Transmission of Barley/Cereal yellow dwarf viruses by aphid vectors

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Abstract

Barley and Cereal yellow dwarf viruses (B/CYDVs) negatively impact the fitness of host plants and have a destabilizing effect on established ecosystems. Plant and B/CYDV pathogen interaction dynamics can be significantly altered by increasing worldwide nitrogen and phosphorous input. Viruses rely on interactions between host plants, insect vectors and environmental factors in order to spread. Relationships between virus transmission and both the level of and ratio between host nitrogen and phosphorous resources are difficult to establish, especially in natural systems, and remain poorly understood. In order to assess the relationships between host resources and vectored-disease spread in controlled conditions, we developed a method of inoculation of healthy plants using aphid vectors that previously fed on virus infected fresh plant leaves. While we originally tested an inoculation method in which aphids fed on a homogeneous virus inoculum obtained by grinding and clarifying infected plant tissues, this resulted in low aphid survival and low virus transmission rate in our lab conditions. We will use the inoculation technique developed in this study to test the effects of host nutrient levels and ratios on virus transmission rate. The results obtained under laboratory conditions will allow us to make predictions on the broad connections between human nutrient loading and rates of viral infection.

Introduction

Viral infections in plants have huge impacts on both agriculture, where they are estimated to cost farmers over $300 billion annually (Anderson et al. 2004) and natural ecosystems (Webster et al. 2007). In natural grasslands in California, *Barley* and *Cereal yellow dwarf virus* (B/CYDV) infections contributed to shift the original state of dominance of native species in favor of exotic annuals through a mechanism of apparent competition (Power et al. 2011,

Malmstrom et al. 2005, Borer et al. 2007). Indeed, native perennials suffered from a greater fecundity reduction than annual plants when infected by BYDV's and annual exotics acted as amplifying hosts for both vectors and viruses, increasing viral prevalence in mixed grasslands over pure native perennial grasslands (Power et al. 2011).

It is imperative, then, that we understand how the processes of viral transmission and emergence are affected by human mediated environmental change. Human activities are currently adding significantly more nitrogen and eight times more phosphorous to global nutrient cycles than natural processes (Rockström et al. 2009). These nutrients are introduced into ecosystems through fertilization and through numerous manufactured products which contain phosphorous (Rockström et al. 2009). Studies suggest that increased levels of nitrogen and/or phosphorous are correlated with increases in viral prevalence in natural host communities (Borer et al. 2010, Seabloom et al. 2010). However, the effects of nutrient availability on disease spread in natural systems are difficult to separate from other environmental factors and covariates such as plant community composition. It is also difficult to examine the mechanistic aspects of this relationship. Manipulative experiments performed in laboratory conditions while controlling for both the level of and ratio between nutrients supplied to hosts will allow us to test whether viral prevalence is directly linked to nutrient availability or is indirectly altered through effects on vector preference and reproduction.

Nutrient Impacts on Plants and Secondary Effects on Higher Trophic Levels

The levels of and ratios between available of nutrients such as carbon (C), nitrogen (N), and phosphorous (P) are thought to change plant tissue chemistry, metabolic activity, plant biomass production, and plant community composition. Total biomass production by individual

plants has been shown to increase with increased levels of N and P (Seabloom et al. 2011). Increasing one of these nutrients can eventually induce limitation by the other unless it was already overly abundant, but increasing both caused a synergistic response in production (Richardson et al. 2002, Elser et al. 2007). These increases in biomass production by individual plants caused by long-term addition of high levels of nitrogen tend to reduce species diversity at the community level by allowing the plants which can take up the most resources to outcompete their neighbors (Haddad et al. 2000). Host nutrient supply has also been shown to alter the fecundity of herbivorous insects such as aphids (Borer et al 2009), which can have large effects on plants as both herbivores and virus vectors. Previously published studies suggested that increased aphid populations under conditions of elevated host nutrient supply were due to the relief of aphid N-limitation (Throop and Lerdau 2004), as well as to increased host attractiveness to herbivores because of decreased leaf mass per unit area and higher N concentration in leaf tissue (Wright et al. 2004). Many of these metabolic changes due to increased nutrients can be explained through the interaction of two nutrient modeling concepts. The first concept is that larger plants require greater support structures, such as vascular systems, and that these support structures have limited nutrient requirements in comparison to metabolically active tissue. This idea is known as the metabolic scaling theory or MST (Elser et al. 2010). The second concept is an understanding of the stoichiometric ratios of nutrients required for metabolically active tissue, known as the biological stoichiometry theory or BST (Elser et al. 2010). Nitrogen and phosphorous are generally the limiting nutrients for metabolic tissue under this theoretical framework. These theories provide a framework to study the ratios of N:P which are optimal for plant growth and metabolic activity. If the concentration of either nutrient falls below the threshold of the N:P ratio required by the plant, that nutrient will be limiting to biomass

production and metabolic activity (Geider and La Roche 2002, Karpinets 2006). On the contrary, as N and P are necessary primary components of RNA, DNA and protein (Elser et al. 2010), increased levels of these nutrients in optimal ratios for a plant resulted in an increased total biomass production and metabolic activity, including higher levels of RNA production (Elser et al. 2010; Elser et al. 2003), which allows for increased production of viral particles as the virus replicates by hijacking a cell's metabolic activity.

