



Trichomes mediate plant–herbivore interactions in two Cucurbitaceae species through pre- and post-ingestive ways

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Abstract

Plant structural defenses such as trichomes exert a significant selection pressure on insect herbivores. However, whether variation in structural defense traits affects common herbivores in related plant species is less understood. Here, we examined the role of trichomes in plant–herbivore interactions in two commonly cultivated members in Cucurbitaceae: bottle gourd (*Lagenaria siceraria*) and cucumber (*Cucumis sativa*). In common garden experiments when the two species were grown together, we observed that they differed in their attractiveness to four major herbivore species (*Trichoplusia ni*, *Acalymma vittatum*, *Diaphania indica*, and *Anasa tristis*) and, consequently, their feeding behavior. We found that *L. siceraria* consistently harbored less herbivores, and the two lepidopteran herbivores (*T. ni* and *D. indica*) were found to take significantly longer time to commence feeding on them, a primary mode of pre-ingestive defense function of trichomes. To tease apart structural and chemical modes of defenses, we first used scanning electron microscopy to identify, quantify, and measure trichome traits including their morphology and density. We found that *C. sativa* has significantly lower number of trichomes compared to *L. siceraria*, regardless of trichome type and leaf surface. We then used artificial diet enriched with trichomes as caterpillar food and found that trichomes from these two species differentially affected growth and development of *T. ni* showing cascading effects of trichomes. Taken together, we show that trichomes, independent of chemical defenses, are an effective pre- and post-ingestive defense strategy against herbivores with negative consequences for their feeding, growth, and development.

Keywords Trichomes · Polyphenol oxidase · *Trichoplusia ni* · Artificial diet · Eclosion

Introduction

Insect herbivory leads to the initiation of a suite of structural and chemical defenses in their host plants. Trichomes, often considered as the first line of defense, play well-established roles—both as constitutive and induced defenses, affecting herbivores, and consequently improving host plant survival and fitness (Pechan et al., 2002; Andama et al. 2020). Trichomes defend against herbivores by feeding (Duffey and

Isman 1981; Weinhold and Baldwin 2011; Kaur et al. 2022a, 2022b) and movement restriction (Dalin et al. 2008; Pieffer et al. 2009; Szyndler et al. 2013; Kariyat et al. 2018), reducing leaf consumption, damaging herbivore gut lining (peritrophic matrix; Kariyat et al. 2017), and even blunting their mouthparts (Riddick and Wu 2011).

Trichomes (glandular) have a bulbous head embodied with sticky exudates and defensive compounds that can entangle herbivores, act as toxins, activate defense gene expression, or selectively attract their predators (Zalucki et al. 2002; Hare 2005; Pieffer et al. 2009; Weinhold and Baldwin 2011; Kaur et al. 2022a, 2022b; Vasquez et al., 2022). Moreover, recent work has also showed that these effects are instar dependent, with early instar caterpillars more affected by pre-ingestive effects since they do not necessarily ingest trichomes, when compared to late instars that mow them down and profusely ingest them (Kariyat et al. 2018). Clearly, even before the onset of defense signaling at the cellular level (Gandhi et al. 2020, 2021), and

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consequent downstream gene expression and defense induction, trichomes guard the leaves and make it challenging for herbivores to feed, grow, and develop on them (Bhonwong et al. 2009; Kariyat et al. 2019).

Due to the tremendous diversity in morphology and chemistry underlying trichomes at species, genus, and family levels in angiosperms (Watts and Kariyat 2021), we still lack a detailed understanding of their roles in plant defense against herbivores that vary in functional and life history traits. Most studies on trichomes tend to scale down trichome morphology in to just glandular and/or non-glandular trichomes (Kariyat et al. 2019; Karabourniotis et al. 2020), with little attention to further differentiate and characterize trichomes into different morphotypes (Levin 1973; Payne 1978) along with their functional diversity (Vargas et al. 2019). For example, non-glandular trichomes can be either stellate or non-stellate, and glandular trichomes can have a short stalk below the bulbous head or a multicellular long stalk (Wagner 1991; Guliani and Bini 2008; Chavana et al. 2021; Watts and Kariyat 2021). These morphological variations can potentially have functional effects since stellate trichomes cover more surface area and can further restrict the commencement of caterpillar feeding (Kariyat et al. 2018, 2019; Watts and Kariyat 2021), and short and long stalks can also affect release of toxins with respect to herbivore size and morphology (Glas et al. 2012).

In general, plants tend to invest their resources among three activities including growth, reproduction, and defense, where allocation tends to be prioritized based on optimum fitness returns under unfavorable conditions, including the constant threat of herbivory (Neilson et al. 2013). This also implies that such investment should select traits that can proactively minimize herbivory, thereby reducing the further synthesis of defenses, that can affect fitness.

Ideally, any defense trait/s that can reduce herbivory before feeding should thus be selected and fixed across species but also tightly regulated based on herbivore feeding pattern and behavior. For example, oviposition by herbivores while not directly affecting plants, clearly signals imminent herbivory. Consequently, plants have evolved a variety of defense traits to either impair larval performance or attract their natural enemies (Hilker and Fatouros 2015; Coapio et al. 2018; Singh et al. 2021). Along these lines, we speculate that the presence of diverse forms of trichomes (morphological and functional variation) and their ability to be induced by wounding and herbivory (Wilkins et al. 1996; Kariyat et al. 2013; Jayanthi et al. 2018; Kaur and Kariyat 2020a), suggests both investment and regulation of such defenses. Our hypothesis is even more plausible since trichome defenses can be even more effective as a preventive pre-ingestive measure than post-ingestion, and thereby

reducing the need for resource allocation toward future defense induction. On the other hand, plants that possess both glandular and non-glandular trichomes should experience less herbivory, as both types are successful in preventing herbivory through different methods (Kariyat et al. 2018, 2019). Taken together, an integrated approach to examine the role of trichomes on herbivore traits including landing, feeding, growth, and behavior is warranted, speculating that their morphological diversity will have functional consequences.

To answer these questions, we used two agriculturally important members of Cucurbitaceae family: garden cucumber (*Cucumis sativa*; Cucurbitales: Cucurbitaceae) and bottle gourd (*Lagenaria siceraria*; Cucurbitales: Cucurbitaceae), two commonly grown, but relatively unexplored species in trichome studies, in a combination of common garden, microscopy, behavioral, and laboratory experiments. We chose these species since they are two common cucurbit species that have similar seasonal growth habits, harbor the same groups of herbivores, and can be planted together. Specifically, our study was aimed to answer the following questions: (a) Can morphological variation in trichomes affect herbivore defenses including incidence and damage, and (b) Can trichomes act as both physical and chemical defenses affecting herbivore growth and development.

