Czech University of Life Sciences in Prague

Faculty of Forestry and Wood Sciences

Department of Forest Genetics and Physiology

Transcriptional response and changes on the level of the secondary metabolites of the poplar after the attack by insect herbivores

Dissertation

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CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Forestry and Wood Sciences

Ph.D. THESIS ASSIGNMENT

Ing. Filip Pastierovič

Forestry Engineering

Forest Biology

Thesis title

Transcriptional response and changes on the level of the secondary metabolites of the poplar after the attack by insect herbivores

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Objectives of thesis

1. The strategic goal is to establish the quantification of the stress response to insect herbivore attack (analysis of the composition and quality, primarily of secondary metabolites).
2. Optimization of methodological procedures/experimental design to integrate physiological measurement techniques, chemical profile analysis of metabolites, and molecular-level responses (gene expression) in cultivated aspen poplar saplings.
3. Transcriptome analysis – description and identification of transcriptional changes in response to insect herbivory, for which metabolic pathways will be identified based on a literature review.
4. Identification and description of unique secondary metabolites specific to the given type of insect herbivory.
5. Validation and integration of generated data from the described approaches within the framework of a manipulative experiment.

Methodology

The study will be carried out as a manipulative experiment. Genetically uniform aspen poplar saplings (Populus tremula), grown in vitro, will be cultivated in growth chambers with controlled environmental conditions, specifically in the ”Walk-in FytoScope. Three sets of individuals will be established from the cultivated poplar saplings: attacked by the gypsy moth (Lymantria dispar); attacked by aphids (Chaitophorus populialbae); and without insect attack (reference set). Twenty saplings will be cultivated in breeding boxes alongside the corresponding insect species. The net material will be selected to ensure at least 90% light penetration without altering its spectral composition, while also preventing the insects from spreading throughout the entire growth chamber. The following instruments will be used to generate physiological data:

- LiCOR 6400XT gas exchange system;
- HACH 6000 spectrophotometer – quantitative evaluation of amino acids (proline);
- HPLC (high-performance liquid chromatography) – analysis of secondary metabolites;

• GC-MS (gas chromatography–mass spectrometry) – analysis of volatile organic compounds;

• transcriptome sequencing – determining differences in gene expression among the three sets of individuals.

The proposed extent of the thesis

100 SP

Keywords

plant-insect interactions; herbivory; transcriptomics; plant defense response; secondary metabolites; plant metabolomics; plant physiology; biotic stress

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Recommended information sources

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Expected date

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The Dissertation Thesis Supervisor

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Supervising department

Katedra genetiky a fyziologie lesních dřevin

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Electronic approval: 31. 08. 2022

prof. Ing. Róbert Marušák, PhD.

Dean

Prague on 01. 10. 2024

Declaration of Honor

With this text, I confirm that this Dissertation thesis "**Transcriptional response and changes on the level of the secondary metabolites of the poplar after the attack by insect herbivores**" was elaborated independently with the use of quoted literature and consultations with my supervisor. I agree with publishing this Dissertation thesis according to Czech law No. 111/1998 Sb. about the universities in its current valid wording. This agreement is independent from the result of the official defense of this thesis.

Prague, 30th September 2024 Ing. Filip Pastierovič

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Abstract

The dissertation focuses on describing the interactive effects on the target species: European aspen (*Populus tremula*) in response to selected insect herbivores: spongy moth (*Lymantria dispar*) and aphids *Chaitophorus populialbae* and *Chaitophorus nassonowi*. Plant tissue was analyzed in the context of three experiments, in which genetically uniform individuals of aspen were grown in growth chambers. Optimal setting of environmental variables allowed for precise isolation of the effects of insect herbivores. Changes in gene expression, phenolics, chlorophyll and proline content, and volatile compounds, in damaged and control leaves of aspen were analyzed. The results reveal distinct metabolic and physiological responses to **sucking** and **chewing** insects, with aphid-infested leaves showing approximately half the chlorophyll content and double the proline amount compared to uninfested leaves, indicating a stronger physiological impact compared to spongy moth herbivory. Phenolic analyses showed that catechin and procyanidin B1 were significantly increased within the first five minutes of spongy moth feeding, followed by a shift to a tolerant strategy, with concentrations returning to control levels after ten minutes. Specific volatile compounds were identified, such as 3-hexenal and 5-methyl-2-furanone for aphids, and trans-α-farnesene for spongy moths, indicating species-specific metabolic patterns. Additionally, saccharide utilization differed, with increased levels in leaves but decreased levels in roots under spongy moth attack, highlighting different resource management strategies. Using multi-omics approaches, significant changes in gene expression were revealed, with 1203 transcripts being differentially regulated after aphid infestation and 272 transcripts after spongy moth infestation. Notably, 5716 transcripts were differentially regulated between aphid and moth infestation, emphasizing the specificity of aspen's defense strategies. Defense-related hormones, transcription factors, and signaling molecules were upregulated, while growth-related genes were downregulated, indicating a prioritization of defense overgrowth. Approximately 37 % of metabolites were associated with growth and defense pathways, further confirming the finely tuned responses of *P. tremula* to different herbivores. Our findings document species-specific fine-tuning of *P. tremula* defense response, showing that the plant maintains resource allocation for defense at the expense of growth.

Key words: metabolomics, plant-insect interactions, plant physiology, transcriptomics

Table of Contents

List of original publications

I. **Pastierovič, F.**; Kalyniukova, A.; Hradecký, J.; Dvořák, O.; Vítámvás, J.; Mogilicherla, K.; Tomášková, I. Biochemical Responses in *Populus tremula*: Defending against Sucking and Leaf-Chewing Insect Herbivores. *Plants* **2024**, *13*, 1243. https://doi.org/10.3390/plants13091243

II. **Pastierovič, F.**; Mogilicherla, K.; Hradecký, J.; Kalyniukova, A.; Dvořák, O.; Roy, A.; Tomášková, I. Genome-Wide Transcriptomic and Metabolomic Analyses Unveiling the Defence Mechanisms of *Populus tremula* against Sucking and Chewing Insect Herbivores. *Int. J. Mol. Sci.* **2024**, *25*, 6124. https://doi.org/10.3390/ijms25116124

III. **Pastierovič, F.**; Čepl, J.; Kalyniukova, A.; Mogilicherla, K.; Hradecký, J.; Bláha, J.; Tomášková, I. Time is of the essence: unveiling the rapid response of *Populus* to insect feeding. *Frontiers in Forests and Global Change*, **2024**, *7*, 1376465. https://doi.org/10.3389/ffgc.2024.1376465

Caterpillars (*Lymantria dispar*) feeding during an experiment on genetically uniform european aspen (*Populus tremula*) in a growth chamber (Pastierovič et al., 2024).

List of figures and tables

Conceptual Framework of Introduction part

The mechanisms of plant metabolic response to insect herbivory are highly diverse, and a detailed review would exceed the scope of this dissertation. Therefore, the conceptual design of the work is structured so that the first part of the introduction addresses the issue of plant-insect interactions, highlighting key aspects on the insect side that significantly influence plant responses.

The subsequent section of the introduction focuses on the processes occurring on the plant side, describing the individual steps in the plant defense cascade. For better clarity, these processes are presented in a chronological and mechanistic arrangement—from herbivore attack detection to the induced production of secondary metabolites.

1 Introduction

1.1 Paleontological Overview of Plant-Insect Herbivore Interactions

The study of plant defense against herbivores is a cornerstone of ecology and evolution, grounded in the theory of coevolution (Ehrlich and Raven, 1964). From a paleontological perspective, the origins of interactions between the taxonomic kingdoms of plants (Plantae) and animals (Animalia), specifically the phylum Arthropoda, can be traced back to the Early Devonian period, as evidenced by studies from Labandeira (2007, 2013) and Fürstenberg-Hägg (2013) (**Figure 1**). The Early Devonian period, which spans approximately 397 to 407 million years ago (Gerrienne et al., 2011), saw the rise of macroscopic organisms with differentiated tissues, such as lichens, bryophytes, primitive vascular plants, and fungi. This development was crucial for the establishment of terrestrial ecosystems, which became trophically organized in the presence of arthropods (Shear and Selden, 2001; Knor et al., 2012). These ecosystems featured primary producers alongside saprophages (arthropods and fungi) responsible for the biotic degradation of tissues. Crawley (1983) and Schowalter (2000) agree that the proportion of saprophagous feeding strategies in these early ecosystems was significantly lower than the proportion of insect herbivory.

The close ecological relationship between arthropods and plants over approximately 407 million years of evolutionary history is reflected in the ratio of described herbivorous insect species to described plant species. Bánki et al., (2024) records 385,753 plant species and 994,284 insect species in the Catalogue of Life (2024) database. Wu and Baldwin (2010) report that approximately 500,000 insect species (about 50 % of all insect species) are directly linked to plants through their feeding strategies. Insect herbivores can influence ecological and evolutionary changes in plant populations in real-time. Studies indicate that plants rapidly develop traits that confer resistance to herbivores when they are present, and conversely, traits that enhance competitive ability when herbivores are absent (Agrawal et al., 2012; Hare, 2012; Züst et al., 2012). While phytophagous insects adapt to exploit their host plants, the plants simultaneously evolve defensive mechanisms to counter herbivore attacks (Anderson and Mitchell-Olds, 2011; Johnson, 2011). Based on these findings, herbivorous insect species are considered a dominant evolutionary force that has directly shaped the development of life on Earth. The extraordinary diversification of herbivorous insects is often attributed to their close

interactive relationships, particularly with angiosperms (Mitter et al., 1988; Farrell, 1998; Marvaldi et al., 2002).

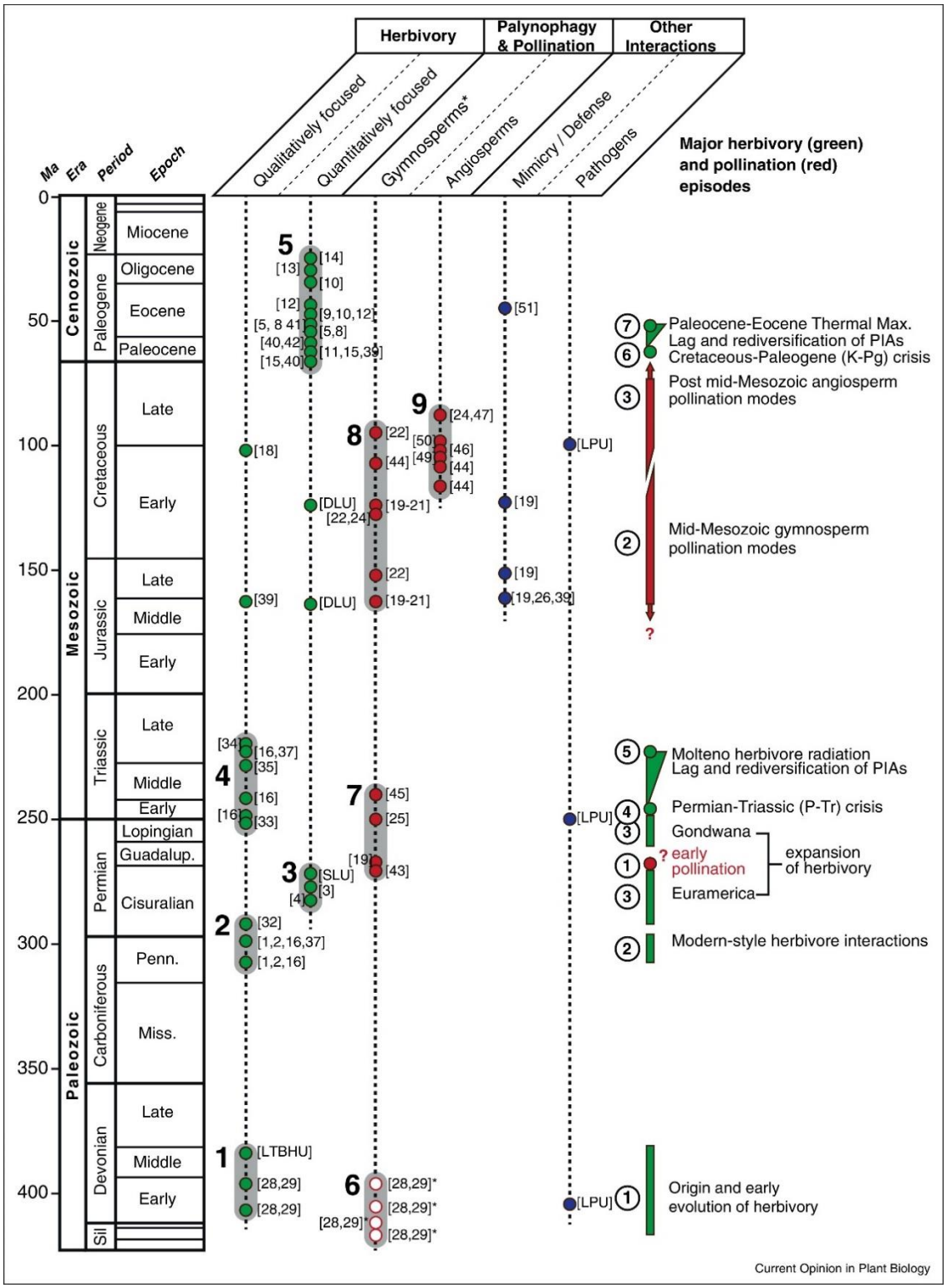

Figure 1 Geochronological distribution of fossil plant-insect association, and the timing of significant episodes of plant-insect associations. Herbivory is marked in green, pollination in red, and other associations in black (adapted from Labandeira, 2013).

1.2 Plant-Insect Interactions

The dynamic interaction between insects and plants provides insects with food, a niche, shelter, and other conditions necessary for the development of insect communities or individuals, thereby meeting their essential needs. On one hand, certain insect species act as pollinators, predators of herbivorous species, or aid in seed dispersal (e.g., myrmecochory) for some plant species. In a balanced state, this relationship is mutually beneficial (Panda and Khush, 1995). On the other hand, depending on the intensity of herbivore infestation, insect feeding can be extremely destructive to the plant, potentially leading to its death. This dynamic system of interactions is constantly influenced and altered by external factors. Although this relationship may appear parasitic, it ultimately benefits both surrounding vegetation and herbivores through the process of nutrient recycling (Coleman and Sollenberger 2007). During population gradations of herbivorous insect species, plants, as sedentary organisms without the possibility of escape, have defense mechanisms.

Over the course of long-term evolutionary interactions between plants and insect herbivores, plants have adapted the ability to recognize chemical molecules indicating the presence of herbivores and subsequently activate signaling pathways of their defense system from damaged cells of plant tissue. As a result, a specific immune response is triggered in plants (Howe and Jander, 2008; Verhage, 2010; Hare, 2011). This minimizes the potential devastation of the original population of affected plant species, thereby exerting a limiting effect on the overpopulated insect species. Consequently, the abundance of the insect pest is reduced to a baseline level (Stiling et al., 1999).

To reduce the intensity of insect infestation, plants have developed defense mechanisms based on mechanical and physical barriers (Fordyce and Agrawal, 2001). In response to insect herbivory, various processes occur in plant tissues that can be qualified and quantified using appropriate analytical approaches. The action of insects (through **leaf-chewing** or **sap-sucking** feeding guild) induces the production of defensive proteins (Haruta et al., 2001; Chen et al., 2007; Fürstenberg-Hägg et al., 2013), volatile organic compounds (Birkett et al., 2000; Holopainen and Gershenzon, 2010; Niinemets, 2010; Rosenkranz and Schnitzler, 2016), and secondary metabolites (Baldwin, 2001; Wink 2018) in plant tissues. Using appropriate tools of molecular genetics and transcriptomics, it is possible to localize changes in gene expression in response to insect herbivory (Silva et al., 2012; Vogel et al., 2014; Birnbaum and Abbot, 2020). Additionally, changes in photosynthesis efficiency, transpiration, and stomatal conductance of leaves can be

determined using an open gasometric system (Rousselin et al., 2018). Spectrophotometric analysis can be used, for example, to determine the contents of chlorophylls a/b, proline, and carotenoids (Golan et al., 2015; Mattioli et al., 2009).

To effectively defend against various attackers, each requiring specific defense strategies, plants have evolved mechanisms over a long period of coevolution with insects that enable them to recognize specific threats (Acevedo et al., 2015). Plants perceive herbivory through the binding and recognition of specific molecular signals. These signals can either originate from mechanical damage to cell walls (especially in leaf-chewing insects), providing general information about plant injury (known as damage-associated molecular patterns or DAMPs), or they can provide more specific information about the identity of the herbivore through herbivore-associated elicitors (HAEs) or herbivore-associated molecular patterns (HAMPs), which are found in the oral secretions of herbivores (more details in chapter **1.4**). The perception and recognition of these specific attackers lead to the activation of defense mechanisms through phytohormonal signaling and extensive reprogramming of gene expression (Acevedo et al., 2015; Schuman et al., 2016; DeFalco and Zipfel, 2021).

Each type of attacker triggers a unique profile of responses involving various phytohormones. Generally, the response to leaf-chewing herbivores, such as caterpillars, is primarily mediated by jasmonic acid (JA), abscisic acid (ABA), and ethylene (ET). In contrast, sap-feeding herbivores, such as aphids, typically induce defense responses mediated by salicylic acid (SA) (more details in chapter **1.5.7**) (Erb and Raymond, 2019).

1.2.1 The Importance of Studying Plant-Herbivore Interactions

Since the majority of organisms that sustain energy flows in food webs rely on the autotrophic production of green plants, it is not surprising that plant defense against natural enemies represents one of the most significant sets of adaptations in the history of life. Across various biomes, habitats, natural and managed ecosystems, including estimates of **leaf-chewing**, **sap-sucking**, and root herbivory, herbivores consume more than 20 % of the annual net primary productivity (Schoonhoven et al., 2005; Rasmann and Agrawal, 2008). The intricate complexity of plant defense in mediating community interactions has made this research area a model in evolutionary ecology.

Plant-herbivore interactions, a highly multidisciplinary field of science, are essential for understanding community dynamics and ecosystem function, as they represent a crucial link between primary production and food webs. Studies of plants and

herbivores also form the backbone of many fields within ecology and evolution, including coevolution (Ehrlich and Raven, 1964; Labandeira, 2013; Johnson et al., 2015), chemical ecology (Hay and Fenical, 1988; Schuman and Baldwin, 2016), nutritional ecology (Wetzel et al., 2016), and ecological stoichiometry (Lemoine et al., 2014; Burkepile and Parker, 2017).

The application of these research findings extends to bioengineering methodologies, including the development of pest-resistant agricultural products (Huang and Osbourn, 2019), the enhancement of protection and plant immunity (Zuo et al., 2024), also with potential applications in pharmacology (Singh et al., 2021). Additionally, these studies support targeted cultivation practices that enhance natural plant defenses, thereby reducing the reliance on ecologically contentious chemical treatments (Divekar et al., 2022). In addition to the direct damage caused by feeding, insects can also harm plants indirectly by transmitting viral, bacterial, and fungal pathogens. Over the past few decades, the primary strategy for crop protection against insects has been the application of chemical insecticides. However, due to the development of insect resistance to pesticides and the negative impact on the environment, the use of such compounds has decreased in recent years (Du et al., 2020). Researchers have begun to uncover the molecular mechanisms underlying plant-insect interactions in order to find more effective methods for controlling these pests (Wang et al., 2023).

The significance of these studies extends beyond their theoretical contributions to our understanding of internal metabolic processes in plants, as well as ecological, evolutionary, and interaction principles. They also have practical applications with the potential to positively impact both natural ecosystems and human society.

1.2.2 Ecological and Economic Impact of *Populus tremula*

The genus *Populus* consists of approximately 30 species that hold significant ecological and economic value (Stettler, 1996). Beyond providing wood products, these species offer a variety of services, including bioenergy production, carbon sequestration, bioremediation, nutrient cycling, biofiltration, and habitat diversification (Brunner et al, 2004). Due to its relatively small genome, rapid growth rate, ease of vegetative propagation, short rotation cycle, high genetic diversity, and amenability to genetic manipulation, this genus has become a model system for studying tree molecular biology (Wullschleger et al., 2002).

Populus tremula, commonly known as aspen, is widespread across the cool temperate regions of Europe and Asia (**Figure 2**), typically found in mountainous areas at high altitudes. This species is highly resilient, capable of withstanding long, cold winters. It has been extensively cultivated for production timber, firewood, and veneer production. Natural populations of *P. alba* and *P. tremula*, or their varieties, often interbreed and have been selected as parent species for artificial hybrid breeding (Qiu et al., 2019).

Figure 2 Chorological map of *Populus tremula* adapted from Caudullo et al., 2017

Ecologically, European aspen is often considered a keystone species due to its critical role in supporting other species. Large aspen trees host hundreds of herbivorous and saprophytic invertebrates, fungi, and epiphytic lichens (Kouki et al., 2004). Dead trees undergo a decay succession, with each stage characterized by saprophagous insect species and fungi (Siitonen and Martikainen, 1994; Vehmas et al., 2009). Additionally, large aspens are vital for maintaining populations of cavity-nesting birds, such as woodpeckers (Gjerde et al., 2005). Aspen is also a highly preferred winter forage for large herbivores (Bergström and Hjeljord, 1987).

1.2.3 Global climate changes: Impact of Rising Carbon Dioxide Levels on Plant-Insect Interactions

Given the trend in atmospheric carbon dioxide (CO_2) concentration over recent decades (**Figure 3**), this factor cannot be overlooked in the context of plant-insect interactions. According to the Climate Change report (2014), the atmospheric CO_2 concentration in 2011 was 391 ppm, representing a 40 % increase compared to the pre-industrial period. By 2018, this concentration had risen to 410 ppm (parts per milion). Tans (2008) reports that the average annual increase in CO_2 concentration is 2 ppm. The

recorded value from the Mauna Loa monitoring station in Hawaii showed an average CO_2 concentration of 426.7 ppm in May 2024.

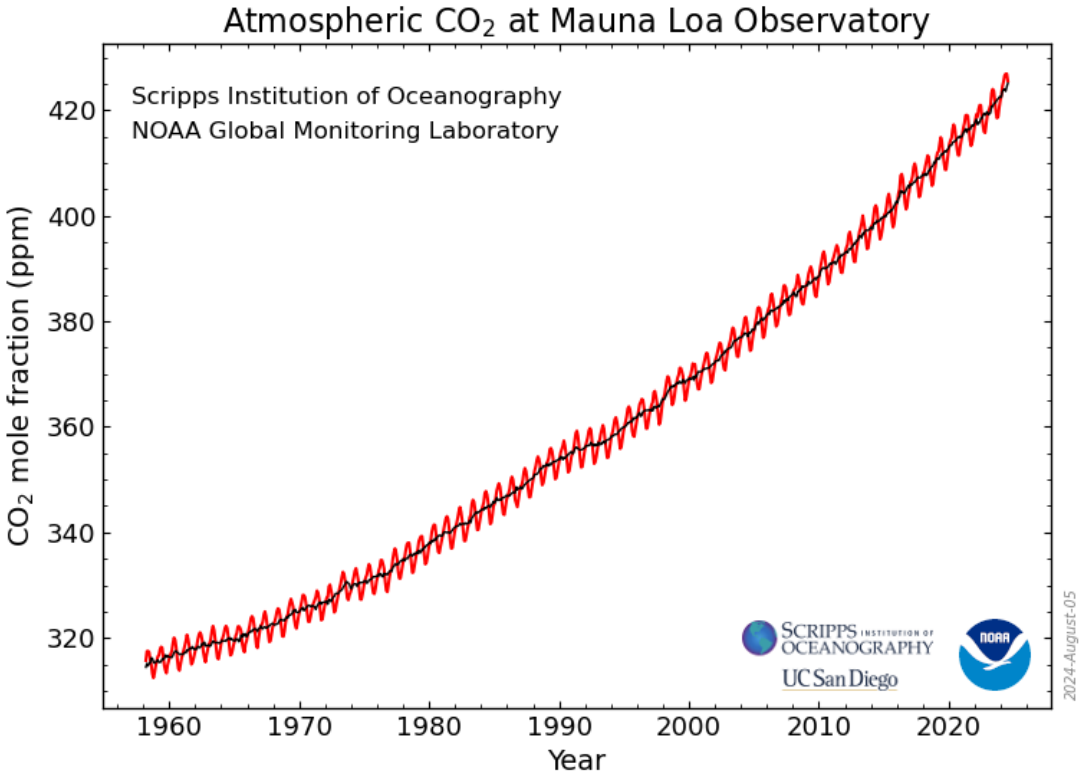

Figure 3 Mauna Loa Atmospheric Baseline Observatory by Lan (2024) NOAA's Global Monitoring Laboratory

Current research suggests that the most significant factor influencing the relationship between insects and plants is the increasing concentration of CO_2. This phenomenon results in higher levels of non-structural carbohydrates and lower nitrogen content in plant tissues (Valkama et al., 2007; Currano et al., 2010). As a consequence, insect herbivores must consume larger quantities of plant material to maintain their metabolic homeostasis, leading to increased herbivory (Agrell et al., 2000; Tuchman et al., 2002; Stiling and Cornelissen, 2007). However, the response of insect herbivores to elevated CO_2 levels varies significantly. For some taxa, such as butterflies (Lepidoptera), there is an average decrease in performance under increased CO_2 levels, whereas aphids (Hemiptera) show an increase in performance (Robinson et al., 2012).

This phenomenon is accompanied by rising average temperatures, which, according to Ayres and Lombardero (2000), trigger typical responses in insect herbivores, including: increased reproductive potential, faster development rates, altered diapause periods, and changes in the number of generations per growing season (voltinism). As a result, there is a growing need for a deeper understanding of interspecies interactions, particularly concerning economically and ecologically valuable tree species in forestry.

1.2.4 The Evolution of Plant Interaction Research: From History to Current Trends

The earliest references to plant sensitivity likely come from Aristotle, who considered plants devoid of sensory perception, setting them apart from animals: “plants live without sensation, and it is by a sensation that we distinguish animal from what is not animal” (Barnes, 1995).

Key figures in plant-insect interactions include Jean-Henri Fabre, who pioneered insect behavior studies (Fabre, 1879); Charles Darwin, who highlighted co-evolution between insects and plants (Darwin, 1877); and Karl von Frisch, who explored insect sensory perception (Frisch, 1953). Early research, such as by Dethier (1941) and Fraenkel (1959), defined plant defense mechanisms and the role of secondary metabolites.

In the 1970s, there was a trend among both scientific and non-scientific communities to anthropomorphize plant characteristics, for example, through polygraph experiments that sought to demonstrate plants' emotional sensitivity (Backster, 1968) or their response to events like the mass death of boiled shrimp (Horowitz et al., 1975). However, these findings were debunked (Galston, 1975).

The first comprehensive summary of plant-insect interaction research was published by Fritz and Simms in 1992. Currently, the study of plant-insect interactions is a rapidly evolving field of science, building on an extensive body of literature. It offers new and significant insights into both the unique molecular determinants of plant-insect interactions and the broader ecological context (Agrawal et al., 2010; Hancock et al., 2015; Singh 2021).

1.3 Insect herbivores: Feeding guilds

Herbivorous insects are phenomenally abundant and diverse. Approximately half of the million known insect species depend on green plant tissues for their survival (Wu and Baldwin, 2010). Insect herbivores employ a range of feeding strategies to obtain nutrients for their growth, development, and reproduction. Some insects are generalists, feeding on a broad spectrum of plant families, while others are specialists, targeting specific families, species, or even particular plant parts or tissues such as leaves or phloem. Of these, half are members of the orders Coleoptera (beetles) or Lepidoptera (caterpillars) as clearly displayed (**Figure 4**), which damage and consume leaves using

mouthparts adapted for **chewing**, tearing, or snipping (Schoonhoven et al., 1998; Will et al., 2013; Bánki et al., 2024).

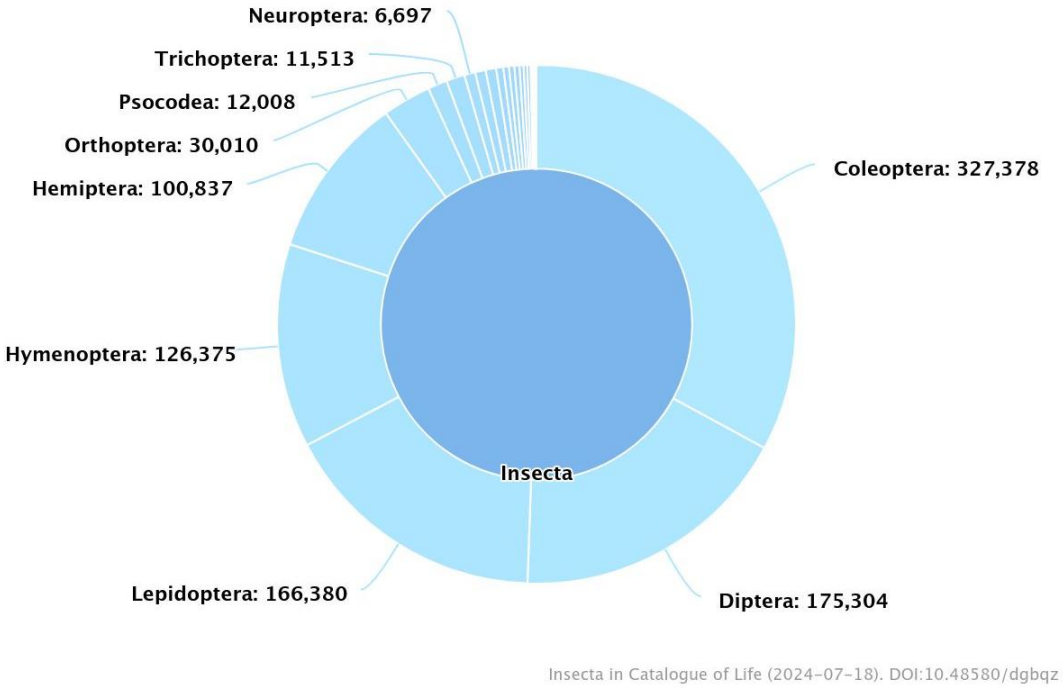

Figure 4 Proportional representation of orders within the class Insecta. Adapted from Bánki et al. (2024) in Catalogue of Life (2024)

In contrast, many hemipterans (such as aphids) use specialized piercing-sucking mouthparts that they insert between cells to **suck** the phloem sap (Howe and Jander 2008).

To study how plant metabolism responds to insect herbivory, it is crucial to understand the feeding guilds and the processes involved in insect feeding, as summarized in **Table 1.**

Table 1 Overview of major insect herbivore orders and specific feeding guilds

Order	Members	Feeding guild	References
Coleoptera	Beetles	**Chewing**, snipping	Schoonhoven et al. (1998)
Diptera	Mosquitoes and flies	**Sucking**	Yoshinaga et al. (2007)
Lepidoptera	Moths and butterflies	**Chewing**, snipping	Schoonhoven et al. (1998)
Hymenoptera	Ants, Bees, Wasps	Nectar and pollen-feeding is the most widespread, **Chewing,** limited **sucking**	Jervis et al. (2000); Quicke (2009); Malagodi-Braga et al. (2019)
Hemiptera	Aphids, leaf hoppers	**Sucking**	Howe and Jander (2008)
Orthoptera	Grasshoppers, crickets	**Chewing**, snipping	Yoshinaga et al. (2007)

According to the above, insects are typically grouped into two main broad categories: **chewing** insects (a typical representative is Lepidoptera: *Lymantria dispar*) and **sucking** insects (Hemiptera: e.g., *Chaitophorus populialbae*) (Bonaventure, 2012; Cranston 2014).

The mechanism of plant tissue damage corresponds to the feeding apparatus of the respective feeding guild. Chewing insects have two mandibles, one on each side of the head. The mandibles are located between the labrum and the maxillae and are typically the largest mouthparts in chewing insects, used for grinding food (**Figure 5A**).

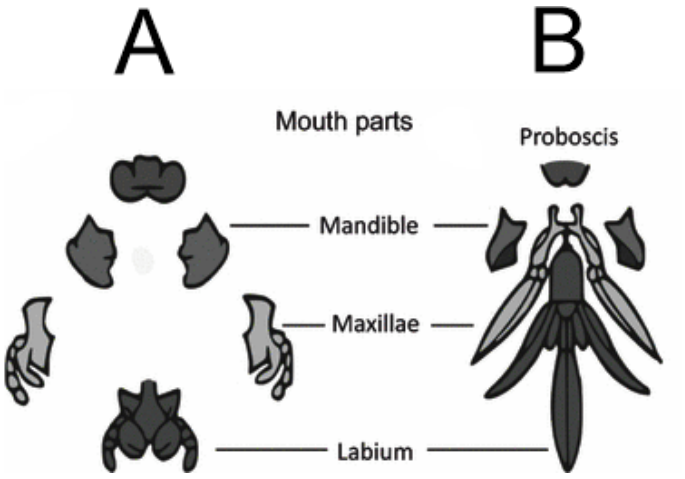

Figure 5 Anatomical structure of the feeding apparatus in the chewing guild (A) and the sucking guild (B) (adapted from Bonaventure, 2012).

Sap-sucking insects have mouthparts where the mandibles and maxillae are modified into a proboscis, encased in a modified labium, capable of piercing tissues and sucking phloem or cellular fluids (**Figure 5B**) (Bonaventure, 2012).

1.3.1 Sap-sucking insect feeding guild

Phytophagous hemipterans employ two primary feeding strategies: salivary sheath feeding, where they extract fluids from plant vascular tissues such as phloem or xylem (commonly known as sap feeders), and cell rupture feeding, which targets the mesophyll (Chuche et al., 2017b). Sap-sucking insects species can be further categorized into two feeding subguilds:

- **Phloem-feeders**, which include most species in the suborder Sternorrhyncha, such as for example aphids (Aphidoidea), and white-flies (Aleyrodoidea);
- **Xylem-feeders**, including among others cicadas (Cicadoidea), spittlebugs (Cercopoidea), and sharpshooter leafhoppers (Cicadellinae) (Douglas, 2006; Bennet and Moran, 2013).

Phloem and xylem tissues have very different properties, and as a result, sap-feeding insects typically specialize in one or the other (Labandeira and Phillips, 1996). However, phloem-feeding insects may occasionally consume xylem sap, likely to regulate osmotic potential (Pompon et al., 2011). This is because phloem sap has a very high concentration of sugars (i.e., osmotic pressure 2–5 times higher than in insect hemolymph) (Douglas, 2006).

Modified mouthparts of hemipteran insects include a slender, beak-like labium, within which are two pairs of long stylets (two outer mandibles and two inner maxillae) that form a bundle (see **Figure 5**). Only the stylets penetrate the plant tissues to pierce and suck. The maxillary stylets contain both a salivary channel and a food channel on their inner surfaces. Through these channels, the insect injects saliva into the plant and simultaneously sucks plant sap into its digestive tract. In sap-feeding insects, the stylet path to the vascular tissue is typically intercellular, following an apoplastic route—between cell walls without entering the cytoplasm. Finally, some intracellular punctures occur for assessing cell content, host acceptance, and positioning the stylets within the plant (Branco et al., 2023).

Aphids produce two types of saliva. During the stylet penetration process, gel-forming lipoprotein saliva is secreted, creating a lubricating and hardening sheath around the stylets. This gel-forming saliva remains in the plant tissues even after the stylets are withdrawn. Additionally, watery saliva is directly injected into the vascular tissues before the sap is ingested. This watery saliva may interfere with the host plant's defensive responses, for instance, through proteins involved in phenol detoxification or by directly interacting with the plant's defense signaling (more details in chapter **1.5**) (Giordanengo et al., 2010; Will et al., 2013; Gullan and Cranston, 2014; Chen and Mao, 2020).

An essential aspect of the plant-insect herbivore interaction that cannot be overlooked is the involvement of the third trophic level: the inherent presence of microorganisms. Given that both phloem and xylem are nutritionally unbalanced food sources, sap-suckings insects rely on symbiotic bacteria to provide essential nutrients missing from their diet (Bennett and Moran 2013). Primary endosymbionts (P-endosymbionts) are obligate mutualistic bacteria found within polyploid host cells of hemipterans (bacteriocytes), which typically cluster into a specialized organ called the bacteriome. These P-endosymbionts are crucial for the host's survival and reproduction and are present in all individuals within the host population (Baumann, 2005; Morrow et al., 2017).

In addition to these essential primary symbionts, sap-sucking insects may also harbor one or more facultative or secondary symbionts (S-symbionts). Secondary symbionts commonly found in aphids include *Serratia* (present in 47 % of studied aphid species), *Wolbachia* (43 %), *Hamiltonella* (34 %), *Regiella* (33 %), *Rickettsia* (29 %), X-type (14 %), *Spiroplasma* (13 %), and *Arsenophonus* (9 %) (Zytynska and Weisser, 2016). These secondary symbionts are generally not required for the host's development and reproduction. S-symbionts can inhabit various tissues other than bacteriocytes, do not typically infect all individuals within a host population, and can be horizontally transmitted among aphid populations (Chuche et al., 2017a; López-Madrigal and Gil, 2017).

1.3.2 Leaf-chewing insect guild

Some insects species, such as moths and butterflies (order Lepidoptera), possess chewing mouthparts only during their larval stage, while adults lack them. During feeding, chewing insects tear plant tissue, triggering a defensive response in the plant that overlaps with the response to mechanical injury (Mithofer and Boland, 2008). Chewing insects have a specialized mouthpart structure (**Figure 5**) that includes the labrum, mandibles, first and second maxillae, hypopharynx, and epipharynx. The labrum is rectangular and flap-like, while the mandibles, which are paired and serrated on the inner surfaces, use transverse muscles to chew food. The first maxillae hold the food, and the second maxillae push the chewed material into the mouth. The hypopharynx features a central, tongue-like projection with the salivary canal opening beneath it. The epipharynx, containing taste buds, is a small membranous structure at the base of the labrum (Felton et al., 1999; Stotz et al., 1999).

As a result, the affected plant undergoes a dual assault: mechanical damage combined with chemical interference from the oral secretions introduced by the feeding organism (Mithofer and Boland, 2008). Oral secretions, consisting of regurgitant and saliva, are excreted from the foregut and midgut of insects to lubricate their mouthparts during feeding (Peiffer and Felton, 2009; Chen and Mao, 2020). Regurgitated saliva includes fatty acid conjugates, plant and insect proteins, insect enzymes, and elicitors (Mori et al., 2003; Chung et al., 2013). In chewing insects, saliva may contain enzymes that pre-digest food before gut enzymes act on it. Oral secretions primarily contain amylase and invertase but may also include enzymes such as glucose oxidase and phospholipase C, which help prevent infection during feeding (Acevedo et al., 2017).

Despite the diversity and economic importance of phytophagous Lepidoptera, only few studies have characterized their saliva (Rivera-Vega et al., 2017). Lepidopteran secretions include not only gut regurgitants but also saliva from labial and mandibular salivary glands, as well as feces (Felton, 2008). Detailed insights into the composition of oral secretions (more details in chapter **1.4**) and their impact on activating plant defense signaling pathways are provided by Arimura (2021) and Rivera-Vega et al. (2017).

In leaf-chewing insect herbivores, the presence and activity of microorganisms also play a significant role. Microbes colonize both the interior and external surfaces of insects, forming mutualistic, commensal, or parasitic relationships (Su et al., 2013). Insect endosymbionts contribute to saliva secretion, elicitor compounds, proteins, and defensive enzymes like glucose oxidase (Wang et al., 2017; Bayendi Loudit et al., 2018). The composition of salivary proteins differs significantly between insects with and without endosymbionts (Zhu et al., 2018). Furthermore, microbes in insect regurgitant can inhibit important plant defensive enzymes, such as polyphenol oxidase, thus benefiting the insect during feeding (Acevedo et al., 2017). Since plant tissues often lack essential nutrients, insects feeding on them receive an unbalanced supply of crucial nutrients like amino acids, nitrogen compounds and lipids, which can negatively impact their health (Douglas, 2003; Chen et al., 2008). Therefore, the association with endosymbionts, which can supplement their nutritionally poor diet, is crucial for their survival (Douglas, 2009).

The aim of the last two chapters is to provide a concise overview of the fundamental knowledge and mechanisms associated with the feeding behaviors of **sap-sucking** and **leaf-chewing** insect herbivores. This overview summarizes the findings of many authors (Mattiacci et al., 1995; Backus et al., 2005; Rivera-Vega et al., 2017; Arimura, 2021), who report that insect saliva contains components such as herbivore-associated elicitors (HAE) or also herbivore-associated molecular patterns (HAMP). Which, alongside the impact of mechanical damage (DAMPs), play a fundamental role in the qualification and quantification of plant metabolic responses.

1.4 Herbivore-associated elicitors/Herbivore-associated molecular patterns

As mentioned in the previous chapters, a crucial factor in the interaction between plants and insect herbivores is a group of substances known as elicitors (HAE/HAMPs). These specialized compounds found in the oral secretions of herbivorous insects act as signals that trigger defensive responses in plants. They can induce the biosynthesis of specific compounds and directly influence cellular processes, making them recognizable

to plant cells (Bonaventure, 2012; Xu et al., 2015). These active molecules play a pivotal role in triggering plant defense responses, and their effects can be analytically distinguished from general mechanical damage, such as cutting a leaf with scissors (Baldwin, 1990; Van Zandt and Agrawal, 2004; Hogenhaut and Bos, 2011; Chen and Mao, 2020).

Defensive responses in plants triggered by insect herbivores are often highly specific, as different insect herbivore species or feeding guilds can induce distinct defensive reactions in plants. This specificity is largely mediated by the unique chemical properties of the particular herbivore-associated elicitor (HAE) (Schmeltz et al., 2006; Xu et al., 2015). According to current knowledge, in the case of Lepidoptera, HAE/HAMP includes:

- **β-glucosidase** (enzymes) - commonly known as GOX (Mattiacci et al., 1995);
- **Fatty acid-amino acids conjugates** (FACs) (for example, volicitin) (Voelckel and Baldwin, 2004);
- **Caeliferins** first isolated by Alborn et al. (2007);
- **Inceptins**, first identified by Schmeltz et al. (2006);
- **Bruchins**, first isolated by (Doss et al., 2000).

The detailed chemical structure of selected compounds can be seen in **Figure 6**.

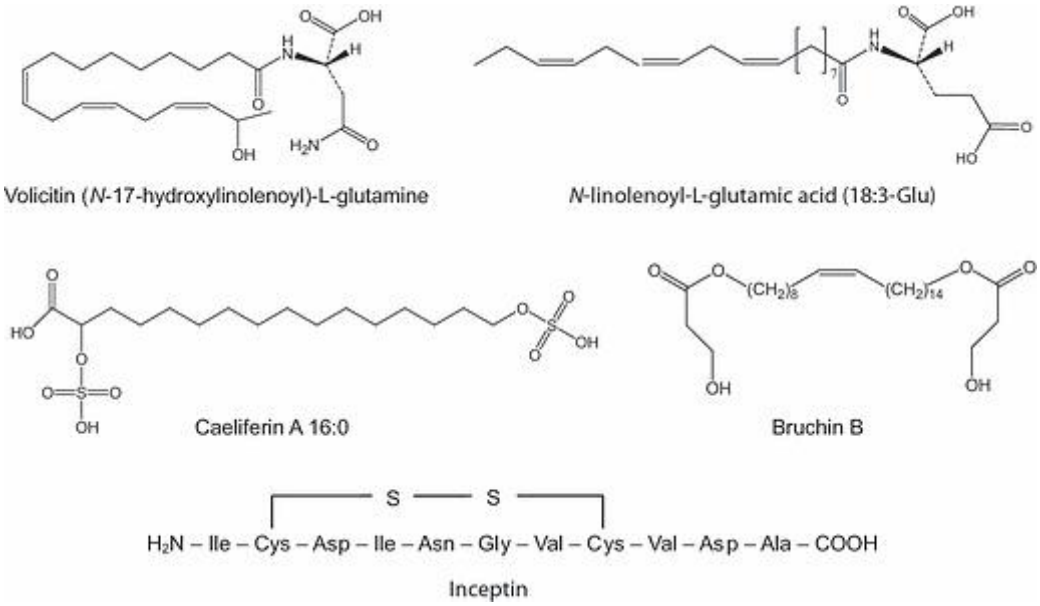

Figure 6 The oral secretions (OS) of lepidopteran larvae from various species contain fatty acid–amino acid conjugates (FACs), including volicitin and N-linolenoyl-glutamic acid (18:3-Glu). Caeliferins are present in the OS of grasshopper species, while bruchins originate from the oviposition fluids of cowpea weevils. Inceptins are formed by the degradation of the plant ATP synthase γ-subunit during folivory by *Spodoptera frugiperda* on cowpea plants (Adapted from Bonaventure, 2012).

1.4.1 β-glucosidase (GOX)

Plants have the ability to recognize HAE and initiate a complex array of defense mechanisms. The first reported elicitor, β-glucosidase, was isolated from the regurgitant of the large white butterfly (*Pieris brassicae*). Leaves treated with p-glucosidase showed an increased emission of volatile compounds that are highly attractive to parasitic wasps (Mattiacci et al., 1995). Glucose oxidase (GOX), present in the saliva of Noctuid caterpillars (*Helicoverpa zea*) and the European corn borer (*Ostrinia nubilalis*), upregulates the expression of genes related to the jasmonic acid (JA) biosynthesis pathway and late-response defenses, such as the proteinase inhibitor 2 (Pin2) in tomatoes (Tian et al., 2012; Louis et al., 2013). The phytohormone JA plays a central role in plant metabolism as a signaling molecule that triggers a cascade of induced defenses (Xu et al., 2015). A specific case of JA induction is provided by Schmelz et al. (2009). This suggests that the specificity of JA accumulation induced by HAE is likely mediated by specific receptor-ligand interactions, although the molecular mechanisms are not fully understood across all species. The specificity of JA accumulation induced by different HAE may also be mediated by hormonal cross-talk (Thaler et al., 2012; Wang et al., 2023).

