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INBREEDING AND HERBIVORY AFFECT PLANT PHYSICAL DEFENSES  
IN HORSENETTLE (*Solanum carolinense* L.)

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## ABSTRACT

Foliar herbivory is a ubiquitous component of natural ecosystems. Herbivory reduces plant nutritional reserves, depletes photosynthetically active leaf area, and reduces plant fitness. Previous studies have suggested that host plant volatiles act as key foraging cues both for insect herbivores and their natural enemies. In this study, Horsenettle (*Solanum carolinense* L., Solanaceae) was used for my studies on plant-insect interactions. This plant is an agriculturally important herbaceous perennial weed found throughout the United States. Earlier studies have suggested that inbreeding decreases plant fitness, resistance to herbivory, production of volatile organic compounds and phytohormones in Horsenettle. This study investigated whether host plant inbreeding, genetic diversity, and herbivory affect plant trichome/spine induction in horsenettle. Three ramets (genetic replicates) were randomly assigned to one of three treatments- control, herbivore damage, mechanical damage from each of two inbred progeny and two outbred progeny from each of the nine maternal families. The larval stage of a solanaceous specialist herbivore, tomato hornworm (*Manduca sexta*) was used in the herbivore damage treatment. Induction of trichomes and internode spines was measured three weeks post-induction in each assigned treatment. The results show that inbreeding affects induced physical defenses such as trichomes and spines, and inbred plants fail to distinguish between mechanical wounding and caterpillar damage probably due to their impaired defense pathways.

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## INTRODUCTION

Foliar herbivory is a ubiquitous component of natural ecosystems. Herbivory reduces plant nutritional reserves, depletes photosynthetically active leaf area, and reduce plant fitness. Due to the variety and variability amongst herbivores (Berenbaum and Zangerl, 2008), plants have evolved a suite of chemical and physical defenses (Duffey and Stout 1996). Physical structural defenses include trichomes and spines (Agren and Schemske 1993). Trichomes are the hair-like protuberances seen on the leaves, floral organs, and epidermis of plants. Trichomes can be either glandular or non-glandular. Non-glandular trichomes impede the movement of arthropods on plant surface whereas glandular trichomes produce toxic exudates that hinder arthropod attack (Kennedy, 2003).

The impact of trichomes on herbivory in Solanaceae has been well documented, although most of these studies are focused on the exudates released from glandular trichomes (Hill et al. 1997). Stellate trichomes are also important as they act as mechanical barriers to movement of early stage larvae (Medeiros & Moreira 2002). Many insect species are found to possess adaptive morphological patterns to anchor their legs and thereby moving above them, as seen in the *Solanum sisymbriifolium*- *Gratiana spadicea* system (Medeiros & Moreira 2002).

Although considered less effective against insects than trichomes (Koricheva and Nykanen, 2004), leaf and internode spines are another group of physical defenses that negatively affect herbivore feeding (Koricheva and Nykanen, 2004). Herbivores have been found to preferentially feed on plants with lower spine density. By “choosing” to

feed on plants with reduced spine density, insects do not spend as much energy on maneuvering around and repairing damage caused with plant spines. Artificial removal of spines has been shown to increase herbivory rates and subsequent total tissue loss, as seen in *Acacia drepanolobium* and many other species (Milewski et al. 1991).

In the absence of herbivory, constitutive expression of plant defenses are unnecessary, using up resources thereby leading to fitness costs (Purrington 2000; Cipollini et al., 2002). Therefore, induced defenses, post-herbivory activated, appear to be more beneficial from a plant perspective, since they can provide an adaptive function as they are activated only after an event of herbivory, thereby imposing lower defense related fitness costs (Karban & Baldwin 1997, Baldwin 1998, 1999, Cipollini 1998, Agrawal 1999, Agrawal et al. 1999b; Cipollini et al., 2002). Taken together it is plausible to expect that constitutive and induced trichome and spine development can play important roles in defense against herbivory.

