

Population size and incidence of virus infection in free-living populations of *Cucurbita pepo*

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Impact assessments of virus resistance transgene introgression into wild, free-living populations are important for determining whether these transgenes present a risk to agriculture or the environment. Transgenic virus-resistant *Cucurbita pepo* ssp. *ovifera* var. *ovifera* L. (squash) cultivars have been commercialized, and may be cultivated in close proximity to cross-compatible wild, free-living relatives (*C. pepo* subsp. *pepo* vars. *ozarkana* and *texana*). Therefore, the potential impact of these virus resistance transgenes was studied by surveying the incidence and fluctuations of virus infection (as assayed by ELISA), virus symptoms (which may not be seen in an infected plant) and population size in forty-three free-living *C. pepo* populations in Illinois, Missouri, Arkansas, Mississippi, Louisiana, and Texas. Ten of these populations were studied over three consecutive seasons. Depending on the year, 61% to 78% percent of the populations had at least one individual infected by at CMV, ZYMV or WMV2, but the median incidence of infection within populations was 13%. The observed infection level in free-living populations was consistent with levels defined as “low” in field plot experiments conducted by others, leading to the conclusion that transgenic virus resistance should not provide a significant fitness advantage to the free-living populations examined. Viral symptoms were detected in only 2% of plants observed, indicating that severity of viral infection was low. CMV, ZYMV, and WMV2 were not the only viruses infecting these populations, further reducing the likelihood that resistance to these viruses would release populations from constraints imposed by virus diseases.

Keywords: transgenic / virus resistance / populations / *Cucurbita pepo* / introgression / gene flow

INTRODUCTION

Transgenic crop species have been commercialized since 1994, and are responsible for significant and demonstrable economic benefits in areas where they have been commercially adopted (Brookes and Barfoot, 2005, 2006; Sankula, 2006; Sankula and Blumenthal, 2004). Thus far, these crops have had a proven record of safety, both from a human/animal health and environmental perspective. Nevertheless, scientific issues surrounding the safety assessment of these crops continue to be of scientific interest. These continuing risk appraisals provide feedback for decision-making by testing the assumptions and conclusions made during the pre-market risk assessment, and thereby improve the quality, efficiency, and effectiveness

of the process used to assess the risk of transgenic crops. Post-market research may generate data that contradict the pre-market risk assessments, and therefore prompt a reconsideration of a previous regulatory decision, or may generate data that confirm those risk assessments and increases the level of confidence in regulatory decisions and approaches.

Of considerable ecological importance is the potential impact that transgenes might have on the structure and competitiveness of free-living (*i.e.* not agriculturally managed) populations into which they are introgressed. In the United States, several transgenic cultivars that have been commercialized or have received regulatory approval have been evaluated for these impacts, including rice, oilseed rape, squash, and cotton (USDA, 1994b, 1994c, 1994d, 1995a, 1995b, 1996b, 1996c, 1997a, 1997b, 1998a, 1998b, 1999a, 1999b,

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2002a, 2002b, 2002c, 2002d, 2003, 2004a, 2004b, 2005, 2006). Commercial transgenic squash has had the longest history of unrestricted regulatory approval, including approval for cultivation in areas where wild relatives may be present (USDA, 1994a). This regulatory approval was not without controversy, and was criticized in two reports written by the United States National Research Council (US National Research Council, 2000, 2002). Therefore, studies that provide further information regarding the potential impact of virus resistance transgenes in free-living populations have continuing relevance.

The deployment of transgenes in squash affords an opportunity to assess the risk of transgene flow from this crop given real patterns of commercialization and the proximity of transgenic cultivars to free-living populations. Commercialized transgenic squash cultivars express resistance to *Cucumber mosaic virus* (CMV), *Zucchini yellow mosaic virus* (ZYMV), and *Watermelon mosaic virus 2* (WMV2) (USDA, 1996a) or only ZYMV and WMV2 (USDA, 1994a). Studies have shown that free-living *Cucurbita pepo* (*C. pepo* ssp. *ovifera* vars. *ozarkana* and *texana*) populations and cultivated squash (*C. pepo* ssp. *ovifera* vars. *ovifera* and *pepo*) can readily exchange genes (Chung 2006; Decker-Walters et al., 1993, 2002; Kirkpatrick and Wilson, 1988; Smith et al., 1992; Wilson, 1990). Moreover, transgenes from cultivars can be introgressed into wild genotypes without a significant decrease in fitness (Fuchs et al., 2004a, 2004b; Spencer and Snow, 2001). Consequently, the possible impact of virus resistant transgene introgression into free-living squash populations continues to be an interesting area for investigation.

