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Review

Driving factors of epiphytic bacterial communities: A review

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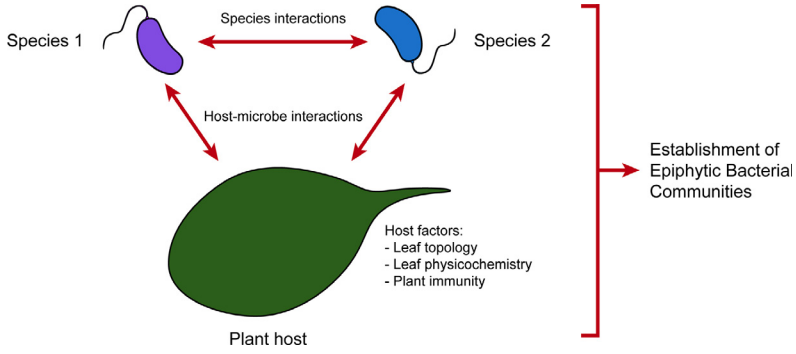
HIGHLIGHTS

- The physicochemistry of leaves is unique and is a major driver of leaf colonisation.
- Competition and cooperation may be major drivers of bacterial colonisation.
- Leaves respond to bacterial colonisation locally and systemically.
- How leaf responses shape bacterial colonisation patterns is unclear.
- Plant-microbe interaction should be studied at the micrometer resolution.

GRAPHICAL ABSTRACT



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ABSTRACT

Bacteria establish complex, compositionally consistent communities on healthy leaves. Ecological processes such as dispersal, diversification, ecological drift, and selection as well as leaf surface physicochemistry and topology impact community assembly. Since the leaf surface is an oligotrophic environment, species interactions such as competition and cooperation may be major contributors to shape community structure. Furthermore, the plant immune system impacts on microbial community composition, as plant cells respond to bacterial molecules and shape their responses according to the mixture of molecules present. Such tunability of the plant immune network likely enables the plant host to differentiate between pathogenic and non-pathogenic colonisers, avoiding costly immune responses to non-pathogenic colonisers. Plant immune responses are either systemically distributed or locally confined, which in turn affects the colonisation pattern of the associated microbiota. However, how each of these factors impacts the bacterial community is unclear. To better understand this impact, bacterial communities need to be studied at a micrometre resolution, which is the scale that is relevant to the members of the community. Here, current insights into the driving factors influencing the assembly of leaf surface-colonising bacterial communities are discussed, with a special focus on plant host immunity as an emerging factor contributing to bacterial leaf colonisation.

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Introduction

All the aboveground surfaces of a plant that represent microbial habitats are referred to as the phyllosphere [1]. In particular, leaf surfaces host a dense population of bacteria (i.e., epiphytes) esti-

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mated to reach 10^7 bacteria per cm^2 of leaf surface [2]. Despite the high cell density, leaf surfaces are a challenging ecosystem to colonise and grow on. Epiphytes must cope with constant ultraviolet (UV) radiation exposure, low water and nutrient availability and large temperature fluctuations throughout the day, making leaves an extreme environment [3].

Recent culture-independent sequencing methods have shown that leaves host bacterial communities that are compositionally consistent within a plant species [4–6]. However, little is known about the factors that shape these communities. Although there is increasing evidence that non-pathogenic leaf-colonising bacteria may stimulate plant growth and provide protection against different stresses [7–14], the functions of most of these bacteria, their dynamics at the community level, and their interactions with the plant host remain largely unknown.

The leaf surface

The leaf is a highly structured and multi-layered plant organ (Fig. 1). Its microtopography is determined by the first cell layer, namely, the epidermis, which consists of different cell types that regulate many aspects of leaf physiology, such as gas exchange, temperature regulation, and water and secondary metabolite secretion [15].

The most common cell type in the epidermis is the pavement cell, which contributes to leaf shape. Within the layer of pavement cells, more specialised epidermal cell types are embedded [15]. Stomata, which are pores formed by two guard cells that act as turgor-driven valves to regulate gas exchange and transpiration, are an important feature of the epidermis [16]. Some plants develop modified stomata called hydathodes, which are pores found at the end of the vasculature on leaf margins [16]. Because these structures cannot regulate their pore aperture, hydathodes maintain a continuous pathway for water and solute secretion, a process known as guttation [17]. Another type of specialised epidermal cell are outgrowths called trichomes, which are either glandular or non-glandular [15]. Glandular trichomes are secretion organs that release a wide spectrum of exudates, such as polysaccharides, salts, lipids, volatile compounds, and proteins, the functions of which are associated with plant-plant, plant-insect and plant-microbe interactions [18,19]. The functions of non-glandular trichomes may include water retention and absorption, light reflection to reduce the impact of UV radiation and heat, and increased freezing as well as drought tolerance [18].

