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Leaf morpho-anatomical traits in the modulation of insect-plant interactions: synthesis, validation, and vibroacoustic mechanisms in the tomato-tomato pinworm system

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Doctor Scientiae

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DIEGO DOS SANTOS SOUZA

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Thesis submitted to the Entomology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Raul N. Carvalho Guedes

Co-advisers: Agustin Zsogon
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"Nada na vida deve ser temido, somente compreendido. Agora é hora de compreender mais, para que possamos temer menos." — Marie Curie

ABSTRACT

SOUZA, Diego dos Santos, D.Sc., Universidade Federal de Viçosa, February, 2026. **Leaf morpho-anatomical traits in the modulation of insect-plant interactions: synthesis, validation, and vibroacoustic mechanisms in the tomato-tomato pinworm system**. Adviser: Raul Narciso Carvalho Guedes. Co-advisers: Agustin Zsogon and Leonardo Morais Turchen.

Plant-herbivore interactions are strongly modulated by leaf morpho-anatomical characteristics, influencing everything from host choice to insect biology, development, and feeding behavior. Although plant resistance is traditionally associated with chemical defenses, there is growing recognition that structural leaf traits are important physical modulators of these interactions. However, the literature presents fragmented results, especially regarding the functional role of internal anatomical traits. This thesis aimed to integrate synthetic and experimental evidence to elucidate how leaf morpho-anatomical traits, with an emphasis on internal characteristics, affect insect-plant interactions, particularly involving leafminer insects. In the first chapter, a systematic review with meta-analyses was conducted, following the PRISMA guidelines, encompassing nine decades of publications. After screening, 123 studies were included in the qualitative synthesis and 43 in the meta-analytical synthesis. Most studies focused on insects of the orders Lepidoptera and Hemiptera and on plants of the families Vitaceae, Solanaceae, and Fabaceae. The meta-analyses demonstrated that internal anatomical traits, such as epidermal and palisade parenchyma thickness, consistently reduce herbivore preference, consumption, and performance. However, the literature remains heavily focused on external traits, especially trichomes and cuticle characteristics, while internal traits remain underexplored. Furthermore, many studies evaluate traits in isolation and within a restricted set of biological systems, limiting generalizations about the structural mechanisms of herbivory resistance. In the second chapter, the role of vascular sheath extensions (BSEs), a poorly studied internal anatomical feature, in the interactions between tomato (*Solanum lycopersicum*) and the tomato leafminer *Phthorimaea* (= *Tuta*) *absoluta* was experimentally investigated. Using isogenic lines with heterobaric (with BSEs) and homobaric (without BSEs) leaves, the effect of this structure on insect consumption, development, and performance was evaluated. The absence of BSEs reduced larval consumption and prolonged development, in addition to resulting in lower pupal mass and delayed adult emergence. Differences in the phytohormone profile, especially jasmonates, indicated that BSEs can modulate physiological

plant defense pathways. These results demonstrate that BSEs act as morphophysiological modulators of insect-plant interactions, contributing to herbivory resistance. In the third chapter, we evaluated whether BSEs impose immediate biomechanical costs on leafminer feeding behavior. The feeding behavior of P. absoluta larvae on heterobaric and homobaric leaves was analyzed using high-resolution videos and laser Doppler vibrometry, using vibratory emission as a proxy for feeding events. The analyses revealed that mesophyll architecture did not affect chewing motor patterns, indicating that motor control of feeding is highly robust, especially in specialist leafminer insects, and does not undergo immediate changes in response to structural heterogeneity in leaf tissue. Taken together, these findings demonstrate that internal leaf morphoanatomical traits play a relevant role in modulating insect-plant interactions, particularly by affecting consumption and performance throughout development, even if they do not impose immediate biomechanical costs on the feeding behavior of specialized herbivores. The results reinforce the importance of integrating internal anatomical traits and physiological responses in the study of plant resistance in different ecological contexts. This approach contributes to a more comprehensive understanding of the underlying mechanisms that structure insect-plant interactions and can provide support for the development of more sustainable pest management strategies.

Keywords: plant-insect interaction; *Phthorimaea absoluta*; *Solanum lycopersicum*; functional leaf traits; herbivory; meta-analysis; biotremology; feeding behavior

RESUMO

SOUZA, Diego dos Santos, D.Sc., Universidade Federal de Viçosa, fevereiro de 2026. **Características morfoanatômicas foliares na modulação das interações inseto-planta: síntese, validação e mecanismos vibroacústicos no sistema tomate-traça do tomateiro**. Orientador: Raul Narciso Carvalho Guedes. Coorientadores: Agustin Zsogon e Leonardo Morais Turchen.

As interações entre plantas e herbívoros são moldadas por características foliares, que influenciam desde a escolha do hospedeiro até a biologia, o desenvolvimento e o comportamento alimentar dos insetos. Embora a resistência vegetal seja ligada a defesas químicas, há um crescente reconhecimento de que traços estruturais das folhas atuam como importantes moduladores físicos dessas interações. No entanto, a literatura apresenta resultados fragmentados, especialmente sobre o papel funcional de traços anatômicos internos. Essa tese teve como objetivo integrar evidências sintéticas e experimentais para elucidar como traços morfoanatômicos foliares, com ênfase em características internas, afetam interações inseto-planta, particularmente envolvendo insetos minadores. No primeiro capítulo, foi feita uma revisão sistemática com meta-análises, seguindo as diretrizes PRISMA, abrangendo nove décadas de publicações. Após a triagem, 123 estudos foram incluídos na síntese qualitativa e 43 na síntese meta-analítica. A maioria dos trabalhos concentrou-se em insetos das ordens Lepidoptera e Hemiptera e em plantas das famílias Vitaceae, Solanaceae e Fabaceae. As meta-análises mostraram que traços anatômicos internos, como a espessura da epiderme e do parênquima paliçádico, reduzem consistentemente a preferência, o consumo e o desempenho dos herbívoros. Contudo, a literatura permanece fortemente focada em traços externos, especialmente tricomas e características da cutícula, enquanto traços internos continuam subexplorados. Além disso, muitos estudos avaliam traços isoladamente e em um conjunto restrito de sistemas biológicos, limitando generalizações sobre os mecanismos estruturais de resistência à herbivoria. No segundo capítulo, investigamos experimentalmente o papel das extensões da bainha vascular (BSEs), uma característica anatômica interna pouco estudada, nas interações entre o tomateiro (*Solanum lycopersicum*) e a traça-do-tomateiro *Phthorimaea* (= *Tuta*) *absoluta*. Utilizando linhagens isogênicas com folhas heterobáricas (com BSEs) e homobáricas (sem BSEs), avaliamos o efeito dessa estrutura sobre o consumo, o desenvolvimento e o desempenho do inseto. A ausência de BSEs reduziu o consumo larval e prolongou o desenvolvimento, além de resultar em menor massa pupal e atraso na emergência dos adultos.

Diferenças no perfil de fitohormônios, especialmente jasmonatos, indicaram que BSEs podem modular vias fisiológicas de defesa vegetal. Esses resultados demonstram que as BSEs atuam como moduladores morfofisiológicos das interações inseto-planta, contribuindo para a resistência à herbivoria. No terceiro capítulo, avaliamos se BSEs impõem custos biomecânicos imediatos ao comportamento alimentar de insetos minadores. O comportamento alimentar de larvas de P. absoluta em folhas heterobáricas e homobáricas foi analisado por meio de vídeos em alta resolução e vibrometria a laser Doppler utilizando a emissão vibratória como proxy para eventos de alimentação. As análises revelaram que a arquitetura do mesófilo não afetou os padrões motores de mastigação, indicando que o controle motor da alimentação é altamente robusto em *P. absoluta*, e não sofre alterações imediatas diante da heterogeneidade estrutural do tecido foliar. Em conjunto, esses achados demonstram que traços morfoanatômicos foliares internos desempenham papel relevante na modulação das interações inseto-planta, sobretudo ao afetarem o consumo e o desempenho, ainda que não imponham custos biomecânicos imediatos ao comportamento alimentar de herbívoros especializados.

Palavras-chave: interação planta-inseto; *Phthorimaea absoluta*; *Solanum lycopersicum*; características funcionais das folhas; herbivoria; meta-análise; biotremologia; comportamento alimentar

SUMMARY

GENERAL INTRODUCTION

Interactions between plants and herbivorous arthropods represent a fundamental evolutionary force that shapes the biodiversity and structure of terrestrial ecosystems (Jander 2014; Shinohara and Yoshida 2021; Hu et al. 2024). This co-evolutionary dynamic is driven by biochemical, physical, and behavioral responses, in which plants develop defenses and arthropods counter-adapt to overcome them (Wu and Baldwin 2010; Mostafa et al. 2022). Understanding the complex nature of these plant defenses is not only a central ecological issue, but also the basis for developing innovative and sustainable management strategies.

Plants employ a wide range of defense mechanisms, classically divided into constitutive and induced (Zhang et al. 2020). For decades, scientific research has focused almost exclusively on chemical defenses and physical surface barriers (Hanley et al. 2007; Agrawal 2011). Historically, these traits have been extensively characterized as acting as a first line of resistance, directly deterring or hindering the colonization and feeding of herbivorous insects (Lewandowska et al. 2020; Wang et al. 2020).

However, this historical emphasis on the exterior of the leaf has created a profound conceptual gap. The role of structural features, especially the internal anatomy of the leaf and the organization of plant tissues, has remained a substantially unexplored territory in the context of pest resistance (Grubb 1992; Schoonhoven et al. 2005; Hanley et al. 2007). The novelty of investigating these hidden layers becomes even more imperative when dealing with leaf-mining insects, a highly specialized group whose larvae feed exclusively within the leaf parenchyma (Sinclair and Hughes 2010). For these organisms, the leaf is simultaneously food and habitat; therefore, and this has been little explored until now, it is necessary to recognize that the internal architecture completely defines the mechanical and nutritional environment of foraging (Clissold 2007; Dai et al. 2011).

Despite the morphoecological importance of this internal interface, the literature has failed to capture the mechanics of this interaction, as studies often assess the impact of internal defenses only through integrated ecological

measures, such as the final performance of the insects (Garvey et al. 2022; Bosorogan et al. 2023), completely neglecting the immediate behavioral processes that generate these results. To break through this methodological barrier, this work introduces a pioneering and revolutionary approach to this system of study: biotremology. This science studies the vibrations transmitted by substrates, which are a critical sensory channel for insects. (Virant-Doberlet and Andrej 2004; Cocroft and Rodríguez 2005; Strauß et al. 2021). Feeding, in particular, generates characteristic vibratory signatures that encode precise information about the insect's mandibular kinematics and the mechanical properties of the food (Appel and Cocroft 2014; Body et al. 2019).

In a completely innovative way, the analysis of vibrational signals offers an unprecedented window to decipher the biomechanics of insect-plant interaction in real time and in a non-invasive manner (Hill and Wessel 2016). The underlying and original hypothesis of this research is that the physical properties of internal plant tissue, determined by its anatomy, can modulate the nature of interactions and consequently vibrations, imposing direct biomechanical costs on the insect during the act of feeding itself. Until now, this mechanistic hypothesis remained largely untested. By using biotremology, this thesis overcomes the critical disconnect between the ecological scale (where traits are only correlated with performance) and the mechanistic scale, allowing, for the first time, direct observation of the physical interaction and access to the proximal mechanism of resistance.

We start from the premise that understanding plant resilience to severe pests, such as the tomato leafminer (*Phthorimaea absoluta*), requires the adoption of unique botanical models and cutting-edge tools to bridge these scales. Thus, this thesis proposes a structured and unprecedented investigation, whose general objective is to establish the role of leaf anatomy, with emphasis on the internal architecture of the leaf, as an active modulator of insect-plant interactions. To this end, we innovatively integrate three approaches: (i) the synthesis of literature data to redefine which leaf morpho-anatomical traits actually modulate this interaction between plants and arthropods; (ii) experimental ecology using unique botanical models to validate the ecological impact of historically neglected internal characteristics; and (iii) the pioneering use of biotremological analysis to elucidate, at high resolution, whether this

architecture acts as a mechanical barrier that manifests itself in immediate adjustments in the insect's kinematics. In this way, we have uncovered, for the first time, the true costs and mechanisms that shape evolution in the proposed biological system.

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CHAPTER 1

Leaf morpho-anatomical traits as bottom-up modulators of plant-arthropod interactions: a meta-analytic synthesis

[Article submitted to the *Pest Management Science*]

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ABSTRACT

BACKGROUND: Morpho-anatomical leaf traits play a central role in the bottom-up modulation of plant-arthropod interactions, influencing herbivore preference, feeding, and overall performance. Yet, inconsistent findings across studies prevent broader generalizations regarding the contribution of these traits to plant resistance. Here, following the PRISMA guidelines, we conducted a systematic and meta-analytic synthesis of nine decades of publications retrieved from the Web of Science and Scopus databases.

RESULTS: Successive screening steps yielded 123 articles for qualitative evaluation and 43 articles for quantitative synthesis. Taxonomic, experimental, and performance-related variables were extracted, and effect sizes (Hedges' g) were estimated using random-effects models. Meta-analyses indicated that internal anatomical traits, particularly epidermal and palisade tissue thickness, significantly reduce herbivore preference and feeding. Meta-regressions further showed that increased expression of these traits prolongs development and reduces survival. For surface structures such as trichomes, consistent patterns emerge in meta-regressions, revealing reductions in feeding and reproduction. Increased cuticular roughness reduced host plant preference, whereas leaf shape and size exhibited no detectable effects on arthropod performance.

CONCLUSION: Collectively, these findings underscore that leaf structural traits act as efficient physical modulators in trophic interactions. However, most available evidence remains concentrated on a narrow set of external features, notably trichomes and cuticle characteristics. Consequently, internal leaf traits remain comparatively underexplored, limiting our understanding of their potential role as defense mechanisms mediating plant-herbivore dynamics. Expanding the functional scope of future studies is therefore essential for advancing ecological insights and applied perspectives on plant resistance.

Keywords: Functional leaf traits; arthropod herbivory; plant-herbivore interactions; leaf anatomy; bottom-up modulation; meta-analysis

1. INTRODUCTION

Plants form the basis of virtually all terrestrial food chains, providing energy and structural resources for a wide diversity of herbivorous arthropods.[1] However,

plants are far from passive in the face of herbivory, deploying a wide array of defenses that modulate both the intensity and the ecological consequences of such interactions.[2,3] Among these defenses, the morpho-anatomical characteristics of leaves are particularly important, as they constitute an initial barrier to herbivorous arthropods functioning as key mediators of trophic interactions.[1, 4–7]

Leaf structural traits — including epidermis thickness, trichome density,[8] cuticle composition, and the proportion of palisade and spongy tissues[4–6] — exert direct influences on arthropod feeding behavior and physiological performance. Leaves with greater mechanical rigidity, for instance, can reduce chewing efficiency,[9,10] depress feeding rates, and lower net energy gain.[11] Likewise, trichomes and cuticular waxes may hinder access to internal tissues,[4,12] interfering directly and indirectly with oviposition, locomotion, and attachment. In this sense, leaf morpho-anatomy acts as a physical and functional filter that conditions herbivore success and ultimately shapes the flow of energy within ecological communities.[13]

These structural mechanisms are embedded within the broader framework of bottom-up effects, a foundational concept in trophic ecology describing how the characteristics and quality of basal resources influence higher trophic levels.[14] By modulating the availability, usability, and profitability of plant resources, leaf traits determine the abundance, diversity, and performance of herbivores, and indirectly of their natural enemies.[11,15,16] This upward regulation stands in contrast to the top-down effects exerted by predators and parasitoids, reflecting the bidirectional and dynamic nature of trophic interactions.[17,18] Understanding the morpho-anatomical determinants of plant resistance is therefore essential for integrating functional defense perspectives and elucidating the eco-evolutionary processes that shape trophic networks.[19]

Despite the conceptual importance of this topic, the literature on leaf traits and herbivory remains fragmented and frequently contradictory. Different studies report divergent outcomes of the same trait, often depending on the biological system examined. For instance, while high trichome density or particular trichome types may reduce herbivore feeding in some systems,[20–23] other studies report no effect or even positive associations,[24] underscoring strong dependence on plant species, arthropod guild, and trait variability. Epidermal thickness[25] and

cuticle properties may act as a physical barrier in some contexts,[26,27] although they elicit stimulatory responses in others.[28] Such heterogeneity has constrained the identification of generalizable patterns and hindered the development of predictive frameworks with practical relevance for pest management.

Several sources of variability contribute to these inconsistencies. The immense taxonomic diversity of both plants and arthropods entails a wide range of structural and behavioral adaptations.[29,30] Chewing, sucking, scraping, and mining herbivores interact with leaf tissues in distinct ways, exploiting different microhabitats and responding to specific selective pressures.[31,32] Environmental conditions, including nutrient availability, light, and water stress, further modulate the expression of morpho-anatomical traits, conferring considerable phenotypic plasticity.[33,34] These biotic and abiotic factors reinforce the context-dependence of bottom-up effects, emphasizing that plant-herbivore interactions are inherently variable across ecological settings.[35]

Another relevant source of inconsistency stems from methodological limitations. Most studies remain qualitative or descriptive,[36,37] which limits direct comparison across systems. Response metrics vary widely (e.g., consumption, growth, survival, fecundity), as do the units and scales used to quantify leaf traits. Moreover, research often targets a narrow subset of species or populations, restricting the generalization of results across the vast diversity of taxa for which we still lack even a rudimentary understanding of trait–herbivore interactions.[18] The absence of methodological standardization and the limited integration across taxonomic and ecological levels ultimately prevent the formulation of a broad and cohesive theoretical framework for understanding the physical drivers of plant-arthropod interactions.

Given the substantial variability and methodological fragmentation across existing literature, a comprehensive synthesis that consolidates and critically evaluates the available evidence on how morpho-anatomical leaf traits modulate plant–arthropod interactions is both timely and necessary. Quantitative synthesis tools — such as meta-analyses and meta-regressions — are especially powerful in this context, as they enable the identification of general trends, estimation of effect sizes, and the evaluation of the consistency and directionality of patterns across diverse experimental settings.[38] By disentangling random variation from systematic effects, these approaches strengthen the empirical basis for theories

of resistance and adaptation. Moreover, systematic reviews and meta-analyses help to reduce publication bias, quantify heterogeneity, identify key moderators, and highlight critical knowledge gaps and emerging research priorities.[38,39]
Here, we present a systematic literature review and meta-analysis spanning nine decades of research on the effects of leaf morpho-anatomical traits on herbivorous arthropods. Our synthesis integrates evidence across diverse plant and arthropod taxa, multiple types of bioassays, and a broad range of ecological contexts. Through meta-regressions and random-effects models, we identify which leaf traits most strongly influence arthropod preference, feeding, survival, development, and reproduction. By quantifying these relationships, we aim to reveal consistent patterns underlying the bottom-up modulation of plant-arthropod interactions. This integrative perspective highlights the role of leaf morpho-anatomical traits as fundamental ecological filters with direct implications for plant resistance and pest management.

2. MATERIALS AND METHODS

The methodological design for the systematic survey and meta-analysis followed PRISMA recommendations (Preferred Reporting Items for Systematic Reviews and Meta-Analyses),[40] with specific procedures detailed below.

