

Identity and Behavior of Xylem-Residing Bacteria in Rough Lemon Roots of Florida Citrus Trees†

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Received 23 November 1981/Accepted 8 February 1982

An aseptic vacuum extraction technique was used to obtain xylem fluid from the roots of rough lemon (*Citrus jambhiri* Lush.) rootstock of Florida citrus trees. Bacteria were consistently isolated from vascular fluid of both healthy and young tree decline-affected trees. Thirteen genera of bacteria were found, the most frequently occurring genera being *Pseudomonas* (40%), *Enterobacter* (18%), *Bacillus*, *Corynebacterium*, and other gram-positive bacteria (16%), and *Serratia* (6%). Xylem bacterial counts fluctuated seasonally. Bacterial populations ranged from 0.1 to 22 per mm³ of root tissue (about 10² to 2 × 10⁴ bacteria per g of xylem) when bacterial counts were made on vascular fluid, but these numbers were 10- to 1,000-fold greater when aseptically homogenized xylem tissue was examined similarly. Some of the resident bacteria (4%) are potentially phytopathogenic. It is proposed that xylem bacteria have an important role in the physiology of citrus.

Little information is available regarding bacteria which are capable of colonizing internal parts of apparently healthy plants, particularly woody perennials (12, 19, 23, 24). These bacteria, if shown to be present, could play a role as potential disease agents under certain stress conditions or, alternatively, as beneficial bacteria serving to fix nitrogen or produce growth factors. This study was initiated to determine the identity, pathogenicity, and seasonal populations of bacteria in xylem vessels of apparently healthy as well as diseased roots of citrus and to explore the possibility that bacteria can survive in planta for extended periods.

Using a sterile vacuum extraction procedure, Feldman et al. (8, 9) recovered a rickettsia-like bacterium (RLB) from the roots and twigs of young tree decline (YTD)-affected citrus trees as well as from several apparently healthy trees. These investigators (9) also found many other bacteria in the xylem fluid, with generally greater numbers in the healthy-appearing roots of YTD-affected trees. Since xylem dysfunction is a characteristic symptom of this destructive disease, the presence of these various bacteria in the xylem fluid of citrus trees and their possible relationship to citrus tree health and vigor prompted a more in-depth investigation of the role of these bacteria in this unique ecological niche.

Our results demonstrate that bacteria normal-

ly reside inside xylem tissues of citrus (rough lemon) roots and that such bacteria are capable of existing compatibly in planta for 1 year. Some of these bacteria are potentially phytopathogenic. This characteristic is lost when the organism is cultured in vitro, but is retained or potentiated by their passage in planta.

MATERIALS AND METHODS

Plant material. Root samples for vascular fluid extraction were collected from 12 citrus groves in the central ridge area and from 3 groves in the flatwoods area of Florida. Trees used were sweet orange (*Citrus sinensis* [L.] Osb.) and grapefruit (*C. paradisi* Macf.) on rough lemon rootstock (*C. jambhiri* Lush.), which is the rootstock most susceptible to YTD. Each week, root samples from two healthy and two YTD-affected trees in an early stage of decline were collected from a single grove. Trees previously sampled were not used for subsequent samplings. A composite of 12 to 14 healthy-appearing sections of root, approximately 1 by 25 cm, was collected from each tree as follows. Pruning shears were stored in 95% ethanol and flamed before each use. Before the intact root or twig was cut, the area to be cut was swabbed with 95% ethanol and flamed. This latter procedure was discontinued after it was shown that it had little effect on bacterial counts. After cutting, the 25-cm section was rinsed with ethanol, wrapped in cheesecloth, and stored on ice. For initial processing, sections were washed with a detergent solution, rinsed with sterile water, and dipped in 95% ethanol for 5 min. They were then placed in a sterile plastic bag and stored at 4°C overnight before vacuum extraction.

Vacuum extraction method. Ten uniform 25-cm root sections were selected, and 5-cm pieces from each end were removed and discarded after dipping in 95%

† Florida Agricultural Experiment Stations journal series no. 3481.

