Accepted Manuscript

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PII: S0944-5013(17)31185-0
DOI: https://doi.org/10.1016/j.micres.2018.01.005
Reference: MICRES 26123

To appear in:

Received date: 29-11-2017
Revised date: 13-1-2018
Accepted date: 20-1-2018

Please cite this article as: del Carmen Orozco-Mosqueda Ma, del Carmen Rocha-Granados Ma, Glick Bernard R, Santoyo Gustavo.Microbiome engineering to improve biocontrol and plant growth-promoting mechanisms.*Microbiological Research* https://doi.org/10.1016/j.micres.2018.01.005

Microbiome engineering to improve biocontrol and plant growth-promoting mechanisms

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ABSTRACT

A plant microbiome includes a microbial community that typically interacts extensively with a plant. The plant microbiome can survive either inside or outside of plant tissues, performing various plant beneficial activities including biocontrol of potential phytopathogens and promotion of plant growth. An important part of the plant microbiome includes plant growth-promoting bacteria (PGPB) that commonly reside in the rhizosphere and phyllosphere, and as endophytic bacteria (inside of plant tissues). As new plant microbiome-manipulating strategies have emerged in recent years, we have critically reviewed relevant literature, chiefly from the last decade. We have analysed and compared the rhizosphere, phyllosphere and endosphere as potential ecosystems for manipulation, in order to improve positive interactions with the plant. In addition, many studies on the bioengineering of the endophyte microbiome and its potential impact on the core microbiome were analysed with respect to five different strategies, including host mediated and multi-generation microbiome selection, inoculation into soil and rhizosphere, inoculations into seeds or seedlings, tissue atomisation and direct injection into tissues or

wounds. Finally, microbiome engineering presents a feasible strategy to solve multiple agriculture-associated problems in an eco-friendly way.

Keywords: Plant microbiome, rhizosphere, endophytic bacteria, PGPB, biological control.

INTRODUCTION

The term ¨microbiome¨ has been defined in two different ways. For example, Bulgarelli et al. (2013) refer to the microbiome as the set of genomes of the microorganisms in a particular habitat, whereas they define the microbiota as the set of microorganisms of a particular habitat. Thus, they employ the term microbiota as a synonym of microbial communities. Some others use the term microbiome to mean all the microbes of a community, and in particular, for the plant microbiome, those microbial communities associated with the plant which can live, thrive, and interact with different tissues such as roots, shoots, leaves, flowers, and seeds (Haney and Ausubel, 2015; Haney et al., 2015; Mendes et al., 2013; Mueller and Sachs, 2015; Nelson, 2017; Turner et al., 2013). Here, we refer to the plant microbiome according to this last definition.

The microbiome is composed of several different types of organisms, including bacteria, fungi, protozoa, archaea, and viruses (Mueller and Sachs, 2015). Plant microbiomes can play a beneficial role, protecting the plant from potential pathogens, at the same time, improving growth, health, and production, as well as can conferring an adaptive advantage to plants (Berg et al., 2016; Haney et al., 2015). In particular, the bacterial microbiome, carrying out beneficial interactions is important for sustainable agriculture and has attracted more attention, compared to the other groups of organisms. Manipulation of the bacterial microbiome and the production of bioinoculants enables scientists to affect plant beneficial activities such as limits to growth, the action of phytopathogens, and promoting plant growth and health, thereby potentially reducing the use of chemical fertilizers (Adesemoye and Kloepper, 2009). Rhizospheric or endophytic bacteria that promote plant growth are known as plant growth-promoting bacteria (PGPB) (Kloepper et al., 1980; Santoyo et al., 2016).

