

拮抗性Pseudomonas fluorescens HP72を用いたベントグラス葉腐病の生物防除とHP72の野外でのモニタリング

誌名	日本植物病理學會報 = Annals of the Phytopathological Society of Japan
ISSN	00319473
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発行元	日本植物病理學會
巻/号	63巻6号
巻号補足	
掲載ページ	p. 437-444
発行年月	1997年12月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council
Secretariat



Biological Control of *Rhizoctonia solani* AG2-2 IIIB on Creeping Bentgrass Using an Antifungal *Pseudomonas fluorescens* HP72 and Its Monitoring in Fields

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Abstract

Bacteria which show antifungal activity were isolated from roots of turfgrass and associated soil. Among the bacteria, a fluorescent pseudomonad (strain HP72) which was identified as *Pseudomonas fluorescens* showed strong antifungal activity and was selected as a candidate for biological control of golf course turfgrass pathogens. Enumeration methods, established to monitor the bacteria in soil, were based on the detection of a DNA fragment (ca. 800 base pairs) which was very specific to strain HP72. The methods called FIA-PCR and MPN-PCR gave very good results in a model inoculation test and were applied to laboratory sod and field survival experiments. Because a background of indigenous bacteria having the same DNA fragment as strain HP72 was not detected in soils used for these experiments, strain HP72 was easily enumerated. When soil temperature was lower than 35°C, the bacteria survived for at least 20 days (ca. 10⁵ cfu per gram soil and roots). However, if soil temperature rose higher than 43°C for 2 hr per day, the bacteria died completely within 7 days. The ratio of strain HP72 to the total fluorescent pseudomonads on turfgrass roots increased up to 5 days after inoculation and was higher than 80% of total fluorescent pseudomonads for at least 20 days. In each greenhouse experiment, creeping bentgrass developed significantly less disease when inoculated with HP72 before inoculation with *Rhizoctonia solani* than when inoculated with the pathogen alone. The level of control provided by HP72 was as good as that by a widely used isoprothiolane-flutolanil fungicide.

(Received March 17, 1997 ; Accepted August 28, 1997)

Key words : *Pseudomonas fluorescens*, *Rhizoctonia solani* AG2-2 IIIB, biological control, PCR, monitoring.

INTRODUCTION

Because increasing fungicide inputs to highly managed golf course turf in Japan attract public concern over the potential for contamination of drinking water, biological control is an attractive means of reducing these inputs^{2,20}. Fluorescent pigment-producing pseudomonads have been used to biologically control plant pathogens^{5,11,13,18,21,23,25} because they readily colonize plant roots and some produce antifungal chemicals. As they are usually applied to plants by seed bacterization or root-dip treatments^{5,11,18,21,25}, these methods can be used when a new golf course is constructed. For established golf courses, "on-site" application is necessary. Repeated on-site application is also necessary for previously bacterized turf to maintain a high bacterial population. However, on-site application has not been

adopted because efficacy assessment of the application is difficult. Enumeration of bacteria in the field, therefore is very important. Frederickson *et al.*⁷ enumerated Tn5-marked pseudomonads. However, the method is not applicable for field applications because environmental release of genetically modified bacteria has not yet been accepted in Japan. Enumerating the introduced bio-control agents by a selective marker such as antibiotic resistance is possible⁴. However, evaluating the loss of the marker and the populations of indigenous strains with possessing resistance to the selected marker is difficult for this method. Khan and Cerniglia¹⁴ used a specific DNA fragment for detecting *Pseudomonas aeruginosa* and Wang *et al.*²⁴ used a 16S rRNA-based probe. However, the detection methods by Khan and Cerniglia¹⁴ and Wang *et al.*²⁴ were not strain-specific.

Rhizoctonia solani, which causes large patch or brown patch disease, is one of the most destructive pathogens

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of turfgrass¹⁷⁾. The disease is widespread geographically and affects both warm- and cool-season turfgrasses. Numerous studies have been attempted to develop effective methods for the biological control of plant disease caused by *R. solani*^{1,6,10,15,19)}. However, few effective biocontrol agents have been reported for turfgrass disease caused by *R. solani*. Brown patch on creeping bentgrass was inhibited by avirulent binucleate *Rhizoctonia* spp., but the inhibitory effects continued only for short periods³⁾. Yuen used *Rhizoctonia* GM460 and *Gliocladium* TRGB for biological control of *R. solani* on tall fescue²⁷⁾.

This paper deals with screening microorganisms for their ability to control brown patch disease of turfgrass caused by *R. solani*. In the present study strain HP72, which was identified as *P. fluorescens*, was selected for its strong antifungal activity, enumerated of strain HP72 and evaluated for its biocontrol activity on turfgrass disease caused by *R. solani*.

