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Fusarium mangiferae localization *in planta* during initiation and development of mango malformation disease

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Mango malformation disease (MMD), caused by *Fusarium mangiferae*, is a major constraint to mango production, causing significant yield reduction resulting in severe economic impact. The present study characterizes fungal localization *in planta* during initiation and development of vegetative and floral malformation. Young mango trees were artificially inoculated with a green fluorescent protein (GFP)-expressing strain of *F. mangiferae*. Shoots and buds were sampled periodically over a period of more than a year and localization of the GFP-expressing fungi was determined using confocal microscopy. Fungal localization appears to be epiphytic: mycelia remained in close contact with the plant surface but did not penetrate the tissue. In vegetative malformation and in young inflorescences, the fungus was confined to protected regions between scales, young leaf bases and buds. Fungal colonization was only very rarely detected on open leaves or on exposed shoot sections. In developed flowers, mycelia were localized mainly to protected regions at the base of the flower organs. Upon development of the inner flower organs, specific mycelial growth occurred around the anthers and the style. Mycelial penetration through the stylar tract into aborting carpels was observed. For several months, mycelia were confined to the surface of the organs and were not detected within plant tissues. Only at later stages, transient saprophytic growth of the fungus was detected causing the malformed inflorescences to senesce and collapse, concurrent with dispersion of conidia. Implications of the present study on MMD in natural field infections are discussed.

Keywords: confocal microscopy, floral buds, flower organs, *Fusarium mangiferae*, green fluorescent protein, vegetative buds

Introduction

Mango (*Mangifera indica*) is one of the most important fruit crops in the world. It is the fifth largest cultivated fruit crop globally with yields of approximately 40 million tonnes, second only to banana among the tropical fruit species (FAOSTAT, 2015). Mango cultivation originated in India and expanded throughout southeast Asia (Mukehrjee & Litz, 2011). Starting from the 15th and 16th centuries, mango was distributed by the Spaniards and Portuguese to many regions of the world. Today it is commonly grown in most tropical and subtropical regions.

Mango cultivation in many countries worldwide is seriously affected by mango malformation disease (MMD) (Kumar *et al.*, 1993; Ploetz & Freeman, 2009). The major causal agent of MMD, *Fusarium mangiferae* within the *Gibberella fujikuroi* species complex, was described as a new species in 2002 (Steenkamp *et al.*, 2000; Britz *et al.*, 2002). Previously, the pathogen was identified as *Fusarium moniliforme* (Summanwar *et al.*, 1966), and has had several synonyms in the literature, including *F. subglutinans* (Marasas *et al.*, 2006). *Fusarium mangiferae* has been identified in China, Egypt, India, Israel, Malaysia, Oman, South Africa, Spain, Sri Lanka and the USA, and appears to be the most common causal agent of MMD worldwide (Freeman *et al.*, 2014c). A second MMD causal agent, *F. sterilihyphosum*, was described in South Africa (Britz *et al.*, 2002) and Brazil (Lima *et al.*, 2009), while another causal agent, *F. mexicanum*, was described exclusively from Mexico (Otero-Colina *et al.*, 2010). A fourth recently described species, *F. tupiense* sp. nov. (resembling *F. sterilihyphosum*), has been shown to cause malformation in Brazil (Lima *et al.*, 2012), Senegal (Senghor *et al.*, 2012) and Spain (Crespo *et al.*, 2016). Most recently, *F. pseudocircinatum* has been described as an additional MMD causal agent in Mexico and the Dominican Republic (Freeman *et al.*, 2014b; García-López *et al.*, 2016). In addition, *F. mangiferae*, *F. proliferatum*, *F. pseudocircinatum* and other *Fusarium* species have been isolated from affected mango in Australia (Liew *et al.*, 2016). All *Fusarium* species responsible for MMD cause similar disease symptoms.

Mango malformation disease affects both vegetative and reproductive structures of the plant. The phenology

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of normal healthy mango growth is not continuous. Vegetative mango growth occurs in several intermittent flushes, separated by resting periods with no apparent growth (Davenport, 2009; Hernández Delgado *et al.*, 2011; Ramírez *et al.*, 2014). Each flush generates a segment of stem with 10–20 leaves generated mainly at the apex. At early flush development, meristems at apical and lateral buds are activated; the buds swell and break, followed by a rapid elongation of the stem and expansion of the leaves.

Although mortality of mango trees does not occur due to MMD, the affected vegetative structures restrict canopy development and the affected inflorescences reduce fruit yield dramatically (Ploetz & Freeman, 2009; Chakrabarti, 2011; Freeman *et al.*, 2014c). Symptoms of vegetative malformation include hypertrophy of young shoots, with an overall tightly bunched appearance of the shoot where swollen apical and lateral buds produce deformed shoots with shortened internodes and dwarfed leaves that curve from the tip back towards the adaxial portion of the petiole (Kumar *et al.*, 1993; Chakrabarti, 2011; Freeman *et al.*, 2014c). The growth of this shoot is arrested and subsequently several similar shoots arise from the same axillary bud, collectively giving rise to a number of bunched shootlets, i.e. the 'bunchy-top' symptom of the disease. Vegetative malformation seriously affects seedlings and small plants in nurseries (Ploetz *et al.*, 2002; Youssef *et al.*, 2007).

The main stages of normal reproductive development are similar to those of vegetative ones, with development of the 'resting' meristems, bud swelling and growth of the inflorescence (Davenport, 2009; Hernández Delgado *et al.*, 2011). The reproductive units are panicles with hundreds to several thousands of flowers. Some of the flowers are hermaphrodite, having a carpel, while others are staminate, where the carpel is absent or not fully developed (Ramírez & Davenport, 2010, 2016). Each flower contains a single fertile developed stamen, and several (usually four) sterile staminoids (degenerated stamens) (Davenport, 2009; Ramírez & Davenport, 2010).

In malformed inflorescences, primary or secondary axes on affected panicles are shortened, thickened and highly branched. Malformed panicles may also produce dwarfed and distorted leaves (exhibit phyllody). Developed inflorescences are condensed deformed, resembling a cauliflower, containing larger and more flowers than usual. Malformed inflorescences remain vital and continue to bear open flowers for several weeks; however, fruit setting is hampered and affected inflorescences usually do not set fruit (Kumar *et al.*, 1993; Noriega-Cantú *et al.*, 1999; Ploetz, 2001; Youssef *et al.*, 2007; Ploetz & Freeman, 2009; Chakrabarti, 2011).

