THE *BARLEY YELLOW DWARF VIRUS- RHOPALOSIPHUM PADI* PATHOSYSTEM: AN EXAMINATION OF HOST PLANT-VIRUS-VECTOR INTERACTIONS

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Abstract

Barley yellow dwarf disease is an important plant disease caused by a complex of plant viruses referred to as *Barley/Cereal yellow dwarf virus* (B/CYDV) which is comprised of eight viral species infecting cereal crops and over 150 other *Poaceae* species. B/CYDV is transmitted exclusively by aphids in a persistent, circulative manner. In the United States, the most widespread species is BYDV-PAV transmitted by *Rhopalosiphum padi* (L.).

The effects of BYDV-PAV infection on host plant preferences were examined for viruliferous (virus-carrying) and nonviruliferous *R. padi*. Viruliferous aphids that acquired virus from either infected barley or through membranes infused with artificial diet containing purified virus, were used to examine direct (membrane feeding) vs. indirect (infected host plant) effects of virus acquisition. Nonviruliferous aphids significantly preferred BYDV-infected plants while viruliferous aphids significantly preferred virus-free plants. The shift in preference from infected to noninfected plants following virus acquisition could accelerate the rate of virus spread and was shown to be the result of direct effects of virus acquisition within the insect vector.

The wheat producing region of northern Idaho and eastern Washington is characterized by fragmented native prairie and Conservation Reserve Program (CRP) habitats embedded in an agricultural matrix. The Palouse Prairie and CRP lands were surveyed to determine in which habitats and grass species B/CYDV occur and what B/CYDV species are present. Ten species of *Poaceae* that have not been reported as hosts of B/CYDVs were evaluated via laboratory inoculations. Nine of these species are found throughout the Pacific

Northwest in Camas and Palouse Prairie habitats or CRP lands and one of these species is being proposed as a candidate for biofuel production.

B/CYDV was found for the first time in Palouse Prairie and CRP habitats and BYDV-SGV and PAV were identified as the predominant viral species occurring in this region. *Achnatherum occidentale*, *A. lettermanii*, *A. thurberianum*, *Danthonia intermedia, Poa fendleriana*, *Sporobolus airoides, S. cryptandrus*, *Ventenata dubia* and *Arundo donax* were identified as new hosts of BYDV-PAV. Transmission of BYDV-PAV from some of these grass hosts to susceptible barley was demonstrated using *R. padi*. The ecological and epidemiological implications are discussed.

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Chapter 1: Introduction

Insects serve as vectors of a large number of economically important pathogens, including viruses of agricultural crops worldwide that cause diseases which result in significant yield losses each year. Attempts to manage these diseases require an understanding of the interactions between components of the pathosystem associated with disease outbreaks. As farmers and land managers struggle to prevent disease outbreaks and crop losses in a changing climate, a better understanding of the interactions among the vector, virus and host plants is required.

Plant viruses, vectors and host plants are believed to have coevolved for periods of time that spans millions of years. Many of these plant viruses have been found to occur in natural ecosystems and maintain a balance between infection and damage (Malmstrom et al. 2005b, Seabloom et al. 2009, Borer et al. 2010). Current agricultural practices which promote the implementation of sustainable agricultural systems provide new challenges for disease management (Conway 1996).

Cereal grains, including wheat (*Triticum aestivum* L.), barley (*Horedeum vulgare* L.) and oat (*Avena sativa* L.) are grown throughout the world as major food staples. The United States is among the largest producers of these crops (Lister and Ranieri 1995). Among these, wheat is a major staple cereal crop used for food consumption and provides nourishment for more people throughout the world than any other food source (Inglett 1973). In 2010, the US harvested over 47 million acres of wheat, with a value of almost $13 billion US (USDA 2010). The US export of wheat accounts for almost half of the total wheat produced in the US, 75% of which consists of winter wheat varieties (Ali 2002). In Idaho, growers harvested 1.34

million acres of wheat in 2010, ranking them nationally as fifth for wheat production and export (Idaho Wheat Commission 2011).

Barley Yellow Dwarf Virus

A large outbreak in the spring of 1951 in California led to the identification of *Barley yellow dwarf virus* (BYDV) disease in the United States (Oswald and Houston 1951). Globally, this is one of the most economically important diseases of crops (Plumb 1983). Barley yellow dwarf disease is caused by a group of viruses belonging to the family *Luteoviridae*. BYDVs are ubiquitous in *Poaceae* species occurring worldwide and infect small grain crops as well as pasture, rangeland and natural grasslands. The most damage occurs globally in wheat, oat and barley. Average yield losses caused by BYDV disease have been reported to range from 11-47% in wheat and 15-33% in barley in various countries around the world (Lister and Ranieri 1995). These values can vary dramatically depending on plant cultivar, location and abiotic stressors. In a greenhouse study in the US, grain yield of winter wheat (4 cultivars) infested with the bird cherry-oat aphid alone was reduced up to 21% and infection of BYDV and aphid damage reduced yield up to 58% (Riedell et al. 1999).

Within the family *Luteoviridae* several viral species and serotypes which cause BYD disease are grouped into two distinct genera: *Luteovirus and Polerovirus* (King et al. 2012). Viruses in the family *Luteoviridae* have virions that are 25-30 nm in diameter, hexagonal in shape with an icosahedral symmetry (King et al. 2012). These viruses are single-stranded RNA viruses. Transmission occurs in a circulative, non-propagative manner by aphid vectors. The members of the genus *Luteovirus* include the species BYDV-PAV, BYDV-MAV and BYDV-PAS. The genus *Polerovirus* includes the species *Cereal yellow dwarf virus* (CYDV)-RPS and CYDV-RPV, which also cause yellow dwarf disease upon infection (Krueger et al.

2013). The previously unassigned species BYDV-RMV has recently been proposed to be closely related to members in the genus *Polerovirus* requiring reclassification of this species (Krueger et al. 2013). The species BYDV-GPV and BYDV-SGV remain unassigned members of the family Luteoviridae. The viral species and serotypes were originally named after their respective, most-efficient aphid vectors. For example, RPV is transmitted most efficiently by *Rhopalosiphum padi*, RMV by *R. maidis*, MAV by *Sitobion avenae* (formerly *Macrosiphum avenae*), SGV by *Schizaphis graminum* and PAV has multiple vectors (*R. padi*, *S. avenae*, and others) (Rochow 1970, Rochow and Muller 1971, Rochow 1979). The vector specificity of each serotype appears to be determined by the interactions between the viral capsid protein and the aphid salivary glands (Rochow 1970, 1975). Advances in molecular biology and immunology provide efficient and affordable ways to test plant samples and discriminate between the various strains of BYDV in infected material (Malmstrom and Shu 2004, Cervená et al. 2009)

PAV and MAV are the serotypes that dominate globally and cause damage to cereal grains, including maize and rice (Lister and Ranieri 1995, Cervená et al. 2009). Transmission efficiency experiments have shown the ability of *R. padi* to transmit BYDV-RPV, RMV, MAV, SGV and PAV but this aphid species is considered to be the most efficient vector of the PAV serotype (Gray et al. 1991, Bencharki et al. 2000). PAV strains historically dominated the northwest United States until 1986, at which time RMV-like strains were found in barley growing in eastern Washington (Hewings and Eastman 1995). SGV strains linked with the vector *S. graminum*, but also vectored by *R. padi* and *S. avenae*, were also predominant in irrigated winter wheat in Idaho and in dryland cereal production in southeastern Idaho in the 1970-80s (Forster et al. 1990, Halbert et al. 1992, Lei et al. 1995). In

the cereal growing regions of South Carolina, North Carolina and Kentucky the most predominant BYDV serotype collected from field samples was PAV, followed by RPV (Gray et al. 1998). The vectors responsible for transmission in these regions include *S. graminum, R. rufiabdominalis, R. padi* and *S. avenae* (Gray et al. 1998).

Virus Impact on Host Plants

Barley yellow dwarf viruses are phloem-restricted and therefore disrupt a variety of physiological processes in the host plant. BYDV infection is variable depending on the identity of the host plant and the abiotic conditions under which they are growing. These factors influence the severity of symptoms and expression of BYDV. The most common symptoms reported for BYDV include stunting, inhibition of leaf elongation and initiation, loss of green color in leaves and a resulting yellowing or purpling along older leaves (D'Arcy 1995). The disease also causes a reduction in grain filling and quality. The discoloration for which the disease was named can usually be seen 7-20 days after infection in most cereal crops, however many varieties of wheat tend to be symptomless. High light intensity and temperatures ranging from 15-18°C can favor the expression of symptoms (D'Arcy 1995). Physiologically, the infected host plant experiences necrotic obliteration of the phloem due to infection, followed by an accumulation of a gum-like material disrupting nutrient transport (Jensen and D'Arcy 1995). The earlier the infection occurs in the host plant, the more severe the damage becomes. The disruption in phloem tissues leads to an overall decline in translocation which can be seen through increases in dry weight due to accumulation of sugars, starch, reduction of chlorophyll content, increased respiration and overall decline in photosynthesis (Jensen and D'Arcy 1995).

Vector Biology

BYDV is transmitted exclusively by aphids in a persistent, circulative manner. Insect vectors acquire virus from an infected host plant during phloem ingestion. Virus particles are ingested into the gut, associated with hindgut epithelial cells and transported into the hemolymph via endocytosis. Luteoviruses are recognized by cellular receptors at the hindgut membrane (Gildow 1993). Virus particles are transported out of the hindgut, through the hemocoel and into the accessory salivary glands (ASG). Once the virus has reached the ASG, the insect becomes an infectious vector. The ASG produces a watery secretion that is used to lubricate the stylet sheath during aphid feeding and transports virus into plant tissues. This pathway is efficient for virus transport because the secretions do not contain enzymes which could degrade virus particles (Gray and Banerjee 1999). The vector-virus specificity among *Luteoviridae* occurs at the ASG basal lamina and is regulated by virus-specific capsid-glycoprotein interactions (Gildow and Gray 1993). Non-transmissible luteovirus species can be found within the gut and hemolymph of non-vectoring aphid species, but not in the ASG.

The amount of time required for an insect to feed on an infected plant before acquiring the virus is termed acquisition access period (AAP). This is followed by a latent period during which persistent viruses circulate within the insect body and move into the ASG. The time required for an infectious insect to feed on a healthy plant in order to transmit the virus is termed inoculation access period (IAP). All of these processes vary depending on the pathosystem. In general, younger leaves of infected plants contain higher virus titer since the replicating tissues in the plant are also where viruses are replicating (Foxe and Rochow 1975). Virus titer has been correlated with acquisition and transmission efficiencies of insect vectors (Gray et al. 1991, van den Heuvel et al. 1991, Jiménez-Martínez and Bosque-Pérez 2004). In

older leaves with low virus titer, AAP of 1-2 hours were required to achieve 50% transmission of BYDV to oats by *R. padi* and *S. avenae* (Gray et al. 1991). Jiménez-Martinez and Bosque-Pérez (2004) examined the influence of wheat genotype on *R. padi* transmission of BYDV-PAV with varying AAP and IAP using soft white winter wheat cv. Lambert and two Lambert-derived transgenic lines, 103.1J and 126.02, which express the coat protein (CP) gene from BYDV-PAV, developed at the University of Idaho (Hansen et al. 1998, Jiménez-Martínez et al. 2004a). They found that acquisition from each genotype resulted in a unique pattern of virus transmission by *R. padi*, with AAP spanning 1, 3, 6, 12, 24 and 48 hours. The lowest rates of transmission were observed on transgenic 103.1J, followed by Lambert and then 126.02 (Jiménez-Martínez and Bosque-Pérez 2004). Variable IAPs of 6, 12, 24 and 48 hours did not affect the transmission efficiencies among the three wheat genotypes tested. The combination of variable AAP transmission efficiencies and lower titer of BYDV in the transgenic plants ultimately resulted in *R. padi* being a less efficient vector after acquisition on transgenic wheat and was attributed to the lower titer in combination with aphid host preferences or feeding behaviors (Jiménez-Martínez and Bosque-Pérez 2004).

Impact of Plant Virus Infection on Aphid Vectors

Virus infection changes host plant physiology and appearance, which have been shown to influence the life history and alter the behavior of insect vectors (Bosque-Pérez and Eigenbrode 2011). Chemical and physical factors contribute to insect host plant selection. Changes in these factors, due to plant breeder selection or virus infection, influence the suitability of host plants. Physical factors such as leaf pubescence, which can be selected and bred for, have been shown to increase resistance to *R. padi* among various cultivars of wheat (Roberts and Foster 1983). Insect vector attraction to virus-infected plants has been attributed

to chlorosis resulting from viral infection (Macias and Mink 1969, Ajayi and Dewar 1983, Eckel and Lampert 1996), but other factors could be at play.

Insect vectors feeding on virus-infected host plants exhibit faster growth rates, higher fecundity, greater longevity and enhanced production of alates (Kennedy 1951, Baker 1960, Gildow 1980, Hodgson 1981, Ajayi 1986, Araya and Foster 1987, Jiménez-Martínez et al. 2004a). The relationships between infection and vector performance vary depending on the species involved. For example, *S. avenae* displayed increased fecundity both in the field and the laboratory on BYDV-infected hosts while *Metopolophium dirhodum* showed no difference in fecundity on BYDV-infected compared to virus-free hosts (Ajayi and Dewar 1983). Enhanced vector performance can be found among noncirculative viruses, which include nonpersistent and semipersistent viruses, and circulative non-propagative viruses (Fereres and Moreno 2009).

Jiménez-Martínez et al. (2004a) examined the influence of BYDV infection on *R. padi* life history traits using four different soft white winter wheat genotypes: susceptible Lambert, the Lambert-derived transgenics 103.1J and 126.02 mentioned earlier, and Caldwell which is a virus-tolerant variety. *R. padi* developmental period was significantly shorter for aphids feeding on BYDV-infected Lambert compared to noninfected plants and plants challenged with healthy aphids (aphid-challenged) Lambert, whereas on the transgenic lines aphid development was significantly longer on BYDV-infected plants compared to noninfected and aphid-challenged plants (Jiménez-Martínez et al. 2004a). *R. padi* reproductive period was significantly longer and post-reproductive period was significantly shorter on BYDV-infected Lambert compared to other plant treatments on Lambert plants (Jiménez-Martínez et al. 2004a). Overall, aphid performance was enhanced on BYDV-infected compared to

noninfected and aphid-challenged plants, adding to the support of enhanced vector performance on virus-infected plants (Jiménez-Martínez et al. 2004a).

Host Plant Selection

Plant volatile emissions have been well documented and shown to elicit responses from a variety of trophic levels within an ecosystem. They are also an important mechanism involved in host plant selection by insects. In general plant volatiles have been shown to attract insects to plants that are potential food sources, used in the location of a mate and during oviposition. These chemical signals may be an evolutionary adaptation of the plant if the behavior induced in the insect serves a beneficial role for the overall plant fitness (i.e. pollination) (Szendrei and Rodriguez-Saona 2010). In a meta-analysis of insect behaviors associated with plant volatile signals, differences in the responses of male versus female insects were observed, with females showing higher attraction to plant volatile baits which may be associated with oviposition selection (Szendrei and Rodriguez-Saona 2010). There was also a difference in the response of various feeding guilds of insects, with chewing insects being most attracted, followed by wood-borers and sap feeders (Szendrei and Rodriguez-Saona 2010).

Plant viruses have been shown to influence the volatile profile of infected plants (Eigenbrode et al. 2002, Jiménez-Martínez et al. 2004b, Bosque-Pérez and Eigenbrode 2011). The arrestment, attraction and/or repulsion of insect vectors due to virus-induced changes in plants have been demonstrated in both persistently and nonpersistently-transmitted viruses. *Potato leaf roll virus* (PLRV), is transmitted in a circulative persistent manner, and induces changes in the volatile profile of infected *Solanum* spp. plants. The preference of *M. persicae* for PLRV-infected leaves over non-infected leaves has been attributed to virus-induced

volatile changes (Eigenbrode et al. 2002, Srinivasan et al. 2006, Ngumbi et al. 2007). On potato, the rate of change due to infection and subsequent insect responses have been shown to differ at various stages of PLRV disease progression (Werner et al. 2009, Rajabaskar et al. 2013).

In the BYDV-*R. padi* pathosystem, *R. padi* was shown to be more attracted to BYDV-infected wheat compared to uninfected or sham-inoculated leaves due in part to virus-induced volatiles (Jiménez-Martínez et al. 2004b, Medina Ortega et al. 2009). Jiménez-Martínez et al. (2004b) was the first to report that BYDV-infection in soft white winter wheat alters the volatile profile of these plants and that the transgenic resistance acquired by integrating the CP-gene of BYDV in the genotype 103.1J results in alterations of the volatile profile. In choice-test bioassays eliminating visual and gustatory cues by placing a mesh barrier between the insect and the test plants and performing tests in the dark, significantly more *R. padi* congregated over BYDV-infected compared to noninfected and sham-inoculated (aphid-challenged) Lambert wheat (Jiménez-Martínez et al. 2004b). There were no significant differences among aphid preferences for BYDV-infected, noninfected or sham-inoculated transgenic 103.1J or between noninfected 103.1J versus noninfected Lambert (Jiménez-Martínez et al. 2004b) The same 20 volatile components were detected in the headspace of all Lambert and 103.1J plant treatments, with differences occurring in the relative composition and overall concentrations being produced by plants. Volatiles detected in the headspace of BYDV-infected Lambert were 2.8 and 3.8-fold greater than noninfected and sham-inoculated treatments respectively, and the total concentration of volatiles from BYDV-infected Lambert was significantly greater than all other Lambert and 103.1J treatments (Jiménez-Martínez et al. 2004b). Expanding on this work, Medina-Ortega et al. (2009) were able to conclude

following a series of immigration, emigration and settling bioassays that increased congregation of *R. padi* on BYDV-infected plants is caused by attraction rather than arrestment. In immigration bioassays isolating specific VOC's produced by wheat and using pure compounds applied to paper leaf models, Medina-Ortega et al. (2009) observed significantly greater numbers of nonviruliferous apterae *R. padi* immigrating to paper leaf models treated individually with nonanal, (Z)-3-hexenyl acetate, decanal, caryophyllene or undecane than to paper leaf controls. In similar bioassays with VOC blends applied to paper leaf models, significantly more nonviruliferous apterae *R. padi* immigrated towards blends mimicking BYDV-infected Lambert than towards blends mimicking noninfected Lambert. These results verify the attractiveness of virus-induced volatiles (ViV) produced by wheat as a result of BYDV infection. The previously described work has been done with nonviruliferous apterae *R. padi*. Medina-Ortega (2009) performed the immigration bioassays using viruliferous *R.padi* and found a significantly lower number of aphids responding to the paper leaf model than either plant treatment, but no differences were observed between sham-inoculated and BYDV-infected Lambert or 103.1J. These results suggest that upon virus acquisition, *R. padi* no longer exhibit a host plant preference for BYDV-infected wheat.

Infection of *Cucurbita pepo* cv. Dixie by the nonpersistent virus, *Cucumber mosaic virus* (CMV), which is transmitted by aphids, increases volatile production by plants (Mauck et al. 2010). The vectors *M. persicae* and *A. gossypii* are initially attracted to virus-infected plants likely as a result of volatiles. Dispersion from these plants occurs rapidly, as they are not qualitatively good hosts (Mauck et al. 2010). Under field conditions, plant volatiles along with visual cues are important for long-range host selection by aphids during events such as migration. Current research suggests that under these conditions aphids would be attracted to

virus-infected plants preferentially over neighboring virus-free plants, regardless of the mode of transmission (nonpersistent or persistent).

It is believed to be evolutionarily advantageous for a viral pathogen to increase host plant attractiveness upon infection (Fereres and Moreno 2009, Medina Ortega et al. 2009, Bosque-Pérez and Eigenbrode 2011). This would increase visitation by vector species facilitating virus spread. Epidemiological modeling has shown that an increased preference for virus-infected plants will increase the rate of spread of the virus (Jeger et al. 2004, Sisterson 2008, Thackray et al. 2009, Roosien et al. 2013). In the case of the nonpersistent CMV, it has been hypothesized that volatile attraction in conjunction with gustatory repulsion due to virus infection may be the mechanism at work within this pathosystem (Mauck et al. 2010). This could potentially be an effective strategy for nonpersistent viruses because probing alone can result in acquisition of viral particles on the stylet of the vector and this association is short lived. However, more research is needed to investigate this mechanistic hypothesis.

Work in the BYDV-*R. padi*-wheat pathosystem has shown that nonviruliferous aphids exhibit a preference for BYDV-infected plants (Jiménez-Martínez et al. 2004b, Medina-Ortega et al. 2009) while viruliferous aphids appear to have no preference between infected and noninfected wheat (Medina Ortega et al. 2009). In this pathosystem, initially attracting nonviruliferous aphids to infected plants will increase the infectious vector population. Once acquired, the aphids will remain viruliferous for the duration of their life, transmitting virus to any subsequent host plants they feed on.

In the *Tomato spotted wilt virus* (TSWV)- *Frankliniella occidentalis* (Pergande)-*Datura stramonium* L. pathosystem, TSWV is transmitted by its thrips vector, *F. occidentalis*,

through probing and successful transmission increases with increased feeding (Stafford et al. 2011). Male thrips that had been infected with TSWV increased their feeding behaviors three-fold compared to uninfected thrips, essentially increasing the likelihood of transmitting the virus (Stafford et al. 2011). TSWV is a member of the viral family *Bunyaviridae* which contains many animal-infecting viruses. There are a variety of parasites, including many viruses, which have been shown to affect the behavior of their vectors upon acquisition (Lefèvre and Thomas 2008). Taken together, the current research examining the relationships between plant viruses, vectors, and host plants are consistent with the evolutionary adaptations that have been proposed in such intricate pathosystems.

Ecology and Epidemiology of BYDV Disease

In order to understand the epidemiology of BYDV a detailed understanding of each of the three biotic elements of the system and how they interact is required. The three elements include: the virus complex with its many different serotypes/species and their interactions with one another, the different aphid species that act as vectors of the various viral serotypes/species, and the cereal grains and other grasses that act as hosts of the virus and/or the vectors (Irwin and Thresh 1990). Thus, multiple interactions influence virus dynamics and create a challenging system to predict and manage. In samples collected from infected fields it is often seen that there are multiple infections from various strains present in a particular field, and that this occurs more often when there are severe epidemics. Mixed infections often cause more severe symptoms (Baltenberger et al. 1987, Irwin and Thresh 1990).

The occurrence of viruses in natural ecosystems is important to understand in order to better predict and manage disease outbreaks in agricultural crops. This includes an understanding of source inoculum and naturally-occurring virus hosts. BYDV commonly

occurs in native grasslands (Grafton et al. 1982). A survey of natural grasslands in California in 1990 revealed that 37 of 56 cool-season grass species were infected with BYDV-PAV, MAV or RPV-like strains in relatively equal proportions (Griesbach et al. 1990). Symptomatic infection is most readily observed among *Avena fatua* and *Bromus diandrus* which appears as bright red or purple discoloration on the leaves (Malmstrom 1998).

In California, BYDV has also been shown to play an important role in the competitive dynamics of native and invasive grasses (Malmstrom et al. 2005a, Malmstrom et al. 2005b, Borer et al. 2007). The presence of exotic annuals increases the prevalence of virus infection and is thought to lower the competitive ability of the native grass species (Seabloom et al. 2009). Native perennial grasses dominate in the absence of disease; however when BYDV is present in the system it persists year to year among the perennial grasses and exotic annual grasses which return disease-free each year are able to establish and coexist with the native perennials (Borer et al. 2007). Consequences of additional environmental stressors were exacerbated as a result of virus infection, which has also been seen in agricultural crops suffering BYDV infection and abiotic stress (Irwin and Thresh 1990, Malmstrom et al. 2006).

Natural grasslands may contribute to virus outbreaks in surrounding agricultural fields. Perennial grasses may serve as a reservoir of inoculum for annual species and emerging agricultural crops. Our knowledge and understanding of insect vector movement between agricultural and natural landscapes is limited. Some studies show that viral species do not overlap between these two ecosystems. For example, while PAV is the most commonly reported strain of BYDV occurring in cereal fields in Kansas, a recent survey of the grass species in the Konza prairie found MAV and SGV to be predominant in these natural grasslands (Garrett et al. 2004). In California, there is a large overlap in the virus species

present in agricultural and natural landscapes suggesting vector utilization of both habitats (Malmstrom 1998).

In dry subtropical climates in the Mediterranean region wild grasses and corn play an important role as reservoirs of BYDV (Irwin and Thresh 1990). In the Pacific Northwest of the United States irrigated corn has been implicated as the source of inoculum and reservoir for *R. padi* and *M. avenae* moving between cereal grains during harvest and planting (Brown et al. 1984). *R. maidis* have been shown to be an important vector in this same region transporting BYDV between cereal grains and barnyard grass (Blackman et al. 1990). While progress is being made identifying reservoir sources, there are still a lot of anomalies between the viral strains being found in natural grasses and those causing disease in crops.