Materials and methods

Study system

Plant material

We used seeds of variety bottle/birdhouse for *L. siceraria* (bottle gourd) and variety Marketmore76 for *C. sativa* (cucumber). Both seed types were purchased online from Amazon.com. These varieties were specifically chosen since they have been previously used for laboratory experiments and have been found to grow and develop under similar conditions and had same herbivores feeding in field conditions. For laboratory experiments, the seeds were sown directly in pots (15-cm diameter) filled with growth media (Sunshine professional growing mix: Sun Gro Horticulture Canada Ltd., Agawam, MA, USA). The pots were kept in growth chamber (16 L:8 D; day/night at 25/22 °C; 65% relative humidity), and after emergence, the seedlings were watered daily. The stems were tied to the wooden stick (Wooden Round Dowels (3/16 inches; Horizon Group, USA)) to support them to stand in upright position as soon as they started to trail on the ground. Plant health was ensured and monitored daily until they were ready for the experiments.

Herbivores

Four herbivores, *Trichoplusia ni* (cabbage looper; Lepidoptera, Noctuidae), *Acalymma vittatum* (striped cucumber beetle; Coleoptera, Chrysomelidae), *Diaphania indica* (cucumber moth; Lepidoptera, Crambidae) [chewing herbivores], and *Anasa tristis* (squash bug; Hemiptera, Coreidae) [sucking herbivore], were the primary species used to observe herbivore feeding and incidence in common garden experiments. The rationale behind choosing four different herbivores with two different guilds were twofold: (1) They are the most commonly found herbivores on these two host plants in South Texas and having two feeding guilds and three orders gave us an (2) opportunity to examine whether trichomes have broad defense functions on herbivore settling and feeding initiation. After common garden experiments, we used, *T. ni* for laboratory experiments as it is an important generalist pest of many plant families, and severe pest of Cucurbitaceae (Rivera-Vega et al. 2017). They cause huge economic losses throughout the continental USA, making them one of the most dangerous pests in the USA. To conduct laboratory experiments, eggs of *T. ni* were brought from commercial vendor (Benzon Research, Carlisle PA, USA). The eggs were allowed to hatch inside the plastic container (35-cm long × 10-cm high × 15-cm wide) covered with a moist paper towel. Once the eggs hatched, the neonate larvae were gently transferred to Petri dishes (90 cm × 15 cm) with a paint brush, where they were kept on a generic Lepidopteran diet (Frontier Scientific Newark, DE, USA). The diet was prepared by boiling 875 ml of water along with 19 g of agar on hot plate stirrer which was mixed rigorously with 144 g of diet powder. Once boiled, the diet was allowed to cool and solidify, to be stored in refrigerator at 4 °C (Watts and Kariyat 2021).

Common garden experiments

For common garden experiments, the seeds of *L. siceraria* and *C. sativa* were directly planted in garden soil in a home garden in McAllen, Texas. To get a sample size of at least 15 plants per species, 45 seeds of each species were planted alternating in groups of three in a 3" deep pit in Spring of 2020, at 2 feet spacing from each other, and watered daily (an area of ~400 m²). The common garden experiment was a makeshift experiment since university garden, commonly used for such experiments, was shut down due to COVID-19. Once the seeds germinated and had the first set of true leaves, one or two seedlings per group (from 3) were removed, and a single seedling was allowed to grow. Both species were planted in a completely randomized design in the garden, and a trellis of 7 feet (50ft × 20ft × 7ft) was installed for the seedlings to climb, spread, and grow over

the spring and summer. Five weeks after planting, we had a well-established common garden with > 15 plants of each species growing in proximity (one *L. siceraria* next to one *C. sativa*) for herbivory assessment experiments. While a few individual plants died 2 weeks after germination, this design gave an opportunity to herbivores in the form of a two-choice assay with multiple individuals of two host plants.

Herbivore incidence in common garden

In the common garden experiment, we examined herbivore incidence on *L. siceraria* and *C. sativa* by counting individual insects of the four herbivore species; *T. ni*, *A. vittatum*, *D. indica*, and *A. tristis*. For incidence assessment, we counted the number of each herbivore present on 30 randomly selected leaves of both plant species. Since both species were allowed to climb on trellis, it was difficult to confirm that only two leaves per plant were used for incidence, so we used the origin of the vine as our guide to ensure we do not pseudo-sample/pseudo-replicate for incidence. Utmost care was taken to not damage leaf surface and break trichomes. In addition to the incidence of the herbivores, we also collected data on the number of *A. tristis* egg clutches using the similar method.

Herbivore feeding (*Trichoplusia ni* on *Lagenaria siceraria* and *Cucumis sativa*)

During our herbivore incidence experiment, we noticed that *T. ni* tend to be on *L. siceraria* in very low numbers. To test whether *T. ni* feeds on *L. siceraria*, we collected 30 *T. ni* caterpillars (first and second instars from the common garden) and were starved for 24 h and then placed on 15 different leaves of both species; two fully opened leaves per species with each leaf having a caterpillar. Only one caterpillar was placed at a time, and they were observed for 5 min to see if they commence feeding (similar to Kariyat et al. 2017, 2018; Watts and Kariyat 2021) to measure their time to start feeding.

Forced feeding of *Trichoplusia ni* and damage assessment on *Lagenaria siceraria* and *Cucumis sativa* plants

To confirm whether the difference in feeding behavior among the two species persists, and if there were any cascading effects on growth, we also did no choice assays in the common garden. Pre-weighed first instar caterpillars were forced to feed on both the plants species by placing a single caterpillar per plant ($n = 30$) on a fully matured leaf. The leaf on which they were

placed was covered with bridal veil bag (Walmart Inc., Bentonville, USA) to prevent them from escaping, and to ensure that their feeding is restricted to the leaves they were placed on. After 48 h of feeding, the caterpillars were recovered, and their mass and mortality on each species were calculated. This mass was used to calculate mass gain with the equation:

$$\text{Mass gain} = (\text{Final mass} - \text{initial mass}) / \text{initial mass}$$

We also analyzed damage inflicted by *T. ni* which was forced to feed on both the types of plants ($n=15$). After 48 h, damage on the leaves on which *T. ni* was tied with bridal veil was rated on a 0–4 scale (0—no damage, 1—25% damage on enclosed leaves, 2—50%, 3—75%, and 4—if all the leaves were completely damaged; Kaur and Kariyat 2020a, 2020b).

