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Biocontrol of Sugar Beet Seedling and Taproot Diseases Caused by *Aphanomyces cochlioides* by *Pythium oligandrum* Treatments before Transplanting

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Abstract

Biocontrol activities in the oospore suspensions of 17 isolates of *Pythium oligandrum* (PO) were examined for their effect against post-emergence seedling diseases. The seeds of sugar beet that had been commercially chemical-pelleted to prevent pre-emergence damping-off were sown in *Aphanomyces cochlioides*-infested soil on trays. PO isolate MMR2 proved most effective in controlling post-emergence damping-off, at a level equivalent to that of applying fungicides. Oospore suspensions stored for 188 days or 379 days at 4℃ were as effective against *A. cochlioides* post-emergence damping-off as chemical control. Treating soil with PO oospores by mixing suspensions into the surface of soil in paper pot nurseries or by drenching seedlings 32 days before transplanting could control *Aphanomyces* root rot in the field without additional applications. The control effect conferred by PO suspensions was also demonstrated *in vitro*. A polymerase chain reaction (PCR) assay used to detect PO indicated that this oomycete could colonize the rhizosphere of sugar beet plants cultivated in the field when applied before transplanting. These results suggest that PO has the potential to control both post-emergence damping-off and root rot caused by *A. cochlioides*.

Discipline: Plant disease
Additional key words: Aphanomyces root rot, biological control, colonization, post-emergence damping-off

Introduction

In Japan, sugar beets (*Beta vulgaris* L.) are only cultivated in the Hokkaido region, where a transplanting technique is commonly employed. This technique entails seeding sugar beets in paper pot nurseries for about 45 days of cultivation in a greenhouse, and then transplanting the seedlings in the field. Commercial fungicide-pelleted seeds are typically used to protect sugar beets from seedling diseases. In the Hokkaido region, *Pythium* spp., *Rhizoctonia solani* and *Aphanomyces cochlioides* are the main causal pathogens at the seedling stage of sugar beet[11]. Infection by *Pythium spp*. primarily causes pre-emergence damping-off, while *R. solani* causes the pre-emergence death of seedlings and damages newly emerged seedlings. On the other hand, *A. cochlioides* causes little or no pre-emergence damping-off, but causes extensive post-emergence damping-off that could result in the destruction of entire fields, especially in warm and wet soil[13]. Fungicide-pelleted seeds can stably control pre-emergence damping-off, but not post-emergence damping-off, and so the latter type of damping-off caused by *A. cochlioides* is the main cause of sugar beet seedling loss. Furthermore, after seedlings are transplanted in the field, *A. cochlioides* can also cause root rot (i.e., Aphanomyces root rot) from late June to the end of the growing season[28, 29]. The pathogen is widely distributed throughout sugar beet production areas, persistent for long periods in the soil, and not easily controlled by chemicals and cultivation practices. As a result, Aphanomyces root rot is a chronic problem that could become particularly severe in certain fields or during years when warm and moist conditions prevail. Therefore, new control measures for both post-emergence

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damping-off and root rot caused by *A. cochlioides* are required. Biocontrol using beneficial microbes that can colonize the rhizosphere offers one avenue of disease control that provides season-long crop protection, without having a negative impact on the environment or resulting in the development of fungicide resistance. Although biocontrol using bacterial species antagonistic to *A. cochlioides* has previously demonstrated the potential to reduce post-emergence damping-off in sugar beet[12, 20, 31], the specific ability of each bacterial species to reduce Aphanomyces root rot has not been clearly shown.

The oomycete *Pythium oligandrum* (PO) — a soil inhabitant originally isolated from discolored pea roots[7] — has received considerable attention as a potential biocontrol agent due to its advantageous biocontrol properties. PO has been shown to colonize the rhizosphere of a wide range of agriculturally important crops[1]. However, there is no direct evidence that PO is a plant pathogen; Martin and Hancock[17] have shown that PO was not pathogenic to 12 species of agriculturally important plants. Moreover, PO reportedly shows antagonistic activity against a wide range of plant pathogenic true fungi and oomycetes via mycoparasitism mediated by intimate hyphal interaction and antibiosis[4, 16]. This oomycete also has indirect effects on host plants, such as the stimulation of defense reactions and the promotion of plant growth. Benhamou et al[3]. provided the first convincing evidence showing that PO has the potential to induce plant defense reactions in the interaction between tomato and *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Root growth and yield are also enhanced by root colonization with PO, due to its auxin compounds[15]. PO has been demonstrated to be an effective biocontrol agent for such soilborne diseases as damping-off of sugar beet caused by *Pythium ultimum*[17], Verticillium wilt of pepper[2], and bacterial wilt of tomato[9]. Recently, our group found the treatment of potato seed tubers with PO oospore suspensions to be a practical control measure for black scurf of potato caused by *R. solani* AG-3 in the field[10]. PO is also effective against leaf diseases caused by such aerial pathogens as gray mold (*Botrytis cinerea*) in tomato[14] and grapevine[21].

