Catch-22 in specialized metabolism: balancing defense and growth

Catch-22 in Specialized Metabolism: Balancing Defense and Growth

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ABSTRACT

Plants are unsurpassed biochemists that synthesize a plethora of molecules in response to everchanging environment. The majority of these molecules considered as specialized metabolites, effectively protect the plant against pathogens and herbivores. However, this defense most likely comes at a high expense, leading to reduction of growth (known as the 'growth-defense tradeoff'). Plants employ several strategies to reduce the high metabolic costs associated with chemical defense. Production of specialized metabolites is tightly regulated by a network of transcription factors facilitating its fine-tuning in time and space. Multifunctionality of specialized metabolites, their effective recycling system by re-using carbon, nitrogen and sulphur, thus, re-introducing them back to the primary metabolite pool allows further cost reduction. Spatial separation of biosynthetic enzymes and their substrates, sequestration of potentially toxic substances and conversion to less toxic metabolite forms are plant's solutions to avoid the detrimental effects of metabolites they produce as well as reduce production costs. Constant fitness pressure from herbivores, pathogens and abiotic stressors lead to honing of specialized metabolite biosynthesis reactions to be timely, efficient and metabolic cost-effective. In this review we assess the costs of specialized metabolites production for chemical defense and the different plant mechanisms to reduce the price of of such metabolic activity in terms of self-toxicity and growth.

Key words: Specialized metabolite, transport, self-toxicity, vacuole, growth/defense tradeoff, herbivore, biosynthesis, regulation

HIGHLIGHT

This review is focused on the costs of plant chemical defense in terms of resourse allocation and self-toxicity in view of mechanisms for fine-tuning the "growth-defense tradeoff".

INTRODUCTION

The unsurpassed ability of plants to synthesize a wide variety of low molecular weight compounds, largely specialized (secondary) metabolites, facilitates their adaptation to a changing environment and battling natural enemies (Wink, 2010; Kessler and Kalske, 2018). It is predicted that the plant kingdom can generate from two hundred thousand to one million specialized metabolites (Wang *et al.*, 2019). These metabolites represent four major structuraly diverse classes; terpenoids, phenolic, alkaloids and sulphur-containing compounds (Guerriero *et al.*, 2018). This huge diversity orginates from basic metabolite skeletons, modified at different positions through glycosylation, methylation, hydroxylation, oxidation, and additional reactions (Wang *et al.*, 2019). The elevation of defense responses in plants typically suppresses their growth; a phenomenon known in ecology as the "growth-defense tradeoff" (Adler and Karban, 1994; Karban, 2019). In addition to metabolic allocation costs (diversion of resources from growth and reproduction), a combination of other factors, such as ecological, opportunity and storage costs need to be taken into account when considering growth and defense (Purrington, 2000). The metabolic allocation cost in the "growth-defense tradeoff" is yet questionable as there are more significant evidences for the opportunity and ecological costs and less evidence for actual tradeoffs supporting metabolic costs (Agrawal *et al*., 2012). In this review we will focus on potential metabolic costs of chemical defence linking it to the penalty in terms of self-toxicity costs and mechanisms of recycling. In the last decade, advances in molecular ecology facilitated validation of "growth-defence tradeoff" concept, using transgenic and mutant plants, although precise calculations of metabolic costs remains elusive (Neilson *et al.*, 2013; Karasov *et al*., 2017).

Estimation of the price tag for specialized metabolites production under different conditions is difficult due to the complexty of the metabolic network as well as our limited knowledge with respect to metabolic fluxes and yet unidentified pathways and reactions. The metabolic cost for chemical defense is primarily associated with assimilation of carbon and/or nitrogen into specialized metabolite molecules (Havko *et al.*, 2016). The biochemical nature of plant specialized metabolites makes them not only toxic for enemies but potentially harmfull to the plants themselves. In other words, plants can rather not overaccumlate defensive specialized metabolites, as at a certain threshold they posses a negative impact on plant fitness. To overcome the toxic effect of plant's own metabolites, these chemicals accumulate to very high levels in specific tissues, organs,

cells and organelles. For example, several cyanogenic plants accumulate nearly 25-50% cyanogens in the seedlings only, and in *Cannabis* spp. upto 25% cannabinoids accumulate in specific glandular trichomes (Adewusi, 1990; Mahlberg and Kim, 2004; Livingston *et al*., 2019). Conversely, some defensive metabolies are specifically biosynthesized only when required. For example, glucosinolates and cyanogenic glucosides don't really track the optimal defense theory given that their bipartite nature, means they need to be accumulated prior to pathogen attack to protect the plant (Pedras and Yaya, 2015). This adds a significant bill to the cost budget as such potentially cytotoxic molecules require sequestration and effect plant development and growth (Shitan, 2016; de Brito Francisco and Martinoia, 2018). In consequence, plants possess several solutions to avoid the harmful effects of metabolites they produce (Figure 1). Mechanisms to avoid self-toxicity in plants include (i) sequestration to either the vacuole, extracellular space or specialized cells, (ii) 'trapping' metabolites in metabolons, (iii) translocation between the site of production and storage site; (v) conversion to less toxic metaboloite forms and (vi) separation between substrates and the correspoding enzyme making a toxic product (Figure 1); [Samanani and Facchini, 2006; Møller, 2010; Jørgensen *et al.*, 2015; Shitan, 2016; Nour-Eldin *et al.*, 2017; Payne *et al.*, 2017; Takanashi *et al.*, 2017; Shitan and Yazaki, 2019].

To interpret mechanisms of plant self-toxicity avoidance the spatio-temporal pattern of biosynthesis and regulation of a given specialized metabolite in the spatiotemporal manner. While the biosynthesis of specialized defense metabolites is well-studied, limited information is available with respect to fine-tuning of their organelle -or tissue-specific accumulation. No information is available on the maximum capacity of specialized metabolism induction in certain tissues and response to stress. There are plenty of studies reporting on different modes of regulation [i.e. transcriptional, post-transcriptional (e.g. miRNAs), translational and post-translational] of plant specialized metabolites. Yet, the link between those modes is barely understood.

