

Ecometabolomics of plant–herbivore and plant–fungi interactions: a synthesis study

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Abstract. The functional adaptive responses of higher plants to biotic interactions with herbivores and fungi have long been topics of research. One constraint to obtaining a comprehensive understanding of the most general plant responses, however, has been the difficulty of studying all plant functional shifts simultaneously due to analytical limitations. Now this is possible with the advent of metabolomics. Using 151 records from the WEB of SCIENCE database, we have analyzed the development and application of metabolomic profiles to ecological studies in the last two decades. We have used meta-analysis and pathway enrichment analyses to assess the whole set of constitutive and inducible defenses. Constitutive defenses against herbivores were mainly based on a significant high level of the metabolism of several amino acids with parallel increases in the concentrations of flavones (phenolics) and saponins (glycosides). Inducible defenses, though, were mainly based on the increases in concentration of methyl-ketone, pantothenate, and Coenzyme A. Butyrate metabolism and the mitochondrial electron-transport chain were upregulated, in agreement with previous reports that herbivory-activated plant chemical defenses were mainly based on jasmonic acid, salicylic acid, and ethylene-associated pathways. The metabolic responses/acclimations to pathogenic fungi were mainly linked with increases in aspartate and pyruvate metabolism, the transfer of acetyl groups within mitochondria, and the upregulation of branched-chain amino acids degradation pathways. These responses/acclimations were accompanied by higher concentrations of the most important groups of secondary metabolites such as phenolics (anthocyanins, flavonoids), quinones, alkaloids, terpenoids, and polyamines and other compounds related to antistress mechanisms such as proline. The leaves of mycorrhized plants accumulated nucleotide sugars, sphingolipids, and methylhistidine. These responses were associated with maintaining the integrity of plant cell membranes under fungal hypha penetration. The responses were accompanied by increases in the concentrations of phenolics, blumenols, and alkaloids and decreases in the concentrations of polyamines, consistent with the mycorrhizal inhibition of polyamines. This summary provides a clear synthesis of the most successful plant strategies selected after millions of years of evolution and will be a very promising tool for the management of crops and ecosystems and for selecting the main lines in breeding studies for future research.

Key words: biotic relationships; ecometabolomics.

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INTRODUCTION

As sessile organisms, plants are forced to adapt rapidly in response to changing environmental conditions, including interactions with newly appearing biota by adapting physiologically and metabolically (Wall 2009, Muday and Brown-Harding 2018). Plant–herbivore and plant–fungal relationships, which emerged soon after plants colonized land, are at the center of such plant responses and have become pivotal in determining the structure and functioning of terrestrial ecosystems (Tucker et al. 2010, Dong et al. 2017, Ponzio et al. 2017, Hill et al. 2018, Salazar et al. 2018, Sedio et al. 2018, Sedio 2019). As such, a very complex set of relationships now exist between plants and herbivores (Bruce 2015, Zhu et al. 2015, Ponzio et al. 2017) and plants and fungi (Redman et al. 2001, Schmidt et al. 2011, Venkateshwaran et al. 2013, Toju et al. 2014). Plant defense mechanisms against herbivores can act either reactively as shifts in DNA expression under herbivore attack (inducible defense) (Walling 2000, Hermsmeier et al. 2001, Anderson and Mitchell-Olds 2011) or constitutively through basal genetic expression (constitutive defense) (Anderson and Mitchell-Olds 2011, Kuzina et al. 2011). Moreover, some chemical compounds synthesized under herbivore attack or under fungi interactions can be emitted by plants and trigger cascading responses that affect multiple trophic levels (Herre et al. 2007, Li et al. 2013, Zhu et al. 2015, Chen et al. 2018a, Frew et al. 2018, Hill et al. 2018). These biotic interactions are clearly important for the evolution and ecology of plants and ecosystems, but they are complex to understand because they occur at the metabolic level, which precludes the use of traditional ecological metrics.

Non-targeted metabolomics analyses are being increasingly used to study the impact of plant–fungi and plant–herbivore interactions by profiling the suite of metabolites responsible for physiological processes (i.e., the metabolome). Metabolomics analyses have provided novel insights into the metabolomic shifts underlying the complex relationships that plants have with herbivores and fungi (Sardans et al. 2011, 2020, Peters et al. 2018, Salazar et al. 2018), being able to detect the signaling metabolites and their

induced metabolomics shifts (Leiss et al. 2011, Piasecka et al. 2019, Williams et al. 2021). However, the complexity of these relationships has made it difficult to draw general conclusions about them from only individual studies. In this study, we synthesize the results of metabolic studies from the last two decades to identify the shifts in foliar metabolic profiles and changes in primary and secondary metabolic pathways in response to herbivore attack and plant–fungal relationships. In doing so, we offer a novel view of the metabolic strategies most frequently used in higher plants as a consequence of long-term evolutionary processes to maintain fitness in the presence of these biotic interactions.

MATERIALS AND METHODS

We used a combination of several terms such as metabolomics, metabolome, metabolism, NMR, MS, LC, GC, gas chromatography, liquid chromatography, plant, fungi, fungal, herbivore, insect, nematode, mammal, bird, lizard, tree, shrub, grass, mass spectrum, inducible defenses, mycorrhiza, constitutive defenses, defense, and stress. We checked all records ($N = 166$) and selected only those that provided explicit information regarding metabolite responses to interactions with herbivores or fungi analyzed by the three main metabolomic platforms: (proton nuclear magnetic resonance spectroscopy [^1H NMR]), liquid chromatography–mass spectrometry [LC-MS] or gas chromatography–mass spectrometry [GC-MS]) ($N = 122$) (Fig. 1). We separated remaining records and their references into four categories based on reported shifts in foliar metabolite concentrations: (1) constitutive defenses against herbivores; (2) inducible defenses against herbivores; (3) responses to pathogenic fungi; and (4) responses to mycorrhizal fungi.

We combined the filtered data with the MetaboAnalyst platform (Chong et al. 2018) using the KEGG database (Kanehisa and Goto 2000, Kanehisa et al. 2021) to identify the primary metabolic pathways that were up- or down-regulated in the four categories (constitutive and inducible defenses against herbivores, pathogen fungi, and mycorrhizas). In each one of these four distinct biotic relationships, we introduced the number of studies that provided information of significant increase or decrease of

