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Are epicuticular waxes a surface defense comparable to trichomes? A test using two Solanum species and a specialist herbivore.

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Are epicuticular waxes a surface defense comparable to trichomes? A test using two *Solanum* species and a specialist herbivore†

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Abstract:

Although plants possess a suite of structural defenses, most studies have focused on trichomes. Trichomes can have both pre- and post-ingestive effects and have been consistently found to reduce herbivory. Along with trichomes, a few studies have focused on epicuticular waxes as an important defense; however, manipulated comparisons examining herbivore growth and development is limited. In this study, using two *Solanum* species (*Solanum glaucescens* and *Solanum macrocarpon*) that vary in both defenses, we tested the hypothesis that variation in defenses will affect herbivore feeding, primarily by restricting feeding commencement. We used electron microscopy together with a series of plant- and diet-based manipulative experiments, using tobacco hornworm (*Manduca sexta*; Lepidoptera: Sphingidae) as the herbivore. We found that *S. glaucescens* leaves had significantly fewer trichomes and significantly higher wax content when compared to *S. macrocarpon*. We also found that *S. glaucescens* waxes acted as a strong physical barrier resulting in lower mass gain and higher mortality of caterpillars compared to *S. macrocarpon*. Artificial diet manipulation experiments also suggested the possible toxicity of waxes. Collectively, we show that epicuticular waxes can play a significant role as a strong surface barrier and should be examined further.

Keywords: trichomes, plant defense, wax, Solanum, *Manduca sexta*, herbivory

Introduction

It is well-established that plants use both physical and chemical defenses to protect themselves against arthropod herbivores (Singh and Kariyat, 2020; Tayal et al., 2020a; 2020b; Singh et al., 2021). Physical defenses mainly include waxes, trichomes and spines. Chemical defenses include numerous secondary metabolites that can both directly and indirectly protect plants (Howe and Jander, 2008). Among physical defenses, trichomes are one of the most important and possibly the most studied of defenses (Levin, 1973; Kariyat et al., 2013; 2017; 2018; 2019; Karabourniotis et al., 2019; Kaur and Kariyat, 2020a; 2020b; Watts et al., 2021; Watts and Kariyat, 2021a; 2021b). Trichomes are epidermal hairs present on various plant parts including leaves, stems and flowers (Kaur and Kariyat, 2020a). Trichomes deter herbivore movement and feeding pre-ingestion but can also cause post-ingestive effects by damaging the caterpillar's inner gut lining (peritrophic matrix) and determine multitrophic interactions by providing cues to herbivore natural enemies (Kariyat et al., 2017; Weinhold and Baldwin, 2011). A higher trichome density is usually associated with reduced herbivory (Watts and Kariyat, 2021b). While trichomes are primarily classified as glandular and non-glandular types, based on the presence or absence of a glandular head, deeper inspection has revealed both inter and intra specific morphological variation (Watts and Kariyat 2021a). This variation includes trichome size, shape, density, and dimensions on abaxial and adaxial leaf surfaces (Watts and Kariyat 2021a; b).

Plant cuticle is a hydrophobic layer of cutin and associated waxes (made of lipids and hydrocarbons) that coats most aerial parts of plants (Konno et al., 2006; Kaur et al., 2022). Along with their role in hampering water loss from the plant surface by protection from excessive transpiration (Jenks and Ashworth, 2010; Sharma et al., 2018), plant waxes also play a major role

in herbivore defense (Jetter et al., 2006) by discouraging the movement and feeding of herbivores by making the leaf surface slippery and filling up crevices (Jetter et al., 2006; Whitney and Federle, 2013). When ingested, herbivores have also been found to spend additional time cleaning their mouthparts when covered with waxes (Shelomi et al., 2010). In one study, Chanchala et al. (2020) demonstrates that epicuticular waxes of sugarcane significantly impact feeding of the leaf hopper, *Deltocephalus menonii* (Hemiptera: Cicadellidae), a serious pest and vector of white leaf disease. Therefore, sugarcane accessions with higher levels of epicuticular wax can potentially be incorporated in integrated disease management of white leaf disease. In this regard, waxes have both pre and post ingestive effects on insect herbivores, similar to trichomes.

