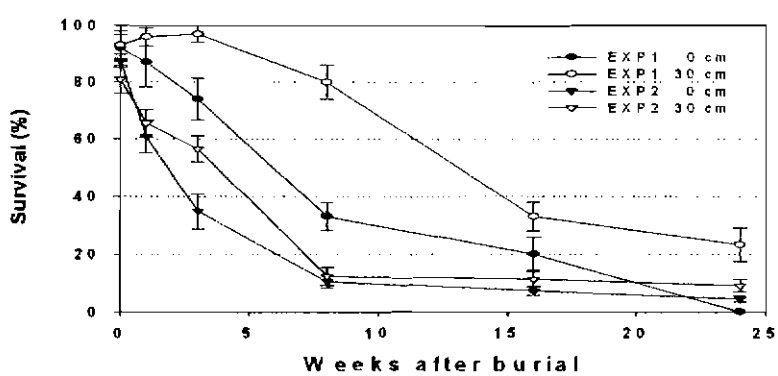
| Isolates | Conditions | Regression equation | $R^2$ | Survival[b] (weeks) |
|---|---|---|---|---|
| 506/2 | Controlled | y = -0.14x + 5 | 0.99 | 17.9 a |
| 506/2 | Winter | y = -0.15x + 4.7 | 0.92 | 14.7 a |
| 506/2 | Summer | y = -1.34x + 4.7 | 0.96 | 1.6 b |
| 135A | Controlled | y = -0.13x + 4.5 | 0.81 | 18.4 a |
| 135A | Winter | y = -0.14x + 4.7 | 0.92 | 15.4 a |
| 135A | Summer | y = -1. 4x + 4.8 | 0.93 | 1.6 b |

[a] Spores were mixed in soil and survival was monitored periodically under controlled (25°C) or average winter and summer months (respective 14°C and 33°C average soil temperatures) conditions.
[b] The survival percentages were calculated from the survival vs. time curves, according to the regression equations for each isolate under the different incubation conditions and the $R^2$ values ranging between 0.81 and 0.99, were significant ($P<0.05$). Columns with a common letter are not significantly different ($P<0.05$), according to paired t-tests between populations.

Survival of *F. mangiferae* in infected panicles

Affected panicles ('Keitt' in Israel and 'Sucharri' in Egypt) were collected from orchards in the Volcani Center, Israel and in Ismailia Governorate, Egypt. Experiments were conducted in the same orchards between May and October, when inoculum from panicles could be dispersed by wind. Panicles were placed between trees in nylon bags, either buried to a depth of 30 cm or left on the surface, or sampled periodically over 180 days to determine viability of the pathogen. Each of three replicate panicles were retrieved from soil or the soil surface, washed under running tap water, surface-sterilized, divided into 10 parts, and plated on a *Fusarium*-specific medium. Percent survival was determined by calculating the number of infected stalks that yielded *F. mangiferae* compared to infection at zero time. The experiment was conducted twice, once in each country. Temperatures on the soil surface and at 30 cm depth, from May to October, averaged 39 ± 7°C and 24 ± 3°C, respectively. Results of both experiments are presented. Pathogen populations in the infected panicles declined more rapidly on the soil surface than in soil in both locations (Fig. 2). In general, survival of populations at both depths in Israel (experiment 1) declined slower than those in Egypt (experiment 2). After 8 weeks, survival of populations in Egypt had declined to approximately 15% whereas those in Israel declined to 80 and 35% at the respective 30 cm and 0 cm depths (Fig. 2). After 24 weeks, populations of the pathogen in panicles on the soil surface declined to negligible levels in both locations, whereas a 20% survival rate was evident in soil in Israel.

Figure 2



Systemic versus local infections

One hundred 1.5-year-old infected, vegetatively malformed 'Sucharri' mango seedlings growing directly under affected 'Sucharri' mature trees (cv. Sucharri) and 40 affected panicles from these trees were sampled from four different plots (25 seedlings and 10 panicles from each plot) in two locations each in Ismailia and Sharkia govenerates in Egypt, in April 2004, to determine whether infection in seedlings was systemic (originated from the seed and soil and evenly distributed within plant tissue) or whether the pathogen was airborne and originated from malformed panicles overhead. Seedlings were propagated in secluded nurseries and replanted under infected mature trees, 9 months after germination. Six months later, all the sampled seedlings were sectioned into six parts each, 10 cm in length (apical infected tissue, 20, 30, 40, 50 cm from top, and root sections). One cm pieces of each infected panicle and each 10 cm portions of the seedlings were further subsectioned into 10 pieces (approximately 1 cm per piece) and then plated on the *Fusarium*-specific medium to determine presence of the pathogen or other *Fusarium* species. Subsequently, samples of all the fungal growth from the different tissues were verified by species-specific PCR for the presence of *F. mangiferae*.

DNA samples, extracted from the fungal cultures that grew from the infected panicles and seedling subsections, were amplified with species-specific primers for *F. mangiferae* to verify presence of *F. mangiferae*. Presence of the pathogen was detected by a 608 base pair fragment (Fig. 3) in 100% of the malformed panicles originating from the mature trees, in 97% of seedling apical meristem section, and in sections 20, 30, 40, 50 cm from top and roots at levels of 30, 17, 4, 5 and 5%, respectively (Fig. 4).

Figure 3

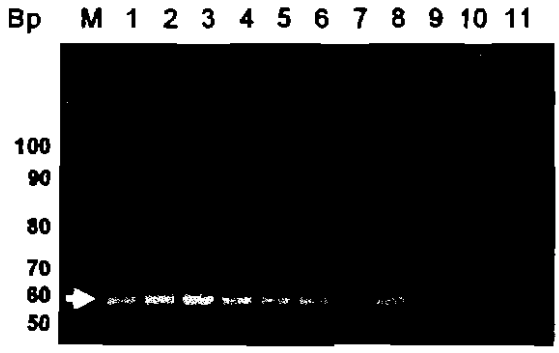
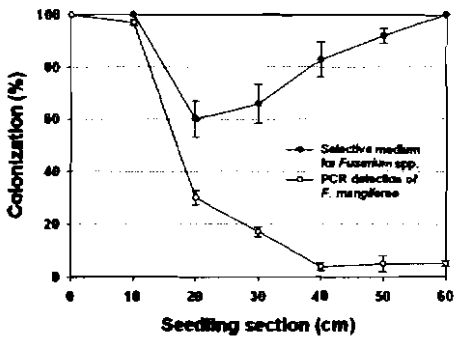



Figure 4




**In summary, it appears that negligible levels of inoculum survive in soil, both as naked spores and in diseased panicles. Since plants did not show symptoms after root infection, soilborne inoculum does not appear to induce foliar symptoms of the disease. In Egypt and Israel, utilization of molecular markers determined that inoculum of the pathogen is airborne with infections in seedlings descending from top to lower stem sections and roots. Although minor infections of roots may occur from inoculum that originates from infected panicles, the pathogen is not systemic.**

vi) Determine whether *bona fide* isolates of *F. oxysporum* can cause malformation after soil and foliar inoculation of the host. Since isolates of *F. oxysporum* have not been recovered from malformed mango trees in Florida and Israel, only isolates from Egypt were to be studied.

**All inoculations with these *bona fide Fusarium* isolates did not yield disease symptoms after seedling inoculation indicating that only *F. mangiferae* is the causal agent of disease in the Middle East region.**

vii) Role of *Aceria mangiferae* in vectoring *F. mangiferae*.
The role that the mango bud mite, *A. mangiferae*, plays in moving *F. mangiferae* within and among seedlings (Stemlicht, and Goldenberg, 1976) was investigated using a pathogenic isolate of the fungus that is marked with a green fluorescent protein (gfp). Mites collected from infested buds in the orchard were exposed to the gfp-marked *F. mangiferae* isolate and, after 24 hours, were removed and mounted for microscopic observation; gfp-marked spores were observed on the mites (Fig. 5).