Previous studies have suggested that PO has great potential as a biological control agent for a wide range of diseases, which prompted us to initiate this study of the ability of PO to control sugar beet seedling and root rot diseases caused by *A. cochlioides*. In this study, we first selected MMR2 as being the most effective of 17 PO isolates obtained from field soils, for use, against *A. cochlioides* post-emergence damping-off. Next, the ability of PO to control Aphanomyces root rot was examined by various applications of PO oospores in paper pot nurseries, *in vitro*, and in a field test. Sugar beet rhizosphere colonization by PO in the field was also evaluated by polymerase chain reaction (PCR), in order to build support for the mechanisms by which PO is hypothesized to act as a biocontrol agent for Aphanomyces root rot in sugar beet.

Materials and methods

1. Fungal cultures

Table 1 lists the PO isolates used in this study. For the production of oospores, the isolates were grown in V-8 broth (100 ml of Campbell's V8 juice, 1.5 g of $CaCO_3$ per liter) containing 0.1% wheat germ oil at 25℃ for 19 to 48 days. The mycelium was harvested by filtration, washed three times with sterile distilled water (DW), and then transferred aseptically to a Waring blender cup. After homogenization at 14,000 rpm for five minutes to disrupt hyphae without causing obvious physical damage to the oospores, the number of oospores was determined using a Thoma haemocytometer, followed by storage at 4℃ until use. For the production of inoculum of the sugar beet pathogen, *Aphanomyces cochlioides* isolate MMRA1 (isolated from sugar beet hypocotyl in Hokkaido prefecture by Dr. Y. Kobayashi, National Agriculture and Food Research Organization [NARO], Kyushu Agricultural Research Center) was cultivated on potato-dextrose agar (PDA) medium at 25℃ for three to four days, and zoospores were prepared according to Parke and Grau[22] for inoculation.

2. Nursery tray experiments for suppression of *A. cochlioides* post-emergence damping-off

Artificially infested soil was produced by three successive plantings of sugar beet seedlings, followed by inoculation with zoospores of *A. cochlioides*. More than 4,000 early stage sugar beet seedlings were planted in a container (603 × 377 × 145 mm) filled with natural soil (Brown Andosol), and then inoculated with a zoospore suspension prepared by incubating a mycelial PDA plate at 25℃ for 24 hours with 200 ml of sterile DW. The containers were placed in a greenhouse until damping-off of seedlings occurred. After the seedlings were removed, the soil was mixed thoroughly, and a new cropping cycle was conducted with seedlings and new *A. cochlioides* inoculum as described above. After the third crop, the soil (including the roots) in each container was mixed thoroughly and used as *A. cochlioides*-infested soil.

Plastic trays (27 × 53 cm) divided into 128 cones (3 × 3 × 5 cm) were filled with natural soil (Brown Andosol) mixed with the same volume of the soil (artificially infested with *A. cochlioides*) as described above. Commercially pelleted sugar beet seeds (cultivar ABEND) containing 5 g of hymexazol per unit (one unit = 100,000

seeds) to control pre-emergence damping-off were seeded at a rate one seed per cone. To assess the suppressive ability of the 17 isolates of PO listed in Table 1 against post-emergence damping-off caused by *A. cochlioides*, 5 ml of oospore suspension (10^4 oospores ml^{-1}) of each isolate were applied to a single cone after seeding. Similarly, 5 ml of water or fungicide solution (0.3 mg active ingredient [a.i.] of hymexazol and 50 µg a.i. of validamycin A ml^{-1}) were applied for control treatments. Plants were grown in a growth chamber at 25℃ during the day and 20℃ at night, with a 16-hour photoperiod. Disease severity (DS) in each treatment was estimated at 28 days after seeding as an index based on a scale of 0 to 5 (0 = no symptoms on hypocotyl; 1 = necrosis of less than 50% of hypocotyl area without constriction; 2 = necrosis of more than 50% of hypocotyl area without constriction or necrosis of less than 50% of hypocotyl area with slight constriction; 3 = necrosis of more than 50% of hypocotyl area with slight constriction; 4 = severe stunting of hypocotyl; 5 = seedling dead). The experiment included 13 to 21 plants in each treatment group for the determination of DS, and was repeated three times. Dunnett's one-sided *t* test was used to determine significant differences between each treatment and the water control ($P<0.05$). Because these experiments were divided over six time periods due to space limitations imposed by the growth chamber, the DS of each isolate treatment was compared with that of the water control in each separate experiment, and control efficacies of the 17 PO isolates were calculated as follows:

