Role of Antibiotic Production in Suppression of Radish Damping-off by Seed Bacterization with *Pseudomonas cepacia**

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Abstract

Strains RB425 and RB3292 of *Pseudomonas cepacia*, isolated originally from roots of lettuce and *Campanula* sp. and known producers of the antibiotics pyrrolnitrin and two pseudane compounds, both were suppressive to damping-off of radish caused by *Rhizoctonia solani* AG4 when applied on radish seeds. Two other naturally occurring strains A2 and A4 of *P. cepacia*, causal agents of bacterial brown spot of *Cymbidium* spp., and known producers of pyrrolnitrin (but not the two pseudanes), also were suppressive to damping-off of radish. A fifth strain, *P. cepacia* ATCC No. 25416, produced no detectable quantity of any of the three antibiotics and was not suppressive to damping-off of radish. Eight mutants of strain RB425, induced by nitrosoguanidine (NTG), were distinguished from the wild-type parent by colony morphology. Three of these mutants produced no detectable quantities of either pyrrolnitrin or the two pseudanes in liquid culture and provided no suppression of damping-off. The other five mutants produced the three antibiotics in amounts similar to the parent and were suppressive to damping-off. Tests with derivative strains resistant to rifampicin and nalidixic acid showed that the mutants were not significantly different from the parent in the ability to colonize the roots of radish seedlings. Both colonization of radish seedlings by *R. solani* and severity of damping-off of radish caused by this fungus were suppressed by about 50% when the radish seeds were coated with either living cells of RB425 (10^7 cfu/seed) or purified pyrrolnitrin ($1.0 \mu\text{g}/\text{seed}$). Damping-off was suppressed only slightly or not at all following seed treatment with either of the two pseudane compounds, even at $40 \mu\text{g}/\text{seed}$. Observations by SEM confirmed that the effective strains multiplied in the spermosphere and the rhizosphere of radish seedlings. These results indicate that pyrrolnitrin plays an important role in suppression of radish damping-off by seed bacterization with *P. cepacia*.

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Key words: antagonist, *Pseudomonas cepacia*, antibiotics, bacterization, biological control, soilborne pathogen.

INTRODUCTION

Some soilborne pathogens have been successfully restricted in their disease-producing activity by bacterization of the planting materials (seeds, tubers, cuttings or roots) of the host plants with root-colonizing bacteria^{1,4)}. Successful control of root diseases by bacterization has been attributed to the ability of the bacteria to rapidly colonize the rhizosphere, effect quantitative and qualitative changes in the rhizosphere microflora, and suppress the activity of pathogens

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in the infection court⁴⁾. Disease suppression has been attributed to the ability of the bacteria to produce either antibiotics, such as pyrrolnitrin¹⁰⁾, pyoluteorin¹¹⁾ or phenazine⁶⁾, or siderophores, e.g. pseudobactin, inhibitory to major or minor pathogens in the rhizosphere^{8,10,11)}. Analytical techniques for detection of antibiotics have been improved greatly during recent years, but direct demonstration of the production of antibiotics by microorganisms in rhizosphere is still difficult. Evidence for a role of antibiotic production by introduced microorganisms in biological control of soilborne pathogens has been obtained by using isogenic mutants that produce less or no antibiotic^{12,21)}, or by applying purified antibiotics isolated from the antagonists to seeds and demonstrating a suppressive effect on disease^{10,11)}, and now also by direct isolation of the antibiotic from rhizosphere soil²¹⁾.

Pseudomonas cepacia (Burkholder) Palleroni et Holmes was initially described as a cause of sour skin of onion bulbs³⁾ but was subsequently reported to provide biological control of certain soilborne pathogens^{8,14)} and to produce several antibiotics^{9,15,18)}. Strains of *P. cepacia* exhibited strong antibiosis to *Fusarium oxysporum* Schl. f. sp. *cepae* (Hanz.) Snyder et Hans. *in vitro* and suppressed the incidence of Fusarium-wilt of onion when introduced on the seed¹⁴⁾. We previously reported⁸⁾ that a strain of *P. cepacia*, obtained from lettuce roots and tested in pots, controlled damping-off of radish caused by *Rhizoctonia solani* Kühn, Verticillium-wilt of eggplant caused by *Verticillium dahliae* Klebahn, and Fusarium-wilt of tomato caused by *F. oxysporum* f. sp. *lycopersici*. This same strain also produced antibiotics in culture, namely pyrrolnitrin and two pseudane compounds⁹⁾.