The distribution of *F. mangiferae* in affected trees indicates that vegetative and floral buds are the primary sites of infection and that systemic colonization does not occur (Gamliel-Atinsky *et al.*, 2009c). In the past, Freeman *et al.* (1999) transformed isolates of *F. mangiferae* from mango with the *uidA* reporter gene (*β*-glucuronidase), and used them to artificially inoculate mango. The results verified that bud and flower tissues of the host are primary infection sites (Freeman *et al.*, 2014a). In recent years, studies on dispersal patterns of conidia of *F. mangiferae* suggest aerial dispersal of inocula as the primary mechanism for fungal dissemination (Noriega-Cantú *et al.*, 1999; Youssef *et al.*, 2007; Gamliel-Atinsky *et al.*, 2009a,c). Gamliel-Atinsky *et al.* (2009c) showed that infections are not systemic, with infections of apical meristems most probably originating and disseminating via conidia from malformed panicles. Malformed inflorescences and malformed vegetative growth serve as sources of inoculum from diseased panicles and malformed vegetative tissue, which disseminate passively in the air as conidia, or fall from dry, malformed inflorescences as dry debris (Gamliel-Atinsky *et al.*, 2009b; Freeman *et al.*, 2014a). After penetration, the pathogen colonizes the bud tissue but does not progress systemically into other plant tissues. Inflorescences from a colonized bud may emerge malformed, suggesting that a hormonal imbalance occurs in affected tissues and that an infection threshold is required for symptom development (Ploetz & Freeman, 2009).

The development of MMD symptoms has, to a large extent, been fully described (Kumar *et al.*, 1993; Ploetz & Freeman, 2009; Chakrabarti, 2011). However, there is still a lack of knowledge regarding the host–pathogen interactions and fungal localization *in planta*, during initiation and development of vegetative and floral malformation. To study the interactions between *F. mangiferae* and mango tissue and its role in MMD, a cell biology approach was applied. Young mango trees were artificially inoculated with a GFP-expressing isolate of *F. mangiferae* and the specific fungal localization was followed during affected vegetative and reproductive developmental stages using confocal microscopy. Presence of the fungus was also determined by growth on selective media in leaves and other plant organs in the greenhouse and under natural field conditions.

Materials and methods

Artificial inoculations with a GFP-labelled *F. mangiferae* isolate

The monoconidial GFP-marked strain (gfp-1) of *F. mangiferae* (MRC 7560; Gamliel-Atinsky *et al.*, 2009a) used in this study was maintained on potato dextrose agar (PDA; Difco) supplemented with chloramphenicol (250 mg mL^{-1}) at 25 °C.

Ten 5-year-old mango trees susceptible to malformation (cultivar Maya grafted on rootstock 13/1), planted in 10 L pots containing local red loam, were used in inoculation experiments. The seedlings were placed in a growth chamber at a constant temperature of 25 ± 2 °C under diurnal 12 h light conditions or kept in a greenhouse under natural conditions. To induce flowering, diurnal temperature growth conditions were changed (16 h at 20 °C day, 8 h at 12 °C night) in March–April 2014 for 8 weeks, to mimic natural conditions.

Two weeks before inoculation (October 2012 and 2013), the seedlings were fumigated twice with dichlorvos (1000 g L^{-1} Divipan; Makhteshim), using a fumigator (Hagarin) to ensure they were devoid of mites and insects. The base of the stem was

ringed with a water-based adhesive (Rimifoot liquid) to prevent infestation by ambulant arthropods (Freeman *et al.*, 2014a). Conidial suspensions were obtained by adding sterile water to 7-day-old culture plates, mixing the suspension and filtering it through a four-layered gauze pad (Gamliel-Atinsky *et al.*, 2009a). Mango seedlings were artificially inoculated by pipetting 20 μL of conidial suspension (10^6 gfp-1 isolate conidia per mL 0.1% water agar) on 80–90 apical buds each, that were covered overnight with plastic bags sprayed with water, according to the method described by Freeman *et al.* (1999). Following inoculations, trees were kept under similar conditions in the greenhouse until March 2014. Approximately 6–18 months later, during March to May 2013 and 2014, approximately 50 newly infected vegetative buds generated from the original apical bud inoculations (bearing typical symptoms of swollen appearance) were collected for confocal microscopy. Approximately 45 reproductive buds were collected during May–July 2014 at different developmental stages for confocal microscopy (see below).

Fungal localization in developing vegetative and reproductive mango organs *in planta*

To determine the initial interactions between the pathogen and different mango organs *in planta*, developing vegetative and reproductive mango buds, sections of inflorescences and flowers were visualized by confocal microscopy. Longitudinal hand sections were analysed using the IX 81 (Olympus) inverted laser scanning confocal microscope (FLUOVIEW 500) equipped with a 488 nm argon-ion laser. GFP was excited by 488 nm light and the emission was collected through a BA 505–525 filter (excitation DM 488/543/633, beam splitter SDM 560). Confocal microscopy of non-infected buds of similar developmental stages was performed to identify any unrelated autofluorescence. The images were colour-coded green for GFP, and red for chlorophyll autofluorescence. The transmitted light images were obtained using Nomarski differential interference contrast (DIC). The three channels (GFP, Chl and bright field) were superimposed to generate final images. For comparison, following confocal microscopy, the same hand sections were observed using a MZFLIII stereomicroscope (Leica) and photographed with a DS-Fi1 digital camera and NIS-Elements software (Nikon).

Fusarium mangiferae colonization of buds, stem and leaf tissues determined on selective growth medium

Five expanded branches infected with the GFP-labelled *F. mangiferae* strain (containing a total of five apical and 87 lateral buds) bearing infected panicles and six apparently healthy leaves from each branch were removed from the trees to evaluate the presence and colonization efficacy of the gfp-1 isolate from the different plant organs (apical and lateral buds, xylem tissue, internodes and leaves). Mango tissues were surface sterilized for 10 s in 70% ethanol and then 3.5 min (for buds and woody tissue) or 3–6 min (for leaves, sectioned into six leaf discs of 1 cm diameter per leaf) in 3% sodium hypochlorite, dried on sterile filter paper and then plated on PDA supplemented with hygromycin B (50 μg mL^{-1}) in certain cases (Gamliel-Atinsky *et al.*, 2009a), to determine percentage fungal colonization of the different tissues.

Two leaves were sampled from approximately 10 cm below each of 10 malformed (infected) and symptomless panicles from a naturally heavily infected orchard located in the northern Negev region of southern Israel, close to Sde Nitzan (31°13′12″ N, 34°24′45″E). Each leaf was sectioned into six discs of 1 cm diameter per leaf and surface sterilized as described, and then plated on PDA supplemented with chloramphenicol (250 mg mL^{-1}), to determine percentage fungal colonization of the tissue.