PGPB may promote plant growth by direct or indirect mechanisms (Glick, 2012; Santoyo et al., 2012). Briefly, direct promotion of plant growth occurs when a bacterium facilitates the acquisition of essential nutrients or modulates the level of hormones within a

plant. Nutrient acquisition facilitated by PGPB usually includes elements such as nitrogen, phosphorus, and iron, which are essential for plant development (Calvo et al., 2017). Modulation of hormone levels may involve PGPB in the synthesis of one or more phytohormones, auxins, cytokinins, and gibberellins (Bhattacharyya et al., 2015, Pérez-Flores et al., 2017). In addition, some PGPB can lower the levels of the phytohormone ethylene by synthesising an enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, that cleaves the compound ACC which is the immediate precursor of ethylene in all higher plants (Glick, 1995; Glick, 2012). Indirect plant growth promotion occurs when a PGPB decreases plant damage after infection with a plant pathogen, including harmful fungi and bacteria. This is usually due to the inhibition of the pathogens by PGPB (Compant et al., 2005, Ryan et al., 2008, Santoyo et al., 2012; Santoyo et al., 2016). The mechanisms include the synthesis and excretion of antibiotics (such as 2,4-diacetylphloroglucinol), proteases, chitinases, bacteriocins, siderophores, lipopeptides (such as iturin A, bacillomycin D, and mycosubtilin), and volatile organic compounds, to mention a few (Leclère et al., 2005; Santoyo et al., 2012, Glick, 2012, Hernández-León et al., 2015, Martínez-Absalón et al., 2014). The above-mentioned capabilities make the microbiome an important component for the plant to carry out physiological functions, and in some cases, the microbiome is a fundamental part of basic processes such as plant development or growth of essential organs such as the root, for improved acquisition of nutrients and water (Gutiérrez-Luna et al., 2010). Though the plant microbiome is composed of numerous organisms, which play relevant roles, this review is mainly focused on bacterial species, particularly those with biocontrol and plant growth promoting activities.

LIVING INSIDE, OUTSIDE AND ON THE PLANT

Figure 1 shows the plant microbiome, as well as the regions or zones where the microbes can interact with the plant, either they live outside (rhizosphere), inside (endosphere) or on (phyllosphere) the plant. The rhizosphere is the soil portion influenced by plant roots (Hartmann et al., 2008). This microecosystem is the major region where chemical communications and the exchange of compounds and nutrients occur between the plant and soil microorganisms (Peiffer et al., 2013, Pieterse et al., 2016). A strategy to improve plant health and development includes the selection and modification of the

rhizosphere microbiome (Chaparro et al., 2012; Mendes et al., 2013; Swenson et al., 2000). The endospheric microbiome can be a subset of the rhizosphere microbiome (Germida et al., 1998; Márquez-Santacruz et al., 2010). The work by Bai et al. (2015) also found a huge overlap between the Arabidopsis leaf and root microbiomes, highlighting the dynamic state of microbes interacting with the plant host. The endosphere is the internal environment of plants wherein endophytes, including bacteria, archaea, fungi, and viruses inhabit, colonise, and survive without causing any harm to the plant (Compant et al., 2010).

Many studies have investigated the colonisation of internal plant tissues by endophytes. In a recent work, Santoyo et al. (2016) reviewed available data about various entry points, the rhizosphere being an important region for microbe entry into the plant because of its nutrient-rich nature, supplied by the root exudates, and hence its high concentration of microbes (Bradi and Vivanco, 2009). There are other areas of entry too, such as the lenticels, stomas, wounds, ruptures, and nodules. Endophytes can also be inherited by vertical transmission, through seeds (Santoyo et al., 2016). Some researchers have proposed that the internal microbiome of plants has an advantage over external bacteria (phyllosphere or rhizosphere) in that it not affected by soil conditions including the presence of bacterial predators. Moreover it has been proposed that (chemical) communication is more effective inside of the plant's tissues (Ali et al., 2012; Coutinho et al., 2015), because lower concentrations of metabolites secreted by the bacterial endophytes may exert a greater effect on the plant, compared to those secreted in an open environment such as the rhizosphere, where biotic and abiotic factors, can counteract and minimise its effect (Santoyo et al., 2017). For example, siderophores of bacterial origin excreted in the rhizosphere can be captured and internalised by other organisms such as fungi, which possess nonspecific transporters of the siderophore-Fe complexes (Philpott, 2006).

