Trends in Plant Science

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Opinion

Ménage à Trois: Unraveling the Mechanisms Regulating Plant–Microbe–Arthropod Interactions

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Plant–microbe–arthropod (PMA) three-way interactions have important implications for plant health. However, our poor understanding of the underlying regulatory mechanisms hampers their biotechnological applications. To this end, we searched for potential common patterns in plant responses regarding taxonomic groups or lifestyles. We found that most signaling modules regulating two-way interactions also operate in three-way interactions. Furthermore, the relative contribution of signaling modules to the final plant response cannot be directly inferred from two-way interactions. Moreover, our analyses show that three-way interactions often result in the activation of additional pathways, as well as in changes in the speed or intensity of defense activation. Thus, detailed, basic knowledge of plant–microbe–arthropod regulation will be essential for the design of environmentally friendly crop management strategies.

Plant–Microbe–Arthropod Interactions and Their Relevance for Plant Health

Plants are central players in complex food webs, with numerous organisms relying on the resources of plants. These plant-associated organisms, including microbes and arthropods, influence plant performance significantly, and determine productivity in agroecosystems [1–4]. Not surprisingly, research on plant–arthropod and plant–microbe interactions has become one of the central topics in plant biology.

Insects, representing the most species-rich group of arthropods, comprise around 6 million species, half of which are herbivorous [5]. The diversity of pathogenic plant microbes is less characterized, but their threat to plants is equally renowned [6]. Besides parasitic interactions, plants establish mutualistic relationships with a plethora of organisms. Those include pollinators, which are attracted to flower volatiles; natural enemies of attacking herbivores, such as predators and parasitoids that are attracted to **volatile organic compounds (VOCs)** (see Glossary) emitted by herbivore-challenged tissues; and beneficial microbes [7,8] found in the **rhizosphere** and **phyllosphere** [9].

Plants often simultaneously or sequentially interact with both microbes and arthropods (Figure 1). The response of plants to either of these threats can substantially change their suitability as a host plant for the other attacker [10]. For instance, plant-associated microbes can change the quality of plants for herbivores by altering plant phenology, morphology, physiology, and chemistry [11–14]. Notably, beneficial microbes can improve plant health and induce resistance against a broad range of pathogens and pests [9,15–17] either directly as antagonists or indirectly by fine-tuning the **plant immune system** to prime plant defenses [18,19]. Likewise, insect

Highlights

Plant-microbe-arthropod (PMA) interactions have important impacts on plant fitness, and recent studies shed light on how plants regulate responses in such complex interactions.

Biosynthetic pathways for the production of defensive and signaling compounds, and the corresponding signaling modules (mostly related to phytohormones) are key regulators both in interactions of the plant with either microbes or arthropods (two-way interactions), or when exposed to both (PMA; three-way interactions).

Most signaling modules regulating two-way interactions of plants with microbes or arthropods also operate in three-way PMA interactions, but changes in their speed or intensity (e.g., defense priming) and/or activation of additional pathways frequently occur.

These differences shape the outcome of PMA interactions and may have implications for ecologically based crop protection.

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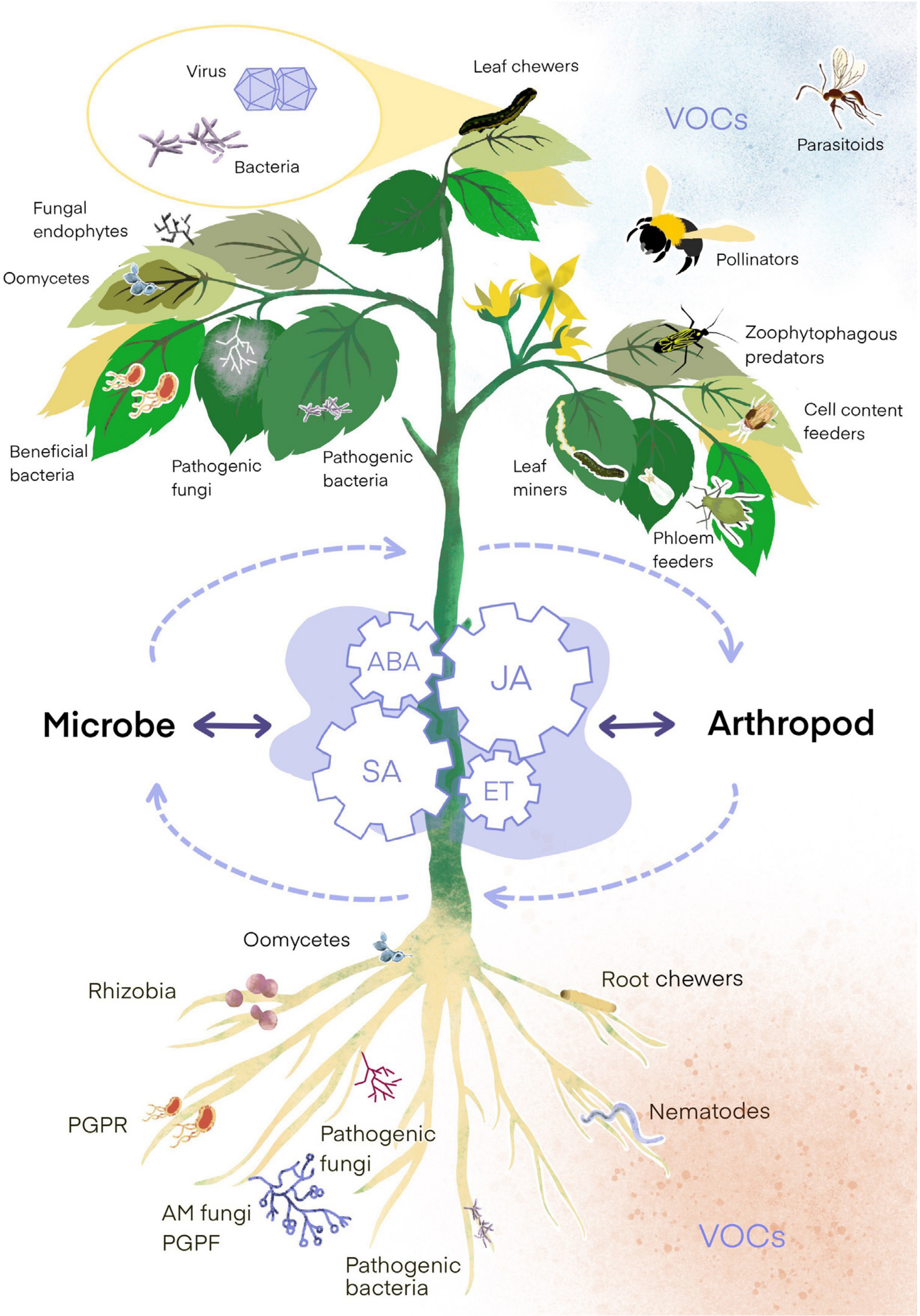

