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Guava Decline: A Complex Disease Involving *Meloidogyne mayaguensis* and *Fusarium solani*

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Abstract

In Brazil, *Meloidogyne mayaguensis* has become a threat to guava production. Approximately a third of the cultivated area is infested, leading almost inevitably to the decimation of the orchards. Because parasitized trees develop rotten roots as the disease progresses, the possibility that a soil-borne pathogen could be involved was investigated. From several nematode-free or nematode-infested orchards, nearly 2000 root fragments were tested for bacteria and fungi. Positive isolations were obtained from nematode-infested areas only and were predominantly identified as *Fusarium* sp. In a 5-month microplot experiment, guava seedlings were uninoculated (control) or were inoculated with *M. mayaguensis* only or with this nematode and 21 days later with one of 11 *Fusarium* sp. isolates. A Scott–Knot analysis of several vegetative variables and of the extent of root rot allowed the generation of a dissimilarity dendrogram that indicated that four *Fusarium* sp. isolates were particularly associated with damage to the seedlings. Upon identification of these isolates as *Fusarium solani*, a 6-month microplot experiment was set up, in which guava seedlings were uninoculated or were inoculated with one of the following: (i) *M. mayaguensis* only, (ii) four *F. solani* isolates, separately, (iii) four *F. solani* isolates separately, combined with physical injury of the roots with a knife, (iv) *M. mayaguensis,* and 21 days later with four *F. solani* isolates, separately. No root rot and virtually no effect on all variables were observed in the seedlings inoculated with the fungus isolates, with or without physical injury. Major root rot and a negative effect on all variables were observed in the seedlings inoculated with *M. mayaguensis* and all four *F. solani* isolates. This characterizes guava decline as a complex disease caused by the synergistic effect of these organisms, in which parasitism by the nematode predisposes the plants to root decay caused by the fungus.

Introduction

In Brazil, guava (*Psidium guajava* L.) decline associated with *Meloidogyne mayaguensis* Rammah and Hirschmann, 1988 has become the main health problem of this fruit crop. Nationwide, the nematode-infested area is estimated to be approximately 5000 ha spread over 16 States, and the economic impact of this disease on guava growers has been calculated to be around US$ 66 million as of 2008 (Pereira et al. 2009).

In Brazil, *M. mayaguensis*-infected guava seedlings and healthy seedlings transplanted into heavily nematode-infested fields often die within months. By contrast, mature guava trees may have galled roots for several months without secondary shoot symptoms. The onset or worsening of shoot symptoms is often associated with (i) an increase in the number of root galls in the entire root system, (ii) following a high production season or (iii) following drastic pruning, which is used by some growers to synchronize production.

Declining trees show chlorosis, scorching of margin, wilting and falling of leaves. Gomes et al. (2008) have shown that these symptoms are associated with foliar deficiency of nitrogen, potassium and phosphorus, a near-deficiency of calcium and magnesium and an unbalanced accumulation of manganese, chlorine and sodium. Underground, declining trees develop progressive rotting of the entire root system. Usually, declining trees do not recover, and death occurs within weeks to a few months. This sequence of symptoms has been described as Stages 1–3 (Gomes 2007). A 2-year experiment conducted in commercial orchards showed high yield losses associated with nematode population densities as low as 25 root galls per 10 g of roots and 10 second-stage juveniles per 100 cc of soil (Gomes et al. 2010).

The severity of guava decline and its associated root rot has triggered an investigation into whether a soil-borne pathogen could be involved in this pathosystem,

as it occurs in several complex diseases involving *Meloidogyne* spp. In addition to causing wilt diseases when associated with *formae speciales* of *Fusarium oxysporum*, *Meloidogyne* spp. are known to cause root rotting when in complex with *Rhizoctonia solani*, *Sclerotium rolfsii*, *Cylindrocladium crotalariae*, *Phytophthora parasitica* f.sp. *nicotianae*, *P. megasperma* f.sp. *medicaginis*, *Thielaviopsis basicola*, *Pythium ultimum or P. aphanidermatum* (Powell 1971; Webster 1985; Hussey and McGuire 1987; Mai and Abawi 1987; Evans and Haydock 1993; Abawi and Chen 1998; Back et al. 2002).

We report here the results of laboratory and two field studies that established the complex nature of guava decline, which is caused by a synergistic association between *M. mayaguensis* and *Fusarium solani* (Mart.) Sacc.

