Molecular characterization and pathogenicity of *Pythium* species associated with damping-off in greenhouse cucumber (*Cucumis sativus*) in Oman

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A study was undertaken in 2004 and 2005 to characterize pathogens associated with damping-off of greenhouse-grown cucumber seedlings in 13 districts in Oman. Identification of *Pythium* to the species level was based on sequences of the internal transcribed spacer (ITS) of the ribosomal DNA. Of the 98 *Pythium* isolates collected during the survey, *Pythium aphanidermatum*, *P. spinosum*, *P. splendens* and *P. oligandrum* accounted for 76%, 22%, 1% and 1%, respectively. *Pythium aphanidermatum* was isolated from all of the districts, while *P. spinosum* was isolated from seven districts. Pathogenicity tests showed inter- and intraspecific variation in aggressiveness between *Pythium* species. *Pythium aphanidermatum*, *P. spinosum* and *P. splendens* were found to be highly aggressive at 25°C. However, the aggressiveness of *P. spinosum* decreased when the temperature was raised to 30°C, which was found to correspond to the lower frequency of isolation of *P. spinosum* in the warmer seasons, compared to the cooler time of the year. *Pythium aphanidermatum* exhibited limited intraspecific variation in the sequences of the ITS region of the rDNA and showed 100% similarity to the corresponding *P. aphanidermatum* sequences from GenBank. The ITS sequence data, as well as morphological characteristics of *P. spinosum* isolates, showed a high level of similarity within and between *P. spinosum* and *P. kunmingense*, and suggested that the two species were synonymous. This study represents the first report of *P. spinosum*, *P. splendens* and *P. oligandrum* in Oman.

**Keywords**: cucumber seedlings, inter- and intraspecific variation, *Pythium aphanidermatum*, *Pythium oligandrum*, *Pythium spinosum*, *Pythium splendens*

Introduction

Soil-based greenhouse production of cucumbers (*Cucumis sativus*) in Oman expanded in the period between 2001 and 2005 at a rate of about 38% per year, making it amongst the fastest-growing agricultural sectors in Oman. Over 95% of greenhouses in Oman are used exclusively to produce cucumbers.

The continuous production of cucumbers in monoculture has been accompanied by an increase in post-emergence damping-off disease, which has become the main limiting biotic factor to profitable production of cucumbers in greenhouses. Damping-off, reported in 77% of greenhouses in the major agricultural areas in Oman, causes up to 75% mortality in cucumber seedlings in the most affected greenhouses (Al-Kiyumi, 2006).

Damping-off in cucumber is a worldwide problem in greenhouse systems. Stanghellini & Phillips (1975) reported up to 87% mortality of cucumber seedlings in untreated greenhouse soil in Abu Dhabi. In Canada, damping-off has been reported as a serious disease of several vegetable crops including cucumber (Howard et al., 1994). Although *Rhizoctonia solani* and some *Fusarium* spp. have been associated with damping-off (Lida et al., 1983; Abbasi et al., 2004), *Pythium* spp. have been shown to be the most frequent cause of damping-off disease of cucumber (Stanghellini & Phillips, 1975; Gubler & Davis, 1996; Deadman et al., 2002). In Oman,
P. aphanidermatum was reported as the pathogen responsible for damping-off of greenhouse-grown cucumber (Al-Hasani, 2004). However, only limited surveys have been conducted and little is known about either the composition of pathogens infecting cucumber seedlings or their distribution.

The genus Pythium has more than 200 described species, these being pathogenic or saprophytic on plants and some mammals and fish (CABI, 2004). Identification to the species level has been based for several decades on morphological and growth characteristics on specific media (Matthews, 1931; Plaats-Niterink, 1981; Dick, 1990). This requires a high level of expertise and is time-consuming as morphological characters are often variable and some Pythium spp. will not or seldom develop reproductive structures in culture. Moreover, misidentification may occur among morphologically similar species (Wang & White, 1997; Godfrey et al., 2003). This has led to the development and implementation of a number of techniques that facilitate faster and more accurate identification of Pythium spp. Several workers have reported the use of monoclonal antibodies (Yuen et al., 1998; Kageyama et al., 2002), isozyme polymorphisms (Chen et al., 1992), species-specific primers (Wang & White, 1996; Godfrey et al., 2003; Wang et al., 2003) and the sequence of the ras-related protein gene (Moorman et al., 2002) to aid identification. However, characterization of Pythium spp. associated with certain crops has largely depended on sequences of the internal transcribed region (ITS) of the ribosomal DNA (rDNA) (Tojo et al., 2001; Moorman et al., 2002; Paulitz & Adams, 2003).