While methods for measuring gene flow have undergone extensive development for application in transgenic crops, the potential consequences of gene flow has not been well studied. The most information has been obtained for sunflowers (Arias and Rieseberg, 1994; Burke and Rieseberg, 2003; Linder et al., 1998; Pilson, 2000; Pilson and Decker, 2002; Reagon and Snow, 2006; Rieseberg et al., 1999; Snow et al., 1998, 2003; Whitton et al., 1997), and even in that system, the potential for negative consequences of transgene introgression into free-living populations has not been well established. The difficulties associated with defining such consequences include the inability to: (1) detect potentially small fitness differences during comparative analyses, and; (2) link observed fitness differences to weediness, invasiveness, or other long-term adverse environmental consequences.

With respect to *C. pepo*, Spencer and Snow (2001) suggested that given the demonstrated lack of barriers to transgene introgression into natural populations of *C. pepo*, it would be useful to document the frequency and severity of viral infections in free-living *C. pepo* populations in order to assist in determining whether a

fitness-related trait such as transgenic virus resistance could enhance the abundance and invasiveness of this species. This approach is consistent with concepts elaborated by Hancock (2003), Pallett et al. (2004), and Raybould and Cooper (2005), who propose ways to assess the potential for transgenic traits to result in ecological release of wild relatives. For example, in the case of transgenes conferring pathogen resistance, which could increase fitness in wild populations, Hancock (2003) recommends that studies should be conducted to determine whether the level of control exerted by a pathogen on its host is significant. If a pathogen does exert significant control, then a gene conferring resistance to the pathogen could release the host from biological constraints that are preventing it from being weedy or invasive. This effect could be manifested by the appearance of larger or faster-growing populations of squash containing the resistance trait, compared with populations of squash that do not have the resistance.

As implied by Spencer and Snow (2001), the extent to which pathogens (*e.g.*, viruses) control free-living populations ought to be a function of infection incidence and severity. Therefore, the benefit of a resistance gene depends very much on what Fuchs et al. (2004a, 2004b) refer to as “virus pressure”. Under conditions of “high virus pressure”, *i.e.* 100% infection of plants in a population by day 32–42 of the season, Fuchs et al. (2004a, 2004b) demonstrated that virus resistance transgenes in transgenic squash cultivars can indeed confer a selective advantage on wild genotypes. However, they detected no benefit under low disease pressure (less than 10% infection by day 32). While the selective advantage reported under substantial disease pressure may be expected to result in the increase in frequency of the virus resistance transgene within populations, virus pressure (incidence and severity) and its effect on population size is the focus of the present study, because it provides a better indicator of ecological release, which is of greater concern for regulatory purposes (Hancock, 2003; Pallett et al., 2004; Raybould and Cooper, 2005).

In support of a petition for the non-regulated status of transgenic virus resistant squash, nine free-living *C. pepo* populations were surveyed to assess the potential threat of transgene introgression (USDA, 1994a, 1994b). Although that survey failed to detect the presence of any viruses against which the transgenic squash crop was resistant, the number of populations examined was relatively small. Thus, the present study was designed to: (1) examine a larger number of wild *C. pepo* populations for the frequency of infection by WMV, ZYMV, CMV, and other viruses; (2) assess the severity of virus disease in infected plants as determined by the presence or absence of symptoms; and (3) to determine whether there is any relationship between the incidence of viral

