

ECOLOGICAL AND EVOLUTIONARY ASPECTS OF INTERACTIONS
BETWEEN MICROBES COINFECTING PLANTS

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ECOLOGICAL AND EVOLUTIONARY ASPECTS OF INTERACTIONS
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Coinfections of one host with multiple pathogen species are common, and have important implications for host health and pathogen fitness. In the research reported here, plant virus systems were used to explore the effects of coinfection on pathogen populations and host responses. Chapter 1 addresses the importance of coinfection timing using *Barley yellow dwarf virus* (BYDV-PAV) and *Barley stripe mosaic virus*. Coinfection timing significantly influenced viral within-host competition. Additionally, simultaneous coinfections were significantly more severe than sequential coinfections, which were only as severe as the most damaging constituent virus. A mathematical model was used to demonstrate that inaccurate projections of disease impacts on host populations can result when the effects of coinfection timing are not taken into account. Chapter 2 explores the effects of coinfections of *Cereal yellow dwarf virus* (CYDV-RPV) and two species of BYDV (PAV and PAS) on pathogen evolution using an experimental evolution approach. Viruses exhibited altered within-host concentrations and transmission after serial passage in coinfections, without altered disease severity. Chapter 3 examines interactions between *Bean common mosaic virus* (BCMV) and *Clover yellow vein virus* (CIYVV), and a microbial mutualist, rhizobia bacteria. The presence of rhizobia allowed CIYVV to reach higher

within-host concentrations in coinfections. Viral transmission was also affected by interactions between coinfection and plant nitrogen source. Viral infection significantly reduced the percentage of nitrogen in plant tissues derived from microbial mutualists, with a greater than additive decrease in coinfections. Chapter 4 assesses the effects of BCMV and CIYVV coinfection and rhizobia colonization on plant primary and secondary metabolism. Increased photosynthetic rates were observed in plants colonized by rhizobia, which were driven by increased maximum rates of electron transport. Infection status, inorganic nitrogen fertilizer, and rhizobia had significant effects on components of plant volatile organic compound (VOC) emissions, with nitrogen source significantly affecting overall VOC profile composition. Chapter 5 analyzes how reductions in rhizobial nitrogen fixation caused by viral infection affect soil fertility, and projects substantial monetary losses for farmers when viral prevalence is high in a legume rotation, either due to additional fertilizer costs or reduced yield of a subsequent non-legume crop.

BIOGRAPHICAL SKETCH

Katherine Myers Marchetto received a B.S. in biology and an M.S. in ecology from the Pennsylvania State University. She studied seed release, dispersal, and population biology of invasive thistles with Katriona Shea for her Masters thesis. She decided to explore an interest in disease ecology, and enrolled at Cornell University to pursue a PhD. in Ecology and Evolutionary Biology. She studied interactions between microbes coinfecting the same host plant with Alison Power for her dissertation.

Dedicated to my grammie, Georgetta Myers, who taught me to love plants.

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Andre Kessler is a collaborator on the project to collect and analyze the volatile organic compounds emitted from plants colonized by viruses and/ or rhizobia

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INTRODUCTION

Infections of one host with multiple pathogens are frequent and can have devastating effects on host health for humans, agricultural species, and wild populations. In a meta-analysis, Griffiths et al. found that coinfection reduced human health in 76% of studies, while worsening infections in 57% of studies (2011). Two prominent examples of coinfections that have profound effects on human health are HIV-tuberculosis and influenza-bacterial pneumonia coinfections. Twenty-five percent of all United States tuberculosis infections are thought to be facilitated by the immunosuppressive effects of HIV infection, and 10% of HIV positive adults die from tuberculosis worldwide (Corbett et al. 2003). In addition, 55% of samples from individuals who died from A/H1N1 in the 2009 pandemic showed histological evidence of bacterial pneumonia (Gill et al. 2010).

Coinfections are rampant in both animal and plant agricultural species. For example, 78% of feedlot cattle with pneumonia also had at least one other pathogen (Shahriar et al. 2002). It is also common in both chickens and pigs for an immunosuppressive virus to greatly increase the damage caused by an otherwise minor parasite (Nunez et al. 2003). Coinfections in agricultural crops can be so devastating that some particularly virulent coinfections have been given their own names. Rice tungro (Hull 1996), corn lethal necrosis (Scheets 1998), and cowpea stunt disease (Pio-Ribeiro et al. 1978) are all caused by viral coinfections. Corn lethal necrosis in particular can reach prevalences of 40% in African maize fields and is a threat to food security in the region (Mahuku et al. 2015). Other coinfections, while less devastating, are even more prevalent. The viral coinfection rate in cultivated

wheat can be as high as 60%, with the mean number of viruses per individual greater than two in most years (Mesterházy et al. 2002).

The prevalence of coinfections is also high in wild populations. For instance, 22% of *Ixodes ricinus* ticks feeding on green lizards were found to carry at least two of the following disease species: *Borrelia lusitaniae*, *Anaplasma* sp., or *Rickettsia* sp. (Václav et al. 2011). Retrovirus coinfections are incredibly high in wild West African red colobus monkeys (*Ptilocolobus badius badius*). Seventy-seven percent of the population were infected with at least one retrovirus, and 45% of the population had all three retroviruses examined (Leendertz et al. 2010). Wild populations of plants are also plagued by high rates of coinfection. In native grasses, the average percentage of viral infections that contain multiple viral species can be 70% (Seabloom et al. 2009).

There are a number of reasons, beyond simple probability, why coinfections are so prevalent. As previously mentioned, pathogens that suppress the host's immune system facilitate the invasion of other pathogen species. In some cases, immunosuppressed hosts can die from pathogens that are at most minor annoyances in healthy hosts (Prado et al. 2009). While most attention is paid to immunosuppression in animals, plant viruses can also facilitate the growth of other virus species by attacking the host's primary defense against viruses, the RNA-silencing pathway (Syller 2011). Coinfection also becomes more likely when, rather than clearing pathogen infections, hosts adopt a tolerance strategy to instead limit damage caused by the pathogen (Medzhitov et al. 2012) or in the case of chronic infections. Shared pathogen vectors and conditions necessary for infection also increase the risk of coinfections. For instance, needle sharing leads to high HIV and hepatitis C

coinfection rates among injection drug users. In plants, many viruses share vector species (Kennedy et al. 1962), which increases coinfection risk. Transmission facilitation is also observed between pathogens, either by sharing of viral coat proteins (Falk and Duffus 1981, Creamer and Falk 1990) or when coinfecting hosts are more attractive to vectors than singly infected hosts (Srinivasan and Alvarez 2007).

Coinfections typically result in increased pathogen damage to hosts, but this is not always the case. There are a number of instances where the coinfection is no more severe than a single infection of the most damaging constituent pathogen (Lohr et al. 2010), or where coinfection of an avirulent pathogen with a virulent one can reduce disease severity (Hargreaves et al. 1975, Woolhouse et al. 2015). It is also important to note that sometimes increases in disease severity during coinfections are more a function of a self-damaging host immune response than the direct actions of the pathogens (Nakamura et al. 2011).

The high prevalence of coinfections should cause pathogens to evolve in response to coinfecting species in addition to host responses. The prevailing wisdom is that in many cases, coinfections should cause increased disease-induced mortality in hosts. The short-sighted evolution hypothesis states that pathogens should increase their within-host accumulation during coinfections in an attempt to outcompete coinfecting species (Levin and Bull 1994). This strategy is short-sighted because increasing within-host growth rates at the expense of host resources could backfire if hosts die more quickly and therefore cannot transmit the pathogen. However, there are a variety of factors that the short-sighted hypothesis does not account for that could change this outcome, such as pathogen phenotypic plasticity, shared pathogen

common goods, and immunopathology (Day et al. 2007, Choisy and de Roode 2010, Alizon and Lion 2011). There are a plethora of theoretical expectations, but few empirical studies to validate their conclusions, especially in intact, multicellular hosts.

Coinfections can also present novel challenges to disease intervention strategies by leading to unexpected effects. For example, the use of an anti-malaria drug can actually increase parasitemia and disease severity when it is used on patients with mixed infections of resistant and susceptible parasites (Harrington et al. 2009). The drug decreases within-host populations of the susceptible parasites, but leads to competitive release of the resistant ones.

Despite recent attention and interest in coinfection, there are many unanswered questions. For example, how do pathogens interact when their transmission strategies conflict? To what extent do pathogen coinfections influence host population dynamics? Does coinfection lead to the evolution of increased virulence? What happens when coinfecting pathogens interact with microbial mutualists? All four of these questions are addressed in this dissertation, using plants as the host study system.

Plants are ideal for the study of interactions between pathogens in coinfections and effects on hosts. Plant pathogens of economic concern are extremely well studied and characterized. Many plants have relatively short generation times, are low maintenance, easy to inoculate, exhibit a complex immune system, and don't present ethical concerns. For these reasons and more, pathosystems of barley (*Hordeum vulgare*) and common bean (*Phaseolus vulgaris*) were explored in this dissertation, which is described chapter by chapter below.

Chapter 1 investigates the effects of coinfection timing on viral competition and disease virulence, as well as model-based projections of the impacts of a two-virus disease system on plant population dynamics. Barley host plants were inoculated with either no virus, *Barley yellow dwarf virus* (BYDV), *Barley stripe mosaic virus* (BSMV), BYDV then BSMV a week later, BSMV then BYDV a week later, or both viruses at the same time. Within-host competition between BYDV and BSMV was evident during sequential coinfections, when at least one virus had a lower within-host concentration in a sequential coinfection than in a single infection. However, no competition was evident during simultaneous coinfections, where both viruses had the same within-host concentration in simultaneous coinfections as in a single infection. Simultaneous coinfections were also significantly more virulent than sequential coinfections, which were only as virulent as a single infection of the most virulent constituent virus (BSMV). Most previous experiments have examined only one type of coinfection timing, usually simultaneous coinfections (but see Kim et al. 2010, Lohr et al. 2010). We used a mathematical model to understand how inaccurate projected disease impacts on host populations could be if differences in the virulence of different types of coinfections were not taken into account. The conditions where these inaccuracies would be expected to be highest were also examined. This work fills a general gap in the literature where effects of coinfections on virulence are measured at the individual level, but rarely extrapolated to potential effects on host population biology.

Chapter 2 describes an experimental evolution experiment addressing the effects of coinfection on pathogen within-host accumulation, virulence, and

transmission. The prevailing expectation is that interactions between coinfecting pathogens should generally lead to pathogens evolving higher virulence than they would if the pathogens only experienced single species infections. However, empirical studies that test this hypothesis are rare, and usually involve either tissue culture or the use of bacteria as hosts. We passaged the RPV species of *Cereal yellow dwarf virus* and either the PAV or PAS species of *Barley yellow dwarf virus* together in barley hosts, then determined how the strains passaged in coinfections compared to strains passaged in single infections. We found that RPV strains with a history of coinfection (coRPV) accumulated significantly more viral particles when alone in a host than strains of RPV with a history of passage in single infections (sRPV). coRPV strains also accumulated higher viral particle concentrations than sRPV strains when coinfecting a host. On the other hand, neither strain history or infection type had much of an impact on PAV viral particle concentrations. Unexpectedly, PAS strains with a history of coinfection accumulated significantly less viral particles during coinfections. Contrary to expectations, there was no effect of passaging strains in coinfections on pathogen virulence despite no constraints on possible virulence evolution. coRPV strains did not have a transmission advantage over sRPV strains. In particular, coRPV transmission was significantly worse during transmission from a single infection as opposed to during transmission from a coinfection with PAV. We suspect that these results may be explained by heterogeneous encapsidation, where PAS and PAV might be encapsidating their RNA using proteins from RPV. These particles appear as RPV capsids using our immunological assay technique, but could contain PAS or PAV genomes.

Chapter 3 examines the interactions between coinfecting viruses and a microbial mutualist. Commensal and mutualistic microbes are ubiquitous within multicellular hosts, and have the potential to influence competition between pathogens. For example, Lysenko et al. (2010) found that in the absence of a commensal/ opportunistically pathogenic bacteria species, avirulent *Streptococcus* strains outperformed virulent strains. However, in the presence of the commensal bacteria, virulent *Streptococcus* strains were more successful than avirulent strains. We examined this scenario in common beans for a combination of two viruses, *Clover yellow vein virus* (CIYVV) and *Bean common mosaic virus* (BCMV), in the presence a microbial mutualist, rhizobia bacteria. We expected that the presence of rhizobia would shift the competitive landscape between CIYVV and BCMV. In addition, host viral infection is known to have negative effects on nitrogen fixation of rhizobia bacteria (Tu 1997). Coinfection with multiple viruses often has a greater than additive negative effect on disease severity, and we hypothesized that the effects on the benefits that the host receives from rhizobia would also be greater than additive. We found that there was a significant positive interaction between the presence of rhizobia and viral coinfection on within-host accumulation of CIYVV. CIYVV only exhibited increased within-host concentrations in coinfections when rhizobia were present. Within-host BCMV concentrations were lower in coinfections, but there was no effect of the presence of rhizobia. However, coinfection and nitrogen source interacted to affect BCMV transmission. Single infections of CIYVV and BCMV both caused a significant reduction in the percentage of nitrogen in host tissues derived from nitrogen fixation. Coinfections in turn had a greater than additive negative effect on

this benefit that plants receive from mutualism with rhizobia. This result was likely partially due to host production of the immune signaling hormone salicylic acid (SA). SA induction has a negative effect on nodulation by rhizobia (Sato et al. 2002). We found that SA concentrations were reduced in plants infected with one virus and colonized by rhizobia in comparison to singly infected plants without rhizobia. However, the presence of rhizobia did not cause SA levels to drop in coinfecting plants. Coinfections also reduced host seed production significantly more than a single infection of either virus.

Chapter 4 focuses on the interacting effects of viral coinfection and rhizobia colonization on host primary and secondary metabolism. Specifically, we examined mechanisms behind increased photosynthetic rates in plants colonized by rhizobia as well as plant volatile organic compound (VOC) emissions. We found a trend towards increased carboxylation rates in plants colonized by rhizobia, and a highly significant increase in the maximum rate of electron transport. Rhizobia also had a significant effect on the composition of VOC emissions. Infection status was important for several individual compounds, but did not adequately describe general VOC profile composition. Two compounds were emitted in significantly higher amounts in coinfecting plants, but not in plants singly infected by either virus. Coinfection with BCMV negated significant effects of CIYVV single infections on three compounds. There were also three compounds where both infection status and the presence of rhizobia interacted to affect emissions. These results are important, because VOC emissions influence herbivore preferences. The presence of rhizobia could have a large impact on feeding by aphids that vector plant viruses such as BCMV and

CIYVV, and therefore disease spread.

Chapter 5 examines the agricultural implications of the results of Chapter 3 that viral infection, and particularly coinfection, has a significant negative effect on the percentage of nitrogen in plant tissue that is derived from nitrogen fixation. Both large-scale and subsistence farmers take advantage of legume rotations to increase soil fertility. Farmers can use less inorganic nitrogen fertilizer in a crop grown following a legume, while getting equal yields. This reduction is known as a nitrogen fertilizer replacement value (NFRV), or nitrogen credit. NFRVs are determined for different legumes, regions, and soil types and used as guidelines for farmers. However, NFRVs do not take into account reductions in plant biomass and the percentage of nitrogen derived from fixation caused by viral infection. We estimated how much nitrogen could be lost for a range of viral prevalences in common beans, clover, and alfalfa. Depending on the legume crop and disease prevalence, losses can be substantial and costly in either prices of inorganic nitrogen fertilizer or yield losses in a subsequent crop. This could be a particular concern for resource-poor farmers, since inorganic fertilizers can be prohibitively expensive for them in many locations.

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CHAPTER 1

COINFECTION SYNCHRONY BETWEEN TWO PLANT VIRUSES INCREASES VIRULENCE IN MULTIPLE INFECTIONS

Abstract

Infections of one host by multiple parasites are common, and coinfecting parasites can have conflicting life histories. For example, the fitness of a macro or micro parasite that is transmitted vertically through host offspring is tied to host fitness, whereas horizontally transmitted parasites do not share this constraint. In such systems, infection timing could influence interaction outcomes. Using a model system of two viruses infecting barley, we examined whether inoculation sequence affects competition between viruses with contrasting transmission modes, one transmitted horizontally among unrelated hosts and one transmitted both horizontally and vertically from parent to offspring. We found that sequential coinfections had lower within-host concentrations of each virus compared to single infections, but simultaneous coinfections exhibited the same concentrations of each virus observed in single infections. Simultaneous coinfections had greater than additive virulence that decreased vertical transmission opportunities, whereas sequential coinfections had the virulence of the most virulent constituent virus. We built a susceptible-infected model to examine whether the observed difference in coinfection virulence could affect host population dynamics under a range of scenarios. Coinfection timing can have an appreciable, but context dependent, effect on projected host population dynamics. Studies that examine only simultaneous coinfections could inflate disease impact predictions.

Introduction

Infections of one host with multiple parasites are common and widespread. For example, 10% of HIV positive adults die from tuberculosis (Corbett et al. 2003). In plants, several important diseases of agricultural crops are actually caused by coinfections of two viruses that are relatively avirulent in single infections, including rice tungro (Hull 1996), corn lethal necrosis (Scheets 1998), and cowpea stunt disease (Pio-Ribeiro et al. 1978). It is becoming increasingly clear that coinfection has important ramifications for both parasite fitness and host health (as reviewed by Mideo 2009; Syller 2011).

The sequence and timing of infection by multiple parasites can alter the strength of interactions between them. We use the general term parasite to describe all organisms that live inside a host, to the host's detriment. We define a sequential coinfection as one where one parasite is introduced into the host and establishes, followed by the subsequent introduction and establishment of another parasite. We define a simultaneous coinfection as an introduction of multiple parasites to the host at the same time, all of which result in infection. Coinfection timing often affects parasite interactions, but in most cases it is difficult to distinguish between specific mechanisms of competition. Parasites may affect each other by competing for limited resources, by disrupting the growth of other parasites, or by stimulating the host immune response. An indirect interaction between parasites mediated through the host immune response can also lead to facilitation if one parasite impairs host immunity (Graham 2008). If a parasite has lower fitness when invading the host second, it is often difficult to determine whether the cause is an already sickly host

with depleted resources or previous activation of the host's immune defenses.

A parasite that infects the host first often gains a competitive advantage (Hood 2003; Hoverman et al. 2013) but this is not always the case (Lohr et al. 2010). Some parasites are such strong competitors that invading second has little effect on their dominance (de Roode et al. 2005; Kim et al. 2010). Simultaneous coinfection can lead to either less competition (Hoverman et al. 2013) or more (de Roode et al. 2005). The most intense form of competition between plant viruses is cross protection, a priority effect where previous infection prevents coinfection with genetically similar viruses (Fulton 1986). However, closely related viruses can infect the same host when inoculated at the same time or with a short delay (Aapola and Rochow 1971). When one plant virus has a particularly strong method of impairing host immunity, viral interactions are often asymmetrical (Syller 2011). For example, a potyvirus suffers most from coinfection when infecting the host after a non-potyvirus, while a non-potyvirus reaches higher within-host concentrations when it invades after a potyvirus has established (Balogun 2008). In other cases, facilitation is highest during simultaneous coinfections (Goodman and Ross 1974).

Infection sequence and timing also can have large effects on host health. Previous infection by an avirulent pathogen strain may prevent later infection by a more virulent strain (Seifi et al. 2012; Sernicola et al. 1999) or lessen its effects on the host (Hargreaves et al. 1975). However, within-host competition between parasites is not necessarily beneficial to the host, and can still lead to increased virulence even when both parasites suffer reduced concentrations in coinfections (Srinivasan and Alvarez 2007). Depending on the host fitness measurement, simultaneous coinfection

can be either worse than sequential coinfection or as bad as a single infection of the most virulent parasite (Balogun 2008; Kim et al. 2010; Lohr et al. 2010).

Most plant coinfection studies using distantly related viruses have only included one type of coinfection timing (e.g. Pio-Ribeiro et al. 1978; Rentería-Canett et al. 2011; Scheets 1998), which makes it difficult to determine how timing may affect interactions between viruses (but see Balogun 2008; Kim et al. 2010).

Sequential coinfections are usually more likely in the field than simultaneous coinfections, because simultaneous coinfections require more specific conditions to occur. For example, simultaneous coinfections are more likely when independent viruses share a vector species (Seabloom et al. 2009), or in helper-dependent viral complexes, where the dependent virus cannot be transmitted by vectors without the helper virus (Falk and Duffus 1981). Sequential coinfections with a delay of less than two days may also yield similar outcomes to simultaneous coinfections, if cross protection studies (such as Aapola and Rochow 1971; Zhang and Melcher 1989) provide a reasonable estimate of how long it takes for a virus to become entrenched or for the host to mount an immune response.

One extreme example of sequential coinfection involves vertical transmission from parent to offspring, which is a feature of several ecologically and agriculturally important viruses of plants (Mink 1993). A vertically transmitted virus can invade a host before horizontal transmission between unrelated hosts is possible. Both hosts and vertically transmitted viruses suffer reduced fitness if the host becomes coinfecting with a horizontally transmitted virus that reduces the number of viable host offspring available for vertical infection. A vertically transmitted virus could retain its fitness

by excluding competitors from invading an occupied host or reducing the virulence of later invading viruses (Haine et al. 2005). Interestingly, many plant viruses that are transmitted vertically are also transmitted horizontally. Even without a head start, if allocation to horizontal vs. vertical transmission is plastic, then a virus with both transmission modes could salvage some fitness in a coinfection with a horizontally transmitted virus by increasing its own horizontal transmission. According to the virulence-transmission trade-off hypothesis, this increased horizontal transmission can only be purchased by increasing within-host accumulation, which increases virulence (Alizon et al. 2009; Froissart et al. 2010) and thereby reduces vertical transmission.

One element that is usually missing in plant coinfection studies is a bridge between empirical results at the level of individual hosts and inferences concerning how much these individual differences could matter at the host population level (Escriu et al. 2003; Péréfarres et al. 2014; Savary et al. 2006; but see Susi et al. 2015; Tollenaere et al. 2016). It would be interesting to find a difference in virulence between coinfections that occur sequentially or simultaneously, but could this affect hosts at the population level, even in a situation where viruses share a vector species and simultaneous coinfections are relatively likely? Mathematical modeling can show what types of dynamics are possible, and can be used to forecast whether and under what conditions significant differences at the individual level would be expected to matter at the host population level.

In this study, we investigated the effects of inoculation timing on the interactions between a horizontally transmitted plant virus (the PAV species of *Barley yellow dwarf virus*) and one that is transmitted both horizontally and vertically (*Barley*

stripe mosaic virus). In order to examine how viral concentration, virulence, and transmission change with coinfection timing, barley host plants were inoculated with each virus individually, both viruses sequentially, or both viruses simultaneously. We then developed a susceptible-infected type model to explore how empirically observed differences in virulence between sequential and simultaneous coinfections would affect host population sizes and fitness in agricultural or wild plant hosts. Although the likelihood of simultaneous coinfection is rather low in our experimental system, we generalized the model to explore cases where simultaneous coinfection would be more likely.

Materials and Methods

Barley yellow dwarf virus (BYDV; Luteoviridae) is composed of a group of positive-sense, single stranded RNA viruses that can infect over 150 species of grasses, including many economically important cereal crops (Miller and Rasochová 1997). The viruses are transmitted horizontally by aphids in a circulative, nonpropagative manner, meaning that while the viruses do not replicate within the aphid, once acquired, vectors can transmit for the rest of their lives (Ng and Perry 2004). We collected a field isolate of the PAV species of BYDV from barley in Ithaca, NY in the fall of 2010 for use in this experiment. We preferred to use a field isolate because passaging in the lab can alter pathogen virulence. Briefly, symptomatic barley samples were collected from the field and tested for multiple species of BYDV and cereal yellow dwarf virus that share *Rhopalosiphum padi* as a vector using double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA, antibodies

from Agdia, Elkhart, IN). Field tissue that tested positive for only the PAV species of BYDV was used to inoculate seedlings in the greenhouse for virus culture using *R. padi* aphids in the manner described below. *R. padi* is a common and widespread aphid species not known to transmit any other viruses that can replicate in barley besides BYDV and CYDV species (Kennedy et al. 1962).

Barley stripe mosaic virus (BSMV; Virgaviridae) is a positive-sense, single stranded RNA virus that infects barley, wheat, and oats (Jackson et al. 2009). BSMV can be spread horizontally by contact and vertically in seeds. The ND18 strain of BSMV, which has been cultured in the lab by horizontal transmission for several generations, was used due to difficulty in acquiring a field strain. The prevalence of coinfections between BYDV and BSMV depends on many factors, but coinfection prevalence can be 7% in randomly sampled wheat plants (Mesterházy et al. 2002).

Inoculations. One maternal line of barley (*Hordeum vulgare* var. Conlon) was used to ensure that all seedlings were at least half sibs. This reduces the effects of host genetics on pathogen growth and virulence. Eight replicates of each inoculation treatment were planted in 15.24 cm diameter pots filled with Metro-Mix (Sun Gro Horticulture, Agawam, MA, USA) for a total of 48 plants. Plants were randomly assigned to treatments, and positions on the greenhouse bench were randomized several times during the experiment. Plants were watered 5 days a week with 150ppm of 21-5-20 fertilizer (Jack's Professional, JR Peters Inc., Allentown, PA, USA).

Six inoculation treatments were used to examine the effects of inoculation timing on viral population dynamics, transmission, and virulence. Control plants received no inoculation. Single virus inoculations of BSMV and BYDV took place

one week after planting. One week after planting, simultaneously inoculated plants were inoculated with BSMV, rinsed, and immediately afterwards aphids carrying BYDV were caged on plants. Sequential inoculations were performed by inoculating with the first virus one week after planting (BYDV or BSMV), and inoculating with the second virus 2 weeks after planting (BSMV or BYDV, respectively). Mock inoculations were not performed both based on previous experience with the study system and because including a mock inoculation for every treatment would nearly double the size of the experiment. In the cases of a few plants that did not become infected after inoculation, there was no difference in plant fitness between uninoculated plants and plants subject to unsuccessful inoculation ($p=0.95$).

BSMV was mechanically inoculated. Leaves from plants that tested positive for BSMV using DAS-ELISA were weighed and ground with a volume of inoculation buffer in ml equal to ten times the weight of the tissue in grams. The inoculation buffer consisted of a solution of 38.9 ml 0.1M KH_2PO_4 , 61.1 ml 0.1M Na_2HPO_4 , and 100 ml deionized water. The youngest leaf of each plant was sprinkled with carborundum powder and rubbed with inoculum. Plants were rinsed with water after 5 minutes to remove inoculum.

BYDV is exclusively transmitted by aphid feeding and was inoculated using *R. padi* aphids. Colonies of *R. padi* were maintained on barley (var. Conlon) in growth chambers at 20°C in 24 hr. light. Adult aphids were fed BYDV infected leaves in dishes for 2 days, and then 3 aphids were caged on each plant to be inoculated. After 2 days the cages were removed and all plants were sprayed with soap (M-Pede, Gowan Company, Yuma, AZ, USA) after the first week of inoculations to kill the

aphids. Soap does not leave residuals on plant leaves that would prevent vector feeding during the second week of inoculations. All plants were sprayed with Talstar insecticide (FMC Professional Solutions, Philadelphia, PA, USA) after the second week of inoculations were complete.

Viral concentration sampling. Viral concentration sampling began 3 weeks after planting and continued weekly for 7 weeks. Tissue samples were taken from a separate, mature leaf each week. Leaf tissue samples (0.5 g) were ground in 5 mL of phosphate buffered saline (PBS). Quantitative DAS-ELISA was used to obtain a relative measure of viral concentration (Pollina 2013). Coat and capture antibodies were purchased from Agdia (Elkhart, IN). Two replicate samples were randomized in location on a 96 well plate for each virus tested. Sample optical density (OD) values were standardized by dividing the OD value of each sample by the OD value of known infected tissue grown in the greenhouse. The standardized ELISA values of replicate samples were then averaged to give the average relative viral concentration for the plant. Transmission success was high, but not 100%. Therefore, we introduce a change in terminology here such that all successful sequential and simultaneous inoculations are termed sequential and simultaneous coinfections, respectively, to distinguish these majority cases from the few instances where one virus did not successfully transmit to the host plant. Infection status, rather than infection treatment, was used as the response variable in all analyses. All analyses were performed in R version 2.10.1 (R Development Core Team 2015). Linear mixed effects models (LME) with a Gaussian error distribution were used to relate viral concentration to the fixed effects plant infection, week of sampling, and their

interaction as well as the random effect of individual plant.