Several studies have shown that the composition of the rhizosphere is more diverse than the endosphere. In a study involving rhizospheric and endospheric 16S rRNA genes from Mexican husk tomato plants (*Physalis ixocarpa*), a higher number of operational taxonomic units (OTUs) were found in a library of clones from the rhizosphere compared to that from the root endosphere (i.e. 86 vs. 17, respectively). It should be noted that the number of clones randomly obtained from the endosphere versus the rhizosphere was statistically similar. The majority of the genera found in the rhizosphere, such as

Stenotrophomonas, *Burkholderia*, *Bacillus,* and *Pseudomonas*, were also detected in the internal tissues of the plant (Márquez-Santacruz et al., 2010). When cultivable bacterial endophytes were isolated and characterised at the molecular level from different agrofields of *Physalis ixocarpa* plants they coincided with the diversity of the OTUs found by Márquez-Santacruz et al (Rojas Solis et al., unpublished results). These results show evidence of the existence of a core microbiome, with beneficial functions in *Physalis ixocarpa* plants, that is inherited through generations.

The phyllosphere comprises the surface and the apoplast of leaf tissue (Vorholt, 2012). The great importance of the phyllosphere microbiome on biocontrol, and the promotion of plant growth, has been suggested for years. In fact, some authors have proposed that the foliar microbiota exerts beneficial activities comparable to that which is observed for rhizospheric and endophytic microbes (Lindow and Brandl, 2003; Peñuelas and Terradas, 2014). For example, many foliar microbiotas can fix nitrogen, this being the main mechanism to provide nitrogen to plants growing in tropical humid ecosystems (Abri et al., 2005). Other beneficial roles of the microbiota residing on the plant surface include the indirect protection against pathogens and the production of plant hormones. Thus, the possibility exists that foliar microbiota could be employed to reduce the use of agrochemicals to control leaf pathogens and promote plant growth (Peñuelas and Terradas, 2014). However, considerable additional research is needed to obtain a better understanding of the functioning of these microorganisms.

WHY MODIFY THE PLANT MICROBIOME?

The aforementioned mechanisms have been extensively studied, and several reports show the potential of PGPB to restrict the growth of phytopathogens and promote plant growth. However, why do we need to modify the microbiome? To the best of our knowledge, every plant that has ever been studied is associated with microorganisms. Moreover, all plants are known to contain endophytic organisms as part of their internal microbiome (Ryan et al., 2008). The plant microbiome is composed of active organisms that alter plant physiology and development and induce resistance systems against pathogens as well as tolerance mechanisms to various types of stress such as drought, saline, or contaminated soils (Santoyo et al., 2017; Yaish et al., 2017; Yuan et al., 2016). However, such functions are

not carried out by 'the whole microbiome', but by one or a few microbial species, as well as because of the additive or synergistic effects between two strains or more (Rojas-Solís et al., 2016; Timm et al., 2016). Therefore, these few bacterial species could be used to 'enhance' or activate defence systems in other plant species (heterologous hosts) that are susceptible to certain pathogens or abiotic factors such as salinity, as well as for other uses such as soil bioremediation or increased fruit yield (Mueller and Sachs, 2015, Yuan et al., 2016).

Different plants may have different tolerance capacities to biotic or abiotic stresses, growth at different latitudes or altitudes, or nutrient-poor soils, where, in many cases, their microbiome allows them to survive in such conditions (Santoyo et al., 2017). Therefore, the general interest is to transfer these capabilities to plants that lack them, including for the biocontrol of potential pathogens (Wicaksono et al, 2017; Yuan et al., 2016;). By transferring one or more microbial species, mainly bacteria, a greater amount of genetic material or gene numbers can be transferred, compared to single gene transfer (i.e., genes coding for *Bacillus thuringiensis cry* toxins) (Romeis et al., 2006), improving several functions at the same time. For example, some PGPB perform multiple direct and indirect growth-promoting activities, such as *Pseudomonas fluorescens* UM270 or *Arthrobacter agilis* UMCV2 (Avilés-García et al., 2016; Hernández-León et al., 2015; Hernández-Salmerón et al., 2016; Orozco-Mosqueda et al., 2013). These PGPB can exert multiple beneficial activities from the rhizosphere or endosphere (Mendes et al., 2013; Santoyo et al., 2016). However, additional studies are necessary to understand the interaction and/or consequences that PGPB could exert on the core microbiome (internal or external) of the plant species to be inoculated.

