

Isolation, Cultivation, and Pathogenicity of *Xylella fastidiosa*, the Causal Bacterium of Pear Leaf Scorch Disease in Taiwan

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ABSTRACT

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Leaf scorch symptoms occurred in 12–20.6% of the pear trees (*Pyrus pyrifolia*) in the low-altitude areas of central Taiwan in 1990. Symptoms appeared on leaves in early July and stayed until the leaves dropped in winter. If unchecked, dieback of twigs and branches, and finally death of the infected trees occurred within a few years. The disease was closely associated with a xylem-limited bacterium. An electron micrograph showed that the bacterium was rod-shaped with rippled cell walls, and measured from 0.2 to 0.5 × 1.1 to 3.4 μm. The bacterium could be cultured on PD2 and PW media but not on general-purpose bacterial media. Colonies were convex, roundish, and creamy white with a smooth margin, and reached 0.1–0.2 mm in diameter after a 14-day incubation at 30 C. Serologically, our culture was not closely related to the *Xylella fastidiosa* MT1 strain originally isolated from tissues with alfalfa dwarf disease. Fifty-five percent of healthy scions grafted onto water shoots of the leaf scorch-infected trees showed leaf scorch symptoms, and 50% of greenhouse-grown seedlings artificially inoculated with a bacterial strain from scorched pear developed scorch symptoms. The bacterium was reisolated from those inoculated symptomatic tissues. Control seedlings inoculated with phosphate-buffered citrate-magnesium solution remained symptomless. Injection of oxytetracycline alleviated disease development.

In Taiwan, pear (*Pyrus pyrifolia* (N.L. Burm.) Nakai) is cultivated in areas with an altitude ranging from 100 to 2,000 m. In lower altitude areas (<800 m), low chilling cultivar Hengshan is widely cultivated. Starting more than a decade ago, scions of high-quality varieties such as Twentieth Century, New Century, Fousui, and Kousui, which were cultivated around 1,700-m-altitude areas, were high grafted to water shoots of Hengshan after flower formation was completed. Fruits of the grafted varieties could be harvested in June and July, compared to the normal harvesting time of September in the high altitude areas. Fruit from the stock Hengshan trees were harvested in September (14). Because of the handsome return for producing high-quality pears early in the season, grafting has become so common that scions of these high-quality varieties have been in short supply in Taiwan. Consequently, desirable varieties have been imported from Japan to satisfy the farmers' demands. In the Chiayi area (100 m), the growers adjusted to harvest the fruit from the Hengshan variety between April and May.

Pear leaf scorch is a hitherto unreported disease that may have occurred in Taiwan for more than a decade. In

the variety Hengshan, leaf scorch symptoms can be observed during the summer and fall. Eventually dieback of twigs and branches, and in severe cases, death of infected trees occur. Since 1988, we have surveyed the disease and studied the association of a fastidious xylem-limited bacterium (XLB) with infected trees. We report here the results of our studies. Preliminary results were reported by Leu and Su in 1990 (13).

MATERIALS AND METHODS

Symptoms and occurrence. Symptom expression was surveyed twice a month from May to November in the Taichung area and once in August in the Chiayi and Lisan areas in 1990. To survey disease incidence, orchards were randomly selected and all trees were examined from August to September in the Taichung area. A total of 2,097 trees, ranging from 18 to 202 in each of 23 orchards, were surveyed. Any trees showing unequivocal leaf scorch symptoms with decline were considered to be infected.

Isolation of bacteria. Twigs with leaves showing typical scorch symptoms were collected in the field along with healthy-appearing controls. Petioles and twigs of 0.5–1 cm in diameter and 5–10 cm in length were immersed in 95% ethanol for 3 min, then passed through the flame of an alcohol lamp. The sap was pressed out with a pair of pliers and collected by a capillary tube, and 10 spots were

pipetted onto a solid-culture medium. Usually, 20 sap samples were obtained from the diseased tissues and 10 from the healthy ones. Isolation was conducted once in October 1989 and again in July and September 1990. Tissues for the last isolation were collected from a different orchard. Media used for isolation were those developed for the isolation of various *Xylella fastidiosa* Wells et al strains (20), such as PD2 (6), CS20 (2), PW (4), and BYCE (21), and those for common phytopathogenic bacteria, such as nutrient agar (NA), yeast extract-dextrose calcium carbonate (YDC) (22), King's medium B (KB) (12), and 523 agar (11). Plates were incubated at 30 C, and colony development was observed under a dissecting microscope weekly for one month.