MATERIALS AND METHODS

Isolation of bacteria with antifungal activity

Fluorescent pseudomonads were isolated from roots of creeping bentgrass, grown on brown patch-suppressive soil. Selection of fluorescent pseudomonads was carried out on P-1 agar (containing KH_2PO_4 , 0.1%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005%; KCl , 0.02%; $(\text{NH}_4)_2\text{SO}_4$, 0.08%; betaine, 0.2%; agar, 1.5% and adjusted pH to 7.2–7.4)¹²⁾. Fungal pathogens were isolated from diseased golf course turf on potato dextrose agar (PDA) (Difco Laboratories, Detroit, Mich.). Isolated pathogens, identified as *R. solani* AG2-2 IIIB, *Sclerotinia homoeocarpa*, *Pythium aphanidermatum* and *Gaeumannomyces graminis*, were used in assays to estimate the antifungal activity of fluorescent pseudomonads. Antifungal activity was estimated by the concomitant cultivation of fluorescent pseudomonads with fungal pathogens on PDA plates as described by Weller²⁵⁾. Bacteria were cultured in potato dextrose broth overnight at 28°C, before spotting 10 μl (ca. 8×10^6 cfu) of bacteria on agar plates at 1 cm from the plate edge. After 2 days incubation at 28°C, a 1.0-cm plug from a fungal culture grown on a PDA plate at 28°C for 3 to 7 days was placed in the center of the plate. The plates were incubated at 28°C and fungal growth inhibition was assessed after 3 to 7 days by measuring the distance between the edge of the bacterial colony and the fungal mycelium. A strain which showed very strong antifungal activity to *R. solani* AG2-2 IIIB was selected after repeated assay. The strain (HP72) was identified as *P. fluorescens* based on morphological, physiological and biochemical characterizations, and was maintained on a King's B slant.

Selection of a strain-specific DNA fragment from *P. fluorescens* HP72 A strain-specific DNA fragment from HP72 was selected by the random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) method^{2,8,9)}. For the RAPD PCR, 20 randomly

designed primers with eight or nine nucleotides were synthesized with an Applied Biosystems model 380A DNA synthesizer. Bacterial DNA was extracted using the phenol extraction method²²⁾. RAPD PCR was carried out in a 50 μl reaction mixture containing 50 ng genomic DNA, 50 mM Tris (pH 8.3), 1.25 mM MgCl_2 , 0.02 mM each deoxy nucleotide triphosphate, 0.1% gelatin, 400 pmol of each primer, and 2.5 units of *Taq* polymerase (Perkin Elmer, Inc., USA). Amplification was performed in a thermal cycler (Perkin Elmer model 480) programmed for one cycle of 4 min at 94°C, 2 min at 35°C, and 3 min at 72°C, followed by 45 cycles of 1 min at 94°C, 1 min at 35°C, and 2 min at 72°C, and a final incubation at 72°C for 5 min. After amplification, 12.5 μl of product was electrophoresed in 1.0% agarose gel and visualized by ethidium bromide staining. To establish the specificity on the DNA fragment, *P. fluorescens* IAM 12001, *P. fluorescens* IAM 12022 (type strain of *P. fluorescens*) and 60 fluorescent pseudomonads isolated from soil used for growing creeping bentgrass were analyzed by RAPD PCR. The specificity of amplification was estimated by comparing the size of the DNA product from HP72 to products amplified from the DNA of control strains. Primer NSRP-6 (5' CGCGTCCAC) produced a DNA fragment of approximately 800 base pairs which was specific to strain HP72. This fragment was cloned into Blue T7 vector (Novagen, Inc., Madison, Wisc., USA). The nucleotide sequence was determined by the dideoxy nucleotide chain termination method using a dye terminator sequencing kit (Applied Biosystems Inc., Foster City, USA) Based on the determined sequence, the following specific primers were designated: primer RP6HP72-1, GCCAATCACGCCCTCAAGCT; and primer RP6HP72-2, GGCAACTGCACAAGCGCCA. The specific DNA fragment of approximately 800 bp was amplified using these primers.

Strategy for Enumeration of *Pseudomonas fluorescens* HP72 Two methods of enumeration were performed. The first procedure was designated the most probable number (MPN)-PCR method. Soil samples (5.0 g) were suspended in 45 ml sterile water, and a 10-fold dilution series was prepared. For root samples, the roots were washed ten times with sterilized water, well macerated with a mortar and pestle, and suspended in sterile 0.01 M phosphate buffer (pH 6.7). One hundred μl of each diluted soil suspension was added to 1 ml of P-4 liquid medium (KH_2PO_4 , 0.1%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005%; KCl , 0.02%; $(\text{NH}_4)_2\text{SO}_4$, 0.08%; inositol, 0.2%; pH to 7.2–7.4)¹³⁾, and incubated for 2–3 days at 28°C. The grown cells were collected by centrifugation ($10,000 \times g$ for 1 min), and the pellet was resuspended in 50 μl sterile water and incubated for 10 min at 95°C. One μl of the solution was used for the PCR reaction. The amplification was performed in a thermal cycler programmed for 1 min at 95°C, followed by 30 cycles at 94°C, 20 sec at 62°C and 90 sec at 72°C, with a final incubation at 72°C for 5 min. After PCR, the size of the amplified DNA was compared with the DNA from HP72 by subjective

