Survival of *Bacillus subtilis* in Silver and Sugar Maple Seedlings over a Two-Year Period

T. J. HALL, Assistant Professor, and W. E. EDGAR DAVIS, Horticulturist, School of Agriculture, Tennessee Technological University, Cookeville 38505

ABSTRACT

Hall, T. J., and Davis, W. E. E. 1990. Survival of *Bacillus subtilis* in silver and sugar maple seedlings over a two-year period. Plant Dis. 74:608-609.

One-year-old seedlings of *Acer saccharinum* (silver maple) and *A. saccharum* (sugar maple) that were inoculated with a Rifampicin-resistant strain of *Bacillus subtilis* were found to contain bacteria resembling *B. subtilis* after 12 and 24 mo. After 2 yr, the bacteria were recovered from the xylem tissue located 30–72 cm and 5–54 cm from the inoculated site in silver and sugar maple, respectively. Therefore, *B. subtilis*, a potential antagonist to *Verticillium dahliae*, can survive and be transported into newly formed xylem tissue of silver and sugar maple trees.

Additional keywords: Verticillium wilt

Strains of *Bacillus subtilis* (Ehrenberg) Cohn have been used to control several types of diseases associated with fruits (4,10,11,14,15,17), vegetables (16,18), field crops (5), and flower crops (12). Antifungal substances produced by *B. subtilis* have been shown to have effects on vascular wilt pathogens (6). However, vascular wilt pathogens like *Verticillium* and *Ceratocystis* are confined to xylem tissue in woody plants and are not likely to be affected directly by surface applications of a bacterial antagonist. Introduction of bacteria into xylem tissue of trees has been used control vascular wilt diseases (8,9,13). *Bacillus* species recovered from *Acer rubrum* L. (red maple) and *A. saccharinum* L. (silver maple) have been shown to affect the incidence of Verticillium wilt in silver maple and *A. platanoides* L. (Norway maple) (3).

The objective of this study was to examine the survival of a Rifampicin (Rf)-resistant strain of *B. subtilis* in silver and sugar maple liners (1- or 2-yr-old seedlings that are lined out in a row) over a 2-yr period and to determine if bacteria could be transported into new xylem tissue as the tree grew.

MATERIALS AND METHODS

Dormant silver and sugar maple bare root nursery stock (30–45 cm × 1 cm) were placed in 1.85-L containers and grown in a commercial bark:sand medium and placed in a greenhouse at 24 C. When plants were in full leaf, stems were fitted with rubber serum caps which held 5 ml of an aqueous cell suspension of *B. subtilis*.

Accepted for publication 23 January 1990.

Bacterial inoculum was prepared from an Rf-resistant mutant of *B. subtilis* that was antagonistic to *Verticillium dahliae* Kleb. in silver and Norway maple (3). Bacteria were grown and harvested as described previously and then resuspended in sterile, distilled water to give a cell suspension of approximately 1×10^6 colony-forming-units per milliliter (cfu/ml) (3).

A stem-wound technique (2) was used to inoculate plants. Five ml of the bacterial cell suspension was placed in each serum cap, and a scalpel blade was used to make a small wound on two sides of the stem to a depth of approximately 1 mm. The incision did not encircle or sever the stem. Sixty plants of each species were moved to a shaded (50%) greenhouse and stems were inoculated with cell suspensions. Serum caps were refilled twice daily with the bacterial suspension over a period of 5 days. Controls were handled as above and consisted of 40 trees that were wounded and treated with only sterile, distilled water. After inoculation, plants were moved to a lath house for the duration of the study. Plants received two applications of 9 g of Osmocote 18-6-12 (Sierra Chemical Co., Milpitas, CA) fertilizer during each year of the study.

Near the end of the dormancy period following each growing season, a sample of 20 trees treated with *B. subtilis* and five non-treated controls of each species were randomly selected from each group of trees. These were allowed to break dormancy and grow until leaves were fully expanded. In the second year of the test, 30 of the remaining 40 silver maple trees treated with the bacterium died from a lack of water during a period of extremely high temperatures. The remaining silver maple and sugar maple specimens received adequate water and were sampled as previously described. Plants were harvested and height measurements were recorded. Stem lengths were measured from the initial inoculation site to the terminal bud region extant at the time of initial bud break. (Measurements did not include tissue formed after bud break.) Stems were debarked and cut serially into sections 2.5 cm long. Stem sections were cut from the point of inoculation upward to the point where the current year's growth began, kept in serial order, frozen, and stored at −15 C.