Microscopic observation of bacteria.

Three randomly selected strains were used for microscopic observation. Bacterial colonies were streaked on PD2 medium and incubated at 30 C for 7 days. Sterile distilled water was added to the cultures to prepare a bacterial suspension for electron microscopic observation. For negative staining, a drop of bacterial suspension (10^8 to 10^9 cells per milliliter) was pipetted onto a Formvar-coated copper specimen grid. After 5 min, the excess bacterial suspension was removed with sterilized filter paper, and a drop of 2% phosphotungstic acid containing 0.1% bovine serum albumin was placed on the coated grid for 1 min, then removed with filter paper and examined with a Hitachi 300 transmission electron microscope (TEM). One hundred cells were photographed and measured. Stained bacteria were observed by phase-contrast microscope (×1,000) under oil immersion to determine Gram reaction (17). For thin-section observation, the bacterial suspension was mixed with 2% melted Bacto agar at 50 C. The solidified agar with the bacterial cells attached was cut into 2-mm blocks, fixed with 2.5% glutaraldehyde and post fixed with 2% osmium tetroxide for 2 hr each, then dehydrated through an acetone series (50, 70, 80, 95, and three times at 100%) for 10 min each. The agar block was embedded with Spurr mixture (18) at 70 C, and after a 4–8 hr infiltration, was polymerized for 8 hr. Sample blocks were sectioned by a glass knife with an ultra-

microtome (Sorvall MT-1). The sections were stained with uranyl acetate and lead citrate and examined by TEM.

Microscopic observation of the diseased tissues. Main and lateral vein tissues of the scorched leaves were cut into approximately 1–2-mm pieces and prepared as above for thin-section observation with a TEM. Some of the same samples were fixed with 5% F.A.A. solution (ethanol 90 ml, acetic acid 5 ml, formalin 5 ml). Fixed samples were dehydrated through an ethanol series and embedded in paraffin medium after approximately 24–36 hr of infiltration. Semithick sections were cut with a microtome (Reichert-Jung 2040), then released into a xylene and an ethanol series. The released paraffin sections were critical-point dried in carbon dioxide, mounted on aluminum stubs with silver paste, coated with gold-aurum in an Ion coater IB.2 (Eiko Engineering, Japan), and examined with a Hitachi S400 scanning electron microscope (SEM).

Serology test. Polyclonal antibodies of pear leaf scorch bacterium (PLSB) (provided by C. P. Lin, Department of Plant Pathology and Entomology, National Taiwan University, Taipei, Taiwan) were purified from antisera collected from BALB/c mice killed for the production of monoclonal antibodies against PLSB (C. P. Lin, unpublished). The antibodies were further diluted to 50 µg of protein per milliliter in phosphate-buffered saline (PBS, pH 7.4) for enzyme-linked immunosorbent assay (ELISA). Indirect ELISA with the phosphatase system described previously (19) was used to differentiate PLSB from other procaryotes. PLSB and the MT1 strain from alfalfa dwarf disease were maintained in PD2 media. Other bacteria included in the ELISA were the following: *Xanthomonas campestris* pv. *campestris*, *X. campestris* pv. *vesicatoria*, *Erwinia carotovora* pv. *carotovora*, *E. chrysanthemi*, *Pseudomonas cichorii*, *P. solanacearum*, *P. fluorescens* (bacteria cultures provided by S. T. Hsu and K. C. Tzeng), *Agrobacterium tumefaciens*, *Bacillus subtilis*, *B. thuringiensis*, *Escherichia coli*, and *Klebsiella pneumoniae* (procaryotes provided by C. P. Lin). Double-sandwich ELISA was performed according to the manufacturer's specifications. All reagents (except distilled water) were provided in the kit (Agdia, Inc). All procedures were done at room temperature. The bacterial samples were adjusted to 5×10^7 cells per milliliter with an extraction buffer by a spectrophotometer at 620 nm wavelength. The prepared samples (200 µl) were added to antibody-coated wells, incubated for 2 hr, and the samples discarded. The plates were washed five times with PBS plus 0.5% Tween 20. The peroxidase-conjugated antibody (200 µl) was added to the wells, and the plates

were incubated for 2 hr and washed as before. Peroxidase substrate was prepared by dipping one o-phenylenediamine dihydrochloride stick (10 mg) in 10 ml of citrate buffer (0.012% H₂O₂, 20 mM citric acid, 50 mM Na₂HPO₄ [pH 5.0]). Substrate (200 µl) was added to each well, and the plates were incubated for 30 min; 70 µl of 3 M sulfuric acid was added to stop the reaction, and optical density was measured at 490 nm.