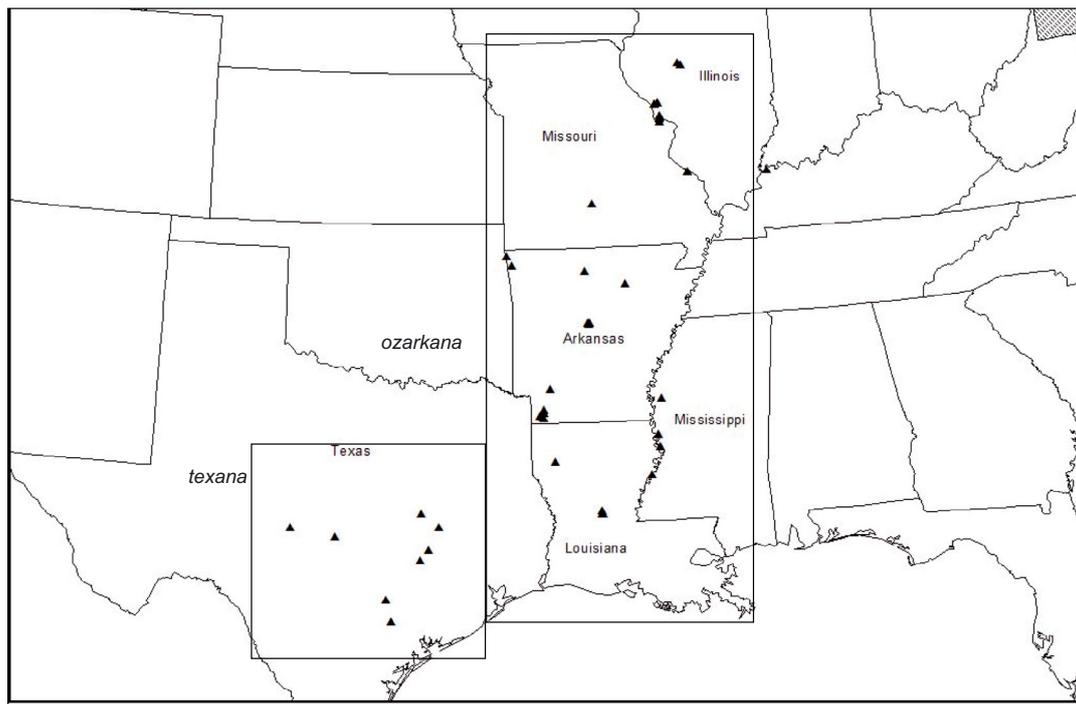


Figure 1. Locations of the *C. pepo* L. populations surveyed from 2000 through 2002. Populations of var. *texana* and *ozarkana* are delimited by boxes.

infection (determined by ELISA) within populations, and population levels in subsequent years. The occurrence of an infection may not have a significant impact on plant mortality or reproduction if symptoms are not expressed. Therefore, the incidence of visual symptoms in free-living populations was also recorded. The information obtained in this study can be linked to experimental studies of transgene flow and fitness in *C. pepo*, to determine which experimental conditions most closely reflect nature, and thereby to draw conclusions regarding the fitness advantage specific virus resistance transgenes might confer on free-living populations. Furthermore, the descriptive approach used in this study will likely be useful for risk assessments of similar interactions between other transgenic crops and their free-living sexually compatible relatives.

RESULTS

Locations

Forty-three *C. pepo* var. *ozarkana* and var. *texana* populations were surveyed for this study. The locations of these populations are shown in Figure 1. Free-living *C. pepo* populations were typically found in disturbed areas on

river or irrigation canal banks, or in river floodplains. Many populations grew in or on the edges of agricultural fields (associated with soybean, maize, cotton, millet, wheat). A molecular marker examination by Chung et al. (2006) of a subset of the populations surveyed in this study provided evidence of introgression from cultivated squash. While Chung et al. (2006) did not determine the presence of transgenes, it is unlikely that any of the free-living populations we surveyed had crossed with transgenic cultivated squash, since these populations were not in the areas where most transgenic squash cultivation occurs (Sankula 2006; Sankula and Blumenthal, 2004). Furthermore, in the three years of surveys, we encountered only one field of transgenic squash. This field was > 50 km from the closest free-living population sampled by us or by Chung et al. (2006).

Population size

Forty-three populations (see Materials and Methods for the definition of “population”) were surveyed through the 2000, 2001, and 2002 growing seasons, yielding 58 estimates of population size. These data are provided in Table 1, and the distribution of population sizes is shown in Figure 2. Excluding population 37 (not depicted

Table 1. Population sample sizes, and virus infection of free-living *C. pepo* L. Populations indicated by shading were studied for three consecutive years.

Population ID	2000				2001				2002
	Number of individuals	Individuals assayed by ELISA	Proportion CZW	Proportion PPCZW	Number of individuals	Individuals assayed by ELISA	Proportion CZW	Proportion PPCZW	Number of individuals
1	2								
2	1								
² 3	Not reported	10	0.3	0.3					
² 4	Not reported	10	0.9	0.9					
5	13								
6	2								
² 7	25	10	0.3	0.5					
¹ 8	5	2	0	0	30	Not sampled			Not revisited
¹ 9	10	1	0	0	10				
10	300				100				
² 11	30	9	0	0					
12	30	8	0	0					
13	1								
14	5				15	12	0.58	0.58	
15	2				30	10	0.4	0.4	
16	19	19	0.47	0.47					
17	3	3	0.67	1					
18	51	32	0.84	0.88					
¹ 9	7	7	0.43	0.71	21	Not sampled			Not revisited
20	6								
21	2								
22	5								
23					2	2	1	1	
24	5								
25	200	64	0.5	0.78					
26	2								
27	2	2	0	0					
28	1	1	0	1					
29	46	46	0.52	0.76					
30	25	25	0.24	0.64					
31	9	9	0.22	0.89					
¹ 32	13	3	0.33	0.67					
33					60	16	0.06	0.06	
¹ 34	21	21	0	0	15	15	0.4	0.47	4
35	16	15	0.13	0.47	0				6
36	23	23	0.13	0.13	0				0
37	10000	32	0	0.25	0				5
38	40	19	0.05	0.37	0				2
39	5	5	0	0	6	6	0.5	0.5	5
40	35	10	0	0	7	7	0.14	0.29	13
41	12	9	0	0	7	7	0	0	9
¹ 42	42	3	0	0	6	6	0	0	
¹ 43	29	2	0	0					