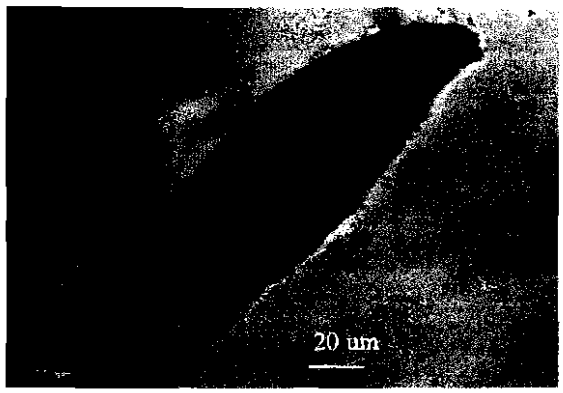



Figure 5. Gfp-marked spores were observed on mites that were placed on agar plugs bearing the marked strain

**Spores of the pathogen did not attach to any particular part of the mite's body, but the mite was clearly capable of bearing these propagules of the pathogen.**

A vectoring experiment was performed on potted mango plants, in a controlled environment growth chamber with 25C°±2 C° and 14:10 L:D. The plants were fumigated twice with dichlorvos 2 weeks before the beginning of the experiment to ensure that they were void of mites or insects. The base of the stem was ringed with a sticky barrier to prevent infestation by ambulant arthropods. Each plant was placed in a disinfested plastic cage and was treated with one of the following four treatments: treatment 1- 100 mites were placed on two $5mm^2$ agar plugs with the gfp-marked isolate. The agar plugs bearing bud mites and the gfp-marked pathogen were then placed on a leaf, approximately 5cm away from an apical bud; treatment 2- 100 mites were placed on two $5mm^2$ agar plugs without the fungus and then placed near an apical bud as described above; treatment 3- two $5mm^2$ agar plugs with the gfp-marked isolate were placed near an apical bud; treatment 4- untreated control. Four apical buds were inoculated in each treatment and the experiment was repeated 5 times. Two days following inoculation the apical buds were inspected under a stereomicroscope and the bud mites were counted. Then, the gfp-marked spores (if present) were washed from the bud bracts and plated on a selective media amended with 50 µg/ml hygromycin. After 5 days, the gfp-marked colonies were enumerated on the plates.
Bud mites were found in 25% of the inoculated buds of treatment 1 (inoculation with bud mite and with gfp-marked spores) and 35% of those in treatment 2 (inoculation with bud mites), showing clearly that the bud mites could orientate themselves from the adjacent leaves to the apical bud. Bud mites were not found in treatments 3 (inoculation with gfp-marked spores) and 4 (untreated control) which confirms that the plants used in these experiments were not infested prior to the initiation of the experiment. The gfp-marked spores were found in bracts of 25% of the inoculated buds in treatment 1 but not in buds from other treatments.

**To conclude, the mango bud mite can carry spores of *F. mangiferae* on its body and transfer them to the infection court of apical buds of the mango host.**

**b. Control**

(i) Investigate controlling malformation with combinations of miticides and different protectant and systemic fungicides.

**From previous and current work it was shown that the bud mite does not cause malformation, and that *F. mangifeare* can cause the disease when the mite is not present. Therefore, chemical control experiments focused on the fungus, not the mite.**

Chemical control of the disease

New fungicides were assessed for their effectiveness in *in vitro* followed by *in vivo* experiments. Numerous experiments were conducted to screen fungicides for the inhibition of *F. mangiferae in vitro*. The most effective were: prochloraz ($ED_{50}$ (50% pathogen mortality) = 0.01 µg/ml); signum (0.7 µg/ml) and famoxadone (1.5 µg/ml). Since prochloraz was most effective, infusions and sprays of this fungicide were assessed for disease control in affected orchards. Two types of applications were tested for impact on the numbers of malformed panicles and yield. In Ein Hachoresh, Sharon coastal region, infusions of 3 L per tree were made three times (summer, winter and spring before flowering) (Table 7). In the Volcani orchard, sprays (250 ppm a.i.) were applied at various times before flowering (Table 8).

Table 7. Control of malformation by prochloraz infusion of infected trees (En Hachoresh, Sharon, 2003)

| Treatment | No. malformed panicles/tree | | | | |
|---|---|---|---|---|---|
| Date | 10/4 | 22/05 | 23/07 | Totals | Yield (fruit/tree) |
| Control | 9.8a | 13.2a | 1.3a | 24.2a | 50.6a |
| Pruning | 9.1a | 14.0a | 2.0a | 25.1a | 50.8a |
| Prochloraz infusion | 10.8a | 14.7a | 1.8a | 27.3a | 52.5a |

Table 8. Prochloraz field experiment #2 for control of malformation (Volcani orchard)

| Treatment | Total panicles | Malformed panicles | % infection |
|---|---|---|---|
| Control | 292 | 51 | 17.5 a |
| Prochloraz spray | 437 | 30 | 6.8 b |

Columns with % infection having a different letter are significantly different ($P<0.05$), according to paired t-tests between treatments.

**No significant reduction in disease was evident due to prochloraz infusion. It appears that the fungicide does not move within the plant, but is adsorbed at the application site in the tree trunk. However, spray treatments appeared to reduce disease incidence.**

Additional experiments with sprays and continuous drip irrigation of this fungicide were conducted for assessing the potential for control in affected orchards. Sprays (250 ppm a.i.) and drip (500 ppm a.i.) at various times before flowering (Tables 9 to 14) were applied to assess impact on the numbers of malformed panicles that developed relative to nontreated controls.

Table 9. Prochloraz field exp. #3 (Cholit, Negev) for control of malformation on 'Keitt' (2005)

| Treatment | Total panicles (av/tree) | Malformed panicles (av/tree) | Infection (%) |
|---|---|---|---|
| Control | 66.0 | 16.0 | 16.7 a |
| Prochloraz (Early sprays) | 70.6 | 7.8 | 11.0 a |
| Prochloraz (Late sprays) | 74.0 | 8.0 | 10.8 a |
| Prochloraz (Early & late sprays) | 73.0 | 8.7 | 11.9 a |

In each treatment, 12 trees were included - three per block in 4 blocks. Average panicles per tree were recorded (Early sprays = 6 sprays: 1/6,14/6, 28/6, 13/7, 27/7, 10/8; Late spray = 6 sprays: 13/1, 26/1, 10/2, 24/2, 13/3, 24/3; Early and later sprays: combination of the above, 12 sprays). Columns with % infection having the same letter are not significantly different ($P<0.05$).

Table 10. Prochloraz field exp. #4 (Cholit, Negev) to control malformation on 'Tommy Atkins' (2005)

| Treatment | Total panicles (av/tree) | Malformed panicles (av/tree) | Infection (%) |
|---|---|---|---|
| Control | 74.8 | 17.4 | 23.3 a |
| Prochloraz (Early sprays) | 40.0 | 10.8 | 26.8 a |
| Prochloraz (Late sprays) | 51.2 | 12.7 | 24.8 a |
| Prochloraz (Early & late sprays) | 68.6 | 11.5 | 16.8 ab |

Each treatment contained 12 trees - three per block in 4 blocks. Average panicles per tree were recorded

In each treatment, 12 trees were included - three per block in 4 blocks. Average panicles per tree were recorded (Early sprays = 6 sprays: 1/6,14/6, 28/6, 13/7, 27/7, 10/8; Late spray = 6 sprays: 13/1, 26/1, 10/2, 24/2, 13/3, 24/3; Early and later sprays: combination of the above, 12 sprays). Columns with % infection having the same letter are not significantly different ($P<0.05$).