METHODS FOR DETECTING ENDOPHYTES WITHIN THE PLANT

When a bacterial strain is inoculated into the bulk soil or into the rhizosphere, with the intention of modifying the existing microbiome, it is relatively easy to confirm its presence by re-isolating the inoculated bacterium. However, the methods to study bacteria within plant tissues are more complex (Mitter et al., 2017). We can classify endophyte localisation methods as quantitative and qualitative (visual) methods. Quantitative methods allow us to determine the actual amount or number of cells that inhabit the tissue (usually expressed as

per gram of tissue). These methods include qPCR (which usually includes amplification of specific genomic regions such as the16S-23S rRNA intergenic spacer regions) (Avilés-García et al., 2016), in order to detect the presence of endophytes at the single cell level. Re-isolation (*de novo*) of endophytes may be a quantitative method. However, it requires researchers to select the pre-inoculated strains and differentiate them from the rest of the microbiome, for example by selecting for resistance to antibiotics because of previously analysed induced or spontaneous mutations (Wicaksono, et al., 2017).

Visual methods include tracking GFP (Green Fluorescent Protein), in situ hybridisation with fluorescence, and gene fusions with β-glucuronidase (GUS) (Avilés-García et al, 2016; Mitter et al, 2017; Patel and Archana, 2017). These methods can be semi-quantitative, because it is possible to quantify the spots where the strains are located in each tissue section. The advantages of such methods include the ability to determine the specific localisation at the level of the tissue, or even, cell type or intercellular zone. An easy and rapid although qualitative method is the detection by traditional PCR. This technique allows a researcher to determine the localisation of the endophyte of interest at the tissue level. However, multiple controls are required to rule out false-positive amplification. See Table 1 for a list of advantages and disadvantages of the methods discussed above.

ENGINEERING OF THE PLANT MICROBIOME

Strategies for inoculating strains to introduce and/or engineer the plant microbiome in plants are diverse. For example, they occur through host-mediated and multi-generation microbiome selection, inoculation into bulk soil, rhizosphere, seeds or seedlings, atomisation into tissues such as stems, leaves, and flowers, and direct injection into tissues or wounds. Some recent relevant studies involving the aforementioned techniques are reviewed below.

Host-mediated and multi-generation microbiome selection

According to Mueller and Sachs (2015), an engineering approach host-mediated microbiome selection is a method that selects microbial communities indirectly through the host and leverages host traits that evolved to influence microbiomes. Pioneering work in

which 'artificial selection of the ecosystem' was attempted on the basis of selecting the highest (high selection lines) or lowest (low selection lines) plant biomass of *Arabidopsis thaliana* previously modified by microbial interaction (bacteria, algae, protozoa and fungi) with the plant over 16 generations, was published by Swenson et al. (2000). In this study, the authors designed an aquatic microcosm that worked at low or high pH and allowed the degradation of the toxic compound 3-chloroaniline. Importantly, these experiments show that selection or bioengineering can be attempted at the ecosystem level rather than for individual organisms, as was previously the case. Unfortunately, the authors did not analyse specific changes in the microbiota across generations, which would have allowed a better picture of the biotic evolution of the microecosystem. More recently, Panke-Buisse et al., (2015) used a technique similar to that of Swenson et al. (2000); they undertook a multi-generation experiment with *Arabidopsis thaliana* Col-0 plants that could select the microbiome that influenced their life events such as late and early flowering. The microbiome was inoculated into 3 different *Arabidopsis* (Ler, Be, RLD) and *Brassica rapa* genotypes. The authors analysed the changes in the microbiome during different generations by sequencing the 16S rRNA genes and found that the plants inoculated with a microbiome associated with late flowering showed consecutive increases in inflorescence biomass for the 3 *Arabidopsis* genotypes, as well as an increase in the total biomass of *Brassica rapa*. Based on the observation that the increase in biomass correlated with an increased activity of the microbial extracellular enzymes associated with nitrogen mineralisation in soils, the authors proposed that their selection results based on different microbial communities have great potential to aid the use of microbiomes to address diverse agronomic and environmental problems.