Nutrient Impacts on Viruses

While nutrient availability has drastic effects on both plants and herbivorous insects, viral infections could also rely on host nutrient availability. Viral pathogens are obligate parasites and thus use host metabolites and cellular machinery to complete their replication cycle. While nutrients supplies have long been known to influence both the severity of and the susceptibility to infectious diseases by mediating immune responses in animal hosts (Field et al. 2002, Smith 2007), several studies show that disease dynamics in plants can also be directly influenced by the host nutritional status (Smith 2007, Alexander 2010). Indeed, under conditions of elevated nutrient supplies, increases in plant metabolic activity and in the production of protein and nucleic acid molecular precursors of new viral particles (Elser et al. 2010) could lead to a higher accumulation rate of viruses. Under optimal plant growth conditions, virus infected plant tissues have been shown to use more nitrogen and phosphorous than healthy plant tissue, and nutrient levels above optimal for the growth of healthy plant tissue appear to increase the development of symptoms during viral infection (Selman and Grant 1957, Merrett and Sunderland 1965). Within-host viral accumulation has been shown to increase with increased nutrient availability (Merret and Sunderland 1965, Bawden and Kassanis 1950, Elser et al. 2007). Taking into account that transmission rate of viruses by insect vectors has been shown to be positively

correlated with within host viral accumulation (Froissart et al. 2010), a potential increase of plant viral RNA content following nutrient addition could result in higher transmission and thus higher prevalence of plant RNA viruses. Experiments by Elser et al. (2007) and Bawden and Kassanis (1950) demonstrated strong correlations between phosphorous availability and viral concentrations. In these same experiments, nitrogen addition alone produced little effect on viral concentration, while nitrogen and phosphorous added together produced stronger effects. These results suggest that not only N and P availability, but also the ratio of available N:P, can be important for viral reproduction.

Viral Infection of Plants

Viral accumulation appears to be correlated to nutrient addition as previously stated. This increased accumulation and reproduction of viral particles could also lead to a higher stress and harm caused to the host by the virus. While diverting host metabolic functions and products for their own replication, viruses can cause numerous modifications in plant physiology, including changes in nutrient availability, phloem transport, an overall reduction in photosynthetic pigments and an increase in abnormal meiotic processes (Adam et al. 1987, Muqit et al. 2007, Afreen et al. 2011), which can ultimately lead to reduced plant survival and seed yield (Kazinczi et al. 2006, Muqit et al. 2007, Froissart et al. 2010, Afreen et al. 2011). Viral reduction of host fitness, referred to as virulence, would appear disadvantageous, but virulence-transmission theory provides a model whereby this interaction might be understood (Singh 1973). Indeed, the well-known 'trade-off' hypothesis, used to explain the evolution of parasite virulence, states that greater rates of within-host replication increases pathogen transmission at the cost of increased detrimental effects on host viability (Anderson and May 1982, Froissart 2010). The model predicts that parasites virulence should evolve to an optimal level that corresponds to a trade-off

between an increased transmission rate and virulence (Alizon 2009). Although the correlation between virulence and within-host accumulation has rarely been demonstrated for plant viruses (Froissart 2010), the expression of virulence has been shown to be dependent on host nutritional status in other systems (Bedhomme 2004, Frost et al. 2008). Studies suggest that the severity these expressed symptoms is correlated with fecundity reductions caused by the viral infection (Singh 1973). Thus the trade-off made by a virus is likely impacted, and could be altered by variation in nutrient availability.