This paper deals with the relation between production of the antibiotics *in vitro* and suppression of disease *in vivo* among several naturally occurring strains and nitrosoguanidine (NTG)-induced mutants of *P. cepacia*, and the relative efficacy of seed treatment with live bacteria versus purified antibiotics on colonization of *R. solani* in the rhizosphere of radish seedlings and on suppression of damping-off caused by *R. solani*.

MATERIALS AND METHODS

Naturally occurring strains and mutants of *P. cepacia*. Five naturally occurring strains of *P. cepacia* were used in this study. Strains RB425 and RB3292 were obtained, respectively, by dilution plating of macerates of washed roots of lettuce grown in Ibaraki Prefecture⁸⁾ and *Campanula* sp. grown in Saitama Prefecture. Strains A2 and A4 were reported to cause a bacterial brown spot of *Cymbidium* spp.²²⁾ The fifth was ATCC No. 25416 of *P. cepacia*.

Bacterial cells of *P. cepacia* RB425 were suspended at 1.5×10^7 cfu/ml in 10, 40, 100, 200 and 400 ppm of 1-methyl-3-nitro-1-nitrosoguanidine (NTG, Sigma Chem. Co., St. Louis, MO 63178, USA) and incubated for 10, 20, 30, 40 and 60 min at 30 C. Mutants were obtained from single colonies grown on the plate of PPGSA⁹⁾ in which about 1% of bacteria survived after spread-plating the treated suspension and incubating at 28 C for 5 days. Eight mutants were selected for further experiments from among 443 isolates obtained after mutagenesis; five were still inhibitory to *R. solani* on Czapek-Dox agar but three were no longer inhibitory in these test. These eight mutants were then used i) to estimate production of the pyrrolnitrin and the two pseudane derivatives [2-(2-heptenyl)-3-methyl-4-quinolinol, HMQ, and 2-(2-nonenyl)-3-methyl-4-quinolinol, NMQ], in culture solution and ii) evaluate the role of these antibiotics in suppression of radish damping-off caused by *R. solani*. Growth of these strains in PPGS solution was verified by optical density (UV 254 nm) after 3 days at 28 C. These mutants were also tested for ability to cause lesions on onion; an onion scale was peeled from a fresh onion bulb and cut into four pieces, and each piece was inoculated by a toothpick with bacterial cells of each of the four strains and incubated in a moist chamber in darkness at 25 C for 3 days.

Four of the original eight NTG-mutants together with the parent were selected for studies of root-colonizing ability. Each antibiotic-resistant strain of RB425 and its NTG-induced mutants, designated as RB425 R^{RNA}, NTG-102 R^{RNA}, NTG-218 R^{RNA}, NTG-348 R^R.

NA^R and NTG-403 R³NA^R, was obtained by successively culturing strain RB425 and the mutants on PPGSA medium containing a ranged concentrations (10, 20, 50, 100 ppm) of rifampicin and nalidixic acid, respectively. Antibiotic-resistant derivative strains were subcultured at 28 C for 2 days on PPGSA medium containing 100 ppm of both antibiotics before use. These strains were used for monitoring the population on radish seeds and roots after sowing in soil.

