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Holly R. Prendeville
University of Nebraska-Lincoln, hp8j@virginia.edu

Xiaohong Ye
University of Nebraska-Lincoln

Thomas Jack Morris
University of Nebraska - Lincoln, jmorris1@unl.edu

Diana Pilson
University of Nebraska - Lincoln, dpilson1@unl.edu

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VIRUS INFECTIONS IN WILD PLANT POPULATIONS ARE BOTH FREQUENT AND OFTEN UNAPPARENT[1]

HOLLY R. PRENDEVILLE[2], XIAOHONG YE, T. JACK MORRIS, AND DIANA PILSON

School of Biological Sciences, 348 Manter Hall, University of Nebraska, Lincoln, Nebraska 68588-0118 USA

- *Premise of the study:* Pathogens are thought to regulate host populations. In agricultural crops, virus infection reduces yield. However, in wild plants little is known about the spatial and temporal patterns of virus prevalence. Thus, pathogen effects on plant population dynamics are unclear. Prevalence data provide necessary background for (1) evaluating the effects of virus infection on plant population size and dynamics and (2) improving risk assessment of virus-resistant transgenic crops.
- *Methods:* We used ELISA and RT-PCR to survey wild *Cucurbita pepo* populations over 4 years for five viruses, aphid-transmitted viruses of the genus *Potyvirus* as a group and PCR to survey for virus-resistance transgenes. In addition, we surveyed the literature for reports of virus prevalence in wild populations.
- *Key results:* In 21 *C. pepo* populations, virus prevalence (0–74%) varied greatly among populations, years, and virus species. In samples analyzed by both ELISA and RT-PCR, RT-PCR detected 6–44% more viruses than did ELISA. Eighty percent of these infections did not cause any visually apparent symptoms. In our samples, the virus-resistance transgene was not present. In 30 published studies, 92 of 146 tested species were infected with virus, and infection rates ranged from 0.01–100%. Most published studies used ELISA, suggesting virus prevalence is higher than reported.
- *Conclusions:* In wild *C. pepo*, the demographic effects of virus are likely highly variable in space and time. Further, our literature survey suggests that such variation is probably common across plant species. Our results indicate that risk assessments for virus-resistant transgenic crops should not rely on visual symptoms or ELISA and should include data from multiple populations over multiple years.

Key words: *Cucurbita pepo*; *Cucumber mosaic virus*; genetically modified organisms; *Papaya ringspot virus*; *Squash mosaic virus*; unapparent virus infection; virus prevalence; *Watermelon mosaic virus*; *Zucchini yellow mosaic virus*; potyvirus.

Pathogens affect host populations by reducing viability, fecundity, and competitive ability, as well as affecting community interactions (Friess and Maillet, 1996; Malmstrom et al., 2005b, 2006; Seabloom et al., 2009). Viruses commonly infect wild plants (MacClement and Richards, 1956; Hammond, 1981; Mackenzie, 1985; Raybould et al., 1999; Tugume et al., 2008). However, virus infection is easily overlooked in wild plant populations. Although infections can be visually unapparent (Oswald and Houston, 1953; Thurston et al., 2001; Remold, 2002), it is frequently assumed that an absence of visual symptoms (such as leaf mottling or malformation) indicates a lack of virus infection. Moreover, symptoms of virus infection are sometimes difficult to distinguish from environmental stresses. For these reasons, in part, virus ecology in natural plant populations has been poorly studied (Cooper and Jones, 2006).

Because so little is known about the prevalence or effects of 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 et al., 1996), resulting in economic losses. Moreover, many virus vectors are difficult to control, and for this reason, genetic resistance to virus infection is often the most practical means of controlling crop losses. The use of transgenic crops with virus resistance offers promise for control of problematic viruses. In the United States, 27 crop species with virus-resistance transgenes have been issued permits for field trials, and a handful of crops have been deregulated for commercial production (i.e., squash, papaya, and potato; Information Systems for Biotechnology, 2012).

The commercial release of virus-resistant transgenic crops has motivated research on plant–virus ecology in natural populations (Cooper and Jones, 2006). Studies investigating plant–virus interactions have focused on a few viruses and have found that virus prevalence can vary with herbivory (Borer et al., 2009) and environment (Funayama et al., 2001; Seabloom et al., 2009). In addition, in wild plants, virus infection can affect plant growth, mortality, and seed production (Friess and Maillet, 1996; Funayama et al., 2001; Fuchs et al., 2004b), but these effects vary among populations (Mackenzie, 1985; Yahara and Oyama, 1993; Thurston et al., 2001), species (Remold, 2002; Malmstrom et al., 2005a), and environments (Seabloom et al., 2009). Although these data indicate that viruses can affect community dynamics and have fitness consequences in many wild plants, remarkably little is known about virus prevalence in wild populations.

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[2]Author for correspondence (e-mail: hp8j@virginia.edu); present address: Department of Biology, University of Virginia, Charlottesville, VA 22904-4328 USA

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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 (Tepfer, 2002; Pilson and Prendeville, 2004; Thompson and Tepfer, 2010). In particular, if an introgressed transgene provides a fitness benefit to the wild population (e.g., resistance to pathogen attack), its frequency will increase by natural selection. If, in addition, the size or dynamics of the wild plant population is limited by pathogen attack, then the plant population may increase in size or become weedier. In this way, natural habitats could be negatively affected by transgenes introgressed into wild populations. For example, in an investigation of transgenic virus-resistance in *Trifolium repens*, Godfree et al. (2007) found that an experimental population into which transgenic virus resistance had been introgressed had a 15% higher intrinsic growth rate in the presence of virus compared to a nontransgenic population. In addition, Godfree et al. (2007) predicted that transgenic populations could expand their range into marginal habitats.

The potential for crop–wild hybridization followed by introgression is a concern when cultivated squash (*Cucurbita pepo* L. var. *pepo* L.H. Bailey) is grown near wild gourd [also *C. pepo* L. var. *texana* (Scheele) D. Decker, *C. pepo* L. var. *ozarkana* D. Decker; Wilson, 1993]. Wild and cultivated plants readily interbreed (Quesada et al., 1996), and gene flow from cultivated nontransgenic plants to wild *C. pepo* 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-cultivated and wild *C. pepo* have produced viable hybrids that express transgenic resistance (Spencer and Snow, 2001; Fuchs et al., 2004a). Moreover, in common garden experiments, virus infection reduces seed production in wild *C. pepo*, and transgenic resistance introgressed into wild plants has fitness benefits in the presence of virus (Spencer and Snow, 2001; Fuchs et al., 2004b; Laughlin et al., 2009; Sasu et al., 2009). These data suggest that if the virus-resistance transgene introgresses into wild *C. pepo* populations, and if virus infection limits wild *C. pepo* population size, then expression of transgenic resistance could allow populations to increase in size.

Although virus-resistant transgenic squash has been commercially available for over 15 years (APHIS-USDA, 1994), wild *C. pepo* populations have not been monitored for transgene introgression. Virus is present in wild *C. pepo* populations (Quemada et al., 2008). However, little is known about the temporal and spatial patterns of prevalence of individual virus species. Furthermore, since the effects of individual virus species are not equivalent (Hull, 2002), it is difficult to predict the effect of transgenic virus-resistance on wild *C. pepo* populations.

