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Research Focus

How Close are We to Crown Gall-Free Nursery Stock?

Tim Martinson[1] and Tom Burr[2]
[1]Senior Extension Associate, Dept. Horticulture and
[2]Professor, Dept. Plant Pathology and Plant-Microbe Biology
NYS Agricultural Experiment Station
College of Agriculture and Life Sciences, Cornell University

Crown gall formation at the graft union (left) on a nursery plant with grafting wax still visible (left) and galls on the trunk of a mature grapevine (right).

Photos by Tom Burr

Crown gall disease, caused by the bacterium *Agrobacterium vitis,* is perhaps one of the most costly trunk diseases affecting grape growers in cool climate regions. Galls produced as a result of *A. vitis* infection can grow rapidly, girdling trunks and weakening or killing the vines. Crown gall has been difficult to eliminate from grapevine nursery stock because it can remain latent within vines for many years before causing significant injury and it persists in the soil.

With support from the National Clean Plant Network, we are developing improved diagnostic tests for crown gall and indexing grapevines for crown gall disease. The resulting crown gall-free grapevines will be housed at Foundation Plant Services in Davis, California, and made available to nurseries for propagation. Availability of improved tests, micro shoot-tip propagated foundation material, and renewed attention to the disease by the nurseries that supply grafted *V. vinifera* and hybrid nursery stock to U.S. growers may help realize the goal of greatly reducing the incidence of crown gall in new vineyards – and associated problems during vineyard establishment.

Key Concepts

- Crown gall is a bacterial disease caused by *Agrobacterium vitis*.
- Wounds and callus tissue activate infection by the bacterium. A plasmid present in *A. vitis* includes genes that are transferred to vines and become expressed in grapevines to produce undifferentiated cells that form crown gall.
- *A. vitis* also causes grape root necrosis.
- *A. vitis* can persist for several years in dead root tissue in the soil.
- New testing methods are being developed to increase the sensitivity and reduce the cost of testing vines for *A. vitis*.
- Shoot-tip meristem culture eliminates crown gall bacteria from grapevines.
- Our program is working with UC Davis Foundation Plant Services to index shoot-tip cultured vines for a new foundation block.
- Availability of crown gall-tested vines should reduce the early incidence of crown gall in commercial vineyards.
- Growers will still need to manage vineyards to minimize crown gall.

Background. Crown gall is important worldwide, but it is most prevalent in regions with cold climates. The causal agent, the bacterium *Agrobacterium vitis,* infects the vine systemically within the water-conducting vascular xylem vessels. Because they affect vascular tissues, crown gall tumors caused by *A. vitis* block the flow of nutrients and water from the roots to the developing buds and shoots. Gall formation is triggered by routine tissue injury and repair processes. Instead of producing new, organized vascular tissue, cambial cells infected with *A. vitis* produce rapidly dividing, disorganized cells that develop into galls. If the galls form higher on the trunk, growers can replace the trunk and keep the vine in production. However, galls can girdle the trunk, killing the vine. Freeze events are a common source of injury in cooler climates, but graft unions are vulnerable to infection and gall formation regardless of climate.

Conditions under which galls form. Galls often appear after damaging winter low temperature episodes, and tend to be more prevalent in heavy soils and low-lying portions of vineyards. Following a winter cold episode in which temperatures plunged from 50°F to -14°F in January 2004, many growers in the Finger Lakes region of New York saw substantial crown gall injury over the next two years as an indirect result of the trunk injury vines sustained during the freeze. Vines planted in heavy, poorly drained soils often have more injury than vines growing in coarse-textured, well-drained soils. Low lying areas or 'frost pockets' with poor air drainage are also prone to more injury. Field observations suggest that overly-vigorous vines that continue vegetative growth past veraison can be more prone to gall formation than moderately vigorous vines.

