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Endophytic *Agrobacterium* in crown gall-resistant and -susceptible *Vitis* genotypes

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Summary: Several methods were used to study endophytic colonization of *Vitis* genotypes by *Agrobacterium vitis* (AV). AV was seldom detected except at inoculated sites, indicating little systemic movement of the bacterium under the conditions of these experiments. AV populations at inoculated sites were evaluated for 10 months following inoculation of crown gall-resistant and -susceptible genotypes. Two months after inoculation, *V. amurensis* selections had significantly smaller populations than *V. vinifera* (Cabernet Sauvignon) or *V. riparia* x *V. rupestris* (C3309). All crown gall-resistant genotypes had significantly lower populations than Cabernet Sauvignon 10 months after inoculation. Examination of vines one year after inoculation indicated that AV populations were much higher at inoculated sites when crown galls developed. However, even when no galls were apparent, Cabernet Sauvignon had significantly higher AV populations than *V. amurensis* 689 (6-fold higher) and C3309 (70-fold higher). Crown gall-resistant genotypes appear to support lower populations of AV than the crown gall-susceptible Cabernet Sauvignon. Freezing followed by a two-day incubation significantly increased recovery of *Agrobacterium* using vascular fluid displacement in naturally-infected and artificially-inoculated vines and therefore increased the sensitivity of indexing for AV in grapevines.

Key words: Vitis, Agrobacterium, crown gall, systemic development, endophytic bacteria.

Introduction

Crown gall is an important disease of grapevines worldwide and is especially severe in regions where vineyards experience freeze injury (BURR 1988). When crown gall is severe, vine vigor and productivity can be significantly reduced (SCHROTH *et al.* 1988) leading to substantial mortality of young vines in cold winter regions (DHANVANTARI 1983). The strains of *Agrobacterium* associated with most grape crown galls are distinct from other crown gall pathogens and had been grouped together as *Agrobacterium tumefaciens* (AT) biovar 3 (KERR and PANAGOPOULOS 1977, SÜLE 1978, BURR and KATZ 1984). These strains are now characterized as *Agrobacterium vitis* (AV), separated from other *Agrobacterium* species by differences in DNA homology and metabolic characteristics (OPHEL and KERR 1990).

AV has been shown to exist systemically within grapevines (LEHOCZKY 1968, BURR and KATZ 1984) in some cases even when no crown gall symptoms are evident (BURR and KATZ 1983, SÜLE 1986). The *Agrobacterium* population in vines is reported to progressively decrease distal to the roots, except in the vicinity of galls (TARBAH and GOODMAN 1986). AV was not detected in green shoots of grape using standard methods (BURR *et al.* 1988). However, dormant first year canes used for propagation can be systemically infected (GOODMAN *et al.* 1987) contributing to widespread distribution of *Agrobacterium* in grape nursery stock. Examination of soils for *Agrobacterium* in three areas of the United States found AV in vineyard soils but not in nonvineyard soils (BURR *et al.* 1987). Together these observations suggest that the initial source of AV in vineyard soils may be contaminated nursery stock. Use of techniques to index grape propagation material (GOODMAN *et al.* 1987) or produce 'clean' plants through shoot tip culture (BURR *et al.* 1988) can provide planting material which could dramatically decrease crown gall at new vineyard sites. However, when 'clean' grapevines are planted on soil containing populations of tumorigenic AV they can become quickly invaded by AV (BISHOP *et al.* 1988).

Use of rootstocks that reduce passage of AV from infested soil into susceptible scions may significantly diminish the incidence of crown gall when clean planting material is used on sites where grapes have been grown. This report is part of a series of experiments directed toward this goal. In earlier reports, genotypes resistant to several aspects of crown gall pathogenesis were identified (STOVER *et al.* 1996, 1997 a, b). The purpose of this study was to characterize endophytic development and vascular movement of AV in crown gall-susceptible and -resistant grape genotypes.