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ethanol and flaming. Two centimeters of bark, including functional phloem and cambium, was removed aseptically from each end. The cut end was dipped for 3 min in ethanol, flamed, and rinsed in sterile distilled water. Sections were then vacuum extracted with 2.0 ml of sterile 0.02 M potassium phosphate buffer, pH 7, as described previously (8, 9).

The extracted fluid was collected directly into sterile 15-ml Corning screw-capped centrifuge tubes, serially diluted, and spread on triplicate plates of JD-3 medium (7) and medium 523 (14). Plates were counted on day 5, and JD-3 plates for isolating fastidious RLB were reexamined on day 10. Populations of bacteria are enumerated as numbers of bacteria per 20 ml of extractant (the amount obtained from 10 15-cm root sections). Monthly counts are means of 8 to 10 healthy or diseased trees representing 4 to 5 different grove locations.

Homogenization extraction method. Root sections (25 cm) were surface sterilized in 33% Clorox (1.9% NaClO₄), dipped in 95% ethanol, flamed for 5 to 10 s, and rinsed in sterile distilled water. Five-centimeter pieces were cut from each end, and bark, including phloem and cambium, was stripped off. Sterilization procedures were repeated. Subsequently, a 5- to 10-cm section was cut, weighed, directly placed in a sterile stainless-steel blender containing 20 ml of sterile 20 mM potassium phosphate buffer, pH 7, and comminuted for 3 to 5 min. A 0.1- or 0.2-ml portion was spread on triplicate plates of Tryptone soy agar (TSA). Colony-forming units (CFU) were scored after 5 days at 28°C because some slow-growing bacteria took more than 3 days to appear.

Since tissue was incompletely homogenized, tissue fragments not passing through a 20-mesh screen were reweighed, and this was subtracted from the original tissue weight. Bacterial numbers are expressed as CFU per gram (fresh weight) of tissue.

Sterility checks and controls. All tools and extraction equipment were sterilized by autoclaving. No contaminating organisms were observed when sterility checks were made periodically on tools and cut surfaces of tissues and when extraction procedures were carried out with autoclaved root sections.

Control experiments were carried out with both ends of each root section sealed with hot paraffin wax immediately after severing from the tree to eliminate the possibility that organisms were introduced into cut ends of tissue sections. We found no differences in numbers of bacteria recovered whether or not ends were sealed. Also, we found no differences in numbers of bacteria recovered by vacuum extraction when all bark was removed from root sections. In the vacuum extraction procedure, it was easier to avoid contamination during handling and extraction if root pieces had 2 cm of bark removed only from upper and lower ends.

In another control experiment, autoclaved root sections were dipped in a 10⁸-cells/ml solution of *Pseudomonas cepacia* (citrus isolate 299RW3 Na^r Rif^r) and subsequently carried through surface sterilization and extraction procedures to test the possibility that bacteria could survive surface sterilization procedures. Contamination with these surface bacteria was not observed.

Identification of bacteria. After vacuum extraction and quantitation, bacterial isolates were randomly

selected from single colonies on JD-3 and 523 media. Colonies were restreaked on 523 medium or TSA until pure cultures were obtained for further characterization.

Isolates were identified according to *Bergey's Manual* (2). Initially, bacterial isolates were tested for Gram stain, oxidase reaction, and morphology. In general, oxidase-negative, gram-negative bacteria were characterized by using the Roche Enterotube II system (Roche Diagnostics, Nutley, N.J.), and oxidase-positive, gram-negative rods were characterized on the Roche OXI/FERM system (13, 21). When the oxidase reaction was variable or weak, both systems were utilized. Additional tests in some cases were made with the API 20E system (Analytab Products, Plainview, N.Y.) (21). Phytopathogenic *Pseudomonas* types which elicited a hypersensitive reaction (HR) in tobacco (*Nicotiana tabacum* L.) (15) were subjected to additional tests (17, 18, 25): utilization of 65 different carbon sources, growth at 40°C, electron microscopy for size, morphology and flagellar insertion, production of fluorescent pigments on Kings B medium, production of lipase (hydrolysis of Tween 80), pectinase (potato soft rot), and protease (hydrolysis of gelatin), production of poly-β-hydroxybutyrate, and resistance to antibiotics.

