LEFT TO RIGHT, TOP TO BOTTOM: Dr. Dimitre Mollov looking at plants of 'Charles Albanel' positive for Rosa rugosa leaf distortion virus; Classic rose mosaic disease symptoms on a plant of 'Olympiad' for sale at a big box store; Necrotic stem lesions on 'Ballerina' due to rose yellow mosaic virus, all photos courtesy David Zlesak.

BEHIND THE SCENES EFFORTS

Keeping Roses Clean of Virus

by Dr. David Zlesak, Master Rosarian

STARTING WITH A HEALTHY ROSE PLANT goes a long way towards speedy establishment and the plant reaching its full potential. We sometimes can nurse a purchased rose back to health depending on the problem (e.g. if it was water stressed at the nursery, broken canes, etc.). However, for challenges like virus infection, there unfortunately isn't a practical way to remove a virus from our plant. The best course of action for virus diseases is to start with a clean plant and keep it clean.

There are more than 25 viruses that have been documented to infect roses. Viruses infect all life forms including animals, plants, bacteria, and fungi. They are relatively simple in structure. They have a protein coat and inside the coat one of two forms of nucleic acid (RNA or DNA). Genes found in the nucleic acid sequence code for things such as making more of the unique protein coat, an enzyme to replicate the nucleic acid, and sometimes movement proteins that help virus particles efficiently move from one cell to the next.

Viruses use their host cell's machinery to multiply themselves since they are so small and lack the infrastructure to do so on their own. Plants have some defense mechanisms to limit the replication of viruses, but not an immune system like us. In some situations virus replication can become so prominent that the cells cannot complete their own functions adequately and become damaged beyond repair. Areas of particularly damaged cells can show up as yellow or dark regions on our rose leaves and stems.

Depending on the specific virus, there are different means by which they typically spread to a new plant. For instance, some viruses are more easily transmitted by a particular insect or mite depending

TOP TO BOTTOM: 'Therese Bugnet' leaf with disrupted expansion from Rosa rugosa leaf distortion virus; Classic rose mosaic disease symptoms on a plant of 'Olympiad' for sale at a big box store, photos David Zlesak. BELOW AND OPPOSITE: Roses in the new Brooks South clean rose block; photos courtesy Regents of the University of CA.

on the feeding pattern of the animal and the type of tissue the virus typically is most concentrated in the plant. Thankfully many common rose viruses are seldom transmitted by insects or pruning tools and are primarily transmitted during propagation by grafting. Graft-transmittable viruses can also spread in our garden over time through underground root grafts between adjacent plants. It's the primarily graft-transmittable viruses that we can have the greatest success limiting in roses with careful attention to propagation practices in production.

Although most viruses aren't lethal (key exception is rose rosette virus which leads to rose rosette disease), they can weaken plants and magnify the effects of other stressors like harsh winters. Since viruses are systemic and move throughout a plant, a challenge is that when cuttings or budwood are used from an infected plant, the new plants are also infected. Reputable nurseries want to sell clean plants to help their customers be as successful as possible and also improve their production efficiency to support their bottom line. For instance, lower propagation success rates and smaller plants at the end of the production cycle have been documented for roses infected with rose mosiac disease (RMD).

The primary way rose viruses have traditionally been spread in the US (like RMD which includes prunus necrotic ringspot virus and apple mosaic virus) has been through budding (bud grafting). Nurseries typically line out hardwood cuttings of rootstock directly in the field late in the season. Cuttings root over the winter and by late spring/early summer the rootstocks are growing vigorously and are ready to be budded (a single bud of a desired cultivar is surgically nestled into the stem of the rootstock). If the bud is infected with virus, the virus can move from the bud into the rootstock. After the first growing season, the top of the rootstock is cut off to force all of the stem growth out of the desired cultivar the second season. To save resources, nurseries frequently use the prunings of the rootstock tops they cut off as material for hardwood cuttings for the next generation of rootstock. Compared to having a separate field for growing clean rootstock solely for harvesting cuttings, this practice saves field space. The virus-infected rootstock cuttings can transmit virus in the next propagation cycle to whatever cultivar is budded onto it the following year. As growers keep harvesting rootstock cuttings and more budwood off of their

own production fields, over time virus can spread throughout their fields. With many newer cultivars being grown own-root versus grafted, this should help tremendously in slowing the spread of many viruses.