Inoculation into the soil and rhizosphere

Inoculation of external strains from bulk or rhizospheric soils may change the structure of the microbial community. In semi-natural conditions, Chihaoui et al. (2015) analysed the effect of inoculation of *Agrobacterium* sp. 10C2 on nodulation, plant growth, and the rhizosphere microbiome of *Phaseolus vulgaris*, a nodule-inhabiting endophyte; this strain does not form nodules and is non-pathogenic. Inoculation with the 10C2 strain promoted a significant increase in the number of nodules and plant biomass. The beans that

were produced showed a significant increase in the contents of phosphorus, polyphenols, and flavonoids, and total antioxidant capacity. To evaluate the effect of the 10C2 strain on the microbiome, the terminal restriction fragment length polymorphism (TRF) technique of PCR-amplified 16S rRNA genes was used. Fifteen days after the inoculation of the 10C2 strain, the TRF richness was unchanged, but the bacterial community structure did change. However, when bean plants were cultivated in these soils for a much longer period of time, i.e. 75 days, both the TRF richness and structure were affected by the 10C2 strain. Interestingly, the TRF richness increased in the rhizosphere soil, but not in the soil without the plant. *Bacillus licheniformis*, *Bacillus pumilus*, *Paenibacillus koreensis*, and the genera *Arthrobacter*, *Microbacterium*, *Actinomyces*, *Brevibacterium*, among others (Chihaoui et al., 2015) have been implicated in the inoculation of 10C2 strain. It is noteworthy that such bacterial groups are widely known as plant growth promoters (Glick et al., 2015; Santoyo et al., 2012). The authors of this study inferred that the 10C2 strain positively influenced the rhizosphere through stimulation of the bacterial species known for their plant growth-promoting abilities.

Inoculation into seeds or seedlings

Studies on the interaction between the rhizosphere and endosphere inhabitants of slow-growing plants such as orchids are few. However, degradation of natural environments such as forests have limited the natural habitat of these plants, and therefore, there have been several attempts to rescue them. Certainly, orchids have specific associations with mycorrhizal fungi, and various studies account for this. However, a recent study (Pavlova, 2017) showed that inoculation of bacterial species such as *Pseudomonas fluorescens* and *Klebsiella oxytoca* into *Dendrobium nobile* Lindl. increases the adaptive, germination, and growth capacity of orchids. In this study, the authors followed the GFP colonisation pattern of *P. fluorescens* and *K. oxytoca* in the seeds, seedlings, and roots of the epiphytic orchid. It would have been interesting to analyse the impact of inoculation of these strains on the endophyte microbiome of orchids.

Recently, Rojas-Solís and colleagues (2018) reported a beneficial additive effect of two endophyte strains, *Pseudomonas stutzeri* E25 and *Stenotrophomonas maltophilia* CR71, when both strains were co-inoculated into the rhizosphere of tomato seedlings

(*Lycopersicon esculentum* cv Saladette), compared to single inoculation. A better plant growth-promoting effect was observed. Interestingly, the volatile cocktails emitted by strains E25 and CR71 were quite similar, highlighting the production of sulphur-containing compounds such as the antimicrobial volatile dimethyl disulphide (DMDS).

Tissue atomisation

The effect that the microbiome has on various phenotypic and physiological characteristics of plants may be due to the multiple mechanisms carried out by various PGPB (Hernández-León et al., 2015). It is common to identify the involvement of more than one mechanism in plant growth promotion (e.g., phytohormones or organic volatile compounds) as well as the biocontrol effect against phytopathogens (e.g., the production of antibiotic compounds) (Hernández-León et al., 2015; Pérez-Flores et al., 2017). On the other hand, the beneficial effects of the microbiome can be attributed to the interaction between individual organisms or functions. Timm et al. (2016) found a beneficial additive effect of two strains of *Pseudomonas* and *Burkholderia* isolated from *Populus deltoides*. The authors observed, by plant inoculation, that the mixture of these strains in *Populus* plants improved root biomass and plant photosynthetic capacity better than either individual inoculation or no inoculation. The plant response was analysed through gene expression (transcriptomic) analysis and it was found that inoculation of each strain turned on genes specific for *Pseudomonas* and *Burkholderia*, as well as those of the mixture, including genes encoding for the responses to ethylene and stresses such as temperature and salinity. Other genes involved in the synthesis of thiamine, sulphate, and lipids were modulated by the mixed inoculum. The authors also analysed the impact of the mixed inoculum on the metabolic profile of the leaf compared to individual inoculation.

