VIRUS ELIMINATION USING TISSUE CULTURE IN ROSES

FROM A SPECK TO A PLANT

Susan T. Sim, Deborah A. Golino,
Foundation Plant Services,
University of California, Davis

Tissue culture is the process of growing cells, tissues, organs and organisms on a defined medium in an artificial environment separate from the organism. In roses and many other crops tissue culture techniques are used for two completely different purposes — micropropagation and virus elimination therapy. The end goal is either thousands of plants in micropropagation or one single, clean plant in virus elimination therapy.

Micropropagation is used to increase the number of plants of a cultivar more rapidly than can be accomplished with traditional techniques. The idea is similar to taking cuttings, except that the cutting for micropropagation consists of a single bud and piece of stem about 20 mm (one inch) long instead of a five-bud cutting about six inches long. This single bud is established in a growth medium in sterile culture and stimulated to produce multiple shoots using growth hormones. Depending on many factors, such as cultivar and amount of hormone, the bud may produce anywhere from five to 20 shoots. Each shoot is then transferred to a new container where it multiplies again to produce more shoots. Each shoot goes through several to many more multiplication stages so that number of plants produced increases exponentially. Over the course of a year or two, a single bud may produce thousands of new shoots. Finally, the shoots are rooted either in (*in*

vitro) or out (*ex vitro*) of a sterile container and are acclimatized in a greenhouse and grown to the final desired size. The use of tissue culture to propagate roses has been much studied and is used commercially (Jacobs et al., 1970; Davies, 1980; Bressen et al., 1982; Carelli and Echeverrigaray, 2002; Kim et al., 2003).

Let's set aside micropropagation now and focus on the use of tissue culture for virus elimination therapy in roses. As we said earlier, the goal in using tissue culture for virus elimination therapy is to produce a single, clean plant from a plant that is infected with virus. This clean plant is then used as the new source plant for propagation, either by traditional means or micropropagation.

Virus elimination therapy is a primary mission at Foundation Plant Services (FPS), a self-supporting center in the College of Agriculture and Natural Resources at the University of California, Davis. At FPS, we produce, test, maintain and distribute premium disease-tested plant propagation material. We have programs for grape, strawberries, fruit and nut trees, roses and sweet potatoes. The rose program at FPS was started in the 1960s by Dr. George Nyland, a faculty member in the Plant Pathology Department at UC Davis. Throughout his career and even after his retirement in 1985 George Nyland was a tireless advocate of virus-tested planting stock. He was convinced that rose mosaic disease was a problem in roses that could and should be addressed by virus testing and a recently developed virus elimination technique using heat treatment. Today, the rose collection at FPS contains over 900 cultivars, 12 rootstocks, covers eight acres and is the largest public collection of virus-tested roses in the US.

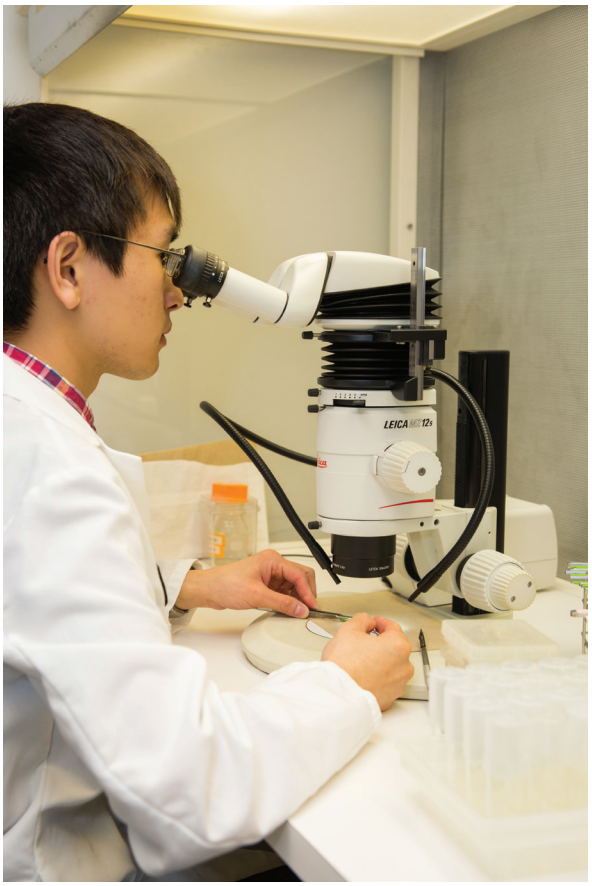
WHY ELIMINATE VIRUSES IN ROSES?