The epidermis is covered by a cuticle, i.e., a waxy layer that provides a physical barrier against abiotic and biotic stresses and determines the physicochemical properties of the leaf surface. The cuticle is formed by an extracellular polymer membrane composed of a matrix of cross-linked polyhydroxy fatty acids and glycerol called cutin. This matrix is interspersed with polysaccharides and a complex mixture of long-chain aliphatic compounds, which are overlaid on and/or impregnated in the matrix (cuticular waxes) [20]. Aliphatic compounds render the cuticle hydrophobic and determine the physicochemical properties of the leaf surface, such as its permeability and wettability, which limits water and solute diffusion from inner cell layers to the leaf surface and the adherence of particles to the surface [21–23].

Impact of leaf topology and physicochemistry on microbial life on leaves

The organisation of leaf epidermal cell types defines leaf physiology and shapes an intricate microtopology that influences the distribution and abundance of microorganisms on the leaf surface [24–26]. The establishment of these microhabitats depends on the physicochemical properties of the leaf surface and the ability of microorganisms to adapt and modify this environment [27]. Epiphytes are often found in aggregates or biofilms, likely because these microenvironments protect bacteria from harsh environmental conditions [24,28]. Large bacterial aggregates have been predominantly found at the bases of trichomes, above veins, and in epidermal cell grooves [29,30], where water and nutrients are more prevalent.

The permeability and wettability of the leaf cuticle is likely to be one of the most important properties of the leaf surface that influences the ability of microbes to colonise this habitat [31]. Cuticular permeability determines the diffusion rate of compounds

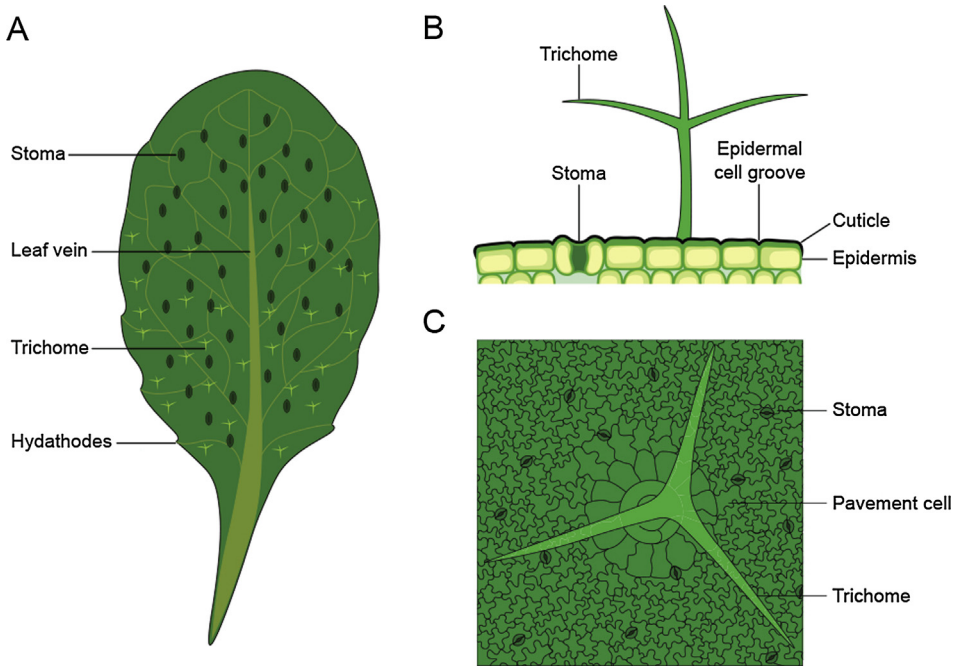

Fig. 1. (A) Representation of an *Arabidopsis thaliana* leaf and its main features. (B) Cross-section and (C) top-view representations of the leaf surface, including different epidermal cell types that make up the leaf's surface relief.

from the apoplast onto the leaf surface, while wettability influences the retention of water droplets on the leaf surface [22,32].

