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Ecological effects of virus-resistant transgenic squash

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ECOLOGICAL EFFECTS OF VIRUS-RESISTANT TRANSGENIC SQUASH

by

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ECOLOGICAL EFFECTS OF VIRUS-RESISTANT TRANSGENIC SQUASH

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Two ecological risks associated with the use of transgenic crops include the effects of transgene products on non-target organisms and the effects of a transgene after it moves from crops into a wild plant population. In work presented here, we specifically investigate the ecological risks of virus-resistant transgenic squash.

We observed pollinator behavior to determine if pollinators are affected by non-target effects of the virus-resistant transgene. We found that pollinator behavior did differ between conventional and virus-resistant transgenic squash due to pleiotropic effects of the transgene. This difference in pollinator behavior can affect plant mating patterns, thereby affecting crop-wild hybridization and transgene introgression into wild squash populations.

For the virus-resistant transgene to confer a benefit in wild squash populations virus must be present. Thus, we surveyed wild squash populations to determine the prevalence of five virus species and members of one virus genus. We found that virus is prevalent in wild squash populations though variable among populations, virus species, and years.

Finally, we focused on the effects of the virus-resistant transgene in wild squash populations. Then, we surveyed wild squash populations for the virus-resistant transgene, which we did not find. Next, we found the population growth rate of wild squash is reduced by virus. However, there is no affect of virus when the virus-resistant transgene is present in wild squash.

We recommend future risk assessments of transgenic crops to examine non-target effects of transgenes on pollinators in different environments as this can affect transgene movement into wild populations. Furthermore, additional wild squash populations should be assayed for the transgene, since our work was not exhaustive. Moreover, to predict when virus affects wild populations, thereby infer when a virus-

resistant transgene is favored by natural selection, additional work examining plant-virus ecology is essential. The results from these studies will allow us to better predict the evolution of transgenic resistance in wild populations and guide policy decisions on the use and deregulation of transgenic crops.

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Summary

Though transgenic crops are grown throughout the world (James 2009) concerns remain about their use. Potential risks associated with the use of transgenic crops include food safety, agronomic risks, and ecological risks. This thesis focuses on the ecological risks associated with the use of transgenic crops. Two ecological risks linked with the use of transgenic crops include potential effects of transgene products on non-target organisms (Pilson and Prendeville 2004, O'Callaghan et al. 2005, Felber et al. 2007) and the effects of transgene movement into wild plant populations (Pilson and Prendeville 2004).

Non-target effects occur when organisms that do not affect crop yield are negatively affected by a product of the transgene construct (Pilson and Prendeville 2004). This can happen when a transgenic crop produces an insecticide that affects beneficial insects (Groot and Dicke 2002). Also, non-target effects can occur through pleiotropic effects of the transgene. Pleiotropic effects occur when a gene or gene products affect the expression of other traits. For instance, flower production is less in transgenic herbicide-resistant canola in comparison to conventional canola (Pierre et al. 2003). A reduction in flower production due to the presence of the transgene may affect pollinator behavior, thereby affecting insect-mediated pollination. Insect-mediated pollination is an important ecosystem service that contributes to the production of the global food supply (Klein et al. 2007). Thus, it is important to understand the potential non-target effects of transgenic crops, on pollinators.

Another ecological risk associated with the use of transgenic crops is movement of transgenes into wild populations. Crop-wild hybridization and subsequent transgene introgression into a wild population may provide wild plants with a novel trait. Novel traits could alter the size and dynamics of wild plant populations. Crop-wild hybridization commonly occurs when crop production fields are near wild relatives (Wilson 1990, Ellstrand 2003). Thus, if transgenic crops are grown near a wild relative, then it is likely that crop-wild hybridization and transgene introgression will occur. Transgenes from transgenic canola (*Brassica napus* L., Hall 2000) and transgenic creeping bentgrass (*Agrostis stolonifera* L., Watrud et al. 2004) have entered feral and wild populations. However, the effects of the transgene in these feral and wild populations are unclear.

Transgenes that are expected to affect wild plant populations are those that confer resistance to natural enemies, such as insects and pathogens, since growth and size of wild plant populations are affected by natural enemies (Alexander and Antonovics 1988, Louda and Potvin 1995, Rose et al. 2005). For instance, one would expect that a virus-resistance transgene would affect wild squash populations, since in field experiments virus reduces fruit and seed number in wild squash (Fuchs et al. 2004b, Laughlin et al. 2009). However, that assumes that viruses occur in and affect wild squash populations. In general, information about virus prevalence and plant-virus ecology in wild plant populations is limited. However, in wild squash populations virus is prevalent, though it is not clear how individual virus species vary in prevalence (Quemada et al. 2008) and their effect on wild squash populations. This lack of information makes it difficult to predict the ecological effects of virus-resistant transgenic crops.

Many studies investigating the ecological effects of transgenic crops have focused on components of individual plant fitness (Fuchs et al. 2004b, Laughlin et al. 2009, Sasu et al. 2009). However, factors other than seed and fruit number may limit wild plant populations, such as germination (Bergelson 1994). It is not clear if benefits conferred by a virus-resistance transgene, such as an increase in seed and fruit production, will lead to an increase in wild squash population size. Thus, to determine if transgenes will affect wild populations, studies must investigate all life-history traits that contribute to population growth and size.

In work presented here, we investigate the ecological effects of virus-resistant transgenic squash. First, we determine if pollinator behavior is affected by non-target effects of the virus-resistant transgene. Particularly, we investigate honey bee and squash bee behavior on transgenic virus-resistant and conventional squash, *Cucurbita pepo* L. (Chapter 1). Next, wild squash populations were surveyed to determine virus prevalence in order to infer the potential benefit a virus-resistant transgene may confer to wild squash (Chapter 2). Then, we constructed deterministic matrix models to estimate the population growth rate of wild squash in the presence and absence of virus infection (Chapter 3). In addition, we estimated the population growth rate of back-cross squash with and without the virus-resistant transgene (Chapter 4) in the presence and absence of virus infection.

In this thesis, we present evidence supporting the existence of ecological risks of virus-resistant transgenic squash. First, we found that pollinator behavior differs between conventional and virus-resistant transgenic squash due to pleiotropic effects of the transgene. This difference in pollinator behavior can affect plant mating patterns, thereby affecting crop-wild hybridization and transgene introgression into wild squash populations. Moreover, this work in conjunction with others indicates that pleiotropic effects can affect traits beyond those conferred by the transgene, thereby complicating our ability to predict the ecological effects of transgenic crops.

Our survey of wild squash populations revealed that virus infection is present though variable among virus species, plant populations, and years. In addition, virus infections in wild squash are frequently asymptomatic, in that there are no visual symptoms of virus infection. A lack of visual symptoms of virus infection is just one factor that has limited investigations of wild plant-virus ecology.

Next, our investigations focused on the effects of virus and the virus-resistant transgene on the population growth rate of wild squash. The virus-resistant transgene can prevent a reduction in population growth rate caused by virus infection. However, even though virus is present and can reduce population growth rates, we did not detect the virus-resistant transgene in wild squash populations. In particular, surveys should focus on wild populations that are close to production fields of transgenic squash. Since our work was not exhaustive, future investigations are necessary to determine if the virus-resistant transgene has introgressed into other wild squash populations. However if it is determined that the virus-resistant transgene has not introgressed into any wild squash populations, then it would be beneficial for future regulation of transgenic crops to understand what factors limited transgene introgression.

In addition, when investigating the effects of virus on wild squash population dynamics, we found that virus species (*Cucumber mosaic virus* and *Zucchini yellow mosaic virus*) differentially affect wild squash populations. Specifically, *Cucumber mosaic virus* and not *Zucchini yellow mosaic virus* reduced wild squash population growth rate. However, when we compared our results to other works by Fuchs et al. (2004) and Laughlin et al. (2009), we noted that the timing of virus infection in

relation to plant development may determine if viruses affect wild squash populations. Specifically, we incorporated seed and gourd production reported in Fuchs et al. (2004) and Laughlin et al. (2009) into the deterministic matrix model with remaining parameters from derived from the common garden experiment. These data suggests that plants infected early in development with *Zucchini yellow mosaic virus* will reduce population growth rate. Of course, other factors such as environmental conditions and plant-virus interactions may account for this difference in population growth rate. Regardless, this comparison underscores our lack of understanding of plant-virus ecology in wild populations.

Recent research in plant-virus ecology indicates that plant-virus genotype interactions mediate the effect of virus on components of plant fitness. In addition, environmental conditions can mediate the effects of virus. Therefore, research enhancing our understanding of plant-virus interactions will aid in predicting the ecological effects of virus-resistant transgenic crops. Furthermore, future investigations assessing the ecological risks of transgenic crops must continue to be collaborative efforts as scientists from many fields (i.e. agronomists, weed scientists, plant ecologists, theoretical ecologists, etc.) are required to appropriately address this issue.

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Chapter 1: Transgenic virus resistance in cultivated squash affects pollinator behavior[♣]

H.R. Prendeville and D. Pilson

Summary

1) Two ecological risks associated with the use of transgenic crops are transgene movement into wild populations and effects on non-target organisms, such as pollinators. Despite the importance of pollinators, and their contribution to the global food supply, little is known about how they are affected by transgenic crops. Pollinator preferences affect plant mating patterns; thus understanding the effects of transgenic crops on pollinators will aid in understanding transgene movement.

2) Honey bee and squash bee visit number and duration were recorded on conventional and transgenic virus-resistant squash *Cucurbita pepo* L. planted in a randomized block design. Floral characters were measured to explain differences in pollinator behavior. The effect of *Zucchini Yellow Mosaic Virus* infection on pollinator behavior was also examined.

3) Honey bees visited female conventional flowers more than female transgenic flowers. Conventional flowers were generally larger with more nectar than transgenic flowers, although floral traits did not account for differences in pollinator visitation.

4) Squash bees visited male transgenic flowers more than male conventional flowers; squash bees also spent more time in female transgenic flowers than in female conventional flowers. Transgenic flowers were significantly larger with greater amounts of sweeter nectar and they were present in greater number. Floral traits accounted for some of the variation in pollinator visitation.

[♣] Chapter 1 has been published in the Journal of Applied Ecology (2009) 46: 1088-1096

5) Squash bee visit number and duration did not differ between virus-infected and healthy plants, but this may be because pollinator behavior was observed early in the virus infection.

6) *Synthesis and applications*. Pollinator behavior controls patterns of plant mating, thus non-target effects of transgenic resistance, such as those observed here, may influence transgene movement into wild populations. These results suggest that transgenic crops should not be planted within the native range of wild relatives because pleiotropic effects may affect crop-wild hybridization and transgene introgression into wild populations.

Chapter 2: Virus infections in wild plant populations are both frequent and often asymptomatic

Holly R. Prendeville, Xiaohong Ye, T. Jack Morris, and Diana Pilson

Summary

1. Viruses commonly infect crop and wild plants, and previous studies indicate that viruses typically reduce plant fitness. However little is known about virus prevalence in wild populations. Prevalence data provide necessary background for evaluating the effects of virus infection on plant population size and dynamics and for improving risk assessment of virus-resistant transgenic crops.
2. We surveyed the literature for reports of virus prevalence in wild plant populations. In addition, we used ELISA and RT-PCR to survey wild squash (*Cucurbita pepo*) populations over 4 years in the south-central US for five virus species, one virus genus, and transgenic virus-resistance.
3. In 28 published studies 56 of 117 tested plant species were infected with virus; infection rates in infected populations ranged from 0.01-100%. Results of our field survey were comparable. In 21 populations sampled from 2004-2007 virus prevalence varied (from 0-74%) among populations, years, and virus species. In samples analyzed by both ELISA and RT-PCR, RT-PCR detected 6-44% more infections (depending on virus species) than did ELISA. Most published studies used ELISA, suggesting that virus prevalence is higher than is typically reported. 80% of infections in wild squash were asymptomatic. The virus-resistance transgene was not present in any of our samples.
4. *Synthesis:* Virus is common in wild plant populations, including wild squash. Although virus can reduce plant fitness, the role of virus infection in wild plant population ecology and community ecology is poorly understood. Limited research on virus infection in wild plants is due to frequent asymptomatic infections and relatively slow and expensive detection methods (e.g. RT-PCR),

both of which make examining the effect of virus on plant population and community dynamics difficult. Studies addressing the effects of virus infection on plant population dynamics and community ecology would contribute to both basic and applied ecology.

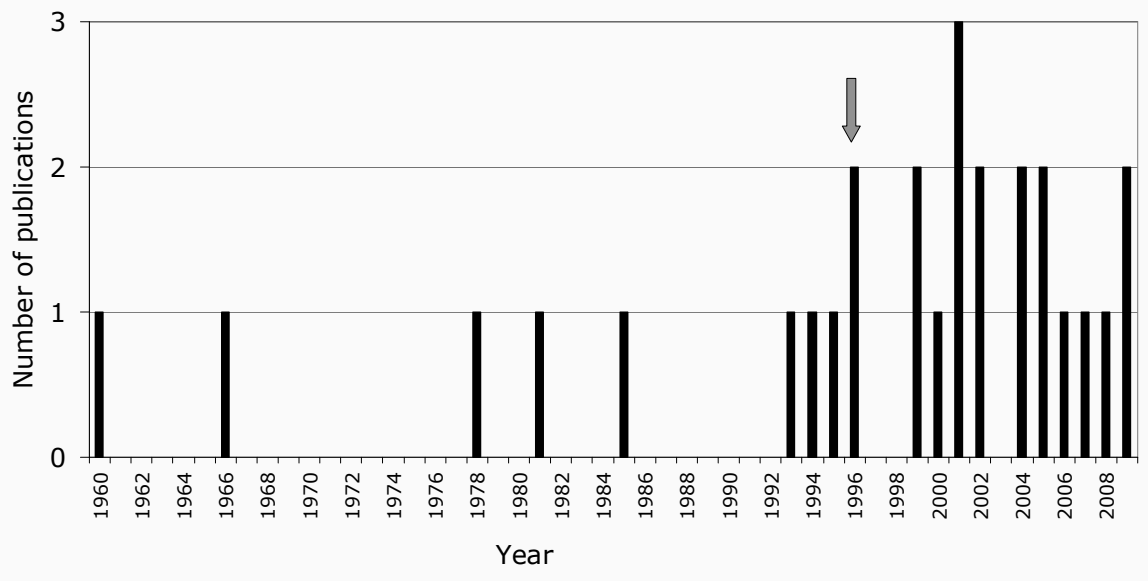
Introduction

Viruses commonly infect plants (MacClement & Richards 1956; Hammond 1981; Mackenzie 1985; Raybould et al. 1999; Tugume, Mukasa, & Valkonen 2008), and virus infection can have large effects on plant fitness and community interactions (Friess & Maillet 1996; Malmstrom et al. 2005b, 2006; Seabloom et al. 2009). However, in natural plant populations virus infection is easily overlooked. Although infections can be asymptomatic (Oswald & Houston 1953; Thurston et al. 2001; Remold 2002) or unapparent it is frequently assumed that an absence of virus symptoms indicates a lack of virus infection. Moreover, symptoms of virus infection are sometimes difficult to distinguish from environmental stresses. For these reasons virus ecology in natural plant populations has been poorly studied (Cooper & Jones 2006).

Because so little is known about virus infection in wild plant populations, much of our understanding of plant-virus interactions comes from economically important plants (e.g. crops, horticultural varieties, and pasture plants). In crops virus infection can reduce plant growth by depressing photosynthesis, changing metabolism (Técsi et al. 1996), and altering resource allocation (Matthews 1991; Radwan et al. 2007). Virus infections can drastically reduce crop yield (Oerke et al. 1994; Picó, Diez, & Nuez 1996) resulting in economic losses. Genetic resistance to virus infection is often the most practical means of controlling crop losses. Thus, the use of virus-resistant transgenic crops offers promise for control of many crop virus problems. In the US, about 20 different virus-resistant transgenic crops have been field tested, and a handful of crops have been deregulated for commercial production (i.e. squash, papaya, and potato; Information Systems for Biotechnology 2010).

The commercial release of virus-resistant transgenic crops has motivated research focused on plant-virus ecology in natural populations (Fig. 2.1). Studies investigating plant-virus interactions have focused on a few viruses, primarily in grasses, and have found that virus prevalence can vary with herbivory (Borer et al. 2009) and environment (Seabloom et al. 2009). In addition, virus infection can affect plant growth, mortality, and seed production in wild plants (Friess & Maillet 1996; Fuchs et al. 2004b), but these effects vary among populations (Mackenzie 1985; Yahara & Oyama 1993; Thurston et al. 2001), species (Remold 2002; Malmstrom et al. 2005a), and environments (Seabloom et al. 2009). Although these data suggest that viruses can affect community dynamics and have fitness consequences in wild plants, remarkably little is known about virus prevalence in wild populations.

Fig. 2.1. Number of publications per year (1960-2009) on plant virus prevalence in natural plant populations. Note: Grey arrow indicates the year virus-resistant transgenic crops were first grown without regulations in the USA.



When transgenic crops are grown in proximity to wild relatives one ecological risk is crop-wild hybridization followed by the introgression of transgenes into wild relatives. For instance, when cultivated squash is grown near native squash populations

(Wilson 1993) wild and cultivated squash can readily interbreed (Quesada, Winsor, & Stephenson 1996). In addition, gene flow from cultivated non-transgenic plants to wild squash has been documented (Decker 1988; Wilson 1990; 1993; Decker-Walters et al. 2002), suggesting that transgenes will similarly move into wild populations. Experimental crosses and natural hybridization in experimental fields between transgenic and wild squash have produced viable hybrids that express the transgene (Spencer & Snow, 2001; Fuchs, Chirco, & Gonsalves 2004a). These data suggest that if the virus-resistance transgene introgresses into wild squash populations, and if virus infection limits wild squash population size, then expression of transgenic resistance could allow populations to increase in size. However, because so little is known about plant-virus ecology, it is difficult to predict the effect of transgenic virus resistance on the size or dynamics of wild squash populations.

Although virus-resistant transgenic squash has been commercially available for over fifteen years (APHIS/USDA, 1994) wild squash populations have not been monitored for transgene introgression. In addition, little is known about the prevalence of individual virus species in wild squash populations (though see Quemada et al. 2008). However, data from common garden experiments suggest that transgenic virus resistance increases fitness in the presence of virus, and thus would be favored by natural selection if virus infection is common in the wild (Fuchs et al. 2004b, Laughlin et al. 2009, Sasu et al. 2009).

In the work presented here, we had three objectives. First, we reviewed literature reporting virus infections in wild plant populations. Second, we surveyed wild squash (*Cucurbita pepo*) populations in the south-central US over 4 years for five virus species and members of one virus genus. Finally, we examined these same wild squash populations for the presence of the virus resistance transgene.

Materials and methods

Literature survey-

Data on virus prevalence in wild plants are dispersed among the fields of ecology, virology, agronomy, plant pathology, and probably others, which makes it difficult to locate all published work. Thus, to compile data on plant virus prevalence in natural ecosystems, we searched for papers with keywords “wild plant virus incidence” and

"wild plant virus prevalence" in three databases: ISI Science Citation Database from 1990-2010, AGRICOLA from 1970-2010, and Google Scholar. In addition, we searched for "virus incidence" and "virus prevalence" in journals of the American Phytopathological Society and in JSTOR within the following categories: Biological Sciences, Botany & Plant Sciences, and Ecology & Evolutionary Biology. Also, we examined all references cited in reviews of plant virus-ecology (Bos 1981; Thresh 1981; Cooper & Jones 1996).

In this literature summary, we only included studies that present data on non-cultivated terrestrial vascular plants. We define non-cultivated plants as those plants growing in the absence of direct human assistance (by seeding, fertilizing, tilling, selective weeding, etc.) to promote growth. Thus, we did not include data from studies of virus prevalence in crops, fallow fields, pastures, botanical gardens, and parks. Other reviews have examined virus prevalence in these habitats (Duffus 1971; Bos 1981; Thresh 1981; Cooper & Jones 2006). Some studies presented data from both cultivated and wild populations of the same species and to the best of our knowledge, we include only data from wild populations (not feral or volunteer crops). In addition, we only included studies in which the sample sizes were a minimum of ten plants per species per site, or if the study explicitly stated that all individuals of a species were collected in a site. Finally, we only included studies in which it was clearly stated that samples were collected regardless of symptoms, randomly, or included both symptomatic and asymptomatic samples.

Data presented here include plant and virus families and species when available. Plant virus families are those recognized by the International Committee on Taxonomy of Viruses (2009). For the purposes of this review, *Barley/Cereal yellow dwarf virus* prevalence is grouped across all serotypes.

Field survey for virus infection and transgenic virus resistance in Cucurbita pepo-

System biology-

Wild squash (*Cucurbita pepo* L. var. *ozarkana* D. Decker and *Cucurbita pepo* L. var. *texana* (Scheele) D. Decker) is native from central and south-western USA and throughout Mexico. Wild squash is an annual herbaceous vine that grows in floodplains, disturbed areas, and roadside ditches, and produces buoyant gourds

which are dispersed by water (Wilson, 1993). Squash depends on animal pollination for fertilization, and outcrossing distances can exceed 1.25 kilometers (Kirkpatrick & Wilson, 1988). In addition, mosaic viruses that commonly infect cultivated summer squash, also *C. pepo*, have been reported in wild squash (Quemada et al. 2008). However, the prevalence of individual virus species in wild squash populations is unknown.

Mosaic viruses that commonly infect cultivated summer squash include three species in Potyviridae: *Papaya Ringspot Virus* (*PRSV*), *Watermelon Mosaic Virus* (*WMV*), and *Zucchini Yellow Mosaic Virus* (*ZYMV*); one in Bromoviridae: *Cucumber Mosaic Virus* (*CMV*); and one in Secoviridae: *Squash Mosaic Virus* (*SqMV*) (Provvidenti, Robinson & Munger 1978; Fuchs & Gonsalves 1999). These mosaic viruses affect a variety of host plants and are non-persistently transmitted by aphids, except *SqMV* is beetle transmitted. Mosaic viruses can drastically reduce yield in cultivated squash (Fuchs & Gonsalves 1995) by stunting growth; causing mottling, discoloration and malformation of leaves, flowers, and fruits; reducing fruit production; and occasionally causing death (Walkey 1991; Fuchs & Gonsalves 1995; Gianessi et al. 2002).

