Overwintering of Plum Leaf Scald Bacteria in Infected Trees*

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Abstract

Isolation of xylem-limited bacteria (XLB) on CS20 agar medium was used to assess bacterial survival in plum trees. Thirty to forty twigs were randomly sampled monthly from each of the three trees with plum leaf scald from October 1982 to October 1984. From December 1983 to May 1984, 16-24 roots were also sampled from three other trees and plated on CS20BN agar medium. Sap obtained by squeezing each surface-sterilized twig or root with pliers was immediately placed on the surface of CS20 or CS20BN agar. Plates were incubated at 30 C and examined weekly with a microscope (50×) for bacterial colonies. Bacteria were isolated each month, except May 1983 and April 1984, from cultivar "Santa Rosa"; each month, except May 1983, February and April 1984, from cultivar "Burbank"; each month from July to December 1983 and August to October 1984 from cultivar "Ozark Premier". XLB were isolated from root samples of each tree from November 1983 through May 1984. Results demonstrate that XLB can survive through winter at Byron, Georgia, and suggest that the XLB may be transmitted to healthy trees anytime vectors are present.

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Key words : phony peach, xylem-limited bacteria.

Introduction

Plum leaf scald (PLS) disease has caused severe loss in Japanese plum (*Prunus salicina* Lindl.) and other plum species. It was first reported in Argentina in 1935³. Recently, PLS was reported in Brasil and Paraguay⁹. In 1977 PLS was reported to cause serious problems on plums in the southeastern United States^{4,11,12}. The PLS bacterium has been successfully cultivated in enriched culture media^{2,12} and its pathogenicity to plums has also been demonstrated⁹.

Although the occurrence of PLS bacteria in several plum species has been investigated by a vacuum infiltration technique to recover PLS bacteria from different tissues of plum, no seasonal isolation of the bacteria from plum tissues has been described. Recovery of the PLS bacteria on a culture medium enables us to understand the seasonal distribution of viable bacteria and hence, control measures can be better devised.

In this report, we describe the monthly recovery rates of bacteria isolated from twigs of three leaf scald infected plum trees for 25 consecutive months from October 1982 to October 1984, and roots of three other leaf scald infected plum trees for 7 consecutive

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months from November 1983 to May 1984.

Materials and Methods

Source of plum trees. Six leaf scald infected plum trees (*Prunus salicina* L.) cvs Santa Rosa, Burbank, Methley, and Ozark Premier, located at the Southeastern Fruit and Tree Nut Research Laboratory, U. S. Department of Agriculture, Byron, GA were used throughout the study.

Isolation medium. CS20 agar consists of deionized distilled water, 1,100 ml; soy peptone (Scott Laboratories, Fiskerville, Rhode Island), 2.0 g; Bactotryptone (Difco, Detroit, Michigan), 2.0 g; hemin chloride (Sigma Chemical Co., St. Louis, Missouri) stock solution (0.1% in 0.05 N NaOH), 15 ml; $(\text{NH}_4)_2\text{HPO}_4$, 0.8 g; KH_2PO_4 , 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g; phenol red stock solution (0.2%), 5 ml; L-glutamine (Sigma), 6.0 g; dextrose (Fisher), 1.0 g; potato soluble strach (J. T. Baker Chemical Co., Phillipsburg, N. J.), 2.0 g; L-histidine HCL (Sigma), 1.0 g; and Bacto-agar (Difco), 12.0 g. All ingredients were added and dissolved in the order given, and autoclaved for 18 min at 15 lbs. CS20 liquid medium was prepared as the above without agar. CS20BN agar was prepared by adding 50 $\mu\text{g}/\text{ml}$ bacitracin and 25 $\mu\text{g}/\text{ml}$ neomycin into CS20 agar medium¹⁹. The final pH was 6.6-6.7 for all the mentioned media.

Isolation of bacteria. Thirty to forty twigs 0.5-1.0 cm diameter and 5-8 cm long were randomly sampled monthly from each tree from October 1982 to October 1984, placed into 95% ethanol for 3 min and briefly flamed. A section was then cut from the center of each surface sterilized twig with alcohol-flamed pruners, and a freshly cut end was squeezed with pliers. The pliers were alcohol-flamed between changes of twigs. The sap which accumulated was immediately withdrawn with sterile capillary pasteur pipets and dropped onto CS20 agar plates. The plates were enclosed in plastic bags and incubated at 30 ± 1 C. The same procedure was used to isolate the XLB from 16-24 root samples each month collected from each of three other plum trees from November 1983 to May 1984. Plants were examined for developing colonies with a binocular dissecting scope (50 $\times$) weekly for a month.

