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Genetic diversity, aggressiveness and metalaxyl sensitivity of *Pythium aphanidermatum* populations infecting cucumber in Oman

A. M. Al-Sa'di[a]*, A. Drenth[b], M. L. Deadman[a] and E. A. B. Aitken[c]

[a]*Department of Crop Sciences, College of Agricultural and Marine Sciences, Sultan Qaboos University, PO Box 34, Al-Khod 123, Oman;* [b]*Tree Pathology Centre, The University of Queensland and Queensland Department of Primary Industries and Fisheries, 80 Meiers Road, Indooroopilly, 4068, QLD; and* [c]*School of Integrative Biology, The University of Queensland, St. Lucia, QLD, 4072, Australia*

Seventy three isolates of *Pythium aphanidermatum* obtained from cucumber from four different regions of Oman and 16 isolates of muskmelon from the Batinah region in Oman were characterized for aggressiveness, sensitivity to metalaxyl and genetic diversity using AFLP fingerprinting. Twenty isolates of *P. aphanidermatum* from diverse hosts from different countries were also included in the study. Most isolates from Oman were found to be aggressive on cucumber seedlings and all were highly sensitive to metalaxyl ($EC_{50} < 0{\cdot}80\ \mu g\ mL^{-1}$). Isolates from cucumber and muskmelon were as aggressive as each other on both hosts ($P > 0{\cdot}05$), which implies a lack of host specialization in *P. aphanidermatum* on these two hosts in Oman. AFLP analysis of all isolates using four primer–pair combinations resolved 152 bands, of which 61 (~40%) were polymorphic. Isolates of *P. aphanidermatum* from Oman and other countries exhibited high genetic similarity (mean = 94·1%) and produced 59 different AFLP profiles. Analysis of molecular variance indicated that most AFLP variation among populations of *P. aphanidermatum* in Oman was associated with geographical regions ($F_{ST} = 0{\cdot}118$; $P < 0{\cdot}0001$), not hosts ($F_{ST} = -0{\cdot}004$; $P = 0{\cdot}4323$). These data were supported by the high rate of recovery (24%) of identical phenotypes between cucumber and muskmelon fields in the same region as compared to the low recovery (10%) across regions in Oman, which suggests more frequent movement of *Pythium* inoculum among muskmelon and cucumber fields in the same region compared to movement across geographically separated regions. However, recovering clones among regions and different countries may imply circulation of *Pythium* inoculum via common sources in Oman and also intercontinental spread of isolates.

Keywords: AFLP, AMOVA, damping-off, genetic differentiation, genetic similarity, phenotypes

Introduction

The rapid expansion in cucumber cultivation in greenhouses in Oman since the mid 1990s and especially since the turn of the century has been accompanied by adoption of cultural practices that were not common in this country in the past. This expansion has been characterized by monoculture and a heavy reliance on inorganic fertilizers and the import of potting mixtures. The expansion has also given rise to an increase in cucumber pests and diseases and subsequently a high reliance on pesticides to manage these problems, with as many as 20 pesticide applications per growing season (Al-Kiyumi, 2006). One of the major diseases is pythium damping-off, which was reported to be the biotic factor most limiting greenhouse cucumber production in Oman, causing up to 75% mortality among cucumber seedlings. It was found to be present in 77% of greenhouses surveyed in the Batinah region between 2000 and 2005 (Al-Kiyumi, 2006). A subsequent study has identified *Pythium aphanidermatum* as the predominant species associated with damping-off in 13 cucumber growing districts in Oman (Al-Sa'di *et al*., 2007). The high incidence of the disease, accompanied by the predominance of *P. aphanidermatum* in all the growing districts, is considered a major concern for most cucumber growers in Oman.

Earliest reports of *P. aphanidermatum* in Oman date back to the early 1990s when it was found to infect more than 12 different plant species including some cucurbits in the Batinah region (Moghal *et al*., 1993). Cucumber was not among the hosts reported to be infected by *P. aphanidermatum* during that survey, however,

*E-mail: alsadi@squ.edu.om

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P. aphanidermatum was commonly found on muskmelon in various parts of Oman. It is unknown whether the occurrence of this pathogen in greenhouses originated from inoculum previously present on other crops or from new introductions of the pathogen. In addition, because of geographical separation of most cucumber-growing regions in Oman by mountains and the relatively large distance between them (50 to 150 km), little is known about phenotypes constituting *Pythium* inoculum in these regions or the rate of movement between regions.

Pythium aphanidermatum is a homothallic diploid oomycete, reproducing sexually to give oospores, and asexually by production of sporangia and zoospores (Plaats-Niterink, 1981). Due to the fact that it is a serious pathogen of many vegetable, fruit, grass and ornamental crops in several parts of the world (Hendrix & Campbell, 1973; Plaats-Niterink, 1981; Moorman *et al*., 2002), a significant body of knowledge has been generated concerning the ecology, identification, transmission, host range, aggressiveness and management of *P. aphanidermatum* (Goldberg & Stanghellini, 1990; Herrero & Klemsdal, 1998; Martin & Loper, 1999; Al-Hasani, 2004). However, little attention has been given to genetic diversity within this species, with the exception of one study. This study reported a low level of genetic diversity within 23 isolates, mostly from the USA, using amplified fragment length polymorphisms (AFLP), in an attempt to develop diagnostic fingerprints for some *Pythium* species (Garzon *et al*., 2005a). Despite the amount of information available on pathogenicity, host range and control of *P. aphanidermatum* in Oman (Deadman *et al*., 2002; Al-Sa'di *et al*., 2003; Al-Hasani, 2004; Al-Sa'di *et al*., 2007), little is known about the levels of diversity within and among populations of *P. aphanidermatum* affecting cucumber.

Population genetic studies have been widely used to provide information useful for integrated disease management strategies (Lamour & Hausbeck, 2001a,b; Huang *et al*., 2004; Ivors *et al*., 2004). These studies were aided in specific cases by several molecular techniques including isozymes (Barr *et al*., 1997), random amplified polymorphic DNA (RAPD) (Francis *et al*., 1994; Matsumoto *et al*., 2000), single-copy restriction fragment length polymorphism (RFLP) (Harvey *et al*., 2000, 2001) and the use of AFLP (Van Der Lee *et al*., 1997; Lamour & Hausbeck, 2001a,b, 2003; Ivors *et al*., 2004; Garzon *et al*., 2005a).