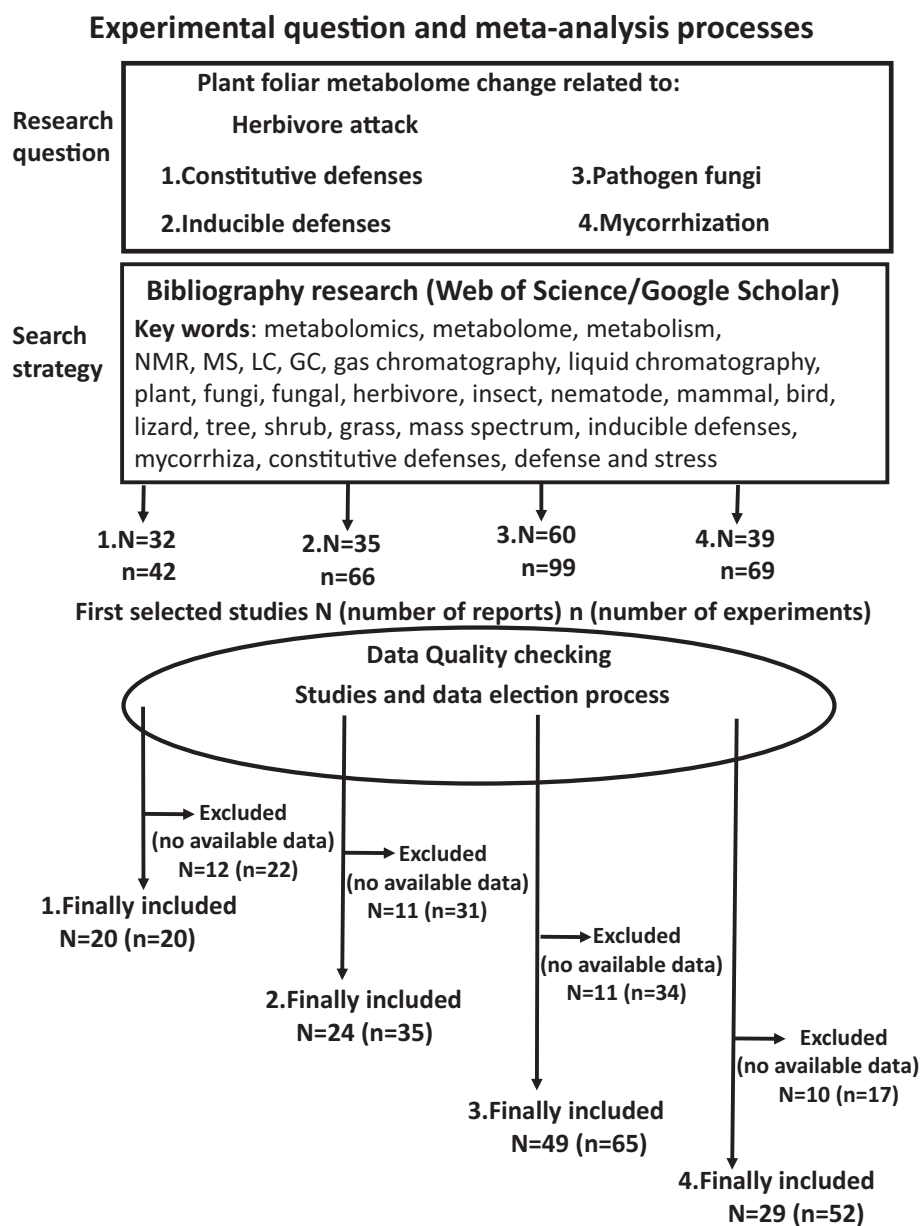


Fig. 1. Meta-analysis process followed in this study.

concrete metabolites when plant was or not submitted to herbivore attack or fungi interaction with respect no interaction in the case of inducible defenses and plant–mycorrhizal and pathogen fungi interactions. In the case of constitutive defenses, we used those metabolites that are in higher or lower concentrations in the genotypes that better adapt to or avoid herbivore attack.

We additionally performed a meta-analysis of the sets of chemical groups of secondary

metabolites that were in higher or lower concentrations and categorized using the chemical groups described by Hedges et al. (1999). We assessed metabolite responses with the ln response-ratio (lnRR):

$$\ln(X_i/X_n) = \ln X_i - \ln X_n$$

where X_i is the metabolite concentration with herbivores or fungi, and X_n is the metabolite concentration in the corresponding control. We then

calculated sample variance for each lnRR using the R package metafor 1.9-2 (Viechtbauer 2010):

$$\ln \left[(1/n_i) \times (S_i/X_i)^2 + (1/n_n) \times (S_n/X_n)^2 \right]$$

where n_i , n_n , S_i , S_n , X_i and X_n are as described by Hedges et al. (1999). Briefly, n_x , S_x and X_x are the sample size, standard deviation, and mean, respectively, of the plants interacting with herbivores or fungi (subscript i) vs. the corresponding control (subscript n). We controlled for variance in ln response ratios between studies by specifying study identity as a random factor in rma models in the R package metafor (Viechtbauer 2010). In all cases, we considered foliar metabolite concentrations to differ significantly between treatments and controls if the 95% confidence interval of lnRR did not overlap zero. All statistical analyses were performed using R 3.1.2 (R Core Team 2015).

RESULTS AND DISCUSSION

Plant metabolome under herbivore attack: constitutive vs. inducible metabolic profiles

A clear separation must be made between constitutive defenses and inducible defenses at the level of overall metabolism. The differentiation between inducible defenses (those triggered when the pathogen/herbivore/parasite effectively interacts with a plant) and constitutive defenses (those that exist previous to pathogen/herbivore/parasite interactions with plants and have deterring and protective functions) are conceptually clear but are never easy to establish experimentally. Studies may thus provide results that do not clearly state whether the observed responses are constitutive or inducible (Lande et al. 2019). Moreover, the situation is complicated by the fact that the induction of defense mechanisms may occur not only in the presence of herbivore or fungi, but also can be induced by other biotic (e.g., another herbivore) or abiotic factors (especially UV radiation). In several cases, the experimental design does not allow to disentangle if the metabolites involved in the response to herbivore attack exist previously to the attack or as a response to it.

We have standardized the selection of the studies within this complex identification of constitutive vs. inducible defenses by using the

concept that constitutive defense is expressed by plants irrespective of external stimuli, whereas induced defenses are activated after, and in response to, external stimuli (War et al. 2011). The first step in the study of constitutive defenses is to identify the species or genotypes within a set that are more or less attacked by an insect or group of insects (Macel et al. 2019). This step can use bioassays (Macel et al. 2019) or simply long-term field observation (Clancy et al. 2017). This information for unattacked leaves of different plant species or genotypes can then be submitted to metabolomic analyses to identify metabolites or metabolic pathways that are in distinct concentrations and thus differentially regulated in different species or genotypes to infer which are involved with successful defenses. Especially, relevant are studies that first submit healthy leaves of different plant species or genotypes to herbivorous attack and that quickly estimate the level of damage and then submit other unattacked leaves to metabolomic analyses, assuming that the differences in herbivore preferences are linked to constitutive defenses, given the short term of the bioassay of herbivorous attack (Mirnezhad et al. 2009). Only Rasmann et al. (2015) have included metabolomic analyses previous to attack and at different times during an attack to clearly identify constitutive or inducible chemical metabolite defenses. This limitation severely impedes the identification of constitutive defenses prior to herbivorous attack relative to those that are induced by insect attack. The number of studies that clearly differentiate constitutive and inducible defenses, however, is fortunately sufficient, despite this structural caveat, allowing us to reach several general conclusions. Several ecometabolomic studies specifically found differences in metabolomic profiles linked to constitutive defenses in leaves prior to a herbivorous attack (generally by an insect) and report clear differences in metabolomic profiles between attacked and unattacked species or genotypes in the presence of herbivores (Leiss et al. 2009a, b, Mirnezhad et al. 2009, Khakimov et al. 2012, Clancy et al. 2017, Macel et al. 2019, Perez-Fons et al. 2019).

Constitutive differences in primary metabolic pathways and secondary metabolites in response to herbivore are shown in Fig. 2 and Appendix S1: Table S1. Specifically, we found