Determination of pyrrolnitrin and pseudane derivatives. To measure production of pyrrolnitrin and pseudane derivatives by the strains, each naturally occurring and mutant strain of *P. cepacia* was cultured in flasks (100 ml) of PPGS or m523¹³ solution on a reciprocal shaker at 28 C for 3 days. These culture solutions were then extracted with ethyl acetate, fractionated, and purified by silica-gel-column chromatography, gel filtration, and high performance liquid chromatography (HPLC, JASCO TRIOTAR-III, Japan Spectroscopic Co. Ltd., Tokyo, Japan). The biological activity of the various fractions was detected following each fractionation by use of *V. dahliae* grown as an indicator fungus cultured directly on the thin-layer chromatographs (TLC, bioautography). Purified materials were identified to pyrrolnitrin and pseudane derivatives based on i) activity against *V. dahliae*, ii) *R_f*-values on TLC, iii) coloration on TLC when sprayed with a 1% methanol solution of *p*-dimethyl-amino-benzaldehyde, and iv) various spectra such as infrared (IR), nuclear magnetic resonance (NMR) and mass⁹.

Extracts for determination of the amounts of the respective antibiotics produced were obtained from 100 ml of m523 culture solution by shaking the culture solution twice with an equal volume of ethyl acetate. The extracts were dehydrated with anhydrous sodium sulfate, filtered, and taken to dryness *in vacuo*. For determination of pyrrolnitrin and pseudane derivatives, the residue was dissolved in 1 ml of 50% benzene in ethyl acetate and then 1 μ l of the solution was injected into a gas-liquid chromatograph (GLC, Shimadzu Gas Chromatograph GC-9AM, Shimadzu Seisakusho Ltd., Kyoto, Japan) equipped with a 2.1 m $\times$ 0.6 cm glass column of 1.5% Silicone OV-17 on chromosorb w. Column temperature was graduated at 5 C/min from 230 C to 300 C with 1 min of initial time; inlet temperature was 340 C. The retention times under this condition were 7.2, 10.8 and 13.4 min for pyrrolnitrin and the two pseudanes (HMQ and NMQ), respectively. When a known amount of the purified antibiotics was added as a mixture to 100 ml of m523 solution, 85, 71 and 74% of pyrrolnitrin, pseudane (HMQ) and pseudane (NMQ) was recovered, respectively, from the solution using this same method of extraction and quantification.

Seed coating with bacterial cells and antibiotics. Radish seeds were coated with either i) bacterial suspensions, ranging from 10⁵ to 10⁸ cfu/ml, ii) pyrrolnitrin solutions at 0.2–10 μ g/ml, or iii) each the two pseudane derivative in solutions at 2–40 μ g/ml, separately mixed either with diatomaceous soil or powder of glass beads (100 μ m in diam.). The coated seeds were then sown in soil artificially infested with *R. solani* (AG-4). The soil was infested with 2% (by volume) of the culture in which the pathogen was grown in vermiculite-bran medium at 25 C for 2 days. The isolate of *R. solani* was obtained from a root lesion on Japanese radish obtained in Kagawa Prefecture, Japan. The extent of colonization of the seeds and radicals of seedling by *R. solani* was estimated by the frequency of hyphal growth of *R. solani* from either 25 seeds or 25 radicals from each of three replicates on water agar. The seed coats or radicals were recovered from the soil, washed in distilled water, blotted free of excess water with filter paper, and then plated on the water agar.

Estimation of bacterial population and SEM observation of *P. cepacia* on radish. Radish seeds were soaked in a suspension of cells of *P. cepacia* in 1% methylcellulose, dried overnight on a clean-air flow bench, and then planted in garden soil (Kureha Engei Bai-Do, Kureha Co. Ltd., Tokyo, Japan) contained in a plastic pot (8 $\times$ 8 cm). These plants were recovered from the soil at 12, 24 and 48 hr after sowing, gently washed to remove soil particles, and then used for SEM observation of *P. cepacia* on the seeds and radicals of radish. The plants were recovered from the soil at 1, 2, 3 and 7 days after sowing the seeds coated with strain RB425 R³NA^R or mutant strain R³NA^R, and then used for estimation of the bacterial population.