Trichome estimation on *Lagenaria siceraria* and *Cucumis sativa*

To estimate the morphological and quantitative variation in trichomes between these species, and on the abaxial and adaxial leaf surfaces (Watts and Kariyat 2021; Watts et al. 2022), a SNE-4500 Plus Tabletop Scanning Electron Microscope was used. We used the following specifications for image acquisition; 30 kV; 110 μA ; secondary electron image detector; high vacuum mode; 29% e-beam spot size; auto-brightness/contrast; pre-centered tungsten filament cartridge electron gun; rotary vane pump; and Pfeiffer HiPace turbo molecular pump for vacuum (Chavana et al. 2021; Watts and Kariyat 2021). Care was taken that all the leaf samples were of same size and similar age (fully opened young leaves). Each leaf sample was carefully cut (without damaging trichomes) into rectangular shape with a pair of scissors and mounted on circular stage (diameter = 1.5 cm) with the aid of carbon double tape and then inserted into the SEM stage. For *L. siceraria*, 15 samples from 15 randomly chosen plants were taken and scanned at 100 \times magnification at 1.85 mm² area. Trichome density for *C. sativa* leaves was analyzed from 10 leaf samples over an area of 3.82 mm² at 70 \times magnification (to facilitate easier counting of trichomes, as the density of trichomes was high on *L. siceraria*). The area was calculated using measurement tools in Nanoeye software (Nanoimages LLC, Pleasanton, California, USA) aligned with SEM. In addition to identifying trichome types (glandular vs. non-glandular), their density was also identified and estimated (for details please see Watts and Kariyat 2021).

Trichome density for *L. siceraria* leaves

$$= \text{Number of trichomes in the image taken at 100} \\ \times \text{magnification (1.85 mm}^2\text{leaf area)}$$

Trichome density for *C. sativa* leaves

$$= \text{Number of trichomes in the image taken at 70} \\ \times \text{magnification (3.82 mm}^2\text{leaf area)}$$

Trichome-added diet experiment in laboratory

Our common garden experiments were geared to understand whether herbivore feeding and incidence were affected by trichomes, primarily through a structural defense mechanism that delays and/or restricts the amount of feeding (Kariyat et al. 2017, 2018; Watts and Kariyat 2021). However, the microscopy data encouraged us to speculate whether these differences also affected in post-ingestive ways—due to the presence of different types of non-glandular trichomes (Andama et al. 2020; Kaur and Kariyat 2020a, 2020b) that can cause negative impacts on development. So, we made trichome enriched artificial diets (Kariyat et al. 2017; 2019) and allowed the caterpillars to grow, develop, and pupate on them. The experiment had three treatments; artificial diet without any addition of trichomes, artificial diet incorporated with trichomes of *L. siceraria*, and artificial diet incorporated with trichomes of *C. sativa*. Eighteen leaves of bottle gourd with a total mass of ~137 gm and 42 leaves of *C. sativa* with a total mass of ~75 gm were used to make trichome-based solution. Due to difference in weight, length, and mass of leaves of two plant species, we had to take different number of leaves, to make a total volume of 400 ml of trichome solution. To extract trichomes from the leaves, leaves were cut into small and uniform size rectangular pieces, dipped in liquid nitrogen for 40–60 s. These leaf samples were then placed into 45-cm falcon tubes and then 8-ml water added in the falcon tube and vortexed for 20 s. After vortexing, leaf samples were taken out, and the remaining trichome solution was passed through the sieve to ensure that no leaf material is present in the extracted water. The same process was continued to obtain 400 ml of water from both the treatments (see Kariyat et al. 2019 for detailed methodology on trichome extraction). Then, 250 ml of deionized water was allowed to boil on hot plate stirrer with 9.5 gm of agar added. After boiling, the prepared mixture was added to blender along with 72 gm of artificial Lepidopteran diet and 250 ml of the extracted trichome solution and thoroughly mixed. The water used for extraction and diet preparations were tepid and was ~40C. The control diet had no added trichomes. The diet was allowed to cool at 45 °C, stored in a refrigerator at 4 °C, and used after 24 h (for extended details on trichome diet preparations, please see Kariyat et al. 2017, 2019; Watts and Kariyat 2021).

Mass and mass gain of *Trichoplusia ni* on different diets

For this experiment, 4 days after hatching, 30 *T. ni* caterpillars were weighed on a microbalance (Accuris Instruments, DX series, USA) and then placed individually on the three different treatment diets. Diet pellets of approximately 1 cm³ were cut and were placed in these plastic cups for caterpillars to feed on them. Data on mortality rate, mass, and mass gain at each instar and total mass gain over the entire larval lifecycle (Tayal et al. 2020) were also estimated by the following equation:

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

SEM images of frass pellets collected from three different treatments

To fortify our point that these trichomes are being ingested by *T. ni* along with the artificial diet and any negative effects observed are possibly due to trichome ingestion, we also took scanning electron microscopy images of frass pellets of *T. ni*, to see if we can locate undigested trichomes in the frass (Kariyat et al. 2017). We collected SEM of the frass pellets collected from all the three treatments. The frass pellets of caterpillars fed on different diets (artificial diet without any addition of trichomes), artificial lepidopteran diet incorporated with trichomes of *L. siceraria* and *C. sativa* leaves, were collected and mounted on circular stage (diameter = 15 cm) to use for SEM imaging using similar methodology detailed in 3.

Pupation

Pupal characteristics such as days to pupation, pupal mass, and pupal volume were also assessed with respect to treatments and sex. Once pupated, pupae from each treatment were weighed sexed, volume measured (Tayal et al. 2020) and pooled together in plastic container (23.19 cm × 15.24 cm × 16.84 cm, Walmart) along with wooden shavings. These plastic containers were kept inside laboratory cabinets to give them dark conditions at room temperature of 27 °C and RH of 65% to eclose (Tayal et al. 2020).

Adult moth survival

We also examined the possible cascading effects of these three-diet treatment on adult moths. We collected data on adult traits such as number of moths emerged from pupae of different treatments, pupal mortality rate, and number of

days survived (collected every evening). The goal of this experiment was to assess whether trichome-added diet and their variation in the two species affects caterpillar growth and development, and whether these differences if any spill over to other life stages including pupa and adult (Tayal et al. 2020).