In sum, physical defenses in plants and their role in deterring herbivores is well-researched from both morphological and molecular perspectives. The fitness impacts and behavioral modifications caused by these physical defenses at various herbivore life stages, as well as the mechanisms underlying the multi-trophic interactions they mediate have been well described. However, to date there are no studies that disentangle the relative importance of trichomes and waxes as defenses against herbivores.

Understanding the relative contributions of waxes and trichomes warrants the use of phylogenetically similar (within the same genus) plant species, exhibiting variation in defenses (epicuticular waxes and trichomes) which are easy to manipulate for experiments. Previous work has used members of the Solanaceae family to understand the effects of inter and intraspecific variation in physical defenses, and their impact on mediating plant-herbivore interactions (Kariyat et al., 2013; Kariyat et al., 2017; Kariyat et al., 2019; Chavana et al., 2021; Watts and Kariyat, 2021b). More recently, we observed that, among 14 species in the *Solanum* genus, *Solanum macrocarpon* (Gboma) has glandular and non-glandular trichomes (Watts and Kariyat, 2021a), but

almost no waxes on the leaf surface. On the other hand, *Solanum glaucescens* (Cuatomate) has a thick wax layer on its leaf surface, but almost no trichomes (Kaur et al., 2022). This natural variation in trichome-wax presence-absence provides an opportunity to test the impact of these defenses together. We used a combination of microscopy, herbivore behavior and growth assays to test our hypothesis that the trichomes and waxes would differentially affect herbivore feeding, with consequences on their growth and development.

Materials

a. Plants: We bought the seeds of two Solanaceae species viz. *Solanum glaucescens* (Product code: Y5SSSOGL) and *Solanum macrocarpon* (Product code: Y5SSSOLG) from rarepalmseeds.com. For details of how the plants were grown, please see Watts and Kariyat (2021a).

b. Insects: Tobacco hornworm (*Manduca sexta*; Lepidoptera: Sphingidae) was used for laboratory-based assays. *M. sexta* is a Solanaceae specialist chewing-type herbivore that feeds voraciously on a variety of Solanaceae species (Watts and Kariyat, 2021b). Caterpillars of *M. sexta* were allowed to feed on artificial diet. For more details on rearing of the insect colony, see Tayal et al. (2020a) and Watts and Kariyat (2020b).

c. Desktop Scanning Electron Microscopy (DSEM): To image leaves for trichome morphology, dimension, and density analysis (n= 3-11 plants per abaxial and adaxial leaf surface per species), a desktop Scanning Electron Microscope (DSEM; SNE-4500 Plus Tabletop; Nanoimages LLC, Pleasanton, California, USA; Watts et al., 2021) was used. For operational procedures and DSEM methodology, see Watts and Kariyat (2021a) and Watts et al. (2021).

Assays

a. Trichome morphology: To determine trichome morphology on both *Solanum* species, fresh leaf samples (n= 3-11 plants per leaf surface per species) were used as described above. The images were captured at 45X to 1000X magnification depending on the trichome type and size, to visualize the finer details of trichome structure (Watts et al. (2021) and Watts and Kariyat (2020a).

***2. Trichome density estimation*:** To determine the trichome density on both *Solanum* species (n= 3-11 plants per leaf surface per species), samples were prepared as described above. The images for trichome count were captured at 60X magnification which contains approximately 5.32 mm^2leaf area measured using 'Nanoeye' software linked to DSEM. We calculated the total trichome number, total glandular trichomes and total non-glandular trichomes per mm^2 of leaf surface area as described (Chavana et al., 2021; Watts and Kariyat, 2021b).

3. Wax quantification: The epicuticular waxes on leaves of both species were quantified. 50 circular leaf discs (0.63 cm in diameter) were collected uniformly from each plant (n=19: *S. glaucescens*; n= 12: *S. macrocarpon*) using a hole puncher. The leaf discs from each plant were placed in a pre-weighed 2 ml microcentrifuge tube containing anhydrous chloroform. The tubes were subsequently vortexed (VWR; ~1500 rpm) for one minute. The leaf discs were removed from the tubes post-vortexing and the tubes containing chloroform+wax solution were left under a fume hood for 24 h to allow the chloroform to evaporate, leaving the wax behind. The tubes were weighed again and the difference in post and pre weight of tubes was recorded as the wax amount (mg) for each sample (Kariyat et al., 2019).