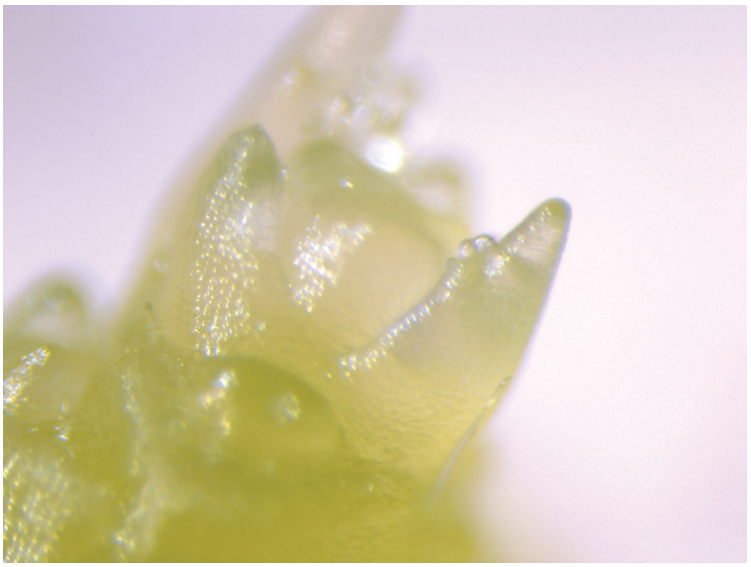
Many viruses infect roses but three of the most common viruses cause rose rosette disease and rose mosaic disease. Rose Rosette Disease is widespread east of the Rocky Mountains in the U.S.; it is transmitted by eriophyid mites and through grafting. Rose Rosette was described in the 1940s but the virus, *Rose Rosette Virus* (RRV), was not discovered until 2011 using a very advanced and powerful technology to sequence nucleic acid known as high throughput sequencing (HTS). Rose Rosette Disease is something you definitely do not want as a commercial grower or in your yard. It causes leafiness, thorniness, leaf malformations, excessive numbers of shoots and eventually plant death.

Rose Mosaic disease is most commonly caused by two viruses either alone or in combination — *Prunus Necrotic Ringspot Virus* (PNRSV) and *Apple Mosaic Virus* (ApMV). Rose mosaic is extremely common everywhere roses are grown. Symptoms of rose mosaic disease include line patterns, vein clearing, mosaic, ringspots and puckering on leaves. Leaf symptoms vary with year, cultivar and temperature, often disappearing in hot weather or only appearing in spring or fall. Often, infected bushes appear symptomless. Infection with PNRSV or ApMV can reduce stem length, stem diameter, grafting success, bush size, and number, size and quality of flowers and rooting success in some cultivars (Pool et al., 1970; Thomas, 1981; 1982; Moran et al., 1988; Stein et al., 1988; Palmer and Horst, 1990; Wong et al., 1988; Manners, 1997; Golino et al, 2007b).

An ounce of prevention is invaluable compared to a cure when the subject is rose viruses. Once a plant is infected with virus, it stays infected — there is no "cure" for

OPPOSITE: Kristen Farrar processing samples for lab testing, Rose leaves are collected, weighed and processed in the spring for lab tests to detect viruses. TOP, LEFT TO RIGHT: The rose collection at Foundation Plant Services, University of California, Davis contains more than 700 cultivars and is the largest virus-tested public collection in the US. Symptoms of Rose mosaic disease, caused by *Apple mosaic virus* and/or *Prunus necrotic ringspot virus*, include line patterns and ringspots on the leaves, seen here.

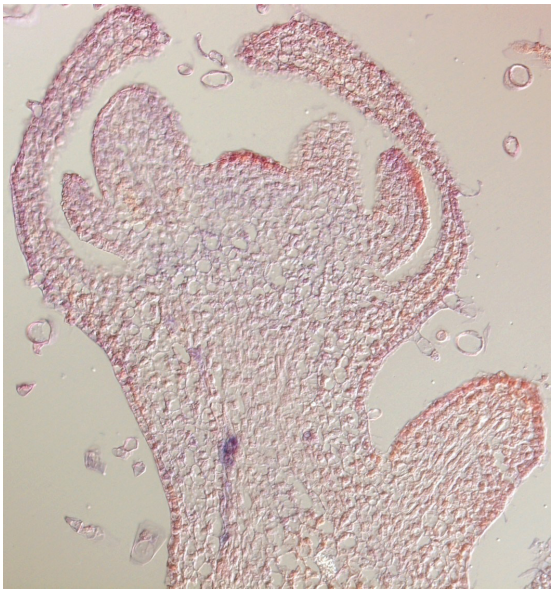




an individual plant; however, it is possible to "clean up" a variety, i.e., eliminate the virus from the propagating stock.