Early gall formation during vineyard establishment. Reports of crown gall in newly-planted vineyards are increasingly common—and frustrating—for growers. Some growers have reported galls in up to one-third of grafted vines in the year of planting or a high incidence of galls in the subsequent growing season. Crown gall disease in newly planted vineyards arrives through infected cuttings. This has proven hard to eliminate or control because the bacterium can be present even in apparently healthy cuttings. Wild grapevines, which do not appear to harbor the tumor-causing bacteria, are not thought to be sources of infection for nearby commercial vineyards. However, in sites where vines were previously planted, the bacterium can persist in live or dead root tissue in the soil for many years.

Crown Gall Bacteria:

Agrobacterium vitis and *Agrobacterium tumefaciens*

Agrobacterium vitis (Ophel and Kerr 1990), the main cause of crown gall of grapevines, belongs to the family Rhizobiaceae. Other bacteria in this family include *A. tumefaciens* (Smith and Townsend 1907, Conn 1942), which causes crown gall in many plant species, and *Rhizobium* species, which cause nitrogen-fixing root nodules in legume plants. *A. vitis* and *A. tumefaciens* have been shown to form biofilms on tomato roots and on other surfaces, a phenomenon that may be associated with both their survival and host infection. Both *A. vitis* and *A. tumefaciens* are gram-negative, rod-shaped bacteria which swim by moving their whip-like flagella.

Not all strains of *A. vitis* and *A. tumefaciens* cause tumors. The ability of strain of *A. vitis* or *A. tumefaciens* to cause disease is associated with the presence of a tumor-inducing plasmid (Ti plasmid), a small circular piece of DNA separate from the regular chromosomes. For *A. vitis*, a second plasmid encoding tartrate utilization is also involved in the host-pathogen interaction. *A. vitis* strains are genetically diverse, and they are generally categorized based on the presence and makeup of their Ti plasmid. All *A. vitis* strains cause necrosis on grape roots, but interestingly the plasmid causes crown gall only on trunks and canes. Necrosis appears to provide a portal for the bacterium to enter vines through soil and a refuge where the bacterium can survive for years in grape root debris.

Colonies growing in the lab on nonspecific culture media are white, convex, circular, glistening and translucent, and the two *Agrobacterium* species are visually indistinguishable. Culture media that are semi-selective make it possible to isolate *Agrobacterium* species from soil and plant tissues. Some of the media are selective for *A. vitis*, while others support the growth of both *A. vitis* and *A. tumefaciens*. Because colonies of tumorigenic and nontumorigenic isolates of both species look identical and grow equally well on selective media, pathogenicity tests are necessary to confirm the pathogenicity of *A. vitis* or *A. tumefaciens* from plant or soils samples. *Agrobacterium* species may be isolated on selective culture media and then tested on indicator plants for pathogenicity, or identified with molecular and ELISA procedures.

Indicator plants commonly used in greenhouse pathogenicity tests for *A. tumefaciens* include tomato, sunflower, and tobacco (particularly *Nicotiana glauca*). For *A. vitis*, grape cuttings must be used to identify some host-specific strains, however most strains produce galls on common *A. tumefaciens* hosts. The overlapping host range of the two species and genetic diversity within species require additional DNA-based tests for identification of the species and strains within them.

Although some vines with crown gall will remain productive for many years, crown gall-infected vines impose additional costs of vine replacement and re-establishing trunks on vineyard owners, which can be especially severe during vineyard establishment because young vines may be less likely than mature vines to survive extensive galling. For these reasons, eliminating crown gall from propagation wood and producing crown gall-free vines is an important goal.