Rationale and Significance

This research will aid in the achievement of a long-term goal to improve the management of BYDV, one of the most important disease-causing viruses of cereal crops worldwide. Wheat is one of the mainstay crops in the Palouse and in the USA. Depending on the year, yield losses have exceeded 70% in individual fields in Idaho due to BYDV infection (Bishop and Sandvol 1984). In order to prevent future epidemics in a changing climate and to be able to predict vector and virus dynamics from year to year we need to understand the intricate relationships between each member of the pathosystem. Extensive work has been done and progress made in identifying secondary natural hosts of a variety of BYDV vectors (Irwin and Thresh 1990). Progress has also been made in the development of cereal varieties with some degree of resistance to BYDV (Irwin and Thresh 1990, Maule et al. 2007, Ordon et al. 2009, Zhang et al. 2009). Advances in these aspects of the pathosystem have enabled the development of epidemiological models that help make predictions of epidemics from year to

year in various regions (Leclercq-Le Quillec et al. 2000, Sisterson 2008, Thackray et al. 2009). Lacking is the knowledge of the specific behavioral traits of vectors that drive and sustain epidemics, specifically including vector movement between infected and noninfected host plants (Irwin and Thresh 1990). A better understanding of the behavior of BYDV vectors and the mechanisms mediating this behavior could provide new strategies to manage the disease in our region and globally.

Research Objectives

1. To compare the behavioral responses of viruliferous and nonviruliferous *R. padi* to BYDV-infected and noninfected plants.
2. To assess the invasive grass *Ventenata dubia* as a potential host and source of BYDV inoculum.
3. To examine Palouse Prairie remnants and Conservation Reserve Program (CRP) sites for the presence of B/CYDV and assess if seasonal variation exists in virus presence.
4. To determine the potential of grass species in Idaho prairie and CRP sites that have not been documented as hosts of BYDV to harbor the virus.
5. To evaluate the potential of *Arundo donax*, an exotic species proposed as a biofuel crop, to serve as a host of BYDV.

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Chapter 2: Plant viruses alter insect behavior to enhance their spread

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Abstract

Pathogens and parasites can induce changes in host or vector behaviors that enhance their transmission. In plant systems, such effects are largely restricted to vectors, because they are mobile and may exhibit preferences dependent upon plant host infection status. Here we report the first evidence that acquisition of a plant virus directly alters host selection behavior by its insect vector. We show that the aphid *Rhopalosiphum padi*, after acquiring *Barley yellow dwarf virus* (BYDV) during *in vitro* feeding, prefers noninfected wheat plants, while noninfective aphids also fed *in vitro* prefer BYDV-infected plants. This behavioral change should promote pathogen spread since noninfective vector preference for infected plants will promote acquisition, while infective vector preference for noninfected hosts will promote transmission. We propose the "Vector Manipulation Hypothesis" to explain the evolution of strategies in plant pathogens to enhance their spread to new hosts. Our findings have implications for disease and vector management.

Introduction

Pathogenic and parasitic organisms interact with their hosts on a variety of cellular and organismal levels that potentially cause changes in host behavior leading to enhanced

transmission[1-5]. This phenomenon led to the emergence of the "Host Manipulation Hypothesis" (HMH)[6]. The HMH and its synonyms the adaptive manipulation[7] and behavioral manipulation[8] hypotheses posit that natural selection on the parasite or pathogen has favored the capacity to elicit host behavior that enhances their transmission. Although examination of the HMH has progressed from descriptive studies to investigations of the mechanisms through which parasites affect host behavior and their consequences for parasite spread[9,10], the field remains predominantly focused on animal pathosystems.

Pathogens or parasites can influence the behavior not only of their primary hosts, but also of their vectors. Arthropods are important vectors of both animal and plant pathogens, transmitting thousands of species of pathogens, including viruses, bacteria, phytoplasmas, trypanosomes and Plasmodia[2,11]. The effects of pathogens on vector biology and behavior have been documented in several pathosystems, including those associated with important human diseases such as malaria, leishmaniasis and sleeping sickness[2,5]. The observed changes in vector behavior include those related to pathogen transmission. For example, mosquitoes infected with the malaria parasite exhibit increased biting frequency and increased attraction to humans infected with the gametocytes of the parasite compared to noninfected humans[1,3].

In contrast to animal pathosystems, plant pathosystems have been less well studied for evidence of host or vector manipulation by pathogens[12]. While animal pathogens can alter the behavior of both hosts and vectors in ways that increase frequency of host-host or host-vector encounters[2,4,5], in plant pathosystems the host is sessile, so the potential for behavioral manipulation is restricted to the vector, the mobile component in these systems. Furthermore, unlike animal pathogens most plant pathogens, including the majority of plant viruses, do not replicate within the vector, so these vectors are not pathogen hosts, *sensu stricto*.

We previously demonstrated that *Barley yellow dwarf virus* (BYDV) infecting wheat and *Potato leafroll virus* (PLRV) infecting potato indirectly induce changes in the host selection behavior of their respective principal aphid vectors, *Rhopalosiphum padi* and *Myzus persicae*[13-16]. We also have shown that plants infected with these viruses have altered volatile organic compound profiles that elicit greater settling of or arrestment by their noninfective vectors[13,14,16,17]. Luteoviruses (viruses in the family Luteoviridae), including BYDV and PLRV are persistently transmitted. They are ingested and pass through the midgut or hindgut into the hemocoel, eventually associating with the accessory salivary glands of the vector[18]. These viruses rely almost exclusively on insect vectors for transmission and require sustained feeding by a vector for their successful acquisition and transmission[19]. After acquisition, the insect remains a vector for life. Although they do not replicate within the vector, persistently-transmitted viruses interact with the vector at the cellular level during movement among tissues and organs[20], with the potential to directly alter vector physiology and behavior.

Preferential settling by vectors onto infected plants, as occurs for BYDV and PLRV, could contribute to enhanced pathogen spread. Models indicate that a preference for infected plants will accelerate pathogen spread, but only when infected plants are rare, not when they are prevalent in a plant population[21]. However, conditional vector preference could enhance pathogen spread regardless of the prevalence of infected plants. Specifically, if noninfective vectors prefer infected plants thereby promoting acquisition, and infective vectors prefer noninfected hosts promoting transmission, overall spread would be accelerated. The possibility of conditional vector preference for pathogen-infected plants has hardly been examined despite its potential importance. Changes in vector behavior that occur after feeding on virus-infected plants could be attributed to direct effects of the acquired virus on the vector, but such direct

effects are difficult to distinguish from indirect ones associated with feeding on virus-infected plants. Here we test the hypothesis that a change in host plant selection behavior by an insect vector is the direct result of virus acquisition by the vector. We provide the first experimental evidence that acquisition of a plant virus through *in vitro* feeding, which eliminates indirect effects of an infected plant host, directly alters subsequent host plant selection behavior of its vector. These findings enhance our understanding of how plant viruses spread to new hosts, with implications for disease and vector management.

Results

We first examined host plant selection preferences of infective (reared on virus-infected plants) and noninfective (reared on virus-free plants) *R. padi*. In dual-choice bioassays using an arena in a platform[22] (Fig. 2.1) infective or noninfective insects were allowed to select BYDV-infected or sham-inoculated wheat plants as their hosts. Sham-inoculated plants are noninfected plants previously fed upon by noninfective aphids and are utilized in our bioassays to account for potential aphid feeding-induced changes in plants[23]. Infective and noninfective insects were tested simultaneously in separate platforms. Each platform contained a leaf from each plant treatment, BYDV-infected or sham-inoculated, onto which aphids could settle and feed throughout the bioassay. We compared the responses of infective and noninfective aphids by examining the proportion of aphids that settled on BYDV-infected or sham-inoculated plants every 12 h for 72 h. A 72 h time period is sufficiently long for virus acquisition by noninfective aphids to occur when exposed to BYDV-infected plants, while a 12-hour time period is unlikely to result in noninfective aphids become infective[24,25]. We therefore compared aphid responses at the first 12-h observation, and after 72 h when responses were pooled over time. The 12-h observation occurs before additional virus acquisition was expected while the 72-h comparison is

more powerful statistically and incorporates a time period more meaningful for transmission dynamics in the field. Noninfective aphids significantly preferred to settle on BYDV-infected wheat compared to infective aphids at the first 12-h observation point (generalized linear model; $\chi^2 = 3.12$, $p = 0.0774$, marginally significant) (Fig. 2.2a, Supplementary Table S1a) and throughout the duration of the experiment (generalized linear model; $\chi^2 = 19.33$, $p < 0.0001$) (Fig. 2.2b, Supplementary Table S2a). In contrast, infective aphids significantly preferred to settle on sham-inoculated wheat compared to noninfective aphids at the first observation point (generalized linear model; $\chi^2 = 3.12$, $p = 0.0774$, marginally significant) (Fig. 2.2a, Supplementary Table S1a) as well as throughout the duration of the experiment (generalized linear model; $\chi^2 = 20.14$, $p < 0.0001$) (Fig. 2.2b, Supplementary Table S2b). The time at which the observations were made was not a significant factor when examining the response to BYDV-infected wheat (generalized linear model; $\chi^2 = 4.96$, $p = 0.4203$) (Supplementary Table S2a) or sham-inoculated wheat (generalized linear model; $\chi^2 = 2.15$, $p = 0.8282$) (Supplementary Table S2b). The results suggest that virus acquisition changes vector host plant selection behavior to favor noninfected plants rather than infected plants.

These behavioral changes could result either from direct effects of acquired virus particles on the aphid, or from insect exposure to cues from virus-infected host plants. To isolate potential direct effects of virus acquisition on the vector we conducted a similar experiment using *in vitro* feeding to obtain infective and noninfective aphids. Insects were first reared on virus-free plants and subsequently transferred to membrane feeding chambers[26] (Fig. 2.3) that contained artificial phloem with either purified BYDV particles or no virus. Host plant selection preferences of infective and noninfective insects were examined every 12 h for 72 h using an arena as described above. Observation time was not a significant factor when examining the response to BYDV-

infected wheat (generalized linear model; $\chi^2 = 2.41$, $p = 0.7906$) (Supplementary Table S2c) or sham-inoculated wheat (generalized linear model; $\chi^2 = 3.66$, $p = 0.5995$) (Supplementary Table S2d). We present the results of the aphid responses at the first 12-h observation point as well as the responses pooled over time. Noninfective aphids significantly preferred BYDV-infected wheat compared to infective aphids at the first observation point (generalized linear model; $\chi^2 = 4.24$, $p = 0.0394$) (Fig. 2.4a, Supplementary Table S1c), as well as throughout the duration of the experiment (generalized linear model; $\chi^2 = 16.18$, $p < 0.0001$) (Fig. 2.4b, Supplementary Table S2c). Similar to the patterns obtained using aphids that acquired virus from plants, infective aphids significantly preferred sham-inoculated wheat compared to noninfective aphids at the first observation point (generalized linear model; $\chi^2 = 5.64$, $p = 0.0176$) (Fig. 2.4a, Supplementary Table S1d), as well as throughout the duration of the experiment (generalized linear model; $\chi^2 = 16.32$, $p < 0.0001$) (Fig. 2.4b, Supplementary Table S2d).

Results from RT-PCR tests verified that our inoculation and acquisition methods were successful (See Supplementary Figures S1-S2). All plants used in the dual choice tests were tested via RT-PCR immediately after the bioassays. Sham-inoculated plants remained virus-free and infected plants remained BYDV-infected, indicating that during the bioassays (72 h) the plant treatments were stable, despite being exposed to potential feeding by infective aphids. Tests of aphids using RT-PCR revealed that infective aphids remained BYDV-infective subsequent to the bioassay, while 25% of noninfective aphids acquired BYDV during the 72-h bioassay when they has access to BYDV-infected plants in the bioassay arena. Although the bioassay design unavoidably results in virus acquisition by some noninfective aphids, the result is a more conservative test of our hypothesis since within-bioassay virus acquisition should to diminish detectable differences between the aphid treatments. Furthermore, the aphid responses

after 72 h in the bioassay arena are consistent with the preferences observed after 12 h, during which time noninfective aphids almost certainly remained noninfective[24,25] . The lack of BYDV infection of the sham-inoculated plants after 72 h of exposure to initially noninfective aphids in an arena with BYDV-infected plants also indicates that these aphids did not become infective during the bioassay.

Discussion

Assays utilizing membrane-fed infective aphids yielded results similar to those obtained using aphids that acquired BYDV from infected plants, confirming our hypothesis that changes in host plant selection by the vector are mediated by direct effects of virus acquisition, rather than indirect effects of feeding on infected host plants. Direct effects of virus acquisition on the vector host plant selection behavior in a manner that will promote the spread of the virus is consistent with an evolved strategy in the pathogen of manipulation of its vector. We propose the "Vector Manipulation Hypothesis" (VMH) to explain the evolution of strategies in plant pathogens that enhance their spread to new hosts through their effects on vectors. Selection should favor both direct and indirect mechanisms producing such effects. Vectors that feed on virus-infected host plants exhibit faster growth rates, higher fecundity, greater longevity and/or enhanced production of alate forms of the vector[27-33], which can lead to increased virus spread and are typically attributed to indirect effects of infections on host quality. Virus infection of the host plant's secondary chemistry also can affect vector behavior. Evidence for such indirect effects of pathogens on vector behavior continues to accumulate and is consistent with the VMH [13-16,34-36]. We provide the first evidence for a direct effect of a plant virus on its vector consistent with VMH, specifically by influencing the vector's host selection behavior to maximize pathogen spread. In our model pathosystem, noninfective vectors are attracted to virus-infected

host plants, which is beneficial as it increases vector fitness[23]. After virus acquisition virus vector preferences shift to noninfected hosts, maximizing pathogen transmission potential by promoting the movement of infective aphids onto noninfected host plants. Our results offer a specific example of a plant virus directly manipulating its vector in a manner that is likely to maximize pathogen transmission potential between hosts, providing support for the VMH.

Results supportive of the VMH also have been reported from work on nonpersistently-transmitted plant viruses examining effects on noninfective vector behavior. Nonpersistently transmitted viruses bind transiently to insect mouthparts[20] and interactions in these pathosystems are likely limited to indirect effects on vectors. Recent work with the non-persistently transmitted *Cucumber mosaic virus* (CMV), which is acquired rapidly during aphid feeding and benefits from rapid vector dispersal, showed that aphids are initially attracted to volatile organic compounds from CMV-infected squash plants, but subsequently prefer to colonize noninfected plants[34]. Attraction to CMV-infected plants appears to be mediated by their increased emission of volatile organic compounds similar to those from healthy plants. Since CMV can be acquired within a few seconds by an aphid probing on an infected plant, these behaviors can act to enhance virus spread[34] and illustrate manipulation of an insect vector by the virus. Interestingly, in addition to manipulating vectors, CMV also may manipulate defensive signaling pathways in plants that could result in enhanced vector survival[37].

Our findings highlight the ecological and evolutionary significance of vector manipulation by pathogens and parasites. Effects like those we document for a plant virus, consistent with the VMH, may be widespread since direct and indirect mechanisms that enhance the spread of plant viruses should be favored by natural selection. Furthermore, similar patterns in behavioral changes among vectors of other plant pathogens, such as bacteria and phytoplasmas, which are

limited to sessile plant hosts, might also occur. Although our results do not address the specific cellular and molecular mechanisms mediating direct plant virus effects on their vectors, they offer strong quantitative evidence for the VMH, providing a foundation upon which to base further studies of pathogen-mediated manipulation of their vectors and the identification of underlying mechanisms. The evolution of host-vector interactions has recently been suggested to be in part, mediated by virus transmission mechanisms[38] underlying the importance of studying such interactions. Greater understanding of host plant-virus-vector interactions has the potential to improve management of vectors and plant diseases in agricultural settings and enhance our understanding of the role plant viruses play in natural settings[39], including their effects on ecological processes at the community and ecosystem levels[38].

Methods

Virus maintenance and insect rearing. The model system for our study was the wheat-*R. padi*-BYDV pathosystem. BYDV is exclusively transmitted among *Poaceae* hosts by aphids, including *R. padi*, in a persistent circulative manner and the virus does not replicate within the vector[40,41]. A Washington State isolate of BYDV-PAV maintained by mass transfer of *R. padi*, the bird-cherry oat aphid, on cv. Sprinter barley plants was used to inoculate wheat plants[23]. *Rophalosiphum padi* is the most efficient vector of the BYDV-PAV serotype[40]. Both the virus and an infective colony of *R. padi* are maintained at the University of Idaho (UI) Agricultural Biotechnology Laboratory. Aphids were originally obtained from Washington State University and are kept virus-infective through serial transfer[23]. A noninfective colony of *R. padi* was derived from the infective colony and is maintained at the UI Manis Entomological Laboratory. Infective and noninfective aphid colonies are reared on Sprinter barley in environmental chambers (20±2 °C; 16 h light photoperiod). Aphids from each colony are examined on a regular

basis using RT-PCR tests to ensure that the respective colonies remain virus-free or BYDV-PAV infected (see Supplementary Methods).

Plant rearing and inoculation. Winter wheat cv. Lambert was used for all bioassays. Seeds were planted at a density of one per pot in 10.2 cm^2 plastic pots. Pots were filled with a mixture of 6:1:0.02 ratio of Sunshine mix #1: sand: Osmocote®, placed on trays in an environmental chamber (20±2 °C; 16 h light photoperiod) and bottom watered. After germination, plants were fertilized using a soluble N-P-K fertilizer (15:30:15) biweekly.

Plant inoculations were done at the 2-3 leaf stage (14-16 days after planting). BYDV-infected plants were obtained by caging 10 adult aphids from the infective colony per plant for a 72 h virus inoculation access period[23]. Cages consisted of a 4-cm long piece of 23 mm dialysis tubing (14.6 mm D, Spectra/Por®) sealed on both ends with a foam stopper. Since BYDV is exclusively insect-transmitted, all BYDV-infected plants are fed-upon by aphids. Insect feeding may induce resistance in plants and potentially affect the response of insects subsequently exposed to such plants[23]. To account for such potential confounding effects sham-inoculated plants were produced and served as virus-free controls. Sham-inoculation was conducted by caging 10 adult aphids from the noninfective colony per plant for 72 h[23].

Infective and noninfective aphid handling. To examine effects of plant virus acquisition from infected plants on host selection behavior, apterous aphids (fourth nymphal stage to early adults) originated from the respective infective and noninfective colonies. Previous research in this pathosystem has focused on aptera[14,15,23]. While alates are important vectors for long-distance dispersal events, apterous aphid behavior can be used to predict severity of epidemics within a field once the virus and vector are established[21]. Future studies will examine alate behavior in response to BYDV-infection. Aphids for each treatment were individually removed from plants

using a number 3 camel's hair brush and placed into vials (2.3x5.5 cm; DxH). Fifty aphids were placed per vial. Vials were capped and aphids starved for one hour prior to the bioassay. A total of 600 aphids of each treatment (infective and noninfective) were tested among 12 replicates of the dual-choice bioassay described below.

Aphids for the experiment to assess the direct effects of virus acquisition originated from the noninfective *R. padi* colony. Tests were conducted using membrane feeding chambers modified after Trębicki et al.[26] (Fig. 2.3), containing artificial diet as described by Ramsey and Jander[42]. After preparation the diet solution was sieved using a bacteria-proof filter (0.2 µm cellulose acetate). To set up membrane feeding chambers, the bottom halves of glass petri dishes (5.5 cm; D) were first sterilized under UV light for 10 min. Aphids (fourth nymphal stage to early adults) were collected from colony plants using a number 3 camel's hair brush and placed in the petri dishes, 35 aphids per dish. Parafilm®, sterilized with 70% ethanol, was used as membrane material. After placing the aphids in the petri dish, the dish was immediately sealed with a layer of Parafilm® stretched tightly across the dish top. After all dishes were sealed with the first Parafilm® layer 100 µL of artificial diet was pipetted onto the membrane and a second layer of Parafilm® was stretched tightly to sandwich the diet[26]. The diet was then spread across the surface of the Parafilm® membrane by applying pressure to the top layer with a fingertip. Dishes were placed in a tray with moistened filter paper and the tray covered with cling wrap and placed inside an environmental chamber (20±2 °C; 16 h light photoperiod) for 24 h. Noninfective aphids were fed on an amino acid and sucrose diet solution. To obtain infective aphids, insects were fed on the same diet solution that was infused with purified BYDV at a concentration of 100 µg/mL. Virus was purified following a method adapted from Hammond et al.[43], and obtained from Dr. Alex Karasev, UI PSES Department. After a 24-h feeding period, aphids were

transferred into a vial as described above, starved for one hour and released in a bioassay arena (see below). A total of 840 aphids of each treatment were placed in membrane chambers, 600 were tested among 12 replicates of the dual-choice bioassay described below. The remaining aphids were stored in 70% ethanol at -20 °C to verify their status (infective vs. noninfective). Virus-infection status of plants and aphids was determined using RT-PCR (See Supplementary Methods and Supplementary Figures S1-S2).

We recognize that purified virus may contain phloem proteins. Such proteins have been reported to occur *in vivo*, and were recently reported to play a role in virus transmission[44]. Additional studies are required to determine if a virus-plant protein complex is present *in vitro* and if such a complex could contribute to behavioral changes in vectors.

Bioassays to assess aphid preferences. Dual-choice bioassays were performed 40-46 days after plant inoculation, utilizing an arena adapted from Castle et al.[22] (Fig. 2.1). The base of the arena was glued into the lid portion of a 15 cm D petri dish. The platform of the arena consisted of the inverted bottom of the petri dish with a 2.5 cm D hole cut in the center. A clear plastic tube (16x2.5 cm; LxD) was inserted into the bottom of the dish and secured with glue. The arena was wrapped in a heavy weight mylar frame (30.5x46.1 cm; WxL) to add stability to the structure. Holes were cut in the mylar, four (2 cm; D) equally spaced around the top of the platform and two (8x8 cm^2) in the bottom to access the arena. One leaf still attached to the plant from each treatment (BYDV-infected and sham-inoculated) was inserted through holes on either side of the arena and held in place with a cotton seal. A vial (5.5x2.5 cm; LxD) containing 50 aphids, starved for one hour, was inserted into the bottom of the plastic tube leading to the arena. Apterous infective and noninfective aphids were released simultaneously into separate arenas. Aphids crawled up the tube and emerged onto a platform with one leaf from each treatment on

either side (3 cm on either side of where aphids entered the arena). Aphids were able to settle on, feed and move between the two leaves. Aphids were released at the start of a dark period and monitored every 12 h (alternating dark and light times) for a 72-h period. The number of aphids on each leaf was counted at each observation, using a red light when monitoring during the dark cycles[14]. Assays were conducted in a growth room (14±3 °C; 12h light photoperiod). One replicate consisted of an arena containing infective aphids paired with another arena containing noninfective aphids, constituting a single block. Twelve replicates were performed across time in a randomized complete block repeated measures design.

Data analysis. The proportion of aphids responding to either the BYDV-infected or sham-inoculated plant treatment was compared using a generalized linear model assuming a binomial distribution and logit transformation (SAS, Proc Genmod). Logit transformation was performed to stabilize the variance and meet the assumptions of normality for the analysis. Aphids not located on either plant leaf in an arena were considered nonresponsive and excluded from the analysis. The partial model examined the main effects of replicate (block; n = 12) and aphid treatment (infective or noninfective). The analysis was conducted separately four times, once for each plant treatment (BYDV-infected or sham-inoculated) for the indirect effects experiment (aphids reared on noninfected plants or virus-infected plants) (Supplemental Table S1a-b) and the direct effects experiment (aphids fed on membrane chambers with or without virus) (Supplemental Table S1c-d). The full model examined the main effects of replicate, aphid treatment and time (n = 6) assuming a compound symmetric correlation. The time variable examined observations made at 12, 24, 36, 48, 60 and 72 h after release using a repeated measures design. Observations made at 12, 36, and 60 h were recorded in the dark. Light and dark observations were examined with the model separately and no significant interactions were

observed, thus results were pooled in the overall analysis (Supplemental Table S2). All statistical tests (likelihood ratio χ^2) were carried out at the alpha = 0.05 level of significance.

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Author contributions

N.B.P., L.L.I., and S.D.E. conceived and designed research; L.L.I. performed research and analyzed data; L.L.I., N.B.P., and S.D.E. interpreted results and wrote the paper.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

Figure Legends

Figure 2.1 Diagrammatic illustration of the dual-choice bioassay arena used in experiments. Adapted from Castle et al.[22].1, BYDV-infected wheat; 2, sham-inoculated wheat; 3, vial (5.5 x2.5 cm; Lx D) initially containing 50 aphids; 4, tube (16x2.5 cm; LxD); 5, platform (15 cm; D); 6, lid enclosing the arena.

Figure 2.2 Mean proportion of infective and noninfective aphids responding in a dual-choice bioassay examining host plant selection preferences to BYDV-infected and sham-inoculated wheat (noninfected plants previously fed upon by noninfective aphids) as influenced by indirect

effects of feeding on virus-infected plants. Each replicate (n = 12) consisted of one arena with noninfective aphids paired with one arena of infective aphids, randomized in a complete block design over time. Statistical analyses compared the response of infective and noninfective aphids to the BYDV-infected or sham-inoculated plant treatment. (a) Aphid responses at the first observation point made 12 h after release. Noninfective aphids preferred BYDV-infected wheat compared to infective aphids (generalized linear model; $\chi^2 = 3.12$, $p = 0.0774$, marginally significant). Infective aphids preferred sham-inoculated plants compared to noninfective aphids (generalized linear model; $\chi^2 = 3.12$, $p = 0.0774$, marginally significant). (b) Aphid responses pooled over time (6 observations). Noninfective aphids significantly preferred BYDV-infected wheat compared to infective aphids (generalized linear model; $\chi^2 = 19.33$, $p < 0.0001$). Infective aphids significantly preferred sham-inoculated plants compared to noninfective aphids (generalized linear model; $\chi^2 = 20.14$, $p < 0.0001$). Data are means ± SE following logit transformation. Errors bars are s.e.m.

