Bacteriocin-producing Strains of *Pseudomonas solanacearum* and Their Effect on Development of Bacterial Wilt of Tomato

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摘　　要

蔡正文、徐世典、陳隆鐘（1985）。**產生細菌素之青枯病菌菌株及其對番茄青枯病發生之影響**。（國立中興大學植物病理學系）

以臺灣各地區六種寄主植物上分離的青枯病菌 52個菌株作爲指示菌株時，所有菌株均能產生細菌素，但產生的細菌素其抑制作用範圍差異甚大，其中以天堂鳥花分離的六個菌株所產生的細菌素最具廣效性，它們能抑制52個菌株中 73～90%菌株的生長。從這六個天堂鳥花菌株再分別選取而得的無致病性菌株仍具有產生細菌素之能力（Avirulent bacteriocin-producing strains, ABPS)。當番茄幼苗根部浸漬於 ABPS 懸浮液（10^9 cfu/*ml*）中 30分鐘後再種植於含有青枯病菌的土壤中時，都能延遲發病或減輕發病程度，但其防治效果因不同的 ABPS 而有差異，其中以 BP5A 的防治效果最佳。如以另一無致病性但不產生細菌素的青枯病菌菌株 PS31A 作同樣防治試驗，則無防治效果。BP5A 雖有良好的防治效果，但其效果不能持續到植株生長的後期。BP5A 接種於番茄根部後，其族群逐漸降低。

（關鍵字：青枯病菌，細菌素，番茄，生物防治）

ABSTRACT

Fifty-two strains of *Pseudomonas solanacearum* collected from six hosts in different parts of Taiwan were assayed for bacteriocin activity using all of the 52 strains as indicators. Each strain was found to produce bacteriocin against at least one of the 52 indicator strains. The test strains varied considerably in their spectra of bacteriocin activity, among them, 6 strains isolated from bird-of-paradise inhibited the growth of a greatest number of *P. solanacearum* strains. These 6 strains were selected for further study and an avirulent strain was obtained from each of the 6 strains. The avirulent strains retained their wide spectra of bacteriocin activity when tested against all of

the 52 strains. Effect of the 6 avirulent bacteriocin-producing strains (ABPS) on development of bacterial wilt of tomato was evaluated in the greenhouse. Disease development was delayed or disease severity was reduced when roots of tomato seedlings were dipped in suspensions of ABPS (10^9 cfu/ml) prior to transplanting in soil infested with virulent strain PS68 (a bacteriocin-sensitive strain) of *P. solanacearum*. The level of disease control, however, varied with the ABPS, of which, BP5A showed the best control. When an avirulent bacteriocin-nonproducing strain (PS31A) selected from virulent strain PS31 was tested for control of the disease by the same root dip method, PS31A was not able to suppress the disease. Although BP5A was effective in protecting tomato seedlings from bacterial wilt, the protection did not persist to the later stages of tomato growth. The population of BP5A on the roots declined gradually after treatment.

(Key words: *Pseudomonas solanacearum*, bacteriocin production, tomato, biocontrol)

INTRODUCTION

Bacterial wilt of tomato caused by *Pseudomonas solanacearum* (Smith) Smith is one of the most important bacterial diseases in Taiwan. The disease has often been the limiting factor in tomato production in summer or warm seasons. The disease is also of major economic importance on several other solanaceous crops[6]. Breeding for disease resistance is the main approach for controlling bacterial wilt in Taiwan. During the past 10 years good progress has been made in breeding tomato for resistance to the disease[11,12,13]. However, highly resistant cultivars have not yet been developed. While breeding for resistance is the most important method for bacterial wilt control, other means of control may be necessary and used with host resistance in an integrated approach to disease management.

The potential use of avirulent bacteriocin-producing bacteria for control of bacterial plant diseases has been suggested[16]. The successful control of crown gall disease of various crops incited by *Agrobacterium tumefaciens* (Smith & Townsend) Conn by preplanting treatment with a nonpathogenic bacteriocin-producing strain 84 of *A. radiobacter* (Beijerinck & van Delden) Conn is a well-known example[10,15]. Recently, Chen et al[1] reported that tobacco seedlings were protected from bacterial wilt by immersion of roots of the seedlings into cell suspensions of avirulent bacteriocin-producing strain of *P. solanacearum* before planting. The purposes of this study were to determine whether strains of *P. solanacearum* collected in Taiwan are bacteriocinogenic and to evaluate the avirulent bacteriocin-producing strains of this bacterium for control of bacterial wilt of tomato.