In another recent work, Mitter et al. (2017) employed a new technique to introduce the endophyte bacterium *Paraburkholderia phytofirmans* PsJN by atomisation into the flowers of mono- and dicotyledonous plants. Importantly, the authors managed to modify the seed microbiome by vertical inheritance as well as the growth characteristics of wheat plants. In either greenhouse or field experiments, it was found that seeds that inherited the PsJN strain showed significant differences in plant development with respect to the controls (plants with an unmodified microbiome). This study also analysed the changes in the endogenous

microbiome of the seeds following the introduction of the endophyte PsJN, and found that certain groups significantly changed their abundance. Such changes were marked by a strong decrease in the population of α- and γ-Proteobacteria and an increase in β-Proteobacteria. The authors point out that one of the challenges of this technique that still needs to be addressed is that the modified microbiome should be inherited for more than one generation, as the plants in the second generation do not inherit the PsJN strain. However, this novel technique raises many expectations for bioengineering of plant microbiome without genetic manipulation of the plant.

Direct injection into tissues or wounds

Some plants are naturally resistant to certain pathogens, while others might be highly sensitive. The medicinal plant Mānuka (*Leptospermum scoparium*) native to New Zealand, produces antimicrobial oils that are effective against the pathogenic bacterium *Pseudomonas syringae*. Therefore, Wicaksone and colleagues (2017) questioned whether this biocontrol effect might be transferrable by inoculating *Actinidia deliciosa* (Kiwifruit) with bacterial endophytes from *L. scoparium*. In this experiment, three endophytic strains of the genus *Pseudomonas* that were originally isolated from *L. scoparium* were inoculated into wounds of *Actinidia deliciosa*; they were able to survive in this new host and confer resistance to *P. syringae* infection. In this way, antimicrobial capacities exerted by endophyte plant bacteria can be transferred to other plants susceptible to attack by pathogens.

Another recent study showed that direct inoculation of a PGPB into a plant, help the bacterium to colonise and survive within the plant. This is observed in the case of the biocontrol agent and PGPB *Arthrobacter agilis* UMCV2 (Orozco-Mosqueda et al., 2013; Velázquez-Becerra et al., 2013), which can survive direct injection into the stem of *Medicago truncatula* plants (Avilés-García et al., 2016). The authors point out that such colonisation may be more successful when nutrients (particularly Fe) in the plant are bioavailable (Avilés-García et al., 2016). Direct injection techniques involving bacterial endophytes have been employed in other plant species such as teocinte, the maize ancestor (*Zea mays*) (Johnston-Monje and Raizada, 2011). Interestingly, the authors observed that two isolates of *Enterobacter asburiae*, inoculated by stem injection, managed to leave the

endophyte environment and also colonised the rhizosphere. The data indicate two aspects of the stages of colonisation: the plant can expel bacteria into the surrounding or rhizospheric soil, as occurs with many low molecular weight compounds exuded by the roots, or the endophytes have their own mechanisms to open and close the entry door to the plant tissues. The two hypotheses have not been clarified until now and this point warrants further detailed study. Understanding such aspects may help us discover the mechanisms of entry and exit into and from the plant tissues for endophytes as well as or pathogenic organisms.

