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COMPETITIVE ADVANTAGE OF AN INTROGRESSED TRANSGENE IN THE PRESENCE OF ZUCCHINI YELLOW MOSAIC VIRUS ON THE MALE FUNCTION OF *CUCURBITA PEPO SSP. TEXANA*

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ABSTRACT

Transgenes are commonly used in the agricultural industry in order to create disease resistant plants. In turn, this reduces the need for pesticides and increases crop yield. However, gene flow between cultivated and wild crops is a well-documented phenomenon, resulting in the escape of the transgene into wild plant populations. The escape of the transgene can potentially have adverse effects on the surrounding environment and nontarget species. We examined the competitive fitness of an introgressed virus resistant transgene in our pathosystem of *Cucurbita pepo ssp texana* (wild gourd) while an epidemic of Zucchini Yellow Mosaic Virus (ZYMV) spread. In a field study utilizing wild gourd that was either introgressed with a virus resistant transgene (VRT) or non-introgressed (virus susceptible), we examined the frequency of seeds sired by the transgene as a viral epidemic of ZYMV circulated in the system. Four identical fields containing both transgenic and nontransgenic wild gourds were established. An epidemic of ZYMV was initiated early in the season within two fields, and spread rapidly among susceptible plants. A viral epidemic was not started in the remaining two fields. Within the virus fields, we found transgenic plants to have greater flower production as compared to virus-infected plants. Using DAS-ELISA, we calculated the transgenic frequency of seeds sired by the transgene within virus and healthy fields, and saw that virus-infected fields had a higher frequency suggesting a selective advantage of the transgene. This study demonstrates the competitive advantage of the VRT in the *Cucurbita* pathosystem in the presence of ZYMV. In a subsequent study, we examined the competitive ability of pollen produced by virus-infected plants in order to determine the effect of disease on male fitness. We pitted virus-infected pollen against healthy pollen, and determined that viral infection significantly reduces pollen competitive ability, suggesting that viral infection adversely impacts male reproductive fitness. The fact that pollen from virus infected plants is less competitive than pollen from uninfected (healthy) plants may partially explain why the transgenic frequency of seeds sired in virus fields is higher than the frequency in healthy fields.

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Chapter 1

Introduction

Background

Over the past twenty years, transgenes have been increasingly utilized in the agricultural industry in order to increase crop yield and reduce financial cost of disease in commercial crops. Via introgression into the genome of a crop, transgenes effectively reduce the plant's susceptibility to disease because they confer resistance to viruses. For instance, plums (genus: *Prunus*) have a transgene conferring resistance against plum poxvirus by insertion of a coat protein gene from the virus and are now immune to potential epidemics (Phillips 2008). Disease resistant transgenes possess an advantage over traditional breeding because transgenes can be introgressed into a plant's genome without altering its phenotypic characteristics. These genes are universal to confer resistance in many plants facing the same diseases (Fuchs and Gonsalves 2007). Therefore, transgenes are not necessarily specific to the plant they are introgressed into but are specific to the diseases they provide resistance against.

Typically, transgenes are effective in creating herbicide-tolerant and virus-resistant crops. The financial benefits of transgenic plants particularly for farmers are numerous because they increase crop yield and reduce pesticide use. However, through gene exchange between cultivated crops and wild plants, transgenes can escape into wild plant populations and have potentially problematic consequences (Kirkpatrick and Wilson 1988; Wilson et al. 1994; Decker-Walters et al. 2002). Transgene escape occurs when pollen from a genetically modified

organism (GMO) pollinates a wild plant creating wild-transgenic hybrid plants, which then pass the transgene to their offspring either via the seeds they produced or via the pollen they donate to neighboring plants of the same species.

Goodman and Newell of Calgene (Davis, California) were among the first in 1985 to publish growing concerns associated with the sexual transfer of genes to weedy plant species. They expressed the issue of wild-transgenic hybrids becoming uncontainable weeds as the greatest risk associated with transgenic crops (Goodman and Newell 1985). These wild genetically modified organisms (GMOs) can breed to create new plants with resistance to herbicides and other chemicals commonly used to control potentially invasive plant species.

With the increasing frequency of transgene escape, ecologists now fear that transgenes will alter genetic diversity within plant populations (Gilbert 2010). Non-target members of the community, including herbivores, pollinators, predators, and nearby plants could also be adversely affected by an escaped transgene. For example, natural pests could become inadvertently resistant to new strategies for control or beneficial insects could be accidentally poisoned altering ecosystem dynamics (Ellstrand 2001). Further assessment of ecological risks caused by transgenic plants must be explored in order to protect biodiversity.

Virus resistant transgenes (VRT) have been used agriculturally for squash plants over the past twenty years to protect against watermelon mosaic virus (WMV), cucumber mosaic virus (CMV), and zucchini yellow mosaic virus (ZYMV). ZW-20 and CZW-3 are virus resistant transgenic lines of *Cucurbita pepo* that have been deregulated by the USDA and are commonly used in agriculture today. ZW-20 plants are resistant to ZYMV and WMV because they express the coat proteins (CP) of these two viruses. CZW-3 line plants express coat proteins of ZYMV, WMV, and CMV, and are resistant to all three viruses. Using gene constructs originated from

multiple viruses as seen in the ZW-20 and CZW-3 lines is called pyramiding and has been effective in conferring virus resistance to *Cucurbita pepo* (Ellstrand 2001).

Transgenes conferring resistance to the above three viruses encode a single stranded RNA coat protein (CP) construct. This VRT is passed to offspring by hemizygous inheritance meaning only 50% of pollen would be expected to carry the transgene. Neomycin phosphotransferase II (*NPTII*) marks the gene and flanks the coat protein. Because *NPTII* is the marker gene, its corresponding protein can be used to detect for the presence of the VRT.