The objectives of this work were (i) to investigate the pathogens associated with damping-off of cucumber in Oman; (ii) to investigate inter- and intraspecific variation in aggressiveness among different pathogen species at different temperatures; and (iii) to determine the geographical distribution of Pythium spp. causing damping-off in cucumber in Oman. Knowledge in these areas will provide basic information for the establishment of strategies to manage pythium damping-off in cucumbers.

Materials and methods

Collection of isolates

A survey during 2004 and 2005 covered 136 greenhouses in the 13 districts where more than 85% of greenhouses in Oman are located (Fig. 1). The sample size varied between times of the year and districts, mainly as a result of variability in intensity of cultivation. In general, the number of greenhouses visited per district varied from three in Bidiyah (BD) to 31 in Barka (BK), with at least 12 greenhouses per district in most of the surveyed districts. Pythium spp. were isolated from roots and crowns of cucumber seedlings showing damping-off symptoms. Isolations were carried out by washing soil debris from roots and crowns. Seedling tissue was then disinfected with 1% NaOCl for 2 min, washed in sterile distilled water and then dried on sterile filter paper. Tissue was transferred to 1·7% corn meal agar (CMA) amended with 5 µg pimaricin, 250 µg ampicillin and 10 µg rifampicin mL⁻¹ (Jeffers & Martin, 1986), as well as on 2·5% potato dextrose agar (PDA), for 1–3 days. Monocultures were obtained by excising hyphal tips and Pythium cultures were maintained on CMA slants at 8°C.

Molecular identification

Identification of Pythium spp. was mainly based on sequences of the internal transcribed spacers (ITS-1 and ITS-2) of the ribosomal DNA. However, morphological examination of some isolates was also conducted on
Polymerase chain reaction (PCR) and sequencing

The ITS region of all Pythium isolates was amplified using the universal primers ITS1 (5'–TCCGTAATGACCT-GCGG-3') and ITS4 (5'–TCTTCGCCTATTGAGATATGC-3') (White et al., 1990). The PCR reaction mixture consisted of 2.5 µL of 10× amplification buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl), 2.5 mM MgCl₂, 0.2 mM dNTPs, 0.4 µM ITS1 primer, 0.4 µM ITS4 primer, 0.2 µL AmpliTaq Gold® polymerase enzyme (Applied Biosystems), 2 µL diluted DNA (~12 ng µL⁻¹) and Milli-Q water to a final volume of 25 µL. Prior to thermocycling, samples were heated at 95°C for 10 min. This was followed by 35 cycles of 95°C for 30 s, 55°C for 10 min and 72°C for 1 min. Successful amplification of the ITS region was checked by running 6 µL of each reaction mixture on a 1.5% agarose gel in 1× Tris-borate-EDTA buffer (TBE) at 110 V for 60 min. 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 for sequencing using BigDye V3.1 (Applied Biosystems) and the same primers used for amplification.

Analysis of sequences

ITS sequences were compared using BLAST with sequences deposited at the National Center for Biotechnology Information (NCBI). Identity of species with sequences having less than 100% similarity to published sequences in GenBank were further analysed using RAUP (phylogenetic analysis using parsimony) version 4.0b8 (Swofford, 1998). All available entries of sequences of the corresponding species and the most closely related species in GenBank were downloaded. The criterion for the selection of closely related species was based on clades of Pythium spp., generated by Lévesque & de Cock (2004). Sequences were first aligned with CLUSTALW (fast) (Thompson et al., 1994) and converted to nexus format using the program ProSeq v2-91 (Filatov, 2002). Gaps were treated as missing data and maximum parsimony analysis was performed using a heuristic search with random stepwise additions, tree bisection reconnection (TBR) branch swapping and the steepest descent option not in effect. Bootstrap 50% majority-rule consensus trees were generated using 1000 replications.

Aggressiveness of Pythium spp.