This review is focused on specialized metabolism in the context of the plant 'growth-defense tradeoff'. We disscuss resource allocation, compartmentalization, phytotoxicity prevention and regulation with respect to different aspects of specialized metabolites production costs.

Cost calculation of specialized metabolite production

Limited resource allocation is the main assumption for the tradeoff between biosynthesis, transport and storage of specialized metabolites at the expense of plant growth and development (Stamp, 2003). Throughout the years attempts were made to identify and accurately calculate the budget allocation for chemical defense

during stress conditions (King *et al.*, 2006; Goodger *et al.*, 2006; Dahlgren *et al.*, 2009; Manzaneda *et al.*, 2010; Paul-Victor *et al.*, 2010; Penuelas *et al.*, 2010; Siemens *et al.*, 2010; Sampedro *et al.*, 2011; Züst *et al.*, 2011; Heinrich *et al.*, 2013). Accurate quantification of the metabolic cost possesses two major challenges: i) the lack of an adequate experimental setup that can consider variations in environmental factors during the life cycle of the plant, since is it increasingly difficult to dissect the role of a single environmental factor on a plant fitness, at a certain time. and ii) the effect of natural selection on specialized metabolism, due to large intervals of time that are necessary for evolutionary changes to occur (Strauss *et al.*, 2002; Paul-Victor *et al.*, 2010). Carbon is considered a unit for metabolic cost assessment that is hypothetically associated with defense *vs*. growth tradeoffs. Oversimplification of carbon budget for defense *vs.* growth dichotomy is challenging as the mode of division in carbon flux to plant chemical defense pathways and growth physiologies is still unclear (Weraduwage *et al.*, 2015).

Several methods exist that could be used to evaluate the costs of chemical defense. Proximal analysis is used for cost assessment of specialized metabolites, synthesized from precursor primary metabolites (De Vries *et al.*, 1974; Gershenzon, 1994). In this method, the total energy requirement for biochemical constituents of a given tissue, including the cost of biosynthesis and transport is examined. Typically there is a high metabolic cost for production of more reduced specialized metabolites such as triterpenoids as compared to carbohydrates and phenolic compounds. It was reported that 3.1g and 1.5g glucose (in the form of acetyl-CoA, ATP and NADPH) is required for the biosynthesis of the monoterpene camphor and phenol glycoside, respectively (Gershenzon, 1994). Calorimetric analysis is another method for analyzing budget allocation, where a calorimeter is used for the combustion of the tissue or whole plant. Although not as informative as proximal analysis, it is a relatively simple approach which offers satisfactory information on total energy stored in the tissue, but not on the energy contribution of each individual metabolite, thus both methods are used for wide-range studies (Havko *et al.*, 2016). Miller *et al.* (2013) used calorimetric analysis to evaluate alteration of carbon partitioning in lipid biosynthesis of Arabidopsis, yet, up to date, no report exists that describes the use of this method to estimate budget allocation during growth-defense tradeoff and specialized metabolism.

As compared to the limited use of the proximal and calorimetric methods, flux balance analysis (FBA) is the most suitable and widely accepted approach for cost estimation of metabolite production. Construction of *in-silico* metabolic network of an entire biochemical pathway is necessary for FBA, and is achieved by combining gene expression data and information on chemical reactions from a particular biosynthetic pathway. In recent years, several studies examined the use of complex network analysis to conduct predictive

modification of specialized metabolism and plant productivity (Mintz-Oron *et al.*, 2012; Fesenko and Edwards, 2014; Liu and Stewart, 2015; Fuentes *et al.*, 2016; Töpfer *et al*., 2017; Delfin *et al.*, 2019; Küken and Nikoloski, 2019). FBA was extensively used to estimate nitrogen assimilation and allocation during specialized metabolite biosynthesis. Stable isotope labeling using ^{15}N revealed the cost of nitrogen allocation in photosynthesis and specialized metabolism during growth *vs*. defense tradeoff (Ullmann-Zeunert *et al.*, 2012; 2013). Ullmann-Zeunert *et al*. (2013) reported that in *Nicotiana attenuata*, herbivory reduced nitrogen assimilation in ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO) and in total soluble proteins (by approximately 90%). Specialized metabolite biosynthesis pathways are highly cross-connected with each other by using the same precursor molecules. The major limitation of FBA is insufficient knowledge regarding the function, localization and regulation of the entire set of enzymes in a single given metabolic pathway, making it increasingly more difficult to link diverse pathways for specialized metabolites biosynthesis (Stavrinides *et al.*, 2015; Wurtzel and Kutchan, 2016; Barros *et al.*, 2019). For example, phenylalanine serving as precursor for numerous and diverse specialized metabolites was known for many years to be synthesized through a plastidial pathway (Widhalm *et al.*, 2015). Recently, Qian *et al*. (2019) demonstrated an alternative, cytosolic pathway for phenylalanine biosynthesis. A combined model using FBA with additional systems biology tools [COBRA model and Systems Biology Markup Language (SBML)] (Rai *et al.*, 2017; Rowe *et al.*, 2018) as well as kinetic models (Guo *et al.*, 2018) will likely provide more accurate estimation regarding the cost of specialized metabolism production. Additionally, lack of fully annotated genome-scale metabolic network(s) is the key problem for accurate estimation of the cost budget for specialized metabolism (Mintz-Oron *et al.*, 2012). For example, A genome-scale metabolic network reconstruction of tomato was developed by Yuan *et al*. (2015) which mostly deals with photorespiratory metabolism, thus this network is inadequate for other metabolic pathways. Providing a comprehensive metabolic network model that accounts for primary as well as secondary metabolite pathways (ideally under changing environmental conditions) would greatly improve understanding the costs associated with the use of primary megtabolites as precusrors for specialized metabolism.