1.4.2 Fatty acid-amino acids conjugates (FACs)

As their name implies, FACs (fatty acid-amino acid conjugates) are composed of two distinct components: a plant-derived fatty acid, such as linolenic acid (18:3) or linoleic acid (18:2), which is released from membranes due to lipase activity or wounding, and an herbivore-derived amino acid, typically glutamate (Glu) or glutamine (Gln) (Halitschke et al. 2001; Yoshinaga et al. 2008). These compounds (FACs) (**Figure 6**) are one of the most common bioactive compounds found in Lepidoptera (Yoshinaga et al., 2010; Kallure et al., 2024). For insects, the effect of FACs is crucial as they help assimilate nitrogen by regulating amino acid levels in the insect's midgut (Yoshinaga et al., 2008) and also function as biosurfactants in the insect gut to improve lipid solubility (Kuhns et al., 2012a). The most significant and well-recognized class of FACs is volicitin, N-(17-hydroxylinolenoyl)-L-Gln (**Figure 6**), which was first isolated from the oral secretions of beet armyworm larvae (*Spodoptera exigua*; Alborn et al., 1997). In addition to volicitin, other fatty acid (FA)-amino acid conjugates have been isolated from various noctuid and geometrid Lepidoptera larvae. The general structure of these compounds has been identified as N-acyl-Gln, with the FA component primarily consisting of linoleic acid (C18:3), linolenic acid (C18:2), and their derivatives (Spiteller et al., 2004). For

instance, in eggplant, two fatty acid amino acid conjugates (FACs), volicitin and N-linolenoyl-Gln, triggered more than a twofold increase in JA levels compared to mechanical wounding alone. However, two other tested HAE, caeliferin A16:0 and inceptin, did not induce JA accumulation.

1.4.3 Caeliferins

Elicitors known as caeliferins are saturated and monounsaturated sulfated α-hydroxy fatty acids (C15-C20) that were first isolated from the regurgitate of the American bird grasshopper, *Schistocerca americana* (Alborn et al., 2007). Like FACs, caeliferins can induce the release of volatile compounds that attract parasitoids when applied to injured plant tissues. However, caeliferins have so far been identified only within the suborder Caelifera (e.g., grasshoppers) and are not as widely distributed as FACs (Alborn et al., 2007). In *Arabidopsis thaliana*, treatment with synthetic caeliferin A 16:0 did not stimulate the emission of volatile compounds but significantly increased the production of both ethylene (ET) and jasmonic acid (JA) within 2 hours, and slightly raised salicylic acid (SA) levels after 4 hours of application to wounded leaves, suggesting a central role of caeliferins in grasshopper oral secretion-induced responses (Schmelz et al., 2009).

1.4.4 Inceptins

Inceptins are small, plant-derived peptides (11–13 amino acids) linked by disulfide bonds, which function as elicitors. These peptides are generated through the proteolytic degradation of the regulatory regions of the ATP synthase gamma-subunit in the insect gut. Inceptins play a crucial role in triggering and amplifying both local and systemic defense responses in plants (Yamaguchi and Huffaker, 2011). The ability to recognize inceptins suggests that plants have evolved not only to detect insects directly through their secretions or movements but also indirectly by recognizing the presence of catabolic products, which indicate effective feeding and digestion by the insect (Schmelz et al. 2009). Inceptins, initially isolated from the oral secretions of Spodoptera frugiperda larvae (Schmelz et al. 2006), were later found in the oral secretions of larvae from various Lepidoptera species (Schmelz et al. 2012). It is evident that inceptins act as chemical signals that trigger specific plant responses following insect attack. These findings strongly suggest that inceptins are genuine peptide elicitors with a high degree of

structural specificity. Their mode of action is most likely receptor-mediated, as they do not directly interact with plant membranes (Maischak et al., 2007).

1.4.5 Brunchins

This group of elicitors, known as bruchins, has been isolated from the pea weevil (*Bruchus pisorum*) and the cowpea weevil (*Callosobruchus maculatus*). Bruchins are long-chain α,ω-diols that are mono- and diesterified with 3-hydroxypropanoic acid (Doss et al., 2000). During oviposition, if female pea weevils come into contact with their host plant, the pea (*Pisum sativum*), bruchins trigger neoplastic growth on the pods of certain pea genotypes at the site where the eggs are laid. This growth, consisting of undifferentiated callus cells, elevates the eggs above the oviposition site, effectively hindering larval entry into the pod tissue. As a result, the larvae are exposed to natural enemies and become susceptible to desiccation, reducing their chances of survival (Doss et al., 2000).

1.4.6 The Intricacies and Challenges of Studying HAE/HAMPs

The characterization of elicitor molecules produced by sap-feeding herbivores has long been hindered by the technical challenges of collecting regurgitant secretions from small arthropods. The availability of genomic, RNA-seq, and proteomic databases has made it possible to characterize salivary gland genes encoding potential elicitors, even in the smallest sap-feeding herbivores. For example, RNA-seq and proteomic analyses of the salivary glands of the brown planthopper (*Nilaparvata lugens*) recently led to the identification of a mucin-like salivary protein (NlMLP) (Shangguan et al., 2018). This protein is highly expressed in the salivary glands and plays a role not only in the formation of salivary sheaths in plants but also acts as an elicitor in host leaves when secreted during feeding (Shangguan et al., 2018).

Similarly, tetranins (Tet1 and Tet2) were recently identified as salivary gland proteins in *Tetranychus urticae*. These two protein elicitors induce a cytosolic influx of Ca^{2+} and membrane depolarization (more details in chapter **1.5.1**), which likely triggers jasmonic acid/salicylic – dependent plant defensive responses (Iida et al., 2019). Bos et al. (2010) report that the salivary proteins secreted by hemipterans exhibit similarities to effectors of plant pathogens. It has been proposed that effectors derived from aphids may interfere with plant defense responses, potentially operating through mechanisms analogous to those activated by the perception of effectors from microbial pathogens.

The complexity of HAE and HAMPs is significantly compounded by the high chemical diversity of oral secretions produced by insect herbivores. Within the saliva of a single insect species, multiple active compounds can be present, each with different effects on plant perception. Although many HAE/HAMPs have been described, research on their interactions has primarily focused on a limited number of these compounds from only a few insect species. Identifying additional active compounds within the same species and across different insect species will help uncover the broader range of evolutionary and ecological mechanisms involved in plant perception of insect herbivory (Bonaventure, 2012; Kallure, 2024).

Further complicating the general description of the role of HAE/HAMPs is the fact that certain elicitors, which typically trigger defensive responses in plants, can also function as **effectors** that suppress these defenses. This phenomenon is species-specific and non-uniform across different plant species (Rivera-Vega et al., 2017; Wang et al., 2023). Moreover, as reported by Chaudhary et al. (2014) and Bonaventure et al. (2011), some HAE/HAMPs are not produced by the insects themselves but originate from microbes—referred to as microbe-associated molecular patterns (MAMPs)—which are carried by the insects on their surfaces, as well as in their intestines and oral secretions. (more details in chapter **1.4**).

Another challenge in applying the knowledge of insect HAE functions is highlighted by Alborn et al. (2000), who found that the biological activity of volicitin—a well-known FACs—depends heavily on its structural composition. Synthetic volicitin conjugated with D-glutamine showed minimal activity in plant metabolic responses, indicating that the biological efficacy of FACs is largely determined by their structural configuration (**Figure 6**). Many researchers agree that despite decades of experiments and analyses, this field is still in its early stages and requires further investigation (Peiffer and Felton, 2009; Rivera-Vega et al., 2017; Wang et al., 2023; Kallure et al., 2024).

Previous chapters have introduced plant-insect interactions, focusing on key mechanisms such as the classification of insect herbivores by feeding guild and the role of chemical compounds (HAE/HAMPs/MAMPs/PAMPs) in oral secretions, which fundamentally stimulate plant metabolism through their signaling defense pathways.

1.5 Mechanisms of Plant Defense Against Insect Herbivores

The following chapters will focus in greater detail on processes related to plant metabolism, activation of defensive signaling pathways, immune responses, and

transcriptional changes. Additionally, the biochemical processes at the level of plant metabolism within the context of plant-herbivore interactions will be explored. To enhance clarity, this section will be structured chronologically, starting from the moment the insect herbivore initiates feeding to the subsequent plant responses (more details in chapter **1.5.2**).

The ecological and evolutionary context of plant-insect interactions always requires individual consideration, as they are dynamic, and what occurs at one moment may not happen at another. Insects are programmed to recognize and quickly respond to host stimuli (plants) (Bruce et al., 2005). Successful plant defense depends on the plant's ability to quickly recognize the attacking insect herbivore. Timely defensive responses require the activation of signaling cascades triggered by the presence of the enemy. The activation of these cascades ensures an induced defensive response that is effective, rapid, and coordinated with other processes within the host cells (Maffei et al., 2007; Bonaventure et al., 2011; Maffei et al., 2012).

1.5.1 Pattern Recognition Receptores by the Plant Immune System

Unlike mammals, plants lack mobile defensive cells and a somatic adaptive immune system. Instead, they rely on the innate immunity of each cell and systemic signals originating from the sites of infection (Chisholm et al., 2006). The induced response to insect herbivory fits within the broader conceptual framework of innate immunity, particularly the plant immune system (Medzhitov and Janeway, 2002; Jones and Dangl, 2006). A successful immune response requires two phases:

- recognition, which involves perceiving non-self or modified self-stimulants;
- an effective response, consisting of defense outputs tailored to the attacker (Upson et al., 2018) (more details in chapter **1.5.2**).

The first step in plant immune recognition molecular patterns against external pathogens, such as bacteria, fungi, or insect herbivores, involves the activation of immune receptors known as Pattern Recognition Receptors (PRRs) (Bonaventure et al., 2011; Maffei et al., 2012; Couto and Zipfel, 2016; Albert et al., 2020). PRRs on the cell surface detect conserved: pathogen-associated molecular patterns (PAMPs), damage- associated molecular patterns (DAMPs) (endogenous, modified plants self-signals from mechanical damage, are involved in plant defense responses), herbivore-associated molecular patterns (HAMPS), microbe-associated molecular patterns (MAMPs) and initiate pattern-triggered immunity (PTI), which helps to limit pathogen virulence. These PRRs are

typically associated with the plasma membrane (PM) and are either receptor-like kinases (RLKs) or receptor-like proteins (RLPs) that lack a kinase domain. To evade or suppress PTI, pathogens have evolved to secrete effector molecules (more details in chapter **1.4**), leading to effector-triggered susceptibility (ETS) (Tanaka and Heil, 2021; Ngou et al., 2022; Wang et al., 2023). The functional diagram according to selected associated patterns is illustrated in **Figure 7**, including the listed elicitors.

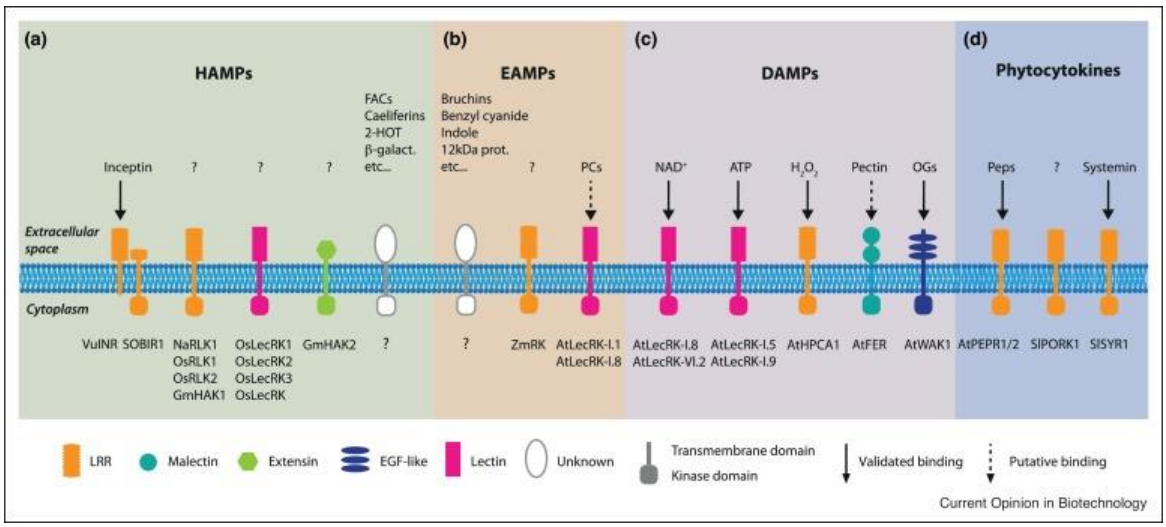

Figure 7 (a) Herbivore-associated molecular patterns (HAMPs) from arthropod oral secretions or salivary glands, (b) egg-associated molecular patterns (EAMPs), and (c) damage-associated molecular patterns (DAMPs) from damaged cells are recognized by pattern recognition receptors (PRRs) located on the plant plasma membrane, which contain various extracellular domains. Upon ligand binding, these PRRs activate downstream immune responses through their kinase domains. (d) Phytocytokines act as secondary danger signals in plants, are secreted into the apoplastic space, are recognized by PRRs, and amplify defense responses. The corresponding PRRs for several HAMPs and EAMPs remain unidentified (indicated in white). Validated binding between a ligand and its corresponding receptor is shown with a solid arrow, while hypothesized binding is indicated with a dotted arrow. Several PRRs still lack an identified ligand. (Adapted from Reymond, 2021)

The high sensitivity of Pattern Recognition Receptors (PRRs) was demonstrated in the study by Alborn et al. (1997), which experimentally confirmed the effect of volicitin, the first purified HAMP. Applying just 300 pmol of volicitin to plants was sufficient to trigger a defensive response.

1.5.2 Sequence of Defense Responses - Cascade of Plant Defense Reactions

Plants have developed mechanisms to recognize and respond quickly to herbivory. These mechanisms include:

- Recognition of molecular patterns (HAE/HAMPs/MAMPs/DAMPs) and defensive effectors (Bonaventure et al., 2011; Maffei et al., 2012) (more details in previous chapter **1.5.1**);
- Depolarization of the transmembrane potential (V_m) of the plasma membrane (Bricchi et al., 2012);

- Increase in cytosolic calcium concentration ([Ca^{2+}]cyt) (Medvedev, 2005; Reddy et al., 2011);
- Activation of NADPH oxidase, and the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Miller and Mittler, 2006; Bricchi et al., 2010; Arimura et al., 2011);
- Activation of mitogen-activated protein kinases (MAPK), and protein phosphorylation (Arimura and Maffei, 2010; Arimura et al., 2011);
- Signaling cascades lead to the increased production of phytohormones such as jasmonic acid (JA) and salicylic acid (SA) (Zipfel, 2009; Consales et al., 2012; Erb et al., 2012; Bricchi et al., 2013);
- Increased production of ethylene (Arimura et al., 2009; González-Garcia et al., 2011; Scala et al., 2013);
- Expression of defense genes involved in the emission of volatile organic compounds (Baldwin, 2010; Wu and Baldwin, 2010; Karban et al., 2010; Maffei et al., 2012);
- Production of secondary metabolites (Karban, 2010).

These defense responses begin locally at the site of damage but can spread systemically throughout the plant (Wu and Baldwin, 2010; Maffei, 2012; Bricchi et al., 2012).

Although the mechanisms and processes in plant metabolism following herbivore attacks have been extensively cataloged individually, the relationships between these events and their interdependencies have received relatively little research attention (Zebelo and Maffei, 2015). The summary of these processes over time is clearly illustrated in **Figure 8**.

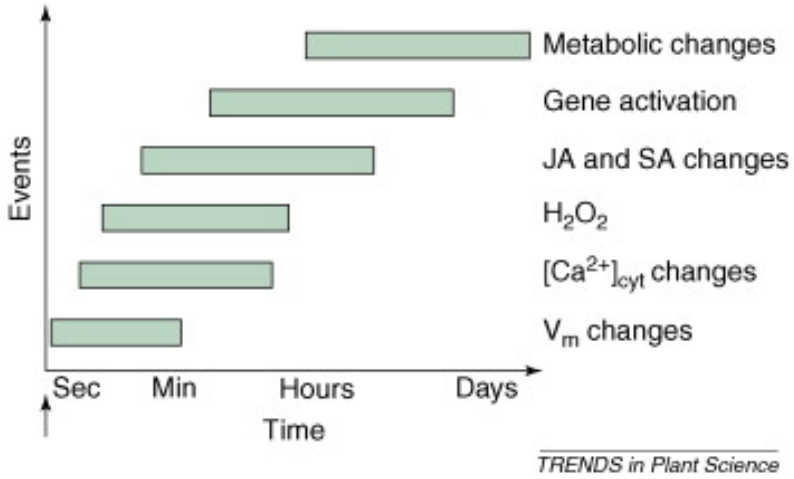

Figure 8 The cascade of sequential events detectable in plant tissues, triggered by insect feeding, (Adapted from Maffei et al., 2007).

Plant defense reaction begins with the earliest measurable changes in membrane potential (V_m) at the plasma membrane. These are immediately followed by alterations in intracellular Ca^{2+} concentration and the production of hydrogen peroxide (H_2O_2). Within minutes, kinases are activated, and the phytohormones jasmonic acid (JA) and salicylic acid (SA) become detectable. Gene activation and subsequent metabolic changes typically become evident around one hour after the initial response (Maffei et al., 2007)

1.5.3 Depolarization of the Transmembrane Potential (V_m)

Bioelectric circuits can operate over long distances within biological tissues, and their activation can lead to various physiological and biochemical reactions (Nordenstrom, 1984). Cells in many organs generate electrical potentials that allow the flow of electric currents, with these impulses capable of spreading to adjacent cells (Volkov et al., 1998). The plasma membrane, which is in direct contact with the environment, acts as a sensory element capable of detecting changes and initiating reactions that lead to specific responses. Changes in the transmembrane potential (V_m) and the modulation of ion fluxes at the plasma membrane level are among the earliest cellular responses to biotic and abiotic stresses (Ebel et al., 1998; Shabala, 2006; Maffei et al., 2007).

Changes in Vm can create a wave of depolarization that propagates to adjacent resting membranes. When the plasma membrane is stimulated, the action potential can spread along the entire cell membrane and through conductive tissue bundles, maintaining a constant amplitude, duration, and velocity (Volkov, 2012). Depolarization of V_m is associated with an increase in calcium ion levels in the cytosol, ion channel activity, and the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). These processes typically occur within seconds to minutes after herbivory and are among the earliest defense responses in plants (Zebelo and Maffei, 2015).

The interaction between elicitors and receptors induces changes in the transmembrane potential (V_m), defined as the difference in the electrochemical gradient between the inside and outside of the cell. These changes can lead to depolarization (more positive V_m values) or hyperpolarization (more negative V_m values), which subsequently trigger signaling cascades (Zebelo and Maffei, 2012). At resting membrane potential, living cells exhibit a difference in electrical potential of several tens of millivolts across the plasma membrane, with the intracellular environment being negative relative to the extracellular fluids. This membrane potential is generated by ion transport mechanisms

that maintain an intracellular ion medium distinct from the extracellular ion medium (Pachú et al., 2023).

Both direct contact with leaf-chewing herbivores and insect oral secretions have been shown to induce rapid depolarization of V_m. In sap-sucking insects, such as *Myzus persicae*, significant V*m* depolarization has been observed in response to almost every stylet puncture during phloem feeding (Maffei et al., 2007; Volkov, 2012b). The main types of electrical signals include action potential (AP), variation potential (VP), and systemic potential (SP) (Zimmerman et al., 2009; Pachú et al., 2023).

Action potential (AP) is a signal that propagates as a transient depolarization with a characteristic pulse shape, with amplitudes ranging from several tens to one hundred millivolts (mV) and durations from a few seconds to several tens of seconds.

Variation potential (VP), also known as "slow wave potential," is a transient depolarization of irregular shape, with an amplitude of several tens of mV and a duration of up to several tens of minutes (Mudrilov et al., 2021).

Systemic potential (SP) is a systemic signal induced by abiotic and biotic factors that propagates and transmits a hyperpolarization event associated with the activation of H+-ATPase in the plasma membrane (Zimmerman et al., 2009).

1.5.4 Increase in Cytosolic Calcium (Ca^{2+})

Calcium ions (Ca^{2+}) play a crucial role as signaling molecules in many plant signaling pathways. In healthy tissues, the concentration of free Ca^{2+} in the cytosol ($[Ca^{2+}]cyt$) is maintained between 100 and 200 nM, which is approximately 10,000 times lower than in the apoplastic fluid and 10,000 to 100,000 times lower than in cellular organelles. This steep gradient drives the import of Ca^{2+} into the cytosol, where it functions as a signaling molecule (Lecourieux et al., 2006; Maffei et al., 2007).

Calcium sensors are essential in calcium signaling during herbivory. According to the standard model, Ca^{2+}-sensitive proteins such as calmodulin (CaM) detect Ca^{2+} signals and subsequently regulate target proteins in the defense signaling cascade (Du et al., 2011). Calmodulin (CaM) is a highly conserved calcium-modulated protein composed of two globular domains, each containing two EF-hand motifs, with each motif capable of binding one Ca^{2+} ion (Noman et al., 2021).

Thus, a single calmodulin molecule can bind up to four Ca^{2+} ions (Norman et al., 2021). The main families of calcium sensors include calmodulins (CaM) and calmodulin-like proteins (CML), Ca^{2+}/CaM-dependent protein kinases (CCaMK), calcium-dependent

protein kinases (CDPK/CPK), calcineurin B-like proteins (CBL), and CBL-interacting protein kinases (CIPK) (Kudla et al., 2018; Ghosh et al., 2022). Upon detecting Ca^{2+} signals, CBL proteins interact with CIPKs to form CBL–CIPK complexes. The CBL–CIPK signaling pathway is regulated by intricate mechanisms in coordination with other signaling pathways (**Figure 9**) (Zebello and Maffei, 2015).

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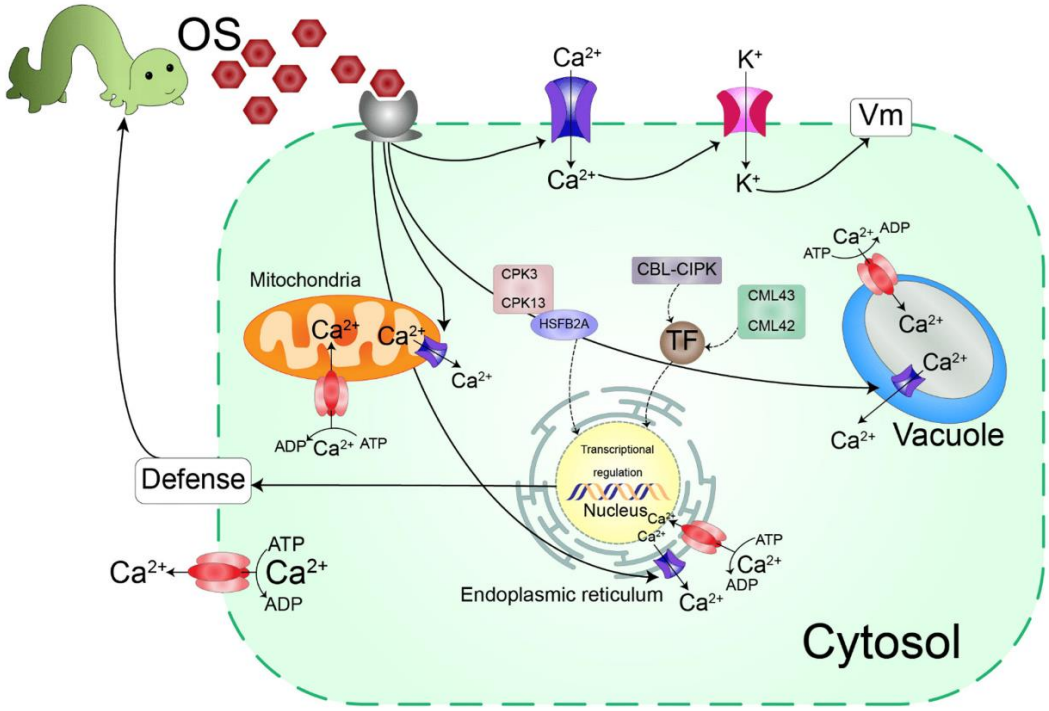

Figure 9 A schematic diagram of the calcium signaling pathway following herbivore attack. Elicitors (from insect oral secretions) bind to specific receptors, causing a rapid increase in cytosolic calcium concentration ([Ca^{2+}] cyt). Calcium channels and ATP-dependent Ca^{2+} pumps in the cell membrane and organelles (e.g., mitochondria, vacuoles, and endoplasmic reticulum) regulate the distribution of Ca^{2+} ions inside and outside the cell and organelles. The influx of Ca^{2+} ions activates potassium (K^+) channels, leading to depolarization of the plasma membrane potential (Vm). Subsequently, various calcium receptors such as CBL–CIPK complexes (calcineurin B-like proteins and their interacting protein kinases), CML42/CML43 (calmodulin-like proteins 42/43), and CPK3/CPK13 (calcium-dependent protein kinases 3/13) are activated, which in turn trigger the activation of transcription factors such as HSFB2A. This transcriptional regulation in the nucleus ultimately induces plant defense mechanisms against herbivores (Adapted from Mostafa et al., 2022).

Insect feeding triggers a dramatic influx of Ca^{2+} ions into the cytosol, concentrated in several layers of cells surrounding the wounded area (Maffei et al., 2004; Howe and Jander, 2008). The fact that neither single nor repeated mechanical damage alone induces significant changes in [Ca^{2+}]cyt suggests that the true triggers are oral secretions (elicitors) associated with insect herbivory (Bonaventure et al., 2011). Insect herbivory and isolated insect-derived elicitors are known to disrupt Ca^{2+} homeostasis through the tight regulation of ion channels and transporters located in the plasma membrane and organelle membranes of plants, as well as through Ca^{2+} sensors like calmodulin (Arimura and Maffei, 2010; Batistic and Kudla, 2012).

1.5.5 Activation of NADPH Oxidase and the Production of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS)

Nicotinamide adenine dinucleotide phosphate oxidase (NADPH), also known as NOX, is an enzyme complex located on the plasma membrane that utilizes NADPH as an electron donor and catalyzes the production of superoxide radicals (O_2^-) in the apoplast (Sagi and Fluhr, 2006). These enzymes, also referred to as respiratory burst oxidase homologs (RBOH), play a crucial role in the production of reactive oxygen species (ROS) in plants. NOX/RBOH are considered molecular "hubs" in ROS-mediated signaling pathways, which has recently brought them considerable attention. Increasing numbers of NOX/RBOH gene homologs have been identified in various plant species (Chang et al., 2016).

Upon interaction with calcium sensors (CaM) the activity of NADPH oxidase RBOH is enhanced through phosphorylation mediated by a kinase. This kinase specifically interacts with the N-terminal domain of RBOH as well as the full-length RBOH protein in plant cells. Additionally, CIPK phosphorylates RBOH, and the coexpression of CBL significantly increases ROS production via RBOH in cells. These findings reveal a direct connection between Ca^{2+} mediated signaling by CBL-CIPK complexes and ROS signaling in plants, providing evidence for the synergistic activation of NADPH oxidase RBOH through Ca^{2+}-induced phosphorylation by CBL–CIPK complexes (Drerup et al., 2013).

As mentioned in the previous paragraph, ROS are primarily generated in plants through the action of NADPH oxidase, which is activated by Ca^{2+} ions. This enzyme produces O_2^-, which is then converted into hydrogen peroxide (H_2O_2) in the plasma membrane (Marino et al., 2012; Poór, 2020). In plants, ROS exist in two main forms: ionic and molecular. The ionic forms include hydroxyl radicals (•OH) and superoxide anions ($O_2^{\cdot-}$), while the molecular forms mainly consist of H_2O_2 and singlet oxygen ($^1O_2$) (Apel and Hirt, 2004; Mittler et al., 2017). Each type of ROS has a specific oxidative capacity and influences different physiological and biochemical processes, regulated by various genes in plants. Singlet oxygen ($^1O_2$), an excited form of oxygen, is typically produced in photosystem II (PSII) in chloroplasts and has strong oxidative properties. Although $^1O_2$ exists in cells for only a very short time and is extremely unstable, its production significantly impacts photosynthesis. Superoxide anion ($O_2^{\cdot-}$) acts as a precursor for various ROS due to its instability and strong oxidative/reductive properties. It also plays a role in maintaining the stability of plant stem cells (Zeng et al., 2017).

However, excessive levels of $O_2\cdot^-$ can lead to an increase in ROS levels, ultimately resulting in cell death (Gill and Tuteja, 2010).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are inevitable byproducts of aerobic metabolism, playing a crucial role in signal transduction across all forms of life (Del Rio, 2015; Turkan, 2018). The precise mechanism of RNS in plants remains unclear; however, nitric oxide (NO) is known to contribute to plant stress tolerance and acts as a signaling molecule during herbivore attacks (Turkan, 2018). While NO was initially associated with plant defense responses against pathogens, its involvement in plant responses to insect attacks has also been recognized (Wuensche et al., 2011a). The chemistry of RNS encompasses a wide range of redox species with diverse functional properties (Turkan, 2018; Del Rio, 2015). Under stress conditions, neutral NO is attacked by various nucleophiles and aromatic compounds, leading to the formation of nitrosonium (NO^+) and nitroxyl anion radical (NO^-) through a series of substitution reactions (Del Rio, 2015). NO can also react with O_2 and several transition metals, such as Fe and Cu, in aqueous solution, resulting in the formation of peroxynitrite via a second-order reaction (Palma et al., 2020). Additionally, RNS can form non-heme transition metal complexes of potential biochemical interest, which simultaneously trigger post-translational modifications (PTM) (Rai, 2023). These PTMs induce significant changes in the localization, structure, function, and stability of proteins, thereby affecting their overall transcriptome (Del Rio, 2015; Lubega et al., 2021).

Reactive oxygen species (ROS), such as superoxide anion ($O_2\cdot^-$) and hydrogen peroxide (H_2O_2), are well-known for their key role in regulating a wide range of biological processes that affect plant development and growth, as well as their adaptation to biotic and abiotic stresses (Kaur et al., 2014). The accumulation of ROS has been observed following both leaf-chewing insect attacks (Louis et al., 2013) and sap-sucking insect infestations (Goggin et al., 2022). The rapid production of ROS, known as a "burst," is a conserved signaling mechanism in immunity across kingdoms. Once plant pattern recognition receptors (PRRs) (more details in chapter **1.5.1**) are activated by PAMPs, an intracellular activation cascade of mitogen-activated protein kinases (MAPK) is triggered (Kawasaki et al., 2017; Wang, 2023).

1.5.6 Activation of Mitogen-Activated Protein Kinases (MAPK)

An early signaling event that occurs after a herbivore attack is the activation of mitogen-activated protein kinases (MAPKs). Growing biochemical and genetic evidence

has revealed that MAPKs play a critical role in plant resistance to herbivores. Upon herbivore attack, plants activate MAPK signaling, which subsequently alters the levels of phytohormones, including jasmonic acid (JA) and ethylene, thereby reshaping the transcriptome and proteome in preparation for defense against the attack (Hettenhausen et al., 2015). MAPKs are part of well-conserved eukaryotic signaling cascades that regulate numerous cellular responses (Herskowitz, 1995). The MAPK cascade occurs in three steps: MAPKKK phosphorylates MAPKK, which then phosphorylates MAPK. Activated MAPKs phosphorylate their substrates, most of which are transcription factors and enzymes, triggering stress-related responses (Hazzalin and Mahadevan, 2002). MAPK cascades are involved in signaling multiple defense responses, including the biosynthesis/signalization of plant stress/defense hormones, production of reactive oxygen species (ROS), stomatal closure, activation of defense genes, phytoalexin biosynthesis, strengthening of the cell wall, and hypersensitive response cell death (apoptosis). However, pathogens use effectors to suppress the activation of plant MAPKs and subsequent defense responses to promote pathogenesis (Meng, 2013). Since their discovery in plants in 1993 (Duerr et al., 1993), numerous components of MAPK pathways have been described in biotic and abiotic stress signaling (**Figure 10**) and developmental processes (Rodriguez et al., 2010).

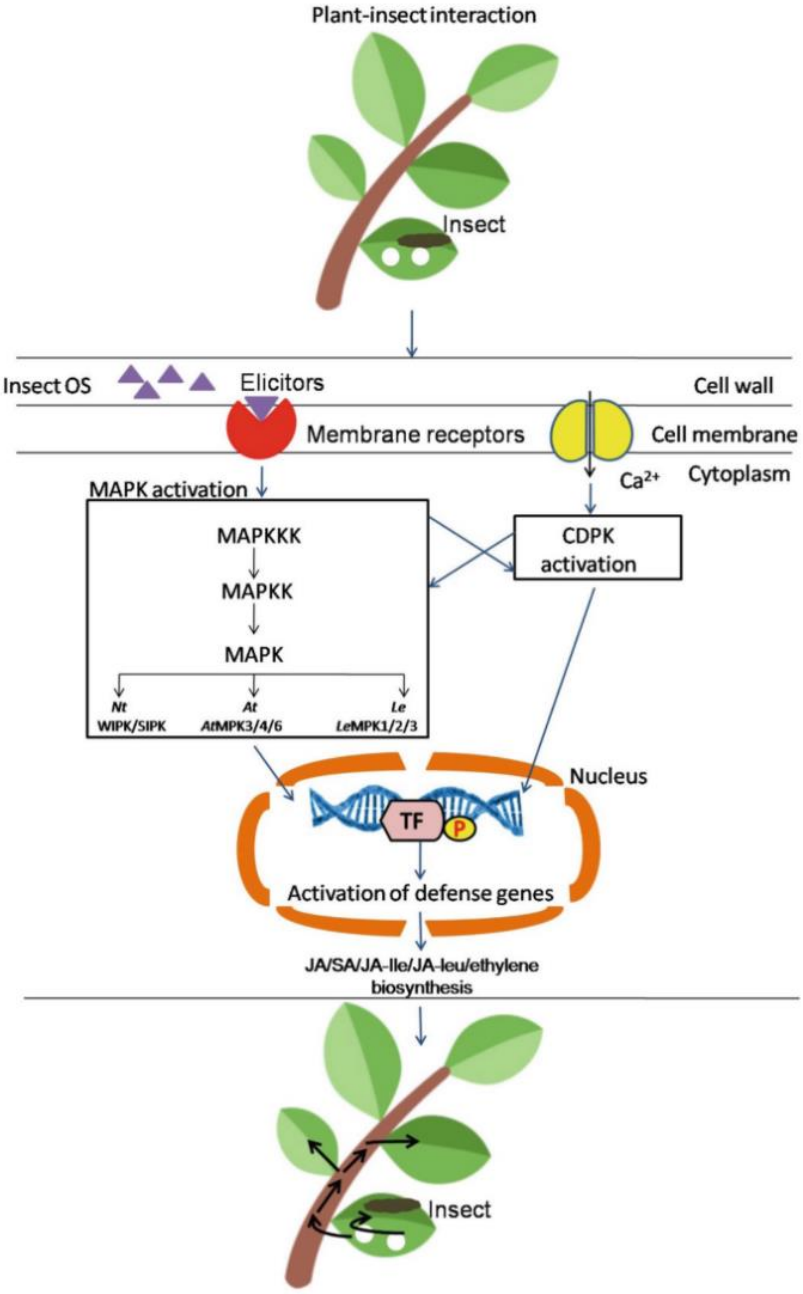

Figure 10 When an insect feeds on plant tissues, it triggers a defense response in the plant. Insect oral secretions (OS) contain elicitors that bind to plant cell surface receptors, activating a signaling pathway called the MAPK cascade. This cascade involves three kinases: MAPKKK (MAPK kinase kinase), MAPKK (MAPK kinase), and MAPK (mitogen-activated protein kinase). Different plants use specific MAPKs, such as WIPK/SIPK in tobacco, MPK3, MPK4, and MPK6 in Arabidopsis, and MPK1, MPK2, and MPK3 in tomato. In parallel, calcium ions (Ca^{2+}) enter the cytoplasm, activating CDPK (calcium-dependent protein kinase), which also affects transcription factors in the nucleus. This leads to the activation of transcription factors (TFs) involved in defense genes and the production of signaling phytohormones such as jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) (Adapted from Manjeet and Yadav, 2021).

Over the years, details have been gradually added that provide a more comprehensive understanding of how the MAPK cascade functions. Specifically, the MAP2Ks MKK4 and MKK5, along with MAPKs MPK3 and MPK6, collectively stimulate ethylene biosynthesis in response to wounding (Li et al., 2018). Early studies also suggested that this MAPK cascade plays a crucial role in regulating the production

of jasmonic acid (more details in chapter **1.5.7**) (Ahmad et al., 2016). In tobacco lines with modified expression of the WIPK (wound-induced protein kinase), it was observed that JA levels increase following wounding with overexpression of WIPK, while they decrease when WIPK is silenced through RNA interference (RNAi) (Seo et al., 2007). Additionally, it was found that JA can modulate MPK6 activity, suggesting a feedback mechanism that fine-tunes the JA balance in plants (Takahashi et al., 2007). MAPKs are classified into four groups (A to D), with MPK3 and MPK6 belonging to clade A, which is typically activated by stress. However, data indicate that even less well-studied members of the MAPK family may play a role in damage signaling. For example, wounding activates MAPK MPK8 from clade D, as well as MPK1 and MPK2 from clade C (Ortiz-Masia et al., 2007; Takahashi et al., 2011).

1.5.7 Increased Production of Phytohormones

The two most important hormonal signaling pathways associated with induced plant defense are the jasmonic acid (JA) and salicylic acid (SA) pathways, as well as ethylene (ET) (Erb, Meldau and Howe, 2012; Howe and Jander, 2008; Wu and Baldwin, 2010). Meanwhile, gibberellin (GA), abscisic acid (ABA), brassinosteroid (BR), and cytokinin are also occasionally involved in plant-insect interactions (Yang et al., 2015). The JA signaling pathway is primarily thought to be involved in defense against leaf-chewing and mining herbivores, necrotrophic pathogens, bacteria, and nematodes, while the SA signaling pathway is mainly associated with defense against sap-sucking herbivores, biotrophic pathogens, and viruses (Thaler et al., 2012). JA and SA have been shown to exhibit an antagonistic relationship, meaning that JA signaling can suppress the SA pathway, and vice versa (Pieterse, 2012). Microbial symbionts provide herbivores with an advantage by inducing the SA pathway through effectors (more details in chapter **1.5.7**) while simultaneously suppressing JA-dependent defense responses. Although this antagonism has been demonstrated in many plant species, it remains controversial whether there is a universal genetic basis for the crosstalk between JA and SA (Thaler et al., 2012). Additionally, jasmonic acid (JA), a major defense signaling molecule (see below), rapidly accumulates in response to mechanical damage (Glauser et al., 2008; Chehab et al., 2012; Cazzonelli et al., 2014).

Jasmonic acid (JA) signaling is induced by chemical signatures like HAMPs, DAMPs, and wounding, leading to increased accumulation of jasmonoyl-L-isoleucine (JA-Ile), which regulates most of the plant's defense responses. JAZ proteins contain a

Jas domain at the C-terminus and a ZIM domain at the N-terminus. Under normal conditions, **JAZ** proteins act as repressors of JA signaling. The C-terminal Jas domain binds to transcription factors such as **CORONATINE INSENSITIVE1** (*COI1*), a component of the ubiquitin E3 ligase **SCFCOI1**, **MYC2**, or the JA-Ile receptor, serving as a protein-protein interaction surface (Yan et al. 2009). These transcription factors are downregulated by the accumulating **JAZ** proteins under non-stress conditions.

Upon mechanical wounding or insect attack, jasmonoyl-L-isoleucine accumulates, causing the **COI1-JAZ** interaction and ubiquitin-dependent degradation of **JAZ** proteins via the 26S proteasome (Koo et al. 2009; Sheard et al. 2010). This degradation releases transcription factors like **MYC2**, which promotes the production of proteins involved in defense responses while inhibiting genes related to vegetative growth. Plant defense against insect attacks comes at a metabolic cost to growth, representing a balancing act to optimize overall plant health (Huot et al. 2014).

Although the JA pathway has been extensively studied, some questions remain unanswered. For example, the connection between early defense signaling and the activation of acyl-lipid hydrolases in chloroplasts to initiate JA biosynthesis is still largely unknown. A relatively recent study in *Arabidopsis* by Yan et al. (2018) demonstrated that Ca^{2+}/CaM-dependent phosphorylation of **JAV1** leads to its degradation by the proteasome, disrupting the nuclear repressive complex JAV1-JAZ8-**WRKY51**, which inhibits the expression of JA biosynthesis genes. Provides the first coherent model linking herbivore-induced cytosolic [Ca^{2+}]. It is worth noting that JA accumulates within 30 seconds in wounded tissues (Glauser et al., 2009), suggesting that the initial burst of JA does not require transcriptional activation of JA biosynthesis genes and that JAV1-mediated regulation may instead serve as a secondary amplification step (Erb and Reymond, 2019).

When placing the induction of phytohormones in the context of the plant defense response cascade, after the depolarization of cell membranes and activation of Ca^{2+} influx (the earliest events), a large number of downstream signaling components, such as CDPK and NADPH oxidases (RBOH), are activated. This activation leads to the production or enhanced accumulation of reactive oxygen intermediates (ROI)/(reactive oxygen species (ROS) (Koo et al., 2009; Sheard et al., 2010; Huot et al., 2014).

MAPKs (e.g., SIPK - Salicylic acid-Induced Protein Kinase and WIPK - Wound-Induced Protein Kinase) are rapidly activated after herbivory to regulate gene expression and JA and ET biosynthesis. Evidence also suggests that MAPK activation is necessary

for activating NADPH oxidases and **WRKY** transcription factors. SIPK is also likely involved in nitric oxide (NO) production. ROS and NO modify amino acids in regulatory proteins as a redox mechanism to convert secondary signals into transcriptional activation of defense-related genes (**Figure 11**). The production and signaling of SA are inherently linked to the formation and signaling of ROS. For example, in *N. attenuata*, LecRK1 suppresses SA bursts triggered by *M. sexta*. GOX (glucose oxidase) in the saliva of some Lepidoptera larvae contributes to H_2O_2 production and suppresses induced defense responses (Bonaventure, 2012).

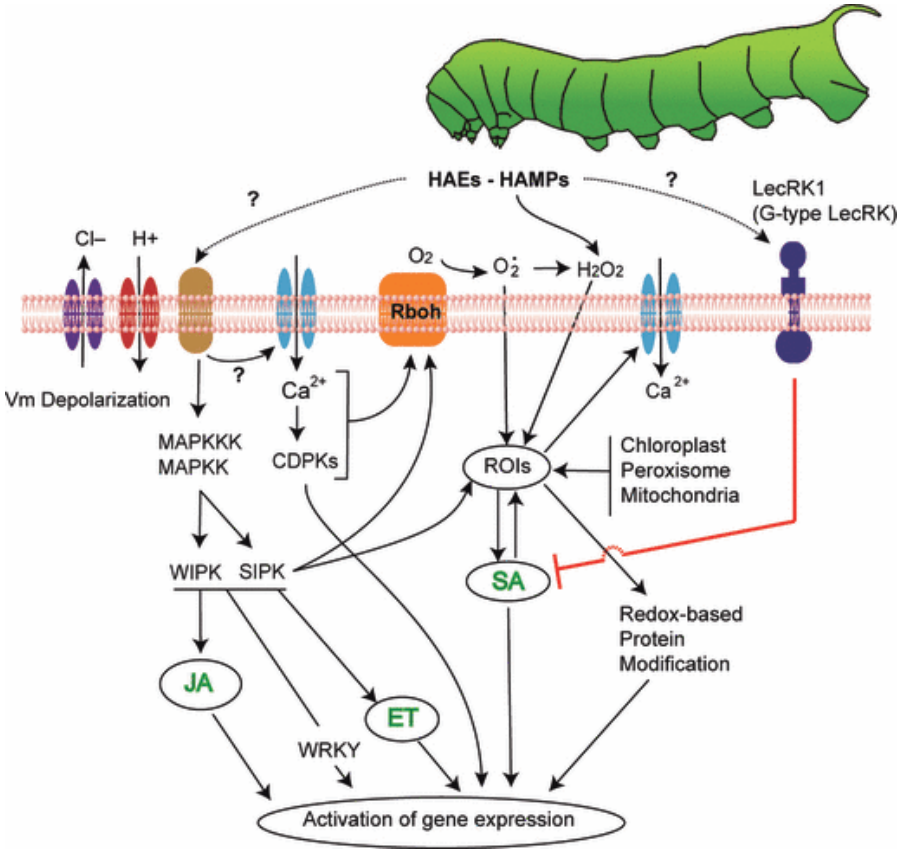

Figure 11 Summary of the main cellular events with the indicated phytohormones signaling cascades activated in plants when attacked by an insect herbivore (Adapted from Bonaventure, 2012).

The salicylic acid (SA) pathway is generally crucial for deterring sap-sucking insects or biotrophic pathogens, and plays a key role in PTI/effector-triggered immunity (ETI; Pieterse et al., 2012; Thaler et al., 2012). SA, a phenolic compound, is primarily synthesized from the precursor chorismate via **ISOCHORISMATE SYNTHASE 1** (*ICS1/SID2*). Activation of the lipase-like protein **ENHANCED DISEASE SUSCEPTIBILITY 1** (*EDS1*) and its interacting partner **PHYTOALEXIN DEFICIENT 4** (*PAD4*) by biotrophic pathogens triggers the accumulation of SA (Vlot et al., 2009).

Functionally, SA regulates basal defense by activating mechanisms associated with systemic acquired resistance (SAR) through R-gene signaling (Gao et al., 2015). SA synthesis, activation, and signaling can result in the accumulation of reactive oxygen species, callose deposition, activation of pathogenesis-related (PR) proteins, and induction of the hypersensitive response (Luna et al., 2011; Voigt, 2014). SA is an essential prerequisite for the activation of SAR (Ellili et al., 2017). Antagonism between SA-mediated and JA/ET-mediated defense pathways has been observed, especially in response to viruses, insects, and necrotrophs (Guerreiro et al., 2016; Yuan et al., 2017). However, there is also evidence of synergism between these pathways (Yang et al., 2015). Phenolic metabolites (more details in chapter **1.5.9**), which contain structures similar to salicylic acid (SA), are the main non-structural components of the leaves, shoots, and roots of *Populus*. These so-called phenolic glycosides (PGs) are taxonomically restricted to the Salicaceae family, where they are known to mitigate insect and animal herbivory. Although common PGs, such as salicin, salicortin, and their derivatives, contain a salicylic group, a direct metabolic relationship between PGs and SA in *Populus* has not been demonstrated (Tsai et al., 2011).