Permeation plays an important role in the growth and survival of epiphytes by allowing the leaching of water and compounds to the phyllosphere, making nutrients accessible for microorganisms. An aqueous pathway contributes to compound permeation across the cuticle with facilitation from aqueous pores preferentially found on cuticular ledges of guard cells, at the base of trichomes, and over the cuticle of anticlinal cell walls [33]. Sites on the leaf surface that are characterised by higher permeation rates are also more densely colonised by bacterial communities [34]. Bacteria can modulate cuticular permeability and wettability through the production of biosurfactants such as syringafactin, which is released by *Pseudomonas syringae* [31,35,36]. Increased cuticular permeability not only affects water diffusion but also alters sugar availability for sustained epiphytic growth [37]. *In situ* fructose availability to the leaf-colonising bacterium *Pantoea eucalypti* 299R (formerly known as *Erwinia herbicola* and *Pantoea agglomerans* [38,39]) in the bean phyllosphere was found in sites containing aqueous pores [32,40,41]. The patchy distribution of carbon sources on bean leaves promotes differentiation of the *P. eucalypti* population into subpopulations differing in access to fructose [40]. Thus, permeation of photosynthates across the cuticle is exploited by epiphytic microorganisms, allowing them to survive and thrive. Besides modulation of leaf physicochemistry by phyllosphere-associated microbes, changes in leaf chemistry can also be attributed to abiotic and biotic soil conditions [42–44]. However, the extent to which plant-soil feedbacks influence the assembly of phyllosphere microbial communities are yet to be determined.

The topography and physicochemical properties of leaves render the phyllosphere an oligotrophic and heterogeneous habitat for epiphytes. It is also possible for microorganisms to construct niches on the leaf surface in response to interaction with the plant [45]. Although discrete hotspots on the leaf form microhabitats in which bacterial populations can be sustained [46], the impact of these microhabitats on the assembly and establishment of bacterial communities is not yet understood.

Composition of phyllosphere-associated microbial communities

Advances in cultivation-independent methods and next-generation sequencing techniques have led to a better understanding of the composition and diversity of plant microbiota. Although plants host a wide variety of microbes, bacteria are far more abundant than eukaryotes and archaea [47,48]. Bacterial communities on plants are dominated by only four phyla: the Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes [1,49,50]. Although bacterial community composition and abundance are host specific, members of the Alphaproteobacteria are predominant and ubiquitous in phyllosphere microbiotas, and within this class, the genera *Methylobacterium* and *Sphingomonas* are consistently detected among different hosts [47]. Plant-colonising bacteria are thought to share mechanisms of adaptation to leaf surfaces, considering the high overlap between the proteome of phyllosphere microbiotas and the identification of a core set of genes potentially involved in adaptation to plant colonisation in over 3000 plant-associated bacterial genomes [47,51,52].

Spatially explicit ecology of bacteria on leaves

Due to the leaf's heterogeneous nature, the composition and abundance of bacterial communities at the whole-leaf scale are not sufficient to understand the drivers of community assembly [46]. Therefore, the importance of spatial information becomes increasingly apparent for understanding community structure (Fig. 2). In the example given in Fig. 2, the same community composition on a whole-leaf scale can be explained by different interspecies correlations such as different levels of co-aggregation, segregation, or random distributions (Fig. 2C, D, or E, respectively). Fluorescence *in situ* hybridisation (FISH), a method commonly used to visualise and identify microorganisms in their environment, has been used to describe the distribution patterns of taxonomically different bacterial groups within the phyllosphere [2]. This study, the first investigation of the spatial distribution of phylogenetically different taxa colonising leaf surfaces under different environmental conditions, estimated the likelihood of bacterial taxa co-aggregation in the *Arabidopsis thaliana* phyllosphere. Bacterial aggregates can be either monoclonal or polyclonal. Monoclonal aggregates represent the offspring of single cells, while polyclonal aggregates are formed by the aggregation of multiple cells in one location [53]. Distantly related taxa can form mixed aggregates, even though members of the same phylogenetic group have the highest probability of co-aggregation. However, due to the technical limitations of the FISH probes used in that study, it is unclear which individual species contribute to the observed aggregation patterns and whether co-aggregation of the same phylogenetic groups is a result of local monoclonal aggregate formation of an individual species or mixed populations. In general, aggregation between different taxa is observed at a distance of less than 5 μm. A similar approach taken to study the spatial distribution and colonisation patterns of two fluorescently tagged bacterial strains (*P. eucalypti* 299R and *P. syringae* B728a) on bean leaves provided similar results [26]. In an investigation of the spatial aggregation between the bacterial strains and topological features of the leaf, the strongest correlations were found between bacterial colonies and epidermal cell grooves within distances of up to 12 μm, and adjacent to glandular and non-glandular trichomes within 60 and 120 μm, respectively. Closer examinations of spatial relationships between bacterial species and their surroundings could shed light on the functional and metabolic diversity within aggregates and communities in the phyllosphere.

