Isolation and Characterization of Opine-Utilizing Strains of *Agrobacterium tumefaciens* and Fluorescent Strains of *Pseudomonas* spp. from Rootstocks of *Malus*

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ABSTRACT

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Media containing opines were used to isolate *Agrobacterium tumefaciens* and fluorescent *Pseudomonas* spp. from rootstock tumors and roots of *Malus*. Strain B49C of *A. tumefaciens* was used to inoculate cultivars Mark, EMLA 7, and Red Delicious seedling apple rootstocks in field trials at two nurseries in Washington state. Crown gall incidence was greater on inoculated than on uninoculated controls at both locations. Of 12 strains of *A. tumefaciens* isolated, three utilized mannopine, four utilized nopaline, and four utilized both mannopine and nopaline as the sole carbon and nitrogen source in culture media. None of the 12 strains utilized octopine, and one utilized none of the three opines tested. Forty-one strains of *Pseudomonas* spp. utilized octopine, 48 utilized nopaline, one utilized both octopine and nopaline, and none utilized mannopine. Seventy-seven of the isolates of *Pseudomonas* spp. inhibited the growth of *A. tumefaciens* in culture.

Crown gall, induced by *Agrobacterium tumefaciens*, is a serious problem of apple rootstocks in Pacific Northwest nurseries where incidence reaches 100% in some years (14). There currently are no known chemical or biological methods for controlling the disease. Strain K84 of *A. radiobacter*, which is very effective in controlling crown gall in *Prunus* and other genera (4,7,8,13,16,20), has not provided consistent control of crown gall in apple in repeated field trials in Washington and Oregon.

Studies on the control and epidemiology of crown gall on apple have been impeded by the difficulty in isolating *A. tumefaciens* from apple rootstock tumors (1,11,18,24). Attempts over a 17-yr period to isolate *A. tumefaciens* from apple rootstocks primarily with semiselective media for biovars 1, 2, and 3 yielded only 30 out of 905 isolates that were pathogenic on tomato, but these strains were only weakly virulent on apple rootstocks in field trials.

Past failures in isolating *A. tumefaciens* from apple rootstock tumors may have been due to the use of semiselective media on which the causal organism would not grow, or to competition or antagonism from other microorganisms within the tumors, with resultant low survival of the pathogen. The second possibility led to a new approach for isolating the pathogen and other microorganisms, using opines as the sole carbon and/or nitrogen source in the isolation media.

Opines were chosen because they are synthesized in plant tumors and can be used by *A. tumefaciens* (21). Genes for opine synthesis are transmitted from the Ti plasmid in *A. tumefaciens* to the plant genome (21). If the strains of *A. tumefaciens* are the only microorganisms capable of utilizing opines, then an exclusive niche is created (2,12,20–22). However, other microorganisms, including fluorescent *Pseudomonas* spp., also utilize opines and may compete with *A. tumefaciens* within a tumor for this nutrient (18,23,24). Another form of competition that can exist within a tumor is antibiotic production by strains of *Pseudomonas* spp. that are antagonistic to *A. tumefaciens* (3).

Our goals in this study were to isolate *Agrobacterium* spp. and *Pseudomonas* spp. from apple rootstocks, using opines as the sole carbon and/or nitrogen source in the isolation media, characterize the strains and test them for opine utilization, test the strains of *Agrobacterium* spp. for pathogenicity, and determine the ability of *Pseudomonas* spp. to inhibit the growth of *A. tumefaciens* in culture.

MATERIALS AND METHODS

Isolations. Isolations were made from Red Delicious apple seedlings in Washington and included three tumors and three roots from one nursery and three tumors and six roots from a second nursery. Plant material was washed in running tap water to remove soil; then a 1-g sample was suspended in 9 ml of sterile distilled water for 1 hr. This suspension was streaked on seven different media. One medium, developed by Hooykaas et al (5), contained (g L^{-1}): KH_2PO_4, 0.09; NaCl, 0.15; $MgSO_4 \cdot H_2O$, 0.50; $CaCl_2$, 0.1, mannitol, 2.0; brom thymol blue, 0.15; and octopine, 0.1. Six other media contained the following basal salts: (g L^{-1}): K_2HPO_4, 10.5; KH_2PO_4, 4.5; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.01; $FeSO_4$, 0.005; $MnCl_2$, 0.002. To the basal medium was added (g L^{-1}) octopine or nopaline, 0.1, alone or in combination with glucose, 5, or ammonium nitrate, 0.15. All of the media contained 20.0 g L^{-1} of Noble agar (Difco Laboratories, Detroit, MI).