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Figure 1. Illustration of Multiway Interactions between Plants, Microbes, and Arthropods (PMA) and the Main Signaling Pathways Orchestrating the Corresponding Plant Responses. Plants must fine-tune their molecular responses to the interaction with a plethora of organisms with different lifestyles. Microbes and arthropods interact and can alter each other's effects on plant health through their modulation of plant responses. Continuous arrows represent the two-way interactions between the plant and the microbe or the arthropod. Discontinuous arrows represent the three-way PMA interactions. Major signaling pathways coordinating plant responses during two-way and PMA

(Figure legend continued at the bottom of the next page.)

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herbivores may impact plant-associated microbial communities by affecting the abundance, accessibility, suitability, and chemistry of the host plant tissue for microbes [20–22]. Such effects may even cascade up and down multiple trophic levels, impacting **multitrophic webs** in ecosystems [14,23,24].

Plant responses in two-way interactions with either microbes or arthropods have been well characterized. These rely on the recognition of interacting organisms and specific activation of immune signaling and the related defense arsenal [25]. This arsenal is diverse, including physical barriers leading to cell wall reinforcement, such as callose accumulation, and the production of repellent, toxic, or digestibility-reducing volatile and nonvolatile compounds and lytic enzymes [25]. Plant immune signaling is regulated by small signaling molecules leading to a network of interconnected pathways, where the **phytohormones** jasmonic acid (JA) and salicylic acid (SA) have key regulatory roles [26]. Other hormones, such as ethylene (ET), abscisic acid (ABA), cytokinins (CK), auxins (AUX), and gibberellins (GA), can interact with the JA–SA backbone in the orchestration of plant defenses [27,28]. Thus, the plant immune system is based on a highly flexible and complex **signaling network**. This flexibility allows plants to integrate multiple signals from their environment into an adaptive response that optimizes plant functions [29]. Only recently have studies begun to unravel how such responses are regulated in more complex three-way PMA interactions [10].

Here, we synthesize current information on plant-defense mechanisms driving PMA three-way interactions to develop a conceptual model of the plant-signaling pathways mediating such tripartite interactions. We identify major regulatory modules and common mechanistic patterns guiding these complex interactions. In addition, we identify and discuss major bias sources and knowledge gaps and provide guidelines for future research.

Mechanism Database for PMA Interactions: Biases in Studied Biological Systems

We generated a database compiling the available information on mechanisms shaping PMA interactions as described in the Material and Methods in the supplemental information online. Briefly, we searched for relevant scientific articles in publicly available databases using specific keywords, consulting experts through the international network COST Action FA1405, and through citation snowballing. The reference list was filtered to include only publications providing experimental evidence for the mechanisms, and the information was structured as shown in Tables S1 and S2 in the supplemental information online. When inspecting the overall data structure, we identified two major sources of bias. The first was related to the taxonomic diversity of the interacting organisms, and the second to the methodological approaches used. Regarding the taxonomic bias, we found mechanistic studies of multiway interactions for plants belonging to nine plant families. Most of the studies (64%) involved two families, Solanaceae (mostly *Nicotiana* and *Solanum* sp.) and Brassicaceae (mostly *Arabidopsis* sp. and *Brassica* sp.), followed by studies involving Poaceae and Fabaceae. The microbes studied were grouped into three functional groups: (i) beneficial microbes, including **plant growth-promoting rhizobacteria or fungi** (**PGPR/PGPF**, see Glossary for full definition); we considered **arbuscular mycorrhizal fungi**

