Characterization of *Agrobacterium* Isolates from Muscadine Grape

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ABSTRACT

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The systemic presence of a host-restricted strain of *Agrobacterium tumefaciens* in muscadine grape (*Vitis rotundifolia*) was confirmed by means of a detached leaf assay procedure using *Agrobacterium*-free plants. Assays of muscadine plants representing vineyards from throughout the region indicated a widespread systemic presence of *A. tumefaciens* in roots and vascular fluids of shoots of symptomatic and asymptomatic plants in Mississippi. Not all isolates from muscadine were clearly classified as biovar 1, 2, or 3. Biovar 3 appeared to predominate among isolates from roots and vascular fluids, but the number of biovar 1 and biovar 3 isolates from galled tissues was almost equal. High percentages of biovar 1, biovar 3, and unclassified isolates were pathogenic.

Additional keywords: *Vitis vinifera*

Crown gall, caused by *Agrobacterium tumefaciens* (E.F. Smith and Townsend) Conn, commonly occurs on *Vitis vinifera* L. and other *Vitis* spp. and is recognized as a notable problem in grape culture internationally. This disease in grape has historically been attributed primarily to *A. tumefaciens* biovar 3 (AT-3), although *A. tumefaciens* biovar 1 (AT-1) has also been implicated (4,5). A sinister aspect of crown gall of grape is that the pathogen may occur systemically in symptomless plants and thus be transmitted by vegetative propagation practices, a fact that perhaps accounts for its present widespread occurrence (5,14,15,21). Certain predisposing factors have been suggested as instrumental in gall development on systemically infested plants, most notably early spring sap rise followed by freezing conditions (15,21). Prior to 1985, however, this disease was not considered to be a significant problem on muscadine grape (*V. rotundifolia* Michx.).

Galling was first noted on muscadine in Mississippi in 1985 in a planting of breeding lines (Georgia 15-5-3 and Georgia 23-45) at the Truck Crops Branch Experiment Station, Crystal Springs, where incidence was nearly 100%. Subsequently, the disease was observed at other locations throughout the state on a number of popular muscadine cultivars. Galls were found most often at the base of the plants but occurred frequently along the length of the cane and sometimes along the cordons. Pathogenicity of *Agrobacterium* isolates obtained from galled plants was shown by inoculating wounded canes of rooted cuttings (8,11). Although galls developed on muscadines inoculated with *Agrobacterium* isolates from muscadine, they sometimes developed on injured, but uninoculated, muscadine check plants. Preliminary assays of symptomless plants being used as sources of cuttings for propagation indicated that a large percentage was systemically infested with *Agrobacterium* spp. Subsequent surveys suggested that most, if not all, commercially grown muscadines are systemically infested with agrobacteria (10,12,13).

With one exception, galling was not induced by muscadine isolates in a number of other artificially inoculated plant species, including those most commonly used in crown gall host range studies (13). This exception was meristem-cultured, *Agrobacterium*-free

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cv. Orlando Seedless grape, wherein galls were produced by inoculation with muscadine isolates (13). Orlando Seedless is a Florida hybrid with parentage from *V. vinifera* and native American grape species. This observation suggests that muscadine isolates of *Agrobacterium* may be pathogenic to other grape species, but further confirmation must be done with known *Agrobacterium*-free plants.

The purpose of this study was to develop *Agrobacterium*-free muscadine plants and to determine: 1) the presence and character of pathogenic agrobacteria in muscadine and 2) the incidence of pathogenic forms of agrobacteria in symptomless and galled commercial muscadine plantings. Preliminary abstracts have been published elsewhere (23,24).

MATERIALS AND METHODS

Development of *Agrobacterium*-free muscadine plants. The methods of Tarbah and Goodman (21) were used to screen 900 rooted cuttings of muscadine from symptomless shoots taken from vineyards. After each of three screenings, plants yielding isolates of *Agrobacterium* were discarded. Unfortunately, after further screening and study, the few remaining plants were also found to be positive (C. H. Graves, Jr., *unpublished*).