Logistics of specialized metabolism as a mechanism to reduce production costs by avoiding self-toxicity and fine-tuning production in time and space

Plants produce an array of active specialized metabolites that represent a ‘chemical language’ mediating interaction with the environment (Isah, 2019; Tohge and Fernie, 2020; Erb and Kliebenstein, 2020). It is likely that most of these metabolites are toxic to plant cells at certain concentration and as noted above regulated

compartmentalization is required for preventing such harmful effects. In addition to preventing toxicity, regulated spatial and temporal localization of metabolites represents the means by which production and accumulation is controlled and costs of production are reduced. Regulating the levels of metabolites in specific compartments is complemented by localization of enzymes, protein complexes as well as their metabolite precursors.

Localization to the vacuole was shown for several groups of potentially toxic specialized metabolites; e.g. alkaloids (nicotine and berberine), glycoalkaloids, saponins, cyanogenic glycosides, and glucosinolates (Saunders and Conn, 1978; Alcantara *et al.*, 2005; Otani *et al.*, 2005; Sirikantaramas *et al.*, 2007; Mylona *et al.*, 2008; Payne *et al.*, 2017; Kazachkova *et al*., 2021). However, only a handful of proteins were experimentally shown to be associated with transport of self-toxic specialized metabolites across the tonoplast membrane. To date NPF (nitrate-peptide transporter) (Payne *et al.*, 2017; Kazachkova *et al*., 2021) and MATE (multidrug and toxic compound extrusion) (Shoji *et al.*, 2009; Morita *et al.*, 2009) family transporters were shown to participate in specialized metabolite transport to the vacuole (Shitan and Yazaki, 2019). Extensive experimental data and reviews are available on the mechanisms of flavonoid transport (Goodman *et al.*, 2004; Poustka *et al.*, 2007; Zhao and Dixon, 2010; Francisco *et al.*, 2013; Chanoca *et al.*, 2015; Behrens *et al.*, 2019). Though, no definite information is available on flavonoid self-toxic effects on plant cells. In addition to transporter-mediated vacuolar import, ER-associated vesicular transport to the vacuole was shown for the alkaloid sanguinarine in cultured opium poppy (*Papaver somniferum*) cells in response to elicitor treatment (Alcantara *et al.*, 2005). Inability to sequester self-toxic specialized metabolites to the vacuole might result in toxicity. The steroidal glycoalkaloid (SGA) exporter *GORKY* (*SlNPF1.5*), excludes the tomato *α*-tomatine and its derivatives from the vacuole to the cytosol during ripening, facilitating the conversion of the entire *α*-tomatine pool to non-toxic and non-bitter forms (Kazachkova *et al*., 2021). Overexpression of *GORKY* in tomato leaves resulted in the accumulation of SGA pathway intermediates in the cytosol causing severe toxicity symptoms in the plant. This observation clearly indicated that sequestration of SGAs in the vacuole serves as a self-protection mechanism (Kazachkova *et al*., 2021). Severe and largely similar phenotypes were observed in tomato plants accumulating tomatidine (the *α*-tomatine aglycon) following down-regulation of GLYCOALKALOID METABOLISM 1 (GAME1)*;* a UDP-glycosyltransferase that carries out tomatidine galactosylation (Itkin *et al.*, 2011).

In *Nicotiana tabacum*, nicotine transport at the tonoplast membrane is mediated by the MATE family transporters NtJAT1, NtJAT2, NtMATE1, and NtMATE2 (Shoji *et al.*, 2009; Morita *et al.*, 2009). In addition to the intracellular compartmentalization of nicotine, upon wounding or herbivore attack, its biosynthesis is

induced in root tissues and transported via xylem to leaves where it serves as an insecticide due to its neurotoxic properties (Baldwin, 1989; Steppuhn *et al.*, 2004; Shitan *et al.*, 2014) (Figure 1). Berberine, a renown alkaloid from *Coptis japonica*, is produced in the roots and translocated to rhizomes where it is localized to the vacuole by the MATE family transporter, *Cj*MATE1 (Takanashi *et al.*, 2017). Several ABC (B-type) transporters were shown to participate in relocation of berberine from roots to rhizomes via xylem transport and through cytosolic membranes of the rhizome cells (Yazaki *et al.*, 2001; Shitan *et al.*, 2003, 2013; de Brito Francisco and Martinoia, 2018).

Catharanthus roseus produces monoterpene indole alkaloids via an intricate pathway that comprises multiple reactions distributed between several cell types and involving intra- and intercellular translocation of pathway intermediates (Verma *et al.*, 2012; Courdavault *et al.*, 2014; De Luca *et al.*, 2014; Qu *et al.*, 2019). To date, close to 30 genes involved in the pathway are known (Qu *et al.*, 2019), including several transporters. *CrTPT2* encodes a plasma membrane exporter that re-locates catharanthine to the leaf surface (Yu and De Luca, 2013). CrNPF2.4, CrNPF2.5 and CrNPF2.6 showed transport activity in *Xenopus laevis* oocytes against the iridoid glycosides and are localized to the plasma membrane suggesting a function in intercellular transport from the apoplast to the cytosol (Larsen *et al.*, 2017). The CrNPF2.9 transporter was shown to export indole alkaloid strictosidine from leaf vacuoles (Payne *et al.*, 2017). Down-regulation of *CrNPF2.9* using virus-induced gene silencing (VIGS) lead to increased accumulation of strictosidine in the vacuole and simultaneous decrease in downstream alkaloids. *NPF2.9*-silenced leaves showed symptoms of toxicity and cell death indicating that disproportionate accumulation of strictosidine may be self-toxic to the plant (Payne *et al.*, 2017) (Figure 1).

