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Evidence of curtovirus competition and synergy in co-infected plant hosts

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Curtoviruses, members of the Geminiviridae, have wide host ranges, including weeds and crops and are often found in mixed infections of different strains. While other members of the Geminiviridae have been demonstrated to interact through competition and synergism in mixed infections in plants, either type of interaction has not been reported in curtoviruses. This research used qPCR to study the interactions between *Beet curly top virus*, pepper curly top strain, isolate BV3 (BCTV-PeCT-BV3) and *Beet curly top virus*, beet severe curly top strain (BCTV-Svr) in three plant hosts. A significant decrease in virus titer in both BCTV-PeCTV and BCTV-Svr in co-infected sugar beets was observed when compared to beets infected with either virus, indicating competition. Chile pepper showed a significant increase in BCTV-PeCT titer in co-infected plants, compared to singly infected plants, indicating synergism. BCTV-PeCT caused severe symptoms and yielded high virus titer in chile, compared to the lack of symptoms and extremely low titer of BCTV-Svr in that plant host. These results indicate that curtovirus symptoms and infection can be host specific and such host may influence mixed infections of virus. Curtoviruses can interact through both competition and synergism and the response may be dependent on the type of host plant.

Key words: *Beet curly top virus*, interspecific competition, host specificity, mixed infections

INTRODUCTION

Curly top disease is caused by members of the genus *Curtovirus*, within the family *Geminiviridae* (Bennett, 1971; Soto et al., 2005; Varsani et al., 2014). Curtoviruses are transmitted among dicot hosts by beet

leafhopper [*Circulifer tenellus* (Baker)] in a circulative persistent manner (Soto and Gilbertson, 2002). Curtovirus host range is broad and includes crops as chile pepper [*Capsicum annuum* (L.)], tomato [*Solanum*

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lycopersicum (L.)] and sugar beet [*Beta vulgaris* (L.)] as well as weeds such as London rocket [*Sisymbrium irio* (L.)], Russian thistle [*Salsola iberica* (Sennen and Pau)], and Kochia [*Kochia scoparia* (L.) Roth] (Creamer et al., 2005; Lam et al., 2009). In the field, infected weeds are usually asymptomatic while crops develop severe symptoms (Lam et al., 2009). Symptoms of curtovirus-infected chile pepper plants are stunting, chlorosis, thickened curled leaves, hyperplastic phloem growth, and reduced fruit set (Creamer et al., 2003), while curtovirus symptoms on sugar beets include stunting, chlorosis, severe leaf curling, and production of punctate growths on leaf veins (Bennett, 1971).

Co-infections with two or more viruses at the same time are commonly found in field samples (Roossinck, 2005) and can be beneficial to at least one of the viruses or antagonistic, where the co-infection is deleterious to one of the viruses. Co-infection is known to affect symptomology, host range, pathogen diversity, transmission rates, and infection prevalence (Lacroix et al., 2014). Since virus replication is an immediate indicator of competition for resources, changes in virus titer has been used to experimentally demonstrate competition between viruses (Roossinck, 2005; Hall and Little, 2013; Salvaudon et al., 2013).

Synergy between virus species is known to occur within the same genus and across virus families. Synergy has been reported between *Cucumber mosaic virus* (CMV) and potyviruses in cucurbits (Wang et al., 2002), potyviruses and *Potato virus X* (PVX) (Bowman-Bance 1991; Gonzalez-Jara et al., 2004), *Potato leafroll virus* and *Potato virus Y* (PVY) (Srinivasan and Alvarez, 2007) and among begomovirus species (Rentería-Canett et al., 2011).

Changes in virus transmission from co-infected hosts could also be an indicator of competition. Joint infection of two criniviruses, *Tomato infectious chlorosis virus* (TICV) and *Tomato chlorosis virus* resulted in the increased concentration and higher transmission efficiency of TICV (Wintermantel et al., 2008).

