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FULL RESEARCH PAPER

Molecular characterization of *Phoma tracheiphila*, causal agent of Mal secco disease of citrus, in Israel

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Abstract Mal secco disease of citrus caused by *Phoma tracheiphila* is a devastating disease in the Mediterranean basin. Susceptible citrus species include lemon, citron, lime and others. Trees attacked by the fungus show characteristic symptoms; the smallest twigs die first, followed by the larger branches. Eventually, the whole tree is killed. The symptoms are clear in the orchards but by the time they are visible the disease is already well established. The need for a sensitive, reliable and rapid diagnostic method for the early identification of the fungus in trees and fruit exists. We have developed a PCR-based method for the identification of *P. tracheiphila* from plant tissues including fruit. Any such method must take into account the genetic variability in the pathogen population. Molecular methods were used to compare different isolates of *P. tracheiphila*. This study found no significant differences between different isolates from different citrus species from different parts of Israel.

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Keywords ApPCR · Citrus · Internal Transcribed Spacer (ITS) region · Mal secco disease

Abbreviations

ApPCR	Arbitrary primed polymerase chain reaction
ITS	Internal Transcribed Spacer
rDNA	ribosomal DNA

Introduction

Mal secco disease of citrus, caused by *Phoma tracheiphila* is a devastating disease of citrus in the Mediterranean basin, around the Black Sea and in Asia Minor (EPPO, CABI and Data sheet on Quarantine pests 1990; Solel 1976; Palm 1987; Timmer et al. 1988). Mal secco was first reported in Israel and neighbouring countries in 1930, but it was most likely present in the area long before then since the typical symptoms of the disease in lemons and citrons had been noted by growers many years earlier (Chorin et al. 1956). Almost all citrus species are susceptible to *P. tracheiphila* when inoculated artificially. In the field, the disease is highly destructive to lemon (*Citrus lemon*), citron (*C. medica*), bergamot (*C. bergamia*), lime (*C. auratifolia*), sour orange (*C. auranitium*), rough lemon (*C. jambhiri*) and others (Graniti and Perrota 1988). The fungus

and the disease have been studied and documented extensively, in works focused on epidemiology, the toxins involved in the pathogenicity and possible methods of disease management (Chorin et al. 1956; Solel et al. 1972; Solel 1976; Palm 1987; Graniti and Perrota 1988; Timmer et al. 1988; Fogliano et al. 1998; Rotem et al. 1998; Nicasio et al. 2000).

In Israel, the pathogen usually infects the host tree by penetrating through wounds in the leaves and branches (Horin et al. 1966). Typical symptoms include venal chlorosis, leaf wilt, red colouration of the xylem and die-back of twigs and branches. The pathogen proceeds slowly downward from the infected young shoots to the branches and main limbs; when the trunk and roots become infected, the tree dies (Palm 1987; Timmer et al. 1988). In rare cases, the fungus infects the base of a trunk or the roots, a phenomenon known as—Mal fulminante (Graniti and Perrota 1988). In these cases, the tree usually dies quickly.

Management efforts are focused on preventing the fungus from spreading to new areas through the use of disease-free propagation material and less susceptible cultivars and uncompromising sanitation of infected trees, including the pruning and burning of diseased plant material (Timmer et al. 1988). In most cases, disease symptoms are visible only when the pathogen is already established in the plant. However, early detection of the fungus may help to prevent the disease from progressing in the plant and spreading to other plants within the orchard or to other orchards. Although *P. tracheiphila* can be isolated from infected trees in the laboratory (Timmer et al. 1988), the need for a rapid, reliable and sensitive method for detection is essential. The use of molecular and immunological methods for identification and characterization of *P. tracheiphila* has been described previously (Nachmias et al. 1979; Barash et al. 1981; Rollo et al. 1990; Gentile et al. 2000). However, the utility of these methods has been limited by their problems with specificity (Rollo et al. 1990) and background interference (Nachmias et al. 1979). A new, improved PCR-based method for the identification of *P. tracheiphila* in woody and leaf tissue was recently described by Balmas et al. (2005) in Italy. There is no method currently available for identifying *P. tracheiphila* in fruits, and such a method is certainly needed. Moreover, our experience shows that the previously proposed PCR conditions may be too permissive when working with plant material (unpublished data).