Transmission tests. Horizontal transmission trials were conducted approximately 10 weeks after planting. Two gram leaf samples were collected from all plants, and the samples from control plants and plants infected with BYDV were placed in individual dishes with aphids for virus acquisition. After two days, three replicate seedlings per experimental plant were inoculated with three aphids each in the manner described above. The experimental control plants were included to test for cross-contamination during the transmission tests. In addition, each plant infected with BSMV, plus control plants, were also used to inoculate three seedlings. BSMV inoculations were conducted by sprinkling the leaves of each uninfected seedling and the infected focal plant with carborundum powder, and then rubbing leaves of both plants together. This approach was used because it is mechanistically similar to field transmission, which occurs by contact and is facilitated by wounding caused by rubbing or abrasion. To control for potential fitness effects of damage close to flowering, all experimental plants, even plants with single infections of BYDV not used in BSMV transmission trials, received equal tissue abrasion. Seedlings were tested for horizontal transmission two weeks after inoculation using DAS-ELISA.

Vertical transmission was quantified by growing out ten seeds from each plant containing BSMV, plus control plants, and determining the proportion of infected seedlings using DAS-ELISA. BSMV horizontal transmission was analyzed using a binomial generalized linear model (GLM). Both BSMV vertical transmission and BYDV horizontal transmission were overdispersed, and evaluated with quasibinomial GLMs (Crawley 2007).

Virulence measurements. Neither virus species tends to kill its host (Catherall 1966; Slykhuis 1976), so virulence was quantified as a reduction in host fitness at the end of the experiment. Dry aboveground vegetative biomass, reproductive biomass, and total number of seeds were quantified for each plant. Total number of seeds produced is a good proxy for fitness, because seed germination rates were high and consistent across treatments. Biomass was analyzed using GLMs with Gaussian error distributions. Quasipoisson GLMs were used to analyze seed counts due to overdispersion in the data. GLMs with quasipoisson error distributions were used to examine the relationship between host seed production and median viral concentration of infected plants.

Host-pathogen model. A susceptible-infected (SI) model was developed to explore whether a difference in virulence between sequential and simultaneous coinfections of the magnitude we observed could affect host population dynamics (Figure 1-1). The best system to see such an effect would be one with a high probability of simultaneous coinfection. For this reason our model makes an assumption that differs from the empirical BYDV-BSMV system: the assumption that the two viruses share a vector. This assumption also makes the model more general, because most plant viruses are vectored (Power and Flecker 2003), and many share vectors (Kennedy et al. 1962).

The model is based on a single virus SI model with explicit vector dynamics by Holt et al. (1997), expanded to include multiple viruses. Parameter definitions and values are given in Table 1-1. Hosts can belong to one of 5 categories: susceptible (S), infected with virus A (I_a), infected with virus B (I_b), coinfecting sequentially (I_{ab}), and

simultaneously coinfecting (J_{ab}). There are no priority effects for sequential coinfections because our empirical results showed no difference between the two types of sequential coinfections on either transmission or virulence (BYDV then BSMV, or BSMV then BYDV). Virus A is vertically and horizontally transmitted, while virus B is only horizontally transmitted. Vector dynamics are modeled explicitly. Vectors are either not carrying any virus (U), carrying virus A (V_a), carrying virus B (V_b), or carrying both viruses (V_{ab}); the order of acquisition (A first or B first) is assumed to not matter for vectors carrying both viruses. There is a virus species-specific probability (f) that vectors feeding on a coinfecting plant will fail to acquire one of the viruses. Vectors are infectious for life after acquiring one or more viruses and die with constant per-capita rate (c). Virulence (α) is modeled in two different ways: a reduction in seed production (parameterized by data) or an increase in host death rate (rates of mortality increase are proportional to observed decreases in seed production). Increased host death rates due to infection would be the expectation for pathogens that kill their hosts.

All hosts are annuals, and exhibit two stages of population change. During the growing season, host and vector population dynamics are described by a system of ordinary differential equations (Eqns. 1-9). A low proportion of vectors that enter the host population at the beginning of the growing season are infectious. The growing season lasts for just over 3 months (100 days). The initial host population size at the beginning of the growing season is influenced both by planting density (θ) and a small number of seeds left in the field from the previous year (Eqns. 10-11). The model is not intended to provide exact disease estimates for the BYDV-BSMV-barley system,

but rather examines situations where differences in virulence due to coinfection timing may be important. The model calculates host and vector dynamics over 100 growing seasons. A sensitivity analysis was performed to determine which parameter values have the most impact on disease dynamics (see Appendix A for full details).

Effects on projected host density and seed yield at the end of the growing season were calculated for three different model assumptions. The first two assumed that all coinfections have the same virulence: either that of a sequential coinfection or that of a simultaneous coinfection. The third had different virulence for sequential and simultaneous coinfections, based on our experimental findings. Proportional differences in host density were determined by dividing the host density at the end of the growing season under each disease scenario by the host density in a healthy population, and averaging over years. Projected seed yield losses were estimated by multiplying host density in each category by the seed production of that category, and dividing by the expected seed production in a healthy host population.

$$\begin{aligned}
dS/dt &= -\beta SV_a - \beta SV_b - \beta SV_{ab} - \mu S & (1) \\
dI_a/dt &= \beta SV_a - \beta I_a V_b - \beta I_a V_{ab} - \alpha_a I_a - \mu I_a & (2) \\
dI_b/dt &= \beta SV_b - \beta I_b V_a - \beta I_b V_{ab} - \alpha_b I_b - \mu I_b & (3) \\
dI_{ab}/dt &= \beta I_a V_b + \beta I_b V_a + \beta I_a V_{ab} + \beta I_b V_{ab} - \alpha_{ab} I_{ab} - \mu I_{ab} & (4) \\
dJ_{ab}/dt &= \beta SV_{ab} - \alpha_{jab} J_{ab} - \mu J_{ab} & (5) \\
dU/dt &= b(U + V_a + V_b + V_{ab}) \times \left(1 - \frac{U + V_a + V_b + V_{ab}}{m(S + I_a + I_b + I_{ab} + J_{ab})}\right) - \beta I_a U - \beta I_b U \\
&\quad - \beta U I_{ab} f_b - \beta U I_{ab} f_a - \beta I_{ab} U(1 - f_a - f_b) - \beta J_{ab} U f_b - \beta U J_{ab} f_a & (6) \\
&\quad - \beta J_{ab} U(1 - f_a - f_b) - cU \\
dV_a/dt &= \beta I_a U - \beta I_b V_a + \beta U I_{ab} f_b - \beta V_a I_{ab}(1 - f_b) + \beta U J_{ab} f_b & (7) \\
&\quad - \beta V_a J_{ab}(1 - f_b) - cV_a \\
dV_b/dt &= \beta I_b U + \beta U I_{ab} f_a + \beta U J_{ab} f_a - \beta I_a V_b - \beta I_{ab} V_b(1 - f_a) & (8) \\
&\quad - \beta J_{ab} V_b(1 - f_a) - cV_b \\
dV_{ab}/dt &= \beta I_{ab} U(1 - f_a - f_b) + \beta I_b V_a + \beta I_{ab} V_a(1 - f_b) + \beta I_a V_b + \beta I_{ab} V_b(1 - f_a) \\
&\quad + \beta J_{ab} U(1 - f_a - f_b) + \beta J_{ab} V_a(1 - f_b) + \beta J_{ab} V_b(1 - f_a) - cV_{ab} & (9) \\
S_{s+1} &= \theta(1 - p) + r(S + w_a I_a(1 - p) + w_b I_b + w_{ab} I_{ab}(1 - p) + w_{jab} J_{ab}(1 - p)) & (10) \\
I_{a(s+1)} &= \theta p + r(w_a I_a p + w_{ab} I_{ab} p + w_{jab} J_{ab} p) & (11)
\end{aligned}$$

Results

Within-host viral performance. Inoculation success was high, and viruses had no trouble infecting a host that had been previously infected by another virus species. Sequential coinfections that received BSMV second had lower BSMV concentration than single BSMV infections ($p=0.004$, Table 1-2, Figure 1-2A), but the BSMV concentration of these sequential infections increased faster over time than BSMV concentration in single infections when the full 7 weeks of sampling were included ($p<0.05$). When weeks 1 and 2 were excluded from the analysis to avoid transient effects, the difference in BSMV concentration in coinfections that received BSMV second was only marginally lower than BSMV single infections ($p=0.08$). During weeks 3-7, BSMV concentration increased over time in all inoculation treatments that included BSMV ($p<0.02$), with no significant interactions between infection treatment and time. BSMV concentrations in simultaneous coinfections and sequential coinfections that received BSMV first were not significantly different from BSMV concentration in single infections ($p>0.1$), whether the full sampling period or only weeks 3-7 were included in the analysis.

BYDV viral concentration analysis results were similar whether the full 7 weeks of sampling were included in the analysis, or the analysis focused on weeks 3-7. Sequential coinfections had lower BYDV concentration than BYDV single infections, regardless of whether BYDV was inoculated first or second ($p<0.05$; Table 1-2, Figure 1-2B). Sequential coinfections where BYDV was inoculated first had higher BYDV concentration than those where it was inoculated second ($p<0.05$). All infection treatments that included BYDV increased in BYDV concentration equally

over time ($p < 0.001$, Table 1-2). Interestingly, BYDV concentration in simultaneous coinfections was not different from BYDV concentration in single infections ($p = 0.80$, Table 1-2, Figure 1-2B).

Virulence. The effects of different infection treatments on host performance were very similar for vegetative biomass, reproductive biomass, and total number of seeds. Therefore, we only present the results from the total number of seeds produced by hosts as the most direct measure of host fitness. Plants infected with only BYDV produced the same number of seeds as controls ($p = 0.75$, Table A3, Figure 1-3). This further shows that early feeding by aphids had no effect on plant fitness. BSMV infection significantly lowered seed production, either as a single infection or as part of either type of sequential coinfection ($p < 0.001$). Therefore, the amount and timing of plant tissue damage during inoculation had no influence on plant fitness. Simultaneous infections resulted in the highest disease severity and lowest seed production ($p < 0.001$). There was no relationship between host seed production and median viral concentration of infected plants for either virus ($p > 0.22$).

Viral transmission. We observed no evidence that BYDV and BSMV transmission rates are affected by coinfection. BYDV and BSMV horizontal transmission rates were the same for coinfections and single infections (Figure A6; $p > 0.75$ and $p > 0.36$, respectively). BSMV vertical transmission was also the same from single and coinfections (Figure A7; $p > 0.47$). However, plants that were simultaneously coinfecting produced many fewer seeds than plants infected with either BSMV alone or a sequential coinfection, so vertical transmission opportunities were reduced.

Host-pathogen model. We used a mathematical model to ask how important it is to distinguish between sequential and simultaneous coinfections when projecting disease impacts on host populations. One can fail to distinguish between different types of coinfections in two ways, either by treating all coinfections as sequential or by treating all coinfections as simultaneous. We therefore compared model simulations that assumed that: 1) all coinfections have the virulence of a sequential coinfection, 2) all coinfections have the virulence of a simultaneous coinfection, or 3) coinfections with different infection timing have different virulence. The model is most sensitive to changes in initial host density (planting rates), which have a large impact on disease prevalence and on the importance of explicitly tracking coinfection timing (Figure 1-4, Figures A1-A3). As planting rate increases, the difference in projected disease impacts between the various modeling scenarios also increases (Figure 1-4). This is most extreme when virulence is expressed as a decrease in seed yield (Figure 1-4A), as opposed to when virulence is expressed as an increase in host death rate (Figure 1-4B). The ability for coinfections to increase in prevalence is limited in the latter case, because coinfecting plants are removed from the population more quickly and therefore contribute less to transmission dynamics.

The model sensitivity analysis sheds light on which situations would benefit most from explicit tracking of coinfection timing (Figures A1-A3). Higher transmission rates tend to have a stronger positive effect on sequential coinfections than simultaneous coinfections, which decreases the proportion of coinfections that are simultaneous. As one would expect, increasing the disease-induced mortality of simultaneously coinfecting hosts also decreases the proportion of coinfections that are

simultaneous. Higher vertical transmission rates increase the proportion of coinfections that are sequential by preventing simultaneous coinfections. The proportion of simultaneous coinfections is positively affected by planting rate, the probability that an aphid feeding on a coinfecting plant will acquire both viruses, and increasing virulence of sequential coinfections. Finally, a small number of volunteer seedlings left in the field from the previous growing season has little effect on disease prevalence.

Discussion

The timing of host inoculation had significant effects on the strength of interactions between viruses and impacts on the host. Priority effects were evident when inoculations occurred sequentially, where the last virus to infect the host always had a lower overall concentration in coinfections than in single infections. However, viruses did not show competitive within-host dynamics when inoculations were simultaneous. At the between-host level, BSMV transmission opportunities were equivalent for single infections and sequential coinfections with BYDV, but vertical transmission opportunities were lower for BSMV in simultaneous coinfections due to the presence of greater than additive, or synergistic, virulence. In sequential coinfections, virulence was equivalent to the most virulent constituent virus.

A difference in virulence between sequential and simultaneous coinfections of the magnitude observed in our empirical work can matter at the host population level, but the strength of the effect on the host population is context dependent. Virulence differences due to coinfection timing lead to the largest projected population level

effects both when virulence is expressed as a decrease in seed production and in cases of higher initial host population density. All else being equal, one would expect to observe higher impacts of coinfection timing in systems where horizontal transmission rates are modest, the probability that vectors acquire multiple viruses when feeding on a coinfecting plant is high, vertical transmission rates are low, host density is high, and where disease-induced mortality due to coinfection is low.

Many coinfection experiments in plants only examine simultaneous coinfections (e.g. Pio-Ribeiro et al. 1978; Rentería-Canett et al. 2011; Scheets 1998), which may lead to an overestimate of the population level effects of multiple infections. However, there is a trend toward incorporating both simultaneous and sequential coinfections in experiments (e.g. Kim et al. 2010), which will provide better estimates of the effects of coinfection on host populations. While we did not observe priority effects in parameters that matter at the population level (transmission and virulence) during sequential coinfections, it is important to continue to include both sequences of coinfection (inoculation with A then B as well as inoculation with B then A). We did observe priority effects on within-host concentration, and in other systems priority effects on within-host concentration could lead to priority effects on transmission and virulence.

The virulence-transmission trade-off hypothesis predicts that within-host parasite accumulation should be positively correlated with virulence and transmission (Froissart et al. 2010). While this prediction has been observed in some systems (as reviewed by Alizon et al. 2009; Sacristan and Garcia-Arenal 2008), other systems yield different results (Escriu et al. 2003; Sacristán et al. 2005). In the present study,

neither transmission success nor virulence were correlated with the within-host concentration of either virus. In fact, three infection treatments had the same virulence despite significant differences in viral concentration. A number of hypotheses have been proposed to explain deviations from the trade-off hypothesis. In the present study, the greater than additive virulence observed in simultaneous coinfections could result from an increased host immune response that is more costly or damaging (Dunoyer and Voinnet 2005; Voinnet 2005). Host tolerance may also obscure the predictions of the trade-off hypothesis (Fraile and Garcia-Arenal 2010), particularly in cases where tolerance reduces virulence nonlinearly (Miller et al. 2006).

The fact that BSMV had higher virulence in single infections than BYDV was surprising. BSMV should theoretically have reduced virulence, since seed transmission is an important part of its epidemiology (Jones et al. 2010). However, the lab strain of BSMV used had a long history of horizontal transmission, which is known to increase virulence in this species (Stewart et al. 2005). A history of vertical transmission may have resulted in decreased BSMV virulence (Pagan et al. 2014; Stewart et al. 2005). The field-collected BYDV isolate, while avirulent in all but simultaneous coinfections in the greenhouse, may exhibit increased host impacts under more stressful field conditions, such as water limitation and low soil fertility. We used the host population model to examine what would happen if the virulence of the horizontally transmitted virus (virus B) and the horizontally and vertically transmitted virus (virus A) were reversed. In this situation, our main result that keeping track of coinfection timing can make a real difference still holds, especially as planting rate/initial population density increases (data not shown). When virus B is more virulent

than virus A, the main effect is a reduction of disease impacts on the host population.

It is becoming increasingly clear that the relative timing of parasite invasion can have significant impacts on both within-host competition and disease severity (de Roode et al. 2005; Hood 2003; Hoverman et al. 2013; Lohr et al. 2010). We observed greater within-host competition when there was a lag between invasions of different pathogen species, either due to a competitive advantage of greater within-host abundance or through apparent competition via previous activation of the host's immune response. Disease severity was highest when hosts were inoculated with both pathogens at once. In order to improve projections of disease impacts at the host population level, coinfection studies should include both simultaneous and sequential inoculations when possible.

Acknowledgments

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	Model parameters	Virulence as death rate	Virulence as seed loss	Units
θ	planting rate	varied	varied	m^{-2}
b	transmission/ acquisition rate	0.004	0.004	vector ⁻¹ day ⁻¹
r	proportion of volunteer seed	0.01	0.01	
u	background host death rate	0	0	day ⁻¹
a_a	death rate due to virus A	0.01433	0	day ⁻¹
a_b	death rate due to virus B	0.0013	0	day ⁻¹
a_{ab}	death rate in sequential coinfections	0.01433	0	day ⁻¹
a_{jab}	death rate in simultaneous coinfections	0.02733	0	day ⁻¹
b	vector birth rate	0.2	0.2	day ⁻¹
m	maximum number of aphids/ plant	50	50	plant ⁻¹
c	vector death rate	0.12	0.12	day ⁻¹
f_a	proportion of potential coinfection acquisitions where only virus B is acquired	0.1	0.1	
f_b	proportion of potential coinfection acquisitions where only virus A is acquired	0.1	0.1	
w_a	proportional decrease in seed production caused by virus A	1	0.57	
w_b	proportional decrease in seed production caused by virus B	1	0.96	
w_{ab}	proportional decrease in seed production caused by sequential coinfections	1	0.57	
w_{jab}	proportional decrease in seed production caused by simultaneous coinfections	1	0.18	
p	proportion of seeds infected with virus A	0.1	0.1	

Table 1-1: Model parameter definitions and values. Parameter values are given both for when virulence is expressed as increased host death rate and when virulence is expressed as decreased host seed production. Initial conditions for the models are θ

susceptible hosts m^{-2} , 0.2 virus A infected hosts m^{-2} , 0.1 virus B infected hosts, and 50 noninfectious vectors. At the beginning of each successive growing season, 50 virus-free vectors, 4 vectors carrying virus A, 4 vectors carrying virus B, and 2 vectors carrying both viruses are added. *Italic parameters are either estimated from experimental data (host seed production) or proportional to these values (disease induced mortality rates).*

	<i>Barley stripe mosaic virus</i>				<i>Barley yellow dwarf virus</i>			
	Estimate	Std. Error	t value	P-value	Estimate	Std. Error	t value	P-value
Intercept	1.11	0.07	16.98	0.000 ***	0.91	0.10	9.09	0 ***
Week	0.00	0.01	0.10	0.920	0.11	0.02	5.77	0 ***
SMYD	0.18	0.11	1.61	0.121	-0.46	0.10	-4.53	0.0002 ***
YDSM	-0.38	0.12	-3.25	0.004 **	-0.22	0.11	-2.09	0.0495 *
J	0.11	0.12	0.96	0.347	-0.03	0.11	-0.26	0.7993
Week x SMYD	-0.04	0.02	-1.57	0.119	N.S.	N.S.	N.S.	N.S.
Week x YDSM	0.05	0.03	2.05	0.042 *	N.S.	N.S.	N.S.	N.S.
Week x J	-0.02	0.03	-0.91	0.362	N.S.	N.S.	N.S.	N.S.

Table 1-2: Linear mixed effects models describing the fixed effects of infection and sampling week on viral concentration.

Coinfections are compared to single infections of a given virus. Plant sampled explained 6.4% of model variation for BSMV and less than 1% of model variation for BYDV. SMYD: infection with BSMV followed by infection with BYDV. YDSM: infection with BYDV followed by infection with BSMV. J: simultaneous coinfections. N.S. not significant, * p<0.05, ** p<0.01, *** p<0.001

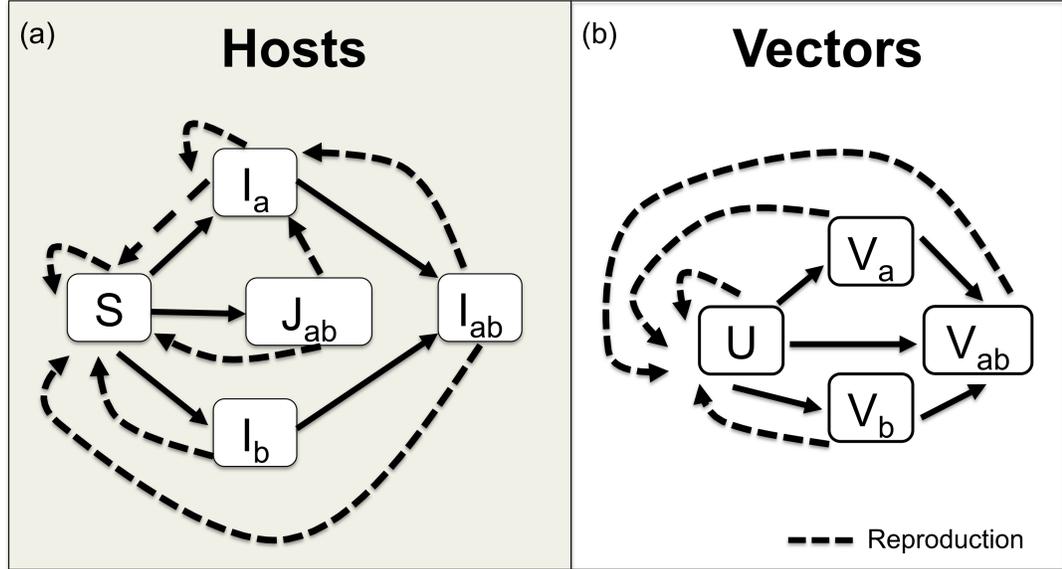


Figure 1-1: Diagram of the state variables and transitions between them. Hosts are either susceptible (S), infected with one virus (I_a or I_b), sequentially coinfecting (I_{ab}), or simultaneously coinfecting (J_{ab}). Virus A can infect seeds. Vectors may not be carrying any virus (U), carrying one virus (V_a or V_b), or carrying both viruses (V_{ab}). All hosts and vectors can die natural deaths, while infected hosts have an additional disease induced mortality rate. Transition arrows describing reproduction are dashed.

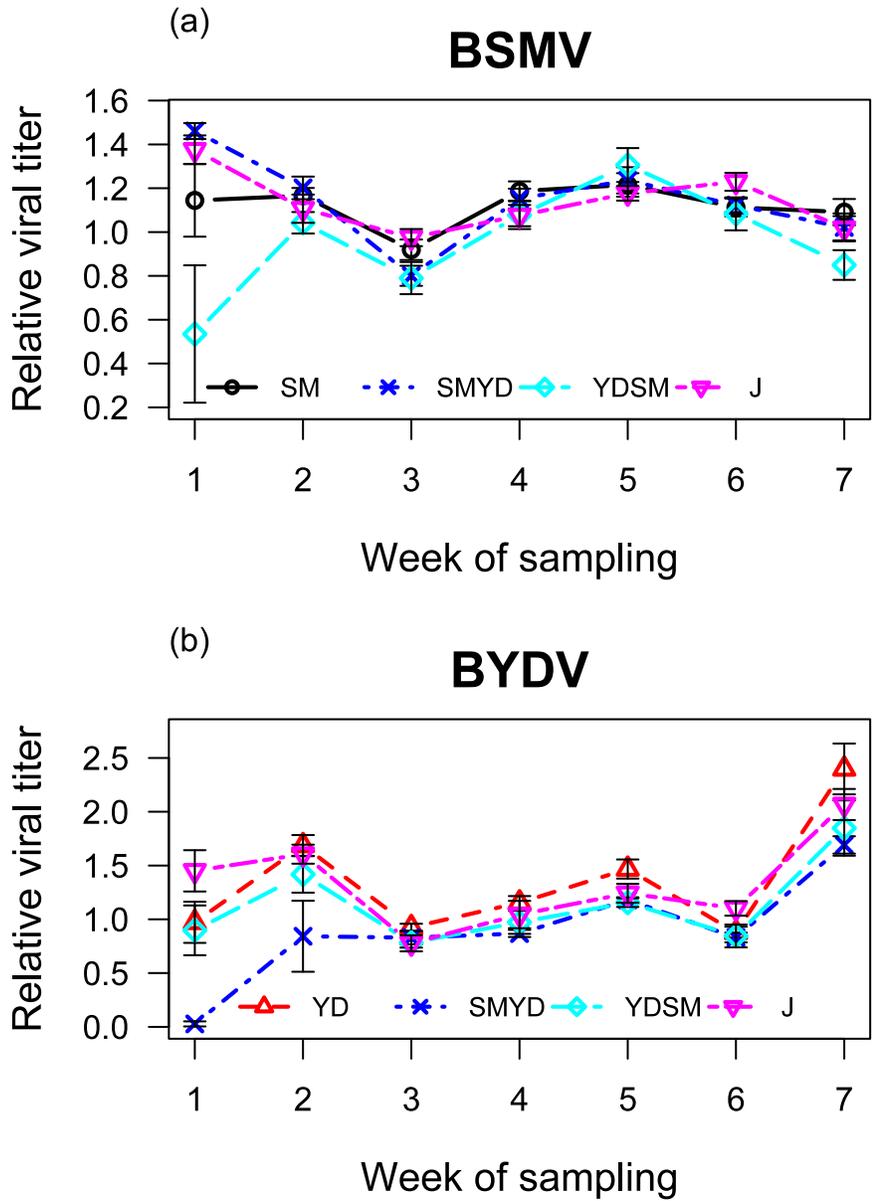


Figure 1-2: Relative viral concentrations over time for a) BSMV and b) BYDV. SM: BSMV single infection, YD: BYDV single infection, SMYD: infection with BSMV followed by infection with BYDV, YDSM: infection with BYDV followed by infection with BSMV, J: simultaneous coinfections. Bars denote standard errors.

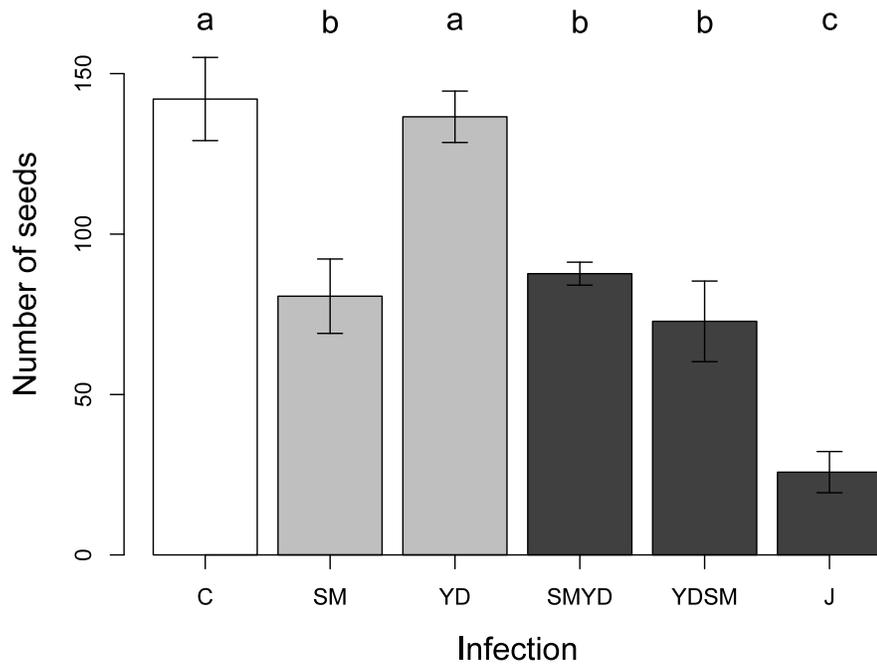


Figure 1-3: Seed production of hosts in each disease treatment. SM: BSMV single infection, YD: BYDV single infection, SMYD: infection with BSMV followed by infection with BYDV, YDSM: infection with BYDV followed by infection with BSMV, J: simultaneous coinfections. Bars denote standard errors.

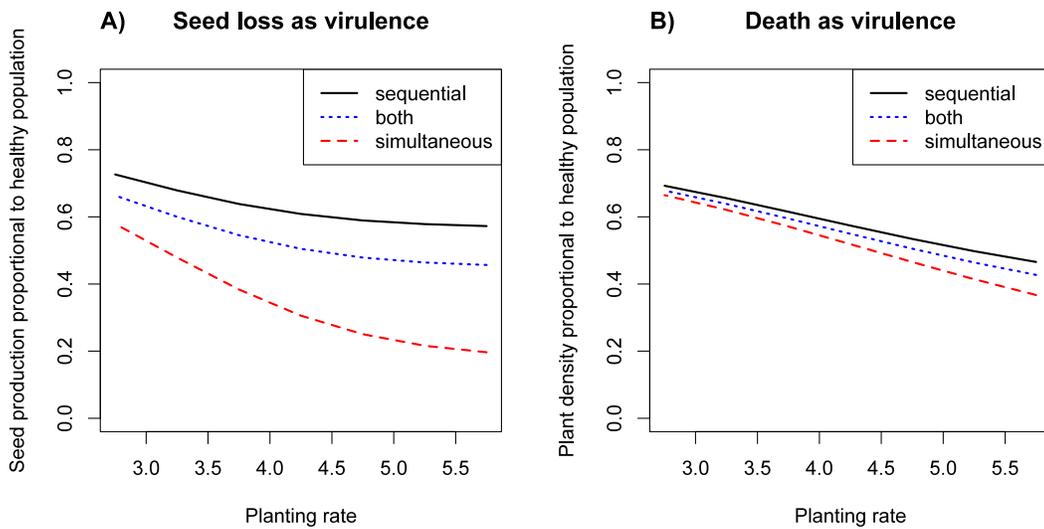


Figure 1-4: Projected proportional declines in seed production (A) and host density (B) in comparison to healthy populations for a range of planting rates when virulence is defined as either a decrease in seed yield (A) or an increase in death rate (B).