(DS of water control – DS of each isolate treatment) / DS of water control × 100

To determine whether the storage of oospore suspension at 4℃ affects biocontrol activity against *A. cochlioides* post-emergence damping–off, oospore suspensions (prepared as described above and stored for 188 days or 379 days at 4℃) were used for this nursery tray experiment. Three replicates of 13 to 17 plants were used for the determination of DS. Dunnett's one-sided *t* test was used to determine significant differences between each treatment and the water control ($P<0.05$).

3. Application of PO oospores to seedlings in paper pot nursery for suppression of Aphanomyces root rot

On 15 March 2005, the commercially pelleted sugar beet seeds described above were sown in soil containing fertilizer (20 g of N, 180 g of P_2O_5, 10 g of K_2O per block) in paper pots (with one seed per paper tube). The paper pots consisted of slender paper tubes, 1.9 cm in diameter and 13-cm deep, glued together into a block (20 × 70 tubes). Seedlings were cultivated in a greenhouse without heating until the transplanting date. During cultivation in the greenhouse, five different treatments were conducted on sugar beet plants: (i) control (untreated); (ii) application of 900 g of total surface soil including approximately 6×10^6 PO oospores per block immediately after seeding (PO-I); (iii) application of 1 L of PO oospore suspension including 7×10^7 oospores per block at 32 days before transplanting (PO-II); (iv) applications of both surface soil and oospore suspension (PO-I + II); or (v) application of 1 L of total fungicide solution (5 mg a. i. ml^{-1} of fluazinam) per block just before transplanting.

4. *In vitro* assay for suppression of Aphanomyces root rot by PO

To assess the ability of PO to control Aphanomyces root rot on sugar beet, seedlings subjected to the five different treatments described above were subsequently tested *in vitro*. On 26 April 2005, the treated seedlings in the paper pots were transplanted into glass tubes (24 mm in diameter and 130 mm in length) with plugged drain tubes 40 mm in length at the bottom (Fig. 3 A). These seedlings were inoculated with *A. cochlioides* by first removing the soil from their root bases and adding two mycelial disks (7 mm in diameter) from the PDA plates into each glass tube. *A. cochlioides*-inoculated seedlings were kept for 48 hours under submerged conditions in the growth chamber described above for zoospore production and infection, and after drainage, the glass tubes were transferred to the greenhouse for cultivation. On 7 July 2005, 10 taproots were harvested from each of four replicates of each treatment group, and their weights were determined.

5. Field test for suppression of Aphanomyces root rot by PO

On 26 April 2005, seedlings subjected to the five different treatments described above were transplanted in the field at NARO Hokkaido Agricultural Research Center, located in eastern Hokkaido, Japan. The field design was a split plot (5.2 m × 8 m) in four replications, with rows 65 cm apart and a single sugar beet transplanted every 20 cm in each row. Because saturated soil conditions allow *A. cochlioides* to infect sugar beet roots and generate Aphanomyces root rot in the field, the *A. cochlioides*-infested soil prepared above was scattered onto the field (16 L soil per split plot), which was irrigated for about two months beginning on 7 June 2005. On 30 September 2005, the sugar beets were harvested from each plot. Disease severity of 28-39 taproots in each plot was estimated as an index based on a scale of 0 to 5 (0 = no symptoms on taproot; 1 = superficial brown symptoms on less than 50% of taproot area; 2 = superficial brown symptoms on more than 50% of taproot area; 3 = internally rotted symptoms on less than 50% of taproot area; 4 = internally rotted symptoms on 50 – 75% of taproot area; 5 = internally rotted symptoms

on nearly 100% of taproot area). Tukey's HSD test was used to determine significant differences between each treatment and the oospore-untreated control ($P<0.05$).

