

## Isolation and Growth of Strains of *Xylella fastidiosa* from Infected Grapevines on Nutrient Agar Media

S. M. FRY, Graduate Research Assistant, R. D. MILHOLLAND, Professor, and PI-YU HUANG, Research Cooperator, Department of Plant Pathology, North Carolina State University, Raleigh 27695-7616

### ABSTRACT

Fry, S. M., Milholland, R. D., and Huang, P. -Y. 1990. Isolation and growth of strains of *Xylella fastidiosa* from infected grapevines on nutrient agar media. *Plant Dis.* 74:522-524.

Two grape strains (FC and C) of the causal organism of Pierce's disease (PD) of grapevine, *Xylella fastidiosa*, were isolated from infected grape petioles onto PD3 medium, nutrient agar (NA), and NA amended with various concentrations of sucrose (NAS). All media supported growth of both strains, however, the C strain grew more slowly than the FC strain. Four additional strains from grape grew as single colonies on each of the media. The FC and C strains were subcultured weekly on PD3 and nutrient agar-2.0% sucrose media for 12 mo. The strains remained pathogenic to the grapevine cultivar French Colombard but became less aggressive after 8 mo of subculturing. Colonies of *X. fastidiosa* were reisolated from leaves with symptoms of Pierce's disease.

The causal organism of Pierce's disease of grapevines, *Xylella fastidiosa* Wells et al (20), is a fastidious, xylem-limited bacterium. *X. fastidiosa* was first isolated in 1978 (6) on a medium designated as JD1. The medium was complex, containing a PPLO broth base, hemin chloride, bovine serum albumin or starch, and agar. Subsequently, an improved complex medium for culture of the bacterium, PD2, was reported in 1980 (7). Modifications of PD2 (PD3 and

PD4) have been reported (8) and are as effective as PD2.

Previous attempts to isolate *X. fastidiosa* from grapevines on standard bacteriological media such as nutrient agar were unsuccessful (10). Moreover, nutrient agar did not support growth of the xylem-limited bacteria responsible for phony peach disease (9,11). The isolation and culture of the fastidious bacteria causing phony peach disease and plum leaf scald required specialized media (BCYE) (21) that contained yeast extract, an amino acid carbon source, inorganic mineral supplements, starch or albumin, a source of soluble iron, and charcoal (5,21).

Recently, Bennett et al (2) used nutrient agar for the primary isolation of a xylem-limited bacterium associated with Sumatra disease of cloves. The bacterium grew best on BCYE; however, the organism also grew on nutrient agar alone and nutrient agar supplemented with hemin chloride or phosphate. The

xylem-limited bacterium that causes mulberry leaf scorch has also been grown on nutrient agar (16).

The objectives of this study were to determine if strains of *X. fastidiosa* from grapevines with Pierce's disease could be isolated on nutrient agar or nutrient agar supplemented with sucrose, to compare growth of *X. fastidiosa* on such media with that on PD3, and to determine if pathogenicity could be maintained during repeated subculturing on both types of media.

### MATERIALS AND METHODS

**Source of strains.** Leaves of *Vitis rotundifolia* Michx. 'Carlos' and *V. vinifera* L. 'French Colombard' with symptoms of Pierce's disease were collected from the Horticultural Crops Research Station, Castle Hayne, North Carolina, and Central Crops Research Station, Clayton, North Carolina, respectively. Sap was expressed from petioles onto PD3 (8). Two strains of *X. fastidiosa* (FC from French Colombard and C from Carlos) were purified by single-colony transfers onto PD3 and confirmed as *X. fastidiosa* by proof of Koch's postulates and by characteristics shown by transmission electron microscopy (13).

**Isolation of bacteria from diseased plants.** Five media were compared for direct isolation of *X. fastidiosa* from diseased grapevines. The media were PD3, nutrient agar (NA) at 23 g/L, and nutrient agar-sucrose (NAS) at 23 g of

Paper 11238 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh 27695-7643.

The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service of the products named or criticism of similar ones not mentioned.

Accepted for publication 5 December 1989 (submitted for electronic processing).

© 1990 The American Phytopathological Society

nutrient agar plus 10, 15, or 20 g of sucrose per liter.