To reduce economic losses associated with virus infection farmers in the US cultivate virus-resistant transgenic squash. Virus-resistant transgenic squash was among the first transgenic crops made available for commercial production without regulation in the US (APHIS/USDA 1994) and has been field tested in Mexico (Alvarez-Morales 1999). Transgenic cultivars contain one of two transgenic constructs, called ZW-20 and CZW-3. Both constructs confer resistance to *ZYMV* and *WMV*; CZW-3 also confers resistance to *CMV* (Tricoli et al. 1995).

Survey of wild squash populations-

In the south-central US, we surveyed wild squash populations for the virus-resistance transgene and virus infection. Wild squash populations were located by searching in and around areas listed in herbarium records, at sites suggested by Hector Quemada (pers. comm.) and John Byrd (pers. comm.), and reported by Decker-Walters et al. (2002). GPS coordinates were noted for all populations (Table 2.S1) and each site was named after the nearest town. Sites with wild squash populations occurred in abandoned and active pastures, agricultural crops, waysides,

road-side ditches, and wild riparian areas. Samples collected from locations within ~3 kilometers are considered a single population due to outcrossing distances (Kirkpatrick & Wilson 1988) and local gourd dispersal.

Because wild squash is a vine and seeds from a single gourd often germinate in close proximity, it is often difficult to distinguish individual plants. For this reason leaf samples were only collected from obvious individuals at a site or only one sample was collected from a cluster of plants. In addition, we noted for each sample if symptoms typical of mosaic viruses were present. When symptoms were present we collected leaves from the symptomatic vine. For each sample, 2-3 unexpanded young leaves were pinched off at the base of the petiole and stored in a 50 mL screw cap tube. Each tube was filled to the 20 mL mark with a desiccant (Drierite, W.A. Hammond Drierite CO. LTD., Xenia, Ohio) and topped with a tissue to separate the desiccant from leaf samples. Drierite was replaced based on indicating color change to permit complete drying of leaf samples.

In 2004, wild squash plants were sampled from Louisiana (2 sites), Missouri (2 sites), and Oklahoma (1 site). In Arkansas, 7 sites were sampled in 2004 and one of these 7 sites was resampled in 2007. In Mississippi, a total of 6 sites were sampled with 2 sites sampled in 2004, 5 sites sampled in 2005, and 6 sites sampled in 2006 and 2007. From these collections, we assayed 1256 leaf samples for the virus-resistance transgene and 1143 leaf samples for virus infection. Most samples were analyzed for both virus infection and the transgene; however, due to limited tissue availability some samples were only analyzed for one or the other.

Transgene detection-

GeneSeek Inc. (Lincoln, Nebraska, USA) assayed 446 samples for the transgene. In 96-well plates DNA was extracted from about 16.5 mm^2 of dried leaf per sample. Each plate had at least two negative controls and four positive controls from cultivated varieties of non-transgenic and transgenic squash, respectively. PCRs were performed using primers designed to amplify a portion of the transgene conferring resistance to *Watermelon Mosaic Virus.* These primers amplify in both lines of virus-resistant transgenic squash (ZW-20 and CZW-3; Wall et al. 2004). GeneSeek Inc. viewed amplicons with an infrared fluorescent system (LI-COR Inc., Lincoln, Nebraska, USA). In the remaining 810 samples, DNA was extracted from

10-20 mg of dried leaf tissue per sample using DNeasy Plant Mini Kits (Qiagen Inc., Valencia, California, USA) and a portion of the transgene conferring resistance to *Zucchini Yellow Mosaic Virus* (found in both lines) was amplified using PCR (Spencer, 2001). Positive and negative controls were present in each round of DNA amplification. Amplicons were viewed with gel electrophoresis using 2% agarose gel with ethidium bromide.

Virus detection-

Wild squash samples were assayed for virus infection using antigen coated plate enzyme-linked immunosorbent assay (ELISA; 545 samples), or reverse transcriptase polymerase chain reaction (RT-PCR; 176 samples), or both methods (422 samples).

For the ELISA 15-20 mg of dried leaf material was sent in 96-well plates with a glass bead for homogenizing to testing services at Agdia Inc. (Elkhart, Indiana, USA). In each plate, we included a positive control for each of the five viruses and three negative controls to which Agdia Inc. was blind. We verified these positive controls using RT-PCR, since RT-PCR is more sensitive than ELISA for RNA virus detection (Hu et al. 1995). To each plate Agdia Inc. added a second set of positive controls for each virus and two negative controls. Agdia homogenized and analyzed samples for five viruses common in cultivated squash fields (*CMV*, *PRSV*, *SqMV*, *WMV*, *ZYMV*) and also all aphid-transmitted viruses within the genus Potyvirus (Poty) by ELISA in a 96-well plate or using Immunostrip tests®, Agdia Inc. for *SqMV*. There are over 143 virus species within Potyvirus; most of these are aphid-transmitted, and at least 10 are known to infect squash (International Committee on Taxonomy of Viruses 2009).

For RT-PCR, total RNA was isolated from each sample by homogenizing 1-5mg of dried leaf and extracting with 1 ml of TriPure isolation reagent (Roche Diagnostics Corporation, Indianapolis, Indiana, USA). The extract was transferred to a 2.0 ml microcentrifuge tube, 0.25 ml of chloroform was added and tubes were twice vortexed for 20 s. The extract was incubated for 10 min at room temperature and centrifuged at 12,000 x g for 20 min at 4°C. Total RNA was precipitated from 0.7 ml of the aqueous phase by adding 0.6 ml of isopropanol and incubating at room temperature for 10 min. RNA was pelleted at 14,000 x g for 20 minutes at 4°C. The RNA pellet was washed with 0.75 ml of 75% ethanol, drained and allowed to air dry. RNA pellet was resuspended in 0.05 ml of RNase-free water. RNA concentration was

quantified using NanoDrop (Thermo Fisher Scientific Inc., Wilmington, Delaware, USA). RNA was amplified using a two-step RT-PCR with oligonucleotide primers specific to each of the five viruses (Lee and Falk, in prep.). Amplicons were viewed in 2% agarose gel electrophoresis. RT-PCR was not used to assay for the Potyvirus genus.

Over four hundred samples were analyzed using both ELISA and RT-PCR. When results differed between tests, the results of RT-PCR are reported since this assay is more sensitive. We used differences in detection between these two methods to provide an estimate of error.

Results

Literature survey –

We found 28 studies that examined virus prevalence in 117 wild plant species. Viruses were detected in 56 of the 117 plant species. Within infected populations between 0.01 and 100% of plants were infected (Table 2.2; Appendix 2.S1).

Approximately 5% of vascular, terrestrial plant families have had at least one species investigated for virus prevalence in a natural setting. More than half of the 117 plant species studied are perennials. Poaceae is the most studied plant family with 68 species examined for virus infection, followed by Fabaceae with 14 species surveyed. Other plant families have had just 1-8 species investigated. More than half of these studies have monitored virus prevalence at fewer than ten sites and for only one year. Overall it is evident that virus-infected plants were present in many of the wild populations studied. However, virus infection is variable among sites, years, plant species, and virus species (Tables 2.1, 2.2; Appendix 2.S1).

Few studies have reported the presence or absence of virus symptoms in wild plant populations. However, from these studies it is clear that not all virus infections produce symptoms, although the frequency of asymptomatic infections varies among plant species, virus species, and among sites (Appendix 2.S1). For instance, 45-86% of *Brassica nigra* (L.) W.D.J. Koch and 50% of *Brassica rapa* L. had asymptomatic infections of *Turnip crinkle virus*, and 60-100% of *B. nigra* and 0% of *B. rapa* had asymptomatic infections of *Turnip yellow mosaic virus* (Appendix 2.S1).

Furthermore, the presence of multiple infections was rarely quantified, but when examined, multiple infections were frequently detected (Appendix 2.S1). For instance, multiple infections were found in 20-100% of *Arabidopsis thaliana* L., 0-24% of *Cucurbita pepo*, 8% of *B. nigra*, 6-16% of *B. rapa*, and 54% of *B. oleracea* in surveyed populations (Appendix S1). Also, in seven grass species multiple serotypes of *Barley/Cereal yellow dwarf virus* were present in 0.9-70% plants in surveyed populations.

Of the 21 virus families that infect terrestrial plants and are currently recognized by the International Committee on Taxonomy of Viruses (2009), 11 have been investigated in wild plant populations. In three of these families (Luteoviridae, Potyviridae, and Alphaflexiviridae) multiple virus species have been surveyed in 10-68 wild plant species (Appendix 2.S1, Tables 2.1, 2.2). The other eight virus families have been less well examined (1-2 virus species per virus family surveyed in 1-4 plant species). To date, four virus species infecting wild plant populations have not been assigned to a virus family.

Survey of wild squash populations-

Transgene assay-

The virus-resistance transgene was not present in any of the 1256 leaf samples of wild squash collected from 21 sites over four years in south-central US.

Virus prevalence-

In 2004 at least one of the surveyed viruses (*CMV*, *WMV*, *ZYMV*, *PRSV*, *SqMV* or aphid-transmitted viruses within the genus Potyvirus) was detected in 12 of the 14 sampled populations. Within these infected populations virus prevalence ranged from 8-74%. 80% of infected plants exhibited no symptoms (Fig. 2.2) and would have been missed in a visual survey of virus prevalence. Prevalence varied dramatically among virus species (Tables 2.3, 2.4). We detected no *PRSV* in 2004. In contrast, *SqMV* was present in 8 of 14 populations, and in these eight populations 2-40% of plants were infected. *CMV*, *WMV*, and *ZYMV* were less common than *SqMV*, and were each found in ≤8% of plants in ≤7 populations (except 1 population in which 33% of plants were infected with *ZYMV*). Viruses in the *Potyvirus* genus, which includes *ZYMV*, *PRSV*, and *WMV*, plus at least seven additional virus species to

which squash is susceptible, were present in 2-70% of sampled individuals in 10 of the 14 populations.

In populations surveyed in Mississippi from 2004-2007, prevalence varied among years (Table 2.4). As in the broader geographic survey, *SqMV* and Potyvirus were more common than *CMV*, *PRSV*, *WMV*, and *ZYMV*. Prevalence varied among sites, but differences among sites were not consistent across years. For example, in Mississippi *WMV* was detected in only two populations, but in different years. Finally, general climatic conditions appear to influence prevalence of virus infection. In 2007, rainfall was below average (National Climatic Data Center 2009), plants were few and small, and none were infected with any of the assayed viruses.

Results of the *Potyvirus* group test were not always consistent with the results of assays for individual species within the *Potyvirus* genus (*PRSV*, *WMV* and *ZYMV*; Table 2.4). Of the 173 samples that tested positive for *PRSV*, *WMV* and/or *ZYMV*, only 58% were positive for the *Potyvirus* test. The difference was not due to virus detection method as the majority of these positive samples were tested with ELISA (161 samples). Thus, these data indicate that *Potyvirus* prevalence is higher than detected by the *Potyvirus* assay.

In addition, comparisons of ELISA and RT-PCR results of positive virus controls indicate that RT-PCR is a more sensitive assay than ELISA. For example, ELISA identified virus infection in 10 of 18 *PRSV* positive controls and 18 of 26 *ZYMV* positive controls. In contrast, RT-PCR correctly detected infection in all of these samples. Overall, RT-PCR detected 6-44% more positive controls than did ELISA (Table 2.5) indicating that ELISA accuracy varied among virus species. For instance, *SqMV* was detected by ELISA in 10 of 10 positive controls while *ZYMV* was detected in 8 of 26 positive controls. These results suggest that our estimates of virus prevalence in wild squash, which are primarily based on ELISA, are underestimates of true prevalence.

Figure 2.2. Prevalence of virus in all plants (black bars) and prevalence of virus in plants exhibiting virus symptoms (gray bars) in wild squash populations in the south-central USA in 2004. Site abbreviations along x-axis are A: Alpena, AR; B: Berryville, AR; C: Bigelow, AR; D: Bradley, AR; E: Canale, AR; F: Cozahome, AR; G: Gilbert, AR; H: Moreland, LA; I: Woodworth, LA; J: Fitler, MS; K: Eagle Lake, MS; L: Simcoe, MO; M: Washburn, MO; N: Park Hill, OK

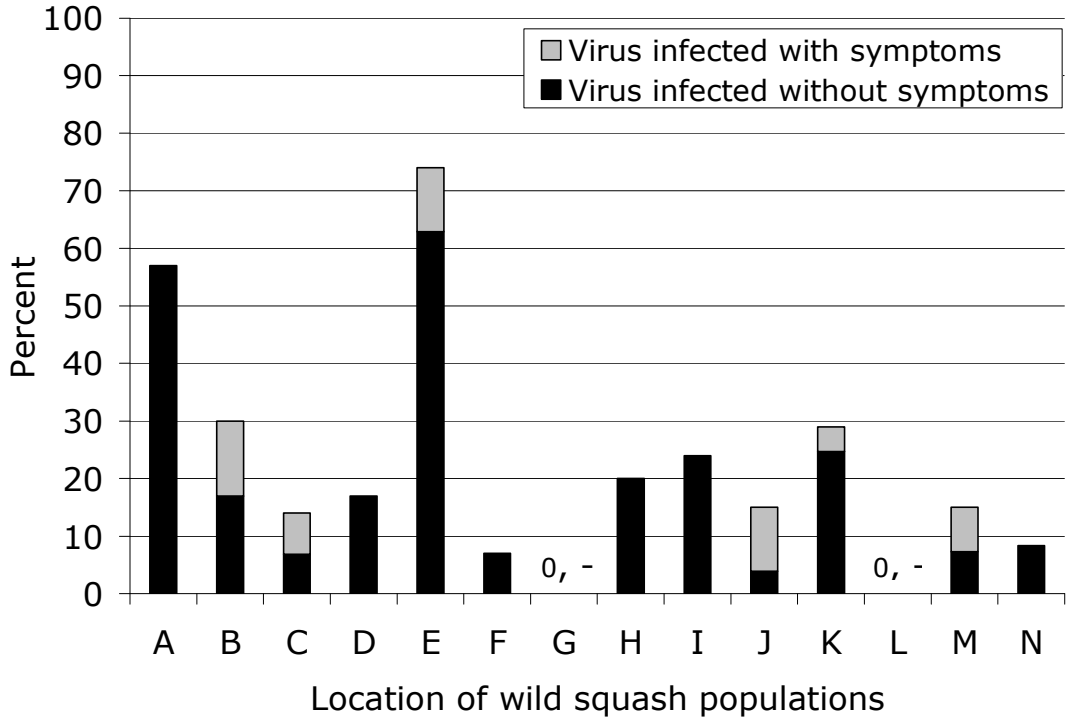


Table 2.1. Summary of literature survey of virus prevalence in wild plant populations. A range is given when the percent of plants infected differed among plant species, virus species, sites, and/or years. Percent of plants infected indicates plants infected with any of the surveyed viruses. More detailed results and citations are presented in Appendix S1.

Plant family	Number of plant species surveyed	Virus Family	Total number of virus species surveyed	Percent of plants infected	Sites	Years	Number of publications
Apiaceae	4	Potyviridae	ǂ	0-100	1	1	1
Araliaceae	1	Caulimoviridae	1	0-30	13	2	1
Asteraceae	1	Geminiviridae	1	0-83	1-15	1-8	2
	2	Luteoviridae	1	0-2	17-24	2	1
Brassicaceae	2	Bromoviridae	1	0-2	4	3	2
	4	Caulimoviridae	1	0-90	3-5	1-3	4
	4	Luteoviridae	1	0-97	2-29	1-3	5
	4	Potyviridae	1	0-80	3-5	1-3	4
	3	Tombusviridae	1	0-36	3-4	1-3	3
	2	Tymoviridae	1	0-76	3-4	1-3	4
	2	Unassigned	1	0-74	3-4	1-3	2
Chenopodiaceae	1	Closteroviridae	1	10-60	30	1	1
	1	Potyviridae	1	12-73	30	1	1
	1	Unassigned	1	0	6	1	1
Cucurbitaceae	1	Bromoviridae	1	0-90ǂǂ	5-15	1-4	1
	2	Luteoviridae	1	0-3	3-28	3	1
	1	Potyviridae	3	0-90ǂǂ	5-15	1-4	1
Ericaceae	1	Secoviridae	1	0-30	6	2*	1
Fabaceae	1	Bromoviridae	1	0-11	11	1	1
	1	Alphaflexiviridae	1	0-1	11	1	1
	13	Potyviridae	2	0-58	1-44	1	3
	1	Tymoviridae	1	0-100	24	2*	1
Geraniaceae	1	Luteoviridae	1	0	2	1	1
Iridaceae	1	Luteoviridae	1	0	5	1	1
Orchidaceae	1	Bromoviridae	1	0-3	2	1	1
	8	Alphaflexiviridae	2	0	2	1	2
	1	Potyviridae	4	0	2	1	1
	1	Virgaviridae	1	0	2	1	1
	1	Unassigned	1	0	2	1	1
Plantaginaceae	1	Alphaflexiviridae	1	39	9	3	1
	1	Potyviridae	1	10	9	3	1
	1	Virgaviridae	1	21	9	3	1
	1	Unknown	2	0.7-8	9	3	1
Poaceae	2	Secoviridae	3	0	1	1	1
	66	Luteoviridae	2	0-100	1-30	1-4	6
	2	Unassigned	1	0	1	1	1
Polemoniaceae	1	Luteoviridae	1	0-1	1-37	2	1
Portulacaceae	1	Luteoviridae	1	0	5	1	1
Primulaceae	1	Secoviridae	3	0-43	5	1	1
	1	Unassigned	1	0	1	1	1
Rosaceae	1	Secoviridae	3	0	1	1	1
	1	Unassigned	1	0	1	1	1
Solanaceae	1	Luteoviridae	1	0-10	6-10	3	1
Zygophyllaceae	1	Luteoviridae	1	0	2	1	1

ǂǂ One study pooled results across virus species

* virus incidence grouped across two years

ǂ Assay for virus family only

Table 2.2. Virus prevalence in wild populations of species with cultivated relatives. Percent of plants infected with virus is given as a range when virus prevalence differed among sites or when the study took place over multiple years. The total number of samples (N) assayed for virus infection across sites and years.

Plant species	Virus Family	Virus species	Percent of plants infected	Samples tested (N)	Sites	Years	Citation
Beta vulgaris subsp. maritima L.	Closteroviridae	*Beet yellows virus*	10-60	>1800	30	1	Gibbs 1960
	Potyviridae	*Beet mosaic virus*	12-73				
Beta vulgaris subsp. maritima L.	Unassigned	*Beet necrotic yellow vein virus*	0	60	6	1	Bartsch et al. 1996
Brassica nigra	Bromoviridae	*Cucumber mosaic virus*	0-2	597	4	3	Thurston et al. 2001
	Caulimoviridae	*Cauliflower mosaic virus*	0-10				
	Luteoviridae	*Beet western yellow virus*	0-68				
	Potyviridae	*Turnip mosaic virus*	0-4				
	Tombusviridae	*Turnip crinkle virus*	0-36				
	Tymoviridae	*Turnip yellow mosaic virus*	0-38				
	Unassigned	*Turnip rosette virus*	0-74				
Brassica oleracea	Caulimoviridae	*Cauliflower mosaic virus*	36-90	211	5	1	Raybould et al. 1999
	Luteoviridae	*Beet western yellow virus*	16-67				
	Potyviridae	*Turnip mosaic virus*	26-80				
	Tymoviridae	*Turnip yellow mosaic virus*	0-62				
Brassica rapa	Caulimoviridae	*Cauliflower mosaic virus*	0-2	2224	3	1[¥]	Pallett et al. 2002
	Luteoviridae	*Beet western yellow virus*	0-15				
	Potyviridae	*Turnip mosaic virus*	0				
	Tombusviridae	*Turnip crinkle virus*	0-5				
	Tymoviridae	*Turnip yellow mosaic virus*	0-76				
	Unassigned	*Turnip rosette virus*	0-7				
Cucurbita pepo	Bromoviridae	*Cucumber mosaic virus* [*]	0-90	398	15, 5	1,2	Quemada et al. 2008
	Potyviridae	*Zucchini yellow mosaic virus* [*] *Watermelon mosaic virus* [*]					
Lupinus angustifolius	Potyviridae	*Bean yellow mosaic virus*	0.1-31	~34000	34	1	Cheng and Jones 1999
Lupinus luteus			0.3-7	~11000	11		
Trifolium repens	Alphaflexiviridae	*White clover mosaic virus*	0-1	1512	11	1	Godfree et al. 2004
	Bromoviridae	*Alfalfa mosaic virus*	0-11				
	Potyviridae	*Clover yellow vein virus*	0-58				
Vaccinium spp.	Secoviridae	*Blueberry leaf mottle virus*	0-30	910	6	2[δ]	Sandoval et al. 1995

[¥] One site was sampled for two years

* Virus assay grouped all viruses listed

[δ] Virus incidence grouped for two year period not shown separately

Table 2.3. Virus prevalence in wild *C. pepo* in 2004. Total number of samples tested (N) at each site. Virus prevalence is the percentage of samples that tested positive for each virus, *Cucumber mosaic virus* (*CMV*), *Squash mosaic virus* (*SqMV*), *Papaya ringspot virus* (*PRSV*), *Watermelon mosaic virus* (*WMV*), *Zucchini yellow mosaic virus* (*ZYMV*), and unspecified virus species within the genus *Potyvirus* (*Poty*). *PRSV*, *WMV*, and *ZYMV* are all within the genus *Potyvirus*.