Figure 2.3 Diagrammatic illustration of a membrane feeding chamber. 1, artificial diet solution (100 µL); 2, upper layer of Parafilm®; 3, bottom layer of Parafilm®; 4, humid chamber; 5, petri dish (5.5 cm; D); 6, moist filter paper.

Figure 2.4 Mean proportion of infective and noninfective aphids responding in a dual-choice bioassay examining host plant selection preferences to BYDV-infected and sham-inoculated wheat plants as influenced by direct effects of virus acquisition following membrane feeding. Each replicate (n = 12) consisted of one arena with noninfective aphids paired with one arena of infective aphids, randomized in a complete block design over time. Statistical analyses compared

the response of infective and noninfective aphids to the BYDV-infected or sham-inoculated plant treatment. (a) Aphid responses at the first observation point made 12 h after release. Noninfective aphids significantly preferred BYDV-infected wheat compared to infective aphids (generalized linear model; $\chi^2 = 4.24$, $p = 0.0394$). Infective aphids significantly preferred sham-inoculated wheat compared to noninfective aphids (generalized linear model; $\chi^2 = 5.64$, $p = 0.0176$). (b) Aphid responses pooled over time (6 observations). Noninfective aphids significantly preferred BYDV-infected wheat compared to infective aphids (generalized linear model; $\chi^2 = 16.18$, $p < 0.0001$). Infective aphids significantly preferred sham-inoculated wheat compared to noninfective aphids (generalized linear model; $\chi^2 = 16.32$, $p < 0.0001$). Data are means ± SE following logit transformation. Errors bars are s.e.m.

Figures

Fig. 2.1

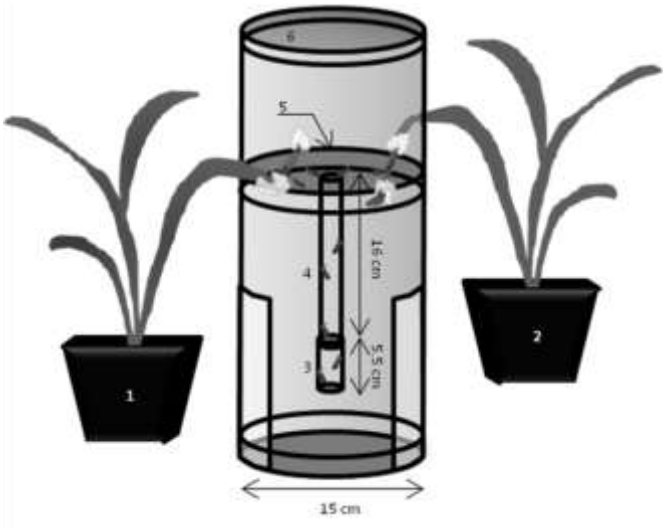
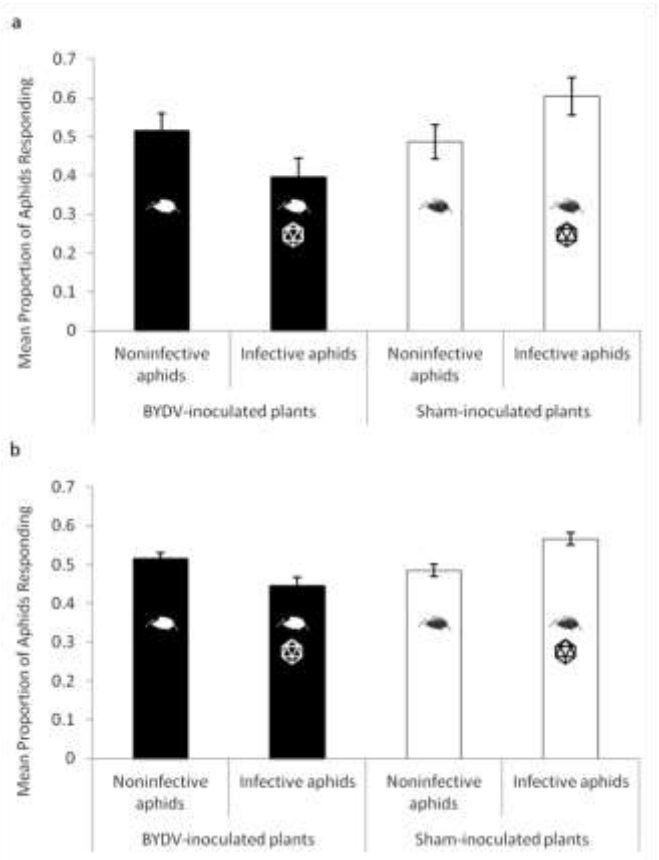


Fig. 2.2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Infective aphids
Noninfective aphids
Infective aphids
BYDV-inoculated plants
Sham-inoculated plants
b
Mean Proportion of Aphids Responding
0.7
0.6
0.5
0.4
0.3
0.2
0.1
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Noninfective aphids
Infective aphids
Noninfective aphids
Infective aphids
BYDV-inoculated plants
Sham-inoculated plants

Fig. 2.3

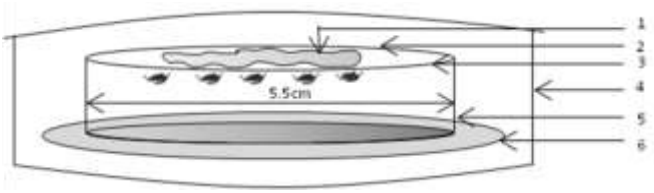
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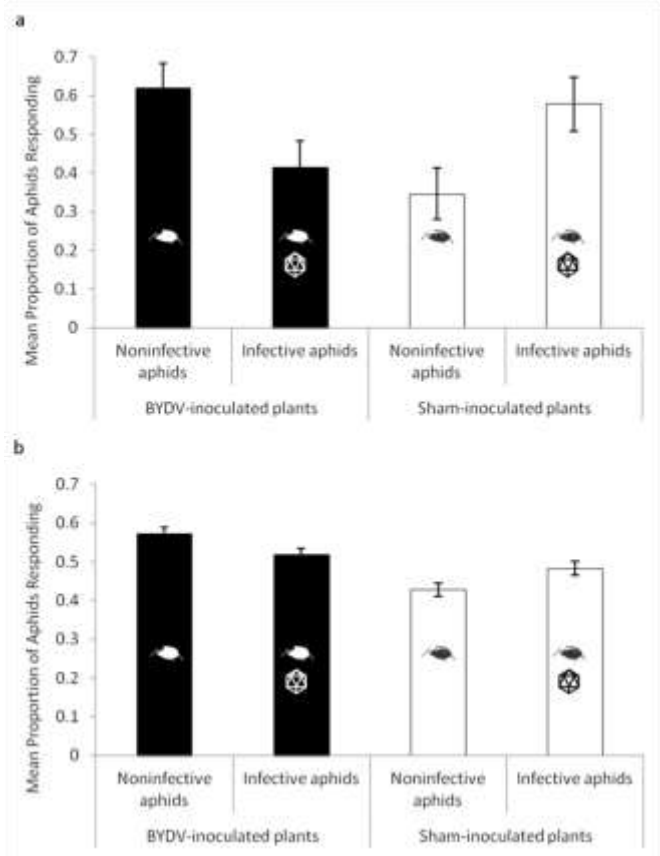
Fig. 2.4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Noninfective aphids
Infective aphids
BYDV-inoculated plants
Sham-inoculated plants
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Mean Proportion of Aphids Responding
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Noninfective aphids
Infective aphids
Noninfective aphids
Infective aphids
BYDV-inoculated plants
Sham-inoculated plants

Supplementary Methods

Determination of virus infection status of plants and aphids. Virus-infection status of plants and aphids was determined using a reverse transcription PCR procedure.

RNA extraction. An adapted Dellaporta nucleic acid extraction method[42] was used to extract total RNA from plants used in the bioassays and samples of aphids from the membrane feeding dishes. For each plant sample, approximately 0.03-0.05 g of tissue were processed. Aphids were individually processed. Samples were placed in a 1.5 mL tube and ground in 400 µL of Dellaporta I extraction buffer (containing 1 mL of 100 mM Tris, pH 8.0, 1 mL of 50 mM EDTA, 1.25 mL 500 mM NaCl, 10 µL β-mercaptoethanol, and 6.75 mL of DEPC water). After grinding, 52.8 µL of 10% SDS was added to each sample, vortexed and incubated at 18 °C for 10 min. After incubation, 128 µL of 5M potassium acetate solution was added to each sample, vortexed and centrifuged at 12000 rpm for 10 min. The supernatant from each tube was removed to a fresh tube and centrifuged for another 10 min at 12000 rpm. The supernatant was transferred to a new tube and 240 µL of cold isopropanol was added. Samples were inverted and held on ice for one hour to allow the RNA precipitation. After one hour, samples were centrifuged for 20 min at 12000 rpm and 10 °C. The supernatant was discarded, 800 µL of cold 70% ethanol added, and centrifuged again for 10 min at 12000 rpm and 10 °C. The supernatant was discarded and the pellet air dried overnight. The RNA was then re-suspended in 80 µL DEPC treated water.

Reverse transcription (RT). The reverse transcription reaction used 2.4 µL of RNA extract from either the insect or plant samples. The RNA was denatured on a Multigene Labnet thermal cycler at 70 °C for 5 min. To each reaction, 6 µL of 5X RNA extraction buffer and 0.6 µL of

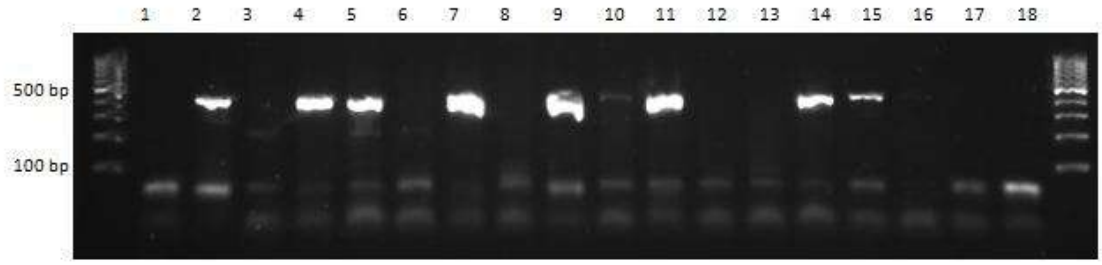
SuperScript II RNase H^- Reverse Transcriptase (Invitrogen Life Technologies, Frederick, MD, USA) was added, along with 12 µL of 2.5 mM dNTPs, 3 µM random hexamer primers, 0.3 µL RNAse Out and 7.5 µL of ddH_2O for a total reaction volume of 30 µL. The samples were then returned to the thermal cycler and ran on an RT-ST program for 90 min. The program was set up as follows: 25°C for 2 min, then increasing the temperature 1°C every 30 s up to 42 °C, hold at 42 °C for 45 min, then increasing the temperature 1 °C every 2 min up to 70 °C, finally holding the samples at 70 °C for 10 min.

Polymerase Chain Reaction (PCR). The total reaction volume was 20 µL. Each PCR reaction included 2 µL of RT product, 2 µL 10X PCR buffer and 0.30 µL My taq (New England Biolabs Inc., Ispwich, MA, USA), 2 µL 2.5mM dNTPs, 1.6 µL of 5 µM forward primer (5'-ATG AAT TCA GTA GGY CGT AGA-3'), 1.6 µL of 5 µM reverse primer (5'-CCC ARG GCT GAT TGC TTG CA-3') and 10.50 µL ddH_20. The primers are designed to produce a band at 411 bp indicating the presence of BYDV-PAV. The samples were amplified on the Labnet Thermal Cycler with the following PCR conditions: 95 °C for 2 min, followed by 11 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 2 min 30 s, followed by 22 cycles of 95 °C for 30 s, 52 °C for 30 s and 72 °C for 2 min 30 s concluded with a hold at 72 °C for 7 min.

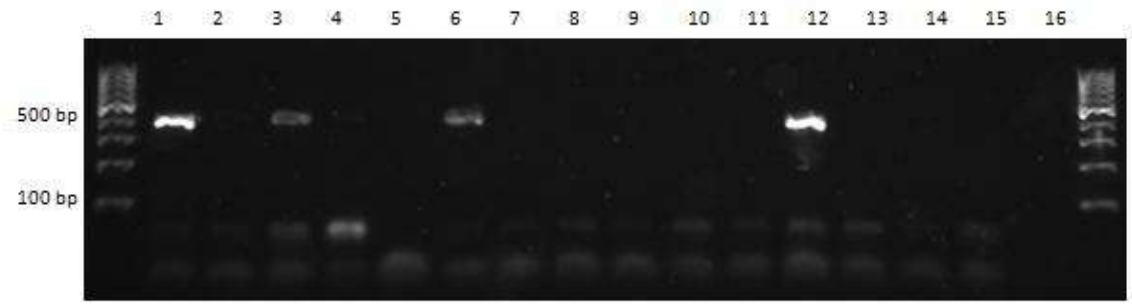
Analysis of amplified product. PCR products were analyzed using gel electrophoresis on a 1.2% agarose gel using GelStar^{TM} nucleic acid gel stain (Lonza Group Ltd, Basel, Switzerland) in a 1X TBE buffer. The products were visualized under UV illumination using AlphaEase FC Software (Alpha Innotech Corporation, Santa Clara, CA, USA). Fragmented sizes were determined by comparison with a 100 bp DNA ladder (Fermentas Life Sciences, Glen Burnie,

MD, USA). Results demonstrated the presence of BYDV in all of the inoculated plants, and no presence of BYDV in sham-inoculated plants (Fig. S1). Samples of aphids obtained from membrane chambers, showed that aphids fed on membranes with diet containing BYDV successfully acquired the virus and that aphids fed only on the amino acid-sucrose solution did not contain BYDV (Fig. S2).

Supplementary figures and captions



Supplemental Figure S1. Agarose gel analysis of a subset of the plants used in the dual-choice bioassays. Lanes 2, 4, 5, 7, 9, 11 and 14 are from samples of plants inoculated with infective aphids showing the 411-bp BYDV-PAV band indicating successful virus inoculation. Lanes 1, 3, 6, 8, 10, 12 and 13 are from samples of plants inoculated with noninfective aphids, representing sham-inoculated plants. Lane 15 is a positive control, using plant tissue from the infective aphid colony. Lane 16 is a negative control, using plant tissue from the noninfective aphid colony. Lanes 17-18 are negative controls from the RT and PCR reactions.



Supplemental Figure S2. Agarose gel analysis of a subset of the individual aphids from membrane feeding assays. Lanes 1, 3 and 6 are from aphids fed on the membrane with amino acid and sucrose solution diet infused with purified BYDV, showing the 411-bp BYDV-PAV band indicating successful virus acquisition. Lanes 2, 4, 5, and 7 to 11 are from noninfective aphids fed on the membrane with amino acid and sucrose solution diet. Lane 12 is a positive control (an infective aphid from the virus-infected colony) showing the 411-bp BYDV-PAV band. Lane 13 is a negative control (a noninfective aphid from the noninfected aphid colony). Lanes 14 and15 are negative controls from the RT and PCR reactions. Lane 16 was left empty.

Supplementary Tables

Supplemental Table S1: Output from the model examining the effects of virus acquisition on host plant selection behavior by aphid vectors at the first observation point, 12 h after aphid release. (PROC GENMOD, binomial distribution, logit link transformation, assuming compound symmetry). Panel A displays indirect effects of virus acquisition and responses to the BYDV-infected plant treatment. Panel B displays indirect effects of virus acquisition and responses to the sham-inoculated plant treatment. Panel C displays direct effects of virus acquisition and responses to the BYDV-infected plant treatment. Panel D displays direct effects of virus acquisition and responses to the sham-inoculated plant treatment.

Score Statistics for Type 3 GEE Analysis

A

Source	DF	Chi-Square	Pr > ChiSq
Rep	11	18.41	0.0726
Aphid	1	3.12	0.0774

B

Source	DF	Chi-Square	Pr > ChiSq
Rep	11	18.41	0.0726
Aphid	1	3.12	0.0774

C

Source	DF	Chi-Square	Pr > ChiSq
Rep	11	20.66	0.0371
Aphid	1	4.24	0.0394

D

Source	DF	Chi-Square	Pr > ChiSq
Rep	11	22.75	0.0192
Aphid	1	5.64	0.0176

The aphid responses at the first observation point in the bioassay reflect the overall response of the insect treatments. The aphid responses are significant (marginally in panel A and B), indicating a difference in host plant preference between infective and noninfective aphids as early as 12 h after release. The replicate factor is significant (marginally in panel A and B), indicating some variation in the response of aphid treatments among the 12 replicates performed.

Supplemental Table S2: Output from the full model examining the effects of virus acquisition on host plant selection behavior by aphid vectors, pooling all observations made throughout the 72 h period. (PROC GENMOD, binomial distribution, logit link transformation, assuming compound symmetry). Panel A displays indirect effects of virus acquisition and responses to the BYDV-infected plant treatment. Panel B displays indirect effects of virus acquisition and responses to the sham-inoculated plant treatment. Panel C displays direct effects of virus acquisition and responses to the BYDV-infected plant treatment. Panel D displays direct effects of virus acquisition and responses to the sham-inoculated plant treatment.

Score Statistics for Type 3 GEE Analysis

A

Source	DF	Chi-Square	Pr > ChiSq
Rep	11	22.14	0.0233
Aphid	1	19.33	<0.0001
Rep*Aphid	11	22.49	0.0208
Time	5	4.96	0.4203
Aphid*Time	5	1.90	0.8626

B

Source	DF	Chi-Square	Pr > ChiSq
Rep	11	21.35	0.0299
Aphid	1	20.14	<0.0001
Rep*Aphid	11	22.36	0.0217
Time	5	2.15	0.8282
Aphid*Time	5	2.26	0.8123

C

Source	DF	Chi-Square	Pr > ChiSq
Rep	11	20.04	0.0448
Aphid	1	16.18	<0.0001
Rep*Aphid	11	21.65	0.0272
Time	5	2.41	0.7906
Aphid*Time	5	4.04	0.5444

D

Source	DF	Chi-Square	Pr > ChiSq
Rep	11	19.56	0.0517
Aphid	1	16.32	<0.0001
Rep*Aphid	11	21.03	0.0331
Time	5	3.66	0.5995
Aphid*Time	5	5.72	0.3349

The majority of the variation in all the models is described by the main effect of the aphid treatment. The replicate and replicate by aphid interactions are significant (marginally in panel D), indicating some variation in the response of aphid treatments among the 12 replicates performed. There were no effects of the time at which observations were made during either of the experiments. Light and dark observations were examined with the model separately and no significant interactions were observed, thus results were pooled in the overall analysis.

Supplementary References

42 Dellaporta, S., Wood, J. & Hicks, J. A plant DNA minipreparation: Version II. *Plant Mol. Biol. Rep.* **1**, 19–21 (1983).

Chapter 3: The invasive grass *Ventenata dubia* is a host to *Barley yellow dwarf virus*

Abstract

Ventenata dubia, a winter annual grass, is a nonnative species invading grasslands, rangelands and pastures throughout the United States. There is little information available on this species, including its suitability as a host to pathogens and insect pests in its invaded range. Field surveys of *V. dubia* in endangered Palouse Prairie and Conservation Reserve Program (CRP) habitats of southeastern Washington and adjacent northern Idaho were conducted to examine for the presence of *Barley yellow dwarf virus* (BYDV) in natural populations of this grass species. Laboratory tests were conducted to examine the suitability of *V. dubia* to host BYDV-PAV following transmission using the aphid vector *Rhopalosiphun padi*. Plant height, number of leaves per plant, number of tillers per plant, and above-ground and below-ground wet and dry weight of tissues were examined to gauge the susceptibility of *V. dubia* to BYDV-PAV. The potential for *V. dubia* to serve as a source of BYDV-PAV inoculum was also examined. Following field surveys immunological and molecular techniques demonstrated infection of *V. dubia* in Palouse Prairie and CRP habitats with two species of BYDV: PAV and SGV. The ability of BYDV-PAV to infect *V. dubia* under controlled conditions in the laboratory and transmission from infected *V. dubia* plants to susceptible barley cv. Sprinter were also demonstrated. BYDV-PAV-infected *V. dubia* showed reductions in plant height, the number of leaves per plant, the number of tillers per plant and the above-ground dry weight of plant tissue suggesting that *V. dubia* is susceptible to BYDV infection. These results demonstrate that *V. dubia* is a host to BYDV and may serve as a virus inoculum source under natural conditions. The

ecological and epidemiological implications of *V. dubia* infection with BYDV need to be explored.

Key words: Palouse Prairie, virus inoculum, virus ecology, virus vectors, Conservation Reserve Program

Introduction

Ventenata dubia (Leers) Coss is a winter annual grass native to southern Europe, western Asia and northern Africa (Scheinost et al. 2009). *V. dubia* was first reported in the United States in the state of Washington in 1952 and within the next 35 years established in 13 counties in Washington and 21 counties in Idaho (Northam and Callihan 1994, Scheinost et al. 2009). It can now be found along roadsides, in hay, pasture, range and conservation reserve program (CRP) fields throughout the western United States (James 2008, Scheinost et al. 2009). *V. dubia* is also found in Wisconsin, New York, Maine and bordering Canadian provinces in the east and west (Scheinost et al. 2009). A weedy and invasive species, *V. dubia* is considered undesirable as it replaces forbs and native perennial grasses and causes the soil to become more prone to erosion due to its shallow root system (Scheinost et al. 2009). In the Pacific Northwest *V. dubia* is now the focus of increased attention, as it is invading an endangered ecosystem, the Palouse prairie.

Southeastern Washington and adjacent northern Idaho are home to the critically endangered Palouse prairie ecosystem which has historically been dominated by bunchgrasses such as Idaho fescue (*Festuca idahoensis* Elmer) and bluebunch wheatgrass [*Pseudoroegneria spicata* (Pursh) Á. Löve] (Noss et al. 1995, Lichtardt and Moseley 1997, Donovan et al. 2009, Scheinost et al. 2009). The prairie that once dominated the landscape is now reduced to small remnants (< 2 hectares) located in areas that could not be farmed and are mostly privately owned

(Hanson et al. 2008, Donovan et al. 2009, Looney and Eigenbrode 2012). Less than 0.1% of the historical prairie habitat remains (Noss et al. 1995, Black et al. 2000). Palouse prairie remnants are surrounded by a landscape matrix dominated by wheat production and CRP perennial grasslands (Donovan et al. 2009, Looney and Eigenbrode 2012). One of the most threatening invasive species present in the region is *V. dubia*, which can be found in almost all prairie remnants (Lichtardt and Moseley 1997, Hanson et al. 2008, Nyamai et al. 2011).

Barley yellow dwarf disease is one of the most economically important plant diseases worldwide and occurs ubiquitously in *Poaceae* species infecting small grain crops as well as pasture, rangeland and natural grasslands (Plumb 1983). The disease is caused by a group of viruses belonging to the family Luteoviridae referred to as *Barley* or *Cereal yellow dwarf virus* (B/CYDV) (King et al. 2012). There are eight different species of B/CYDV that make up this viral complex, five of which occur in the U.S.: BYDV-PAV, -MAV, -SGV, CYDV-RPV and CYDV-RMV (Fauquet et al. 2005, Krueger et al. 2013). Historically, each strain was named after its most efficient aphid vector species (Rochow 1969, Rochow and Muller 1971).

The Pacific Northwest of the U.S. is one of the top global producers of wheat (*Triticum aestivum* L.) (Lister and Ranieri 1995) where yield losses due to BYDV infection could range between 11-47% on average (Bishop and Sandvol 1984). BYDV is transmitted in a persistent circulative manner (Gildow 1993) by at least 25 species of aphids (Halbert and Voegtlin 1995) including *Rhopalosiphum padi* L., *R. maidis* Fitch, *Metopolophium dirhodum* Walker, *Schizaphis graminum* Rondani, and *Sitobion* (formerly *Macrosiphum*) *avenae* Fabricius, which are commonly encountered in the western U.S. (Gildow and Rochow 1983, Halbert and Pike 1985, Schotzko and Bosque-Pérez 2000, Bosque-Pérez et al. 2002).

BYDV infection has also been reported in nonmanaged grass systems throughout the U.S. (Malmstrom 1998, Garrett et al. 2004, Malmstrom et al. 2007). In these nonmanaged systems, BYDV infection has been shown to influence competitive dynamics between native and invasive grass species and facilitate invasion by nonnative annual grasses (Malmstrom et al. 2005, Malmstrom et al. 2006, Borer et al. 2007, Borer et al. 2010).