***4. Time to first bite*:** Leaf surfaces can hinder the movement and feeding of herbivores due to the presence of surface defenses, even before the herbivore has started feeding (Wilkens et al., 1996;

Kariyat et al., 2017; Despland, 2019). To test whether the caterpillar takes a longer time to initiate feeding on one species compared to the other, first instar caterpillars (n= 15 per species) were used. Twenty minutes was set as a limit to stop each observation if the caterpillar does not start feeding since a starved caterpillar starts feeding within few minutes (Kariyat et al., 2018; 2019).

***5. Mass gain and mortality by caterpillars*:** This experiment was performed to estimate if caterpillars gain higher or lower mass and mortality when fed on a species with higher waxes or a control diet. First instar *M. sexta* caterpillars on *S. glaucescens* (n=15) and artificial diet (n=30) were used. Pre-weighed 1st instar caterpillars were placed on plants and allowed to feed. Pre-weighed caterpillars were placed on a diet pellet in a separate petri-plate as a control. The caterpillars were allowed to feed for 24 h and then collected to weigh. After recording their mass, the caterpillars were placed back on their respective treatments and were weighed again after 24 h (48 h in total). While recording mass gain at 24 h and 48 h, caterpillar mortality was also recorded under treatment and control conditions. Data of mass gain by caterpillars was recorded as following:

$$\textit{Mass gain} = (\textit{Final Mass} - \textit{Initial Mass}) / \textit{Initial Mass}$$

6. Mass gain and mortality by caterpillars on diet pellets coated with waxes: Waxes extracted from the wax quantification experiment were used to determine the mass gain and mortality of caterpillars on species with different amounts and/or possibly different composition of waxes. Five 2-ml microcentrifuge tubes containing extracted waxes of each species were selected randomly. 200 µl of chloroform was added to each tube as the solvent and the tubes were vortexed (VWR; ~1500rpm) for one minute to dissolve the waxes in chloroform. The dissolved waxes were coated thrice on artificial diet pellets using a paint brush. There were two treatments: diet pellets coated with waxes extracted from *S. glaucescens* leaves and diet pellets coated with waxes extracted from

S. macrocarpon leaves. Additionally, a third set of diet pellets had only chloroform coated on them and lastly, control pellets had neither chloroform nor waxes as a coating. Pre-weighed 1st instar caterpillars (n=15 per treatment) were allowed to feed for 24 h and were then weighed. After weighing caterpillars at 24 h, they were put back on diet pellets and weighed again after another 24 h (48 h in total). At 48 h, all four batches of caterpillars were moved to an artificial control diet allowed to feed for another 24 h (72 h in total), and then weighed again. Mass gain by caterpillars at 24 h, 48 h and 72 h were recorded as described above. The mortality of caterpillars was recorded for each timepoint.

7. *Mass gain and mortality by caterpillars on a diet of leaf tissue*: This experiment was performed to test the effects of leaf tissue composition on caterpillar mass gain and mortality. 50 g of leaf tissue from each species (from 8 different plants) was cryo-dried. The dried was ground (Mitton et al., 1979) and added into 0.5 liter of artificial diet. 0.5 liter of control artificial diet was prepared in the similar manner, but without leaf tissue. Pre-weighed 1st instar caterpillars (n=30) were allowed to feed on diet pellets for all three treatments. The mass and mortality of caterpillars was recorded after 24 h and 48 h. The mass gain of caterpillars was recorded as described above (Kariyat et al., 2019).

