Isolation of *Agrobacterium tumefaciens* Biovar 3 from Grapevine Galls and Sap, and from Vineyard Soil

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ABSTRACT

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Agrobacterium tumefaciens biovar 3 (AT 3) was isolated from grapevine galls, sap of "bleeding" vines, and from vineyard soil by using a selective medium. AT 3 was the predominant biovar isolated from galls. The bacterium was recovered from sap from seven of 24 infected vines and one of 17 apparently healthy vines. Ten sap isolates were identified as AT 3 and all were pathogenic on grapevine and sunflower. Two hundred forty-four typical colonies of *Agrobacterium* were selected from dilution plates from 30 vineyard soil samples. Five of these strains were pathogenic, three being AT 3 and two similar to *A. tumefaciens* biovar 1. Almost all of the AT 3 strains from galls, sap, and soil caused galls on tomato, sunflower, and grapevine inoculated in greenhouse tests.

Crown gall of grapevine can cause extensive losses in New York vineyards, particularly on cold-sensitive cultivars (2). Galls usually occur on the trunk just above the ground line; however, aerial galls up to 1 m above the soil have been observed. Reports from Greece (12,13) and Hungary (20), and preliminary reports from the United States (3,15) have identified *Agrobacterium tumefaciens* biovar 3 (AT 3) as the predominant biovar on grapevines. It is well documented that *Agrobacterium* spp. survive in soil (17,18) and it has generally been assumed that inoculum in vineyards is splashed from soil onto freeze cracks or other wounds and incites galls (14). Lehoczky, however, has recovered *A. tumefaciens* (AT) from grapevine sap (8) and symptomless tissues (9) and believes the pathogen survives systemically in grapevines.

Thus far, chemical (5) and biological (7) controls for crown gall of grapevine have been ineffective. It appears that a thorough understanding of the biology of AT in vineyards is required for the development of effective controls. This paper reports the predominance of AT 3 in grapevine galls and sap and its presence in vineyard soils in New York State.

MATERIALS AND METHODS

Isolations from galls of infected grapevine. Isolations were made from fresh galls on naturally infected vines collected from the end of June through September 1978–1981. Galls were washed thoroughly in running tap water and blotted dry. Small sections of surface tissues were removed with a sterile scalpel, cut into smaller pieces in a drop of distilled water on a microscope slide, and observed microscopically. Five to ten sections of tissue were observed per gall, and when large masses of bacteria were seen, a loopful of the suspension was streaked on nutrient agar (NA) and potato-dextrose agar (PDA) (Difco Laboratories, Detroit, MI 48232). Additional isolations were made from galls by using NKS selective medium (see below). All isolation plates were incubated 4–6 days at 28 C, before typical colonies of *Agrobacterium* were selected for pathogenicity tests.

Medium for isolation of AT 3 from galls, sap, and soil. Preliminary tests showed that many microorganisms can interfere with the detection of *Agrobacterium* spp. in soil and grapevine sap samples. A previous report (7) indicated that AT 3 does not grow on the medium of Schroth (16) (SCH) or that of New and Kerr (11) (NK). In addition, it was found that New York grapevine gall strains do not grow on the medium of Kado and Heskett (6) and that seven days are required before minimal growth is visible on the medium of Clark (4). Therefore, NK was modified by substituting sucrose for erythritol as the primary carbon source. This medium (NKS) allows the growth of AT biovars 1, 2, and 3.

Isolations from vineyard soil. Thirty-six soil samples from five vineyards were collected during the 1980 and 1981 growing seasons. All samples were taken from the root zones of vines with fresh or dried galls on their trunks. Samples collected from June to September were taken beneath vines that had fresh galls on them. Several small soil samples containing roots were collected from a 5-to 10-cm-depth around the base of the vines, and were combined to make a total sample of 1 kg. Samples were refrigerated and processed within 24 hr of collection.

Soil samples were thoroughly mixed and 10-g subsamples were stirred in 100 ml sterile, distilled water for 15 min. Serial water dilutions were made and 0.1 ml spread on SCH, NK, and NKS media. Plates were incubated for 5 days at 28 C before suspect colonies of *Agrobacterium* were saved and tested for pathogenicity.

Isolations from grapevine sap. Grapevine sap samples were collected from 19 cultivars of "bleeding" vines from four vineyards at seven different times during April and May in 1981 (Table 1). Twenty-four infected and 17 apparently healthy vines were sampled by surface sterilizing areas of trunks with 70% ethanol, making small cuts into the wood and allowing sap to flow for 30 sec prior to collection. Apparently healthy vines were sampled 75 cm above the soil line and infected trunks 50 cm above the galled area. Sap (0.5–5.0 ml) was collected in sterile Pasteur pipettes and placed in vials for transport to the laboratory. All samples were refrigerated and processed within 24 hr after collection. Serial water dilutions of the sap were plated on SCH, NK, and NKS. Plates were incubated for 5 days at 28 C before suspect colonies of *Agrobacterium* were selected for pathogenicity tests.