Materials and Methods

Field samplings

Four nematode-free and four nematode-infested commercial orchards (cv. Paluma) located in the municipality of São João da Barra, southeast Brazil (21°38′24″S, 41°03′03″W) were sampled on different occasions from January through November 2006. In each orchard, 500 g root samples were collected separately from eight different guava trees. In the laboratory, 24 root fragments from each of the 64 root samples were randomly selected from the main, secondary and tertiary roots in equal proportions, totalling 1536 fragments that were used for bacterial and fungus isolations.

Additionally, seven root samples from declining orchards were received from seven municipalities in the States of Paraná, Mato Grosso do Sul and Pernambuco, in the south, midwest and northeast regions of Brazil, respectively.

Bacterial isolation

Seven hundred and sixty-eight root fragments (12 from each tree sampled) were cleansed, disinfested in 50% alcohol for 25 s and Qboa® commercial bleach (*c*. 2% sodium hypochlorite) for 3 min and rinsed with sterile distilled water (SDW) (Romeiro 2001). The fragments were individually macerated in drops of SDW and then placed on culture medium 523 in Petri dishes (Kado and Heskett 1970). The dishes were incubated for 3 days at 25°C and 12-h photoperiod before being examined for bacterial growth.

Fungi isolation and identification

Seven hundred and sixty-eight root fragments were cleansed, disinfested in 70% alcohol for 1 min and Qboa® for 1 min and rinsed with SDW (Dhingra and Sinclair 1995). The fragments were incubated for 7 days at 27°C and 12-h photoperiod in Petri dishes with potato-dextrose-agar (PDA) medium amended with 500 p.p.m. of streptomycin sulphate. The resulting fungal colonies were subcultured and, upon sporulation, they were identified at the genus or species level in lactophenol glass slides observed under light microscope, with or without staining with cotton blue or fuchsine, according to the descriptions by Ellis (1971, 1976), Barnett and Hunter (1972) and Sutton (1980).

Selection and identification of *Fusarium* sp. isolates

Of the fungi isolated from the roots of declining guava trees (see Results), *Fusarium* sp. was the only genus that was further investigated. Based on the morphology of the colonies in Petri dishes with PDA medium, macro and microconidia and chlamydospores, 11 *Fusarium* sp. isolates (referred to hereafter as UENF/CF 160–170) were selected as representatives of the diversity of the 68 isolates obtained from the orchards in São João da Barra. These 11 isolates were used in the first field experiment.

Based on the results of the first experiment (see below), a second experiment was conducted with the isolates UENF/CF 160, 163, 164 and 170. These isolates were first subcultured from single spores and identified as *F. solani* based on their morphology by Dr. Ludwig H. Pfenning (Dept of Phytopathology, Universidade Federal de Lavras, Brazil). This identification was confirmed by PCR amplification of the rDNA ITS region, using primers for ITS4 and ITS5, by Dr. Robert Miller (Universidade de Brasília, Brazil).

Preservation of *Fusarium* sp. isolates and inoculum production

All isolates were preserved in glass tubes with PDA medium at 7°C, on wheat grains stored in glass vials maintained at 4°C, and at room temperature mixed with silica gel in the dark (Dhingra and Sinclair 1995).

The inoculum was produced following a modification of the method by Burgess et al. (1994): 50 g of whole wheat grains was embedded in distilled water overnight at 5°C, placed in plastic bags and autoclaved. A mixture of hyphae and conidia was collected from the colonies maintained on PDA, diluted in autoclaved distilled water and applied to the wheat grains. The plastic bags were incubated at 25°C and 12-h photoperiod until the wheat grains were entirely colonized by the fungus.

Microplot experiment 1

To investigate whether *Fusarium* sp. was involved in the decline of guava, 52 cv. Paluma seedlings (30–40 cm high) were transplanted into 25-l plastic pots partially buried in a nematode-infested orchard. To avoid using either nematode- or *Fusarium*-infested soil, the pots were filled with sandy soil from a nearby area with natural vegetation. During the experiment, care was taken to avoid soil spills into the pots. The irrigation water was treated with Qboa® 24 h prior to use. The plants were fertilized monthly with nitrogen, potassium, copper, borum and zinc following recommendations by Pereira (1995), and they were drip irrigated as required.