Materials and methods

Plant material: *Vitis* genotypes for laboratory and greenhouse studies were obtained from the USDA *Vitis* Germplasm Repository in Geneva, NY, USA (*V. amurensis* 689, *V. amurensis* 1295, *V. cinerea* 236, *V. solonis* x *V. amurensis* 725), the Foundation Plant Material Service in Davis, CA, USA (*V. riparia* x *V. rupestris* C3309, *V. riparia* x *V. rupestris* 101-14 Mgt, *V. vinifera* Cabernet Sauvignon, *V. berlandieri* x *V. rupestris* P775), and from Dr. Dennis Gray of the University of Florida (*V.* complex hybrid Blanc du Bois, *V. labruscana* x *V. rufotomentosa* Tampa,

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V. cordifolia, *V. tiliafolia*). Accession numbers are included for selections from the USDA *Vitis* Germplasm Repository. Cuttings were rooted under mist and were maintained in soilless mix (Pro-Mix BX™, a proprietary blend composed principally of peat moss, perlite, and vermiculite) in the greenhouse throughout each experiment. Plants were maintained at 18-27 °C and received supplemental lighting when grown during the fall and winter. Plants used in an existing vineyard for one experiment were obtained from the New York Fruit Testing Association.

Inoculation with AV: Tab. 1 outlines the origins and identities of AV strains used in this study. Strains were maintained at -80 °C in cryogenic-storage medium (nutrient broth containing 22.5 % (w/v) glycerol). Two days prior to inoculation these cultures were streaked onto potato dextrose agar (PDA) (Difco) and grown at 28 °C. Colonies from PDA were suspended at about 10^9 colony forming units (cfu) per ml in sterile deionized water (SDW). Actively growing young plants in the greenhouse were inoculated after the lower portion of stems developed a brown periderm. Typically shoots were 1 to 2 m long at the time of inoculation. Inoculated sites were chosen to include nodes that were still green and those with a brown periderm. 5 µl of bacterial suspension were applied to 1 mm diameter holes drilled through shoot nodes with typically 5 sites in green tissue and 5 in mature tissue for each vine. Inoculated sites were wrapped in parafilm and plants were grown in the greenhouse.

Isolation and identification of *Agrobacterium*: Vines were tested for the presence of endophytic AV at various times after inoculation. Several different methods for sampling AV populations were used; they are described below. In all cases, samples were dilution-plated on modified ROY and SASSER (RS) medium which is semi-selective for *Agrobacterium* (BURR *et al.* 1987). *Agrobacterium* in general and AV in particular can be identified on RS by characteristic colony morphology. Colonies which were convex and slightly mucoid, with red centers and a narrow white margin after 4 d at 28 °C were identified as AV (BURR *et al.* 1987). Where noted, colonies which had the characteristic appearance of *Agrobacterium* on RS were subcultured on potato dextrose agar (Difco) and were challenged with an AV-specific monoclonal antibody using an immunoblot procedure (BISHOP *et al.* 1989). One colony of each morphology consistent with *Agrobacterium* was tested from each plate.

Sampling *Agrobacterium* in sap exuded through natural root pressure: In two experiments, natural root pressure exudate ("bleeding sap") was collected from freshly pruned vines and assayed for presence of *Agrobacterium* spp. and AV. This procedure was used with actively growing vines two months after inoculation with single strains or mixtures of AG57, NW180, CG471, CG612, and 339-6. Additional vines subjected to the same inoculations were tested after emerging from dormancy. These vines were grown in the greenhouse for 8 months after inoculation and transfered to a 4 °C chamber through the winter. Vines were moved outdoors in the spring when there was no threat of freezing. To collect exudate, canes were cut 20 cm above the most distal inoculated site and supported so that bleeding sap could be collected in sterile tubes.

Sampling *Agrobacterium* through homogenizing nodal tissue: Both inoculated nodes and nodes adjacent to inoculated nodes were homogenized as another method of quantifying endophytic AV. For each sample, 1 cm of shoot on either side of the tested node was excised and macerated with a razor blade. Tissue pieces were placed in 3 ml of SDW and homogenized using a polytron (Brinkman Instruments). Samples were processed in thin glass tubes on ice with intermittent cooling and homogenization to minimize heating.