6. PCR detection of PO from the rhizosphere of sugar beets cultivated in the field

To evaluate the efficiency of rhizosphere colonization by PO, the rhizosphere soils of field-cultivated sugar beet plants were examined for the presence of PO using PCR. On 15 June 2005, two plants per treatment were gently uprooted from two split plots in the field, and then shaken to remove clumps of soil around the roots. For the collection of rhizosphere soils, these plants were then shaken in a plastic bag to collect as many soil particles adhering to root surfaces as possible. Collected rhizosphere soil was mixed well and part of the soil sample was used to determine soil dry weight after incubation at 70℃ for 48 hours. Total soil DNA was extracted from 0.2 to 0.3 g of soil (expressed as dry weight) using a Bio 101 FastDNA SPIN kit as described by the manufacturer (Qbiogene, Inc., Irvine, CA) with some modifications[26], and then used for the detection of PO DNA by PCR. Soil sample DNA concentrations were adjusted to correspond to the amount of DNA extracted from 25 mg of soil (expressed as dry weight) in 2 µl of solution. PO-specific primers P.OLIG.F1 and P.OLIG.R04 designed by Godfrey et al[8]. were used to produce a 384-bp amplicon from within the internal transcribed spacer (ITS) region of ribosomal (r) DNA. The PCR reaction mixture, with a total volume of 20 µl, consisted of 0.5 unit of Ex Taq DNA polymerase (Takara Shuzo, Otsu, Japan), 2 µl of soil DNA solution, 2 µl of 10 × PCR buffer, 0.5 M of each primer, and 100 µM of dNTP mixture. The thermal cycling conditions for PCR were as follows: after initial denaturation at 94℃ for five minutes, samples were amplified for 35 cycles by touchdown PCR[6] with denaturation at 94℃ for one minute, annealing on a decline ramp of 0.5℃ from 70 to 60℃ every second cycle over the first 21 cycles, and then annealing at 60℃ for one minute for the last 14 cycles, followed by primer extension at 72℃ for one minute. Finally, the reaction mixture was incubated at 72℃ for five minutes. The reaction solution was then subjected to electrophoresis on 2% agarose gel and stained with SYBR Green I (Molecular Probes, Eugene, OR, USA).

Results

1. Effect of application of PO isolates on suppression of *A. cochlioides* post-emergence damping-off in the nursery tray experiment

Seventeen isolates of PO, which were isolated from soils in Hokkaido or Osaka prefecture (Table 1), were assessed for their suppressive ability against *A. cochlioides* post-emergence damping-off in nursery tray experiments (Fig. 1). When the commercial fungicide-pelleted seeds were sown in the infested soil, 73.3 to 91.6% (82.7% on average) of the seeds emerged, and the disease severity scores for the newly emerged seedlings 28 days after seeding in water controls were 1.18 to 2.90 (2.23 on average). There was a significant difference in disease suppressive ability among PO isolates; applications of MMR 2, 3, and 13 significantly reduced the disease severity as compared with the water control, but other isolates showed no significant reduction in disease severity compared with the water control (Fig. 1). Because MMR2 showed the highest control efficacy among 17 PO isolates and its efficacy level was similar to that of fungicide (hymexazol and validamycin A), only this isolate was used for subsequent experiments.

To determine whether the storage of oospore suspensions at 4℃ affects biocontrol activity against *A. cochlioides* post-emergence damping–off, oospore suspensions of MMR2 stored for either 188 days or 379 days were used for this tray experiment. The application of oospore suspensions stored for either 188 days or 379 days at 4℃ could both significantly reduce disease severity as well as could MMR2 when not stored at 4℃, as shown in Fig.1 (Fig. 2). These results indicate that storing oospore sus-

Table 1. Isolates of *Pythium oligandrum* used in this study

Isolate	Habitat	Origin
MMR1	Soybean soil	Memuro, Hokkaido
MMR2	Soybean soil	Memuro, Hokkaido
MMR3	Wheat soil	Biei, Hokkaido
MMR7	Wheat soil	Kunneppu, Hokkaido
MMR8	Wheat soil	Kunneppu, Hokkaido
MMR10	Wheat soil	Memanbetsu, Hokkaido
MMR11	Alfalfa soil	Kitami, Hokkaido
MMR13	Alfalfa soil	Kitami, Hokkaido
MMR14	Alfalfa soil	Kitami, Hokkaido
MMR15	Timothy soil	Kitami, Hokkaido
MMR17	Timothy soil	Kitami, Hokkaido
MMR18	Wheat soil	Kamikawa, Hokkaido
MMR19	Wheat soil	Kamikawa, Hokkaido
MMR20	Wheat soil	Kamikawa, Hokkaido
MMR21	Wheat soil	Kamikawa, Hokkaido
NBRC32559	Vegetable soil	Sakai, Oosaka
OPU425[1)]	Vegetable soil	Sakai, Oosaka