			Virus prevalence (%)					
State	Site	N	*CMV*	*SqMV*	*PRSV*	*WMV*	*ZYMV*	*Poty*
Arkansas	Alpena	30	0	40	0	0	3	27
	Berryville	23	0	13	0	0	0	22
	Bigelow	14	0	14	0	0	0	0
	Bradley	12	0	0	0	8	0	17
	Canale	27	0	4	0	0	4	70
	Cozahome	137	0	0	0	0	0	0
	Gilbert	10	0	2	0	0	0	2
Louisiana	Moreland	5	0	20	0	0	0	0
	Woodworth	49	0	2	0	8	0	24
Missouri	Simcoe	13	8	0	0	8	0	8
	Washburn	8	0	0	0	0	0	0
Oklahoma	Park Hill	12	0	0	0	0	0	8

Table 2.4. Virus prevalence in wild *C. pepo* in sites in Mississippi and one site in Arkansas collected 2004-2007. Virus prevalence is presented as a percentage (%) of the total number of wild squash samples (N) at each site in each year that tested positive for each virus (*Cucumber mosaic virus* (*CMV*), *Squash mosaic virus* (*SqMV*), *Papaya ringspot virus* (*PRSV*), *Watermelon mosaic virus* (*WMV*), *Zucchini yellow mosaic virus* (*ZYMV*), and unspecified virus species within the genus *Potyvirus* (*Poty*)).

		Virus prevalence and sample size											
		CMV		*SqMV*		*PRSV*		*WMV*		*ZYMV*		*Poty*	
Site	Year	%	(N)	%	(N)	%	(N)	%	(N)	%	(N)	%	(N)
Fitler	2004	0	(22)	0	(6)	0	(23)	6	(17)	33	(21)	17	(6)
	2005	0	(91)	4	(68)	0	(91)	0	(90)	7	(91)	18	(68)
	2006	18	(55)	2	(55)	0	(55)	0	(55)	11	(55)	9	(55)
	2007	0	(6)	0	(6)	0	(6)	0	(6)	0	(6)	0	(6)
Eagle Lake	2004	0	(18)	29	(7)	0	(18)	0	(18)	0	(18)	14	(7)
	2005	0	(108)	12	(66)	1	(108)	0	(85)	4	(107)	8	(66)
	2006	0	(160)	40	(131)	0	(131)	0	(131)	1	(160)	1	(131)
	2007	0	(7)	0	(7)	0	(7)	0	(7)	0	(7)	0	(7)
Vaiden	2005	2	(61)	18	(55)	2	(60)	2	(61)	0	(61)	18	(55)
	2006	22	(86)	0	(75)	0	(76)	0	(75)	5	(86)	3	(75)
	2007	0	(25)	0	(25)	0	(25)	0	(25)	0	(25)	0	(25)
Yazoo	2005	0	(10)	25	(4)	0	(10)	0	(4)	0	(10)	0	(4)
	2006	0	(3)	0	(2)	0	(3)	0	(2)	0	(3)	50	(2)
	2007	0	(8)	0	(8)	0	(8)	0	(8)	0	(8)	0	(8)
Redwood	2005	2	(50)	3	(36)	0	(50)	0	(50)	0	(50)	0	(36)
Port Gibson	2006	13	(22)	0	(19)	4	(22)	0	(19)	41	(19)	4	(22)
Onward	2006	0	(4)	0	(4)	0	(4)	0	(4)	0	(4)	0	(4)
	2007	0	(6)	0	(6)	0	(6)	0	(6)	0	(6)	0	(6)
Mayersville	2007	0	(4)	0	(4)	0	(4)	0	(4)	0	(4)	0	(4)
Lollie (AR)	2007	0	(35)	0	(35)	0	(35)	0	(35)	3	(35)	0	(35)

Table 2.5. Comparison of ELISA and RT-PCR. Each block of four entries indicates the number of samples in which a particular virus (*Cucumber mosaic virus* (*CMV*), *Squash mosaic virus* (*SqMV*), *Papaya ringspot virus* (*PRSV*), *Watermelon mosaic virus* (*WMV*), and *Zucchini yellow mosaic virus* (*ZYMV*)) was detected by both methods (+, +), neither method (-,-) or one of the two methods (+,-; -,+). Sixty-eight samples were positive by RT-PCR and negative by ELISA while just 9 samples were positive by ELISA and negative by RT-PCR, indicating that RT-PCR is the more sensitive method.

			ELISA									
			CMV		*SqMV*		*PRSV*		*WMV*		*ZYMV*	
			+	-	+	-	+	-	+	-	+	-
RT-PCR	Wild samples	+	0	32	.	.	0	0	0	0	0	17
		-	1	388	.	.	1	335	1	234	2	403
	Positive controls	+	14	1	10	0	10	8	8	2	18	8
		-	0	1	2	0	0	0	2	4	0	0

Discussion

Our literature survey clearly demonstrates that wild plant populations are commonly infected with viruses (Tables 2.1, 2.2; Appendix 2.S1). This result is consistent with reviews of virus prevalence in plants near cultivated fields (Duffus 1971; Bos 1981; Thresh 1981; Cooper & Jones 2006), which also show that virus infection is common. It is also evident from our literature survey that wild plant populations typically host multiple virus species (Table 2.2, Appendix 2.S1), and occasionally multiple virus species infect individual plants (Appendix 2.S1). Another pattern that emerges from the literature is that the prevalence of virus infection varies dramatically among years, virus species, plant species, and populations. Moreover, this variation is apparent even among populations well within the dispersal distances of virus vectors (Taylor 1979).

The patterns we observed in our literature survey are similar to those we observed in wild squash populations in the south-central USA. At least one of the five viruses surveyed was present in 17 of 21 wild squash populations and prevalence ranged from 4-43%. Among wild squash populations sampled in the south-central USA in 2004 the median virus prevalence was 16.5% and the average virus prevalence was 23% (Table 2.3). Similar trends were observed in four populations surveyed over 3-4 years in Mississippi: a median virus prevalence of 25% and an average virus prevalence of 24% (Table 2.4). Furthermore, multiple viruses were present within

populations (Tables 2.3, 2.4) and within individual host plants. In addition, virus prevalence varied among years, wild squash populations, and virus species. These results are consistent with those reported by Quemada et al. (2008) who sampled 15 *C. pepo* populations for *ZYMV*, *WMV*, and *CMV* and found virus prevalence ranging from 0-90%.

Another finding from the literature and our population surveys is that asymptomatic virus infections are common in wild populations (Appendix S1, Fig. 2.2; Muthukumar et al. 2009). In our field survey 80% of infections were asymptomatic. Interestingly, virus symptoms are not consistently related to virus concentration (Pallett et al. 2002; Thurston et al. 2001), and virus concentration and plant fitness are not always correlated (Pagán, Alonso-Blanco, & García-Arenal 2007). In addition, asymptomatic virus infection can increase or decrease plant fitness relative to the fitness of uninfected plants (Remold 2002). Taken together, these data suggest that studying the effect of virus infection on wild plant fitness or population dynamics will require frequent serological and/or molecular assays for infection throughout the growing season.

Little is known about processes affecting virus prevalence in natural plant populations. However, in agricultural systems, virus prevalence varies due to virus competition within host plants and vectors; host genetic diversity; and vector transmission efficiency, abundance, and behavior (Power 1991; 1996; Hull 2002). Some of these processes are affected by environmental variables such as air temperature, wind speed, and solar radiation (Klueken et al. 2009). In wild populations host genetic diversity is likely to be greater than in agricultural fields, suggesting that virus prevalence may be more variable as well. In addition, in wild populations the biotic community in which the host exists in is also likely to affect virus infection (Malstrom et al. 2006; Seabloom et al. 2009), as it does for herbivory (Stiling and Rossi 1996). Thus, virus infection rates are probably at least as, if not more, variable in wild populations. The results of our literature review and survey of wild squash populations support this suggestion.

Genetic variation for resistance to virus infection almost certainly contributes to variation in virus prevalence. Genetic variation for resistance is common in plant-pathogen systems (Thrall, Burdon, & Bever 2002; Caicedo 2008; Salvaudon, Giraud,

& Shykoff 2008) and has been documented for virus resistance in wild populations of *A. thaliana* (Pagán, Alonso-Blanco, & García-Arenal 2007; 2008). Genetic variation for resistance can affect virus concentration and the degree to which the virus infection affects plants (Pagán, Alonso-Blanco, & García-Arenal 2009; Pagán et al. 2010). Several studies have found that virus-infected plants have reduced fitness relative to healthy plants (e.g. Friess & Maillet 1996; Fuchs et al. 2004b; Pagán, Alonso-Blanco, & García-Arenal 2009). These data suggest that resistance alleles should increase in frequency. However, some studies have found either direct (Tian et al. 2003) or indirect (Sasu et al. 2009) costs of resistance to pathogen infection. If such costs are present then balancing selection may act to maintain variation for resistance in populations (Bergelson et al. 2001). Moreover, genetic variation for resistance to attack by insect vectors will also contribute to variation in virus prevalence, and natural selection for reduced virus infection could act indirectly through vector resistance as well as directly through virus resistance itself.

Plant community composition can also affect pathogen incidence in plants (Burdon & Chilvers 1982; Alexander 2010). In field experiments, both species richness and presence of a dominant species were negatively correlated with pathogen load (Mitchell et al. 2002). In addition, the presence of an invasive species indirectly increased virus incidence in a native species, whereas in the invasive species there was no relationship between virus incidence and the native species (Malmstrom et al. 2005b). As plant species are introduced and climate changes occur each will alter plant community composition (Vitousek et al. 1997; Walther et al. 2002). However, it is unclear how alterations in plant community composition will affect virus incidence (though see Garrett et al. 2006).

In addition, knowledge of how single or multiple virus species affect wild plant population and community dynamics is limited. In general, multi-species interactions can have demographic and evolutionary consequences for populations that differ from outcomes predicted by pairwise interactions (Hougen-Eitzman & Rausher 1994; Iwao & Rausher 1997). For instance, within a plant infected with multiple virus species there can be synergistic or antagonistic effects, thus either enhancing or reducing the effects of infection (Hammond, Lecoq, & Raccah 1999). In addition, virus infection may also affect the growth and reproduction of insects feeding on infected plants (Hull 2002).

Most of our understanding of plant-virus interaction is derived from cultivated plants. However ecology of agroecosystems frequently differs from natural ecosystems, such that interactions that are common in managed systems may be rare in wild systems. For instance, viruses that commonly infect horticultural orchids were absent in wild populations of orchids (Appendix 2.S1, Zettler et al. 1978; Kawakami, Fuji & Miyoshi 2007). In addition, *PRSV* is widespread in cultivated squash (Davis & Mizuki 1987; Ullman, Cho & German 1991), but was rarely present in wild squash populations (Tables 2.3, 2.4). Furthermore, viruses that may be detrimental in agricultural crops may provide benefits to plants in natural ecosystems (Remold 2002). Therefore, our knowledge of viruses from managed systems should be cautiously extrapolated to wild plant populations. This is of particular importance when trying to predict the ecological risks associated with using virus-resistant transgenic crops.

An ecological risk associated with the use of transgenic crops is crop-wild hybridization followed by the introgression of transgenes into wild populations (Darmency 1994; Pilson & Prendeville 2004). Because experimental work suggests that virus infection has negative effects on wild squash fitness (Fuchs et al. 2004b), it seems likely that transgenic resistance would be selected for in wild populations. In addition, there is no direct fitness cost of the virus-resistance transgene in squash (Laughlin et al. 2009), although there may be an indirect cost due to herbivores and other pathogens (Sasu et al. 2009). For this reason, we assayed hundreds of wild squash plants for the virus-resistance transgene. However, the virus-resistance transgene was not present in our samples. Many factors likely contributed to this result.

First, none of the sites we sampled were in close proximity to fields of virus-resistant transgenic squash. This may be typical for most wild squash populations since about 90% of USA squash production occurs outside of the range of wild squash (USDA/NASS 2008) and transgenic varieties consist of only 18% of total summer squash production (Johnson, Strom & Grillo 2007). Moreover, in regions of overlap, the distance between wild squash populations and squash production fields is often further than pollinators travel (pers. obs.), thus limiting the opportunities for cross pollination. Another factor limiting hybridization is a reduction of wild squash populations. Personal observations and anecdotal evidence from farmers suggest

that the number and size of wild squash populations have declined due to the use of herbicide-resistant transgenic crops (e.g. Round-up Ready cotton) and farming in riparian areas.

The data presented here indicates that virus infection is common in wild squash which contradicts the ecological risk assessment used to deregulate virus-resistant transgenic squash (APHIS/USDA 1994). In the ecological risk assessment an unstated number of wild squash plants from 14 sites in five counties in Arkansas, Louisiana, and Mississippi were visually assessed for virus symptoms and samples were collected to test on indicator plants and by ELISA for seven viruses, which included the five viruses assayed in this study. In contrast to the risk assessment, we found virus prevalence of 0-56% in wild squash at sites that were in or near four of the five counties surveyed as part of the risk assessment. In addition, in the ecological risk assessment, it was assumed that there is a low probability of asymptomatic infection. However data presented here indicates that 80% of virus infections in wild squash are asymptomatic (Fig. 2.2). The conclusion of the risk assessment, that virus is rare in wild squash populations, is clearly incorrect.

Understanding patterns of virus infection depends on accurate detection tools. Only recently have cost-effective serological and molecular tools become available to allow large numbers of plants to be assayed for virus infection (reviewed in Lopez et al., 2003; James et al. 2006). However, as reported here and elsewhere (Figueira, Domier & D'Arcy 1997; Berniak, Malinowski & Kamińska 2009), the accuracy of virus detection varies among serological and molecular methods. In general, serological methods such as ELISA are less sensitive than molecular methods, such as RT-PCR or PCR (Hu et al. 1995). In our literature survey 20 studies used ELISA while three studies used either RT-PCR alone or in conjunction with ELISA (Appendix 2.S1). Since ELISA is less sensitive than RT-PCR it seems likely that virus prevalence is higher than is typically reported. This conclusion is consistent with our data. In wild squash samples analyzed by both ELISA and RT-PCR overall virus prevalence was 1.4% by ELISA and 11.6% by RT-PCR (Table 2.5). Even though the accuracy of virus detection can differ among methods by an order of magnitude this difference is rarely noted in studies of virus prevalence. As detection methods are refined and developed (e.g. pyrosequencing, Roossinck et al. 2010) so will the accuracy of detecting virus incidence in wild plant populations.

Conclusions

Viruses are common in wild plants and virus infection varies among years, sites, plant species, and virus species. Although virus can reduce plant fitness, the role of virus in wild plant population and community ecology is poorly understood. For this reason, it is difficult to predict the ecological risks associated with the use of virus-resistant transgenic crops. Furthermore, because infections are frequently asymptomatic and sensitive detection methods do not provide immediate results it is difficult to accurately detect virus infections rapidly in ongoing field studies. Studies addressing the effects of virus and transgenic virus-resistance on plant population dynamics would contribute to both basic and applied ecology.

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Table 2.S1. Collection sites of wild squash collected in 2004-2007 including GPS coordinates. Samples collected from locations within ~3 kilometers are considered a single population and are named for nearby towns.

City	State	Latitude	Longitude	8 July - 23 Aug 2004	12 Sept- 14 Oct 2005	20 July - 20 Sept 2006	6 Aug - 5 Sept 2007
Eagle Bend	Mississippi	32.46800	-90.98000	x	x	x	x
Eagle Bend	Mississippi	32.47800	-90.98200	x	x	x	x
Eagle Bend	Mississippi	32.48838	-91.04198	x	x	x	x
Eagle Bend	Mississippi	32.50073	-91.06134	x	x	x	x
Fitler	Mississippi	32.72179	-91.02126	x	x	x	x
Fitler	Mississippi	32.72200	-91.02400	x	x	x	x
Fitler	Mississippi	32.72200	-91.02500	x	x	x	x
Fitler	Mississippi	32.73158	-91.02602	x	x	x	x
Fitler	Mississippi	32.72440	-91.03091	x	x	x	x
Vaiden	Mississippi	33.34358	-89.76036		x	x	x
Vaiden	Mississippi	33.34854	-89.76099		x	x	x
Vaiden	Mississippi	33.34854	-89.76099		x	x	x
Vaiden	Mississippi	33.34224	-89.76231		x	x	x
Vaiden	Mississippi	33.34222	-89.76234		x	x	x
Vaiden	Mississippi	33.34949	-89.7627		x	x	x
Vaiden	Mississippi	33.34949	-89.76270		x	x	x
Vaiden	Mississippi	33.34174	-89.76414		x	x	x
Vaiden	Mississippi	33.34168	-89.76491		x	x	x
Yazoo City	Mississippi	32.894339	-90.373531		x	x	x
Yazoo City	Mississippi	32.80645	-90.3158		x	x	x
Port Gibson	Mississippi	31.99600	-90.97700			x	
Redwood	Mississippi	32.47205	-90.84035		x		
Onward	Mississippi	32.749081	-90.926819			x	x
Mayerville	Mississippi	32.005931	-91.044531				x
Park Hill	Oklahoma	35.83300	-94.91700	x			
Simcoe	Missouri	36.616589	-94.32745	x			
Simcoe	Missouri	36.588419	-94.389411	x			
Washburn	Missouri	36.5834	94.116739	x			
Washburn	Missouri	36.583108	94.120128	x			
Washburn	Missouri	36.583081	-94.115911	x			
Woodworth	Louisiana	31.18100	-92.39900	x			
Woodworth	Louisiana	31.18200	-92.39900	x			
Moreland	Louisiana	31.23100	-92.41600	x			
Moreland	Louisiana	31.22300	-92.41700	x			
Moreland	Louisiana	31.23200	-92.41700	x			
Woodworth	Louisiana	31.17400	-92.42300	x			
Alpena	Arkansas	36.302289	-93.347667	x			
Berryville	Arkansas	36.338569	-93.548961	x			
Bigelow	Arkansas	34.99645	-92.591	x			
Bigelow	Arkansas	34.985142	-92.589142	x			
Bigelow	Arkansas	34.986	-92.59	x			
Bigelow	Arkansas	34.996261	-92.582289	x			x
Bigelow	Arkansas	35.007	-92.577	x			
Bigelow	Arkansas	35.014	-92.572	x			
Bradley	Arkansas	33.12895	-93.730919	x			
Bradley	Arkansas	33.143139	-93.078844	x			
Bradley	Arkansas	33.149	-93.749	x			
Bradley	Arkansas	33.15	-93.744	x			
Canale	Arkansas	33.109167	-93.811942	x			
Canale	Arkansas	33.108289	-93.81435	x			
Canale	Arkansas	33.102619	-93.834689	x			
Canale	Arkansas	33.102631	-93.838333	x			
Cozahome	Arkansas	36.04917	-92.57639	x			
Cozahome	Arkansas	36.05244	-92.57692	x			
Cozahome	Arkansas	36.04621	-92.57729	x			
Cozahome	Arkansas	36.06276	-92.58437	x			
Cozahome	Arkansas	36.05426	-92.58453	x			
Cozahome	Arkansas	36.03417	-92.58699	x			
Cozahome	Arkansas	36.04181	-92.58905	x			
Gilbert	Arkansas	35.9967	-92.73755	x			
Gilbert	Arkansas	35.99158	-92.74105	x			
Gilbert	Arkansas	35.98205	-92.75259	x			
Gilbert	Arkansas	35.98325	-92.75387	x			
Gilbert	Arkansas	35.98605	-92.75563	x			
Gilbert	Arkansas	35.99005	-92.76292	x			
Gilbert	Arkansas	35.98355	-92.7803	x			
Gilbert	Arkansas	35.98417	-92.7817	x			
Gilbert	Arkansas	35.97667	-92.7947	x			

Appendix 2.S1: A summary of published reports of virus prevalence in wild plant populations. The percent of wild plants infected (% Inf) with virus across multiple sites (# Sites) and/or years (# Years). The total number of samples assayed for virus infection (N). When the number of years that sites were examined differed within a study we present all information given in the publication separated by a comma. For instance, # Site 15,1 and # Years 1,2 indicates 15 sites were assayed in one year and one site was assayed for two years. Life cycle indicates the plant life history (annual: A; periennial: P: and/or biennial: B. Method indicates the method used to detect virus infection and includes: visualizing symptoms (S); serological assays (Se); inoculating indicator plant species from collected leaf samples (M); electron microscopy (E); enzyme-linked immunosorbent assay (ELISA); reverse-transcriptase polymerase chain reaction (RT-PCR); hybridization via 32P-UTP-labelled RNA probes (H); Tissue-blot immunoassay (TBI); and Ouchterlony double immunodiffusion (ODI). Information about the percent of infected plants that had visual symptoms (% Sym) and the percent of plants with multiple infections (% Mult) is presented. Location indicates the country of the virus survey. n/a indicates data was not available.

