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The importance of the microbiome of the plant holobiont

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Summary

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Plants can no longer be considered as standalone entities and a more holistic perception is needed. Indeed, plants harbor a wide diversity of microorganisms both inside and outside their tissues, in the endosphere and ectosphere, respectively. These microorganisms, which mostly belong to Bacteria and Fungi, are involved in major functions such as plant nutrition and plant resistance to biotic and abiotic stresses. Hence, the microbiota impact plant growth and survival, two key components of fitness. Plant fitness is therefore a consequence of the plant *per se* and its microbiota, which collectively form a holobiont. Complementary to the reductionist perception of evolutionary pressures acting on plant or symbiotic compartments, the plant holobiont concept requires a novel perception of evolution. The interlinkages between the plant holobiont components are explored here in the light of current ecological and evolutionary theories. Microbiome complexity and the rules of microbiotic community assemblage are not yet fully understood. It is suggested that the plant can modulate its microbiota to dynamically adjust to its environment. To better understand the level of plant dependence on the microbiotic components, the core microbiota need to be determined at different hierarchical scales of ecology while pan-microbiome analyses would improve characterization of the functions displayed.

I. Introduction

The association between plants and microorganisms is known to be ancient, and arbuscular mycorrhizal (AM) mutualism is believed to have played a key role in the terrestrialization process and to have accompanied the evolution and diversification of plant photo-trophs (Selosse & Le Tacon, 1998; Heckman *et al.*, 2001). The

community of mycorrhiza colonizing a plant has been extensively studied over several decades. However, it was only in 2002 that the diversity of fungi colonizing the plant root was shown to be much greater than previously believed (Vandenkoornhuyse *et al.*, 2002), indicating a gap in our knowledge concerning microorganisms colonizing roots. Over the last few years, research into plant microbiota (i.e. the diversity of plant-associated microorganisms

within the so-called plant microbiome) has progressed significantly. Analysis of the plant microbiome involves linking microbial ecology and the plant host's biology and functioning, and viewing microorganisms as a reservoir of additional genes and functions for their host. Even if, at first sight, the interaction might appear to be symptomless, the additive ecological functions supported by the plant microbiome are acknowledged to be a major trait extending the plant's ability to adapt to many kinds of environmental conditions and changes (Bulgarelli *et al.*, 2013), which is of primary significance in view of the sessile lifestyle of plants. Deciphering the various types of interaction between plants and their microbiomes is a hot topic for research in ecology as well as in plant sciences and agronomy. The use of molecular approaches based on high-throughput sequencing is dramatically extending our knowledge of plant microbiome diversity. Nevertheless, our understanding of the role of the plant microbiome, in terms of ecology and function, remains limited, although analytical studies of the interactions between plants and microorganisms have proliferated in recent years (Kiers *et al.*, 2011; Werner *et al.*, 2014). We are thus only just beginning to comprehend the ecological functions of the plant microbiome. The upshot of this better understanding will have substantial impacts on a variety of research investigations and applications, for example possible innovations for crop production.

We present herein a synthesis of recent research on the plant microbiome and current prospects. We first focus on knowledge of the aboveground and belowground compartments of the plant microbiome, clarifying, in the latter case, the distinction between epiphytes and the rhizosphere. We discuss differences between these plant compartments and related differences in microbiome composition and function, together with the advantages and limits of the molecular tools used to study the plant microbiome. We also explore novel theoretical and empirical ideas to better comprehend the interactions occurring between the plant and its microbiome. We limit this synthesis to the holobiont definition, the host organism and all its symbiotic microbiota (Margulis, 1993; Knowlton & Rohwer, 2003; Gordon *et al.*, 2013) (Fig. 1). The pathogenic fraction of the plant microbiome, including bacteria, oomycetes, other protists and viruses, is not addressed in this review, although its inclusion from a holistic perspective would make sense. Where necessary, references to comprehensive reviews on specific aspects are provided.

II. Plants as holobionts

Decades of research have demonstrated the importance of microorganisms in plant health. The difficulty of culturing transplants of different species in the absence of bacteria and fungi is widely known (Hardoim *et al.*, 2008), which strongly implies the importance of such microorganisms in plant growth. It is readily acknowledged that cooperative microbial symbionts play an important role in their host's life and fitness (Kiers & van der Heijden, 2006). Thus, a plant can be regarded as a holobiont comprising the host plant and its microbiota (Zilber-Rosenberg & Rosenberg, 2008) (Fig. 1). The concept of holobiont requires a collective view of the functions and interactions existing between a macroorganism host and its associated microorganisms (i.e. a single

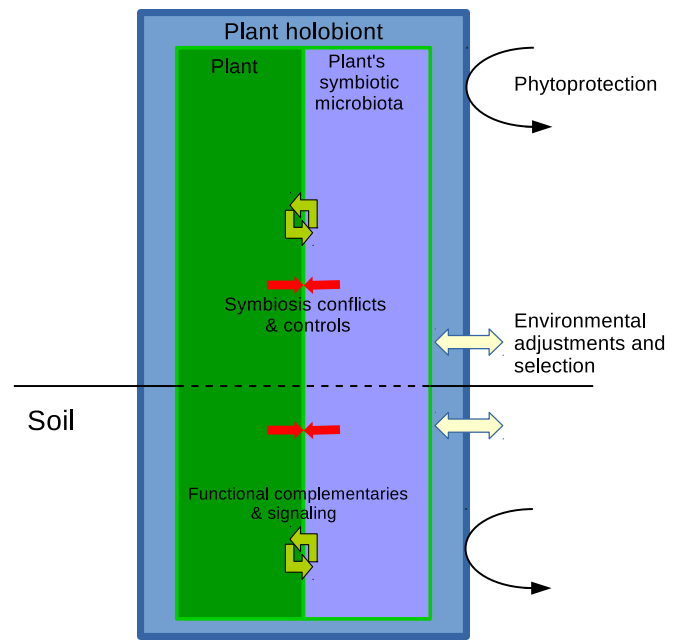


Fig. 1 Scheme of the plant holobiont and related key interaction aspects both in term of evolution and functioning.