“Sequential” and “Simultaneous” describe scenarios where all coinfections were modeled as having the virulence of either a sequential coinfection or a simultaneous coinfection, respectively. The “Both” scenario captures the actual dynamics of the system, where sequential and simultaneous coinfections have different virulence.

APPENDIX A

Model Sensitivity Analysis

Methods

A sensitivity analysis was performed for the coinfection model to determine which parameters have the largest effects on model outcomes. Latin hypercube sampling was used to choose sets of parameter values evenly throughout the parameter space (Blower and Dowlatabadi 1994; Mao-Jones et al. 2010). Parameter minima and maxima examined are given in Table A1. Fifteen hundred parameter sets were randomly chosen for each of the model scenarios using the lhs function in R (R Development Core Team 2012). The model scenarios included the cases where the only effect of the disease was a reduction in host seed production, where the only effect of the disease was an increase in host mortality, and where both disease induced mortality and fitness loss were allowed to vary. Only cases where the total disease prevalence was greater than 1% were used for sensitivity analysis. Partial rank correlation coefficients were used to describe the sensitivity of the model output to each parameter value (Blower and Dowlatabadi 1994) using the epiR function in R. Parameter sensitivities for models where disease either only affects death rate or affects both death rate and host seed production were similar, so only the models where disease affects either only death or only seed production are shown.

Results

Host initial population size/ planting density was a consistently important parameter in the case where disease reduces host seed production (Figure A1). The total number of hosts was additionally affected by host death rate. Transmission/ acquisition and the rate of vertical transmission by virus A were important determinants of both total disease prevalence and the prevalence of simultaneous coinfections. Total prevalence was affected by host death rate and vector carrying capacity. The prevalence of simultaneous coinfections was influenced by the rate at which vectors fail to pick up both viruses from a coinfecting host.

When virulence is expressed as an increase in host death rate, transmission/ acquisition, host planting rate, death rates of sequentially coinfecting hosts, and the rate of vertical transmission by virus A were consistently important parameters (Figure A2). The total number of hosts was also affected by the death rates of hosts infected with single infections of virus A and coinfections. Total disease prevalence was sensitive to the death rate of hosts singly infected with virus A and vector carrying capacity. Finally, the prevalence of simultaneous coinfections was influenced by the death rate of hosts infected with simultaneous coinfections and by the rate at which vectors fail to pick up both viruses from a coinfecting host.

The proportion of coinfections that were simultaneous rather than sequential was increased by decreasing transmission/ acquisition rates, decreasing planting rates, lower rates in which aphids feeding on a coinfecting plant fail to transmit both viruses, decreasing vertical transmission rates, increasing virulence for sequential coinfections, and decreased disease-induced mortality of simultaneously coinfecting plants (Figure

A3).

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Parameter	Maximum	Minimum
β	0.03	0.003
r	0.1	0.001
θ	8	1
u	0.005	0.00005
a_a	0.1	0
a_b	0.1	0
a_{ab}	0.1	0
a_{jab}	0.1	0
b	0.25	0.15
m	200	25
c	0.17	0.11
f_a	0.4	0
f_b	0.4	0
w_a	1	0
w_b	1	0
w_{ab}	1	0
w_{jab}	1	0
p	0.8	0

Table A1: Maximum and minimum parameter value ranges used in the sensitivity analyses. Parameter definitions are given in Table 1 of the main text.

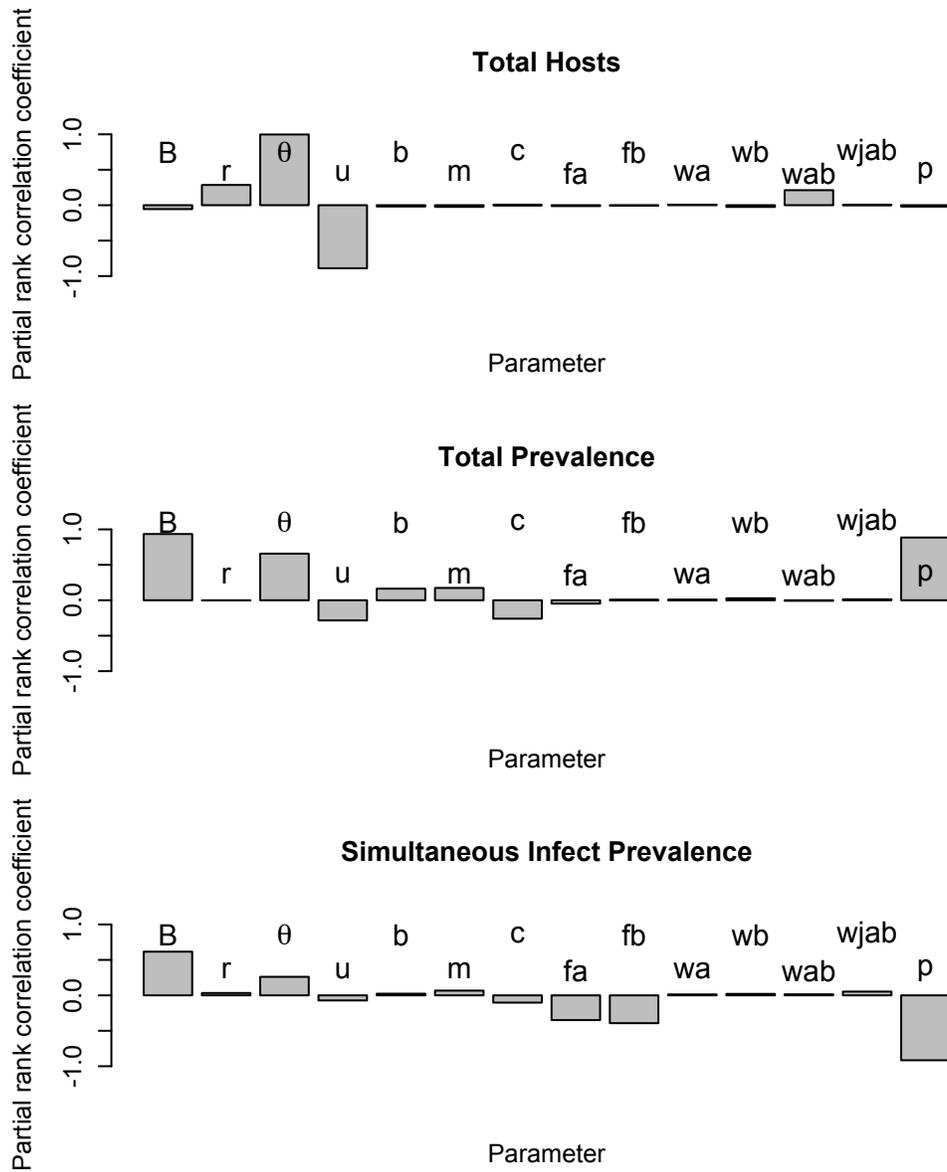


Figure A1: Model sensitivity analysis when the only effect of disease is a decrease in seed production. Parameter definitions are given in Table 1 of the main text. Model sensitivities are shown for total disease prevalences of 1% of the host population and up.

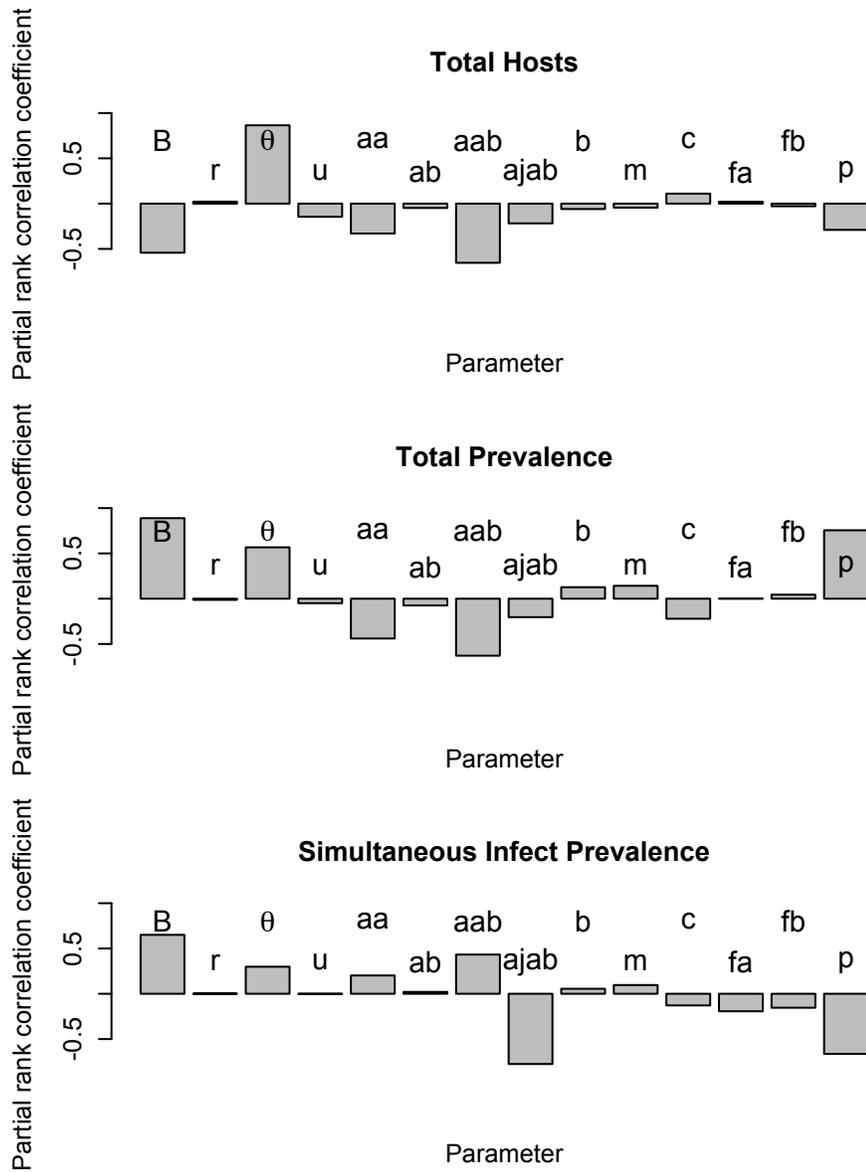


Figure A2: Model sensitivity analysis when the only effect of the disease is an increase in host mortality. Parameter definitions are given in Table 1 of the main text. Model sensitivities are shown for total disease prevalences of 1% of the host population and up.

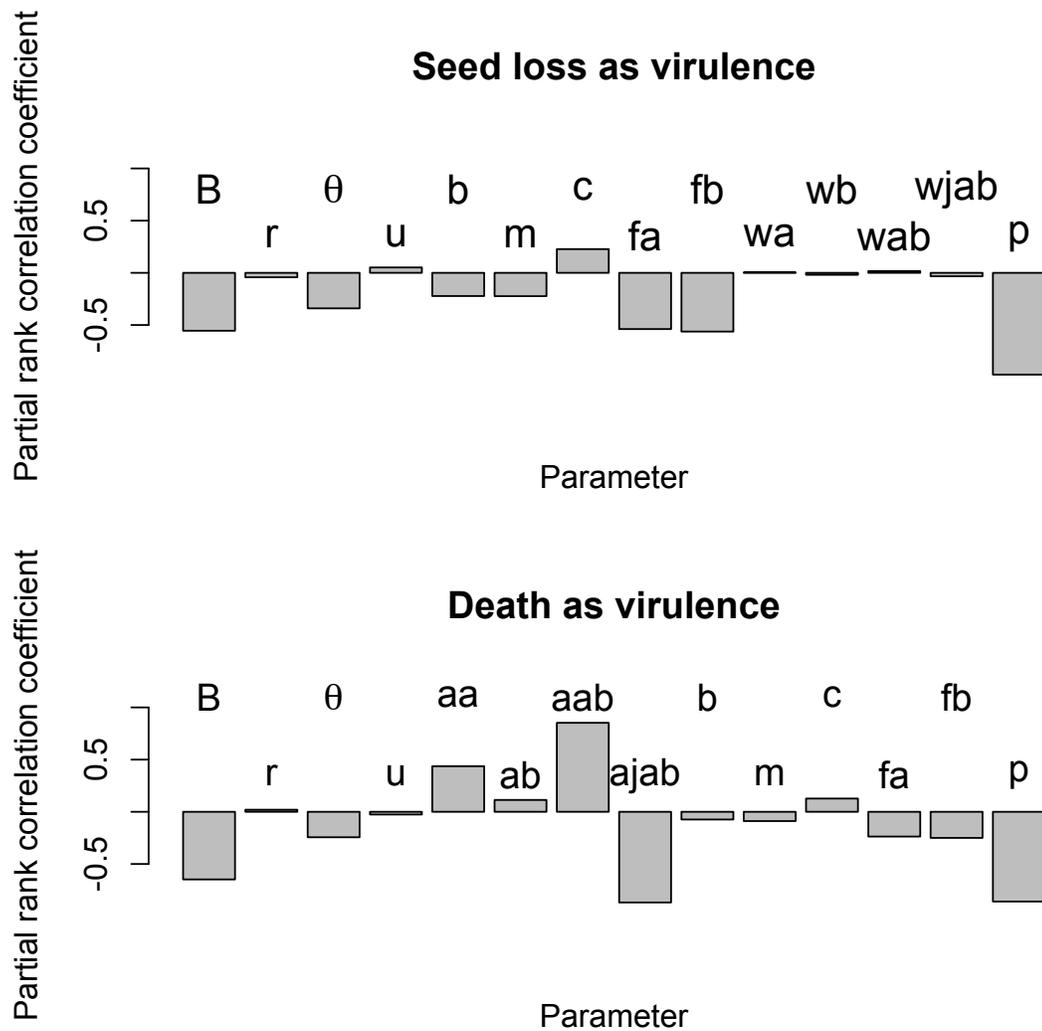


Figure A3: Model sensitivity analysis for the proportion of coinfections that were simultaneous rather than sequential. Parameter definitions are given in Table 1 of the main text. Model sensitivities are shown for total disease prevalences of 1% of the host population and up.

Supporting Tables and Figures

	Estimate	Std. Error	t value	P-value	
Intercept	4.96	0.08	62.31	$< 2 \times 10^{-16}$	***
SM	-0.57	0.14	-4.17	0.000	***
YD	-0.04	0.12	-0.32	0.748	
SMYD	-0.48	0.16	-2.95	0.005	**
YDSM	-0.67	0.19	-3.53	0.001	**
J	-1.71	0.30	-5.69	1.11×10^{-6}	***

Table A2: Generalized linear model describing the relationship between infection and the total number of seeds produced by the host. Quasipoisson errors were used to account for over-dispersed count data. All infection treatments are compared to uninfected controls. SM: BSMV single infection, YD: BYDV single infection, SMYD: infection with BSMV followed by infection with BYDV, YDSM: infection with BYDV followed by infection with BSMV, J: simultaneous coinfections. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

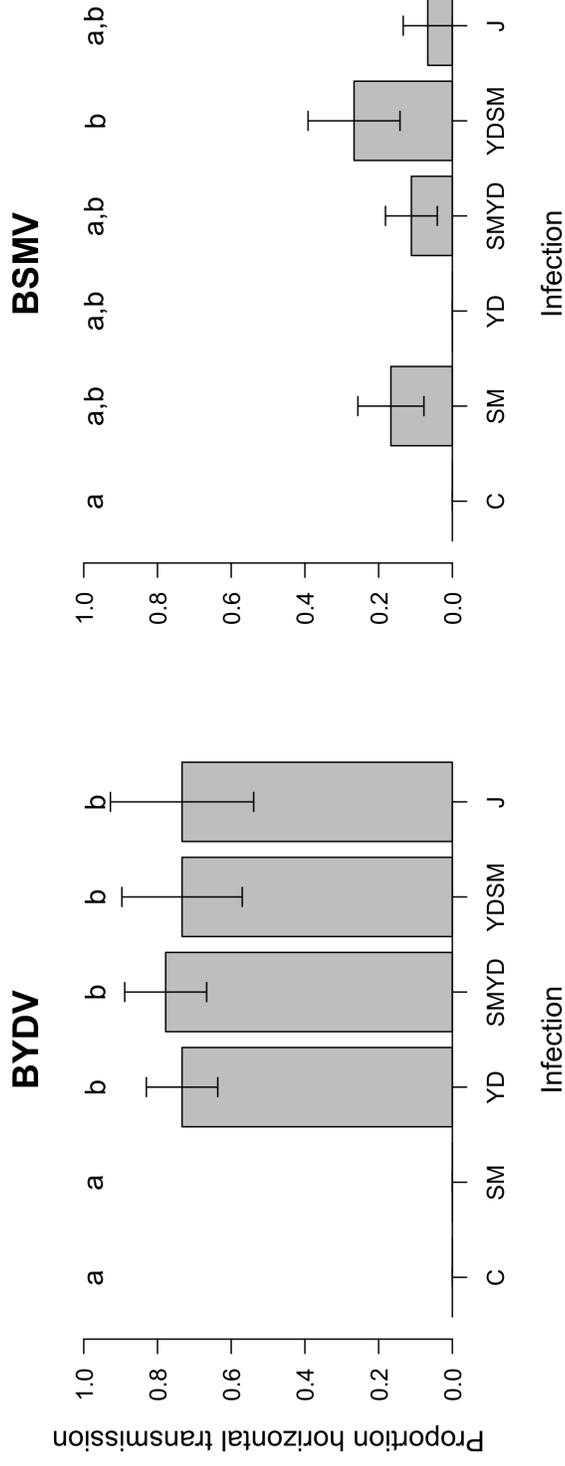


Figure A4: Horizontal transmission rates by infection treatment for *barley yellow dwarf virus* (BYDV; left panel) and *Barley stripe mosaic virus* (BSMV; right panel). C: control, SM: BSMV single infection, YD: BYDV single infection, SMYD: infection with BSMV followed by infection with BYDV, YDSM: infection with BYDV followed by infection with BSMV, J: simultaneous coinfections. Bars denote standard errors.

BSMV

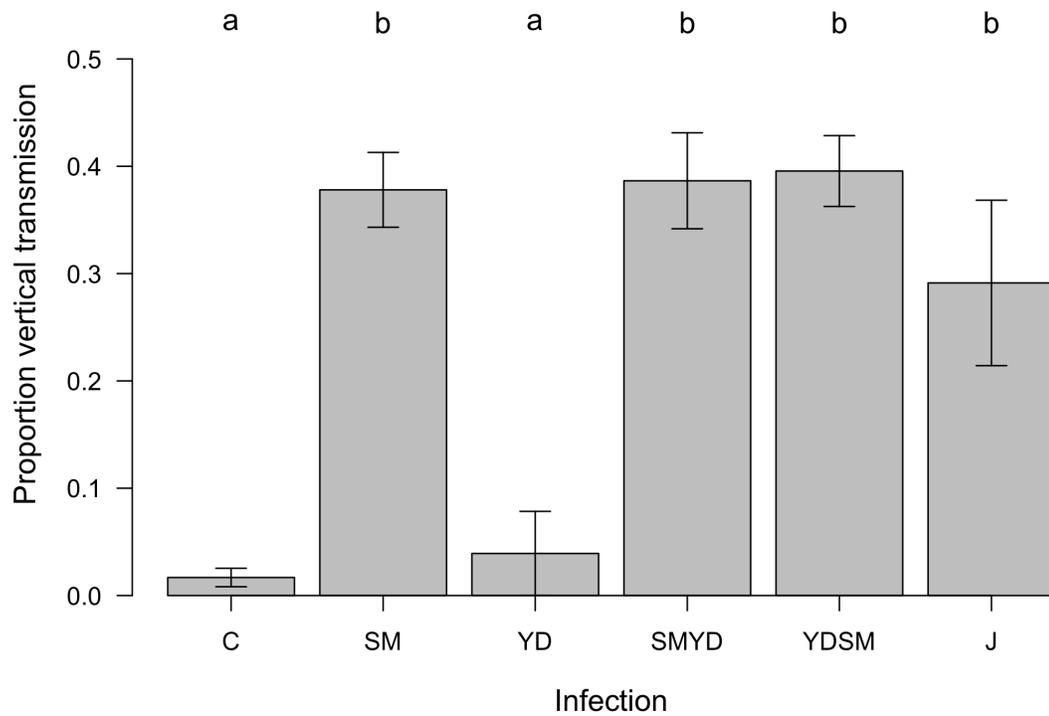


Figure A5: *Barley stripe mosaic virus* vertical transmission rates by infection treatment. C: control, SM: BSMV single infection, YD: BYDV single infection, SMYD: infection with BSMV followed by infection with BYDV, YDSM: infection with BYDV followed by infection with BSMV, J: simultaneous coinfections. Bars denote standard errors.

CHAPTER 2

EXPERIMENTAL EVOLUTION OF TWO RNA VIRUSES DURING COINFECTION IN PLANT HOSTS

Abstract

Pathogen evolution takes place in a complex environment where hosts are often concurrently infected by multiple pathogen species. There has been intense theoretical interest in how multiple infections influence the evolution of pathogen virulence. The overarching expectation is that in most cases, multiple infection should lead to the evolution of increased virulence. However, relevant empirical studies are few and typically involve cell culture or single-celled hosts rather than more complex, multicellular hosts. We conducted an experimental evolution study to determine how passaging in coinfections affects virulence, within-host accumulation, and transmission of two RNA viruses, *Cereal yellow dwarf virus* and *Barley yellow dwarf virus*, in barley hosts. We found no change in pathogen virulence, despite significant changes in both within-host accumulation and transmission by *Cereal yellow dwarf virus*. Our results suggest that more experimental evolution studies employing multicellular hosts are needed to evaluate the assumptions and expectations of theoretical models of the effects of coinfection on pathogen evolution.

Introduction

Infections of one host with multiple species of pathogens are common in nature. For example, in cultivated wheat and native grass species the average percentage of viral infections that contain multiple viral species can be quite high, 60% and 70%, respectively (Mesterházy et al. 2002, Seabloom et al. 2009). Whether

in cultivated or natural settings, pathogens typically evolve in the context of within- and between-host competition with cohabiting microbes.

Much theoretical work has focused on the evolution of pathogen virulence. The way virulence is defined is discipline-specific, but in most theoretical work virulence is defined as an increase in disease-induced mortality. In single infections, the prevailing hypothesis is the virulence-transmission trade-off hypothesis (Anderson and May 1982). This hypothesis is predicated on the assumption that increased within-host concentrations drive increased transmission rates, but also lead to increased disease-induced mortality (Alizon et al. 2009). The expectation of this model is that pathogens with the highest fitness should exhibit intermediate virulence. However, models that contain more than one species of pathogen competing within hosts typically lead to different conclusions.

One of the first hypotheses addressing the effects of within-host competition on the evolution of pathogen virulence was the short-sighted evolution hypothesis (Levin and Bull 1994). Here, the expectation is that pathogens that accumulate higher within-host concentrations will be able to compete more strongly for host resources, which leads to increasing virulence. This type of evolution would be short-sighted because competition at the within-host level has a negative effect on transmission through increased host death rates. There is some empirical evidence for the assumptions of this model. For instance, more virulent strains of *Plasmodium chabaudi* tend to have a greater negative effect on competitors while also causing greater host red blood cell loss (de Roode et al. 2005a); however, results have been mixed as to whether within-host numerical dominance is associated with increased transmission (Taylor et al. 1997, de Roode et al. 2005b, Pollitt et al. 2011).

The short-sighted evolution hypothesis is not perfect. Theoretical work on this topic suggests that many different types of dynamics are possible even with similar

basic assumptions. For example, Nowak and May (1994) examined a model in which the most virulent strain to infect a host is assumed to completely outcompete the other strains. While average pathogen virulence is higher than the optimal virulence for a single pathogen system, the population can support a range of pathogen virulence levels (Nowak and May 1994).

While the prevailing expectation is that coinfection should increase pathogen virulence, there are a number of biologically relevant considerations that affect our assumptions about how coinfection could influence viral evolution. First, the degree of pathogen phenotypic plasticity, or to what extent pathogens can alter within-host accumulation and virulence in single vs. coinfections, is expected to drastically affect predictions of the effect of pathogen competition on virulence evolution (Choisy and de Roode 2010). In addition, the level of virulence associated with a coinfection is not always correlated with within-host pathogen concentration. There are cases of multiple infections that are less virulent than a single infection of the most virulent component pathogen (Thomas et al. 2003, Kim et al. 2010), or at least no more virulent than the most virulent component pathogen (Lohr et al. 2010). It is expected that in cases where coinfection leads to reduced virulence, hypervirulent strains can persist in a host population (Alizon 2008). The sequence and timing in which different pathogens invade hosts can also influence virulence (Chapter 1, de Roode et al. 2005a, Kim et al. 2010, Lohr et al. 2010).

In addition, virulence is not only a function of pathogen genes (Råberg et al. 2009). Host traits, including tolerance mechanisms and immune system aggressiveness, also affect virulence (Day et al. 2007, Ayres and Schneider 2012). Heterogeneity in virulence is also caused by host responses to the presence of microbial mutualists, as well as gene expression by these mutualists (Zamioudis and Pieterse 2012). Finally, many pathogens, especially those of plants, are not lethal

under ideal conditions (Slykhuis 1976). Theoretical work suggests that coinfection can select for reduced virulence when pathogens reduce host growth rate, at least in the case where pathogen transmission is related to host size (Schjørring and Koella 2003). For instance, a larger animal could support a larger within-host parasite population, or a larger plant could be more apparent to insects that vector viruses.

Facilitation, and even cooperation, between coinfecting pathogens is also expected to influence pathogen evolution (Leggett et al. 2014). In some cases, transmission of one or both pathogens is increased in coinfecting hosts in comparison to singly infected hosts due to changes in vector preference or performance (Srinivasan and Alvarez 2007, Salvaudon et al. 2013). Another important assumption is the exchange of common goods between coinfecting pathogens (Brown et al. 2002). Common goods are products that are costly to produce, and can be utilized by multiple pathogens. Examples include bacterial siderophores and viral coat proteins. The influence that common goods are expected to have on the evolution of virulence in systems with multiple pathogens depends on assumptions about the relationship between transmission and virulence, and under certain conditions could lead to either reduced or increased virulence (Alizon and Lion 2011).

These complex theoretical explorations of coinfection and virulence suffer from the scarcity of empirical data. More experimental evolution and long-term studies are needed to help test assumptions and validate the predictions made by theoretical work (Alizon et al. 2013). We address this gap using an experimental evolution approach to examine how coinfection affects viral strains that had been in single infections for more than 15 years. Within-host concentrations of viral particles, virulence, and insect-vectored transmission were assessed for pathogen strains with different serial passaging histories, either in single or mixed infections. We observed significant changes in strain characteristics after serial passaging in coinfections, but

these results are not well predicted by current theoretical models.

Materials and Methods

Study species: *Barley yellow dwarf virus* (BYDV; Luteovirus) and *Cereal yellow dwarf virus* (CYDV; Polerovirus) are positive-sense, single stranded RNA viruses in the family *Luteoviridae*. Both species share a host range of over 150 species of grasses, including economically important cereal crops (Miller and Rasochová 1997). Infections of one host with multiple BYDV and CYDV species are common in the field (Seabloom et al. 2009). Our experiments were conducted with the RPV species of CYDV and both the PAV and PAS species of BYDV. Viral strains were originally isolated from central New York, USA, and have been maintained in the laboratory in single species infections for more than 15 years. Coinfections were conducted between the RPV species of CYDV and one of the BYDV species.

Both BYDV and CYDV are exclusively aphid transmitted in a non-propagative, circulative manner. Viruses do not replicate within the aphid, and once acquired can be transmitted for the life of the vector (Ng and Perry 2004). PAV, PAS, and RPV are transmitted at high efficiency by *Rhopalosiphum padi*. Colonies of *R. padi* used for inoculations were maintained on barley (*Hordeum vulgare* var. Conlon) in growth chambers at 20°C in 24 hr. light.