Several cannabinoids in *Cannabis sativa*, including tetrahydrocannabinolic acid (THCA), cannabigerolic acid (CBGA) and cannabichromenic acid (CBCA) were shown to induce cell death of tobacco BY-2 and *Cannabis* leaf cells causing severe mitochondrial damage (Sirikantaramas *et al.*, 2007; Morimoto *et al.*, 2007). Therefore to avoid self-damage, cannabinoid biosynthesis is exclusively reserved to glandular trichomes (Sirikantaramas *et al.*, 2005). Moreover, extracellular production of toxic THCA in the storage cavity of glandular trichome acts as a mechanism to avoid self-toxicity (Sirikantaramas *et al.*, 2007).

Assembly of biosynthetic enzymes in metabolons serves as additional mechanism of avoiding cytotoxicity of metabolic pathway intermediates (Winkel, 2004; Laursen *et al.*, 2016; Obata, 2019). Rapid channeling of reaction products between catalytic sites of enzymes united into a metabolon facilitates their accumulation in higher concentrations and quick turnover to more stable and less toxic metabolites without damaging the cells (Winkel, 2004). It is anticipated that biosynthetic enzymes of many specialized metabolites such as alkaloids,

flavonoids, cyanogenic glycosides and possibly others are organized in metabolons (Hemm *et al.*, 2003; Winkel, 2004; Jørgensen *et al.*, 2005; Møller, 2010; Weis *et al.*, 2014). Biosynthesis of dhurrin, the tyrosine-derived cyanogenic glucoside from *Sorghum bicolor* seedlings, is carried out in a metabolon, facilitating rapid conversion of labile highly toxic aldoxime intermediates to more stable and less toxic ones (Jørgensen *et al.*, 2005; Sakurada *et al.*, 2009; Laursen *et al.*, 2016; Obata, 2019). Clustering of dhurrin enzymes into metabolon facilitated engineering of the whole pathway in Arabidopsis without significant adverse effects on the plant (Tattersall *et al.*, 2001; Kristensen *et al.*, 2005) indicating that metabolon formation effectively encapsulates toxic intermediates in heterologous systems.

Another mechanism to avoid self-toxicity is separation of potentially self-toxic metabolites and the enzymes that can cause metabolite breakdown to toxic products. However, when plant cells are wounded or attacked by herbivores, cellular compartmentalization disintegrates; the enzymes and the substrate(s) come in contact and finally release toxic defense compounds. For example, dhurrin, stored in the vacuoles in intact tissues is hydrolyzed by a chloroplastic β-glucosidase (dhurrinase), forming a cyanohydrin that is further cleaved by the cytosolic hydroxynitrile lyase to form aldehyde or ketone and highly toxic HCN (Morant *et al.*, 2008; Clausen *et al.*, 2015; Laursen *et al.*, 2016). Similarly in the case of glucosinolates, thioglucosides found in Brassicaceae, and their hydrolytic enzymes, myrosinases (specific class of β-thioglucosidases), are stored in separate cells. Upon wounding, myrosinases come in contact with glucosinolates, resulting in the production of biologically active isothiocyanates, nitriles, and thiocyanates (Rask et al, 2000; Halkier and Gershenzon, 2006; Winde and Wittstock, 2011) (Figure 1).

Reduce, reuse and recycle in plant specialized metabolism.

Biosynthesis of a specialized metabolites requires significant investment in energy and resources (Neilson *et al.*, 2013). Thus, in addition to tight regulation of production, recycling of superfluous specialized metabolites back into the primary metabolite pool, minimizes production costs (Neilson *et al.*, 2013; Erb and Kliebenstein, 2020). Throughout the life cycle, the demand of plants for defensive specialized metabolites is dynamically changing and remobilization into the primary metabolite pool serves as a strategy to reuse the available resources and reduce energy costs. Although evidence exists that plants recycle specialized metabolites, through channeling of degradation products into the primary metabolite pool, it is difficult to estimate the contribution of these degradation products to the primary metabolite content. To date, cyanogenic glucosides recycling and reuse as a nitrogen source (without release of toxic HCN), is probably the best studied example (Selmar *et al.*, 1988; Jenrich *et al.*, 2007; Pičmanová *et al.*, 2015) (Figure 2). Comparative metabolite profiling

of three plant species producing cyanogenic glycosides (i.e. almond, cassava and sorghum), pointed to a similar turnover pathway leading to re-assimilation of nitrogen and carbon into the primary metabolite pool (Pičmanová *et al.*, 2015). In *Sorghum bicolor*, three members of the NITRILASE 4 (NIT4) family, NIT4A, NIT4B1, and NIT4B2 were shown to form heterodimeric complexes that can effectively detoxify dhurrin degradation products thereby replenishing the pool of nitrogen in the plants and simultaneously avoiding formation of toxic HCN (Jenrich *et al.*, 2007) (Figure 2). Similarly in Arabidopsis, three nitrilase isoenzymes are postulated to participate in glucosinolate turnover by conversion of nitriles to carboxylic acids (Vorwerk *et al.*, 2001). Sulphur-containing glucosinolates could potentially serve as an additional source of sulphur for plants. Arabidopsis *glucosinolate transporters* (*gtr1gtr2*) double knockout plants fail to transport glucosinolates to reproductive tissues and the seedlings exhibit lower biomass under sulphur-deficient conditions (Nour-Eldin *et al.*, 2012, 2017). Legumes accumulate a non-protein amino acid, *L*-canavanine, that is degraded during seed germination, providing the developing seedling with additional source of carbon and nitrogen (Rosenthal, 1990).

Anthocyanin biosynthesis is well studied, however, not much is known about anthocyanin degradation and its potential role as an additional carbon source for plants (Liu *et al*., 2018). In *Brunfelsia calycina* flowers are rapidly changing color from purple to white in course of time due to anthocyanin degradation, that was shown to require *de novo* mRNA and protein synthesis (Vaknin *et al*., 2005). However, it remains elusive whether and to what extent such anthocyanin degradation products get re-icorporated into the primary metabolite pool in anthocyanin producing plants.

