SENSITIVITY OF TWO *PODOSPHAERA APHANIS* POPULATIONS TO DISEASE CONTROL AGENTS

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SUMMARY

Powdery mildew (*Podosphaera aphanis*) causes severe losses in strawberry crops. The two aims of this study were to evaluate the efficacy of powdery mildew control agents and compare two geographically distinct populations of the pathogen (from northern Italy and Israel). Germination and germ tube elongation of *P. aphanis* conidia were tested in the presence of several control agents using both leaf and glass slide bioassays. The leaf bioassay was found to be more suitable for comparing the efficacy of control agents. Agents found effective were used to compare the relative sensitivities of samples from the Italian and Israeli populations. Various chemical agents, including those containing sulphur, strobilurin and triazole fungicides, were more effective than salts and biocontrol agents on both populations.

The Italian and Israeli *P. aphanis* populations were found to be similar in sensitivity to all the agents. Similarly, DNA analyses showed that the Israeli and Italian samples were nearly identical (100-99% similar) based on sequence analyses of a 359 bp fragment of the ITS 1-2 region and 6 random fragments totalling 2800 bp. ITS sequence analysis of an isolate of the synonymous species *Sphaerotheca aphanis* from strawberry was 100% identical to *P. aphanis*, compared to 97, 85 and 67% for *S. pannosa*, *S. fuliginea* and *Leveillula taurica*, respectively.

Key words: powdery mildew, fungicide, biocontrol agent, molecular marker.

INTRODUCTION

Powdery mildew of strawberry is caused by the obligate parasite *Podosphaera aphanis* (Wallr.) U. Braun *et* S. Takam. [formerly *Sphaerotheca macularis* (Wall. ex Fries) Jacz f. sp. *fragariae* (Peries)], which affects leaves, flowers and fruits of strawberry worldwide. It causes severe losses in traditional strawberry cropping systems in Mediterranean climates and in greenhouse soil-free systems, which are more common in central Europe and in some areas of Northern Italy. Leaf infections reduce photosynthesis, cause necrosis or even defoliation and, consequently decrease fruit yield (Maas, 1998; Spencer, 1978). Mild infection on fruit causes slight discoloration and shortened shelf life, while more severe infection causes deformation and cracking in fruit. Crops grown in warm, dry Mediterranean climates are particularly vulnerable (Amsalem *et al.*, 2006).

Growing strawberries in raised beds under high tunnels or in greenhouses can positively affect fruit quality and shelf life. These systems also allow growers to schedule their harvests to coincide with periods of market demand (Lieten, 1996; D'Antuono *et al.*, 2000). The method also helps to control several important diseases like grey mould, fruit rots and root rots (Freeman *et al.*, 1997; Xiao *et al.*, 2001; Legard *et al.*, 2002). However, without the inhibitory effect of rain on conidia germination, sheltered crops tend to have more powdery mildew infection (Pertot *et al.*, 2001; Xiao *et al.*, 2001; Legard *et al.*, 2002). Long periods at around 20°C and the high relative humidity in tunnels provide favourable conditions for *P. aphanis* (Amsalem *et al.*, 2006). These infections can appear early and develop quickly, especially on sensitive cultivars, like Elsanta and Tamar, the most common strawberry varieties in the two studied environments.

Controlling strawberry powdery mildew in the typical production system of northern Italy, soil-less production in tunnels, generally requires at least seven or eight fungicide treatments per growing cycle (Pertot *et al.*, 2004). Crops grown in the open may require 12 to 16 sprayings per season (Amsalem *et al.*, 2004). Regular, intensive fungicide applications contrast with the goals of integrated pest management, so a decision support system (DSS) for applying pesticides only when strictly needed is desirable. Development and maintenance of a DSS is costly, therefore, any new DSS should be designed to be valid over a large area. As strawberry is a relatively minor crop, a DSS should cover several production areas which have similar problems in order to

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maximize its economic value.

Although information is available on the efficacies of pesticides and biocontrol agents against *P. aphanis* under field conditions (Pertot *et al.*, 2002, Pertot *et al.*, 2004), it is not known if strawberry powdery mildew populations, adapted to different environmental conditions, differ in sensitivity to particular fungicides. Different sensitivities have been documented for subpopulations of *Phytophthora infestan*s and *Uncinula necator* (Day and Shattock, 1997; Hsiang *et al.*, 1997; Svircev *et al.*, 2000; Savocchia *et al.*, 2004), suggesting that disease models and control strategies should be adapted to particular geographical regions. Even if different pathogen populations are consistently sensitive to sulphur treatments (the oldest fungicide applied against powdery mildews), different pathogen populations may differ in their sensitivities to new fungicides or biocontrol agents. Active ingredients based on triazole chemistry, like penconazole or miclobutanil, strobilurines (*e.g.* azoxystrobin or kresoxym-methyl), or pyrimidines (fenarimol) are expected to provide consistently effective disease control (Hollomon and Wheeler, 2002). However, local differences may exist, reflecting differences in usage patterns, genetic differences among pathogen subpopulations or interactions between the compounds and the local climactic conditions. Among low impact control agents used against powdery mildews, mineral salts, such as monobasic potassium phosphate (Agosteo *et al.*, 2002), plant extracts, resistance inducers such as benzothiadiazole (Oostendorp *et al.*, 2001; Cambell and Latorre, 2004), and microbial antagonists, such as *Ampelomyces quisqualis* (Stuart *et al.*, 1995), *Bacillus subtilis* (EPA, 2005) and *Trichoderma harzianum* (Elad *et al.*, 1998), have been tested or developed as commercial products for control of powdery mildews on other crops. However, little is known of their efficacies against strawberry powdery mildew. There are no reports on genotypic variability and sensitivity to fungicides among populations.