Cellular morphology and ultrastructure. Triple-cloned isolate PLSB1, originally isolated from plum tree "Santa Rosa" in Dec. 1982, was grown in CS20 broth medium for 7 days. Cells were centrifuged at 15,600 $\times g$ for 15 min and the pellet was covered with a few drops of melted 2% Bacto-agar. The solidified agar, with the pelleted cells attached, was removed from the centrifuge tube and fixed with 2% glutaraldehyde and post fixed with 2% osmium tetroxide for 45 min each. It was then stained with 2% uranyl acetate in 10% ethanol for 5 min before dehydration in a series of ethanol concentrations (50%, 75%, 95% and 100%) for 5 min each. The agar pellet was embedded in Spurr Mixture⁸⁾ at 68 C overnight after two propylene oxide/ Spurr mixture (1:1) treatments for 30 min. Sample blocks were sectioned with a diamond knife and ultra-microtome (Sorvall MT-1) and sections were stained with lead citrate and examined with a transmission electron microscope (Zeiss EM-10A).

Pathogenicity test. Two triple-cloned isolates, PLSB1 and PLSB2, were grown

separately in 20 ml CS20 broth medium for 7–10 days at 30 C with constant shaking. Cells were centrifuged at $15,600 \times g$ for 15 min, and the pellet was then resuspended in 5 ml fresh CS20 broth medium to $3-4 \times 10^9$ cfu/ml. One and one half-year-old Santa Rosa plum seedlings were inoculated with the bacterial suspension by needle injection. About 0.1 ml of this inoculum was injected into each pulvinus. Five different pulvini from base to tip were injected per seedling, and six seedling were injected with each isolate. Six control seedlings were similarly injected with fresh CS20 broth medium. The inoculated plum seedlings were kept in the greenhouse and watered and fertilized regularly.

Reisolations of bacteria from inoculated plums were conducted 6–9 months after injection using the same method and medium described above. Colony and cellular morphology of reisolated bacteria were compared with those of the original isolates.

Indirect immunofluorescent staining (IF). Indirect immunofluorescence was performed using cells from CS20 agar plates. The colonies of PLSB1 and PLSB2 [originally isolated from plum tree “Burbank” in Dec. 1982] were removed with a loop and suspended in phosphate buffer saline (PBS) containing formalin. The cells were then heat-fixed on the slides and stained with immunoglobulin G (IgG) to ribosomes of phony peach bacteria (PP-1) or IgG to membrane of Pierce’s disease bacteria (PD-2). The slides were rinsed, dried and counterstained using FITC conjugated anti-rabbit goat IgG (GIBCO, Grand Island, New York). They were then rinsed, dried, and mounted. The IgG was obtained from each antisera by Sephadex A-500 column chromatography. A block test⁹⁾ was used to determine the optimal dilution of IgG and anti-rabbit goat IgG. Staining was performed as described⁹⁾, and slides were examined with a Zeiss standard incident-light fluorescent microscope. Bacteria were observed under a Planapo 100 $\times$ oil-immersion objective. Cells of *Xanthomonas campestris* pv. *campestris* B24 and antiserum against the ribosomes of *X. campestris* pv. *campestris* B24 were used for comparison.

Results

Isolation and cultivation of plum leaf scald bacterium in culture

The xylem-limited bacterium associated with plum leaf scald (PLS) disease was isolated on CS20 or CS20BN agar medium. Colonies of the bacterium appeared visible using a dissecting microscope (50 $\times$) after 10–14 day’s incubation. The colonies were convex, opalescent white, circular and ranged from 0.15 to 0.75 mm in diameter with entire margins (Fig. 1).

Seasonal isolation rate of plum leaf scald bacterium

The isolation rate of PLS bacterium from twigs of each tree, and the average monthly air temperature are shown in Fig. 2. Bacteria were isolated each month from October 1982 to October 1984 except May 1983 and April 1984 for cultivar “Santa Rosa”; each month from November 1982 to October 1984 except May 1983, February 1984 and April 1984 for cultivar “Burbank”; each month from July 1983 to December 1983, and August 1984 to October 1984 for cultivar “Ozark Premier”. The highest isolation rate of bacteria from cultivars “Santa Rosa” and “Burbank” peaked during November and December in 1982 and 1983, while isolation rate from “Ozark Premier” peaked during Septem-

Fig. 1. Colonies (arrows) developed after 10-14 days incubation from sap extracted from scalded plum twig (72×).