CONCLUSIONS AND PERSPECTIVES

The microbiome is proposed to be the "second genome" of various organisms by many groups (Clavel et al., 2016; Grice and Segre, 2012; Zmora et al., 2016), while others have proposed the term "holobiont" to define the broad role that is played by the microorganisms associated with plant or animal hosts (Zilber-Rosenberg and Rosenberg, 2008). What it is clear is that the microbiome plays a transcendental role in host interaction. The plant and animal intestine microbiome can perform similar functions such as participating in nutrient absorption, modulating gene expression, acting as biocontrol pathogens, and improving growth and health in general (Hacquard et al., 2015; Mendes and Raaijmakers, 2015; Ramírez-Puebla et al., 2013; Xue et al., 2015). The plant microbiome, though not discussed here, includes not only the bacterial communities, but also several other organisms, such as viruses, archea, fungi or nematodes, which have important effects on host biology. In some cases, the microorganisms may develop forced symbioses with their host (Gil, Latorre, and Moya, 2004). The microbial communities are highly active and influence the metabolism and physiology of the host. Some microbes are able to colonize the inner plant tissues, and therefore modify the core microbiome, which can be inherited in the subsequent generation of the plant; but the multi-generation inheritance of endophytic genome components in plants is one of the challenges involved in the use of endophytes. Microbiome engineering can have a significant impact on agricultural production (Mitter et al., 2017). Therefore, development of a (modified) microbiome with these desired characteristics is necessary.

The role of interaction between multiple biotic and abiotic factors in ecosystems such as the rhizosphere or the endosphere (although more stable) warrants more studies (Santoyo et al., 2017). It is necessary to select highly competitive organisms with good colonisation abilities (Rojas-Solís et al., 2016). These strains may have an advantage over other organisms, since they must compete for nutrients, displace other organisms, and survive in an environment as challenging as the rhizosphere. Breaking the barrier of the plant cell as well as colonising the tissues and producing compounds that positively modulate plant physiology or genetics can be equally challenging.

As far as we know, there are only a limited number of studies with the intention of engineering the foliar microbiome or the phyllosphere (Wu et al., 2013). Diverse difficulties have been proposed since a few decades ago and only a few cases exist with the intention to biocontrol some pathogens in this habitat; however, these approaches have met with little success (Andrews, 1990). One major challenge in this regard is the effect of abiotic or environmental factors, which can significantly modulate the phyllosphere biodiversity (Santoyo et al., 2017; Vacher et al., 2016). And, although the atomization of bacteria initially includes the colonization of the phyllosphere, the intention is that such strains can colonize the internal tissues of the plant. The goal is clear, to modify the internal microbiome such that the beneficial effects of this modification are transgenerational. Here, it is worth mentioning that Bodenhausen et al. (2014) found that the genotype of a plant can modify the foliar microbiota. Thus, two mutants of *Arabidopsis thaliana*, in the *acs2* and *pec1* genes, affected in cuticle formation and ethylene signalling, showed marked changes in the diversity of phyllosphere microorganisms compared to the wild-type plant. This work suggests that it is necessary, when studying plant-microbe interactions, to focus on both how the bacterial microbiome affects the plant but also on how the plant affects the bacterial microbiome.

Several strategies involving PGPB can modify the plant microbiome. However, a major challenge is to colonise the internal tissues of the plant. The strategies and methods of localisation employed by strains that successfully colonise and inhabit various plant tissues have their own advantages and disadvantages (Mitter et al., 2017; Peiffer et al., 2013). The objective of each study varies; in some cases, only the process of colonisation is followed, and in other instances, the growth-promoting effect or biocontrol activity is

observed. Therefore, it is advisable to re-isolate the introduced species from the soil, rhizosphere, or endosphere, as well as to locate and corroborate their endophytic capacity (Avilés-García et al., 2016; Mitter et al., 2017; Pavlova et al., 2017; Patel and Archana, 2017). The latter provides certainty about the beneficial effect of inoculating new strains into the core microbiome. It is of equal importance to analyse the impact exerted by the colonisation of the rhizosphere or endosphere on the plant microbiome.

Recently, many studies have reported that some endophytic strains can modify the structure and species richness within plant tissues (Chihaoui et al., 2015; Patel and Archana, 2017; Timm et al., 2016,). To the best of our knowledge, only very few studies have assessed the plant internal microbiome several generations following the introduction of a selected strain(s) (Mitter et al., 2017). Similarly, little work has been done on the manipulation and long-term persistence of the microbiome to control plant diseases and the impact on biocontrol of potential phytopathogens, although certain populations in disease-suppressive soils are known to play an important role in this activity (Mendes et al., 2011; Xue et al., 2015). From a practical perspective, it would be extremely useful to identify a stable stress tolerance microbiome that could improve crop production capacity in different types of soils and in different climates (Muller et al., 2016). Finally, bioengineering of the plant microbiome is an interesting option to improve or increase the biological capabilities of the plant, a strategy that, while in its infancy, might be of enormous agricultural importance.