2.1. Data collection

The data were obtained through a systematic search in two databases: Web of Science and Scopus. The search strategy employed general keywords ("morphology" and "leaf") combined with ("arthropods", "mite*", or "insect*"). This allowed the recovery of a comprehensive dataset encompassing scientific outputs published from January 1935 to May 7, 2025. he metadata (author, year, title, journal, abstract, etc.) from both databases were imported into the R environment, initially in BibTeX (.bib) format, and unified into a single dataframe. The “revtools” [41] package was used to process and remove duplicate records. Duplicate records were removed based on the title and Digital Object Identifier (DOI). After consolidation, the final database, containing the unique references, was exported in .xlsx format for subsequent analysis. (Fig.1).

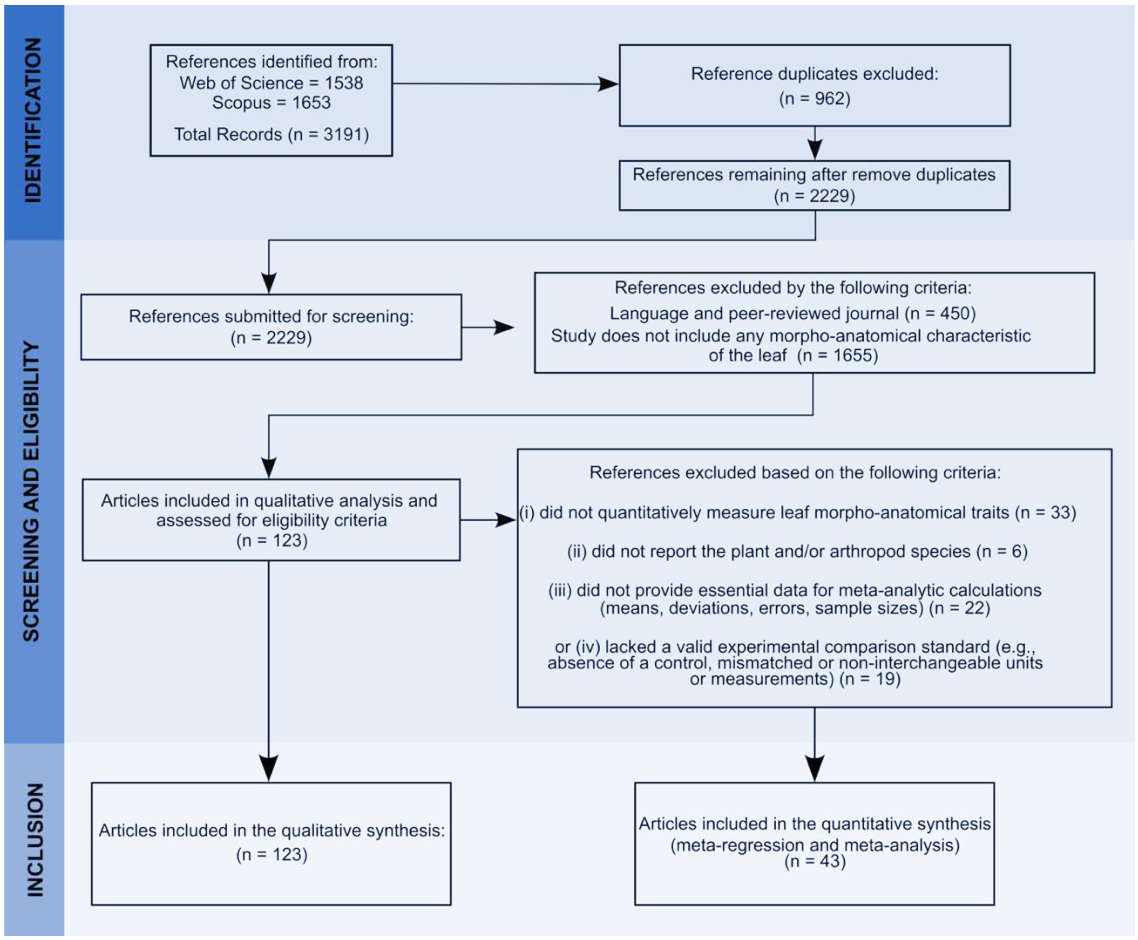


Figure 1. Flowchart of the selection and exclusion steps applied to scientific papers obtained from the systematic literature search in the Web of Science and Scopus databases covering the period from January 1935 to May 2025.

2.2. Screening and eligibility

The screening proceeded in two stages (Fig. 1). In the first stage, we excluded articles not written in English, those not published in peer-reviewed journals, and other publication types (e.g., narrative reviews, case reports, patents, protocols, editorials, books and book chapters, theses, dissertations, and conference/meeting proceedings). Publications that did not address the morpho-anatomical characteristics of leaves were also removed. The remaining studies were included in the qualitative synthesis.

The second screening stage involved a more detailed assessment, excluding studies that does not meet the following criteria: (i) did not quantitatively measure leaf morpho-anatomical traits; (ii) did not report the plant and/or arthropod species; (iii) did not provide essential data for meta-analytic calculations (means, deviations, errors, sample sizes); or (iv) lacked a valid experimental comparison standard (e.g., absence of a control, mismatched or

non-interchangeable units or measurements). This additional screening ensured that only studies suitable for meta-analysis and meta-regression were retained.

2.3. Data extraction

Complementary information was extracted from the selected studies, including geographic coordinates of the first author's address, country where the work was conducted, plant species and family, arthropod species, family and order, morpho-anatomical leaf traits, type of bioassay, arthropod developmental stage, and quantitative descriptors (means, standard deviations, standard errors, and sample sizes for treatments and controls). Because studies reported a wide range of leaf traits and bioassay types, these variables were grouped into broader analytical categories (Fig. 2). Interchangeable measurement units were standardized when necessary. All extracted data are provided in the supplementary table (Table S1).

BOX1 - GROUPING CATEGORIES*

Grouping of leaf morpho-anatomical traits:

1. Internal anatomy and mechanical resistance: Encompasses structural traits related to tissue thickness and stiffness (e.g., epidermis and palisade tissue thickness).
2. Surface structures: Encompasses traits related to the presence, density, type, and size of trichomes on the leaf surface
3. Waxes and cuticle: Encompasses traits related to the deposition of secretions on the outer leaf surface, including physicochemical and microstructural properties such as arithmetic mean roughness.
4. Shape and size: Encompasses geometric leaf traits such as area, linear dimensions, and architecture.
5. Others: Includes functional and physiological variables not covered by the previous groups, such as stomatal density, guard cell traits, and leaf age or coloration.

Grouping of experimental bioassays:

1. Feeding: Groups studies related to direct foliar consumption (weight or area consumed) and indirect consumption (frass and leaf damage).
2. Preference: Includes studies involving the selection and/or retention of the arthropod on the host, provided there is a possibility of choice among hosts.
3. Survival: Includes studies that assess arthropod mortality or longevity.
4. Development: Covers studies that consider at least two stages of the arthropod life cycle.
5. Reproduction: Studies related to reproductive success and species propagation, such as fecundity and oviposition.
6. Other: Studies not classified above but relevant to ecological interactions (e.g., locomotion, predation).

Grouping of arthropod developmental stages:

1. Egg: Initial embryonic stage, distinct from the active phases.
2. Juvenile Stages: All immature stages post-egg and pre-adult, regardless of the type of metamorphosis.
3. Adult: Final stage, reproductive and morphologically stable.
4. Complete Life Cycle: Studies that addressed transitions across multiple stages to assess overall development.

****All categories are not mutually exclusive.***

Figure 2. Description of the categories used to group scientific articles for qualitative and quantitative analyses.

2.4. Qualitative analysis

The qualitative analysis summarized: (i) temporal and spatial trends in publication output; (ii) the major arthropod orders and families investigated, along with plant families; (iii) the main leaf morpho-anatomical traits evaluated; (iv) the predominant types of bioassays employed; and (v) the arthropod developmental stages most commonly assessed.

2.5. Quantitative analysis

Quantitative analyses involved both meta-regression and meta-analysis. Because individual studies often reported multiple assays, only data from at least three independent studies within each morpho-anatomical characteristic and bioassay type were included. Effect sizes (Hedges' g) were calculated based on the Standardized Mean Difference (SMD), a suitable metric for integrating results obtained across different measurement scales and for correcting small-sample bias.[39,42] Meta-regression was used to investigate whether the morpho-anatomical traits influenced arthropod responses (e.g., preference, feeding, survival, development, and reproduction). Both study and arthropod species were treated as random factors in the model.

Meta-analyses were then employed to assess the magnitude and consistency of overall trends across studies, accounting for study-level effects within each bioassay and morpho-anatomical trait. Random-effects models were used due to methodological and sampling heterogeneity. Variance among studies (τ^2) was estimated using the inverse-variance method with a Restricted Maximum Likelihood Estimator (REML). All analyses were conducted in the R version 4.4.1 [43], using the packages "meta",[44] "metafor",[45] "dplyr",[46] and "emmeans".[47] Graphical outputs were generated with "ggplot2",[48] and the 'forest' function, and finalized for publication in Inkscape.[49]

3. RESULTS

3.1. Overview of the bibliographic research

The article selection process is summarized in the flowchart in figure 1. The initial bibliographic search in the Web of Science (n= 1538) and Scopus (n= 1653), covering approximately 90 years of publication, yielded 3191 records. We identified 962 duplicate references across the two databases. After removing

duplicates, a total of 2229 unique articles remained. These were screened by title and abstract to verify their consistency with the eligibility criteria. In the first screening, 450 articles were excluded because they did not meet language or peer-review criteria, and an additional 1655 were removed for not examining any morpho-anatomical leaf traits. The remaining 123 articles were included in the qualitative synthesis, allowing exploration of spatiotemporal publication trends and the taxa most frequently studied. A second, more detailed screening applied four additional exclusion criteria, resulting in the removal of 80 articles (see Figure 1 for details). Ultimately, 43 studies met all criteria and were included in the quantitative synthesis (meta-regressions and meta-analyses).

3.2. Qualitative analysis

Spatiotemporal trends in publications

Although our search encompassed literature from the past 90 years, the first study meeting the adopted criteria was published only in 1974.[50] From that year onward, publication frequency exhibited cumulative growth, with a modest increase through the 1970–2000 period (< 15 articles; Sup.Fig. 1A), followed by a marked acceleration in the subsequent decades following an exponential trend and reaching 124 publications by May 2025.

Geographical patterns (Sup.Fig. 1B,C) reveal a broad global distribution of studies. However, North America and Europe show the most substantial fractional contribution, each representing 29% [n= 36 papers] of the total of publications. Asia accounts for 19% [n= 23 papers] and South America for 17% [n= 21 papers], while Oceania and Africa contribute more modestly (3% [n= 4 papers] and 2% [n= 3 papers], respectively). At the national level, the United States was the leading contributor [n= 31 papers], followed by Brazil [n= 17 papers] and Germany [n= 9 papers]. Other nations, such as India [n= 6 papers], China [n= 6 papers], Japan [n= 5 papers], and England [n= 5 papers], also provided relevant but smaller contributions (Sup.Fig. 1D).

Categories of publications

Grouping studies by thematic categories revealed a strong concentration on leaf surface structural traits (n= 44). Most experiments focused on arthropod preference and feeding, particularly for juvenile and adult stages (Sup.Fig. 2).

Arthropod and plant taxa

The 123 studies included taxa across nine insect orders and three mite orders (grouped here as Acari) (Fig. 3A). Research focused predominantly on Lepidoptera (29.1% of studies [n= 34 papers]) and Hemiptera (25.2% [n= 30 papers]), followed by Acari (17.3% [n= 20 papers]), Coleoptera (12.6% [n= 16 papers]), and Thysanoptera (5.5% [n= 7 papers]). Leaf morpho-anatomical traits were examined in 53 botanical families, with Vitaceae (16.48% [n= 30 papers]), Solanaceae (9.89% [n= 18 papers]), Fabaceae (7.69% [n= 14 papers]), Poaceae (6.59% [n= 12 papers]), Brassicaceae (5.49% [n= 10 papers]), and Cucurbitaceae (3.84% [n= 7 papers]) being the most frequently represented (Fig. 3B).

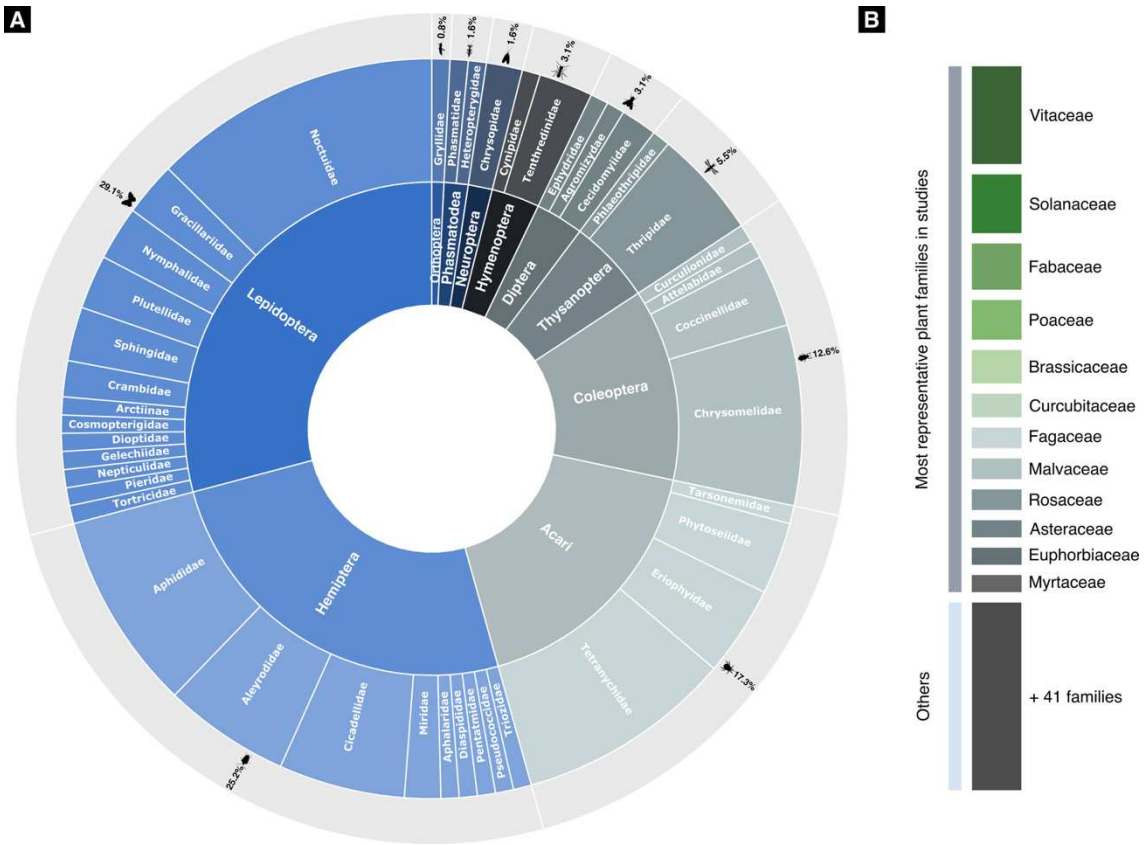


Figure 3. Systematic categories of arthropods and plants that were most frequently studied. A) Arthropod orders and families with the highest number of studies and their respective percentages; and B) Plant families with the highest number of species for which leaf morpho-anatomical traits have been examined. Note: 124 articles were included, but counts exceed this number because individual articles may report multiple plant and/or arthropod species (n = 127 for A; n = 182 for B).

3.4. Quantitative trends: Meta-analysis and meta-regressions

Leaf morpho-anatomical traits — including internal anatomy and mechanical resistance (e.g., epidermis and palisade thickness), surface structures (e.g., trichome abundance), wax and cuticle roughness (e.g., arithmetic mean roughness), and shape and size (e.g., leaf width and length) — are consistently associated with variation in the performance of herbivorous arthropods. These traits impose physical and mechanical barriers predicted to alter host plant preference, feeding capacity, and arthropod development, survival, and reproduction. To investigate these relationships, we applied two complementary quantitative approaches: (i) meta-analyses to evaluate overall effects across studies; and (ii) meta-regressions to test whether the increasing magnitude of these traits explains variation in arthropod responses.

Internal anatomy and mechanical resistance

Internal anatomical features such as epidermis and palisade thickness produced distinct effects on arthropod performance metrics. Meta-analysis indicated significant negative effects on host plant preference (SMD = -2.86; p = 0.0367; Fig. 4A) and feeding (SMD = -1.54; $p < 0.0001$; Fig. 4B). No significant effects were detected for survival (SMD = -4.62; p = 0.1923), development (SMD = 0.80; p = 0.2783), or reproduction (SMD = -0.24; p = 0.2962) (Fig. 4C-E). All meta-analyses exhibited high heterogeneity ($I^2 > 65.3\%$; $p < 0.05$).

Meta-regressions revealed no relationship between increasing leaf anatomical thickness and host plant preference (β = -0.1326; p = 0.5605; Fig. 4A). However, increasing these traits significantly reduced feeding (β = -0.2020; $p < 0.0001$; Fig. 4B), decreased survival (β = -17.7598; p = 0.0130; Fig. 4C), and prolonged development time (β = 14.7686; $p < 0.0001$; Fig. 4D). Reproduction remained unaffected (β = 0.4582; p = 0.4416; Fig. 4E).