¹ Included in the analysis of population size but not in the analysis of virus incidence for 2000 (see Materials and Methods).

² Collected by Dr. Amanda Neill, Botanical Research Institute of Texas. Population size was estimated by visual appraisal.

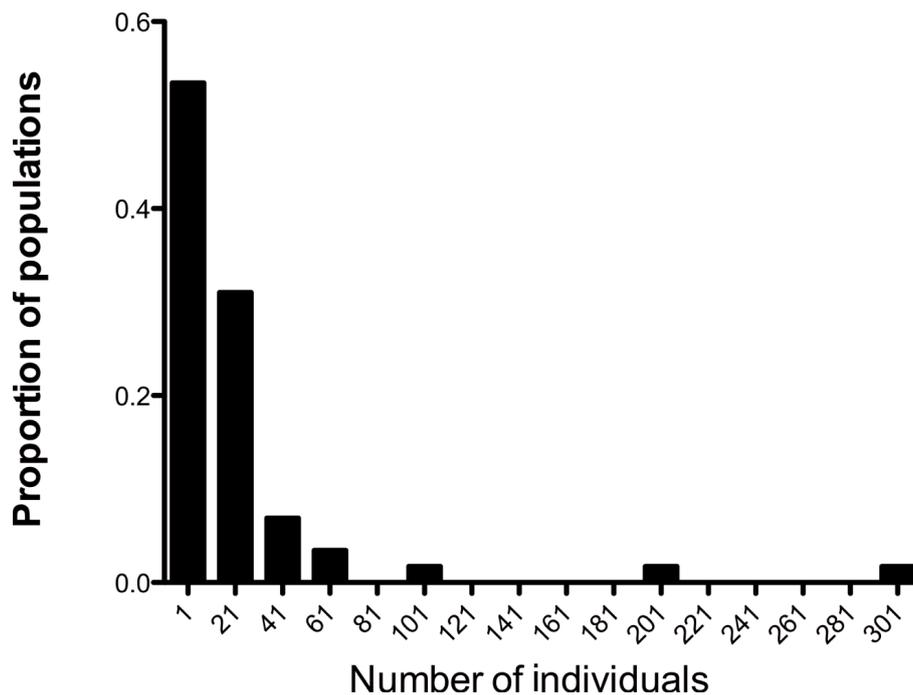


Figure 2. The distribution of population sizes of free-living *C. pepo* L. surveyed for this study, excluding population 37.

in Fig. 2), free-living *C. pepo* populations were relatively small, with a median number of 10. Only four of the populations consisted of more than 100 individuals. These small populations were an indication that free-living *C. pepo* may not present as much of a weed problem as they have in the past (Boyette et al., 1984; Bridges, 1992).

Rates and patterns of infection in natural populations

The number of samples and plants infected with CMV, WMV2, and ZYMV either singly or in combination are also given in Table 1. The numbers of plants infected by these three viruses, in addition to papaya ringspot virus (PRSV) and other unidentified potyviruses are also presented therein. For the populations surveyed and sampled in 2000 and for which data were included for analysis (see Materials and Methods), sixty-one percent (14 out of 23) populations contained at least one individual infected by at least one of the viruses for which transgenic resistance has been developed (CMV, ZYMV, or WMV2). If PRSV and other potyviruses are considered in addition to these viruses, 70% of populations were infected. For the populations that were re-surveyed and re-sampled in 2001, 78% of populations were infected with the viruses assayed.

The distribution of virus incidence among populations in 2000 is shown in Figure 3, which illustrates the low incidence of virus infection within populations. Figure 3 shows that uninfected populations comprise the largest class, while 100% infected populations are rare. The median incidence of infection by CMV + ZYMV + WMV2 (CZW) is 0.13, but was higher for PRSV + ZYMV + WMV2 + other potyviruses (PPCZW) at 0.47. In 2001, the median incidence of infection for both CZW and PPCZW infections was 0.4. Because of the smaller number and more limited geography of populations assayed in 2001, it is not possible to draw meaningful conclusions based on a comparison of virus infection in 2000 versus 2001. However, it is clear that other viruses against which no transgenic resistance has been deployed are also present in free-living populations, and may also exert an impact on these populations.