dynamic entity). The holobiont (i.e. the host genome and associated microbiome) can be seen as 'the genomic reflection of the complex network of symbiotic interactions that link an individual of a given taxon with its associated microbiome' (Guerrero *et al.*, 2013). This supposes a novel comprehension of evolution acting on the entire holobiont, the holobiont being the unit of selection processes and adaptation (Zilber-Rosenberg & Rosenberg, 2008) (Fig. 1). Under this theoretical framework, holobiont evolution could lead to variations in either the host or the microbiotic genomes (Zilber-Rosenberg & Rosenberg, 2008). In addition, related implications of the holobiont concept also need to be examined.

- The observed heterogeneity in the plant microbiome within a given organ is the consequence of adaptation processes and adjustments to given environmental conditions of the holobiont and allows rapid buffering of environmental changes (see Section III). Short-term responses related to modifications of the symbiotic microbial community (i.e. plant microbiota) can therefore be expected.
- Heterogeneity in the symbiotic community could be related to transitory associations while other microbiotic components might form long-lasting interactions and involve vertical transmission through seeds (Cankar *et al.*, 2005) and/or pseudo-vertical transmission (Wilkinson, 1997). If it exists, the plant 'core microbiome' (see Section IV) must be functionally significant for the plant holobiont.
- The diversity of the endospheric microbiota should be limited by the plant's innate immune defense system (see Section V) and at the same time the plant microbiota also contribute to the immune system by producing antimicrobial compounds and eliciting plant defense mechanisms (Berendsen *et al.*, 2012). Coordination of these two co-occurring components of plant defense is assumed to occur (see Section V).

- From an evolutionary perspective, in the context of symbiosis conflicts, there must be mechanisms within the plant holobiont that allow fine-tuning of host and symbiont behaviors. The sorting mechanisms of the cooperators need to be efficient to maximize plant holobiont fitness (see Section V).

III. Recruitment of the plant microbiota: what are the driving factors?

We can hypothesize that interactions between plants and soil microorganisms lead to the gradual enrichment of a subset of soil microorganisms in the defined continuum of habitats (or compartments) that extends from the bulk soil to the root internal tissues.

1. The soil, a 'seed bank' for root microbiota

Root-associated microorganisms are mainly recruited from the surrounding soil. The root microbiota are strongly influenced by the composition of the soil microbial species pool that is present in the vicinity of the roots. Several studies, using high-throughput amplicon sequencing, have demonstrated the strong connection between the soil bacterial communities and root-associated bacteria in *Arabidopsis thaliana* (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012; Schlaeppi *et al.*, 2014). Also, the importance of local environmental parameters, notably soil properties, as determinants of soil microbial community composition and root microbiota has been demonstrated in various studies (Shakya *et al.*, 2013; Schreiter *et al.*, 2014). Soil microbial community structure and the associated environmental parameters appear to be more important drivers of root-associated bacterial community structure than plant genotype or species (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012; Shakya *et al.*, 2013; Schlaeppi *et al.*, 2014). However, a plant's evolutionary history can significantly influence the formation of root-inhabiting bacterial assemblages when different cultivars or species or distinct genotypes of plants are grown in the same soil (Manter *et al.*, 2010; Ofek *et al.*, 2013; Bouffaud *et al.*, 2014). Similarly, the structure of fungal communities is determined more by soil origin than by plant host species (Bonito *et al.*, 2014).

2. The rhizosphere, a 'growth chamber'

At the periphery of the roots, the rhizosphere constitutes the first plant-influenced habitat encountered by soil microorganisms. This thin layer of soil that surrounds roots is profoundly influenced by plant metabolism through the release of oxygen and secretion of a highly complex array of exudates including not only carbon-rich molecules that can be used as energy sources by microorganisms but also antimicrobial compounds. Overall, this makes the rhizosphere between soil and roots a highly dynamic environment and a differentiation of microbial communities has been shown to occur accordingly (Peiffer *et al.*, 2013; Schreiter *et al.*, 2014). For example, the 'recruitment' in the rhizosphere of fungi with antagonistic activity toward the soilborne plant pathogen *Verticillium dahliae* has been reported (Berg *et al.*, 2005). A recent

work elegantly shows that differences between microbial communities are explained by an enrichment of microbial functional capabilities in the rhizosphere/rhizoplane which is dependent on the plant species (Ofek-Lalzar *et al.*, 2014). However, other recent comparisons of the microbial communities residing in the rhizosphere of *A. thaliana* and in the surrounding bulk soil revealed a weak 'rhizosphere effect', as only slight differences in taxonomic composition and community structure could be detected (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012; Schlaeppi *et al.*, 2014), and similarly in oak (*Quercus* sp.) (Uroz *et al.*, 2010). In these studies, the rhizosphere microbiota appear to be largely similar to the microbial communities present in the surrounding soil. As there is no physical limit between the soil and the rhizosphere, this could be attributable to sampling methodologies that do not allow the isolation of genuine rhizospheric microorganisms from the overwhelming majority of soil microorganisms. In addition, high-throughput amplicon sequencing does not, to date, allow detailed taxonomic affiliation of sequences, because of the relatively short 16S rRNA gene sequences obtained, which in turn prevents the detection of fine differences between the compartments. It is worth noting, nevertheless, that the specific richness of bacteria in the rhizosphere seems to be less than in the microbial communities in the surrounding soil (Bulgarelli *et al.*, 2012).