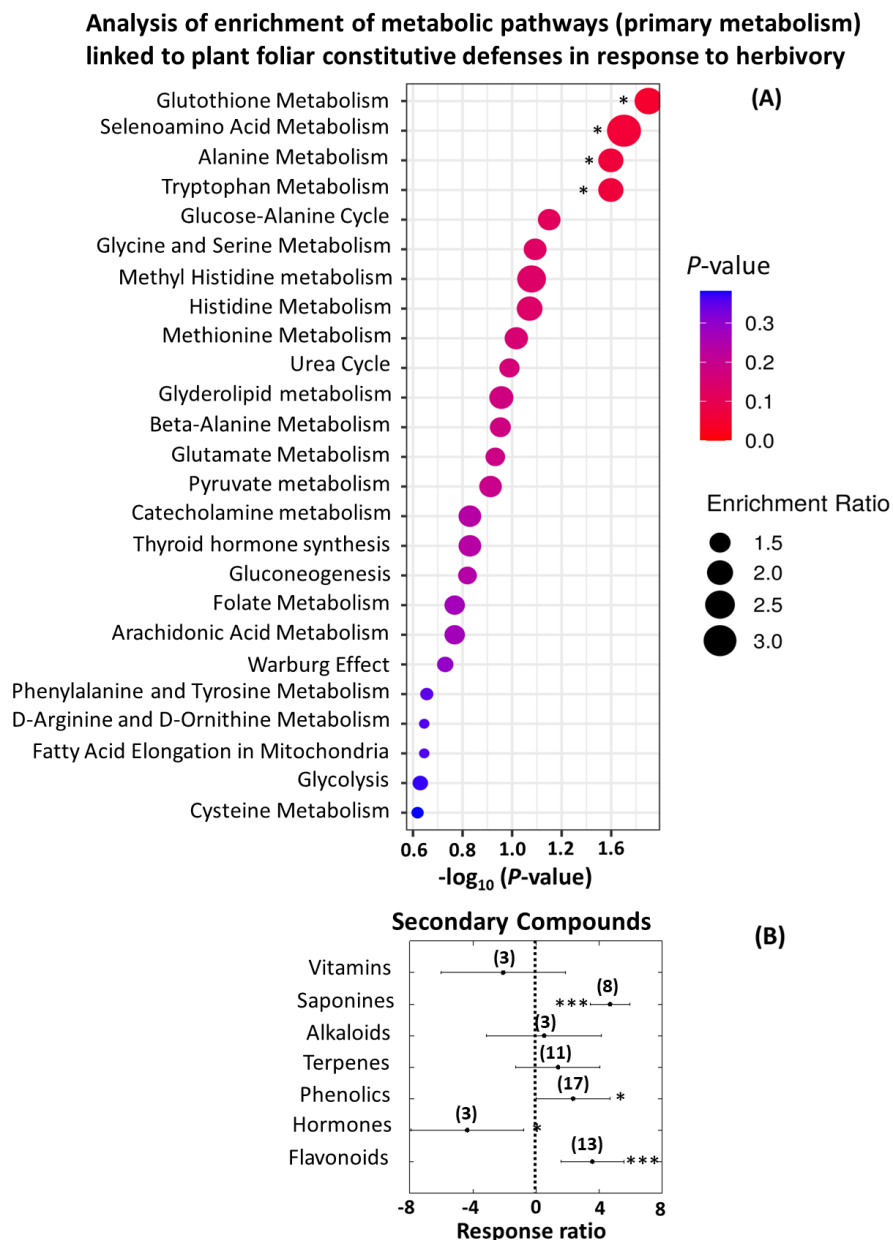


Fig. 2. Results of the analyses of enrichment pathways of foliar primary metabolites in studies that tested the metabolic differences of genotypes with higher constitutive defenses relative to other genotypes with no or fewer constitutive defenses in response to herbivory (A). Meta-analysis of groups of foliar secondary metabolites between genotypes with higher constitutive defenses relative to other genotypes with less constitutive defenses in response to herbivory. The number of studies is provided within parentheses for each group of secondary compounds (B). * $P < 0.05$, *** $P < 0.001$.

that metabolisms of glutathione and selenoamino acids were significantly enhanced in genotypes with lower levels of herbivore attack. The accumulation of selenoamino acids is well known to

be a deterrent mechanism against herbivores in plants (Bell 2003, Ding et al. 2017, Gupta and Gupta 2017, Schiavon and Pilon-Smits 2017). Glutathione is one of key antioxidants in plant

metabolome (Hameed et al. 2014, Hasanuzzaman et al. 2017) and specifically helps to maintain the reduced state of alpha-tocopherol and zeaxanthin (Hasanuzzaman et al. 2017). Glutathione also acts favoring the conversion of seleniates into reduced selenium, which is the chemical form of selenium incorporated into amino acids (Gupta and Gupta 2017). Individually, most studies reported lower concentrations of essential amino acids and higher phenolics concentrations (especially flavonoids) linked to lower levels of herbivore attack. Accordingly, our analyses consistently detected a significant ($P < 0.05$) increase in the metabolism of several amino acids across studies (Fig. 2A), and our meta-analysis also detected increases in the concentrations of phenolics (and in particular flavonoids) and saponins as constitutive metabolic defenses (Fig. 2B), whereas a decrease of hormones is also observed. Together, our findings suggest that plants allocate carbon (C) to a broad spectrum of C-rich compounds, as well as reducing allocation of C to amino acids and proteins to improve its level of constitutive defenses as claimed by the cost-benefit paradigm in environments with a great herbivore pressure (Cipollini et al. 2014). The degradation and alkylation of amino acids reduce plant protein nutritional quality, and this acts as deterrents (Zhou et al. 2015). The catabolism of lysine and branched-chain amino acids affects the resistance of plants to specific classes of pathogens, inducing the formation of pipecolic acid that maintains large basal systemic defense (Zeier 2013, Yang and Ludewig 2014). It is important to note, however, that phenolics are the most widespread group of defensive compounds in higher plants (War et al. 2012), and recent studies have shown that phenolics and saponins, in particular, are linked to constitutive chemical defenses in tropical plants (Bixenmann et al. 2016). Thus, the synthesis of current overall metabolomic studies showed that constitutive defenses are related to higher level of glutathione and selenoamino acid metabolism, to carbon shifts from amino acids to secondary metabolites, and to the formation of deterrent and antioxidant compounds such as selenoamino acids, pipecolic acid, and glutathione (Fig. 3).

Many studies have used metabolomics approaches to investigate inducible defenses by comparing attacked and unattacked plants of the

same initial phenotype and living in otherwise identical conditions (Tzin et al. 2015, 2017, Alagna et al. 2016, Rivas-Ubach et al. 2016a, b, 2017, Wang et al. 2016, Kato et al. 2017, Rodríguez et al. 2018, Kang et al. 2019). These studies have collectively identified specific biomarkers of herbivore attack, as well as discovering that entire pathways can be up- or down-regulated (Leiss et al. 2011, 2013, Kersten et al. 2013, Clancy et al. 2017). Studies of the full metabolome have also shown that two broad metabolic strategies are triggered in plants under herbivore. One strategy relates to the production of metabolites to counteract physical (i.e., broken tissues) and physicochemical (i.e., increase of oxidative potential) damage to tissues. The other strategy relates to the upregulation of metabolites or pathways that deter further attack (Mohanta et al. 2012). Generally, inducible defenses require mobilization of sugars from unattacked parts of the plant (Gómez et al. 2012), consistent with the different local and systemic metabolomically induced changes due to herbivore attack (Rivas-Ubach et al. 2016a, b), despite at this moment there is a lack of sufficient number of reports that have studied the local and systemic defense at once to reach a more general conclusion. The complexity of the shift in metabolic profiles induced by herbivores is even greater when considering that a single plant can be attacked by several herbivores at once. Errard et al. (2015) reported that plant responses to simultaneous attacks by different herbivores were not additive, but that multiple infestations elicited positive synergistic effects on carotenoid concentrations in leaves and stems.

The results of the enrichment analysis of the up- and down-regulated primary metabolic pathways and of the meta-analysis of the secondary groups of metabolites involved in inducing metabolic responses to herbivore attack are shown in Fig. 4 and Appendix S1: Table S2. We found that ketone metabolism was the most significantly upregulated pathway. Methyl-ketone synthesis has been widely associated with jasmonate-dependent induced defenses in response to herbivore attack (Chatzivasilieiadis and Sabelis 1997, Farmer et al. 2003, Benítez et al. 2017). While less dramatic than the response of ketone metabolism, we also detected upregulation of butyrate metabolism (Fig. 4A). Plants are well

General and significant changes observed in metabolomic analyses of plant foliar metabolism related to:

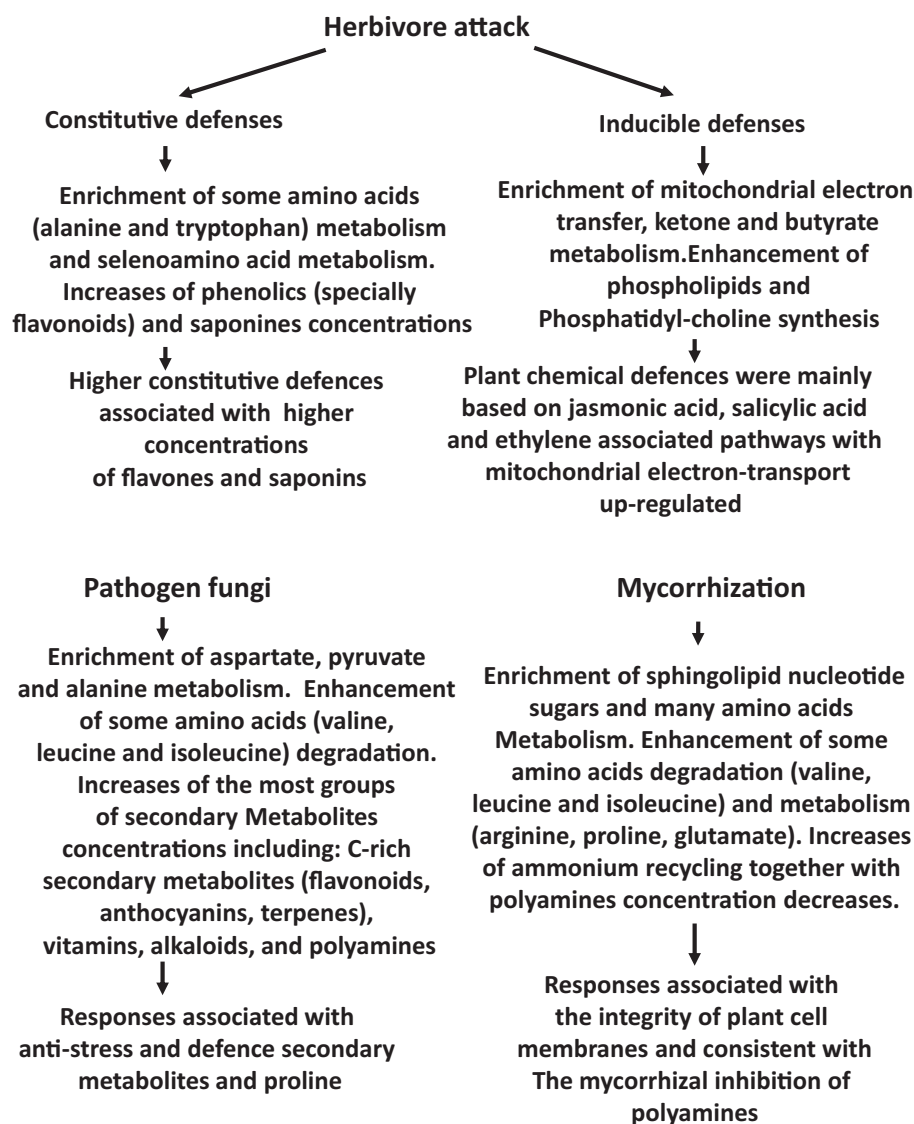


Fig. 3. Main overall conclusions of this study.

known to emit butyrate derivatives, such as (Z)-3-hexenyl butyrate and (Z)-3-hexenyl 2-methylbutyrate, from leaves in response to herbivore that act attracting predators to herbivore-damaged plants (Loughrin et al. 1994, McCall et al. 1994, Kessler and Baldwin 2001). Moreover, we found in the enrichment pathway analyses of primary metabolism that pantothenate and acetyl coenzyme A biosynthesis, as well as the mitochondrial electron-transport chain, were

additionally upregulated. This is consistent with the observed in some target studies where greater CoA synthesis has been associated with increases in jasmonate immediately following herbivore attack (Li et al. 2005, Fürstenberg-Hägg et al. 2013, Chen et al. 2019) thus making necessary higher amounts of CoA for the synthesis of this compound. The biosynthesis of jasmonic acid in plant peroxisomes requires the action of acyl-CoA oxidase (Xin et al. 2019).

Analysis of enrichment of metabolic pathways (primary metabolism) linked to plant foliar inducible defenses in response to herbivory

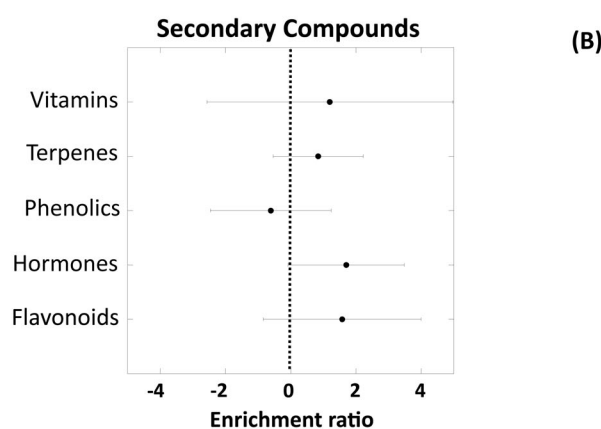
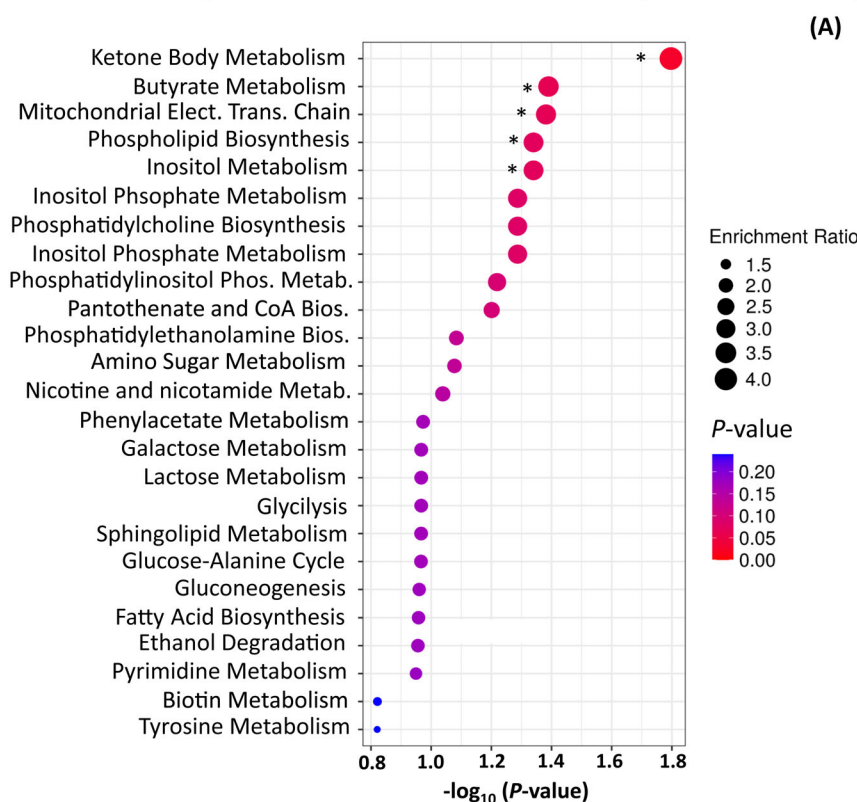


Fig. 4. Results of the analyses of enrichment pathways of foliar primary metabolites in studies that tested the metabolic differences of genotypes with higher inducible defenses relative to other genotypes with no or fewer inducible defenses in response to herbivory (A). Meta-analysis of groups of foliar secondary metabolites between genotypes with higher inducible defenses relative to other genotypes with no or fewer inducible defenses in response to herbivory. The number of studies is provided within parentheses for each group of secondary compounds (B). * $P < 0.05$.

Thereafter, Jasmonic acid derivatives Methyl-JA, a volatile compound, synthesis compete with the production of the bioactive form JA-Ile (Li et al. 2016). Then, these two molecules are involved in the stimulating plant defenses (War et al. 2011, Benevenuto et al. 2019). Moreover, the biosynthesis of flavonoids is based in Acetyl-CoA throughout p-coumaroyl-CoA and malonyl-CoA intermediates (Schmidt et al. 2005, Zhao et al. 2020). This is also consistent with other studies reporting increased pantothenate concentrations as an inducible leaf defense against herbivore (von Dahl et al. 2006, Rivas-Ubach et al. 2016a, b). Moreover, triggering inducible defenses is energetically costly (Kant et al. 2015, Yactayo-Chang et al. 2020), which may explain why we found that mitochondrial electron transfer was additionally upregulated. An increase in the metabolism of inositol phosphate is also consistent with previous experimental studies, which have reported increases in concentrations of inositol pyrophosphates associated with induced defensive mechanisms triggered by jasmonate signaling under herbivore attack (Abreu and Aragao 2007, Shah et al. 2017, Freed et al. 2020, Lorenzo-Orts et al. 2020). Other studies have similarly observed an upregulation of the expression of several genes coding for phospholipases (Pandey et al. 2017) and higher phospholipid concentrations (Fauconnier et al. 2003, Zhou et al. 2015) in plants under herbivore attack, which is consistent with our finding that phospholipid synthesis was also upregulated.

