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Insect Mediated Species Interactions: Examining Methods to Improve Artificial Buzz Pollination and Testing the Effects of Plant Based Bioactive Compounds on Herbivore Life-history Traits

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INSECT MEDIATED SPECIES INTERACTIONS: EXAMINING METHODS TO IMPROVE ARTIFICIAL BUZZ POLLINATION AND TESTING THE EFFECTS OF PLANT BASED BIOACTIVE COMPOUNDS ON HERBIVORE LIFE-HISTORY TRAITS

A Thesis

by

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ABSTRACT

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Pollination and herbivory are two major interactions in insect-plant ecology. Specialized pollination systems such as buzz pollination where pollen grains have to be extracted by bees with special thoracic vibrations and indirect flight muscles, is observed in ~6% of all flowering plants. Breeding and research programs in these species demand artificial pollination, but natural buzz pollinators are unreliable for this purpose. To find an alternative, we tested the pollen extraction efficiency of using inexpensive electric toothbrush over tuning fork (another commonly used device) in two buzz-pollinated species (Tomato and Silverleaf nightshade) at different buzzing frequencies and multiple buzzing time intervals. Our results show that species and extraction time significantly influenced pollen extraction, while there were no significant differences for the different vibration frequencies and more importantly, the use of a toothbrush over tuning fork. We conclude that electric toothbrushes can be used as a viable and inexpensive alternative to tuning forks for pollen extraction. As the second most important insect mediated species interaction, herbivory, is one of the major threats in crop production and food security. Although synthetic chemicals and pesticides are used to manage insect-pests, their use have led to major concerns of resistance development, pest resurgence as well as toxicity to non-target organisms. Plant-based bioactive compounds are good alternatives, but their use is limited by complicated and expensive

extraction and purification methods. We tested the effects of polyphenol rich purple corn pericarp extract (extracted inexpensively) on the growth and development of *Manduca sexta*, a damaging herbivore. We found that pericarp extract negatively affects egg hatching, mass gain, developmental time and these effects cascade through pupal, adult and next generation offspring suggesting its potential suitability as a biopesticide. Taken together, our findings of inexpensive pollen extraction and sustainable pest management methods can have implications in improving agricultural practices.

DEDICATION

I dedicate my thesis to my parents Sunita Rani and Ram Kumar, my sisters Rabina Bansal and Priyanka Garg, brothers-in-law Yashpal Bansal and Parul Garg , my brother Gagandeep Tayal and my mentors. Without their unconditional support and motivation, my thesis would haven’t been possible to complete. Thank you for your love, guidance and patience.

.

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CHAPTER I

INTRODUCTION

In insect-plant interactions, pollination and herbivory are two major areas of interest (Kearns, et al., 1998; Masters, et al., 1993; Weisser & Siemann, 2013). Pollination, the key mutualism responsible for reproduction and fitness of many domesticated and wild plants, is largely carried out by insect pollinators (McGregor 1976; Klein et al., 2006). Over the millions of years of co-evolution, plants and their pollinators have evolved specialized pollination syndromes and behavioral adaptations leading to a wide range of interesting and intriguing interactions. Among these, buzz pollination is a specialized type of pollination found in ~6% of flowering plants (Cardinal et al., 2018; Correa et al., 2019). Having concealed pollen inside poricidal anthers, these are usually pollinated by special buzz pollinators such as bumble bees : *Bombus* spp., carpenter bees : *Xylocopa* spp., and sweat bees : *Lasioglossum* spp. (Buchmann & Hurley, 1978). It is interesting to note that honeybees (Apis *spp*.) are incapable of buzz pollination, due to their inability to use thoracic muscles to extract pollen by buzzing at the right frequency. Although a number of studies have addressed the ecologically relevant questions on buzz pollination (Harris, 1905), recent studies have been more focused on understanding the biomechanics, pollinator physiology and behavior in relation to buzzing (Vallejo-Marín, 2018; Correa et al., 2019; De Luca et al, 2019). In general, these studies demand consistent and efficient pollen extraction which by artificial methods by manipulating poricidal anthers. In addition, most of the breeding programs and variety trials in buzz pollinated species also depend on artificial pollen extraction (Pessarakli & Dris, 2004). This is even more important in the plant family Solanaceae, which

houses economically important species such as Tomato (*Solanum lycopersicum*), Potato (*S. tuberosum),* Eggplant (*S. melongena),* and Peppers (*Capsicum spp.*), to name a few. Traditionally scientists use tuning forks in artificial buzz pollination (Buchmann & Hurley, 1978; King & Buchmann, 1996; Williams, 1998) but they tend to have some concerns such as easiness to break, comparatively expensive, and inability to get replaced in a timely fashion under field conditions.

To find an alternative for tuning fork, we examined the efficiency of pollen extraction efficiency using electric toothbrush, which are cheaper, easier to find, and more reliable without being damaged. In chapter 1 we tested the pollen extraction efficiency of electric toothbrush and tuning fork in two buzz-pollinated species (Tomato: *S. lycopersicum* and Silverleaf nightshade: *S. elaeagnifolium*) at different buzzing frequencies (low, medium, high) for different buzzing time intervals (3 sec and 16 sec) and found interesting results.

While pollination is mutualism, insect herbivory is a form of predation, and is one of the major threats in crop production accounting for ~18-20% of total production every year (Oerke, 2006). In order to manage insect herbivores, we have been relying heavily on synthetic chemicals and pesticides. However, the continued use of these chemicals has resulted in various concerns such as resistance, residue problems, pest resurgence, toxicity to non- target organisms etc., to name a few, and consequently endangering ecosystem functioning and sustainability (Coats, 1994; Jeyasanker & Jesudasan, 2005; Datta et al., 2019). Clearly, in addition to the various sustainable management practices, there is an earnest need for alternative safe and ecofriendly pest management strategies. As a potential alternative, we had been in search of natural products to be used as biopesticides (Williams & Mansingh, 1993; Zounos et al., 1999; Sampson et al., 2005; Atawodi & Atawodi, 2009; Ahmed et al., 2018; Aljaghthmi et al., 2018; Campos et al., 2019).

Previous studies had shown that plant-based bioactive compounds are biodegradable and eco-friendly alternatives which can be incorporated in integrated pest management (Dimetry, 2012; Kedia et al., 2015). Although, there are plethora sources of bioactive compounds, their complicated and expensive extractions and purification of these compounds limits their use (Mandana et. al., 2011; Sasidharan et. al., 2011; Raks et. al., 2017). A good example is anthocyanins, a group of flavonoid compounds used in food and pharmaceutical industry are usually isolated from blueberries, black carrots, sweet potatoes etc. (Bridgers et al., 2010: Somavat et al., 2016). While effective, these are expensive and tedious to extract.

Alternatively, purple corns rich in polyphenols (majorly anthocyanins) can be used as potential sources of bioactive compounds as their extraction and purification are highly efficient and economical (Li et al., 2017, Somavat et al., 2018). Well known for their antioxidant (Fernandes et al., 2014; Reque et al., 2014), anti-inflammation (Esposito et al., 2014), pharmaceutical properties (Pellecchia & Reed, 2004; Hatano et al., 2005) as well as their role in insect plant defense (Lee et al., 1987; Close & Beadle, 2003; Lev-Yadun & Gould 2008), in chapter 2, we tested whether anthocyanins and polyphenols rich purple corn pericarp extract has any insecticidal properties against insect-herbivores. Following this in chapter 3, we examined the role of the extract on growth and development as well its cascading and transgenerational effect on Tobacco hornworm (*Manduca sexta; Lepidoptera; Sphigidae*) caterpillars. Easy laboratory rearing and quick development as well as its size make *M. sexta* an ideal study system for physiological, developmental and behavioral studies (Kingsolver, 2007; Kariyat et. al., 2012; 2018; 2019).

CHAPTER II

EFFICIENCY OF USING ELECTRIC TOOTHBRUSH AS AN ALTERNATIVE TO THE TUNING FORK FOR ARTIFICIAL BUZZ POLLINATION IS INDEPENDENT OF INSTRUMENT BUZZING FREQUENCY

Abstract

Breeding programs and research activities where artificial buzz-pollinations are required have primarily relied upon using tuning forks, and bumble bees. However, these methods can be expensive, unreliable, and inefficient. To find an alternative, we tested the efficiency of pollen collection using electric toothbrushes and compared it with tuning forks at three vibration frequencies- low, medium, and high and two extraction times at 3 seconds and 16 seconds- from two buzz – pollinated species (*Solanum lycopersicum* and *Solanum elaeagnifolium*). Our results show that species, and extraction time significantly influenced pollen extraction, while there were no significant differences for the different vibration frequencies and more importantly, the use of a toothbrush over tuning fork. More pollen was extracted from *Solanum elaeagnifolium* when compared to *Solanum lycopersicum*, and at longer buzzing time regardless of the instrument used. Our results suggest that electric toothbrushes can be a viable and inexpensive alternative to tuning forks, and regardless of the instrument used and buzzing frequency, length of buzzing time is also critical in pollen extraction.

Introduction

In another wonderful example of convergent evolution, it is estimated that around 6% of flowering plants, comprising of species from multiple plant families, are primarily buzz-pollinated (Arroyo-Correa et al., 2019; Cardinal et al., 2018). Among these species, the most common anther type is poricidal, where pollen grains tend to be stored inside non-dehiscent anther tubes with small pores at the tip (Corbet and Huang, 2014). Concealing pollen grains inside poricidal anthers conserves pollen, and has also led to specialized pollinators, commonly known as buzz pollinators. More interestingly, these pollinators mainly include bumble bees (*Bombus* spp.), carpenter bees (*Xylocopa* spp.), and sweat bees (*Lasioglossum* spp.), among others but not honeybees (*Apis* spp.) (Buchmann & Hurley, 1978). Unlike other insect pollinators (e.g., Lepidoptera), buzz pollinators produce floral vibrations using their thoracic muscles and use their other body parts including mandibles, head and abdomen to release the pollen from these anthers (Michener, 1962; Buchmann, 1983; King and Buchman 2003; Switzer et al., 2015; Arroyo-Correa et al., 2019), an ability confined to few insect genera. Although studies on ecology and evolutionary biology of buzz pollination has been carried out for more than a century (Harris, 1905), the biomechanics, pollinator physiology and behavior in relation to buzzing have only recently gained an increased interest (Vallejo-Marín, 2018; Arroyo-Correa et al., 2019, De Luca et.al., 2019).

Solanaceae is one of the major plant families that are predominantly buzz-pollinated. They include crops such as tomato (*Solanum lycopersicum*), peppers (*Capsicum* spp.), eggplant (*Solanum melongena*), and weeds such as horsenettle (*Solanum carolinense*), buffalo bur (*Solanum rostrum*) and silverleaf nightshade (*Solanum elaeagnifolium*) to name a few. Equally important for crop husbandry purposes and ecological research, pollination experiments in these

species essentially require the manipulation of poricidal anthers to collect pollen. For example, both *S. carolinense* and *S. elaeagnifolium* are obligate outcrossing species with gametophytic self-incompatibility but will undergo selfing under certain circumstances such as lack of foreign pollen and increase in floral age (Mena-Ali & Stephenson, 2007), and any manipulative empirical studies on these require pollen extraction at our convenience. In cultivated species such as *S. lycopersicum* and *S. melongena*, most breeding programs and variety trials require the extraction and analysis of pollen, and subsequent artificial pollination (Pessarakli & Dris, 2004; Sidhu, et. al., 2005). Previous studies show that synthetic stimuli construction (De Luca, et. al., 2012), vibrations produced by transducers (Harder & Barclay, 1994) and tuning forks (Buchmann & Hurley, 1978; King & Buchman, 1996; Williams, 1998) can be used in artificial pollen extraction. Among these, tuning forks are common tool, employed in most of studies for pollen extraction. For such extractions, the tuning fork is allowed to vibrate and held close to the anthers, thereby releasing the pollen which is collected into a tube for further use (Buchmann & Hurley, 1978). However, tuning forks can be expensive, hard to find at the right frequency for field experiments, and more importantly, tend to break if struck hard before initiating the vibration cycle (personal observation). Since a significant part of ecological research is done in field which limits the access to finding appropriate replacement for tuning forks in a timely fashion, this can severely hamper the experiments.

To find an alternative for tuning forks, we tested the pollen extraction efficiency of electric toothbrushes, which are cheaper, easier to find, and much more reliable without being damaged. However, pollen extraction through buzzing could also be affected by species variation, time of buzzing and also by the frequency of vibrations. For example, it has been shown that vibrations at high frequencies (450-1000 Hz) ejects more pollen as compared to the

low frequency (100-400 Hz) vibrations (Harder & Barclay, 1994). To account for these factors, we carried out an experimental design where we collected pollen from two Solanaceous species, an invasive weed Silverleaf nightshade (*S. elaeagnifolium),* and tomato (*Solanum lycopersicum*). In addition, we tested the efficiency of pollen removal at multiple buzzing frequencies for both electric toothbrushes and tuning forks, at two-time intervals. Since floral vibrations produced by bees are substrate-borne vibrations affected by time and frequency (Arroyo-Correa et al., 2019), we hypothesized that both instruments would extract similar amounts of pollen. In addition, we also hypothesized that both frequency and time of collection would significantly affect pollen extraction, also affected by the plant species.

Material and Methods

Study species

For the experiments detailed below, we used two buzz-pollinated *Solanum* species, i.e. silverleaf nightshade (*S. elaeagnifolium*) and tomato (*S. lycopersicum)*. Silverleaf nightshade is a worldwide invasive perennial weed, native to the southwestern United States and Mexico (Boyd et. al., 1984). The flowers are usually blue lilac in color, nectar-less, hermaphrodite and have poricidal anthers mostly visited by buzz pollinators (carpenter bees: *Xylocopa* spp., bumble bees: *Bombus* spp., sweat bee: *Lasioglossum* spp.) for pollen transfer and reproduction success (Petanidou et. al., 2018). It acts as ruderal, colonizes disturbed sites and is also toxic to livestock (Boyd et. al., 1984). However, tomato is an herbaceous, economically important agricultural crop widely cultivated throughout the world. The flowers are nectar-less, yellow in color and anthers are laterally bound together with pore-like openings at the apical end (Teppner, 2005). Flower

agitation either by wind or natural pollinators (bumble bee, sweat bee, carpenter bee) is crucial for pollen removal (Franceschinelli et. al., 2013).

Plant material

The plant species used in the study were either grown in controlled conditions (*S. lycopersicum*) or sampled (*S. elaeagnifolium*) from the local native population. We used F1 tomato hybrid seeds (Variety: Valley Girl, Product ID 741, Johnny's Selected Seeds, Maine, USA) sown in growth media (Sunshine professional growing mix: Sun Gro Horticulture Canada Ltd., MA, USA) in the plastic trays (51.435cm * 25.4cm) and covered with thin transparent film to maintain optimum temperature, i.e. 27^0C for germination. At 2-4 leaf stage, the seedlings were repotted individually to bigger pots (15.24 cm diameter) and kept in greenhouse at 25^0C and 65% RH. Plant nutrient requirements were met by applying OMRI (Organic Material Review Institute, OR, USA) listed organic fish emulsion fertilizer (NPK 5:1:1, Alaska Fish Fertilizer, Pennington Seed, Inc., USA) once every two weeks. Plant growth and health was maintained until flowering and plants were ready for experiment.

On the other hand, for *S. elaeagnifolium*, we used flowers from multiple native populations in the City of Edinburg, Texas (26°18'25.8"N 98°12'10.9"W). In synchronization to the tomato flowers, we selected silverleaf nightshade plants with at least 5 fully opened new flowers, and the plants were uprooted using a pair of pruning shears. After collecting the plants with flowers, they were immersed in water up to 7~8 cm and were immediately brought back to the lab. The plant sampling was done early in the morning before pollinators visits to avoid any prior floral visits (personal observations).