Plant resistance to herbivory has been commonly found to vary among individuals within populations (Hare et al., 2003; Weinig et al., 2003); which suggests that the benefits acquired through resistance may also vary based on the intensity of herbivory and the genetic background of the plant (Simms 1992; Hare et al., 2003).

Inbreeding is common in flowering plants and has major impacts on fitness. It is estimated that more than half of all flowering plants self-pollinate more than 20% of the time and inbreeding has been found in a wide variety of species possessing a range of mating systems, (Charlesworth and Charlesworth 1987; Johnston 1992; Uyenoyama et al.

1993; Willis 1993; Carr and Dudash 1995; Husband and Schemske 1996; Johnston and Schoen 1996; Willis 1999). Because inbreeding reduces heterozygosity, thereby exposing deleterious recessive alleles to selection while decreasing the contribution of overdominance to fitness, most species show a significant loss of fitness with inbreeding. Inbreeding depression, the reduction in fitness of inbred progeny relative to outbred progeny, is a potent evolutionary force that is thought to play major roles in the evolution of plant mating systems and in the extinction of small populations. Inbreeding can potentially have both direct and indirect effects on plant resistance to herbivores. Increased homozygosity could adversely affect the function of any of the several hundred genes known to directly alter the speed and/or magnitude of the responses of inducible plant defenses.

This study examines (a) Whether physical defenses production (trichomes and spines) is affected by inbreeding and (b) Whether herbivory and mechanical wounding cause similar changes in the induced physical defense phenotype.

To address these questions, common horsenettle was used (*Solanum carolinense* L; Solanaceae), a noxious perennial herbaceous weed, as the host plant and the tobacco hornworm caterpillar (*Manduca sexta* L; Sphingidae, specialist on Solanaceae) as the damaging herbivore. Horsenettle exhibits a variety of physical and chemical traits that play a role in defense against herbivores. The leaves and stem are covered with spines and the leaves are covered with stellate trichomes. All plant parts including leaves, flowers and fruits contain constitutive toxic secondary compounds (glycoalkaloids)

(Cipollini et al., 2002). Previous studies in this system also indicate that horsenettle emits volatile organic compounds (VOCs) in response to herbivore feeding (Delphia et al. 2009) and that horsenettle is attacked by many important herbivores and pathogens that are also known to attack closely related crops in the genus *Solanum* (e.g. tomato and potato) (Delphia et al. 2009).

## METHODS

### Study Organism:

*Solanum carolinense* L. is an herbaceous perennial weed that colonizes agricultural fields, crop pastures and disturbed sites throughout southeastern Canada and central and eastern United States (Britton & Brown 1970). After initial establishment, *S. carolinense* spreads via rhizomes that may extend a meter or more from the parent stem, enabling the invasion and spread of the parent in newly colonized areas (Ilnicki et al. 1962). The plant has an indeterminate growth and reproductive pattern with flowers that look similar in to potato and tomato. The flowers measure approximately 2 cm in diameter, with five partially fused white to violet petals and five yellow anthers. The inflorescences consist of 1–20 flowers that mature acropetally. The fruits are round, yellow berries that measure 2–3 cm in diameter and typically contain 60–100 seeds. The majority of the flowers are perfect and functionally hermaphroditic. However, some of the flowers usually located at the tip of the raceme exhibit reduced non-functional pistils and are considered functionally staminate (Solomon 1985; Mena-Ali et al., 2008).