Petioles were removed from 10–15 diseased leaves of French Colombard or Carlos plants inoculated with the FC and C strains of *X. fastidiosa*, respectively. The petioles were cut into 1-cm sections, submerged in 1% sodium hypochlorite for 3 min, and washed in four changes of sterile distilled water. A drop of sap was squeezed from each of four petiole sections onto separate areas of each plate. Each medium-strain combination was replicated four times. Plates were arranged in a randomized complete block design and incubated at 28 C. The test was performed three times.

Plates were examined daily up to 11 days with a stereoscope at  $\times 30$  or  $\times 60$  for formation of colonies. The time between the isolation procedure to colony formation, the percentage of attempted isolations that yielded typical colonies on a given day, and the maximum percentage of successful isolations were recorded. The results are presented as the average of the means of four plates per medium-strain combination per test for three repeated tests.

**Dilution plating.** Dilution plating was employed to test the ability of PD3, NA, and 1.0%, 1.5%, and 2.0% NAS to support growth of six strains of *X. fastidiosa*. The strains included FC and C; two strains, CHC and SC, obtained from naturally infected Carlos grapevines located at the Horticultural Crops Research Station in Castle Hayne, and the Sandhills Experiment Station, Jackson Springs, North Carolina, respectively; and two strains obtained from the American Type Culture Collection, ATCC 35879 (isolated from a grapevine in Florida), and ATCC strain 35877 (from a grapevine in Napa Valley, California) (1).

The FC and C strains were isolated from artificially inoculated grapevines, whereas the CHC and SC strains were isolated from naturally infected grapevines. Five petioles from a diseased grapevine were cut into 2-cm sections, surface-sterilized, and rinsed as previously described. The sections were ground in 10 ml of succinate-citrate-phosphate (SCP) buffer (12) with a mortar and pestle. The suspension was filtered through four layers of cheesecloth into a 15-ml centrifuge tube, and the filtrate was centrifuged at 4,000 g for 15 min. The pellet was resuspended in 2 ml of SCP buffer. A 10-fold dilution series in SCP buffer was made for each strain, and 0.1 ml of each of three dilutions were plated onto each of 12 plates per medium (four replications per dilution).

The ATCC strains were initially reisolated onto BCYE (21), then transferred and maintained on PD3. In addition, the SC strain was maintained on PD3 after being single-colony

isolated. Five- to 6-day-old cultures of the ATCC and SC strains were suspended in SCP buffer, diluted to an absorbance of 0.20 at  $A_{484}$ , serially diluted, and plated onto the different media as previously described.

Plates for each strain were arranged in a randomized complete block design in a 28 C incubator. Colonies were counted after 15–22 days. Dilution series for all strains except CHC were performed at least three times. Dilutions with the CHC strain were performed once.

Results of dilution plating of the FC, C, CHC, SC, and ATCC strains onto the various media are presented as the average of the means of three plates per medium-strain combination per test for three repeated tests. A difference in populations of 10-fold or greater was considered significant. The experiment was designed to compare populations on media only within each strain.

In one test, the diameters of colonies of the FC strain were measured on plates with well-isolated colonies. Four colonies at the center of each quadrant of a plate were measured with an ocular micrometer at  $\times 40$  or  $\times 100$ . Measurements were recorded for four replications, resulting in the measurement of 64 colonies per media.

Identity of colonies growing on the various media was confirmed through the use of a double antibody sandwich enzyme-linked immunosorbent assay (ELISA). The assay was performed according to the manufacturer's instructions (Agdia Inc., Mishawaka, IN). Polyclonal antibodies were produced in New Zealand white rabbits to a strain of *X. fastidiosa* (PCE-50) provided by Dr. C. J. Chang, University of Georgia. The antibodies were conjugated to horse radish peroxidase, and *o*-phenylenediamine served as the substrate. ELISA plates were read at 490 nm on a Titertek Multiscan MC (Flow Laboratories Inc., McLean, VA). Absorbances three times that of negative controls were used to define a positive reaction.