Bacterial populations on seed surfaces and radicals of radish were estimated by dilution plate counting using a spiral plater (Spiral System Inc., Cincinnati, OH 45244, USA) on PPGSA medium containing 100 ppm each of rifampicin and nalidixic acid. The medium also contained 50 ppm cycloheximide to inhibit fungal growth.

For SEM observation, the radish seedlings were recovered from soil and immediately fixed by immersion for more than 2 hr in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0). The materials were dehydrated in a graded ethanol and isoamyl acetate series, dried by a critical point dryer (Hitachi HCP-2, Hitachi Koki Co. Ltd., Tokyo, Japan), coated with a platinum paradium layer by an ion sputter-coating apparatus (Eiko Ion Coater IB-5, Eiko Engineering Co. Ltd., Tokyo, Japan), and then observed under SEM (Hitachi S-450, Hitachi Ltd., Tokyo, Japan).

RESULTS

Relation between antibiotic production and disease suppression

Strains RB425 and RB3292 each produced abundant pyrrolnitrin and the two pseudane compounds (HMQ and NMQ) in both of PPGS and m523 solution (Table 1). Strains A2 and A4 produced similar amounts of pyrrolnitrin, compared with RB425 and RB3292, but production of pseudane derivatives was not detected in any of several trials with either of these strains using both PPGS and m523 solution. Neither pyrrolnitrin nor the two pseudanes were detected in culture solutions of strain ATCC No. 25416. The incidence of damping-off in response to seed coating was 73.7 and 71.2% lower than the check for strains RB425 and RB3292, respectively, 45 and 53.3% lower than the check for strains A2 and A4, respectively, and only 13.7%

Table 1. Antibiotic production by naturally occurring strains and NTG-induced mutants of *Pseudomonas cepacia* and the effect of seed-coating with these isolates on severity of radish damping-off caused by *Rhizoctonia solani*

Strain of <i>P. cepacia</i>	Antibiotic production ^{a)}			% Disease ^{b)} suppression
	Pyrrolnitrin	Pseudane (HMQ)	Pseudane (NMQ)	
Naturally occurring strains				
RB425	1.47	6.88	4.29	73.7 ab
RB3292	1.65	11.98	7.94	71.2 abc
A2	1.94	ND	ND	45.0 e
A4	1.82	ND	ND	53.3 de
ATCC No. 25416	ND	ND	ND	13.7 f
NTG-induced mutants of RB425				
NTG-34	ND	ND	ND	0 g
NTG-70	3.32	14.47	11.08	76.2 a
NTG-102	2.02	6.64	4.58	61.2 cd
NTG-218	1.84	3.80	2.46	63.7 bcd
NTG-233	2.15	6.94	5.31	60.0 cd
NTG-348	ND	ND	ND	6.2 fg
NTG-369	2.22	7.78	6.60	58.7 cd
NTG-403	ND	ND	ND	0 g
Control inoculation	—	—	—	0 g
Control non	—	—	—	100 h

a) $\mu\text{g/ml}$ of culture solution of m523 after shaking at 28 C for 5 days. The production of antibiotics as estimated in ethylacetate extracts from the culture solution by gas liquid chromatography. ND: not detected.

b) Decrease (%) of damping-off of radish comparing with the unprotected check. Values followed by the same letter are not significantly different at $P=0.05$ according to Duncan's multiple range test.

lower (not significant at $P=0.05$) than the check for isolate ATCC No. 25416.

Of the eight mutants selected from among 443 isolates obtained from strain RB425 after NTG mutagenesis, five were inhibitory against *R. solani* on Czapek-Dox agar medium and three were less inhibitory than the parent strain. In addition, colonies of the parent strain RB425 exhibited a dull surface and was yellow green in color, whereas colonies of the eight mutants exhibited a dull to glistening surface and were cream white to yellow-green in color. All mutant strains showed a growth pattern in PPGS solution similar to strain RB425 and all eight, like the parent strain, produced water-soaked lesions on an onion scale in response to toothpick-inoculation with bacterial cells. The range of variation in antibiotic production among the eight mutants in culture solution was 0(ND)–3.32 $\mu\text{g/ml}$ for pyrrolnitrin, 0(ND)–14.47 $\mu\text{g/ml}$ for pseudane HMQ, and 0(ND)–11.08 $\mu\text{g/ml}$ for pseudane NMQ (Table 1). The incidence of radish damping-off caused by *R. solani*, when seeds were coated with these respective mutants, ranged from as much as 76.2% lower than the check (no bacterization) to no different than the check (Table 1). The correlation coefficients were $r=0.898$, 0.776 and 0.752 ($P<0.01$) between the disease suppression and the production of pyrrolnitrin, pseudane (HMQ) and pseudane (NMQ), respectively.

