Universität Rostock Traditio et Innovatio

Increasing culturability of plant microbiome towards core microbiome manipulation and engineering

Kumulative Dissertation

zur Erlangung des akademischen Grades
Doktor der Agrarwissenschaften (doctor agriculturae (Dr. agr.))

an der Agrar- und Umweltwissenschaftlichen Fakultät
der Universität Rostock

vorgelegt von
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geb. am 01.03.1993 in Giza, Egypt

Rostock, 17.08.2020

https://doi.org/10.18453/rosdok_id00003155

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Jahr der Einreichung: 2020

Jahr der Verteidigung: 2020

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Chapter 1: General Introduction

General Introduction

Plant ecosystem

Plants, as living organisms, are not sterile entities, but rather harboring different life forms, that end in highly complicated ecosystems (Partida-Martinez and Heil 2011). Such ecosystems involve abiotic factors, e.g. soil, water, air, temperature, and pH, as well as biotic interactants, e.g. bacteria, archaea, fungi, protozoa, worms, and insects (Schenk et al. 2008, Agler et al. 2016, Benckiser et al. 2018). Such factors and interactants allow various modes of interactive relationships, which at the end impact on plant fate. For instance, some bacteria, e.g. *Rhizobium* spp. (family: Rhizobiaceae) and fungi, e.g. Arbuscular mycorrhiza, form symbiotic relationships with plant roots that positively promote plant vitality, i.e. via improving nitrogen-fixation and nutrient absorption. However, some bacterial genera such as *Candidatus* Liberibacter, that belong to the same family of *Rhizobium* spp., Rhizobiaceae, are obligate insect/plant parasites and cause remarkable losses in agricultural production (Wang et al. 2017, Coyle et al. 2018). Therefore, unveiling such crucial interactions, particularly of bacteria, is of principal importance for improving plant productivity and quality.

Plant-microbe interactions

Efforts to studying microorganisms inhabiting plant ecosystems are back to the beginning of the twentieth century, when Lorenz Hiltner proposed the term “Rhizosphere” as the soil area that is influenced directly by plant roots (Hiltner 1904). After that, further research focused on studying microorganisms that have higher affinity to rhizosphere, i.e. rhizobacteria, revealing their ecological roles in promoting plant vitality or even causing plant diseases (Raaijmakers et al. 2009). Several plant growth-promoting rhizobacteria (PGPR), e.g. *Azotobacter* spp., *Azospirillum* spp., and *Rhizobium* spp., were isolated and investigated for potential application in agricultural practices to reduce the use of chemical fertilizers (Caron 1901, Selim 1930, Tarrand et al. 1978). Such developments resulted in a prominent achievement, in which soybean cultivation currently does not rely on mineral nitrogen but rather on biological nitrogen fixed by symbiotic rhizobia (Seneviratne et al. 2000, Nyoki and Ndakidemi 2018).

Moreover, microbial ecological studies on plants revealed remarkable differences in microbial loads within the plant itself, classifying the plant into different spheres. As the plant roots can be divided, from the inside out, into endorhizosphere, rhizoplane, and ectorhizosphere. Likewise, the vegetative parts of the plant are divided into endophyllosphere, phylloplane, ectophyllosphere, and even caulosphere (stems).

Additionally, other temporal compartments develop throughout the plant lifetime, viz. anthosphere (flowers), carposphere (fruits), and spermosphere (seeds). Correspondingly, plant microbiome is of a great diversity of endophytic bacteria and archaea, as well as endophytic fungi, that colonize different plant spheres and organs according to their environmental needs, e.g. pH, oxygen concentrations, and nutrient availability, and perform positive/negative interactions related to plant nutrition and fitness (Partida-Martinez and Heil 2011). These interactions, between plant spheres and their associated microorganisms, are primarily mutualistic (both parties are benefitted), commensalistic (one party is benefitted), and/or parasitic (one party is harmed). Indeed, the plant microbiota play crucial roles in enhancing growth of host plants by fixing atmospheric nitrogen, siderophore production, phosphate and potassium solubilization, and phytohormone production as well as production of other growth promoting factors. In turn, they are benefitted with nutrients and dwelling within the host plant spheres. Also, they can confer resistance against pathogens, pests, and insects, as well as tolerance to drought, salinity, and high-temperatures (Compant et al. 2019).

Biotic stresses, caused by phytopathogens of bacteria, fungi, and viruses, are responsible for ca. 20-40% of global annual losses of economic crops (Savary et al. 2019). The rapid development of pathogen counter-resistance, due to the intensive and irrational use of synthetic chemicals as well as genetic engineering technologies, imposes utilizing biological resources, of biocontrol agents, in agricultural management (Dangl et al. 2013). Therefore, employing benign microorganisms in crop protection is one of the rising trends in modern agriculture (Compant et al. 2019). Unlike chemicals, such approach lacks to standardization and reproducibility, which can delay large-scale application (Thomashow et al. 2019).

Recent studies in plant microbiomes pointed their crucial roles in improving plant fitness and health, despite the limited knowledge on the mode of action of plant responses to beneficial microorganisms (Vannier et al. 2019). The primary signaling molecules in plants are phytohormones, i.e. plant hormones, that regulate development and organization of several defense responses, e.g. salicylic acid, ethylene, and jasmonic acid. Thus, systemic acquired resistance (SAR) can be induced by microbe-associated molecular patterns (MAMPs), e.g. flagella and chitin, which can bind to membrane bound pattern recognition receptors (PRRs), to stimulate MAMP triggered immunity (MTI) (Beneduzi et al. 2012). Other mechanisms involve stimulation of effector triggered immunity (ETI) that cause programmed cell death, and upregulation of pathogenesis-related (PR) genes (Rosier et al. 2018, Vannier

et al. 2019). Recent studies suggested microbiota-mediated disease resistance in plants, through designing synthetic microbial communities (SynComs) with predictable beneficial functions for plants (Vannier et al. 2019) (Figure 1.1).

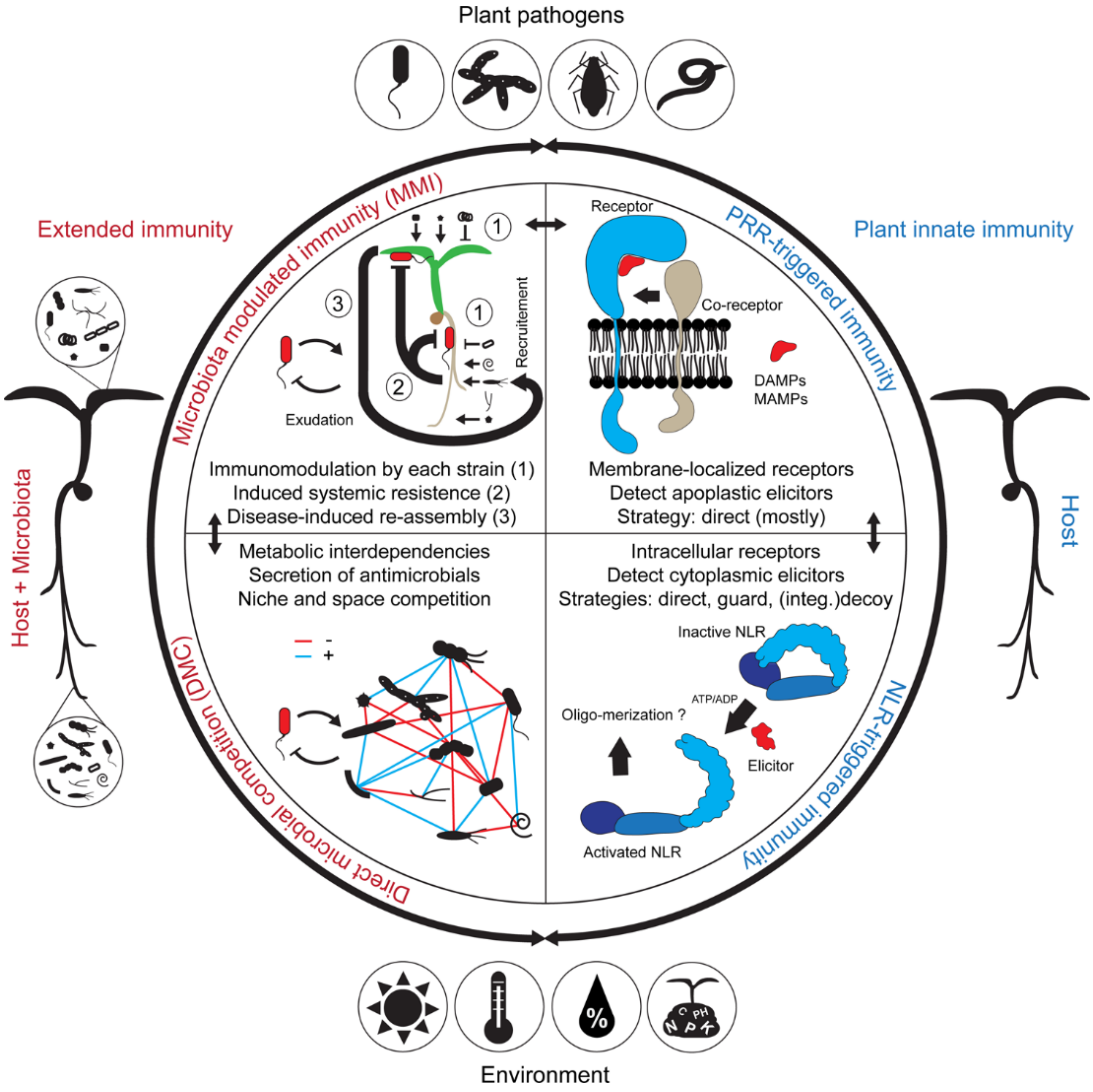

Figure 1.1: Model of microbiota-mediated extension of the plant immune system, proposed and illustrated by Vannier et al. (Vannier et al. 2019). MMI, microbiota-modulated immunity; PRR, pattern recognition receptor; MAMP, microbe-associated molecular pattern; DMC, direct microbial competition; DAMP, damage-associated molecular pattern; NLR, nucleotide-binding domain and leucine-rich repeat-containing receptor.

Hence, studies on plant-microbe interactions have become a crucial aspect of improving plant adaptation and crop production (Vassilev et al. 2015, Mhatre et al. 2018). Currently, those multi-functional groups of the plant microbiome, in particular nitrogen-fixers and plant growth-promoting rhizobacteria (PGPR), are under ambitious research and development programs to maximize their inputs towards agricultural sustainability and good agricultural practices (GAP).

Great plate count anomaly and metagenomics

Great contradictions between microscopic microbial counts and agar plate counts were exhaustively reported in numerous studies; the phenomenon that is known as "*Great plate count anomaly*". Counts of the latter were usually 2-3 logs less than counts of the former (Butkevich 1932, Jannasch and Jones 1959). This implies that the majority of microbes, in different ecosystems, are still unexplored and huge potentials are also yet to be discovered (Stewart 2012). Therefore, cultivation-independent techniques were rapidly developed to characterize microbial communities in different environments without the need for *in vitro* cultivation. These methods are based on profiling microbial communities according to specific microbial marker genes, usually ribosomal RNA genes. The significant advancement in microbial ecology and microbiome analyses came after the completion of the human microbiome project (HMP) and the introduction of next-generation sequencing (Turnbaugh et al. 2007). Therefore, microbial ecology research has expanded and enabled microbiologists to characterize microbial communities' compositions and functions in a high-resolution culture-independent manner. In metagenomics, shotgun sequencing is applied to sequence millions of randomly fragmented genomes, directly obtained from environmental samples (Daniel 2005, Tringe et al. 2005).

Another high-throughput technology is the PhyloChip microarray technology, that has been developed by Second Genome Inc. (San Francesco, CA, USA). This technology, in its latest version "G3", is able to detect 16S rRNA bacterial and archaeal probe sequences from any sample in a parallel and high throughput system as it covers 2 domains, 147 phyla, 1,123 classes, 1,219 orders, and 10,993 subfamilies. It can also detect up to 50,000 different prokaryotic species belonging to both culturable and unculturable taxa (Brodie et al. 2006, Weinert et al. 2011).

Later, these high-throughput technologies led to the launch of one of the biggest scientific projects ever, the Earth Microbiome Project (EMP) which combined multidisciplinary efforts in order to analyze microbial community structures and functions across the globe (earthmicrobiome.org). The EMP aimed to analyze 200,000 samples from these communities using culture-independent methods of metagenomics, meta-transcriptomics, and amplicon sequencing to generate a "global gene atlas" (Gilbert et al. 2014).

All of the previous advancements culminated in a massive changes in the tree of life since the first version sketched by Charles Darwin (Figure 1.2a) (Darwin 1859, 1987).

However, using the DNA sequences to construct phylogenetic trees remains the most influential innovation that highly impacted the classification of living organisms, enabling precise definition of the three domains of life, viz. Bacteria, Archaea, and Eukarya (Figure 1.2b) (Woese and Fox 1977, Woese et al. 1990). Thus, cultivation-independent genomic approaches, that retrieve sequences directly from environmental samples, have amended the tree with many new lineages. Specifically, since 1987, more than 100 novel bacterial phyla have been discovered and added to the bacterial databases, most of which are not-yet-cultured (Achtman and Wagner 2008, Parks et al. 2018). Consequently, the culturable bacterial community does not represent the total phylogenetic diversity, and the huge biotechnological potential of such unexplored populations is still concealed within unculturable populations. This encouraged further developments in genomic tools that can provide comprehensive metabolic insights without the need for axenic cultures (Youssef et al. 2015, Yeoh et al. 2016, Castelle and Banfield 2018) (Figure 1.2).

Although developments of DNA sequencing technologies and molecular biology techniques broadened the gaps between the cultured and uncultured lineages, they provided high resolution characterization of the functional potential of those uncultured, which in turn streamlined their isolation. Most importantly, those metagenomic techniques unveiled the mysterious reasons behind their unculturability.

Reasons behind bacterial unculturability

Latest versions of the tree of life displayed remarkable distinct clusters, containing only unculturable lineages (Figure 1.2c), e.g. candidate phyla radiation (CPR) (Hug et al. 2016, Castelle and Banfield 2018, Parks et al. 2018). Multi-omics derived data highly suggests that members belonging to those phyla are of streamlined genomes and thus of limited metabolic capacities (Yeoh et al. 2016). Such organisms depend mainly on other organisms, usually eukaryotes, to complement their defective metabolic networks, and thus are obligate symbionts that are constrained with their host’s life cycle. In general, evolution moves towards economization, in terms of genome size and specialized metabolic pathways, through the creation of an organism-organism dependence fate (Dutta and Paul 2012, Martínez-Cano et al. 2015).

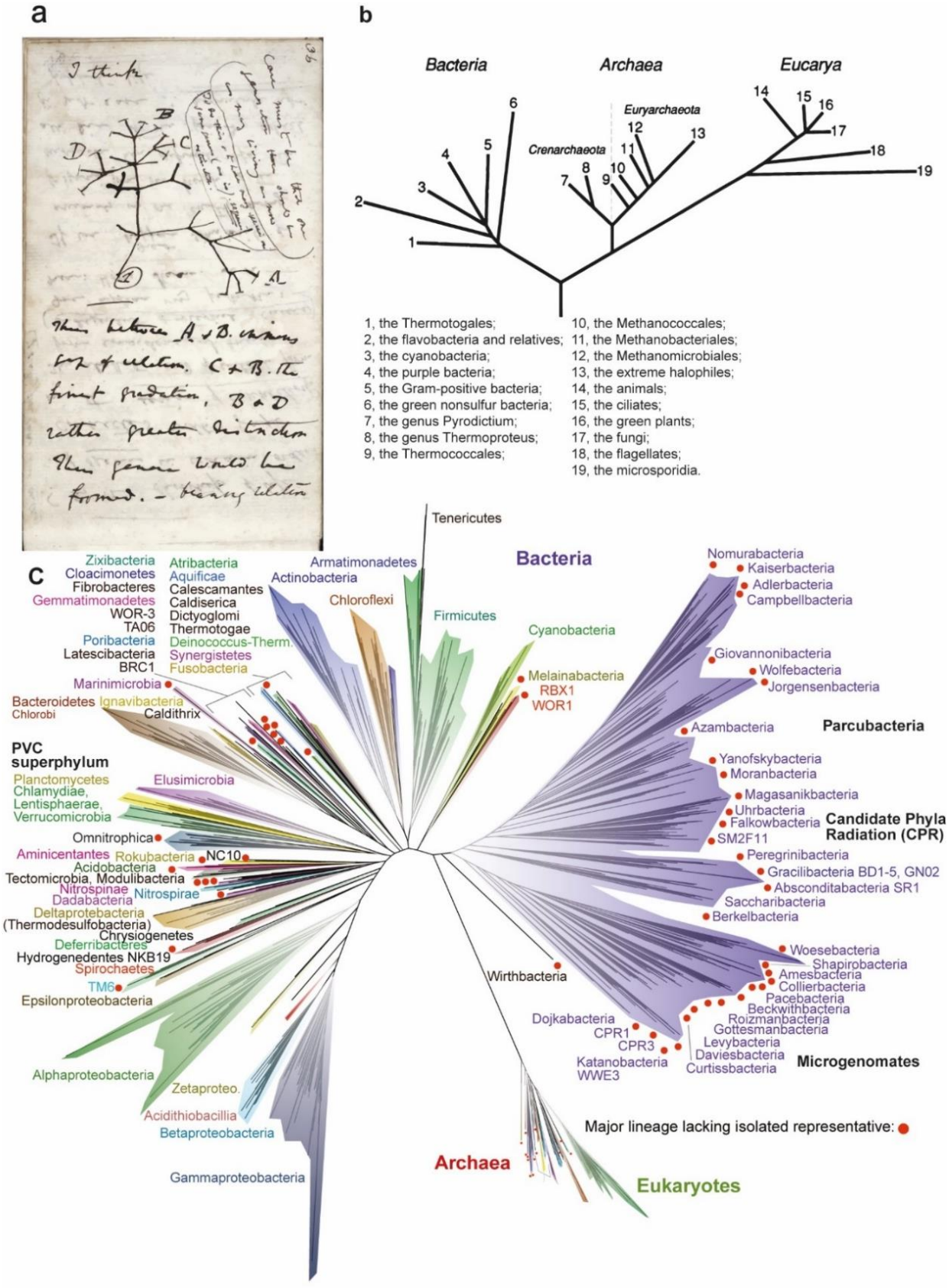

Figure 1.2: Development of phylogenetic tree of life overtime: a, The first evolutionary tree sketched by Charles Darwin in 1837 (Darwin 1987); b, Phylogenetic tree showing the three domains of life, based on rRNA sequence comparison (Woese et al. 1990); c, A modern tree of life detailing the bacterial culturable and unculturable bacterial phyla, based on sequences of ribosomal proteins [adapted from Hug et al. (2016)].

Unculturability of such enormous range of bacteria, ca. 90 phyla, represents one of the very simple reasons for their unculturability, that they are different. In other words, they are highly diverse in their physiology, metabolism, ecological role/niche, and genome composition, and consequently their nutritional requirements are tremendously variable (Stewart 2012). Therefore, one of the very useful tools to gain information on this is the multi-omics data analyses (e.g. metagenomics, meta-transcriptomics, and meta-proteomics); which can reveal important information related to culture media formulation, culturing conditions, and nutritional requirements (Oberhardt et al. 2015, Gutleben et al. 2018).

In plants, one successful example is the cultivation of the *Xylella fastidiosa,* the fastidious xylem-inhabiting pathogenic bacterium (de Macedo Lemos et al. 2003). Genome sequence of such bacterium revealed lack of enzymes required for biosynthesis of some amino acids, viz. methionine, cysteine, and serine. This is in addition to the missingness of the enzyme inositol monophosphatase, that is crucial for all inositol-dependent processes such as protein secretion, DNA synthesis, and glucose response pathways (Roberts 2006). Consequently, supplementation of culture media with myo-inositol and those amino acids resulted in faster growth of *X. fastidiosa* (de Macedo Lemos et al. 2003). Despite such low-resolution annotation, this example shows the power of genome-based metabolism prediction in achieving cultivation of fastidious bacteria.

Another example is the bacterial phylum Saccharibacteria, previously known as TM7. The ubiquitous abundance of this bacterial group was reported throughout the last decade (Ferrari et al. 2014), only through metagenomic 16S rRNA sequences without any axenic culture. The genome sequence of TM7 is highly reduced (~705 kb) and suggested: i) resistance to the antibiotic streptomycin, due to a highly typical single-base substitution in the 16S rRNA (Hugenholtz et al. 2001), and ii) complete lack of amino acids anabolic pathways. This information enabled the first enrichment and isolation of the human oral TM7 bacterium, in a co-culture with *Actinomyces odontolyticus*, as an obligate epibiont. Thus, obtaining an axenic culture of TM7 was not successful in absence of *A. odontolyticus* (Hugenholtz et al. 2001, Soro et al. 2014, He et al. 2015).

Other detailed reasons for bacterial unculturability, related to genome size and composition, are treated in chapter 5 of this thesis (Sarhan et al. 2019).

The potential of unculturable bacteria and the power of pure cultures

In general, bacteria are the primary source of ca. 50% of the commercially-available pharmaceuticals compounds such as antibiotics, cosmetics, and anticancer products (Demain and Sanchez 2009). Specifically, soil Actinobacteria have been the major source of novel antibiotics during the “golden era” of antibiotics, from the 1940’s to the 1960’s (Lewis 2013). However, the rate of antibiotic discovery has remarkably decreased thenceforth, to the extent that the year 1987 marked the last novel class of antibiotics that has been released for clinical therapeutics; this phenomenon was known as “discovery void”. In 2015, a new class of antibiotics was discovered, which broke that “discovery void”, by culturing a novel bacterial species using the technique of “i-Chip” (Nichols et al. 2010). The bacterium belonged to the Betaproteobacteria, and named *Eleftheria terrae*, and the antibiotic named “teixobactin”. Of interest is that teixobactin was shown to kill *Staphylococcus aureus* and *Mycobacterium tuberculosis* without any detectable resistance (Ling et al. 2015).

Therefore, there is a resurgent attention for isolating novel microorganisms to expand the current library of antibiotics and pharmaceuticals. It is worth to mention that, the potential natural products from bacteria are not limited to antibiotics, but also extended to secondary metabolites serving as insecticides, fungicides, and antiparasitic compounds or even more.

On the other side, the phytopathogenic bacteria can be of immense significance as well, since some pathogenic bacteria resist cultivation *in vitro*. Those bacteria, despite having full genome sequence, are considered to be problematic due to the lack of axenic culture, which is the key to their characterization, classification, and nomenclature. Therefore, microbial taxonomists proposed the term “*Candidatus*” to describe the bacterial taxa that are characterized based on genome sequence and/or phenotyping (Murray and Stackebrandt 1995). In addition to the taxonomical assignment, the ability of culturing those ‘*Candidatus*’ organisms will help in: i) fulfilling Koch’s postulates; ii) screening efficient antibacterial compounds; iii) understanding the basis of biological interactions with their hosts, plants, and insects; and iv) developing effective control methods based on the molecular plant-pathogen-insect interactions. For example, *Candidatus* Liberibacter, a genus of Gram-negative bacteria that belongs to the family Rhizobiaceae, is one of the most damaging bacterial phytopathogens. It can infect economic vegetable- and fruit crops, e.g. potato (zebra chip disease) and citrus (Huanglongbing disease, HLB). Members of this genus are plant pathogens typically transmitted by psyllids. Moreover, due to the lack of pure isolates of these pathogens, there is no curative remedy for control, but only preventive measures. Detection of the *Candidatus* Liberibacters is based mainly on PCR of their 16S

rRNA gene, using specific primers. Notably, pathogenic Liberibacters have undergone cumulative reductive genome evolution with current average genome size of 1.2 Mb (Duan et al. 2009). Another example is the *Candidatus* Phytoplasma, an obligate bacterial plant/insect parasite that is transmitted from plant to plant by insect vectors such as psyllids. Phytoplasmas lack cell walls and have: i) pleiomorphic or filamentous shape; ii) diameter less than 1 µm; and iii) very small genome (~ 0.7-1.5 Mb). They infect >1000 plant species, including agriculturally important crops such as sesame, coconut, sugarcane, and grapevine, in which they cause a wide variety of symptoms ranging from mild yellowing to death (Seemüller et al. 2002). Furthermore, they are not amenable to culturing in any cell-free culture medium; routine cultivation in an artificial medium thus remains to be a major challenge. However, recent studies reported successful cultivation of *Candidatus* Phytoplasmas *in vitro*, but no experimental repetition has been yet reported (Contaldo et al. 2015, Contaldo et al. 2016).

In conclusion, if you do not have the microbe in culture, you cannot study it. Metagenomics is powerful enough to: i) measure phylogenetic diversity; ii) predict ecological functions; and iii) postulate the main reasons of the unculturability. However, obtaining a pure culture is an indispensable aim to gain the maximum potential behind these not-yet-cultured bacteria. The key to overcoming the imposing challenges is to increase the rate at which novel bacterial taxa are being isolated. This could be achieved through developing innovative techniques, known as “Culturomics”, by which multi-omics derived information can be combined with high throughput culturing strategies to gain access to such unculturable entities (Lagier et al. 2016, Marx 2017, Lagier et al. 2018, Seck et al. 2018). The rise and development of culturomics are extensively treated in chapter 5 “*Culturomics of the plant microbiome and the dawn of plant-based culture media*”.