In the work presented here, we had three objectives. First, we surveyed wild *C. pepo* populations in the south-central United States over 4 years for five virus species and species within one virus genus. Second, we examined these same populations for the presence of virus-resistance transgenes. Finally, to determine whether prevalence patterns observed in *C. pepo* are similar to patterns observed in other species, we reviewed literature reporting virus infections in wild plant populations and then compared our virus-incidence data to infection rates reported for other species.

MATERIALS AND METHODS

Field survey for virus infection and transgenic virus resistance in* Cucurbita pepo—System biology*—Wild gourd [*Cucurbita pepo* L. var. *ozarkana* D. Decker and *Cucurbita pepo* L. var. *texana* (Scheele) D. Decker] is native to central and southwestern United States and throughout Mexico (Wilson, 1993). Approximately 10% of commercial squash, also *C. pepo*, is grown within the native range (USDA-NASS, 2011). Wild gourd 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). *Cucurbita pepo* depends on animal pollination for fertilization, and outcrossing distances can exceed 1.25 km (Kirkpatrick and Wilson, 1988). In addition, the viruses that commonly infect and cause mosaic symptoms on cultivated summer squash have been reported in wild *C. pepo* (Quemada et al., 2008). However, the prevalence of individual virus species in wild *C. pepo* populations is poorly understood.

The viruses that commonly cause mosaic diseases/symptoms on 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 et al., 1978; Fuchs and Gonsalves, 1999). The first four viruses infect a variety of host plants and are nonpersistently transmitted by aphids. SqMV is beetle transmitted. These viruses can drastically reduce yield in cultivated squash (Fuchs and 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 and Gonsalves, 1995; Gianessi et al., 2002).

One strategy used by farmers in the United States to reduce economic losses associated with virus infection is the cultivation of virus-resistant transgenic squash. Virus-resistant transgenic squash was among the first transgenic crops made available for commercial production without regulation in the United States (APHIS-USDA, 1994) and has been field tested in Mexico (Alvarez-Morales, 2000). 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.

Survey of wild C. pepo populations—In the south-central US, we surveyed wild *C. pepo* populations for the virus-resistance transgene and virus infection. Populations were surveyed in July through September when plants were flowering and gourd production was underway (collection dates and locations in Appendix S1 [see Online Supplemental Data with the online version of this article]). Wild *C. pepo* populations were located by searching in and around areas listed in herbarium records, at sites suggested by John Byrd (Mississippi State University, personal communication), Karen Laughlin (Environmental Protection Agency, personal communication), Leon Shipman (Gilbert, Arkansas, personal communication), and Hector Quemada (Donald Danforth Plant Science Center, personal communication) and reported by Decker-Walters et al. (2002). GPS coordinates were noted for all populations (online Appendix S1), and each site was named after the nearest town. Wild *C. pepo* populations occurred in abandoned and active pastures, agricultural crops, waysides, roadside ditches, and wild riparian areas. Samples collected from locations within ~3 km are considered a single population due to outcrossing distances (Kirkpatrick and Wilson, 1988) and local gourd dispersal.

Because wild *C. pepo* is a vine and seeds from a single gourd often germinate in close proximity, it can be difficult to distinguish individual plants. For this reason, leaf samples were only collected from obvious individuals at a site or only one sample was haphazardly collected from a cluster of plants. Sample sizes at each site are listed with virus prevalence data (see Results). In addition, we noted for each sample whether typical symptoms of mosaic diseases were present. 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, Xenia, Ohio, USA) 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 *C. pepo* plants were sampled from Louisiana (two sites), Missouri (two sites), and Oklahoma (one site). In Arkansas, seven sites were sampled in 2004, and one of these seven sites was sampled in 2007. In Mississippi, a total of six sites were sampled, with two sites sampled in 2004, five sites sampled in 2005, and six sites sampled in 2006 and 2007. From these collections, we assayed 1143 leaf samples for virus infection and 1256 leaf samples for the virus-resistance transgene. Most samples were analyzed for viruses and the transgene; however, due to limited tissue availability, some samples were only analyzed for one or the other.

Virus detection—Wild *C. pepo* samples were assayed for viruses using antigen-coated plate enzyme-linked immunosorbent assay with an alkaline phosphatase label (ACP-ELISA; Agdia, Elkhart, Indiana, USA; 545 samples), or reverse transcriptase polymerase chain reaction (RT-PCR; 176 samples), or both methods using duplicate samples (422 samples).

For the ELISA, 15–20 mg of dried leaf material was added to 96-well plates with a glass bead and sent to testing services at Agdia. In each plate, we included a positive control for each of the five viruses and three negative controls to which Agdia 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 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* by ELISA in a 96-well plate or using lateral flow immunoassay (Immunostrip tests, Agdia) for SqMV. Although there are over 143 *Potyvirus* species, only ~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 1–5 mg of dried leaf and extracting with 1 mL of TriPure isolation reagent (Roche Diagnostics, 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 × *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 × *g* for 20 min at 4°C. The RNA pellet was washed with 0.75 mL of 75% ethanol, drained, and allowed to air dry. The pellet was then resuspended in 0.05 mL of RNase-free water. RNA concentration was quantified using NanoDrop (Thermo Fisher Scientific, Wilmington, Delaware, USA). RNA was amplified using a two-step RT-PCR with oligonucleotide primers specific to each of the five viruses using methods and primer sequences provided by Bryce Falk, University of California-Davis (for primers and RT-PCR protocol see Appendix S2 with online Supplemental Data). Amplicons were viewed in 2% agarose gel electrophoresis. RT-PCR was not used to assay for the potyviruses as a whole group.

Over 400 samples were analyzed for virus 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.

Transgene detection—In 810 samples, DNA was extracted from 10–20 mg of dried leaf tissue per sample using DNeasy Plant Mini kits (Qiagen, Valencia, California, USA) and a portion of the transgene conferring resistance to ZYMV, which is found in both lines of virus-resistant transgenic squash (ZW-20 and CZW-3), was amplified using PCR (primer sequences and protocol in 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. To expedite the analysis of samples, we had the company GeneSeek (Lincoln, Nebraska, USA) assay the remaining 446 samples for the transgene. In 96-well plates, DNA was extracted from ca. 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 nontransgenic and transgenic squash, respectively. PCRs were performed using primers designed to amplify a portion of the transgene conferring resistance to WMV (found in both lines; primer sequences and protocol in Wall et al., 2004). GeneSeek tested a subset of samples with both the Spencer (2001) and Wall et al. (2004) primer sets and then used the primers amplifying a portion of WMV as it gave more consistent results with their system. GeneSeek viewed amplicons with an IR fluorescent system (LI-COR, Lincoln, Nebraska, USA). Positive controls, negative controls, and 102 samples were analyzed using both ZYMV transgene primers in our laboratory and WMV transgene primers by GeneSeek and yielded the same results.

***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 1950 to 2011, AGRICOLA from 1970 to 2011, 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 and Plant Sciences, and Ecology and Evolutionary Biology. Also, we examined all references cited in reviews of plant-virus ecology (Bos, 1981; Thresh, 1981; Cooper and Jones, 2006).