The aim of this work was to compare the sensitivities of two geographically distant *P. aphanis* populations (from Italy and Israel) to chemical and biological control agents under laboratory conditions. Conidium germination and germ tube elongation were used as indicators. Two bioassays were compared to identify the best approach for comparing the sensitivities of the two pathogen populations. The first assay measured the accumulation of mycelial biomass (percentage of germinated conidia × average germ tube length) on glass slides. This assay specifically assessed the direct toxic effect of each active ingredient on the pathogen. In the second assay, pathogen/control agent interactions were examined on strawberry leaves, emphasizing possible control mechanisms in which the host plant is involved (*e.g.* induced resistance). The most suitable bioassay was used to evaluate the sensitivities of the two naturally occurring *P. aphanis* populations to a wide spectrum of control agents. Genetic divergence between the two populations was assessed using universal ITS primers and six randomly selected genome regions (Saenz and Taylor, 1999; Mori *et al.*, 2000; Hirose *et al.*, 2005).

MATERIALS AND METHODS

Inoculum sources. The inocula used were collected from several hundred infected leaves in commercial high tunnels in Italy (Trentino region) during July 2003 and fields in central Israel during September 2004 (named “Volcani” in the DNA analysis). These pooled field populations were maintained on strawberry plants in greenhouses (within their native countries) and conidia were obtained from fresh sporulating lesions whenever needed.

For the DNA analyses, *P. aphanis* conidia were collected from naturally infected leaves (cv. Tamar, susceptible to disease) from strawberry production fields located in six locations in central Israel (cv. Tamar, locations: Givat Chen, Kadima, Porat, Volcani Center, Kfar Ma’as and Zofit) and one location in Trentino region, Italy (cv. Elsanta, location: Valsugana). Each batch of conidia was treated as an individual sample (7 pooled population samples).

Leaf and glass-slide bioassays. Leaf and glass-slide tests, were done in Italy (using Italian inoculum) during August 2003. The control agents were grouped into six categories: salts, biocontrol agents (BCAs), compounds containing sulphur, triazoles, strobilurins and other chemicals (Table 1). In the bioassays, fully expanded two to three week-old, detached strawberry leaves (*Fragaria* x *ananassa*, cv. Elsanta) and microscope glass slides were used.

Aqueous solutions/suspensions (7 ml) of the various products were applied to the leaves and slides, using a Potter tower (Burkard Manufacturing, Rickmansworth, UK) to obtain uniform distributions.

All commercial products were applied according to manufacturers’ instructions. The experimental BCAs, *Bacillus subtilis* F77 and *Cladosporium tenuissimum*, were grown in nutrient broth (Oxoid, Basingstoke, UK) for 48 hours and then sprayed directly onto leaves and slides. When the applied solutions/suspensions were dry (approx. one hour after application), inoculation was done by shaking leaves with heavy sporulating lesions over the glass slides and the detached leaves. Each leaf or slide sample was then placed in a Petri dish with moist paper, and incubated at 20 $\pm$ 1°C for 48 hours under 12:12 hour light: dark conditions. Subsequently, the percentage of germination and germ tube length were measured, as described by Miller *et al.* (2003). A 48 hour incubation period had been previously found to

allow for optimal germination. In order to observe the germination, *P. aphanis* conidia and germlings were removed from each leaf surface using a 2 × 4 cm piece of transparent adhesive tape. The conidia and germlings on the tape (for the leaf assay) and glass slides (for the leaf-free assay) were stained with cotton blue to arrest further development, following the method of Peries (1962). There were three replicates of each treatment in both the leaf and glass slide tests. Germination percentage was determined by observing conidia under a light microscope. Two hundred conidia were evaluated for each slide or leaf replicate. Conidia were scored as germinated when their germ tube exceeded their lateral radius, as suggested by the APS, Committee on Standardization of Fungicidal Tests (1943). The germ tube length was calculated (Boesewinkel, 1980). Hyphal biomass was calculated by multiplying the germination percentage by the average germ tube length for each replicate.

Comparison of sensitivities to control agents for the Italian and Israeli fungal populations. Comparison of the sensitivities of the two populations to control agents was based on the leaf assays described above. This evaluation included a subset of the control agents from the previous test (Table 1). In this set of experiments, we tested only the higher dosages of K bicarbonate and *A. quisqualis*. The experimental wetting agent KF 643 was excluded due to its toxic effect on strawberry leaves. Benzothiadiazole (BTH) was the only resistance inducer included in this test. The different control agents were grouped according to their chemical nature, as done for the leaf and glass slide tests. During August 2003, experiments were performed in Italy on leaves of the cultivar Elsanta. During the winter of 2004, experiments were performed in Israel on leaves of the cultivar Tamar. In Israel, the control agents were applied using a hand sprayer instead of a Potter tower.

Statistical analyses. For all analyses, data was grouped according to the chemical nature of the control agents. The germination rate data was arcsin transformed prior to analysis. The inhibitory effects of the control agents on germination and germ tube elongation, relative to the untreated controls in the two populations, was calculated. Values were analysed using ANOVA and means were separated according to Tukey's HSD test ($\alpha = 0.05$). The relative sensitivities of the two pathogen populations to the different control agents were compared using the non-parametric Wilcoxon paired-sample test (Wilcoxon and Wilcoxon, 1964), after the efficacy data had been transformed into ranks. All the statistical analyses were performed using the software package "Statistica, version 7" (StatSoft, Tulsa, OK, USA).