Four seedlings were kept as uninoculated controls, being transplanted into autoclaved soil. At the transplant, the remaining 48 seedlings were inoculated with 10 g of chopped tomato roots with *M. mayaguensis*-induced galls, which were mixed with the soil. Twenty-one days later, isolates UENF/CF 160–170 were separately inoculated on four seedlings each by mixing

10 g of colonized wheat grains into the top layer of the soil. The 13 treatments (T1, uninoculated control; T2, nematode inoculated; T3–T13, inoculated with the nematode and the fungus) were arranged in randomized blocks, with four replicates.

Five months after transplantation, the plants were carefully removed from the pots, and the soil was washed off the roots. The following variables were assessed: number of leaves per plant, fresh and dry weight of shoot and root, root system depth (distance from the plant's collar region to the most distal rootlet), root system volume (total, rotted and unrotted, calculated through water displacement in a graduated cylinder) and estimated root system length. This estimate was obtained by applying the line-intersect method (Tennant 1975) to three 10 g root aliquots from the top, medium and lower regions of the plant's root system. The average length obtained was related to the weight of the plant's entire root system.

At the end of the experiment, eight 1 cm long root fragments were randomly selected from each of the 52 experimental plants and processed for fungus re-isolation as described before. When *Fusarium* sp. was re-isolated, it was subcultured on PDA medium, and its morphological characteristics were compared to the previously inoculated isolate.

The original data (not transformed) was analysed using ANOVA and compared by the Scott–Knot method at 5% probability. The results were also analysed by the Ward method to generate a dissimilarity dendrogram based on Euclidean distance (Mardia et al. 1997).

Microplot experiment 2

This experiment was conducted with the most damaging *F. solani* isolates in the first experiment: UENF/CF 160, 163, 164 and 170. Eighty-four cv. Paluma seedlings (30–40 cm high) were transplanted into partially buried 25-l plastic pots filled with washed river-bed sand. The plants' fertilization and irrigation, the nematode and fungus inoculations and the fungus re-isolation at the end of the experiment were conducted as in the first experiment.

Six seedlings were kept as uninoculated controls (T1), being transplanted into autoclaved sand. At the transplantation, the following treatments were established: T2 (inoculation with *M. mayaguensis* only), T3–T6 (inoculation with isolates UENF/CF 160, 163, 164, and 170 only, separately), T7–T10 (inoculation with the fungus isolates cited above, combined with physical injury of the roots inflicted by four knife strokes around each seedling, at the depth of 0–20 cm), and T11–T14 (inoculation with *M. mayaguensis*, followed 21 days later with the fungus isolates cited above). All treatments were arranged in randomized blocks, with six replicates.

Six months after transplant, the plants were carefully removed from the pots, and the soil was washed off the roots. The following variables were assessed: fresh weight of shoot and root, root system volume (total, rotted and unrotted, calculated through water displacement in a graduated cylinder), foliar area and chlorophyll content, using a SPAD-502® (Minolta, Tokyo, Japan) chlorophyll meter. The original data (not transformed) were analysed using ANOVA and compared by the Tukey test at 5% probability.

Results

No bacteria were isolated from root tissues collected in São João da Barra orchards. In addition, no fungi were isolated from nematode-free orchards. In nematode-infested orchards, the following fungi were isolated: *Fusarium* sp., *Chaetomium* sp., *Penicillium* sp., *Trichoderma* sp., *Aspergillus niger*, *Nigrospora* sp., *Monilia* sp. and *Rhizoctonia* sp. Of the 68 *Fusarium* sp. isolates obtained, 46 originated from guava trees that were in Stage 3 of decline, while trees with mild symptoms (Stage 1) yielded only eight isolates.

From the samples received from different Brazilian regions, 448 root fragments were randomly selected and incubated. Fifty per cent were positive for *Fusarium* sp. Based on the morphology of the colonies in Petri dishes, 68 isolates (UENF/CF 234–301) were preserved for future studies (see Discussion).

The shoot and root system variables assessed in the first experiment are shown in Table 1. These data were also analysed by the Ward method and arranged in a dissimilarity dendrogram based on Euclidean distance (Fig. 1).

The shoot and root system variables assessed in the second experiment are shown in Table 2. The typical effect of *M. mayaguensis* and *F. solani* on the root system is shown in Fig. 2.