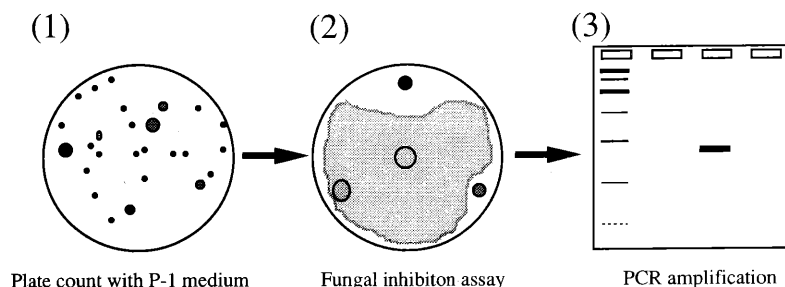


Fig. 1. Scheme of FIA-PCR method. In step (1) appropriately diluted soil or root suspension was poured onto a P-1 agar plate, for incubation at 28°C for 3 days. In step (2) strains which showed fungal growth inhibitory activity were selected for PCR amplification (step (3)) using primers RP6HP72-1 and RP6HP72-2. When the specific DNA fragment was amplified, the strain was identified as strain HP72.

amplified fragments to gel electrophoresis. After amplification for five replicates of each dilution, the number of cells was inferred from the most probable number for three suitable dilutions.

The second procedure, FIA (fungal inhibition assay)-PCR, is illustrated in Fig. 1. An appropriately diluted soil or root suspension was poured onto a P-1 agar plate, and the plates were incubated at 28°C for 3 days. All bacteria, which were growing at the highest dilution plates, were subjected to the fungal growth inhibition assay. If they did not exhibit fungal growth inhibitory activity similar to that of HP72, we used bacterial colonies growing on plates of lower dilution. The strains which showed the same degree of fungal growth inhibition as HP72 were subjected to PCR amplification with the specific primers RP6HP72-1 and RP6HP72-2. When the specific DNA fragment was amplified, the strain was identified as strain HP72. In this PCR reaction, cells were harvested from each colony with a sterile needle, suspended in sterile water, then incubated at 95°C for 10 min to release the DNA. Conditions for the amplification reaction and the method for detection of a specific DNA fragment were the same as those described for MPN-PCR.

Evaluation of the two enumeration methods

Cells of strain HP72, which were counted on a P-1 plate, were added to non-sterile soil, then the cell numbers were counted by the two methods as soon as possible with five replicates. Soil used was a sandy soil from a putting green (pH 6.7, total nitrogen 0.12%, and total carbon 1.98%) with 1.6×10^6 cfu/g of indigenous fluorescent pseudomonads as background. The target DNA fragment was not detected in the background fluorescent pseudomonads.

Conditions for inoculation with *P. fluorescens* HP72

The bacterial cells were added to soils as a compost-zeolite mix (CZ-HP72 mix) which was prepared as follows. Strain HP72 was grown in 200 ml King's B broth for 2 days at 28°C. Cells were collected by centrifugation ($10,000 \times g$ for 10 min) and suspended in 100 ml phosphate buffered saline (PBS)⁽²⁰⁾. The bacterial suspension was mixed well with $4000 \times g$ of autoclaved (120°C for 1 hr) compost-zeolite mixture (containing

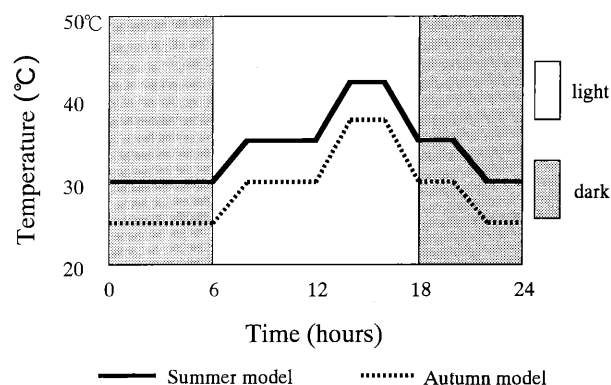


Fig. 2. The growth condition cycle for sod experiment. Light: 15,000 lux.

compost, zeolite, and water at a ratio of 1:2:1). After mixing with a mortar mixer, the material was incubated at 28°C for 3 days and dried under warmed air (40°C) until water content reached 10%.