HR. Tobacco, cv. Bottom Special, was inoculated with a suspension of 10⁸ to 10⁹ cells/ml according to established procedures (15). A necrotic reaction within 24 h was taken as a reliable indication that the bacterium was a potential phytopathogen.

Antimetabolite production. Production of antimetabolites was evaluated by using *Escherichia coli* strain K-12 (University of Florida, Gainesville, culture collection no. AB259) growing on minimal media as described by Gasson (11). Briefly, young (24 to 48 h) colonies were transferred to minimal agar media containing 10⁸ cells of *E. coli* per ml by stabbing the agar with a loopful of inoculum. A clear zone of inhibition after 48 h at 28°C indicated antimetabolite production. Antimetabolite production was evaluated weekly from 32 randomly selected single colonies.

Other methods. For dye distribution in root sections, aqueous solutions of 0.1% acid fuchsin and 0.1% Congo red were vacuum infiltrated into 150 by 58-mm root segments, and the latter were immediately cut in several sections and examined.

Bacteria resistant to the antibiotics nalidixic acid (Nal), neomycin (Neo), and rifampin (Rif) were selected in a serial fashion as spontaneous mutants growing on 75 μg of each of the antibiotics per ml. *P. cepacia* Na^r Rif^r and Neo^r Rif^r mutants were inoculated into rough lemon seedlings and into budlings of Pineapple sweet orange on rough lemon rootstock by cutting the root system and dipping it into a 5 × 10⁸-cells/ml suspension of bacteria. A vacuum (625 mm of Hg) was placed on the cut of the stem for 5 min. Plants were then repotted in Lakeland sand with added dolomite and maintained in an insect-proof screenhouse. For recovery of antibiotic-resistant mutants, a 10-cm segment of the lower stem was cut out and carried through the homogenization extraction procedure. Dilutions of the homogenate were plated on TSA containing 150 μg of cycloheximide per ml and 50 to 75 μg of respective antibiotics per ml.

For testing growth of anaerobic bacteria, xylem exudates were plated on JD-3, TSA, and 523 agar

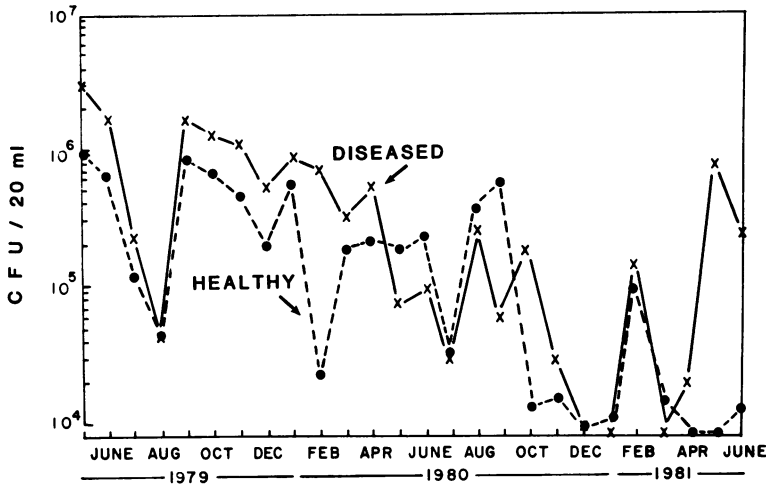


FIG. 1. Bacterial populations from surface-sterilized, vacuum-extracted rough lemon roots from apparently healthy (O) and YTD-affected (x) trees. Colony-forming units were tabulated each week from a total of 20 ml of xylem extract from 10 15-cm root sections. Each point represents a monthly average of 4 to 5 weekly determinations.

media (plus and minus nitrate) in an anaerobic chamber (GasPak; BBL Microbiology Systems, Cockeysville, Md.).

