Virus Studies in the Geneva® Apple Rootstock Breeding Program

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Some Geneva® rootstocks (G.814 and G.16) have displayed hypersensitivity to viruses while others (G.935 or G.969) have displayed poor growth or slow decline with certain virus-laden scion cultivars. Other Geneva® rootstocks (G.41, G.202, G.222, G.214, and G.890) have not displayed sensitivity to viruses. The Geneva apple rootstock breeding program is committed to providing virus free plant material to rootstock producers worldwide. To do that we have combined heat therapy with cryotherapy to eradicate even one of the most recalcitrant viruses.

Viruses that infect apple trees cause losses in apple production mostly due to tree decline and death, graft union incompatibility, decreased tree growth, deformation of branches and roots, and by making fruit unmarketable (Campbell, 1981; Sampson and Johnstone, 1974). Thanks to recent advances of DNA and RNA high throughput sequencing technologies, a number of viruses and viroids have been discovered in apple tissues and apple orchard related material (Liu et al., 2021; Umer et al., 2019; Wright et al., 2020). However, only a few of these have been directly implicated with adverse symptomatology including the latent viruses Apple chlorotic leaf spot virus (ACLSV, *Trichovirus*), Apple stem grooving virus (ASGV, *Capillovirus*), Apple stem pitting virus (ASPV, *Foveavirus*), Tomato ring spot virus (ToRSV, *Nepovirus*), Tobacco ring spot virus (TRSV, *Nepovirus*), and Apple mosaic virus (ApMV, *Ilarvirus*) (Hu et al., 2019; Keshavarz and Hajnajari, 2019; Koike et al., 1993; Lana et al., 1983; Li et al., 2020; Stouffer et al., 1977; Xing et al., 2018). Other viruses and viroids like Apple hammerhead viroid (AHVd, *Pelamoviroid*) and Citrus concave gum virus (CCGaV, *Coguvirus*) (Liu et al., 2021; Serra et al., 2018; Wright et al., 2018) have only suspected associations with symptoms that include trunk splitting, mosaic, necrosis, shoot decline, and dieback (Lim et al., 2019; Messmer et al., 2017; Nabi and Baranwal, 2020; Sanderson and James, 2019; Szostek et al., 2018; Wright et al., 2020). Because these viruses are often found in conjunction (mixed infections) with other apple viruses, more research is needed to assess the influence of each when it is the only virus present in an apple tree.

Our understanding to date is that most viruses are spread by grafting, where infected clonal rootstocks or scions are the media for transmission from one tree to another (Barba et al., 2015; Li et al., 2020; Rubio et al., 2020; Wood, 2000); however, a recent report suggests the possibility of pollen transmission of ASGV (Isogai et al., 2022). While the goal of most apple industries throughout the world has been to work only with material that has been certified tested free of viruses, phytoplasmas and other adverse graft-transmissible agents, the eradication of these agents has been elusive due to propagation practices of some nurseries, growers and homeowners that use infected sources of budwood (Fuchs, 2016; Fuchs et al., 2018). Millions of trees propagated prior to the discovery of viruses affecting apple trees, the transport of these trees across continents, and propagation on common virus infected apple rootstocks like M.9, M.7, MM.111, B.118, M.8 etc. have all contributed to the historical spread of these viruses and viroids around the world. Nurseries in the Netherlands may have been some of the first to adopt virus elimination as a practice after several experiments demonstrated the adverse nature of viruses in apple rootstocks (Baumann and Louis, 1980; Oosten, 1975a, b, c, 1979; Robitaille and Carlson, 1973). To complicate things, as we have been reminded lately with the COVID 19 pandemic, viruses, including the ones that affect apple trees, mutate and may form many strains within a certain type. Some of these strains may be more or less virulent depending on the individual type of apple (or apple rootstock) being exposed (Howell et al., 1996). Some wild species of apple seem to react severely to the presence of viruses (Kirby et al., 2001; Silva et al., 2008), hence they have been used as live indicators (biological indexing) for the presence of viruses in budwood. In apple rootstocks, some of these wild species have been the source of positive traits like resistance to fire blight (*Malus robusta, M. floribunda*) and cold tolerance (*M. baccata*) (Gardner et al., 1980; Warner et al., 1984). One of the best apple rootstocks to survive extreme cold events 'Ottawa 3' displays susceptibility to at least ASGV (James et al., 1997). Several apple rootstocks released by the Geneva® breeding program (G.935, G.214, G.890, G.969, G.814) are derived from parents 'Ottawa 3' and 'Robusta 5' and