HOW IS VIRUS ELIMINATION DONE?

Tissue culture therapy has been used successfully for decades to eliminate virus in many horticultural crops. There are several techniques for eliminating virus from an infected cultivar. The two most common techniques are heat therapy and tissue culture. Heat therapy was used for many decades at FPS. In hot conditions, many plant viruses fall apart, lose the ability to infect cells and cannot replicate themselves. In the heat therapy process, potted bushes of rose cultivars infected with virus are subjected to constant 100°F temperatures in a heat chamber for a minimum of four weeks. Buds are removed from the plant and budded onto *Rosa multiflora* 'Burr' understock. This plant is observed and tested to determine whether the virus is eliminated. Heat treatment works well for many cultivars but others die in the heat or viruses are still present in the buds that were removed.



Tissue culture therapy has been successfully for decades to eliminate virus in many horticultural crops — orchids, grapes, sweet potatoes, and garlic to name a few. Tissue culture therapy involves removing a miniscule shoot tip from the growing point and regenerating a whole new plant from it. This shoot tip is just a speck of a piece of tissue! You can just barely see it with the naked eye. It is referred to as a microshoot tip; it measures less than 0.5mm and consists of the meristematic dome surrounded by a few leaf primordia. The meristematic

tissue has a unique potential to regenerate a new plant with a minimum chance of mutation, or genetic change, in comparison to plants derived from other tissues. It is not known exactly why virus is eliminated, but one reason it may be successful is because cells in the microshoot tip are growing faster than the virus can infect them. Additionally, the microshoot tip does not have a direct connection to the vessels in the plant, where many viruses are located. From a practical point of view, the smaller the microshoot that can be cultured, the greater the chance of eliminating virus. Unfortunately, the smaller it is, the more difficult it is to regenerate a plant. The microshoot tip is excised using a microscope under sterile conditions and placed on a growth medium inside a sterile test tube. We have found that rosebuds differentiate into flower buds very early in development. The bud is very flat and petals can be seen surrounding it. Sadly, flowerbuds usually die within two weeks in culture. The vegetative bud cultures are then maintained in a growth chamber which provides controlled growing conditions during the critical establishment phase. Once well developed, which may require eight to 12 months or longer, plants are transferred into soil, acclimated to normal light and air, moved into greenhouses and screen houses and, finally, to outdoor conditions.

At FPS, heat treatment has been replaced or supplemented by microshoot tip therapy for grapevines, strawberries and sweet potatoes. In those crops, virus is successfully eliminated in 90-100 percent of cultivars that are treated. In 2007 we reported successful elimination of *Apple mosaic virus* (ApMV) and *Prunus necrotic ringspot virus* (PNRSV) using microshoot tip culture for six cultivars. However, six other cultivars that were treated did not survive (Golino et al, 2007b). In 2015, we received funding from the National Clean Plant Network (NCPN), (more about that below) and started to treat modern, heirloom and species roses. We are now working to optimize rose virus elimination therapy using microshoot tip culture. We hope that this technique becomes as routine for roses as it is for other crops we use it on. If successful, this would greatly increase the availability of virus-free roses throughout the U.S.

DISEASE TESTING

Viruses in plants can be very difficult to detect. They are unevenly distributed in a plant so they may be in one bud but not another. Viruses also do not always cause symptoms and they can go latent, hiding in specific tissues and then causing disease just when you think everything is growing and well established. As a result, we use many different kinds of tests that act as checks on each other to determine the virus status of a plant. The oldest tests we use are biological indexes. We also use several laboratory tests.

Biological indexes have been used for decades to detect rose viruses. Various sensitive "indicator" plants are inoculated with buds of candidate plants, and the indicators are then monitored for disease symptoms. The development of symptoms on the indicator means that the candidate was virus infected. Indicator varieties are chosen for their ability to display relatively rapid, distinct disease symptoms when infected. Biological tests for rose viruses are very reliable, but they may require up to three years before results are obtained. Two biological tests used at FPS for rose viruses are the *R. multiflora* 'Burr' field index and the Shirofugen cherry index.