### Maternal Families, Inbred and Outbred Lines:

Rhizome cuttings of 16 plants were taken in the field. Plants sampled were at least 5 meters apart from each other in order to reduce the probability of taking more than one rhizome sample from the same plant. The genets (sampled clones) selected were part of a large population located near State College, Pennsylvania. The rhizome cuttings were then planted in 4L pots and allowed to resprout and grow to flowering within a greenhouse. Plants were then pruned back to the soil line and the rhizomes were placed in

a cold room (4°C) to vernalize for 6-8 weeks. Following this period of time, the rhizomes were returned to the greenhouse (16: 8 L: D; 25°C: 22°C Day: Night; 65% RH). 5-6 ramets (clones) were then taken from each of these plants and replanted in a 4L pot and allowed to resprout and grow. The flowers produced on one ramet from each of the original 16 field collected plants were outcrossed until a total of 40 flowers per ramet were pollinated (for details see Mena-Alí, 2006; Mena-Alí & Stephenson, 2007). Flowers from a second ramet from each of the 16 original genets were self-pollinated until a total of 40 flowers per ramet were pollinated. A sample of the resulting seeds from self- and cross-pollinations were germinated and grown in the greenhouse. The purpose of this complicated design was to minimize the impact of cross-generational environmental maternal effects on our experimental genets and the ramets produced from them.

Of the original 16 maternal genets, I randomly selected a subset of nine genets for this study. I then selected 2 self- and two cross-pollinated progeny from each of the 9 maternal families for a total of 36 genets (i.e. genotypes). Three ramets were then created from each of the 36 genets by dividing the rhizomes. Rhizomes were allowed to sprout in flats of general-purpose potting soil (Pro-Mix, Premier Horticulture Inc., Quakertown, PA) in a growth chamber chamber (16: 8 L: D; 25°C: 22°C Day: Night; 65% RH). After 2 weeks, the sprouts were then individually transplanted to 2L pots and placed in a controlled greenhouse (16: 8 L: D; 25°C: 22°C Day: Night; 65% RH). On the day of transplanting, the plants received an application of fertilizer (50ppm; 8-45-14 NPK, plus micronutrients, Scotts® Company, Marysville, OH) and iron chelate (Sprint 138 at 6%, Becker Underwood, Ames, IA), followed by an application of 200ppm fertilizer (above)

once a week thereafter until the end of the experiment. At approximately 8 weeks of age, the plants had produced enough leaves to begin the experiments.

### Insects

*Manduca sexta* L. eggs were obtained from the Carolina Biological supply (Carolina biological supply, NC). Eggs were allowed to hatch in Petri dishes (90mm x 15mm; Becton Dickinson and Company, Lincoln Park, NJ) on moist filter paper (Whatman® 1; 90mm) in a growth chamber (16:8 L: D; 25°C: 22°C Day:Night; 65% RH). Larvae were then moved to large plastic containers (approx. 35cm long x 10cm high x 15cm wide) located in the same growth chamber for mass rearing on artificial casein diet. Newly molted 3rd-instar larvae were used for the damage treatments.

### Physical Defense Induction:

One ramet per genet was randomly assigned to one of the three treatments” control, herbivore damaged, and mechanical damaged. The first sets of 36 plants (1 ramet per genet) was assigned as controls and were spatially separated from the other plants within the same greenhouse room (~ 10ft to minimize any possible effects of priming; 16:8 L: D; 25°C; 70% RH). The second set of plants was assigned as mechanical damage treatment, and using a hole punch (# 13, 2cm diameter) 2 leaf disks each were removed from 2 fully developed leaves, opposite to each other (Fig 1). The plants were then allowed to continue growing for another 3 weeks. The third set of 36 plants was assigned as caterpillar damaged treatment. One 3rd instar *Manduca* each was placed on 2 fully developed opposite leaves (comparable to mechanically damaged leaves) and allowed to

feed for 6-8 hours. To ensure that the younger leaves did not get eaten, a paper coffee filter was cut from the edge to the center and was wrapped around the stem right above the treated leaves. This was also done for both control plants and mechanically damaged plants. After 6-8 hrs of damage, the caterpillars were removed and the plants were allowed to continue their growth. Three weeks post treatments leaf samples were taken for trichome analysis. A leaf disk (# 13 hole punch, 2cm diameter) was removed from the youngest fully developed leaf on each plant and was used for the trichome count. Each leaf disk was immediately placed into a Petri dish (90 mm x 15mm; Becton Dickinson and Company, Lincoln Park, NJ) lined with moistened filter paper (Whatman® 1; 90 mm) for digitization and trichome count.