8. ***Polyphenol oxidase (PPO) assay:*** Polyphenol oxidases are widely distributed enzymes known to play an important role in plant defense against diseases and herbivores (Constabel and Barbehenn, 2008). In this experiment, we quantified the PPO content (U/mg) in both *Solanum* species to test for the presence of defensive compounds and herbivore resistance. Fresh leaves from the central part of the plant were excised from both *Solanum* species (n=8 per species) and used for PPO quantification. The PPO assay performed as described in the Polyphenol Oxidase Assay Kit manual (Catalog# MBS822343; MyBioSource). Quantification of PPO was performed

using the equation in the Polyphenol Oxidase Assay Kit manual (Catalog# MBS822343; MyBioSource).

$$PPO\ (U/g) = (OD_{Sample} - OD_{Control}) \times V_{Total} / (W \times V_{Sample} / V_{Assay}) / 0.01 / T = 233.3 \times (OD_{Sample} - OD_{Control}) / W$$

Where OD stands for calorimetric readout of optical density at 410 nm, V_{Total} is the volume of sample (0.35 ml), W is weight of the sample (0.1 g of plant tissue), V_{Sample} is the volume of sample (0.05 ml), V_{Assay} is the volume of Assay buffer (1 ml) and T is the reaction time (3 minutes).

Analysis

We identified and classified the trichomes in *S. glaucescens* and S. *macrocarpon* according to Watts and Kariyat (2021a). The trichome number (total, total glandular trichomes and total non-glandular trichomes), wax quantity (in mg) and first bite time by first instar caterpillars (in minutes) was analyzed using a Wilcoxon two-sample test with species as the explanatory variable. Mass gain by caterpillars at 24 h and 48 h on plant and artificial diets was analyzed using a one-tailed t test. The mortality of caterpillars in the mass gain experiment at 24 h and 48 h timepoints was analyzed using Logistic Regression with binomial distribution (alive/dead). The mass gain of caterpillars fed on pellets coated with waxes at 24 h was analyzed using one-way ANOVA with Tukey's post-hoc test. At 48 h and 72 h, because the data did not follow a normal distribution, a Kruskal-Wallis test pairwise comparison was done using a Wilcoxon test. The mortality of caterpillars on pellets coated with waxes was analyzed using Logistic Regression with binomial distribution (alive/dead) at all timepoints. The data for mass gain by caterpillars on a diet containing leaf tissue (both 24 h and 48 h) was analyzed using a Kruskal-Wallis test and pairwise

comparison was done using a Wilcoxon test. The PPO data was normally distributed and thus a one-tailed t-test was used for analyzing the data. All analyses were carried out using JMP statistical software (SAS institute, Cary, NC, USA). Plots were built using GraphPad Prism (La Jolla, CA, USA).

Results

1. ***Trichome morphology***: The leaves of both the species were examined using scanning electron microscopy (SEM). We observed that *S. glaucescens* had two types of trichomes: glandular hairs with a globular head; and acuminate glandular hairs with a multicellular stalk and a small glandular tip. *S. macrocarpon* had three types of trichomes: glandular hairs with a globular head and single stalk cell; attenuate basilatus glandular hairs with a small glandular tip; and subulate non-glandular hairs with a pulvinate base and pedestal (Figure 1).

2. ***Trichome density assessment***: For *S. macrocarpon*, we observed more trichomes compared to *S. glaucescens*. As expected, we found a significantly higher total trichome number (Wilcoxon 2 sample test; $p= 0.0049$), total glandular trichome number (Wilcoxon two-sample test; $p= 0.0073$), and total non-glandular trichome number (Wilcoxon two-sample test; $p= 0.0084$) in *S. macrocarpon* compared to *S. glaucescens* (Figure 1).

3. ***Wax quantification***: In SEM images of leaves, we observed a significantly thicker layer of epicuticular waxes on the leaf surface of *S. glaucescens* (10.19 ± 0.50 mg; average ± SE) compared to *S. macrocarpon* (4.06 ± 0.57 mg; average ± SE) (Figure 1).

4. ***Time to first bite***: After quantification of physical defenses in both species, we found that 1st instar caterpillars took a significantly longer time to initiate feeding on *S. glaucescens* compared to *S. macrocarpon* leaves (Wilcoxon 2 sample test; $p<0.0001$) (Figure 2). This result indicates a

stronger physical barrier offered by the *S. glaucescens* leaf surface compared to *S. macrocarpon* in the inhibition of feeding.