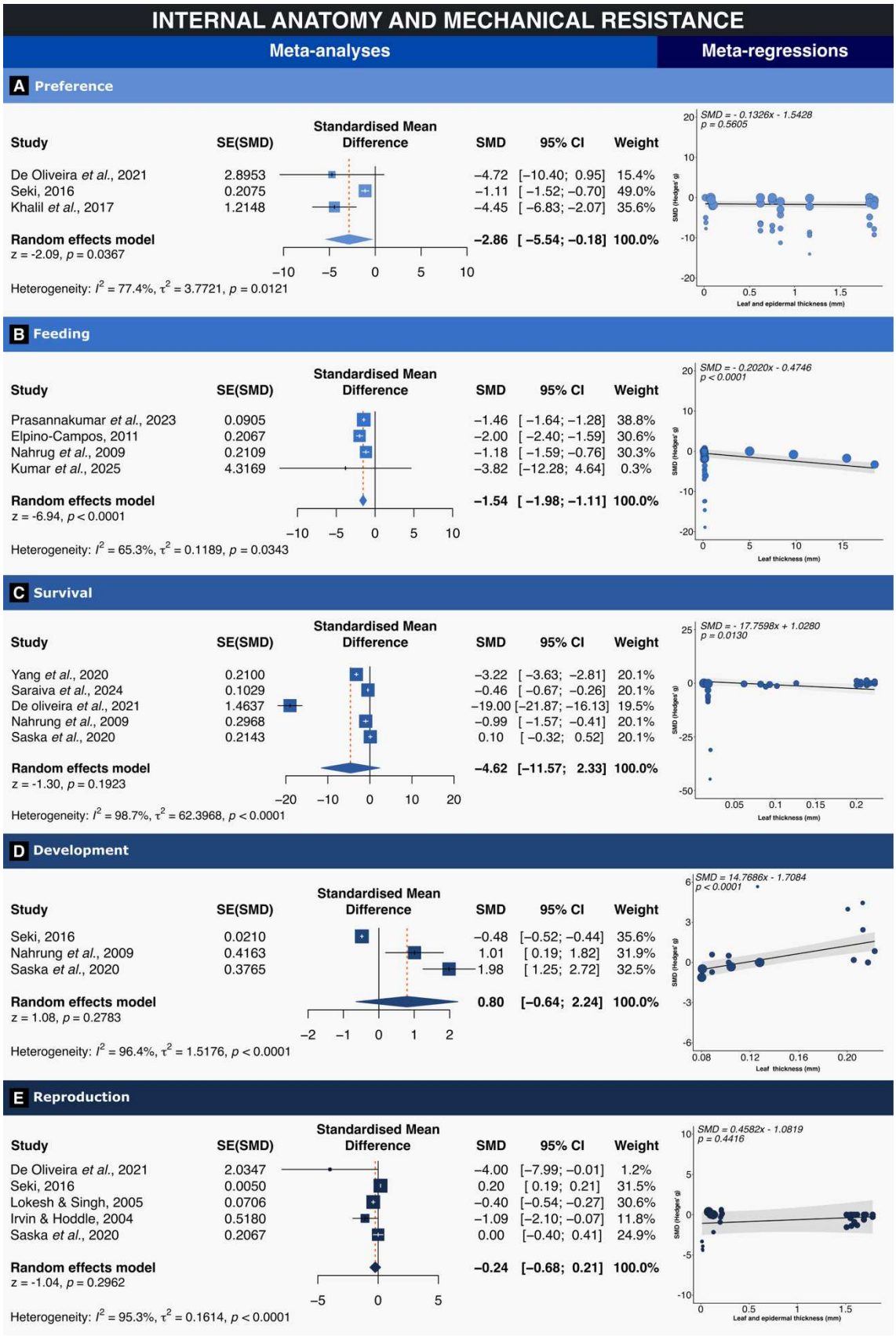


Figure 4. Forest and scatterplots summarizing the results of meta-analyses and meta-regressions, respectively. Each panel is organized by arthropod biological parameter or bioassay: (A) Preference; (B) Feeding; (C) Survival; (D) Development; and (E) Reproduction. The forest plots display, for each study, the standardized mean difference (SMD) and its 95% confidence interval (95% CI). Each box represents the SMD (vertical line) and its 95% CI (horizontal line),

with box size proportional to the inverse of the variance of the study. The diamond indicates the overall effect size, with its width corresponding to the 95% CI. The red dashed vertical line shows the pooled estimated effect across all studies, whereas the solid black vertical line represents the null effect (= 0). Statistical results for heterogeneity and the random-effects model are also provided. In the scatterplots, each point represents an SMD calculated for a given leaf trait, with the circle size proportional to the magnitude of the effect. Solid lines represent the predicted trend from the meta-regressions, and the gray shaded area indicates the 95% CI.

Surface structures

Surface structures such as trichomes may act as physical or chemical barriers by hindering arthropod movement, feeding, oviposition, or by releasing toxic metabolites.[51,52] Meta-analysis did not detect significant effects of trichomes on herbivore preference (SMD = -0.26; $p = 0.7093$), feeding (SMD = -0.36; $p = 0.2868$), survival (SMD = -2.74; $p = 0.2358$), development (SMD = 0.27; $p = 0.4011$), or reproduction (SMD = -0.40; $p = 0.2970$) (Fig. 5A-E). Heterogeneity was consistently high ($I^2 > 93.50\%$; $p < 0.001$).

In contrast, meta-regressions revealed clearer patterns. Increasing trichome density reduced feeding ($\beta = -0.2190$; $p < 0.0001$; Fig. 5B), shortened development time ($\beta = -0.1307$; $p = 0.0042$; Fig. 5D), and decreased reproduction ($\beta = -0.1021$; $p = 0.0115$; Fig. 5E). No significant effects were detected for preference ($\beta = -0.0620$; $p = 0.0653$; Fig. 5A) or survival ($\beta = 0.0136$; $p = 0.2358$; Fig. 5C).

SURFACE STRUCTURES

Meta-analyses

Meta-regressions

A Preference

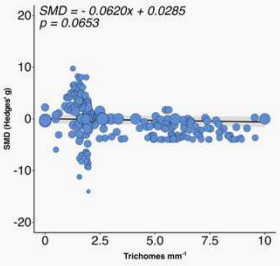
Study	SE(SMD)	SMD	95% CI	Weight
Li *et al.*, 2017	0.9868	−1.20	[−3.13; 0.74]	9.2%
Fernandes *et al.*, 2015	1.3092	−2.34	[−4.91; 0.22]	8.0%
Zeist *et al.*, 2021	0.8534	−2.17	[−3.85; −0.50]	9.6%
Salerno *et al.*, 2018	0.1001	−0.05	[−0.25; 0.14]	11.2%
Savi *et al.*, 2021	0.2114	−0.19	[−0.60; 0.23]	11.2%
Kang *et al.*, 2010	0.1142	0.75	[0.53; 0.98]	11.2%
Le Guigo *et al.*, 2011	0.0672	−0.17	[−0.30; −0.04]	11.3%
Bisht *et al.*, 2022	0.1520	1.66	[1.37; 1.96]	11.2%
Santana Lima *et al.*, 2024	1.0940	4.51	[2.36; 6.65]	8.8%
Khalil *et al.*, 2017	1.2148	−4.45	[−6.83; −2.07]	8.3%
Random effects model		**−0.26**	**[−1.61; 1.09]**	**100.0%**

Standardised Mean Difference

−6 −4 −2 0 2 4 6

z = -0.37, $p = 0.7093$

Heterogeneity: $I^2 = 95.5\%$, $\tau^2 = 4.2162$, $p < 0.0001$



B Feeding

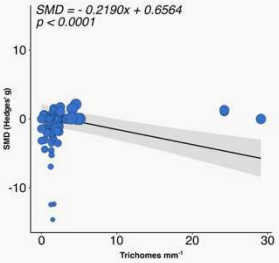
Study	SE(SMD)	SMD	95% CI	Weight
Kang *et al.*, 2010a	0.1227	0.59	[0.35; 0.83]	11.4%
Hall *et al.*, 2020	0.2302	−1.21	[−1.67; −0.76]	11.0%
Biru *et al.*, 2021	0.4084	−1.46	[−2.26; −0.66]	9.9%
Stack *et al.*, 2023	0.1904	−0.73	[−1.10; −0.36]	11.1%
Tian *et al.*, 2012	0.1405	0.42	[0.15; 0.70]	11.3%
Kang *et al.*, 2010b	0.1264	0.70	[0.45; 0.95]	11.4%
Mathur *et al.*, 2011	0.1419	−0.51	[−0.79; −0.23]	11.3%
Ghebremariam *et al.*, 2014	0.0365	0.74	[0.67; 0.81]	11.5%
Kumar *et al.*, 2025	4.3169	−3.82	[−12.28; 4.64]	0.6%
Dakshayani *et al.*, 1993	0.3146	−1.91	[−2.52; −1.29]	10.5%
Random effects model		**−0.36**	**[−1.01; 0.30]**	**100.0%**

Standardised Mean Difference

−10 −5 0 5 10

z = -1.07, $p = 0.2868$

Heterogeneity: $I^2 = 96.7\%$, $\tau^2 = 0.9673$, $p < 0.0001$



C Survival

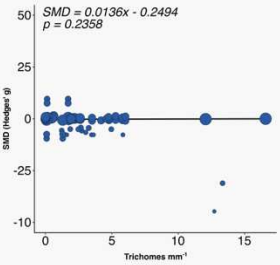
Study	SE(SMD)	SMD	95% CI	Weight
Cho *et al.*, 2027	0.2042	−0.28	[−0.68; 0.12]	12.7%
De Oliveira *et al.*, 2021	1.4637	−19.00	[−21.87; −16.13]	12.1%
Li *et al.*, 2017	0.0704	0.43	[0.29; 0.56]	12.7%
Zeist *et al.*, 2021	1.7707	−4.34	[−7.81; −0.87]	11.8%
Savi *et al.*, 2021	0.0394	0.21	[0.13; 0.28]	12.7%
Amini *et al.*, 2021	0.4622	0.00	[−0.91; 0.91]	12.6%
Le Guigo *et al.*, 2011	0.0677	0.09	[−0.05; 0.22]	12.7%
Saska *et al.*, 2020	0.2143	0.10	[−0.32; 0.52]	12.7%
Random effects model		**−2.74**	**[−7.27; 1.79]**	**100.0%**

Standardised Mean Difference

−20 −10 0 10 20

z = -1.19, $p = 0.2358$

Heterogeneity: $I^2 = 96.5\%$, $\tau^2 = 42.0507$, $p < 0.0001$



D Development

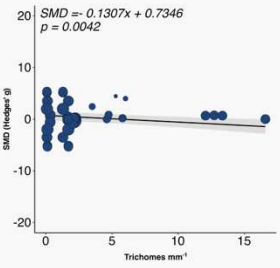
Study	SE(SMD)	SMD	95% CI	Weight
Saraiva *et al.*, 2024	0.1046	0.52	[0.32; 0.73]	17.1%
Savi *et al.*, 2021	0.0404	−0.14	[−0.22; −0.06]	17.4%
Tian *et al.*, 2012	0.0678	0.06	[−0.08; 0.19]	17.3%
Amini *et al.*, 2021	0.0807	0.00	[−0.16; 0.16]	17.3%
Mathur *et al.*, 2011	0.1417	−0.50	[−0.78; −0.22]	16.9%
Saska *et al.*, 2020	0.3765	1.98	[1.25; 2.72]	14.1%
Random effects model		**0.27**	**[−0.36; 0.90]**	**100.0%**

Standardised Mean Difference

−2 −1 0 1 2

z = 0.84, $p = 0.4011$

Heterogeneity: $I^2 = 93.5\%$, $\tau^2 = 0.5896$, $p < 0.0001$



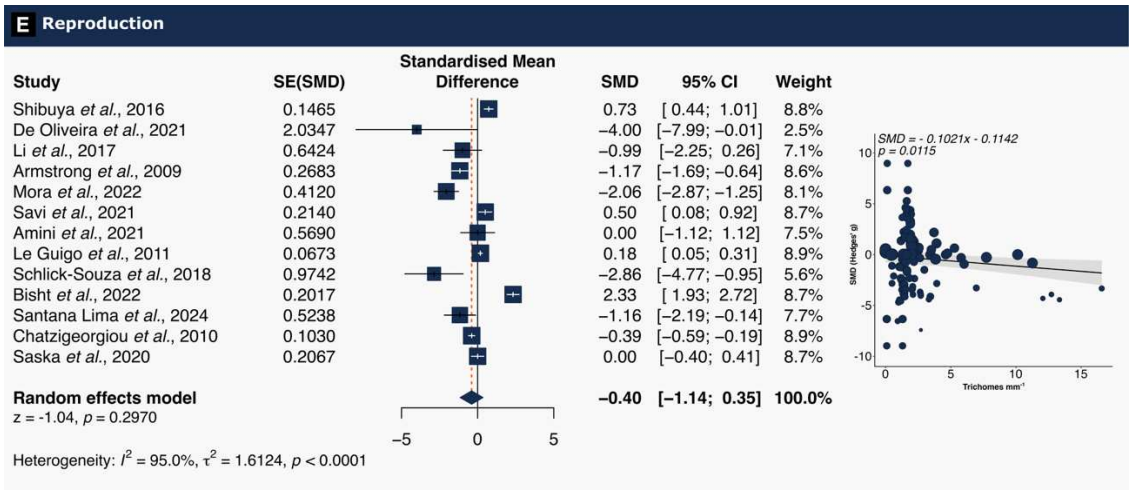


Figure 5. Forest and scatterplots summarizing the results of meta-analyses and meta-regressions, respectively. Each panel is organized by arthropod biological parameter or bioassay: (A) Preference; (B) Feeding; (C) Survival; (D) Development; and (E) Reproduction. The forest plots display, for each study, the standardized mean difference (SMD) and its 95% confidence interval (95% CI). Each box represents the SMD (vertical line) and its 95% CI (horizontal line), with box size proportional to the inverse of the variance of the study. The diamond indicates the overall effect size, with its width corresponding to the 95% CI. The red dashed vertical line shows the pooled estimated effect across all studies, whereas the solid black vertical line represents the null effect (= 0). Statistical results for heterogeneity and the random-effects model are also provided. In the scatterplots, each point represents an SMD calculated for a given leaf trait, with the circle size proportional to the magnitude of the effect. Solid lines represent the predicted trend from the meta-regressions, and the gray shaded area indicates the 95% CI.

Wax and cuticle

The leaf cuticle, covered by epicuticular waxes, creates a microstructured surface that contributes strongly to surface roughness and can reduce arthropod adhesion or affect locomotion. Meta-analysis showed that leaves with greater roughness were less preferred by arthropods (SMD = -0.94; p = 0.0009). Meta-regression further demonstrated that increasing arithmetic mean roughness intensified this pattern, reinforcing its role in modulating host plant preference (β = 1.2382; p < 0.0001; Fig. 6).

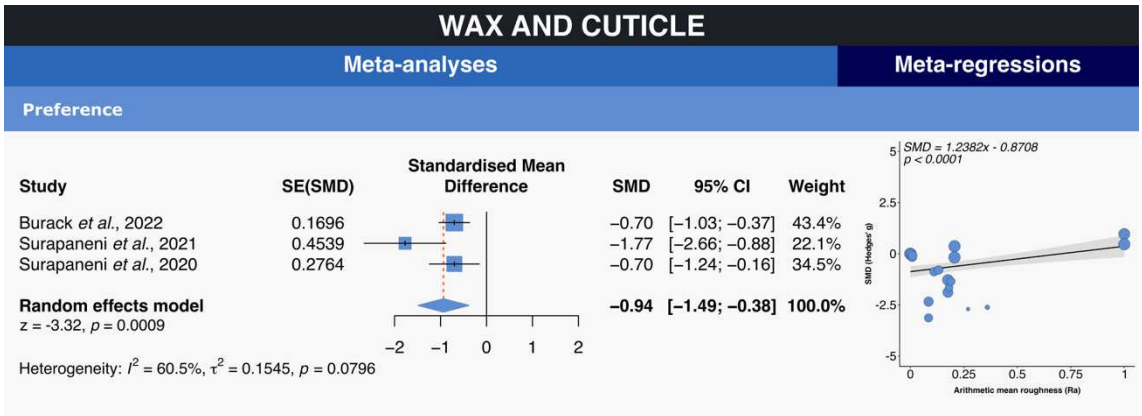


Figure 6. Forest and scatterplots summarizing the results of meta-analyses and meta-regressions, respectively. The panel displays the results for wax and cuticle for the arthropod preference bioassay. The forest plots display, for each study, the standardized mean difference (SMD) and its 95% confidence interval (95% CI). Each box represents the SMD (vertical line) and its 95% CI (horizontal line), with box size proportional to the inverse of the variance of the study. The diamond indicates the overall effect size, with its width corresponding to the 95% CI. The red dashed vertical line shows the pooled estimated effect across all studies, whereas the solid black vertical line represents the null effect (= 0). Statistical results for heterogeneity and the random-effects model are also provided. In the scatterplots, each point represents an SMD calculated for a given leaf trait, with the circle size proportional to the magnitude of the effect. Solid lines represent the predicted trend from the meta-regressions, and the gray shaded area indicates the 95% CI.

Leaf shape and size

Morphological characteristics linked to leaf dimensions — such as shape, width, and length — are traditionally considered relevant because they affect ecological and physiological processes in phytophagous arthropods. However, meta-analysis indicated no significant effects on feeding (SMD = -0.71; p = 0.5914) or reproduction (SMD = 0.05; p = 0.8488) (Fig. 7A-B). Similarly, meta-regression detected no relationship between increasing leaf size and feeding (β = 0.0005; p = 0.5522) or reproduction (β = 0.0002; p = 0.8301).

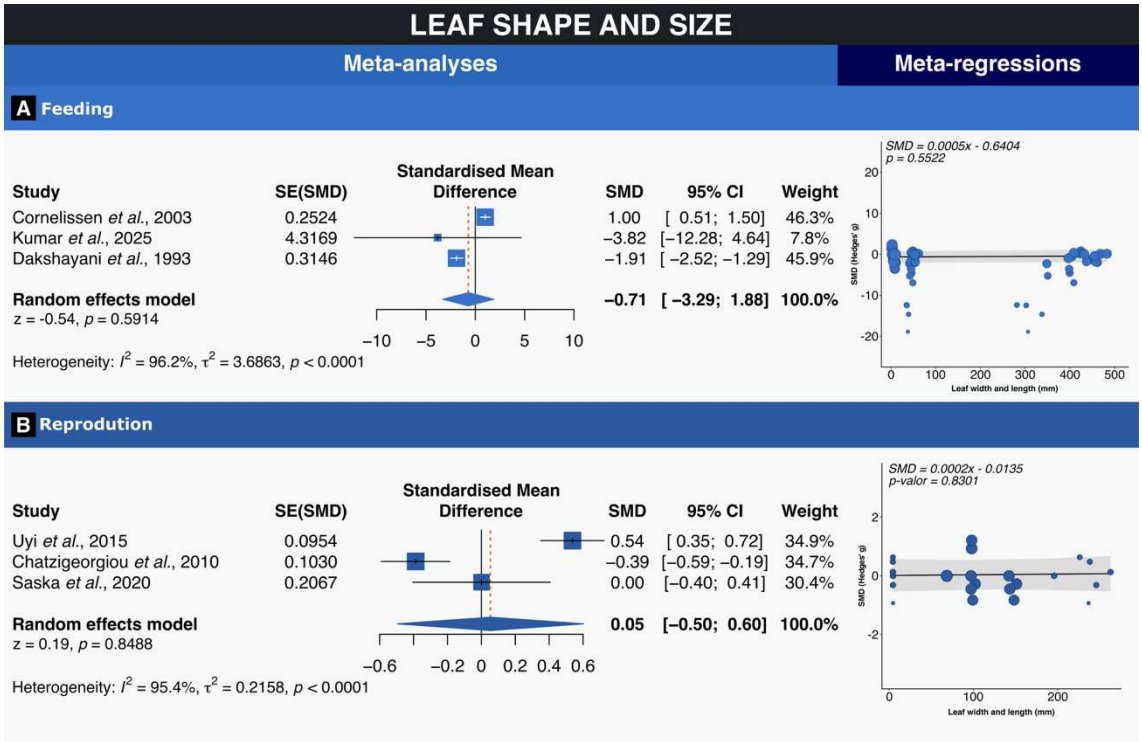


Figure 7. Forest and scatterplots summarizing the results of meta-analyses and meta-regressions, respectively. Each panel is organized by arthropod biological parameter or bioassay: (A) Feeding and (B) Reproduction. The forest plots display, for each study, the standardized mean difference (SMD) and its 95% confidence interval (95% CI). Each box represents the SMD (vertical line) and its 95% CI (horizontal line), with box size proportional to the inverse of the variance of the study. The diamond indicates the overall effect size, with its width corresponding to the 95% CI. The red dashed vertical line shows the pooled estimated effect across all studies, whereas the solid black vertical line represents the null effect (= 0). Statistical results for

heterogeneity and the random-effects model are also provided. In the scatterplots, each point represents an SMD calculated for a given leaf trait, with the circle size proportional to the magnitude of the effect. Solid lines represent the predicted trend from the meta-regressions, and the gray shaded area indicates the 95% CI.