Citrus species exhibit a wide range of responses to *P. tracheiphila*. Citron (*C. medica*) was found to be the most sensitive species to Mal secco in Israel (Horin et al. 1966; Shpigel and Solel 1973), followed by lemon, lime, mandarin (*C. deliciosa*), tangelo and tangors. Grapefruit (*C. paradisii*) and sweet orange (*C. sinensis*) are rarely infected (Horin et al. 1966; Palm 1987; Timmer et al. 1988). The response of different citrus species to *P. tracheiphila* may vary with their location. For example, sour orange (*C. aurantium*) was reported to be highly susceptible in Italy, but it was only moderately affected in Israel (Palm 1987). These differences in susceptibility may be due to differences in the interaction or compatibility of the pathogen and its citrus hosts. It is possible that more then one variant of *P. tracheiphila* exists in Israel. If there is more then one variant, the question of the respective abilities of variants to infect different citrus species needs to be examined. It is also possible that only one variant, capable of attacking all susceptible types of citrus, exists. In the latter case, the variability in the severity of the disease in different citrus species may reflect differences in host susceptibility.

Information about variability in the population of *P. tracheiphila* is still lacking. Isolate variability can be determined through the use of molecular tools such as apPCR patterns and comparisons of the ITS1, 5.8S and ITS2 rDNA region sequences.

The first objective of this study was to develop a rapid, reliable and highly specific PCR-based molecular method for the identification of *P. tracheiphila* from plant tissues. The second objective was to characterize, molecularly, the variability in *P. tracheiphila* isolates collected from different citrus species and different production areas in Israel.

Material and methods

Fungal isolation identification and storage

Bark, leaves and fruit were collected from a variety of citrus species from orchards in several locations in Israel (Table 1). Plant material was collected from

trees exhibiting disease symptoms and from suspected plants with no definite symptoms. The plant material was kept in plastic bags on ice until it could be processed in the laboratory. Woody material (bark) was surface-sterilized by dipping the tissue in 100% ethanol and burning it for a few seconds. Leaves were sterilized by immersion in a 1% hypochlorite solution for 45 to 60 s, and immediately rinsed in distilled water for 5 min. Fruits were washed under running water and then surface-sterilized by spraying with hypochlorite solution and rinsed with distilled water. A few isolates of the fungus were sampled and archived from each of the sampled trees (data not shown). One isolate per tree was chosen for further investigation.

Table 1 Isolates of *Phoma tracheiphila* and other fungi used in this study

Isolate ID			
Host	Location	Isolate	Accession number
Citron (*C. medica*)	Eytan (S)	Ipt11	DQ993287
	Galiya (S)	Ipt8	DQ792932
Cook (*C. lemon*)	Shova (S)	Ipt17	DQ993289
Helen (*C. lemon*)	Nitzanim (S)	Ipt5	DQ993285
	Nitzanim (S)	Ipt7	DQ993286
Lemon (*C. lemon*)	Eytan (S)	Ipt10	DQ792933
	Nir Eliyahoo (S)	Ipt32	DQ993290
	Nir Oz (S)	Ipt30	DQ792942
Lime (*C. auratifolia*)	Yehiam (N)	Ipt1	DQ792928
	Ramat Yohanan (N)	Ipt13	DQ993287
	Rosh Anikra (N)	Ipt18	DQ792937
	Snir (N)	Ipt26	DQ792940
Limoneira (*C. lemon*)	Shova (S)	Ipt15	DQ792936
	Tzrifin (C)	Ipt16	DQ993288
Nectar (*C . paradisi)*	Yaad (N)	Ipt2	DQ792929
Newhall Navel Orange	Eytan (S)	Ipt24	DQ792939
Or -3 (*C. temple* × *C. tangerina*)	Yaad (N)	Ipt3	DQ792930
Orange Shamouti	Nir Oz (S)	Ipt27	DQ792941
Star ruby (*C. paradisi*)	Kfar Hasidim (N)	Ipt14	DQ792935
Troyer Citrange	Tzrifin (C)	Ipt12	DQ792934
Villafranca (*C. lemon*)	Yaad (N)	Ipt4	DQ792931
Volckamer lemon (*C. volckameriana*)	Ramat Yohanan (N)	Ipt20	DQ792938