A number of meristem or shoot-tip tissue culture methods were tested in an effort to produce *Agrobacterium*-free muscadines, beginning with methods used successfully in previous studies of tissue culture of grapes (9). Although a few muscadine plants were propagated by these methods, acceptable shoot proliferation was difficult to achieve. However, a sufficient supply of *Agrobacterium*-free plants was obtained to permit successful pathogenicity assays for this study. These plants produced by meristem micropropagation were assayed and found to be free of *Agrobacterium*, using methods described by Tarbah and Goodman (21). Leaves were further checked by placing leaf disks or spreading surface-sterilized leaves crushed in sterile distilled water on *Agrobacterium*-selective media, modified New and Kerr (NKS) (4,18) and Roy and Sasser (RS) (19). A more successful meristem tissue culture procedure has since been developed (22).

Sources of *Agrobacterium* isolates. *Agrobacterium* spp. used in this study were randomly selected from a collection of over 500 cryopreserved isolates obtained from various muscadine cultivars in vineyards throughout Mississippi. These included isolates from roots and vascular fluids of shoots from symptomless plants and from galled tissues.

Isolates of *Agrobacterium* were obtained from the vascular fluids of current-season muscadine shoots using methods described by Tarbah and Goodman (21), i.e., the water pressure method of Bennett et al (2) for extracting vascular fluids. Sources from roots were isolated by surface-sterilizing the tissue in 1% NaOCl (20% bleach) for 15 min, rinsing in sterile distilled water, and cutting horizontally with a sterile scalpel as described by Burr et al (6). The cut surface was streaked on NKS and RS media. Additionally, root samples were assayed by placing surface-sterilized, rinsed, and chopped roots in sterile distilled water in a Waring blender at high speed for 1 min (6). Platings of resultant water samples were made on NKS and RS media. Isolates from galls were obtained by dissecting the gall from the trunk or cordon, then surface-sterilizing, rinsing in sterile water, and grinding the gall tissue in a sterile Waring blender for 5 min (1-min bursts) or with a mortar and pestle. The ground tissue was diluted and plated on NKS and RS media.

After being streaked for purity, all cultures were stored in nutrient broth plus 10% glycerol at −80 C. These cryopreserved cultures were recovered by scraping cell suspensions with a sterile loop and inoculating nutrient glucose (2.5%) agar (NGA) plates. All cultures were incubated at 28 C.

Isolates used as positive controls included CG49 and Ag57-81 (AT-3 strains) from *V. vinifera*, obtained from T. J. Burr, New York State Agricultural Experiment Station, Cornell University, Geneva; and Ag63 (AT-3) from *Prunus amygdalus* Batsch and FACH (AT-1) from *V. vinifera*, obtained from R. N. Goodman, University of Missouri, Columbia. *A. radiobacter* used as negative controls included K84, also obtained from R. N. Goodman, and ATCC 31700, acquired from the American Type Culture Collection, Rockville, Maryland.

Detached leaf pathogenicity assay procedure. Muscadine leaves were collected from greenhouse-grown *Agrobacterium*-free plants produced by meristem tissue culture procedures, transported to the laboratory, and stored at 4 C. Petioles were trimmed to about 20 mm. Leaves were soaked in sterile water for about 10 min, then surface-sterilized as follows: 0.05–0.1% Ivory detergent, 1 min; 70% ethanol, 3 min; 1% NaOCl, 3 min; two rinses in sterile double-distilled water, 2 min each; sterile double-distilled water plus 0.05% Tween 20, 2 min; 1% sterile ascorbic acid plus Tween 20, 2 min; and 1% sterile ascorbic acid, 2 min. Leaf edges were trimmed to fit leaves into petri dishes, and petioles were trimmed to about 5–7 mm in the presence of the ascorbic acid solution. The leaves were inoculated and aseptically placed on the surface of petri plates containing agar. Water agar and woody plant medium (WPM) (16) with modified soft agar (0.05% [w/v] agar plus 0.2% [w/v] Gelrite) were compared for maintainence of the leaves. Incubation under light was found to be necessary to maintain leaves on water agar, whereas leaves on WPM could be incubated in dark or light. The WPM procedure was judged to be superior and was used for this study.