Survival of *P. fluorescens* HP72 in laboratory sod Creeping bentgrass sod (ca. 100 cm²) was removed from the putting green of a golf course and grown in a growth chamber. These turfs contained ca. 10^6 cfu/g indigenous fluorescent pseudomonads. Each turf was mowed to a height of 7 mm. Growth conditions were based on model of temperature fluctuations during the summer and autumn in the Kanto area of Japan (Fig. 2). CZ-HP72 was applied on top of the turf on the first day to segments of the sod at the rate of 150 g/m² with approximately 7.2×10^7 cfu/g. Ten sod segments were used for each condition. For each measurement the numbers from two sod segments were averaged. Soil samples were removed from the sod with a 1.0-cm-diameter cork borer. The soil core was ca. 1.5 cm deep and contained leaves, roots, rhizomes, thatch and soil. The samples were processed within 2 hr. Soils were gently teased from roots until only a small amount of adhering rhizosphere soil remained. The roots were washed with excess sterile water 10 times, macerated with a mortar and pestle, then enumerated by MPN-PCR and FIA-PCR methods after 0, 1, 2, 4, 7 and 14 days incubation.

Survival of *P. fluorescens* HP72 in the field

Field plots were established in 1994 on a creeping bentgrass putting green at the Hitotonoya Country Club golf course (Tochigi, Japan). The putting green was about 3 years old, constructed on soil and mowed to a height of 5 mm. In 1994, this green received a total of approximately 22 g/m² nitrogen-fertilizer input. The soil used for the survival test contained approximately 3×10^6 cells of fluorescent pseudomonads (measured on P-1 plates). The DNA fragment specific to strain HP72 was not detected in the indigenous bacteria. For the summer test, a *P. fluorescens* HP72 inoculum was applied on August 24 to 5.0 m² plots at the rate of 150 g/m² with three replicates. Inoculum bacteria were applied once with CZ-HP72 containing 6.4×10^7 cells/g. The inoculum was distributed by hand as uniformly as possible over the plot area and lightly rubbed to distribute it into the turf canopy. Several plots to which no *P. fluorescens* HP72 inoculum was applied were used as controls. All plots were irrigated by applying 0.5 cm of tap water. At each sampling, 10 cores were removed at random from each of the replicate plots with a 1.0-cm-diameter cork borer. Cores were removed to a depth of ca. 1.5 cm, transported to the laboratory, and enumerated within 4 hr after collection. Enumeration of HP72 was carried out by the same procedure as described in the laboratory sod test. Survival of HP72 was assessed using MPN-PCR and FIA-PCR methods after 0, 1, 2, 4, 7, and 14 days. For the autumn test, inoculum of *P. fluorescens* HP72 inoculum was applied on October 11, 1994. *P. fluorescens* HP72 had approximately 4.5×10^7 cells/g. Bacterial numbers were measured after 0, 1, 2, 4, 7, 14 and 20 days.

Use of *P. fluorescens* HP 72 for biological control of turfgrass pathogen

Biocontrol activity on brown patch by strain HP72 was assessed in pot and sod segment tests. In pot tests bentgrass seed was surface sterilized by immersion in 70% ethanol for 5 min, rinsed in sterile distilled water, then dried overnight under a sterile air stream. Strain HP72 cells were grown in 500 ml liquid King's B medium for 3 days at 28°C, collected by centrifugation ($5000 \times g$, for 10 min), suspended in 30 ml of 1.0% methylcellulose and mixed with 10 g bentgrass seeds. The bacterized seeds were placed in petri plates as a thin layer, dried overnight under a sterile air stream, and were separated very well prior to planting. The bacterized seeds contained approximately 10^7 – 10^8 cells per gram. Bentgrass was seeded at approximately 10 g/m² into plastic trays 15 cm wide $\times$ 22 cm long $\times$ 6 cm deep containing 95% (w/w) fine sand and 5% (w/w) vermiculite and maintained at a height of approximately 1.5 cm. Fertilization and irrigation were similar to those used for bentgrass golf course putting greens¹⁶. Pots were placed in a growth chamber, and maintained at 25°C with a 12-hr photoperiod for 45–50 days.

R. solani strain AG2-2 III B was cultured on a sterile solid material which was made of equal weights of bran and vermiculite and contained ca. 50% moisture. The

cultures, for 5–7 days at 28°C, were used as inoculum for the pot test, by placing a block (approximately 5.0 g) of above mentioned inoculum below the sand surface at the center of the pot. Then, the pots were placed in plastic bags to keep humidity high and to prevent moisture loss during incubation in a growth chamber at 28°C with a 12-hr photoperiod. Non-inoculated bentgrass was used as a control. The fungicide, isoprothiolane-flutolanil mixture (Nihon-nohyaku Inc.) containing 25% isoprothiolane and 20% flutolanil, was applied to the turf as a drench at 2.0 g/m².