5. ***Mass gain and mortality by caterpillars***: The mass gain by caterpillars on *S. glaucescens* was significantly lower compared to control artificial diet at 24 h (one-tailed t-test; $p<0.0001$) and 48 h (one-tailed t-test; $p<0.0001$). Consistent with these data, the mortality of caterpillars on *S. glaucescens* was significantly higher compared to control diet (Generalized Regression; $p<0.0001$) at both 24 h and 48 h (Generalized regression; $p<0.0001$) (Figure 2). This result suggests a strong negative effect of caterpillar feeding on *S. glaucescens* (species with higher leaf surface waxes) compared to the artificial control diet with no surface or embedded waxes.

6. ***Mass gain by caterpillars on diet pellets coated with waxes:*** When diet pellets were coated with waxes, mass gain by 1st instar caterpillars was significantly different for all four treatment groups at 24 h (one-way ANOVA; $R^2= 21.4134$; DF= 59; $p= 0.0084$). Post-hoc tests showed that caterpillars placed on pellets coated with *S. glaucescens* waxes had a significantly lower mass gain compared to caterpillars placed on the control diet (Tukey's test; $p=0.0062$). Other interactions between treatments (such as diet pellets with waxes of *S. macrocarpon* and control diet, diet pellets with chloroform and control diet) were non-significant (Tukey's test; $p>0.05$). At 48 h, there was no significant difference among all four treatments (Kruskal-Wallis test; $p=0.1217$). At 72 h, there was significant difference among all treatments (Kruskal-Wallis test; $p=0.0306$). Caterpillars that were initially placed on pellets coated with *S. glaucescens* waxes (Wilcoxon test; $p=0.0320$) and *S. macrocarpon* (Wilcoxon test; $p=0.0141$) had a significantly lower mass gain compared to caterpillars that were placed on the artificial control diet. However, there was no significant difference in the mortality of caterpillars among all treatments at 24 h, 48 h and 72 h (Generalized Regression; $p>0.05$) (Figure 3). While this experiment suggests that the waxes of both species

decrease the growth of caterpillars, chloroform consistently reduced the mass gain by caterpillars. This factor likely resulted in lower mass gain of caterpillars feeding on artificial diet + chloroform than artificial diet without chloroform at all timepoints (24 h, 48 h and 72 h; Figure 3).

7. ***Mass gain by caterpillars on diet containing leaf tissue***: When plant tissue was added into the artificial diet, there was no significant difference in caterpillar mass gain among diet treatments at both 24 h (Kruskal-Wallis test; $p=0.7698$) and 48 h (Kruskal-Wallis test; $p=0.6952$). There was no significant difference between treatments at 24 h and 48 h (Wilcoxon test; $p>0.05$), and no mortality was recorded for any of the treatments at both times. These data indicate that the composition of leaves has little or no effect on caterpillar growth and suggests that the resistance in these plants to *M. sexta* is mainly due to trichomes and/or surface waxes.

8. ***Polyphenol oxidase (PPO) assay***: There was no significant difference in PPO content between both plant species (one-tailed t-test; $p=0.8463$). This result suggests that surface defenses, rather than leaf tissue containing defensive compounds (e.g., PPO) are responsible for differential effects on herbivore growth and mortality for the two *Solanum* species in the study.

Discussion

Our collective results show that although *S. glaucescens* and *S. macrocarpon* have different types of trichomes, trichome density is higher on *S. macrocarpon* leaves and wax quantity is higher on *S. glaucescens* leaves. Behavioral assays show that *M. sexta* caterpillars tend not to initiate feeding on *S. glaucescens* compared to *S. macrocarpon* and have lower mass and high mortality on the *S. glaucescens* leaf surface with greater wax amount compared to an artificial diet. Caterpillars also gained a lower mass compared to control diet up to 48 h after ingestion of artificial pellets coated with *S. glaucescens* waxes. When the artificial diet was supplemented with leaf tissue from either

species, no differential effects on herbivore growth were observed. Polyphenol oxidase, one of the major enzymes involved in providing defense to the plants against herbivorous arthropods, was not significantly different between the two *Solanum* species in this study. These findings support our hypothesis that defense against herbivores is independent of leaf tissue components, but dependent on surface defenses including waxes and trichomes for the plant species used in the study.