Plant family	Plant species	Virus Family	Virus[H]	Percent of plants infected	N	Sites	Years	Life cycle	Method	% Sym	% Mult	Location	Citation
Apiaceae	*Actinotus helianthii* Labill.	Potyviridae		0-100	12	1	1	A	ELISA, RT-PCR	some	n/a	Australia	Moran et al. 2002
	Apium prostratum Labill.			2	38			B/P					
	Foeniculum vulgare Mill.			20-100	10			P					
	Hydrocotyle spp.			0	16			P					
Araliaceae	*Stilbocarpa polaris* (Homb. et Jacq.) Gray	Caulimoviridae	*Stilbocarpa mosaic bacilliform virus*	0-30	13	13	2	B/P	S, E, PCR	n/a	.	Australia	Skotnicki et al. 2003
Asteraceae	*Eupatorium makinoi* L.	Geminiviridae	*Tobacco leaf curl virus*	0-73	485	15,1	1,2	P	S	100	.	Japan	Yahara and Oyama 1993
Asteraceae	*Eupatorium makinoi* L.	Geminiviridae	*Tobacco leaf curl virus*	0-83	~1300	1,2	4,8	P	S	100	.	Japan	Funayama et al. 2001
Brassicaceae	*Arabidopsis thaliana* L.	Bromoviridae	*Cucumber mosaic virus*	0-100	971	6	4	A	H, ELISA	100	20-100	Spain	Pagán et al. 2010
		Caulimoviridae	*Cauliflower mosaic virus*	0-60						100			
		Potyviridae	*Turnip mosaic virus*	0-67						100			
		Tombusviridae	*Turnip crinkle virus*	0-60						100			
		Tymoviridae	*Turnip yellow mosaic virus*	0-38						100			
Brassicaceae	*Brassica nigra* (L.) W.D.J. Koch	Bromoviridae	*Cucumber mosaic virus*	0-2[δ]	597	4	3	A	ELISA	n/a	8	UK	Thurston et al. 2001
		Caulimoviridae	*Cauliflower mosaic virus*	0-10						76-100			
		Luteoviridae	*Beet western yellows virus*	0-68						n/a			
		Potyviridae	*Turnip mosaic virus*	0-4						n/a			
		Tombusviridae	*Turnip crinkle virus*	0-36						14-55			
		Tymoviridae	*Turnip yellow mosaic virus*	0-38						0-40			
		Unassigned	*Turnip rosette virus (Sobemovirus)*	0-74						60-100			
Brassicaceae	*Brassica rapa* L.	Caulimoviridae	*Cauliflower mosaic virus*	0-2	2224	3	1[δ]	A/B	ELISA	100	6-16	UK	Pallett et al. 2002
		Luteoviridae	*Beet western yellow virus*	0-15						0			
		Potyviridae	*Turnip mosaic virus*	0						100			
		Tombusviridae	*Turnip crinkle virus*	0-5						50			
		Tymoviridae	*Turnip yellow mosaic virus*	0-76						100			
		Unassigned	*Turnip rosette virus (Sobemovirus)*	0-7						n/a			
Brassicaceae	*Brassica oleracea* L.	Caulimoviridae	*Cauliflower mosaic virus*	36-90	211	5	1	P	ELISA	n/a	54	UK	Raybould et al. 1999
		Luteoviridae	*Beet western yellow virus*	16-67									
		Potyviridae	*Turnip mosaic virus*	26-80									
		Tymoviridae	*Turnip yellow mosaic virus*	0-62									
Brassicaceae	*Raphanus raphanistrum* L.	Luteoviridae	*Beet western yellows virus*	0-97	520	18,2	1,2	A/B	ELISA, TBI	40-76	.	Australia	Coutts and Jones 2000
Chenopodiaceae	*Beta vulgaris subsp. maritima* L.	Closteroviridae	*Beet yellows virus*	10-60	>1800	30	1	A/B	M, S	100	n/a	UK	Gibbs 1960
		Potyviridae	*Beet mosaic virus*	12-73									
Chenopodiaceae	*Beta vulgaris subsp. maritima* L.	Unassigned	*Beet necrotic yellow vein virus* (Benyvirus)	0	60	6	1	A/B	ELISA	n/a	.	Italy	Bartsch et al. 1996
Cucurbitaceae	*Cucurbita pepo* L.	Potyviridae	*Zucchini yellow mosaic virus*[‡] *Watermelon mosaic virus*[‡]	0-90	398	15, 5	1,2	A	ELISA	2	n/a	US	Quemada et al. 2008
		Bromoviridae	*Cucumber mosaic virus*[‡]										

Ericaceae	*Vaccinium spp.*	Secoviridae	*Blueberry leaf mottle virus*	0-30	910	6	2*	P	ELISA	n/a	.	US	Sandoval et al. 1995
Fabaceae	*Trifolium repens* L.	Potyviridae	*Clover yellow vein virus*	0-58	1512	11	1	P	ELISA, M	n/a	n/a	Australia	Godfree et al. 2004
		Bromoviridae	*Alfalfa mosaic virus*	0-11						n/a	n/a		
		Alphaflexiviridae	*White clover mosaic virus*	0-1						n/a	n/a		
Fabaceae	*Lupinus angustifolius* L.	Potyviridae	*Bean yellow mosaic virus*	0.1-31	~34000	34	1	A	S, ELISA	0.1-31	.	Australia	Cheng and Jones 1999
	L. luteus L.			0.3-7	~11000	11		A		0.3-7	.		
Fabaceae		Potyviridae	*Bean yellow mosaic virus*		850	1-44	1		ELISA, M			Australia	McKirdy et al. 1994
	Acacia alata R.Br.			0	14	1		P		.	.		
	Acacia moirii E. Pritz.			0	10	1		P		.	.		
	Chorizema glycinifolium (Sm.) Druce			0	21	1		P		.	.		
	Daviesia incrassata Sm.			0	11	1		P		.	.		
	Erythrina indica Lam.[¥] (*Erythrina variegata* L.)			0	92	4		P		.	.		
	Gastrolobium parvifolium Benth.			0	24	1		P		.	.		
	Hardenbergia comptoniana (Andrews) Benth.			0	94	11		P		.	.		
	Kennedia coccinea Vent.			0-33	256	30		P		1	.		
	Kennedia prostrata R.Br.			0-0.1	316	44		P		3	.		
	Pultenaea strobilifera Meisn.			0	12	1		P		.	.		
Fabaceae	*Ononis repens* L.	Tymoviridae	*Ononis yellow mosaic virus*	0-100	~788	24	2*	P	Se, M, E	.	.	UK	Gibbs et al. 1966
Orchidaceae	*Calanthe izu-insularis* (Satomi) Ohwi et Satomi	Alphaflexiviridae	*Clover yellow mosaic virus*	0	104	2	1	P	RT-PCR	n/a	.	Japan	Kawakami et al. 2007
			Cymbidium mosaic virus	0						n/a	.		
		Potyviridae	*Calanthe mild mosaic virus*	0						n/a	.		
			Bean yellow mosaic virus	0						n/a	.		
			Turnip mosaic virus	0						n/a	.		
			Calanthe mosaic virus	0						n/a	.		
		Bromoviridae	*Cucumber mosaic virus*	0-3						n/a	.		
		Virgaviridae	*Odontoglossum ringspot virus*	0						n/a	.		
		Unknown	*Orchid fleck virus*	0						n/a	.		
Orchidaceae	*Epidendrum anceps* Jacq.	Alphaflexiviridae	*Cymbidium mosaic virus**	0	17	1	1	P	E, ODI	n/a	.	US	Zettler et al. 1978
	E. cochleatum L.[¥] (*Prosthechea cochleata* (L.) W.E. Higgins)	Virgaviridae	*Odontoglossum ringspot virus**	0	11	1		P		n/a	.		
	E. conopsem[¥] (*E. magnoliae* Muhl.)			0	15	1		P		n/a	.		
	E. difforme[¥] (*E. boricuarum* Hágsater & Sánchez)			0	15	1		P		n/a	.		
	E. nocturnum Jacq.			0	13	1		P		n/a	.		
	E. rigidum Jacq.			0	16	1		P		n/a	.		
	E. tampensis (Lindl.) Small			0	32	2		P		n/a	.		
Plantaginaceae	*Plantago lanceolata* L.	Virgaviridae	*Ribgrass mosaic virus*	21	130	9*	3*	A/B/P	E, M	n/a	.	UK	Hammond 1981
		Potyviridae	*Plantain virus A*	10						n/a	.		
		Alphaflexiviridae	*Plantain virus X*	39						n/a	.		
		Unknown	*Plantain virus 7*	6						n/a	.		
			Plantain virus 8	0.7						n/a	.		
			Plantain virus 6	0.7						n/a	.		
			Plantain virus 4	8						n/a	.		
			Plantain virus 5	0.7						n/a	.		

Poaceae	Luteoviridae	*Barley / Cereal yellow dwarf viruses*	0-40	2293	1-11	n/a	A/P	ELISA, M, S			New Zealand	Davis and Guy 2001
Agrostis capillaris L.			12	110	11		P		0	.		
Agrostis muelleriana Vickery			0	40	2		A		.	.		
Anemanthele lessoniana (Steud.) Veldkamp			6	35	2		P		0	.		
Anthoxanthum odoratum L.			0	70	7		P		.	.		
Arrhenatherum elatius (L.) P. Beauv. ex J. Presl et C. Presl			0	50	3		P		.	.		
Bromus catharticus Vahl			0	50	3		A/P		.	.		
Chionochloa crassiuscula (Kirk) Zotov			0	40	2		P		.	.		
Chionochloa macra Zotov			0	40	2		P		.	.		
Chionochloa rigida (Raoul) Zotov			1	160	8		P		0	.		
Chionochloa rubra Zotov			0	110	6		P		.	.		
Chionochloa teretifolia (Petrie) Zotov			0	60	3		P		.	.		
Dactylis glomerata L.			34	100	10		P		0	.		
Deyeuxia avenoides [¥]			0	126	7		P		.	.		
Calamagrostis avenoides (Hook. f.) Cockayne												
Echinochloa crus-galli (L.) P. Beauv.			0	15	1		A		.	.		
Elymus rectisetus (Nees in Lehm) A. Love et Connor			0	118	8		P		.	.		
Festuca multinodis Petrie et Hack.			30	20	1		P		0	.		
Festuca novae-zelandiae (Hack.) Cockayne			2	125	8		P		.	.		
Hierochloe novae-zelandiae Gand.			0	33	3		P		.	.		
Hierochloe redolens (Vahl) Roem. et Schult.			11	47	3		P		0	.		
Holcus lanatus L.			0	100	9		P		.	.		
Hordeum murinum L.			3	40	3		A		0	.		
Lachnagrostis lyallii (Hook.f.) Zotov			0	20	1		P		.	.		
Lachnagrostis pilosa (Buchanan) Edgar			20	15	1		n/a		0	.		
Lolium perenne L.			13	110	10		P		0	.		
Microlaena avenacea (Raoul) Hook.f.			0	87	5		P		.	.		
Paspalum dilatatum Poir.			40	52	3		P		0	.		
Phleum pratense L.			0	20	2		P		.	.		
Poa annua L.			10	50	3		A		0	.		
Poa cita Edgar			2	105	6		P		0	.		
Poa colensoi Hook.f			12	110	6		P		0	.		
Poa kirkii Buchanan			0	20	1		P		.	.		
Rytidosperma gracile (Hook. f.) Connor et Edgar			0	60	4		P		.	.		
Rytidosperma pumilum (Kirk) Connor et Edgar			0	40	2		P		.	.		
Rytidosperma setifolium (Hook.f.) Connor et Edgar			2	65	4		P		0	.		
Trisetum antarcticum (G.Forst.) Trin.			0	40	2		P		.	.		
Trisetum spicatum (L.) K. Richt.				10	1		P					
Poaceae	Luteoviridae	Barley / Cereal yellow dwarf viruses		2077	n/a	n/a		ELISA			Australia (Tasmania)	Guy et al. 1987
Agropyron repens			0	20			P		.	.		
Agrostis capillaris			0	47			P		.	.		
Bromus cebadilla			0	15			P		.	.		
Danthonia tenuior			25	27			P		0	.		
Digitaria sanguinalis			4	25			A		0	.		
Echinochloa crus-galli			19	42			A		0	.		
Ehrharta erecta			33	15			P		0	.		
Ehrharta longiflora			27	11			A		0	.		
Eragrostis cilianensis			0	12			A		.	.		
Panicum capillare			0	11			A		.	.		
Paspalum dilatatum			0	24			P		.	.		
Phleum pratense			0	50			P		.	.		
Phragmites australis			0	63			P		.	.		
Poa annua			0	10			A		.	.		
Poa gunnii			0	12			P		.	.		
Poa labillardieri			0	13			P		.	.		

		Luteoviridae	*Beet western yellows virus*		11244	2-37	3		TBI			Australia	Coutts et al. 2006
Asteraceae	*Conyza spp.*			0-2	3169	17-24	2	n/a		n/a	n/a		
	Sonchus oleraceus L.			0	589	6-8	3	A		n/a	n/a		
Brassicaceae	*Raphanus raphanistrum*			0	2127	5-29	3	A/B		n/a	n/a		
Cucurbitaceae	*Citrullus lanatus* (Thunb.) Matsum. et Nakai			0-3	794	7-28	3	A		n/a	n/a		
	Cucumis myriocarpus E. Mey. ex Naud.			0	504	3-14	3	A		n/a	n/a		
Geraniaceae	*Pelargonium sp.*			0	31	2	1	n/a		n/a	n/a		
Iridaceae	*Homeria spp.*			0	440	5	1	n/a		n/a	n/a		
Polemoniaceae	*Navarretia squarrosa* (Eschsch.) Hook. & Arn.			0-1	3201	1-37	2	A		n/a	n/a		
Portulacaceae	*Portulaca oleracea* L.			0	67	5	1	A		n/a	n/a		
Solanaceae	*Solanum nigrum L.*[¥] *Solanum americanum* Mill.			0-10	277	6-10	3	A/P		n/a	n/a		
Zygophyllaceae	*Tribulus terrestris L.*			0	45	2	1	A		n/a	n/a		

[¥] Indicates change in scientific name since publication, which is found in the next row

[H] Virus species presented if family unassigned then virus genus presented parenthetically in the next row

[Φ] only tested in 1999 142 samples at 3 sites

[δ] one site was sampled for two years

[‡] Virus assay grouped together listed viruses

[β] tested for BYDV only

* virus incidence grouped across two years

n/a indicates when potential data was not provided

. indicates when data is not possible (i.e. multiple infections when study assayed for one virus)

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Chapter 3: Environment and virus affect wild squash population dynamics

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Introduction

A fundamental goal of ecology is to understand factors that control population size and dynamics. Often it is assumed that components of individual plant fitness scale up to the population, thus affecting population size and dynamics. For example, it is sometimes assumed that reductions in components of individual plant fitness, i.e. due to herbivore or pathogen damage, result in reduced population size or growth rate. However, a reduction in components of individual plant fitness does not always reduce population growth rate or size. This is because factors, other than seed production, such as space or dispersal limitation, may limit recruitment and population size (Bergelson 1994). Thus, to understand how biotic and abiotic factors affect population dynamics, it is essential to examine these factors on all life history traits.

Biotic factors, such as pathogens, are thought to regulate host populations (though see Holmes 1982). Although the ecology of agroecosystems frequently differs from natural ecosystems, much of our understanding of natural plant-pathogen interactions is inferred from studies of pathogens in agricultural crops. However, extrapolating our knowledge of the effects of pathogens in agricultural crops to wild plants can lead to erroneous predictions. One reason is that viruses common in managed plants can be rare in wild systems (Zettler et al. 1978, Davis and Mizuki 1987, Ullman et al. 1991, Kawakami et al. 2007, Prendeville et al.-Ch 2). In addition, in agricultural crops virus infections typically present symptoms, whereas in wild plant populations virus infection is frequently asymptomatic (Prendeville et al.-Ch 2). In agricultural crops, it is clear that pathogens such as viruses can stunt plant growth, cause deformity in leaves, fruits, and flowers, reduce plant survival and limit seed production.

Although pathogens also have negative effects on wild plants, this is not always the case. Pathogens may have no effect (Jarosz and Burdon 1992, Malmstrom et al. 2005a) or positive effects on wild plants. For instance, biomass, seed germination, seedling establishment, components of female fitness, and tolerance to abiotic stress have been reported to increase in some wild plants following pathogen infection (Ferris et al. 1989, Remold 2002, Eviner and Chapin III 2003, Xu et al. 2008).

Abiotic conditions affect both plant and pathogen performance (Colhoun 1973, Hull 2002), and environmental conditions can mediate the effects of virus on components of individual plant fitness (Agrios 1969, Remold 2002, Seabloom et al. 2009a). For instance, plants susceptible to virus under one set of environmental conditions may be resistant under another (Hull 2002).

Although very few studies have examined the effects of pathogens on wild plant populations (though see Alexander and Antonovics 1988), pathogens are often assumed to regulate plant populations. This assumption is made even though not all effects of pathogens on individual plants scale up to plant populations (Alexander and Mihail 2000). Therefore to investigate the effects of different biotic and abiotic factors on the size and dynamics of plant populations, matrix models are commonly used to project future population size and dynamics from vital rates measured on individuals (Caswell 1989, 2001).

In the work presented here, we used wild squash as a model system to examine factors influencing population size and dynamics. In particular, we used data from common garden experiments to parameterize matrix models. Using these models we investigated: 1) if either virus infection or environmental conditions affect population growth rate, 2) if natural selection favors different life history stages in the presence and absence of virus infection, and in drought and normal precipitation years, and 3) if life history transitions differ in their contribution to the population growth rate in differing virus or environmental conditions.

Materials and Methods

Study system

Wild squash (*Cucurbita pepo* L. var. *ozarkana* D. Decker and *Cucurbita pepo* L. var. *texana* (Scheele) D. Decker) is native from central and south-western USA throughout Mexico. This annual herbaceous vine grows in floodplains, disturbed areas, and roadside ditches. Wild squash is monoecious and therefore requires insect-mediated pollination for reproduction. Flowers are produced for several weeks; however individual flowers last for less than 1 day, opening at dawn and closing around noon, depending upon environmental conditions. These plants produce buoyant gourds, which are dispersed by water (Wilson 1993). Seeds can remain viable within gourds for more than 1 year, but gourds must break open before seeds can germinate. Germination starts in early spring, seedlings establish and flowering starts about 4-8 weeks later. Flower and gourd production can continue until the first frost or severe drought.

Wild squash is susceptible to virus infection and mosaic viruses have been reported in wild populations (Quemada et al. 2008, Prendeville et. al-Ch. 2). The prevalence of virus in wild squash populations ranges from 0-100% with a median virus infection at 25% (Prendeville et. al-Ch. 2). In wild squash populations, 80% of virus infections are asymptomatic (Prendeville et. al-Ch.2). Mosaic viruses that infect wild squash include *Squash Mosaic Virus* (*SqMV*, Secoviridae), *Zucchini Yellow Mosaic Virus* (*ZYMV*, Potyviridae), and *Cucumber Mosaic Virus* (*CMV*, Bromoviridae; Provvidenti et al. 1978, Fuchs and Gonsalves 1999, Fuchs 2008). *SqMV* is known to infect at least four host families, probably has a world-wide distribution, and is non-persistently transmitted by beetles. *ZYMV* has a moderate host range (10 host plant families), but infects mostly cucurbits. *CMV* has a very broad host range and is known to infect 85 host plant families (reviewed by Palukaitis et al. 1992). Both *ZYMV* and *CMV* are non-persistently transmitted by aphids.

Mosaic viruses can drastically reduce yield in cultivated squash by stunting growth; causing mottling, discoloration and malformation of leaves, flowers, and fruits; reducing fruit production; and occasionally causing death (Walkey 1991, Fuchs and Gonsalves 1995, Gianessi et al. 2002). In wild squash, mosaic viruses reduce male and female flower production, gourd and seed number, and biomass (Fuchs et al.

2004b, Laughlin et al. 2009). However, it is not known if this decline in gourd and seed number due to virus infection results in a reduced population growth rate.

Model parameterization: Common garden experiments

2006 Common Garden- On 7 February 2006, we planted a common garden experiment at the Delta Conservation Demonstration Center in Metcalfe, Mississippi. We used a randomized block design that included 32 spatial blocks, seeds from three Mississippi populations, and three virus treatments. Seeds were collected near the towns of Yazoo City, Eagle Lake, and Vaiden. Following germination and seedling establishment plants experienced one of three virus treatments: inoculated with *SqMV*, inoculated with *ZYMV*, or non-inoculated. In each block, each population by virus inoculation treatment was replicated once, but each population by non-inoculated treatment was replicated twice. The non-inoculated treatment was repeated twice because we expected that some plants would become naturally infected during the experiment. Thus, the design included 384 plants. To limit contact between wild squash plants later in the growing season each planting location was six meters away from any other location. However, experimental plants did experience competition from other species present in the field.

At each planting location, we sowed four seeds from the assigned population. If multiple seeds germinated in a location then seedlings were either transplanted to empty locations or thinned to one plant. Germination and seedling survival was low enough that the block design was incomplete. Seeds began to germinate in April and flowering began in May. Plants in the virus-infected treatments were inoculated on 12 July 2006 by rubbing two-three new leaves with ~1 ml of phosphate buffer with celite and homogenized squash leaf tissue infected with either *SqMV* or *ZYMV*.

In the 2006 growing season, precipitation was below average (National Climatic Data Center 2009), squash plants stopped flowering in July, and all plants were dead by 28 July. Since all plants died shortly after virus inoculation, we were unable to collect leaf tissue to verify virus infection.

At each location, we recorded germination, seedling survival, and plant survival to flowering on a weekly basis throughout the growing season. Flower production by

sex was noted each day since flowers are only open for one day. Gourds were collected following plant death.

2007 Common Garden- On 28 March 2007 in the same field previously used, we planted a second common garden experiment. Our 2007 design was similar to our 2006 design except that we used seeds from Onward, MS instead of seeds from Yazoo, MS, and we planted 24 randomized blocks (instead of 32), for a total of 288 planting locations. In May 2007, precipitation was well below normal (and was even lower than May 2006). To simulate a normal precipitation year and to improve seedling establishment and survival, we flood irrigated the field once in June. In late June 2007 natural precipitation increased and for the remainder of the season precipitation was higher than in the 2006 growing season (National Climatic Data Center 2009).

Plants in the virus-infected treatment were inoculated with either *CMV* on 10 July or *ZYMV*, on 14-15 July as previously described. Virus inoculations were verified with Immunostrip tests (Agdia, Indiana, USA) performed in the field and Antigen Coated Plate-Enzyme-Linked Immunosorbent Assay (ELISA) performed by Agdia testing services. To limit aphid populations, and reduce natural virus spread, we sprayed plants with Sevin (Bayer Company) on 28-31 May and 31 July. The impact of the pesticide on pollinators was limited by spraying in the evening when pollinators were not active. Also, wild squash flowers are only open for one day and open for in the morning thus pesticide was not applied to surfaces pollinators frequently contact.

Germination did not occur at all locations; although we transplant seedlings when possible the block design was incomplete. We monitored germination, seedling survival, plant survival to flowering, and flower production as previously described. Prior to virus treatment application on 21 May 2007, we counted all expanded leaves on each plant as an estimate of plant size. Gourds were collected following plant death in November.