DNA analyses of *P. aphanis* isolates. Genetic analyses were conducted to compare the Israeli and Italian samples. Conidia and mycelial fragments of the samples were collected in sterile, filtered 1 ml pipette tips using a diaphragm vacuum pump (Vacuubrand, Wertheim Germany).

The material was lyophilised and 60 mg of each sample were used for DNA extraction according to Freeman *et al.*, (2001). Each DNA sample was dissolved in 0.5 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) to an approximate concentration of 200-500 µg/ml and diluted to a final concentration of 10-100 ng/ml for PCRs reactions.

The rDNA-ITS fragment (ribosomal ITS1, ITS2 and subunit 5.8s) was amplified using ITS4- (5'-TCCTCCGCTTATTGATATGC-3') and ITS5- (5'-GGAAGTAAAAGTCGTAACAAGG-3') primers (White *et al.*, 1990). The reaction mixture contained 1.5 µl DNA, 2 µl 10X buffer, 2 µl $MgCl_2$, 0.5 µl ITS4, 0.5 µl ITS5 and 0.2 µl Taq polymerase (Freeman *et al.*, 2001). Sterile dH_2O was added to 20 µl volume. The PCR program consisted of a 5 min denaturation at 95°C, 40 cycles of 30 s each at 95°C, 30 sec at 50°C, 90 s at 72°C and a termination step at 72°C lasting 10 min.

Amplification of rDNA from the *P. aphanis* populations using the primers ITS 4 and ITS 5 (White *et al.*, 1990) resulted in a product of ca. 590 bp which was extracted from agarose gels using a DNA isolation kit (Biological Industries, Beit Ha'emek, Israel). Ligation and cloning into the competent *E. coli* DH5a were done using the pGEM-T Easy Vector System® kit (Promega, Minipreps DNA Purification System® (Promega, Madison, WI, USA). The Big Dye Terminator® DNA sequencing kit (Perkin-Elmer Inc., Branchburg, NJ) was used for determining the sequence of the amplicon (ITS 1 and 2 regions including the 5.8 rDNA subunit) using the vector primers SP6 and T7 (White *et al.*, 1990). The sequences of both strands of the DNA were determined using an ABI prism 377 DNA sequencer (Applied Biosystem Inc., Foster City, CA, USA). Sequencing was performed at Tel Aviv University.

Sequence similarity tests and cluster analyses were performed for the rDNA-ITS 1, 2 and 5.8s sequences of *Podosphaera* samples and related species. Each sequence, including the ones obtained from GenBank, was processed and refined for multiple alignment, using the Chromas, version 1.41® program (Conor and McKarthy, Brisbane, Australia). The results were analyzed and processed after reducing the overlapping primer ends and identical 5.8S fragment, and a fragment of 359 bp, representing the most diverse regions of the ITS 1-2 fragment was aligned for comparisons. Manual alignment was carried out for accurate adjustment while cluster analysis was performed using the Neighbour Joining method (http://www.ebi.ac.uk/clustalw/) and viewed using TreeView, version 1.6.6® (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Table 1. Control agents included in this study.

Active Ingredient (A.I.)	Commercial name	Company	A.I. (%)	Dosage (g/l)***	C**
K phosphate monobasic	K phosphate monobasic	Sigma	99	2.00	SAL
Ca carbonate	Ca carbonate	Sigma	99	4.00	SAL
K bicarbonate	K bicarbonate	Sigma	99.5	4.00	SAL
Na bicarbonate	Bicarbonato	Solvay	99	4.00	SAL
Ultrafine oil	UFO	Intrachem-bio Italia	98.8	1.00 *	SAL
K bicarbonate + Ultrafine oil	K bicarbonate + UFO	Sigma Intrachem-bio Italia	99.5 98.8	2.00 1.00 *	SAL
K bicarbonate + Ultrafine oil	K bicarbonate + UFO	Sigma Intrachem-bio Italia	99.5 98.8	4.00 1.00 *	SAL
Na bicarbonate + Ultrafine oil	Bicarbonato + UFO	Solvay Intrachem-bio Italia	99 98.8	4.00 1.00 *	SAL
Ampelomyces quisqualis	AQ10	Intrachem-bio Italia	58	0.08	BCA
Ampelomyces quisqualis + Ultrafine oil	AQ10 + UFO	Intrachem-bio Italia Intrachem-bio Italia	58 98.8	0.08 1.00 *	BCA
Trichoderma harzianum T39	Trichodex	Makhteshim	20	4.00	BCA
Bacillus subtilis QST 713	Serenade	Intrachem-bio Italia	1.46	4.00	BCA
Bacillus subtilis F77	Experimental	SafeCrop Centre	-	no dil.*	BCA
Cladosporium tenuissimum	Experimental	CNR, Firenze	-	no dil.*	BCA
Sulphur	Heliosoufre	Intrachem-bio Italia	51.1	1.50*	CHE
Sulphur	Thiovit	Syngenta	80	3.00	CHE
K phosphate monobasic + Sulphur	K phosphate monobasicThiovit	Sigma Syngenta	99 80	2.00 1.50	SAL
Tetraconazole + Sulphur	Domark Combi	ISAGRO	1 + 40	5.0	CHE
Azoxystrobin	Ortiva	Syngenta	23.2	0.80 *	CHE
Kresoxim methyl	Stroby	BASF-AGRO	50	1.0	CHE
Pyraclostrobin + Nicobifen	BASF (Signum)	BASF-AGRO	6.7+ 6.7	1.0	CHE
Hexaconazole	Anvil 5	Syngenta	4.80	0.50 *	CHE
Miclobutanil	Thiocur	Dow Agrosciences	13.4	0.40 *	CHE
Penconazole	Ophir	Syngenta	10.5	0.40 *	CHE
Penconazole	Topas	Syngenta	10.5	0.40 *	CHE
Triadimenol	Bayfidan 25	Bayer	25	0.20 *	CHE
Tetraconazole	Domark	ISAGRO	10	1.0 *	CHE
Acylbenzolar-S-methyl	Bion	Syngenta	50	1.00	OTH
Fenarimol	Rubigan	Dow Agrosciences	11.4	0.30 *	OTH
Mancozeb + Famoxadon	Clipman	Du Pont	5 + 6.25	1.5	OTH
Polyoxin AL	Polar	Kaken Pharm.	50	0.50	OTH
Untreated	Water				NNN