Order in which viruses are acquired can influence transmission, in that sequential transmission of two viruses, can favor transmission of one virus over another. For example, when whitefly vectors were allowed to acquire two strains of *Tomato yellow leaf curl virus* sequentially, 75% of the whiteflies transmitted both strains of the begomovirus and 25% only the first strain that they acquired (Ohnishi et al., 2011).

Host specificity also plays a role in curtovirus infection. *Beet curly top virus* spinach curly top strain (BCTV-Sp) infects a wide host range including spinach, sugar beet, bean and *Nicotiana benthamiana*, but not tomato or Samsun tobacco (Baliji et al., 2004). *Beet curly top virus* Worland strain (BCTV-Wor) causes mild symptoms on sugar beet, but severe symptoms on a variety of dicot species including tomato and pepper (Stenger et al., 1990). In contrast BCTV-Svr causes severe symptoms

on sugar beet and mild symptoms on most other hosts (Bennett et al., 1971).

Mixed infections of curtovirus are common (Stenger and McMahon, 1997; Strausbaugh et al., 2008; Lam et al., 2009; Chen et al., 2010). Mixed infections of curtoviruses from sugar beet nursery samples showed that, more than one strain or type was easily identified in mixed infections (Stenger and Ostrow, 1996). In New Mexico, *Beet curly top virus*, severe pepper strain (BCTV-SvrPep), BCTV-PeCT, *Beet curly top virus*, Worland strain (BCTV-Wor), and *Beet curly top virus*, pepper yellow dwarf strain (BCTV-PeYD) are prevalent in chile peppers, often in mixtures (Lam et al., 2009; Varsani et al., 2014). Preliminary research on serial acquisition of two of these viruses showed that once leafhoppers acquired BCTV-Svr, they could no longer acquire BCTV-PeCT. These results prompted a series of experiments designed to assess the potential for intrahost interaction as measured by changes in virus titer in crops and weed hosts.

MATERIALS AND METHODS

Plants, viruses, and leafhopper sources and maintenance

Plants used for greenhouse experiments were grown from seed. Plants used were Chile (*Capsicum annuum*) var. NM 6-4, which is susceptible to curtoviruses, Sugar beet [*Beta vulgaris* (L.)] highly susceptible to curtoviruses and a host of the beet leafhopper, grown from seed that was a gift of Robert Lewellen, USDA, Salinas, CA, USA and Kochia [*Kochia scoparia* (L.) Roth], a weed, known to be susceptible to curtoviruses and a host of the beet leafhopper, grown from seed collected near Las Cruces, NM, USA.

Beet leafhoppers were collected from Idaho (gift from Carl Strausbaugh, Kimberly, ID, USA) and maintained on BCTV-Svr-infected sugar beets and Kochia in a growth chamber at 29°C with 15 h of light.

BCTV-Svr(US-SVR-Cfh), abbreviated in this paper as BCTV-Svr was obtained from Idaho (gift of Carl Strausbaugh) and was maintained on sugar beets. BCTV-PeCT (US-NM-Pep-05) was collected from infected sugar beets near Las Cruces, NM, maintained on sugar beets, and used only for preliminary studies. BCTV-PeCT (US-CA- BV3) (GenBank JX487184.1) abbreviated in this paper as BCTV-PeCT-BV3, was originally isolated from tomato in California by Chen and Gilbertson (2009). This isolate was used for all but in the initial experiments. All strain designations were confirmed by PCR and sequencing prior to their use (Lam et al., 2009).

Agroinoculation of plants with BCTV-PeCT-BV3

Agro inoculation was used to infect plants with BCTV-PeCT-BV3 because leafhoppers are not able to acquire the virus from chile plants due to the toxicity of the plants to the insects (Sedano et al., 2012). An infectious clone (EHA105-BV3) of BCTV-PeCT isolate BV3 was produced by inserting a tandem dimeric construct into a binary vector pCGN1547 (Chen and Gilbertson, 2009). The clone was maintained in *Agrobacterium tumefaciens*. Cultures were incubated at 27°C for a minimum of 2 h prior to inoculation (Sedano et al., 2012).