Figure 1. G.935 apple rootstock grafted with a virus laden strain of 'Honeycrisp' (left) and a virus cleaned version of the same strain (right) planted in Ephrata, WA (Willow Drive Nursery).

display differential sensitivity to apple viruses — G.814 gives significant early signs of sensitivity to ASGV, while others, like G.935 and G.969, have displayed sensitivity to a combination of strains of latent viruses (possibly ASGV, ASPV, and ACLSV). This sensitivity was only discovered when rare scions possessing all virulent virus strains were used for graftwood (Figure 1). Apple rootstock G.16 (progeny of *M. floribunda* and 'Ottawa 3') shows combined virus sensitivity likely derived from both parents and is hypersensitive to latent viruses, causing nursery trees to decline and die within 2 years of grafting with virus-infected wood (Figure 2). In some cases, the demise of apple trees due to the presence of viruses can be slow and display a gradual decline caused by graft union necrosis among certain rootstock/scion combinations in the presence of ToRSV (Tuttle and Gotlieb, 1985), as observed in MM.106 rootstock grafted with 'Delicious' scion. The bottom line is that viruses and sensitivity to viruses are detrimental and should be avoidable by the implementation of elimination practices and perhaps the discovery and selection against the genes that cause hypersensitivity in apples.

Figure 2. Comparison of virus-positive (left) and virus-negative (right) 'McIntosh' grafted on G.16 rootstock in 2003.

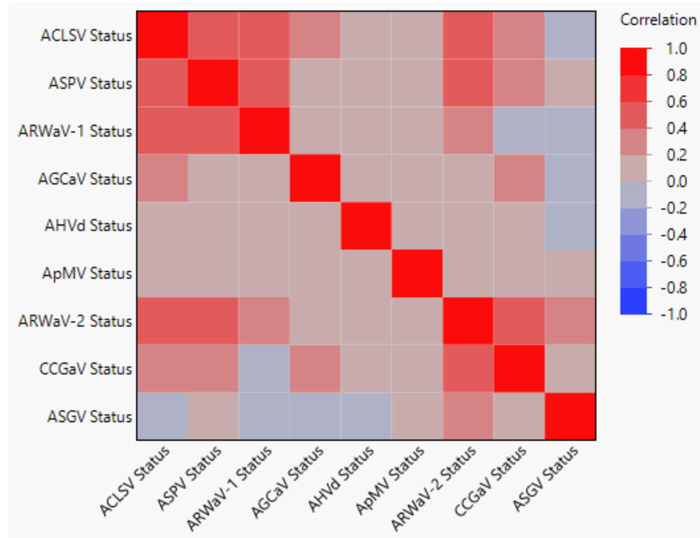

Figure 3. Clustered correlation coefficients showing the co-presence of viruses in breeding lines of the Geneva® apple rootstock breeding program.

Virus research in the Geneva® apple rootstock breeding program has taken many forms throughout the years including nursery trials in 2003 comparing G.16 and other advanced apple rootstocks grafted with the same scion cultivar infected with viruses or cleaned (Figure 2), the testing of 50 rootstocks in the Hudson Valley Lab and at Virginia Tech planted in 2012 to identify sensitivity to ToRSV, and other field trials throughout the U.S. that featured scion-wood loaded with diverse virus types. More recently, incidents of decline of G.935 rootstock when grafted with certain strains of 'Delicious' and 'Honeycrisp' cultivars urged the need to develop experiments that would reveal the viral causes of this decline and their genetic components in apple rootstocks in order to identify other rootstocks in the breeding program which may suffer similar problems. Furthermore, as the breeding program releases additional rootstocks for industry use, it needs to make sure that the material distributed is free of viral agents that may compromise the industry, therefore experiments aimed at the detection and elimination of apple viruses have been conducted. In this article we describe some of these experiments and the results obtained so far.

Breeding Program Testing for the Presence of Viruses and Viroids in Elite Breeding Lines

