Rapid Detection of *Agrobacterium tumefaciens* in Grapevine Propagating Material and the Basis for an Efficient Indexing System

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ABSTRACT

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Sap from hardwood stem pieces of five grape cultivars and 10 rootstock selections was collected by a water-displacement procedure and plated on a medium selective for the *Agrobacterium tumefaciens* biovar 3. Of 36 strains of *A. tumefaciens* recovered, 86% were identified as biovar 3 according to their physiological and virulence characteristics; 90% of the apical portions of grape canes sampled were free of *A. tumefaciens*. Our data provide the basis for a simple, yet rapid indexing system for the detection of low populations of unevenly distributed *A. tumefaciens* cells in grape wood. With this procedure, it should be possible to obtain *A. tumefaciens*-free propagating material.

Crown gall, caused by *Agrobacterium tumefaciens* (Smith & Townsend) Conn., is a common disease of grapes (*Vitis* L.) in many regions, e.g., Australia and Eastern Europe (13), South Africa (11), and the United States (3,4). The pathogen induces gall formation on the roots; however, it more seriously affects the aerial parts, girdling the main cane and killing cold-sensitive cultivars (3). Panagopoulos and Psallidas (13) and Panagopoulos et al (14) in Greece, Süle (17) in Hungary, and recently, Burr and Hurwitz (4) and Burr and Katz (5) in the United States have demonstrated that a distinct biovar 3 of *A. tumefaciens* is the usual cause of crown gall of grapevine. Lehoczky isolated *A. tumefaciens* from the xylem exudate of diseased vines (8), from tumor-free vines (9), and from grape root systems (10). He suggested that under moist conditions in the spring, root pressure causes xylem fluids to sweep cells of *A. tumefaciens* from the root system toward wound sites caused internally by low-temperature injury. Tumors then form at these sites. Neither chemical nor biological control for grape crown gall has been effective (6), hence pathogen-free propagating material may reduce the severity of the problem. Grape growers extensively use propagating material from vineyards assumed to be free of the crown gall organism; however, we present data suggesting that much propagating material may be contaminated. This paper describes a procedure for detecting and indexing *A. tumefaciens* in woody propagating material of grapes and proposes a simple indexing procedure.

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MATERIALS AND METHODS

Sample collection. During 1983 and 1984, canes from dormant vines and growing shoots were collected from a vineyard and a nursery in Missouri. The experiments conducted in 1983 were of a preliminary nature through which the indexing procedures were established. Data from samples taken in 1984 from matured (October) or dormant (December) canes are presented. Twenty-six dormant canes 100 cm long were sampled from each of five cultivars: Chancellor, Seyval Blanc, Vidal Blanc, Catawba, and Riesling. In another sampling, 15 dormant canes 100 cm long of Vidal Blanc were obtained from five plants 1 yr after they were started from greenwood tip cuttings. An additional 18 dormant canes 100 cm long were taken from 2-yr-old Chancellor plants from the nursery and from a rootstock collection in Virginia. The rootstocks were collected by Leslie McCombs (Virginia Polytechnic Institute, Horticulture Department Farm, Blacksburg). These too were dormant canes; however, they were about 50 cm long and the position on the plants from which they were taken was not noted.

Collection of vascular fluids from stem segments. All canes were divided into three 12- to 15-cm-long segments: basal, middle, and apical. Each was surface-sterilized by flame after immersion in 95% ethanol. A water-displacement procedure (1) was used to obtain vascular fluids with which sterile distilled water was forced basipetally at 0.1 MPa through the stem segment and fluid was collected at the apical end with a sterile capillary pipette. The first 1.5–2.0 ml of fluid collected was placed in a sterile tube and subsequently examined for *A. tumefaciens* by spreading 0.1 ml of each sample in each of three petri plates containing agar medium selective for biovar 3 (15). The plates were incubated for 5 days at 28 C. Representative colonies from each plate were transferred to YTSA medium (yeast extract 5 g, tryptone 5 g, sucrose 50 g, K_2HPO_4 2 g, and agar 15 g in 1,000 ml of distilled water, pH 6.6) to obtain inoculum for pathogenicity tests.