Sugar beet, chile, and Kochia were planted in Metro-Mix 360 (Sun Gro Horticulture), maintained in an insect-free greenhouse and inoculated 10 to 15 days after germination by vascular puncture with 4 µl of culture containing the clone (Sedano et al.,

2012). After inoculation, plants were incubated in a growth chamber at 28°C with 15 h of light for 2 days, and then transferred to a greenhouse for 14 days.

Leafhopper transmission of BCTV-Svr

Plants were inoculated with BCTV-Svr by exposing them to feeding by, five adult leafhoppers that were reared on BCTV-Svr-infected sugar beets. Insects were inserted in leaf cages and allowed to feed for 16 to 18 h. Plants were incubated 14 days in a growth chamber at 28°C with 15 h of light.

Plants previously inoculated with BCTV-PeCT-BV3 were inoculated with BCTV-Svr after 14-day recovery incubation in the greenhouse. Plants were not assessed for virus presence after the initial inoculation and prior to the second inoculation due to their small size.

Primer design

Primers were developed that amplify the viral coat protein genes (CP) of BCTV-PeCT-NM (GenBank EF501977.1), BCTV-PeCT-BV3 (GenBank JX487184.1) and BCTV-Svr (Genbank U02311.1). The primer set PeCTVIIF (5'-GGAGTGCCTCGAGAGAACAAC-3') and PeCTVIIR (5'-GCTTAGTAACGGTTATATTGTTGG-3') amplify a 400 bp fragment of the BCTV-PeCT CP that was used for detection by conventional PCR. CPIIF (5'-GTATCCATCAAGAGATAGAG-3') and CPIIR (5'-CGTCACAGTAACGTTCTTC-3') amplify a 402 bp CP fragment from BCTV-Svr which was used for both conventional and qPCR. PeCTVIIF and BV3R (5'-CCCTCGTGAGAGGACGT-3') amplify a 254 bp CP fragment from BCTV-PeCT-BV3 that was used for qPCR.

Virus detection and quantification by PCR

To determine if plants were infected with curtovirus, total DNA was extracted from young plant tissue, 14 days post infection (dpi) by alkaline lysis (Dellaporta et al., 1983) and quantified with Nanodrop ND-1000 (Thermo Scientific).

PCR reactions were carried out using GoTaq Flexi DNA Polymerase (Promega), following the manufacturer's guidelines. PCR parameters were as follows for CPIIF-CPIIR and PeCTVIIF-BV3R reactions: 94°C for 3 min, followed by 35 cycles consisting of 94°C for 30 s, 55/65°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min.

Virus titer was determined for all infected plants using quantitative PCR. Two independent groups of plants were incubated for 28 days post inoculation (dpi) and 56 dpi to allow symptom development before DNA extraction. BCTV-PeCT-BV3 and BCTV-Svr titers from plants infected with both viruses were quantified in independent qPCR reactions.

qPCR parameters were as follows: 94°C for 3 min, followed by 35 cycles consisting of 94°C for 30 s, 55°C (CPIIF/CPIIR) or 56.5°C (PeCTVIIF/BV3R) for 30 s, 30 s at 72°C for 30 s, followed by a final 72°C for 10 min. The temperature range for melting curve analyses was from 55 to 95°C rising 0.5 °C for 0.5 s for both primer pairs. Reactions were carried out in an IQ5 thermocycler (Bio Rad) using IQ SYBR green supermix (Bio Rad) at a final reaction volume of 20 µL with 2 µL of template and 0.5 µM primers.

The qPCR amplification products were used as reference amplicons for standard curves. After electrophoresis on an agarose gel, BCTV-Svr and BCTV-PeCT-BV3 PCR amplicons were extracted with a QIAquick gel extraction kit (Qiagen) and virus DNA concentration determined. Virus titer was calculated through the amplification of a five fold dilution series of viral DNA for BCTV-PeCT-BV3 from 1.027×10^7 ng/µL to 3.286×10^3 ng/µL and

BCTV-Svr from 8.020×10^6 ng/µL to 2.566×10^3 ng/µL.