Polyphenol Oxidase (PPO) assays of *L. siceraria* and *C. sativa* leaves

To test the activity of plant defensive proteins, as an index of chemical defenses, we also measured the polyphenol oxidase activity of both the cucumber and bottle gourd leaves ($n=6$ for each; Singh et al. 2021). We used the General Polyphenol Oxidase Assay Kit from My BioSource.com (San Diego, CA, USA). To measure PPO activity from samples, we used the following procedure. About 10 ml of distilled water was first added to substrate buffer. Then, 0.1 gm of leaf from both the species ($n=6$) is mixed with 1 ml of assay buffer in the tube. The tubes were placed immediately placed on ice. The tubes were then centrifuged at 8000 rpm 4 °C for 10 min. Supernatant was taken in a new tube, and 50 µL of sample was prepared and boiled, and we used distilled water as control. About 150 µL of reaction buffer and 50 µL of substrate buffer were subsequently added to the sample, and all the reagents were mixed and placed in oven for 37 °C for 3 min. After adding 100 µL of stop solution, the mixture is homogenized and centrifuged at 10,000 rpm for 5 min. Supernatant (200 µL) was used for microplate reading using 96 well plate (Thermo Fisher Scientific, Waltham, NA), and the absorbance was read at 410 nm.

Statistical analysis

All the analysis were performed using the statistical software JMP (Statistical Analysis Software Institute, NC, USA), and the plots were built using GraphPad Prism (La Jolla, CA, USA). For data sets that failed to meet normality assumptions after transformations, non-parametric tests were used, count data were analyzed with generalized regression. The presence of *T. ni* on both the Cucurbitaceae species was analyzed using Mann–Whitney test, while the data for *T. ni* behavior were done by Fisher's exact test. The data of *A. vittatum* presence, *D. indica* presence, *A. tristis* egg clutch, and *A. tristis* adult's presence were also analyzed using Mann–Whitney tests. One-way ANOVA followed by Sidak multiple comparisons tests was used to analyze the mass gain of *T. ni* on forced feeding experiment in the common garden. To estimate if there is any variation in density of trichomes present in two different Cucurbitaceae species, we ran generalized regression test followed by Poisson Maximum Likelihood Ratio test. Forced feeding and mass gain

assay was analyzed using one-way ANOVA. Generalized regression model was used to analyze the data of mass gain of different instars of *T. ni* fed on artificial diet comprising of three treatments, i.e., control (with no trichomes) and diet incorporated with *L. siceraria* and *C. sativa* trichomes, followed by all pairwise comparison through *post hoc* Tukey–Kramer test. The same model was further used to analyze different interactions such as pupal mass, volume, sex, and interactions between different diets on sex of *T. ni*. We also analyzed total lifespan in days for *T. ni* fed on different diets using generalized regression model followed by Poisson Maximum Likelihood test. And *t*-test (unpaired) was used to analyze the PPO activity between two Cucurbitaceae species.

Results

Lagenaria siceraria had less herbivores and lower herbivory levels

The results from common garden experiments revealed that the herbivores preferred *C. sativa* plants over *L. siceraria* plants. *T. ni* was present significantly more

on *C. sativa* plants (Mann–Whitney U-test; $U = 202.5$; $P < 0.0001$, Fig. 1A), and observations on feeding preference of *T. ni* also showed similar pattern, where significantly lower number of *T. ni* started feeding on *L. siceraria* compared to *C. sativa* (Fisher’s exact test; $P < 0.0001$, Fig. 1B). We observed that the starved caterpillars of *T. ni* were unable to breach the trichome rich surface of *L. siceraria* and were found to wander when compared to *C. sativa* where they readily commenced feeding. Consistent with our observations, other insect herbivores also significantly preferred *C. sativa* plants as observed in case of *D. indica*—was observed in significantly more numbers on *C. sativa* plants when compared to *L. siceraria* (Mann–Whitney U-test; $U = 42.50$; $P < 0.0001$, Fig. 1D). However, there was no significant difference in case of *A. vittatum* (Mann–Whitney $U = 333$, $P = 0.0701$, Fig. 1C). Interestingly though, *A. tristis* laid significantly more eggs on *L. siceraria* plants (Mann–Whitney U-test; $U = 240$; $P = 0.0001$, Fig. 1E) with more adults also observed (Mann–Whitney test; $U = 341$; $P = 0.0553$, Fig. 1F) on *L. siceraria* (for more details on results and statistics, refer Table 1). Clearly, non-lepidopteran specialists (*A. vittatum* and *A. tristis*) were not affected by the trichome-rich surface of *L. siceraria*.

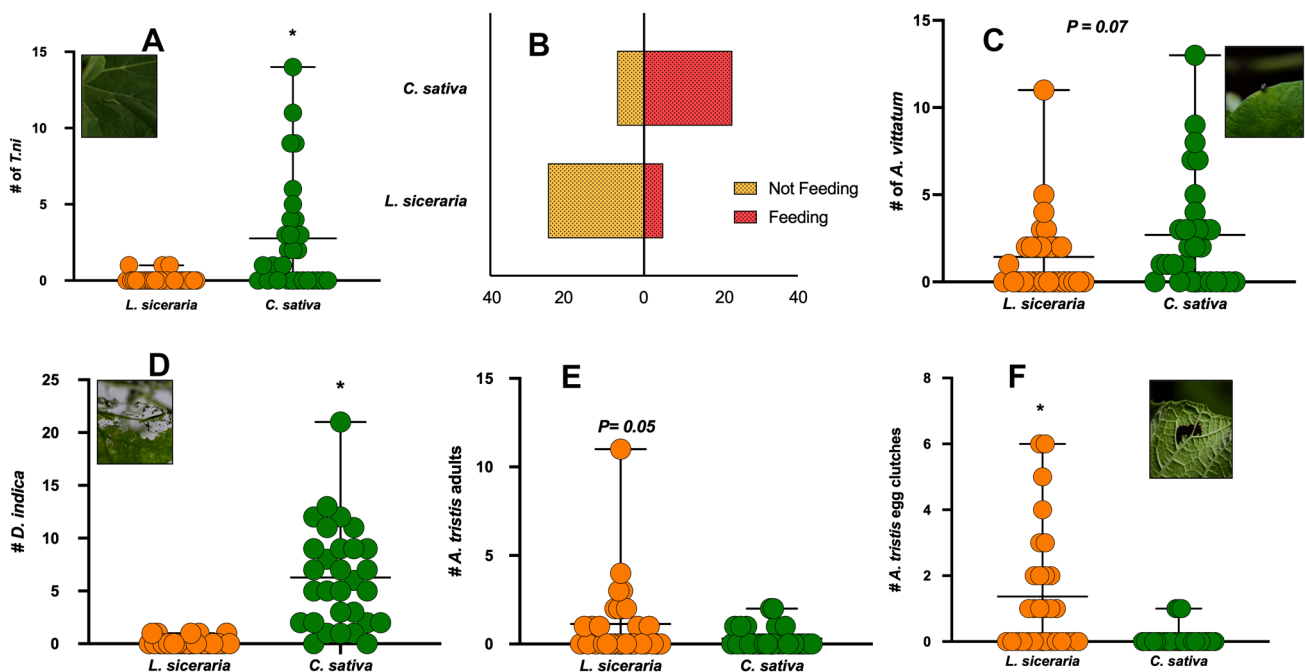


Fig. 1 Results of herbivore incidence and feeding on *L. siceraria* ($n = 15$) and *C. sativa* ($n = 15$) in common garden experiments (A) *T. ni* incidence, (B) *T. ni* feeding preference, (C) incidence of *A. vittatum* (D), E incidence of *D. indica*, F incidence of *A. tristis* adults, and G incidence of egg clutches of *A. tristis*. The asterisk mark