1) Provided by M. Tojo, University of Osaka prefecture,Japan.

pensions at 4℃ for at least one year does not affect their subsequent biocontrol activity.

2. *In vitro* effect on Aphanomyces root rot suppression by PO MMR2 oospores previously applied to seedlings in paper pot nurseries

An *in vitro* assay was first used to examine the efficacy of different applications of MMR2 oospores to paper pot nurseries for controlling Aphanomyces root rot. When the seedlings were inoculated with *A. cochlioides* and then grown in glass tubes for 72 days, the growth of taproots was reduced. However, when the seedlings were pretreated with fungicide solution, the average taproot weight was significantly higher than that of the control (Fig. 3). Although the average weights of taproots in PO-I and PO-II treatments did not differ significantly from that of the control, taproots from the fungicide and PO-I + II treatments showed significantly higher weight than the control.

3. Effect of applying PO to seedlings in paper pot nurseries on suppression of Aphanomyces root rot in the field

Next, a field experiment was conducted to examine the efficacy of different applications of PO oospores to seedlings in paper pot nurseries for controlling Aphanomyces root rot. The field was irrigated for about two months from the beginning of June to provide high soil moisture favorable for zoospore production and the migration of *A. cochlioides* from the scattered infested soil. Therefore, the average disease severity of the control was 2.62, which corresponded to a medium to heavy level of disease (Table 2). Disease severity of the PO-I treatment was lower than that of the control, but the difference was statistically insignificant. The PO-II and PO-I + II treatments significantly reduced disease severity as compared with the control. There was no difference in the rate of reduction of disease severity among the PO-I, PO-II, PO-I + II, and fungicidal treatments.

4. Rhizosphere colonization of PO on sugar beet plants cultivated in the field

The efficiency of PO colonization of the rhizosphere of sugar beet plants pretreated with oospores in the paper pot nurseries was determined by PCR 50 days after transplanting in the field. Although low levels of PO were detected by PCR in the rhizosphere soil of water control plants in field plot 1, there was no PCR product from the rhizosphere soil samples of water control plants in field

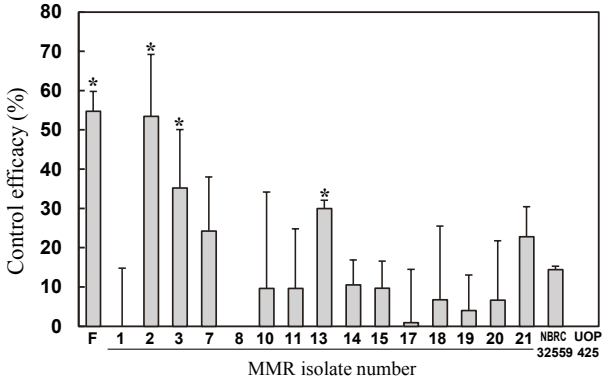

Fig. 1. Effect of application of oospore suspensions of *Pythium oligandrum* isolates or fungicide (F: hymexazol and validamycin A) on control efficiency regarding *Aphanomyces cochlioides* post-emergence damping-off on sugar beet

Disease severity was estimated 28 days after sowing in *A. cochlioides*-infested soil by an index explained in **Materials and methods**. Asterisks indicate significant differences (P<0.05) compared to the water control according to Dunnett 's one-sided *t* test. Control efficacies of different isolates were calculated with disease severity of the water control for each separate experiment.