interactions are represented, namely volatile organic compounds (VOCs), jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), and ethylene signaling (ET). Major groups of arthropod and microbe lifestyles are illustrated by particular examples, microbes on the left side of the figure, arthropods on the right. The third trophic level (**indirect interaction**) is also represented by parasitoids and predators of arthropods. The insert represents arthropod-associated microbes impacting the arthropod interaction with the plant. Drawing by J. Lidoy, V. Lidoy, and J. Lidoy. Abbreviations: AM fungi, arbuscular mycorrhizal fungi; PGPF, plant growth-promoting fungi; PGPR, plant growth-promoting rhizobacteria.

Glossary

Arbuscular mycorrhizal fungi (AMF): soil fungi that established an ancient symbiotic association with plant roots with important benefits for plant performance. They are obligate biotrophs and the interaction (called mycorrhiza) is finely regulated.

Defense priming: preconditioning of the plant immune system leading to an activation of an 'alert state' that prepares the plant for more efficient activation of defenses upon attack by potentially deleterious organisms or abiotic challenges (usually through faster/stronger defense responses).

Indirect interactions: interaction in which one organism affects another organism through changes in the properties of a third organism, such as a shared host plant.

Multitrophic webs: food webs involving organisms from different trophic levels.

Parasitoid/predator: natural enemies of phytophagous arthropods. They negatively impact their hosts by laying their eggs in or on the host body followed by host consumption by the developing larvae (parasitoid) or by directly preying on them (predator).

Phyllosphere: leaf surface or total aboveground surfaces of a plant when referred to as a habitat for microorganisms.

Phytohormones: molecules produced in plants that, when perceived in extremely small concentrations by the corresponding receptors, produce physiological effects locally and in distal parts of the plant.

Plant growth-promoting rhizobacteria or fungi (PGPR/PGPF): bacteria or fungi, usually located in the rhizosphere that improve plant growth by improving their nutrition and/or their resistance to biotic and abiotic stress.

Plant immune system: defense system comprising many biological structures and processes to protect the plant against diseases. It comprises elements for the detection/recognition of other organisms and signaling networks that modulate plant responses, shaping plant interactions with other organisms.

Rhizosphere: plant–soil interface that comprises the narrow region of soil that is directly influenced by root exudates; represents an enriched habitat for microorganisms that closely interact with the plant.

(AMF) separately from other PGPF due to their specific relationship with the plant and their high representation in the database [30]; (ii) pathogenic microbes (fungi, bacteria, and viruses); and (iii) arthropod-associated microbes that influence the plant–arthropod interaction (Figure 1). Beneficial microbes were most frequently studied in PMA interactions, with AMF ranking first, followed by PGPR and other PGPF [mostly *Trichoderma* and *Piriformospora* (now *Serendipita*)]. Remarkably, in some families (Fabaceae, Vitaceae, Plantaginaceae, Salicaceae, and Fagaceae), AMF were almost the only microbes studied in a three-way context. There was a clear focus on three insect orders, Lepidoptera ranking first, followed by Hemiptera and Coleoptera, mostly including herbivorous insects. A limited number of studies considered interactions with beneficial insects, such as **parasitoids/predators** of herbivorous arthropods. Among the beneficial arthropods, parasitoids were more frequently studied than predators (Table S1 in the supplemental information online).

Biases in Methodological Approaches

Some studies reported conflicting information for the same set of interacting organisms. This can arise from differences in the experimental setup (Box 1) or from the methodologies applied. To address the latter source of bias, we compared the outcomes of studies using **targeted and untargeted methodologies**, since the analysis of untargeted methodologies is agnostic to the researchers' assumptions.

From the 86 publications analyzed, 34 used untargeted methodologies. Regarding metabolite detection, VOCs are generally analyzed through untargeted methodologies, while targeted methodologies are used for phytohormone analyses. Thus, the comparison between targeted and untargeted analysis was not possible for these groups of metabolite. Therefore, targeted and untargeted analyses were compared only for transcriptional data. Untargeted transcriptomic studies were not biased towards specific processes, while targeted ones usually focused on molecular markers of major stress-related pathways. Only Brassicaceae and Solanaceae families had sufficient numbers of mechanistic studies to merit an objective targeted–untargeted approach comparison. Contrary to our expectations, untargeted methods did not reveal additional processes. For example, more hormonal signaling pathways were reported in targeted than in untargeted studies in Solanaceae (Figure S1 in the supplemental information online). To test whether all significant changes were reported in untargeted studies, we reanalyzed microarray and RNA-seq transcriptomics studies in these two families [three arabidopsis (*Arabidopsis thaliana*) and one potato (*Solanum tuberosum*) study]. We used the Wilcoxon Sum Rank test implemented in MapMan [31] to obtain the lists of regulated pathways and compared them with the pathways reported. Most of the pathways significantly altered according to our test were highlighted in the publications (Table S3 in the supplemental information online). We conclude that, at the transcriptomics level, the major pathways triggered in three-way interactions are already known, and are well covered by targeted approaches allowing more precise quantifications. Thus, the transcriptomics methodology applied does not appear as a major source of bias regarding the pathways regulating PMA interactions. Nonetheless, the –omics approaches reveal new elements in those pathways and can shed light on the mechanisms governing their crosstalk (e.g., [32]).

