A Root-Specific Decay of Grapevine Caused by *Agrobacterium tumefaciens* and *A. radiobacter* Biovar 3

T. J. Burr, A. L. Bishop, B. H. Katz, L. M. Blanchard, and C. Bazzi

First four authors: Department of Plant Pathology, New York State Agricultural Experiment Station, Cornell University, Geneva 14456; fifth author: Institute of Plant Pathology, University of Bologna, Italy.

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ABSTRACT

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Tumorigenic (T) and nontumorigenic (NT) biovar 3 strains of *Agrobacterium tumefaciens* and *A. radiobacter*, respectively, were isolated from dark, sunken lesions on roots of grapevines grown in a commercial nursery. Roots of the grape cultivars Concord and Pinot Chardonnay decayed after inoculations with T and NT biovar 3 strains. Thirty-five strains of biovar 3 from several geographic areas worldwide reacted identically. Decay was restricted to the roots of grapes, whereas tumors were incited by T strains on grape shoots and on other hosts. No decay resulted when T and NT strains of biovars 1 and 2 were inoculated to grape roots. In addition, none of the strains decayed bean or sunflower roots. Root decay may provide sites for entrance of *Agrobacterium* into the grape vascular system and may cause detrimental effects on root development and vine growth.

Crown gall of grapes, caused by *Agrobacterium tumefaciens* (Smith and Townsend), occurs worldwide and is particularly severe on cultivars of *Vitis vinifera* when they are grown in cold climates (3,8,11). The predominant biovar of *A. tumefaciens* on grape is biovar 3 (4,12,15,19,20). Tumors usually develop in the summer on trunks of vines near the soil line. Crown gall is most prevalent after winters when trunks are freeze injured (3,11). Biovar 3 strains of *A. tumefaciens*, which are tumorigenic (T), and of *A. radiobacter* (Beijerinck & van Delden), nontumorigenic (NT), coexist within the vascular system of galled and apparently healthy grape canes (4,5,13,20) and therefore are spread in vegetative propagation material. They are also rhizosphere inhabitants of grape (6). We recently reported the isolation of T and NT biovar 3 from dark sunken lesions on roots of grape cultivars Merlot and Sauvignon Blanc grown in field soil (6).

We now report a previously undescribed decay caused by *A. tumefaciens* and *A. radiobacter* biovar 3 that is specific to grape roots.

MATERIALS AND METHODS

Isolation from lesions. T and NT strains of biovar 3 were isolated from lesions on grape roots as previously described (6). Samples included roots from nongrafted vines of the cultivars, Merlot (two samples), Sauvignon Blanc, White Riesling, Pinot Chardonnay, Chenin Blanc, and Semillon collected from commercial nurseries. Approximately 50 g of roots of each cultivar was collected by randomly cutting them from bundled vines in nursery storages. Each root sample was collected from vines that were propagated from specific sources of cuttings. Roots were refrigerated in plastic bags before assaying for *Agrobacterium*.

For each sample, six to 10 root segments that were about 10 cm in length were washed under running tap water to remove adhering soil. Roots were then soaked in 0.5% sodium hypochlorite for 10 min to reduce surface microflora. The bleaching also made root lesions easily observable. After a final rinsing in sterile distilled water, cross-sectional cuts were made through the lesions, and the cut ends were streaked once across a culture medium (RS) that is semiselective for biovar 3 (6,18). Plates were incubated at 28 C for 4–6 days, at which time typical *Agrobacterium* colonies were noted. Colonies were subcultured on potato-dextrose agar (PDA) (Difco), tested for tumorigenicity by inoculation on *Nicotiana glauca* Graham (6) and *Helianthus annuus* L. (5), and identified to biovar as previously described (5,6).

Root decay assays. To determine if strains were capable of inducing root decay, the roots of 2-wk-old Concord (*V. labrusca* L.) grape seedlings were dipped for 1 min in suspensions of two T strains (II-5, III-1) and one NT strain (I-4) of biovar 3 that had been isolated from root lesions of Sauvignon Blanc. Inoculum consisted of bacteria grown on PDA for 48 hr and suspended in sterile distilled water to about 10^8 colony-forming units (cfu)/ml. The seedlings were then replanted in a Cornell potting mixture (2) in the greenhouse. Fifteen seedlings were inoculated per strain, and fresh weights of surviving seedlings were recorded after 2 wk.