The Stephenson Laboratory has been researching the fitness impacts of the VRT in the *Cucurbita pepo ssp. texana* pathosystem. The purpose of this thesis is (1) to examine the changing frequency of transgenic seeds in the next generation sired under conditions with and without ZYMV and (2) to determine if pollen competitive ability is affected by virus infection. These results are part of a larger experiment involving factors associated with disease spread, transgene inheritance, plant reproductive ability during viral outbreaks, and pollen performance.

Impact of the transgene in the presence of a virus was studied by tracking the frequency of seeds sired by the transgene over time in our experimental fields. Chapter 2 discusses the different frequency of transgenic sired seeds in virus-infected fields as compared to no virus (or low virus) fields. In Chapter 3, we examine whether the increase in the number of seeds sired by transgenic plants in fields with ZYMV are due to differences in pollen production between susceptible and transgenic plants or whether the difference is due to pollen competitive ability. Therefore, we performed a pollen competition experiment.

The Study System

Both experiments utilized the Cucurbita pathosystem. The wild gourd, *Cucurbita pepo ssp. texana* is an annual monoecious vine with indeterminate growth and reproduction (Arriaga et al. 2006). The plants undergo some vegetative growth before they produce large yellow flowers (can be either male or female). Flowers bloom for only one morning and are pollinated by bees, especially squash bees.

Cucumber beetles are the primary herbivore in this system, as they are adapted to feed on the bitter cucurbitacins that are produced by the plant. Beetles feed and cause plant damage, which can reduce yield and reproductive output of the wild gourd. Beetles are also the vectors of *Erwinia tracheiphila*, the causative agent of bacterial wilt disease. They increase exposure to the pathogen because *Erwinia* lives in the gut of the beetle. Fecal pellets containing *Erwinia* from the beetles land on wounds at sites of feeding damage, transmitting the disease. The bacteria proliferate once inside the gourd and secrete a mucilaginous matrix in the xylem, which cuts off water supply, resulting in its characteristic wilting symptoms. Wilt disease is fatal after several weeks of infection (Sasu 2010).

Aphids are also a component of the *Cucurbita* pathosystem. They vector the virus of interest in this study, Zucchini Yellow Mosaic Virus (ZYMV). ZYMV is a single stranded RNA potyvirus and is transmitted in a non-persistent manner when the aphid's stylet probes a plant. The virus causes symptoms of blisters, necrotic lesions, branches with short internodes, and small highly serrate leaves (Harth et al. 2014).

Previous field studies using this system have shown that viral diseases such as ZYMV do not impact survivorship of the plants, but do decrease flower number. Furthermore, virus infected plants have decreased exposure to wilt disease because beetles are more attracted to

healthy larger plants (Stephenson et al. 2004; Hayes et al. 2004; Avila- Sakar and Stephenson 2006; Ferrari et al. 2006; Du et al. 2008).

Chapter 2

Changes in Transgene Frequency from One Generation to the Next

Introduction

The Stephenson Laboratory has been studying the impact of an introgressed VRT in wild *Cucurbita pepo. ssp. texana* (wild gourd) since 2002. Previous studies involving the wild gourd, its primary herbivores (cucumber beetles and aphids), and diseases vectored by the herbivores (wilt disease and ZYMV) provided the foundation for my research.

Under viral conditions, reproductive output of transgenic and non-transgenic plants is affected. In a 2010 study, it was found that in the absence of Zucchini yellow mosaic virus (ZYMV), reproductive output of transgenic plants was not significantly different from non-transgenic plants output (Sasu et al. 2010). As expected, non-transgenic susceptible plants had decreased male and female function with a viral outbreak compared to susceptible (non-transgenic) plants. A previous study by Fuchs et al. (2004) compared seed production between wild squash and commercial transgenic squash in the presence of low and high disease. The study found that transgenic plants produced significantly more seeds under viral conditions than squash without the transgene. This suggests that the transgene confers a selective advantage within settings where virus is present.

The study presented here examines the fitness impacts of a VRT on wild gourd in the presence of ZYMV. ZYMV negatively impacts susceptible plants by reducing flower counts, which ultimately reduces the ability of virus-infected plants to donate pollen (Sasu 2010). In

previous experiments, we noted that there was a higher percentage of transgenic plants in the next generation than what was originally expected. This could suggest a selective advantage to the transgene; therefore, we examined the number of seeds sired by transgenic plants on susceptible plants in our experimental fields in which ZYMV was introduced either early or late into the fields. We hypothesize that the proportion of seeds sired by the transgene would be greater in the early virus-infected fields compared to the fields infected later in the season. Furthermore, seeds sired by the transgenic introgressives in late virus fields would be constant during the field season while seeds sired by the transgene would increase as virus spreads in the inoculated fields.

Methods

Field Experimental Design

Using large-scale field experiments, we were able to calculate the changing frequency of seeds sired by the transgene in virus-infected fields. We also obtained a control frequency by calculating the transgenic frequency in late virus fields (hereafter termed the healthy fields).

Seeds were germinated in a greenhouse in early May 2013. Once the third leaf was produced, a small section of the leaf was removed and analyzed using DAS-ELISA in order to test for the virus resistant transgene (VRT). During earlier research within the Stephenson laboratory, the VRT transgene was backcrossed into our study system. Using the Liberator III, a commercially available squash cultivar with the transgenic coat protein-based resistance to WMV, ZYMV, and CMV, the transgene was backcrossed into 20 families of *Cucurbita texana*. The Liberator III contains the *NPTII* marker gene and VRT; the NPTII protein can be detected

using DAS-ELISA, which then indicates the presence of the VRT. The detection of the NPTII protein allows the transgene to be tracked as it is transmitted from generation to generation.

Four, one acre fields were utilized—two fields were adjacent and were more than a kilometer away from the other adjacent two fields. Into each field, we transplanted 18 wild gourd plants, 9 BC8 (eighth generation back crossed from the Liberator III squash) transgenic plants, and 9 BC8 non-transgenic plants from each of five families. There were 180 plants total per field and 25% of them were transgenic.