Table 11. Prochloraz field exp. #5 (Mishmar Hasharon, Sharon) to control malformation on 'Tommy Atkins' (2005)

| Treatment | Total panicles (av/tree) | Malformed panicles | Infection (%) |
|---|---|---|---|
| Control | 195.5 | 53.6 | 27.4 ab |
| Prochloraz spray | 243.5 | 56.7 | 23.3 a |
| Prochloraz drip | 193.3 | 63.4 | 32.8 b |

Each treatment contained 42 trees, 7 trees per block, total of 6 blocks. Average panicles per tree were recorded. Prochloraz spray and drip treatments were as follows: Sprays conducted: 25/11; 3/12; 12/12; 20/12; 27/12; 10/1; 23/1; 16/2; 23;2; 7/3. Drip of 1 L 80 ppm/tree conducted: 14/12; 2/1; 20/1; 9/2; 27/2; 16/3; 5/4; 20/4. Columns with % infection having the same letter are not significantly different ($P<0.05$).

Table 12. Prochloraz field exp. #6 (Volcani) for control of malformation (2005)

| Treatment | Total panicles (av/tree) | Malformed panicles (av/tree) | Infection (%) |
|---|---|---|---|
| Control | 151.3 | 9.2 | 6.1 b |
| Prochloraz spray | 145.7 | 3.1 | 2.1 a |
| Prochloraz drip | 186.8 | 7.2 | 3.9 a |

Table 13. Prochloraz field exp. #7 (Mishmar Hasharon, Sharon) for control of malformation on 'Tommy Atkins' (2006)

| Treatment | Total panicles (av/tree) | Malformed panicles | Infection (%) |
|---|---|---|---|
| Control | 3834 | 2130 | 55.6 a |
| Prochloraz spray | 1190 | 595 | 50.0 a |
| Prochloraz drip | 3565 | 1364 | 38.3 b |

Each treatment contained 42 trees, 7 trees per block, total of 6 blocks. Average panicles per tree were recorded. Prochloraz spray and drip treatments were as follows: Sprays conducted every 2 weeks; Drip of 1 L 80 ppm/tree conducted continuously. Columns with % infection having the same letter are not significantly different ($P<0.05$).

Table 14. Prochloraz field exp. #8 (Cholit, Negev) for control of malformation (2007).

| Treatment | Malformed panicles (% of total) | |
|---|---|---|
| | Tommy cv. | Keitt cv. |
| Control | 19.6 a | 26.3 a |
| Prochloraz spray | 13.4 b | 19.3 b |
| Reduction (%) | 31.7 | 26.4 |
| Significance | $P$=0.0013 | $P$=0.02 |

Columns with % infection having the same letter are not significantly different ($P<0.05$).

**In summary, prochloraz, applied as a spray or drip, has the potential to reduce disease in the field, but must be applied at the correct time. Critical periods when infection occurs need to be determined, as these could focus application schedules.**

(ii) Determine whether *A. mangiferae* facilitates infection and disease development, and if its populations fluctuate during different seasons or phenologic stages of the host.

Facilitating fungal penetration - quantitative evaluation

The experiment was performed on potted plants in a controlled environmental chamber. Each plant was placed in a disinfested plastic cage and 40 apical buds were inoculated with a $10^6$ gfp-marked spore suspension and either amended or not amended with bud mites that were collected from an orchard (50 mites per bud). Three weeks post-inoculation apical buds were separated into bracts, surface-sterilized and placed on *Fusarium*-selective media. Fungal growth was evaluated after 5 days and two parameters were measured: 1. Frequency of infected buds calculated by the percentage of infected buds in the treatment using a Pearson statistical test to compare the two treatments ($P<0.05$); 2. Severity of infection by calculating the average number of infected bracts per bud and the means of the two treatments were compared using Tukey-Kramer analysis ($P<0.05$).

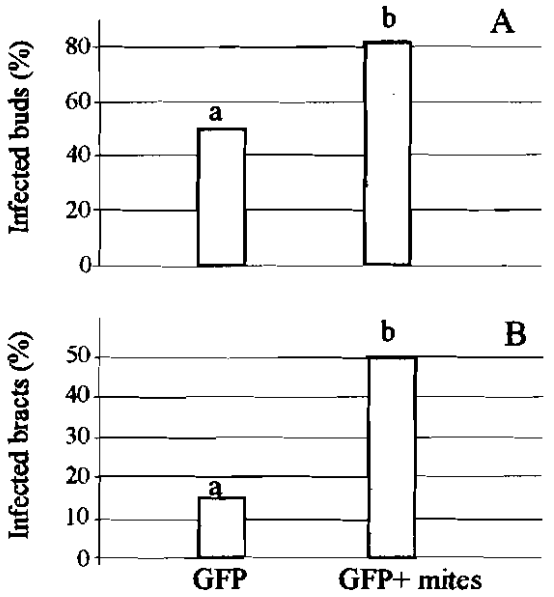
Significantly more apical buds (Fig 6A) and bracts (Fig 6B) were infected when mites were added after inoculation with the gfp-marked strain of *F. mangiferae*.

***Aceria mangiferae* enhances penetration of mango bud tissue by *F. mangiferae*, perhaps through wounds it creates on the bud bracts while feeding. However, the pathogen is capable of penetrating and colonizing tissue without assistance from the mite.**

Aerial dissemination of spores

Fungal spores were trapped in the Volcani mango orchard using two methods: 1. A Burkard™ volumetric spore trap, which sucked air at 10 liters per minute over adhesive-coated transparent plastic tape, for periods of 7 days. The adhesive tape was then washed and mounted on the *Fusarium*-

Figure 6

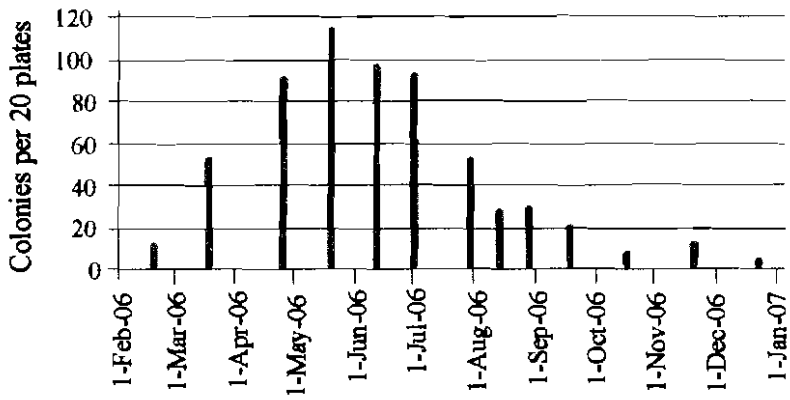



selective medium to enumerate fungal colonies. 2. Petri dishes of the selective medium were opened and exposed to the orchard's environment overnight, then brought back to incubate in the lab and examined for the presence of *F. mangiferae* colonies.

To monitor wind-blown mites in the orchard, a free-rotating wind trap, made of a 9-cm-diameter PVC pipe, was mounted on a pole attached to a wind vane. The pipe's floor was covered with 70 sticky slides that were replaced monthly, then inspected under a stereo-microscope for the presence of mites and placed on the selective medium for pathogen detection.

Spores of *F. mangiferae* were trapped using both methods. An annual peak of dissemination was detected with method 2 in the spring/early summer months (Fig. 7). Similar results were obtained with the Burkard™ trap where higher numbers of spores were caught early in the summer months (May/June) and declined towards the end of the summer. *A. mangiferae* was trapped throughout the season, but *F. mangiferae* was not detected when the mites were placed on the selective medium.

Figure 7

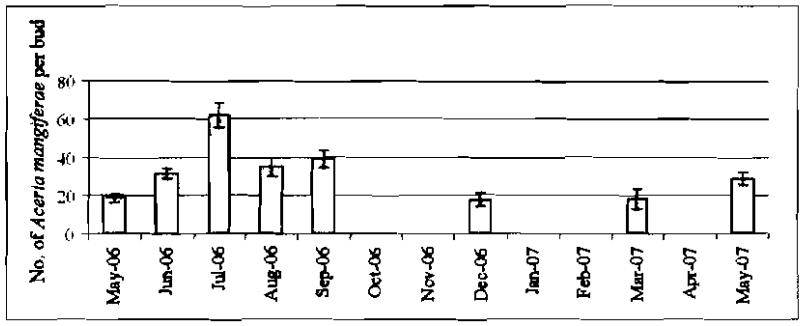



**Spores of *F. mangiferae* can reach mango buds independent of the bud mite. Thus, the mite may not play an essential role in disseminating the pathogen among trees.**

Fluctuations of bud mite populations during the growing season

Mites were counted in closed buds that were collected during the growing season. There appeared to be an increase in populations in the warmer months. Each bar in Fig. 8 represents an average of 60 apical buds.

