Original Article

Fate of *Xanthomonas campestris* pv. *poae*, a Biological Control Agent for Annual Bluegrass, in Soil

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(Received March 17, 1997; Accepted July 2, 1997)

The fate of a rifampicin tolerant mutant (strain Rif-482) of *Xanthomonas campestris* pv. *poae* JT-P482 in soil and the effect of strain Rif-482 on soil microorganisms were examined. Two mascarene grass turf fields were used as test fields, one of which had clay soil and the other had sandy loam soil, and each soil was used in pot tests in an incubator. In sterile sandy loam soil the population of strain Rif-482 rapidly declined from over 1.0×10^6 colony forming unit (cfu)/g dry soil to the detection limit (1.0×10^3 cfu/g dry soil) 3 days after incubation. However, about 1.0×10^6 cfu/g dry soil of strain Rif-482 existed in sterile clay soil 3 weeks after incubation. In the case of nonsterile conditions in pots and in the mascarene grass turf fields, the population of strain Rif-482 in both kinds of soil rapidly declined. The number of soil bacteria and soil fungi in the two mascarene grass turf fields did not change drastically after application of strain Rif-482. Strain Rif-482 and the wild type (JT-P482) showed no antagonistic activity to several microorganisms on potato semi-synthetic agar plate in a dual culture. These results suggest that *X. c.* pv. *poae* is neither able to persist nor to grow over the detection limit in soil in the natural ecosystem, and that *X. c.* pv. *poae* shows little effect on soil microorganisms.

INTRODUCTION

Xanthomonas campestris pv. *poae* is a causal agent of bacterial wilt of the *Poa* genus. *X. c.* pv. *poae* was first reported as a pathogen of rough bluegrass (*Poa trivialis* L.) in Switzerland.^{1,2)} Recently, new strains of *X. c.* pv. *poae* which show stronger aggressiveness to annual bluegrass (*P. annua* L.) than to rough bluegrass were found in Japan³⁾ and in the United States.⁴⁾ Recently identified *X. c.* pv. *poae*, strain JT-P482 from Japan and strain MB218 from the United States, have been investigated⁵⁻⁸⁾ as biological control agents against annual bluegrass in intensively managed turf fields e.g. golf courses.

Strain JT-P482 is able to kill annual bluegrass in plant growth chamber conditions when the inoculum concentration is over 1.0×10^6 colony forming units per ml (cfu/ml).⁹⁾ High temperature (ca. 30°C) and low air humidity (ca. 30%) accelerate the activity of strain JT-P482 as a biological control agent.⁹⁾ Annual bluegrass in mascarene grass (*Zoysia tenuifolia* Willd. ex Trin.) turf fields in natural conditions have been well controlled by strain JT-P482.⁷⁾ Results of inoculation tests in the greenhouse in which JT-P482 was inoculated to 9 major crop species and 8 other turf grass species showed no adverse effects.⁶⁾ However, the fate of strain JT-P482 in the

environment after application has not been investigated.

To elucidate the fate of *X. c.* pv. *poae* in soil, we produced a rifampicin tolerant mutant of strain JT-P482. This paper reports the fate of a rifampicin tolerant mutant of *X. c.* pv. *poae* in soil, in laboratory conditions and in field conditions, and the population dynamics of soil bacteria and soil fungi after the application of rifampicin tolerant mutant of *X. c.* pv. *poae* to turf fields.

MATERIALS AND METHODS

1. Bacteria

A spontaneous mutant (strain Rif-482) of *X. c.* pv. *poae* JT-P482, tolerant to the antibiotic rifampicin, was used in this study. Host ranges, aggressiveness to annual bluegrass and bacteriological properties of both strain JT-P482 and strain Rif-482 were similar apart from their tolerance limit to rifampicin. Strain Rif-482 grew on medium YNA³⁾ containing 30 µg/ml of rifampicin but strain JT-P482 did not. Pathogenicity of both strains are shown in Table 1. Pathogenicity of both strains to 8 grasses were decided from the symptoms of test plants which were inoculated with 1.0×10^8 cfu/ml of bacterial suspension by leaf clipping-inoculation.³⁾ Inoculated plants were grown at 25°C/20°C (day/night) for 4 weeks in a greenhouse. The bacteriological properties of both strains were examined by the Biolog MicroStation Sys-

Table 1 Comparison of pathogenicity between *Xanthomonas campestris* pv. *poae* wild type (strain JT-P482) and the rifampicin tolerant mutant (strain Rif-482) to 8 grasses.

