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DISEASE CONTROL

Characterization of *Pythium nunn* newly recorded in Japan and its antagonistic activity against *P. ultimum* var. *ultimum*

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Abstract Two isolates of *Pythium nunn*, newly recorded in Japan, were obtained from soils in Nagano and Fukuoka prefectures, characterized, and tested for antagonistic activity against *P. ultimum* var. *ultimum*. The morphology of both isolates corresponded with those of the original description of *P. nunn*. The rDNA-ITS sequences of the two isolates were identical to each other and had a high similarity with the sequences of the type strain of *P. nunn*. The two *P. nunn* isolates were mycoparasitic toward *P. ultimum* var. *ultimum* and suppressed damping off of cucumber seedlings caused by the pathogen at an early stage of plant growth.

Keywords Antagonistic activity · Mycoparasitism · *Pythium nunn* · *Pythium oligandrum* · *Pythium ultimum* var. *ultimum* · Cucumber

Introduction

Pythium nunn Lifshitz, Stanghellini & Baker is a potential biocontrol agent first recorded from a grassland soil in Colorado, USA (Lifshitz et al. 1984a). This species, which has never been recorded as a plant pathogen (Lifshitz et al. 1984b, c), efficiently suppresses pre-emergence damping off of cucumber seedlings caused by *P. ultimum* Trow var. *ultimum* in greenhouse conditions (Adams 1990; Lifshitz et al. 1984b; Paulitz and Baker 1987a, b, 1988b; Paulitz et al. 1985, 1990). It also suppresses root rots of azalea and sweet orange caused by *Phytophthora cinnamomi* Rands, *P. citrophthora* R. E. & E. H. Smith and *P. parasitica* Dastur (Fang and Tsao 1995). These studies, however, have been limited to a few isolates from soils in the USA.

We recently obtained *P. nunn*-like isolates from soils in Japan. The objectives of this study were to identify them and to characterize their antagonistic activity against *P. ultimum* var. *ultimum*.

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Isolation and identification

Two isolates of *P. nunn*, UZ041 and UZ415, used in this study were recovered from soils by a baiting technique using cucumber seeds as a baiting substrate (Watanabe 1981). Isolate UZ041 was recovered from soil in a vegetable field in Nagano Prefecture in June 2003. Isolate UZ415 was recovered in Fukuoka Prefecture in April 2007 from soil in a deciduous forest dominated by Japanese little leaf box (*Buxus microphylla* Sieb. et Zucc. var. *japonica* Rehd. et Wils.).

The two isolates were cultured on corn meal agar (CMA) prepared according to Tojo et al. (1998) and grass blade culture prepared according to Martin (1992). Both isolates were maintained on CMA until use. Morphological identification was based on the keys of van der Plaats-Niterink (1981) and the species description of Lifshitz et al. (1984c). The cardinal temperature for hyphal growth of the isolates UZ041 and UZ415 was determined on plates of potato–carrot agar (PCA) (van der Plaats-Niterink 1981) in darkness at temperatures from 4 to 40°C at 3°C intervals and at 42°C. Based on the morphologies and growth

temperatures (Table 1; Fig. 1), both isolates were identified as *P. nunn*. Zoospore production was not reported in the original description of *P. nunn* (Lifshitz et al. 1984c), but was found in the present isolates (Table 1). The diameter of spherical sporangia differed between isolates UZ041 and UZ415 (Table 1). Isolate UZ041 readily formed sporangia and zoospores, but isolate UZ415 rarely formed them. Zoospore productivity does vary within a species of *Pythium* spp., especially in species that form spherical sporangia (van der Plaats-Niterink 1981). The present result, with earlier works (Lifshitz et al. 1984c; van der Plaats-Niterink 1981), suggests that zoospore productivity of *P. nunn* varies among the isolates of the species. The cardinal temperatures for hyphal growth were similar for the two *P. nunn* isolates, but differed from those in the original description (Table 1). This biological property is also thought to vary intraspecifically in *P. nunn* as we discuss later. Isolates UZ041 and UZ415 were deposited at the National Institute of Agrobiological Sciences Genebank, Japan as accessions MAFF 241106 and MAFF 241787, respectively.