**Maintenance of pathogenicity through repeated subculturing.** The strains FC and C were isolated from surface-sterilized petioles onto PD3 and 2.0% NAS. Cultures were grown at 28 C and transferred weekly for 12 mo. Greenhouse-grown French Colombard plants with 2- to 4-wk-old shoots were inoculated with cultures that were subcultured for 8, 18, 30, 39, and 51 wk. Six petioles on each of two plants were inoculated with each medium-strain combination or SCP buffer alone. Four- to 5-day-old cultures were suspended in SCP buffer, and the suspension was adjusted to an absorbance of 0.27 at  $A_{484}$ . Ten  $\mu$ l of the suspension were placed on each petiole and the petioles pricked with a pin to allow uptake of the inoculum

into the vascular system. Plants were kept in the greenhouse at 25 to 30 C. Symptom expression was recorded and isolations from all plants made on PD3.

## RESULTS

**Primary isolation of bacteria from diseased plants.** Little or no growth of nontarget microbes was observed on the different media. Round, dome-shaped, white colonies (0.5–1.0 mm diam.) with entire margins developed on plates of PD3, NA, and NAS within 9 days. The bacteria were gram negative and rod-shaped.

Colonies of the FC strain were observed on the various media about 4 to 5 days after isolation and continued to appear up to an average of 6 days (Table 1). Colonies of the C strain were first observed about 4 to 6 days after isolation on PD3 and NAS, however, 9 days elapsed before colonies appeared on NA. The maximum percentage of successful isolations of the C strain was observed after 5 to 9 days.

On the first day that colonies were visible under the stereoscope, the frequency of isolation of the FC strain was 71%, 56–58%, and 38% on PD3, NAS, and NA, respectively (Table 1). In contrast, the frequency of isolation of the C strain was 54%, 46–58%, and 16% on PD3, NAS, and NA, respectively.

The average maximum frequency of successful isolations ranged from 45% to 90% on the media tested. The frequency of isolation was lowest on NA (45%) because of the poor growth of the C strain on this medium.

**Dilution plating.** Colonies developed on PD3, NA, and NAS inoculated with suspensions of the FC, C, SC, CHC, and two ATCC strains. Higher populations of the C strain and ATCC 35879 were recovered on PD3 ( $1 \times 10^6$ ,  $1 \times 10^8$  cfu/ml), compared to NA ( $1 \times 10^5$ ,  $5 \times 10^6$  cfu/ml) and 1.0% NAS ( $1 \times 10^5$ ,  $1 \times 10^7$  cfu/ml). Addition of sucrose at levels of 1.5% and 2.0% had no effect on the growth of the bacteria. Higher populations of the FC strain were recovered on PD3 ( $3 \times 10^7$  cfu/ml)

**Table 1.** Colony formation by grape strains of *Xylella fastidiosa* on bacteriological media<sup>a</sup>

Media	Days <sup>b</sup>		Frequency <sup>c</sup>	
	FC	C	FC	C
PD3	4	4	71	54
NA	5	9 <sup>d</sup>	38	16 <sup>d</sup>
1% NAS	4	5	56	58
1.5% NAS	5	6	56	48
2% NAS	5	5	58	46

<sup>a</sup>Data presented are the average of the means of four plates per medium-strain combination per test for three repeated tests.

<sup>b</sup>Minimum number of days that elapsed between inoculation of the plates and colony formation.

<sup>c</sup>Percentage of successful isolations per four attempted isolations.

<sup>d</sup>Average of four plates from one test.

compared to 1.0%, 1.5%, or 2.0% NAS ( $6 \times 10^4$ ,  $2 \times 10^6$ ,  $1 \times 10^5$  cfu/ml). Growth on nutrient agar alone was significantly less ( $1 \times 10^4$  cfu/ml).

Strains SC, CHC, and ATCC 35877 developed colonies on all media but not consistently. ATCC 35877 consistently grew only on PD3, whereas primary isolation of the SC strain was observed on PD3, NA, and NAS. Higher populations of the SC strain were recovered on PD3 ( $5 \times 10^4$  cfu/ml), compared to NA and NAS ( $3 \times 10^3$ ,  $5 \times 10^3$  cfu/ml). However, after the SC strain had been repeatedly subcultured, it grew consistently only on PD3 and 1.5% NAS. There was no difference in the population of the SC strain on PD3 ( $3 \times 10^7$  cfu/ml) compared to 1.5% NAS ( $7 \times 10^6$  cfu/ml).