For sod tests, HP72 was inoculated onto turf with the CZ-HP72 mix at least 7 days before *R. solani* inoculation. The same procedures as used for the pot tests were carried out to evaluate biocontrol activity.

In sod and pot tests disease severity was measured after 7 and 15 days and rated on a scale from 0 to 9, for which 0=no disease, 1=less than 5%, 2=5 to 10%, 3=11 to 20%, 4=21 to 30%, 5=31 to 40%, 6=41 to 50%, 7=51 to 65%, 8=66 to 80%, and 9=over 80% brown patch. Each experiment contained five replicates and was repeated at least three times. Data were analyzed by Duncan-Waller Bayesian least significant difference test²⁷.

RESULTS AND DISCUSSION

Selection of a specific DNA fragment by RAPD PCR

Among 20 primers tested, the primer NSRP-6 (5'-CGCGTCCAC) amplified a DNA fragment specific to *P. fluorescens* HP72 (Fig. 3). The target DNA was approximately 800 base pairs. PCR primers, RP6HP72-1 and RP6HP72-2, which were used to detect of the DNA fragment, were designed based on the sequence of the target DNA fragment. When the primer annealing temperature was 62°C, DNA with the same size was not detected from *P. fluorescens* IAM 12001, *P. fluorescens* IAM 12022, and more than 1000 isolates of fluorescent pseudomonads from sandy green of several golf courses. No target DNA was found in indigenous fluorescent pseudomonads isolated from several golf courses.

Evaluation of the two enumeration methods

Strain HP72 (3.72×10^3 , 4.71×10^4 , 5.56×10^5 , 6.62×10^6 and 7.46×10^7 cfu/ml) was added to non-sterile soil, then the cell numbers were counted by the two methods (Table 1). The lowest detection limit of the FIA-PCR method was 10^4 , as opposed to a limit of 10^3 for the MPN-PCR method. Each method recovered more than 85% of total inoculated cells.

Both the FIA-PCR and the MPN-PCR methods provided satisfactory results for enumerating strain HP72. Although the FIA-PCR method is more time consuming than MPN-PCR, the FIA-PCR method more closely agreed with plate count data (Table 1). This fact is quite reasonable because identification for the FIA-PCR is based on the plate count. Furthermore, the FIA-PCR method makes good use of both phenotype (FIA) and

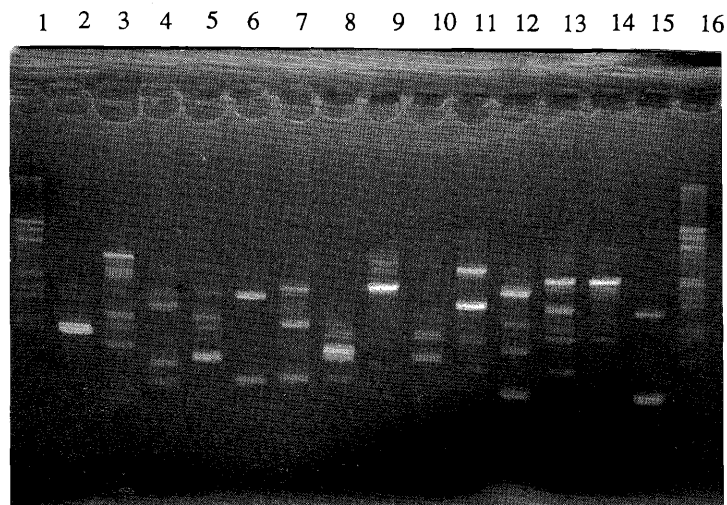


Fig. 3. Comparison of amplified DNA with primer NSRP-6 among fluorescent pseudomonads. Lanes 1 and 16 are size markers (*Eco*RI and *Hind*III cut of lambda phage DNA). Lane 2, *P. fluorescens* HP72; lane 3, *P. fluorescens* IAM 12001; lane 4, *P. fluorescens* IAM 12022; and lanes 5 to 15, fluorescent pseudomonads isolated from sandy soil of a golf course.

Table 1. Evaluation of most probable number (MPN)-PCR and fungal inhibition assay (FIA)-PCR methods for counting the cell numbers of HP72

Method	Log. of HP72 cells g ⁻¹ dry weight of soil samples				
	Soil No. 1	Soil No. 2	Soil No. 3	Soil No. 4	Soil No. 5
FIA-PCR	—	4.66 (0.25)	5.72 (0.12)	6.76 (0.07)	7.32 (0.17)
MPN-PCR	3.18 (0.41)	4.35 (0.33)	5.36 (0.20)	6.28 (0.22)	7.19 (0.13)
Plate count	3.72 (0.11)	4.71 (0.12)	5.56 (0.08)	6.62 (0.15)	7.46 (0.09)

Numbers in parentheses refer to standard deviations of the log values.