MATERIALS AND METHODS

Bacterial strains Strains of *P. solanacearum* were obtained from the stock culture collection maintained in the Department of Plant Pathology at the

National Chung Hsing University. They were isolated from various host plants in different parts of Taiwan and maintained in sterile distilled water in screw cap tubes at room temperature. The sources of the 52 strains tested for bacteriocin production and sensitivity were as follows: PS9, 11, 21, 27, 31, 38, 41, 50A, 50B, 64, 65, 66, 67, 68, 69, 73, 79 and 82 (from tomato collected in Taichung area); PS44, 45, 48 and 51 (tobacco, Taichung); PS60 and 98 (potato, Nantou); PS61 (tomato, Nantou); PS63 (sweet pepper, Nantou); PS70 (tomato, Changhua); PS71 and 72 (tomato, Yunlin); PS75 (tomato, Tainan); PS76 and 77 (eggplant, Pingtung); PS78 (tomato, Pingtung); PS84 and 86 (sweet pepper, Taichung); PS88 and 89 (tomato, Hsinchu); PS90 (tomato, Kaohsiung); PS91 (sweet pepper, Kaohsiung); PS92 (potato, Taichung); PS94, 95 and 96 (eggplant, tomato and sweet pepper, respectively, Taipei); BP1, 2, 3, 4, 5, 6 and 8 (bird-of-paradise, Nantou), PS99 (eggplant, Changhua), and T145 (tobacco, Chiayi). Before being used in each experiment, the stock cultures were streaked on Kelman's TZC agar plates[8] and incubated for 48 hr at 30C. A single fludal colony from each strain was then transferred to YDC agar[4], incubated for 18-24 hr at 30C and used as the inoculum.

Bacterioncin production and detection The 52 strains of *P. solanacearum* were assayed on CPG agar medium[3] for bacteriocin production by the method of Vidaver et al[17] using all of the 52 strains as indicators. The surface of each CPG agar plate was spot-inoculated with 10 different test strains. The inoculated plates were incubated for 36 hr at 30C and used as master plates. Colonies on the master plates were then replica plated to fresh CPG plates and incubated further for 48 hr at 30C. The plates were inverted and exposed to vapor from 3 ml chloroform for 1 hr to kill the bacterial cells. Residual chloroform vapors were removed by placing the plates with lids off in a laminar flow for 30-60 min. Each plate was then overlaid with 4 ml molten (45C) CPG soft agar[3] containing 0.2 ml (10^7 colony forming units (cfu)/ml) of an indicator strain. After incubation for 24 hr at 30C, inhibition zones were observed and recorded.

Selection of avirulent strains from bacteriocin-producing strains Avirulent strains were selected from virulent strains of *P. solanacearum* showing broadspectrum of bacteriocin activity according to the procedure of Kelman and Hruschka[9]. The strains were cultured in TYG broth [9] for 5-10 days at room temperature. The bacterial cells were then removed from surface layer of the broth, streaked on the TZC plates, and incubated for 48 hr at 30C. Appearance of small butyrous colonies was considered as formed by avirulent cells[8]. A single butyrous colony was selected for each strain and purified by repeated platings on TZC medium. The aivurlent strains thus obtains were maintained in sterile distilled water in screw cap tubes at room temperature.

Pathogenicity tests were made on tomato seedlings grown in pots by stem-inoculation[7] with the avirulent strains to confirm their avirulence on tomato. The production of bacteriocin by the avirulent strains was also tested against

all indicator strains by the method described above.

Disease control experiments with avirulent bacteriocin-producing strains (ABPS) Effect of ABPS on bacterial wilt development was evaluated in a greenhouse[1]. Tomato (*Lycopersicon esculentum* Mill, "Shuang Hsi") plants were grown in clay pots containing sandy loam soil, one plant per pot. When the seedlings reached 15-18 cm high, they were removed from pots and roots were washed free of soils. Roots were then immersed in a water suspension of ABPS (10^9 cfu/ml) for 30 min and transplanted to 13-cm clay pots containing soils infested with 1.1×10^6 cfu/g dry soil of a virulent bacteriocin-sensitive strain PS68 of *P. solanacearum*. Roots similarly treated with sterile distilled water were served as controls. At 5-day intervals for 30 days after transplanting, the disease development was recorded using the disease index described previously[7]. Each treatment consisted of 5 plants and the experiment was repeated twice.