Efforts to protect and restore the Palouse prairie have increased recently (Donovan et al. 2009, Looney et al. 2009). Previous studies have examined plant, earthworm and arthropod diversity in these habitats (Hatten et al. 2004, Looney et al. 2004, Hanson et al. 2008, Sánchez de León and Johnson-Maynard 2009, Nyamai et al. 2011, Hatten et al. 2013, Xu et al. 2013). There have been no investigations of plant pathogens or their insect vectors in Palouse prairie remnants or neighboring CRP lands. The potential for *V. dubia* to host BYDV has implications for the conservation of Palouse prairie remnants as well as viral dynamics in crop lands and adjacent habitats, all of which contain *V. dubia*.

In order to evaluate the impact of *V. dubia* in its invaded range a better understanding of its susceptibility to BYDV and suitability for its insect vectors is needed. The objectives of this study were to: 1. assess if *V. dubia* populations in Palouse prairie and CRP habitats harbor BYDV and determine which viral species are present in the landscape, 2. determine if *V. dubia* could be infected with BYDV under controlled conditions using an aphid vector, 3. examine if *V. dubia* could serve as an inoculum source by conducting virus transmission studies from it to a susceptible crop host via aphids, and 4. measure the impact of BYDV infection on *V. dubia* growth.

Materials and Methods

Field sampling Natural populations of *V. dubia* were sampled at eight different sites located along the Idaho-Washington border (Figure 3.1). Three of the sites sampled are remnants of native Palouse Prairie habitat and the other five sites are CRP habitats. Sites were sampled in the spring of 2011 and spring of 2012. Up to 15 samples, each sample comprised of four leaves from multiple plants, were collected at each site. Samples were collected along a transect extending the width of the fragment/habitat and parallel to the slope if located on a hillside. Observations were made to determine if aphids were present in sampled plants. Reverse transcription polymerase chain reaction (RT-PCR) (see below) was used to assess virus infection of plant samples. Viral species were determined via cloning and sequencing (see below).

Virus and vector BYDV-PAV, the historically dominant species of BYDV in the Pacific Northwest (Hewings and Eastman 1995) was maintained by serial transfer on barley (*Hordeum vulgare* L.) cv. Sprinter using the aphid *R. padi* at the University of Idaho Agricultural Biotechnology Laboratory. The colony was maintained in an environmental growth chamber (20 ±2°C; 16:8 L:D). A nonviruliferous colony of *R. padi*, derived from the viruliferous colony, was maintained on Sprinter barley in an environmental growth chamber (same regime) at the University of Idaho Manis Entomological Laboratory. Barley plants for the colonies were bottom watered and fertilized using a soluble N-P-K fertilizer (15:30:15) biweekly.

Barley control plants Barley cv. Sprinter served as a positive control for virus inoculation and acquisition experiments. Barley was planted at a density of one seed per pot in 10.2 cm^2 plastic pots filled with a mixture of 6:1:0.02 ratio of Sunshine mix #1 (Afco Distribution, Spokane, WA):sand:Osmocote®. Pots were placed on trays in an environmental chamber (20±2°C; 16:8

L:D), bottom watered and fertilized biweekly with a soluble N-P-K fertilizer (15:30:15). Twenty-four barley plants were used as controls in the virus-infection tests, 12 were inoculated with BYDV-PAV to serve as positive controls and 12 remained virus and aphid free to serve as negative controls (described below).

***Ventenata* test plants** *V. dubia* seeds were obtained from field-collected populations in northern Idaho. The surface of seeds was sterilized by soaking them in sterile water for 30 min, decanting the water and adding 95% ethanol for a 5-min soak, decanting the ethanol and adding 10% bleach for a 5-min soak and finally rinsing the seeds 5 times using sterile water (sterilization protocol as described by LEHLE Seeds, Tucson, AZ). Seeds were germinated on sterile blotter paper moistened with sterile water. Upon germination 24 seeds were transplanted to 10.2 cm^2 plastic pots filled with the soil mixture described above, one seed per pot. Pots were placed on trays and maintained in environmental chambers as described above.

Virus-infection tests Plants were removed from environmental chambers and placed on the laboratory bench at 20±2°C for virus inoculation at the 2-3 leaf stage (Zadoks et al. 1974) which occurred approximately 29 days after planting (DAP) for *V. dubia* and 18 DAP for Sprinter barley. Twelve plants of each species served as healthy controls and were not exposed to aphid feeding. For virus inoculation of the remaining plants, 15 viruliferous *R. padi* were caged on each of 12 *V. dubia* and 12 barley plants using a 4-cm long piece of 23-mm dialysis tubing (14.6 mm D, Spectra/Por®) inserted over the entire plant (*V. dubia*) or a single leaf (barley) and sealed with foam stoppers on each end (Jiménez-Martínez et al. 2004). Aphids were caged on *V. dubia* for a 96-h inoculation access period (IAP), a sufficient time period for aphids to transmit the virus to susceptible hosts (Jiménez-Martínez and Bosque-Pérez 2004). At the end of the IAP, cages and aphids were removed and plants returned to environmental chambers. Safer®

insecticidal soap was used to kill aphids after the IAP. Plants were monitored weekly for symptom development. *V. dubia* was sampled for virus presence 47 days after inoculation (DAI). Barley develops faster than *V. dubia* and therefore was sampled earlier (31 DAI). A composite sample of multiple leaves from each single plant (ca. 0.50 g of *V. dubia* tissue and 2 g of barley tissue) collected 47 and 31 DAI, respectively, was sent to Agdia, Inc. (Elkhart, IN) to be tested for BYDV-PAV using indirect triple antibody sandwich-enzyme-linked immunosorbent assay (TAS-ELISA). To confirm ELISA results, *V. dubia* tissue was sampled 57 DAI and reverse transcription (RT)-PCR performed (see below). Plant height, number of leaves per plant and number of tillers per plant were recorded 47 DAI for *V. dubia* and 31 DAI for barley. The above-ground wet and dry weight of barley tissues were recorded 31 DAI. *V. dubia* plants were harvested 88 DAI and the wet and dry weight of above and below-ground tissue recorded.

Statistical analysis The mean plant height, mean number of leaves per plant, mean number of tillers per plant, mean above-ground and below-ground wet and dry tissue weights of BYDV-infected and non-inoculated plants were compared using independent samples t tests. *V. dubia* and barley were tested separately. Statistical tests were performed using PROC TTEST (SAS Institute 9.2) with a 95% confidence interval (alpha = 0.05).

Virus-transmission tests To examine the ability of aphids to transmit BYDV-PAV from infected *V. dubia* to other susceptible hosts, transmission tests were conducted using nonviruliferous *R. padi*. Plants were removed from environmental chambers and placed on the laboratory bench (22±2°C) during tests. Five BYDV-PAV-infected *V. dubia* were used as source plants along with one virus-free *V. dubia*, which served as a negative control. All *V. dubia* plants originated from the tests described above. Approximately 74 DAI, 25 nonviruliferous *R. padi* were caged using dialysis tube cages on each *V. dubia* plant for a 72-h virus acquisition access

period (AAP) (Jiménez-Martínez and Bosque-Pérez 2004). Sprinter barley served as the susceptible indicator host. Barley was planted at a density of four seeds per pot in 10.2 cm^2 plastic pots and maintained following the methods described above. After the 72-h AAP, five aphids were transferred to each barley plant, and caged using dialysis tube cages as described above for a 72-h virus IAP. Two pots of barley, with three to four plants per pot, were used for each BYDV-infected *V. dubia* source plant, resulting in a total of 32 barley indicator plants. Four noninfected barley plants served as the negative control. Aphids from the viruliferous colony were caged on eight barley plants (five aphids/plant) for a 72-h virus IAP, and these plants served as a positive control. At the end of the 72-h virus IAP, aphids and cages were removed and barley plants remained on the lab bench for the remainder of the experiment. Indicator plants were sampled (ca. 1 g of tissue per plant) 36 DAI and sent to Agdia Inc. (Elkhart, IN) to be tested for BYDV-PAV using indirect TAS-ELISA. Samples were considered positive if the optical density (A_{405}nm wavelength) was two times greater than the negative controls.

RNA extraction Total nucleic acids were extracted using a modified Dellaporta method (Pappu et al. 2005). Plant tissue was sampled by punching four pieces of leaves using the lid of a 1.5 mL microfuge tube. One mL of buffer (100 mM Tris, pH 8.0, 50 mM EDTA, 500 nM NaCl and 10 mM mercaptoethanol) was added to each tube and samples ground with a micropestle. To each ground sample, 140 μL of 10% SDS was added, samples were vortexed and incubated at 65°C for 15 min. Samples were then transferred to ice and 250 μL of 8M potassium acetate added. Samples were inverted to mix and held on ice for 10 min. Samples were then centrifuged at 13,000 rpm for 10 min and the supernatant transferred to a new microfuge tube, 600 μL of isopropanol added, tubes inverted to mix and incubated on ice for 7 min. Samples were then centrifuged at 13,000 rpm and 10°C for 7 min. The supernatant was discarded and the pellet

washed with 1 mL of 70% ethanol. The pellet was air-dried overnight and resuspended in 60 µL of DEPC water.

Reverse transcription Reverse transcription (RT) was carried out using M-MLV Reverse Transcriptase (Promega Corp., Madison, WI, USA) following the manufacturer's guidelines with random hexamer primers. A master mix of water and 10 µM random hexamer primers was added to the plate, along with 1.2 µL of nucleic acid extract and denatured for 5 min at 70°C on a Multigene Labnet thermal cycler (BioExpress, Kaysville, UT, USA). To each reaction, 4 µL of 5X RNA extraction buffer and 0.5 µL of M-MLV Reverse Transcriptase (Promega Corporation, Madison, WI) was added, along with 0.5 mM dNTPs, 0.3 µL RNasin® Ribonuclease Inhibitor (Promega Corporation, Madison, WI) and 38mg/mL of T4 Gene 32 Protein (New England Biolabs Inc., Ipswich, MA, USA) for a total reaction volume of 20 µL per tube. The samples were then returned to the thermal cycler and held at 25°C for 60 min followed by 95°C for 5 min.

Polymerase Chain Reaction (PCR) Virus infection and transmission test plants that were inoculated with BYDV-PAV were tested using primers designed to detect a region of the coat protein of the PAV species of the virus described in Ingwell et al. (2012; see Suppl. Mat.). A total of 1 µL of RT product was used in the polymerase chain reaction in addition to 2 µL 10X PCR buffer, 2mM $MgCL_2$, and 0.30 µL My taq (New England Biolabs Inc., Ipswich, MA), 0.4 mM dNTPs, 0.12 µM each of the forward and reverse primers, 34.2 µg/mL of T4 Gene 32 Protein (New England Biolabs Inc., Ipswich, MA) and ddH_2O to reach a total reaction volume of 20 µL. The samples were amplified on a Labnet Thermal Cycler with the following PCR conditions: 95°C for 2 min, followed by 11 cycles of 95°C for 30s, 62°C for 30s and 72°C for

1min 30s, with the annealing temp decreasing 0.5°C each cycle, followed by 22 cycles of 95°C for 30s, 56°C for 30s and 72°C for 1 min 30s concluded with a hold at 72°C for 7 min.

Field collected *V. dubia* samples were tested using primers designed to detect multiple species of B/CYDV. The forward primer (5'-CGGACARTGGTTRTGG-3') and reverse primer (5'-TGGTAGGACTTRAGTAYTCC-3') were modified by C.L. Lacroix from previously published reports (Robertson et al. 1991, Chomič et al. 2010). Samples infected with BYDV-SGV, RMV, PAV and MAV will produce a band 224 bp in size while samples infected with CYDV-RPV produce a band 227 bp in size. The protocol described above, using the generic primers at a concentration of 0.1 μM each was performed. The PCR conditions were as follows: 95°C for 30s followed by 40 cycles of 95°C for 30s, 47°C for 45s and 68°C for 1 min concluded with a hold at 68°C for 5 min. The PCR products were viewed using gel electrophoresis following the procedure described in Ingwell et al. (2012).

Cloning and sequence analysis A subset of *V. dubia* field-collected samples was selected for cloning and sequencing to confirm B/CYDV infection and determine viral species. The product from the above PCR procedure was purified on a low melting point (LMP) agarose gel with ethidium bromide staining using the Wizard® SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI). The purified PCR product was cloned into pGEM® T-Easy vectors following the manufacturer's protocol (Promega Corp., Madison, WI) and transformed using *E.coli* cells DH5α. Clones were screened using EcoRI restriction enzymes following the manufacturer's protocol (New England Biolabs Inc., Ipswich, MA) by identifying plasmids containing inserts 224-227 bp in size. Four independent clones from each sample were sequenced in the forward direction by GENEWIZ, Inc. (South Plainfield, NJ) using the M13F (-

21) primer. Sequence results were analyzed performing a nucleotide BLAST search on the National Center for Biotechnology's (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Field surveys *V. dubia* samples were collected from two Palouse Prairie sites and four CRP habitats in the spring of 2011 and from two Palouse Prairie sites and two CRP habitats in the spring of 2012. BYDV was detected at seven of the eight sites sampled (Table 3.1). The only location where virus was not detected was at the CRP located near Kamiak Butte, which was sampled in the spring of 2011. One prairie site, Paradise Ridge, and one CRP site, a private CRP, were sampled both years and virus detected among the samples at each site and each year sampled (Table 3.1). The proportion of samples infected ranged from 11-87% depending on location and sampling time (Table 3.1). Two species of BYDV were identified from field-collected specimens, BYDV-PAV and BYDV-SGV. PAV was detected at the private CRP and at the Smoot Hill upper CRP in the spring of 2011 (Table 3.1). SGV was detected at Paradise Ridge Prairie and at Smoot Hill lower CRP in the spring of 2011. SGV was also detected at the private CRP, Paradise Ridge Prairie, Kramer prairie, and Kramer CRP in the spring of 2012 (Table 3.1). This is the first report of BYDV-PAV and BYDV-SGV infection of *V. dubia*. No aphids were found infecting *V. dubia* plants.

Virus-infection tests There were no visual disease symptoms (i.e., discoloration or lesions) observed on BYDV-inoculated *V. dubia* plants, however infection was detected in ten out of ten plants that survived. Two BYDV-inoculated *V. dubia* plants died prior to the end of the experiment and were not tested for virus infection. When sampled 47 DAI, ten of the ten inoculated *V. dubia* were positive using TAS-ELISA and RT-PCR performed 57 DAI confirmed

infection in all ten BYDV-inoculated *V. dubia* plants (Table 3.2). The Sprinter barley that served as a control exhibited 100% inoculation success (Table 3.2). All of the healthy plants (12/12 *V. dubia* and 12/12 Sprinter barley) remained virus-free confirming there was no cross-contamination in the assay.

BYDV-PAV-infected *V. dubia* exhibited reduced plant height (independent t tests, $p = 0.001$), reduced number of leaves per plant (independent t tests, $p = 0.011$) and a reduction in the number of tillers per plant (independent t tests, $p = 0.009$) when measured 47 DAI (Table 3.3). While there was no change detected in the below-ground biomass or the above-ground wet weight, there was a decrease in the above-ground dry weight of BYDV-PAV-infected *V. dubia* compared to healthy plants 88 DAI (independent t tests, $p = 0.045$; Table 3.4). BYDV-PAV-infected barley exhibited a reduction in the above-ground wet weight 34 DAI (independent t tests, $p = 0.032$; Table 3.4) but no differences in height, the number of leaves or tillers per plant were observed at this early stage of infection (Table 3.3).

Transmission tests BYDV-PAV infection was detected in 94% (30/32) of barley plants when using infected *V. dubia* as the virus source plants (Table 3.2). BYDV-PAV infection was detected in 88% (7/8) of inoculated barley when infected Sprinter barley as the virus source plants (Table 3.2). Elevated absorbance readings were recorded using indirect TAS-ELISA for all infected plants. This is the first report of transmission of BYDV-PAV from *V. dubia* to a susceptible host.

Discussion

This is the first report of BYDV infection in *V. dubia.* Field sampling of *V. dubia* populations in Palouse prairie remnants and CRP fields showed that this invasive grass is a host

of BYDV-PAV and BYDV-SGV. Experimental inoculations using the aphid vector *R. padi* confirmed BYDV-PAV infection in *V. dubia*. These results document the first report of BYDV in Palouse prairie and CRP habitats in northern Idaho and adjacent Washington. Examining the seasonality of viral species collected in field samples, it appears that PAV was prevalent in the spring of 2011 at two of the eight sites sampled while SGV was prevalent in both the spring of 2011 and 2012 at five of the eight sites sampled.

This is also the first report documenting transmission of BYDV from *V. dubia* to a susceptible host. These findings have important implications for the ecology and epidemiology of BYDV in areas where *V. dubia* is present. In the Pacific Northwest, *V. dubia* has been increasing in abundance and can be found along roadsides, in agricultural fields, CRP fields and Palouse prairie remnants (Scheinost et al. 2009), creating a bridge of BYDV-inoculum among these diverse habitats.

V. dubia appears to be susceptible to BYDV. While there were no physical symptoms of BYDV-PAV infection observed in laboratory inoculations (i.e., discoloration) there were significant declines observed in plant height, number of leaves, number of tillers and above-ground biomass dry weight. In order to evaluate the impact of BYDV infection on the invasiveness of *V. dubia* further studies examining the fitness response to virus infection as well as its ability to compete with other grass species are needed.

Since its introduction to the U.S. in 1952, little attention has been paid to *V. dubia* until recently (Scheinost et al. 2009). With increased efforts to conserve the critically endangered Palouse Prairie located in southeastern Washington and adjacent northern Idaho (Lichtardt and Moseley 1997) researchers have been looking at ways to suppress this invasive annual grass

(Northam and Callihan 1994, James 2008, Nyamai et al. 2011). Along with *Bromus tectorum* L. (cheatgrass), *V. dubia* is the main threat to these plant communities and can be found at almost all remnant sites studied previously (Hanson et al. 2008, Nyamai et al. 2011). Annual grasses have an advantage in grasslands where plant viruses, such as BYDV, are present because they return disease free each year while viral infections persist in the root stock of perennial hosts (Borer et al. 2007). BYDV has been shown to influence the competitive dynamics of native and invasive grass species and facilitate invasion in California grasslands (Malmstrom et al. 2005, Borer et al. 2007, Power et al. 2011). The presence of exotic annual grasses, such as *Avena fatua* L., was shown to increase the prevalence of BYDV infection in native *Elymus glaucus* Buckley (Malmstrom et al. 2005). Exotic annual grasses, such as *A. fatua,* have been shown to be superior and preferable host for vectors leading to increases in their population when this host is present (Malmstrom et al. 2005, Borer et al. 2009). Annual grass invasion has been facilitated in California by BYDV infection which reduces the benefits of perennial longevity among the native species (Borer et al. 2007, Power et al. 2011). There is a need to examine if invasion of the Palouse Prairie by *V. dubia* is in part mediated by BYDV infection of native perennial grasses.

The vector specificity that occurs among the BYDV-complex can lend insight to vector population dynamics and host utilization between habitats. In the region of study, the historically prevalent BYDV species that have been identified among cereal crops include PAV and RMV in eastern Washington (Halbert and Pike 1986), PAV and RPV in low elevation areas of western Idaho (Gildow 1990) and SGV in dry-land cereal production in Southeastern Idaho (Rochow et al. 1987). Surveys of field-collected aphid species in this study region have found that the most abundant species include *M. dirhodum, S. avenae,* and *R. padi* (Schotzko and Bosque-Pérez

2000, Bosque-Pérez et al. 2002), all vectors of BYDV. During the field-sampling conducted in the current study, aphids were never detected on *V. dubia* plants and were rarely seen in prairie or CRP habitats in general. The aphid *Metopolophium festucae cerealium* Stroyan is newly discovered in this region (Halbert et al. 2013) and its ability to transmit BYDV is unclear. BYDV-PAV is transmitted most efficiently by *R. padi* and *S. avenae,* after which it was named, and less efficiently by *S. graminum* (Rochow 1969). BYDV-SGV is transmitted most efficiently by *S. graminum* (Rochow 1969) which is rarely encountered in the study region (Schotzko and Bosque-Pérez 2000, Bosque-Pérez et al. 2002). However, historical reports of virus infection and transmission in Idaho have shown that the Idaho strains of SGV have been transmitted by *R. padi* and *S. avenae,* in addition to *S. graminum* (Halbert et al. 1992, Lei et al. 1995). The rare encounter of aphids in prairie and CRP remnants suggests that BYDV detected in these habitats is a result of non-colonizing aphids.

The results of this study provide the first evidence that the invasive species *V. dubia* is a host to BYDV, both the PAV and SGV species, and may serve as an inoculum source to surrounding susceptible host species. Future work should be directed at examining the impacts of virus infection on the competitive ability of *V. dubia* and neighboring plant species. Studies should also be conducted to evaluate vector use of *V. dubia* as an alternate host and the movement of vector species between habitat types among the agricultural matrix in which this study was conducted. The implications of virus infection among plants in the endangered Palouse Prairie should also be explored.

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Tables

Table 3.1 Location, proportion infected and viral species detected in field-collected *V. dubia* samples.

Site[a]	Date Sampled	# Samples	% infected	Viral Species
Private Prairie	Spring 2011	18	11.1	NT
Private CRP	Spring 2011	15	33.3	PAV
	Spring 2012	15	80	SGV
Kamiak Butte CRP	Spring 2011	15	0	NT
Paradise Ridge Prairie	Spring 2011	15	13.3	SGV
	Spring 2012	15	46.6	SGV
Kramer Prairie	Spring 2012	7	71.4	SGV
Kramer CRP	Spring 2012	15	46.7	SGV
Smoot Hill Upper CRP	Spring 2011	15	33.3	PAV
Smoot Hill Lower CRP	Spring 2011	15	86.7	SGV

[a] CRP = Conservation Reserve Program fields.
Proportion infected determined by RT-PCR. Viral species identified through cloning and sequencing of PCR product. NT = not determined

Table 3.2 Results of BYDV-inoculation and transmission tests. To determine the proportion of infected plants *V. dubia* was tested 47 days after inoculation (DAI) using indirect TAS-ELISA and 57 DAI using reverse transcription PCR. *H. vulgare* was tested 34 DAI using TAS-ELISA. Transmission was detected using *H. vulgare* as the indicator host and plants were tested 36 DAI using indirect TAS-ELISA.

Species	Common Name	Proportion Infected	Host	Transmission[a]
Ventenata dubia	North Africa grass	10/10[b]	Yes	30/32
Hordeum vulgare[c]	barley cv. Sprinter	24/24	Yes	7/8

[a] proportion transmitted from species at left to *H. vulgare*
[b] two plants died, only ten were examined for infection
[c] cultivated species used as positive control

Table 3.3 Comparison of BYDV-PAV infected and healthy plant growth characteristics measured 34days after inoculation (DAI) for *H. vulgare* and 47 DAI for *V. dubia*.

		Mean ± SE		
Species	Infection treatment	Plant height (mm)	No. leaves/plant	No. tillers/plant
Ventenata dubia	Noninfected	114.58±3.50	119.25±8.65	45.92±3.69
	Infected	95.30±3.56 ***	89.00±5.41 **	32.50±2.50 **
Hordeum vulgare[a]	Noninfected	485.13±9.14	57.04±3.63	14.38±0.85
	Infected	459.42±10.96	49.83±5.53	12.58±1.57

[a] cultivated species used as positive control

** $p < 0.01$, *** $p < 0.001$ Results were analyzed using independent samples t tests comparing noninfected and infected plants within each species. Data reported are means ± s.e.m.

Table 3.4 Plant weight measured 34 (*H. vulgare*) and 88 (*V. dubia*) DAI.