The process of breeding apple rootstocks includes the maintenance of thousands of individual breeding lines and the evaluation of thousands of apple trees grafted with different scion varieties. While the program has the goal to keep the original seedling trees alive and well and maintain their "seedling-virus-free" status, at times these seedlings were lost and we had to rescue the rootstocks from finished trees for which the virus status was unknown. Hence there was a need to conduct a virus census of all the elite breeding lines being propagated in the program to identify the ones that needed to be cleaned up. Iterations of this census were attempted at different times in the history of the program, however, in 2021, thanks to a cooperation between USDA-APHIS Plant Germplasm Quarantine Program's (PGQP) and the Geneva breeding program and under the leadership of Abe Steinberger (currently a PhD student at the University of Minnesota) the program was able to utilize testing protocols established at APHIS PGPQ to identify apple rootstock breeding lines that had been compromised by viruses and viroids. Having established in-house RT-PCR and qRT-PCR testing methods for eight apple viruses and one viroid [ACLSV, Apple green crinkle associated virus (AGCaV), AHVd, ApMV, Apple rubbery wood-associated virus type 1 (ARWaV-1), Apple rubbery wood-associated virus type 2 (ARWaV-2), ASGV, ASPV, and CCGaV] the census found that out of 1,395 tests only 186 were positive. Most times, the same rootstock was infected by more than one virus (mixed infections). Analysis of the co-presence of viruses in these rootstocks with mixed infections (Figure 3) revealed that ACLSV, ASPV, ARWaV-1, ARWaV-2, and CCGaV were often found together, whereas ASGV was not associated with these except for ARWaV-2. We are utilizing this information to target elite apple rootstock lines for eradication.

Cryotherapy and Thermotherapy Experiments to Eradicate ASGV, ACLSV and AHVd from Elite Rootstock Breeding Lines

The Geneva breeding program has adopted a procedure to establish "clean" propagation material at key centers and micropropagation laboratories prior to release of elite breeding lines into the commercial stream as rootstock cultivars. While preparing to release a set of new apple rootstocks for the U.S. industry, the program collaborated with Foundation Plant Services in Davis, CA and APHIS PGQP in Beltsville, MD to index plant material with what is known as High Throughput Sequencing (HTS) or Deep Sequencing, which is a powerful technology that allow the detection of known viruses that also include variants that might escape regular RT-PCR testing, including novel viruses. This procedure found that some of the stocks were infected by viruses. At the same time, Dr. Bettoni and Dr. Volk at USDA ARS National Laboratory for Genetic Resources Preservation (NLGRP), were working on discovering new ways to eradicate viruses from apple germplasm destined for cryopreservation. As a result, a collaboration ensued between the Geneva breeding program and NLGRP to investigate whether thermotherapy or cryotherapy alone or in combination could effectively eradicate ACLSV, ASGV and AHVd from *in vitro* cultures of four apple rootstocks developed in the Cornell-Geneva apple rootstock breeding program (CG.2034, CG.4213, CG.5257, and CG.6006) (Figure 4). For thermotherapy treatments, *in vitro* plants were treated for four weeks at 36°C (day) and 32°C (night). Plant vitrification solution 2 (PVS2) and cryotherapy treatments included a shoot tip preculture in 2 M glycerol + 0.8 M sucrose for 1 day and then exposure to PVS2 for 60 or 75 min at 22°C, either without or with liquid nitrogen (LN, cryotherapy) exposure. Combinations of thermotherapy and PVS2/cryotherapy treatments were also performed. Shoot tips were then warmed, recovered on growth medium, transferred to the greenhouse, grown, placed in dormancy inducing conditions, and then grown again prior to sampling leaves for the presence of viruses and viroids. Overall, thermotherapy combined with cryotherapy treatments resulted in the highest percentage of virus- and viroid-free plants. The work was published in the journal *Plants* in early 2022 (Bettoni et al., 2022). Although the efficacy of the combination of thermotherapy with cryotherapy has been reported for eradication of some apple viruses, to the best of our knowledge, this is the first study reporting success in eradicating of AHVd from infected *in vitro*-cultured apple rootstock plants. This combination of procedures has great potential for producing virus and viroid-free planting materials for the apple industry. Furthermore, it could also be a valuable tool to support the global exchange of apple germplasm. We are in the process of replicating the eradication procedure in Geneva, NY with some promising results.