Individual bacterial cells can significantly change the concentration of solutes, such as nutrients in their environments, within a distance of approximately ten times their cell's diameter. This distance can be effectively interpreted as an "interaction distance", where fluxes of compounds and metabolites can diffuse from cell to cell [54]. This interaction distance of cells without direct physical contact is in good agreement with the observed scales of bacterial aggregation in the phyllosphere. Recently, cell-to-cell interactions on porous surfaces were found to be higher when the aqueous phase was fragmented, which increased the probability of direct physical interactions between cells [55]. These findings can be extrapolated to the leaf surface, as its segregated nature should result in a high prevalence of cell-to-cell interactions [56]. Evidently, community assembly on leaves is strongly determined by factors acting at a micrometre resolution [46].

Ecological drivers of bacterial community assembly

Recently, a framework of community assembly was proposed for microbial communities in the phyllosphere [57]. The framework proposes that community structure is driven by four main processes: (1) dispersal, (2) diversification, (3) ecological drift, and (4) selection (Fig. 2A). First, dispersal is the immigration of microorganisms onto the leaf surface, which can occur via seed inoculation, rainfall, animal transmission vectors, bud burst colonisation, and bioaerosols [57]. Second, the emergence of new genetic variation through evolutionary diversification may affect community diversity. As UV radiation and/or reactive oxygen species can

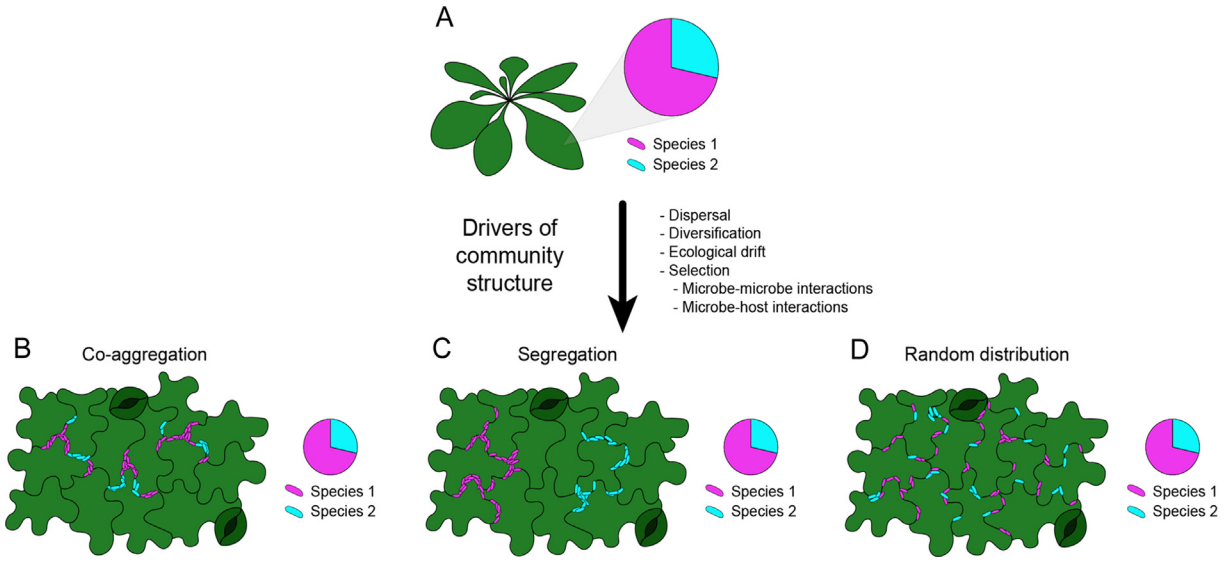

Fig. 2. Relevance of spatial patterns in bacterial community structure on the leaf surface. (A) Considering a plant host with a defined microbial composition and relative abundance of species 1 and 2, factors driving community structure, i.e., dispersal, diversification, ecological drift, and selection, can lead to different aggregation patterns of these species while maintaining the same bacterial diversity and abundance. (B) Bacterial community of species 1 and 2 with a strong spatial co-aggregation pattern influenced by, for example, cooperation, resource partitioning, stochastic processes, and/or priority effects (niche modification). (C) Bacterial community of species 1 and 2 with a strong spatial segregation pattern influenced by resource overlap-driven exploitation competition, antibiosis (interference competition), stochastic processes, and/or priority effects (niche preemption). (D) Bacterial community of species 1 and 2 showing a random distribution, which might indicate benign interactions between the species.