Inoculation procedure. Bacterial cultures acquired from cryopreserved (−80 C) cell suspensions were subcultured on NGA plates at 28 C for 24 hr, then stored at 4 C until used. To prepare inoculum, fresh nutrient glucose broth (25 ml/125-ml flask) was inoculated with one loop of bacterial culture and incubated with rotary aeration for 18 hr at room temperature. Approximately 0.05 ml of inoculum from such an overnight culture was injected into the base of each petiole. At least three leaves per isolate per cultivar (cvs. Carlos and Summit)

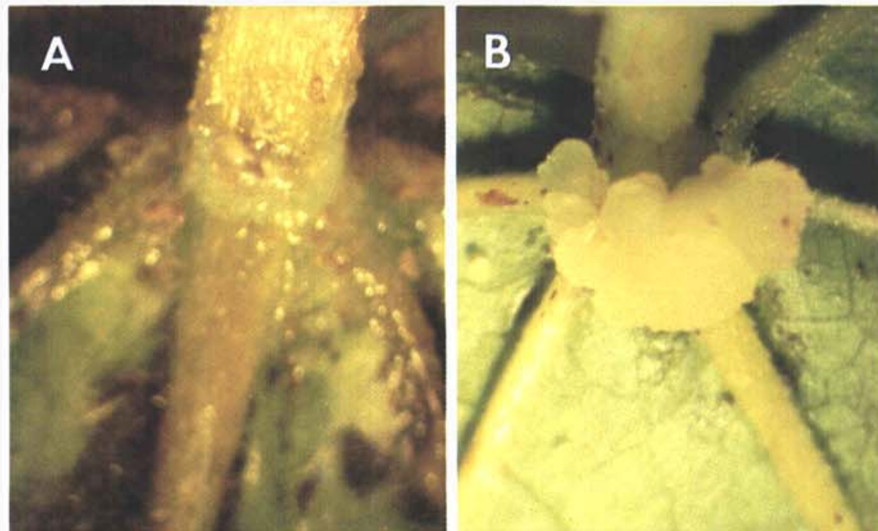

Fig. 1. Detached leaf assay method for determining pathogenicity of *Agrobacterium* isolates from muscadine: **(A)** Representative *Agrobacterium*-free muscadine leaf inoculated with a negative control, *A. radiobacter*, and **(B)** leaf inoculated with an isolate of *A. tumefaciens* from muscadine.

were inoculated in each study.

Controls for each experiment included inoculation with sterile nutrient glucose broth in the same manner as with bacterial suspensions. The FACH AT-1 strain was chosen as the positive control for inclusion in each experiment because it consistently caused gall formation on the detached muscadine leaves. For comparison, the ATCC 31700 *A. radiobacter* strain was chosen as a negative control.

Plates with inoculated leaves were wrapped with Parafilm and incubated for 3 wk at 26–28 C with a 16-hr photoperiod. Beginning at 3 wk, leaves were checked weekly for galling at the site of inoculation.

Galling produced by muscadine isolates was compared to controls in serially conducted experiments, each experiment including no more than 12 test isolates. Isolates were classified as pathogenic or nonpathogenic on the basis of the presence or absence of gall formation at the site of inoculation (Fig. 1). Normal callus formation was observed occasionally on the cut petiole ends, but this white, friable callus was distinctive from the more compact, often olivaceous *Agrobacterium*-induced galls at the site of inoculation. Confirmatory reisolations were made.

The detached leaf pathogenicity assay method was validated by repeating the procedure at least three times with 40 of the isolates used in this study.

Biovar classification. Biovar classification of *Agrobacterium* isolates from muscadine plants was determined first by growth of the isolates on New and Kerr, NKS, RS, and Schroth's (20) media. Additional diagnostic tests, as described by Moore et al (17), included: growth in litmus milk, sodium chloride tolerance, production of 3-ketolactose, acid production from erythritol or melezitose, alkali production from malonate, and growth on nutrient agar at 35 C. Classifications were repeated for all isolates in this study, using selective media. When biovar classification was in question, the above tests were repeated and the mucic acid test (17) was added.