		Mean ± SE			
		Above-ground tissue		Below-ground tissue	
Species	Infection treatment	Wet weight (g)	Dry weight (g)	Wet weight (g)	Dry weight (g)
Ventenata dubia	Noninfected	3.89±0.42	1.32±0.19	1.54±0.30	0.14±0.05
	Infected	2.96±0.66	0.79±0.15 *	1.82±0.58	0.27±0.11
Hordeum vulgare[a]	Noninfected	20.66±1.30	1.64±0.11	-	-
	Infected	10.42±0.80 *	-	-	-

[a] cultivated species used as positive control

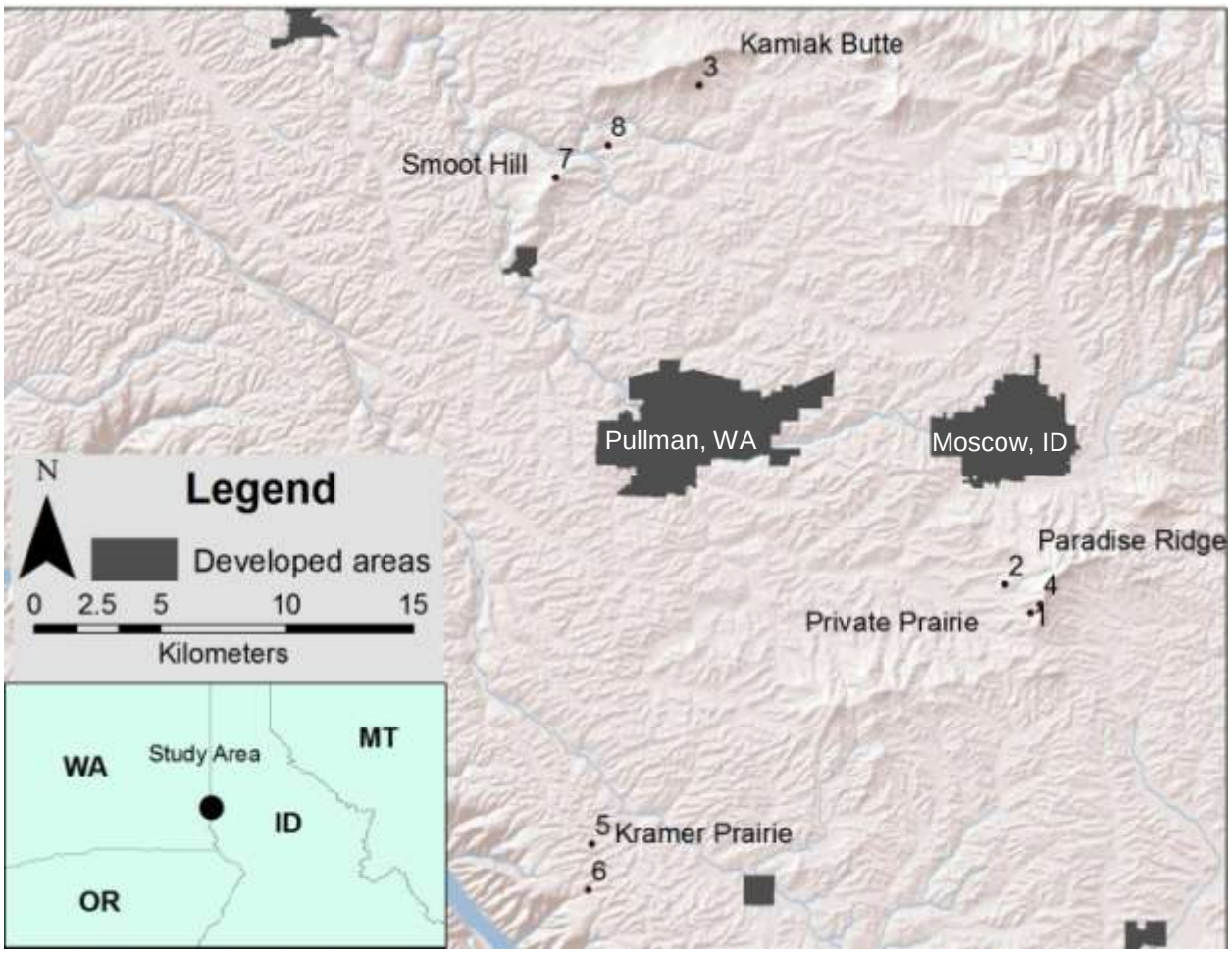
* $p < 0.05$ Results were analyzed using independent samples t tests comparing noninfected and infected plants within each species. Data reported are means ± s.e.m.

Figure Legend

Figure 3.1 A map of the locations at which *V. dubia* samples were collected. 1, Private Prairie; 2, Private CRP; 3, Kamiak Butte CRP; 4, Paradise Ridge Prairie; 5, Kramer Prairie; 6, Kramer CRP; 7, Smoot Hill Upper CRP; 8, Smoot Hill Lower CRP.

Figure

Fig. 3.1



Chapter 4: Virus infection in an endangered grassland habitat

Abstract

The Palouse Prairie is a critically endangered habitat located in one of the most productive wheat-growing regions of the world. *Barley/Cereal yellow dwarf virus* (B/CYDV) is one of the most economically important disease-causing agents of small grain cereal crops, such as wheat, and is known to infect over 150 *Poaceae* species. Included in the host range of this virus are many of the grass species which occur in the Palouse Prairie as well as Conservation Reserve Program (CRP) lands in the Pacific Northwest U.S. Disease incidence has never been examined in these two susceptible habitats which may play an important role in the disease ecology of the region. Field surveys of the *Poaceae* species in Palouse Prairie and CRP habitats of southeastern Washington and adjacent northern Idaho were conducted to examine for the presence of B/CYDV among potential hosts. Sampling was conducted at four separate times to examine the seasonality of infection. Aphid vectors were sampled to identify the species present in each habitat and tested to determine virus presence. Viral species were identified using cloning and sequencing techniques. Over 2,000 samples were collected containing 30 different species of *Poaceae*. BYDV infection was discovered at every CRP and prairie remnant sampled. The overall infection rate was 46%. BYDV-SGV and BYDV-PAV were the two viral species identified. Infection rates did not differ between annual and perennial species. Aphids were encountered only once, found on five plants at one CRP location, suggesting that non-colonizing aphids are responsible for disease dynamics in these habitats. Three of the five aphid samples tested positive for virus. These results demonstrate that BYDV infection is prevalent among CRP and Palouse Prairie habitats in eastern Washington and adjacent northern Idaho. Vector

utilization of Palouse Prairie and CRP habitats need to be explored. The ecological and epidemiological implications are discussed.

Key Words: Palouse Prairie, Conservation Reserve Program, *Barley yellow dwarf virus*, virus ecology, virus inoculum, virus vectors

Introduction

The Palouse Prairie is a critically endangered ecosystem with less than 0.1% remaining (Noss et al. 1995). Like many of the natural grasslands of the United States, the Palouse Prairie is a highly fragmented landscape that is under pressure from a land-use perspective but is also being invaded by many introduced species (Noss et al. 1995, Lichtardt and Moseley 1997, Looney and Eigenbrode 2012). This prairie ecosystem is located in southeastern Washington and adjacent northern Idaho, home to one of the highest wheat (*Triticum aestivum* L.) producing regions in the U.S. (Lister and Ranieri 1995). It is characterized by rolling hills and deep loess soils. Conservation efforts have increased recently, with research focusing on earthworms, pollinators, beetles and the plant community (Hanson et al. 2008, Donovan et al. 2009, Looney et al. 2009, Nyamai et al. 2011, Hatten et al. 2013, Xu et al. 2013).

Barley yellow dwarf disease is one of the most economically important diseases of crops globally (Plumb 1983), it infects cereals such as wheat and barley and is ubiquitous in *Poaceae* species worldwide (D'Arcy 1995). Barley yellow dwarf disease is caused by a complex of viruses belonging to the family *Luteoviridae* referred to as *Barley* or *Cereal yellow dwarf virus* (B/CYDV) (Fauquet et al. 2005). B/CYDV is transmitted exclusively by aphids in a persistent circulative manner (Gildow 1993), they are not transmitted mechanically or through seed. There are at least 25 different species of aphids reported as vectors of B/CYDV (Halbert and Voegtlin

1995). The most common aphid species in the Pacific Northwest include *Metopolophium dirhodum* Walker, *Sitobion avenae* F., *Rhopalosiphum padi* L., *R. maidis* Fitch and *Schizaphis graminum* Rondani (Halbert and Pike 1985, Schotzko and Bosque-Pérez 2000).

The occurrence of viruses in natural ecosystems is important to understand in order to better predict and manage disease outbreaks across the landscape. This includes an understanding of source inoculum and naturally-occurring virus hosts. One region of nonmanaged grasslands that has been extensively studied is the native grasslands of California. BYDV commonly occurs in these native grasslands (Grafton et al. 1982). For example, a survey in 1990 revealed that 37 of 56 cool-season grass species were infected with BYDV-PAV, MAV or RPV-like strains in relatively equal proportions (Griesbach et al. 1990). BYDV has also been shown to play an important role in the competitive dynamics of native and invasive grasses in these communities (Malmstrom et al. 2005a, Malmstrom et al. 2005b, Borer et al. 2007). The presence of exotic annuals increases the prevalence of virus infection and is thought to lower the competitive ability of the native grass species (Seabloom et al. 2009). Native perennial grasses dominate in the absence of disease, however when BYDV is present in the system it persists year to year among the perennial grasses, and exotic annual grasses, which return disease-free each year, are able to establish and coexist with the native perennials (Borer et al. 2007). Virus infection can also exacerbate the consequences of environmental stressors which have been documented in agricultural crops and natural plant communities (Irwin and Thresh 1990, Malmstrom et al. 2006).

Natural grasslands may contribute to virus outbreaks in surrounding agricultural fields or they may harbor their own community of pests and pathogens distinct from the surrounding agricultural crops. Perennial grasses may serve as a reservoir of inoculum for annual species and

emerging agricultural crops. Our knowledge and understanding of insect vector movement between agricultural and natural landscapes is limited. Some studies show that viral species do not overlap between these two ecosystems, suggesting distinct pathosystems. For example, while PAV is the most commonly reported strain of BYDV occurring in cereal fields in Kansas, a recent survey of the grass species in the Konza Prairie found MAV and SGV to be predominant in these natural grasslands (Garrett et al. 2004). In California, there is a large overlap in the virus species present in agricultural and natural landscapes suggesting vector utilization of both habitats (Malmstrom 1998).

Included in the host range of B/CYDV are many of the grass species found in the Palouse Prairie as well as conservation reserve program (CRP) lands. The CRP program was established by the USDA Farm Service Agency in 1985. It is a collaborative conservation effort coordinated among private land owners intended to improve water quality, reduce soil erosion and increase undisturbed habitats for threatened or endangered species (www.fsa.usda.gov). These habitats may play an important role in the dynamics of plant viruses among neighboring susceptible crops and may have ecological and epidemiological significance in plant disease dynamics. While both Palouse Prairie and CRP habitats harbor susceptible species, these habitats have never been examined as reservoirs of plant viruses, such as B/CYDV.

To better understand the epidemiology of BYDV among cropping systems, potential reservoir hosts need to be identified. Furthermore, to manage and conserve endangered grasslands a better understanding of the interactions among members of the community, including pathogens, is required. Therefore, the objectives of this study were to survey Palouse Prairie remnants and CRP habitats to gain a better understanding of the prevalence of B/CYDV among grass hosts, examine the seasonality of viral infection among host species, identify the

species of B/CYDV present at each habitat and examine the aphid species present to determine potential vectors within Palouse Prairie and CRP habitats.

Materials and Methods

Site selection and sampling Five Palouse Prairie remnants and six adjacent CRP habitats were selected based on availability, location and accessibility for this study (Fig. 4.1). Each prairie site was paired with one adjacent CRP except for Smoot Hill. This location is unique because it sits on a large preserve maintained by Washington State University where weed and other management efforts are performed. Two CRP fields were selected at this site: a large CRP field planted with hard fescue (*Festuca brevipila* Tracey) located on the hillside above the prairie remnant and an additional CRP located farther from the prairie surrounded by conventionally managed agricultural fields, more similar to the other study sites. Each site was sampled four times, fall 2010, spring 2011, fall 2011 and spring 2012, with the exception of the private prairie and CRP and the Kamiak Butte CRP, which were sampled only on the last three dates. Sampling in fall 2010 began on September 28 and ended October 14. Sampling in spring 2011 began on May 25 and ended on June 28. Sampling in fall 2011 began on October 25 and ended on November 8. Sampling in spring 2012 began May 21 and ended July 11, with a break occurring May 23 through July 5.

Each prairie and CRP was sampled along two parallel transects running perpendicular to the slope of each fragment. *Poaceae* species were identified and 15 unique samples per species, comprised of four leaves from four individual plants, was collected at each site and sampling time. Transects acted as a guide but did not restrict sampling. Tissue collection was made at the time of flowering to aid in species identification. Aphids encountered during sampling were also

collected and identified to species using available keys. Plant species were identified in the Lambert-Erickson Weed Herbarium and the Stillinger Herbarium, each located at the University of Idaho. Grass and aphid samples were processed using reverse transcription polymerase chain reaction (RT-PCR) (see below) to determine viral infection. Cloning and sequence analysis (see below) was used to identify the viral species present in infected samples.

Nucleic acid extraction For each plant sample, approximately 0.03-0.05 g of tissue were ground in liquid nitrogen and total nucleic acid extraction performed using an adapted Dellaporta method (Pappu et al. 2005). Aphids were processed individually or three individuals from a single host plant pooled if more than one was present, and ground with a pestle directly in one mL of extraction buffer (100 mM Tris, pH 8.0, 50 mM EDTA, 500 nM NaCl and 10 mM mercaptoethanol). For plant samples, the buffer was added directly to the ground tissue after the liquid nitrogen evaporated. Following the addition of buffer for both aphid and tissue samples, 140 µL of 10% SDS was added, samples were vortexed and incubated at 65°C for 15 min. Samples were placed on ice and 250 µL of 8M potassium acetate added. Samples were inverted to mix and incubated on ice for seven min. They were then spun at 13,000 rpm for 10 min. The supernatant was transferred to a new tube containing 600 µL of isopropanol, mixed and incubated on ice for seven min. Samples were spun at 13,000 rpm for seven min. at 10°C, supernatant discarded and the pellet washed with 1 mL of 70% ethanol. The pellet was air-dried and resuspended in 60 µL of DEPC-treated water.

Reverse transcription Reverse transcription (RT) reactions were done using M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Along with 1.2 µL of RNA extract from either the insect or plant samples, water and 0.14 µM random hexamers were added and the mixture was placed on a Multigene Labnet

thermal cycler (BioExpress, Kaysville, UT, USA) at 70 °C for 5 min to denature the RNA. To each reaction, 4 µL of 5X RNA extraction buffer and 0.5 µL of M-MLV Reverse Transcriptase (Promega Corporation, Madison, WI) was added, along with 0.5 mM dNTPs, 0.3 µL RNasin® Ribonuclease Inhibitor (Promega Corporation, Madison, WI). Finally, 38mg/mL of T4 Gene 32 Protein (New England Biolabs Inc., Ipswich, MA, USA) was added to reduce inhibitor effects that may be present in the variety of noncultivated grass species sampled. The samples were then returned to the thermal cycler and held at 25°C for 60 min followed by 95°C for 5 min to complete the construction of cDNA to be used in Polymerase Chain Reaction **(**PCR) tests.

Polymerase Chain Reaction (PCR) A unique set of primers designed by C.J. LaCroix was used to detect multiple species of B/CYDV. The forward primer (5'-CGGACARTGGTTRTGG-3') and reverse primer (5'-TGGTAGGACTTRAGTAYTCC-3') were modified from previously published reports (Robertson et al. 1991, Chomič et al. 2010). BYDV-SGV, -RMV, -PAV, and -MAV infection produce a band 224 bp in size while CYDV-RPV produces a band 227 bp in size, which are visually indistinguishable on the agarose gel. A master mix containing 1X PCR Buffer, 2 mM $MgCl_2$, 0.3 µL per sample of My Taq (New England Biolabs Inc., Ipswich, MA), 0.4mM dNTPS, 34.2 µg/mL Protein T4gp32 and 0.1µM each forward and reverse primers and nuclease-free water to make a total reaction volume of 20 µL each was aliquoted onto each PCR plate. To this mixture, 1 µL of cDNA from the RT reaction was added. The samples were amplified on the Labnet thermal cycler with the following PCR conditions: 95°C for 30s followed by 40 cycles of 95°C for 30s, 47°C for 45s and 68°C for 1 min concluded with a hold at 68°C for 5 min. The PCR products were viewed using gel electrophoresis following the procedure described in Ingwell et al. (2012; see Suppl. Mat.)

Cloning and sequence analysis A subset of samples were selected for cloning and sequencing to confirm B/CYDV infection and determine viral species. Samples were selected to include a variety of site, hosts and collection times in order to examine geographic and seasonal variability as well as gauge the efficiency of the primers on a diverse set of host plant species. PCR products were purified on a low melting point (LMP) agarose gel with ethidium bromide staining using the Wizard® SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI). Purified product was resuspended in 25-50 μL of nuclease-free water depending on the intensity of the band. The purified PCR product was cloned into pGEM® T-Easy vectors following the manufacturer's protocol (Promega Corp., Madison, WI) and transformed using *E.coli* cells DH5α. Clones were screened using EcoRI restriction enzymes following the manufacturer's protocol (Promega Corp., Madison, WI) by identifying plasmids containing inserts 224-227 bp in size. A minimum of three independent clones from each sample were sequenced in the forward direction by GENEWIZ, Inc. (South Plainfield, NJ) using the M13F (-21) primer. Sequence results were analyzed performing a nucleotide BLAST search on the National Center for Biotechnology's (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

A total of 2,271 plant samples was collected comprised of 30 species of grasses from the eleven prairie and CRP sites (Table 4.1). Number of samples collected at each site ranged from 101 (Paradise Ridge CRP) to 316 (Kramer Prairie). Among the samples, 46.9% tested positive for B/CYDV infection. Virus infection was detected in 28 of the 30 different species collected (Table 4.1) with infection rates varying from 2 to 93% depending on species. In addition to the species listed here, the invasive grass *Ventenata dubia* was also collected and found to be infected with B/CYDV (Chapter 3). Virus infection was detected at every site sampled and

showed little variation between prairie and CRP sites within a location, except for Kramer where infection at the prairie site was 56% compared to 37% at the CRP (Fig. 4.2).

The average infection rate among annual grasses, all of which are introduced species, was 40% and among perennial grasses, which are equally mixed between native and introduced species, was 43% showing little variability between growth habits. The average infection rate among introduced species was 46%, slightly higher than the average rate of infection among native species which was 40%.

Observations on the seasonality of infection rates among perennial hosts indicated that the infection rate in the perennial introduced host *Arrhenatherum elatius* [(L.) P. Beauv. ex J. Presl & C. Presl] (tall oatgrass) remained high throughout the sampling times at the Smoot Hill lower CRP. Infection in the perennial host *Bromus inermis* (Leyss.) (smooth brome) was relatively low, but increased during the last sampling period at the Smooth Hill upper CRP (Fig. 4.3). Infection rates among annual hosts showed similar patterns of increase as *B. inermis*. The infection rate among the annual introduced host *B. tectorum* (L.) (cheatgrass) was approximately 25% during the first two sampling times at the Kramer CRP, but increased to 60% during the last sampling time in the spring of 2012 (Fig. 4.4). Infection rates also increased among the annual introduced host *B. racemosus* (L.) (bald brome) at the private CRP and *Aegilops cylindrica* (Host) (jointed goatgrass) at the Kamiak Butte CRP throughout the sampling times (Fig. 4.4).

The viral species identified through sequencing include BYDV-PAV and BYDV-SGV. While BYDV-SGV was the dominant species identified, BYDV-PAV was detected at the earlier sampling times at the Kramer CRP and Paradise Ridge Prairie and at the last sampling time at the Smoot Hill Upper CRP (Table 4.2). A mixed infection was detected at one site in one sample

of the perennial native *Pseudoregneria spicata* [(Pursh) Á. Löve] (bluebunch wheatgrass) in the fall of 2011 (Table 4.2). The seasonality of viral species present in the samples of the perennial host *B. inermis* show that in the fall of 2010 and 2011 BYDV-SGV was present at the Smoot Hill Upper CRP (Fig. 4.3). In the spring of 2012 BYDV-PAV was identified in *B. inermis* at the same site (Fig. 4.3). The viral species identified among the annual host *B. tectorum* at Kramer CRP was BYDV-PAV in the fall of 2010 and BYDV-SGV in the spring of 2012 (Fig. 4.4). BYDV-SGV was also detected from a sample of *A. cylindrica* collected at Kamiak Butte CRP in the spring of 2012 (Fig. 4.4).

Aphids were encountered only once during the entire sampling period. In July of 2012 aphids were found on five different plant samples at the Kamiak Butte CRP. All were *Sitobion avenae* except for a single *Metopolophium dirhodum* found on one sample of *Aegilops cylindrica*. The *S. avenae* were found on two samples of *Avena fatua* L. and two samples of volunteer wheat. A pooled sample of three aphids was tested from each sampled plant. The *M. dirhodum* sample tested negative for virus infection while the host plant it was collected from tested positive for virus. Another sample of *A. cylindrica* from the same site and sampling time was sequenced and determined to be infected with BYDV-SGV. One of the pools of *S. avenae* collected from *A. fatua* tested positive for virus and the other tested negative; neither of the sampled plants was infected. Both pools of *S. avenae* collected from volunteer wheat tested positive for virus while only one of the host plant was virus positive.

Discussion

This is the first comprehensive sampling of *Poaceae* species in Palouse Prairie and CRP habitats documenting infection of B/CYDV among a large variety of plant species. The presence

of B/CYDV among prairie and CRP habitats may influence agricultural management practices and conservation efforts for Palouse Prairie restoration.

Previous work in a California grassland system has shown that the invasive annual grasses, under disease-free conditions, would not be able to persist among the competitively-dominant native species without some sort of perturbation or land-use change (Seabloom et al. 2003, Corbin and D'Antonio 2004). More recent work examining the effects of BYDV-infection on the competitive interaction among native and exotic species in this community have demonstrated the crucial role that plant diseases play in shaping community structure (Borer et al. 2007). We have shown that BYDV is prevalent in both the native and exotic grass species among all of the Palouse Prairie sites sampled. Future work should examine the effects of BYDV infection on the competitive interactions between native and exotic grasses in Palouse Prairie habitats, as this may hinder restoration efforts.

In dry subtropical climates in the Mediterranean region wild grasses and corn play an important role as reservoirs of BYDV (Irwin and Thresh 1990). In the Pacific Northwest of the United States irrigated corn has been implicated as a source of inoculum and reservoir for *R. padi* and *M. avenae* moving between cereal grains during harvest and planting (Brown et al. 1984). *R. maidis* have been shown to be an important vector in this same region transporting BYDV between cereal grains and barnyard grass (Blackman et al. 1990). While progress is being made identifying reservoir sources, there are still many anomalies between the viral strains being found in grasses and those causing epidemic in crops.

For instance, in Idaho five species of BYDV (RPV, MAV, PAV, RMV, SGV) have been detected (Forster 1983), but historically the most consistently identified species among wheat in

southeastern Idaho has been SGV linked with the vector *S. graminum* (Forster et al. 1990). This creates a unique situation for Idaho, as this species of BYDV has not played an important role among other regions of the U.S. (Gildow 1990). In the western regions of Idaho and Washington states, PAV and RPV are the dominant viral species found in cereal crops and these viral outbreaks are associated with large populations of the vectors *R. padi* and *M. dirhodum* (Halbert and Pike 1985, Gildow 1990). The most recent survey of cereal aphids in wheat conducted in and around our study region in 2011-2013 found *S. avenae* and *Metopolophium festucae cerealium* Stroyan to be the most abundant aphid species (Halbert et al. 2013 for 2011 and 2012, unpublished for 2013). *S. avenae* is an efficient vector of BYDV-MAV but has also been shown to transmit BYDV-PAV (Rochow 1969). *M. festucae cerealium* recently was reported for the first time in the U.S. (Halbert et al. 2013) and its ability to transmit BYDV is unknown at this time. Among our study sites, located in north central Idaho and adjacent eastern Washington, we have identified PAV and SGV to be the most prevalent virus species in nonmanaged grasslands. Results from our samplings and data from aphid collections in wheat fields would suggest that *S. avenae* could be utilizing both habitats and transmitting PAV but there is no apparent SGV vector that has been linked between these two habitats.

Among the Palouse Prairie and CRP grass hosts harboring PAV, the majority is perennial species, making it difficult to deduce when the infection may have occurred or when PAV vectors were present in the landscape. In the fall of 2010 BYDV-PAV was identified in one sample of cheatgrass collected from Kramer CRP (Table 4.2). We could conclude from these results that in the fall of 2010 there were PAV aphid vectors present in this habitat and actively transmitting the virus among susceptible hosts. The source of inoculum for this infection could have arisen from an infected perennial host within the CRP or prairie, or from neighboring cereal

crops. Virus infection was detected in annual hosts at each site during each sampling period, indicating the presence of vector species within these habitats. Exploring the viral and vector species found among annual hosts can lend insight to yearly variations in vector composition and abundance in the community.

Given that very few aphids were encountered in only one of the CRP sites and none in the prairie remnants, the virus infection that we have documented can be attributed to non-colonizing aphid species. The infection rate of 40% among annuals indicates that there are aphid vectors within these habitats from year-to-year contributing to BYDV epidemiology in these systems. The aphids responsible for transmission in CRP and prairie habitats are not forming colonies in these habitats but are using the grasses as host for a period of time long enough for the extended feeding required to transmit the virus to occur (Power et al. 1991).

The BYDV infection rate we observed, around 46%, is relatively high compared to the 0-4% reported for grasses along field margins in Canada (Paliwal 1982) or 21% detected in California as a result of natural inoculum (Griesbach et al. 1990). This is most likely a result of improved detection methods, such as RT-PCR utilized in our study, which can detect much lower amounts of virus compared to ELISA and may be more reliable than the older methods of using vectors to conduct transmission studies from field-collected specimens (see Paliwal 1982). We acknowledge that while we implemented measures to minimize nonspecific amplification during the RT-PCR process, nonspecific amplification did occur in an optimal fashion as we were trying to optimize the procedure for a wide breadth of host species. Therefore, our report of 46% infection may include nonspecific RT-PCR amplifications, such that actual infection rates are lower in the field. The cloning and sequencing procedure performed to verify infection and determine viral species revealed that in most situations, viral species were identified. Nonspecific

amplification was encountered among the host species *Poa bulbosa* L. (bulbous bluegrass) and *B. tectorum* where we could not identify virus among some of the amplified products.