Pathogenicity test. Artificial inoculation with cultured bacterium. A bacterial strain obtained in March 1991 from scorch-affected pear was used. Bacterial cells grown on PD2 plate for 7–10 days at 30 C were washed and suspended in phosphate-buffered citrate-magnesium solution (PBCM), and the concentration of the cell suspension was adjusted to 10⁸ cells per milliliter. In April 1991, 10 greenhouse-grown seedlings of 1-yr-old self-rooted Hengshan variety, approximately 80 cm tall, were inoculated with the cell suspensions by the following methods: 1) Root uptake inoculation (2). Three seedlings were unpotted, and a main root of each was cut. The remaining cut end was connected by a short piece of Tygon tubing to a 5-ml pipette containing 5 ml of the bacterial suspension. Seedlings were repotted with the pipette reservoirs connected to the root. Most of the cell suspension was absorbed within 2 days. 2) Injection inoculation (5). Three seedlings were inoculated with the bacterial suspension by a syringe needle injection. About 0.1 ml of cultural

suspension per seedling was injected into two branches at the third and the fifth node. Four other seedlings were inoculated with PBCM as controls, two by root uptake inoculation and two by injection inoculation. All inoculated seedlings were kept in the greenhouse. Watering and fertilization were according to usual practices. Seedlings were observed for symptom development through October 1992.

Transmission test by grafting. On 5 January 1991, 40 scions of healthy Hengshan pear were high grafted on the water shoots of four diseased trees of the same variety. The developing twigs were covered with insect-proof nylon nets for protection. All nylon nets were removed after leaf scorch symptoms appeared on some of the twigs in July of the same year.

Control. Three leaf scorch-infected trees in each of five orchards were injected with oxytetracycline solution (4% 4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,5,6,10,12,12a-hexahydroxy 6-methyl 1, 11-dioxonaphthalene-2 carboxamide, J. J. Mauget product, Los Angeles, CA) in October 1989 and again in May 1990. This was similar to the method used by Chang et al (3). For a control, three diseased trees and three asymptomatic trees in the same orchard were not injected. The number of water shoots was counted in October 1990. The total square area of the tree was figured as the diameter of the tree canopy multiplied by the diameter of the trunk 20 cm above ground level.