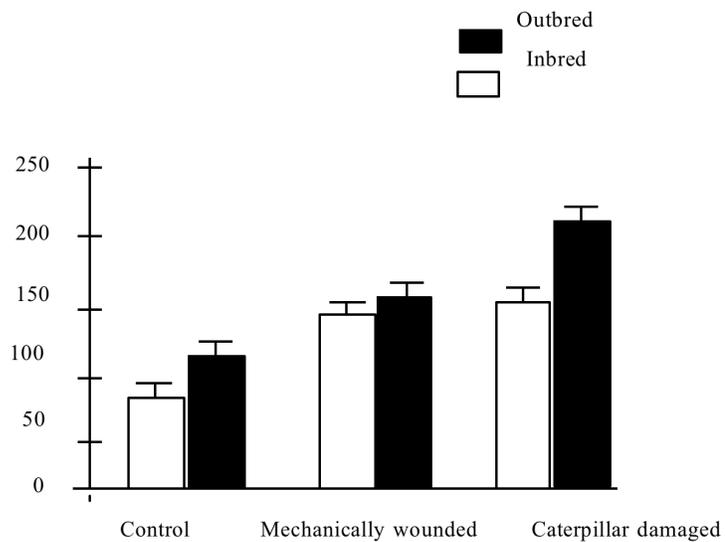
#### Trichomes and Spine Numbers:

The leaf disks were converted into digital Images for storage and subsequent analyses. This was done by converting them into .jpeg files using a Monochromatic digital camera (Sting Ray F504B; Allied vision Technologies, Germany) attached to a dissecting microscope (Olympus SZX 16, Objective lens SDF PLAPO 1X PF, Japan). The camera used the ring light system of the scope to capture the image with a total area of 35 sq. mm which was converted into a digital .jpeg file (1224 X 1028 pixels; 96 dpi) using the accompanying software (AVT Smart View version 1.8.1; Allied Vision Technologies, Germany). Each file was then opened in Microsoft Paint© (MS Paint 2010; Microsoft Corporation, USA) and partitioned into 8 rectangular grids for the ease of counting. The grids were assigned random numbers and 4 out of the 8 grids were randomly selected and were counted for the number of trichomes. Trichome counts were independently carried

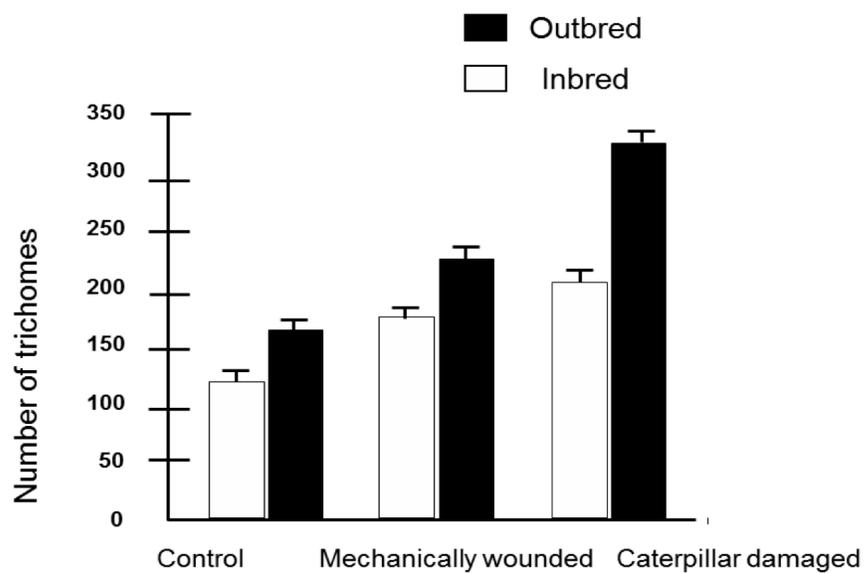
out by three people (Rupesh Kariyat, Christopher Balogh, and I) and the mean of the three counts was used as the final count. This mean was then multiplied by 2 to get the total trichome number for the total area of 35 sq mm. Trichome counts were carried out for both abaxial and adaxial leaf surfaces using the same method (Figure 2). After collecting the leaf disks for trichome counts, data on the numbers of spines on the two internodes, one below and one above the sampled leaf was also collected.