In experiments evaluating AV population development at inoculated nodes, 2 plants of each genotype were assayed at each time point with 2 samples (each representing 2 inoculated nodes) assayed for each plant, except for evaluations 10 to 12 months after inoculation when 4–6 samples were evaluated per plant.

Table 1

Agrobacterium vitis strains used in this study

Strain	Source[a]	Plasmid type	Isolator
CG230	sap, Cabernet Sauvignon[b], NY	vitopine[d]	T. BURR
CG471	roots, Sauvignon blanc[b], WA	nopaline[d]	T. BURR
CG612	roots, Pinot noir[b], NY	unknown	T. BURR
339-6	gall, Kerner[b], Spain	unknown	M. LOPEZ
NW180	gall, Riesling[b], Germany	octopine[e]	E. BIEN
K1059	cutting, K5140[c], Australia	octopine[f]	T. BURR
AG57	gall, Crete	octopine, LHR[g]	C. PANAGOPOULOS

[a] grape tissue from which isolated, and state or country where isolated
[b] cultivars of *Vitis vinifera*
[c] rootstock hybrid of *V. champinii* x *V. riparia*
[d] OTTEN *et al.* (1996)
[e] SCHULTZ *et al.* (1993)
[f] MATSUMOTO *et al.* (1992)
[g] LHR = limited host range; PANAGOPOULOS and PSALLIDAS (1973)

Sampling *Agrobacterium* through vascular fluid displacement: In several experiments endophytic AV was measured in inoculated vines by displacing (flushing) vascular fluid from shoots using SDW as described by GOODMAN *et al.* (1987). 1 ml of SDW was flushed through each cutting. To measure AV that had systemically moved from the inoculation sites, shoots were sampled just above the highest (distal to the roots) inoculated node. These shoots were cut so that the node above the highest inoculated node was the base of the sample. Recovery of AV from inoculated nodes using this technique was also tested. In these experiments, shoots were cut so that the basal node in the sample tissue was the highest inoculated node.

The effect of freezing cuttings on the recovery of *Agrobacterium* through vascular fluid displacement was tested. After an initial flushing, shoots were either frozen (at -20 °C or through application of crushed dry ice) or left unfrozen. Shoots were incubated 48 h at 28 °C before being flushed again. Similar experiments were also conducted with canes from a crown gall-infested vineyard. Canes were collected from dormant Chardonnay and Cabernet Sauvignon vines and C3309 root suckers in February and were kept at 4 °C until being tested in May. With these shoots the effect of flushing immediately after freezing was also tested. Other cuttings of Chardonnay and C3309 were collected in October before freezing temperatures were recorded and were evaluated without storage.

Results and Discussion

Evaluation of *Agrobacterium* in sap exuded through natural root pressure: We initially tested the possibility of analyzing sap from inoculated vines to screen genotypes for suppression of systemic AV development. Eighteen plants representing 11 *Vitis* genotypes were tested for *Agrobacterium* in sap from pruning cuts two months after inoculation with AV strains. Two of the plants yielded colonies typical of *Agrobacterium* on the semi selective modified RS medium (BURR *et al.* 1987). However, one of 7 uninoculated vines tested also yielded *Agrobacterium*. Presence of vascular endophytes, including *Agrobacterium*, before vines were inoculated, complicates the interpretation of results throughout these experiments.