Methods to increase culturability and development of the plant-based culture media

The previously mentioned explanations for bacterial unculturability infer that the empirical approach to solve the culturability dilemma is to replicate the natural environmental conditions of a given environment into an *in vitro* emulating system. Another approach is to combine all possible conditions by varying different factors, e.g. pH, temperature, osmosis, etc., which will result in thousands of possibilities which make this approach less adaptable in microbiology laboratories. However, studies that applied selective conditions together with copying the remaining factors attained remarkable successes. For example, extensive dilution of culture media and extending incubation periods enabled isolation and identification of hundreds of novel species (Overmann et al. 2017a). Moreover, other

mimicry approaches, e.g. diluting bacterial inocula prior to encapsulation in gel microdroplets (GMD) (Zengler et al. 2002, Stewart 2012, Dichosa et al. 2014), co-culturing with associate microorganisms (Vartoukian et al. 2016), soil-substrate membranes and diffusion-chambers (Svenning et al. 2003, Ferrari et al. 2005, Ferrari et al. 2008, Ferrari and Gillings 2009), and *in situ* cultivation of bacteria in micro-well chips, iChip (Piddock 2015, Berdy et al. 2017, Lodhi et al. 2018), assisted in recovery of novel taxa and recovery of organisms that were not even detectable with cultivation-independent techniques.

Formulating a general culture medium, for recovery of broad range of bacteria, particularly plant associated PGPR, is a major challenge for microbiologists, and therefore, tremendous efforts are being exerted to achieve it (Pham and Kim 2012). In accordance with the approach of replicating natural environments *in vitro*, several studies reported improved culturability of endophytic fungi and bacteria when supplementing traditional culture media with plant extracts (Zhao et al. 2010, Martyniuk and Oron 2011, Murphy et al. 2015). For example, addition of plant extracts to standard culture media resulted in significant increase in diversity of endophytes from pumpkin (Eevers et al. 2015). Furthermore, some leguminous seed extracts successfully substituted beef extract of the MRS culture media and supported growth of probiotic bacteria (Pathak and Martirosyan 2012). Interestingly, similar approach was applied to develop vegetarian culture media to obtain biomass of probiotic species free of animal-derived ingredients such as beef-extract (Heenan et al. 2002).

Aim of the study and hypothesis

Recently, culture media solely based on plant materials, i.e. without any chemical supplements, were developed to cultivate the plant associated bacteria (Nour et al. 2012, Osman et al. 2012, Sarhan et al. 2016, Youssef et al. 2016, Mourad et al. 2018). Different formulations were prepared accordingly, e.g. juices, saps, slurries, as well as dehydrated powders, and termed as “plant-based culture media” (Youssef et al. 2016). Such plant-based culture media displayed exceptional potentials on recovering rhizobacteria and producing biomass comparable to artificial rich culture media. Additionally, culture media made of dehydrated plant-powders displayed higher culturability percentages of clover root associated bacteria compared with the different artificial culture media (Sarhan et al. 2016). Furthermore, molecular community fingerprinting of culturable rhizobacterial populations exhibited closer clustering of rhizobacteria developed on plant-based culture media to the original culture-independent root population, than the standard culture media. It is hypothesized that the complexity of nutrients presents in the plant-based culture media, in terms of diversity and quantity, is somehow mimicking the original plant root environments.

The present study aimed to optimize the use of the plant-based culture media for exploration of the plant-associated microbiota and isolation of not-yet-cultured bacterial taxa. This is in addition to meticulous characterization of total culturable and unculturable plant-associated prokaryotic microbiota, using one of the high throughput microbiome characterization tools, with particular focus on the unculturable bacterial phyla/divisions.

Chapter 2: Plant-fed versus chemicals-fed rhizobacteria of Lucerne: Plant-only teabags culture media not only increase culturability of rhizobacteria but also recover a previously uncultured *Lysobacter* sp., *Novosphingobium* sp. and *Pedobacter* sp.

Plant-fed versus chemicals-fed rhizobacteria of Lucerne: Plant-only teabags culture media not only increase culturability of rhizobacteria but also recover a previously uncultured *Lysobacter* sp., *Novosphingobium* sp. and *Pedobacter* sp.

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Abstract

In an effort to axenically culture the previously uncultivable populations of the rhizobacteria of Lucerne (*Medicago sativa* L.), we propose plant-only teabags culture media to mimic the nutritional matrix available in the rhizosphere. Here, we show that culture media prepared from Lucerne powder teabags substantially increased the cultivability of Lucerne rhizobacteria compared with a standard nutrient agar, where we found that the cultivable populations significantly increased by up to 60% of the total bacterial numbers as estimated by Quantitative Real-time Polymerase Chain Reaction (qRT-PCR). Cluster analysis of 16S rDNA Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) of cultivable Colony-Forming Units (CFUs) revealed a more distinct composition and separation of bacterial populations recovered on the plant-only teabags culture media than those developed on a standard nutrient agar. Further, the new plant medium gave preference to the micro-symbiont *Sinorhizobium meliloti*, and succeeded in isolating a number of not-yet-cultured bacteria, most closely matched to *Novosphingobium* sp., *Lysobacter* sp. and *Pedobacter* sp. The present study may encourage other researchers to consider moving from the well-established standard culture media to the challenging new plant-only culture media. Such a move may reveal previously hidden members of rhizobacteria, and help to further explore their potential environmental impacts.

Introduction

It has been long established that more than 90% of environmental microorganisms are not amenable to cultivation on/in standard laboratory media [1]. Bacterial databases are continually updated with new bacterial taxa, most of which are not yet cultured. Microbial ecologists struggle to recover and maintain these unculturables as viable entities grown as pure cultures. The last decade has witnessed the development of culture media and conditions for culturing new bacterial members from various environments [2–8]. In 2012, we proposed the use of plant-only culture media as a new approach for culturing rhizobacteria that might resist conventional cultivation. Crude plant juices, saps and powders, without any amendments, supported good growth and recoverability of rhizobacteria, comparable with the standard culture media [9–11]. For ease of application and practicability, tea bags packed with plant powders were successfully introduced to obtain the plant infusions necessary to prepare culture media [11].

The host plant tested was Lucerne (alfalfa, *Medicago sativa* L.), being the well-established crop available in the experimental open fields of IGZ-Großbeeren. Among other perennial and/or annual legume crops, it is known to be the most common grown forage because of its ability to be grown over a range of climatic conditions, efficient yield, nutritional qualities and biotic (e.g. pests) and abiotic stress-tolerance (e.g. salinity and soil acidity) [12]. Globally, Lucerne is used as a major source of protein for livestock and in crop rotation as pasture and organic/green manure fertilization. It is of very significant contribution to the N-status and biofertility of the plant-soil system via the consortium of rhizobacteria, namely the nodule-forming rhizobia and associated plant growth-promoting bacteria.

As to the molecular aspects of macro- and micro-symbiosis, *Medicago sativa* and its relative *Medicago truncatula* (Gaertn.) are commonly used as model legume plants. Very much emphasis was particularly devoted to the level of Alphaproteobacteria species *Sinorhizobium meliloti* [13], not to the whole bacterial community composition. Later, Pini *et al*. [14] used culture-independent techniques (T-RFLP, qPCR and 16S rRNA sequencing), and showed a high diversity; more than 7 classes with the distinguished dominance by members of Alphaproteobacteria in the plant-soil system including soil and plant shoots. Among the Alphaproteobacteria families, Sphingomonodaceae and Methylobacteriaceae were abundant inside plant tissues, including aerial parts, while in soil they were represented by Hyphomicrobiaceae, Methylocystaceae, Bradirhizobiaceae and Caulobacteraceae. On the species level, *S. meliloti* were detected with higher values in root nodules compared to those reported in soil and aerial tissues of leaves and stems. Further, the species is proved

to behave as an endophytic strain colonizing all plant compartments, besides being a root symbiont of legumes [15]. Also, PCR-based 454 pyrosequencing confirmed the decreasing bacterial diversity from unplanted soil to root tissues of Lucerne, and that the root tissues were mainly inhabited by Alphaproteobacteria associated by Gammaproteobacteria, as well as Betaproteobacteria, Flavobacteria and Actinobacteria, which may have some additional plant-growth-promoting activities [14,16]. Of interest was the study of Schwieger and Tebbe [17] who compared the products of cultivated isolates and community profiles as determined by Cultivation-independent-SSCP (Single Strand Conformation Polymorphism) and indicated that the most dominant groups identified by cultivation were also detected in the community profiles. However, there was one exception where the most dominant group, which represented 24% of the total cultivated isolates and was related to the genus *Variovorax*, was not detected. These results together with those of Dunbar *et al.* [18] raise questions and reservations towards the inherent problems and limitations of the existing culture-independent techniques. And, strongly encourage the on-going sincere efforts to improve culturability of the plant microbiome.

In the present study, we tested the suitability of culture media prepared from teabags packed with varying quantities of dehydrated powder of Lucerne (*Medicago sativa* L.) for culturing host rhizobacteria. Quantitative real-time PCR (qPCR) was applied to measure the total bacterial copy numbers present on Lucerne roots to assess the relative efficiencies of cultivability on tested culture media. A replica plating technique was used to test reproducibility of developed CFUs on various culture media. PCR-DGGE analysis of cultured bacterial populations and 16S rDNA sequencing were carried out to identify and compare the cultivable rhizobacterial community composition.

Material and Methods

Host plant and sampling

Intact shoots and roots of Lucerne plants (*Medicago sativa* L.) were randomly sampled from the experimental fields of Leibniz institute of vegetable and ornamental crops (IGZ, 52°20'56.2"N - 13°19'00.6"E), Großbeeren, Germany. Roots were initially washed with tap water and aliquoted for bacterial CFU (fresh root material) and qPCR measurements (freeze dried). Root dry weight was determined (drying at 65 °C for 24 hrs.) to calculate the bacterial counts per gram dry root. The plant vegetative parts were kept for the preparation of the plant-only teabags culture media.

Culture-independent quantification of Lucerne rhizobacteria

Total bacterial quantification using quantitative real-time PCR

Total DNA (bacterial and plant DNA) was extracted from freeze dried Lucerne roots, using QIAGEN DNeasy plant mini kit (QIAGEN, Hilden, Germany) according to the manufacturers' instructions. The quality of DNA isolated from roots using DNeasy Plant MiniKit (Qiagen) was determined photometrically by the 260/280 ratio calculation to be above 1.9, and the A_{230} measurement was nearly 0 and quantified at 260 nm. Detection and quantification of bacterial 16S rDNA copy numbers were performed by quantitative real-time PCR (qPCR) using the CFX96 Touch™ Detection System (Bio-Rad Inc., CA, USA) in optical grade 96 well plates. The PCR reaction was performed according to Sarhan *et al.* [11] in a total volume of 25 µl using SYBR® green master mix (Bio-Rad) containing 10-30 ng genomic DNA and 8.25 pmol of each primer [19]; the universal forward 519f (CAGCMGCCGCGGTAANWC) and reverse 907r (CCGTCAATTCMTTTRAGTT). The cycling program and both standard and melting curves construction were done according to Sarhan *et al.* [11]. Bacterial cell numbers were indirectly calculated assuming an average 16S rDNA copy number of 3.6 per bacterial cell [20,21].

Culture-dependent quantification and characterization of Lucerne rhizobacteria

Culture media preparation

Plant-only teabags were prepared according to Sarhan *et al.* [11]. Fresh vegetative parts, including leaves and stems, of Lucerne were oven dried at 65 °C for 18 hrs. Then the dry plant material was mechanically ground and passed through a 2.0 mm sieve to obtain a fine dehydrated powder. Teabags were packed with different quantities of the prepared dehydrated Lucerne powder: 0.25, 0.5, 1.0 and 4.0 g per pouch. The teabags were soaked in lukewarm distilled water to obtain plant infusions of 0.25, 0.5, 1.0 and 4.0 g l^{-1}, and pH was neutralized to 7.0. The teabags were left during autoclavation for further extraction. Agar culture media were prepared by adding agar (2%, w/v) and autoclaved at 121 °C for 20 min. A schematic illustration of plant-only teabags and culture media preparation is presented in Fig. (1).

The nutrient agar Standard-I, with the addition of glucose (1.0 g l^{-1}), (Merck KGaA, Darmstadt, Germany) was used as the control.

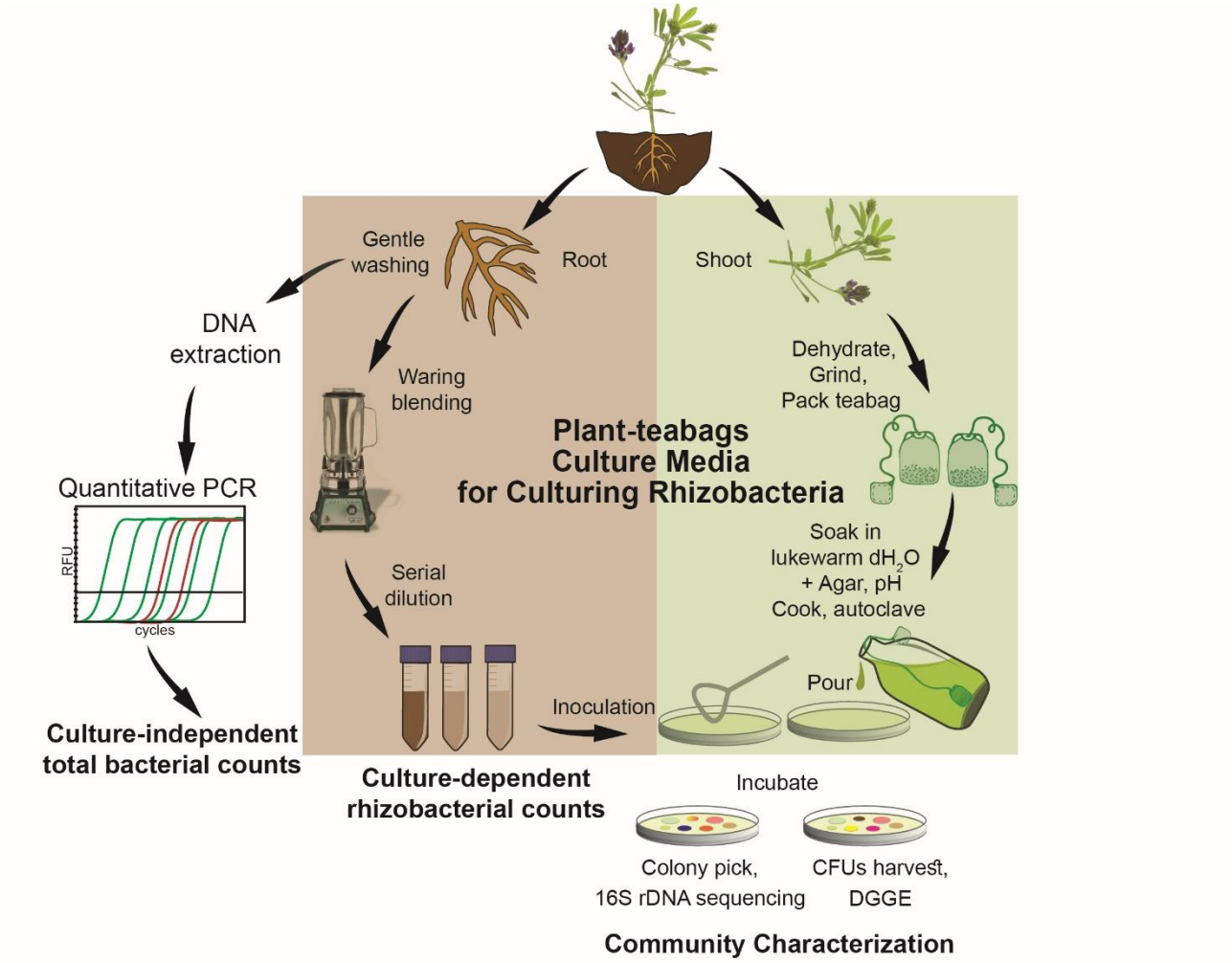

Fig. 1. Schematic illustration for the preparation of plant-only teabags culture media, and the work flow of culture-dependent and culture-independent analyses of Lucerne rhizobacteria.

Culture-dependent recovery of *in-situ* rhizobacteria of Lucerne

Plant roots were initially washed with tap water, then with sterilized water before crushing in a previously autoclaved Warring blender, with an adequate volume of autoclaved basal salts of the yeast mannitol agar (YMA) medium [22] as diluent. Further serial dilutions were prepared, and 200 µl aliquots of suitable dilutions were surface inoculated on agar plates, with 3 replicates, prepared from the plant-only teabags (0.25, 0.5, 1.0, 4.0 g l^{-1}) and the Standard-I nutrient agar (Merck, Darmstadt, Germany). The culture plates were incubated at 28 °C and CFUs were counted throughout the incubation period at two, four and fifteen days. Bacterial counts were log transformed, tested for equality of variances, and a 2-way ANOVA was performed using STATISTICA 10 (Statsoft inc. 2011).

Harvest of CFUs and DNA extraction

For DNA extraction, all CFUs developed on representative agar plates (> 30 – 300 CFU $plate^{-1}$) of the tested culture media were washed using 0.05 M NaCl solution, and collected by centrifugation for 10 min. at 9500 *g*. DNA was extracted from collected CFUs

pellets using the QIAGEN DNeasy plant mini kit (QIAGEN, Hilden, Germany) according to the manufacturers' instructions. DNA concentrations and quality were checked as previously described.

16S rDNA PCR-DGGE fingerprinting

Bacterial DNA, extracted from the total CFUs harvested from the agar plates, was used to amplify the whole 16S rDNA gene using the primers 9bfm (GAGTTTGATYHTGGCTCAG) and 1512r (ACGGHTACCTTGTTACGACTT) [23]. The reaction was performed using the Bio-Rad C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA) in a total volume of 25 µl using QIAGEN TopTaq master mix (Qiagen Inc., Hilden, Germany), containing 10-30 ng genomic DNA and 8.25 pmol of each primer. The PCR thermal cycling program was adjusted according to Sarhan *et al.* [11]. A QIAquick PCR Purification Kit (Qiagen Inc., Hilden, Germany) was used to purify the PCR product, according to the manufacturers' instructions. For amplification of the V3 region, a nested PCR was performed using 341f-GC (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGCCTACGGGAGGCAGCAG) and 518r (ATTACCGCGGCTGCTGG) primers [23,24]; the reaction conditions and thermal cycling program used were as described in Sarhan *et al.* [11]. PCR products of the V3 region were heated at 95 °C for 5 min and stored at 65 °C before loading onto the gradient gel. The products of both PCR reactions were tested on 1.5% agarose gel to ensure single products of the expected size.

DGGE was performed using the Dcode Mutation Detection System (Bio-Rad Inc., CA, USA). PCR products (10 µ1 of 10-15 ng PCR products mixed with loading dye) were electrophoresed on 8% polyacrylamide gel containing 30 to 70% denaturing gradient of formamide and urea with 1x TAE buffer. DGGE was conducted at 60 °C for 20 hrs at a voltage of 50 V. The gel was stained with SYBR® Gold Nucleic Acid Gel Stain (Life Technologies Inc., Germany) and recorded with a UV gel documentation system (Biometra GmbH, Goettingen, Germany). A self-created standard was constructed according to Sarhan *et al.* [11]. The DGGE fingerprints were analyzed using Phoretix 1D pro software (TotalLab Ltd. v.11.4). Principal coordinates analysis (PCoA) was performed using GenALEx 6.5 [25].

Replica plating and 16S rDNA sequencing

Plates with confined colonies (≤ 30 plate^{-1}), developed with the longer incubation of 15 days, were replicated on agar plates of homologous and heterologous culture media [26].

With incubation at 29 °C, reproduced colonies were further subcultured on agar plates of the corresponding culture medium. The two groups of both common colonies and those only confined to plant-only teabags culture media were subjected to 16S rDNA sequencing (Eurofins MWG Operon, Ebersberg, Germany). To obtain the nearest phylogenetic neighbours, 16S rDNA sequences were compared with their closest matches using BLASTn tool (blast.ncbi.nlm.nih.gov/Blast.cgi)[27] and the classifier tool of Ribosomal database project database (rdp.cme.msu.edu/classifier/classifier.jsp)[28], executed on April 05, 2016. The Sequence alignment and the phylogenetic tree were constructed using ClustalW and the Neighbors-Joining method based on the Jukes-Cantor model implemented in MEGA 6.0 [29]. *Bacillus* species were selected as an out-group. The bootstrap values were calculated after 1000 replicates and indicated at each node. The 16S rDNA sequences identified in this study have been deposited in the GenBank database under the accession numbers KP411236, KR911852, KP411237, KR911853, KP411238, KR911855, KR911854, KP411239, KP411240, KP411241, KP339867 and KP411242.

Results

Quantification of Lucerne rhizobacteria using culture dependent and independent techniques

Surface-inoculated agar plates were used to estimate the cultivable population (CFUs numbers) of rhizobacteria of Lucerne plants. Agar plates were prepared from both Standard I nutrient agar and plant-only teabags culture media, where teabags contained 4.0, 1.0, 0.5 or 0.25 gram dehydrated Lucerne powder per liter. Compared with the Standard I nutrient agar, all tested plant powder contained greater quantities of nutrients, and supported excellent development of macro- and micro-colonies of rhizobacteria. We found numerous transparent/translucent and slimy bacterial colonies growing on the 4.0 g l^{-1} Lucerne powder medium, while a greater number of smaller colonies developed on the 1.0 g l^{-1} Lucerne powder medium. In contrast, the colonies growing on the Standard I nutrient agar were remarkably tinted and relatively sparse (Fig 2). Numbers of CFUs were significantly different ($P < 0.05$) between plant-only teabags culture media and Standard I nutrient agar, and increased with incubation time particularly on plant-only teabags culture media (Table 1). Longer incubation (15 days) increased the numbers of micro-colonies, particularly on plant-only teabags culture media and culminated in significant increases in the total number of CFUs. Mean log CFU counts were highest for plant-only teabags containing 1.0 g l^{-1} (8.73 ± 0.018 log CFU g^{-1} root) followed by 0.50, 0.25 and 4.0 g l^{-1} (8.51 ± 0.023 log CFU g^{-1} root), and were lowest for Standard I nutrient agar (7.31 ± 0.055 log CFU g^{-1} root) (Table 1). Total

bacterial cell numbers were estimated using qPCR analysis of 16S rDNA copy numbers per gram of dry Lucerne roots. Assuming an average of 3.6 16S rDNA copy numbers per bacterial cell [20,21], the mean total bacterial cell numbers amounted to 8.94 ± 0.041 per gram of Lucerne root. Relating CFU numbers to the total bacterial numbers measured by qPCR revealed higher culturability efficiencies on plant-only teabags culture media (62-71%) compared to the Standard I nutrient agar (2-16%), particularly with the extension of incubation period up to 15 days (Table 1).

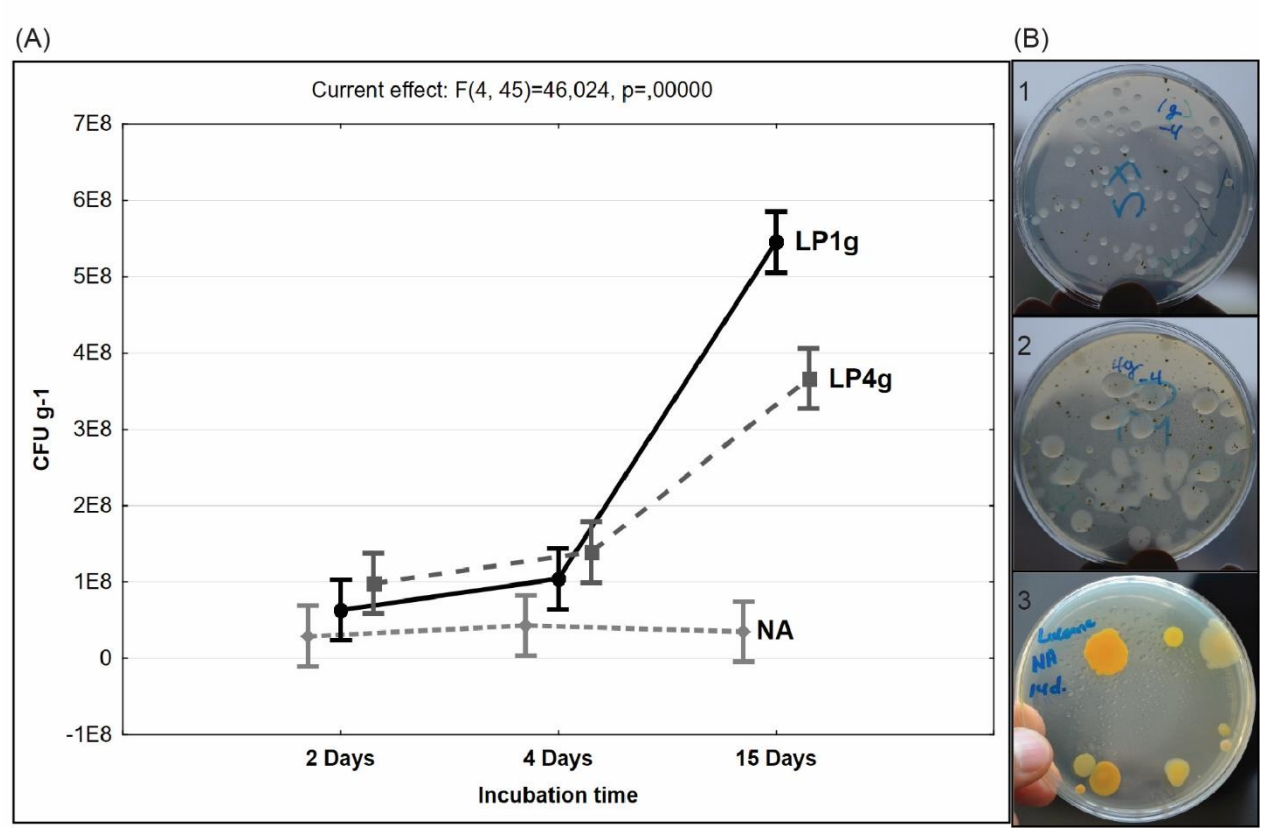

Fig 2. Cultivable rhizobacteria of Lucerne roots (CFUs g^{-1} root) developed on all tested culture media. A, ANOVA analysis (2-way interactions) of CFUs numbers of Lucerne rhizobacteria as affected by culture media and incubation period. NA, nutrient agar; LP1g, teabags of Lucerne powder 1.0 g l^{-1}; LP4g, teabags of Lucerne powder 4.0 g l^{-1}. B, Morphology of CFUs developed on agar plates of culture media prepared from teabags-only-containing Lucerne powder (4.0 g l^{-1} and 1.0 g l^{-1} culture media) as well as nutrient agar, inoculated with aliquots of 200 µl of the same root suspension (10^{-4}): B1, confined/limited and transparent/translucent colonies on LP1.0 g l^{-1}; B2, numerous slimy colonies on LP4.0 g l^{-1}; B3, large tinted colonies on NA.