Acknowledgements

G.S. thanks Coordinación de la Investigación Científica-Universidad Michoacana de San Nicolás de Hidalgo (2018-2019) and Fundación Santoyo for financial support.

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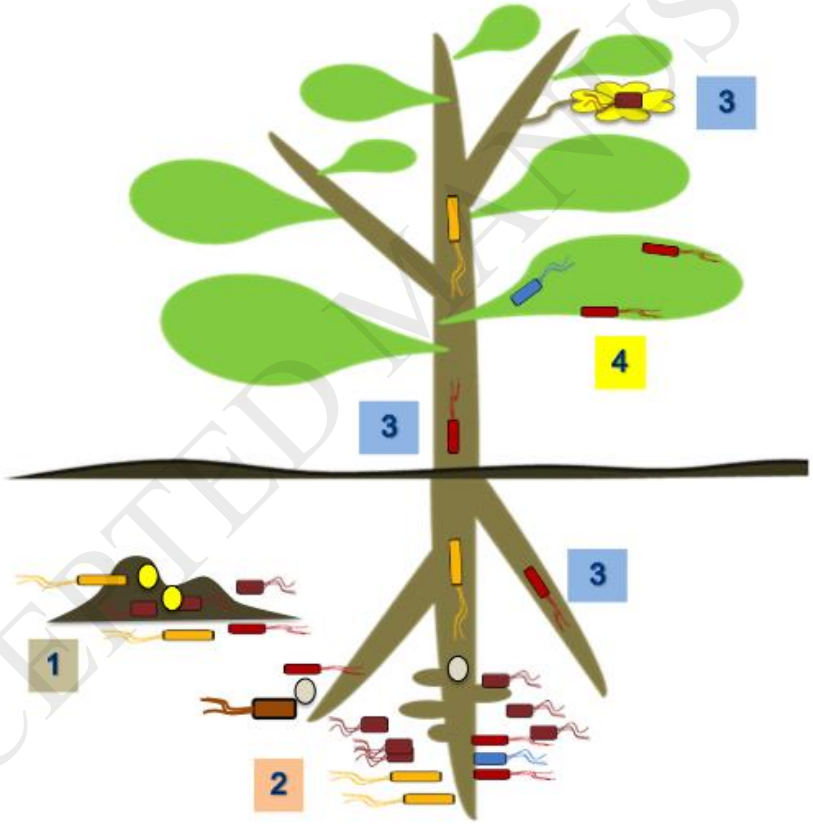
Figure Legend.

Figure 1. The plant microbiome. The figure shows the main regions involved in plant-microbe interactions. (1) Bulk soil: ¨the soil outside the rhizosphere where the root metabolites cannot influence the microbes but where the microbes can nevertheless exert some influence on the plant roots". (2) Rhizosphere: "a narrow zone of soil where microbes are found in high concentrations and are influenced by plant exudates". (3) Endosphere: "the internal regions of plant tissues inhabited by endophytic microbes". (4) Phyllosphere: "the surface and apoplast of leaf tissues".



METHODS FOR DETECTING ENDOPHYTES WITHIN THE PLANT		
Quantitative	**Advantages**	**Disadvantages**
qPCR (Quantitative real-time PCR)	✓ Highly reliable ✓ Quantifiable at the single cell level	• High cost (relative) • No visual detection of endophytes
Re-isolation of endophytes (CFU/gm tissue)	✓ Can be used for further analysis (inoculation, bioinoculants, etc.) ✓ Experimental evidence as endophytes	• Additional work is required to confirm the identity of the strains
Qualitative/Visual		
GFP (Green fluorescent protein) **GUS** (β-glucuronidase) **FISH** (Fluorescence in situ hybridisation)	✓ Visual detection in specific tissues ✓ Strong evidence as endophytes	• High cost of equipment (relative) • Semi-quantitative, in some cases
PCR (Polymerase chain reaction)	✓ Easy detection of endophytes	• Not quantitative • Needs multiple controls