Figure 8




**In summary, *A. mangiferae* can carry spores of *F. mangiferae* on its body and move them into apical buds, which are important infection courts for the pathogen. The mite also increases infection of buds and bud bracts by the pathogen via its feeding wounds. Finally, since the fungus was not recovered from wind-blown mites it does not appear that they play a significant role in aerial dissemination of spores. Mites are present most of the year but in higher numbers during hotter months. These results support previous speculations on the involvement of *A. mangiferae* in the epidemiology of mango malformation (Ploetz, 2001).**

(iii) Resistance/susceptibility of mango genotypes

To assess the resistance/susceptibility of different mango genotypes to malformation, several experiments were conducted with single-spored isolates of *F. mangiferae* and grafted plants. Inoculations utilized previously described protocols (Freeman et al., 1999). A pronounced relationship

was observed between the length of time the inoculated apical bud remained dormant after inoculation and whether, and the extent to which, disease developed (Figure 9 and data not shown). Isolates varied on virulence (Table15a and data not shown), and latency (the time between inoculation and symptom development) ranged from 40 to >200 days, further complicating this objective. Overall, there were pronounced differences in disease development on different Egyptian and Israeli cultivars (Table 15b). In agreement with anecdotal reports, little malformation developed on the Egyptian cultivar 'Zebda' in these experiments. Reasons for variable reports on the susceptibility of 'Ewais' (its susceptibility ranged from low to high) were not clear; in these experiments it was moderately susceptible (Table 15b). In contrast to previous reports, embryony was not related to disease reaction (Table 15b).

Figure 9

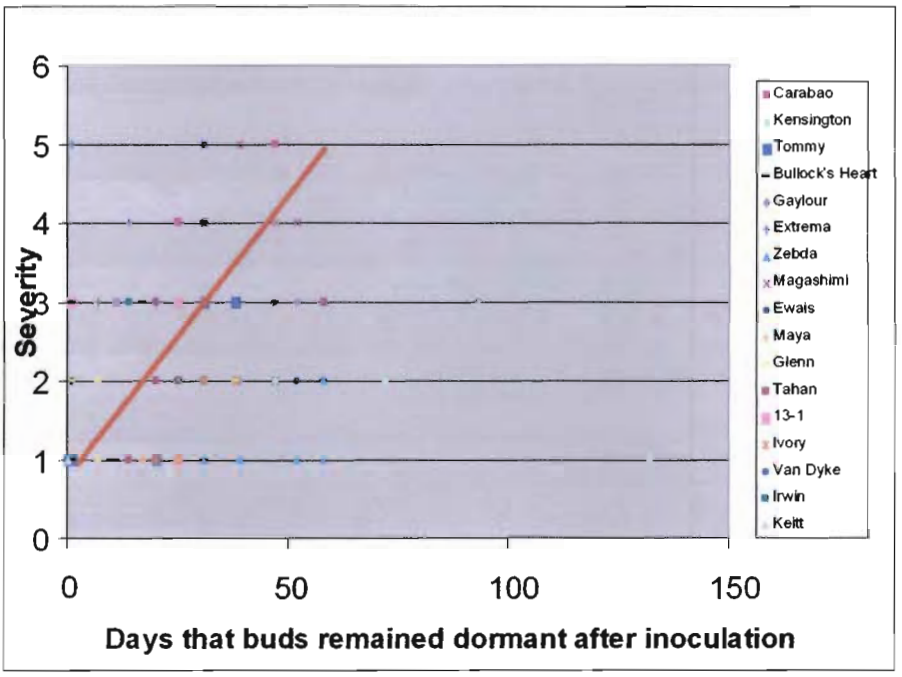


Table 15a. Malformation development on Florida mango cultivars 192 days after inoculation[a]

| | | Inc[b] | Sev[c] | | Inc[b] | Sev[c] | | Inc[b] | Sev[c] | Cultivar means | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Cultivar | Isolate | (0, 1) | (1-5) | Isolate | (0, 1) | (1-5) | Isolate | (0, 1) | (1-5) | Inc[b] | Sev[c] |
| Glenn | Cg2-5 | 1 | 2.5 | Cg2-14 | 0.5 | 2 | FS5 | 0 | 1 | 0.5 b | 1.8 b |
| Haden | Cg2-5 | 0.5 | 1.5 | Cg2-14 | 1 | 3 | FS5 | 0.5 | 1.5 | 0.7 ab | 2 b |
| Irwin | Cg2-5 | 0.5 | 2 | Cg2-14 | 1 | 2.5 | FS5 | 0.5 | 1.5 | 0.7 ab | 2 b |
| Keitt | Cg2-5 | 1 | 4 | Cg2-14 | 1 | 2.5 | FS5 | 0.5 | 1.5 | 0.8 ab | 2.7 a |
| Tommy Atkins | Cg2-5 | 1 | 3.5 | Cg2-14 | 1 | 3.5 | FS5 | 1 | 2 | 1 a | 3.2 a |
| Van Dyke | Cg2-5 | 1 | 2 | Cg2-14 | 1 | 4 | FS5 | 1 | 2 | 1 a | 2.8 a |
| Zill | Cg2-5 | 0.5 | 2 | Cg2-14 | 1 | 4 | FS5 | 1 | 2 | 0.8 ab | 2.2 ab |
| Isolate means | | 0.8 ns | 2.4 a | | 0.9 ns | 3 a | | 0.6 ns | 1.5 b | | |

Table 15b. Malformation development on mono- and polyembryonic mango cultivars from Egypt, Israel and elsewhere[a]

| Cultivar | Embryony | Origin | Published susceptibility | Incidence[b] | Severity[c] | n |
|---|---|---|---|---|---|---|
| 13-1 | polyembryonic | Israel | n/a | 0.8 | 2.6 ab | 5 |
| Bullock's Heart | polyembryonic | Egypt | moderate | 0.6 | 1.9 b | 18 |
| Carabao | polyembryonic | Philippines | n/a | 0.5 | 1.8 b | 12 |
| Ewais | polyembryonic | Egypt | low – high | 0.9 | 2.5 ab | 8 |
| Extrema | polyembryonic | Brazil | n/a | 0.8 | 2.8 a | 9 |
| Gylour | polyembryonic | Egypt | high | 0.8 | 2.0 ab | 12 |
| Ivory | polyembryonic | Thailand | n/a | 0.3 | 1.3 b | 7 |
| Kensington | polyembryonic | Australia | n/a | 0.5 | 1.8 b | 10 |
| Zebda | polyembryonic | Egypt | low | 0.4 | 1.5 b | 12 |
| | | | means: | 0.69 | 1.97 | 94 |
| Glenn | monoembryonic | Florida | n/a | 0.5 | 1.6 b | 42 |
| Magshimim | monoembryonic | Israel | n/a | 0.9 | 3.6 a | 9 |
| Maya | monoembryonic | Israel | n/a | 0.8 | 2.0 ab | 6 |
| Tahar | monoembryonic | Israel | n/a | 0.8 | 2.1 ab | 11 |
| Tommy Atkins | monoembryonic | Florida | n/a | 0.8 | 2.1 ab | 9 |
| | | | means: | 0.65 | 1.99 | 77 |

Legends for Tables 15 a and 15b.

[a]Potted, grafted plants were wound inoculated with three isolates of *Fusarium mangiferae* as described by Freeman et al. (*Phytopathology* 89:456-461). Mean disease ratings among cultivars within a column are either not significant (ns) or differ when not followed by the same letter (DMRT $P < 0.05$).