Sequence analysis

The nucleotide sequences of the ITS region including 5.8S rDNA of isolates UZ041 and UZ415 were determined as described previously (Uzuhashi et al. 2008). Both sequences were 875 bp long, and they were identical each other. The sequences had high similarity (98.6%) with those of *P. nunn* type isolate CBS 808.98 (AY598709; Lévesque and de Cock 2004; Lifshitz et al. 1984c). The results support the morphological identification of the isolates. The sequence differences between the present isolates and the type isolate were related to difference of growth temperature (Table 1). Although it is difficult to explain why the sequence differences relate to growth temperatures in *P. nunn*, such intraspecific variation in the ITS sequences relating to growth temperature is widely known for *Pythium* spp. (Kurokawa and Tojo 2010; Perneel et al. 2006; Uzuhashi et al. 2009). The sequences of isolates UZ041 and UZ415 have been deposited in GenBank as accessions AB468771 and AB537557, respectively.

Table 1 Morphology and hyphal growth of study isolates of *Pythium nunn* from Japan and from reference data

Trait	Isolate UZ041	Isolate UZ415	*Pythium nunn* (Lifshitz et al. 1984b)
Width of hyphae (μm)	<5.0	<5.0	<6.5
Sporangium shape (μm)	Globose or subglobose	Globose or subglobose	(Not formed)
Sporangium position (μm)	Terminal or intercalary	Terminal or intercalary	(Not formed)
Globose sporangium, diameter (μm)	11.0–17.0 (avg. 14.0)	10.0–13.0 (avg. 11.2)	(Not formed)
Size of subglobose sporangia (μm)	11.0–17.0 × 8.5–15.5 (avg. 13.7 × 12.1)	11.0–16.0 × 10.0–14.0 (avg. 13.3 × 11.6)	(Not formed)
Length of discharge tube (μm)	6.0–33.0 (avg. 14.2)	6.0–18.0 (avg. 12.1)	(Not formed)
Encysted zoospore, diameter (μm)	7.0–10.0 (avg. 8.8)	8.0–10.0 (avg. 8.8)	(Not formed)
Shape of hyphal swellings	Globose, oval or lemon	Globose, oval or lemon	Globose, oval or lemon
Position of hyphal swellings	Terminal or intercalary	Terminal or intercalary	Terminal or intercalary
Globose hyphal swellings, diameter (μm)	10.0–21.0 (avg. 14.4)	11.0–21.0 (avg. 14.8)	12.2–22.5 (avg. 16.2)
Size of subglobose hyphal swellings (μm)	11.0–16.0 × 8.5–14.5 (avg. 13.2 × 11.4)	10.0–16.5 × 8.5–14.0 (avg. 12.9 × 10.6)	(Not reported)
Oogonium diameter (μm)	15.3–24.3 (avg. 19.7)	13.0–23.0 (avg. 18.7)	18.5–23.0 (avg. 20.8)
Oogonium position	Terminal or rarely intercalary	Terminal	Terminal
Plerotic or aplerotic oospore	Aplerotic	Aplerotic	Aplerotic
Oospore diameter (μm)	12.6–22.2 (avg. 17.5)	12.5–20.5 (avg. 16.7)	18.0–20.5 (avg. 19.2)
Oospore wall thickness (μm)	<2.2	<2.0	<2.0
Shape of antheridial stalk	Crook or inflated	Crook or inflated	Crook or inflated
Monoclinous or diclinous antheridium	Monoclinous or diclinous	Monoclinous or diclinous	Monoclinous or diclinous
No. of antheridia/oogonium	1–4	1–4	1–5
Cardinal temperatures for hyphal growth	10–40°C (31°C optimum)	10–40°C (31°C optimum)	7–42°C (34°C optimum)
Daily growth rate at 25°C (mm)[a]	13.7	14.1	14.0