Instruments and treatments

Our experimental design was to examine the effects of buzzing instrument, buzzing time, and frequency differences on pollen removal from these two species. To accomplish that we used tuning forks (Tuning fork aluminum alloy, Lot No: 3200-x, Ward's Science, New York, USA) cost ranges $8-$11 each of different frequencies, i.e. low (256 HZ), medium (320 HZ) and high (512 HZ). We also used the electric toothbrushes, which cost ranging from $4-$6 each of different strokes i.e. 14000/ minute (233/sec or 233 Hz) (Oral-B 3d White Action Power Toothbrush), 20000/ minute (333/sec or 333 HZ) (Colgate 360 powered toothbrush, Colgate Co. Pvt. Ltd.) and 30000/ minute (500/sec or 500 HZ) (Vivid Sonic Clean toothbrush) We used a digital acoustic recorder (Tascam DR-100 MK-III: TEAC America, Inc., CA, USA) to record each of their vibration frequencies (see Additional file 1a, 1b, 1c) and then analyzed the files in Audacity v. 2.1.3 (https://sourceforge.net/projects/audacity/) by examining the spectrogram using 'Spectrogram' function (FFT = 8192 Hz, Hamming window). We found a different range of frequencies than those advertised. (See Supplementary Table 1). The tuning fork vibrational frequencies (see Additional file 2a, 2b, 2c) were also verified in this software, but were found to be consistent with the advertised frequencies (Supplementary Table 1).

Detailed methodology

As mentioned above, the *S. elaeagnifolium* plants were sampled and brought to the lab on each day of the experiment. *S. lycopersicum* plants with newly opened flowers were moved from greenhouse to the lab. Both species were tested in tandem. At first, the tuning fork of low frequency (259 Hz) was used for 3 seconds to extract the pollen. For this, the tuning fork was hit once on the lab countertop, and then it was brought close to the flower without making contact. The resulting pollen was collected in 0.5ml PCR tubes (Pryme PCR: Midwest Scientific, MO, USA). The same procedure was repeated for same frequency but for a different time (16 sec)

interval. For the other half of the plants, we followed the same methodology, except that an electric brush was used instead of the tuning fork. The bristle head of the brush was removed, and anthers were vibrated by bringing metal nub near to the anthers. The same procedure was repeated for other frequencies, i.e. medium and high in both species. To collect enough pollen for better weight measurement, we pooled pollen from three flowers for each treatment, and then weighed the sample. An empty 0.5ml tube was weighed and the PCR tubes containing pollen were weighed to get pollen weight. Weight measurements were carried out using an advanced digital balance (Accuris *Series Dx*, Model: W3101A-220, Benchmark Scientific, NJ USA). A schematic of the experiment is detailed in Figure 2.

Statistical analysis

Due to the non-normal nature of the data set, the raw data were transformed using Squareroot+1transformation prior to analysis of variance. We used the weight of pollen collected as our response variable and instrument, species, time, and frequency, and their interactions as our fixed factors. Means were separated and pairwise comparisons were carried out using the post-hoc Tukey tests at $p < 0.01$. All analyses were carried out using the statistical software JMP (Statistical Analysis Software (SAS) Institute, Cary, NC).

Results

We found significant differences among treatments for pollen extraction (Table 1A). Among the factors, we found that plant species, and length of vibration time were statistically significant. We extracted significantly more pollen from *S. elaeagnifolium* when compared to *S. lycopersicum* (Figure 1A), and among time intervals, 16 seconds of vibration significantly

extracted more pollen when compared to 3 seconds (Figure 1B). More interestingly, we found that there was no significant difference between the use of tuning fork and electric toothbrush even at multiple time intervals and vibration frequencies for these two species (Figure 1C). We also found that different frequency levels of both instrument vibrations did not affect pollen extraction (Figure 1D). Even the extreme comparison of high-frequency electric toothbrush with low frequency tuning fork extracted almost equal amounts of pollen (Figure 1E). Among the interactions, only instrument X species was significant, where using an electric toothbrush on *S. elaeagnifolium* extracted more pollen (Table 1B) than electric tooth brush and tuning fork on *S. lycopersicum*, and tuning fork on *S. elaeagnifolium* extracted more pollen than electric tooth brush and tuning fork on *S. lycopersicum*, although the instrument difference did not affect pollen extraction within the species.

Discussion

The major takes away from our results is that we didn't find any significant difference in the amount of pollen collected using an electric brush over a tuning fork, which was our primary factor of interest. As tuning forks are expensive (cost ranges $8-$11 each), less durable and difficult to replace in the field, our results clearly show that they can be substituted with an inexpensive (cost ranges $4-$6 each), and durable electric toothbrush. In addition, our results clearly show that the species and buzzing time are significant factors in pollen extraction in artificial buzzing regardless of the vibration frequency and type of instrument. The greater the buzzing duration, the more pollen is extracted, and this result aligns with the previous work that showed a positive correlation of high amplitude and buzzing duration on pollen ejection in *S. rostratum* (De Luca et. al., 2013), a species with similar floral traits as *S. elaeagnifolium* and *S.*

lycopersicum. This is primarily because with longer buzzing time, vibrations are generated and transmitted for a longer time and consequently, release more pollen. However, the discrepancy found between claimed and observed toothbrush frequency restricted us in comparative frequency analysis among both instruments. Between the two species tested, we extracted the higher amount of pollen in *S. elaeagnifolium* as compared to *S. lycopersicum*. The presence of more pollen in *S. elaeagnifolium* might also contribute to high fruit set (Petanidou et al., 2018) and colonization success of this weed species. Our results also showed no differences in pollen amount extracted among different frequency levels. This was somewhat surprising because, recently, it has been found that larger bees that generate high floral vibration frequencies extract more pollen when compared to small bees in a given foraging effort (De Luca et. al., 2019), also suggesting that there may be additional effects of pollinator-specific buzzing that affect pollen removal (Arroyo-Correa et al., 2019).

The Solanaceae plant family is a model for studying self-incompatibility (SI) and the species that exhibit it tend to be obligate outcrossers, and in some cases, SI breaks down with floral age Mena Ali & Stephenson, 2007) leading to selfing, and consequently inbreeding depression, (Kariyat et al., 2011) which plays a significant role in the evolution of mating systems (Kariyat et al., 2013). Most studies on inbreeding and/or genetic variation and their effects on fitness traits require pollen extractions, pollen trait measurements, and controlled pollinations (Nihranz et al., 2019). In the case of tomatoes and other economically important crops, breeding programs also require the use of such methods for pollen extraction and subsequent selection studies. Bumble bees and tuning forks have traditionally been employed for these respectively, but here we show that cheap and easily available electric toothbrushes can be used as a viable alternative to these methods, producing similar results. However, one concern we had was for *S. elaeagnifolium*, the

flowers were collected from the field early in the morning, assuming they weren't pollinated yet (personal observations). Ideally, we would want to grow them also as an experimental population in controlled conditions. Future research should also involve comparative studies on insect pollinators and artificial methods to tease out the differences in the characteristics that separate them, and their consequences on pollen removal and plant fitness. Although a disparity in manually calculated frequency and software calculated frequency was observed in electric toothbrushes, it didn't affect our experimental results showing pollen collection is independent of buzzing frequencies in artificial buzzing.

Table 1: **ANOVA for the pollen extraction**

1A

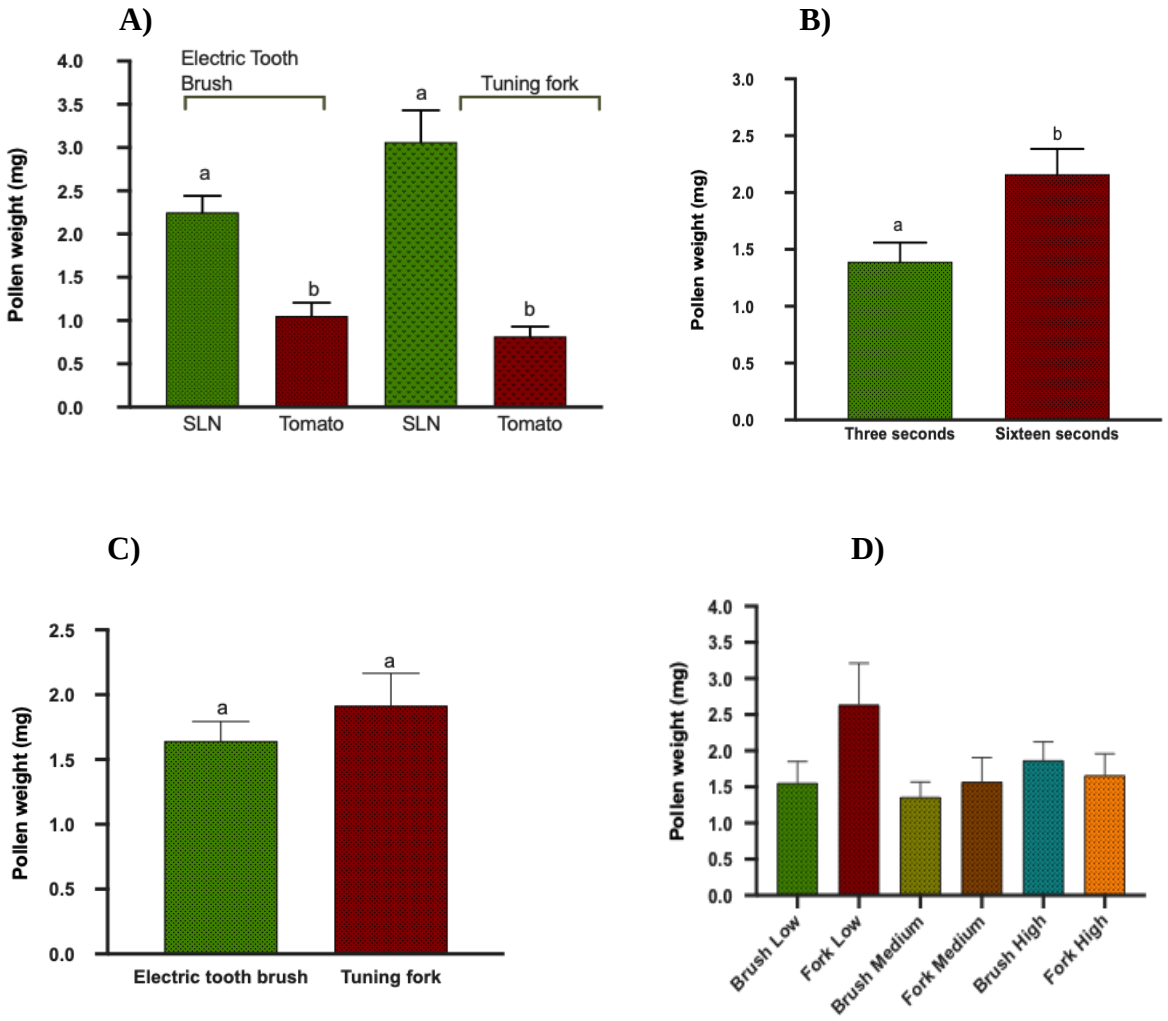
Source	***DF***	***F Ratio***
Model	11	10.7507
Error	84	**Prob > F**
C. Total	95	**<.0001***

1B

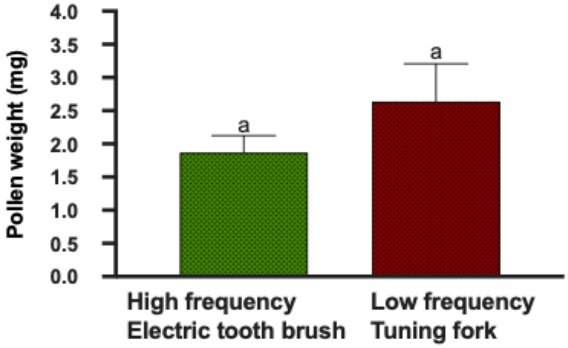
Source	***DF***	***F Ratio***	***Prob > F***
Instrument	1	0.6431	0.4249
Species	1	87.5024	**<.0001***
Time	1	18.4352	**<.0001***
Frequency	2	1.4225	0.2469
Instrument*Species	1	5.3229	**0.0235***

Species*Time	1	0.6864	0.4097
Time*Frequency	2	1.1406	0.3245
Species*Time*Frequency	2	1.0708	0.3474

Figure 1: A-E: The amount of pollen extracted in different treatments



E)



Post hoc Tukey's test ($p < 0.01$) for pollen extraction from A) Tomato and Silverleaf nightshade B) different time intervals C) electric toothbrush and tuning fork at D) different instrument vibrations frequency levels and E) low frequency tuning fork and high frequency electric toothbrush. Means followed by same letters are not significantly different at $p < 0.01$. Different letters show means are significantly different ($p < 0.05$).

Figure 2: Schematic representing artificial buzz pollination using a toothbrush and a tuning fork.

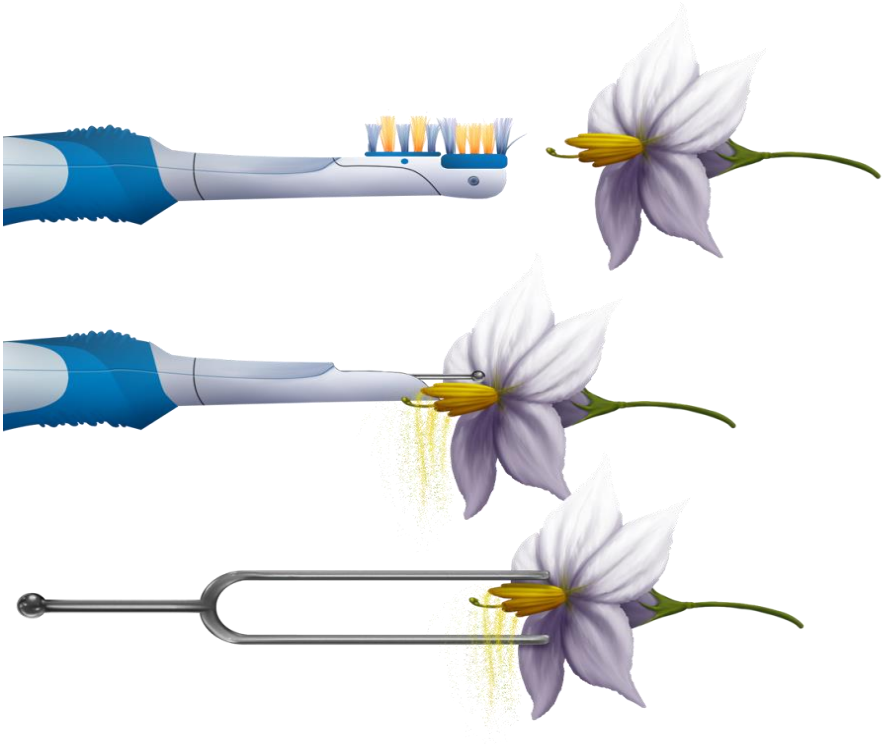


Figure 2: Schematic representing artificial buzz pollination using a toothbrush and a tuning fork. The bristle head of the toothbrush is removed, and the metal nub is held near the anthers to vibrate. In the case of tuning fork, the prongs are held over the anthers or near them. Cartoon by Annette Diaz, University of Texas Rio Grande Valley

CHAPTER III

POLYPHENOL RICH PURPLE CORN PERICARP EXTRACT ADVERSELY IMPACTS HERBIVORE HATCHING,GROWTH AND DEVELOPMENT

Abstract

Plant secondary metabolites play various functional roles in plants including pigmentation, foliar and floral volatile synthesis, hormonal regulation, and direct and indirect defenses. They include terpenoids, phenolics, peptides, and nitrogen and sulphur containing compounds. Among these, phenolic compounds constitute a major group that are common but varies in their specific compound/s distribution among different plant families. Polyphenols, including anthocyanins and tannins are widely distributed and has been well documented for their roles in plant pigmentation and plant defense against herbivores. However, commercialization of any of these compounds are severely hampered by expensive and time-consuming extraction protocols. Using a recently developed inexpensive and easy extraction method using the byproducts of pigmented corn processing, we examined whether the pericarp extracts from cultivated purple and regular yellow corn can affect growth and development traits of *Manduca sexta*, a commonly used herbivore for such studies. Our findings clearly show that regardless of the concentrations, purple corn pericarp extract containing polyphenols (quantified using spectrophotometric assays), negatively affected *M. sexta* egg hatching, larval mass gain, and prolonged developmental time compared to yellow corn extract or control diet. We also found that *M. sexta* caterpillars gained less body mass when allowed to feed on tomato plants sprayed with the

purple corn extract. Purple corn pericarp, an inexpensive by product of corn milling industry can yield a valuable product demonstrating excellent potential bioinsecticide.