[b]Inc = disease incidence, the proportion of plants that developed symptoms (0 = no disease; 1 = any disease).

[c]Sev = disease severity, which was estimated on a visual 1 (no symptoms) to 5 (severe) scale.

**Time, length of bud dormancy, isolate and cultivar all affected disease development. Virulent isolates, patience and sufficient replication (no fewer than six plants per treatment) are needed to successfully conduct screenings for response to malformation. Futnre work should assess response of a greater range of cultivars, and investigate what attributes are related to and might predict resistance to this disease.**

**c. Population biology and pathogenicity**

Investigate the population biology and pathogenic diversity of *F. mangiferae*.
*F. mangiferae* from Israel (140 isolates) and Egypt (447 isolates) were collected from representative locations in each country where malformation is present. Vegetative compatibility grouping (VCG) was conducted on isolates from each country. In Israel, a single VCG (VCG5) was confirmed whereas in Egypt multiple VCGs (VCG1, 2, 3 and 4) were found (Zheng and Ploetz. 2002). DNA was extracted for population diversity studies. Species-specific primer identification of *F. mangiferae* and arbitrarily primed-PCR for genetic diversity studies (Freeman et al., 1993) was performed on representative isolates from different regions in each country (Fig. 9, Egypt; Fig. 10, Israel). Variation in pathogenicity of certain isolates was evident (see previous section on resistance/susceptibility of mango genotypes).

**The polymerase chain reaction amplification of DNA using 1-3 F/R specific primer pair reaction (Zheng and Ploetz. 2002) was very reliable in identifying *F. mangiferae* in both Israel and Egypt. In Egypt *F. mangiferae* populations are genetically diverse (VCG and molecular) suggesting multiple introductions of the pathogen into the country may have occurred. In contrast, a uniform/clonal exists in Israel indicating a single introduction.**

**d. Diagnosis**

(i) Isolation of species-specific primers based on ap-PCR and ITS sequence analysis
Species-specific primers (1-3 F/R specific primer pair; Forward -TGCAGATAATGAGGGTCTGC and Reverse - GGAACATTGGGCAAAACTAC) from the lab of Ploetz (Zheng and Ploetz. 2002) were developed and successfully adapted in the labs of all partners. These primers were used to confirm the identity of the species *F. mangiferae* (see Figs. 9 and 10).

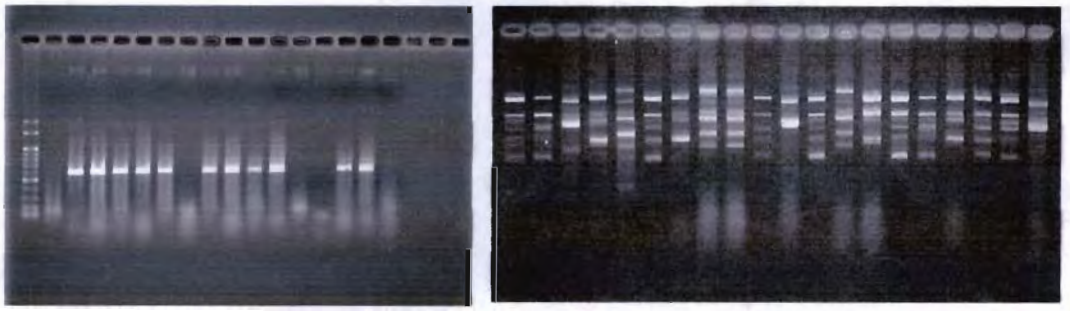
(ii) Isolation of species-specific primers based on genomic and cDNA libraries
The designated species-specific primers are working successfully and reliably for the identification of *F. mangiferae* therefore it was not necessary to develop additional specific primers based on genomic and cDNA libraries.

(iii) Pathogen detection within host tissue.
In cooperative work with the USDA/ARS subtropical research station in Miami, *in planta* assays for *F. mangiferae* were conducted with the described 1/3 FR PCR primer pair. Three different types of tissue were assayed: 1) branch material from previously malformed trees that had been pruned to remove malformed tissues; 2) branch and root collar samples from plants that had been planted in soil infested with a *nit* mutant of *F. mangiferae*; and 3) branch and bud material from plants that had been artificially inoculated with *F. mangiferae*. Only one of the plants in sample set 3 had symptoms of malformation.

# Figure 9

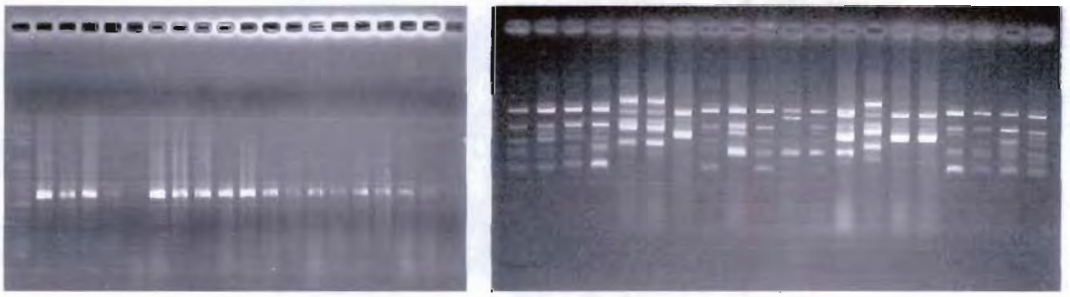
Alexandria Governorate

A B



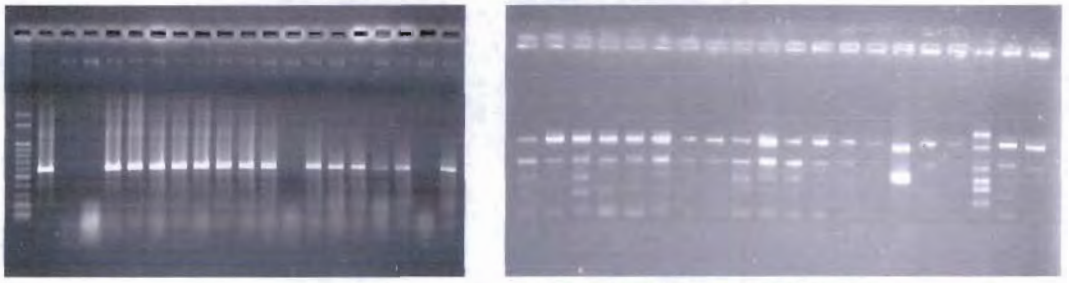
Beni-Sweif Governorate

A B



El Menia Governorate

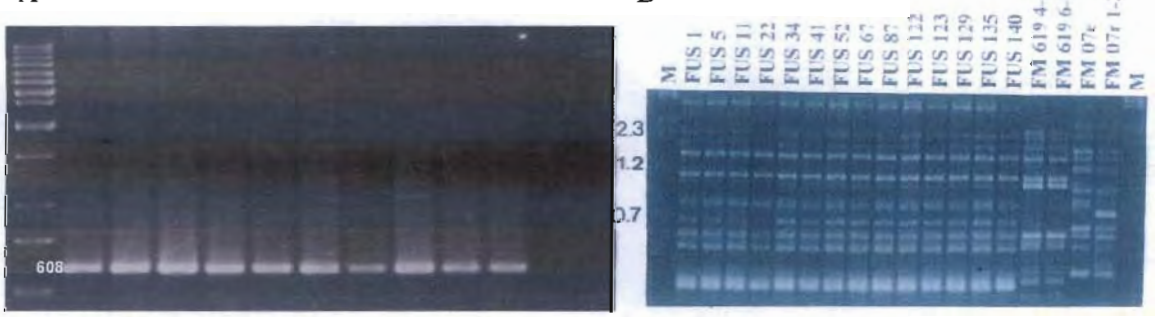
A B



# Figure 10

PCR ANALYSIS USING PRIMER $(CAG)_5$ OF *FUSARIUM* CAUSING MANGO MALFORMATION

A B




Figs. 9 and 10. (A) Polymerase chain reaction amplification of DNA using 1-3 F/R specific primer pair reaction with amplification of the 608 bp single band (Zheng and Ploetz. 2002) and (B) RAPD-PCR products of different isolates of *F. mangiferae* collected from various Governorates in Egypt and Israel.