Results

Localization of *F. mangiferae* in developing vegetative buds

Six to eighteen months following inoculation, most of the buds produced typical vegetative MMD symptoms. Buds were sampled at different developmental stages and analysed using confocal microscopy. Fungal localization was always external and did not penetrate the plant tissue of leaves, scales or stems. The mycelia were confined to specific protected regions within the buds, between scales and developing leaf primordia (Fig. 1a,b). The mycelia developed between these organs reaching the dome of the apical meristem (Fig. 1c). Although approximately 50 buds were carefully examined, no penetration of the mycelia into the apical meristem itself was detected. Mycelia were only very rarely detected in outer, exposed regions of the buds. Few mycelial filaments or conidia were detected on exposed leaves, associated with trichomes, or on more developed leaves or exposed sections of the stem (data not shown).

Localization of *F. mangiferae* in reproductive buds and during inflorescence development

Upon induction of flowering, reproductive buds developed into malformed inflorescences. These could be identified at early stages by their thicker appearance (data not shown). The malformed inflorescences were stunted, and the flowers remained viable for several weeks, forming cauliflower-like structures. Fruitlets did not develop or aborted at early pea-sized stages. As in the vegetative buds, at early stages of reproductive inflorescence development, *F. mangiferae* mycelia were detected in many protected regions, between different developing florets and protecting scales. Similar to vegetative buds, mycelia reached the inner sections of the developing flowers, and were observed between the innermost scales (Fig. 2). However, no mycelia were detected penetrating the leaf or primordial tissues.

As florets grew, their internal organs developed. As long as petals and sepals remained closed, protecting the inner organs, mycelial growth was detected between all enclosed flower organs (Fig. 3a,b). However, specific interactions and growth were detected in several closed flowers around both fertile and degenerated stamens (Figs 3b & 4f). Although mycelia did not grow and develop within exposed leaves and stems, they remained viable surrounding the fully developed and exposed anthers of the mature flower (Fig. 3c,d).

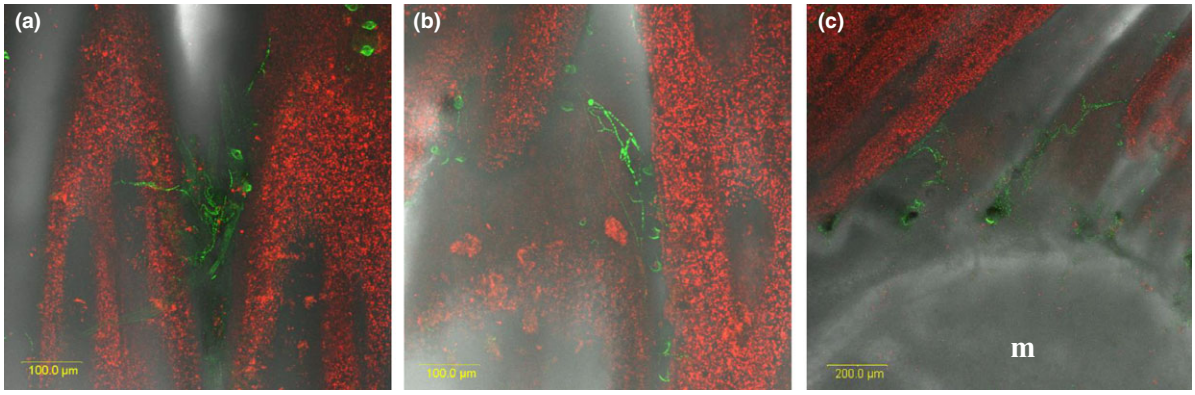

Figure 1 Confocal images of *Fusarium mangiferae* localization in developing vegetative mango buds. (a,b) Growth of mycelia between leaf primordia and developing leaves, without penetration into the leaf tissue; (c) growth of mycelia between leaf primordia, to the meristematic region, without penetration of the plant meristematic tissue. Green: GFP-labelled *F. mangiferae* mycelia; red: chlorophyll autofluorescence in developing leaves; m: meristematic region. Scale bars: (a,b) = 100 µm, (c) = 200 µm. [Colour figure can be viewed at wileyonlinelibrary.com]

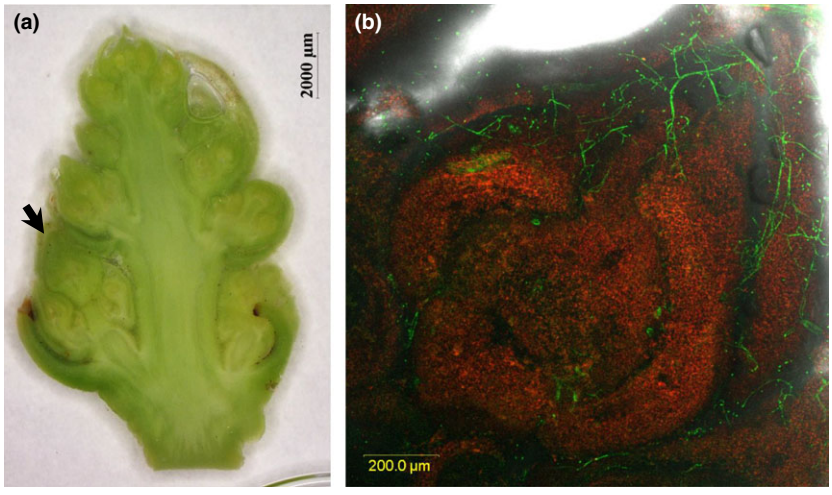

Figure 2 *Fusarium mangiferae* localization in an early developing reproductive mango bud. Mycelial growth between scales and developing organs of the flower without penetration into the internal tissues. (a) Stereomicroscope image of a hand section of a developing inflorescence. (b) Confocal image of a developing flower in the same hand section. Green: GFP-labelled *F. mangiferae* mycelia; red: chlorophyll autofluorescence in developing plant organs; arrow denotes the specific developing flower presented in (b). Scale bars: (a) = 2000 µm, (b) = 200 µm. [Colour figure can be viewed at wileyonlinelibrary.com]

Similar interactions were observed with *F. mangiferae* mycelia and female flower organs, the gynoecium and the style (Fig. 4). The mycelia present between the inner floret organs also surrounded the developing carpels (Fig. 4b). Specific growth could be detected around the style (Fig. 4b). As in the anthers, mycelia remained viable encompassing the fully developed styles for weeks after flower blooming and anthesis (Fig. 4h). However, unlike other tissues, specific growth of the fungal filaments within the developing style was detected (Fig. 4b,d), penetrating into the developing carpels (Fig. 4d). In some cases, mycelia were present within the inner carpelate space surrounding the ovule (Fig. 4f). This phenomenon was manifested by specific interactions of mycelia with the style and their penetration into the inner carpelate space in several pea-sized aborted fruitlets, several weeks post-anthesis. A continuum of mycelial filaments was observed growing along the surface of the style, through its central transducing tract into the inner carpelate space (Fig. 5).