lead to increased mutation rates, low-abundance taxa might be a genetic reservoir for horizontal gene transfer; furthermore, bacteria can exhibit dormancy [58]. Third, ecological drift relates to changes in the abundance of taxa due to stochastic events. This process is assumed to have greater effects on low-abundance taxa, which may become extinct at local scales [59]. Lastly, selection is the deterministic fitness differences between species within a community, which can be due to internal and external determinants, such as species interactions and environmental factors, respectively.

Microbe-microbe interactions

In general, the effect of microbial interactions in shaping community structure can be divided into cooperation, parasitism and competition. Cooperation describes interactions that are beneficial to at least one species and do not cause harm to the other, while the latter type refers to interactions that are detrimental to at least one species. Mutualism is a type of cooperative interaction in which both species benefit from each other, while commensalism is an interaction between two organisms in which one partner benefits while the other is not impacted. Microorganisms would be commensals when, for example, one microbe produces non-metabolisable substrates and/or growth factors that positively affect the commensal [60,61]. However, to be considered a true commensal, the organism should not influence their interaction partner at all, which although theoretically possible, is highly unlikely in practice. The alternative term "tritagonist" has been proposed for these organisms instead [62]. The effect of cooperative interactions in shaping the structure of phyllosphere bacterial communities has not yet been investigated. However, an example of cooperation has been observed using synthetic communities from the maize rhizosphere, in which the removal of a keystone species led to the collapse of other bacterial populations [63]. Another kind of mutualism may occur between fast growing bacterial species and fungal pathogens infecting host plants, as the latter seemed to increase bacterial richness and diversity [64]. However, this depends on specific microbe-microbe interactions, as the fungal and oomycete species *Dioszegia* sp. and *Albugo* sp., respectively, have been shown to decrease the bacterial species diversity of the *A. thaliana* leaf microbiota [45].

Parasitism in the phyllosphere is mostly driven by virus-bacterium interactions. Bacterium-infecting viruses (i.e., bacteriophages or phages) are found in most (if not all) ecosystems and can alter community dynamics by influencing bacterial diversity, nutrient cycling, and species interactions [65]. Phages impose strong selection on bacterial members of the leaf microbiota at a local scale and in short time periods, affecting the microbiota composition [66,67].

Competitive relationships involve detrimental effects for at least one species, which may be a result of interference or exploitation competition. When competition is the result of active mechanisms of species exclusion, this interaction is known as interference. The most common example of interference is antibiosis, in which a species secretes compounds that are toxic to the other. This effect is also the case for epiphytes; for example, *P. agglomerans* E325 has been shown to suppress the growth of the phytopathogen *Erwinia amylovora* on apple flowers through antibiotic activity [13]. Exploitation competition is the dispute for shared resources, such as nutrients or space. In this relationship, one of the species has compromised population growth, resulting in either complete spatial exclusion or coexistence [68]. Fructose and sucrose, for example, are limiting resources during phyllosphere colonisation [40]. In bean leaves, the amount of available sugars on the leaf surface decreases rapidly to a tenth of the initial concentration upon colonisation of *Pseudomonas fluorescens* A506 [69]. The availability of sugars is also limited by its permeation from the apoplast to the leaf surface, and sugars may not be replenished at rates supporting the survival of large bacterial populations [40].

Effect of resource overlap on species interactions

Cooperative and competitive interactions greatly depend on the metabolic capabilities of the members of a microbial community, that is, the potential of the microbes to uptake, metabolise, and

secrete an array of nutrients and other compounds (e.g., siderophores or antimicrobials). The degree of shared nutritional requirements between species, i.e., nutrient or resource overlap, may shape microbial communities, as shown by the fact that nutrient overlaps in naturally occurring bacterial communities are higher than expected based on null models [70]. When estimating nutrient overlaps between over 6000 pair of bacterial species, Freilich et al. (2011) found a positive correlation with the competition potential between species in a community and negatively with the cooperation potential, suggesting that the higher the nutrient overlap between the species in a community is, the more likely these species are to compete for shared nutrients and, consequently, the less likely they are to cooperate with each other [71]. However, nutrient overlaps cannot solely predict the nature of species interactions e.g. it has recently been shown that close relatedness and similarity in gene expression between pairs of algal species, and thus similarity in their metabolisms, was correlated to facilitation, stabilising coexistence [72]. In addition, other factors such as priority effects can influence species interactions through, for example, niche preemptions or niche modifications [73]. Resource utilisation accounts for only a fraction of a species' niche; thus, the spatial context, environmental conditions, and biotic relationships should be considered when positioning species in their ecosystems.