3. The rhizoplane, a specific habitat or a transitional boundary?

The rhizoplane, or root tissue surface, is often defined as a separate habitat from the rhizosphere, colonized by microorganisms that are firmly attached to the root surface. The most well-known root-epiphytic microorganisms are fungi forming ectomycorrhizas (EcMs) with ligneous plants. EcMs are mostly involved in enhancing soil nutrient mobilization and uptake while, in turn, the host plant provides carbohydrates (Courty *et al.*, 2010; Cairney, 2011). Despite the large amount of literature pertaining to EcMs, the community structure and dynamics of the root-epiphytic compartment still need to be determined. Again, there is no obvious boundary between the rhizosphere and the rhizoplane. Hence, selective extraction and analysis of the adhering root-epiphytic compartment using culture-independent molecular methods and high-throughput sequencing are very challenging from a technical point of view. Notwithstanding these methodological hindrances, it must be stressed that the rhizoplane constitutes the point of entry to the internal root tissues and that the epiphytic state can be considered as a transition stage between life outside and inside the roots. Catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) has been efficiently employed to characterize the abundance and spatiotemporal dynamics of bacteria and archaea colonizing the rhizoplane in rice (*Oryza sativa*) (Schmidt & Eickhorst, 2013). This has led to identification of the sites of preferential colonization by microorganisms such as the root tips and lateral root cracks which can serve as portals for microorganisms to enter the roots (Hardoim *et al.*, 2008). Also in rice, GFP-tagged Rhizobia were used to follow the patterns of colonization of plant tissues (Chi *et al.*, 2005). Analysis

of the dissemination dynamics of Rhizobia revealed an initial colonization of the rhizoplane followed by colonization of the root endosphere. Similarly, in *A. thaliana*, CARD-FISH allowed the detection of bacterial phyla at the root surface that had been shown by massive amplicon sequencing to be dominant in the endosphere compartment, therefore strengthening the hypothesis of a recruitment of endosphere microbiota from outside the roots (Bulgarelli *et al.*, 2012).

4. The endosphere, a restricted area

Microorganisms able to penetrate and invade the root internal tissues form the endosphere microbiote. In the vast majority of land plants, the root endosphere is colonized by AM fungi (Smith & Read, 2008) along with other fungi (Vandenkoornhuysen *et al.*, 2002), Bacteria (Reinhold-Hurek & Hurek, 2011) and, to a lesser extent, Archaea (Sun *et al.*, 2008). Some of these microorganisms are clearly interacting with their host plant. Vandenkoornhuysen *et al.* (2007) used RNA stable isotope probing to identify previously unknown root-inhabiting microorganisms that receive and consume labeled photosynthetates from their host plants. Combining stable isotope labeling with high-throughput sequencing now provides a powerful means of distinguishing the obligate symbionts truly interacting with their host from the facultative, transient endophytes. In contrast to the rhizosphere and the rhizoplane, the plant's endospheres feature highly specific microbial communities. In this habitat, the microbiota are very different from the microbial community in the adjacent soil. Diversity is much lower than that estimated for microbial communities outside the root (Bulgarelli *et al.*, 2012; Schlaeppli *et al.*, 2014). Lundberg *et al.* (2012) identified only 97 Operational Taxonomic Units (OTUs) of Bacteria (out of 256 OTUs whose abundances were significantly different between plant and bulk soil) that were consistently more abundant in the endosphere of *A. thaliana* grown in two different soils compared with the ectosphere. Comparisons of taxonomic profiles indicate broad changes in the microbial community structure as one goes from the rhizosphere to the endosphere. The above-mentioned OTUs were mainly affiliated to a small number of bacterial families including the *Streptomyetaceae* (Actinobacteria), which dominate the libraries of 16S rRNA gene amplicons. Interestingly, Actinobacteria are known to be producers of a huge variety of antimicrobial compounds (Lazzarini *et al.*, 2000). The selective enrichment of OTUs belonging to *Streptomyetaceae* in the endosphere of *A. thaliana* was also detected in another study using a similar experimental set-up but involving controlled soils as well as soils from natural sites (Bulgarelli *et al.*, 2012). Similarly, when wider sets of plant hosts affiliated to *Arabidopsis* and *Cardamina* species were examined, sequences assigned to Actinobacteria, *Betaproteobacteria* and Bacteroidetes dominated the data sets obtained for the rhizosphere and endosphere samples (Schlaeppli *et al.*, 2014). Taken together, these findings seem to indicate that the selection of Actinobacteria within the endosphere of distinct species of *Arabidopsis*, as well as in the *Arabidopsis* relative *Cardamina hirsuta*, is robust against differences in soil microbial community structure, local environmental parameters or host genotypes.