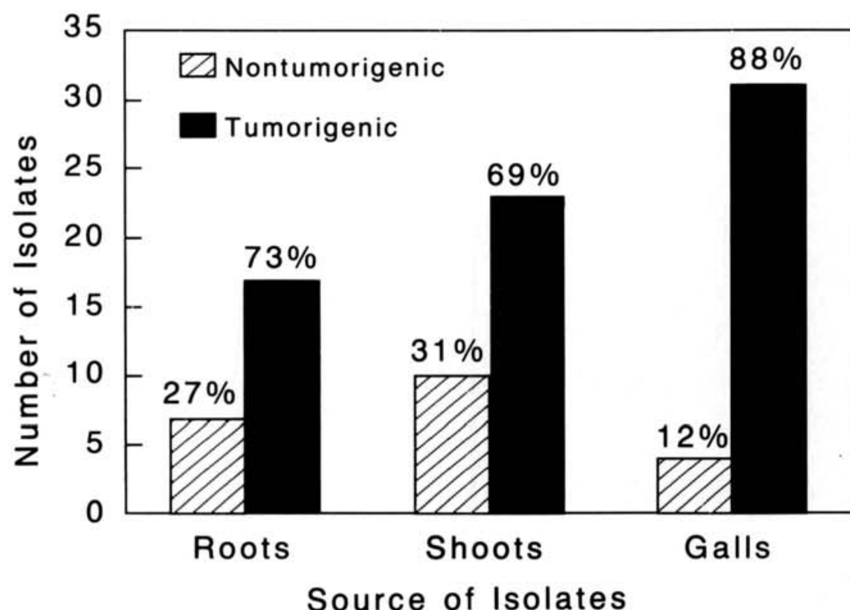

Fig. 2. Pathogenicity of *Agrobacterium* isolates from muscadine determined by a detached leaf assay. Isolates from roots and vascular fluids of shoots were from symptomless plants.

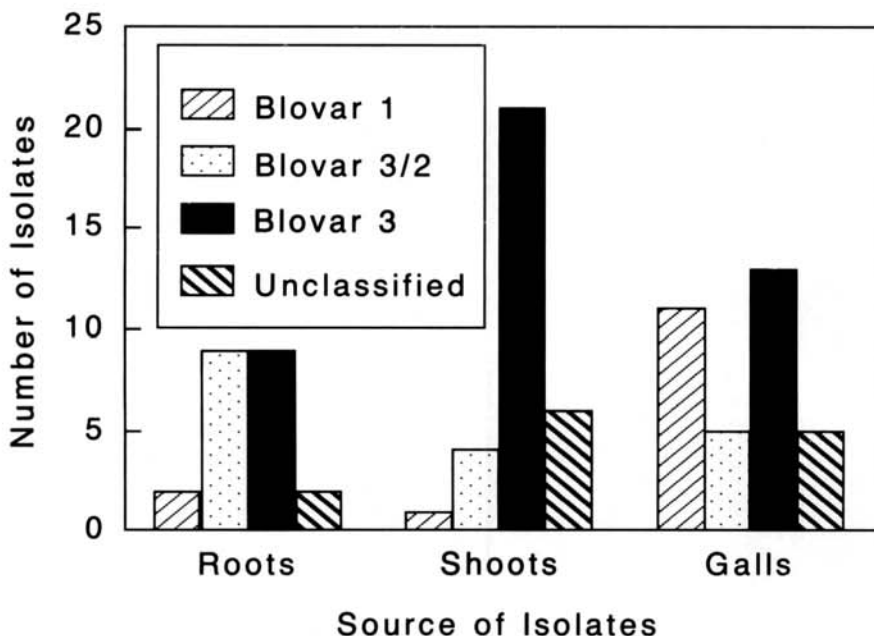

Fig. 3. Biovar classification of *Agrobacterium* isolates from muscadine. Isolates designated as 3/2 showed positive characteristics for biovar 3 but also attributes of biovar 2, such as growth on erythritol and New and Kerr medium. Unclassified isolates were positive in at least one of each of the three biovar classification tests.