N—Northern part of Israel; C—Central part; S—Southern part

In order to isolate *P. tracheiphila* from the plant material, small pieces of the sterilized samples were placed on potato dextrose agar (PDA) (Acomedia, Inc. Michigan, USA) with tetracycline (12 mg ml^{-1}) (Sigma, S.Louis,MO, USA) and on PTSM media (PDA with Rovral (Bayer CropScience, EU) 0.2 mg ml^{-1} and tetracycline 25 µl ml^{-1}). The plates were kept at 24°C for a few days and fungal growth was monitored daily. Fungi showing *P. tracheiphila* characteristics were collected and replated on fresh PDA plates. Fruit samples were taken by cutting small pieces from the top, inner part of the fruit. Attempts were made to include as much tracheotic tissue as possible in the tissue samples.

Phoma tracheiphila was identified visually, based on recognition of specific phenotypic characteristics and microscopic fungal structures (e.g. unique phialoconidia, characteristic pigmentation and colony structure). Fungi, visually identified as *P. tracheiphila*, were grown for at least 7 days on fresh PDA plates before examination under the microscope. The cultures were examined for typical *P. tracheiphila* phialoconidia. Suspected fungi were investigated further; ITS sequencing was used for molecular identification.

Phoma tracheiphila isolates and other fungal isolates used as controls were kept on PDA plates for routine work. The controls were *Pythium ultimum*, *Fusarium* spp. (isolated from citrus), *Colletotrichum* spp. (isolated from citrus), *Sclerotinia sclerotiorum*, *Phytophtora* spp., *Alternaria alternata* (isolated from citrus) and some unidentified fungi isolated from citrus trees. Isolates were kept in 20% glycerol at −80 or 4°C or were maintained on PDA at room temperature. Fungal cultures were transferred every two weeks to fresh plates and incubated at suitable temperatures.

DNA extraction

Fungal DNA for PCR was extracted from cultured fungi or infected plant material. DNA was isolated from pure cultures growing on PDA using the Rapid Homogenization: Plant leaf DNA Amplification Kit (Cartagen; Washington, USA). Squares of the cul-

tured mycelia (0.5 cm^2) were cut from one week-old cultures. The agar was scraped from the bottom of the pieces, in order to exclude as much agar as possible. The pieces were placed into 1.5 ml eppendorf vials and incubated for about 10 min at −80°C. DNA was then extracted according to the instructions of the kit manufacturer. Extracted DNA was diluted (1:9) in double-distilled, sterile water; 1 µl samples were used for PCR. Plant and fruit material prepared for fungal isolation was used for direct DNA extraction. For plant samples, one piece of plant material was randomly selected from the pieces prepared for the fungal isolation; about 0.3 to 0.6 mg of plant tissue were used for each sample Direct extraction of fungal DNA from plant tissues, including fruit, was performed using the Genomic DNA Extraction Kit: Plant Samples (Cartagen; Washington, USA) according to manufacturer's instructions. One-half µl samples of the extracted DNA were used for PCR.