An operational approach for comparing species' nutrient utilisation profiles is to determine a resource overlap index between pairs of species. In phyllosphere studies, Wilson and Lindow introduced the niche overlap index in 1994 [74], which relates to the number of carbon sources utilised by two species as a proportion of the total carbon sources used by one of the species. Two issues arise from this formulation: (1) the index is asymmetrical, such that one species can have total overlap while the other only partial overlap, as is the case for specialist and generalist species, respectively; and (2) by measuring only the ability of a strain to consume a carbon source independently, information is lost about the species' affinity for that resource, which may lead to false interpretation of the potential for two species to coexist based on their nutrient preferences. Instead, symmetrical indices that account for the proportions of utilised nutrients are more informative and less biased [75].

The major drivers of community assembly in the phyllosphere are currently unclear. As the phyllosphere is generally oligotrophic, cooperation and competition may have a major impact on community assemblies in the phyllosphere.

The host as a driver of bacterial community structure

Plants provide habitats that support different bacterial communities [4,76]. Although bacteria share a core set of adaptation mechanisms to colonise and thrive on plants [4,47,51], community composition is, to some degree, host specific [6,76–79]; thus, changes in bacterial diversity, abundance, and community structure can be attributed to host factors and specific bacterial adaptation mechanisms. For example, bacterial community composition is influenced by host species, genotype, plant traits (e.g., cuticle composition), leaf age and host developmental stage; the latter two factors are often indistinguishable from seasonal effects [25,79–84].

Plant responses to microbial colonisation

In addition to providing a habitat for microorganisms, plants interact with their associated microbiota. Recent findings suggest that the plant immune system plays a role in shaping the bacterial community [80,85], thereby indicating an active role of the host plant in modulating the composition of its associated microbiota.

The plant immune system consists of two layers. The first layer of immunity, pattern-triggered immunity (PTI), is elicited by conserved molecular structures such as microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) or damage-associated molecular patterns, which are perceived by plasma membrane-localised pattern recognition receptors [86,87]. A prominent example of pattern recognition receptors is the receptor flagellin sensing 2 (FLS2), which recognises a 22-amino-acid peptide of bacterial flagellin (flg22) [88]. The significance of MAMP/PAMP recognition in limiting pathogen growth was shown in a *fls2* mutant exhibiting enhanced susceptibility to the bacterial pathogen *P. syringae* pathovar (pv.) *tomato* DC3000 (*Pst* DC3000) [89]. Recently, comparison of plant responses to the growth-promoting rhizobacterium *Pseudomonas simiae* WCS417 and its cognate flg22 peptide showed that the whole organism elicited only about half of the plant transcriptional responses compared to the purified flg22 peptide alone. Genes that were only upregulated in the flg22-treated plants were enriched for defence-related transcriptional responses, suggesting that *P. simiae* WCS417 suppressed a significant number of defence-associated genes [90]. Whether plants recognise non-pathogenic bacteria and modulate their response to limit unnecessary costly defence are still unknown.

To subvert PTI, microbial pathogens release so-called effector proteins [91]. Effector proteins from different microbes target overlapping sets of plant proteins. Most of the targeted plant proteins are considered hubs of highly interconnected protein-protein interaction networks. Hence, targeting these proteins will likely result in strong perturbations of the host's immune response [92,93]. As a countermeasure, plants have evolved additional intracellular receptors, which are often nucleotide-binding leucine-rich repeat proteins (NLRs), that directly or indirectly recognise effector proteins, thus forming the second layer of plant immunity, designated effector-triggered immunity (ETI) [94,95]. Recently, a study on microbial genes important for adaptation to the plant environment identified 64 plant-resembling plant-associated and root-associated domains (PREPARADOs). Some PREPARADOs resemble NLR domains. This finding suggests that bacterial proteins containing PREPARADOs might compete with NLRs for effector binding, thereby restricting bacterial detection by the plant [51].