Multiplication of ABPS on tomato roots Tomato plants grown in pots in a greenhouse were removed and washed free of soils. Roots of the plants were dipped in a water suspension (2×10^9 cfu /ml) of ABPS (BP5A) for 30 min prior to transplanting in pots containing soils. The treated plants were returned to the greenhouse. Immediately and 5-day intervals for 30 days after treatment, three plants were removed for each determination. The roots were washed thoroughly with running water and rinsed with sterile distilled water thrice. The roots were blotted with tissue papers, excised, weighed and then ground with 10 ml sterile distilled water in motars. Samples (0.1 ml) of appropriate dilutions of the macerated suspensions were spread on a selective medium modified from that of Granada and Sequeira[5]. The modified medium contained TZC medium supplemented with 5 ppm crystal violet, 100 ppm polymyxin B sulfate, 20 ppm tyrothricin, 5 ppm chloromycetin, and 5 ppm cycloheximide. After 48 hr incubation at 30C, colonies were counted, and the number of colony forming units per g wet root was calculated. Multiplication of a virulent strain PS68 on the root was similarly determined.

RESULTS

Bacteriocin-producing strains The 52 strains of *P. solanacearum* tested were found to produce bacteriocins on CPG medium when all of the strains were used as indicator strains (Fig. 1). However, bacteriocins produced by different strains differed considerably in their spectrum of inhibitory activity; bacteriocins produced by some strains (e. g. PS21, PS51, PS67, PS68, PS70, PS71, PS91) were active against only one or a few indicator strains, while those produced by others (e. g. PS38, PS98, BP1, BP2, BP3, BP4, BP5, BP6, BP8) inhibited the growth of most of the indicators. All strains from bird-of-paradise (BP strains) were notably to have a broad spectrum of inhibitory activity and were selected for further study. To determine whether the inhibition zones observed on the plates were due to activity of phages, spot tests were carried out[14]. Agar blocks (0.5×0.5 cm^2 each) were picked out from the inhibition zones on CPG agar produced by the BP strains (BP2,

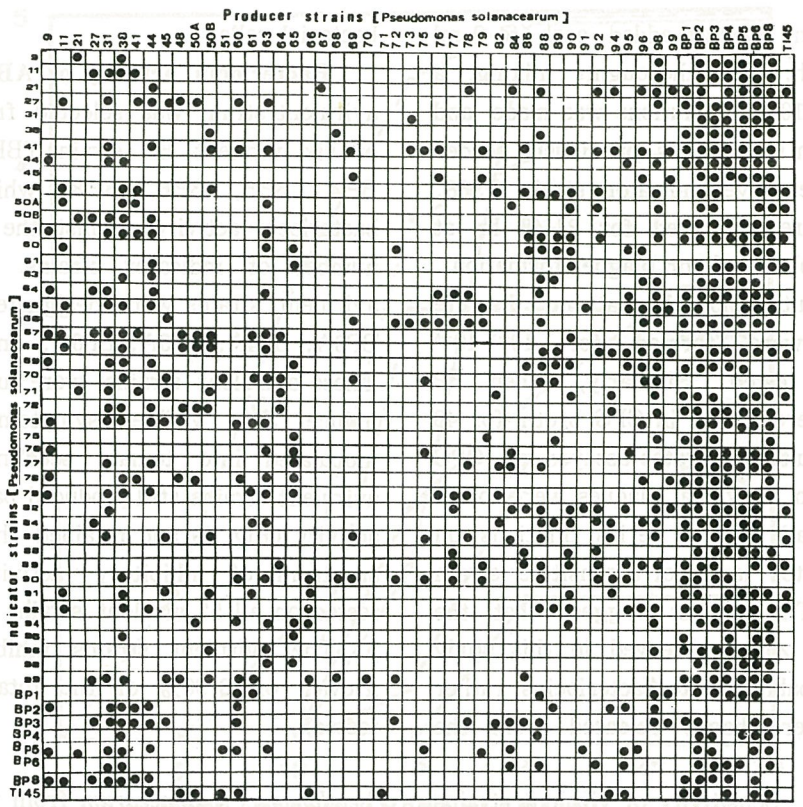


Fig. 1. Bacteriocins production by 52 strains of *Pseudomonas solancearum* on CPG agar medium using each of the 52 strains as an indicator. Squares with black dots indicate presence of inhibition zones, and squares without black dots indicate absence of inhibition zones.