denotes the significant difference between the treatment groups when measured at ($p = 0.05$) level of significance, where orange color indicates incidence/feeding on *L. siceraria* leaves and green color on *C. sativa* leaves, and error bars depict standard error. For statistical details, see Table 1

Lagenaria siceraria* have significantly more glandular and non-glandular foliar trichomes than *Cucumis sativa

We found that there is significant difference in numbers of total trichomes between the two species, *L. siceraria* had more trichomes (Poisson Maximum Likelihood Test; $P < 0.001$, Fig. 2B). Consistent with our observations, there was also significant difference ($P < 0.001$, Fig. 2C) between the glandular trichomes of *C. sativa* and glandular trichomes of *L. siceraria* and between non-glandular trichomes of *L. siceraria* and non-glandular trichomes of *C. sativa*; Fig. 2C. For different morphotypes with detailed description of each type of trichomes present in these two species, see Fig. 3A–G. For line diagrams, of each morphotype, refer supplementary fig. 2. In addition, our analyses also revealed that the number of trichomes found on abaxial and adaxial surface was marginally non-significant.

***Trichoplusia ni* caterpillars gained more mass and caused higher damage on *C. sativa* in forced feeding experiments**

We found that when *T. ni* caterpillars were forced to feed on the species in no-choice assays, on *C. sativa* plants, they gained significantly more mass (one-way ANOVA; $F = 10.45$; $P < 0.0001$, Fig. 4A) after 48 h as compared to *L. siceraria* plants (Sidak multiple comparisons test). We also found that there was significantly more damage inflicted on *C. sativa* leaves when *T. ni* caterpillars were forced to feed on both the types of plants (Mann–Whitney U-test, $U = 57.5$; $P = 0.0002$, Fig. 4B).

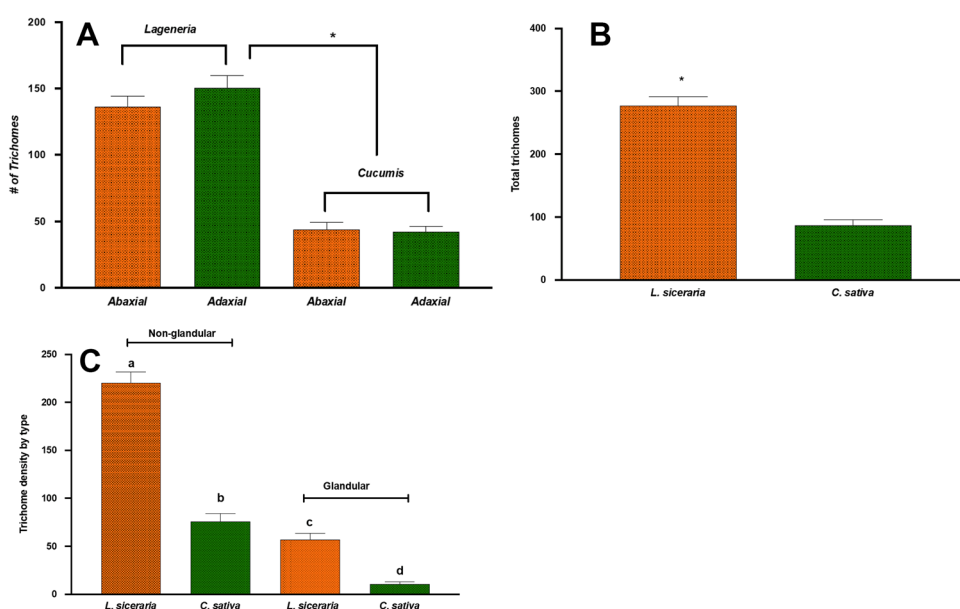
Life history traits of *Trichoplusia ni* shows variation on different diets, more pronounced in later life stages

We did not find any significant difference in mass of *T. ni* when fed on three different treatment diets. We also analyzed mass gain on trichome-added diet which showed that in general, trichome diet variation affected mass gain across

Table 1 Details of statistical analysis of incidence and feeding of different types of herbivores on *L. siceraria* and *C. sativa* in common garden experiments. Values in bold denote statistical significance at $P < 0.05$

Type of herbivore	Plant preference	Test	Test statistics	P value
<i>T. ni</i> presence	<i>C. sativa</i>	Mann–Whitney test	Mann–Whitney $U = 202.5$	< 0.0001
<i>A. vittatum</i> presence	<i>C. sativa</i>	Mann–Whitney test	Mann–Whitney $U = 333$	0.0701
<i>D. indica</i> presence	<i>C. sativa</i>	Mann–Whitney test	Mann–Whitney $U = 42.50$	< 0.0001
<i>A. tristis</i>	<i>L. siceraria</i>	Mann–Whitney test	Mann–Whitney $U = 341$	0.0553
<i>A. tristis</i> egg clutch	<i>L. siceraria</i>	Mann–Whitney test	Mann–Whitney $U = 240$	0.0001

Fig. 2 Results of the statistical analysis of trichomes on *L. siceraria* and *C. sativa* leaves: **A** density of trichomes found on abaxial and adaxial leaf surface, **B** comparison of total number of trichomes, where orange color indicates density of trichomes on *L. siceraria* leaves and green color on *C. sativa* leaves, and **(C)** comparison between total number of glandular and non-glandular trichomes present. Here, asterisk mark and different letters denote significant level of differences at $P < 0.05$, and error bars depict standard error, where orange color indicates density of trichomes on *L. siceraria* leaves and green color on *C. sativa* leaves