In order to determine if different species of Pythium show different levels of aggressiveness on cucumber, tests were conducted at 25 and 30°C. Five randomly selected isolates of each species were used to inoculate cucumber seedlings in pots containing California mix (Baker, 1957). Seven cucumber seeds (cv. RS 164695; Seminis) were sown in plastic pots and emerging seedlings were thinned to five plants of approximately the same size. On day 7, 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 (Herrero et al., 2003). The pots were incubated in growth chambers under 14-h days and 70% relative humidity. Seedlings were irrigated once every 2 days for 2 weeks. The experiment was repeated twice using five pots at each incubation temperature of 25 and 30°C. The progress of damping-off disease was plotted against time and the area under disease progress curve (AUDPC) was determined using the formula described by Campbell & Madden (1990)

\[
AUDPC = \sum_{i=1}^{n-1} \left[ \frac{y_i + y_{i+1}}{2} \right] (t_{i+1} - t_i)
\]

where \(n\) is the number of readings (for 5 days), \(y\) is the percentage of cucumber seedlings showing damping-off symptoms at each reading and \(t\) is the time in days between each reading.

Results

Occurrence and distribution of Pythium spp.

Isolations from cucumber seedlings exhibiting damping-off symptoms yielded 98 isolates of Pythium. Generated sequences of the ITS region of the rDNA for all isolates separated them into four species: P. aphanidermatum (75 isolates); P. spinosum (21 isolates); P. splendens (1 isolate); and P. oligandrum (1 isolate). Isolates of P. aphanidermatum were found in every district surveyed, while P. spinosum was isolated from seven districts (Fig. 1). Most P. spinosum isolates were isolated during the cooler time of year, while P. aphanidermatum was consistently isolated in the autumn, winter and spring (Table 1). No attempts were made to collect samples in summer as most cucumber growers suspend cucumber production during this time because of the high ambient temperature. Representative isolates of each species were deposited at the Centraal-bureau voor Schimmelcultures (CBS) in the Netherlands (Table 2).
Pythium and cucumber damping-off in Oman

Molecular characterization of *Pythium* isolates

ITS1 and ITS4 primers amplified the entire ITS rDNA region (ITS-1, 5.8S, ITS-2) and parts of the 18S small and 28S large subunit rDNA. With the exception of two isolates of *P. aphanidermatum* (DQ298522 and DQ298523), which lacked one base pair in the ITS-1, 73 out of 75 *P. aphanidermatum* isolates were identical in the sequence of the ITS region. Similarly, the 21 *P. spinosum* isolates were identical in the sequence of the ITS region. Comparisons to sequences of the relevant species in the GenBank database revealed 100%, 99%, 99% and 100% nucleotide (nt) similarity to *P. aphanidermatum* (AY151180), *P. spinosum* (AY598701), *P. splendens* (AY598655) and *P. oligandrum* (AY598618) sequences, respectively.

Although it is known that *P. splendens* is closely related to *P. ultimum* based on sequences of the ITS region, the ITS sequence of isolate P091 showed 99% (nt) similarity to *P. splendens* AY598655 and clustered with a high bootstrap value (100%) with sequences of reference isolates of *P. splendens* (Fig. 2). It is interesting to note that the ITS sequence of *P. kunmingense* clustered within sequences of *P. spinosum* (Fig. 3). Isolate P006 from Oman formed a subclade with *P. kunmingense* (AY598700) and *P. spinosum* (CBS 290-31), but only with moderate bootstrap support (61%).

Morphological characterization of *P. spinosum*

Because the sequence of the ex-type strain of *P. kunmingense* clustered within sequences of *P. spinosum* isolates, two randomly selected isolates of *P. spinosum* (P006 and P017) were characterized by morphology. Morphological characteristics revealed that the two isolates shared some characters in common with *P. spinosum* (e.g. size of hyphal swellings) and others in common with *P. kunmingense* (e.g. main hyphal diameter). The two isolates were also found to differ from each other by possessing some characteristics common with either of the two species. To be more specific, the size of oogonia of P017 (17–21 µm, mean 20·7 µm) was similar to that of *P. kunmingense* (15–26 µm, mean 21 µm), while the size of oogonia of isolate P006 (15–20 µm, mean 18 µm) was similar to the size of oogonia of *P. spinosum* (17–21 µm, mean 18·5 µm). However, isolates from Oman were distinct from *P. kunmingense* and *P. spinosum* in having mostly smooth, intercalary oogonia with aplerotic (to plerotic) oospores (Table 3).