Discussion

The failure to isolate bacteria or fungi in samples from nematode-free orchard areas is consistent with the lack of reports of soil-borne bacteria and fungi parasitizing guava trees on their own in Brazil, despite the nation-wide cultivation of this crop (Marques et al. 1994; Piccinin and Pascholati 1997). It is important to notice that the procedures and culture media used for bacterial and fungi isolation are well established for a wide range of root pathogens. In contrast, several fungi were isolated from nematode-parasitized roots, with the frequency of *Fusarium* sp. reaching up to 50% of the root fragments randomly picked for incubation.

In the first experiment, a fungus-only treatment was not included. Nonetheless, this experiment was important in unveiling the nature of guava decline. No root rot or root galls occurred in the control plants (T1) (Table 1), whereas abundant root galls and some rotting occurred in the plants inoculated with the nematode only (T2). *Trichoderma* sp., *Penicillium* sp. and *Rhizoctonia* sp., but not *Fusarium* sp., were re-isolated from these plants. In contrast, *Fusarium* sp. was abundantly re-isolated from T3 to T13. The combination of the nematode and the 11 *Fusarium* sp. isolates caused various degrees of damage to the guava plants, with more damage being associated with more root rot.

Table 1
Root and shoot variables of guava plants grown in microplots in the field in São João da Barra, Brazil, 5 months after inoculation with *Meloidogyne mayaguensis* alone or combined with *Fusarium solani*

	Root system					Shoot weight (g)		Root weight (g)	
Treatments[a]	Estimated length (m)[b]	Depth (cm)[c]	Total volume (ml)[d]	Unrotten volume (ml)[d]	Number of leaves	Fresh	Dry	Fresh	Dry
T1	13.7 a[e]	56 a	113.2 a	113.3 a	82.2 a	98.1 a	53.5 a	107 a	23.4 a
T2	11.2 a	55.7 a	140 a	92.5 a	72.7 a	113.2 a	70.3 a	105.8 a	21.3 a
T3	7.3 b	55.5 a	149.2 a	74.3 b	44.7 b	95.7 a	53.6 a	138.5 a	27.7 a
T4	5.1 b	55.5 a	136 a	52.5 b	36.5 b	66.9 b	37.6 b	116.8 a	24.1 a
T5	13.2 a	52.4 a	141.2 a	64.3 b	61.5 a	114.8 a	60 a	126.3 a	25.3 a
T6	5.5 b	52 a	114.8 a	55.3 b	42.5 b	69.9 b	33.6 b	101.4 a	18 a
T7	1.9 c	40 b	97.5 a	8 b	20.7 c	27.2 b	14.4 c	81.9 b	13 b
T8	15.9 a	58 a	192.2 a	135.3 a	74.2 a	127.6 a	61 a	172.5 a	39.7 a
T9	6.4 b	50 a	142.5 a	54 a	46 b	115 a	47.8 b	131.2 a	27.5 a
T10	12.9 a	57.5 a	165.5 a	119.5 a	73.2 a	138 a	62.7 a	151.7 a	31.9 a
T11	11.7 a	52.6 a	165 a	99 a	71 a	132.4 a	61.2 a	136.6 a	28.2 a
T12	6.5 b	58.2 a	161.2 a	100 a	66.2 a	106.1 a	45.4 b	128.1 a	25.7 a
T13	5.5 b	53 a	139.7 a	53.5 b	22.7 c	57.5 b	26.9 b	129 a	25 a
Calculated F	4.38	2	1.07	2.68	3.19	2.47	1.57	1.89	2.24
CV (%)	22.7	6.8	17.3	36.3	24.7	24.9	30.1	13.7	17.1

Values followed by the same letter in the columns are not significantly different when compared through the Scott–Knot method at P = 0.05. Treatment degrees of freedom (Df) = 12; Df for error = 39; total Df = 51.
[a]T1: uninoculated control; T2: nematode inoculation; T3–T13: inoculation with a combination of the nematode and one of the isolates UENF/CF 160–170.
[b]Calculated through the intersect method (Tennant 1975).
[c]Distance from the plant's collar region to the most distal rootlet.
[d]Calculated through water displacement in a graduated cylinder.
[e]Values are average of four replicates (plants).