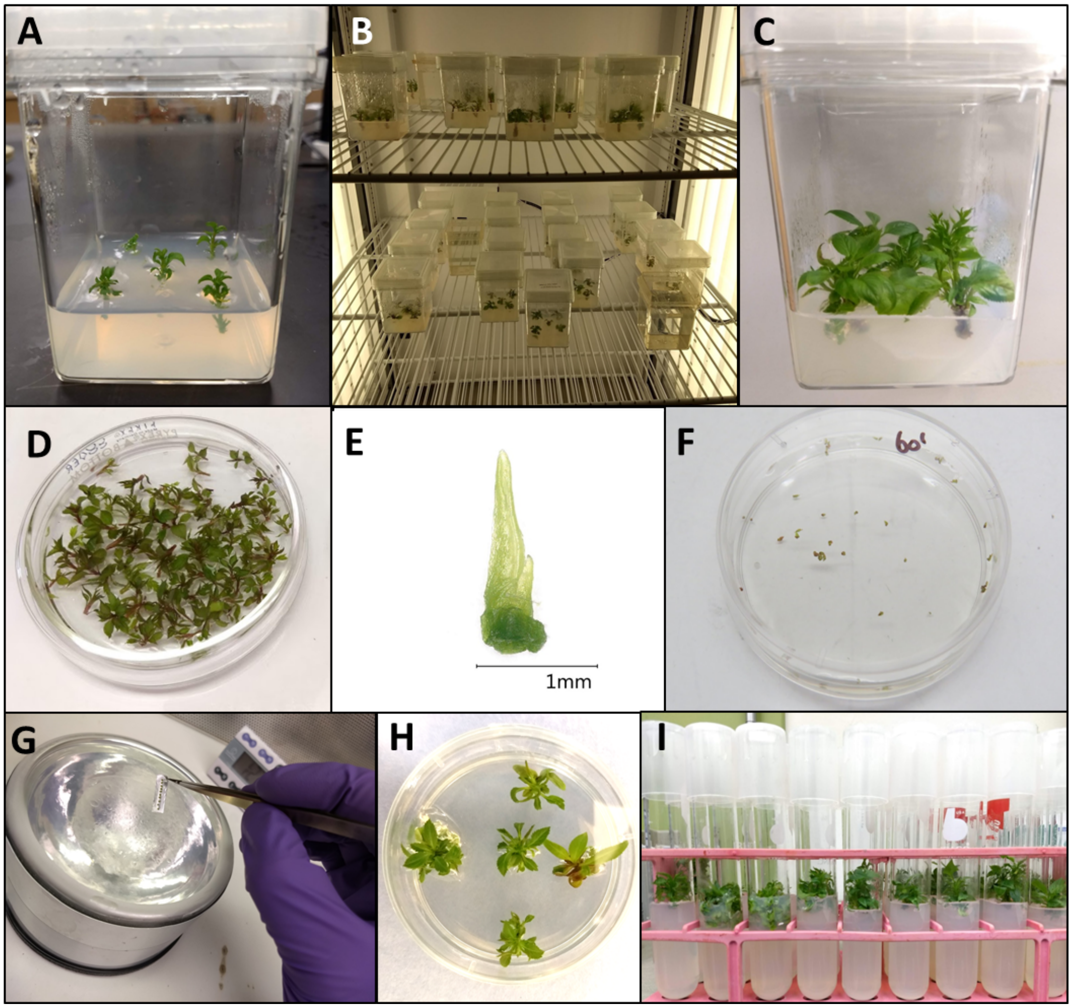

Figure 4. In *vitro* infected *Malus* plants undergoing therapy treatments; A) CG 5257 prior to thermotherapy exposure; B) plants during thermotherapy treatment; C) CG 5257 after 4 weeks of thermotherapy; D) CG 6006 prior to shoot tip excision; E) 1 mm shoot tip excised from CG 5257; F) shoot tips incubated on plant vitrification solution 2 (PVS2); G) PVS2-treated shoot tips placed onto a thin layer of PVS2 on sterile aluminum foil strips and then plunged into liquid nitrogen; H) CG 5257 exhibiting regrowth after eight weeks; and I) treated in *vitro* plants prior to shipment to Geneva, New York for greenhouse plant establishment.

Investigation on the Genetics of Sensitivity to Viruses in the Geneva Apple Rootstock Breeding Program

As a result of the issues discovered with the rootstock 'G.935' when grafted with certain strains of 'Honeycrisp' and 'Red Delicious' that had been found to contain a somewhat rare mixture of viruses and viroids (Wright et al., 2020), the Geneva apple rootstock breeding program initiated a collaboration with Willow Drive Nursery to test how widespread the sensitivity was within some of the elite germplasm of the breeding program and to utilize some of the breeding populations to determine the genetics of sensitivity. Willow Drive Nursery had obtained virus-free and virus-laden material from the same 'Honeycrisp' strain that was associated with the slow decline experienced with 'G.935' (Figure 1) and grafted that material on a set of 12 rootstocks that represented some of the elite material available at that time (2017). Overall, the preliminary trial revealed that the presence of the virus cocktail inhibited growth on most rootstocks; however, some were more affected than others (Figure 5). In 2020, these preliminary results led to the preparation and planting of a larger replicated experiment featuring 165 different rootstock breeding lines grafted with both virus free and virus laden scions of the same 'Honeycrisp' strain. The experiment is in progress and has already produced some preliminary growth data that will be used to discover genetic links to virus sensitivity. These links will enable the breeding program to preselect material that is not hypersensitive to viruses and perhaps discover the genes underlying such hypersensitivity in the Geneva apple rootstock breeding program. Root systems of G.935 and G.969 are obviously being compromised by viruses, and in an effort to understand the mechanism by which root growth is being inhibited by viruses we have initiated another experiment (Figure 6) using aeroponics to test if root growth is being repressed by viruses and featuring