Conclusions

The endangered Palouse Prairie and CRP habitats have been shown to host B/CYDV species, with an infection rate of 46%. The viral species identified include BYDV-PAV and BYDV-SGV. Very few aphids were encountered in these habitats during sampling, suggesting that non-colonizing aphids may be responsible for transmission. The movement of aphids between agricultural crops and prairie or CRP habitats needs to be examined. The implications of viral infection on the competitive interactions of native and introduced species in prairie habitats should also be examined, as it may impact the effectiveness of restoration efforts in this endangered ecosystem.

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Tables

Table 4.1 A comprehensive list of all grass species collected at Palouse Prairie and CRP sites.

Plant Species	Growth Habit[a]	Native Status[b]	Host	Proportion Infected with B/CYDV
Aegilops cylindrica	A	I	Y	10/30
Agropyron cristatum	P	undetermined	Y	17/62
Alopecurus pratensis	P	I	Y	44/154
Arrhenatherum elatius	P	I	Y	123/185
Avena fatua	A	I	Y	12/30
Bromus inermis	P	I,N	Y	66/180
Bromus japonicus	A	I	N	0/15
Bromus racemosus	A	I	Y	27/65
Bromus tectorum	A	I	Y	55/150
Dactylis glomerata	P	I	Y	9/30
Elymus elymoides	P	N	Y	15/49
Elymus glaucus	P	N	Y	3/6
Elymus repens	P	undetermined	Y	8/15
Elymus sp.			Y	17/30
Festuca brevipila	P	I	Y	27/60
Festuca idahoensis	P	N	Y	152/293
Festuca sp.			N	0/1
Koeleria macrantha	P	N	Y	23/40
Lolium perenne	P/A	I	Y	2/6
Phalaris arundinacea	P	N	Y	25/45
Poa bulbosa	P	undetermined	Y	11/51
Poa pratensis	P	N	Y	42/84
Poa secunda	P	N	Y	1/42
Poa secunda var. ampla	P	N	Y	17/60
Poa sp.			Y	10/15
Pseudoroegneria spicata	P	N	Y	213/348
Thinopyrum intermedium	P	I	Y	83/122
Triticum aestivum	A	I	Y	18/30
Morphospecies 1	P		Y	11/15
Morphospecies 2	P		Y	23/59
Total				1064/2271

[a] A = annual, P = perennial

[b] I = introduced, N = native, according to USDA Plants Database for the lower 48 U. S.

Table 4.2 Seasonality and identity of viral species among selected hosts.

Plant Host	Location	Virus Species	Collection Time
Aegilops cylindrica (A)	Kamiak Butte CRP	SGV	July 2012
Bromus inermis (P)	SH Upper CRP	SGV	October 2010
	SH Upper CRP	SGV	October 2011
	SH Upper CRP	PAV	July 2012
	Kramer Prairie	SGV	May 2012
Bromus tectorum (A)	Kramer CRP	PAV	October 2010
	Kramer CRP	SGV	May 2012
Elymus glaucus (P)	Private Prairie	SGV	July 2012
Festuca idahoensis (P)	Paradise Ridge Prairie	PAV	June 2011
	SH Prairie	SGV	July 2012
Pseudoregneria spicata (P)	Private Prairie	PAV/SGV mix	November 2011
Morphospecies 1 (P)	Kramer CRP	SGV	October 2010

SH = Smoot Hill

Figure Legends

Figure 4.1 A map of the locations at which samples were collected. 1, Private Prairie; 2, Private CRP; 3. Kamiak Butte Prairie; 4. Kamiak Butte CRP; 5. Paradise Ridge Prairie; 6. Paradise Ridge CRP; 7. Kramer Prairie; 8. Kramer CRP; 9. Smoot Hill Prairie; 10. Smoot Hill Upper CRP; 11. Smoot Hill Lower CRP.

Figure 4.2 The proportion of samples infected at each prairie and CRP site, pooled across sampling times and the viral species identified at each location.

Figure 4.3 Seasonal variability in infection rate and viral species between two different perennial host species at two locations. SH = Smoot Hill

Figure 4.4 Seasonal variability in infection rate and viral species among three different annual host species at three locations. KB = Kamiak Butte

Figures

Fig. 4.1

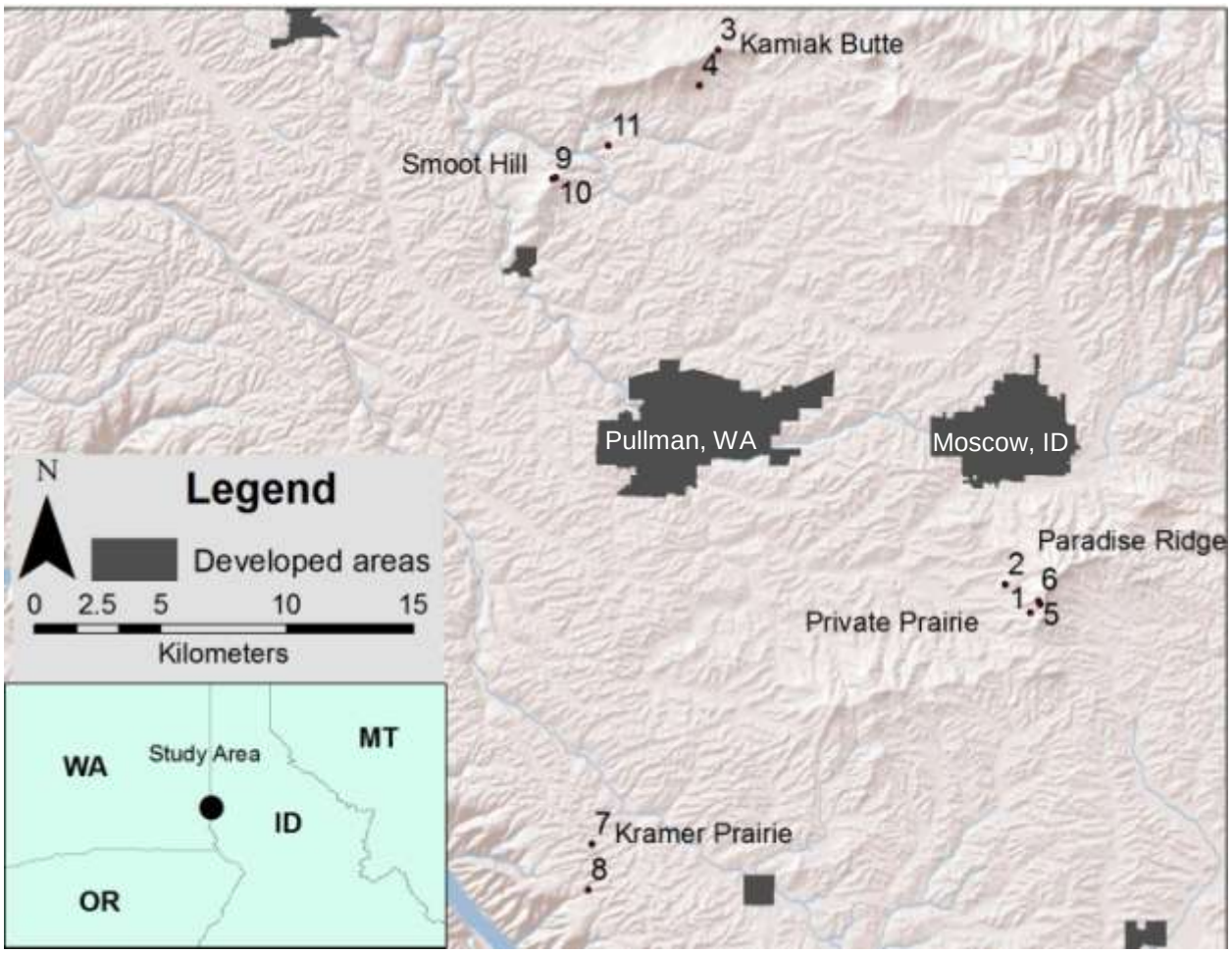


Fig. 4.2

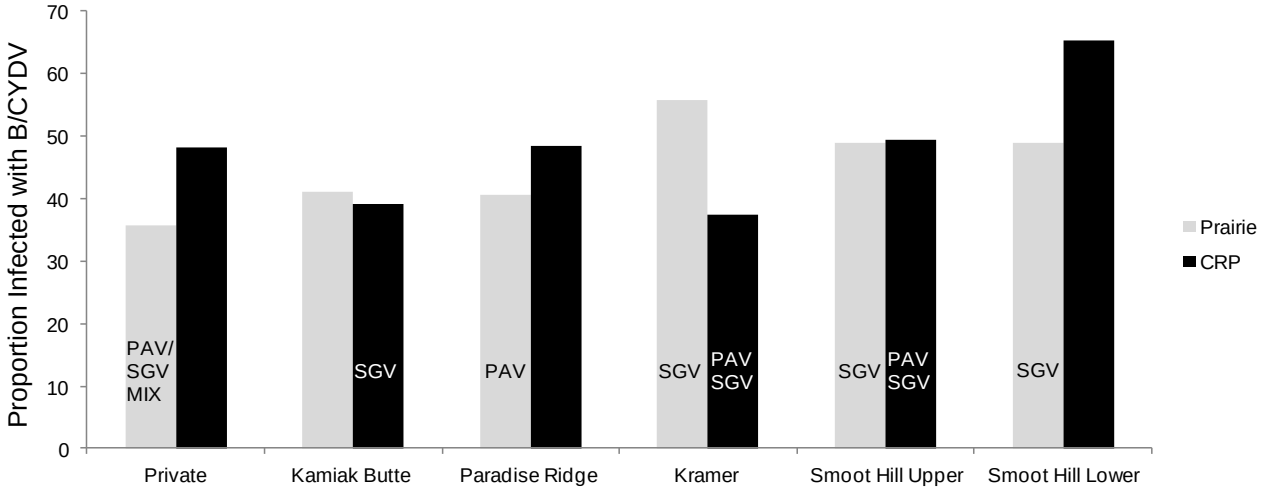

Proportion Infected with B/CYDV
70
60
50
40
30
20
10
0
PAV/ SGV MIX
SGV
PAV
SGV
PAV SGV
SGV
PAV SGV
SGV
Private
Kamiak Butte
Paradise Ridge
Kramer
Smoot Hill Upper
Smoot Hill Lower
Prairie
CRP

Fig. 4.3

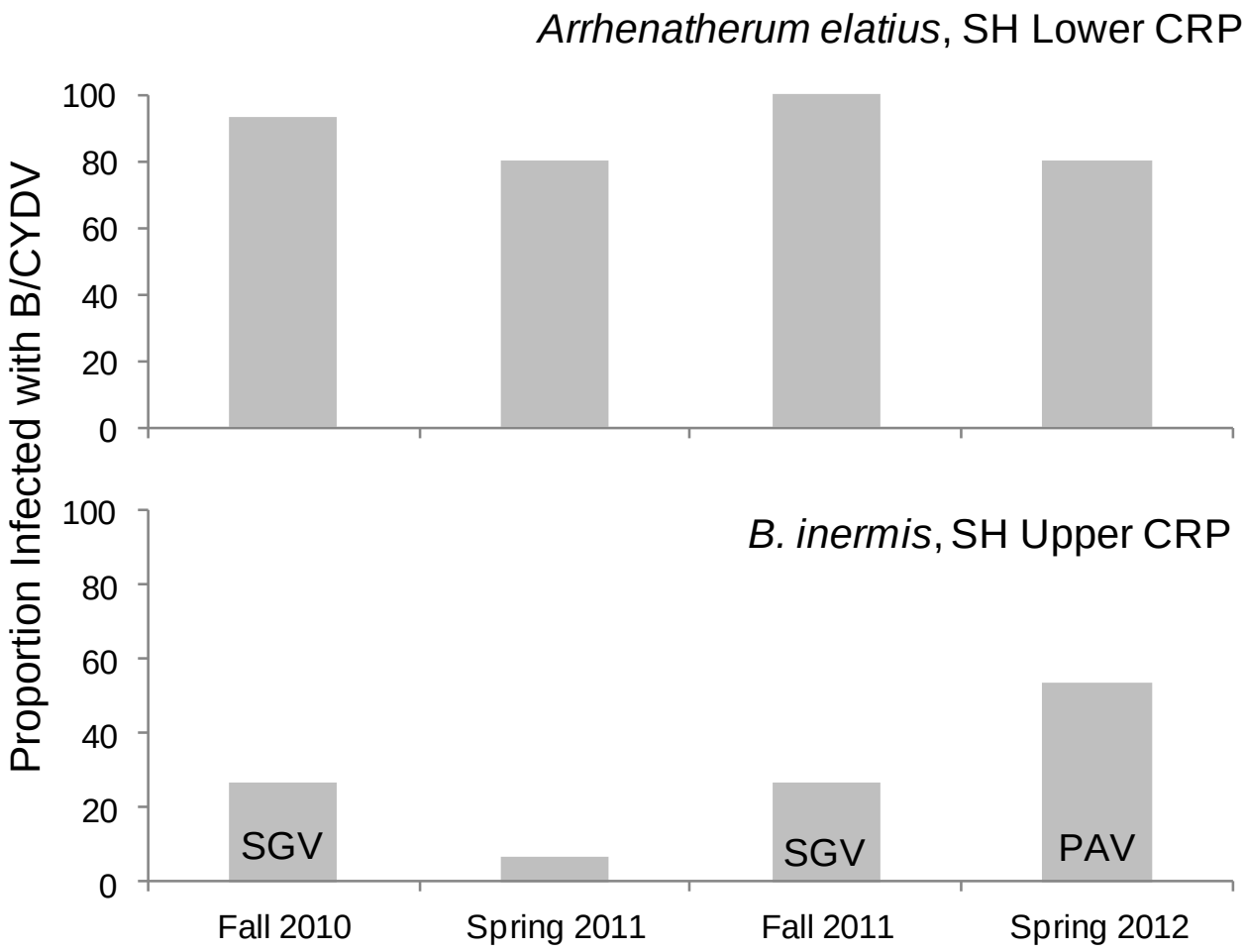
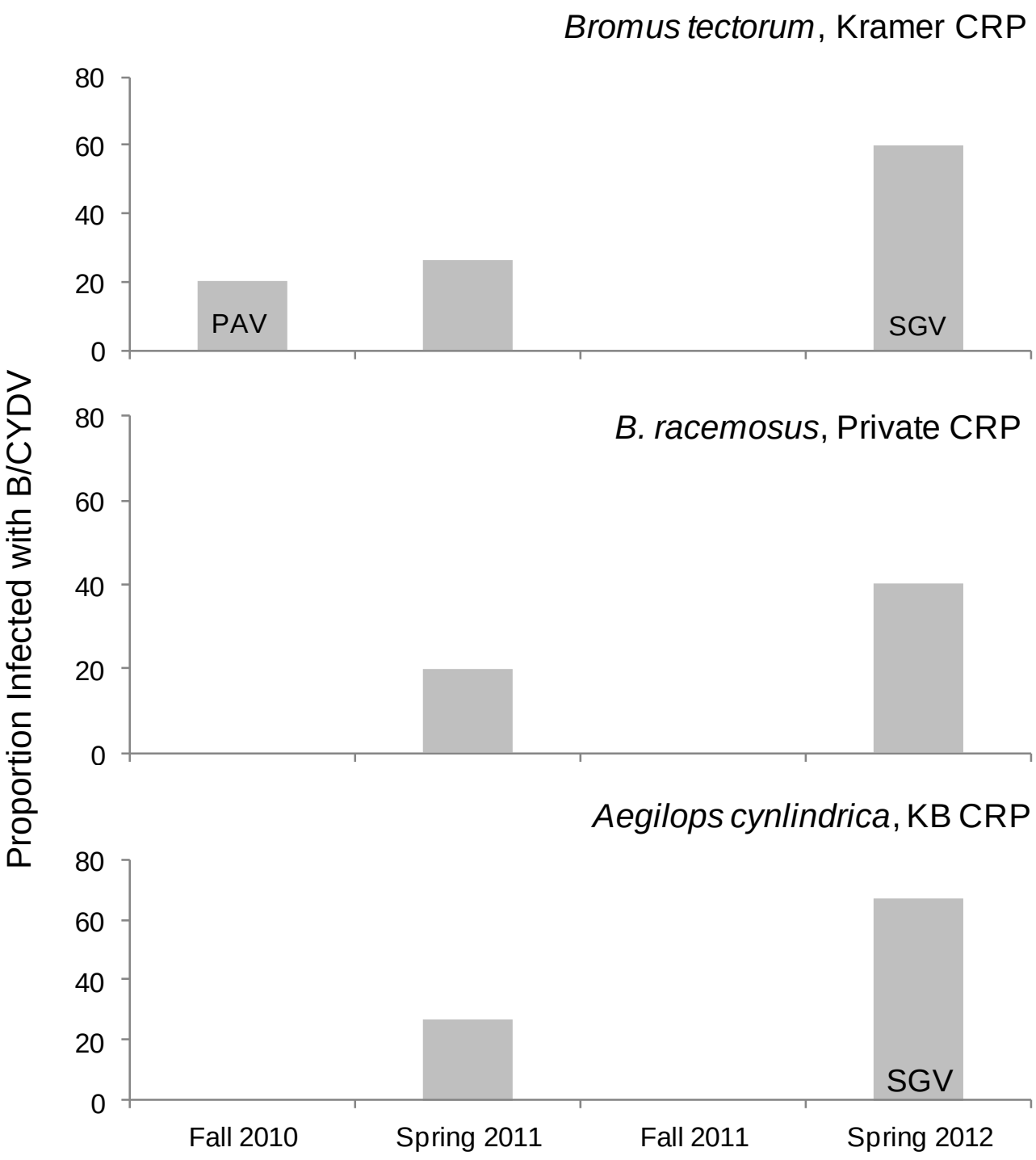

Arrhenatherum elatius, SH Lower CRP
100
80
60
40
20
0
Proportion Infected with B/CYDV
B. inermis, SH Upper CRP
SGV
SGV
PAV
Fall 2010
Spring 2011
Fall 2011
Spring 2012

Fig. 4.4


Bromus tectorum, Kramer CRP
B. racemosus, Private CRP
Aegilops cynlindrica, KB CRP
Proportion Infected with B/CYDV
80
60
40
20
0
PAV
SGV
SGV
Fall 2010
Spring 2011
Fall 2011
Spring 2012

Chapter 5: New experimental hosts of *Barley yellow dwarf virus* among wild grasses

Abstract

This study was conducted to examine species of grasses that have not been reported as hosts to *Barley yellow dwarf virus* (BYDV) and are commonly encountered in nonmanaged grasslands throughout the United States and Canada. Laboratory inoculations with the aphid *Rhopalosiphum padi,* a vector of BYDV-PAV were performed to determine the ability of 14 grass species to be infected with the virus, eight of which were not previously documented as potential hosts. Triple antibody sandwich-enzyme linked immunosorbent assays (TAS-ELISA) and reverse transcription polymerase chain reaction (RT-PCR) confirmed BYDV-PAV infection in 12 of the 14 species. This is the first report of BYDV infection of *Achnatherum occidentale, A. lettermanii, A. thurberianum, Danthonia intermedia, Poa fendleriana*, *Sporobolus airoides* and *S. cryptandrus*. Infection was confirmed in *Bromus inermis*, *Elymus elymoides, P. bulbosa, P. secunda* and cultivated barley, *Hordeum vulgare*, which served as controls. As a result of BYDV infection, reductions in plant height were observed for *P. bulbosa* and *P. fendleriana*. *Poa secunda* infected with BYDV had fewer leaves per plant compared to healthy plants of the same species. BYDV-infected *A. lettermanii* exhibited reduced dry weight in both below-ground and above-ground tissue. *Elymus wawawaiensis* was examined for the first time and did not become infected with BYDV-PAV using the vector *R. padi*. The implications of virus infection among the new hosts described here are discussed.

Key Words: virus vectors, virus ecology, Camas Prairie, Palouse Prairie, Conservation Reserve Program

Introduction

Barley yellow dwarf disease is one of the most economically important diseases of crops globally (Plumb 1983). The disease is caused by a group of viruses belonging to the family Luteoviridae referred to as *Barley/Cereal yellow dwarf virus* (B/CYDV, hereafter referred to as BYDV). BYDV has been documented to infect over 150 *Poaceae* species worldwide (D'Arcy 1995). The most economic damage as a result of BYDV infection occurs globally among wheat, barley and oat with average yield losses of 17%, 15% and 25%, respectively (Lister and Ranieri 1995).

BYDV is transmitted exclusively by aphids in a persistent, circulative, non-propagative manner. There are 25 species of aphids known to vector BYDV (Halbert and Voegtlin 1995). The predominant species in the U.S. include *Rhopalosiphum padi* L., *R. maidis* Fitch, *Metopolophium dirhodum* Walker, *Schizaphis graminum* Rondani, and *Sitobion* (formerly *Macrosiphum*) *avenae* Fabricius (Gildow 1990). These aphids have been recorded in agricultural and nonmanaged habitats (Halbert and Voegtlin 1995, Malmstrom 1998, Schotzko and Bosque-Pérez 2000, Bosque-Pérez et al. 2002), however their movement between habitats is not well understood.

BYDV is found in both agricultural habitats and nonmanaged grasslands. Nonmanaged grasslands may contribute to virus outbreaks in surrounding agricultural fields. Perennial grasses can serve as inoculum for annual species and emerging agricultural crops. For example, a survey of the Konza prairie in Kansas found BYDV infection prevalent in three of the four most

common tallgrass species, which include *Schizachyrium scoparium* (Michx.) Nash, *Panicum virgatum* L., and *Andropogon gerardii* Vitman (Garrett et al. 2004). In California, the presence of *Avena fatua* L., an exotic species, was shown to increase the prevalence of BYDV in nonmanaged grasslands where at least 66% of the species present have been shown to host the virus (Griesbach et al. 1990, Malmstrom et al. 2005a, Malmstrom et al. 2005b). *A. fatua* and other exotic annual grasses have been shown to be superior and preferred hosts of the aphid vector species *R. padi*, *R. maidis*, and *S. avenae* (Malmstrom et al. 2005b, Borer et al. 2009, Power et al. 2011).

Among the habitats occurring in the agricultural matrix of the Pacific Northwest of the United States there is an extensive amount of *Poaceae* diversity in nonmanaged grasslands, such as the Palouse and Camas Prairies (Lichtardt and Moseley 1997) and Conservation Reserve Program (CRP) lands. The Palouse Prairie is an endangered ecosystem with less than 0.1% remaining in small fragmented patches which are mostly privately owned (Noss et al. 1995, Looney and Eigenbrode 2012). The Palouse prairie is home to many threatened or endangered species and has become the focus of regional conservation efforts (Hanson et al. 2008, Sánchez de León and Johnson-Maynard 2009, Nyamai et al. 2011, Hatten et al. 2013). CRP is a USDA Farm Service Agency program that has been established to protect land from soil erosion, improve water quality and provide undisturbed habitats for threatened or endangered species. Producers can enroll for a 10-15 yr period during which time they plant cropland with resource-conserving vegetative covers. Among the species suggested and included in these seed mixes are a variety of grass species that have not been examined as potential hosts to BYDV.

This paper describes host range studies of BYDV using 14 grass species commonly encountered in nonmanaged grasslands, including prairies and CRPs. Virus inoculations were

performed under controlled conditions using an aphid vector. Enzyme-linked immunosorbent assay (ELISA) and reverse transcription polymerase chain reactions (RT-PCR) were used to confirm infection among new hosts. The impact of BYDV infection on plant growth was measured among species that served as hosts to BYDV. The implications for infection among new hosts and on the epidemiology of BYDV are discussed.

Materials and Methods

Virus and vector The historically dominant species of BYDV encountered in the US Pacific Northwest is PAV (Hewings and Eastman 1995) and it was therefore used in our study. BYDV-PAV is maintained by mass transfer in barley (*Hordeum vulgare*) cv. Sprinter using the aphid *R. padi* L. (Jiménez-Martínez et al. 2004). Viruliferous (virus carrying) colonies of *R. padi* are maintained in environmental growth chambers (20 ±2°C; 16:8 L:D) on barley and new plants added every three weeks to renew host material while maintaining virus inoculum. Plants are bottom watered and fertilized using a soluble N:P:K fertilizer (15:30:15) biweekly.