Little is known about variation in aggressiveness in the current *P. aphanidermatum* population infecting cucumber in Oman, in spite of studies indicating host specificity and differences in levels of aggressiveness among isolates of *Pythium* on different hosts (Hampton & Buchholtz, 1962; Morgan & Hartwig, 1964).

A number of cultural and chemical control tactics are currently employed by growers in Oman to manage pythium damping-off. Solarization of greenhouse soil in summer when no cucumbers are grown and frequent soil replacement are common cultural practices. Metalaxyl is a commonly used fungicide for the control of *Pythium*-induced diseases in many greenhouses in Oman (Al-Kiyumi, 2006). However, decreased efficacy of metalaxyl to control damping-off disease in Oman has recently been noticed, with disease incidences in some greenhouses as high as in non-treated greenhouse crops (Deadman *et al*., 2002; Al-Kiyumi, 2006).

There are different explanations for the decrease in the efficacy of metalaxyl. Firstly, development of resistance to metalaxyl could be one factor as reported in other oomycete plant pathogens such as *Pseudoperonospora cubensis* (Reuveni *et al*., 1980), *Phytophthora infestans* (Davidse *et al*., 1981) as well as *Pythium aphanidermatum* in Pennsylvania, USA (Sanders, 1984). Second, it has been found that metalaxyl can become subject to rapid biodegradation in soil, lowering its effective half life from 82 days to only 10 days (Davison & McKay, 1999).

This paper examines specific hypotheses: (i) to determine if *P. aphanidermatum* populations in Oman consist of a single phenotype or a large number of different phenotypes; (ii) to test for genetic differentiation among *P. aphanidermatum* populations from four regions, and from cucumber and muskmelon from the Batinah region; (iii) to test for differentiation in aggressiveness and metalaxyl sensitivity between *P. aphanidermatum* isolates obtained from different regions and hosts; and (iv) to test if failure in disease control using metalaxyl is due to the presence of metalaxyl resistant isolates.

Materials and methods

Sources of *Pythium* isolates

Seventy-three isolates of *Pythium aphanidermatum* were obtained from cucumber seedlings showing damping–off disease in Oman in 2004 and 2005 (Al-Sa'di *et al*., 2007). Isolates were collected from cucumber in 11 administrative districts and were assigned to one of four geographical regions: Sharqiya north (SC), Interior (IC), Muscat (MC) and Batinah (BC). These regions are separated from each other by mountains and are at least 50–150 km apart, except for Muscat and Batinah which are adjacent (Fig. 1). Variation in the number of isolates among regions (17 from Sharqiya, five from Interior, four from Muscat and 47 from Batinah) reflects the intensity of greenhouse cucumber cultivation and prevalence of damping-off in 2004 and early 2005. Each of the 73 isolates was obtained from a different greenhouse except for two isolates from the same greenhouse in Batinah region and two from the same greenhouse in Sharqiya region. Fourteen isolates were also obtained from muskmelon showing wilt symptoms from the Batinah region (BM), in order to compare muskmelon isolates with isolates obtained from cucumber from the same region (BC). In addition, two isolates were obtained from a culture collection at Sultan Qaboos University. These were from wilting muskmelon, collected in 1996 and 1997 from the Batinah region. In order to compare isolates in Oman to other populations, 15 isolates of *P. aphanidermatum* were obtained from the USA and five from Norway.

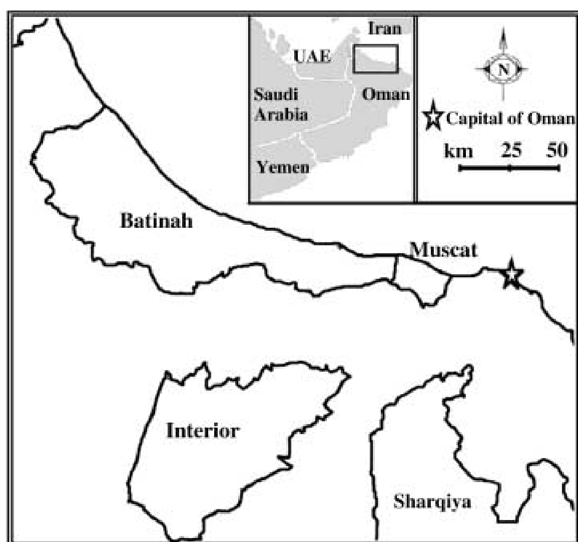

Figure 1 Four regions in Oman (Batinah, Muscat, Interior and Sharqiya) from which isolates of *Pythium aphanidermatum* were obtained. Borders indicate the surveyed areas in each region and do not represent administrative divisions.

Identification of *P. aphanidermatum* isolates obtained from muskmelon

Isolates of *P. aphanidermatum* from cucumber were identified in a previous study using sequences of the internal transcribed spacer (ITS) of the ribosomal DNA (Al-Sa'di *et al*., 2007). The 16 isolates of *P. aphanidermatum* obtained from muskmelon were identified to the species level using sequences of the ITS region. DNA was extracted using the protocol of Lee & Taylor (1990) modified as described by Al-Sa'di *et al.* (2007). The ITS region (ITS 1, 5·8S and ITS 2) of the 16 *Pythium* isolates was amplified using the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al*., 1990) as described by Al-Sa'di *et al.* (2007).

The PCR products were purified from primers and dNTPs using the UltraClean PCR Clean-up kit (Mo-Bio Laboratories) according to the manufacturer's protocol. Samples were submitted to the Australian Genomic Research Facility (AGRF) in Brisbane (Australia) for sequencing using BigDye V3·1 (Applied Biosystems). Sequencing was performed in both directions using the same primers used for amplification.