Experimental evolution experiment: Serial passages were initiated by inoculating barley seedlings with single infections (PAV or RPV only) or coinfections of BYDV and CYDV (PAV-RPV). Barley seedlings were from the same maternal line to reduce between-host differences in immune response. Seeds were planted in Metro-Mix (Sun Gro Horticulture, Agawam, MA, USA) and plants received 150ppm of 21-5-20 fertilizer (Jack's Professional, JR Peters Inc., Allentown, PA, USA). *R.*

padi aphids were allowed to feed on tissue from singly infected plants for 2 days prior to inoculation for viral acquisition. Inoculations took place eight days after planting. Mock-inoculated controls and single infections received 5 aphids each, and coinfections received 5 aphids carrying each coinfecting virus species. There were sixteen replicates per treatment. Each plant was caged individually, and aphids were allowed to feed for 5 days before they were killed using the insecticide Talstar (FMC Professional Solutions, Philadelphia, PA, USA). Inoculation success was confirmed using enzyme-linked immunosorbent assays (ELISA) using antibodies from Agdia Diagnostics (Elkhart, IN, USA).

Plantings for subsequent serial passages occurred at approximately 50 day intervals. The passaging timing and protocol were the same as for the first passage with a few exceptions. In order to establish independent viral lineages, each original infected plant received a name and tissue from each of the original plants was used to inoculate 2 new seedlings. Two replicates were used to insure against imperfect inoculation success or host mortality. New seedlings were inoculated with a total of 5 aphids that had fed on tissue from a specific and independent lineage. Serial passages continued for a total of 12 host generations. However, experiments to determine the competitiveness and virulence of strains occurred at multiple points during this time span. Strain competitiveness experiments for PAS-RPV coinfection strains occurred after 4 and 6 serial passages, while PAV-RPV experiments occurred after 7 and 8 passages. Transmission trials for PAV-RPV coinfection strains occurred after 12 serial passages.

Strain competition experiments: In order to assess strain competitiveness and virulence, strains that had been passaged in coinfections were split briefly into single species infections. Tissue from passaged plants was fed to aphids for restricted acquisition periods (4-24 hours) and single aphids were used to inoculate seedlings.

Viral infection status was confirmed using ELISA before tissue was used in strain competition experiments.

Inoculation treatments for strain competition experiments comprised control plants, and plants with every combination of single and coinfection (see Table 2-1 for abbreviations, descriptions, and replication). The experiment was repeated twice for each for PAV-RPV and PAS-RPV. Inoculations were carried out in the same manner as during the initiation of serial passaging (5 aphids from each viral species to be inoculated).

Within-host viral concentrations were assessed 2 weeks after inoculation using quantitative ELISA (Pollina 2013). Briefly, 0.5g leaf tissue samples were ground in 5mL phosphate-buffered saline. All plants were tested for both PAV and RPV. For each viral species test, two samples were randomly located on two separate 96-well plates. Optical density (OD) values were standardized by dividing the OD value of each sample by the average OD value for the positive control on each plate. The standardized OD values for each plant from each replicate plate were then averaged. During the first PAS-RPV experimental replicate, optical density values were abnormally high for the PAS positive controls. Therefore, PAS optical density values were standardized by the average optical density of the sPAS samples on each plate. This has no effect on the statistical results, but it makes the data in both experimental replicates comparable for visualization. Data were analyzed using linear mixed effects models in R with spatial block nested in experimental replicate as random variables (R Development Core Team 2015).

Neither BYDV nor CYDV tend to kill their hosts under good growing conditions (Catherall 1966), so plant dry biomass was used as a proxy for virulence. Aboveground plant parts were harvested approximately 40 days after planting, dried, and weighed.

Transmission trials: Transmission trials were conducted for sPAV and sRPV as well as for 6 lineages of coPAV, coRPV, and coPR. Aphids were fed on tissue from infected plants of each lineage for an acquisition period that lasted 12 hours and 5 minutes for all samples. Twenty seedlings per infection type and viral strain lineage were inoculated with 3 aphids each. Aphids were allowed to feed for 5 days before being killed using insecticide. Student's t-tests were used to compare differences in the mean proportion of viral transmission events from each infection type. Transmission trials were not carried out for PAS-RPV strains.

Results

Within-host viral particle concentration: RPV strains with a history of coinfection accumulated higher viral particle concentrations in single infections than strains with a history of single infections ($p < 0.05$, Table 2-2, Figure 2-1A). While sRPV strains had significantly lower viral particle accumulations in coinfections with either sPAV or coPAV ($p < 0.0001$), coRPV retained the same viral particle accumulations in coinfection with sPAV or coPAV as a single infection of sRPV ($p < 0.57$). Similar results were seen for RPV with a history of coinfection with BYDV-PAS (Table 2-3, Figure 2-2A).

There were few significant differences in PAV viral particle concentration (Table 2-2, Figure 2-1B). However, single infections of coPAV strains did have significantly higher viral particle concentrations than the PAV concentration of coPAV-sRPV or sPAV-coRPV ($p < 0.047$). This contrasted with the results for PAS strains passaged in coinfections with RPV. When paired with either sRPV or coRPV, coPAS viral particle concentrations in coinfections were lower than sPAS concentrations in single infections ($p < 0.025$, Table 2-3, Figure 2-2B).

Virulence: Any infection generally caused a significant reduction in host dry

aboveground biomass in comparison to controls, and coinfections tended to have a greater impact on hosts than single infections (Figure 2-3). However, there was no consistent effect of strain history on virulence for PAV-RPV strains. The PAS-RPV strain data are similar, but single infections and coinfections tended to have more similar effects on hosts (Figure 2-4).

Transmission: Viral transmission was lower for strains with a history of coinfection than strains with a history of single infection for both RPV and PAV ($p < 0.002$, Figure 2-5). coRPV strains had a significantly lower transmission rate when the host plant was coinfecting than when the host was singly infected ($p < 0.01$). However, coPAV strains transmitted just as well from either coinfections or single infections ($p = 0.26$).

Discussion

Our results provide experimental evidence that evolution does not necessarily favor increased virulence when pathogens have a history of coinfection. While coinfections tended to have a greater negative impact on hosts than single infections, strain history had no effect on host aboveground biomass. Strains were transmitted to new hosts regardless of the effect on the host, so there was no constraint on virulence evolution. Despite no change in pathogen virulence, strains of RPV with a history of coinfection (coRPV strains) accumulated more viral particles during infections than strains with a history of single infections (sRPV strains). This effect was evident in as little as four passages, each corresponding to a host generation. coRPV strains also showed greater viral particle accumulation in coinfections with PAV than sRPV strains. However, these increases in viral particle accumulation for coRPV strains did not result in greater transmission success. Transmission was particularly low for coRPV strains transmitting from a coinfecting host, as opposed to a single infection.

Our results, while puzzling from a traditional theoretical standpoint, are consistent with the hypothesis that passaging in coinfection affects the accumulation and scavenging of a viral coat proteins, a common good in this system. Heterogeneous encapsidation, when one virus encapsidates its genome using coat proteins from another viral species, is common in CYDV and BYDV species (Creamer and Falk 1990). ELISA responds to the identity of the coat proteins in the viral capsid, rather than the identity of the encapsidated genome. Some of the results presented here have a different interpretation depending on whether heterogeneous encapsidation is taken into account. For example, the observation of increased RPV viral particle accumulation after a history of coinfection may mean that coRPV produces more coat proteins rather than replicating additional genomes. Some of those capsids could enclose PAV or PAS genomes, hiding them from observation. Conflict over the production and use of coat proteins could explain why RPV produces more viral capsids after a history of coinfection, but transmits poorly, especially when transmitting from a coinfecting plant. It also provides an explanation why coPAS strains would produce less viral capsids during coinfections; coPAS strains may be using RPV coat proteins to encapsidate their genomes and producing less coat proteins themselves.

The majority of our key results are not influenced by whether or not heterogeneous encapsidation is occurring in this system. For instance, we observed a clear result that serial passage in coinfections did not increase the virulence of any viral species examined. Viruses with a history of coinfection show clear differences after passaging in coinfections consistent with evolutionary change, it is only the exact nature of these changes that remains in question. Finally, the difference in transmission rates for coRPV when leaving a coinfecting plant still stands, but is much harder to explain in the absence of heterogeneous encapsidation.

These results also shed light on the amount of plasticity in viral particle production. First, RPV viral particle production is not plastic. coRPV strains produced more viral particles in single infections than sRPV strains, despite a lack of competition from PAV. PAV also showed a lack of plasticity. PAV viral particle production was relatively constant across strain histories and infection types, even though reduced transmission of coRPV from coinfections in comparison to single infections indicates that PAV may have stolen capsids from RPV. PAS viral production, on the other hand, may be plastic. sPAS and coPAS had the same viral particle accumulation during single infections; however, coPAS viral particle accumulation dropped during coinfections with RPV. Most models of virulence evolution assume that pathogens cannot plastically respond to the presence of other pathogens in coinfections. However, several pathogens are known to respond plastically to their social environment (Buckling and Brockhurst 2008, Reece et al. 2008, Leggett et al. 2013). It is critical to determine under what circumstances the assumption of plasticity holds, and when it does not, because it can strongly influence our inferences about how coinfection affects virulence evolution (Choisy and de Roode 2010).

Despite considerable theoretical investigation of the effects of coinfection on virulence, experimental evolution studies are rare, and most have been carried out in tissue culture or prokaryote hosts, rather than in intact, multicellular hosts. These studies tend to show higher multiplication and/ or virulence when separate strains of the same pathogen species are passaged in coinfections as opposed to passage in single infections (Carrillo et al. 2007, Leggett et al. 2013). One study that examined the effects of coinfection on the evolution of virulence in diamondback moths found that when virulent and avirulent strains of *Bacillus thuringiensis* were passaged together, both virulence and pathogen fitness were reduced (Garbutt et al. 2011). The authors

suggest that social interactions between the bacterial strains likely played a role in this outcome, because growth inhibition of competitors and common goods (Cry toxins) are both features of this biological system. It is notable that experimental evolution studies involving coinfections of separate pathogen species are extremely difficult to find. Most focus on coinfections between strains of the same pathogen species.

Empirical studies are necessary that examine experimental evolution of coinfecting pathogens of different species, including those in separate biological kingdoms, using multicellular hosts. At the same time, models featuring a range of assumptions regarding the ecological effects of coinfection could shed light on which assumptions would be expected to have the greatest impacts on pathogen evolution. Models that address how coinfection effects on vector preference and performance influence pathogen evolution would yield additional insights that are difficult to glean from experimental evolution studies. In a complex world of interacting organisms, both empirical and theoretical approaches can improve our understanding of the long-term outcome of coinfections in a range of systems.

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PAV and RPV passaged in coinfections

<i>Treatment Abbreviation</i>	<i>Description</i>	<i>Experiment replication 1</i>	<i>Experiment replication 2</i>
C	Control; no virus	10	10
sPAV	A single infection of PAV with a history of single infections	10	10
sRPV	A single infection of RPV with a history of single infections	10	10
coPAV	A single infection of PAV with a history of coinfections	10	10
coRPV	A single infection of RPV with a history of coinfections	10	10
sPAV-sRPV	A coinfection of PAV and RPV where both strains have a history of single infections	10	10
coPAV-sRPV	A coinfection where PAV has a history of coinfections and RPV has a history of single infections	28 (7 for each of 4 lineages)	42 (7 for each of 6 lineages)
sPAV-coRPV	A coinfection where PAV has a history of single infections and RPV has a history of coinfections	28 (7 for each of 4 lineages)	42 (7 for each of 6 lineages)
coPAV-coRPV	A coinfection of PAV and RPV where both strains have a history of coinfections	28 (7 for each of 4 lineages)	42 (7 for each of 6 lineages)

PAS and RPV passaged in coinfections

<i>Treatment Abbreviation</i>	<i>Description</i>	<i>Experiment replication 1</i>	<i>Experiment replication 2</i>
C	Control; no virus	20	20
sPAS	A single infection of PAS with a history of single infections	20	20
sRPV	A single infection of RPV with a history of single infections	20	20
coPAS	A single infection of PAS with a history of coinfections	20	20
coRPV	A single infection of RPV with a history of coinfections	20	20
sPAS-sRPV	A coinfection of PAS and RPV where both strains have a history of single infections	20	20
coPAS-sRPV	A coinfection where PAS has a history of coinfections and RPV has a history of single infections	20 (5 for each of 4 lineages)	20 (5 for each of 4 lineages)
sPAS-coRPV	A coinfection where PAS has a history of single infections and RPV has a history of coinfections	20 (5 for each of 4 lineages)	20 (5 for each of 4 lineages)
coPAS-coRPV	A coinfection of PAS and RPV where both strains have a history of coinfections	20 (5 for each of 4 lineages)	20 (5 for each of 4 lineages)

Table 2-1: Strain competition experiment treatment abbreviations, descriptions, and treatment replicates for each experimental replicate.

<i>Cereal yellow dwarf virus- RPV</i>						<i>Barley yellow dwarf virus- PAV</i>					
	Value	Error	DF	t-value	p-value	Value	Error	DF	t-value	p-value	
Intercept	1.21	0.20	303	6.07	0	1.11	0.37	307	3.02	0.003	
coRPV	0.18	0.08	303	2.30	0.0221	0.03	0.09	307	0.34	0.737	
sPAV-sRPV	-0.38	0.10	303	-3.93	0.0001	-0.07	0.12	307	-0.58	0.560	
coPAV-sRPV	-0.35	0.08	303	-4.51	0	-0.10	0.09	307	-1.03	0.305	
sPAV-coRPV	-0.11	0.08	303	-1.48	0.1402	-0.11	0.09	307	-1.15	0.251	
coPAV-coRPV	-0.04	0.08	303	-0.56	0.5777	-0.07	0.09	307	-0.73	0.467	

Table 2-2: Within-host concentrations for *Cereal yellow dwarf virus-RPV* and *Barley yellow dwarf virus-PAV* strains in single and mixed infections. The random effects used in the model were block nested in experimental replicate. * p<0.05, ** p<0.01, ***

p<0.001

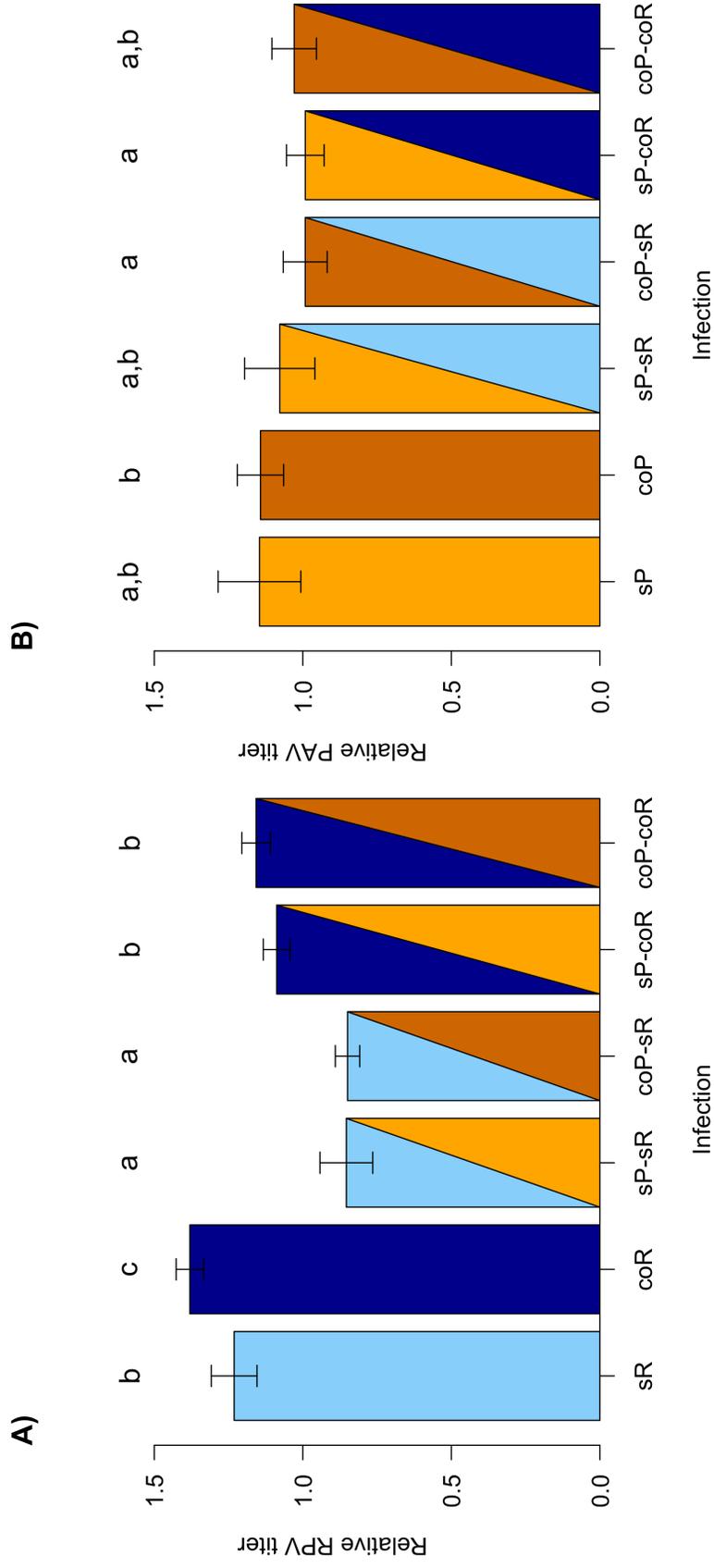


Figure 2-1: Within-host viral particle concentrations for A) RPV and B) PAV during strain competition trials. Bars denote standard errors. Within a panel, bars with different letters are significantly different at the $p < 0.05$ level. R:RPV, P:PAV, s: strain with a history of single infections, co: strain with a history of coinfections, -: coinfection between two strains.

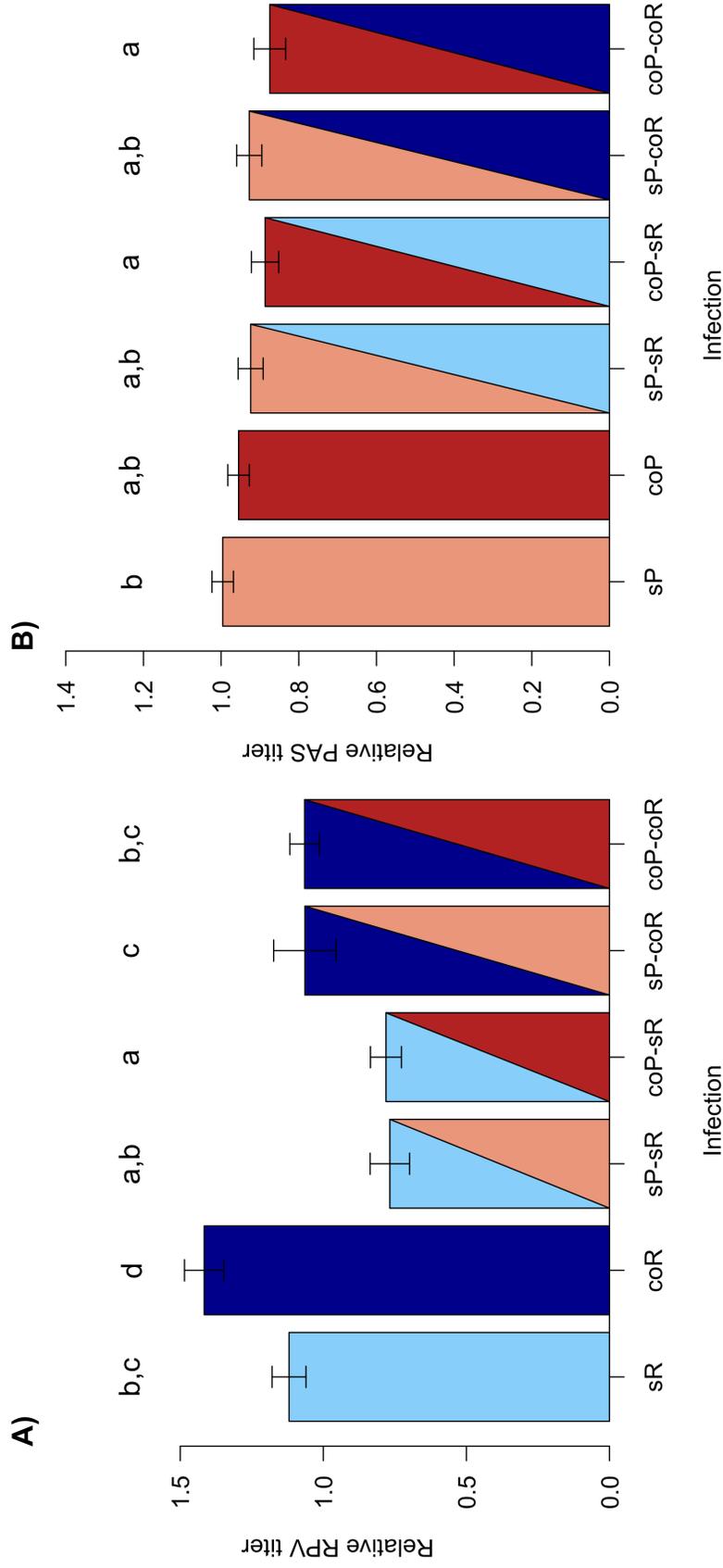


Figure 2-2: Within-host viral particle concentrations for A) RPV and B) PAS during strain competition trials. Bars denote standard errors. Within a panel, bars with different letters are significantly different at the $p < 0.05$ level. R:RPV, P: PAS, s: strain with a history of single infections, co: strain with a history of coinfections, -: coinfection between two strains.

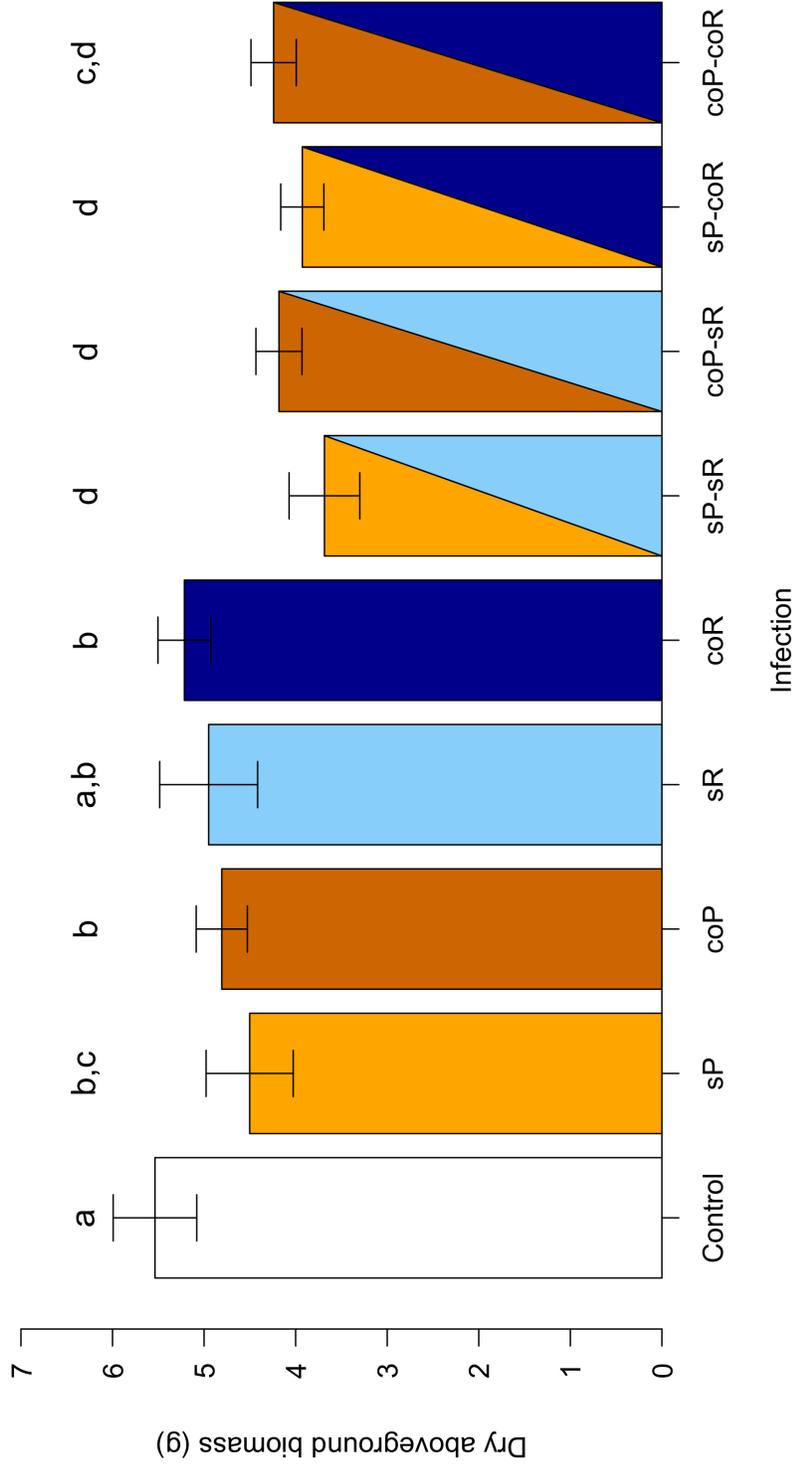


Figure 2-3: Virulence of single and coinfections of all strains used in the PAV-RPV strain competition experiments. Bars denote standard errors. Within a panel, bars with different letters are significantly different at the $p < 0.05$ level. R:RPV, P:PAV, s: strain with a history of single infections, co: strain with a history of coinfections, -: coinfection between two strains.

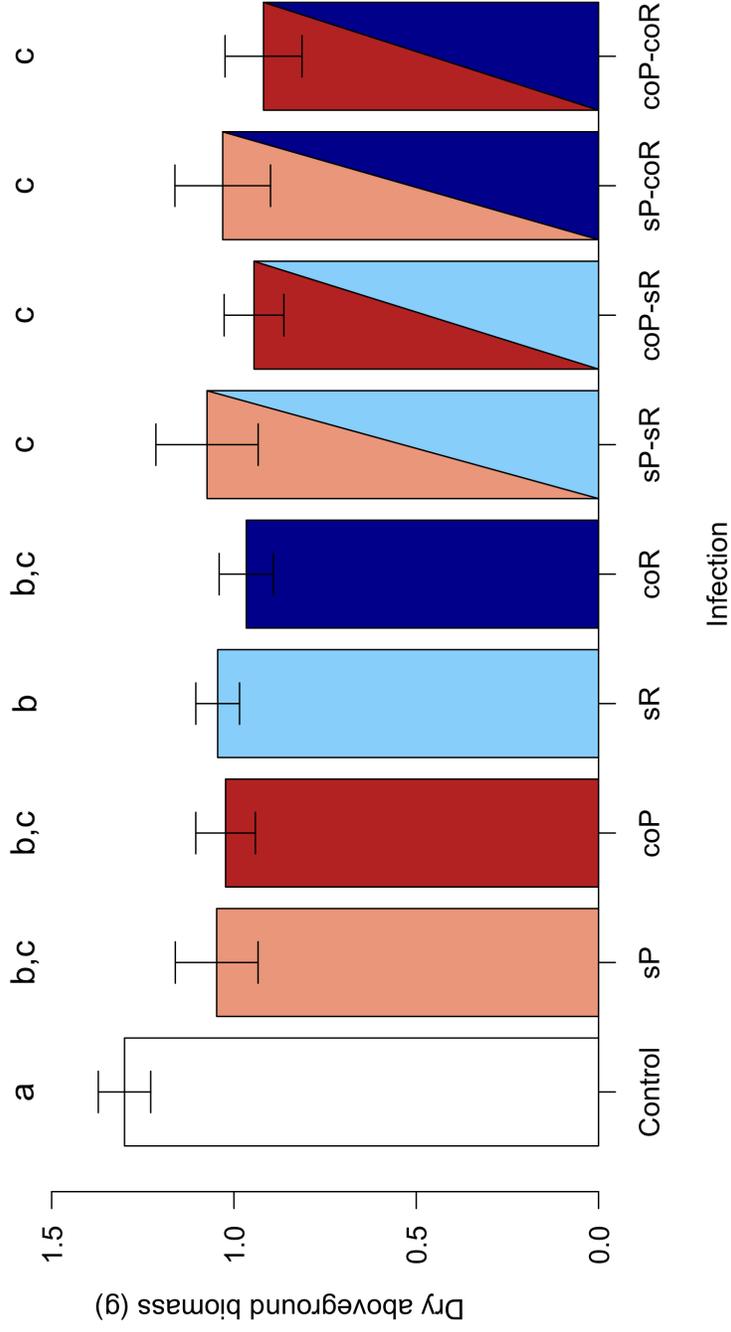


Figure 2-4: Virulence of single and coinfections of all strains used in the PAS-RPV strain competition experiments. Bars denote standard errors. Within a panel, bars with different letters are significantly different at the $p < 0.05$ level. R:RPV, P:PAS, s: strain with a history of single infections, co: strain with a history of coinfections, -: coinfection between two strains.