How much is too much? Constitutive *vs*. induced specialized metabolism

During their life cycle plants withstand a remarkable diversity of potential threats, of both biotic and abiotic origin; specialized metabolite production is one of the strategies they employ to survive in this rapidly changing environment (Karban and Baldwin, 1997; Heil and Baldwin, 2002). Specialized metabolites peform their key function in protection from pathogens and herbivores through several startegies including i) direct toxicity to herbivores and pathogens ii) attraction of natural enemies of the herbivores, iii) repellent action and iv) reduction of tissue digestibility and nutritional profile (Kessler, 2015). Their production can be either constitutive, irrespective of herbivore threat presence, or inducible, when biosynthesis occurs only in response to herbivory (Heil and Baldwin, 2002). In response to the stressor, changes in both primary and specialized metabolism occur that not only induces plant resistance against the herbivore, but also simultaneously transmits the information to the neighboring organisms, mostly by volatile organic compounds (Heil and

Baldwin, 2002; Heil and Karban, 2010; Kessler and Kalske, 2018). Interestingly, plants that were not previously exposed to the herbivores had a lower baseline of specialized metabolite production levels (Agrawal *et al.*, 2012). Although direct comparison of metabolic costs upon constitutive *vs*. induced specialized metabolism is difficult to perform, several studies have shown that the costs of constitutive production of defense compounds is rather similar to retaining early herbivory endogenous recognition signaling pathways that mediate herbivore-induced metabolic responses (Steppuhn *et al.*, 2008; Abreu and Munné-Bosch, 2009). Additional factors need to be taken into account in such calculations; such as the multiple roles of plants specialized metabolites (Erb and Kliebenstein, 2020). For instance, glucosinolates and benzoxacioids, well-known defense compounds, were shown to have a role in regulating accumulation of other classes of specialized metabolites (Kim *et al.*, 2015; Zhou *et al.*, 2018; Li *et al.*, 2018). The possible mechanism of crosstalk between the glucosinolate and phenylpropanoid pathways is related to the degradation of the enzyme phenylalanine ammonia-lyase catalyzing the first step of the phenylpropanoid biosynthesis pathway (Kim *et al.*, 2020).

To date, three plant defense theories exist that provide theoretical background explaining specialized metabolites accumulation patterns for plant fitness. The moving target hypothesis indicates that the change in specialized metabolite production in response to herbivore attack leads to a change of the plant biochemical phenotype which itself serves as a defense strategy (Adler and Karban, 1994; Kessler, 2015). The information transfer hypothesis suggests that herbivore-induced specialized metabolite production also serves as chemical cues that plants transmit to their environment (neighboring plants, insects, bacteria, etc) (Kessler, 2015). The optimal defense hypothesis states that in the absence of herbivores, plant overall specialized metabolite accumulation should be low, therefore allowing the plants to produce costly metabolites only when needed (McKey, 1974; Denno, 2012). Optimal defense theory is used to predict the spatial distribution of specialized metabolite levels in plant tissues, depending on how valuable certain tissues are to the plant as a whole. Plant reproductive tissues, like fruit and flowers typically accumulate significant amounts of specialized metabolites (Howe and Jander, 2008). Similarly young leaves are more valuable to the plant therefore contain higher levels of protective metabolites (Darrow and Bowers, 1999; Kozukue *et al.*, 2004). Several studies also compared the spatial allocation of specialized metabolite induction in response to above- and belowground-herbivores (Bezemer *et al.*, 2004; Bezemer and van Dam, 2005; Kaplan *et al.*, 2008). Intuitively, specialized metabolites are likely to accumulate in specific tissues based on the possible risk of future attack. Indeed, in *N. tabacum*, leaf-chewing insects elicited strong response in leaf tissues but had no impact on roots (Kaplan *et*

al., 2008). Interestingly plant responses to root herbivory did not follow the same pattern; root nematodes caused changes in specialized metabolome of both, roots and shoots in tobacco plants.

In addition to the biotic stress, abiotic factors also have a significant impact on the accumulation of several classes of specialized metabolites (Dixon and Paiva, 1995; Yang *et al.*, 2018; Li *et al.*, 2020). A number of studies focused on the alteration to individual specialized metabolite or an isolated class of molecules in response to one or several changing environmental conditions (Yang *et al.*, 2018; Li et al., 2020). Although these studies provide valuable information, they have significant limitations since analyzing changes in controlled environment may not provide adequate representation of the processes occurring in a rapidly changing natural environment (Mittler, 2006; Zandalinas *et al.*, 2020). Harmonized regulation of resourse allocation between plant grows and defense is crucial for pant fitness nature and are primarily carried out by phytohormone crosstalk.

Controlling the balance between growth and defense

The activation of multifaceted immunity demands nutrient resources to support the biosynthesis of defense metabolites; such resource utilization frequently suppresses normal growth and development. To ensue such trade-offs between growth and defense, plants control carbon fluxes between primary and specialized metabolism (Cheynier *et al.*, 2013). Radiolabeling studies showed that biotic stress alters normal metabolic flux of carbon or nitrogen to allow the integration of these resources into defense specialized metabolites (Engelsdorf *et al.*, 2013; Ullmann-Zeunert *et al.*, 2013). The molecular mechanisms controlling this trade-off involve a vast number of different factors including hormones such as salicylic acid (SA) and jasmonic acid (JA) (Wasternack and Strnad, 2019; Ali, 2020). Biotic stress responses are carried out either by systemic acquired resistance (SAR) or induced systemic resistance (ISR), depending on the site of induction and the nature of the pathogen (Vlot *et al.*, 2020). SAR triggers a broad-spectrum of disease resistance in uninfected tissues in response to a local infection, which plays a crucial role in balancing growth and immunity of the plant (van Butselaar and Van den Ackerveken, 2020). Accessibility of carbon was shown to be critical for SA-regulated defense. Previous work on a starch-free mutant of Arabidopsis showed delayed production of SA-regulated camalexin, resulting in increased susceptibility to the hemi-biotrophic pathogen *Colletotrichum higginsianum* (Engelsdorf *et al.* 2013).

