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Minireview

The plant endosphere world – bacterial life within plants

Stéphane Compant[1], **Marine C. Cambon**[2], **Corinne Vacher**[2], **Birgit Mitter**[1], **Abdul Samad**[3] **and Angela Sessitsch**[1*]

[1]*Center for Health and Bioresources, Bioresources Unit, Konrad Lorenz Straße 24, AIT Austrian Institute of Technology, Tulln, A-3430, Austria.*

[2]*INRAE, Univ. Bordeaux, BIOGECO, Pessac, F-33600, France.*

[3]*Natural Resources Canada, Canadian Forest Service, Laurentian Forestry Centre, Québec, G1V4C7, Canada.*

Summary

The plant endosphere is colonized by complex microbial communities and microorganisms, which colonize the plant interior at least part of their lifetime and are termed endophytes. Their functions range from mutualism to pathogenicity. All plant organs and tissues are generally colonized by bacterial endophytes and their diversity and composition depend on the plant, the plant organ and its physiological conditions, the plant growth stage as well as on the environment. Plant-associated microorganisms, and in particular endophytes, have lately received high attention, because of the increasing awareness of the importance of host-associated microbiota for the functioning and performance of their host. Some endophyte functions are known from mostly lab assays, genome prediction and few metagenome analyses; however, we have limited understanding on *in planta* activities, particularly considering the diversity of micro-environments and the dynamics of conditions. In our review, we present recent findings on endosphere environments, their physiological conditions and endophyte colonization. Furthermore, we discuss microbial functions, the interaction between endophytes and plants as well as methodological limitations of endophyte research. We also provide an outlook on needs of future research to improve our understanding on the role of microbiota colonizing the endosphere on plant traits and ecosystem functioning.

Received 3 July, 2020; revised 11 September, 2020; accepted 16 September, 2020. *For correspondence. E-mail angela.sessitsch@ait.ac.at; Tel. +43(0) 50550-3509; Fax +43(0) 50550-3666.

Introduction

For a long time, the scientific community thought that plants that do not show symptoms of diseases are free of microorganisms, particularly from bacteria. There were few early reports on bacterial colonization of the plant endosphere (Galippe, 1887; Laurent, 1889); however, in the 19th century, the general belief was that healthy plants are free of microorganisms, following the postulates of Louis Pasteur (Compant *et al*., 2012). Bacteria occupying root nodules of leguminous plants, nowadays well known as rhizobia being responsible for fixing atmospheric nitrogen, were discovered by Martinus Willem Beijerinck in 1888 (Beijerinck, 1888). In the same year, Hellriegel and Wilfarth (1888) reported that leguminous plants are independent on mineral N, further indicating the importance of the N-fixing symbiosis between plants and rhizobia.

Active research on the plant endosphere as a habitat for non-pathogenic bacteria started in the 1990s, triggered by the increasing number of reports on the beneficial effects of plant growth-promoting rhizobacteria (PGPR). The pioneering work of Johanna Döbereiner on specific bacteria which, like *Herbaspirillum seropedicae*, colonize the endosphere of sugarcane and fix nitrogen (Baldani *et al*., 1986; Boddey and Döbereiner, 1988) stimulated, given their importance for the Brazilian economy, further research on bacteria colonizing the plant endosphere. The increasing interest in studying microbial communities in the environment together with the development of molecular, cultivation-independent tools (like the DNA fingerprinting tools 16S rRNA gene-based denaturating gradient gel electrophoresis or terminal restriction fragment length polymorphism analysis) to study their community structure also triggered research on endosphere microbiota.

In the early 1990s, definitions came up on the term ‘endophyte’, mostly referring to microorganisms that inhabit internal plant tissues, at least some time of their

lifecycle, without causing apparent harm or disease to their host (Petrini, 1991; Wilson, 1995). Although this definition has been used in many studies and represents a pragmatic distinction between endophytes and pathogenic colonizers, it has been recently revised by Hardoim *et al*. (2015). A revised definition was needed due to the understanding obtained in the last years showing that pathogenicity or mutualism of microorganisms may depend on many factors including the plant genotype, the environment and the co-colonizing microbiota (Brader *et al*., 2017). Therefore, a clear distinction between non-pathogenic microorganisms (i.e. endophytes) and pathogens is often not feasible without detailed functional analysis. Also, functional assignment of endophytes studied purely by molecular tools, e.g. by microbiome analysis based only on phylogenetic markers, is usually not possible. Therefore, Hardoim *et al*. (2015) suggested that the term endophyte should refer to the habitat only and include all microorganisms, which for all or part of their lifetime colonize internal plant tissues. In the present review, the term endophyte refers to any microorganism that can colonize internal tissues of plants, including pathogens.

In the last decade, host-associated microbiota have gained increasing attention, triggered by spectacular findings on the role of the human microbiome for human health, behaviour and well-being. Already in 1994, Jefferson postulated that the evolutionary selection unit is not the macro-organism (e.g. the plant) but the macro-organism and all its associated microorganisms that act in concert as a holobiont (Jefferson, 1994). This hypothesis was further elaborated in the highly debated hologenome theory of evolution (Zilber-Rosenberg and Rosenberg, 2008; Theis *et al*., 2016). The rhizosphere is considered as an important component of the plant holobiont, but endophytes have received increasing attention due to their intimate interaction with plants. Despite this increasing awareness of microbial life within plants, the endosphere is often recognized as one habitat without considering the variety of microenvironments and dynamics of microenvironment conditions. We aim here to review the multiple facets of the plant endosphere environment for bacterial colonization and life and pinpoint to the methodological limitations and knowledge gaps, which need to be addressed to further elucidate the role of bacterial endophytes in plant physiology and ecosystem functioning.

Microbiota in different plant compartments

Plant compartments and physiological conditions

Plants host diverse microbiota in different compartments and tissues, i.e. vegetative organs like roots, stems and leaves and also reproductive/disseminating organs (flowers, fruits/seeds). Bacterial densities inside plant tissues typically range from 10^5 to 10^7 of cultivable cells per gram of root to 10^3–10^4 in leaves and stems. In flowers, fruits and seeds typically 10^2–10^3 cells per gram tissue are found (Compant *et al*., 2010). These numbers pinpoint to the soil environment as a major reservoir of potential endophytes. However, the plant immune system may control the abundance of endophytes and maintain the most 'plant-favorable' bacterial density in the different organs (Liu *et al*., 2017). Pathogens can overcome plant defense and can therefore reach higher cell numbers than non-pathogenic strains (Brader *et al*., 2017). High bacterial cell density can be detrimental for the host organs. For instance, high cell density is known to induce quorum sensing (i.e. cell-density dependent) regulated processes such as virulence and pathogenicity but are also important for beneficial functions (Braeken *et al*., 2008; Hartmann *et al*., 2014). Seeds usually show low bacterial numbers and do not provide suitable conditions for microbial growth. When seeds germinate, the bacterial cell density increases, seed-derived endophytes colonize the different plant tissues of the emerging plant (Mitter *et al*., 2017).