Can We Predict Mechanisms in Multiway Interactions based on Two-Way Interactions?

The overview of the mechanisms responding in two- and three-way interactions shows that the complexity of responses increases in the three-way interactions, because more mechanisms were reported in three-way compared with two-way interactions in 56% of the studies (Figures 2 and 3, and Figures S2–S5 in the supplemental information online). To compare the

Signaling module: part of the signaling network that is tightly interconnected.

Signaling network: network of molecular components that transmits the signal from the environment to the cell function.

Systems biology: studies of biological systems using a holistic approach through the combination of biological experimental techniques and mathematical modeling.

Targeted/untargeted methodologies: techniques focusing on the analysis of molecules (metabolites, nucleotides, or proteins) either predetermined (targeted, when looking for particular molecules) or untargeted (when looking for a range of unknown molecules).

Volatile organic compounds (VOCs): low-molecular-weight organic compounds that remain in the vapor phase at environmental temperatures. They can have important roles in chemical communication between organisms.

Box 1. Guidelines for Experimental Design: Treatments, Minimal Standards for Controls, and Information Needed

Appropriate experimental setups are essential to reach robust conclusions on the molecular mechanisms governing PMA interactions. Here, we highlight some key aspects to take into account.

Appropriate Controls

To identify differential regulation in three-way versus two-way interactions, it is crucial to determine the changes occurring in each individual interaction. The basal state of the host must also be determined. Thus, multiway studies should include four treatments: plants alone ('control'); plants interacting with the microbe (O1); with the arthropod (O2); and interacting with both (O1O2) (Figure I).

Moreover, control treatments should be carefully considered for each experimental system to avoid misleading results derived from the inoculation method or accompanying microbes [57].

Order of Challenge

The order of the interactions is relevant for the final outcome and the operating mechanisms [58]. Therefore, interaction timing should be determined depending on specific research questions.

Age–Developmental Stage of the Interacting Organisms

Plant responses are age/developmental stage dependent [59–61]; hence, the stage must be described for the plants under study. The arthropod developmental stage is also important. Arthropod instar, age, as well as density should be clearly stated. Similarly, the type and concentration of the microbe inocula and stage of the interaction should be taken into consideration.

Plant Growth Conditions

Plant responses to biotic interactions are highly context dependent. Environmental factors, such as light quality and intensity, temperature, humidity, nutrient, and water availability, can influence the interaction outcome. Hence, these parameters should be described in detail.

Plant Organs/Tissues to Sample

Plant organs act both in an autonomous and coordinated way in response to biotic stimuli. For example, root or shoot responses to the same stimuli may differ substantially. Responses can be local, can appear in distal, not-treated tissues (systemic responses), or both. Details regarding the anatomical and developmental characteristics of the plant material sampled for analysis may be crucial for the interpretation of the results.

Timing of Sampling

The plant integrates multiple signals leading to early and transient signaling events clearly differentiated from medium or late sustained responses. For example, dynamic changes in hormone content occur during infectious processes. Therefore, time-course experiments are informative to understand plant responses in multiway interactions.

Quantifying Responses

The speed and intensity of plant defense responses are essential for their efficiency. In fact, defense priming appears to be a common mechanism. This can only be addressed through quantitative analysis comparing the intensity of the response in the three-way versus two-way interactions.

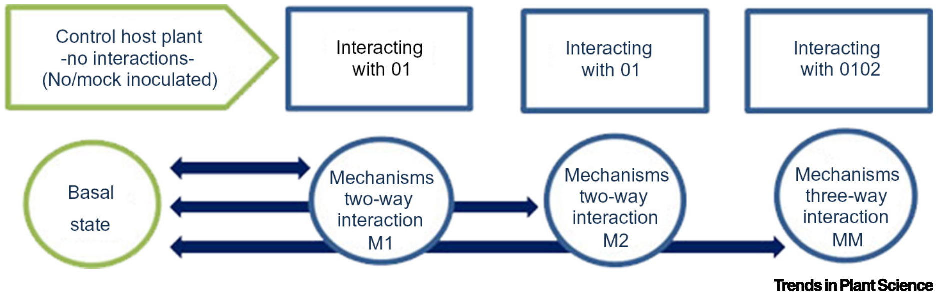

Figure I. Overview of Optimal Experimental Design to Study Mechanisms Shaping Plant–Microbe–Arthropod (PMA) Interactions.

