Populations of *Agrobacterium* in Vineyard and Nonvineyard Soils and Grape Roots in Vineyards and Nurseries

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ABSTRACT

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Higher populations of biovar 3 of *Agrobacterium tumefaciens* and *A. radiobacter* were isolated from roots of grapevines with crown gall than from roots of noninfected vines or from nonrhizosphere soils sampled from infected and noninfected vineyards. Biovar 3 was not detected by plating serial dilutions of nonvineyard soils on a selective medium, indicating that populations were less than 100–1,000 colony-forming units per gram of soil. Roots of apparently healthy rootstocks and nongrafted grapevine cultivars sampled from nursery storages were contaminated with tumorigenic and nontumorigenic biovar 3. When specific sections of roots were assayed for biovar 3, it was predominantly isolated from small, dark, sunken lesions on the roots.

Agrobacterium tumefaciens biovar 3 (AT3) has been reported throughout the world as an important pathogen of grapevine (2,4,11,15,16,22). Lehoczky (9), Burr and Katz (3), and Tarbah and Goodman (23) have shown that grape scion and rootstock cuttings used for propagation are often systemically infested with the pathogen. The same authors proposed that the disease may be controlled by propagating and planting noninfested vines. Prerequisite to this approach, however, is a clear understanding of the soil ecology of AT3 and the potential for soil populations to function as inoculum. The purpose of this study was to examine populations of *Agrobacterium* in vineyard and nonvineyard soils and grape roots in vineyards and in nurseries.

MATERIALS AND METHODS

Vineyard soil and root samples. Soils and grape roots were collected from the Finger Lakes Region of New York State, from southeastern Washington State, and from the Demming area of New Mexico in the spring and summer of 1985. Samples consisted of soil and grape roots from vineyards with and without crown gall and from nonvineyard sites. The nonvineyard sites in Washington and New Mexico were fields that were proposed for future vineyards and were at least 500 m from existing vineyards. In New York, it was a field that was planted to vegetables for at least 10 yr and was about 5 km from the nearest vineyard. In diseased vineyards, 70–90% of the vines had galls. Soils were sampled to a depth of 20 cm with a small shovel. Several subsamples (about 100 g each) were collected from each of five vines per vineyard. In vineyards, soils were collected within 20 cm of trunks of infected or noninfected vines. Nonvineyard soils were sampled by collecting several samples (about 50 g each) at random from 5 to 20 cm deep for a total of about 1 kg.

To collect roots, soil was first removed from around the bases of the vines to a depth of 20 cm to expose them. About 30 g of lateral roots less than 4 mm in diameter were collected from each vine. Root samples from New York and New Mexico were collected from the same vines around which soil samples were taken, whereas Washington roots and soil were not necessarily collected from the same vines. Soils and roots were stored in plastic bags, transported to the laboratory in an ice chest, and stored in a refrigerator until isolations were made. The soil type, cultivar, and rootstock of samples are given in Table 1.

Isolations were made on a modification of a selective medium that was developed by Roy and Sasser (RS) (18). The medium is composed of (g/L), $MgSO_4$, 0.20; K_2HPO_4, 0.90; KH_2PO_4, 0.70; adonitol, 4.0; yeast extract, 0.14; NaCl, 0.20; boric acid, 1.0; and agar, 15.0. The pH is adjusted to 7.2, and after autoclaving and cooling to 50 C, the following (g/L) are added by filter sterilization: triphenyl tetrazolium chloride, 0.08, D-cycloserine, 0.02, trimethoprim, 0.02, and cycloheximide, 0.25. Cycloheximide replaces the chlorothalonil in the original recipe.

Soils were mixed in plastic bags, and a 50-g subsample was placed in 500 ml of sterile distilled water (SDW). Ten grams of roots were subsampled from each root sample and added to 100 ml of SDW. The water suspensions of soils and roots were then shaken on a reciprocal shaker for 20 min at 125 rpm, and 0.1 ml of serial water dilutions were plated in triplicate on RS. Plates were incubated for 4 days at 28 C, and typical colonies of *Agrobacterium* were counted. Each soil and root sample was assayed at least twice on different dates.