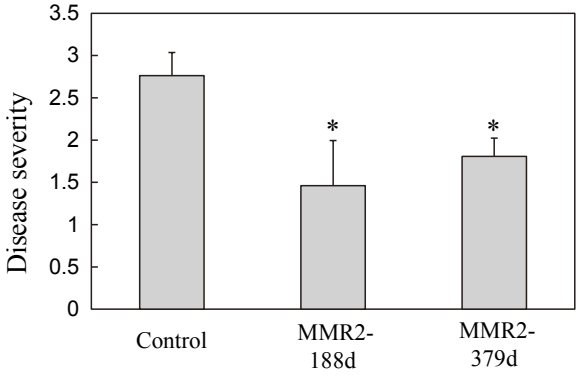

Fig. 2. Effect of water or oospore suspension of *Pythium oligandrum* isolate MMR2 stored at 4℃ for 188 days (MMR2-188d) or 379 days (MMR2-379d) on disease severity of *Aphanomyces cochlioides* post-emergence damping-off in sugar beet

Disease severity was estimated as an index explained in **Materials and methods**. Values are the means of three replicates and asterisks indicate significant differences (P<0.05) compared to the water control according to Dunnett's one-sided *t* test.

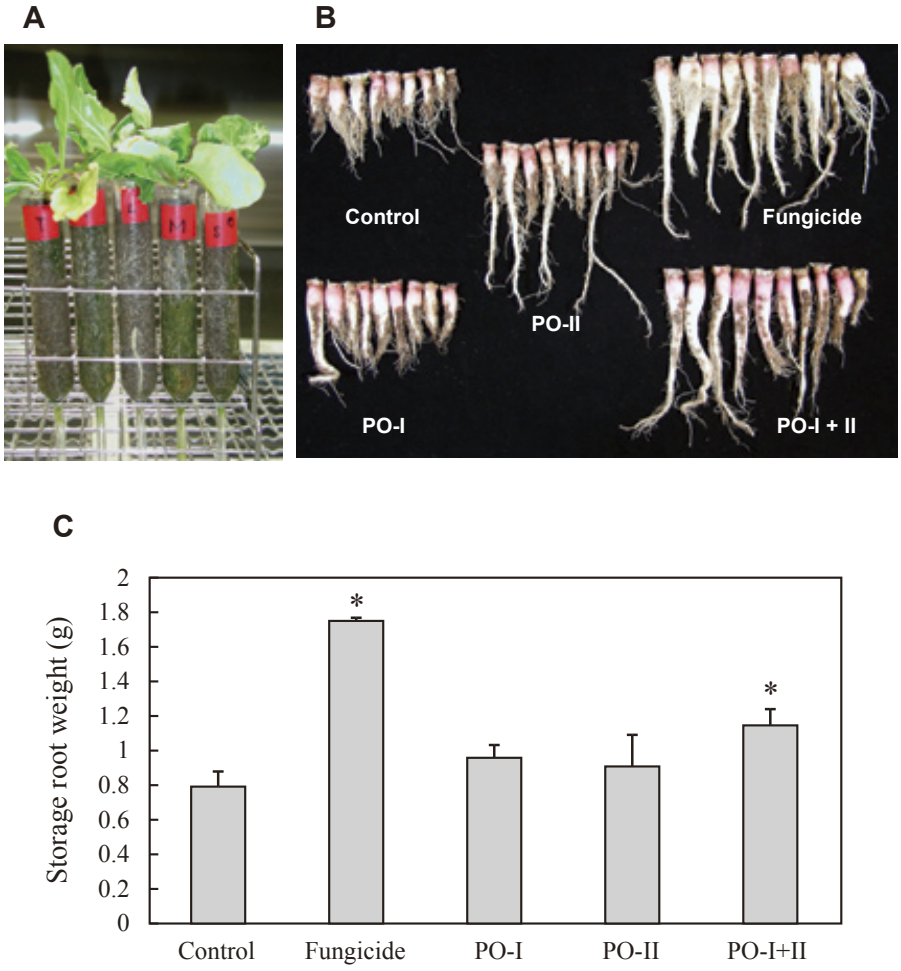

Fig. 3. Effect of treatment with *Pythium oligandrum* (PO) oospores on disease severity due to Aphanomyces root rot on sugar beet plants in an *in vitro* assay

Treatment with PO oospores was conducted by mixing PO oospore suspensions with surface soil in paper pot nurseries (PO-I) drenching seedlings with PO oospore suspensions 32 days before transplanting (PO-II), or using both applications (PO-I + II). The fungicide (fluazinam) treatment was conducted by drenching just before transplanting. (A) The treated seedlings were transplanted into glass tubes. (B) A representative sample of taproots from the assay conducted in glass tubes. (C) Average taproot weight was estimated for a comparison of disease severity. Values denoting the means of ten replicates and asterisks indicate significant differences ($P<0.05$) compared to the water control according to the least significant difference.

plot 2, or from fungicide-treated plants in either field plot (Fig.4). PO was detected in the rhizosphere soil samples of PO oospore-treated plants after PO-I, PO-II, and PO-I + II treatments. A PCR product (~380 bp) was strongly detected in rhizosphere soils from PO-I + II treatment in both field plots.