The occurrence, abundance and activities of individual bacterial taxa depend on the micro-environment provided by the plant compartment (Compant *et al*., 2010; Brader *et al*., 2017), the plant genotype and physiology as well as the surrounding environment (Turner *et al*., 2013; Barret *et al*., 2015; Hardoim *et al*., 2015; Afzal *et al*., 2019). Roots, stems, leaves, flowers, fruits and seeds show different chemical conditions, in terms of organic acids, carbohydrates, vitamins, sugars, but also hormones, amino acids, fatty acids, flavonoids, glucosinolates, as well as phenolic compounds, pH and water, which are essential for plant growth, development, stress adaptation and defense (Hounsome *et al*., 2008). Each chemical environment enables the growth of specific microorganisms showing appropriate metabolic activities to colonize and inhabit plant organs and tissues, leading to different microbial assemblages (Compant *et al*., 2010; Vorholt, 2012; Sasse *et al*., 2018).

From the soil environment, microorganisms reach the rhizosphere, which is known to be directly influenced by root exudates and constitutes a hotspot for the establishment and development of microbial communities (Hiltner, 1904; Lemanceau *et al*., 2017). Roots can release about 10%–40% of their total photosynthetically fixed carbon through organic and inorganic forms (Newman, 1985). These exudates include secretions and diffusates that are chemically very diverse, including mucilage, cellulose, organic acids, amino acids, fatty acids, phenolics, plant growth regulators, nucleotides, sugars, putrescine, sterols and vitamins (Jones *et al*., 2009; Sasse *et al*., 2018). Root environment, root morphology and root

exudates have a tremendous influence on shaping the soil microbiome, but also on the establishment of bacterial endophytes in roots (Pfeiffer *et al*., 2017; Sasse *et al*., 2018) as well as in flowers, fruits (Compant *et al*., 2011) and seeds (Escobar Rodríguez *et al*., 2018a). However, exudates and leachates of other plant organs than roots can also facilitate establishment and colonization of bacterial endophytes, from the outer to inner tissues, as shown, for instance, with the leaf environment (Vorholt, 2012; Vacher *et al*., 2016), but also with flower and fruit environments showing specific exudate composition (Compant *et al*., 2010).

In organs like seeds, not only the chemical environment is important. Seeds are desiccated tissues that enable only specific microorganisms to thrive as endophytes (Hardoim *et al*., 2015; Truyens *et al*., 2015) albeit pathogenic strains can also destroy seeds. Further, starch accumulation and drying during seed maturation only allows primarily bacterial endophytes to establish and survive if they can tolerate high osmotic pressure (Mano *et al*., 2006; Rana *et al*., 2020). Plant emergence, cultivar and pathogens further shape the seed microbial community (Barret *et al*., 2015, 2016). These seed endophytes can be acquired from the soil environment, but also transmitted from birds or other animals to plant tissues (Berg and Raaijmakers, 2018; Escobar Rodríguez *et al*., 2018a). Furthermore, vertical transmission of endophytes to the next generation may be possible, e.g. when microorganisms colonize flowers and then migrate into the developing seed (Mitter *et al*., 2017). Cultivation-independent analysis also suggests that some endophytes may be transferred from seed to seed (Johnston-Monje and Raizada, 2011; Escobar Rodríguez *et al*., 2018b). The composition of seed microbiota can impact seed quality and ultimately plant fitness (Shade *et al*., 2017; Escobar Rodríguez *et al*., 2020).

Plant compartments and bacterial microbiota composition

In comparison to other plant organs, root bacterial microbiota often (but not always) contain the highest diversity of microorganisms (Amend *et al*., 2019). Mostly found taxa are Acidobacteria, Verrucomicrobia, Bacteroidetes, Proteobacteria, Planctomycetes and Actinobacteria and most of them can be also found in the rhizosphere (Hardoim *et al*., 2015). However, a subpopulation of rhizosphere microbiota can enter roots. The most abundant phyla often found were, for instance, in grapevine roots, Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes, Verrucomicrobia, Planctomycetes, Chloroflexi, Firmicutes and Gemmatimonatedes (Samad *et al*., 2017). For maize, Proteobacteria, Firmicutes, and Bacteroidetes have been also found as predominant phyla inside roots (Correa-Galeote *et al*., 2018). Different plant species usually host different microbiota composition, but also the plant genotype and developmental stage affect diversity and shape community structure. Furthermore, the soil environment, its history, as well as biotic or abiotic stresses lead to a shift in the diversity and abundance of the different taxa inhabiting roots (Brader *et al*., 2017; Correa-Galeote *et al*., 2018; Sasse *et al*., 2018; Xu *et al*., 2018). The presence of different other microorganisms like fungi or mycorrhizae-like fungi and microbial interactions can additionally influence the composition of root endophytic microbiota (Deveau *et al*., 2018).

Above-ground organ microbiota such as in stems, fruits or seeds have been shown to be influenced by several factors, including the soil environment (Rasche *et al*., 2006; Klaedtke *et al*., 2015; Zarraonaindia *et al*., 2015; Escobar Rodríguez *et al*., 2020). Interestingly, Harrison and Griffin (2020) recently reported that variation in endophyte assemblages between below- and above-ground tissues varied with the host growth habit. The authors showed that in woody plants stems hosted the richest endophyte communities, whereas in graminoids roots had the richest communities (Harrison and Griffin, 2020). Inside leaves, different endophytic assemblages have been found depending on external factors, such as climate and microclimate, as well as plant species and genotypes (Turner *et al*., 2013; Robinson *et al*., 2016; Vacher *et al*., 2016; Mina *et al*., 2020). Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes have been isolated from maize seeds (Johnston-Monje and Raizada, 2011; Rana *et al*., 2020). Also, molecular community analysis evidenced these taxa within seeds, e.g. of *Brassicaceae* (Barret *et al*., 2015) or of *Setaria* (Escobar Rodríguez *et al*., 2018a).

Colonization pattern of endophytes

Colonization patterns and niches of plant-associated microorganisms from the soil to aerial organs have been studied for decades for both pathogenic and non-pathogenic strains. Roots have been widely studied where microorganisms could be endophytic crossing from the rhizosphere to the rhizodermis before reaching the cortical cell layers, the central cylinder (stele) and the xylem vessels (Compant *et al*., 2010; Brader *et al*., 2017) (Fig. 1). Different pathways have been described to explain crossing from the rhizosphere to root internal tissues (Kandel *et al*., 2017). Microorganisms can enter roots at the root tip and root hair level (Kandel *et al*., 2017), and root hair inner colonization has been demonstrated (Mercado-Blanco and Prieto, 2012; Mercado-Blanco, 2015). This is, for instance, the case for