Fig. 2. The isolation rates of plum leaf scald bacteria on CS20 agar medium from twigs of three scalded plum trees from October 1982 to October 1984.

Table 1. Isolation of xylem-limited bacteria from roots of plums showing leaf scald symptoms

Time isolated	Soil temp (C) ^{a)}	Number of isolation attempted	Isolation rate (%) ^{b)}		
			Ozark Premier	Ozark Premier	Methley
Nov. 1983	14.0	24	33.3	40.0	50.0
Dec. 1983	8.7	24	30.8	35.7	45.5
Jan. 1984	6.6	16	28.6	33.3	41.2
Feb. 1984	10.0	24	20.0	23.1	28.6
Mar. 1984	13.5	16	26.7	26.7	33.3
Apr. 1984	18.1	24	25.0	30.1	40.0
May. 1984	24.7	24	30.8	36.4	42.9

a) Monthly mean temperature at 10.2 centimeters.

b) Isolation rate (%) = $\frac{\text{positive isolations}}{\text{number of isolations attempted}} \times 100$.

Fig. 3. Cellular morphology of plum leaf scald bacteria. (A) Cells streaked from colonies as viewed under phase contrast microscope. Bar=3.0 μm . (B) Electron photomicrograph of plum leaf scald bacteria. Cells exhibit rippled walls. Bar=0.5 μm .

Table 2. Indirect fluorescent-antibody staining of plum leaf scald (PLS) bacteria from agar culture.

Cells	Immunoglobulin G to :		
	PP-1 ribosome	PD-2 membrane	<i>Xanthomonas campestris</i> ribosome
PP-1	+++ ^{a)}	++	—
PD-2	++	+++	—
PLSB1	+++	++	—
PLSB2	+++	++	—
<i>Xanthomonas campestris</i>	—	—	+++

a) symbols : —=no fluorescence (cells not visible), ++=green fluorescence, +++ & ++++=bright green fluorescence.

ber and October, 1983. The highest bacterial isolation rates from "Santa Rosa", "Burbank", and "Ozark Premier" were 72.5%, 89.5%, and 50%, respectively.

The isolation rate of PLS bacterium from roots of each tree is shown in Table 1. PLS bacterium was isolated from each tree from November 1983 through May 1984 with the highest isolation rate in November 1983 and the lowest in February 1984.

Pathogenicity test and reisolation

Nine of the 12 injected Santa Rosa plums developed leaf scald and rosetting symptoms 6-7 months after inoculation. None of the controls developed leaf scald symptoms.

The leaf scald bacteria were re-isolated in 10 of 12 attempts from inoculated plums. All controls were negative. Colony and cellular morphology of the re-isolated bacterium were identical to those of the original isolates.

Cell morphology

Cells of plum leaf scald bacteria from colonies as viewed with a phase contrast microscope are shown in Fig. 3A. Electron photomicrographs (Fig. 3B) showed that cells of PLS bacterium are rod-shaped with an average size measuring 0.35-0.44 μm wide and 2.1-6.5 μm long. The cell wall topography appears to be rippled.

Indirect immunofluorescent staining

Cells of two isolates of the PLS bacterium isolated from cultivar "Santa Rosa" and "Burbank" in Dec. 1982 gave a strong fluorescence when reacted with IgG to ribosome of the phony peach bacterium (PP-1) or with IgG to membrane of Pierce's disease bacterium (PD-2) (Table 2). Cells of PLS bacterium failed to fluoresce when reacted with IgG to ribosomes of *Xanthomonas campestris*. Reciprocal tests were also negative.