The resulting ITS sequences (ITS 1, 5·8S and ITS 2) were compared with sequences deposited at the National Center for Biotechnology Information (NCBI) and with sequences generated by Al-Sa'di *et al.* (2007) from the *P. aphanidermatum* isolates in Oman. Earlier studies have reported limited intraspecific variation in the ITS region of *P. aphanidermatum* (Moorman *et al*., 2002; Lévesque & de Cock, 2004; Al-Sa'di *et al*., 2007), and so sequences generated in this study were compared directly with sequences of reference isolates from GenBank by aligning all sequences using the program Clustal W (fast) (Thompson *et al*., 1994).

AFLP fingerprinting

In order to test if the *P. aphanidermatum* population consists of a single or multiple phenotypes, DNA fingerprinting using AFLPs was conducted. The AFLP protocol was modified from Vos *et al.* (1995) using Hex-labelled *Eco*RI-AC (5′-Hex-GACTGCGTACCAATTCAC-3′) and *Eco*RI-AG (5′-Hex-GACTGCGTACCAATTCAG-3′) selective primers. Genomic DNA, extracted using the protocol of Lee & Taylor (1990) modified by Al-Sa'di *et al.* (2007), was digested for 90 min at 37°C using *Eco*RI and *Mse*I restriction enzymes (2·1 μL of 10× One-Phor-All buffer Plus (OPA$^+$) (Amersham Biosciences), 2 U *Eco*RI; 2 U *Mse*I, ~100 ng of genomic DNA, and Milli-Q water up to a volume of 17·5 μL). A 2·5 μL ligation mixture consisting of 0·3 μL of 10× OPA$^+$ buffer, 2·5 ρmol *Eco*RI adaptor (5′-CTCGTAGACT-GCGTACC/AATTGGTACGCAGTC-3′), 25 ρmol *Mse*I adaptor (5′-TACTCAGGACTCAT/GACGATGAGTCCT-GAG-3′), 0·5 U T4 DNA ligase (USB Corporation) and 100 μM of ATP-lithium salt (Roche Diagnostics) was added to the digested DNA and incubated for 90 min at 37°C. The restriction/ligation (R/L) mix was diluted at a ratio of 2 (R/L):1 ($TE_{0·1}$). AFLP fingerprinting was first performed on a random sample of at least 10 *P. aphanidermatum* isolates using 16 primer pair combinations (2 *Eco*RI+2 × 8 *Mse*I+1, 2 or 3). Out of these, the four which gave good resolution after size fractioning using polyacrylamide gels were chosen for analysis of the whole population of *P. aphanidermatum* (Table 1). Pre-selective amplification reaction mixtures consisted of 2·0 μL of 10× PCR buffer, 0·40 μL of 10 mM dNTPs, 1·2 μL of 25 mM $MgCl_2$, 0·54 μL of 10 μM *Eco*RI+A and 0·54 μL of 10 μM *Mse*I+C/A, 0·18 μL of 5·5 U/μL *Taq*1 (Fisher Biotec), 3·0 μL of the diluted (R/L) mix and 12·14 μL Milli-Q water. PCR was performed on a Mastercycler Gradient-5331 (Eppendorf) with the following temperature profile: 4 min at 94°C, followed by 28 cycles of 94°C for 30 sec, 60°C for 1 min and 72°C for 1 min. A final extension step was performed at 72°C for 5 min. The PCR product was then diluted at a ratio of 1 (PCR product):16 ($TE_{0·1}$). The selective amplification reactions were as above except using 0·15 μL of 10 μM *Eco*RI selective primers, 0·5 μL of 10 μM *Mse*I selective primers and 5 μL of the diluted pre-selective amplification product. The cycling profile was 94°C for 4 min followed by 94°C for 30 sec, 65°C for 30 sec, 72°C for 1 min, then nine cycles of 94°C for 30 sec, 65°C for 30 sec, 72°C for 1 min with a stepwise lowering of the annealing temperature by 1°C in each cycle, followed by 26 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min and a final extension step for 5 min at 72°C. Selective amplification products were diluted 1:1 in blue dextran formamide, denatured for 2 min at 94°C and then 1 μL of the mixture was loaded on a 5% denaturing polyacrylamide gel. GeneScan-500 TAMRA (Applied Biosystems) was used as a size standard and

Table 1 Description of the four primer–pair combinations used in AFLP fingerprinting of 109 *Pythium aphanidermatum* isolates

Primer pair combination	N[a]	P (%)[b]	Polymorphic bands[c]
*Eco*RI+AC/*Mse*I+C	64	24 (38)	50, 58, 72, 86, 110, 136, 153, 155, 158, 178, 213, 215, 218, 238, 240, 245, 256, 260, 302, 304, 333, 363, 405, 541
*Eco*RI+AG/*Mse*I+A	50	19 (38)	73, 100, 128, 199, 206, 224, 225, 243, 247, 255, 259, 279, 321, 347, 349, 378, 433, 437, 477
*Eco*RI+AG/*Mse*I+CA	19	9 (47)	79, 99, 166, 171, 254, 263, 266, 376, 408
*Eco*RI+AG/*Mse*I+CT	19	9 (47)	56, 67, 70, 74, 84, 97, 106, 135, 419
Overall	152	61 (40·1)	

[a]N = number of unambiguously scored loci.
[b]P (%) = number (percentage) of polymorphic loci.
[c]Values indicate fragment sizes in base pairs.

electrophoresis was performed at 1200 V for 90 min in a Gel Scan-2000 (Corbett Research). Electropherogram signals produced by the GeneScan-2000 software were scored manually as binary data with 1 indicating the presence and 0 indicating the absence of a band at a given location in a lane. Assuming each band represents a unique locus, the size of each band was determined in base pairs (bp).

Reproducibility of AFLP profiles was examined starting from the DNA extraction step, the restriction-ligation step and the pre-selective amplification step for at least 10, 35 and 90 isolates, respectively. Repetitions of AFLP analysis from the DNA extraction step were performed on three separate occasions with DNA extracted in January 2005, November 2005 and June 2006. Repetitions performed on 90 isolates from the pre-selective amplification step were done two to six times each. In addition, at least two positive control isolates were included in every run for each of the four primer-pair combinations.