In addition to SA, N-hydroxy-pipecolic acid (NHP), a lysine derivative, was shown to act as another key signal for SAR in diverse plants (Chen *et al.*, 2018; Hartmann and Zeier, 2018; Homes *et al.*, 2019).

Chen *et al*. (2018) reported that NHP can be detected in an O-glucosylated conjugated form (NHPG) upon biotic stress suggesting that its activity might be at least in part controlled by the sugar substitution. Overaccumulation NHP in Arabidopsis leaves results in a dwarf phenotype that correlates with constitutive defense responses in Arabidopsis. In contrast, overaccumulation of NHPG displays more vigorous growth phenotype but hypersusceptibility to pathogen infection (Cai *et al.*, 2021). Recent work by Cai *et al.* (2021), highlighted the importance of the NHP to NHPG ratio in the growth-defense trade-off. According to their model, at a certain NHP threshold level, UGT76B1 glycosylates NHP attenuating its activity and decreases SAR response, thus reestablishing normal plant growth and development (Figure 3).

In contrast to SAR, ISR response is predominately regulated by JA (Van der Ent *et al.*, 2009). In response to necrotrophic pathogens or phytophagous insects, JA biosynthesis is increased leading to production of an array of defense compounds, along with a strong growth inhibition (Havko *et al.*, 2016). Previous work in *N. attenuata* showed the function of the JA-signaling pathway is re-allocation of nitrogen from RuBisCO into nicotine and phenolamide compounds during plant-herbivore interactions (Ullmann-Zeunert *et al.*, 2013). JA signaling activates the biosynthesis of several classes of specialized metabolites including nicotine, artemisinin, terpenoid indole alkaloids, withanolides and SGAs (Chen *et al.*, 2019; Wasternack and Strnad, 2019; Goklany *et al.*, 2013; Sharma, *et al.*, 2019, Min *et al*., 2020). In contrast, constitutively active JA signaling leads to suppressed plant growth (Campos *et al.*, 2016; Guo *et al.*, 2018; Major *et al.*, 2020). The coordination of chemical defense and plant growth is primarily achieved through crosstalk between JA and gibberellic acid (GA) signaling pathways. It involves MYC2, the JA signaling repressor JAZ and the GA response repressor DELLA (Navarro *et al.*, 2008) (Figure 3). Hong *et al.* (2012) reported that in Arabidopsis upregulation of DELLA sequesters the JA responsive MYC2 protein and this results in transcriptional suppression of the *TPS21* and *TPS11* sesquiterpene synthase genes. Loreti *et al.* (2008) found that JA signaling positively and GA metabolism negatively regulate anthocyanin biosynthesis in Arabidopsis. In *N. attenuata* a pair of calcium-dependent protein kinases (CDPK4 and CDPK5) were found to regulate specialized metabolism by suppression of JA biosynthesis, and at the same time modulating plant growth through interaction with core GA biosynthetic enzymes (GA20-OXIDASE and possibly GA13-OXIDASE) (Heinrich *et al.*, 2013). Additionally, Machado *et al.* (2013; 2017) demonstrated that herbivore induced nicotine biosynthesis in *N. attenuata* led to strong growth suppression, while GA complementation rescued plant growth. In tomato, the JA perception *jai1* mutant possesses low SGAs levels (Abdelkareem *et al.*, 2017).

Schubert *et al.*, (2019) showed that the tomato *jai1* mutant has lower JA, but higher GA levels as compared to wild-type plant.

Arabidopsis *della* mutants exhibited elevated GA response, but reduced JA level (Cheng *et al.*, 2009). Pauwels and Goossens (2011) suggested DELLA-mediated modulation of JA responses in Arabidopsis following competition with MYC2 for JAZ binding. In Arabidopsis, low GA levels allows DELLA to bind with JAZ consequently enabling MYC2 mediated transcription of JA response genes (Hou *et al.*, 2010). JAZ proteins inhibit MYC2 activity not only by recruiting corepressors but also by blocking the binding of MYC2 to target promoters (Pauwels and Goossens, 2011). In Arabidopsis, MYC2 was shown to transcriptionally regulate the expression of one of the *DELLA* gene (*RGL3*) to modulate plant growth (Wild *et al.*, 2012) (Figure 3). Additionally, Yang *et al.*, (2012) reported that active JA signaling interfere with GA-mediated DELLA degradation in Arabidopsis. Wang *et al.* (2020) identified diverse DELLA proteins throughout the plant kingdom that are possibly responsible for restricting plant development. JA-mediated growth suppression was likely oversimplified as most of the focus was on DELLA-dependent growth inhibition (Yang *et al.*, 2012; Davie`re and Achard, 2016; Jang *et al.*, 2020). However, recently Major *et al.* (2020) demonstrated that in Arabidopsis, JAZ mutations inhibit plant growth likely independent of DELLA. Thus, the molecular mechanism of JAZ-MYC2-DELLA-mediated JA-GA crosstalk and regulaton of the growth *vs*. defense tradeoff remains controversial.