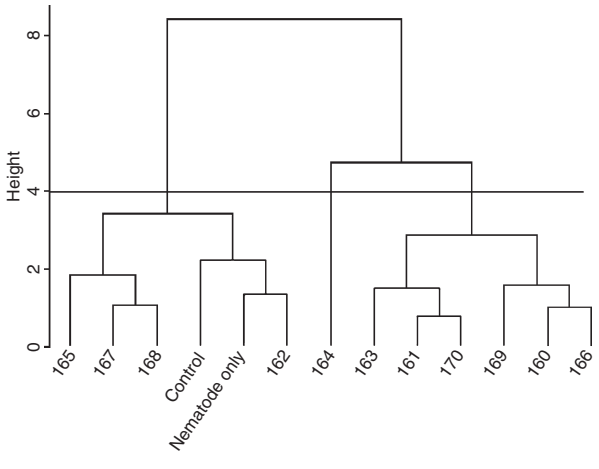

Fig. 1 Dissimilarity dendrogram based on Euclidean distance, calculated by the Ward method and based on the variables assessed in guava plants grown in microplots in the field, 5 months after inoculation with *Meloidogyne mayaguensis* alone or in combination with one of the *Fusarium* sp. isolates UENF/CF 160–170, in São João da Barra, Brazil. Uninoculated plants served as controls

The dissimilarity dendrogram (Fig. 1) indicated which *Fusarium* sp. isolates should be further investigated.

In the second experiment, no root rot occurred in the control plants (T1) (Table 2), whereas abundant root galls, some rotting and damage occurred in the plants inoculated with the nematode (T2). The fungi *Trichoderma* sp., *Penicillium* sp. and *Rhizoctonia* sp., but no *Fusarium* sp., were isolated from these plants. No rot occurred in the plants abundantly inoculated with the four isolates of *F. solani* (T3–T10), with virtually no effect on the other variables assessed. In sharp contrast, significant ($P < 0.05$) root rot and damage occurred when *M. mayaguensis* and *F. solani* were combined (T11–T14) (Fig. 2).

The synergistic effect of *M. mayaguensis* and *F. solani* in causing guava decline is clear, and this combination reflects the complex nature of the disease. Interestingly, a significant distinction ($P < 0.05$) between T7–T10 and T11–T14 suggests that a physiological factor is involved in the susceptibility of *M. mayaguensis*-parasitized guava plants to root rot, because the physical injuries inflicted on the roots did not enable *F. solani* to cause decay. Furthermore, this study indicates that in guava decline it is *F. solani*, not *M. mayaguensis*, the agent that actually defines the extension of the damage caused to the plant. The mechanism(s) by which *M. mayaguensis* predisposes guava trees to *F. solani* decay is presently under investigation.

One aspect of guava decline that remains unsolved is the possible, although unlikely, secondary role of other soil-borne fungi in this complex disease. Indeed, *Trichoderma* sp., *Penicillium* sp. and *Rhizoctonia* sp. have occasionally been isolated from guava orchards, and they may have been responsible for the modest yet statistically significant ($P < 0.05$) root rot observed in the second experiment (treatment T2), together with a reduction in foliar area and shoot weight.

The widespread incidence of guava decline in Brazil prompted the authors to request root samples from other Brazilian regions. The samples' late arrival precluded the use of the resulting *Fusarium* sp. isolates (UENF/CF 234–301) in the field experiments. A less time-consuming laboratory assay has been devised, which assesses the damage caused by these *Fusarium*

Table 2
Root and shoot variables of guava plants grown in microplots in the field in São João da Barra, Brazil, 6 months after inoculation with *Meloidogyne mayaguensis* or *Fusarium solani* alone or combined

Treatments[a]	Foliar		Root system volume (ml)[b]		Fresh weight (g)	
	Chlorophyll content[c]	Area (cm^2)	Total	Unrotten	Shoot	Root system
T1	48.9 a[d]	2171.7 a	139.3 a	139.3 a	125.2 a	144 a
T2	46 a	1425.5 b	104.5 a	93.8 b	76.3 b	112 ab
T3	49.3 a	1966.1 ab	122.8 a	122.8 ab	115.3 a	128 ab
T4	46.9 a	1914.2 ab	103.3 a	103.3 b	111.4 a	104.7 ab
T5	48.7 a	1673.3 ab	111.5 a	111.5 ab	110.6 a	113.1 ab
T6	49.4 a	1966.1 ab	111.8 a	111.8 ab	106 ab	117.6 ab
T7	48.3 a	1913.5 ab	110.3 a	110.3 ab	111.5 a	114.2 ab
T8	47.6 a	1873.4 ab	102.7 a	102.7 b	106.6 ab	103.1 b
T9	47.7 a	1968.7 ab	105.3 a	105.3 ab	119.4 a	118.1 ab
T10	48.6 a	1868.4 ab	115.2 a	115.2 ab	109.4 ab	124.7 ab
T11	38.7 b	364.5 c	35.8 b	2.7 c	28.6 c	43.1 c
T12	39.9 b	284.5 c	44.8 b	2.7 c	29 c	51.3 c
T13	39.6 b	239.9 c	28.8 b	0.3 c	21 c	37.3 c
T14	37.2 b	420.6 c	37 b	2.2 c	30.3 c	45.4 c
Calculated F	21.55	30.12	22.20	51.29	33.78	20.13
CV%	5.21	23.21	21.14	22.34	19.63	20.31