RESULTS

Bacterial populations in citrus roots. Bacteria were consistently isolated from the xylem of healthy and YTD-affected trees. Bacterial populations monitored in 15 different citrus groves for a 2-year period ranged from 10⁴ to 3.3 × 10⁶ CFU per 20 ml of xylem fluid from 150-cm³ root tissue (Fig. 1). Each monthly data point represents 8 to 10 trees (10 root samples per tree) from 4 to 5 groves per month. Fluctuations in bacterial populations, in view of the somewhat limited sample size, may have been due to inherent variation in samples as well as to complex environmental factors. For instance, in 1980 to 1981, rainfall was 42% lower than in 1979 to 1980. Temperature extremes in 1980 to 1981 were also greater. This pattern coincided with lower average bacterial numbers in 1980 to 1981 (Table 1).

The average bacterial count for diseased trees was higher than counts for healthy trees in 1979 to 1980 and in 1980 to 1981 (Table 1). Vessel plugging, which is characteristic of YTD-affected trees, would tend to bias these data in favor of healthy trees by restricting bacterial recovery during vacuum extraction. About 8% of healthy trees exhibited YTD symptoms within 3 to 9 months after initial sampling, and these trees had higher than average bacterial counts. No differences in bacterial genera or species were

found between isolates from the two groups of trees.

Effect of extraction method. Vacuum extraction was found to be an inefficient technique for quantitative extraction of bacteria. Xylem tissue, when stripped of cambium and phloem and homogenized aseptically, yielded 10- to 1,000-fold-higher bacterial counts (Table 2). Essentially, this is in accord with more direct evidence obtained by electron microscopy (J. M. Gardner, et al., manuscript in preparation) that most bacteria do not exist freely within xylem vessels but become entrapped in fibrous occlusions along border pits and vessels walls.

Bacterial counts in twigs and trunkwood of mature trees. In contrast to root samples, bacterial numbers were low in twig and trunkwood samples. Bacteria were not detected in 40% of the twig samples nor in 75% of the trunkwood

TABLE 1. Average annual bacterial counts for healthy and diseased citrus, 1979 to 1980 and 1980 to 1981

Group	CFU recovered ^d /cm ³ of root (×10 ³)		
	1979-1980	1980-1981	1979-1981
Diseased	6.20 ^b	2.70 ^c	4.45 ^d
Healthy	0.90 ^e	0.71	0.80 ^f
Total	3.55	1.70	2.62

^a Bacteria were recovered by the vacuum extraction method.

^{b-f} The following pairs are significantly different (*P* = 0.5) according to Fisher's least significant difference test: (*b*, *c*); (*b*, *d*); (*d*, *f*).

TABLE 2. Quantitation of bacteria from citrus xylem from twigs and roots and comparison of two methods of extraction^a

Tree no.	CFU/g of xylem (fresh wt)				Ratio, ^a H/V
	V		H		
	Root ($\times 10^2$)	Twig ($\times 10^3$)	Root ($\times 10^5$)	Twig ($\times 10^2$)	
1	0.7	<10	0.03	3.0	43
2	0.7	50	0.15	2.0	214
3	10.0	<10	0.32	0.0	32
4	2.0	<10	0.08	2.0	40
5	1.0	80	0.73	1.0	730
6	4.0	40	>10	7.0	>250
7	180.0	<10	4.00	1.0	22
8	30.0	<10	5.00	1.0	166
9	6.0	<10	1.10	5.0	183
10	1.0	<10	2.10	1.0	2,100
11	5.0	30	1.20	1.0	240
Avg	2.2	>50	2.25	2.0	365

^a V, Vacuum method; H, homogenation method.

^b Ratio for root samples only (most twig counts were negligible).

samples. The average yield of bacteria from twig samples by homogenization was about 5×10^2 /g of xylem tissue. Thus, bacteria appear to be restricted more to the root system, and motility in xylem vessels is apparently limited.