Nursery samples. Roots from grafted and nongrafted vines in storage were collected from a nursery in Washington State (A) and two nurseries in New York State (B and C) during the winter of 1985–1986. All vines had established good root systems the previous season and had no visible galls. Four nongrafted

Table 1. Soil and root samples assayed for biovar 3 strains of *Agrobacterium*

Sample[a]	Cultivar/rootstock/condition[b]	Soil type
NY1S, NY1R	Chardonnay/3309 C/healthy	Sandy loam
NY2S, NY2R	Chardonnay/3309 C/galled	Clay loam
NY3S	Nonvineyard	Clay loam
NM1S, NM1R	Ugni Blanc/5 BB/healthy	Sandy loam
NM2S, NM2R	French Colombard/own/galled	Sandy loam
NM3S	Nonvineyard	Sandy loam
WA1S, WA1R	White Riesling/own/healthy	Sandy loam
WA2S, WA2R	White Riesling/own/galled	Sandy loam
WA3S	Nonvineyard	Sandy loam

[a] The first two letters of the sample represent the abbreviation of the state from which it was collected, and the last letter indicates whether soil (S) or roots (R) were sampled. Soils were collected from around the trunks of vines to a depth of 20 cm by combining several about 100-g samples from five vines per vineyard. About 30 g of small lateral roots were collected from each of the vines.

[b] Soil and roots were collected from vineyards that had high incidence of crown gall or were apparently healthy.

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samples were collected from the Washington nursery, including one each from the cultivars Chenin Blanc and Sauvignon Blanc and two from Merlot. Five samples were collected from New York nursery B, including the following cultivar/rootstock combinations: Cabernet Sauvignon/3309 C, White Reisling/3309 C, Pinot Noir/SO 4, NY47616/5 A, and Einset/5 BB. Three 3309 C rootstock samples were collected from New York nursery C; one was grafted to Pinot Noir. Roots were collected from the nursery storages by cutting about 500 g of roots from randomly selected bundles of 1-yr-old vines of different cultivars or rootstocks. Roots were transported to the laboratory in plastic bags in an ice chest and stored in a refrigerator until processing. All roots that were collected from nurseries were less than 2 mm in diameter.

Three methods were used to assay the roots from the Washington nursery. Ten-gram root samples from each of the cultivars were shaken with 200 ml of SDW in flasks as described earlier for the vineyard roots, or the root-water mixtures were chopped at high speed in a Waring Blendor for 1 min. In addition, 10-g samples were soaked in 0.525% sodium hypochlorite for 1 hr, rinsed thoroughly in distilled water, and chopped in 200 ml of SDW. Platings of root washes were made of root samples before and after soaking to test the effect of the sodium hypochlorite surface sterilizaton on AT3 recovery. Dilutions of all samples were plated as described earlier.

Attempts to recover *A. tumefaciens* from different sections of roots of the Washington samples were also made after soaking 10-cm root pieces for 1 hr in 1.05% sodium hypochlorite and rinsing thoroughly in distilled water. Cross-sectional cuts were made about every 3 mm of root length with a sterile scalpel, and the cut end was streaked once across an RS plate. It was recorded on the isolation plates when streaks were made from necrotic areas or from apparently healthy areas of the roots.

All New York nursery root samples were processed by washing loose soil from the roots under running tap water, chopping a 10-g subsample in 200 ml of SDW in a blender as described previously, and plating dilutions on RS.

Identification of strains. Strains were initially identified by growth rate, colony color, and morphology on RS. Up to 10 typical colonies were selected per isolation plate, streaked on potato-dextrose agar (Difco), and tested for tumorigenicity in the greenhouse with the indicator host, *Nicotiana glauca* L., which was previously demonstrated highly sensitive for AT3 (5). Pinprick inoculations through a heavy smear of bacteria were made into plant stems, and results were recorded after 2 wk. All tumorigenic strains and several nontumorigenic strains were identified to biovar by spotting for growth determinations on the selective media of Schroth et al (19) for biovar 1 and of New and Kerr (13) for biovar 2 and RS, on which all biovars grow. The suitability of this method for biovar identification was tested by comparing the results of more than 117 known culture collection strains including 11 biovar 1 strains, 10 biovar 2 strains, and 96 AT3 strains with results from more detailed testing schemes (8,14,22). The specific tests that were conducted included: production of 3-ketolactose (1), growth in 2% NaCl (12), action on litmus milk (8), acid production from erythritol and melezitose (8), and alkali production from malonate (8). Twelve tumorigenic and nontumorigenic strains were identified to genus using the API Rapid NFT strips (API Analytab Products, Plainview, NY).

RESULTS

The selective medium RS was very effective for isolating *Agrobacterium* from soil and grape roots. In most cases, soil dilutions of 1:10 were plated with almost total suppression of microbes other than *Agrobacterium*. Typical AT3 colonies are convex and slightly mucoid and have red centers with a narrow white margin after 4 days at 28 C. Biovars 1 and 2 also grow on the medium but usually at a slower rate than AT3, with colony development (resembling that of AT3) taking 6–8 days at 28 C. Variation in colony morphology and color was observed among AT3 strains, however, and some biovar 1 colonies could not be visually distinguished from AT3 on RS even at 4 days. Therefore, biovar and pathogenicity tests were necessary to confirm AT3 strains.