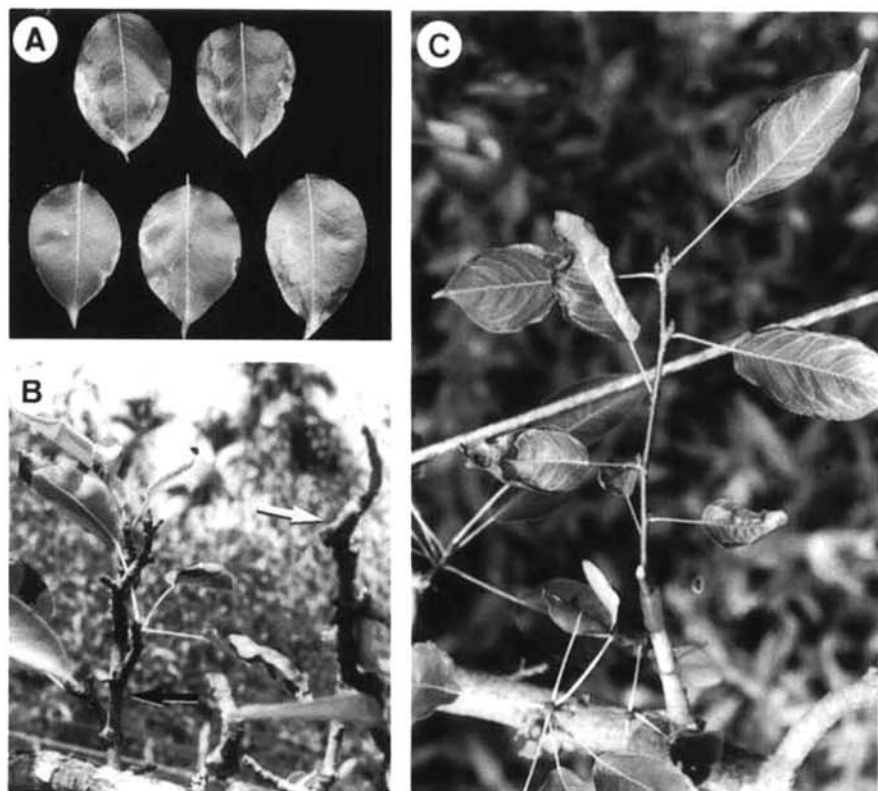


Fig. 1. Symptoms of pear leaf scorch disease. (A) Different degrees of scorch symptoms on leaves. (B) Declining (black arrow) and dead spur (white arrow). (C) Leaf scorch symptoms on the twig developed from grafted healthy scion on the diseased tree.

RESULTS

Symptoms and occurrence. Leaf scorch symptoms were observed in early July, 6 mo after the sprouting of the dormant buds. Brown tissue with yellow margins appeared in one or several areas. The brown tissue expanded and coalesced. Yellow margins usually disappeared as the scorched areas became well developed. The scorched tissues frequently started from the apical and the marginal areas and spread downward and inward. Scorched areas could affect half of the total leaf area in severe cases (Fig. 1A). Earlier leaf drop occurred in the fall. Infected spurs declined and died (Fig. 1B) and the branch died back. In water shoots, scorch symptoms usually appeared in August or later. Some upper leaves remained symptomless during leaf drop in late fall. Water shoots also decreased in number and vigor, followed by die-back of the twigs. The leaf scorch symptoms appeared in one or a few of the branches and then spread to all parts of the tree. If disease progress was not checked, the average 10–20-yr-old tree that became infected died within 3–6 yr. Occurrence of pear leaf scorch was scattered throughout a field. The 1990 survey showed that the disease was common in 23 out of 24 orchards in the low altitudes of central Taiwan. The percentage of infected trees was 1.4–41%, with an average of 12.2–20.6% per

orchard (Table 1). The disease was also found in Chiayi in the southern part of the country, and in Lisan, in the higher altitudes of central Taiwan where the New Century variety is grown. Histological studies, however, failed to reveal XLB bacteria from the Lisan samples.

Microscopic observation. Both TEM and SEM showed the presence of rod-shaped bacteria in xylem vessels. Xylem vessels were partially or almost completely occluded by the bacteria (Fig. 2A, B, and C). Strandlike structures could be observed in the partially occluded vessels (Fig. 2D). Under higher magnification, rod-shaped, ripple-walled, xylem-limited bacteria appeared to be of the same size and character as those in vitro and from the scorch samples.

Isolation and morphology of the bacterium. With PD2 and PW media, the bacterium was consistently isolated from pear leaf scorch-infected trees except those from Lisan. The colony was convex, roundish, and creamy white with a smooth margin, and measured 0.1–0.2 mm in diameter on PD2 medium after 14 days incubation at 30 °C. Strains of the bacterium did not grow on BYCE, CS20, KB, NA, YDC, and 523 media. The bacterium showed the same colony pattern after serial transfers. It could multiply in the liquid culture of PD2 and PW media. The bacterium was gram-negative and rod-shaped with rippled cell

walls, measuring 0.2–0.5 μm in width and 1.1–3.4 μm in length (Fig. 3).

Serology test. ELISA showed all eight strains of the PLSB reacted only slightly with antibodies produced against bacteria isolated from tissues with alfalfa dwarf disease; no reaction was recorded in a reciprocal study. All other common phytopathogenic bacteria and some other prokaryotes tested did not react with either of the two antibodies (Table 2). The results showed that our isolate was probably not closely related serologically to the alfalfa dwarf bacterium that causes Pierce's disease in grape (7).

Pathogenicity test. Artificial inoculation with cultured bacterium. One of the three root-uptake-inoculated and two of the three injection-inoculated seedlings developed characteristic leaf scorch symptoms on leaves 16–17 mo after inoculation. The bacterium was observed microscopically and was reisolated from all seedlings showing scorch symptoms. In contrast, no symptoms developed on any of the four PBCM-inoculated control trees.