Analysis of AFLP data

AFLP fragments ranging in size from 50 to 600 bp that could be scored unambiguously were included in the analysis. The percentage polymorphic loci were determined for each primer pair combination. Genotypic diversity ($\hat{G}$) within each population was determined as described by Stoddart & Taylor (1988). To allow comparison of genotypic diversity between populations of different sample sizes, $\hat{G}$ was scaled by the number of phenotypes (g) (Grünwald *et al*., 2003). Binary data were loaded in POPGENE (v 1·32, Yeh & Boyle, 1997) which was used to generate a dendrogram based on Nei's unbiased measures of genetic identity (Nei, 1978) using the unweighted pair-group mean analysis (UPGMA). Tree branch stability was assessed using bootstrap analysis with 1000 replications using PAUP (Phylogenetic Analysis Using Parsimony) version 4·0b8 (Swofford, 1998). The AFLP data were also examined by analysis of molecular variance (AMOVA) using ARLEQUIN (v 3·1, Excoffier *et al*., 2005), where partitioning of molecular variation was performed by considering AFLP banding patterns as haplotypes with an unknown gametic phase. Total genetic variance was partitioned among and within populations based on hosts (cucumber and muskmelon from Batinah region) and cucumber growing regions in Oman (Sharqiya, Interior, Muscat and Batinah).

Aggressiveness

The level of aggressiveness of *P. aphanidermatum* isolates from cucumber and muskmelon was determined by inoculating 6-day-old cucumber seedlings var. RS 164695 (Seminis) with *Pythium* plugs in a growth chamber at 25°C and 65–70% relative humidity as described by Al-Sa'di *et al*. (2007). Seven cucumber seeds were sown in plastic pots and emerging seedlings were thinned to five plants. On day seven, seedlings were inoculated with 10 mm agar–plugs obtained from the edge of a 3-day-old *Pythium* culture placed 2 mm away from each seedling. The pots were incubated for 2 weeks in a growth chamber in a completely randomized design. The experiment was replicated by testing each isolate at least twice using five pots (five seedlings per pot) for each isolate per replicate. The level of aggressiveness of each isolate was determined as the percentage of seedlings showing damping-off symptoms within 4 days of inoculation. In addition, 20 randomly selected isolates of *P. aphanidermatum* from the Batinah region were compared for aggressiveness on muskmelon with the 16 isolates of *P. aphanidermatum* from muskmelon.

Data generated from aggressiveness levels of different isolates on cucumber and muskmelon were used to test whether populations from different regions, AFLP phenotypes and hosts showed different levels of aggressiveness. Statistical analysis of this data was conducted using Tukey's Studentized Range test (SAS v8). In addition, Kolmogorov-Smirnov two-sample test (Sokal & Rohlf, 1995) was used to test for differences in frequency distribution of aggressiveness levels of *P. aphanidermatum* populations from cucumber and muskmelon.

Sensitivity of *P. aphanidermatum* isolates to metalaxyl

In order to test if reduced efficacy of metalaxyl was due to fungicide resistance in the pathogen population, the

effective dose at which 50% (EC_{50}) growth reduction *in vitro* takes place was determined. A pilot study was used to estimate the range of sensitivities of five randomly selected isolates to metalaxyl. Based on those initial results, a dilution series of 0, 0·1, 0·5, 1, 5 and 20 μg mL^{-1} of a.i. 97% metalaxyl (Novartis) in 1·7% corn meal agar (CMA) was used for the main experiment. A 5-mm-diameter disc was transferred from the margin of a 3-day-old *Pythium* culture to the edge of each plate. Four replicate plates were used for each treatment and each isolate was tested twice. Plates were incubated at 25°C in a completely randomized design. Linear growth was measured after 24, 48, 72 and 96 h. Metalaxyl concentration resulting in 50% growth inhibition (EC_{50}) of *Pythium* was estimated from the fitted regression line of the percent inhibition plotted against log-transformed fungicide concentration. Eleven standard isolates of *P. aphanidermatum* of known degree of sensitivity to mefenoxam (an isomer of metalaxyl), obtained from Prof. Gary Moorman (University of Pennsylvania, USA), were included in the test and one more concentration of metalaxyl, 100 μg mL^{-1}, was used in addition to the standard dilution series used previously, to be able to accurately determine the EC_{50} of the resistant isolates.

In addition to testing whether reduced efficacy of metalaxyl is due to the presence of less sensitive populations of *P. aphanidermatum*, data were also used to compare sensitivity to metalaxyl of populations from different regions, AFLP phenotypes and hosts. Data were analysed using Tukey's Studentized Range test (SAS, v8) and Kolmogorov-Smirnov two-sample test (Sokal & Rohlf, 1995).

Results

Identity of *P. aphanidermatum* isolates from muskmelon

The identity of the 16 *P. aphanidermatum* isolates obtained from muskmelon was confirmed using sequences of the entire ITS region (ITS-1, 5·8S, ITS-2) of the rDNA (777 base pairs) through amplification using the ITS1 and ITS4 primers. All sequences from different isolates showed 100% nucleotide similarity to each other and also to previously published sequences of *P. aphanidermatum* in GenBank (e.g. AY151180 and DQ298521). Therefore, only one sequence of a representative isolate (P007) was deposited in GenBank under Accession number DQ872464.

AFLP analysis of *P. aphanidermatum* populations

AFLP fingerprinting yielded 152 clearly resolved and unambiguously scored bands ranging in size from 50 to 540 bp, of which 61 (40·1%) were polymorphic (Table 1). Repeated AFLP analysis from the DNA extraction step, restriction step and pre-selective amplification step confirmed reproducibility of all fragment patterns produced except for two bands which disappeared from two isolates after repetitions, and were therefore not included in the analysis. Very limited variation was observed in the intensity of the electropherogram signals between separate DNA extractions and between reactions repeated from the restriction step.

