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TERMINAL REPORT

USAID PROJECT: 6.032

BIOLOGICAL CONTROL OF SEED-BORNE BACTERIAL PATHOGENS WITH BACTERIOCINS

I. *XANTHOMONAS CAMPESTRIS* PV. *VESICATORIA*
II. *X. CAMPESTRIS* PV. *MALVACEARUM*, AND
III. *X. CAMPESTRIS* PV. *GLYCINES*

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1. DESCRIPTIVE TITLE: BIOLOGICAL CONTROL OF SEED-BORNE BACTERIAL PATHOGENS WITH BACTERIOCINS.
   I. *XANTHOMONAS CAMPESTRIS* PV. *VESICATORIA*
   II. *X. CAMPESTRIS* PV. *MALVACEARUM*, AND
   III. *X. CAMPESTRIS* PV. *GLYCINES*

2. ORGANIZATION WHEREIN RESEARCH WAS CONDUCTED:

   College of Agriculture, University of the Philippines at Los Baños (UPLB-CA)

3. PROJECT IDENTIFICATION NUMBER: 6.032

4. PRINCIPAL INVESTIGATOR AND CO-INVESTIGATOR:

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5. PROJECT INITIATION, DURATION, AND TOTAL COST:

Project Started	-	July 1, 1986
Project Ended	-	March 31, 1989
Duration	-	Two years and 9 months
Total Cost	-	US$ 69,782

6. OVERALL AIM AND SPECIFIC OBJECTIVES:

The overall aim of the project was to study the biological control of bacterial spot of tomato (*Xanthomonas campestris* pv. *vesicatoria*), bacterial blight or angular spot of cotton (*X. campestris* pv. *malvacearum*) and bacterial pustule of soybean (*X. campestris* pv. *glycines*) (Figs. 1, 2, and 3), three of the

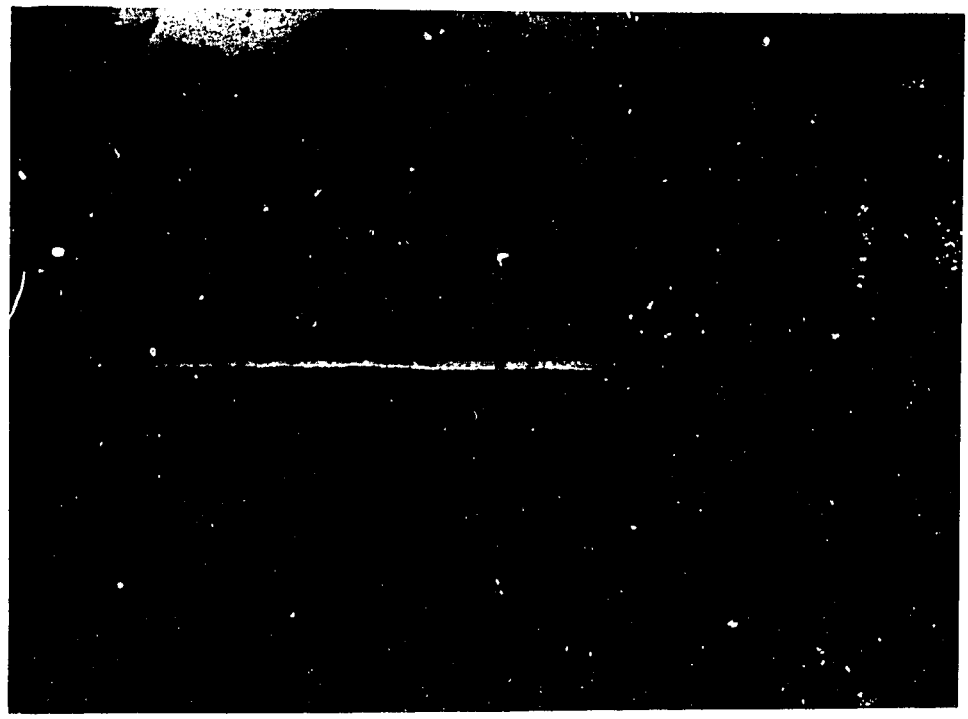


Fig. 1. Bacterial pustule of soybean caused by *Xanthomonas campestris* pv. *glycines*.

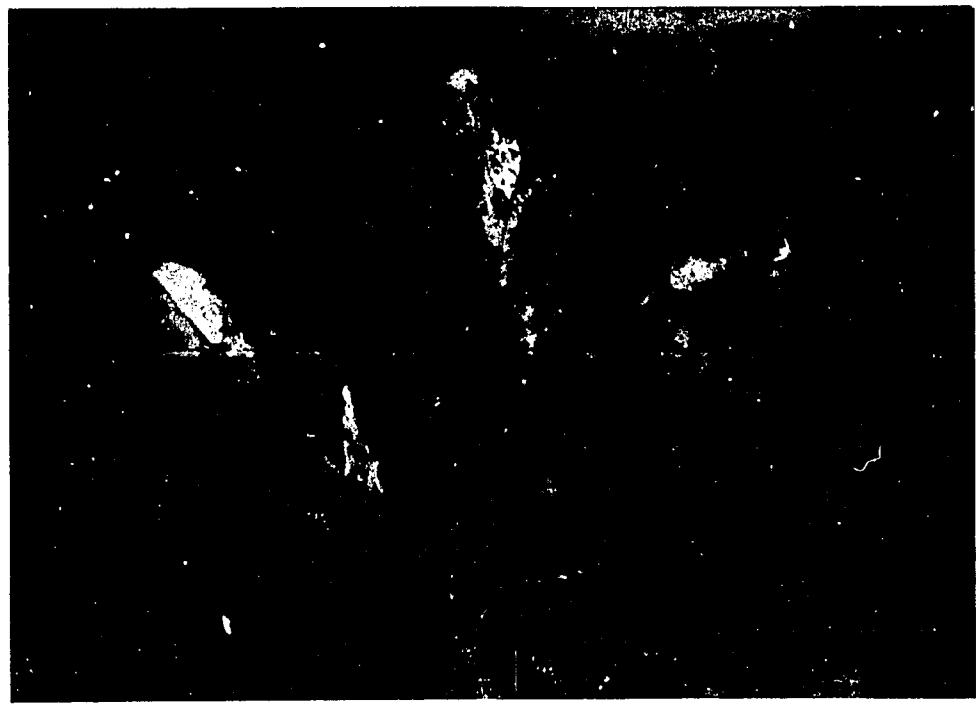


Fig. 2. Bacterial spot of tomato caused by *Xanthomonas campestris* pv. *vesicatoria*.

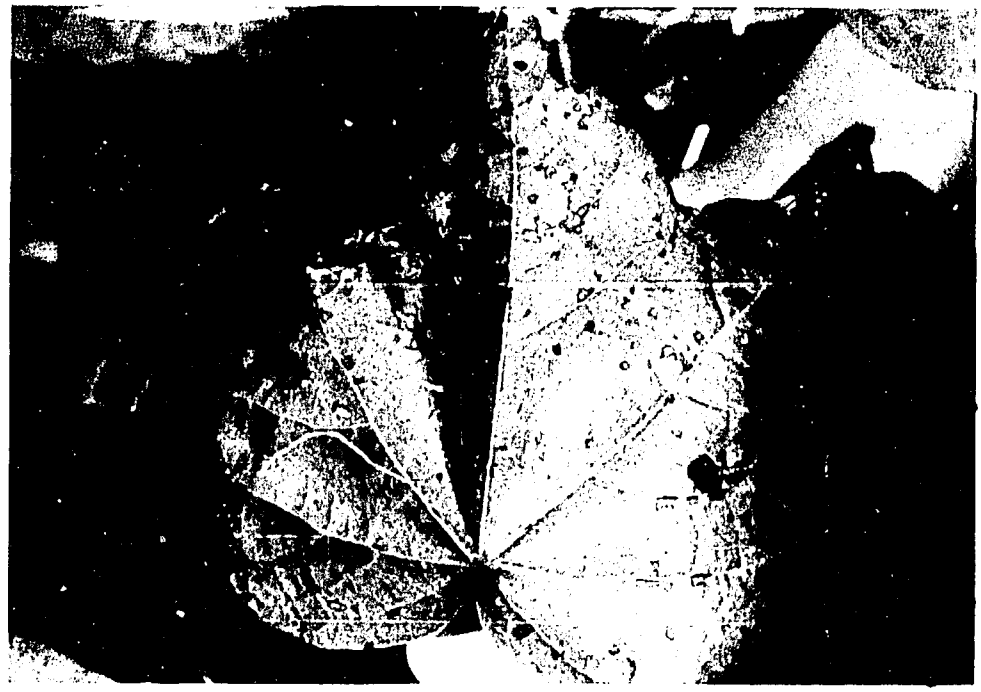


Fig. 3. Bacterial blight on angular spot of cotton caused by *Xanthomonas campestris* pv. *malvacearum*.

most destructive seed-borne bacterial diseases in the humid tropics, with bacteriocin producer strains. Its specific objectives were to (1) isolate avirulent mutants of bacteriocin producer strains (AVBPS) of X. campestris pv. vesicatoria, pv. malvacearum, and pv. glycines; (2) evaluate in the greenhouse and/or in the field the biocontrol efficacy of these AVBPS and (3) study the possible limitations of the AVBPS as biocontrol agents.

7. RELEVANCE TO DEVELOPMENT

In less developed countries (LDC) particularly in the humid tropics bacterial diseases of plants cause tremendous crop losses because resistant varieties which offer the best control measures for these diseases are practically non-existent in these countries for lack of disease resistance breeding programs specifically design for them. Besides, disease resistance breeding programs are long and expensive processes and seldom if ever less developed countries are able to support much less sustain these activities. Some pesticides (some antibiotics and copper-based fungicides) which can be used to control bacterial diseases are all imported by less developed countries and are seldom if ever used by farmers in these countries; majority of these farmers are subsistence farmers and, therefore, they can not afford expensive inputs in their crop farming.

Antibiotics are commonly used in the United States in plant bacterial disease control but it is the only country in the western world that allows the same antibiotics for human and plant disease

control. This practice, however, is not likely to continue because of the recognition by both the scientific community and environmental regulatory agencies of the destructive effects of antibiotics on the ecosystems.

The use of avirulent bacteriocin producer strains which are highly specific biocontrol agents offers an innovative and a cheap alternative to the expensive and often ecologically destructive pesticides or difficult to arrive at resistant varieties for the control of bacterial diseases of plants.

Low input technology that will minimize crop losses due to seed-borne bacterial diseases such as the use of avirulent bacteriocin producer strains certainly if proven effective will be a welcome development in crop farming in many less developed countries where crop plants are continously damage or threaten by destructive bacterial diseases.

8. INNOVATIVE ASPECTS

The only bacteriocin producer strain which is now used commercially for controlling a bacterial disease is a non-pathogenic strain of *Agrobacterium radiobacter* (strain K84). This strain selectively controls most pathogenic soil-borne Agrobacteria (*Agrobacterium tumefaciens*) that cause crown gall on stone fruits. Pre-plant dip treatment of cuttings or roots of young plants in *A. radiobacter* strain K84 suspension to control crown gall is now commercially practiced in Australia, New Zealand, Italy, South Africa, and the USA (1).

The use of bacteriocin producer strains as biocontrol agents for bacterial diseases of plants has distinct advantages over pesticides (some antibiotics and copper containing inorganic compounds) that are used for controlling these diseases. Firstly, while the phytobactericidal pesticides are non-specific in their actions, killing both harmful and beneficial organisms, the bacteriocin producer strains are highly specific in their actions; the bacteriocins they produce kill only their closely related strain or species (i.e. usually only the target virulent bacterial pathogen strain). Secondly, the bacteriocin producer strain for biocontrol purposes normally is a non-virulent strain of the bacterial pathogen and, therefore, it could exist in the same ecological niche as its wild type-disease causing virulent strain relative; theoretically, the non-virulent bacteriocin producer strain can continuously guard the plant against the wild type-disease causing virulent strain. Thirdly, biocontrol of seedborne bacterial diseases by seed treatment with non-virulent bacteriocin producer strain, if it works, would be an inexpensive, low level technology which could be easily adopted by farmers in less developed countries.

9. BACKGROUND AND RATIONALE

Bacteriocins are non-replicating bactericidal protein-containing substances produced by bacteria that kill other strains of the bacteriocin producer or its closely related species (2). Many bacteria including plant pathogens of the genera Agrobacterium, Corynebacterium, Erwinia, Pseudomonas, and Xanthomonas are

known to have bacteriocin producer strains (see Ref. 3, 4, 5, 6).

Phytopathogenic bacteria produce a diverse array of bacteriocins ranging from low molecular weight trypsin-sensitive, thermostable bacteriocins to heat-sensitive or trypsin-resistant or both high molecular weight bacteriocins that may even resemble bacteriophage components (3, 7). Many of these bacteriocins, however, remain to be characterized.

To date, there is no uniform system of naming bacteriocins (8). The most commonly used method is to use the root of the species plus "cin" followed by a strain designation which is usually a number. For example, the bacteriocin produced by Pseudomonas syringae pv. syringae strain 4A is called syringacin 4A.

Bacteriocins are heterogenous compounds and as expected they have several modes of action. They require specific receptor site on the bacterial cell wall or an attachment site in the outer membrane. A site independent of specific attachment site is believed to be also involved in the adsorption process indicating that two stages are needed in the adsorption phase of the bacteriocin by the cell prior to killing. Most bacteriocins affect protein synthesis, DNA synthesis, energy flux, or membrane integrity (8).

Of the several bacteriocins produced by phytopathogenic bacteria only the modes of action of Agrocin 84 (from Agrobacterium radiobacter K84) and Carotovoricin (from Erwinia

carotovora) have been studied. Agrocin 84 specifically inhibits DNA synthesis (9) while Carotovoricin has phospholipase activity that affects cytoplasmic membrane integrity (10).

Bacteriocin production may be controlled by plasmids as in the case of Agrocin 84 (11) or chromosomal genes as in the case of some bacteria (8).

Non-pathogenic or non-virulent bacteriocin producer strains have been demonstrated both experimentally and commercially to effectively control certain bacterial diseases. A non-pathogenic bacteriocin-producing strain of Agrobacterium radiobacter (strain K84) has been demonstrated to control grown gall of peaches (12, 13, 14), cherries (12, 15, 17), pecan (18), and rose (19). This strain is now used commercially in the biological control of crown gall of stone fruits in Australia, Italy, New Zealand, South Africa and in the USA (1).

Other successful experiments on control of bacterial diseases with bacteriocin producer strains include control of bacterial wilt of tobacco (20), tomato canker (21), and fire blight of apples (22). Purified and crude bacteriocin preparations have also been demonstrated to have prophylactic effectiveness against certain bacterial diseases (3, 23).

The greatest potentials of bacteriocins as biocontrol agents, however, are on seed-borne bacterial diseases (24) (e.g. bacterial blight of cotton caused by Xanthomonas campestris pv. malvacearum bacterial spot of tomato caused by X. campestris pv. vesicatoria: bacterial pustule of soybean caused by X. campestris pv. glycines).

In the humid tropics, the pathogens of these diseases can not survive between cropping seasons in the soil or plant debris in the soil and seldom if ever have alternate host plants in the field. They survive between planting seasons mainly in contaminated or infected seeds. They are carried in or on the seeds and, therefore, spread and develop foci of infections primarily through these planting materials. Seed treatment with the bacteriocin producer will ensure contact of bacteriocin with the pathogen during the early phases of infection and thus it will prevent the disease cycle to start. As a supplementary and prophylactic treatment the bacteriocin producer strain may be sprayed on germinating seeds or developing seedling to eradicate pathogen propagules that may have survived seed treatment. The bacteriocin producer exists in the same ecological niche as its target pathogen and, therefore, theoretically, it can continuously guard the plant from the latter.

The prospects of using bacteriocin producer strains for controlling seed-borne bacterial plant pathogens have not been critically explored. Hence, this study.

10. PROJECT PRESENTATION

For purposes of clarity of presentation and to emphasize the systematic approach used in pursuing the objectives of the project, the various studies in the project are presented in 5 parts as follows:

PART I - Isolation of wild type bacteriocin producing strains of *Xanthomonas campestris* pv. *glycines*, pv. *vesicatoria*, and pv. *malvacearum*

PART II - Isolation of avirulent bacteriocin producer mutants of virulent wild type bacteriocin producer strains of *Xanthomonas campestris* pv. *glycines*, pv. *vesicatoria*, and pv. *malvacearum*

PART III - Biological control of bacterial pustule of soybean, angular spot of cotton, and bacterial spot of tomato by seed treatment with avirulent bacteriocin producer strains of their respective causal bacteria

PART IV - Survival of avirulent bacteriocin producer strains of *Xanthomonas campestris* pv. *glycines*, pv. *vesicatoria*, and pv. *malvacearum* on seed and leaf surfaces

PART V - Effects of seed treatment and soil fungicides and insecticides on avirulent bacteriocin producer strains of *Xanthomonas campestris* pv. *glycines*, pv. *vesicatoria*, and pv. *malvacearum*

PART I

ISOLATION OF WILD TYPE BACTERIOCIN PRODUCING STRAINS OF *XANTHOMONAS CAMPESTRIS* PV. *GLYCINES*, PV. *VESICATORIA*, AND PV. *MALVACEARUM*

INTRODUCTION

The specific objectives of this first part of the project were to (1) isolate virulent wild type strains of *X. campestris* pv. *glycines*, pv. *vesicatoria*, and pv. *malvacearum*, (2) determine the frequency of bacteriocin producing strains among the isolates, and (3) identify wild type bacteriocin producing strains for induction and isolation of avirulent bacteriocin producing strains for the biocontrol studies.

MATERIALS AND METHODS

Sources and isolations of virulent wild type strains

Fresh diseased specimens of soybean bacterial pustule, tomato bacterial spot, and cotton angular spot were collected from the market (in case of tomato fruits) and fields in different parts of the Philippines. All isolations were made at the Department of Plant Pathology, UPLB. Modified SB medium (5 g peptone; 5 g sucrose; 1 g sodium glutamate; 5 g yeast extract; 16 g agar; 1 L dist. water) or peptone sucrose agar (PSA) (10 g peptone; 10 g sucrose; 1 g sodium glutamate; 10 g agar (Oxoid No. 3); 1 L dist. water; pH 7.0) were used in isolation works. If immediate isolation from fresh specimens was not possible, tissues from advancing margins of young lesions were cut out and kept in vials

containing 2 ml sterile distilled water or phosphate buffer saline solutions. Isolations were made from the resulting suspensions. Cultures were purified by repeated agar streaking and isolating of single colony isolates. Incubations were made at room temperature.

Pathogenicity tests

Forty-eight hr-old PSA slant cultures of Xcg or Xcv or Xcm suspended in sterile water (10 ml/slant; approx. 1×10^8 cfu/ml) were used in inoculations. About 3-week old tomato (C-132 cv); cotton (Deltapine 16 cv), and soybean (BPI Sy4 or UPL Sy2 cvs) seedlings raised in baked soils were lightly dusted with carborundum dust then inoculated by swabbing the leaves with cotton bud previously dipped in the inoculum suspension (Fig. 4 and 5). Control plants were swabbed similarly with sterile water alone. The plants were covered with plastic bags for 2 days and observed for symptoms in the greenhouse. All plants received standard fertilizer and pesticide treatments.

Maintenance of cultures

Pure cultures were maintained as suspensions in sterile distilled water (pH 7) at 10°C or in skimmed milk (medium (100 g skimmed milk; 1.5 g sodium glutamate; 1 L dist. water) in a refrigerator freezer until further used. Cultures for routine used were grown on PSA.

Isolation of virulent wild type bacteriocin producer strains (WBPS)

Forty-eight hr-old PSA cultures of the wild type strains were spot inoculated on fresh PSA plates individually or by using an improvised manual spot inoculator . After 48 hrs incubation

Fig. 4. Dusting carborundum on lower soybean leaf surface.