In contrast to the incidence of infected plants detected by ELISA, the incidence of symptomatic plants (by visual inspection of a total of approximately 11 000 plants in 2000 and 2001 combined) was 2% over all locations. Therefore, the severity of virus infection in free-living populations was too low to elicit consistent detectable visual symptoms. As discussed in the following section, this lack of symptoms indicates that despite the detection of virus infection in free-living populations, the physiological impact of these infections is low, and therefore the impact on population levels is likely to be low as well.

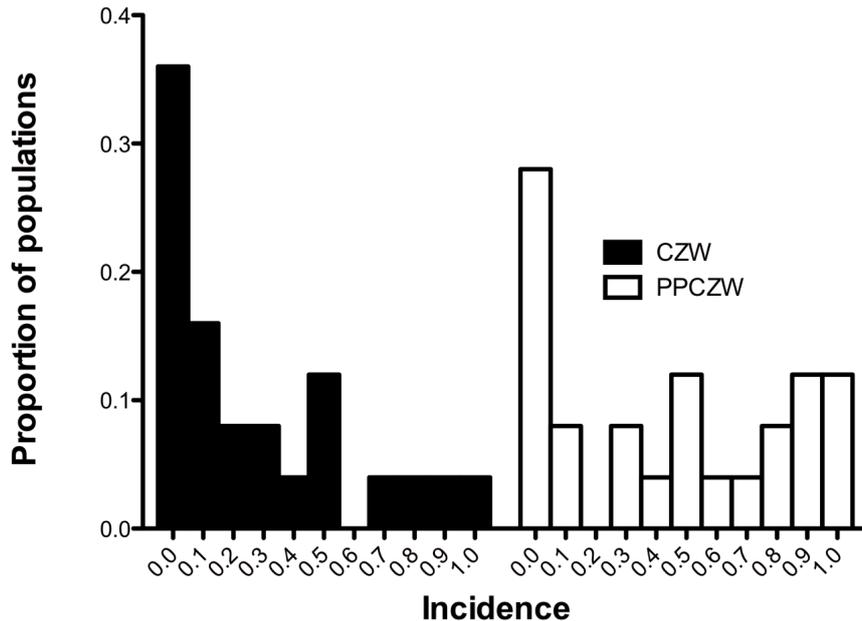


Figure 3. The distribution of virus incidence in *C. pepo* L. populations in 2000. Filled columns indicate the incidence of CMV + ZYMV + WMV2 infection, while unfilled columns indicate the incidence of all viruses assayed (Potyvirus + PRSV + CMV + ZYMV + WMV2).

Infection and population size in sequential years

Population size and the incidence of virus infection varied greatly from year to year (Tab. 1). We were able to repeat surveys of a subset of populations over three years (indicated by shading in Tab. 1). Most of the populations (34 through 41), located near the Illinois and Mississippi rivers in Illinois and Missouri, decreased in size or remained constant over 2000, 2001 and 2002. In contrast, the size of two populations (population 8 in Louisiana and population 19 in Arkansas), increased from 2000 to 2001.

Examination of population size and virus incidence in Table 1 failed to reveal a discernable association between fluctuations in population size and the incidence of virus infections in the populations studied. Because of the relatively few populations followed over three seasons, meaningful statistical analyses of any correlations were not possible. Therefore the analysis presented here should be viewed as a starting point for more extensive surveys in the future. The very large data sets necessary to provide a rigorous test of correlations between virus infection and changes in populations will require a more significant effort than was possible within the resources available for this study.

Populations 34 through 41 declined from 2000 to 2001 in the absence of virus infection. Populations 34, 39, 40, and 41 were infected in 2001, but only population 34 showed a large decline in population number during the investigation period. Population 39 remained stable (5 to 6 individuals) despite the lack of infection in 2000 and a 50% incidence of infection in 2001. This population was reported to have remained stable at this level for at least 10 years prior to our survey (D. Asch, New York State Museum, personal communication, 2000). Although populations 35 through 38 were infected in 2000, and then were completely absent in 2001, this absence was due to conditions other than virus infection (see Discussion). The complete disappearance of population 37 ($n > 10\,000$) is particularly interesting, given that only 25% of individuals were infected with viruses, including viruses other than CMV, ZYMV, or WMV2. ELISA assays detected primarily PRSV infections in this population, and one individual tested positive for an unidentified potyvirus. There was no obvious reason for this disappearance, since the site appeared undisturbed between 2000 and 2001. The presence of five individuals in population 37 in 2002 was clearly attributable to influx of new individuals rather than from the seed bank, since those individuals were found clustered around the remnants of a fruit that was found at the margin of a recently dried pool