In short, analysis of enriched pathways from metabolomic studies agrees well with previous reports claiming that plant chemical defenses activated by insect and arachnids herbivorous attack are mainly based on jasmonic acid, salicylic acid, and ethylene (Ponzio et al. 2017). However, it would not have been possible to reach this conclusion from any study individually. Nevertheless, our meta-analysis identified a trend (but not significant) of higher flavonoid and phenolic concentrations, because individual studies disagreed on whether the concentrations of phenolics and flavonoids were up- or down-regulated under herbivore attack (Appendix S1: Table S2). This disagreement among individual studies can be explained and related to the Alarm–Resistance–Exhaustion phases claiming for different plant responses depending on time

and intensity of herbivore attack. If the stress in the alarm phase is too strong (very high herbivore infestation), the plant is not able to reach the resistance phase which leads to acute damage. In this case, the constitutive flavonoids are oxidized and their content even decreases. On the other hand, if the herbivore attack is small, it leads to signaling (by jasmonic acid, salicylic acid, ethylene, and Reactive Oxygen Species [ROS]) and induced synthesis of protective compounds (flavonoids, anthocyanins, etc.). However, if the stress (herbivore attack) increases, the plant is not able to synthesize more defensive compounds which leads to exhaustion (associated with decrease of defense compounds). There are many compounds indicating the exhaustion phase (oxidative stress) such as lipid peroxides and chlorophyll catabolites (see the review of Noctor et al. 2015). So the intensity and duration of the stress are crucial. The threshold (tipping point) from which is decided between acute damage or resistance, however, depends on the environment. The synthesis of secondary metabolites requires energy and thus higher light intensity means usually higher resistance and vice versa. On the contrary, higher N availability reduces the resistance threshold. Therefore, the C:N balance theory is generally useful but it does not apply unconditionally. There is a lot of other factors affecting the defense triggering. Identifying the responses of secondary metabolism to herbivore attack thus warrants future research using metabolomics platforms and new programs and more complete databases allowing to analyze shifts in overall metabolome including primary and secondary metabolic pathways.

Metabolomics is a useful approach for identifying phenotypes resistant to herbivorous attack. One constraint in breeding plants to defend against plant herbivores and pathogens has generally been the need to combine several analytical methods to obtain an overview of total secondary metabolism (Wink 1988). Ecometabolomic analyses are excellent tools to advance the study of effective treatments against herbivorous pests in plants (Chen et al. 2020). Chen et al. (2020) recently reported that the application of jasmonic acid to all leaves or only the apical leaves of chrysanthemums (*Chrysanthemum × morifolium*) increased the resistance to thrips, whereas the application to basal leaves was not

effective against the thrip pest (Chen et al. 2020). Extra doses of ultraviolet radiation can induce defenses mediated by jasmonic acid, thereby increasing the resistance to thrips in *Chrysanthemum × morifolium* (Escobar-Bravo et al. 2021). The dosage depended on time and genotype but demonstrated the possibility of some management strategies to efficiently stimulate natural plant defenses against arthropod herbivores.

Metabolomic analyses of plant-herbivore relationships can also identify sophisticated mechanisms of plant defense and detect the nutritional quality of a plant species to understand the resistance of some plant species against generalist herbivores. These abilities are especially essential for economically important crops and when the herbivore is a vector of plant diseases. The fly *Diaphorina citri*, the vector of huanglongbing infestation produced by the bacterium *Liberibacter asiaticus*, an important infestation in citrus plants, prefers the phloem sap of *Citrus sinensis* to that of *Murraya paniculata* and *Bergara koenigii* due to its higher concentrations of soluble sugars and amino acids (Killiny 2016, Killiny and Nehela 2017). This preference allows us to identify the metabolites that favor herbivorous attacks from a metabolic point of view, a tool indispensable to perform further adequate breeding efforts to reduce herbivorous and associated pathogenic damage.

An increase in photosynthetically active radiation in *Solanum lycopersicum* induced increases in the concentrations of abscisic acid and auxin in leaves, even though the concentrations of jasmonic and salicylic acids were unaltered in an upregulation of genes of the jasmonic acid pathway (Escobar-Bravo et al. 2018). The most recent studies using metabolomic analyses have thus observed the direct or indirect induction of chemical molecular defenses involving jasmonic acid, which can be easily induced and can effectively deter herbivores.

Thus, overall results from metabolomics platforms in a synthesis of a great taxonomical plant spectrum showed that plant chemical inducible defenses were mainly based on jasmonic acid, salicylic acid, and ethylene-associated pathways with mitochondrial electron-transport upregulated (Fig. 3).

Our synthesis of results from studies that have used metabolic platforms to investigate constitutive and inducible defenses against herbivorous

attack at the level of metabolomic profiles clarifies the more widespread changes to metabolic pathways used across several plant species to minimize the negative effects of herbivore. Few studies, however, using metabolomics platforms have compared various constitutive and inducible defenses at once among plant genotypes in response to attacks by the same herbivore (Sato et al. 2013, Cai et al. 2017, Escobar-Bravo et al. 2021, Perez-Fons et al. 2019). Untargeted analyses that used ecometabolomics have demonstrated a large chemical diversity that can structure natural communities into metabolic subgroups to create high small-scale heterogeneity for specialized herbivores (Clancy et al. 2017). This can constitute a new line of research that can shed light, for example, in explaining the high α - and β -diversity of some ecosystems such as of tropical rainforests.

Metabolomic analyses in the study of plant metabolic shifts to herbivore attack in trophic networks

Plants are frequently attacked by multiple herbivores but can concurrently establish other trophic relationships with other organisms, such as mutualisms or symbioses. Some studies have observed that herbivore attacks shift the metabolome profiles of both the plant and the corresponding herbivore species (Widarto et al. 2006, Errard et al. 2015, Sanchez-Arcos et al. 2019). Metabolomics offers a holistic view of local and systematic profiles of plant metabolomes under simultaneous attacks by different herbivores and on the interactions between plant function and response to herbivore attack, depending on the establishment of other simultaneous biotic relationships. For example, Kutyniok and Müller (2012) observed that attack on *Arabidopsis thaliana* leaves by the specialist aphid *Brevicoryne brassicae* promoted systemic changes in the plant that considerably reduced the attack of roots by the generalist nematode *Heterodera schachtii*, whereas the nematode attack did not affect the aphid attack. On the contrary, the suppression of specific monoterpenes and flavonoids in *S. lycopersicum* due to an attack of the whitefly *Bemisia tabaci* led to even more attacks by other whiteflies (Su et al. 2017). At the same time, certain associations of plants with microbes can improve the capacity of plants to chemically defend against herbivores (Khanna et al. 2019). For instance, the tomato plant,

Lycopersicon esculentum, if inoculated with the rhizobacteria *Pseudomonas aeruginosa* and *Burkholderia gladioli*, has been shown to possess higher concentrations of metabolic deterrents and has antioxidant capacity under attack of the herbivorous root nematode *Meloidogyne incognita* (Khanna et al. 2019). Other studies have reported upregulation of molecular defenses in microbe-inoculated plants, which increase the efficiency of their defenses against herbivores (Kang et al. 2018). In other words: The current ecometabolomic studies indicate that modulation of plant metabolism represents a key link for explaining antagonistic and mutualistic interactions between plants and herbivores. Fatouros et al. (2012) observed that eggs of the specialist cabbage butterfly *Pieris brassicae* deposited on the leaves of *Brassica nigra* generated an emission of plant volatiles that attracted parasitoid wasp species *Trichogramma brassicae* and *Cotesia glomerata*. The eggs of generalist moths, however, had no effect on parasitoid or predator communities, indicating that intense and long-term interactions between plants and their specialist herbivores have generated very specific mechanisms that influence ecosystem food web chains. In this regard, metabolomic studies are consistent with previous studies in this field but provide a more integral view of overall metabolism shifts that confers a more consistent support to these findings.