Discussion

The plum leaf scald (PLS) bacterium was successfully isolated from plum twigs on CS20 agar plates in each of twenty-five months, and from roots in each of seven months. CS20 medium represents another medium for the primary isolation and growth of PLS bacteria. CS20 medium differs from the BC-YE medium¹²⁾ and PW medium²⁾ in the composition and in the preparation procedure. The BC-YE medium, used for the primary isolation of plum leaf scald and phony peach bacteria, consists of Aces buffer, yeast extract, KOH, activated charcoal, L-cysteine HCl, ferric pyrophosphate, and Bacto-agar. Among them, L-cysteine and ferric pyrophosphate were sterilized by millipore filter. The PW medium that was formulated for the isolation of plum leaf scald and phony peach bacteria includes phytone peptone, trypticase peptone, K_2HPO_4 , KH_2PO_4 , hemin chloride, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, agar, phenol red, glutamine, and bovine serum albumin fraction V (BSA). Glutamine and BSA were sterilized by millipore filtration. All the ingredients included in the CS20 medium were sterilized by autoclave. This makes the medium preparation simpler especially when a large quantity of culture medium, such as those used in this study, is needed. The colony morphology of the bacterium that developed on CS20 agar plates from cultivars "Santa Rosa" and "Burbank" was studied and used as criteria for future determination of a positive or negative isolation. Two isolates, PLSB1 and PLSB2, originally isolated from cultivar "Santa Rosa" and "Burbank" respectively, were triple-cloned and found to be serologically related to an isolate of the phony peach bacterium (PP-1). The latter has been shown to cause plum leaf scald symptom

on Santa Rosa plum (C. J. Chang, unpublished). Both PLSB1 and PLSB2 proved to be pathogenic to plums. The determination of a positive or negative recovery of the PLS bacterium for the entire time period was based on these two facts: (i) the isolation technique used for twigs gave an extremely low contamination, and (ii) contaminants, if they developed, were fast growing and easily differentiated from the PLS bacterium. In the isolation of the PLS bacterium from roots, contamination was minimized by the antibiotics present in the CS20BN medium permitting PLS bacterial colonies to develop. Chang and Schaad¹⁰ showed that the minimum inhibitory concentrations (MICs) of bacitracin and neomycin to PP and PLS bacterial isolates were 400 and 160–320 $\mu\text{g/ml}$, respectively. The incorporation of bacitracin (50 $\mu\text{g/ml}$) and neomycin (25 $\mu\text{g/ml}$) in CS20 medium made it possible to isolate the bacteria from highly contaminated samples such as roots. The isolation rates from plum roots in November and December of 1983 and January of 1984 were lower than those from twigs of the same months. Whether the two antibiotics were inhibitory to the bacteria in the initial isolation was not determined. Nevertheless, survival of bacteria in the root system through the winter was demonstrated.

The isolations curve was the same for both "Santa Rosa" and "Burbank" cultivars, while "Ozark Premier" was different. According to studies by Latham and Norton⁷, the bacterial counts obtained from both roots and twigs of "Santa Rosa" and "Burbank" cultivars were higher than those of "Ozark Premier" cultivar. The "Ozark Premier" cultivar is probably more tolerant than the other two cultivars. A tolerant cultivar might limit the multiplication of PLS bacteria *in situ*, and this may have resulted in lower isolation of bacteria from the "Ozark Premier" cultivar. Further investigation by increasing the number of each test variety is needed before a conclusion about varietal tolerance is made.

For the "Santa Rosa" and "Burbank" cultivars, bacteria were recovered on agar plates for 23 out of the 25 months. No bacteria were recovered during May 1983 or April 1984. The only high rate of contamination in 25 months may have resulted in no isolation in April, 1984. Our results differed greatly from those of Wells *et al.*¹¹). By using vacuum infiltration of root and twig sections of peach trees, they concluded that the XLB counts were higher in May as compared to those obtained in February, August, or November. Whether different hosts (plum vs. peach) and different methods (cultivation vs. vacuum infiltration) used in the two studies contributed to the conflicting results is unknown and warrants further investigation.

The isolation curves indicate that active multiplication and spread of the PLS bacteria in "Santa Rose" and "Burbank" began between mid-May and June and peaked in November or December, while in "Ozark Premier" it began in mid-July and peaked in September and October. This coincides with the appearance of leaf scald symptoms (mid-June to July). The time of rapid increase in PLS bacteria in "Ozark Premier" was about two months later than in either "Santa Rosa" or "Burbank", indicating that "Ozark Premier" is probably more tolerant to PLS bacteria than the other cultivars. Similar results were shown in the study of the accumulation of Pierce's disease bacterial occlusions in xylem vessels of leaves of susceptible "Schuyler" bunch grapes and