Table 1. The culturability of Lucerne rhizobacteria on various culture media.

Experiment 1. Nutrient agar compared to Lucerne teabags of 1.0 and 4.0 g l^{-1} culture medium				
Culture media	log CFU count g^{-1} root (at 2-day incubation time) **	Culturability (%)*	log CFU count g^{-1} root (at 15-day incubation time)**	Culturability (%)*
Nutrient agar	7.180 ± 0.055^{d}	2%	7.309 ± 0.035^{d}	2%
Lucerne powder 4.0 g l^{-1}	7.911 ± 0.099^{c}	9%	8.511 ± 0.023^{b}	37%
Lucerne powder 1.0 g l^{-1}	7.548 ± 0.037^{cd}	4%	8.737 ± 0.018^{a}	62%
Experiment 2. Nutrient agar compared to Lucerne teabags of decreasing quantities of Lucerne powder, 1.00, 0.50, 0.25 g l^{-1} culture media				
Culture Media	Log CFU g-1 root at 15 days of incubation**			Culturability (%)*
Nutrient agar	8.03 ± 0.085^{b}			16%
Lucerne powder 1.00 g l^{-1}	8.71 ± 0.042^{a}			71%
Lucerne powder 0.50 g l^{-1}	8.69 ± 0.048^{a}			71%
Lucerne powder 0.25 g l^{-1}	8.64 ± 0.055^{a}			66%

Note:

* Culturability calculated as numbers of CFUs developed on agar plates and related to the total bacterial numbers measured by quantitative real-time PCR. The mean value of qPCR cell numbers indirectly obtained for 8 replicates is log 8.93 ± 0.041 g^{-1} root dry weight (2 plant biological samples with 4 technical replicates, and assuming that the average 16S rDNA copy number per bacterial cell is 3.6 [20,21].

** Data are log means ± standard error (SE), n = 3. Statistically significant differences are indicated by different superscript lowercase letters (*P* value ≤ 0.05, n =3).

PCR-DGGE fingerprinting of Lucerne culturable rhizobacteria

Based on UPGMA cluster analysis of 16S rDNA PCR-DGGE band patterns obtained for the cultivable CFU populations, a distinct separation at a similarity level of 60% was distinguished between Standard I nutrient agar and all Lucerne plant-only teabags culture media. With higher levels of similarities, 70% and >85%, successive separations were attributed to the tested quantities of plant powders in the teabags used for the preparation of culture media (Fig 3A). In addition, the principal coordinate analysis of the obtained DGGE fingerprints revealed three main clusters along the first axis representing 31.86% of variation; one cluster included the lower quantities of Lucerne powder (1.0, 0.50, and 0.25 g l^{-1}) and the second cluster for the higher quantity of 4.0 g l^{-1}. The third cluster was for the Standard I nutrient agar alone. The second axis representing 22.88% variation showed the same trend of clustering (Fig 3B). This is a strong indication of the independent effects of culture media type and concentrations of nutrients on the culturability and community composition of rhizobacteria.

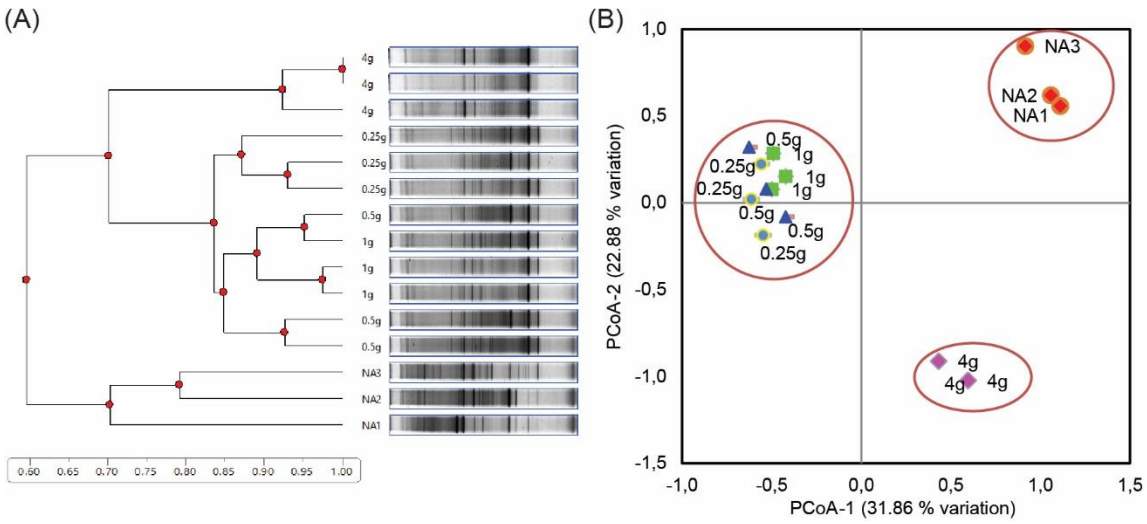

Fig 3. Cluster analysis of culturable Lucerne rhizobacteria (CFUs) developed on all tested culture media. A, UPGMA cluster analysis, with Euclidean distances, of 16S rDNA-DGGE pattern obtained for culturable populations (CFUs) developed on agar plates of culture media prepared from Lucerne powder-only-teabags (4.0 g l^{-1}, 1.0 g l^{-1}, 0.5 g l^{-1} and 0.25 g l^{-1} Lucerne powder) as well as standard I nutrient agar (NA). B, The principal coordinate analysis of the obtained DGGE fingerprints.

Replica plating of rhizobacteria colonies and 16S rDNA sequencing

All of the tested 122 colonies grown on Standard I nutrient agar were successfully reproduced on the plant-only teabags culture medium (1.0 g l^{-1}). In general, colonies developed on plant-only teabags culture media were transparent to translucent, and when reproduced on nutrient agar exhibited colored pigmentation. Among 184 of those colonies originally developed on such plant-only teabags medium, some failed to reproduce on Standard I nutrient agar. A total of 12 morphologically different colonies were traced and followed: ten grew only on plant-only teabags culture media and the other two were able to grow on either media.

16S rDNA sequencing of these pure isolates confirmed that among the ten colonies that only reproduced on plant-only teabags media, eight (LP1.1, LP1.3, LP3.1, LP3.3, LP3.4, LP3.5, LP3.7, and LP3.8) were identified as *Sinorhizobium meliloti*, phyla: Proteobacteria, class: Alphaproteobacteria. The remaining two colonies (LP1.2 and LP3.2) were identified as phyla: Proteobacteria, class: Alphaproteobacteria, and both were matching closely, according to BLASTn tool of the Genbank database, to an uncultured Bacterium (AM697055.1) and/or to an uncultured *Novosphingobium* sp. (HM438566.1) (Fig 4). RDP-classifier tool confirmed their affiliation to the genus *Novosphingobium*. The other two colonies that were originally isolated on plant-only teabags culture media and re-cultivated on Standard I nutrient agar were identified as follows: the isolate LP3.11 affiliated to phyla: Proteobacteria, class: Gammaproteobacteria, closely matching to an uncultured Bacterium (HF586982.1) and to *Lysobacter pocheonensis* (EU273938.1); the isolate LP2.2 affiliated to

phyla: Bacteriodetes, class: Sphingobacteria, closely matching to the uncultured *Pedobacter* sp. (JN697525.1) (Fig 4). These two colonies most likely represent a fraction of fastidious rhizobacteria that were not originally developed on Standard I nutrient agar, being possibly overgrown by a faster growing population. In contrast, the plant-based culture media provided them with an appropriate nutrient matrix (in terms of complexity, concentration and diversity) which allowed such a distinct fraction to be unmasked upon culturing.

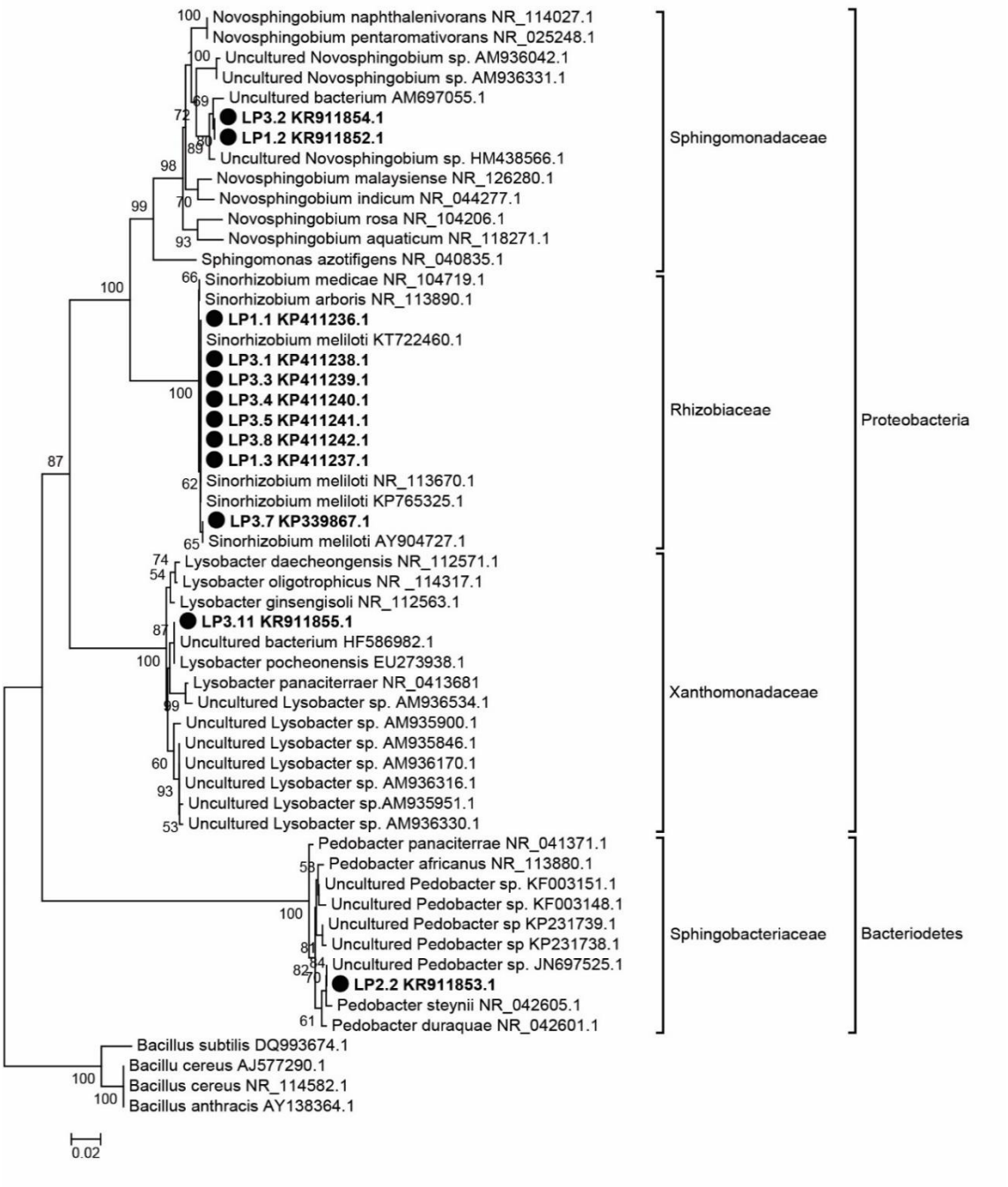

Fig 4*. Medicago sativa* rhizobacteria isolates phylogeny based on 16S rDNA sequences. The isolates of this study are indicated by solid circle (●) (LP1.1, LP1.2, LP1.3, LP2.2, LP3.1, LP3.2, LP3.3, LP3.4, LP3.5, LP3.7, LP3.8, and LP3.11), and closest matches obtained from the GenBank database are indicated by accession numbers after organism name, using Neighbor-Joining method with calculated Jukes-Cantor distances. *Bacillus* spp. were included as an out-group to root the tree. The node numbers return the bootstrap values after 1000 replicates.

Discussion

Throughout the recent history of microbiological research, different types of culture media have been designed to meet the nutritional requirements of microorganisms cultured *in vitro*. While these media have been mostly successful for the general cultivation of a broad range of microorganisms, they have been less successful when it comes to meeting the requirements for cultivating endophytes. We have addressed this issue by using a novel approach which focuses on the use of plant-based culture media for the recovery and cultivation of bacterial endophytes. The proposed plant-only culture medium is of a complex nature that mimics the nutritional matrix present in the plant root and/or rhizosphere. Such plant-only-based culture media contain relatively well-balanced amounts of nutrients [9–11], being of natural origin, concentration and diversity. We found that a culture medium with concentrations as low as 0.25 g plant powder per liter supported the cultivability of a large number of Lucerne rhizobacteria, to the extent that no statistically significant differences were observed between the tested concentrations of plan-only teabags culture medium (0.25, 0.5, 1.0, 4.0 g l^{-1}). The population densities of rhizobacteria, in terms of total CFUs, were higher on plant-only teabags media compared with the rich Standard I nutrient agar; recovering up to 62-71% and 2-16% of the total bacterial numbers (as measured by qPCR), respectively. This result corresponds with a number of other techniques that have been proposed to develop culture media with low nutrient concentrations in order to facilitate culturing the unculturables [30–32]. Further, the community composition of rhizobacteria recovered on plant-only teabags culture media was substantially different, being clustered apart from those fed on Standard I nutrient agar. In fact, the use of plant-only teabags culture media resulted in the development of more diverse populations of Lucerne rhizobacteria and extended the range of cultivability among its microbiome. This enabled us to easily recover pure isolates of globally distributed rhizobacteria, predominantly *Sinorhizobium meliloti*. It appeared that the plant-only teabags culture media gave preference to culturability and recovery of the specific micro-symbiont of Lucerne, namely *S. meliloti*. Moreover, they were able to reveal fastidious not-yet-cultured communities of Lucerne rhizobacteria (Table 2). Both of our two isolates LP1.2 and LP3.2 represent a high similarity to the uncultured Bacterium (AM697055) detected in the dust of indoor environments in Finland [33], and the uncultured *Novosphingobium* sp. (HM438566) which have been detected in a PCR-amplified 16S rDNA sequence from Mexican soil [34]. Two more isolates were originally isolated on plant-only teabags culture media and were subsequently able to regenerate on Standard I nutrient agar. The isolate LP3.11 was most closely (99% similarity) related to an

uncultured bacterium (HF586982.1), detected in a batch sample of activated sludge (Germany), and *Lysobacter pocheonensis* (accession no. EU273938) isolated from the soil of ginger and ginseng fields in South Korea and China. The second isolate LP2.2 was found to be most closely related (99% similarity) to an uncultured *Pedobacter*- JN697525 [35], and the nearest cultivated type strain is *Pedobacter steynii* isolated from a spring of the Westerhofer Bach, Westerhof, Germany [36]. So far and to the best of our knowledge, those 3 genera (*Novosphingobium*, *Lysobacter*, and *Pedobacter*) brought into cultivation via plant-only teabags culture media were not reported in literature as culturable endophytes of Lucerne.

Table 2. Growth performance of a group of rhizobacteria isolates of Lucerne, originally developed on Lucerne-powder culture medium, on commonly-used chemically-synthetic culture media.

Isolates (codes)	Culture media of original isolation	Successive growth on other culture media	Comments
Rhizobia isolates			
• ***S. meliloti*** - LP1.1, LP1.3, LP3.1, LP3.3, LP3.4, LP3.5, LP3.7, and LP3.8	Plant medium (+)	NA (-)	*In vitro* free-living growth of *S. meliloti* requires growth factors (amino acids/vitamins [37]) that are naturally present with balanced amounts in the plant medium, compared to ambiguous quantities in the yeast extract added to the chemically-synthetic culture media of YEMA, LB, TY [37]
		CCM (-)	
		YEMA (+/-)	
Non-rhizobia isolates*			
• ***Novosphingobium* sp.** - LP1.2 and LP3.2	Plant medium (+)	NA (-)	As root stimulating bacteria, cultivable isolates of *Novosphingobium* sp. brought to culture in wood plant culture medium (WPM) amended with casein hydrolysate, nicotinic acid, pyridoxine, thiamine, glycine, asparagine and glutamine [38]
		CCM (-)	
		YEMA (+/-)	
• ***Pedobacter* sp.** - LP2. 2	Plant medium (+)	NA (+)	To optimize growth of culturable isolates of *Pedobacter* sp., rich amendments of tryptone, yeast extract, and NH_4Cl are required [39]
		CCM (-)	
		YEMA (+)	
• ***Lysobacter* sp.** - LP3.11	Plant medium (+)	NA (+)	To bring the very low populations of terrestrial and aquatic *Lysobacter* sp. into cultivation, need to be enriched in culture media containing yeast cells, in addition to antibacterial and antifungal drugs inhibiting other microorganisms [40]
		CCM (-)	
		YEMA (+)	

+, good growth; +/-, scant growth; -, no growth
NA, nutrient agar
YEMA, yeast extract mannitol agar, representing a number of yeast extract-containing culture media (LB, TY, R2A) commonly used to culture rhizobia
CCM, N-deficient combined carbon sources culture medium used for culturing rhizobacteria other than rhizobia [41], grouped with similar culture media, e.g. M9, and supplemented with yeast extract and/or defined amino acids/vitamins.
Note: *Such non-rhizobia three genera, representing many other fastidious rhizobacteria associated/satellite to rhizobia, emerged/enriched on the plant culture media because of its contents of a wide array of natural amounts of C and N compounds as well as growth factors (amino acids and vitamins); But, they are smeared/masked by rather big and slimy colonies of fast-growing bacteria invasive to chemically-synthetic culture media (NA and YEMA,LB, TY, TSA, R2A) because of their copious contents of peptones, casein, beef and yeast extracts, sugars, alcohols,…etc.

It is known that free-living growth of *S. meliloti* is somewhat problematic, as they reported to require growth factors, such as amino acids, i.e. methionine, cysteine, and histidine as well as members of vitamin B group [37]. These nutritional demands are usually met via the inclusion of yeast extract in the commonly used rich chemically-synthetic culture media, e.g. YEMA, LB, and TY. The major problem of using such rich yeast extract-amended culture media is the unusual over growth of big slimy colonies of rhizobia as well as other fast-growing bacteria, mainly representatives of Firmicutes and Gammaproteobacteria that smear or mask the satellite fastidious rhizobacteria [38–41]. This is not the case when using the plant-based culture medium because of the balanced Lucerne's biochemical repertoire, having proteins (>3.0-5.0 %), Carbohydrates (>6.0-17.0 %), 7 essential and 11 non-essential amino acids (>0.05-0.56 %); in addition to macro- (>0.3-6.0 %) and micro- (>13.0-70.0 µg/g) nutrients [42]. This particular nutritional matrix mimics the root milieu, and exceptionally satisfies both growth requirements, not only of specific rhizobia but also of other associated rhizobacteria, especially those hard to culture, e.g. the species of *Lysobacter* sp., *Pedobacter* sp., and *Novosphingobium* sp. that we reported for first time among the culturable microbiome of Lucerne.

We further used the G3 PhyloChip microarray to characterize the composite culturable rhizobacteria populations developed on culture media (unpublished data). Compared to nutrient agar, populations recovered on plant-based culture media included several unculturable microorganisms of the phyla Acidobacteria, Actinobacteria, Armatimonadetes, Chlorobi, Chloroflexi, Cyanobacteria, Gemmatimonadetes, Planctomycetes, Tenericutes, Synergistetes, Fibrobacteres, and Fusobacteria. We even found a greater culturability and abundance of representatives of the previously unculturable candidate phyla Atribacteria (OP9), Dependentiae (TM6), Gracilibacteria (GN02), Latescibacteria (WS3) Omnitrophica (OP3) and BRC1.

In conclusion, the data presented here, together with those reported earlier [9–11], strongly support the use of plant-only-based culture media as a new method for culturing rhizobacteria. It opens a new horizon towards exploring the unculturable world of authentic plant-fed rhizobacteria, with the possibility of uncovering bacteria with a great potential for improving plant nutrition and health. We are following in the footsteps of one of the great microbiology pioneers Hans Christian Gram (1853-1938), who stated that "the method is published, although we are aware that as yet it is very defective and imperfect; but it is hoped that in the hands of other investigators it will turn out to be useful" (www.schaechter.asmblog.org).

Acknowledgements

Hegazi and Sarhan acknowledge the financial support of Alexander von Humboldt Foundation, Bonn, during their 2014 research stay in Germany. They are grateful to all kinds of support provided by Prof. Eckhard George in his capacity as the research director of IGZ. Thanks are also extended to Ann-Christin Scherwinski and Birgit Wernitz for their excellent technical and lab support. We appreciate the support of our graduate student Ahmed Tarek for his excellent schematic illustration, and Hassan Daanaa for editing support.

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Chapter 3: An inoculum-dependent culturing strategy (IDC) for the cultivation of environmental microbiomes and the isolation of novel endophytic Actinobacteria

An inoculum-dependent culturing strategy (IDC) for the cultivation of environmental microbiomes and the isolation of novel endophytic Actinobacteria

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Abstract

The recent introduction of plant-only-based culture media enabled cultivation of not-yet-cultured bacteria that exceed 90% of the plant microbiota communities. Here, we further prove the competence and challenge of such culture media, and further introduce “the inoculum-dependent culturing strategy, IDC”. The strategy depends on direct inoculating plant serial dilutions onto plain water agar plates, allowing bacteria to grow only on the expense of natural nutrients contained in the administered inoculum. Developed colonies are successively transferred/subcultured onto plant-only-based culture media which contains natural nutrients very much alike to those found in the prepared plant inocula. Because of its simplicity, the method is recommended as a powerful tool in screening programs that requires microbial isolation from a large number of diverse plants. Here, the method comfortably and successfully recovered several isolates of endophytic Actinobacteria represented by the six genera of *Curtobacterium* spp., *Plantibacter* spp., *Agreia* spp., *Herbiconiux* spp., *Rhodococcus* spp., and *Nocardioides* spp. Furthermore, two of the isolates are most likely novel species belonging to *Agreia* spp. and *Herbiconiux* spp.

Keywords:

Actinobacteria / Endophytes / Plant microbiome / Source inoculum effect phenomenon / Inoculum-dependent cultivation / Plant-only-based culture media

Not-yet-cultured populations represent diverse groups of microbes that account for more than 90% of a given ecosystem that are still under shadow (Libby and Silver 2019). To gain insights into their unknown functions and exploit their potentials, different approaches were recently introduced to culture such not-yet-cultured bacteria (Berdy et al. 2017, Lagier et al. 2018). In this respect, the plant-only-based culture media were presented as natural culture media to replace myriad formulas of synthetic culture media, and strongly recommended to increase the cultivability of the plant microbiota (Sarhan et al. 2016, Youssef et al. 2016, Hegazi et al. 2017, Saleh et al. 2017, Mourad et al. 2018, Sarhan et al. 2018, Sarhan et al. 2019). To alleviate the stress of disproportionate nutrients, present in common culture media, we aimed at culturing maize and sunflower microbiota on the natural nutrients present in the plant inoculum itself, and related to highly diluted plant-only-based culture media and standard R2A. The rationale of the study is to allow the existing plant microbiota to grow on nutrient-deficient water agar (WA) depending on the proportionate nutrients present in the administered inoculum itself. Expressly, the plant inoculum is used as a dual source of enclosed microbes and inherent nutrients.