#### Statistical Analyses:

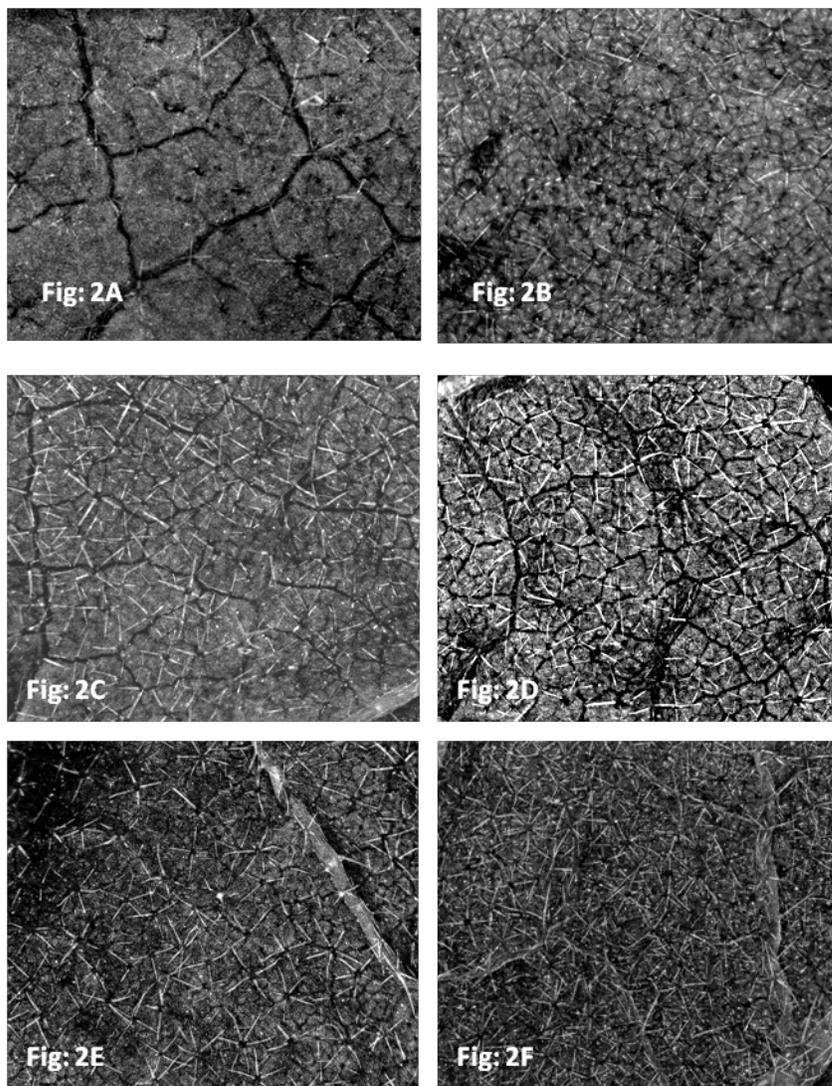
To determine the effects of damage (caterpillar damage, mechanical wounding or control), breeding, and family on the induction of physical defenses (abaxial and adaxial leaf trichomes and internode spines) I used a mixed effects model ANOVA. This was done by using treatment (caterpillar damage, mechanical wounding, or control) and breeding (inbred/outbred) as fixed effects and family as the random effect. The mixed effects model ANOVA also included the effects of interaction of treatment by breeding, treatment by family, family by breeding and treatment by breeding by family. Raw data were log transformed to attain normality and then back transformed and reported (Minitab Version 15, Minitab Inc, State College, PA).