5. The aboveground compartment

In addition to the studies on plant microbiota associated with belowground plant organs, evidence is accumulating to suggest substantial abundance and diversity of microbial denizens residing either inside or outside the aerial tissues of plants (Peñuelas & Terradas, 2014). Few studies have investigated the microbiome composition in reproductive organs (i.e. the anthosphere, carposphere and spermosphere), although this could have important implications for vertical transmission (Lopez-Velasco *et al.*, 2013). The leaf internal tissues constitute an attractive environment for a large diversity of bacteria and fungi. Analysis of *A. thaliana* (Bodenhausen *et al.*, 2013) and *Solanum lycopersicon* L. (Romero *et al.*, 2014) indicated very different leaf endosphere microbiotic compositions. Based on the comparison of microbial communities located in the ectosphere and endosphere of the above- and belowground organs of plants tissues, the colonization of the leaf internal tissues by bacteria originating from either shoots or roots (i.e. microbial endosphere continuity) has been hypothesized (Bodenhausen *et al.*, 2013; Romero *et al.*, 2014). In spite of evidence of within-plant dissemination of endophytes, one cannot rule out the possibility that most endophytic dwellers found inside leaf tissues originated from the external environment and first colonized the leaf surface.

The global abundance of leaf epiphytic microorganisms has been estimated to be as high as *c.* 10^{26} cells (Vorholt, 2012) as a consequence of the huge cumulated surface area represented by leaves. In contrast to root-associated habitats, the foliar surface is characterized by much harsher conditions such as oligotrophy, exposure to UV radiation and desiccation. Microbial epiphytes, able to thrive on the leaf surface, have evolved different adaptations to cope with these extreme conditions. A phototrophic lifestyle on the leaf surface has been suggested based on the identification of microbial rhodopsins (Atamna-Ismael *et al.*, 2011; Vorholt, 2012). Some of these proteins could act as proton pumps, providing additional energy to diverse members of the phyllosphere. Interestingly, these proteins could preferentially absorb green light, thus avoiding an overlap with plant pigments (Atamna-Ismael *et al.*, 2012).

Methylotrophic bacteria have repeatedly been identified on the leaf surface. About 10^{24} g yr^{-1} of methanol, a plant metabolic waste product, is emitted from leaves (Galbally & Kirstine, 2002). It is thus not surprising that methylotrophs such as *Methylobacteriaceae* (*Alphaproteobacteria*) often dominate the microbial communities on the leaf surface (Vorholt, 2012). Comparison of *Methylobacterium* communities collected on leaves of several plant species, including *A. thaliana*, originating from five different sites, revealed that the local environmental conditions were more important drivers of community composition than plant species (Knief *et al.*, 2010).

Bacterial light harvesters and methylotrophs discussed above can be seen as beneficiaries of stable ecological habitat and niches provided by the host plant. Under this assumption, evolution of these microorganisms is expected to lead to the selection of specialized microorganisms able to efficiently colonize the leaf

surface. Thus, these microorganisms may induce competitive exclusion of other microorganisms including plant pathogens.

IV The plant holobiont: an existing core plant microbiota?

1. General concepts

Defining 'core' plant microbiota From the ecological perspective of the plant holobiont, the results obtained from analyzing the 'core' microbiota (Fig. 2) can be misleading if the ecological scale of the analysis has not been clearly defined and addressed. Different dimensions of the core microbiota can be defined. The simplest dimension is the individual plant holobiont (Fig. 2), where the core microbiota represent all the microorganisms found in different samples of the given host plant (i.e. this dimension allows the control of possible microbiotic heterogeneity). The core microbiota of a plant population represent the fraction of microorganisms shared between plants of the studied population. The core microbiota of a plant species are the fraction of microorganisms shared between plants from different populations (Fig. 2). Thus, the core microbiome composition is expected to become less and less complex as the hierarchical level of ecological organization increases (i.e. from individual to ecosystem). In addition to these core microbiotic dimensions, additional layers of complexity need to be addressed, especially changes occurring through time and space.

Taxonomic ranking matters in defining the core microbiota When comparing microbiomes, the grain of observation also matters. Saying that a Poaceae community is associated with bacteria and fungi, even if true, contains less information than saying that this Poaceae community is associated with *Glomus clarum* and *Burkholderia cepacia*. This exaggerated example indicates the importance of taking into account the taxonomic ranking level

in order to properly describe a microbiome and make assumptions about its functioning. Furthermore, the observation that *G. clarum* and *B. cepacia* occurred together would lead to questions about the rules governing assemblage. Thus, the lowest possible level of taxonomic ranking analysis is expected, and phylogenetic analyses of sequence data would be required to properly describe the microbiome. A clear definition of the core microbiome would be obtained by examining the intersection between sequence analyses for different samples.

A functional definition of core microbiota? In addition to the taxonomical approach to core microbiome definition, a functional approach is also feasible. In this case, the required data are generated from metagenomic or metatranscriptomic analyses and the obtained sequences are used to predict functions. At a given core microbiome dimension (Fig. 2), the core microbiome is defined by the shared predicted functions. This makes it possible to link diversity with the functions displayed, and to compare observations with theory. Different hypotheses linking diversity and functions have been developed (Naeem *et al.*, 2002). Among these, the hypothesis of key species (Paine, 1969) assumes that a given function is sustained by one species only. Conversely, the hypothesis of functional redundancy supposes that a diversity of organisms contributes to a function (Walker, 1992). Loss of a key species leads to a loss of function while, in the case of functional redundancy, observed differences in the taxonomic composition of the microbiome are not synonymous with function loss. These hypotheses to explain the relationships between diversity and function could account for a possible elasticity in core microbiotic community composition.

In both taxonomic and functional analyses of the core microbiome, we are making the assumption that the higher the ecological dimension, the greater the plant's dependence on the core microbiome. At high levels of ecological hierarchy, analysis of the core microbiota probably highlights key component species (i.e. high plant dependence).