Test plants	Strains	
	JT-P482	Rif-482
Annual bluegrass (<i>Poa annua</i> L.)	++ ^{a)}	++
Rough bluegrass (<i>P. trivialis</i> L.)	+	+
Kentucky bluegrass (<i>P. pratensis</i> L.)	—	—
Italian rye-grass (<i>Lolium multiflorum</i> Lam.)	—	—
Timothy grass (<i>Phleum pratense</i> L.)	—	—
Tall oat-grass (<i>Arrhenatherum elatius</i> L.)	—	—
Creeping bent (<i>Agrostis stolonifera</i> L. cv. Pencross)	—	—
Rice (<i>Oryza sativa</i> L. cv. Koshihikari)	—	—

^{a)} ++, severe symptom; +, moderate symptom; —, no symptom.

tem (Biolog, Inc., U.S.A.) which was designed for bacterial identification based on carbon utilization.¹⁰⁾

Strain Rif-482 was cultured by shaking in liquid medium YNB³⁾ containing 30 µg/ml of rifampicin for 2 days at 28°C. Bacterial cells were collected by centrifugation and were rinsed once with sterile distilled water to remove the medium. The bacterial cells were stored in 10% glycerol solution at -80°C. The concentration of Rif-482 cells in frozen storage was 5.5×10^{11} cfu/ml. The frozen stocks were diluted to the appropriate bacterial concentrations with sterile distilled water for use as inocula.

2. Counting the Number of Strain Rif-482 in Soil

For the isolation of strain Rif-482 from soil samples a new semi-selective medium, TYD5, was developed with reference to medium D5 for *Xanthomonas* sp. by Kado *et al.*¹¹⁾ The medium TYD5 consists of cellobiose 5 g, K₂HPO₄ 3 g, NaH₂PO₄ 1 g, NH₄Cl 1 g, MgSO₄·7H₂O 0.3 g, yeast extract (Difco) 0.5 g, methyl green 40 mg, cycloheximide 50 mg, nystatin 20 mg, rifampicin 30 mg, agar 15 g and distilled water 1 l.

Fifty grams of wet soil sample were mixed with 450 ml of sterile distilled water for 3 min at 10,000 rpm in a blender (Nihonseiki, Japan; model DX-7) to isolate the microorganisms in soil. The suspension was diluted serially and spread onto TYD5 plates. Seven days after incubation at 28°C, the number of Rif-482 colonies which were viscous and greenish yellow in color, were counted.

Table 2 Soil samples.

	A-field (Ibaraki Prefecture)	B-field (Fukuoka Prefecture)
Soil property	Clay soil	Sandy loam soil
Clay content	29.1%	3.8%
Kind of clay	Allophane	Halloysite
Carbon content	4.92%	1.0%
Cation exchange capacity	32.4 me/100 g	8.1 me/100 g
Maximum water holding capacity	103.1	35.3
Phosphate absorption coefficient	1980	350

3. Counting the Number of Soil Bacteria and Soil Fungi

Microorganisms were isolated from 50 g of wet soil sample by the same method as that used to count the number of strain Rif-482 cells. The soil suspension was diluted serially and spread onto medium YG¹²⁾ (glucose 1 g, yeast extract (Difco) 1 g, K₂HPO₄ 0.3 g, KH₂PO₄ 0.2 g, MgSO₄·7H₂O 0.2 g, agar 15 g, cycloheximide 50 mg and distilled water 1 l; pH 6.8) plates and onto medium Rose Bengal Agar¹²⁾ (glucose 10 g, peptone (Difco) 5 g, KH₂PO₄ 1 g, MgSO₄·7H₂O 0.5 g, rose bengal 33 mg, streptomycin 30 mg and distilled water 1 l; pH 6.8) plates for counting bacteria and fungi, respectively.

4. Field Test

Mascarene grass turf fields in Ibaraki Prefecture (A-field) and in Fukuoka Prefecture (B-field) were used as test fields. The soil in A-field was clay and the soil in B-field was sandy loam. The detailed soil properties of both test fields are shown in Table 2.