Study System: Barley and Cereal Yellow Dwarf Viruses

Barley and Cereal Yellow Dwarf Viruses (B/CYDVs) are RNA plant viruses belonging to the *Luteoviridae* family. These viruses are distributed worldwide, and are transferred via aphid vector in a persistent and circulative manner during the vector feeding process (Gray and Gildow 2003, Rasochova et al. 1997). The aphid *Rhopalosiphum padi* is the main vector for both BYDV-PAV and CYDV-RPV, the two viral species used in this study. B/CYDVs only replicate within host phloem cells, and this replication is associated with symptoms including dwarfing and yellowing or reddening (Perry et al. 2000). These symptoms can reduce host fecundity and lifespan, and have contributed to significant agricultural losses, especially within oats and other small grains (Anderson et al. 2004). Outside of agricultural plants, B/CYDVs are known to infect 150 species within family *Poaceae*, and have been recognized as the agents of a dramatic shift in the species composition of native California grasslands (Borer et al. 2007, Malmstrom et al. 2005).

Our Experiment

In order to better understand the outcomes of nutrient addition on plant-virus interactions, we will use a factorial combination of two levels of nitrogen and phosphorous to fertilize a cultivated host species of oats, *Avena sativa*. Along this gradient of N and P supply rates and ratios, we will quantify the effect of the replication of two viral species in the Barley and Cereal Yellow Dwarf Virus group (BYDV-PAV and CYDV-RPV) on plant fitness and development of symptoms as well as the rate of transmission of virus to healthy plants using aphid vectors (Gray 2008). This protocol will make possible separate examinations of the effects of nutrient supply level and supply ratio on viral transmission, symptoms, and virulence. The information gained from this study could give important insights about the implications of human mediated nutrient loading of nitrogen and phosphorous into both wild ecosystems and agricultural systems. Our future studies may show a relationship between human loading of nitrogen and phosphorous, and an increase in viral prevalence in grasses, potentially leading to decreased agricultural yield and increased invasion of native ecosystems by exotic grass species.

Infection of healthy plants with phloem-restricted viruses such as B/CYDV cannot be performed with mechanical inoculation methods and require the direct introduction of virus particles in host phloem cells using their insect vectors. While the latter method is often used to study various aspects of virus-plant interactions, the transmission efficiency of vectors for different virus species, including B/CYDVs, can vary widely due to variation in both biotic and abiotic factors (Halbert and Voetglin 1995, Power and Gray 1995, Miller and Rasochova 1997, Bencharki et al. 2000, Smyrnioudis et al. 2001, Dedryver et al. 2005). The previously published methods (Gray 2008), initially tested in this study for virus inoculation, yielded in low and inconsistent transmission efficiencies of BYDV-PAV and CYDV-RPV virus species. These results highlighted the need of optimized protocols for B/CYDV inoculations in our lab

conditions. The experiments described in this paper therefore focus on testing multiple variants of virus inoculation techniques, as well as on the relative strengths and weaknesses of these techniques. The developed and presently described method will be used to examine virus-nutrient interactions in a laboratory setting.

Materials and Methods

Aphid Colonies

We raised *Ropalosiphum padi* aphids in 15 cm x 15 cm pots, each planted with 15 individuals of the cultivated oat, *Avena sativa* in Sunshine brand MVP potting soil (Sun Gro Horticulture, Agawam, MA). Each pot was covered with a mesh hood designed to keep the aphids on the plants. Colonies were maintained in a growth chamber (16/8 hrs day/night at 19-21°C) and were watered twice each week with 300mL tap water. To keep the aphid colonies healthy, ~30 aphids were transferred bi-weekly to ~10 cm tall *A. sativa* plants.

Acquisition of viral particles

In Vitro

We utilized an *in vitro* protocol developed by Gray (2008). Aphids were removed from colony pots, placed into glass petri dishes and starved for two hours to maximize subsequent feeding on infected tissue and thus acquisition of viral particles. Virus inoculum was obtained from infected plant tissues by grinding and homogenizing with liquid nitrogen followed by centrifugation for 20 min at 3550g, 4°C. The resulting inoculum was mixed with a 50% sucrose solution (V/V) and was placed on top of a first parafilm layer stretched over each petri dish. A

second layer of parafilm was then stretched over the first layer to contain the virus inoculum and to simulate plant tissue layers. The initial parameters of this inoculation method used 300 µl inoculum containing 10% sucrose per petri dish, with aphids allowed to feed for 72 hrs. Subsequent changes to increase aphid survival shortened the AAP from 72 to 48 hours and increased inoculum amounts to 450 µl of 30% sucrose or 900 µl of 50% sucrose.

In Vivo (Initial protocol)

Aphids were removed from colonies and starved in glass vials for two hours. A leaf of infected tissue was then added to glass petri dishes. The dishes were sealed using parafilm. Aphids were allowed to feed for 48 hours. After this acquisition period, aphids were randomly distributed onto uninfected plants. This distribution served to control for differences created by aphids feeding on plant tissue non-homogenous for viral titer.