Identification of strains to AT3 could be made accurately by determining growth on the three selective media. All of the 96 known AT3 strains grew on RS medium but not on the media of Schroth or New and Kerr. Strains of other biovars grew on their respective selective media with the exception of some biovar 1 strains that grew on New and Kerr medium. Biovar 2 strains did not grow on Schroth's medium. Biovars were confirmed using the additional tests that were described. The API Rapid NFT strips characterized all 12 known tumorigenic AT3 strains to genus. Of the 12 nontumorigenic strains suspected of being AT3 by growth reaction on selective media, 10 were characterized as *A. radiobacter* with the API test strips.

Recoveries of AT3 were made from all three root samples taken from infected vineyards and from one of three apparently healthy vineyards (Table 2). Although trunks of vines were severely infected, galls were not observed on roots collected from any of the vineyards. Only one of three soils from an infected vineyard and none of the other six yielded AT3. No tumorigenic biovar 1 or 2 strains were detected in any of the root or soil samples. The lowest levels of *Agrobacterium* were detected in nonvineyard soils except for New York soil, NY3S (previously planted to various vegetable crops), which contained a high level, 1.2×10^5 colony-forming units (cfu) of nontumorigenic biovar 1 per gram of soil. Most of the nontumorigenic strains detected in other samples were also identified as biovar 1. The highest populations of *Agrobacterium* were

Table 2. Isolations of *Agrobacterium tumefaciens* and *A. radiobacter* strains from grapevine roots and vineyard and nonvineyard soils

Sample/condition[a]	Log_{10} cfu/g soil or root[b]	No. T. strains/ no. tested[c]	Biovars of T strains[d]	Biovars of NT strains[c]
NY1S/healthy	3.81	0/7	···	1
NY1R/healthy	5.42	1/6	3	ND[e]
NY2S/galled	4.28	3/9	3	2
NY2R/galled	5.62	1/10	3	1
NY3S/nonvineyard	5.08	0/19	···	1
NM1S/healthy	5.26	0/8	···	1
NM1R/healthy	7.80	0/16	···	1
NM2S/galled	5.28	0/19	···	1,3
NM2R/galled	7.04	11/50	3	1,2
NM3S/nonvineyard	2.34	0/2	···	1
WA1S/healthy	2.26	0/5	···	ND
WA1R/healthy	4.73	0/13	···	ND
WA2S/galled	2.74	0/5	···	ND
WA2R/galled	6.40	4/25	3	ND
WA3S/nonvineyard	···	···	···	···

[a] Soil and roots were collected from vineyards that had high incidences of crown gall or were apparently healthy.
[b] Serial dilutions of 50 g of soil in 500 ml water or 10 g of roots blended in 100 ml of water were plated on RS medium.
[c] Tumorigenicity tests were conducted on *Nicotiana glauca*. T = tumorigenic and NT = nontumorigenic.
[d] Biovars were determined with previously reported testing schemes (7,12,19) and/or by growth on selective media (11,15,16).
[e] ND = not determined.

recovered from roots from healthy and galled New Mexico vineyards.

AT3 was recovered from apparently healthy grape roots from both nongrafted and rootstock vines from all three commercial nurseries (Table 3). Galls were not apparent on any of the vines sampled. Up to 80% of the strains tested from a single sample were tumorigenic, but the percentage was usually much lower. Nontumorigenic AT3 was recovered from all 12 root samples, and nontumorigenic biovar 1, from two.

Tumorigenic and nontumorigenic AT3 were isolated from the Washington nursery samples regardless of method used. Surface disinfestation of roots with sodium hypochlorite did not noticeably affect isolations. AT3 was recovered from surface washes before and after this treatment, indicating that it may be present in adhering rhizosphere soil, on root surfaces, or in cracks on the surfaces of roots as well as systemically within them.

Necrotic lesions were observed on roots of all three cultivars sampled from Washington State. The lesions were black and sunken and ranged up to 5 mm long and occasionally girdled the entire root. They were dispersed along the entire length of the roots and frequently extended into vascular tissues. By streaking cut ends of roots on RS, it was shown that tumorigenic and nontumorigenic AT3 were concentrated at locations where necrotic lesions occurred on roots (Table 4) and may coexist in the same lesions. The New York samples were not inspected for the presence of root lesions.