1.5.8 Transcriptomics and Expression of Defense Genes

Transcriptomics and the expression of defense genes are key areas of plant biology focused on understanding how plants respond to environmental stresses, such as insect herbivory, pathogen attacks, or physical damage, at the molecular level over time (Lowe et al., 2017; Li et al., 2021). The term "transcriptome" is defined as "the complete complement of mRNA molecules produced by a cell or population of cells." The term was first proposed by Charles Auffray in 1996 (Piétu et al., 1999) and was first used in a scientific publication in 1997 (Velculescu, 1997). Transcriptomics belongs to the family of "omics" analytical techniques, such as genomics, proteomics, metabolomics, phenomics, epigenomics, microbiomics and ionomics (Cavill et al., 2015; Shen et al., 2023).

Inside organisms, genes are transcribed and spliced (in eukaryotes) to produce mature mRNA transcripts (**Figure 12**). mRNA is extracted from the organism, fragmented, and reverse transcribed to generate stable double-stranded cDNA (ds-cDNA). The ds-cDNA is sequenced using high-throughput sequencing methods with short reads. These sequences can then be compared to a reference genomic sequence to reconstruct which genomic regions have been transcribed (Tachibana, 2015).

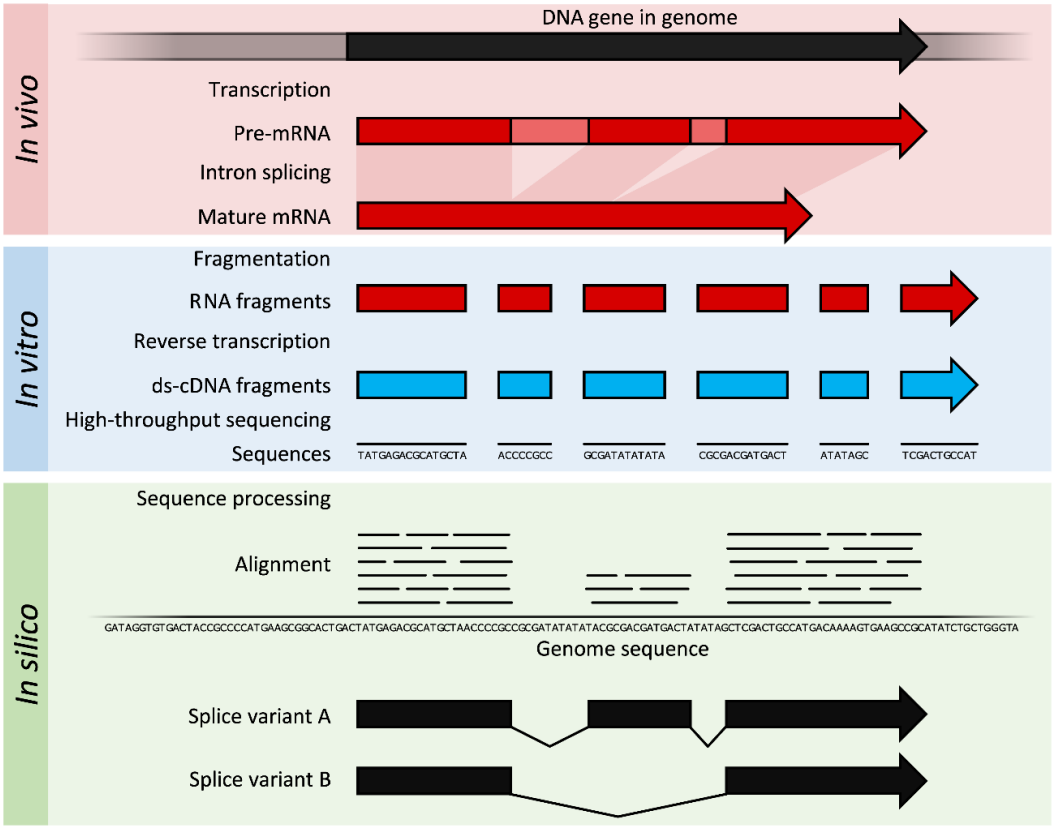

Figure 12 Summary of RNA Sequencing process (Adapted from Lowe et al., 2017)

This data can be used to annotate where expressed genes are located, their relative expression levels, and any alternative splicing variants (Lowe et al., 2017; Tachibana, 2015).

Many omics strategies have been used to assess plant-aphid and plant-insect interactions, either individually or in combination (e.g. Sanchez-Acros et al., 2019; Erb and Reymond, 2019; Zogli et al., 2020; He et al., 2020). These studies typically reveal extensive changes in transcriptomes and metabolomes in response to insect herbivory. A common feature across many of these interactions is the documentation of changes in plant hormone levels, such as jasmonic acid (JA), its active form JA-isoleucine (JA-Ile), and the JA precursor 12-oxo-phytodienoic acid (OPDA), along with other hormones like salicylic acid (SA) and abscisic acid (ABA). These hormonal changes subsequently influence the expression levels of transcription factors (TFs), which, as several studies suggest, are regulated by microRNA (miRNA) (Lima et al., 2012; Koroban et al., 2016; Samad et al., 2017). miRNAs are a class of small (20-24 nucleotide long), non-coding RNAs that play a major role in the post-transcriptional regulation of gene expression by either degrading their target mRNA or inhibiting the translational machinery (Baulcombe, 2004).

Transcription factors (TFs) are sequence-specific DNA-binding proteins that identify and bind specifically to cis-regulatory sequences in the promoter regions of target genes. They can either activate (upregulate) or repress (downregulate) the expression levels of these genes in response to developmental and external stimuli (Patra et al., 2013). In plants, TFs code for up to 10% of the total genes at various stages, thereby regulating signal-mediated gene expression (Baillo et al., 2019). Major TF families known for their role in regulating plant defense networks include **WRKY**, **MYB**, **ERF**, **NAC**, **bHLH**, and **bZIP**. These families control the expression levels of their related genes and pathways. The outcome of these changes in TF expression levels is the modulation of primary and secondary metabolic pathways, which trigger defense responses to mitigate herbivory (Maag et al., 2015; Castano-Duque and Luthe, 2018).

The most significant **WRKY** TF family has been extensively studied in plants under stress conditions. The inducible expression pattern of **WRKY** genes supports their involvement in modulating the biosynthesis of defense-related secondary metabolites (SM). Activation of **WRKY** TFs, in conjunction with various signaling cascades, such as those involving salicylic acid (SA), abscisic acid (ABA), jasmonic acid (JA), ethylene (ET), mitogen-activated protein kinases (MAPK), calcium-dependent protein kinases (CDPK), and reactive oxygen species (ROS), triggers defense mechanisms against environmental stimuli (Goyal et al., 2023). **WRKY** family members possess a conserved 60-amino acid domain responsible for gene regulation and interaction with W-boxes in target promoters. Plant **WRKY** TFs are largely involved in stress responses; they can be regulated by wound signaling or jasmonic acid and alter the expression of genes involved in the biosynthesis of various SMs, such as alkaloids, terpenoids, and their subclasses (Phukan et al., 2016; Long et al., 2023). Javed et al. (2022) and Song et al. (2023) agree that the **WRKY** family may act as a major regulator balancing growth and defense.

The second most discussed family is the **MYB** proteins, which are among the most abundant transcription factor families in the plant kingdom. Among various TFs, **MYB** proteins are involved in the biosynthesis of secondary metabolites (SM) and participate in various biological processes in plants, such as growth, reproduction, and stress responses. **MYB** TFs are characterized by different numbers of DNA-binding domains consisting of 50–53 amino acids with four imperfect repeats. They can be categorized into four subclasses: R1, R2, R3, and R4, depending on the repeats of the DNA-binding domain. The R2R3 **MYB** TF family is significantly associated with the regulation of various SM pathways in different plant species. For example, *AtMYB113*, *AtMYB114*,

AtMYB75, and *AtMYB90* in *Arabidopsis thaliana* are potentially involved in regulating anthocyanin residues through changes in the phenylpropanoid pathway (Gonzales et al., 2008). In poplars, for instance, the gene *PtMYB115* binds to the promoter regions of the *ANR1* and *LAR3* genes to enhance their expression, resulting in higher accumulation of proanthocyanidins and increased resistance to pathogens. However, little is known about the characterization and functions of **MYB**-related proteins in *Populus*, an important model and commercial tree species (Wang et al., 2017; Yang et al., 2021).

Through the regulation of transcription factors and genes involved in the response to insect herbivory, secondary metabolites are typically produced in plants; however, their biosynthesis can be stimulated by external signals, enhancing plant resistance or tolerance to stress conditions. This chapter aims to demonstrate that selected specific genes and transcription factors (such as **WRKY**/**MYB**) regulate responses to specific biotic stress.

1.5.9 Metabolomics and Production of Secondary Metabolites

Metabolomics (part of the "omics" tools group) is a complex interdisciplinary research field requiring knowledge in biosciences, analytical chemistry, organic chemistry, chemometrics, and informatics. Metabolomics involves comprehensive profiling of metabolites present in an organism (Okazaki and Saito, 2012). In terms of metabolic function, it follows proteomics and transcriptomics (previous chapter), which are considered as the flow of media related to genetic information (**Figure 13**). In contrast, metabolomics should be viewed as focusing on the phenotype (Fukusaki and Kobayashi, 2005). The term metabolomics has entered common usage, and it is often defined by Fiehn (2008) as focusing on a better understanding of biological networks through precise and extensive research of metabolism.

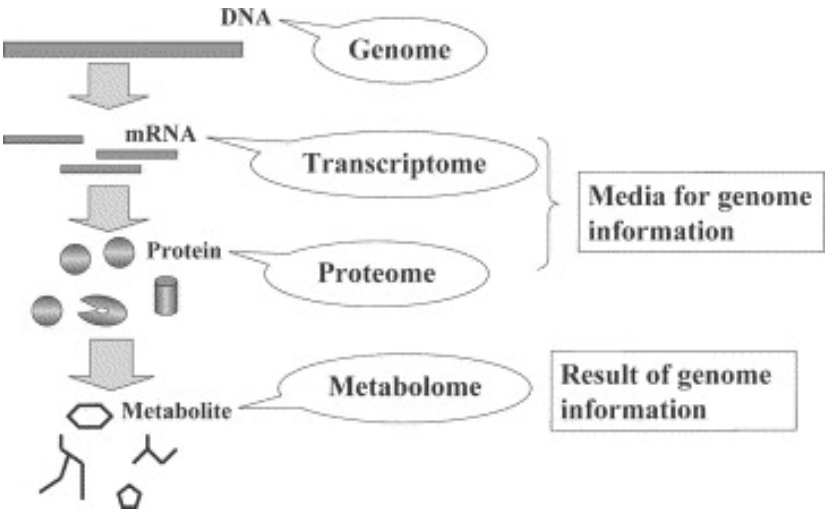

Figure 13 While transcriptomics and proteomics focus on genetic information and its expression in RNA and proteins, metabolomics provides information about the resulting phenotype, namely the metabolic outputs of these processes. (Adapted from Fukusaki and Kobayashi, 2005)

In the plant defense cascade in response to insect herbivory, the production of secondary metabolites represents the peak of induced defense (**Figure 12**) (Maffei et al., 2007; Karban, 2010). Secondary metabolic pathways generate compounds that do not directly contribute to plant growth but are crucial for their survival in the environment. These pathways utilize resources and biosynthetic enzymes derived from primary metabolism, which is responsible for plant growth, development, and reproduction and is constitutively stored in plant cells (Fernie and Tohge, 2017; Velu et al., 2018; Movahedi et al., 2021). Although induced responses have certain metabolic costs (Agrawal et al., 2002), they are essential for mitigating immediate stress because some of these chemicals are produced in response to herbivore attacks (Miranda et al., 2007).

Secondary metabolites are classified based on their chemical structure and biochemical effects (Movahedi et al., 2021). Induced defense mechanisms render plants phenotypically plastic, reducing the chances of attacking insects adapting to these chemicals (Agrawal et al., 2011). Nearly 200,000 secondary metabolites have been isolated and characterized, a small number compared to the total number of described plant species (Willis, 2017). Plants indirectly defend themselves against herbivory by releasing a mixture of volatile and non-volatile compounds. Volatile organic compounds (VOCs) emitted by plants in response to herbivory (HIPVs – herbivore-induced plant volatiles) play a crucial role in plant defense by either attracting natural enemies of herbivores or acting as deterrents to feeding and/or oviposition (Dudareva et al., 2006).

Plant secondary metabolites are generally classified into three broad categories:

- Phenolics;
- Terpenoids;
- Sulfur-containing metabolites;
- Nitrogen-containing metabolites.

Phenolic compounds (polyphenols) represent the largest, most diverse, and most widespread class. There are thousands of polyphenolic compounds found in plants, synthesized through phenylpropanoids and/or polyketides derived from shikimic acid (**Figure 14**) (Cheynier et al., 2013; Patra et al., 2013; Divekar et al., 2022). They have a basic structure consisting of a benzene ring with an attached hydroxyl group, without any nitrogen-based functional groups (Lattanzio, 2013; Jan et al., 2021).

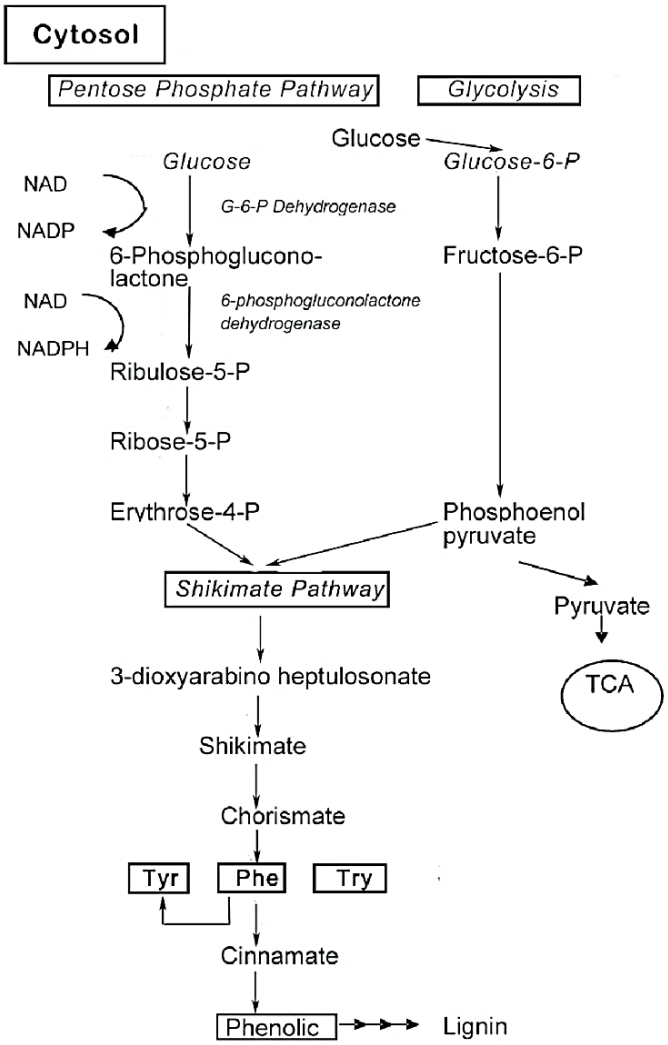

Figure 14 Biosynthesis of phenolic compounds in the pentose phosphate, shikimate and phenylpropanoid pathways in plants (Adapted from Lin et al., 2010)

L-phenylalanine is a primary compound in this pathway, serving as the precursor for the subsequent synthesis of other polyphenols (Singh et al., 2021). Additionally, it

exhibits antioxidant properties against oxidative damage in plants, such as that caused by harmful UV radiation. Furthermore, flower and leaf pigmentation is due to a complex phenolic molecule known as anthocyanin. Flavonoids (a subclass of phenols) play a crucial role: they act as attractants for pollinators, quench ROS, can serve as anti-feedants, and may reduce palatability for insects (Sosa et al., 2004; Crozier et al., 2006; Butelli et al., 2008).

It has also been found that plants sequester phenolic compounds in the cell vacuole to rapidly respond to any future attacks (Beckman, 2000; Brillouet et al., 2014). These compounds are not only toxic to herbivores but have also been shown to be toxic to plants themselves. As a result, plants tend to store them in specialized structures known as phenyloplasts—thylakoid membrane cells produced through the redifferentiation of primary cells. To store polyphenols in these cells, they are first detoxified by conjugation with glycosides to form phenylglycosides, making them hydrophilic and reducing their toxicity (Gachon et al., 2005). Once these molecules are filled with polyphenolic compounds, they move inside the vacuoles. At the onset of herbivory, signaling molecules such as reactive oxygen species (ROS) are produced by insect oral secretions (more details in chapter **1.4**), leading to oxidative stress within the cell. This stress causes phenyloplasts to break down their outer protective layer, releasing various polyphenolic compounds (Gachon et al., 2005). These compounds are released from the vacuole, cross-link and/or polymerize the cell wall, providing the plant with mechanical strength and rigidity, creating a tougher barrier for herbivores to continue leaf-chewing and sap-sucking. Additionally, phenolic compounds act as proteinase inhibitors, binding to several essential enzymes within the insect's body, thus impairing critical physiological processes, digestive abilities, and nutrient uptake (Kessler and Baldwin, 2002).

Terpenes are among the most diverse known secondary metabolites, synthesized from acetyl-CoA and glycolytic intermediates. More than 30,000 different terpenes are known (Keeling and Bohlmann, 2006). They are formed by the fusion of 5-carbon units called isoprenes, resulting in a branched backbone. Terpenes are categorized into monoterpenes, diterpenes, sesquiterpenes, triterpenes, tetraterpenes, and polyterpenes, each playing a significant role in defense against pathogens and herbivores. Their biosynthesis involves two main pathways: the mevalonic acid pathway and the methylerythritol phosphate pathway, which occur in plastids and are known to produce both isopentenyl diphosphate and dimethylallyl diphosphate, the basic building blocks for terpene synthesis (Khare et al., 2020). Some terpenes, such as gibberellins (diterpenes)

and brassinosteroids (triterpenes), are recognized as hormones and play crucial roles in growth and development. Terpenes like limonene and menthol also serve as deterrents against herbivores (Lin et al., 2017).

Nitrogen-containing secondary metabolites and precursors, including amino acids such as lysine, tyrosine, and tryptophan, are significant. These primarily include cyanogenic glycosides and non-protein amino acids. Alkaloids such as morphine, berberine, vinblastine, and scopolamine have pharmaceutical properties, while cocaine, caffeine, and nicotine exhibit sedative and stimulating effects. Most alkaloids are toxic; for instance, pyrrolizidine alkaloids contribute to defense against microbial infection and herbivory (Irmer et al., 2015; Stella et al., 2018).

Sulfur-containing secondary metabolites represent a group of plant secondary metabolites (PSMs) that includes approximately 200 compounds. These sulfur-containing PSMs encompass glucosinolates, glutathione, glycosphingolipids, phytoalexins, alliin, thionins, and defensins (Venditti and Bianco, 2020). They are directly associated with plant defense against microbial pathogens. These metabolites are known for their diverse biochemical structures and modes of action, which are reported to provide plants with a broad range of chemical defense mechanisms to protect against various potential enemies (Khare et al., 2020). Glucosinolates are nitrogen- and sulfur-containing glycosides that are effective against herbivores, competitors, and parasites (Bloem et al., 2007). Damage to plant cells caused by herbivores leads to the breakdown of glucosinolates by myrosinases, resulting in the production of toxic metabolites such as nitriles, thiocyanates, and isothiocyanates. These glucosinolate breakdown products are as effective as synthetic insecticides (Liu et al., 2000).

This concludes the introduction of this dissertation, which aims to provide the reader with a comprehensive overview of plant-insect interactions. Key aspects of chemical ecology in this relationship were presented, including the role of insect oral secretions, the mechanisms by which plants perceive specific chemical molecules, the activation of signaling pathways, and the subsequent defense cascade that leads to the production of secondary metabolites. The introduction was designed to lay the foundation for understanding the complexity of these biological interactions, which are discussed in greater detail in the following scientific publications.

2 Objectives of the study

1. The strategic goal is to quantify the stress response to insect herbivore attacks (analyzing the composition and quality of primarily secondary metabolites);

2. Optimization of methodological procedures/experimental design to enable the integration of physiological measurements, analysis of the chemical profile of metabolites, and molecular-level responses (gene expression) in cultivated aspen poplar saplings;

3. Transcriptome analysis – description and delineation of transcriptional changes in response to insect herbivory, with the metabolic pathway identified based on a review of the scientific literature;

4. Identification and description of unique secondary metabolites specific to the type of insect herbivory;

5. Validation and integration of generated data from the described approaches within a single manipulative experiment.

3 Materials and Methods

The aim of this chapter is to summarize the development of methodologies and approaches throughout the course of the study. It focuses on tracking the sequence of events and adjustments to the methodology that were not detailed in the published articles. A detailed methodological description for each sub-experiment is provided in the publications.

3.1 Optimization of Experimental Design

Based on the review of scientific literature and existing knowledge, critical areas were identified that needed to be addressed before starting the actual experiment:

- Selection of the model plant species
- Elimination of unwanted biotic influences
- Optimal abiotic environmental factors
- Provision and selection of insect herbivores
- Technical equipment for the experiment
- Experimental design
- Sample collection – verification of methodology

3.2 Selection of the model plant species

The original target species were chosen for their economic significance and value: English oak (*Quercus robur*) and European beech (*Fagus sylvatica*). However, they proved unsuitable due to relatively slow growth, susceptibility to pathogens (*Erysiphe alphitoides*), and contaminated soil with various types of arthropods. They were replaced with the general model species, European aspen (*Populus tremula*). To enhance statistical reliability, the plants were propagated in sufficient quantities and under sterile conditions using somatic embryogenesis tools in vitro (Murashige and Skoog, 1962). During ex vitro cultivation, the plants were fertilized with NPK fertilizer every two weeks during the growing season.

Throughout the experiment, strict adherence to the issues discussed by Trethewey (2004) was maintained, such as rationalizing procedures to minimize variations between plants and ensuring uniformity (manipulation, watering, fertilization, and placement of plants in growth chambers) of all cultivated specimens. Fukusaki and Kobayashi (2005)

confirm this in their publication, emphasizing that a careful approach and expert "know-how" are essential for this type of experiment. Inappropriate interventions or insufficient care of plants can significantly affect analysis results by altering their metabolic profile, which may lead to incorrect interpretation of results. Additionally, it would undermine the principle of reproducibility, which is essential for ensuring that experimental results can be reliably repeated.

3.3 Elimination of unwanted biotic influences

The transfer of in vitro sterile European aspen seedlings to ex vitro conditions was carried out in a laboratory environment. A steam-sterilized growth substrate was chosen to eliminate the possibility of contamination by biotic pathogens (fungi, microbiota, arthropods). Immediately after the transfer to ex vitro conditions, the seedlings were placed in growth chambers Step-In FytoScope FS-SI (Photon Systems Instruments, Drašov, Czech Republic).

3.4 Optimal abiotic environmental factors

The aspens placed in the growth chambers were always arranged so that they did not shade each other and had sufficient space around them. The abiotic variables (spectral composition of light, proportion of far-red light, temperature, intensity and composition of photosynthetically active radiation (PAR), light and dark periods, CO_2 concentration, and relative humidity) were set to match the optimal conditions for the target species.

3.5 Provision and selection of insect herbivores

When selecting insect species for the study, their affiliation with feeding guilds (more details in chapter **1.3**) was considered. Therefore, two of the most common feeding strategies were chosen: **leaf-chewing** and **sap-sucking** representatives.

The leaf-chewing insect selected was the well-known lepidopteran *Lymantria dispar*. Eggs for rearing were kindly provided by the University of Natural Resources and Life Sciences, Vienna. Upon hatching, the larvae were cultivated in Petri dishes and fed with boiled nutrient agar (*Lymantria dispar* agar, Southland Products Inc., Newark, DE, USA).

Originally, *Corytucha arcuata* was intended as the representative of the sap-sucking feeding guild. Unfortunately, rearing this invasive and currently discussed species proved impossible in the growth chamber facilities, as the insects clustered at the

top of the rearing cage (seemingly attempting to approach the ceiling lights) and did not consume the provided food, leading to their eventual death. In a test experiment conducted under natural daylight, the species thrived and was able to reproduce. Due to the change in the model plant species, aphids *Chaitophorus populialbae* and *Chaitophorus nassonowi* were captured in the wild as sap-sucking insects. These aphids were long-term reared in sterile plastic containers and were provided with fresh aspen seedlings every three days.

3.6 Technical equipment for the experiment

In addition to the equipment listed in the Materials and Methods sections of the individual publications, it was necessary to construct rearing boxes (**Figure 15, G**) for the part of the experiment where aspens were exposed to sap-sucking insect herbivores. The primary purpose of these boxes was to prevent the aphids from escaping and contaminating the growth chamber/laboratory environment. Given the defined environmental conditions—such as minimizing variations among different groups of aspens—the mesh material had to meet technical specifications for light transmission, allowing at least 90% light penetration without affecting its spectral composition, and also had to prevent the insects from accessing the entire space of the growth chamber.

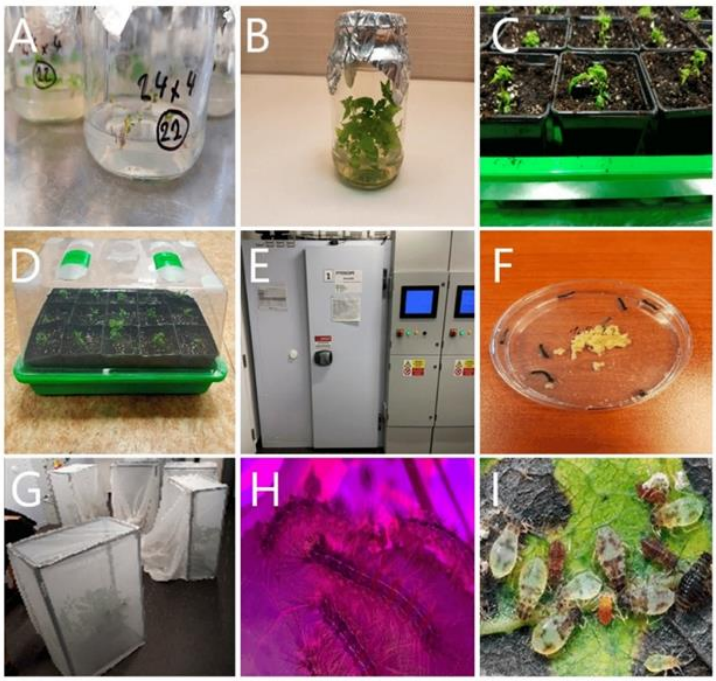

Figure 15 Experimental setup and plant treatment with insect feeding. (A) Poplar tissue propagation on MS medium. (B) In vitro growth of genetically uniform poplars. (C, D) Transfer of in vitro poplars to ex vitro. (E) Poplar plants are grown in the growth chamber. (F) Spongy moth larvae. (G) Aphid treatment setup. (H) Spongy moth feeding on poplar leaf. (I) Aphids feeding on poplar leaf (Adapted from Pastierovič et al., 2024)

3.7 Experimental design

In line with the objectives of the study, the experimental design was developed and consists of three distinct sub-experiments: **1)** Determination of chlorophyll a/b content, proline, selected polyphenolic compounds, and volatile organic compounds (VOCs); **2)** Integration of two 'omics' approaches—transcriptomics and non-target metabolomics; **3)** Implementation of time-segmented sample collection, non-invasive gasometric measurements, quantification of selected polyphenolic compounds, and measurement of carbohydrates in roots and leaves.

This division was essential for conducting the analyses—each leaf was examined after being exposed to herbivore activity for a specific period, which required sufficient plant tissue (from a single leaf) for each type of analysis. Additionally, it was necessary to tailor the sample collection for each sub-experiment to the intended analysis (e.g., transcriptomics and non-target metabolomics) (see the following chapter for details). All sub-experiments utilized clones of a single aspen species (genetically uniform), ensuring consistency across the experiments. All sub-experiments shared a common structure:

- Moths infested;
- Aphids infested;
- Control (without damage).

The strategic goal of the experiment was to precisely and exclusively qualify and quantify changes in plant metabolism in response to target insect species (leaf-chewing and sap-sucking herbivores). It was therefore essential that the aspen individuals entered the experiment in an optimal health state. To prevent chemical communication between aspens, all experimental treatments were strictly separated within individual growth chambers.

The methods for statistical data analysis are detailed in the respective publications (more details in chapters: **4.1**; **4.2** and **4.3**). Depending on the type of data generated, these included hypothesis testing procedures using multifactorial ANOVA. Alternatively, a mixed linear model (more details in chapter **4.3**) was employed using the R software (R Core Team, 2021). "Omics" data were bioinformatically processed using specialized software (SYMCA, OmicsBox transcriptomics module (ver 1.4.11)).

3.8 Sample collection – verification of methodology

The sample collection methodology strictly adhered to the recommendations of Fukusaki and Kobayashi (2005), who emphasized that sample collection is one of the

most critical steps and requires **careful** attention to minimize experimental error. Improper sampling can cause significant experimental variation that may sometimes exceed biological variation.

To minimize phenotypic variation, leaf samples were collected only when they reached full tissue maturity and development. Additionally, circadian and diurnal rhythms were taken into evidence, with samples always collected at the same time of day (a two-hour window was established for sampling). Collected leaves were immediately recorded after cutting with a sterile tool, placed in 50 ml Falcon tubes, and stored in liquid nitrogen before being kept at -80°C.

Before conducting the main experiments, each individual analysis was validated through a series of trial experiments. These trials were specifically designed to optimize the methodological approach, ensuring that all variables were controled and the analytical tools were functioning correctly. This step helped confirm the efficacy and reliability of the techniques used, thus minimizing the risk of experimental errors during the actual sample collection. By conducting these pre-experimental validations, we ensured that the methodologies employed were robust, repeatable, and could accurately capture the biological phenomena under investigation.

4 Results

This dissertation consists of three scientific articles, of which I am the first author, and which were submitted to the editorial boards of scientific journals this year. All articles underwent independent peer review, were accepted, and have been published.

Article I (Pastierovič et al., 2024a). It shows the specific responses of European aspen (*Populus tremula*) to **leaf-chewing** and **sap-sucking** insects. It summarizes the differences in plant metabolic responses to these major types of insect herbivory at the level of induction of selected polyphenolic compounds and volatile organic compounds, as well as changes in the concentrations of proline, chlorophyll a/b, and carotenoids. Biological interpretation and functionality of these changes are provided in relation to the specific insect herbivores.

Article II (Pastierovič et al., 2024b). It builds upon the previous publication, offering a more detailed insight into the restructuring of the transcriptome and changes at the level of all captured metabolites in response to insect herbivory. Utilizing tools from the "omics" family, it integrates results from transcriptomics and metabolomics. Non-targeted metabolic analysis quantifies and qualifies the overall metabolic profile of each treatment in the experiment and provides a cluster analysis of the most important compounds for separation. The transcriptomic section identifies differentially expressed genes and contextualizes them according to their functions within the metabolic pathways of *P. tremula*.

Article III (Pastierovič et al., 2024c). The study presents results from an unconventional methodological approach in experimental plant physiology, where changes in gasometric parameters, photosynthetic efficiency, and concentrations of phenolic compounds and sugars were analyzed at defined time intervals within the first 60 minutes of *Lymantria dispar* feeding.

4.1 Article I.: Biochemical Responses in *Populus tremula*: Defending against Sucking and Leaf-Chewing Insect Herbivores

Published as

Pastierovič, F.; Kalyniukova, A.; Hradecký, J.; Dvořák, O.; Vítámvás, J.; Mogilicherla, K.; Tomášková, I. Biochemical Responses in *Populus tremula*: Defending against Sucking and Leaf-Chewing Insect Herbivores. *Plants* **2024**, *13*, 1243. https://doi.org/10.3390/plants13091243

Author Contributions

F.P.: conceived the presented idea, carried out the experiment, preparation, and processing of laboratory protocols, and wrote the manuscript; A.K.: liquid chromatography and data evaluation; J.H.: gas chromatography and data evaluation; O.D.: carried out the experiment; J.V.: carried out the in vitro preparatory work; K.M.: wrote the manuscript; I.T.: wrote the manuscript All authors have read and agreed to the published version of the manuscript.

Extended summary

The biochemical properties of poplar leaves were examined under the influence of aphids and caterpillars. In European aspen (*Populus tremula*), changes in proline, polyphenolic compounds, chlorophyll a/b, carotenoids, and volatile compounds were monitored between infested and uninfested leaves.

Among nine phenolic compounds, only catechin and procyanidin showed significant differences. GC-TOF-MS analysis revealed distinctions between control leaves and those infested with aphids or caterpillars. Key compounds for aphid-infested leaves were 3-hexenal and 5-methyl-2-furanone, while for caterpillar-infested leaves, they were trans-α-farnesene and 4-cyanocyclohexane. Aphid-infested leaves contained half the chlorophyll and twice the proline compared to uninfested leaves, indicating a greater impact of aphids on plant physiology.

Connection with the objectives of the dissertation

In the context of the **objectives** of the dissertation, this publication reflects the following: **No. 1** quantification of the stress response by analyzing carotenoids, chlorophyll a/b and proline within treatments; **No. 2** Optimizes the methodological procedure by modifying laboratory protocols (microextraction) and a synergistic approach of applied analytical methods; **No. 4** Within the GC-MS separation model, determine the TOP 10 the most decisive volatile compounds for the given treatments.

Implications

Despite the technical capability to identify individual phenolic compounds, hypotheses are often based on total phenolic content (Scogings et al., 2021; Salazar-Mendoza et al., 2024). Our study reveals significant variations in individual phenol concentrations, challenging the interpretation of "total phenolic content." Specific biological impacts on insect herbivores are attributed to individual phenols, highlighting the need for separate analysis.

The separation model identified ten key volatile organic compounds (VOCs) for different herbivory strategies, guiding further research.

A unique laboratory protocol was used, involving microextraction for individual analyses of chlorophyll, proline, VOCs, and phenols from single leaf samples.

plants

MDPI

Article

Biochemical Responses in *Populus tremula*: Defending against Sucking and Leaf-Chewing Insect Herbivores

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Abstract: The main biochemical traits were estimated in poplar leaves under biotic attack (aphids and spongy moth infestation). Changes in the abundance of bioactive compounds in genetically uniform individuals of European aspen (*Populus tremula*), such as proline, polyphenolic compounds, chlorophylls *a* and *b*, and volatile compounds, were determined between leaves damaged by sucking insects (aphid—*Chaitophorus nassonowi*) and chewing insects (spongy moth—*Lymantria dispar*) compared to uninfected leaves. Among the nine analyzed phenolic compounds, only catechin and procyanidin showed significant differences between the control leaves and leaves affected by spongy moths or aphids. GC-TOF-MS volatile metabolome analysis showed the clear separation of the control versus aphids-infested and moth-infested leaves. In total, the compounds that proved to have the highest explanatory power for aphid-infested leaves were 3-hexenal and 5-methyl-2-furanone, and for moth-infested leaves, trans-α-farnesene and 4-cyanocyclohexane. The aphid-infested leaves contained around half the amount of chlorophylls and twice the amount of proline compared to uninfected leaves, and these results evidenced that aphids influence plant physiology more than chewing insects.

Keywords: aphids; carotenoids; chlorophylls *a* and *b*; polyphenolic compounds; proline; spongy moths

Citation: Pastierovič, F.; Kalyniukova, A.; Hradecký, J.; Dvořák, O.; Vítámvás, J.; Mogilicherla, K.; Tomášková, I. Biochemical Responses in *Populus tremula*: Defending against Sucking and Leaf-Chewing Insect Herbivores. *Plants* **2024**, *13*, 1243. https://doi.org/10.3390/plants13091243

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1. Introduction

Global climate change has far-reaching effects on all levels of ecosystems, with a predominantly adverse impact on forests around the world. On a global scale, more than 50% of tree damage can be attributed to biotic factors, with insect herbivores emerging as significant stressors in this context [1]. Although the complex interplay between host trees and insect herbivores is difficult to predict, there is a consensus that the negative changes favour insects [2]. Higher temperatures and drought weaken the trees, thus promoting insect abundance and geographical spread, as well as usually shortening their generation time [3]. Nevertheless, plants have the constitutive and induced defence needed to cope with unpredictable changes in the environment. The constitutive defence is based mainly on the substances that form a mechanical barrier against insects (suberin in cell walls or a waxy layer in the epidermis). Induced defences suggest that individual plants have the capacity to alter their chemical phenotype in reaction to biotic stress, thereby potentially exerting a decisive influence on species interactions over ecological and evolutionary timescales, through modifications in interactions [4]. Research has largely focused on exploring these interactions between a plant and a single attacker, representing a pivotal initial stage in unraveling the chemical ecology of plants. However, as our technical capabilities advance, it becomes imperative for us to delve deeper [5].

Photosynthetic pigments are an essential part of the primary phase of photosynthesis, where ATP and NADPH are generated to later fix CO_2 into carbon products. Therefore, a decrease in chlorophyll *a* and *b* due to various stress factors endangers the survival of the plant by decreasing the assimilation rate and stomatal conductance [6]. On the other hand, the total concentration of carotenoids can increase by up to five times under higher irradiance or in response to biotic attack, as the conversion of violaxanthin to zeaxanthin within the xanthophyll cycle helps to scavenge newly emerging oxygen radicals [7]. Proline, a proteinogenic amino acid with signal function, is on the rise in the case of insect larvae that feed on *Populus* leaves [8]. The higher level of proline enhances the NADP+/NADPH ratio, increasing the oxidative pentose phosphate pathway (OPPP) and producing phenolic compounds directly involved in plant defence [9]. Through underground and aboveground communication channels in the plant, proline serves as a stress marker in the aboveground biomass after root damage caused by, for example, *Melolontha melolontha* [8]. Despite the recorded positive effect of proline on the development of aphid populations, it appears that at higher levels, it acts as a limiting factor [10–12].

The induced defence is based on the metabolites that emerge several minutes after an insect attack [13]. From the four basic pathways of the secondary metabolism (i.e., phenols, flavonoids, terpenes and nitrogen/sulfur) [14], most of the structural and defence compounds are metabolized via the shikimate–phenylpropanoid pathway. Phenolic glycosides, hydroxycinnamates, flavonoids, and condensed tannins are accumulated in the case of biotic stress [15]. Every plant has its typical profile of secondary metabolites with antifeeding activity or another adverse effect on larval development. In poplar (*Populus*) leaves, phenolic glycosides are very common compounds. Catechin, rutin, and quercetin are responsible for a reduced *Lymantria* larvae weight or prolonged larval development [16]. Although these substances are generally present, their concentration increases after an insect attack [17]. Several studies have also confirmed the induction of phenolic glycosides after an insect attack [15,18–20]. The induction of phenolics in trees depends on several factors, including the specific tree species, its genotype, and the species of insect herbivore responsible for the attack [17].

Plants, and especially trees, are the largest source of volatile organic compounds (VOCs) worldwide, both in stressed and non-stressed conditions [19,21]. These compounds are important in plant–insect (including pollinators) or plant–plant communication [22–28]. Insect herbivory changes the rate of plant VOC emission and the types of compounds emitted [29]. Herbivore-induced plant volatiles (HIPV) are stress-induced VOCs released as a response of the plant metabolism to herbivory [11,17]. The type of feeding damage affects the VOCs produced; leaf chewing generally induces jasmonic acid production, while phloem-sucking insect herbivores tend to induce salicylic acid-mediated signalling pathways. Causality between the induction of VOCs after an attack by an insect herbivore has been demonstrated many times [30–32].

Biotic stress alters the pigment, proline, and phenolic compounds in Fabaceae species [33], *Ulmus* [34], and *Populus* [35]. Nonetheless, a number of unresolved issues persist with respect to the variability in responses to the different feeding-type behaviours exhibited by insects, as well as the explanatory power of particular primary and secondary metabolites. Based on this, we established an experiment using a genetically uniform line of European aspen (*Populus tremula*). In this study, we attempt to provide material for a thorough understanding of the metabolic manifestations following an attack by an insect herbivore, starting at the level of changes in photosynthetic pigments, progressing to changes in the concentration of the proteinogenic amino acid proline and changes in the induction of secondary metabolism products like phenolic compounds and volatile organic compounds.

2. Results

2.1. Photosynthetic Pigments

The control values for chlorophylls *a* and *b* showed the highest value of all treatments. The content of chlorophyll *a* was up to 50% lower in leaves infested by aphids (2.1 ± 0.5 mg.g^{-1} of FW) compared to the control leaves (3.8 ± 0.6 mg.g^{-1} of FW), and about 20% in leaves infested by moths, with the same pattern in chlorophyll *b*. In contrast, carotenoids had the lowest values in the control. The carotenoid content was double in leaves infested by moths and higher by about 40% in aphid-infested leaves (Figure 1). There are significant differences ($p < 0.01$) among the control, aphid and spongy moth treaments for both chlorophylls and carotenoids, with the exception of the control and months-infested treatments for chlorophyll *a* (LSD Fisher's test).

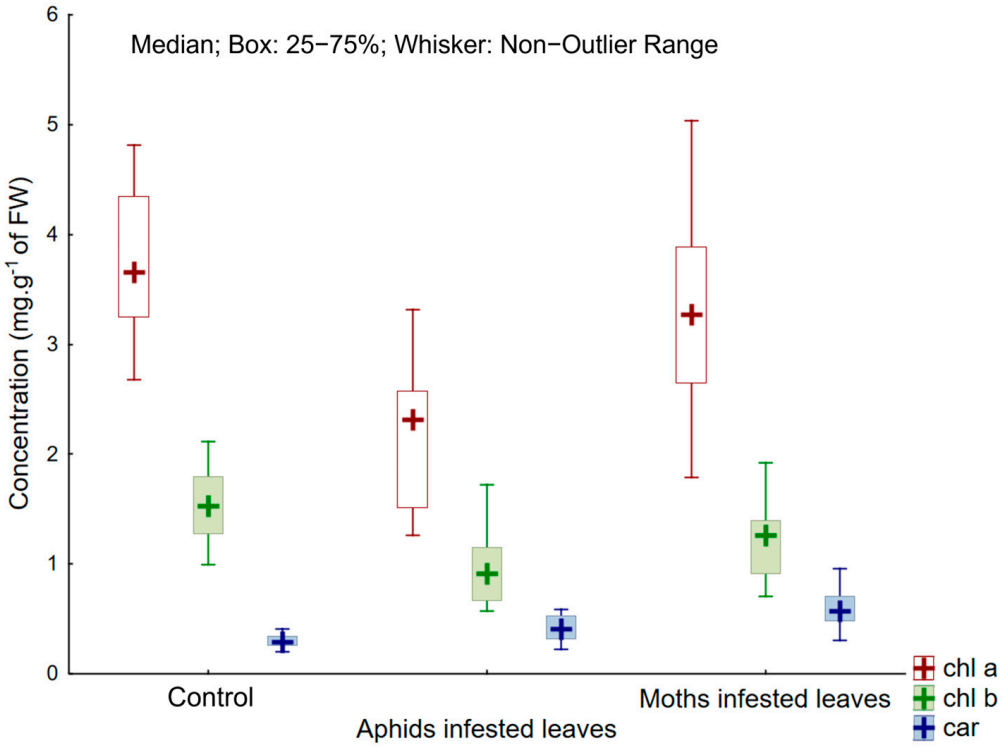

Figure 1. Concentration of chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*) and carotenoids (car) in aphid-infested leaves (A) and moth-infested leaves (M) compared to the control leaves (C).

2.2. Proline

For proline, the values in moth-infested leaves were lower compared to the control leaves (10.0 ± 3.4 and 15.8 ± 5.1 μg.g^{-1}, respectively). But for aphid-infested leaves, a proline content was twice as high as that of the control leaves was recorded. No significant differences between the control leaves and moth-infested leaves were found (Figure 2). The difference between the control and aphid-infested leaves was significant.

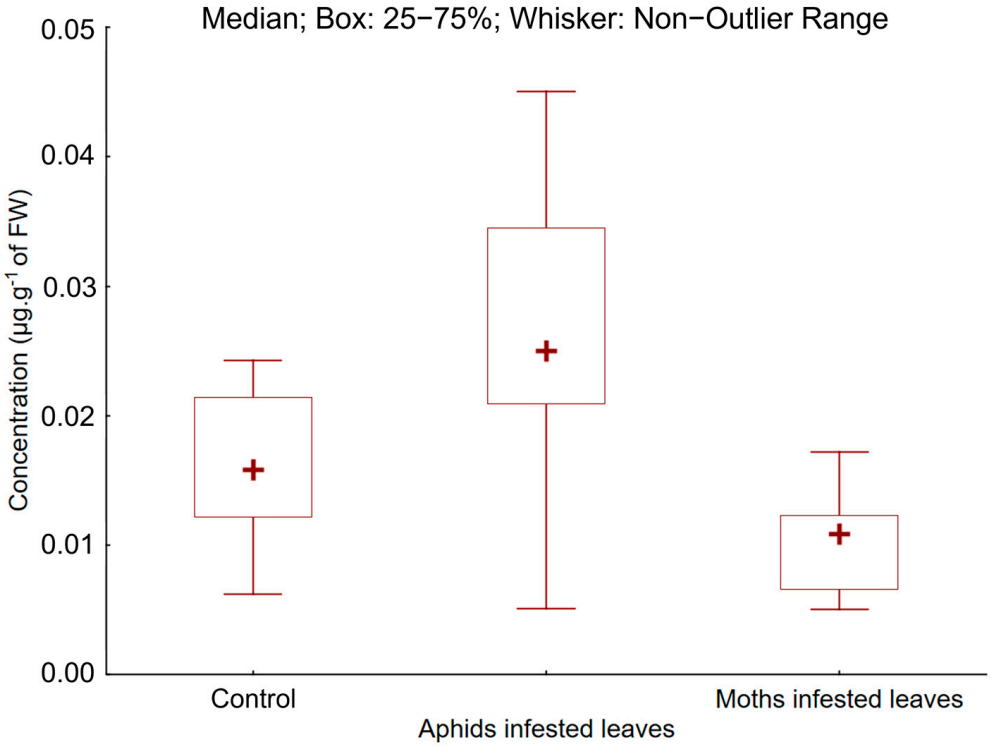

Figure 2. Concentration of proline in aphid-infested leaves (A) and moth-infested leaves (M) compared to the control leaves (C).