Introduction

Plants produce a wide range of secondary metabolites that serve a gamut of functions. These include their role as floral scent that attracts pollinators (Theis & Lerdau, 2003; Raguso, 2004; Ayasse, 2006; Dobson, 2006), foliar volatiles that act as attractants and/or repellants for herbivores and predators (Runyon, 2006; Turlings et al., 2012; Kariyat et al., 2012; 2014; Kariyat et al., 2019), defensive toxins against herbivory (Bennett & Wallsgrove, 1994; Wittstock & Gershenzon, 2002; Harborne, 2007; Rattan, 2010; Campbell et al., 2013), and pigments such as anthocyanins and flavonoids (Lev-Yadun & Gould, 2008; Andersen & Jordheim, 2010) to name a few. Among these functions, role of secondary metabolites (e.g., plant volatiles) in insect mediated species interactions (e.g., herbivory and pollination) have been well documented in various study systems, and these studies have collectively found that these interactions, either mutualistic or antagonistic are affected by a wide range of factors, both abiotic and biotic in nature (Adler et al., 2001; Irwin et al., 2004; Barber et al., 2012; Gorden & Adler, 2013). Moreover, these compounds are also tightly regulated and species specific; both in synthesis and emission (Dudareva et al., 2000; Pott, et al., 2002; Schnepp & Dudareva, 2018). However, we still lack a complete understanding of their potential use as defense compounds in plant-insect interactions.

In addition to the various organic volatile compounds and their well-documented effects, plants also produce phenolics, nitrogen and sulphur containing compounds (Harborne, 2007;

Kumar, 2017). These compounds, while ubiquitous in angiosperms, are also found to vary both qualitatively and quantitatively among various plant families (Balasundaram et al., 2016). For example, coumarins (1, 2-benzopyrones) are common in many dicotyledonous families, including the *Apiaceae, Asteraceae, Fabaceae, Moraceae, Rosaceae, Rubiaceae*, and *Solanaceae* while catechol (1, 2-di-hydroxybenzene) and phloroglucinol (1,3,5-trihydroxybenzene) have been found in leaves of *Gaultheria* species (*Ericaceae*), and as a glucoside in the peel of various citrus fruits (*Rutaceae*; Lattanzio, 2013). These compounds play an important role in cell division, hormonal regulation, various plant biochemical and physiological activities as well as protects them from biotic and abiotic stresses (Zobel & Brown, 1995; Pourcel et al., 2007; Silva et al., 2013). Besides their benefits to the plants against biotic and abiotic stresses, they also impart color to plant tissues. Anthocyanins are vacuolar pigments primarily found in floral organs (e.g. Cyanidin, delphinidin, peonidin, petunidin, pelargonidin and malvidin) and imparts red, blue, black and purple hues (Winkel-Shirley, 2002). Biosynthetically similar to anthocyanins, tannins constitute another group of polyphenols, synthesized via ubiquitous flavonoid pathways (Marles et al., 2003; Xie & Dixon, 2005; Barbehenn & Constabel, 2011). These are structurally $(C6\text{-}C3\text{-}C6)_n$ molecules consisting of two or more flavan-3-ols, majorly found as two structural forms of condensed tannins and hydrolyzable tannins (Bernays et al., 1989). Known for their potential defensive role against insect herbivores (Barbehenn & Constabel, 2011), there is a limited understanding of methods to commercially extract and use them for biopesticide development.

Besides flowers and fruits (Lawrence et al., 1939; Tanaka et al., 2008; Andersen & Jordheim, 2010; Mazza, 2018), colored corns (red and purple varieties) are also a rich source of polyphenols including anthocyanins and tannins (Li et al., 2008; Li et al., 2017). Andes regions

of Peru has been reported to be the origin of purple corn, however, these are produced and consumed across the Argentina, Bolivia, Ecuador, Peru and United States (Lao & Giusti, 2018). Due to its richness in purple pigments, it has long been used to color food and beverages (FAO, 2013; Lao & Giusti, 2018). While a large body of literature has documented the effects of phenolic compounds against microbial and herbivore attacks (Daniel et al., 1999), most of these studies have either used purified compounds (Babbar et al., 2011; Datta et al., 2019), or crude extracts from wild non-cultivated species for testing these effects (Datta et al., 2019). But these compounds in general, are expensive to purify, and /or extremely difficult to extract at an industrial size operation. However, Somavat et al. (2018) developed an efficient and economical methodology to isolate and quantify these compounds from purple corn pericarp, which is essentially a waste product of corn processing. Due to high biological activity of these compounds (anthocyanins in particular) as an antioxidant (Fernandes et al., 2014; Reque et al., 2014), anti-obesity (Esposito et al, 2015; Johnson et al., 2016) anti-cancer (Fernandes et al., 2014) and anti-inflammation compounds (Esposito et al., 2014), it is plausible to expect that they may also have other effects- including insecticidal properties. Few other studies have explored their possible applications as an anti-herbivore compound (Lee et al., 1987; Close & Beadle, 2003; Lev-Yadun & Gould 2008). Collectively, they have found that these compounds protect plants through different characteristics like aposematic coloration, defensive mimicry, camouflage, and as direct toxins etc. (Close & Beadle, 2003; Lev-Yadun & Gould 2008; Freeman & Beattie, 2008). However, none of these studies have used anthocyanin extracts to test its effectiveness against insect herbivores.

Continuing the long quest of identifying bioactive compounds with insecticidal activity (Williams & Mansingh, 1993; Zounos et al., 1999; Sampson et al., 2005; Moreira et al., 2007,

Atawodi & Atawodi, 2009; Zoubri & Baaliouamer, 2014; Ahmed et al., 2018; Aljaghthmi et al., 2018; Campos et al., 2019), we hypothesized that the pericarp extracts in its semi-processed form may also negatively affect herbivore growth and development. We have consistently used such methodology to disentangle the effects of plant defenses (direct and indirect; Kariyat et al., 2012; Kariyat et al., 2013; Portman et al., 2015; Kariyat et al., 2017; Kariyat et al., 2019) on herbivore traits.

To test our hypotheses, we used Tobacco hornworm caterpillars (*Manduca sexta* L.), a commonly used herbivore for examining growth and development traits (easy to rear in lab with short lifecycle and distinct growth stages; Kariyat et al., 2014; 2017; 2019). We designed these experiments with three treatments of a) artificial diet enriched with purple corn pericarp extract b) artificial diet enriched with yellow corn pericarp extract, as a positive control c) a control diet without any extract added as an additional control, to remove any extract effects.

Material and Methods

Corn dry milling for pericarp recovery

One kg lab-scale dry milling protocol proposed by Rausch et al. (2009) was used with minor adaptation to recover purple and yellow corn pericarp. An electronic moisture meter (GAC 2500-UGMA, DICKEY-john, Auburn, IL) was used to estimate the corn moisture content prior to the processing. The amount of water required to increase the corn moisture content to 23.5% was first determined. The corn kernels were then mixed with appropriate amount of water and tempered in sealed 1-gallon plastic bottles rotated horizontally at 1 rpm for 20 min to allow for complete water absorption by the kernels. After tempering, the kernels were passed through a

specially designed custom-made lab-scale horizontal drum degerminator. The degermed fractions were collected in two plastic boats and dried in a convection oven for 2 hr at 49°C. After conditioning, the degermed fractions were sifted on a 5-mesh screen for 3 min using a lab scale sifter (Model RX-812, WS Tyler, Mentor, OH).

The fraction collected at the top of the screen (+5) and the material which passed through the screen (-5) were separated and roller milled twice using a lab scale roller mill (Micromill, Apollo Machine & Products Ltd., Saskatoon, Canada). The roller milled +5 fraction was further sieved on a 10-mesh screen for 3 min and +10 fraction was collected as germ/pericarp while -10 fraction constituted large grits. Similarly, -5 fraction was also sieved for 3 min on a 10-mesh screen and +10 material was collected as germ/pericarp and added to the same fraction collected earlier. The -10 fraction was sifted on a 25-mesh screen and +25 fraction was collected as small grits while -25 fraction constituted as fine. Finally, the germ and pericarp collected from +5 and -5 fractions were sifted using a lab scale aspirator (Model 6DT4, Kice Industries, Wichita, KS) to separate pericarp from the germ fraction (Fig. 3).

Pericarp extract preparation

Purple and yellow dent corn pericarp samples (5 g) recovered from dry milling were steeped in glass bottles containing 100 mL deionized water. These bottles were incubated at 52°C for 24 hr and stirred at 100 rpm to prepare liquid extracts as reported by Li et al. (2019). After steeping, the extracts were centrifuged at 5000 rpm for 5 min and the filtrate was collected for mixing in caterpillar diet.

Quantification of anthocyanins and polyphenols:

The pH differential method was used to quantify the amount of total monomeric anthocyanins present in purple corn pericarp extract using a 96 well microplate reader (Multiskan Sky Microplate Spectrophotometer: #51119600, Thermo Fisher Scientific, MA, USA) in 6 replicates (Lee et al., 2005; Li et al., 2017). Dilutions of pericarp extract (1:20) was made by taking 50 extract and 950 µ buffers at two different pH ranges (pH 1.0, 0.025 M KCl, and pH 4.5, 0.40 M sodium acetate). These diluted solutions at each pH were transferred 6 replications to a 96 well plate and absorbance was read at 520 nm and 700 nm. The concentration of total monomeric anthocyanins (mg/L) was calculated by using the following formula and reported as mg of cyanidin-3-O-glucoside (C3G) equivalent per kg pericarp:

Total monomeric anthocyanins (mg/L) = (A*MW*D*1000)/(ε*0.45*PL)

where A = (A520nm – A700nm) at pH 1.0 – (A520nm –A700nm) at pH 4.5, MW is molecular weight, i.e., = 449.2g/mol, D is the dilution factor, 1000 is the conversion factor from grams to milligrams, ε is molar extinction coefficient for C3G = 26900 L/mol, PL = 1 cm path length, and 0.45 was used as a conversion factor for adapting the established method to a plate reader.

The polyphenol concentration of purple and yellow corn pericarp extracts was measured in six replicates employing Folin-Ciocalteu's method adapted to a microassay as reported by Heck et al. (2008). Extracts were diluted (1:10) using deionized water, and 50 µl of diluted extract, standard and blank were added to 125 µl of Folin-Ciocalteu's phenol reagent. After 5 min, 750 µl of 20% Na_2CO_3 was added and the solution was allowed to react for 10 min. The absorbance was measured at 690 nm using Multiskan Sky Microplate Spectrophotometer (Thermo Fischer Scientific, MA, USA). The polyphenol concentration was expressed as g of gallic acid/ kg pericarp.

Insect colony

Manduca sexta L. eggs were bought from a commercial vendor (Great Lake Hornworm Ltd. Romeo, Michigan, USA) and were allowed to hatch in Petri dishes (8.8 cm diameter, Mid Sci, MI, USA) on a moist filter paper in a growth chamber (16 h light/8 h dark, 25^0C day/22^0C night, 65% RH). We also have an active colony in the lab, and we periodically introgress with wild caught *M. sexta* (native to the region) to minimize inbreeding effects. After hatching, the larvae were moved to plastic containers (35 cm long x 10 cm high x 15 cm wide) and reared on an artificial diet (Kariyat et al., 2019). Newly molted larvae were used according to the experiment requirements.

Artificial diet

M. sexta caterpillars were allowed to feed on a wheat germ-based artificial diet prepared as per recommendations from the supplier (Frontier Scientific Services, DE, USA). We boiled 1000 ml of water to 85 °C then added 200 g of artificial diet and mixed thoroughly until there were no more clumps (Kariyat et al., 2019).

Plant Material

Tomato seeds (*Solanum lycopersicum*: Variety: Valley Girl), were bought from an online vendor (Johnny's Selected Seeds, Maine, USA). Seeds were in sown in growth media (Sunshine professional growing mix: Sun Gro Horticulture Canada Ltd., MA, USA) filled in the plastic trays (51.435 cm * 25.4 cm) and covered with thin transparent film to maintain optimum temperature i.e. 27^0C for germination. At 2-4 leaf stage, the seedlings were repotted individually to bigger pots (15.24 cm diameter) and kept in green house at 25^0C and 65% RH. Plant nutrient requirements were met by applying OMRI (Organic Material Review Institute, OR, USA) listed

organic fish emulsion fertilizer (NPK 5:1:1, Alaska Fish Fertilizer, Pennington Seed, Inc., USA) once every two weeks. Plant growth and health was maintained until plants were ready for experiment.

Detailed experimental design

We used three diets differing by either the presence or absence of pericarp extracts. Out of three groups, one diet group without any pericarp extract was used as a control and the other one group was fed diet containing yellow dent corn pericarp extract (Fig. 4). Purple corn and yellow corn pericarp extracts were prepared by steeping 5 g pericarp of each corn type in 100 ml of water (5 g/100 ml), respectively while control diet was prepared without adding any extract. In order to prepare each treatment diet, we boiled 1000 ml of water to 85^0C, then added 200 g of artificial diet (Frontier Scientific Services, DE, USA) and mixed them thoroughly. During mixing, we added 1 g of agar and 40 ml of respective pericarp extracts. The diet was allowed to cool before adding the extracts and mixed thoroughly until there were no lumps. The diet was allowed to solidify and then made into ~1 cm^3 cubes and used for the experiments.

Bioassays:

All diet-based caterpillar assays were conducted in lab, under room temperature of 27°C and RH of 65%. To begin with, different concentration diets were allocated separately to different petri dishes comprising respective treatments. For each individual petri dish, diet was cut into ~1cm^3 cubes and placed in corresponding petri dishes and labeled by treatment and date. Different caterpillar growth and development parameters were examined as explained below:

a. **Egg hatching:** To test the effect of purple corn pericarp extract on egg hatching, 2-day old viable eggs were kept on top of ~1cm^3 cube of diet in different petri dishes. After 24 hrs, the

eggs were examined for hatching rate. The same data was recorded after 48 hrs and 72 hrs as well. Each treatment had a sample size of 30.

b. First instar survival: Newly hatched first instar caterpillars (N = 15) were allowed to feed on three treatment diets. After 24 hours, the larval movement was used as an index to determine if the larvae were dead or alive. We recorded the number of alive larvae for three days until they became second instar.

c. Mass: We pre-weighed twenty-five (N = 25) first instar caterpillars and put them on each treatment diets and then recorded caterpillar weight once every other day until they had reached the 5^{th} Instar larval stage and were ready for pupation.

d. Mass gain: Using the following equation we calculated two types of mass gain: i) instar specific mass gain over previous mass and ii) Final mass gain over initial mass

$$\text{Mass gain} = \frac{(\text{Final mass} - \text{Initial mass}) \times 100}{\text{Initial mass}}$$

e. Mortality: While recording mass, the caterpillars were also examined to identify whether they were alive or dead. Caterpillar mortality was recorded by checking their body movements and the dead specimens were removed from the experiment.

f. Feeding behavior: Apart from examining larval mass, we also examined their diet feeding preference. For this, on every other day just before the mass measurements, we observed if the caterpillars were feeding on the diet pellet or not. The caterpillars feeding on diet were recorded as on diet or off the diet.

g. Ethovision: To examine in detail whether the caterpillars were able to distinguish two different treatment diets based on color, odor or texture, we also ran a behavioral assay experiment using the automatic video tracking Noldus Ethovision set-up (EthoVision XT 14: Noldus Information Technology, Wageningen, The Netherlands) mounted on an open field-testing arena. The arena

(74.93 x 59.18 cm) was made up of wooden material (Thermo Fisher Scientific, MA, USA) with bottom side covered with paper sheet from inner side to avoid any friction (Denninger, 2018). In this experiment, 1 cm^3 cubes of each diet was kept 10 cm apart in the center of an arena. Each trial consisted of 4 hr starved two newly molted third instar caterpillars (reared on artificial control diet) released at an equal distance away from diet cubes and by recording their movement, and behavior parameters for an hour. These parameters included total distance (cm) travelled by each caterpillar, their velocity (cm/s) as well as feeding preferences. After every trial, whole arena was wiped with ethanol (50% v/w) and diet positions were interchanged. We ran a total of ten trails constituting twenty caterpillars for both treatments.

h. **Time to pupate:** Once the larvae reached 5^{th} Instar, and started to clearly show the dark pulsating vein on dorsal side, stopped feeding and were wandering in the petri dishes, larvae were transferred to plastic container with wood shavings (Natural Aspen small animal bedding: Petco Animal Supplies, Inc., San Diego, CA, USA) to provide them a dark environment to undergo pupation. Time to pupate was recorded for each caterpillar counting from the date of transferring to plastic boxes to the date when they get pupated (collected every day in morning).

i. **Diet switch assay:** In addition to the mass-based assays with three treatments, we also ran a diet switch assay. For this, 45 first instar *M. sexta* caterpillars were pre-weighed and 30 of them were placed on a control diet and other 15 on a purple corn extract diet and were allowed to feed and develop. After they molted to third instar, we removed 15 caterpillars from control diet, weighed and transferred them to anthocyanin rich diet, and moved all 15 caterpillars from purple corn extract diet back to control diet. The three new diet treatments (control, control moved to purple corn extract diet, and purple corn extract diet moved to control diet) were again continuously monitored and their mass measured at regular intervals. The reasoning for this experiment was to

test whether the effects of purple corn diet, if any can be compensated by switching them to regular diet or vice versa. Ideally, we should have additional treatments with different growth stages for the switch experiment, but logistical constraints prevented us from doing so.

j. **Spray experiment:** To test whether pericarp extract has any insecticidal properties against *M. sexta* larvae when applied on plants, we conducted a spray experiment in which tomato plants were sprayed twice with different pericarp extracts. The experimental design was consisted of three treatments (N=8) of i) 4% diluted purple corn extract (v/v) ii) 4% diluted yellow corn extract (v/v) and iii) water (control). To start with, 4 weeks old tomato plants were fully sprayed from all directions with treatment solutions at least 24 hours before starting the experiment. We put pre-weighed two third instar caterpillars per plant comprising a total of 8 caterpillars per treatment. After 48 hrs, the caterpillars were removed from plants and weighed again to calculate mass gain. Afterwards, plants were again sprayed with treatment extracts and same caterpillars were allowed to feed for the next 48 hours.