Fusarium mangiferae saprophytic stage during inflorescence senescence, necrosis and collapse

Malformed inflorescences, forming cauliflower-like clusters, remained viable for several weeks post-anthesis. Throughout this period, specific localization of *F. mangiferae* filaments was detected with the anthers and styles. At a certain stage, the inflorescences started to senesce, collapse and dry (Fig. 6). During this process, necrotic lesions started to appear, concurrent with rapid fungal growth from the surface of organs toward the centre of the tissue (Fig. 6a,b). At this stage, a mass of conidia was produced and conidia were dispersed from the drying malformed inflorescences (Fig. 6c,d).

Fusarium mangiferae colonization of bud, stem and leaf tissues as determined on selective media

As mentioned previously, most of the buds produced typical floral and vegetative MMD symptoms. Microscopic

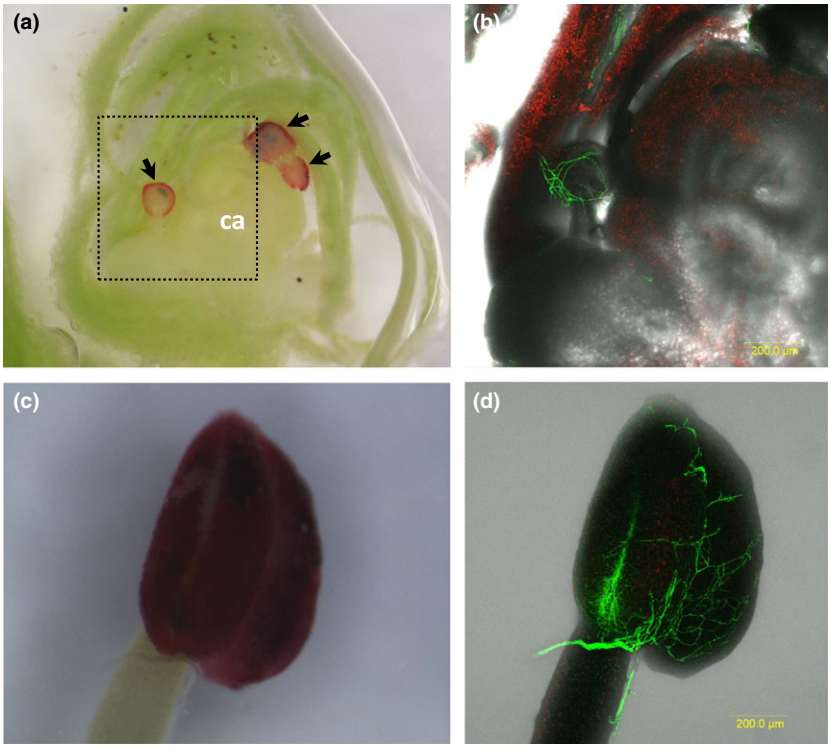

Figure 3 Specific interactions of *Fusarium mangiferae* and mycelial growth surrounding developing and mature stamens. (a,b) Fungal localization in a developing closed mango flower; (c,d) mycelial growth surrounding the locales of fully developed mature and fertile stamens. (b,d) Confocal images of green GFP-labelled *F. mangiferae* mycelia and red chlorophyll autofluorescence in developing flower organs. (a,c) Stereomicroscope images of the same flowers presented in (b) and (d), respectively. Arrows denote developing stamens, ca, developing carpel. Dashed box in (a) represents approximate region presented in (b). Scale bars: (b,d) = 200 µm. [Colour figure can be viewed at wileyonlinelibrary.com]