Frozen stocks of strain Rif-482 were diluted 1000 times with distilled water to prepare an inoculum in which the bacterial concentration was 5.5×10^8 cfu/ml. Bacterial suspensions were sprayed at the rate of 200 ml/m² on 10 m² of the test fields on April 10 (A-field) and on April 18 (B-field) in 1995. To collect the soil samples, the test fields were bored from the land surface to a depth of 100 mm and a diameter of 40 mm, at four separate points. Bored soil samples were mixed and sifted out with a sieve (pore size, 4.75 mm) to remove plant material. The existence of strain Rif-482, soil bacteria and soil fungi in the soil samples was investigated before treatment, immediately after treatment and 1, 3, 7, 14, 21, 28 and 35 days after treatment.

5. Pot Tests in an Incubator

In the pot tests, the two kinds of soils from A-field and B-field were used. The soil was sifted out with a sieve (pore size, 4.75 mm) to remove plant material. The moisture content of the soil samples was adjusted to 60%

of the maximum water holding capacity of each soil and the soil samples were incubated at 28°C for 7 days to stabilize the microcosms. Two milliliters of bacterial suspension (2.8×10^7 cfu/ml) of strain Rif-482 were added to 50 g of sterile or nonsterile wet soil samples in a 200 ml tall-beaker with an aluminum foil cover. Inoculated soil samples were placed in a plant growth chamber and were kept at a relative humidity (RH) of 85% at 28°C under dark conditions. The existence of strain Rif-482 in the soil samples was investigated before treatment, immediately after treatment and 1, 3, 7, 14 and 21 days after treatment.

6. Antagonistic Activity of *X. c. pv. poae* to Microorganisms

To test the antagonistic activity of *X. c. pv. poae* JT-P482 and rifampicin tolerant mutant Rif-482 to microorganisms, the growth inhibitions of 5 species of bacteria and 1 species of fungus were examined in a dual culture on potato semi-synthetic (PS) agar plates¹³⁾ (Table 4). The activity was measured 7 days after incubation at 25°C.

RESULTS AND DISCUSSION

In sterile sandy loam soil from B-field in Fukuoka Prefecture, the population of strain Rif-482 rapidly declined from over 1.0×10^6 cfu/g dry soil to the detection limit (1.0×10^3 cfu/g dry soil) 3 days after inoculation (Fig. 1). However, about 1.0×10^6 cfu/g dry soil of strain Rif-482 still existed in the sterile clay soil, from the A-field in Ibaraki Prefecture, 21 days after inoculation (Fig. 1). In nonsterile conditions in pot tests (Fig. 2), and in the mascarene grass turf fields in field tests (Fig. 3), the population of strain Rif-482 in both kinds of soil rapidly declined to the detection limit 7 days after inocu-

Fig. 1 Persistence of *Xanthomonas campestris* pv. *poae* Rif-482 in two kinds of sterile soil.

Two milliliters of bacterial suspensions (2.8×10^7 cfu/ml) of *X. c. pv. poae* Rif-482 were added to 50 g of sterile clay soil (●) and sterile sandy loam soil (○) in a 200 ml beaker with an aluminum cap, and the soils were incubated in a plant growth chamber which was kept at 28°C, at 85% relative humidity. Bars indicate standard errors ($n=3$).

lation. However, in B-field in Fukuoka Prefecture, the population of strain Rif-482 in the soil increased to about 4.5×10^4 cfu/g dry soil 15 days after inoculation and then declined to the detection limit 21 days after inoculation. In Fukuoka Prefecture, 30 mm of rainfall had been observed 14 days after inoculation (data not shown). It was supposed that heavy rain washed out the cells of strain Rif-482 from plant materials, e.g. annual blue-grass, to soil.

Liang *et al.*¹⁴⁾ reported that the behavior of microorganisms in natural ecosystems could be distinguished into three patterns. (I) Microorganisms decline rapidly in sterile and nonsterile samples of the natural ecosystems. (II) Microorganisms decline rapidly in non-

Fig. 2 Persistence of *Xanthomonas campestris* pv. *poae* Rif-482 in two kinds of nonsterile soil.

