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Isolation of *Pythium acanthicum*, *P. oligandrum*, and *P. periplocum* from soil and evaluation of their mycoparasitic activity and biocontrol efficacy against selected phytopathogenic *Pythium* species

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Abstract

Mycoparasitic *Pythium* species with spiny oogonia were surveyed in 50 Palestinian agricultural fields subject to different cropping practices using the Sclerotia Bait Technique (SBT) and the Surface-Soil-Dilution-Plate method (SSDP) with the selective VP3 medium. The mycoparasitic *Pythium* species were obtained from 21 (42%) soils using the SSDP method and from 37 (74%) soils using SBT. *Pythium acanthicum* and *P. oligandrum* were isolated by both methods, whereas *P. periplocum* was isolated only by the SBT. Using a newly modified dual plate culture method (MDPCM), the three mycoparasites showed varying antagonistic performance against several *Pythium* host species under a range of *in vitro* conditions. However, *P. periplocum* and *P. oligandrum* were found to be active biocontrol agents against *P. ultimum*, the damping-off organism of cucumber. This pathogen was antagonized, on thin films of water agar, by the three mycoparasites, and was moderately susceptible to *P. periplocum* while slightly susceptible to *P. acanthicum* and *P. oligandrum*. In direct application method in which antagonistic mycoparasites were incorporated into peat/sand mixture artificially infested with *P. ultimum* under growthroom conditions, *Pythium oligandrum* and *P. periplocum* (at 500 CFUg^{-1}) significantly improved seedling emergence and protected seedlings from damping-off. In the seed coating method, biocontrol by two types of seed dressing (homogenate- or oospore coated seeds), was comparable to that achieved by direct application.

Key words: antagonism, biological control, modified dual plate culture method (MDPCM), *Pythium acanthicum*, *P. oligandrum*, *P. periplocum*, *P. ultimum var. ultimum*, sclerotia bait technique

Introduction

Four *Pythium* species with spiny oogonia have been shown to have mycoparasitic activity against other fungi including other *Pythium* species. These mycoparasites are *P. acanthicum* Drechsler, *P. oligandrum* Drechsler, *P. periplocum* Drechsler, and *P. acanthophoron* Sideris. Another two mycoparasitic *Pythium* species with smooth oogonia, *P. nunn* Lifshitz, and *P. mycoparasiticum* Deacon, have also been shown to have mycoparasitic activity [1]. However, little is known about the ecology of these species in soil [2–8]. This was partly attributed to the lack of efficient isolation methods needed for ecological studies of these fungi [7]. Methods that have been used for detecting mycoparasitic *Pythium* spp. in soil include direct plating of soil onto host-colonized agar, and baiting of soil with mycelia, sclerotia or resting spores of a host then isolation from baits onto selective media [8]. A CMA–based semiselective medium (POSSM) was recently developed and used for the enumeration of *P. oligandrum* in soil by Al-Rawahi and Hancock [3].

Of the six known *Pythium* mycoparasitic species, only *P. oligandrum* Drechsler, and *P. nunn*, have been studied in detail in terms of mode of parasitism and potential for the biocontrol of plant pathogens [e.g., 1, 4, 9–12] *Pythium periplocum*, was first evaluated as biocontrol agent against damping-off of cucumbers induced by *P. aphanidermatum* [13] and no other works had been reported on the use of this antagonist to control diseases incited by phytopathogenic fungi. No attempts had been made to evaluate the mycopa-

rasitic species, *P. acanthicum*, as a biocontrol agent against plant pathogenic fungi. However, research on mycoparasitic *Pythium* species as biological control agents of damping-off diseases caused by phtyopathogenic *Pythium* species is limited, and still restricted to experimental work mostly under *in vitro* conditions [4, 13–15].

The present work was therefore, aimed at: (1) studying the occurrence of mycoparasitic *Pythium* species in fifty field soils in the West Bank using both the SBT and the SSDP with the selective VP3 medium to give a more accurate qualitative and quantitative picture of the occurrence of these species in field soil; and (2) evaluating the possibility of their use in the biocontrol of cucumber seedling damping-off caused by *P. ultimum* var. *ultimum*.

Materials and methods

Sampling sites. Fifty fields distributed in the West Bank were carefully selected on the basis of cultural practices and geographical locations. The soils surveyed can be placed in the following cropping categories: A. permanent irrigated soils under vegetables (9 fields); B. irrigated citrus orchards (10 fields); C. non-irrigated soils under vegetables (11 fields); D. non-irrigated vineyards (9 fields); E. non-irrigated fruit trees (6 fields); F. non-cultivated soils (5 fields). These sites were expected to provide the widest range of soil characteristics, vegetation types and cultural practices.

Collection and processing of soil samples. Soil was collected from a depth of 0–10 cm. Soil sample consisted of 4 aliquots each of approximately 250-g soil chosen randomly from an area of 4 m^2. The four aliquots were thoroughly mixed in a single plastic bag as a composite sample. Two 50-g aliquots of soil were weighted out of the composite sample and put into an oven and dried overnight at 105 °C to determine soil moisture content. The remaining composite sample was divided into three equal parts and a suspension prepared from each using *ca* 50-g sub-samples.

Determination of physical factors. Soil water content, soil pH, and organic matter were determined using standard procedures [16, 17].