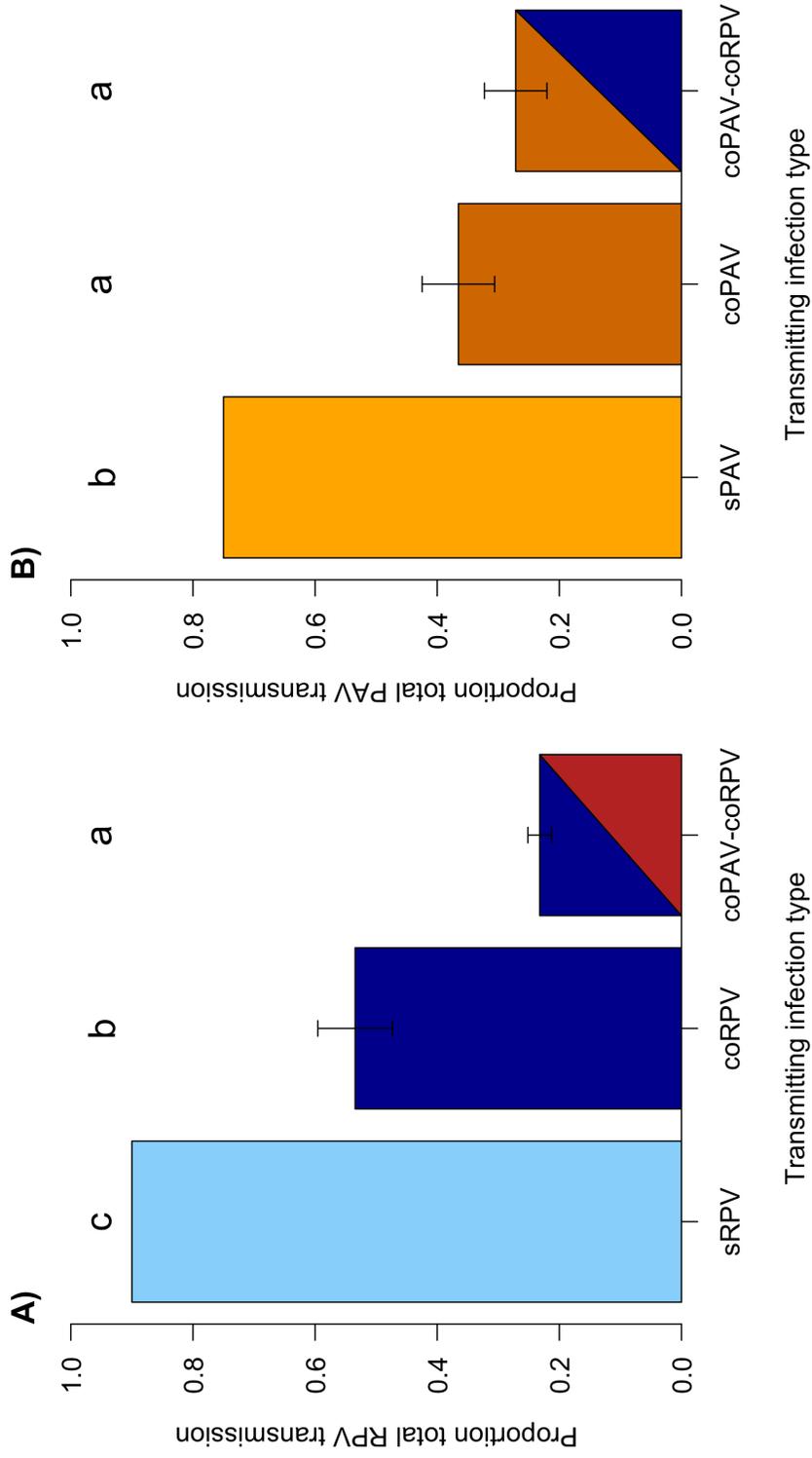


Figure 2-5: Transmission success of A) RPV and B) PAV strains with different histories. Bars denote standard errors. Within a panel, bars with different letters are significantly different at the $p < 0.05$ level.

CHAPTER 3

CONTEXT-DEPENDENT INTERACTIONS BETWEEN COINFECTING PATHOGENS AND MUTUALISTS AFFECT PATHOGEN FITNESS AND MUTUALIST BENEFITS TO HOSTS

Abstract

Many organisms host microbial mutualists, while infections of one host with multiple pathogens are common. However, few experimental studies examine higher-order interactions of more than two microbes of different types within hosts. Here, we examined to what extent a microbial mutualist could alter competition between coinfecting pathogens, and how coinfection impacts the benefits that hosts receive from microbial mutualists. We manipulated the presence of *Clover yellow vein virus* (CIYVV), *Bean common mosaic virus* (BCMV), and rhizobia bacteria in common beans (*Phaseolus vulgaris*). CIYVV reached higher concentrations within hosts during coinfections with BCMV, but only in the presence of rhizobia. BCMV within-host concentrations decreased in coinfections with CIYVV. Different aspects of viral transmission were influenced by coinfection and host access to a source of supplemental nitrogen (inorganic fertilizer or rhizobia). Coinfection had greater than additive negative effects on the amount of nitrogen that plants received from rhizobia.

Introduction

Individual plants and animals host communities of microbial symbionts, whose interactions with the host can range from parasitic to mutualistic (Bosch & McFall-Ngai 2011). These microbial symbionts also interact with each other, either directly or through their shared interactions with the host (Mihaljevic 2012). Tripartite

interactions between a host and two species of microbial symbionts (either two pathogens, two mutualists, or a pathogen and a mutualist) are receiving increasing attention (Larimer et al. 2010; Bordes & Morand 2011). Here, we provide a rationale for the exploration of higher-order interaction networks, specifically interactions between two species of coinfecting pathogens and a microbial mutualist that share a host.

Coinfection of one host with multiple pathogens is common in nature, and can lead to changes in pathogen fitness and disease severity. For example, coinfections of *Maize chlorotic mottle virus* (MCMV) and *Sugarcane mosaic virus* (SCMV) have reached prevalences of 40% in African maize fields (Mahuku et al. 2015). Coinfections of MCMV and any Potyvirus, such as SCMV, are a classic example of viral synergism resulting in increased within-host viral concentration and increased symptom severity (Syller 2011). Interestingly, greater than additive disease symptoms do not always result from increases in within-host replication in coinfections; in some cases the within-host concentrations of each virus in a coinfection can be lower than in their respective single infections, yet virulence is still increased (Srinivasan & Alvarez 2007). In other cases, coinfection may result in lower within-host concentration for one component virus, and lower overall symptom severity (Kim et al. 2010).

In addition to within-host concentration, pathogen transmission rates can also be modified by multiple infections. For plant viruses, a classic example is helper dependence, where one virus species can only be transmitted by a vector if it is encapsulated in the protein coat of a second virus species (Hull 2002). Coinfection can also allow independent viruses to be transmitted by novel vectors during heterogeneous encapsidation when the genome of one virus species is packaged within the protein coat of a separate viral species (Rochow & Gill 1978). In some instances, one virus can receive a transmission advantage when coinfecting a host with a virus

that is more attractive to vectors (Salvaudon et al. 2013). In other cases, coinfecting hosts can be more attractive to vectors than hosts that are singly infected with either component virus (Srinivasan & Alvarez 2007).

Just as pathogens that share a host can interact, pathogens and microbial mutualists sharing a host can also influence each other with consequences for host health. Microbial mutualists and symbionts are responsible for priming animal and plant immune systems, influencing the speed and strength of host responses to pathogens (Selosse et al. 2014). Symbionts can also decrease the transmission of pathogens and increase the survival probability of infected hosts (Scarborough et al. 2005). Other microbial symbionts take a more aggressive stance, such as fungal endophytes of grasses, which produce antimicrobial compounds against competitors that can reduce pathogen growth and decrease pathogen severity (Clay et al. 1989; Siegel & Latch 1991). It is possible for colonization by rhizobia bacteria to either decrease (Elsheikh & Osman 1995) or increase (Mayoral et al. 1989) the severity of viral infections. On the other hand, pathogens can also affect microbial mutualists. For example, infection of red clover by *White clover mosaic virus* reduces not only nodule numbers and rhizobia cell populations, but also decreases nitrogenase activity that rhizobia use to fix atmospheric nitrogen into a biologically accessible form (Khadhair et al. 1984).

All multicellular organisms can form commensal or mutualistic relationships with microbes, while multiple infections of one host with multiple pathogens are common. Therefore, many opportunities exist for coinfecting pathogens to affect microbial mutualists, and for commensal or mutualistic microbes to influence competition between multiple coinfecting pathogen species. In mice, there is evidence that the presence of a third party pathogenic microbe, *Haemophilus influenzae*, can alter within-host competition between virulent and avirulent strains of *Streptococcus*

pneumoniae (Lysenko et al. 2010). In the absence of *H. influenzae*, avirulent strains of *S. pneumoniae* are more competitive than virulent strains, but when *H. influenzae* is present, virulent strains of *S. pneumoniae* outcompete avirulent strains (Lysenko et al. 2010). If a facultative commensal or mutualistic microbe can similarly shift competitive outcomes between coinfecting pathogens, then competition experiments conducted in the absence of common facultative microbes may provide incomplete or misleading results (Alizon et al. 2013). It is also unclear whether the presence of multiple species of coinfecting pathogens with greater than additive effects on the host also have greater than additive effects on microbial mutualists.

We used a study system of common beans (*Phaseolus vulgaris*), rhizobia bacteria, *Clover yellow vein virus* (CIYVV), and *Bean common mosaic virus* (BCMV) to explore the interactive effects of the presence of microbial mutualists and multiple species of coinfecting pathogens. We hypothesized that the presence of rhizobia could alter within and between host competition between the coinfecting pathogens, while sharing a host with multiple pathogen species could reduce the benefits that hosts receive from rhizobia.

Materials and Methods

Clover yellow vein virus (CIYVV) and *Bean common mosaic virus* (BCMV) are both positive sense, single-stranded RNA viruses in the family Potyviridae. Both CIYVV and BCMV are transmitted non-persistently by aphids, meaning that vectors quickly acquire and transmit viruses without needing long feeding periods (Ng & Perry 2004). Neither virus replicates inside the vector. BCMV is also vertically transmitted through seeds. The BCMV strain used was collected from a field of common beans near Brooktondale, NY. We used the laboratory-cultured strain CIYVV-NY due to difficulties in isolating a recent CIYVV field strain.

The experiment was conducted in two growth chambers to minimize the risk of rhizobia contamination. One chamber was assigned to rhizobia-inoculated plants at the beginning of each experimental repetition, and chamber treatment assignment was changed between experimental repetitions to control for chamber effects. The experiment was repeated five times, and the chambers were thoroughly cleaned with Green-Shield disinfectant (BASF, Research Triangle Park, NC, USA) between experimental repetitions. Chambers were set at 26°C during the day and 22°C at night with a day length of 12.5 hours.

Plants were grown in a 4:1:2:2 mixture of topsoil, sandy loam, sand, and vermiculite. All soil was autoclaved after mixing to kill any native bacteria. After autoclaving, the soil had a pH of 7.4 and contained 27.1 ppm nitrate nitrogen, 21.6 kg ha⁻¹ phosphorus, 304.5 kg ha⁻¹ potassium, 6681.8 kg ha⁻¹ calcium, and 333 kg ha⁻¹ magnesium.

One maternal line of *Phaseolus vulgaris* var. taylor horticultural seeds was used throughout the experiment to minimize variation in host immune response. Seeds were surface sterilized prior to planting by soaking in 1% hydrogen peroxide for 10 minutes (Kempel et al. 2009). Seeds were planted in 15 cm diameter pots, with the addition of 5 mL of either active or autoclaved granular peat inoculum. The strain of rhizobia used was USDA 2667 (National Rhizobium Germplasm Resource Collection). Active inoculum contained approximately 1×10^8 bacterial cells/ mL. An inorganic nitrogen fertilization treatment was added for the last two experimental repetitions to examine the role of nitrogen fertilization per se or in combination with the immunological effects of rhizobia colonization. The number of plants in the non-rhizobia treatments were doubled, and half received 150 ppm nitrogen fertilizer weekly.

Mechanical viral inoculations took place 8 days after planting. Leaf tissue

from singly infected plants was ground with a volume of inoculation buffer in mL equal to ten times the weight of the leaf tissue in grams. The inoculation buffer consisted of a solution of 38.9 ml 0.1M KH₂PO₄, 61.1 ml 0.1M Na₂HPO₄, and 100 ml deionized water. Equal amounts of CIYVV and BCMV inoculum were combined and mixed to create inoculum for mixed infections. Control plants were mock inoculated with healthy leaf tissue ground in inoculation buffer. Plants were inoculated by sprinkling one leaf with carborundum powder and rubbing with inoculum. Inoculum was rinsed off the plants using water after 10 minutes.

Plants were sampled for phytohormone analysis 4.5 hours, 24 hours, and 4 weeks after viral inoculation during two experimental replicates. Approximately 200mg of leaf tissue was removed from an uninoculated leaf from each plant (including mock-inoculated plants), weighed, and flash frozen in liquid nitrogen. During extraction, leaf samples were ground in 1 mL extraction solvent (2:1:0.005 ratio of iso-propanol: H₂O: HClconc.) and 100 µL internal standard. The internal standard consisted of 800pg µL⁻¹ each of D6- abscisic acid, D4- salicylic acid, D5- jasmonic acid, and D5- indole-3-acetic acid. Samples were extracted using dichloromethane, dried, and dissolved in methanol for analysis by liquid chromatography-tandem mass spectrometry using a triple quadrupole LC-MS system (Pan et al. 2008).

Within-host viral concentrations were measured using quantitative enzyme-linked immunosorbent assays (qELISA) (Pollina 2013), using antibodies purchased from A.C. Diagnostics (Fayetteville, AR, USA). Tissue samples (0.5g) were collected 2 weeks after viral inoculation and ground in 5 mL of phosphate-buffered saline (PBS). The positions of two replicate samples per virus assayed were randomized on 96 well plates. Optical density (OD) values were standardized by dividing the sample OD by the average OD of positive control (infected) tissue from the same well plate.

The standardized OD values of both replicate samples were then averaged to give a relative measure of viral concentration.

At time of flowering, leaf samples were collected and dried for stable isotope analysis. $\delta^{15}\text{N}$ content was analyzed by the Cornell University Stable Isotope Laboratory using a Thermo Delta V isotope ratio mass spectrometer interfaced to a NC2500 elemental analyzer. Two controls were used for the stable isotope data: barley growing in the same soil mixture as the experimental plants, as well as bean plants growing hydroponically with only rhizobia as a nitrogen source. These controls can be used to calculate the percentage of nitrogen in plant tissue derived from the atmosphere (%NDFA) rather than from the soil (Shearer & Kohl 1986).

Immediately following stable isotope sampling, one third of plants across all treatments were sub-sampled for root nodule quantification during four experimental replicates. All nodules were counted, removed from the root system, dried, and weighed.

During one experimental replicate, horizontal transmission from each experimental plant was assessed. Transmission trials were conducted by starving aphid vectors (*Myzus persicae*) for two hours, allowing them to feed on excised leaf tissue for 5-10 minutes, and then inoculating bean seedlings with 3 aphids each. Three bean seedlings were inoculated per control plant, and five seedlings were inoculated per virus-infected plant. Aphids were sprayed with Talstar insecticide (FMC Professional Solutions, Philadelphia, PA, USA) after 24 hours. Plants were assayed for viral infection 2.5 weeks after inoculation using ELISA.

Plants not sampled for nodule counts grew until senescence, when vegetative and reproductive biomass were collected separately, dried, and weighed. Seeds were also counted and weighed separately. At this point, every plant in the rhizobia-free treatment was checked for the presence of nodules, and contaminated plants were

removed from the analysis. Finally, up to 10 seeds from each plant were tested for vertical transmission of BCMV by growing them in the greenhouse and testing the seedlings for BCMV using ELISA.

Data were analyzed using linear mixed effects models in R (R Development Core Team 2015). Random effects included in the models were experimental replicate, growth chamber identity, and the interaction between experimental replicate and growth chamber identity. Within-host concentration data were log-transformed to reduce heteroscedasticity. Percentage of nitrogen derived from the atmosphere was modeled using a beta distribution for percentage data using the *glmmadmb* package. Salicylic acid concentration data were square root transformed to improve normality. Vertical and horizontal transmission rates were modeled as success/failure data using a binomial distribution. Seed production was analyzed with a poisson distribution for count data. We also investigated the expected number of vertical transmission opportunities per host plant by multiplying host plant seed production by vertical transmission rate.

Results

Coinfection and the presence of rhizobia had differing effects on within-host concentrations of CIYVV and BCMV (Figure 3-1, Table 3-1). The main effects of coinfection, fertilizer, and rhizobia had no significant effect on CIYVV within-host concentration on their own, but there was a positive interaction between coinfection and the presence of rhizobia ($p < 0.05$, Table 3-1). CIYVV within-host concentration only increased in coinfections when rhizobia were present (Figure 3-1A). The presence of fertilizer or rhizobia had no effect on the within-host concentration of BCMV, but coinfection had a significant negative effect ($p < 0.01$, Figure 3-1B, Table 3-1).

Viral infection had a substantial impact on the mutualism between rhizobia and the host (Figure 3-2, Table B1). Leaf nitrogen content derived from the atmosphere rather than the soil declined by 12% in single infections of each CIYVV or BCMV in comparison to uninfected plants (significance of effects $p < 0.01$ for CIYVV and $p < 0.05$ for BCMV Table B1). Coinfection led to greater than additive declines in nitrogen derived from the atmosphere (a 35% reduction in comparison to uninfected plants, significance of effect $p < 0.001$). Reductions in the number of nodules per plant by infection status show similar trends (data not shown).

The effects of microbial treatments on the defense hormone, salicylic acid (SA), differed greatly with time following viral inoculation (Figure 3-3, Table B2). At 4.5 hr after viral inoculation, viral treatment had no effect on the amount of SA in leaf tissue (Figure 3-3A, Table B2). The presence of rhizobia, on the other hand, led to a significant increase in SA at both 4.5 hours after viral inoculation ($p < 0.001$) and 24 hours after viral inoculation ($p < 0.001$, data not shown). In contrast, 4 weeks after viral inoculation CIYVV and BCMV singly infected plants colonized by rhizobia had lower SA content than CIYVV and BCMV singly infected plants not colonized by rhizobia (interaction $p < 0.05$ for both viruses). On the other hand, during the same interval SA concentrations for coinfecting plants did not drop if plants were colonized by rhizobia in comparison to uncolonized plants.

Despite the fact that both CIYVV and BCMV are vectored by *Myzus persicae*, we only analyzed the horizontal transmission rates of BCMV due to extremely poor transmission of CIYVV. Coinfection significantly lowered horizontal transmission rates by BCMV (Figure 3-4, Table B3, $p < 0.05$). The trend was influenced most by plants receiving supplemental nitrogen either from fertilizer or rhizobia (Figure 3-4). The addition of fertilizer led to significant reductions in BCMV horizontal transmission (fertilizer: $p < 0.01$), and addition of rhizobia showed a similar, but

nonsignificant, trend.

While coinfection decreased horizontal transmission of BCMV, coinfection significantly increased vertical transmission in the absence of supplemental nitrogen from fertilizer or rhizobia ($p < 0.01$, Figure 3-5A, Table B4). However, if supplemental nitrogen was present then the vertical transmission rate of BCMV was the same in coinfections as in single infections (significant negative interaction terms both $p < 0.05$). The number of vertical transmission opportunities from each infected host depends on both the vertical transmission rate and seed production. Single infections of either CIYVV or BCMV caused similar significant reductions in seed production ($p < 0.001$ for each virus, Figure 3-5B, Table B5). Coinfection with both viruses caused an even greater reduction in seed production ($p < 0.001$). Fertilizer and rhizobia both increased seed production ($p < 0.001$, Figure 3-5, Table B5), and there was no difference in seed production between plants receiving different sources of supplemental nitrogen. Seed germination rates were high and consistent across treatments (data not shown). The number of expected vertical transmission opportunities per BCMV infection was influenced by coinfection and both types of supplemental nitrogen (Figure 3-5C, Table B4). Expected vertical transmission opportunities for hosts that either received fertilizer or were colonized by rhizobia were lower when plants were coinfecting ($p < 0.05$, Figure 3-5C, Table B4), while coinfection did not significantly influence vertical transmission opportunities for plants without supplemental nitrogen sources.

Discussion

Our results demonstrate that interactions between a microbial mutualist and two coinfecting pathogens can affect pathogen competition and the benefits that hosts receive from microbial mutualists. We found that the presence of a microbial

mutualist, rhizobia bacteria, can elevate within-host concentrations of the plant virus CIYVV during coinfections, but not during single CIYVV infections. This could increase the overall competitive ability of CIYVV, while at the same time influencing the fitness landscape for another plant virus, BCMV. While the presence of rhizobia affected CIYVV within-host concentration, the presence of two viruses in turn had a greater than additive effect on the reduction in the benefits that hosts receive from rhizobia colonization. One mechanism for this effect could be mediation by the plant defense hormone, salicylic acid (SA). Both viral infection and the addition of exogenous SA have been shown to reduce nodule initiation (Tu 1997; Sato et al. 2002). Hypothetically, if the production of SA has a negative effect on rhizobia, then plants colonized by rhizobia could suppress SA production in order to not harm their mutualists (Mabood & Smith 2007). In our system, this was not the case 4.5 hours after viral inoculation when the symbiosis with the rhizobia was relatively new, but by 4 weeks after viral inoculation SA was significantly lower in singly infected plants colonized by rhizobia compared to plants without rhizobia. Interestingly, this did not occur in coinfecting plants; the SA content of coinfecting plants remained high 4 weeks after viral infection whether or not plants were colonized by rhizobia. The sustained high concentrations of SA in coinfecting plants with or without rhizobia may indicate a chronic immune response to multiple infection. If so, then the greater than additive reduction in benefits from rhizobia during coinfection may be partly due to the host immune response rather than the actions of either virus.

We found that horizontal transmission by BCMV follows the predictions of the virulence-transmission trade-off hypothesis (Alizon et al. 2009), in that coinfecting plants had lower BCMV concentrations while coinfection had a negative overall effect on horizontal transmission. It is also noteworthy that this trend was more pronounced for plants receiving supplemental nitrogen. Immune responses rely heavily on a

number of proteins, and the nitrogen necessary to build these proteins would be more readily available to plants with a greater nitrogen supply. Therefore, non-exclusive hypotheses to explain reduced BCMV horizontal transmission from coinfecting plants include both exploitative competition between BCMV and CIYVV for host resources and apparent competition via increased host immune responses.

BCMV vertical transmission shows a different trend, where in the absence of supplemental nitrogen vertical transmission rates are higher for coinfecting plants. However, low seed production by coinfecting hosts blunts the effect of this increased vertical transmission rate on overall potential transmission opportunities. There are a few other reports of coinfection increasing vertical transmission rates (Kuhn & Dawson 1973; de Assis Filho & Sherwood 2000), but little is known about this topic and especially the potential mechanism behind it. In general, the mechanisms that allow viruses to infect seed tissues are poorly understood even for single-virus infections (Simmons & Munkvold 2014).

Our data show no difference in disease severity between plants receiving nitrogen from fertilizer or from rhizobia, but other outcomes are also possible. In other systems, plants colonized by rhizobia have shown either decreased (Elsheikh & Osman 1995) or increased (Mayoral et al. 1989) disease severity. Hypothetically, the presence of rhizobia could benefit the host through increased nitrogen reserves or immune system priming. On the other hand, in our case the presence of rhizobia depresses defense hormone signaling for single pathogen infections, which may increase vulnerability to subsequent infections. The metabolic demands of hosting both rhizobia and pathogen growth may also be overwhelming for hosts. The study of how coinfection influences virulence evolution is an expanding field (Alizon et al. 2013), but less thought has been given to how the presence of facultative mutualists influences pathogen virulence. Fundamentally, the presence of a

facultative mutualist leads to an increase in host heterogeneity, but the time scales in which pathogens and microbial mutualists undergo genetic change are similarly faster than the time scales necessary for host evolutionary change. The presence of a pathogen could change the cost-benefit ratios for both partners in a facultative mutualism, which could have complex effects on the cooperative status of the mutualism.

In the case of a nutritional mutualism like that between legumes and rhizobia, changes in the benefits that hosts receive from microbial mutualists during disease outbreaks could have ecosystem-level consequences. Plants associated with nitrogen-fixing bacteria play crucial roles in ecological succession (Chapin et al. 1994; Li et al. 2010). It is known that herbivores of legumes can alter rates of succession, mainly through their effects on population growth rates (Bishop 2002). Pathogen epidemics could have even greater effects by both reducing population growth rates and reducing the accumulation of soil nitrogen. From a more applied standpoint, viral outbreaks can be expensive for farmers using legume rotations to improve soil fertility in terms of either costs of supplemental nitrogen or yield losses in a subsequent crop.

Our results support recent calls to take the microbiota of organisms into account during experimental design and analysis (Bleich & Hansen 2012). Studies on interactions between coinfecting pathogens and their implications for the evolution of virulence could provide misleading results in cases where additional symbionts change the outcome of interactions (Alizon et al. 2013). Given the ubiquity of complex microbial communities within hosts, more studies are warranted on host-symbiont networks with more than two microbial symbionts.

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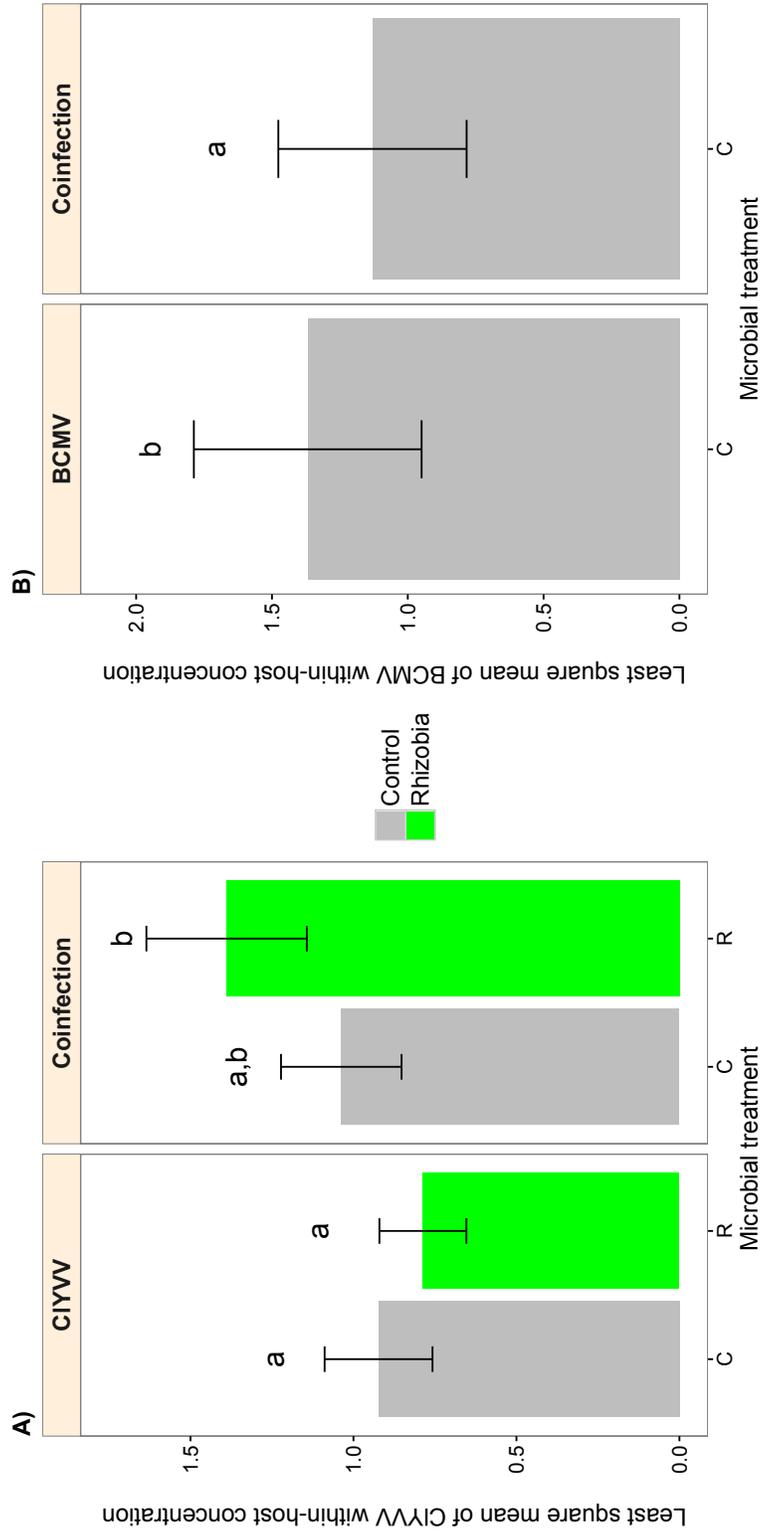


Figure 3-1: Effects of viral infection and rhizobia on within-host concentrations of A) *Clover yellow vein virus* and B) *Bean common mosaic virus*. Least square means are presented in order to take random effects into account. Error bars represent standard error values for least square mean estimates. Within a panel, bars that share the same letter designation are not significantly different from each other at the $p > 0.05$ level.