Statistical Analysis

Due to non-normal nature of our data set, we used nonparametric tests followed by post hoc pairwise comparisons to analyze most of our data. For anthocyanins, polyphenols, and tannin analyses we used Mann-Whitney tests. Larval diet treatments were factors whereas caterpillar egg hatching, survival, caterpillar mass, mass gain, time to pupation, feeding preference were response variables. Separate analyses for caterpillar mass and mass gain were also carried out at each point of their development. On the other hand, feeding preference (On/Off) data was analyzed using chi-square ($\chi 2$) test (Kariyat et al., 2019). For Ethovision experiments, the distance and velocity data were analyzed using unpaired t-test. All the data sets were analyzed

using the statistical softwares, JMP (SAS institute, NC, USA), and GraphPad PRISM (La Jolla, California).

Results

Quantification of anthocyanins, tannins and total polyphenols

Based on quantification methods described above, we found that purple corn pericarp extract contained significant higher concentration of anthocyanins (Mean ± SE: 4710.08 ± 43.13 mg C3G equivalent/kg pericarp; (Mann-Whitney U Test, p = 0.0022; Fig 5A) and total polyphenols (Mean ± SE: 186.59 ± 1.03 g gallic acid equivalent/kg pericarp; Mann-Whitney U Test, p = 0.0022; Fig. 5B).. In addition, no anthocyanins were detected in yellow corn pericarp extract (Mean ± SE: -5.69 ± 4.23 mg C3G equivalent/ kg pericarp) (Fig. 5A). The results for anthocyanins quantified in yellow corn extract were negative. There were no anthocyanins present in yellow dent corn extract and since the spectrophotometric method for the quantification of anthocyanins utilized absorbance differences at two different wavelengths, these results came out with negative numbers. Results of tannin quantification also followed similar trends. Results of tannin quantification was also similar. Purple corn extract had more than 14 times the amount of tannins (Mean ± SE; 36964.23 ± 9879.10 mg catechin equivalent/kg pericarp) when compared to yellow corn extract (Mean ± SE; 2573.30 ± 687.74; Mann-Whitney U Test, p <0.0001; Fig. 5B). Collectively, it was clear that purple corn pericarp extract contained higher amounts of phenolic compounds when compared to yellow corn extract. Total polyphenol concentration of pericarp extract from yellow dent corn was four times lower

compared to purple corn pericarp (Mean ± SE: 47.77 ± 0.58 g Gallic acid equivalent/ kg pericarp) (Fig. 5C).

A total of 40 ml purple corn pericarp and yellow corn pericarp extract were mixed in respective insect diets. The diet prepared from purple corn extract contained approximately 0.94 mg C3G equivalent anthocyanins, 373.18 mg gallic acid equivalent of polyphenols and 73.92 mg catechin equivalent of tannins. On the other hand, the diet prepared from yellow corn pericarp extract contained 95.54 mg gallic acid equivalent of polyphenols, 5.14 mg catechin equivalent of tannins and no anthocyanins.

Bioassay results

a) Effects of purple corn pericarp extract on egg hatching and Ist instar mortality

We found that egg hatching proportion on diet having purple corn pericarp extract was significantly low (F value = 26.04, $p < 0.0001$) compared to other treatments (eggs kept on yellow corn extract and control diets; Fig. 6A). Only 6% eggs were hatched on purple corn extract diet whereas yellow corn and control diet had higher egg hatching rates of 70% and 54%, respectively. However, we didn't find any significant difference (F value = 6.286, $p = 0.1529$) in mean number of first instar caterpillars surviving on different treatment diets, implied that there was no significant effect of pericarp extract on Ist instar mortality (Fig. 6B).

b) Effects of purple corn pericarp extract on larval mass

We recorded larval mass once every other day until caterpillars reached 5^{th} Instar comprising a total of 9 mass measurements throughout whole larval stage. Except for the Ist mass, we found a significantly low larval mass throughout whole larval stage for specimens feeding on purple corn

extract diet compared to yellow corn extract and control diet (Supplementary Table 2) (Fig. 7A-7B).

c) Effects of purple corn pericarp extract on larval mass gain

We calculated total eight mass gains (mass gain over previous mass and mass gain over Ist instar) through whole larval stage. A significant negative impact of purple corn pericarp extract on mass gains compared to yellow* corn extract and control diet was detected (Supplementary Table 3 and Table 4) (Fig. 8A-8C). However, purple corn pericarp extract fed fifth instar caterpillars gained significantly more mass ($p = 0.0004$) compared to individuals fed on control diet (Fig. 8C).

d) Effects of purple corn pericarp extract on larval feeding preference

Starting from first instar to fifth instar larval stage, we recorded total eight feeding preference observations on every other day. In this, we examined caterpillar position in the petri plate (On/Off diet). It was found that a significant number (F value = 26.49, $p < 0.0001$) of purple corn pericarp extract fed caterpillars stayed away from the diet compared to those on yellow corn extract and control diets, implying that purple corn pericarp extract was their least preferred diet compared to yellow corn extract and control. However, we didn't find any significant difference among yellow corn extract and control diet preference ($p = 0.8563$) (Fig. 9).

e) Ethovision

In ethovision experiments, there was no difference in velocity and distance travelled by larvae for any diet preference. However, when we visually analyzed the video clips of each trial, we observed that at first both caterpillars were moving towards the diets but once they tasted purple corn pericarp extract diet, they moved away from the diet and no longer fed on it (see video 1).

This clearly shows that caterpillars were completely unable to distinguish both treatment diets based on color, odor and texture (Fig. 4I-4J).

f) Effects of purple corn pericarp extract on pupation time

Examining the effect of purple corn pericarp extract on pupation time, we found that purple pericarp fed larvae took significantly greater number of days (F value = 50.91, p <0.0001) compared to yellow corn extract and control diet fed caterpillars. The pupation time in purple pericarp extract fed caterpillars was increased to 34 days whereas control and yellow corn extract fed caterpillars had 30 and 27 days, respectively (Fig. 11).

g) *M. sexta* mass on switched diet experiment

Similar to other caterpillar mass results, we found that feeding on purple corn extract diet affected caterpillar mass (t = 2.226, p = 0.0329), although, they had similar mass at the start of the experiment (Fig. 12A). More interestingly, once the diets were switched, it was found that control diet fed caterpillars continued to havegreater mass compared to caterpillars switched to purple corn extract diet from control diet and vice-versa (Fig. 12B). The data was not significantly different from each other during first data collection (F value = 3.445, p = 0.0508) but then it was significantly different after 3 days (F value = 4.740, p= 0.0226), showing the continued effects of early exposure to purple corn extract diet (Fig. 12B). Both pre and post exposure to purple corn extract diet in relation to control diet had adverse consequences for caterpillar growth.

h) Effects of plant sprayed purple corn pericarp extract on larval mass gains

Larval mass gain after first spray was significantly different only between purple corn and yellow corn treatments (p = 0.0036) (Fig. 13A). However, there was no significant difference in purple corn and control larval mass gains (mass gain 1: p = 0.4884 and mass gain 2: P = 0.279)

(Fig. 13A-13B) after both sprays. The mass gain after spray 2 was not different, with more mass gain for caterpillars fed on yellow corn extract sprayed plants (F value = 5.276, p = 0.0671) (Fig. 13A-13B).

Discussion

There were negative effects of feeding anthocyanin/polyphenol rich purple corn pericarp extract on growth and development of *M. sexta* caterpillars. We tested the effects of pericarp extract throughout the larval stages including egg hatching, first instar survival, feeding preference, mass, mass gain, and time to pupation. As eggs are the earliest life stage on which subsequent stages depend, low egg hatching percentage (6%) due to diet enriched with purple corn pericarp extract signifies its importance to limit subsequent larval growth and development (Fig. 6A). This could possibly be due to developmental abnormalities induced by phenolic compounds present in their diet, previously documented in plant-based diet experiments (Mira & Bernays, 2002). Similarly, researchers in a previous study have reported that the presence of acyl sugars in artificial diet (isolated from glandular phenotype of *D. wrightii*) caused reduced growth of *M. sexta* (van Dam & Hare, 1998b).

However, we didn't find any significant difference on Ist instar survival rates on different diets (Fig. 6B). A possible explanation for neonate survivorship and egg mortality, might be due to increased enzyme production in early instars induced by secondary metabolites present in diet. For example, in gypsy moth (*Lymantria dispar*) larval neonates, the consumption of phenolic glycosides led to increased activity of esterase enzymes, resulting in higher survival rates (Lindroth & Weisbrod, 1991; Zalucki et al., 2002). Moreover, the first instar larvae of *P.*

glaucus were not affected by the neolignans (4,4'-diallyl-2'3'-dihydroxybiphenyl ether: a biphenyl ether) isolated from magnolia (*Magnolia virginiana*) hosts (Nitao et al., 1992; Zalucki et al., 2002).

Similar to the results of Datta et al. (2019), we found that larvae fed on purple corn pericarp extract containing diet had low mass and mass gain in early larval stages, suggesting its effectiveness as a potential biopesticide (Fig. 7A-7B, 8A-8C). The low mass and mass gain might be primarily due to either the negative impact of pericarp extract ingested by them through diet or its antifeedant properties (Koul et al., 1997; Koul et al., 2000; Bullangpoti et al., 2012; Kaur et al., 2016; Dastranj et al., 2018; Datta et al., 2019). To confirm this, we did a choice assay with ethovision in which we found that there were no differences in caterpillar movement and choice (velocity and the distance travelled) between purple corn pericarp extract and control diet (Fig. 10A-10B), suggesting that the impacts are possibly not due to olfactory or tactile cues. However, analyzing video recordings (see video 1) collected during Ethovision experiment, it was found that the caterpillars readily fed on pericarp extract diet, but possibly due to unpalatability of the food, they stopped feeding, which eventually resulted in affecting their growth and development. These results imply that larvae were unable to distinguish between two types of diets based on color, odor and texture. In addition, the presence of significantly low number of caterpillars on pericarp diet found in our feeding preference analysis (on/off data; Kariyat et al., 2017) are possibly due to the antifeedant properties of pericarp extract diet and hence they stayed away (Fig. 9). Significant reduction in larval mass in response to nutritional stress is speculated to negatively affect their adult fitness and reproduction. Moreover, Diamond and Kingsolver (2010) found that low-quality host food resulted in decreased immune response in *M. sexta* larvae making them more susceptible to pathogens.

Surprisingly, significant increase in mass gain of fifth instar pericarp extract larvae (Fig. 8C) suggests their compensatory growth in response to food stress. As previous studies have shown that nutritionally stressed caterpillars make some physiological adjustments, i.e. producing detoxifying enzymes (Brattsten 1988; Snyder et al. 1994) or altering food efficiency use (Slansky and Feeny 1977; Slansky and Wheeler 1989, 1991; Woods et al., 1999), we also speculate similar mechanisms resulted in rapid growth of late instar caterpillars. However, the possible toxicity encountered during the early stages significantly derailed their progress in mass gain and other traits, even after compensatory feeding in later stages. These findings are consistent with Dam et al. (2000) who found the compensatory feeding and increased larval mass in *M. sexta* after shifting MeJA (defensive compound) treated plants to untreated control plants. Adding to the compensatory growth, we also found that larvae fed on pericarp extract took significantly longer time to pupate (Fig. 11) compared to other treatments, suggesting developmental delay. Previous studies have reported similar effects of toxic diets. For example, *Myrtillocactus geometrizans* extracts (rich in sterols and triterpenoids) delayed pupation and moulting in fall armyworm (*Spodoptera frugiperda;* Torres et al., 2003; Cespedes et al., 2005). A possible explanation for the increased developmental time (time to pupate) may also be due to midgut phenoloxidase inhibtion and moulting sclerotization toxicity, caused by phenolics (Kubo & Kinst-Hori, 1999; Kubo, 2000). It is well understood that resource acquisition in larval stage plays a critical role in an individual's adult condition and henceforth in life history and fitness (McNamara & Houston 1996; Saastamoinen et al., 2013), we speculate that pericarp extract might have potential cascading effects on pupal mass, adult fitness, and dispersal as well as their mating success, an area we are currently examining.

In spray experiment, although we didn't find any significant difference in caterpillar mass gain among three treatments (Fig. 13A-13B), we did see a pattern of low mass of larvae on purple corn sprayed plants suggesting its partial effectiveness. The insignificant difference may be due to low sample size and lower extract solution concentration (4%), same as that was mixed in caterpillar diet. Also, since artificial diet is highly concentrated and directly ingested by the larvae whereas in plants, these low concentration extracts might not stick to the plant and not ingested sufficient amounts to be effective. Future studies could explore dose dependent effects of pericarp extract against *M. sexta* larvae.

In addition, since purple corn contains anthocyanins and a number of other polyphenolic compounds such as tannins, phenolic acids and flavonoids (Ramos-Escudero et al., 2008), more research is needed to study whether these effects are due to an individual compound or/and due to a synergistic effect of these compounds in plant-herbivore interactions. Different compounds present in the extract should be isolated and their dose dependent effects should be individually studied in all possible combinations. The ability of generalist herbivores to exhibit plasticity in response to diet quality variations gives us directions to study if pericarp extract affects integrated compensatory responses of insect herbivores (Orlando et al. 2009; Couture et al., 2016). Also, apart from lepidoptera pests, it will be interesting to test the effects of extract against pests with different feeding habits i.e. aphids, white flies etc. And finally, future studies are also required for better understanding of the underlying mechanisms/mode of action of anthocyanin and polyphenol rich purple corn pericarp extract at the molecular level, some of the areas we are currently exploring.

Table 2 Details of Statistical tests used to study the effect of defense metabolites rich pericarp extract on egg hatching, growth and development of Tobacco hornworm (*Manduca sexta* L.) Significant results with P values <0.05 are in bold.