Detection of mycoparasitic Pythium spp in soil. The surface-soil-dilution-plate method (SSDP) together with the selective VP3 medium was used for the isolation of *Pythium* species from soil samples [18]. Colony counts were made as the number of *Pythium* colony forming units (C.F.U.) g^{-1} D.W.

Sclerotia bait technique (SBT). The method of Ribeiro and Butler [7] was used for the isolation of mycoparasitic species of *Pythium* with spiny oogonia from soil. Sclerotia of *Sclerotinia sclerotiorum* (Lib) de Bary that was previously isolated from cottony rot diseased cucumber fruits and maintained on PDA plates, were produced on oat grain-water agar medium (OGWA). A soil sample of 60 g was weighed from the composite sample and divided into 3 sub-samples each of 20 g. Each sub-sample was placed separately in a 90 mm diameter Petri dish and mixed with 20 ml of sterile distilled water. Each Petri dish was then inoculated with 12 air-dried sclerotia both on the surface of the soil and buried in it. To maintain the moisture level during 48-hour incubation period at 25 °C, 9 cm diameter sterile wet filter paper was placed in the lid of each Petri dish. After incubation, sclerotia were transferred to 3P medium plates (cornmeal agar amended with 50-mg polymixin-b, 50-mg penicillin, and 5-mg pimaricin per liter). Four sclerotia were transferred to each of three 3P medium plates used for each sub-sample (i.e., thirty-six sclerotia were used for each soil sample assayed). Plates were checked for the presence of *Pythium* colonies, and growing colonies were transferred on to 2P medium (3P medium-pimaricin) plates.

Identification of Pythium species. *Pythium* cultures were identified and classified with the aid of keys of Plaats-Niternik [19], Ali-Shtayeh [20], and Dick [21].

Fungal interactions. Fungal isolates used in this study are shown in Table 1. All fungal cultures were maintained on CMA or in water cultures.

Interactions on agar plates. A modified dual plate culture method (MDPCM) using sunflower seed extract agar (SSEA) was employed. This method was a modification of that of Deacon and Henry [22] and was designed as to give an indication of the distance that the mycoparasite had grown over the host colony (i.e., mycoparasitic activity). Fungal interactions on agar plates were assessed using two different media; Oxoid potato-dextrose agar (PDA) and Difco CMA. The media were chosen partly to study their effect on the mycoparasitic activity. Plates were inoculated at the

Table 1. Fungal isolates used in the current work

Fungal Isolates	FCCAU* No.	Source
Mycoparasites		
Pythium acanthicum Drechsler	201.1	Soil, Jenin
P. oligandrum Drechsler	202.1	Soil, Nablus
P. periplocum Drechsler	203.1	Soil, Jenin
Host fungi		
P. aphanidermatum (Edson) Fitzp	102.2	*Cucurbita* sp. roots, Nablus
P. debaryanum Hesse	104.1	Soil, Nablus
P. debaryanum Hesse	106.6	*Vicia fabae* roots, Nablus
P. deliense Meurs	105.4	Soil, Jericho
P. intermedium de Bary	107.2	*Rosa* sp. roots, Nablus
P. irrigulare Buisman	108.3	*Vicia fabae* roots, Nablus
P. lutarium Ali-Shtayeh	110.8	Soil, Tulkarm
P. oedochilum Drechsler	5710	Soil, South Africa
P. paroecandrum Drechsler	117.3	Soil, Beithlehem
P. rostratum Butler	119.2	*Zea mays* roots, Tulkarm
P. sylvaticum Campbell & Hendrix	132.4	*Lens culinaris* roots, Nablus
P. ultimum var ultimum Trow	204.1	*Cucumis sativus* roots, Tulkarm
P. vexans de Bary	126.9	*Allium cepa* roots, Nablus

* Fungal Culture Collection of An-Najah University, Nablus.

margin with 5-mm diameter agar disks cut out from the edge of a growing colony of the host fungus. The plates were then incubated at 25 °C to allow the host to colonize the plates until they were just completely covered. A five-mm diameter disk of the mycoparasite was then transferred on to the periphery of the host colony (i.e., the youngest part of the host colony) and the plates were reincubated at 25 °C. After 10 days of incubation, a strip of agar, 5 mm wide, was removed from the zone of interaction, so that it contained the host and the mycoparasite. The strip was then cut into 5-mm long segments. These segments were placed separately in series on 10-mm diameter disks of SSEA medium. This medium was prepared as follows: sun-flower seeds (100 g) were boiled in 500 ml distilled water, for one hr., strained through muslin, agar (17 g) was added until dissolved, the suspension made up to 1L and then autoclaved at 15 psi for 20 min. The medium was amended with 50 mg of penicillin after autoclaving, and after it had cooled down to about 50 °C. After seven days of incubation on SSEA disks at 25 °C, the segments were examined for the presence/absence of spiny oogonia produced by the mycoparasites. Using this method, it was easy to assess growth of a mycoparasite to the nearest 5-mm, because the presence of spiny oogonia indicated that the pathogen had been colonized by the mycoparasite. Six replicate plates of each agar medium were used for each mycoparasite-pathogen combination.