Model parameterization: Dormancy

In February 2006 and March 2007, we placed seeds and gourds (that had been produced the previous fall) into the field (next to the common garden) so we could assay seed viability and gourd integrity through time. In February 2006, we

individually caged 15 gourds per population (Yazoo, Vaiden, and Peanut) and in March 2007, we individually caged 15-20 gourds per population (Vaiden, Peanut, and Onward). Cages were made from chicken wire tacked to the ground with wire stakes. During the 2006 and 2007 growing seasons, we monitored gourd integrity on a weekly basis. Once a gourd opened, then all seeds were collected and stained with tetrazolium to assess seed viability. Gourds that remained intact through the sampling period were collected in April 2008 and seeds were tested for viability. Some gourds were lost due to animal disturbance. The proportion of gourds lost to rodents was included as a parameter in our demographic model.

In February 2006 and again in March 2007, we buried 60 open-topped mesh boxes (20 cm x 20 cm x 10 cm deep), each with 50 seeds from one of three populations (2006: Peanut, Vaiden, Yazoo; 2007: Peanut, Vaiden, Onward). Seeds had been collected in the previous growing season and were buried ~1 cm deep. To prevent seeds from dispersing outside of the box the open top of each box was ~0.5 cm higher than the soil surface. During the growing seasons in 2006 and 2007, we monitored germination on a weekly basis. Following germination seedlings were cut at the stem to reduce soil disturbance. Two-four baskets from each population and year-buried combination were collected from the field in August 2006, May 2007, and January 2008; the remaining 7-14 boxes in each population and year-buried combination were collected in April 2008. Some boxes were destroyed by animals and were not recovered. After box collection seeds were removed from the soil and stained with tetrazolium to assay seed viability.

The proportion of seeds surviving to the next growing season is probably less in nature than observed in this experiment. In this experiment, seed survival was likely increased by planting seeds into disturbed bare soil in February or March. In general, estimating the probability a seed will survive to the next growing season is difficult as the fate of a seed must be tracked from the fruit to entry into the soil.

Statistical analysis

2006 Common Garden- MANOVA was used to examine the effect of population on total female and male flower production per plant, average number of gourds per plant, average number of seeds per gourd (all log transformed), the proportion of seeds that germinated, the proportion of seedlings surviving to flower, and the

proportion of adult flowering plants that produced gourds (SAS 9.1, SAS Institute 2003). MANOVA indicated a significant effect of population (P=0.0016), thus we used generalized linear models with the appropriate error distribution for each demographic parameter to analyze each parameter individually. In all of these analyses population is considered a fixed effect. Virus treatment was not included in analyses of the 2006 common garden because all plants died shortly after inoculation.

2007 Common Garden- We used MANOVA to examine the effect of virus treatment, population, and the virus*population interaction on male and female flower production per plant, average number of gourds per plant (all log transformed), total seeds per plant, the proportion of seedlings surviving to flower, and the proportion of flowering plants that produced gourds. In this analysis, we included leaf number as a covariate since the plant size and fecundity are usually correlated. MANOVA indicated significant effects of population (P=0.0073) and the population*virus treatment interaction (P=0.0186). Therefore, we conducted univariate analyses of each demographic parameter using generalized linear models with appropriate error distributions. In all of these analyses virus, population, and the virus*population interaction were considered fixed effects.

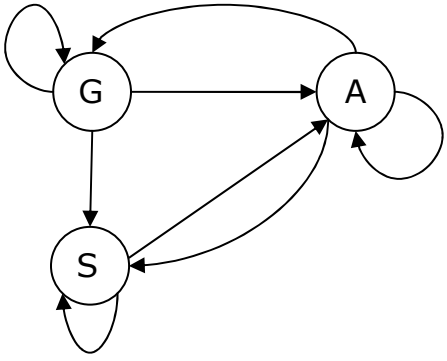
2006/2007 Comparison- We conducted MANOVAs to determine if demographic parameters within a population were different in 2006 and 2007, presumably because environmental conditions differed between these years (2009). Only plants from the Peanut and Vaiden populations were grown in both years. Therefore we compared demographic parameters estimated for plants grown in 2006 to those parameters derived for plants in the no virus treatment grown in 2007 from either the Peanut or Vaiden population. In these analyses, response variables were the proportion of seeds that germinated, the probability a seedling survived to flower, the proportion of flowering plants that produced gourds, average number of gourds per plant (log-transformed) and average number of seeds per plant (log-transformed). The remaining parameters related to dormancy minus dormant seeds less than one year old were not included. These parameters related to dormancy were not included since we used the same estimates of these parameters to calculate population growth rate in both years. MANOVAs indicated a significant effect of year (Peanut P<0.0001 and Vaiden P<0.0001); therefore we conducted

univariate analyses for each of the demographic parameters analysed in the MANOVA. To determine the effect of year on the proportion of seeds that are dormant less than one year from seed maturation, we conducted a generalized linear model with a binomial error distribution. This parameter was not included in the MANOVA, since a separate experiment was used to estimate seed dormancy.

Modeling

To calculate the population growth rate (lambda, λ), we constructed a stage-structured matrix model (Lefkovitch model) with a pre-breeding census, birth-pulse process using an annual time step (Caswell 2001). The population is censused in the fall after plants have flowered, but before gourds are produced. At this time individuals are either adult flowering plants (A), gourds in the gourd bank (G), or seeds in the seed bank (S; Figure 3.1). Since *C. pepo* does not reproduce continuously through the year, and because it is an annual species, a birth-pulse process and annual time step are biologically appropriate (Figure 3.1). Following the census, adults produce gourds that either die, remain in the gourd bank, open to release seeds that remain in the seed bank, or open to release seeds that germinate. Although the transition probabilities differ, gourds present in the gourd bank at the time of the population census have the same potential fates as gourds released from plants after the census. Finally, seeds in the seed bank at the time of the census can die; remain in the seed bank; or germinate. Germination usually occurs in early spring and afterwards seedlings may establish, flower, and produce gourds or die during this process.

Figure 3.1. Life-history diagram of wild squash (*Cucurbita pepo*) population model. The arrows indicate transitions within and between stage classes: adult plants (A), gourd bank (G), and seed bank (S).



We parameterized stage-structured matrix models for each population, year (2006 and 2007), and virus treatment (in 2007 only) combination using estimates of germination, dormancy, establishment, and reproduction from the 2006 and 2007 common garden and dormancy experiments. If a life history trait did not differ among virus treatments or among populations, we used pooled data, as appropriate, to estimate the trait for model analysis (Table 3.1A). Parameters were estimated as least square means derived from a generalized linear model with the appropriate error distribution with population, virus treatment (2007 only), and the population*virus interaction (2007 only) as fixed effects. When appropriate, the data were transformed before analysis; back-transformed parameter estimates were used in the model.

Table 3.1. Life-history parameters included in population projection models of wild squash. A) Variables used to estimate each element of the transition matrix and their symbols. Significant effects of population, virus, or year are indicated by ◇ (P=0.0501), * (P<0.05), ** (P<0.01), or *** (P<0.0001). Not all traits were evaluated for a virus or year effect; "." indicates that a trait was not included in a particular analysis either because the virus treatment was not applied to those traits (Virus effect) or the parameter was not estimated for each year (Year effect). B) Parameters multiplied to calculate each element of the transition matrix for wild squash. Symbols defined in (A).

A

Demographic parameters	Symbols	Population effect 2006	Population effect 2007	Virus effect 2007	Year effect Peanut	Year effect Vaiden
Proportion of seeds less than 1 year old that are dormant	D	***	*	.	***	
Proportion of seeds more than 1 year old that are dormant	D.old			.	.	.
Average number of gourds per plant	F.g			**		*
Average number of seeds per gourd	F.s	*				*
Number of seeds more than 1 year old that are viable	F.s.old			.	.	.
Proportion of seeds more than 1 year old that germinate	Germ.old	**	*	.		
Proportion of flowering plants that produce a gourd	G.prod				◇	*
Proportion of viable gourds more than 1 year old	G.viable			.		
Proportion of seeds that germinate	Germ	**	*	.	***	***
Proportion of gourds that open	Open			.	.	.
Proportion of gourds not consumed by rodents	Rod.g			.	.	.
Proportion of seedling that survive to flower	S.f	**	**		***	***

B

	Gourd	Seed	Adult
Gourd	Rod.g * (1-Open) * G.viable	.	G.prod * F.g * Rod.g * (1-Open) * G.viable
Seed	Open * Rod.g * G.viable * F.s.old * D.old	D.old	G.prod * F.g * Rod.g * Open * F.s * D
Adult	Open * Rod.g * F.s.old * Germ.old * S.f	Germ.old * S.f	G.prod * F.g * Rod.g * Open * F.s * Germ * S.f

All possible transitions in the demographic model include multiple life history traits measured in the field (Tables 3.1A,B). In particular, 12 life history traits (Table 3.1A) were used to estimate elements of the transition matrix (Table 3.2). For instance, the transition from an adult plant at time t to a new adult plant at time $t+1$ is calculated by multiplying eight life history traits (proportion of flowering plants that produce a gourd *average number of gourds per plant * proportion of gourds not consumed by rodents * proportion of gourds that open *average number of seeds per gourd * proportion of seeds that germinate * proportion of seedlings that survive to flower). Within elements of the transition matrix, we estimated the proportion of flowering plants that produce a gourd, which excludes flowering plants with no gourd production. Therefore the average number of seeds per gourd was derived only from plants that produced gourds. Since there were no seeds more than a year old from the Onward population we used an estimate of germination for seeds more than a year old from the Peanut population.

We developed deterministic matrix models rather than stochastic matrix models because we had data to estimate the transitions for two years stochastic matrix models should only be used if at least five years of demographic data are available (Doak et al. 2005).

Wild squash population growth rates from literature

To determine if population growth rates estimated from our deterministic matrix models were comparable to growth rates observed in wild squash populations, we compiled population census data from the literature (Quemada et al. 2008). Population growth rates were estimated by dividing the number of wild squash plants in year $t-1$ by the number of wild squash plants in year t. We were able to calculate population growth rates in 14 wild squash populations that were each monitored over 2-4 growing seasons.

Analysis of population growth rates

We examined the effects of population, virus treatment (2007 only), and year on population growth rate using sampled randomization tests (Sokal and Rohlf 1995, Caswell 2001). We used sampled randomization tests, to randomly sample raw data

without replacement 5,000 times, to determine if observed differences were due to random chance or the effects of population, virus treatment, or year. All demographic parameters were used to estimate the population growth rate, but only parameters that were significantly different due to virus treatment, population, or year were randomized (Table 3.1A). For instance, to determine if virus treatments differed in 2007, we compared population growth rates estimated from randomly sampling raw data without replacement for the average number of gourds produced per plant from the compared virus treatments within the same population. Since there was no effect of virus treatment on the remaining parameters, then we estimated these parameters by pooling data across the compared virus treatments within a population. Also, randomization tests were used to compare λ between populations in 2006 and between populations in the no virus treatment for 2007. In addition, randomization tests were used to compare λ between years by estimating demographic parameters for each appropriate population (Vaiden or Peanut) from all plants in 2006 and from all plants with no virus in 2007. Again, randomization of raw data occurred for only those parameters that differed due to year (Table 3.1A).

We tested for global effects of population, virus treatment, or year on population growth rates using the z-transform approach (Whitlock 2005). The z-transform method combines p-values by summing the quantiles of the standard normal distribution for each p-value and then divide this sum by the square root of the number of combined p-values. For instance, to test for an effect of virus on population growth rate, we combined all p-values from each population that compared the difference in population growth rates of no virus to *CMV* and no virus to *ZYMV*. Similarly, to determine if there was an overall effect of year, we combined p-values from comparisons of Vaiden 2006 to 2007 and Peanut 2006 to 2007 using the z-transform method (Whitlock 2005). In addition, to test for the effect of population on population growth rate, we combined p-values for all comparisons of population within a year and the no virus treatment across both years.

To determine the sensitivity of λ to changes in each element in the transition matrix, we conducted sensitivity analyses (Caswell 2001). The sensitivity of λ to changes of an element in the transition matrix is analogous to the selection gradient and determines the dependence of λ on that life history transition represented in the transition matrix element (Lande 1982, Stearns 1992). Therefore sensitivity

analyses indicate the strength and direction of natural selection. Sensitivity (***S***) is the slope of the asymptotic growth rate, λ, as a function of a matrix element, a_{ij}, which is calculated by taking the partial derivative of the asymptotic growth rate as a function of a matrix element, a_{ij}, $\frac{\partial \lambda}{\partial a_{ij}}$. We conducted sensitivity analyses to determine if the pattern of natural selection on life history transitions differed among virus treatments or between years (Caswell 1989, 2001), but see (Demetrius et al. 2007).

Since each matrix element is composed of a number of parameters (see **Modeling**; Table 1B), we also conducted lower level sensitivity analyses (LLS). LLS is the sum of the partial derivative of the asymptotic growth rate as a function of the partial derivative of parameter, x, $\left(LLS = \sum_{i,j} \frac{\partial \lambda}{\partial a_{ij}} \frac{a_{ij}}{\partial x} \right)$.

In addition, to determine if virus (2007 only) or year affects the proportional contribution of life-history transition to the population growth rate we conducted elasticity analyses. The elasticity of λ to changes in a life history transition indicates the proportional contribution of that life history transition to the population growth rate. Specifically, an elasticity analysis quantifies the change in λ with a small proportional change in a life history transition (a_{ij}) when other transitions are held constant (de Kroon 1986, Caswell 2001). An elasticity matrix (***E***) is $\left(\frac{a_{ij}}{\lambda} \frac{\partial \lambda}{\partial a_{ij}} \right)$ and the sum of each transition within an elasticity matrix equals one. Since each matrix element is composed of a number of parameters, we also calculated lower level elasticities (LLE) to determine if virus or year affects the contribution of each parameter to the population growth rate, λ. LLE is $\left(\frac{x}{\lambda} \frac{\partial \lambda}{\partial x} \right)$ do not sum to 1 since λ is not expected to be a homogenous function of x (Caswell 2001). These calculations, randomization tests, and modeling were completed using R software (2.11.1).

Results

2006 Common Garden- MANOVA indicated a significant effect of population (P=0.0016), therefore we used univariate analyses to evaluate the effect of population for each parameter. In 2006, male flower production per plant differed among populations (P=0.0001) with plants from the Vaiden and Yazoo populations producing more male flowers than plants from the Peanut population. However female flower production did not differ among populations (P=0.8547). Vaiden and Yazoo plants produced more gourds per plant, more seeds per gourd, and seedlings survived to flower more often than did plants from the Peanut population (P=0.0074, P=0.0433, and P=0.0064, respectively Table 3.1A). There was only a marginally significant difference due to population in the proportion of flowering plants that produced gourds (P=0.0721). Also in 2006, we found population differences for the proportion of seeds that germinate (P=0.0318), the proportion of seeds that are dormant (P<0.0001), and the proportion of seeds more than 1 year old that germinate (P=0.0006, Table 3.1A). Since populations differed in demographic parameters, we estimated population growth rates for each population.

2007 Common Garden- MANOVA indicated significant effects of population (P=0.0073), the population*virus treatment interaction (P=0.0186), and the covariate leaf number (P=<0.0001) on demographic parameters. Also, there was a significant effect of leaf number*virus*population interaction (P=0.0304), but there was no effect of either the leaf number*virus or leaf number*population interactions (P=0.2694 and P=0.1850, respectively).

Univariate analysis indicated a marginally significant effect of virus (P=0.0540) and a significant effect of the covariate, leaf number (P=0.0079), on the average number of gourds produced per plant. *Post hoc* tests indicated that plants with no virus produced more gourds than did plants infected with *CMV* (65 vs. 21 gourds; P=0.0354) and more gourds than plants infected with *ZYMV* (39 gourds, P=0.0463). There was no difference in gourd production between plants infected with either *CMV* and *ZYMV* (P=0.9266). Also, there was a significant effect of virus treatment on the total number of seeds produced per plant (P=0.0241, Table 3.1A). *Post hoc* tests indicated that plants with no virus produced more seeds (1959 seeds) than plants infected with *CMV* (725 seeds, P=0.0162) and plants infected with *ZYMV* (1392

seeds, P=0.0264). There was no difference in seed production between plants infected with *CMV* and *ZYMV* (P=0.8723). Since demographic parameters differed due to virus and population, we calculated population growth rates separately for each population by virus treatment combination.

Comparison between 2006 and 2007- Demographic parameters differed between years as detected using MANOVA for both the Peanut and Vaiden populations (P<0.0001 and P<0.0001, respectively). Univariate analyses indicated an effect of year among some demographic parameters within each population (Table 3.1A).

Population growth rates

Using the z-transform method to combine p-values calculated from randomization tests, we found an overall effect of population, year, and virus treatment on population growth rates of wild squash (Table 3.2).

2006- In 2006, randomization tests indicated that the population growth rates did not differ between the Peanut and Vaiden populations, the Peanut and Yazoo populations, and the Vaiden and Yazoo populations (Table 3.2, Fig. 3.2).

2007- Under normal precipitation experienced in 2007, randomization tests indicated that the population growth rates of Onward, Peanut, and Vaiden were each reduced when infected with *CMV* in comparison to no virus or *ZYMV* (Fig. 3.2, Table 3.2). In each population, the population growth rates did not differ when plants were infected with *ZYMV* in comparison to plants with no virus (Fig. 3.2, Table 3.2). Also, the Onward population grew faster than both the Peanut and Vaiden populations (Fig. 3.2, Table 3.2). However, the population growth rates of plants from the Peanut and Vaiden populations did not differ (Fig. 3.2, Table 3.2).

Comparison between 2006 and 2007- Since precipitation differed between 2006 and 2007, we used randomization tests to investigate the effect of different environmental conditions on population growth rate. For both the Peanut and Vaiden populations, we found λ was greater in 2007 than 2006 (Fig. 3.2, Table 3.2).

Population growth rates from the literature

From 14 wild squash populations, we found that population growth rates ranged from 0-6, with an average of 1.35 and a median of 0.6 (Fig. 3.3). Seed and gourds from four of these 14 populations were used in the common garden experiments presented here. In these four populations λ ranged from 0-6, with an average λ=1.71 and a median λ=1.45 (Fig. 3.3).

Table 3.2. Overall population, year, and virus effects determined using z-transform method (Whitman 2005) indicated bold font. Statistical differences in λ for each comparison determined by sampled randomization tests.

	Difference in λ	P-values
Overall population effect		**0.0058**
2006 Populations		
Peanut-Vaiden	-0.003	0.4037
Peanut-Yazoo	0.002	0.4705
Yazoo-Vaiden	-0.005	0.4635
2007 Populations		
Onward-Peanut	5.731	0.0030
Onward-Vaiden	4.381	0.0200
Peanut-Vaiden	-1.350	0.1672
Overall year effect		**<0.0001**
Vaiden 2007-2006	3.63	0.0001
Peanut 2007-2006	0.82	0.0001
Overall virus effect		**<0.0001**
Onward		
No virus - *CMV*	3.60	0.0238
No virus - *ZYMV*	0.55	0.4123
ZYMV - CMV	3.05	0.0366
Peanut		
No virus - *CMV*	1.17	0.0240
No virus - *ZYMV*	0.18	0.4081
ZYMV - CMV	0.99	0.0500
Vaiden		
No virus - *CMV*	1.74	0.0245
No virus - *ZYMV*	0.27	0.4073
ZYMV - CMV	1.47	0.0414

Figure 3.2. Wild squash population growth rates for 2006 and 2007. In 2006, wild squash from Peanut, Vaiden, and Yazoo were grown in drought conditions. In 2007, wild squash from Peanut, Vaiden, and Onward experienced one of three virus treatments (no virus (black bars), *Cucumber mosaic virus* (*CMV*, grey bars), or *Zucchini yellow mosaic virus* (*ZYMV*-white bars) and were grown in normal precipitation conditions.

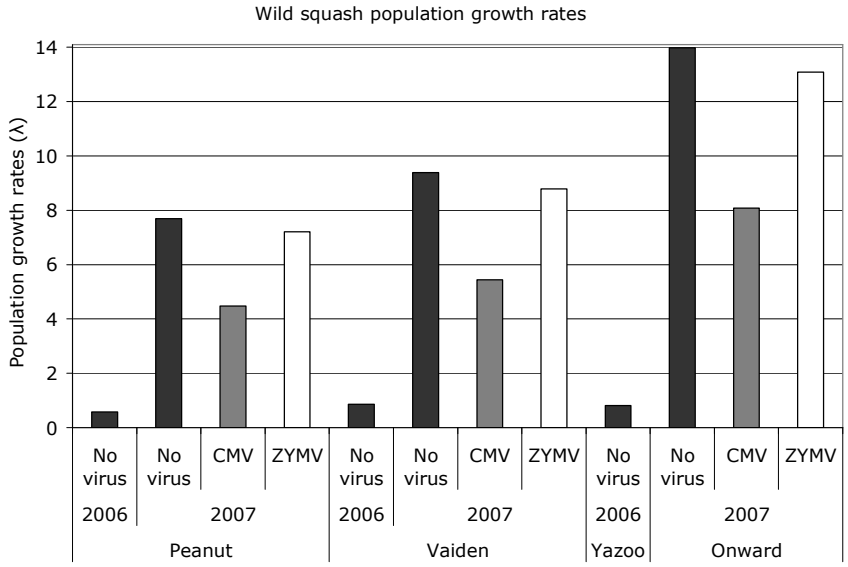
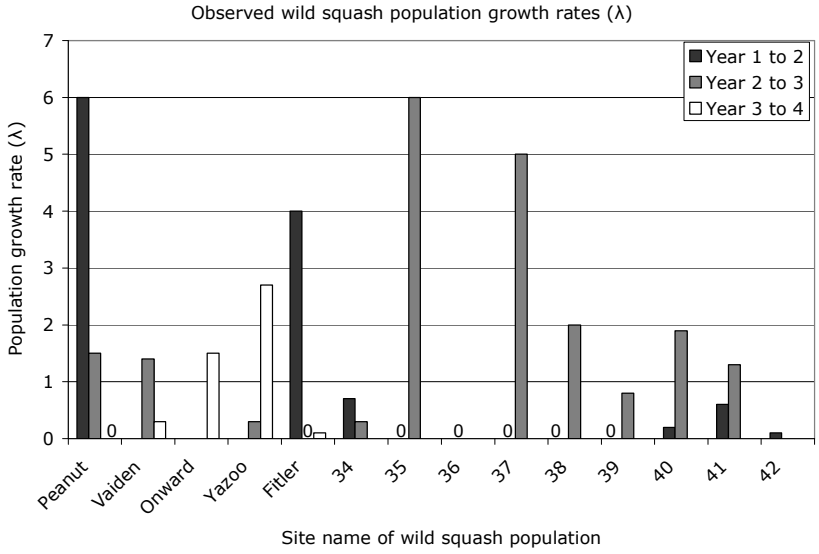


Figure 3.3. Wild squash population growth rates estimated from published population estimates. Data from populations identified as Peanut, Vaiden, Onward, Yazoo, and Fitler are from Prendeville et al.-Ch. 2; population sizes were estimated in 2004-2007. Seeds from most of these populations were used in the common garden study presented here. Data from populations identified by number are from Quemada et al. 2008; population sizes were estimated in 2000-2002. Zeros indicate that lambda was zero (i.e. a population went extinct); no bar or zero indicates that lambda could not be estimated since population size was not documented.