Plant species A total of 14 species (Table 5.1) was examined, including the cultivated host barley (cv. Sprinter), which served as a control. Species were selected based on reports of their occurrence in prairie and CRP habitats in the US Pacific Northwest (Lichtardt and Moseley 1997, Ogle et al. 2009) and non-documented susceptibility to BYDV. The noncultivated species *Alopecurus pratensis, Elymus elymoides, Bromus inermis, Poa secunda* and *P. bulbosa* served as additional controls because previous reports of BYDV infection have been made among these species (Oswald and Houston 1953, Watson and Mulligan 1960, Paliwal 1982, Guy et al. 1987, Malmstrom 1998). All seeds were surface sterilized prior to germination by soaking in sterile water for 30 min, followed by a 95% ethanol soak for 5 min, 10% bleach soak for 5 min and then

rinsing the seeds thoroughly five times with sterile water (LEHLE Seeds, Tucson, AZ). Seeds were germinated either on top of blotters or soil and transplanted, one seed per pot, to a 10.2 cm^2 plastic pot filled with a mixture of 3:1 Sunshine mix #1 (Afco Distribution, Spokane, WA): sand upon germination. A total of 24 plants of each species were used in inoculation tests except for *Elymus wawawaiensis* and *Sporobolus cryptandrus* which had lower germination rates (six and four plants tested, respectively). Barley was planted directly into pots (same size pots and soil mix as above) at the start of the experiment and again 41 days later to have control plants throughout the duration of the experiment. A total of 48 barley plants were tested. All plants were placed on trays, grown in an environmental chamber (22 ±2°C; 16:8 L:D), bottom watered and fertilized biweekly with a soluble N:P:K fertilizer (15:30:15).

Inoculation tests Plants were inoculated with BYDV-PAV at the 2-3 leaf stage (Zadoks et al. 1974), ranging from 14 to 92 days after planting (DAP) depending on the species. Most species were inoculated by 35 DAP, with the exception of *S. airoides* (72 DAP) and *S. cryptandrus* (92 DAP). Plants were removed from environmental chambers and inoculations performed on the laboratory bench (20±2°C). Half of all plants were inoculated while the other half remained aphid and virus-free to serve as healthy controls. Healthy controls remained on the laboratory bench during inoculations. Inoculations were done by caging 15 viruliferous *R. padi* on each plant using a 4-cm piece of 23 mm dialysis tube (14.6 mm D, Spectra/Por®) cage sealed on each end with a foam stopper. Aphids were allowed to feed on plants for a 96-hr inoculation access period (IAP), which is ample time for BYDV transmission (Jiménez-Martínez and Bosque-Pérez 2004). At the end of the IAP, aphids and cages were removed and plants returned to environmental chambers. Safer® insecticidal soap was used to kill aphids after inoculation.

Plants were monitored for symptom development. Plant height, the number of leaves and the number of tillers on each plant were recorded 31-51 days after inoculation (DAI), depending on species. Plants were harvested and the wet weight of above and below-ground tissues weighed 31-105 DAI, depending on species. Harvested plant tissues were dried in an oven at 70°C for 72 hrs and dry weights recorded.

Data Analysis The differences in growth parameters of BYDV-PAV infected and noninfected plants were compared using independent samples t tests for each species. Growth parameters compared include the mean plant height, mean number of leaves per plant, mean number of tillers per plant, and mean above-ground and below-ground wet and dry weight of plant tissue. Statistical tests were carried out using PROC T TEST (SAS Institute 9.2). Confidence levels were examined at the 0.05, 0.01 and 0.001 level to strengthen comparisons.

ELISA Triple antibody sandwich enzyme-linked immunosorbent assays (TAS-ELISA) using BYDV-PAV polyclonal antibodies were used for virus detection (Agdia Inc., Elkhart, IN). Tissue was sampled (ca. 0.1-2.0g) at the time that physical measurements were recorded, 31-51 DAI, depending on species. Tissue was placed in mesh sample bags (Agdia, ACC 00930) and sent directly to the Agdia testing laboratory for processing. Absorbance values were read at A_{405}nm wavelength. Samples were considered positive if the optical density was two times greater than the negative controls.

Nucleic acid extraction A subset of samples was tested using RT-PCR to verify ELISA test results. Total nucleic acid extraction was performed using a modified Dellaporta method (Pappu et al. 2005). Each plant was sampled by making four punches with the lid of a 1.5 mL microfuge tube and then ground in liquid nitrogen with a micropestle. To each ground sample, one mL of

Dellaporta buffer (100 mM Tris, pH 8.0, 50 mM EDTA, 500 nM NaCl and 10 mM mercaptoethanol) and 140 µL of 10% SDS was added; samples were vortexed and incubated at 65°C for 15 min. Samples were mixed every 3 min during incubation. To each sample 250 µL of 8M potassium acetate was added, tubes inverted to mix and held for 10 min on ice. Samples were centrifuged for 10 min at 13,000 rpm and the supernatant transferred to a new tube. To precipitate nucleic acids, 600 µL of isopropanol was added and samples were incubated for 7 min on ice. Samples were centrifuged for 7 min at 13,000 rpm and 10°C. The resulting pellet was washed with cold 70% ethanol, air-dried overnight and resuspended in 60 µL of DEPC water.

Reverse transcription-polymerase chain reaction (RT-PCR) Reverse transcription was performed using M-MLV Reverse Transcriptase (Promega Corp., Madison, WI) following the manufacturer's guidelines with random hexamer primers. In order to minimize the effects of potential inhibitors in noncultivated species, 38 mg/mL of T4 Gene 32 Protein (New England Biolabs Inc., Ipswich, MA) was added to each sample. PCR was performed using a total of 1µL of RT product and My taq (New England Biolabs Inc., Ipswich, MA) and 34.2 µg/mL of T4 Gene 32 Protein. Primers were used that have been designed to detect a region of the coat protein for BYDV-PAV (Ingwell et al. 2012; see Suppl. Mat.). The samples were amplified on a Multigene Labnet thermal cycler (BioExpress, Kaysville, UT, USA) with the following PCR conditions: 95°C for 2 min, followed by 11 cycles of 95°C for 30s, 62°C for 30s and 72°C for 1min 30s decreasing 0.5°C each cycle, followed by 22 cycles of 95°C for 30s, 56°C for 30s and 72°C for 1 min 30s concluded with a hold at 72°C for 7 min. PCR products were amplified following Ingwell et al. (2012; see Suppl. Mat.).

Results

BYDV-PAV infection was detected in 100% of the cultivated host species, *H. vulgare,* verifying inoculation and detection techniques were effective (Table 5.1). Virus infection was detected in 23% of *B. inermis*, 33% of *E. elymoides*, 50% of *P. secunda*, and 75% of *P. bulbosa*, which served as control species;, however virus infection was not detected in *A. pratensis,* the remaining control species (Table 5.1). Of the other 10 species examined, BYDV-PAV infection was detected using TAS-ELISA and verified with RT-PCR among *A. occidentale*, *A. lettermanii*, *A. thurberianum*, *D. intermedia*, *P. fendleriana*, *S. airoides* and *S. cryptandrus* (Table 5.1). This is the first report of BYDV infection of these grass species. BYDV-PAV infection was not detected in *E. wawawainesis* (Table 5.1). The number of individuals tested among *E. wawaiensis* and *S. cryptandrus* was low because of poor germination and survival rates.

Plant height was significantly reduced among BYDV-PAV-infected *P. bulbosa* (independent t tests, df = 19, p = 0.015; Table 5.2) recorded 51 DAI and among *P. fendleriana* (independent t tests, df = 15, p = 0.052; Table 5.2) recorded 47-51 DAI. BYDV-PAV-infected *P. secunda* had significantly fewer leaves than healthy individuals of the same species (independent t tests, df = 6.254, p = 0.037; Table 5.2) recorded 47 DAI. Dry weight was significantly reduced in both above-ground (independent t tests, df = 6, p = 0.001; Table 5.3) and below-ground tissue (independent t tests, df = 6, p = 0.039; Table 5.3) of *A. lettermanii* as a result of BYDV-PAV infection recorded 105 DAI. While not statistically comparable, the BYDV-infected *Sprobolus airoides* and *S. cryptandrus* had increased above-ground biomass (Table 5.3). The cultivated control, *H. vulgare*, had significant reductions in BYDV-PAV-infected above-ground wet weight (independent t tests, df = 33.66, p = 0.031; Table 5.3) recorded 36 DAI.

Discussion

The cultivated control species, *H. vulgare*, confirmed that our inoculation and detection techniques were successful. Additional, noncultivated controls were included to verify that ELISA and RT-PCR techniques were able to detect infection in a variety of host plants containing diverse metabolites and potential inhibitors. Virus detection among *B. inermis, E. elymoides, P. secunda,* and *P. bulbosa* confirms previous reports of these species as hosts to BYDV (Oswald and Houston 1953, Guy et al. 1987, Malmstrom 1998) and indicate that the techniques used could detect infection among noncultivated species. The noncultivated species *A. pratensis,* which was included as an additional control was not detected as a host of BYDV-PAV in our experiment. Historically, this species has been shown to host a virulent isolate of BYDV recovered in Great Britain (Watson and Mulligan 1960) but not host three other isolates of *Barley/Cereal yellow dwarf virus* that have been examined experimentally (Oswald and Houston 1953, Bruehl and Toko 1957, Watson and Mulligan 1960).

Symptoms such as yellowing or purpling of leaves did not occur in any of the nine new hosts of BYDV reported here. Reductions in plant growth, such as stunting or reduced biomass, indicate that *A. lettermanii*, *P. fendleriana* and *P. secunda* are susceptible to BYDV-PAV. *A. thurberianum*, *A. occidentale, D. intermedia, E. elymoides*, *S. airoides* and *S. cryptandrus* are symptomless hosts that appear to be tolerant to BYDV-PAV infection. The observed increase in above-ground plant biomass among the *Sporobolus* sp. may be the result of accumulation of sugars and starches in the tissues, which have been recorded as a result of BYDV-infection (Jensen and D'Arcy 1995). These results suggest that while infection may occur in the wild, it may be difficult to visually identify.

All of the species documented here as new hosts of BYDV are perennial grasses native to the U.S. *A. lettermanii, A. thurberianum, P. fendleriana* and *S. cryptandrus* are all commonly used as conservation species in seed mixes for CRP lands in the intermountain west (Ogle et al. 2012). *D. intermedia*, and *A. occidentale* are found among the endangered Camas and Palouse Prairie grasslands (Lichtardt and Moseley 1997) and throughout the western half of the United States and Canada. *S. airoides* is found throughout the western U.S., Missouri, Arkansas, South Carolina and New York. It is commonly used as a forage crop for horses and cattle and is seeded to re-colonize oil well pits and saline waste sites because of its high salt tolerance (Brakie 2007). Field surveys and vector sampling need to be done to examine the new hosts documented here under field conditions.

Our understanding of insect vector movement between agricultural and nonmanaged landscapes is limited. Vector specificity among the B/CYDV complex contributes to differences in the predominant viral species among hosts and geographic regions depending on vector populations (Rochow 1979, Rochow and Carmichael 1979) and can lend insight into habitat use of vector species. Some studies show that viral species do not overlap between agricultural and nonmanaged ecosystems, suggesting vector discrimination between the two habitats. For example, while PAV is the most commonly reported species of BYDV occurring in cereal fields in Kansas, a recent survey of the grass species in the Konza prairie found MAV and SGV to be predominant in these nonmanaged grasslands (Garrett et al. 2004). In contrast, in California, there is a large overlap in the virus species present in agricultural and nonmanaged habitats landscapes suggesting vector utilization of both habitats (Malmstrom 1998). In a survey of cereal fields and adjacent grassland margins in Canada, researchers found low populations of vectors and an overall low incidence of virus among grasslands compared to neighboring crops and

concluded that aphids prefer crop hosts (Paliwal 1982). To better predict the implications of nonmanaged grasses to act as vector reservoirs and potential sources of inoculum to susceptible crops, studies should be conducted at local scales to examine virus species and vector movement between habitats.

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Tables

Table 5.1 Grass species tested in BYDV-PAV host range experiments and results from inoculation tests.

Species	Common Name	Growth Habit[a]	Seed Source[b]	Proportion Infected	Host Y/N
Achnatherum occidentale subsp. *occidentale*	Western needlegrass	P	1	3/13	Y
Achnatherum lettermanii	Letterman's needlegrass	P	1	4/12	Y
Achnatherum thurberianum	Thurber's needlegrass	P	1	5/12	Y
Alopecurus pratensis	Meadow foxtail	P	1	0/14	N
Bromus inermis subsp. *Inermis*	Smooth brome	P	1	3/13	Y
Danthonia intermedia	Timber oatgrass	P	1	12/13	Y
Elymus elymoides	Squirreltail	P	1	4/12	Y
Elymus wawawaiensis	Snake River wheatgrass	P	1	0/4	N
Poa bulbosa	Bulbous bluegrass	P	1	9/12	Y
Poa fendleriana	Muttongrass	P	3	5/17	Y
Poa secunda	Sandberg bluegrass	P	1	6/12	Y
Sporobolus airoides	Alkali sacaton	P	1	1/12	Y
Sporobolus cryptandrus	Sand dropseed	P	1	1/1	Y
Hordeum vulgare[c]	Barley cv. Sprinter	A	2	24/24	Y

[a] A= Annual; P =Perennial

[b] Seed Source: 1= USDA, ARS, NPGS Western Regional Plan Introduction & Research Station, Washington State University, Pullman, WA 99164-6402, 2 = Westbred, a Unit of Monsanto Company, St. Louis, MO 63167, 3 = Wind River Seed, Manderson, WY 82432

[c] cultivated species used as positive control

Table 5.2 Plant growth characteristics measured for BYDV-PAV host species tested. The mean of infected and noninfected plants within each species were compared using independent samples t tests. Data reported are means ± s.e.m.

Species	Infection Treatment	Mean ± SE		
		Plant Height (mm)	No. leaves/plant	No. tillers/plant
Achnaterum occidentale subsp. *occidentale*	Noninfected	754.4±49.10	16.7±4.31	6.3±1.49
	Infected	736.0±83.05	17.7±3.76	6.0±1.00
Achnatherum lettermanii	Noninfected	507.3±20.15	10.6±0.85	5.8±0.50
	Infected	519.6±22.59	10.6±1.50	5.0±0.71
Achnatherum thurberianum	Noninfected	406.3±22.73	6.7±0.56	3.3±0.30
	Infected	423.0±14.51	6.2±0.37	3.8±0.20
Bromus inermis subsp. *Inermis*	Noninfected	540.3±40.43	32.6±7.37	6.9±0.99
	Infected	538.3±68.67	34.0±5.03	7.3±1.45
Danthonia intermedia	Noninfected	271.8±18.35	19.5±1.62	5.8±0.60
	Infected	287.4±14.43	19.7±1.37	6.1±0.47
Elymus elymoides	Noninfected	439.0±19.97	39.3±4.74	14.0±1.73
	Infected	399.3±7.79	31.8±1.80	10.5±0.29
Poa bulbosa	Noninfected	133.1±6.61	16.0±2.33	-
	Infected	**107.8±6.61****	14.0±1.76	-
Poa fendleriana	Noninfected	209.8±20.47	29.9±3.68	13.4±1.90
	Infected	**136.2±21.77***	29.2±7.62	10.6±2.50
Poa secunda	Noninfected	138.5±8.17	18.7±2.24	7.2±1.52
	Infected	120.0±8.65	**36.5±6.39***	11.7±2.51
Sporobolus airoides	Noninfected	347.5±32.36	6.8±0.59	2.6±0.23
	Infected	425	15	5
Sporobolus cryptandrus	Noninfected	341.0±31.00	13.5±0.50	3.0±0.00
	Infected	555	11	2
Hordeum vulgare[a]	Noninfected	485.1±9.14	57.0±3.63	14.4±0.85
	Infected	459.4±10.96	49.8±5.53	12.6±1.57

[a] cultivated species used as positive control
* $p < 0.05$, ** $p < 0.01$

Table 5.3 Biomass of above and below-ground tissue in BYDV-PAV infected and noninfected host plants. The mean weight of noninfected and infected plants within each species was compared using independent samples t tests. Data reported are means ± s.e.m.

		Mean ± SE			
		Above-ground tissue		Below-ground tissue	
Species	Infection treatment	Wet weight (g)	Dry weight (g)	Wet weight (g)	Dry weight (g)
Achnaterum occidentale subsp. *occidentale*	Noninfected	8.89±0.77	1.30±0.33	2.73±0.38	0.20±0.13
	Infected	5.90±1.87	0.93±0.34	2.02±0.50	0.18±0.10
Achnatherum lettermanii	Noninfected	10.75±0.55	3.04±0.18	8.50±0.90	1.49±0.17
	Infected	5.26±1.00	**1.22±0.34*** **	4.97±1.22	**0.71±0.19***
Bromus inermis subsp. *Inermis*	Noninfected	12.41±3.77	2.13±0.82	9.97±2.62	1.14±0.43
	Infected	10.56±1.73	1.47±0.41	14.22±1.25	2.00±0.53
Danthonia intermedia	Noninfected	4.35±0.81	1.07±0.20	2.77±0.49	0.23±0.06
	Infected	4.39±0.69	0.80±0.16	2.17±0.42	0.17±0.05
Poa fendleriana	Noninfected	4.13±2.17	0.76±0.59	1.50±0.60	0.15±0.08
	Infected	2.25±0.29	0.39±0.03	0.74±0.14	0.08±0.04
Poa secunda	Noninfected	0.97±0.20	0.17±0.05	2.20±0.62	0.34±0.11
	Infected	0.94±0.21	0.22±0.07	1.96±0.43	0.32±0.09
Sporobolus airoides	Noninfected	0.87±0.20	0.19±0.04	0.63±0.10	0.05±0.01
	Infected	2.16	0.5	0.51	0.07
Sporobolus cryptandrus	Noninfected	0.73±0.09	0.17±0.04	0.36±0.01	0.04±0.00
	Infected	2.24	0.3	0.37	0.04
Hordeum vulgare[a]	Noninfected	20.66±1.30	-	-	-
	Infected	**10.42±0.80***	-	-	-

[a] cultivated species used as positive control

* $p < 0.05$, *** $p < 0.001$

Chapter 6: *Arundo donax* infection with *Barley yellow dwarf virus* has implications for biofuel production and nonmanaged habitats

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Abstract

Arundo donax, a perennial grass, is being considered as a renewable-fuel feedstock. *A. donax* reproduces vegetatively and can produce up to 30 metric tons/hectare of biomass, making it an ideal candidate for biofuel. There is limited information on the susceptibility of *A. donax* to pests and pathogens and the implications for biofuel production. This study examined the ability of *Barley yellow dwarf virus* (BYDV-PAV) to infect *A. donax* under controlled conditions using the aphid vector *Rhopalosiphum padi* and the impact of BYDV-PAV on *A. donax* growth. Virus systemicity and the potential for *A. donax* to serve as a BYDV-PAV inoculum source were also examined. Immunological techniques confirmed BYDV-PAV infection of *A. donax*, but there were no significant differences in height, number of leaves and number of shoots per rhizome between infected and healthy plants, suggesting *A. donax* is tolerant to BYDV-PAV. BYDV-PAV infection was determined to be systemic within the host, resulting in virus detection in multiple shoots of the same rhizome. Transmission from infected *A. donax* to susceptible barley plants was demonstrated using the vector *R. padi*. These results demonstrate that *A. donax* is an alternative host to BYDV-PAV. Large *A. donax* monocultures may provide a perennial inoculum source to neighboring small grain crops, like wheat and barley, as well as turf and forage grasses. Field studies are needed to examine the movement of insect vectors between *A. donax*,

susceptible crops and nonmanaged ecosystems to assess the ecological and epidemiological implications of these findings.

Key Words: giant reed; virus ecology; virus inoculum; virus vectors

Introduction

Energy production that uses renewable biobased feedstocks rather than fossil fuels continues to be a high priority in regions of the United States (US) and internationally. Success of using such biobased fuels will depend, among other factors, on identifying feedstocks that are sustainable and have a guaranteed dependable supply chain. Commercial agricultural production provides the most reliable source of biomass feedstocks. There is also a desire that the production of biomass feedstocks not displace food crops. Therefore, the selection of a species to be used for biomass production requires careful consideration. Species that will be economical for the production of biomass will likely be perennials that produce biomass quickly under low-input conditions. It is critical that the benefits of producing a species for biomass production not outweigh the risks to agriculture and the environment.

Arundo donax L. (giant reed), a large size perennial grass that is native to Asia and was introduced into the US in California in the 1800s [1], can now be found in all of the US southern states and extends into Nevada and Utah in the west. *A. donax* has the potential to produce more than 30 metric tons of dry biomass per hectare [2]. Under ideal growing conditions with warm temperatures and ample water, *A. donax* has been reported to grow up to 0.3-0.7 m per week and typically reaches a height of 8 meters, making it an ideal candidate for biofuel [3, 4]. In the U.S., *A. donax* reproduces vegetatively via rhizomes and rooting of nodes [4].

Arundo donax is currently in production or testing stages as a biofuel crop in Italy and Australia [5, 6]. In the US, the Environmental Protection Agency has approved the production of *A. donax* as a renewable fuel feedstock [7]. Florida, Texas and North Carolina are preparing to use *A. donax* as a biofuel crop [8, 9]. In the US Pacific Northwest, Portland General Electric (PGE) is evaluating *A. donax* as a potential fuel replacement at its coal powered plant in Boardman, Oregon. PGE estimates that the Boardman plant would require 25,000 to 36,000 hectares of *A. donax* within an 80 kilometer radius of the Boardman plant [10]. If *A. donax* is to be used for biomass production, the potential impacts of its production on the environment and on the existing agricultural sectors must be assessed.

The most frequent objection to *A. donax* as a biofuel is the potential for it to become invasive [9, 11]. In California, *A. donax* was originally introduced for erosion control on riverbanks but the plant is now an invasive weed. It is widely spread in riparian areas causing wildlife habitat loss, increasing fire and flooding hazards and affecting water conservation [12, 13]. In addition to the potential to become an invasive species, there are other risks that need to be examined in relation to *A. donax* production to prevent potential negative impacts to established cropping systems. One such risk is the potential for *A. donax* to become infected with pathogens that could affect neighboring crops.

Barley yellow dwarf virus (BYDV; family *Luteoviridae*) causes one of the most devastating diseases of cereal crops worldwide [14]. In addition to cereal crops there are over 150 documented hosts of BYDV, all belonging to the family Poaceae [15]. The greatest economic damage as a result of BYDV infection occurs in crops such as wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). Average yield losses due to BYDV disease range from 11-47% in wheat and 15-33% in barley [16]. BYDV is transmitted exclusively by aphids in

a persistent, circulative manner whereby the virus is transported across membranes within the vector ultimately residing in the accessory salivary glands and resulting in a vector that can transmit the virus for life [17]. There are at least 25 species of aphids that transmit BYDV [18], including *Rhopalosiphum padi* L., *R. maidis* Fitch, *Metopolophium dirhodum* Walker, *Schizaphis graminum* Rondani, and *Sitobion* (formerly *Macrosiphum*) *avenae* Fabricius, which are commonly encountered in the US Pacific Northwest, including Oregon and neighboring states [19, 20].

The pathosystem described above has been reported in nonmanaged ecosystems throughout the United States, where disease impact on plant communities is less well understood [21, 22]. Field surveys of nonmanaged grasslands in the US have detected BYDV infection among a variety of native and introduced *Poaceae* species [21-24]. BYDV infection of grasses in nonmanaged ecosystems has been shown to impact the competitive dynamics between native and invasive grasses and has been suggested as a facilitator of nonnative grass invasion in California grasslands [25-28].

Under greenhouse conditions, *R. maidis* were observed feeding on *A. donax* (C. Mallory-Smith, personal observations), which raised the question as to whether *A. donax* could be infected with BYDV. If *A. donax* is a host to BYDV and is fed upon by vectors in the field, it could serve as a green bridge to spread the disease to other susceptible hosts such as wheat, barley, turf and forage grasses and corn (*Zea mays* L.). BYDV currently overwinters in grasses, volunteer cereals, irrigated maize and winter wheat [29] and the perennial growth habit of *A. donax* could provide an additional overwintering host and potential inoculum source for both fall and spring sown crops.

In order to better evaluate the implications of *A. donax* as a biofuel crop its interactions with BYDV and its insect vectors need to be examined. The objectives of this study were to: 1. determine if *A. donax* could be infected with BYDV under controlled conditions using aphids as vectors, 2. assess the impact of BYDV infection on *A. donax* growth, 3. determine if BYDV is systemic in *A. donax*, and 4. examine if *A. donax* could serve as an inoculum source by conducting virus transmission studies from it to another host via aphids.

Materials and Methods

Virus and vector BYDV-PAV is the historically dominant species of BYDV in the US Pacific Northwest [29] and for this reason was used in our studies. A Washington state isolate of BYDV-PAV, maintained by mass transfer of *R. padi* [30] on barley cv. Sprinter at the University of Idaho Agricultural Biotechnology Laboratory in environmental growth chambers (20 ±2°C; 16:8 L:D) was used for all experiments. Viruliferous (virus carrying) and nonviruliferous colonies of *R. padi* are maintained by mass transfer on virus-infected or non-infected Sprinter barley plants, respectively and kept in environmental growth chambers (20 ±2°C; 16:8 L:D) at the University of Idaho.

Barley control plants Barley cv. Sprinter served as a positive control in all experiments. Barley was planted at a density of three seeds per pot in 10.2cm^2 plastic pots filled with a mixture of 6:1:0.02 ratio of Sunshine mix #1 (Afco Distribution, Spokane, WA, US):sand:Osmocote®. Pots were placed on trays in an environmental chamber (22 ±2°C; 16:8 L:D) and bottom watered. Upon germination, plants were thinned to one per pot and fertilized biweekly with a soluble N:P:K fertilizer (15:30:15). Four barley plants were inoculated at the same time as *A. donax*

plants (20 days after planting: DAP) following the procedure described in the section below. One barley plant remained aphid and virus free to serve as a healthy control.