2.3. Polyphenolic Compounds Allocated in Damaged Poplar Leaves

Compared to a significant decrease in photosynthetic pigments and an increase in carotenoids, the response of phenolic compounds in damaged leaves was not uniform. Procyanidin and catechin showed statistically significant differences for aphid and moth-infested leaves compared to the control leaves. The significantly lowest level of procyanidin was recorded in leaves infested by moths. The procyanidin content was less than half of that of the control leaves. Similarly, the concentration of catechin in moth-infested leaves was lower compared to the control leaves, but not significantly different between the control and aphid-damaged leaves. There was a significant difference in procyanidin between the aphid and spongy moth infestations, where the aphid-infested leaves had the highest concentration (Table 1).

Table 1. Overview of the estimated concentrations of the investigated phenolic compounds in leaves in µg.g $^{-1}$ of dry weight (DW). N = 20. Asterisks denote a statistically significant difference.

Compound	Mean ± Standard Error (µg g^{-1} of DW)		
	Control Poplar Leaves	Moth-Infested Leaves	Aphid-Infested Leaves
4-coumaric acid	21.4 ± 2.4	20.1 ± 3.7	30.5 ± 5.7
Rutin	29.6 ± 1.6	31.6 ± 2.1	29.1 ± 1.4
Catechin	37.6 ± 4.2	9.6 ± 1.5 *	41.0 ± 6.6 *
Taxifolin	2.6 ± 0.7	0.32 ± 0.03	1.5 ± 0.3
Procyanidin B1	23.4 ± 2.6	10.5 ± 1.3 *	45.6 ± 10.3 *
Chlorogenic acid	1231.6 ± 62.2	1569.7 ± 83.7	1231.0 ± 84.5
Ferulic acid	20.1 ± 3.6	25.1 ± 3.3	14.3 ± 1.9
Kaempferol	0.7 ± 0.1	0.6 ± 0.1	0.8 ± 0.1
Quercetin	2.4 ± 0.5	2.3 ± 0.3	2.2 ± 0.4

2.4. Volatile Compounds Released from Poplar Leaves

The profiles of the volatile compounds emitted from leaves and measured via SPME-GC×GC-TOF-MS were aligned and a data table was created, where the area of the quantification ion (unique mass from deconvoluted mass spectrum of signal) is provided for each of the 304 recorded chromatographical signals. This data table was then evaluated using PCA and OPLS-DA. The initial PCA, explaining 50% of the variance in data, showed a tendency for the separation of samples according to infestation (Figure 3).

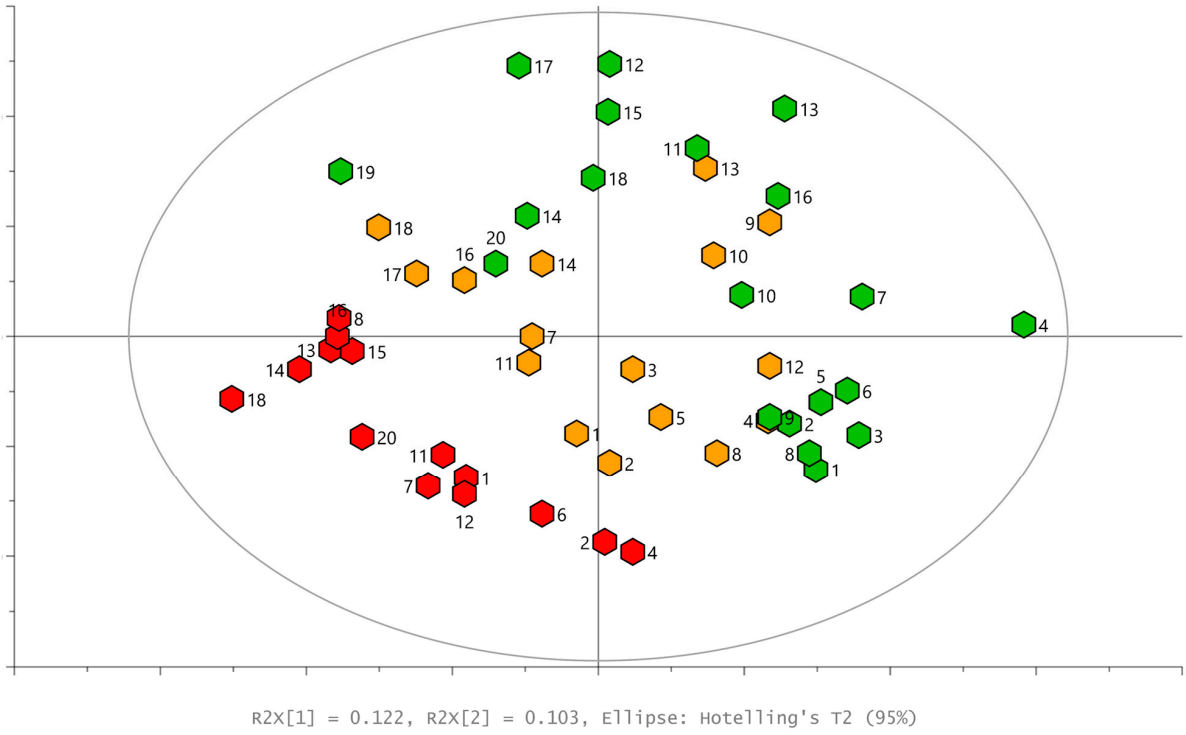

Figure 3. PCA scores plot, visualized according to the first two principal components; the numbers of samples are provided close to each hexagon, presenting an individual sample: green—control leaves, red—aphid-infested leaves, orange—moth-infested leaves. Hotteling's T^2 ellipse $\alpha = 0.05$.

OPLS-DA models were created focusing on the difference between the control samples and those infested by spongy moths or aphids. By comparing the control and aphid-infested samples (Figure 4), the model parameters ($R^2X_{cum} = 0.23$, $R^2Y_{cum} = 0.97$, $Q^2_{cum} = 0.90$) show the good separation and predictive power (based on internal cross-validation) of the model. This indicates strong and reproducible damage to poplar leaves by aphids. The ten compounds most responsible for this separation were selected from the VIP plot (Variable Importance Plot, not shown). The VIP values are reported together with their standard error (Table 2). Interestingly, the variability of some compounds (high in control samples) decreased after infestation, showing that infestation with aphids changes the profile of volatiles to a more homogenous one.

The same approach was also used for the spongy moth-infested samples (Figure 5). OPLS-DA in this case also provided good separation power but showed a much lower predictive ability, as can be seen from the model parameters after internal cross-validation ($R^2X_{cum} = 0.15$, $R^2Y_{cum} = 0.91$, $Q^2_{cum} = 0.49$); this probably shows that the damage of foliage was not as extensive as in the case of aphids. The compounds with the most decisive power for the presented separation are listed in Table 3.

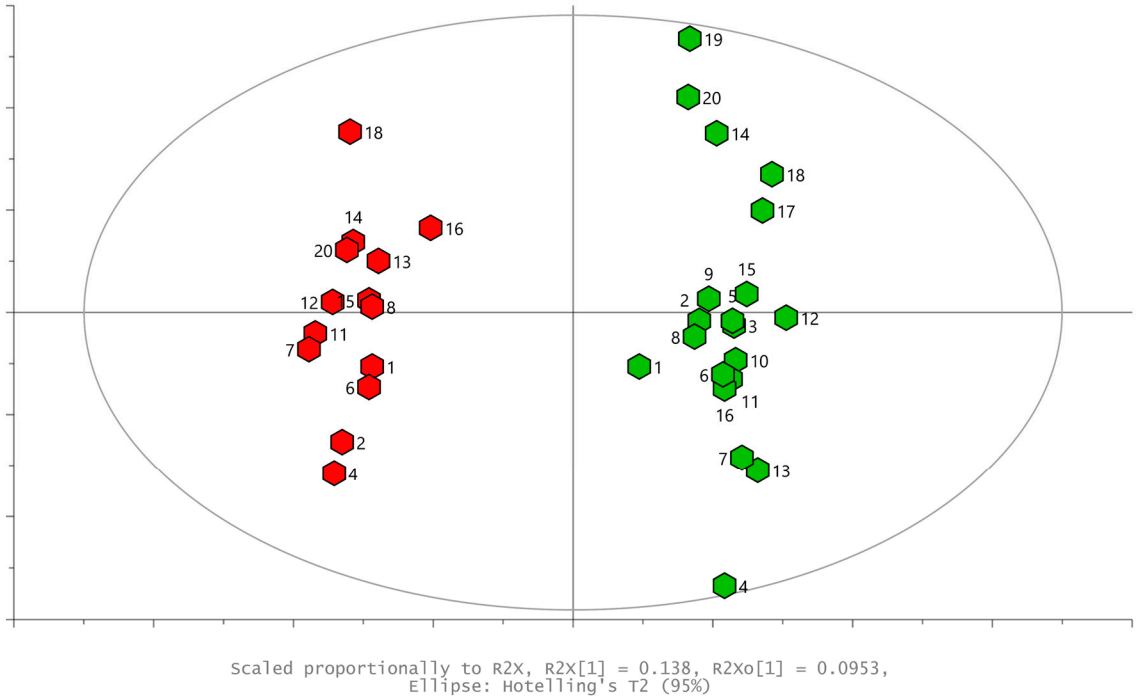

Figure 4. OPLS-DA scores plot showing separation of control leaves (green) and aphid-infested (red) volatiles in poplar leaves; model parameters (R^2X_{cum} = 0.23, R^2Y_{cum} = 0.97, Q^2_{cum} = 0.90); Hotteling's T^2 ellipse α = 0.05.

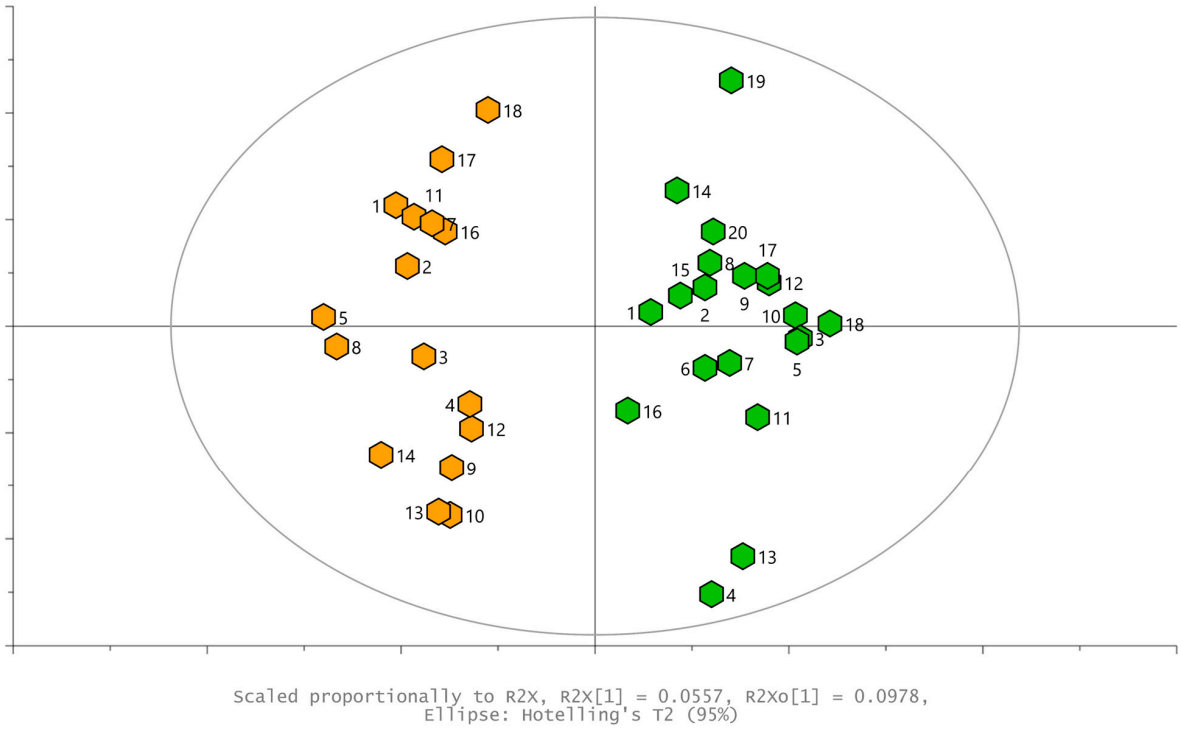

Figure 5. OPLS-DA scores plot showing separation of control (green) and moth-infested (yellow) poplar leaves; (R^2X_{cum} = 0.15, R^2Y_{cum} = 0.91, Q^2_{cum} = 0.49); Hotteling's T^2 ellipse α = 0.05.

Table 2. The most decisive volatile compounds for the control leaves and aphid-infested leaves. The "*" symbol represents multiplication.

Compound	VIP	VIP cvSE * 2.44693	Spectral Similarity (%)	RI (calc)	RI (NIST)
3-Hexenal	2.80	0.82	93	800	800
5-Ethyl-2(5H)-furanone	2.74	1.01	81	962	963
Unknown (RI 1358)	2.59	0.46	-	1358	-
2-Hexenal	2.33	0.69	89	848	847
trans-α-Farnesene	2.31	1.47	84	1514	1511
Dendrasaline	2.30	1.06	78	1586	1579
trans-2,4-Hexadienal	2.30	0.83	92	919	913
Hexyl acetate	2.26	0.45	71	1010	1013
Unknown (RI 962)	2.14	0.81	-	962	-
Ethyl 2-oxopropionate	2.11	0.84	71	770	774

Table 3. Most decisive volatile compounds for the control leaves and moth-infested leaves. The "*" symbol represents multiplication.

Compound	VIP	VIP cvSE * 2.44693	Spectral Similarity (%)	RI (calc)	RI (NIST)
trans-α-Farnesene	2.76	1.38	84	1514	1511
4-Cyanocyclohexene	2.60	0.82	78	1024	1027
Indole	2.50	1.83	80	1306	1300
2-Hexenyl acetate	2.43	1.45	90	1014	1017
Dendrasaline	2.43	1.00	78	1586	1579
Hexyl acetate	2.32	1.35	71	1010	1013
Dihydromyrcenol	2.17	1.19	74	1079	1072
Germacrene D	1.99	1.63	83	1500	1489
3-Hexen-1-ol	1.85	1.22	95	852	856
2-Pentanone	1.75	1.82	88	686	689

3. Discussion

After insect attack, the allocation of organic substances changes. The primary metabolism (the main consumer of carbon in plants) ensures the conditions for an efficient secondary metabolism, on which the plant's defence depends [36]. Hughes [37] hypothesized that plants are constitutionally equipped with a flavonoid metabolism to defend against herbivores, which was later confirmed at the level of mammalian herbivory [38] and more recently at the level of insect herbivory [17]. Rutin formed via the phenylalanine pathway, together with quercetin, coumaric acid, kaempferol, and chlorogenic acid, exhibit a constant concentration independent of herbivore attack [39]. Rutin is a flavone glycoside known to delay insect moulting and cause death [40]. This compound can also prolong the developmental cycle of Lepidoptera and cause higher larval mortality [41,42].

Considering other polyphenolic compounds, chlorogenic acid significantly contributes to constitutive resistance in insects, as found in thrips-resistant chrysanthemums [43]. The polyphenolics produced by the plant and digested by the moth larvae cause an increase in oxygen radicals in the larvae midgut, using the redox balance of glutathione (GSH) towards a higher percentage of its oxidized form (glutathione disulfide; GSSG). The higher ratio of GSSG/total GSH in third-instar moth larvae than in fourth-instar moth larvae suggests a difference in sensitivity to chlorogenic acid or phenolics generally [18]. The enduring interplay between insects and plants, as extensively documented by Agrawal [4], underscores the pivotal role that fundamental genome interactions play in the development of both plant species and herbivores. A common response to insects is an increase in tannin for chewing insects and also for aphids [44]. The more we understand about the plant metabolism under stress, the more apparent it is that the product of the plant metabolism undergoes dynamic coherent processes, of which only a fraction of the many associated individual secondary metabolites and biosynthetic pathways are observable [45]. Nevertheless, the online monitoring of all discussed variables is complicated.

The ability of aspen genotypes to synthesize, accumulate, and store catechin as well as procyanidin (condensed tannins) is genetically determined [46], determining the extent of condensed tannin induction within the population or genome [47]. The results of our research confirm that aphid infestations have many similarities with fungal pathogens [48], whereby sucking phloem sap aphids also induce the salicylic acid–hormonal pathway and thus generate reactive oxygen species [49]. The contribution of flavan-3-ol (whose parts are catechin and procyanidin) to plant defence was proved for microbial pathogens, insects and mammalian herbivores with the direct involvement of salicylic acid [23,50,51]. In mature poplar, spongy moths have only a marginal effect on the accumulation of low-molecular-weight flavan-3-ols, with an increase of 10% in the bark and even a decrease of 10% in the leaves [52]. A decrease in catechin and procyanidin was observed in the case of leaves infested by spongy moths, probably because of an interruption of the central or lateral veins of the leaf. This phenomenon was initially documented during the leaf-feeding activities of various leaf-chewing insect species [53–55]. This mechanical damage to the veins interrupts the flow of phloem, toxins, antifeedants, and other secretions. According to the Herms and Mattson [56] theory, in addition to mechanical damage, the allocation of resources for defence in the case of huge and quick damage at the level of a single leaf can also be a persistent dilemma for plants. Phenolic compounds derived via the common phenylpropanoid pathway perform as a signalling molecule and can act as agents in plant shielding [57]. The result of taxifolin induction in our study shows the same dynamics in the treatment of aphids as in the attack by fungal pathogens, which is observed in the works of Ullah et al. [51] and Hammerbacher et al. [58]. According to the metabolic pathway, taxifolin originates from the metabolism of the phenylalanine pathway and is a precursor of catechin, proanthocyanidins and quercetin [51]. The results of the decrease in the concentration of these substances in the treatment of aphids show the activation of this defense mechanism in *Populus tremula*. In contrast, a decrease in the concentration of these metabolically related compounds was observed in the treatment of moths. The treatment of moths according to the increased concentration of ferulic acid shows the activation of hydroxycinnamic acid amides (HCAAs). This group of coumpounds is widely distributed in plant secondary metabolites and is often referred to as one of the major phenylpropanoid metabolites [59]. HCAAs, including ferulic acid, have been discovered to exert inhibitory effects on acetylcholinesterase, an enzyme crucial in the molting process of the rice weevil (*Sitophilus oryzae* L.) [60]. Despite the general consensus, the combined mixture of allelochemicals significantly improves plant defense against insect herbivores. Nevertheless, there are populations of *Lymantria dispar* that show excessive tolerance to the tannins contained in plant tissues [61].

Sesquiterpene (E,E)-α-farnesene was one of the most important volatile compounds for both types of infestation in our research, and was also previously found in leaves after spongy moth damage [15]. Other investigations also indicate that this substance is a fairly typical VOC produced in response to insect herbivory [62–64]. However, the biological interpretation of the effect is still unclear. James and Grasswitz [65] gives examples of parasitic wasps (*Anagrus* spp.) attracted by farnesene. Furthermore, studies by Beale et al. [66] mention the possibility of the influence of farnesene on reducing the occurrence of viruses transmitted by aphids. This compound was the most important for differentiation between the control and spongy moth-infested leaves, while the fifth most important for aphid-infested poplars versus the control ones. A similar behaviour was observed for dendrolasin, a compound derived from farnesene via biosynthesis [67]. In our study, the presence of green leaf volatiles (GLVs) like 3-hexanal indicates its metabolic availability in the poplar genome. Due to the high sensitivity of the olfactory system of insect herbivores [68], they may act as active attractants to the natural enemies of insect herbivores [69]. Interestingly, the higher emission of 3-hexanal observed during the night hours corresponds to the nocturnal activity of *Lymantria dispar* caterpillars [70]. There is not much information about the biological mechanisms and the effect on insect herbivory. Dendrolasin, as a component of essential oils, performs antimicrobial activity in plants [71,72].

Moreover, the production of primary metabolites is also compromised, as at least some chlorophyll must be renewed daily. Chlorophyll *a* and *b,* in response to phytophagous insects, is decreasing in many different plant species [33,73]. For example, the decrease in chlorophyll *a* and *b* observed in *Citrus* leaves correlates positively with the density of colonization of *Coccus hesperidus* [74]. As we observed, the chlorophyll decrease depends on the insect species. The negative impact of aphids was greater on the content of chlorophylls compared to spongy moths, with a 50% decrease recorded compared to a 15% chlorophyll loss in moths. The chlorophyll content in plant tissues is a key factor in interactions between plants and insects [74]. Changes in chlorophyll concentrations occur in response to a wide range of stresses, including biotic stresses such as insect feeding and pathogenic infections [33]. Our results support the observations of Huang et al. [75], as we confirmed a relative decrease in the chlorophyll content in *Populus tremula* in response to both sucking and chewing insect attacks. Photosynthesis is the main source of reactive oxygen species (ROS) in light, and reactions in chloroplasts produce a variety of ROS forms, including singlet oxygen, superoxide, and hydrogen peroxide, at high rates even under optimal conditions [76,77]. ROS have a detrimental effect on the plant DNA and protein complexes. At the same time, insect herbivory has a negative impact on the leaf water status, resulting in stomatal closure [78]. Therefore, it is possible that plants suffering from insect herbivory may also experience excess excitation energy caused by an excessive concentration of singlet oxygen [79]. Given the extensive crosstalk between light, ROS, and hormonal signalling, this phenomenon is likely to have a strong impact on plant responses to insect herbivores. Considering this, increasing concentrations of certain phenolic compounds, known for their scavenging activity in eliminating ROS, appear to be advantageous [80]. Next to both chlorophylls, carotenoids are also involved in the trapping of light in the first phase of photosynthesis. However, carotenoids participate in other physiological functions, e.g., antioxidative activity is usually enhanced after an insect attack [81]. The response of carotenoids is not as straightforward as in the case of chlorophyll, depending on tree species and insect density [74]. The level of carotenoids follows an irregular curve, with an increase after infestation and a decrease over a prolonged time [74], or simply a decrease [73]. In our study, the carotenoid level was enhanced in both aphid- and moth-attacked leaves, and their level on moth-attacked leaves was double compared to the control. The increase in carotenoids was 40% higher in aphid-attacked leaves compared to the control leaves. Other studies have shown that the proline content and peroxidase activity reached their peak after 7 days of exposure to sucking insects from the *Pseudococcus* family [82]. Peroxidase scavenges oxygen radicals, as do carotenoids [83]. Consistent with the published results, proline increased after insect attack: the proline level increased by two times in aphid-attacked leaves, while the increase in proline in moth-attacked leaves was lower (by 40%). The work of Lackner et al. [8] provides insight into the dynamics of proline in *Lymantria dispar*, which, according to their observations, has a phagostimulatory effect; this implies that leaves with higher levels of proline are preferred by spongy moths. According to our results, the activity of this herbivore does not induce increased levels of proline in *Populus tremula*. Proline is considered a reliable marker indicating plant stress due to drought [84,85], reaching up to three times the concentration [86]. In this context, there is a potentially greater risk of spongy moth attack in poplar stands suffering from drought. As part of the aphid treatment, there was apparently a significant increase in the proline content due to the consumption of phloem sap by aphids.

4. Materials and Methods

4.1. Plant Material

European aspen (*Populus tremula*) seeds were used as the initial plant material. Genetically homogeneous individuals were employed to achieve statistically significant results. These individuals were propagated utilizing somatic embryogenesis techniques. The seeds were obtained by the controlled crossing of parent trees (locations: Czech Republic, Sušice (Svatobor), and Ore Mts. (Fláje), 40–50 years old), which was carried out in early spring

2019. From five to seven days after seed collection, the seed material was used for in vitro propagation. In total, 218 seedlings sprouted, from which individual number 22 was selected because it propagated best in in vitro culture. Seeds of *P. tremula* were washed in 200 mL of distilled water with the addition of 1–2 drops of Tween 20® (Sigma-Aldrich) for 10–15 min. The seeds were then sterilized in 0.1% $HgCl_2$ for 6 min, rinsed three times in sterile distilled water, and placed in 230 mL jars containing 30 mL of Murashige and Skoog (MS) medium [87] solidified with 8 g.L^1 Danish® agar (Carl Roth), containing 100 mg·L^{-1} myo-inositol, and supplemented with 1 mg·L^{-1} 6-benzylaminopurine (BAP). After the adjustment of pH to 5.7, the medium was sterilized in an autoclave at 121 °C and 118 kPa for 30 min. The explants were cultivated under a 16/8 h light/dark photoperiod (photosynthetic photon flux density 35 ± 2 µmol. M^{-2}·s^{-1} cool white fluorescent light), at a temperature of 22 ± 1/20 ± 1 °C (light/dark). The first seeds began to germinate after 1 week on the medium. Most germinated in 2–3 weeks after deployment on the MS medium. Over the next 2–3 months, the plants formed new longer shoots, which were further used for multiplication. The germinated seeds and newly sprouted shoots were regularly subcultured every 2–3 weeks on the same medium until sufficient plant material was obtained for rooting. Dry, brown, and contaminated explants were discarded during the in vitro cultivation.

In vitro rooting was performed on segments about 1.5–2.5 cm long with at least three buds. The shoots developed in vitro were rooted on half-strength MS medium (Murashige and Skoog, 1962) with the addition of 0.5 mg·L^{-1} indole-3-butyric acid (IBA). The first roots began to develop after about 4 weeks on rooting medium and, after 6–8 weeks of cultivation, the rooted shoots were used for ex vitro transfer.

Well-rooted shoots were removed from the cultivation jars and the roots were washed with water to remove residues from the culture medium. The plants were transferred into a sterile substrate (peat and perlite, Forestina, Czech Republic) within plastic pots (7 × 7 × 8 cm), watered, and then treated with 1% Previcur Energy®, Bayer Garden, Germany). The plants were cultivated in an air-conditioned room under a photoperiod of 16/8 h (day/night, photosynthetic photon flux density of 35 ± 2 µmol·m^{-2}·s^{-1} cool white fluorescent light) and a temperature of 22 ± 1/20 ± 1 °C (day/night). They were acclimated by gradually decreasing the air humidity from 95% to 60%. One month after ex vitro transfer, the plants were transferred to cultivation boxes. During ex vitro cultivation, the plants were fertilized using NPK fertilizer once every 2 weeks during vegetation growth.

4.2. Insect Breeding

Eggs of spongy moth (*Lymantria dispar*) were obtained from sterile laboratory cultures from the University of Natural Resources and Life Sciences, Vienna. After hatching, the larvae were fed in sterile petri dishes with nutritionally balanced agar. In the experiment, caterpillars were used after the fourth molting. To enhance the feeding activity, they were incubated in darkness and deprived of food for 48 h, considering their nocturnal behavior. The stockbreeding of aphids (*Chaitophorus nassonowi*) was established by catching adult individuals in the wild during the colonization of aspen. Then, the aphids were cultured in sterilized plastic containers for several months. In vitro cultures of poplar individuals were used as food for the aphids. This method of breeding minimizes the variance in possible phytopathological contamination (especially the development of moulds and fungi).

4.3. Experimental Design

The strategic goal of this experimental design was to arrange a process that minimized any other effects on aspen individuals. Therefore, through the action of model insect species (leaf-sucking and leaf-chewing insect species), the influence of insect herbivory was the only factor that affected the plant metabolism. The experiments were established in Step-In FytoScope FS-SI growth chambers (Photon Systems Instruments, Drasov, Czech Republic). In the growth chambers, the conditions set simulated the optimal environmental conditions (humidity: 75%; average intensity of Photosynthetic active radiation: 250 µmol.m^{-2}·s^{-1};

CO_2 concentration: 415 ppm; day and night period: 2 h of dawn, 10 h of light, 2 h of twilight, 10 h of darkness). The basic structure of the experiment (Figure 6) was made up of three treatments of aspen, where each group had 20 individuals. To prevent intraspecific chemical communication, each group was grown in a separate growth chamber. Group markings: (C) Control—individuals without damage; (A) individuals attacked by aphids (leaf-sucking); (M) individuals attacked by spongy moths (leaf-chewing).

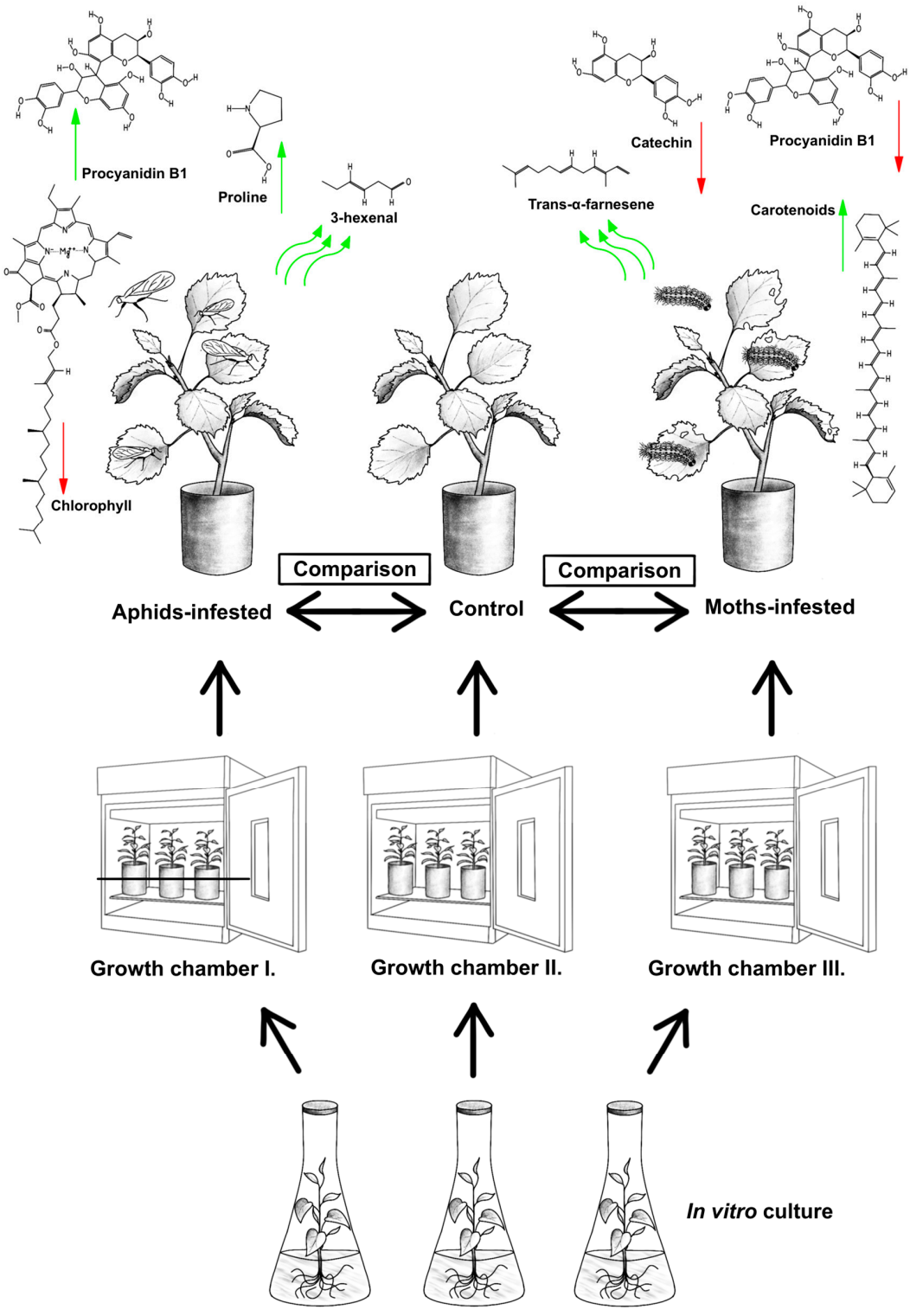

Figure 6. Graphical representation of the experimental design. Green arrows show individual compounds increase, red arrows indicate decrease.

4.4. Sample Collection

Sample collection took place five months after the transfer of poplars to ex vitro. Leaf collection took place during the treatment of the damage caused by the 5 spongy moths at the moment at which about 30% of the leaf was eaten within 30 min. The samples in the aphid treatment were harvested after about 4–5 days of colonization (the number of aphids was around 100 in different stages of development) when signs of damage were visible on the leaf. Aphids were removed from the leaf with prepared individual brushes with natural fibres (washed repeatedly in chloroform and stored in sterile aluminium foil). The leaves were cut off and immediately put into liquid nitrogen. In order to minimize the influence of circadian cycles on the plant metabolism, samples were always collected within a time interval of 6 h after dark, and the collection was stopped 2 h before dark in the growth chambers.

4.5. Spectrophotometric Measurement

For photosynthetic pigment estimation, the common method of extraction and analysis was used [88], but several steps were modified. First, 10 mg of deep-frozen plant tissue was placed into a 2 mL test tube and homogenized using a mill crusher (Retsch mill, Haan, Germany) for 3 min and 30 oscillations per second. Then, 1.5 mL of acetone and several crystals of magnesium carbonate (to stop the pheophytin formation) were added. Before centrifugation, the pigments were left to elute from the tissue for a few minutes. Then, the samples were centrifuged for 3 min at 13,000 rpm. The supernatant was collected in the new test tube and supplied with 5 mL of acetone prior to spectrophotometric analysis. The absorbance was measured at 663 nm, 646 nm, and 470 nm for the estimation of chlorophylls *a*/*b* and the total carotenoid content using a spectrophotometer DR6000 (HACH Company, Loveland, CO, USA).

The following equations were used for calculation:

$$c_{chl\ a} = \mathbf{12.25}\, A_{662} - \mathbf{2.81}\, A_{647}$$

$$c_{chl\ b} = \mathbf{20.13}\, A_{647} - \mathbf{5.03}\, A_{664}$$

$$c_{total\ carotenoids} = (\mathbf{1000}\, A_{470} - \mathbf{3.27}\, c_{chl\ a} - \mathbf{104}\, c_{chl\ b})/\mathbf{198}$$

The proline content was determined according to Bates [89] with the following modifications. For the analysis, 20 mg of the plant tissue was weighed (fresh weight FW) and homogenized in the mill crusher for 3 min. Then, 300 µL of 3% sulfosalicylic acid was added and mixed well using a vortex for 30 s. Next, the samples were centrifugated for 5 min at 13,000 rpm. Then, 160 µL of supernatant was transferred to the new test tube. After this, 160 µL of glacial acetic acid and 160 µL of ninhydrin solution were added (ninhydrin solution was prepared from 0.25 g of ninhydrin, 6 mL of glacial acetic acid, and 4 mL of phosphoric acid). The solution was vortexed and inserted into a heat block (Major Science, Taoyuan City, Taiwan) at 95 °C for one hour. Then, the samples were left to cool down. Once cool, 320 µL of toluene was added and vortexed for 30 s for the extraction of proline to the organic phase. After the phase separation, the absorbance of the upper phase was measured at 520 nm against toluene.

4.6. Extraction Procedure for Determination of Polyphenolic Compounds

Each sample consisted of 20 mg of lyophilized and homogenized plant tissue, meticulously weighed. Subsequently, 500 µL of a methanol/water solution (70:30 *v*/*v*, sourced from Honeywell, Offenbach, Germany) was added to each sample. Then, the sample was mixed well using a vortex and placed into an ultrasonic bath with ice for 10 min. After, the samples were centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was collected and filtered through a PTFE syringe filter (0.22 µm) prior to LC-MS-qTOF analysis.

4.7. LC-MS-qTOF Analysis of Polyphenolic Compounds

LC-MS-qTOF analysis was carried out using an Agilent 1290 Infinity II coupled with an Agilent 6546 LC/MS QTOF system (Agilent, Santa Clara, CA, USA) and Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8 µm), (Agilent, USA). Mobile phase A contained 0.05% formic acid and mobile phase B consisted of acetonitrile. The gradient elution was as follows: 0–1 min, 100% A; 1–7 min, 35% A; 7–8 min, 100% A; 8–8.01, 100% A; and 8.01–10 min, 100% A. The flow rate of the mobile phase was set at 1.1 mL minute^{-1} [35]. The column temperature was set at 35 °C. The injection volume was 1 µL. The system was operated in negative ionization mode. The optimization of qTOF parameters was previously performed using the standards of polyphenolic compounds. The qTOF parameters were as follows: scan range, 100–1000 m/z; drying gas temperature, 350 °C; sheath gas flow rate, 12.0 L/minute; sheath gas temperature, 400 °C; capillary voltage, 5.0 kV; nozzle voltage 0.9 kV; fragmentor, 140 V; and collision energy at 10, 20 and 40 eV. MS/MS data were acquired at a scan range of 50–800 m/z using a 0.5 min retention time window, an isolation window of 1.3 amu, and an acquisition rate of 2 spectra s^{-1}. During the analysis, two reference masses (112.9855 m/z and 966.0007 m/z) were continuously measured for mass correction. Data collection was carried out using Agilent Mass Hunter Acquisition software Workstation Plus 11.0. Data analysis was performed using Qualitative Analysis 10.0 and Q-TOF Quantitative analysis.

4.8. GC×GC-TOF-MS Metabolomic Analysis

The fingerprints of poplar leaf volatiles were collected using solid-phase microextraction (SPME) coupled to two-dimensional comprehensive gas chromatography and time of flight mass spectrometry (GC×GC-TOF-MS). From each freeze-dried sample, 200 mg was placed into a 10 mL headspace glass vial and sealed with a screw lid with PTFE septum. Volatiles from the sample headspace were collected after 10 min of incubation at 50 °C using an SPME fiber with an divinylbenzene/carboxen/polydimethylsiloxane coating from Supelco (Bellefonte, PA, USA).

An agilent gas chromatograph 7890B (Agilent Technologies, USA) was equipped with a HP-5MS UI capillary column (30 m, 0.25 mm i.d., 0.25 µm film thickness) for one-dimensional separation and coupled to BPX-50 (SGE, Victoria, Australia, 1.5 m, 0.1 mm i.d., 0.1 µm film thickness) for two-dimensional separation using a consumable free modulator. Helium at a flow of 1 mL min^{-1} was used as a carrier gas. The spitless injection was applied to a hot (265 °C) split/splitless injector. After 2 min of solvent delay, the oven temperature was increased from an initial 40 °C at a rate of 10 °C min^{-1} to 280 °C. The second dimension GC oven and modulator followed this temperature program, with 5 °C and 15 °C offset, respectively. With a hold time of 1 min, the GC run took 27 min.

In initial data processing, the peak find and automated spectral deconvolution algorithms were performed in ChromaTOF SW (LECO, St. Joseph, MI, USA). Then, the peak alignment tool (Statistical Compare, LECO) was used to align signals with S/N higher than 150, with a maximal retention time deviation between samples of no more than 5 s and consequently a spectral similarity between signals in samples higher than 75%.

For the tentative identification of compounds, a spectral comparison of the measured and deconvoluted spectrum with mass spectra in the National Institute of Standards and Technology mass spectral library (NIST 2017) library was performed. To support this identification, retention indexes from NIST were used.

4.9. Statistical Evaluation

A statistical evaluation of the GC pre-cleaned and centered log-ratio (CLR) transformed metabolomic data was performed using Simca 17.0 SW (Sartorius Stedim Data Analytics AB, Umeå, Sweden), where Principal Component Analysis (PCA) and Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) were used. PCA was used mainly to judge the obtained data concerning the measurement quality and to remove outlying samples.

For marker selection, OPLS-DA was used. In this model, samples were classified based on the species of insect.

One-way ANOVA was used for the estimation of statistically significant differences among the phenolic compounds, proline and photosynthetic pigments. Data normality and variance homogeneity were tested before the analysis. To reveal the differences between the control and treatments with aphids and moths, a post hoc Scheffe test was applied. Statistica 14.1.0 (Tibco Software Inc., Palo Alto, CA, USA) was used to test the differences at the level of 0.05.

5. Conclusions

In this investigation, our focus was on providing a comprehensive understanding of plant responses to insect herbivory, spanning from the level of photosynthetic active pigments (the primary energy contributors to metabolism) to alterations in the proteinogenic amino acid proline, and extending to the most metabolically intricate compounds in the context of herbivory by both sucking and leaf-chewing species (graphical abstract). Even in the case of an in vitro experiment designed with genetically uniform plants for an exact description, the complexity of the connections, flow, and interaction of substances and influences is evident. Currently, a comprehensive systematic framework is lacking. However, with the impact of genetic interactions, chemotypes, and metabolic strategies, there are ample opportunities to enhance our understanding of the dynamics in plant host–herbivore interactions. Despite the abundance of publications and studies, there are many opportunities to expand our knowledge of plant host–herbivore interaction dynamics. In this study, we observed lower concentrations of chlorophyll a and b in *Populus* leaves attacked by moths and aphids. At the same time, the leaves damaged by moths and aphids had twice the concentration of carotenoids compared to the control. In addition, it was found that in leaves attacked by aphids, an increased concentration of proline was observed compared to the control. We also observed the same concentrations of flavan-3-ol compounds (like catechin and procyanidin), whereas trans-α-farnesene and 3-hexenal were the most differentiated compounds in moth and aphid-infested leaves compared to the control leaves, respectively.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants13091243/s1. Table S1: List of compounds monitoring by LC-MS-qTOF in negative ionization mode.

Author Contributions: F.P.: conceived the presented idea, carried out the experiment, preparation, and processing of laboratory protocols, and wrote the manuscript; A.K.:liquid chromatography and data evaluation; J.H.: gas chromatography and data evaluation; O.D.: carried out the experiment; J.V.: carried out the in vitro preparatory work; K.M.: wrote the manuscript; I.T.: wrote the manuscript I.T. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data are contained within the article and Supplementary Materials.

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Conflicts of Interest: The authors declare no conflicts of interest.

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4.2 Article II.: Genome-Wide Transcriptomic and Metabolomic Analyses Unveiling the Defence Mechanisms of *Populus tremula* against Sucking and Chewing Insect Herbivores

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Author Contributions

F.P. conceived the presented idea, designed the experiment, carried out the experiment, prepared and processed laboratory protocols, and wrote the manuscript; K.M. processed laboratory protocols, conducted transcriptomic data evaluation, and wrote the manuscript; A.K. laboratory work—LC-MS analysis and metabolomic data evaluation; J.H. metabolomic data evaluation; O.D. carried out the experiment; A.R. transcriptomic data analysis and wrote the manuscript; I.T. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Extended summary

Based on available information, no comparable study has integrated metabolomic and transcriptomic data for *Populus tremula* across the two main insect herbivore feeding strategies. Both sap-sucking and leaf-chewing insects trigger significant changes in gene expression and metabolism in *P. tremula*.

Integrated analysis revealed that flavonoid and isoflavonoid biosynthesis pathways are notably enriched in response to insect infestation. Additionally, pathways related to plant hormone signaling (salicylic and jasmonic acids), PAMP-triggered immunity, and MAPK signaling are crucial for plant resistance. Insect infestation also impacts primary metabolism, particularly carbohydrate and amino acid pathways. These findings highlight a species-specific, finely-tuned response to herbivory in *P. tremula* leaves.

Connection with the objectives of the dissertation

All five objectives of this dissertation were successfully achieved within the scope of this publication.

Implications

The methodology of the study, which involved harvesting a single leaf after insect herbivory and analyzing it through homogenization for metabolomic and transcriptomic data, effectively captured the plant's state at a specific time point. This approach challenges the claim by Cavill et al. (2015) about the infeasibility of this method and supports the use of replicated study designs.

The non-target metabolomic screening revealed new compounds in *Populus spp.*, including plumieride (an antifungal iridoid glycoside), heptamethoxyflavone (a polymethoxyflavone), and veronicoside (an antioxidant iridoid glycoside).

Differential gene expression (DGE) analysis showed that approximately 272 transcripts were differently regulated in response to leaf-chewing herbivores, and 1203 transcripts were differently regulated in response to sap-sucking herbivores, compared to controls. Additionally, 5716 transcripts were regulated differently between aphid and moth infestations, indicating species-specific and finely tuned responses based on the type of herbivore feeding strategy.

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Genome-Wide Transcriptomic and Metabolomic Analyses Unveiling the Defence Mechanisms of *Populus tremula* against Sucking and Chewing Insect Herbivores

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Abstract: Plants and insects coevolved as an evolutionarily successful and enduring association. The molecular arms race led to evolutionary novelties regarding unique mechanisms of defence and detoxification in plants and insects. While insects adopt mechanisms to conquer host defence, trees develop well-orchestrated and species-specific defence strategies against insect herbivory. However, current knowledge on the molecular underpinnings of fine-tuned tree defence responses against different herbivore insects is still restricted. In the current study, using a multi-omics approach, we unveiled the defence response of *Populus tremula* against aphids (*Chaitophorus populialbae*) and spongy moths (*Lymantria dispar*) herbivory. Comparative differential gene expression (DGE) analyses revealed that around 272 and 1203 transcripts were differentially regulated in *P. tremula* after moth and aphid herbivory compared to uninfested controls. Interestingly, 5716 transcripts were differentially regulated in *P. tremula* between aphids and moth infestation. Further investigation showed that defence-related stress hormones and their lipid precursors, transcription factors, and signalling molecules were over-expressed, whereas the growth-related counterparts were suppressed in *P. tremula* after aphid and moth herbivory. Metabolomics analysis documented that around 37% of all significantly abundant metabolites were associated with biochemical pathways related to tree growth and defence. However, the metabolic profiles of aphid and moth-fed trees were quite distinct, indicating species-specific response optimization. After identifying the suitable reference genes in *P. tremula*, the omics data were further validated using RT-qPCR. Nevertheless, our findings documented species-specific fine-tuning of the defence response of *P. tremula*, showing conservation on resource allocation for defence overgrowth under aphid and moth herbivory. Such findings can be exploited to enhance our current understanding of molecular orchestration of tree responses against herbivory and aid in developing insect pest resistance *P. tremula* varieties.