Fig. 2. Inhibition zones on a lawn of an indicator strain PS68 of *Pseudomonas solanacearum* caused by bacteriocin(s) produced by virulent (BP5) and avirulent (BP5A) strains of the same bacterium.

4, 5, 6 and 8) and added each to 9 ml CPG broth, after thoroughly mixing, a series of 10-fold dilutions was made and spotted on CPG plates previously seeded with a sensitive indicator strain PS68. Plates were incubated for 24-48 hr at 30C and observed for plague formation. The results showed that no discrete plaques were formed for 10^{-1}-10^{-5} dilutions tested. Similarly, when BP strains were grown in CPG broth for 48 hr, and culture filtrates assayed on CPG plates, no individual plaques were observed for all 10-fold series dilutions on the indicator lawns of a sensitive strain PS68. The results suggest that the inhibition zones observed in this study were probably due to bacteriocins rather than bacteriophages released from the bacterial cells.

Bacteriocin activity of ABPS An avirulent strain was selected from each of six virulent BP strains (BP2, BP3, BP4, BP5, BP6, BP8) which have bacteriocin activity against the greatest number of indicator strains. The six avirulent strains were designated BP2A, BP3A, BP4A, BP5A, BP6A, and BP8A. These avirulent strains were unable to induce any visible symptoms when inoculated into tomato seedlings. The avirulent strains still produced bacteriocin activity (Fig. 2) and retained their wide spectrum of inhibitory activity when tested on all 52 virulent strains (Table 1). The avirulent strains inhibited the growth of 73-90% of the total strains tested.

Table 1. Sensitivity of virulent strains of *Pseudomonas solanacearum* from various hosts collected from Taiwan to avirulent bacteriocin-producing strains (ABPS) of the bacterium

ABPS[1]	No. of bacteriocin-sensitive strains isolated from							Percentage of total
	Tomato (28)[2]	Tobacco (5)	Potato (3)	Sweet pepper (5)	Eggplant (4)	Bird-of-paradise (7)	Total (52)	
BP2A	25	5	2	5	4	6	47	90%
BP3A	23	4	2	4	4	5	42	81%
BP4A	19	4	2	5	4	4	38	73%
BP5A	20	3	3	5	4	3	38	73%
BP6A	21	5	3	5	4	2	44	85%
BP8A	22	5	3	1	4	5	40	77%

1) The avirulent strains were selected from virulent strains of *P. solanacearum* isolated from bird-of-paradise.

2) Numbers in parentheses indicate the number of strains from each host tested for bacteriocin sensitivity.

Effect of ABPS on bacterial wilt development of tomato Symptom expression was delayed or disease severity was reduced on tomato plants treated with ABPS. The level of disease control, however, varied with ABPS (Fig. 3 and