4. DISCUSSION

Understanding the structural complexity of leaves is essential to elucidating the mechanisms underlying plant–herbivore interactions across diverse ecosystems. Our study provides the most comprehensive synthesis to date on how morpho-anatomical leaf traits modulate plant–herbivore interactions. Across nine decades of research, our integrated analysis demonstrates that structural features of leaves exert measurable – though highly variable – effects on key biological parameters of herbivorous arthropods. Collectively, these findings reinforce the central hypothesis that leaf structural properties function as bottom-up defense mechanisms, influencing processes from host selection to the physiological performance of herbivores.[6,51]

The marked increase in publications over recent decades reflects a growing recognition of the ecological importance of functional leaf traits in both plant resistance research and interaction ecology. Yet, this temporal expansion contrasts with a persistent geographical bias, with most studies concentrated in temperate regions, particularly North America and Europe. Such asymmetry limits the inferential power of global ecological generalizations. Although the latitudinal gradient hypothesis predicts higher herbivory pressure and greater guild diversity in tropical regions,[52,53] empirical patterns remain variable and heavily dependent on the specific herbivore guild, plant clade, and defense traits examined.[54,55] The scarcity of studies from tropical ecosystems, therefore, constrains our capacity to evaluate the universality, direction, and magnitude of structural defense effects at a global scale.

The predominance of studies on leaf surface traits, rather than internal anatomical attributes, likely reflects the greater methodological complexity of evaluating internal structural defenses. Likewise, the taxonomic concentration on Lepidoptera, Hemiptera, and Acari mirrors the applied relevance of these groups, but also underscores the need for broader phylogenetic coverage. The substantial heterogeneity in methodological approaches and trait quantification

further highlights the value of meta-analytical tools capable of extracting generalizable patterns from disparate datasets.

Our results for internal anatomy and mechanical resistance reveal that increased thickness of the epidermis and palisade tissue significantly reduces host preference and feeding performance. These patterns accord with the leaf mechanical resistance theory, which associates dense, thick tissues with structural defense and extended leaf longevity.[56] Greater tissue resistance likely imposes a mechanical barrier that hinders stylet penetration, chewing efficiency, and nutrient acquisition. The negative associations between leaf thickness and herbivore survival, coupled with prolonged development times, suggest that mechanically resistant leaves impose physiological costs that can ripple through trophic networks. Prolonged development increases exposure to natural enemies,[57] thereby reinforcing indirect defenses mediated through bottom-up processes that interact with top-down pressures.[58,59] Yet the absence of a consistent effect on reproduction suggests potential compensatory strategies, behavioral or morphological, which may mitigate fitness losses in certain taxa.

Surface structures, especially trichomes, exhibited contrasting outcomes between meta-analyses and meta-regressions. While overall effects were non-significant, meta-regressions revealed that increasing trichome density significantly reduces feeding, development, and reproduction. Such divergence likely reflects the functional heterogeneity of trichomes. Glandular trichomes release adhesive or toxic secretions, whereas non-glandular trichomes function primarily as physical obstacles.[60,61] The weak or absent effects on preference suggest that trichomes often do not deter initial contact or oviposition but act predominantly by affecting post-contact performance. Moreover, trichome effects are inherently species-specific and strongly conditioned by mouthpart morphology; sucking insects may circumvent surface barriers that strongly affect chewing herbivores.[6,32,62] These findings underscore the non-linear, context-dependent nature of mechanical defenses.

Cuticular waxes and surface roughness showed a consistent reduction in arthropod preference, with meta-regressions indicating that greater roughness amplifies this effect. This result aligns with biomechanical studies demonstrating that epicuticular microstructures disrupt insect adhesion, impair locomotion, and hinder oviposition.[63–65] Depending on scale, surface roughness can markedly

reduce adhesion forces in walking insects,[66–69] offering a defensive mechanism largely independent of chemical deterrents and highlighting the multifunctional nature of leaf waxes.

By contrast, leaf shape and size did not significantly influence feeding or reproduction, likely because these traits operate indirectly by altering microenvironmental conditions (e.g., temperature, hydration, or irradiance) or influencing visual host-detection cues.[70,71] Dimensional traits may also covary with other functional attributes (e.g., specific leaf area, lignin content), potentially masking direct effects in synthetic analyses.[72]

The consistently high heterogeneity across all meta-analyses reflects a complex interplay of methodological variation, taxonomic differences, environmental context, and trait interactions. Rather than diminishing the ecological relevance of these defenses, this variability emphasizes that bottom-up effects emerge from multidimensional trait architectures in which anatomical, chemical, and physiological traits intersect dynamically.[73] Structural defenses rarely act in isolation; instead, they integrate into coordinated defense portfolios shaped by evolutionary and ecological pressures.

From a broader ecological perspective, our synthesis supports the view that morpho-anatomical leaf traits are fundamental determinants of plant defensive strategies, with cascading consequences for trophic structure, consumer specialization, and community dynamics. By modulating herbivore behavior and performance, these traits shape not only local interactions but also macroecological patterns of herbivore abundance and diversity. Nonetheless, the study's limitations warrant acknowledgement. The prevalence of qualitative data and lack of methodological standardization restricts direct comparability across studies. High heterogeneity further suggests that future research should develop integrative, mechanistic approaches combining detailed morpho-anatomical measurements with controlled bioassays to more precisely identify causal pathways. Expanding research in tropical regions and across contrasting taxa and environmental contexts is also essential for resolving biogeographical gaps and evaluating the robustness of observed global patterns.

In summary, this meta-analysis indicates that leaf morpho-anatomical traits function as ecological filters that modulate plant–arthropod interactions in hierarchical, context-dependent ways. While some traits exhibit consistent

negative effects on herbivore performance, the complexity and variability of responses highlight that structural defenses operate through integrated mechanisms that underpin key bottom-up processes shaping plant resistance and trophic community organization.

Author contribution statement

DSS, LMT, and RNCG conceived and designed the study. AS and RNCG provided the materials and resources required for its execution. DSS and LMT collected the data and performed the analyses. DSS and RNCG structured and prepared the initial manuscript draft, which was read, revised, and approved by all authors. All authors contributed to the final revision of the material.

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. RNCG serves as an Executive Editor of *Pest Management Science*, but was not involved in the peer-review process of this manuscript.

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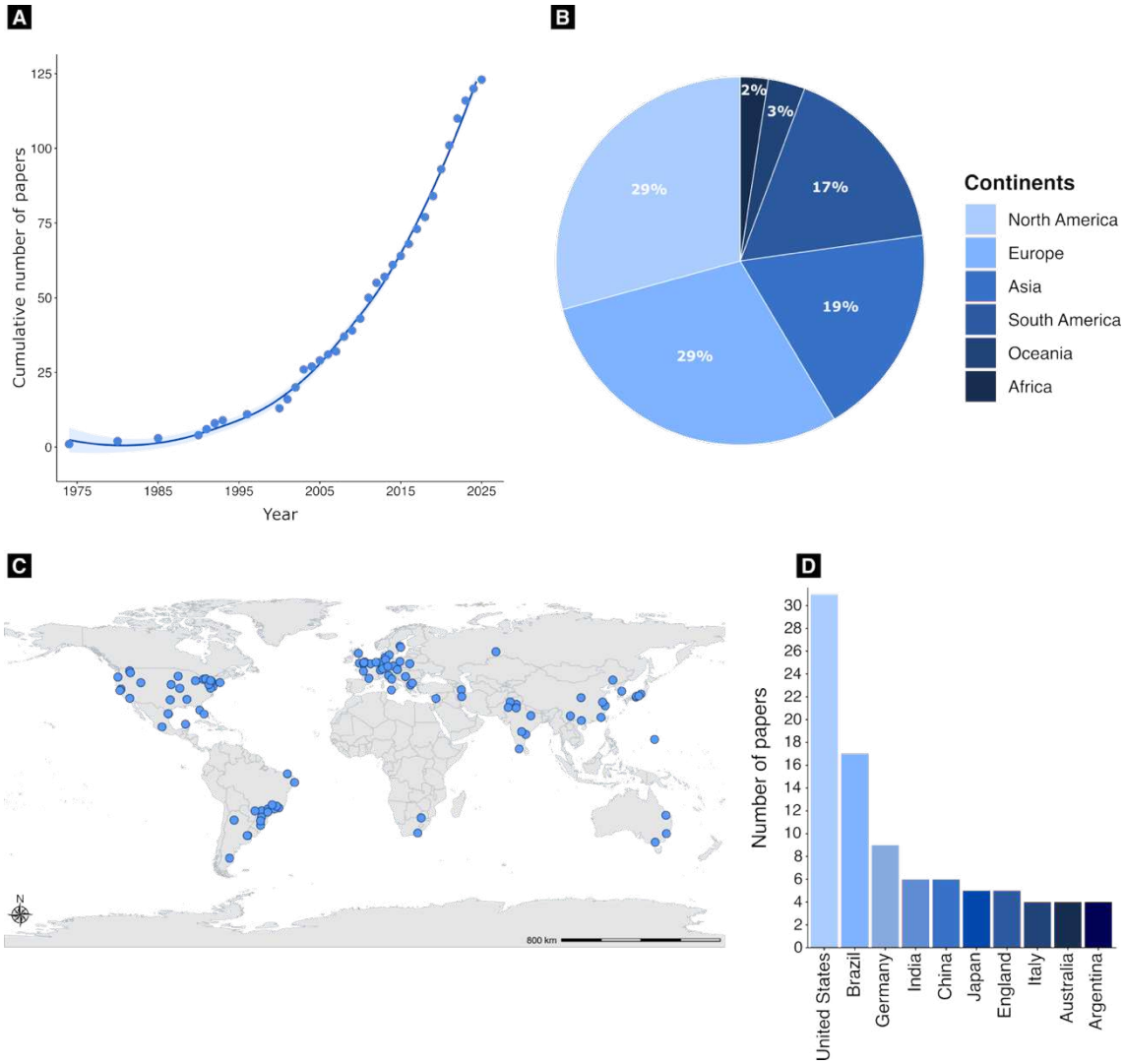
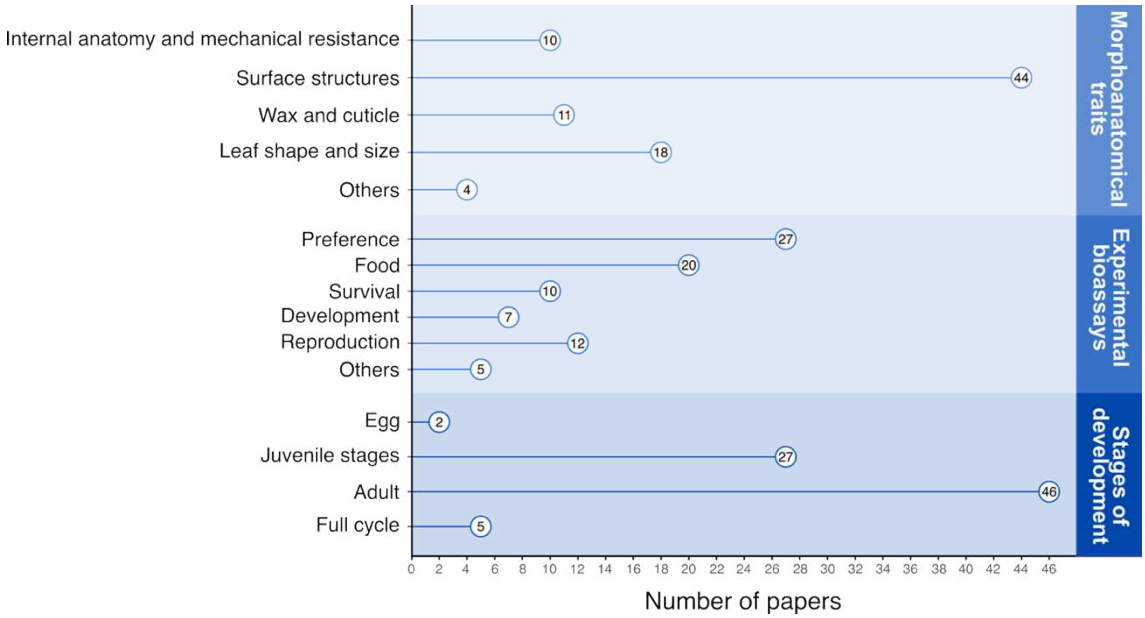


Supplementary Figure 1. Research output on bottom-up effects of leaf structures on herbivorous arthropods. A) Cumulative number of published papers from 1935–2025; B) Percentage of papers published by continent; C) Geographic distribution of scientific production; and D) Scientific production by country.



Supplementary Figure 2. Number of papers produced according to the categorization scheme adopted for qualitative and quantitative analyses.

CHAPTER 2

Leaf vascular bundle sheath extensions modulate tomato interactions with the tomato pinworm

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ABSTRACT

Leaf anatomical traits can play a pivotal role in mediating plant resistance to herbivory and shaping pest dynamics in agroecosystems. Understanding how leaf anatomical traits shape insect-plant interactions is essential to elucidate these relationships and to develop more sustainable pest management strategies. In this context, we investigated an underexplored leaf structural feature - the vascular bundle sheath extension (BSE). Using tomato (*Solanum lycopersicum* L.) isogenic lines harboring the *obscuravenosa* (*obv*) mutation, we assessed how the presence (+) or absence (-) of BSEs impacts interactions with the tomato pinworm, *Phthorimaea* (= *Tuta*) *absoluta* (Meyrick) (Lepidoptera: Gelechiidae), a major invasive pest of the tomato. The absence of BSEs did not influence oviposition preference or egg incubation time, but it significantly reduced larval leaf consumption and prolonged larval development, without affecting survival. Pupal development was also altered in BSE- plants, resulting in lower body mass and delayed adult emergence, while sex ratio remained unaffected. Phytohormonal profiling revealed differences between BSE+ and - BSE plants, particularly in jasmonate levels, suggesting that BSEs may influence plant defense signaling pathways. Together, these findings highlight the potential of BSEs as morphophysiological modulators of insect–plant interactions, emphasizing that internal leaf anatomical traits, alongside chemical defenses, contribute significantly to herbivory resistance.

Keywords: Plant-insect interaction, *Phthorimaea absoluta*, *Solanum lycopersicum*, herbivory, leaf anatomy

Key message

- Leaf anatomy remains underexplored in insect-plant interactions and crop protection.
- We tested how vascular bundle sheath extensions (BSEs) affect an important tomato pest species.
- Mutant leaves without BSEs slowed larval growth and reduced leaf consumption.
- Phytohormone profiles differed, linking leaf traits to defense signaling pathways.

- Internal leaf traits may offer novel targets for pest-resistant crop development.

Author contribution statement

DSS, AZ and RNCG conceived and designed the study. AZ and RNCG provided the materials and tools. DSS performed the experiments and analyzed the data. DSS and RNCG structured and wrote the manuscript. All authors revised the material.

INTRODUCTION

Plants have evolved a wide array of defenses in response to herbivore pressure across ecosystems, allowing them to resist insect attacks throughout their evolutionary history (Johson 2011; Poelman and Kessler 2016; Bobadilla et al. 2022; Endara et al. 2023). To better understand these defenses and explore alternatives to insecticide use, examining the anatomical and morphological traits of leaves – particularly lesser-studied features in the context of insect-plant interactions, such as vascular bundle sheath extensions (BSEs) - has become increasingly relevant. In this regard, plant genetic engineering provides access to numerous genes with potential for manipulation and improvement of desirable characteristics, such as enhanced pest resistance (Zhou and Jander 2021; Taggar et al. 2024; Vasantha-Srinivasan et al. 2025).

Plant resistance to insects involves both chemical and morphological defenses, comprising constitutive traits that are expressed permanently and inducible traits activated upon attack, such as the synthesis of defense-related metabolites (Karban and Baldwin 1997). Among the former, most studies have focused on external physical barriers, including trichomes (Therezan et al. 2021, Almeida et al. 2023, Vendemiatti et al. 2022, 2024), cuticle thickness (Heredia et al. 2024), and epicuticular waxes (Eigenbrode and Espelie 1995b; Haliński et al. 2015), which serve as the first line of defense against insect attack.

Internal anatomical traits, particularly leaf vein architecture, have received relatively little attention in the context of plant resistance to insect pests. Emerging evidence suggests that bundle sheath extensions (BSEs) may play important roles in structural defense against herbivory, as discussed by Sack and Scoffoni (2013). Other anatomical features of the leaf blade have also been associated

with defense mechanisms and can act as physical barriers, potentially influencing plant-herbivore interactions and contributing to pest resistance (Malishev and Sanson 2015). This is the case with the cuticle, which plays multiple roles in the plant protection against stresses (Müller and Riederer 2005). Bundle sheath extensions (BSEs) have been highlighted as key components of this interaction (Sack and Scoffoni 2013).

BSEs, present in so-called heterobaric leaves (hereafter referred to as BSE+), are non-photosynthetic cells that extend from the vascular bundles to the epidermis, physically separating the vascular tissues (xylem and phloem) from the surrounding parenchyma (Evert and Eichhorn 2006; Sack and Scoffoni 2013). This structural arrangement compartmentalizes the intercellular spaces (Liakoura et al. 2009), potentially restricting the mobility and feeding of leaf-feeding herbivores, especially those with endophytic habits such as leaf miners. BSEs also contribute to important physiological processes, including gas exchange and the transport of water and nutrients (Choong et al. 1992; Buckley et al. 2011; Kawai et al. 2017).

By contrast, homobaric sheets (hereafter BSE-), which lack these extensions, exhibit a more uniform internal structure with increased connectivity between intercellular spaces (Terashima 1992, Liakoura et al. 2009). These leaves tend to show reduced photosynthetic efficiency, which in turn affects photoassimilate production and nutrient translocation (Jones et al. 2007; Zsögön et al. 2015). Such differences and herbivory stress imposed to the plants may exhibit hormonal mediation, which is also deserving of attention. Regardless, given these differences, greater mesophilic compartmentalization of heterobaric leaves suggests that BSE+ plants may offer enhanced protection against herbivory, acting as an additional barrier to leaf-mining insects. In contrast, BSE- leaves may be more vulnerable due to their accessible mesophyll, although there is no recognized evidence of the association between BSE and leaf mechanical and structural properties (Kawai et al. 2017).

The *obscuravenosa* (*obv*) mutant of tomato (*Solanum lycopersicum*) presents a valuable model for testing this hypothesis. In *obv* (BSE-) plants, the absence of BSEs results in homobaric leaf anatomy (Jones et al. 2007, Moreira et al. 2022). This mutant allows for the controlled investigation of BSE function in insect resistance while minimizing the variation from other morphophysiological

traits (Barbosa et al 2019). The effect of this trait is expected to be particularly relevant against endophytic herbivores that interact directly with the leaf mesophyll.

The tomato pinworm *Phthorimaea* (= *Tuta*) *absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is native to South America and is current regarded as the most important tomato pest worldwide (Campos et al. 2017; Biondi et al. 2018). It has invaded numerous regions—including Europe, Africa, the Middle East, and Asia—where it has become the dominant tomato pest species (Campos et al 2017; Srinivasulu 2017; Biondi et al. 2018), causing severe yield losses under favorable humidity and temperature conditions (Guedes e Picanço 2012), and exhibiting difficulties in its management (Guedes et al., 2019).