In this literature summary, we only included studies that present data on noncultivated terrestrial vascular plants. We define noncultivated plants as those plants growing in the absence of direct human assistance (e.g., by seeding, fertilizing, tilling, selective weeding to promote growth). Thus, we did not include data from studies of virus prevalence in crops, fallow fields, pastures, botanical gardens, and parks, as other reviews have examined virus prevalence in these habitats (Duffus, 1971; Bos, 1981; Thresh, 1981; Cooper and Jones, 2006). Some studies presented data from both cultivated and wild populations of the same species, and to the best of our knowledge, we included 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 10 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/unapparent 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 yellow dwarf virus* and *Cereal yellow dwarf virus* prevalence is grouped across all serotypes.

RESULTS

Survey of wild* C. pepo *populations*—Virus prevalence*—In 2004, at least one of the surveyed viruses (CMV, WMV, ZYMV, PRSV, SqMV, or aphid-transmitted viruses in the genus *Potyvirus*) was detected in 12 of the 14 sampled populations. Within the infected populations virus, prevalence ranged from 8–74%, but was typically less than 30% (Fig. 1). Furthermore, 80% of infected plants exhibited no visual symptoms (Fig. 1) and would have been missed in a visual survey of virus prevalence. Prevalence varied dramatically among virus species (Tables 1, 2). We detected no PRSV in 2004. In contrast, SqMV was present in eight 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 one population in which 33% of plants were infected with ZYMV). One or more potyviruses (including ZYMV, PRSV, and WMV) were present in 2–70% of sampled individuals in 10 of the 14 populations.

In populations surveyed in Mississippi from 2004 to 2007, prevalence varied among years (Table 2). As in the broader geographic survey, SqMV and potyviruses as a group were more common than CMV, PRSV, WMV, and ZYMV separately.

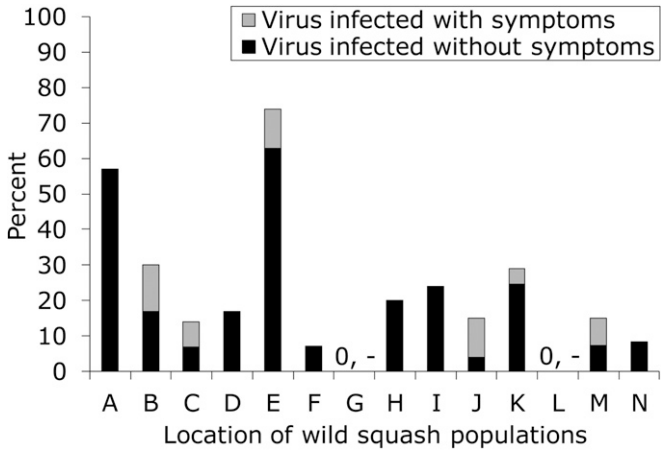

Fig. 1. Total virus prevalence in all plants of *Cucurbita pepo* equals the sum of unapparent infections (black bars) and apparent infections with visual symptoms (gray bars) in wild *C. pepo* populations in the south-central United States in 2004. Site abbreviations along *x*-axis are A: Alpena, Arkansas (AR); B: Berryville, AR; C: Bigelow, AR; D: Bradley, AR; E: Canale, AR; F: Czahome, AR; G: Gilbert, AR; H: Moreland, Louisiana; I: Woodworth, Louisiana; J: Fitler, Mississippi; K: Eagle Lake, Mississippi; L: Simcoe, Missouri; M: Washburn, Missouri; N: Park Hill, Oklahoma.

TABLE 1. Virus prevalence in wild *Cucurbita 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.

			Virus prevalence (%)					
State	Site	*N*	CMV	SqMV	PRSV	WMV	ZYMV	Potyviruses
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

Notes: Cucumber mosaic virus (CMV), *Squash mosaic virus* (SqMV), *Papaya ringspot virus* (PRSV), *Watermelon mosaic virus* (WMV), *Zucchini yellow mosaic virus* (ZYMV), and unspecified potyviruses (PRSV, WMV, and ZYMV are potyviruses).

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. In 2007, rainfall was below average and was lower than annual rainfall in years 2004 to 2006 (National Climatic Data Center 2009), wild *C. pepo* were few and very 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 potyviruses (PRSV, WMV, and ZYMV; Table 2). Of the 173 samples that tested positive for PRSV, WMV, and/or ZYMV, only 58% were also positive for the potyvirus group test. The difference was not due to the virus detection method because 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, when comparing virus detection methods, we found 68 samples tested positive with RT-PCR, but negative with ELISA, while nine samples tested positive with ELISA, but negative with RT-PCR. These data are consistent with other reports indicating that RT-PCR is more sensitive than ELISA (Vunsh et al., 1990). For example, ELISA did not identify virus in 44% and 30% of PRSV and ZYMV positive controls, respectively (Table 3). In contrast, RT-PCR detected virus in all positive controls of CMV, PRSV, and ZYMV, but missed 16% of positive controls of both SqMV and WMV. Overall, RT-PCR detected 6–44% more positive controls than ELISA (Table 3), indicating that the accuracy of ELISA varies among virus species.

TABLE 2. Virus prevalence in wild *Cucurbita pepo* in sites in Mississippi and one site in Arkansas (AR) collected in 2004–2007. Virus prevalence is presented as a percentage (%) of the total number of wild *C. pepo* samples (*N*) at each site in each year that tested positive for each virus.

		Virus prevalence and sample size											
		CMV		SqMV		PRSV		WMV		ZYMV		Potyviruses	
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	0	86	0	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	0	35	0	35

Notes: *Cucumber mosaic virus* (CMV) *Squash mosaic virus* (SqMV), *Papaya ringspot virus* (PRSV), *Watermelon mosaic virus* (WMV), *Zucchini yellow mosaic virus* (ZYMV), and unspecified potyviruses (PRSV, WMV, and ZYMV are potyviruses).

TABLE 3. Comparison of virus detection in wild plant samples and positive controls by 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 virus detection methods (+, +), neither method (–, –), or one of the two methods (+,–; –, +). Sixty-eight samples were positive by RT-PCR and negative by ELISA; nine 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	
Method	Sample	Presence	+	–	+	–	+	–	+	–	+	–
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

While our finding that RT-PCR is more sensitive than ELISA is consistent with previous work (Vunsh et al., 1990), our results also suggest that neither method is 100% accurate. Positive controls not detected by RT-PCR could be due to human error during inoculation or analysis. Nonetheless, these results suggest that our estimates of virus prevalence in wild *C. pepo*, which are primarily based on ELISA, are underestimates of true virus prevalence.

Transgene assay—The virus-resistance transgene was not present in any of the 1256 leaf samples of wild *C. pepo* collected from 21 sites over 4 years in south-central United States.

***Literature survey*—**We found 30 studies that examined virus prevalence in 146 wild plant species. Viruses were detected in 92 of the 146 plant species. Within infected populations, between 0.01 and 100% of plants were infected (Table 4; online Appendix S3).