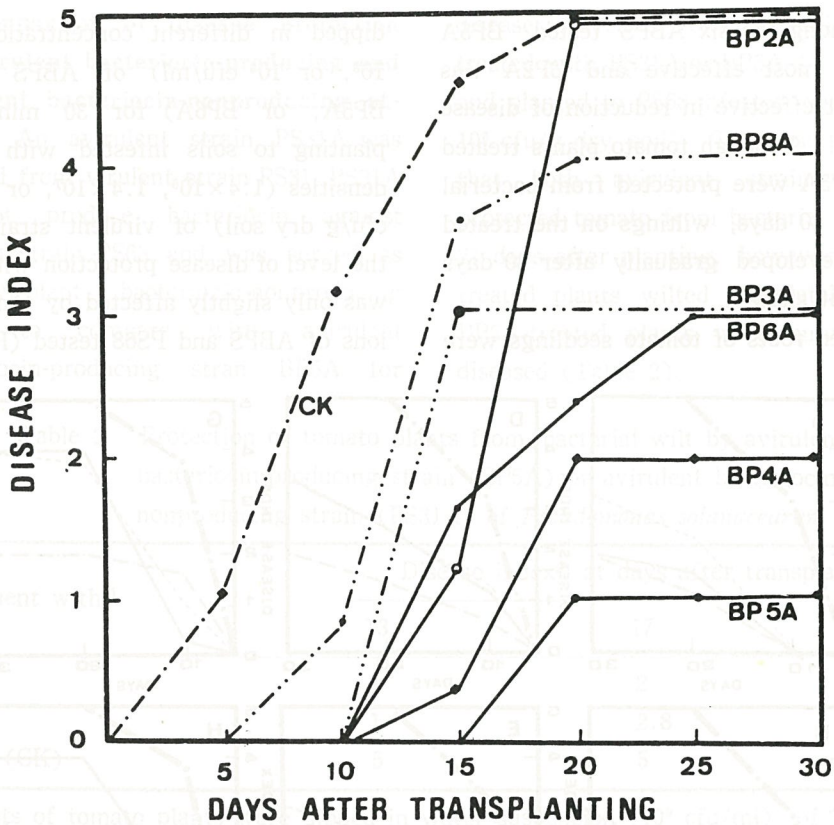


Fig. 3. Development of bacterial wilt of tomato plants treated with avirulent bacteriocin-producing strains (ABPS) of *Pseudomonas solanacearum*. Roots of tomato seedlings were dipped in a water suspension (10^9 cfu /ml) of ABPS (BP2A, BP3A, BP4A, BP5A, BP6A, or BP8A) or water (CK) for 30 min prior to transplanting in soil infested with virulent strain PS68 of *P. solanacearum* (1.1×10^6 cfu/g dry soil).



Fig. 4. Control of bacterial wilt of tomato by avirulent bacteriocin-producing strains (ABPS) of *Pseudomonas solanacearum*. Roots of tomato seedlings were immersed in ABPS (BP2A, BP3A, BP5A, BP6A or BP8A) suspension (10^9 cfu/ml) or water (CK) for 30 min before planting to soils infested with virulent strain PS68 (1.1×10^6 cfu/g dry soil) of the same bacterium.

4). Among the six ABPS tested, BP5A was the most effective and BP2A was the least effective in reduction of disease severity. Although tomato plants treated with BP5A were protected from bacterial wilt for 30 days, wiltings on the treated plants developed gradually after 30 days (data not shown).

When roots of tomato seedlings were dipped in different concentrations (10^7, 10^8, or 10^9 cfu/ml) of ABPS (BP4A, BP5A, or BP6A) for 30 min before planting to soils infested with various densities (1.4×10^6, 1.4×10^7, or 1.4×10^8 cfu/g dry soil) of virulent strain PS68, the level of disease protection with ABPS was only slightly affected by concentrations of ABPS and PS68 tested (Fig. 5).