Arbitrary Primers PCR profiling (apPCR)

The apPCR procedure was carried out in a 14 µl reaction mix containing 1 µl DNA extracted from pure fungal culture (1:9 dilution), 1 µl primer (Table 2), 7 µl RedMix™ plus PCR mix with 1.5 mM $MgCl_2$ (GeneChoice, Inc., Maryland, USA) and 5 µl ddH_2O PCR grade (Fisher Scientific, Wembley, Western Australia, Australia). PCR amplification was performed in a Biometra personal cycler (Goettingen, Germany): 96°C for 5 min followed by 35 cycles of 95°C for 45 s, 48 to 63°C according to the primers used (Table 2) for 45 s and 72°C for 45 s, followed by a 72°C cycle for 5 min. The PCR products were separated and examined using gel electrophoresis, on a 1.3% agarose gel for 30 min at 100 V with TAE buffer. (GelXLUltra V-2 from Labnet International, Inc., (Woodbridge, NJ, USA) or Wealtec GES cell system (Wealtec Inc., Georgia, USA). Gels were soaked in a 0.5 µg ml^{-1} ethidium bromide solution for 5 min and then washed in distilled water for 5 min. Gel imaging was performed under UV light in a Bio-Imaging System (model 202D; DNR-Imaging Systems, Kiryat Anavim, Israel). DNA from the same preparation was used for amplification with all apPCR primers (Table 2) (Freeman et al. 1993). Results were visually compared for differences in patterns.

ITS, 5.8S ribosomal DNA sequencing

DNA extracted from a pure fungal culture was used for the amplification of the ITS1, 5.8S, ITS2 region of the ribosomal DNA. Amplification of the rDNA was performed as described for apPCR except for the use of universal primers ITS1 and ITS 4 (White et al. 1990) (Table 2) and an annealing temperature of 50°C. A ~500 bp PCR product was examined using gel electrophoresis and purified using the UltraClean PCR Clean Up DNA Purification Kit (MO BIO Laboratories, Inc., California, USA). Purified products were sent for direct PCR sequencing. Sequencing was performed on both strands of the PCR product using ITS1 and ITS4 primers. Sequencing was performed using a BigDye Terminator Cycle Sequencing Kit from ABI (Hay Laboratories, Ltd., Rehovot, Israel). Sequences were submitted to the gene bank on the NCBI web site (http://www.ncbi.nlm.nih.gov). Accession numbers are listed in Table 1.

Table 2 Primers used in the study

Primers	Sequence	Length of product	Reference
ITS1	5′ TCCGTAGGTGAACCTGCGG 3′	~500 bp	White et al. (1990)
ITS4	5′ TCCTCCGCTTATTGATATGC 3′	~500 bp	White(1990)
P.trachITSF	5′ CAGGGGATGGGCGCCAGCC 3′	~500 bp	This study
P.trachITSR	5′ CCGTCCTGCACAAGGGCAGTGG 3′	~500 bp	This study
Ap CAG	5′ CAGCAGCAGCAGCAG 3′		Freeman et al. (1993)
Ap AGG	5′ AGGAGGAGGAGGAGG 3′		Freeman et al. (1993)
Ap GACAC	5′ GACACGACACGACAC 3′		Freeman et al. (1993)

Sequence analysis

Phoma tracheiphila sequences obtained in this study were compared to those in the gene bank database using the BLAST software on the NCBI web site (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence alignments among isolates (ITS sequences) and between *P. tracheiphila* and other species were performed using the ClustalW software on the EMBL-EBI web site (http://www.ebi.ac.uk/clustalw/) and the BioEdit sequence alignment editor version 7.0.5.3. (2005) (Hall 1999).

Design of *P. tracheiphila* specific primers

Phoma tracheiphila ITS, 5.8S region sequences were aligned with sequences of other fungi from the data bank at the NCBI web site. Two regions, at the 3′ and 5′ ends of the sequence, which were most dissimilar, were chosen as primers. Primers PtrachITSF (5′CAGGGGATGGGCGCCAGCC 3′) and PtrachITSR (5′CCGTCCTGCACAAGGGCAGTGG 3′) were synthesized by IDT, Inc. (Iowa, USA) or Syntezza (Jerusalem, Israel) (Table 2).