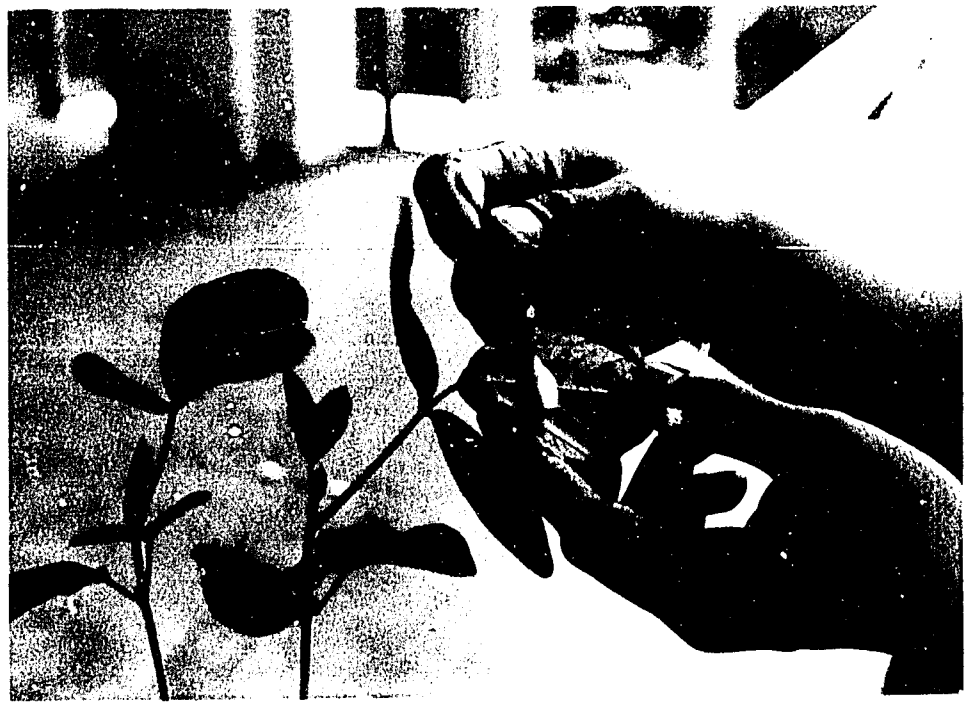


Fig. 5. Inoculating soybean leaf dusted with carborundum using a cotton bud swab previously dipped into bacterial suspension.

the culture plate (bottom plate) was inverted on another bottom plate containing 2 ml chloroform. The colonies were exposed to chloroform fumes for 1 hr, after which the cover plate of the culture was replaced partially open, to allow the chloroform to evaporate for another hour. The cultures were then overlaid with 4 ml of 0.7% molten water agar (about 42°C) seeded with 0.5 ml of 48-hr old test indicator strain (approx 1×10^7 cfu/ml). The plates were incubated at room temperature and diameter of zones of growth inhibition of the indicator isolate (i.e. the isolate in the water agar overlay) by the bacteriocins produced by the producer isolates (i.e. the isolates spot inoculated on PSA plates and treated with chloroform) were recorded after 24 hrs. All tests for ability of isolates to produce bacteriocins as required in the succeeding studies followed these agar overlay technique on chloroform treated colonies.

RESULTS AND DISCUSSION

Sources and isolations of virulent wild type strains

The survey of tomato bacterial spot, cotton bacterial blight and soybean bacterial pustule was conducted from August 1986 to January 1987. Two hundred eighty nine strain of putative *Xanthomonas campestris* were isolated from soybean, tomato and cotton from 50 municipalities/cities in 18 provinces of the Philippines (Table 1); Based on colony morphology and pathogenicity tests, 95 isolates from soybean were identified as *X. campestris* pv. *glycines* (Xcg), 147 from tomato as *X. campestris* pv. *vesicatoria* (Xcv), and 22 from cotton as *X. campestris*

Table 1. Places where specimens of soybean bacterial pustule, tomato bacterial spot and cotton bacterial blight or angular spot were collected.

Province	Municipality/City
Laguna	Calamba, Los Banos, Alaminos, San Pablo City, San Pedro, Binan
South Cotabato	Tupi, Tampacan, Polomolok, Gen. Santos City
Cavite	Indang, Carmona, Bacoor
Bukidnon	Musuan
Agusan del Sur	Tagmamarkay, Bayugan
Agusan del Norte	Butuan, Cabadbaran, Jabonga, Antikula, Santiago
Davao del Sur	Bago Oshiro, Davao City
Batangas	Lipa City, Tanauan
Misamis Oriental	Balingon, Bunao, Claveria, Balingasag, Cagayan de Oro City
Capiz	Roxas City
Iloilo	Jaro, Alimodian, Cabatuan, Pavia
Ilocos Sur	San Juan, Vigan
Ilocos Norte	Marcos
Tarlac	Gerona
Bulacan	San Miguel
Nueva Ecija	Munoz, Sta. Rosa, Talugtog, Talavera, Gapan, Palayan, Cabanatuan City, Guimba
Leyte	Ormoc
Negros Occidental	La Carlota City

pv. malvacearum as (Xcm) (Table 2). The distribution of these isolates are shown in Figure 6.

Isolation of virulent wild type bacteriocin producer strains (WBPS)

A total of 204 isolates were evaluated for bacteriocin production. All Xcg, Xcv and Xcm isolates were tested as producer strains respectively, against all Xcg, Xcm and Xcv isolates as indicator strains.

Forty six out of 96 Xcg strains produced bacteriocins (Fig. 7). Two strains (Xcg 11 and Xcg 38) were very potent producing larger inhibition zones and inhibiting a wider range of Xcg indicator strains compared with that of other Xcg producer strains. Another isolate (Xcg 103) also produced large inhibition zones but had a narrow spectrum of activity (Table 3). Some of the Xcv and Xcm strains used as indicator isolates were also sensitive to 9 Xcg producer strains particularly to Xcg 11 and Xcg 38 (Table 4).

Nine Xcv producer strains were detected from 147 test strains 147 Xcv isolates. Only one strain, Xcv 42, however, was highly active producing larger inhibition zones and inhibiting 56 Xcv indicator strains (Table 5). Six Xcg and 6 Xcm indicator strains were also sensitive to Xcv 42 producer strain (Table 6).

In Xcm, 10 out of 22 test strains were bacteriocin producers but these were active only against 2 Xcm indicator strains (Table 7). The Xcm producer isolates also had a wider range of activity against Xcv indicator strains than Xcm indicator strains (Table 8).

The bacteriocin assay data suggest that bacteriocin producer strains are more common in Xcg (46/96 or 48%) (Table 3) than in

Table 2. Pathogenic isolates of *Xanthomonas campestris* pv. *glycines* (Xcg), *X. campestris* pv. *vesicatoria* (Xcv) and *X. campestris* pv. *malvacearum* (Xcm) collected from different areas of the Philippines.

ISOLATE CODE	DATE DISEASED SPECIMEN COLLECTED	HOST VARIETY	PLACE COLLECTED
SOYBEAN			
Xcg_2	8-1-86	Pb-1	Plant Pathology, Greenhouse, UPLB
Xcg_3	8-1-86	IPB Sy 138-28	Plant Pathology, Greenhouse, UPLB
Xcg_5	8-5-86	6001 (WF)	BPI, Economic Garden, Los Banos, Laguna
Xcg_6	8-5-86	TK 5 x D67-4601	BPI Economic Garden, Los Banos, Laguna
Xcg_7	8-5-86	SRF 400 X L-114X Jupiter	BPI Economic Garden, Los Banos, Laguna
Xcg_8	8-5-86	G0010 (WP)	BPI Economic Garden, Los Banos, Laguna
Xcg_9	8-5-86	TK 5 X SANKVO (PBS)	BPI Economic Garden, Los Banos, Laguna
Xcg_{10}	8-5-86	BPI Var X_1	BPI Economic Garden, Los Banos, Laguna
Xcg_{11}	8-5-86	BPI Sy 4	BPI Economic Garden, Los Banos, Laguna
Xcg_{12}	8-5-86	G0089 (DPGP)	BPI Economic Garden, Los Banos, Laguna
Xcg_{13}	8-5-86	L114 X SRF 307	BPI Economic Garden, Los Banos, Laguna
Xcg_{14}	8-5-86	PI 7937	BPI Economic Garden, Los Banos, Laguna
Xcg_{15}	8-5-86	BPI Var X2	BPI Economic Garden, Los Banos, Laguna
Xcg_{16}	8-5-86	PI 6987	BPI Economic Garden, Los Banos, Laguna
Xcg_{17}	8-5-86	GC 3026 25-2-18-17	BPI Economic Garden, Los Banos, Laguna
Xcg_{18}	8-13-86	UPL Sy 2	Blk 1006, IPB, Tranca, College

Table 2 continued ...

ISOLATE CODE	DATE DISEASED SPECIMEN COLLECTED	HOST VARIETY	PLACE COLLECTED
Xcg_{19}	8-13-86	7521-26-2	Blk 1005, IPB, Tranca, College
Xcg_{20}	8-13-86	IPB Sy 152-14	Blk 205, IPB, Tranca, College
Xcg_{21}	8-13-86	IPB Sy 178-10	Blk 1038, IPB,Tranca, College
Xcg_{22}	8-13-86	GC 81090-10	Blk 1272,IPB, Tranca, College
Xcg_{23}	8-13-86	GC 00036-1-37	Blk 1211, IPB, Tranca, College
Xcg_{24}	8-13-86	IPB Sy 138-28	Blk 1044, IPB, Tranca, College
Xcg_{25}	8-13-86	IPB Sy 152-51	Blk 320, IPB, Tranca, College
Xcg_{26}	8-13-86	IPB Sy 178-37	Blk 1175, IPB, Tranca, College
Xcg_{28}	8-13-86	IPB Sy 178-14	Blk 98, IPB, Tranca, College
Xcg_{29}	8-13-86	GC 00002-51-2	Blk 17, IPB, Tranca, College
Xcg_{30}, Xcg_{34}			Blk 908, IPB, Tranca, College
Xcg_{36}, Xcg_{40}, Xcg_{37}	8-13-86	IPB Sy 178-9 B-3037	Blk 103, IPB, Tranca, College
Xcg_{38}	8-13-86	IPB Sy 178-3	Blk 71, IPB, Tranca, College
Xcg_{39}	8-13-86	TGX 311-14C	Blk 8, IPB, Tranca, College
Xcg_{41}	8-13-86	IPB Sy 178-13	Blk 4, IPB, Tranca, College
Xcg_{43}	8-13-86	IPB Sy 178-19	Blk 1032, IPB, Tranca, College
Xcg_{44}	8-13-86	IPB Sy 178-18	Blk 5, IPB, Tranca, College
Xcg_{45}	8-13-86	IPB Sy 178-21	Blk 6, IPB, Tranca, College
Xcg_{46}	8-13-86	TGX-888-48-C	Blk 10, IPB, Tranca, College
Xcg_{47}	8-13-86	TGX-293-GIE	Blk 7, IPB, Tranca, College
Xcg_{48}	8-13-86	UPL 136-38	Blk 105, IPB, Tracan, College
Xcg_{49}	8-13-86	No. 89	Blk , IPB, Tranca, College
Xcg_{50}	8-13-86	EG Sy 73	Blk 1001, IPB, Tranca, College
Xcg_{54}	10-10-86	EG Sy 26	Tupi Seed Farm, South Cotabato
Xcg_{55}	10-10-86	Sy 4	Tampacan, South Cotabato
Xcg_{56}	10-10-86	unknown	Tampacan, South Cotabato
Xcg_{57} Xcg_{60}	10-10-86	SG 4	Tupi, South Cotabato

Table 2 continued .....

ISOLATE CODE	DATE DISEASED SPECIMEN COLLECTED	HOST VARIETY	PLACE COLLECTED
Xcg_{58}, Xcg_{59}	10-10-86	unknown	Orba, Filipro, Tupi, South Cotabato
Xcg_{62}, Xcg_{63}	10-10-86	unknown	Tupi, South Cotabato
Xcg_{64}	10-10-86	unknown	Polomolok, South Cotabato
Xcg_{66}	10-4-86	TGX 239-77E	SMARC, Kabacan
Xcg_{67}, Xcg_{69}	10-21-86	unknown	DSAC, Indang, Cavite
Xcg_{68}	10-29-86	ETP #4	CMU, Musuan, Bukidnon
Xcg_{70}, Xcg_{72}, Xcg_{78}	10-28-86	unknown	CMU, Musuan, Bukidnon
Xcg_{71}	10-28-86	SG 4	CMU, Musuan, Bukidnon
Xcg_{73}	11-1-86	unknown	Tagmamakay, Agusan del Sur
Xcg_{74}, Xcg_{75}	10-31-86	unknown	Cabadbaran, Agusan del Norte
Xcg_{76}, Xcg_{77}	10-31-86	unknown	Santiago, Agusan del Norte
Xcg_{79}	10-28-86	unknown	BPI Dalwangan
Xcg_{81}, Xcg_{87} Xcg_{98}, Xcg_{104}	1-21-87	LG Sy 82-08-18	BPI, La Granja, La Carlota City
Xcg_{82} Xcg_{90}, Xcg_{93}, Xcg_{97}, Xcg_{100}	1-21-87	LG Sy-82-07-16	BPI, La Granja, La Carlota City
Xcg_{83}	1-21-87	LG Sy 82-08-7	BPI, La Granja, La Carlota City
Xcg_{84}, Xcg_{85} Xcg_{94}, Xcg_{95}, Xcg_{109}	1-21-87	LG Sy-07-8	BPI, La Granja, La Carlota City

Table 2 continued ...

ISOLATE CODE	DATE DISEASED SPECIMEN COLLECTED	HOST VARIETY	PLACE COLLECTED
Xcg_{86}, Xcg_{103}, Xcg_{107}	1-21-87	Clark 63	BPI, La Granja, La Carlota City
Xcg_{89}, Xcg_{96}, Xcg_{99}	1-21-87	LG Sy 82-08-11	BPI, La Granja, La Carlota City
Xcg_{91}, Xcg_{106}	1-21-87	LG Sy 82-17-5	BPI, La Granja, La Carlota City
Xcg_{92}, Xcg_{108}	1-21-87	TK 5	BPI, La Granja, La Carlota City
Xcg_{101}, Xcg_{105}	1-21-87	LG Sy 82-04-10	BPI, La Granja, La Carlota City
Xcg_{110}	1-21-87	LG Sy 82-03-9	BPI, La Granja, La Carlota City
TOMATO			
Xcv_2	8-11-86	Marikit	BPI Economic Garden, Los Banos, Laguna
Xcv_3	8-11-86	CL 1131-00-43-8-1	BPI Economic Garden, Los Banos, Laguna
Xcv_4	8-11-86	CL 1131-0-0-7-2-0	BPI Economic Garden, Los Banos, Laguna
Xcv_5	8-11-86	CL 5915-153 (G) D4-2	BPI Economic Garden, Los Banos, Laguna
Xcv_6	8-19-86	G 132	Plant Pathology Greenhouse, UPLB
Xcv_7	9-5-86	VC 11	Plant Pathology Greenhouse, UPLB
Xcv_8	9-5-86	unknown	Botany Greenhouse, UPLB
Xcv_9 to Xcv_{11}	9-5-86	unknown	Agronomy, UPLB, Laguna
Xcv_{12} to Xcv_{18}	9-12-86	unknown	Calamba, Market, Laguna
Xcv_{19} to Xcv_{21}	9-15-86	unknown	Carmona, Cavite
Xcv_{23}, Xcv_{24}	9-15-86	unknown	San Pedro, Laguna

Table 2 continued ...

ISOLATE CODE	DATE DISEASED SPECIMEN COLLECTED	HOST VARIETY	PLACE COLLECTED
Xcv_{25} to Xcv_{27}	9-15-86	unknown	Platero, Biñan, Laguna
Xcv_{28} to Xcv_{33}	10-2-86	unknown	Aga , Davao City
Xcv_{34} to Xcv_{40}	10-3-86	unknown	Horti plot, USM, Kabacan
Xcv_{35}	10-3-86	unknown	USM Housing, Kabacan
Xcv_{36} to Xcv_{39}	10-10-86	unknown	Tupi Public Market, South Cotabato
Xcv_{37}, Xcv_{41}	10-10-86	unknown	Gen. Santos City
Xcv_{38}	10-10-86	unknown	Polomolok Public Market, Gen. Santos City
Xcv_{42}	10-10-86	unknown	Town Market, Bago Oshiro, Davao City
Xcv_{43}	10-21-86	Bonanza	Hortanova Farm, Lipa, Batangas
Xcv_{44}, Xcv_{49}, Xcv_{52}, Xcv_{60}	10-21-86	unknown	Tanauan, Batangas
Xcv_{45}, Xcv_{46} Xcv_{58}, Xcv_{61}	10-21-86	unknown	DSAC, Indang, Cavite
Xcv_{47}	10-21-86	unknown	San Pablo
Xcv_{48}	10-21-86	Cross 1	Hortanova Farm, Lipa, Batangas
Xcv_{94}, Xcv_{97} to Xcv_{101}, Xcv_{108}	12-15-86	unknown	Mabut, Marcos, Ilocos Norte
Xcv_{50}	10-21-86	unknown	Alaminos Market, Laguna
Xcv_{51}, Xcv_{53} Xcv_{54}, Xcv_{63}	10-21-86	unknown	Lipa, Batangas
Xcv_{55}	10-20-86	unknown	Bacoor, Cavite

Table 2 continued ...

ISOLATE CODE	DATE DISEASED SPECIMEN COLLECTED	HOST VARIETY	PLACE COLLECTED
Xcv_{56}	10-21-86	Marglobe	Hortan va Farm, Lipa, Batangas
Xcv_{57}	10-21-86	unknown	Hortanova Farm, Lipa, Batangas
Xcv_{59}	10-21-86	Cross 2	Hortanova Farm, Lipa, Batangas
Xcv_{62}	10-21-86	Cross 3	Hortanova Farm, Lipa, Batangas
Xcv_{64}, Xcv_{65}	10-31-86	unknown	Libas, Jabonga, Agusan del Norte
Xcv_{66}, Xcv_{86} Xcv_{91}, Xcv_{93}	10-31-86	unknown	Ampoyan, Butuan City
Xcv_{67}	10-31-86	unknown	Balingon, Misamis Oriental
Xcv_{68}, Xcv_{69}	11-1-86	unknown	Antikula, Market, Agusan del Norte
Xcv_{71}, Xcv_{73}, Xcv_{74}, Xcv_{84}, Xcv_{90}	11-1-86	unknown	Lumbia, Cagayan de Oro City
Xcv_{73}, Xcv_{75}, Xcv_{89}	10-31-86	unknown	Bunao, Misamis Oriental
Xcv_{76}	10-31-86	unknown	Cabadbaran Market, Agusan del Norte
Xcv_{77}, Xcv_{88}	10-31-86	unknown	Claveria, Misamis Oriental
Xcv_{78}, Xcv_{80}	11-1-86	unknown	Puntod, Cagayan de Oro City
Xcv_{79}	11-1-86	unknown	Carmen, Cagayan de Oro City
Xcv_{82}	11-1-86	unknown	Antikula, Agusan del Norte
Xcv_{83}	11-1-86	Amprove Pope CV	Calamba, Laguna
Xcv_{85}	10-31-86	unknown	Balingasag, Misamis Oriental
COTTON			
Xcm_{3}	Isolate from IPB Plant Path Dept.	Deltapine 16	IPB, College, Laguna
Xcm_{1}, Xcm_{2}, Xcm_{4}, Xcm_{5}	12-6-87	CBR-4	San Juan, Ilocos Sur
Xcm_{6}, Xcm_{7}	12-6-87	CBR-9	San Juan, Ilocos Sur
Xcm_{9}	12-10-87	DP 6	IPB, College, Laguna

Table 2 continued .....