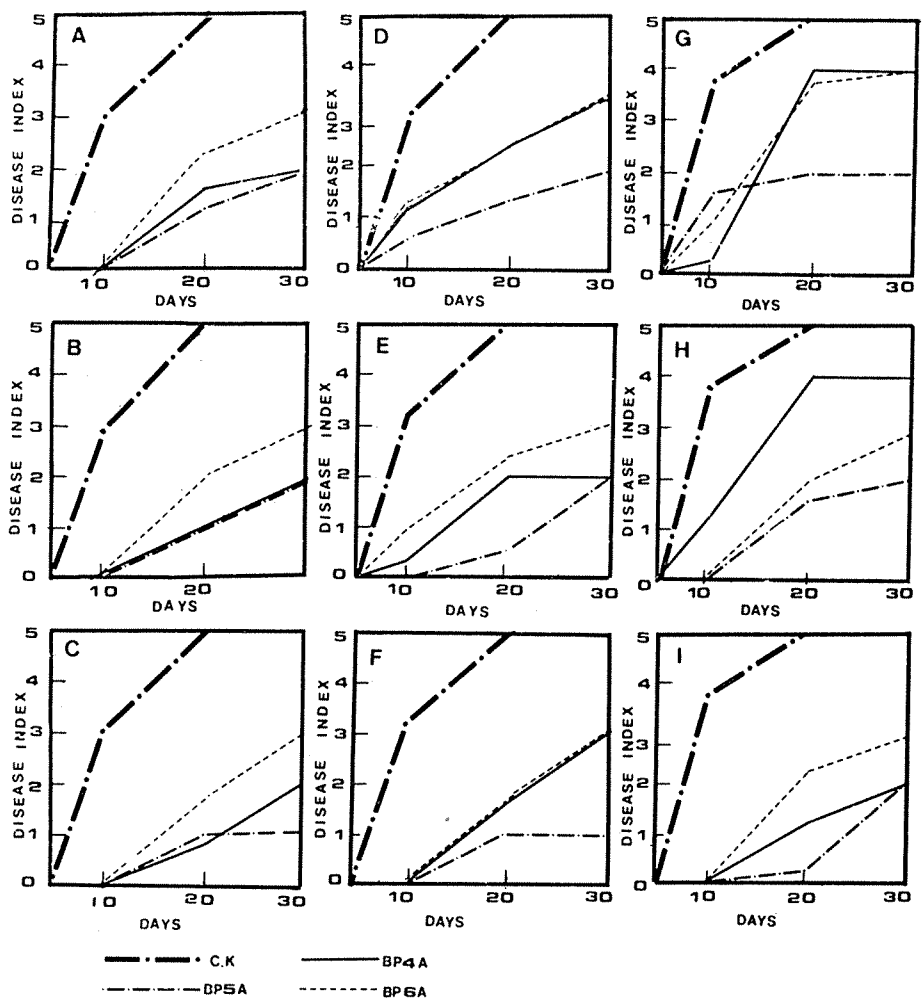


Fig. 5. Effect of concentration of avirulent bacteriocin-producing strains (ABPS) of *Pseudomonas solanacearum* on control of bacterial wilt of tomato. Roots of tomato seedings were dipped in 10^7 (A. D. G.), 10^8 (B. E. H.) or 10^9 cfu/ml (C. F. I.) of ABPS (BP4A, BP5A or BP6A) suspension or water (CK) prior to transplanting in soils infested with 1.4×10^6 (A, B, C), 1.4×10^7 (D. E. F.) or 1.4×10^8 cfu/g dry soil (G. H. I.) of virulent strain PS68 of *P. solanacearum*.

Comparison of disease protection by avirulent bacteriocin-producing and avirulent bacteriocin-nonproducing strains An avirulent strain PS31A was selected from virulent strain PS31. PS31A did not produce bacteriocin against virulent strain PS68 and was served as an avirulent bacteriocin-nonproducing strain to compare with avirulent bacteriocin-producing stran BP5A for disease protection. Tomato seedlings were treated with PS31A or BP5A (10^9 cfu/ml) and planted to PS68-infested soils (1.4×10^6 cfu/g dry soil). The result showed that both avirulent strains initially protected tomato from bacterial wilt. At 21 days after planting, however, PS31A-treated plants wilted completely, while BP5A-treated plants were only slightly diseased (Table 2).

Table 2. Protection of tomato plants from bacterial wilt by avirulent bacteriocin-producing strain (BP5A) or avirulent bacteriocin-nonproducing strain (PS31A) of *Pseudomonas solanacearum*

Treatment with[1)]	Disease index[2)] at days after transplanting		
	13	17	21
BP5A	1	2	2
PS31A	1	2.8	5
Water (CK)	5	5	5

1) Roots of tomato plants were dipped in water suspension (10^9 cfu/ml) of BP5A or PS3 1A, or in water only for 30 min prior to transplanting in soils infested with virulent strain PS68 of *P. solanacearum* (1.4×10^6 cfu/g dry soil).
2) Disease index: 0-5 (no symptoms-plant dead).