Parameter	**Test**	**Test Statistics**	**P- value**
Total Anthocyanins	Mann-Whitney test	Mann-Whitney U=0	**0.0022**
Total Tannins	T test	t statistic = 33.33	**<0.0001**
Total Polyphenols	Mann-Whitney test	Mann-Whitney U=0	**0.0022**
Egg hatching	Kruskal-Wallis Test	Kruskal-Wallis Statistic = 26.04	**<0.0001**
1st Instar survival	Kruskal-Wallis Test	Kruskal-Wallis Statistic = 3.756	0.1529
Larval survival	Kruskal-Wallis Test	Kruskal-Wallis Statistic = 6.557	**0.0377**
Time to pupate	Kruskal-Wallis Test	Kruskal-Wallis Statistic = 50.91	**<0.0001**
Ethovision	t-test	t statistic = 0.3063	0.7629
Diet switch (larval mass)	One Way ANOVA	F value = 4.740	**0.0226**
Larval mass gain Spray	Kruskal-Wallis Test	Kruskal-Wallis Statistic = 10.54	**0.0051**
Larval frass after Spray	One Way ANOVA	F = 5.414	**0.014**

Fig. 3 Process schematic of lab scale dry milling protocol (adapted from Rausch 2009).

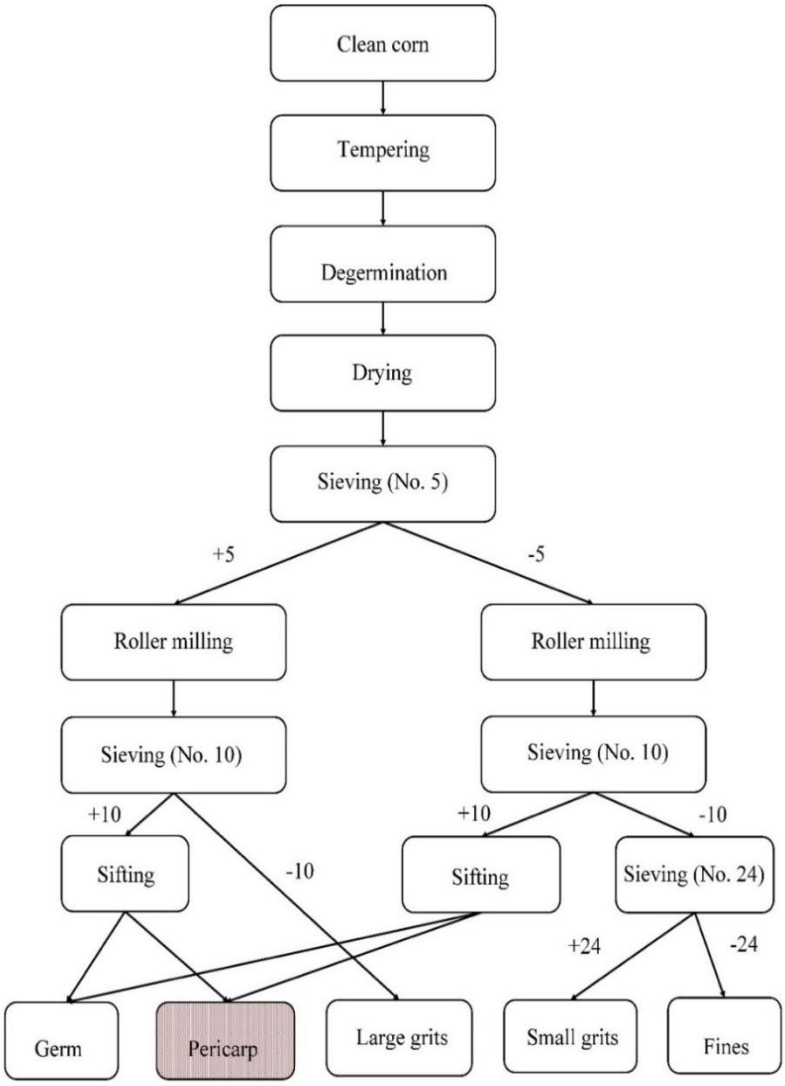
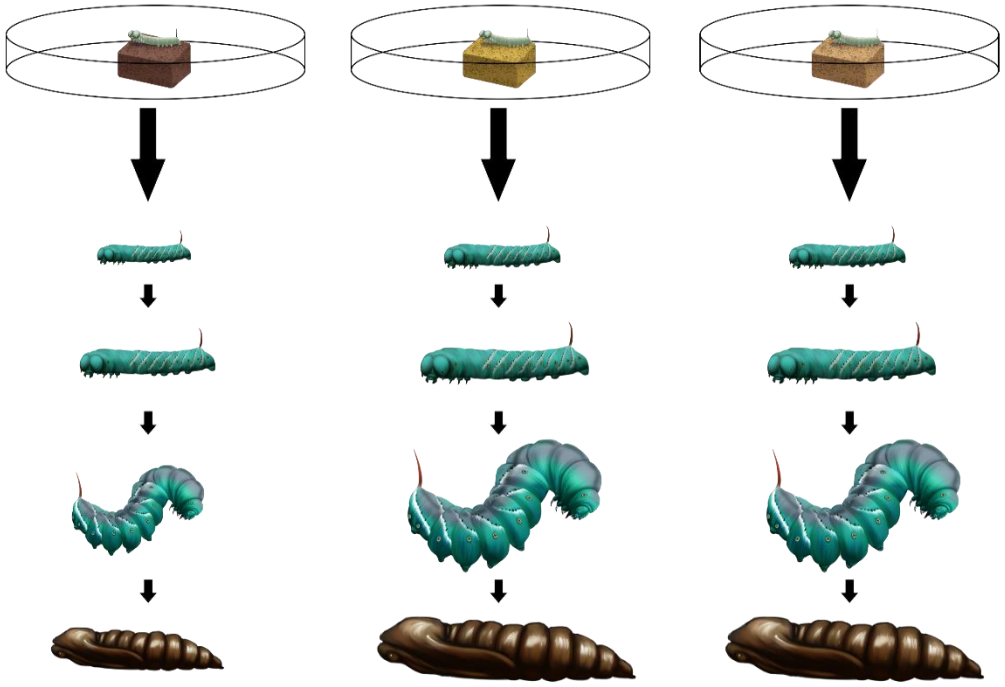
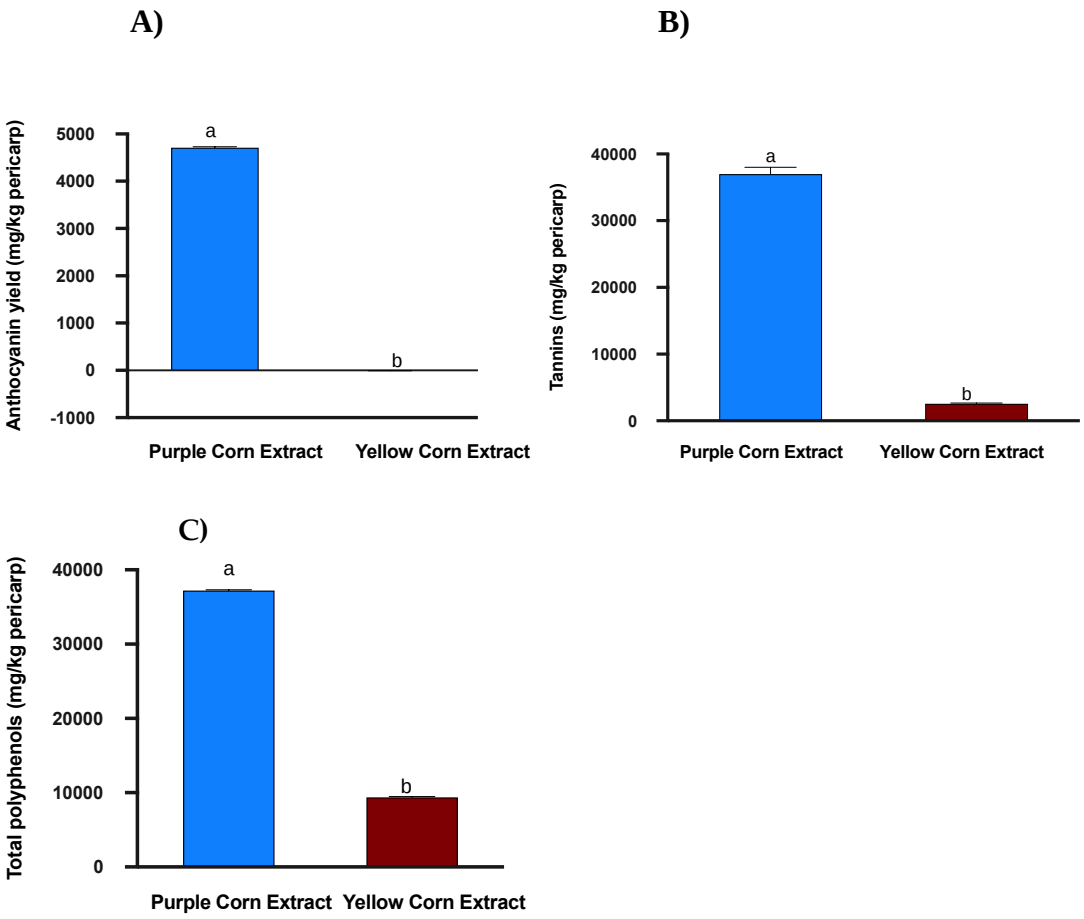


Fig. 4 Schematic representation of the experimental design to test the effects of pericarp extract containing diets on *M. sexta* caterpillar growth and development.



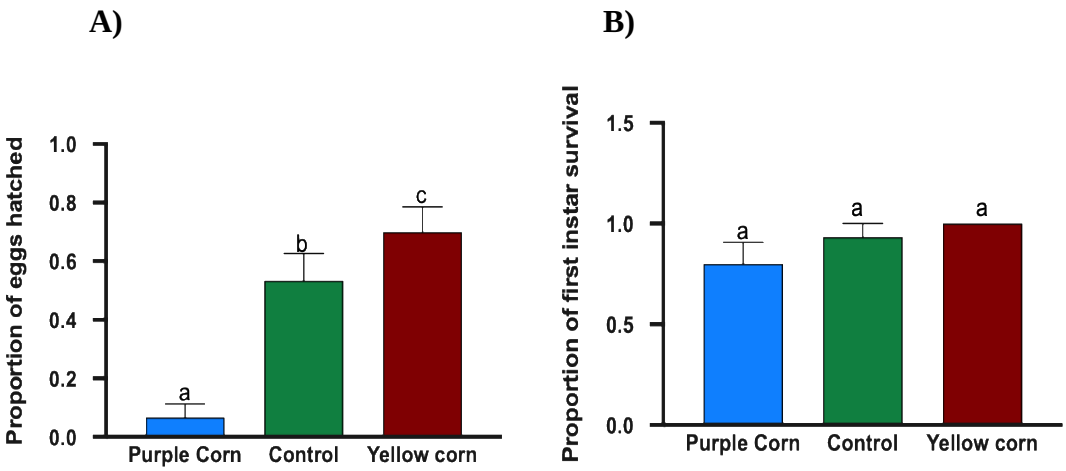
Schematic representation of the experimental design to test the effects of pericarp extract containing diets on *M. sexta* caterpillar growth and development. The caterpillars were randomly assigned to one of the three treatments (diet with anthocyanin rich purple corn pericarp extract added, diet with yellow corn pericarp extract added, and control diet). Schematic drawn by Annette Diaz (UTRGV, Edinburg, Texas, USA)

Fig. 5. Quantification of anthocyanins and total polyphenols



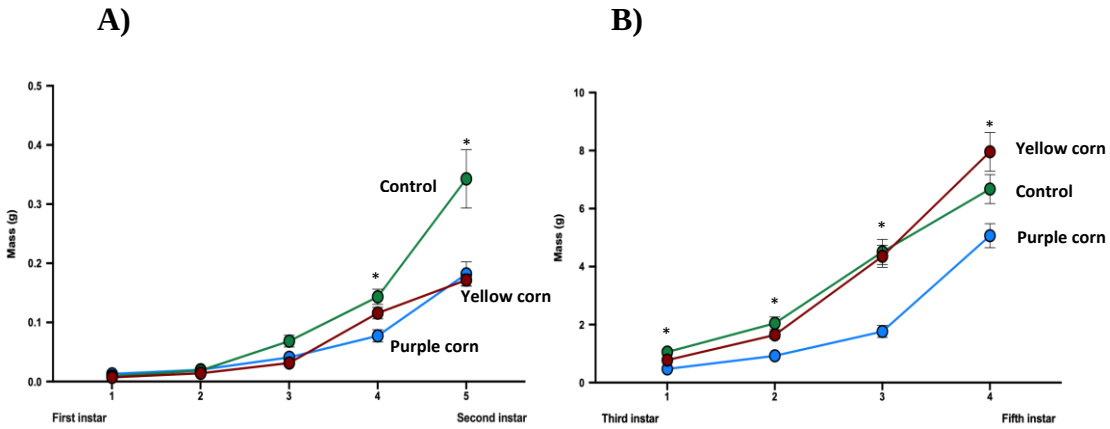
Results of Mann-Whitney Statistical Analysis Test for quantification of A) anthocyanins and B) tannins and C) total polyphenols present in purple corn and yellow corn pericarp extracts. Means followed by different letters are significantly different at $p < 0.05$

Fig. 6. Effects of purple corn pericarp extract on egg hatching and Ist instar mortality



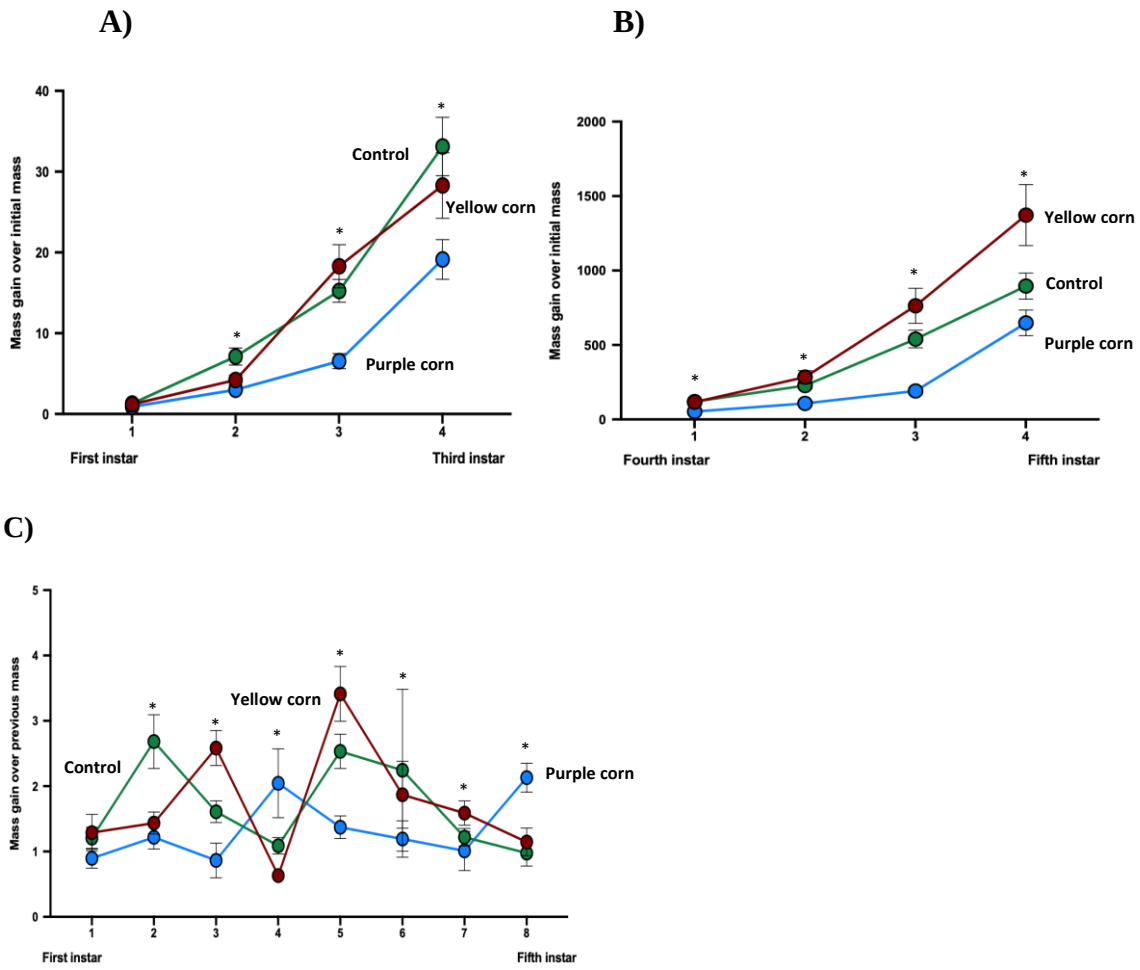
Results of Kruskal-Wallis tests, Dunn's multiple comparison test ($p < 0.05$) for the effect of purple corn pericarp extract on mean A) number of eggs hatched B) Ist instar survival. Means followed by same letters are not significantly different at $p < 0.05$ while different letters show means that are significantly different at $p < 0.05$

Fig. 7. Effects of purple corn pericarp extract on larval mass



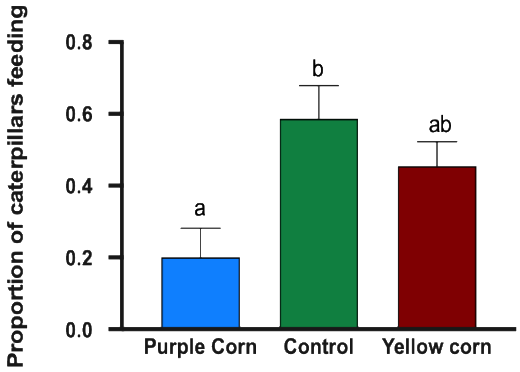
Results of Kruskal-Wallis tests, Dunn's multiple comparison test ($p < 0.05$) for the effect of purple corn pericarp extract on mean larval mass from first instar to fifth instar stage. Due to huge variation of larval mass scale from ist instar to fifth instar, we splited larval mass analysis into two parts i.e. A) Ist instar to second instar larval mass B) third instar to fifth instar larval mass. Groups with *statistical significance at p value of 0.05 for the model.