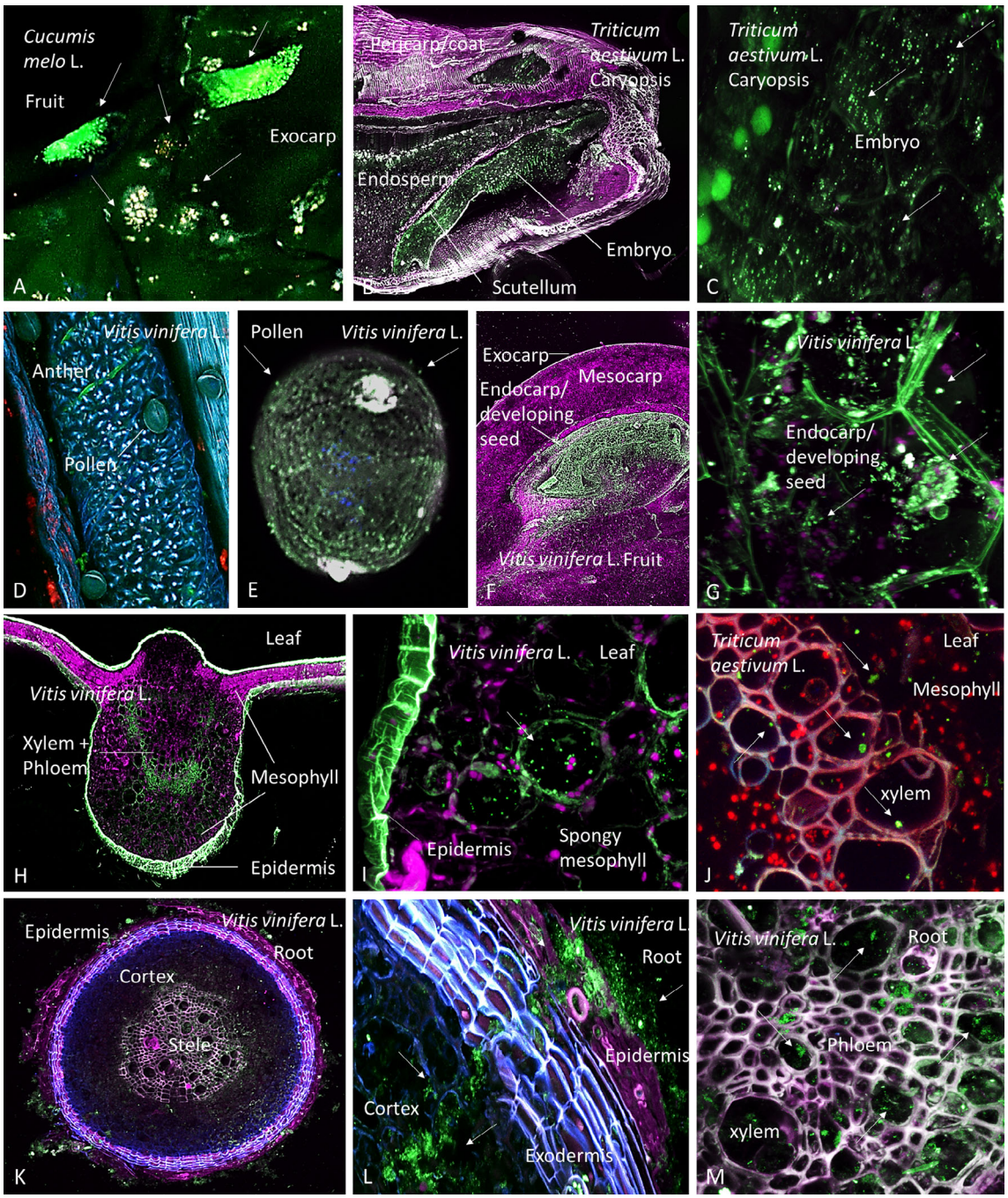

Fig. 1. Confocal microscopy pictures of reproductive/disseminating or vegetative plant organs and bacteria.
A. *Cucumis melo* L. fruit.
B and C. Caryopsis of *Triticum aestivum* L.
D. Anther of *Vitis vinifera* L.
E. Pollen of *Vitis vinifera* L.
F and G. Developing fruit and seed of *Vitis vinifera* L.
H and I. Leaf of *Vitis vinifera* L.
J. Leaf of *Triticum aestivum* L.
K, L and M. Root of *Vitis vinifera* L. Transversal section (A, H-M), longitudinal section (B and C, F and G), surface (D, E). Bacteria (arrows) appear as green, orange, white (A) by fluorescence *in situ* hybridization using general and specific probes targeting bacterial taxa or green (C, E, G, I, J, L, M) due to general staining with Syto9®. Scale of a bacterium: 1–2 μm.

some plant beneficial endophytes like strains belonging to *Pseudomonas* spp. in olive roots (Mercado-Blanco and Prieto, 2012; Mercado-Blanco, 2015). Root crack colonization has been further described. Microorganisms can enter root tissues at cracks of the secondary root emergence zone enabling a non-active process for bacteria to be present inside root tissues (Compant *et al*., 2010; Hardoim *et al*., 2015; Compant *et al*., 2019). Bacteria enter roots at the junctions of rhizodermal cells through plant cell wall degradation using plant cell wall-degrading enzymes, including polymer-degrading cellulases, cellobiohydrolases, endoglucanases and xylanases (Liu *et al*., 2017). Furthermore, pathogens and insects may open a path for non-pathogenic bacteria to enter roots (Compant *et al*., 2005, 2010). All these routes of penetration have been demonstrated with inoculated non-pathogenic or pathogenic microbial strains, but evidence has also been obtained by microscopy analysis of samples obtained from natural environments (Compant *et al*., 2016) (Fig. 1). Comparative analysis of root colonization has been performed for beneficial and pathogenic *Pseudomonas syringae* strains on pepper plants (Passera *et al*., 2019). Both strains showed a preference for colonization at the secondary root emergence site. The beneficial strain was also found in high density on the surface of primary roots, while the pathogenic strain was found more often on secondary roots. The latter strain also colonizes strongly damaged roots and locates over the xylem zones, whereas the beneficial strain did not (Passera *et al*., 2019).

Albeit most endophytic bacteria colonize intercellular spaces, also intracellular colonization as well as the combination of both colonization types have been observed. This has been described for instance with the beneficial endophyte *Paraburkholderia phytofirmans* strain PsJN in grapevine roots, where the strain colonizes mostly intercellular spaces, but it was also found to colonize intracellularly in the endodermis as well as in some cortical cells (Compant *et al*., 2008). Intracellular colonization has been also demonstrated with other model endophytes such as strains of *Azoarcus*, *Gluconacetobacter*, *Herbaspirillum* and *Klebsiella* spp. (Turner *et al*., 2013). Inside stems, several bacterial taxa have been described to colonize mostly intercellular spaces but also intracellular spaces, with presence in the vascular system, in the parenchyma layer as well as inside the epidermis. The cells inside the vascular system such as xylem vessels derive mostly from the roots, although translocation to the vascular system has been shown additionally for microorganisms deriving from insects (Hardoim *et al*., 2015) such as in the case of *Phytoplasma* (Brader *et al*., 2017). For the stem part residing in soil, such as tubers and stolons, several bacterial taxa were shown to inhabit these organs and to derive from the surrounding soil as it has been described for roots (Kõiv *et al*., 2015).

In leaves, several reviews have focused further on the distribution, range and bacterial taxa with presence inside substomatal chambers, parenchyma and the vascular system. As for stems, microorganisms have different routes of colonization, the ones inside the vascular system mostly derive from root xylem vessels, while those close to the surface derive from the external environment of leaves (Vacher *et al*., 2016). Sabaratnam and Beattie (2003) compared colonization characteristics of the pathogen *Pseudomonas syringae* pv. *syringae* B728a and a non-pathogenic strain, *Pantoea agglomerans* BRT98, on and inside leaves of bean and maize. *P. syringae* B728a could colonize the leaf interior, while the *Pantoea* strain could not. On pear leaves, *P. syringae* pv. *syringae* multiplied on the leaf surface, before colonizing internal leaf tissues through the trichome base and fissures in the cuticular cell layer (Mansvelt and Hattingh, 1987). This pathogen also showed to colonize xylem vessels and the leaf apoplast by using syringolin A that suppresses plant resistance by blocking SA signalling (Misas-Villamil *et al*., 2013). However, also non-pathogenic strains can colonize leaves endophytically as seen, for instance, with *Methylobacterium* PA1 (Peredo and Simmons, 2018).