ISOLATE CODE	DATE DISEASED SPECIMEN COLLECTED	HOST VARIETY	PLACE COLLECTED
Xcm_{10}	12-10-87	Lamcot 37	IPB, College, Laguna
Xcm_{11}	12-10-87	Strain 72	IPB, College, Laguna
Xcm_{12}	12-10-87	unknown	IPB, College, Laguna
Xcm_{13}	12-10-87	DES 810-125	IPB, College, Laguna
Xcm_{15}	12-10-87	138-F	IPB, College, Laguna
Xcm_{16}	12-10-87	BC-1017-10	IPB, College, Laguna
Xcm_{17}	12-10-87	Gm 10	IPB, College, Laguna
Xcm_{18}	12-10-87	EC-1579	IPB, College, Laguna
Xcm_{19}	12-10-87	DES 88-29	IPB, College, Laguna
Xcm_{20}	12-6-87	CBR-4a	San Juan, Ilocos Sur
Xcm_{21}	12-6-87	unknown	San Juan, Ilocos Sur
Xcm_{22}	1-26-87	unknown	Tiring, Cabatuan, Iloilo City
Xcm_{23}	1-26-87	PORA	Binalud, Alimodian, Iloilo City
Xcm_{25}	1-26-87	UPLC 3	Binalud, Alimodian, Iloilo City

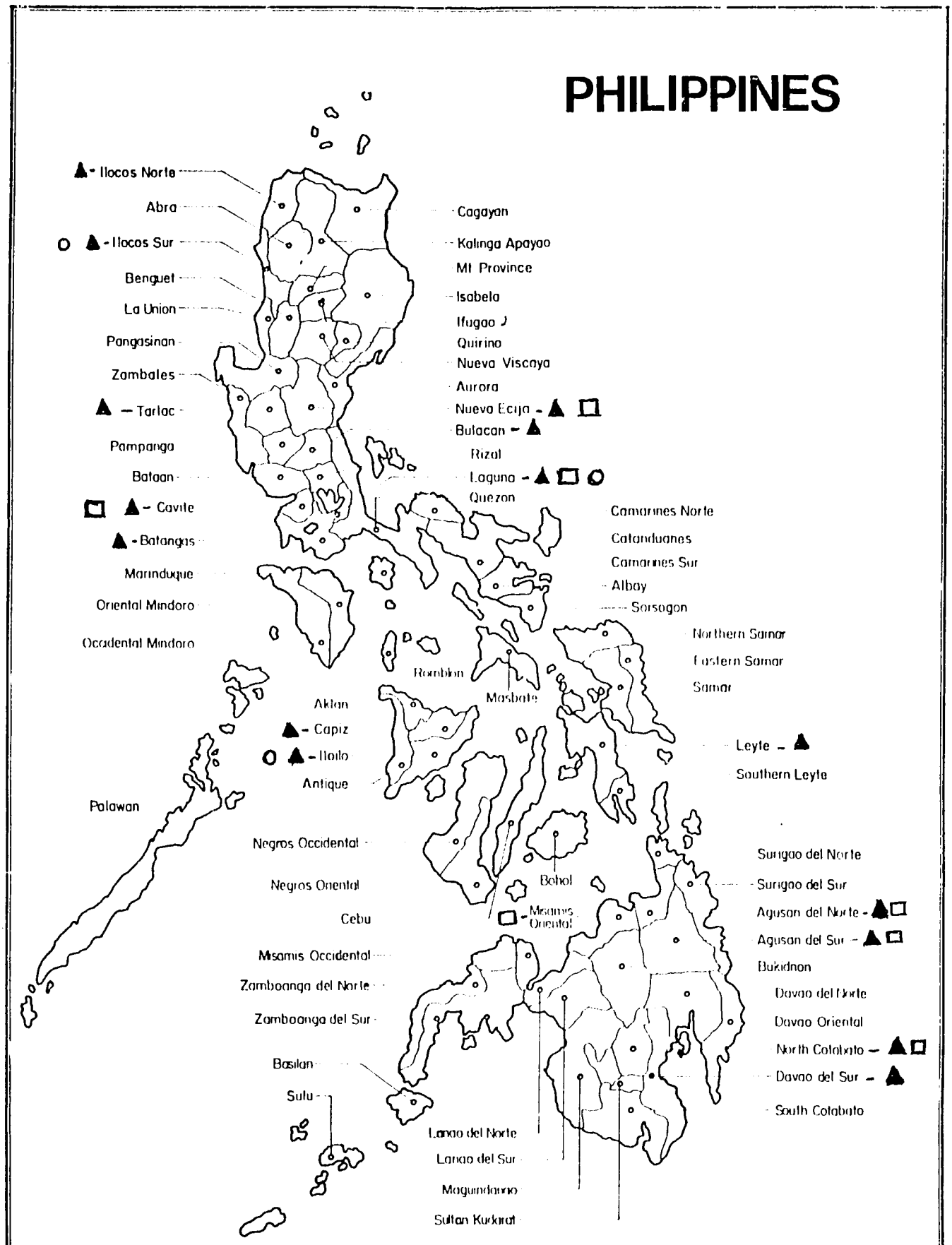


Fig. 6. Philippine map showing the origin of X. campestris pv. glycines (□), pv. malvacearum (○), and pv. vesicatoria (▲) used in the study.

Fig. 7. Bacteriocin assay of virulent wild type strains of X. campestris pv. glycines (Xcg). Colonies with inhibition zones are bacteriocin producers. Xcg cultures were spot inoculated on PSA plate using a mechanical spot inoculator, incubated, treated with chloroform fumes, seeded with an indicator Xcg strain, incubated and observed for zones of inhibition.

Table 3. Detection and efficiency of bacteriocin producer strains of *Xanthomonas campestris* pv. *glycines* (Xcg) using Xcg indicator strains.

PRODUCER STRAINS	SENSITIVE INDICATOR STRAINS[a]	DIAMETER OF INHIBITION ZONES (mm)[b]
Xcg_{103}	Xcg_{77}, Xcg_{59}, Xcg_{66}	23-25
Xcg_{24}	Xcg_{43}, Xcg_{57}, Xcg_{71}, Xcg_{78}, Xcg_{81}	10-16
Xcg_{84}, Xcg_{86}, Xcg_{100}	Xcg_{66}, Xcg_{68}, Xcg_{74}, Xcg_{77}, Xcg_{82}, Xcg_{110}	10-16
Xcg_{30}	Xcg_{66}, Xcg_{68}, Xcg_{77}	10-16
Xcg_{11}, Xcg_{38}	Xcg_{5}, Xcg_{19}, Xcg_{28}, Xcg_{39}, Xcg_{41}, Xcg_{48}, Xcg_{50}, Xcg_{59}, Xcg_{66}, Xcg_{68}, Xcg_{74}, Xcg_{77}, Xcg_{82} to Xcg_{86}, Xcg_{90}, Xcg_{95}, Xcg_{96}, Xcg_{98}, Xcg_{99}, Xcg_{101} to Xcg_{110}	20-30
Xcg_{93}, Xcg_{96}, Xcg_{104}, Xcg_{108}	Xcg_{82}, Xcg_{110}	10-16
Xcg_{16}, Xcg_{20}, Xcg_{40}, Xcg_{41}, Xcg_{49}, Xcg_{56}, Xcg_{58}, Xcg_{81}, Xcg_{87}, Xcg_{88}, Xcg_{99}	Xcg_{74}, Xcg_{77}, Xcg_{66}, Xcg_{68}	10-16
Xcg_{81} to Xcg_{83}, Xcg_{85}, Xcg_{87} to Xcg_{92}, Xcg_{94}, Xcg_{95}, Xcg_{97} to Xcg_{99}, Xcg_{101}, Xcg_{102}, Xcg_{105} to Xcg_{107}, Xcg_{109}	Xcg_{110}	10-16

[a] No. of indicator strains tested: 75

[b] Range of zones produced against sensitive indicator strains.

Table 4. Detection of *X. campestris* pv. *glycines* (Xcg) bacteriocin producer strains using the pathovars *vesicatoria* (Xcv) and *malvacearum* (Xcm) as indicator strain.

PRODUCER STRAINS (Xcg)	Sensitive Indicator Strains (Xcv)	Total	(Xcm)	Total
Xcg_{24}	Xcv_{153}	1	none	0
Xcg_{44}	Xcv_{61}, Xcv_{101}, Xcv_{112}, Xcv_{122}	4	Xcm_{20}, Xcm_{10}	2
Xcg_{45}, Xcg_{10} Xcg_{91}	Xcv_{23}, Xcv_{58}, Xcv_{61}, Xcv_{63}, Xcv_{47}, Xcv_{85}, Xcv_{99}, Xcv_{103}, Xcv_{104}, Xcv_{152}, Xcv_{153}, Xcv_{154}	12	Xcm_{20}, Xcm_{10}	2
Xcg_{103}, Xcg_{56}	same as above and including Xcv_{10}	13	Xcm_{20}, Xcm_{10}	2
Xcg_{11}	Xcv_{4} to Xcv_{6}, Xcv_{10} to Xcv_{12}, Xcv_{14}, Xcv_{17}, Xcv_{18}, Xcv_{20}, Xcv_{21}, Xcv_{23}, Xcv_{24}, Xcv_{27} to Xcv_{29}, Xcv_{32} to Xcv_{40}, Xcv_{43}, Xcv_{46} to Xcv_{51}, Xcv_{54}, Xcv_{58}, Xcv_{61}, Xcv_{63}, Xcv_{65}, Xcv_{74}, Xcv_{77}, Xcv_{85}, Xcv_{99}, Xcv_{101}, Xcv_{103}, Xcv_{108}, Xcv_{112}, Xcv_{137}, Xcv_{142}, Xcv_{148}, Xcv_{149}, Xcv_{152}, Xcv_{154}	53	Xcm_{9} to Xcm_{12}, Xcm_{19}, Xcm_{20}	6
Xcg_{38}	same as above except Xcv_{10}, Xcv_{148} and Xcv_{74}	50	Xcm_{9} to Xcm_{12} Xcm_{3}, Xcm_{4}, Xcm_{19}	7

Table 5. Detection and efficiency of bacteriocin producer strains of *Xanthomonas campestris* pv. *vesicatoria* (Xcv) using Xcv indicator strains.

PRODUCER STRAINS	SENSITIVE INDICATOR STRAINS[a]	DIAMETER OF INHIBITION ZONES (mm)[b]
Xcv_{5}	Xcv_{58}	7.5
Xcv_{90}	Xcv_{23}	6.0
Xcv_{137}	Xcv_{34}	7.0
Xcv_{145}	Xcv_{28}, Xcv_{33}	5.5-6.3
Xcv_{77}	Xcv_{23}, Xcv_{28}, Xcv_{36}, Xcv_{71}	5.0-7.0
Xcv_{48}	Xcv_{9} to Xcv_{11}, Xcv_{43}	8.0-17.0
Xcv_{56}	Xcv_{18}, Xcv_{23}, Xcv_{28}, Xcv_{33}, Xcv_{57}, Xcv_{58}, Xcv_{60}, Xcv_{95}, Xcv_{149}, Xcv_{154}	21.0-28.3
Xcv_{34}	Xcv_{9} to Xcv_{11}, Xcv_{17}, Xcv_{18}, Xcv_{23}, Xcv_{31}, Xcv_{39}, Xcv_{43}, Xcv_{45}, Xcv_{49}	15.0-24.5
Xcv_{42}	Xcv_{10}, Xcv_{11}, Xcv_{16} to Xcv_{21}, Xcv_{23}, Xcv_{28}, Xcv_{31}, Xcv_{33}, Xcv_{34}, Xcv_{36}, Xcv_{37}, Xcv_{39}, Xcv_{43} to Xcv_{45}, Xcv_{48}, Xcv_{49}, Xcv_{54}, Xcv_{57}, Xcv_{58}, Xcv_{60} to Xcv_{63}, Xcv_{66}, Xcv_{71} to Xcv_{74}, Xcv_{77}, Xcv_{85}, Xcv_{88}, Xcv_{94}, Xcv_{99}, Xcv_{101} to Xcv_{103}, Xcv_{106}, Xcv_{108}, Xcv_{100}, Xcv_{112}, Xcv_{122}, Xcv_{137}, Xcv_{142}, Xcv_{144}, Xcv_{146}, Xcv_{148}, Xcv_{149}, Xcv_{151} to Xcv_{154}.	12.0-26.0

[a]No. of indicator strains tested: 147

[b]Range of zones produced against sensitive indicator strains.

Table 6. Detection of *X. campestris* pv. *vesicatoria* (Xcv) bacteriocin producer strain using the pathovars *glycines* (Xcg) and *malvacearum* (Xcm) as indicator strains

PRODUCER STRAINS (Xcv)	SENSITIVE INDICATOR STRAINS (Xcg)	(Xcm)
Xcv_{42}	Xcg_{59}, Xcg_{66}	Xcm_{9}, Xcm_{10},
	Xcg_{68}, Xcg_{74}	Xcm_{11}, Xcm_{12}
	Xcg_{77}, Xcg_{110}	Xcm_{19}, Xcm_{20}

Table 7. Detection and efficiency of bacteriocin producing strains of *Xanthomonas campestris* pv. *malvacearum* (Xcm) using Xcm indicator strains

PRODUCER STRAINS	SENSITIVE INDICATOR STRAINS[a]	DIAMETER OF INHIBITION ZONES (mm)[b]
Xcm_1, Xcm_2, Xcm_4	Xcm_{20}, Xcm_{21}	8.0-15.0
Xcm_5, Xcm_6, Xcm_7,		
Xcm_9, Xcm_{10}, Xcm_{15},		
Xcm_{19}		

[a]No. of indicator strains tested: 22

[b]Range of zones produced against sensitive indicator strains.

Table 8. Detection of *X. campestris* pv. *malvacearum* (Xcm) bacteriocin producer strain using the pathovar *vesicatoria* (Xcv) as indicator strain.

PRODUCER STRAINS (Xcm)	SENSITIVE INDICATOR STRAINS (Xcv)	TOTAL
Xcm_3	Xcv_4	1
Xcm_4	Xcv_{49}, Xcv_{103}, Xcv_{93}, Xcv_{10}	4
Xcm_5	Xcv_{11}, Xcv_{49}, Xcv_{103}	3
Xcm_6	Xcv_{11}, Xcv_{49}, Xcv_{103}, Xcv_{124}	4
Xcm_9	Xcv_{11}, Xcv_{93}, Xcv_{124}	3
Xcm_{11}	Xcv_{49}	1
Xcm_{15}	Xcv_{11}, Xcv_{10}, Xcv_{28}, Xcv_{49}, Xcv_{103}, Xcv_{124}	7
Xcm_{19}	Xcv_{49}, Xcv_{11}, Xcv_{107}	3
Xcm_2, Xcm_7, Xcm_{12}	Xcv_{11}	1

Xcv (9/147 or 6%) (Table 5). In Xcm, while the frequency of bacteriocin producers was high (10/22 or 45%) (Table 7) the bacteriocin activity spectra was very narrow (2/22 or 9%).

The sensitivity of some Xcv and Xcm strains to bacteriocins produced by some Xcg strains (Table 4) or some Xcg and Xcm strains to an Xcv bacteriocin (Table 6) indicate close relationships among these pathovars.

Xcg 11 and Xcg 38 which produced bacteriocins active against 33% (25/75) of the test Xcg strains (Table 3) and Xcv 42 whose bacteriocins were potent against 44% (65/147) of the test Xcv strains (Table 5) appeared to be the best possible sources of avirulent bacteriocin producer mutants for biocontrol studies. Xcm 15, although it was the best among the Xcm bacteriocin producers has doubtful potential because of its limited biological activity.

PART II

ISOLATION OF AVIRULENT BACTERIOCIN PRODUCER MUTANTS OF VIRULENT WILD TYPE BACTERIOCIN PRODUCER STRAINS OF *XANTHOMONAS CAMPESTRIS* PV. *GLYCINES*, PV. *VESICATORIA*, AND PV. *MALVACEARUM*

INTRODUCTION

After identifying the best among the virulent wild type bacteriocin producer strains (Part I) in terms of in-vitro biological activity of the bacteriocins (zone of growth inhibition of indicator strains and or number of isolates sensitive to the bacteriocins of the producer strains), the next logical step was to produce from these strains avirulent mutants that would retain the ability to produce bacteriocins. Selected strains of these mutants which were referred to as avirulent bacteriocin producer strains (AVBPS) from here on were used in field biocontrol studies (Part III).

The specific objectives of this part of the project were to (1) induce and isolate avirulent bacteriocin producer mutants from selected virulent wild type bacteriocin producer strains, (2) determine the activity spectra of these mutants against virulent wild type strains, (3) identify the mutants (AVBPS) for biocontrol studies, and (4) determine the stability of the AVBPS selected for biocontrol studies.

MATERIALS AND METHODS

Virulent wild type bacteriocin producer strains (WBPS) used

Xcg-11 (*X. campestris* pv. *glycines* isolate no. 11) and Xcg-38, respectively, from BPI Economic Garden, Los Baños, Laguna and

Tranca, Bay, Laguna, Xcv-42 (X. campestris pv. vesicatoria) from Bago Oshiro, Davao City, and Xcm-15 (X. campestris pv. malvacearum) from IPB, College, Laguna were used in the study. These isolates were the best possible sources of AVBPS for biocontrol studies based on studies presented in Part I of the project.

Induction of avirulent bacteriocin producer strains from WBPS

a. N-methyl-N-nitro-N-nitrosoguanidine (NG) mutagenesis

The selected WBPS was grown in 50 ml peptone sucrose broth (PSB) for 24 hr at room temperature. Five ml cell suspensions were centrifuged at 3000 rpm for 15 mins. The cells were then washed twice with 0.1 M sodium citrate/citric acid buffer (pH 5.5) and resuspended in 5 ml of the same buffer. To the cell suspension, 0.1 ml of freshly prepared NG (Sigma) (1 mg in 2 ml citrate buffer) was added. The tubes were left at room temperature for periods of 10, 20, 30 and 40 minutes. After exposure to the mutagen, cells were centrifuged at 3000 rpm for 15 minutes, washed twice with 0.1 M sodium phosphate buffer (pH 7.0) and resuspended in 5 ml of the same buffer.

Serial dilutions and surface plating on PSA were performed for each batch of mutagenized cells. All plates were incubated at room temperature for 48 hours. The number of colonies were counted for each exposure period and the % survival and % kill were computed using the formula:

$$\% \text{ survival} = \frac{\text{no. of surviving cells for each exposure pd.}}{\text{no. of cells exposed}} \times 100$$

$$\% \text{ kill} = 100\% - \% \text{ survival}$$

Cells (colonies) from the exposure period that gave a 99.9% kill, were considered possible mutants; these colonies were isolated, multiplied and tested for pathogenicity and ability to produce bacteriocins as described in Part I of the project.

b. Acridine Orange Mutagenesis

Acridine Orange was used to mutagenize Xcv 42 and Xcm 15. The same procedures as in NG mutagenesis were followed except that Acridine Orange (Sigma) was used instead of NG.

c. Ultraviolet (UV) Light Mutagenesis

UV light mutagenesis was used on Xcv 42, Xcm 15, Xcg 11 and Xcg 38. The same procedure of cell preparation as mentioned in NG mutagenesis was done except that UV light was used as the mutagen. The exposure to UV light (GE) (253 nm) was 0, 2, 4, 6, 8, and 10 nm at a fixed distance of 1 ft. Isolation of UV light-induced mutants was done from treatments giving a 99.9% kill. Isolates were assayed for bacteriocin and tested for pathogenicity.

d. Spontaneous Mutation

This technique was used on Xcv 42, Xcm 15, Xcg 11 and Xcg 38. Weekly transfers of the cultures on nutrient agar + 5% sucrose were performed for several weeks. Occasional transfers on tetrazolium chloride agar (25) were also done. Colonies with different morphologies were cultivated on PSA, assayed for bacteriocin, and

RESULTS AND DISCUSSION

N-methyl-N-nitro-N-nitrosoguanidine (NG) mutagenesis

Two of the most effective bacteriocin producing Xcg isolates (Xcg 11 and Xcg 38), 4 Xcv isolates (Xcv 42, Xcv 45, Xcv 34 and Xcv 77) and 1 Xcm isolate (Xcm 15) were exposed to NG mutagen. There were 96 possible mutants isolated, 30 isolates from Xcg 11, 15 from Xcg 33, 3 from Xcv 45, 10 from Xcv 42, 12 from Xcv 34, 16 from Xcv 77, and 10 from Xcm 15. Most of these mutants were light yellow in color and were not producing mucous as much as the parent wild type strains. There were also some mutant isolates that totally lost the yellow pigmentation but retained the mucoidal properties. A few of the isolates were observed to be both non-mucoidal and non-pigmented.