* = ml/l; no dil.= no dilution: *B. subtilis* F77 >$5\cdot10^8$ CFU, *C. tenuissimum* >$1\cdot10^6$ CFU.
**category in which the a.i. was included: SAL=salt, BCA=biocontrol agent, SUL=compounds containing sulphur STR= strobilurin, TRI=triazole, OTH=other chemicals, UNT= untreated.
*** On glass slides, additional dosages of K bicarbonate at 2.00 g/l + Ultrafine oil at 1.00 ml/l, *A. quisqualis* (AQ10) at 0.04 g/l, sulphur (Heliosoufre) at 4.00 g/l, *T. harzianum* T39 (Trichodex) at 0.20 g/l. For the OTH group of treatments, an experimental wetting agent based on silicones (KF 643, CBC Europe, Italy) at 1.00 ml/l (OTH) and Harpin, a known resistance inducer (Messenger, Eden Bioscience, USA) at 0.64 g/l (OTH) were also tested.

Development of *P. aphanis* SCAR (RAPD-derived) markers. DNA for marker development was extracted from *P. aphanis* collected in Italy (Valsugana), as described above. RAPD-PCR reactions using 94 arbitrary decamers Operon Technologies (Alameda, CA, USA) were used to generate random fragments. RAPD-PCR amplification was carried out in reaction mixtures containing: 1 µl of non-quantified DNA, 0.1 mM dNTP, 1.5 mM $MgCl_2$, 0.3 mM of each RAPD primer, 0.07 U Taq polymerase in 1X PCR buffer (Amersham/Pharmacia Biotech, Uppsala, Sweden), in a total volume of 11 µl. Amplification conditions were as follows: two cycles of 30 s each at 94°C, 30 s at 36°C and 2 min at 72°C; 20 cycles, each consisting of 20 s at 94°C, 15 s at 36°C, 15 s at 45°C and 2 min at 72°C; 18 cycles, each consisting of 20 s at 94°C (one extra second at the end of each cycle), 15 s at 36°C, 15 s at 45°C and 2 min at 72°C (three extra seconds at the end of each cycle). After the 40th cycle, there was a final extension step of 10 min at 72°C. All the amplicons derived from RAPD reactions were pooled in a single tube, purified using the Wizard SV Gel Purification System® (Promega, Madison, WI, USA) and ligated to the PCR 2.1 TOPO vector. One Shot TOP10® cells were transformed using the TOPO XL PCR Cloning Kit System® (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendations. A colony PCR using the transformed One Shot TOP10® cells (Invitrogen Life Tecnologies, Carlsbad, CA, USA) was performed in 10 µl of PCR mix using the M13 universal primers supplied with the cloning kit.

Each of these PCR mixtures consisted of a final volume of 10 µl including: 0.1 mM dNTP, 1.5 mM MgCl2, 0.2 mM of each M13 primer and 0.7 U Taq polymerase in 1X PCR buffer (Amersham/Pharmacia Biotech, Uppsala, Sweden). The reaction conditions were as follows: denaturation at 94°C for 5 min followed by 38 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min. The reaction was terminated with an incubation at 72°C for 10 min. PCRs were carried out using a Geneamp 9600® thermocycler (Perkin-Elmer, Foster City, CA, USA). The products were purified using the Wizard SV Gel Purification System® (Promega Corp, Madison, WI, USA) according to the manufacturer's instructions.

Sequencing of both strands of the products was carried out using the M13 forward and reverse universal primers in an ABI 3100 Prism Sequencer® (Applied Biosystems Inc., Foster city, CA, USA). Specific primers were designed and synthesized on the basis of the sequences obtained. Primer specificity was tested on DNA extracted from *P. aphanis* mycelia, infected strawberry leaves and uninfected strawberry tissue.

Polymorphism of *P. aphanis* loci was successively tested on DNA extracted from *P. aphanis* infected strawberry leaves collected in 2003 in Israel (one sample each from Givat, Kadima, Volcani, Porat and Zofit) and in Italy (San Michele, four samples from the same site). Each sample consisted of 1 cm^2 of infected strawberry leaf. Each PCR mixture had a final volume of 11 ml, including 0.1 mM dNTP, 1.5 mM $MgCl_2$, 0.2 mM each of forward and reverse primer and 0.7 U HotStarTaq® polymerase in 1X PCR buffer (Qiagen, Valencia, CA, USA).

The reaction consisted of denaturing at 94°C for 15 min followed by 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min and a termination step of 10 min at 72°C. Sequencing, alignment and comparison of the *P. aphanis* specific markers were performed as described for the ITS regions.