Transmission test by grafting. Healthy scions of the Hengshan variety high grafted on water shoots of the leaf scorch-infected trees in January 1990 showed leaf scorch symptoms from which XLB could be isolated (Fig. 1C). From the four trees grafted with 10 scions each, 3, 5, 6, and 8 scions, or 22 out of 40, developed twigs that showed symptoms. Ten healthy scions grafted onto each of two healthy trees showed no

Table 1. Occurrence of pear leaf scorch disease in low-altitude areas of central Taiwan

District	No. of orchards surveyed	No. of trees with symptoms/ no. of trees surveyed (%)
Cholan	9	87/607 (14.3)
Tungshuh	8	223/1,080 (20.6)
Hsinshe	6	50/410 (12.2)

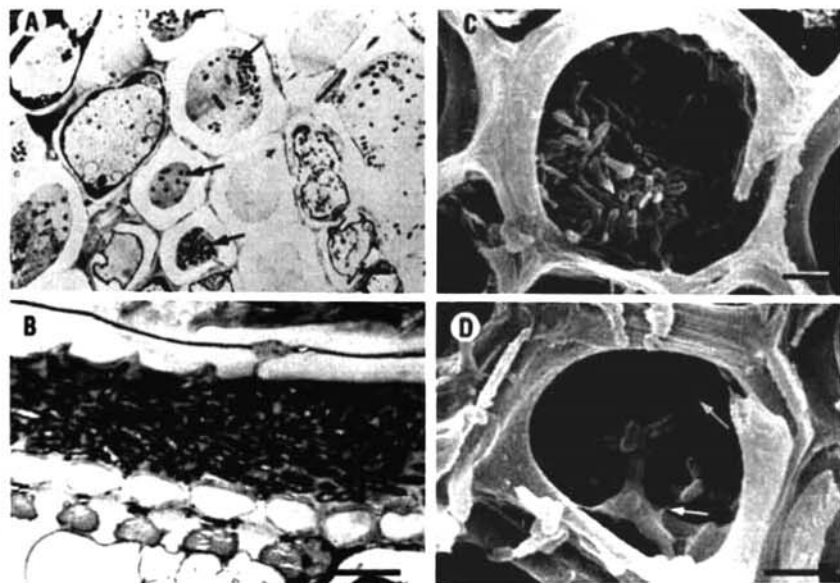


Fig. 2. Micrographs of xylem-limited bacteria found in pear tissues with leaf scorch symptoms. (A) Cross section shows bacterial aggregate in tracheids (arrows) of leaf lateral vein tissue; and (B) longitudinal section shows total occlusion of tracheid by bacterial aggregate (transmission electron microscope). (C) Cross section shows partial occlusion by bacterial aggregate; and (D) strandlike structure (arrows) associated with xylem-limited bacteria (scanning electron microscope). (Bar = 3 μm).

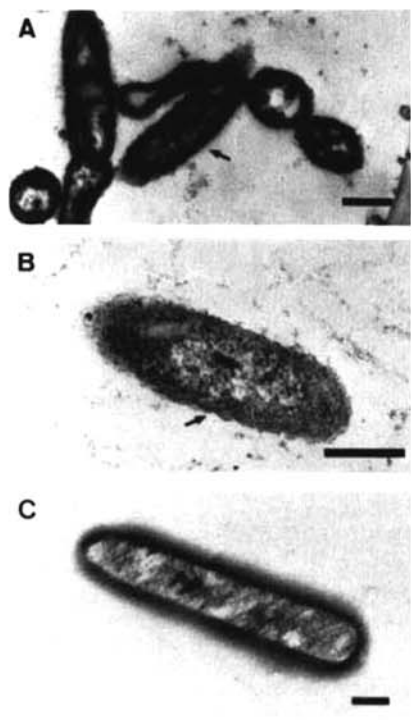


Fig. 3. Micrographs of xylem-limited bacteria associated with pear leaf scorch disease. Ultrathin sections show rod-shape and ripple-walled cells (arrow) in (A) diseased tissue and (B) axenic culture. (C) Cultured bacterium by negative stain. (Bar = 0.3 μm).

symptoms at all.

Control. Injection with oxytetracycline resulted in trees with greater vigor and more water shoots than noninjected diseased trees, although they still were not comparable to healthy trees (Table 3). Recovery of the tree's vigor with the disappearance of symptoms in some branches was observed. The number of water shoots suitable for high grafting increased. It is encouraging that the injection of oxytetracycline solution partially alleviated disease symptoms.

