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Brief Report

Manipulating the structure of citrus tristeza virus populations

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Abstract
Interaction between viruses is one of the major factors that determines viral population structure or equilibrium, which is a determinant of virus pathogenesis. If we could manipulate virus interactions, we could potentially limit the effects of disease. Using citrus tristeza virus (CTV) as a model, we examined if we could alter the equilibrium of a population by adding different CTV genotypes or other citrus pathogens. We found that population structure could be altered through the addition of specific CTV genotypes, disrupting existing interactions and selectively changing the titer of specific genotypes, while the addition of other citrus viruses or viroids did not have an effect.

Keywords: CTV genotypes, Population equilibrium, Pathogens interaction

Introduction
Interaction between viruses is, along with the fitness of individual viruses and the host species, one of the major factors that determines the viral population structure (the ratio of one virus to another) and/or equilibrium (Harper et al. 2015a). The equilibrium reached is important for it has been demonstrated that the structure of a population is a determinant of pathogenesis for both animal (Domingo et al. 2012) and plant viruses (Syller and Grupa 2014). While many virus-virus interactions synergistically increase virus virulence or pathogenicity (Harper et al. 2015a, 2015b; Karyeija et al. 2000; Scheets 1998; Untiveros et al. 2007), others produce the opposite effect: preventing movement, accumulation, or expression of the pathogenic isolates, and limiting the effects of disease (Capote et al. 2006; Harper et al. 2015a, 2015b; Syller and Grupa 2014).

If we could induce negative virus-virus interactions, or disrupt existing synergisms, we could potentially limit the effects of disease. The non-random nature of virus populations (Harper et al. 2015a) highlights the feasibility of manipulating virus-virus interactions against pathogenic isolates. However, understanding the conditions under which competition or antagonism occur within a population is a requirement to manipulate a population in a predictable manner.

We have been using citrus tristeza virus (CTV) as a model to study the dynamics of virus populations as this virus has 8 genetically distinct genotypes or “strains” that show marked differences in infectivity and transmissibility (Harper 2013; Harper et al. 2015b; Yokomi et al. 2018), and are frequently found to occur as mixed populations in the field (Brlansky et al. 2003;

Scott et al. 2013). We previously have demonstrated that specific genotypes of CTV are capable of positive interaction, for example, complementation of genotypes such as T36 to allow systemic infection of selective host species (Harper et al. 2015a; 2015b). This interaction was both genotype- and host-specific and provided a means for the survival and spread of tropism-limited genotypes in the environment (Harper et al. 2015b).

But what of negative interactions? Are there conditions under which CTV populations may be manipulated and potentially pathogenic genotypes suppressed? We previously reported that abiotic factors such as elevated temperature can shift population structure. Yet this effect is temporary; the population will revert once the stimulus is removed (Cowell et al. 2016). In contrast, the interactions between viruses are more stable, tending towards equilibrium unless new, potentially interacting viruses are introduced (Harper et al. 2015a). Therefore, in this study we examined whether the addition of either new CTV genotypes, or other citrus viruses or viroids, could alter the population equilibrium in a field-derived CTV isolate in a selective host.

Materials and Methods

Given the effect population composition has on equilibrium in CTV-genotype selective hosts, we examined whether we could force a change in an established population through the introduction of another CTV genotype. We graft inoculated 26 *Citrus sinensis* cv. Valencia sweet orange seedlings (30 to 40 cm in size) with isolate FS627, which was originally obtained from a citrus grove in central Florida and contains CTV genotypes T36, T30, and VT (Brlansky 2003). The population was left to equilibrate for 12 weeks under greenhouse conditions, with an ambient temperature of 25 to 30°C. Samples were then taken from leaf midrib and young flush growth from around each plant and pooled for total RNA extraction using Trizol reagent (LifeTechnologies, Carlsbad, CA), as per the manufacturer's instructions. The successful introduction of FS627 was confirmed by RT-qPCR as per Harper et al. (2015a) and isolate T68-1 was then introduced into half of the plants through graft inoculation, with the other half of the plants left un-challenged as controls. The population was again left to equilibrate for 12 weeks under greenhouse conditions. The population structure was quantified again as previously described except where, because VT and T68 are both amplified with the same ORF1b-p33 primer/probe set, additional primer/probe sets targeting genotype-specific sites in ORF1a were used instead. T68 titer was quantified using generic ORF1a primers: (Sense: 5'-TCGATGGTCGCYRTCCCRGTGC-3' and antisense: 5'-GTYTCAGCSGCATGRTAGTY-3'), and T68 specific probe (5'-6-FAM-AGCATTGCCCACT ACGGCTTGG-BHQ1-3'), while VT was quantified using primer/probe set VT-2 from Ananthakrishnan et al. (2010). Differences between challenged and un-challenged CTV populations were examined by one-way analysis of variance, followed by Tukey's post-hoc test.