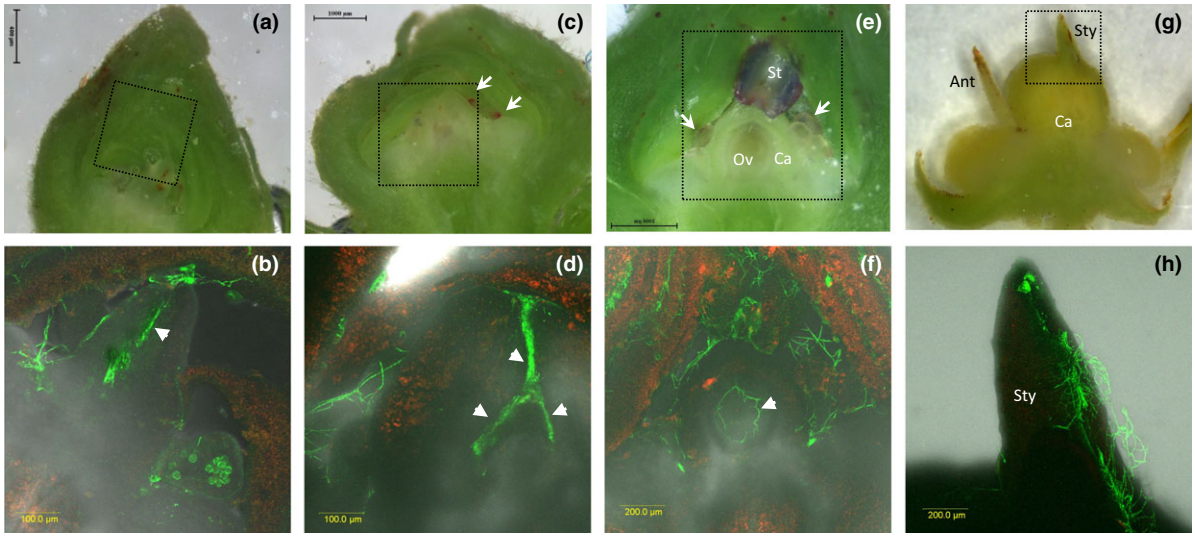

Figure 4 Specific interactions of *Fusarium mangiferae* with mango gynoecium during different stages of flower development. Stereomicroscope images of hand sections of flowers at different developmental stages (a,c,e,g), and confocal images of regions of the same flowers, respectively (b, d,f,h). Green: GFP-labelled *F. mangiferae* mycelia; red: chlorophyll autofluorescence in developing flower organs. Mycelial growth detected at the inner space of closed flowers (b,d,f), and on the developing carpels (b,d). Mycelia detected in the centre of the developing style (b,d), and surrounding the ovule within the inner carpel space (f). Specific interaction detected on the style of a fully open flower with developed internal organs in a malformed inflorescence (boxed region of g shown in h). *Fusarium mangiferae* mycelia also detected in protected places between sepals and petals (d,f), and surrounding degenerating (d) and fertile developing (f) stamens. Ca, carpel; Sty, style; Ov, ovule; St, fertile stamen; Ant, anther of a previously fertile stamen in a developed malformed inflorescence. Arrows denote degenerating stamens (c,e). Arrowheads highlight specific penetration of mycelia into developing style (b,d) or carpel (f). Dashed box in (a,c,e,g) represent approximate region presented in (b,d,f,h), respectively. Scale bars: (a) = 400 µm, (b,d) = 100 µm, (f,h) = 200 µm, (c,e) = 1000 µm. [Colour figure can be viewed at wileyonlinelibrary.com]

results of fungal localization in vegetative and reproductive buds were compared to their colonization in the tissue using PDA medium supplemented with hygromycin in five malformed shoots. Of all the vegetative sampled tissues, colonization by the pathogen was confirmed in 100% of the apical buds (5/5), 98% of the lateral buds

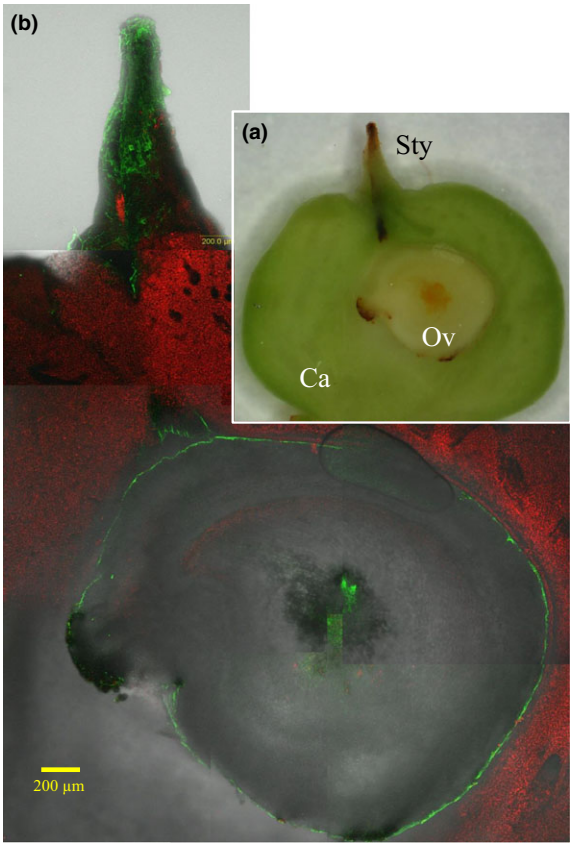

Figure 5 *Fusarium mangiferae* localization in degenerated pea-size mango fruitlet on a fully developed malformed panicle. Mycelial growth on the surface of the style and through the transducing stylar tract penetrating the inner carpel space, between the ovule and the inner carpelate wall. (a) Stereomicroscope image of a hand section of the degenerated fruitlet. (b) Confocal images of the same fruitlet; eight different confocal series were performed on different regions of a single mango fruitlet at approximately the same field depth. Images were manually aligned to form a composite image of the entire ovule within the carpel. Green: GFP-labelled *F. mangiferae* mycelia; red: chlorophyll autofluorescence; Ca, carpel; Sty, style; Ov, ovule. Scale bars in (b) = 200 µm. [Colour figure can be viewed at wileyonlinelibrary.com]

(85/87), 13.2% of the xylem tissue (9/68), 12.8% of the internodes (12/129) and 83.8% of leaves (166/198 discs from 33 leaves) (Fig. 7a).

For comparison, leaves were collected from a heavily infected orchard in Sde Nitzan. The pathogen was not isolated from any of the sampled leaf discs (0/80) that originated below symptomless panicles. However, 18.8% discs from leaves (15/80) sampled below panicles with MMD symptoms were colonized by the pathogen (Fig. 7b).

Discussion

The current study followed the localization of *F. mangiferae* and its interaction with mango organs during both vegetative and reproductive development. A summary of fungal localization in parallel to stages of plant vegetative and reproductive development is presented in Table 1. In general, fungal localization appears to be epiphytic. During most of the vegetative or reproductive development, mycelia of the pathogen remained in close contact with the plant surface but did not penetrate the tissue, unless in localized damaged areas such as wounds. These results are in contrast to several studies that reported *F. mangiferae* mycelial growth and penetration between and into cells of infected tissues (Usha *et al.*, 1997; Babu & Rao, 1999; Iqbal *et al.*, 2010). However, the methods used in this study, both the high resolution of confocal microscopy as well as fresh and non-fixed infected plant material, support the accuracy of the findings. Under different environmental conditions, tissue necrosis may occur at earlier stages of flower development, which may allow the penetration of fungal hyphae into developing tissue.

It appears that the interaction of *F. mangiferae* with its mango host tissue is epiphytic by nature. However, fungal growth is usually confined to specific protected regions, between the scales of vegetative (Fig. 1) and reproductive (Fig. 2) buds, between the developing florets of inflorescences and between the primordia and various organs of closed flowers (Figs 3 & 4). All these locations are niche environments, protected from outside conditions. They provide appropriate environmental conditions, specifically with high humidity, enabling survival and growth of mycelia. Once plant growth occurs and the organs (shoots, leaves, petals or sepals) expand and become exposed to external conditions, fungal growth ceases. Only a few mycelial cells were detected on these exposed organs, mostly associated with damaged tissue, or with leaf trichomes, that may provide some protection for the fungus. *Fusarium mangiferae* mycelia and conidia senesce and die rapidly when exposed to adverse conditions, as shown by limited survival of conidia, from 2 to 4 h, when exposed to direct UV sunlight (Freeman *et al.*, 2014b).

Specific interactions of fungal mycelial filaments with the flower reproductive organs, stamens (Figs 3 & 4) and gynoecium (Fig. 4), were observed. Mycelial growth, surrounding and exterior to these organs, was detected at early developmental stages. Moreover, unlike the external flower organs, mycelia continued to grow on stamens and styles even after the flowers opened and were exposed to external conditions (Figs 3d & 4h). Similar results pertaining to specific interactions of mycelia with essential flower organs (stamen and gynoecium) were previously detected by histopathology methods (Babu & Rao, 1999). Mycelia on the fully developed anthers and styles remained viable for several weeks, throughout the period of extended viability of the malformed clusters, until necrosis and final collapse. These interactions with the stamens and gynoecium are in contrast to mycelial disappearance from other flower organs once they become exposed to external conditions.