[a] Growth rate was examined on potato–carrot agar for isolates UZ041 and UZ415 and on V-8 juice agar in reference

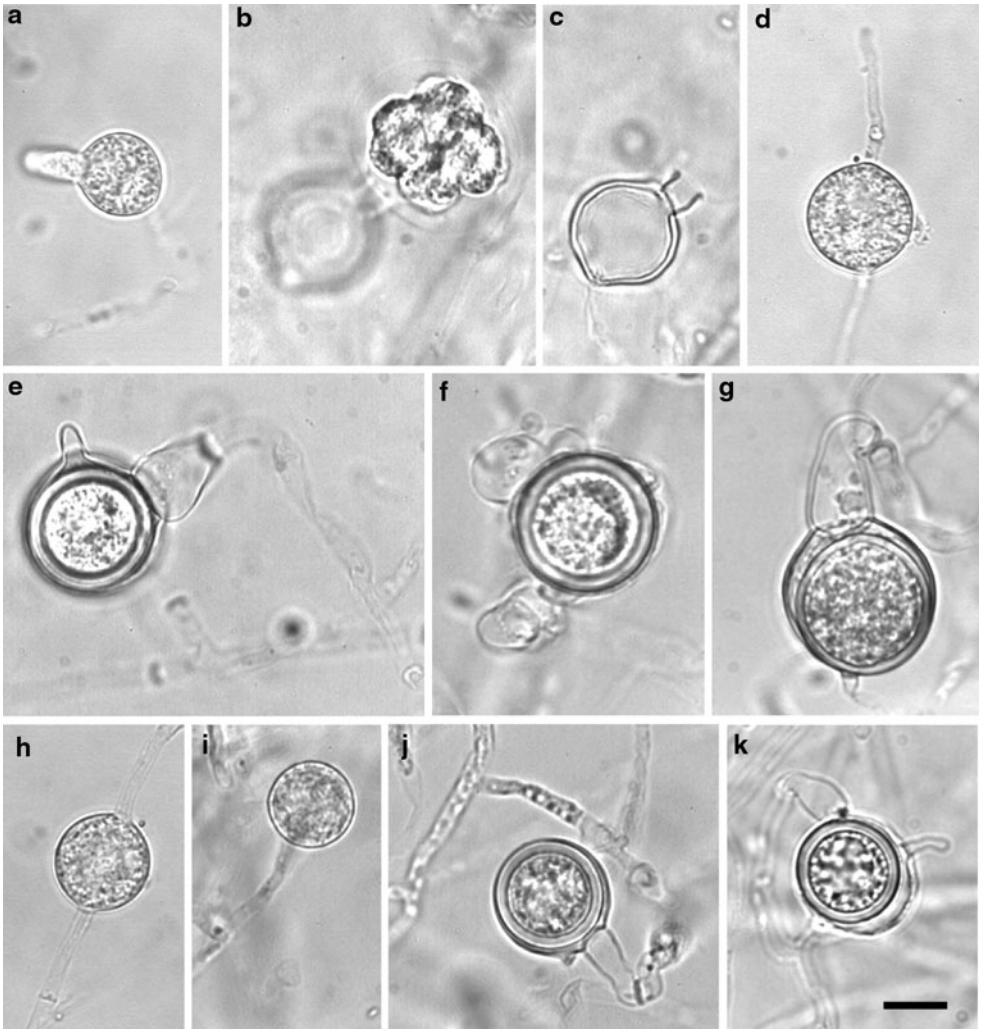

Fig. 1 Morphological characteristics of *Pythium nunn* isolated from Japan. **a**–**g** Isolate UZ041, **h**–**k** isolate UZ415. **a** Sporangium with germ tube. **b** Vesicle with zoospores. **c** Empty sporangium after dispersion of zoospores. **d** Globose, intercalary hyphal swelling. **e** Oogonium with a spine and an antheridium with an inflated stalk. **f** Terminal oogonium with two antheridia. **g** Terminal oogonium with an antheridium having crook stalk. **h** Globose, intercalary hyphal swelling. **i** Globose, terminal hyphal swelling. **j** Terminal oogonium and an antheridium that has an inflated stalk. **k** Oogonium with one spine and a monoclinous antheridium having crook stalk. *Bar* 10 μm