For each tree, a sample of frozen sections was selected at 7.5-cm intervals beginning at the inoculation site. Each frozen section was surface-sterilized by immersion in boiling water for 1–2 sec. Sections were then split open, and the flat longitudinal surface was placed face down on a sucrose-nutrient agar (SNA) medium amended with 100 ppm of Rf and 2,3,5-triphenyl-2H-tetrazolium chloride (TPTZ), and incubated in the dark at 24 C for a minimum of 14 days (3,7). The remaining sections were stored frozen and used to confirm bacteria recovery in adjacent stem sections. Bacteria from colonies associated with the wood sections were streaked onto the selective medium; individual colonies that resembled the stock culture of the Rf-resistant strain of *B. subtilis* were sampled and purified on the selective medium and tested for gram reaction and the presence of endospores. Gram stains were prepared with the use of the Hucker method (1). For detection of endospores, cultures were grown on nutrient agar for 5 days and stained with a 7% aqueous nigrosin stain (1). Analysis of variance and the *F* test were used to analyze plant height and extent of bacterial recovery in the stem.

RESULTS AND DISCUSSION

Over the duration of the study, the Rf-resistant strain of *B. subtilis* was recovered from 97% (29/30) and 100% (40/40) of the treated silver and sugar maple trees, respectively. Most of the bacteria isolated from wood sections exhibited the same culture characteristics as stock cultures of the introduced strain of *B. subtilis*. Colonies produced by the stock culture typically were round to irregular in shape with dull or crusty surfaces and wrinkled and cream-colored margins. Colonies also typically had a spreading

Table 1. Effect of inoculation of silver and sugar maple liners with *Bacillus subtilis* on stem height and recovery of the bacterium after 12 and 24 mo

Treatment	Silver Maple		Sugar Maple	
	12 mo	24 mo	12 mo	24 mo
B. subtilis				
Number of trees	20[x]	10	20	20
Hardwood height[y]	23.8 a	50.1 a	22.6 a	40.5 a
Range	15–45	30–72	9–45	19–60
Stem recovery[z]	18.1	42.0	20.7	31.5
Range	0–45	30–72	9–45	5–54
Control				
Number of trees	5	5	5	5
Hardwood height	26.0 a	47.4 a	15.6 a	33.6 a
Range	24–30	36–60	12–24	27–39
Stem recovery	0	0	0	0

[x]For each column, means followed by the same letter are not significantly different using the *F* test. All measurements are in centimeters.
[y]Hardwood height refers to stem length originating at the point of inoculation and ending in the region where softwood of the current season's growth begins.
[z]Stem recovery refers to the extent of the hardwood height from which *B. subtilis* was recovered.

type of growth pattern with the center exhibiting a dull red color attributed to the reduction of tetrazolium. Strains recovered from the trees after 12 and 24 mo and the stock culture of *B. subtilis* were Rf-resistant, gram-positive, rod shaped, TPTZ positive, and spore formers.

Rf-resistant bacteria not resembling the strain used for inoculation were recovered from both treated and nontreated trees. These bacteria were rods or cocci, non-spore formers, and gram-positive or gram-negative. In culture, colonies of these strains were variable in color and morphology. They produced smooth or mucoid colonies which were translucent or opaque and yellow or cream colored. Some strains reduced tetrazolium to form a glossy or dull red colony with or without a cream-colored margin.

All liners used in the study originated from outcrossed seed sources; therefore, considerable variation in growth would be expected. However, inoculation with *B. subtilis* had no effect on the increase in height of the trees (Table 1). The Rf-resistant strain of *B. subtilis* was recovered from tissues that were extant at the time of inoculation, and new tissue formed after 12 and 24 mo. The test strain was not recovered from stems of the controls. After 12 mo, the bacterial strain was recovered from silver and sugar maple stem tissue at a level of ≥75% of the stem height in 11 of 20 and 17 of 20 trees, respectively. After 24 mo, recovery from silver and sugar maples at a level of ≥75% of the stem height was 9 of 10 and 14 of 20 trees, respectively. It is apparent that the *B. subtilis* strain was transported in xylem tissue into newly produced stem tissue.