The next group of vines were taken through dormancy before assaying sap for AV, since systemic AV has been frequently recovered in vines as they emerge from dormancy (LEHOCZKY 1968, BURR and KATZ 1983, GOODMAN *et al.* 1987), and few of the actively growing vines in the first experiment displayed systemic AV. During pruning sap from fresh cuts of 5 *Vitis* genotypes inoculated with each of 4 AV genotypes and an SDW control were collected as plants emerged from dormancy. Most vines that were inoculated with AV strains had crown galls at one or more of the inoculation sites, and large galls were present on Cabernet Sauvignon. Undiluted sap from 34 % of the plants produced colonies characteristic of *Agrobacterium* on RS but only 5.3 % (two plants) yielded colonies that reacted with the antibody specific to AV. Colonies which did not react with the antiserum did not have the morphology typical of AV on RS medium and all known AV strains tested with this antibody have reacted positively, so it is unlikely that AV isolates went undetected. Infrequent recovery of AV in sap of heavily galled plants indicated that xylem transport of AV did not readily occur under our experimental conditions and evaluation of AV in sap would be a poor method for comparing systemic development in grape genotypes. We suspect that recovered *Agrobacterium* colonies which were not AV are nontumorigenic strains of *A.tumefaciens* biovar 1 which are frequently recovered from grapevines (BURR *et al.* 1987, BISHOP *et al.* 1988). No consistent difference was seen between crown gall-resistant (C3309, 101-14 Mgt, and *V. amurensis* 689) and -susceptible (Cabernet Sauvignon and P775) genotypes in this experiment. Response by strain was variable but some plants inoculated only with SDW also produced colonies of *A. tumefaciens*. Interestingly, the two plants found to have AV in sap were both inoculated with K1059. In an earlier report, this strain induced significantly more disruption of *Vitis* stem tissue than other strains studied (STOVER *et al.* 1997 b). Of the two AV-positive vines, one was a highly crown gall-susceptible Cabernet Sauvignon and the other a crown gall-resistant C3309 vine (STOVER *et al.* 1997 a).

Evaluation of systemic *Agrobacterium* movement using tissue homogenates: Experiments in which root pressure-induced sap was assayed suggested that AV did not readily move through the xylem elements in the plants that were examined. This technique only detects free AV in vascular fluid so bacteria that are attached or in other tissues may remain undetected. Therefore, samples of homogenized plant tissue were assayed. Nodal tissue was tested for AV in all studies using homogenates since C. BAZZI (University of Bologna, Italy, personal communication) has determined that AV concentrates at the nodes in grapevines.

Four months after inoculation with CG230 and NW180, vines were assayed for endophytic AV by homogenization of nodal tissue. Highly crown gall-susceptible genotypes with large galls (Cabernet Sauvignon and Tampa) were tested along with crown gall-resistant genotypes C3309, 101-14 Mgt, *V. amurensis* 689, and *V. amurensis* 1295 (STOVER *et al.* 1997 a). No consistent differences were seen between resistant and susceptible genotypes (data not shown). Of all inoculated nodes tested, 79 % produced colonies typical of *Agrobacterium* species on modified RS medium, but only 12 % of inoculated nodes produced colonies that reacted to antibody specific to AV. When nodes apical to inoculated nodes were tested, *Agrobacterium* spp. were recovered from 46 % of samples 2 nodes beyond inoculated nodes, 31 % of nodes 3 nodes above inoculated nodes, and 9 % of nodes 5 nodes beyond inoculations. However, no colonies were identified as AV by immunoblot except those from nodes which had been inoculated. Colonies were tested repeatedly with antibody after further isolation to verify this conclusion. It is interesting that colonies of *Agrobacterium* spp. and bacteria other than *Agrobacterium* were recovered much more frequently from inoculated nodes than from other nodes

(data not shown). It is possible that wounding the plants during inoculation encouraged proliferation of opportunistic bacterial species including endophytic *Agrobacterium*. As in the sap analyses, this experiment indicated little systemic development of AV under the conditions studied.

Evaluation of AV development at inoculated sites: Endophytic development of AV at inoculation sites on different *Vitis* genotypes was studied because of the apparent lack of systemic movement in artificially-inoculated vines. AV populations were measured in crown gall-susceptible and -resistant *Vitis* genotypes at several time points after inoculation with strain K1059 (Figure). Numerous typical AV colonies were consistently observed on RS following isolation from inoculation sites. An increase in AV was observed in most *Vitis* genotypes 2 weeks after inoculation, similar to a transient peak reported by Bauer *et al.* (1994) when monitoring AV in Riesling and Müller-Thurgau. After one month, all genotypes still contained an average of at least 20 % of the original inoculum population. The *V. amurensis* selection 1295 had significantly smaller AV populations than were found in Cabernet Sauvignon at all time points from 2 weeks after inoculation while AV populations in *V. amurensis* 689 were significantly smaller than those in Cabernet Sauvignon from 2 months after inoculation. All of the resistant genotypes studied (*V. amurensis* 689 and 1295, C3309 and 101-14 Mgt) had significantly lower AV populations at inoculated sites than Cabernet Sauvignon at 10 months after inoculation. The seasonal fluctuation in AV population observed by Bauer *et al.* (1994) was not seen in this study, possibly because vines were grown in the greenhouse rather than exposed to external environmental conditions.