Further testing of the root decay activity of biovar 3 strains utilized a laboratory inoculation procedure. Seeds of Concord grape were stratified and then germinated in moistened sterile sand in petri dishes. Two to four days after germination, seedlings were harvested from the sand, rinsed in sterile distilled water, and placed on the surface of moistened, sterile perlite in petri dishes. Three seedlings per dish were inoculated by making a puncture in the crown area with a fine insect-mounting needle and placing a drop of a suspension of a test strain on the wound. Inoculum suspensions consisted of about 10^8 cfu/ml of 48-hr-old strains grown on PDA, suspended in sterile distilled water. Control seedlings were wounded and inoculated with sterile distilled water. Inoculated seedlings were incubated on the laboratory bench for 96 hr. Attempts to reisolate biovar 3 were done at this time by triturating a small piece of tissue from around the inoculation sites in 1 ml of sterile distilled water and streaking the suspension on RS.

Strain specificity of root decay. Sixty-three strains of *Agrobacterium* and three other bacteria were tested for root decay activity by inoculation of seedlings as described above. These represented T and NT biovars 1, 2, and 3 and single strains of *Pseudomonas fluorescens* Migula, *Hafnia alvei* Moller, and *Salmonella* spp. The latter three strains were included because they had previously been identified as rhizosphere inhabitants of fruit crops (7). All inoculations were repeated at least twice.

Root decay activity was also tested on excised roots of cultivar Pinot Chardonnay of *V. vinifera* that were grown in tissue culture (9). Roots were harvested from plants growing in a solid tissue culture medium and rinsed in sterile distilled water. Three 5-cm root segments were inoculated per strain by first wounding them at

three or four sites with a needle and then placing a drop of inoculum on each wound as previously described. Roots were incubated on moistened sterile perlite in petri dishes for 96 hr. Inoculations were repeated once.

Host and tissue specificity of root decay. Susceptibilities of grape roots and shoots were compared using the seedling assay. One-week-old seedlings were punctured with a needle at 1-cm intervals starting from the root tip and extending up the shoot to the cotyledons. One drop of inoculum was placed on each wound and the seedlings were incubated as in previous experiments. Four T and NT biovar 3 strains were tested and three seedlings were inoculated per strain. This experiment was repeated once.

Host and biovar specificity of decay was examined by comparing the results obtained on grape to inoculations on the roots and shoots of sunflower (*H. annuus*) and snap bean (*Phaseolus vulgaris* L.). Seedlings of both hosts were grown in Cornell potting mixture in the greenhouse. One week after emergence, root inoculations were made by excising about 50% of the roots with sterile scissors and then dipping the seedling roots into a sterile distilled water suspension of test strains. Seedlings were immediately replanted into the Cornell mix. Shoot inoculations were made at a single needle-wound site about 1 cm below the cotyledons. Inoculum for all experiments consisted of a sterile distilled water suspension of each strain containing about 10^8 cfu/ml. Two T and NT biovar 3 and one T and NT biovar 1 and 2 strains were tested. Plants were grown in the greenhouse for 1 mo, at which time they were examined for decay or gall formation on roots and shoots. The experiment was repeated once.

Root decay activity on bean and sunflower was also tested using the same strains. The taproots of 4-day-old seedlings of each were wounded with a needle and inoculated, as described for grape seedlings. Seedlings were incubated in moistened sterile perlite for at least 5 days at 26 C, and the experiment was repeated once.

RESULTS

Lesions were observed on the roots of all of the grape cultivars examined. They were dark, slightly sunken, and often encompassed more than half of the root cross section. Lesions were up to 5 mm in length and were found along the entire length of current season roots (Fig. 1). The relative numbers of lesions on roots of different cultivars were not measured, but they appeared to be greatest on Sauvignon Blanc. Tumorigenic biovar 3 strains were isolated from lesions on Merlot-1 (3 of 14), Merlot-2 (1 of 18), Sauvignon Blanc (7 of 25), Chenin Blanc (1 of 10), Pinot Chardonnay (0 of 11), White Riesling (0 of 14), and Semillon (0 of 11).