In flowers, various bacteria were found to colonize the epidermis, the xylem vessels, the ovary, the ovules and stigma as well as other parts such as the flower receptacle, the petal, sepal and anthers with their filaments as well as pollen (Compant *et al*., 2011). Firmicutes, Actinobacteria and Proteobacteria strains have been, for instance, isolated from these organs (Shi *et al*., 2010; Compant *et al*., 2011; Fürnkranz *et al*., 2012). Colonization is related to the surface of the flowers for bacteria inhabiting niches close to the surfaces of the organs, while other routes involve colonization from roots to the aerial plant parts as discussed with pathogenic (Maude, 1996) or non-pathogenic strains (Compant *et al*., 2011). In fruits, bacteria have been also visualized as colonizing the exocarp, the mesocarp and the endocarp in grapevine or melon as well several plants (Compant *et al*., 2011; Glassner *et al*., 2015) and in the parenchyma or xylem vessels. Inside fruits, seed tissues inhabiting microorganisms are the outer and inner seed coat parts, the embryo hypocotyl root-axis, the plumule and the cotyledon as well as the endosperm (Escobar Rodríguez *et al*., 2018b; Glassner *et al*., 2018) (Fig. 1). Seeds may also host pathogens, which may enter via the floral pathway as seen for instance with *Xanthomonas citri* subsp. *fuscans* in bean seeds (Darsonval *et al*., 2008). This pathogen was observed entering seeds through vascular elements and parenchyma of funiculus, but also via the micropyle and testa. The same pathogen was also found inside seeds on radicle surfaces, in

cotyledons and plumules (Darrasse *et al*., 2018). Also, the beneficial endophyte *P. phytofirmans* strain PsJN can colonize the seed embryo of pepper, tomato, wheat and maize, using the floral pathway (Mitter *et al*., 2017).

Bacterial traits enabling life in the endosphere

Several bacterial traits enable life in the plant endosphere (Compant *et al*., 2010; Pinski *et al*., 2019). Lipopolysaccharides, flagella, pili, and twitching motility (Duijff *et al*., 1997, Dörr *et al*., 1998, Böhm *et al*., 2007; Tadra-Sfeir *et al*., 2011) have been linked to endophytic colonization and are also important for rhizosphere competence (Compant *et al*., 2010). Bacteria have also to enter inside plants and this requires uptake and degradation of plant-derived compounds. Pathogens are known for using multiple tools including carbohydrate active enzymes to enter plants (Brader *et al*., 2017), and the same type of enzymes are utilized by non-pathogenic strains. For example, a mutant of *Azoarcus* sp. BH72 devoid of endoglucanase activity had a decreased ability to colonize rice (Reinhold-Hurek *et al*., 2006). Comparative genomics of endophytic bacterial strains revealed that genes related to motility, chemotaxis, signal transduction, transcriptional regulators, stress-related enzymes, transporters and secretion systems are important for colonization of host plant internal tissues (Hardoim *et al*., 2015). Furthermore, expansins could be also associated with endophytic colonization as seen with a mutant of *Bacillus subtilis* 168 (Ampomah *et al*. 2013). Once inside plants chemotaxis towards L-arabinose present in xylem vessels of cucumber has been shown for strains of *Pseudomonas* spp. (Malfanova *et al*., 2013). Bacteria also have to adapt to the rather stressful environment inside plants and detoxification mechanisms have been shown in several endophytes (Compant *et al*., 2010). Furthermore, siderophore and biocontrol metabolites may have a role for endophytic colonization as well as secretion systems. For instance, it has been shown that a knock-out mutant of *Kosakonia* lacking the type 6 secretion system (T6SS) showed significantly reduced rhizosphere and endosphere colonization (Mosquito *et al*., 2020). *Herbaspirillum rubrisubalbicans* M1 T3SS mutants were also less successful in endophytic colonization (Schmidt *et al*., 2012).

Functions exhibited by non-pathogenic endophytes in the plant endosphere

Plant-associated microorganisms are known for various functions, which are particularly important for the plant host and which have been reviewed recently (Hardoim *et al*., 2015). These functions involve N cycling, phosphate mobilization, plant defense induction, antibiotic production, out-competition of pathogens, as well as improving plant tolerance to biotic and abiotic stresses (Turner *et al*., 2013). These activities have been widely demonstrated, either by lab assays or genome analysis of individual strains or by metagenome analysis (Sessitsch *et al*., 2012; Saminathan *et al*., 2018; Carrión *et al*., 2019). However, most of these analyses indicate potential functional activities or show activity in lab assays, but there is only scarce information on activities *in planta*, especially when plants are grown in the field. The benefits of plant-associated microorganisms, particularly of non-pathogenic endophytes, for the health and growth of their host have been demonstrated in numerous studies inoculating plants with individual microbial strains. However, inoculation experiments or application also often fails under field or more natural conditions, which may be due to limited colonization and/or due to no or low activity. In some studies, *in situ* activity has been analyzed by expression or transcriptome analysis, which can indicate functional activities (Schenk *et al*., 2012). Sessitsch *et al*. (2012) showed, for example, that different N-cycling genes are expressed in rice roots indicating N fixation, nitrification and denitrification processes *in planta*. More recently, Carrión *et al*. (2019) showed the transcriptional and functional analysis of disease-suppressive bacterial consortia demonstrating the role of secondary metabolites produced by the endophytic root microbiome. Furthermore, Xu *et al*. (2018) demonstrated that drought increases Actinobacteria populations in sorghum roots and has a significant effect on the transcriptional activity of the root-associated microbiome. Genes associated with carbohydrate and amino acid metabolism and transport showed an increased expression under drought, and the shift was largely due to actinobacterial activity and function. Along these lines, Sheibani-Tezerji *et al*. (2015) showed by transcriptome analysis that the endophyte *Paraburkholderia phytofirmans* PsJN senses and responds to plants under osmotic stress. Also, soil contamination was shown to influence the transcriptome of willow associated microbiome (Yergeau *et al*., 2018). Saminathan *et al*. (2018) demonstrated by metagenomic and metatranscriptomic analysis the role of the endophytic fruit microbiome in carbohydrate metabolism and ripening of watermelon fruits.