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 146 plant species studied are perennials. Poaceae is the most studied plant family with 77 species examined for virus infection, followed by Fabaceae with 14 species surveyed. Other plant families have had just one to eight species investigated. More than half of these studies have monitored virus prevalence at fewer than 10 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 was variable among sites, years, plant species, and virus species (Table 4; online Appendix S3).

Few studies have reported the presence or absence of visually apparent virus symptoms in infected wild plants. However, from these studies it is clear that not all virus infections produce visual symptoms. In addition, the frequency of visually unapparent infections varies among plant species, virus species, and among sites (Appendix S3). For instance, 45–86% of *Brassica nigra* (L.) W.D.J. Koch and 50% of *Brassica rapa* L. had visually unapparent infections of *Turnip crinkle virus*, and 60–100% of *B. nigra* and 0% of *B. rapa* had visually unapparent infections of *Turnip yellow mosaic virus* (Appendix S3).

Furthermore, the presence of multiple infections was rarely quantified, but when examined, multiple infections were frequently detected (Appendix S3). For instance, multiple infections were found in 20–100% of *Arabidopsis thaliana* L., 0–24% of *C. pepo*, 8% of *B. nigra*, 6–16% of *B. rapa*, and 54% of *B. oleracea* in surveyed populations (Appendix S3). Also, in many grass species, multiple serotypes of *Barley yellow dwarf virus* and *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 (i.e., *Luteoviridae*, *Potyviridae*, and *Alphaflexiviridae*), multiple virus species have been surveyed in 10–77 wild plant species (Appendix S3; Table 4). The other eight virus families have not been examined as well, with one to two virus species per virus family surveyed in one to four plant species. To date, six virus species infecting wild plant populations have not been assigned to a virus family, but were included in this literature survey.

DISCUSSION

Virus infection is frequent in wild *C. pepo.* At least one of the five viruses surveyed was present in 17 of 21 wild *C. pepo* populations and prevalence ranged from 4–74%. Among wild *C. pepo* populations sampled in the south-central United States in 2004, the median virus prevalence was 16.5%, and the average virus prevalence was 23% (Table 1). Similar trends were observed in four populations surveyed over 3 to 4 years in Mississippi: a median virus prevalence of 25% and an average virus prevalence of 24% (Table 2). These results are consistent with those of Quemada et al. (2008), who presented virus prevalence pooled for CMV, WMV, and ZYMV. Furthermore, multiple viruses were present within populations (Table 2) and within individual host plants. In addition, virus prevalence varied among years, wild *C. pepo* populations, and virus species.

These results are similar to patterns found in our literature survey, where 63% of tested plant species were infected, and prevalence ranged from 0.1–100% in infected populations (Appendix S3). In addition, our empirical results, as well as our literature survey, are consistent with reviews of virus prevalence in plants near cultivated fields (Duffus, 1971; Bos, 1981; Thresh, 1981; Cooper and Jones, 2006). Clearly, virus infection is common in wild plant populations. Moreover, similar patterns of annual and spatial variation are apparent in both our empirical survey of wild *C. pepo* populations and in our literature survey. Among wild *C. pepo* populations, spatial variation is apparent even among populations well within the dispersal distances of virus vectors (Taylor, 1979).

In our field survey, 80% of infections were visually unapparent (Fig. 1). Visually unapparent infections were common in the literature survey as well (Appendix S3; Muthukumar et al., 2009).

TABLE 4. Summary of literature survey of virus prevalence in wild plant populations. A range is given when the percentage of infected plants 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 S3 (see Supplemental Data with the online version of this article).

Plant family	No. of plant species surveyed	Virus family	Total no. of virus species surveyed	Percent of plants infected	Sites	Years	No. of publications
Apiaceae	4	*Potyviridae*	1 [a]	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	0–90 [b]	5–15	1–4	1
	2	*Luteoviridae*	1	0–3	3–28	3	1
	1	*Potyviridae*	3	0–90 [b]	5–15	1–4	1
Ericaceae	1	*Secoviridae*	1	0–30	6	2 [c]	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 [c]	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	Unassigned	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

[a] Results pooled across virus species.
[b] Assay for virus family only.
[c] Virus incidence grouped across 2 yr.

It might be argued that visually unapparent infections have little to no effect on plant fitness. But interestingly, virus symptoms are not consistently related to virus concentration (Thurston et al., 2001; Pallett et al., 2002), and virus concentration and plant fitness are not always correlated (Pagán et al., 2007). In addition, visually unapparent virus infection can increase or decrease plant fitness relative to the fitness of uninfected plants (Remold, 2002). Taken together, it is not clear how unapparent virus infections affect plant fitness. Therefore, 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.

Moreover, similarly variable amounts of herbivore damage (Louda and Potvin, 1995; Maron and Simms, 1997), fungal pathogen infection (Alexander and Antonovics, 1988; Carlsson and Elmqvist, 1992; Fowler and Clay, 1995), and virus infection in *Eupatorium* (Funayama et al., 2001) are known to affect plant population growth. Furthermore, seed addition experiments find that populations are seed-limited about 50% of the time (reviewed in Turnbull et al., 2000). Thus, it seems likely that virus infection has population-level consequences in many wild plant species, including wild *C. pepo*. For example, in a common garden experiment, the population growth rates of wild *C. pepo* from three different populations infected with CMV are reduced in comparison to plants from the same population without virus (Prendeville, 2010). However, the effect of ZYMV on wild *C. pepo* varies among populations, indicating that plant–virus interactions are idiosyncratic among virus species and plant populations.

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 typically 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 (Malmstrom et al., 2006; Seabloom et al., 2009), as it does for herbivory (Stiling and Rossi, 1996). Thus, in comparison to agricultural systems, virus infection rates are probably at least as, if not more, variable in wild populations. Our data and literature review demonstrate highly variable infection rates in wild populations and thus, are consistent with 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 et al., 2002; Caicedo, 2008; Salvaudon et al., 2008) and has been documented for virus resistance in wild populations of *A. thaliana* (Pagán et al., 2007, 2008) and *Trifolium repens* (Godfree et al., 2007). Genetic variation for resistance can affect virus concentration and the degree to which the virus infection affects plants (Pagán et al., 2009, 2010). Several studies have found that virus-infected plants have reduced fitness relative to healthy plants (e.g., Friess and Maillet, 1996; Fuchs et al., 2004b; Pagán et al., 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 and 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 plant community composition will be altered (Vitousek et al., 1997; Walther et al., 2002). However, it is unclear how alterations in plant community composition will affect virus incidence (but see Garrett et al., 2006).