RESULTS

Comparison of leaf and glass-slide bioassays. In the absence of any control agents, germination rates were higher on leaves (31.7%) than on glass slides (11.9%). Additionally, germ tubes grew longer (36.1 mm) on the leaves than on the slides (15 mm). These results are presented in terms of mycelial biomass (Table 2). Ultrafine oil did not affect the accumulation of biomass. All of the tested salts reduced the accumulation of fungal biomass on leaves. On the slides, the salts were generally less effective, and only Na bicarbonate, with and without ultrafine oil, and K bicarbonate at the higher dosage reduced biomass accumulation of the BCAs evaluated, only *T. harzianum* T39 at the higher concentration and the two *Bacillus* species (QST 713 and F77) were effective on leaves. None of the BCAs significantly affected biomass accumulation on slides.

On leaves, all the tested sulphur-based products reduced biomass accumulation. Strobilurin products, such as kresoxym-methyl and pyraclostrobin + nicobifen, reduced biomass, but the effect of azoxystrobin was not significantly different from that of the untreated control. All of the triazole fungicides reduced biomass accumulation on leaves. On glass slides, the effects of sulphur-containing agents, strobilurins and azoles on mycelial biomass were lower and not always significant. The other chemicals tested reduced biomass accumulation on leaves.

Interestingly, induced resistance agents (Harpin and BTH) failed to control *P. aphanis* conidium germination on slides (Table 2) and even resulted in greater accumulation of biomass.

Due to these differences in the results of the two bioassays, we chose to use the leaf bioassay in our comparison of the sensitivities of the two *P. aphanis* populations to the control agents.

Comparison of sensitivities of the Italian and Israeli populations to control agents. Conidia from the Israeli population had lower germination rates (13.2%) and shorter germ tubes (10.9 µ) than the Italian conidia

Table 2. Hyphal biomass* of *Podosphaera aphanis* treated with control agents on leaves and glass surfaces.

Treatment	Hyphal biomass			
	Leaf		Glass slide	
Salts:				
Untreated	1691.00		161.32	a
Ultrafine oil (UFO)	1562.92	a	74.00	ab
Ca carbonate	657.59	ab	73.72	ab
K bicarbonate (2 g/l) + UFO	455.99	a	67.72	ab
K bicarbonate (4 g/l) + UFO	297.73	b	59.31	ab
K bicarbonate	276.52	b	21.23	b
Na bicarbonate + UFO	257.22	b	36.78	b
Na bicarbonate	226.84	b	24.99	b
K phosphate monobasic	143.05	b	130.63	ab
Biocontrol agents:				
Untreated	1691.00	a	161.32	ab
Trichoderma harzianum T39 (0.2 g/l)	1436.25	a	30.45	b
Ampelomyces quisqualis (0.08 g/l)	922.15	ab	38.34	b
A. quisqualis (0.08 g/l)+ UFO	685.87	ab	190.42	ab
A. quisqualis (0.04 g/l)	596.73	ab	146.22	ab
T. harzianum T39 (4.00 g/l)	440.27	b	15.26	b
Bacillus subtilis QST 713	74.31	b	7.98	b
B. subtilis F77	17.00	b	275.92	a
Sulphur products:				
Untreated	1691.00	a	161.32	a
Sulphur (Tiovit)	230.175	b	46.50	ab
Sulphur (Heliosoufre)	212.04	b	29.72	ab
Sulphur (s-700)	77.85	b	14.22	b
K phosphate monobasic + Sulphur	76.79	b	78.66	ab
Tetraconazole + Sulphur	2.16	b	99.40	ab
Triazole fungicides				
Untreated	1691.00	a	161.32	a
Triadimenol	403.25	b	44.64	bc
Tetraconazole	490.96	b	86.66	abc
Penconazole (Topas)	189.55	b	61.30	abc
Miclobutanil	184.59	b	28.21	c
Penconazole (Ophir)	123.45	b	18.05	c
Hexaconazole	56.57	b	126.38	ab
Strobilurin fungicides:				
Untreated	1691.00	a	161.32	ab
Azoxystrobin	826.68	ab	45.46	b
Kresoxim methyl	64.82	b	57.80	b
Pyraclostrobin + Nicobifen	14.05	b	304.01	a
Other chemical fungicides:				
Untreated	1691.00	a	161.32	ab
Benzothiadiazole	269.04	b	339.15	ab
KF-643	229.90	b	197.69	ab
Harpin	211.61	b	500.51	a
Fenarimol	209.54	b	160.24	ab
Polyoxin	93.13	b	83.83	ab
Mancozeb + Famoxadon	25.81	b	30.46	b

* Treatments were sprayed on leaves or slides (three replicates for each treatment) immediately before conidia inoculation. The assessment was done by evaluating germination and germ tube elongation for 200 conidia per replicate after 24 hours of incubation at 20°C and >90 % relative humidity. Biomass was calculated by multiplying conidia germination by germ tube elongation.

**Numbers in the same column and the same group (salts, biocontrol agents, compounds containing sulphur, strobilurins, triazoles, other chemicals) followed by the same letter do not differ significantly at $P \leq 0.05$.

(31.3% and 36.1 µ). For comparisons of the two populations, data was expressed as percentage of inhibition of germination and germ tube elongation. The two populations did not significantly differ in their sensitivities to control agents. This was shown by comparing the inhibition of germination and germ tube elongation of the two populations within each group of control agents using ANOVA and Tukey's HSD test. The results for the two populations were also compared using the non-parametric Wilcoxon paired-sample test (Table 3).

Ultrafine oil was ineffective against both *P. aphanis* populations. In general, salts were more likely to affect germ tube elongation than germination itself. K and Na bicarbonate were the most effective control agents, in both Israel and Italy. Except for its partial inhibition of germ tube elongation, Ca carbonate was not effective against *P. aphanis* conidia.