Fig.8. Effects of purple corn pericarp extract on larval mass gain



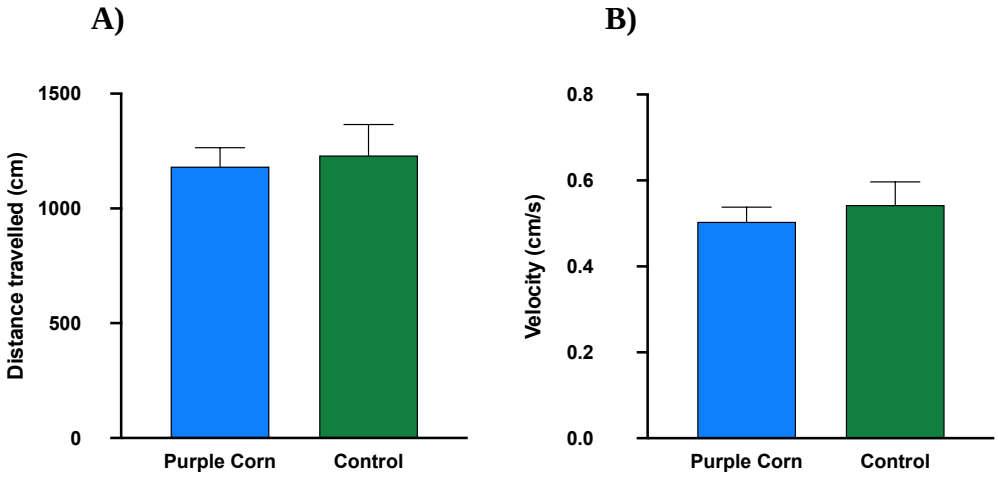
Results of Kruskal-Wallis tests, Dunn's multiple comparison test ($p < 0.05$) for the effect of purple corn pericarp extract on mean larval mass gain over initial mass from first instar to fifth instar stage. Due to huge variation of larval mass scale from ist instar to fifth instar, we splited larval mass gain analysis into two parts i.e. A) mass gain from Ist instar to third instar B) mass gain from third instar to fifth instar. C) mean larval mass gain over previous mass. Groups with *statistical significance at p value of 0.05 for the model

Fig. 9. Effects of purple corn pericarp extract on larval feeding preference



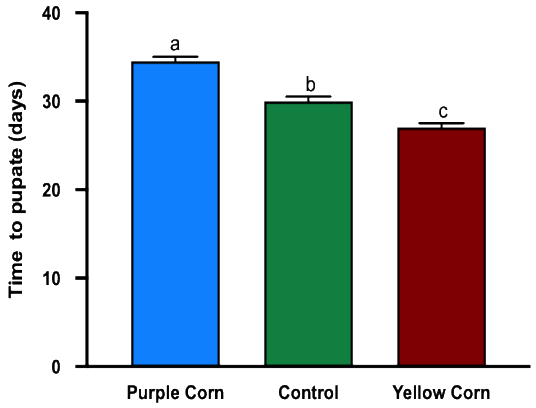
Results of Kruskal-Wallis tests, Dunn's multiple comparison test ($p < 0.05$) for the effect of purple corn pericarp extract on lraval feeding prefence. Means followed by same letters are not significantly different at $p < 0.05$ while different letters means that they are significantly different at $p < 0.05$

Fig. 10. Ethovision



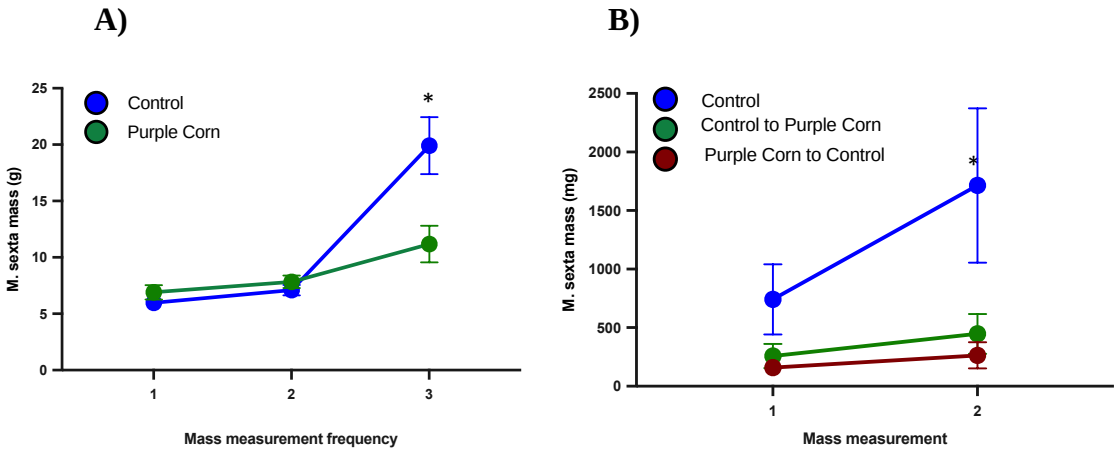
Unpaired t test ($p < 0.05$) analysis of ethovision experiment results I) distance travelled by third instar caterpillars J) velocity of caterpillars in diet preference studies. Bars with different letters are statitically significant at p value < 0.05

Fig. 11. Effects of purple corn pericarp extract on pupation time



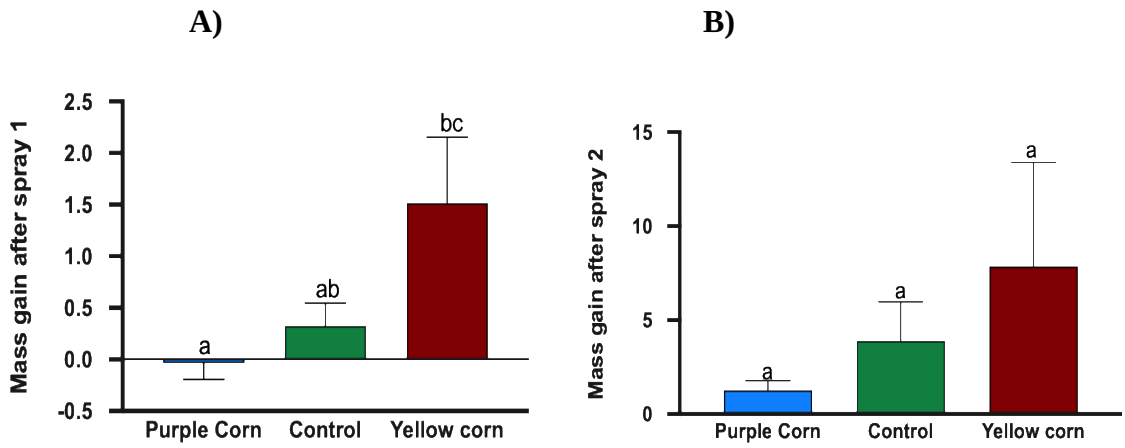
Results of Kruskal-Wallis tests, Dunn's multiple comparison test ($p < 0.05$) for the effect of feeding purple corn pericarp extract on mean time to pupate of M. sexta caterpillars. Means followed by same letters are not significantly different at $p < 0.05$ while different letters show means that are significantly different at $p < 0.05$

Fig. 12. *M. sexta* mass on switched diet experiment



Results of One-way ANOVA, unpaired t-test, Tukey's HSD test ($p < 0.05$) of pericap extract diet om mean larval mass L) before diet switch and M) after diet switch. Groups with *statistical significance at p value of 0.05 for the model

Fig. 13. Effects of plant sprayed purple corn pericarp extract on larval mass gains



Results of Kruskal-Wallis tests, Dunn's multiple comparison test ($p < 0.05$) for the effect of plant sprayed purple corn pericarp extract on mean N) larval mass gain after first spray O) larval mass gain after second spray. Means followed by same letters are not significantly different at $p < 0.05$ while different letters show means that are significantly different at $p < 0.05$

CHAPTER IV

CASCADING EFFECTS OF POLYPHENOL RICH PURPLE CORN PERICARP EXTRACT ON PUPAL, ADULT AND TRANSGENERATIONAL OFFSPRING OF TOBACCO HORNWORM

Abstract

In past, quite a few studies have examined the insecticidal properties of plant-based bioactive compounds against various insect pests. However, a major bottleneck in commercialization of such products is that these extraction methods are complex, time consuming and even highly expensive. Using a recently developed inexpensive extraction and quantification methodology to isolate polyphenols (including anthocyanins and condensed tannins) from commercially grown purple corn (pericarp), we examined their effects on *Manduca sexta*, a common damaging insect herbivore. Following up on our previous work which demonstrated negative impacts of polyphenol rich extract on larval stages, we further examined whether there were any cascading effects on subsequent life stages including any possible transgenerational effects. It was found that polyphenol rich purple corn extract fed caterpillars had significantly lower pupal mass and survival rate, but eclosed earlier compared to a yellow corn extract diet devoid of anthocyanins and low on polyphenols, and an artificial control diet. Moreover, adult moths regardless of their sex also had lower mass when reared on the purple corn extract diet. To test whether there were any transgenerational effects, we allowed male and female adults fed on purple corn extract diet

and control diet to mate and lay eggs in a full factorial design experiment. Although, purple corn extract fed adults (males and females) laid a lower number of eggs, the numbers weren't statistically significant compared to other treatments. However, we found that second instar *M. sexta* caterpillars from any mating combination with at least one parent reared on purple corn extract, gained significantly lower mass compared to caterpillars with both parents reared on control diet. There were cascading negative effects of feeding purple corn pericarp extract on pupal, adult and second generation, reaffirming its potential application as an inexpensive biopesticide. Future studies should be focused on testing its effects in other study systems, as well as understanding the underlying principles of causation.

Introduction

Globally, insect pests account for ~18% loss of total crop yield production (Oerke, 1994; Oerke, 2006). The use of synthetic insecticides/pesticides to manage these pests while delivering positive results have also produced a different set of concerns including resistance development, residue build up, biomagnification, and toxicity to non-target organisms (Jeyasanker & Jesudasan, 2005; Oerke, 2006; Datta et al., 2019). Alternatively, a growing body of researchers has examined the possible insecticidal properties of plant based bioactive compounds against various insect pests (Sampson et al., 2005). For example, Azadirachtin (C-*seco*-limotriterpenoid), extracted from neem plant *(Azadirachta indica)* has been shown to exhibit larvicidal effect against horn fly (*Haemotobia irritans*, L), stable fly (*Stomoxys calcitrans*) and house fly (*Musca domestica*; Miller & Chamberlain, 1989) as well as fifth instar larvae of tobacco hornworm (*Manduca sexta*) (Lay et al., 1993; Mitchell et al., 1997; Atawodi & Atawodi, 2009). Volatile oils (3-methyl-6-(1-methyl- ethylidene)-cyclohex-2-en-1-one) isolated from

Lippia javanica have been found to possess insecticidal properties against major legume pests (aphids, mites) (Muzemu et al., 2011; Mwanauta et al., 2014). Generally, these compounds act as antifeedant, repellent, anti-ovipositor and in some cases as toxins that can kill insects (Atawodi & Atawodi, 2009; Mwanauta et al., 2014).

To test their efficacies against insect pests, these compounds are generally used either as crude extracts or pure compounds (Zounos et al., 1998; Sampson et al., 2005; Moreira et al., 2007; Atawodi & Atawodi, 2009; Hasheminia et al., 2011; Datta et al., 2019). However, due to the presence of other undesirable compounds in plant matrix, it becomes tedious to extract and purify these compounds efficiently and economically (Mandana et al., 2011; Sasidharan et al., 2011). In other words, there is a never-ending quest for sourcing biologically active compounds that can satisfy the abovementioned properties to be potentially incorporated into sustainable pest management practices (Sampson et al., 2005).

Colored corns (purple, blue, red) – originated in Andres regions of Peru, are widely cultivated and consumed across the Argentina, Bolivia, Ecuador and Peru (Betran et al., 2001; Lao & Giusti, 2018). Purple corn (*Zea mays* L.) is considered to be one of the richest sources of polyphenols including anthocyanins and tannins (Li et al., 2017). These compounds have been shown to possess insecticidal properties in various study systems (Felton et al., 1992; Pourcel et al., 2006; Barbehenn & Constabel, 2011; Rashid War et al., 2012). Having longer storage life and significant amounts of such compounds (Li et al., 2017), these corn varieties can potentially be explored as a source of inexpensive polyphenolic compounds for utilization as bioinsecticides. In addition, Somavat et al. (2018) developed an efficient and inexpensive methodology to isolate them from corn pericarp, which is essentially a waste product in corn processing. This is indeed a significant step since, most of current compound recovery methods

from natural sources are plagued by complicated and expensive extraction methods, lower recovery of active ingredients, and also tend to be time consuming (Liu et al., 2011; Mandana et al., 2011; Sasidharan et al., 2011; Raks et al., 2017). Known for its antioxidant (Fernandes et al., 2014; Reque et al., 2014), anti-obesity, (Esposito et al, 2015; Johnson et al., 2016) anti-cancer, (Fernandes et al., 2014) as well as anti-inflammatory properties (Esposito et al., 2014), these compounds (especially the combination of anthocyanins, polyphenols and tannins) can potentially have multiple pharmacological uses, but also potential insecticidal properties (Lee et al., 1987; Close & Beadle, 2003; Lev-Yadun & Gould 2008; Kariyat et al., 2019).Although, the precise mechanism behind these effects is little understood.

In previous study, we found the larvicidal effects of purple corn pericarp extract on growth and development of different *Manduca sexta* (tobacco hornworm; Sphingidae; Lepidoptera) larval stages (egg hatching, caterpillar mass, caterpillar mass gain and time to pupation; Tayal et al., 2020). It was found that the purple corn pericarp extract diet significantly decreased egg hatching rate as well lowered the mass and mass gain compared to control diets. Moreover, the purple corn pericarp fed larvae showed significantly lower preference to feed on that diet compared to control diets, took longer to pupate (Tayal et al., 2020). We used *M. sexta* as a study system because of its size, rapid growth, easiness of laboratory rearing and their longtime use as a study system for physiological, developmental, and behavioral studies (Kingsolver, 2007; Kariyat et al., 2012; 2018; 2019).