Colony sizes of the FC strain were 0.13–1.20 mm on PD3, 0.05–0.68 mm on 1.0% NAS, 0.06–0.45 mm on 1.5% NAS, and 0.13–0.77 mm on 2.0% NAS after 2 wk. At 15 days, colonies on PD3 were often much larger, rougher, and whiter than those on the simple media, which were smaller and smoother. In the ELISA test, typical colonies growing on all media reacted positively with polyclonal antibodies to xylem-limited bacteria.

**Pathogenicity test.** Grapevines inoculated with freshly isolated strains of *X. fastidiosa* developed typical symptoms of Pierce's disease (necrosis of leaf margins) within 5 wk. In most plants, symptoms were systemic and all plants were dead approximately 5 mo after inoculation. Leaves of plants inoculated with strains that had been subcultured for 1 yr exhibited slight marginal leaf burn 8 wk after inoculation and developed systemic symptoms after 3 mo. Control plants remained healthy. No medium-strain combination was associated with enhanced aggressiveness. The pathogen was reisolated from diseased leaves onto PD3 and appeared as typical colonies within 4 days. Buffer-inoculated plants did not yield the pathogen. Typical colonies of *X. fastidiosa* reacted positively with polyclonal antibodies to xylem-limited bacteria.

## DISCUSSION

Several strains of *X. fastidiosa* were isolated from diseased grapevines on NA and NAS or subcultured on NAS. All media supported similar amounts of growth of the FC and C strains; however, the C strain tended to grow more slowly on nutrient agar than the FC strain. Despite differences in the ability of strains of *X. fastidiosa* to grow on nutrient agar, the simpler bacteriological media appear to be useful in the primary

isolation of *X. fastidiosa* from grapevines.

Dilution plating revealed that six grape strains were able to grow on NA and NAS whether isolated directly from plant material or from cultures. The ATCC cultures, one being a type culture, were transferred on BCYE 12 to 16 times before being submitted to ATCC (1). The cultures were transferred several times on PD3 before use in experiments in this study. These strains of *X. fastidiosa* were still able to grow on simple media after being cultured on complex media for an extended period of time. Thus, repeated subculturing on complex media did not select for increased fastidiousness.

Huang et al (13) reported a loss of aggressiveness of a strain of *X. fastidiosa* that was subcultured on PD3 for 8 mo. The aggressiveness of the FC and C strains used in our tests remained constant over weekly transfers on NAS and PD3 for 6 mo but decreased after 8 mo. As the age of cultures increased, the incubation period increased.

Wells et al (20) indicated that different strains of xylem-limited bacteria, including strains from grapevines, are nutritionally fastidious and will not grow on simple bacteriological media. The xylem-limited bacteria causing phony peach disease (5,21), plum leaf scald (5,21), periwinkle wilt (5), and sycamore (17) and maple leaf scorch (18) have been cultured on PW and BCYE. Like the xylem-limited bacteria of phony peach disease, the xylem-limited bacterium causing ragweed stunt grows well on PW, BCYE, and BCZE but does not grow on PD2 (19). The xylem-limited bacterium causing oak leaf scorch has been isolated on PW (15) and CS20 media (3,4) whereas the xylem-limited bacterium associated with elm leaf scorch has been grown on S-8 and PD2 (14).

We have been able to isolate several strains of *X. fastidiosa* from grapevines infected with Pierce's disease onto nutrient agar and nutrient agar supplemented with sucrose through the use of primary isolation techniques and dilution plating. Strains did vary in their ability to grow on the simpler media. Two strains were subcultured on bacteriological media for 12 mo and did not lose their pathogenicity. Aggressiveness, though, did decrease after about 6 mo. We believe that use of nutrient agar or nutrient agar-sucrose media for isolations may be useful and time-saving techniques for diagnosticians and those interested in primary isolation of the bacterium from grapevines.