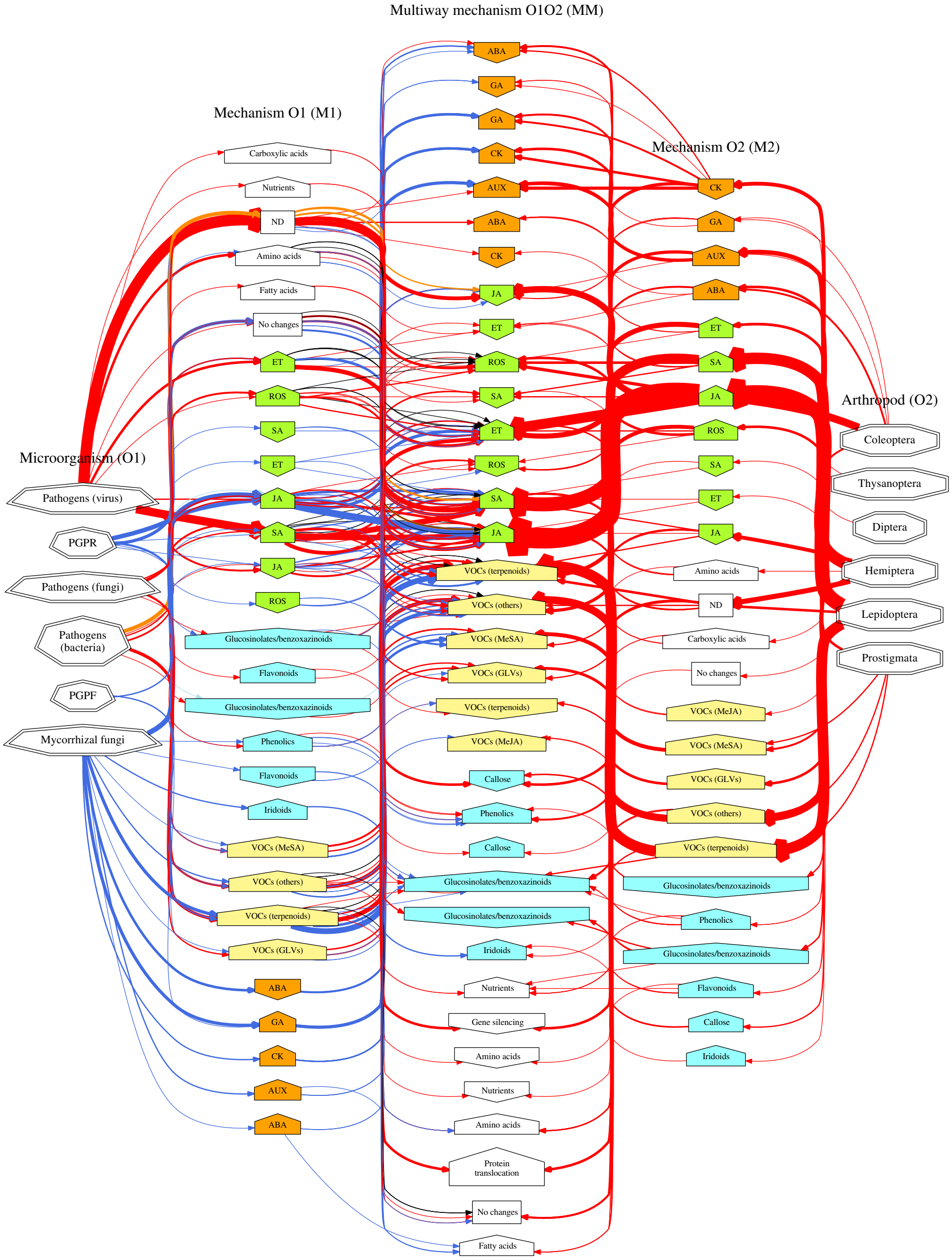

Figure 2. Overview of Reported Mechanisms Responding in Plants when Exposed to Different Microbes (Left), Arthropods (Right) or to a Combination of Both (Center). Heptagons, octagons, and pentagons are used to represent microorganisms, arthropods, and interaction mechanisms as graph nodes, respectively. On the left-hand side, the microbes included in the experiments are shown (O1), grouped according to their functional and higher taxonomy level, and linked with an arrow with the nodes representing the mechanisms that respond in the plant when exposed to the microorganism (M1). On the right-hand side, a similar representation is given for the taxonomically structured arthropods (O2) and the plant response to the infestation (M2). In the center, the nodes (MM) represent the mechanisms responding in plants when exposed to both organisms (O1O2). Coloring of the mechanism nodes reflects their function: yellow, volatile organic compounds (VOCs); orange, growth-related hormones; green, stress-related hormones; and light blue, secondary metabolism. O1O2 nodes are linked to both O1 and O2 nodes if the information comes from the same experimental