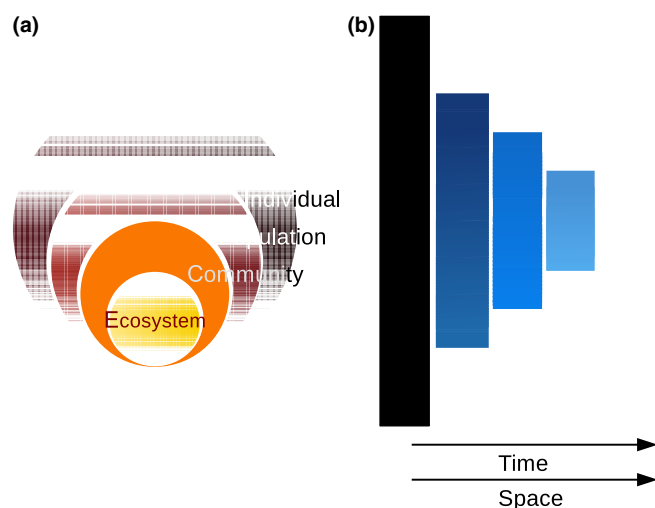


Fig. 2 The concept of core microbiome in the light of the different hierarchical ecological levels (i.e. expected Venn diagram) (a) and at a given ecological level, changes of the core microbiome through time and space (b).

2. Attempts to define the core microbiome

Analyses of plant core microbiota are still in their infancy. A finer perception of the different core microbiome dimensions is required to elucidate the possible rule(s) of organization. An initial attempt to define the core microbiome of *A. thaliana* has been published recently (Lundberg *et al.*, 2012). This study identified the consistent presence of OTUs mostly affiliated to *Streptomycetaceae* (Actinobacteria) in the endophytic compartment, which could constitute a potential bacterial core microbiome for *A. thaliana*. This study focused on a taxonomically based description of the core microbiota. Although one can speculate about possible functions, a clear functional description of the core microbiome of *A. thaliana* remains elusive.

The presence of a common set of protein-coding genes can reveal assembly rules based on functions rather than on species compositions, as underlined recently (Ofek-Lalzar *et al.*, 2014). One interesting example has emerged from the analysis of epiphytic microbial communities of the green macroalga *Ulva australis*

(Burke *et al.*, 2011). Community membership analysis highlighted high phylogenetic variability and low similarity in microbial taxonomic composition between the algal samples (i.e. 15% similarity), whereas they demonstrated high similarities in functional composition (70% similarity), which suggests an existing functional redundancy.

Toward a pan-microbiome analysis? The measured plant microbiotic compositions do show heterogeneity (i.e. Lundberg *et al.*, 2012). This observed variance can be related, among other things, to sampling bias, heterogeneity between plant tissues and heterogeneous plant growth conditions. The definition of the core microbiome corresponds to the intersection across all the samples analyzed at a given level of ecological hierarchy. From a theoretical point of view, if the heterogeneity between samples is high, (1) a limited core microbiome can be expected and (2) the defined core microbiome composition will decline as the number of samples is increased. This theoretical reduction of the core microbiome, taken alone, could by itself result in important components of the microbiota being overlooked. To circumvent this drawback, it might be helpful to relax the definition of 'core microbiome' at a given ecological scale. A pan-microbiome perspective refers to the core microbiome plus the microbiome fraction shared between different samples of a given modality (i.e. eco-microbiome) plus the unshared fraction. According to this concept, those microorganisms and functions belonging to the core are presumed to be essential to the host taxon. Conversely, the 'accessory' microbiome (i.e. eco-microbiome as defined above) would be expected to contain more dispensable functions or microorganisms whose presence is related to interactions with the surrounding environmental conditions. The pan-microbiome concept would thus be helpful to take into account the observed heterogeneity that does exist among plant microbiota and to thereby improve our understanding of the rules of assembly governing plant microbiotic composition.

V. The plant and its microbiome: what controls what?

The question of possible control of the microbiome by the plant or, reciprocally, control of the plant by its microbiome is important to address as it impacts our understanding of fitness (i.e. survival and reproduction) at the level of the plant holobiont and of the endosphere and ectosphere microbial populations.

Likely key components for understanding what controls what in the plant holobiont include the modulation of functional plasticity of the microbiota to adjust the plant holobiont to its environment, the plant immune system and also the symbiosis behavior.

1. Functional plasticity of the plant holobiont

Trophic interactions among (micro)organisms are accepted to be key drivers controlling community assembly (Tilman, 1982; Mittelbach, 2012), which mostly takes into account competition processes. However, the positive interactions among same trophic guilds, referred to as facilitation, are key processes in ecosystems. It is now argued that facilitation (i.e. positive

interactions) is an omnipresent driver of biodiversity (McIntire & Fajardo, 2014). Facilitation involves different processes such as (1) the mitigation of abiotic stresses, (2) the creation of novel habitats, (3) heterogeneity and habitat complexity, (4) service sharing, and (5) the best possible access to resources (McIntire & Fajardo, 2014). All of these phenomena help to maintain or improve spatial and local diversity. The plant holobiont concept provides a new perspective on and understanding of facilitation. In fact, the plant microbiota can be seen as a facilitator component providing additional genes to the host, which are involved in the adjustment to local environmental conditions. This view is supported by various lines of evidence. First, plant microbiome composition is more dependent on the soil type in which a given plant genotype has been grown rather than on the plant genotype *per se* (Bulgarelli *et al.*, 2012), which suggests that the microbiome composition trajectory is environmentally dependent. Secondly, some host-adapted microbes may have been selected as they provide a selective advantage for their host, and there is growing evidence of recruitment by the plant of microorganisms from the ectosphere to fight pathogens (for a review, see Berendsen *et al.*, 2012), and/or to improve its nutrition and growth (e.g. via mycorrhiza formation). Thirdly, the colonization of a plant by particular microorganisms can lead to substantial phenotypic modifications (Streitwolf-Engel *et al.*, 1997, 2001; Wagner *et al.*, 2014). Because facilitation and related plant functional plasticity embrace multiple mechanisms primed by the plant microbiota, it is clear that a plant can no longer be seen as a standalone entity. Plants require the means to dynamically adjust to biotic and abiotic constraints on account of their sessile lifestyle. The use of resources by a plant necessarily leads to an alteration in the available multidimensional niches (McIntire & Fajardo, 2014). We are proposing the hypothesis that the plant microbiome is the powerhouse of the adjustment to local conditions.