## LITERATURE CITED

1. American Type Culture Collection, Rockville, MD. Application form for submission of cultures.

2. Bennett, C. P. A., Jones, P., and Hunt, P. 1987. Isolation, culture and ultrastructure of a xylem-limited bacterium associated with Sumatra disease of cloves. *Plant Pathol.* 36:45-52.
3. Chang, C. J., and Walker, J. T. 1983. Isolation of bacteria from oak trees showing leaf scorch symptoms. (Abstr.) *Phytopathology* 73:807.
4. Chang, C. J., and Walker, J. T. 1988. Bacterial leaf scorch of northern red oak: Isolation, cultivation, and pathogenicity of a xylem-limited bacterium. *Plant Dis.* 72:730-733.
5. Davis, M. J., French, W. J., and Schaad, N. W. 1981. Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. *Curr. Microbiol.* 6:309-314.
6. Davis, M. J., Purcell, A. H., and Thomson, S. V. 1978. Pierce's disease of grapevines: Isolation of the causal bacterium. *Science* 199:75-77.
7. Davis, M. J., Purcell, A. H., and Thomson, S. V. 1980. Isolation media for the Pierce's disease bacterium. *Phytopathology* 70:425-429.
8. Davis, M. J., Whitcomb, R. F., and Gillaspie, A. G. 1981. Fastidious bacteria of plant vascular tissue and invertebrates (including so-called rickettsia-like bacteria). Pages 2172-2188 in: *The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria*. M. P. Starr, H. O. Stolp, H. G. Triper, A. Balows, and H. G. Schlegel, eds. Springer-Verlag, Berlin.
9. French, W. J., Christis, R. G., and Stassi, D. L. 1977. Recovery of rickettsialike bacteria by vacuum infiltration of peach tissues affected with phony disease. *Phytopathology* 67:945-948.
10. Hopkins, D. L., and Mollenhauer, H. H. 1973. Rickettsia-like bacterium associated with Pierce's disease of grapes. *Science* 179:298-300.
11. Hopkins, D. L., Mollenhauer, H. H., and French, W. J. 1973. Occurrence of a rickettsia-like bacterium in the xylem of peach trees with phony disease. *Phytopathology* 63:1422-1423.
12. Hopkins, D. L., and Thomson, C. M. 1981. Multiplication of virulent and avirulent Pierce's disease bacterial isolates in grapevine tissue. *Proc. Int. Conf. Plant Pathog. Bact.* 5th 225-234.
13. Huang, P. -Y., Milholland, R. D., and Daykin, M. E. 1986. Structural and morphological changes associated with the Pierce's disease bacterium in bunch and muscadine grape tissues. *Phytopathology* 76:1232-1238.
14. Kostka, S. J., Serald, J. L., Hearon, S. S., and Rissler, J. F. 1981. Cultivation of the elm leaf scorch-associated bacterium (ESB). (Abstr.) *Phytopathology* 71:768.
15. Kostka, S. J., Serald, J. L., and Tattar, T. A. 1984. Culture of fastidious, xylem-limited bacteria from declining oaks in the northeastern states. (Abstr.) *Phytopathology* 74:803.
16. Kostka, S. J., Tattar, T. A., Serald, J. L., and Hurtt, S. S. 1986. Mulberry leaf scorch, new disease caused by a fastidious, xylem-inhabiting bacterium. *Plant Dis.* 70:690-693.
17. Serald, J. L., Hearon, S. S., Kostka, S. J., and Morgan, D. L. 1983. Sycamore leaf scorch: Culture and pathogenicity of fastidious xylem-limited bacteria from scorch-affected trees. *Plant Dis.* 67:849-852.
18. Serald, J. L., Wells, J. M., Hurtt, S. S., and Kostka, S. J. 1987. Association of fastidious, xylem-inhabiting bacteria with leaf scorch in red maple. *Plant Dis.* 71:930-933.
19. Timmer, L. W., Brlansky, R. H., Lee, R. F., and Raju, B. C. 1983. A fastidious, xylem-limited bacterium infecting rag weed. *Phytopathology* 73:975-979.
20. Wells, J. M., Raju, B. C., Hung, H. Y., Weisburg, W. G., Parl, L. M., and Beemer, D. J. 1987. *Xylella fastidiosa* gen. nov. sp. nov.: Gram-negative, xylem-limited, fastidious plant bacteria related to *Xanthomonas* spp. *Int. J. Syst. Bacteriol.* 37:136-143.
21. Wells, J. M., Raju, B. C., Nyland, G., and Lowe, S. K. 1981. Medium for isolation and growth of bacteria associated with plum leaf scald and phony peach diseases. *Appl. Environ. Microbiol.* 42:357-363.