DISCUSSION

Since the first XLB associated with Pierce's disease of grapevine was described in 1973 (7,9) and later cultured in axenic in 1978 (5), XLB have been found in other host plants, such as alfalfa, almond, peach, plum, elm, sycamore, mulberry, oak, periwinkle, red maple, and citrus (8). For the first time, pear is described as a new host for XLB. Several strains associated with the leaf scorch of pear were isolated from diseased trees and maintained in axenic

culture on PD2 and PW media. Based on the consistent isolation from diseased trees, the pathogenicity test, and the reisolation of the bacterium from inoculated seedlings, we concluded that this leaf scorch disease is caused by an XLB. Because it requires specific media such as PD2 and PW for in vitro growth, possesses rippled cell walls, and resides only in xylem tissues, this bacterium could well be a strain of *X. fastidiosa*, as reported by Wells et al (20), even though our serological comparison showed no close relationship between pear leaf scorch strains and alfalfa dwarf strain. The relatedness of pear leaf scorch strains to other strains of *X. fastidiosa* is unknown and warrants further investigation.

While most XLB causing leaf scorch disease were found in North and South America, as mentioned by Hopkins (8), the pear leaf scorch XLB represents the second one to be found in Asia, following the report by Jindal and Sharma (10) of the outbreak of almond leaf scorch in India in 1987. This finding adds important information in the under-

standing of the geographical distribution of XLB.

Transmission of the pear leaf scorch XLB in the orchards is likely carried out by insect vectors (1,15,16), because the occurrence of the disease is scattered and symptoms usually start with one or two twigs. The identification of the insect vectors is currently under investigation by ELISA. Suppression and remission of the disease symptoms by oxytetracycline injection not only supports the association of the XLB to the disease, but also provides a possible control measurement for the disease.

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Table 2. Serological relatedness of pear leaf scorch bacterium (PLSB) to alfalfa dwarf bacterium (ADB) and other bacteria by enzyme-linked immunosorbent assays (ELISA)

Antigen ^v	ELISA A reading at 490 nm	
	ADB ^w	PLSB ^x
ADB-MT1 ^y	1.467 a ^z	0.017 b
PLSB (8 strains)	0.336 b	1.142 a
<i>Xanthomonas campestris</i> pv. <i>campestris</i>	0.016 c	0.016 bc
<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>	0.008 c	0.013 bcd
<i>Erwinia carotovora</i> pv. <i>carotovora</i>	0.012 c	0.005 cd
<i>Erwinia chrysanthemi</i>	0.010 c	0.006 cd
<i>Pseudomonas cichorii</i>	0.001 c	0.004 e
<i>Pseudomonas solanacearum</i>	0.009 c	0.004 e
<i>Pseudomonas fluorescens</i>	0.005 c	0.007 de
<i>Agrobacterium tumefaciens</i>	0.011 c	0.009 cde
<i>Bacillus subtilis</i>	0.005 c	0.004 e
<i>Bacillus thuringiensis</i>	0.013 c	0.007 de
<i>Escherichia coli</i>	0.021 c	0.004 e
<i>Klebsiella pneumoniae</i>	0.005 c	0.009 bcde

^v Antigen concentration used for ELISA was 5×10^7 cells/ml for each bacterial suspension.

^w Antiserum prepared against ADB.

^x Antiserum prepared against PLSB.

^y Strain MT1 was originally isolated from alfalfa tissues with dwarf symptoms provided by M. Davis, Tropical Research and Education Center, University of Florida, Homestead 30031.

^z Values are the average of two replicates. Means in column followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

Table 3. Control of pear leaf scorch disease by injection of oxytetracycline solution^{v,w}

Treatment ^x	No. of water shoots/m ² ^y	
	Range	Average
Diseased trees		
without injection	0.00-24.97	12.58 a ^z
with injection	6.16-36.76	16.41 b
Healthy trees		
without injection	9.25-39.76	20.10 c

^v Trees were injected with oxytetracycline solution twice: on 5 October 1989 and on 17 May 1990. Each time 0.48-0.72 g of oxytetracycline in 12-18 ml of solution was injected per tree. The survey was conducted on 23 October 1990.

^w Oxytetracycline capsules were provided by J. J. Mauget Co., Los Angeles, California.

^x 15 Trees were injected in each treatment.

^y Total square meter area of each tree was represented as the diameter of canopy multiplied by the diameter of trunk 20 cm above ground level.

^z Means in column followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

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