Effects of endophytes on plants and effects of plants on endophytes

Many efforts have been put on studying the possible effects of endophytes on plants and following the current definition of endophytes (Hardoim *et al*., 2015), the interaction ranges from mutualism to pathogenicity. While the elucidation of the effects of endophytes on plants is the aim of an ever faster growing number of studies focusing

on stimulation of plant growth through the production of plant hormones, increasing the availability of nutrients and minerals, fighting or outcompeting plant pathogens, and eliciting resilience to biotic and abiotic stresses (reviewed, e.g. in the study by Bulgarelli *et al*., 2013; Mitter *et al*., 2013; Hardoim *et al*., 2015), we still have limited understanding on the effects of plants on their microbial inhabitants. The plant is an environment for microorganisms with different ecological niches, interconnected at the spatial and temporal scale. Moreover, plants are under constant influence from external (environmental) and internal (developmental) factors, resulting in a dynamic system of adaptive responses of physiological processes. Thus, existence in the plant may require microorganisms to be highly adaptable, both on community and cellular level. From studies employing community sequencing, we know that the endophytic communities are dynamic and change during plant development (Shi *et al*., 2014; Ren *et al*., 2015; Borruso *et al*., 2018), and in response to biotic (Bulgari *et al*., 2012; Kõiv *et al*., 2015; Wemheuer *et al*., 2019) and abiotic stimuli (Kandalepas *et al*., 2015; Ren *et al*., 2015) of plants. The observed shifts in the endophyte communities, which may be explained by different abilities of individual species to adapt to changes in the plant environment as well as by interactions between species within the community. In this context, we may speculate that the ecological concept of species-sorting (Leibold *et al*., 2004; Holyoak *et al*., 2005) applies also to microbial communities inside plants. This implies that each plant compartment with its specific environmental conditions is colonized by a metacommunity of microorganisms and the different compartments with their metacommunities are connected through bacterial dispersal in xylem vessels. However, changes in the composition of endophytic bacterial communities must begin with a reaction of microorganisms on a cellular level to specific stimuli in the plant.

In plants, the response to changing environmental conditions and development processes often cause a shift in the cellular redox state. In a previous study, we analyzed the genetic response of the endophyte *P. phytofirmans* PsJN to osmotic stress of the host plant (Sheibani-Tezerji *et al*., 2015). The gene expression pattern indicated that the bacterium noticed and reacted to the changed redox conditions in the plant. Among other genes involved in the defense against oxidative stress, a cell surface signalling system was activated, which is involved in adjusting the iron acquisition to the redox status (Sheibani-Tezerji *et al*., 2015). Similarly, the endophyte *Gluconacetobacter diazotrophicus* PAL5 showed increase expression of genes encoding for ROS-detoxifying enzymes such as superoxide dismutase and glutathione reductase during the colonization of rice roots (Alquéres *et al*., 2013). It also has been shown that H_2O_2 breakdown by endophytes might also be involved in the adaption of bacteria to the redox conditions during germination in seed and seedling (Gerna *et al*., 2020). Hydrogen peroxide might also shape the microbial community in floral nectar. In principle, nectar contains everything microorganisms need for growth, i.e. different sugars, amino acids and organic acids. However, growth of microorganisms is controlled by the activity of specialized nectar proteins (nectarins), which maintains a redox cycle, allowing for high levels of peroxide in nectar (Carter and Thornburg, 2004). Taking all these findings into consideration, it is not surprising that based on metagenomics data genes for the detoxification of reactive oxygen species are prevalent among endophyte communities (Sessitsch *et al*., 2012). Apart from this rather general response to the plant environment, which is comparable to oxidative stress response in any other habitat, endophytes adjust their behavior also specifically in response to plant signals. This might be particularly important during plant colonization.

Most endophytes derive from the soil and the root is the initial contact point with the plant, and the detection of signal molecules in the root exudates often initiate the symbiosis between plants and endophytes. For example, plant flavonoids activate a series of bacterial genes, which results in production and secretion of the nod-factors in rhizobia necessary for successful interaction between plant and bacterial cells (Geurts and Bisseling, 2002). Similarly, rice root exudates induced the expression of genes involved in adherence and signal transduction, while flagella synthesis was downregulated in the endophyte *Azoarcus* sp. BH72, indicating that the bacterium was primed for the switch from the rhizosphere to the plant endosphere (Shidore *et al*., 2012). Furthermore, chemoattraction by plant-released oxalate is involved in the early steps of colonization of lupin and maize by *P. phytofirmans* PsJN (Kost *et al*., 2014). Interestingly, only plant-associated members of the '*Burkholderia* complex' were found to grow on oxalate while plant pathogenic and human opportunistic species could not use it as carbon source, indicating a role of this sugar in communication between plant and beneficial endophytes (Kost *et al*., 2014). Volatile organic compounds (VOCs) play a similar role in the specific selection of microbial colonizers by plants in the rhizosphere, phyllosphere as well as anthosphere by either suppressing the growth of microorganisms or serving as carbon source for the growth of others (reviewed by Junker and Tholl, 2013). Although there is already some indication that endophytic bacteria actively sense the plant environment and adjust their behavior in response to changing conditions and plant signals, we still are at the very beginning to understand the interplay between

plants and endophytes on a molecular level. Given the growing evidence, that the composition of endophytic communities may influence plant phenotypic traits (Li *et al*., 2019a), obtaining comprehensive understanding of the selective forces in the plant environment and the mechanisms of adaptation to this environment by endophytes and endophytic communities may open new avenues to optimize plant productivity and performance via modulating the endophytic communities.

Improving the performance of endophytes in promoting plant growth and health

Adaptation of strains to environmental conditions

Due to the many beneficial functions of endophytes for plant growth and health, there is a huge interest in applying them for a more sustainable crop production, e.g. as biofertilizers of crop protection agents. Bacterial endophytes have a great capability to adapt to changing environments and can express different phenotypes in different conditions such as pH, temperature, and host species. Rapid adaptation of endophytes may be a prerequisite for the plant holobiont to better adapt to stress conditions. Many studies have shown that plants growing in unfavorable conditions like hot, drought, saline, and heavy metal contaminated environments can develop different adaptation capacities to stresses, which is partially due to their associated microorganisms (Vandenkoornhuyse *et al*., 2015; Li *et al*., 2019a). As discussed above, endophytes need to show a number of characteristics to colonize plants internally such as motility and chemotaxis functions or degradation of reactive oxygen species. Moreover, transcriptome analysis of the endophyte *Bacillus mycoides* EC18 showed that an upregulation of genes involved in amino acids metabolism, transcriptional regulators, and signal transduction can play an important role in the adaptation of endophytic strains to their ecological niche (Yi *et al*., 2017). Scheuerl *et al*. (2020) addressed also the question on how the surrounding bacterial community affects evolutionary trajectories of strain adaptation to new niches by studying environmental samples in artificial micro-ecosystems. They found that the diversity of surrounding bacterial communities as well as characteristics of the strain such as genome size are important factors for strain adaptation to new environmental conditions (Scheuerl *et al*., 2020). Although this study addressed environmental microorganisms (and not specifically endophytes), this kind of experimental evolutionary approach can help to better understand the required traits for successful penetration and internal plant colonization by endophytes.

In endophytes, horizontal gene transfer (HGT) can be an important natural evolutionary mechanism for host adaptation and acquisition of novel genes. Sufficient experimental evidence has proven the implication of HGT for ecological behavior and biotechnological application. For example, novel functions acquired by endophytic strains during HGT events played role in toluene biodegradation and disease control in corn and wheat (Wang *et al*., 2010). Moreover, HGT events conferred novel traits in endophytes, which are important for the degradation of volatile organic contaminants (Weyens *et al*., 2009) and for the resistance against heavy metals (He *et al*., 2020). In particular, bacteriophage-mediated HGT may be a powerful pathway for adaptation and acquisition of new traits (Obeng *et al*., 2016; Harrison and Brockhurst, 2017). Lately, the role of phages has been primarily addressed in the human gut and a healthy human gut phageome has been proposed (Manrique *et al*., 2016). The role of phage-mediated HGT in the plant environment is under-investigated but is likely to play an important role for the adaptation of endophytes to different plant compartments or physiological conditions (Pratama *et al*., 2020). Understanding the key functions involved in bacterial adaptation including the role of HGT will be of further importance to benefit from endophytes to enable holobiont adaptation to stressful conditions.