Apart from MYC2, other TFs such as MYB21, MYB24 and PHYTOCHROME INTERACTING FACTORS (PIFs) were shown to interact with DELLAs, and might play a role in the crosstalk between the JA and GA signaling pathways (De Lucas *et al.*, 2008; Feng *et al.*, 2008; Hong *et al.*, 2012; Li *et al.*, 2016; Pham *et al.*, 2018; Huang *et al.*, 2020). In Arabidopsis, PIFs were found to regulate the JA-GA crosstalk either by reducing JA-Ile biosynthesis or by competing with DELLA for JAZ binding, consequently modulating MYC2 activity (Li *et al.*, 2016; Pierik and Ballaré, 2020; Fernández-Milmanda *et al.*, 2020) (Figure 3). In a recent study, isoprene (i.e. 2-methyl-1,3-butadiene) was found to pair GA-mediated growth regulation and JA-mediated defense responses in *Eucalyptus globulus* by the modulation of JAZ, MYC2, DELLA and PIFs (Zuo *et al.*, 2019). In transgenic poplar, heterologous overexpression of Arabidopsis *DELLAs* lead to compromised plant growth, due to reduced carbon flux through lignin metabolism (Busov *et al.*, 2006; Ribeiro *et al.*, 2012).

Apart from the JA-GA crosstalk, JA interrelates with the biosynthesis of growth-promoting brassinosteroids (BRs; Ren *et al.*, 2009). Kim *et al.* (2013) reported that JA treatment can represses

transcript accumulation of DWARF4, a key BRs biosynthesis enzyme, in a CORONATINE INSENSITIVE 1 (COI1)-dependent manner. Previous reports demonstrated that two BRs responsive transcription factors BRASSINAZOLE-RESISTANT 1 (BZR1) and BRI1-EMS-SUPPRESSOR 1 (BES1) can interact with DELLA, PIF and MYB proteins, hence modulating Arabidopsis glucosinolate metabolism (Guo *et al.*, 2013; Bruyne *et al.*, 2014; Liao *et al.*, 2020). Taken together, –hormone signaling cascade coordinately modulates biosynthesis of plant specialized metabolietes and cosequently suppresses plant growth (Figure 3).

The role of specialized metabolites in the regulation of the plant defense *vs.* growth tradeoff

Hormone-mediated regulation of the defense *vs.* growth tradeoff has been studied extensively (Huot *et al.*, 2014; Guo *et al.*, 2018). Yet, specialized metabolites and/or their breakdown products were also found to participate in the regulation of plant development and might take a direct part in balancing such tradeoff. Malinovsky *et al.* (2017) showed that 3-hydroxypropylglucosinolate fine-tunes the target of rapamycin pathway in Arabidopsis to suppress root growth. Although the exact target of 3-hydroxypropylglucosinolate is still unknown, indole-3-carbinol, a breakdown product of glucosinolate was reported to bind the auxin receptor TRANSPORT INHIBITOR RESPONSE 1, and regulate auxin-mediated root growth. Furthermore, the Arabidopsis auxin repressors (auxin-responsive protein; IAA5, IAA6, and IAA19) were found to regulate 4-(methylsulfinyl)butylglucosinolate (4-MSO; glucoraphanin) level and consequently control stomatal aperture (Salehin *et al.*, 2019). Khokon *et al.*, (2011) demonstrated the role of the glucosinolate breakdown product allyl isothiocyanate in stomatal closure through ROS production. In addition to stomatal closer, 4-MSO was found to regulate the circadian clock and flowering time in Arabidopsis by transcriptional regulation of circadian genes (Kerwin *et al.*, 2011).

Apart from glucosinolates, flavonoids are also known to regulate plant growth by modulating auxin transport. The Arabidopsis *transparent testa 4* mutant possesses elevated auxin transport and increased ROS levels in the guard cells, which can be recovered by the addition of naringenin, a flavonoid precursor (Murphy *et al.*, 2000; Brown *et al.*, 2001). Several studies demonstrated the mechanistic link between flavonoid biosynthesis, auxin transport, ROS accumulation and plant growth (Peer and Murphy, 2007; Santelia *et al.*, 2008; Hernández *et al.*, 2009; Muhlemann *et al.*, 2018). According to Erb and Kliebenstein (2020), flavonoids could be the possible signaling molecules as they are reported to change the oxidative state of cells by ROS activation which can modify the disulfide bridge formation

between partner proteins of cell signaling. In addition to flavonoids, *β*-carotene derived *β*-cyclocitral is known to regultate root stem cell behavior in diverse plant species (Dickinson *et al.*, 2019). Recently Mitra *et al*. (2021) found that herbivor interaction in Arabidopsis induces *β*-cyclocitral-mediated volatile signal which elevates plant resistance but inhibits the MEP pathway that is significant for plant growth. The direct involvement of specialized metabolism in growth suppression and the growth-defense tradeoff is not yet clear and needs to be critically examined.

CONCLUDING REMARKS

The concept of secondary metabolism was first described by Albrecht Kossel, Nobel Prize winner for physiology and medicine in 1910 (Jones, 1953). From that time, the advancement of analytical, genetic and molecular tools facilitated understanding and manipulation of specialized metabolite production in a large number of plant species. A large body of information was collected with respect to biosynthesis, transport and regulation of different classes of specialized metabolites. There are a few reports from Baldwin and his colleagues (Zavala and Baldwin, 2004; Zavala *et al.*, 2004) that assessed the fitness costs and benefits in tobacco under control as well as during herbivory event. Baldwin and colleagues showed that the plants lacking trypsin protease inhibitors (TPIs) were more vigorous compared to the TPI-producing genotypes, indicating the high costs of TPI production to the plant (Zavala and Baldwin, 2004; Zavala *et al.*, 2004). However, during herbivory the benefits of TPI biosynthesis are becoming higher that the costs (Zavala *et al.*, 2004). However, additional studies are necessary to investigate the role of TPIs in the control of primary metabolism and plant fitness. For instance, it was shown that PIF factors regulate specialized metabolism in addition to light-regulated development, but do specialized metabolites have any effect on modulation of the plant photosynthetic capacity? Additionally, numerous reports describe the “growth-defense tradeoff”, predicting the plant ‘budget’ for specialized metabolites. Yet, it is difficult and currently impossible to integrate the ‘cost’ of multiple stresses imposed constantly on plants in an everchanging environmnet.