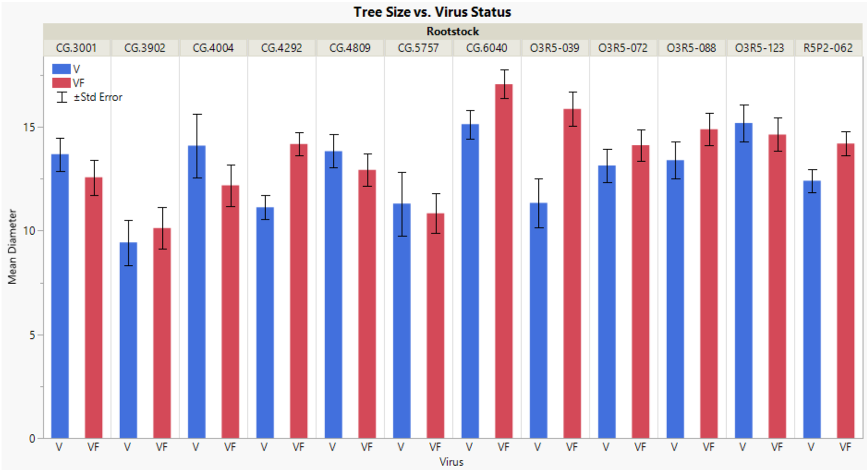

Figure 5. Preliminary results showing the comparative tree growth (mm diameter) of experimental apple rootstocks grafted with virus-laden (V) and virus-free (VF) versions of a 'Honeycrisp' strain. Mention age of tree?

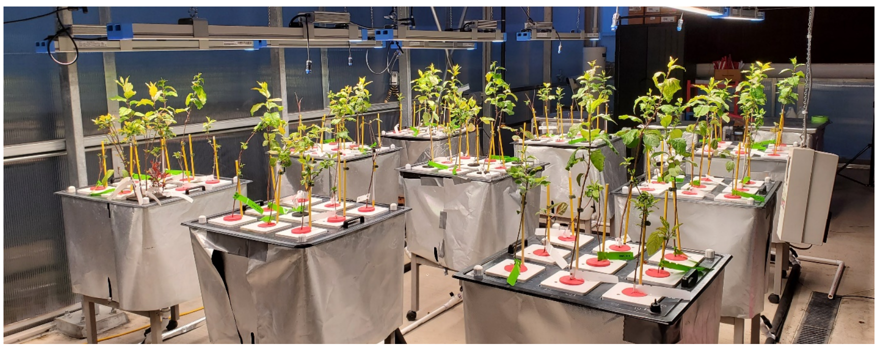

Figure 6. Several apple rootstocks including G.890, G.935, and G.969 grafted with virus-laden (green tags) and virus-free (white tags) versions of a 'Honeycrisp' strain being grown in aeroponics to test if root growth is being repressed by viruses.

Figure 7. Apple trees grown in aeroponics to gain easy access to roots during experiments.

several rootstocks including G.890 (symptomless), G.935, and G.969 grafted with virus-laden (V) and virus-free (VF) versions of a 'Honeycrisp' strain. The aeroponic system allows easy access to roots (Figure 7) for studying gene expression and measuring growth.

Conclusions

Viruses are detrimental for apple production and the nursery industry; therefore, they should be avoided whenever possible. Geneva® rootstocks G.41, G.202, G.222, G.214, and G.890 have not displayed the hypersensitivity of G.814 and G.16 or the slow decline that G.935 or G.969 experiences with certain virus-laden scion cultivars. Recent experiences with G.969 grafted with virus-laden 'Granny Smith' have shown that G.969 seems to have similar sensitivity as G.935 to one or a combination of latent viruses. Interestingly, in a current field trial with virus-laden 'Granny Smith' which includes G.969 and G.814, only G.969 is struggling, whereas G.814 seems to be growing well, perhaps indicating some genetic specificity to the viral factors in this strain of 'Granny Smith'. The discovery that heat therapy combined with cryotherapy is able to eradicate even one of the most recalcitrant viruses is a big step toward the eradication of viruses from the breeding program. The Geneva rootstock breeding program is committed to understanding the genetic basis of this phenomenon and to providing virus free or 'cleaned' material to the industry.

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