of more tolerant "Carlos" and "Welder" muscadines. Hopkins and Thompson⁶⁾ concluded that the accumulation in "Schuyler" bunch grapes occurred two months earlier than in "Carlos" and "Welder" muscadines. In the same report,⁶⁾ the highest vessels infestation occurred in September or October for bunch grapes and muscadines. Less than 20% of vessels were infested in May for "Schuyler" bunch grapes and in May, June, and July for "Carlos" and "Welder" muscadines as compared to 80% or more in September or October for all three varieties. Probably, the reason vessel infestation was low in May, June, and July was because the PD bacterium does not develop in actively growing juvenile tissue⁶⁾. This would explain why the isolation rates were lower in the month of April and May. The PLS bacteria continued to increase and disseminate through the tree from August to November in "Santa Rosa"; to December in "Burbank"; and to October in "Ozark Premier" even though the air temperatures were declining. The isolation rates then dropped through May, even when temperature from January to May were increasing. The recovery rate sharply dropped in January and February, 1984, possibly because unusually cold temperatures occurred in Georgia in December, 1983 (6.8 C versus 13.0 C of December, 1984) and January, 1984 (5.7 C). The PLS bacteria may survive unusually cold temperatures in the root system. However, with near normal or mild temperatures, PLS bacteria could possibly survive the winter in twigs as well.

Apparently, PLS bacteria do not develop well in actively growing tissues and the population is lower during April and May. Therefore, April and May appear to be ideal times for application of chemotherapeutics by tree injection for best chemical uptake to inhibit the low bacterial population.

Literature cited

1. Chang, C. J. and Schaad, N. W. (1982). *Phytopathology*: 72 : 1006.
2. Davis, M. J., French, W. J. and Schaad, N. W. (1981). *Curr. Microbiol.* 6 : 309-314.
3. Fernandez-Valiela, M. V. and Bakarcic, M. (1954). *In*: Inf. Invest. Agric. (IDIA) No. 84, Inst. Nac. Toen. Agropec. (INTA), Buenos Aires, Argentina. pp. 2-6.
4. French, W. J., Latham, A. J. and Stassi, D. L. (1977). *Proc. Am. Phytopathol. Soc.* 4 : 223.
5. French, W. J. and Kitajima, E. W. (1978). *Plant Dis. Rep.* 62 : 1035-1038.
6. Hopkins, D. L. and Thompson, C. M. (1984). *Hort. Sci.* 19 : 419-420.
7. Latham, A. J. and Norton, J. D. (1980). *Agric. Exp. Stn. Bull.* 525. Auburn Univ., Auburn, AL, USA. 15 pp.
8. Raju, B. C., Wells, J. M., Nyland, G., Brlansky, R. H. and Lowe, S. K. (1982). *Phytopathology* 72 : 1460-1466.
9. Schaad, N. W. (1978). *Ibid.* 68 : 249-252.
10. Spurr, A. R. (1969). *J. Ultrastruct. Res.* 26 : 31-43.
11. Wells, J. M., Weaver, D. J. and Raju, B. C. (1980). *Phytopathology* 70 : 817-820.
12. Wells, J. M., Raju, B. C., Nyland, G. and Lowe, S. K. (1981). *Appl. Environ. Microbiol.* 42: 357-363.

和 文 摘 要

C. J. CHANG・C. E. YONCE : 罹病樹内におけるスモモ葉焼病菌の越冬

スモモ樹内での木部局在性細菌 (XLB) の生存を評価するために CS20 寒天培地を用いて分離を行なった。1982年10月から1984年10月にわたり葉焼病罹病樹3本からそれぞれ30~40本の小枝を毎月、無作為に採取した。1983年12月から1984年5月には他の3樹から16~24本の根を採取し CS20BN 寒天培地で分離を行なった。

それぞれ表面殺菌した枝および根から得た搾汁液を直ちに CS20 又は CS20BN 寒天培地上に置き、30℃で培養し細菌集結の形成を検鏡(×50)により毎週観察した。品種“Santa Rosa”からは1983年5月と1984年4月を除く毎月、“Burbank”からは1983年5月と1984年2月、4月を除く毎月、および“Ozark Premier”からは1983年6月～12月 および1984年8月～12月の各月にそれぞれ細菌が分離された。XLB は各樹の根からも1983年11月から1984年5月にわたり分離された。以上の結果から XLB がジョージア州バイロンで冬期間生存可能であること、および常に健全樹への伝搬を可能にする何らかのベクターが存在することが示唆された。