Pathogenicity tests. Strains suspected of being AT were streaked on PDA to check for purity and to prepare inoculum for pathogenicity tests. Inoculations were made on two or more of the following hosts: sunflower (*Helianthus annuus* L.), tomato (*Lycopersicon esculentum* Mill.), grapevine (*Vitis vinifera* L.), and carrot slices (*Daucus carota* L.). A heavy smear of cells from each test strain was spread with a sterile toothpick onto stems of 2- to 3-wk-old sunflower and tomato plants and shoots of potted 6-wk-old grapevines in the greenhouse. Subsequently the stems or shoots were repeatedly punctured through the inoculum with a fine, sterile needle. Carrot disks were placed in petri dishes with moist filter paper and heavy inoculum suspensions were spread across each disk. Plates were kept at room temperature in the dark and the filter

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paper was periodically remoistened. At least three plants or disks were inoculated with each strain and each test was repeated at least once.

In addition to the above hosts, 13 strains from grapevine galls were inoculated on *Bryophyllum* sp. and *Datura stramonium* L. by using the stem puncture method. Final pathogenicity readings on all hosts were made after 1 mo.

Characterization of strains. Strains that produced typical colonies on culture media and produced galls on one or more of the indicator plants were further characterized to biovar by using the following key tests (Table 2): growth on the selective media SCH (16) and NK (11), production of 3-ketolactose (1), growth in 2% NaCl (10), litmus milk test (7), acid production from erythritol, ethanol, and melezitose (7), and alkali production from malonate and tartrate (7). All tests were repeated at least twice.

To determine if strains from sap were host specific, 120 colonies were selected from isolation plates and checked for pathogenicity on sunflower and grapevine. They were classified to biovar on the basis of their growth on SCH, NK, NKS, their ability to produce 3-ketolactose, and their sensitivity to Agrocin 84 (19).

RESULTS

Isolations from galls. AT 3 was isolated from grapevine galls using NA, PDA, and NKS. Twenty-one strains were obtained from seven New York and one Michigan vineyard from the following cultivars: Baco Noir, Chancellor, Chenin Blanc, Chelois, Gamay Beaujolais, Gewurtztraminer, Olivette Noir, Pinot Chardonnay, Sultana Moscata, and White Riesling. Two pathogenic strains of an intermediate biovar were recovered from a Niagara vineyard. These two strains resembled biovar 1 except they did not grow on SCH, were negative for 3-ketolactose production, and were not inhibited by Agrocin 84. Nineteen strains from galls were characterized as AT 3 using the scheme shown in Table 2. These 19 strains responded identically except in acid production from ethanol, for which a variable response was obtained.

Almost all of the gall strains were pathogenic to all of the indicator hosts (Table 2). The results of the carrot disk test, however, were inconclusive since false positive reactions sometimes developed on check disks, which were indistinguishable from reactions of known pathogenic strains.

Isolations from soil. Only five of 244 strains selected from typical colonies of *Agrobacterium* on soil dilution plates were pathogenic to sunflower. All five strains were detected using NKS medium. Three of these were identified as AT 3 and two agreed with all the criteria of biovar 1 (Table 2). Pathogenic *Agrobacterium* spp. were only isolated from samples collected during the period when fresh galls were present on trunks (June–September).

Isolations from sap. Although *Agrobacterium* spp. were recovered from sap on all three media used, more strains were recovered on NKS than on SCH or NK media. Pathogenic AT strains were obtained from sap from seven of 24 infected and one of 17 apparently healthy vines (Table 1). In most cases, almost pure cultures of AT grew from sap dilutions on NKS. Ten strains, representing at least one from each of the eight positive sap samples, were identified as AT 3, as shown in Table 2. All strains were pathogenic on grapevine and sunflower, and nine of the 10 were pathogenic on tomato.

Of the 120 strains of *Agrobacterium* selected from sap isolation plates, 84% were identified as AT 3 based on pathogenicity tests, growth on NKS but not SCH or NK, lack of 3-ketolactose production and lack of inhibition by Agrocin 84. Two percent of the strains were identified as either AT 1 or 2 and the remaining 14% were not pathogenic on sunflower or grapevine; presumably these are *Agrobacterium radiobacter*.

DISCUSSION

Systemic survival of *A. tumefaciens* in sap and symptomless grapevine tissues has been reported in Hungary (8,9). This phenomenon may be extremely important as a means of spreading

TABLE 1. Recovery of *Agrobacterium tumefaciens* (AT) from sap[a] of grapevines with and without crown gall

Vineyard	Cultivar	Sampling date (1981)	Frequency of AT recovery from vines[b] With galls	Without galls
A	Olivette Noir	3 April	0/1	···
	Sultana Moscata	13 April	2/2	···
	Barbebleue	13 April	0/1	···
	Chenin Blanc	27 April	0/1	···
	Semillon	27 April	0/1	···
	Flora	5 May	1/1	···
	Muscat Hamburg	5 May	0/1	···
	Baco Noir	5 May	0/1	···
	Colobel	5 May	0/1	···
	P. I. 10868	5 May	0/1	···
B	Gamay Beaujolais	20 April	0/2	···
		27 April	···	0/1
		14 May	···	0/1
		19 May	···	0/2
	Chenin Blanc	20 April	3/3	···
		14 May	0/1	···
		19 May	0/1	···
	Pinot Chardonnay	20 April	1/1	···
		27 April	0/1	···
	Pinot Noir	27 April	···	0/1
	Delaware	21 April	···	0/1
	Cabernet Sauvignon	27 April	···	1/1
		14 May	···	0/2
		19 May	···	0/1
	White Riesling	27 April	···	0/1
		14 May	0/1	···
C	Niagara	27 April	0/1	0/1
	Concord	27 April	···	0/2
	Cayuga White	7 May	0/1	0/3
D	White Riesling	20 April	0/1	···