Interactions on thin films of agar. The method was based on that of Laing and Deacon [11]. Host – mycoparasite pairings were made on sterile glass coverslips coated with 2% water agar (20 g Difco agar/1L distilled water). Microscopic observations were made throughout the coated coverslip at regular intervals. Three replicate slides were used for each host-parasite interaction, and the entire experiment was repeated twice.

Evaluation of host susceptibility to parasitism by mycoparasitic *Pythium* species was based on Ribeiro and Butler's rating system [23].

Biological efficacy trials

Preparation of planting mixes. The peat/sand plant mix consisted of 50% peatmoss and 50% fine sand amended with 1% (w/w) ground rolled oats [24] was used for experiments on disease suppression under growth room conditions. Indigenous *Pythium* species, including mycoparasites, were eliminated by moistening the soil and autoclaving for 1 hour. The soil was then exposed to the air for one week before use.

Preparation of initial inocula. The method of Paulitz and Baker [25] was used for inoculum preparation. Initial inocula of *P. ultimum* and the three isolates of mycoparastitc *Pythium* species were prepared by adding five 5 mm disks obtained from the margins of an actively growing colony to the planting mix. Resterilization was accomplished by autoclaving for 1 hour on each of two consecutive days. The inoculum was incubated under aseptic conditions at 25 °C in 2-L beakers covered with aluminum foil. Control preparations received agar disks only. After three weeks, the population density of each isolate in these inocula expressed as colony forming units per gram of soil (CFU g^{-1}), was determined by the SSDP technique on VP3 selective medium. These colonized mix preparations, at calculated amounts, were then used as the inocula to infest large quantities of the plant mix to reach the desired concentrations, which were used in experiments on disease suppression. The preparations contained 500 CFU g^{-1} of each of the mycoparasitic isolates and 50 CFU g^{-1} of the phytopathogenic *P. ultimum* isolates. All preparations were incubated at 25 ± 1 °C in large plastic bags and kept until use.

(A) Direct application treatment. All experiments were conducted in plastic pots (200 ml-volume) under growth room conditions, ten replicate pots per treatment. Seven cucumber seeds (*Cucumis sativus* L. 'Oscar') were planted in each pot after soil was moistened. Plants were grown in a constant-temperature growth room at 26 ± 2 °C with a 12 hour light/dark cycle. The following treatments were used: *P. ultimum* plus *P. acanthicum*; *P. ultimum* plus *P. oligandrum* and *P. ultimum* plus *P. periplocum*. Control treatments consisted of pots with sterilized plant mix only, pots containing plant mix infested with *P. ultimum* alone, or only with the mycoparasite. All pots received adequate watering (twice daily) with distilled water and percent emergence were recorded seven days after planting. After that, all seedlings were removed and pots were replanted at 10 and 20 days.

(B) Seed coating treatment. Preparation of seed coated with the mycoparasitic *Pythium species* was carried out as follows. Two kinds of seed coating were prepared. The first preparation, termed 'homogenate' was obtained by growing each mycoparasitic species in cornmeal extract liquid culture. Aliquots of 20 ml of this medium were dispensed into 2.5 cm-diameter culture tubes, which were then each inoculated using a single 5 mm diameter disc cut out from the margin of each 7-day old mycoparasitic colony grown on cornmeal agar. The cultures were incubated for 7 days at 25 °C with vigorous shaking by hand each day to ensure mixing of the contents. This preparation contained mycelial fragments and intact oospores, which was stored at 4 °C until use. A second preparation, termed 'oospore suspension' was made as follows: A 5 mm diameter disc cut out from the margin of each mycoparasitic colony was dispensed into 2.5 cm-diameter culture tubes containing 20 ml of sunflower seed extract liquid medium. After an incubation period of seven days, the mycelial mats were dried in a laminar flow bench overnight at 20–25 °C to kill vegetative hyphae. The mycelial mats were then homogenized by mixing with sterile distilled water. The suspension was then filtered through five layers of sterile cheesecloth to form a dense oospore solution that was stored at 4 °C until required for use. Homogenate and oospore suspension preparations were each used to coat cucumber seeds. Twenty ml of each preparation was poured over 50 cucumber seeds (which had previously been surface-sterilized in 0.5% sodium hypochlorite for 2 minutes followed by rinsing in sterile distilled water) in 90 mm-diameter petridish. After being allowed to soak for 5 minutes in each preparation, the seeds were removed, spread on to a sterile filter paper and allowed to air-dry for 1 hour. Seeds were then ready for use in biological control experiments.

Plastic pots containing 200 g of planting mix infested with 50 CFU g^{-1} of *P. ultimum* were planted each with ten cucumber seeds placed 1.5 cm apart and approximately 2 cm deep. Five replicates were used for each type of seed coating and all pots were incubated in growth room environment at 26 ± 2 °C, with alternating periods of 12 hour light and dark. All pots were irrigated twice daily with distilled water and percent emergence was recorded seven days after planting. The treatments used were: untreated seeds planted in planting mix infested with 50 CFU g^{-1} of inoculum of *P. ultimum*; homogenate-coated seeds of each mycoparasite planted in planting mix infested with *P. ultimum*; and oospore-coated seeds of each mycoparasites planted in planting mix infested with *P. ultimum*. For control treatments, seeds coated with either homogenate or oospore suspension were planted in sterile planting mix.