Plants can alter their metabolism due to herbivore attack, but herbivore attack can also be reinforced when herbivores have access to specific plant metabolites (Hopkins et al. 2017, Bell et al. 2019). For example, the presence of xylan in root exudates can stimulate synthesis of plant cell wall-degrading enzymes in root herbivore nematodes (Bell et al. 2019). At the same time, herbivores must adapt to changes in the availability, quality and quantity of plant nutritional quality and quantity. Ecometabolomics has been used less to study the impacts of plant food sources on herbivore metabolome. Rivas-Ubach et al. (2019) found a difference in variance of 10% in overall metabolic profiles between caterpillars of the processionary moth (*Thaumetopoea pityocampa*) feeding on two subspecies of *Pinus sylvestris* in the same forest.

Plant–fungi metabolism: The narrow limits between mutualism and parasitism

Symbiotic relationships between plants and fungi describe a continuum ranging from

mutualism to commensalism and parasitism (Redman et al. 2001, Schmidt et al. 2011). As such, many studies have struggled to determine the nature of specific plant–fungi associations (Kogel et al. 2006, Schmidt et al. 2011). Metabolomics is a unique tool for helping to determine the nature of such associations, since it can accurately assess the overall metabolic shifts of plants when associated or not with fungi (Kaur and Suseela 2020). We investigated how primary metabolic pathways and groups of secondary metabolites are up- or down-regulated in plants infested by known pathogenic fungi (Fig. 5 and Appendix S1: Table S3). We found that aspartate and pyruvate metabolism, as well as the transfer of acetyl groups within mitochondria, increased significantly in the presence of pathogenic fungi (Fig. 5A). We also found that the concentrations of all secondary metabolite groups were higher (Fig. 5B). Our analysis of primary metabolic pathways also indicated that the degradation of the branched-chain amino acids valine, leucine, and isoleucine was upregulated when pathogenic fungi were present, albeit only marginally significantly ($P < 0.1$). The catabolism of branched-chain amino acids has previously been associated with a higher resistance to specific pathogens by modulating defensive pathways regulated by salicylic acid and jasmonate (Sonderby et al. 2010, Zeier 2013). In fact, the shifts in primary metabolism observed here provide clear evidence of a widespread plant metabolic defense strategy against pathogenic fungi, whereby enhanced aspartic acid metabolism drives biosynthesis of lysine, isoleucine, and other amino acids (Stuttman et al. 2011). In turn, amino acids participate in the biosynthesis of other metabolites, such as glucosinolates (Sonderby et al. 2010) and pipecolic acid (Zeier 2013) that is an intermediate in the synthesis of N-hydroxypipecolic acid, which thereafter activates a set of plant immune genes involved in systemic acquired resistance (SAR) (Hartmann and Zeier 2018, Cai et al. 2021). This has proved to protect plants from pathogenic fungi (Bednarek et al. 2009) by triggering SAR via stimulation of pathways regulated by salicylic acid and jasmonate (Zeier 2013, Yang and Ludewig 2014). Our synthesis efforts thus suggest that the conversion of aspartate to other amino acids facilitates biosynthesis of defensive molecules in the presence of

Analysis of enrichment of metabolic pathways (primary metabolism) Linked to plant foliar metabolism response to pathogen fungi attack

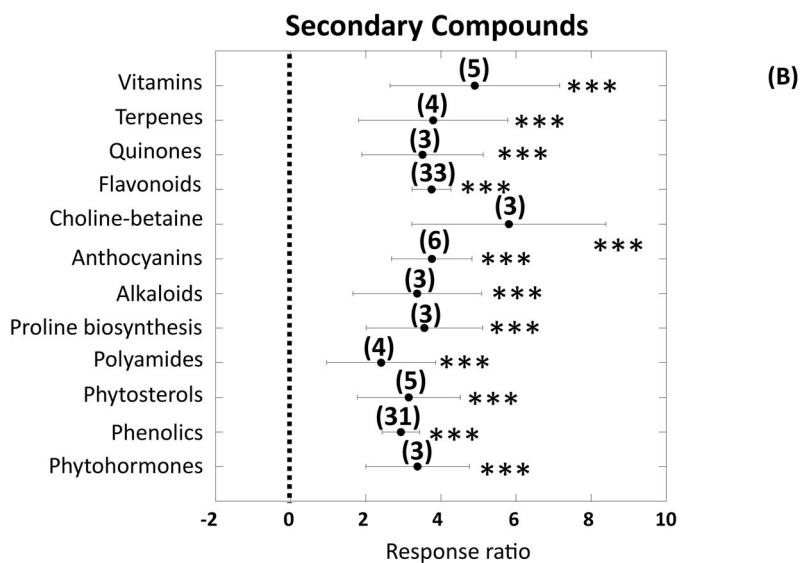
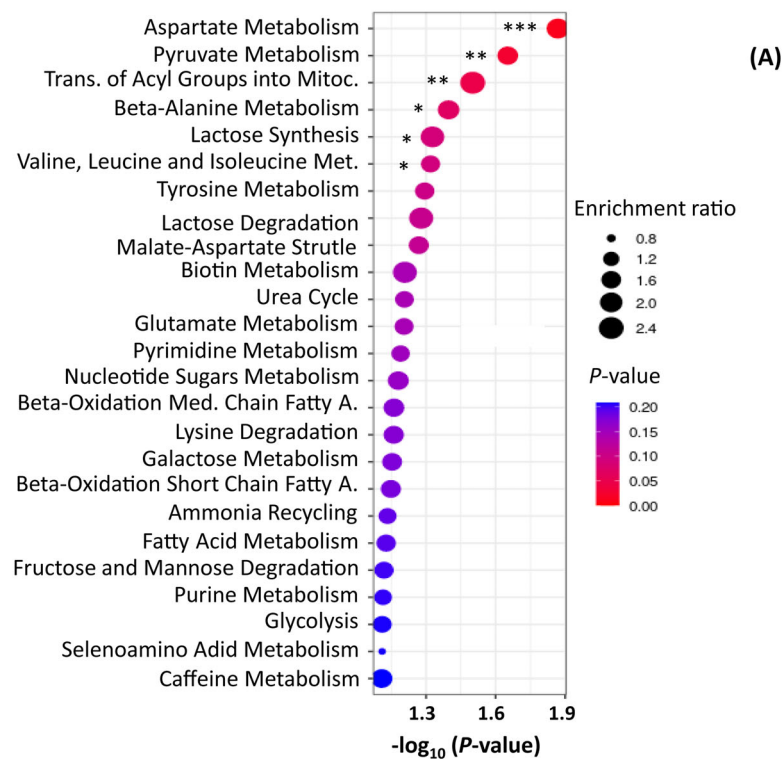


Fig. 5. Results of the analyses of enrichment pathways of foliar primary metabolites in studies that tested the metabolic differences of plants submitted to fungal pathogenic attack relative to other plants of the same species without fungal pathogenic attack (A). Meta-analysis of groups of foliar secondary metabolites between plants submitted to fungal pathogenic attack relative to other plants of the same species without fungal pathogenic attack. The number of studies is provided within parentheses for each group of secondary compounds (B). *** $P < 0.001$.

pathogenic fungi. This mechanism may indeed occur generally in higher plants. Specifically, the upregulation of plant genes involved in the degradation of amino acids has been observed as an inducible plant response to a wide array of pathogens (Ward et al. 2010, Rojas et al. 2014). Moreover, plants have multiple pathways for synthesizing lysine and isoleucine from aspartate (Galili 2011). Finally, the response of plants to fungal attack is extremely energy demanding (Heil et al. 2000), implying that an increase in pyruvate metabolism is necessary to activate the tricarboxylic acid (TCA) cycle (Strumilo 2005, Bolton 2009, Rojas et al. 2014) and intense load and transfer of acetyl-CoA in the TCA cycle (Ferne et al. 2004, Bolton et al. 2008), as found here. When only the data from forbs plants were analyzed, we observed only a marginal significant trend toward a rise in some amino acids metabolism (Appendix S1: Fig. S1).