Keywords: *Populus tremula*; aphids; spongy moth; transcriptome and metabolomics; reference gene analysis; RT-qPCR; induced defence

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1. Introduction

Over approximately 350 million years, plants and insects have coevolved, resulting in a spectrum of beneficial and detrimental interactions between the two groups [1,2]. Beneficial interactions encompass insect-mediated processes such as pollination and seed dispersion, providing mutual advantages to both interaction partners. Conversely, negative interactions involve insect predation, frequently damaging the host [1,2]. To repel

insect pests and pathogens, plants have evolved a variety of morphological and biochemical defences, such as constitutive and induced defence, that are highly effective and dynamic [3–10]. Because plant development and defence are mutually exclusive, induced defence is more frequently triggered in response to herbivore attacks than constitutive defence to optimally utilize the energy budget [4,5,10,11]. After plants perceive the herbivory, damage-associated molecular patterns or herbivore-associated molecular patterns trigger the herbivore-inducible defences.

The repercussions of global climate change extend across all layers of ecosystems, predominantly manifesting in adverse impacts on forest stands worldwide. Over 50% of tree damage worldwide is attributed to biotic causes, with insect herbivores being one of the principal stressors [12]. It corresponded that the negative alterations favour insects, even though the intricate interactions between insect herbivores and host trees are unpredictable [13]. Drought and higher temperatures weaken the tree defence, encouraging insect abundance and dispersal across larger geographic areas while reducing the duration of their generation [14,15].

Interestingly, trees are constantly under pressure to resist multiple herbivores of the same or different feeding guild. Current research indicates that trees can tailor defences against different insect herbivores and their sequential attacks using a plastic defence strategy [16]. However, understanding the tree defence tailoring for two herbivores from different feeding guilds (i.e., sucking and chewing) is very limited. Next-generation sequencing (RNA-Seq) and metabolomics have emerged as valuable tools for unveiling plant signalling networks in response to herbivory [17,18]. Hence, the current study attempted to understand how *P. tremula* responds to herbivory by insects from different feeding guilds. To delve into this, we employed a combination of transcriptomics and metabolomics on *Populus* leaves, comparing those with and without sucking and chewing insect infestation. By combining transcriptomic and metabolomic data, we quantitatively map transcripts to specific metabolic pathways involved in resistance against both types of insect feeding. It has been found that insect infestation has led to notable alterations in tree primary metabolism, affecting photosynthesis, carbohydrate and amino acid pathways, and secondary metabolites related to flavonoids. Nevertheless, these findings deepen our understanding of different herbivore-induced plant defences in *P. tremula*, providing insights for developing strategies against pests like aphids and spongy moths.

2. Results

2.1. Metabolomic Analysis

Initial principal component analysis (PCA) revealed a tendency for clustering (explained variance by the first two principal components was 76%) (Figure 1A–C). Identified metabolites in the sucking and chewing insect treatment groups differed significantly from the control group, as demonstrated by PCA of the differentially accumulated metabolites. Following Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) showed clear separation ($R^2X(cum) = 72\%$, $R^2Y(cum) = 85\%$, $Q^2(cum) = 56\%$) (Figure 1). For two-group models, in the case of aphid vs. control comparison, the resulting OPLS-DA parameters were $R^2X(cum) = 65\%$, $R^2Y(cum) = 99\%$, $Q^2(cum) = 75\%$), for moth vs. control $R^2X(cum) = 98\%$, $R^2Y(cum) = 100\%$, $Q^2(cum) = 98\%$) and for aphid vs. moth $R^2X(cum) = 97\%$, $R^2Y(cum) = 100\%$, $Q^2(cum) = 99\%$). Despite the relatively low number of samples measured from the created models, it can be concluded that damage by the selected pests strongly affected the non-volatile metabolome of tested leaves. Lower parameters for the model considering control vs. aphid-infested samples result from larger variability in between samples of aphid-infested leaves, also resulting in lower predictive power ($Q_2(cum)$; based on multiple internal model cross-validation). A total of 78 differentially regulated metabolites were found across different treatments and comparisons (VIP > 1 and log2FC ≥ 1) (Figures 1 and 2). We discovered that among the differential abundance

metabolites (DAMs), fructose phosphate and benzoic acid were the most significantly enriched; we also displayed the top 10 metabolites in individual treatments compared to each other (Figures 1D–F and 2, Table 1).

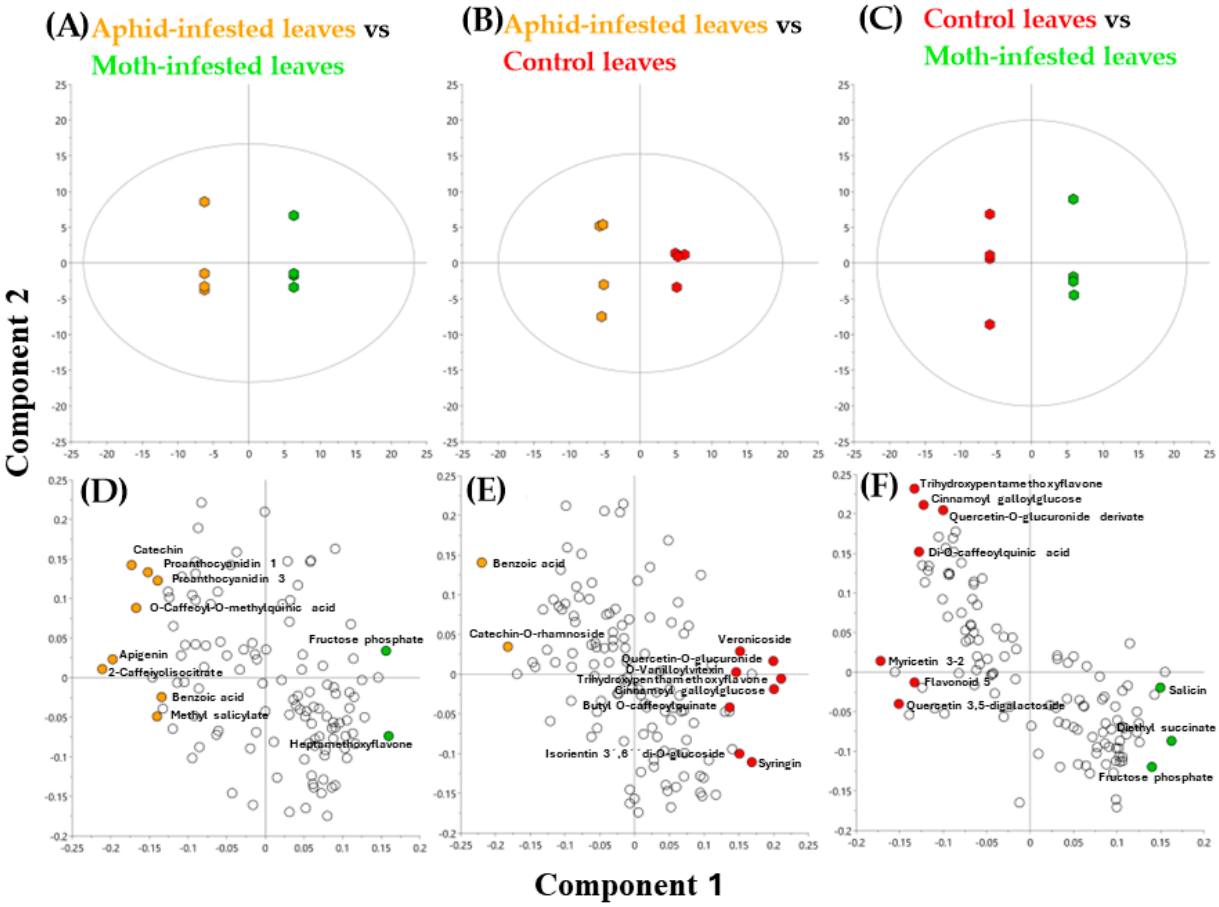

Figure 1. Xy OPLS-DA score plots for two groups models: (**A**) aphid-infested leaves vs. moth-infested leaves, (**B**) aphid-infested leaves vs. control leaves, and (**C**) control leaves vs. moth-infested leaves. Hotteling ellipse 95%. (**D–F**) OPLS-DA loading plots for models (**A–C**) with the ten most important compounds for separation highlighted, selected from variable importance plot. The colours of highlighted compounds correspond to sample classes in respective models.

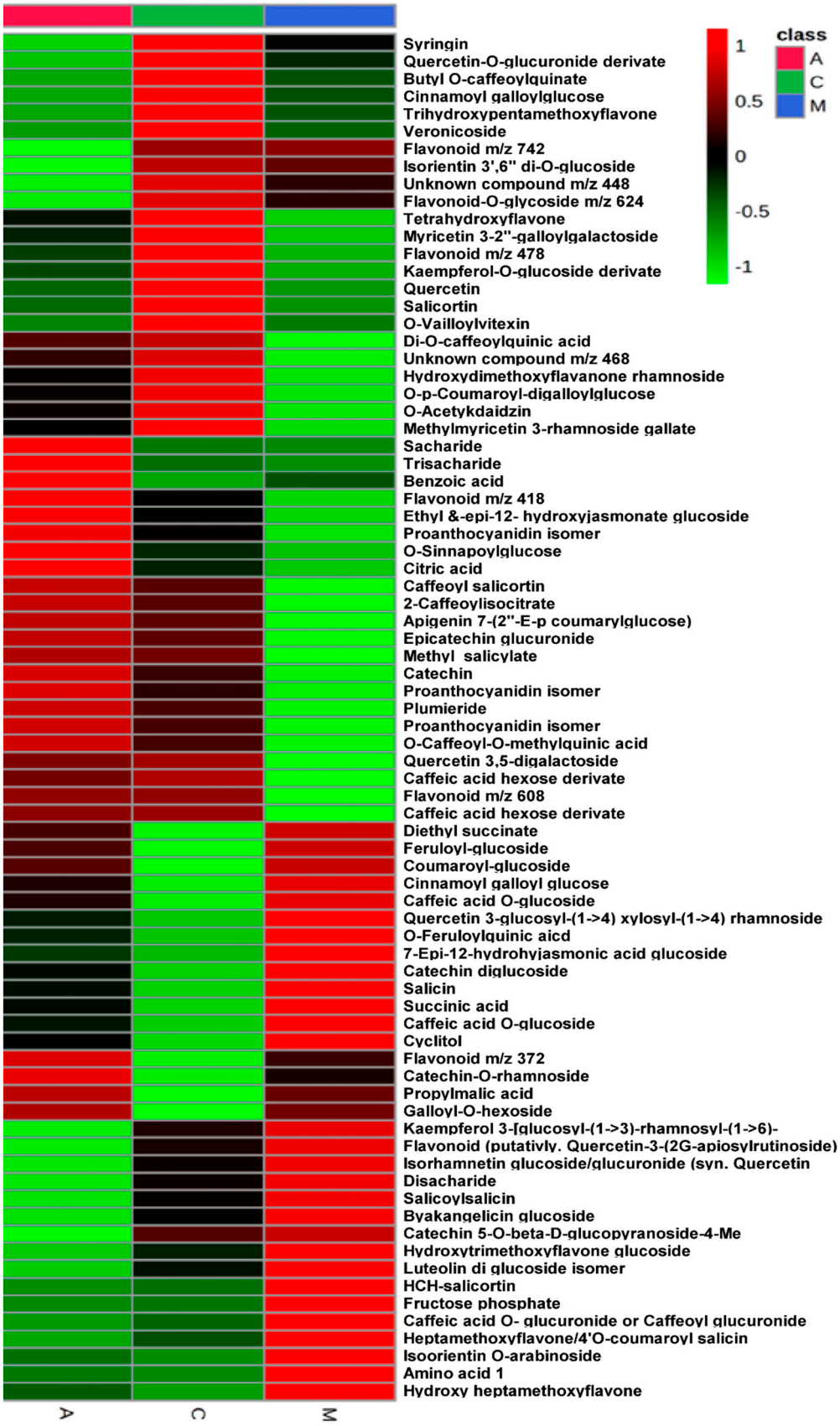

Figure 2. Heatmap for differentially abundant metabolites (DAMs) in *Poplar* samples tested in the current study. A—Aphid-infested treatment, C—control, M—Moth-infested treatment.

Table 1. Top 10 metabolites differentially abundant between treatments.

Control Leaves vs. Moth-Infested Leaves			Control Leaves vs. Aphid-Infested Leaves			Aphid-Infested Leaves vs. Moth-Infested Leaves		
Metabolite	**VIP Value**	**KO Number**	**Metabolite**	**VIP Value**	**KO Number**	**Metabolite**	**VIP Value**	**KO Number**
Myricetin 3-2	1.7	00941	Trihydroxypentamethoxyflavone	2.2	00941	Catechin	1.8	00941
Trihydroxypentamethoxyflavone	1.6	00941	Benzoic acid	2.2	00362	2-Caffeoylisocitrate	1.7	00940
Diethyl succinate	1.6	00020	Cinnamoyl galloylglucose	2.1	00941	Proanthocyanidin 1	1.6	00942
Cinnamoyl galloylglucose	1.6	00941	Quercetin-O-glucuronide derivate	2.1	00941	Fructose phosphate	1.6	00010
Flavonoid 5	1.5	00941	Catechin-O-rhmnoside	1.8	00941	Heptamethoxyflavone	1.5	00941
Salicin	1.4	00940	Syringin	1.7	00940	Proanthocyanidin 3	1.5	00942
Quercetin 3,5-digalactoside	1.4	00941	Veronicoside	1.6	00941	Benzoic acid	1.4	00362
Fructose phosphate	1.4	00010	Butyl O-caffeoylquinate	1.5	00940	Methyl salicylate	1.3	00940
Di-O-caffeoylquinic acid	1.4	00940	O-Vanilloylvitexin	1.5	00940	Apigenin	1.3	00941
Quercetin-O-glucuronide derivate	1.4	00941	Isorientin 3′,6′di-O-glucoside	1.5	00941	O-Caffeoyl-O-methylquinic acid	1.3	00940

KO Numbers: ko00941—Thiamine metabolism; ko00020—Citrate cycle (TCA cycle); ko00940—Phenylpropanoid biosynthesis; ko00010—Glycolysis/Gluconeogenesis; ko00362—Benzoate degradation; ko00942—Anthocyanin biosynthesis.

2.2. DGE Analysis on Poplar Leaves Infested by Sucking and Chewing Pests

We investigated the gene expression profiles of *P. tremula* leaves infected by aphids and moths and their corresponding control samples. We analysed the differentially expressed genes in leaf samples attacked by sucking and chewing insects and compared them with their respective control groups. Five biological replicates were employed for each treatment, yielding fifteen samples. We obtained 13.5 Gb of data, with each sample generating more than 12.5 Gb of clean data and a Q30 base percentage exceeding 80% (Supplementary File S1). A total of 7191 differentially expressed genes (DEGs) were found following treatment with sucking and chewing insect attacks; off these, 216 and 56 were up- and downregulated in spongy moth infestation and whereas 624 and 579 were up- and downregulated when compared to respective control leaf samples, respectively. Between the aphid and spongy moth comparison, 2422 and 3294 genes were differentially regulated (p-adjust value < 0.05 and log2FC > 1) (Figure 3A). Furthermore, hierarchical clustering was performed to illustrate the expression pattern of DEGs. Poplar response against aphids and spongy moths feeding revealed significant differences, suggesting that the poplar gene expression level varies depending on the insect feeding style (Figure 3E). The Venn diagrams also endorse the unique plant response to different insect feeding as different genes were up or down-regulated after moth and aphid feeding (Figure 3B–D).

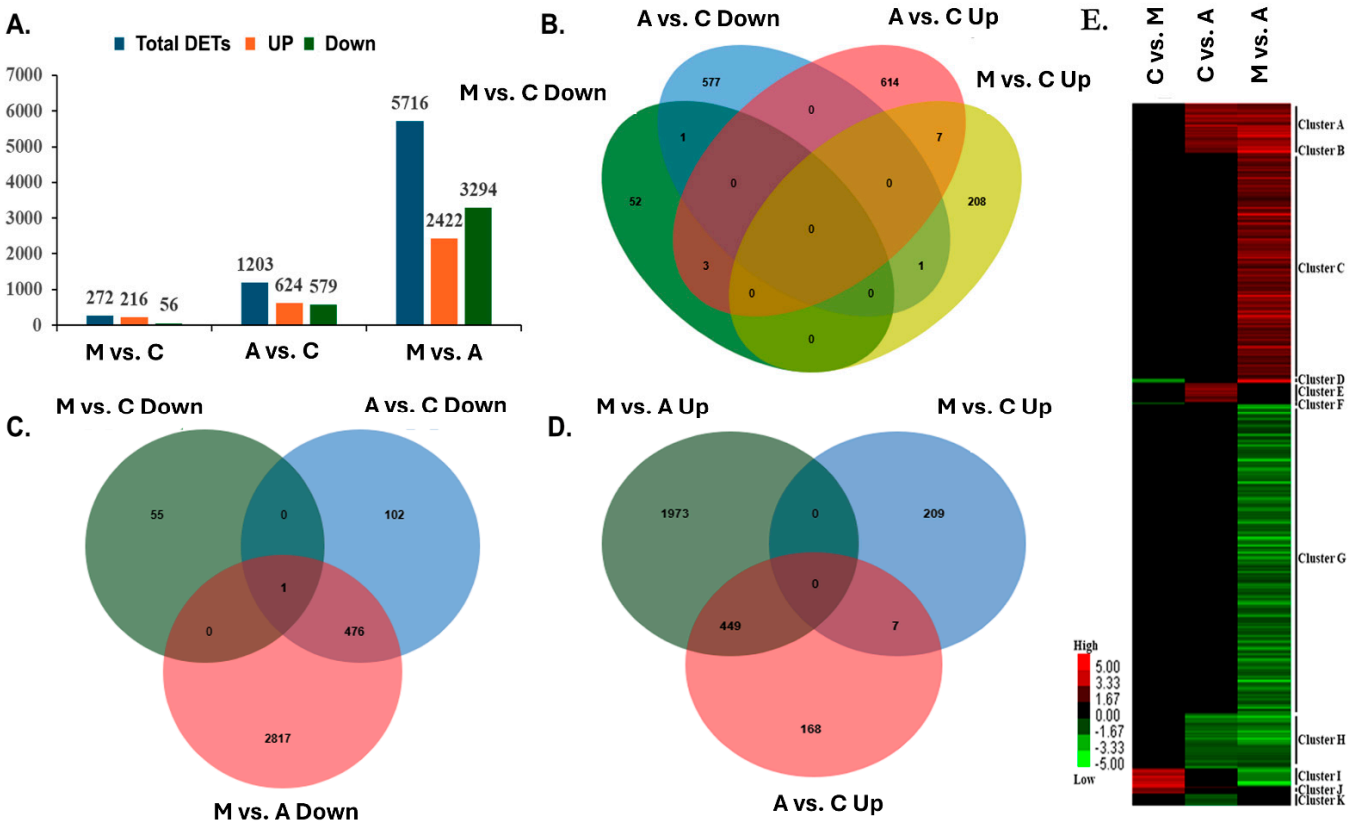

Figure 3. Differential gene expression and cluster analysis (treatment legend: A—aphid-infested treatment, C—control, M—moth-infested treatment). (**A**) Number of genes differentially regulated in various comparisons. (**B**) Venn diagram showing comparisons between different treatments. (**C**) Venn diagram showing comparisons between all down-regulated genes. (**D**) The Venn diagram shows comparisons between all upregulated genes. (**E**) Hierarchical cluster analysis of DEGs in three comparisons. The letters (A–K) indicate major groups identified by cluster analysis. Red colour indicates upregulation (>2.0 fold), green colour indicates downregulation (<−2.0 fold), and black indicates no change as compared to respective controls.

Further examination revealed that identified DEGs distributed across comparisons of distinct KEGG pathways (Figures 4–7). Specifically, DEGs were associated with pathways involved in genetic information processing, encompassing processes such as folding, sorting, degradation, transcription, and translation. Additionally, DEGs were linked to various metabolic pathways, including carbohydrate metabolism, lipid metabolism, amino acid metabolism, and biosynthesis of secondary metabolites (Figure 7). Furthermore, DEGs were implicated in cellular processes, environmental adaptation, and signal transduction pathways. In spongy moths, compared to the control group, there was an upregulation in genes associated with oxidoreductase activity, DNA binding, transcription regulatory activity, and DNA-binding transcription factor activity. At the same time, starch and sucrose metabolism increased, and carbon fixation in photosynthetic metabolism decreased (Figure 4). Similarly, when compared to spongy moths with aphids, thiamine metabolism had an upregulation and a significant downregulation in starch and sucrose metabolism (Figure 5). In aphids, compared to the control group, genes related to transferase activity, glycosyl transferase activity, lipid metabolic process, and hydrolase activity were upregulated, while Glycerophospholipid metabolism increased, and arginine and proline metabolism, fructose and mannose metabolism, and carbon fixation in photosynthetic organism metabolism were notably downregulated (Figure 6). Complete visualization of PEA (pathway enrichment analysis) in all treatments is shown in Figure 7.

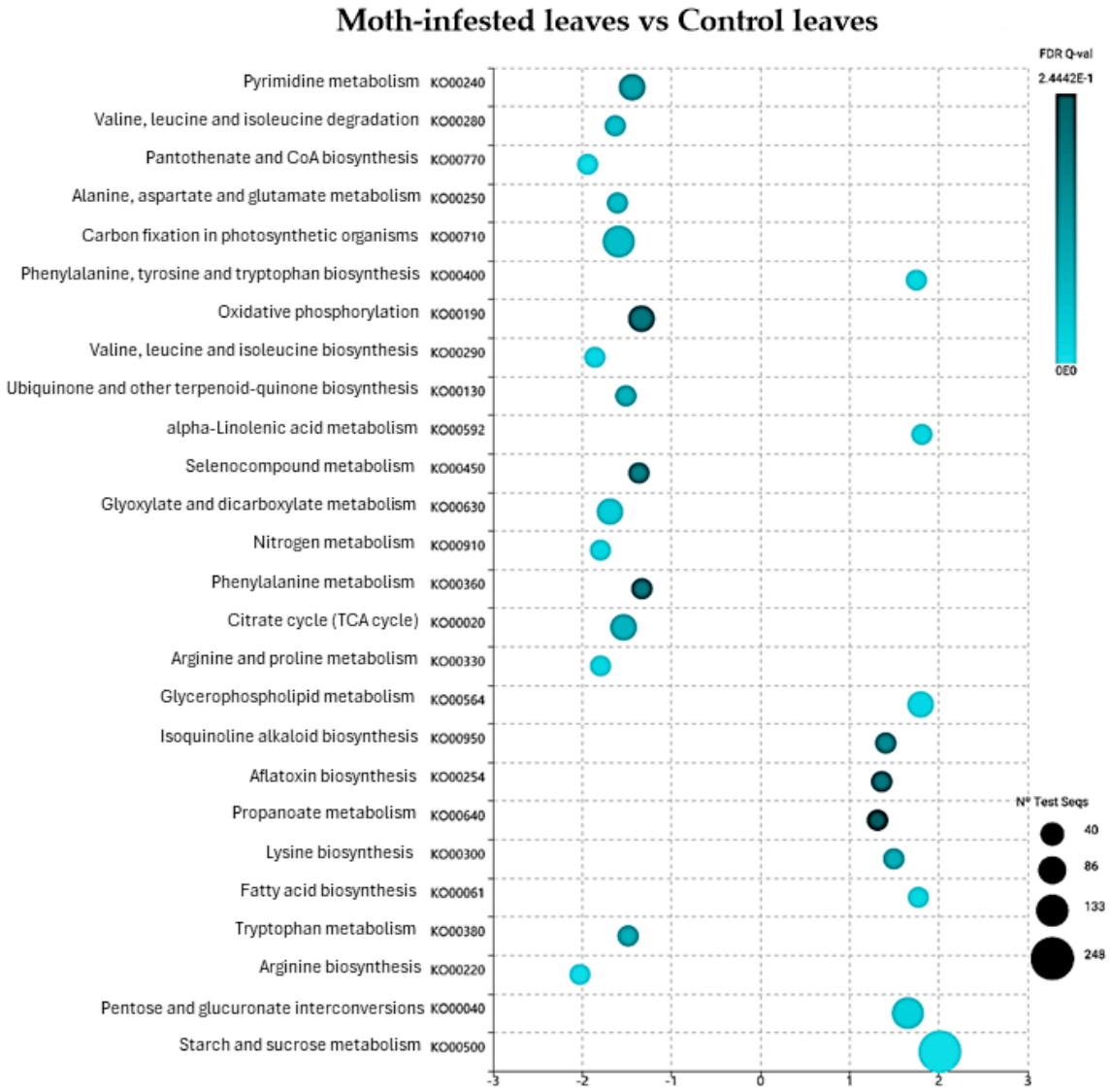

Figure 4. Pathway enrichment in the treatment of moth-infested leaves compared to control leaves according to the number of identified DEGs for individual metabolic pathways.

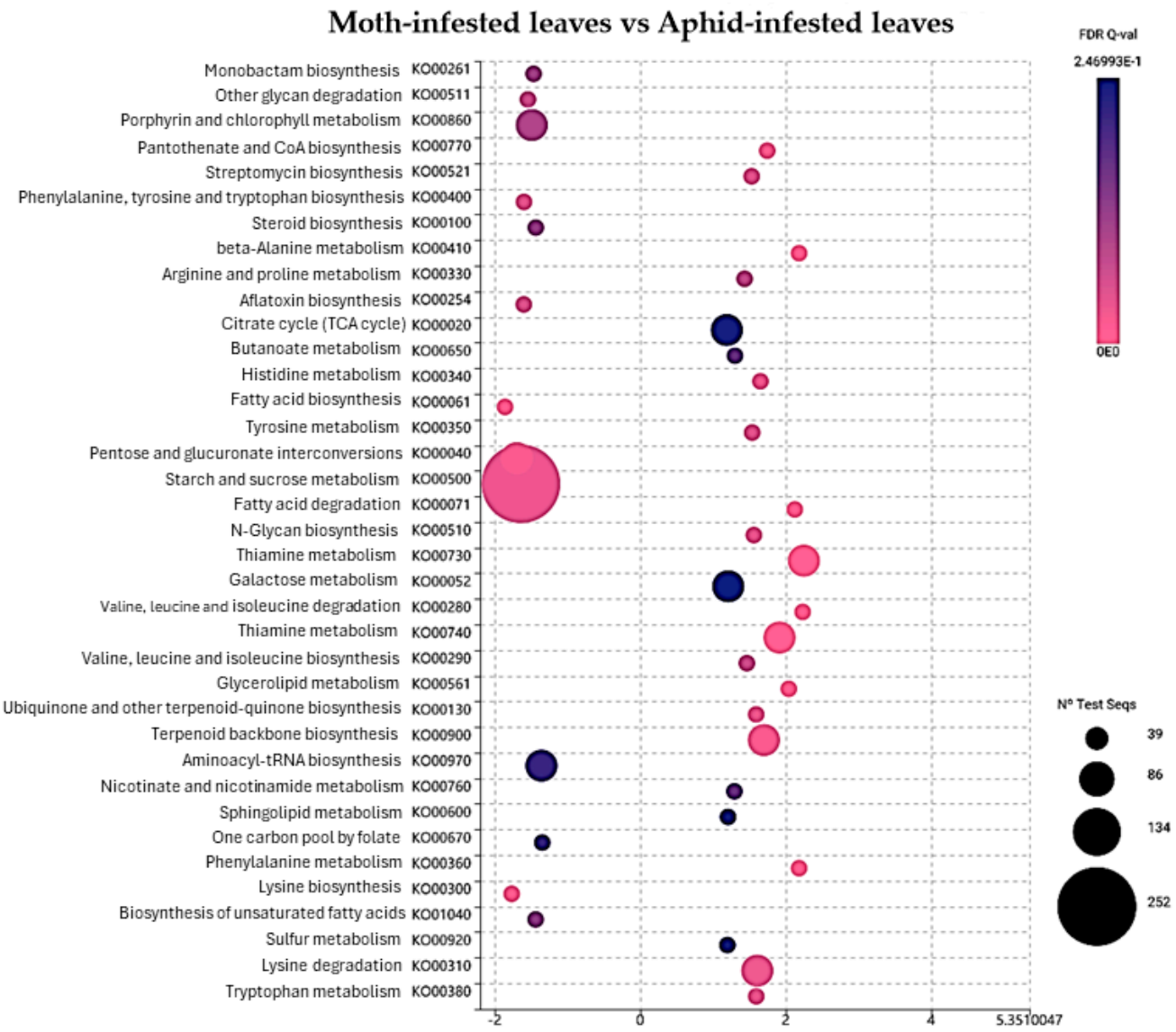

Figure 5. Pathway enrichment in the treatment of moth-infested leaves compared to aphid-infested leaves according to the number of identified DEGs for individual metabolic pathways.

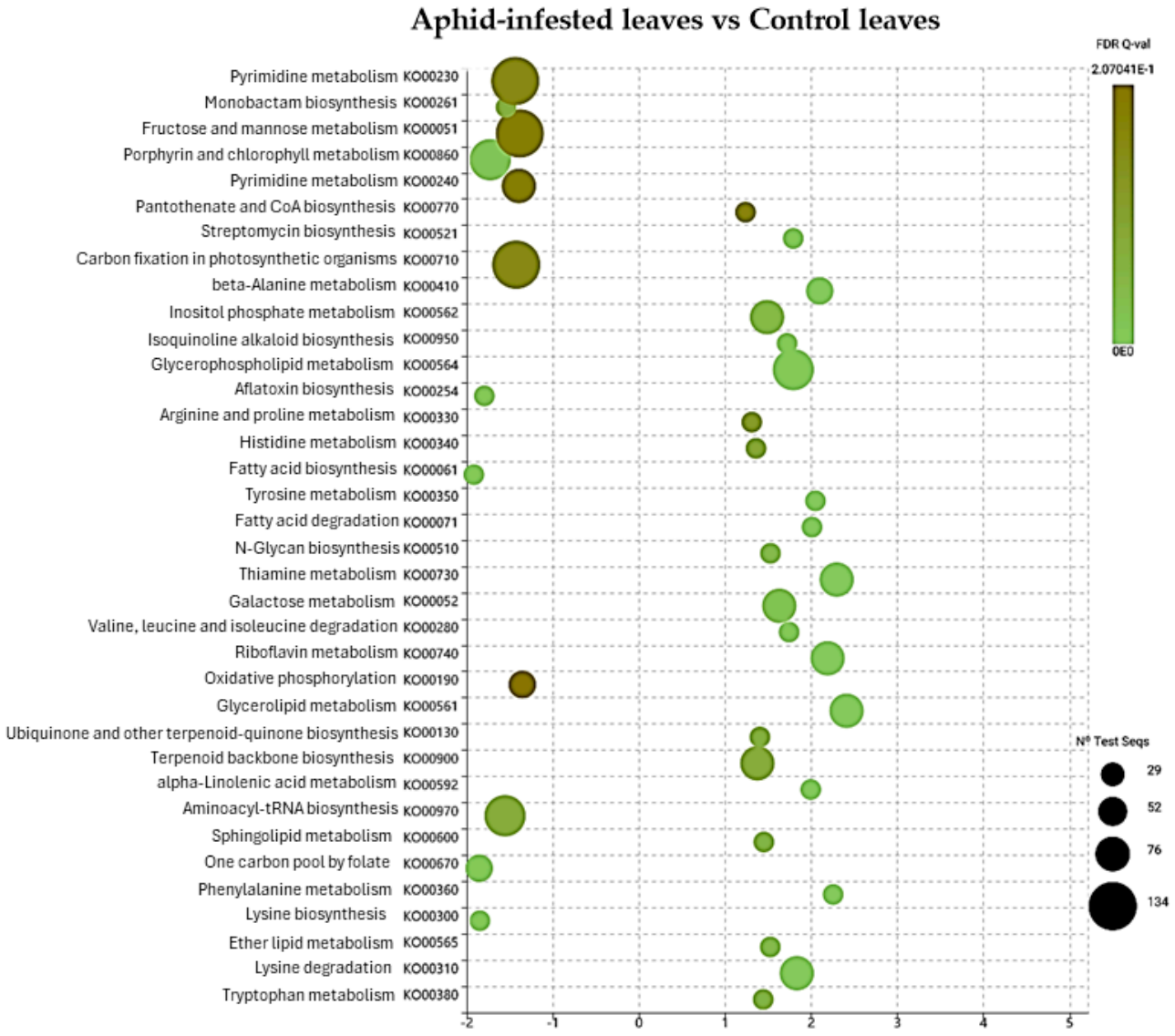

Figure 6. Pathway enrichment in the treatment of aphid-infested leaves compared to control leaves according to the number of identified DEGs for individual metabolic pathways.

Pathway Enrichment Analysis (KO00010-KO00380)				
Biological process / KO annotation		Insect guild Treatment		
Identity	Metabolic pathway	Control leaves vs Moths infested leaves	Control leaves vs Aphids infested leaves	Aphids infested leaves vs Moths infested leaves
KO00010	Glycolysis / Gluconeogenesis	x		x
KO00020	Citrate cycle (TCA cycle)			x
KO00030	Pentose phosphate pathway	x		x
KO00040	Pentose and glucuronate interconversions	x	x	x
KO00051	Fructose and mannose metabolism	x	x	x
KO00052	Galactose metabolism	x		x
KO00053	Ascorbate and aldarate metabolism	x	x	x
KO00061	Fatty acid biosynthesis	x		x
KO00062	Fatty acid elongation			x
KO00071	Fatty acid degradation	x		x
KO00073	Cutin, suberine and wax biosynthesis	x		x
KO00100	Steroid biosynthesis			x
KO00130	Ubiquinone and other terpenoid-quinone biosynthesis	x		x
KO00140	Steroid hormone biosynthesis	x		x
KO00190	Oxidative phosphorylation	x		x
KO00195	Photosynthesis			x
KO00220	Arginine biosynthesis	x	x	x
KO00230	Purine metabolism	x	x	x
KO00232	Caffeine metabolism			x
KO00240	Pyrimidine metabolism		x	x
KO00250	Alanine, aspartate and glutamate metabolism	x	x	x
KO00254	Aflatoxin biosynthesis	x		x
KO00260	Glycine, serine and threonine metabolism	x		x
KO00261	Monobactam biosynthesis			x
KO00270	Cysteine and methionine metabolism	x		x
KO00280	Valine, leucine and isoleucine degradation	x		x
KO00281	Geraniol degradation			x
KO00290	Valine, leucine and isoleucine biosynthesis	x		x
KO00300	Lysine biosynthesis	x		x
KO00310	Lysine degradation			x
KO00330	Arginine and proline metabolism	x		x
KO00340	Histidine metabolism	x		x
KO00350	Tyrosine metabolism	x	x	x
KO00360	Phenylalanine metabolism	x		x
KO00362	Benzoate degradation			x
KO00380	Tryptophan metabolism	x		x

Pathway Enrichment Analysis (KO 00400-00627)				
Biological process / KO annotation		Insect guild Treatment		
Identity	Metabolic pathway	Control leaves vs Moths infested leaves	Control leaves vs Aphids infested leaves	Aphids infested leaves vs Moths infested leaves
KO00400	Phenylalanine, tyrosine and tryptophan biosynthesis	x	x	x
KO00401	Novobiocin biosynthesis			x
KO00405	Phenazine biosynthesis	x		x
KO00410	beta-Alanine metabolism	x		x
KO00430	Taurine and hypotaurine metabolism	x		
KO00450	Selenocompound metabolism	x		x
KO00460	Cyanoamino acid metabolism			x
KO00480	Glutathione metabolism	x		x
KO00500	Starch and sucrose metabolism	x	x	x
KO00510	N-Glycan biosynthesis			x
KO00511	Other glycan degradation	x		x
KO00513	Various types of N-glycan biosynthesis	x		x
KO00515	Mannose type O-glycan biosynthesis			x
KO00520	Amino sugar and nucleotide sugar metabolism	x		x
KO00521	Streptomycin biosynthesis	x		x
KO00524	Neomycin, kanamycin and gentamicin biosynthesis			x
KO00531	Glycosaminoglycan degradation	x		x
KO00532	Glycosaminoglycan biosynthesis - chondroitin sulfate			x
KO00534	Glycosaminoglycan biosynthesis - heparan sulfate			x
KO00540	Lipopolysaccharide biosynthesis			x
KO00541	O-Antigen nucleotide sugar biosynthesis	x		x
KO00561	Glycerolipid metabolism	x		x
KO00562	Inositol phosphate metabolism			x
KO00564	Glycerophospholipid metabolism	x	x	x
KO00565	Ether lipid metabolism	x	x	x
KO00590	Arachidonic acid metabolism			x
KO00591	Linoleic acid metabolism			x
KO00592	alpha-Linolenic acid metabolism	x	x	x
KO00600	Sphingolipid metabolism	x		x
KO00601	Glycosphingolipid biosynthesis - lacto and neolacto			x
KO00603	Glycosphingolipid biosynthesis - globo and isoglobo	x		x
KO00604	Glycosphingolipid biosynthesis - ganglio series	x		x
KO00620	Pyruvate metabolism	x	x	x
KO00623	Toluene degradation			x
KO00625	Chloroalkane and chloroalkene degradation	x		x
KO00626	Naphthalene degradation			x
KO00627	Aminobenzoate degradation			x

Pathway Enrichment Analysis (KO00630-01040)				
Biological process / KO annotation		Insect guild Treatment		
Identity	Metabolic pathway	Control leaves vs Moths infested leaves	Control leaves vs Aphids infested leaves	Aphids infested leaves vs Moths infested leaves
KO00630	Glyoxylate and dicarboxylate metabolism	x	x	x
KO00640	Propanoate metabolism	x		x
KO00643	Styrene degradation			x
KO00650	Butanoate metabolism	x		x
KO00660	C5-Branched dibasic acid metabolism	x		x
KO00670	One carbon pool by folate			x
KO00680	Methane metabolism	x		x
KO00710	Carbon fixation in photosynthetic organisms			x
KO00720	Carbon fixation pathways in prokaryotes	x		x
KO00730	Thiamine metabolism	x		x
KO00740	Riboflavin metabolism	x	x	x
KO00760	Nicotinate and nicotinamide metabolism	x	x	x
KO00770	Pantothenate and CoA biosynthesis	x	x	x
KO00785	Lipoic acid metabolism			x
KO00790	Folate biosynthesis			x
KO00830	Retinol metabolism	x		x
KO00860	Porphyrin and chlorophyll metabolism	x		x
KO00900	Terpenoid backbone biosynthesis		x	x
KO00901	Indole alkaloid biosynthesis	x		x
KO00903	Limonene and pinene degradation	x		x
KO00908	Zeatin biosynthesis	x		x
KO00909	Sesquiterpenoid and triterpenoid biosynthesis			x
KO00910	Nitrogen metabolism	x	x	x
KO00920	Sulfur metabolism	x		x
KO00930	Caprolactam degradation			x
KO00942	Anthocyanin biosynthesis	x		x
KO00950	Isoquinoline alkaloid biosynthesis	x	x	x
KO00960	Tropane, piperidine and pyridine alkaloid biosynthesis	x		x
KO00966	Glucosinolate biosynthesis			x
KO00970	Aminoacyl-tRNA biosynthesis	x		x
KO00980	Metabolism of xenobiotics by cytochrome P450	x		x
KO00981	Insect hormone biosynthesis			x
KO00982	Drug metabolism - cytochrome P450	x		x
KO00983	Drug metabolism - other enzymes	x	x	x
KO00984	Steroid degradation			x
KO00997	Biosynthesis of various secondary metabolites - part 3			x
KO01040	Biosynthesis of unsaturated fatty acids			x

Figure 7. Pathway enrichment analysis. The "X" means that a DEG has been identified in the individual metabolic pathway.

2.3. Reference Gene Selection

Using a standard curve created with StepOne™ Software v2.3 and a known concentration of cDNA template, seven reference genes were filtered out based on their PCR amplification efficiency (Supplementary Table S1). The genes are expressed in *P. tremula*, according to the amplified product examined on the agarose gel. An expected amplicon size determined each gene's specificity, and the amplification efficiency range was 80–120%. For these reference genes, the coefficient of determination (R2) varied from 0.91 to 1.00 (Supplementary Table S1). The single peak was shown by the amplification specificity of each gene in the RT-qPCR examined using a melt curve analysis (Supplementary Figure S3). All potential reference genes had average Cq values between 21 and 26 (Figure 8).

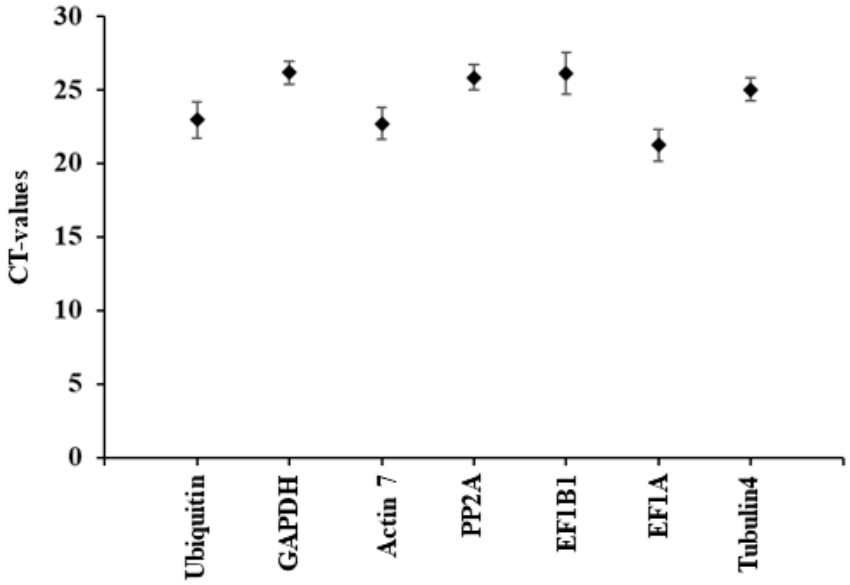

Figure 8. Identification of reliable housekeeping genes. Seven housekeeping genes' Ct values in control, aphid- and moth-infected *Populus* leaf samples varied. Total RNA was obtained and converted to cDNA to calculate Ct values. The cDNA and gene-specific primers were then utilized in RT-qPCR. Ct values are displayed as the mean ± SE.

Based on the overall ranking by geNorm, NormFinder, BestKeeper, ΔCt, and RefFinder, PP2A, GAPDH, and Act7 were designated as the highly stable genes across the treatments (Table 2; Supplementary Figures S2 and S3). EF1B1 and EF1A were the least stable genes as calculated by all the algorithms (Table 2; Supplementary Figures S2 and S3).

Table 2. The candidate housekeeping genes are ranked according to their stability value by geNorm, NormFinder, BestKeeper, ΔCT, and RefFinder analysis. M—the gene expression stability measure; SD—standard deviation value; SV—stability value; GM—Geomean value; and R—Ranking.

Gene Name	geNorm		NormFinder		BestKeeper		ΔCT		Comprehensive	
	M	R	SV	R	SD	R	SD	R	GM	R
Ubiquitin	1.32	3	1.334	5	2.95	6	2.14	4	4.68	5
GAPDH	0.841	1	1.002	3	2	2	2.08	3	2.06	2
Act7	1.078	2	0.785	2	2.48	5	1.9	2	2.78	3
PP2A	0.841	1	0.42	1	1.95	1	1.82	1	1	1
EF1B1	1.662	5	2.079	6	3.31	7	2.57	6	6.24	7
EF1A	2.468	6	4.331	7	2.44	4	4.48	7	6.09	6
Tub4	1.47	4	1.29	4	2.2	3	2.28	5	4.16	4

2.4. Gene Expression Validation

To corroborate the transcriptome results, RT-qPCR was performed on twenty physiologically significant DEGs associated with defence (Figure 9). Even though certain changes in the RT-qPCR data were not statistically significant, there was sufficient agreement between the transcriptomic and RT-qPCR data regarding the expression patterns of the twenty identified DEGs. The results showed how reliable the transcriptome discoveries were made in this study.

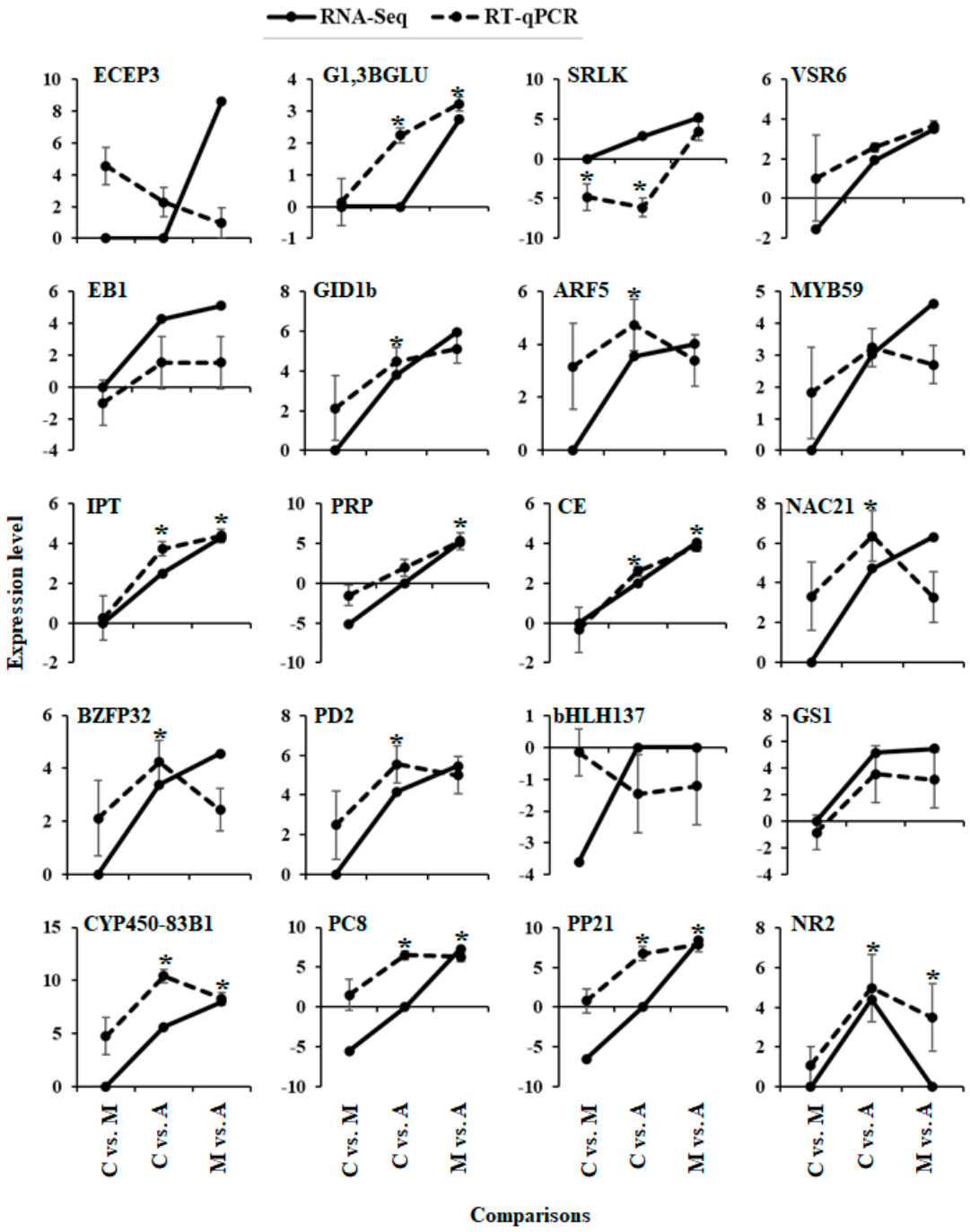

Figure 9. Comparison of transcriptome and RT-qPCR data for the expression of 20 genes in different feeding insects attack Poplar leaf samples (treatment legend: A—aphid-infested treatment, C—control, M—moth-infested treatment). The *x*-axis represents different comparisons of analysed samples and the *y*-axis represents the $\log^2$ fold change of RNA seq ($n = 4$) and RT-qPCR ($n = 4$). * represents $p < 0.05$.