Aggressiveness of *Pythium* spp.

With the exception of isolates P017 and P024 of *P. spinosum* and isolate P025 of *P. aphanidermatum*, which were found to exhibit significantly lower levels of aggressiveness than other isolates, no significant differences were found between *P. aphanidermatum* and *P. spinosum* in aggressiveness at 25°C (P < 0·05). *Pythium splendens* was found to be the most aggressive at 25°C, but this was only on the basis of the single isolate obtained from the survey (P091). However, *P. aphanidermatum* isolates were significantly more aggressive at 30°C than *P. spinosum* isolates. The aggressiveness of *P. aphanidermatum* isolates also significantly increased after increasing the temperature from 25 to 30°C. Thus, *P. aphanidermatum* and *P. spinosum*

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**Table 1** Frequency of isolation of *Pythium aphanidermatum*, *P. spinosum*, *P. splendens* and *P. oligandrum* during different times of the year in Oman

<table>
<thead>
<tr>
<th>Time of year</th>
<th>Sample size</th>
<th>Districts surveyed</th>
<th>No. of isolates collected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>P. aphanidermatum</em></td>
</tr>
<tr>
<td>Feb–Mar 2004</td>
<td>16</td>
<td>BR, MH, MS, SB</td>
<td>9</td>
</tr>
<tr>
<td>Apr–May 2004</td>
<td>19</td>
<td>BK, BR, MH, MS, SB</td>
<td>15</td>
</tr>
<tr>
<td>Sep–Nov 2004</td>
<td>35</td>
<td>BK, BL, KB, MS, NZ, SB</td>
<td>27</td>
</tr>
<tr>
<td>Dec 2004–Feb 2005</td>
<td>42</td>
<td>All districts*</td>
<td>24</td>
</tr>
</tbody>
</table>

*Districts are presented in Fig. 1.

**Table 2** GenBank and CBS accession numbers of representative isolates of *Pythium* species collected from greenhouse-grown cucumber seedlings in Oman for this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate accession no.</th>
<th>Location</th>
<th>GenBank accession no.</th>
<th>CBS no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aphanidermatum</em></td>
<td>P002</td>
<td>Barka</td>
<td>DQ298521</td>
<td>CBS 118745</td>
</tr>
<tr>
<td><em>P. aphanidermatum</em></td>
<td>P025</td>
<td>Nizwa</td>
<td>DQ298522</td>
<td>--</td>
</tr>
<tr>
<td><em>P. aphanidermatum</em></td>
<td>P063</td>
<td>Mudaybi</td>
<td>DQ298523</td>
<td>--</td>
</tr>
<tr>
<td><em>P. spinosum</em></td>
<td>P006</td>
<td>Barka</td>
<td>DQ381807</td>
<td>CBS 119116</td>
</tr>
<tr>
<td><em>P. spinosum</em></td>
<td>P017</td>
<td>Mahdah*</td>
<td>--</td>
<td>CBS 119117</td>
</tr>
<tr>
<td><em>P. splendens</em></td>
<td>P091</td>
<td>Seeb</td>
<td>DQ381808</td>
<td>CBS 118747</td>
</tr>
<tr>
<td><em>P. oligandrum</em></td>
<td>P010</td>
<td>Nizwa</td>
<td>DQ381809</td>
<td>CBS 118746</td>
</tr>
</tbody>
</table>

*No CBS numbers provided.*

*The ITS sequence of isolate P017 was identical to that of P006.*
had similar levels of aggressiveness at 25°C, but *P. aphanidermatum* was more aggressive at 30°C. Significant intraspecific variation was observed in aggressiveness among isolates of the same species at both temperatures (Fig. 4).

**Discussion**

Damping-off is a serious disease of greenhouse-grown cucumbers in Oman (Deadman *et al.*, 2003). In this study, damping-off was found to occur in all 13 surveyed districts, on average in 82% of the 136 visited greenhouses. Incidence levels up to 50% were observed in some greenhouses within 3 weeks after transplanting seedling stock to the greenhouse; however, an incidence of 4–8% was found to be common in most greenhouses. Stanghellini & Phillips (1975) reported 87% mortality in cucumber seedlings within 17 days after transplanting in untreated soil in greenhouses in Abu Dhabi. Root-rot and wilt symptoms on mature cucumber plants were also found to be prevalent in several greenhouses, especially towards the senescence stage. In addition to damping-off and wilt symptoms, fusarium fruit rot, downy mildew and root-knot diseases were found to be common in some greenhouses of the surveyed districts.