**RESULTS**

**Figure 1.1:** Mean (+SE) of the number of abaxial trichomes present in 35mm sq leaf area of horsenettle genotypes 21 days post treatments (control, mechanically wounded and caterpillar damaged).



**Figure 1.2:** Mean (+SE) of the number of adaxial trichomes present in 35mm sq leaf area of horsenettle genotypes 21 days post treatments (control, mechanically wounded and caterpillar damaged).



**Figure 2:** Monochromatic digital camera attached microscope image of abaxial leaf trichomes found in different treatments of inbred and outbred horsenettle plants. (2a). Inbred control plant, (2b) Outbred control plant, (2c). Mechanically wounded inbred plant 21 days post wounding, (2d). Mechanically wounded outbred plant 21 days post wounding, (2e). Caterpillar damaged inbred plant 21 days post damage. (2f). Caterpillar damaged outbred plant 21 days post damage. All images constitute a leaf area of 35sq.mm.

Outbred plants produced significantly more abaxial trichomes than inbred plants (abaxial; outbred =  $142.2 \pm 1.0$ ; inbred =  $108.9 \pm 1.0$ ; back transformed Least Square Means  $\pm$  Standard Error; LSMeans  $\pm$  SE; Appendix A: Table 1,  $P = 0.003$ ) and adaxial trichomes (adaxial; outbred =  $226.5 \pm 1.0$ ; inbred =  $159.2 \pm 1.0$ ; back transformed Least Square Means  $\pm$  Standard Error; LSMeans  $\pm$  SE; Appendix A: Table 1,  $P < 0.001$ ). There were also significant difference for the treatments in trichome production, with the caterpillar damaged plants producing more trichomes than the mechanically wounded plants, which was higher than the control plants ( $P < 0.001$ ). There was no interaction for maternal family X breeding ( $P = 0.147$ ) and maternal family X damage ( $P = 0.656$ ) for abaxial trichome production, where as breeding X damage ( $P = 0.051$ ; Fig 2) was marginally insignificant and maternal family X breeding X damage ( $P = 0.014$ ) had a significant effect. These results suggest that although maternal family has a small effect on abaxial trichome production, the major effects are contributed by inbreeding and caterpillar damage. In the case of adaxial trichomes, we found no significant interaction effects.

The total number of internode spines post treatments were found to be significantly higher on outbred plants compared to the inbred plants (outbred =  $13.50 \pm 1.0$ ; inbred =  $10.4 \pm 1.0$ ; back transformed Least Square Means  $\pm$  Standard Error; LSMeans  $\pm$  SE; Appendix A: Table 1,  $P = 0.001$ ). There was also a significant treatment effect with caterpillar damaged plants producing significantly higher number of internode spines than both mechanically wounded and control plants ( $P < 0.001$ ). There was no significant effect for the maternal families ( $P = 0.473$ ). There was however a marginal effect for damage X breeding interaction ( $P = 0.058$ ), but the maternal family X breeding

interactions was insignificant, suggesting that inbreeding and caterpillar damage were the major factors influencing the production of spines.

## **DISCUSSION**

Evolution of plant defense traits is thought to be heavily influenced by selection exerted by the herbivores that attack them (Agren and Schemske, 1994; Janzen 1979; Futuyma 1983; Marquis 1991; Futuyma and Keese 1992) and also due to the amount of genetic variation present within and between plant populations (Jones et al. 1978; Pollard and Briggs 1982; Berenbaum et al. 1986; Fritz and Price 1988; Simms and Rausher 1989; Marquis 1990). Plant tissue damage caused by insect herbivores and various microorganisms is a universal phenomenon, and there have been conflicting reports on whether mechanical wounding (e.g., razor blade, clippers) can simulate actual insect herbivory (Maffei et al., 2007). These reports also raise the question about whether plants are able to perceive these signals separately and then specifically activate subsequent systemic defense responses.