Statistical analysis. Analysis of variance was performed on combined data by using a SPSS computer program. Separation of means was based on Duncan's multiple range test. Unless otherwise indicated, data

from repeated experiments were pooled for statistical analysis.

Results

Occurrence of mycoparasitic Pythium spp in soil. Data on the mycoparasitic *Pythium* species recovered from soil by the SSDP technique with the selective VP3 medium, and the SBT, are shown in Table 2.

Pythium species were detected in 47 (94%) and 49 (98%) of the soils assayed using the SSDP and the SBT, respectively. Using the SBT, three mycoparasitic *Pythium* species, *P. oligandrum*, *P. acanthicum*, and *P. periplocum* were recovered from 26 (52%), 16 (32%), and 4 (8%) soils, respectively. On the other hand, using the SSDP, only *P. oligandrum*, and *P. acanthicum* were detected in 15 (30%) and 6 (12%) soils, respectively.

Three other non-mycoparasitic *Pythium* species with spiny oogonia were also detected in 8 soils using the SBT whereas only one non-mycoparasitic species with spiny oogonia was recovered using the SSDP.

Taking into account the two isolation methods used in this study (SBT and SSDP), *P. oligandrum* was the most frequently isolated species. It was found in 70% of soils yielded mycoparasites. The next most commonly isolated mycoparasitic species of *Pythium* was *P. acanthicum*, detected in 43% of soils. *Pythium periplocum* was detected in only 11% of these soils. Mycoparasitic pythiums were detected in 74% (37) and 42% (21) of all the soils studied using the SBT and the SSDP, respectively.

Using the SSDP, population levels of mycoparasitic *Pythium* species in soils studied ranged from 0.0–53.0 CFU g^{-1} D.W. Unlike non-mycoparasitic pythiums, which showed higher population levels in soils under irrigation, mycoparasitic *Pythium* species showed higher mean population densities in non-irrigated soils under vines, vegetables or under fruit trees (14.6, 8.5 and 2.7, respectively) than in irrigated soils under vegetables or citrus (5.6 and 6.4, respectively).

When taking cropping practices in the occurrence of mycoparasitic pythiums into account, *P. oligandrum* was shown to be more frequent in non-irrigated soils under vine (present in 89% and 67% of these soils assayed by the SBT and the SSDP, respectively), followed by non-irrigated soils under vegetables (73%, SBT; 27%, SSDP) and irrigated soils under citrus (50%, SBT; 40%, SSDP).

Pythium acanthicum showed also similar pattern of occurrence. On the other hand, *P. periplocum* was more frequent in irrigated than in non-irrigated soils and was recovered only by the SBT. Mycoparasitic *Pythium* species were much less frequent in non-cultivated than in cultivated soils, only *P. oligandrum* was isolated from non-cultivated soils by using the SBT.

Interactions on agar plates (Modified Dual Plate Culture Method). Table 3 shows the distance that the mycoparasite had grown over the host colony indicated by the presence of spiny oogonia. These results showed that all mycoparasites had made little growth across colonies of *Pythium* host species on PDA plates compared with those on CMA plates, and were much less effective against all tested hosts on PDA plates. *P. periplocum* grew more progressively across colonies of most tested *Pythium* species than did the other two mycoparasites, *P. acanthicum* and *P. oligandrum*, on both types of media. The phytopathogenic isolate of *P. ultimum* was parasitized more efficiently by the three mycoparasites on CMA, and oospores of *P. periplocum* and *P. oligandrum* being visible very close to the pathogen inoculum after 10 days. Whereas, *P. acanthicum* grew to a less extent into the pathogen incoulum. *P. vexans* was the most effectively parasitized host species on CMA medium by both *P. periplocum* and *P. oligandrum*. *P. aphanidermatum* and *P. deliense* did not enable the three mycoparasites to grow across their colonies, and the mycoparasites failed to produce oospores or to be reisolated further than its own inoculum. *P. debaryanum* was the only host species to be effectively parasitized on both PDA and CMA by the three mycoparasites.

Interactions on thin film of agar. The three mycoparasites showed identical mechanisms of parasitism during interaction with *P. ultimum* on water agar films. After contact, the hyphae of the mycoparasites continued to extend beyond the contact points with the pathogen hyphae, and produced lateral branches which, in turn, coiled around, penetrated and grew within the pathogen mycelia. Extensive coiling around host hyphae and internal growth within the host mycelium had commonly been seen during interactions between *P. ultimum* and mycoparasites.

Microscopic examination had also revealed that the three mycoparasites differ in aggressiveness towards the host fungus. *P. periplocum* was found to be a very efficient mycoparasite during interaction on

Table 2. Isolation of *Pythium* species (numbers and percentages of +ve soils) using the surface-soil-dilution plating (SSDP) method and the sclerotia bait technique (SBT)