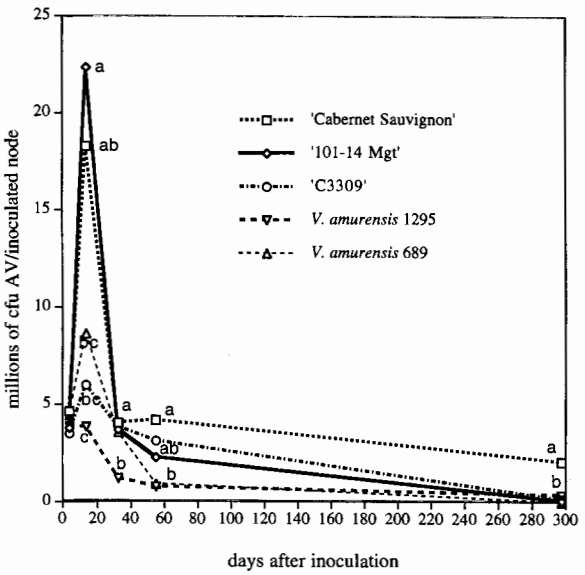

Figure: Population dynamics of *Agrobacterium vitis* at inoculated nodes in crown gall-resistant and -susceptible *Vitis* genotypes. Each node was initially inoculated with $5x10^6$ colony forming units of K1059. Nodes were homogenized at indicated times after inoculations and AV populations were estimated by plating samples on RS medium and counting colonies with AV morphology. Genotypes inoculated were the crown gall-resistant *Vitis amurensis* 1295, *V. amurensis* 689, 101-14 Mgt., C3309, and the crown gall-susceptible Cabernet Sauvignon. Means at each time point marked with the same letter were not significantly different: p=0.05.

Crown gall tissue was excised and analyzed separately when galls were apparent at inoculated sites. At 2 months after inoculation, galls from Cabernet Sauvignon contained 2–11 % of the *Agrobacterium* present at the inoculated sites. Most of the gall tissue was dead 10 months after inoculation, however, one Cabernet Sauvignon gall remained alive and contained 50 times as many *Agrobacterium* colony forming units as the rest of the tissue from that node ($2x10^8$ *vs.* $4x10^6$).

Colonies from homogenized nodes were very uniform in appearance at the first two sampling times. By 5 weeks after inoculation, plates from some 101-14 Mgt, C3309, and Cabernet Sauvignon nodes included from 1 to 10 % colonies larger than AV but with morphology typical of *Agrobacterium*. These probably represent endophytic nontumorigenic AT biovar 1 colonies. All genotypes except C3309 regularly produced putative AT1 and other bacterial colonies at populations similar to AV by 10 months after inoculation.

Populations of K1059 were determined at inoculated sites after one year in another set of vines. In this experiment, Cabernet Sauvignon and some *V. amurensis* 689 produced galls that were still alive after one year. The effect of gall formation on AV population was compared in this material (Tab. 2). C3309, which formed no galls, had much lower AV populations at inoculated sites than the other genotypes. When all inoculated nodes were compared, *V. amurensis* 689 had 12 % of the AV populations observed in Cabernet Sauvignon. Despite the much greater gall formation in Cabernet Sauvignon, when only nodes without visible galls were compared, Cabernet Sauvignon contained AV levels 6-fold greater than *V. amurensis* 689. For nodes