3. Discussion

Poplar stands out as a key forest species due to its remarkable economic significance, rapid growth, simple vegetative reproduction, and ample genomic data, rendering it a prime candidate for the study of forest genetics, genomics, and breeding [19]. However, as a tree cultivated in open fields, poplar faces escalating environmental risks, particularly heightened biotic stresses, including insect attacks exacerbated by global warming [20]. However, *Populus* trees in southeast Asia, northeast Africa, Europe, and the East and West American continent suffer harm from over 100 insect species that belong to different insect groups, including Lepidoptera, Hemiptera, Diptera, Hymenoptera, and Coleoptera [21]. Therefore, it is critical to manage insects that harm *Populus* quantity and quality by using cultivars that are resistant to them. Plants have developed robust defence strategies against insect attacks, encompassing morphological traits, mechanistic barriers like trichomes and hairs, as well as chemical defences involving genes and pathways associated with various mechanisms [22–25]. Plant memories of previous biotic stresses can often facilitate its quick response to insect feeding [26].

The recent reports of high-quality genomes of spongy moths, aphids, *P. tremula*, and other "omics" technologies open up the scope for a higher understanding of the interactions between numerous feeding behaviour insects and plants [27–32]. Information on genetic diversity in the host response to insect infestation is needed for developing plants against pest resistance and insect control [5,33]. In this study, we examined the genes and metabolites expressed and accumulated differently by analysing the transcriptome and metabolome of leaves attacked by aphids and spongy moths compared to control leaves. We aimed to investigate the mechanisms underlying tree resistance to insects, specifically those that suck and chew on trees.

3.1. Response of Hormones Signalling Pathways after Aphid and Spongy Moth Infestation

The primary signal-transduction pathways in plants that underlie induced defence against herbivorous insects are jasmonic acid (JA) and salicylic acid (SA), which often exhibit both signalling pathways that can function additively or synergistically, though they typically behave antagonistically [34–36]. Sap-sucking insects such as aphids and whiteflies, when they attack plants, activate genes related to SA metabolism, leading to SA accumulation in infected plants. For instance, the green peach aphid (*Myzus persicae*) feeding induces the accumulation of SA-inducible transcripts in *Arabidopsis thaliana* [37–39], whereas silverleaf whitefly (*Bemisia tabaci*) infestation increases SA-inducible gene transcripts in both *A. thaliana* and tomato plants [40,41]. SA has been observed to exert a detrimental effect on the growth of phloem-feeding aphids and plays a crucial role in activating plant defences against these insects [42–45]; however, salicylic acid has also been found to elicit either a neutral or even a beneficial effect on the growth of numerous other phloem-feeding insects [40,46–48]. In this study, in aphid infestation, we observed significant upregulation of key genes involved in salicylic acid (SA) transduction, including NPR1 (BTB/POZ domain and ankyrin repeat-containing protein NPR1, BTB/POZ domain, and ankyrin repeat-containing protein NPR1-like), PR-1 (pathogenesis-related protein-1 and pathogenesis-related protein 1-like), and TGA (transcription factor TGA1-like, transcription factor TGA2-like isoform X1, transcription factor TGA2.3-like isoform X1, transcription factor TGA7-like, and transcription factor TGA9-like isoform X1) when compared to spongy moth infestation (Figure 10A).

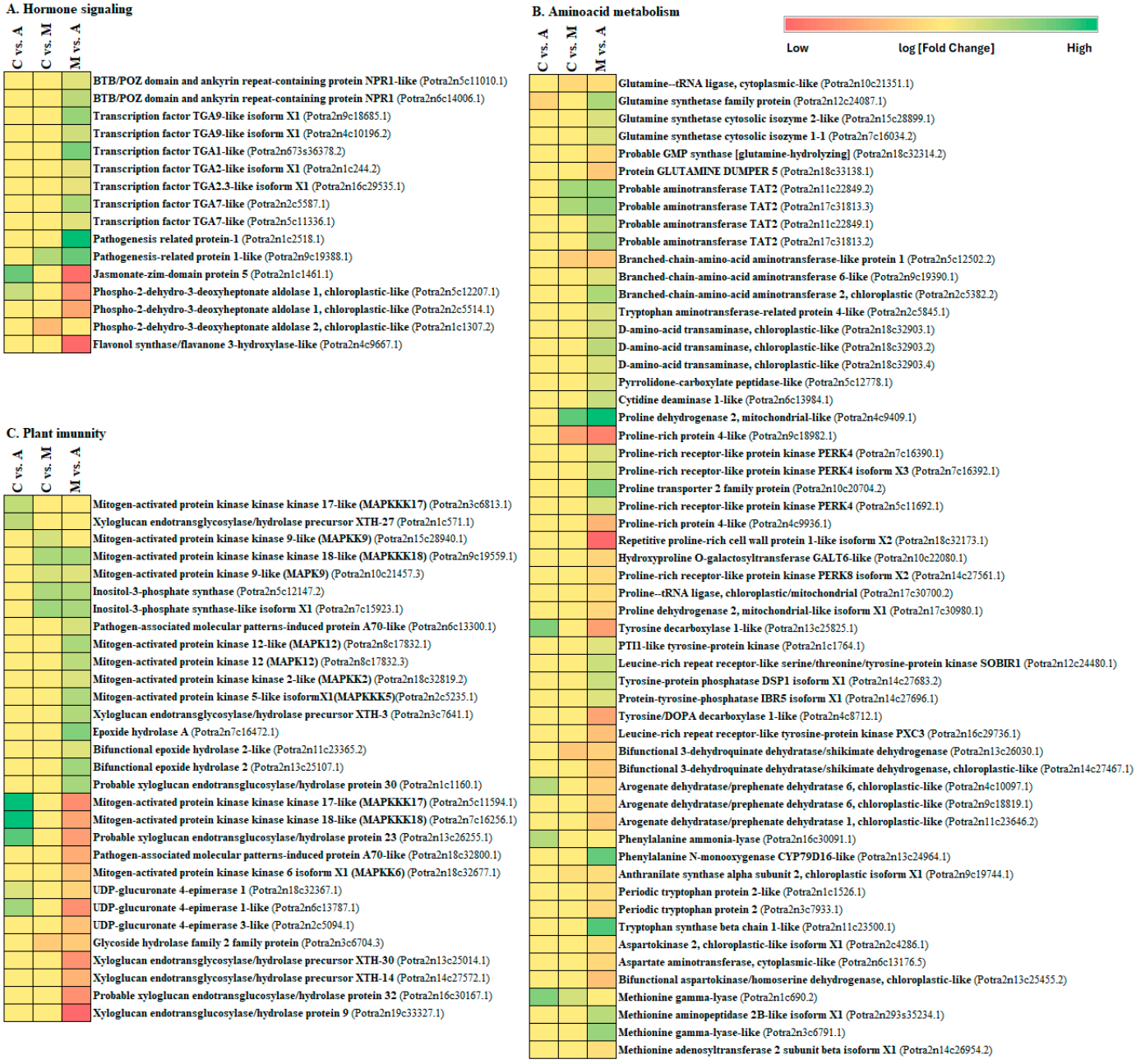

Figure 10. Impact of insect feeding treatment on key gene expression in *Poplar* leaves (treatment legend: A—aphid-infested treatment, C—control, M—moth-infested treatment). Genes are differentially regulated in (**A**) hormone signalling, (**B**) plant immunity, and (**C**) Amino acid metabolism pathways.

Plants have developed adaptive defences against chewing insects by inducing proteins, including polyphenol oxidases (PPOs) and proteinase inhibitors (Pis), which interrupt insect feeding and hinder insect growth [49,50]. Exposure of potato and tomato plants to Colorado potato beetles (*Leptinotarsa decemlineata*) prompts the activation of proteinase inhibitors (PIs), effectively inhibiting the activity of digestive proteinases within the insect's gut [51]. Jasmonic acid (JA), induced by chewing insects and wounding, triggers the expression of defensive proteins like Pis and PPOs [49,52]. Caterpillars feeding JA-deficient tomato mutants exhibit higher survivorship and weight gain than wild-type plants [49,53]. Exogenous application of JA or methyl jasmonate (MeJA) boosts plant resistance to herbivores and stimulates defensive protein expression in tomatoes [54–58]. This highlights JA's pivotal role in regulating plant defences against herbivores, with a JA-mediated

pathway identified from insect attack to defensive gene expression in plants [39,47,59–61]. In this study, our findings align with prior research, indicating that feeding by spongy moth insects triggers the activation of the JAZ (jasmonate-zim-domain protein 5) gene, which plays a vital role in JA biosynthesis. (Figure 10A, Supplementary File S2). When plants activate a JA signalling pathway, they become resistant to phloem-feeding and chewing insects [40,62,63]. Given that SA and JA exhibit antagonistic behavior, with SA inhibiting the buildup of JA and JA-inducible gene expression [64–66], it is theorized that numerous phloem-feeding insects have developed a strategy to dampen or undermine JA-inducible plant defences by stimulating the SA-inducible pathway [40,46,48,67].

The phospho-2-dehydro-3-deoxyheptonate aldolase enzyme catalyses the first of seven steps of chorismite biosynthesis and the final common precursor of all three aromatic amino acids as well as PABA, ubiquinone, and menaquinone. An essential component of the flavonoid biosynthesis pathway, flavanone 3-hydroxylase (F3H) regulates the accumulation of anthocyanidins and flavonols. When the sugarcane aphid (*Melanaphis sacchari*) infected the susceptible and resistant sorghum plants, it was observed that the infected plants' expression of flavonoid 3′-5′hydroxylase (F3′5′H) at day 10 and 15horismi-2-dehydro-3-deoxyheptonate aldolase at days 10 and 15 was lower than that of the uninfested control plants [68]. In this study, genes related to 15horismite biosynthesis and flavonoid biosynthesis pathways, including Phospho-2-dehydro-3-deoxyheptonate aldolase 1, Phospho-2-dehydro-3-deoxyheptonate aldolase 2, and Flavonol synthase/flavanone 3-hydroxylase-like, exhibit downregulation during aphid infestation whereas upregulation during spongy moth infestation (Figure 10A). In addition to direct feeding damage, aphids are vectors for plant diseases such as tospoviruses. Some studies suggested that viruses can manipulate plant defences by interacting with the SA and JA signalling pathways [69]. Our findings underscore the pivotal roles played by the SA and JA signalling pathways in the induction of plant defence mechanisms in response to sucking and chewing insect feeding.

3.2. Plant Immune Defence against Aphid and Spongy Moth Infestation

The interactions between plants and pathogens and plants and insects are known to share certain responses. Plants can fend off a pathogenic invasion and protect against insect damage and predation because of receptors on their cells that recognize pathogen-associated molecular patterns (PAMPs) and herbivore-associated molecular patterns (HAMPs) and then activate defence signalling pathways in the plant [36]. When insects attack plants, mitogen-activated protein kinases (MAPKs) get activated and regulate the plant defence induction, phytohormonal dynamics, transcription of genes relevant to defence, and synthesis of defence metabolites [70]. The MAPK cascade progresses through three sequential steps: MAPKKK phosphorylates MAPKK, which subsequently phosphorylates MAPK, and this activation of MAPK initiates a downstream cascade of events, influencing changes in plant hormone levels, restructuring the transcriptome and proteome, ultimately fortifying the plant's defence mechanisms against insect attacks [71]. In this study, the PAPM (pathogen-associated molecular patterns-induced protein A70-like) and MAPK (MAPKKK18) associated signalling pathways were identified as differentially expressed during the sucking and chewing insects attack (Figure 10C, Supplementary File S2). However, in spongy moth infestation, MAPKKK17 and UDP-glucuronate 4-epimerase 1-like genes show upregulation, whereas in aphid infestation, MAPKK9, MAPK9, MAPK12, MAPKK2, MAPKKK5, Inositol-3-phosphate synthase, Epoxide hydrolase A, and Bifunctional epoxide hydrolase 2 genes exhibit upregulation (Figure 10C). These genes exhibit activities linked to various plant defence mechanisms, including programmed cell death, maintenance of homeostasis, accumulation of reactive oxygen species, hypersensitive response, cell wall reinforcement, and induction of defence-related genes through stomatal closure. These findings underscore the significance of DEGs related to MAPK signalling and plant–pathogen interactions in facilitating plant-induced

defence against both sucking and chewing insect attacks, aligning with prior research findings and corroborating existing literature [5,72].

3.3. Primary Metabolism Alteration after Aphid and Spongy Moth Infestation

Insect attacks trigger a range of alterations in plant primary metabolism, including carbohydrate and nitrogen processes, as well as the composition and levels of amino acids that influence a plant's ability to withstand insect infestations [73,74]. Many plants synthesize defensive compounds from amino acid precursors like secondary metabolites and glucosinolates because amino acids are a primary nitrogen source, and their abundance in sap is a critical determinant of insect survival [75–77]. For instance, in *Arabidopsis*, caterpillar feeding activated the genes involved in amino acid biosynthesis and sulfur assimilation, which are critical for cysteine and methionine production and can lead to the accumulation of defence-related compounds like glucosinolates, mainly derived from methionine and tryptophan [74,78]. Similarly, tomato plants respond to foliar herbivory by accumulating tryptophan in systemic tissues, potentially fueling the production of defensive molecules [79]. Thus, herbivore-induced amino acid biosynthesis likely facilitates the synthesis of defence compounds in certain plant defence scenarios. Insects that feed on plants often trigger changes in gene expression within the plants, leading to alterations in amino acid metabolism. This is particularly noticeable with aphids, where substantial evidence indicates that their feeding directly increases the levels of free amino acids. For instance, the green bugs (*Schizaphis graminum*) known to cause chlorosis in wheat plants have been shown to boost the essential amino acid content in the plant's phloem sap [80]. Similarly, Japanese rowan (*Sorbus commixta*) leaves infested with apple-grass aphids (*Rhopalosiphum insertum*) exhibit a significant increase in amino acid excretion compared to unaffected leaves [81]. However, the precise mechanisms behind these observations are not fully understood, and it is likely that these herbivores enhance their diet's amino acid content through a combination of mechanisms such as increased amino acid production, accelerated leaf ageing leading to protein breakdown, or manipulation of nutrient transport within the plant. This study revealed the induction of numerous genes related to amino acid metabolism and its derivatives in response to infestations by both sucking and chewing insects (Figure 10B, Supplementary File S2). During aphid infestation, glutamine synthetase family protein genes, *probable aminotransferase TAT2*, *tryptophan aminotransferase-related protein 4-like*, *tryptophan synthase beta chain 1-like*, *D-amino-acid transaminase*, *pyrrolidone-carboxylate peptidase-like*, *cytidine deaminase 1-like*, *proline dehydrogenase 2*, *proline-rich receptor-like protein kinase PERK4*, *PTI1-like tyrosine-protein kinase*, *leucine-rich repeat receptor-like serine/threonine/tyrosine-protein kinase SOBIR1*, *tyrosine-protein phosphatase DSP1 isoform X1*, *protein-tyrosine-phosphatase IBR5 isoform X1*, *phenylalanine N-monooxygenase CYP79D16-lik*, and *methionine aminopeptidase 2B-like isoform X1* genes are upregulated. Whereas, in spongy moth infestation, tyrosine decarboxylase 1-like, arogenate dehydratase/prephenate dehydratase 6, and phenylalanine ammonia-lyase genes are upregulated (Figure 10B).

Lipids are an essential class of primary metabolites that perform structural, storage, and signaling roles and serve as precursors for compounds like jasmonic acid involved in plant defense. The investigation into how maize lipids respond to feeding by Egyptian cotton worms revealed notable alterations in lipid compositions [82]. Moreover, extracts of epicuticular lipids from plants, along with specific lipid components like cutin and wax, play crucial roles in plant defense against insects by influencing oviposition, movement, and feeding behavior [83–85]. Lipid signalling plays a crucial role during biotic and abiotic stresses in plants [86]. An intriguing discovery from our study revealed that genes involved in lipid metabolism (*Biotin carboxyl carrier protein of acetyl-CoA carboxylase*, *Omega-3 fatty acid desaturase*, *Acyl-lipid omega-3 desaturase (cytochrome b5)*, *Fatty acid amide hydrolase isoform X1*, *Fatty-acid-binding protein 1*, *Protein FATTY ACID EXPORT 4*, *Dihydroceramide fatty acyl 2-hydroxylase FAH1 isoform X1*, *Fatty acyl-CoA reductase 2-like isoform X*, *3-ketoacyl-CoA synthase 1-like*, *Acyl-CoA-sterol O-acyltransferase 1-like*, *Long chain acyl-CoA synthetase 2*

isoform X1, *Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase PASTICCINO 2*, *Non-specific lipid-transfer protein 1-like*, *Lipid transfer protein*, *Lipid phosphate phosphatase 2*, *Sphingolipid delta(4)-desaturase DES1-like*, *Phospholipid-transporting ATPase 3-like isoform X1*, etc.) were uniformly downregulated in sucking insect attacks, suggesting a negative correlation between lipid levels and the induced plant defense against these types of insect feeding (Figure 11A, Supplementary File S2).

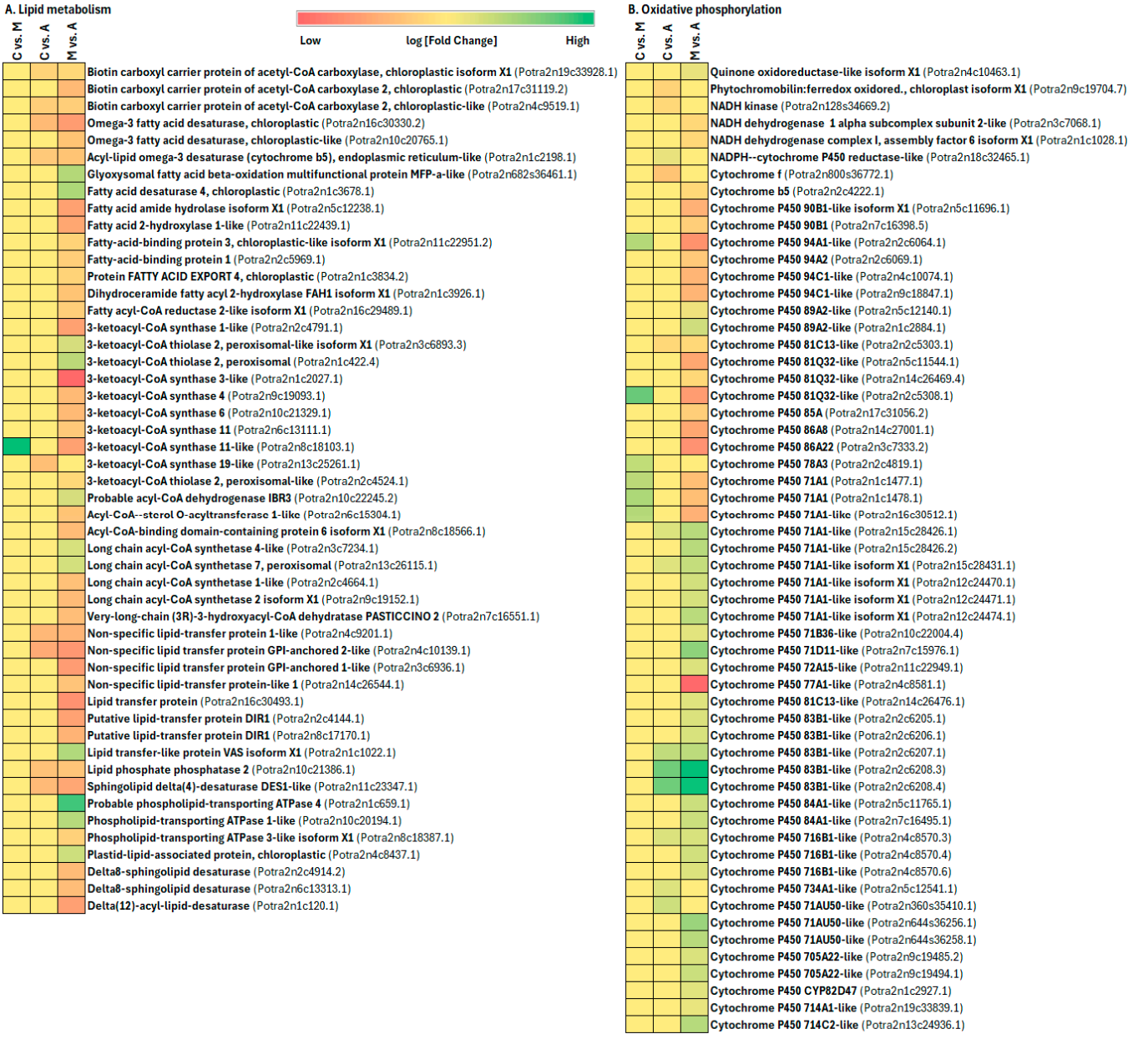

Figure 11. Impact of insect feeding treatment on physiologically important gene expression in Poplar leaves (treatment legend: A—aphid-infested treatment, C—control, M—moth-infested treatment). Genes are differentially regulated in (**A**) Lipid metabolism and (**B**) Oxidative phosphorylation.

Enhanced photosynthesis and localized carbohydrate breakdown can fuel plant defenses during interactions with herbivores [87,88]. Evidence for decreased photosynthesis due to herbivory is backed by direct measurements of alterations in photosynthesis rate, gene expression linked to photosynthesis, or the synthesis of proteins integral to the photosynthetic machinery [74,78,89]. The chewing herbivores, which consume leaf material,

and phloem-feeding insects, which extract nutrients from the phloem, trigger decreased expression of genes associated with photosynthesis [87,90]. Even cues of insect presence, such as oviposition or exposure to volatile compounds emitted by infested plants, can diminish photosynthetic capacity without causing direct damage [91,92]. These findings suggest that the decline in photosynthetic activity is a deliberate response by the plant rather than merely a byproduct of metabolic constraints during herbivory. In this study, we found that many genes involved in energy metabolism (including oxidative phosphorylation and carbon fixation in photosynthetic organisms) and carbohydrate metabolism were induced by sucking and chewing insect infestation (Figures 11B, 12A and 13, Supplementary File S2). In aphid infestation, oxidative phosphorylation genes cytochrome P450 71A1-like, cytochrome P450 71B36-like, cytochrome P450 71D11-like, cytochrome P450 72A15-like, cytochrome P450 81C13-like, cytochrome P450 83B1-like, cytochrome P450 84A1-like, cytochrome P450 716B1-like, cytochrome P450 734A1-like, cytochrome P450 71AU50-like, cytochrome P450 705A22-like, cytochrome P450 CYP82D47, cytochrome P450 714A1-like, and cytochrome P450 714C2-like gene were upregulated, whereas, in spongy moth infestation, cytochrome P450 94A1-like, cytochrome P450 81Q32-like, cytochrome P450 78A3, and cytochrome P450 71A1 genes are upregulated (Figure 11B). In aphid infestation, carbon fixation-related genes like NADPH-dependent aldo-keto reductase, Glutamate receptor 2.9-like, Digalactosyldiacylglycerol synthase 1, Malate synthase, Pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit alpha, Chlorophyllase-2-chloroplastic isoform X1, Glutamate receptor 2.8-like, etc., were upregulated (Figure 12A, Supplementary File S2).

When herbivores attack plants, they disrupt the usual carbohydrate supply from photosynthesis, whereas to compensate, plant cells often turn to alternative carbon and energy sources to produce defensive compounds. Many plants facing herbivore threats boost the breakdown of energy storage compounds like sucrose or starch locally. For example, a study on *Arabidopsis* involving four insect herbivores found increased expression of invertases and genes responsible for breaking down complex carbohydrates [78]). Similarly, in grain amaranth (*Amaranthus cruentus*), leaf herbivory led to a rise in cytoplasmic invertase and amylolytic enzyme activities and decreased monosaccharides concentrations like sucrose and starch in the affected tissues in the days following herbivore infestation [93]. In the present study, aphid infestation, carbohydrate metabolism-related genes like galactinol synthase 1-like, galactokinase, transaldolase isoform X1, sucrose synthase 6-like, raffinose synthase family protein, glucuronokinase, pyruvate-phosphate dikinase, phosphoenolpyruvate carboxykinase family protein, D-lactate dehydrogenase, isocitrate dehydrogenase, aldehyde dehydrogenase family 2 member B7, aldehyde dehydrogenase family 7 member B4, bifunctional UDP-glucose 4-epimerase and UDP-xylose 4-epimerase, UDP-glucose 4-epimerase 2-like, cellulose synthase-like protein G1, stachyose synthase-like, etc., were upregulated (Figure 13, Supplementary File S2).

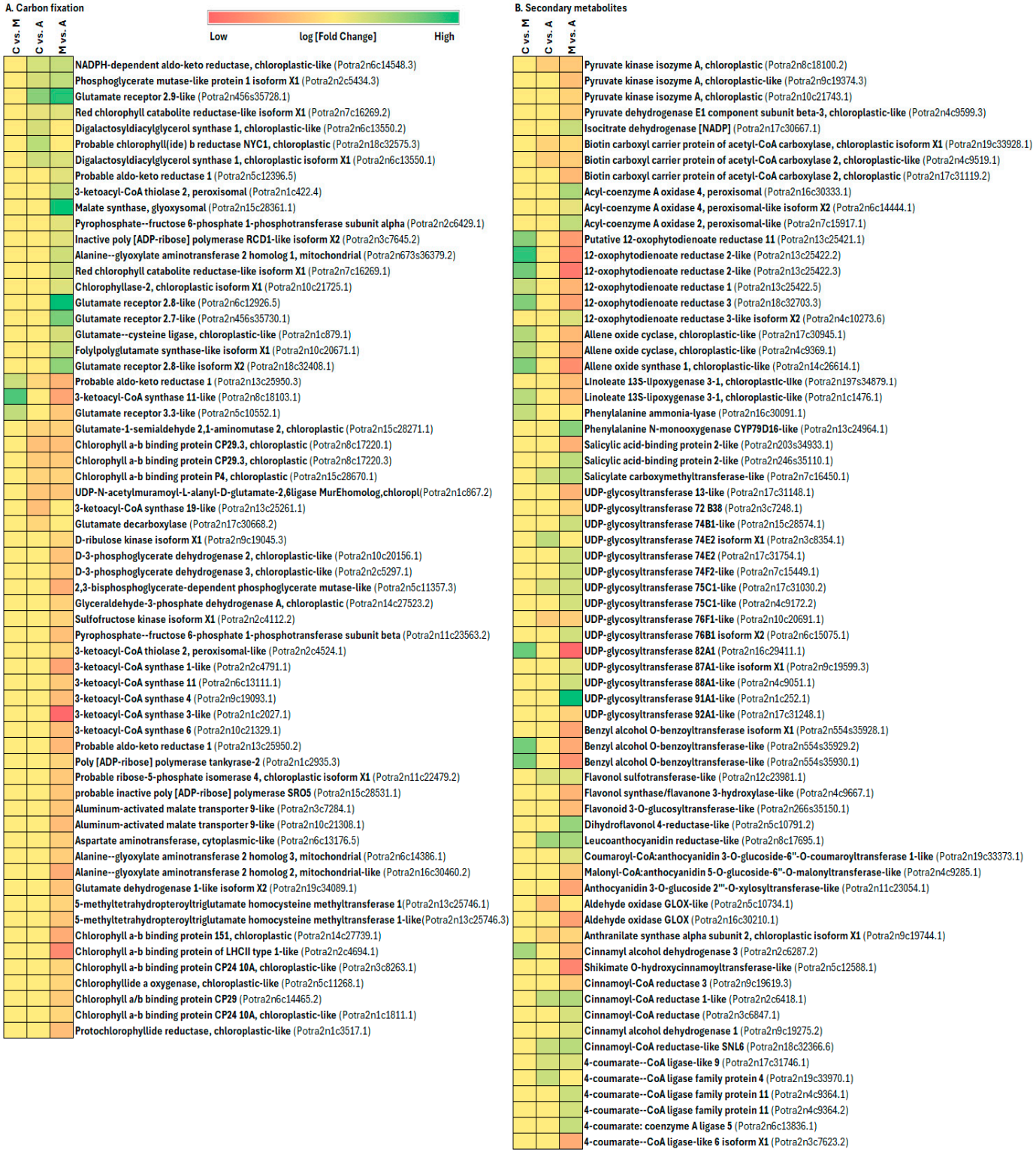

Figure 12. Impact of insect feeding treatment on genes related to (**A**) carbon fixation, (**B**) defense (secondary metabolite). Treatment legend: A—aphid-infested treatment, C—control, M—moth-infested treatment.

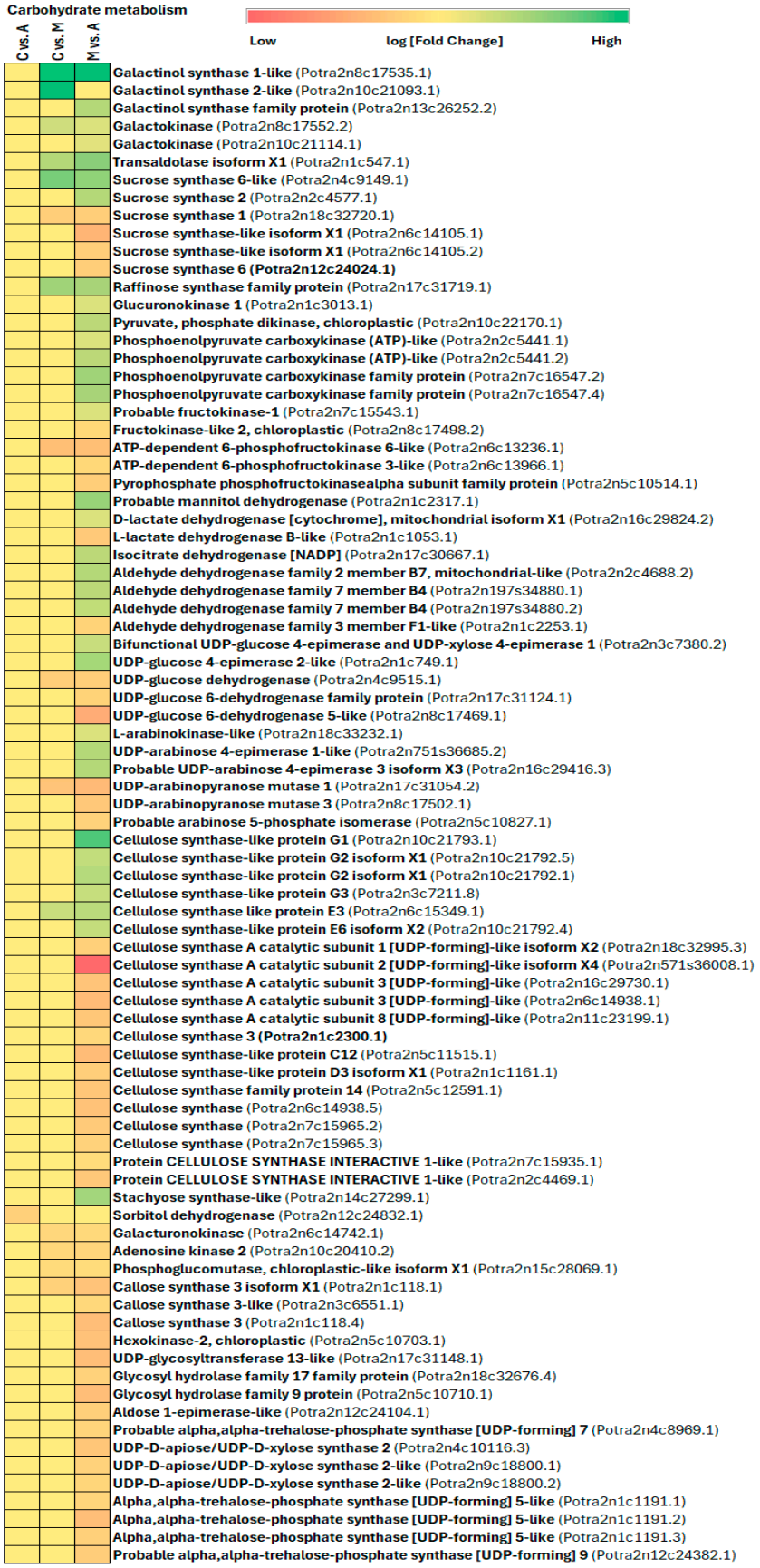

Figure 13. Impact of insect feeding treatment on genes related to carbohydrate metabolism and energy production under biotic stress. Treatment legend: A—aphid-infested treatment, C—control, M—moth-infested treatment.

These findings underscore the significant involvement of primary metabolite pathways, such as carbohydrate, lipid, amino acid metabolism, oxidative phosphorylation, and carbon fixation, in the defense response of *P. tremula* to sucking and chewing insects.

3.4. Plant Secondary Metabolism Alteration Due to Aphid and Spongy Moth Infestation

Insect pests have evolved several adaptive defense mechanisms to survive the morphological and biochemical phenomena that plants have created to endure their damage. The two most crucial plant defensive characteristics in terms of enhancing protection against insects are the plant's nutritional content and its inducible and constitutive chemical barriers [94]. Plants have evolved special behaviors and life cycles to overcome the mechanical barrier, but because the chemical defense is so dynamic and costly, it is more challenging to adopt. Plants continually generate secondary metabolites as a defense mechanism, diminishing their vulnerability to insect herbivores or negatively influencing insect biology and behaviour [9]. Plant secondary metabolites are categorized according to their structural composition, and the varied pathways involved in their biosynthesis include terpenes, phenolics, as well as nitrogen- and sulfur-containing compounds, showcasing a diverse array of chemical defences [95]. While plant secondary metabolites, such as alkaloids, glucosinolates, or phenolic compounds, play a crucial role in defending against insects, their content and distribution vary significantly among plant genotypes [96–99]. Flavonoids are crucial as secondary metabolites in safeguarding plants against pathogens, herbivores, and ultraviolet radiation [100]. When herbivores attack tea plants, a large number of genes involved in the biosynthesis of flavonoids are activated, leading to an increase in the contents of flavonols, dihydroflavonols, flavan-3-ols, anthocyanidins, flavones, and flavonoid glucosides, including myricetin, rutin, dihydroquercetin, and dihydromyricetin. In contrast, some flavonoid precursors and derivatives are decreased [101,102]. Subsequent research revealed that an artificial diet supplied with quercetin glucoside decreased the larval growth rate and that an *Ectropis grisescens* infestation markedly enhanced the accumulation of quercetin glucosides generated from quercetin catalysed by UGT89AC1 [103]. Additionally, during *Empoasca onukii* infestation, the levels of tricetin, kaempferol 3-O-glucosylrutinoside, and methyl 6-Ogalloyl-b-D-glucose, along with the expression of key genes involved in flavonoid biosynthesis, were significantly increased [104]. The present study demonstrated that, in comparison to aphid infestation, spongy moth infestation led to a notable abundance of secondary metabolites such as catechin, 2-caffeoylisocitrate, proanthocyanidin 1, fructose phosphate, heptamethoxyflavone, proanthocyanidin 3, benzoic acid, methyl salicylate, apigenin, and O-caffeoyl-o-methylquinic acid (Table 1). The findings of our transcriptome and metabolome analysis demonstrated that the infestation of sucking and chewing insects stimulated the pathways involved in the manufacture of flavonoids and phenylpropanoids (Figure 12B). In aphid infestation, secondary metabolites related genes like acyl-coenzyme A oxidase 4, acyl-coenzyme A oxidase 2, Phenylalanine N-monooxygenase CYP79D16-like, Salicylate carboxymethyltransferase-like, UDP-glycosyltransferase 74B1-like, Flavonol sulfotransferase-like, Dihydroflavonol 4-reductase-like, Leucoanthocyanidin reductase-like, Cinnamoyl-CoA reductase, Cinnamyl alcohol dehydrogenase 1, 4-coumarate--CoA ligase family protein 4, etc., were upregulated (Figure 12B). Whereas, in spongy moth infestation, putative 12-oxophytodienoate reductase 11, 12-oxophytodienoate reductase 1, Allene oxide cyclase, Allene oxide synthase 1, Linoleate 13S-lipoxygenase 3-1, Phenylalanine ammonia-lyase, UDP-glycosyltransferase 82A1, Benzyl alcohol O-benzoyltransferase-like, and cinnamyl alcohol dehydrogenase 3 genes were up-regulated (Figure 12B). A comprehensive diagram of captured up/downregulated genes for specific metabolic pathways is shown (Figure 14).

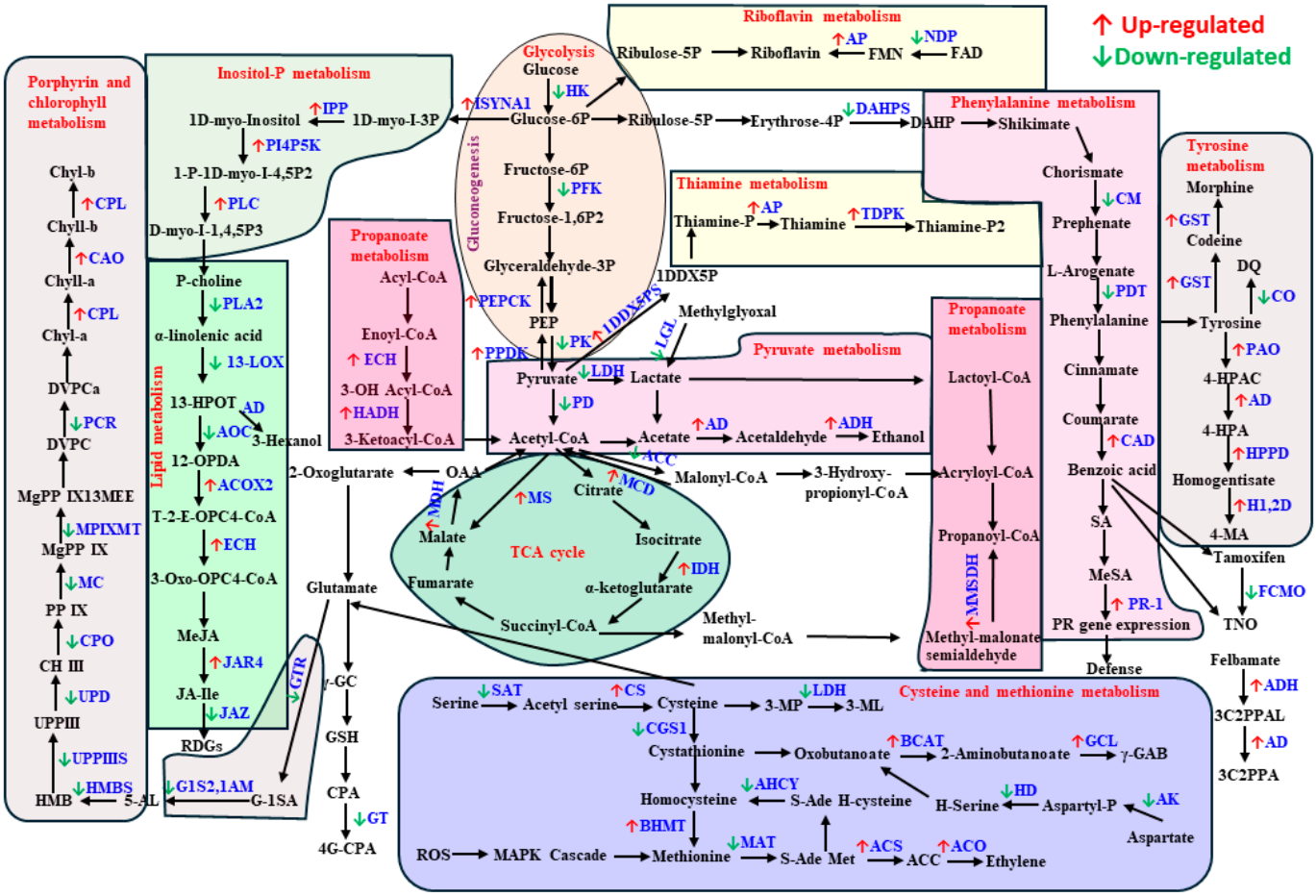

Figure 14. Diagrammatic representation of DEGs analysis in the aphid-infected leaf samples compared to spongy moth-infected leaf samples. The red (↑) and green (↓) arrows represent the up- and down-regulated genes (p-value < 0.05) in aphid-infested leaf samples. The DEGs coding enzymes are mainly related to carbohydrate metabolism (glycolysis-gluconeogenesis, pyruvate metabolism, citrate cycle, propanoate metabolism, inositol phosphate metabolism) lipid metabolism (alpha-linolenic acid), amino acid metabolism (phenylalanine, tyrosine, and tryptophan biosynthesis, cysteine and methionine metabolism, tyrosine metabolism), metabolism of cofactors and vitamins, other secondary metabolites synthesis (isoquinoline alkaloid biosynthesis) and xenobiotics biodegradation and metabolism (drug metabolism-cytochrome p450). Abbreviations: HK—Hexokinase; PFK—6-phosphofructokinase; PK—Pyruvate kinase; PPDK—Pyruvate, phosphate dikinase; PD—Pyruvate dehydrogenase; AD—Aldehyde dehydrogenase; ADH—Alcohol dehydrogenase; LDH—L-lactate dehydrogenase; PEPCK—Phosphoenolpyruvate carboxykinase; LGL—Lactoylglutathione lyase; ACC—Acetyl-CoA carboxylase; MS—Malate synthase; MDH—Malate dehydrogenase; IDH—Isocitrate dehydrogenase; MMSDH—Methylmalonate-semialdehyde dehydrogenase; MCD—Malonyl-CoA decarboxylase; HADH—3-hydroxyacyl-CoA dehydrogenase; ECH—Enoyl-CoA hydratase; ACOX2—Acyl-coenzyme A oxidase 2; IPP—Inositol-phosphate phosphatase; PLC—Phospholipase C; ISYNA1—Inositol-3-phosphate synthase 1; PI4P5K—1-phosphatidylinositol-4-phosphate 5-kinase; AOC—Allene-oxide cyclase; PLA2—Phospholipase A2; ECH—Enoyl-CoA hydratase; 13-LOX—13-lipoxygenase; JAR4—Jasmonoyl—L-amino acid synthetase JAR4; JAZ—Jasmonate-zim-domain protein 5; PDT—Prephenate dehydratase 6; DAHPS—Phospho-2-dehydro-3-deoxyheptonate aldolase 1; CM—Chorismate mutase; CAD—Cinnamyl alcohol dehydrogenase; CGS1—Cystathionine gamma-synthase 1; CS—Cysteine synthase; MAT—Methionine adenosyltransferase; ACO—1-aminocyclopropene-1-carboxylate oxidase; ACS—1-aminocyclopropane-1-carboxylate synthase; BHMT—Betaine--homocysteine S-methyltransferase; AHCY—Adenosylhomocysteinase; HD—Homoserine dehydrogenase; AK—Aspartate kinase; BCAT—Branched-chain-amino-acid transaminase; GCL—Glutamate--cysteine ligase; SAT—Serine O-acetyltransferase; LDH—L-lactate dehydrogenase; CO—Catechol oxidase; H1,2D—Homogentisate 1,2-dioxygenase; PAO—Primary-amine oxidase; HPPD—4-hydroxyphenylpyruvate dioxygenase; AP—Acid phosphatase; TDPK—

Thiamine diphosphokinase; NDP—Nucleotide diphosphatase; HMBS—Hydroxymethylbilane synthase; MPIXMT—Magnesium protoporphyrin IX methyltransferase; CPO—Coproporphyrinogen oxidase; UPD—Uroporphyrinogen decarboxylase; G1S2,1AM—Glutamate-1-semialdehyde 2,1-aminomutase; UPPIIIS—Uroporphyrinogen-III synthase; GTR—Glutamyl-tRNA reductase; CAO—Chlorophyllide a oxygenase; CPL—Chlorophyllase; PCR—Protochlorophyllide reductase; MC—Magnesium chelatase; GST—Glucuronosyltransferase; FCMO—Flavin-containing monooxygenase; GT—Glutathione transferase. PR-1—Pathogenesis related protein-1. G-1SA—Glutamate-1-semialdehyde; 5-AL—5-Amino-levulinate; HMB—Hydroxymethylbilane; UPPIII—Uroporphyrinogen III; CHIII—Coproporphyrinogen III; PPIX—Protoporphyrin IX; MgPPIX—Mg-protoporphyrin IX; MgPPIX13MEE—Mg-protoporphyrin IX 13-monomethyl ester; DVPC—Divinylproto-chlorophyllide; DVPCa—Divinylproto-chlorophyllide a; Chyl-a—Chlorophyll a; Chyll-a—chlorophyllide a; Chyll-b—chlorophyllide b; Chyl-b—Chlorophyll b.