Given that citrus pathogens are rarely found in the field as single infections, we examined whether we could force a change in an established CTV population through the introduction of other common citrus-infecting pathogens. To investigate this FS627 was challenged with Florida isolates of citrus leaf blotch virus (CLBV) and citrus tatter leaf virus (CTLV) in *Citrus aurantium* cv. California Standard sour orange and citrus exocortis viroid (CEVd) in *Citrus medica* cv. Etrog citron. This investigation was carried out as described above, except where petiole and leaf blade tissues were included in addition to samples from leaf midrib and young flush growth, and RT-qPCR of CLBV, CTLV, and CEVd, was carried out using published assays from Cowell et al. (2018), Cowell et al. (2017), and Monger et al. (2010) respectively.

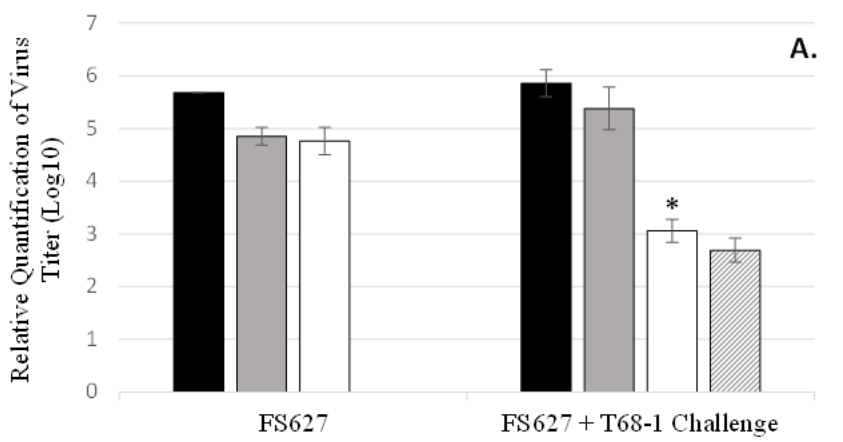
Results and Discussion

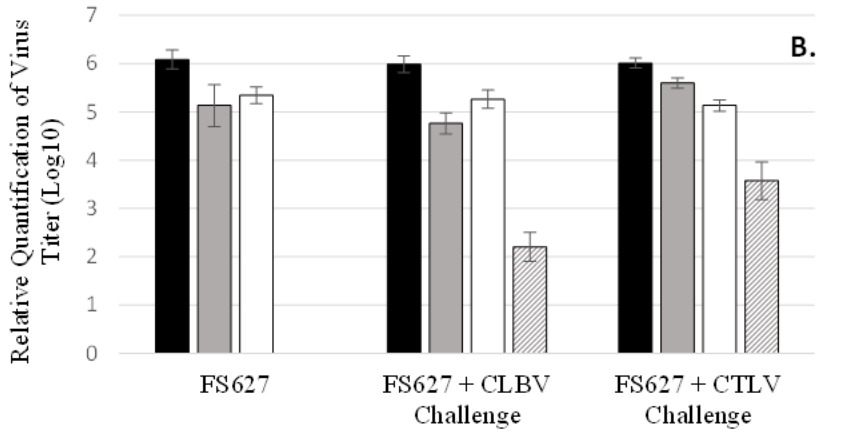
We had previously reported that the addition of challenge isolates can cause a shift in the equilibrium of a CTV population (Harper et al. 2015a). However, these were artificially constructed populations made from well-characterized single-genotype isolates. We wondered whether the same would hold true of a field-derived population whose components had equilibrated over time when challenged by an additional CTV genotype. We also examined whether the introduction of other citrus-infecting viruses or viroids could affect the CTV population structure because, given their long lifespan, individual field-grown citrus accumulate a number of viral species and other pathogens over time (Cowell et al. 2018).