From our literature survey and field survey of *C. pepo*, it is evident that many wild plant populations typically host multiple virus species (Tables 1, 2, 4), and occasionally multiple virus species infect individual plants (online Appendix S3). Knowledge of how single or multiple virus species affect wild plant populations and community dynamics is limited (but see Funayama et al., 2001). In general, multispecies interactions can have demographic and evolutionary consequences for plant populations that differ from outcomes predicted by pairwise interactions (Hougen-Eitzman and Rausher, 1994; Iwao and 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 et al., 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 interactions is derived from cultivated plants. However the 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 S3; Zettler et al., 1978; Kawakami et al., 2007). Similarly, PRSV is widespread in cultivated squash (Davis and Mizuki, 1987; Ullman et al., 1991; Yuki et al., 2000), but was rarely present in wild *C. pepo* populations (Tables 1, 2). In addition, viruses that typically reduce crop yield may provide fitness benefits to plants in natural ecosystems. For example, Remold (2002) found that wild populations of *Setaria lutescens* infected with serotypes of *Barley yellow dwarf mosaic virus* had 25% greater fitness in one year and similar fitness in another year compared to uninfected plants. However, the mechanism underlying this benefit has not been investigated. Finally, infection by an avirulent virus may reduce susceptibility to infection by additional viruses (cross protection: Wen et al., 1991), resulting in complicated patterns of infection and fitness consequences of the infection. For all of these reasons, our knowledge of viruses from managed systems should be cautiously extrapolated to wild plant populations, particularly 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; Tepfer, 2002; Pilson and Prendeville, 2004). Because experimental work with *C. pepo* suggests that virus infection can reduce seed production by 80–100% (Fuchs et al., 2004b; Laughlin et al., 2009), it seems likely that natural selection would favor transgenic resistance if it were present in wild populations. Thus, if *C. pepo* populations are seed-limited, population size could increase in populations with transgenic resistance. In addition, there is no direct fitness cost of the virus-resistance transgene in wild *C. pepo* (Laughlin et al., 2009), although there may be an indirect cost due to herbivores and other pathogens (Sasu et al., 2009).

To examine potential transgene introgression, we assayed hundreds of wild *C. pepo* plants for the virus-resistance transgene. However, the virus-resistance transgene was not present in any of our samples. Many factors likely contributed to this result. First, none of the sites sampled were in close proximity to fields of virus-resistant transgenic squash. This may be typical because in the United States only about 18% of total summer squash production consists of transgenic varieties (Johnson et al., 2007) and about 90% of squash production occurs outside the range of wild squash (USDA-NASS, 2011). Moreover, in regions of overlap, the distance between wild *C. pepo* populations and squash production fields is often farther than pollinators travel (H.R. Prendeville, personal observation), thus limiting the opportunities for cross pollination. Another factor limiting hybridization is a reduction of wild *C. pepo* populations. Personal observations and anecdotal evidence from farmers suggest that the number and size of wild *C. pepo* 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 demonstrating that wild *C. pepo* is frequently infected with virus is in contrast to the ecological risk assessment used to deregulate virus-resistant transgenic squash (APHIS-USDA, 1994). In the ecological risk assessment, an unstated number of wild *C. pepo* plants from 14 sites in five counties in Arkansas, Louisiana, and Mississippi were visually assessed for virus symptoms, and samples were collected to further test for seven viruses, which included the five viruses assayed in this study. This ecological risk assessment found no visual symptoms of virus infection (except one population with slight chlorosis), and no virus was found in any of the analyzed samples. In contrast, we found virus prevalence of CMV, WMV, and ZYMV as high as 54% in a single population of wild *C. pepo*, although low to no virus prevalence was observed in some years and populations. Furthermore, it is incorrect to assume that infections can be detected visually (APHIS-USDA, 1994), since data presented here indicates that unapparent virus infections are frequent (Fig. 1). Since virus prevalence is variable and symptoms are frequently unapparent, it is necessary to monitor multiple populations over multiple years to appropriately assess the ecological risk of using transgenic crops with virus resistance.

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 López et al., 2003; James et al., 2006). However, as reported here and elsewhere (Figueira et al., 1997; Berniak et al., 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 since these techniques can detect virus at low concentrations (Hu et al., 1995; Shang et al., 2011). In our literature survey, 20 studies used ELISA, while three studies used either RT-PCR alone or in conjunction with ELISA (Appendix S3). Thus, it seems likely that virus prevalence is higher than is typically reported. This conclusion is consistent with our field survey. In wild *C. pepo* samples analyzed by both ELISA and RT-PCR, overall virus prevalence was 1.4% by ELISA and 11.6% by RT-PCR. 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 *C. pepo* populations and indeed in many wild plant populations. In addition, virus prevalence varies among years, sites, plant species, and virus species. In wild *C. pepo*, the demographic effects of virus (which are known to be substantial in field experiments) are likely to be variable in space and time. Visually unapparent infections are very common, and this is probably one reason that the effect of virus in wild plant populations is underappreciated. Risk assessments for virus-resistant transgenic crops should not rely on visual symptoms, and should include data from many populations over multiple years.

LITERATURE CITED

ALEXANDER, H. 2010. Disease in natural plant populations, communities, and ecosystems: Insights into ecological and evolutionary processes. *Plant Disease* 94: 492–503.

ALEXANDER, H. M., AND J. ANTONOVICS. 1988. Disease spread and population dynamics of anther-smut infection of *Silene alba* caused by the fungus *Ustilago violacea. Journal of Ecology* 76: 91–104.

ALVAREZ-MORALES, A. 2000. Mexico: Ensuring environmental safety while benefiting from biotechnology. *In* G. J. Persley and M. M. Lantin [eds.], Agricultural biotechnology and the poor: Proceedings of an international conference, 90–96, 1999, Washington, D.C., USA. Consultative Group on International Agricultural Research, Washington, D.C., USA. Available online http://www.cgiar.org/biotech/rep0100/morales.pdf [accessed 18 May 2012].

APHIS-USDA [ANIMAL AND PLANT HEALTH INSPECTION SERVICE-U.S. DEPARTMENT OF AGRICULTURE]. 1994. Availability of determination of nonregulated status for virus resistant squash. *Federal Register* 59: 64187–64189.

BERNIAK, H., T. MALINOWSKI, AND M. KAMI SKA. 2009. Comparison of ELISA and RT-PCR assays for detection and identification of *Cucumber mosaic virus* (CMV) isolates infecting horticultural crops in Poland. *Journal of Fruit and Ornamental Plant Research* 17: 5–20.

BERGELSON, J., M. KREITMAN, E. A. STAHL, AND D. TIAN. 2001. Evolutionary dynamics of plant *R*-genes. *Science* 292: 2281–2285.

BORER, E. T., C. E. MITCHELL, A. G. POWER, AND E. W. SEABLOOM. 2009. Consumers indirectly increase infection risk in grassland food webs. *Proceedings of the National Academy of Sciences, USA* 106: 503–506.

BOS, L. 1981. Wild plants in the ecology of virus diseases. *In* K. Maramarosch and K. F. Harris [eds.], Plant diseases and vectors: Ecology and epidemiology, 1–28. Academic Press, New York, New York, USA.

BURDON, J. J., AND G. A. CHILVERS. 1982. Host density as a factor in plant disease ecology. *Annual Review of Phytopathology* 20: 143–166.

CAICEDO, A. L. 2008. Geographic diversity cline of *R* gene homologs in wild populations of *Solanum pimpinellifolium* (Solanaceae). *American Journal of Botany* 95: 393–398.

CARLSSON, U., AND T. ELMQVIST. 1992. Epidemiology of anther-smut disease (*Microbotryum violaceum*) and numeric regulation of populations of *Silene dioica. Oecologia* 90: 509–517.