Hyphal interaction

Pythium nunn isolates UZ041 and UZ415, and *P. ultimum* var. *ultimum* isolate OPU774 (Kida et al. 2007) were used. Agar plugs (4 mm diameter) were cut with a cork borer from the margins of actively growing colonies on CMA. The plugs were transferred to opposite sides of a Petri dish (90 mm diameter) with a cellophane film disk (90 mm diameter) placed on water agar. The plates were incubated for 3 days at 25°C, then a strip of the film (15 mm^2) was removed from the crossing zone between the mycelia and was examined with a compound light microscope. Hyphae of both *P. nunn* isolates had penetrated the hyphae of the *P. ultimum* var. *ultimum* isolate (Fig. 2). In the penetrated hyphae, cytoplasm had disappeared, and hyphal septa had formed (Fig. 2). These phenomena are consistent with previous observations of hyphal interactions between *P. nunn* and *P. ultimum* var. *ultimum* (Adams 1990; Lifshitz et al. 1984a).

Disease inhibition

A pot inoculation test was used to test the ability of *P. nunn* to inhibit disease caused by *P. ultimum* var. *ultimum*. *Pythium nunn* isolates UZ041 and UZ415, *P. oligandrum* isolate UOP399 (Kinoshita et al. 1994), and *P. ultimum* var. *ultimum* isolate OPU774 were used. *Pythium oligandrum* was used as comparison for biocontrol with *P. nunn*, because *P. oligandrum* has been well documented as a biocontrol agent on *P. ultimum* var. *ultimum* (Martin and Hancock 1987; Mcquilken et al. 1992) and on many other

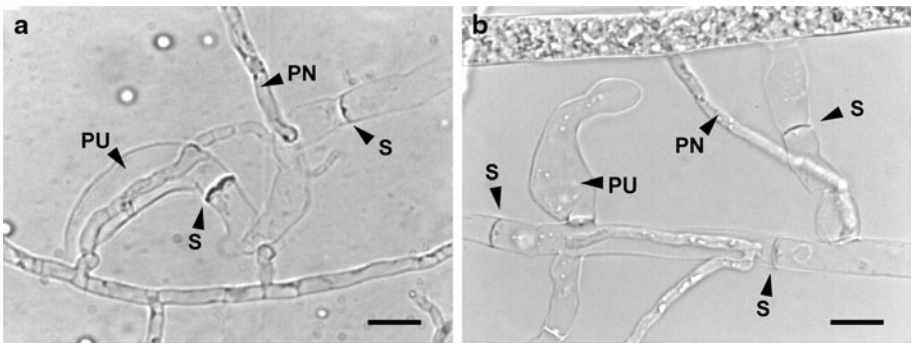

Fig. 2 Hyphal interaction between *Pythium nunn* and *P. ultimum* var. *ultimum*. **a** *P. nunn* isolate UZ041 versus *P. ultimum* var. *ultimum*. **b** *P. nunn* isolate UZ415 versus *P. ultimum* var. *ultimum*. *Arrowheads* indicate hyphae of *P. nunn* (PN), *P. ultimum* var. *ultimum* (PU), and septa (S) formed in hyphae of *P. ultimum* var. *ultimum*. *Bar* 10 μm

Table 2 Effects of *Pythium nunn* or *P. oligandrum* on percentage of healthy cucumber seedlings at 4 and 10 days after sowing in nursery soil infested with *P. ultimum* var. *ultimum*