Our meta-analysis of metabolomic studies also indicated a strong upregulation of several groups of plant secondary metabolites under infestation with pathogenic fungi, which is consistent with established literature. In particular, we found increases in phenolics (Lattanzio et al. 2006, Singh et al. 2007, Shalaby and Horwitz 2015), especially flavonoids (Ribera and Zuñiga 2012, Camargo-Ramírez et al. 2018) and anthocyanins (Chalker-Scott 1999), alkaloids (Singh et al. 2007, Ribera and Zuñiga 2012), terpenes (Singh et al. 2007, Ribera and Zuñiga 2012, Chen et al. 2018b), proline (Chen and Dickman 2005, Zeier 2013, Saima and Wu 2019), quinones (Leatham et al. 1980, Kiselevsky et al. 2014), and polyamides (Zeier 2013). Our findings for terpenes, however, differed from some other studies, which reported lower terpene concentrations under fungal infestation (Rodríguez et al. 2011).

In summary, ecometabolomic studies have provided a general view of the most widespread metabolically induced plant defenses in response to pathogenic fungal attack. Most mechanisms involve a transfer of C from primary to secondary metabolism driven by the metabolization of several primary compounds, including some amino acids. These amino acids then stimulate the activity of the TCA cycle, which releases stored energy in C resources and facilitates the activation of pathways controlled by jasmonate and acetylsalicylic acid. This results in increases

of secondary metabolite concentrations and proline in affected plant tissues (Fig. 3).

In comparison with pathogenic fungi, plant metabolic responses to associations with mycorrhizal fungi are less clear (Schweiger and Müller 2015). Indeed, shifts in metabolite concentrations under mycorrhization can be plant species specific, making it difficult to make general inferences for all plants (Schweiger et al. 2014). Here, our meta-analysis indicated that mycorrhization leads to an upregulation of nucleotide sugar, sphingolipid, and methylhistidine metabolisms (Fig. 6A). This is relevant because arbuscular mycorrhizal symbioses are an important component of the global C cycle, with plants allocating up to 20% of their photosynthates to the establishment and maintenance of mycorrhizae (Keymer et al. 2017). A major part of C transferred from plants to fungi is in the form of lipids (Wang et al. 2012, Gerlach et al. 2015, Bravo et al. 2017), which is consistent with our analysis that lipid metabolism is upregulated under mycorrhization. Sphingolipids, in particular, have been associated with the defense of the integrity of plant cell membranes under fungal hypha penetration and the accommodation of fungal hyphae within plant tissues (Mortimer and Scheller 2020). Sphingolipids have also been linked to hypha growth within host tissues (Berkey et al. 2012, Ghirardo et al. 2020). High concentrations of nucleotide sugars have also been observed under rhizospheric penetration (Shu et al. 2016, Shtark et al. 2019). Importantly, however, under mycorrhization nucleotide sugars are thought to be involved in the recycling of sugars (Bar-Peled and O'Neill 2011). Our results thus suggest that the increase in foliar concentrations of nucleotide sugars under mycorrhizal formation could be due to their role in the recycling of sugars released from the restructuring of cell walls under hypha penetration. In contrast to lipids and nucleotide sugars, investigation of methylhistidine in plants under mycorrhization has received little attention. Yang et al. (2020) observed higher concentrations of methylhistidine in the grass *Puccinellia tenuiflora* inoculated with arbuscular mycorrhizal fungi (AMF), but only under alkaline stress. Moreover, the same study found that mycorrhized plants had a higher ability to tolerate high pH conditions, which was attributed to the antistress properties

**Analysis of enrichment of metabolic pathways (primary metabolism)
Linked to plant foliar metabolism shifts under mycorrhization**

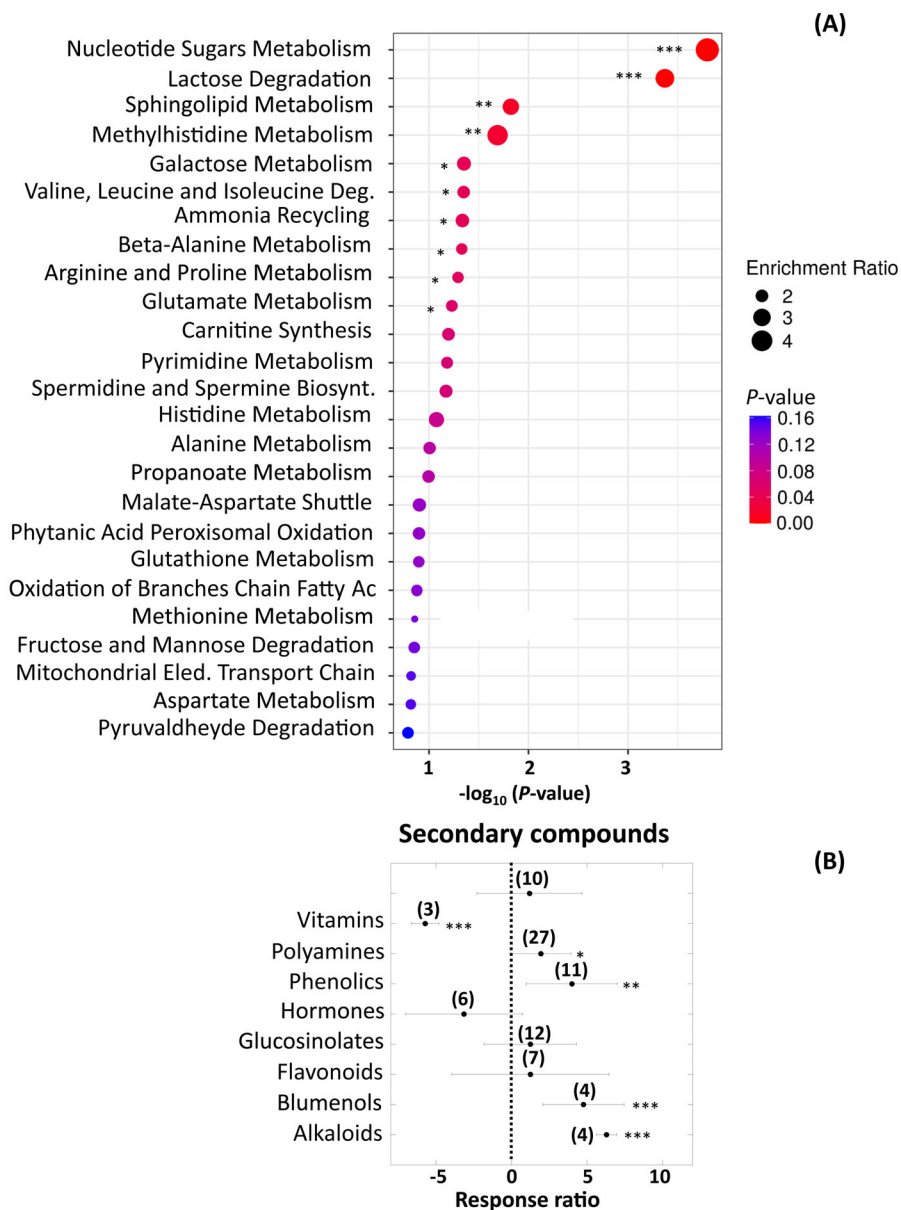


Fig. 6. Results of the analyses of enrichment pathways of foliar primary metabolites in studies that tested the metabolic differences of mycorrhized genotypes relative to the same genotypes not mycorrhized (A). Meta-analysis of groups of foliar secondary metabolites between mycorrhized genotypes relative to the same genotypes non-mycorrhized (B). The number of studies is provided within parentheses for each group of secondary compounds. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

of methylhistidine. The metabolism of several amino acids was also increased, but despite this, our pathway enrichment analysis did not find significant changes in any pathway directly

associated with most amino acids, sugars, and organic acids. This is in agreement with a recent review of the effects of arbuscular mycorrhizae on some groups of metabolites (Kaur and Suseela

2020), which found that sugars, organic acids, and amino acids are differentially regulated in plant species by different AMF species. The metabolomic enrichment analyses with only data from plant forbs (excluding *Arabidopsis*) showed a general enhancement of some amino acid metabolism (Appendix S1: Fig. S2) suggesting that the plant metabolomic shifts under mycorrhization can also depend of plant species, but all the data strongly suggest a clear change in amino acid metabolism in plants under mycorrhization.