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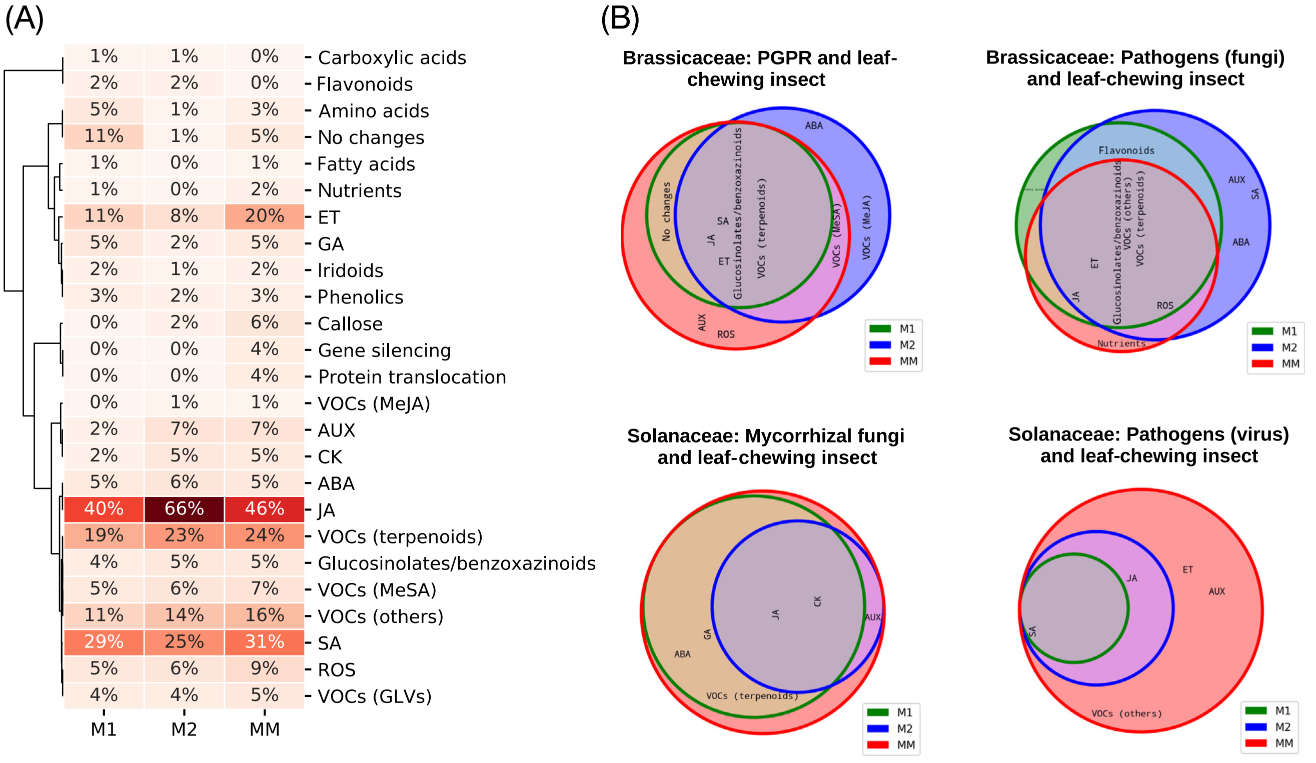

Trends in Plant Science

Figure 3. Mechanisms Regulating Plant Responses when Exposed to Multiple Organisms Are More than the Sum of the Responses in Two-Way Interactions. (A) Heatmap representing the frequency of a given mechanism in publications analyzing Plant–Microbe–Arthropod (PMA) interactions. Percentages of articles reporting changes in individual mechanisms during two-way and three-way interactions among the total number of articles compiled are given. The mechanisms are clustered using cosine distance and complete linkage. For heatmaps of individual families, see Figure S4 in the supplemental information online. (B) Comparison of mechanisms triggered when plants of different families encounter leaf-chewing insects and beneficial or harmful microbes of the same taxonomic group. Euler graphs were generated from information in the database (Table S1 in the supplemental information online). Abbreviations: ABA, abscisic acid; AUX, auxins; CK, cytokinins; ET, ethylene; GA, gibberellins; GLV, green leaf volatiles; JA, jasmonic acid; M1, mechanisms triggered in plants interacting with the microbe; M2, mechanisms triggered in plants interacting with the arthropod; MeJA, methyl jasmonate; MeSA, methyl salicylate; MM, mechanisms triggered when plants interact with both organisms; PGPR, plant growth-promoting rhizobacteria; ROS, reactive oxygen species; SA, salicylic acid; VOCs, volatile organic compounds. The complete set of Euler diagrams is available in Figure S7 in the supplemental information online.

diversity of processes triggered in PMA interactions, we counted the overall number of pathways responding during the plant interaction with either the microbe or the arthropod alone or when exposed to both organisms. The diversity of responding pathways in the three-way interactions increased by 25% and 41% compared with the plant responses to microbes and arthropods alone, respectively (Figure 2). The regulation of JA, SA, and ET signaling pathways, as well as the production of VOCs or glucosinolates, had a dominant role in three-way interactions. Upregulation of the JA signaling pathway and, to a lesser extent, the SA and ET pathways,