Producing crown gall-free vines. *A. vitis* can be eliminated from propagation wood through either micro- or macro-shoot tip culture (Sim and Golino, 2010). In shoot tip culture, the apical meristem of a grapevine shoot is excised and placed in tissue culture medium to generate new plants. Because cells from the apical meristem lack organized vascular tissue, the viruses and bacteria that rely on it for transport are not present and are thus eliminated. In both cases, the excised shoot tip is placed in sterile tissue growth medium and grown until root and shoot tissue about 10 cm long is produced. The difference between micro- and macro-shoot tip culture is largely the size of tissue excised from the growing tip and the time required— micro-shoot tip culture takes from 3 to 15 months longer—but micro-shoot culture has the added benefit of eliminating viruses in addition to *A. vitis.*

These methods are routine and reliable. The challenge in producing crown-gall free commercial cuttings has been keeping vines gall-free as they move from the laboratory and foundation planting into field increase blocks and commercial nurseries. Two significant hurdles are preventing reinfection in field plantings and increase blocks and developing sensitive, reliable testing methods to track the infection status of nursery vines.

Challenges in the nursery. Nurseries face several challenges in propagating crown gall-free nursery stock.

Latency. The crown gall bacterium remains latent in apparently healthy plants until events (e.g., winter injury) trigger expression and gall formation in vines. For nurseries and growers in warmer areas, even infected wood rarely presents visible crown gall symptoms. Lack of symptoms means that it is impossible to 'rogue out' infected vines based on visual assessment.

Nursery incentives. Until recently, there has been little incentive for nurseries to minimize crown gall. Because most vines are produced and sold in regions where crown gall symptom expression is rare, nurseries and foundation plantings have not made crown gall elimination or minimization a goal. A safe assumption is that, although undoubtedly some materials remain clean, a significant proportion of propagation wood from established nursery and increase blocks is infected with the crown gall bacterium. This has changed in the past few years: Increased sales to cool and cold-climate regions have created market demand for crown gall-free vines, and new emphasis on crown gall elimination has come about under the auspices of the National Clean Plant Network.

Biological Control of Crown Gall

Field testing of Strain F2/5. Some strains of *A. vitis* do not cause galls and can provide protection from the tumor-causing strains of the bacterium when applied as a biocontrol agent. Our group is testing the effectiveness of non-tumorigenic *A. vitis* strain F2/5. In collaboration with Dr. Tom Zabadal at Michigan State University, pathogen-free grapevines were propagated by shoot tip culture. These vines were treated by soaking crowns and root systems in either water or a suspension of F2/5 prior to planting in commercial sites that were previously planted to grapevines with crown gall. At our test vineyard at the New York State Agricultural Experiment Station, Cabernet Franc, grafted to 3309 (also shoot-tip cultured) was planted in 2009. Data collected in the fall of 2011 showed 7 of 60 control vines had crown gall compared to a single F2/5 treated vine.

Although results of this experiment are promising it was also previously discovered that F2/5 application at graft unions it has a deleterious effect on graft take. We have not observed reduced vine vigor in our field trial where roots and crowns were soaked in F2/5 prior to planting, however because of the reaction that occurs at graft unions, further evaluations are warranted.

Persistence in the soil. A. vitis can persist for several years on root tissue remaining in the soil. Therefore, any site previously planted to grapes is likely to harbor the bacterium, which will be able to move through the soil to reinfect crown gall-free vines planted at the site. Nurseries wishing to establish and maintain crown-gall free increase blocks would need to establish plantings in a virgin site and maintain some degree of separation from other commercial vineyard blocks to prevent reinfection.

Testing Methods. Developing reliable testing methods to diagnose and confirm infection status of vines in the field has been challenging, for several reasons.

Until recently, the standard testing method involved taking cuttings from vines, producing callus tissue (in which *A. vitis* will multiply), culturing the bacterium on a selective growth medium, isolating *A. vitis* colonies, then confirming the tumorigenic nature of the 'strain' through molecular probes. Monoclonal antibodies in ELISA tests were traditionally used but do not differentiate tumorigenic from nontumorigenic forms of *A. vitis*. Recently the use of DNA sequences has allowed the differentiation of tumorigenic strains. These testing methods take several weeks to complete and are not conducive for use in development of a clean plant program.

While these tests are very specific and diagnostic, they have not been sensitive enough to reliably detect *A. vitis* in mature vines with visible galls. One challenge is that the bacterium is not evenly distributed within the vine. So even if galls are present on the lower trunk or crown, *A. vitis* may be absent or present in very low numbers in canes and new growth. As a result, tests with vines in the field are often inconclusive – and often provide false negative results even in vines showing visible galls.