Among the BCAs, *A. quisqualis* performed poorly regardless of whether or not it was applied together with ultrafine oil. The BCAs performed better against the Italian pathogen samples than the Israeli ones, despite the genetic similarity of the two populations. The two *Bacillus* species performed consistently against the two populations.

Sulphur was effective against both pathogen populations. The new formulation Heliosoufre® (with a reduced concentration of sulphur as compared to Thiovit®) showed good activity. Monobasic K phosphate with sulphur used at lower concentration (Thiovit®) had lower activity. All the triazoles were effective against the Israeli population but in the Italian population, the effects of triadimenol on germination and germ tube elongation were not significantly different from those of the untreated control. Tetraconazole also had low activity against germination in the Italian population. Among the strobilurins tested, azoxystrobin was more effective against germination than germ tube elongation across both populations. In Israel, there was no significant difference between Polyoxin AL and the untreated control. BTH was found to effectively inhibit germination and germ tube elongation in both populations (Table 3).

DNA analysis of *P. aphanis* isolates. The genetic similarity of *P. aphanis* populations from Israel and Italy was analyzed according to the ITS 1-2 sequence and randomly selected sequences. The aligned 359 bp fragments used for comparison were nearly identical (99-100%). The six randomly selected fragments (Table 5) covering 2800 bp were 100% identical between the five Israeli samples and the Italian sample. Identity of 100% was also found when comparing the ITS1-2 aligned 359 bp fragment to a GenBank submitted sequence of an *Sphaerotheca aphanis* isolate from Japan, also collected from strawberry (Takamatsu *et al.*, 2000). An isolate of *S. pannosa*, collected from *Rosa* sp. which is a close relative of strawberry, was 97% similar in sequence to the *P. aphanis* populations samples. Other species of powdery mildew were compared with our samples, including those affecting cucurbits (*S. fuliginea*) and tomato (*L. taurica*) which were 85 and 67% similar. These findings illustrate the utility of this method for species differentiation (Table 4).

Phylogenetic analysis of the ITS 1-2 sequence was performed using the Neighbour Joining method. Cluster analysis was performed using Clustalw and viewed using TreeView®. The results of these analyses are presented in Figure 1. Removing highly variable positions from the sequence analysis did not affect tree topology. ITS 1-2 sequence analysis was conducted on six Israeli *P. aphanis* samples (collected from strawberry crops at Givat Chen, Kadima, Porat, Volcani Center, Kfar Maas and Zofit), one Italian sample (Italy) and submitted GenBank sequences of isolates of the powdery mildews *S. aphanis* (synonym of *P. aphanis*) (AB 026136), *S. pannosa* (AB 011323), *S. fuliginea* (AB 040332) and *Leveillula taurica* (AB 044349). Sequence analysis confirmed the identification of the powdery mildew samples from Israel and Italy as *P. aphanis*.

RAPD-derived *P. aphanis* specific SCAR markers were successfully developed. Thirty-two transformed One Shot TOP10 cells were randomly selected among the hundreds of colonies obtained. The insert in the PCR 2.1 TOPO vector was sequenced and 32 primer pairs were designed. Six primer pairs were specific for *P. aphanis* (Table 5), one amplified strawberry DNA and the remaining 25 did not amplify any sequence. One of the six specific sequences contained the $(CA)_{15}$ microsatellite motif. The polymorphism of the six markers was examined on five Israeli- and four Italian *P. aphanis* samples, but none of the six markers showed any polymorphisms: the 2800 bp sequenced, including the microsatellite marker, were 100% monomorphic.

DISCUSSION

The leaf bioassay was the most suitable for testing the efficacy of control agents and for comparing the sensitivities of different populations to control agents. Conidial germination and growth in the untreated control was higher on leaves than on slides (Table 2). Previous research has indicated that powdery mildew conidia do not to rely on external nutrients for germination (Spencer, 1978), but our result may indicate an interaction between leaf surface metabolites and/or leaf morphology and the fungus that is important for the initial stages of infection. In the leaf bioassay, the effects of control agents were less variable among replicates and there were more significant differences between treatments as compared to the glass-slide bioassay. Two possible hypotheses may explain these results: an interac-

Table 3. Effects of control agents* on conidial germination and germ tube elongation for the Italian and Israeli *Podosphaera aphanis* populations.