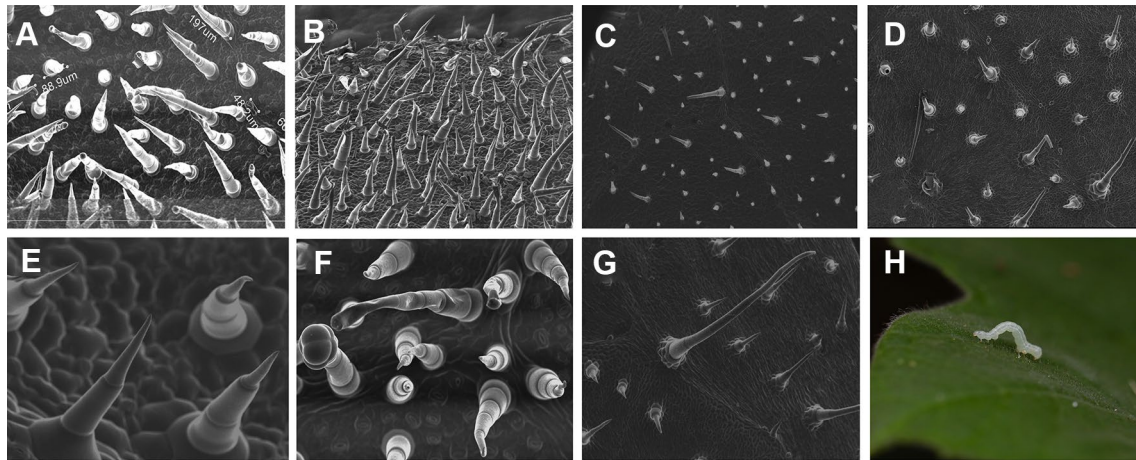


Fig. 3 Scanning electron microscopy (SEM) images of trichomes present on *L. siceraria* leaves taken at 100× magnification (**A**, **B**), *C. sativa* leaves at 70× magnification (**C**, **D**), morphotypes of glandular and non-glandular trichomes present on *L. siceraria* leaves (**E**, **F**), Type III uniseriate non-glandular trichome (**E1**), subulate deflexed non-glandular trichome (**E2**), hooked non-glandular trichome, **F3**,

Type IV colleter (trichomes with multicellular globular secretory head with multicellular stalk) glandular trichome (**F4**), SEM image of awl-shaped uniseriate non-glandular trichome found on *C. sativa* leaves (**G**), and second instar *T. ni* struggling to walk on trichome-rich leaf surface of *L. siceraria* (**H**)

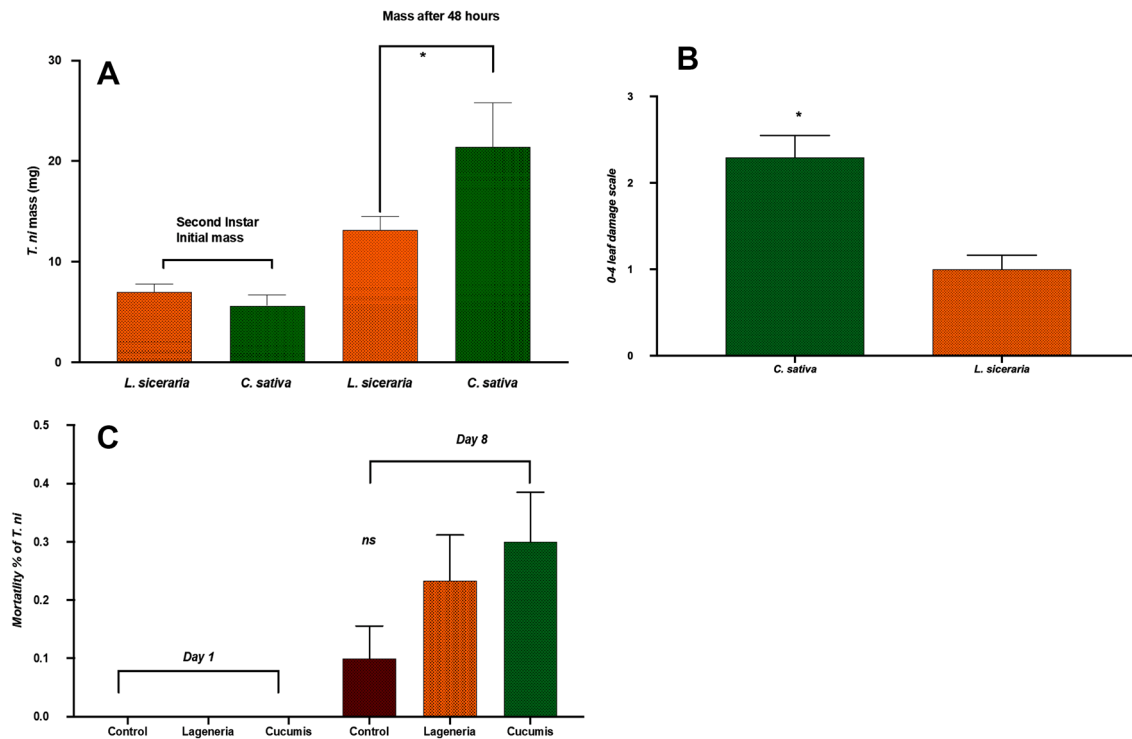


Fig. 4 Forced feeding experiments of *T. ni* on *L. siceraria* and *C. sativa* (**A**) Results of mass gain of second instar *T. ni* when forced fed on *C. sativa* and *L. siceraria* plants on the 1st day and mass after 48 h with asterisk mark representing the significant difference between the groups, **4B** shows damage assessment by *T. ni* when forced fed on both the plants (**4C**) shows the percentage of *T. ni* that died observed

after 8 days. The asterisk mark represents significant difference at $P < 0.05$, while ns represents non-significant results, and error bars depict standard error. In this figure, orange color bar graph denotes to attributes related to *L. siceraria* leaves and green color to *C. sativa* leaves and brown color for control parameters

all the instars ($P < 0.001$). However, *post hoc* Tukey pairwise comparisons showed no differences for instar (F ratio = 2.15, $P = 0.09$, supplementary fig. 1A and C), diet (F ratio = 0.59, $P = 0.55$), and diet X instar (F ratio = 1.073 $P = 0.37$, supplementary fig. 1B and C). We also analyzed mass (supplementary fig. 1A) mortality ratio of *T. ni* larvae when fed on three different diets; however, we did not find any significant difference in this well (supplementary fig. 1D). We followed up this experiment to analyze pupal parameters such as volume, mass, and sex to observe if trichome-based diet might have effect on these parameters (Fig. 5). Although we did not observe any significant effects during larval stage, we found significantly higher pupal volume (Fig. 5A) for *T. ni* fed on control diet, and they also pupated early, although not significant different from other treatments. However, we did not observe any significant difference in pupal mass. We also did not observe any significant difference in sex of pupae fed on different treatment diets as well ($P = 0.47$). More interestingly, we found out that almost half of the moths emerged that fed on *L. siceraria* trichome-based diet were deformed, whereas from the other two treatments, no deformation was observed (Fig. 5C). We also found difference in the lifespan of *T. ni* fed on three different types of diet with control fed having longer lifespan ($P = 0.07$, Fig. 5D), with marginally

non-significant difference as confirmed by *post hoc* Dunnett's test. For more details of individual statistical tests, please refer the supplementary table 2.