Two milliliters of bacterial suspensions (2.8×10^7 cfu/ml) of *X. c. pv. poae* Rif-482 were added to 50 g of nonsterile clay soil (●) and nonsterile sandy loam soil (○) in a 200 ml beaker with an aluminum cap, and the soils were incubated in a plant growth chamber which was kept at 28°C, at 85% relative humidity. Bars indicate standard errors ($n=3$).

Fig. 3 Persistence of *Xanthomonas campestris* pv. *poae* Rif-482 in soils in two test fields, A-field (●) in Ibaraki Prefecture and B-field (○) in Fukuoka Prefecture, which were covered with mascarene grass turf.

Bacterial suspensions (5.5×10^8 cfu/ml) were sprayed at the rate of 200 ml/m² on 10 m² of the A-field on April 10 in 1995 and the B-field on April 18 in 1995. Bars indicate standard errors ($n=3$).

sterile samples but persist or grow in sterile samples. (III) Microorganisms decline slowly and often reach stable populations in nonsterile samples, and persist or grow in sterile samples. Liang *et al.*¹⁴⁾ also reported that the microorganisms which behaved according to pattern (I) were susceptible to abiotic factors (e.g. pH or inorganic or organic inhibitors) in samples or were not able to obtain enough nutrients from the habitat to grow. Microorganisms which behaved according to pattern (II) were able to obtain enough nutrients to grow in the absence of competition and were resistant to abiotic factors but were susceptible to predation, parasitism, or metabolites of indigenous microorganisms, or were not able to obtain the nutrients from the habitat due to competition with indigenous microorganisms. The behavior of strain Rif-482 in the sandy loam soil of the B-field followed pattern (I), and in the clay soil of A-field it followed pattern (II). This suggests that the sandy

loam soil of B-field is not suitable for *X. c. pv. poae* Rif-482 to persist or to grow, and that the clay soil of A-field is suitable for *X. c. pv. poae* Rif-482 to persist. However, strain Rif-482 may fail to persist in the presence of other indigenous microorganisms in clay soil. Almost all phytopathogenic bacteria, except for *Pseudomonas solanacearum* and *Agrobacterium tumefaciens* etc., are unable to persist in nonsterile soil due to competition with other microorganisms.¹⁵⁾ Protozoa are one of the agents for such decline of phytopathogenic bacteria, (e.g. *X. c. pv. campestris*).¹⁶⁾

The number of soil bacteria and soil fungi in both test fields did not change drastically after application of strain Rif-482 (Table 3). Strain JT-P482 and strain Rif-482 did not show antagonistic activity to 5 genera of bacteria and 1 genus of fungus on PS agar plates (Table 4). From our results, it is concluded that *X. c. pv. poae* JT-P482 (or Rif-482) is not able to persist or to grow over

Table 3 Population dynamics of soil bacteria and soil fungi after application of *Xanthomonas campestris* pv. *poae* Rif-482 on mascarene grass turf fields.

Location/ microorganism	The number of cells ^{a)}					
	Before application	After application	1 ^{b)}	7	21	35
A-field/ Bacteria	1.4×10^8 $\pm 4.9 \times 10^6$	1.2×10^8 $\pm 8.2 \times 10^6$	1.2×10^8 $\pm 4.2 \times 10^6$	1.9×10^8 $\pm 6.1 \times 10^7$	2.7×10^8 $\pm 3.6 \times 10^7$	2.5×10^8 $\pm 3.9 \times 10^7$
Fungi	3.6×10^5 $\pm 2.3 \times 10^4$	4.6×10^5 $\pm 2.2 \times 10^4$	4.6×10^5 $\pm 4.0 \times 10^3$	7.0×10^5 $\pm 9.7 \times 10^3$	6.5×10^5 $\pm 5.2 \times 10^4$	4.1×10^5 $\pm 1.4 \times 10^4$
B-field/ Bacteria	4.4×10^7 $\pm 3.9 \times 10^6$	4.6×10^7 $\pm 5.7 \times 10^6$	1.2×10^8 $\pm 9.5 \times 10^6$	4.4×10^7 $\pm 5.4 \times 10^6$	6.8×10^7 $\pm 2.0 \times 10^6$	4.6×10^7 $\pm 3.4 \times 10^6$
Fungi	1.5×10^5 $\pm 2.4 \times 10^3$	2.2×10^5 $\pm 8.4 \times 10^3$	1.8×10^5 $\pm 1.2 \times 10^4$	1.8×10^5 $\pm 1.6 \times 10^4$	1.7×10^5 $\pm 1.6 \times 10^3$	1.6×10^5 $\pm 2.7 \times 10^3$

^{a)} Each value is the mean colony forming unit/g dry soil (cfu/g dry soil) $\pm$ standard error of 3 soil samples.