Grape seedlings that were dipped in suspensions of T and NT biovar 3 were visibly stunted within 4 days of inoculation. Up to 30% of the inoculated seedlings died within 1 wk, and those that survived sustained severe root decay and significant weight reductions as compared to the controls (Table 1).

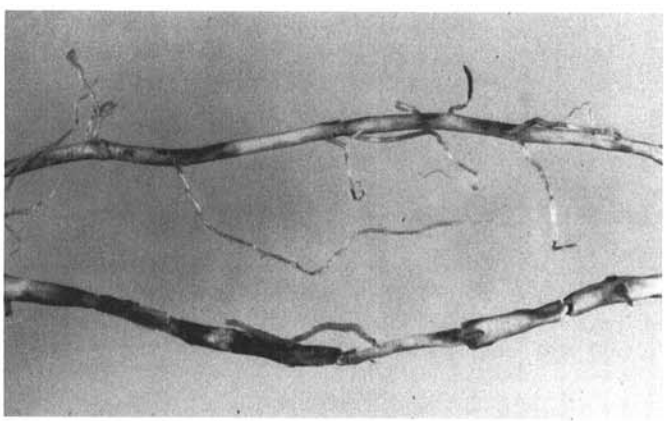

Fig. 1. Naturally occurring root lesions associated with *Agrobacterium tumefaciens* biovar 3 dispersed along current-season roots of cultivar Sauvignon Blanc. Lesions are up to 5 mm in length.

All of the 35 biovar 3 strains, regardless of source or tumorigenicity, caused root decay of grape (Table 2). Host reactions varied slightly with respect to the extent and rapidity of the response, but all biovar 3 strains caused noticeable decay within 72 hr (Fig. 2A). Root decay progressed basipetally until the entire root system was decayed. Biovar 3 was always reisolated from the decayed areas on RS medium. Decay did not progress from roots to green pigmented portions of hypocotyls. Inoculations of wound sites on green shoot tissues resulted in no reaction or the development of restricted necrotic areas. In addition to Concord roots, the excised roots of Pinot Chardonnay were also decayed by T and NT strains of biovar 3.

In contrast, no decay developed on Concord or Pinot Chardonnay roots that were inoculated with T or NT strains of biovars 1 or 2 (Fig. 2B) or with strains of *P. fluorescens*, *H. alvei*, or *Salmonella* spp. A negative reaction consisted of the puncture wound that remained white or developed slight browning during the incubation period.

The decay caused by biovar 3 could only be demonstrated on roots of grape. No decay developed on sunflower and bean roots, but tumors were induced by T strains on sunflower roots (Table 3).

Tumors also developed on stems of sunflower and/or bean inoculated with all T strains except for CG 414, which is only tumorigenic on *Nicotiana glauca* (B. H. Katz, *unpublished*).

TABLE 1. Effect of tumorigenic (T) and nontumorigenic (NT) biovar 3 strains of *Agrobacterium* on Concord seedling growth

Strains[x]	Tumorigenicity[y]	Mean fresh weight of seedlings (g)[z]
I-4 (NT)	−	0.17 a
III-1 (T)	+	0.25 ab
II-5 (NT)	−	0.28 bc
Control		0.35 c

[x] Strains were obtained from lesions on grape roots collected from a commercial nursery. Inoculations were made by dipping seedling roots in about 10^8 cfu/ml suspension for 1 min before planting.

[y] As measured by stem inoculations on *Nicotiana glauca* and *Helianthus annuus*.

[z] Means of 30 seedlings that survived the treatments followed by different letters are significantly different based on Fishers F-protected $LSD_{0.05}$ = 0.10.

TABLE 2. Decay of grape seedling roots by *Agrobacterium*

Biovar	Tumorigenicity[a]	Root decay[b]	Strains tested (no.)
1	+	−	5[c]
1	−	−	15[d]
2	+	−	3[e]
2	−	−	5[f]
3	+	+	27[g]
3	−	+	8[h]

[a] Determined by stem inoculation on *Nicotiana glauca* and *Helianthus annuus* L.