BioRad IQ5 software automatically calculated the fluorescence threshold and a regression line was calculated with the threshold cycle ($C_{(t)}$) of serial dilution of standards. This equation used the molecular weight of curtovirus genomes to estimate the virus copy number per nanogram of total DNA in the samples.

Statistical analysis

SAS 9.2 (SAS Institute Inc., Cary, NC, USA 1989-2007) was used to conduct a regression analysis of qPCR standard curve amplification $C_{(t)}$ values and to compare the virus titer in infected plants. Simple linear regression was used by BioRad IQ5 software to produce a linear equation that relates RFU to $\Delta(C_{(t)})$. The same data was used to produce a linear regression analysis in SAS and calculate the confidence intervals of the regression at $\alpha=0.05$.

Normal distribution of virus titre data sets was tested by Kolmogorov-Smirnov goodness of fit test. Homogeneity of variance between data sets was tested with Levene's test. Correlation between BCTV-PeCT-BV3 and BCTV-Svr titers in co-infected beets and chile were tested, using the Spearman's non-parametric correlation analysis. A two-way factorial ANOVA was run to determine the relationship between the virus titer, virus species and infection status on sugar beets using JMP (SAS Institute Inc., Cary, NC, USA 1989-2007). Pairwise comparison between virus titers in co-infected chile plants was done using Kruskal-Wallis rank sum test. The significance level for all the analyses was set at $\alpha=0.05$.

RESULTS

When initial leafhopper transmission tests were done by allowing leafhoppers carrying BCTV-Svr to acquire BCTV-PeCT-NM from infected sugar beets, and transmit to either sugar beets or chile, only BCTV-Svr was transmitted. The results were identical independent of the number of leafhoppers/plant (5 or 1) used for transmissions or source sugar beet plant (four attempted) used.

BCTV-PeCT-BV3 and BCTV-Svr were quantified using qPCR. Plants infected by a single virus were used to establish a titer baseline to determine if, co-infection influenced viral titer in sugar beet, chile, and kochia (Table 1). Melting curve analysis of the amplicon obtained from BCTV-PeCT-BV3 gave a primary peak at 84°C while that of BCTV-Svr gave a primary peak at 82°C (Figure 1). Neither amplicon produced secondary peaks indicating that, there was no primer dimer formation or nonspecific amplification. Quantification of BCTV-PeCT-BV3 was effective within the range of 1.027×10^7 to 1.6432×10^4 ($r^2=0.995$), while BCTV-Svr was quantified within the range of 8.020×10^7 to 1.2832×10^4 ($r^2=0.994$) (Figure 2).

Virus titer was significantly linked to both infection status and virus species ($p \leq 0.0001$), where the infection status is defined as a plant being infected by one virus alone or in combination of both (Supplementary Table 1S). While 22 out of 28 sugar beet plants pre-inoculated with BCTV-PeCT-BV3 were positive for both viruses, the remaining six did not become infected with BCTV-Svr. BCTV-PeCT-BV3 titer was significantly different in co-

Table 1. Range of viral titers based on infection type and host.

Virus	Infection	Host	Number infected plants/total	Titer log ₁₀ copies/ng	
				Range	Average ±SEM
BCTV-PeCT	Single	Beet	10/10	3.50 - 4.70	4.25±0.2
BCTV-Svr	Single	Beet	10/11	5.79 - 7.52	6.70±0.2
BCTV-PeCT	Mixed	Beet	22/28	1.72 - 4.00	3.28±0.1
BCTV-Svr	Mixed	Beet	22/28	3.37 - 6.74	5.47±0.1
BCTV-PeCT	Single	Chile	15/30	4.99 - 5.34	4.71±0.1
BCTV-Svr	Single	Chile	4/11	-	BT
BCTV-PeCT	Mixed	Chile	8/15	4.22 - 5.15	5.14±0.0
BCTV-Svr	Mixed	Chile	8/15	-	BT
BCTV-PeCT	Single	Kochia	8/384	-	BT
BCTV-Svr	Single	Kochia	3/10	-	BT
BCTV-PeCT	Mixed	Kochia	0/8	-	-

SEM=standard error of the mean, BT= below quantification threshold, BCTV-PeCT=BCTV-PeCT-BV3.