Pythium species inoculated	Healthy seedlings (%)[a]	
	4[b]	10
P. nunn (UZ041[c]) + *P. ultimum* var. *ultimum*	51.8 ± 3.8 B[d]	8.9 ± 3.8 B
P. nunn (UZ415) + *P. ultimum* var. *ultimum*	55.4 ± 5.7 B	16.1 ± 8.7 B
P. oligandrum + *P. ultimum* var. *ultimum*	94.6 ± 2.6 A	83.9 ± 8.7 A
P. ultimum var. *ultimum*	1.8 ± 1.8 C	1.8 ± 1.8 B
Noninoculated	96.4 ± 2.3 A	96.4 ± 2.3 A
Least significant difference ($P < 0.05$)	14.3	24.0

[a] Healthy seedlings were counted 4 and 10 days after planting

[b] Days after sowing

[c] Isolate numbers of *P. nunn*

[d] Data given as mean ± standard errors ($N = 8$). Values followed by the same letters in a column do not differ significantly according to a Tukey–Kramer honestly significant different (HSD) test

plant pathogens (e.g., Takenaka et al. 2008). A CMA plug containing mycelia of one of the *Pythium* isolates was transferred to a 300 ml Erlenmeyer flask containing 3 g of autoclaved seeds of tall fescue (*Festuca arundinacea* Schreb. cv. Davinchi) and a 12 ml of distilled water. After 10 days of incubation at 25°C in darkness, 15 g of these colonized seeds were mixed with 150 g of commercial nursery soil (Aisai-1, Katakura Chikkarin, Tokyo, Japan) in a mortar. The soil infested with *P. ultimum* var. *ultimum* was prepared with mixing 15 g of the inoculum and 3 kg of the nursery soil in a polyethylene bag. A single layer of a water-soluble paper (Scottie toilet tissue, Nippon Paper Crecia, Tokyo, Japan) was cut into a disk (7 cm diameter) and was placed on 90 ml of the *P. ultimum* var. *ultimum* infested soil in a ceramic pot (inner diameter 7 cm, inner depth 6.5 cm). Either the *P. nunn*-infested or the *P. oligandrum*-infested soil (40 ml) was placed on the paper disk, and seven seeds of cucumber (*Cucumis sativus* L. cv. Jibai) were placed in the soil. The seeds were covered with the paper disk, then 30 ml of the *P. ultimum* var. *ultimum* infested soil was placed on top. Each pot was enclosed in a double layer of polyethylene bag and kept at 25°C under continuous light (73 μmol/m^2/s measured at plant levels) in a growth chamber. The soil in the plates was watered every day to keep the moisture near −10 kPa. The percentage emergence was recorded at 4 and 10 days after sowing, and again at 20 days (data not shown). The experiments were repeated eight times with one pot per treatment. Analysis of variance was conducted for the percentage emergence data of different treatments using JMP software (version 8; SAS Institute, Cary, NC, USA). Means of the data were compared by the least significant difference based on a Tukey–Kramer honestly significant different (HSD) test ($P < 0.05$).

Pythium nunn isolates UZ041 and UZ415 significantly ($P < 0.05$) supported the stand of the cucumber seedlings planted in the soil infested with *P. ultimum* var. *ultimum* at 4 days after sowing (Table 2; Fig. 3). They did not support the stand after 10 days of the sowing, resulting in post-emergence damping off of all seedlings (Table 2). *Pythium oligandrum*, on the other hand, inhibited damping off through 10 days after the sowing. The result demonstrated that the present isolates of *P. nunn* are potential biocontrol agents for *P. ultimum* var. *ultimum*. The lower effectiveness of *P. nunn* than *P. oligandrum* may be related to a unique mechanism for the disease inhibition by *P. nunn*.