Bacterial counts in seedlings. Substantial bacterial counts were obtained from aboveground,

main shoots of seedlings. An average bacterial count from 10 different cultivars (Sour orange, Hamlin, Carrizo, Volkameriana, Pineapple, Valencia, Rough lemon, Grapefruit, Milam, and *Trifoliata*) of citrus seedlings from five different commercial nursery sources was 7×10^4 CFU/g of xylem (data not shown). Care was taken to select samples from healthy seedlings that had not been wounded. This relatively high bacterial count from seedlings indicates that xylem vessels harbor resident bacteria from an early stage of development.

Identity of bacteria and frequency of occurrence. Of the 13 genera of bacteria identified in a survey of 556 isolates, *Pseudomonas*, *Enterobacter*, *Bacillus* plus other gram-positive bacteria, and *Serratia* were most predominant, in that order (Table 3). There appeared to be no difference in frequency of occurrence of specific genera of bacteria between healthy and diseased trees. Overall, gram-negative bacteria accounted for 84% of the 556 isolates identified. *Pseudomonas* species accounted for 40% of the bacteria that were randomly selected, and of these approximately 95% were nonfluorescent species.

In contrast to the above, bacteria in the rhizosphere were, in many cases, predominately (>50%) fluorescent pseudomonads. The fluorescent isolates were identified as *P. putida*, *P. fluorescens*, and *P. aeruginosa*, usually in that order of predominance (data not shown). Since

TABLE 3. Frequency of isolation of various genera and species derived from xylem fluids of rough lemon rootstock

Genus	Species ^a	No. identified	% of total
<i>Pseudomonas</i>	<i>cepacia</i> , other nonfluorescent spp., <i>putida</i> <i>aeruginosa</i> , <i>fluorescens</i>	224	40.3
<i>Enterobacter</i>	<i>agglomerans</i> , <i>cloacae</i> , <i>sakazakii</i> , <i>aerogenes</i>	103	18.5
Gram-positive species ^b			
<i>Bacillus</i> , <i>Corynebacterium</i> , <i>Arthrobacter</i> , <i>Actinomycetes</i>		88	15.8
<i>Serratia</i>	<i>marcescens</i> , <i>liquefaciens</i>	32	5.8
<i>Yersinia</i>		20	3.6
<i>Acinetobacter</i>	<i>lwoffii</i>	16	2.9
<i>Citrobacter</i>	<i>freundii</i>	14	2.5
<i>Alcaligenes-Moraxella</i>		13	2.3
<i>Klebsiella</i>		8	1.5
<i>Shigella</i>		6	1.1
<i>Achromobacter</i>		5	0.9
<i>Providencia</i>		3	0.3
<i>Flavobacterium</i>		2	0.4
<i>Vibrio</i>		2	0.4
Unknown ^c		20	3.6
Total		556	99.9

^a Most probably species as indicated by OXI/FERM or Enterotube tests, including other standard criteria (2). Colonies were chosen through random selection.

^b Identified only by Gram reaction, colony morphology, and microscopic morphology.

^c Isolates not fitting any code number for OXI/FERM or Enterotube and otherwise of uncertain identity.

TABLE 4. Inhibition of *E. coli* growth on minimal media by xylem-residing bacteria from rough lemon roots

Month	% of isolates inhibiting <i>E. coli</i>	
	Healthy	Diseased
March	19.0	27.6
April	11.9	19.9
May	20.3	21.0
June	27.3	30.9
July	25.6	37.6
Avg	20.8	27.4 ^a

^a Significantly different from healthy at 5% probability (least significant difference at 0.05 = 5.5).

so few of the isolates from xylem were fluorescent pseudomonads, this would argue against the possibility of isolates from root tissue being rhizosphere contaminants.