Pathogenicity tests. Colonies resembling *A. tumefaciens* biovar 3 were streaked on YTSA and examined for purity; however, in most instances, these appeared as pure cultures. To inoculate plants, a heavy smear of 48-hr-old bacteria from a single colony was stab-inoculated with a sterile needle into surface-sterilized stems of tomato (*Lycopersicon esculentum* Mill cv. Rehovot 13), castor bean (*Ricinus communis* L.), and grape cultivar Chancellor. Two plants of each host were inoculated with each isolate in the greenhouse, and evidence of disease development was recorded after 3 wk. Strains that were virulent were further characterized to specific biovar by the determinative procedures of Kerr and Panagopoulos (7). These tests include maximum temperature for growth, action on litmus milk, sodium chloride tolerance, production of ketolactose (2), production of acid from erythritol and melezitose, production of alkali from malonate and tartrate (7), and growth on selective media of Schroth et al (16).

RESULTS

Data from sap collection experiments with dormant cutting wood from a commercial vineyard appear in Table 1. Of the five cultivars examined, pathogenic *A. tumefaciens* biovar 3 was recovered from basal, middle, and apical segments of Chancellor, Seyval Blanc, and Catawba. No bacteria were detected in the apical portions of either Riesling or Vidal Blanc stem segments. Of the vascular fluids of the 26 Chancellor canes examined, two samples were free of *A. tumefaciens*. Samples from four Seyval, six Catawba, 20 Riesling, and 16 Vidal Blanc canes also appeared to be free of the pathogen (Table 1). Low numbers of bacteria were detected in one of five basal segments from Vidal Blanc plants started from greenwood tip cuttings. Canes from six 2-yr-old Chancellor plants started from dormant hardwood cuttings,

Table 1. Isolation of *Agrobacterium tumefaciens* from expressed vascular fluids of dormant grape cuttings

Cultivar	Total cfu/0.1 ml/26 segments[a]		
	Basal	Middle	Apical
Chancellor	786[b]	220	13
Seyval Blanc	490	184	2
Catawba	553	174	9
Riesling	15	3	0
Vidal Blanc	150	63	0

[a] Colony-forming units per 0.1 ml of vascular fluids per 26 segments. Fluid was obtained by forcing sterile distilled water through vessels under pressure (0.1 MPa). The aliquot tested was from 1.0 to 1.5 ml collected from the apical end of each segment.
[b] Of the 26 Chancellor canes examined, 2 were free of *A. tumefaciens* and 4 Seyval Blanc, 6 Catawba, 20 Riesling, and 16 Vidal Blanc were similarly bacteria-free.

Table 2. Isolation of *Agrobacterium tumefaciens* from 2-yr-old grape plants selected at random in a commercial nursery

Cultivar	Propagation method	Total cfu/0.1 ml/segment[a]		
		Basal	Middle	Apical
Vidal Blanc	Green tip cutting[b]	1/5	0/5	0/5
Chancellor	Dormant hardwood cutting[b]	19/6	3/6	1/6

[a] Colony-forming units per 0.1 ml of vascular fluids per segment. Fluid was obtained by forcing sterile distilled water through vessels under pressure (0.1 MPa). The aliquot tested was from 1.0 to 1.5 ml collected from the apical end of each segment.
[b] Vidal Blanc canes were mature (October 1984), whereas Chancellor canes were dormant (December 1984) when sampled.

Table 3. Numbers of *Agrobacterium tumefaciens* cells detected in vascular fluids expressed from dormant grape canes of rootstocks

Rootstock cultivars	Position[a]		
	Basal	Middle	Apical
Teleki 5C	29[b]	11	5.0
101-14	22	10	0.0
3309	25	10	7.0
Cosmos-2	14	11	6.0
Cosmos-10	31	16	0.0
5BB	20	9	0.2
5BBCL-13	33	18	9.0
5BBL-114	34	16	0.7
5C-516	20	0	0.0
420-A	31	13	3.0

[a] Basal, middle, and apical segments of the canes that were indexed.
[b] Average number of colony-forming units per 0.1 ml of vascular fluids expressed from segments of two to five canes per rootstock cultivar.

selected at random from the nursery row, contained bacteria in their vascular fluids, with the basal segments containing the highest number (Table 2). *A. tumefaciens* biovar 3 was also detected in the basal, middle, and apical segments of most rootstock selections (Table 3).