2. Plant immunity and microbiota

Plants have mechanisms to fight colonization by microorganisms. A focus on the molecular dialogue between the plant and microorganisms is important to better understand how microorganisms can live on or in their host plants.

Plant innate immunity overview Plants rely on innate immunity. Two forms exist: (1) pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), formerly called horizontal resistance, and (2) effector-triggered immunity (ETI), also called R-gene-based immunity and previously known as vertical resistance (Jones & Dangl, 2006).

In PTI, microbe-associated molecular patterns (MAMPs) and damage-associated molecular patterns (DAMPs) induce corresponding pattern recognition receptors (PRRs) (i.e. host plant receptors) (Boller & Felix, 2009). MAMPs are molecular signatures of the microbe world, the best known probably being the elongation factor and flagellin, which are slowly evolving bacterial features. Bacterial flagellin elicits PTI when it is sensed as a MAMP by the leucine-rich repeat (LRR) domains of the Flagellin-Sensing 2

(FLS2) receptor (for a review, see Boller & Felix, 2009). The plant promptly responds by producing reactive oxygen species (ROS), activating defense genes and thickening its cell walls by callose deposition (Boller & Felix, 2009).

Plant pathogens can use effectors, which are tools or weapons to manipulate their hosts by suppressing PTI mechanisms (Kamoun, 2006). Reciprocally, plants have developed the perception of danger signals through the recognition of effectors. ETI corresponds to a direct or indirect interaction between a pathogen effector and the product of a specific plant resistance gene, which leads to a strong response (i.e. hypersensitive response) consisting of cell apoptosis and local necrosis. In this case, nuclear binding sites (NBSs) interact with a variety of LRR domains to initiate cell death (Boller & Felix, 2009). It must be underlined that the knowledge corpus of plant innate immunity is mostly based on reductionist analyses focusing on one host and one microbe. The phytoprotection is also a consequence of biotic interactions where the plant microbiota are suggested to play an important role, such as the case of the fungal leaf endophyte *Neotyphodium coenoccephalum* (*Clavicipitaceae*) in tall fescue (*Festuca arundinacea*), which provides a fitness advantage to its host. The success of this fungus-mediated phytoprotection is so great that it has resulted in the formation of an invasive plant cultivar of tall fescue, 'Kentucky31', in North America (Selosse *et al.*, 2004).

Plant defense and microbiota, 'hand in hand' As explained in the previous section, MAMPs and DAMPs in PTI and effectors in ETI are interpreted as danger signals by plants (Boller & Felix, 2009). Thus, it can be assumed that MAMPs, DAMPs and effectors are necessarily 'bypassed' by the plant microbial endosphere. In agreement with this assumption, it has been suggested that, upon attack by a pathogen, plants are able to recruit protective microorganisms from their ectosphere reservoir (for a review, see Berendsen *et al.*, 2012), procuring a clear modulation of host plant immunity by beneficial microbes (Zamioudis & Pieterse, 2012), which induces systemic resistance primed for accelerated activation (Conrath *et al.*, 2006; Bakker *et al.*, 2007; Berendsen *et al.*, 2012). To date, the recruitment mechanisms of microorganisms from the ectosphere remain poorly understood, while understanding of the mechanisms by which the plant microbiota avoid the plant's defenses is growing.

Microbiota and plant defense 'manipulation' Hormonal modulation of plant immunity (for a review, see Pieterse *et al.*, 2012) is especially effective through salicylic acid (SA) and jasmonic acid (JA) acting as backbone cellular signal molecules interplaying with complex networks of regulation (Pieterse *et al.*, 2012). Like some successful plant pathogens, certain root endophytes have been shown to be able to 'hijack' or manipulate plant hormone signaling in different ways, by:

- synthesizing auxins and auxin analogs along with gibberellins (GAs) (Sirrenberg *et al.*, 2007; Ben Lugtenberg & Kamilova, 2009), which probably attenuate SA signaling (Pieterse *et al.*, 2012);
- using effectors which modify the hormonal signaling pathways, as is the case for mycorrhizal fungi (Kloppholz *et al.*, 2011; Plett *et al.*, 2011);

- transient accumulation of JA at an early stage of mycorrhiza formation and root-nodule formation, supposedly to 'bypass' the SA-triggered response (Gutjahr & Paszkowski, 2009).

Both the PTI and EPI innate immunity responses are related to the host plant encoding small RNAs involved in silencing processes of pathways leading to targeted post-transcriptional gene silencing or transcriptional gene silencing (for a review, see Peláez & Sanchez, 2013). For example, interactions between bacteria-responsive miRNAs and hormone signaling have been reported to occur after inoculation with symbiotic nitrogen-fixing bacteria and during nodule development (De Luis *et al.*, 2012; Reynoso *et al.*, 2013). Despite this growing knowledge, the molecular bases of the signaling dialogue which allows an endophytic lifestyle within the host plant are still mostly unknown (Reinhold-Hurek & Hurek, 2011), and progress in this fascinating research area is expected from comparative metagenomic and metatranscriptomic studies.