A promising approach to induced resistance in agriculture is achieved by administering 4-fluorophenoxyacetic acid, a synthetic plant strengthner, that induces formation of flavonoid polymers in plant cell walls suppressing the sap-sucking insect population and therefore increasing the yield without compromising plant growth (Wang *et al*., 2020). However, from a breeder’s point of view, the ultimate goal of research into the “growth-defense tradeoff” and budget allocation, is to develop cultivars that can be simultaneously high yielding and resistant to a broad spectrum of plant’s natural enemies. Neverthless, the

current research points to mutual exclusiveness of growth and defense, thus, it seems difficult to identify a "soft spot" and obtain plants that would be optimized for sustainable agriculture.

DATA AVAILABILITY

No experimental data is associated with this review.

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AUTHOR CONTRIBUTION

SP, YK, AA wrote the manuscript.

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Figure legends

Figure 1. Mechanisms to reduce costs of specialized metabolites production by controlling biosynthesis and avoiding self-toxicity of intermediates and final products. A, Metabolon formation facilitates rapid channeling of reaction products between catalytic sites of enzymes, allowing their quick turnover to more stable and less toxic metabolites without damaging cells. Schematic representation of the *Sorghum bicolor* metabolon involved in dhurrin biosynthesis (Laursen *et al.*, 2016). It is comprised of two cytochrome P450 isoforms (CYP79A1 and CYP71E1), NADPH-dependent cytochrome P450 oxidoreductase (POR) and a soluble glycosyltransferase (UGT85B1). **B,** In *N. tabacum*, nicotine biosynthesis enzymes are localized in roots, and nicotine is transported via the xylem to leaves and acts as an insecticide. **C,** Sequestration to the vacuole was reported for several groups of specialized metabolites. Known exporter and importer proteins and their target molecules are shown. *Cj*, *Coptis japonica*, *Cr*, *Catharanthus roseus*, *Nt*, *Nicotiana tabacum*, *Sb*, *Sorghum bicolor*, *Sl*, *Solanum lycopersicum*. Additionally, ER-associated vesicular transport to the vacuole is depicted for the alkaloid sanguinarine produced by *Papaver somniferum.* **D,** Spatial separation of potentially self-toxic metabolites and the enzymes that catalyze metabolite breakdown to toxic products. Upon wounding and herbivore attack damaged cells containing enzymes and substrates join and release toxic defense compounds. The glucosinolate–myrosinase defense system is found in plants of the *Brassicaceae* family. Glucosinolates and myrosinases, their hydrolytic enzymes (specific class of *β*-thioglucosidases), are stored in separate cells in intact tissues. Upon tissue disruption, myrosinases are encounter and act on glucosinolate substrates, forming an unstable aglycone, that, depending on pH and other conditions is converted to nitriles and potent products. ESP, epithio specifier protein. For additional information, see Rask *et al.*, (2000). **E**, In *C. sativa* toxic cannabinoids are produced in the storage cavity of glandular trichomes.

Figure 2. Biological activation, detoxification and recycling of the cyanogenic glycoside dhurrin in *Sorghum bicolor*. Bioactivation. In response to wounding or herbivore attack, dhurrin is hydrolyzed by a specific *β*-glucosidase, dhurrinase (DHR), forming an unstable cyanohydrin, which releases toxic HCN, either spontaneously or mediated by the enzyme hydroxynitrile lyase (HNL). **Detoxification.** HCN is detoxified by formation of *β*-cyanoalanine (by *β*-Cyanoalanine synthase; CAS) that can be converted to Asn, Asp and ammonia by heteromers of nitrilases A and B (NIT4A/B). **Recycling**. In Dhurrin, either spontaneouly or catalyzed by an unknown glutathione transferase (GST), glucose is replaced by a glutathione moiety. This results in formation of a glutathione conjugate of *p*-hydroxyphenyl acetonitrile (GS-pOHPACN). It is next cleaved by GSTs converting reduced GSH to its oxidized form (GSSG) and releasing GS-pOHPACN. NIT4

heteromers catalyze hydrolysis of *p*-hydroxyphenyl acetonitrile to *p*-hydroxyphenylacetic acid and free ammonia that can be reincorporated into the primary metabolite pool. For additional information, see Bjarnholt *et al.*, (2018).

Figure 3. A schematic representation of the crosstalk between hormone signaling pathways and their roles in chemical defense *vs* plant growth strategies. Transcriptional activation is highlighted in green, while inhibition in red. Broken lines indicate indirect manipulation of processes or unknown mechanisms. [GA, gibberellic acid; JA-Ile, jasmonate-isoleucine; SA, salicylic acid; BR, brassinosteroid; COI1, CORONATINE INSENSITIVE 1 receptor protein; JAZ, JASMONATE ZIM DOMAIN proteins; GID1, GIBBERELLIN INSENSITIVE DWARF1 receptor protein, SLY1, SLEEPY1; PIFs, PHYTOCHROME INTERACTING FACTOR proteins; BZR1, RASSINAZOLE-RESISTANT 1 protein; BES1, BRASSINAZOLE-RESISTANT 2/BRI1-EMS-SUPPRESSOR 1 protein; HY5, LONG HYPOCOTYL 5; PAP1, PRODUCTION OF ANTHOCYANIN PIGMENT1; NPR1, NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1; NHP, N-hydroxy-pipecolic acid; NHPG, O-glucosylated N-hydroxy-pipecolic acid]. The GLYCOALKALOID METABOLISM 9 (GAME9) TF is known to work synergistically with MYC2, to transcriptionally activate SGA biosynthetic genes (Cárdenas *et al.*, 2016). Similarly, anthocyanin pigment 1 (PAP1)/MYB75 from Arabidopsis is known to transcriptionally activate anthocyanin biosynthesis genes (Shin *et al.*, 2015).