of water in the field. Free-living *C. pepo* fruits are water-dispersed (Smith et al., 1992). Arkansas population 19 trebled in number despite the relatively high level of virus infection present (43% CMV + ZYMV + WMV2; 71% Potyvirus + PRSV + CMV + ZYMV + WMV2). Lastly, population 8 increased in the absence of virus infection between 2000 and 2001. This population was the only one followed from year to year that showed the change expected if the viruses assayed in this study were restricting the size of these free-living populations.

DISCUSSION

Although the recent commercialization of numerous transgenic crops has provided many benefits for U.S. agriculture, continued research is needed to monitor the potential risks posed by the introduction of engineered traits into free-living relatives of these crops. Data presented herein identified free-living squash populations in the southern U.S. not previously described, in order to assess the potential impact of virus infection on these populations.

Population survey

Free-living *C. pepo* populations generally consist of relatively few individuals (median number = 10). The present small size of free-living *C. pepo* populations suggests that this plant does not pose the same weed problem that it might have in the past. In the populations studied, the numbers of individuals varied from year to year (Tab. 1). However, we were not able to attribute the fluctuations in population size to virus infection, since no relationship could be discerned between the incidence of virus and the number of individuals in subsequent generations.

On the other hand, we were able to determine that the decline in the size of Illinois and Missouri populations 33 through 41 (Tab. 1) was associated with the agricultural practices employed in and around the fields where these populations were found. In particular populations 34 through 36 and 38, which disappeared in 2001, were in areas where evidence of weed control was observed (mowing or herbicide). Growers interviewed during the 2000 survey and sampling trip attributed the elimination of problems caused by “weedy gourds”, a significant pest in fields previously planted with soybean and cotton, to the use of Roundup® herbicide. These observations were corroborated during a 2006 survey of free-living *C. pepo* populations in the central and southern U.S. (C. Calderon, University of Wisconsin-Madison, personal communication, 2006). Therefore, it is likely that agricultural weed control practices have a significant influence in limiting free-living *C. pepo* populations in agricultural settings.

Additionally, drought, herbivory (insects as well as mammals), and other diseases (bacteria and fungi) were also contributing factors identified during the 2000 survey trips that may also have influenced the size of free-living populations in the sites examined (data not presented).

Impact of virus on population size

In contrast to a previous survey conducted prior to the commercial release of transgenic squash (USDA, 1994b), the 2000 and 2001 surveys showed that free-living *C. pepo* plants are indeed infected by viruses (Tab. 1, Fig. 3). Given a median viral incidence of for CMV + ZYMV + WMV2 of 0.13 to 0.4 within populations, depending on the year, most plants in the populations examined would be expected to remain uninfected by these viruses, and thus should not benefit from introgressed virus resistance transgenes. Furthermore, the impact of virus infection on individuals that are infected may be affected by other environmental or evolutionary factors, since the incidence of virus infection was not a clear predictor of population increase or decrease in subsequent seasons (Tab. 1). The general dearth of individuals in most of the populations surveyed does not allow for a quantitative analysis of the impact of virus infection on population size. This was even more difficult for determining any correlation between the incidence of symptoms and changes in population size, which, due to the low incidence of symptomatic plants, could not be tested. However, for our dataset, fluctuations (or constancy) of population size cannot be qualitatively explained by the presence or absence of viruses. It is possible, however, that following more populations over a longer period of time would reveal patterns that our dataset did not enable us to detect.

We have considered the possibility that the reason for the low incidence of infection, especially in 2000, may be the result of a high infection rate at the seedling stage that then leads to the death of infected individuals before sampling. This explanation does not appear likely under natural conditions. Fuchs et al. (2004a, 2004b) showed that uninoculated seedlings of *C. pepo* did not show symptoms until at least 30 days post-planting. Spencer and Snow (2001) report the appearance of symptoms in *C. pepo* populations at day 45 post-planting, even though sources of inoculum were nearby. Furthermore, Klas et al. (2006) conducted experiments in which two *C. pepo* plots, each consisting of 200 plants, were planted as seedlings, surrounded by inoculated plants as a source of inoculum. Under this high level of virus disease pressure, 399 of the 400 plants survived to the end of the experiment (74 days post-planting). Therefore, it is not likely that seedlings are exposed to virus infection early in the season, and even if they were, survival rates would be high.