THE CLEAN ROSE PROGRAM AT FOUNDATION PLANT SERVICES

In the 1960s Dr. George Nyland, a plant pathologist that loved roses, began the rose clean plant program at Foundation Plant Services (FPS; part of UC-Davis). His efforts and the efforts of those that came after him at FPS have had a huge impact limiting the amount of virus-infected roses entering the marketplace. As resources allowed, Dr. Nyland and colleagues worked to test popular rose varieties for the key rose viruses known at the time. Testing has traditionally been done by a process called virus indexing and is not equivalent with the term "virus-free." When we see "virus-free" in a catalog it should be a red flag since it is hard to prove and pretentious.

Each kind of virus has unique properties to its protein coat and nucleic acid sequence. Indexing allows one to search specifically for a unique feature of the virus' protein coat or nucleic acid to learn if the specific virus is present or absent. There is a test called enzyme-linked immunosorbent assay (ELISA) that uses antibodies and can test for a unique protein, and traditionally polymerase chain reaction (PCR) is used to test for unique patterns in nucleic acids. For virus indexing, one can only find what one is looking for. There may be another virus present, but without the proper tools to specifically test for the unique features of that virus, we wouldn't be able to detect it. Therefore, there may be additional viruses in our sample that what is routinely tested for. When companies market their plants as "virus-indexed", they should be able to tell us which viruses they had indexed when we ask.

FPS is committed to have their clean rose collection as clean as possible (it is the largest clean rose collection in the world). Therefore, they are concerned

RIGHT: Prepared budwood from FPS ready to ship; BELOW: Walk-in growth chamber with controlled temperature and lighting for plant tissue culture, photos courtesy Regents of the University of CA.

about the possibility of additional viruses that may be present and are not among those being indexed. To help protect their collection, before entering all roses have had to not only pass the laboratory tests for key viruses of concern using ELISA and/or PCR tests, but also two biological tests. Buds of the plants in question are grafted onto both *Rosa multiflora* 'Burr' and 'Shirofugen' cherry. Although rose and cherry are not graft compatible for the long term, within just a few weeks if there is virus present, the cherry tends to produce a lot of gummy sap and discoloration at the graft site. *Rosa multiflora* 'Burr' tends to readily show symptoms of virus infection. The *R. multiflora* 'Burr' plants are budded in the field and observed for two seasons (the rootstock above the graft is not fully removed in order to observe its foliage and stems for virus symptoms). If plants of a rose cultivar pass all the tests, the rose cultivar can enter the isolated clean rose collection (aka rose stock block). Plants within this collection are periodically retested with laboratory tests to make sure they aren't inadvertently reinfected. The clean rose collection is isolated far from other roses to help those plants remain clean.

Budwood and cuttings from the clean rose collection at FPS are available for sale (currently 813 cultivars and eight rootstocks are available on the website). A minimum of five stems per cultivar ($1 per stem) are required (rootstocks are cheaper) and the minimum order is $100. Commercial nurseries start with a limited amount of clean propagation material from FPS and carefully multiply it up at their facilities being as clean as possible. Eventually from those plants they generate enough propagation material to meet their full production needs. Some commercial growers reorder clean material of rose cultivars on a routine basis (typically starting with fresh material from FPS every few years) to be proactive.

In the unfortunate situation when no individual plant of an important cultivar tests clean, attempts are made to generate a clean plant of it through relatively slow and tedious means. Dr. Nyland used heat therapy for this task. Infected plants were grown under high

LEFT: Growing point of a rose stem exposed and ready to be excised for tissue culture; BELOW: Different stages of development of isolated shoot tips in tissue culture, photos courtesy Regents of the University of CA.

heat (continually at ~100F for multiple weeks) to reduce virus concentration. High heat stressed the plants, but theoretically stressed viruses more. Buds with as little extra material as possible were isolated from the heat-treated plants and budded onto clean rootstock. When the resulting plants matured and were indexed, periodically Dr. Nyland could find a clean plant.

FPS transitioned to using shoot tip (meristem) isolation and tissue culture instead of heat therapy to generate clean stock of infected cultivars since it tends to have a higher success rate. Tissue culture is a highly controlled environment where nutrition, temperature, light, and moisture can be optimized. Most viruses move from cell to cell using straw-like channels called plasmodesmata. The very youngest cells in shoot tips have not yet developed plasmodesmata and are typically not yet infected. These very small groups of dividing cells (typically aiming for 0.5mm or smaller) can be carefully excised and placed onto a nutrient rich medium. Months later some of these small groups of cells have developed into plantlets. These plantlets can be taken out of culture, grown on, and indexed to hopefully find some that are clean. Some viruses are more difficult to rid than others (e.g. prunus necrotic ringspot virus). Unfortunately, not all roses grow

TOP TO BOTTOM: Budding of rose scions onto a 'Shirofugen' cherry stem; *Rosa multiflora* 'Burr' index with examples of healthy and virus infected plants; Symptoms of virus infection on 'Shirofugen' cherry stem one month after budding, photos courtesy Regents of the University of California.

well in culture and efforts to uniquely optimize the nutrient and growing conditions for finicky cultivars may be needed.