Both *Solanum* species used in the study have glandular and non-glandular trichomes. However, non-glandular trichomes are present in considerably greater number on *S. macrocarpon* versus *S. glaucescens* leaves. Previously, non-glandular trichomes have been found to have deterrent effects on caterpillar feeding (Kariyat et al., 2017) especially in earlier instars (Kariyat et al., 2018). The trichome density of all trichome types (total, glandular and non-glandular) is higher in *S. macrocarpon*, creating a comparatively stronger physical barrier against stresses. Higher trichome density is usually associated with decreased herbivory (Fordyce and Agrawal, 2002; Eaton and Karba, 2014; Pastório et al., 2019; Watts and Kariyat, 2021b) and increased abiotic stress tolerance (Liakoura et al., 1997; Li et al., 2018). Thus, we expected *S. macrocarpon* to be more resistant to herbivore feeding than *S. glaucescens*, but this was not the case. Waxes are also considered as a strong physical defense against herbivores (Müller and Reiderer, 2005; Daoust et al., 2010; Whitney and Federle, 2013; Kaur et al., 2022). *S. glaucescens* has a thick epicuticular wax layer and higher wax content compared to *S. macrocarpon*. Our findings show that surface waxes can be a significant physical deterrent against herbivory.

Herbivore feeding behavior assays showed that starved 1st instar caterpillars took much longer to initiate feeding on species with more waxes (Varela and Bernays, 1988; Shelomi et al., 2010). In our experiment, the caterpillars did not start feeding on *S. glaucescens* for at least 20 minutes (some

feeding was observed afterwards). Additionally, caterpillars failed to gain any mass or even lost mass, possibly due to desiccation, after placement on *S. glaucescens*. Mortality of the caterpillars was also very high (more than 90%) on *S. glaucescens* plants, with almost no mortality on the artificial diet (Wójcicka, et al., 2016). Given that *S. glaucescens* leaves have more epicuticular wax and lower trichome numbers compared to *S. macrocarpon*, these results suggest that waxes act as a stronger physical barrier for caterpillars, independent of trichomes (Pelletier et al., 1999). Plants tend to use a combination of different defense strategies to mount "an efficient defense phenotype" based on their ancestry and evolutionary history (Agrawal and Fishbein, 2006). In this study, epicuticular waxes outperformed trichomes: feeding on *S. macrocarpon* leaves with a high trichome density and thin wax layer failed to hinder caterpillar feeding in comparison to *S. glaucescens*.

When artificial diet pellets were coated with plant waxes, the mass gain by caterpillars placed on pellets containing waxes from *S. glaucescens* was lower compared to caterpillars on a control diet (pellets without waxes and chloroform) at 24 h and 48 h. The mortality for all treatments was similar, indicating an inhibitory effect of waxes. This inhibitory effect was more pronounced on leaves since caterpillars on *S. glaucescens* failed to gain mass and grow after 24 h. Later, when caterpillars from all four treatments were moved to an artificial diet, the mass gain by caterpillars initially placed on pellets smeared with plant waxes was lower compared to caterpillars placed on artificial diet pellets with no coating. These data indicate an effect of chemical composition (Eigenbrode and Espelie, 1995) on caterpillars' mass gain and growth (Johnson, 2021). Toxins in the leaf wax may contribute to this inhibitory effect (Belete, 2018). For instance, Negin et al. (2021) found that fatty alcohols in the epicuticular waxes of *Nicotiana glauca* (Solanaceae) tend to reduce caterpillar growth. In our experiment, mass gain was lower in caterpillars placed on *S.*