Plant defense and microbiota, toward emerging ideas Because of the great complexity of the endospheric plant microbiota (Lundberg *et al.*, 2012), we can postulate the existence of a generic mechanism across plant and microorganism species rather than specific signaling and plant defense silencing for each given pair of plant species and endospheric microorganism. Bacteria in the exosphere that are able to switch to a life within the endosphere probably possess the required key genetic machinery. It has been suggested that the mechanisms of attraction, recognition and association involve, to some extent, the ancient and widespread common symbiosis (SYM) pathway (Venkateshwaran *et al.*, 2013) and that this root colonization is modulated by plant ethylene concentration (for a review, see Hardoim *et al.*, 2008).

In other situations, different nonpathogenic rhizobacteria are known to be able to trigger systemic plant resistance via the activation of a variety of genes induced by pathogen attack (Conrath *et al.*, 2006). A fraction of the plant endophytic microbiota are also known to synergistically enhance plant health by producing a variety of defensive chemicals (for a review, see Friesen *et al.*, 2011). Thus, in addition to the plant's innate immunity systems, the idea is emerging that the actual plant microbiota can be seen as a component of plant defense.

3. Symbiosis behavior

Symbiosis and especially mutualism are usually regarded as commonplace. However, evolutionary theory predicts that cooperation should not be the norm (Schwartz & Hoeksema, 1998). If a 'black sheep' becomes less cooperative but keeps receiving the same advantage from its host or symbiont, a fitness gain in comparison to the 'wild type' should occur. From a theoretical point of view, such 'black sheep' cheaters thus rapidly invade the population with the result that mutualism would be unstable.

Different hypotheses have been proposed to explain the observed stability of mutualism. Luxury resource exchange, when resources are not limited and exchange is based upon emitted surplus (Kiers & van der Heijden, 2006), could provide shared advantages to the interacting (micro)organisms. Indeed, if both host and symbiont transfer resources in excess, the cost of symbiosis is lower, which is

beneficial for both parties. However, it is well known that nutrients can be stored at low cost and that they do not exist in infinite amounts. A second hypothesis relates to partner choice. In this case, partners are able to discriminate based on their functional trait, which can result in an immediate fitness benefit to the interacting individuals (Sachs *et al.*, 2004). The watermark of partner choice is the evaluation of the interaction effectiveness and a related 'decision' to keep interacting or to enter into an interaction (Sachs *et al.*, 2004) requiring complex molecular signaling. This hypothesis would probably apply to the fraction of microbial endophytes selected from the rhizosphere by plants (van Overbeek & van Elsas, 2008; Berendsen *et al.*, 2012). Conversely, a simpler process would be based on the rate of nutrient exchanges. In this context, a third hypothesis relates to the possibility of forced cooperative behavior if the interacting individuals are able to reward good partners with nutrients and simultaneously sanction the less cooperative ones. In the case of rhizobium–legume symbiosis, plants have been shown to detect and penalize Rhizobia defective in N₂ fixation by reducing resource allocation (Kiers *et al.*, 2003). Similarly, AM symbiosis is stabilized both by the control of carbon embargo against AM fungal cheaters and by the transfer of phosphorous to plants conditioned by a plant carbon allocation (Kiers *et al.*, 2011).

Despite this growing evidence, which supports the host and symbiont sanction hypothesis to explain cooperative behavior, it is clear that 'free riders' (i.e. cheaters) do exist in nature. This can be explained in several different ways.

- For example, strains of Rhizobia providing little benefit to their host plant have been shown to be able to circumvent the plant's sanctions by producing rhizobitoxine, an ethylene inhibitor, which promotes an increase in lipid allocation from the host (Ratcliff & Denison, 2009). Furthermore, recent evidence has empirically confirmed the selection for cheating in the legume–rhizobium partnership (Porter & Simms, 2014).
- It is hypothesized that the plant sanction trait is no longer selected in modern plant crops selected and grown in nutrient-enriched soils using conventional agricultural practices and that, in consequence, an increase in the number of cheaters can be expected in conventionally farmed agricultural soils (Duhamel & Vandenkoornhuys, 2013), a trend that has already been indirectly demonstrated (Johnson, 1993).
- Assuming that the plant microbiota are in continuous transition, 'free riders' might be seen as maintainers of plant health mechanisms.

All the endophytes within the complex microorganism community could adapt their interaction behaviors (cooperator versus cheater) with the host plant in a way that 'selfishly' maximizes their own fitness. Thus, in addition to better understanding the ecological functions of plant endophytes, a key question is how the interaction behaviors of both the plant and microorganisms have evolved. In relation to these key issues, the question of selfishness must be meticulously addressed, especially regarding the biotic interactions within the complex microbiota. As emphasized by Kemen (2014), an improvement in fitness for different microorganisms sharing the same habitat can result from collaboration which can be achieved by sharing common goods (e.g. a detoxifying enzyme, a nutrient produced by a given (micro)

organism) and genes. From a more theoretical perspective, the Black Queen Hypothesis (Morris *et al.*, 2012) explains how possible cooperation among species can be 'automatic' and based upon purely selfish trajectories. This theory is based on two main assumptions: first, bacterial functions are often leaky, leading to common good production by so-called helpers, which is consistent to some extent with the idea raised by Kemen (2014); secondly, beneficiaries, by adopting a specialist trajectory, become dependent on the presence of helpers and undergo loss of genes and functions rendered dispensable through the production of common goods. This theory, developed for free-living microorganisms, may be extended to the interactions among microorganisms of the plant microbiota but needs experimental support.