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system. Blue and red arrows indicate an interaction with an organism that is either beneficial or harmful to the plant, respectively. Light blue represents neutral effects and orange unknown effects on the plant (mostly insect symbionts that were not tested in direct interaction with the plant). The width of the arrow is proportional to the number of studies with the given observation. The up- and downregulation of the mechanism represented in a node is depicted by the shape pointing either upward (upregulation) or downward (downregulation). For the full names of the mechanism nodes, see Table S1 in the supplemental information online. Abbreviations: ABA, abscisic acid; AUX, auxins; CK, cytokinins; ET, ethylene; GA, gibberellins; GLV, green leaf volatiles; JA, jasmonic acid; MeJA, methyl jasmonate; MeSA, methyl salicylate; ND, mechanisms not determined; PGPF, plant growth-promoting fungi; PGPR, plant growth-promoting rhizobacteria; ROS, reactive oxygen species; SA, salicylic acid.

was the most common molecular responses reported in the three-way interactions (Figures 2 and 3). The diversity of processes regulated in three-way interactions was not just the sum of processes regulated in each of the two-way interactions. Processes not regulated in two-way interactions can respond when the plant is exposed to multiple interactors. For example, modulation of ET and AUX signaling was detected only in the three-way potato–Colorado potato beetle–potato virus Y interaction (*Solanum tuberosum*–*Leptinotarsa decemlineata*–*Potato virus Y*) [32]. Additionally, the responses of the pathways triggered in two-way interactions also differed in their strength when the plant was exposed to multiple organisms. For example, JA signaling was induced less when Arabidopsis was exposed to *Pieris rapae* and *Botrytis cinerea* compared with *Pieris rapae* alone [33].

A noticeable node in the two-way interactions was 'not determined' (ND) (Figure 2, and Figure S2 and Table S1 in the supplemental information online), illustrating the extent of missing two-way controls in three-way studies (Box 1). Out of 32 studies involving bacteria, 31% and 13% did not report the mechanism of the two-way interaction with the microbe or the arthropod, respectively. Similarly, 22% of the studies involving fungi did not describe the mechanisms operating in the two-way plant–fungus interaction. For viruses, 39% of the studies lacked information about their interaction with the plant. This hinders the generation of predictive models on three-way PMA interactions based on two-way interaction studies.

The analysis of the available data revealed a stronger influence of the response to the insect than to the microbe in the three-way interaction (Figure 3, and Figures S4 and S6 in the supplemental information online). To determine the influence of either the microbe or the insect on the three-way interactions, we compared the overlap in the processes triggered in all possible combinations of insect feeding style, microbe type, and plant family (26 combinations when we took into account only the four most studied families). In 46% of the studied combinations, the overlap of processes triggered in three-way interaction and the interaction with the insect only was higher than the overlap of processes triggered by the microbe alone. By contrast, the influence of the microbe was stronger in only 11% of the studied combinations (Figure S7 in the supplemental information online).

Integrated analysis also showed that plant responses reported in three-way interactions were different when considering harmful or beneficial microbes. Considering harmful microbes, SA signaling was almost exclusively induced in the case of viruses, while reactive oxygen species (ROS), JA, VOCs, or secondary metabolites were also stimulated by fungal or bacterial pathogens. A more diverse set of signaling pathways was reported during interactions with beneficial microbes (Figure 2, and Figures S2 and S6 in the supplemental information online). Interestingly, induction of CK, GA, and AUX and downregulation of ABA were only recorded in three-way interactions with beneficial microbes, while induction of ABA and ROS were only recorded in entries from harmful interactions. These patterns appear to be in agreement with plant growth-related effects of beneficial microbes and activation of stress responses in deleterious interactions [26,34]. The remaining mechanisms were induced by both beneficial and harmful interactions, but likely with different timings and intensity of response.

Beneficial microbes, such as PGPRs and PGPFs, not only promote growth, but can also induce **defense priming** [19,35–38]. For example, tomato plants colonized by the mycorrhizal fungi *Funneliformis mosseae* were more resistant to chewing caterpillars through primed accumulation of JA and JA-regulated defenses in response to the herbivore [39,40]. Defense priming was evaluated in 43% of the studies compiled here and, interestingly, priming was confirmed in almost 50% of those, highlighting its relevance in three-way interactions (Tables S4 and S5 in the supplemental information online). Most of the studies addressing priming dealt with beneficial microbes (73%). Regarding arthropods, chewers were the most abundant category (65%). From the studies confirming priming, the most abundant combinations were AMF or PGPR with chewers (45%), followed by beneficial microbe–nematode interactions (28%). Even in some studies not addressing priming, the data provided pointed to primed defenses in the three-way interaction (e.g., [41,42]).

We also considered studies (ten in total) including arthropod-associated microbes (endosymbionts/entomopathogens) and plants (Figure 1 and Figure S5 in the supplemental information online). They encompassed Prostigmata and three insect orders (Coleoptera, Lepidoptera, and Hemiptera) on the arthropod side, and three plant families (Solanaceae, Rosaceae, and Poaceae). These interactions could not be conclusively evaluated due to the insufficient number of studies, but again, phytohormone pathways (JA, CK, and SA) were triggered in the three-way interactions, with JA signaling being the pathway most consistently reported (Figure S5 in the supplemental information online).