In Vivo (Optimized protocol)

Aphids were removed from colonies and starved in glass vials for two hours (15 per vial). A leaf of infected tissue was hung in the glass vial, which was then tightly corked. Aphids were allowed to feed for 48 hours. Following acquisition, aphids were distributed to uninfected plants in the same manner as the original *in vivo* protocol.

Transmission

Following either *in vitro* or *in vivo* acquisition, we assigned three aphids to each experimental *Avena sativa* plant. These aphids were introduced in a mesh cage (2cm x5cm) placed on the youngest leaf of each test plant. The aphids were then allowed to feed on plant

tissue for 48 hours, after which the plants were sprayed with an organic insecticide. Twenty-four hours later, the cages were removed and any remaining aphids were killed by hand.

After two weeks, which corresponds to the time when the viral titer of BYDV-PAV is the highest (Chain et al. 2005), we collected all plant tissue from each experimental plant, subsampled 0.05g of tissue from each plant for RNA extraction and RT-PCR detection procedure. The remaining plant tissues were stored at-20°C.

RNA extraction

In order to test for the presence of B/CYDV's in our samples, we first extracted RNA from the collected plant tissue. Total RNA extraction was performed based on a phenol-chloroform protocol using Trizol (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. Briefly, 1ml of Trizol and a copper bead were added to 2ml screw cap tubes each containing an individual plant sample. Plant tissues were then ground using bead-beater. The supernatant was collected after a 10 min centrifugation step (12000g, 4°C) which separated proteins, lipids and nucleic acids from most of the solid plant structures. RNAs were then isolated from the aqueous phase obtained after the addition of 400µl chloroform and a 15 min centrifugation step (12000g, 4°C). RNAs were precipitated in 500µl isopropanol after a 10 min centrifugation step (12000g, 4°C) and the recovered pellets washed with 1ml ethanol. After the removal of ethanol, nucleic acids were resuspended in RNAse-free water, the RNA concentration was quantified using a nanodrop spectrophotometer and RNA extracts were stored at -20°C.

Reverse Transcription

Following extraction from plant tissues, 1µg of each RNA extract was combined with 4µl of 5X ImProm-II reaction buffer (Promega), 1.5mM $MgCL_2$ (Promega), 0.5mM dNTPs

(Promega), 20U RNasin (Promega) and 1µl of ImProm-II Reverse Transcriptase (Promega), and generic B/CYDV primer PCRR1 (5'TGGTAGGACTTRAGTAYTCC3') designed by Christelle Lacroix (unpublished) in a final volume adjusted to 20µl with RNase-free water. These primers target a gene coding for the proteic capsid in the sequence of five B/CYDV viral species. These reactants were then held in a thermocycler for 5 min at 25°C and then 60 min at 45 °C. Following a deactivation stage of 15mn at 70°C, cDNA was stored at -20 °C.

Polymerase Chain Reaction and Gels

The transcribed cDNA products were combined with 1 µl of 10X PCR Buffer (Qiagen), 3.5mM MgCL2 (Promega), 0.4mM dNTPs (Promega), 0.75U HotStart Taq (Qiagen) and 0.07µM and 0.02µM of generic B/CYDV forward and reverse primers PCRR1 ('TGGTAGGACTTRAGTAYTCC3') and PCRF4 (5'GGACARTGGTTRTGG3') (designed by C. Lacroix - unpublished) respectively in a final volume adjusted to 10µl with RNase-free water. After a 15 min 95°C activation period of the Taq polymerase, cDNA fragments were amplified following 35 repetitions of a sequence of 30s 95°C, 45s 45°C and 1 min 72°C. After a final extension time of 10 min 72°C, DNA products were store at -20°C.

To assess the presence of B/CYDVs in our samples, we analyzed the PCR products using a gel of Agarose-1000 (Invitrogen) at 2% (W/V). SybrSafe dye (Invitrogen) was added to the gel at a ratio of 1:10000. Loading dye (Genesee) was added to the PCR products, which were then run in the gel with a DNA ladder marker (Genesee). The gel was then photographed using a UV-light EZ doc system, and the image analyzed for the presence of viral bands (220-230 base pairs in length).

Results

In a first small scale trial of *in vitro* and *in vivo* inoculation methods (Gray 2008), in which we exposed 8 plants to viruliferous aphids in June 2012, we achieved a 100% transmission rate on plants infected by aphids using the previously published *in vivo* method (n = 2 plants for each viral species, Fig. 1). In contrast, *A. sativa* inoculated with aphids that had acquired virus using the published *in vitro* acquisition method showed 100% transmission for RPV, but 0% transmission for PAV (n = 2 plants for each viral species, Fig. 1).