In this study we inoculated *Citrus* spp. seedlings with field isolate FS627, comprised of genotypes T36, T30, and VT, and once equilibrated, introduced isolate T68-1, the type-isolate of genotype T68 (Harper 2013), CLBV, CTLV, or CEVd. These two viruses and viroid were selected on the basis of their prevalence in Florida citrus (Cowell et al. 2018) and source availability, and hosts for each of these challenge experiments were selected to favor the accumulation of the challenge virus or viroid (Harper et al. 2014; Bernad et al. 2009). At 12 weeks post-challenge we found that the addition of T68 altered the population structure relative to unchallenged controls, and produced a significant decrease of approximately 67 fold in the titer of the VT genotype; the T36 and T30 genotypes were not significantly affected (Figure 1). This would suggest that, as with artificial populations, field isolates can be disrupted by the introduction of an interacting or competing CTV genotype, raising the intriguing possibility of controlling disease through manipulation of the virus population.

In contrast, the addition of CTLV, CLBV, or CEVd had no significant effect on structure or overall titer of the co-infecting CTV population (Figure 1). This may be due to an inability to interact due to different tissue or cellular tropism, though this is not necessarily a barrier to interaction. Phloem-limited sweet potato chlorotic stunt virus has been shown to be able to enhance the accumulation of sweet potato feathery mottle virus in dual-infected sweet potato (Karyeija et al. 2000). Instead, the gene products of the other viruses may not be able to interact directly or indirectly with CTV in a meaningful way, such as through co-suppression of host defenses (Karyeija et al. 2000; Syller 2012). The highly specialized and host-specific nature of CTV and its gene products (Tatineni et al. 2008) may preclude interaction with anything other than different CTV genotypes, as we have observed here. It may also be that the challenge viruses or viroid were unable to alter the CTV population equilibrium as they themselves did not cause disease during the experimental observation period or interact with the host in a manner that effected the coinfecting CTV population.

Irrespective of the mechanism, the ability to reduce the titer of specific genotypic strains or variants, and to disrupt beneficial or synergistic interactions within a population provides us with a tool to manipulate virus populations. Through empirical testing it may be possible to build stable cross-protective populations for the prevention of disease - if you have the appropriate variants. Furthermore, our work demonstrates the need to identify and map the viruses that can interact within a specific host, for one cannot assume that all viruses will interact, and of those that do, not all interactions may have a desired outcome.


A.
Relative Quantification of Virus Titer (Log10)
0
1
2
3
4
5
6
7
*
FS627
FS627 + T68-1 Challenge


B.
Relative Quantification of Virus Titer (Log10)
0
1
2
3
4
5
6
7
FS627
FS627 + CLBV Challenge
FS627 + CTLV Challenge

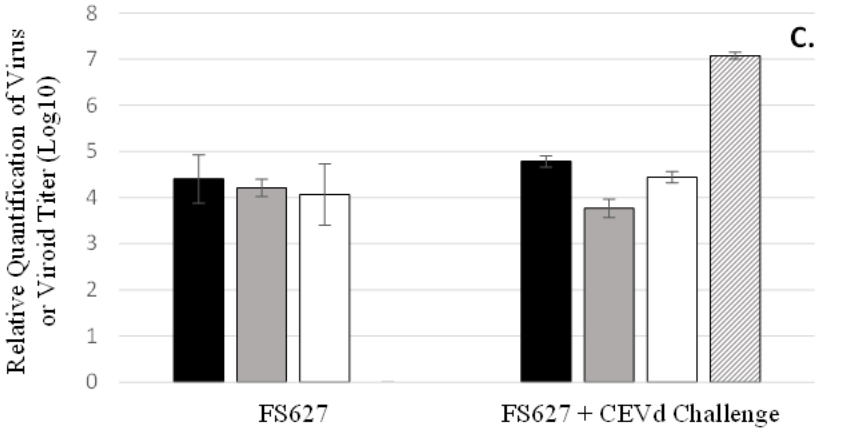

C.
Relative Quantification of Virus or Viroid Titer (Log10)
0
1
2
3
4
5
6
7
8
FS627
FS627 + CEVd Challenge

Figure 1. Comparison of the titer of CTV genotypes T36 (black), T30 (grey), and VT (white) from isolate FS627 in sweet orange (A.), sour orange (B.), or citron (C.) plants challenged (grey diagonal) with CTV genotype T68, citrus leaf blotch virus (CLBV), citrus tatter leaf virus (CTLV), or citrus exocortis viroid (CEVd) at 12 weeks post-challenge inoculation. Significant changes ($P<0.05$) are indicated with an asterisk (*).

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