We hand inoculated 60 of the susceptible plants (40 wild gourd plants and 20 ntBC8) with ZYMV prior to field transplanting using the carborundum rub method. Briefly, the leaves were wounded with carborundum and ZYMV suspended in a buffer was rubbed over the wounds to induce disease. These plants were transplanted into only two of the adjacent fields (virus infected fields/inoculated fields). Virus was not introduced into the remaining two fields (healthy fields).

Virus symptoms were recorded over the course of the field season weekly. Wilt disease symptoms and beetle damage was also tracked. Reproductive output was evaluated by counting male and female flowers every week and fruits at the end of the field season.

In order to determine if the probability that pollen from a transgenic plant will sire a seed on a susceptible plant changes as ZYMV infection increases in the fields, ten female flowers on susceptible plants in each field were tagged on three dates between July 1 and August 15 (early, mid, and late field season). When the field season was over, these fruits were collected. The seeds were then removed and stored. Seeds from healthy fields were separated from inoculated field seeds. A random sample of these seeds was germinated from each of the three dates. Leaf tissue was harvested from the seeds. Then, using DAS-ELISA, the seeds were scored for the

presence of the transgene (NPTII) using a commercially available kit (Agdia Inc. Elkhard, Indiana).

Statistical Analysis

We calculated mean male and female flower production per plant for transgenic and susceptible plants in both virus and healthy fields. Analysis of variance (ANOVA) was performed to test for significance between flowers produced by transgenic and susceptible plants in healthy and virus-infected fields. Differences in transgene frequency (between the two field types and overtime within each field type) were tested using Chi-Square Analysis.

Results

On average, transgenic and susceptible plants produced very similar numbers of male and female flowers in the healthy fields (Figure 1 and Figure 2). In the virus-infected fields, transgenic plants produced more male and female flowers on average as compared to the flower production of susceptible plants (Figure 3 and Figure 4).