Genetic similarity among isolates of *P. aphanidermatum*

AFLP analysis of the 73 isolates from cucumber in Oman produced 40 different AFLP phenotypes. Most *P. aphanidermatum* populations were found to have a high phenotypic diversity as measured by $\hat{G}/g$% (Table 2). However, a high level of genetic similarity was found among isolates from Oman (mean = 96·6%) and from other countries (mean = 94·1%) (Fig. 2). Ten clones occurred more than once in the *P. aphanidermatum* population in Oman. Among these ten, four were recovered from more than one region, representing 10% of the total phenotypes recovered from cucumber. Only two (C4 & C6) were found to be shared among populations from different countries, with clone C4 made up of isolates from Oman, the USA and Norway and clone C6 having isolates from Oman and the USA (Table 3). Interestingly, about 69% of *P. aphanidermatum* isolates from muskmelon

Table 2 Phenotypic diversity within populations of *Pythium aphanidermatum*

Origin	Population	Region	Host	N[a]	%P[b]	g[c]	$\hat{G}$[d]	$\hat{G}/g$%[e]
Oman	SC	Sharqiya	Cucumber	17	11·8	16	15·2	95
	IC	Interior	Cucumber	5	5·3	5	5·0	100
	MC	Muscat	Cucumber	4	4·6	4	4·0	100
	BC	Batinah	Cucumber	47	15·8	20	6·7	34
	BM	Batinah	Muskmelon	16	8·6	11	8·5	77
USA	USA	–	–	15	23·7	12	9·8	82
Norway	Norway	–	–	5	16·5	5	5·0	100
	Overall	–	–	109	40·1	59	–	–

[a]N = sample size.
[b]%P = percentage of polymorphic loci (out of 152).
[c]g = number of different phenotypes recovered.
[d]$\hat{G}$ = Stoddart and Taylor's measure of genotypic diversity.
[e]$\hat{G}/g$% = percentage of maximum diversity obtained in each population.

Table 3 Analysis of *Pythium aphanidermatum* clones recovered from Oman, the USA and Norway

Clone No.[a]	No. of isolates	Country	Region/state[b]	Host[c]	Year	EC_{50} (mean)[d]	Aggressiveness (mean)[de]
1	19	Oman	B, M	C, M	04, 05	0·30 a	75 ab
2	7	Oman	B	C, M	04, 05	0·30 a	71 ab
3	2	Oman	B	C	04	0·32 a	86 a
4	15	Oman, USA, Norway	B, I, N, P, S	C, M, P	02, 04, 05	> 7·02 a	74 ab
5	3	Oman	B	C, M	04, 05	0·28 a	75 ab
6	5	Oman, USA	C, P, S	C, R, H	03, 04, 05	> 20·28 a	80 ab
7	2	Oman	B, M	C	04	0·25 a	78 ab
8	3	Oman	B	C, M	97, 04	0·31 a	72 ab
9	2	Oman	B	C, M	96, 05	0·27 a	76 ab
10	2	Oman	B, S	C	04	0·25 a	54 b

[a]Only those phenotypes that consist of two isolates or more are listed in this table.
[b]B = Batinah, I = Interior and S = Sharqiya in Oman; C = California, P = Pennsylvania in the USA; N = Norway.
[c]C, H, M, P and R refer to cucumber, chrysanthemum, muskmelon, poinsettia and pepper, respectively.
[d]Means with the same letter in the same column are not significantly different at $P < 0·05$ (Tukey's Studentized Range test, SAS, V8).
[e]Represent aggressiveness means (% seedling mortality) for isolates from Oman.

produced AFLP profiles identical to those produced by *P. aphanidermatum* isolates from cucumber in the same region (Fig. 2), which accounted for 24% of the total phenotypes recovered from the Batinah region.

Clustering of isolates, as well as clones, was not related to geographical region, host, metalaxyl sensitivity, aggressiveness or year of isolation (Fig. 2, Table 3). For example, clone C4 contained isolates obtained in different years (2002, 2004 and 2005) from all countries, three regions in Oman (Batinah, Sharqiya and Interior), two hosts (cucumber and muskmelon), metalaxyl sensitive (P037; $EC_{50} = 0·27$ μg mL^{-1}) and metalaxyl resistant (P125; $EC_{50} > 100$ μg mL^{-1}) isolates. The mean aggressiveness level of clone C10 was significantly less than clone C3, but only on the basis of two isolates in either of the clones (Table 3).

Partitioning of genetic variation in *P. aphanidermatum*

Analysis of molecular variance of *P. aphanidermatum* populations from cucumber from four different regions in Oman partitioned 12% of the total variance among regions. On the other hand, host grouping (cucumber and muskmelon) did not account for significant AFLP variation (Table 4). Based on F_{ST} values, most populations from different geographical locations (except Muscat) were found to differ significantly from one another (Table 5). Cluster analysis was also in agreement with AMOVA and F_{ST} values, where most of the clones recovered were found to be shared among host groups in the same location compared to the limited clones recovered among populations from different geographical locations (Fig. 2).

Aggressiveness of *P. aphanidermatum* isolates

Analysis of *P. aphanidermatum* populations from Oman on cucumber showed high levels of aggressiveness towards cucumber. Out of 89 isolates tested for aggressiveness on cucumber seedlings, 78 (87·6%) were found to result in more than 60% mortality within 4 days of inoculation (Fig. 3). Only two isolates from cucumber were found to be weak pathogens, resulting in 16–18% mortality within 4 days of inoculation.

Table 4 Variation as measured using AFLPs among and within populations of *Pythium aphanidermatum* in Oman based on hierarchical analysis of molecular variance (AMOVA)

Source of variation[a]	Df[b]	Sum of squares	Variance component	Percent variation	F_{ST}	P[c]
Host						
Among populations	1	1·405	–0·006	–0·37	–0·004	0·4323
Within populations	61	94·118	1·543	100·37		
Cucumber growing regions (Oman)						
Among populations	3	13·666	0·226	11·83	0·118	< 0·0001
Within populations	69	115·978	1·681	88·17		

[a]Analysis of variation among and within hosts (cucumber and muskmelon) and cucumber growing regions in Oman (Sharqiya, Interior, Muscat and Batinah) (see Table 2 for populations).
[b]Degrees of freedom.
[c]P = the probability of obtaining a more extreme variance component estimate by chance alone (1000 permutations).