Plant responses are finely tuned

Although the same signaling networks seem to be employed during PTI and ETI, they are used in a different manner, generating specific outputs of immunity level. While the relationships between different signaling pathways in PTI are partly synergistic and partly compensatory, they are exclusively compensatory during ETI [96,97]. As MAMPs can be found on pathogenic as well as non-pathogenic bacteria, it seems reasonable that PTI is less robust or more tunable than ETI, allowing the host to avoid recurrent fitness costs [98]. Synergism between signaling pathways enables the plant to elevate the output of its immune response when multiple MAMPs, providing more information than a single MAMP, signal a pathogen.

Different MAMPs have been shown to activate different immune signaling pathways with varying strengths, leading to diverse immune outputs (Fig. 3) [99]. Such differential use of the immune signaling network likely allows plants to induce appropriate defence mechanisms against pathogens with different lifestyles during PTI. For example, salicylic acid signaling is known to be effective against biotrophs, pathogens that feed on living host tissue, and hemibiotrophs, pathogens that first feed on living host tissue and later feed on dead host tissue. In contrast, jasmonic acid and ethylene signaling is effective against necrotrophs, pathogens

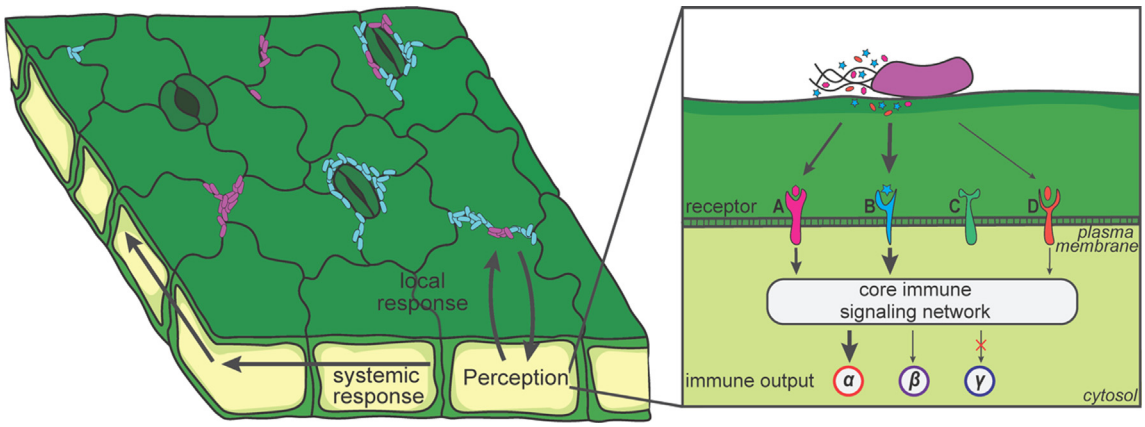

Fig. 3. Plant responses to bacterial colonisation. Leaf-colonising bacteria elicit local and systemic responses. As shown on the left-hand side, a cell type-specific response to prevent bacteria from entering the apoplast is stomatal closure. As shown on the right-hand side, plants perceive bacteria via receptors localised in the plasma membrane (A, B, C, D), which recognise microbe-associated molecular patterns (depicted by hexagons, ovals and stars around the bacterium). Downstream signaling of these receptors is integrated in a highly interconnected immune signaling network. Integration of varying receptor inputs leads to specific immune outputs (α, β, γ).

that feed on dead host tissue [100]. Furthermore, tunability of the immune network presumably enables plants to limit costly immune responses to non-pathogenic colonisers.

Recently, two non-pathogenic bacteria were shown to elicit unique transcriptional and metabolic responses that differed from those of a pathogenic bacterium, indicating that plants differentiate bacteria [7,101]. Since the availability of carbohydrates clearly affects bacterial community composition [102,103] and plants actively deprive the apoplast of monosaccharides upon pathogen encounter to limit pathogen growth [104], plants likely also supply certain carbohydrates by sugar exporters [105] to support beneficial bacterial populations. Such responses are likely to be spatially explicit, as the bacterial composition on the leaf is heterogeneous and the same carbohydrate might promote the growth of spatially separated populations of beneficial and non-beneficial bacteria. However, plant responses at a high spatial resolution are currently underinvestigated in the context of plant-microbe interactions. Local plant responses may have important implications for leaf surface-colonising bacteria and bacterial community composition.