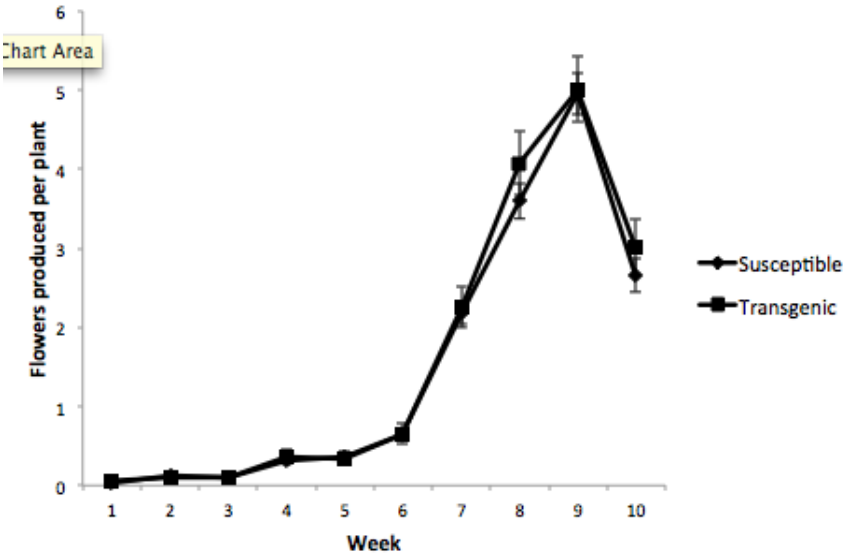

Figure 1: Mean Male Flower Count Per Plant in Healthy Fields

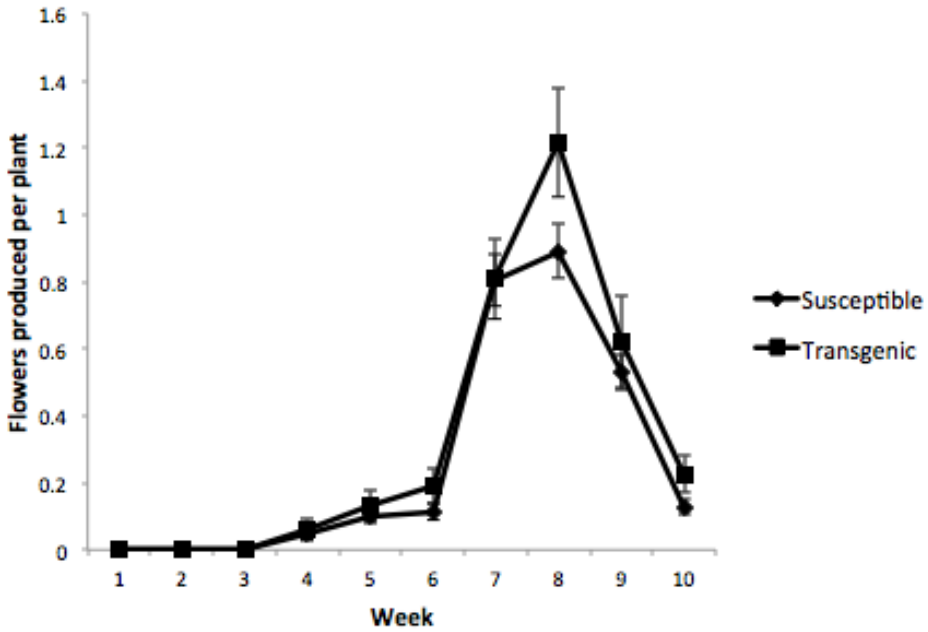

Figure 2: Mean Female Flower Count Per Plant in Healthy Fields

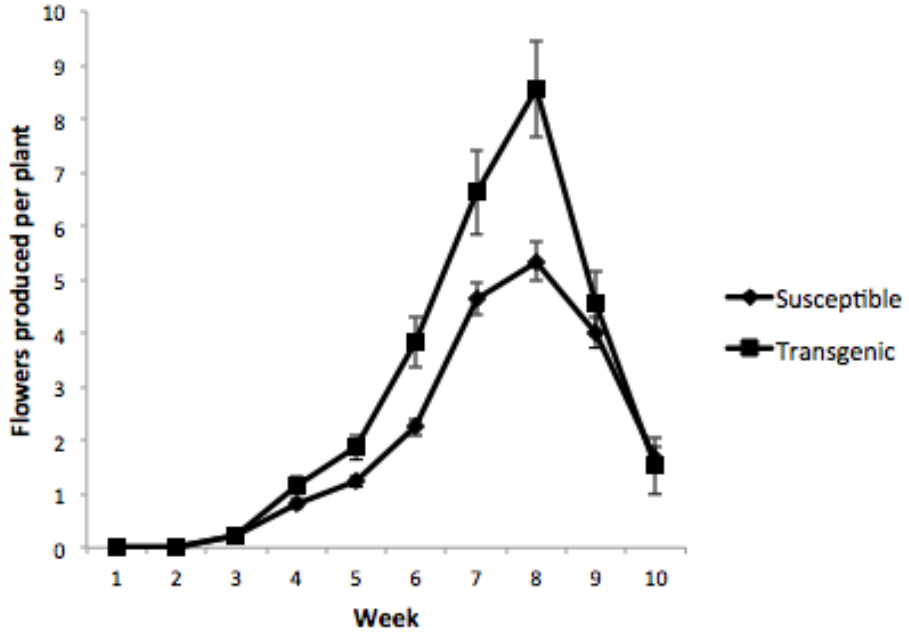

Figure 3: Mean Male Flower Count Per Plant in Virus Fields

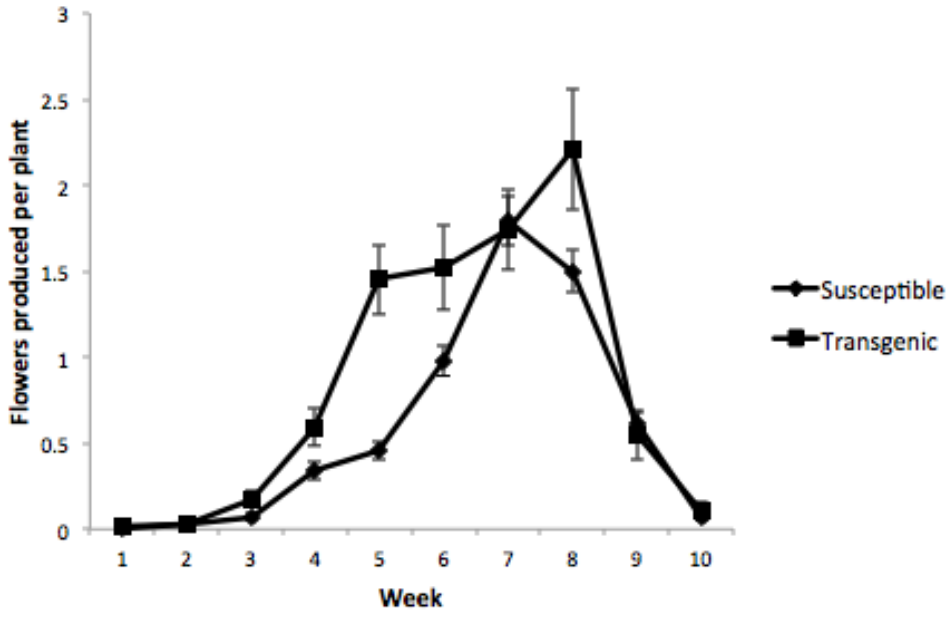

Figure 4: Mean Female Flower Count Per Plant in Virus Fields

There was no significant difference between transgenic (19.2 ± SE) and susceptible (17.5 ± SE) flowers per plant for total flower production in the healthy fields ($F = 0.99$, $df = 1$, $P > 0.05$). However, there was a significant difference in the virus-infected fields for total flower production between transgenic (36.7 ± SE) and susceptible (26.1 ± SE) flowers per plant ($F = 12.05$, $df = 1$, $P < 0.05$).

Virus incidence in the healthy fields was not seen until very late in the field season when virus eventually made its way into the field (Figure 5). In the inoculated fields, an epidemic of ZYMV started very early in the field season and continued until late in the field season (Figure 6). Transgenic plants in both fields did not show symptoms of ZYMV infection (Figure 5 and Figure 6). Peak flower production coincided with peak ZYMV spread in the inoculated fields (Figure 6).