Two larger inoculation trials run in August 2012 using the previously published *in vitro* method, in which aphids were fed on PAV, RPV or mock (uninfected) inoculum (300µl inoculum per dish, 10% sucrose) for 72 hours prior to exposure to the *A. sativa* plants, resulted in very poor aphid survival and inoculation success. In the first trial, 32 out of 32 plants tested negative for B/CYDV infection (results not shown). In the second trial, too few aphids survived the 72 hour acquisition access period to run a subsequent inoculation trial.

In order to improve aphid survival during the acquisition access period (AAP), we decreased the time of the AAP from 72 to 48 hours, we increased the volume of inoculum distributed on each dish from 300 to 450µl as well as the concentration of sucrose in this solution from 10 to 30%. The average aphid survival after 48 hours of AAP of two different trials performed using these parameters was 27.9% (Fig. 2). A further test realized with a volume of 900µl of inoculum containing 50% of sucrose resulted in a 55.4% aphid survival after 48 hours AAP (fig. 2). However, subsequent inoculation using these aphids was still unsuccessful: all plants tested negative for viral infection. Thus, the *in vitro* method was inefficient in our laboratory conditions, even with modifications.

Given our results using the *in vivo* protocol (Fig. 1), we retested that protocol for use in our larger experiment. When aphids were fed on pieces of infected tissue for 48 hours before

being exposed to uninfected plants, only 10.2% of aphids survived. During the acquisition phase, the infected tissue dried and became brittle (results not shown), likely causing high aphid mortality. All plants exposed to the remaining aphids tested negative for viral infection.

A modified version of the *in vivo* method (sealed tubes containing fresh leaves hung vertically), developed to give the aphids a more natural feeding environment and to increase moisture retention led to a 70.9% aphid survival rate (Fig. 2). Subsequent PAV and RPV transmission was 75% (N=8/10) and 83.3% (N=10/12), respectively (Fig. 3). None of the mock-inoculated plants (N=7) were infected.

Discussion

Our final trial showed conclusively that the hanging leaf method is effective for aphid survival, and that acquisition of viral particles by aphids using this protocol is likely to yield successful infection of subsequent host plants. With the method proposed by Gray (2008), as well as our own variants on those methods in our laboratory conditions, we observed low aphid survival rates during the acquisition period, and extremely low rates of viral transmission from the remaining aphids. Studies by both Power et al. (1991) and Gray et al. (1991) have demonstrated the effectiveness of long (>72 hrs) acquisition access periods (AAPs). However, due to the low survival rates of aphids during these lengthy AAPs, it was unlikely that we would be able to successfully apply the *in vitro* protocol to our own experiments. The modified *in vivo* hanging tissue method, on the other hand, showed moderate vector survival and high viral transfer rates. This suggests the method we developed here will be the most effective technique for the rest of our experiments.

Studies of BYDV transmission by several aphid species including *R. padi* have demonstrated significant variance in efficiency of viral transmission based on genetic differences

both in viral strain and in clonal aphid colonies, as well as variance based on variation of abiotic parameters such as temperature during inoculation (Du et al. 2007, Zavaleta et al. 2001, Bencharki et al. 2000, Dedryver et al. 2005, Smymioudis et al. 2001). Prior to testing for these effects in our initial experiment by replacing either the aphids or virus strain being used, we decided to perform simpler alterations of the initial *in vitro* and *in vivo* protocols by modifying several experimental parameters (e.g. inoculum volume, sucrose concentration, and humidity levels in vials and dishes) aimed at improving aphid survival and virus transmission with the biological material already available. We initially implemented the *in vitro* protocol because it guaranteed that aphids would be feeding on inoculum of homogenous viral titer. One concern in developing these new protocols was to control for aphids feeding on tissue of varying viral titer. During an infection, each leaf on a plant can differ in the concentration of viral particles. This can affect inoculation success as well as viral accumulation in the subsequent host. Exposing test plants to higher or lower concentrations of viral particles could result in differential rates of viral reproduction, which might be mistaken for an effect of nutrient addition in our final experiments. Randomization of aphids from *in vivo* AAP trials prior to their exposure to uninfected test plants solved this concern by reducing the experimental bias introduced by this varying viral concentration. In order to increase aphid survival, we first shortened the AAP to 48 hours while increasing the volume and sucrose concentration of inoculum. Studies suggested that 48 hours was the minimum AAP that would return consistent transmission results (Gray et al. 1991). We had hoped that this would result in higher aphid survival as well as more time spent feeding on the inoculum, which would increase transmission rates in the surviving aphids. This increased aphid survival, although not to the levels we had hoped for, but still failed to result in efficient virus transmission. We thought that virus concentrations in our inoculum might be too low, or

that virus particles might be decaying due to exposure. Following these trials, we went back to our initial *in vivo* method, and modified it to better maintain humidity and keep the plant leaves from drying out through the use of corked vials. This produced the highest levels of aphid survival and the most stable transmission rates seen throughout our experiments. Our work has demonstrated greater effectiveness of the modified *in vivo* method of hanging tissue in tightly sealed vials than either the *in vitro* Gray protocol (2008) or our original *in vivo* protocol. With the success of the modified *in vivo* method, we will be able to transmit B/CYDVs and continue with the originally planned experiment.