Uncharacterized pathogen populations associated with damping-off of cucumber in Oman have been considered one of the major limitations to successful management of this disease. Isolations from diseased cucumber seedlings exhibiting damping-off symptoms showed that *Pythium* spp. are the main pathogens associated with this disease in Oman. *Pythium aphanidermatum* was found to be the pathogen most frequently associated with cucumber damping-off, making up 76% of the isolates and distributed all over the 13 surveyed districts in Oman. The dominance of *P. aphanidermatum* among other *Pythium* spp. infecting greenhouse-grown cucumbers is not surprising. In a survey in the early 1990s by Moghal *et al.* (1993) in
the northern part of Oman, *P. aphanidermatum* was found to infect more than 14 different plant species, resulting in severe damping-off, root rot, wilt, fruit rot and seedling rot diseases. It is therefore most likely that the predominance of this species in Oman in the past is one of the reasons behind its high rate of occurrence (76%) compared to the newly reported species *P. spinosum* (22%). In addition, the reported higher incidence of *Pythium* root diseases of cucumber in the warmer months of the year (Al-Kiyumi, 2006) may suggest infection by the predominant high-temperature-loving species *P. aphanidermatum* (Plaats-Niterink, 1981). On a worldwide basis *P. aphanidermatum* is considered one of the pathogens most consistently associated with damping-off of cucumber (Stanghellini & Phillips, 1975; Howard *et al.*, 1994).

*Pythium spinosum*, *P. splendens* and *P. oligandrum* are reported here for the first time in Oman, with *P. splendens* being the first heterothallic *Pythium* sp. reported in Oman. Apart from *P. oligandrum* which is a mycoparasite, *P. spinosum* and *P. splendens* are considered important pathogens of cucumber (Kao & Ko, 1986; Lo & Lin, 1990). However, the low rate of isolation of *P. splendens* (1%) suggests that it contributes less to epidemics of cucumber damping-off in Oman.

It is unclear whether the second most predominant species *P. spinosum* (22%) has only recently been introduced to Oman or has been present for sometime. Al-Hasani (2004) did not report it in a previous study, most likely because of the limited sample size (only 17 greenhouses sampled). The distribution pattern of *P. spinosum* in districts from the far northern part of Oman to the southern part where greenhouses are concentrated, together with the isolation frequency of 22%, may suggest that this pathogen has been in Oman for a relatively long period of time. However, when and how *P. spinosum* was introduced in Oman cannot be determined at this stage because

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**Figure 3** A phylogram representing the relationship of isolate P006 to *Pythium spinosum* and *P. kunmingense* based on ITS rDNA sequences using maximum parsimony analysis inferred by a heuristic tree search. Numbers within the tree represent bootstrap values (values above 50% are indicated; 1000 replications). Of 938 total characters, 816 were constant, 106 were variable and parsimony-uninformative and 16 were parsimony-informative. The tree was rooted to *P. sylvaticum* (AY598645).
of the lack of studies on the level of genetic diversity within this species and the lack of studies characterizing the potential sources of *Pythium* inoculum in greenhouse systems in Oman.

Comparison of the ITS sequences of the rDNA from isolates obtained in this study with sequences deposited in GenBank readily identified *P. aphanidermatum* and *P. oligandrum*. Limited intraspecific variability was found in the ITS sequence of 75 isolates of *P. aphanidermatum*. Similar findings were reported for this species by Lévesque & de Cock (2004) and Moorman et al. (2002). Clustering of the ITS sequence of isolate P091 with *P. splendens* sequences and the high bootstrap support (100%) for separation from *P. ultimum*, the closest species based on ITS sequences (Lévesque & de Cock, 2004), confirmed the identity of isolate P091 as *P. splendens*. In addition to molecular identification, the identity of *P. splendens* was also confirmed by morphological characteristics (results not presented). However, 21 isolates of *Pythium*, which were later confirmed to be *P. spinosum*, showed high nucleotide similarity to *P. kunmingense* followed by *P. spinosum* (∼99%). As all isolates showed an identical ITS sequence, one sequence (P006) was utilized for subsequent analysis with all entries of the two species in GenBank. The maximum parsimony tree showed clustering of sequence P006 with *P. spinosum* sequences.