Natural selection

2006- Sensitivity analyses indicated that in the drought conditions experienced in 2006 natural selection acted most strongly on the seed bank to adult transition, followed by the adult to adult transition in the Peanut population (Fig. 3.4A). While for the Vaiden and Yazoo populations natural selection favored the adult to adult transition the most. (Fig. 3.4A)

Lower level sensitivity analyses indicated the strength of selection on each lower level parameter that was used to estimate each element of the transition matrix (Table 3.1A,B). In the Peanut population natural selection was greatest on the proportion of seedlings surviving to flower followed by the proportion of seeds surviving to the next growing season (Fig. 3.5A). In the Vaiden and Yazoo populations natural selection was greatest on the proportion of seeds surviving to the next growing season followed by the proportion of seedlings surviving to flower (Fig. 3.5A).

Figure 3.4. Sensitivity (left column) and elasticity (right column) values. Panels A and B: 2006 comparisons among Peanut, Vaiden, and Yazoo. Panels C and D, E and F, and G and H: 2007 comparisons among virus treatments (*CMV: Cucumber mosaic virus, ZMYV: Zucchini yellow mosaic virus*, and No virus*)* in Onward, Peanut, and Vaiden populations, respectively. Sensitivities and elasticities are presented for matrix transitions from time t to time t+1: GG, a gourd remains in the gourd bank; AG, adult produces a gourd that enters gourd bank; GS, gourd in the gourd bank opens and seed enters seed bank; SS, seed remains in seed bank; AS, adult produces a gourd which opens and seed enters the seed bank; GA, gourd in gourd bank opens and seed develops into adult; SA, seed in seed bank germinates and develops into adult; and AA, adult produces an adult.

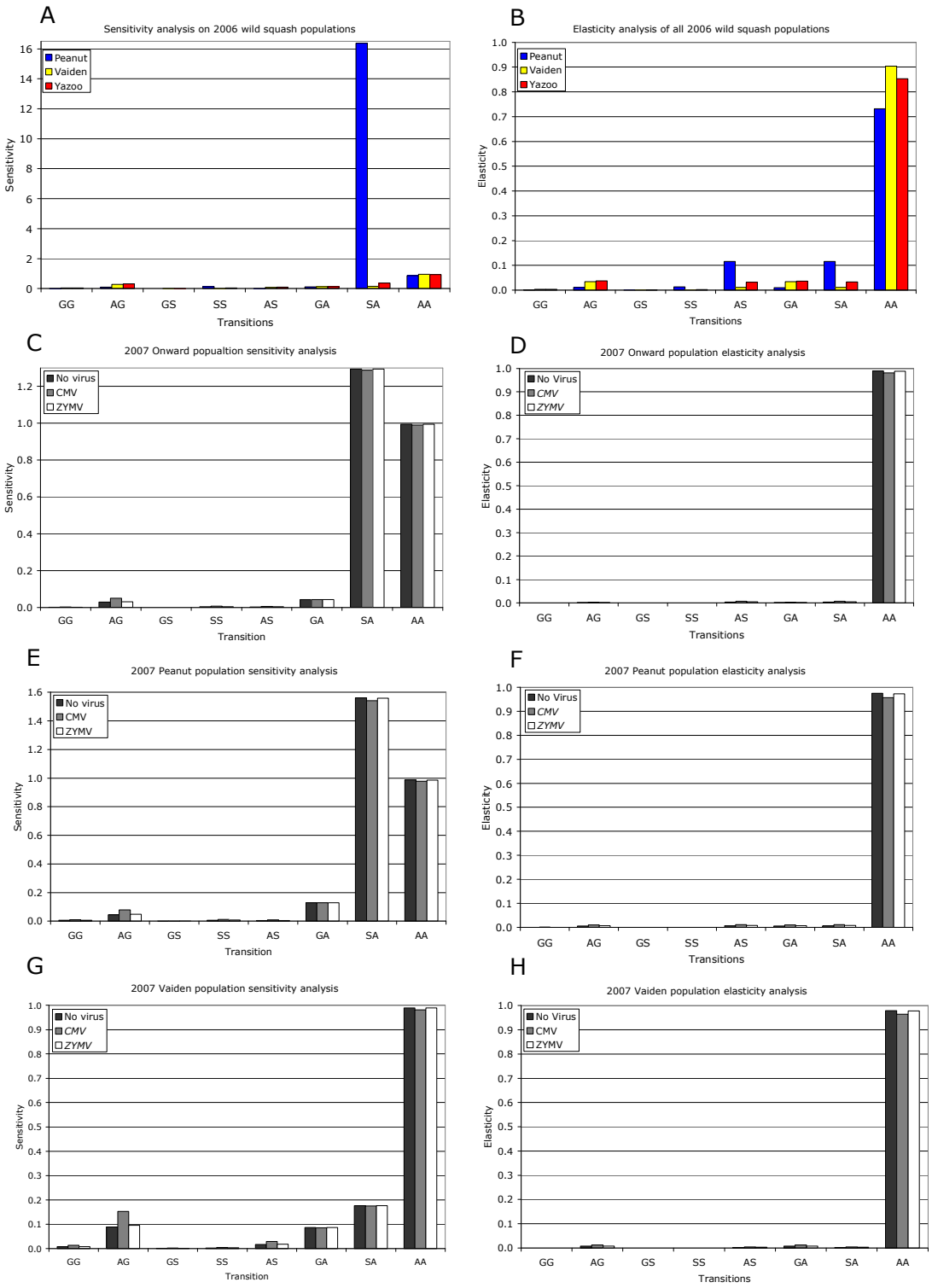
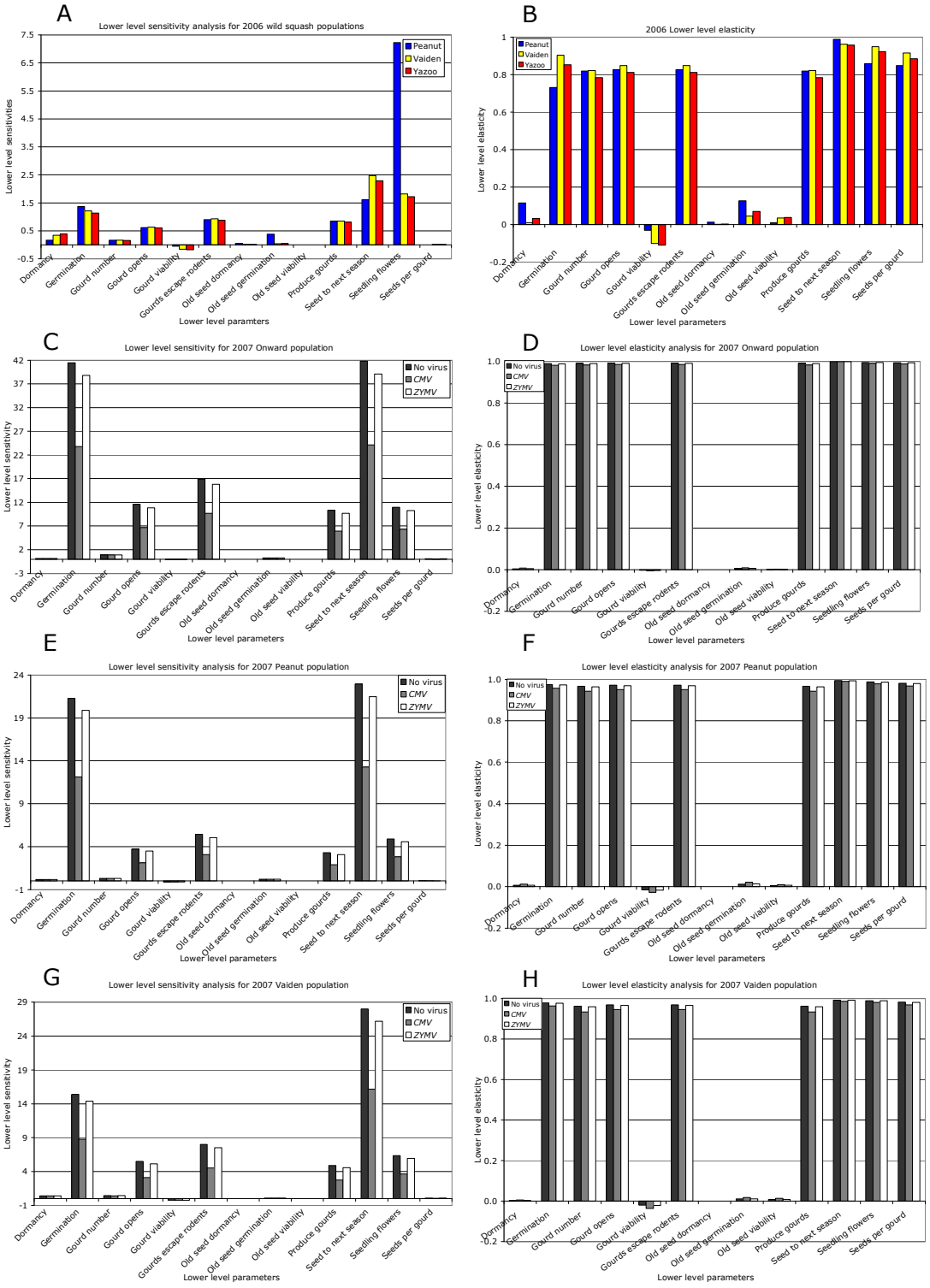

A
Sensitivity analysis on 2006 wild squash populations
Peanut
Vaiden
Yazoo
Sensitivity
Transitions
GG AG GS SS AS GA SA AA
B
Elasticity analysis of all 2006 wild squash populations
Peanut
Vaiden
Yazoo
Elasticity
Transitions
GG AG GS SS AS GA SA AA
C
2007 Onward popualtion sensitivity analysis
No virus
CMV
ZYMV
Sensitivity
Transition
GG AG GS SS AS GA SA AA
D
2007 Onward population elasticity analysis
No Virus
CMV
ZYMV
Elasticity
Transitions
GG AG GS SS AS GA SA AA
E
2007 Peanut population sensitivity analysis
No virus
CMV
ZYMV
Sensitivity
Transition
GG AG GS SS AS GA SA AA
F
2007 Peanut population elasticity analysis
No Virus
CMV
ZYMV
Elasticity
Transitions
GG AG GS SS AS GA SA AA
G
2007 Vaiden population sensitivity analysis
No virus
CMV
ZYMV
Sensitivity
Transition
GG AG GS SS AS GA SA AA
H
2007 Vaiden population elasticity analysis
No Virus
CMV
ZYMV
Elasticity
Transitions
GG AG GS SS AS GA SA AA

Figure 3.5. Lower level sensitivity (left column) and elasticity (right column) values for each lower level parameter. Panels A and B: 2006 comparison among Peanut, Vaiden and Yazoo populations. Panels C and D, E and F, and G and H: 2007 comparisons among virus treatments (*CMV: Cucumber mosaic virus, ZMYV: Zucchini yellow mosaic virus*, and No virus*)* in Onward, Peanut, and Vaiden populations, respectively.



2007- For each population and virus treatment grown in 2007, we calculated sensitivity matrices to determine if natural selection differed in strength and/or direction among virus treatments. There was no difference in the direction of natural selection among virus treatments though the magnitude of selection differed among virus treatments (Figs. 3.4C, 3.4E, 3.4G). For instance, the magnitude of natural selection was greater on gourds releasing seeds to become adults in the next growing season for plants infected with *CMV* than for plants with no virus or infected with *ZYMV*. The magnitude of natural selection did differ among wild squash populations suggesting that different traits are favored by natural selection in the different environments in which these populations occur. For instance, in the Peanut and Onward populations natural selection was greatest on seeds in the seed bank germinating and becoming adults in the next growing season, whereas in the Vaiden population natural selection favored the adult to adult transition (Fig 3.4G).

For all three populations, lower level sensitivity analyses indicated that natural selection was greatest on the proportion of seeds that survive to the next growing season followed by the proportion of seeds that germinate (Figs. 3.5C, 3.5E, 3.5G). The magnitude of natural selection differed among virus treatments. However the rank order of parameters did not differ indicating that the dependence of the population growth rate on each lower level parameter does not differ with virus treatment. In all three populations, the magnitude of selection was less for all parameters of plants infected with *CMV* in comparison to plants with no virus or infected with *ZYMV* (Figs. 3.5C, 3.5E, 3.5G).

Life history trait contributions to population growth rates

2006- Elasticity analyses indicated how a life history transition contributes to the population growth rate. In 2006, the adult to adult transition had the greatest contribution to the population growth rate for all three populations. The remaining transitions contributed much less to the population growth rate and differed in contribution among populations (Fig 4B).

To determine how each lower level parameter (Table 3.1A,B) contributed to the population growth rate we conducted lower level elasticity analyses. We found that many parameters made large contributions to the population growth rate (Fig. 3.5B)

particularly those parameters used to estimate the probability an adult produces a new adult in the next year. Contribution to the population growth rate was minimal from dormant seeds in the seed bank and gourd bank (Fig. 3.5B). In addition, the probability of a gourd remaining viable in the next growing season had a negative contribution to λ, indicating that an increase in this parameter reduces λ.

2007- To determine if the contribution of each life history transition (Table 3.2) to the population growth rate differed among virus treatments, we calculated elasticity matrices. For all three populations and all three virus treatments the adult to adult transition had the greatest contribution to the population growth rate (Figs. 3.4D, 3.4F, 3.4H). There was no difference in contribution from each life history transition to the population growth rate among virus treatments for each of the wild squash populations.

In addition, we calculated lower level elasticities for each lower level parameter (Table 3.1A) to determine if parameters differed in their contribution to λ due to virus treatment. For all three populations and virus treatments, those parameters used to estimate the adult to adult transition made a positive contribution to the population growth rate (Figs. 3.5D, 3.5F, 3.5H). There was a minor difference in the magnitude of contribution made by each of these parameters with plants infected with *CMV* contributing less to the population growth rate in comparison to plants with no virus or infected with *ZYMV* (Figs. 3.5D, 3.5F, 3.5H).

Discussion

Pathogens are thought to regulate host plant populations. However in wild squash a reduction in population growth rate due to virus depends on the virus species. Though both *CMV* and *ZYMV* reduced gourd and seed production compared to plants with no virus only *CMV* reduced gourd production to the extent that population growth rates were affected. Therefore it is not appropriate to assume a reduction in components of fitness will always result in a reduction in population growth or size as populations may be affected by other factors (Bergelson 1994).

The notion that a reduction in individual plant fitness leads to a reduction in population growth rate has been built upon to develop hypotheses to explain plant ecology. For instance, this assumption influences the enemy release hypothesis, which assumes natural enemies (i.e. plant diseases), particularly specialists, limit wild plant population size within its native range. Therefore when a plant species enters a new environment it escapes these natural enemies and is able to rapidly grow to invade the new environment. Studies testing the enemy release hypothesis have primarily focused on the effects of enemies on components of individual plant fitness and not on populations (Keane and Crawley 2002, Mitchell and Power 2003, Agrawal et al. 2005, Joshi and Vrieling 2005). However both *CMV* and *ZYMV* are present in wild squash populations (Prendeville et al.-Ch 2), but only *CMV*, a generalist, reduced the population growth rate of wild squash, not *ZYMV* which has a limited host range (Hull 2002). Thus, if wild squash enters a new environment void of *ZYMV*, our results suggest that wild squash population would not increase in size, which counters the predictions of the enemy release hypothesis.

However, a study by Laughlin et al. (2009) found *ZYMV* reduced gourd and seed production in wild squash 80-100%, whereas in this study *ZYMV* reduced gourd and seed production by 29-40%. In the Laughlin study (2009), plants with four leaves or less were inoculated with virus prior to transplanting in the field. However in the experiment presented here wild squash were inoculated with *ZYMV* much later in the growing season when most plants were established. The timing of virus infection in relation to plant development mediates the effect of virus on wild squash populations in that virus infections in smaller plants have a greater effect on plant performance than virus infections in larger plants (Pagán et al. 2007). From crop systems, it is clear that the timing of virus infection varies from year to year (Rowell et al. 1999). However it is not clear how the timing of virus infection differs in wild plant populations and what effect this may have on wild plant population growth and dynamics. It is likely that common garden experiments that inoculate plants at a small size may represent the maximal effect of virus, but it is not clear how well such experiments represent natural populations.

In wild plant populations, virus infections are frequently asymptomatic (Muthukumar et al. 2009), Prendeville et al.-Ch 2) in that there are no visual virus symptoms. Thus virus infections in wild plant populations are largely ignored as a factor in plant

ecology (though see Malmstrom et al. 2005a, Malmstrom et al. 2005b, Malmstrom et al. 2006, Seabloom et al. 2009a, Seabloom et al. 2009b). In this experiment, symptoms were present in 16% of plants infected with *CMV* and 15% of plants infected with *ZYMV*. Even though *CMV* infection frequently was asymptomatic, it reduced the population growth rate of wild squash population in comparison to no virus. Other studies have also documented that asymptomatic virus infections affect components of individual plant fitness relative to the fitness of uninfected plants (Remold 2002). Furthermore, the relationship between virus symptoms and virus concentration can be inconsistent (Thurston et al. 2001, Pallett et al. 2002), and not always correlated with plant fitness (Pagán et al. 2007).

Natural selection on life history traits did not differ in direction, but in magnitude among virus treatments. Therefore natural selection favors the same traits regardless of the presence or absence of virus infection. Similarly, lower level elasticity analyses indicated that the contribution of life history traits to population growth rates differ slightly in magnitude among virus treatments. Therefore the same traits contribute to the population growth rate regardless of the presence or absence of virus. Interestingly, when comparing traits favored by natural selection and that contributed to the population growth rate, we see that the magnitude of natural selection favoring the average number of gourds per plant or the average number of seeds per gourd is very small though these traits make large contributions to the λ. In addition, other traits made large contributions to λ (i.e. germination, proportion of gourds that open, probability a gourd escapes rodent herbivory, proportion of flowering plants that produce gourds, proportion of seeds that survive to the next season, and the proportion of seedlings that produce flowers; Figs 3.5B, 3.5D, 3.5F, 3.5H), but were not favored by natural selection.

In our common garden experiment, we only examined the effects of virus on a few parameters related to the adult plant. Therefore we did not investigate the effects of virus on germination, dormancy, and gourd integrity. However work by Fuchs et al. (2004a) indicates that virus can affect germination. In analyses, not presented here, we lowered the germination rate alone in the deterministic model, which resulted in a reduction in the population growth rate of wild squash. Since it is not clear how virus affects dormancy and seed mortality, we did not present these results. Regardless, it is interesting that if virus affects other traits in the absence of reducing

seed and gourd production that this would result in a reduction in population growth rate.

From demographic parameters estimated from a common garden experiment, we calculated λ between 0.49-8.5, which was somewhat higher than those derived from the literature (Fig. 3.3). Our over estimation of population growth may be due to not incorporating rodent herbivory on seeds as well as seed loss when gourds open and seeds are incorporated into the seed bank in the deterministic matrix model. Since we did not have an accurate estimate of the mean and variance of seed loss, we excluded these parameters from our model. Regardless this does not change our interpretation that virus and environment affect wild squash population growth rate assuming that rodents do not differentially consume seeds with or without virus or from plants in normal or low precipitation years.

We found that λ differed between years probably due to different environmental conditions (National Climatic Data Center 2009). Natural selection favored the same traits in each environment, but the magnitude of selection differed between the two environments. With different environmental conditions the contributions of life history traits to the population growth rate differed in magnitude such that dormant seeds contributed more to the population growth rate in drought conditions than in normal precipitation conditions.

We have shown that even though virus can reduce components of individual plant fitness this reduction does not always lead to a decrease in population growth rate. Thus, extrapolation from components of individual fitness does not always scale up to population level effects. Therefore, studies examining the ecological risks of transgenic virus-resistant squash in wild squash populations must examine the effects of the virus-resistant transgene and virus at the population level. This point is pertinent beyond risk assessment of transgenic crops and applies to all aspects of plant ecology and evolution.

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Chapter 4: The idiosyncratic effects of the virus-resistance transgene and virus infection on wild squash populations

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Introduction

Transgenic crops have been commercially available since 1992 and are grown throughout the world (James 1998). Since the development of transgenic crops, there have been concerns about their use and the potential effects transgenic crops could have on natural ecosystems. Two ecological risks associated with the use of transgenic crops are effects of transgene products on non-target organisms and the effects of transgene introgression into wild plant populations (Pilson and Prendeville 2004, Felber et al. 2007). Effects on non-target organisms occur when organisms that do not reduce yield are negatively affected by products of the transgene (Pilson and Prendeville 2004).

The second ecological risk associated with transgenic crops is transgene introgression into a wild population. Transgenic crop-wild hybridization and subsequent introgression would confer a novel trait to wild plants, which could alter the size and dynamics of wild plant populations. When a crop and wild relative co-occur then crop-wild hybridization is common (Wilson 1990, Ellstrand 2003). Thus, transgene introgression from transgenic crops to wild populations is likely and has been reported. For instance, in canola (*Brassica napus* L., Hall 2000) and creeping bentgrass (*Agrostis stolonifera* L., Watrud et al. 2004) transgenes have entered feral and wild populations.