Identity of potentially phytopathogenic pseudomonads. Approximately 850 isolates, including most of the 556 that were identified, were screened for ability to elicit HR in a standard indicator plant (tobacco) as a means of identifying potential phytopathogens (15). Of these, 48 isolates gave a definite positive reaction. Over 90% of those that were HR positive were pseudomonads, and about 95% of these were nonfluorescent. The few fluorescent species most closely resembled *P. putida* and *P. fluorescens*, which are normally saprophytic bacteria and not phytopathogenic species such as *P. syringae*. Identification at species level is in progress, but at least 15 isolates (33% of the pseudomonads) have been identified as *P. cepacia*. These isolates had standard characteristics for this species: nonfluorescent diffusible pigment (in most cases), oxidase positive (occasionally weak or variable), growth at 40°C, arginine dihydrolase negative, presence of betahydroxybutyrate granules, protease and lipase positive, utilization of a very broad spectrum of carbon sources (data not shown), nonutilization of characteristic substrates, characteristic protein sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles (data not shown), protease and lipase positive, and resistance to ampicillin, carbenicillin, polymyxin B, and streptomycin. The OXI/FERM code numbers of 0051 and 0050 were obtained for many isolates, although 0151, 0150, 4151, and 4150 occurred in other cases. Mainly, these code numbers denote *P. cepacia*, undefined *Pseudomonas* spp., or both. Unlike standard *P. cepacia* strains (2), our isolates were mostly nitrate reductase negative. Many of our strains produced greater amounts of an antimetabolite inhibiting *E. coli* than did standard strains of *P. tabaci* and *P. phaseolicola*.

Antimetabolite production. *Pseudomonas* anti-

metabolites are often plant disease toxins (11) and, in other cases, metal chelators (4). The toxins could contribute to xylem dysfunction, and the production of chelates could explain the zinc accumulations characteristic of YTD-affected trees. About 80% of the pseudomonads that induced HR also produced antimetabolites which inhibited growth of *E. coli* on minimal media; however, only 20% of the total bacterial population (or approximately 50% of the total *Pseudomonas* population) from xylem produced antimetabolites (Table 4). Isolates from diseased trees had a somewhat higher ($P = 0.05$) incidence of *E. coli* antimetabolite production than did isolates from healthy trees. Most of the isolates producing antimetabolites were *Pseudomonas* species, although some *Enterobacter* and *Serratia* isolates were also obtained. Some of the latter isolates selected by this test may also produce colicins.

Action of the antimetabolite from *P. cepacia* strains was suppressed or completely reversed by methionine (40 µg/ml) and glutamine (40 µg/ml) but not affected by other amino acids.

Fluorescent pseudomonads which were isolated in large numbers from the rhizosphere produced antimetabolites that were reversed by 20 µg of Fe²⁺ ml. These isolates were rarely found in xylem exudates.

Survival of *P. cepacia* isolates in planta. Survival of *P. cepacia* (strain 112) in xylem tissue and in soil was monitored. Doubly antibiotic-resistant mutants resistant to rifampin and nalidixic acid (or neomycin) were constructed for unambiguous identification of recovered bacteria. Resistance to these antibiotics was stable over a period of 1 year. Resistance to both rifampin (rifamycin derivative) and nalidixic acid is thought to be chromosomal rather than plasmid borne (6).

Results of these experiments clearly indicate that *P. cepacia* is able to survive compatibly for up to 11 months within xylem tissue and favorably so in comparison with populations obtained from the rhizosphere (Table 5). Although multiplication of bacteria per se has not been demonstrated in these experiments, observations on pathogenicity of these isolates support the view that long-term survival in planta is dynamic. The original inoculum gave a weak or negative HR test. This is in accord with observations on our other *P. cepacia* isolates in that most lost their ability to produce HR within 3 to 5 months after isolation from citrus and subsequent maintenance on nutrient agar media at 24 or 28°C (with intervals of storage at 4°C). However, when the doubly antibiotic-resistant strain was isolated, a predominant number of colonies from xylem tissue tested gave a strong, positive HR. Fewer numbers of rhizosphere isolates also regained an

TABLE 5. Recovery of *P. cepacia* Nal^r Rif^r and Neo^r Rif^r isolates from citrus xylem and rhizosphere