One exception to the epiphytic nature of *F. mangiferae* and interaction with the host is its specific penetration

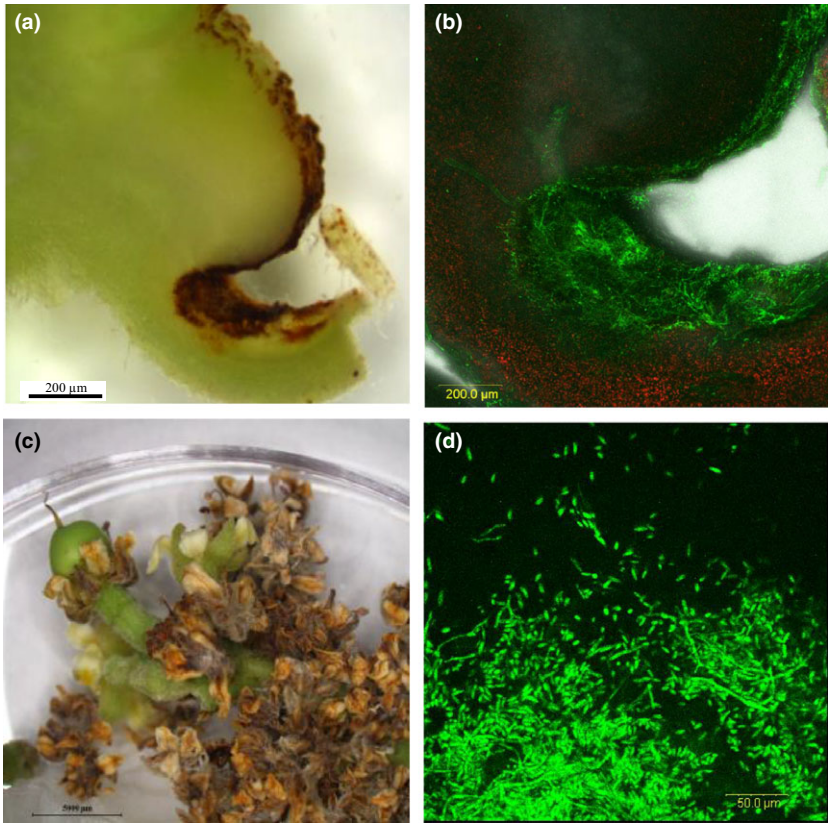

Figure 6 Saprophytic growth of GFP-labelled *Fusarium mangiferae* during senescence, necrosis and collapse of 3-month-old malformed inflorescences; (a,b) massive fungal growth from the surface of organs toward the centre of the tissue on a malformed flower; (c) senescent panicles with necrotic lesions, collapsed and dried tissues; (d) fungal growth and conidia on panicles. Scale bars: (a) = 500 µm, (b) = 200 µm, (c) = 5000 µm and (d) = 50 µm. [Colour figure can be viewed at wileyonlinelibrary.com]

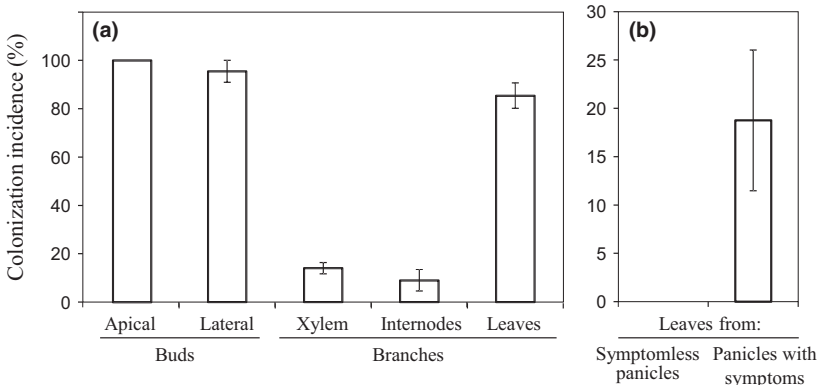

Figure 7 Colonization of mango organs by *Fusarium mangiferae*, causal agent of mango malformation disease, as determined on selective medium. (a) Plant organs originated from symptomless shoots that were artificially inoculated at the apical buds with the GFP-expressing isolate; all plant organs, besides the apical buds, were symptomless. (b) Leaves sampled from a heavily infected orchard below panicles with or without symptoms. Vertical bars indicate standard error of the mean ($P < 0.05$).

into the carpel's inner space in aborted fruitlets (Fig. 5). Even in this case, mycelia did not penetrate directly through the surface of the carpel wall, but only via a natural aperture, the stylar tract. Such penetration was also detected during development of the style (Fig. 4b,d) and in the growing carpel of closed flowers (Fig. 4f). Specific infection of mycelia into the carpel through the stylar tract was also observed in another histopathology study (Babu & Rao, 1999). The structure of this channel allows penetration and growth of pollen tubes into the carpel, through the style toward the ovule. However, because affected fruitlets usually abort, localization of the fungus within carpels is not expected to play a major role in epidemiology of MMD. Previous studies in heavily infected orchards detected infection in the skins of fruits but not the seeds, seed coats or flesh (Youssef *et al.*, 2007). Recently, *F. mangiferae* was isolated from seed coats and cotyledons of apparently healthy mango fruits from heavily infected mango orchards in Egypt (M. A. A. Sattar, S. Youssef, The Agriculture Research Center (ARC), Giza, Egypt, personal communication). However, the implications of fungal dissemination via these structures will need additional, detailed work.