**Table 3** Morphological comparison of two *Pythium spinosum* isolates (P006 and P017) from Oman with the ex-type strains of *P. kunmingense* and *P. spinosum*

<table>
<thead>
<tr>
<th>Character</th>
<th><em>P. spinosum</em></th>
<th><em>P. kunmingense</em></th>
<th>P006</th>
<th>P017</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main hyphal diameter (µm)</td>
<td>2.5–5 (–7)</td>
<td>Up to 8.6</td>
<td>up to 9</td>
<td>up to 8</td>
</tr>
<tr>
<td>Sporangia</td>
<td>not formed</td>
<td>not formed</td>
<td>not formed</td>
<td>not formed</td>
</tr>
<tr>
<td>Hyphal swellings</td>
<td>present</td>
<td>present</td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td>Size of hyphal swellings (µm)</td>
<td>≤33</td>
<td>13–23</td>
<td>≤34</td>
<td>≤31</td>
</tr>
<tr>
<td>Oogonia position</td>
<td>terminal and</td>
<td>terminal and</td>
<td>intercalary and</td>
<td>intercalary and</td>
</tr>
<tr>
<td></td>
<td>intercalary</td>
<td>intercalary</td>
<td>occasionally terminal</td>
<td>occasionally terminal</td>
</tr>
<tr>
<td>Oogonia size (µm)</td>
<td>17–21</td>
<td>15–26</td>
<td>15–20</td>
<td>17–23</td>
</tr>
<tr>
<td></td>
<td>(mean 18.5)</td>
<td>(mean 21)</td>
<td>(mean 20.7)</td>
<td>(mean 20.7)</td>
</tr>
<tr>
<td>Oogonia ornamentation</td>
<td>varying</td>
<td>rarely smooth</td>
<td>mostly smooth,</td>
<td>mostly smooth,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>occasionally 1–2 spines</td>
<td>occasionally 1–4 spines</td>
</tr>
<tr>
<td>Antheridia number</td>
<td>1 (–3)</td>
<td>1–3</td>
<td>1–2</td>
<td>1 (–3)</td>
</tr>
<tr>
<td>Antheridia origin</td>
<td>monoclinous,</td>
<td>monoclinous,</td>
<td>monoclinous,</td>
<td>monoclinous and</td>
</tr>
<tr>
<td></td>
<td>occasionally</td>
<td>rarely</td>
<td>occasionally</td>
<td>diclinous</td>
</tr>
<tr>
<td></td>
<td>diclinous</td>
<td>smooth</td>
<td>smooth</td>
<td>diclinous</td>
</tr>
<tr>
<td>Oospore type</td>
<td>pellerotic</td>
<td>pellerotic</td>
<td>pellerotic</td>
<td>pellerotic</td>
</tr>
<tr>
<td></td>
<td>occasionally</td>
<td>aplerotic to</td>
<td>aplerotic</td>
<td>aplerotic to almost</td>
</tr>
<tr>
<td></td>
<td>aplerotic</td>
<td>almost</td>
<td>pellerotic</td>
<td>pellerotic</td>
</tr>
</tbody>
</table>

*Morphological descriptions of *P. kunmingense* and *P. spinosum* were obtained from (Yu, 1973) and (Plaats-Niterink, 1981), respectively.