Out of the 45 possible Xcg mutants, 32 retained the ability to produce bacteriocins. However, pathogenicity tests of these strains on soybean showed that only 3 were avirulent; all these 3 avirulent bacteriocin producing strains (AVBPS) came from Xcg 11 and were small, circular, non-pigmented (white) and non-mucoidal (Fig. 7); they were designated Xcg 11-5, Xcg 11-9, and Xcg 11-11. Further tests for bacteriocin production showed that these mutants were effective not only against other Xcg strains but also against Xcv and Xcm indicator isolates (Table 9). These mutants were also active against a wider range of pathogenic indicator strains than the wild type producer strain (Table 10). The zones of inhibition produced by the mutants were also comparable or sometimes larger than their parent strains (Table 11) (Figs. 8, 9 and 10). The mutants also

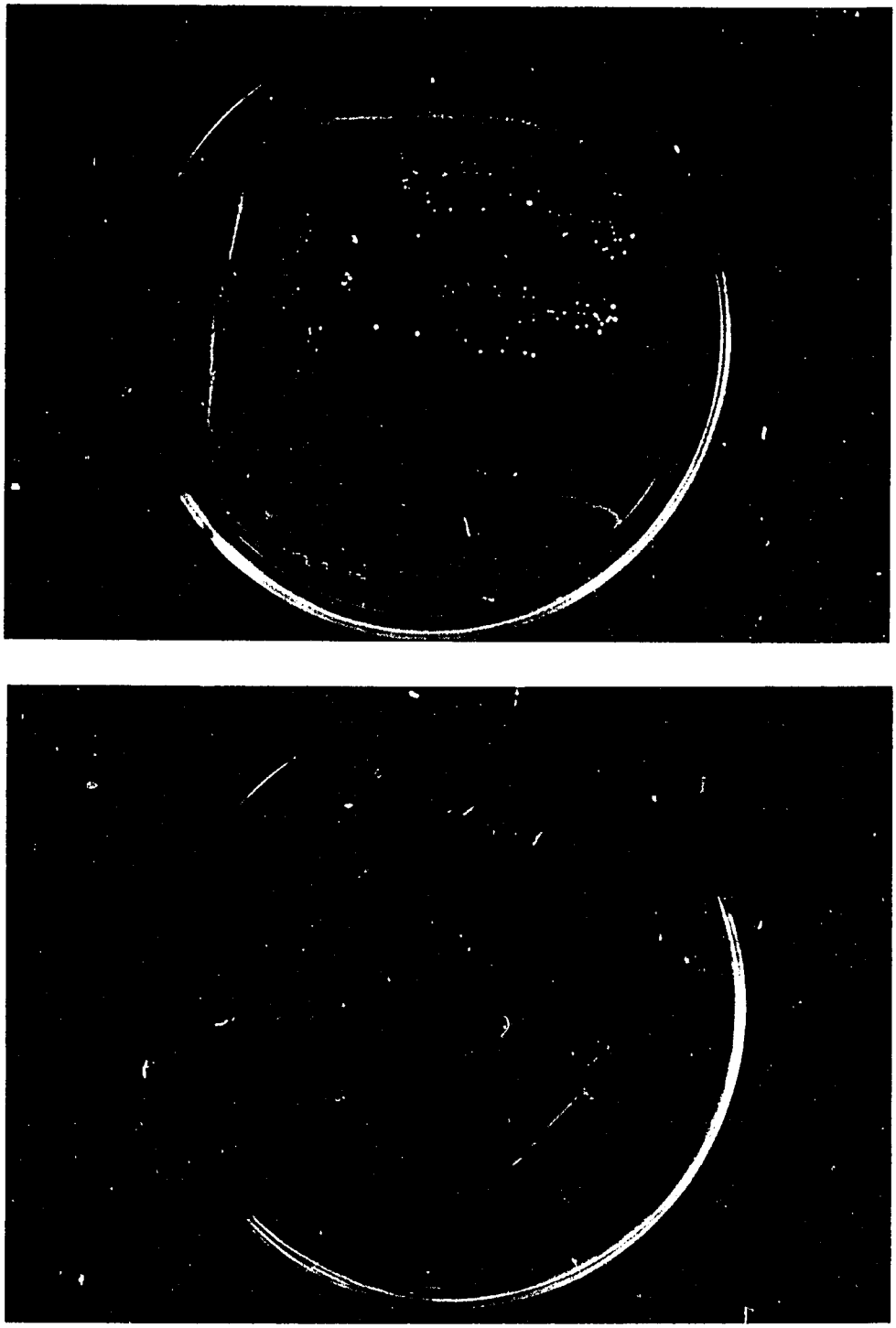


Fig. 7. Pure cultures of 48-hr old NG-induced avirulent bacteriocin producer strain (AVBPS) of X. campestris pv. glycines (Xcg 11-9) (A) and its parent virulent wild type bacteriocin producer strain (Xcg 11) (B) on PSA.

Table 10. Bacteriocin production of wild type and NG-induced mutant strains of *X. campestris* pv. *glycines* (Xcg 11 and Xcg 38) against wild type pathogenic *X. campestris* pv. *vesicatoria.*

PRODUCER STRAINS[1]	Diameter of zones of inhibition (mm)[2]										
	Indicator Strains										
	Xcv_4	Xcv_{38}	Xcv_{29}	Xcv_{37}	Xcv_{53}	Xcv_{27}	Xcv_{43}	Xcv_{20}	Xcv_{34}	Xcv_{47}	Xcv_{46}
Xcg_{11}	30.0	0	27.5	24.0	20.0	26.0	29.5	27.0	26.0	27.5	27.0
Xcg_{38}	28.5	0	29.0	24.0	29.0	29.0	29.5	26.0	25.5	26.5	26.5
Xcg_{11}-9	20.5	19.0	18.5	22.0	25.0	26.0	17.5	0	13.5	22.0	22.5
Xcg_{11}-5	19.5	15.0	12.0	20.0	17.5	17.0	17.5	16.5	16.0	14.0	18.5
Xcg_{11}-11	12.5	13.5	22.5	13.0	17.5	25.0	16.0	16.0	26.0	23.5	16.5

Table 10 continued .....

PRODUCER STRAINS 1/	Diameter of zones of inhibition (mm) 2/ Indicator Strains											
	Xcv_{49}	Xcv_{12}	Xcv_{12}	Xcv_{43}	Xcv_{5}	Xcv_{11}	Xcv_{51}	Xcv_{35}	Xcv_{6}	Xcv_{36}	Xcv_{17}	Xcv_{18}
Xcs_{11}	26.0	23.0	0	29.0	27.0	25.5	29.0	26.0	11.0	29.0	22.5	28.5
Xcs_{38}	30.0	27.0	0	23.5	26.5	29.5	28.5	28.5	11.5	27.0	27.0	26.5
Xcs_{11}-9	25.0	22.5	18.5	14.0	12.0	22.0	21.0	23.0	17.5	15.0	12.0	19.0
Xcs_{11}-5	28.0	13.0	16.0	11.0	11.0	15.0	21.0	18.0	22.5	15.5	15.5	13.50
Xcs_{11}-11	12.0	17.5	25.5	20.5	14.5	13.0	22.0	20.0	20.0	15.0	10.0	21.0

Table 10 continued .....

PRODUCER STRAIN[1/]	Diameter of zones of inhibition (mm)[2/] Indicator Strains											
	Xcv_{54}	Xcv_{40}	Xcv_{65}	Xcv_{24}	Xcv_{39}	Xcv_{9}	Xcv_{21}	Xcv_{32}	Xcv_{30}	Xcv_{50}	Xcv_{28}	Xcv_{14}
Xcg_{11}	23.0	24.5	26.5	26.0	28.0	0	26.5	28.5	29.0	28.5	28.0	26.5
Xcg_{38}	27.5	25.5	27.0	27.0	26.5	0	26.5	27.5	27.0	0.0	28.5	30.0
Xcg_{11}-9	22.5	20.0	14.0	15.0	12.0	19.0	22.5	15.0	17.0	13.5	20.0	15.0
Xcg_{11}-5	15.5	13.5	16.5	11.0	20.5	13.5	17.5	17.0	11.0	12.5	15.5	16.0
Xcg_{11}-11	22.0	17.5	11.5	12.0	12.0	12.5	20.0	20.0	12.0	14.5	15.0	12.0

1/ Virulent wild type bacteriocin producer strains (WBPS) - Xcg 11, Xcg 38; Avirulent bacteriocin producer strains (AVBPS) - Xcg 11-9, Xcg 11-5, Xcg 11-11.

2/ Average of 2 measurements; zones of inhibition indicates bacteriocin production.

teriocin production of wild type and NG-induced mutant strains of X. campestris pv. glycines
g 11 and Xcg 38) against wild type pathogenic X. c. pv. glycines.

Diameter of zones of inhibition (mm)																			
Indicator Strains																			
Xcg44	Xcg59	Xcg48	Xcg26	Xcg39	Xcg30	Xcg47	Xcg50	Xcg40	Xcg41	Xcg25	Xcg49	Xcg45	Xcg19	Xcg23	Xcg64	Xcg63	Xcg16	Xcg25	Xcg36
0	15.0	14.5	0	13.0	0	0	16.3	0	0	20.0	0	13.0	13.0	15.0	0	0	0	14.5	0
0	16.0	17.5	0	17.0	0	0	18.0	0	0	20.0	0	0	16.5	17.0	0	0	0	14.0	0
17.3	16.0	10.0	14.0	17.5	15.0	15.3	19.3	17.5	18.5	15.0	17.5	17.0	15.5	15.0	12.5	21.0	25.5	16.5	14.0
17.3	12.0	12.0	15.7	17.6	12.7	12.3	21.3	11.5	22.5	15.5	12.5	19.0	13.0	14.0	15.5	19.5	19.5	19.5	17.5
20.3	13.0	13.5	12.6	14.0	13.0	14.3	17.7	16.0	15.5	22.5	17.0	19.0	16.5	20.0	15.0	21.0	18.0	15.5	17.5

: of 2 measurements; zones of inhibition indicates bacteriocin production.
Xcg 11, Xcg 38; AVBPS - Xcg 11-9, Xcg 11-5, Xcg 11-11.

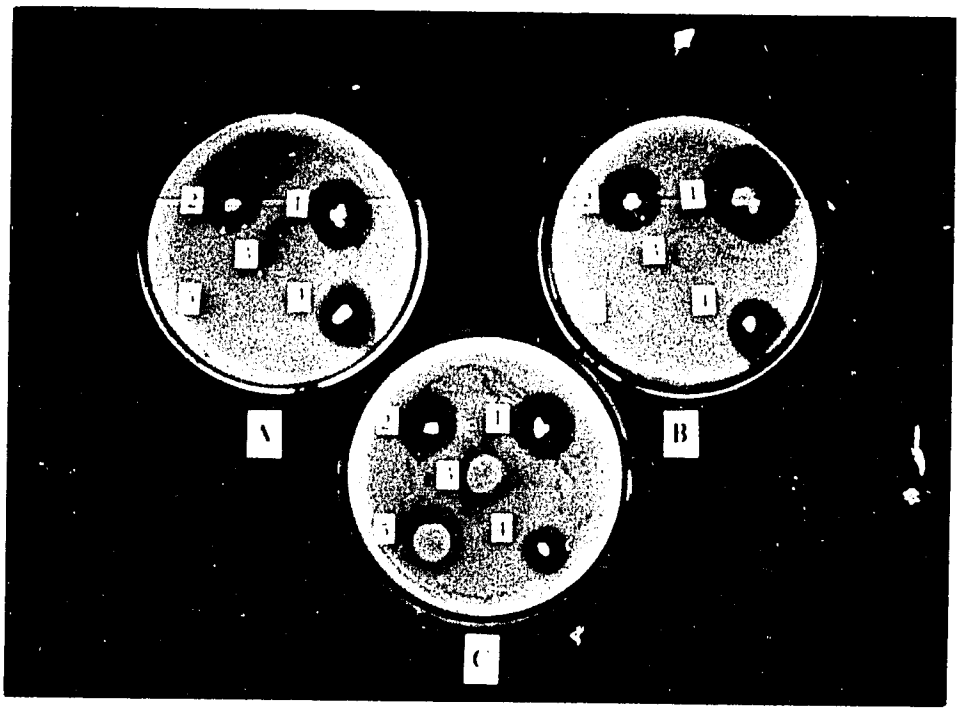


Fig. 8. Bacteriocin production of (1) Xcg 11-9, (2) Xcg 11-5, (3) Xcg 38, (4) Xcg 11-11, and (5) Xcg 11 on indicator (A) Xcg 44 (B) Xcg 16 and (C) Xcg 5.

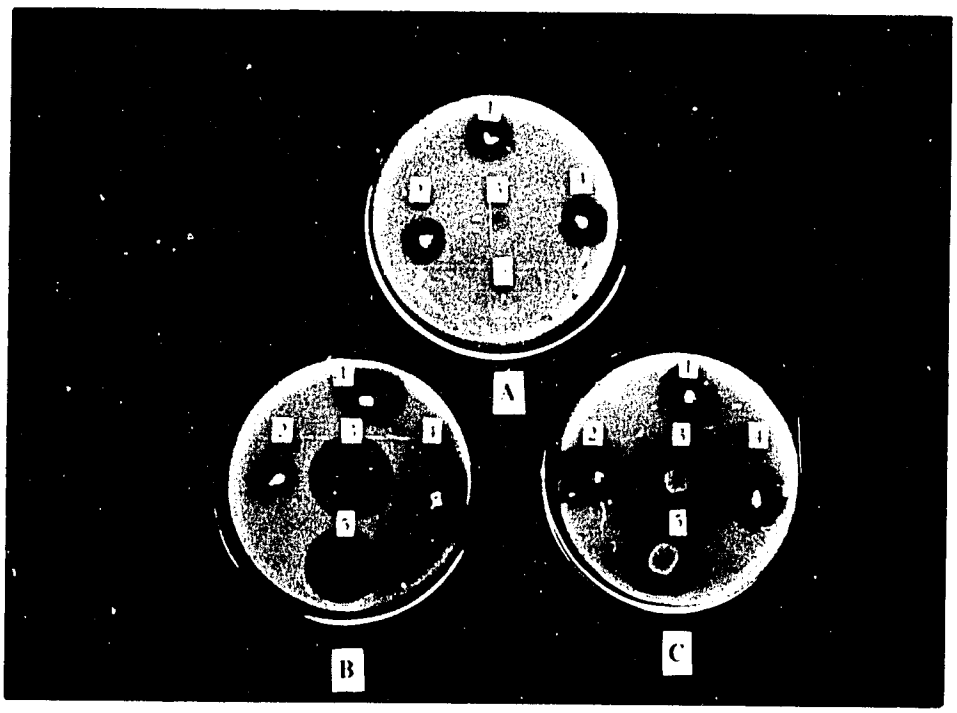


Fig. 9. Bacteriocin production of (1) Xcg 11-9, (2) Xcg 11-5, (3) Xcg 38, (4) Xcg 11-11 and (5) Xcg 11 on indicator (A) Xcv 13, (B) Xcv 51 and (c) Xcv 29.

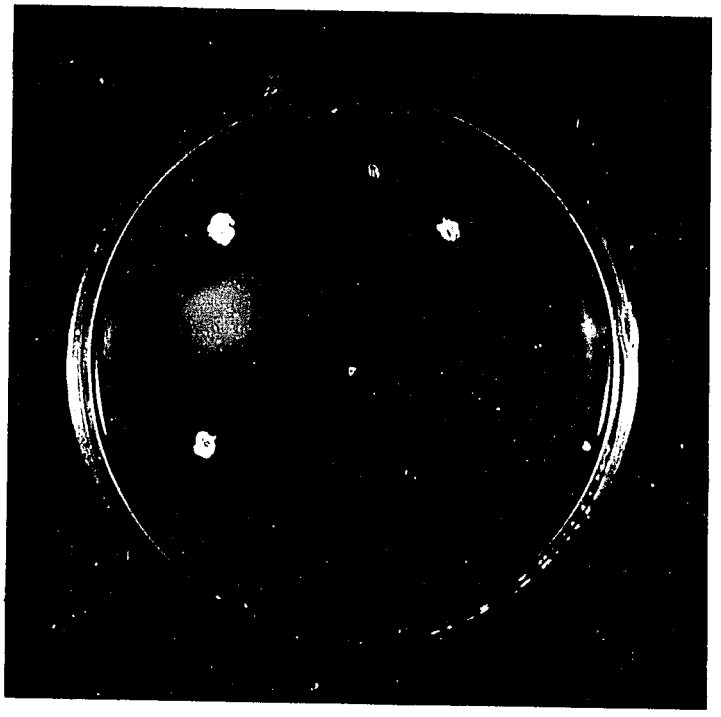


Fig. 10. Bacteriocin assay of NG-induced avirulent bacteriocin producer strains (AVBPS) (white colonies) of X. campestris pv. glycines (Xcg) and their virulent wild type bacteriocin producer parent strains (yellow colony) against a wild type Xcg strain.

retained their bacteriocin producing ability and non-pigmented and non-mucoidal properties even after more than 1 year of weekly transfers in PSB and PSA. Moreover, repeated pathogenicity tests of these cultures on soybean proved negative.

NG mutageneses to produce AVBPS of Xcv and Xcm were not successful. The possible mutant isolates either lost their ability to produce bacteriocins and or retained their virulence (pathogenicity) to their respective host plants.

Acridine Orange mutagenesis

For failure to produce AVBPS of Xcv and Xcm by NG mutagenesis, Acridine Orange G was used to mutagenize Xcv 42 and Xcm 15; a concentration of 40 ug/ml was used after preliminary trials showed that it induced low survival rates. Morphotypes similar to those that were induced on Xcg by NG mutagenesis were observed. Out of 45 possible mutants of Xcm 15, 17 retained the ability to produce bacteriocins while all 11 mutant isolates of Xcv 42 were bacteriocin producers. Pathogenicity tests of these mutant bacteriocin producers showed that they were all avirulent; the colonies of these AVBPS were small, circular, non-pigmented (white) and non-mucoidal, very similar to AVBPS of Xcg (Fig. 11). Further bacteriocin assays of these AVBPS showed that they (their bacteriocins) were active against both intra- and inter-pathovar strains. They were also active against a wider range of indicator strains than that of their parent virulent wild type bacteriocin producer strains (WBPS)(Fig. 12, 13 (Tables 12, 13, 14, 15, 16, 17). They retained their bacteriocin producing ability, colony characters, and avirulent nature after more than one year of weekly transfers in PSB or PSA.

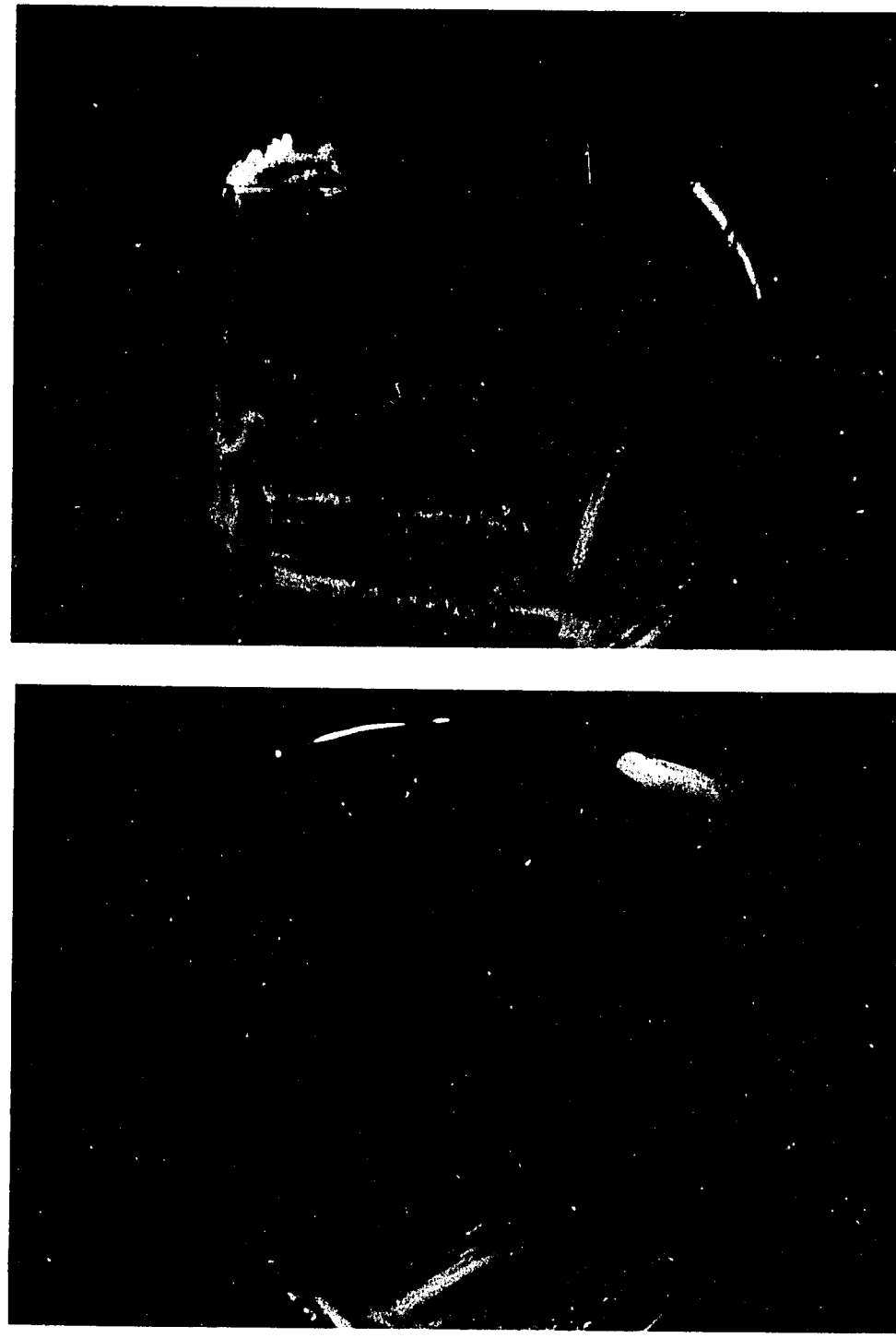


Fig. 11. Pure cultures of 72-hr old Acridine orange-induced avirulent bacteriocin producer mutants of X. campestris pv. vesicatoria (Xcv 42-4) and pv. malvacearum (Xcm 15-16) on PSA.