To test this hypothesis, appropriate serial dilutions of surface-sterilized roots (rhizosphere) and shoots (phyllosphere) of maize plants (*Zea mays* L.) were surface-inoculated on plain water agar (WA), principally used to examine the growth of CFUs of plant endophytes depending on the nutrients contained in the inoculum itself. For comparisons, i.e. bench marking, other sets of agar plates were prepared as well from: a) 1/200 diluted autoclave-sterilized maize juice (AJ, v/v), b) 1/200 diluted filter-sterilized maize juice (FJ, v/v, filtration through 0.2 µm Sartorious membrane filters), and c) 1/100 diluted (v/v) R2A culture medium. The plant juices were prepared by juicing carefully-washed shoots of full-grown plants using a sugarcane juicer. By incubation at 25°C, counts of colony-forming units (CFUs) of the rhizosphere, log 8.13-8.75 g^{-1}, markedly exceeded those reported for phyllosphere, log 5.23-5.51 g^{-1} (Fig. 1a). In general, CFUs counts were lowest at 4 days of incubation, and prolonging the incubation period up to 32 days resulted in slight, but not always significant, increases in CFU counts for either spheres. The extension of incubation time significantly stimulated the development of microcolonies (i.e. µCFU, < 1 mm dia.). Notably, the development of such microcolonies was very much reported for the rhizosphere compartment by the end of the incubation period (32 days), representing > 20 % out of total CFU counts (Fig. S1). Such phenomenon of microcolonies development was brought into focus while introducing novel approaches for increasing culturability of environmental microbiomes (Overmann et al. 2017b, Gutleben et al. 2018, Lagier et al. 2018). Indeed, the

development of microcolonies was significantly enhanced by new methodologies for culturing the uncultured bacterial populations, e.g. the use of overlay agar technique for plating (Nour et al. 2012), the diffusion-chamber-based techniques, encapsulation of cells in gel microdroplets under low nutrients flux conditions, and soil slurry membrane system that combines a polycarbonate membrane as a growth support and soil extract as substrate (Zengler et al. 2002). Majority of microcolonies developed by such techniques were isolated in pure cultures and identified as not-yet-cultured/novel species bacteria (Zengler et al. 2002). The recurrent development of such microcolonies was experienced in the present study, depending on the combined effect of prolonged incubation time and the proportionate nutrient concentrations present either in water agar receiving plant nutrients contained in the plant inoculum itself, or the extensively-diluted plant juice and R2A agar. In other words, the limited nutrients, present in such extensively-diluted culture media, don't satisfy the growth of fast-growing bacteria, but allow the recovery of fastidious ones developed in form of microcolonies.

Table 1. Log CFU counts of phyllosphere bacteria of sunflower plants developed on all tested culture media over incubation time.

Culture media	Incubation time (Days)	
	4 days	9 days
Plain water agar (WA)	$3.94\pm0.23^{d*}$	4.20 ± 0.26^{bcd}
Standard R2A	4.45 ± 0.20^{ab}	4.52 ± 0.18^{ab}
Plant-only-juice culture media (AJ)	4.05 ± 0.32^{cd}	4.39 ± 0.29^{abc}
Plant-only-juice culture media (FJ)	4.48 ± 0.20^{ab}	4.60 ± 0.15^{a}

*Statistically significant differences are designated by different letters based on Tukey's Honestly Significant Differences (HSD, $P \leq 0.05$, n = 4)

Although the limitations of PCR-DGGE of the 16S rRNA gene, the method is nonetheless in practice for studying microbial diversity (Orlewska et al. 2018). Consequently, we used culture-dependent PCR-DGGE of the 16S rRNA gene to analyze the community compositions of the culturable endophytes of maize rhizosphere and phyllosphere (Edenborn and Sexstone 2007, Sarhan et al. 2016). PCR-DGGE analysis of CFUs harvest clearly differentiated the rhizosphere populations from those inhabiting the phyllosphere at similarity level of 48% (Fig. 1b). This might be attributed to the differential composition of the nutrient pools of either plant compartments. No explicit separations were reported as due to the culture media effect. Hence, it is suggested that by using highly diluted culture media, the inoculum has stronger impact in shaping culturable community composition than the culture medium by itself. This is in consistency with the findings of Hegazi et al. (Hegazi et al. 2017). They reported gradual separations of DGGE profiles of culturable population of lucerne rhizobacteria according to nutrient concentrations and origin; at the very low

concentrations, i.e. 0.25 – 1.00 g L^{-1} of plant powder, no further separations were observed. Such phenomenon, of source inoculum effect, was defined as the influence of the inoculum on the growth of its pertinent load of bacterial cells.

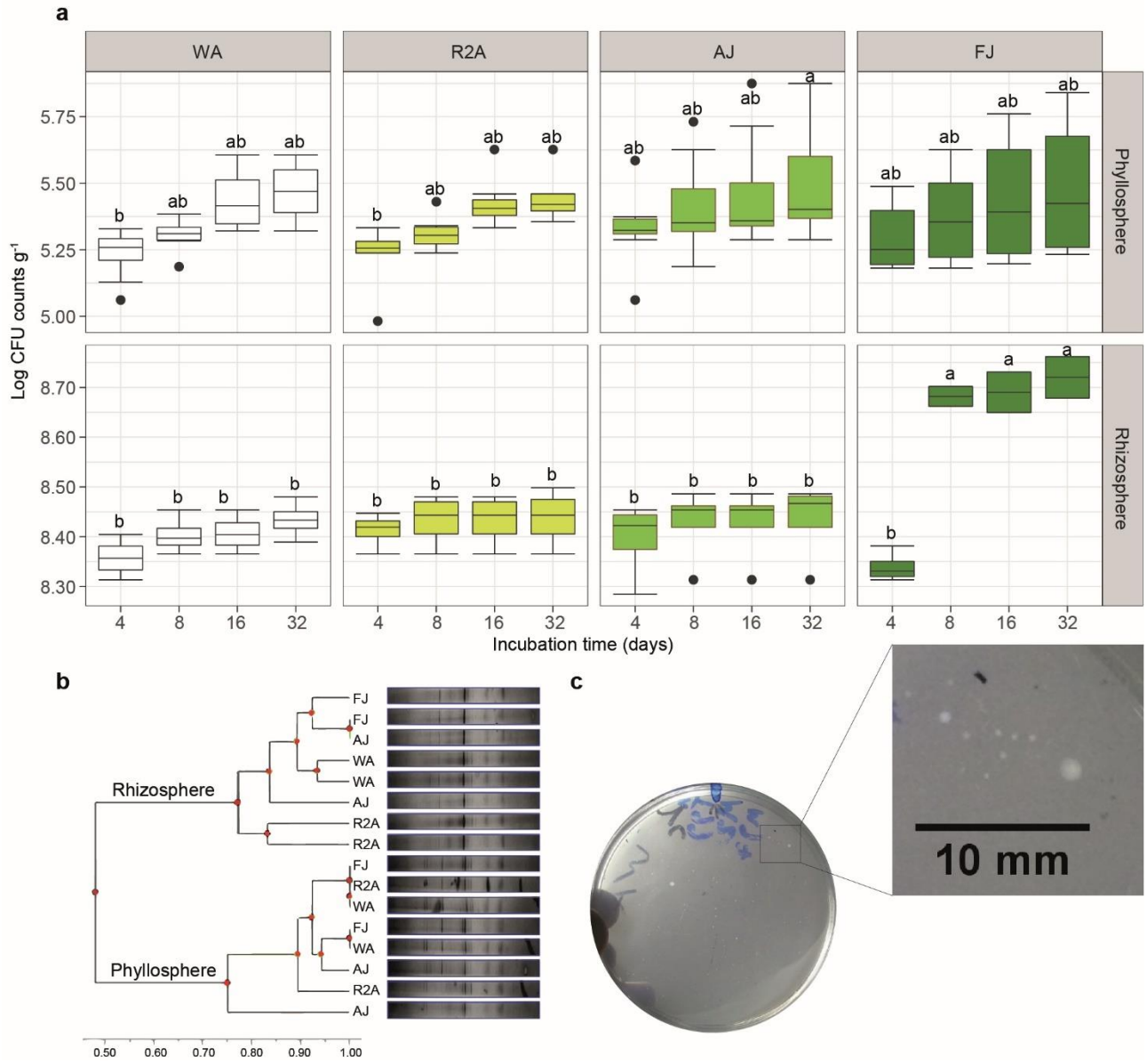

Fig. 1 Culture-dependent bacterial community analysis of maize rhizosphere and phyllosphere. **a**, Log CFU counts developed on the tested culture media over time. Statistically significant differences are designated by different letters based on Tukey's Honestly Significant Differences (HSD, $P \leq 0.05$, n = 4); **b**, DGGE analysis of 16S rRNA gene profiling of culturable bacterial community recovered on the tested culture media after 32 days of incubation, clustered with UPGMA method based on Euclidean distances (totallab.com/home/cliqs); **c,** Morphologies of colonies grown on WA together with zoom in section of macro- and micro-colonies – scaling was carried out using ImageJ software (imagej.nih.gov/ij). WA, water agar; R2A, 1/100 diluted (v/v) R2A; AJ, autoclaved 1/200 diluted (v/v) maize juice agar; and FJ, filtered 1/200 diluted (v/v) maize juice agar culture media.

To confirm such phenomenon, another experiment was carried out with maize phyllosphere. This is primarily to test the pretreatment of inocula suspensions to eliminate as much as possible their contents of nutrients' traces. This pretreatment included low-speed centrifugation for the removal of coarse plant debris and high-speed centrifugation

for collecting pellets of bacterial cells, together with successive washings (Supplementary methods). Results indicated highly significant differences (P = 2.24 ×10^{-6}) attributed only to the inoculum pretreatment (Fig. S2). The significant decreases in counts of CFUs developed on WA not AJ is a clear indication on the affinity between the nutrients and the bacterial load of the inocula.

In view of the previous results obtained, we further analyzed the phyllosphere bacteria of sunflower plant (*Helianthus annuus* L.) using the IDC strategy to confirm the microcolonies phenomenon and specifically analyze their taxonomic affiliation. In general, counts of CFUs developed on culture media of AJ and FJ of sunflower, diluted R2A, and plain WA were in the range of log 3.9 to log 4.6 g^{-1}. With prolonged incubation (9 days), one-way ANOVA displayed no significant differences, and counts of CFUs developed on the plain water agar (WA) were very much comparable to those developed on the conventional culture media based on plant juices (AJ, FJ) or chemically synthetic nutrients (R2A) (Table 1). Again, the pervasive occurrence of microcolonies was reported on both WA and FJ. Randomly, we picked representatives of those microcolonies (µCFUs) for further subculturing and taxonomic identification using 16S rRNA gene sequencing. Isolates derived from WA plates failed to further sub-culturing on plain WA. Interestingly however, all of the isolates, including those recovered from WA, successfully-maintained culturability on FJ semi-solid and agar culture media for up to 4-6 subsequent generations. Furthermore, it proves that IDC is a workable culturing strategy of potential application as a culturomic tool in future. As to the microcolonies, some retained their status (<1 mm dia.), while others developed into macrocolonies (>1 mm dia.), with varying chromogenic phenotypes. This is contrary to what have been earlier reported by previous investigations, where microcolonies were irreproducible, i.e. they were not able to grow when subcultured on the same original culture media of isolation (Da Rocha et al. 2010, Youssef et al. 2016, Overmann et al. 2017b). It appeared that microcolonies require further and careful domestication/passage process to sustainably grow in standard Petri dishes (Nichols et al. 2010). In our case, microcolonies were able not only to just survive after subculturing, but also proliferate and turn into macrocolonies when further subcultured on the related plant-only juice culture media (FJ).

The 16S rRNA gene sequences of secured isolates were deposited in the GenBank under the accession numbers MK100479-MK100506, and compared to the databases of GenBank (ncbi.nlm.nih.gov) and EZBioCloud (ezbiocloud.net). The analysis revealed the domination of endophytic Actinobacteria species, with single incidences of each of the phyla Proteobacteria (*Methylobacterium mesophilicum*) and Firmicutes (*Bacillus aryabhattai*). The

twenty-six isolates of the phylum Actinobacteria represented the genera *Curtobacterium* spp. (18 isolates), *Plantibacter* spp. (1 isolate), *Agreia* spp. (1 isolate), and *Herbiconiux* spp. (1 isolate) of the family Microbacteriaceae, as well as the genus *Rhodococcus* spp. (2 isolates) of the family Nocardiaceae, and the genus *Nocardioides* spp. (2 isolates) of the family Nocardioidaceae (Fig. 2). Interestingly, the isolates F19 and F20 are most likely to represent novel species - their highest matching scores were < 98.7%. This was confirmed as Maximum Likelihood (ML) phylogeny, with bootstrapping of 1000 replicates, displayed significant separation of such two isolates away from all of the deposited members of the genera *Herbiconiux* and *Agreia* (Fig. 2).

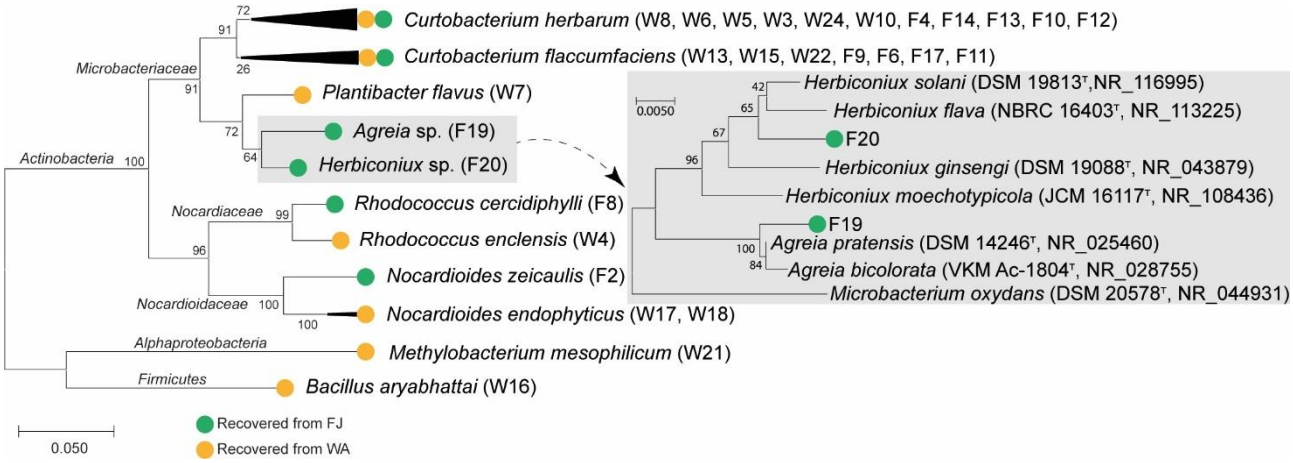

Fig. 2 Maximum Likelihood (ML) phylogenetic tree of the bacterial isolates recovered from WA (15 isolates) and FJ (13 isolates) culture media, based on 16S rRNA gene sequences - The subtree includes the isolates F19 and F20 with all reported species of the genera *Agreia* and *Herbiconiux*, including *Microbacterium oxydans* as an outgroup. Bootstrapping was performed for each tree with 1000 replicates; the percentage of trees in which the associated taxa clustered together is shown next to the branches. Phylogenetic analyses were conducted in MEGA X. WA, water agar; R2A, 1/100 diluted (v/v) R2A; AJ, autoclaved 1/200 diluted (v/v) sunflower juice agar; and FJ, filtered 1/200 diluted (v/v) sunflower juice agar culture media.

Our results highlight the endophytic nature of Actinobacteria as well as their wide occurrence in plant tissues, being widely known as a potential bio-repertoire of natural products. To facilitate their isolation and cultivation, tens of culture media were developed and enabled isolation of wide range of Actinobacteria, as described in literature (Table S1). They all contain one or more of diversified carbon and nitrogen sources as well as growth factors, e.g. soluble starch, lignin, chitin, cellulose, glycerol, asparagine, casein, yeast extract, soil extract, humic acid, and consortia of vitamins and amino acids (Jiang et al. 2016b, Wei et al. 2018). Culture media derived from plant materials (e.g. oatmeal medium, YIM21) are also recommended (Jiang et al. 2016b). It appeared that a prerequisite of culturing fastidious Actinobacteria is to suppress fast-growing microbes and eliminate competition of Gram-positive and Gram-negative bacteria, as well as fungi. This is achieved through use of complex C and N sources, salt concentrations, pH levels, and appropriate antibiotics and chemicals. This is imperative to keep mining the rich store of antibiotic, active

compounds and secondary metabolites of newly isolated Actinobacteria (Barka et al. 2016, Goodfellow et al. 2018).

As the water agar does not support growth of most prokaryotes *per se* (Silva et al. 2003), the reported ability of WA to recover CFU counts implies that the applied inocula contained both microbes (biomass) and nutrients (plant juice). In theory, the constitutive effect of the source inoculum on CFU development is considered of an intrinsic nature. When using conventional rich culture media, such effect is suppressed, i.e. being recessive. Employing the host plant materials (e.g. juices, concentrates and powders) in the preparation of plant-based culture media, as in Mourad et al. and Hegazi et al. (Hegazi et al. 2017, Mourad et al. 2018), the effect appears as synergic. In case of using IDC or highly diluted culture media, the effect appears as dominant. This persuades us to reconsider previous reports on the use of diluted culture media and the resulted increases in culturability (Armanhi et al. 2017, Hegazi et al. 2017). We may extrapolate that such increases are most likely considered as an inoculum effect. This supports the growing interest in strategies of increasing cultivability of *in situ* bacterial communities by boosting the recovery of microcolonies via dilution of nutrients present in the recommended standard culture media and/or adjusting *in vitro* atmosphere of the surroundings (Da Rocha et al. 2010, Lagier et al. 2018).

In conclusion, our results encourage the use of ultra-diluted plant-only-based culture media, contained in the plant inoculum itself (IDC), for *in vitro* cultivation of the plant microbiota. The method guarantees providing real-time nutrients of the tested homologous host plants with their unique diversity, complexity, and concentrations. This strategy is a simple, practical, and convenient approach to mine for the hidden and novel members of the plant microbiota, particularly those of biotechnological potential like Actinobacteria. It is a promising culturomic tool, and highly recommended for future screening programs that require isolation of large number of isolates from diverse plants, as well as multiple analysis of other environmental microbiomes.

Acknowledgement

The present work was funded by the German-Egyptian Research Fund (GERF-STDF 5032). Hegazi acknowledges the support of Alexander von Humboldt Foundation for equipment subsidies and financing his research stays in Germany, at IGZ. Our gratitude is extended to Prof. Eckhard George, the IGZ research director, for his continuous support and cooperation. The junior authors appreciate the financial aid provided by edX foundation for pursuing the online courses 7.QBWx, BIF001x BIF002x, and BIF003x, through edX platform (edx.org).

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Supplementary materials

Supplementary methods

Host plant sampling

Representative samples of the whole root system and the leaves of full-grown maize plant (*Zea mays*) were obtained from the experimental fields of the Faculty of Agriculture, Cairo University, Giza, Egypt. The sunflower plants (*Helianthus annuus*) were obtained from the experimental fields of the Leibniz Institute of Vegetables and Ornamental Crops (IGZ), Großbeeren, Germany. Microbiological analyses were conducted within 2 hours of sampling.

Preparation of culture media

Plant-culture media, based on filter- and autoclave-sterilized juices: The maize juice of vegetative shoots and leaves was extracted by small electrical sugarcane juicer, while sunflower leaves were crushed in Waring blender. Crude juices were then pre-filtered through Whatman No. 1 filter papers and stored at -20°C until usage. Two formulations of plant-only-based culture media were prepared from sterilized juices, either autoclaved (for 20 min at 121°C) or filtered (through Sartorius membrane filters of 0.2 µm pore size). The prepared juices were added to autoclaved water agar to a final concentration of 1:200 (v/v) to prepare plant-only-based culture media: filter-sterilized juice **(FJ)** and autoclave-sterilized juice **(AJ)**. The measured pH of both culture media was 7.0 ± 0.1.

Water agar culture medium (WA): Bacteriological Agar (residue on ignition (Ash) < 6.5%, Sigma-Aldrich A5306, MO, USA) was added to distilled water (1.2%, w/v), then autoclaved for 20 min at 121°C.

Reasoner's 2A agar culture medium (R2A): The R2A culture medium was prepared according to Reasoner and Geldreich (Reasoner and Geldreich 1985), then diluted with distilled water 1:100 (v/v).

All tested culture media were tested for sterility before use.

Recovery of endophytic bacterial populations:

Microbiological analyses were carried out for both the endorhizosphere and endophyllosphere communities of maize plants and the phyllosphere of sunflower. Initial suspensions of tested plant spheres were prepared by adding 5 g of the respective plant samples to 45 mL of the sterilized ½ strength basal salts of CCM (Hegazi et al. 1998), and carefully blended with mortars and pestles. Further serial dilutions were prepared, and aliquots of 100 µL of suitable dilutions were surface-inoculated on agar plates prepared from

tested plant-only-based and R2A culture media. Incubation took place at 25°C for up to 32 days for maize and at 28°C for up to 9 days for sunflower.

Elimination of traces of nutrients present in plant inocula

In an attempt to remove as much as possible of plant nutrients in the original maize phyllosphere suspension, one mL of the suspension was transferred into a 1.5 mL microcentrifuge tube and low-speed centrifuged (at 3,000 rpm for 30 sec) for the removal of coarse plant debris. The supernatant was transferred into another microcentrifuge tube and re-centrifuged at 10,000 rpm for 10 min. The resultant supernatant was discarded and the pellet was re-suspended in ½ strength basal salts of CCM; the last two steps were repeated thrice (Dong et al. 1994). Then, further serial dilutions were prepared, and aliquots of 100 µL of suitable dilutions were surface inoculated on agar plates of AJ and WA culture media. Incubation took place at 25°C for up to 32 days. Developed colonies were differentiated to macro-colonies (MCFU, ≥ 1 mm in diameter) and micro-colonies (µCFU, < 1mm in diameter).

DGGE analysis of culture-dependent bacterial communities

Maize endophytic CFUs developed on agar plates (>30–300 CFUs plate^{-1}), in 2 replicates representing the various culture media, were harvested using 4 mL of 0.05 M NaCl solution. CFUs harvest suspensions were centrifuged for 10 min at 13000 rpm. DNA extraction was carried out from the resulting pellets using i-genomic DNA Extraction Mini Kit (iNtRON Biotchnology Inc., Kyungki-Do, Korea). DNA concentration was measured by BioDrop µLITE Spectrophotometer (BioDrop Inc., Warwickshire, UK).

Amplification of whole 16S rRNA gene and reamplification of the V3-region were carried out according to Sarhan et al. (Sarhan et al. 2016), using the universal primers of Muyzer et al. and Mühling et al. (Muyzer et al. 1993, Mühling et al. 2008).

DGGE was performed according to Sarhan et al. (Sarhan et al. 2016), with VS20WAVE-DGGE (Cleaver Scientific, Warwickshire, UK). DGGE fingerprints were analyzed using CLIQS 1D software (TotalLab, Newcastle upon Tyne, UK).

DNA extraction and 16S rRNA gene sequencing of representative isolates of sunflower phyllosphere

Surface-inoculated plates were prepared for tested sunflower phyllosphere, representing various tested culture media, of FJ, AJ and R2A. After 9 days of incubation at 28°C, CFUs were carefully monitored. Representative micro-colonies (< 1.0 mm in diameter) particularly developed on water agar (16 colonies) and FJ culture media (16 colonies) were further

subcultured on agar plates of plant-juice-based culture media. On FJ semi-solid culture media and agar plates, all of the isolates were successfully subcultured and maintained growth for several passages. They were subjected to DNA extraction and 16S rRNA gene sequencing (Eurofins MWG Operon, Ebersberg, Germany). To obtain the nearest phylogenetic neighbours, 16S rRNA gene sequences were compared with their closest matches using EZBioCloud identification tool (ezbiocloud.net/identify). The Sequence alignments and the phylogenetic tree were constructed using ClustalW and the Neighbour-Joining method based on the Jukes-Cantor model implemented in MEGA X (Kumar et al. 2018). The 16S rRNA gene sequences identified in this study have been deposited in the GenBank database under the accession numbers MK100479-MK100506.

Statistical analysis

The following R-CRAN packages (cran.r-project.org): “Agricolae” for calculating the Honest Significant Differences (HSD) and “ggplot2” for plotting.