Here, using a series of controlled experiments, it is shown that mechanical wounding and insect feeding on older leaves can cause a systemic signaling response in younger leaves and internodes, activating induced physical defenses (Figures 1.1, 1.2, 2; Appendix A: Table 1). Although both mechanical wounding and caterpillar damage induced physical defenses, the magnitude of induction differed (Appendix A: Table 1). Since previous studies from the Stephenson Group have already shown the effects of inbreeding and herbivory on induced chemical defenses (Delphia et al., 2009), these results lend further support to the notion that inbreeding impairs defense signaling.

In contrast with previous studies (Gouinguéné et al., 2001), we find that most of the defense traits observed here were influenced very little by maternal families (Appendix A: Table 1). Resource allocation theory predicts that resource allocation to defense is a function of damage (Agren and Schmeske, 1993). This theory also suggests that induced defenses are extremely expensive to produce (Simms and Fritz, 1990; Agren and Schmeske, 1993).

Despite family not having a strong effect by itself, we found strong interaction effects with family X breeding, family X damage, breeding X damage and family X breeding X damage on trichome induction. This suggests that of population level differences in breeding history and herbivory can impact plant response to herbivory.

As suggested by Agren and Schmeske (1993), plants with lower constitutive trichome density are expected to have a higher fitness than plants with higher constitutive trichome numbers in the absence of herbivores, since defense production is expensive; conversely, post herbivory the difference is expected to be reversed, or at least reduced, since trichomes and spines have been found to deter herbivore feeding. In addition to this, one would also expect reduced trichomes and reduced fitness in undamaged inbred plants compared to outbred plants due to inbreeding depression. It was also expected to see reduction in induced trichomes and increased fitness traits in inbred plants when compared to induced outbred plants. Interestingly, the data suggests that, regardless of herbivory or mechanical wounding, inbred plants have significantly lower trichome density than outbred plants. The data also shows that when induced, inbred plants

produce higher trichome numbers than constitutively present, though the trichome density is still lower than induced outbred plants. Similar trend was also found for internode spine numbers.

It was my original goal to track the fitness traits of the plants from all the treatments. A powdery mildew outbreak limited the observations of fitness and physiological traits to 3.5 weeks post induction. In contrast to other findings (Agren and Schemske, 1993) these limited results suggest that induction of higher trichome numbers in both inbred and outbred damaged plants compromises growth. A controlled field experiment would be interesting and will further help to uncouple the effects of physical defense induction and associated costs that affect fitness.

These results on damage-induced changes in plant physical defenses support previous findings that plants perceive mechanical and caterpillar induced damage differentially (Pare and Tumlinson, 1997). Although plant perception of damage and related defense response is well documented, specificity of defense response against herbivory and its costs on growth has not been well studied in the light of genetic variation and population level differences. Although a few recent studies have examined the effects of inbreeding on plant resistance to herbivores this is the first study to examine the effects of inbreeding on the physical defenses of plants.

Insect herbivory is a critical factor that affects natural plant populations. Specialist and generalist herbivores have been found to decrease fitness in a wide variety of plant species both at field and lab conditions. Since inbreeding compromises plant vigor, inbred plants have fewer resources to deploy toward defense. Also, increased homozygosity may expose deleterious recessive alleles for any of the hundreds of genes known to be involved in plant defenses against natural enemies. As a result of these genetic factors, inbreeding depression appears to be a critical component in understanding plant-herbivore-defense mechanisms.

This study comprehensively shows that inbreeding affects induced physical defenses such as trichomes and spines, because inbred plants fail to distinguish between mechanical wounding and caterpillar damage (Figures 1.1, 1.2) probably due to their impaired inducible defense pathways.