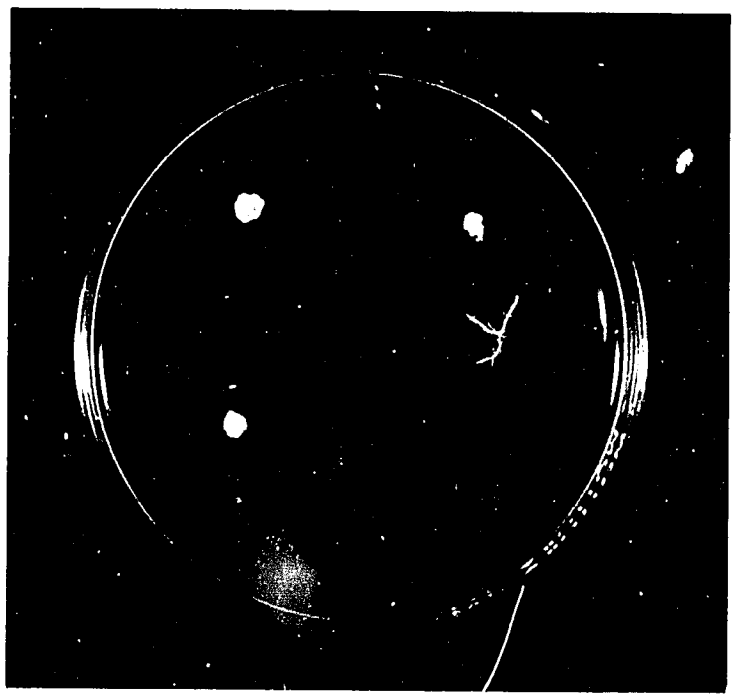


Fig. 12. Bacteriocin assay of Acridine orange-induced avirulent bacteriocin producer strains (AVBPS) (white colonies) of X. campestris pv. malvacearum (Xcm) and their virulent wild type bacteriocin producer parent strain (yellow colony) against a wild type Xcm strain.

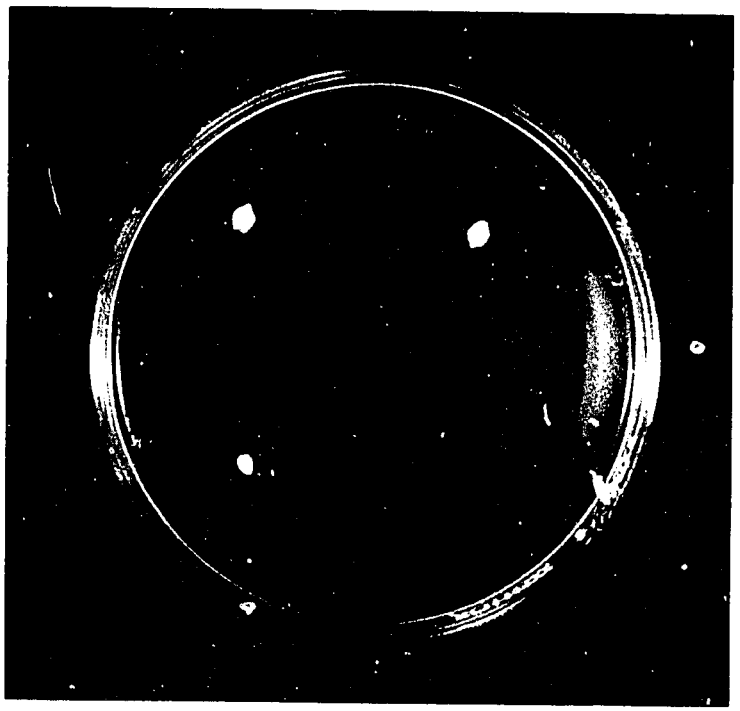


Fig. 13. Bacteriocin assay of Acridine orange-induced avirulent bacteriocin producer strains (AVBPS) (white colonies) of X. campestris pv. vesicatoria (Xcv) and their virulent wild bacteriocin producer parent strain (yellow colony) against a wild type Xcv strain.

Table 12. Bacteriocin production of wild type and Acridine Orange-induced mutant strains of X. campestris pv. vesicatoria (Xcv 42) against wild type pathogenic X. campestris pv. vesicatoria (Xcv) strains.

Producer strain[1]	Diameter of Inhibition Zones (mm)[2]									
	Indicator Strains									
	Xcv 33	Xcv 10	Xcv 122	Xcv 25	Xcv 31	Xcv 21	Xcv 154	Xcv 48	Xcv 148	Xcv 57
Xcv_{42}	18.5	17.5	18.5	19	14.5	18.5	26	19	18.5	19.5
Xcv_{42-1}	20.5	18.5	17	16.5	26	20.5	27.5	21	25.1	18
Xcv_{42-2}	21	21.5	14	24.5	20	20.5	18	17.5	22.5	25
Xcv_{42-3}	23	17	14	19.5	19.5	19.5	26.5	18	24	23.5
Xcv_{42-4}	25.5	21.5	17	22.5	23.5	21.5		27.5	25	24.5

1/ All mutant producer strains were 4.5 mm in diameter while the wild type producer strains were 6.5 mm in diameter. WBPS - Xcv 42; AVBPS - Xcv 42-1 to 4.

2/ Average of 2 measurements; zone of inhibition indicates bacteriocin production.

Table 12 continued .....

Producer strain[1]	Diameter of Inhibition Zones (mm)[2]								
	Indicator Strains								
	Xcv 23	Xcv 16	Xcv 49	Xcv 43	Xcv 42	Xcv 12	Xcg 11	Xcg 45	Xcg 56
Xcv_{42}	19	14	17	19	0	0	0	0	0
Xcv_{42-1}	16.5	24	22.5	18.5	19	35.5	32	10	0
Xcv_{42-2}	13.5	25.5	16	20	21.5	35.5	4.5	25	28
Xcv_{42-3}	22.5	17.5	23	17.5	24.5	35.5	6.5	6.0	0
Xcv_{42-4}	26.5	22	23.5	20	29	35.5	34	21.5	25

Table 12 continued .....

Producer strain[1]	Diameter of Inhibition Zones (mm)[2]					
	Indicator Strains					
	Xcg 66	Xcg 68	Xcg 74	Xcg 77	Xcg 88	Xcg 91
Xcv_{42}	0	0	0	0	0	0
Xcv_{42-1}	25	11	13.5	0	6.5	19.5
Xcv_{42-2}	30.5	27	5.5	8.5	8.5	26
Xcv_{42-3}	20.5	25	13.5	-	-	6.0
Xcv_{42-4}	26.5	32.5	33.0	21.5	10.5	18.5

Table 13. Activity of X. campestris pv vesicatoria (Xcv) avirulent bacteriocin producer strains (mutants) against Xcv and the pathovars glycines (Xcg), and malvacearum (Xcm) wild types

Avirulent Xcv Bacteriocin Producer Mutants	No. sensitive Xcv wild type / Total No. isolates tested	No. sensitive Xcg wild type / Total No. isolates tested	No. sensitive Xcm wild type / Total No. isolates tested
Xcv_{42}-1	16/16	-	-
Xcv_{42}-2	16/16	-	-
Xcv_{42}-3	16/16	-	-
Xcv_{42}-4	70/71	2/2	9/9

- no data

Table 14. Bacteriocin production of wild type and Acridine Orange induced mutant strains of X. campestris pv. malvacearum (Xcm 15) against some wild type pathogenic X. campestris pv. glycines (Xcg) strains.

Producer strain[1]	Diameter of Inhibition Zones (mm)[2]						
	Indicator Strains						
	Xcg 11	Xcg 45	Xcg 60	Xcg 74	Xcg 77	Xcg 84	Xcg 88
Xcm 15	0	0	-	0	0	0	0
Xcm 15-11	16.5	0	18	15	0	13.5	10
Xcm 15-12	34.5	14	16.5	8	13.5	12	14
Xcm 15-14	0	22	22.5	19	14.5	15	12.5
Xcm 15-16	35	-	28.5	36	33	23	29
Xcm 15-18	23	18	18	8.5	14.5	0	12.5
Xcm 15-19	3.5	9	12.5	12.5	13.0	0	13.5

1/ WBPS - Xcm 15; AVBPS - Xcm 15-11, -12, -14, -16, -18, -19.

2/ Average of 2 measurements; zone of inhibition indicates bacteriocin production.

Table 15. Activity of *X. campestris* pv *malvacearum* (Xcm) avirulent bacteriocin producer mutants against Xcm and the pathovars *glycines* (Xcg), and *vesicatoria* (Xcv) wild types

Avirulent Xcm Bacteriocin Producer Mutants	No. sensitive Xcm wild types / Total No. isolates tested	No. sensitive Xcg wild types / Total No. isolates tested	No. sensitive Xcv wild types / Total No. isolates tested
Xcm_{15}-11	9/9	-	-
Xcm_{15}-12	9/9	-	-
Xcm_{15}-14	9/9	-	-
Xcm_{15}-16	17/18	2/2	55/55
Xcm_{15}-18	9/9	-	-
Xcm_{15}-19	9/9	-	-

- no data

Table 16. Bacteriocin production of wild type and Acridine Orange-induced mutant strains of X. campestris pv. malvacearum (Xcm 15) against wild type pathogenic X. campestris pv. malvacearum (Xcm) strains.

Producer strain 1/	Diameter of Inhibition Zones (mm) 2/ Indicator Strains								
	Xcm 5	Xcm 19	Xcm 6	Xcm 4	Xcm 10	Xcm 9	Xcm 7	Xcm 11	Xcm 15
Xcm 15	0	0	0	0	0	0	0	0	0
Xcm 15-11	22	15.5	7.5	11	14.5	10.5	11.5	20.5	19.5
Xcm 15-12	24	14.5	12.5	16.5	13.5	7.5	10	26.5	11
Xcm 15-14	20	13.5	8.5	15	17	15.5	16	24.5	16.5
Xcm 15-16	24	15.5	15	15.5	15	17.5	14	34.5	18.5
Xcm 15-18	9.5	11	13.5	6.5	7.5	16	10	25.5	20.5
Xcm 15-19	20.5	16.5	6.5	21.5	8	14.5	15.5	30.5	8.5

1/ All mutant producer strains were 3.5 mm in diameter while the wild type producer strains were 5.5 mm in diameter. WBPS - Xcm 15; AVBPS - Xcm 15-11, -12, -14, -16, -18, -19.

2/ Average of 2 measurements; zone of inhibition indicates bacteriocin production.

Table 17. Bacteriocin production of wild type Xcv and its mutant Xcv 42-4 and Xcm 15 and its mutant Xcm 15-16 against wild type pathogenic Xcv, Xcm and Xcg strains.

Producer strain[1]	Diameter of Inhibition Zones (mm)[2]									
	Indicator Strains									
	Xcv 37	Xcv 102	Xcv 99	Xcv 25	Xcv 48	Xcv 149	Xcv 23	Xcv 11	Xcv 21	Xcv 9
Xcv_{42}	26.5	23	21	16	26	0	29	20	21	0
Xcm_{15}	6	5	8	6	7	0	6	5	9	0
Xcv_{42-4}	28.5	16.5	16.5	24.5	15.5	17.5	8.5	31.5	31.5	14.5
Xcm_{15-16}	6.5	12.5	10.5	6.5	8.5	13.5	12.5	12.5	26.5	6.5

1/ All mutant producer strains were 3.5 mm in diameter while the wild type strains were 9 mm in diameter. WBPS - Xcv 42, Xcm 15; AVBPS - Xcv 42-4, Xcm 15-16.

2/ Average of 2 measurements; zone of inhibition indicates bacteriocin production.

Table 17 continued .....

Producer strain	Diameter of Inhibition Zones (mm)									
	Indicator Strains									
	Xcv 19	Xcv 108	Xcv 35	Xcv 102	Xcv 16	Xcv 10	Xcv 31	Xcv 61	Xcv 18	Xcv 154
Xcv_{42}	21	16	2	23	9	21	19	21	19	0
Xcm_{15}	3	4	0	8	1	6	7	5	0	0
Xcv_{42-4}	11.5	11.5	10.5	0	21.5	26.5	31.5	16.5	15.5	18.5
Xcm_{15-16}	16.5	6.5	13.5	11.5	16.5	18.5	21.5	16.5	17.5	11.5

Table 17 continued .....

Producer strain	Diameter of Inhibition Zones (mm)									
	Indicator Strains									
	Xcv 43	Xcv 100	Xcv 33	Xcv 57	Xcv 103	Xcv 124	Xcv 54	Xcv 122	Xcv 31	Xcv 75
Xcv_{42}	23	26	18	0	20	21	16	16	16	21
Xcm_{15}	7	4	6	0	6	1	8	8	11	7
Xcv_{42-4}	26.5	16.5	13.5	32.5	11.5	14.5	23.5	21.5	26.5	16.5
Xcm_{15-16}	12.5	14.5	14.5	11.5	13.5	16.5	15.5	14.5	7.5	11.5

Table 17 continued .....

Producer strain	Diameter of Inhibition Zones (mm)									
	Indicator Strains									
	Xcv 101	Xcv 66	Xcv 76	Xcv 18	Xcv 17	Xcv 16	Xcv 20	Xcv 28	Xcv 34	Xcv 36
Xcv_{42}	26	23	25	21	18	16	19	26	22	21
Xcm_{15}	1	6	10	0	8	10	5	0	0	0
Xcv_{42-4}	26.5	25.5	31.5	14.5	21.5	23.5	24.5	28.5	22.5	16.5
Xcm_{15-16}	10.5	16.5	16.5	4.5	17.5	18.5	21.5	23.5	15.5	11.5

Table 17 continued .....

Producer strain	Diameter of Inhibition Zones (mm)									
	Indicator Strains									
	Xcv 39	Xcv 44	Xcv 45	Xcv 49	Xcv 58	Xcv 60	Xcv 62	Xcv 63	Xcv 71	Xcv 106
Xcv_{42}	18	16	18	20	15	26	28	19	18	17
Xcm_{15}	3	11	4	6	11	6	0	10	0	11
Xcv_{42-4}	26.5	31.5	23.5	15.5	17.5	32.5	26.5	27.5	21.5	12.5
Xcm_{15-16}	12.5	18.5	20.5	15.5	19.5	16.5	18.5	17.5	17.5	14.5

Table 17 continued .....

Producer strain	Diameter of Inhibition Zones (mm)								
	Indicator Strains								
	Xcv 146	Xcv 30	Xcv 23	Xcv 70	Xcv 57	Xcm 15	Xcm 12	Xcm 10	Xcm 5
Xcv_{42}	13	0	0	0	0	0	0	22	0
Xcm_{15}	4	0	0	3	0	0	-	0	0
Xcv_{42-4}	25.5	16.5	21.5	16.5	32.5	18.5	10.5	6.5	28.5
Xcm_{15-16}	12.5	8.5	13.5	14.5	11.5	16.5	-	12.5	11.5

Table 17 continued .....

Producer strain	Diameter of Inhibition Zones (mm)						
	Indicator Strains						
	Xcm 6	Xcm 2	Xcm 7	Xcm 11	Xcm 4	Xcg 36	Xcg 91
Xcv_{42}	0	0	0	0	0	2	0
Xcm_{15}	0	0	0	0	0	0	0
Xcv_{42-4}	21.5	26.5	16.5	20.5	24.5	26.5	15.5
Xcm_{15-16}	16.5	20.5	14.5	21.5	10.5	11.5	7.5

Ultraviolet Mutagenesis and Spontaneous Mutation

Ultraviolet mutageneses and spontaneous mutations failed to produce AVBPS from Xcv 42, Xcm 15, Xcg 11 and Xcg 38. The experiments were carried out because earlier attempts to produce AVBPS from Xcv and Xcm by NG mutagenesis failed.

The data on mutageneses indicate the differences in the sensitivity of the different Xanthomonas campestris pathovars (i.e. Xcg, Xcv, and Xcm) to NG and Acridine Orange mutagens. Xcg WBPS seem to be easier to induce for AVBPS than the WBPS of Xcv and Xcm. Both Xcv and Xcm WBPS, however, are amenable to Acridine Orange mutagenesis. The AVBPS produced by both mutagens are stable enough in so far as the avirulent character and the ability to produce the bacteriocins are concerned. The mutagen treatment affects not only the biological activity spectra of the bacteriocin in terms of the number of strains that are sensitive to it but also its potency. Molecular genetic studies of bacteriocin production of the pathovars may elucidate these phenomena.

On the basis of biological activity data Xcg 11-9, Xcv 42-4, and Xcm 15-16 avirulent bacteriocin producing strains (the first number is the WBPS code, the second number is the code of the mutant from the WBPS) can be tested for their biocontrol efficacy against sensitive virulent wild type strains.

PART III

BIOLOGICAL CONTROL OF BACTERIAL PUSTULE OF SOYBEAN, ANGULAR SPOT OF COTTON, AND BACTERIAL SPOT OF TOMATO BY SEED TREATMENT WITH AVIRULENT BACTERIOCIN PRODUCER STRAINS OF THEIR RESPECTIVE CAUSAL BACTERIA

INTRODUCTION

Having identified the potential avirulent bacteriocin producer strains (AVBPS) for biocontrol studies (Part II), the next step was to find out whether or not these strains (the AVBPS) would indeed control the diseases initiated by bacteriocin sensitive virulent wild type strains.

The specific objective of this part of the project, therefore, was to determine if bacterial pustule of soybean, angular spot of cotton, and bacterial spot of tomato can be effectively controlled by seed treatment with AVBPS of their respective bacterial causal agents. The seed treatment procedure for applying the AVBPS was selected because it is cheap and the bacterial agents of the diseases referred to are seedborne and, therefore, theoretically amenable to biopesticide seed treatment.

MATERIALS AND METHODS

Avirulent bacteriocin producer strains (AVBPS) used

Xcg 11-9 (*Xanthomonas campestris* pv. *glycines* AVBPS iso. 9 from wild type Xcg iso. 11),

Xcm 15-16 (*X. campestris* pv. *malvacearum* AVBPS iso. 16 from wild type Xcm iso. 15) and

Xcv 42-4 (*X. campestris* pv. *vesicatoria* AVBPS iso. 4 from wild type Xcv iso. 42) were used.

AVBPS inoculum

The inoculum consisted of 24-hr old PSB shake culture of the desired AVBPS (300 ml PSB (per dextrose bottle) seeded with one 48-hr old PSA slant culture suspended in 10 ml sterile water).

Production of soybean, cotton, and tomato seeds naturally infected respectively with Xcg, Xcm, and Xcv

Soybean (BPI SY4 cv), cotton (Deltapine 16 cv) and tomato (Marikit cv) were grown at the UPLB-CES fields according to standard cultural management practices. At flowering time the plants were inoculated with the desired pathogens (Xcg 16 on soybean, Xcm 11 on cotton, and Xcv 12 on tomato). The diseases were allowed to develop unhampered. The infected fruits (soybean pods, tomato fruits, cotton bolls) were harvested and the seeds were processed following recommended practices. The seeds were stored in the refrigerator until further used.

Experimental plots and design for soybean and cotton

The experimental plots for soybean and cotton at the UPLB-CES were plowed, disked, harrowed twice, rotovated, and furrowed at 0.75 m. All the experiments were installed in RCB design. The treatment blocks were replicated thrice with four rows per block. There were 4 rows of corn between blocks to prevent inoculum transfer between blocks.

Planting and care of plants

Four rows of corn (Lagkitan var.) were planted along borders and between experimental blocks, 2 weeks before the sowing of cotton

and soybean seeds or transplanting of tomato seedlings. The corn plants served as a carrier to inoculum transfer between treatment blocks.

Recommended cultural management practices including fertilizer application and cultivation were followed for each crop. Herbadox was used as pre-emergence herbicide and handweeding was done whenever necessary. Several combinations of insecticides and fungicides (e.g. Thiodan, Sevin, Decis, Benlate, Daconil, Dithane M-45) were used as necessary at recommended rates.

Soybean seed treatment

Soybean seeds (BPI SY4) naturally infected with Xcg 11 were used.