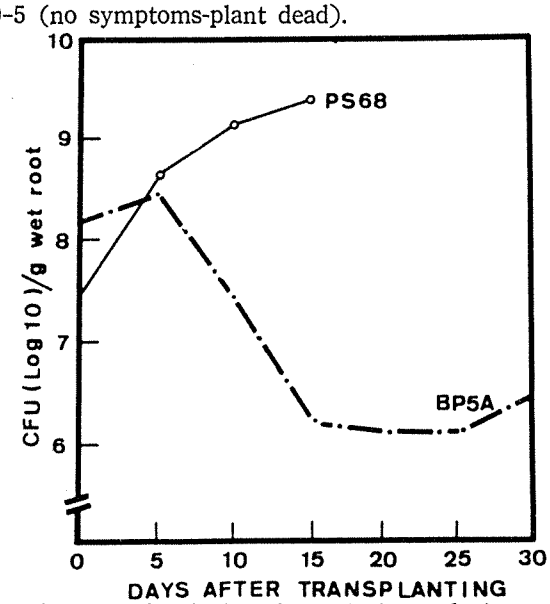


Fig. 6. Populations changes of avirulent bacteriocin-producing strain BP5A or virulent strain PS68 of *Pseudomonas solanacearum* on or in roots of tomato plants. Roots were dipped in suspension of BP5A (2×10^9 cfu/ml) or PS68 (1.3×10^9 cfu/ml) for 30 min before planting to the steam-treated soils.

Multiplication of BP5A and PS68 on roots Colonization of tomato roots by BP5A and PS68 is illustrated in Fig. 6. Population of BP5A slightly increased to 4.9×10^8 cfu/g wet root at 5 days after transplanting, then decreased sharply to 2.5×10^6 cfu/g wet root by 15 days, and remained more or less constant thereafter. For virulent strain PS68, the population increased gradually after planting and reached 3.5×10^9 cfu/g wet root at 15 days. Determination of the population was terminated after 15 days, because of the plants entirely wilted.

DISCUSSION

Bacteriocin production appears to be widespread in strains of *P. solanacearum*. Cuppels[2] reported that 48 strains of the bacterium from different hosts and regions of the world were bacteriocinogenic for one or more of the 53 indicator strains. Chen et al[1] tested 149 strains and found 121 strains had bacteriocin activity against 22 indicator strains. The results of the present investigation also revealed that 52 strains of *P. solanacearum* collected from Taiwan produced bacteriocins when all of the 52 strains were used as indicators. Bacteriocin-producing strains, however, differed greatly in their spectrum of inhibitory activity. In our study, notably, all strains from bird-of-paradise were found to have inhibitory activity against a greatest number of *P. solanacearum* strains. Sensitvity patterns suggest that different bacteriocins might be produced by *P. solanacearum*. However, a more detail study is required to reveal the nature of the bacteriocins produced. A nonsedimentable bacteriocin produced by avirulent *P. solanacearum* B1 has recently been isolated and characterized [3].

Avirulent bacteriocin-producing strains (ABPS) have been used or tested for control of bacterial plant diseases[16]. In this study, we demonstrated that preplanting treatment of tomato roots with ABPS derived from BP strains of *P. solanacearum* protected the plants from bacterial wilt. Similar results were also obtained by Chen et al in the control of bacterial wilt of tobacco with ABPS[1]. Therefore, ABPS appear to have the potential for use as biological control agents in control of bacterial wilt disease. The control mechanism was not determined in the present investigation. The data of the experiment on avirulent bacteriocin-nonproducing strain (PS31A) suggest that control is probably not due to a simple physical blocking of infection sites, since PS31A was not effective as ABPS (BP5A). However, not all ABPS were effective in disease control, even the most effective one, BP5A, did not protect tomato plants from bacterial wilt at later stages of tomato growth. The reasons for the different control efficacy observed among ABPS are not certain. There is possibility that some ABPS may not produce or produce insufficient amount of bacteriocins on the roots to combat the pathogen. Since effective biological control agents must be able to colonize the root systems, the difference in their colonization ability may also account for the different efficacy of ABPS to control the disease. Population assays indicated that BP5A did not multiply on the treated tomato roots. The inability to multiply and colonize the new growths of the root

system may be one of the reasons for the failure of protection by BP5A at later stages of plant development. Therefore, further selection of specific ABPS which can readily establish and multiply profusely on root systems is essential if effective and lasting control is to be achieved. The biocontrol agents should also produce sufficiently high concentrations of bacteriocins on the treated plants and are effective against most strains of the pathogen existing in the control areas concerned.

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