PCR assay conditions for *P. tracheiphila* detection

Phoma tracheiphila detection *in planta* or from cultured tissue was performed on total DNA extracted, as described above**.** PCR was carried out in a 14 µl reaction mix containing: 1 µl DNA extraction, 0.5 µl of each specific primer (Table 2), 7 µl RedMix^{TM} plus PCR mix with 1.5 mM $MgCl_2$ (GeneChoice Inc. Maryland, USA) and 5 µl PCR grade ddH_2O (Fisher Scientific, Wembley, Western Australia , Australia). PCR amplification was performed in a Biometra personal cycler (Goettingen, Germany): 96°C for 5 min followed by 10 cycles of 95°C for 45 s, 74°C for 60 s, followed by 30 cycles of 95°C for 45 s, 75°C for 60 s, followed by a 72°C cycle for 5 min. PCR products were evaluated using gel electrophoresis, as described for apPCR. Infected plant material, for which isolation and characterization had already shown to harbour *P. tracheiphila*, was used as a positive control. Uninfected lemon and grape vine tissues were used as negative controls for the presence of fungi and for possible reaction with the plants' ITS sequences. PCR under the described conditions was performed on different pathogenic and citrus-related fungi, using the specific primers designed.

Results

Development of a specific identification method for *P. tracheiphila*

Phoma tracheiphila was isolated from symptomatic and diseased citrus trees from different regions in Israel. Two different fungal phenotypes were observed: chromatic isolates producing characteristic red pigment and achromatic isolates lacking the red pigment (data not shown). Occasionally, chromatic and achromatic isolates were collected from the same tree.

Different ITS, 5.8S region sequences of plant pathogenic fungi were collected from the gene bank. The sequences were aligned to the *P. tracheiphila* ITS 5.8 sequence using the ClustalW software. Two short sequences in the 5′ and 3′ *P. tracheiphila* sequence, showing obvious differences in comparison to the other fungal sequences, were selected as primers for PCR (Fig. 1). PCR performed with DNA extracted from the different fungi showed that these primers were very specific to the target DNA (Fig. 2a). A 450 bp product could be detected, under the specified temperature conditions, only when *P. tracheiphila* DNA was present. None of the other plant pathogens or other fungi isolated from citrus reacted with these primers. In a control PCR reaction, using the same DNA samples with universal ITS 5.8S region primers, these less-specific primers reacted with all fungi tested (Fig. 2b).

The primers designed for *P. tracheiphila* detection were used to develop a protocol for the direct PCR detection of the fungus in woody and fruit tissue. A rapid DNA isolation kit was used for the isolation of the fungal DNA directly from the plant tissue. The presence of the fungus could be detected in different tissues, different citrus species and from symptomatic and asymptomatic trees (Fig. 3, lanes 2–7). The negative controls, a non-contaminated host (lemon), a non-host plant (grape vine), another plant pathogen (*P. ultimum*) and water, did not react with the primers (Fig. 3, lanes 8–11).