Discussion

Among the 17 PO isolates examined in this study, MMR2 showed the highest control efficacy for *A. cochlioides* post-emergence damping-off, when its oospore suspension was applied to soil in a tray seeded with commercial hymexazol-pelleted seeds of sugar beet. McQuilken et al[18]. had previously investigated the effect of PO on the control of *A. cochlioides* damping-off in sugar beet by coating each seed with oospores. Coating the seeds with PO oospores controlled the disease to a level equivalent to hymexazol seed-coating treatment at low inoculum potentials of the pathogen. However, neither PO nor hymexazol seed-coating adequately controlled disease at high inoculum potentials of *A. cochlioides*. Coating seeds with oo-

spores is less effective for controlling disease during high inoculum potential, and may result from a lower degree of root colonization by PO in the rhizosphere. PO populations may not easily increase in the rhizosphere during root expansion when inoculated by coating the surface of sugar beet seeds with oospores[17]. In this study, the combined treatments of drenching the soil with an oospore suspension and using commercial hymexazol-pelleted seeds effectively controlled *A. cochlioides* post-emergence damping-off even at relatively high inoculum potentials, where the average disease severity was 2.23, even when using commercial fungicide-pelleted seeds (Fig. 1). Therefore, drenching the soil with an oospore suspension is apparently more effective against post-emergence damping-off than seed treatment due to the improved distribution of oospores in the rhizosphere by direct application to the soil. Al-Rawahi and Hancock[1] reported that PO could colonize the rhizosphere of 11 crop species including sugar beet grown in PO-infested soil.

A long shelf life and minimal specialized storage requirements are desirable attributes for successful biocontrol agents. The study of the effect of storage on the biocontrol activity of PO indicates that the storage of oospore suspension at 4℃ for at least one year does not affect subsequent biocontrol activity (Fig. 2). The previous study by McQuilken et al[19]. showed that oospore inocula of this fungus can be stored for periods of at least one year at 15℃, with little loss of biocontrol activity against *P. ultimum* when subsequently used to coat cress seeds. These results indicate that PO oospores with great longevity would be appropriate for a commercial biofungicide. A biofungicide containing PO oospores has already been registered under the name Polyversom® *or* Polyversom™ and is prepared in the Czech Republic[5].

Table 2. Disease severity of Aphanomyces root rot on field-grown sugar beet plants treated with oospores of *Pythium oligandrum* (PO) or fungicide before transplanting

Treatment[1)]	Disease severity[2)]
Control (water)	2.62 ± 0.44[a]
Fungicide (fluazinam)	2.06 ± 0.47[b]
PO-I	2.28 ± 0.38[ab]
PO-II	1.88 ± 0.21[b]
PO-I + II	2.00 ± 0.41[b]

1) Treatment of PO oospores was conducted by mixing with the surface soil of paper pot nurseries (PO-I), drenching to seedlings 32 days before transplanting (PO-II), or using both applications (PO-I + II). The fungicide treatment was conducted by drenchting just before transplanting.
2) Disease severity of storage roots was estimated as the index explained in Materials and methods. Values denoting the means ± standard error values of four plots, followed by a different letter, are significantly different using Tukey's HSD test ($P < 0.05$).