Table 3. Assays of roots of grapevines from nursery storages for *Agrobacterium*

Sample[a]	Log_{10} cfu/g root[b]	No. T strains/ no. tested[c]	Biovars of T strains[d]	Biovars of NT strains[c]
WA/A/Chenin Blanc	5.40	1/10	3	3
WA/A/Merlot, 1	5.46	1/10	3	3
WA/A/Merlot, 2	4.11	1/10	3	3
WA/A/Sauvignon Blanc	5.46	5/10	3	3
NY/B/3309 C/1	5.70	0/10	···	3
NY/B/3309 C/2	5.15	0/6	···	3
NY/B/5 BB	5.00	0/10	···	3
NY/B/SO 4	5.26	0/6	···	3
NY/B/5 A	5.82	8/10	3	3
NY/C/3309 C/1	3.98	6/29	3	1,3
NY/C/3309 C/2	4.08	0/24	···	1,3
NY/C/3309 C/3	4.08	1/35	3	3

[a] State from which sample was collected/nursery designation/cultivar or rootstock from which the roots were collected/sample number.
[b] Ten grams of roots were blended in 100 ml of water, and serial dilutions were plated on RS.
[c] Tumorigenicity was determined on *Nicotiana glauca*. T = tumorigenic strains and NT = nontumorigenic strains.
[d] Biovars were determined using standard schemes (7,12,19) and/or by growth on selective media (11,15,16).

DISCUSSION

AT3 strains were consistently isolated from roots of grapevine. Although nontumorigenic biovar 1 strains were common from all samples, the only tumorigenic strains recovered were AT3. This and other reports (2,14,17) illustrate the ecological specialization of AT3 for grape. AT3 was most frequently isolated from roots of crown gall-diseased vines. In contrast, relatively few AT3 were detected in nonrhizosphere soils, indicating that, like other agrobacteria, they survive preferentially in the rhizosphere (7,20,21).

The failure to detect AT3 strains in nonvineyard soil is significant because it supports the strategy of planting pathogen-free vines in such soils for control of grape crown gall. The production of *Agrobacterium*-free vines has been proposed by Tarbah and Goodman (23) and Burr and Katz (3). The success of this strategy will depend on the rate of reinfestation of the vines. Indexing methods (3,9,23) have demonstrated widespread contamination of propagation material, and it is likely that root residues in old vineyards and nurseries may harbor the bacteria systemically (10) or superficially for years. Preliminary tests have shown that when AT3-free vines are planted into artificially infested soils, they rapidly become reinfested (A. L. Bishop, *unpublished*). It may be possible, however, to establish clean plantings for sources of propagation material by planting AT3-free vines in noninfested soils. Further investigations on the population dynamics of AT3 are needed. The sensitivity threshold of the assay methods used was between 100 and 1,000 cfu/g soil, and the possibility of nondetectable levels of the pathogen surviving in soil exists.

In addition to the previously reported systemic survival and spread of AT3, we determined that the bacterium may be transported with rhizosphere soil or on the rhizoplane of apparently healthy nursery vines. This means of dissemination has also been suggested for *Agrobacterium* on other hosts (6).

Although RS was very useful in our studies, we could not rely totally on colony morphology and color for identifying biovars or tumorigenicity. Of 194 potential AT3 colonies selected for further testing from vineyard soil and root samples, only about 10% were identified as AT3. Most of the nontumorigenic strains belonged to biovar 1.

The association of AT3 with lesions on grape roots suggests a means by which the pathogen may invade the plant. Some lesions extended into the vasculature of roots and may thus be a point of entry for the pathogen into the vascular system of the vine. Subsequent testing of AT3 strains from root lesions and from our culture collection has demonstrated the ability of all of them to cause nontumorigenic root infections (T. J. Burr, *unpublished*) on grape. The effects of root decay by AT3 on root development, vine growth, and pathogen establishment in the plant are under investigation.

Table 4. Association of *Agrobacterium tumefaciens* biovar 3 with lesions on grape roots

Cultivar	No. AT3 recovered/ no. lesions assayed[a]	No. AT3 recovered/ no. healthy areas assayed
Sauvignon Blanc	2/11	0/18
Sauvignon Blanc	1/3	0/18
Sauvignon Blanc	5/14	0/27
Chenin Blanc	0/11	0/12
Merlot, 1	6/10	1/10
Merlot, 2	1/11	0/9

[a] Soil was washed from the surface of root segments with running tap water; they were soaked in 1.05% sodium hypochlorite for 1 hr and rinsed thoroughly. Cuts were made through lesions or apparently healthy areas of roots with a sterile scalpel, and the cut ends were streaked once across RS medium. Typical colonies were subcultured and tested for tumorigenicity and biovar.

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