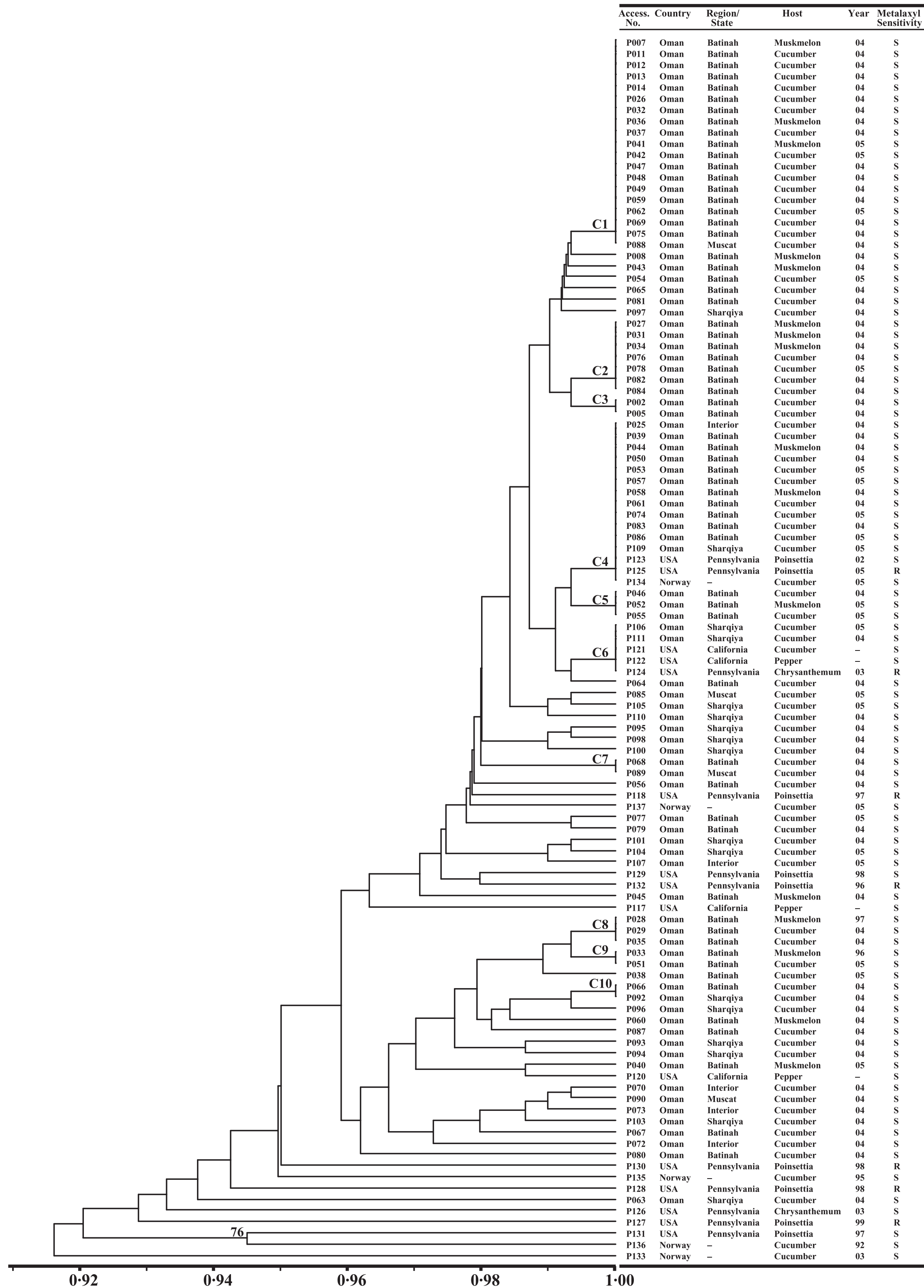

Figure 2 Unweighted pair-group mean analysis (UPGMA) dendrogram illustrating Nei's genetic similarities (Nei, 1978) and characteristics of 109 isolates of *Pythium aphanidermatum* from diverse hosts and geographical locations. C1 to C10 represent clones of *P. aphanidermatum*, while S and R in the metalaxyl sensitivity column represent sensitive (EC_{50} < 100 μg mL^{-1}) and resistant (EC_{50} > 100 μg mL^{-1}) isolates of *P. aphanidermatum*. Values over branches represent bootstrap support values (values above 50% are indicated; 1000 replications).

	Cucumber				Muskmelon	Multiple crops	
	Sharqiya (SC)	Interior (IC)	Muscat (MC)	Batinah (BC)	Batinah (BM)	USA	Norway
SC	–	0·266	0·458	0·001	0·001	0·035	0·003
IC	0·025	–	0·434	0·002	0·012	0·041	0·036
MC	–0·007	–0·005	–	0·860	0·681	0·247	0·144
BC	**0·133**	**0·264**	–0·073	–	0·432	0·000	0·000
BM	**0·104**	**0·209**	–0·045	–0·004	–	0·002	0·005
USA	**0·045**	**0·102**	0·019	**0·161**	**0·120**	–	0·097
Norway	**0·151**	**0·152**	0·070	**0·309**	**0·205**	0·059	–

Below diagonal: F_{ST} values; above diagonal: probabilities of having more extreme F_{ST} values than observed by chance alone. Values in bold indicate significant differences at $P < 0·05$.

Table 5 Pairwise genetic differentiation among populations of *Pythium aphanidermatum* based on F_{ST} values

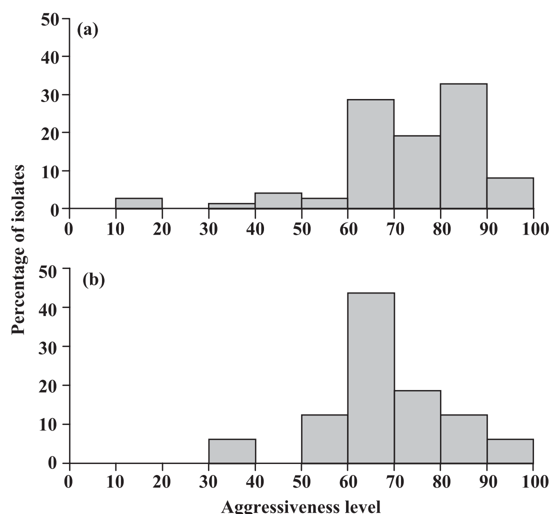

Figure 3 Distribution of aggressiveness levels on cucumber for 73 isolates of *Pythium aphanidermatum* from cucumber (a) and 16 isolates from muskmelon (b) in Oman. Aggressiveness level represents the percent seedling mortality within 4 days of inoculation. No significant differences were found in frequency distribution of aggressiveness levels between the populations from cucumber and muskmelon ($P > 0·05$; Kolmogorov-Smirnov two-sample test).