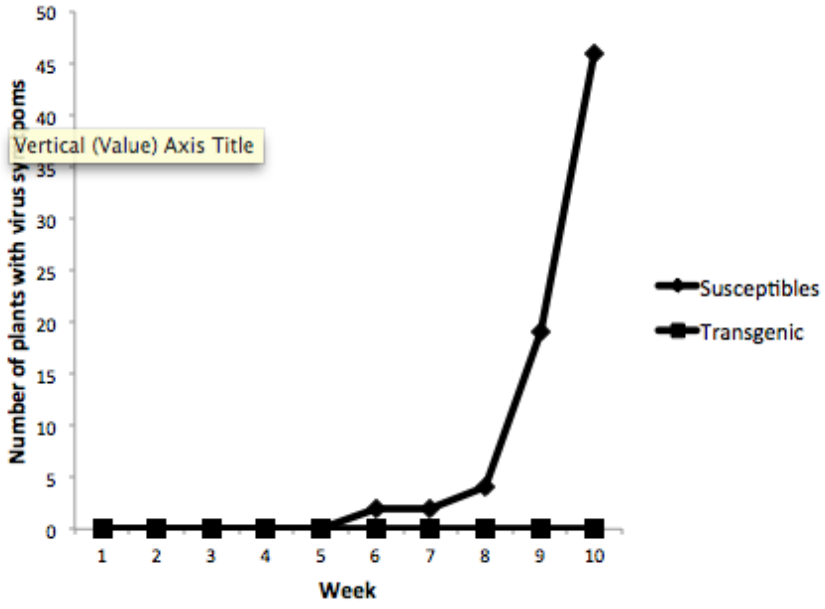

Figure 5: Virus Incidence - Healthy Fields

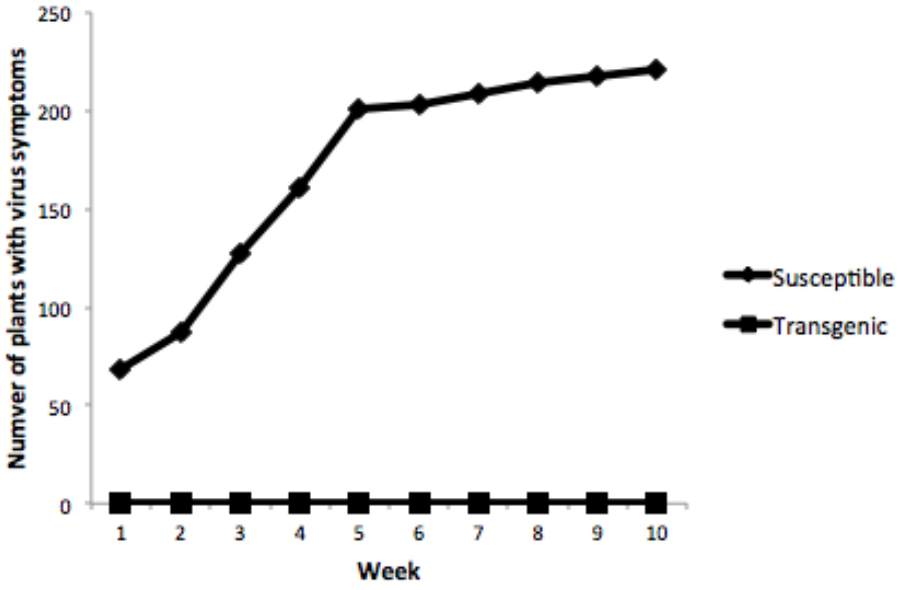

Figure 6: Virus Incidence - Virus Fields

We scored 760 seedlings for the presence of the transgene from the virus and healthy fields—380 seedlings each. We found that the proportion of seeds sired by the transgene was not independent of the field type. More seeds were sired by the transgene in the virus fields (32%) than in the healthy fields (21%) (Table 1). However, we also found that the proportion of seeds sired by pollen with the transgene did not increase in the virus-infected fields as the number of plants with virus symptoms increased (early, to mid, to late in the field season). The proportion of the seeds sired by the transgene in the virus-infected fields decreased significantly over the growing season (Table 2). The proportion of seeds sired by the transgene in the healthy fields was expected to be constant over the field season; however, the results were more sporadic than anticipated (Table 3).

Table 1: Seedlings sired by the transgene in virus and healthy fields

	Transgenic (%)	Non-transgenic (%)
Virus Fields	123 (32%)	257 (68%)
Healthy Fields	80 (21%)	300 (79%)
Chi-square (X^2) = 12.428, df = 1, P < 0.001		

Table 2: Seedlings sired by the transgene in virus-infected fields over time (early to mid to late in the fields season)

	Transgenic (%)	Non-trangenic (%)
Early	42 (44%)	53 (56%)
Mid	30 (32%)	65 (68%)
Late	51 (27%)	139 (73%)
Chi-square (X^2) = 8.763, df = 2, P < 0.05		

Table 3: Seedlings sired by the transgene in healthy fields over time (early to mid to late in the field season)

	Transgenic (%)	Non-transgenic (%)
Early	30 (32%)	65 (68%)
Mid	21 (6%)	169 (94%)
Late	29 (31%)	66 (69%)
Chi-square (X^2) = 24.32, df = 2, P < 0.001		

Discussion

The experimental design of this study allowed us to examine the frequency of the transgene on seeds sired under viral conditions compared to the frequency without viral conditions. Because ZYMV has adverse effects on male flower production, this creates an opportunity for pollen from healthy plants to sire more seeds (Sasu 2010).

We found that susceptible and transgenic plants in the healthy fields produce a very similar number of flowers throughout the field season; however, in the inoculated fields, transgenic plants produce significantly more flowers than susceptible plants. This supports previous research showing that the virus hinders flower production, but also suggests a competitive advantage to having the transgene. Transgenic plants produce more flowers than virus-infected plants, which could correlate to these plants having more pollen as well.

Due to the virus's effects on flower production, we predicted that the transgene in virus-infected fields would sire more seeds than it would in healthy fields—this hypothesis was confirmed with experimentation. Over the course of the field season, the transgene in virus-infected fields consistently sired more seeds when compared to the healthy fields. This suggests that plants with the transgene have a fitness benefit in the presence of ZYMV infection, and that their pollen may be more competitive than pollen from virus-infected plants. This benefit could be due to one (or both) of two factors: transgenic plants make more pollen and/or the pollen from transgenic plants is competitively superior after it lands on a stigma.

Twenty five percent of the plants in each field were transgenic and since the transgene is passed on through hemizygous inheritance, we expected ~12.5% of seeds produced from susceptible plants would be transgenic given that all plants generate the same amount of pollen and all pollen has uniform ability to fertilize the ovules after pollinating the stigmas. However,

in all of our fields (healthy and virus-infected), the transgenic frequency was much higher than the expected 12.5% (virus-infected: 32%, healthy: 21%). The difference in the number of seeds sired by the transgene between healthy and virus fields was significant; therefore, the proportion of seeds sired by the transgene was not independent of the field type. The variance between the fields provides further evidence of the transgene's advantage under viral conditions. The increased frequency could suggest a selective advantage to the transgene, resulting in conferred virus resistance to the next generation of plants.

We were particularly interested in the greater than expected proportion of transgenic seeds in our healthy fields (21% vs. 12.5%). This could suggest a larger fitness advantage than originally predicted among transgenic plants in terms of pollen production or pollen competitive ability. The higher transgenic frequency in our healthy fields also provides evidence for selection of the transgene.