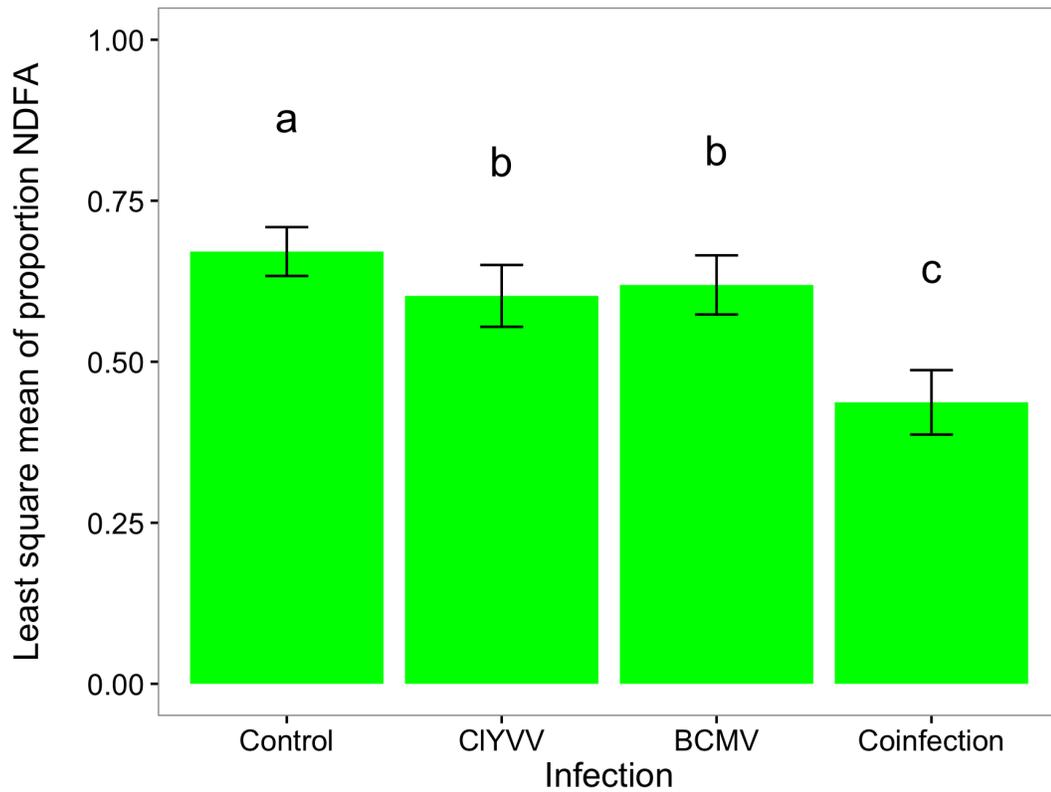


Figure 3-2: Effect of viral infection status on the percent nitrogen derived from the atmosphere (%NDFA) of leaf tissue from plants colonized by rhizobia. Least square means are presented in order to take random effects into account. Error bars represent standard error values for least square mean estimates. Bars that share the same letter designation are not significantly different from each other ($p>0.05$).

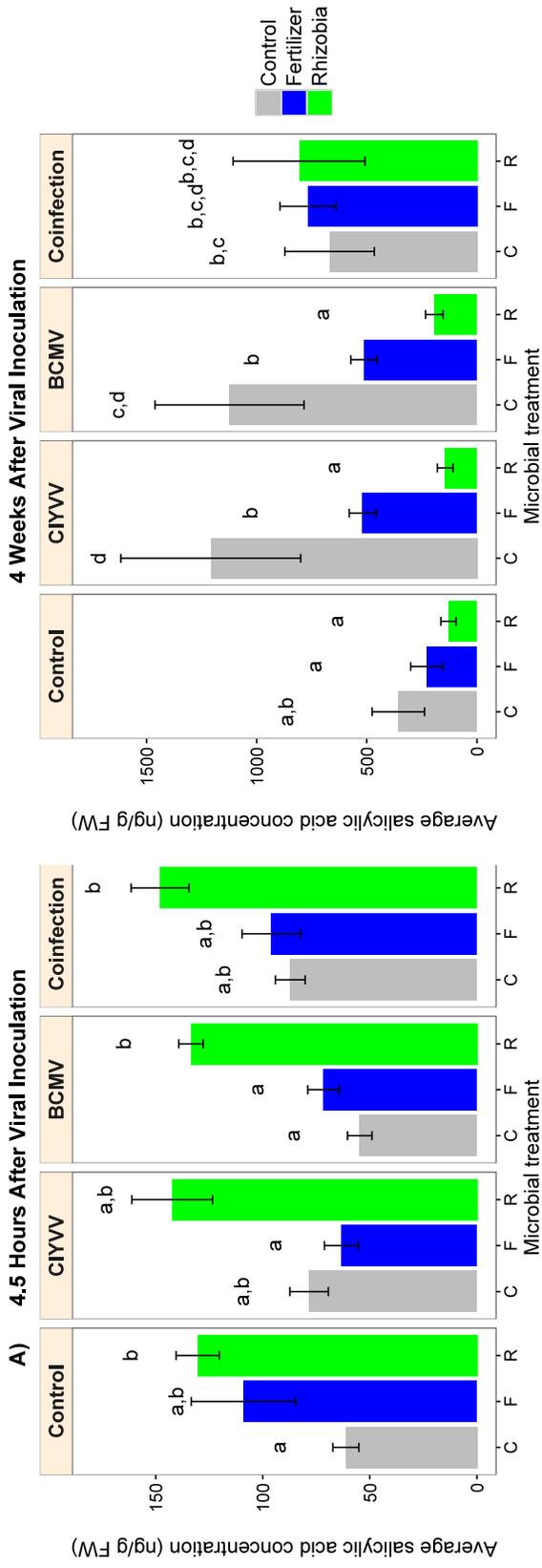


Figure 3-3: Effects of viral infection, inorganic nitrogen fertilizer, and rhizobia on leaf salicylic acid concentration A) 4.5 hours after viral inoculation and B) 4 weeks after viral inoculation. Error bars represent standard errors. Within a panel, bars that share the same letter designation are not significantly different from each other at the $p < 0.05$ level.

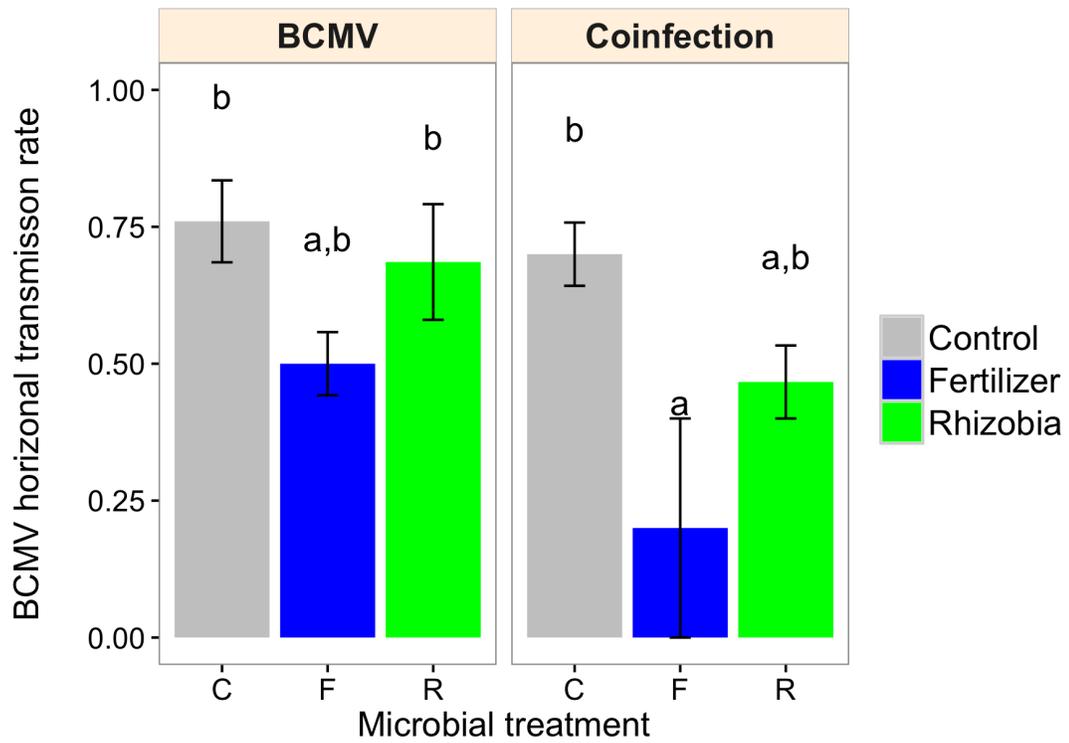


Figure 3-4: Effects of viral infection, inorganic nitrogen fertilizer, and rhizobia on horizontal transmission rates of *Bean common mosaic virus*. Error bars represent standard errors. Within a panel, bars that share the same letter designation are not significantly different from each other at the $p < 0.05$ level.

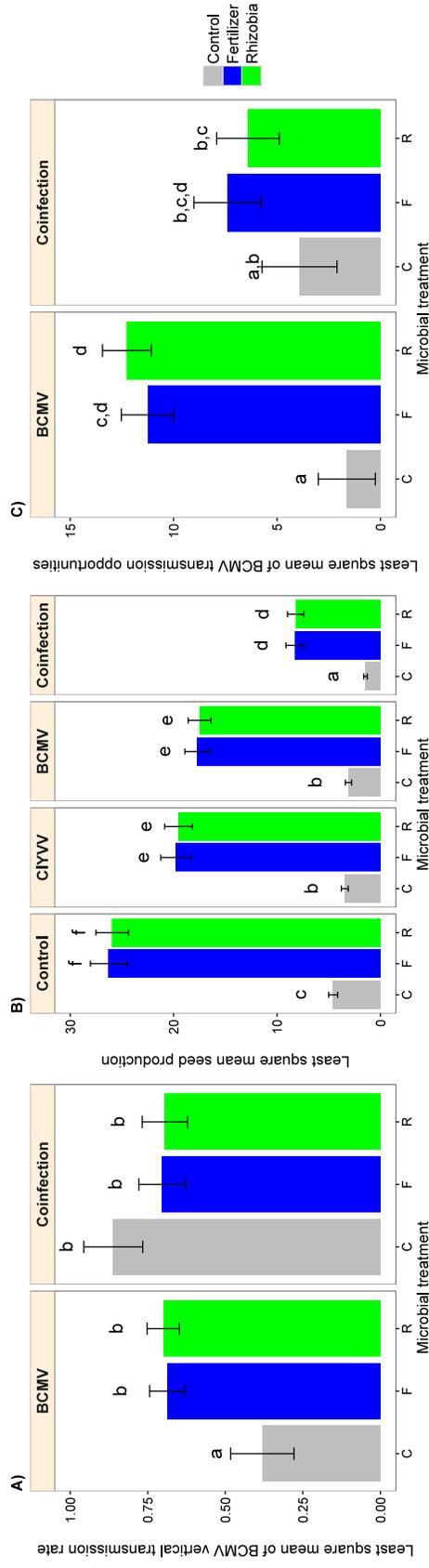


Figure 3-5: Effects of viral infection, inorganic nitrogen fertilizer, and rhizobia on A) vertical transmission rates of *Bean common mosaic virus*, B) seed production, and C) *Bean common mosaic virus* transmission opportunities. Least square means are presented in order to take random variables into account. Error bars represent standard error values for least square mean estimates. Within a panel, bars that share the same letter designation are not significantly different from each other at the $p < 0.05$ level.

APPENDIX B

Supporting Tables

	Estimate	Std. error	z-value	p-value	
Intercept	0.71	0.17	4.15	3.3×10^{-5}	***
CIYVV	-0.30	0.10	-2.87	0.004	**
BCMV	-0.23	0.09	-2.46	0.014	*
Coinfection	-0.97	0.11	-8.77	$< 2 \times 10^{-16}$	***

Table B1: Effects of viral infection on the percentage of nitrogen in plant tissues derived from the atmosphere for plants colonized by rhizobia. Data were modeled using a beta distribution for percentage data. Random effects included in the model were experimental replicate, growth chamber identity, and the interaction between experimental replicate and growth chamber identity. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

	4.5 Hours After Viral Inoculation				4 Weeks After Viral Inoculation			
	Estimate	Std. Error	t-value	p-value	Estimate	Std. Error	t-value	p-value
Intercept	8.28	0.85	9.73	5.1x10 ⁻¹⁴ ***	17.91	3.00	5.97	1.8x10 ⁻⁷ ***
CIYVV	-0.65	0.93	-0.69	0.49	15.50	4.50	3.44	0.001 **
BCMV	-0.52	0.84	-0.62	0.54	14.32	4.24	3.37	0.001 **
Coinfection	0.76	1.00	0.76	0.45	6.06	4.24	1.43	0.159
Fertilizer	0.66	0.83	0.80	0.43	-3.78	4.06	-0.93	0.356
Rhizobia	3.19	0.83	3.85	2.8x10 ⁻⁴ ***	-7.23	3.93	-1.84	0.071
CIYVV*Fertilizer	N.S.	N.S.	N.S.	N.S.	-7.03	6.06	-1.16	0.251
BCMV*Fertilizer	N.S.	N.S.	N.S.	N.S.	-6.18	5.48	-1.13	0.264
Coinfection*Fertilizer	N.S.	N.S.	N.S.	N.S.	7.31	6.37	1.15	0.256
CIYVV*Rhizobia	N.S.	N.S.	N.S.	N.S.	-14.60	5.98	-2.44	0.018 *
BCMV*Rhizobia	N.S.	N.S.	N.S.	N.S.	-11.64	5.48	-2.12	0.038 *
Coinfection*Rhizobia	N.S.	N.S.	N.S.	N.S.	10.10	5.98	1.69	0.097

Table B2: Effects of viral infection status, inorganic nitrogen fertilizer, and rhizobia on the concentration of salicylic acid in leaf tissues either 4.5 hr. after viral infection or 4 weeks after viral infection. Data were square-root transformed. Interaction terms without statistical significance were removed. * p<0.05; ** p<0.01; *** p<0.001

	Estimate	Std. error	z-value	p-value
Intercept	1.40	0.41	3.46	0.001 ***
Coinfection	-0.80	0.41	-1.97	0.049 *
Fertilizer	-1.56	0.52	-3.00	0.003 **
Rhizobia	-0.66	0.46	-1.44	0.151

Table B3: Effects of coinfection, inorganic nitrogen fertilizer, and rhizobia on horizontal transmission rates of bean common mosaic virus by *Myzus persicae*. Data were modeled using a binomial distribution. Interaction terms without statistical significance were removed. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

	BCMV Vertical Transmission Rate			BCMV Vertical Transmission Opportunities				
	Estimate	Std. Error	z-value	p-value	Estimate	Std. Error	t-value	p-value
Intercept	-0.49	0.43	-1.12	0.261	1.63	1.38	1.18	0.300
Coinfection	2.31	0.87	2.65	0.008 **	2.29	1.88	1.21	0.232
Fertilizer	1.27	0.45	2.84	0.004 **	9.63	1.40	6.87	2.4x10 ⁻⁸ ***
Rhizobia	1.33	0.44	3.02	0.003 **	10.63	1.49	7.15	1.000
Coinfection*Fertilizer	-2.23	0.95	-2.35	0.019 *	-6.15	2.49	-2.46	0.018 *
Coinfection*Rhizobia	-2.33	0.94	-2.48	0.013 *	-8.14	2.40	-3.39	0.002 **

Table B4: Effects of coinfection, fertilizer, and rhizobia on bean common mosaic virus vertical transmission rate and expected vertical transmission opportunities. Vertical transmission rates were modeled with a binomial distribution. Random effects were experimental replicate, growth chamber identity, and the interaction between experimental replicate and growth chamber identity. * p<0.05; ** p<0.01; *** p<0.001

	Seed Production			
	Estimate	Std. Error	z-value	p-value
Intercept	1.52	0.09	16.02	< 2x10 ⁻¹⁶ ***
CIYVV	-0.28	0.05	-5.32	1.1x10 ⁻⁷ ***
BCMV	-0.39	0.05	-8.69	< 2x10 ⁻¹⁶ ***
Coinfection	-1.15	0.09	-13.47	< 2x10 ⁻¹⁶ ***
Fertilizer	1.75	0.08	20.64	< 2x10 ⁻¹⁶ ***
Rhizobia	1.74	0.11	16.14	< 2x10 ⁻¹⁶ ***

Table B5: Effects of viral infection status, inorganic nitrogen fertilizer, and rhizobia on *Phaseolus vulgaris* seed production. Data were modeled using a poisson distribution. Random effects included in the model were experimental replicate, growth chamber identity, and the interaction between experimental replicate and growth chamber identity. *** p<0.001

CHAPTER 4

EFFECTS OF VIRAL COINFECTION AND RHIZOBIA BACTERIA ON PRIMARY AND SECONDARY METABOLISM OF COMMON BEANS (*PHASEOLUS VULGARIS*)

Abstract

1. Within-host microbial communities are diverse, and have profound effects on host functioning and health. However, in a majority of studies the effects of individual microbes are examined in isolation, which does not allow the examination of interacting effects of microbes on host metabolic processes.
2. We performed an experiment to examine the response of common bean (*Phaseolus vulgaris*) to colonization by a microbial mutualist, rhizobia bacteria, in combination with either single infections or coinfections of *Bean common mosaic virus* (BCMV) and *Clover yellow vein virus* (CIYVV). We focused on two aspects of plant primary and secondary metabolism: carbon isotope discrimination during photosynthesis and the emission of volatile organic compounds (VOCs). VOC emission is not only indicative of the action of several host biosynthetic pathways, it also is known to affect attraction of herbivorous insects that vector plant viruses.
3. Colonization by rhizobia led to ^{13}C enrichment in plant tissues, but there was no effect of viral infection. When the mechanism behind the difference in carbon isotope discrimination was examined using gas exchange measurements, we found a significant increase in maximum rates of electron transport in plants colonized by rhizobia in comparison to plants receiving inorganic nitrogen

fertilizer. Stomatal conductance, on the other hand, was not affected by rhizobia.

4. Plant nitrogen source had a significant effect on the overall composition of VOC profiles, but neither nitrogen source nor viral infection influenced total VOC production. The emissions of 47 individual compounds were significantly affected by viral infection and/or nitrogen source.
5. These results highlight the importance of studying how the presence of multiple microbes affects host metabolic processes. More work is necessary to explore how changes in VOC emissions caused by interacting rhizobia and viruses affect viral vector preference and viral transmission.

Introduction

Plants and animals do not live in isolation; each individual hosts communities of multiple mutualistic, commensal, and parasitic microbes. Both the expression of microbial genes and host responses to the presence of microbes greatly influence host metabolism, immunity, and health (Friesen et al. 2011, Zamioudis and Pieterse 2012). However, our knowledge of the effects of microbes on host metabolism is greatly biased towards the study of individual microbes, which does not allow the investigation of potential interactions when hosts are colonized by multiple microbes. In particular, situations where a host with a microbial mutualist is coinfecting with more than one pathogen species are rarely studied despite the commonality and consequences of pathogen coinfections.

Plants in the *Fabaceae* family, legumes, have both a well-known mutualism

with nitrogen-fixing rhizobia bacteria, and also many reports of extensive coinfection with multiple species of viruses. For instance, viral coinfection rates for pasture legumes such as clover and alfalfa are generally greater than 10% (Barnett and Gibson 1975, McLaughlin and Boykin 1988) and coinfection rates for common beans can reach 25% in years of high viral prevalence (Shah et al. 2006).

Viral infection and the presence of rhizobia are known to affect each other, with implications for host health. Single viral infections tend to reduce nitrogen fixation by rhizobia (Khadhair et al. 1984, Wroth et al. 1993, Tu 1997), and coinfections of two viruses cause a greater than additive reduction in the percentage of nitrogen in plant tissues derived from nitrogen fixation by rhizobia (Chapter 3). On the other hand, the presence of rhizobia can either decrease (Elsheikh and Osman 1995) or increase (Mayoral et al. 1989) viral disease severity. Viral coinfection and rhizobia colonization can also interact to affect plant phytohormone concentrations. Four weeks after viral inoculation the presence of rhizobia causes a significant reduction in salicylic acid production in plants infected with one virus, but this reduction is not evident in coinfecting plants (Chapter 3).

We focus here on the effects of rhizobia and viral coinfection on two aspects of plant primary and secondary metabolism: carbon isotope discrimination during photosynthesis and the emission of volatile organic compounds (VOCs). Plants colonized by rhizobia are typically enriched in ^{13}C (Knight et al. 1995), and show increased rates of photosynthesis, which are mediated in soybeans by increased maximum carboxylation rates and/ or higher rates of photosynthate export from chloroplasts (TPU, triose phosphate use) (Zhou et al. 2006, Kaschuk et al. 2012).

Virus infection, on the other hand, can lead to reduced photosynthetic rates. For instance, infection by *Pea enation mosaic virus* causes the break-down of photosynthetic pigments and reduces the efficiency of photosystem II chemistry (Kyseláková et al. 2011).

The emission of VOCs from plants provides a window into changes in primary and secondary metabolism, while playing a key ecological role by releasing chemical information into the air that can be perceived by other organisms such as insect herbivores. VOC emissions are influenced by chemical energy provided by plant primary metabolism, nutrient availability, and the regulation of several biosynthetic pathways such as the lipoxygenase (LOX), mevalonic acid (MVA), methylerythritol phosphate (MEP), and Shikimate pathways (Dudareva et al. 2013). VOC emissions may be constant (constitutive) or induced by the regulation of plant genes under circumstances such as herbivory. (Baldwin et al. 2006). There is also a hypothesis that viruses manipulate the VOC blends emitted by hosts (Ingwell et al. 2012). For instance, infections of several virus species are related to shifts in plant VOC emissions that is attractive to vector insects (Mauck et al. 2010, Bosque-Pérez and Eigenbrode 2011), although it has not been proven if these changes are truly active host manipulation by the viruses.

We conducted an experiment that crossed nitrogen source (un-augmented, inorganic nitrogen fertilizer, or rhizobia) with viral infection status (mock-inoculation, single infection with *Bean common mosaic virus*, single infection with *Clover yellow vein virus*, or coinfections of both viruses) in order to look for interactive effects of rhizobia and viral infection on host primary and secondary metabolism. We expected

to see a significant interaction between the presence of rhizobia and viral infection on carbon isotope content of plant tissue. We also examined the mechanism behind observed ^{13}C enrichment in *Phaseolus vulgaris* plants colonized by rhizobia. We expected to see reduced stomatal conductance and/ or an increase in the maximum rate of carboxylation. We also expected to find changes in VOC emission consistent with observations of VOC shifts caused by viral infection in other systems, with possible modifications of compound emissions due to the presence of rhizobia. We hypothesized that VOCs related to nitrogen balance would be affected by both inorganic nitrogen fertilizer and the presence of rhizobia, while other compounds would be affected only by host responses to the microbial mutualist separate from the nitrogen the mutualists provide.

Materials and Methods

Study system. One maternal line of common bean seeds (*Phaseolus vulgaris*, var. taylor horticultural) was used in experiments to reduce variation in host response. *Bean common mosaic virus* (BCMV) and *Clover yellow vein virus* (CIYVV) are viruses in the family *Potyviridae* that are transmitted non-persistently by aphids. This means that vectors acquire virus particles quickly, and can transmit the virus within seconds to minutes of feeding (Ng and Perry 2004). BCMV is also transmitted vertically through seeds. Neither virus replicates within the vector. The BCMV strain used in experiments was collected from a bean field near Brooktondale, NY, USA. A laboratory-cultured strain of CIYVV was used due to difficulties in isolating a strain from the field. The rhizobia inoculum contained approximately 1×10^8 bacterial cells/

mL of strain USDA 2667 (National Rhizobium Germplasm Resource Collection).

Plant growth conditions. Plants were grown in growth chambers to minimize risk of rhizobia contamination. Chamber conditions were 26°C daytime temperature, 22°C nighttime temperature, and 12.5 hour day length. During each experimental replicate, one chamber was assigned to the rhizobia treatment, and one chamber was assigned to the no rhizobia treatment. Chambers were sterilized using Green-Shield disinfectant (BASF, Research Triangle Park, NC, USA) between experimental replicates, and growth chambers were alternated for each of 5 replicate experiments. Multiple experimental replicates allow us to avoid pseudoreplication and to control for growth chamber identity during analysis.

The soil used consisted of a 4:1:2:2 mixture of topsoil, sandy loam, sand, and vermiculite. After mixing, the soil was autoclaved to kill any native bacteria. Once autoclaved, the soil had a pH of 7.4 and contained 27.1 ppm nitrate nitrogen, 21.6 kg ha⁻¹ phosphorus, 304.5 kg ha⁻¹ potassium, 6681.8 kg ha⁻¹ calcium, and 333 kg ha⁻¹ magnesium. Seeds were planted in 15 cm diameter pots after soaking for 10 minutes in 1% hydrogen peroxide (Kempel et al. 2009). All pots received 5 mL of either active or autoclaved granular peat rhizobia inoculum. A subset of plants was harvested 3 weeks after viral inoculation for the quantification of dry aboveground vegetative biomass. At the end of the experiment, all plants in no rhizobia treatments were checked for contamination. Rare contaminated plants were excluded from the analysis.

A synthetic nitrogen fertilizer treatment was added during the last two replicates to determine which plant responses could be accounted for by additional

nitrogen availability, and which were influenced by rhizobia colonization itself. The number of plants in the non-rhizobia treatment were doubled, and half received 150 ppm nitrogen fertilizer once a week.

Viral inoculations. Plants were mechanically inoculated 8 days after planting. Leaf tissue from singly infected plants was ground in a volume of inoculation buffer in mL equal to ten times the weight of the leaf tissue in grams. The inoculation buffer consisted of a solution of 38.9 ml 0.1M KH_2PO_4 , 61.1 ml 0.1M Na_2HPO_4 , and 100 ml deionized water. Equal volumes of BCMV and CIYVV inoculum were mixed to create the inoculum used for two-virus inoculations. Control plants were mock-inoculated in the same fashion, but by grinding a healthy leaf in inoculation buffer. One leaf per plant was sprinkled with carborundum powder and rubbed with inoculum. Plants were rinsed after 10 minutes to remove inoculum. Virus infection was confirmed two weeks after viral inoculation using enzyme-linked immunosorbent assay (ELISA), using antibodies purchased from A.C. Diagnostics (Fayetteville, AR, USA).

VOC collection and analysis. Dynamic headspace volatile collection was used to collect VOCs from single leaves on adsorbent charcoal filters following the method of Kessler and Baldwin (2001). Charcoal traps without samples were included for use as blanks. The duration of VOC collection was 8 hours. Each leaf used in VOC collection was excised and photographed in a common location using an object of known size for scale. When leaflets were curled by symptoms of viral infection, cuts were made in the leaflets until they could lay flat. The surface area of each leaf was calculated using ImageJ software.

Charcoal traps were stored at -20°C until elution. Tetraline was added as an internal standard, then traps were eluted using dichloromethane. Samples were run on a Varian gas chromatograph-mass spectrometer using a wax column. Chromatograph peaks were located that were either absent on the respective blank charcoal filters, or that were detected in lower quantity on blanks. The area under the chosen peaks was integrated with respect to the internal standard.

VOC data were blanked using air controls to adjust for ambient chamber volatiles. A permutational multivariate analysis of variance (using the *adonis* function in the R package *vegan*) was used to determine the effects of infection treatment and nitrogen source on the overall VOC compound profiles emitted by plants (R Development Core Team 2015). Linear mixed effects models were used to determine the effects of infection treatment and nitrogen source on the emission of individual compounds (function *lmer* in R). In all analyses, leaf area was used as a covariate, and data were blocked by experimental repetition. Insignificant interaction terms were removed from models.

Stable isotope analysis. Leaf samples were collected and dried for stable isotope analysis. $\delta^{13}\text{C}$ was analyzed by the Cornell University Stable Isotope Laboratory using a Thermo Delta V isotope ratio mass spectrometer interfaced to a NC2500 elemental analyzer. $\delta^{15}\text{N}$ and nitrogen content were also analyzed, but the $\delta^{15}\text{N}$ results are described elsewhere (Chapter 3). Data were analyzed using linear mixed effects models with the fixed effects infection status and nitrogen source and the random effects growth chamber identity, experimental repetition, and the interaction between chamber identity and experimental replicate (function *lmer* in R).

Measurement of photosynthetic parameters. In order to investigate the mechanism behind differences in $\delta^{13}\text{C}$ data between plants with and without rhizobia, a LI-COR LI-6400 was used to construct curves of photosynthetic rates (A) for different concentrations of intercellular CO_2 (c_i). Tests were conducted at 22°C and a light intensity of $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$. Four A- c_i curves each were constructed for plants receiving supplemental inorganic nitrogen fertilizer and plants colonized by rhizobia. The R *plantecophys* package was used to generate A- c_i curves and to calculate the maximum rate of carboxylation (V_{cmax}) and the maximum rate of electron transport (J_{max}) for each curve (Duursma 2015). T-tests were used to determine differences in parameter means between treatments.