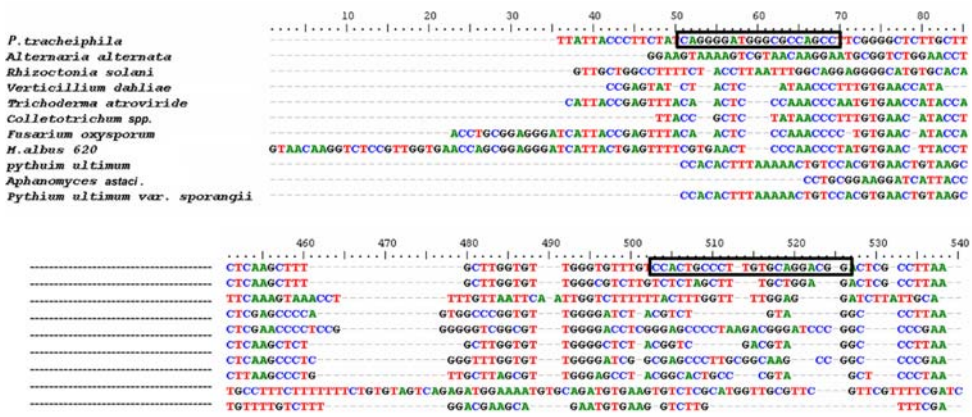

Fig. 1 rDNA ITS1, 5.8S, ITS2 region alignment of the different fungi to the *Phoma tracheiphila* sequence. Ptrach-ITSF and Ptrach ITSR primers are in rectangles. *Phoma tracheiphila* (DQ792928), *Alternaria alternata* (AJ853759), *Verticillium dahliae* (AY566606), *Trichoderma atroviride* (AB249680) *Colletotrichum* sp. (AB255294), *Fusarium oxysporum* f. sp. *nelumbicola* (DQ002550), *Muscodor albus* isolate 620 (AY244622), *Pythium ultimum* (AY986953), *Aphanomyces astaci* (AY683896), *Pythium ultimum* var. *sporangiiferum*(AY986961)

Variability in *P. tracheiphila* isolates in Israel

The apPCR profiling showed very similar patterns for all the isolates compared (Fig. 4). All three primers (Table 2) were used on all the tested isolates. Similar patterns were found for isolates from different citrus species (Figs. 4a and b), from different locations and from different tissues (Fig. 4c).

Comparison of rDNA (ITS, 5.8S regions) sequences reinforced the results of the apPCR. No significant differences were found between the different isolate sequences. ClustalW multiple sequence alignment of the Israeli isolates shows that isolate IPT7 and isolate IPT8 are different from the rest of the isolates aligned, by one nucleotide; isolate IPT30 has one additional nucleotide. The rest of the isolates have 100% identity (Data not shown).

The sequences received were used for final identification of the isolates as *P. tracheiphila* by comparison to the gene bank. All isolates were very similar to the Italian isolates published by Balmas et al. (2005).

Discussion

The Mal secco disease of citrus has been the subject of numerous research articles. The disease is highly

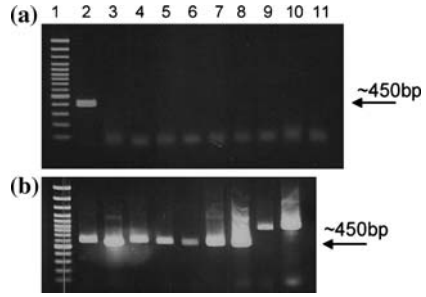

Fig. 2 PCR results from fungi isolated from citrus trees with: (**a**) *P. tracheiphila* specific primers, (**b**) ITS1/ITS4 universal primers. Lanes: 1. PCR Marker; 2. *P. tracheiphila* control (+); 3. *Fusarium* spp.; 4. *Colletotricum* spp.; 5. *Alternaria* spp.; 6. unidentified fungus isolated from citrus plants: TC-5*; 7. RL-7*;8. TC-6*; 9. *P. ultimum*; 10. *Phytophthora* spp.; 11. *Sclerotinia sclerotiorum*

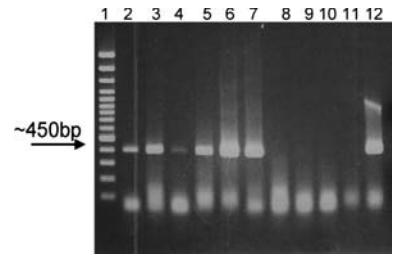

Fig. 3 Direct PCR amplification of *Phoma tracheiphila* from plant tissue with *P. tracheiphila* specific primers. 1. PCR Marker; 2. Lemon twig; 3. Orange Shamouti twig; 4. Lime twig asymptomatic; 5. Lemon fruit; 6. Lemon twig, asymptomatic; 7. Fungal DNA from Troyer Citrange; 8. H_2O control (−); 9. Lemon control (−); 10. Grapevine control (−); 11. *Pythium ultimum* control (−); 12. *P. tracheiphila* control