These tests have been much more reliable in verifying the *A. vitis* status of propagation wood that has been through shoot tip culture in preparation for planting in foundation blocks.

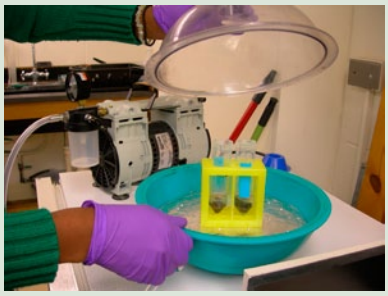
Developing more sensitive tests. Funding through the USDA National Clean Plant Network has allowed us to focus our efforts on developing more sensitive tests. We have adapted a method called *magnetic capture hybridization* (see box) that appears to be highly sensitive and rapid. It relies on utilization of very specific DNA fragments for detection of tumorigenic *A. vitis*, and separating its DNA from that of other organisms in grapevine tissues. Our preliminary results indicate this test is sensitive enough to detect as few as 10 tumorigenic *A. vitis* cells in samples—and results can be obtained in a few days instead of several weeks. This new tool offers researchers—and potentially nurseries and growers—a more rapid, reliable way to determine the infection status of vines.

Testing Material for the National Clean Plant Network. Foundation Plant Services, located at the University of California at Davis, is in the process of establishing a new foundation block based on accessions which have undergone micro shoot-tip culture for elimination of crown gall and plant viruses (Sim and Golino, 2010). The first vines at this new location, called the Russell Ranch, were planted in 2011 (Cunningham, 2011), and the goal is to make the

More Efficient Crown Gall Testing

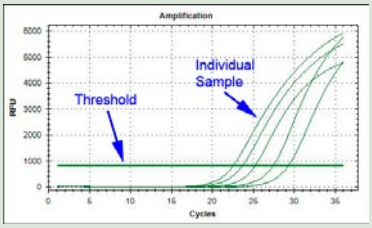
A new method for testing for crown gall, using a technique called *magnetic capture hybridization* is under development in the Burr laboratory. It can detect as few as 10 *A. vitis* cells in a grape tissue sample, and greatly improves the sensitivity of tests available for *A. vitis.* Previous tests involved callusing cane tissue (6 wk), incubating on selective media (1-3 wk), and amplifying and testing DNA from colonies to verify identity of *A. vitis*. Detection limit was around 1,000,000 (10^6) bacteria. This new method should improve testing accuracy, and results are available in 3-4 days.

1. Nodes from test canes are cut and put in vials containing buffer solution to soak tissue.



2. Vials placed in vacuum to extract Agrobacterium vitis from vine tissue. Nutrient broth is inoculated with PBS containing extracted Agrobacterium and incubated for 3-4 days.

3. DNA is extracted and placed in small tubes with small beads coated with a matching DNA probe that selects for A. vitis DNA and leaves behind DNA from the plant and other microorganisms that could interfere with the test.



4. Real-time PCR: Samples are placed in a DNA Polymerase Chain Reaction (PCR) cycler (left), which also provides a read out of the amount of crown gall DNA produced (right). Each green line represents a single sample; a positive result is recorded when the amount exceeds a threshold (horizontal green line).

highest level of crown gall and virus-tested material available to nurseries and growers nationwide, under the auspices of the National Clean Plant Network funded by the USDA.

Dr. Thomas Burr's laboratory has indexed over 380 vines for this program since 2008, with another 320 accessions slated for this spring. The crown gall-free vines we've screened will become available to nurseries across the United States within the next several years. In addition, we've tested over 500 samples from a variety of nurseries and growers for crown gall since 2009.