COOPER, J. I., AND R. A. C. JONES. 2006. Wild plants and viruses: Underinvestigated ecosystems. *Advances in Virus Research* 67: 1–47.

DARMENCY, H. 1994. The impact of hybrids between genetically modified crop plants and their related species: Introgression and weediness. *Molecular Ecology* 3: 37–40.

DAVIS, R. F., AND M. K. MIZUKI. 1987. Detection of cucurbit viruses in New Jersey. *Plant Disease* 71: 40–44.

DECKER, D. S. 1988. Origin(s), evolution, and systematics of *Cucurbita pepo* (Cucurbitaceae). *Economic Botany* 42: 4–15.

DECKER-WALTERS, D. S., J. E. STAUB, S.-M. CHUNG, E. NAKATA, AND H. QUEMADA. 2002. Diversity in free-living populations of *Cucurbita pepo* (Cucurbitaceae) as assessed by random amplified polymorphic DNA. *Systematic Botany* 27: 19–28.

DUFFUS, J. E. 1971. Role of weeds in the incidence of virus diseases. *Annual Review of Phytopathology* 9: 319–340.

FIGUEIRA, A. R., L. L. DOMIER, AND C. J. D'ARCY. 1997. Comparison of techniques for detection of *Barley yellow dwarf virus*-PAV-IL. *Plant Disease* 81: 1236–1240.

FRIESS, N., AND J. MAILLET. 1996. Influence of *Cucumber mosaic virus* infection on the intraspecific competitive ability and fitness of purslane (*Portulaca oleracea*). *New Phytologist* 132: 103–111.

FOWLER, N. L., AND K. CLAY. 1995. Environmental heterogeneity, fungal parasitism and the demography of the grass *Stipa leucotricha. Oecologia* 103: 55–62.

FUCHS, M., E. M. CHIRCO, AND D. GONSALVES. 2004a. Movement of coat protein genes from a virus-resistant transgenic squash into a free-living relative. *Environmental Biosafety Research* 3: 5–16.

FUCHS, M., E. M. CHIRCO, J. MCFERSON, AND D. GONSALVES. 2004b. Comparative fitness of a free-living squash species and free-living × virus-resistant transgenic squash hybrids. *Environmental Biosafety Research* 3: 17–28.

FUCHS, M., AND D. GONSALVES. 1995. Resistance of transgenic squash Pavo ZW-20 expressing the coat protein genes of *Zucchini yellow mosaic virus* and *Watermelon mosaic virus 2* to mixed infections by both potyviruses. *Nature Biotechnology* 13: 1466–1473.

FUCHS, M., AND D. GONSALVES. 1999. Risk assessment of gene flow from a virus-resistant transgenic squash into a wild relative. *In* K. Ammann,

Y. Jacot, V. Simonsen, and G. Kjellsson [eds.], Methods for risk assessment of transgenic plants. III. Ecological risks and prospects of transgenic plants, 141–143. Birkhaeuser Verlag, Basel, Switzerland.

FUNAYAMA, S., I. TERASHIMA, AND T. YAHARA. 2001. Effects of virus infection and light environment on population dynamics of *Eupatorium makinoi* (Asteraceae). *American Journal of Botany* 88: 616–622.

GARRETT, K. A., S. P. DENDY, E. E. FRANK, M. N. ROUSE, AND S. E. TRAVERS. 2006. Climate change effects on plant disease: Genomes to ecosystems. *Annual Review of Phytopathology* 44: 489–509.

GIANESSI, L. P., C. S. SILVERS, S. SANKULA, AND J. E. CARPENTER. 2002. Plant biotechnology: Current and potential impact for improving pest management in U.S. agriculture. An analysis of 40 case studies. National Center for Food and Agricultural Policy, Washington, D.C., USA.

GODFREE, R. C., THRALL, P. H., and YOUNG, A. G. 2007. Enemy release after introduction of disease-resistant genotypes into plant-pathogen systems. *Proceedings of the National Academy of Sciences of the United States of America* 104: 2756–2760.

HAMMOND, J. 1981. Viruses occurring in *Plantago* species in England. *Plant Pathology* 30: 237–243.

HAMMOND, J., H. LECOQ, AND B. RACCAH. 1999. Epidemiological risks from mixed virus infections and transgenic plants expressing viral genes. *Advances in Virus Research* 54: 189–314.

HOUGEN-EITZMAN, D., AND M. D. RAUSHER. 1994. Interactions between herbivorous insects and plant–insect coevolution. *American Naturalist* 143: 677–697.

HU, J. S., H. P. LI, K. BARRY, AND M. WANG. 1995. Comparison of dot blot, ELISA, and RT-PCR assays for detection of two *Cucumber mosaic virus* isolates infecting banana in Hawaii. *Plant Disease* 79: 902–906.

HULL, R. 2002. Matthews' plant virology, 4th ed. Academic Press, New York, New York, USA.

INFORMATION SYSTEMS FOR BIOTECHNOLOGY. 2012 [continuously updated]. U. S. Department of Agriculture-Animal and Plant Health Inspection Service data on environmental releases in the United States. Website http://www.isb.vt.edu/data.aspx [accessed 17 January 2012].

INTERNATIONAL COMMITTEE ON TAXONOMY OF VIRUSES. 2009. Virus taxonomy: Release 2009. Website http://www.ictvonline.org/virusTaxonomy.asp?version=2009 [accessed 7 August 2011]

IWAO, K., AND M. D. RAUSHER. 1997. Evolution of plant resistance to multiple herbivores: Quantifying diffuse coevolution. *American Naturalist* 149: 316–335.

JAMES, D., A. VARGA, V. PALLAS, AND T. CANDRESSE. 2006. Strategies for simultaneous detection of multiple plant viruses. *Canadian Journal of Plant Pathology* 28: 16–29.

JOHNSON, S., S. STROM, AND K. GRILLO. 2007. Quantification of the impacts on US agriculture of biotechnology-derived crops planted in 2006. National Center for Food and Agricultural Policy, Washington, D.C., USA.

KAWAKAMI, K., S.-I. FUJI, AND K. MIYOSHI. 2007. Endangered wild populations of endemic *Calanthe* orchids on an isolated Japanese island tested for viruses. *Australian Journal of Botany* 55: 831–836.

KIRKPATRICK, K. J., AND H. D. WILSON. 1988. Interspecific gene flow in *Cucurbita*: *Cucurbita texana* vs. *Cucurbita pepo*. *American Journal of Botany* 75: 519–527.

KLUEKEN, A. M., B. HAU, B. ULBER, AND H.-M. POEHLING. 2009. Forecasting migration of cereal aphids (Hemiptera: Aphididae) in autumn and spring. *Journal of Applied Entomology* 133: 328–344.

LAUGHLIN, K. D., A. G. POWER, A. A. SNOW, AND L. J. SPENCER. 2009. Risk assessment of genetically engineered crops: Fitness effects of virus-resistance transgenes in wild *Cucurbita pepo*. *Ecological Applications* 19: 1091–1101.