Infection of *F. mangiferae* during MMD development in the host is not systemic, but rather localized (Gamliel-Atinsky *et al.*, 2009c; Freeman *et al.*, 2014c). It was

Table 1 Summary of *Fusarium mangiferae*–mango interactions at different stages of vegetative and reproductive malformation development, and during inflorescence senescence, necrosis and collapse

Developmental stage	Fungus location
(a) Vegetative malformation development	
Dormant bud	Bud is colonized prior to inflorescence development. Mycelia located between scales
Bud swelling	Mycelia located between scales
Bud break. Early growth and development of vegetative bud	Mycelia grow in protected sites between scales and developing leaves
Shoot growth, stem elongation and leaf expansion	Mycelia are limited to apical meristems and developing axillary buds (or within tissue at damaged sites)
Fully exposed leaves	Limited survival of hyphae on leaves, sometimes in association with trichomes
(b) Reproductive malformation development	
Dormant bud	Bud is colonized prior to inflorescence development. Mycelia located between scales
Bud swelling	Mycelia located between scales
Bud burst, differentiation and development of floral primordia, and early panicle development	Mycelia grow in protected sites between and within flowers and scales
Development of inner plant organs stamens and gynoecium	Mycelia grow between internal organs
Flower maturation, carpel growth and flower opening	Mycelia grow surrounding internal organs, stamens and style
Development and growth of malformed inflorescence	
Flower organs remain viable for several weeks up to a few months	Mycelia are established on internal flowers organs
Aborted fruitlets (3–8 mm) remain on the inflorescences, and do not drop	Penetration of hyphae into the carpel (through the style and transducing tract and growth within the carpel)
(c) Inflorescence senescence, necrosis and collapse	
Necrotic regions appear on flowers and inflorescence organs	Dense growth of mycelia in association with necrotic tissues
Inflorescence senescence and collapse	Saprophytic growth, generation and formation of conidia

shown that the primary sites of infection of the pathogen are the vegetative and floral buds, while systemic colonization of older, subtending tissues does not occur (Ploetz & Freeman, 2009). Similarly, in this study, it was shown that the pathogen typically colonized the apical and lateral buds (95–100%) while colonization of the xylem and internode branch tissues were significantly lower (5–15%), probably due to samples originating close to affected buds (Fig. 7), but not due to a systemic nature of infection. Leaves from both GFP-inoculated plants and those sampled and growing under panicles with symptoms from a naturally infected orchard, showed significant percentages of colonization, in contrast to limited detection of the GFP-marked strain from leaves using confocal microscopy (Fig. 7). This may be explained by the limited area covered by microscopy, as opposed to plate assays, in which even very small quantities of fungal structures (mycelium and/or conidia) are sufficient for pathogen isolation and detection. In addition, detection of the GFP-marked strain in leaves was visualized by confocal microscopy associated with trichomes, where it may survive. These structures in leaves may provide protection for the fungus allowing identification in plate assays, after surface sterilization. In contrast, fungal cells can survive within buds of the mango host for many years, as long as they are protected. Thus, dormant buds can remain quiescently infected for many years (Freeman *et al.*, 2014c). Because the pathogen does not colonize the plant systemically within the vasculature, or by continuous growth throughout entire plant organs, the few mycelial cells detected on exposed stems or leaf sections may only be remnants of the mycelia that grew on these organs during their development. While mycelia were only sporadically visualized by fluorescent microscopy of exposed organs (such as leaves), isolation of the pathogen on selective media or identification by PCR may enable more sensitive detection from such infected tissues.

In general, the level of infection of plant material by *F. mangiferae* is low. Only minute amounts of the pathogen were detected in buds or flowers during most stages of growth. However, these very low fungal levels are sufficient to cause dramatic effects on the morphology and viability of the infected inflorescences, causing the typical cauliflower form of infected panicles and vegetative symptoms of infected hypertrophied, tightly bunched young shoots with dwarfed leaves. Although mycelia of the pathogen do not infect systemically, the intricate interactions of the fungus with the plant at the cellular level appears to involve secretion of hormones and/or effectors that modify and cause an internal imbalance in the infected tissues without actual penetration into the host tissues (Nicholson & van Staden, 1988; van Staden & Nicholson, 1989; Chakrabarti, 2011; Freeman *et al.*, 2014c). The close proximity of mycelia to meristems, the sites where differentiation of organs and their pattern formation occurs, may facilitate the deformation of these organs even under low pathogen levels. Contrary to the

infection process of *F. mangiferae*, the fungal pathogen *Taphrina deformans* causing leaf curl of stone fruits by way of indole acetic acid (IAA) production, is reported to infect host tissue by intercellular mycelial colonization of host tissues (Ogawa *et al.*, 1995).

The last stage of MMD infection involves necrosis of the malformed inflorescence. This parallels rapid growth and spread of a mass of mycelia. Fungal proliferation developed at the edge of healthy tissue and progressed together with necrosis. A close interaction of the pathogen with its host, even during necrosis, suggests that the fungus plays an active role in controlling the process of inflorescence collapse. *Fusarium mangiferae* modifies the hormonal balance of the plant to enable its prolonged viability (Ploetz, 2001; Freeman *et al.*, 2014c). It is speculated that a change in the hormones or effectors secreted by the fungus terminates the ability of the inflorescence tissue to retain its viability, thus inducing its collapse. However, further studies are required to assess this hypothesis.

In summary, the interaction of *F. mangiferae* with mango tissue is mainly epiphytic. Mycelial growth occurs only at protected niches in vegetative and reproductive buds, and usually does not survive in exposed organs. However, specific interactions with stamens and gynoecium allow extended survival of the pathogen on developing malformed panicles. The present results support the limited local infection mechanism of *F. mangiferae* in mango buds.

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References

Babu AK, Rao K, 1999. Histopathology of floral organs of *Mangifera indica* L. (*Anacardiaceae*) as affected by *Fusarium moniliforme* J. Sheld. *Phyton* **39**, 239–49.

Britz H, Steenkamp ET, Coutinho TA, Wingfield BD, Marasas WFO, Wingfield MJ, 2002. Two new species of *Fusarium* section *Liseola* associated with mango malformation. *Mycologia* **94**, 722–30.

Chakrabarti DK, 2011. *Mango Malformation*. Dordrecht, Netherlands: Springer.

Crespo M, Cazorla FM, de Vicente A *et al.*, 2016. Genetic diversity of *Fusarium tupiense*, the main causal agent of mango malformation disease in orchards in the south of Spain. *Plant Disease* **100**, 276–86.

Davenport T, 2009. Reproductive physiology. In: Litz RE, ed. *The Mango: Botany, Production and Uses*. Wallingford, UK: CABI, 97–169.

FAOSTAT, 2015. FAO Statistics Division. [http://faostat3.fao.org/home/E]. Accessed 14 November 2016.

Freeman S, Maimon M, Pinkas Y, 1999. Use of GUS transformants of *Fusarium subglutinans* for determining etiology of mango malformation disease. *Phytopathology* **89**, 456–61.

Freeman S, Maymon M, Biton A, Levin AG, Shtienberg D, 2014a. Management of mango malformation disease based on a novel strategy of timing of fungicide applications combined with sanitation. *Crop Protection* **61**, 84–91.