Isolation plates were incubated at 26 C for 1 wk, when colonies were selected from each plate and streaked onto King's medium B (9). All isolates that produced a diffusible pigment that fluoresced in the medium under ultraviolet light at 350 nm (Gelman-Camag Universal UV Lamp, Wilmington, NC) and all of those having the colony morphology of *Agrobacterium* were transferred to potato-dextrose agar (PDA). Colonies were suspended in sterile distilled water, restreaked to purity on fresh PDA, and then stored at 4 C on PDA slants supplemented with 0.5% $CaCO_3$.

Opine utilization. A basal medium used for screening isolates contained (g L^{-1}): K_2HPO_4, 7.2; KH_2PO_4, 2.8; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2$, 0.01; and $FeSO_4 \cdot 7H_2O$, 0.0005, and Gelrite gellan gum (Kelco Div., Merck & Co., Inc., San Diego, CA), 4.0. The medium was supplemented with 5 mM octopine, nopaline, or mannopine. Control plates contained basal medium plus glucose and $(NH_4)_2SO_4$ at 0.4 and 1.6 g L^{-1}, respectively.

Isolates to be tested for opine utilization were grown on mannitol glutamate (MG) slants for 48 hr (6). Bacteria then were suspended in 2 ml of sterile 0.85% NaCl solution and spotted onto plates with the three opine media. Plates were examined

for growth 3 and 7 days later. Strains known to use opines also were spotted to each opine medium.

All isolates that grew on solid medium containing opines were grown in liquid media of the same composition to confirm opine utilization, thus eliminating isolates capable of utilizing the solidifying agent, Gelrite, as a carbon or nitrogen source. Transfers for liquid media were made from a 48-hr bacterial culture grown on MG. Bacteria were suspended in a 0.85% sterile solution of NaCl to a concentration of 0.16–0.18 absorbance at 600 nm cm^{-1} in a Spectronic 20 colorimeter (Bausch & Lomb Inc., Rochester, NY). Colorimeter tubes were filled with 3 ml of the opine salts medium, and 0.3 ml of the bacterial suspension was transferred to each tube. Tubes were placed on a rotary shaker at 25 C and observed for growth at 16 and 38 hr. A colorimeter reading of 0.20 at 600 nm was considered positive for growth. Isolates that had not grown by 38 hr were incubated for 76 hr. All opine utilization tests were repeated once.

Ten isolates of *Pseudomonas* that could utilize nopaline were tested for octopine (5 mM) utilization, with a low level (0.05 mM) of nopaline in the medium. Ten octopine-utilizing isolates were tested in a similar way on 0.05 mM octopine in 5 mM nopaline medium.

Identification and pathogenicity of isolates. All opine-utilizing isolates were identified by standard biochemical and physiological tests (10,15). For *A. tumefaciens*, these tests included 3-ketolactose production, growth in 2% NaCl, acid or alkali production in litmus milk, acid production from erythritol and melezitose, alkali production from L-tartaric acid, growth on ferric ammonium citrate, L-tyrosine utilization, and cytochrome oxidase activity. Tests used to identify isolates of *Pseudomonas* were cytochrome oxidase activity, levan production, arginine dihydrolase activity, nitrate reduction, gelatin liquefaction, utilization of mannitol, utilization of trehalose, utilization of sorbitol, and utilization of sucrose. All tests were repeated once.

All isolates of *Agrobacterium* were tested for pathogenicity on tomato (*Lycopersicon esculentum* Mill. 'Bonnie Best') and sunflower (*Helianthus annuus* L. 'Mammoth') plants in the greenhouse according to standard methods (15). Stems of three tomato and three sunflower seedlings were wounded and inoculated with bacteria grown for 48 hr on MG slants. Inoculated plants were observed for the presence of tumors after 4 wk. Known strains of *A. tumefaciens* also were inoculated.

All isolates that produced tumors on the herbaceous hosts were inoculated to cultivars Emla 7 and Red Delicious seedling apple by dipping root-pruned trees in a suspension of 10^7 colony-forming units (cfu) ml^{-1} of each isolate for 20 sec. Each isolate was inoculated to three trees, which then were planted in 3.8-L pots in the greenhouse. Trees were examined for tumor production 6 mo after inoculation.