Each tissue sample was split longitudinally in two, one half of which was taken to the USDA station for PCR analysis, and the other half of which was processed for isolation of *F. mangiferae*. PCR assays were conducted with total DNA that was extracted from samples, amplified with the 1/3 FR primer pair, and run on an automated DNA sequence analyzer. Results from these assays confirm that the 1/3 FR primer pair amplifies DNA of *F. mangiferae* in culture (mycelium), as well as in total DNA isolated from infected plant tissue, even when that tissue did not display symptoms. However, these results were inconsistent in that there were three times when the fungus was isolated in culture, but was not detected in total DNA by PCR; conversely, the pathogen was detected in one sample from which it was not isolated. Although the reasons for these inconsistencies are not clear, they may relate to the inconsistent, patchy distribution of the pathogen in infected tissue.

**It was confirmed that the 1/3 FR primer pair amplifies DNA of *F. mangiferae* in culture as well as in total DNA isolated from infected plant tissue, even when that tissue did not display disease symptoms. However, inconsistencies were evident, probably due to sampling method and uneven distribution of the pathogen within infected plant tissue. Future work must address this problem if *in planta* diagnosis of this pathogen using these primers is to become reliable.**

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## 6. Impact Relevance and Technology Transfer

Mango malformation disease is the most important disease of mango in the region being a major constraint to a successful crop in both Egypt and Israel. All mango growers, especially in Egypt and Israel, have and will continue to benefit greatly from the improved knowledge of etiology, basic epidemiology and spread of the disease; knowledge on cultural control, and use of new chemicals for disease control; and the accurate diagnostic tool for pathogen detection. An additional benefit, not directly associated with the original project aims, was to distribute a guide to approximately 3000 local Egyptian farmers on how to cultivate disease-free propagation material. It was observed that infected seedlings originating from infected mature orchards were the major source of disease spread when establishing new orchards with this propagation material. The impact of this knowledge will certainly contribute to expanding mango productivity in Egypt and lead to improving yields due to reduction of disease incidence in new plantings.

The laboratory of Dr. Shalaby at the Agricultural Research Center in Egypt is now excellently equipped with all the modern molecular diagnostic equipment mostly due to generous funding from this MERC project (M21-030). All the scientists involved in the project, from Israel and the United States worked very closely with their Egyptian counterparts to establish the most appropriate technologies for diagnostics, and understanding epidemiology and control of the disease. Israeli and Egyptian students conducted part of their theses on the disease epidemiology, fungal-mite interaction and molecular typing of the Egyptian isolates. Substantial interaction between the Egyptian and Israeli research teams took place during all stages of the project. At least five annual meetings were arranged during the project lifespan, in Egypt and the US, with another meeting taking place in the main mango region of Spain. Dr. Freeman traveled extensively to Egypt within the context of the mango project (M21-030) and will continue to travel regularly to Egypt as part of another ongoing MERC project (M24-022; Disease-indexing and mass propagation of superior strawberry cultivars) with a projected termination date in 2012. Therefore, continued meetings with Dr. Shalaby will take place on some of these occasions.

Solving the problems associated with mango malformation will not be limited to the lifetime of this proposal. Mango malformation is a serious disease known and researched for over a century worldwide, therefore, an active continued exchange of research ideas and results will be fostered by this project. In addition, local decisions will have major impact at the regional level mainly due to similar climatic and biological conditions in common in both Israel and Egypt. Therefore, mutual future decision making based on the results of this research project, at the agricultural levels in the field, will certainly be adopted by both countries. These decisions will have long term implications for the future of mango cultivation in both countries since mango is a long-term crop which will outlive the lifetime of this project.

The information derived from the project has been disseminated by the Israeli and Egyptian Ministries of Agriculture and by their bulletins. People directly involved in the project, researchers and students alike, have been regularly in direct contact with growers and the grower's organizations, and have disseminated information by lectures, visits and articles in trade and international journals. Impact of the project's results will have a direct effect on production by elevating yields, producing disease-free propagation material and contributing to extensive new field planting.

Future studies are ongoing and a continuation research proposal with the current researchers within the context of the USAID-MERC program is now in the planning.

## 7. Project Activities/Outputs

Meetings within the context of the project:

1. The 2003 annual meeting took place at UFL, Homestead, Florida from 6-12 July, 2003, attended by Drs. Freeman, Palevsky, Ploetz, Shalaby and Sattar. We were most fortunate to have Dr. Jorge Pena of UFL participate in our discussions and contribute his knowledge on mite infestation of mango. At this meeting the workplan was discussed and experiments planned.

2. The 2004 meeting was held from 19 to 22nd February, 2004 to discuss progress according to the workplan, assess Egyptian studies in the lab, greenhouse, and field, and to collect mites and infected material. Participants included Daniel O. Hastings (Second Secretary) US Embassy; Dr. A. Reinhart (USAID-MERC); Drs. Sattar and Shalaby (Egypt), and Drs. Freeman and Palevsky (Israel).

3. Dr. Freeman traveled to Egypt from 16th to 23rd April, 2004 to conduct field experiments in Sharkia and Ismailiya with Shalaby, to determine whether the source of distribution of malformed material is via seedlings.

4. Dr. Freeman traveled to Egypt to coordinate results and plan future experiments with Shalaby during the period from 23-26th December, 2004.

5. The 2005 annual meeting took place in Alexandria, Egypt from 28th to 31st March, 2005. In this meeting, research progress, coordination of results and mutual publications were discussed. Exceptional cooperation and collaboration was expressed. All participants were present at the meeting: Drs. Freeman and Palevsky (Israel), Ploetz (USA), Shalaby, Sattar, 3 other senior scientists, and 5 postdocs and students from Egypt. In attendance was also Dr. A. Reinhart and Dr. H. Hamroush from the USAID mission.

6. Dr. Freeman traveled to Egypt 5-8th January, 2006 to finalize mutual co-authored publications with Shalaby.

7. The 2006 annual project meeting took place from 13-17th September at the Estación Experimental La Mayora, Algarrobo-Costa (Málaga), Spain. At the meeting, the group interacted with Spanish researchers working on mango malformation, participated in field excursions to mango growing areas and exchanged views and ideas which were very beneficial to all participants.

8. The 2007 annual meeting took place from 16-18th May 2007, at the University of Florida, TREC, Homestead facilities. At the meeting, the group interacted with researchers at the UFL campus. The participants also toured the Williams Farm containing a worldwide mango collection and deals with research at the Fairchild Tropical Gardens. Dr. Richard Campbell was the host. An additional tour was conducted to the USDA-ARS National Germplasm repository for mango, where Dr. David Kuhn and Mike Winterstein were the hosts.

9. Dr. Freeman will meet again with Shalaby from 8-15th November 2007 (while traveling to Egypt within the context of MERC project M24-022) to discuss future plans and proposal submission to USAID for continuation of the project.

Training

Two training periods were conducted.
1. Drs. Palevsky traveled with Freeman to Egypt in February 2004 to collect mite sampled in mango orchards. During this visit the "art" of sampling and the methods of mite detection were demonstrated in the field.

2. During a visit to Egypt in April 2004, Dr. Freeman set up field experiments and demonstrated methods of isolating the fungal pathogen from infected tissue while working in the lab of Dr. Shalaby with students and other personnel.

Joint, co-authored publications

1. A pamphlet was prepared (see below) for distribution to over 3000 Egyptian farmers to educate them in cultivation of seedlings separate from mature orchards since the pathogen is spread in the orchard to the seedlings and this way the disease is further exacerbated.