[b] Determined by inoculation on *Vitis labrusca* 'Concord' seedling roots.

[c] From *Vitis* spp.

[d] From lesions on grape roots (two strains), surfaces of healthy grape roots (10 strains), and *Vitis* spp. (three strains).

[e] From vineyard soil (one strain), *Rosa* spp. (one strain), and *Chrysanthemum* sp. (one strain).

[f] From lesions on grape roots (two strains), surfaces of healthy grape roots (two strains), and vineyard soil (one strain).

[g] From lesions on grape roots (three strains), surfaces of healthy grape roots (eight strains), and *Vitis* spp. (16 strains). Biovar 3 strains were isolated in the U.S. and Italy. In addition the authors wish to acknowledge the following researchers for strains that were isolated in their respective countries: Spain (M. M. Lopez, Ministerio de Agricultura INIA, Moncada, Valencia), West Germany (E. Bien, Landes-Lehr-und Forschungsanstalt, Breitenweg), and Greece (C. G. Panagopoulos, Athens College of Agriculture Science, Athens).

[h] From lesions on grape roots (four strains) and surfaces of healthy grape roots (four strains).

DISCUSSION

Root decay by biovar 3 is a new pathological syndrome that has not been previously reported for *Agrobacterium*. Practical implications of the infection are suggested by the severe root decay of grape seedlings obtained in the greenhouse and by the aggressive nature of the decay. The infections are potentially detrimental to root development and subsequent vine growth and productivity in vineyards.

TABLE 3. Tissue, host, and biovar specificity of root decay by *Agrobacterium*

Strain	Biovar	Tumorigenicity[a]	Response on shoot/root		
			Bean[b]	Sunflower[b]	Grape[c]
CG49	3	+	G/G[d]	G/G	G/D
CG975	3	+	−/−	G/−	G/D
II-5	3	−	−/−	−/−	−/D
WR-6	3	−	−/−	−/−	−/D
CG90	1	−	−/−	−/−	−/−
CG628	1	G/−	G/G	G/−	
CG423	2	−	−/−	−/−	−/−
CG414[e]	2	+	−/−	−/−	−/−

[a] Determined by stem inoculations on *Nicotiana glauca* and *Helianthus annuus*.
[b] Inoculations were made in 1-wk-old seedlings in the greenhouse. Half of the roots from each seedling were cut with sterile scissors and seedlings were dipped in bacterial suspensions (about 10^8 cfu/ml) and then immediately replanted in Cornell mix. Stem inoculations were made by placing a drop of inoculum on a single needle wound site on the stems.
[c] Stem inoculations were made on 1-mo-old vines in the greenhouse. Root inoculations were made on 4-day-old Concord seedlings by placing a drop of bacterial suspension (about 10^8 cfu/ml) on a single wound site on the seedling taproot where lateral roots are initiated.
[d] G = gall produced, D = decay of tissue, − = healthy response.
[e] CG414 is only tumorigenic on *N. glauca*.