With the exception of significant differences in aggressiveness observed between the cucumber population from Muscat and the muskmelon population from the Batinah region ($P < 0·05$), no significant differences were observed among the other populations from different regions in their aggressiveness on cucumber seedlings (Table 6). In addition, isolates from muskmelon were found to be as aggressive as isolates from cucumber on cucumber seedlings ($P > 0·05$), with a mean level of aggressiveness of 69% and 72% for muskmelon and cucumber isolates from the same region, respectively. No significant differences were found in frequency distributions of aggressiveness levels between *P. aphanidermatum* populations from cucumber and muskmelon ($P > 0·05$; Fig. 3). When 20 isolates obtained from cucumber in the Batinah region were compared with muskmelon isolates for aggressiveness on muskmelon, the mean levels of aggressiveness for cucumber and muskmelon isolates were not significantly different (55% and 51%, respectively; $P > 0·05$). Repetition of aggressiveness tests for all isolates showed no significant differences between the two replicates for most of the isolates, except for seven isolates from cucumber and one isolate from muskmelon, for which the tests were repeated three times to confirm the results obtained (results not presented).

Metalaxyl sensitivity

All *P. aphanidermatum* isolates from Oman were found to be sensitive to metalaxyl, with EC_{50} values ranging from less than 0·01 to 0·76, with a mean of 0·29 μg mL^{-1} (Fig. 4). No significant differences were observed among populations from different regions and from different hosts in their sensitivity to metalaxyl in Oman ($P > 0·05$) (Table 6). In addition, the Kolmogorov-Smirnov test indicated no significant differences in frequency distributions of EC_{50} values between cucumber and muskmelon populations ($P > 0·05$; Fig. 4). The population of *P. aphanidermatum* from Norway was also found to be sensitive to metalaxyl, while seven US isolates of known resistance to mefenoxam were found to be resistant to metalaxyl ($EC_{50} > 100$ μg mL^{-1}). No significant difference ($P > 0·05$) was observed between the repetitions of the two experiments.

Discussion

AFLP fingerprinting revealed a relatively high level of phenotypic diversity in the *P. aphanidermatum* population in Oman, expressed as a high number of phenotypes recovered from different regions. A high level of genetic similarity was observed among isolates and phenotypes of *P. aphanidermatum* from Oman and other countries, which is consistent with the homothallic nature of this pathogen. It also corresponds to the overall high levels of genetic similarity reported for sexually inbreeding diploid species such as *P. aphanidermatum* (Garzon *et al.*, 2005a) and *Phytophthora cactorum* (Huang *et al.*, 2004; Bhat *et al.*, 2006), compared to the lower levels reported for the

Table 6 Mean values of aggressiveness on cucumber and metalaxyl sensitivity of populations of *P. aphanidermatum* from Oman

Population	Region	Host	Aggressiveness (mean % seedling mortality)[a]	EC_{50} (μg mL^{-1})[a]
SC	Sharqiya	Cucumber	73 ± 19 ab	0·28 ± 0·16 a
IC	Interior	Cucumber	74 ± 5 ab	0·34 ± 0·13 a
MC	Muscat	Cucumber	82 ± 9 a	0·24 ± 0·10 a
BC	Batinah	Cucumber	72 ± 15 ab	0·29 ± 0·09 a
BM	Batinah	Muskmelon	69 ± 14 b	0·30 ± 0·12 a

[a]Means (± SD) in the same column with the same letter are not significantly different at $P < 0·05$ (Tukey's Studentized Range test, SAS, V8).

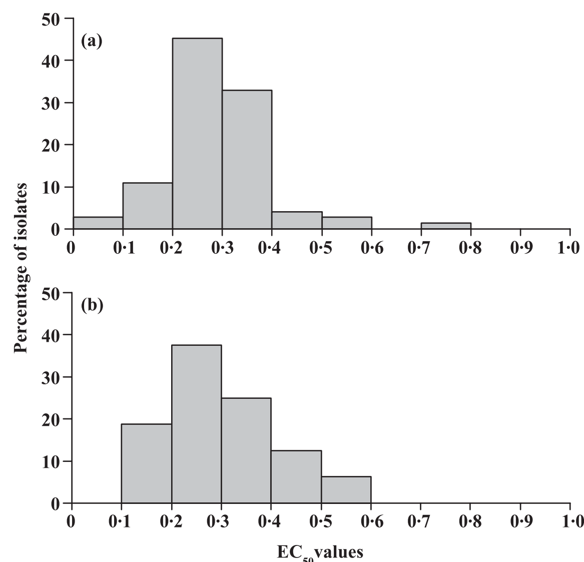

Figure 4 Variation in metalaxyl sensitivity among isolates of *Pythium aphanidermatum* from Oman. (a) Distribution of EC_{50} values for 73 isolates of *P. aphanidermatum* from cucumber and (b) 16 isolates from muskmelon. No significant differences were found in frequency distribution of metalaxyl sensitivity levels between the two populations ($P > 0·05$; Kolmogorov-Smirnov two-sample test).

heterothallic outcrossing *Phytophthora infestans* (Flier *et al.*, 2003) and *Phytophthora capsici* (Lamour & Hausbeck, 2001b). However, genetic similarity can be low for some homothallic *Pythium* species. Barr *et al.* (1997) and Harvey *et al.* (2001) indicated that *P. irregulare*, a homothallic species, may undergo some outcrossing, thus resulting in low levels of genetic similarity as reported by some workers (Harvey *et al.*, 2000; Garzon *et al.*, 2005a,b).

Populations of *P. aphanidermatum* from Oman exhibit uniformity in the level of aggressiveness and metalaxyl sensitivity. Most isolates of *P. aphanidermatum* were found to be aggressive, with only two isolates showing relatively weak levels of aggressiveness. No significant differences were found in aggressiveness among two populations of *P. aphanidermatum* from cucumber and muskmelon on either host, which confirms previous reports that isolates obtained from one host are not necessarily more aggressive on their host of origin compared to isolates obtained from another host (Harvey *et al.*, 2000).