Most studies examining the effects of transgene introgression into wild plant populations have focused on how the transgene affects components of individual plant fitness (Fuchs et al. 2004b, Laughlin et al. 2009, Sasu et al. 2009). However, components of individual plant fitness do not always scale-up to the population (Alexander and Mihail 2000). Therefore, it is not clear how transgene introgression

will affect wild plant population dynamics (although see Claessen et al. 2005a, Claessen et al. 2005b, Warwick et al. 2008).

Transgenes that are expected to affect wild plant populations are those that confer resistance to natural enemies, such as insects and pathogens, since natural enemies affect wild plant population growth and size (Alexander and Antonovics 1988, Louda and Potvin 1995, Rose et al. 2005). This may be the case for the virus-resistance transgene, since virus reduces wild squash fruit and seed number (Fuchs et al. 2004b, Laughlin et al. 2009). However, factors other than fruit number may limit wild plant populations, such as germination (Bergelson 1994). Therefore, it is not clear if benefits conferred by the virus-resistance transgene, such as an increase in fruit production, will lead to an increase in wild squash population size.

To determine if virus and the virus-resistant transgene affect wild squash population dynamics, we used deterministic matrix models to calculate population growth rates. We calculated population growth rates of back-cross generation two (BC2) squash populations with and without the virus-resistance transgene in the presence and absence of virus using a deterministic matrix model. By estimating the population growth of BC2 squash, we assume transgenic crop-wild hybridization and virus-resistance transgene introgression have occurred within an experimental population. With this experimental population, we were able to investigate if 1) virus reduces the population growth rate of BC2 plants, 2) if the virus-resistance transgene in the absence of virus affects the population growth rate of BC2 plants, and 3) if the virus-resistance transgene confers a benefit to the BC2 population in the presence of virus. In addition, we estimated population growth rates of additional experimental BC2 populations using some of the demographic parameters reported in the literature. We compare population growth rates derived from other studies to our estimates of population growth rate to infer general effects of virus and the virus-resistant transgene on wild squash populations.

Materials and Methods

Natural history

Cultivated and wild summer squash *Cucurbita pepo* L. are monoecious annuals that require insect-mediated pollination for reproduction. Squash plants produce flowers

for several weeks. However individual flowers last for less than 1 day, opening at dawn and closing around noon, depending upon environmental conditions. Wild squash occurs in south-central US and Mexico, which overlaps with commercial squash production (Wilson 1993). Cultivated squash readily interbreeds with its wild progenitor (also *C. pepo*), and non-transgenic cultivated alleles have been identified in wild squash populations (Wilson 1990, Wilson 1993, Decker-Walters et al. 2002).

Both wild and cultivated squash are susceptible to mosaic viruses, which are transmitted by aphids and beetles. Mosaic viruses common in cultivated squash are found in wild squash populations (Quemada et al. 2008). In squash, mosaic viruses cause mottling and deformity of fruits, leaves, and flowers, and can drastically reduce fruit production (Fuchs and Gonsalves 1995, Gianessi et al. 2002, Laughlin et al. 2009). The reduction in yield by viruses lead to the development of virus-resistant transgenic squash, which was deregulated in the US and made available for commercial use in 1994 (USDA/APHIS 1994, 1996). One variety of virus-resistant transgenic squash, Destiny III (Seminis Vegetable Seeds, Inc., Saint Louis, Missouri), has a transgenic construct, CWZ-3, which confers resistance to two potyviruses (*Zucchini yellow mosaic virus* and *Watermelon mosaic virus*) and a cucumovirus (*Cucumber mosaic virus*; Tricoli et al. 1995). Also, CWZ-3 has a selectable marker, neomycin phosphotransferase II (npt-II), which confers antibiotic resistance.

Backcross generation two squash (BC2)

In this experiment, we developed backcross generation two (BC2) squash that segregate for the CWZ-3 transgenic construct (Fuchs et al. 2004a). Specifically, we used pollen from Destiny III to hand pollinate plants collected from wild squash populations in 2005 to develop F1 plants. This movement of pollen from virus-resistant transgenic squash to wild squash simulates the most likely direction of introgression. We identified F1 plants with the transgene using PCR (Spencer 2001, Wall et al. 2004, Prendeville et al.-Ch. 2) and then backcrossed these F1 plants to wild squash collected in 2005 to create a backcross one generation (BC1). Again, we used PCR to identify BC1 plants with the transgene and used these plants to backcross into wild squash collected in 2006 to create the BC2 generation. BC2 plants segregate 1:1 for the transgene. To minimize the potential effects of particular genetic backgrounds, we used at least five different wild parents per cross

and individual F1 and BC1 plants were crossed with different parents. All crosses were completed in the greenhouses at the University of Nebraska-Lincoln.

Model parameterization

Common garden experiments

A common garden experiment was planted on 28 March 2007 at the Delta Conservation Demonstration Center in Metcalfe, Mississippi. We used a randomized block design with 24 spatial blocks. In each block, we planted BC2 seeds that we created from two populations collected in Mississippi near the towns of Yazoo City and Vaiden. These seeds then germinated and had one of three virus treatments: inoculated with *Cucumber mosaic virus* (*CMV*), inoculated with *Zucchini yellow mosaic virus* (*ZYMV*), or non-inoculated plants. In each block each population, each transgene status (present, absent), and virus inoculation treatment was replicated once, except for each population, transgene status, and non-inoculated treatment combination was replicated twice. The non-inoculated treatment was repeated twice because we anticipated some plants becoming naturally infected during the experiment. Thus, in total the experiment had 288 planting locations.

Wild squash were planted six meters apart to limit contact between wild squash plants later in the growing season. However, this experiment did occur with competition such that wild squash did come in contact with other plants that occurred naturally in the field. At each location four seeds of a particular population were planted to increase the probability of a plant with the appropriate transgene status was present in each location. If more than one seed germinated then seedlings were transplanted to empty locations or thinned to one plant per location. Germination did not occur at all locations therefore the block design was incomplete. Seeds began to germinate in April and flowering began in May.

In the field, we determined transgene status in BC2 squash using a leaf bleach assay. A leaf bleach assay tests for the presence of the selectable marker, which confers antibiotic resistance, present in Destiny III. To test for antibiotic resistance, we injected a small amount of 0.05% antibiotic solution (Paromomycin sulfate, MP Biomedicals, Inc., Solon, Ohio) into the underside of wild squash cotyledons (Cheng et al. 1997, Freitas-Astua et al. 2003). We pierced the lower epidermis of the

cotyledons by using the end of a paperclip so that the antibiotic solution entered the parenchyma. Within 2-3 days the injected area either died and looked bleached indicating absence of the transgene or did not change indicating presence of the transgene. A few plants were not identified in the field due to herbivory on the cotyledons. Leaf samples from all plants were sent to GeneSeek Inc. (Lincoln, Nebraska) to verify transgene status (methods described in Prendeville et al.-Ch.2). As a control for damage due to handling, piercing the leaf, and injection, we created a hole and inject ddH_2O in the other cotyledon. Control leaves looked normal a few days post ddH_2O infusion.

Plants in the virus-infected treatment were inoculated with either *CMV* on 10 July or *ZYMV* on 14-15 July, by rubbing two-three new leaves with ~1 ml of phosphate buffer with celite and homogenized squash leaf tissue infected with *CMV* or *ZYMV*, respectively. Virus inoculations were verified in the field with Immunostrip tests (Agdia, Indiana, USA) and after the field season with Antigen Coated Plate-Enzyme-Linked Immunosorbent Assay (ELISA) performed by Agdia testing services. We sprayed plants with Sevin (Bayer company, Monheim am Rhein, Germany) on 28-31 May and 31 July to limit aphid populations, thus reduce natural virus spread. The impact of the pesticide on pollinators was limited by spraying in the evening when pollinators were not active.

At each location, we recorded germination, seedling survival, and plant survival to flowering on a weekly basis throughout the growing season. Male and female flower production was noted daily since flowers are only open for one day. Gourds were collected following plant death in November.

Dormancy

In March 2007, we assessed BC2 seed viability in the seed bank. We buried 10 open-topped mesh boxes (20 cm x 20 cm x 10 cm deep) in a randomized design to assess seed viability in the seed bank (using methods described in Prendeville et al.-Ch.3). We mixed all viable seeds from the BC2 Vaiden population and buried 50 seeds ~1 cm deep into the soil to simulate seeds entering the seed bank. We monitored boxes for germination on a weekly basis. Boxes were collected from the field in January and April 2008. Animal disturbance affected some boxes; thus, only 7 of the 10 boxes were recovered. Following collection seeds were removed from the soil and stained with tetrazolium to assay seed viability.

Statistical analyses

We examined the effects of population, virus-resistance transgene, and virus treatment on demographic parameters of BC2 squash using MANOVA. Specifically, we examine how these factors and their interactions affected the average number of gourds per plant (log transformed), total seed number, male and female flower production (log transformed), the proportion of seedlings that survived to flower, and the proportion of flowering plants that produced a gourd. In this model, we used leaf number as a covariate since plant size is correlated with fruit production. There was a significant effect of the population*virus treatment*transgene interaction and a main effect of population (MANOVA $P= 0.0188$, $P<0.0001$, respectively). Thus, we used univariate ANOVA to examine population, virus treatment, virus-resistant transgene, and interactions on each demographic parameter. To evaluate the effect of population, virus-resistant transgene, virus treatment and interactions on the proportion of seedlings that survived to flower and the proportion of flowering plants that produced a gourd, we used separate general linear models each with a binomial distribution (GLMM; SAS 9.1; SAS Institute 2001). We examined the effects of these factors on the average number of gourds produced per plant and the total number of seeds produced per plant (log transformed) using separate GLMMs with a log normal distribution and a normal distribution, respectively, and with leaf number as a covariate. Also, to examine the effects of the population and the virus-resistant transgene on the proportion of seeds that germinated, we used a GLMM with a binomial distribution. In all of these analyses population, virus-resistant transgene, and virus treatment were fixed effects.

Modeling

To estimate the population growth rate (lambda, λ) of BC2 squash, we combined vital rates from a pre-breeding census considering a birth-pulse process (Caswell 2001) using an annual time step. *C. pepo* does not reproduce continuously throughout the year and is an annual species. Therefore a birth-pulse process and annual time step are biologically appropriate. We assume that adults produce gourds that are released after the population census. Once released gourds can open and seeds enter the soil to survive over the winter or die. Germination usually occurs in early spring and seedlings may establish to produce flowers and gourds or die during this process. Unlike wild squash, we found no seed dormancy for BC2 squash plants and we assumed there is no gourd dormancy. Therefore, there is only

one stage for BC2 squash plants, flowering adults that produce a gourd (A) (Table 4.1).

For each BC2 squash population, transgene status, and virus treatment, we estimated vital rates, specifically germination, dormancy, establishment, and reproduction from the common garden experiment (Table 4.1). Parameter estimates were calculated as least square means from a general linear model that included population, transgene status, virus treatment, and interactions as fixed effects. To estimate the average number of gourds per plant and average number of seeds per gourd (log transformed), we used general linear model with a log normal distribution and normal distribution, respectively. Back-transformed parameter estimates were used in each matrix model. All other parameters were proportions. Thus, we estimated these parameters as least square means from general linear models using binomial distribution (Table 4.1). In the dormancy experiment, we did not have enough BC2 Yazoo seeds to test seed viability. Therefore, we used estimates of seed viability from BC2-Vaiden seeds in BC2-Yazoo deterministic models. Also, to estimate the proportion of gourds that open in a growing season, we used estimates from another experiment. In this experiment, wild gourds collected in the previous growing season were caged and monitored weekly (Prendeville et al.-Ch.3). From these gourds, we also estimated the proportion of gourds that escaped rodent herbivory. Parameters that differed between populations, between transgene statuses, or among virus treatments were estimated separately, whereas those parameters not significantly affected by these factors were estimated using pooled data (Table 4.1).

To determine the effect of transgene status we estimated population growth rate of plants with and without the virus-resistant transgene in the presence and absence of virus. Therefore, we multiplied eight life history traits (the proportion of gourds not consumed by rodents * the proportion of gourds that open * proportion of seeds that survive to the next growing season * the proportion of seeds that germinate * the proportion of seedlings that survive to flower * the proportion of flowering plants that produce a gourd * the average number of gourds per plant * the average number of seeds per gourd, Table 4.1).

Table 4.1. Demographic parameters used to calculate BC2 population growth rate with results of statistical analyses evaluating the effect of population, virus treatment, virus-resistance transgene and the interactions on these demographic parameters. * represents P<0.05. x represents parameters not evaluated for each main effect and interaction effect since the treatment(s) was not applied to the parameter.

Parameters	Population	Virus	Transgene	Population *Virus	Population* Transgene	Virus* Transgene	Population* Virus* Transgene
Proportion of gourds that open		x	x	x	x	x	x
Proportion of gourds that escape rodent herbivory		x	x	x	x	x	x
Proportion of seeds that survive over winter	x	x	x	x	x	x	x
Proportion of seeds that germinate		x		x		x	x
Proportion of seedlings that survive to flower							
Proportion of flowering plants that produce gourds					*		
Average number of gourds per plant							*
Average number of seeds per plant					*		

Modeling using parameters from the literature

To estimate population growth rates under different experimental conditions, we incorporated two life history traits obtained from the literature. Both Fuchs et al. (2004) and Laughlin et al. (2009) report the effects of virus and the virus-resistant transgene in BC2 and BC3 squash on components of individual plant fitness. From these studies, we incorporated the average number of gourds produced per plant and the average number of seeds produced per gourd for each study with the other vital rates derived by pooling data from both BC2 Vaiden and BC2 Yazoo plants in our common garden experiment. Thus, we assume that pooled data from BC2 Vaiden and BC2 Yazoo represent life history traits in Fuch et al. (2004) and Laughlin et al. (2009) BC2 populations and that life history traits do not differ between BC2 and BC3 plants.

Analysis of population growth rate

Using randomization tests, we examined the effects of transgene status and virus treatment on the population growth rates (Caswell 2001). With randomization tests, we randomly sampled data without replacement 5,000 times in order to determine if our observed differences due to virus treatments or transgene status differed from those generated from randomized data. To determine if transgene status affected BC2 population growth rates, we compared plants with and without the virus-resistant transgene for each virus treatment. To evaluate the effect of virus treatment on BC2 population growth rates, randomization tests were conducted within each population between each virus treatment. All demographic parameters

were used to estimate the population growth rate, but only parameters that were significantly different due to transgene status or virus treatment were randomized within a population (Table 4.1). The remaining parameters not affected by transgene status or virus treatment were estimated by pooling data across the factor of interest.

Global effects of virus treatment and virus-resistant transgene status on population growth rates were tested using the z-transform approach (Whitlock 2005). The z-transform method combines p-values by summing the quantiles of the standard normal distribution for each p-value and then divide this sum by the square root of the number of combined p-values. In this case, p-values calculated from randomization tests are combined for all comparisons of the factor of interest. For instance, to test for an effect of virus on population growth rate, we combined all p-values from each population and transgene status that compared the difference in population growth rates of plants with no virus to plants with *CMV* as well as of plants with no virus to plants with *ZYMV*. Similarly, we determined if there was an overall effect of virus-resistant transgene status. Specifically, we used the z-transform method to combine p-values generated from randomization tests from both populations that compared population growth rates of plants without the virus-resistant transgene to plants with the virus-resistant transgene (Whitlock 2005).

Next, we conducted sensitivity analyzes. The sensitivity of an asymptotic population growth rate (lambda, λ) to changes in life history traits is analogous to natural selection on a life history trait and determines the dependence of λ on that life history trait (i.e. selection gradient, Lande 1982, Stearns 1992). Therefore, we used these analyses to determine if natural selection differed among life history traits in BC2 populations with different virus treatments and with and without the virus-resistant transgene (Caswell 1989, 2001, but see Demetrius et al. 2007). We followed the methods outlined by Caswell (2001). Sensitivity analyzes and modeling were completed using R software (2.11.1).

Results

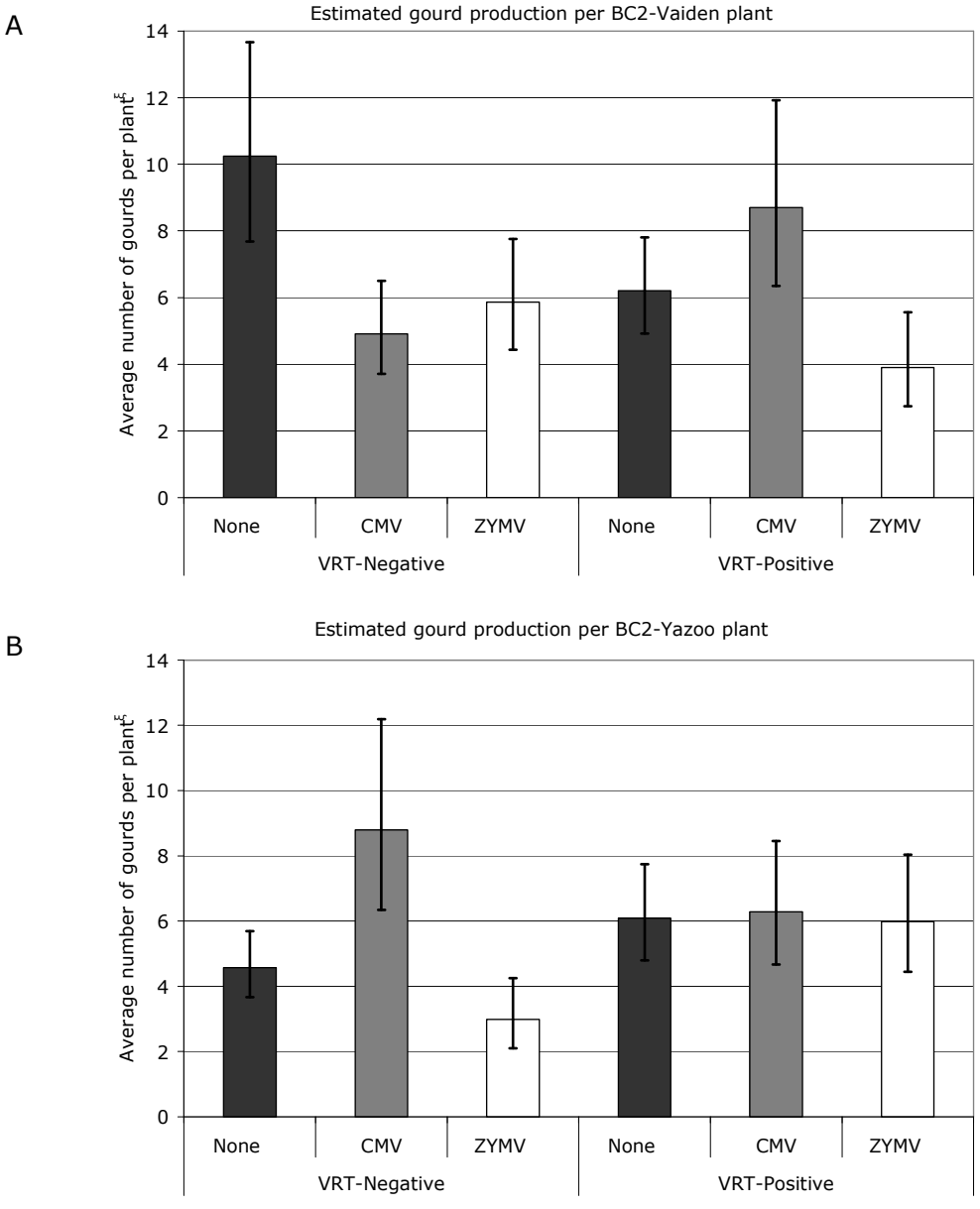
Parameters

We evaluated the effect of population, the virus-resistance transgene, virus treatment and interactions of these factors on demographic parameters of BC2 squash (Table 4.1). MANOVA (of average number of gourds per plant, total number of seeds, male and female flower production, proportion of flowering plants that produced a gourd, proportion of seedlings that flowered) indicated a significant effect of population*virus treatment*transgene interaction and a main effect of population (MANOVA $P=0.0188$, $P<0.0001$, respectively). The covariate, leaf number, had a significant effect on these demographic parameters ($P<0.0001$).

Univariate ANOVA indicated a significant effect of the population*virus treatment*transgene interaction on the average number of gourds produced per plant ($P=0.0375$, Fig. 4.2A/B, Table 4.1). The population*transgene interaction and the covariate, leaf number, had significant effects on the total number of seeds produced per plant ($P=0.0239$ and $P<0.0001$, respectively). There was no difference in the proportion of seedlings that survived to flower due to population, virus treatment or transgene status. The proportion of seeds germinating did not differ due to transgene status or population. However there was a population*transgene interaction effect on the proportion of flowering plants that produced a gourd ($P=0.0463$). There was no effect of population on the proportion of gourds that were not consumed by rodents and the proportion of gourds that opened (Prendeville et al.-Ch 2).

In addition, for the matrix model, we used the total number of gourds produced per plant from all plants that produced gourds. Univariate analysis indicated a significant effect of population*virus treatment*transgene interaction on the average number of gourds produced per plant ($P=0.0429$). Therefore we estimated gourd number for each population, virus treatment, and virus-resistant transgene. Also, for the matrix model, we used the average number of seeds produced per gourd, which was affected by the population*transgene interaction and covariate ($P=0.0250$, $P<0.0001$, respectively). Therefore, we estimated the average number of seeds per gourd for each population and transgene status combination.

Figure 4.2. The average number of gourds produced per plant (back-transformed and with standard error bars) for A) BC2 Vaiden plants and B) BC2 Yazoo populations with (VRT-Positive) and without (VRT-Negative) the virus-resistant transgene (VRT) in the presence of no virus (None), *Cucumber mosaic virus* (*CMV*), and *Zucchini yellow mosaic virus* (*ZYMV*). Estimates were used in the deterministic matrix model to estimate population growth rates. [ξ] The average number of gourds per plant is estimated from only plants that produced gourds since in the deterministic matrix model the proportion of flowering plants that produced gourds accounts for plants that flowered and did not produce any gourds.