Field category*	Number of fields	Number of positive soils (%)							
		P. oligandrum		*P. acanthicum*		*P. periplocum*		Other species	
		SSDP	SBT	SSDP	SBT	SSDP	SBT	SSDP	SBT
A	9	1 (11)	2 (22)	3 (33)	3 (33)	0 (0)	3 (33)	9 (100)	9 (100)
B	10	3 (30)	5 (50)	3 (30)	3 (20)	0 (0)	0 (0)	10 (100)	10 (100)
C	11	3 (27)	8 (73)	1 (9)	6 (55)	0 (0)	0 (0)	11 (100)	11 (100)
D	9	6 (67)	7 (78)	0 (0)	4 (44)	0 (0)	0 (0)	9 (100)	9 (100)
E	6	1 (17)	2 (33)	0 (0)	1 (17)	0 (0)	1 (17)	6 (100)	6 (100)
F	5	0 (0)	2 (40)	0 (0)	0 (0)	0 (0)	0 (0)	2 (40)	4 (80)
All fields	50	14 (28)	26 (52)	7 (14)	16 (32)	0 (0)	4 (8)	47 (94)	49 (98)

* A, permanent irrigated soils under vegetables; B, irrigated citrus orchards; C, non-irrigated soils under vegetables; D, non-irrigated vineyards; E, non-irrigated fruit trees; F, non-cultivated soils.

Table 3. Extent of growth (mm) by *P. acanthicum*, *P. oligandrum* and *P. periplocum* after 10 days at 25 °C, on CMA and PDA plates pre-colonized by other *Pythium* species. Means ± SE of six replicates

Host on pre-colonized agar plates	*P. acanthicum*		*P. oligandrum*		*P. periplocum*	
	CMA	PDA	CMA	PDA	CMA	PDF
P. aphanidermatum (Edson) Fitzp	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P. debaryanum Hesse	60.8 ± 3.8	47.5 ± 2.7	65.0 ±3.2	50.8 ± 2.0	67.5 ± 2.7	55.8 ± 2.0
P. debaryanum Hesse	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P. deliense Meurs	34.2 ± 3.8	N.D	32.5 ± 2.7	N.D	54.2 ± 3.8	N.D
P. intermedium de Bary	50.8 ± 2.0	N.D	60.8 ± 5.8	N.D	63.3 ± 5.2	N.D
P. irrigulare Buisman	34.2 ± 2.0	2.5 ± 2.7	23.3 ± 2.6	0.83 ± 2.0	43.3 ± 4.1	4.2 ± 6.6
P. lutarium Ali-Shtayeh	51.7 ± 2.6	30.8 ±3.8	53.3 ± 6.1	34.2 ± 2.0	64.2 ± 4.9	35.0 ± 3.2
P. oedochilum Drechsler	57.5 ± 7.6	33.3 ± 2.6	65.0 ± 4.5	31.7 ± 2.6	64.2 ± 4.9	40.8 ± 2.0
P. paroecandrum Drechsler	53.3 ± 8.8	34.2 ± 4.9	58.3 ± 10.3	49.2 ± 9.2	60.0 ± 11.4	52.5 ± 7.6
P. rostratum Butler	65.8 ± 3.8	35.8 ± 3.8	67.5 ± 2.7	40.8 ± 2.0	67.5 ± 2.7	43.3 ± 2.6
P. sylvaticum Campbell & Hendrix	63.9 ± 4.9	31.7 ± 2.6	68.9 ± 2.2	39.4 ± 3.0	68.3 ± 2.5	45.0 ± 5.0
P. ultimum var ultimum Trow	60.8 ± 5.8	35.0 ± 4.5	69.2 ± 2.0	41.7 ± 2.6	70.0 ± 0.0	42.5 ± 2.7
P. vexans de Bary	53.3 ± 4.1	33.3 ± 2.6	56.7 ± 5.2	20.0 ± 0.5	60.0 ± 9.5	45.8 ± 4.9

N.D., not determined.

thin film of agar. Coiling of *P. periplocum* hyphae around host hyphae was noticeably more than either *P. acanthicum* or *P. oligandrum* hyphae. Internal colonization by this mycoparasite was abundant and well developed throughout the host mycelium. *P. oligandrum* and *P. acanthicum* also made extensive coiling around host hyphae, however, internal colonization by these mycoparasites occurred infrequently and restricted to only few infected host hyphae. In only a few instances, lysis of *P. ultimum* hyphae by *P. oligandrum* could be observed.

Generally, three types of hyphal interactions were observed: coiling around the host hyphae; penetration of the host hyphae; and subsequent lysis of the infected hyphae.

Biocontrol efficacy

(A) Direct application treatment. In planting mix infested with *P. ultimum* (50 CFU g^{-1}), this pathogen gave significant decrease (P = 0.05) in cucumber seedling stand; damping-off percentage was about 33%, seven days after planting (Table 4). *Pythium acanthicum*, *P. oligandrum*, and *P. periplocum* applied to the planting mix (500 CFU g^{-1}) infested with *P. ultimum* resulted in significant increase (P = 0.05) in seedling stand in the first growth cycle compared to treatment with *P. ultimum*, but was not effective as the un-inoculated control.

Efficacy of *P. oligandrum* and *P. periplocum* in the suppression of cucumber damping-off was signi-

Table 4. Percent emergence of cucumber seeds untreated and treated with *P. acanthicum*, *P. oligandrum*, or *P. periplocum* planted in a planted mix. Numbers represent the percent of healthy seedlings after 7 days. Treatments followed by the same letter are not significantly different (p = 0.05) according to Duncan's multiple range test

Treatment	Direct application planting		
	1st	2nd	3rd
Control (-ve)	93* A	94 A	91 A
P. ult	33 D	30 D	23 D
P. ult + P. acan	54 C	34 D	24 D
P. ult + P. olig	67 B	43 C	25 D
P. ult + P. per	69 B	47 C	29 D
P. acan	89 A	90 A	90 A
P. olig	90 A	89 A	87 A
P. per	87 A	86 A	87 A

* Mean of ten replicate pots each with seven seeds.