To confirm pathogenicity under field conditions, one of the highly virulent isolates, B49C, was chosen for field testing. Within each of two nursery sites in Washington, three separate experiments were conducted, one for each apple rootstock (Emla 7, Mark, and Red Delicious). Treatments consisted of 100 inoculated and 100 uninoculated trees. A spontaneous mutant of B49C that was resistant to rifampicin (100 mg L^{-1}) was grown on antibiotic MG medium with 1 g L^{-1} of yeast extract (MGY) and used to make a suspension of 10^7 cfu ml^{-1}. Root-pruned trees were dipped for 20 sec in the bacterial suspension or in water. Treatments were arranged in a randomized complete block design and replicated four times. Each plot within a block, therefore, contained 25 trees. Trees were spaced at 15-cm intervals within rows 120 cm apart. Field plots were established during the third week of April 1988 at both locations. All trees were dug the following October and examined for tumors.

Treatments were compared within each rootstock and within each nursery by analyzing the proportion with tumors. Two-sided tests were used to analyze the data both parametrically (*t*-tests on arcsine square root-transformed proportions) and nonparametrically (Wilcoxon rank-sum and Kolmogorov-Smirnor tests) with the NPARIWAY procedure developed by the SAS Institute (19). The *P*-values from the Kolmogorov-Smirnov tests are presented here. Isolations were made from tumors and roots on rifampicin-amended MGY medium. Isolates from the antibiotic plates then were tested for pathogenicity on tomato stems in the greenhouse as previously described.

Antagonism of isolates of *Pseudomonas* to *A. tumefaciens*. Antagonism of fluorescent isolates of *Pseudomonas* was tested in vitro. Isolates of *Pseudomonas* were grown on MG slants for 48 hr at 26 C. Ten microliters of a dilute suspension (10^6 cfu ml^{-1}) were spotted onto the center of two plates, one containing MG medium and one containing MG medium amended with 20 mg L^{-1} of FeCl to prevent siderophore production (3). Cultures were incubated for 48 hr at 26 C; then a suspension of the test strain of *A. tumefaciens* (10^8 cfu ml^{-1}) was sprayed onto the surface of the medium with an aerosol propellant (Sigma Chemical Co., St. Louis, MO). The plates were incubated at 26 C for 48 hr, and zones of inhibition were measured.

RESULTS

Isolation and opine utilization. The use of opines in the medium was effective for isolating both *A. tumefaciens* and *Pseudomonas* spp., which utilize opines. Thirty-eight of 221 bacterial isolates from roots and tumors on opine media had the colony morphology of *Agrobacterium*, that is, white, domed, glisteny, mucoid, and spherical, with intact margins. Three of the 38 isolates utilized mannopine, four utilized nopaline, and four utilized both mannopine and nopaline. The remaining 27 isolates were unable to utilize any of the three opines. On King's medium B, 183 isolates produced a fluorescent pigment characteristic of the fluorescent pseudomonads. Ninety of the 183 isolates of *Pseudomonas* spp. (49%) were opine utilizers: 41 utilized octopine, 48 utilized nopaline, and one isolate utilized both opines as the sole carbon and nitrogen source. None of the isolates of *Pseudomonas* that utilized nopaline grew in octopine in the presence of low concentrations of nopaline, nor did octopine utilizers grow in nopaline in the presence of low concentrations of octopine.

All opine-utilizing isolates of *Agrobacterium* were obtained from plant tumors, whereas the isolates of *Pseudomonas* were obtained in similar numbers from roots and tumors. Nine of 11 opine-utilizing isolates of *Agrobacterium* spp. originally were isolated on nopaline medium, whereas 78 of 90 opine-utilizing isolates of *Pseudomonas* spp. were isolated on octopine medium (Table 1).

The number and kinds of bacteria isolated varied considerably among the tumors and roots sampled. No opine-utilizing bacteria were isolated from tumors of seven of the 15 plants sampled. In contrast, nine octopine- and four nopaline-utilizing isolates of *Pseudomonas* and two nopaline- and three mannopine- and nopaline-utilizing isolates of *Agrobacterium* were obtained from a single tumor. This tumor was the only tissue from which isolates of *Agrobacterium* and *Pseudomonas* capable of utilizing the same opine were obtained.