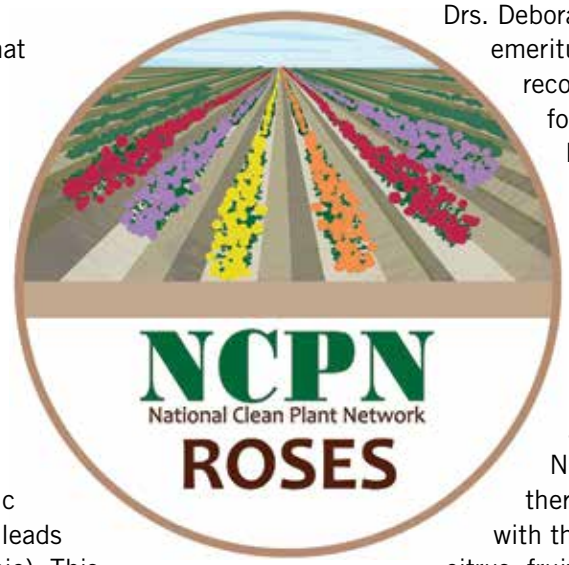
Fortunately, over the years industry members have valued the FPS clean rose program and were able to support it financially. The program has been funded not only through the sale of the modestly priced cuttings and budwood, but also by periodic donations from especially the industry-led Garden Rose Council, Inc. The size of the collection, until recently, has been limited to eight acres as that is what the Garden Rose Council, Inc. could commit to helping fund. As new roses were added to the collection, some plants of another, less popular cultivar were removed to generate space. Typically, there are between five to 20 plants of each cultivar. Once a cultivar is in the collection, the goal is to at least maintain five plants of it in case it is ever needed again.

THE NATIONAL CLEAN PLANT NETWORK (NCPN)

The NCPN is a USDA program that began with the 2008 Farm Bill and is meant to help support clean plant centers like FPS. The mission of the NCPN is to support the security of our national agriculture. This is accomplished through supporting the generation and availability of clean propagation material along with improved diagnostic tools to test for the presence of critical viruses and other systemic pathogens (i.e. the bacteria that leads to crown gall can become systemic). This is a new direction for the USDA. The USDA is known for its involvement with plant inspection and destruction of plants that are positive for problematic pests to prevent their spread. USDA inspections occur at multiple points of movement including when plants enter and leave our nation and at various points during inter- and intrastate commerce movement.

The USDA NCPN funding is tremendously helpful for clean plant centers to continue to do their work and ensure growers can access clean propagation material of important cultivars. Investing in current clean plant centers versus the USDA starting their own, leverages and builds upon resources already in place. Many clean plant centers (typically associated with universities) are struggling to keep their programs going. It can be hard to find stable and ongoing support from grower associations with the volatilities in the marketplace. Initially, congress appropriated $5 million annually to the NCPN. With the very successful outcomes of the NCPN early on, the NCPN quickly garnered strong bipartisan support in congress and has since become a permanent program in the Farm Bill. The current directive is that at least $7.5 million be allocated to the NCPN annually. Each crop approved under the NCPN umbrella has an advisory board (aka Tier 2 advisory board) made up of stakeholder representatives (e.g. industry members, pathologists, breeders, etc.) that meet regularly. At the meetings, needs and priorities are determined that help guide the proposals written that compete for a portion of the overall NCPN funding.