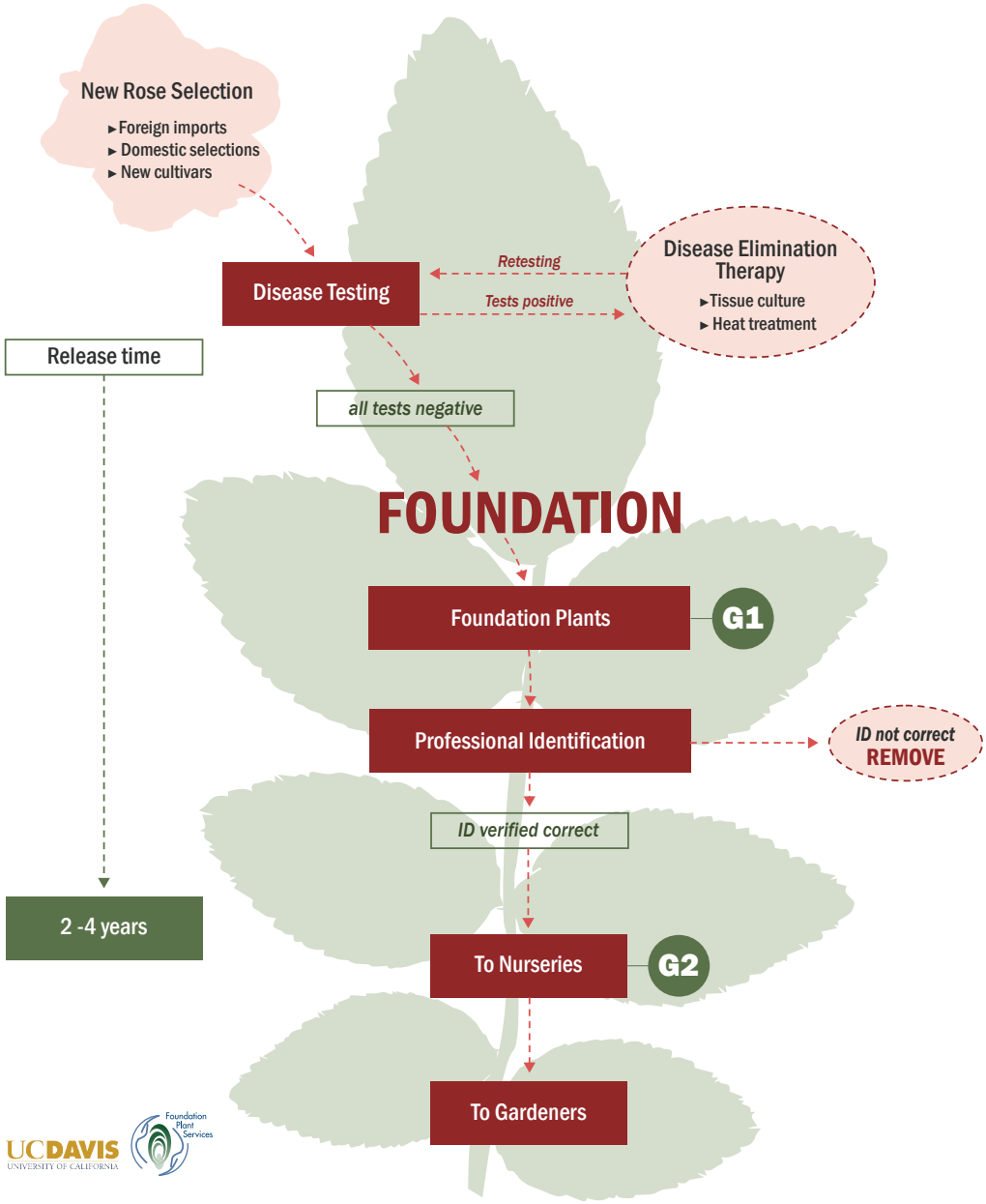
Rosa multiflora 'Burr' understock is used as an indicator variety for the detection of rose mosaic, rose spring dwarf and rose yellow mosaic. For this test, buds from candidate cultivar are T-budded into one year old *R. multiflora* plants in a field (Fig.6). As the buds heal into the multiflora, viruses that may be present in the candidate buds will multiply and move into the multiflora indicator plants. The multiflora plants are observed for symptoms twice a year for two to three years. Symptoms may include vein clearing, ringspots, line patterns, mosaics, and leaf distortion and puckering. Symptoms are most clearly expressed in March and April, fading dramatically as seasonal daytime temperatures increase.