2. A joint publication entitled "Mango malformation: source of disease in seedlings cultivated in infected orchards in Egypt" by S.A. Youssef, M. Maymon, A. Zveibil, D. Klein-Gueta, A. Sztejnberg, A.A. Shalaby and S. Freeman was presented by Freeman during the 2006 International Mango Symposium, held from 5$^{th}$-10$^{th}$ February in South Africa.

3. The proceedings of this study are currently in press in the international publication Acta Horticulturae:
S.A. Youssef, A.A. Shalaby, A. Sztejnberg, M. Maymon, A. Zveibil, D. Klein-Gueta, and S. Freeman. 2007. Mango Malformation: Source of Disease in Seedlings Cultivated in Infected Orchards in Egypt. Acta Hort. (in press).

4. A peer reviewed article was published in the prestigious British Society of Plant Pathology journal, Plant Pathology (see below):
Youssef, S.A., Maymon, M., Zveibil, A., Klein-Gueta, D., Sztejnberg, A., Shalaby, A.A., and, Freeman, S. (2007). Epidemiological aspects of mango malformation disease caused by *Fusarium mangiferae* and source of infection in seedlings cultivated in orchards in Egypt. Plant Pathology 56:257-263.

5. Additional mutual publications in preparation:

A. Genetic diversity of *Fusarium* isolates causing mango malformation in Egypt and Israel.
B. Interaction of the mite, *Aceria mangiferae* and the fungus *Fusarium mangiferae* in mango malformation disease.

## 8. Project Productivity

All the proposed goals of the project were accomplished besides the *in planta* diagnosis of the fungus in infected tissue. Diagnostic inconsistencies were evident, probably due to the sampling method and uneven distribution of the pathogen within infected plant tissue. Future work must address this problem if *in planta* diagnosis of the pathogen using the current primers is to become reliable.

It should be further emphasized that during excursions in the fields of Egyptian mango farms, the researchers observed that nurseries containing seedlings were established in infected mature orchards under malformed canopies. This led to infection of these seedlings and subsequent spread of disease via the seedlings that were sold to establish new orchards. A plan to limit the spread of disease via the infected seedlings was implemented by distribution of a pamphlet to approximately 3000 small Egyptian farm holders, and establishment of new nurseries void of disease has begun.

## 9. Future Work

1. Determine the nature of seed infection in Egypt, crucial for maintaining disease-free propagation material.

2. Determine whether mature fruit is latently infected by spores that have germinated and penetrated the skin or whether the spores are passively "resting" on the surface and can be washed off or surface decontaminated. This is critical for export of fruit to areas where mango malformation does not exist (e.g. Australia).

3. Effect of temperature and humidity on mite/fungal interaction.

4. Determine whether internal infection of buds can take place via ingestion of spores via the mite's body.

5. Determine whether presence of mite predators can influence bud mite populations and affect levels of disease incidence.

6. Does growth rate affect disease severity and bud mite feeding damage? If yes, could this be manipulated by root stock and growth hormones? Perhaps this manipulation could be important in developing and standardizing the methodology for evaluating cultivar susceptibility.

7. What affect do bud mites have on disease development in floral buds?

8. Continue sensitivity/resistance of mango germplasm to malformation with emphasis on mechanisms of resistance.

9. Monitor pathogen spread in orchards and determine whether a window period is present which may be crucial for pathogen infection of buds.

10. Can protection of buds from inoculum of the pathogen reduce and or limit disease incidence ?

11. Continue genetic diversity studies on Egyptian isolates in comparison with current data indicating that multiple *Fusarium* species from various origins worldwide are involved in causing disease.

## Appendices

**First Annual MERC Meeting which took place from 6-12th July 2003 at UFL, Homestead, FL, USA (left to right: S. Freeman, E. Palevsky, R. Ploetz, A. Shalaby and M. Sattar).**

**Field trip to infected mango plantations during visit in Egypt (22nd February, 2004): Including, Daniel O. Hastings (2nd Secretary) US Embassy; Dr. A. Reinhart (USAID-MERC); Project investigators: Dr. M. Sattar, Dr. A. Shalaby (Egypt) and Dr. S. Freeman, Dr. Palevsky (Israel)**

**Field trip collecting mites during visit in Egypt (20th February, 2004): (L-R) Dr. S. Freeman (PI); Dr. S. Youssef (Egyptian research assistant); and (co-PI's) Dr. E. Palevsky, Dr. A. Shalaby and Dr. M. Sattar**

**Second Annual MERC Meeting, Alexandria, Egypt March 28-31st, 2005**

**Third MERC project participants (bottom row: L-R, Dr's Freeman, Shalaby, Youssef, Ploetz and Sattar) held their annual meeting at La Mayora, Algarrobo-Costa, Spain, 13-17th September, 2006. Spanish participants (top row)**

**Fourth annual MERC meeting held at UFL-TREC, Homestead Florida, 16-18th May, 2007.**

**MERC project participants: L-R, Dr's Palevsky, Reinhart (USAID representative), Freeman, Ploetz, Shalaby, Youssef, and Sattar)**

# Pamphlet distributed to Egyptian farmers

التوصيات الخاصة بالحد من ظاهرة اصابة شتلات المانجو المنزرعة تحت الاشجار الكبيرة بمرض التشوه الخضرى والزهرى.

١- عدم إقامة مشاتل للمانجو أسفل الأشجار المصابة وزراعة شتلات المانجو فى مناطق بعيدة عن الأشجار المصابة.

٢- عدم استخدام أقلام تطعيم مأخوذة من أشجار مصابة.

٣- لايتم تقليم الشتلات المصابة حيث ان الخطر المسبب للمرض يعيش فى الأجزاء السفلى من الشتلة وايضا الجذور

٤- لايتم استخدام تربة مأخوذة من المناطق التى تظهر فيها الإصابة لاستخدامها كمهد لزراعة الشتلات.

٥- التوصية بإتباع إجراءات حجر زراعى صحى داخلى من شأنها عدم السماح بنقل الشتلات المصابة من المناطق الموجودة فيها الى المناطق الجديدة التى لم تظهر فيها الإصابة بعد خاصة مناطق الأقصر وأسوان والخالية من الإصابة بالتشوه.

٦- زراعة شتلات من الأصناف المقاومة لظاهرة التشوه مثل الفجرى كلان و لانجرا بنارس و الزبدة و الجولوك البرتقالى والكيت والكنت ومعلومة على اصل سكرى ابيض لتفادى الإصابة.

البرنامج الحالي المتبع من خلال المشروع لانتاج شتلات مانجو خالية من التشوه.

يقوم المشروع حاليا باتباع برنامج لإنتاج شتلات مانجو من الأصناف فجرى كلان و لانجرا بنارس و الزبدة و الجولوك البرتقالى والكيت والكنت والتى أكدت نتائج التقييم لها من خلال المشروع مقاومتها لظاهرة التشوه حيث يتم تطعيم هذه الأصناف على اصل سكرى ابيض لما يتميز به من مقاومة لظاهرة تشقق القلف ويعطى مجموع جذرى قوى ويؤدى فى النهاية الى الحصول على شتلات قوية متجانسة النمو و خالية من التشوهات على ان تكون هذه الشتلات متاحة للمشاتل الأهلية و الحكومية وكذلك القطاع الخاص وبأسعار رمزية.

فريق العمل بالمشروع

لمزيد من المعلومات

الاتصال بمشروع تطوير انتاجية المانجو ٩ شارع جامعة القاهرة مركز البحوث الزراعية تليفون ٥٦٩٣٣٣٧

و بريد الكترونى aashalaby@link.net

وزارة الزراعة و استصلاح الاراضى

مركز البحوث الزراعية

معهد بحوث أمراض النباتات

## مشروع تطوير إنتاجية المانجو

## (ميرك)

التوصيات الخاصة زراعة شتلات المانجو الخالية من التشوه الخضرى و الزهرى

نشرة رقم ١

مدير المشروع

أ.د / عبد الباسط احمد شلبى

المشروع ممول من هيئة المعونة الامريكية

MERC-TA-MOU-02-M21-30

ARC/ARO/FLU

مقدمة:

يعتبر مرض التشوه الخضرى و الزهرى (التكتل) Mango malformation الذى يصيب اشجار وشتلات المانجو مرضا اقتصاديا هاما حيث انه يزداد عاما بعد عام ويؤثر على إنتاج الأشجار مما يقلل من كمية وجودة المحصول الناتج وينتشر هذا المرض فى جميع مناطق زراعة المانجو فى العالم بالاضافة الى مصر.