4. Fitness of the plant holobiont

A plant can classically adapt to changing environment through changes in intrinsic plant traits. However, as described in Section III, a plant can also adapt to the perceived environment by involving different components of its microbiome. Plants can select at least part of their ecto- and endospheric microbiota to better adapt to environmental constraints. Partner choice corresponds to the enrollment of a cooperative partner X by an individual Y (X and Y being a microorganism and a plant, respectively), which enables Y to improve its own fitness and to promote the evolution of cooperation processes (Sachs *et al.*, 2004). Despite the evolution of these processes of cooperation, the enrollment of X is not subjected to vertical transmission and so X is not necessarily present in the following generation of Y. Conversely, another fraction of the microbiota can be vertically transmitted. One example is the case of the fungus *Epichloë festucae*, in *Festuca rubra*, which impacts the plant's nutrient balance (Vázquez-de-Aldana *et al.*, 2013). This vertical transmission allows partner fidelity feedback (Frederickson, 2013) or 'automatic' fitness feedback (Sachs *et al.*, 2011), given the fact that vertical transmission tightly correlates symbiont and host reproductive interests (Sachs *et al.*, 2004). On a broader scale, the feedback between partners in cooperative interactions is often in alignment with their respective fitness interests (Friesen, 2012; Frederickson, 2013).

The plant microbiota are complex and consist of a number of partners of different origins and evolutionary trajectories. Because of the broad diversity of microbiota, it can be speculated that each individual component of the microbiota does not have distinct functions but instead that a functional redundancy exists providing opportunities for effective and rapid adaptation.

The fitness measurement of the microbiota components is possible using specific molecular targets combined with detailed spatial and temporal dynamic analyses. Measuring plant fitness *per se* (i.e. the fitness of the plant alone, excluding the contribution of the microbiome) is much more difficult, even impossible, because this would require axenic growth and therefore a disconnection from most of the environmental factors. In consequence, measures of plant fitness generally include both the endosphere and ectosphere and correspond in fact to the fitness of the plant holobiont. Thus, plants are already viewed intrinsically as holobiont entities. However, the change is in our perception of this fact

and our perception of the deep impact of the microbiota in the measurement of plant holobiont fitness. This perception can be extended to measures of a plant phenotypic trait (Streitwolf-Engel *et al.*, 2001), which is not the consequence solely of plant genome expression under particular constraints (Wagner *et al.*, 2014). One important paper reported that a given AM fungus colonizing different host plants was able to differentially impact the above-ground biomass, a proxy of plant fitness (van der Heijden *et al.*, 1998), thereby impacting plant community structure and productivity (van der Heijden *et al.*, 1998; Vogelsang *et al.*, 2006). Feedbacks between soil communities and plants drive plant community dynamics by modifying the intensity and nature of plant competition (Pendergast *et al.*, 2013).

VI. Concluding remarks and prospects

The species richness and diversity of the plant microbiota are greater than was believed just a few years ago. A new field of research into the intricacies of the plant holobiont is opening up. Certain key issues still need to be addressed. These include: (1) description of the core microbiome and rules of community assemblage, (2) the functions of the microbiota, (3) the molecular interactions occurring between the host plant and its symbionts, and (4) the link between symbiont diversity and functions. Because of our lack of understanding of these issues, we do not yet know how and why the microbiota are so complex. It should be possible to tackle this enigma and related research questions through the use of modern tools such as metagenomics and metatranscriptomics, applying innovative strategies and performing experiments to obtain a detailed description of the microbiome and its expression along with the processes controlling plant genome expression. These molecular analyses could be combined with image analyses and the exchange processes tracked by stable isotope probing approaches, for example. A complementary analysis of the plant holobiont is to manipulate the microbial communities to elucidate the interactions involved, including synergies and exclusions, and also to better understand the ecological functions displayed. This reductionist strategy, applied under controlled conditions using gnotobiotic plants (i.e. mutants germinated in sterile conditions and manipulated microbial communities as in Bodenhausen *et al.* (2014)), would allow (1) identification of the genetic factors that shape the associated microbial community (Bodenhausen *et al.*, 2014) and (2) elucidation of the importance of the microbiota in plant phenotypic plasticity (Wagner *et al.*, 2014) and the possible adjustment of the plant holobiont to environmental conditions (Panke-Buisse *et al.*, 2014).

The complexity of the microbiota can be seen as a consequence of: active recruitment of microorganisms by plants; opportunist transfer of nonpathogenic microorganisms through cracks; and pseudo-vertical and vertical transmission. For the latter, and despite the existence of a large cohort of studies mainly concerning model fungal endophytes, knowledge of the vertically transmitted microbiotic fraction is scarce, and the related co-evolutionary processes explaining partner fidelity remain poorly investigated.

The plant microbiota are also expected to change through time depending on abiotic and biotic environmental constraints.

Among the possible explanations of plant microbiotic complexity that have received little attention is the impact of microbial viruses, which are able to provoke drastic dynamic changes (Buée *et al.*, 2009), and the network of interactions within the microbiota (Bakker *et al.*, 2014; Desirò *et al.*, 2014). Improved knowledge of the microbiome component of the plant holobiont could also lead to a number of important applications in crop production (Rodriguez *et al.*, 2008) and start-up companies are already emerging. Nevertheless, the importance of this change in perception of the plant microbiota has, as yet, not been fully taken into account in crop selection and production. However, a 'neodomestication' of plants, which takes full advantage of the mutualist plant compartment and thus considers the plant holobiont as a whole, is feasible in innovative and ecologically intensive agriculture (Duhamel & Vandenkoornhuise, 2013).

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