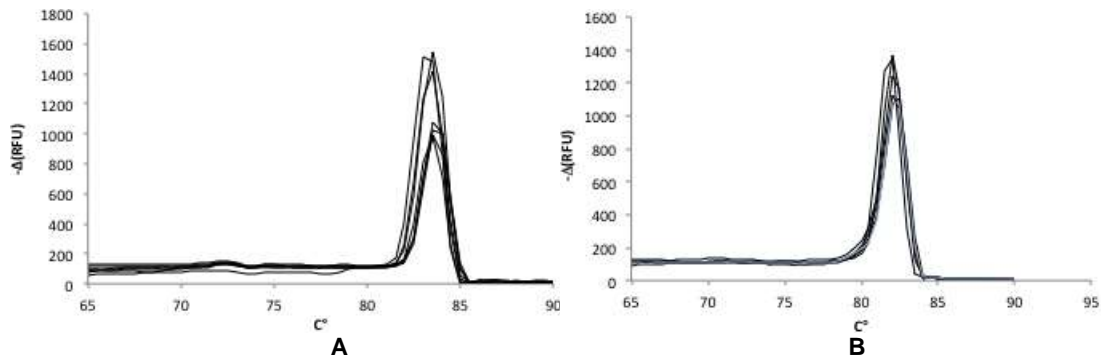


Figure 1. Melting curve analysis of (a) BCTV-PeCT-BV3 amplification indicating the melting point at 84°C (upper) and (b) BCTV-Svr amplification indicating the melting point at 82°C (lower).

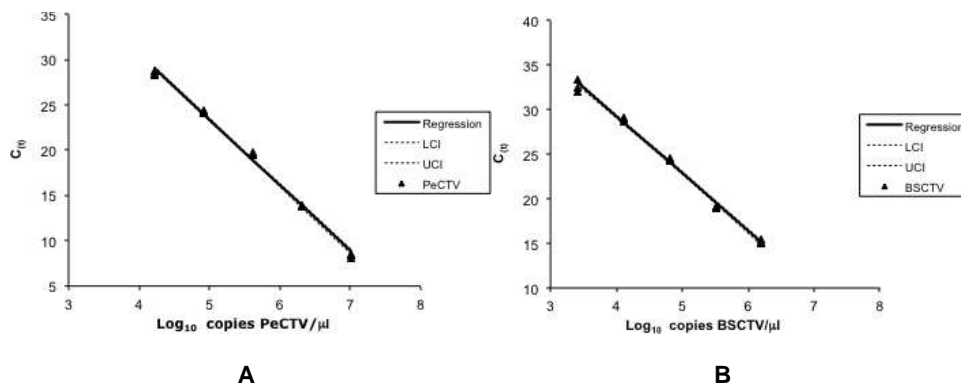


Figure 2. Representative qPCR standard for (a) BCTV-PeCT-BV3 (PeCTV) (upper) and (b) BCTV-Svr (BSCTV) (lower).

infected plants (2.8838×10^3 copies/ng) (p -value =0.0020) when compared to singly infected plants (2.6504×10^4

copies/ng) (p -value ≤ 0.0001). BCTV-Svr titer was significantly different in co-infected plants (1.1365×10^6