Expt no.	Inoculum	Recovered ^a from:	Period in planta (mo)	CFU/g of xylem or soil ^e ($\times 10^2$)	HR positive (%)
1	112 Nal ^r Rif ^r	Lyophilized ^b			0
	112 Nal ^r Rif ^r	Xylem ^c	10.5	5.0	73 (11/15)
	112 Nal ^r Rif ^r	Rhizosphere ^d	10.5	2.5	75 (6/8)
2	112 Nal ^r Rif ^r	Lyophilized ^b			0
	112 Nal ^r Rif ^r	Xylem ^c	11	97.0	43 (3/7)
	112 Nal ^r Rif ^r	Rhizosphere	11	4.5	0 (0/8)
3	112 Neo ^r Rif ^r	Original ^b			0
	112 Neo ^r Rif ^r	Xylem ^c	8	60.0	87 (7/8)
			11	102.0	43 (6/14)
	112 Neo ^r Rif ^r	Rhizosphere	8	33.0	63 (5/8)

^a Recovered isolates were confirmed to be *P. cepacia* and to have appropriate Nal and Rif antibiotic resistance markers.

^b Original cultures were HR negative after storage in glycerol at -20°C or after lyophilization.

^c Source plant was Pineapple sweet orange on rough lemon rootstock. Recovery was from the rootstock by the homogenization method.

^d Soil adhering to roots was used for determination of rhizosphere bacteria.

^e Source plant was a Valencia sweet orange on rough lemon rootstock. Recovery was from the lower scion and rootstock.

ability to produce HR, but were usually less virulent than in planta isolates (Table 5).

Dye distribution studies. Solutions of 0.1% acid fuchsin and 0.1% Congo red were vacuum infiltrated into root segments (1-cm diameter) and immediately examined for areas of dye uptake to determine what tissues were being extracted by vacuum. Dye was restricted to xylem elements, and there was little if any lateral distribution of dye into cambium and phloem. In late summer, dye was uniformly distributed throughout the central core of xylem, whereas during winter, distribution was restricted to younger peripheral vessels.

DISCUSSION

The consistent recovery of bacteria from xylem of citrus roots is significant considering their potential roles in the physiology and disease status of citrus. Bacteria from diverse genera and species normally inhabit xylem vessels of mature citrus trees and young seedlings. About 5% of these bacteria are potentially phytopathogenic as evidenced by a positive hypersensitive test in tobacco. The pathogenicity of several citrus isolates has been documented (A. W. Feldman and J. M. Gardner, Proc. Soil Crop Sci. Soc. Fla., in press). Moderate to severe stunting of rough lemon rootstock and scion dieback seem to be characteristic symptoms elicited by these isolates. The number of bacterial cells found in diseased trees would be sufficient to cause xylem blockage, particularly if localized multiplication to high numbers occurred under conditions of stress. Although similar types of bacteria appeared to be present in both diseased and healthy trees, this may simply

indicate that most trees harbor potentially pathogenic species.

The topic of resident bacteria in apparently healthy plants, particularly perennials, has not been adequately addressed. Perhaps this has been due mainly to a healthy skepticism as to effectiveness of surface sterilization and other procedures of isolation on the assumption that the organisms obtained were contaminants (10). There is some evidence that so-called contaminants are often normal microflora in plant tissues (12, 19, 23). Many plant tissues are indeed aseptic, but there is often opportunity for bacteria to enter through protective barriers into intercellular spaces or xylem vessels and to reside there for extended periods.

Mundt and Hinkle (19) isolated 19 genera of bacteria (*Bacillus* sp., *Enterobacter agglomerans*, *Flavobacterium* sp., and *P. fluorescens* predominating, in that order) from seeds and ovules of 27 species of plants. Infestation of these plant tissues was apparently nonspecific; i.e., many of the colonizers reflected the general soil bacterial population. However, the majority (70 to 85%) of ovules and seeds were apparently sterile, indicating that normal protective mechanisms, physical or metabolic (or both), exist to exclude bacteria. We have also found this to be true with citrus seeds and fruit; most are sterile unless normal protective barriers are overcome (data not shown). The latter would most likely occur during germination and radicle development. In this respect, we have found that over 90% of citrus seedlings have resident xylem bacteria. We can only speculate that these resident bacteria may multiply and remain with the tree through development.