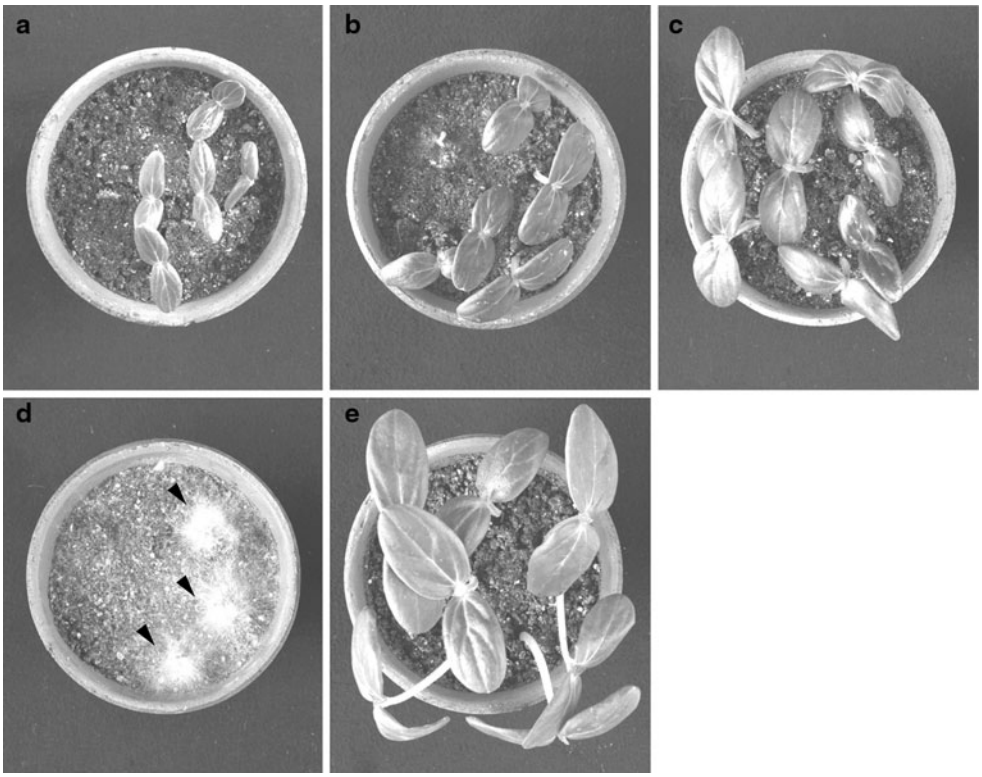

Fig. 3 Effects of *Pythium nunn* or *P. oligandrum* on cucumber seedlings planted in a commercial nursery soil infested with *P. ultimum* var. *ultimum* at 4 days after planting. **a** *P. nunn* isolate UZ041 + *P. ultimum* var. *ultimum*. **b** *P. nunn* isolate UZ415 + *P. ultimum* var. *ultimum*. **c** *P. oligandrum* + *P. ultimum* var. *ultimum*. **d** Noninoculated soil + *P. ultimum* var. *ultimum* (*arrowheads* indicate the diseased plants covered by mycelia of *P. ultimum* var. *ultimum*). **e** Noninoculated

Lifshitz et al. (1984b) reported that the antagonistic activity of *P. nunn* against *P. ultimum* var. *ultimum* was dependent on organic substrates that contained high levels of labile carbon substrates, such as green leaves. Paulitz and Baker (1987a, 1988a) also reported that disease suppression against *P. ultimum* var. *ultimum* on cucumber operated only if organic amendments such as bean leaves or rolled oats were also added to the soil, along with a low inoculum density of *P. nunn*. Moreover, *P. nunn* is a relatively slow-acting mycoparasite compared with *P. oligandrum* (Laing and Deacon 1991). These previous reports suggest that *P. nunn* is a slow-acting antagonist that requires organic amendments for activity. The effect of organic amendments on the antagonistic activity has not yet been determined for the present isolates of *P. nunn* and will be examined in a future study.

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