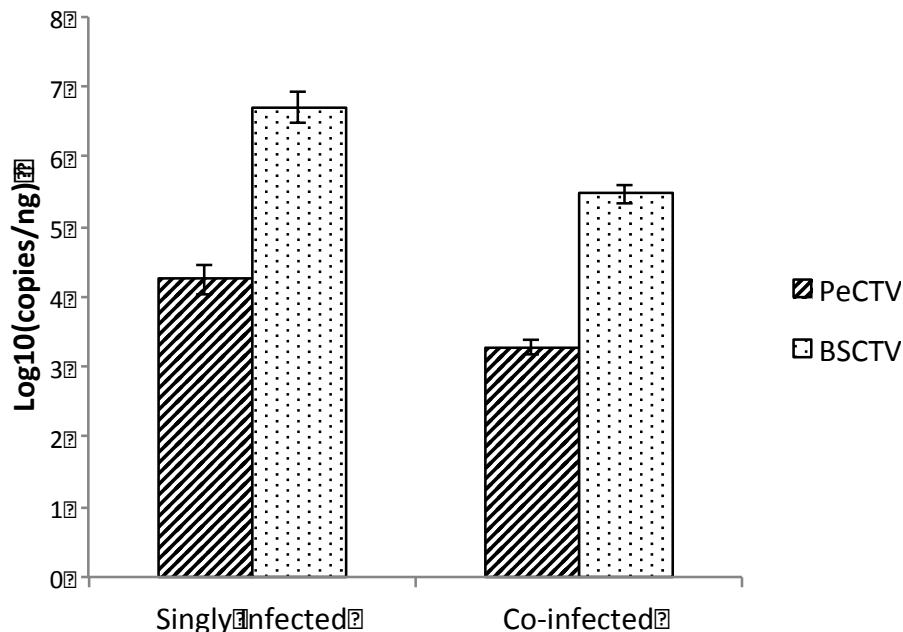


Figure 3. Comparison of average virus titer for BCTV-PeCT (PeCTV) and BCTV-Svr (BSCTV) in singly and co-infected beet plants $\alpha=0.05$.

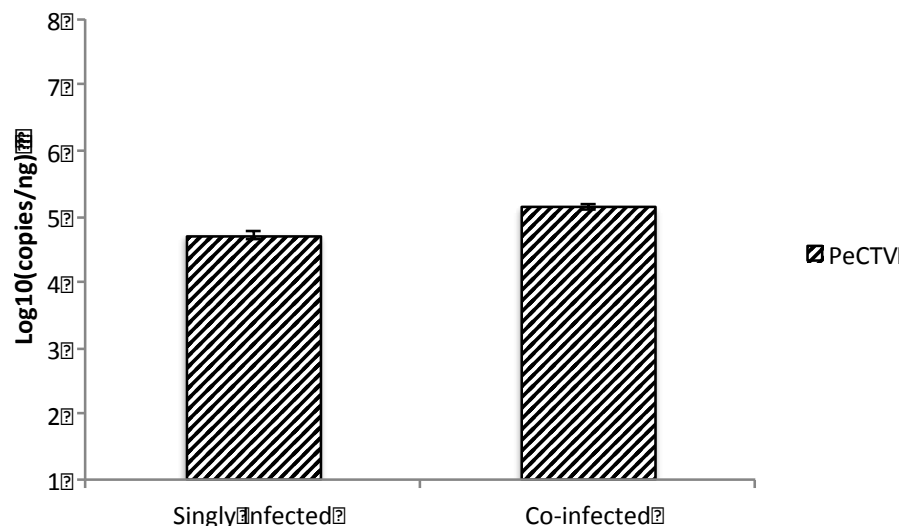


Figure 4. Comparison of average titer for BCTV-PeCT (PeCTV) in singly and co-infected chile plants. $\alpha=0.05$.

copies/ng) when compared to singly infected plants (1.1806×10^7 copies/ng) (p -value ≤ 0.0001) (Figure 3).

In contrast, 8 out of 15 chile plants pre-inoculated with BCTV-PeCT-BV3 were positive for both viruses. BCTV-PeCT-BV3 titer was lower in singly infected plants (5.8642×10^4 copies/ng) when compared to co-infected plants (1.4541×10^5 copies/ng) ($p=0.0003$, $DF=1$, $\chi^2=13.0538$) (Figure 4). Chile plants infected with BCTV-Svr alone or with both BCTV-Svr and BCTV-PeCT-BV3 tested positive for BCTV-Svr with PCR but fell below the

quantification threshold in qPCR. Chile plants infected with BCTV-PeCT-BV3 alone or in a mixed infection exhibited severe infection symptoms, and no change in symptoms was observed when infected with both viruses.