We also thought that with virus spread in the inoculated fields, the transgene frequency would also increase; however, the frequency decreased with each measurement from early to mid to late in the field season (44% early, 32% mid, 27% late). In mid-July, naturally occurring diseases, such as wilt disease and powdery mildew, present themselves into our system. Previous studies have shown that transgenic wild gourd suffers greater exposure to bacterial wilt disease under viral conditions because the beetles that vector wilt disease prefer larger healthier plants (Sasu et al. 2009). Recent research proposes that plants with ZYMV are more resilient to powdery mildew than healthy plants. Furthermore, these studies suggest powdery mildew adversely affects male flower production (Harth et al. 2014). Because of the effects of wilt disease and powdery mildew on transgenic plants, this could potentially explain why transgenic frequency decreases over time in our results.

It would have been expected that the transgenic frequency over the field season in our healthy fields would have stayed relatively constant because there was no virus in these fields to impact flower production. However, the changing transgenic frequency in the healthy fields was inconsistent and varied over the field season [from early (32%) to mid (6%) to late (31%)]. The frequencies between the early and late calculations are almost identical, but the mid calculation is much lower. Because our early and late calculations are so similar, we believe the mid season calculation may be due to unusual field or weather conditions or possibly experimental error. Repeating this part of the experiment would confirm or refute the results presented here.

Our results confirmed that transgenic frequency was much higher in virus-infected fields as compared to healthy fields; however, we were skeptical if the results were completely due to virus impacts on male flower production or if the virus impacted pollen performance as well. Therefore, we continued our study by examining pollen performance by comparing the performance of pollen from virus-infected and non-infected plants.

Chapter 3

Pollen Competitive Ability

Introduction

Viral diseases negatively impact the fitness of plants by decreasing leaf area, disrupting cellular and transport processes, and inducing various biochemical defense systems. These effects in turn can be costly to the plant in terms of energy and nutrient usage (Burdon 1987a; Conner et al. 1996; Ryals et al. 1996). Pathogens also negatively impact reproductive output of plants. Many studies analyze the effects of disease on female flower function in Cucurbits; however, there are far fewer looking at the impacts of disease on male flower function. Under pathogenic conditions, wild gourds have limited growth, survival, and reproductive output (Harth et al. unpublished data). Because Cucurbits are hermaphroditic, their fitness relies 50% on female function and 50% on male function. Therefore, pathogenic impacts on male function are equally important as impacts on female function. Previous studies have shown reduced male flower count under viral conditions (Sasu 2010); however, to our knowledge, there are no prior studies examining fitness impacts of virus on pollen performance.

Because male flower production is reduced in the presence of ZYMV infection, this results in less net pollen from virus-infected plants circulating in the fields. However, from our previous studies looking at transgenic frequency, it is unclear if changing transgene frequency was solely due to virus effects on male flower count or if possible virus effects on pollen performance contributed as well. Therefore, we examined the performance of pollen from non-

infected and virus infected plants. Based on our previous results, we wanted to find what the specific impact of ZYMV was on male flower performance and if it affected competitive ability of pollen. If the virus affects pollen performance, we would expect healthy pollen to sire more seeds than virus-infected pollen. We would also expect that in a competition between two types of healthy pollen, both types of healthy pollen would sire an approximately equal amount of seeds.

Methods

To compare the competitive ability of pollen collected from healthy plants versus virus-infected plants, we grew 25 Golden Yellow Zucchini plants in a small field at Rock Springs. Plants were sprayed with an aphid specific pesticide to prevent virus infections. In July, when peak virus spread occurs in our experimental fields, we collected male flowers from virus infected and non-infected plants and also collected male flowers from the zucchini plants. We created two 50:50 mixtures of pollen: virus infected wild gourds + healthy zucchini and healthy wild gourd + healthy zucchini. We did not use transgenic pollen from transgenic flowers in the competition because we wanted to show that the virus negatively impacted pollen and that is was not the transgene giving a selective advantage to pollen; therefore, healthy pollen from Golden Yellow Zucchini was used. We hand pollinated 18 female flowers on the Golden Yellow Zucchini plants with these two pollen mixtures. The resulting fruits were collected from 17 of the 18 pollinations—one fruit did not reach maturity before the first frost. Seeds were removed from the collected fruits.

During the following field season in early June 2014, these seeds were germinated in a field at Rock Springs in order to determine the proportion of seeds sired by pollen from virus-infected and healthy plants. 150 seeds from each of the 17 fruits were planted in rows spaced 0.5 meters apart at 0.4 meter intervals.

The plants were allowed to grow to maturity and were then scored by ovary shape. Golden Yellow Zucchini produces club shaped ovaries and *Cucurbita pepo ssp. texana* produces round shaped ovaries; therefore, we were able to visually determine which type of plant sired the seeds by examining ovary shape—club shaped or round. Plants sired by wild gourd pollen also have tendrils and a vine like pattern of growth as compared to plants sired by zucchini, which do not have these characteristics and are more bush like. Using these visual characteristics, we were able to determine if there was a competitive difference between pollen from healthy and virus infected gourd plants. Differences between plant types were analyzed using chi-square.

Results

Ovary shape was identified for 1,040 plants out of the 2,550 seeds that were planted. Prior to germination, birds or animals ate some seeds. Other seeds failed to germinate and a few plants did not survive to maturity, making scoring of these plants impossible. A chi-square test of independence showed the number of seeds sired by wild gourd pollen depended upon whether the pollen came from virus infected or virus free plants. We found that there was a greater proportion of wild gourd seeds sired in the healthy mixture than there were sired in the virus mixture (Table 4).

Table 4: Number of zucchini and wild gourd seeds sired by each pollen donor

Pollen Donor	Zucchini (%)	Texana (%)
Healthy + Zucchini	325 (59.1%)	225 (40.9%)
Virus + Zucchini	327 (66.7%)	163 (33.2%)
Total	652 (62.7%)	388 (37.3%)
Chi-square (X^2) = 6.492, df = 1, P < 0.011		

Discussion

After analysis of our pollen competition results, we concluded that the virus does impact pollen performance. In both types of pollen competition, the zucchini pollen sired significantly more seeds than the texana pollen. However, the texana pollen from the healthy plants sired a significantly higher proportion of the seeds in competition with zucchini pollen than did the texana pollen from the virus-infected plants (Table 4).