Local and systemic plant host responses to microorganisms

Plant immune responses to pathogens can be divided into local and systemic responses (Fig. 3). Local immune responses at the infection site comprise early calcium ion (Ca^{2+}) influx and MAP kinase and calcium-dependent protein kinase activation within minutes, followed by reactive oxygen species production, defence gene activation and after several hours, callose deposition and hypersensitivity response resulting in programmed cell death, which is regarded as a hallmark of ETI [106]. A cell type-specific local response is stomatal closure, which occurs within an hour after bacterial recognition to restrict the bacteria from entering the apoplast (Fig. 3). However, some bacterial pathogens, such as *Pst* DC3000, are able to modulate stomatal aperture [107]. In addition to local responses, pathogens also elicit systemic responses, such as systemic acquired resistance, conferring broad spectrum resistance against biotrophic pathogens to plant parts that have not been in contact with the pathogen [108]. Recently, the impressively fast propagation of systemic Ca^{2+} signaling throughout the plant in *A. thaliana* (approximately 1 mm/s) was shown to be mediated by glutamate, which is released upon wounding [109].

Studying plant epigenetic, transcriptional, proteomic, and metabolomic changes in microorganisms at the whole-tissue level does not allow us to study local and systemic responses separately. Moreover, tissues are mixtures of different cell types that react differently to microorganisms. In a recent study, local responses to the biotrophic pathogen *Hyaloperonospora arabidopsis* were shown to differ markedly from systemic responses [110]. This finding highlights the importance of performing -omic studies at a high spatial (potentially single-host cell) resolution to identify the nature of plant responses. To perform cell type-specific and single-cell studies, the cell type of interest must be isolated from the surrounding tissue. Root hairs are likely the simplest cell type to isolate as they can be separated from frozen root tips by stirring with a glass rod [97]. Other cell types demand more sophisticated techniques for isolation; these types can be divided into two groups. Microscopy-assisted techniques such as capillary extraction [111], atomic force microscopy-based extraction [112] and laser capture microdissection [113] allow the isolation of cells or cell material with direct spatial context. With regard to plant-microorganism interactions, capillary extraction was used to study transcriptional changes of barley cells infected by powdery mildew [114]. Cell/nuclei sorting or affinity purification methods such as fluorescence-activated cell sorting of protoplasts [115], fluorescence-activated nuclei sorting [116], isolation of nuclei tagged in specific cell types [117], and "translating ribosome affinity purification" (TRAP) [118] allow the isolation of larger numbers of cells or more cell material. However, the spatial information is only indirect, and the techniques rely on cell type-specific markers.

Plants shape their associated microbiotas. Consequently, plants need to be considered as a driving factor of community composition in bacterial ecology studies. To study the role of the plant host in shaping microbiota, new controls must be found that distinguish between fast active plant signaling and comparably slow changes in physicochemical properties of leaves. To that end, studies can be performed on artificial plants, ranging from plastic tomato plants in a field context [119] to artificial leaves in the laboratory [120–123]. Such studies should progress to a more refined spatial scale, as bacterial colonisation is heterogeneous, plant responses are likely also heterogenous.

Conclusions and future perspectives

The relatively static physicochemistry of the leaf surface as well as dynamic microbe-microbe, microbe-plant and plant-mediated microbe-microbe interactions are drivers of plant microbiota community composition. These factors do not homogeneously influence the bacterial communities on leaves; instead, there might be markedly different communities on the same leaf. Such spatial

heterogeneity of leaf-colonising microbial communities is not only due to the heterogeneity of the leaf surface and heterogeneous microbial colonisation but also likely due to plant responses that may differ between individual cells of the same leaf. Since the plant immune network is highly tunable, it presumably enables plants to limit costly immune responses to non-pathogenic colonisers, and on a more refined spatial scale to respond differently to distinct microbial colonisers. Heterogeneous colonisation patterns are thus likely to translate into heterogeneous plant response patterns.

In the future, more emphasis should be placed on spatial heterogeneous differences in colonisation and plant responses to further our understanding of the underlying mechanisms that drive bacterial community composition and assembly. By resolving (1) the effect of microbe-microbe interactions, (2) the function of non-pathogenic bacteria on the plant and (3) the role of the host in shaping community structure, will give bottom-up insights into the intimate interplay of plant hosts and their bacterial communities.

Conflict of interest

The authors have declared no conflict of interest.

Compliance with Ethics requirements

This article does not contain any studies with human or animal subjects.

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