Effect of purified antibiotics on seed and root colonization by and on pathogenicity of R. solani

When radish seeds coated with either increasing concentrations of live cells (10^5 – 10^8 cfu/seed) of strain RB425 or purified pyrrolnitrin (0.2–10.0 $\mu\text{g/seed}$) were sown in soil artificially infested with *R. solani*, colonization of the seeds and roots by *R. solani* and the incidence of damping-off caused by *R. solani* all were suppressed in accordance with the concentration of bacterial cells or pyrrolnitrin (0.2–10.0 $\mu\text{g/seed}$) tested (Table 2). On the other hand, coating the seed with either of the two pseudane derivatives (HMQ and NMQ) at 2.0–40.0 $\mu\text{g/seed}$ gave no suppression of either colonization of seeds and roots by *R. solani* or incidence of damping-off (Table 2).

Colonization of radish seeds and roots by P. cepacia

Radish seeds were coated with three different concentrations of parent of strain RB425 $R^{\text{B}}\text{NA}^{\text{B}}$; the resultant populations on the seed before sowing, counted on the PPGSA containing 100 ppm of each rifampicin and nalidixic acid, was 9.4×10^6 , 4.7×10^5 and 9.4×10^4 cfu/seed, respectively. By 2 days after sowing, these populations increased by 1.2-, 1.7- and 6.9-fold, respectively, on the seed (Table 3), and were estimated at 4.6×10^6 , 1.8×10^4 and 5.3×10^3 cfu/radical, respectively. At 7 days after sowing, the populations were estimated at 10.2×10^6 , 1.2×10^5 and 3.5×10^4 cfu/radical. The population of NTG-mutants 102, 218 and 343 $R^{\text{B}}\text{NA}^{\text{B}}$ increased 2.5- to 4.5-fold on the seed by 2 days after planting, but the population of NTG-403 $R^{\text{B}}\text{NA}^{\text{B}}$ decreased slightly (0.75-fold) during the same 2-day-period. The population of NTG-mutants $R^{\text{B}}\text{NA}^{\text{B}}$ on individual radicals ranged from 1.2 to 6.8×10^5 cfu/plant by 2 days after sowing and increased 3.1- to 11.9-fold by 7 days after sowing.

SEM observation of P. cepacia in rhizosphere

Radish seeds were coated with bacterial cells of the strain RB425 of *P. cepacia* at 10^8 cfu/seed using 0.1% methylcellulose as a carrier and then were sown in soil artificially infested with *R. solani*. Plants were recovered from the soil 24 and 48 hr after sowing and observed under SEM. Several layers of bacterial rods were observed on the seed before and after sowing in soil (Fig. 1). The rod-shaped bacteria, possibly *P. cepacia* RB425, were also observed along the junction of epidermal cells of young root immediately after emergence from the seed (Fig. 2). These rods were observed to stick to the surface of root cells with fine threads (Fig. 2) and sometimes to be embedded in mucilaginous materials (Fig. 3). Colonies of the similar rod-shaped bacteria were randomly located on the juvenile root surface, except at the root apex. Without bacterization, many hyphae of *R. solani* were observed to produce numerous short branch-like lobate appressoria⁵⁾ and to form a hyphal mat on the surface of seeds and radicals newly emerged from the seeds. In contrast, with seed bacterization, only a few hyphae of *R.*