While over 380 Kochia seedlings were inoculated with BCTV-PeCT-BV3 infectious clone, only 8 plants tested positive for BCTV-PeCT-BV3, and none of these were infected with BCTV-Svr after leafhopper inoculation. The BCTV-PeCT-BV3 titer in these eight plants fell below the quantification threshold. Infection with BCTV-Svr alone

was determined in three *Kochia* plants by PCR, but the estimated virus titer was below the quantification threshold by qPCR.

DISCUSSION

A variety of factors influence the infection of a plant host by more than one virus. Mechanism of transmission, host range, order of infection, and viral interactions can affect, if a plant will be infected, the titer of the viruses, and the symptoms induced. This study views the effect of coinoculation of curtoviruses onto different hosts. Presence of BCTV-PeCT-BV3 in chile pepper and beets had a significant impact on the likelihood of the host to be infected by BCTV-Svr. In beets infected with both viruses, the titer of both was lower when compared to plants infected by either one. The BCTV-Svr titers in plants infected with BCTV-Svr and BCTV-PeCT-BV3 were 1 to 2 orders of magnitude lower than those of plants infected with BCTV-Svr alone. This is similar to the preliminary results of Wintermantel (2011) which found BCTV-Svr titer alone in beets for at least 100 times higher than in plants infected with both BCTV-Svr and BCTV-Wor. The decrease in titer of both viruses in co-infected beets is suggestive of within-host competition, which had not been reported for BCTV-PeCT.

Viral interactions between species have been shown to change infection rates of each viral species compared with single species inoculations for closely related potyviruses and luteoviruses, but rarely for geminiviruses. Alves et al. (2009) found that *Tomato yellow spot virus* (ToYSV) established infection and accumulated to higher concentration earlier than *Tomato rugose mosaic virus* (ToRMV) in tomato and tobacco. Interestingly, ToRMV appears to interfere with ToYSV only during early infection. Mixed infections of potyviruses, *Watermelon mosaic virus* (WMV) and *Zucchini yellow mosaic virus* (ZYMV) were asymmetrical in that, while ZYMV replicated similarly in single and mixed infections, WMV accumulated to significantly lower levels in the presence of ZYMV than in single infections (Salvaudon et al., 2013). Studying mixed infections of luteoviruses, Lacroix et al. (2014) found that BYDV-PAV reduced the infection rate of CYDV-RPV, while CYDV-RPV had no effect of the infection rate of BYDV-PAV. Hall and Little (2013) found that BYDV-PAV dominated all mixed infections of wheat with BYDV-PAS, independent of the order of inoculation or length of time between inoculations. BYDV-PAV was also more likely to be aphid transmitted from a mixed infection. The authors speculated that the strength of the cross protections might be related to dose of the first virus or rate of movement through the phloem from site of inoculation.

Order of inoculation appeared to have an effect only in the preliminary experiments. For those experiments, allowing leafhoppers to acquire BCTV-Svr first appeared

to interfere with acquisition of BCTV-PeCT-NM. However, for the balance of the experiments, order of inoculation did not appear to have had an effect on level of virus in our experiments, although it was not specifically tested. BCTV-PeCT-BV3 was inoculated first onto beets and was still dominated by BCTV-Svr in the mixed infection on this plant.

Inoculum delivery could have potentially been a factor in our results since BCTV-PeCT-BV3 was inoculated by agro inoculation and BCTV-Svr was inoculated using leafhoppers. However, Chen and Gilbertson (2009) showed that both inoculation methods, agroinoculation and leafhopper transmission, gave similar results when inoculating BCTV-Svr onto a range of plants.