Treatment	Inhibition of germ tube elongation (%) **		Inhibition of germination (%)**	
	Israel	Italy	Israel	Italy
Salts:				
K phosphate monobasic	71.4 cd***	71.8 cd	67.1 abc	56.6 abc
Ca carbonate	54.1 bcd	31.4 abcd	50.6 abc	18.1 ab
K bicarbonate	82.3 d	58.0 bcd	83.5 c	41.6 abc
Na bicarbonate	40.6 abcd	66.5 cd	41.8 abc	49.0 abc
Ultrafine Oil (UFO)	16.2 abc	-11.2 a	6.8 ab	13.7 ab
K bicarbonate + UFO	76.7 d	55.9 bcd	68.4 bc	41.7 abc
Na bicarbonate + UFO	82.7 d	56.9 bcd	70.9 bc	47.2 abc
Untreated	0.0 ab	0.0 ab	0.0 a	0.0 a
Biocontrol agents:				
A. quisqualis	59.1 bc	35.6 ab	63.2 bcd	20.4 ab
T. harzianum T39	62.2 bc	35.1 ab	60.9 bcd	41.9 abc
B. subtilis QST 713	58.9 bc	71.8 bc	62.5 bcd	76.7 cd
B. subtilis F77	53.9 abc	88.3 c	69.3 bcd	88.1 d
A. quisqualis +UFO	60.0 bc	39.4 ab	62.1 bcd	0.5 a
Untreated	0.0 a	0.0 a	0.0 a	0.0 a
Sulphur products:				
Sulphur (Heliosulfre)	96.9 b	72.3 ab	96.4 bc	39.0 ab
Sulphur (Tiovit)	98.1 b	56.9 ab	98.6 c	53.5 abc
K phosphate monobasic + Sulphur	58.5 ab	68.1 ab	65.8 abc	80.2 bc
Tetraconazole + Sulphur	88.1 b	95.2 b	86.7 bc	96.5 bc
Untreated	0.0 a	0.0 a	0.0 a	0.0 a
Triazole fungicides:				
Hexaconazole	76.8 b	78.7 b	68.3 bc	76.8 bc
Miclobutanil	77.2 b	63.8 ab	90.0 c	57.1 abc
Penconazole (Ophir)	68.2 b	67.0 b	60.0 abc	68.1 bc
Penconazole (Topas)	78.1 b	54.3 b	76.7 bc	65.1 bc
Tetraconazole	72.0 b	42.0 ab	71.7 bc	27.3 ab
Triadimenol	75.9 b	20.2 ab	70.0 bc	42.0 abc
Untreated	0.0 a	0.0 a	0.0 a	0.0 a
Strobilurin fungicides:				
Azoxystrobin	43.2 abc	23.9 ab	37.9 b	33.0 b
Pyraclostrobin + Nicobifen	96.3 d	86.7 cd	95.7 d	90.8 cd
Kresoxim-methyl	80.3 cd	76.1 cd	77.1 c	76.5 c
Untreated	0.0 a	0.0 a	0.0 a	0.0 a
Other chemical fungicides:				
Benzothiadiazole	89.7 bc	53.7 b	82.8 bc	49.7 b
Fenarimol	67.5 bc	64.4 b	66.4 bc	49.6 b
Mancozeb + Famoxadon	99.5 c	84.0 bc	99.3 c	86.9 bc
Polyoxin	55.5 ab	73.4 bc	32.1 ab	77.0 bc
Untreated	0.0 a	0.0 a	0.0 a	0.0 a

*Treatments were sprayed on leaves (three replicates for each treatment) immediately before conidia inoculation. The assessment was based on evaluations of germination and germ tube elongation for 200 conidia per replicate after 24 hours of incubation at 20°C and > 90 % relative humidity.

**Percentage of reduction was calculated relative to the average germination and germ tube elongation of the untreated controls, which were 31.3% and 36.1 µ for the Italian population and 13.2% and 10.9 µ for the Israeli population.

***Statistical analyses of the two populations were performed separately for the inhibition of germ tube elongation and for the germination in each group of control agents (salts, biocontrol agents, compounds containing sulphur, strobilurins, triazoles, other chemicals) on both populations. Numbers in both columns (Italy and Israel) and in the same section (germination or germ tube elongation inhibition) in each group of control agents, followed with the same letter do not differ significantly differ at $P \leq 0.05$.

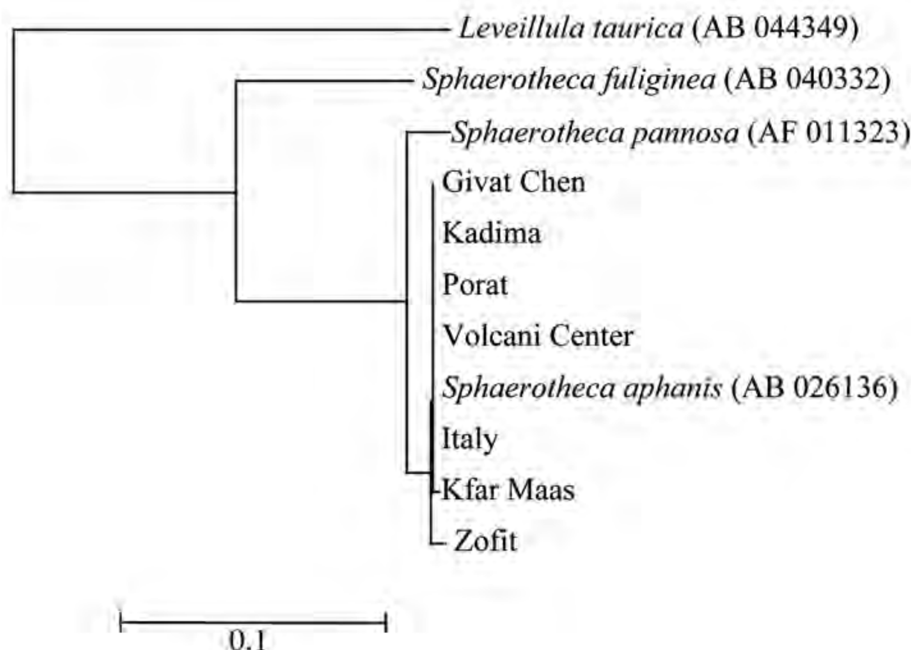

Fig. 1. ITS 1-2 based phylogenetic tree of *Podosphaera aphanis* populations from strawberry and published sequences of related *Sphaerotheca* and other species. The tree was produced using the neighbour-joining algorithm. Scale bar indicates estimated 10% sequence divergence.

tion between conidia and leaf tissues may influence the effects of the control agents on germination and germ tube elongation, or the presence of leaf exudates and/or the process of transpiration may affect the distribution of the active ingredients on the leaf surface.