***A. donax* plants** *A. donax* plants were obtained from Boo-Shoot Gardens, LLC (Mount Vernon, WA, US) and were planted in 13.5cm^2 (2.6 L) plastic pots filled with the soil mix described above. Pots were placed on trays in the same growth chamber as barley, bottom watered and fertilized biweekly with a soluble N:P:K fertilizer (15:30:15). Each pot contained one to four shoots initially. All shoots were cut back to the soil level to obtain similar aged and sized shoots at the start of the experiment. Twelve days after being cut, one shoot in each of 11 pots was inoculated with BYDV. Four pots remained aphid and virus free to serve as controls.

Virus-infection tests Plants were removed from environmental chambers and inoculations were performed on the laboratory bench at 20±2°C. For virus inoculation of both *A. donax* and barley controls, 15 viruliferous *R. padi* were caged per plant for a 72-hour virus inoculation access period (IAP), which is ample time for *R. padi* to settle, feed and transmit BYDV-PAV [31]. Aphids were secured to barley plants in cages consisting of a 4-cm long piece of 23 mm dialysis tubing (14.6 mm D, Spectra/Por®) sealed on each end with a foam stopper [30]. Aphids were secured to *A. donax* using clip cages (2.5 cm D, 2 cm H) because the leaves were too broad for dialysis cages. Cages and aphids were removed after 72 hrs and plants returned to environmental chambers. Safer® insecticidal soap was used to kill any aphids that might have escaped from cages during the IAP. The inoculated leaf was marked on the underside using a permanent marking pen. Plants were then monitored for symptom development. Tissue was sampled and tested for virus presence using enzyme-linked immunosorbent assay (ELISA) [32] 21 and 31 days after inoculation (DAI) (see below). Symptoms were described and photographed 12 and 21 DAI. The number of shoots per rhizome, height of the tallest shoot, height of the inoculated

shoot, height of all shoots per rhizome and the number of leaves on the tallest shoot, the inoculated shoot and every shoot per rhizome were recorded 46 DAI for *A. donax* plants only.

Transmission tests To evaluate the potential for *A. donax* to act as a source of inoculum of BYDV-PAV the ability of *R. padi* to acquire the virus from infected *A. donax* plants and transmit it to a susceptible cultivated host, barley cv. Sprinter was examined. Nonviruliferous *R. padi* were used in this experiment. Plants were removed from environmental chambers and placed on the laboratory bench (22±2°C) during transmission tests. Six previously BYDV-infected *A. donax* (see virus infection tests above) were used as source plants along with one BYDV-infected Sprinter barley and one virus-free *A. donax*, which served as positive and negative control plants, respectively. Multiple shoots among the six BYDV-infected *A. donax* plants served as inoculum sources resulting in a total of 12 shoots of BYDV-infected *A. donax* used in the experiment. Multiple shoots per pot were used to evaluate the systemicity of virus among shoots from the same rhizome. Approximately 74 DAI, aphids were caged on source plants for a virus acquisition access period (AAP) of 72 hours [31]. A total of 720 aphids were used (30 aphids per cage, 2 cages per shoot among 12 shoots) in the AAP from BYDV-infected *A. donax*. A total of 60 aphids were used (30 aphids per cage, 2 cages per shoot/plant) in the AAP on each of the control plants. Clip cages were used to secure aphids on *A. donax* while dialysis tube cages were used on barley plants. After the 72-hr AAP, aphids were transferred from source plants to indicator plants for a 72-hr IAP. Indicator plants were Sprinter barley planted and grown as described in section 2.2. Aphids were caged on barley plants 14 DAP (2-3 leaf stage) using dialysis tube cages as described above. A total of 43 barley plants were inoculated; two with aphids from the healthy *A. donax* source plant, five with aphids from the BYDV-infected barley source plant and 36 with aphids from BYDV-infected *A. donax* source plants. After the 72 hr

IAP, aphids and cages were removed and barley plants returned to environmental chambers (22 ±2°C; 16:8 L:D) for virus incubation.

ELISA In order to determine virus infection, tissue was collected from *A. donax* and Sprinter barley control plants from the virus infection tests 21 and 31 DAI. One sample from each barley control plant (ca. 0.30 g of tissue) was tested along with tissue from each shoot of healthy *A. donax* plants (0.30-0.50 g of tissue). For BYDV-inoculated *A. donax*, tissue was collected from the inoculated leaf ($\leq$ 0.30 g), the youngest leaf ($\leq$ 0.30 g) on the inoculated shoot and leaves from each shoot (0.3-0.5 g of tissue) and tested individually. A pooled sample of all symptomatic *A. donax* leaves in each pot ($\leq$ 0.50 g) was also tested.

Indirect triple antibody sandwich ELISA (TAS-ELISA) was performed using commercially available antibodies for BYDV-PAV utilizing a kit from Agdia Inc. (Elkhart, IN, USA) following the manufacturer's protocol. Tissue was ground using Agdia sample mesh bags (ACC 00930) and a homogenizer and was diluted 1:10 in general extraction buffer (Agdia: ACC00955). In addition to the test samples and the positive and negative controls from the experiments, each ELISA plate contained positive controls provided by Agdia (LPC 27500). Samples were duplicated on each plate. Testing was conducted the same day that tissue was ground 21 and 31 DAI. Tests were repeated three days post-grinding at the 31 DAI sampling. Microtiter plates were read in an ELISA plate reader at $A_{405\ nm}$ at daily intervals with incubation at 4°C between readings for four days. Absorbance values are reported.

To examine barley indicator plants from the virus transmission tests, one sample from each barley plant (ca. 0.30 g of tissue) was sampled 33-37 DAI into sample mesh bags (Agdia,

ACC 00930). Samples were sent directly to Agdia Inc. for testing using TAS-ELISA due to time constraints and their ability to optimize the ELISA procedure.

Statistical analysis Independent-samples t tests were used to analyze the differences in the growth parameters measured between non-inoculated and BYDV- infected *A. donax* plants. The mean number of shoots per rhizome, mean height of the tallest shoot, overall mean height of the rhizome, mean number of leaves on the tallest shoot and total mean number of leaves per plant were compared. Statistical tests were performed using PROC TTEST (SAS Institute 9.2) with a 95% confidence interval (alpha = 0.05).

Results

Virus-infection tests *R. padi* readily survived on *A. donax* during the 72 hr IAP and transmitted the virus. Symptoms of BYDV-PAV were first observed on inoculated *A. donax* 12 DAI (Fig. 6.1A). They began as yellowing lesions noted initially on the inoculated shoot in leaves above the inoculated leaf. As time progressed they appeared on non-inoculated shoots and developed into necrotic lesions (21 DAI, Fig. 6.1B). Symptomatic tissue developed on seven of the eleven inoculated plants. Sprinter barley, used as a control in these experiments, is an asymptomatic host.

ELISA tests performed 21 DAI and 31 DAI using plant extracts immediately after grinding resulted in the detection of virus in inoculated Sprinter barley and Agdia controls only. BYDV-PAV infection was detected in 100% of the inoculated barley controls and the healthy controls remained virus-free (Table 6.1). BYDV is a phloem-limited virus, often present in low titer in host plants leading to difficult detection, especially if ELISA tests have not been optimized for a given host. In order to enhance detection of the virus in *A. donax*, diluted extracts

from samples 31 DAI were stored at 4°C and ELISA tests repeated after 3 days. Plants were considered to be infected if absorbance values were two-fold or higher than the value of noninfected plants. Elevated absorbance readings were detected in six of the eleven inoculated *A. donax* plants (Table 6.1) resulting in a 54.5% inoculation success (Table 6.2). Virus was detected in the inoculated shoot of one of the infected plants, among the pooled samples of symptomatic tissue in three of the infected plants and in secondary shoots of inoculated rhizomes in five of the inoculated plants indicating that virus infection in *A. donax* is systemic and difficult to detect. This is the first report of BYDV-PAV infection of *A. donax* plants.

There were no differences (independent t tests $p > 0.05$) detected in any of the growth parameters measured 46 DAI between infected and noninfected *A. donax* plants (Table 6.2).

Transmission tests Transmission efficiency from the positive control plant (BYDV-PAV-infected Sprinter barley) was 80% (4/5 plants were infected). When BYDV-PAV infected *A. donax* served as the virus source, transmission efficiency was 17% (6/36 plants infected). No transmission was observed from the negative control (virus-free *A. donax*) (0/2 plants), confirming there was no cross-contamination in the assay. This is the first report of transmission of BYDV-PAV from *A. donax* to a susceptible small grain cereal.

Discussion

This is the first report of BYDV-PAV infection of *A. donax* and the first report of infection of *A. donax* in North America with any BYDV strain. A previous study reported infection of *A. donax* by BYDV (a MAV-like type strain) in two samples, one from a collection of *Arundo* Italian ecotypes and one from a plant in Hungary [33]. Barley yellow dwarf disease,

which results from BYDV infection in susceptible plants, is caused by a complex of eight different viral species [34]. The MAV-like type detected in the Italian and Hungarian samples [33] belongs to the same genus, *Luteovirus*, as the PAV species used in this study [34]. *A. donax* was reported as an experimental host of *Sugarcane mosaic virus* (SCMV; Family *Potyviridae*, Genus *Potyvirus*), following mechanical sap inoculation in the laboratory in Italy [35]. *A. donax* was also mentioned as an alternative host to a chlorotic streak disease of sugarcane in experiments examining transmission via ratooning [36]. The ability of *A. donax* to be infected by other plant viruses in addition to BYDV-PAV has implications for biofuel production and disease ecology and epidemiology.

We have also shown that BYDV-PAV can be transmitted from *A. donax* to barley by the aphid *R. padi*. The low transmission rate reported here (17%) may be a result of lower virus titer in *A. donax* compared to barley, making acquisition of the virus by the vector more difficult. The low rate may also be a result of virus movement among the tissues of *A. donax*, reducing its potential as an inoculum source. Regardless of the rate of transmission, results suggest that large acreages of *A. donax* could provide a perennial source of virus inoculum in an agricultural landscape where BYDV-susceptible crops such as corn and small grain cereals are economically important. In Oregon in particular, there are large acreages of small grains and corn grown near the proposed biofuel site. Planting large monoculture stands of *A. donax* in this region may increase the incidence of BYDV, which is known to infect and cause disease outbreaks on susceptible hosts in the region [37, 38]. More detailed studies of virus movement within *A. donax* and vector transmission efficiency are required to fully assess the impact of *A. donax* as a source of inoculum in the landscape.

Symptom development and ELISA results indicate that BYDV-PAV infection in *A. donax* is systemic, moving throughout the rhizome and detected in secondary shoots 31 DAI. Given the systemic nature of BYDV-PAV in *A. donax* documented here and its perennial growth pattern, single origins of virus infection have the potential to create widespread persistent disease and source of inoculum for neighboring susceptible crops. Previous virological investigations of *A. donax* in Europe suggest that different clones may vary in their susceptibility to viruses [33]. More intensive examination of *A. donax* susceptibility to viral diseases needs to be conducted, together with studies on the genetic variability with respect to disease susceptibility of *A. donax* clones being considered for large-scale biofuel production in the US and worldwide.

Infection of *A. donax* also may impact the quality and quantity of the crop for biofuel production. Overall *A. donax* appears to be tolerant to BYDV-PAV-infection as indicated by the lack of decline in any of the growth parameters measured, the lower rates of virus infection and the lower virus titer detected on this host compared to barley. However, we did not examine BYDV-PAV effects on plant biomass due to space limitations and future studies are required to assess this. Our study was conducted in an environmental growth chamber where ample light and water were provided. Under field conditions, the host plant will most likely encounter a variety of biotic and abiotic stresses. Virus infection may exacerbate the impact from these stresses and could impact the quality of the product for biofuel. Field studies, similar to those conducted in Italy [2, 39], are required to evaluate the impact of virus infection as well as other abiotic and biotic stresses on the productivity and yield of *A. donax* as a biofuel crop. We are unaware of any studies that have assessed the impacts of disease on *A. donax* productivity. Such effects, if any, will be an important factor to incorporate when calculating the inputs and outputs attainable using *A. donax* for biofuel. It is also important to consider that while virus tolerance may be

beneficial for biofuel production, it can result in virus inoculum build-up for neighboring susceptible hosts.

Interest on the impact of plant virus infection in nonmanaged ecosystems, such as the grasslands that once dominated much of the US, has increased recently [40]. In California, BYDV has been shown to play an important role in the competitive dynamics of native and invasive grasses [26, 28]. The presence of exotic annuals increases the prevalence of virus infection and is believed to lower the competitive ability of native grass species [23]. Native perennial grasses dominate in the absence of disease, however when BYDV is present it persists year to year among the perennial grasses, and exotic annual grasses which return disease-free each year are able to establish, coexist with and in some cases outcompete the native perennials [28].

The aphids *R. padi*, *R. maidis*, *M. dirhodum*, *S. graminum* and *S. avenae* are the dominant vector species associated with BYDV in California and much of the US Pacific Northwest [18, 23, 41]. Adding large monoculture stands of *A. donax* to the landscape, which we have shown here to be a host of BYDV-PAV, has the potential to impact surrounding nonmanaged systems that must also be considered when examining the potential use of *A. donax* as a biofuel crop. Field surveys should be conducted throughout the current range of *A. donax* in the U.S. to determine if BYDV is present among this species. If *A. donax* is found to be infected with BYDV, studies on potential virus spread from *A. donax* to other plant hosts would be merited.

In order to understand the potential impact of *A. donax* on the epidemiology and ecology of BYDV throughout the current and proposed *A. donax* range, studies on the life history and virus transmission efficiency of aphids are required. Insect vectors feeding on virus-infected host

plants often exhibit faster growth rates, higher fecundity, greater longevity and enhanced production of alates [30, 42, 43]. The relationship between infection and vector performance varies depending on the species involved [44]. The transmission efficiency of the insect vector varies depending on the vector species, virus species and titer of the virus in the host plant [31, 45, 46]. For example, Jiménez-Martinez and Bosque-Pérez [31] examined the role of acquisition access periods and inoculation access periods in the transmission efficiency of BYDV-PAV by *R. padi* among genetically diverse winter wheat genotypes and found that AAP and virus titer in the host plant influence transmission. Vector fitness and virus titer on the host plant are important variables impacting the ecology and epidemiology of BYDV disease and information on these variables is lacking for BYDV in *A. donax*.

Similarly, studies on the ecology and movement of aphid vectors between host species need to be conducted. In cropping systems, aphid monitoring and immunological techniques have been used to track the movement of aphids across the landscape [20, 47]. Modeling techniques incorporating field data are used to map and predict disease dynamics and outbreaks in the field [48, 49]. The movement of aphids between crops and nonmanaged grass systems is not well understood. The host preferences and insect movement between *A. donax*, susceptible crops and nonmanaged grasses needs to be examined in order to evaluate the potential of *A. donax* to serve as inoculum of plant viruses such as BYDV in the proposed biofuel regions of the US and other areas of the world.

Conclusion

We have demonstrated that the biofuel crop, *A. donax*, is a host to BYDV-PAV following virus transmission tests under controlled conditions using the aphid vector *R. padi*. *A. donax*

appears to be tolerant to virus infection suggesting that production as a biofuel product might not be heavily impacted by BYD disease in the field, but additional studies are required to confirm this. In laboratory transmission studies, *A. donax* was proved to be a source of BYDV-PAV inoculum that was transmitted to barley using *R. padi*. These results suggest that under field conditions, large plantations of *A. donax* have the potential to influence BYDV ecology and epidemiology in areas of production and could affect neighboring crops. Field surveys examining virus incidence and vector utilization of *A. donax* are required to fully understand the implications of BYDV infection in this biofuel crop.

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Tables

Table 6.1 BYDV-PAV absorbance ($OD_{405\ nm}$) values observed 31 DAI.

Species	Treatment	Absorbance[a]
Arundo donax	Noninfected	0.183 ± 0.015
Arundo donax	Infected	0.411 ± 0.043
Hordeum vulgare	Noninfected	0.411 ± 0.164
Hordeum vulgare	Infected	2.328 ± 0.317

[a]OD_{405nm}, reported mean ± s.e.m.
Ground tissue extracts were incubated for 3 days at 4°C and absorbance readings reported were measured after 4 days of plate incubation at 4°C. Plants were considered infected if the absorbance values were two-fold or higher than the noninfected plants

Table 6.2 Results from BYDV-PAV inoculation and physical measurements of healthy and BYDV-PAV-infected *Arundo donax* plants measured 46 days after inoculation (DAI).

Parameter	Treatment[a]	
	Non-inoculated	BYDV-infected
Percent plants infected	-	54.5
Mean no. shoots per rhizome	3.75 ± 0.85	4.00 ± 0.52
Mean height (mm) tallest shoot	875.25 ± 180.65	846.5 ± 108.14
Mean height (mm) inoculated shoot	-	808.5 ± 112.32
Mean height (mm) all shoots per rhizome	632.38 ± 113.48	561.19 ± 123.01
Mean no. leaves on tallest shoot	13.75 ± 0.47	15.33 ± 0.80
Mean no. leaves on inoculated shoot	-	15± 0.89
Mean no. leaves per rhizome	9.67 ± 0.98	9.42 ± 0.69

[a] Means were compared using independent samples t tests (alpha = 0.05) and no statistically significant differences were detected for any measured parameter.
Data reported are means ± s.e.m.

Figure caption

Figure 6.1 Symptoms of *Barley yellow dwarf virus* infection on *Arundo donax*, observed 12 DAI (A) and 21 DAI (B) following transmission with the vector *Rhopalosiphum padi* and the serotype BYDV-PAV. Symptoms began on the inoculated shoot in leaves above the inoculated leaf and progressed onto non-inoculated shoots. Non-inoculated *A. donax* plants (C) served as controls and were photographed 12 DAI (left and top right) and 21 DAI (bottom right).

Figure

Fig. 6.1

Chapter 7: Summary and Implications

The host plant preferences of the aphid vector *Rhopalosiphum padi* L. were examined. The response of viruliferous (virus-carrying) and nonviruliferous *R. padi* were compared simultaneously in response to host plant selection of BYDV-PAV-infected or noninfected winter wheat cv. Lambert. Viruliferous aphids were obtained through feeding on BYDV-PAV-infected barley cv. Sprinter or fed on membranes infused with artificial diet solution containing purified virus. Nonviruliferous aphids were obtained through feeding on noninfected barley or membranes infused with artificial diet solution only. Under both acquisition scenarios, nonviruliferous aphids significantly preferred BYDV-infected wheat while viruliferous aphids significantly preferred noninfected wheat. The behavioral change documented here promotes the optimal strategy of pathogen spread by promoting acquisition via the preferences for noninfected host plants by nonviruliferous vectors, and optimizing spread via the change in preference of viruliferous vectors for noninfected host plants.

This is the first report that acquisition of a plant virus directly alters host selection behavior by its insect vector. The "Vector Manipulation Hypothesis" (VMH) was proposed to explain the evolution of strategies in plant pathogens to enhance their spread to new hosts (Ingwell et al. 2012). Results supporting the VMH have been reported from work on other plant viruses (Mauck et al. 2010, Stafford et al. 2011, Ziebell et al. 2011, Mauck et al. 2012, Carmo-Sousa et al. 2013) and bacteria (Mann et al. 2012). These findings highlight the evolutionary and ecological significance of vector manipulation by pathogens and parasites. A greater understanding of host plant-virus-vector interactions impacts the way we predict and manage disease outbreaks in agricultural settings. Incorporating changes in vector preference into

ecological and epidemiological models (see Roosien et al. 2013) can help improve our methods of prediction and mitigate outbreaks in the field.

An exotic invasive species threatening the endangered Palouse Prairie is *Ventenata dubia* (Leers) Coss. It can be found along roadsides, in hay, pasture, range and Conservation Reserve Program (CRP) fields, as well as prairie remnants, throughout the western United States (James 2008, Scheinost et al. 2009, Nyamai et al. 2011). The susceptibility of *V. dubia* to BYDV infections was examined in natural populations and under controlled conditions. Infection of *V. dubia* by BYDV-PAV and BYDV-SGV was determined in three Palouse Prairie remnants and four CRP habitats. Laboratory inoculations of BYDV-PAV using the aphid vector *R. padi* confirmed the ability of *V. dubia* to host BYDV. As a result of BYDV-PAV infection, *V. dubia* plants exhibit reductions in plant height, number of leaves per plant, number of tillers per plant and above-ground dry weight compared to healthy plants, suggesting that *V. dubia* is susceptible to BYDV infection. The transmission of BYDV-PAV from *V. dubia* to susceptible barley cv. Sprinter using *R. padi* demonstrated that *V. dubia* is a suitable host to this aphid vector and may serve as a reservoir of BYDV-inoculum under natural settings. Further studies are needed to examine the impact of BYDV infection on the invasiveness of *V. dubia* and its role in BYDV epidemiology.

The Palouse Prairie and CRP habitats in northern Idaho and eastern Washington were surveyed to identify the *Poaceae* species present in these systems and examined to determine the presence and identity of *Barley/Cereal yellow dwarf virus* (B/CYDV) viral species. Twenty-eight species of grasses were collected from five prairie and six CRP sites. Virus infection was detected in 27 of the 28 species collected. BYDV was detected at every site sampled, with an average infection rate of 46% among the sites. The two viral species detected were BYDV-PAV

and BYDV-SGV. Aphids were only encountered during one sampling time at one CRP location and in very low abundance, suggesting that the aphids responsible for viral infection among these two communities are non-colonizing aphid species. These reports are the first evidence of BYDV in Palouse Prairie and CRP habitats. B/CYDV and their aphid vectors have been shown to play an important role in the community structure of plant species in California grasslands (Power et al. 2011). Future work should be aimed at identifying the vector species present in Palouse Prairie and CRP habitats and determining their utilization of these habitats. Furthermore, more research is needed on the implications of viral infection on the competitive interactions of native and introduced species in Palouse Prairie habitats, as it may impact the effectiveness of management tactics aimed at restoring the native plant communities.

The ability of BYDV-PAV to infect 14 grass species, eight of which were not previously documented as potential hosts, was examined performing laboratory inoculations with the aphid vector *R. padi*. The grass species examined can be found in Palouse and Camas Prairie habitats as well as CRP lands (Lichtardt and Moseley 1997, Ogle et al. 2012). BYDV-PAV infection was detected in 12 of the 14 species examined using triple antibody sandwich-enzyme linked immunosorbent assays (TAS-ELISA) and reverse transcription polymerase chain reaction (RT-PCR). The first report of BYDV infection of *Achnatherum occidentale, A. lettermanii, A. thurberianum, Danthonia intermedia, Poa fendleriana, Sporobolus airoides* and *S. cryptandrus* was made. All of these new hosts are perennial grasses native to the US and can be found beyond our study region. Field surveys and vector sampling need to be done to examine these new hosts under natural conditions. Perennial grasses are reservoirs of plant viruses such as BYDV and can be a source of inoculum in nonmanaged and agricultural grasslands (Paliwal 1982, Seabloom et al. 2003, Malmstrom et al. 2005a, Malmstrom et al. 2005b). Studies should be conducted at local

scales to examine virus incidence and vector habitat utilization to better understand the role that the new hosts play in BYDV ecology and epidemiology.

Lastly, the ability of BYDV-PAV to infect *Arundo donax* L. (giant reed), an introduced perennial grass that is being considered as a biofuel crop, was examined under controlled conditions using the aphid vector *R. padi*. Symptoms appeared as yellow lesions 12 days after inoculation (DAI) and developed into necrotic lesions. Using TAS-ELISA, BYDV-PAV infection was detected 31 DAI in inoculated shoots and secondary shoots that developed off of the inoculated shoot, indicating that *A. donax* is a host to BYDV-PAV infection and that the infection is systemic throughout the rhizome. Transmission from infected *A. donax* to susceptible barley cv. Sprinter was achieved under controlled conditions using the insect vector *R. padi*, indicating that *A. donax* is a potential reservoir to neighboring susceptible crops in proposed biofuel production regions. These results are the first report of BYDV-PAV infection in *A. donax* and the first report of BYDV-PAV transmission from *A. donax* to a susceptible small grain cereal. Field surveys are required to confirm laboratory inoculations and transmission and to evaluate the impact of virus infection on the productivity and yield of *A. donax* as a biofuel crop.

Based on the findings presented in this dissertation there has been a new hypothesis presented, the Vector Manipulation Hypothesis, which highlights the ability of pathogens to exploit the various contenders in the pathosystem for which they are an integral part. These findings also highlight the importance of investigating the interactions between hosts, vectors and plant viruses to better understand the ecology and epidemiology of the diseases they cause. A number of new grass species have been added to the host range of BYDV which provides more information on potential virus reservoirs in natural settings. The Palouse Prairie and CRP habitats in the Pacific Northwest have been shown to harbor BYDV with infection rates around

46%. Detection of the virus in annual grass species from 2010-2012 suggest that there is an active vector population and virus reservoir in the region. These results provide insight into BYDV disease dynamics in the Pacific Northwest. These findings also provide opportunities to examine the BYDV pathosystem in novel environments (Palouse Prairie) and new agricultural environments (*A. donax* biofuel production).

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