SEM images of frass pellets show undigested trichomes

SEM of frass pellets of *T. ni* larvae fed on three different diets (supplementary fig. 3) showed that the trichomes are ingested by the *T. ni* larvae but mostly undigested. The undigested trichomes were found embedded in frass pellets in case of *T. ni* larvae (supplementary fig. 2B) when fed on *C. sativa* and *L. siceraria*, but obviously absent on the ones fed on control diet.

Polyphenol Oxidase (PPO) activity in *Lagenaria siceraria* and *Cucumis sativa* leaves

Unpaired t-test analysis of PPO activity showed that there was no significant difference in polyphenol oxidase (PPO) between the two treatment groups ($df = 13$, $P = 0.52$,

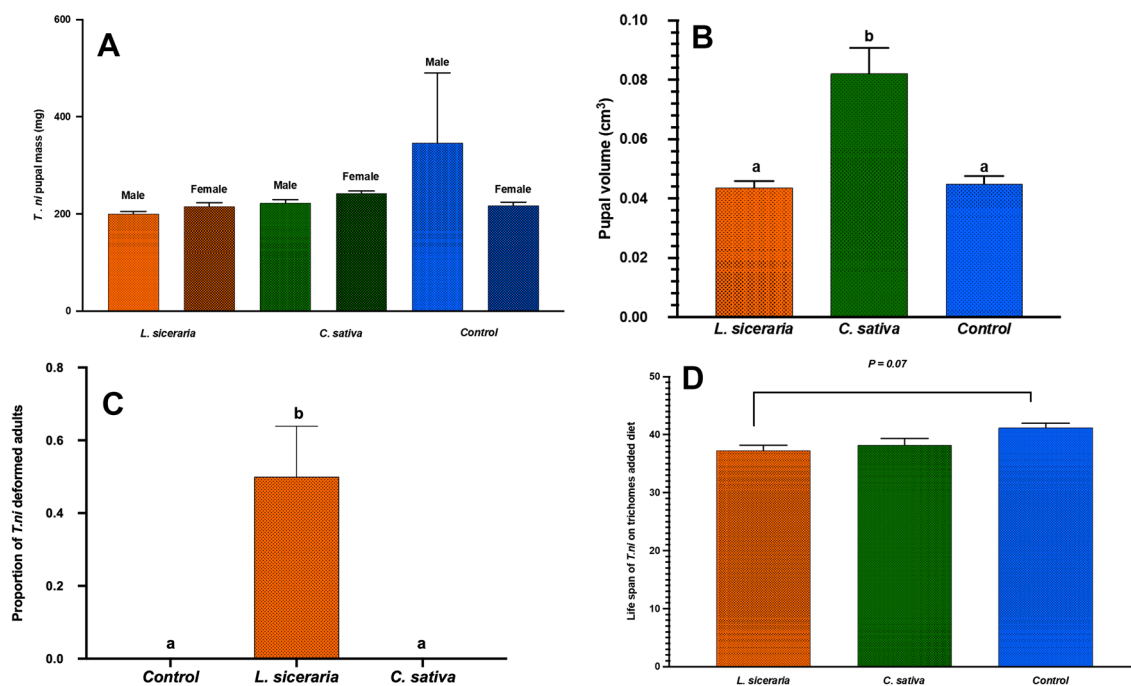


Fig. 5 Graphical representation of the effects of different types of diet (control, diet incorporated with *L. siceraria* trichomes, and diet incorporated with *C. sativa* trichomes) on pupal and adult stages of *T. ni* (A) Mass of male and female pupae when fed on three different diets, (B) volume in cm^3 of pupae of caterpillar when fed on three different diets, and (C) proportion of deformed moths emerged when fed on three different diets. D Lifespan in days of *T. ni* when fed on

three different diets. The asterisk mark denotes the significant difference between the treatment groups when measured at ($p = 0.05$) level of significance, and error bars depict standard error. In this figure, orange color bar graph denotes to attributes related to *L. siceraria* leaves and green color to *C. sativa* leaves and blue color for control parameters

two-tailed) (supplementary fig. 2A), suggesting similar levels of chemical defenses in both species, but will need deeper examination to confirm using other compounds such as cucurbitacins, which are the primary defense metabolites in the family.

Discussion

Through a series of field, laboratory, and greenhouse experiments, we examined the role of trichomes in mediating plant–herbivore interactions in two agriculturally important species in Cucurbitaceae. In our studies, we observed the incidence and feeding ability of four common insect herbivores, and our data show that *T. ni* and *D. indica*, which are chewing herbivores (caterpillars), preferred to feed on *C. sativa* which had lower density of both glandular and non-glandular trichomes, when compared to *L. siceraria*. However, contrasting results were found in case of *A. tristis*, a sucking herbivore which preferred *L. siceraria* to lay more egg clutches and more adults was also found on them. While sucking herbivores such as aphids have been documented to be impacted by trichomes (Wang et al. 2021; Blanco-Sánchez et al. 2021), it is expected that sucking herbivores use their stylet to pierce epidermis, and can potentially insert stylet between trichomes, unlike caterpillars who must constantly maneuver through the trichome mat to commence feeding (Kariyat et al. 2017). *A. vittatum*, a highly damaging herbivore on most of the cucurbit crops (Brzozowski et al. 2019), did not show any preference among the species, as it is a specialist herbivore and have possibly managed to evolve counter defenses against them (Weber 2018; Sparks et al. 2020; Haber et al. 2021), an area we are currently exploring. Interestingly, our observations from the common garden experiments collectively showed hesitation and repulsion behavior for the two caterpillar species on *L. siceraria* (supplementary videos 1 and 2), and even *A. vittatum* was also found to vigorously clean their head and mouthparts after brushing through the trichomes (supplementary video 3). Clearly, these observations suggested that chewing herbivores disliked *L. siceraria*, and the species may even possess additional repulsion traits possibly through repulsive volatiles or bitter cucurbitacins. These results clearly align with the previous studies which showed that trichomes limit herbivore feeding (Hanley et al. 2007; Kaplan et al. 2009; Karley et al. 2016; Kariyat et al. 2017, 2018, 2019).