Although, the larval stage of herbivorous insects such as *M. sexta* is very critical from crop husbandry perspective (caterpillars cause damage and adults are usually pollinators), it is imperative that other growth stages are also investigated to examine the possible lingering or cascading effects of feeding bioactive compounds to them. This is especially important since

adult females can lay over 200 eggs (Kariyat et al., 2013), and oviposition can be considered as the first sign of herbivory (Paschalidou et al., 2015). Moreover, it has been shown that the diet and energy requirements for holometabolous insects change between life stages (Bauerfeind & Fischer, 2005). And, the life cycle modularity and rapid compensation also allows insects to uncouple the effects of environmental disturbances on physiology in one step to the next (Simpson & Simpson 1990; Yang & Joern 1994; Woods 1999; Potter et al., 2011). For example, the food impoverished individuals of Glanville fritillary butterfly (*Melitaea cinxia*) had maintained their high fecundity rate through compensatory increased developmental time (Saastamoinen et al., 2013). In damselflies, reduced larval size due to a temporary food limitation triggers a compensatory increase in growth rate, so that initial size differences are resolved by adulthood (Dmitriew & Rowe 2005; Potter et al., 2011). It has been reported that insect maternal effects on second generation offspring in response to parent diet quality are critical for their growth and can also vary (Morris, 1967; Boggs, 1997; Bauerfeind & Fischer, 2005; Myers et al., 2011; Boggs & Niitepold, 2014; Woestmann & Saastamoinen, 2016). For example, in *Drosophila melanogaster*, the parents reared on poor larval food laid heavier but smaller eggs than control parents showing adaptive and maladaptive effects of parental stress (Vijendravarma et al., 2010). In other words, it is plausible to expect that adults emerging from stressed caterpillars (in this case the purple corn extract) when allowed to mate- can possibly produce offspring that are compromised in their growth and/or development.

Keeping this in mind, we designed a set of experiments where we continued to examine the effects of feeding pericarp extract on different pupal (pupal mass, pupal survival rate, pupal duration) and adult (adult mass, adult wingspan) parameters. And, to study transgenerational effects of pericarp extract, we allowed controlled mating of adult moths on purple corn extract

diet and control diet treatments in a full factorial design experiment (see figure 14 for details) and allowed them to lay eggs and develop.

Material and Methods

Insect Colony: The pupae and adult moths used in this study were collected from our previous experiment in which their respective larvae were allowed to feed on different treatments (purple corn pericarp extract: n=58, yellow corn pericarp extract: n=28, control; n=51) at room temperature. At fifth instar stage, when the caterpillars stopped eating and started wandering in petri dishes with the dark black pulsating vein on dorsal side clearly visible (Supplementary video 1), they were transferred to plastic containers (23.19 cm x 15.24 cm x 16.84 cm; Aqua culture pet carrier: # 564356887, Walmart) with wood shavings (Natural Aspen small animal bedding: Petco Animal Supplies, Inc., San Diego, CA, USA) for pupation. Once pupated, measurements were taken, and the pupal containers were then moved to lab cabinets and kept under dark conditions at room temperature and RH of 65%.

Pericarp extract and its quantification

Pericarp extracts used in different caterpillar diet treatments (purple corn, yellow corn) were obtained by steeping 5 gm of respective pericarps in 100 ml of deionized water followed by stirring and centrifugation at 5000 rpm for 5 minutes (Somavat et al., 2017; Tayal et al., 2020). The resultant filtrate was used for mixing in caterpillar food. A pH differential method using 96 well microplate reader (Multiskan Sky Microplate Spectrophotometer: #51119600, Thermo Fisher Scientific, MA, USA) was used to quantify the amount of total monomeric anthocyanins present in extracts. It was found that purple corn pericarp extract contained greater amounts of

total monomeric anthcyanins, total polyphenols, and tannins compared to yellow corn pericarp extract which contained no anthocyanins and lower amounts of total polyphenols and tannins (Tayal et al., 2020)

Experiment Methodology

All healthy pupae from the three different treatments (purple corn pericarp extract, yellow corn pericarp extract, control) were allowed to develop and eclose. Mass of each pupae was recorded by weighing them on a digital balance (Accuris Series Dx, Model: W3100-210, Benchmark Scientific, NJ USA). Once they eclosed, we calculated the days from pupation to eclosion, recorded as pupal duration (all data was collected at late night and/or early morning to be consistent). Pupae that didn't eclose after 45 days and stopped moving (Supplementary video 2) were considered as dead and were used to calculate pupal survival rate. Once eclosed, we recorded the adult mass as well as wingspan. While adult mass was calculated by using digital balance (Accuris Series Dx, Model: W3100-210, Benchmark Scientific, NJ USA), adult wingspan i.e. length from tip of one wing to tip of other wing when fully expanded, was measured with a ruler (Ruler, White Vinyl: # 70260, North Carolina Biological Supply Co., NC, USA). We had a sample size of pericarp extract n=40, control=19, yellow corn=22 for these measurements.

To examine the transgenerational effects of pericarp extract, we mated adult moths from purple corn pericarp extract diet and control diet treatments to each other in all possible combinations (Purple ♀ x Purple ♂; Purple ♀ x Control ♂; Control ♀ x Purple ♂; Control ♀ x Control ♂; Fig. 14 (N= 5-7/ treatment), for mating details see (Kariyat et al., 2013). Following the crossing design, adult moths (one newly eclosed male and female each) were placed in popup cages (Popup rearing cage: #1466AB, BioQuip Products, Inc., CA, USA) along with orange flavored

Gatorade (Kariyat et al., 2013) as their food and a 6 weeks old tomato plant (Variety: Valley Girl, Product ID 741, Johnny's Selected Seeds, Maine, USA) as a host for oviposition (Kariyat et al., 2013). Cages of different crosses were monitored every day for eggs until the female died. Collected eggs were moved to regular artificial control diet at room temperature (Kariyat et al., 2019). Once the hatched larvae molted into 2nd instar, we recorded larval mass to see any transgenerational effects.

Statistical analysis

We used ANOVA as well as non-parametric tests depending upon the nature of data (normal or non-normal) to analyze the results. As pupal mass, pupal duration, adults mass followed normal distribution, we ran One Way ANOVA (diets as factor) to analyze these. However, non-normal distributed data of pupal survival, adult wingspan, number of eggs laid, and 2nd instar larval mass was analyzed with non-parametric Kruskal-Wallis test. Post hoc pairwise comparisons of all treatments were obtained with Tukey's HSD and Dunn's multiple comparison tests, respectively. All data was analyzed using the statistical software, JMP (SAS institute, NC, USA), and plots were built using GraphPad PRISM software (La Jolla, California). Detailed statistics are described in table 3.

Results

a) Effects of pericarp extract on pupal stages of *M. sexta*

Both pupal and adult mass results showed similar trends. Results from the Two-Way Anova for pupal mass showed that all the main effects were significant; diet treatment ($F = 23.88$, $p < 0.0001$), sex ($F = 7.65$, $p = 0.0067$) and interaction (sex X treatment; $F = 4.53$, $p = 0.01$) (Table

1). Pairwise comparisons showed that pupae from caterpillars reared on yellow corn diet were significantly heavier than both control and purple corn (Fig. 15A). We also found that regardless of the treatments, male pupae from yellow corn and control diets were heavier than purple corn and control female pupae, which was surprising (Fig. 15B).

Pupal survival was significantly low ($F = 9.885$, $p = 0.0105$) for purple corn reared pupae compared to control (Fig. 15C). However, yellow corn reared pupal survival was similar to purple corn and control pupae ($F = 9.885$, $p = 0.0852$). Interestingly, we found that pupae reared on purple and yellow corn diet had significantly low pupal duration (in days) ($F = 5.665$, $p = 0.0047$) and eclosed earlier compared to control pupae (Fig. 15D).

b) Effects of pericarp extract on adult *M. sexta*

For adult mass, we found that both treatments (diets; $F = 14.15$, $p < 0.0001$ and sex; $F = 6.971$, $p = 0.010$) significantly affected the mass, while their interaction was non-significant ($F = 1.73$, $p = 0.183$) (Table 3). Upon close examination with pairwise comparisons, we found that adults emerged from caterpillars fed on yellow corn diet and control diet were significantly heavier than the ones from purple corn diet (Fig. 16A). In addition, we also found that regardless of the treatments, male moths were heavier compared to females (Fig. 16B). However, there was no significant difference in wingspan (F value = 2.38, $p < 0.3037$) across the treatments (Fig. 16C).

c) Effect of pericarp extract on adult fitness and offspring mass

Although, there were no significant differences (F value = 4.83, $p = 0.1847$) in mean number of eggs laid by different crosses, a pattern of lower number of eggs laid was observed when where both parents i.e. male and female were fed on purple corn extract diet (Fig. 17A). The egg laid spread/pattern is shown in Fig. 17B. Interestingly, significantly lower 2nd instar

larval mass (F value = 27.3, $p < 0.0001$) for each cross was observed where one of the parents was from purple corn extract diet compared to artificial diet control (both parents reared on control diet) which had higher mass among all the comparisons (Fig. 17C).

Discussion

In continuation of our recent study demonstrating the negative effects of purple corn pericarp extract on different larval stages (egg hatching, larval mass, mass gain and feeding preference etc.) of *M. sexta* (Tayal et al., 2020), current study clearly demonstrates similar effects cascading through pupal, adult and next generation offsprings. The lower mass observed in purple corn pericarp pupae is a direct result of purple corn pericarp-fed larvae that had gained lower larval mass, clearly indicating larval nutritional stress had negative effects on subsequent life stages. Similar results have been reported on lower pupal mass of starved caterpillars, for example in Squinting bush brown (*Bicyclus anynana*), which signifies the importance of diet on their post larval life history traits (Braby & Jones, 1995; Fischer et al., 2004; Bauerfeind & Fischer, 2005; Ferkau & Fischer, 2006; Bauerfeind & Fischer, 2009; Kehl & Fischer, 2012).

The significant decline in pupal survival and developmental time of purple corn pupae directly affected adult mass and fecundity similar to predictioned by life-history models (Berrigan & Charnov, 1994; Gotthard & Nylin, 1995; Arendt, 1997; Blanckenhorn, 1999; Bauerfeind & Fischer, 2005). Its well documented that juvenile and ecdysteroid hormone levels direct postembryonic insect development (Bollenbacher, 1988), we speculate that purple corn pericarp extract might have affected the interendocrine regulation, resulting in possible low survival rates. In our recent work, we found a large number of larvae stayed away from pericarp extract diet (Tayal et al., 2020), and were reluctant to feed on it compared to control diets. It is possible that

polyphenol rich diet may have anti-feedant properties which resulted in starvation (or reduced intake) and consequently decreased larval and pupal mass.

In addition, the findings of decreased pupal duration are consistent with previous studies which have also documented reduced lifespan in Glanville fritillary butterfly (*Melitaea cinxia*) due to early larval food stress (Saastamoinen et al., 2013). On the other hand, reduced lifespan and faster development may also result in poor growth which can affect subsequent stages. Parallel to this assumption, we found significantly lower mass for adults of purple pericarp extract group compared to control diet and yellow corn pericarp extract group, reflecting their inability to compensate. These findings are also in agreement with Velde et al. (2013) and many others, who reported a strong correlation between pupal mass and adult mass at eclosion. The presence of reduced mass pattern in larval, pupal and adult mass show cascading negative effects of early larval food stress on subsequent stages (Bauerfeind & Fischer, 2005; Kehl & Fischer, 2012; Tayal et al., 2020).

While examining the sex-specific effects of pericarp extract on male and female individual masses, lack of any significant differences among treatment x sex interaction indicates that these effects are sex-independent. In 2005, Bauerfeind & Fischer also didn't find any significant interactions among larval food stress treatments and sex in Squinting bush brown (*Bicyclus anynana*) although, the female pupae had higher mass than male pupae. Previous studies have reported that the adverse effects of poor diet quality are more severe if both the parents and earlier generations experience them, as demonstrated in this study (Triggs & Knell 2011; Keena et al., 1998; Woestmann & Saastamoinen, 2016). Having a strong correlation of pupal mass on female longevity, fecundity, and egg size (Bauerfeind & Fischer, 2009), it is understandable that low female pupal mass would directly affect their reproductive ability.

Moreover, in relation to the individual size and mass, we also expected that polyphenol rich pericarp extract-fed adults may have smaller wings and hence lower wingspan (Boogs & Freeman, 2005) although, the results were not on expected lines. However, since wings are an important part for foraging, dispersal, mate searching (Wong et al., 2016), it would be interesting to study these effects on molecular level i.e. muscle molecular composition and their flight capacity (Marden et al., 2008; Portman et al., 2015). We speculate that there could be possible effects of polyphenol rich pericarp extract on the alternate splice forms of Troponin T (Portman et al., 2015).

Although, we didn't find any significant differences in mean number of eggs laid among different crosses, when both parents belonged to purple corn pericarp extract diet, they indeed had the lowest number of eggs. Number of eggs (123 out of total 905 ~13%) demonstrated the effects of early larval stress on fecundity- possibly due to poor food quality that affected insect physiology and fitness for reproductive investment, coupled with short pupal development (Morris, 1967; Bauerfeind & Fischer, 2005; Myers et al., 2011; Woestmann & Saastamoinen, 2016). Reduced sample size per treatment (~24 mating pairs) and high variability in egg laying (ranging 0 to 447) might explain the reasons we could not resolve many possible pairwise differences for egg laying in this study. Since oviposition is the first step in future herbivory and herbivory related defense induction (Seino et al., 1996; Pashalidou et al., 2010; Pashalidou et al., 2013; Desurmont & Weston, 2011), a reduction in fecundity of adults could be a management strategy againt herbivory (Ketoh et al., 2000; Zhao et al., 2008).

Interestingly, the low second instar larval mass of offsprings from a mating where at least one parent was from pericarp extract diet confirms the transgenerational effects of purple corn pericarp extract. In the past, different studies have shown the role of quality nutrition in female

reproduction and indirectly, its negative transgenerational effects (Bauerfeind & Fischer, 2005; Karl et al., 2007; Rotem et al., 2003; Geister et al., 2008; Carisey and Bauce, 2002; van Asch et al., 2010). In contrast, the fitness buffering ability of individuals may also result in adaptive responses against sensitive environmental stresses (Mousseau & Dingle, 1991; Agrawal et al., 1999; Boogs, 2003, Boggs & Freeman, 2005; Woestmann & Saastamoinen, 2015). For example, it has been reported that mothers exposed to stress through natural enemies may produce more resistant offsprings (Agrawal et al., 1999; Saastamoinen et al., 2013). However, our results are in contrast to this response and demonstrated decreased offspring mass of nutritionally stressed parents. Also, as neonates have to cope with different factors such as plant surfaces, plant defenses, predators, pathogens and parasitoids to establishing themselves on food plant (Zalucki et al., 2002; Kariyat et al., 2017; 2018), we speculate that the reduction in early instar larval masses makes them more susceptible. Future studies should be focused to identify whether any adaptive effects (any resistant individuals) of pericarp nutritional stress are transmitted to next generations.

Plants produce a variety of secondary metabolites (alkaloids, terpenes, phenolics, nitrogen and sulfur containing compounds) for defending themselves against herbivores either by direct toxicity or by indirectly attracting their parasites and predators (Turlings et al., 1990; Dicke et al., 1990; Turlings & Tumlinson, 1992; Vet & Dicke, 1992; Kessler & Baldwin, 2001; Dudareva et al., 2006; Kariyat et al., 2012). It has also been shown that these defenses are upregulated in response to chemical elicitors present in herbivore and pathogen oral secretions (Pare et al., 2005). For example, Cai et al., (2011) reported an increased level of anthocyanins and resveratrol in *Vitis vinifera* cell suspension cultures in response to *M. sexta* saliva, suggesting the possibility that anthocyanins are also a part of herbivore defense mechanism, and are inducible, suggesting an

evolutionary history of such compounds in plant defense, further supporting the merits in our exploration of these extracts for pest management.