For the Shirofugen cherry index, candidate buds are T-budded into the branches of a mature Shirofugen cherry tree in the field. If the candidate buds are infected, the cherry will rapidly kill the surrounding tissue, effectively isolating the bud and preventing virus from infecting the tree. This response causes a lot of visible gumming and necrosis within 30 days. If a bud is not infected, healthy callus tissue heals over the wound caused by budding. Enzyme-Linked Immunosorbent Assay (ELISA) is a sensitive and rapid laboratory serological method for detecting

OPPOSITE, CLOCKWISE FROM TOP LEFT: Virus elimination therapy using tissue culture techniques requires a steady hand and is done in a sterile hood using a stereoscope, Ninh Khuu meristemming. The microshoot tip of a vegetative rose bud is approximately 0.3mm in diameter. The meristem tissue and several leaf primordia of a microshoot tip of a vegetative rose bud is the preferred material for regenerating a new plant, hopefully one that is not infected with viruses. A series of roses of different ages from two weeks to eight months after excising the microshoot tip from an infected plant. This photo, taken through a microscope, shows a virus-infected shoot tip with the meristem dome, leaf primordia, a fluorescently-labelled virus (in purple). When the microshoot tip is taken out and cultured in sterile medium, the virus is left behind and a whole new plant is regenerated which then becomes a clean source of propagation material. Photo courtesy Li-Fang Chen and R. Gilbertson, UCDavis

viruses. ELISA technology is sensitive, but its scope is limited to detecting viruses for which antibodies are available. FPS laboratory staff routinely uses ELISA to test roses for *Prunus Necrotic Ringspot Virus* (PNRSV), *Apple Mosaic Virus* (ApMV), *Arabis Mosaic Virus* (ArMV) and *Prune Dwarf Virus* (PDV).

Another test done in the FPS laboratory is the quantitative Polymerase Chain Reaction test (qPCR). qPCR tests rely on knowledge of the genome of a plant virus and involves the selective amplification of a small part of the virus' genome. If the virus is present in a plant sample, even in very low amounts, the amplification steps in PCR allow for its detection. It is this amplification that makes PCR such a sensitive test. PCR only detects specific viruses, but it is much more sensitive than ELISA in most cases.



ABOVE: Flowchart outlining the steps a rose takes through FPS from introduction to the foundation block and finally to nurseries and growers.

In special cases, we may use HTS to assess the virus status of a cultivar. HTS sequences all the nucleic acids present in a plant – the plant genome as well as the genomes of any viruses, fungi or other organisms that may be in the plant tissue. HTS has the advantage of being able to look for a pathogen without knowing its sequence or having an antibody specific to it. It can lead to discovery of new, previously unknown viruses and other pathogens; we suspect there may be a number of previously undiscovered viruses and virus-like organisms in roses that HTS will identify, as was the case with Rose rosette disease.

Finally, the work that we do on roses at FPS has been greatly enhanced by support from the NCPN-Roses (NCPN-R). The NCPN-R was established in 2015 and is supported by the US Department of Agriculture. This funding makes it possible for FPS to test and treat many non-patented cultivars, including heirloom roses and to collaborate with other institutions and researchers to continually optimize our methods for greatest efficiency and success. More information can be found at nationalcleanplantnetwork.org and in an excellent article by Dr. David Zlesak in the Summer, 2015 issue of Rose Hybridizers Association Newsletter (Zlesak, 2015).

We love beautiful, healthy plants and urge everyone to request virus-tested, clean roses for your most beautiful planting ever.

Overview of the FPS Rose Program from New Selection to Commercial Distribution

1. New introductions, called "candidate selections," are typically sent to FPS as dormant, bareroot plants. Candidate selections may be AARS winners, advanced selections of a rose hybridizer, the proprietary selection of a commercial rose nursery or historic or "heritage" roses.

2. Disease testing for viral pathogens is the first step for inclusion of a candidate selection into the collection. In March and April, samples of young leaf tissue from the candidate plants are tested by the Enzyme-Linked Immunosorbent Assay (ELISA), a serological test conducted in the laboratory. Then, after the first flowering, mature budwood from each candidate rose is graft-inoculated onto both Shirofugen cherry and Burr multiflora rose for biological indexing. Combining the results of these tests gives a greater accuracy for virus detection than relying on any single test. Two to three years are required to complete all tests.

3. When a rose cultivar is found to be virus-infected it is subjected to virus elimination therapy using microshoot tip tissue culture. After treatment, the plants are re-tested to be sure the treatment successfully eliminated viruses.

4. Only when the candidate selection is found negative for virus by each testing method are the rooted cuttings finally planted in the FPS rose collection. Whenever possible, new introductions are planted as cuttings on their own roots to reduce the possibility of introducing virus through an infected understock and to prevent the problems associated with rootstock suckering. The FPS rose collection is regularly retested by ELISA for reoccurrence of virus. The plants are also visually inspected in the spring for virus symptoms. As the selections mature, the rose collection is carefully examined for correct identification and trueness to type.

5. Dormant budwood is cut and shipped from the FPS rose collection in early Nov. Leafy cuttings of scion and understock varieties can be harvested upon request during the summer months for green propagation by customers.

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