Table 2. Effect of seed coating with live cells of *Pseudomonas cepacia* strain RB425 or its purified antibiotics on colonization of radish seedlings by *Rhizoctonia solani* and *Rhizoctonia*-induced damping-off of radish

Strain or antibiotic ^{a)}	Concentration/seed	% Colonization ^{b)}		Damping-off Index ^{c)}
		Seed	Root	
RB425	10 ⁵ cfu	82.7 d	72.0 a	100.0 a
	10 ⁶	96.0 ab	54.0 bc	94.2 abc
	10 ⁷	48.0 f	22.0 d	52.5 d
	10 ⁸	12.0 gh	18.0 d	41.7 e
Pyrrolnitrin	0.2 µg	72.0 e	48.0 c	90.0 c
	1.0	46.7 f	56.0 bc	52.5 d
	2.0	16.0 g	20.0 d	40.8 e
	10.0	4.0 h	8.0 d	11.7 f
Pseudane (HMQ)	2.0 µg	96.0 ab	84.0 a	100.0 a
	10.0	84.0 cd	76.0 a	92.5 bc
	20.0	100.0 a	68.0 ab	95.0 abc
	40.0	92.0 abc	76.0 a	92.5 bc
Pseudane (NMQ)	2.0 µg	96.0 ab	80.0 a	96.7 abc
	10.0	88.0 bcd	72.0 a	100.0 a
	20.0	100.0 a	82.0 a	100.0 a
	40.0	100.0 a	76.0 a	94.2 abc
Control	—	100.0 a	82.0 a	98.3 ab

a) Pseudane HMQ and NMQ; 2-(2-heptenyl)-3-methyl-4-quinolinol and 2-(2-nonenyl)-3-methyl-4-quinolinol, respectively.

b) % of seeds or young roots from which hyphae of *R. solani* grow when the plant parts were placed on water agar plate. Each value was the average of three replicates and 25 plants were used in each replicate.

c) Damping-off index = $\frac{(2A+1B+0.5C)}{2T} \times 100$

A, pre-emergence damping-off; B, post-emergence damping-off; C, infected plants but not damping-off; and T, total number of plants used.

Values followed by the same letter in each column are not significantly different at $P=0.05$ according to Duncan's multiple range test.

Table 3. Population of *Pseudomonas cepacia* RB425 R^{BNAR} and NTG-induced mutants R^{BNAR} on the seeds and young roots of radish

Strain of <i>P. cepacia</i>	Population (cfu) per plant part and days ^{a)}			
	Seed		Young root	
	Before	2 days	2 days	7 days
RB425 R ^{BNAR}	9.4 × 10 ⁶	11.6 × 10 ⁶	4.6 × 10 ⁶	10.2 × 10 ⁶
	4.7 × 10 ⁵	8.2 × 10 ⁵	1.8 × 10 ⁴	1.2 × 10 ⁵
	9.3 × 10 ⁴	6.4 × 10 ⁵	5.3 × 10 ³	3.5 × 10 ⁴
NTG-102 R ^{BNAR}	7.0 × 10 ⁶	17.8 × 10 ⁶	4.5 × 10 ⁵	1.4 × 10 ⁶
NTG-218 R ^{BNAR}	5.4 × 10 ⁶	23.0 × 10 ⁶	6.2 × 10 ⁵	7.4 × 10 ⁶
NTG-348 R ^{BNAR}	3.8 × 10 ⁶	17.0 × 10 ⁶	6.8 × 10 ⁵	6.0 × 10 ⁶
NTG-403 R ^{BNAR}	10.4 × 10 ⁶	7.8 × 10 ⁶	1.2 × 10 ⁵	1.4 × 10 ⁶

a) Population was counted on PPGSA medium containing 100 ppm each of rifampicin and nalidixic acid, and 50 ppm of cycloheximide by using a spiral plater apparatus. Population on seeds and young roots was counted before sowing and 2 days after sowing in soil, and 2 and 7 days after sowing, respectively.

solani were observed on the seed and root surface and many rod-shaped bacteria were observed around and on the hyphae of *R. solani* (Fig. 4). The rods also appeared to adhere to the hyphae of *R. solani* by fine thread-like structures.