We tested how competitive texana pollen from non-infected plants is compared to pollen from infected plants, and saw that the pollen from healthy plants is able to better compete with zucchini pollen. However, when comparing the performance of pollen from healthy texana with pollen from virus-infected texana, the pollen from virus-infected texana sired significantly less seeds. Therefore, we can assume the major decline in seeds sired by pollen from virus-infected plants compared to zucchini pollen is the result of adverse effects on pollen performance from the virus. Because ZYMV causes interruption of nutrient transport, disruption of cellular processes, and induction of costly biochemical defenses, it was likely that virus-infected pollen

received fewer or lower quality nutrients and energy products during development (Burdon 1987a; Conner et al. 1996, Ryals et al. 1996).

This study shows that disease does not just affect the flower count and size of plants, but it also has a much more subtle effect on the reproductive ability of the plant. Virus lowers the plants ability to pollinate by lowering the performance of pollen. As a result, the pollen from healthy plants provides a selective benefit to the plant. These results could contribute to the increased transgenic frequency seen in the virus-infected fields from our first experiment. Adverse virus effects on male flower count may not be solely responsible for the increased transgenic frequency—virus effects on pollen performance could play a role as well.

In the future, it would be interesting to conduct a study pitting healthy pollen against transgenic pollen in order to examine if a selective benefit exists among transgenic pollen. This research could provide further evidence supporting a competitive advantage for transgenic plants.

Appendix A

Field Diagrams

	A	B	C	D	E	F	G	H	I	J	K	L
1	I2-X	I2 BC T	J5-X	I2 BC	OZ-X	I2 BC T	J3-X	I2 BC	D2-X	I2 BC T	I2-X	I2 BC
2	OZ BC T	J5-X	OZ BC	OZ-X	OZ BC T	J3-X	OZ BC	D2-X	OZ BC T	I2-X	OZ BC	J5-X
3	OZ-X	D2 BC	J3-X	D2 BC T	D2-X	D2 BC	D2-X	D2 BC T	OZ-X	D2 BC	J3-X	D2 BC T
4	J3 BC	J3-X	J3 BC T	OZ-X	J3 BC	J5-X	J3 BC T	I2X	J3 BC	J5-X	J3 BC T	I2X
5	D2-X	J5 BC T	D2-X	J5 BC	J3-X	J5 BC T	OZ-X	J5 BC	J5-X	J5 BC T	I2-X	J5 BC
6	I2 BC T	J3-X	I2 BC	D2-X	I2 BC T	D2-X	I2 BC	I2-X	I2 BC T	J5-X	I2 BC	OZ-X
7	I2-X	OZ BC	J5-X	OZ BC T	OZ-X	OZ BC	J3-X	OZ BC T	I2-X	OZ BC	J5-X	OZ BC T
8	D2 BC	D2-X	D2 BC T	OZ-X	D2 BC	J3-X	D2 BC T	J3-X	D2 BC	OZ-X	D2 BC T	D2-X
9	J5-X	J3 BC T	I2X	J3 BC	J5-X	J3 BC T	I2X	J3 BC	J3-X	J3 BC T	OZ-X	J3 BC
10	J5 BC T	OZ-X	J5 BC	J5-X	J5 BC T	I2-X	J5 BC	D2-X	J5 BC T	D2-X	J5 BC	J3-X
11	D2-X	I2 BC	I2-X	I2 BC T	J5-X	I2 BC	OZ-X	I2 BC T	J3-X	I2 BC	D2-X	I2 BC T
12	OZ BC	J3-X	OZ BC T	I2-X	OZ BC	J5-X	OZ BC T	I2-X	OZ BC	J5-X	OZ BC T	OZ-X
13	J3-X	D2 BC T	J3-X	D2 BC	OZ-X	D2 BC T	D2-X	D2 BC	D2-X	D2 BC T	OZ-X	D2 BC
14	J3 BC T	I2X	J3 BC	J3-X	J3 BC T	OZ-X	J3 BC	J5-X	J3 BC T	I2X	J3 BC	J5-X
15	I2-X	J5 BC	D2-X	J5 BC T	D2-X	J5 BC	J3-X	J5 BC T	OZ-X	J5 BC	J5-X	J5 BC T

Virus Infected Fields

	A	B	C	D	E	F	G	H	I	J	K	L
1	I2-X	I2 BC T	J5-X	I2 BC	OZ-X	I2 BC T	J3-X	I2 BC	D2-X	I2 BC T	I2-X	I2 BC
2	OZ BC T	J5-X	OZ BC	OZ-X	OZ BC T	J3-X	OZ BC	D2-X	OZ BC T	I2-X	OZ BC	J5-X
3	OZ-X	D2 BC	J3-X	D2 BC T	D2-X	D2 BC	D2-X	D2 BC T	OZ-X	D2 BC	J3-X	D2 BC T
4	J3 BC	J3-X	J3 BC T	OZ-X	J3 BC	J5-X	J3 BC T	I2X	J3 BC	J5-X	J3 BC T	I2X
5	D2-X	J5 BC T	D2-X	J5 BC	J3-X	J5 BC T	OZ-X	J5 BC	J5-X	J5 BC T	I2-X	J5 BC
6	I2 BC T	J3-X	I2 BC	D2-X	I2 BC T	D2-X	I2 BC	I2-X	I2 BC T	J5-X	I2 BC	OZ-X
7	I2-X	OZ BC	J5-X	OZ BC T	OZ-X	OZ BC	J3-X	OZ BC T	I2-X	OZ BC	J5-X	OZ BC T
8	D2 BC	D2-X	D2 BC T	OZ-X	D2 BC	J3-X	D2 BC T	J3-X	D2 BC	OZ-X	D2 BC T	D2-X
9	J5-X	J3 BC T	I2X	J3 BC	J5-X	J3 BC T	I2X	J3 BC	J3-X	J3 BC T	OZ-X	J3 BC
10	J5 BC T	OZ-X	J5 BC	J5-X	J5 BC T	I2-X	J5 BC	D2-X	J5 BC T	D2-X	J5 BC	J3-X
11	D2-X	I2 BC	I2-X	I2 BC T	J5-X	I2 BC	OZ-X	I2 BC T	J3-X	I2 BC	D2-X	I2 BC T
12	OZ BC	J3-X	OZ BC T	I2-X	OZ BC	J5-X	OZ BC T	I2-X	OZ BC	J5-X	OZ BC T	OZ-X
13	J3-X	D2 BC T	J3-X	D2 BC	OZ-X	D2 BC T	D2-X	D2 BC	D2-X	D2 BC T	OZ-X	D2 BC
14	J3 BC T	I2X	J3 BC	J3-X	J3 BC T	OZ-X	J3 BC	J5-X	J3 BC		J3 BC	J5-X
15	I2-X	J5 BC	D2-X	J5 BC T	D2-X	J5 BC	J3-X	J5 BC T	OZ-X	J5 BC	J5-X	J5 BC T