— means not tested.

Experiments were performed for five replicates of each samples and three times.

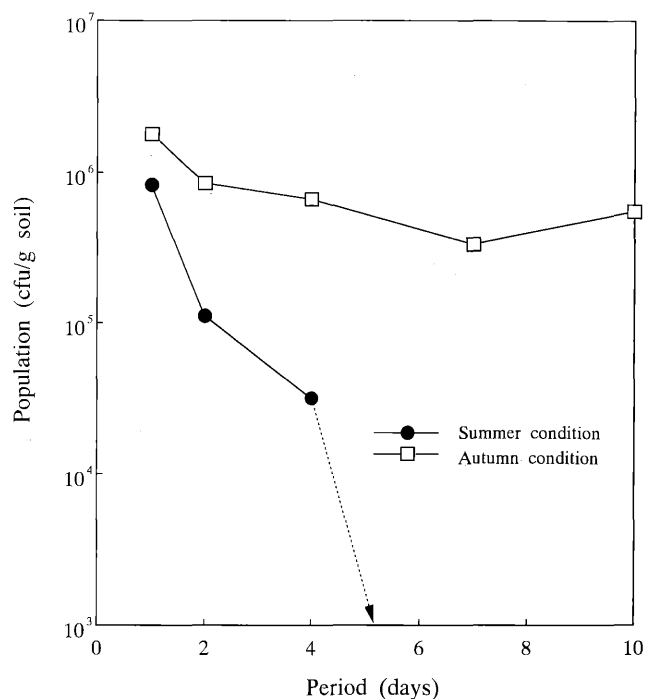


Fig. 4. Survival of *P. fluorescens* HP72 in surface soil in laboratory sod test ---> means HP72 was not detected.

genotype (PCR). Detection by the MPN-PCR method, however, is based only on genotype. Therefore, if indigenous wild strains in a soil sample have the same DNA fragment, the method can not be used.

Because the FIA-PCR and MPN-PCR methods will not be effective, if wild strains having the same genetic arrangement are ubiquitously distributed in soils, we looked for strain HP72 in several kinds of soils. HP72-like bacteria were not detected in any of the tested soil samples. We isolated more than 1000 fluorescent pseudomonads during the screening of effective antifungal bacteria; from none of these did we amplify that specific DNA fragment. We are now trying to isolate the same strain from various kinds of soil and plant roots in order to reveal the ecological importance of the strain. However, we have never found the target DNA fragment in the indigenous fluorescent pseudomonads isolated from sandy soil from several golf course greens.

Survival of *P. fluorescens* HP72 in laboratory sod

The survival of strain HP72 in laboratory sod is shown in Fig. 4. When the sods were maintained at summer model temperatures, the bacteria were not detected after 7 days. When the sods were maintained under the autumn model, however the bacteria survived

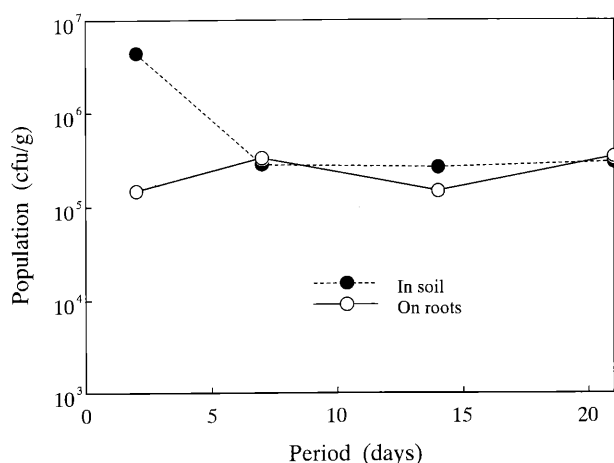


Fig. 5. Survival of *P. fluorescens* HP72 in surface soil and on the turfgrass roots under the autumn model conditions in laboratory sod test.

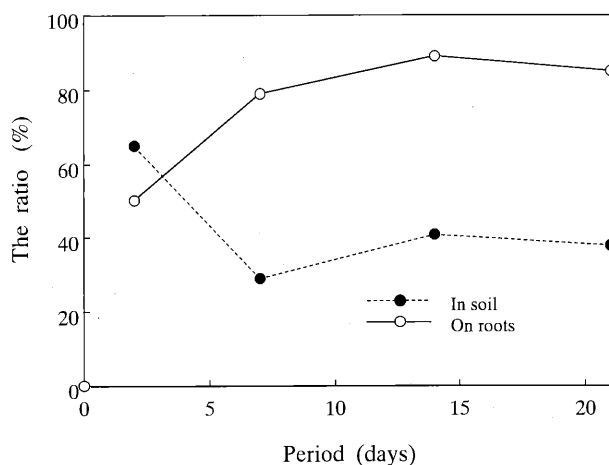


Fig. 6. Ratio of *P. fluorescens* HP72 to the total fluorescent pseudomonads in surface soil and on the turfgrass roots under the autumn model condition at laboratory sod test.

at more than 10^5 cells per gram dry soil for at least 20 days. We have determined that *P. fluorescens* HP72 dies quickly at temperatures over 43°C (data not shown) in soil. In the experimental model for summer, the maximum temperature of sod reached over 43°C ; strain HP72 probably died quickly.