Since *T. ni* caterpillars are highly mobile, we conducted a forced feeding experiment to see if they would feed less on *L. siceraria*, when compared to *C. sativa*, and we found forced fed *T. ni* caterpillars gained more mass on *C. sativa* (Fig. 4A) and consequently inflicted significantly more damage on *C. sativa* leaves (Fig. 4B), indirectly suggesting the presence of better/more structural defenses (and chemical

defenses) in *L. siceraria* possibly delayed or restricted the feeding—a typical feature of trichome defense (Kariyat et al. 2018; Watts and Kariyat 2021). Interestingly, Coapio et al. (2018) have recently shown that the length and density of non-glandular trichome (which *L. siceraria* have in high density) is one of the deciding factors for oviposition decision of *T. ni* females. It is well-known fact, oviposition is crucial step which decides the larval success (Krug and Sosa 2019), and we speculate that the reduced oviposition is the primary reason for lower incidence of *T. ni* found on *L. siceraria*. To confirm that the trichomes play an inevitable role in affecting *T. ni* feeding (Mymko and Avila-Sakar 2019; Meneses-Arias et al. 2020), we dissociated trichomes from rest of the defenses by adding trichomes of both the plant species in the diet to observe the effects on different life stages (Kariyat et al. 2019). However, unlike the previous studies on Solanaceae—*M. sexta* study systems (Kariyat et al. 2019; Watts and Kariyat 2021), no significant effects were observed in the mass and mass gain of *T. ni* fed on these three different diets (Fig. 5), but the effects were more pronounced in the adults, where half of the moths emerged were deformed which fed on *L. siceraria* trichome diet. We speculate that there are four possible reasons for these results; (1) due to high dilution of diet, only a smaller number of trichomes are present in diet to produce any significant effect in their larval stages, (2) the non-glandular trichomes that cause physical damage to caterpillar gut are mostly stellate (Kariyat et al. 2017), whereas these trichomes are non-stellate and were not effective enough (no branching to cause peritrophic matrix tear), and (3) trichome-mediated defenses in *L. siceraria* are independent of the chemical toxins in glandular trichomes to cause larval growth and development inhibition unlike in *Solanum lycopersicon* (Kariyat et al. 2019). And (4) trichome-mediated diet effects magnify over life stages and while they were not detectable in larval stages, these effects accrued and caused mass differences and deformities in adults (Fig. 5).

A significant body of research on plant trichomes has collectively reported that the exudates of glandular trichomes can increase the larval development period, and decrease pupal weight as observed in *Phthorimaea operculella* (Lepidoptera: Gelechiidae) feeding on glandular foliage trichomes of *Solanum berthaultii* (Solanaceae) (Malakar and Tingey 2000). Consistent with these observations, we also found that *T. ni* fed on control diet had pupated faster (Fig. 5A) and had more pupal mass (supplementary fig. 1D) as compared to *T. ni* fed on trichome enriched diets. It has been reported that the delay in pupation has significant effect of *M. sexta* fitness (Kariyat et al. 2019; Tayal et al. 2020), as evident in the marginal non-significant ($P=0.07$) effect on life span where *T. ni* lived longer on control diet (Fig. 5D).

The partially significant but clear trends of trichome-based defenses playing a role in *T. ni* were in agreement

with SEM images that revealed *L. siceraria* has significantly more glandular and non-glandular trichomes, thereby having a comprehensive advantage over *C. sativa* in both pre- and post-ingestive trichome-based defenses (Kariyat et al. 2017; Andama et al. 2020; Kaur and Kariyat 2020b). Having a higher density of glandular trichomes can possibly lead to direct toxicity (Hare 2005; Lucini et al. 2015; Wang et al. 2020) or activation of jasmonic acid-mediated defense signaling cascade (Peiffer et al. 2009) leading to long-term local and systemic defenses (Wang et al. 2009; Biswas et al. 2009). And non-glandular trichomes could have delayed the feeding, and when ingested causing post-ingestive effects which we confirmed with SEM of frass (Kariyat et al. 2017). And finally, to rule out (or account for) the possibility that chemical defenses might be responsible for clear difference in herbivory presence in these two species, we also ran polyphenol oxidase (PPO) test, where we found similar levels of PPO activity, a key enzyme in defense against insect herbivores (Acevedo et al. 2015) among the two species (supplementary fig. 2B). While this is not conclusive evidence for the lack of chemical defenses (since we did not estimate cucurbitacins or other secondary metabolites content), this suggests that trichome-mediated defenses, independent of chemical defenses, can exert significant selection against herbivores.

The variation in herbivore behavior between the two species on leaf surface also points to another important trichome trait—the volatiles from glandular trichomes. Herbivore grooming and repulsion behavior (supplementary videos 1 and 2) suggest the possibility of differences in volatile blend, an area we are currently exploring. The future experiments should be conducted to identify different types of volatiles along with their quantity being emitted by these two species and then identifying the specific blend of volatile which is responsible for repulsion of herbivores in case of *L. siceraria*.

In conclusion, we show that trichomes act as both physical (non-glandular trichomes) and chemical barrier (glandular trichomes) in protecting the plant against insect herbivores in a density-dependent manner. These defenses can restrict, delay, and negatively affect herbivore feeding and development, with even cascading effects of adult traits. However, we also show that these defenses are herbivore species and possibly feeding guild dependent and warrant further enquiry. Most trichome studies have used lines that either overexpress trichomes or mutants that have no trichomes, especially in model plant species. Here, we used two related species that have natural, but consistent differences in trichome density and type to show how these differences have effects ranging from herbivore incidence to adult traits, with possible consequences for fitness and dispersal. Future studies should focus on observing different morphotypes of different types of glandular and non-glandular

trichomes with emphasis on their functional diversity and examine how induction of these trichomes correlates with the intensity of herbivore presence on these plant species with possible implications for sustainable pest management. And more detailed examination of chemical constituents of glandular trichomes and immune response due to non-glandular trichomes, coupled with innate plant defense chemistry will unlock more detailed mechanistic understanding of trichome-mediated defenses, thought to be evolved primarily against abiotic stress (Kaur and Kariyat 2020a, 2020b).

Author Contributions

IK and RK designed the research and cowrote the manuscript, IK conducted the experiments, and IK and RK analyzed the data. Both authors read and approved the manuscript.

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Data availability All data will be made available on a data sharing repository.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no conflicts of interest.

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