Genetic improvement of endophytes

Genetic manipulation of non-pathogenic endophytic strains could be a useful method as an alternative to genetic modification of the plant (Li *et al*., 2017). Genes related to endophytic colonization and adaptation, plant growth promotion, biocontrol of plant pathogens and insects can be introduced into strains to confer new traits. For instance, the *Bacillus thuringiensis* Bt gene was introduced to the endosphere colonizer *Clavibacter xyli* subsp. *cynodontis* to produce the endotoxin for insect control (Tomasino *et al*., 1995). Similarly, silkworms were controlled by an insecticidal protein, which was produced by the endophytic *Burkholderia pyrrocinia* JK-SH007 transformed with the Bt endotoxin gene (Li *et al*., 2017). Moreover, an antifungal gene was incorporated into the genome of the endophyte *Pseudomonas putida* WCS358r for biocontrol of pathogenic *Fusarium* spp. (Glandorf *et al*., 2001). The capability of endophytic colonization of *B. thuringiensis* has also received special attention for the development of new type of insect-resistant crops (Sauka, 2017). Although genetic engineering of endophytes is promising to improve the performance of individual microbial strains, the release of genetically engineered microorganisms is not permitted in many countries.

Genome editing tools, i.e. the use of the CRISPR-Cas9 system has been shown to be a rapid and efficient tool for genome engineering and has been utilized efficiently

for genome editing in various organisms including plants, fungi and bacteria (Deng *et al*., 2017). However, optimization is still needed to be used in most microbial taxa. More recently, Li *et al*. (2019b) presented an advanced multiplex site-specific genome engineering (aMSGE) tool for incorporating multi-locus biosynthetic gene clusters encoding for natural products in actinomycetes. This highly efficient approach may be applied to a wide range of bacteria to enhance synthesis of important biomolecules.

Design of endophyte consortia

The design and application of microbial consortia are a new and promising approach to enhance the positive effects of inoculation on plants. Well-selected microbial consortia may adapt more rapidly to diverse conditions by making positive population-level associations, such as biofilms and microbial mats. Several studies have proven the advantages of consortia over single strain inoculation in several agronomic crops (reviewed in the study by Compant *et al*., 2019). Multiple strains in endophyte consortia have also been shown to promote plant growth and to mitigate abiotic stresses in tree species (Aghai *et al*., 2019). For many years, microbial strains have been combined in a non-targeted manner on a trial and error basis obtaining variable results. However, recently, different, knowledge-based approaches have been proposed to develop microbial consortia showing improved benefit for their host, either by colonizing more efficiently and/or by showing enhanced plant growth-promoting activities. One approach, which has been suggested is to base the design of microbial consortia on microbial network analysis, as also outlined by Vannier *et al*. (2019). Particularly, core microbiome taxa, i.e. a subset of the microbiota associated with a given host irrespective of the macroenvironment (Lemanceau *et al*., 2017), are considered to be highly important for plant fitness and to have established through evolutionary mechanisms for selection and enrichment of taxa containing key functional traits (Lemanceau *et al*., 2017). Along these lines, Kong *et al*. (2018) suggested simplified microbial consortia (SMC) comprising core microbial strains identified by microbial community sequencing and network analysis. It has been further demonstrated that the development of SMC from complex microbial communities can be feasible by combining enrichment with the adapted dilution-to-extinction approach to obtain stable and functional microbial consortia (Kang *et al*., 2019). However, also satellite taxa and rare microbiome taxa have been reported to have important functions (Hol *et al*., 2015). Therefore, a more detailed understanding on the role of core and satellite taxa to different microbiome functions is required to further improve a microbiome-based design of microbial consortia.

A second approach for the design of microbial consortia is to make use of microbial consortia with synergistic or complementary functions including high persistence in the target environment. Here, a deep functional understanding of microbial genomes will help to design such consortia. Secondary metabolites produced by endophytes, for example, play an important role in biofilm formation, plant colonization and in suppressing plant diseases or by activating plant defense (Brader *et al*., 2017). The rapid evolution of bacterial genomes and metagenomes data has led increasingly to genome mining to identify mechanisms and novel bioactive compounds. For instance, Belbahri *et al*. (2017) used various genome mining tools to predict core and accessory gene clusters in all sequenced *Bacillus amyloliquefaciens* genomes to evaluate their potential to synthesize secondary metabolites and to promote plant growth. They found that particularly the accessory part of the analyzed genomes harbor gene clusters largely related to secondary metabolite production, whereas genes in the core genome were suggested to play an important role in strain adaptation to plant-associated habitats (Belhabri *et al*., 2017). Improved genome prediction of microbial functions in relation to plant performance but also in relation to survival and activity under different, ecological conditions will lead to the improved design of microbial consortia.

A third approach, which has been proposed, is based on an ecological framework (Hu *et al*., 2016, 2017). The authors showed that the survival of randomly combined, closely related *Pseudomonas* strains increased with increasing consortium diversity. Increased *Pseudomonas* diversity applied onto potato plants also led to increased out-competition and antagonism of the plant pathogen *Ralstonia solancearum* (Hu *et al*., 2016) as well as to a higher production of plant hormones, siderophores and assimilation of nutrients (Hu *et al*., 2017). This approach needs to be further tested and validated for other taxa and applications. Nevertheless, irrespective of the approach how to design microbial consortia, appropriate formulations and carrier/delivery systems to the crops should be considered to overcome the environmental constraints, which might affect the functioning of consortia in the field conditions (Sessitsch *et al*., 2019).

Methodological challenges and limitations to study endophytes

Endophyte research has received increasing attention in the last years. However, due to the presence of the plant host organelles resembling bacterial cells as well as generally rather low bacterial densities, methodological

considerations are important to address. An overview on the experimental flow for hypothesis-driven and cross-disciplinary endophyte research is shown in Fig. 2.

The first step of endophyte studies, surface-sterilization, should be standardized

Endophytes can be studied at various biological organization levels, from the strain to the community. Regardless of the level, the first step is to remove epiphytes from the surface of plant organs, without altering the endophytic community. This step is important, but there is no consensus on how to remove epiphytic bacteria and no standardized protocols for each plant organ. There is also less quality control at this stage than at stages that require more advanced techniques, such as next generation sequencing (NGS) techniques. Recently, Chen *et al*. (2020a) surface-sterilized leaves of *Arabidopsis thaliana* with 75% ethanol for 1 min before isolating endophytes, or with 5% bleach (NaClO) for 1 min before metabarcoding analysis. Morella *et al*. (2019) surface-sterilized tomato fruits by soaking them in 75% ethanol for 20 min, and surface-sterilized tomato seeds by first sonicating seeds to remove epiphytes, then sterilized them with 2.7% bleach for 20 min. They verified, by plating, that no culturable bacteria were present in post-sterilization washes with sterile water. Bergna *et al*. (2018) surface-sterilized tomato roots by soaking them in 3% bleach for 5 min and imprinted them on agar plates as a sterility check. Wemheuer *et al*. (2019) used serial washes of ethanol and bleach to surface-sterilize maple tree leaves and checked the effectiveness of sterilization by PCR amplification of the 16S rRNA gene in final washes. Tween surfactants are sometimes used to improve the detachment of epiphytes (Rodríguez *et al*., 2019), and combinations of cultivation and PCR have been used for checking epiphyte removal (Wemheuer *et al*., 2020). Surface-sterilization by flaming has also