Our study also shows evidence of viral synergy. In chile, BCTV-PeCT-BV3 titers were higher in the presence of BCTV-Svr compared with chile infected with BCTV-PeCT-BV3 alone. In contrast, in beets, both viruses showed decreases in virus titer in mixed infections. Synergism has been reported to occur with begomoviruses such that when *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV) co-infect a host, both symptom severity and EACMV titer increased due to trans-complementation by ACMV (Fondong et al., 2000). Rentería-Canett et al. (2011) found that co-infection of *Pepper huasteco yellow vein virus* and *Pepper golden mottle virus* had a synergistic effect that increased disease symptoms as well as the titer of both viruses. Similarly, Morilla et al. (2004) found that co-infection of begomoviruses *Tomato yellow leaf curl virus* and *Tomato yellow leaf curl Sardinia virus* (TYLCSV) increased symptom severity and infected even the same nuclei. BCTV has been shown to have a positive synergistic effect on virus titer (but not the symptom severity) of TYLCSV due to the replication enhancer of BCTV (Caracuel et al., 2012).

Quantification of both virus species in co-infected and singly infected plants showed clear indications that both BCTV-PeCT-BV3 and BCTV-Svr exhibit host specificity. BCTV-Svr titer was significantly higher than BCTV-PeCT-BV3 by three orders of magnitude in beets (Table 1). Chile plants infected with BCTV-Svr by leafhoppers in greenhouse and growth chambers routinely test positive for infection but fail to develop severe symptoms, if any at all (Sedano et al., 2012). In contrast, all chile plants that were infected with BCTV-PeCT-BV3 exhibited symptoms of infection. BCTV-PeCT-BV3 appears to be better adapted to peppers and replicates to high levels in this host. In our laboratory setting, BCTV-Svr was maintained in sugar beets and transmitted by leafhoppers. BCTV-Svr is known to be better adapted to infection of sugarbeets, and causes much more severe symptoms on sugar beets than on other plant hosts (Strausbaugh et al., 2008). BCTV-Svr had a low rate of infection in chile, and did not develop high titers.

Curtovirus species are known to accumulate differently depending on the host plant (Chen and Gilbertson, 2009;

Strausbaugh et al., 2008; Wintermantel, 2011). Chen and Gilbertson (2009) showed that while most cucurbits (cantaloupe, honeydew, watermelon) had low rates of infection and mild or symptomless phenotypes when inoculated with BCTV-Wor, tomatoes and shepherd's purse (a mustard weed) had high rates of infection and were highly symptomatic when inoculated with the same virus. In comparison, all of these plants are likely to be infected and show symptoms when inoculated with BCTV-Svr.

A significant difference in the accumulation of virus in agricultural hosts versus Kochia, a weed host was found in this work. This difference in virus titer between agricultural and weed hosts has been previously observed in a field survey of curtovirus (Creamer et al., 1996), and by Chen and Gilbertson (2009) which found that infected shepherd's purse had about half the titer of BCTV-Wor as the infected by sugar beets. Since Kochia serves as a reservoir host for BCTV, BCTV-Wor, BCTV-SvrPep, BCTV-PeCT, and BCTV-PeYD (Lam et al., 2009) in the wild, we expected it to be easily infected in the laboratory. Both viruses in Kochia could be detected, but well below the quantification threshold. Since beet leafhoppers prefer Kochia as a feeding and reproductive host (Hudson et al., 2010), feeding preference does not account for the very low titers found. The method of inoculation, vascular puncture is destructive to young seedlings. This may have led to the very low numbers of infected plants.

Evidence of synergism, competition, and host preference were reported here. This is the first reports for synergism and competition in curtoviruses, it presents additional evidence for host preference within the virus group. Further studies should focus on the molecular mechanisms underlying these relationships, and the implications for these on mixed infections in field settings.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Supplementary Table S1. Two-way factorial ANOVA: The effects of the factors virus species (BCTV-PeCT-BV3, BCTV-Svr) and infection status (singly infected, co-infected) ($\alpha=0.05$).

Source	DF	Sum of squares	F Ratio	Pr>F
Virus	1	73.98	143.43	≤ 0.0001
Infection Source	1	16.61	32.21	≤ 0.0001
Virus * Infection Status	1	0.22	0.442	0.5087