The number of opine-utilizing bacteria isolated differed between the two nursery sites. At one nursery site, 43 of 69 isolates (62%) of opine-utilizing bacteria were obtained from tumors. At the second nursery site, only eight of 41 isolates (20%) were from tumors; the remaining isolates were from roots.

Identification of strains. All isolates of *A. tumefaciens* were biovar 2 strains based on biochemical tests and comparison with a known biovar 2 strain (data not shown). There were at least two major taxonomic groups of the fluorescent isolates as determined by biochemical tests (data not shown). None of the isolates completely matched with published descriptions for *Pseudomonas* spp. (10), but the octopine utilizers most closely resembled *P. fluorescens* biotypes C and E, and the nopaline isolates were closely related to *P. putida* (10).

Isolate pathogenicity. Twelve of 38 isolates of *Agrobacterium* were pathogenic on tomato and sunflower stems. Eight of 12 isolates were pathogenic on both Emla 7 and Red Delicious seedling apple rootstock in greenhouse pot trials. In field inoculations with strain B49C, incidence of crown gall was significantly greater ($P < 0.01$) for uninoculated Mark and Red Delicious

TABLE 1. Use of various combinations of opine media for isolation of opine-utilizing bacteria

Medium[a]	Octopine	Nopaline		Octopine and Nopaline	Mannopine	Mannopine and Nopaline
	Pseudomonas	*Pseudomonas*	*Agrobacterium*	*Pseudomonas*	*Agrobacterium*	*Agrobacterium*
Hooykaas (octopine)	6[b]	1	0	1	0	0
Octopine	16	14	0	0	0	0
Octopine plus 5 g L^{-1} glucose	11	21	1	0	1	0
Octopine plus 0.15 g L^{-1} $(NH_4)NO_3$	2	6	0	0	0	0
Nopaline	6	6	3	0	2	4
Nopaline plus 5 g L^{-1} glucose	0	0	0	0	0	0
Nopaline plus 0.15 g L^{-1} $(NH_4)NO_3$	0	0	0	0	0	0
Total	41	48	4	1	3	4

[a] Medium on which strains were isolated (see Materials and Methods section).
[b] Number of strains that utilized this opine. No octopine-utilizing *Agrobacterium* spp. were isolated. No mannopine-utilizing isolates of *Pseudomonas* spp. were obtained.

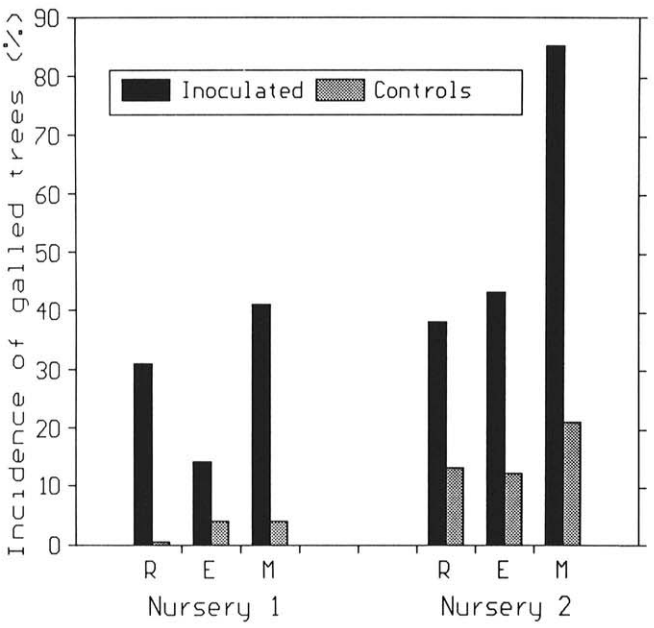

Fig. 1. Incidence of tumor formation at two field sites after inoculation with strain B49C of *Agrobacterium tumefaciens* onto three cultivars of apple rootstock: Red Delicious seedling (R), Emla 7 (E), and Mark (M). Tumor formation on plants inoculated with B49C was significantly different ($P < 0.01$) from controls, except for the Emla 7 rootstock in nursery 1 ($P = 0.11$).

seedling rootstocks at both nurseries, as compared with uninoculated controls (Fig. 1). At nursery two, inoculated Emla 7 rootstocks showed a much higher incidence of tumors compared with controls ($P < 0.01$), but there was a smaller difference at nursery one ($P = 0.11$). Therefore, in five of the six experiments in which strain B49C was inoculated, the incidence of trees with tumors was significantly greater than on uninoculated controls. Another indication of the pathogenicity of this strain in the field was the recovery of pathogenic strains from tumors induced with strain B49C. Eighty-two percent of the isolates of *Agrobacterium* from trees inoculated with strain B49C recovered on rifampicin medium were pathogenic when tested on tomato.