Results

$\delta^{13}\text{C}$ isotope data and A- c_i curve analysis. Plants with rhizobia were enriched in ^{13}C relative to plants either receiving no external nitrogen or plants fertilized by inorganic nitrogen fertilizer (LME, t-value: 16.463, $p < 0.01$, Figure 4-1). Infection status, on the other hand, had no effect on $\delta^{13}\text{C}$ values. In order to explore the mechanism behind the enrichment of ^{13}C in plants colonized by rhizobia, gas exchange measurements were taken and A- c_i curves were calculated. Photosynthetic rates were significantly higher for plants colonized by rhizobia at ambient concentrations of CO_2 than for plants receiving inorganic nitrogen fertilizer ($p = 0.02$, t-value = -3.87, Table 4-1). The magnitude of the decrease in observed CO_2 concentrations inside leaves of plants colonized by rhizobia was physiologically relevant, but not statistically significant ($p = 0.26$, t-value = 1.35). However, this

decrease was not linked to differences in stomatal conductance, which was not significantly different in plants receiving nitrogen from either rhizobia or inorganic fertilizer. Rather, the physiologically relevant difference in mean internal CO₂ concentrations was related to significantly higher J_{max} values (p=0.02, t-value= -4.13) and an increase in Vc_{max} that was physiologically relevant but not statistically significant (p=0.48, t-value= -0.80) in plants colonized by rhizobia. While healthy plants colonized by rhizobia had higher percent nitrogen content than healthy plants receiving inorganic nitrogen fertilizer (Figure 4-2), this does not fully explain the ¹³C enrichment of plants colonized by rhizobia. In all viral infection treatments, percent nitrogen content was the same for plants receiving nitrogen either from inorganic fertilizer or from rhizobia. Finally, while both plants receiving inorganic fertilizer and plants colonized by rhizobia had greater biomass than controls (p<0.01, LME, t-value> 3.33), the biomass of plants colonized by rhizobia was lower than the biomass of plants receiving inorganic fertilizer (p<0.001, LME, t-value= -4.652).

Volatile organic compound emissions. Nitrogen source had a strong influence on the composition of VOC profiles released by plants (p=0.001, Table 4-2, Figure 4-3A). While virus infection status had a significant effect on some individual compounds (Table 4-3), there was no significant effect of infection status on the overall VOC profiles of hosts (Table 4-2, Figure 4-3B).

Neither nitrogen source nor infection status influenced the total emission of VOCs from plants; rather, the emissions of 47 individual compounds showed both increases and decreases in relation to treatments (Table 4-3). Rhizobia had the largest effect on overall changes in the emissions of individual compounds, with a significant

positive effect on 16 compounds and a significant negative effect on 14 compounds. The use of inorganic nitrogen fertilizer had a positive effect on the emissions of 11 compounds. There was only one compound that was influenced by both inorganic nitrogen fertilizer and rhizobia.

Single infections of CIYVV had a significantly negative effect on the emission of 3 compounds, which was erased during coinfection with BCMV. Coinfection itself significantly increased the emission of 2 compounds, which were not significantly altered by single infections of either virus. We did not observe any main effects of BCMV infection on VOC emissions, but there were three compounds with significant interactions between BCMV infection and the presence of rhizobia (2 negative and one positive).

We have identified two affected compounds that are known to be perceived by aphids (Yan et al. 1994, Webster et al. 2008). The emission of methyl salicylate was significantly reduced by single infection with CIYVV (Table 4-3). Colonization by rhizobia had a positive effect on emissions of trans-caryophyllene.

Discussion

Both viral coinfection and colonization by rhizobia significantly modified plant metabolism. Carbon isotope discrimination was influenced solely by rhizobia colonization without any effects of viral infection, so we focused gas exchange measurements on a comparison between plants either receiving inorganic nitrogen fertilizer or colonized by rhizobia. As seen in other studies (e.g. Kaschuk et al. 2009), increased photosynthetic rates allowed plants colonized by rhizobia to supply

mutualists with photosynthate, while producing the same seed yields (Chapter 4) with less aboveground vegetative biomass than plants fertilized with inorganic nitrogen fertilizer. However, the mechanism behind the observed increases in photosynthesis is unusual. In soybeans, the maximum quantum yield of photosystem II is the same in control and rhizobia-inoculated plants, while carboxylation efficiency is significantly increased (Zhou et al. 2006). By comparison, the largest effect of rhizobia we observed in common beans was a significant increase in maximum rates of electron transport of photosystem II, with a suggestion of increased maximum rates of carboxylation. Common beans colonized by rhizobia have greater rates of uptake of manganese and iron (Ndakidemi et al. 2011), which are required in photosystem electron transport chains and may partially explain observed increases in electron transport rates. Neither our study nor that of Zhou et al. (2006) in soybeans showed a difference in stomatal conductance for plants inoculated with rhizobia; however, the effects of rhizobia colonization on stomatal conductance and transpiration are known to vary with host genotype and species (Matiru and Dakora 2005, Pule-Meulenberget al. 2011). The ^{13}C enrichment observed in plants colonized by rhizobia was not due to a restriction of CO_2 into leaves, but rather faster rates of CO_2 utilization.

VOC production in coinfecting hosts may be driven by one constituent virus (Salvaudon et al. 2013) or by interactive effects of multiple viruses (Peñaflor et al. 2016). Peñaflor et al. (2016) found that coinfecting plants emitted significantly less monoterpenes and methyl salicylate than singly infected plants. We found an opposite response, where coinfecting plants emitted significantly higher amounts of two specific VOCs than singly infected plants. Moreover, coinfection with BCMV erased

differences in emissions of three VOCs that are significantly under-produced in CIYVV single infections. One of these compounds was methyl salicylate, which is repellent to the BCMV vector *Aphis fabae* (Hardie et al. 1994). However, it is important to note that VOC blends, rather than single constituent compounds, tend to have the largest effects on aphid preferences (Ngumbi et al. 2007).

Colonization by rhizobia has been shown to negatively affect VOC emission in lima beans (Ballhorn et al. 2013), which contrasts with our finding of no effect of rhizobia on total VOC emissions. However, we also conducted a pilot study that took place only 1-2 weeks after viral inoculation, where we observed substantial decreases in VOC emissions from plants colonized by rhizobia (data not shown). The effect of rhizobia on VOC emission, like the effect of rhizobia on plant salicylic acid concentrations (Chapter 4), likely changes over time.

Rhizobia colonization was by far the most important factor influencing VOC blend composition. Only one compound was significantly affected by both inorganic nitrogen fertilizer and rhizobia, so the effect of rhizobia on 31 other compounds was specific to the presence of the microbial mutualist rather than plant nitrogen balance. There were 10 compounds that were differently emitted only from plants receiving inorganic nitrogen fertilizer. Since percent tissue nitrogen was similar between plants receiving inorganic fertilizer and those colonized by rhizobia in most cases, nitrogen balance was similar between these two treatments. Therefore, the difference in emission of these 10 VOCs either was not related to nitrogen balance, or the presence of rhizobia erased differences in the emission of VOCs associated with high tissue nitrogen.

We found three VOCs whose emissions were significantly affected by the interaction between single infections of BCMV and the presence of rhizobia. This signifies a potential interaction in one or more metabolic pathways due to the joint presence of BCMV and rhizobia, but more work is necessary to understand the biological processes involved or any potential ecological consequences.

Changes in VOC profiles are known to influence herbivore feeding behavior, including that of aphids that vector plant diseases (Ballhorn et al. 2013, Peñafior et al. 2016). Aphids often prefer to feed on infected plants (Bosque-Pérez and Eigenbrode 2011), even when VOC profile changes are modest (Peñafior et al. 2016). We found extensive changes in VOC profiles in plants colonized by rhizobia, including significant interactions between the presence of rhizobia and virus infection. The effects of rhizobia colonization on aphid preference are less well studied than the effects of virus infection; most work in this area focuses on the effects of rhizobia on aphid performance (e.g. Kempel et al. 2009). One study found fewer aphids on plants colonized by rhizobia in the field, but whether this was due to effects on aphid preference or performance is unknown (Dean et al. 2009). Studies of the effects of rhizobia colonization on aphid preference represent a critical next step, both in terms of the costs and benefits of mutualism and also for farmers deciding whether to inoculate their fields with rhizobia or use inorganic nitrogen fertilizers.

The communities of microbes that live in plants and animals have repercussions on host health that are just beginning to be elucidated. The microbiome is considered to in essence provide hosts with an “extended genome” (Kinross et al. 2008) of thousands of novel genes. However, the expression of microbial genes is

driven by fitness considerations that may or may not align between microbial species. A greater understanding of the ways that microbes interact to affect host metabolism could yield important insights into the maintenance of host health.

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Primary Nitrogen Source	C _i	g _s	A _{max}	V _{cmax}	J _{max}
Rhizobia	270.2 +/- 4.0	0.15 +/- 0.04	14.8 +/- 1.5	50.6 +/- 11.4	74.3 +/- 5.1
Fertilizer	300.0 +/- 21.6	0.12 +/- 0.03	8.7 +/- 0.4	40.3 +/- 5.8	49.5 +/- 3.1

Table 4-1: Photosynthetic parameters for *Phaseolus vulgaris* plants receiving nitrogen either from rhizobia or inorganic nitrogen fertilizer. Bolded parameters were found to be significantly different at the p<0.05 level.

	Df	Sums of Sqs	Mean Sqs	F value	R ²	P value
Infection treatment	3	0.85	0.28	1.08	0.03	0.156
Nitrogen Source	2	1.71	0.85	3.26	0.06	0.001 ***
Leaf Area	1	0.58	0.58	2.21	0.02	0.173
Residuals	100	26.19	0.26		0.89	
Total	106	29.33			1.00	

Table 4-2: Permutational multivariate analysis of variance of the effects of infection treatment and nitrogen source on volatile organic compound profiles emitted from *Phaseolus vulgaris* plants. Leaf area was used as a covariate, and data were blocked by experimental replicate. *** p<0.001

Compound ID	Retention time (min.)	Retention							Leaf Area	BCM V x Rhizobia	Putative ID
		BCM V	CIYVV	Co-infection	Fertilizer	Rhizobia	Leaf Area	BCM V x Rhizobia			
B	7.141	3.39	-8.06	9.27	-3.28	-17.55	**	-0.27	-	aliphatic compound	
D	7.388	-1.29	-8.36	3.03	1.96	-4.67		-0.04	-	aliphatic compound	
E	7.555	-2.15	-9.45	5.67	1.78	-5.48		-0.01	-	aliphatic compound	
F	7.602	-0.20	-1.23	-1.14	2.07	6.41	**	-0.01	-	aliphatic compound	
G	7.674	-3.22	-5.66	2.83	0.08	-6.27	*	-0.03	-	aliphatic compound	
H	7.927	5.52	3.80	-0.38	-1.33	14.42	***	-0.07	-	aliphatic compound	
J	8.419	1.49	-3.25	6.20	* -3.92	-9.22	***	0.00	-	aliphatic compound	
K	8.482	-0.21	-8.26	2.08	0.35	-11.45	*	0.04	-	1,4 dimethyl benzene (p-xylene)	
L	8.631	-0.91	-20.93	9.29	1.76	-20.14	*	0.12	-	o-xylene	
P	9.421	30.33	10.02	1.41	14.71	40.99	*	-0.82	-	unknown, possible benzene group	
Q	9.58	0.75	-7.20	3.78	-2.38	19.44	**	0.19	-	isocineole	
T	10.484	-1.06	-15.49	9.48	-3.76	-17.60	*	0.00	-	1-ethyl-2-methyl benzene	
V	10.905	2.29	-1.18	-0.22	-0.27	11.46	***	0.12	-	aliphatic compound	
Y	11.089	1.80	-5.71	5.04	0.38	-10.17	*	0.14	-	styrene	
AB	11.54	-1.35	-18.68	8.87	-2.41	-22.05	*	0.01	-	1 methyl ethyl benzene	
AC	11.68	5.29	0.91	-0.95	1.84	9.11	*	-0.17	-	aliphatic compound	

AD	11.851	4.54	-0.16	-2.08	-0.27	8.18	**	-0.03	-	aliphatic compound
AF	11.959	1.53	-10.31	10.00	-1.59	-14.75	*	-0.08	-	1 methyl 3 propyl benzene unknown, possible
AG	12.021	16.86	-18.73	23.79	22.41	41.72	***	0.40	-	aliphatic compound
AH	12.193	33.83	-3.73	12.42	47.29	*	***	1.05	*	cis 3 hexenyl acetate
AI	12.405	-0.46	-6.11	3.92	-0.19	-8.72	**	-0.05	-	1,2 diethyl benzene
AK	12.766	-1.17	-9.70	4.60	-1.36	-9.42	*	0.00	-	4 ethyl, 1,2 dimethyl benzene
AL	12.874	-1.58	-10.42	5.23	-1.17	-10.36	*	-0.02	-	2 ethyl, 1,4 dimethyl benzene
AN	13.11	-0.91	-1.08	12.53	**	7.53		0.11	-	2,4,6 trimethyl pyridine (collidine)
AQ	13.602	2.67	0.91	-0.93	0.65	7.63	***	-0.03	-	unknown, possible aliphatic compound
AR	13.663	-3.99	-3.38	6.39	6.84	*		0.13	-	1,2,4,5 tetramethyl benzene, or a similar
AS	13.926	-10.70	-6.08	17.26	18.62	*		0.41	-	tetramethyl benzene
AU	14.208	20.20	-2.33	-0.70	3.45	28.38	*	0.13	-	unknown
AV	14.316	-10.87	-6.42	13.93	16.42	*		0.35	-	1-octen-3-ol unknown

AW	14.768	6.56	-5.67	5.68	-12.58	-20.33	**	-0.25	-	4,4 dimethyl-2,2-bis(1,1 dimethylethyl)-1-pentanol
AX	16.079	-10.10	-6.15	19.68	20.83	*		0.38	-	2 methoxy-6 methyl ethyl phenol
AY	16.481	1.97	-2.86	9.29	2.17	17.13	**	0.27	-	trans beta caryophyllene
AZ	16.777	-3.49	-3.43	3.34	5.52	*		0.12	-	unknown, possible aliphatic compound
BC	17.394	4.76	2.73	0.57	0.52	4.87		0.01	-9.11	unknown, possible aliphatic compound
BD	17.614	5.68	1.81	0.52	0.82	13.25	***	-0.17	-	aliphatic compound
BE	17.968	3.05	0.88	1.12	-0.79	5.87	**	-0.05	-	aliphatic compound
BF	18.746	3.26	0.82	-0.28	1.62	7.54	**	-0.06	-	unknown compound
BI	19.309	-7.38	-18.82	-8.82	6.91	6.07		0.19	-	methyl salicylate
BK	19.927	6.46	-13.81	12.88	-21.12	-38.14	***	-0.32	-	4-(1-methylethyl)-,methyl ester benzoic acid
BS	23.017	17.74	17.25	29.78	-7.15	49.19	*	-0.29	-	aliphatic compound, possible long chain alcohol
BT	23.454	-3.51	-2.47	5.15	5.77	*		0.12	-	unknown, possible aliphatic compound
BU	23.81	-1.72	-2.33	-0.27	1.74	5.74	**	0.06	-	unknown compound
BZ	25.125	-4.91	-4.68	8.59	8.70	*		0.21	*	unknown compound
CB	25.384	-9.44	-6.91	14.86	15.34	*		0.21	-	unknown compound

CC	25.626	-1.37	-1.63	5.52	6.25	*	-1.49	-0.01	-	unknown possibly methyl ester
CE	26.217	-5.28	-1.22	10.49	10.28	*	-5.80	0.14	-	hexadecanoic acid
CG	26.524	-6.42	-3.15	-4.46	-0.63		-2.79	-0.01	8.68	unknown aliphatic compound *

Table 4-3. Volatile organic compounds emitted by *Phaseolus vulgaris* plants significantly affected by viral infection, nitrogen source, or both.

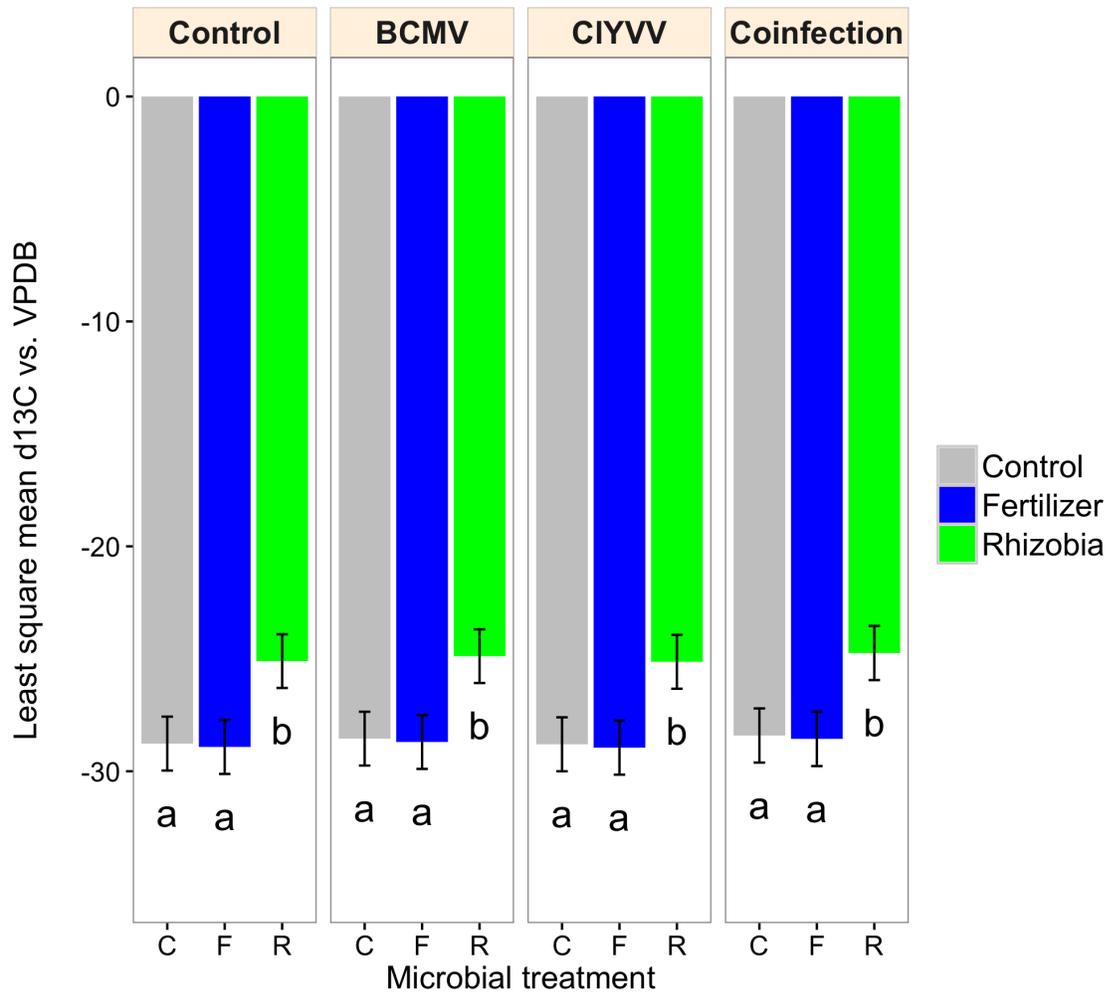


Figure 4-1: Effects of infection treatment and nitrogen source on $\delta^{13}C$. Bars indicate model standard errors. Different letters indicate a significant difference at the $p < 0.05$ level. VPDB: Vienna Pee Dee Belemnite.

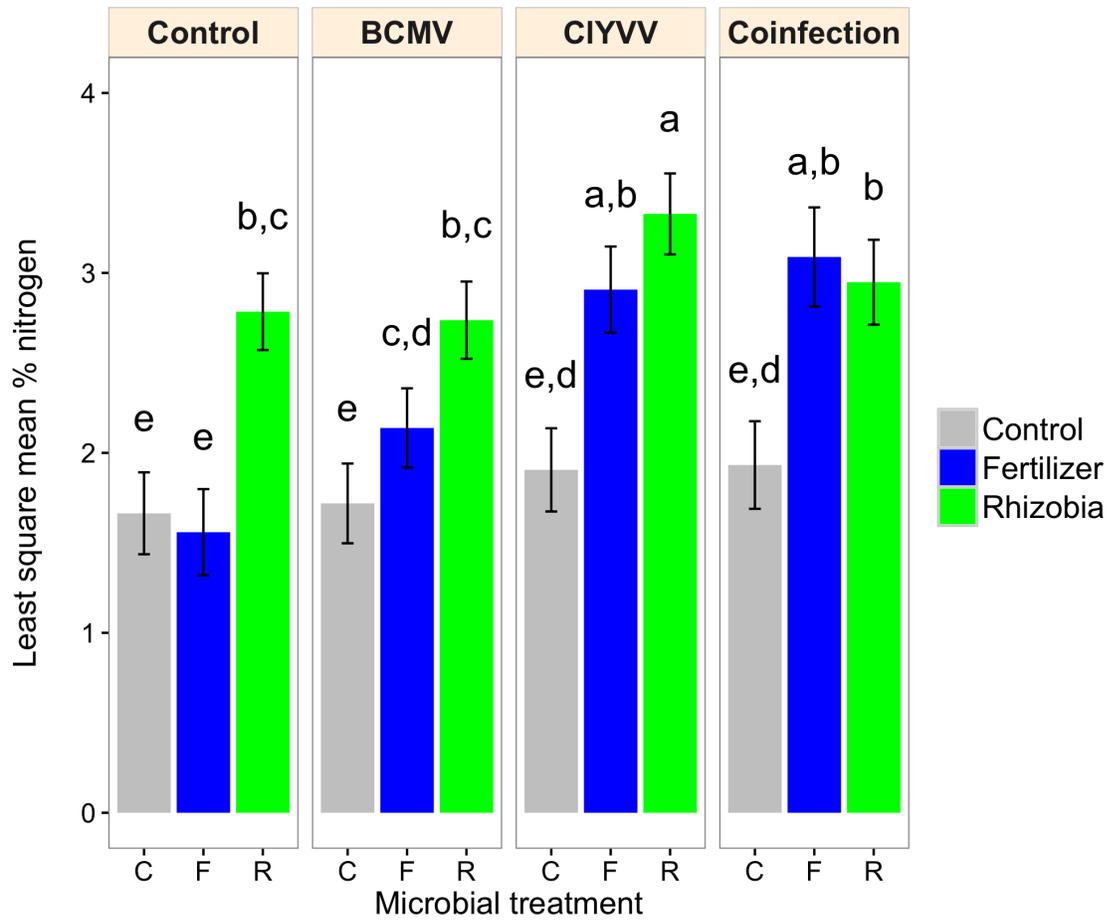


Figure 4-2: Effects of infection treatment and nitrogen source on the percentage of nitrogen in leaf tissue. Bars indicate model standard errors. Different letters indicate a significant difference at the $p < 0.05$ level.

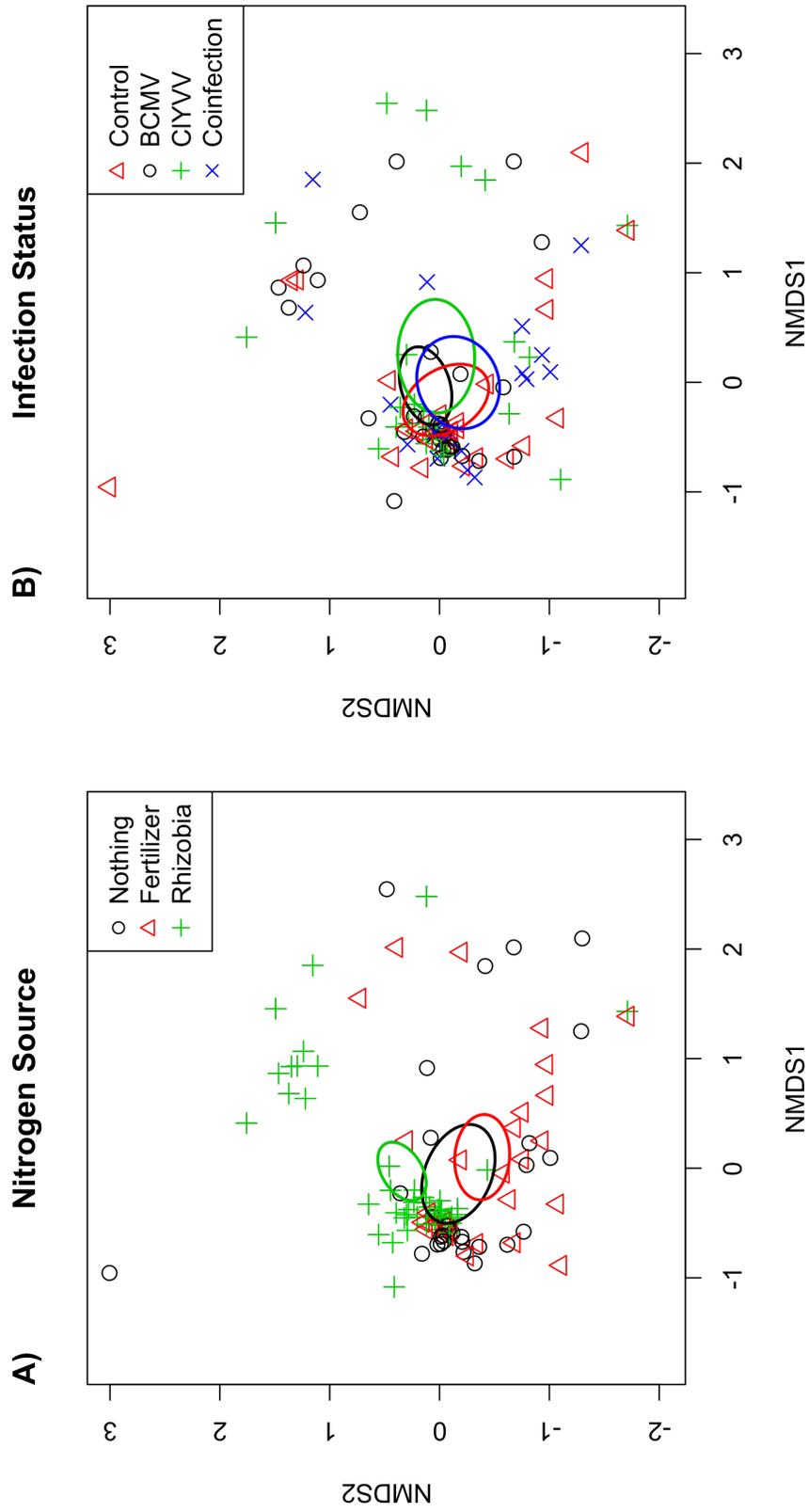


Figure 4-3: Non-metric, multi-dimensional scaling of volatile organic compound emission profiles by A) nitrogen source and B) infection status. Circles represent 95% confidence ellipses.

CHAPTER 5

VIRAL INFECTION CAN REDUCE THE NITROGEN FERTILIZER REPLACEMENT VALUES OF LEGUME ROTATION AND COVER CROPS

Abstract

Legumes are used in crop rotations by both large-scale and subsistence farmers alike to increase soil fertility, especially before high nitrogen demanding crops such as corn. Legume crop residues and green manures are rich in nitrogen due to the mutualistic bacteria, rhizobia, that live in their roots and convert atmospheric nitrogen into a biologically available form. Growers can obtain recommendations from local extension offices about how much less inorganic nitrogen fertilizer needs to be added to a subsequent crop following different legume rotations and for the predominant soil type (the nitrogen fertilizer replacement value, or NFRV). Due to the intimate relationship between legumes and rhizobia, conditions that affect plant health can also affect the rhizobia and how much nitrogen they provide. We use a combination of empirical data and published values to estimate how much lower NRFVs would be under outbreaks of plant viruses of varying severity. We also use historical fertilizer prices to examine the impacts of this lost fertilizer for farmers. We found that fertilizer losses are highest for crops that fix high amounts of nitrogen, such as clover and alfalfa as opposed to common bean. The economic impact on farmers is controlled by the proportion of plants with viral infections and the price of synthetic fertilizer. In outbreak years with high fertilizer prices, growers could be losing US\$20 or greater per hectare in lost fertilizer or reduced yields of subsequent crops. In a year

of high disease prevalence, attention is normally focused on the yield of the diseased crops. We suggest that farmers growing legumes in crop rotations should be concerned about yields of subsequent crops as well.

Introduction

The synthesis of inorganic nitrogen fertilizer consumes approximately 1.3% of the world's energy budget and provides the most energy intensive input to modern agricultural production (Crews and Peoples 2004). The burning of fossil fuels that provides energy to this process results in substantial greenhouse gas emissions (Robertson et al. 2000). While inorganic nitrogen fertilizers are necessary to feed the world population (Erisman et al. 2008), crop rotations incorporating nitrogen-fixing legumes increase the sustainability of many types of agricultural systems, including large-scale agriculture (for example, soybean-corn rotations), organic agriculture, and subsistence farming in areas where inorganic fertilizers are prohibitively expensive (Crews and Peoples 2004).