If the apparent lack of control of populations by virus infection is corroborated by further study, this conclusion would not be surprising in light of the results of Fuchs et al. (2004b), who were only able to show a fitness benefit from viral transgenes under high disease pressure. That high level of disease pressure was not found in the natural settings observed in this study, confirming the opinion of Fuchs et al. (2004a) that high disease pressure is “unlikely to be consistently present throughout the United States, particularly in the Southern parts”. Moreover, Spencer and Snow (2001) reported that even when viral symptoms are apparent and the leaf area of plants is greatly reduced by such infection, plants produced fruits with high quality seed in abundance. Consistent with this observation of Spencer and Snow (2001), our field impact assessment of introgressed transgenic virus resistance on fitness and fecundity in free-living *C. pepo* genotypes was not able to find a clear fitness benefit conferred by these transgenes (Strehlow and Quemada, data not presented, manuscript in preparation).

Disease levels found in the populations studied closely matches the low disease pressure conditions reported by Fuchs et al. (2004b) who evaluated the beneficial effects of transgenes in several wild *C. pepo* backgrounds. Fuchs et al. (2004b) achieved their low disease pressure (due to CMV, ZYMV or WMV2) by transplanting healthy plants to locations removed from sources of infection. Such a situation would be similar to that found in free-living *C. pepo* populations, because of the non-persistent mode of transmission (*i.e.*, transmitted within minutes of acquisition by their aphid vector) of the viruses studied. Logically, non-persistent transmission requires that in each season, emerging seedlings must be re-infected from nearby infected plants. The small numbers of individuals in most free-living *C. pepo* populations, with intervening plants not susceptible to CMV, ZYMV, or WMV2, should therefore effectively isolate these populations from exposure to virus infection.

Low disease pressure in free-living *C. pepo* populations is also evidenced by the fact that only a few of the populations sampled were 90–100% infected by CMV + WMV2 + ZYMV (Tab. 1). At the life stage when most of the free-living plants in this study were sampled (*i.e.*, probably more than six weeks post-germination), the high-disease pressure conditions imposed by Fuchs et al. (2004b) would likely have produced 100% infection, based on the disease progress reported by those researchers. Instead, the proportion of infected plants observed in this study is consistent with the level of infection under the low disease pressure conditions they described.

Low disease pressure experienced by natural *C. pepo* populations is further mitigated by the low severity of the infections detected. The incidence of symptomatic

infections, compared to the incidence of infections detected by ELISA, is dramatically lower. The discrepancy between these numbers indicates that in populations studied, the physiological impact of caused by virus infection (and consequently the impact on fitness) is likely to be low. This expectation is supported by studies that have shown minor reductions in yield (presumably a good indicator of the physiological status of a plant) in *C. pepo* cultivars inoculated with a ZYMV strain that produces mild symptoms (Spence et al., 1996). Similarly, in another cucurbit, *Cucumis melo*, inoculation with a weak (symptomless) strain of ZYMV resulted in no reduction in yield compared with uninfected plants (Perring et al., 1995). Studies in tomato, with *Tomato aspermy virus*, have also shown only minor yield reductions in plants infected with a strain producing mild symptoms (Kuti and Moline, 1986). Therefore, it appears to be a general phenomenon that virus infections that do not produce symptoms also lack significant physiological impact on infected plants.

Analysis of the virus composition of the infected populations examined shows that PRSV and other potyviruses are detectable in free-living *C. pepo* populations (Tab. 1). There are no transgenes deployed against such viruses in commercial squash, and therefore, even if resistance to CMV, ZYMV and WMV2 were to benefit free living *C. pepo*, other cucurbit viruses such as PRSV and *Squash mosaic virus* (which we did not assay) would continue to affect these populations. These populations would also continue to be susceptible to other disease constraints, and abiotic stresses as well.

The opportunity for gene flow between squash and free-living populations has been demonstrated (Chung, 2006; Decker-Walters et al., 1993, 2002; Kirkpatrick and Wilson, 1988; Smith et al., 1992; Wilson, 1990). Therefore, there would be potential for introgression of transgenes into free-living *C. pepo* populations if there were an overlap with the areas where commercial transgenic crops are grown. Commercial adoption of virus resistant transgenic squash in 2001 and 2003 occurred primarily in Florida and Georgia (17 and 19%, respectively; Sankula and Blumenthal, 2004). In 2006, however, transgenic, virus-resistant squash expanded, comprising 22, 20, 5, 25, 10, 20, and 20% of the total planted acreage in Florida, Georgia, Michigan, New Jersey, North Carolina, South Carolina, and Tennessee, respectively (Sankula, 2006; Sankula and Blumenthal, 2004). While these states account for 70% of the U.S. commercial squash acreage (Sankula, 2006), they are not part of the reported geographic distribution of free-living *C. pepo* (Decker, 1988; Smith et al., 1992). Furthermore, the transgenic squash acreage in these states is relatively low (at most 25%). Therefore the opportunity for gene flow between transgenic and free-living *C. pepo* populations is low.