Bacteria and fungi have been associated with discolored heartwood in many types of trees (5, 24). Yet, to our knowledge, reports of bacteria as normal inhabitants in xylem tissue have not been published. In roots, many opportunities exist for bacteria to penetrate through bark, phloem, and cambium and into xylem vessels. In time, these bacteria may multiply and mobilize, depending on resistance pressures of the host, the latter being influenced by many external stresses, i.e., water deficits, temperature extremes, nutritional unbalances, and microbial toxins.

Nemec (20) isolated many genera of bacteria from rough lemon roots. The frequency of occurrence of specific genera described by Nemec contrasts with our results, perhaps due to different sources and to isolation procedures. He reported only 20% gram-negative isolates versus 84% in our study, and 8% *Pseudomonas* sp. versus 40% in our study. However, he considered all but one isolate to be saprophytic bacteria.

Of 44 potentially phytopathogenic bacterial isolates giving a strong positive HR in tobacco, more than 90% were *Pseudomonas* spp. *Pseudomonas* is characteristically restricted to aerobic environments, although some species are known to grow anaerobically at the expense of nitrate reduction. However, since most of our isolates do not reduce nitrate, including *P. cepacia*, we speculate that oxygen tension of xylem is sufficient to support these bacteria. This must also be true with the xylem-limited RLB (8), which are obligate aerobes and multiply in large numbers within xylem vessels of grape and other plants. We failed to find significant numbers of anaerobic bacteria in citrus xylem over 4 months of testing, which included the use of several types of media.

It is difficult to ascribe fluctuations in bacterial populations to any environmental factors. Sampling of trees was limited by necessity. We cannot propose at this time any causal relationship between bacterial numbers and disease incidence, even though statistically higher populations of bacteria were observed in presymptomatic and in YTD-affected trees in 1979 to 1980. The distinctly lower bacterial populations overall in 1980 to 1981 compared with 1979 to 1980 (Table 1) may have been related to high temperatures combined with drought (58% lower rainfall in 1980 to 1981).

It is clear from other studies (Gardner et al., manuscript in preparation) that xylem-residing bacteria exert detrimental effects on xylem conductance and induce plugging. Bacteria are trapped and occluded in xylem vessels soon after being introduced therein, making it very difficult to observe bacteria by scanning electron

microscopy. It is not surprising, therefore, that up to 1,000-fold more bacteria can be extracted by homogenizing tissue as opposed to vacuum extraction of xylem. Cross sections of vessel occlusion material reveal cells closely resembling *P. cepacia* and also RLB. The failure to culture the fastidious RLB may be due to their low numbers and lack of appropriate isolation medium (8). Since *Pseudomonas* spp. in particular can be extracted in a viable form, it would appear likely that these bacteria continually multiply and subsequently induce and become enveloped by occlusion material, which is a characteristic feature of xylem vessels in YTD-affected trees (3).

Although direct proof for multiplication of bacteria in xylem is not presented, results on long-term survival of the pseudomonads and changes in their aggressiveness in planta suggest that their association with the host is a compatible and dynamic one. Passage through citrus potentiates the ability of these isolates to induce HR. The degree to which this occurs might be influenced by particular environmental stress conditions which weaken specific resistance mechanisms of the host.

Xylem bacteria in roots may have a significant long-term influence on vigor and nutrition of the tree. *Enterobacter* sp. isolated from citrus such as *Enterobacter cloacae* and *E. herbicola* are nitrogen fixers in rhizosphere and inside epidermis of grass roots (22). Rhizobacteria on plant roots can have growth-promoting effects (1, 16). Experiments to test possible long-term beneficial as well as phytopathogenic roles of the xylem-residing bacteria are in progress.

ACKNOWLEDGMENTS

We are indebted to the technical assistance of Juan Chandler, Sheree Pratt, William Alexander, and Megan Cassidy. We thank Clarence Kado, Robert Stall, Suresh Patil, R. Durbin, and Mortimer Starr (International Collection of Plant Pathogenic Bacteria) for bacterial cultures. We also appreciate review of the manuscript and appropriate suggestions by R. Stall and L. W. Timmer.

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