Table 5. Effect of seed coating with homogenate or oospores suspension of mycoparasitic pythiums on percent emergence of cucumber seedlings. Numbers represent the percent of healthy seedlings after 7 days. Treatments followed by the same letter are not significantly different (p = 0.05) according to Duncan's multiple range test

Treatment	Seed-coating	
	homogenate-coated	Oospore-ccoated
Control (-ve)	98* A	96 A
P. ult	36 C	36 C
P. ult + P. per	44 B	40 B
P. ult + P. acan	60 C	66 C
P. ult + P. ol	62 B	58 B

* Mean of ten replicate pots each with seven seeds.

ficantly higher than that of *P. acanthicum* (P = 0.05); difference in efficacy was not significant between the two mycoparasites (Table 4). In the second growth cycle, disease suppression by *P. oligandrum* and *P. periplocum* was significant (P = 0.05), whereas it was lost when *P. acanthicum* was applied. Disease suppression was lost for the three *Pythium* mycoparasites in the third growth cycle.

The results also showed that cucumber seeds planted in planting mix inoculated with *Pythium* mycoparasites, or in sterilized planting mix, gave comparable seedling stand counts.

(B) Seed-coating treatment. In soil artificially infested with *P. ultimum*, untreated cucumber seeds gave a stand count of 36% after seven days. However, seeds coated with oospore or homogenate suspensions of *P. acanthicum*, *P. oligandrum* or *P. periplocum* and planted in similar planting mix gave higher stand counts (significant only for the latter two species, P = 0.05) (Table 5). However, no significant difference between seedling emergence in treatments using homogenate- or oospore-coated seeds was evident with all mycoparasites tested.

Discussion

Like most *Pythium* species, the mycoparasitic pythia are common inhabitants of cultivated soils in the West Bank [26]. *P. oligandrum* was the most common mycoparasitic *Pythium* species in the soils surveyed. The ability of this mycoparasite to colonize pre-colonized substrata in which there are relatively low levels of available nutrients accompanied with its ability to exploit a wide range of fungi, may explain the common and widespread occurrence of *P. oligandrum* in soil habitats [2, 27].

Pythium acanthicum was detected relatively less frequently than *P. oligandrum* in this survey. *Pythium periplocum* was isolated much less frequently than the two other mycoparasitic species by using the SBT. However, the successful isolation of this mycoparasite was significant, because it was rarely isolated by the conventional soil dilution methods [6, 28].

The present work also demonstrates that mycoparatistic *Pythium* species differ in their spectra of occurrence in soil in relation to different cropping practices. In contrast to most non-mycoparasitic *Pythium* species, *P. oligandrum* was found to be associated more with non-irrigated soils than irrigated ones. The ability of this species to utilize nutrients mobilized via the extra-cellular enzymes of host, as well as, of non-host species [27] may enable it to survive the relatively unfavorable conditions of the non-irrigated soils. In contrast, *P. periplocum* seems to be associated with irrigated soils. Stanghellini [29] reported that high moisture content might enhance zoospore production and mobility by *Pythium* species with filamentous or lobulate sporangia, which readily produce zoospores. This in part may explain the frequent occurrence of *P. periplocum*, which has lobulate sporangia, in the wetter irrigated soils.

This is the first study that attempts to compare baiting with sclerotia of *S. sclerotiorum* (SBT) with SSDP on the VP3 medium for the isolation of mycoparasitic

Pythium species from soil. Each of the SSDP and SBT approaches has distinct advantages in different contexts. The former provides quantitative and qualitative data on *Pythium* population densities in the soil. On the other hand, the SBT is more suitable when only qualitative data is needed; but can however, provide rough quantitative information.

Comparison of the two isolation techniques showed that, usually more species of mycoparasitic pythia were recorded by SBT than SSDP on VP3 plates. This was attributed to the presence of certain compounds, such as amino acids, amino-sugars or glucose, in the sclerotial cell wall, which may be responsible for the attraction of mycoparasitic *Pythium* spp. with spiny oogonia [7]. This explanation was based on the findings of Foley and Deacon [30] who reported that mycoparasitic *Pythium* species require an organic nitrogen source and thiamin or one of its components for growth, whereas, all non-mycoparasitic species can utilize inorganic nitrogen and are self-sufficient for thiamin. However, the use of VP3 medium, which contains an organic nitrogen source as well as thiamin, in this study, did exclude the possibility of the nutrient requirements explanation, because there was a significant difference in the number of mycoparasitic species obtained by the two methods, despite the availability of all nutritional requirements for these parasites in the selective medium.

The efficiency of SBT in the isolation of mycoparasitic species could be attributed to the production of a specific active factor(s) from sclerotia essential for the attraction of swimming zoospores of these parasites. This explanation is supported by the recent findings of Mischke et al. [31] who reported the isolation of such a compound from the sclerotia of *Sclerotinia minor*, that may act as a specific signal to stimulate germination of the mycoparasite, *Sporidesmium sclerotivorum*.