NATIONAL CLEAN PLANT NETWORK-ROSES (NCPN-R)



Drs. Deborah Golino (FPS Director emeritus) and David Byrne (Texas A&M) recognized the need/opportunity for more stable funding for the FPS rose clean plant program and improving virus diagnostic efforts. They started the process in 2013 that allowed for roses to be added officially as the first (and still only) ornamental crop in the NCPN in 2015. They continue to serve as vice-chair and chair of the NCPN-R, respectively. Currently there are seven crops in the NCPN with the other six being berries, citrus, fruit trees, grapes, hops, and sweet potatoes. Successful inclusion of roses in the NCPN was possible because of the efforts already in place at FPS to generate and house virus-indexed roses along with the strong ongoing support of the rose industry. Other clonally-propagated ornamental crops (e.g. hydrangeas, boxwood, etc.), unfortunately, don't have the same degree of industry collaboration and clean plant center resources in place. With roses included in the NCPN as of 2015, this has allowed proposals for roses specifically to be submitted each year and considered for funding. In recent years the NCPN-R has been able to routinely secure just over $400,000 out of the overall ~$7.5 million of NCPN annual funding.

ABOVE: Drs. David Byrne and Deborah Golino, chair and vice-chair of the NCPN-R, photo Regents of the University of CA.

The NCPN-R meets twice per year (Tier 2 advisory board, invited speakers, and other stakeholders)- one meeting is in person (typically in summer) and the second is held virtually in winter. The purpose of these meetings includes reviewing updates to the overall NCPN program and larger scale initiatives benefitting all the crops (e.g. educational materials, economic impact studies regarding clean plants, etc.), the priorities we set for roses, the accomplishments over the past year, and hearing presentations from experts on topics relevant to the NCPN-R (e.g. advances in virus detection techniques, rose rosette disease, etc.). The meetings are a great opportunity for rose stakeholders (e.g. industry, scientists, public garden professionals, etc.) to network and strengthen relationships.

KEY ACCOMPLISHMENTS TO DATE

• The renovation of the clean collection of roses at FPS as recently been completed. The previous eight acre location (called the Nyland Block to honor Dr. George Nyland) and was planted in 1995. Many of the plants were over 20 years old and declining. Funding allowed for propagation of all the roses in the collection and the young plants being planted on a larger section of land (~16 acres). This new area for roses was previously planted in grapes and is called Brooks South. Brooks South still has room for the collection to grow in size.

• Many cultivars have been added to the collection over the past few years. There are now over 800 rose cultivars that have passed all the tests and are in the clean block with more part way through the testing process. New additions include recently introduced cultivars as well as older cultivars still important to industry that weren't in the collection before this point. Many new industry members are connecting to FPS to have their roses included. More comprehensive nurseries (they sell a wide range of plants) are developing their own exclusive lines of roses (e.g. Bailey Nurseries, Spring Meadow Nursery, etc.) and are excited to have their roses included at FPS.

• In recent years there has been great advancements in virus detection. Although the NCPN does not directly fund characterization of newly identified viruses in crops, it does provide funding for technology transfer and optimization of detection of current viruses of concern. Improved detection tools are not only useful for clean plant centers, but also diagnostic clinics all over the country helping them with improved and unified protocols. Most states have plant disease clinics through their land grant university that accept and run samples for growers and homeowners for a fee. One giant leap forward in diagnostics is a technique called High Throughput Sequencing (HTS; sometimes also called Next Generation Sequencing). HTS is very powerful in that it sequences all the nucleic acid in a tissue sample (that of the plant and any pathogen that may be in the plant). Tens of millions of short sequences (aka reads) are generated from the sample. These short reads should represent all the nucleic acids present and do so multiple times. The short sequences are then assembled into longer sequences that can be used to determine what pathogens may be present. One can think of the process as a very complex puzzle. The short sequences are lined up to generate the longer sequences based on the overlapping ends. This is done using powerful computers. Many viruses have had their full genomes sequenced and their sequences are maintained in public databases. As more virus genomes are added to these databases, that will only improve what can be gleaned from HTS sequence data. HTS data is very powerful in that it should be able to detect any virus in the sample, unlike ELISA and PCR and the process of virus-indexing. Even when a specific virus' sequence isn't in a database yet, families of viruses have unique sequence patterns in areas of their genome and one can leverage that information with HTS data to begin to characterize a new virus. The challenge with using HTS for finding new viruses is that it is important next to understand the biological impact the virus may be having on the plant and if it truly is something of concern. The impact may be minimal or only significant when in the presence of other specific

viruses (mixed infections). HTS not only can help answer a lot of questions, but can lead to many more questions as well.

HTS data is able to be funded under the NCPN umbrella because it is being explored for improved detection of previously characterized viruses. Thankfully, HTS data that points to new viruses is a byproduct of the work and can be leveraged by researchers to seek other funding sources to continue to study them. If the impact of a new virus identified is determined to be significant to growers and consumers, it may someday fall under the umbrella of NCPN funding.