316 *glaucescens* waxed pellets at 24 h compared to the control diet, but it was lower at 72 h on *S.*
317 *macrocarpon* waxed pellets as well, indicating that waxes on *S. glaucescens* act as stronger barrier,
318 possibly due to higher wax content. Although the caterpillars were switched to a control diet after
319 48 h, caterpillars that were initially placed on pellets coated with plant waxes were lower in mass
320 at 72 h compared to caterpillars that were initially placed on control pellets, emphasizing the
321 impact of waxes beyond the treatment period. While our results show that *S. glaucescens* waxes
322 are a stronger barrier compared to *S. macrocarpon* waxes, we note that chloroform had a negative
323 effect on caterpillar growth all timepoints. In previous studies, chloroform extracts have been
324 found to possess antifeedant properties against caterpillars (Nebapure et al., 2015). Thus, the
325 chemical composition of waxes in both plant species should be explored further to extract and
326 identify anti-herbivore compounds, an area that we are currently focusing on.
327 We also tested if leaf tissue composition acts as a significant chemical barrier (Matsuki and
328 Maclean, 1994; Lill and Marquis, 2001) in herbivore growth. Leaf tissues from both species was
329 added to an artificial diet and caterpillars were allowed to feed (Kariyat et al., 2017; Watts and
330 Kariyat, 2021b). The caterpillars gained similar mass on both treatment and control diet pellets.
331 These results suggest that the composition of leaf tissue has no negative or positive effects on
332 caterpillars' mass gain. Thus, surface defenses have a larger effect on the mass gain of caterpillars.
333 However, these effects should be further studied over a longer period, growth stages, or even
334 generations (Tayal et al., 2020b; Portman et al., 2020). For example, Lill and Marquis (2001) found
335 that low quality oak leaves (*Quercus alba*; Fagaceae) lead to reduced survivorship of the first
336 generation of leaf tying caterpillars (*Psilocorsis quercicella*; Lepidoptera: Despressariidae), but
337 the second generation feeding on the same leaves were unaffected.

Taken together, waxes acted as a comparable, if not stronger surface defense compared to trichomes in this study. Previously, surface defense studies have been more focused on trichomes but epicuticular waxes demand higher attention (Kaur et al., 2022). Additional work should also focus on understanding wax-trichome interplay and how other contributing factors could influence the efficiency of these surface defenses under both favorable and harsh environmental conditions (Lewandowska et al., 2020), and with different herbivores (White and Eigenbrode, 2000) and their natural enemies (Yao et al., 2021).

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Competing Interests Statement

The authors declare there are no competing interests

Data Availability Statement

Data generated or analyzed during this study are available from the corresponding author upon reasonable request.

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Figures

Figure 1. Scanning electron micrographs of adaxial leaf surfaces at 500X magnification (A, B), and abaxial leaf surface at 60X (C, D) of (A, C) *Solanum glaucescens* (Solanaceae) displaying a thick wax layer and glandular trichomes, and (B) *Solanum macrocarpon* (Solanaceae) with no visible wax layer, but glandular and non-glandular trichomes. The trichome number (n= 3-11 plants per leaf surface per species) in scanning electron microscopic images at 60X magnification (5.32 mm^2 of leaf surface area) was significantly higher in *S.* macrocarpon compared to *S. glaucescens* for (E) total trichomes, (F), total glandular trichomes, and (G) total non-glandular trichomes. Additionally, (H) Epicuticular waxes were present in significantly higher amount on *S. glaucescens* (n=19) leaves compared to *S. macrocarpon* (n=12) leaves. Different letters on the bars represent significant differences.

Figure 2. (A) The time taken by starved 1st instar *Manduca sexta* (Lepidoptera: Sphingidae) caterpillars (in minutes) to initiate feeding on leaf surface was higher on *S. glaucescens* compared to *S. macrocarpon*. (B) *M. sexta* 1st instar caterpillars gained significantly higher mass (in mg) on a control artificial diet compared to *S. glaucescens* plants after 24 h of feeding. (C) Survival (0-dead; 1-alive) of *M. sexta* caterpillars was significantly higher on control artificial diet compared to *S. glaucescens* plants at 24 h and 48 h during the mass gain experiment. Different letters (a, b) on the bars for each experiment and during each timing (24 h and 48 h; independent) represent a significant difference.

Figure 3. Caterpillars had significant difference in mass gain (in mg) among four treatments (artificial diet pellets coated with waxes extracted from *S. glaucescens*; artificial diet pellets coated with waxes extracted from *S. macrocarpon*, artificial diet pellets coated with chloroform, and artificial diet pellet with no waxes or chloroform) at 24 h and 72 h. None of the differences were

564 significant at 48 h. Post-hoc analysis revealed a significantly lower mass gain by caterpillars placed
565 on pellets with *S. glaucescens* waxes compared to caterpillars placed on the control diet at 24 h
566 and 48 h. Caterpillars that were initially placed on *S. glaucescens* and *S. macrocarpon* waxed had
567 a significantly lower mass gain compared to caterpillars on the control diet throughout the
568 experiment.

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