Evidence from field studies suggested a trend of increasing Barley and Cereal Yellow Dwarf Virus presence with increased levels of phosphorous input, and showed little effect of nitrogen input (Borer et al. 2010). Other evidence suggests that increased nutrient levels shift dominance of ecosystems to plants which grow quicker and are more efficient at taking up nutrients (Cronin et al. 2010). These plants are generally more susceptible to herbivory and infection than their competitors. These data did not, however, rule out the possibility that these changes came about due to changing N:P ratio rather than overall level of these nutrients. With the transmission methods developed in this study, we will be able to examine the evidence for each of these hypotheses in a controlled environment, allowing us greater insight into which effect dominates in nature and what factors are most important in viral spread. The cascade of biological changes caused either by nutrient addition or change in nutrient ratio affect interactions throughout all trophic levels of the ecosystem (Elser et al. 2003, Throop and Lerdau 2004, Elser et al. 2010). These trophic interactions will likely have similar outcomes for barley/cereal yellow dwarf viruses as for other viral species. In this way, the results of our larger

study will be applicable to understanding not only B/CYDVs, but aphid-vectored plant viruses in general.

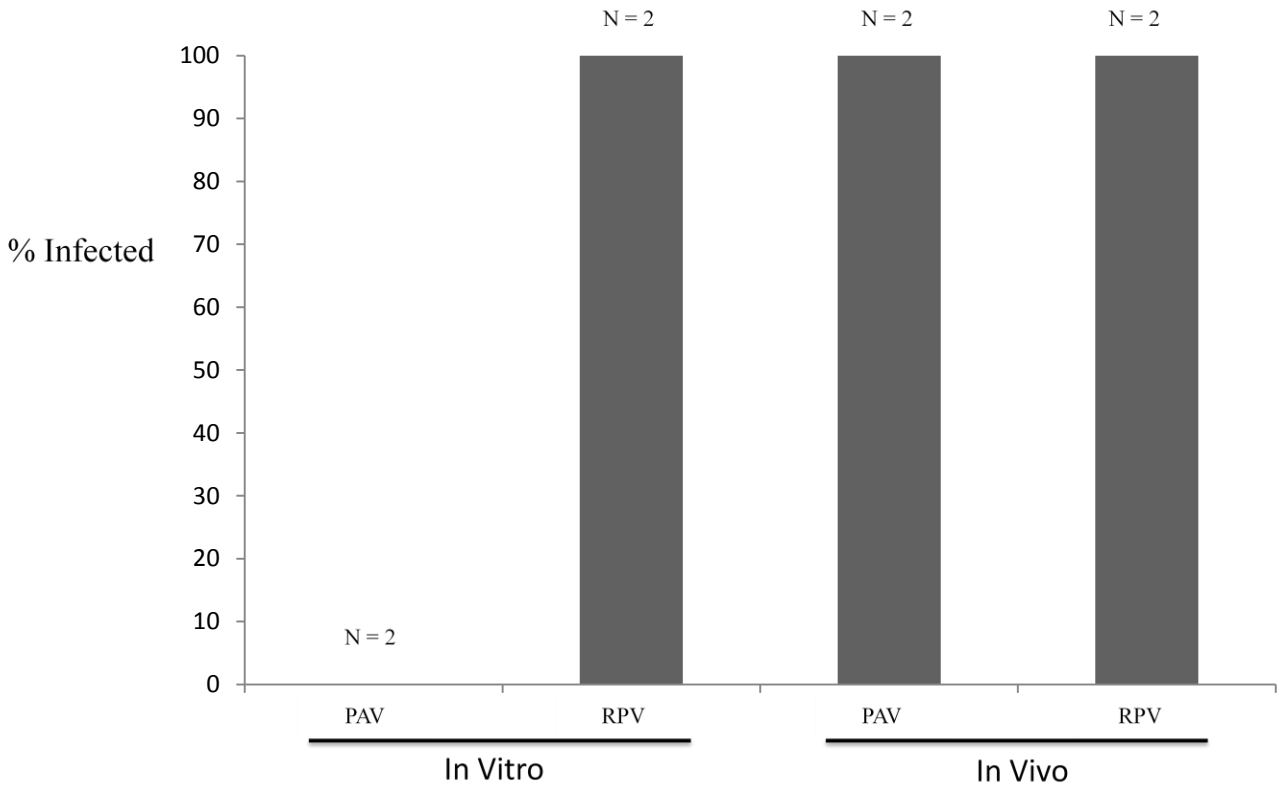

Figure 1. Test of protocols for B/CYDV virus transmission. Percentage of plants infected after exposure to aphids that fed on infected media using initial *in vitro* (300 µl of 10% sucrose + inoculum) and *in vivo* (fresh plant tissue laid in dishes) procedures. N denotes the number of plants exposed to viruliferous aphids. Infection was identified 14 days after inoculation. All mock inoculated plants tested negative for virus infection.

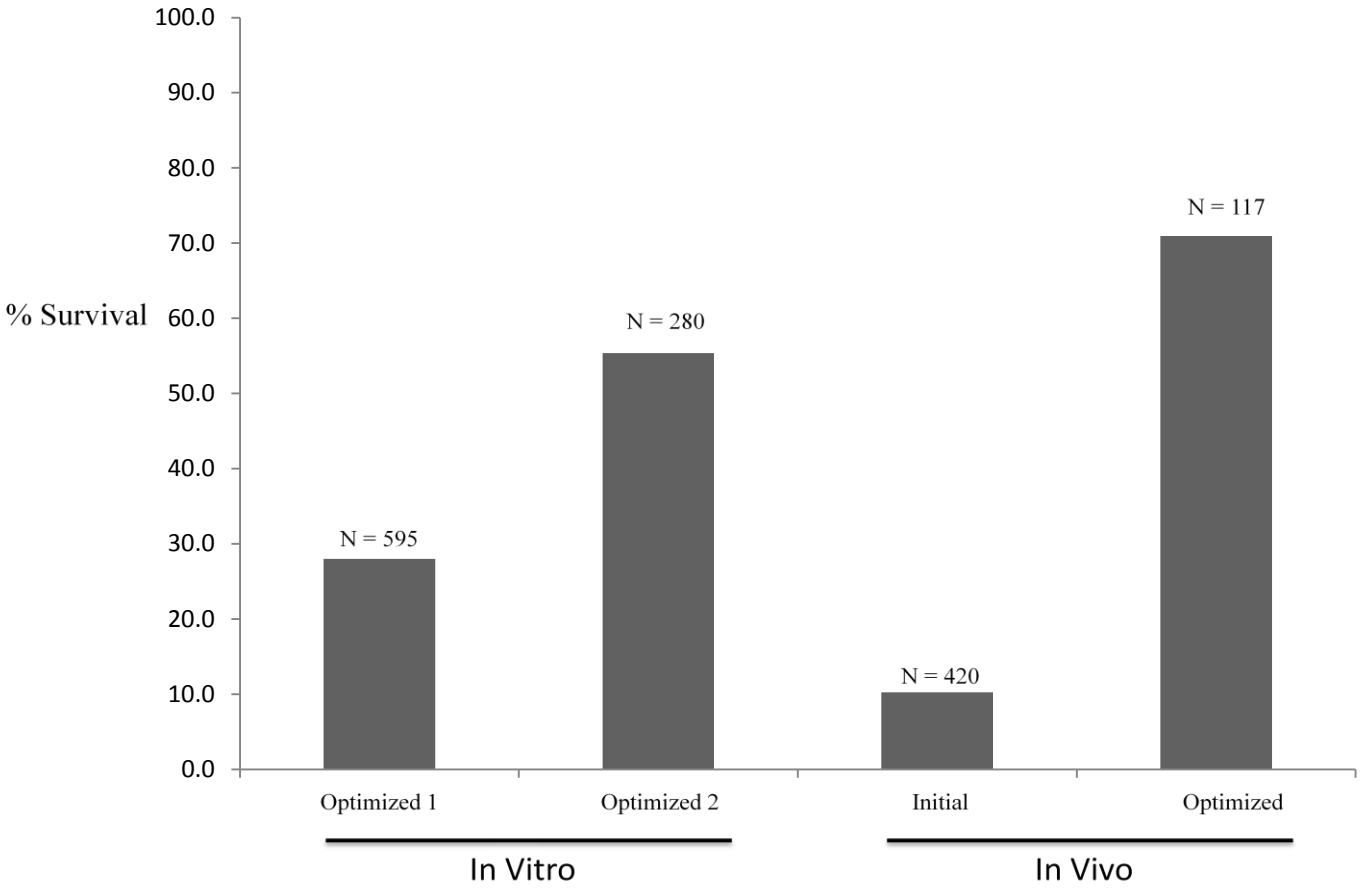

Figure 2. Aphid survival rates during Acquisition Access Period (AAP). Percent aphid survival under various feeding methods. *In vitro* optimized 1 and 2 represent tests using 450 µl of 30% sucrose + inoculum and 900 µl of 50% sucrose + inoculum, respectively. *In vivo* initial and optimized represent tests using infected tissue laid in petri dishes and hung in sealed tubes. N denotes the number of aphids used in the AAP.