However, if the adoption of transgenic squash were to increase and spread to areas where gene flow to free living populations were more likely, then the information provided in this report regarding the potential interplay between virus resistance and population size in free-living *C. pepo* would strengthen existing assessments of risks associated with introgression of virus resistance transgenes. Data on the incidence and severity of virus infection do not support the hypothesis that introgressed virus resistance would release wild squash populations from restraints imposed by the presence of viral pathogens.

MATERIALS AND METHODS

Virus surveys

Surveys of free-living *C. pepo* populations were conducted in 2000, 2001, and 2002. Four hundred eighty-one plants collected from 32 populations were assayed by ELISA for virus infection in 2000 and 2001. However, for some samplings, fewer than five individuals were taken. Consequently, unless the five or fewer samples represented the entire population surveyed, we excluded those data points from further analyses.

Areas explored for the presence of populations were determined by the study of known *C. pepo* ssp. *ovifera* var. *ozarkana* and var. *texana* geographic distributions (Decker, 1988; Smith et al., 1992), and through consultation with Dr. Bruce Smith (Smithsonian Institution, Washington, DC), who subsequently took part in the 2000 survey. Sampling locations were recorded using a Magellan 315 global positioning system (GPS) receiver (Magellan Navigation, Inc., San Dimas, CA). GPS coordinates were validated with Google Earth (<http://earth.google.com/>), and are available upon request. A cluster sampling strategy was used to sample for virus incidence at each location. In cluster sampling, each sampling unit contains n individuals, and all sampled individuals are assayed. There is no requirement that n is identical in each sampling unit (Madden and Hughes, 1999). This strategy is especially suited for populations of free-living *C. pepo*, which are extremely variable in size.

Population counts and samples for ELISA were collected from late July through September of each year (with the exception of two populations that were surveyed and sampled in late June, 2000). This timeframe was based on previous records and consultations with local experts, which led to the conclusion that the typical growing season was May through November. Therefore, the surveys were conducted during the middle months of the growing season. Only individuals that were clearly established were counted and sampled for ELISA.

The furthest reported incidence of pollen-mediated gene flow between free-living *C. pepo* plants is 1300 meters (Kirkpatrick and Wilson, 1988). Thus, when sampling spatially distinct plant groups in a survey area in our study, groups of individuals within 2 km of each other were arbitrarily regarded as belonging to the same population since there was potential for gene exchange *via* pollen. We recognize that this classification does not take into consideration gene flow *via* seeds.

In our study, population size estimates were accurate to within 5, 10, and 50 plants for populations possessing up to 50, 100, and 300 plants, respectively. When plant numbers were sufficiently low and individuals could be sufficiently separated to allow accurate counts, all individuals were counted. Nevertheless, the precision of populations > 300 plants could not be rigorously established.

Plants were scored visually as being either symptomatic or asymptomatic, without quantifying the severity of symptoms. Because there is no reliable method of distinguishing between viruses based on symptoms, especially when there are mixed infections, we also did not classify infection by different viruses based on symptoms.

ELISA assays

Leaves for ELISA assays were sampled from randomly chosen plants, without regard to symptoms. Because of high variability in the number of plants in each population, and varying degrees of separation between plants in a population, it was not possible to predetermine either a specific sampling pattern, or a threshold representative number. However, in most populations of up to 25 individuals, each individual was sampled. Voucher specimens of some populations were collected, pressed, and archived (Herbarium of the Department of Biological Sciences, Western Michigan University, Kalamazoo, Michigan, USA).

ELISA assays for CMV, general potyvirus, ZYMV, WMV2, and PRSV were conducted using commercial kits, using protocols supplied by the manufacturer (Agdia, Elkhart, IN). Samples were initially assayed with the CMV kit, and then with the general potyvirus assay. To determine the spectrum of virus infection, samples that scored positive in the general potyvirus screen were subsequently assayed for the presence of ZYMV, WMV2, and PRSV. Thresholds for distinguishing positive from negative reactions were those established by Frey et al. (1998). Data analyses were performed using GraphPad Prism version 5.0a for Mac OS X, GraphPad Software, San Diego, California, USA, www.graphpad.com.

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