This result is in line with recent research that found sensitive and resistant plant cultivars to spongy moths, and aphid infestations can control the expression of genes in the flavonoid biosynthesis pathways to produce the production of defence genes and proteins [105–107]. Comparable outcomes have been found in cotton plants with various pest infections [5,108,109]. Therefore, the activation of genes and metabolites linked to flavonoid production in *P. tremula* showed their possible role in inducing plant defence in *P. tremula* in response to both sucking and chewing insects.

4. Materials and Methods

4.1. Plants and Insects

The experiments utilized genetically uniform *Populus tremula* individuals aged eight months. The seeds were obtained by controlled crossing of parent trees [locations: Krušné hory (Fláje, 50.6653750N, 13.5753711E), Czech Republic, 40–50 years old]. The material from one seed was used for the in vitro propagation of genetically uniform individuals.

Seeds of *P. tremula* were washed in 200 mL distilled water with 1–2 drops of Tween 20® for 10–15 min, then sterilized in 0.1% $HgCl_2$ for 6 min [110]. After rinsing, seeds were placed in jars with Murashige and Skoog [111] (MS) medium solidified with Danish® agar and supplemented with myo-inositol and 6-benzylaminopurine (BAP). The pH-adjusted medium was autoclaved, and explants were cultivated under 16/8 h light/dark with a temperature of 22 ± 1/20 ± 1 °C (Figure 15A,B). Germination occurred within 1–3 weeks. Shoots were subcultured every 2–3 weeks until sufficient material was obtained. In vitro, rooting was done on segments with at least three buds using a half-strength MS medium supplemented with indole-3-butyric acid (IBA). Roots developed after about 4 weeks, and after 6–8 weeks, rooted shoots were transferred ex vitro (Figure 15C). Rooted shoots were washed and transferred to a sterile substrate in plastic pots, treated with Previcur Energy®, and cultivated under controlled conditions (Figure 15D). Humidity was gradually decreased, and plants were fertilized bi-weekly during growth.

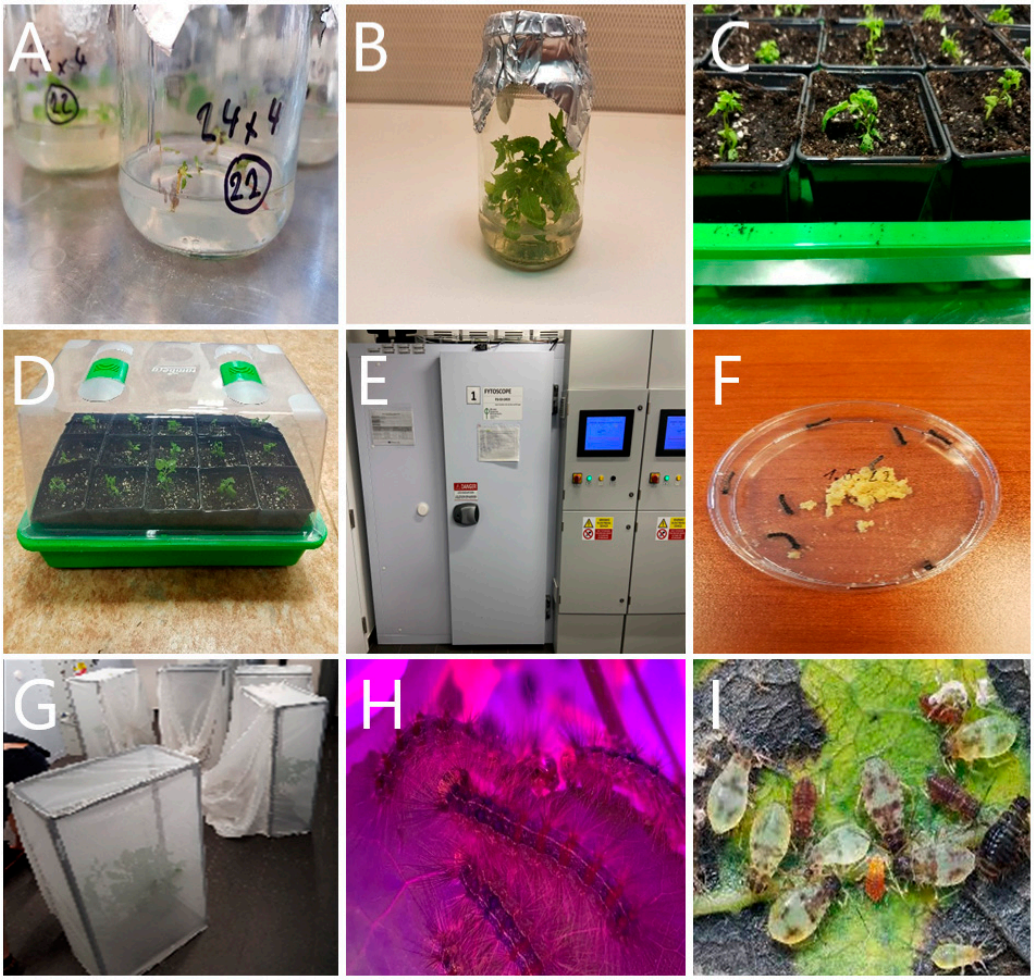

Figure 15. Experimental setup and plant treatment with insect feeding. (**A**) Poplar tissue propagation on MS medium. (**B**) In vitro growth of genetically uniform poplars. (**C**,**D**) Transfer of in vitro poplars to ex vitro. (**E**) Poplar plants are grown in the growth chamber. (**F**) Spongy moth larvae. (**G**) Aphid treatment setup. (**H**) Spongy moth feeding on poplar leaf. (**I**) Aphids feeding on poplar leaf.

Poplars were grown on a high-temperature steam-disinfected substrate without fungi, mold, and insect contamination (Forestina, Czech Republic) in growth chambers Step-In FytoScope FS-SI (Photon Systems Instruments, Drasov, Czech Republic) (Figure 15E). The growth chamber environment simulated optimal conditions for growth, parameters: humidity: 75%; intensity of Photosynthetic Photon Flux density: 250 μmol·m^{2}·s^{-1}; CO_2 concentration: 415 ppm; day and night period: 2 h dawn, 10 h light, 2 h twilight, 10 h dark. The basic features of the experiment are the tripartite design, which includes control, leaf-chewing (*Lymantria dispar*), and phloem-feeding (*Chaitophorus populialbae*). To prevent chemical communication between different experimental plants (Poplars), 20 plants for each treatment were placed in separate growth chambers throughout the experiment. We used the spongy moth (*Lymantria dispar*, Lepidoptera: Erebidae) as a representative species of leaf-chewing insect guild. Eggs of spongy moths (*Lymantria dispar*) were supplied by the Institute of Forest Entomology, Forest Pathology, and Forest Protection at the University of Natural Resources and Life Sciences in Vienna from sterile laboratory cultures. After hatching, larvae were given a nutritionally balanced agar diet (*Lymantria dispar* agar, Southland Products Inc., Newark, DE, USA) in sterile Petri dishes (Figure 15F,H).

As a phloem-sucking insect, we used *Chaitophorus populialbae*, (Hemiptera: Aphididae), which was caught in the wild while sucking on *P. tremula* and incubated in sterile rearing containers before being placed in the growth chamber. In vitro cultures of poplar individuals served as a food source for aphids, which were replaced with fresh ones every

3 days, and at the same time, new and disinfected rearing containers were replaced (Figure 15I). This breeding method effectively reduced the risk of phytopathological contamination, especially the overgrowth of mold and fungi.

4.2. Experimental Design

The strategic goal of this experimental design is to ensure that the influence of insect herbivory will be the only factor that affects plant metabolism. The division into treatment groups is as follows: Control—individuals without any damage; individuals attacked by aphids (leaf-sucking); individuals attacked by a spongy moth (leaf-chewing) (Supplementary Figure S1). Before starting the experiment, five healthy (8-month-old) Poplars were selected for each group based on phenotypic characteristics. They were placed in three growth chambers separately for each group. At this stage, the poplars from the leaf-sucking treatment were placed in prepared boxes (40 cm × 100 cm × 120 cm), and the walls were made of very fine mesh (<0.01 mm), which is certified for use in the food industry (without emission of chemical substances) and does not change the spectral properties of light. The plants were left for 14 days in the climate chambers for acclimatization before the start of the experiment.

4.3. Poplar Tissue Feeding, Collection and Processing

The leaf-chewing treatment was formulated to capture initial occurrences of gene expression while reducing discrepancies due to leaf age and the extent of insect damage. At the same trunk level, each individual was assigned a leaf on which five spongy moth caterpillars were placed. It has been determined that approximately 30% of the leaf area must be eaten within one hour. In the test experiments, it proved critical to ensure the feeding activity of the caterpillars. They were incubated in the dark without food for 48 h to increase feeding activity, considering their nocturnal behaviour [112]. During the experiment, caterpillars were held onto the selected leaf using a size 0 goat hair brush and washed thrice in chloroform. After feeding, the leaf was cut with disinfected scissors, placed in a 50 mL falcon tube, and placed in a liquid nitrogen bath.

Treatment with aphids (*Chaitophorus populialbae*) was different due to the significantly weaker and different effect compared to caterpillars on the plant [113,114]. Part of the aphids were moved from the reared colonies using a prepared size 0 goat hairbrush (treated with chloroform and adequately ventilated). Part of the aphids were moved from the reared colonies using a prepared size 0 goat hairbrush (treated with chloroform and adequately ventilated). After 2 days, the same old, fully matured leaf was taken from each poplar. Aphids and remnants of aphid bodies were removed using a goat hairbrush—it was then immediately placed in a 50 mL falcon tube and placed in a liquid nitrogen bath.

Plant tissue samples were stored at −80 °C for further processing. After lyophilization and homogenization, the processed plant tissue was divided into two halves. One half was intended for non-targeted metabolomics analysis, and the other was used for RNA isolation and subsequent transcriptomic analysis and RT-qPCR validation.

4.4. Metabolomics Non-Targeted Analysis

4.4.1. Extraction Procedure

Accurately 10 mg of freeze-dried and homogenized plant tissue was weighed into a 2 mL microcentrifuge tube before adding 0.5 mL of 70% cold methanol. After 30 s of vortexing, the test tube was placed into an ultrasonic bath with ice for 10 min. The solution was then centrifugated for 10 min at 13,000 rpm and 4 °C. The supernatant was filtered using a 0.22 µm PTFE filter before LC-MS-qTOF analysis. All manipulations with samples were performed on the ice.

4.4.2. LC-MS-qTOF Metabolomic Analysis

Metabolomic analysis using LC-MS-qTOF was performed utilizing an Agilent 1290 Infinity II system coupled with an Agilent 6546 LC/MS QTOF instrument (Agilent, Santa Clara, CA, USA). A column of InfinityLab Poroshell 120 EC-C18 (2 × 150 mm, 2.7 μm) from Agilent (USA) was employed. The mobile phase consisted of two components: mobile phase A containing 0.1% formic acid and 0.005 M ammonium fluoride, and mobile phase B comprising acetonitrile and 0.01% formic acid. The gradient elution program consisted of the following proportions: 0–4 min, 85% A; 4–7 min, 75%; 7–9 min, 68% A; 9–16 min, 60% A; 16–22 min, 45% A; 22–28 min, 5% A; 28–30 min, 5% A. The flow rate of the mobile phase was set to 0.5 mL min^{-1}, and the column temperature was maintained at 35 °C. A 1 μL injection volume was used. The system operated in both positive and negative ionization modes. The QTOF parameters were configured as follows: scan range of 100–1000 *m*/*z*; the drying gas temperature at 160 °C; sheath gas flow rate of 12.0 L/min; sheath gas temperature at 400 °C; capillary voltage set to 5.0 kV; nozzle voltage at 2.0 kV; fragmentor set to 140 V; collision energy employed at 10, 20, and 40 eV. MS/MS data were acquired with a scan range of 50–800 *m*/*z*, a retention time window of 0.5 min, an isolation window of 1.3 amu, and an acquisition rate of 3 spectra per second. For mass correction, the analysis monitored two reference masses, 112.9855 *m*/*z*, and 966.0007 *m*/*z*.

The raw data files were processed using Mass Hunter Profinder 10.0 software for time alignment and feature extraction. Parameters for time alignment were set as minimal intensity 1000 counts and maximum time shift 0.5 min plus 0.3%. For feature extraction, the parameters were *m*/*z* range 100–1000, minimal intensity 1000 counts, retention time tolerance 0.25 min, and mass tolerance 20 ppm plus 2 mDa.

The obtained data were exported to Metabolanalyst (https://www.metaboanalyst.ca/ (accessed on 13 November 2021)) for statistical analysis and visualization. The data were filtered by interquartile range, normalized by a median, log-transformed, and mean centering on identifying metabolite target MS/MS analyses. Metabolite identification was performed by comparing data from the Metline Database, internal library, and literature according to the retention time and MS/MS fragmentation.

4.4.3. Statistical Evaluation of LC-MS-qTOF Data

Separated signals were aligned, and data from three injections of each sample were averaged. Constant sum normalization was performed, followed by the centred log-ratio (clr) transformation. Principal Component Analysis (PCA) and Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) of Pareto-scaled data were created in Simca 17.0 SW (Sartorius Stedim Data Analytics AB, Sweden). To select the most affected metabolites, separate OPLS-DA models were constructed for control vs. moth-infested, moth vs. aphid-infested, and control vs. aphid-infested leaves. From the variable importance for projection (VIP) plot, compounds with a VIP value higher than 1, at least in one of the two-group models, were selected for their metabolism pathways evaluation.

4.5. Transcriptomics Analysis

4.5.1. Total RNA Isolation

For the RNA isolation, leaf samples (50 mg) were put into 2 mL Eppendorf Safe-Lock tubes containing three steel grinding balls and frozen under liquid nitrogen. Subsequently, the tissue was ground with Retsch Mixer Mill 400. Total RNA was extracted with Epicentre MasterPure RNA Purification Kit (Epicentre). After extraction, the total RNA underwent DNase I treatment using the TURBO DNase Kit (Ambion, Austin, TX, USA). Subsequently, the integrity of the purified total RNA was assessed on a 1.2% agarose gel, and its concentration was determined using the NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, MA USA).

4.5.2. NGS Sequencing and Data Analysis

Transcriptome libraries were constructed using leaf samples infected with aphids, spongy moths, and respective control leaf samples (Supplementary Figure S1). To enrich mRNA, oligo (dT) beads were employed, followed by cDNA library preparation using the NEB Next® Ultra™ RNA Library Prep Kit and Illumina Novaseq6000 sequencing, resulting in 30 million reads (150 paired ends) per sample. Each sample had five biological replicates. Differential gene expression analysis (DGE) was conducted by mapping raw reads to the *P. tremula* reference genome [27] using the OmicsBox transcriptomics module (ver 1.4.11) following the developer protocol as described thoroughly in our latest publication [115]. DGE was performed using the edgeR software package (Bioconductor project) [116], deploying a negative binomial Generalized Linear Model (GLM) for multi-factorial statistical analysis to identify differentially abundant transcripts, with FDR corrected p-value < 0.05 and fold change ±2 as thresholds for differentially expressed transcripts (DETs). To illustrate the expression pattern using Cluster 3.0, hierarchical clustering was performed using the average linkage approach with Euclidean distance based on log fold change data [117].

4.5.3. Reference Gene Selection for RT-qPCR

To identify the optimal reference gene for gene expression validation and perform RT-qPCR studies, preliminary studies were conducted, considering genes previously reported and commonly utilized in *P. tremula*. Seven genes were chosen from the transcriptomic data of *P. tremula*, comprising *polyubiquitin* (Ubiquitin), *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH), *Actin 7* (Act7), *serine/threonine-protein phosphatase 2A* (PP2A), *elongation factor 1-beta 1* (EF1B1), *elongation factor 1-alpha* (EF1A), and *tubulin beta-4 chain-like* (Tubulin 4) (Supplementary Table S1). The sequences retrieved underwent a BLASTx search against the NCBI database to corroborate their annotations. One microgram of total RNA was utilized for cDNA synthesis employing the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems-Life Technologies, Waltham, MA, USA), stored at −20 °C. Before usage as a template in RT-qPCR experiments, the cDNA samples underwent a 10-fold dilution. Each RT-qPCR assay involved four biological replicates per sample. Primer design was conducted using the IDT PrimerQuest software (IDT, Belgium, https://sg.idtdna.com/pages/tools/primerquest?returnurl=%2FPrimerquest%2FHome%2FIndex (accessed on 13 November 2021)) (Supplementary Table S1). RT-qPCR analyses were conducted for all samples, including controls and treatments. The 10 µL RT-qPCR reactions comprised 5.0 µL SYBR® Green PCR Master Mix (Applied Biosystems), 1.0 µL cDNA, 1.0 µL of 10 µM forward and reverse primers, and 3.0 µL RNase-free water (Invitrogen, Waltham, MA, USA). Reactions were conducted in an Applied Biosystems™ StepOne™ Real-Time PCR System (Applied Biosystems) under the following conditions: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and dissociation curve analysis with temperature increasing from 60 to 95 °C. Target gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method [118].

The selection of the most effective reference gene was based on assessing their expression stability using standard algorithms described by earlier studies [119]. Utilizing algorithms such as geNorm, Normfinder, Bestkeeper, Delta CT, and RefFinder, the stability of gene expression was assessed to identify the most suitable reference genes for precise normalization of target gene expression across leaf samples affected by aphids and spongy moths and control samples.

4.5.4. Gene Expression Validation by RT-qPCR

To verify the expression of target genes across treatment and control samples, we selected twenty genes linked to both up- and downregulation such as *Endochitinase EP3* (ECEP3), *Glucan endo-1,3-beta-glucosidase, basic isoform* (G1,3BGLU), *Symbiosis receptor*

(SRLK), *Vacuolar-sorting receptor 6* (VSR6), *Expansin-like B1* (EB1), *Gibberellin-Insensitive Dwarf1* (GID1b), *Auxin response factor 5.2* (ARF5), *Transcription factor MYB59-like isoform X2* (MYB59), *Probable inorganic phosphate transporter 1* (IPT), *Pathogenesis-related protein* (PRP), *Caffeoylshikimate esterase* (CE), *NAC domain-containing protein 21/22-like isoform X2* (NAC21), *B-box zinc finger protein 32* (BZFP32), *Proline dehydrogenase 2, mitochondrial* (PD2), *Transcription factor bHLH137-like isoform X1* (bHLH137), *Galactinol synthase 1* (GS1), *Cytochrome P450 83B1* (CYP450-83B1), *Probable carboxylesterase 8* (PC8), *Protein P21* (PP21) and *Probable nucleoredoxin 2 isoform X2* (NR2) in the transcriptomic data (Supplementary Table S2). The RT-qPCR study was performed using four biological replicates from each treatment using the same protocol described before. The RT-qPCR expression data were normalized using the PP2A reference gene. A one-way ANOVA test was performed to evaluate the significance of gene expression differences in RT-qPCR.

5. Conclusions

Sucking and chewing insects feeding on *P. tremula* trigger notable changes in the *P. tremula* physiology. Through an integrated analysis of both transcriptome and metabolome (Figure 16), it was observed that pathways related to flavonoid and isoflavonoid biosynthesis are significantly enriched in response to sucking and chewing insect infestation.

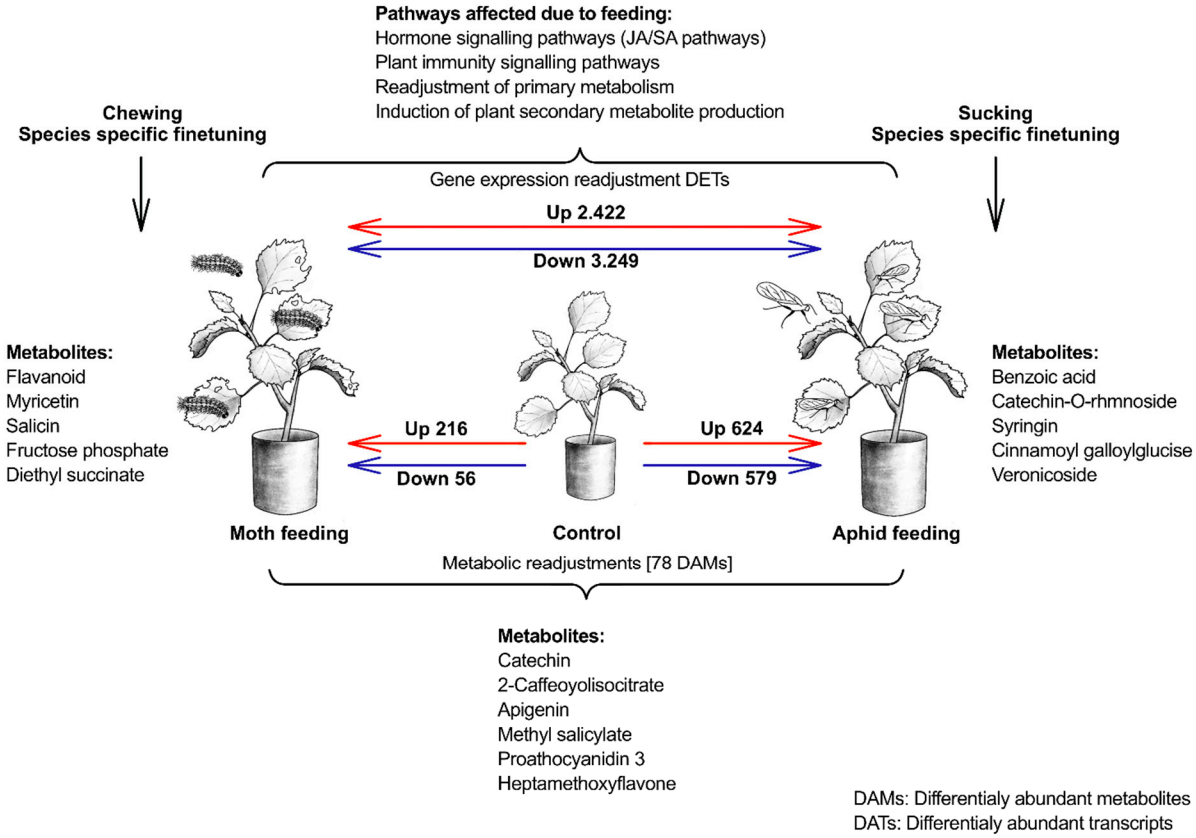

Figure 16. Summary of the poplar defence against two different insects (i.e., spongy moth and aphid) obtained from current metabolomic and transcriptomics study. Our finding indicates that distinct metabolic pathways and gene expression from key physiological pathways in poplar leaves are altered after insect attack, suggesting a species-specific, fine-tuned response.

Moreover, crucial pathways like plant hormone signal transduction (salicylic acid and jasmonic acid), PAMP-triggered immunity, and MAPK signalling pathway–plant interactions play pivotal roles in inducing plant resistance against both sucking and chewing insects in *P. tremula*. Additionally, insect infestation prompts various alterations in plant primary metabolism, particularly in carbohydrate and amino acid pathways, compared to non-infested plants. These findings enhance our current understanding of how plants respond to herbivore-induced stress and offer insights for developing strategies to combat aphids and spongy moths in *P. tremula*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms25116124/s1.

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4.3 Article III.: Time is of the essence: unveiling the rapid response of *Populus* to insect feeding

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Author Contributions

F.P. conceived the presented idea, designed the experiment, carried out the experiment, prepared and processed laboratory protocols, and wrote the manuscript; A.K. laboratory work: LC-MS analysis and metabolomic data evaluation; K.M. processed laboratory protocols; J.H. metabolomic data evaluation; J.B. carried out the experiment; I.T. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Extended summary

This publication focuses on plant metabolism in response to insect herbivory. Genetically uniform individuals of European aspen (*Populus tremula*) were subjected to repeated feeding by spongy moths (*Lymantria dispar*) at specific intervals. The study quantified physiological changes and levels of phenolic compounds and carbohydrates during the first hour. The experimental design revealed unexpected dynamics in plant metabolism, including a strong activation of induced defense after 5 minutes, evidenced by increased catechin and procyanidin B1. After 10 minutes, the plant shifted to a tolerance strategy, with compound levels returning to control levels. Differences in transpiration between affected and unaffected plants were observed only after 10 minutes. Additionally, following moth infestation, carbohydrate levels increased in leaves but decreased in roots.

Connection with the objectives of the dissertation

In the context of the **objectives** of the dissertation, this publication reflects the following:

No. 1 quantification of the stress response by LC-MS analysis of selected phenolics and sugars;
No. 2 optimizes the methodological procedure by integrating time-segmented experimental design.

Implications

The unique experimental design of this study focuses on the temporal aspect of insect herbivory effects on plant metabolism.

Results show that timing is crucial for analyzing and quantifying plant responses to herbivory. Phenolic compounds were categorized into three groups based on their content trends over different time segments.

The study challenges the findings of many previous studies and highlights potential shortcomings in their conclusions, demonstrating that the concentrations of individual phenols change dynamically over time, within minutes and tens of minutes.

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Time is of the essence: unveiling the rapid response of *Populus* to insect feeding

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Plant metabolism response to insect herbivores is the central theme of this publication. Genetically uniform individuals of European aspen (*Populus tremula*) were exposed to recurrent feeding by spongy moths (*Lepidoptera*) at specific time intervals. Changes in physiology, contents of phenolics and saccharides were quantified over the first hour. The unconventional experiment design, integrating analytical methods, and timeline led to the revealing of unexpected dynamics in plant metabolism. The time interval between herbivory initiation and sample collection revealed a pivotal moment, with induced defense activating strongly after 5 min of chewing resulting in an increase in catechin and procyanidin B1. After 10 min, a shift to a tolerant strategy occurs and induced substance concentrations return to control levels. Delayed physiological response was recorded as the first significant difference in transpiration between affected and nonaffected plants and was found after 10 min. A different strategy in exploitation of saccharides after spongy moths infestation was applied because the pool of selected saccharides was rising in the leaves but decreasing in the roots. Placing our results in the context of existing knowledge highlights the uncertain conceptual basis behind the often rigid and definitive classifications in induced plant defense or tolerance strategy.

KEYWORDS

induced defense, net photosynthesis, phenolics, resource allocation, spongy moth, transpiration

Introduction

From a paleontological perspective, the origins of interactions between taxonomic kingdoms of plants (*Plantae*) and animals (*Animalia*), specifically within the phylum *Arthropoda*, extend back to the geological period of the early Devonian, as evidenced by findings in the studies of Labandeira (2007, 2013) and Fürstenberg-Hägg et al. (2013). The early Devonian period is defined by a temporal interval ranging from approximately 397 to 407 million years ago (Gerrienne et al., 2011). While phytophagous insect species, during the course of this coevolutionary relationship, adapted to exploit their host plants, plants simultaneously developed defense systems in response to herbivore attacks (Anderson and Mitchell-Olds, 2010; Johnson, 2011).

Insect herbivory triggers a cascade of processes occurring in plant tissues that can be qualified and quantified. When insects interact with plants, there is an induction of: defense proteins (Haruta et al., 2001; Fürstenberg-Hägg et al., 2013; War et al., 2021); volatile organic

compounds (Holopainen and Gershenzon, 2010; Rosenkranz and Schnitzler, 2016); secondary metabolites (Smith, 2007; Wink, 2018; Khare et al., 2020); changes in gene expression (Vogel et al., 2014; Birnbaum and Abbot, 2020); changes in the level of photosynthesis and gas exchange (Garcia and Eubanks, 2018); and substance transport and resource allocation (Gomez et al., 2012; Schultz et al., 2013).

The outcome of hundreds of millions of years of coevolution between insects and plants suggests that both participants have equipped themselves with the ability for rapid temporal response. Decisions made by insects in selecting a host plant occur on a time scale of tens to hundreds of milliseconds (Bruce and Pickett, 2011). Moreover, scent plumes with an uneven structure, encounter the chemical molecules of the plants only for fractions of a second (Webster et al., 2010). However, the range of descriptions of plant responses to herbivory over time is limited. Insect herbivory in plant tissues causes the accumulation of reactive oxygen species and changes in Ca^{2+} concentrations in the cytoplasm, thereby triggering a chain of defense reactions (Pandey et al., 2000; Medvedev, 2005). The earliest is a change in membrane potential at the plasma membrane, immediately followed by changes in intracellular Ca^{2+} concentration and H_2O_2 formation. Within minutes, the kinases and phytohormones jasmonic acid (JA) and salicylic acid (SA) are detectable and gene activation and subsequent metabolic changes are first noticeable after about 1 h (Maffei et al., 2007).

The focal point of any discussion on carbon allocation in response to herbivory is photosynthesis, which serves as the primary source of nearly all saccharides in green plants (Zhou et al., 2015). Although the impacts of herbivory on photosynthetic efficiency are generally perceived as negative (reducing efficiency), a relatively recent meta-analysis (Garcia and Eubanks, 2018) identified 67 plant species that exhibit a certain degree of overcompensation in response to insect herbivory. Additionally, arguments advocating the implementation of management strategies to enhance market yield using insect herbivores are documented (Poveda et al., 2013, 2017). Spongy moth (*Lymantria dispar*) caterpillars have a substantial impact on the net photosynthetic rate (Pn) of poplar (*Populus* sp.) leaves and these attacks lead to reduced photosynthetic activity due to extensive defoliation, ultimately resulting in a decrease in the leaf area available for photosynthesis (Zhang et al., 2022). Hindering of stomatal conductance (Gs) and transpiration (Tr) can potentially disrupt the equilibrium between water loss and carbon dioxide absorption, and this not only impacts the water-use efficiency of poplar trees but also affects their overall physiological performance (Pilipoviš et al., 2015). Nevertheless, the response in Pn to a chewing insect is a dynamic process depending on the scale of damage. Even moderate feeding with removing of 10% of the leaf area results in 12% Pn reduction in oak (*Quercus* sp.) (Copolovici et al., 2017). Intracellular CO_2 concentration (Ci) increases slightly in oak leaves in response to moth chewing even though Gs decreased about 50% similarly to Pn (Copolovici et al., 2017).

As Delaney (2008) points out, many studies have focused on whole plant processes and fewer studies have included analysis of changes at the physiological level – i.e. gas exchange, photosynthetic changes. According to the studies of Visakorpi et al. (2020) and Fyllas et al. (2022), a combination of several analytical approaches appears to be logically consistent for further revealing the metabolic responses of plants to the attacks of insect herbivores. Gomez et al. (2012) contribute valuable insights into the complex relationships within the primary metabolism of both aboveground and belowground plant components, emphasizing critical aspects of resource allocation essential for plant resistance and tolerance. Primary metabolism assumes a key role in plant resistance and tolerance, closely linked to the efficiency of the formation of defense substances (constitutive defense/induced defense). This link arises from the fact that products of primary metabolism, namely amino acids and saccharides, act as precursors and substrates for the biosynthesis of defense metabolites (Hanik et al., 2010a). An approach describing the dynamics of physiological and biochemical traits over time during herbivore feeding is still missing because, as it is well known, plant-insect interactions are both dynamic processes.

The research's uniqueness of the present study stems from exploring the interplay between biochemical and physiological responses, primary and secondary metabolism, specifically within the initial hour following an insect attack. By concentrating on the timing of these responses, we aim to offer a comprehensive understanding of the complex interactions between European aspen (*Populus tremula*) and polyphagous spongy moth (*Lymantria dispar*).

Materials and methods

Plant material

For the minimization of genotype influence and standardization of experimental conditions, genetically uniform individuals of *Populus tremula* produced through somatic embryogenesis were employed. *Populus tremula* seeds were selected as the starting plant material for the experiment. The seeds were obtained through controlled crossing of parent trees aged 40–50 years, located in the Czech Republic, specifically in Sušice (Svatobor) and Krušné hory (Fláje). The controlled crossing took place in early spring 2019. Within 5–7 days after seed collection, the seed material was utilized for *in vitro* propagation. A total of 218 seedlings sprouted and, out of them, individual number 22 was chosen due to its superior performance in *in vitro* culture.

Seeds of *P. tremula* were washed in 200 mL distilled water with 1–2 drops of Tween 20® for 10–15 min, then sterilized in 0.1% HgCl2 for 6 min. After rinsing, seeds were placed in jars with Murashige and Skoog (MS) medium solidified with Danish® agar and supplemented with myo-inositol and 6-benzylaminopurine (BAP). The pH-adjusted medium was autoclaved, and explants were cultivated under 16/8 h light/dark with a temperature of 22 ± 1 / 20 ± 1°C. Germination occurred within 1–3 weeks. Shoots were subcultured every 2–3 weeks until sufficient material was obtained. *In vitro* rooting was done on segments with at least three buds using half-strength MS medium supplemented with indole-3-butyric acid (IBA). Roots developed after about 4 weeks, and after 6–8 weeks, rooted shoots were transferred *ex vitro*. Rooted shoots were washed and transferred to sterile substrate in plastic pots, treated with Previcur Energy®, and cultivated under controlled conditions. Humidity was gradually decreased, and plants were fertilized bi-weekly during growth. Forty elite individuals were selected from the genetically uniform *in vitro* culture based on phenotypic characteristics. These plants were transplanted into round flowerpots with a diameter and height of 20 cm each during the transfer from *in vitro* to *ex vitro* conditions at the somatic

embryogenesis laboratory. The soil was prepared by sterilizing the substrate through steam treatment, and the plants were then placed into growth chambers. A specialized commercial mixture for sowing and growing plants was used as the soil substrate. The mixture is carefully formulated from a blend of selected light and dark peat, adjusted to achieve the desired pH level. Perlite is then added as an additional component. Furthermore, this substrate is enriched with a comprehensive range of essential and trace nutrients by adding fertilizers (Forestina, Czech Republic). The start of the experiment took place when the plants reached the age of 6 months, the leaves were fully mature and the individuals were approximately 1.2 m high. Throughout the duration of the experiment, all *Populus* individuals showed good health and growth was observable.

Insect breeding

The collection of spongy moths (*Lymantria dispar*) was delivered by Institute of Forest Entomology, Forest Pathology and Forest Protection at the University of Natural Resources and Life Sciences, Vienna from sterile laboratory cultures. Upon hatching, the larvae were provided with a nutritionally balanced agar diet (*Lymantria dispar* agar, Southland products Inc., United States) in sterile Petri dishes.

Lymantria dispar eggs were placed in sterile Petri dishes in a climatic chamber with a day and night regime (12 h dark/12 h light) with a temperature oscillation during the day of 24°C and a drop to 20°C at night. The moment the eggs started to hatch into caterpillars, they were given nutritionally balanced boiled agar. Once every 2–3 days, the caterpillars were transferred to new, clean Petri dishes in a sterile environment.

In the experiment, caterpillars were used after the fourth molting. To enhance feeding activity, they were incubated in darkness and deprived of food for 48 h, considering their nocturnal behavior (Hajek, 2001).

Experimental facility

For increased reproducibility and comparability of results, standardization of experimental conditions was implemented using growth chambers: Step-In FytoScope FS-SI (Photon Systems Instruments, Drasov, Czech Republic). The growth chambers were set to simulate optimal environmental conditions, including a humidity level of 75%, an average Photosynthetic Photon Flux Density of 250 $\mu mol.m^2.s^{-1}$, a CO_2 concentration of 420 ppm, and a light–dark cycle of 2 h of dawn, 10 h of light, 2 h of twilight, and 10 h of darkness.

An Agilent 1,290 Infinity II (Agilent, USA) liquid chromatography system coupled with an Agilent 6,546 LC/MS quadrupole time-of-flight (qTOF) system (Agilent, USA) was used for non-volatile compounds analysis.

For gasometrical measurement, an open portable photosynthesis system with an infrared gas analyzer LI-6400 XT (LICOR, Lincoln, NE, USA) was used. The light of 1,500 $\mu mol.m^{-2}\ s^{-1}$ overlapping the point of light saturation (usually round 450 $\mu mol.m^{-2}\ s^{-1}$) has been measured to obtain a net photosynthetic rate (Pn), transpiration (Tr), stomatal conductance (Gs), and internal-to-ambient CO_2 concentration ratio (Ci/Ca). The measurement referred to the ambient CO_2 concentration of 420 $\mu mol.m^{-2}\ s^{-1}$. A standard leaf chamber with a red/blue LED light source was used. The samples were taken from 11 am to 2 pm. Measurements were performed inside the chamber so as not to change microclimatic conditions.

Experimental design

Out of the initial pool of 40 individuals, which underwent a 6-month incubation period under optimal conditions in the growth chamber, the top 20 elite individuals were chosen for the experiment. For clarity in understanding the methodology, the experiment design (Figure 1) and procedures will be detailed for a single individual plant among the selected 20. The experimental framework drew on previous research such as the work of Boeckler et al. (2013) and Stevens and Lindroth (2005), which provided empirical support for the chosen approach.

The biochemical response of the *Populus* tree was investigated at four time moments in the first 60 min of moth caterpillar insects feeding on one individual poplar leaf. In the first phase, the selected individual was isolated for 10 days after selection (Figure 1), in order to stabilize the stress reactions to handling. Subsequently, measurements were conducted on a specific fully mature leaf located in the midsection of the trunk, using an open gasometric system. The spongy moths were put on the leaf using aseptic entomological tweezer from the Petri dishes and after chewing in individual time segments removed back from the leaf. The investigated leaves were always under the full sunlight with defined light in the growth chambers (LI-6400 XT). Immediately after the measurement, the leaf was aseptically cut with sterile scissors and placed in a sterile 50 mL test tube, which was pre-frozen in a liquid nitrogen bath. After closing the falcon tube in which the leaf was inserted, it was immediately placed in a bath of liquid nitrogen. This was followed by a 10-day rest period, allowing the metabolism to stabilize after leaf cutting.

In the second phase, another leaf of comparable quality and age was selected, and three spongy moths individuals were introduced. The precise moment of the first bite was recorded, and after 5 min of continual feeding, the caterpillars were removed. The leaf was promptly sealed in the LI-6400 XT measurement chamber. Gas exchange was assessed on intact leaves via a chamber measuring 2×3 cm, capturing CO_2 uptake from the entire area. The leaf completely filled the chamber area to ensure accurate gas exchange measurements. Following completion of measurements, the leaf was cut with sterile scissors and immediately placed in a 50 mL test tube, subsequently stored in liquid nitrogen. Another 10-day period was granted to restore metabolic homeostasis. In order to eliminate the influence of the circadian rhythm of the plants, the collection of samples was always carried out at the same time of a day (i.e., from 11 am to 2 pm) at an interval of 3 h.

Subsequent stages replicated the entire process, with the only variation being the measurement time, set at 5, 10, 30, and 60 min from the first bite.

In the final step, root samples were obtained from the designated individual, both as a control and after 60 min of feeding. Throughout, the methodology was strictly followed to ensure active caterpillar feeding and their presence on the selected leaf. This procedural approach was consistently applied to all selected individuals and in all time segments. Throughout the experiment, everything was done in a

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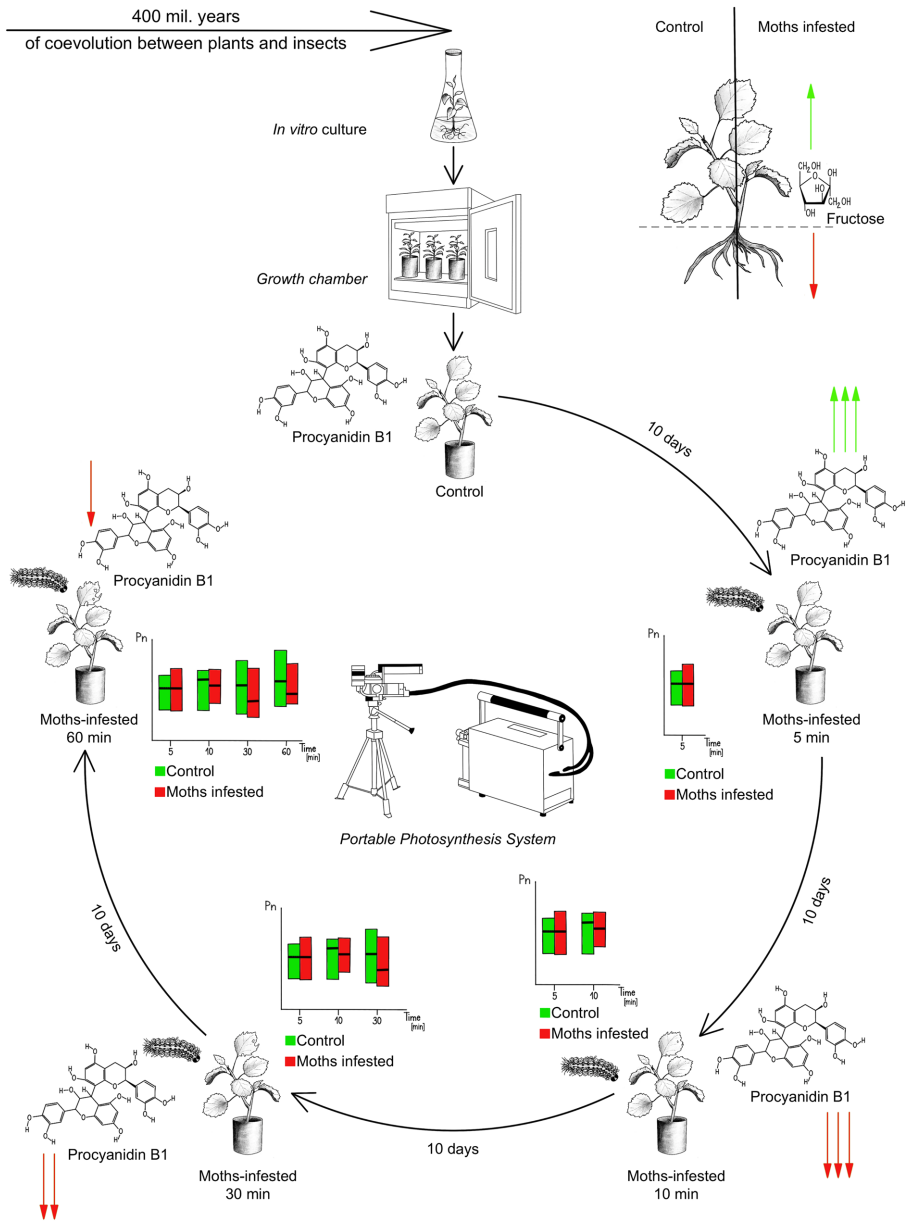

FIGURE 1
Experimental design. Genetically uniform poplar seedlings from *in vitro* culture were transplanted into pots and maintained under controlled conditions. The cycle of one plant within the experiment is shown. Early events in plant metabolism were investigated in time segments after 5, 10, 30, 60 min continuous feeding of *Lymantria dispar*. There were 10-day intervals between individual segments for plant recovery. Green arrows and their number graphically show the rate of increase in concentration, red arrows show the decrease in procyanidin B1 concentration over time.

growth chambers environment to eliminate the effect of handling the plant or the effect of different spectral composition of light on the plant. A total of 4 growth chambers were used, which were appropriately combined in order to eliminate the influence of intraspecific chemical communication between poplars. Before starting the laboratory work, the samples were stored in a −80°C cooling box.

For root saccharides analysis, roots were collected simultaneously with leaf control samples. Each plant was carefully removed from its pot, and approximately 50 mg of roots of various sizes were collected using sterile scissors. Following collection, the roots were promptly washed in demineralized water and stored in a microtube in liquid nitrogen for immediate preservation. The root samples were processed in the same way when the sample was harvested after 60 min of continuous feeding, when the leaf was harvested first and then the roots.

Chemical analysis

Extraction of phenolic compounds in leaves

A freeze-dried and homogenized sample (10 mg) was placed into a test tube, followed by the addition of 0.5 mL of 70% chilled methanol. After vortexing for 30 s, the test tube was then immersed in an

ultrasonic bath with ice for a duration of 10 min. The resultant solution was centrifugated at 13,000 rpm and 4°C for 10 min. Prior to LC–MS-qTOF analysis, the supernatant was filtered using a 0.22 µm PTFE filter. After 5 µL in concentration of 25 µg mL^{-1} of internal standard indole 3-acetic acid was added. All sample handling procedures were conducted on ice. The prepared samples were stored at a temperature of −80°C.

Extraction procedure for determination of saccharides in leaves and roots

Freeze-dried homogenized sample (30 mg) was introduced into a 2-mL test tube, and 1.5 mL of methanol:water (80:20 v/v) was added. The tube was vortexing and placed into a thermoshaker for 30 min at 50°C and 1,000 rpm. Then, the samples underwent centrifugation at 12,500 rpm for 10 min. The resulting supernatant was carefully collected and subsequently filtered through a PVDF syringe filter (0.22 µm) prior to LC-qTOF-MS/MS analysis (Šulc et al., 2021).

LC-qTOF-MS/MS saccharides analysis in leaves and roots

Saccharides analysis was performed on Agilent 1,290 Infinity II liquid chromatography system, coupled with an Agilent 6,546 LC/MS quadrupole time-of-flight (qTOF) detector (Agilent, USA). for LC-qTOF-MS/MS analysis. Chromatographic separation was executed on a Supel Co apHera NH^2 Polymer column (150×2 mm, 5 µm) maintained at 30°C. The mobile phase consisted of acetonitrile (A) and water (B). The gradient elution started at 80:20 (A:B) for the mobile phase, transitioning to 55:45 (A:B) from 0.5 to 13 min, and returning to 80:20 (A:B) from 14 to 15 min, at a flow rate of 0.2 mL min^{-1}. The injection volume was set to 1 µL. The system operated in negative ionization mode (Madsen et al., 2015).

Optimized QTOF parameters, established using glucose, sucrose, fructose, and mannitol standards, were as follows: scan range of 100–1,000 m/z, drying gas temperature of 280°C, sheath gas flow rate at 12.0 L/min, sheath gas temperature of 400°C, capillary voltage set to 2.0 kV, fragmentor at 120 V, and collision energy at 10, 20, and 40 eV. MS/MS data were acquired within a scan range of 50–800 m/z. Throughout the analysis, reference masses at 112.9855 m/z and 922.0098 m/z were continuously monitored for mass correction. Agilent Mass Hunter Acquisition software was employed for data collection, while Qualitative Analysis 10.0 and Q-TOF Quantitative analysis tools were utilized for data analysis (Madsen et al., 2015). External calibration curves using standards of target compounds (fructose, glucose, sucrose and mannitol) were used for quantification.