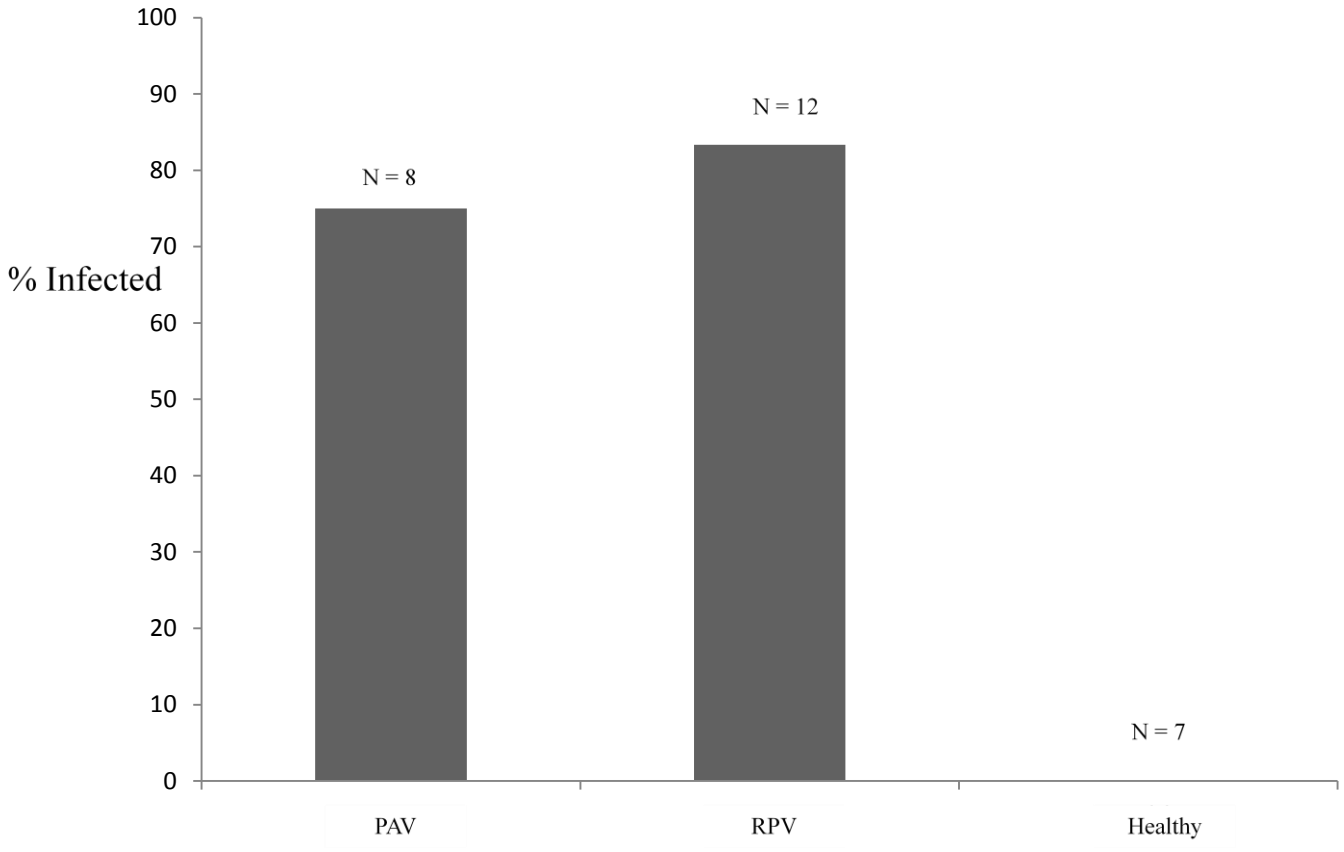

Figure 3. Virus Transmission of B/CYDV under optimized *in vivo* protocol. Percentage of plants infected after exposure to aphids fed on tissue infected with PAV or RPV and hung in sealed tubes (optimized *in vivo* method). N denotes the number of plants exposed to viruliferous aphids. Infection was identified 14 days after inoculation. All mock inoculated plants were tested negative for virus infection.

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