Much of the benefit legumes provide to sustainable agriculture comes from their association with rhizobia bacteria, which live in nodules in roots and convert atmospheric nitrogen into a biologically available form. Growers can take advantage of this association by adding either forage or grain legumes to their crop rotations. For example, a previous alfalfa rotation can provide all of the nitrogen fertilizer needed by a subsequent corn crop (Yost et al. 2012). Legume green manures and cover crops can also provide substantial amounts of nitrogen, as much as 87-184 kg/ha for red clover green manure preceding corn, saving an estimated 104-274 m³/ha of natural gas that

would have been needed to synthesize the same amount of inorganic nitrogen fertilizer (Liebman et al. 2012). Grain legumes provide lower amounts of nitrogen, because the nitrogen content of the seeds is removed from the field, but also provide the additional benefit of a sustainable protein source. The grain legumes that provide the greatest benefits to soil fertility, such as fava bean, field pea, and lentil, are ones with the highest tissue percentage of nitrogen derived from the atmosphere (%Ndfa), sourcing the greatest percentage of their nitrogen from nitrogen fixation rather than the soil (Walley et al. 2007). However, soybean in rotation with corn still provides substantial increases in sustainability in large scale agriculture (Varvel and Wilhelm 2003).

Less inorganic nitrogen fertilizer needs to be added to a non-legume crop following a legume rotation to achieve the same yields as a non-legume crop grown continuously. The magnitude of this reduction in the addition of inorganic nitrogen fertilizer is referred to as a nitrogen fertilizer replacement value (NFRV) or nitrogen credit. The NFRV incorporates both rotation and non-rotation effects of the legume crop, some of which can be replicated by adding more fertilizer and some of which cannot (Lory et al. 1995). The contribution of nitrogen from legume residues to the soil is a large component of the NFRV, but additional benefits include scavenging of available soil N (George et al. 1994), interruption of pest lifecycles, and improved soil characteristics.

One consequence of the intimate mutualistic association between legumes and nitrogen-fixing rhizobia is that variables affecting the plant host can also affect the rhizobia and how much nitrogen they provide. For instance, nitrogen fixation

significantly decreases when a legume is infected by a virus (Chapter 3, Ohki et al. 1986; Khadhair et al. 1984). This will decrease the NFRV of virus-infected legume fields, but this possibility is usually overlooked. Approximations exist for the amount that plant diseases decrease current crop yield or aboveground biomass, but estimates of how much nitrogen could be lost to subsequent crops due to disease in a legume rotation are nonexistent. It is also important to consider infections of one host with multiple viruses, which are common in field settings (Demski et al. 1988; Mahuku et al. 2015; Karyeija et al. 2000; Pio-Ribeiro et al. 1978), especially during disease outbreaks. Multiple infections can cause a greater than additive reduction in the amount of nitrogen supplied by rhizobia (Chapter 3).

We use a mixture of empirically derived data and estimations based on published values to quantify how much of a legume crop's NFRV could be lost due to viral infection. For illustrative purposes, we make the assumption that each crop has two major viruses, and calculate NFRV estimates for common bean, clover, and alfalfa at different levels of disease prevalence.

Materials and Methods

We examine estimated NFRVs for common bean (*Phaseolus vulgaris*), crimson clover (*Trifolium incarnatum*), and alfalfa (*Medicago sativa*). Data on the proportional negative effect that infection with one or two viruses has on host growth and nitrogen fixation by rhizobia come from an experiment involving *Bean common mosaic virus* (BCMV) and *Clover yellow vein virus* (CIYVV) infection of common beans (*Phaseolus vulgaris*). Briefly, common bean plants were grown in growth

chambers and received fully factorial treatment combinations of rhizobia inoculation (yes or no) and virus inoculation (control, infected with BCMV, infected with CIYVV, or infected with both viruses). All plants in the no rhizobia treatment were examined for rhizobia contamination, and discarded from analysis if they contained rhizobia nodules. For detailed information about the experimental conditions, see Chapter 3. These two viruses do not both infect clover and alfalfa, but proportional declines in growth and nitrogen fixation caused by viral infection can be used to model the impacts of other viruses, especially when key information is unavailable. Two viruses that are known to reduce nodule function of clover and alfalfa are *White clover mosaic virus* and *Alfalfa mosaic virus*, respectively (Ohki et al. 1986; Khadhair et al. 1984).

While NFRVs encompass more effects than simply the addition of nitrogen from crop residues, we found that calculating the amount of nitrogen derived from the atmosphere per area that would be added by aboveground crop residues provides estimates which are in many instances close in value to empirically determined NFRVs (Midwestern USA: Bundy et al. 1997; Shapiro et al. 2008). However, nitrogen credits can vary with soil type, environmental conditions, and the species of the subsequent non-legume rotation.

The estimated NRFV (eNFRV) for common beans describes a flux of nitrogen added to the soil through atmosphere-derived nitrogen in aboveground crop residues and nitrogen that is removed from the soil through seed harvest (Eqns. 1-3, Table 5-1). The model assumes that there are two major viruses that infect the bean crop, in this case *Bean common mosaic virus* (BCMV) and *Clover yellow vein virus* (CIYVV). Coinfections, where a plant is infected by both viruses, occur at random based on the

prevalences of each virus. N_{in} is the summation over all disease classes (i) of the amount of nitrogen in the crop residue that was sourced from nitrogen fixation. N_{in} is determined by the proportion of plants in each disease class (π_i), the plant density (d), the percentage nitrogen content of vegetative and pod tissue (N^{veg}_i and N^{pod}_i , respectively), the biomass of these tissues per plant (v_i and p_i , respectively), and the percentage of the nitrogen in these tissues that is derived from nitrogen fixation (f_i). N_{out} is the summation over all disease classes of the amount of soil nitrogen removed from the system with the seed harvest, where s_i is seed mass per plant and N^{seed} is the percentage of nitrogen in seeds. While the model assumes that pod tissue is left in the field with the vegetative crop residue after in-field combining, the nitrogen content of the pods is so low that it doesn't make much difference if a farmer instead threshes the beans at a separate location.

$$N_{in} = \sum_i \pi_i d f_i (v_i N^{veg}_i + p_i N^{pod}_i) \quad \text{Eqn. 1}$$

$$N_{out} = \sum_i \pi_i d s_i N^{seed} (1 - f_i) \quad \text{Eqn. 2}$$

$$eNFRV_{bean} = N_{in} - N_{out} \quad \text{Eqn. 3}$$

The estimated NFRV for clover contains no term for nitrogen leaving the system through harvest (Eqn. 4); the clover is considered to be a green manure. Viral infection related reductions in plant growth and rhizobia function in clover were estimated from known effects of *Bean common mosaic virus* (BCMV) and *Clover*

yellow vein virus (CIYVV) infections of common beans. The percentage reduction in dry biomass estimated from CIYVV infections in common beans is similar to that shown by Gibson et al. for CIYVV infection in white clover (1981). BCMV does not infect clover, but other viruses would likely have effects of a similar magnitude. Here, a biomass per area term (B_i) is used rather than a term for plants per area.

$$eNFRV = \sum_i \pi_i B_i N_i^{veg} f_i \quad \text{Eqn. 4}$$

Alfalfa is usually harvested for animal fodder, and almost all of the crop residue is belowground, which makes calculation of estimated NFRVs more difficult. In order to evaluate how NFRVs for alfalfa would change in a two-virus system, we first applied percentage differences in estimated clover NFRVs caused by BCMV and CIYVV to an average estimate of 180 kg/ ha NFRV for a healthy field of alfalfa in the Midwestern USA (Shapiro et al. 2008; Bundy et al. 1997; Yost et al. 2012). However, BCMV does not infect alfalfa, and CIYVV does not reach economically significant yield losses in alfalfa due to low disease prevalence. *Alfalfa mosaic virus* (AMV) is a seed-transmitted virus that can reach prevalences of greater than 90% in susceptible alfalfa, and is known to reduce nodule function and both above and belowground root biomass (Jones 2013; Ohki et al. 1986; Wroth et al. 1993). For this reason, we also estimated expected reductions in alfalfa NFRVs for a range of *Alfalfa mosaic virus* (AMV) prevalences based on published data. We used Eqn. 4, but applied it to root biomass (including fine root demography) and percentage of nitrogen in root tissue (Table 5-1). Percentage reductions in root growth in AMV infected plants came from

Ohki et al. (1986) and were applied to root biomass (Bohl 1981) and fine root turnover estimates (Goins and Russelle 1996). The percentage of nitrogen in root tissue was estimated by applying a percentage reduction in shoot nitrogen in AMV infected plants (Wroth et al. 1993) to the percentage of nitrogen in alfalfa root tissue (Justes et al. 2002). Reductions in %NDFa for alfalfa were estimated by applying the percentage reduction in acetylene reduction caused by AMV (Wroth et al. 1993) to the %NDFa value for healthy alfalfa (Chen et al. 2004). The estimate we calculated for the amount of nitrogen in healthy alfalfa root systems was only a little more than half of the empirical estimates for alfalfa NFRV in the Midwestern USA (Bundy et al. 1997; Shapiro et al. 2008; Yost et al. 2012). This could be for a number of reasons, including the difficulty in quantifying root biomass, root exudation, aboveground crop residues, and non-nitrogen components of NFRVs such as breaking up of pest cycles and changes to soil structure. For this reason, we calculate a percentage reduction in eNFRV for a certain prevalence of AMV in comparison to a healthy crop and apply it to an estimate of 180 kg/ha NFRV for alfalfa in the Midwestern USA (Bundy et al. 1997; Shapiro et al. 2008; Yost et al. 2012).

Finally, in order to determine how much a reduction in NFRV due to viral infection could matter to farmers, we calculated how much money it would cost to replace the virus-induced loss of nitrogen fixation per ha in heavily infected fields with supplemental urea for a range of historical fertilizer prices (Economic Research Service 2013).

Results and Discussion

The importance of reductions in legume nitrogen fixation caused by viral infection depends on the type of legume crop, viral prevalence, the cost of nitrogen fertilizer, and the type of subsequent crop. Common beans provide low NFRVs in most soils due to a combination of nitrogen harvested in seeds and generally lackluster %NDF values. However, a high viral prevalence year could lead to a total loss of NFRV for bean crops (Figure 5-2), in addition to bean yield losses. Clover and alfalfa are much more productive legumes for the purposes of NFRV, and can easily lose 10% of their NFRV benefits even under low prevalence conditions (Figure 5-3, 5-4).

Historically, even high disease prevalences would have had little effect on farmers with access to inexpensive sources of inorganic nitrogen (Figure 5-5). However, as fuel costs rise the loss of NFRV from virus-infected crops becomes increasingly expensive. These expenses remain low for common beans, since this crop does not contribute much nitrogen availability for the following crop. In contrast, the cost of reduced nitrogen fixation becomes progressively higher as the NFRV of a healthy legume crop increases (Figure 5-5). For example, when fertilizer prices are high the cost of lost fertilizer for alfalfa could reach US\$20 per ha in a field where 40% of plants are infected with AMV (Figure 5-5). This is a moderate prevalence for AMV, because it is seed-transmitted and tends to accumulate in seed over multiple generations. An alfalfa field with high prevalence (80-90%) could potentially cost farmers US\$50/ha.

If a farmer plans to fertilize his or her field at the economic optimum nitrogen rate, then the amount of money the farmer would lose in either additional fertilizer

costs or lost yields in a subsequent crop would be roughly equal for low viral prevalences (data not shown). This happens because at the economic optimum nitrogen yield, the last amount of nitrogen added to the field is just enough to cause an increase in crop yield that will pay for the added nitrogen (Colwell 1994). However, in an outbreak year a farmer would lose less money if he or she knew to add more nitrogen fertilizer to the subsequent crop to compensate for the loss of NFRV. Exactly where this tipping point occurs for a given crop depends on the shape of the relationship between crop yield and nitrogen added.

The estimations we present here for the magnitude of costs caused by virus-induced losses in NFRV are only a first step, and demonstrate a critical need for more information. The magnitude of the reduction in %NDFA and nitrogen content of crop residues is unknown for most combinations of agriculturally important legume and virus species. It is also possible that cultivars of a given legume or strains of rhizobia could vary in the extent to which viral infection impairs the mutualistic relationship. Additional research is also needed to empirically determine expected losses in NFRV for a variety of crops, virus species, soil types, and climates.

This information is crucial as we look forward, because despite a dip in the price of oil in 2015-2016, using legumes as a sustainable nitrogen source will become increasingly important in the future as fossil fuels are depleted, organic produce increases in market share, and countries impose caps on greenhouse gas emissions (Crews and Peoples 2004). Empirical estimates of lost NFRVs during viral outbreaks are also critical information for agencies working with subsistence farmers to alleviate poverty. In an outbreak year of a virus that infects legume rotation crops, any farmers

growing legumes during the outbreak are likely to see yield reductions in their next crop if their fertilization practices remain constant.

Conclusion

Legume rotations are an important tool to increase the sustainability of agriculture. They provide an alternative to the energy-intensive process of making inorganic nitrogen fertilizer, and in the case of grain legumes provide a sustainable protein source for humans. We demonstrate that it is possible for viral infections to reduce the soil fertility benefits of legume rotations, leading to potentially costly increases in inorganic fertilizer use or reductions in subsequent crop yields.

Additional research is urgently needed on this topic to better determine the breadth and depth of this problem for a variety of crop species and settings. This knowledge will provide a way to mitigate, or at least anticipate, yield losses in subsequent non-legume crops.

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Common Beans	Healthy	BCMV-Infected	CIYVV-Infected	Coinfected
Plants per hectare (d)	210040 (a)	210040 (a,b)	210040 (a,b)	210040 (a,b)
Mass of vegetative biomass per plant in kg (v)	0.006 (c)	0.008 (b,c)	0.005 (b,c)	0.005 (b,c)
Mass of pods per plant in kg (p)	0.005 (c)	0.005 (b,c)	0.003 (b,c)	0.002 (b,c)
Percent nitrogen content of vegetative biomass (N ^{veg})	3.25% (b)	2.80% (b)	3.60% (b)	3.20% (b)
Percent nitrogen content of pods (N ^{pod})	0.69% (b)	0.69% (b)	0.69% (b)	0.69% (b)
Percent nitrogen content of seeds (N ^{seed})	3.09% (b)	3.09% (b)	3.09% (b)	3.09% (b)
Percent nitrogen derived from atmosphere (f)	67.5% (b)	61.3% (b)	60.2% (b)	43.9% (b)
Seed mass per plant in kg (s)	0.012 (b,c)	0.012 (b,c)	0.0083 (b,c)	0.0067 (b,c)
Clover	Healthy	BCMV-Infected	CIYVV-Infected	Coinfected
Dry biomass in kg/ha (B)	3500 (d)	3986.5 (b,d)	2394 (b,d)	2054.5 (b,d)
Percent nitrogen content of vegetative biomass (N ^{veg})	3.34% (d)	2.91% (b,d)	3.67% (b,d)	3.30% (b,d)
Percent nitrogen derived from atmosphere (f)	85% (d)	77.19% (b,d)	75.81% (b,d)	55.28% (b,d)
Alfalfa	Healthy	AMV-Infected		
Dry root biomass in kg/ha, including fine root demography (B)	3420 (e,f)	2691.54 (e,f,g)		
Percent nitrogen content of root biomass (N ^{veg})	3.30% (h)	3.00% (b,h)		
Percent nitrogen derived from atmosphere (f)	90% (i)	67.32% (g,i)		

Table 5-1: Parameters used to calculate estimated nitrogen fertilizer replacement values (eNFRVs) for common bean, clover, and alfalfa. Letters in parentheses refer to parameters in Eqn. 1-4. Data citations: (a) Kandel 2014, (b) Chapter 3, (c) Clark and Carpenter 2000, (d) Mueller and Thorup-Kristensen 2001, (e) Bohl 1981, (f) Goins and Russelle 1996, (g) Wroth et al. 1993, (h) Justes et al. 2002, (i) Chen et al. 2004.



Figure 5-1: Nodulation of A) healthy common beans, or common beans infected with B) one virus: *Bean common mosaic virus*, or C) two viruses: *Bean common mosaic virus* and *Clover yellow vein virus*. Nodulation and the percentage of nitrogen in plant tissues derived from the atmosphere decline as the number of viral species infecting a plant increases from zero to two (Chapter 3).

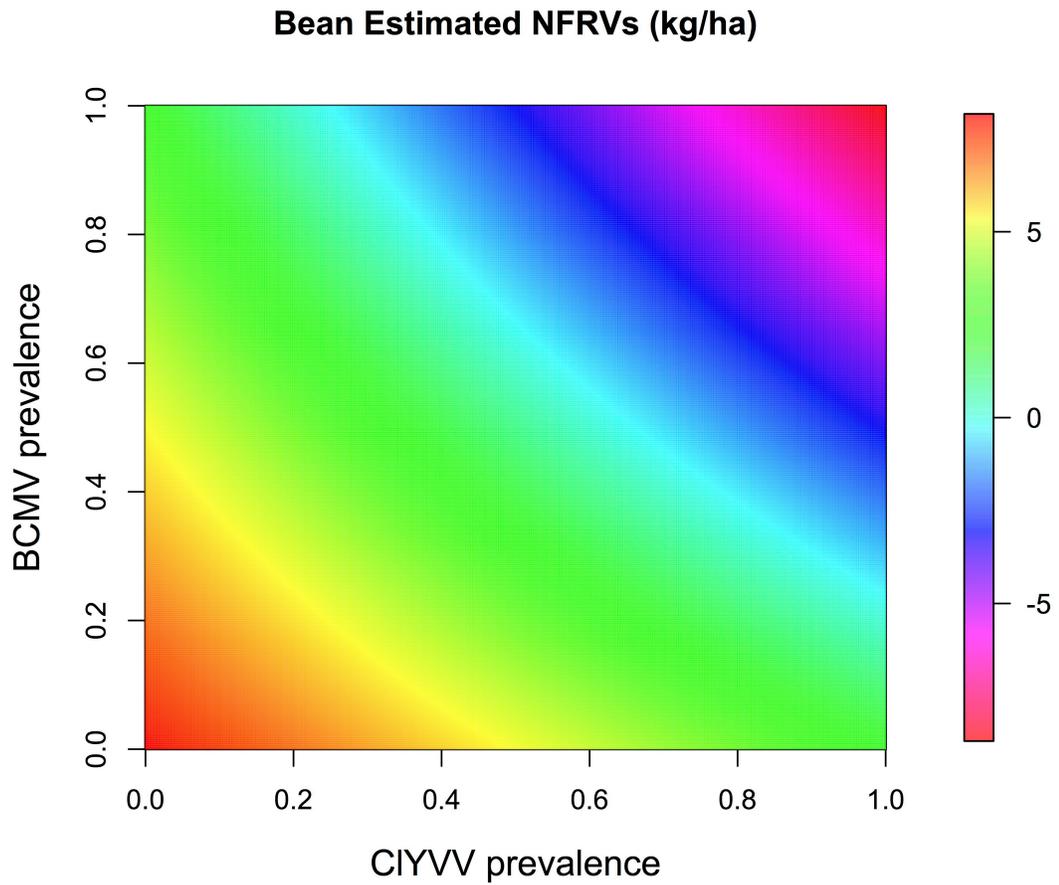


Figure 5-2: Estimated nitrogen fertilizer replacement values for common beans at all possible combinations of *Bean common mosaic virus* (BCMV) and *Clover yellow vein virus* (CIYVV) prevalences. For reference, high but realistic prevalence levels for BCMV and CIYVV in susceptible *Phaseolus vulgaris* varieties are 0.6 and 0.2-0.5, respectively (Omuniyin et al. 1995; Shah et al. 2006; Lisa and Dellavalle 1983).

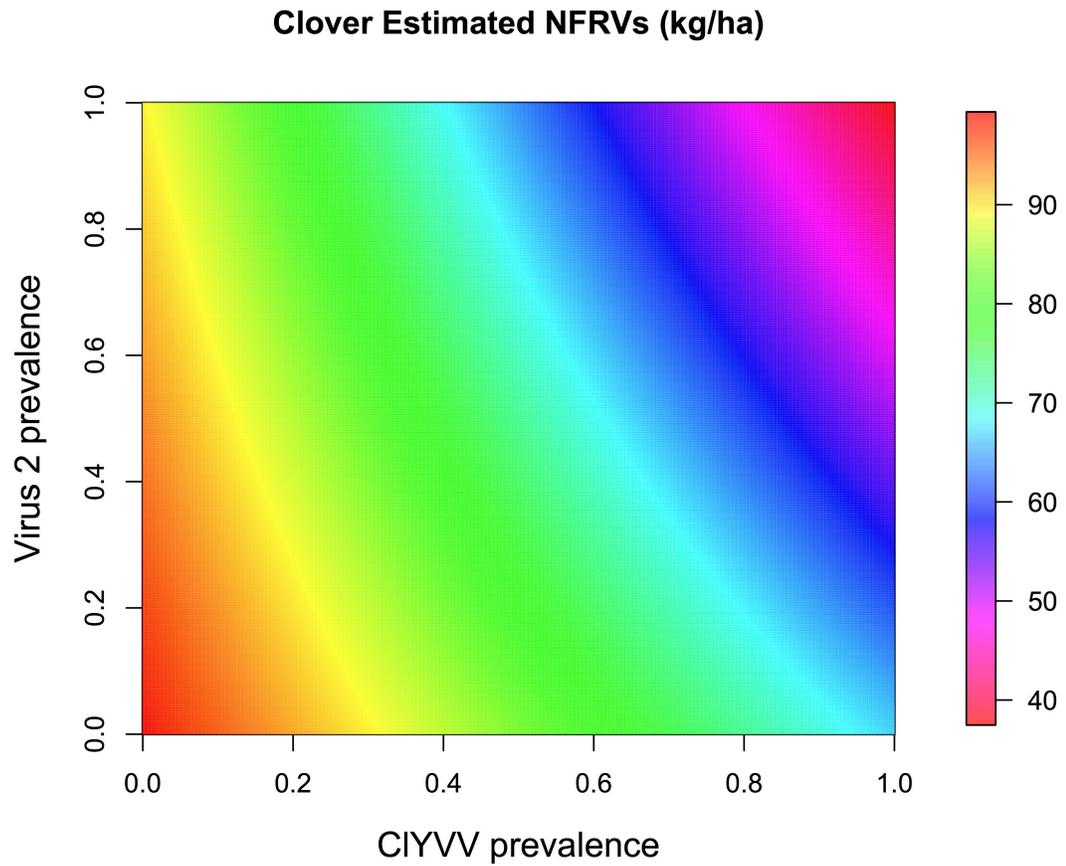


Figure 5-3: Estimated nitrogen fertilizer replacement values for clover at all possible combinations of *Clover yellow vein virus* (CIYVV) and a second virus infecting clover with similar effects as *Bean common mosaic virus*. For reference, high but realistic prevalence levels for CIYVV in susceptible clover varieties are 0.3-0.59 (Barnett and Gibson 1975; Godfree et al. 2004).

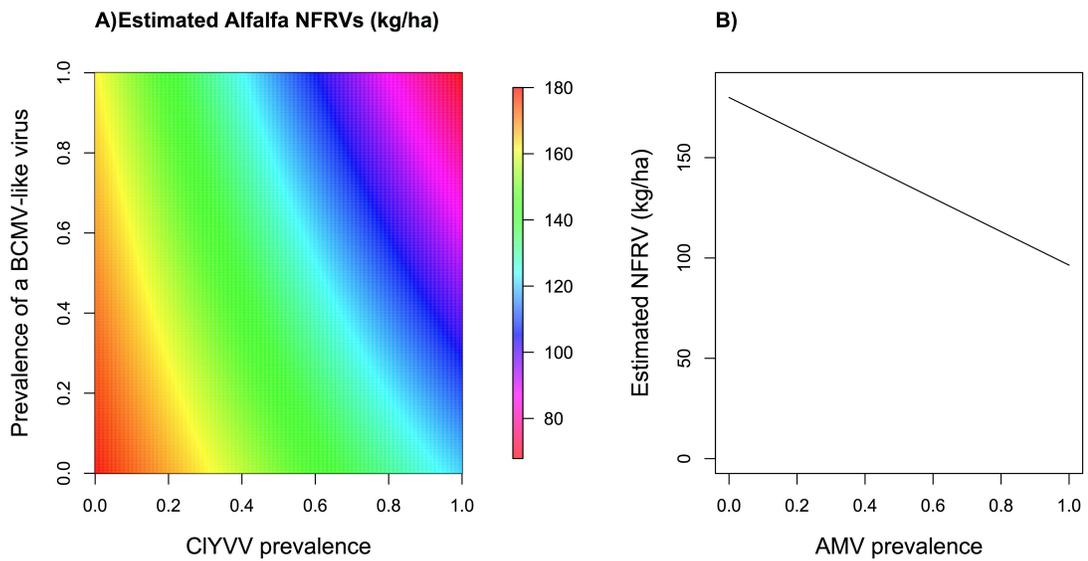


Figure 5-4: Estimated nitrogen fertilizer replacement values (NFRVs) for alfalfa A) at all possible combinations of *Clover yellow vein virus* (CIYVV) and a second virus infecting clover with similar effects as *Bean common mosaic virus* as well as B) all prevalences of *Alfalfa mosaic virus*. CIYVV is not known to cause significant economic damage in alfalfa, but *Alfalfa mosaic virus* decreases nodule function and can reach prevalences of greater than 90% (Jones 2013).

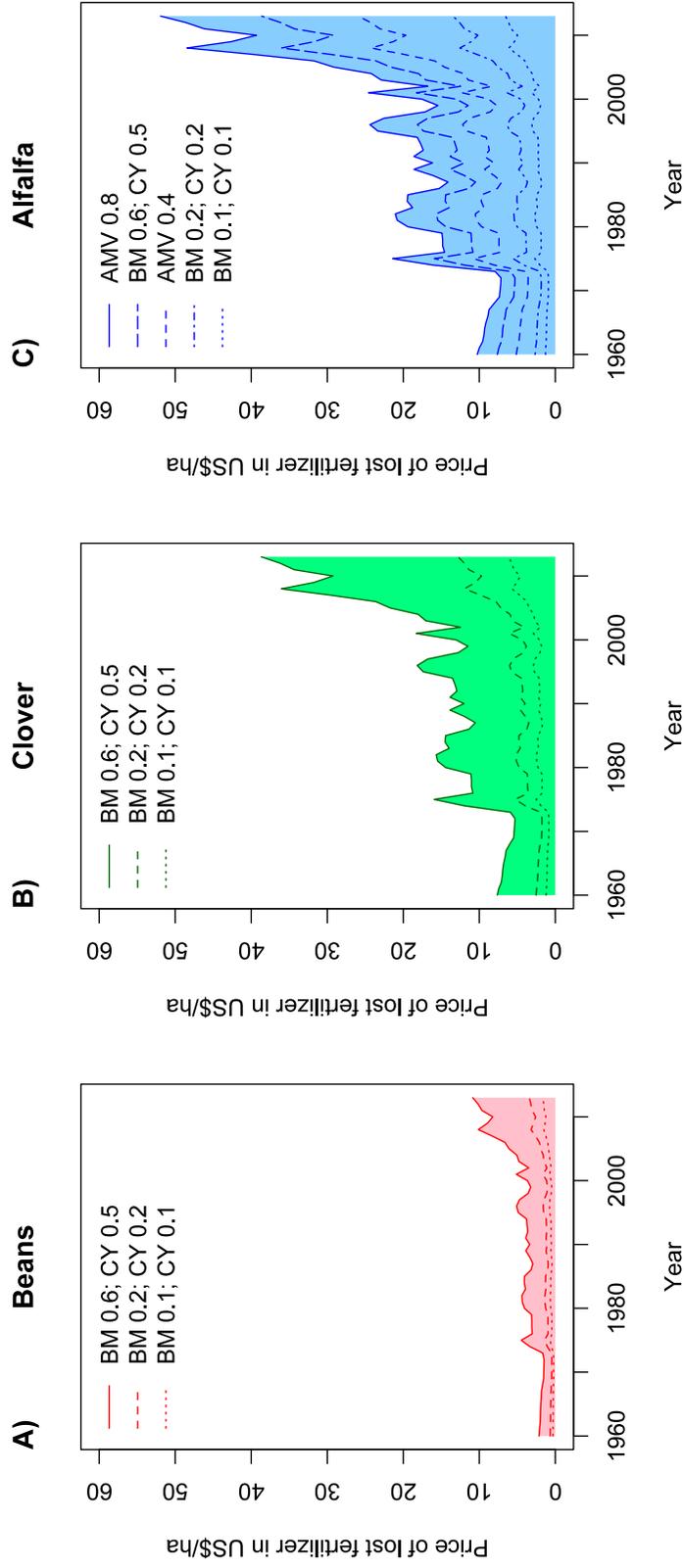


Figure 5-5: Estimated cost of fertilizer in US\$/ha lost due to viral infection for A) beans, B) clover, and C) alfalfa over time for different disease prevalences. Prices assume that the source of supplemental nitrogen is urea.