Larval feeding by the tomato pinworm primarily damages leaves, but can also affect stems, flower buds, and fruits (Medeiros et al. 2009; Desneux et al. 2010). Although insecticides remain the most widely used control method against the tomato pinworm, particularly in open tomato fields, frequent applications have led to the emergence of resistant populations (Guedes et al. 2019; Misra et al. 2021; Ong'onge et al. 2023). Thus, due to the species' capacity for causing crop failure and the growing difficulty in managing its populations, sustainable alternatives that harness innate plant defenses are urgently needed (Guedes e Picanço 2012; Guedes et al. 2019; Pereira et al. 2023).

In this study, we investigated the effects of the absence of BSEs caused by the *obv* mutation in tomato plants as an innate anatomical trait that can modulate resistance to the tomato leaf miner. We hypothesized that the presence of BSEs in heterobaric leaves acts as an internal physical barrier that restricts larval access and feeding in the leaf mesophyll. Although the potential association between BSE and leaf mechanical and physical properties were not observed among woody plant species (Kawai et al., 2017), it may take place for herbaceous species such as tomato plants, either directly, or indirectly. Thus, BSE may potentially impact the quality of the tissue consumed by the insects, particularly leaf miners, hindering their development and minimizing their damage to the host plant.

MATERIAL AND METHODS

Insects

The initial population of the tomato pinworm (*P. absoluta*) was collected in 2020 from tomato fields in the municipality of Coimbra (20°51'12"S'; 42°48'03"W), state of Minas Gerais (MG), Brazil (BR). The insects were reared in aphid-proof cages at 25 ± 1 °C and 65 ± 5% relative humidity under a 12:12 h light-dark photoperiod. Adults were maintained in cages (60 × 50 × 50 cm) with a 10% glucose solution provided *ad libitum*, and tomato leaves offered daily for oviposition. Egg-bearing leaves were collected daily and transferred to larval rearing cages (45 × 45 × 45 cm), where larvae were fed *ad libitum* with fresh leaves of the 'Santa Clara' tomato cultivar (IC11 5500) until the pupation.

Plants

Seeds of tomato cv. M82, harboring either the wild-type allele of *OBSCURAVENOSA* (heterobaric, BSE+) or the loss-of-function allele *obv* (homobaric, BSE-) (Fig. 1; fully characterized by Barbosa et al. (2019)), were sown individually in vermiculite-filled cells. Once the first true leaf emerged, seedlings were transplanted into 5 L pots containing commercial substrate (Plantmax HT, Eucatex, Brazil) enriched with 8 gL^{-1} of NPK (4:14:8) and 4 gL^{-1} of dolomitic limestone ($MgCO_3$+$CaCO_3$). Nutrient supplementation was repeated weekly with the same NPK (4:14:8) and limestone formulation, following Gasparini et al. (2021). Plants were cultivated under semi-controlled greenhouse conditions and irrigated via an automated drip system.

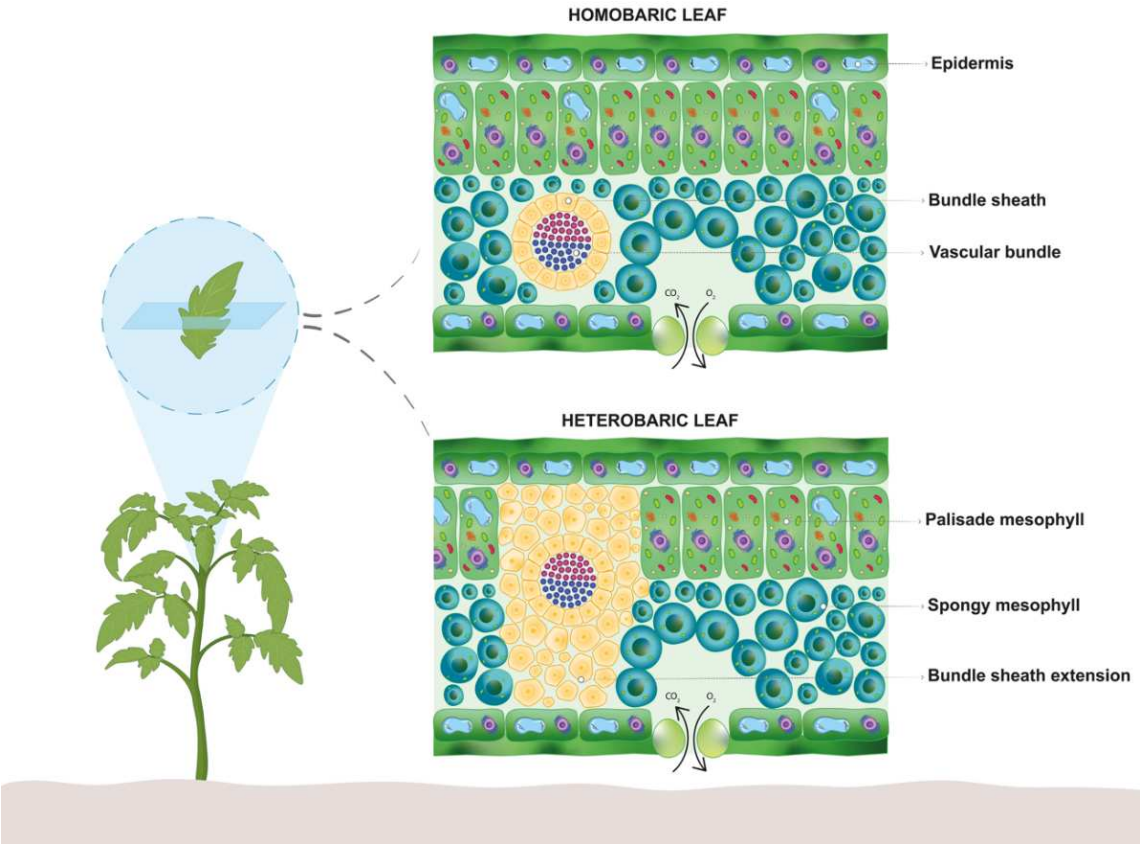

Fig. 1. Illustrative representation of the internal anatomical differences of heterobaric (BSE+) and homobaric (BSE-) leaves, highlighting the presence or absence of vascular bundle sheath extensions.

Egg-laying bioassay

Oviposition preference was assessed using no-choice bioassays in wooden cages (30 × 30 × 30 cm) lined with insect-proof mesh. A tomato leaf (BSE^+ or BSE^-) with three fully developed leaflets was placed inside each cage with their petioles embedded in a water–agar solution to keep the leaves turgid. Ten virgin *P. absoluta* pairs (24 hours old) were released per cage. The number of eggs laid was recorded at 24, 48, 72, and 96 hours post-release. A 10% glucose solution was provided throughout the assay. Ten replicates were performed for each tomato genotype.

Leaf consumption bioassay

To assess leaf consumption, adult insects were allowed to oviposit on 'Santa Clara' leaves for 24 h. Thereafter, individual leaflets from each genotype (BSE+ and BSE-) were placed in Petri dishes with their petioles embedded in a water–agar solution. Excited leaves were used instead of the offering of leaves

of whole plants due to limitations on the amount and quality of the tomato genotypes available, which would be preferable. Thus, we set priority on the quality and standardized material since BSE is a morphological trait, and therefore less prone to physiological influence due to leaf excision. Eggs (<24 h old) were transferred individually to each leaflet using a fine paintbrush. Dishes were kept in a controlled environment chamber (Percival I30VLC8, Percival Scientific, Inc., Perry, IA, USA) at 25 ± 1 °C, 65 ± 5% RH, and a 12:12 h light–dark cycle. Egg development was monitored daily. Upon larval emergence, daily radiographs were taken for seven days to assess leaf consumption following the method of Souza et al. (2024). Radiographs were captured using a digital system (LX60; Faxitron Corp., Tucson, AZ, USA) at 25 kV with a 15-second exposure. Images were analyzed using ImageJ software (Schneider et al. 2012). Each genotype had 50 replicates, with one larva per leaflet.

Insect development bioassay

Eggs from oviposited 'Santa Clara' leaves were transferred to BSE+ and BSE- leaves under the same conditions as the consumption assay. Development was monitored daily until adult emergence. The durations of egg incubation, larval and pupal stages, as well as pupal body mass, were recorded. Larval mortality and sex of emerged adults were also noted. Fifty replicates were used for each genotype (BSE+ and BSE-).

Phytohormone profile

Leaves from BSE+ and BSE- plants were collected, flash-frozen in liquid nitrogen, and stored at –80 °C. Tissues were ground in liquid nitrogen and 150 mg of powdered tissue was extracted with 400 µL of a methanol:isopropanol:acetic acid (20:79:1) solution (Rogachev and Aharoni 2011, with modifications (Vital et al. 2019). The samples were shaken four times for 20 seconds, kept on ice and sonicated in ultrasound for 10 minutes, followed by 30 minutes of resting on ice. Samples were vortexed (4 × 20 s), sonicated for 10 min, and rested on ice for 30 min. Following centrifugation at 13,000g for 10 min at 4 °C, the supernatant was re-centrifuged at 20,000 g for 5 min and filtered through a 0.22 µm PVDF membrane. A 600 µL aliquot was reserved for Ultra-High Performance Liquid Chromatography (UHPLC-MS) analysis.

Aliquots (5.0 µL) were injected into an Agilent 1200 Infinity UHPLC system coupled to an Agilent 6430 triple quadrupole mass spectrometer coupled to a triple quadruplex mass spectrometer (QqQ, Agilent 6430, Agilent Technologies, Santa Clara, CA, US). The system operated in both positive and negative ionization modes and scanned compounds using Multiple Reaction Monitoring (MRM), following Gómez et al. (2020). Analyses were conducted at the Biomolecule Analysis Center of the Federal University of Viçosa (UFV), Brazil, using three biological replicates. Mass spectra were processed in Skyline software (MacCoss Lab, University of Washington, USA), and peak areas were quantified and converted into ng/g tissue using standard phytohormone curves.

Statistical analysis

Data normality and homoscedasticity were evaluated using Shapiro–Wilk and Levene's tests, respectively. Oviposition data were analyzed via a Generalized Linear Model (GLM) with a quasipoisson distribution and log link function, using the number of eggs as the dependent variable and exposure time and plant genotype as explanatory variables.

Leaf consumption and larval duration were analyzed using a Generalized Linear Mixed Model (GLMM) with Gamma distribution and a logarithmic link function, including the number of individuals as a random effect to account for larval mortality. Daily leaf consumption was analyzed using repeated measure analysis of variance performed using the *lme4* (Bates et al. 2015) and *lmerTest* (Kuznetsova et al. 2017) packages, to verify the effect of days on larval consumption and compare the differential consumption in tomato plants over the seven days of analysis.

The duration of the incubation period and larval period in each type of tomato plant were compared using the nonparametric Mann-Whitney test. A simple linear model was used to assess pupal mass, with pupal weight (mg) as the response variable and total leaf consumption, larval duration, and plant genotype as predictors. Individual predictor effects were evaluated via ANOVA ($p < 0.05$). The sex ratio between plant types was compared using chi-square tests, and binomial exact tests were applied within each plant type. Larval mortality was analyzed via Kaplan-Meier estimators and χ^2 Log-Rank test using the *survival*

(Therneau 2024) and *survminer* (Kassambara et al. 2021) packages. Differences in phytohormone levels were tested using one-way ANOVA ($p < 0.05$).

To explore associations between insect performance and hormonal profiles, principal component analysis (PCA) was used to reduce the dimensionality of the hormonal data. Because the performance and hormonal assays were run separately, the insect performance variables (total leaf consumption, larval duration, and pupal weight) were averaged per plant group, corresponding to the three biological replicates used for hormonal analysis. The principal component scores from each biological replicate were then correlated with the insect performance averages using Spearman's correlations ($p < 0.05$).

All statistical analyses were conducted in R (R Core Team, 2025). The graphs were created using the *ggplot2* package (Wickham, 2016), and design edits were made using Inkscape 1.3.2 software (Inkscape Project, 2023).

RESULTS

Egg-laying

The assessment period significantly affected cumulative oviposition by the tomato pinworm ($t = 9.01$, $df = 65$, $p < 0.001$), with a progressive increase in egg-laying over the 96-hour period (Fig. 2A). Although females laid more eggs on BSE- plants (111.50 ± 18.11 eggs) compared to that on BSE+ plants (93.37 ± 11.93 eggs), this difference was not significant ($t = 1.35$, $df = 65$, $p = 0.18$) (Fig. 2 B).

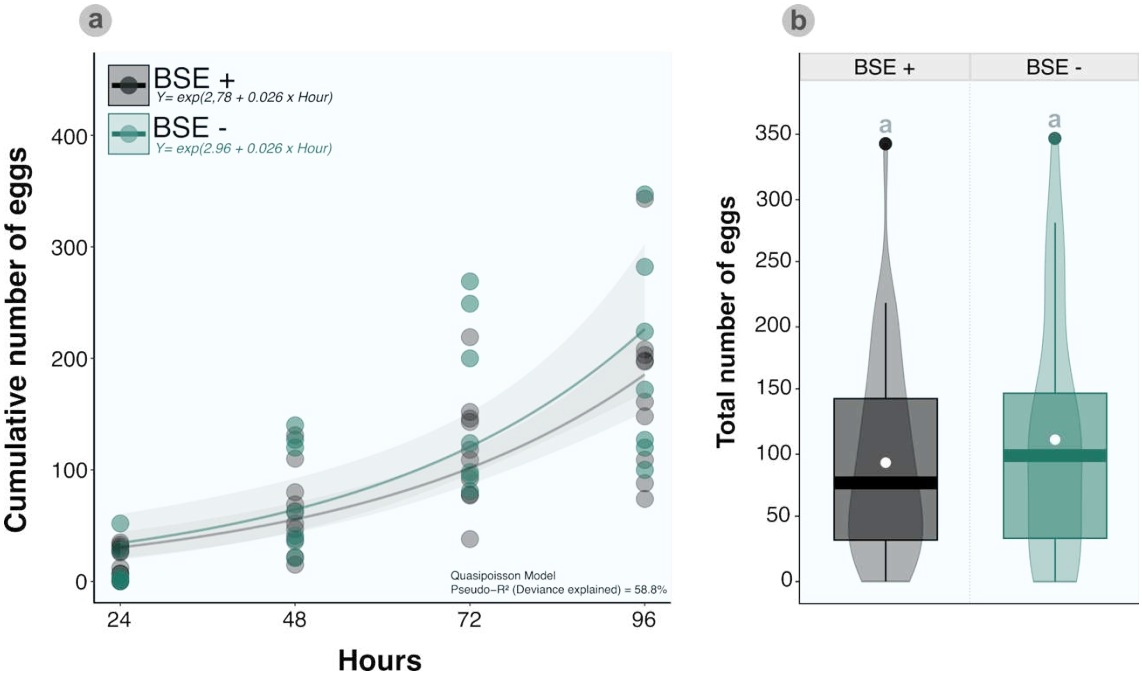

Fig. 2. Egg-laying of tomato pinworm adult females (*Phthorimaea absoluta*) on BSE+ and BSE- plants. A: Cumulative number of eggs laid over 96 hours after adult release on different plant genotypes. B: Total number of eggs laid on BSE+ or BSE- plants after 96 hours. Colored lines in A represent the fitted quasipoisson model with shaded confidence intervals. Boxplots in B display median (solid line), interquartile range (box), lower and upper bounds (whiskers), mean (white circle), and outliers (solid-colored dots). Different lowercase letters denote significant differences ($p < 0.01$).

Leaf consumption

The *obv* mutation significantly influenced larval feeding (GLMM Gamma with random repeat effect: $\chi^2_{(1)} = 7.18$, $p = 0.004$). Larvae consumed less foliage (248.71 ± 15.24 mm²) than those on BSE+ leaves (282.55 ± 14.90 mm²), a reduction of approximately 12% (Fig. 3A and B).

A significant time effect on consumption was also observed ($F = 106.75$, $df = 6$, $p < 0.001$), with statistical differences emerging from the fifth day onward ($F = 4.34$, $df = 1$, $p = 0.04$), coinciding with the transition of most larvae to the third instar.

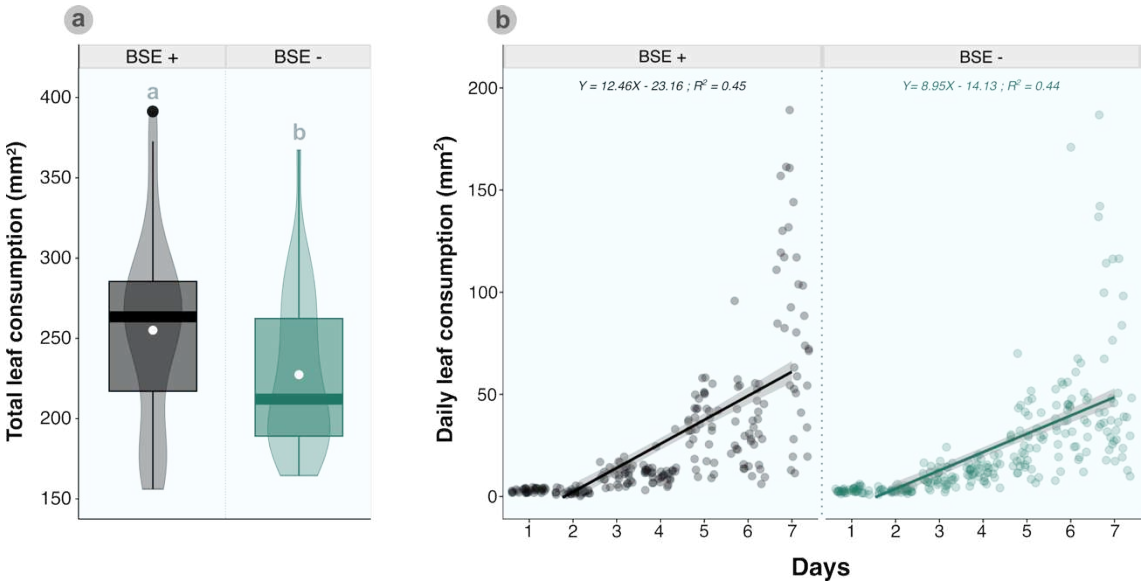

Fig. 3. Leaf consumption of the tomato pinworm (*Phthorimaea absoluta*) on BSE+ and BSE- plants. A: Total consumption over 7 days. B: Regression of daily consumption. Colored lines in A represent the fitted quasipoisson model with shaded confidence intervals. Boxplots in B display median (solid line), interquartile range (box), lower and upper bounds (whiskers), mean (white circle), and outliers (solid-colored dots). Different lowercase letters denote significant differences ($p < 0.01$).

Insect development and mortality

The incubation period of eggs did not differ significantly between BSE+ and BSE- plants ($W = 726.50$, $p = 0.44$) (Fig 4 A). However, larval development was significantly extended on BSE- plants ($W = 361.50$, $p < 0.001$) (Fig 4 B).