DISCUSSION

It appears from the high correlation between the ability of strains of *P. cepacia* to produce antibiotics and the ability of the same strains to suppress disease caused by *R. solani* on radish that the antibiotic production plays an important role in the suppression of radish damping-off by these strains introduced by seed bacterization. Strains RB425 and RB3292, grown in liquid culture produced 1.5–1.7, 6.9–12.0 and 4.3–7.9 $\mu\text{g/ml}$ of pyrrolnitrin and two pseudane compounds, respectively, and were the most suppressive of all strains tested against damping-off of radish caused by *R. solani*. Strains A2 and A4 produced similar amounts of pyrrolnitrin (1.94 and 1.82 $\mu\text{g/ml}$) as strain RB425 but did not produce either of the pseudane derivatives and exhibited less activity than strain RB425 against radish damping-off (Table 1). Natural and mutant strains, deficient for production of both pyrrolnitrin and the pseudanes, showed little or no activity against damping-off. Of the three antibiotics, pyrrolnitrin may be more important in disease suppression than either of the two pseudane derivatives, although the latter also may have some effect against damping-off of radish caused by *R. solani*. Further tests are needed with strains that produce pseudanes but not pyrrolnitrin to answer this question.

Several researchers have used antibiotic-deficient strains of antagonists to obtain evidence for the significance of antibiotic production in disease suppression; *Pythium ultimum* was controlled by a diketopeptide antibiotic, gliovirin, produced by *Gliocladium virens*, but a gliovirin-deficient mutant was overgrown by *P. ultimum* in culture and did not protect cotton seedlings from damping-off in soil infested with *P. ultimum*¹²⁾. Gurrusiddaiah *et al.*⁶⁾ isolated a phenazine-type antibiotic from cultures of *P. fluorescens* 2-79 suppressive to take-all caused by *Gaeumannomyces graminis* var. *tritici*. Subsequent work revealed that inactivation of phenazine production in *P. fluorescens* 2-79 by Tn5 mutagenesis resulted in greatly reduced ability of the strain to suppress take-all and restoration of antibiotic producing ability by complementation with DNA from the wild-type strain coordinately restored ability of the mutant strains to suppress take-all²¹⁾. Gutterson *et al.*⁷⁾ showed that antifungal substances produced by *P. fluorescens* HV37a *in vitro* was at least partially responsible for limiting the incidence of preemergence damping-off of cotton seedlings caused by *P. ultimum*. While direct demonstration of the production of antibiotics by microorganisms in rhizosphere is very difficult, genetic manipulation of antagonists is a useful method for providing experimental evidence for the significance of antibiotic production to disease suppression in the rhizosphere.

Our conclusion, that antibiotic production plays an important role in suppression by microorganisms of *Rhizoctonia*-induced damping-off of radish, is further supported by the evidence that purified pyrrolnitrin coated on radish seed inhibited colonization of radish seeds and young roots by *R. solani* and suppressed the incidence of damping-off caused by *R. solani* (Table 2). This confirms the findings of Howell and Stipanovic^{10,11)}, who demonstrated that purified pyrrolnitrin and pyoluteorin produced by a strain of *P. fluorescens* gave significant protection against *Rhizoctonia*- and *Pythium*-induced damping-off of cotton seedlings when coated on the seeds. The purified pseudane derivatives exhibited little or no effect against colonization of radish by the pathogen (Table 2), and provided no protection against disease, suggesting that pyrrolnitrin is more important than the pseudanes in suppression of damping-off of radish by *P. cepacia*.