Recovery data represent hardwood tissues only. Bacteria were not recovered from softwood tissue associated with new growth following brief immersion in boiling water; evidently all microorganisms growing in softwood tissue were killed by the boiling water treatment. We would expect that *B. subtilis* spores would survive this treatment. However, recovery from softwood xylem sap by using a pressure bomb technique for measuring water potential in cuttings was attempted and could be used to detect Rf-resistant bacteria on the selective medium. Results from sap extraction culture were incomplete and are not reported in this study, although future tests that use this technique are underway.

The recovery of an introduced strain of *B. subtilis* from xylem tissue of silver and sugar maple after 12 and 24 mo is evidence that the organism may persist in vascular tissue of a perennial host and influence vascular wilt disease beyond 1 yr. However, this remains to be demonstrated.

The procedures described herein and previously (3) can be used to screen potential bacterial antagonists for efficacy against *Verticillium* and other wilt diseases of woody plant species. However, additional research is needed on more efficient methods for establishing bacterial antagonists in woody plants.

LITERATURE CITED

1. American Society of Microbiology. 1981. Manual of Methods for General Bacteriology. The Society: Washington, D.C. 524 pp.
2. Gregory, C. F. 1969. A technique for inoculating plants with vascular pathogens. Phytopathology 59:1014.
3. Hall, T. J., Schreiber, L. R., and Leben, C. 1986. Effects of xylem-colonizing *Bacillus* spp. on Verticillium wilt in maples. Plant Dis. 70:521-524.
4. Jordon, V. W. L., and Tarr, H. S. 1987. Inoculum suppression of *Verticillium dahliae*. Ann. Appl. Biol. 89:139-141.
5. Kommendahl, T., and Mew, I. C. 1975. Biocontrol of corn root infection in the field by seed treatment with antagonists. Phytopathology 65:296-300.
6. Krause, C. R., Ichida, J. M., Schreiber, L. R., and Gregory, G. F. 1987. Morphological and cytological effects on *Ceratocystis ulmi* of an antifungal antibiotic produced by *Bacillus subtilis*. (Abstr.) Phytopathology 77:1771.
7. Leben, C. 1978. Biological control of decay fungi: A wood disk evaluation method. For. Sci. 24:560-564.
8. Murdoch, C. W., Campana, R. J., and Hock, J. 1984. On the biological control of *Ceratocystis ulmi* with *Pseudomonas fluorescens*. (Abstr.) Phytopathology 74:805.
9. Myers, D. F., and Strobel, G. A. 1983. *Pseudomonas syringae* as a microbial antagonist of *Ceratocystis ulmi* in the apoplast of American elm. Trans. Br. Mycol. Soc. 80:389-394.
10. Pusey, P. L., and Wilson, C. L. 1984. Postharvest biological control of stone fruit brown rot by *Bacillus subtilis*. Plant Dis. 68:75-756.
11. Pusey, P. L., Wilson, C. L., Hotchkiss, M. W., and Franklin, J. D. 1986. Compatibility of *Bacillus subtilis* for postharvest control of peach brown rot with commercial fruit waxes, dicloran, and cold-storage conditions. Plant Dis. 70:587-590.
12. Rytter, J. L., Lukezic, F. L., Craig, R., and Moorman, G. W. 1989. Biological control of geranium rust by *Bacillus subtilis*. Phytopathology 79:367-370.
13. Scheffer, R. J., 1983. Biological control of Dutch elm disease by *Pseudomonas* species. Ann. Biol. 103:21-31.
14. Singh, V., and Deverall, B. J. 1984. *Bacillus subtilis* as a control agent against fungal pathogens of citrus fruit. Trans. Br. Mycol. Soc. 83:487-490.
15. Swineburn, T. R. 1978. The potential value of bacterial antagonists for the control of apple canker. Ann. Appl. Biol. 89:94-96.
16. Thirumalachar, M. J., and O'Brien, M. J. 1977. Suppression of charcoal rot in potato with a bacterial antagonist. Plant Dis. Rep. 61:543-546.
17. Utkhede, R. S. 1984. Effect of bacterial antagonists on *Phytophthora cactorum* and apple crown rot. Phytopathol. Z. 109:169-175.
18. Utkhede, R. S., and Rahe, J. E. 1983. Chemical and biological control of onion white rot in muck and mineral soils. Plant Dis. 67:153-155.