The sclerotia baiting technique was highly effective for the isolation and detection of mycoparasitic *Pythium* species with spiny oogonia from soils assayed. This method has a number of advantages over other potential methods. It requires no antimicrobial agents, which makes it a less expensive method. It is almost completely selective for the mycoparasitic *Pythium* species. It is highly sensitive, especially when inoculum levels are low. The isolated species could usually be sub-cultured as pure cultures directly on to CMA from their advancing margins around the selerotia bait.

The efficiency of this procedure was possibly improved in the current work by increasing the amount of soil sampled, increasing the amount of water mixed with the soil, and by burying the sclerotia in the soil in order to increase the contact area with the soil.

No direct observable correlation was found between the occurrence of *P. oligandrum*, *P. acanthicum*, *P. periplocum* with the type of vegetation, soil organic matter, or soil pH in this study.

The three mycoparasitic *Pythium* species with spiny oogonia *P. acanthicum* Drechsler, *P. oligandrum* Drechsler, and *P. periplocum* Drechsler, were shown to be potential antagonists of *P. ultimum* Trow, a major damping-off pathogen, and other *Pythium* host species, using a range of *in vitro* techniques.

In the MDPCM, all three mycoparasites were much less effective against *Pythium* host species on PDA plates, and made little growth across precolonized PDA plates than they did on CMA plates. These results are in consistent with those of Whipps [32] who reported that for the majority of dual culture interaction, pathogen-antagonist combinations usually do not show the same colony interaction on all media, and that the medium has a significant effect on the growth of the mycoparasite.

The reduction in the mycoparasitic activity on PDA plates may be attributed to the presence of inhibitory components in the commercial potato extract, which inhibit the mycoparasites but not the phytopathogenic *Pythium* species [33]. The results are also in consistent with those of Bradshaw et al. [34] who also found that *P. oligandrum* growth was reduced during interactions with fungal host species on PDA medium. On the other hand, these results are in contrast with those obtained by Deacon [9] who reported that *P. oligandrum* grew as rapidly across PDA plates precolonized by *Phialophora radicicola* as it did across PDA plates alone.

Colony overgrowth by mycoparasitic *Pythium* species can not be used as a general indication of host susceptibility but perhaps better assesses the ability of a mycoparasite to derive nutrients or tolerate growth-inhibitory products of other fungi [1]. Therefore, the results from the MDPCM in this study do not permit direct comparisons of susceptibility between *Pythium* host species.

In this work, disks cut out from SSEA medium was sufficient to support oogonial production by mycoparasitic *Pythium* species when present in dual cultures with other oomycetous fungi. This makes the MDPCM a more practical and less time consuming method than that of Deacon's [9] in which disks of the highly susceptible host *Phialophora radicicola* Cain

were used to support oogonial production by these mycoparasites. This study is the first to report a successful alternative method for supporting the production of spiny oogonia by the mycoparasitic *Pythium* species when present in dual culture with members of oomycetous fungi.

Hyphal interactions between antagonists and *P. ultimum* as shown in the present work have not followed the usual mechanisms reported by Laing and Deacon [11] who found that a typical sequence of parasitic events was: host stoppage, followed by cytoplasmic coagulation, then parasite branching and penetration. Instead, hyphal coiling around host hyphae followed by intrahyphal growth were the predominant events of the mycoparasitic interactions. Penetration and subsequent lysis of hyphae were clearly observed during the interaction with *P. periplocum*.

In this study, hyphal coiling and internal colonization were taken as an indication of host susceptibility. On the basis of these criteria *P. ultimum* was found to be moderately susceptible to *P. periplocum* and slightly susceptible to the two other mycoparasites. However, some workers considered hyphal coiling as an indication of host resistance and therefore, concluded that *P. ultimum* Trow was moderately resistant to mycoparasitim by *P. oligandrum* [9, 33]. However, whether hyphal coiling may be a symptomatic of a host defense mechanism or an indication of the host responding positively to attack by mycoparasites, such contradictory statements remain open for further investigation.

The inability of the mycoparasitic *Pythium* species to overgrow colonies of *P. aphanidermatum* in this study, was consistent with the recent work of Jones and Deacon [35] who reported that this fungus was not affected by the mycoparasites, *P. oligandrum*, *P. acanthophoron*, *P. mycoparasiticum*, and *P. periplocum*. In fact the fungus was shown by these workers to have a mycoparasite-like behaviour against several other fungi under *in vitro* conditions.

Pythium deliense Meurs was also shown to be immune to parasitism by the mycoparasites tested in this study, this may be explained by the fact that this species is closely related to *P. aphanidermatum*, and may well have the same mycoparasitic behavior.

In the present study, the mycoparasitic *Pythium* species with spiny oogonia, *P. acanthicum*, *P. oligandrum* and *P. periplocum* were evaluated for their ability to suppress damping-off disease incited by *P. ultimum*. Increased seedling emergence, in the presence of *P. ultimum*, as the result of treatments with mycoparasitic Pythia was interpreted as a suppression of damping-off disease by the biocontrol agent.