Values followed by the same letter in the columns are not significantly different when compared through Tukey test at P = 0.05. Treatment degrees of freedom (Df) = 13; Df for error = 70; total Df = 83.
[a]T1: uninoculated control; T2: nematode inoculation; T3–T6: inoculation with *F. solani* isolates UENF/CF 160, 163, 164, or 170, separately; T7–T10: inoculation with the fungus isolates cited above, combined with physical injury of the roots with a knife; T11–T14: inoculation with the nematode, followed 21 days later with the fungus isolates cited above.
[b]Calculated through water displacement in a graduated cylinder.
[c]Measured with a chlorophyll metter SPAD-502® (Minolta).
[d]Values are average of six replicates (plants).

Fig. 2 Root system of guava seedlings at conclusion of a 6-month microplot experiment in São João da Barra, Brazil. From left to right: uninoculated control; inoculated with *Fusarium solani* only; inoculated with *F. solani* only combined with physical injury of the roots with a knife; inoculated with *Meloidogyne mayaguensis* only; and inoculated with *M. mayaguensis*, followed 21 days later with *F. solani*

sp. isolates to guava stem cuttings, having the *F. solani* isolates UENF/CF 160, 163, 164 and 170 as pathogenic standards. The results (unpublished) confirm the nationwide role of *F. solani* in guava decline.

Other suspected associations between nematodes and fungi affecting guava have been reported. In Venezuela, Suárez et al. (1998) reported the concomitant incidence of *Meloidogyne arenaria*, *Meloidogyne incognita*, *Meloidogyne hapla*, *Meloidogyne javanica*, *Macrophomina phaseolina*, *F. oxysporum*, *Pythium* sp. and *Phytophthora* sp. Suarez et al. (1999) claimed a synergistic effect of *Macrophomina* sp. and *Fusarium* sp. with *Meloidogyne* spp., although their experimental results do not support their claim of a disease complex involving these pathogens because no significant difference ($P < 0.05$) was observed between the different combinations of those pathogens.

In India, Dwivedi and Dwivedi (1999) considered guava wilt to be a national problem, causing yield losses estimated to be *c*. 30%. This disease is reported to occur in nurseries and orchards. In the former, the leaves die acropetally, the stem stains blackish brown, and the seedlings die in patches throughout the nursery. In orchards, a partial or complete wilting of the tree is followed by drying and cracking of the hanging fruits, cracking of the tree bark and death. White rhizomorphs and black spots may be seen on the roots. From this report, it seems clear that guava wilt is a disease distinct from guava decline caused by *F. solani* and *M. mayaguensis* in Brazil.

According to Dwivedi and Dwivedi (1999) and Khan et al. (2001), the aetiology of guava wilt is uncertain, with the following fungi being listed as causal agents by different authors: *F. oxysporum* f.sp. *psidii*, *F. solani*, *F. longipes*, *F. moniliforme*, *Macrophomina phaseoli*, *M. phaseolina*, *Rhizoctonia bataticola*, *Cephalosporium* spp., *Cylindrocarpon* sp. and *Myxosporium psiddi*.

Some authors have suspected that nematodes, particularly *Helicotylenchus dihystera*, could be involved with guava wilt (Khan et al. 2001). Although this nematode has been reported to cause damage to seedlings on its

own (Willers and Gretch 1986), surveys conducted in healthy and diseased orchards in five Indian States, as well as experiments under controlled conditions, failed to obtain convincing evidence that *H. dihystera* is involved in guava wilt. In conclusion, accurate studies seem necessary to better characterize the aetiology of guava wilt and to establish the role (if any) of plant-parasitic nematodes.

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