[a] Sap was collected by suction with a sterile Pasteur pipet from surface-sterilized wounded grapevine trunks. Serial water dilutions of the sap were spread on selective media (SCH, NK, and NKS) and suspect strains were tested for pathogenicity on sunflower and grapevine.
[b] Ratios represent the number of vines from which AT was isolated divided by the number of vines sampled. ··· = not sampled.

TABLE 2. Characterization of *Agrobacterium tumefaciens* (AT) strains from grapevine galls or sap, and vineyard soil

Test	AT biovars[a] 1	2	3	Strains[b] from Gall	Sap	Soil
Growth on Schroth medium	+	−	−	0/21	0/10	2/5
Growth on New-Kerr medium	−	+	−	0/21	0/10	0/5
Prod. of 3-ketolactose	+	−	−	0/21	0/10	2/5
Growth in 2% NaCl	+	−	+	21/21	10/10	5/5
Litmus milk	Alk	Acid	Alk	Alk	Alk	Alk
Acid from erythritol	−	+	−	0/21	0/10	0/5
Acid from ethanol	+	−	−	9/21	0/10	5/5
Acid from melezitose	+	−	−	2/21	0/10	2/5
Alkali from malonate	−	+	+	19/21	10/10	3/5
Alkali from tartrate	V[c]	+	+	19/21	10/10	5/5
Pathogenicity on sunflower	+	+		21/21	10/10	5/5
Pathogenicity on grapevine	+	+		19/21	10/10	4/5
Pathogenicity on tomato	+	+		17/21	9/10	2/5
Pathogenicity on *Bryophyllum*	+	+		11/13		
Pathogenicity on *Datura*	+	+		12/12		

[a] Results of characterization tests for AT biovars 1 and 2 are from experiments conducted in our laboratory with previously identified strains supplied by R. S. Dickey, Cornell University, Ithaca, NY. Results of tests for biovar 3 are from Moore et al (10).
[b] Number of positive strains divided by the total number of strains tested.
[c] V = variable response.

the pathogen in propagation material. Our findings support this idea since we detected AT 3 in grapevine sap from both infected and apparently healthy vines. However, our samplings were limited to trunks, and it is still uncertain if AT 3 moves into growing shoots and would be present in propagation cuttings taken in winter. Although Lehoczky (9) has made one isolation of AT from callus of symptomless cuttings (from an infected vine) that were forced in sterile sand, the cultivar sampled is not common in the U.S. Grapevine cultivars differ in susceptibility to crown gall (2,5) and the ability of the pathogen to survive and move systemically in different cultivars may vary also. Further studies using sensitive detection techniques are needed to clarify this point.

Although *Agrobacterium* spp. are reported to survive in soil, the ratio of pathogenic to nonpathogenic strains is usually low (17,18). Our results agree with these findings since only five of 244 colonies selected from isolation plates were pathogenic. It is interesting that AT 3 and strains similar to *A. tumefaciens* biovar 1 (AT 1) were detected in vineyard soil and that the pathogenic strains were only detected during the months when fresh galls were present on infected trunks. This may indicate that the populations we detected were washed from the galls into the soil. Although AT 3 was detected, the duration of its survival in the soil is still unknown. Considering the infrequency with which pathogenic strains were obtained, it seems unlikely that soil provides a significant source of inoculum for grapevine crown gall. In Long Island, NY, for instance, high percentages of young vines have become infected at new vineyard sites that historically were planted to potatoes.

Panagopoulos and Psallidas reported that AT 3 strains from grapevine in Greece would not cause galls on indicator hosts other than grapevines in the greenhouse (12). In a subsequent report, Panagopoulos (13) found a few grapevine strains that would infect tomato, but still concluded that biovar 3 strains have a much narrower host range than biovar 1 or 2. Süle (20), however, found grapevine strains from Hungary that would initiate galls on sunflower and tomato. The majority of our strains also initiated galls on a range of indicator hosts. Some variability among host range was noted, but when 120 *Agrobacterium* strains isolated from grapevine sap were tested for pathogenicity to sunflower and grapevine, only 10% were specific to grapevine.

We found that our known AT 1 and 2 strains readily initiated galls on potted grapevine. The galls we observed were indistinguishable from those produced by AT 3. Although we observe this apparent lack of host specificity with our strains in the greenhouse, it is interesting that AT 3 is almost the only biovar recovered from grapevine in the vineyard. This suggests there may be a natural selection for AT 3 in grapevines.

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