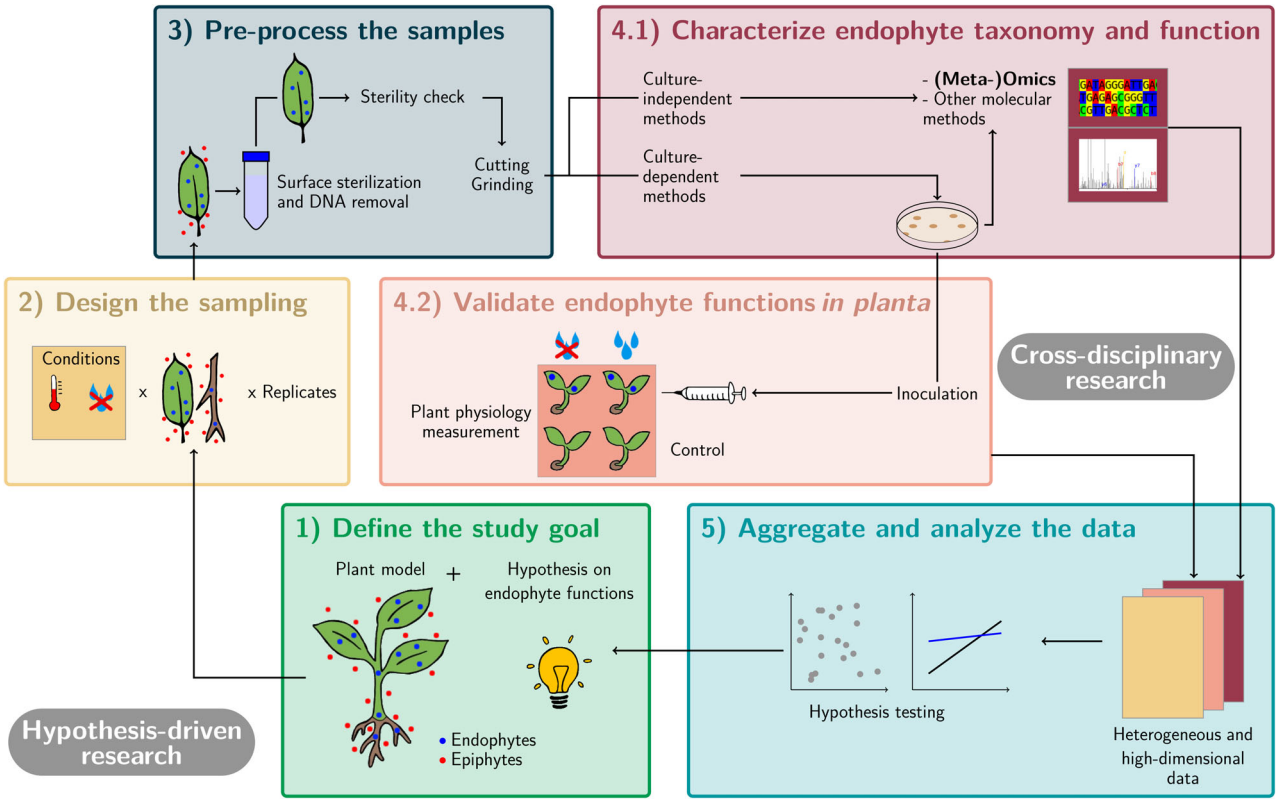

Fig. 2. Experimental flowchart for hypothesis-driven and cross-disciplinary endophyte research. To unravel plant endophyte functions, 1) the study goal needs to be defined, including the hypothesis to test. 2) The sampling design should allow to test the hypothesis or to collect the endophytic strains that will be necessary to test the hypothesis. 3) Plant tissue samples should be surface-sterilized to remove epiphytic microbes and their DNA, and the effectiveness of sterilization should be checked. This step, which relies on low-cost and low-tech methods, is often overlooked. 4.1) Taxonomic and functional information about endophytes are usually required to test the hypothesis. Meta-omic approaches are part of the multitude of methods available, but they are not the only ones. Their use cannot by itself justify a study. 4.2) Culture-dependent approaches are complementary to meta-omic approaches. Isolated strains or synthetic communities of endophytes can be inoculated to assess their influence on plant physiology. 5) The data should be analyzed statistically to test the hypothesis. This step requires advanced bioinformatic and statistical methods because meta-omic approaches provide heterogeneous and high-dimensional datasets that need to be reduced and integrated to make sense.

been used for studying the stem endosphere in grapevine (Deyett and Rolshausen, 2020). Recently, Binetruy *et al*. (2019) showed that the choice of sterilization protocol has a major impact on the measure of microbial diversity associated with insects and even suggested a reconsideration of results of previous studies. We should, therefore, not underestimate the variability triggered by protocols of surface-sterilization and sterility check (or bacterial DNA absence check) in the studies of plant endophytes. This variability will induce biases in meta-analyses of sequence datasets, which will increasingly be used in the future to get generic results on the plant microbiome (Rocca *et al*., 2018). A standardization of this first step (Fig. 2), at least for major crops and model plant species, therefore seems highly important, also because the following steps are generally costly and time-consuming, especially if they require NGS. Recently, Saldierna Guzmán *et al*. (2020) performed a comparison of leaf sterilization protocols for two species, an angiosperm and a gymnosperm. They found that complete removal of the cuticle was required to achieve the sterilization of leaf surface in both species, but that the most effective reagents to remove the cuticle without damaging the integrity of leaf tissues differed between species. They also demonstrated that scanning electron microscopy (SEM) is more efficient than PCR and imprints in checking sterility. Further studies, along the lines of this first study, are needed to determine whether the results are generalizable to other species in the angiosperm and gymnosperm groups.