Of the three mycoparasitic species evaluated, *P. periplocum* and *P. oligandrum* were more effective than *P. acanthicum* in suppressing damping-off disease in all growth room experiments. These two mycoparasites applied to the planting mix (500 CFU g^{-1}), increased the number of healthy seedlings by 103 and 109% respectively, whereas *P. acanthicum* resulted in an increase by 64%, seven days after planting. One possible reason for the difference in the effectiveness of disease suppression by the antagonistic isolates is that the population buildup of *P. oligandrum* and *P. periplocum* that developed in the planting mix were much greater than that of the other mycoparasite, *P. acanthicum*. This explanation is supported by the fact that the mean daily growth rate (DGR) of *P. oligandrum* and *P. periplocum* are very similar, while the DGR of *P. acanthicum* is significantly smaller than that of the former two parasites [23]. This greater growth rate gives the introduced antagonistic mycoparasites the ability to establish themselves and proliferate in the soil or other substrates and thus, the ability to compete for nutrients and space. The ability of a mycoparasite to colonize or establish a large population in the rhizophere is a crucial factor that determines a successful biological control [36, 37]. Martin and Hancock [38] reported that increasing the population density of the mycoparasite in the soil may suppress disease by reducing the saprophytic activity of the pathogen, and thus eliminating the explosive buildup of inoculum density of *P. ultimum*.

The poor performance of *P. acanthicum* as a biocontrol agent against *P. ultimum* may be also related to the observations reported in the present work (*in vitro* experiments) which showed that *P. acanthicum* was relatively less aggressive mycoparasite compared with the two other mycoparasitic pythia, therefore, mycoparasitism by *P. acanthicum* was not sufficient enough to accomplish disease suppression in biological control tests.

Damping-off induced by *Pythium* species has been controlled by coating seeds or by direct application of a variety of antagonistic microorganisms into the soil [15, 39–41]. No attempts, to the best of our knowledge, have been made to compare between the two delivery methods. However, Elad and Chet [37] indicated that inoculation of seeds would be an effective control method against soil-borne diseases since antagonist would have the opportunity of being the first colonizers of the roots, the most important site of inter-

action with plant-rotting *Pythium* species. Harman et al. [42] and Windels [43] have also reported that seed colonization by the mycoparasite may act as physical barriers to the pathogen, or may reduce or alter the diffusing of nutrients exuding into the soil. On the other hand, McQuilken et al. [41] have shown that coating sugar beet seeds *with P. oligandrum* acted as a simple barrier to early infection with *P. ultimum*, and provided a temporary protection for only a short period of time. However, application of antagonists to seed were suggested in a number of publications [e.g. 15, 42] as an alternative approach to introducing them into the soil, since the former method requires smaller amounts of biocontrol materials than in broadcast applications.

In the present work, seed coating with either homogenate or oospore suspension was as effective as direct application of mycoparasitic pythia into the soil (comparison has been made only in the first planting, since seed treatment requires repeated applications for every planting cycle). In direct application treatment, incorporation of mycoparasitic pythia into the planting mix amended with ground rolled oats resulted in suppression of cucumber damping-off disease induced by *P. ultimum*. These encouraging results may be attributed to the fact that, in this way, the mycoparasites would have more opportunity to compete for the organic food base, which is very important for population increase in the mix, and may be a pre-requisite to the maintenance and efficacy of the introduced antagonists [25, 44]. Indeed, Paulitz and Baker [45] reported that soil amendment with organic substrates was a crucial factor in the suppression of damping-off disease by the mycoparasite, *P. nunn*, and this mycoparasite was not effective in pathogen suppression in soil without an organic food base.

Another possible mechanism of disease control may be due to direct hyphal interactions between the mycoparasite and *P. ultimum*. Lutchmeah and Cooke [27] proposed hyphal interference as the mechanism of antagonism. However, mycoparasitism alone could not be a primary mechanism of disease control in the soil, because mycoparasitism is not an immediate process [1, 11]. For this reason, it would be very difficult for mycoparasitism alone to prevent seed and hypocotyl infection by *P. ultimum*, which has the ability to colonize seed coats within 24 hr [46]. However, it appears more likely that the mechanism (s) of disease suppression, in this system, could involve competition for nutrients or space, since mycoparasitic pythia and *P. ultimum* are ecologically related microorganisms and may occupy similar ecological niches [38]. Hyphal interference (mycoparasitism) could also be involved in disease suppression, since microscopic observations revealed that mycoparasitic *Pythium* species parasitize hyphae of *P. ultimum* under *in vitro* conditions [23, 27, 40, 41]. However, competition for nutrients or space and mycoparasitism may not be the only biological control mechanisms.

The efficacy of the tested mycoparasites to suppress damping-off disease decreased with successive plantings. *Pythium oligandrum* and *P. periplocum* lost their efficiency after the second growth cycle, whereas for *P. acanthicum* it was lost after the first planting. This decline in the efficacy may be attributed to the decline in the populations of the added mycoparasites. There are several possible explanations for population decline, including the lack of proper nutrients, depletion of the food base that could sustain increased growth of the antagonist, the presence of toxic substances in the root exudation, or the presence of antagonistic or competing microorganisms, such as bacterial contamination, that may compete with the biocontrol agent for nutrients or produce metabolites toxic to mycoparasitic species.

Finally, taking practical biocontrol in field soil into account, we conclude from our results that *P. oligandrum* and *P. periplocum* were promising as biological control agents of damping-off induced by *P. ultimum* under growthroom conditions.

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