There were 2 treatments: The first treatment (T1) consisted of seeds soaked for 12 hrs in the AVBPS Xcg 11-9 biocontrol agent inoculum· the second treatment (T2), the control treatment, consisted of seeds soaked for 12 hrs in sterile PSB. The seeds were drained and planted right after the treatments.

Cotton seed treatment

Naturally infected and artificially inoculated (with Xcm 11) cotton (Deltapine 16 cv) seeds were used.

a. Naturally infected seeds

Two treatments similar to those described for soybean but using AVBPS Xcm 15-16 as the biocontrol agent were installed.

In another trial AVBPS Xcg 11-9 was used to treat the seeds.

b. Artificially inoculated seeds

Commercial seeds (Deltapine 16 cv) were used. The seeds were first inoculated by soaking them in a 24-hr old Xcm 15 suspension in sterile distilled water (approx. 10^8 cfu/ml) for 12 hrs. After inoculation the seeds were air dried for 12 hrs. One-half of the seeds were then soaked in sterile PSB (T1). The other half were soaked for 12 hrs in the AVBPS Xcm 15-16 inoculum (T2). For the control treatment (T3), a batch of uninoculated seeds were first soaked in sterile PSB for 12 hrs, air dried for 12 hrs, then soaked in plain PSB for 12 hrs. The seeds were drained and planted right away after the treatments.

Tomato Seed Treatment

a. Naturally infected seeds

Tomato seeds (Marikit cv) naturally infected with Xcv 12 were used. Two treatments similar to those described for soybean but using AVBPS Xcv 42-4 as the biocontrol agent, were installed. In this test the seeds were sown in seedboxes with baked soil. Five seedboxes with 100 seeds per seedbox were used for each treatment. The seedboxes were placed in the greenhouse and the seedlings were observed for bacterial spot. Observations were made up to 45 days after sowing. Proper seedling management was followed during the experiment.

b. Artificially inoculated seeds

Tomato seeds (Marikit cv) were inoculated by soaking in 24-hr old PSB cultures of WBPS Xcv 12 for 12 hrs, air-dried for 12 hrs at room temperature, then inoculated, also by soaking, with 48 hr-old PSB cultures of AVBPS Xcv 42-4 for 12 hrs. After air-drying for 12 hrs, the seeds were sown in flats with sterile soil. In another set, the seeds were soaked first in AVBPS Xcv 42-4 then in WBPS Xcv 12. For the control treatment, the seeds were soaked in PSB alone. There were 3 replications with 500 seeds per replicate, per treatment. In another test, the same procedures were used except that the drying time of seeds was reduced from 12 hrs to 30 min. Seedlings were observed for bacterial spot symptoms in the greenhouse.

RESULTS AND DISCUSSION

Soybean Seed Treatment with Xcg AVBPS Using Naturally Infected Seeds

a. First trial (November 1987 to March 1988, a UPLB Central Experiment Station (CES))

Figure 14 shows a portion of the experimental field. A very low percentage of plant population from seeds treated with AVBPS Xcg 11-9 (1.6%) were infected by pustule compared with those from non-treated seeds (40.2%).

Fig. 14a. Field set-up of the experiment on the control of bacterial pustule of soybean by seed treatment with an avirulent bacteriocin producer strain of X. campestris pv. glycines (Xcg 11-9). Note the rows of corn that separated the treatments in an RCB design.

Fig. 14b. Field set-up of the experiment on the control of angular spot of cotton by seed treatment with an avirulent bacteriocin producer strain of X. campestris pv. malvacearum (Xcm 15-16). Note the rows of corn that separated the treatments in an RCB design.

Ninety Xcg strains isolated from 90 infected plants collected at random from the AVBPS treatment plots and assayed for bacteriocin sensitivity were all sensitive to the bacteriocins of Xcg 11-9; these observations show that infection of plants from those plots were initiated by Xcg inocula that were able to escape the AVBPS Xcg 11-9 seed treatment. Yield data also showed that the AVBPS treatment increased seed yield by 62.3% (Table 18).

It is clear from the data that the AVBPS seed treatment can reduce seedborne Xcg inoculum resulting in reduction of disease (bacterial pustule) severity and substantial improvement in soybean seed yield.

b. Second trial (June 1988 to October 1988, UPLB CES)

Bacterial pustule infections in this trial were very low. The disease was noted more than 2 months after planting making infection ratings impractical. The low incidence of the disease could have been due to low Xcg seedborne inocula. Nevertheless, yield data showed that the AVBPS Xcg 11-9 treatment increased seed yield by 31.3% (Table 19); yield improvement due to the treatment may be attributed to increased in number of pods (Table 20).

The data confirmed the observations on the first trial.

Table 18. Effect of seed treatment with avirulent bacteriocin producer strain (AVBPS) of *X. campestris* pv. *glycines* (Xcg 11-9) on bacterial pustule incidence and soybean (BPI-SY4) seed yield (November 1987 - March 1988, UPLB-CES).

Treatments	No. plants infected 1/ Total No. Plants REP I	II	III	Ave.	Ave. % Population Infection	Seed yield 2/ REP I	II	III	Ave. 3/	% Inc. in yield 4/
AVBPS	10/715	9/845	20/870	13/810	1.6	570	610	570	583.3 a	62.3
Control	348/1,199	106/779	766/1,054	407/1,010	40.2	327	365	385	358.1 b	-

1/ Data recorded 1 1/2 mos. after sowing.

2/ Based on 40 sample plants (10 sample plants from each of 4 rows per replicate).

3/ DMRT analysis.

4/ % increase in yield = $\frac{\text{Ave. AVBPS-treated seed yield} - \text{Ave. control seed yield}}{\text{Ave. control seed yield}} \times 100$

Table 19. Effect of seed treatment with avirulent bacteriocin producer strain (AVBPS) of X. campestris pv. glycines (Xcg 11-9) on bacterial pustule incidence and soybean (BPI-SY4) seed yield (June 1988 - November 1988, UPLB-CES).

Treatments	Seed weight per plants (g)[1/] Replicate I	II	III	Ave.[2/]	% Increase in yield[3/]
AVBPS	34.2	22.7	23.3	26.7 a	31.3
Control	22.8	18.5	19.7	20.3 b	-

[1/] Average of 40 sample plants (10 sample plants from each row of 4 rows per replicate).

[2/] DMRT analysis.

[3/] % increase in yield = $\frac{\text{Ave. AVBPS-treated seed yield} - \text{Ave. control seed yield}}{\text{Ave. control seed yield}} \times 100$

Table 20. Effect of seed treatment of soybean (BPI SY4) with AVBPS of X. campestris pv. glycines (Xcg 11-9) on pod number (June 1988 - November 1988, UPLB-CES).

Treatments	Number of pods per plant Replicate I	II	III	Average (pods/plant)[1/]
AVBPS	118.2	93.8	95.75	102.6 a
Control	83.6	75.3	76.5	78.5 b

[1/] Based on 40 sample plants per replicate. DMRT analysis.

Cotton Seed Treatment with Xcg and Xcm AVBPS Using Naturally and Artificially Infected Seeds

a. First trial (November 1987 to March 1988, UPLB-CES)

This experiment which used AVBPS Xcg 11-9 (from soybean) was conducted at that time when AVBPS of Xcm was not yet available. Xcg 11-9 was selected for the trial because its bacteriocin was active against virulent wild type isolates of Xcm. (Part II).

Angular spot infections were not severe. Few spots were observed on leaves of infected plants 1-1/2 months after planting. Nevertheless, a lower percentage of plant population from seeds treated with AVBPS Xcg 11-9 (9.9%) were infected with angular spot compared with that of the non-treated control treatment (17.4%) (Table 21).

b. Second trial (June 1988 to November 1988, UPLB-CES)

In the experiment with naturally infected seeds, the average percent plant population infection was considerably lower in the AVBPS Xcm 15-16 seed treatment (10.2%) compared with that of the control treatment (38.9%) (Table 22); the average number of infected leaves per plant was also lower in the AVBPS seed treatment than the control (Table 23). With artificially inoculated seeds, percent plant population infection (with Xcm) was only 14.9% in the

Table 21. Effect of seed treatment with avirulent bacteriocin producer strain (AVBPS) of X. campestris pv. glycines (Xcg 11-9) on cotton (Deltapine 16) angular spot incidence (Nov. 1987 - March 1988, UPLB-CES) 1/

Treatments	No. plants infected / Total No. Plants R E P I	II	III	Ave.	Ave. % population infection
AVBPS	28/392	51/350	30/375	36/372	9.9
Control	220/765	258/590	29/337	169/564	17.4

1/ X. campestris pv. malvacearum naturally infected seeds were used. Data taken 2 months after sowing.

Table 22. Effect of seed treatment with avirulent bacteriocin producer strain (AVBPS) of X. campestris pv. malvacearum (Xcm 15-16) on cotton (Deltapine 16) angular spot incidence (June 1988 - November 1988, UPLB-CES) 1/

Treatments	No. of plants infected 1/ / Total No. Plants R E P I	II	III	Ave.	Ave. % population infection
AVBPS	20/224	25/239	25/222	23.3/228.3	10.2
Control	119/254	113/246	58/241	96.6/247	38.9

1/ Xcm naturally infected seeds were used. Data taken 2 months after sowing.

Table 23. Effect of seed treatment of cotton (Deltapine 16) with AVBPS of X. campestris pv. malvacearum (Xcm 15-16) on angular leaf spot infection [1/]

Treatments	No. infected leaves/plant R E P I	II	III	Ave.
AVBPS	1.75	1.85	1.8	1.8
Control	2.3	3.3	2.8	2.8

[1/] Based on 20 plants per replicate. Data taken 2 months after sowing.

Table 24. Effect of seed treatment with avirulent bacteriocin producer strain (AVBPS) of X. campestris pv. malvacearum (Xcm 15-16) on cotton (Seltapine 16) angular spot incidence (June 1988 - November 1988, UPLB-CES) [1/]

Treatments [2/]	No. plants infected / Total No. Plants R E P I	II	Ave.	Ave. % population infection
AVBPS	40/420	76/359	58/389.5	14.9
WBPS	210/334	276/342	243/338	71.9
Control	0/479	0/493	0/486	

[1/] Data taken 2 months after sowing.

[2/] AVBPS - seeds treated (inoculated) with avirulent bacteriocin producer strain (Xcm 15-16)

WBPS - seeds treated (inoculated) with virulent wild type bacteriocin producer strain

Control - seeds treated with PSB.

AVBPS Xcm 15-16 seed treatment compared with 71.9% of the non-treated seed control treatment; there was no infection of plants from healthy control seeds (Table 24). In both experiments no yield data was taken because most of the bolls rotted during the rainy periods that occurred long before harvest.

While no yield data were recorded in both experiments and also in the first trial, it was apparent that treatment of cotton seeds with AVBPS of Xcm or Xcg would reduce seed-borne Xcm inocula, thereby, limiting angular spot severity.

Tomato Seed Treatment with Xcv AVBPS Using Naturally and Artificiall Infected Seeds

Experiments utilizing seeds from tomato fruits naturally infected with Xcv failed to yield infected seedlings. This may be attributed to low percentage of seeds infected with Xcv in the seedlot used in the experiment.

In tests involving artificial seed inoculations, seedlings that developed from seeds treated with either AVBPS Xcv 42-4 or virulent wild type bacteriocin producer (WBPS) strain (sensitive to AVBPS Xcv 42-4) did not contract the disease. The experiment was repeated 2 times using other virulent WBPS with the same results. For some reasons the virulent WBPS on the seeds failed to infect the seedlings. Under normal conditions it takes 4-5 days after sowing before the seedling

emerges from the soil. It is possible that the WBPS failed to survive long enough on the seed coat to infect the developing seedlings (see Part IV).

Further tests should be conducted under conditions where Xcv seedborne inocula can induce bacterial pustule infections on developing seedlings.

Tomato Spraying with Xcv AVBPS

This test was conducted because of the failure to induce bacterial spot in the seed treatment experiment. Healthy leaves of 35-day old tomato plants (Marikit cv) were spray-inoculated at 15 psi using an electric sprayer, with 48-hr old PSB culture of AVBPS Xcv 42 mixed with carborandum dust (0.5 g/100 ml culture). After treatment, separate plants were inoculated with virulent WBPS Xcv 12 (1×10^8 cfu/ml) 1 hr and 2, 4, and 7 days after Xcv 42-4 application. Four plants were used per treatment. In another set of similar experiment AVBPS Xcv 42-4 was sprayed on the leaves using a hand sprayer instead of the electric sprayer. The first set of experiment (where AVBPS inoculum plus carborandum dust was sprayed using an electric sprayer) represented a situation were the AVBPS was introduced on injured leaves. All plants were covered with plastic bags for 48 hrs then incubated in the greenhouse and observed for bacterial spot development. Proper plant management were followed for the duration of the experiments.

Results showed that the AVBPS protected (fewer bacterial spots) the uninjured leaves only when it was applied up to 2 days before inoculation with the WBPS; in injured leaves (injured with carborundum dust)the AVBPS provided protection on the leaves only when it was applied just before inoculation with the WBPS (Table 25). These observations indicate the very limited residual activity of the bacteriocins and perhaps the failure of the AVBPS to multiply on the leaf surface to amply protect the leaves from the WBPS (see Part IV). The data also indicate that spray application of live cells of AVBPS may offer very little protection of tomato plants from the virulent Xcv.

Table 25. Effect of avirulent bacteriocin producer strain (AVBPS) of *X. campestris* pv. *vesicatoria* (Xcv 42-4) applied as spray material on tomato (Marikit cv.) on bacterial spot incidence.

Days after inoculation with Xcv [1]	Number of spots per leaf	
	Non-injured [2] (13 days)	Injured [3] (9 days)
1 (hr)	8.6	0.3
2	9.0	15.75
4	107.4	33.0
7	85.9	17.15
Control Xcv	138.4	23.3
Control, AVBPS	0	0

[1] Plants inoculated with virulent Xcv.

[2] Inocula sprayed on plants without pressure. Mean of 16 leaves from 4 plants per treatment.

[3] Inocula mixed with carborundum dust sprayed with pressure on plants. Mean of 20 leaves from 5 plants per treatment.

PART IV

SURVIVAL OF AVIRULENT BACTERIOCIN PRODUCER STRAINS OF *XANTHOMONAS CAMPESTRIS* PV. *GLYCINES*, PV. *VESICATORIA*, AND PV. *MALVACEARUM* ON SEED AND LEAF SURFACES

INTRODUCTION

This part of the project was conducted to determine if the avirulent bacteriocin producer strains (AVBPS) can survive on host plants of the virulent wild type strains. This is important to know because the effectivity of a biocontrol agent in nature depends on its ability to multiply on host surfaces where the target virulent pathogen maybe present or may land and eventually colonize to initiate disease.

MATERIALS AND METHODS

Survival on seed surfaces

Soybean (BPI-SY4 cv), cotton (Deltapine 16 cv) and tomato (Marikit cv) seeds were soaked separately in 48 hr old AVBPS suspension in sterile water for 10 min. After soaking, the seeds were placed in sterile petri plates lined with moist sterile filter paper and then incubated at room temperature. The population change of AVBPS on the seed surface was determined on seed samples taken from the plate after 1 hr, and 1, 2, and 3 days after soaking as follows: The seed samples were shaken vigorously for 3 min in 10 ml sterile water in a test tube, after which the wash water was serially diluted and

plated on PSA. The plates were incubated at room temperature and observed for AVBPS colonies. When identification of AVBPS colonies was in doubt, chloroform treatment and assay for bacteriocin production using specific indicator strains were performed.

Survival on leaf surfaces and in leaf tissues

The same soybean, cotton, and tomato cultivars and AVBPS isolates used in the seed survival test were used in this study.

Three week old plants were sprayed with 48 hr old AVBPS suspension in sterile water (approx 10^8 cfu/ml). After spraying, the plants were covered with plastic bags to maintain a humid atmosphere around the leaves and then incubated in a cool corner of the greenhouse. The AVBPS population was determined on leaf samples taken from the plants 1 hr and 1, 2, and 3 days after inoculation as follows: Three 1 cm dia leaf samples were taken from each of 3 sample leaves from each of 3 treatment plants using a cork borer. The samples from each leaf were shaken vigorously for 3 min in 10 ml sterile water in a test tube, after which the wash water was serially diluted and plated on PSA. The plates were incubated at room temperature and observed for AVBPS colonies as described above.

In another set of experiments, the AVBPS suspension was infiltrated into the leaf by using a no. 28 hypodermic syringe. Samples were taken from the infiltrated leaves and the population of the AVBPS was assayed as described above.

RESULTS AND DISCUSSION

Survival of AVBPS of Xcg on Soybean and Leaf

On seeds, the population of AVBPS Xcg 11-9 increased from an initial 3.5×10^4 cfu/ml to 4.1×10^6 cfu/ml after 3 days. However, on leaves the initial population of 5.4×10^3 cfu/ml dropped to zero after the same period (Table 26) (Fig. 15). These data clearly indicate that Xcg 11-9 can survive and multiply on soybean seeds but not on leaves.

Survival of AVBPS of Xcm on Cotton Seed and Leaf

AVBPS Xcm 15-16 increased in number on seeds from an initial population of 2.6×18^6 cfu/ml to 1.4×10^7 cfu/ml after 3 days. However, on leaves, it disappeared after only 1 day from inoculation (Table 27). These observations suggest that Xcm 15-16 can survive and multiply on cotton seeds but not on the leaves.

Survival of AVBPS of Xcv on Tomato Seed and Leaf

AVBPS Xcg 42-4 did not multiply on either seed or leaf. It was not detected on seeds after 3 days while on leaves, it disappeared only after one day froms praying. In a separate test where Xcv 42-4 was introduced into the leaf by the leaf infiltration technique, it was not detected 2 days after it was introduced into the leaf (Table 28).

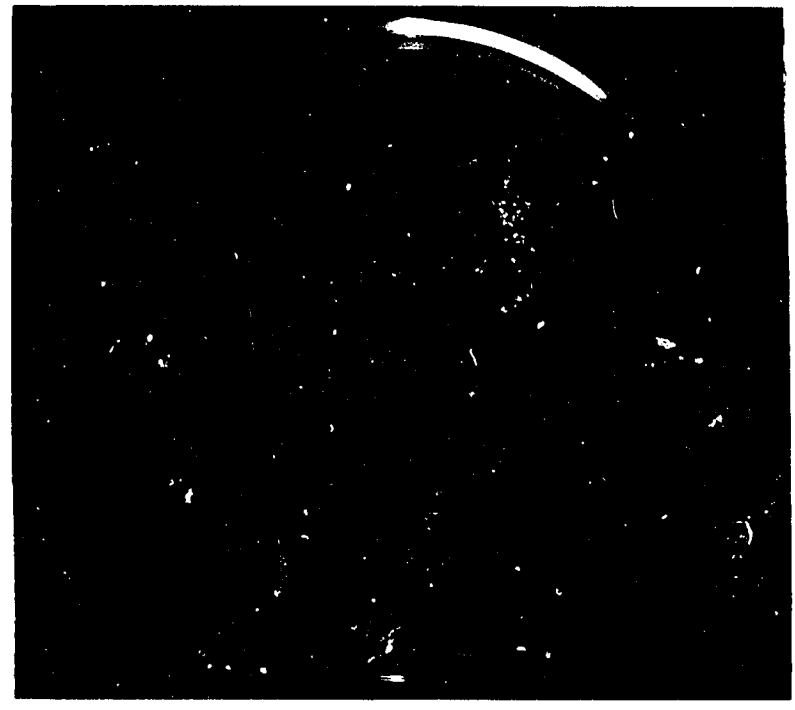


Fig. 15. Avirulent Bacteriocin Producer strains (AVBPS) of Xanthomonas campestris pv. glycines (Xcg 11-9) from soybean seeds (A) and X. campestris pv. malvacearum (Xm 15-16) from cotton seeds (B) detected by plating sensitive wild type indicator strains. Note the clear inhibition zones a around AVBPS colonies.

Table 26. Survival of AVBPS of X. campestris pv. glycines (Xcg 11-9) on soybean seeds and leaves.

Days after inoculation[1]	AVBPS population (cfu/ml) Seed[2]	Leaf surface[3]
0	3.51×10^4	5.4×10^3
1	7.45×10^5	4.7×10^3
2	1.75×10^6	2.24×10^2
0	4.15×10^6	0

[1] Seeds were soaked for 10 min in AVBPS suspension then placed on sterile filter paper disc moistened with sterile distilled water in a Petri dish.