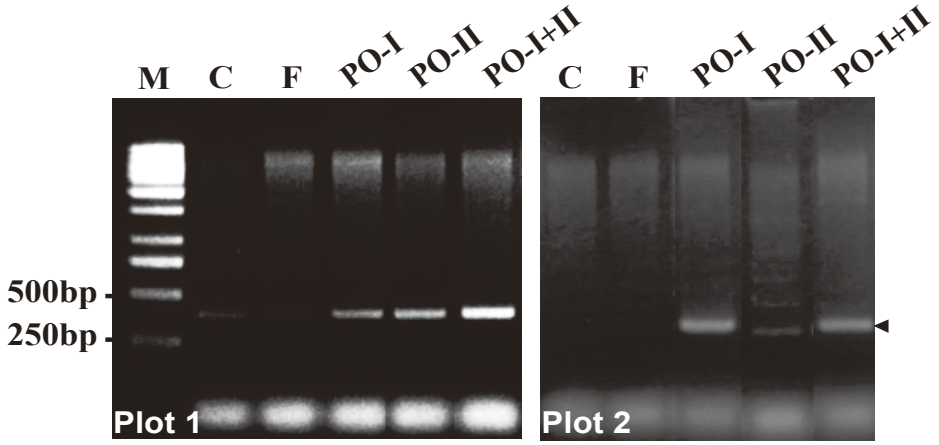

Fig. 4. Polymerase chain reaction (PCR) detection of *Pythium oligandrum* (PO) in rhizosphere soils from sugar beet plants cultivated in two plots (Plots 1 and 2) of a field for 50 days

Plants were pretreated with water (C); with fungicide, by drenching with fluazinam just before transplanting (F); with PO, by mixing PO oospore suspensions with surface soil in paper pot nurseries (PO-I); by drenching seedlings with PO oospore suspensions 32 days before transplanting (PO-II); or using both applications (PO-I + II). The arrowhead indicates PCR products of PO DNA fragments (ca. 380 bp). M is a DNA standard marker (Sigma-Aldrich Japan, Tokyo, Japan).

The field test showed that applications of PO oospores to the paper pot nurseries (PO-II and PO-I + II) also conferred significant control of Aphanomyces root rot in the field without additional applications (Table 1), with an efficacy level similar to that of fluazinam - a representative registered fungicide used for Aphanomyces root rot in Japan. Although the PO-II treatment did not significantly reduce disease severity as compared with the water control during *in vitro* assay, the PO-I + II treatment showed effective control in the same assay. These results suggest that combined treatment by both mixing PO oospores with surface soil in paper pot nurseries and drenching seedlings with PO oospore suspensions is an effective disease-control alternative to the use of chemical fungicides. Other PO oospore application methods are worthy of study and development to improve the control of Aphanomyces root rot in the field. This study apparently represents the first published observation that PO can control Aphanomyces root rot in naturally infested soil, and demonstrates a rare example of biocontrol for the disease. These results suggest that PO has the potential to control both post-emergence damping-off and root rot caused by *A. cochlioides*.

Aphanomyces root rot usually occurs from late June to the end of growing season, but the pathogen typically infects the lateral roots by the end of May, about one month before the first symptoms appear[29]. It is likely that PO must have already colonized the rhizosphere of sugar beets at this stage for protection against infection by *A. cochlioides*. The PCR method used here indicated that PO had colonized the rhizosphere of sugar beet plants pretreated with oospores by 50 days after the seedlings were transplanted in the field (Fig. 4). The PCR product characteristic of PO was strongly detected in rhizosphere soils from the PO-I + II treatment in two field plots. Low levels of PO were detected in the rhizosphere soil of the water control in field plot 1, indicating the likely presence of an indigenous PO isolate. This would not be unexpected as the soil oomycete PO occurs rather commonly in various climates, and was originally isolated from cultivated soils[30]. The biocontrol exerted by PO is a complex process, and appears to be both pathogen- and host plant-dependent through both direct effects (e.g., mycoparasitism with or without antibiosis, competition for colonization) and indirect effects (e.g., stimulation of plant defense reactions, promotion of plant growth)[23]. Dual culture tests on the interactions between PO and *A. cochlioides* demonstrated that hyphal interactions such as coiling and penetration by PO were not observed (Takenaka, *unpublished data*). Other important factors include competition for space and nutrients, and the triggering of active defense reactions in root tissues. Martin and Hancock[17] reported that early colonization of sugar beet seeds by PO apparently protected seedlings against subsequent infection by *P. ultimum*, and was more likely due to competition for space and nutrients. Our group has shown that the cell wall protein fraction (CWP) of PO contains high levels of elicitor proteins (POD-1 and POD-2), and that the application of these elicitor proteins to sugar beet plants induced plant defense reactions[24,25,27], and increased resistance against seedling diseases caused by *A. cochlioides*[25] and *R. solani* AG2-2[24], and against Cercospora leaf spot caused by *Cercospora beticola*[27]. Taken together, these results suggest that the primary mechanisms by which PO protects roots from infection by *A. cochlioides* include early colonization of the rhizosphere prior to pathogen establishment, resulting in competition with the pathogen for space and nutrients, and the subsequent triggering of active defense reactions in root tissues. Further experimentation should be conducted in order to test these hypothesized mechanisms.

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