Population growth rates

Overall, when we combined probabilities there was an effect of transgene status on population growth rates of BC2 squash (Table 4.2). In the BC2-Vaiden experimental population, randomization tests indicated that there was no significant difference in population growth rates due to transgene status regardless of virus treatment (Fig. 4.3A, Table 4.2). However in the BC2-Yazoo population, plants with the virus-resistant transgene and infected with *ZYMV* had a greater population growth rate than plants without the virus-resistant transgene (Fig. 4.3B, Table 4.2).

In addition, combined probabilities using the z-transform method indicated an overall effect of virus (Table 4.3). The population growth rate of BC2-Vaiden plants without the virus-resistant transgene was greater for plants with no virus than plants with *CMV* (Table 4.3; Fig. 4.3A). There was no difference in population growth rates for plants with either no virus or *CMV* compared to plants infected with *ZYMV*. There was no difference in population growth rates for BC2-Vaiden plants with the virus-resistant transgene in any of the virus treatments. Plants from the BC2-Yazoo population had a lower population growth rates when not infected with virus or infected with *ZYMV* than those plants infected with *CMV* (Fig. 4.3B, Table 4.3). However, there was no effect of virus on the population growth rates of BC2-Yazoo plants with the virus-resistant transgene (Fig. 4.3 B, Table 4.3).

Table 4.2. Overall effect of virus-resistant transgene status on population growth rates of BC2 Vaiden and BC2 Yazoo populations in the presence and absence of virus treatment. P-values are calculated from sampled randomization tests and bold indicates significance at P<0.05 using a z-transform method.

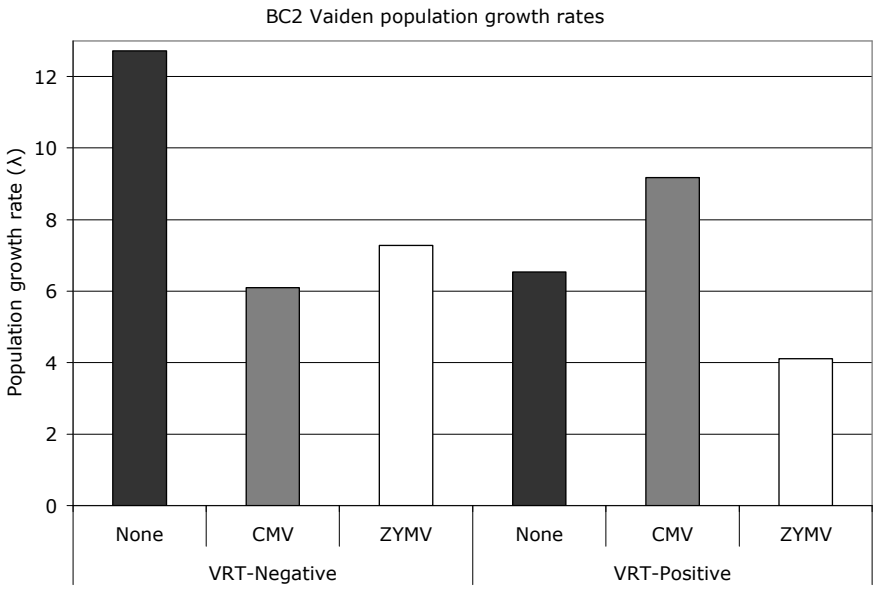
	Difference in λ	P-value
Overall transgene effect		**0.0045**
BC2-Vaiden		
Negative VRT-Positive VRT		
None	6.18	0.1034
CMV	-3.08	0.2639
ZYMV	3.16	0.1562
BC2-Yazoo		
Negative VRT-Positive VRT		
None	-2.22	0.1436
CMV	0.69	0.4209
ZYMV	-3.27	0.0128

Table 4.3. Overall effect of virus on population growth rates of BC2 Vaiden and BC2 Yazoo populations with and without the virus-resistant transgene. P-values are calculated from sampled randomization tests and bold indicates significance at P<0.05 using a z-transform method.

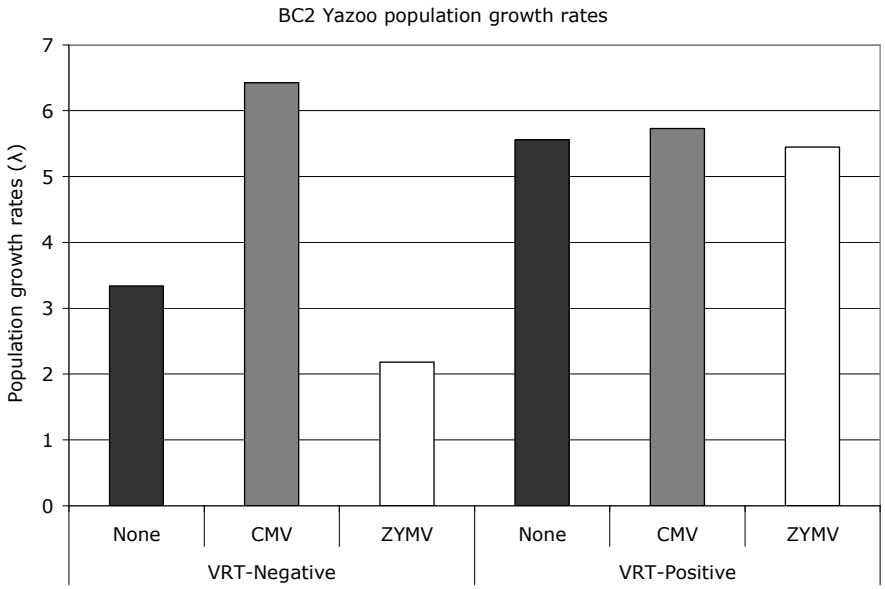
	Difference in λ	P-value
Overall virus effect		**0.0001**
BC2-Vaiden		
Negative VRT		
None-*CMV*	6.62	0.0472
None-*ZYMV*	5.44	0.0878
ZYMV-CMV	1.18	0.3629
Positive VRT		
None-*CMV*	-2.64	0.2076
None-*ZYMV*	2.42	0.1636
ZYMV-CMV	-5.06	0.0542
BC2-Yazoo		
Negative VRT		
None-*CMV*	-3.08	0.0136
None-*ZYMV*	1.16	0.1296
ZYMV-CMV	-4.24	0.0084
Positive VRT		
None-*CMV*	-0.17	0.4629
None-*ZYMV*	0.11	0.4825
ZYMV-CMV	-0.28	0.4467

Figure 4.3. Population growth rates of A) BC2-Vaiden and B) BC2-Yazoo populations with (VRT-Positive) and without (VRT-Negative) the virus-resistant transgene (VRT) in the presence of no virus (None), *Cucumber mosaic virus* (*CMV*), and *Zucchini yellow mosaic virus* (*ZYMV*). Statistical comparisons of population growth rates presented in Tables 4.2 and 4.3.

A



B



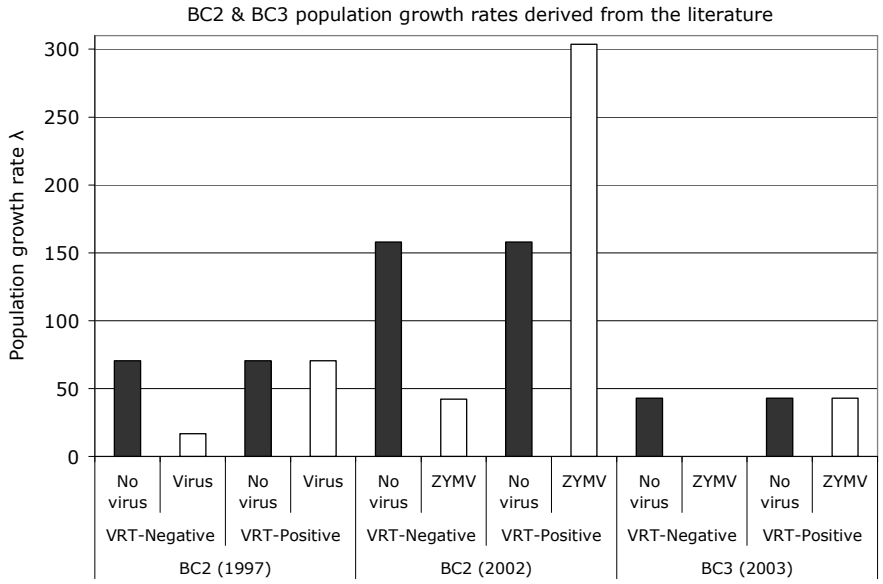
In the BC2-Vaiden population, growth rates did not differ for plants with or without the virus-resistant transgene in the presence or absence of virus infection (Fig. 4.3A, Table 4.2A). Population growth rates did not differ between BC2 Yazoo plants with or without the virus-resistant transgene in the absence of virus or presence of *CMV* (Fig. 4.3B, Table 4.2B). However the population growth rate of BC2 Yazoo plants with the virus-resistant transgene infected with *ZYMV* was greater than the population growth rate for plants without the virus-resistant transgene and infected with *ZYMV* (Fig. 4.3B, Table 4.2B).

Population growth rates from the literature

To calculate population growth rates of additional back-crossed squash populations, we used gourd and seed production of BC2 and BC3 squash reported in the literature (Fuchs et al. 2004, Laughlin et al. 2009) with pooled estimates from our common garden experiment for remaining traits. We are only interested in relative differences among population growth rates due to the effects of virus and the virus-resistant transgene. When comparing relative differences between population growth rates, we found that a population with a mix of virus species (*CMV*, *Watermelon mosaic virus*, and *ZYMV*) reduces λ of BC2 squash plants in comparison to no virus (Fig. 4.4). However, a mix of viruses does not affect the population growth rate of BC2 plants with the virus-resistant transgene. Similarly, *ZYMV* reduced population growth rates of BC2 and BC3 plants in comparison to plants with

no virus. However *ZYMV* did not affect population growth rates of plants with the virus-resistant transgene (Fig. 4.4). In field experiments from Laughlin et al. (2009), BC2 plants with the virus-resistant transgene and infected with *ZYMV* had higher seed and gourd production, which resulted in a greater population growth rate in comparison to plants not infected with virus (Fig. 4.4).

Figure 4.4. Population growth rates of BC2 and BC3 plants in the presence and absence of virus and with and without the virus-resistant transgene (VRT). Population growth rates were calculated using gourd and seed production from published reports and the remaining parameters were derived from pooling across data derived this common garden experiment. BC2 (1997) plants were reared in New York in 1997 in the presence of no virus or a mix of virus species (Fuchs et al. 2004). BC2 (2002) and BC3 (2003) plants were reared in New York in 2002 and 2003 respectively in the presence of no virus or *ZYMV* (Laughlin et al. 2009).



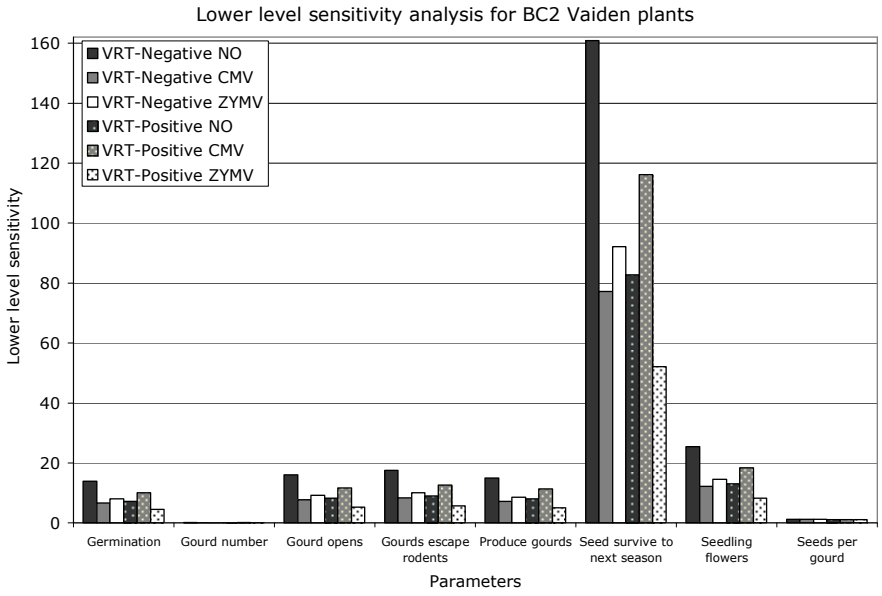
Natural selection

We conducted sensitivity analyses to determine if virus treatment or transgene status affected natural selection in favoring different life history traits. For both BC2 Vaiden and BC2 Yazoo populations, the proportion of seeds surviving to the next growing season was favored by natural selection among all three virus treatments and in the presence and absence of the virus resistant transgene (Fig. 4.5A, B). When comparing among virus treatments and between transgene presence and

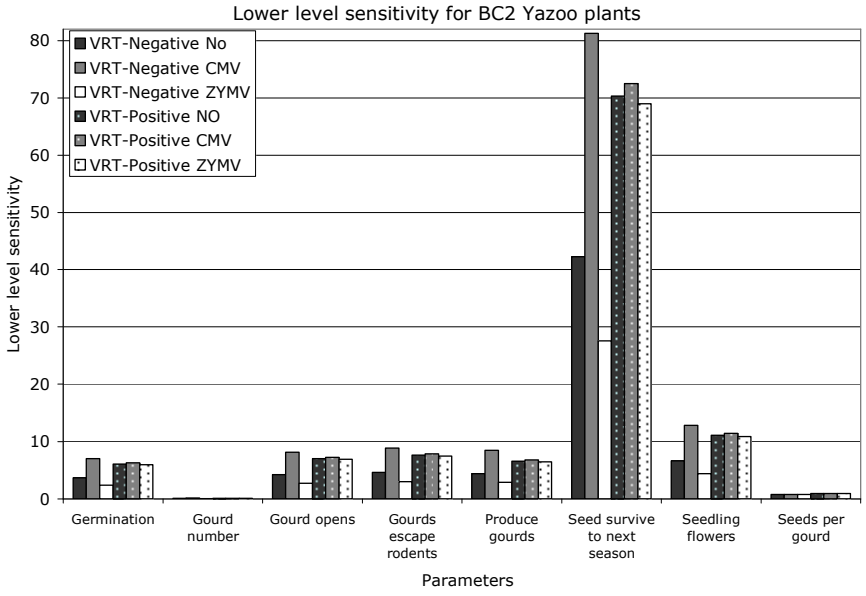
absence, there are differences in the magnitude of selection favoring life history traits. However, there is no difference in the rank order of sensitivities within BC2 Vaiden and BC2 Yazoo populations indicating that natural selection favors the same life history traits in the presence and absence of virus and the virus-resistance transgene (Fig. 4.5A, B).

Figure 4.5. Sensitivity analyses of demographic parameters from A) BC2 Vaiden and B) BC2 Yazoo plants for each virus treatment and transgene status. Solid bars are BC2 plants without the virus-resistant transgene and stippled bars are BC2 plants with the virus resistant transgene.

A



B



Discussion

Virus can reduce the population growth rate in wild squash populations, although the effect of virus depends on the virus species (Prendeville et. al-Ch 3). However, in BC2 experimental populations the effect of virus depends on the wild squash population, thus indicating an interaction with virus species and a population specific genotype. Similar results were found in another study in which the effects of virus on components of individual plant fitness differed among virus strains and plant genotypes (Pagán et al. 2007). In addition, when comparing population growth rates between plants with and without the virus-resistant transgene in the presence and absence of virus, we found the effect of the transgene depended on plant population and virus infection. Thus, the interactions of virus species, status of the virus-resistant transgene, and plant population make it difficult to predict the ecological effects of virus-resistant transgenes. These data indicate the virus-resistant transgene may confer a selective advantage, though this selective advantage will be idiosyncratic and depend on the plant population and virus species.

Moreover, we examined the relative effects of virus and the virus-resistant transgene on population growth rates using gourd and seed production derived from published works. In general, virus reduced population growth rates of BC2 and BC3 plants without the virus-resistant transgene and virus had no affect on plants with the virus-resistant transgene (Fig. 4.4). *ZYMV* reducing population growth rate in plants without the virus-resistant transgene conflicts with our results. This disparity of the effect of *ZYMV* on population growth rate may be due to a variety of factors such as differences in environmental conditions (our experiment occurred in Mississippi, whereas the other experiments occurred in New York), in natural virus resistance between wild squash populations used to make back-crossed squash, and in the timing of virus infection relative to plant development. This disparity highlights the importance of conducting experiments assessing ecological risks of transgenic crops in multiple environments and with many plant populations.

Furthermore in this experiment, we inoculated plants when the majority were at a large reproductive stage. Inoculating plants at a larger stage may dampen the effects of virus on wild squash. For instance, in a laboratory study virus was more severe when plants are infected at a younger and smaller vegetative stage than an

older and larger reproductive stage (Pagán et al. 2007, 2008). Studies by Fuchs et al. (2004b) and Laughlin et al. (2009) suggest that this is the case since in these experiments plants were infected at an earlier developmental stage in these studies, which resulted in 60-73% reduction in gourd production in BC2 plants without the transgene. In general, the timing of virus infection in relation to plant development in wild plant populations is unknown. Thus, it is unclear which scenario (early or late virus infections) best represents natural systems.

Results presented here, as well as from previous works (Fuchs et al. 2004, Laughlin et al. 2009), did not find a cost of the virus-resistant transgene. Therefore natural selection for the virus-resistant transgene should be neutral in the absence of virus pressure. Thus, the virus-resistance transgene would be subject to genetic drift (assuming natural selection is not acting upon an pleiotriopic effects of the transgene, though see Prendeville and Pilson 2009). Then genetic drift could result in the virus-resistant transgene being lost from a wild squash population after hybridization and introgression, if virus pressure is absent from a population. Surveys of wild squash populations indicate that virus infection is absent in some years (Laughlin 2006, Quemada et al. 2008) and is variable among plant populations and virus species (Prendeville et al.-Ch 2). In addition, our data indicate that natural selection favoring transgenic virus-resistance depends on plant population and virus species, which together may explain why the virus-resistance transgene has not been identified in wild squash populations (Prendeville et al.-Ch 2).

When virus is present, then the magnitude of natural selection favoring the virus-resistance transgene will depend on virus pressure within a population. Many factors contribute to virus pressure within a population, such as number of plants within a population infected with virus, virulence of a virus species or strain, and timing of virus infection in relation to plant development. Surveys of virus in wild squash populations indicate that viruses are present, but variable among plant populations, virus species and over time (Prendeville et al.-Ch 2). In particular, *CMV* and *ZYMV* prevalence were monitored over three years and occurred in 0-27% of wild plants in the Vaiden population, but these viruses were not present in the Yazoo population (Prendeville et al.-Ch 2). The presence of virus in the Vaiden population implies that natural selection may have favored natural virus resistance traits. Thus, if natural virus resistance traits are present in the Vaiden population, then this may explain the

lack of effect of the virus-resistant transgene in BC2-Vaiden plants. Likewise, the absence of virus in the Yazoo population suggests the lack of natural selection favoring natural virus resistance traits in this population. Thus, Yazoo may be lacking natural virus resistance traits, which may explain the benefit conferred by the virus-resistant transgene in the presence of virus.

A limitation of this study is that we focused on the effects of virus on the vegetative stage. Thus, we did not examine the effects of virus on germination, dormancy, gourd integrity, rodent herbivory on gourds, and seed survival to the next growing season. If virus affects these other demographic parameters, then this may lead to a more profound effect of virus on population growth rates. For instance, virus can reduce seed germination rates (Fuchs et al. 2004a), which can reduce population growth rates (results not presented here). However, it is not clear how virus affects dormancy and mortality. Thus, we did not include the effect of virus on germination into the deterministic matrix model.

In this study, we are concerned with the relative population growth rates among virus treatments and transgene status. Here we have reported estimates of population growth rates that are much greater than one indicating a very quickly growing population. There are many factors that may contribute to these large population growth rates. First, 12.5% of the genes in BC2 plants are from cultivated squash, which are conventionally bred to produce a high fruit number and tolerate pests. In BC2 squash plants, conventional crop alleles alone can increase components of plant fitness in comparison to wild plants (Laughlin et al. 2009). Higher fruit and seed number can affect BC2 squash population growth rates (as seen in Fig. 4.4). In addition, wild squash are disturbance specialists, thus wild squash populations can have tremendous spurts of growth in good growing conditions (Prendeville et al.-Ch. 3) after periods of disturbance. Population growth rates similar to those of BC2-Vaiden and BC2-Yazoo population have been documented in wild squash populations, cultivated species, and an invasive species (Tozer et al. 2008, Schutzenhofer et al. 2009, Prendeville et al.-Ch 3). Another factor that may have lead to an over estimate of wild squash population growth rate is due to the difficulty in estimating overwintering success of seeds. Therefore, our estimate of seed survival may not represent what occurs within a natural system. Overall, many naturally occurring and a few assumed factors contributed to elevated

population growth rates of BC2 plants. Regardless, these effects are consistent among virus treatments and between plants with and without the virus-resistant transgene. Therefore, our conclusions of virus and the virus-resistant transgene affecting BC2 squash population growth rates are still valid.

From data presented here and other studies (Fuchs et al. 2004b, Laughlin et al. 2009), it is clear that the virus-resistant transgene can confer a selective advantage to BC2 squash populations in the presence of virus. In addition, virus reduces wild squash population growth rates (Prendeville et. al-Ch. 3) and introgression of the virus-resistant transgene is possible even under low disease pressure (Fuchs et al. 2004a). Therefore, to reduce ecological risk of transgene introgression into wild plant populations, we suggest reducing opportunities for crop-wild hybridization and subsequent transgene introgression by limiting transgenic crop production to areas beyond natural ranges of wild relative. In the US, this would be a minor limitation for cultivated squash, since the majority of squash production occurs beyond the wild squash range (USDA/NASS 2010). However, for other transgenic crops this may be more severe limitation and regulation agencies would need to determine if the cost of transgene introgression is outweighed by the benefit of using the virus-resistant transgenic squash.

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