The population dynamics of the strain under autumn conditions is shown in Fig. 5. The population size on roots was almost the same as the population size in soil. The ratio of HP72 to the total fluorescent pseudomonads under autumn conditions was much higher on roots than that in soil (Fig. 6). Strain HP72 is a rhizobacterium²⁶⁾ for which a large population on roots is considered to be reasonable. However, the ratio of strain HP72 on roots to the total fluorescent pseudomonads is extremely high (over 80%). Such a high ratio is somewhat unexpected because the flora of rhizobacteria would be expected to be stable and not disturbed so drastically by inoculation with an introduced microorganism.

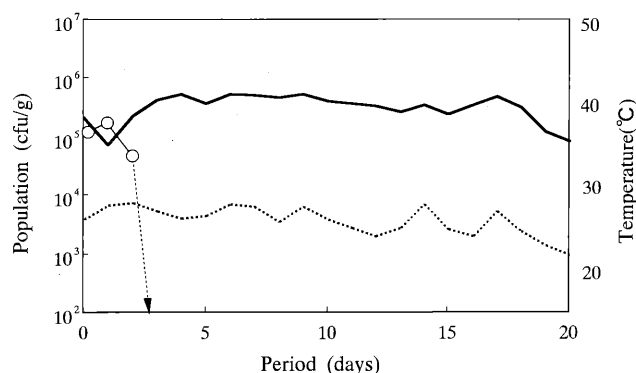


Fig. 7. Survival of *P. fluorescens* HP72 (○) in surface soil in summer field test (maximum temperature, —; minimum temperature, ----).

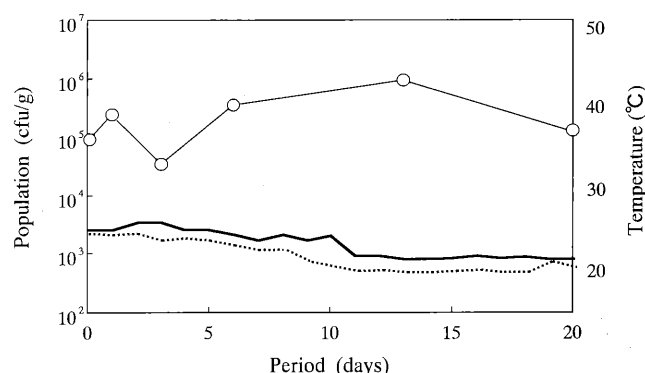


Fig. 8. Survival of *P. fluorescens* HP72 (○) in surface soil in autumn field test (maximum temperature, —; minimum temperature, ----).

Survival of *P. fluorescens* HP72 in the field

In a summer survival test (Aug. 24–Sept. 12, 1994), strain HP72 died within four days after inoculation (Fig. 7). For the survival test we collected the upper 2 cm of soil. Because the recorded temperature at a depth of 3 cm was over 35°C under natural sunlight, the surface temperature must be over 43°C . In summer experiment, strain HP72 may die before they can reach and survive in the rhizosphere. When tested in autumn (Oct. 11–Nov. 1, 1994), the strain survived at a population higher than 10^5 per gram soil for 20 days (Fig. 8). The temperature of the soil remained below 25°C .

P. fluorescens HP72 for biological control of turfgrass pathogens

Prior to evaluating biocontrol activity by strain HP72, we determined that the brown patch on turfgrass was actually caused by *R. solani*; *R. solani* was isolated from all turf which was inoculated and diseased (data not shown). In pot tests, a total of 15 pots of turf were cultivated for each treatment. Turfgrass from strain HP72-inoculated seeds developed significantly less (Duncan multiple range test, $p=0.05$) brown patch than non-treated plants (Table 2). After 15 days as much as 68% control of *R. solani* disease on creeping bentgrass

Table 2. Number of *P. fluorescens* HP72 and effect of HP72 and isoprothiolane-flutolanil fungicide mixture on the suppression of brown patch of creeping bentgrass in growth chamber experiments