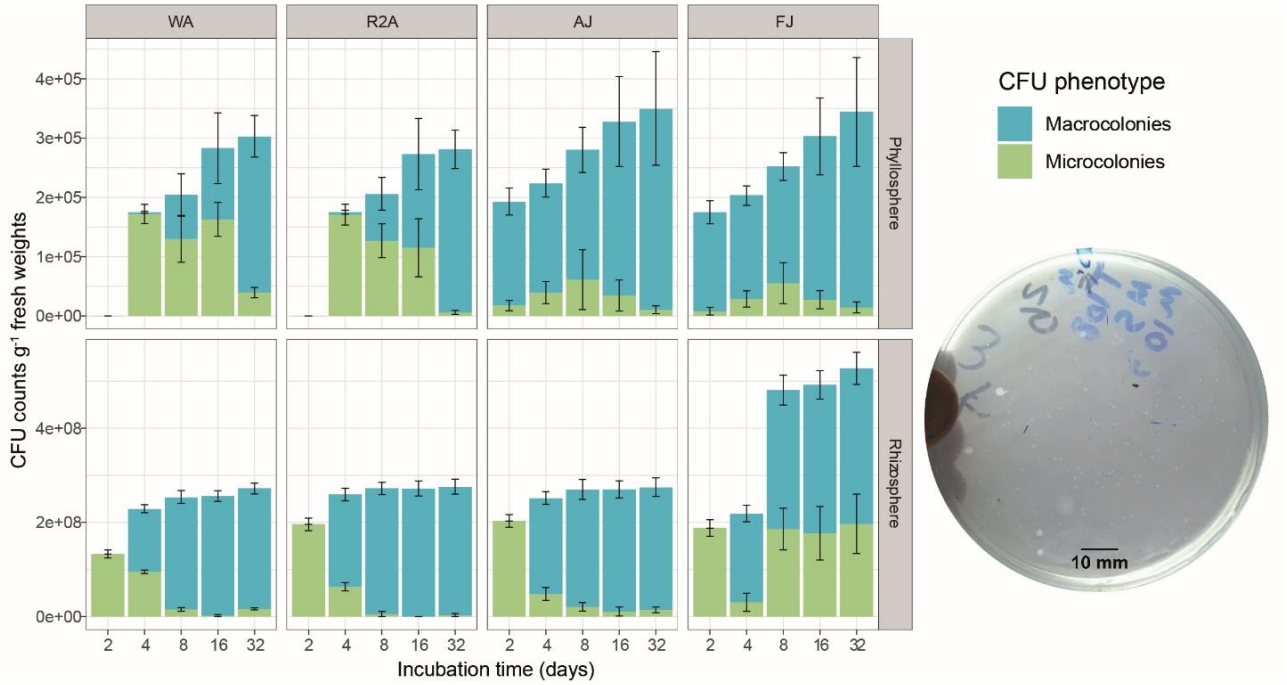

Fig. S1 CFU counts of bacterial community analysis of maize rhizosphere and phyllosphere. Bars indicate the proportion of micro- and macro-colonies. Error bar indicates the standard error (n = 4). Inserted is a plate showing the development of macro- and micro-colonies on water agar (WA).

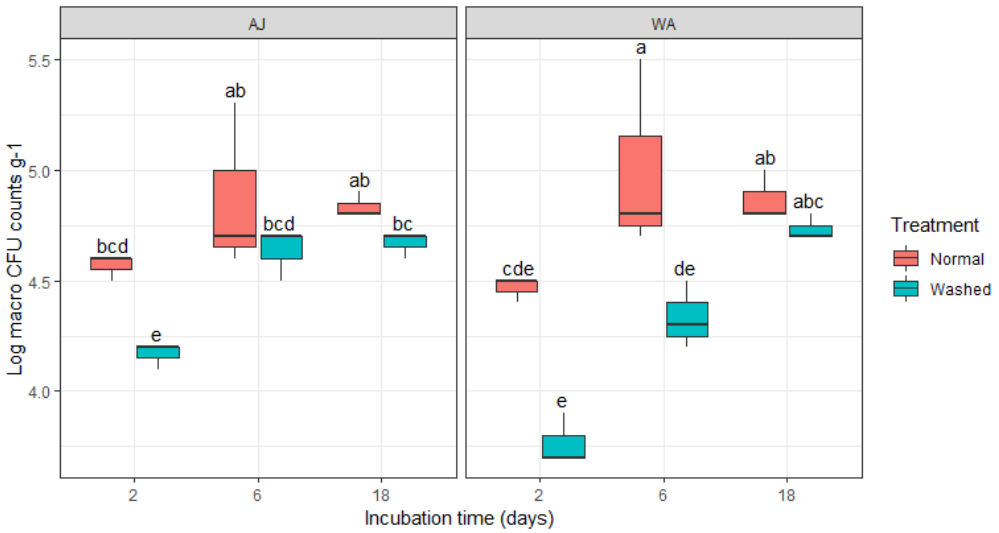

Fig. S2 Culture-dependent bacterial community analysis of maize phyllosphere: The effect of pre-treatment of inoculum suspension to remove as much as possible of the nutrients traces available in the inocula. Log CFU counts developed on the tested culture media overtime.; statistically significant differences are designated by different letters based on Tukey's Honestly Significant Differences (HSD, $P \leq 0.05$, n = 3).

Table S1 Identification of sunflower rhizobacteria isolates* based on 16S rRNA gene sequences and closest hits obtained from the EZBioCloud database, and their corresponding isolation source.

Top-hit taxon (Isolates Codes*)	Similarity (%)	Isolation Media	Environment/Host
• *Agreia* sp. (VKM Ac-1783) - F19	99.61	Trypticase Soy Agar (TSA)	Gall of *Elymus repens* induced by Nematode (Evtushenko et al. 1994)
• *Bacillus aryabhattai* - W16	99.65	Nutrient agar	Air, upper atmosphere (Shivaji et al. 2009)
• *Curtobacterium flaccumfaciens* - W22, W15, W13, F9, F6, F17, and F11	≥ 99.57	Congo-red Sucrose Agar	Beans stem (Hedges 1922)
• *Curtobacterium herbarum* - W8, W6, W5, W3, W24, W10, F4, F14, F13, F10, and F12	≥ 99.36	Nutrient agar II	Mulch of sward (Behrendt et al. 2002)
• *Herbiconiux flava* - F20	98.61	Corynebacterium agar	Phyllosphere of *Carex* sp. (Hamada et al. 2012)
• *Methylobacterium mesophilicum* - W21	99.48	Glucose Yeast Extract Agar	Epiphyllosphere of *Lolium perenne* (Andreote et al. 2006)
• *Nocardioides endophyticus* - W18 and W17	≥ 99.14	Reasoner's 2A agar (R2A)	Endorhizosphere of *Artemisia princeps* (Kim et al. 2016)
• *Nocardioides zeicaulis* - F2	98.96	Trypticase Soy Agar (TSA)	Maize stem (Kämpfer et al. 2016)
• *Plantibacter flavus* - W7	99.64	Nutrient agar	Mulch of sward (Behrendt et al. 2002)
• *Rhodococcus cercidiphylli* - F8	99.77	Tap-water Yeast Extract Agar [5]	Endophyllosphere of *Cercidiphyllum japonicum* (Li et al. 2008)
• *Rhodococcus enclensis* - W4	99.26	Marine agar	Marine sediment (Dastager et al. 2014)

* W, codes of isolates recovered from water agar plates; F, codes of isolates recovered from sunflower filtered juice (FJ) agar plates.

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Chapter 4: G3 PhyloChip Analysis Confirms the Promise of Plant-Based Culture Media for Unlocking the Composition and Diversity of the Maize Root Microbiome and for Recovering Unculturable Candidate Divisions/Phyla

G3 PhyloChip analysis confirms the promise of plant-based culture media for unlocking the composition and diversity of the maize root microbiome and for recovering unculturable candidate divisions/phyla

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Abstract

The rapid development in high throughput techniques and expansion of bacterial databases accelerate the efforts to bring plant microbiomes into cultivation. We introduced the plant-only-based culture media as a successful candidate to mimic nutritional matrices of plant roots. Here, we employed G3 PhyloChip microarray to meticulously characterize culture-dependent and -independent bacterial communities of maize root compartments, endo- and ecto-rhizosphere. Particular emphasis was given to the preference of growth of the unculturable candidate divisions/phyla on plant-only-based culture media compared with standard culture media (nutrient agar). A total of 1818 different operational taxonomic units (OTUs) were resolved representing 67 bacterial phyla. The plant-only-based culture media displayed particular affinity towards recovering the endophytic compared with ectophytic rhizobacteria. This was shown by the tendency of higher CFUs recovery for endophytes on the plant-only-based culture media (26%) compared with the standard culture media (10%) as well as higher taxa richness and numbers of exclusive families of unculturable divisions/phyla. Out of 30 bacterial phyla (comprising > 95% of the whole population), 13 were of significantly higher incidence on the plant-only-based culture media, 6 phyla of which were not-yet-cultured (*Atribacteria*, OP9; *Dependentiae*, TM6; *Latescibacteria*, WS3; *Marinimicrobia*, SAR406; *Omnitrophica*, OP3; BRC1). Furthermore, the plant-only-based culture media significantly enriched the less abundant and/or hard-to-culture bacterial phyla (*Acidobacteria*, *Gemmatimonadetes*, and *Tenericutes*). Our results present conclusive evidence of the ability of the plant-only-based culture media to bring the plant-fed *in situ* microbiome into the status of plant-fed *in vitro* cultures, and to widen the scope of cultivation of heretofore-unculturable bacterial divisions/phyla.

Key words: Plant microbiome – Plant-based culture media – Unculturable bacteria – *Candidatus* Phytoplasma – Candidate phyla/divisions

Introduction

Plant roots are very active integrated ecosystems, harboring massive numbers of bacterial species composing the root microbiome. These bacteria are essentially contributing to plant nutrition and health (3, 38, 39, 50). So, the study of the root microbiome is of great importance for various biotechnological applications, *e.g.* plant growth promotion, biological control, and/or the production of bioactive compounds. Unfortunately, most of these plant inhabiting bacterial species (> 90%) are non-culturable and they may play principal and often unique roles in root ecosystem functions (3, 25, 58). So far, the highly diverse bacterial community has been partially explored using cultivation-based methods (49), and the use of culture-independent methods has deciphered unknown members of plant microbiomes, resulting in high throughput data relating to the unculturable bacterial components of the plant microbiome (2).

The recent development of metagenomics powered a series of articles studying the functional analyseses of entirely uncultured bacterial groups (5, 40, 46, 53, 60, 63). However, culturing of those bacteria is essential for functional understanding and further microbiological and biotechnological applications. Therefore, efforts are continually exerted towards bringing these not-yet-cultured organisms into axenic cultures by developing new growth media with various substrate compositions and modifying existing culture media and cultivation methods. These modifications include the addition of nutritional supplements and dilution of nutrient concentrations, together with prolongation of the incubation period (23, 51). Imitating the natural environments within the culture media is another major approach (6, 18, 19). Recently, we proposed the usage of plant materials as the sole source of nutrients in culture media to explore a wider range of the plant microbiome (43, 51, 62).

The majority of bacterial clades in the newly introduced tree of life (26) are only identified through analysis of bulk environmental samples and metagenomics, and the number of environmental 16S rRNA gene sequences has greatly surpassed the number of cultured microorganisms; therefore, taxonomic assignment of these sequences lags behind (11, 61). Consequently, the precise number of bacterial phyla that may be recoverable is not known. According to the greengenes database (greengenes.lbl.gov), estimates of bacterial phyla numbers currently range from 31 to 88, depending on which of the five different taxonomy systems are used (Pace, Hugenholtz, Ludwig, RDP, or NCBI). The main reason behind this discrepancy is the inability to culture a number of bacterial phyla in order to accommodate them within a systematic classification. Therefore, continuous efforts of *in*

vitro culturing of representatives of those candidate phyla/divisions remains a valid approach to achieve a unified bacterial taxonomy.

In this study, we analyzed the microbiome of the maize (*Zea maize* L.) root compartments (endo- and ecto-rhizospheres), to compare the culture-dependent versus culture independent bacterial communities, in terms of CFU counts versus total bacterial qPCR counts, and the resulting comparative taxonomic characterization of these communities. We employed G3 PhyloChip microarrays to explore the taxonomic affiliation of culture-dependent bacterial communities, developed on the newly plant-only-based culture media versus the standard artificial nutrient agar, in relation to root culture-independent bacterial communities. Our primary objective was to highlight the culture media effect on the incidence and abundance of the unculturable candidate bacterial phyla/divisions. The G3 PhyloChip used in this study accommodates 1.1 million DNA probes able to categorize all known bacteria and archaeal operational taxonomic units (OTUs) into over 50,000 taxa using 59,959 clusters of 17 nucleotides as probes representing 147 phyla, 1123 classes, 1219 orders and 1464 families, for a total of 27938 OTUs (20).

Materials and Methods

Plant sampling

Samples representing the vegetative parts and root system of fully mature maize plants (*Zea mays* L.) were obtained from the experimental fields of the Faculty of Agriculture, Cairo University, Giza, Egypt (30°01’03.7”N 31°12’19.1”E). They were transferred to the laboratory in plastic bags, and used directly for culture-dependent and culture-independent analyses.

Culture-independent quantification of total bacterial numbers (qPCR)

To quantify total bacterial numbers in the endo- and ectorhizosphere samples, the16S rRNA gene copy numbers were measured using the universal primers 519f and 907r specific for the bacterial domain (33) and the real-time PCR analysis with SybrGreen I as an intercalating dye. The quantification and cycling program were performed according to the detailed methodology described by Sarhan *et al.* (51). The data calculation was carried out using CFX^{TM} optical systems software and a calibration curve established by a tenfold dilution (range between 10^9 to 1 copy per µL) of specific *Escherichia coli* PCR product. The quality of the quantification method was verified using a melting profile, giving one specific melting peak at 87°C, and running an agarose gel detecting one single band at 407 bp size. The bacterial cell numbers were calculated indirectly by assuming that the average rRNA operon copy number per cell is 3.6 (31, 52).

Culture-dependent analysis

For the ectorhizosphere (outer root compartment representing rhizoplane together with closely adhered rhizosphere soil), plant roots were shaken vigorously and a known weight transferred to half strength basal salts of CCM liquid medium as a diluent (22). Further serial dilutions were prepared (10^{-2}-10^{-7}). For the endorhizosphere (inner root compartment), plant roots were carefully washed with tap water, surface-sterilized with 95% ethanol for 30 sec followed by 3% sodium hypochlorite for 30 min, then thoroughly washed five times with sterile distilled water. Five grams of the surface-sterilized roots were blended for 5 min. in a Waring blender using 45 mL of half strength basal salts of CCM liquid medium as a diluent, and further serial dilutions were prepared. Aliquots of 200 µL of suitable dilutions representing both root compartments (ecto- and endo-rhizosphere samples) were surface-inoculated on agar plates of tested culture media. Agar plates were incubated at 28 °C for 2-10 d, and developed colony forming units (CFUs) were periodically examined and counted.

Culture media

Plant-only based culture media (51): We used clover plant (*Trifolium alexandrinum* L.) because of its diverse nutrient composition, with regard to C and N compounds, macro- and microelements, and amino acids and vitamins (51). This nutrient composition supports its ability to satisfy the growth of rhizobacteria associated to various host plants (43, 51). The plant teabags culture media were prepared according to Sarhan *et al.* (51). Shoots of fully-grown clover (*Trifolium alexandrinum* L.) were dehydrated in the sun for 24 h, and then oven dried at 70 ºC for 24 h. The dehydrated plant materials were mechanically ground to pass through a 2.0 mm sieve to obtain fine dehydrated powder. Teabags were prepared by packing two grams of the dehydrated powder into each bag and sealing by stapling. Two teabags (each containing 2 g) were added to 1 liter of distilled water to obtain the liquid plant infusion. Solid culture media were prepared by adding agar (2%, w/v), pH adjusted to 7.0, then autoclaved for 20 min at 121 °C. The teabags were left in the culture media during autoclaving for further plant extraction. Media were tested to ensure sterility before use.

Nutrient agar: A standard nutrient agar was used, consisting (g L^{-1}): beef extract, 3.0; peptone, 5.0; glucose 1.0; yeast extract 0.5 g L^{-1}.

Agar culture media were prepared by adding agar (2%, w/v), and autoclaved at 121 °C for 20 min. All culture media were proved to ensure sterility before use.

N-deficient combined carbon-source medium (CCM) basal salts by Hegazi *et al.* (23): This formulation comprises (g L^{-1}): K_2HPO_4, 0.4; KH_2PO_4, 0.6; $MgSO_4$, 0.2; NaCl, 0.1; $MnSO_4$, 0.01; 0.2; KOH, 1.5; $CaCl_2$, 0.02; $FeCl_3$, 0.015; Na_2 MoO_4, 0.002; in addition to $CuSO_4$, 0.08 mg L^{-1}; $ZnSO_4$, 0.25 mg L^{-1}.

Sample preparation for PhyloChip hybridization

To characterize the composition of culture-dependent and culture-independent microbial communities, three representative samples of each root compartment (endo- and ectorhizospheres) were included: 1) The cultured community of tested plant-based culture media; 2) The cultured community grown on standard nutrient agar; and 3) The native microbial community of maize roots without enrichment. For each sample, three replicates were included in the analyses.

Harvest of microbial colonies and DNA extraction

For DNA extraction of culture-dependent communities (samples 1 and 2), all CFUs developed on representative 10 d-incubated agar plates of tested culture media were washed using 0.05 M NaCl solution, and collected by centrifugation for 10 min at 9500*x* g. DNA was extracted from collected CFU pellets using the QIAGEN Dneasey plant mini kit (QIAGEN, Hilden, Germany) according to the manufacturers' instructions. Likewise, and for culture-independent analyses (sample 3), total DNA was extracted from 5 mL aliquots of the original root suspensions of ecto-and endo-rhizosphere samples (three replicates each) that were previously prepared for making serial dilutions for culture-dependent analysis. DNA concentrations were measured at 260 nm and DNA quality was checked photometrically by A_{260}/A_{280} ratio calculation to be above 1.9, and A_{320} measurement was nearly 0 using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., MA, USA).

16S rRNA gene amplification and PhyloChip hybridization

Using the DNA from the plates' harvest and original root suspensions, the bacterial 16S rRNA genes were amplified using the degenerate forward primer 27f: AGRGTTTGATCMTGGCTCAG, and the non-degenerate reverse primer 1492r: GGTTACCTTGTTACGACTT. Thirty-five cycles of amplification were performed, and the amplified products were fragmented, biotin labeled, and hybridized to the PhyloChip™ Array, version G3 following the described procedures of Hazen *et al.* 2010 (20). PhyloChip arrays were washed, stained, and scanned using a GeneArray® scanner (Affymetrix) (Fig S1). Each scan was captured using standard Affymetrix software (GeneChip® Microarray

Analysis Suite). Samples were processed in a Good Laboratory Practices (GLP) compliant service laboratory running Quality Management Systems for sample and data tracking. The laboratory (Second Genome's service laboratory, South San Francisco, CA, USA) implements detailed standard operating procedures (SOPs), equipment and process validation, training, audits and document control measures. Quality control (QC) and quality assurance (QA) metrics were maintained for all sample handling, processing and storage procedures. The detailed PhyloChip protocol is described in the Methods S1.

Data summarization and statistical analysis

After the taxa were identified for inclusion in the analysis, the values used for each taxa-sample intersection were populated in two distinct ways; Hybridization Scores (HybScores, Fig. S1) used directly to denote for abundance (Data S4, available upon request for data depository), and incidence scores to denote for presence/absence (Data S5, available upon request for data depository). Detailed information for the data summarization are provided in the Methods S1.

Second Genome's PhyloChip processing software, Sinfonietta, was used to inter-compare all samples' profiles in a pair-wise fashion, and the UniFrac distances (36) were utilized to determine the metric distance between tested communities. For Weighted UniFrac, the OTU abundance was additionally considered, whereas UniFrac was used for presence/absence data. Two-dimensional ordinations Principal Coordinates Analysis (PCoA) and hierarchical clustering maps of the samples in the form of dendrograms were created to graphically summarize the inter-sample relationships. For whole microbiome significance testing, the Adonis test was utilized for finding significant differences among weighted and unweighted data.

We used the following R-project's packages (https://cran.r-project.org/): "pvclust" was used for calculation of the bootstrap probability percentages. "VennDiagram" was used for construction of Venn diagrams to represent the overlapping of taxa using the incidence data based on family-level, "gplots-heatmap.2" function was used to construct heatmaps of abundance of phyla and OTUs.

Student's t-test was applied across the tested samples, and heatmaps were generally used to display the OTUs of significant differences (P value ≤ 0.05) in abundance. Among the 1160 OTUs, representing 206 families, we constructed the heatmap of Fig. 3B to compare the abundance of 218 OTUs of significant differences among plant-based culture and nutrient agar. The circular tree presented in Fig. S5 was constructed using the reference number of each bacterial group in PhyloT (http://phylot.biobyte.de/), based on NCBI

taxonomy. Taxonomy labels and abundance data are rendered in iTOL (34) (http://itol.embl.de/). The rings around the tree comprise a heatmap: Blue indicates the OTU was more abundant in that sample than in the mean of the baseline samples, and yellow indicates the OTU was less abundant, and the color saturation indicates the degree of difference from the mean value of the baseline samples.

Results

Culture-dependent and independent quantification of the maize root microbiome

We compared the culture-dependent (plant-based culture media and nutrient agar) versus the culture-independent (qPCR) bacterial communities of the inner (endorhizosphere) and the outer (ectorhizosphere) compartments of maize roots. Significantly less culture-independent bacterial cell numbers were reported in the endorhizosphere (log 8.7 ± 0.061 bacterial cells g^{-1} root) compared with the ectorhizosphere (log 9.29 ± 0.06 bacterial cells g^{-1} root) (Fig. 1). In general, the culture-dependent populations represented < 30% of the culture-independent total bacterial numbers in the endorhizosphere, with significantly higher values for the plant-based culture media (26%) compared with only 10% for the nutrient agar (Fig. 1). While the culturable populations in the ectorhizosphere represented only 4-5% of the culture-independent bacterial numbers, no significant differences were attributed to the tested culture media. The plant-based culture media revealed a remarkable development of microcolonies (µCo, <1 mm diameter discriminated with 40x magnification, Fig. S2).

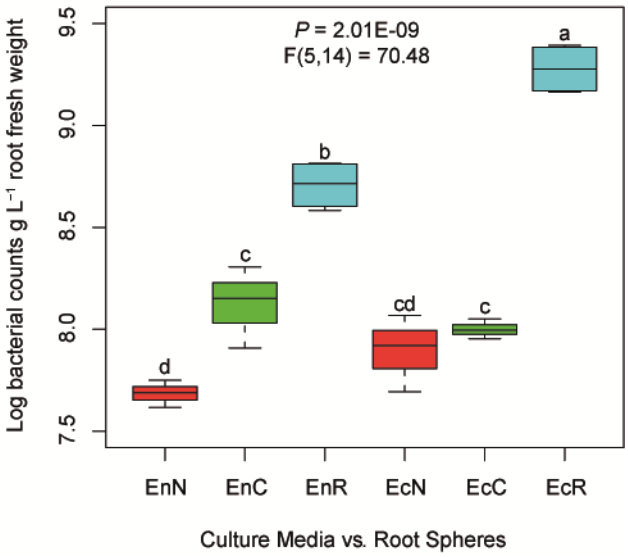

Fig. 1. Culture-dependent and culture-independent recovery of rhizobacteria associated with maize root compartments. Log numbers of CFUs of culturable rhizobacteria in endorhizosphere (En) and ectorhizosphere (Ec) as developed on nutrient agar (EnN, EcN), and Plant-based culture medium (EnC, EcC). Total numbers of rhizobacteria measured by qPCR in maize root compartments of endorhizosphere (EnR) and ectorhizosphere (EcR). Statistical significant differences are indicated by different letters at $P \leq 0.05$ (Tukey's HSD).

G3 PhyloChip-based culture-dependent and independent prokaryotic community analysis

G3 PhyloChip microarrays were used to analyze the culture-dependent (CFUs-harvest of representative agar plates) and the culture-independent (original suspensions of the tested root compartments) bacterial and archaeal communities of the endorhizosphere and the ectorhizosphere of the maize plant. A total of 1818 different OTUs, comprising both bacteria and archaea, were detected in all of the samples analyzed (Fig. S3). Bacteria were represented by 1769 OTUs (67 phyla, 146 classes, 260 orders, 423 families, 704 genera, and 987 species), and Archaea corresponded to 49 OTUs (2 phyla, 8 classes, 12 orders, 17 families, 26 genera, and 32 species) (Fig. S3). Considering the presence/absence (incidence, unweighted data) and the abundance (weighted data) of the total 1818 OTUs, significant differences were attributed to the root compartments, endorhizosphere versus ectorhizosphere, and the methods of analysis, culture-dependent versus culture-independent communities (Table S1). Principal Co-ordinates Analysis (PCoA) and hierarchical clustering revealed clear separation between the culture-dependent (samples representing both culture media) and the culture-independent communities (samples representing plant roots), with subsequent separations among culture-dependent communities according to the root compartments, endorhizosphere and ectorhizosphere (Fig. 2A, B).

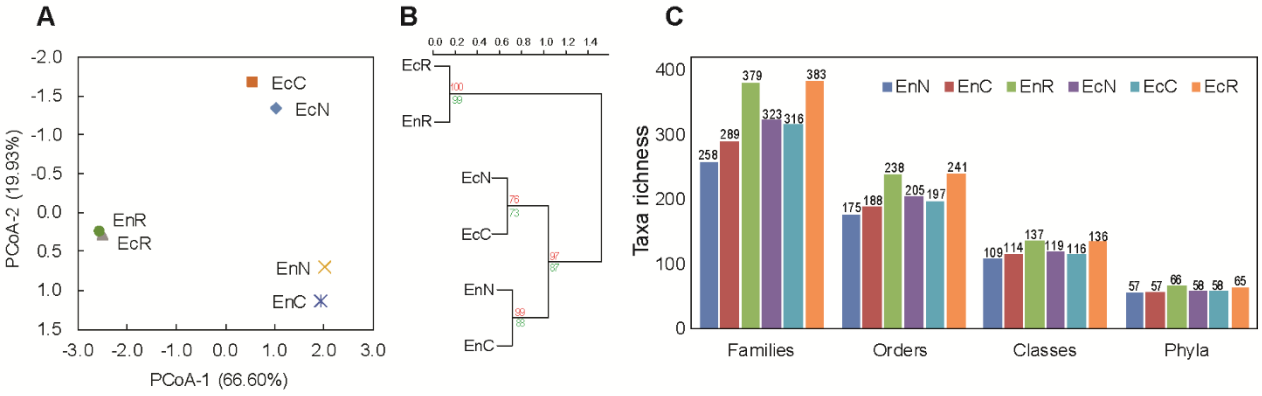

Fig. 2. Analysis of culture-dependent (CFUs) and culture-independent (maize roots) bacterial and archaeal community composition of maize root compartments based on G3-PhyloChip technology. **A**, Principal Co-ordinates Analysis (PCoA); **B**, Hierarchical clustering, bootstrap probability (%) are indicated in green and approximately unbiased" *p*-value indicated in red; **C**, bacterial richness at different taxonomic levels (phyla, classes, orders, families). **En**, endorhizosphere; **Ec**, ectorhizosphere; **R**, culture-independent root sphere; **C**, plant-based culture media; **N**, nutrient agar culture media.