LÓPEZ, M. A., E. BERTOLINI, A. OLMOS, P. CARUSO, M. T. GORRIS, P. LLOP, R. PENYALVER, AND M. CAMBRA. 2003. Innovative tools for detection of plant pathogenic viruses and bacteria. *International Microbiology* 6: 233–243.

LOUDA, S. M., AND M. A. POTVIN. 1995. Effect of inflorescence-feeding insects on the demography and lifetime fitness of a native plant. *Ecology* 76: 229–245.

MACCLEMENT, W. D., AND M. G. RICHARDS. 1956. Virus in wild plants. *Canadian Journal of Botany* 34: 793–799.

MACKENZIE, S. 1985. Reciprocal transplantation to study local specialisation and the measurement of components of fitness. Ph.D. dissertation, University of College of North Wales, Bangor, Gwynedd, UK.

MALMSTROM, C. M., C. C. HUGHES, L. A. NEWTON, AND C. J. STONER. 2005a. Virus infection in remnant native bunchgrasses from invaded California grasslands. *New Phytologist* 168: 217–230.

MALMSTROM, C. M., A. J. MCCULLOUGH, H. A. JOHNSON, L. A. NEWTON, AND E. T. BORER. 2005b. Invasive annual grasses indirectly increase virus incidence in California native perennial bunchgrasses. *Oecologia* 145: 153–164.

MALMSTROM, C. M., C. J. STONER, S. BRANDENBURG, AND L. A. NEWTON. 2006. Virus infection and grazing exert counteracting influences on survivorship of native bunchgrass seedlings competing with invasive exotics. *Journal of Ecology* 94: 264–275.

MARON, J. L., AND E. L. SIMMS. 1997. Effect of seed predation on seed bank size and seedling recruitment of bush lupine (*Lupinus arboreus*). *Oecologia* 111: 76–83.

MATTHEWS, R. E. F. 1991. Plant virology, 3rd ed. Academic Press, London, UK.

MITCHELL, C. E., D. TILMAN, AND J. V. GROTH. 2002. Effects of grassland plant species diversity, abundance and composition on foliar fungal disease. *Ecology* 83: 1713–1726.

MUTHUKUMAR, V., U. MELCHER, M. PIERCE, G. B. WILEY, B. A. ROE, M. W. PALMER, V. THAPA, ET AL. 2009. Non-cultivated plants of the Tallgrass Prairie Preserve of northeastern Oklahoma frequently contain virus-like sequences in particulate fractions. *Virus Research* 141: 169–173.

NATIONAL CLIMATIC DATA CENTER. 2009. Climatological data: Mississippi: vol. 114(4-6) [online]. National Climatic Data Center, U.S. Department of Commerce, Washington, D.C., USA. Website http://www7.ncdc.noaa.gov/IPS/cd/cd.html [accessed 7 August 2011].

OERKE, E.-C., H.-W. DEHNE, F. SCHÖNBECK, AND A. WEBER. 1994. Crop production and crop protection: Estimated losses in major food and cash crops. Elsevier Science B.V., Amsterdam, Netherlands.

OSWALD, J. W., AND B. R. HOUSTON. 1953. The yellow-dwarf virus disease of cereal crops. *Phytopathology* 43: 128–136.

PAGÁN, I., C. ALONSO-BLANCO, AND F. GARCÍA-ARENAL. 2007. The relationship of within-host multiplication and virulence in a plant–virus system. *PLoS ONE* 2: e786.

PAGÁN, I., C. ALONSO-BLANCO, AND F. GARCÍA-ARENAL. 2008. Host responses in life-history traits and tolerance to virus infection in *Arabidopsis thaliana*. *PLoS Pathogens* 4: e1000124.

PAGÁN, I., C. ALONSO-BLANCO, AND F. GARCÍA-ARENAL. 2009. Differential tolerance to direct and indirect density dependent costs of viral infection in *Arabidopsis thaliana*. *PLoS Pathogens* 5: e1000531.

PAGÁN, I., A. FRAILE, E. FERNANDEZ-FUEYO, N. MONTES, C. ALONSO-BLANCO, AND F. GARCÍA-ARENAL. 2010. *Arabidopsis thaliana* as a model for the study of plant–virus co-evolution. *Philosophical transactions of the Royal Society, B, Biological Sciences* 365: 1983–1995.

PALLETT, D. W., M. I. THURSTON, M. CORTINA-BORJA, M.-L. EDWARDS, M. ALEXANDER, E. MITCHELL, A. F. RAYBOULD, AND J. I. COOPER. 2002. The incidence of viruses in *Brassica rapa* spp. *sylvestris* in southern England. *Annals of Applied Biology* 141: 163–170.

PICÓ, B., M. J. DIEZ, AND F. NUEZ. 1996. Viral diseases causing the greatest economic losses to the tomato crop. II. The Tomato yellow leaf curl virus—A review. *Scientia Horticulturae* 67: 151–196.

PILSON, D., AND H. R. PRENDEVILLE. 2004. Ecological effects of transgenic crops and the escape of transgene into wild populations. *Annual Review of Ecology, Evolution and Systematics* 35: 149–174.

POWER, A. G. 1991. Virus spread and vector dynamics in genetically diverse plant populations. *Ecology* 72: 232–241.

POWER, A. G. 1996. Competition between viruses in a complex plant–pathogen system. *Ecology* 77: 1004–1010.

PRENDEVILLE, H. R. 2010. Ecological effects of virus-resistant transgenic squash. Ph.D. dissertation, University of Nebraska, Lincoln, Nebraska, USA.

PROVVIDENTI, R., R. W. ROBINSON, AND H. M. MUNGER. 1978. Resistance in feral species to six viruses infecting *Cucurbita. Plant Disease Reporter* 62: 326–329.

QUEMADA, H., L. STREHLOW, D. S. DECKER-WALTERS, AND J. E. STAUB. 2008. Population size and incidence of virus infection in free-living populations of *Cucurbita pepo. Environmental Biosafety Research* 7: 185–196.

QUESADA, M., J. A. WINSOR, AND A. G. STEPHENSON. 1996. Effect of pollen competition on the reproductive output of F_1 *Cucurbita* hybrids. *Canadian Journal of Botany* 74: 1113–1118.

RADWAN, D. E., K. A. FAYEZ, S. Y. MAHMOUD, A. HAMAD, AND G. LU. 2007. Physiological and metabolic changes of *Cucurbita pepo* leaves in response to *Zucchini yellow mosaic virus* (ZYMV) infection and salicylic acid treatments. *Plant Physiology and Biochemistry* 45: 480–489.

RAYBOULD, A. F., L. C. MASKELL, M. L. EDWARDS, J. I. COOPER, AND A. J. GRAY. 1999. The prevalence and spatial distribution of viruses in natural populations of *Brassica oleracea. New Phytologist* 141: 265–275.

REMOLD, S. K. 2002. Unapparent virus infection and host fitness in three weedy grass species. *Journal of Ecology* 90: 967–977.

ROOSSINCK, M. J., P. SAHA, G. B. WILEY, J. QUAN, J. D. WHITE, H. LAI, F. CHAVARRÍA, ET AL. 2010. Ecogenomics: Using massively parallel pyrosequencing to understand virus ecology. *Molecular Ecology* 19: 81–88.