Plot Area

Healthy Fields

Appendix B

DAS-ELISA Protocol

This protocol is used in the Stephenson laboratory to perform a Double Antibody Sandwich- ELISA, and is adapted from the Agdia protocol. This procedure allows for the detection of the VRT within the *Cucurbita pepo* system. The ELISA is used for quick and qualitative identification of a large sample set. The test is used before the field season by sampling germinated stock seedlings to identify the presence of the transgene within the specific plants so that a predetermined dispersal of transgenic plants can be presence in the field. Fruits are collected and germinated at the end of the field season, then tested for the transgene using the ELISA. By comparing the test results to the initial transgenic population the fitness advantage of the transgene can be calculated.

Necessary materials:

- Agdia NPTII Kit:

  Cold Kit
  1) ELISA 96 Well Plate
  2) MRS-2 Component
  3) Enzyme Conjugate A
  4) Enzyme Conjugate B
  5) TMB

  Room Temperature Kit
  1) PBST Packet
  2) PEB1 Extraction Buffer
  3) Sulfuric Acid

- Glassware:

  1) 2 L Erlenmeyer for PBST
  2) 250 ml Erlenmeyer PEB1 Extraction Buffer
  3) 2-50 ml Erlenmeyer For TMB and Enzyme
  4) 250mL Plastic Wash Bottle for PBST washing
  5) 10mL Graduated Cylinder for measuring enzyme and TMB
  6) 100mL Graduated Cylinder for measuring PEB1

  *All glassware must be autoclaved before use.

- Pipette Tools:

  1) 200 microliter pipettes

2) 1000 microliter pipette
3) Autoclaved yellow pipette tips (200 microliters)
4) Autoclaved blue pipette tips (1000 microliters)

- Grinding Materials:

1) Grinding Bags
2) Grinding Tool
3) Scissors

- Miscellaneous:

1) Lab Gloves
2) Ethanol
3) Timer
4) Camera
5) Deionized Water (dH_20)
6) Paper Towels
7) Incubation Container
8) Markers

- Solutions:

1) Inert Buffer
    a. 1 packet PBST in 1L of distilled water.
    b. Phosphate buffer solution with tween (PBST) is a wash buffer used to remove plant material and other chemicals or compounds from the plate.
2) Extraction Buffer 10x
    a. 10ml PEB1 and 90ml of water
    b. PEB1 is the extraction buffer used to dilute plant extracts.
3) Coenzyme
    a. Enzyme dilute (10ml)
        i. 1.67ml MRS2 component
        ii. 8.33ml PBST buffer
    b. Enzyme conjugate (must be kept in refrigerator)
        i. 100microL A
        ii. 100microL B
    c. Magnesium Homeostasis Factor Homolog combined with PBST to form the dilute enzyme. The conjugate combines with the diluents to form the coenzyme.
4) Florescent Substrate
    a. 15ml TMB (1x solution, light sensitive)
    b. Tetramethylbenzidine will exhibit a blue reaction product and is activated by the presence of the co-enzyme.

ELISA Procedure:

- Gather samples of plant material. The protein we are looking for is most active in the hypocotyls of the plant.
- Place one sample in each grinding bag. Label the bags if necessary.
- Place plant material toward the end of the bag where there is easy pipette access. The bag has abrasive netting that will lyse the cells.
- Make 10x extraction buffer (PEB1) and stir extraction to mix.
- Pipette 500microL of the extraction buffer into each bag with the plant material.
- Using the grinding tool, grind the sample until a green foam forms and the sample is broken down.
- Using a pipettor, extract 150 microL of each tissue sample and transfer it to 96 well ELISA plate (have well #96 be your control—just extraction buffer)
- Store the plate for 2 hours or overnight in 4 degrees Celsius in a humid box (lay down dampened paper towels in a plastic container and close the container
- While waiting, prepare the PBST buffer and co-enzyme.
- Wash the plate with PBST buffer 4-6 times. During the first wash, pipette 200 microL PBST buffer into each well as to not contaminate. For the remaining washes, you may load a wash bottle with PBST and spray into each well.
- Add 95 microL of the co-enzyme to each well.
- Dispense 15 ml of TMB (LIGHT SENSITIVE) and store in a dark place. The solution must be at room temperature.
- Store the plate for another two-hour incubation.
- Once the plate has incubated, wash it again 4-6 times with PBST.
- Add 100 microliters of TMB to each well (some wells will turn blue—these are transgenic).
- Incubate 5 minutes in humid box at room temp.

- While incubating, watch the cells as they change color. Make note of any ambiguous wells.
- Label the plate with the number of the field and take a photo of the plate.
- Count the wells and mark them on paper. Record results.

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Publications and Papers

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