اعتاد كثيراً من المزارعين على إكثار شتلات المانجو أسفل أشجار المانجو الكبيرة والتى تظهر عليها أعراض التكتل مما يؤدى إلى انتقال الإصابة منها إلى هذه الشتلات ومما يؤدى أيضاً إلى زيادة انتشار المرض فى البساتين الحديثة وتظهر الأعراض على صورة نموات خضرية صغيرة ومتزاحمة أو قد تظهر فى صورة افرع صغيرة متقزمة ضعيفة النمو ويموت نسبة كبيرة منها فى المشتل والشتلات الباقية تستمر ضعيفة النمو وغالباً ماتموت بعد نقلها للمكان المستديم أو أن تنقل مصدر للإصابة سواء فى المشتل أو فى المكان المستديم وبالتالى فان اتباع برنامج لانتاج شتلات مانجو خالية من التشوه من الأصناف المقاومة لهذة الظاهرة يعد من الأهداف الهامة للمشروع على أن تكون هذه الشتلات متاحة وبأسعار رمزية للحد من ظاهرة انتشار مرض التشوه سواء فى المشاتل أو فى بساتين المانجو المستديمة.

شتلات مانجو منزرعة تحت الأشجار الكبيرة.

شتلات مانجو منزرعة تحت الأشجار الكبيرة و تظهر عليها الاصابة.

شتلة مانجو يظهر عليها أعراض التشوه الخضرى فى الحقل.

شتلة مانجو يظهر عليها أعراض التشوه الخضرى فى الصوبة.

شمراخ زهرى مصاب

شمراخ زهرى سليم

# Mutual publication in Plant Pathology, co-authored by Egyptian and Israeli scientists

*Plant Pathology* (2007) 56, 257–263 Doi: 10.1111/j.1365-3059.2006.01548.x

## Epidemiological aspects of mango malformation disease caused by *Fusarium mangiferae* and source of infection in seedlings cultivated in orchards in Egypt

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Mango malformation, caused by the fungus *Fusarium mangiferae*, is one of the major diseases of this crop occurring worldwide. This study was conducted to investigate aspects of the epidemiology, survival and spread of the pathogen in general and specifically in seedlings, the majority of which are cultivated in infected orchards in Egypt. Survival of conidia of a representative isolate (506/2) declined very rapidly in soil under summer conditions (1·6 weeks for 50% population decline), but significantly less in controlled and winter conditions (17·9 and 15·0 weeks, respectively, for 50% population decline). Likewise, inoculum survival in naturally infected panicles on the soil surface declined faster than in those buried at 30 cm depths. Natural infections were evaluated on fruits and seeds in a heavily infected and a healthy orchard. In infected trees, the skins of all sampled fruits within a 2-m radius of infected panicles were infected, but the pathogen was not detected in the seeds, seed coats or flesh. The pathogen was not detected in any parts of fruits from a healthy orchard. Vegetatively malformed mango seedlings, growing under infected trees bearing infected panicles, were sampled in two locations in Egypt to determine whether infection in seedlings was systemic (evenly distributed within plant tissue) or whether the pathogen originated from malformed panicles. According to PCR-specific primer amplification, the pathogen was detected in 97% of seedling apical meristems, declining gradually to 5% colonization in roots. It was concluded that inoculum of the pathogen originates from infected panicles and affects seedlings from the meristem, with infections descending to lower stem sections and roots. Minor infections of roots may occur from inoculum originating from infected panicles, but the pathogen is not seedborne.

*Keywords:* flowers, fruit, *Fusarium mangiferae*, *Mangifera indica*, mango malformation, polymerase chain reaction

### Introduction

Inflorescence and vegetative malformation of mango, *Mangifera indica*, occurs in many mango-growing countries worldwide and is one of the most important diseases of this crop (Kumar *et al.*, 1993; Ploetz, 2001). The disease causes serious losses since malformed inflorescences do not bear fruit. Although mango malformation was first reported over a century ago in India, disease epidemiology is poorly understood and conflicting reports still exist regarding the causal agent (Kumar *et al.*, 1993; Ploetz *et al.*, 1994). However, many studies have shown *Fusarium mangiferae* to be the pathogen responsible for mango malformation disease and Koch's postulates have been completed successfully with this fungus in various countries (Summanwar *et al.*, 1966; Varma *et al.*, 1974; Ibrahim *et al.*, 1975; Crookes & Rijkenberg, 1985; Manicom, 1989; Kumar *et al.*, 1993; Ploetz & Gregory, 1993; Freeman *et al.*, 1999). Recently, additional taxa of *Fusarium* have been implicated in disease symptoms, although Koch's postulates have not yet been performed with these isolates (Marasas *et al.*, 2006). Presence of the eriphoid mite, *Aceria mangiferae*, has been claimed to cause mango malformation, but certain studies indicate that the mite may only play a role in wounding and transfer of the fungal pathogen to and from infection sites (Kumar *et al.*, 1993; Ploetz *et al.*, 1994).

Malformation may be dispersed by grafting infected budwood, which is a common means by which the disease is moved to new areas (Kumar *et al.*, 1993). Spread on a small scale has also been clearly demonstrated in nurseries (Prakash & Srivastava, 1987). In Egypt, non-grafted

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## Direct beneficiaries from the project (personnel, students, farmers)

### Israel

Personnel:

Technicians:
Mrs. Marcel Maymon "Genetic diversity of *Fusarium mangiferae* isolates".
Mrs. Aida Zveibil "Survival of *Fusarium mangiferae* propagules in soil".

Students:
Mrs. Danit Klein-Gueta (MSc.): "Epidemiology of the fungus *Fusarium subglutinans* responsible for mango malformation disease".
Mr. Zvi Neuman (MSc.): "Field experiments for the control of mango malformation caused by the fungus *Fusarium mangiferae*".
Mrs. Efrat Gamliel-Atinsky (Ph.D): "Interaction of the mango mite bud, *Aceria mangiferae* with the fungal causal agent of mango malformation disease, *Fusarium mangiferae*".

Farms where field experiments were conducted:
Ein Hachoresh, Sharon region, Central Israel
Mishmar Hasharon, Sharon region, Central Israel
Volcani Experimental Station, Central Israel
Cholit, Negev region, Southern Israel
Sufa, Negev region, Southern Israel

### Egypt

Personnel:
Researchers:
Dr. Ms. Sahar Youssef
Dr. Hanan El Marzouky

Technicians:
Miss. Lydia Admound
Miss. Hend Mohamed
Miss. Wesam Hosny
Miss. Eman Abdella

Students:
Mr. Sam El Hamamsy
Mr. Ahmed Mahmoud
Mr. Nour Saied
Mr. Sherif Diab

All these researchers, technicians and students participated in the study of all project objectives i.e., epidemiology, survival, field experiments for the control of mango malformation, genetic diversity of *Fusarium mangiferae* isolates and interaction of the mango mite bud, *Aceria mangiferae* with the fungal causal agent of mango malformation disease.

Farms where field experiments were conducted:
Abdel-Hady Tantawy, El-Ferdan region, Ismailia Governorate
Nabil Shehata, Ezz El-Din region, Ismailia Governorate
Heba Mostafa Heba, Sarabium region, Ismailia Governorate
Mohamed El-Asherey, El Sawah, AbuHamad, Shrkia Governorate
Mohamed Ayoub, El-Sanagra region, Sharkia Governorate.