Our meta-analysis revealed that mycorrhized plants generally had higher foliar phenolic concentrations (Fig. 6B; Appendix S1: Table S4). Again, these findings were consistent with most previous studies, which report higher concentrations of phenolic metabolites in multiple organs and tissues of mycorrhized vs. non-mycorrhized plants (Ceccarelli et al. 2010, Jugran et al. 2015, Lima dos Santos et al. 2017, Assel et al. 2019). Increase of phenolic compounds in plants induced by mycorrhizas could account for the established role of mycorrhizae in protecting entire plants, including aboveground organs such as leaves, against pathogens (Devi and Reddy 2002, Herre et al. 2007, Pozo and Azcon-Aguilar 2007) and various leaf-mining insects (Gange et al. 2005). Phenolic acids also act as signaling compounds in plant-microbial interactions (Mandal et al. 2010) and as antioxidants that protect plants against many stress conditions. A higher abundance of phenolic acids also increases the quality of plant production for human consumption by their antioxidant properties (Ghasemzadeh and Ghasemzadeh 2011). For example, the leaves and roots of peanut plants inoculated with AMF had higher abundances of phenolic compounds (Devi and Reddy 2002).

We additionally found that foliar blumenol and alkaloid concentrations were higher in mycorrhized plants (Fig. 6B). Higher blumenol concentrations are consistent with the reported role of blumenols as AMF molecular markers (Takeda et al. 1997, Hill et al. 2018, Wang et al. 2018, Fiorilli et al. 2019). Blumenols likely originated in roots but have also been widely observed in leaves of mycorrhized plants (Wang et al. 2018). Blumenols may additionally be involved in the synthesis of signaling molecules, the auto regulation of fungal colonization, and the protection of plants against pathogens and reactive oxygen

species (Fester et al. 2005, Kaur and Suseela 2020). Higher alkaloid concentrations (mainly pyrrolizidine alkaloids) in plant organs, including leaves, have also been reported in mycorrhized than non-mycorrhized plants (Andrade et al. 2013, Hill et al. 2018). Our meta-analysis also confirmed an overall increase of foliar hormone concentrations in mycorrhized plants (Fig. 6B). Current studies have reported general increases in the concentrations of gibberellins, strigolactones, cytokinins, auxins, and ethylene in mycorrhized plants, whereas other hormones such as jasmonic acid, salicylic acid, and abscisic acid can be up- and down-regulated in mycorrhized plants, likely due to growth conditions or species-specific effects (Foo et al. 2013, Foo 2020). Indeed, salicylic acid is known to increase in concentration during initial stages of colonization but to decrease later to facilitate AMF colonization. Arbuscular mycorrhizal fungi also prime the defensive system regulated by jasmonic acid, in that the jasmonic acid signaling pathway is important for AMF to increase the production of defensive compounds (Jung et al. 2012). However, such priming of the defensive system can vary among AMF genotypes (Pozo et al. 2009), which may explain why our findings were not conclusive. The concentrations of other plant hormones such as cytokinins can also be increased, which could be due to the benefits of phosphorus provided by the AMF (Adolfsson et al. 2017).

Some studies have shown that polyamides inhibit mycorrhizal formation (El Ghachtouli et al. 1996), which is in agreement with our findings that mycorrhized plants have consistently lower foliar concentrations of polyamides (Fig. 6B). Some studies, however, have not found clear links between polyamides in the leaves of mycorrhized relative to non-mycorrhized plants (Kytöviita and Sarjala 1997). Metabolomic studies have also reported that some metabolites upregulated in roots colonized by AMF have upregulated some metabolites (e.g., carnitine derivatives), favoring further colonization (Laparré et al. 2014) and also enhancing the chemical defensive capacity against herbivores (Hill et al. 2018).

Thus, summarizing metabolomics analyses of mycorrhize impacts of foliar plant metabolism across a wide spectrum of plant species provides evidence that mycorrhization strongly affects

amino acid metabolism, increasing ammonium recycling coupled with a decrease of polyamides concentrations, and with metabolic responses associated with cell membranes integrity (Fig. 3).

Collectively, our metabolomic results provide a view of the more general differences in shifts of plant metabolomics in response to parasitic vs. symbiotic/mutualistic fungal associations (Figs. 5, 6). In general, we observed a shift of C resources from primary to secondary defensive metabolites in response to pathogenic fungi. By contrast, groups of secondary plant metabolites were enhanced differently under mycorrhization, but this occurred in absence of a decrease in the concentrations and pathways of most primary metabolites. Indeed, in some cases primary metabolites were upregulated under mycorrhization, potentially providing fuel for mycorrhizal growth, penetration, and accommodation. Such foliar metabolic shifts thus represent the overall positive effect that mycorrhizae have on plants in providing soil resources. For example, the metabolic changes due to the favorable colonization of *Populus trichocarpa* by the fungus *Laccaria bicolor* are characterized by metabolic shifts in aromatic acids, organic acids, and fatty acids, with large increases in the concentrations of metabolites such as arbutin, some alkaloids, catechol, glutamic acid, myoinositol, pyruvic acid, stearic acid, stigmasterol, succinic acid, syringic acid, trehalose, and oxalomalic acid and with decreases in the concentrations of other metabolites such as caffeoylquinic acid and benzyl glycoside (Tschaplinski et al. 2014). Tschaplinski et al. (2014) reported that the more recalcitrant colonization of *Populus deltoides* by *L. bicolor* was associated with many fewer changes in plant metabolomic profiles, indicating the suitability of ecometabolomic studies for characterizing the plant–fungus interaction along the symbiosis–parasitism scale.

Ectomycorrhizae can shift metabolism from constitutive defenses to specialized protective compounds, providing a wider array of defensive compounds in leaves and roots and demonstrating the importance of belowground plant–microbe associations in mitigating below- and/or aboveground biotic stress (Bi et al. 2007, Hartley and Gange 2009, Tian et al. 2014, Kaling et al. 2018, Coppola et al. 2019). The arbuscular mycorrhizal colonization of *Triticum* sp. by *Funneliformis mosseae* increased the concentrations of

several plant hormones and metabolites involved with oxidative stress and osmotic homeostasis under drought conditions, such as gibberellic acid, abscisic acid, and metabolites associated with brassinosteroids, gibberellins, auxins, and jasmonic acid (Bernardo et al. 2019). The application of metabolomic studies to root extracts coupled to the behavior of pathogenic and symbiotic fungi near the roots (Zhi-lin et al. 2007) also warrants further research.

Future advances

Despite current bibliography has allowed to observe some general patterns in some metabolomic pathways related to constitutive and inducible defenses in response to herbivory or pathogenic fungi and mycorrhization (Fig. 3), there is still a lot of knowledge gaps in the context of metabolomics analyses in these fields of research. Future studies should design experimental protocols to provide clearer responses to ecological questions, clearly distinguishing the types of relationships to be studied. It is especially necessary well-designed studies to clearly differentiate inducible vs. constitutive defenses and plant–fungi mutualism–symbiosis vs. pathogenic relationships. Labeled ^{13}C substrates to study the metabolomic shifts in plants and fungi and/or herbivores by applying metabolomic ^{13}C NMR to follow metabolites from the pathogenic or symbiotic fungi to the plants or the herbivores is an example of useful technical approaches to advance in the knowledge of these interactions. Shachar-Hill et al. (1995) used ^{13}C -labeled glucose in plant roots to follow the use of plant glucose by AMF. This line of research has unfortunately not been followed in the metabolomic field. The current improvements in NMR and HPLC analytical tools will provide more accurate information of shifts in metabolic pathways and resulting metabolomes. Finally, given that plant–herbivore and plant–fungal interactions involve the interplay between primary and secondary metabolisms, there is an urgent need for platforms that facilitate the simultaneous interrogation, analysis, and interpretation of the entire metabolome.

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