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## APPENDIX A

Trait	Source of variation	SS	F	P
(a) Abaxial Trichomes	Family	0.0467	2.36	0.175
	Breeding	0.368326	16.75	<b>0.003</b>
	Damage	0.868282	88.04	<b>&gt; 0.001</b>
	Family X Breeding	0.021984	1.82	0.147
	Family X Damage	0.009863	0.82	0.656
	Breeding X Damage	0.043565	3.6	<b>0.051</b>
	Family X Breeding X Damage	0.012097	2.24	<b>0.014</b>
(b) Adaxial Trichomes	Family	0.04343	3.55	0.155
	Breeding	0.62593	75.07	<b>&gt; 0.001</b>
	Damage	0.66838	34.33	<b>&gt; 0.001</b>
	Family X Breeding	0.00834	0.54	0.813
	Family X Damage	0.01947	1.25	0.33
	Breeding X Damage	0.01799	1.16	0.34
	Family X Breeding X Damage	0.01557	1.19	0.309
(c) Internode Spines	Family	0.013676	1.14	0.473
	Breeding	0.343591	26.76	<b>0.001</b>
	Damage	0.321135	34.38	<b>&gt; 0.001</b>
	Family X Breeding	0.012842	1.26	0.33
	Family X Damage	0.00934	0.92	0.569
	Breeding X Damage	0.034935	3.42	<b>0.058</b>
	Family X Breeding X Damage	0.010207	1.27	0.253

**Table 1:** Results of ANOVA and Chi-Squares tests for the effect of maternal family (random), breeding and damage (fixed; caterpillar/mechanical/control) on trichome and internode spine counts.

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Honors Thesis: The effects of inbreeding depression and herbivory on plant physical defenses in Horsenettle (*Solanum carolinense* L.)  
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Herbarium Curator Assistant, Penn State University Herbarium  
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REU Research Intern, Smithsonian National Museum of Natural History, Department of Botany  
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Dean's List, Penn State, every semester, 2008-2012  
Academic Excellence Scholarship, 2008-2012  
Martin Goldhaber Renaissance Scholarship, 2008-2012  
Horatio Alger Pennsylvania State Scholarship, 2008-2012

## **Presentations**

### **Poster** (*Presenting author\**)

- Moraski, RP\***, Wen, J. A molecular phylogeny of the grape family (Vitaceae) based on plastid and nuclear sequences. Smithsonian National Museum of Natural History Summer Research Symposium. Washington DC. August 2011.
- Kariyat, RR\*, Mauk, KE, **Moraski, RP**, Balogh, C, Mescher, MC, De Moraes, CM, Stephenson, AG. Plant defense signaling against herbivores is affected by inbreeding depression. The 18th Penn State Plant Biology Symposium. University Park, PA. May 2011.
- Moraski, RP\***, Kariyat, RR, Scanlon, S, Mescher, MC, De Moraes, CM, Stephenson, AG. The effects of inbreeding on plant-insect interactions in Horsenettle (*Solanum carolinense* L.). The annual Penn State Undergraduate Research Exhibition. University Park, PA. April 2011.
- Kariyat, RR, Scanlon, S, **Moraski, RP**, Balogh, C\*, Mescher, MC, De Moraes, CM, Stephenson, AG. Effects of host plant (Horsenettle; *Solanum carolinense* L) genetic diversity and inbreeding on feeding preference and oviposition behavior of specialist herbivore (Tobacco hornworm; *Manduca sexta* L). The Entomological Society of America, Eastern Branch Meeting. Harrisburg, PA. March 2011.
- Moraski, RP\***, Kariyat, RR, Scanlon, S, Mescher, MC, De Moraes, CM, Stephenson, AG. The effects of inbreeding on plant-insect interactions in Horsenettle (*Solanum carolinense* L.). The National Meeting of the International Association of Students in Ag and Related Sci. Athens, GA. January 2011.

## **Publications**

- Kariyat, RR, Balogh, C, **Moraski, RP**, De Moraes, CM, Mescher, MC, Stephenson, AG. Herbivory induced physical defenses affect growth and flowering time in inbred Horsenettle (*Solanum carolinense* L).  
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## **Professional Affiliations**

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