^{b)} Days after application.

Table 4 Antagonistic activity of *Xanthomonas campestris* pv. *poae* JT-P482 and Rif-482 to microorganisms.

Microorganisms	Strains ^{a)}	Features ^{b)}	Inhibition activity ^{c)}	
			JT-P482	Rif-482
<i>Agrobacterium radiobacter</i>	IFO13532 (ATCC19358)	GN ; Type strain	—	—
<i>Bacillus subtilis</i>	IFO13719 (ATCC6051)	GP ; Type strain	—	—
<i>Bradyrhizobium japonicum</i>	IFO14783 (ATCC10324)	GN ; Type strain	—	—
<i>Pseudomonas fluorescens</i>	IFO14160 (ATCC13525)	GN ; Type strain	—	—
<i>Xanthomonas maltophilia</i>	MAFF301689	GN ; Isolate from soil	—	—
<i>Fusarium oxysporum</i> f. sp. <i>dianthi</i>	JT-F108 ¹⁷⁾	F	—	—

^{a)} IFO, Institute for Fermentation, Osaka, Japan ; MAFF, Ministry of Agriculture, Forestry and Fishery, Tsukuba, Japan.

^{b)} GN, Gram negative bacterium ; GP, Gram positive bacterium ; F, Fungus.

^{c)} —, growth inhibition was not observed in a dual culture.

the detection limit (1.0×10^3 cfu/g dry soil) in soil in natural ecosystems, and shows little effect on soil micro-organisms.

ACKNOWLEDGMENTS

The authors wish to thank Dr. S. Nagae and Dr. Y. Takeuchi, The Nishinohon Turfgrass Research Institute, and Dr. S. Gonda, The Japan Association for Advancement of Phyto-Regulators for providing soil samples. We also thank Dr. M. Yamada, Ms. J. Nishino, Mr. M. Honda and Ms. R. Hokabe for their valuable advice and assistance to our research.

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要 約

スズメノカタビラの微生物防除剤である *Xanthomonas campestris* pv. *poae* の土壌中での消長

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Xanthomonas campestris pv. *poae* JT-P482 株の抗生物質リファンピシン耐性突然変異株 (Rif-482 株) を作出し, Rif-482 株の土壌中での残留性およびその土壌微生物に対する影響を調査した。埴土よりなるコウライ芝地と砂壤土よりなるコウライ芝地において野外試験を行い, それぞれの圃場の土壌を用いて容器内試験を行った。その結果, 滅菌した砂壤土中では Rif-482 株はその菌数を維持することができず, 細菌懸濁液を添加直後には乾土 1 g あたり 1.0×10^6 コロニーフォーミングユニット (cfu) 以上存在した菌数が, 添加後 3 日目には検出限界 (乾土 1 g あたり 1.0×10^3 cfu) 近くまでその菌数を減少させた。また, 滅菌した埴土中では Rif-482 株はその菌数を維持し, 細菌懸濁液を添加後 21 日目においても乾土 1 g あたり約 1.0×10^6 cfu の菌数を維持していた。しかし, 容器内の非滅菌土壌中および野外試験では土壌の種類に関わりなく, Rif-482 株はその菌数を維持することができず, 添加後 7 日目には検出限界近くまでその菌数を減少させた。また, 野外試験実施中の土壌細菌数および土壌糸状菌数に顕著な変化は見られず, 対峙培養試験においても細菌および糸状菌に対する拮抗阻害活性は見られなかった。以上の結果から, *X. c.* pv. *poae* は土壌微生物に対して影響を与える可能性は低く, 土壌中の他の微生物の影響により, 速やかにその土壌中における菌数を減少させることが示された。