Keeping crown gall-tested vines clean. As foundation material tested for crown gall and viruses becomes available, it will be important for nurseries to take steps to keep it clean. For nurseries, doing so may involve locating a site that has not been previously planted to grapevines and is geographically isolated from any commercial vineyards that have crown gall. Wild vines are not thought to harbor crown gall: Tumorigenic *A. vitis* strains are exclusively associated with cultivated grapevines and in vineyard soils. If tumorigenic *A. vitis* are not present in the soil, 'clean' vines should be able to remain uninfected by crown gall.

What will growers get with crown gall-tested vines? Crown gall-tested vines will offer growers the best possible start for their vineyards. Currently, the safest assumption for growers is that nursery stock will come with some level of crown gall infection that depends upon the unknown status of propagation wood collected for both the rootstock and scion components of grafted vines. Starting with crown gall-tested material should greatly lower the risk of crown gall disease during the establishment phase, allowing vines to reach maturity. In sites that have never been planted to grapevines, we believe it possible that growers will be able to maintain crown gall-free vines.

Unfortunately, in replant situations or sites near existing vineyards, there is no guarantee that vines won't be re-infected by tumorigenic *A. vitis* and eventually develop galls. In soils where *A. vitis* is present, planting crown gall-tested vines should still delay re-infection of vines and limit the incidence of galls until vines are well-established. Growers won't see one-third of their young vines with galls the year after planting, as has been the experience of some growers in recent years. Crown gall-free nursery stock is only part of the picture. Growers will still need to manage their vineyards to prevent or minimize crown gall.

Management practices for minimizing crown gall. Antibiotics and soil fumigation have not been effective for eradicating *Agrobacterium* from soils. Because crown gall bacteria reside systemically in plant tissues, they may not come into direct contact with topically-applied compounds. The following management practices are recommended to minimize crown gall:

- *Preventing trunk injury.* Grape crown gall generally develops following attachment of the bacterium to cells at plant wounds. On grapevines these wounds are commonly initiated by freeze injuries on trunks. All practices that help to prevent trunk injury are important in the management of crown gall. These include selecting suitable planting sites with good air and water drainage to reduce the chance of freeze injuries and using cultural practices that are less likely to result in trunk injuries.

- *Training vines with multiple trunks.* Maintaining three to five trunks per vine allows the removal of galled trunks while maintaining productivity from the established trunks. Multiple trunks are generated through the training of suckers.

- *Hilling above the graft union.* Hilling above the union on grafted vines protects buds from freezing and ensures the development of new scion shoots that may be needed for trunk renewal the following season. This is a common practice in regions where low temperatures cause winter injury, but it may be as important for grafted vines during vineyard establishment even in warmer climates. Soil insulates the graft union from daily temperature fluctuations, which may be especially important in the first three to five years while the graft union is growing and strengthening.

- *Internal soil drainage.* Avoiding water-saturated soils may be as important in the dormant season as it is during the growing season. Water expansion associated with freezing may cause tissue injury to trunks at or near the soil line, particularly in heavy clay soils. Subsurface drainage tiles remove excess water and empty out the pore spaces in the soil, allowing space for freezing water to expand without injuring trunks.

- *Cultivar selection.* Grape varieties differ in their susceptibility to crown gall (See table 3.1.2 in NY/PA Grape IPM Guidelines (http://ipmguidelines.org/grapes/). For example, wine grapes belonging to *Vitis vinifera* are generally quite susceptible, whereas *V. labrusca* and hybrid varieties are generally more resistant. Therefore, it is sometimes possible to select grape rootstock and scion varieties that are relatively resistant. Select cultivars that will not be subject to frequent winter injury in your climate.

Although growers will still need to actively manage their vineyards to minimize the economic impact of crown gall, we're confident that new indexing methods and the NCPN-Grapes-supported effort to produce and maintain

crown gall and virus-tested foundation vines will reduce the risk of early, severe appearance of crown gall in vineyards. As more accessions become available to nurseries, and as nurseries make it a priority to minimize crown gall during propagation, growers should see early problems with widespread galling during vineyard establishment dramatically reduced.

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