LC-qTOF-MS/MS analysis of polyphenolic compounds in leaves

The same instrument was used for phenolics analysis as in the case of saccharide analysis, but with Zorbax Eclipse Plus C18 column (2.1×50 mm, 1.8 µm) (Agilent, United States). Mobile phase A contained 0.05% formic acid, while mobile phase B consisted of acetonitrile. The gradient elution protocol was as follows: 0–0.1 min, 95% A; 0.1–8 min, 72% A; 8–9.1 min, 25% A; 9.1–11 min, 95% A. The mobile phase flowed at a rate of 1.1 mL min^{-1}, and the column temperature was maintained at 35°C. A 1 µL injection volume was used, and the system was operated in negative ionization mode.

Prior to analysis, qTOF parameters were optimized using standards. The qTOF parameters were set as follows: scan range of 100–1,000 m/z; drying gas temperature of 350°C; sheath gas flow rate at 12.0 L min-1; sheath gas temperature of 400°C; capillary voltage set to 5.0 kV; nozzle voltage at 0.9 kV; fragmentor set to 140 V; collision energy set at 10, 20, and 40 eV. MS/MS data were acquired within a scan range of 50–800 m/z, with a retention time window of 0.5 min, an isolation window of 1.3 amu, and an acquisition rate of 2 spectra per second. Throughout the analysis, reference masses of 112.9855 m/z and 966.0007 m/z were continuously monitored for mass correction.

Agilent Mass Hunter Acquisition software was used for data collection, while data analysis was performed using Mass Hunter Qualitative Analysis 10.0 and Q-TOF Quantitative analysis tools (Agilent, USA). External calibration curves using standards of target compounds (catechin. Epigallocatechin, ferulic acid, chlorogenic acid, procyanidin B1, gallic acid, rutin, kaempferol, quercetin, taxifolin) were used for quantification.

Statistical analysis

For the statistical analyses and data visualization, R statistical software (R Core Team, 2021) was used.

Since a single plant was measured multiple times, paired t-test and linear mixed models for repeated measures were employed.

Saccharides from roots were analyzed using paired t-test (as default by *t.test* function from R's stat package).

Linear mixed models were fitted using ASReml-R v4.1. For gasometry data, the fixed effects were specified as:

$$\sim \boldsymbol{Time} + Treatment + \boldsymbol{Time} : Treatment$$

where *Time* has 4 levels of 5 min, 10 min 30 min and 60 min of infestation and Treatment has two levels: infested and control.

Random factors were specified in a model as:

$$\sim IDplant + IDplant \boldsymbol{: Time}$$

Where ID_{plant} has 20 levels for each plant.

For each *Time:Treatment* combination, mean values, standard errors, and significance for pairwise comparison were extracted using predictPlus function form asremlPlus package. *p*-values were then adjusted using the false discovery rate method. The effect size of differences was calculated using Cohen's *d* approach (difference of means divided by pooled standard deviation; so $d = 1$ means that means are 1 standard deviation apart).

Residuals were checked for normality and constancy of variances.

For phenolic compounds the fixed effects were specified as:

$$\sim Time$$

where *Time* has five levels of 0 (pre-infestation control), 5 min, 10 min, 30 min, and 60 min of infestation.

Repeated measures were tackled specifying heterogenous correlation in residuals specifying:

$$residual = \sim id\left(IDplant\right) : corgh\left(\boldsymbol{Time}\right)$$

Where ID_{plant} has 20 levels for each plant.

Again, the significance for pairwise comparison was extracted using predictPlus function form asremlPlus package. p-values were then adjusted using FDR method. Effect size of differences was calculated using Cohen's d approach.

Results

In leaves, the concentration of fructose and glucose together with mannitol rose in moth-infested treatment in comparison to the control during the whole investigated period of 60 min. The concentration of sucrose – the most important saccharide - did not change in the investigated period and reached 253 ± 13 μg/g in moth-infested treatment and 238 ± 49 μg/g in the control group (Figure 2). Excluding sucrose, the differences between the control and moth-infested treatment were statistically significant from 10 min for glucose and mannitol. The concentrations of both monosaccharides were 5x higher after 60 min of chewing than in the control group. These shifts indicate possible saccharides transport between the roots and leaves.

Regarding the response of root sugar concentrations to hourly feeding by spongy moths, the results are shown in the graph (Figure 3). Sugars involved in the fructose and mannose metabolic pathway (namely glucose, fructose, and mannitol) showed a consistent pattern. Specifically, root fructose concentration significantly decreased from the control level of 66 ± 35 μg/g after feeding to 33 ± 23 μg/g, reflecting a reduction to 49% of the control value. Glucose showed an 85% decrease from the original concentration in the moth-infested treatment, while mannitol decreased to 87.5% of its concentration. In

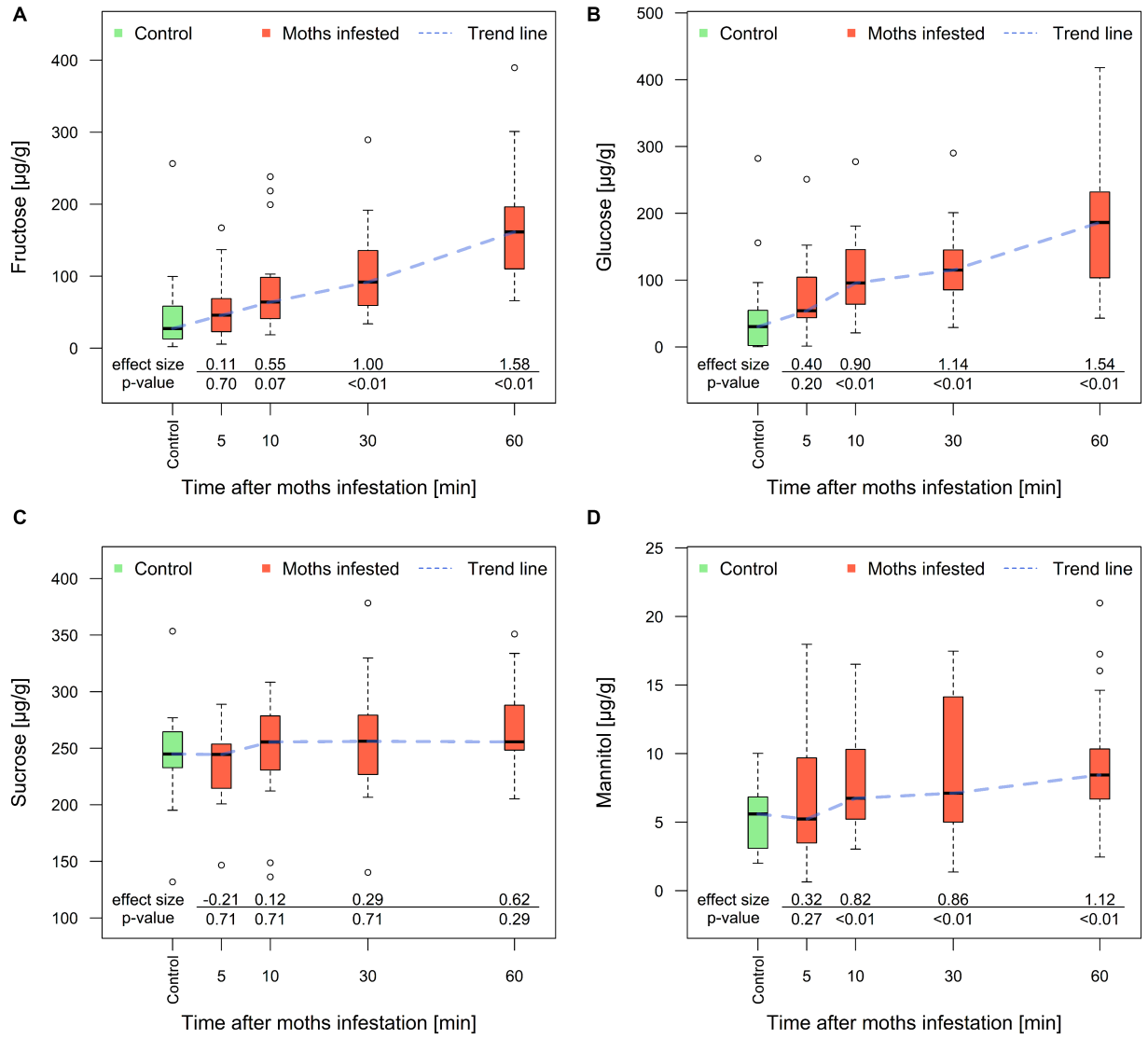

FIGURE 2
Concentration of fructose **(A)**, glucose **(B)**, sucrose **(C)**, and mannitol **(D)** in poplar leaves obtained after 5, 10, 30, and 60 min of spongy moth chewing. The Y-axis represents the relative shift from the control group on a natural logarithmic (ln) scale. Thick line in the box represents median values, the box range represents lower and upper quartile value, whiskers minimum and maximum values, and circles extreme values. The effect size is a quantitative measure of the magnitude of the experimental effect (Cohen's *d*).

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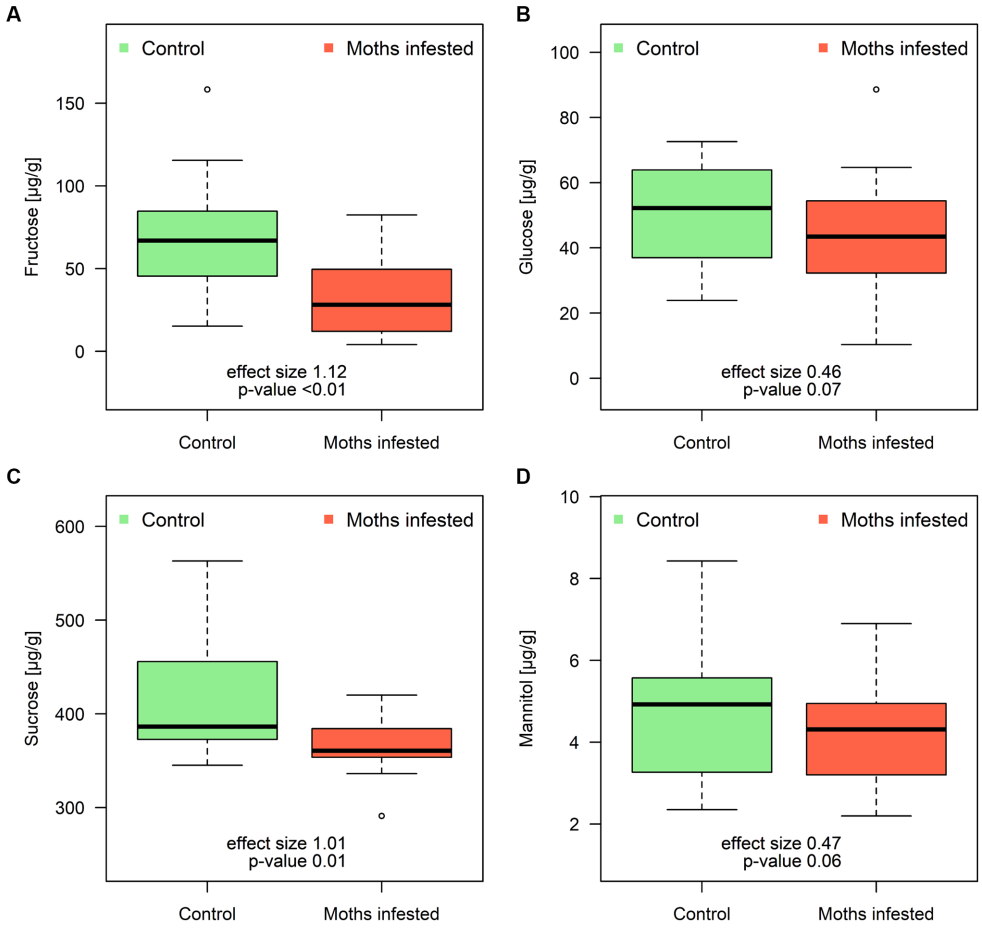

FIGURE 3
Concentration of fructose **(A)**, glucose **(B)**, sucrose **(C)**, and mannitol **(D)** in poplar roots obtained after 60 min of spongy moth chewing. Thick line in the box represents median values, the box range represents lower and upper quartile value, whiskers minimum and maximum values, and circles extreme values. The effect size is a quantitative measure of the magnitude of the experimental effect (Cohen's *d*).

the context of the galactose metabolic pathway, there was a significant decrease in sucrose, which dropped from control values of 418 ± 66 µg/g to 368 ± 29 µg/g (12% difference between treatment and control group) after 60 min of individual leaf feeding by spongy moth.

Physiological response of *Populus* to the moths

The physiological response of poplar to chewing insects was very fast and maintained even after the investigated time. The Pn in damaged plants was comparable to the control group at 5 and 10 min of feeding. Nonetheless, compared to the control, a substantial reduction ($p < 0.01$) in Pn was noted following 30 min of insect chewing (Figure 4A). The control group maintained Pn at 11 ± 4 µmol. m^{-2} s^{-1} but the group with spongy moth damage only reached 9.9 ± 3.7 µmol.m^{-2} s^{-1}. Similar trends were observed for Gs (Figure 4B) with a significant drop off at 30 min (0.50 ± 0.23 mol.m^{-2} s^{-1} in control group and 0.46 ± 0.23 mol.m^{-2} s^{-1} in moth-infested group, respectively). The significant decrease in Tr (Figure 4C) started even after 10 min of chewing (2.66 ± 0.89 mmol.m^{-2} s^{-1} and 2.58 ± 0.71 mmol. m^{-2} s^{-1}, respectively). The reduction in Pn, Gs, and Tr remained similar at 30 and 60 min, ranging between 8 and 12% when compared to the control group. Interestingly, no significant changes were observed in the intercellular CO_2 concentration (Ci) (Figure 4D), which implies that the ratio of Ci to ambient CO_2 concentration (Ca) remained constant throughout the entire investigation period (0.85 ± 0.05 µmol CO_2 mol^{-1}). The stable Ci/Ca ratio indicates that the impact of a moth feeding on the studied physiological processes was not mediated through alterations in intercellular CO_2 concentration.

Biochemical response of *Populus* to the moths

Three distinct patterns of phenolic compound production were observed in the moth-infested leaves, where the concentration of different phenolic compounds changed over time in three different ways (Figure 5) when compared to the control group:

1 The first group (Figure 5A) consisting of procyanidin B1, kaempferol, catechin, epigallocatechin, chlorogenic acid, and rutin increased its concentration several times higher after 5 min of chewing than in the control group. The standard

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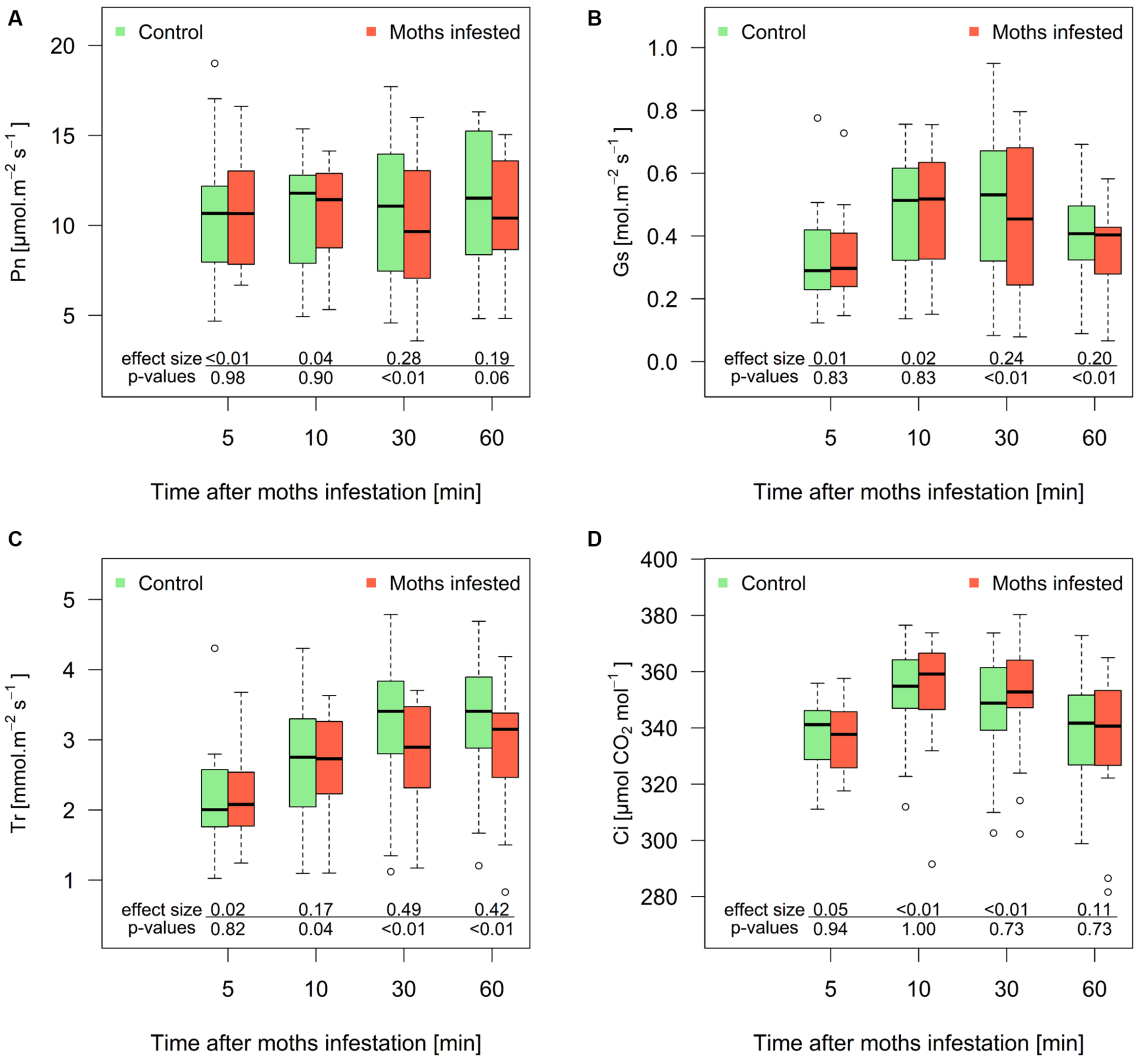

FIGURE 4
Course of net photosynthesis **(A)**, stomatal conductance **(B)**, transpiration **(C)**, and intracellular CO_2 concentration **(D)** in poplar leaves obtained after 5, 10, 30, and 60 min of spongy moth chewing. Values of the Y axis represent the relative shift from the control group. Thick line in the box represents median values, the box range represents lower and upper quartile value, whiskers minimum and maximum values, and circles extreme values. The effect size is a quantitative measure of the magnitude of the experimental effect (Cohen's *d*).

deviations (e.g., catechin and procyanidin B1) increased almost 10 times compared to their concentration in the moths-infested treatment immediately after 5 min of chewing. There was a significant increase (43%) in chlorogenic acid (639 ± 275 µg/g and 914 ± 275 µg/g) and kaempferol (0.16 ± 0.08 µg/g and 0.28 ± 0.18 µg/g, an increase of 75% when compared to control group). Subsequently, this group of substances began to decrease in concentration from 5 min to 60 min reaching control level. In addition to catechin, chlorogenic acid and procyanidin B1 showed lower concentrations than the control group.

2 The second group (Figure 5B) contained quercetin and ferulic acid; these two phenolic compounds had exactly the inverse course in investigated time than the previous group. After the start of feeding, the concentrations started to decrease, reaching a minimum value at 30 min after the start of feeding. Between 30 and 60 min, increases in the concentration of both these phenolic compounds were recorded, reaching approximately half the concentration compared to the control group (30.7 ± 7.8 µg/g). Concentration of ferulic acid was lower during the whole investigated periods when compared to control group. Significant differences were recorded after 10 min (24.3 ± 7.2 µg/g, a decrease of 20%), 30 min (21.7 ± 6.1 µg/g, a decrease of 42%), and 60 min (23.5 ± 8.1 µg/g, an increase of 8%).

3 The third group (Figure 5C) of analyzed phenolic compounds contains gallic acid and taxifolin; the concentration of these substances was stable and did not show any significant response during investigated time segments.

In Figure 6, we show the resulting chromatogram showing selected phenolic substances. By retention time on the x-axis and response rate in mass spectrometry analysis on the y-axis.

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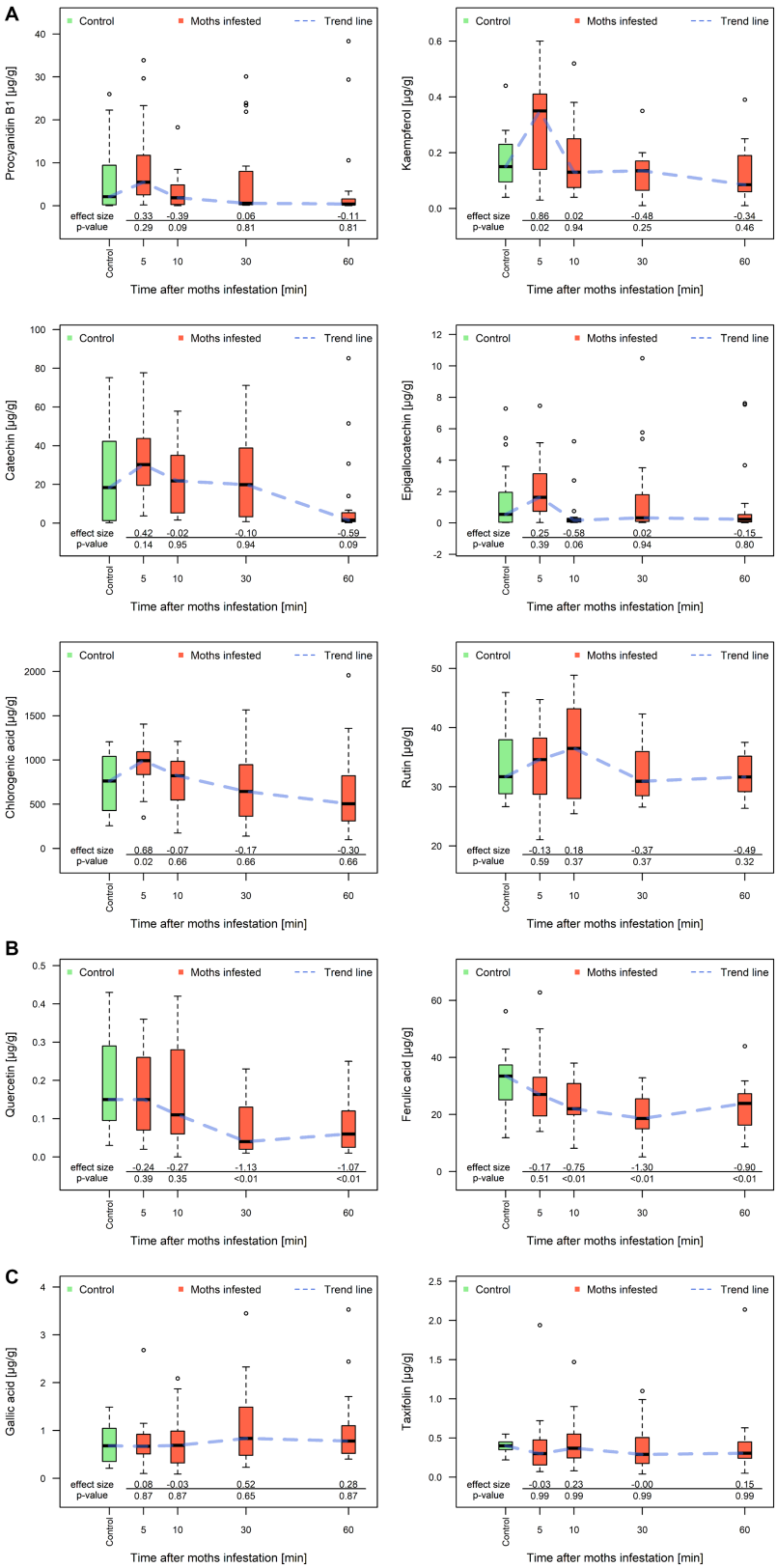

FIGURE 5
Concentration of phenolic compounds of the three groups according to a different patterns of development over time. First group **(A)** procyanidin B1, kaempferol, catechin, epigallocatechin, chlorogenic acid and rutin; second group **(B)** quercetin and ferulic acid; third group **(C)** gallic acid and taxifolin in poplar leaves obtained after 5, 10, 30, and 60 min of chewing by fungus gnats. Y-axis values represent the relative shift from the control group. The thick line in the box represents the median values, the range of the box represents the lower and upper quartile value, the minimum and maximum whisker values, and the circular extreme values. The blue dashed line represents the general trend of compound concentration over time. Effect size is a quantitative measure of the size of an experimental effect (Cohen's *d*).

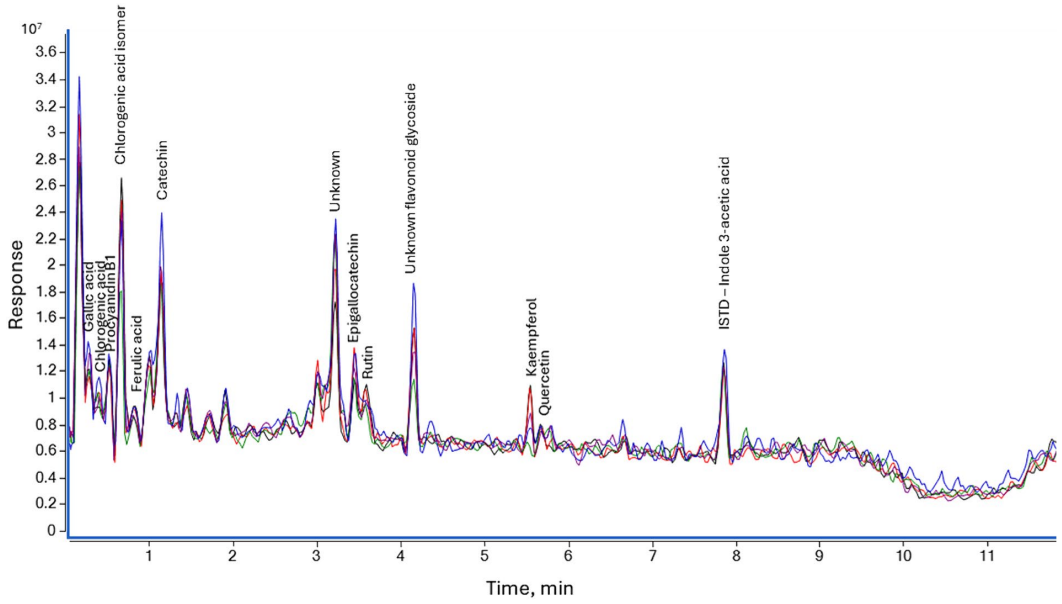

FIGURE 6
Total ion chromatograms of *Populus tremula* polyphenolic compounds: violet – control sample; green – after 5 min; black – after 10 min; red – after 30 min; blue – after 60 min.

Discussion

The current understanding of interactions between plants and insects is potentially vast, as indicated by numerous studies (Arimura, 2021; Mostafa et al., 2022; Wari et al., 2022), and supported by ample data available for meta-analyses (Zebelo and Maffei, 2014; Garcia and Eubanks, 2018; Wallis Ch and Galarneau, 2020). However, it is evident that accurately describing the dynamic relationship between a plant and an insect herbivore is highly complex and constrained. While some attention has been given to the temporal aspect of herbivory events, such as diurnal rhythms and emissions of volatile organic compounds (Kunert et al., 2002), the daily feeding patterns (Doghri et al., 2022), circadian rhythms (Jander, 2012), and early stages of interaction (Maffei et al., 2007), there is a lack of comprehensive studies considering the time perspective in the early phase of insect herbivore attacks.

In this study, we investigated the precise timing of physiological responses and changes in selected secondary metabolites to attacks by sponge moths, resulting in immediate changes in sugars and polyphenols and gasometric parameters. Our findings confirmed previous research by Allison and Schultz (2005) and Schultz et al. (2013) regarding primary metabolite redistribution from roots to damaged leaves in Populus. However, there have been studies documenting the opposite response in other plant species, suggesting that results depend on a variety of factors, including plant condition, resource availability, genetic predisposition, infestation intensity. and the specificity of the plant's defense response (Gómez et al., 2010; Orians et al., 2011). It seems that the well-known law of compensation and growth equilibrium by Saint-Hilaire (1818) generally applies. It says: *"The budget of nature is fixed; but she is free to dispose of particular sums by an appropriation that may please her. In order to spend on one side, she is forced to economize on the other side"* which Schultz et al. (2013) aptly point out in their study.

Taking a closer look at the gasometrical parameter Pn beforehand, the physical damage caused by caterpillar chewing can significantly alter the structure and function of the leaf. The loss of leaf tissue and damage to cell integrity may result in malfunctioning chloroplasts related to photosynthesis, thus reducing the capacity for photosynthetic activity. According to Zhou et al. (2015), a central aspect of any discourse concerning carbon allocation in response to herbivory is photosynthesis, which serves as the primary source of nearly all carbohydrates in green plants. However, the direct effect of leaf biomass disappearance typically does not show a linear correlation with Pn (Bueno et al., 2009). The defensive reactions often incur a cost in terms of reduced photosynthesis, as resources allocated to photosynthetic activities become constrained, leading to lower photosynthetic rates. This shift in energy and resource allocation favors defense mechanisms, as discussed by Arnold et al. (2004) and Gomez et al. (2012), while also restricting carbon fixation.

Physical damage caused by caterpillar feeding results in disruption of the leaf surface. The disruption of the plant tissue thus creates openings, which is usually the explanation for the decrease in transpiration. These physical wounds, when already sealed, serve as barriers, decreasing water vapor's ability to escape from leaf surfaces and lowering transpiration rates (Herms and Mattson, 1992).

At the level of secondary metabolism, trees of the *Salicaceae* family are known to be among the most prolific producers of phenolic compounds (Boeckler et al., 2013). Poplar leaves contain phenolic chemicals with an anti-herbivory function, including procyanidin B1, catechin, chlorogenic acid, quercetin, kaempferol, rutin, taxifolin, and epigallocatechin (Pietarinen et al., 2006; Sobuj et al., 2020). In addition to physiological adjustments, insect bites can induce the production and accumulation of phenolic chemicals in poplar leaves as a defense mechanism. Depending on the extent and duration of insect damage, the concentration of phenolic chemicals can change. The differences in the dynamics of concentration development in our study are in

direct contrast to the studies of Lahtinen et al. (2006). They reported that levels of phenolic compounds tend to rise in response to insect feeding, with higher amounts occurring when damage lasts longer. The activation of defensive signaling pathways causes changes in the amounts of polyphenols in poplar leaves. These signaling molecules can govern the expression of genes related to defense, thereby promoting the creation of defense proteins, protease inhibitors, and various other defensive substances (Walling, 2000). The comparison of our results with the time diagram presented in the study (Maffei et al., 2007) was surprising when we cannot confirm the given values with respect to our results. In particular, metabolic changes (induction of defense substances, allocation of resources) took place in our experiment demonstrably already in the first 5 min of feeding. As part of the results from our other yet unpublished study, the dynamics of changes in gene expression show similar findings. The response of phenolic compounds in *Populus* leaves to spongy moth caterpillars exhibits a notable level of diversity, and the phenolic data exhibited considerable variability even between clones, which is consistent with works (Donaldson et al., 2006; Smith et al., 2011). According to studies, several *Populus* spp. demonstrate diverse patterns of variation in phenolic content in response to caterpillar feeding, with some showing no significant change or even a decrease (Lämke and Unsicker, 2018; Zhang et al., 2020). This significant variation underscores the intricate nature of plant defense strategies and implies that various *Populus* species have developed unique biochemical mechanisms to contend with herbivore assaults. What was particularly noticeable was that this same extensive variability was observed within identical *Populus* clones originating from *in vitro* plant material in the present study.

The majority of prior studies examining the ecological functions of tannins have not specifically investigated individual chemical compounds. Instead, they have relied on general precipitation or colorimetric tests to measure the levels of "total phenolic compounds" or "total hydrolyzable/condensed tannins" (Appel et al., 2001; Boeckler et al., 2013). Given the immense chemical diversity of plant polyphenols, a limiting factor may have been researchers' attempts to pinpoint a singular (primary) effect and define it as the "raison d'être" of the entire class of polyphenols. In fact, it appears more probable that different compounds play distinct roles in plant interactions with their environment (Moctezuma et al., 2014).

Flavan-3-ols, including monomeric catechin and polymeric proanthocyanidins (referred to as condensed tannins), are prevalent phenolics in *Populus* spp. (Ullah et al., 2019; Bandau et al., 2021). Functionally, this group of substances has been shown to induce oxidative stress in the gut of insects (Barbehenn and Constabel, 2011). According to the original work by Feeny (1968), it was generally assumed that higher levels of condensed tannins would reduce the preference of insect herbivores (Forkner et al., 2004). However, studies by Peters and Constabel (2002), Tsai et al. (2006), and Boeckler et al. (2014) suggest that adapted insects, or certain genotypes, may be attracted to higher levels of condensed tannins. The findings of Hjältén and Axelsson (2015) suggest that through coevolution, condensed tannins may even act as stimulants for insect herbivores. Based on the above, the dynamics of tannins appear unclear and inconsistent. When considering the results of our study, which detail the concentration changes of this group of substances after *Lymantria dispar* attack, the situation becomes even more ambiguous.

Quercetin, a key flavonol, is widely distributed in the plant kingdom (Zhang et al., 2020). It plays a complex role in mediating interactions between herbs and insects. It can stimulate insect feeding and promote growth at low concentrations (Rahden-Staron et al., 2001); however, some studies have shown that quercetin may inhibit the activities of antioxidant and detoxification enzymes, resulting in increased mortality of insect herbivores (Gómez et al., 2020). In light of our recorded dynamics, we find agreement with the study by Jing et al. (2024), where a decrease in quercetin and a significant increase in quercetin-3-*O*-glucoside were observed over the course of hours. As noted by Jing et al. (2024), quercetin-3-*O*-glucoside has a crucial negative impact on larval development. and free quercetin had no significant effect on larval growth. Thus, it seems that glucosylation of quercetin should be the subject of further research.

While the antioxidant properties of gallic acid have been observed in other plant species, such as soybean (*Glycine max*), where it has been shown to reduce the level of total ascorbate and glutathione (Ozfidan-Konakci et al., 2019), a contrasting phenomenon is observed in tomato (*Solanum lycopersicum*), where ascorbate content increases (Farghaly et al., 2021). In our study, gallic acid together with taxifolin, belongs to the group of substances with almost no response in the response of *Populus tremula* to *Lepidoptera* attack. Regarding the evolution of gallic acid content, we agree with Zhang et al. (2020), while acknowledging the limited understanding of the impact of this polyphenolic compound on plant resistance to herbivores.

As a crucial factor, hypothetically, along with: plant health vitality, resource availability, genotypes, chemotypes and coevolutionarily given interactions, time appears to be essential for selecting appropriate strategies (and their various combinations) in response to insect herbivory.

Based on our current understanding, there appears to be numerous studies describing the impact of phenolic compounds added to the diet for *Lepidoptera* (Diaz Napal and Palacios, 2015; Su et al., 2017; Wang et al., 2019; Gao et al., 2022); however, no studies focusing on the response of *Populus* spp. mentioning the early metabolic response to *Lepidopteran* attack by phenolic compounds have been found. The general textbook terminology regarding the uncertain classification of plant metabolites into primary and secondary, such as that proposed by Erb and Kliebenstein (2020), appears to shape the thinking and application of approaches. Similarly, this is true according to the results of our study when using the terminology of a strict division into induced defense reaction and tolerant strategy in response to insect herbivory, which may be misleading. It is very common to overlook the dynamic development of the plant's metabolic response. This limitation restricts the characterization of the dynamic trajectory of the metabolic response over time to the identification of a particular response type at a specific point (time) along the developmental curve of the reaction. Based on this, it can be inferred that the approach of many studies (across all taxa) does not define a metabolic strategy but rather a state at specific time - when the samples were collected.

The ability of broadleaves to recover from defoliation and adjust their chemical defense composition according to external conditions is promising in future predictive models shaping forests.

Limitations

Due to the well-documented problems associated with the inherent biological variability of living organisms, various factors were carefully considered in the creation of the experimental design. These factors included the genetic predisposition of the species studied, the potential interplay of temporal effects within individual treatment periods and the cumulative effect of herbivory, the application of multidisciplinary methodologies, and the complexity of elucidating the dynamics of interspecies interactions. Despite careful attention to detail, there is still a chance of inaccuracies or phenomena that are difficult to grasp.

To mitigate these challenges, rigorous measures were implemented: including the use of genetically uniform individuals (*Populus tremula*), setting optimal environmental conditions in growth chambers (especially the elimination of the influence of varying intensity of irradiance), using multiple separate growth chambers to prevent chemical communication between poplars, systematics of manual works, sample collection and laboratory protocols.

Acknowledging the inherent constraints in our study, we believe that our research provides fresh insights into the intricacies of plant-insect interactions. We hope that our findings can spur further investigation in this fascinating field.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

FP: Writing – original draft, Writing – review & editing. JČ: Writing – original draft, Writing – review & editing. AK: Writing – original draft, Writing – review & editing. KM: Writing – original draft, Writing – review & editing. JH: Writing – original draft, Writing – review & editing. JB: Writing – original draft, Writing – review & editing. IT: Writing – original draft, Writing – review & editing.

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Conflict of interest

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.

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5 Discussion

This dissertation, incorporating elements of observational study (analytical and descriptive sections), focused on the comprehensive capture of the response of genetically uniform *Populus tremula* to insect herbivory through various in vitro experiments. All available instrumentation and analytical methods were employed. The plant's response was assessed through qualification and quantification of changes across different levels: **physiological** (e.g., gas exchange parameters such as Pn, Tr, Gs, Ci), carotenoid, proline, and chlorophyll a/b content; **metabolic** (secondary metabolite products measured via non-targeted metabolomic profiling and selected primary metabolites); and **genomic** (describing changes in gene expression).

These changes were then interpreted in the context of metabolic pathways, providing a comprehensive view of the plant's response to stress induced by two prevalent feeding strategies of insect herbivores—namely, leaf-chewing and sap-sucking strategies.

To avoid repeating the discussion sections from published articles (more details in chapters **4.1**; **4.2** and **4.3**) that address results from applied analyses and were accepted and published in scientific journals just a few months prior to this work, this discussion section focuses primarily on scientific approaches within the context of the field, research methodology, future directions, and the development of this scientific domain.

The current understanding of plant-insect interactions, which has developed over hundreds of millions of years of co-evolution (Labandeira, 2013), is potentially very broad, as evidenced by numerous recent studies (e.g., Arimura, 2021; Mostafa et al., 2022; Wari et al., 2022). This is further supported by extensive data from meta-analyses (Zebelo and Maffei, 2014; Garcia and Eubanks, 2018; Wallis and Galarneau, 2020).

Plant defense strategies against herbivores evolve in response to the complexity of attacks from multiple types of herbivores (feeding guilds), but in recent decades, they have largely been studied in isolation. Very few studies have progressed beyond the complexity of experiments involving two stressors (Mathur et al., 2013; Kroes et al., 2016; de Bobadilla et al., 2022). It is well-known that the genotype of aspen is a key determinant of its phytochemistry, as demonstrated in numerous previous studies (e.g., Philippe and Bohlmann, 2007; Lindroth and St. Clair, 2013). However, the factors of morphotype and chemotype, which are also crucial, are often overlooked, with only a fraction of studies addressing them (e.g., Buell et al., 2023; Okińczyć et al., 2024). The methodological approach should always consider the technical and informational

characteristics of the chosen analytical methods and their combinations. As emphasized by Fukusaki and Kobayashi (2005) in the context of metabolomics, metabolomics is primarily based on the phenotype, rather than inherent genomics, unlike approaches such as proteomics and transcriptomics, which are mediated by genomic information.

In this context, a fundamental and challenging issue is the significant interdisciplinarity of studying interactions, which, according to established terminology in multidisciplinary system research as described by Dalton et al. (2021), can be classified as a "*metaproblem*." The study of plant-insect interactions transcends the boundaries of individual disciplines and becomes a collaborative effort across fields such as plant physiology, experimental methods, entomology, microbiology, molecular genetics, biochemistry, analytical chemistry, data processing and integration, bioinformatics, and statistics. This list includes not only specialized knowledge but also the ability to work with often very complex and costly equipment.

The central issue described above often appears as a key explanatory factor in the methodological approaches of many studies, which are burdened by fundamental errors. For instance, regarding the dynamics of the development of phenolic compounds (as more details in chapter **4.3**), where the concentration of phenols strongly correlates with the precise timing of sample collection—down to the minute after the initiation of herbivorous attack. This finding challenges commonly held conceptual conclusions about defined constitutive or induced defenses (e.g., Kaplan et al., 2008 and Rehman et al., 2012). This observed phenomenon raises questions about the results of previous studies that focus on determining the concentrations or integration curves of secondary metabolism products. The findings related to resource allocation (primary metabolites) in Article III (chapter **4.3**) are supported by studies by Kaplan et al. (2008) and Gomez et al. (2012), which suggest that the "choice" of resistance strategy or defense activation depends on the plant's energy resources/vitality and is combined as needed and possible (de Bobadilla et al., 2022).

Other examples include studies that describe biochemical changes without considering abiotic environmental factors, circadian and diurnal rhythms of plants, or methodologies for determining total groups of compounds, such as the well-known "total polyphenols" or "monoterpenes (VOCs)" (e.g., in studies by Fabish et al., 2019; Cotrozzi et al., 2021; Scogings et al., 2021; Salazar-Mendoza et al., 2024). In the context of the results from Article I (more details in chapter **4.1**), it is evident that the concentration of certain metabolites increases in response to specific feeding styles of insects, while others

decrease; furthermore, some metabolites are present in orders of magnitude of tenths and others in thousands of the same units. This logically undermines this approach. While this method may indicate a general trend in "total" content changes, it can be objectively biased and entirely disregards the specific biological impact of individual metabolites on insect herbivores, as described in studies such as Fürstenberg-Hägg et al. (2013); Mostafa et al. (2022); Singh et al. (2021); Ito et al. (2023).

Additionally, strongly related to the above is phenotypic plasticity (even within genetically uniform plants), as noted by Fukusaki and Kobayashi (2005) and Trethewey (2004). Along with the independent, multi-level variation of individual plants, this phenomenon is an unavoidable aspect of these types of experiments, which can be exacerbated by inappropriate methodological approaches and may obscure biologically relevant deviations. The aforementioned authors, along with studies by Moreira et al. (2018) and Sardans et al. (2021), highlight the need to develop a generally accepted experimental design and a cohesive methodology with the potential for replication. Such an approach would help streamline experimental procedures and better control input variables, ensuring an objective analysis of plant responses to insect herbivory. These challenges have been addressed and reflected in the methodological approaches of this dissertation.

In line with the assertion by de Bobadilla et al. (2022), controlled experiments should focus on how plants cope with variations in herbivore density, timing, and order of arrival; the organs targeted; and differences in herbivore traits (e.g., leaf chewers, phloem chewers, cell content feeders; specialists, generalists). Experiments should be designed by comparing attack patterns that differ only in one of these aspects at a time, to evaluate the importance of each individual parameter.

5.1 Future perspectives

After a thorough review of the scientific literature, it appears that despite decades of intensive research, a general consensus on a holistic understanding of plant-insect interactions remains elusive. This is evidenced by the retrospective reflections of prominent authors and the redefinition of previously stated conclusions or hypotheses (Agrawal et al., 2011).

A central issue, as discussed, is the methodological approach. For instance, Wari et al. (2022) highlight the need to develop new methods to ensure plant protection and gain a deeper understanding of plant chemical defenses. Another suggested direction for future research, as proposed by Sing et al. (2023), is to move beyond model organisms and investigate wild and native plant species and their interacting insects. This would help to understand, quantify, and extract plant secondary metabolites and examine their roles in mediating these interactions both in vivo and in vitro.

Many confusing situations, such as unsystematic cross-talk between metabolic and signaling pathways, inconsistent and divergent results for the same species, or inconsistent terminology, stem from the need to integrate individual studies into a coherent system. This suggests that future scientific work should increasingly favor an inductive approach over a deductive one.

It can be inferred that combining an inductive approach (i.e., localized knowledge about interaction dynamics) with practical applications (such as forestry, plant protection, pest management, agriculture, bioengineering, or the extraction of valuable compounds for pharmaceutical/cosmetic use) would benefit from the well-established principles and procedures of Integrated Pest Management (IPM) or bioengineering strategies.

Applying this approach in the future would require greater access to expensive equipment, a deeper understanding of interaction links, and, most importantly, technologies/products for targeted manipulation of internal plant chemistry and defense mechanisms.

5.2 Limitations

Given the well-documented issues related to the biological variability of living organisms, several factors were carefully considered in the experimental design. These factors included the genetic predisposition of the studied species, potential temporal effects within individual treatments, the cumulative effect of herbivory, the application of multidisciplinary methodologies, and the complexity of elucidating interspecies interactions. Despite thorough preparation, inaccuracies or difficult-to-grasp phenomena may still arise.

To minimize these issues, measures were taken such as using genetically uniform individuals (*Populus tremula*), ensuring consistent conditions in growth chambers (including stable light intensity), employing separate chambers to prevent chemical communication between poplars, and adhering to standardized procedures for sample collection and laboratory analysis.

6 Conclusion

As we reach this concluding section, I realize that, despite our best efforts, we have only scratched the surface of the complex defense systems that plants have evolved. Ecological interactions between plants and insects are so intricate and dynamic that what occurs in one system at a given time may not be replicated in another environment or under different conditions. Each interaction between a plant and an insect has its unique characteristics.

Although we have attempted to thoroughly capture the finely tuned defensive responses of aspen trees to attacks by leaf-chewing and sap-sucking insect herbivores, fully understanding the complexity of these mechanisms remains a challenge that exceeds our current capabilities. Even after more than three decades of intensive research using the latest technologies, it appears that the creativity and diversity of defense strategies, shaped by hundreds of millions of years of co-evolution, have yet to be fully explored. New applied approaches and discoveries not only offer fresh answers but often raise new questions, creating opportunities for multidisciplinary research to delve deeper into and untangle the complex relationships within this fascinating field of science.

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