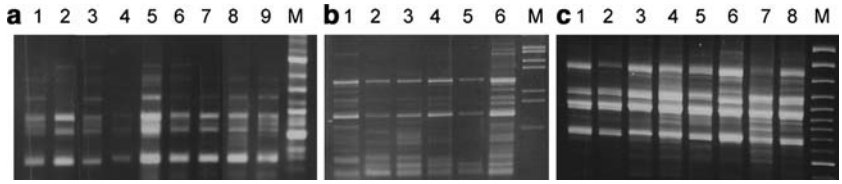

Fig. 4 Arbitrary primer PCR patterns from different *Phoma tracheiphila* isolates: (**a**) Isolates from different citrus species: 1.Orange Shamouti; 2. Newhall Navel Orange; 3.Volckamer lemon (*C. volckameriana*); 4. Limoneira (*C. lemon*); 5. Limoneira (Shova); 6. Star ruby (*C .paradisii*); 7. Troyer Citrange; 8. Or -3 (*C. temple* × *C. tangerina*); 9. Nectar (*C. paradisii*); M. DNA marker. apPCR primer—CAG × 5 (**b**) 1. Sour orange (*C. aurantium*) Bizaron (Achromatic isolate); 2. Lime (*C. auratifolia*) Rosh Anikra; 3. Lime (*C. auratifolia*) Ramat Yohanan; 4. Citron (*C. medica*) Eytan; 5. Citron (*C. medica*) Galiya; 6. Lime (*C. auratifolia*) Yaad; M. DNA marker. apPCR primer—AGG × 5 (**c**) Lemon (*C. lemon*) isolates collected from different locations in Israel: 1. Villafranca (*C. lemon*) Yaad; 2. Helen (*C. lemon*) Nizanim; 3. Helen (*C. lemon*) Nizanim from fruit petal; 4. Lemon (*C. lemon)* Eytan; 5. Cook (*C. lemon*) fruit; 6. Lemon (*C. lemon*) Nir Akiva; M. DNA marker. apPCR primer—GACAC × 3

destructive, but restricted to the citrus orchards in the Mediterranean basin around the Black Sea and in Asia Minor. Most of the work performed in the past was not molecularly oriented. With the development and advancement of the field, new approaches to the study of the disease, the casual agent and the molecular relations between the host and the pathogen have been reported. Our aim in this work was to find a simple, reliable, high fidelity method for the identification of the casual agent in the laboratory. A rapid method for identification of the fungus may help in the control of the disease in nurseries, preventing contaminated propagation material from being distributed to clean areas. It may also serve as a method of verification for disease-free fruit being exported to countries demanding assurances of the product's health and cleanliness. The detection of the fungus in the orchard in the early stages of the disease may help farmers to manage the disease through rational sanitation efforts long before disease symptoms are visible.

PCR-based methods for the identification of *P. tracheiphila* have been presented in the past (Rollo et al. 1990; Gentile et al. 2000). These methods have not been used on a large scale, probably due to their limited specificity and problems resulting from high background interference. Our experience with the primers and protocols previously published (Rollo 1990) has taught us that many plant pathogens and other citrus-related fungi may be mistakenly identified as *P. tracheiphila* by these methods. Recently, a new, improved method was described by Balmas et al. (2005). We have not tested their primers, but there is a very close similarity between the primers used in that study and the set of primers we developed for this study (Table 2). The main difference between our PCR method and Balmas's protocol is in the annealing temperature used for the PCR reaction. We found that lower temperatures gave non-specific, false-positive results with other fungi. Therefore, we believe that the two-step method we have developed has advantages for accurate identification of the fungus. This method enables detection of *P. tracheiphila* even if there are only preliminary symptoms on the tree. This method, being molecular and PCR-based, is very sensitive; under the conditions described in this paper, the method was highly specific and did not falsely identify any of the other tested plant pathogens or citrus-related fungi (Fig. 2a). The target fungi reacted with the universal primers ITS1 and ITS4 and resulted in a PCR product, as expected (Fig. 2b). This method relies on the design of a very specific set of primers, unique to the target organism, for the amplification of a DNA fragment that is unique to that fungus. The ITS 5.8 region of the rDNA is a conserved region of fungal DNA, therefore it is often used for molecular identification of fungi. The use of this region for molecular diagnostic purposes, through the use of a short yet unique sequence that may serve as primer for the PCR reaction is not obvious. By comparing this region in a variety of fungi, we were able to find short, specific sequences that were suitable to serve as specific primers for *P. tracheiphila* (Fig. 1).