The genetic tests suggested that the two populations are part of a single larger population. Indeed, regarding the sensitivity of the two populations, several reactions common to both can be highlighted. Although mineral oil has been found to be effective against other powdery mildews (Elad *et al.*, 1998), it did not significantly affect *P. aphanis* germination. Ultrafine oil did not improve the efficacy of salt-based products. The activity of bicarbonates and K phosphates seemed to be related more to a reduction in fungal growth (inhibition of germ tube elongation) than to a direct toxic effect (inhibition of germination). This may be due to the effects of salinity and pH on pathogen growth. *T. harzianum* and the two tested bacilli were less effective against *P. aphanis* when tested on glass slides, as compared to leaves. Since these organisms have also been reported to act as plant resistance inducers (De Meyer *et al.*, 1998; Guetsky *et al.*, 2002), the reduced efficacy on glass can be explained by the absence of BCA-originating inhibitory products from leaves.

The BCAs revealed some differences between the two populations, unlike other groups of agents. Lack of consistency is a common problem among biocontrol systems based on the activities of antagonistic organisms (Elad and Freeman, 2002). *A. quisqualis* was not very effective. This result may be attributed to this hyperparasite's inability to complete its infection cycle under the conditions tested. If so, these bioassays may not have been suitable for evaluating the effects of this hyperparasite. *B. subtilis* (both commercial and experimental strains) had previously been shown to be effective against other powdery mildew species. These bioassays confirmed its activity against *P. aphanis*.

All of the sulphur-containing compounds dramatically reduced mycelial biomass, confirming the toxic effect of this element on *P. aphanis*. Unlike sulphur, which performed similarly against both populations, some triazoles (like triadimenol and tetraconazole) had lower activity against the Italian population. This may indicate the presence of less sensitive strains within the larger Italian population. This is important information essential for preventing the establishment of triazole-resistance. In Italy, growers should be advised to implement control strategies which reduce the risk of developing fungicide resistance. Within integrated management programs, strobilurins may be alternated with triazoles in order to slow, and hopefully prevent, the development of fungicide resistance. Polyoxin, which is used in Israel, but not registered in Italy, showed reduced activity in Israel. This may indicate the presence of resistant strains in Israel.

The genetic diversity of different populations of *P. aphanis* from Israel and Italy was analyzed using ITS 1-2 sequences and sequences of random selected fragments.

Table 4. Percent similarity according to internal transcribed spacer (ITS) 1-2 nucleotide sequences among representative *Podosphaera aphanis* populations from strawberry from Israel and Italy, compared with powdery mildews from other hosts.

	Givat Chen	Kadima	Porat	Volcani	Kfar Maas	Zofit	Italy	*S. aphanis* AB 026136	*S. pannosa* AB 011323	*S. fuliginea* AB 040332
Kadima	100									
Porat	100	100								
Volcani	100	100	100							
Kfar Maas	99	99	99	99						
Zofit	99	99	99	99	99					
Italy	100	100	100	100	99	99				
S. aphanis AB 026136	100	100	100	100	99	99	100			
S. pannosa AF 011323	97	97	97	97	97	96	97	97		
S. fuliginea AB 04033	85	85	85	85	85	85	85	85	85	
L. taurica AB 044349	67	67	67	67	67	67	67	67	67	68

Table 5. Sequence name, primer sequence, melting temperature (Tm) and expected amplicon size (Eas) of six *Podosphaera aphanis* loci. The marker SF_ms017 includes a micro-satellite with the repeat $(CA)_{15}$. Each fragment was reverse and forward sequenced.

Marker name	Primer sequence (5'-3')	Tm	Eas
SF_as001	f: GACCCAGAGACAGAATAGATAGAGC	60	633 bp
	r: AGACCCAGAGAATACTGTTTATGAAT		
SF_as005	f: GGCTGGTTCCTAATATGGGATA	60	486 bp
	r: CCTTGCAACGTACGTAGAGACA		
SF_as041	f: GAAACAAGAGAGATCGGTTGG	60	625 bp
	r: TGCATATTCATTAGCATGAAACA		
SF_as066	f: GGCTGGTTCCTAATATGGGATA	58	413 bp
	r: CCAGATTAAAGTCTAGGACACCTC		
SF_as089	f: GGAGAGACTCTAGGTGGAAACAA	60	643 bp
	r: TGCATATTCAATTAGCATGAAACA		
SF_ms017	f: GCTGAATATCGCACAAAGATGA	60	109 bp
	r: GGCCCACAACAGACGATAAA		

ITS sequences have been used very consistently as an accurate tool for differentiating between populations and species of certain fungi in situations where classical morphology cannot be utilized (Sreenivasaprasad *et al.*, 1996; Freeman *et al.*, 2001). In our study, certain populations of *P. aphanis* from Israel and Italy were identical in sequence while other pairs showed 99% sequence similarity. Perfect matching of the sequences of 6 fragments, totalling 2800 bp, indicates that the isolates analysed can be considered as part of a single population.

Identity of 100% was also observed between our sequences and a GenBank ITS sequence from *S. aphanis* (synonymous to *P. aphanis*) isolated from strawberry in Japan (Takamatsu *et al.*, 2000). The species *S. pannosa*, isolated from *Rosa* sp., which is a close botanical relative of strawberry, was 97% similar in sequence to the *P. aphanis* isolates. Other species of powdery mildew were more distant, such as those affecting cucurbits and tomato which had 86 and 65% similarity. These findings confirm the utility of this method for species differentiation. These laboratory results suggest that the *P. aphanis* populations from Italy and Israel are similar enough to allow common systems to be developed for optimising the application of strawberry powdery mildew control agents. However, further studies are necessary to confirm these results under field conditions.

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