All isolates were found to be highly sensitive to metalaxyl ($EC_{50} < 0·80$ μg mL^{-1}) compared to the resistant *P. aphanidermatum* isolates from the USA ($EC_{50} >$ 100 μg mL^{-1}). This rejects the hypothesis that the reduced efficacy of metalaxyl to manage pythium root diseases of cucumber in Oman is related to development of resistance to metalaxyl among populations of *P. aphanidermatum*. An alternative hypothesis of enhanced biodegradation of metalaxyl in soil should be tested.

The current study highlights a number of characteristics of *P. aphanidermatum* populations in Oman. No genetic differentiation was found between the two host groups (cucumber and muskmelon) from the same geographical region, indicating a lack of influence of host association on the genetic variation observed. The lack of genetic differentiation among host groups was supported by the high number of muskmelon isolates (69%) producing AFLP profiles identical to profiles produced by cucumber isolates from the same region. This supports the concept of lack of host specialization in populations of *P. aphanidermatum* in Oman as reported elsewhere (Harvey *et al.*, 2000).

Growers in Oman follow a traditional farming system, characterized by growing multiple crops in different plots on the same farm (Moghal *et al.*, 1993). Many growers have by inheritance acquired several small–size farms in the same area. In addition, the expansion in greenhouse cucumber production by about 40% annually has meant that land is now used for cucumber which used to be cultivated with other crops, including muskmelon, a host of *P. aphanidermatum* in Oman (Moghal *et al.*, 1993). It is therefore possible that establishment of greenhouses on land previously cultivated with other crops may have contributed to infections by the same phenotypes on both crops. In addition, there is expected movement of *Pythium* inoculum between greenhouses and muskmelon plots in the same field or between fields in the same area. The reported occurrence of pythium damping-off in newly imported soil in greenhouses (Al-Kiyumi, 2006), which is assumed to be free of pathogens, may also be related to some extent to the introduction of *Pythium* inoculum in greenhouses from adjacent plots or fields.

Most AFLP genetic variance among populations of *P. aphanidermatum* was attributed to geographical

regions. The relatively moderate to high levels of genetic differentiation among most regions in Oman may establish that movement of *Pythium* inoculum between regions is limited compared to movement between host groups in the same area. This is supported by the small (10%) percentage of identical phenotypes recovered among regions and is consistent with the nature of the four regions in Oman, where most (except Batinah and Muscat) are separated from each other by mountains, and ownership in different regions by the same grower is uncommon. It is also consistent with the lack of aerial dissemination of the pathogen over long distances. However, the limited number of identical phenotypes recovered across regions in Oman may still imply that a common source has contributed to disseminating *Pythium* inoculum. The observation of one clone having isolates from the USA, Norway and most growing regions in Oman provides evidence for the hypothesis that there are some phenotypes of worldwide distribution. Huang *et al.* (2004) related the tight clustering and intermixing of isolates of *Phytophthora cactorum* obtained from strawberry from different locations in North America within dendrogram clusters to the introduction of isolates via a common source. Similarly, recovering genotypes of *Phaeomoniella chlamydospora* on grapevines in different countries by Mostert *et al.* (2006) was explained through single introduction events from the same inoculum source, which was hypothesized to be grapevine cuttings. Although irrigation water (Pettitt *et al.*, 2002), dust accumulation on greenhouse roofs (Sanchez *et al.*, 2001), insects (Goldberg & Stanghellini, 1990) and soil (Stanghellini & Phillips, 1975) are reported as sources of *Pythium* inoculum in greenhouse systems, their contribution to the long distance movement of inoculum among regions in Oman is unclear. Potting mixtures which are frequently used by cucumber growers in Oman (Al-Kiyumi, 2006) and which have been reported as a source of *Pythium* inoculum (Favrin *et al.*, 1988; Cartwright *et al.*, 1995; Davison *et al.*, 2006), may represent one possible source. However, the low recovery of identical phenotypes across regions in Oman suggests a low level of contamination of potting mixtures.

In addition to recovery of few identical clones among different regions in Oman, a high number of different phenotypes infecting cucumber was found, which may support multiple infections from different inoculum sources (Mostert *et al.*, 2006). These phenotypes may have originated from inoculum previously present in soil or fields, introduced with the increase in cucumber cultivation in Oman via unknown sources (e.g. potting mixtures) or introduced from adjacent fields or plots in the same field. It may therefore be necessary to investigate the relative importance of different factors that contribute to the introduction of *Pythium* inoculum in greenhouse systems in Oman.

Isolates belonging to the same phenotype do not necessarily have to share an identical set of physiological characteristics. Studies, including this one, have found that isolates of *Pythium* or *Phytophthora* species with the same genetic background can show variation in sensitivity to metalaxyl (Knapova & Gisi, 2002; Wangsomboondee *et al.*, 2002; Garzon *et al.*, 2005a) or other fungicides (Stein & Kirk, 2003). One explanation for this is that phenotypes with the same genetic background have been exposed for different lengths of time to metalaxyl. In addition to several factors influencing the appearance of fungicide resistance, large scale application of fungicides and duration of exposure may contribute to the build-up of resistance (Staub & Sozzi, 1983; Skylakakis, 1987). Variation in physiological traits within the same phenotypes has also been reported for *P. irregulare* (Harvey *et al.*, 2000), *P. sylvaticum* and *P. ultimum* (Lévesque *et al.*, 1993) and some *Phytophthora* species (Abu-El-Samen *et al.*, 2003).

The current study establishes essential information on the genetic structure of *P. aphanidermatum* populations in Oman. It provides evidence for the existence of a large number of phenotypes infecting cucumber in Oman and circulation of *Pythium* inoculum both within and, less frequently, across regions. No evidence was found for the presence of resistance to metalaxyl among *P. aphanidermatum* isolates from all regions in Oman. Further studies are in progress regarding the potential sources of *P. aphanidermatum* inoculum in greenhouse systems in Oman, as well as investigating factors contributing to the reduced efficacy of metalaxyl to manage pythium damping-off.

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