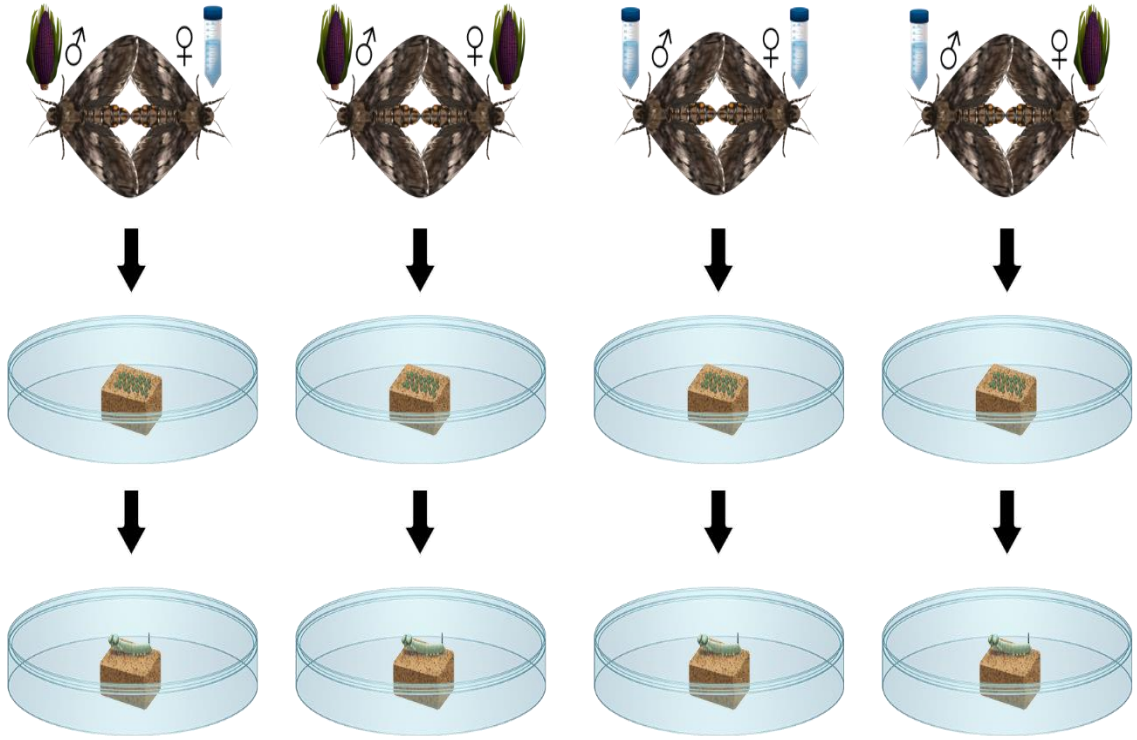
Conclusions and future studies

It was found that polyphenol rich purple corn extract, an inexpensive by product of corn processing has negative impact on pupal and adult fitness and these effects can cascade through transgenerational stages of *M. sexta*. In previous work, we found the larvicidal effects of this extract on egg hatching, caterpillar mass, developmental time, suggesting anti-herbivore effects of polyphenolic compounds and its overall suitability as a biopesticide. Associated complexities and difficulties in bioactive compound extraction mainly limit their commercialization potential. However, polyphenol rich purple corn extract can prove to be an economically viable biopesticide. In addition, since purple corn pericarp extract is a diverse mix of anthocyanins, tannins and other polyphenols, additional research is needed to ascertain whether these effects are due to a specific bioactive compound or due to a synergistic effect of all these compounds. In addition, apart from lepidoptera pests, it would be interesting to test the efficacy of polyphenol rich purple corn extract against pests with different feeding habits i.e. aphids, white flies etc. And finally, future studies are required to identify the mechanisms/mode of action of polyphenol rich pericarp extract at molecular levels, an area we are currently exploring.

Table 3 Statistical test details used to analyze the effect of different pericarp extracts on pupal, adult and transgenerational traits. Significant results with P < 0.05 are in bold.

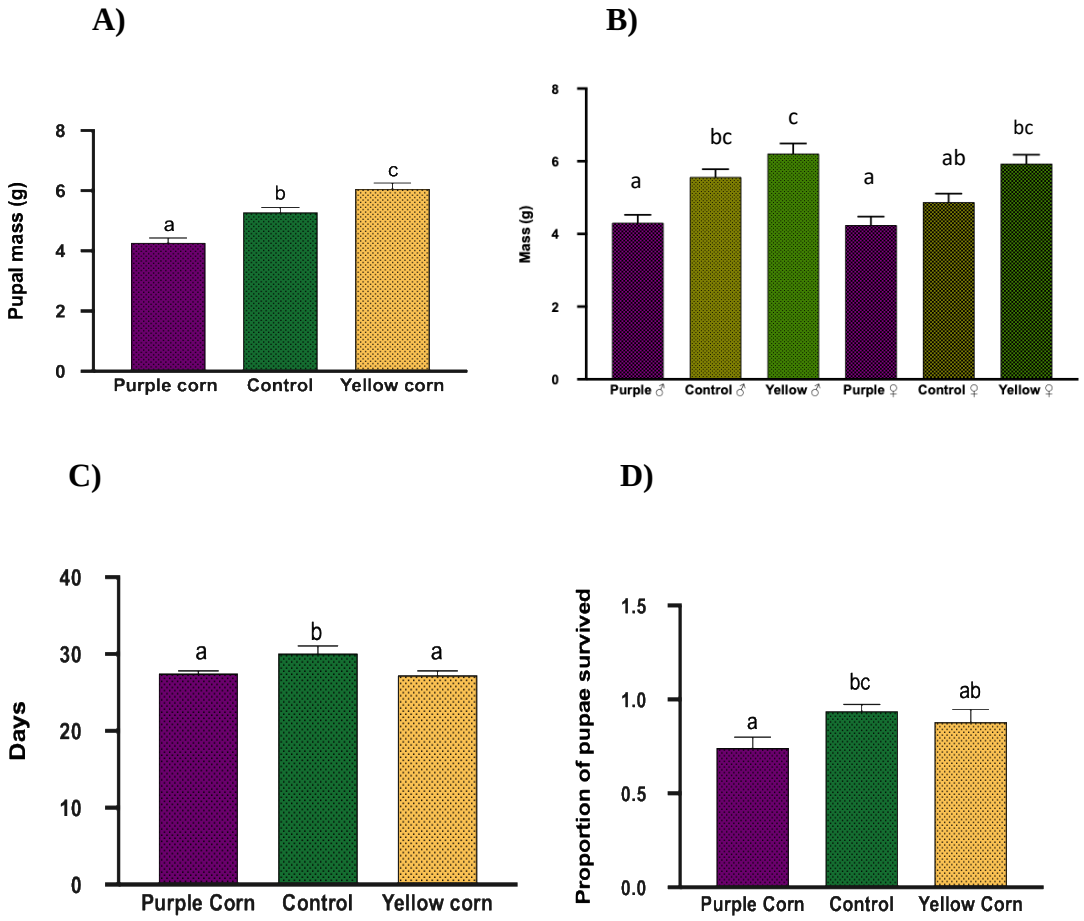
Parameter	**Test**	**Df/groups**	**Test Statistics**	**p- value**
Pupal mass	Two Way ANOVA	2	Treatment (diet) F = 23.88	**<0.0001**
		1	Sex (male and female) F = 7.65	**0.0067**
		2	Interaction F = 4.53	**0.013**
Adult mass	Two Way ANOVA	2	Treatment (diet) F = 14.58	**<0.0001**
		1	Sex (male and female) F = 6.97	**0.010**
		2	Interaction F = 1.73	0.183
Pupal survival	Kruskal-Wallis Test	3	Kruskal-Wallis Statistic = 9.88	**0.0071**
Pupal duration	One Way ANOVA	2	F statistic = 5.665	**0.0047**
Adult wingspan	Kruskal-Wallis Test	3	Kruskal-Wallis Statistic = 2.38	0.3037
Eggs laid	Kruskal-Wallis Test	4	Kruskal-Wallis Statistic = 4.83	0.1847
First instar larval mass	Generalized Regression	3	Wald Chi-Square = 9.56	**0.0226**

Fig. 14. Schematic representation of the experimental design to study effects of pericarp extract on fecundity and mass of second instar offspring larvae



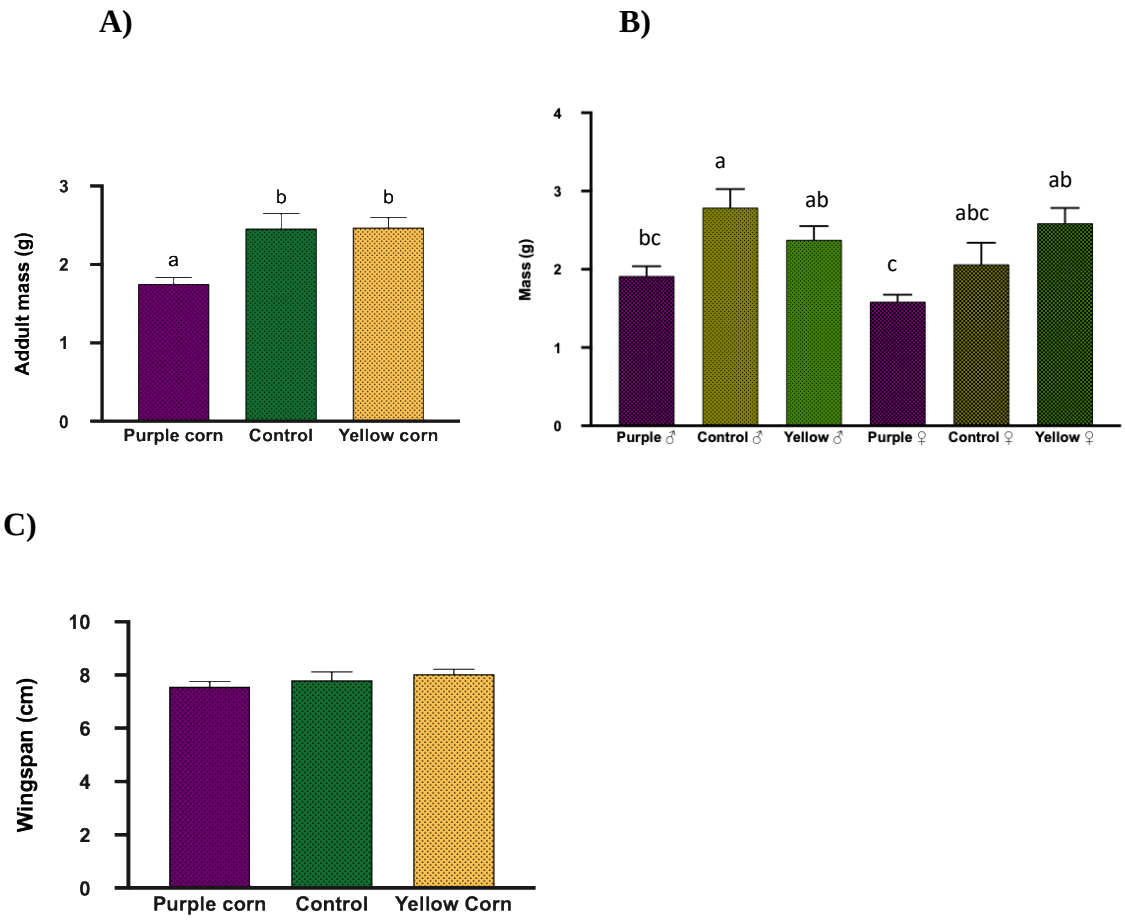
Schematic representation of the experimental design to study effects of pericarp extract on fecundity and mass of second instar offspring larvae. Adult moths were crossed in all possible combinations a) Control ♀ x Purple ♂ b) Purple ♀ x Purple ♂ c) Control ♀ x Control ♂ and d) Purple ♀ x Control ♂. All the collected eggs were counted and placed on artificial control diet and larvae were allowed to feed until they molted to 2nd instar. Caterpillar mass at 2nd instar was measured and used to compute mass gain.

Fig. 15. Effects of pericarp extract on pupal stages of *M. sexta*



Results of One-way ANOVA, Two-way ANOVA, Kruskal-Wallis tests, and the post-hoc Tukey's HSD and Dunn's multiple comparisons ($p < 0.05$) for the effects of pericarp extract diet on mean a) pupal mass b) pupal mass: treatment x sex c) pupal duration d) pupal survival. Means followed by different letters are significantly different at $p < 0.05$.

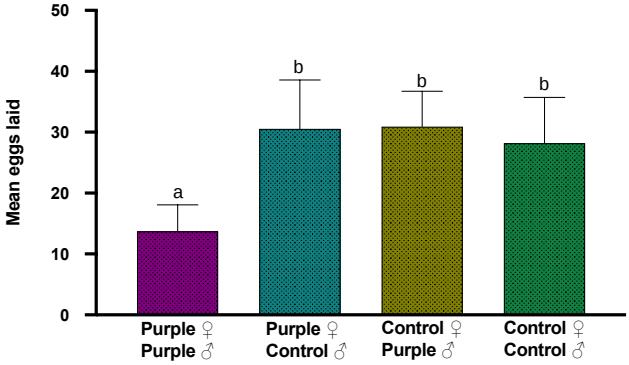
Fig. 16. Effects of pericarp extract on adult *M. sexta*



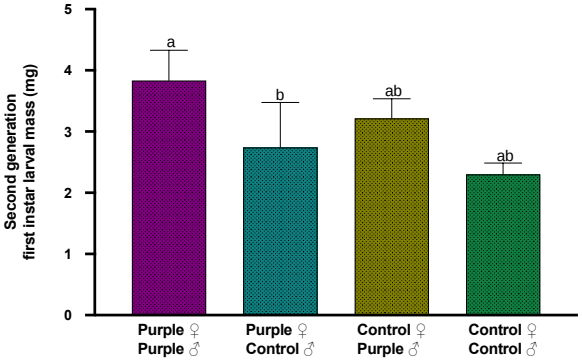
Results of One-way ANOVA, Two-way ANOVA, Kruskal-Wallis tests, Post-hoc Tukey's HSD test ($p < 0.05$) for the effect of pericarp extract diet on mean a) adult mass b) adult mass: treatment x sex c) adult wingspan. Means followed by different letters are significantly different at $p < 0.05$.

Fig. 17. Effect of pericarp extract on adult fitness and offspring mass

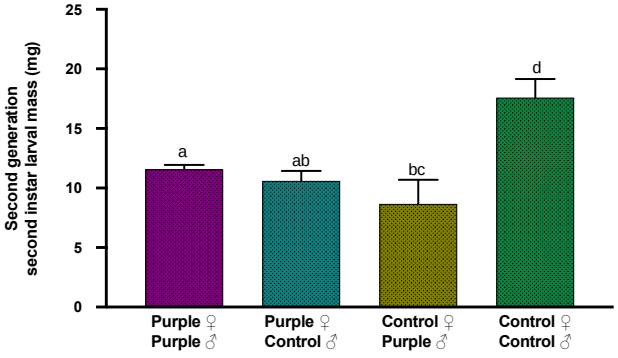
A)



B)



C)



Results of the Kruskal-Wallis tests, Dunn's multiple comparison test ($p < 0.05$) for the effect of pericarp extract diet on a) mean number of eggs b) first instar offspring larval mass d) second instar offspring larval mass. Means followed by different letters are significantly different at $p < 0.05$.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

In the buzz pollination efficiency experiment, we concluded that electric toothbrush is an inexpensive and may be even more durable and viable alternative for tuning fork. Although artificial pollen extraction is not affected by type of instrument and their buzzing frequency, we found that length of buzzing time is a critical factor to be considered. Future studies should be focused on comparing the natural and artificial buzz-pollination with revealing the characteristics factors/strategies employed by pollinators in pollen extraction.

In the insecticidal activity experiments, we concluded that purple corn pericarp extract rich in anthocyanins and polyphenols has severe negative effects on growth and development of Tobacco hornworm (*M. sexta*). The larvicidal effects of pericarp extract on egg hatching, caterpillar mass, caterpillar mass gain and resulted developmental delay evidently indicate their potential candidacy to manage a number of crop insect-pests. In addition to this, the cascading effects of pericarp extract on subsequent (pupal and adult) stages as well as next generation offspring larval mass would also add in pest management by affecting adult fitness and reproduction.

Since we are continuously looking for safe and ecofriendly alternatives of synthetic chemicals to manage insect-pests, our studies demonstrate that byproducts of corn processing

industry yields valuable compounds which has the potential to be used as biopesticide. Future studies should be focused on separating different compounds present in the pericarp and find if these effects are either due to individual compounds or their combination. However, the specific mechanism underlaying these effects should be further investigated. Testing their insecticidal properties against other major insect-pest (e.g. aphids, whiteflies) will be new areas of research for developing new natural and sustainable insecticides.

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BIOGRAPHICAL SKETCH

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