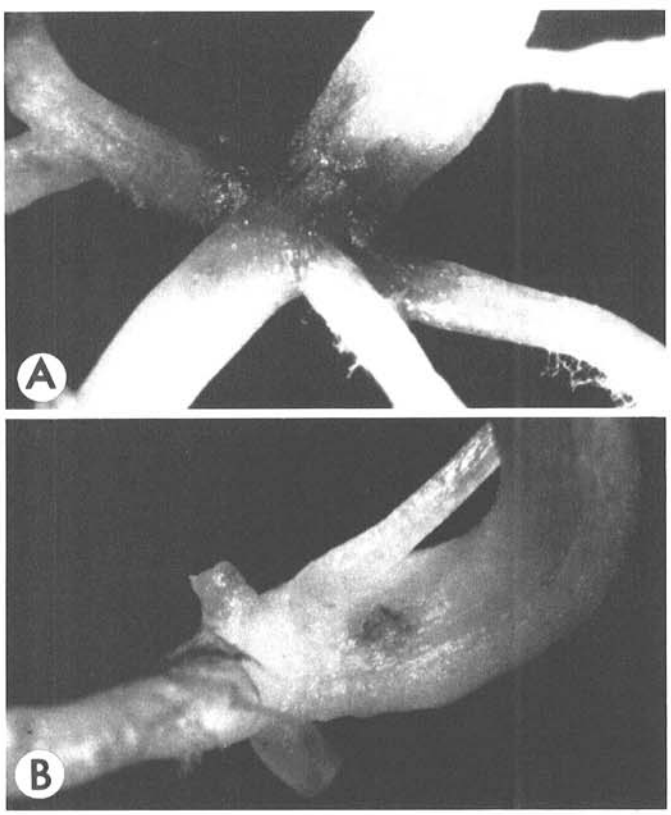

Fig. 2. A, Root decay on Concord grape seedling caused by tumorigenic and nontumorigenic biovar 3 strains of *Agrobacterium*. **B,** Typical wound response following inoculations with T and NT strains of biovars 1 and 2 or with water.

Naturally occurring lesions resemble those caused by root-infecting fungi, and, therefore, cannot be positively attributed to biovar 3, unless isolations are made. In fact, biovar 3 was not isolated from the majority of the root lesions from field samples. This could be due to our isolation methods, or be a factor of lesion age. When isolation attempts were made by streaking several cut surfaces per lesion, they did not all yield biovar 3, suggesting that the pathogen may die out in older lesions or in some areas of lesions. (T. J. Burr, *unpublished*).

A hypersensitive reaction on grape, caused by wide host range strains of *A. tumefaciens*, has recently been reported by Yanofsky et al (21). The hypersensitive reaction is associated with the *vir* C locus on the Ti plasmid and is prevented by mutations to that locus. It is difficult to determine if the root decay phenomenon that we report is related to the hypersensitive response. Yanofsky's assay was conducted on grape shoots, in a different environment than our tests were done. We did not observe rapid decay of shoots. However, inoculations to seedling shoots occasionally resulted in localized necrotic areas that may be interpreted as a hypersensitive response. The root decay is an aggressive decay that develops rapidly and is water soaked, suggesting the action of tissue-degrading enzymes or toxins produced by the pathogen. Further research on the genetics of root decay is necessary. Our results indicate that it is not associated with tumorigenicity (16), because NT biovar 3 strains decayed roots, whereas T and NT strains of biovars 1 and 2 did not.

Inoculum for root decay may come from various sources. Biovar 3 survives systemically in vines (1,4,5,13,14,20) and can be spread in apparently healthy propagation material (5,20). The bacterium is also a rhizosphere inhabitant of grape (6) and, therefore, may persist in soil in association with grape roots. After harvesting of vines from nursery fields, abundant roots remain in the soil and may harbor the pathogen (6,14) resulting in inoculum buildup. Grapes replanted into those same fields are likely to be affected by grape replant disease (10), an important problem of unknown etiology. Interestingly, lesions on the roots of replant disease-affected vines appear similar to those caused by biovar 3. The role of *Agrobacterium* in replant disease is being investigated.

The root lesions that we observed often penetrated into the root vasculature and, therefore, may be sites where *Agrobacterium* first enters the vine, resulting in systemic infestations. The bacterium may then spread through the xylem vessels of the vine and incite tumors at wound sites, as suggested by Lehoczky (14). Tests in our laboratory have shown that when *A. tumefaciens*-free vines are planted into biovar 3-infested soil, the pathogen enters the vine and becomes established in the vascular system (A. L. Bishop, *unpublished*).

A thorough study of differential cultivar and rootstock susceptibility to root decay has not been done. Our results with seedling and excised root inoculations, however, show that root infections may be important on cultivars that do not typically have high incidences of crown gall, such as Concord. Research on the significance of root decay on vine growth is needed.

Agrobacterium-induced decay of roots is limited to biovar 3 and to grape. This is the first indication of physiological specialization of biovar 3 for grape and may play a role in the ecological specialization that has been previously described (4,12,15,17,19,20). The root decay assay that we describe is a simple laboratory test that may also prove useful as a taxonomic tool for identification of biovar 3 strains.

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