Our results indicated that the richness of bacterial communities in the ectorhizosphere was slightly higher than the endorhizosphere, whether considering the culture-dependent or the culture-independent communities. Disparities among tested culture media were not detected at the phylum-level but started to appear in lower taxonomic levels to orders and

families. Such disparities were highly distinguished in the endorhizosphere, particularly associated with the plant-based culture media (Fig. 2C).

Commonalities and differences among the culture-dependent and independent bacterial communities

Data obtained pointed to the limitations of the PhyloChip method in securing continuous taxonomic annotation beyond families down to species level. Therefore, we restricted our analysis to the family level, and used the richness (number of families) to analyze the overlap between the culture-dependent and the culture-independent bacterial communities, i.e. culture media versus root compartments. Regardless of the method used, a total of 414 and 418 different bacterial families were detected in all of the tested samples representing endo- and ectorhizospheres respectively (Fig. 3A, Fig.S4 and Data S1, available upon request for data depository).

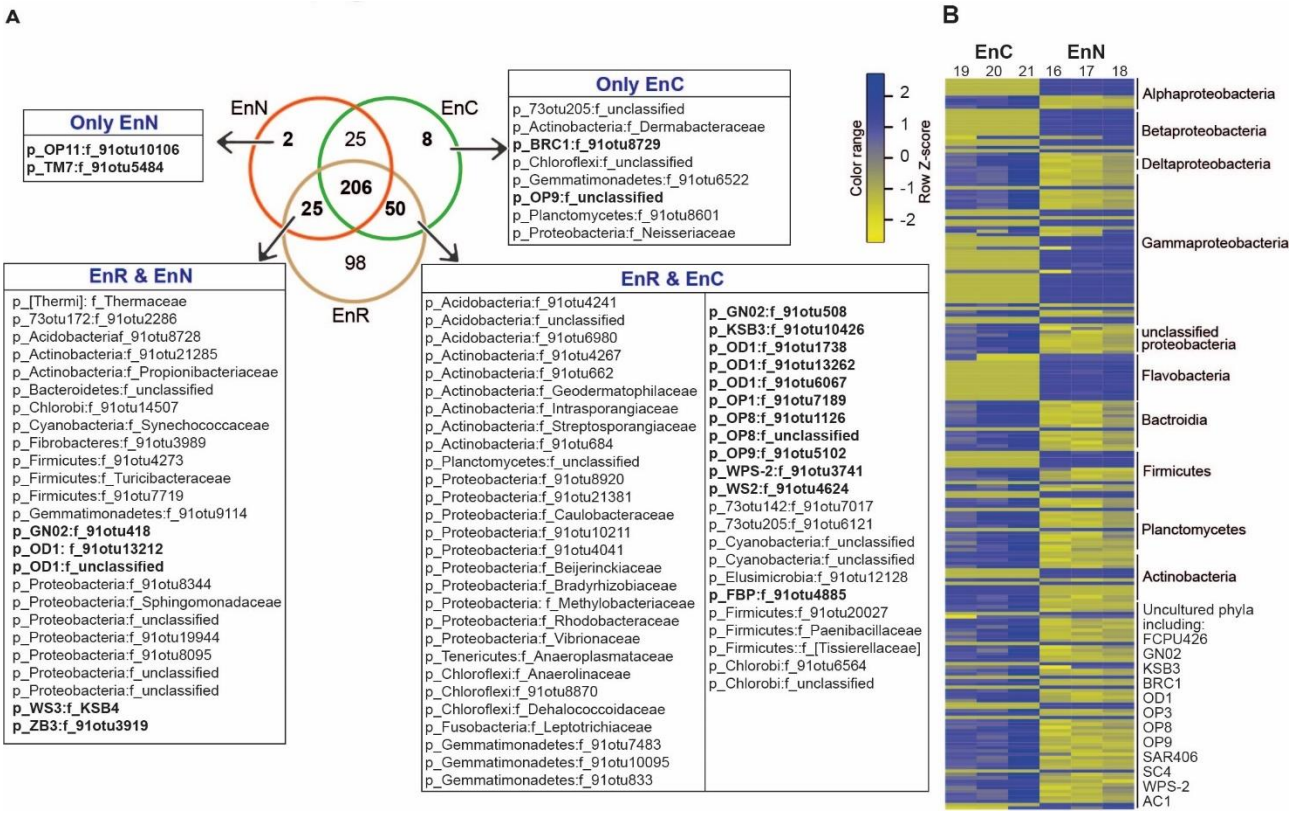

Fig. 3. Overlapping of culture-dependent (on plant-based culture medium and nutrient agar) and culture independent bacterial communities of maize root endorhizosphere. **A**, Venn diagram at family level for bacterial communities displaying the unique and overlapping families; families exclusively grown on only one of our tested media are shown in the linked boxes, and not-yet-cultured candidate divisions are marked in bold; **B**, heatmap of weighted abundance of the OTUs which displayed significant differences among the 206 families commonly grown on both culture media (**EnN**, nutrient agar; **EnC**, plant-based culture medium, three replicates shown for each medium) For detailed information, please refer to Data S3 (available upon request for data depository).

Of 414 families detected in the endorhizosphere, 98 (representing 23.7% of total families detected) were exclusively detected by the culture-independent method. On the

other hand, 35 families were only resolved by the culture-dependent method; of these, 25 were common among the plant-based culture media and the nutrient agar, 8 were unique for the plant-based culture media (2 belonged to the uncultured phyla/divisions BRC1 and *Atribacteria*, OP9) and 2 for the nutrient agar (belonged to *Microgenomates*, OP11 and *Saccharibacteria*, TM7). The plant-based culture media shared 50 families with the culture-independent communities which were not able to grow on the nutrient agar. Twelve of those families belong to the unculturable candidate phyla/divisions of FBP, *Gracilibacteria* (GN02), *Modulibacteria* (KSB3), *Parcubacteria* (OD1), *Acetothermia* (OP1), *Aminicenantes* (OP8), *Atribacteria* (OP9), WPS-2, and WS2. This is in addition to a number of rarely and/or difficult-to-isolate families belonging to the phyla of *Proteobacteria* (*Caulobacteraceae*, *Beijerinckiaceae*, *Bradyrhizobiaceae*, *Methylobacteriaceae*, *Rhodobacteraceae*, and *Vibrionaceae*), *Actinobacteria* (*Geodermatophilaceae*, *Intrasporangiaceae*, and *Streptosporangiaceae*), *Chloroflexi* (*Anaerolinaceae* and *Dehalococcoidaceae*), *Firmicutes* (*Paenibacillaceae* and *Tissierellaceae*), *Fusobacteria* (*Leptotrichiaceae*), and *Tenericutes* (*Anaeroplasmataceae*) (Fig. 3A). On the other side, the nutrient agar shared lower numbers of families, only 25 families, with the culture-independent communities, which were not detectable on the plant-based culture medium. Five of these families belong to the unculturable candidate phyla/divisions *Gracilibacteria* (GN02), *Parcubacteria* (OD1), *Latescibacteria* (WS3), and ZB3 (Fig. 3A). Two-hundred and six families were commonly detected by both methods, and cultured on both culture media. Hence, and since we are interested in the effect of culture media, we used the abundance of OTUs representing these families to compare the enrichment potential of both tested culture media and their suitability for recovering specific bacterial groups. These 206 families were represented by 1160 OTUs, 218 of which displayed significant differences among plant-based culture medium and nutrient agar (Fig. 3B). We found that the OTUs belonging to *Betaproteobacteria*, *Gammaproteobacteria*, and *Flavobacteria* were especially enriched on the nutrient agar. In contrast, the plant-based culture media preferentially recovered taxa of *Deltaproteobacteria*, *Bactroidia*, *Actinobacteria*, *Tenericutes*, *Fibrobacteres*, *Chlamydiae*, *Cyanobacteria*, *Elsumicrobia*, *Gemmatomonadetes*, *Nitrospirae*, as well as a number of uncultured candidate phyla/divisions (FCPU426, *Gracilibacteria* (GN02), *Modulibacteria* (KSB3), BRC1, *Parcubacteria* (OD1), *Aminicenantes* (OP8), *Atribacteria* (OP9), *Marinimicrobia* (SAR406), SC4, and WPS-2) (Fig. 3B, Data S1, available upon request for data depository).

When we examined the overlaps in the ectorhizosphere, 61 families were only detected by the culture-independent methods. However, only 35 families were resolved by the culture-dependent methods; 5 on the plant-based culture media, 7 on the nutrient agar,

and 23 on both. Comparing the efficiency of tested culture media, in terms of exclusive families of the uncultured phyla/divisions detected, the plant-based culture media exclusively recovered 3 families belong to BRC1, *Microgenomates* (OP11), and ZB3. The plant-based media also shared with the culture-independent communities 3 families belonging to FBP, LCP, *Parcubacteria* (OD1) (Fig. S4 and Data S1, available upon request for data depository).

Pairwise comparisons among the culture media based on individual OTU abundance

We compared the abundance of the individual OTUs to assess the culture media potential in supporting the growth of the naturally less abundant bacteria, *i.e.* all bacterial phyla except *Proteobacteria*, *Firmicutes*, and *Bacteroidetes*. For these analyses, we considered only the OTUs whose abundances were significantly different between the nutrient agar and the plant-based culture media.

In the endorhizosphere, out of the total 1747 different OTUs detected, 520 (29 %) displayed significant differences in abundance between the plant-based culture media and the nutrient agar. Among these OTUs, 345 exhibited higher abundance on the plant-based culture media samples compared to 175 on the nutrient agar (Fig. S5A). Principal Co-ordinates Analysis (PCoA) disclosed distinct separation between the communities of the nutrient agar and the plant-based culture media along PCoA-1 and PCoA-2, which explain variations of 56% and 23% respectively (Fig. S5B). The abundance-based heatmap-annotated dendrogram of these significantly different OTUs, in total or when eliminating the biggest three phyla (*Proteobacteria*, *Firmicutes*, and *Bacteroidetes*), clearly demonstrated distinctive higher abundances in the plant-based culture media (Fig. S5C, D). We further selected and clustered the OTUs belonging to the unculturable candidate phyla/divisions (54 OTUs) for a closer view (Fig. S5E). We found that 46 of these were particularly enriched on the plant-based culture media. They belonged to the unculturable candidate divisions/phyla: *Parcubacteria* (OD1), *Microgenomates* (OP11), *Omnitrophica* (OP3), *Saccharibacteria* (TM7), *Latescibacteria* (WS3), *Dependentiae* (TM6), *Gracilibacteria* (GN02), *Modulibacteria* (KSB3), *Hydrogenedetes* (NKB19), *Cloacimonetes* (WWE1), *Atribacteria* (OP9), AC1, BRC1, FBP, GAL15, and LCP (Fig. S5E, the outer 3 rings).

A somewhat similar trend was noticed in the ectorhizosphere. Out of 1755 different OTUs, 180 OTUs, representing 10%, showed significant differences in abundance between the tested culture media (Fig. S6), where 108 were significantly higher on the plant-based media and the rest on the nutrient agar. Principal Co-ordinates Analysis (PCoA) and

heatmaps clustering displayed clear separations of abundance of such OTUs among the tested culture media (Fig. S6).

Pairwise comparisons among culture media based on phyla abundance

We employed Student's t-test to measure the significance of abundance at the phylum level, considering the sum of hybridization scores of all OTUs of each phylum. In the endorhizosphere, the nutrient agar only displayed significant higher abundances ($P \leq 0.05$) in the two major phyla: *Proteobacteria*, and *Bacteroidetes*. In contrast, the plant-based culture media significantly enriched 13 different phyla compared to the nutrient agar (Fig. 4). They include 6 not-yet-cultured candidate divisions/phyla: BRC1, *Omnitrophica* (OP3), *Atribacteria* (OP9), *Dependentiae* (TM6), *Latescibacteria* (WS3), and *Marinimicrobia* (SAR406). In addition to 7 fastidious not-easily-cultured phyla: *Acidobacteria*, *Chloroflexi*, *Cyanobacteria*, *Elusimicrobia*, *Gemmatimonadetes*, *Planctomycetes*, and *Tenericutes* (which includes the "*Candidatus* Phytoplasma") (Fig. 4). Of particular note here was the recovery of the *Candidatus* Phytoplasma genus, a plant pathogen that is known to lack a single representative isolate; these were specifically enriched with higher abundance on the tested plant-based culture media for both root spheres (Data S3, available upon request for data depository).

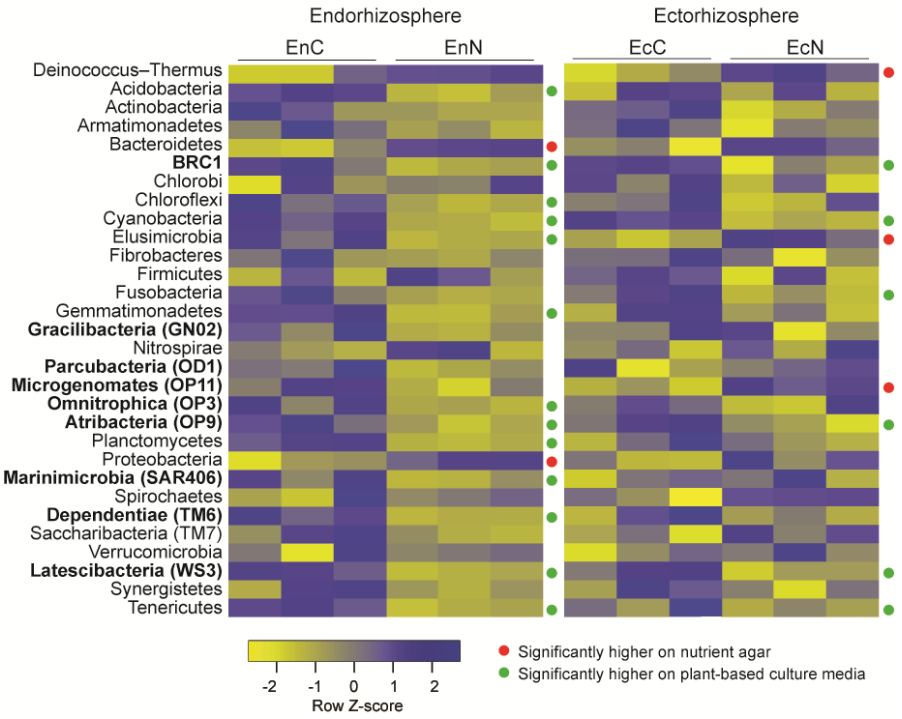

Fig. 4. Heatmap representing the significant differences between the tested culture media (nutrient agar and plant-based culture medium) in respect of weighted abundance of the 30 phyla, representing more than 95% of all detected OTUs, in the maize compartments of endorhizosphere and ectorhizosphere. The phyla indicated in bold are those who lack to even single representative isolate by far.

In the ectorhizosphere, the differences among the tested culture media was confined to only 9 phyla; 6 were significantly enriched on the plant-based culture media (BRC1, *Cyanobacteria*, *Atribacteria* (OP9), *Latescibacteria* (WS3), *Tenericutes*, and *Fusobacteria*)

and 3 on nutrient agar (*Deinococcus-Thermus*, *Elusimicrobia*, and *Microgenomates* (OP11)) (Fig. 4).

Discussion

The horizontal spatial divergence in bacterial community structure across the soil-root system was attributed to the proximity to the root core, and considered to be the key factor that mediates the variations within the root-associated microbial communities (13, 25, 37). Therefore, the nutrient diversity and balance provided within the plant milieu drive natural selection towards a characteristic microbiome fingerprint that identifies a given plant host. In this respect, the problem of selecting/predicting a suitable culture media for either *in vitro* cultivation or *in situ* recovery of elements of the plant microbiome is an acute one, even with the recent databases which guide the selection of compatible culture media (45). We believe that the use of culture media based on the nutritional make-up of the natural environment is the proper approach to explore the existing microbial population without discriminating against the less-abundant phyla. Accordingly, bringing the environment into the laboratory is a profitable approach and has already served as the basis for one of the most heralded success stories of bringing bacteria into culture (6, 16, 35, 54, 56, 59).

Plant materials were successfully introduced as the sole source of nutrients (plant-only-based culture media) for culturing rhizobacteria. Such culture media have proved to be advantageous in the cultivation of rhizobacteria compared with other standard artificial culture media (23, 43, 51, 62). One of the main reasons for the success of plant-based culture media to widen the scope of culturing not-yet-cultured rhizobacteria is their particular make-up of C and N compounds, and their richness in metal ions, vitamins and cofactors (51). These are the largest differentiators among defined growth conditions for strains within a given genus or family (45). This concept can be further extended to include other environmental interactions, such as quorum sensing molecules and siderophores in bacterial co-cultures that enable growth of other unculturable organisms (56, 59).

Many previous studies which compared the culture-dependent and independent methods ignore the paramount effect of the culture media – in terms of the origin, nature, and concentrations of nutrients – on the composition of bacterial communities (2, 28, 48, 49, 55). Therefore, we utilized the plant-only-based culture media because of the complexity and diversity of nutrients delivered by plant materials. In this study, and for the first time in the literature, we used the high throughput “G3-PhyloChip Microarray” to characterize the bacterial community composition resolved by both culture-dependent and independent techniques. We extensively compared the incidence and abundance of the culturable

bacterial communities of maize root compartments, emphasizing the effect of culture media and highlighting the range of not-yet-cultured candidate bacterial phyla/divisions and rarely-isolated phyla.

In conformity with our previous results based on PCR-DGGE analysis (51), our PhyloChip assay confirmed higher taxa richness on the plant-based culture media than on the nutrient agar; particularly in the endorhizosphere when compared with the ectorhizosphere. This supports our theory that the plant-based culture media creates unique balanced and supportive environments more suitable for the endophytes than the ectophytes, and emphasizes the strong imprint of the plant on its microbiome. This effect was partially observed when plant extracts were added as a supplement to standard culture media (14, 15, 41).

Comparisons of the overall phyla abundance among the tested culture media clearly demonstrated the significant enrichment of the plant-based culture media to not-yet-cultured candidate phyla/divisions: BRC1, *Omnitrophica* (OP3), *Atribacteria* (OP9), *Dependentiae* (TM6), *Latescibacteria* (WS3), and *Marinimicrobia* (SAR406) and other fastidious not easily-cultured phyla: *Acidobacteria*, *Chloroflexi*, *Cyanobacteria*, *Elusimicrobia*, *Gemmatimonadetes*, *Planctomycetes*, and *Tenericutes* (Fig. 4). The enrichment capacity of the plant-based culture medium was further demonstrated by the ratio of statistically significant enriched OTUs belonging to the not-yet-cultured candidate phyla/divisions (Fig. S5E). Of the 54 OTUs detected, the vast majority (46 OTUs) displayed higher abundance on the plant-based culture medium compared with the nutrient agar (8 OTUs), represented by *Parcubacteria* (OD1), *Microgenomates* (OP11), *Omnitrophica* (OP3), *Latescibacteria* (WS3), *Dependentiae* (TM6), *Gracilibacteria* (GN02), *Modulibacteria* (KSB3), *Hydrogenedetes* (NKB19), *Cloacimonetes* (WWE1), *Atribacteria* (OP9), AC1, FBP, GAL15, BRC1 and LCP. As the results of our DNA analysis targeted 10-days old grown CFUs, the detection of unique taxa with the used plant-based culture media points mainly to the potentiality of such culture media in increasing culturability, but probably may not guarantee stable successive subculturing, *i.e.* temporal recovery. We experienced this particular situation while culturing the microbiota of cactus "*Aloe arborescens*", where 40% of microcolonies (µ-colonies) that developed on homologous plant-based culture media failed to be successively subcultured (51, 62). It seems that to secure their successive subcultures, they require more defined endophytic growth conditions, *e.g.* nutrient complexity, long-term incubation, adjustable gas phases, and/or co-culturing conditions.

In agreement with Stewart (56), we believe that an “unculturable-status” does not imply that such an unculturable population “can never be cultured” but rather signifies that we lack critical information on their biology and physiology. This obstructs our efforts in the *in vitro* culturing of such populations because of three principle mechanisms: the very poor development in the form of overlooked microcolonies, the change in state to “Viable But Not Culturable (VBNC)”, and the lack of endosymbiosis (Table S2).

Contrary to culture-independent methods that expose the wide diversity of the plant microbiome, most of culture-dependent studies recover bacterial members belonging to the big-three phyla (*Proteobacteria*, *Firmicutes*, and *Bacteroidetes*) (2, 6, 15, 28, 48). Therefore, we introduced the plant-based culture media with the objective of recovering the fastidious bacterial groups at the expense of the fast-growing opportunistic bacteria. Obviously, the plant-based culture media provide the culturable microbiome with nutrients of diversity and concentrations very much mimicking the root milieu. Additionally, the plant materials in the form of plant-based culture media (51, 62) or as a supplement to enrich standard artificial culture media (44) proved to impair the swarming motility of bacteria on agar plates, halting their expansive progression and restricting the dominance of the larger slimy colonies. In many cases, such slimy colonies masked the presence of some small colonies and/or microcolonies. We noticed that the natural make up of nutrients of the plant-based culture media results in the unique advantage of supporting the relatively slow-growers of confined µ-colonies (Fig. S2). In this respect, representatives of unculturable bacteria were reported to develop in the form of microcolonies, *i.e.* successfully enriched but not visible to the naked eye (17, 21). Such microcolonies have attracted the attention of several studies, where fluorescence and on-chip-microscopy were used successfully to facilitate the assessment of microbial abundance (30, 32). One of the highlights of such explored microcolonies was the cultivation of the first representative isolate of the phylum TM7. After 20 years of trials, this candidate division was physiologically and genetically investigated, then successfully grown *in vitro* in the form of 20-200 µm microcolonies and designated as “*Saccharibacteria*” (1, 12, 54). As microcolonies were detected among the culturable population of our tested plant-based culture media, we strongly believe that the microcolonies phenomenon should attract more attention, and should be further investigated on plant-only-based culture media.

It is proposed that some cells that seem to lose viability during growth-arrested states are actually entering “Viable But Not Culturable (VBNC)” states, which are characterized by the inability to form colonies under nutrient stress conditions but with continual maintenance of the proton motive force (PMF) (4, 8, 24). The widespread existence of these VBNC states

in the environment, and the lack of understanding of the triggers for recovering from such states has been proposed as one of the reasons why many bacteria remain uncultured in the laboratory (16). Thus, it is not a state of unculturability but rather the inability to form a visible colony under routine standard cultivation conditions due to growth-arrested state, low metabolic activity and simultaneously increasing doubling time (4). Therefore, it is believed that the higher abundance of many such uncultured taxa on our tested plant-based culture media is possibly attributed to activating their growth and metabolic activity, leading to the emergence from a growth-arrested state (4, 9). In fact, the use of diluted culture media together with an extension of the incubation period of up to three months successfully proved such a theory (10, 27, 29, 57, 64). An example is the isolation of the first representative member of the candidate division OP10 "*Armatimonadetes*" as soon as the concentration of nutrients decreased down to 1% of the commonly used Trypticase Soy Agar (TSA) culture media. The secured isolate by itself resisted cultivation and subculturing on full strength Luria-Bertani (LB) and TSA culture media (57).

Based on the data derived from single-cell genomics as well as genome-resolved metagenomics, the majority of the unculturable candidate divisions/phyla are of limited metabolic capacities because of their small-sized genomes (Table S2). Many of them are compelled to be symbionts with other higher organisms (1, 5, 26, 42).

A striking example is the *Candidatus* Phytoplasma spp. whose genomes lack basic genes responsible for the tricarboxylic acid cycle, and sterol, fatty acid and most amino acid biosynthesis (47). According to the ATCC and DSMZ culture collections, this genus hasn't a single representative isolate. In fact, they are known to be plant pathogens and phloem symbiont/inhabitants which allows them to obtain their metabolic needs. Being resolved with significant enrichment on our tested plant-based culture media, we strongly believe that the complexity and diversity of nutrients delivered through such plant-based culture media fulfills their nutritional requirements, mimicking conditions that prevail in their natural habitat, the plant phloem (47). This was also confirmed by the successful *in vitro* recovery of this bacterium by constructing a complex culture medium to satisfy their nutritional requirements (7).

Conclusions

Our results present conclusive evidence and strong support for the potential of using plant-based culture media for culturing the plant microbiome, both for the purpose of increasing culturability and for deciphering the not-yet-cultured candidate phyla/divisions. As to future

work, adopting the novel methods associated with naturally-formulated culture media for further experiments is justified (23, 56). Rather than focusing on populating subsets of organism–media matrices for uncovering key growth principles of such unculturables (45), such methods will help to improve the success rate for recovering as-yet-uncultivated divisions *in vitro* for exploring/fostering their environmental impacts. We can now confidently predict that future progress with these methods will steer us towards the foreseen target of tailoring protocols and various strategies to increase culturability and isolate as many isolates as possible, known as “culturomics”, in plant microbiome studies.