Most colonies that grew on the Roy-Sasser medium (15) were typical of biovar 3 in appearance. Of these, 36 single colonies (one from each of the 36 vascular fluid samples) were selected at random, and 31 were virulent on grape and identified as biovar 3 (Table 4). One isolate was not pathogenic on grape; however, it was positive for acid production from melezitose and negative for alkali from both malonate and tartrate substrates and was considered to be biovar 1. Four isolates were presumably *A. radiobacter*, because they were nonpathogenic on grape, tomato, and castor bean.

DISCUSSION

Our findings indicate that apparently healthy grape cuttings of several cultivars carry *A. tumefaciens* in their vascular elements, presumably in their xylem vessels. Our data confirm reports by Lehoczky (8,9), Burr and Hurwitz (4), and Burr and Katz (5) that the route of systemic movement of *A. tumefaciens* in grape is in the vascular system. In this regard, the movement of *A. tumefaciens* biovar 3 from artificially inoculated roots of vinifera-hybrid Chancellor and the induction of aerial tumors at aseptically wounded stem tissue were ascertained by using a streptomcyin-rifampicin-resistant mutant Ag 63 of biovar 3.

The water-displacement method (1) used in this study for detecting *A. tumefaciens* in propagating material should provide the basis for an indexing system to detect apparently low populations of unevenly distributed *A. tumefaciens* in grape wood. Our results suggest that contaminated, yet symptomless propagating material may be an important, if not the preeminent, means of dissemination of *A. tumefaciens* as others (4,5) have suggested and constitute a major problem in grape propagation and production in general. The systemic spread of *A. tumefaciens* in the vascular system of grapes (4,5,8,9) makes the procedure of removing infected trunks and canes and bringing up new suckers of questionable usefulness for controlling crown gall in grapes. The procedure of bringing up suckers is totally without merit where cultivars are on rootstocks. Nevertheless, grape growers and nurseries still use hardwood cuttings for propagation. In many instances, these are taken from presumably healthy vines (showing no aerial tumors) that may, nevertheless, carry the pathogen.

Failure to detect *A. tumefaciens* in propagating material by the water-displacement procedure described here does not prove that the cutting is bacteria-free. Research in progress continues to index shoots from rooted cuttings that were indexed by the water-displacement procedure in order to develop "mother" plants that are free of crown galls.

The preliminary examination of nursery stock described in Table 2 and the comparatively fewer bacteria in apical segments of hardwood cuttings (Table 1) suggest that greenwood tip cuttings might be satisfactory starting material for propagating grape plants free of crown galls. Finally, our cursory examination of a small collection of rootstocks and the detection of *A. tumefaciens* therein (Table 3) accentuates the need to give careful, individual attention to propagules of grape rootstocks as well as scion wood.

The indexing method used in this study appears practical in application and can be used to rapidly process large numbers of cuttings. The initial indexing procedure takes 3 wk from time of sampling to proof of pathogenicity of isolated bacteria on grape plants. The selective medium used (14) proved ideal for direct isolation of biovar 3 from dormant cuttings.

Table 4. Characteristics of 36 strains of *Agrobacterium tumefaciens* obtained from vascular fluids expressed from hardwood grape cuttings

Characteristics	Biotypes[a] 1	2	3	Grape strains[b]
Physiological				
Growth on media of				
Roy and Sasser (15)	–[c]	–	+	36
New and Kerr (12)	–	+	–	0
Schroth et al (16)	+	–	–	5
Growth at 35 C	+	–	+	36
2% NaCl tolerance	+	–	+	36
3-Ketolactose	+	–	–	1
Litmus milk	Alk.	Acid	Alk.	Alk.
Acid production from				
Erythritol	–	+	–	0
Melezitose	+	–	–	5
Alkali from:				
Malonate	–	+	+	5
Tartrate	–	+	+	5
Phytopathological				
Pathogenicity on				
Grapevine	–	–	+	31
Tomato	+	+	+	29
Castor bean	+	+	v	27

[a] Characterization tests for biovars 1, 2, and 3 were performed in our laboratory with previously identified strains 1 (Ag 5) and 2 (Ag 222) supplied by C. G. Panagopoulos (Athens, Greece) and 3 (16/6, Ag 63) by S. Süle (Hungary).
[b] Number of positive isolates of 36 tested.
[c] + = Positive, – = negative, and v = variable.

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