Going towards Systems Biology

Studies of multiway interactions have mainly focused on the qualitative evaluation of a particular process in the plant response and the signaling pathway activated (the so-called '**signaling module**'). The combination of signaling modules elicited in each specific interaction differs in composition, magnitude, and timing [43], leading to specific signal signatures that can eventually benefit either the plant or the attacker. Indeed, several studies noted that the same arrays of genes are activated in compatible and incompatible plant–microbe interactions, but that they differ in the timing and intensity of the response [44–49]. This also occurs during three-way interactions: For example, pre-infestation of *Arabidopsis* plants with larvae of *P. rapae* delayed the induction of ET and SA signaling and ROS responses when the plant was infected with *B. cinerea*. Additionally, the strength of the repression of GA signaling was higher [49]. Thus, data collected at only one time point, or lacking quantitative evaluation, may be misleading. So far, a few experiments have addressed the dynamics of plant responses quantitatively and with high time resolution, but only during the interaction with a single pathogen [50] or by triggering a specific signaling module [51,52]. The results of these studies revealed several waves of gene expression triggered even within a 24-h period, indicating the precise control of the response dynamics, likely shaping the specificity of the response. Thus, disentangling the complexity of the immune signaling network will be crucial to understand the dynamics and quantitative properties of the system. However, most studies analyzed plant responses in multiway interactions at only one time point. Consequently, the changes in network properties cannot be discerned from the existing data sets. The temporal aspect is receiving increasing attention and, thus, more accurate estimations of network properties are expected in the future. Future experiments should be carefully designed to take into account, if possible, precise quantification and the time component, while considering limitations in both laboratory space and budget (Box 1). Some new approaches may help, such as that reported by La Manno and coworkers [53] for inferring dynamics of gene expression from single time-point data by comparing the ratios of unspliced and spliced transcript counts.

A common approach to studying mechanisms regulating interactions is the use of plant mutant/genetically modified lines. Although such a reductionist approach is powerful in building base knowledge hypotheses and testing them, it is difficult to discern gene function in a complex network solely from such studies. The behavior of the system may depend heavily on complex interactions between components within it [54,55]. Thus, we recommend combining different approaches to identify small differences relevant to the network responses. Ideally, they should include untargeted analysis, quantitative analysis of selected components, and carefully designed functional experiments including mutant/genetically modified/edited lines.

Additionally, plant responses are coordinated at different levels, from cellular to tissue and organ responses. Different parts of the plant are usually exposed to different organisms and, while systemic responses in the plant are common, this response in distal tissues differs from local responses [56]. However, this aspect was only partially covered in the studies reported so far.

Concluding Remarks and Future Perspectives

Understanding plant responses to environmental factors and interactions with multiple organisms is crucial for biotechnological improvement of plant resilience and, consequently, to achieve efficient and sustainable crop management practices. Our synthetic review reveals that the information on the molecular mechanisms governing plant interactions with other organisms is still fragmented, and that further systematic studies are required to understand the regulation of plant responses. We found important literature biases regarding studied organisms and experimental designs, so that some mechanisms perhaps remain undiscovered. Taken together, drawing conclusions on the mechanisms involved in multiway interactions is more complex than expected. Nonetheless, our analysis points to phytohormone modules as major regulatory hubs in both two-way and three-way interactions, but the responses are fine-tuned in both timing and strength when plants are exposed to multiple interactors. Improved predictions will require **systems biology** approaches that merge mathematical modeling with experimental data sets encompassing the dynamics of the responses (see also Outstanding Questions).

Outstanding Questions

Are there specific plant responses or network patterns shaping three-way interactions?

If the same responses/mechanisms are activated in three-way and two-way interactions, are timings and intensity of response different?

How should future studies be designed to improve the precision of PMA outcome predictions?

How do changes in environmental conditions impact PMA? What are the mechanisms mediating such impacts?

Are the current scientific approaches adequate to reveal the complexity of the plant signaling system in these multiplayer interactions? Will new 'omics technologies, such as single cell or microscale spatial 'omics, in combination with continuous monitoring techniques and mathematical modeling of responses, produce a significant advance in our understanding of the mechanisms involved in shaping three-way interactions?

What are the contributions to PMA regulation of the less explored levels of regulation, such as noncoding RNAs, DNA modifications, protein post-translational modifications, and protein–protein interactions?

Can the results of laboratory- and greenhouse-based three-way interaction studies be extrapolated to natural and agroecosystems?

Are studies of three-way interactions a significant advancement over two-way studies for improving microbial inoculants for agroecological applications?

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Author Contributions

K.G., M.J.P., J.L., M.P., V.F., K.K.P., and M.L.P contributed to the conception and design of the study; J.L., V.P., M.P., K.G., and M.J.P. retrieved data and performed analyses; K.G., M.J.P., J.L., M.P., V.F., K.K.P., M.L.P., A.M.M., and A.B. contributed to the interpretation of data; K.G., J.L., M.P., V.F., K.K.P., M.L.P., A.M.M., and M.J.P. drafted the manuscript; and E.B. and A.B. revised it. All authors approved the submitted version and agreed both to be personally accountable for their own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even those in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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