Traditional methods have still a role to play in endophyte studies

NGS techniques have revolutionized our understanding of the plant microbiome over the past decade and their benefits no longer need to be demonstrated. Metabarcoding approaches, in particular, have become standard in studying the structure of plant endophytic bacterial communities (e.g. Gdanetz and Trail, 2017; Donald *et al*., 2019; Vergine *et al*., 2019; Wemheuer *et al*., 2019; Chen *et al*., 2020a, 2020b; Deyett and Rolshausen, 2020; Kuźniar *et al*., 2020), because they encompass both the cultivable and non-cultivable fraction of the bacterial diversity. However, these approaches have biases and limitations (Beckers *et al*., 2016; Lucaciu *et al*., 2019; Zinger *et al*., 2019). A major issue, in the specific case of bacterial endophytes, is the presence of plant chloroplasts. Specific primers and blocking primers were developed to avoid their amplification (Chelius and Triplett, 2001; Redford *et al*., 2010; Fitzpatrick *et al*., 2018) but despite these developments, amplification of genes from bacterial endophytes is still challenging. Therefore, more traditional methods, such as isolation, culture and fingerprinting, have been maintained and are becoming popular again. For instance, traditional fingerprinting methods, such as terminal restriction fragment length polymorphism (T-RLFP), can yield results comparable to recent metabarcoding approaches regarding the structure of plant bacterial endophytic communities, but their cost is much lower, making them accessible to a larger scientific community (Johnston-Monje and Mejia, 2020). Compared to second-generation metabarcoding approaches, isolation and culture of bacterial endophytes permit more accurate taxonomic identification through the sequencing of longer portions of marker genes (Asghari *et al*., 2019) or whole-genome sequencing (WGS) (López-Fernández *et al*., 2015; Chaudhry *et al*., 2017; Jauri *et al*., 2019; Eida *et al*., 2020). They also permit the phenotypic characterization of isolates and the identification of the genes and metabolic pathways governing the phenotype. Moreover, culture-dependent approaches pave the way for experimentation on plant–endophyte interactions. For example, isolated strains can be reassembled to form synthetic communities (SynCom) (Paredes *et al*., 2018; Carlström *et al*., 2019; Liu *et al*., 2019; Vannier *et al*., 2019), which can then be re-inoculated to assess their role on plant growth and health. Chen *et al*. (2020a) used such experimental approach, in *A. thaliana*, to demonstrate the causal role of the foliar endophytic bacterial community in plant health. Several recent studies combined metabarcoding approaches with culture-dependent approaches to go beyond the simple taxonomic description of endophytic communities associated with plant organs. Donald *et al*. (2019) combined metabarcoding data with co-occurrence analyses and co-cultures, to decipher interactions between foliar endophytes of tropical palms. Gdanetz and Trail (2017) also combined metabarcoding approaches with microbiological assays to identify antagonists of a major fungal pathogen in wheat. However, those culture-dependent methods are still challenging as many plant endophytes are yet unculturable or need specific plant-based culture media (see Sarhan *et al*., 2019 for a review). In the future, it may be useful to invest in the development of plant-based culture media to isolate and characterize more endophyte species, and to re-invest in low-throughput culture-dependent methods and traditional fingerprinting methods to promote the study of endophytes of a larger range of plant species, including those from low-income countries. In parallel, where possible, we should of course continue to use and develop -omics technologies to better understand the role of bacterial endophytes for the plant response to biotic and abiotic stresses.

Multi-omic approaches are the future the field but conceptual frameworks should develop in parallel

A multitude of -omics tools – including (meta)genomics, (meta)transcriptomics, (meta)proteomics, metabolomics, and also culturomics – are now available to understand the functional role of endophytic communities (Kaul *et al*., 2016; Levy *et al*., 2018; Sarhan *et al*., 2019). Levy *et al*. (2018) recently reviewed the objectives, strengths and limitations of each of these tools. A shared feature of all these tools is that they evolve extremely fast and provide high dimension and heterogeneous datasets that need to be reduced and integrated to elucidate valuable information, using advanced bioinformatics and statistical approaches (Rohart *et al*., 2017). All these tools give complementary insights into the function of bacterial endophytes. For instance, Sessitsch *et al*. (2012) pioneered in the field of metagenomics, also called shotgun metagenomics, by identifying the genes, traits and metabolic processes that are important for the endophytic lifestyle. Recently, Terra *et al*. (2019) combined a transcriptomic and proteomic approach, to identify the metabolic pathways and functions that are activated, in an endophytic bacterial strain that promotes growth in sugarcane, when it is exposed to the apoplast fluid. These -omics methods can be complemented by other approaches, such as confocal microscopy, which, after inoculation of a strain into an axenic plant, makes it possible to verify that the strain is indeed an endophyte and to identify the tissues it colonizes (Bünger *et al*., 2020). Agtuca *et al*. (2020) combined confocal and fluorescence imaging with *in situ* metabalomics to discover the metabolites that are over-expressed in plant tissues colonized by an endophytic bacterium. The future challenge in plant endophyte research is, however, not only to master and combine advanced tools, but also to link research questions to conceptual frameworks (Saikkonen *et al*., 2020). Multi-omics approaches are undoubtedly the future of the field and will provide microbiology-based innovations in the field of agriculture (Compant *et al*., 2019). However, endophyte research should not only be driven by technical developments, fluxes of data and bioeconomy, but also by hypotheses in the fields of ecology and evolution (Saikkonen *et al*., 2020). To this end, we need to develop conceptual frameworks that consider the effects of the plant microbial community on the plant phenotype (and *vice et versa*), and upscale these results across space and time. Such a framework has to consider the whole system, i.e. the host plant, the surrounding microbiome, other organisms in the plant environment as well as the environment with all structural and functional aspects and multitrophic interactions. The development of these frameworks will require interdisciplinary approaches that go beyond -omics methods, by integrating evolutionary ecology, plant physiology and pathology, plant genetics and epigenetics, and Earth and ecosystem sciences. They will allow us to better understand the functioning and evolution of plant holobionts and to predict their response and feedback effects on environmental changes, at the global scale (Saikkonen *et al*., 2020; Zhu and Penuelas, 2020).

Conclusions and future prospects

The plant endosphere represents a highly diverse and dynamic environment for bacteria, providing multiple niches in different plant compartments for proliferation. Due to plant development as well as due to fluctuating environmental conditions endophytes are exposed to highly dynamic conditions as plants respond to changing conditions associated with physiological changes. In the last years, we have accumulated knowledge on microbial community dynamics in the various plant micro-environments, however, full understanding on which physiological conditions favor which microorganisms is still lacking. Here, also advanced knowledge on the identity and dynamics of plant metabolites will be needed as well as the linkage between microbiomes, plant and microbial metabolites and environmental/physiological conditions.

Similarly, we have obtained knowledge on the potential activities on plant-associated microorganisms including endophytes, either by lab assays, genome or metagenome analysis. However, the activities performed *in planta* are poorly understood, particularly considering the diversity of micro-environments and conditions endophytes are exposed to. To fully understand the role and contribution of endophytes to plant performance and functioning we need to address *in planta* activities, linking colonization and cell numbers in various plant tissues and at various plant growth stages with activity levels. Activity levels may be assessed, e.g. by transcriptome analysis, however, other complementary -omics approaches like metabolomics will be further useful to understand microbial functions and activities in the endosphere. Furthermore, a better understanding of the plant response to endophytes, particularly in context of environmental conditions, will be required as well as a better understanding on the interplay between plants and endosphere microbiota at a molecular level. Also, microbiome research has to move from analyzing structural composition of microbiota to understanding functional aspects, e.g. of core taxa being consistently associated with a given plant species and satellite microbiota.

Understanding microbial communities and their activities in the plant endosphere is challenged by methodological constraints. The presence of transcriptomes and genomes from the plant host or from microorganisms in the phyllosphere and rhizosphere challenges any *in*

planta analysis of endophytes. These constrains combined with huge data amounts starting to arise from -omics studies lead to a real need for the standardization of protocols, e.g. for surface sterilization or sample preparation. Hypothesis-driven concepts and research approaches combined with multi-disciplinary analysis (Fig. 2) will lead to a better understanding of endophyte activity and adaptation to their host plant, plant–endophyte interactions, and overall, the evolution of the plant holobiont. This knowledge will play a key role in the design of microbial consortia showing synergistic activities to contribute to a more sustainable, microbe-based crop production.

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