Acknowledgements

Hegazi and Sarhan acknowledge the financial support of the Alexander von Humboldt Foundation, Bonn, Germany, during their research stay at IGZ, Germany, the Cairo University, Giza, Egypt and the Leibniz-institute of Vegetable and Ornamental Crops, Grossbeeren, Germany. They are grateful to all kinds of support provided by Prof. Eckhard George in his capacity as the scientific director of IGZ. Thanks are also extended to Birgit Wernitz and Ann-Christine Scherwinski for their excellent technical and lab support. We acknowledge the excellent G3-PhyloChip services and cooperation of Luisa Chan of Second Genome Inc. (South San Francisco, USA). We also appreciate the support of our graduate student Hassan Daanaa for technical support.

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Supplementary methods

Amplified products were then concentrated using a solid-phase reversible immobilization method for the purification of PCR products and quantified by electrophoresis using an Agilent 2100 Bioanalyzer®. PhyloChip Control Mix™ was added to each amplified product. Bacterial 16S rRNA gene amplicons were fragmented, biotin labeled, and hybridized to the PhyloChip™ Array, version G3. PhyloChip arrays were washed, stained, and scanned using a GeneArray® scanner (Affymetrix). Each scan was captured using standard Affymetrix software (GeneChip® Microarray Analysis Suite). From each of the purified PCR products, 500 ng were fragmented and hybridized. Assuming an average GC content of 54% (based on Greengenes database of 16S rRNA genes) and an amplicon length of 1,465 bps, 3.3+E11 (330 billion) molecules were assayed from each sample (11).

Second Genome's PhyloChip processing software, Sinfonietta, was used to execute a multi-stage process (11). The first stage of pixel summarization of the florescent image and array scaling were conducted as previously described by Hazen *et al.* (5). Array fluorescence intensity (FI) of each pixel and image was collected as integer values ranging from 0 to 65,536 providing 216 distinct FI values.

The summary of FI for each single probe feature on the array was calculated by ranking the FI of the central 9 of 64 image pixels and using the value of 75th percentile. Background was defined separately for each G+C class as the median feature FI of all non-16S control probes for a given G+C class. Next, all probes on the array were scaled by multiplication with a single factor so that average FI of the probes perfectly matching the PhyloChipTM Control Mix of non-16S spikes were equal. FI values from redundant probes were averaged to generate the simple probe-level table representing the responses of 994,980 unique 25-mers across all samples. Pairs of probes are two probes with similar but non-identical sequences which align along ≥ 23 bases with ≥ 1 mismatch or gap as determined by blastn (2). (-word_size 8 -dust no -perc_identity 92 –e value 0.005 -penalty -1). Although all probes can produce minor fluorescence from non-specific hybridization, if a sequence-specific hybridization has occurred the probe complementing the target will be brighter than its mis-matching mate as has been observed in 70% of controlled experiments (6). As a general caution, perfect matching probes (PM) were considered positive if they fulfilled the following criteria in comparison to their corresponding mis-matching probes (MM). A) (PM minus MM) > 3*(G+C class-specific background) AND (PM minus MM)/(PM plus MM) > 0.189.

Only PM FI from probes observed as positive in at least 4 experiments were exported from all experiments then rank-normalized in Sinfonietta software and used as input to empirical probe-set discovery. Probes were clustered into probe-sets based on both correlations in FI across all biological samples and taxonomic relatedness. Where multiple clustering solutions were available, higher correlation coefficients were favored over lower, taxonomic relatedness at the species level was favored over higher ranks, and sets composed of more probes were favored over less. All probe sets contained ≥ 5 probes. The empirical OTU (eOTU) tracked by a probe set was taxonomically annotated against the May 2013 release of Greengenes from the combination of the 8-mers contained in all probes of the set (7). The mean FI for each eOTU and each sample was calculated and then rank-normalized within each sample. These values are referred to as the hybridization score (HybScore) used in abundance-based analysis. The proportion of probes for an eOTU that are observed as positive in a sample is referred to as the positive fraction (pf). An eOTU was considered present in a sample where pf ≥ 0.8, and only eOTUs passing the pf cutoff are included in the AT and BT tables below.

Table S1. Adonis test and significance summary between OTUs of culture-dependent and independent microbial communities, characterized by G3-PhyloChip microarray analysis, based on weighted and unweighted UniFrac distance matrices. Significant differences ($P \leq 0.05$) are indicated by *.

column_name	Comparison_between	sample_counts	WUniFrac	UniFrac
Rootsphere	Ec | En	9 | 9	0.009*	0.016*
Milieu	C | N | R	6 | 6 | 6	0.001*	0.001*
comcategor	EcC | EcN | EcR | EnC | EnN | EnR	3 | 3 | 3 | 3 | 3 | 3	0.001*	0.001*
RvsC	C | R	6 | 6	0.003*	0.004*
RvsN	N | R	6 | 6	0.005*	0.002*
NvsC	C | N	6 | 6	0.023*	0.065

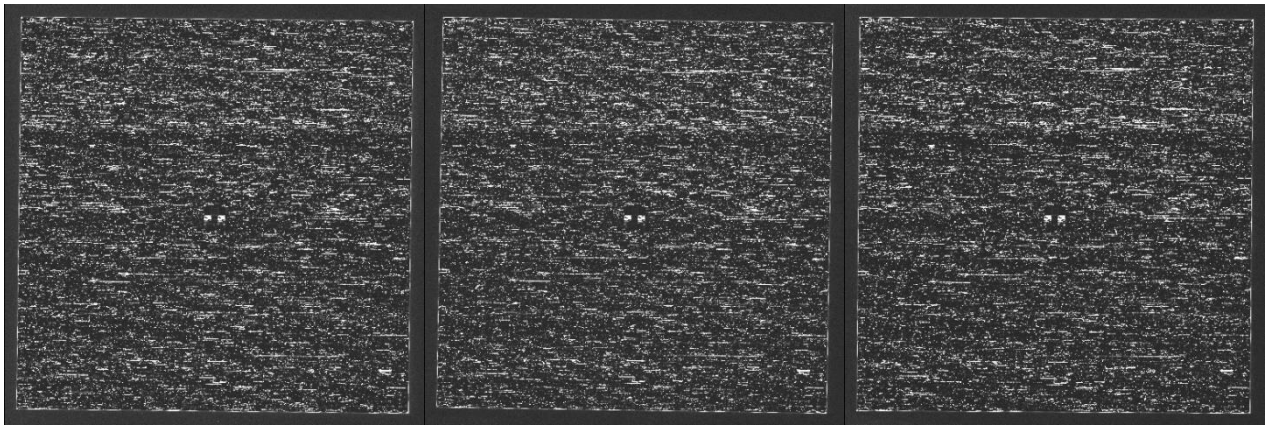

Fig. S1. Scan images showing the hybridization scores (HybScores) of the analyzed G3-PhyloChip microarrays used in the study and carried out by Second Genome Inc., San Francisco, CA, USA.

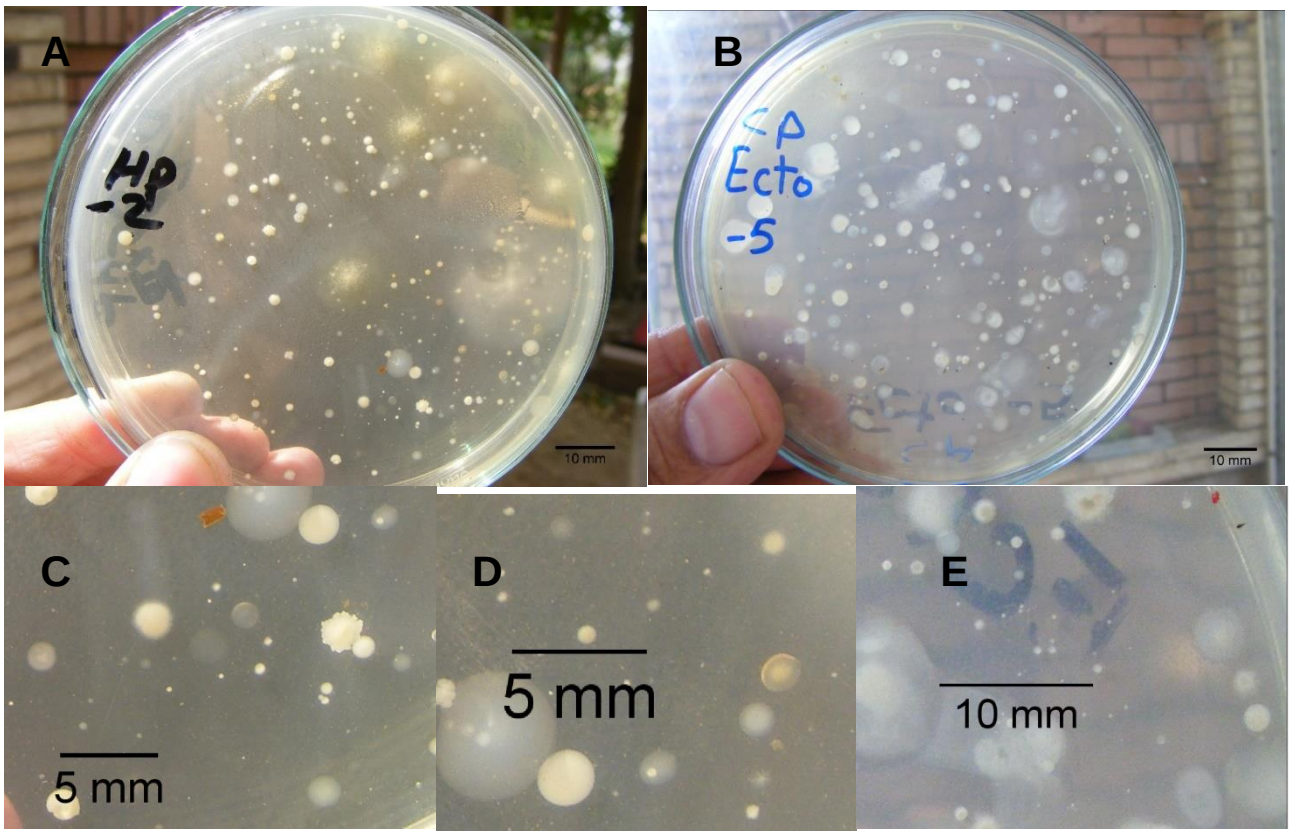

Fig. S2. Microcolonies developed on plant-only-based agar plates incubated for more than 10-days: A & B, overall predominance of microcolonies; C, D, & E, close-ups of microcolonies together with scale-bar to illustrate their microscale diameter.

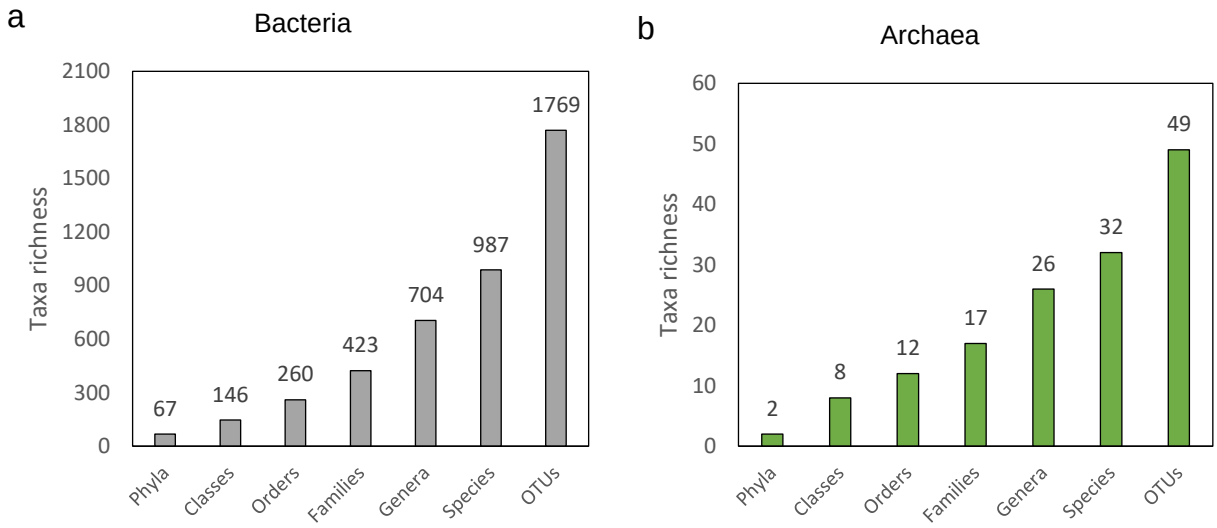

Fig. S3. Whole bacterial (a) and archaeal (b) richness of the maize root microbiome, detected using culture dependent and culture independent methods, at different taxonomic levels.

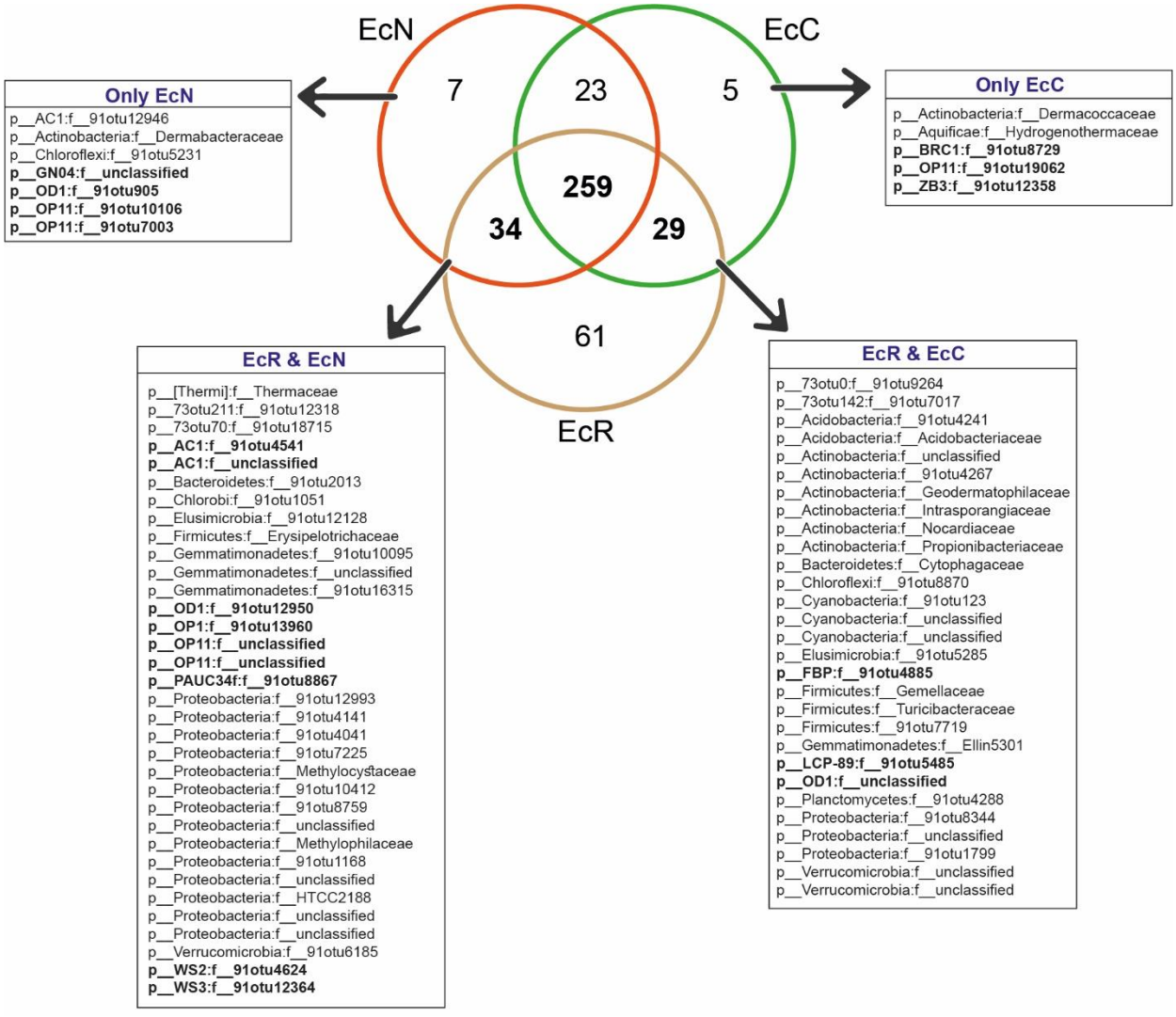

Fig. S4. Overlapping of culture-dependent (on plant-based culture medium and nutrient agar) and culture independent bacterial communities of maize root ectorhizosphere. Venn diagram at family level for bacterial communities displaying the unique and overlapping families; families exclusively grown on only one of our tested media are shown in the linked boxes, and not-yet-cultured candidate divisions are marked in bold. (**EcN**, nutrient agar; **EcC**, plant-based culture medium; **EcR**, maize ectorhizosphere).

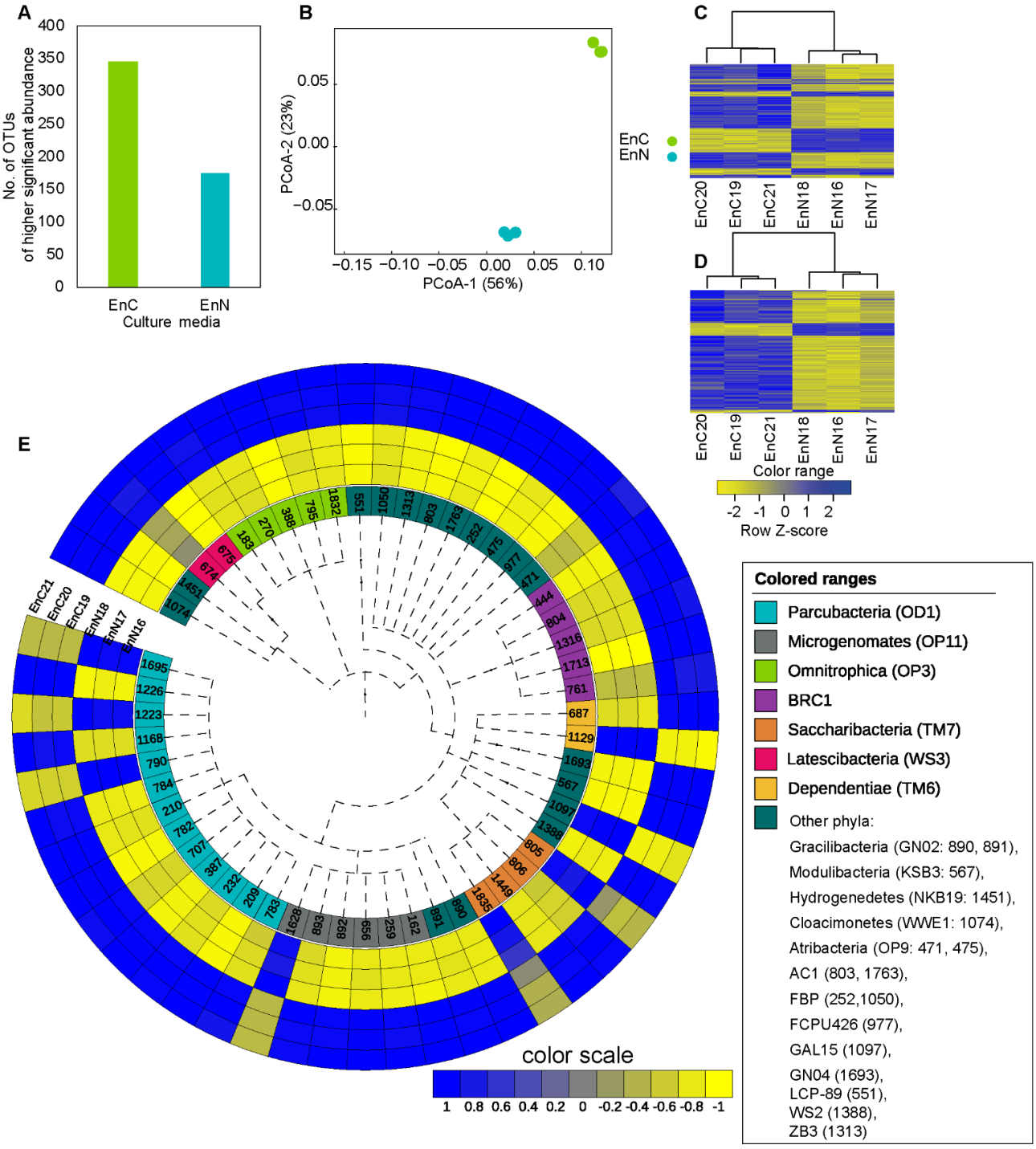

Fig. S5. The total 520 OTUs of the maize root Endorhizosphere (350 OTUs on plant-based medium and 170 OTUs on nutrient agar) that displayed significant differences in abundance among all of the three tested samples of nutrient agar (EnN16, EnN17 and EnN18) and the three tested samples of plant-based culture medium (EnC19, EnC20 and EnC21). **A**, bar-graph representing the absolute number of OTUs of significant higher abundance in either culture media; **B**, Principal Coordinates Analysis (PCoA); **C**, heatmap displaying the weighted abundance of the 520 OTUs; **D**, heatmap displaying the weighted abundance excluding the OTUs of the big-three phyla (*Proteobacteria*, *Firmicutes*, *Bacteroidetes*); **E**, Circular phylogenetic tree representing the 54 OTUs of uncultured bacterial phyla, annotated with heatmap of weighted abundance. Numbers of the inner ring refer to OTUs numbers present in the Data S1 (available upon request for data depository). Numbers between parentheses in “colored range” box indicate the OTU codes used in the circular dendrogram.

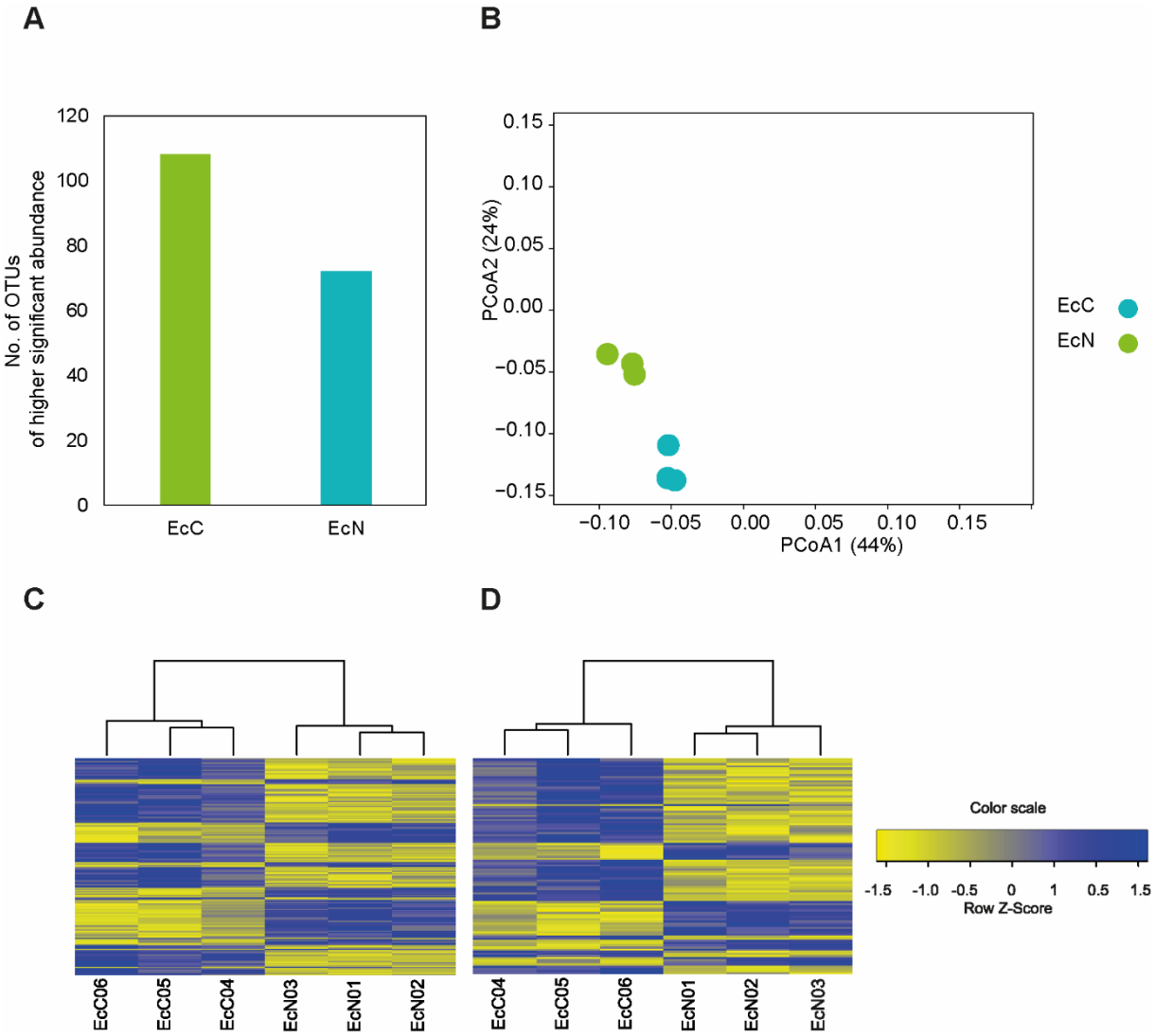

Fig. S6. The OTUs of the maize ectorhizosphere that displayed significant differences in abundance the three tested samples of nutrient agar (EcN01, EcN02 and EcN03) and the three tested samples of plant-based culture media (EcC04, EcC05 and EcC06). **A**, bar-graph representing the absolute number of OTUs of significant higher abundance; **B**, Principal Coordinates Analysis (PCoA); **C**, heatmap representing all OTUs of significant abundance; **D**, heatmap representing all OTUs of significant abundance excluding the big-three phyla (*Proteobacteria, Firmicutes, Bacteroidetes*).