Table 2

Analysis of *Agrobacterium vitis* (AV) populations in inoculated *Vitis* nodes (AV/node) one year after inoculation with $5x10^6$ cfu of K1059. Populations were compared: at all nodes of each genotype, at nodes where no galls formed, and at nodes where galls formed. When galls were present they were cut away from node tissue and assayed separately. Figures indicate colony forming units (cfu $x10^3$)

	Mean values		
Genotype	for all nodes including gall tissue	for nodes when no gall formed	when gall is removed from node
Cabernet Sauvignon			
Mean	9198	675	2794
Standard Error	4224	110	1204
Median	6146	720	1438
V. amurensis 689			
Mean	1101	104	180
Standard Error	575	59	53
Median	194	24	155
C3309			
Mean	9.4	9.4	na*
Standard Error	4.5	4.5	
Median	2.5	2.5	

* not applicable - no galls were observed on C3309 in this study.

with crown galls, separate evaluations were made for galls and remaining nodal tissue after galls were excised. After gall removal, nodes of Cabernet Sauvignon had AV populations 15 times those observed in *V. amurensis* 689.

These experiments indicate that the crown gall-resistant genotypes support lower AV populations than the crown gall-susceptible Cabernet Sauvignon. Crown gall formation enhances AV populations in both susceptible and resistant genotypes. When resistant genotypes form galls, populations of AV in the nodal tissues remain much lower than in Cabernet Sauvignon and this may affect the extent of endophytic colonization. However, it is interesting to note that high populations of AV can survive at inoculated sites of both crown gall-resistant and -susceptible *Vitis* genotypes for more than a year without inducing visible crown gall symptoms.

Evaluation using vascular fluid displacement: Vines inoculated with CG471 were assayed for systemic *Agrobacterium* by forcing vascular fluids out of shoot sections with SDW (GOODMAN *et al.* 1987). To quantify only AV which had moved beyond the point of inoculation, test shoots were cut so that the sample basal node was the node immediately above the most apical inoculated site. 17 vines, 10 with crown galls, representing 10 *Vitis* genotypes were used in this investigation. Vascular fluid plated on RS revealed typical colonies of *Agrobacterium* spp. from 3 vines but none was identified as AV by immunoblot.

C3309 and *V. amurensis* shoot sections containing a basal inoculated site were subjected to the same procedure but none of the vascular extracts yielded *Agrobacterium* spp. on RS plates, even though the basal inoculated sites were found to contain about 10^6 cfu of AV/node when analyzed by tissue homogenization. Once again, these studies indicated little systemic development of AV under our conditions.

Effect of freezing on *Agrobacterium* recovery using vascular fluid displacement: The systemic nature of AV is well documented in *Vitis* (LEHOCZKY 1968, BURR and KATZ 1984, SÜLE 1986) and some researchers report very rapid movement of *Agrobacterium* through grapevines (TARBAH and GOODMAN 1986). The data reported here show clearly that vascular movement of AV is neither common nor rapid in inoculated vines growing under greenhouse conditions. This apparent discrepancy might result from tissue localization of AV at wound inoculation sites or may be due to the use of greenhouse- rather than field-grown material. Freeze injury is typically associated with initial gall development in cold climate viticulture regions (BURR 1988, GOODMAN *et al.* 1987) and it is possible that exposure to freezing temperatures in the vineyard might affect systemic movement of AV in grapes. AV has been detected in xylem sap collected from vines in early spring in cold climate regions (BURR and KATZ 1983, LEHOCZKY 1968). This is consistent with CHAMBERLAIN's (1962) conclusion that high soil moisture and spring freezes are factors that contribute to the development of aerial crown gall tumors.