RESULTS AND DISCUSSION

Pathogenicity screening. Of 88 muscadine *Agrobacterium* isolates evaluated by means of the detached leaf assay method, 77% caused gall development. No galling was observed on controls inoculated with sterile nutrient broth or on negative controls inoculated with *A. radiobacter*. Galling was present on the positive controls inoculated with the FACH AT-1 strain in each experiment. Of the 54 isolates from roots or vascular fluids of shoots from symptomless plants, 71% initiated galls.

These results, the failure to find *Agrobacterium*-free plants among the 900 rooted cuttings screened, and previous findings relative to the apparent universal systemic *Agrobacterium* presence in muscadine (10,12,13) hold particular significance in light of an increased interest in muscadine grape culture in the southeastern United States. Much of this region experiences weather conditions, namely, late spring freezes following sap rise, considered conducive for crown gall development (15,21). Therefore, developing a system for providing the muscadine industry with a means of producing *Agrobacterium*-free plants is important. Use of vines free of *A. tumefaciens* as an approach to control has been proposed by Tarbah and Goodman (21) and Burr et al (5,7). Work by Burr et al (6) and Bishop et al (3) supports the idea that *A. tumefaciens*-free plants established in nonvineyard soils may remain free of the crown gall pathogen. Nevertheless, the goal of providing disease-free plants for vineyard establishment seems logical and could perhaps be achieved by one of two ways. First, since muscadines are customarily propagated by rooting cuttings, the establishment of an *A. tumefaciens*-free foundation planting as a source of cuttings for the industry may be an option (23). Second, a system of meristem culture as mentioned above has potential as an

efficient means for commercial propagation (22).

The percentages of pathogenic isolates from roots (73%) and vascular fluids of shoots (69%) from symptomless plants were essentially the same, whereas 88% of the isolates from active galls proved pathogenic (Fig. 2). Isolates providing a negative response (no gall formation) in the detached leaf assay system were assumed to be *A. radiobacter*.

In preliminary comparisons of AT-3 isolates from other species (CG49, Ag57-81, and Ag63), galls were produced on the detached leaves of *Agrobacterium*-free muscadine, suggesting that isolates from other species are infectious on muscadine.

Biovar classification. Of the 88 muscadine isolates evaluated by selective media and biochemical tests, 43 were classified as biovar 3, 14 as biovar 1, and 18 as biovar 3/2 (positive characteristics for biovar 3 but also attributes of biovar 2, such as growth on erythritol and New and Kerr medium). Thirteen isolates were positive in at least one of each of the three tests for biovar classification and therefore could not be classified.

When isolates were compared as to source, biovar 3 predominated among those from vascular fluids and equaled the number of biovar 3/2 from roots. Few biovar 1 isolates were obtained from roots and vascular fluids, but the number of biovar 1 isolates from galls almost equaled the number of biovar 3 isolates (Fig. 3).

Thirteen of 14 biovar 1, 32 of 43 biovar 3, and 14 of 18 biovar 3/2 isolates were shown to be tumorigenic in the detached leaf assay. Of the 13 unclassifiable isolates, nine were tumorigenic and four were not.

The presence of AT-1 as well as of AT-3 in muscadine is a point of interest. Our data suggest that biovar 1 is a prominent part of the *A. tumefaciens* presence in muscadine, which is in contrast to the consensus of findings of those working on wine and table grapes. Further comparative host range studies with isolates representing biovars 1 and 3 from muscadine are needed. Confirmation of host specificity for both would further suggest that the bacterium should be regarded as a "host-vector" for the tumor-inducing (Ti) plasmid and that perhaps the presence of the Ti plasmid should not enter into taxonomic considerations for the bacterium, as has been suggested (1).

In summary, the presence of *A. tumefaciens* in muscadine is confirmed. More important, the sampling suggests a widespread systemic presence of *A. tumefaciens* in symptomless plants and in both root and vascular fluids of shoots of current-season growth, indicating a need for propagation systems to produce *Agrobacterium*-free plants for new vineyard establishment.

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