Experiment system	Treatment	HP72		Disease rating ^{W)}	
		Number ^{U)}	Ratio (%) ^{V)}	After 7 days	After 15 days
Pot tests	<i>R. solani</i>	N.D. ^{Z)}	—	6.8a ^{X)}	7.9a
	<i>R. solani</i> + isoprothiolane-flutolanil ^{Y)}	N.D.	—	0.6b	1.1b
	<i>R. solani</i> + <i>P. fluorescens</i> HP72	27.4	95	1.1b	1.8b
	<i>P. fluorescens</i> HP72	15.6	100	0.0c	0.0c
	Non-inoculated control	N.D.	—	0.0c	0.0c
Sod tests	<i>R. solani</i>	N.D.	—	7.2a	8.6a
	<i>R. solani</i> + isoprothiolane-flutolanil ^{Y)}	N.D.	—	1.1bc	2.4b
	<i>R. solani</i> + <i>P. fluorescens</i> HP72	5.8	92	1.7b	2.0b
	<i>P. fluorescens</i> HP72	6.7	83	0.5c	0.8c
	Non-inoculated control	N.D.	—	0.3c	1.2bc

U) Number of HP72 on roots (cfu $\times$ 105 per 1.0 g of roots) after 15 days.

V) The ratio of HP72 on roots to the total fluorescent pseudomonads (%).

W) Based on a scale of 0-9, for which 0=no disease and 9=over 80% brown patch.

X) Numbers followed by the same letter are not significantly ($p=0.05$) different according to Duncan-Waller Bayesian least significant difference test. Each values is the mean of five replications.

Y) Pots and sods drenched with isoprothiolane-flutolanil (Grassten).

Z) Not detected.

was obtained with HP72 in contrast to non-treated pots. The fungicide controlled up to 76% *R. solani* disease in contrast to non-treated pots. So the level of biological control provided by strain HP72 was significantly (Duncan multiple range test, $p=0.05$) as effective as that provided by the isoprothiolane-flutolanil fungicide. Strain HP72 survived on roots at a population greater than 10^6 cfu per 1.0 g of fresh roots for 55 days after seeding. Apparent differences between sod tests and pot tests were not found (Table 2). *P. fluorescens* HP72 is not pathogenic for creeping bentgrass, because the treatment with HP72 alone did not damage creeping bentgrass as did the treatment of non-inoculated *R. solani* (data not shown).

The number of bacteria that can be maintained on healthy turfgrass roots is limited due to the finite amount of available nutrients. On bentgrass roots in the field, the limit to the number of fluorescent pseudomonads is approximately 10^5 to 10^6 cells per gram of root. Because of the high ratio of strain HP72 to the total fluorescent pseudomonads on the turfgrass roots, we expected strain HP72 to cause some effect on the growth of brown patch fungus and turfgrass.

We thank Dr. T. Ichitani, Green Research Center, Takarazuka, for valuable advice during this work and Dr. K. Kageyama, Lab. of Plant Pathology Faculty of Agriculture Gifu University, who identified *R. solani* AG 2-2 IIIB. We are indebted to Dr. L.S. Thomashow, Root Disease and Biological Control Research Unit, ARS, USDA, Pullman, for the critical reading of the manuscript.

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和 文 摘 要

村上浩二・神前 健・岡田憲三・松本 聡・小柳津広志：拮抗性 *Pseudomonas fluorescens* HP72 を用いたベントグラス葉腐病の生物防除と HP72 の野外でのモニタリング

芝草の根や根圏土壌より糸状菌に対し拮抗性を有する細菌を分離した。それらの中で、*Pseudomonas fluorescens* と同定された HP72 株は特に強い拮抗性を示し、ゴルフ場の芝草病害を生物防除可能な菌として選抜された。また、HP72 株に特異的な約 800 bp の DNA 断片を用いて土壌中での挙動をモニタリングする方法を開発し、FIA-PCR 法、MPN-PCR 法と名付けた。モデル実験において、HP72 菌株を土壌より回収できることを確認した。これらの方法を用いて実験室内の芝草ソッドやゴルフ場のグリーンでの HP72 の生残性を調べた。実験に供した土壌中の土着菌の中には HP72 株に特異的な約 800 bp の DNA 断片を持つものは検出されなかったために、HP72 株は容易にモニタリングできた。地温が 35°C 以下の時は、少なくとも 20 日間は比較的高い菌数で生残した。また、HP72 は土壌も接種後 5 日以内に根面の全蛍光性 *Pseudomonas* の 80% 以上を占め、この状態は少なくとも 20 日間は続いた。一方、1 日の中で 2 時間以上地温が 43°C 以上に達する季節では、7 日以内に検出限界以下に減少した。室内実験において HP72 株が根に良好に定着しているとき、*Rhizoctonia solani* によるベントグラスの葉腐病に対して化学農薬の isoprothiolane-flutolanil と同等の防除効果が認められた。