Antagonism of isolates of *Pseudomonas* to *A. tumefaciens*. Seventy-seven of the 90 fluorescent, opine-utilizing isolates of *Pseudomonas* inhibited the growth of *A. tumefaciens* on low-iron medium. Eight of these 77 isolates produced an inhibitory compound when grown on high-iron medium.

DISCUSSION

Crown gall of apple rootstocks is poorly understood, in part because many researchers have been unable to isolate *A. tumefaciens* from apple galls. The use of opines in media resulted in more isolates of *A. tumefaciens* than obtained in previous attempts. In one other effort to isolate pathogens with opines (23), isolations were carried out in a liquid medium in contrast to our use of solid medium. Solid medium containing opines may be superior to liquid medium for the isolation of opine-utilizing *A. tumefaciens*.

Identification of the highly virulent strain B49C of *A. tumefaciens* will be useful for further studies to evaluate strategies for control of crown gall in apple rootstocks. Potential antagonists and chemical compounds must be field tested for prevention of crown gall. If strains of *A. tumefaciens* are available that produce crown gall on at least 50% of the trees inoculated, then the effectiveness of various treatments can be more accurately evaluated. Natural field infections vary widely from year to year and may be as low as 5%.

Interestingly, most of the opine-utilizing isolates of *Pseudomonas* in this study were obtained with octopine as the sole carbon and/or nitrogen source. More nopaline isolates were obtained from octopine medium than from nopaline medium. Nopaline-utilizing strains of *A. tumefaciens* are able to use octopine after synthesis of the nopaline-catabolizing enzyme is induced by nopaline (17). If the nopaline-catabolizing enzymes in strains of *Pseudomonas* are similar to those in *A. tumefaciens*, then nopaline-catabolizing enzymes, once induced, could use octopine. However, the 10 nopaline-utilizing isolates of *Pseudomonas* tested in this study were unable to grow in octopine liquid medium with an inducing level of nopaline. Nutrient carryover from plant tumors might account for the isolation of nopaline strains on octopine medium; however, one would expect to find approximately equal numbers of octopine isolates from nopaline medium in this case. Instead, 78% of all the opine isolates were recovered from octopine medium, and 22% were recovered from medium supplemented with nopaline (Table 1).

There were no observable patterns descriptive of the opine-utilizing isolates of *Pseudomonas* with respect to planting site or plant part. All 12 isolates of *A. tumefaciens*, however, were recovered from tumors and none from roots, suggesting that plant tumors provide a more favorable environment for their survival. If opine-utilizing species of *Agrobacterium* and *Pseudomonas* coexist in tumors and the rhizosphere, one would expect to obtain both species from each plant sample, assuming equivalent distribution and abilities to colonize tissues in the field. Instead, of the 24 plant samples from which bacteria were isolated, only four yielded isolates of both *A. tumefaciens* and *Pseudomonas* spp. that could use opines. This may be because bacteria compete for opines within tumors and only one of the species survives.

Although octopine and nopaline utilization was widespread among the isolates of *Pseudomonas*, no mannopine-utilizing isolates were obtained. The inability to use mannopine could put the isolates of *Pseudomonas* at a competitive disadvantage with mannopine-utilizing *A. tumefaciens* in tumors where mannopine has been synthesized. The fact that several isolates of *A. tumefaciens* obtained in this study were capable of utilizing both mannopine and nopaline might indicate that these isolates have a greater selective advantage over those isolates that utilize only one of the opines, especially in tumors where competition for

nopaline may be a factor.

In addition to the substrate competition for opines by strains in this study, many isolates of *Pseudomonas* inhibited the growth of *A. tumefaciens* in culture. Such isolates of *Pseudomonas* might be useful in biological control of crown gall of apple.

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