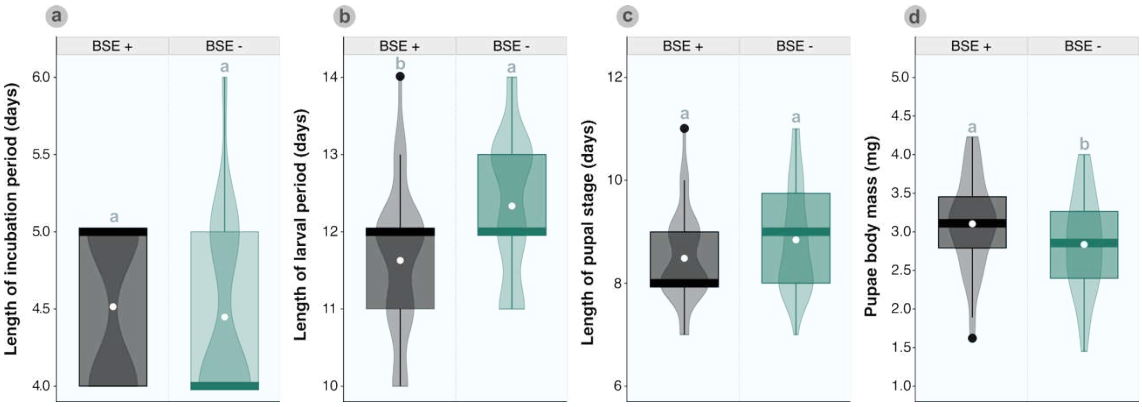

Fig. 4. Developmental parameters of the tomato moth (*Phthorimaea absoluta*) on BSE+ and BSE- plants. A: Incubation period. B: Larval period. C: Pupal period. D: Pupal body mass. Box plots indicate the median (solid line) and spread (lower and upper quartiles) of consumption, the vertical line indicates the lower and upper limits, white circle indicates the mean of the data and solid colored circles in each treatment indicate outliers. Different lowercase letters indicate significant difference at $p < 0.01$.

Pupal duration averaged 8.48 ± 0.15 days on BSE+ plants and 8.84 ± 0.17 days on BSE- plants, with no significant difference (t = 1.65, df = 69, p = 0.10) (Fig. 4C). However, pupal body mass was significantly affected by plant genotype (F = 825.17, df = 2, $p < 0.001$): larvae on BSE- plants yielded lighter pupae (2.84 ± 0.1 mg) than those on BSE+ plants (3.10 ± 0.1 mg), a reduction of approximately 0.26 mg (Fig. 4D).

Larval survival did not differ between genotypes (*Log-Rank* χ^2 = 0.4, df = 1, p = 0.55). No significant differences in adult sex ratios were observed between plant types (χ^2 = 0.32, df = 1, p = 0.57). Binomial tests within each plant type also indicated no deviation from a 1:1 sex ratio: 48.57% male on BSE- ($p > 0.05$), and 57.89% male on BSE+ (p = 0.42). Thus, plant genotype did not affect the sex ratio of the tomato pinworm.

Phytohormone profiles

Chromatographic analysis of plant extracts revealed differences in phytohormonal profiles between BSE+ and BSE- plants, although no significant differences were observed ($p > 0.05$), except for the auxin indoleacetic acid (AIA-2; $F_{1,4}$ = 12.00, p = 0.03) (Table 1). Jasmonic acid concentrations were higher in BSE- plants (17.12 ± 7.42 ng/g) than in BSE+ plants (13.09 ± 1.78 ng/g), suggesting greater induction of defense pathways. Methyl jasmonates showed variable patterns.

Abscisic acid (ABA) levels were similar between genotypes (BSE+: 50.82 ± 4.30 ng/g; BSE-: 55.97 ± 11.95 ng/g). Salicylic acid (SA) was also elevated in BSE- plants (129.12 ± 39.47 ng/g vs. 106.7 ± 60.19 ng/g). Auxin (AIA) levels were lower in BSE- plants (0.16 ± 0.01 ng/g) compared to BSE+ (0.45 ± 0.14 ng/g). Ethylene precursors (ACC-1 and ACC-2) were more abundant in BSE- leaves. *Cis*-zeatin, a cytokinin, was slightly lower in BSE- plants, while brassinolide levels were markedly reduced in BSE- plants (2.04 ± 0.98 ng/g vs. 5.11 ± 2.67 ng/g).

Table 1. Phytohormone concentrations (mean ± standard error) in tomato plants with and without vascular bundle sheath extensions (BSE+ and BSE-), determined via UHPLC-MS.

Category	Molecules	Plants	
		BSE+	BSE-
		Concentration (ng/g)	
Jasmonates	Jasmonic acid (AJ)	13.09 ± 1.78	17.12 ± 7.42
	Methyl jasmonate (MeJA)	1.41 ± 1.29	0.32 ± 0.007
	Methyl jasmonate (MeJA2)	0.24 ± 0.045	0.25 ± 0.06
Abscisic acid (ABA)	ABA (Quant)	50.82 ± 2.48	55.97 ± 6.9
	ABA^{-2}	16.26 ± 1.24	17.09 ± 2.22
Salicylic acid (SA)	Salicylic acid (Quant)	106.7 ± 34.75	129.12 ± 22.8
	Salicylic acid (Neg2)	12.43 ± 4.12	14.89 ± 2.75
Auxins	Indoleacetic acid (AIA^{-1})	0.51 ± 0.053	0.58 ± 0.22
	Indoleacetic acid (AIA^{-2})	0.45 ± 0.082	0.16 ± 0.007
Ethylene	1-Aminocyclopropane-1-carboxylate (ACC^{-1})	271.14 ± 16.14	318.46 ± 35.12
	1-Aminocyclopropane-1-carboxylate (ACC^{-2})	10.37 ± 4.82	18.06 ± 2.4
Cytokinins	*Trans*-Zeatin	0.18 ± 0.034	0.16 ± 0.053
	Cis-Zeatin	3.51 ± 0.108	2.55 ± 0.84
Brassinosteroids	Brassinolide	5.11 ± 2.67	2.04 ± 0.98

Relationship between leaf phytohormones and insect responses

Principal component analysis (PCA) of phytohormone profiles separated BSE+ and BSE- plants, with PC1 and PC2 explaining 72.97% of the total variance (PC1: 45.72%; PC2: 27.26%) (Fig. 5A). PC1 was primarily associated with defense-related hormones (jasmonic acid, ABA, and ACC), while PC2 was

dominated by growth regulators such as brassinolide, methyl jasmonate, and *trans*-zeatin (Fig. 5B).

Spearman correlation analysis revealed a significant negative association between PC1 and total leaf consumption, suggesting that defense-related hormonal profiles reduced feeding activity (Fig. 6). Moreover, pupal weight was negatively correlated with larval development duration, indicating that prolonged development was associated with reduced pupal mass.

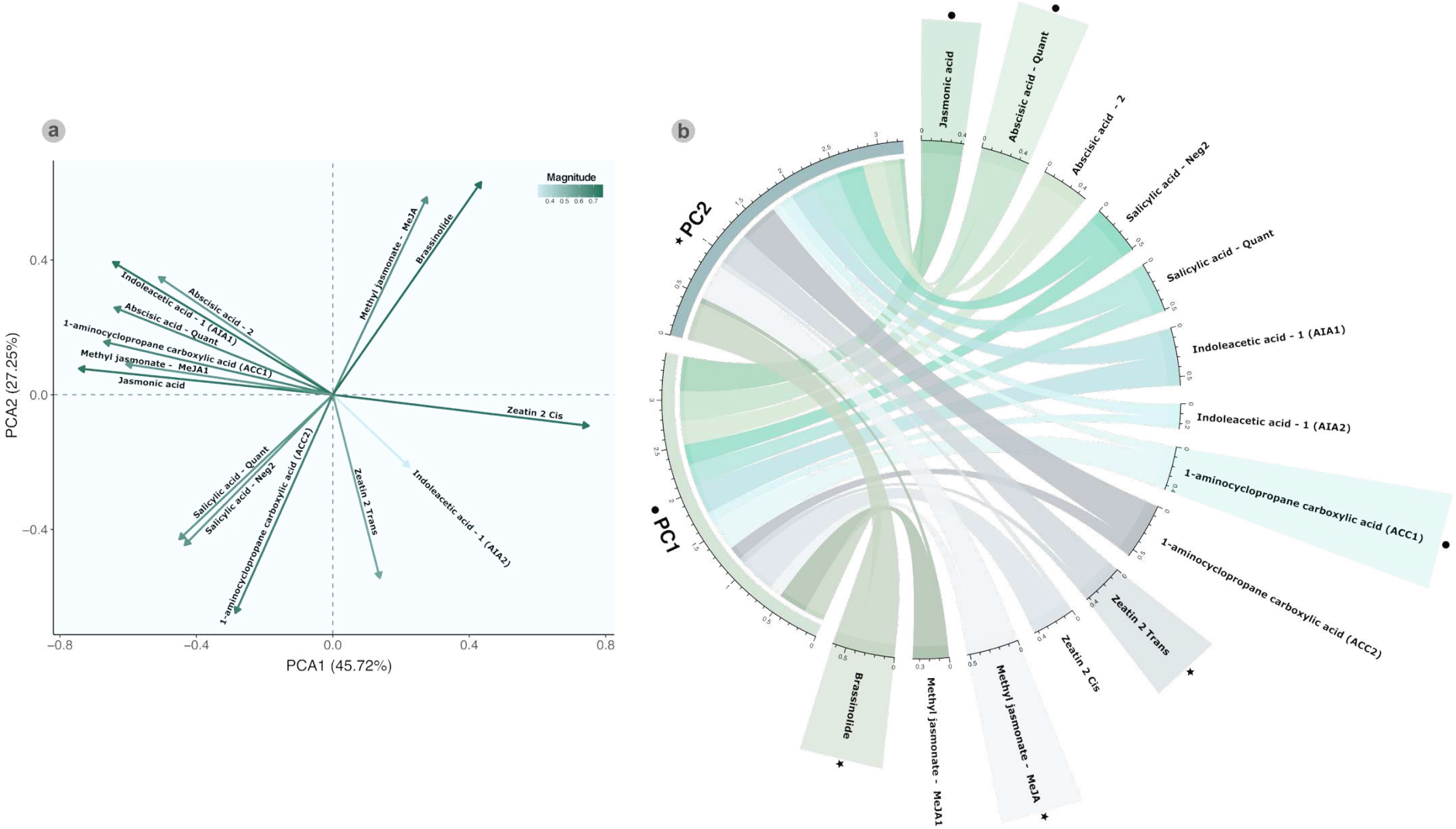

Fig. 5. Principal component analysis (PCA) of phytohormone profiles in BSE+ and BSE- tomato leaves. A: PCA biplot of hormone distributions across genotypes. B: Absolute loading scores for PC1 and PC2. Shaded phytohormones with solid black circles indicate those with the greatest association with PC1, while shaded phytohormones with stars indicate those with the greatest association with PC2.

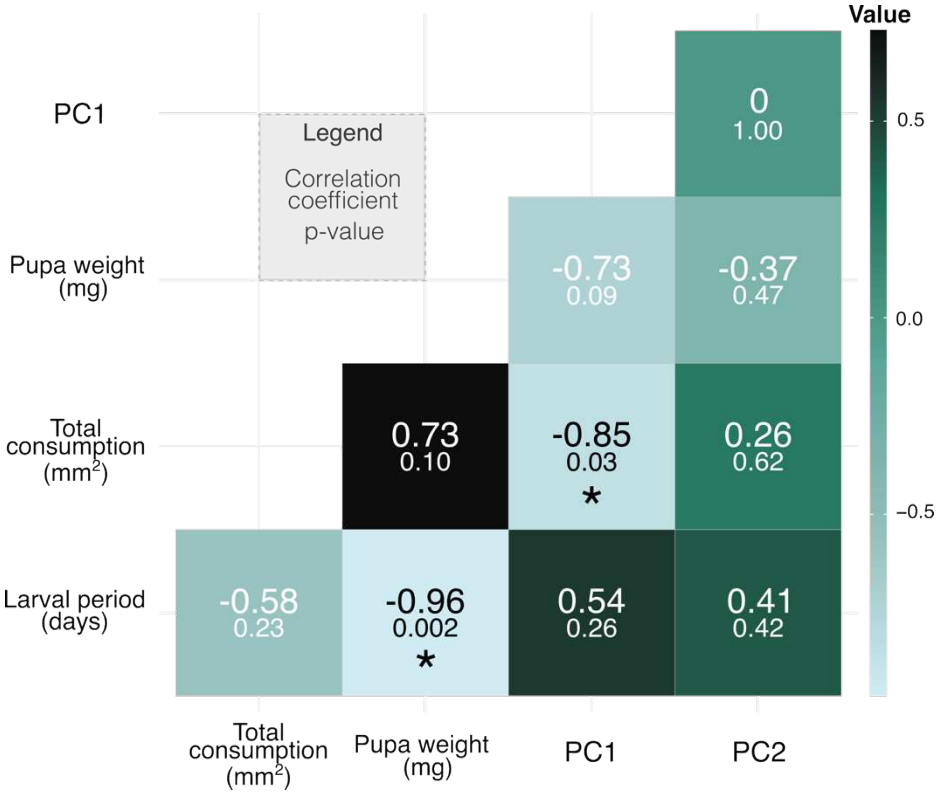

Fig 6. Correlation matrix between principal component scores of the leaf phytohormones and the biological performance variables of the tomato pinworm (*Phthorimaea absoluta*) in BSE+ and BSE- plants. The values presented represent Spearman's correlation coefficients, ranging from -1 to 1, where values close to 1 or -1 indicate strong associations (positive or negative, respectively), and values close to 0 suggest absence of correlation. Lower values represent the *p*-value. * indicates statistically significant correlation at $p < 0.05$.

DISCUSSION

Here, we tested whether leaf bundle sheath extensions (BSEs), an anatomical trait that defines heterobaric (BSE+) and homobaric (BSE-) plants species, would impact the tomato response to the tomato pinworm, a generalist leaf miner. Our results indicate that the presence of the *obscuravenosa* (*obv*) mutation in tomato (BSE-), negatively affects the tomato pinworm *P. absoluta* by reducing leaf consumption and delaying larval development. We initially hypothesized that the compartmentalization of leaf tissue promoted by BSEs, present in heterobaric leaves, would hinder leaf miner development by creating an additional physical barrier to leaf consumption. Notably, our data suggest that the opposite may be the case – namely, that the absence of BSEs in homobaric leaves (BSE-) favors resistance to herbivory. This indicates that the interaction between leaf anatomy and plant physiology may significantly affect feeding behavior and larval development in this pest species, albeit distinctly from our initial expectation.

Understanding the impact of host plant traits on insect development is fundamental for effective pest management (Idriss et al. 2020). Previous studies have highlighted how secondary metabolites and leaf structural properties can affect herbivore performance and fitness throughout their life cycle (Simpson and Raubenheimer 2001; Chen 2008; Cotter et al. 2011; Wilson et al. 2019a; Wilson et al. 2019b). However, the influence of specific structural traits, such as bundle sheath extensions (BSEs), on insect performance remains poorly understood. In this context, the present study is the first to evaluate the role of the *obv* mutation in tomato, which eliminates BSEs as a modulator of plant–insect interactions and a potential source of resistance to herbivores.

Female egg-laying preference was not affected by leaf phenotype, indicating that the main effects of the *obv* mutation manifest during the larval stage. Although prior larval experience can sometimes influence adult oviposition behavior (Idriss et al., 2020), in this study, insects were reared on a different tomato cultivar from those used in the bioassays, thus minimizing any pre-imaginal conditioning effects and lending robustness to our findings. Likewise, the incubation period of the eggs was unaffected by plant genotype, with similar hatching success in both cases.

The *obv* mutation significantly reduced larval leaf consumption, which in turn prolonged larval development. Larvae feeding on BSE- plants had a longer developmental cycle and produced pupae with significantly smaller body masses, suggesting that BSEs themselves do not act as a direct barrier to herbivory. Instead, their absence may compromise gas exchange, water transport, and nutrient assimilation or digestibility, indirectly impairing insect growth potentially compromising molt and metamorphosis, which requires high energy inputs (Simpson and Douglas 2013). Thus, indirect rather than direct effects are likely the main drivers of BSE effects on the tomato leaf miner, which will require the direct assessment of the effects leaf nutritional and digestibility qualities associated with BSEs on leaf miners to ascertain of such relationship.

One factor that should be noted is that our performance assays were conducted using excised leaves, an approach that allows greater experimental control and standardization of evaluation conditions. However, this procedure may affect plant physiological processes, including water relations and defense responses, which can affect plant–herbivore interactions. Thus, although our results provide valuable insights into the potential role of BSEs in plant–insect interactions under controlled conditions,

future studies employing intact plants in the field will be essential to confirm the ecological and agronomic relevance of these interactions.

Homobaric leaves exhibit lower hydraulic conductance, operating with reduced stomatal conductance and, consequently, lower photosynthetic assimilation rates, which affect nutrient translocation and carbohydrate production (Jones et al., 2007; Zsögön et al., 2015). Since protein synthesis in plants strongly depends on carbohydrate availability, changes in primary metabolism can alter the nutrient balance available to herbivores. Considering that herbivorous larvae require adequate carbohydrate and protein intake for growth (Simpson and Douglas 2013), such alterations may have relevant consequences.

Another aspect to consider is that nutritional requirements vary substantially among insect species; while some herbivores demand higher protein intake, others—particularly folivores—are often more limited by carbohydrate supply than by protein (Lee et al. 2008). Thus, any shift in the protein:carbohydrate ratio may have distinct implications for the performance of different herbivore groups. These nutritional constraints could partially explain the extended larval duration and reduced pupal mass observed in this study, but as we did not quantify these macronutrients, further studies are needed to confirm this hypothesis. Similar effects have been reported in other systems, where host plant nutritional quality influences insect development, pupal weight, and even diapause induction (Tauber and Tauber 1982; Rausher 1986; Mousseau and Roff 1989; Carriere et al. 1995).

The observed herbivory response may be linked to the differences in phytohormone profiles between BSE+ and BSE− plants, either due to their basal differences, or due to their herbivore induction, the latter of which was not tested in this study. Thus, considering only the basal phytohormone profile of the tested plant genotypes, BSE- plants showed a marked reduction in indoleacetic acid (AIA), possibly indicating a suppression of growth signaling pathways. This could lead to increased cell wall stiffness or other anatomical alterations affecting larval feeding (Moore 1979; Gull et al. 2023). Previous work has shown a strong connection between *OBV* and the auxin signaling pathway (Moreira et al 2022). *OBV* encodes a C2H2 Zn-finger transcription factor that may potentially interact with auxin signaling components, such as Aux-IAA and ARF proteins. Notably, downregulating or knocking out *ARF4* leads to homobaric leaves in tomato (Bouzroud et al, 2020). Additionally, increased levels of jasmonic acid (JA) in BSE- plants may enhance the induction of defense genes and

secondary metabolites that deter herbivores (Williams 1999; Borrego and Kolomiets 2016).

The elevated levels of 1-aminocyclopropane-1-carboxylate (ACC), an ethylene precursor, in BSE- plants further support the idea of hormone-mediated resistance. Ethylene often acts synergistically with jasmonates to activate the production of toxic or antinutritional compounds (Liu and Timko 2021), which could help explain the observed reduction in larval leaf consumption observed in BSE- plants. This aligns with central paradigms in nutritional ecology: when critical resources such as energy and nitrogen are limited or diluted by antinutritional compounds, organisms are forced into compensatory trade-offs (Hairston et al. 1960). Such trade-offs can reduce allocation to growth and development—particularly detrimental for leaf-mining insects like *P. absoluta* that rely on continuous nutrient uptake from mesophyll tissues (Price et al. 1980; Kidd 1994; Williams 1999).