Recent research (supported by the NCPN) compared traditional virus detection methods (ELISA and PCR) with HTS using multiple crops in the NCPN. The same plants were tested using HTS initially and using the traditional means (ELISA and PCR) over two growing seasons at different times of each growing season. The data revealed HTS was much more sensitive than ELISA and PCR. Although a virus may be present, sometimes it can be in such low concentrations that ELISA and PCR can miss detecting it. At different times of the season and stages of growth, plants can suppress virus concentrations to a greater or lesser extent. When ELISA and PCR tests were positive for virus, HTS was positive too, but not always visa versa. The recommendation for those using conventional tests (plant disease clinics, those without access to HTS, etc.) is to test plants in spring and fall over two years to be very confident a plant is clean and one isn't getting a false negative. Right how HTS is still relatively expensive, the equipment needs careful maintenance, and analysis of the data takes specialized expertise, so most plant disease clinics do not use it. With the incredible sensitivity and relative speed of HTS (compared to the two-year period of using R. multiflora 'Burr' as a biological index), efforts are underway for HTS to be able to replace the biological tests and speed along the process of getting plants into clean collections as well as

TOP TO BOTTOM: Prepping of samples for HTS; Erin Hsu, Tissue Culture Laboratory Manager at FPS, isolating shoot tip cells, photos Regents of the University of CA.

satisfy some USDA regulatory requirements. Dr. Maher Al Rwahnih at FPS is an expert in HTS and has taken a major role in this research for the NCPN.

• Dr. Dimitre Mollov took the lead in establishing a collection of roses positive for key viruses (using HTS) with the goal that they can be used as positive controls for conventional tests at plant disease clinics. He characterized new rose viruses for his Ph.D. thesis working with Dr. Ben Lockhart. It was rewarding to help with their research years ago. In his current position he works with viruses in berry crops and still is able to do some limited work with roses. As clinics test customer samples for particular viruses, having a sample positive for the virus in question to run side by side (positive control) is valuable to ensure the test worked effectively. I have kept roses that are positive for various viruses from Dr. Mollov's research in pots in my rose collection since his doctorate in case there was ever a need for them. Thankfully they are being put to good use again. He has also obtained additional symptomatic roses from industry members, public gardens, and private collections for this effort. Tissue samples of roses positive for different known viruses are being freeze dried and can be requested.

• Old garden roses (OGRs) are valued and loved by gardeners, but their limited sales make it hard to justify a lot of NCPN effort towards them. With the vast numbers of them, they can quickly fill up the remaining space at FPS and eventually limit opportunity for recent introductions with higher demand to be included. FPS does include some OGRs, especially if they are stronger sellers like those designated as Earth-Kind® cultivars (e.g. 'Mutabilis'). Dr. Malcolm Manners at Florida Southern College (FSC) has been working to clean OGRs of virus for many years using heat therapy. Plants that test clean are propagated and planted on the FSC campus. He and his students have used them for propagation experiments and have been very generous donating plants for historical gardens in New York City and elsewhere. The NCPN-R has been able to fund the retesting of roses in his collection (Dr. Kevin Ong, Director of the Texas Plant Disease Diagnostic Laboratory, is taking a significant role in the testing). Plants that test clean are being propagated and planted at the Antique Rose Emporium as a backup location. They are used by the Antique Rose Emporium for propagation and sales, and others can access the material as well.

Dr. Malcolm Manners has cleaned many old garden roses of virus using heat therapy, photo Malcolm Manners.

The whole rose community benefits from the behind the scenes efforts supported by NCPN-R funding to generate and make available virus-indexed roses. As ARS members, we can be a part of sharing this incredible story and helping more industry members and others connect to and benefit from these great resources. Links to NCPN and FPS resources are below. There are a number of educational outreach items (i.e. brochures and fact sheets) that can be useful handouts at ARS sponsored and other events. Dr. Brent Pemberton and Kristen Farrar (along with Natalie Anderson and Pamela Hornby from Texas A&M who serve as NCPN-R co-coordinators and manage the day to day program details) have led the way in the educational outreach components for the NCPN-R.

HELPFUL LINKS:

• NCPN website:
https://www.nationalcleanplantnetwork.org/
• NCPN-R page:
https://www.nationalcleanplantnetwork.org/roses
• NCPN Outreach Materials: Outreach Materials | NCPN (nationalcleanplantnetwork.org)
• Foundation Plant Services-Rose program:
https://fps.ucdavis.edu/roses.cfm

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