Freeman S, Otero-Colina G, Rodríguez-Alvarado G *et al.*, 2014b. First report of mango malformation disease caused by *Fusarium pseudocircinatum* in Mexico. *Plant Disease* **98**, 1583.

Freeman S, Shtienberg D, Maymon M, Levin AG, Ploetz RC, 2014c. New insights into mango malformation disease epidemiology lead to a new integrated management strategy for subtropical environments. *Plant Disease* **98**, 1456–66.

Gamliel-Atinsky E, Freeman S, Sztejnberg A *et al.*, 2009a. Interaction of *Aceria mangiferae* with *Fusarium mangiferae*, the causal agent of mango malformation disease. *Phytopathology* **99**, 152–9.

Gamliel-Atinsky E, Sztejnberg A, Maymon M, Shtienberg D, Freeman S, 2009b. Inoculum availability and conidial dispersal patterns of *Fusarium mangiferae*, the causal agent of mango malformation disease. *Phytopathology* **99**, 160–6.

Gamliel-Atinsky E, Sztejnberg A, Maymon M, Vintal H, Shtienberg D, Freeman S, 2009c. Infection dynamics of *Fusarium mangiferae*, causal agent of mango malformation disease. *Phytopathology* **99**, 775–8.

García-López E, Mora-Aguilera JA, Nava-Díaz C *et al.*, 2016. First report of *Fusarium pseudocircinatum* causing mango malformation disease in Dominican Republic. *Plant Disease* **100**, 1501.

Hernández Delgado PM, Aranguren M, Reig C *et al.*, 2011. Phenological growth stages of mango (*Mangifera indica* L.) according to the BBCH scale. *Scientia Horticulturae* **130**, 536–40.

Iqbal Z, Hameed S, Anjum MA, Dasti AA, Saleem A, 2010. Cytology of infection of *Fusarium mangiferae* Britz in different malformed reproductive parts of mango. *European Journal of Plant Pathology* **127**, 391–8.

Kumar J, Singh US, Beniwal SPS, 1993. Mango malformation: one hundred years of research. *Annual Review of Phytopathology* **31**, 217–32.

Liew ECY, Laurence MH, Pearce CA *et al.*, 2016. Review of *Fusarium* species isolated in association with mango malformation in Australia. *Australasian Plant Pathology*. doi: 10.1007/s13313-016-0454-z.

Lima CS, Monteir JHA, Crespo NC, Costa SS, Leslie JF, Pfenning LP, 2009. VCG and AFLP analyses identify the same groups in the casual agents of mango malformation in Brazil. *European Journal of Plant Pathology* **123**, 17–26.

Lima CS, Pfenning LH, Costa SS, Abreu LM, Leslie JF, 2012. *Fusarium tupiense* sp. nov., a member of the *Gibberella fujikuroi* complex that causes mango malformation in Brazil. *Mycologia* **104**, 1408–19.

Marasas WFO, Ploetz RC, Wingfield MJ, Wingfield BD, Steenkamp ET, 2006. Mango malformation disease and the associated *Fusarium* species. *Phytopathology* **96**, 667–72.

Mukehrjee SK, Litz RE, 2011. Introduction: botany and importance. In: Litz RE, ed. *The Mango: Botany, Production and Uses*. Wallingford, UK: CABI, 1–18.

Nicholson RID, van Staden J, 1988. Cytokinins and mango flower malformation. I. Tentative identification of the complement in healthy and malformed inflorescences. *Journal of Plant Physiology* **132**, 720–4.

Noriega-Cantú DH, Téliz D, Mora-Aguilera G *et al.*, 1999. Epidemiology of mango malformation in Guerrero, Mexico, with traditional and integrated management. *Plant Disease* **83**, 223–8.

Ogawa JM, Zehr EI, Bird GW, Ritchie K, Uriu K, Uyemoto JK, 1995. *Compendium of Stone Fruit Diseases*. St Paul, MN, USA: APS Press.

Otero-Colina G, Rodríguez-Alvarado G, Fernández-Pavía S *et al.*, 2010. Identification and characterization of a novel etiological agent of mango malformation disease in México, *Fusarium mexicanum* sp. nov. *Phytopathology* **100**, 1176–84.

Ploetz RC, 2001. Malformation: a unique and important disease of mango, *Mangifera indica* L. In: Summerell BA, Leslie JF, Backhouse D, Bryden WL, eds. Fusarium: *Paul E. Nelson Memorial Symposium*. St Paul, MN, USA: APS Press, 233–47.

Ploetz RC, Freeman S, 2009. Foliar, floral and soilborne diseases. In: Litz RE, ed. *The Mango: Botany, Production and Uses*. Wallingford, UK: CABI, 231–302.

Ploetz R, Zheng QI, Vazquez A, Abdel Sattar MA, 2002. Current status and impact of mango malformation in Egypt. *International Journal of Pest Management* **48**, 279–85.

Ramírez F, Davenport TL, 2010. Mango (*Mangifera indica* L.) flowering physiology. *Scientia Horticulturae* **126**, 65–72.

Ramírez F, Davenport TL, 2016. Mango (*Mangifera indica* L.) pollination: a review. *Scientia Horticulturae* **203**, 158–68.

Ramírez F, Davenport TL, Fischer G, Pinzón JCA, Ulrichs C, 2014. Mango trees have no distinct phenology: the case of mangoes in the tropics. *Scientia Horticulturae* **168**, 258–66.

Senghor AL, Sharma K, Kumar PL, Bandyopadhyay R, 2012. First report of mango malformation disease caused by *Fusarium tupiense* in Senegal. *Plant Disease* **96**, 1582.

van Staden J, Nicholson RID, 1989. Cytokinins and mango flower malformation. II. The cytokinin complement produced by *Fusarium moniliforme* and the ability of the fungus to incorporate (8-^{14}C) adenine into cytokinins. *Physiological and Molecular Plant Pathology* **35**, 423–31.

Steenkamp ET, Britz H, Coutinho TA *et al.*, 2000. Molecular characterization of *Fusarium subglutinans* associated with mango malformation. *Molecular Plant Pathology* **1**, 187–93.

Summanwar AS, Raychaudhuri SP, Phatak SC, 1966. Association of the fungus *Fusarium moniliforme* Sheld. with the malformation in mango (*Mangifera indica* L.). *Indian Phytopathology* **19**, 227–8.

Usha K, Goswami AM, Sharma HC, Singh B, Pande PC, 1997. Scanning electron microscopic studies on floral malformation in mango. *Scientia Horticulturae* **71**, 127–30.

Youssef SA, Maymon M, Zveibil A *et al.*, 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**, 257–63.