In summary, our findings suggest that the absence of BSEs in homobaric leaves (BSE-) confers greater resistance to *P. absoluta* by reducing leaf consumption and delaying larval development. This resistance appears to stem from a combination of anatomical modifications, possible reduced nutritional quality, and enhanced defense signaling—particularly elevated JA and ACC levels, along with decreased auxin content. These results highlight the importance of internal structural features of leaves in mediating herbivory responses and support the notion that resistance is not solely driven by external or chemical defenses. Understanding these interactions opens promising venues for sustainable pest management. Incorporating resistance traits such as those linked to the *obv* mutation into commercial tomato cultivars could reduce reliance on synthetic insecticides and promote integrated strategies to manage key pests like *P. absoluta*, especially in open-field tomato production.

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Compliance with ethical standards

Conflict of interest: The authors declare that they have no known conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals unduly performed by the authors.

Consent to Participate: Not applicable.

Consent for publication: All authors are in accord with the submission.

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CHAPTER 3

Leaf mesophyll architecture does not alter chewing rhythms in larvae of the tomato pinworm (*Phthorimaea* (= *Tuta*) *absoluta*)

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Abstract

Leaf mesophyll traits influence the architecture and mechanical properties of plant tissue and are often assumed to shape plant–herbivore interactions. For example, homobaric leaves have a relatively continuous mesophyll, whereas heterobaric leaves possess vascular bundle sheath extensions that partition the mesophyll into discrete compartments, thereby increasing structural heterogeneity. Based on this contrast, we hypothesized that heterobaric leaves could impose a mechanical barrier to leafminer caterpillars, potentially raising foraging costs and causing immediate, effort-related changes in chewing behavior. To test this, we compared the feeding behavior of tomato pinworm caterpillars, *Phthorimaea* (= *Tuta*) *absoluta* (Lepidoptera: Gelechiidae), using tomato (*Solanum lycopersicum* L.) isogenic lines harboring the *obscuravenosa* (obv) mutation. These tomato isolines differ in mesophyll architecture by the absence (homobaric) or presence (heterobaric) of vascular bundle sheath extensions. Larval feeding activity was recorded using synchronized high-resolution video and laser Doppler vibrometry. The resulting vibratory output was used as a proxy for mandibular closure events, allowing quantification of fine-scale temporal and spectral features of feeding activity. Contrary to our predictions, mesophyll architecture did not appear to influence larval chewing motor patterns, as no differences were detected in event duration, dominant frequency, amplitude, or mandibular rhythm. Taken together, these results suggest that larval chewing motor control is remarkably robust and largely insensitive to this specific type of substrate variation, possibly reflecting biomechanical or behavioral adaptations that maintain feeding efficiency across structurally heterogeneous tissues. From an applied perspective, the lack of an immediate biomechanical cost indicates that management strategies that rely solely on modifying mesophyll traits are unlikely, on their own, to deter this specialized pest. These points highlight the need to integrate anatomical traits with other plant defense mechanisms for effective pest management.

Keywords: Caterpillars, Biotremology, Insect-plant interaction, Homobaric leaves, Heterobaric leaves, Feeding behavior.

Introduction

Substrate-borne vibrations constitute a fundamental sensory channel for insects, mediating inter- and intraspecific interactions within complex ecological networks (Virant-Doberlet and Cokl 2004; Strauß et al. 2021; Turchen et al. 2022; Virant-Doberlet et al. 2023). Plants, in particular, serve as solid transmission media, allowing insects and other arthropods to propagate vibrations through leaves, stems, and roots (Bell 1980; Michelsen et al. 1982; Hill 2008; Čokl 2009). At the same time, plant tissues are not mechanically uniform, and variation in structural traits is expected to influence the generation, transmission, and attenuation of vibrations, with direct consequences for organisms interacting on plant substrates (Michelsen et al. 1982; Cocroft et al. 2006; Cokl 2009; Joyce et al. 2014; Velilla et al. 2020).

Leaf internal architecture is particularly variable, differing in mesophyll organization, intercellular air space distribution, and the presence of anatomical barriers (Wright et al. 2004; Onoda et al. 2011). A particularly well-defined contrast exists between heterobaric leaves, in which vascular bundle sheath extensions subdivide the mesophyll into discrete compartments (Esau 1977; Evert 2006; Sack and Scoffoni 2013), and homobaric leaves, in which intercellular spaces are widely interconnected (Terashima 1992; Liakoura et al. 2009). These anatomical differences affect gas diffusion, leaf hydraulics, and the mechanical properties of the leaf tissue (Buckley et al. 2011; Zsögön et al. 2015; Kawai et al. 2017). From a biomechanical perspective, heterobaric leaves exhibit increased internal compartmentalization, which may locally increase tissue stiffness and modify pathways for force dissipation within the mesophyll (Liakoura et al. 2009; Barbosa et al. 2019; Palermo et al. 2023). Such features have been proposed to influence herbivore performance by modifying the mechanical context of feeding (Clissold 2007).

Indeed, the absence of vascular bundle sheath extensions has been shown to modify leaf consumption rates and larval development in the tomato pinworm, *Phthorimaea* (= *Tuta*) *absoluta* (Lepidoptera: Gelechiidae) (Souza et al. 2026), indicating that homobaric mesophyll architecture can influence herbivore performance. However, these effects reflect integrated outcomes of feeding and do not necessarily indicate that the fundamental mechanics of chewing are directly altered. Performance differences could instead arise from cumulative energetic costs, altered tissue accessibility, or downstream physiological constraints rather than from immediate

changes in mandibular motor output. As a result, whether these internal anatomical differences are sufficient to elicit immediate, effort-related adjustments in chewing behavior remains unresolved, particularly for leaf-mining caterpillars that feed entirely within the mesophyll.

Chewing is a highly conserved, rhythmic motor behavior that serves as the primary mechanical interface between caterpillars and their food, providing a direct pathway through which internal leaf structure might influence insect chewing behavior (Wagner and Hoyt 2022). In caterpillars, chewing consists of rhythmic mandibular movements that generate substrate-borne vibrations encoding both motor output and the mechanical resistance of the food (Appel and Cocroft 2014; Body et al. 2019; Kollasch et al. 2020). The temporal and spectral properties of these vibrations, such as duration, frequency, amplitude, and regularity, often vary with substrate stiffness and internal architecture and may reflect compensatory adjustments in feeding dynamics (Gaston et al. 1991; Reavey 1993; Clissold 2007). In this sense, chewing-generated vibrations provide a sensitive and immediate readout of how insects respond to fine-scale mechanical heterogeneity within their feeding substrate.

Within this context, vibrational analysis offers a powerful and non-invasive approach to link leaf biomechanics with insect feeding behavior (Clissold 2007; Body et al. 2019; Kollasch et al. 2020; Velilla et al. 2020). By quantifying temporal, spectral, and amplitude characteristics of feeding-induced vibrations, it is possible to assess whether mesophyll heterogeneity is sufficient to perturb chewing motor behavior or whether larvae maintain stable chewing dynamics despite structural variation. Despite this potential, this approach remains underutilized in studies of internal herbivory, such as that of the tomato pinworm, a highly specialized leaf-mining species whose larvae spend most of their life cycle feeding within the mesophyll (Desneux et al. 2011; Biondi et al. 2018).

Here, we tested whether variation in mesophyll architecture between homobaric and heterobaric tomato leaves imposes immediate biomechanical constraints on the chewing motor behavior of tomato pinworm larvae. We predicted that heterobaric compartmentalization, by increasing structural complexity and local stiffness, would be likely to alter the vibrational signatures of mastication, reflecting increased biomechanical costs and trade-offs in feeding dynamics. To test this prediction, we combined genetically controlled variation in leaf anatomy with high-resolution behavioral observations and laser vibrometry to compare larval feeding on homobaric

and heterobaric tomato leaves. More specifically, we evaluated whether chewing rhythms respond to internal structural compartmentalization or remain invariant, pointing to robust motor control. By integrating leaf anatomy, feeding behavior, and substrate-borne vibrations, this study helps fill a conceptual gap between behavioral ecology and the biomechanics of herbivory in plant tissues. More broadly, it highlights the utility of vibroacoustics analysis as a non-invasive tool for studies of insect–plant interactions and provides new insight into the robustness of chewing motor control in leaf-mining insects facing structural variation in their feeding substrate.

Material and Methods

Plants

Tomato plants (cv. M82), harboring either the wild-type allele of *obscuravenosa* (heterobaric, BSE+) or the loss-of-function allele *obv* (homobaric, BSE−), previously characterized by Barbosa et al. (2019), were cultivated under semi-controlled greenhouse conditions and irrigated using an automated drip system, following the protocol described by Souza et al. (2026). Seeds were sown individually in vermiculite-filled cells and, after emergence of the first true leaf, seedlings were transplanted into 5L pots containing commercial substrate (Plantmax HT, Eucatex, Brazil) supplemented with 8 $g.L^{-1}$ of NPK fertilizer (4:14:8) and 4 $g.L^{-1}$ of dolomitic limestone ($MgCO_3$ + $CaCO_3$). Additional supplementation was provided as needed.

At the vegetative growth phenological stage (or when leaves reached 5 cm in length), individual leaflets from each genotype (BSE+ and BSE−) were excised and placed in Petri dishes with their petioles embedded in a water–agar solution for experimental use. Excised leaves were used instead of intact plants due to limited availability and uniformity of tomato genotypes, with priority given to standardized, high-quality plant material. Because BSE is a structural morphological trait, it is considered relatively less susceptible to physiological changes associated with leaf excision.

Insect rearing

The initial insect population consisted of adults of the tomato pinworm (*P. absoluta*), collected from tomato fields in Coimbra county (MG, Brazil). Adults were maintained in cages containing tomato plants (cv. Santa Clara – IC11 5500) for

oviposition and were supplied with a 10% glucose solution. Female moths were allowed to lay eggs on ‘Santa Clara’ leaves for 24 h. Eggs (< 24 h old) were individually transferred to leaflets of each tomato genotype (BSE+ and BSE−) using a fine paintbrush.

Petri dishes containing the infested leaflets were maintained in a controlled-environment chamber (Percival I30VLC8, Percival Scientific, Inc., Perry, IA, USA) at 25 ± 1°C, 65 ± 5% relative humidity, and a 12:12h (light: dark) photoperiod. Egg hatching and larval development were monitored daily, following the protocol described by Souza et al. (2026). When larvae reached the third instar, they were used in experiments to record feeding activity.

Vibration and video recording set up

Leaf vibrations and insect behavior were recorded simultaneously using a laser Doppler vibrometer (PVD-100; Polytec, Waldbronn, Germany) and a high-resolution camcorder (HDR-CX455; Sony, Tokyo, Japan). Petri dishes containing a single tomato leaflet with one third-instar larva were placed on an LED ceiling panel mounted on an anti-vibration table (63-500 Series Micro-g; TMC, Peabody, MA, USA). The laser vibrometer was configured with a velocity range of 20 $mm.s^{-1}$ (5 $mm.s^{-1}$. V^{-1}), with the high-pass filter disabled and the low-pass filter set to 22kHz.

The laser beam was positioned perpendicular to a reflective tape (6 mm diameter) affixed to the adaxial leaf surface, approximately 5 mm from the larval mine. The analog output of the vibrometer was routed to the input of an audio interface (Focusrite Scarlett Solo 4thGen, High Wycombe, England), which was connected via USB-C to a laptop computer (MacBook Air M1; Apple Inc.) for audio recording in WAV format using Audacity® (v. 3.7.7) (Audacity Team 2025). Recordings were acquired at a sampling rate of 48kHz with 24-bit resolution. To synchronize audio and video, the audio interface output was connected to the camcorder's microphone input. Video recordings were acquired at 1920 × 1080 pixels at 30 frames s^{-1}, with an audio sampling rate of 48 kHz and 24-bit resolution. A total of 20 leaves were recorded, with 10 replicates per leaf type. Each replica was recorded for 20 minutes.

Vibroacoustic analysis of feeding behavior

Larval feeding behavior was investigated using synchronized video and audio recordings, allowing a direct association between mandibular movements and the

vibratory cues observed during feeding. Video recordings were analyzed at reduced playback speed and compared with the corresponding acoustic waveforms in Audacity®, enabling accurate identification and temporal scoring of feeding activity. Events were operationally defined and validated through video-audio synchronization.

Feeding periods were defined as continuous temporal intervals comprising groups of vibratory events associated with feeding behavior, separated from adjacent feeding periods by intervals of ≥10 s without detectable vibratory activity. Each feeding period began with the onset of the first vibratory event and ended with the final event preceding a ≥10 s inactive interval. This threshold was established based on preliminary observations indicating that shorter inactive intervals reflected larval body repositioning rather than true interruptions of feeding. Feeding periods could include multiple chewing events. A chewing event was defined as a sequence of ≥3 consecutive vibratory peaks within a feeding period, visually corresponding to continuous mandibular opening and closing movements, usually separated by brief pauses of approximately 1s.

A total of 6.6 h of recordings from 20 larvae (10 per substrate type) were analyzed. For each audio–video recording, all feeding periods and chewing events were annotated. Temporal, spectral, and amplitude parameters associated with feeding behavior were quantified using Audacity® software by measuring: (i) duration of feeding periods, defined as the continuous interval during which larvae actively engaged in leaf tissue excision; (ii) interval between consecutive feeding periods, the time elapsed between the end of one feeding period and the onset of the next; (iii) number of chewing events per feeding period, determined by counting chewing events within each feeding period; (iv) duration of individual chewing events; (v) interval between consecutive chewing events; (vi) number of peaks per chewing event, corresponding to the total number of vibration peaks detected in the substrate during a single chewing event; (vii) inter-peak interval (or mandibular rhythm) within chewing events; (viii) dominant frequency of chewing event, extracted from the spectral component with the highest power using a Fast Fourier Transform (sampling rate 48 kHz, Hanning window, 1024-point resolution, 50% overlap); and (ix) maximum relative amplitude, defined as the peak amplitude of the substrate-borne vibration during each chewing event.

Statistical analyses

To test whether mesophyll architecture (homobaric vs. heterobaric) influenced pinworm feeding behavior, larval chewing rhythms were compared between leaf types using generalized linear mixed models (GLMMs). Leaf type was included as a fixed effect, and audio file identity was treated as a random effect to account for repeated measurements per individual. Continuous variables related to feeding period or chewing events were modeled assuming Gaussian or Gamma distributions, whereas count variables were modeled with a Poisson distribution. The final choice of distribution for each variable was performed based on inspection of model residuals for normality and homoscedasticity using the *DHARMa* package.

Effects of leaf type were assessed via deviance analyses, and estimated marginal means were calculated using the *emmeans* package. All analyses were performed in R (version 4.4.1; https://www.R-project.org/) using the RStudio interface (version 2025.09.2+418; http://www.rstudio.com/).

Results

Larval activity of the tomato pinworm within the leaf mesophyll, such as locomotion, feeding, and postural adjustments, can generate a variety of mechanical disturbances in the leaf substrate. In this study, however, we restricted our analyses to substrate-borne vibrations produced during feeding and explicitly excluded vibratory cues associated with non-feeding movements. This restriction is justified because caterpillar feeding reliably generates vibrations arising primarily from rhythmic mandibular movements during plant tissue excision (Clissold 2007; Kollasch et al. 2020). Because these vibrations originate directly from the physical interaction between the mandible-plant contact, they provide a direct and reliable mechanistic proxy for chewing activity.

To contextualize and illustrate the source of these vibrations, we briefly describe the external morphology of third-instar larvae, focusing on the head capsule and mandibular apparatus that are directly involved in vibration production (Figure 1A–H; Video S1). Overall, third-instar larvae exhibit the elongated and segmented body typical of actively feeding lepidopteran larvae (Figure 1A, D). The cephalic capsule contains robust, heavily sclerotized mandibles, whose articulation allows rapid, repetitive opening and closing against the leaf substrate (Figure 1B-G). These

mandibular movements, together with coordinated contractions of head and anterior thoracic musculature, generate well-defined mechanical impulses that propagate through the leaf tissue as substrate-borne vibrations (Figure 1H; Video S1).

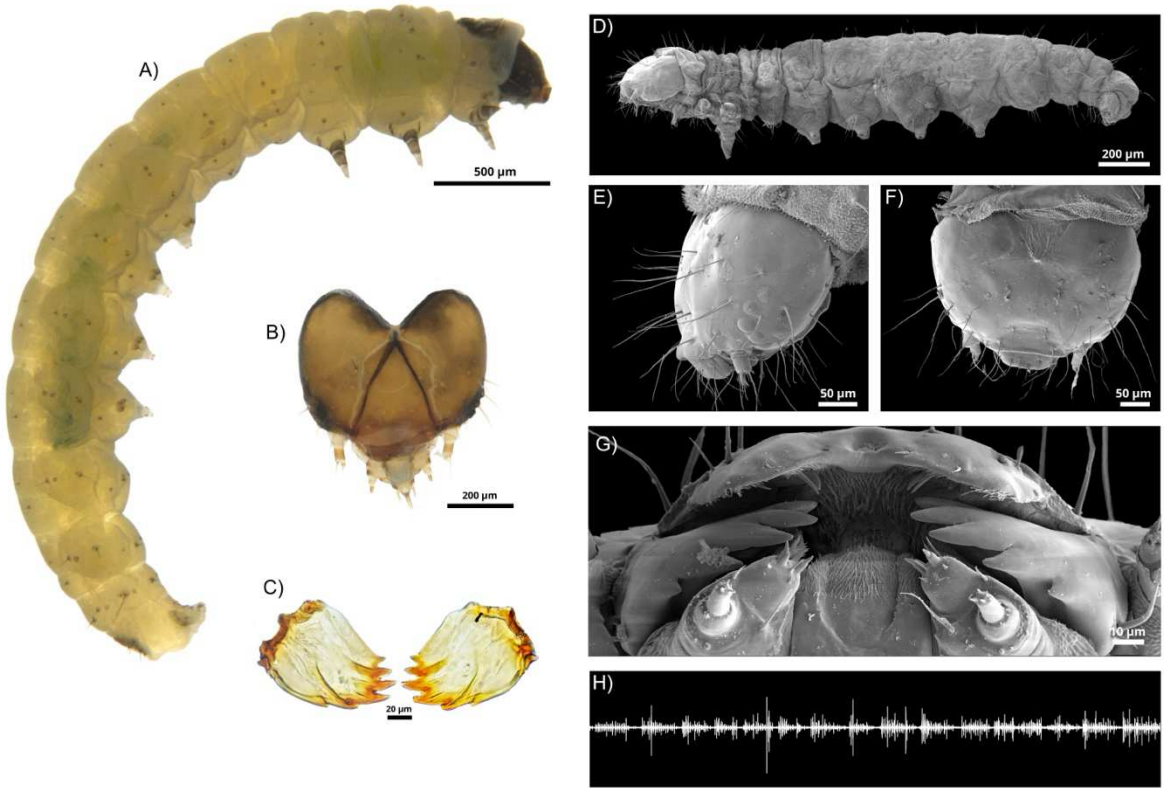

Figure 1. External morphology and feeding-related vibratory signal of third-instar tomato pinworm larvae, *Phthorimaea absoluta*. (A) Whole larva in lateral view, showing the segmented body and thoracic appendages. (B) Isolated head capsule in frontal view. (C) Paired mandibles dissected from the head capsule. (D) Field Emission Gun Scanning Electron Micrograph (FEG-SEM) of the larva in lateral view. (E, F) FEG-SEMs of the head capsule in lateral (E) and frontal (F) views, showing surface sensilla and setae. (G) High-magnification FEG-SEM of the anterior head region, highlighting mouthpart articulation and fine cuticular structures. (H) Representative oscillogram of feeding-associated vibratory cues recorded during larval chewing. Scale bars are indicated in each panel.