Direct identification of the fungus in the plant material was done using a DNA extraction kit designed for total DNA extraction from leaves and other soft plant tissue with some modifications. The results were, in most cases, compatible with the Petri plate isolation test (Fig. 3; other data not shown). In some cases, the PCR amplified a fragment indicating the presence of *P. tracheiphila* in the sample while the fungus could not be isolated on the PDA plates (data not shown). This result supports the PCR-based method's superior sensitivity as compared with the classic Petri plate isolation method. There were no reactions with any of the negative controls, demonstrating the high specificity of this method (Fig. 3). The use of a simple commercial kit enabled the development of a rapid method, which produced results within 3–4 h of the samples arriving in the laboratory. This method was proven to be also useful for the identifying the fungus from fruits (Fig. 3).

Phoma tracheiphila is a quarantine organism in most citrus-importing and citrus-producing countries of the world. Many citrus-importing countries have a great interest in preventing the Mal secco disease from entering their orchards. These countries require certification that imported fruit is free of any *P. tracheiphila*. Trying to isolate the fungus from contaminated fruit is not an easy task; trying to isolate it from suspected fruit is even harder. The presented method has been shown to amplify a PCR fragment from contaminated fruits. We believe that the ability of the method to produce answers rapidly is a significant advantage. Fruits intended for export cannot wait for a week in the warehouse until results from plate isolation tests are acquired. A rapid, sensitive and reliable method of pathogen identification, like our PCR-based method, is the best solution for this problem.

Different isolates of *P. tracheiphila* collected from a wide variety of citrus species exhibited similar morphological characteristics when grown in vitro. The only difference was the inability of some isolates from lime and sour orange to express the red/orange pigment associated with the fungus. Nevertheless, these achromatic isolates were no different in their ITS rDNA sequences than the chromatic isolates. It should be noted that chromatic and achromatic isolates were occasionally isolated from the same trees. Furthermore, results obtained from the apPCR experiments indicate no differences between the examined isolates. All isolates were amplified with all three different apPCR primers and compared (Fig. 4, other data not shown). ITS1-5.8S-ITS2 sequence data was collected. Multiple comparisons found no significant differences between the isolates. This homogeneity is expressed in a multiple sequences alignment. Most of the isolates form one group. Three isolates (numbers 7, 8 and 30) were excluded from the major group, but a close examination revealed that the difference between these two isolates and the isolates in the main group is just a single nucleotide (data not shown). This difference is not enough to differentiate these isolates from the others.

This morphological and genetic homogeneity suggests that the entire Israeli population of the fungus is probably descended from one common ancestor. Furthermore, it seems likely that there is one main variant of the fungus in Israel, able to induce the disease in all susceptible citrus species. Balmas et al. (2005) claim that the Italian isolates which they examined are genetically homogeneous. We have compared the sequences obtained from the Israeli ITS1-5.8S-ITS2 sequences and the published Italian sequences and found no remarkable differences. This suggests that the Israeli population is not only homogeneous, but also very similar to the *P. tracheiphila* population in Italy. It may be the case that the Italian and the Israeli variants have a common origin. This is only an assumption based on the data in the gene bank. It will be interesting to compare ITS sequences isolated from the fungus in Israel and Italy with those of other Mediterranean countries.

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