Leaves of 14-day old soybean plants were sprayed with AVBPS suspension

[2] Average AVBPS count per seed based on 3 seeds. Figures are averages of 2 plates.

[3] Average AVBPS count per leaf based on 3 leaves from each of 3 plants. One-cm dia leaf disc sample per leaflet was used. Figures are averages of 2 plates.

Table 27. Survival of AVBPS of *Xanthomonas campestris* pv. *malvacearum* (Xcm 15-16) on cotton seeds and leaves

Day after inoculation[1]	Seed[2]	AVBPS population (cfu/ml) Leaf surface[3]	
		Spray method	Injection method
0	2.57×10^6	1.27×10^5	1.2×10^3
1	2.75×10^6	0	0
2	4.13×10^6	0	0
3	1.39×10^7	0	0

1/ Seeds soaked for 10 min in AVBPS suspension then placed in sterile filter paper disc moistened with sterile distilled water in a sterile Petri dish

2/ Average AVBPS count per seed based on 3 seeds. Figures are averages of 2 plates

3/ Average AVBPS count per leaf based on 3 leaves from each of 3 plants. One-cm dia leaf disc sample per leaf was used. Figures are averages of 2 plates

Leaves of 3 week-old cotton plants were sprayed or infiltrated by injection with AVBPS suspension.

Table 28. Survival of AVBPS of *Xanthomonas campestris* pv *vesicatoria* (Xcv 42-4) on tomato seeds and leaves

Days after inoculation[1]	ABPM population (cfu/ml) Seed[2]	Leaf surface[3] Spray method	Leaf surface[3] Injection method
0	1.56×10^4		TNC, 10^{-2}
1	3.4×10^3	0	4.78×10^4
2	5.7×10^2	0	0
3	0	0	0

1/ Seeds soaked for 10 min in AVBPS suspension then placed on sterile filter paper disc moistened with sterile distilled water in a sterile Petri dish

2/ Average AVBPS count per seed based on 3 seeds. Figures are averages of 2 plates

3/ Average AVBPS count per leaf based on 3 leaves from each of 3 leaves. One cm dia leaf disc samples per leaflet was used. Figures are averages of 2 plates

Leaves of 3 weeks-old tomato plants were sprayed or infiltrated by injection with AVBPS suspension.

These data suggest that Xcv 42-4 has very poor survival ability.

The ability of AVBPS to survive and multiply on seed surfaces is a desirable characteristic of a good seed treatment biocontrol agent. And this might explain the successful control by seed treatment of bacterial pustule of soybean by Xcg 11-9 described in Part III of this project.

PART V

EFFECTS OF SEED TREATMENT AND SOIL FUNGICIDES AND INSECTICIDES ON AVIRULENT BACTERIOCIN PRODUCER STRAINS OF *XANTHOMONAS CAMPESTRIS* PV. *GLYCINES*, PV. *VESICATORIA*, AND PV. *MALVACEARUM*

INTRODUCTION

This study was conducted based on a suggestion by a UPLB Research Review Panel who reviewed the progress of the project in February 1989 in connection with the yearly University-wide evaluation of on-going research projects of the UPLB Faculty.

The objective of the study was to determine whether or not fungicides and insecticides that may be used for treating seeds of soybean, cotton, and tomato or as pre-plant soil treatment pesticides would affect the growth of AVBPS which can be used as seed treatment material for controlling seedborne bacterial pathogens of these crops.

MATERIALS AND METHODS

Six fungicides namely Daconil 50 WP, Benlate 50 WP, Dithane M-45, Rovral, Arasan, and Captan and four insecticides namely Malathion, Decis, Sevin, and Furadan 3 G were used.

The undiluted formulated fungicides (all were wettable powders) and Furadan 3G (granular formulation) were spot seeded on PSA plates, freshly inoculated before plating with selected AVBPS. The plates were incubated at room temperature and observed for bacterial growth (AVBPS) inhibition by the pesticides.

The insecticides (with the exception of Furadan 3G) were prepared as suspension (Sevin) or emulsion (Malathion and Decis) at 10, 100, and 1000 ppm a.i. Filter paper disc (Whatman No. 2, 5 mm dia) were dipped into the suspension or emulsion and then placed on PSA plates freshly seeded with the test AVBPS. The plates were incubated at room temperature and observed for bacterial growth inhibitions around the insecticide-impregnated filter paper discs. For comparison, virulent wild type strains were similarly tested.

RESULTS AND DISCUSSION

The results are summarized in Table 29 and shown in Figures 16 to 19. Among the test fungicides, only Arasan inhibited the growth of X. c. pv. glycines (WBPS Xcg 11). X.c. pv. vesicatoria (WBPS Xcv 155) and pv. malvacearum (WBPS Xcm 11) were inhibited by Rovral, Arasan, Captan, and Dithane M-45 but not by Daconil or Benlate.

None of the test fungicides inhibited AVBPS Xcv 42-4 while only Daconil and Benlate failed to inhibit AVBPS Xcm 15-16 and Xcg 11-9. The AVBPS Xcg 11-9 was more sensitive to the fungicides than its parent virulent WBPS Xcg 11 as it were inhibited not only by Rovral but also by Arasan, Captan, and Dithane M-45.

All the 3 WBPS test isolates were not affected by the insecticides except the WBPS Xcv 155 which was inhibited by Sevin. The AVBPS (Xcv 42-4 and Xcm 15-16) apparently were more sensitive to the insecticides than the wild type strains.

Table 29. In-vitro growth inhibition of virulent wild types and avirulent bacteriocin producer strains (AVBPS) of X. campestris pv. glycines (Xcg), pv. vesicatoria (Xcv) and pv. malvacearum (Xcm) by various pesticides.[1]

Treatments[2]	Wild Type Isolates			AVBPS		
	Xcg 11 (Soybean)	Xcv 155 (Tomato)	Xcm 11 (Cotton)	Xcg 11-9 (Soybean)	Xcv 42-4 (Tomato)	Xcm 15-16 (Cotton)
A. Fungicides[3]						
Daconil	-	-	-	-	+	-
Benlate	-	-	-	-	+	-
Rovral	+	+	+	+	+	+
Arasan	-	+	+	+	+	+
Captan	-	+	+	+	+	+
Dithane M-45	-	+	+	+	+	+
B. Insecticides						
Furadan[3]	-	-	-	-	-	+
Malathion[4]						
10 ppm	-	-	-	-	-	-
100 ppm	-	-	-	-	+	-
1000ppm	-	-	-	-	+	-
Decis[4]						
10 ppm	-	-	-	-	-	-
100 ppm	-	-	-	-	+	-
1000ppm	-	-	-	-	+	+
Sevin[3]	-	+	-	-	-	-

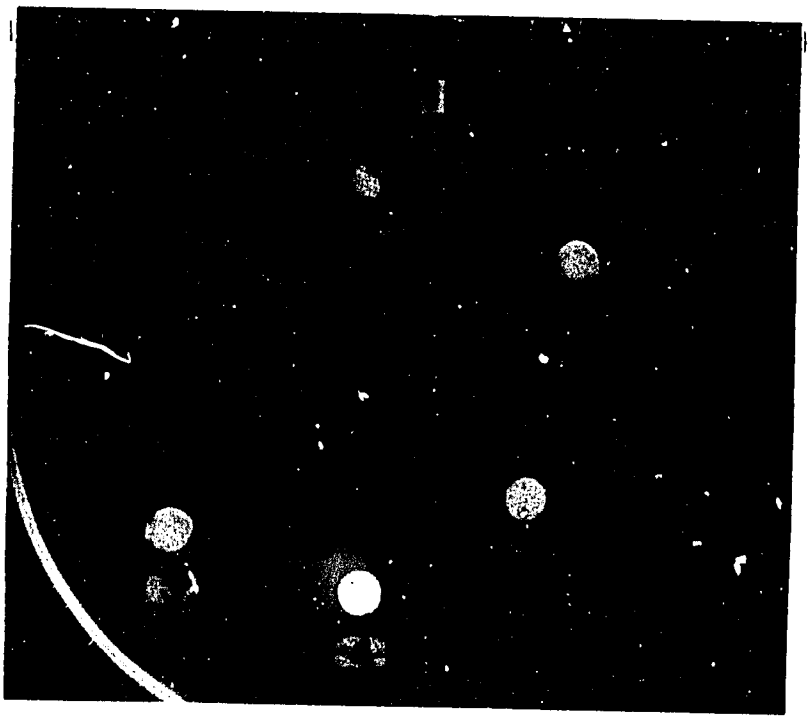
[1] Data based on 3 plates per treatment recorded 48 hrs after incubation. - = no growth inhibition; + = with growth inhibition

[2] Daconil - chlorothalonil
Benlate - benomyl
Rovral - iprodione
Arasan - thiram
Captan - captan
Dithane M-45 - mancozeb or manzeb
Furadan - carbofuran
Malathion - malathion

[3] Undiluted formulated chemical spot-seeded on PSA plates freshly seeded with test isolates.

[4] Filter paper disc impregnated with chemical planted on PSA plates freshly seeded with test isolates.

Fig. 16a. Virulent wilt type X. campestris pv. glycines (WBPS Xcg 11),
Fig. 16b. X. c. malvacearum (WBPS Xcm 11), and
Fig. 16c. X. c. vesicatoria (WBPS Xcv 155) growing on PSA plates with filter paper discs impregnated with various concentrations of insecticides: 1) 10 ppm Malathion, 2) 100 ppm Malathion, 3) 1000 ppm Malathion, 4) 10 pp,m Decis, 5) 100 ppm Decis, 6) 1000 ppm Decis and 7) Sevin (actual material) Note: no growth inhibition.



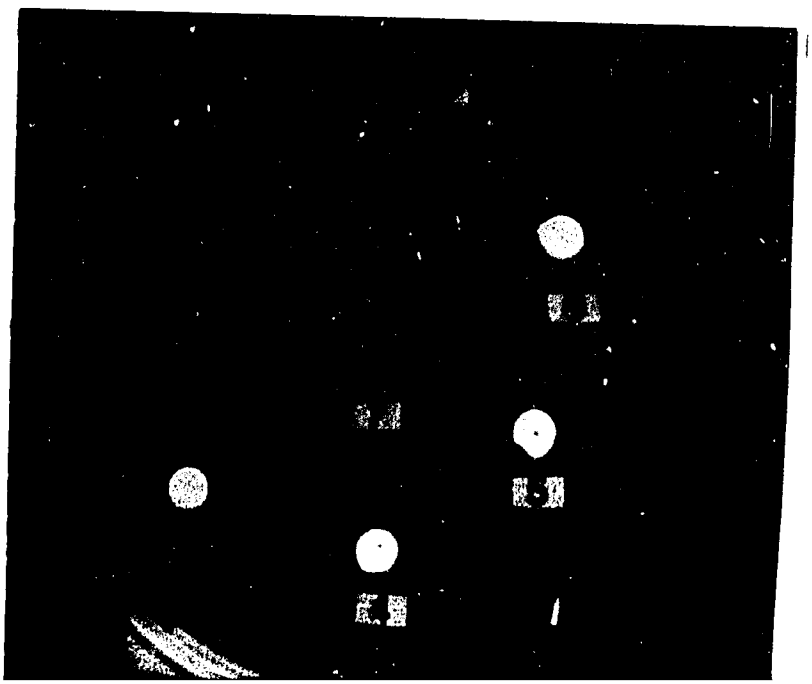
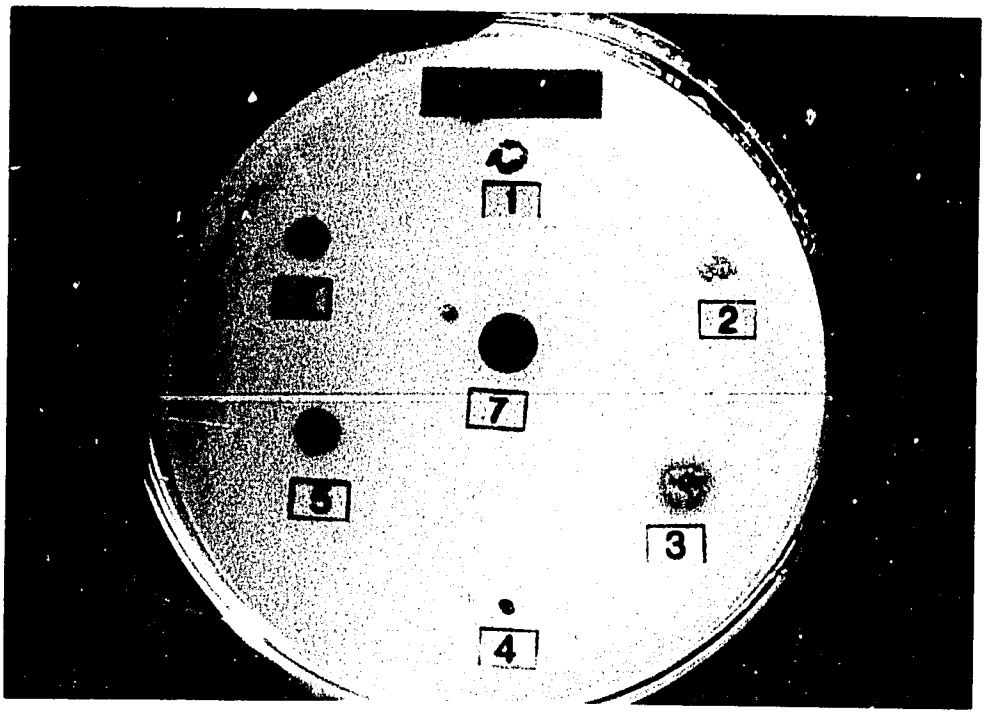


Fig. 17a. Avirulent bacteriocin producer strain (AVBPS) of *X. campestris* pv. *glycines* (Xcg 11-9),
Fig. 17b. AVBPS of *X. c.* pv. *malvacearum* (Xcm 15-16), and
Fig. 17c. AVBPS of *X. c.* pv. *vesicatoria* (Xc 42-4) growing on PSA plates seeded with various pesticides: 1) Daconil, 2) Benlate, 3) Rovral, 4) Furadan, 5) Arasan, 6) Captan, and 7) Dithane M-45. Note: zones of growth inhibition around some pesticides.

Fig. 18a. Avirulent bacteriocin producer strains (AVBPS) of X. campestris pv. glycines (Xcg 11-9),
Fig. 18b. AVBPS of X.c. pv. malvacearum (Xcm 15-16), and
Fig. 18c. AVBPS of X.c. pv. vesicatoria (Xcv 42-4) growing on PSA plates with filter paper discs impregnated with various concentrations of insecticides: 1) 10 ppm Malathion, 2) 100 ppm Malathion, 3) 1000 ppm Malathion, 4) 10 ppm Decis and 7) Sevin (actual material). Note: zones of growth inhibitions.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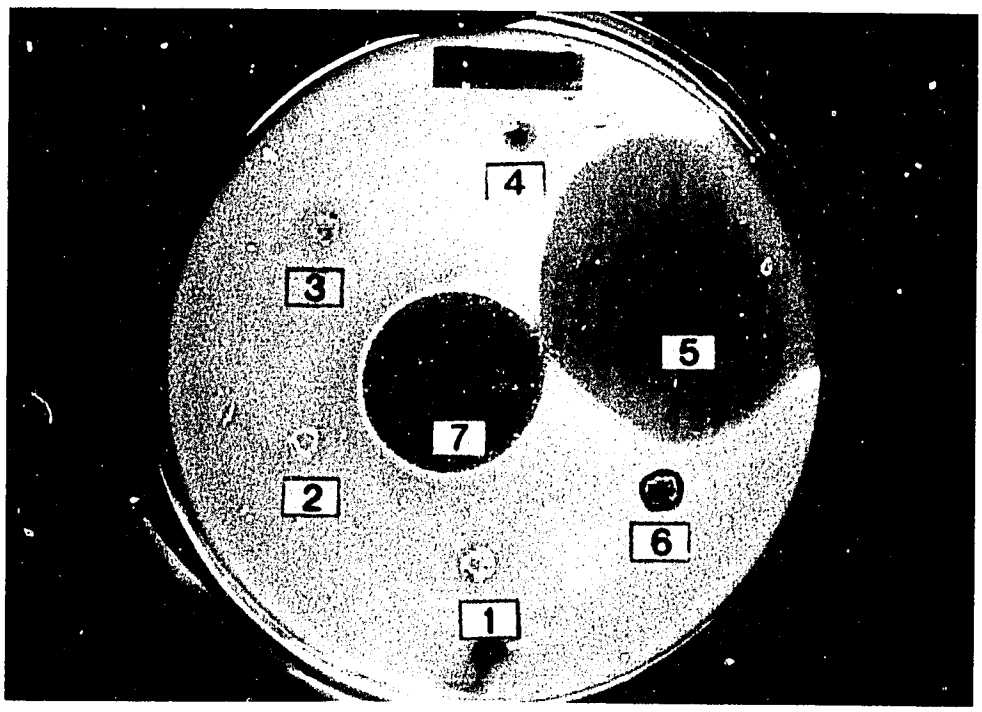
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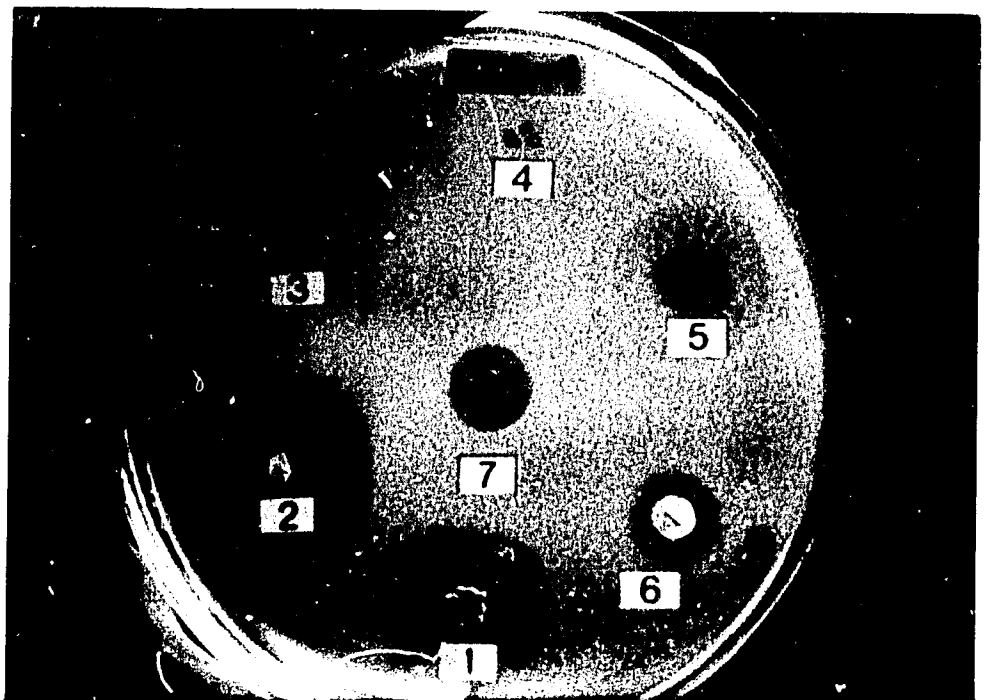
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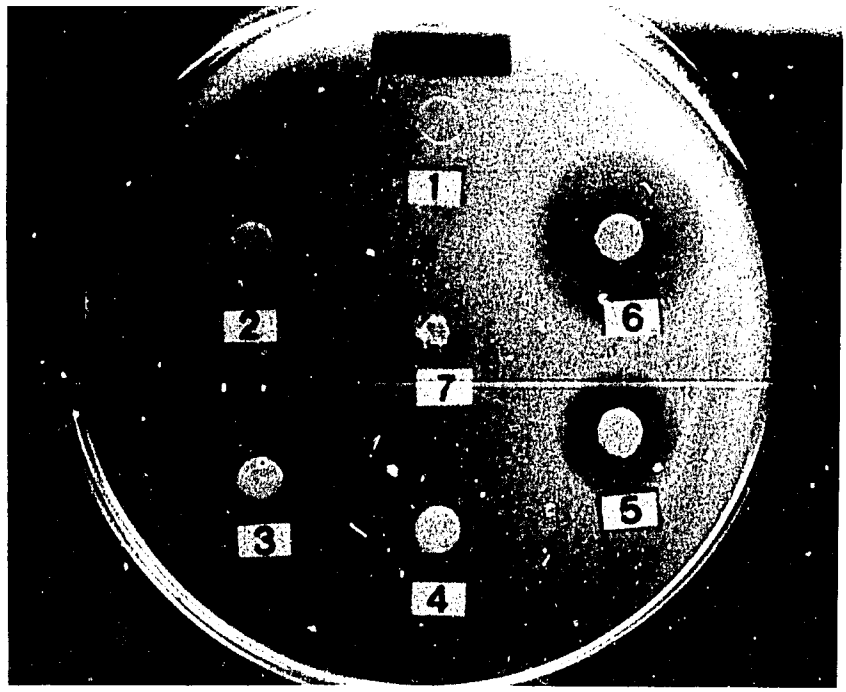
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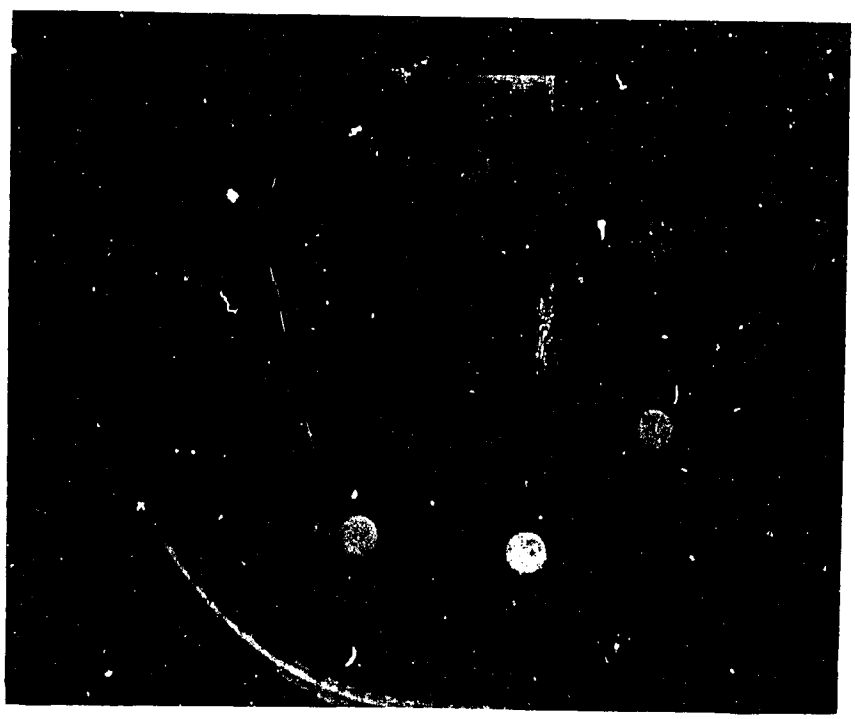