Feeding-generated vibratory cues were characterized and compared between heterobaric (BSE+) and homobaric (BSE−) tomato leaves to test whether chewing-related vibratory patterns vary with leaf mesophyll architecture. Feeding occurred in prolonged periods of continuous vibratory output separated by intervals of inactivity (see Figure 2A). No detectable effect of leaf type was observed on the temporal organization of feeding behavior. Feeding period duration did not differ between substrates (χ^2= 0.68, *P*= 0.41; Figure 3A), averaging 220 ± 57s in BSE+ leaves and 162 ± 43s in BSE- leaves. Similarly, the intervals between successive feeding periods

did not differ between substrates (χ^2= 1.51, *P*= 0.22; Figure 3B), with average intervals of 69.2 ± 21.9s in BSE+ leaves and 120.9 ± 37.9s in BSE- leaves.

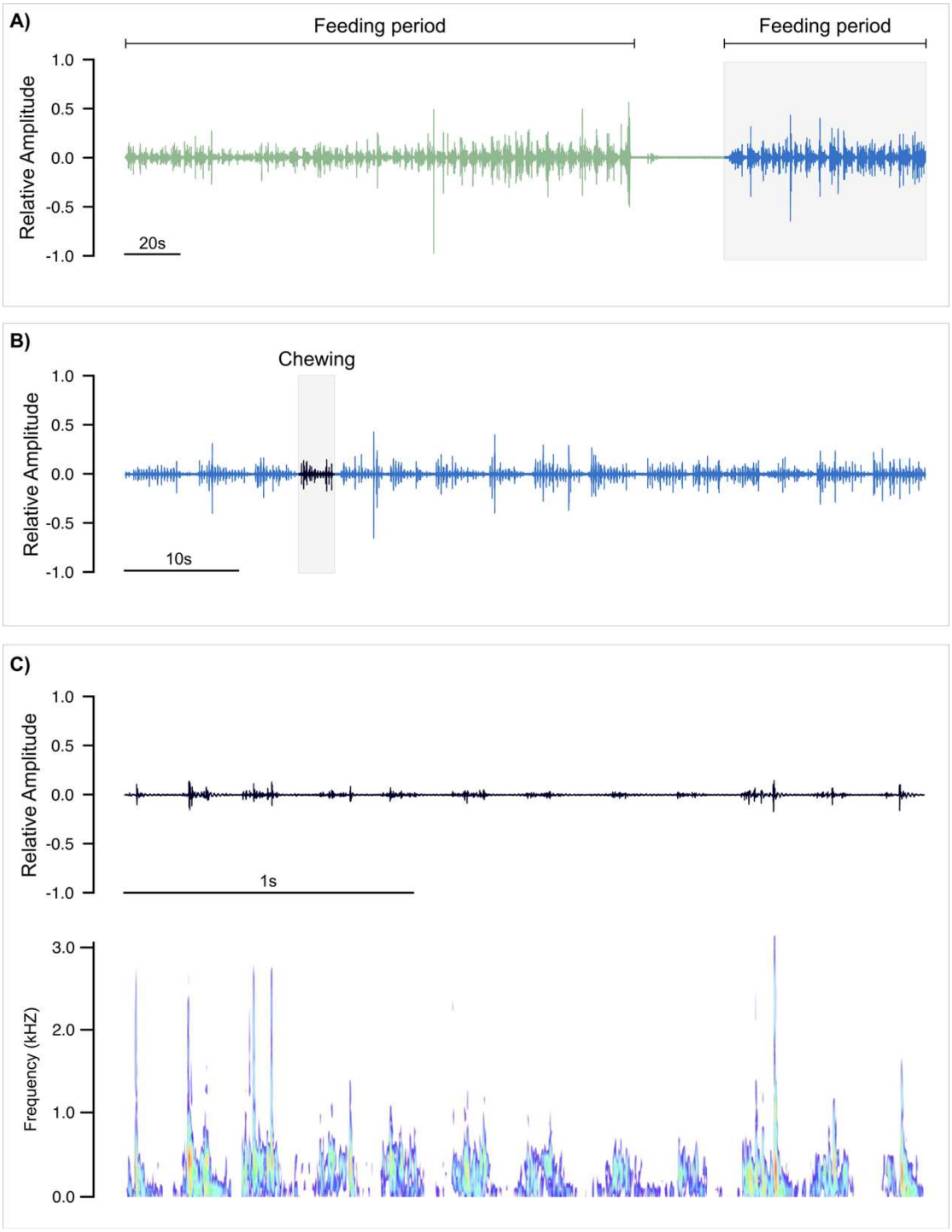

Figure 2. Vibratory cues associated with larval feeding behavior. (A) Representative oscillogram showing substrate-borne vibrations recorded during two complete feeding periods separated by a non-feeding interval. The grey shaded region highlights one feeding period. (B) Expanded oscillogram of a single feeding period, showing multiple chewing events; the shaded region highlights one chewing event. (C) Further expansion of an individual chewing event, showing multiple vibratory peaks associated with mandibular movements. The upper panel shows a high-resolution oscillogram, and the lower panel shows the corresponding spectrogram, illustrating the vibration's temporal structure and

dominant frequency components. Relative amplitude is normalized across panels. Time scales are indicated within each panel.

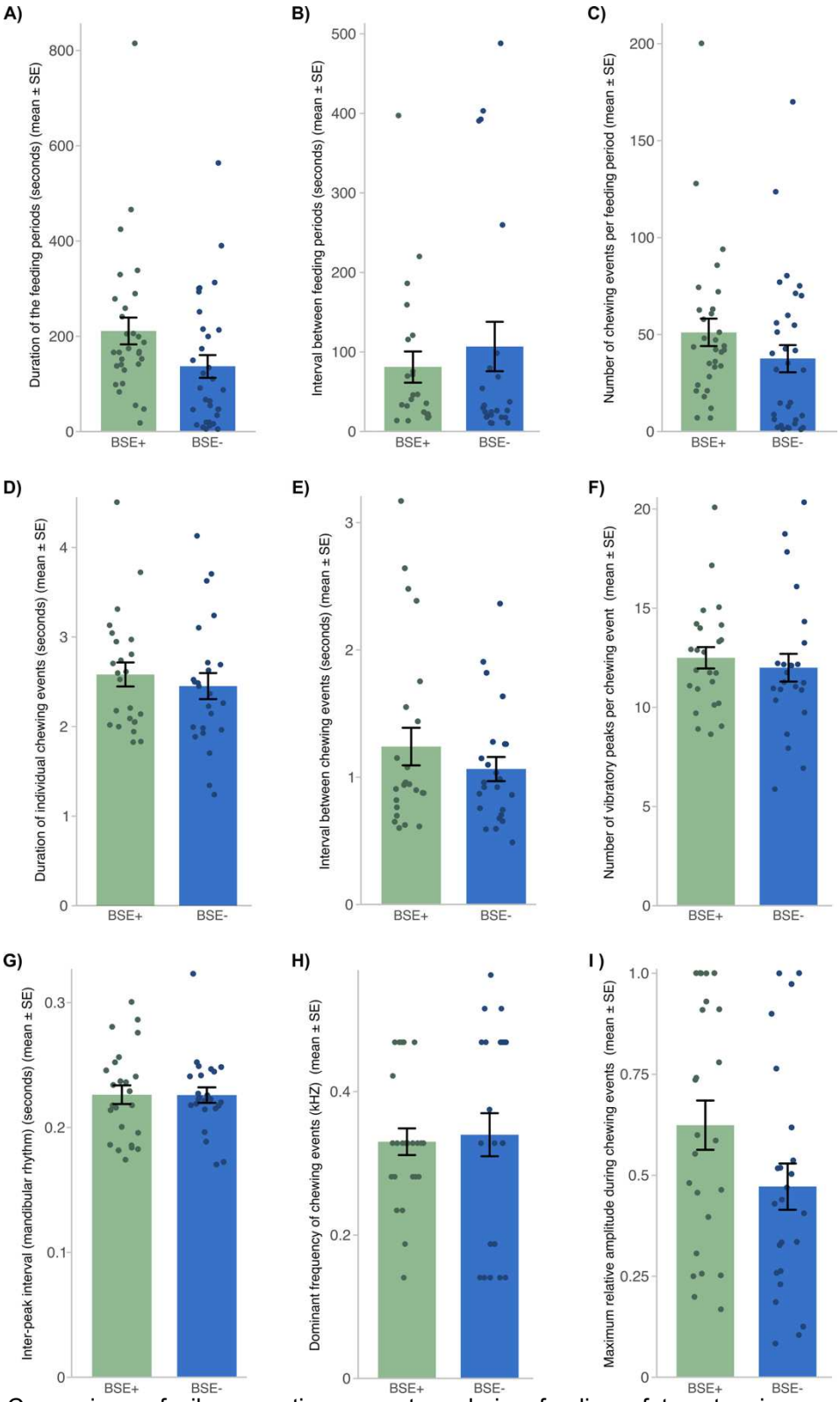

Figure 3. Comparison of vibroacoustic parameters during feeding of tomato pinworm larvae on heterobaric (BSE+) and homobaric (BSE−) tomato leaves. (A) Duration of feeding periods. (B) Interval between consecutive feeding periods. (C) Number of chewing events per feeding period. (D) Duration of individual chewing events. (E) Interval between consecutive chewing events. (F) Number of vibratory peaks per chewing event. (G) Inter-peak interval (mandibular rhythm) within chewing events. (H)

Dominant frequency of chewing events. (I) Maximum relative amplitude during chewing events. Bars represent mean values for each leaf type, vertical lines indicate the standard error, and points correspond to individual observations. No statistically significant differences were detected between treatments using deviance analysis ($P > 0.05$).

Within feeding periods, sequences of at least three consecutive vibratory pulses, corresponding to continuous mandibular opening and closing movements separated by brief pauses, were defined as chewing events (Figure 2B). Individual pulses were short in duration, occurred at regular inter-peak intervals, and showed relatively consistent amplitudes within chewing events (Figure 2C). The number of chewing events per feeding period did not differ between leaf type ($\chi^2 = 0.23$, $P = 0.64$; Figure 3C), averaging 52.8 ± 12.7 events in BSE+ leaves and 45.1 ± 10.8 events in BSE- leaves. Taken together, these results indicate that the overall feeding activity, as captured by chewing event frequency, does not depend on mesophyll architecture.

Leaf architecture also appeared not to influence the structure of individual chewing events. Chewing event duration was similar across substrates ($\chi^2 = 0.28$, $P = 0.60$; Figure 3D), averaging 2.63 ± 0.31s in BSE+ leaves and 2.41 ± 0.28 s in BSE- leaves. Similarly, the intervals between successive chewing events did not differ between substrates ($\chi^2 = 0.05$, $P = 0.81$; Figure 3E), with average intervals of 1.10 ± 0.19s in BSE+ leaves and 1.04 ± 0.19s in BSE- leaves. The number of vibratory peaks per chewing event was likewise similar between leaf types ($\chi^2 = 1.06$, $P = 0.30$; Figure 3F), with an average of 13.1 ± 0.81 peaks in BSE+ leaves and 11.9 ± 0.76 peaks in BSE- leaves. Overall, these consistent chewing patterns indicate that the fine-scale structure of mandibular activity remains stable across leaf mesophyll architectures, implying a conserved motor program for feeding.

Mandibular rhythm, quantified as the mean inter-peak interval within chewing events, did not differ between leaf types ($\chi^2 = 0.04$, $P = 0.83$; Figure 3G), with an average inter-peak interval of 0.22 ± 0.01s in BSE+ leaves and 0.23 ± 0.01s in BSE- leaves. Spectral and amplitude properties of feeding-generated vibrations were likewise unaffected by substrate type. Dominant frequency averaged 0.31 ± 0.06 kHz in BSE+ leaves and 0.30 ± 0.06 kHz in BSE− leaves and did not differ between leaf types ($\chi^2 = 0.01$, $P = 0.90$; Figures 2C; 3H). Vibration intensity, measured as maximum relative amplitude, also did not differ between substrates ($\chi^2 = 0.59$, $P = 0.44$; Figure 3I); with an average of maxima relative amplitude of 0.56 ± 0.10 in BSE+ leaves and 0.43 ± 0.10 in BSE- leaves. Collectively, these results indicate that the temporal and spectral

structure of chewing-related vibratory output is invariant with respect to mesophyll architecture.

Discussion

Our results demonstrate, somewhat unexpectedly, that the differences between the heterobaric and homobaric architecture of the leaf mesophyll, while previously shown to modulate other ecological interactions in this system (Souza et al. 2026), did not impose measurable adjustments on the short-duration vibratory patterns generated during larval chewing of the tomato pinworm. The absence of significant differences in parameters such as chewing event duration, dominant frequency, and mandibular rhythm between heterobaric and homobaric leaves suggests that the motor control of feeding, at the scale of individual chewing events, is robust to this specific variation in substrate architecture.

This robustness can be interpreted as a high degree of behavioral canalization (Waddington 1942) in a critical life-sustaining activity such as feeding in a specialist leafminer insect. Once a chewing event begins, mandibular rhythmic patterns appear to operate in a relatively fixed manner, possibly optimized for maximum efficiency in fragmenting parenchymatous tissue, regardless of subtle cellular barriers. This supports the idea that, for endophytic (“indoor”) herbivores, the mechanical predictability of the intrafoliar environment may have selected for stereotyped motor programs with low immediate behavioral plasticity (Clissold 2007). The mandibular rhythm remained highly stable, suggesting that the basic motor pattern of chewing is tightly regulated and relatively insensitive to local variations in substrate resistance. This stability is consistent with the idea that mandibular rhythmic patterns are generated by relatively conserved central neural circuits, with limited immediate peripheral modulation (Marder and Bucher 2001; Clissold 2007; Hückesfeld et al. 2015). Nevertheless, inferences about neural control or behavioral canalization based solely on vibrational outputs remain speculative and should be treated as hypotheses for future investigation.

Another relevant aspect is that the vibratory cues analyzed in this study are mechanical byproducts of feeding, rather than evolutionarily shaped communication signals. Therefore, they directly reflect the physical interaction between insect and substrate, with minimal scope for intentional behavioral modulation. In this context, the

similarity of vibrational signatures across leaf types therefore reinforces the interpretation that the mechanical properties actually encountered by larvae during chewing are comparable between heterobaric and homobaric leaves, at least within the mined regions explored.

The initial hypothesis that mesophyll compartmentalization via vascular bundle sheath extensions would function as a mechanical barrier imposing higher biomechanical costs was not supported by the vibrational data. This outcome suggests several non-mutually exclusive explanations. The additional stiffness associated with bundle sheath extensions may be negligible relative to the shear forces exerted by larval mandibles, or any localized increase in resistance may be compensated for by physiological or biomechanical adjustments during tissue processing. Alternatively, the effects of mesophyll architecture on larval performance may operate primarily through physiological or nutritional pathways rather than through acute mechanical constraints, as previously suggested by Souza et al. (2026).

These findings contrast with studies demonstrating clear mandibular adjustments in surface-chewing herbivores in response to coarse hardness gradients (Bowdan 1995). This discrepancy highlights the fundamentally different scales at which mechanical perception operates in leaf miners versus external feeders. Whereas surface-feeding herbivores interact with integrated mechanical properties of the leaf blade, leaf miners engage with a comparatively homogeneous cellular matrix at the micrometer scale. At this scale, compartmentalization by vascular bundle sheath extensions may not represent a sufficiently discrete or abrupt obstacle to elicit acute motor adjustments.

Despite the apparent insensitivity at the level of individual chewing events, it is important to emphasize that our study does not exclude effects operating over broader temporal scales. Larval adaptability and feed conversion efficiency, integrated over hours or days, may still be impacted by leaf architecture through mechanisms not detected by our high-resolution vibrational analysis, such as changes in time allocation between feeding and resting, or in the total energy cost of tissue processing (Trier and Mattson 2003).

From an applied perspective, the resilience of the chewing motor pattern suggests that resistance based solely on altering the mesophyll architecture are unlikely to impose immediate behavioral costs sufficient to deter pest species. However, management approaches that combine structural traits with chemical or

induced defenses may prove more effective, particularly if they exploit trade-offs between plant responses to mechanical damage and biotic stress (Karban and Baldwin 2007; War et al. 2012; Mitchell et al. 2016).

In conclusion, this work demonstrates that the tomato pinworm's specialization as a leafminer includes a robust feeding motor program that is insensitive to variation in mesophyll microarchitecture. Using direct vibrational measurements, our study provides the first empirical evidence for decoupling leaf anatomical structure from immediate mandibular kinematics in this system. Future studies should examine whether this robustness persists under combined mechanical and chemical stressors and integrate direct measures of larval energy expenditure to resolve the longer-term costs imposed by plant tissue architecture - costs that may not be evident from vibrational signatures alone

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Statements and Declarations

Author contributions
D.S.S. contributed to conceptualization, data curation, formal analysis, investigation, methodology, software, validation, visualization, and writing (original draft). **A.Z.** contributed to conceptualization, methodology, funding acquisition, resources, supervision, and writing (review and editing). **R.N.C.G.** contributed to conceptualization, funding acquisition, project administration, resources, supervision, and writing (review and editing). **L.M.T.** contributed to conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, software, resources, validation, visualization, and writing (original draft; review and editing).

Conflict of interest statement
The authors declare no conflicts of interest.

Data availability statement
The data supporting the findings of this study are available within the article and its supplementary material.

Ethics statement

All applicable international, national, and institutional guidelines for the care and use of animals were considered in the present investigation.

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GENERAL CONCLUSIONS

This thesis clarifies the still little-explored role of internal morpho-anatomical leaf characteristics as key modulators of insect-plant interactions. We conclude that the literature has long prioritized external barriers, largely neglecting internal leaf anatomy. The absence of vascular bundle sheath extensions proved to be a determining factor in the resistance of tomato plants to the tomato leafminer (*Phthorimaea absoluta*). The differences in architecture between heterobaric and homobaric leaves, associated with the presence of these extensions, do not impose immediate and measurable biomechanical restrictions on larval feeding nor alter the motor pattern of chewing; thus, the reduced performance of the larvae cannot be attributed to direct mechanical inhibition, but probably to post-ingestive factors. In general, the results position the internal anatomy of the leaves as an active component of plant defense, capable of modulating plant physiology and host quality. Finally, we conclude that biotremology constitutes a powerful approach to investigating insect-plant interactions, offering a new way to understand the costs and trade-offs that shape these evolutionary relationships.