The effect of freezing shoots on subsequent recovery of AV was tested by assaying vascular extracts from a group of inoculated shoots (Cabernet Sauvignon, C3309, 101-14 Mgt, and *V. amurensis* 1295), freezing half of them, incubating all shoots for 2 d at 28 °C, and repeating the vascular sap sampling. Collected vascular sap was dilution plated and AV colonies were counted after 5 d. Most shoots yielded no AV from initial extracts, but recovery was greater for all shoots following incubation at 28 °C. To meaningfully compare increase in recovery, shoots with no colonies recovered from initial extracts were assumed to have 10 cfu of AV/ml of extract. Shoots that were not frozen displayed a mean 57-fold (median 19) increase in AV recovery, whereas frozen shoots revealed a mean 41,000-fold (median 556) increase in recovery. The freezing and incubation treatment had no significant effect on AV population at inoculated sites when assayed by homogenizing and dilution plating (data not shown). The physical effect of freezing and increased free water as tissue thaws may enhance movement of AV. VIGOUROUX (1989) has proposed that spread of *Pseudomonas* in stone fruits is increased through this mechanism.

Canes of Cabernet Sauvignon, Chardonnay, and C3309 were collected from a crown gall-infected vineyard in February and then again in the following October prior to freezing. The effect of freezing on recovery of *Agrobacterium* spp. from these naturally-infected cuttings was tested (Tab. 3). As with the artificially-inoculated vines, freezing followed by a 2-d incubation significantly increased recovery of *Agrobacterium* spp. using the vascular fluid displacement technique. However, a significant increase in recovery was not observed when canes were assayed immediately after thawing (Tab. 3).

Chardonnay canes collected before the first natural freeze in October had higher levels of *Agrobacterium* spp. than canes collected the previous winter (Tab. 3), though none of the tested colonies were found to be AV. While differences in storage before testing prevents a direct comparison of these experiments, these results are not indicative of freezing being critical to vascular *Agrobacterium* development. High levels of nonpathogenic *Agrobacterium* spp. that are not AV are frequently observed in grapevines. Since these strains are recovered so easily from vascular fluid, it appears that these strains may become systemic more readily than AV. Various factors may influence the ability of some *Agrobacterium* spp. to systemically colonize grape, such as the ability to metabolize tartrate (GALLIE *et al.* 1984). The increase in recovery observed in *Agrobacterium* spp. after artificially freezing shoots may mirror the increase that would be observed in a much smaller population of pathogenic AV.

Freezing prior to vascular flushing substantially increases the recovery of AV and other *Agrobacterium* spp. in grapevines and may be a valuable step to include when indexing cuttings using vascular fluid displacement. In related research, it was possible to recover AV from heat-treated cuttings using this prefreezing modification, even though cuttings had been scored as "AV-free" by indexing prior to the freezing treatment (data not shown).

Interestingly, cambial tissue, from which most crown galls originate (STOVER *et al.* 1996) is also one of the most freeze-sensitive tissues in dormant grape canes (GOFFINET

Table 3

Effect of a freezing pretreatment on recovery of *Agrobacterium* spp. from grape cuttings. Canes were collected in February and October from vines naturally infected with crown gall (Cabernet Sauvignon and Chardonnay) or from root suckers (C3309 from vines where scions were destroyed by crown gall and/or cold injury). Shoots collected in February were flushed in May after storage at 4 °C. Shoots collected in October were flushed within 2 weeks. Vascular sap was collected using vascular displacement technique. All cuttings were tested before the freeze treatment. Shoots collected in February were frozen at -20 °C and then flushed immediately after thawing. All shoots were incubated 48 h at 28 °C and flushed again. Only 2 of 62 vines yielded bacterial colonies that were identified as AV by immunoblot

		Colony forming units recovered		
Genotype	Collected	before freeze	immediately after freeze	after freeze plus 48 h at 28 °C
Cabernet Sauvignon	February	96	98	1660
Chardonnay	February	2.3	1.5	64
C3309	February	447	886	6003
Chardonnay	October	249	nd	25432
C3309	October	432	nd	40000

nd = not determined

et al. 1991). The presence of galls may make the cambium a repository for *Agrobacterium* spp. that can readily enter the vascular system when freeze damage occurs. This would facilitate movement of AV to multiple wound sites where the probability of crown gall-induction will be high. If freeze injury is an important factor in systemic AV development in the field, it may be possible to reduce AV populations in nursery stock by collecting propagation wood before freezing occurs in the fall.

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