**Figure 4** Aggressiveness of *Pythium aphanidermatum*, *P. spinosum* and *P. splendens* on cucumber seedlings at 25°C (a) and 30°C (b). Codes starting with the letter P represent isolate numbers of each species. Isolates with the same letters in brackets in each figure were not significantly different in aggressiveness from each other at *P* < 0.05 (according to Tukey’s Studentized range test on AUDPC values). Isolates with (+) or (–) after the letters in (b) indicate a significant increase (+) or decrease (–) in aggressiveness of each isolate at 30°C compared to aggressiveness at 25°C at *P* < 0.05 (according to Tukey’s Studentized range test on AUDPC values).
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interestingly, the sequence of the CBS isolate 550-88, the ex-type strain of *P. kunmingense* (AY598700), clustered within *P. spinosum* sequences. Together with the *P. spinosum* isolate CBS 290-31, they clustered with isolate P006 with moderate bootstrap support (61%). Phylogenetic clusters of *Pythium* spp. generated by Lévesque & de Cock (2004) showed that *P. spinosum* and *P. kunmingense* were very closely related compared to other *Pythium* species. In the same study, the ITS sequence of *P. kunmingense* fell within the variation observed for *P. spinosum*. *Pythium spinosum* was first described in 1926, and *P. kunmingense* in 1973 from soil in Kunming, China (Plaats-Niterink, 1981). At that time, *P. kunmingense* was differentiated from *P. spinosum* by a number of characters such as the spines on the oogonia walls and the size of hyphae and oogonia (Yu, 1973; Plaats-Niterink, 1981). Morphological characterization of isolates P006 and P017 showed that they possessed some characters in common with *P. spinosum*, some in common with *P. kunmingense* and others distinct from the two species. The identical ITS sequence between isolates P006 and P017 together with the lack of distinct differences in morphology and phylogeny between *P. spinosum* and *P. kunmingense* may be sufficient to consider the two species synonymous and the morphological differences as widening the range of intraspecific variation within this species. Based on the rules of the International Code of Botanical Nomenclature the name *P. spinosum* predates *P. kunmingense* and therefore takes precedent as a name for this species.

*Pythium spinosum* isolates do not produce zoospores in culture (Plaats-Niterink, 1981); agar plugs were therefore used in this study to compare pathogenicity of the four species. Although this type of *Pythium* inoculum has been found to affect the timing of infection and severity of symptoms (Spencer & Cooper, 1967; Martin & Loper, 1999), the ranking of a given species or isolate in terms of pathogenicity has not been found to be affected by the type of inoculum used (Moulin et al., 1994). These authors reported the production of similar symptoms and unchanged ranking of *Pythium* isolates when three methods of inoculation (agar plugs, mycelium fragments and zoospores) were used. Agar plugs were also used by Herrero et al. (2003) and Tojo et al. (2001) to test pathogenicity of *Pythium* on cucumber seedlings.

Pathogenicity values, expressed as AUDPC, were consistent after repeated tests and mortality in cucumber seedlings did not change following day 5. Pathogenicity tests revealed considerable variation in aggressiveness between and within the isolated *Pythium* spp. at 25 and 30°C. *Pythium splendens* and most *P. aphanidermatum* and *P. spinosum* isolates were highly aggressive on cucumber at 25°C. At 30°C, most *P. spinosum* isolates became less aggressive, while all *P. aphanidermatum* isolates became more aggressive. *Pythium aphanidermatum* is known to be favoured at high temperatures, while *P. spinosum* is favoured by lower temperatures (Plaats-Niterink, 1981). It has been suggested that the environmental conditions of a given production area may influence the rhizosphere populations of *Pythium*, as well as favouring the predominance of the most aggressive species at certain temperatures (Rabin & Turlil, 1995). This study provides evidence for the higher frequency of *P. spinosum*-induced damping-off in the cooler seasons of the year and the comparable levels of aggressiveness to *P. aphanidermatum* at 25°C. Aggressiveness data explain why *P. aphanidermatum* was predominant at all times in the surveyed districts, while *P. spinosum* was mostly isolated during the cool season. The predominance of certain species of *Pythium* in certain seasons was documented by Bates & Stanghellini (1984). In their study on the pathogens associated with root rot of spinach in Arizona, *P. aphanidermatum* was found to be more prevalent in summer, *P. dissotocum* in winter. Variation in aggressiveness among *P. aphanidermatum* and *P. spinosum* isolates was also documented in the present study, supporting previous findings of pathogenic variations within the same *Pythium* species (Zhang & Yang, 2000; Al-Saadi et al., 2003; Herrero et al., 2003).

The present study demonstrates the association of three pathogenic *Pythium* spp. with damping-off of greenhouse-grown cucumber seedlings in Oman. It provides evidence for the presence of diversity in populations of *Pythium* spp. infecting greenhouse cucumbers. However, because the number of isolates involved in the pathogenicity study was limited, a detailed study on the diversity of *Pythium* spp. will be necessary to establish data on the populations of *Pythium* spp. infecting cucumber in Oman. Investigating the origin and distribution of *Pythium* inoculum in greenhouses may help reducing the primary inoculum of the pathogen.

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