It is clear from the SEM observations and by the monitoring the population of bacteria on radish seedlings, that the bacteria introduced on radish seeds multiplied in the spermosphere and then colonized the surface of young roots newly emerged from the coated seed. The behavior of *P. cepacia* on radish seedlings was very similar to that reported for *P. fluorescens* and *P. putida* on roots of potato^{3,16)}, wheat²⁴⁾, sugar beet²⁰⁾ and maize¹⁹⁾ following bacterization

of the seeds of these plants. *P. fluorescens* and *P. putida* multiplied in the spermosphere and then colonized seedlings of soybean¹⁷⁾. The early events of colonization of the surface of young roots of radish by the bacteria introduced in seeds was similar to the pattern reported by van Vuurde *et al.*²³⁾ for rhizobacteria on wheat, namely, colonization of longitudinal junctions of epidermal cells first followed by spreading over the cell surface. It is also clear that without bacterization, *R. solani* rapidly colonized the seed of radish, attacked young roots as they emerged from the seeds, and killed the seedling within 48 hr after sowing, whereas with bacterization, the colonization of young plants by *R. solani* was inhibited by the bacteria and damping-off was reduced. These results suggest that *P. cepacia* colonized and occupied the infection court, including the surface of seeds and young roots, and produced the antibiotics inhibitory to the pathogen as a colonist of young plants.

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和 文 摘 要

本間善久・鈴木孝仁：*Pseudomonas cepacia* の種子バクテリアゼーションによるダイコン苗立枯病の抑制における抗生物質生産の意義

Pseudomonas cepacia RB425 および RB3292 は、抗生物質ピロールニトリンおよびシューダン (HMQ, NMQ) を生産し、ダイコン種子にコーティングすることによって、*Rhizoctonia solani* による苗立枯病を抑制した。*Cymbidium* spp. の褐色斑点細菌病菌 *P. cepacia* A2 および A4 は、シューダンは生産しないがピロールニトリンを生産し、発病抑制効果が認められた。*P. cepacia* ATCC No. 25416 は、いずれの抗生物質も生産せず、抑制効果がなかった。ニトロソグアニジンで誘導した RB425 の突然変異株 8 菌株は抗生物質生産性に変異が認められ、培地上の 3 種の抗生物質生産性と、*R. solani* の幼苗への着生率抑制および発病抑制能との間に高い相関関係が認められた。種子当り 10^7 cfu の RB425 の生菌または、 $1.0\ \mu\text{g}$ の純化したピロールニトリンを種子にコーティングすることによって、およそ 50% の発病抑制率が得られた。シューダンを種子当り $40\ \mu\text{g}$ コーティングした場合には、ほとんど抑制効果がなかった。RB425 のリファンピシンおよびナリジキン酸耐性菌株を用いて播種後の菌数を測定したところ、種子当り 9.4×10^6 , 4.7×10^5 および 9.4×10^4 cfu コーティングした場合、7 日目に幼根 1 g 当り 4.6×10^5 , 1.8×10^4 および 5.3×10^3 cfu であった。種子コーティングした RB425 は、播種後、幼根表皮細胞の縫合部に沿って生育し、根圏で増殖するのが SEM によって観察された。これらの結果から、*P. cepacia* RB425 はダイコン幼苗根圏で増殖でき、種子コーティングによるダイコン苗立枯病の抑制効果にピロールニトリンが重要な役割を有すると考えられた。

Explanation of plate

Plate I.

Scanning electron micrographs of *Pseudomonas cepacia* and *Rhizoctonia solani* on seeds and young roots of radish grown in garden soil; 1. Bacterial cells of *P. cepacia* several layers thick on seed surface at 20 hr after sowing in soil (bar= $5\ \mu\text{m}$). 2. Rods, possibly the cells of strain RB425 of *P. cepacia*, along the junction of epidermal cells of young roots and attached to root cells by fine threads at 20 hr after sowing in soil (bar= $6\ \mu\text{m}$). 3. Rods embedded in mucilaginous materials on the surface of young roots at 48 hr after sowing (bar= $10\ \mu\text{m}$). 4. Hyphae of *R. solani* on the seed with bacterization and colonized by rod-shaped bacteria at 48 hr after sowing in the soil infested with *R. solani* (bar= $20\ \mu\text{m}$).

Plate I