SALVAUDON, L., T. GIRAUD, AND J. A. SHYKOFF. 2008. Genetic diversity in natural populations: A fundamental component of plant–microbe interactions. *Current Opinion in Plant Biology* 11: 135–143.

SASU, M. A., M. J. FERRARI, D. DU, J. A. WINSOR, AND A. G. STEPHENSON. 2009. Indirect costs of a nontarget pathogen mitigate the direct benefits of a virus-resistant transgene in wild *Cucurbita. Proceedings of the National Academy of Sciences, USA* 106: 19067–19071.

SEABLOOM, E. W., E. T. BORER, A. JOLLES, AND C. E. MITCHELL. 2009. Direct and indirect effects of viral pathogens and the environment on invasive grass fecundity in Pacific Coast grasslands. *Journal of Ecology* 97: 1264–1273.

SHANG, H., Y. XIE, X. ZHOU, Y. QIAN, AND J. WU. 2011. Monoclonal antibody-based serological methods for detection of *Cucumber green mottle mosaic virus*. *Virology Journal* 8: 228.

SPENCER, L. J. 2001. Hybridization and introgression in *Cucurbita pepo*: An examination of transgenic virus resistance on wild–crop hybrids. Ph.D. dissertation. Ohio State University, Columbus, Ohio, USA.

SPENCER, L. J., AND A. A. SNOW. 2001. Fecundity of transgenic wild–crop hybrids of *Cucurbita pepo* (Cucurbitaceae): Implications for crop-to-wild gene flow. *Heredity* 86: 694–702.

STILING, P., AND A. M. ROSSI. 1996. Complex effects of genotype and environment on insect herbivores and their enemies. *Ecology* 77: 2212–2218.

TAYLOR, L. R. 1979. The Rothamsted Insect Survey: An approach to the theory and practice of synoptic pest forecasting in agriculture. *In* R. L. Rabb and G. G. Kennedy [eds.], Movement of highly mobile insects: Concepts and methodology in research, 148–185. North Carolina State University, Raleigh, North Carolina, USA.

TÉCSI, L., A. M. SMITH, A. J. MAULE, AND C. RICHARD. 1996. A spatial analysis of physiological changes associated with infection of cotyledons of marrow plants with *Cucumber mosaic virus*. *Plant Physiology* 111: 975–985.

TEPFER, M. 2002. Risk assessment of virus-resistant transgenic plants. *Annual Review of Phytopathology* 40: 467–491.

TIAN, D., M. B. TRAW, J. Q. CHEN, M. KREITMAN, AND J. BERGELSON. 2003. Fitness costs of R-gene-mediated resistance in *Arabidopsis thaliana. Nature* 423: 74–77.

THOMPSON, J. R., AND M. TEPFER. 2010. Assessment of the benefits and risks for engineered virus resistance. *Advances in Virus Research* 76: 33–56.

THRALL, P. H., J. J. BURDON, AND J. D. BEVER. 2002. Local adaptation in the *Linum marginale–Melampsora lini* host–pathogen interaction. *Evolution* 56: 1340–1351.

THRESH, J. M. 1981. The role of weeds and wild plants in the epidemiology of plant virus diseases. *In* J. M. Thresh [ed.], Pests, pathogens and vegetation, 53–70. Pitman Books, London, UK.

THURSTON, M. I., D. W. PALLETT, M. CORTINA-BORJA, M.-L. EDWARDS, A. F. RAYBOULD, AND J. I. COOPER. 2001. The incidence of viruses in wild *Brassica nigra* in Dorset (UK). *Annals of Applied Biology* 139: 277–284.

TUGUME, A. K., S. B. MUKASA, AND J. P. T. VALKONEN. 2008. Natural wild hosts of *Sweet potato feathery mottle virus* show spatial differences in virus incidence and virus-like diseases in Uganda. *Phytopathology* 98: 640–652.

TURNBULL, L. A., M. J. CRAWLEY, AND M. REES. 2000. Are plant populations seed-limited? A review of seed sowing experiments. *Oikos* 88: 225–238.

ULLMAN, D. E., J. J. CHO, AND T. L. GERMAN. 1991. Occurrence and distribution of cucurbit viruses in the Hawaiian Islands. *Plant Disease* 75: 367–370.

USDA-NASS [U.S. DEPARTMENT OF AGRICULTURE-NATIONAL AGRICULTURAL STATISTICS SERVICE]. 2011. Vegetables 2010 Summary, January 2011. Website http://usda.mannlib.cornell.edu/usda/nass/VegeSumm//2010s/2011/VegeSumm-01-27-2011.pdf [accessed 14 February 2012]

VITOUSEK, P. M., C. M. D'ANTONIO, L. L. LOOPE, M. REJMÁNEK, AND R. WESTBROOKS. 1997. Introduced species: A significant component of human-caused global change. *New Zealand Journal of Ecology* 21: 1–16.

VUNSH, R., A. ROSNER, AND A. STEIN. 1990. The use of the polymerase chain reaction (PCR) for the detection of *Bean yellow mosaic virus* in gladiolus. *Annals of Applied Biology* 117: 561–569.

WALKEY, D. 1991. Applied plant virology, 2nd ed. Chapman and Hall, London, UK.

WALL, E. M., T. S. LAWRENCE, M. J. GREEN, AND M. E. ROTT. 2004. Detection and identification of transgenic virus resistant papaya and squash by multiplex PCR. *European Food Research and Technology* 219: 90–96.

WALTHER, G.-R., E. POST, P. CONVEY, A. MENZEL, C. PARMESANK, T.J.C. BEEBEE, J.-M. FROMENTIN, ET AL. 2002. Ecological responses to recent climate change. *Nature* 416: 389–395.

WEN, F., R. M. LISTER, AND F. A. FATTOUH. 1991. Cross-protection among strains of *Barley yellow dwarf virus*. *Journal of General Virology* 72: 791–799.

WILSON, H. D. 1990. Gene flow in squash species. *Bioscience* 40: 449–455.

WILSON, H. D. 1993. Free-living *Cucurbita pepo* in the United States: Viral resistance, gene flow, and risk Assessment. U. S. Department of Agriculture-Animal and Plant Health Inspection Service, Hyattsville, Maryland, USA.

YAHARA, T., AND K. OYAMA. 1993. Effects of virus-infection on demographic traits of an agamospermous population of *Eupatorium chinense* (Asteraceae). *Oecologia* 96: 310–315.

YUKI, V. A., J. A. M. REZENDE, E. W. KITAJIMA, P. A. V. BARROSO, H. KUNIYUKI, G. A. GROPPO, AND M. A. PAVAN. 2000. Occurrence, distribution, and relative incidence of five viruses infecting cucurbits in the state of São Paulo, Brazil. *Plant Disease* 84: 516–520.

ZETTLER, F. W., G. R. HENNEN, W. H. BODNARUK JR., H. T. CLIFFORD, AND T. J. SHEEHAN. 1978. Wild and cultivated orchids surveyed in Florida for the Cymbidium mosaic and Odontglossum ringspot viruses. *Plant Disease Reporter* 62: 949–952.