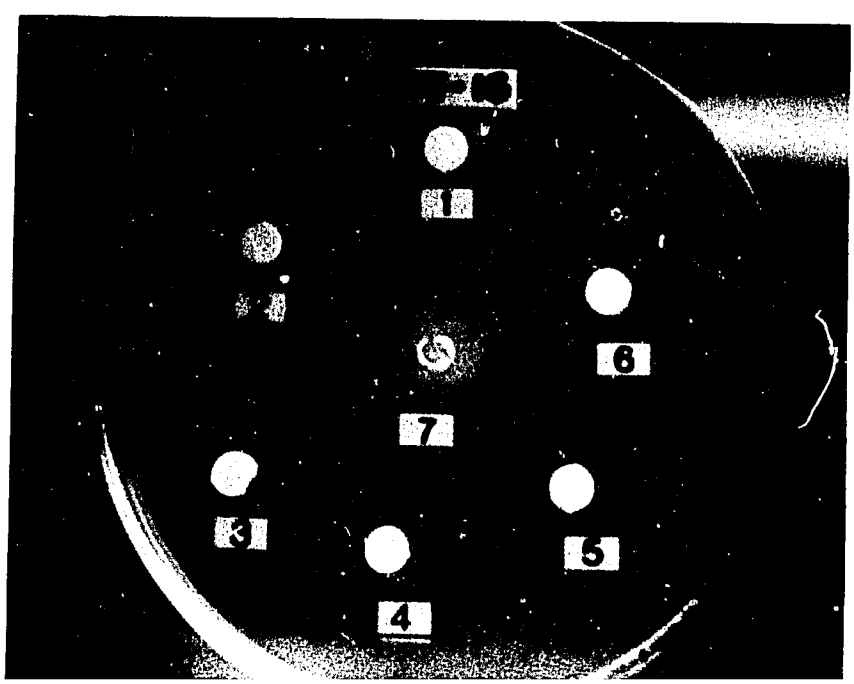
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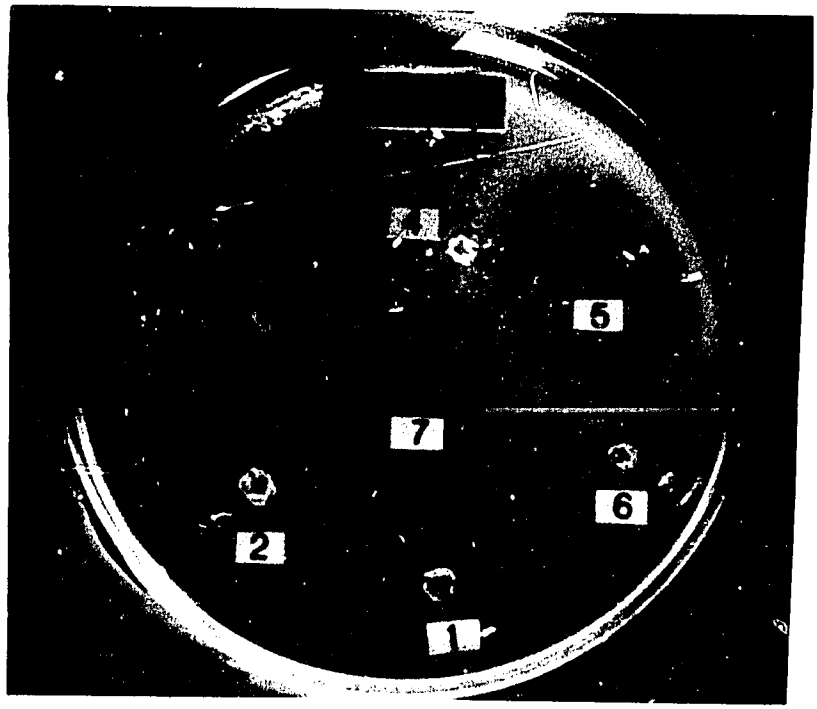
Fig. 19a. Virulent wilt type *X. campestris* pv. *glycines* (WBPS Xcg 11),
Fig. 19b. *X. c.* pv. *malvacearum* (WBPS Xcm 11), and
Fig. 19c. *X. c.* pv. *vesicatoria* (WBPS Xcv 155) growing on PSA plates seeded with various pesticides: 1) Daconil, 2) Benlate, 3) Rovral, 4) Furadan, 5) Arasan, 6) Captan and 7) Dithane M-45. Note: Zones of growth inhibition.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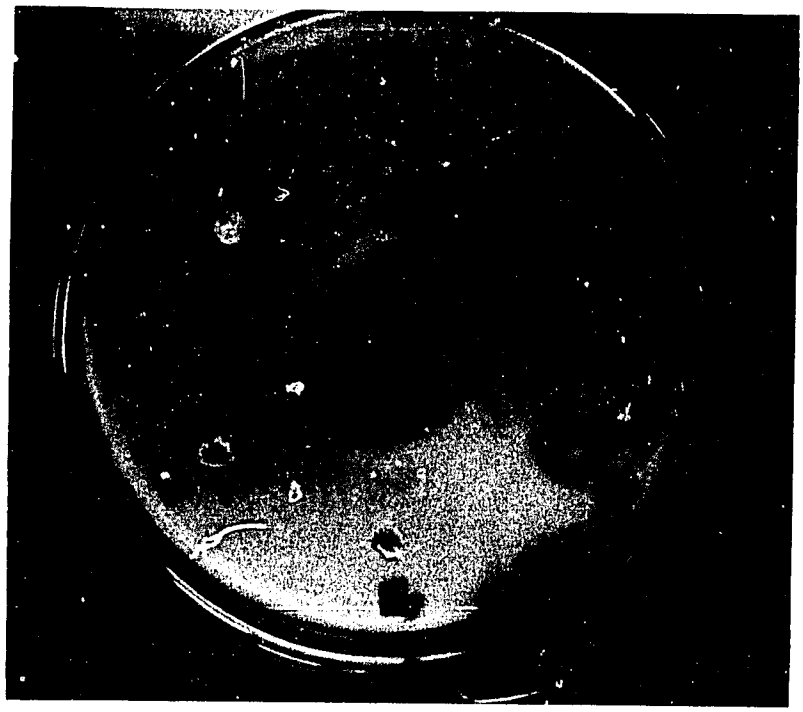

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The data suggest that the survival of AVBPS may be adversely affected by fungicides and insecticides used as seed treatment or spray pesticides. The implication of this observation is that if the AVBPS will be used as a seed treatment or spray bio-pesticide it would be important to consider its compatibility with commonly used seed treatment or spray pesticides.

SUMMARY AND CONCLUSIONS

Bacterial pustule of soybean, angular spot of cotton, and bacterial spot of tomato are among the most destructive plant diseases in the humid tropics. Their causal bacteria, respectively, Xanthomonas campestris pv. glycines, pv. malvacearum, and pv. vesicatoria have quite specific host ranges and are seedborne. For this reason, in the field, these diseases almost always start from contaminated seeds. The use of disease-free seeds or decontaminated seeds (by physical or chemical treatment) is among the most important control measures for these diseases.

In less developed countries of the world like the Philippines, disease-free seeds are often impossible to get. Similarly, decontaminating seeds by physical or chemical treatments is either not effective or not practical, or expensive to apply. Besides, the use of chemical pesticides by and large is not ecologically sound.

Bacteriocin are "highly specific non replicating bactericidal protein-containing substances produced by bacteria that kill other strains of the bacteriocin producer or its closely related species". The project explored the possibility of using avirulent bacteriocin producing strains (AVBPS) of the virulent wild type X. campestris pv. glycines (Xcg), pv. malvacearum (Xcm), and pv. vesicatoria (Xcv) as seed treatment or spray biopesticide as an alternative to the physical and chemical methods of controlling the diseases they induce. Specifically live AVBPS cells were used as seed treatment materials to reduce seedborne inocula of Xcg, Xcm, and Xcv with the objective

of suppressing initial disease and thereby, limiting disease severity and losses.

A survey for bacteriocin producing strains of Xcg, Xcm, and Xcv collected from 50 municipalities/cities representing 18 provinces of the Philippines showed that 48% of Xcg, 45% of Xcm, and 6% of Xcv wild type strains were bacteriocin producers. Sensitivity of indicator strains varied among producers strains. The most potent wild type bacteriocin producer strain of Xcg had 33% (based on 75 strains) indicator strains while that of Xcv and Xcm had 44% (based on 147 strains), and 9% (based on 22 strains), respectively. There were cross reactions in bacteriocin sensitivity of indicator strains in the 3 pathovars indicating their close relationships.

The AVBPS were produced from selected virulent wild type bacteriocin producer strains (WBPS) by nitrosoguanadine (NG) and acridine orange (AO) mutageneses. Xcg AVBPS were induced by NG but not by AO while both Xcv and Xcm AVBPS were induced by AO but not by NG. The AVBPS of Xcg, Xcm, and Xcv were all non-pigmented and possessed similarly colony morphologies. They retained their avirulent and bacteriocin producing characters even after more than one year of weekly culture transfers in nutrient media. The bacteriocins of some of the AVBPS had wider activity spectra and more effective than their parent wild type strains. Cross reactions among AVBPS and virulent wild type indicator strains were also observed.

In two field trials on soybean with AVBPS of Xcg used as seed treatment material for Xcg-naturally infected seeds, seed yield

increased by 37 to 62% due to the suppression of initial disease (bacterial pustule) by the AVBPS treatment. In cotton, 2 field trials with AVBPS of Xcm or Xcg similarly used as seed treatment material or Xcm-artificially or -naturally infected seeds, per cent disease control based on per cent population affected by angular spot was from 74 to 79%. However, in tomato the seed treatment experiments were not successful because of the failure of seedborne Xcv (artificially inoculated or naturally infected seeds) to induce bacterial spot on tomato seedlings.

In greenhouse trials where the AVBPS were sprayed on tomato before inoculations with a virulent wild type strain, bacterial spot development was not significantly controlled. Similar results were observed in trials with AVBPS of Xcg and Xcm (data not shown in report).

The AVBPS of Xcg and Xcm multiplied on seed surfaces but not on leaves of soybean and cotton, respectively. This will explain the successful field control of bacterial pustule of soybean and angular spot of cotton by seed treatment with the AVBPS of Xcg and Xcm, respectively; presumably the AVBPS suppressed initial disease from seedborne inocula of Xcg and Xcm. On the other hand, unsuccessful disease control by foliar application of AVBPS may be attributed to inability of the AVBPS to multiply and survive on leaf surfaces and in leaf tissues. However, since such failure could be a simple question of bacteriocin concentration on the leaf surface it would be interesting to find out whether or not foliar applications of isolated bacteriocin preparations from either the AVBPS or WBPS will control

the diseases.

The AVBPS and wild type strains including the AVBPS parent strains, differ in their sensitivity to pesticides that are commonly used as seed treatment (e.g. Thiram, Captan, Malathion, Pyrethrin), soil treatment (e.g. Carbofuran, Iprodione) or foliar spray (e.g. Benomyl, Chlorothalonil, Mancozeb, Malathion, Pyrethrin) suggesting that the kind of pesticides that are used on the target crops of the AVBPS may adversely affect the degree of disease suppression.

Another problem in the use of the AVBPS in controlling Xcg, Xcm or Xcv is the biocidal specificity of the bacteriocins they produce. It is clear from the data that no single AVBPS is active against all virulent wild type strains. Theoretically, typing isolates of AVBPS can be produced and formulated as biopesticide to cover all wild type strains of a particular bacterial pathogen. But whether or not this is in fact feasible remains to be determined. Nevertheless, for specific geographic areas where a dominant virulent wild type bacterial pathogen strain occur, a single AVBPS active against such a strain can be effectively used.

The project has generated sufficient benchmark information that define the potential of AVBPS for the biological control of seedborne bacterial pathogens particularly the pathogens of bacterial pustule of soybean and angular spot of cotton. The findings, however, open more research challenges in so far as the prospects of commercializing the technology for farmer's use in the countryside/of less developed countries.

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APPENDIX: PUBLICATIONS FROM THE PROJECT

1. In Abstracts of papers for the 18th Anniversary and annual convention of the Pest Control Council of the Philippines. May 5-8, 1987, Davao City, Philippines.

INDUCTION AND ISOLATION OF AVIRULENT BACTERIOCIN-PRODUCING MUTANT OF *XANTHOMONAS CAMPESTRIS* PV. *GLYCINES*

A.J. Quimio, A.K. Raymundo, V.G. Cadiz, A.G. Coroza and A.C. Cueto

A bacteriocin producing *X. campestris* pv. *glycines* (Xcg 11) strain active against pathogenic *X. campestris* pv. *glycines* and pv. *vesicatoria* were exposed to N-methyl-N-nitro-N-nitrosoguanidine (NG) at different periods. The isolate was observed to be sensitive to 0.01 mg/ml NG at 10 minutes resulting to 99.92% kill. Of the colonies that survived the treatment, 10% retained the ability to produce bacteriocins. These isolated mutants (Xcg 11-9, Xcg 11-5, Xcg 11-11) lost the yellow pigmentation, mucoidal properties and virulence on soybean plants typical of the wild type strain. Assay of bacteriocin production against 20 pathogenic *X. campestris* pv. *glycines* and 30 pathogenic *X. campestris* pv. *vesicatoria* indicated that all the pathogenic indicator strains can be controlled by the mutants. The bacteriocins of the mutants were also more potent producing larger inhibition zones than those of their parent wild type bacteriocin producer strains. The non-pigmented, non-mucoidal and avirulent characteristics of these mutants were retained even after the cells were grown for several generations in a rich medium. This indicates the stability of the mutants and their potential as biological control agents against pathogenic *X. campestris* pv. *glycines* and *X. campestris* pv. *vesicatoria*.

2. In Abstracts of papers for the 18th Anniversary and annual convention of the Pest Control Council of the Philippines. May 5-8, 1987. Davao City, Philippines.

ISOLATION OF BACTERIOCIN-PRODUCING STRAINS OF XANTHOMONAS CAMPESTRIS PV. GLYCINES, X. CAMPESTRIS PV. VESICATORIA AND X. CAMPESTRIS PV. MALVACEARUM

A.J. Quimio, A.K. Raymundo, A.G. Coroza, A.C. Cueto and V.G. Cadiz

A total of 290 strains of putative Xanthomonas campestris pv. glycines (Xcg 96 strains), pv. vesicatoria (Xcv 147 strains) and pv. malvacearum (Xcm 22 strains) were identified by pathogenecity to their respective host plants - soybean, tomato and cotton and their characteristic morphology on modified Silva-Buddenhagen medium and peptone sucrose agar (PSA). These strains isolated from over 400 diseased specimens collected from 43 municipalities and 8 cities in 17 provinces of the Philippines were assayed for bacteriocin production.

About 40.6%, 27.2% and 45.4% respectively of Xcg, Xcv and Xcm isolates produced bacteriocins. In Xcg, 5% of the bacteriocin producers were active against 31, 58.9% against 1 and 17.9% against 2 indicator isolates. In Xcv, 25% of the producer strains were active 41, 5% against 1, 2.5% against 2 and another 2.5% against 4 indicator isolates. In Xcm, all the producer strains were actively against only 2 indicator strains.

The data indicate that bacteriocin producer strains are not common in Xanthomonas campestris pathovars glycines, vesicatoria and malvacearum and the bacteriocins produced by a few strains generally had a narrow spectrum of activity.

3. In Abstracts of Papers presented at the 5th International Congress of Plant Pathology p. 107, Aug. 20-27, 1988 Kyoto International Conference Hall, Kyoto, Japan.

2-48

BIOLOGICAL CONTROL OF BACTERIAL PUSTULE OF SOYBEAN WITH AVIRULENT BACTERIOCIN PRODUCER MUTANT OF XANTHOMONAS CAMPESTRIS PV. GLYCINES

A. J. Quimio and A. K. Raymundo, Department of Plant Pathology, U.P. at Los Baños, College, Laguna, Philippines 3720

An avirulent bacteriocin producer dry white colony - mutant (ABPM) produced artificially by nitrosoguanadine mutagenesis from a wild type bacteriocin producer strain of X. campestris pv. glycines (Xcg) was used to control soybean bacterial pustule. Soybean needs naturally infected with Xcg soaked for 12 hr in peptone sucrose broth (PSB) culture of ABPM before planting in the field had 1.6% of the plant population infected with pustule compared with 38.4% of the control; all Xcg isolates from the control were sensitive to the ABPM indicating they were escapes. It had the same strain-spectrum indicators as its wild type parent but more effective. It did not revert to virulent form after more than one year of frequent transfer on PSB or PSA. It was not recovered on leaves 3 days after introduction. On seeds its population increased by 148 folds after 3 days. Data indicate the potential of ABPM for the biological control of soybean bacterial pustule.

4. In Proceedings 7th International Conference on Plant Pathogenic Bacteria, June 11 to 17, 1989, Budapest, Hungary.

BIOLOGICAL CONTROL OF SEEDBORNE BACTERIAL PATHOGENS WITH AVIRULENT BACTERIOCIN PRODUCER MUTANTS

Quimio, A. J. (Dept. of Plant Pathology, Univ. of the Philippines at Los Baños, College, Laguna 4031 Philippines)

Avirulent bacteriocin producer mutants (ABPM) of Xanthomonas campestris pv. glycines (Xcg) and pv. malvacearum (Xcm) artificially produced by nitrosoguanidine and orange G mutagenesis of wild type virulent bacteriocin producer strains, respectively, were used as seed-dip treatment material for the biocontrol of bacterial pustule of soybean and bacterial blight of cotton. In field trials, plants from Xcg-naturally infected soybean seeds soaked overnight in PSB culture of ABPM-Xcg before sowing, had lower infection index and higher seed yield (31 to 62% higher) than the untreated controls. In similar experiments, plants from cotton seeds artificially inoculated with virulent Xcm before the seed-dip treatment with ABPM-Xcm had significantly lower (10%) diseased plants than the untreated controls (39%). Both ABPM-Xcg and ABPM-Xcm were stable in the laboratory, and multiplied well on their respective host seed surfaces but not on leaf surfaces. The data indicate the potential of artificially produced ABPM as selective biocontrol agents for seedborne bacterial pathogens.