Table S2. Candidate division/phyla particularly those enriched on plant-only-based culture media in this study, and previously reported in literature via metagenomics analyses among plant microbiomes. Related references are indicated in parentheses.

Candidate phyla/division	Updated taxonomic nomenclature	Number of OTUs reported in this study	Previous metagenomics reports on plant microbiomes	Available Genome-derived information regarding the recalcitrant culturability
AC1	NA	4	Cotton rhizosphere (12)	NA
BRC1	NA	12	Cotton rhizosphere (12)	NA
FBP	NA	3	Cotton rhizosphere (12)	NA
GN02	Gracilibacteria	9	Cotton rhizosphere (12)	NA
NKB19	Hydrogenedentes	1	Cotton rhizosphere (12)	NA
OD1	Parcubacteria	29	Cotton rhizosphere (12)	Gene sets for biosynthesis of cofactors, amino acids, nucleotides, and fatty acids are absent entirely or greatly reduced. They also lack some activities conserved in almost all other known bacterial genomes, including signal recognition particle, pseudouridine synthase A, and FAD synthase (8).
OP9	Atribacteria	5	Cotton rhizosphere (12)	Atribacteria' are likely to be heterotrophic anaerobes that lack respiratory capacity (9).
OP8	Aminicenantes	4	Cotton rhizosphere (12)	NA
OP3	Omnitrophica	10	Cotton rhizosphere (12)	NA
OP10	Armatimonadetes	11	Maize rhizosphere and several species of angiosperms (5, 10)	NA
OP11	Microgenomates	12	Cotton rhizosphere (12)	NA
TM6	Dependentiae		Cotton rhizosphere (12)	Lack complete biosynthetic pathways for various essential cellular building blocks including amino acids, lipids, and nucleotides. These and other features identified in the TM6 genomes such as a degenerated cell envelope, ATP/ADP translocases for parasitizing host ATP pools, and protein motifs to facilitate eukaryotic host interactions indicate that parasitism is widespread in this phylum (13).
TM7	Saccharibacteria	17	Maize rhizosphere (3, 10)	Absence of key genes for the Embden-Meyerhof (phosphofructokinase) and Entner-Doudoroff (KDPG aldolase) pathways suggest that TM7 can use only the pentose phosphate and heterolactic fermentation pathways for which all key genes were identified (1).
WPS-2	NA	3	several species of angiosperms (5)	NA
WS2	NA	3	Cotton rhizosphere (12)	NA
WS3	Latescibacteria	6	Rice root rhizoplane, rhizosphere, endosphere maize rhizosphere (4, 10)	Assumed to have slow growth rate due to possession of a relatively large genome size and a single rRNA operon (14).
ZB3	Ignavibacteriae	2	Cotton rhizosphere (12)	NA

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Chapter 5: Culturomics of the plant prokaryotic microbiome and the dawn of plant-based culture media–A review

Culturomics of the plant prokaryotic microbiome and the dawn of plant-based culture media – A review

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Abstract

Improving cultivability of a wider range of bacterial and archaeal community members, living natively in natural environments and within plants, is a prerequisite to better understanding plant-microbiota interactions and their functions in such very complex systems. Sequencing, assembling, and annotation of pure microbial strain genomes provide higher quality data compared to environmental metagenome analyses, and can substantially improve gene and protein database information. Despite the comprehensive knowledge which already was gained using metagenomic and metatranscriptomic methods, there still exists a big gap in understanding *in vivo* microbial gene functioning *in planta*, since many differentially expressed genes or gene families are not yet annotated. Here, the progress in culturing procedures for plant microbiota depending on plant-based culture media, and their proficiency in obtaining single prokaryotic isolates of novel and rapidly increasing candidate phyla are reviewed. As well, the great success of culturomics of the human microbiota is considered with the main objective of encouraging microbiologists to continue minimizing the gap between the microbial richness in nature and the number of species in culture, for the benefit of both basic and applied microbiology. The clear message to fellow plant microbiologists is to apply plant-tailored culturomic techniques that might open up novel procedures to obtain not-yet-cultured organisms and extend the known plant microbiota repertoire to unprecedented levels.

Keywords: Plant microbiome; Metagenomics; Plant-based culture media; Culturomics; Unculturable bacteria; Candidate Phyla Radiation (CPR)

Introduction: The birth and development of *in vitro* cultivation and pure culture studies

Since the discovery of microorganisms, *in vitro* cultivation and isolation of bacteria in pure cultures has represented one of the major pillars in developing the science of microbiology. Introducing their pioneer work on the germ-disease theory, both Louis Pasteur and Robert Koch, and their associates, were able to present their nutrient broth "Bouillon, Nährflüssigkeit" and solid culture media, together with single colony isolation and pure cultures studies (Koch 2018). The well-known solid culture media consisting of meat extract, peptones and agar, were developed by the 1890s. With extensive progress in selectivity profiles, diagnostic properties, chromogenic reactions, pre- and selective enrichment power, culture media were the main tools to estimate viable counts, enrich, select and differentiate groups of bacteria. In addition, individuals were isolated in pure cultures to identify, study properties, test for secondary metabolites, and determine the genetic composition (britannica.com/science/pure-culture) (Atlas 2010, Basu et al. 2015). Further environmental adaptation techniques are discussed in the section "*From synthetic to environmental cultivation of microbiomes*".

From plate count anomaly to candidate phyla

Nutrient agar and many other derived culture media, with their major components of meat extract and peptone developed for the isolation of pure isolates of human pathogens, have been continually used for culturing various types of microbiomes irrespective of the nature of their environments, whether humans, animals or plants (Bittar et al. 2014, Bai et al. 2015, Lagier et al. 2018). Additionally, many of the earlier methods continued to be used, while discovering the major differences between the numbers of cells from natural environments that form viable colonies on agar media and the numbers observed by microscopy. This observation noted at the dawn of microbiology (Winterberg 1898) was called "the great plate count anomaly" by Staley and Konopka (Staley and Konopka 1985), and continued to be researched by microbiologists over the years (Amann 1911, Cochran 1950, Epstein 2013, Sura-de Jong et al. 2015). The phenomenon was brought sharply into focus, leading to the realization just how diverse and unexplored microorganisms are, as a result of analyzing microbial small subunit ribosomal RNA (SSU or 16S rRNA) gene sequences directly from environmental samples (Baker et al. 2003).

Historically, until the mid-1980s, most of the available microbial ecology knowledge was based on cultivation techniques and microscopy or enzyme activities measured in

laboratories after substrate induction (Jansson et al. 2012). Then, Muyzer et al. (Muyzer et al. 1993) introduced the denaturing gradient gel electrophoresis (DGGE) technique, designed to separate specific PCR-amplified gene fragments, to analyze microbial communities without the need of culturing microorganisms. As a procedure, DNA samples extracted directly from the environment were targeted to amplify gene regions such as 16S rRNA for bacterial or ITS regions for fungal communities. Concomitantly, terminal restriction fragment length polymorphism (T-RFLP) was introduced to produce fingerprints of microbial communities (Marsh et al. 2000). The emergence of improved sequencing techniques, and the entailed increase of database-stored sequence information in combination with the development of *in situ* hybridization probes provided new methods for microbial community profiling, especially in the 90s, like the full-cycle or cyclic rRNA approach (Amann et al. 1990, Amann et al. 1995, Alm et al. 1996).The major limitation of these methods, including the 16S rRNA gene-based high throughput sequencing of PCR amplicon libraries and the PhyloChip microarray technology of 16S rRNA amplicons to oligonucleotide probes hybridization (Brodie et al. 2006), is the PCR-biased amplification efficiency. This is affected by sample origin, DNA extraction method, primer specificity, and the proportion of target genes within the sample background, which usually favor highly abundant targets (Hansen et al. 1998). Nevertheless, data obtained by these methods revealed that members of the “rare” biosphere are actively attracted by specific environments, and may play an important role despite their low abundance (Dawson et al. 2017).

Newer next generation sequencing techniques (NGS) did enable and simplify metagenomic and metatranscriptomic approaches that partially alleviate the PCR-related problems for just a single or a combination of taxonomic/phylogenetic marker genes by sequencing all genomic variants within an environmental sample (Sergaki et al. 2018). This results in a highly comprehensive dataset of sequenced microbial reads representing genomic fragments or transcripts, that aimed to be assigned to operational taxonomic units (OTUs) and/or specific genes, to describe microbial taxonomic diversity and to estimate functional variety or activity of a certain taxonomic level, optimally of single strains. Although progresses have been achieved in extracting DNA/RNA from environmental samples to reduce contamination and increase purity, there are still limiting factors: (i) restrictions in sequencing methods (e.g. error rate); (ii) direct assignment of reads to their corresponding genes; (iii) gene assembly with the risk of chimaera production among other problems, and (iv) the quality and availability of annotated genes and gene families in the databases; which often lead to genes of unknown functions and consequently to unknown taxa (Prosser 2015).

To overcome the issues above, a huge variety of bioinformatic tools have been developed to prioritize read quality control and processing (e.g. FastQC, FastX, PRINSEQ, Cutadapt), contamination filtering (e.g. BMTagger), and chimaera detection (e.g. Uchime2). Further tools are applied to assign a specific read to its corresponding gene or protein, function or taxon, that can be alignment-based (e.g. BLASTn/x, DIAMOND, LAST, RAPSearch2) or alignment-free (e.g. KRAKEN); the latter mostly uses k-mers to minimize database inadequacies. Currently, comprehensive tools for taxonomic and/or functional classification of reads are exemplified by MEGAN6, MG-RAST, MetaPhlAn2 and Qiita. Notably, some of these metagenomic tools (e.g. MEGAN-LR) deal with the output of long-read sequencing techniques, such as of Pacific Biosciences (PacBio) or Oxford Nanopore Technologies (ONT) (Huson et al. 2018). Those gain of interest in metagenomic research due to the fact that taxonomic and its functional annotation do not rely anymore on single genes covered by multiple short reads (approx. 50-300 bp) and their gene copy number issues (e.g. 16S rRNA) but on multiple genes covered by long reads, with an average read length of 5 to 50 kb, whereof approx. 50% of the reads are larger than 14 kb (Driscoll et al. 2017).

Continuous advances in high throughput genomic sequencing technologies, metagenomics and single cell genomics, have contributed new insights into uncultivated lineages. Several of the known microbial phyla, ~120 bacterial and 20 archaeal phyla, contain few cultivated representatives (ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi). Moreover, phyla exist that are composed exclusively of uncultured representatives, referred to as Candidate Phyla (CP) (Brown et al. 2015, Solden et al. 2016). Such an uncultivated majority, approx. 90 bacterial candidate phyla, defined as microbial dark matter, exists in various environmental microbiomes (Philippot et al. 2013, Bittar et al. 2014, Hacquard et al. 2015, Banerjee et al. 2018). Remarkably, metagenomics and microbiome analyses have detected so many candidate phyla, and phylogenetic analyses have revealed such a close relationship among many of them that the term “Candidate Phyla Radiation” (CPR) was coined for a group of uncultured bacteria that share evolutionary history (Attar 2016, Hug et al. 2016, Parks et al. 2018).

The number of newly discovered candidate phyla is increasing due to further developments in metagenomic techniques and continual updating of genomic databases, and representing a striking challenge to the scientific community (Hedlund et al. 2014, Solden et al. 2016). With increased metagenomic sampling and analysis, taxonomic boundaries and nomenclature are constantly being reassessed. Meanwhile, scientists have

realized that bacterial and archaeal phyla without a single cultivated representative comprise the majority of life's current diversity (Hug et al. 2016, Solden et al. 2016, Parks et al. 2018). Certainly, the current knowledge about the microbial world, not only the substantial roles played by microorganisms in the function of the biosphere but also their reservoir of novel bioactive compounds, is profoundly challenged by what have been cultivated in the laboratory (Hedlund et al. 2014). So far, physiologic and genomic information has been confined to pure cultures and dominated by representation of the Proteobacteria, Firmicutes, Actinobacteria, and Bacteriodetes within the Bacteria and of methanogens and halotolerant members of the Euryarchaeota within Archaea (Rinke et al. 2013).

From synthetic to environmental cultivation of microbiomes

Today, it is established that culture media tailored for *in vitro* cultivation of microorganisms, including CP microorganisms, must provide environmental and nutritional conditions that resemble their natural habitats, combined with long incubation times (Henson et al. 2016). Further attempts towards improving culture media to grow novel species depended mainly on supplementing macro- and micronutrients in the medium as well as manipulating cultivation conditions (Table 1). Conspicuous developments and higher throughput methods have been applied to marine and terrestrial environments (Fig. 1, Table 2), adopting a number of approaches reviewed by Epstein et al. (Epstein et al. 2010): for example, lowering nutrient concentrations in standard media together with longer incubation (Davis et al. 2005), diluting to extinction to minimize the influence of fast growers and facilitate growth of oligotrophs (Button et al. 1993), co-incubating cells individually encapsulated into microdroplets under low flux nutrient conditions (Zengler et al. 2002), adding signaling compounds and/or co-cultivation to trigger microbial growth (Bruns et al. 2002, D'Onofrio et al. 2010).

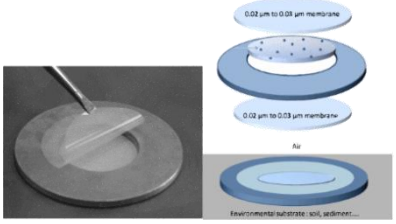
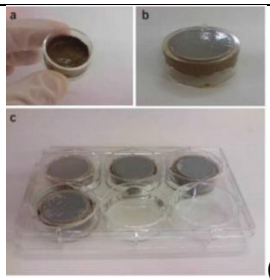
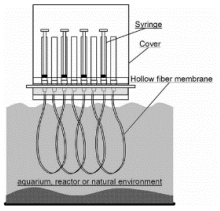
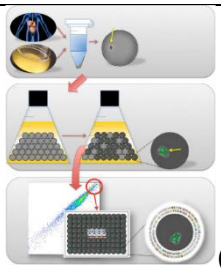
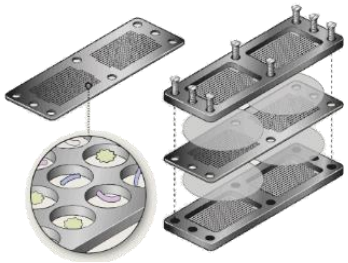
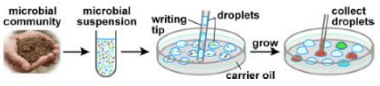
Table 1. Progressive supplements of culture media to improve culturability of environmental microbiomes

Culture media supplementation	Recovered taxa
Basal medium supplemented with isoleucine and yeast extract (Baena et al. 2000)*	*Aminobacterium mobile*
Basal medium supplemented with yeast extract (Prokofeva et al. 2000)	*Acidilobus aceticus*
Nitrogen-free LGI-P medium supplemented with sugarcane juice (Reis et al. 2004)	*Burkholderia tropica*
10-fold-diluted Difco marine broth 2216 supplemented with yeast extract (Biebl et al. 2006)	*Hoeflea phototrophica*
Postgate's medium B supplemented with yeast extract (Nielsen et al. 2006)	*Desulfitibacter alkalitolerans*

MPN soil solution equivalent (SSE) supplemented with pectin, chitin, soluble starch, cellulose, xylan, and curdlan as carbon sources (Koch et al. 2008)	*Edaphobacter modestus* and *Edaphobacter aggregans*
Basal medium supplemented with humic acid and vitamin B (HV medium) (Kaewkla and Franco 2011)	*Pseudonocardia eucalypti*
TSA, casein-starch, and 869 culture media supplemented with plant extracts (Eevers et al. 2015)	*Kaistia* sp. and *Varivorax* sp.
Peptone-Yeast extract-Glucose medium (PYG) supplemented with Resuscitation-promoting factors (Rpf) (Yu et al. 2015)	*Arthrobacter liuii*
Modified Biebl and Pfennig's medium (Lakshmi et al. 2015)	*Thiorhodococcus fuscus*
Culture media based on extracts of potato, onions, green beans, black beans, sweet corn, sweet potato, or lentils (Khalil et al. 2016)	Biomass production of *Pseudomonas fluorescence*
Selective King's B medium supplemented with lichens extract (Biosca et al. 2016)	Resulted in higher endo-lichenic and ecto-lichenic bacterial CFU counts
Basal medium supplemented with sugarcane bagasse (Mello et al. 2016)	Higher CFU recovery compared with other standard media
Fastidious anaerobic agar and blood agar media supplemented with siderophores-like molecules (Vartoukian et al. 2016)	*Prevotella* sp., *Fretibacterium fastidiosum*, *Dialister* sp., and *Megasphaera* sp.
Minimal medium supplemented with peels of orange, potato, or banana (Rane et al. 2017)	Biomass production of *Bacillus subtilis*
PBS buffer supplemented with pig fecal slurry or dried grass hay as carbon sources (Ayudthaya et al. 2017)	*Streptococcus caviae*
MRS and TSB supplemented with Titania (TiO_2) nanoparticles (Timmusk et al. 2018)	Enhanced biocontrol performance of PGPR strains against *Fusarium culmorum*
Modified 80% ethanol soil extract culture media (Nguyen et al. 2018)	18 novel species including isolates belonging to *Verrucomicrobia* and *Elusimicrobia*

*Numbers between brackets refer to related references.

Table 2. Developed novel methods to increase culturability of environmental microbiomes

Developed methods	Recovered taxa	Method illustration
Diffusion Chamber (Bollmann et al. 2007)*	Deltaproteobacteria, Verrucomicrobia, Spirochaetes, and Acidobacteria	 (Bollmann et al. 2007)
Soil substrate membrane system (SSMS) (Ferrari et al. 2005, Ferrari and Gillings 2009)	Enrichment of uncultured Proteobacteria and TM7, as well as isolation of *Leifsonia xyli* sp. nov.	 (Ferrari et al. 2005, Ferrari and Gillings 2009)
Hollow-Fiber Membrane Chamber (HFMC) (Aoi et al. 2009)	Enrichment of uncultured Alphaproteobacteria, Gammaproteobacteria, Betaproteobacteria, Actinobacteria, Spirochaetes, and Bacteroidetes	 (Aoi et al. 2009)
Single cell encapsulation in gel microdroplets (GMD) (Dichosa et al. 2014)	Enrichment of uncultured Gammaproteobacteria, Betaproteobacteria, Alphaproteobacteria, Bacteroidetes, and Planctomycetes (Ben-Dov et al. 2009)	 (Dichosa et al. 2014)
Isolation chip (Ichip) (Nichols et al. 2010)	Enrichment of Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria, Gammaproteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Planctomycetes, and Verrucomicrobia	 (Nichols et al. 2010)
Single-Cell Cultivation on Microfluidic Streak Plates (Jiang et al. 2016a, Zhou et al. 2018)	Enrichment of uncultured Proteobacteria, Firmicutes, Actinobacteria, Bacteroides, Acidobacteria, Planctomycetes, and Verrucomicrobia, in addition to isolation of novel *Dysgonomonas* sp.	 (Jiang et al. 2016a, Zhou et al. 2018)

*Numbers between brackets refer to references related.

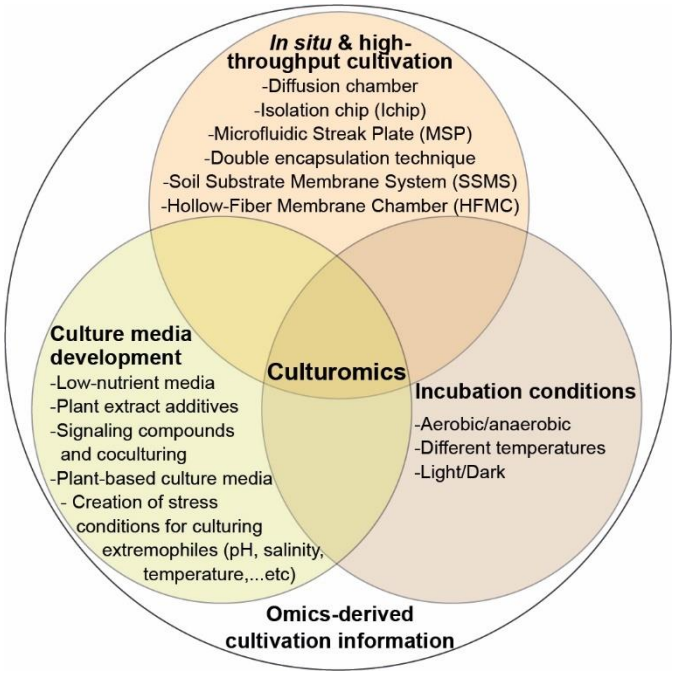


Fig. 1. Toolbox of strategies developed for improving culturability of environmental microbiomes. High throughput culturomics adopt various combinations of the specific methods of the 4 major strategies of *in situ* and high throughput cultivation, culture media development, incubation conditions, and genome-derived cultivation. For further details, please refer to Table (1).

Novel *in situ* cultivation techniques, e.g. diffusion chambers, have been introduced to mimic natural conditions and provide access to critical growth factors found in the environment and/or supplied by neighboring species. This allowed the cultivation of variants that otherwise would not grow *ex situ* (Epstein 2013). Some of the resulting chamber-reared populations were spontaneously lab-domesticated to acquire the ability to grow *in vitro* (Aoi et al. 2009). Undoubtedly, the newly advanced cultivation technologies have unraveled the existence of new species *en masse*. However, microbiologists should be able and continue to minimize the gap between the microbial richness in nature and the number of species in culture, for the benefit of both basic and applied microbiology (Epstein 2013).

Culturomics in place and the progress achieved

Realizing the imperative importance of bringing more bacterial isolates of environmental microbiomes into cultivation, the strategy of “culturomics” was introduced by the group of Didier Raoult and Jean-Christophe Lagier (Samb-Ba et al. 2014, Lagier et al. 2015, Lagier et al. 2016, Lagier et al. 2018). They developed a high throughput strategy of cultivation to study the human microbiota using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) and/or 16S rRNA amplification and sequencing to identify the growing colonies. The principals of culturomics are based on the diversified and multiple combinations of various growth media, culturing conditions, atmospheres and times

of incubation, that were reduced to only 18 culture conditions to standardize culturomics, and to complement the culture-dependent and culture-independent analyses (reviewed in Lagier et al. (Lagier et al. 2015); Table 3). The extensive application of MALDI-TOF-MS for rapid and high throughput identification of rare and new species allowed the group to dramatically extend the known human gut microbiome to levels equivalent to those of the pyrosequencing repertoire. Lagier et al. (Lagier et al. 2016) identified > 1000 prokaryotic species, thereby adding > 500 species that are representing > 50% increase in the total number of microorganisms known in the human gut. Furthermore, they were able to extend culturability of archaea without an external source of hydrogen to recover human archaeal species (Khelaifia et al. 2016).

Table 3. The basic principles and techniques of culturomics of human microbiota and results obtained at URMITE, Marseille, France*

1. Out of seventy culture conditions, 18 were defined for culturomics standardization, based on the following:	
Various combinations of culture media used for: - pre-enrichment in broth cultures, followed by - inoculating onto different agar plates for single colony isolation	Various combinations of: - blood culture, rumen fluid, sheep blood, stool extract - Tryptic Soy Broth (TSB), marine broth
Culture conditions	- Aerobic, anaerobic atmospheres - Thermic shock at 80 ℃ - Specific supplements (e.g. lipids, ascorbic acid)
Incubation temperature	Ranging from 4-55 ℃
Incubation time	From 1 to 30 days
2. Challenges faced and specific answers to isolate rare species	
Growth of bacteria having different physiological properties	Various incubation temperatures and gas phases (aerobe, anaerobe, microaerophile)
Overgrowth of fast growers	Kill the winners by: - diverse antibiotics, and inhibitors (e.g. bile extract, sodium citrate, sodium thiosulphate) - heat shock (65 ℃ and 80 ℃) - active and passive filtration - phages
Fastidious bacterial species	Pre-incubation (in selective blood culture bottles, rumen fluid)
3. Performance of identification of thousands of developed colonies	
Majority of colonies	MALDI-TOF and comparisons with URMITE databases
Confirmatory analyses for unidentified colonies	16S rRNA gene or rpoB sequencing
Colonies representing potential new taxa	Taxonomogenomics: polyphasic approach of both phenotypic (e.g. primary phenotypic characteristics) and genotypic data (e.g. genome size, G+C content, gene content, RNA genes,

	mobile gene elements…etc) and compared with closely related type strains
4. Total of 531 species were added to the human gut repertoire	
Major phyla reported	Firmicutes, Actinobacteria, Bacteriodetes, Proteobacteria, Fusobacteria, Synergisetes, Lentisphaerae, Verrucomicrobia, Dinococcus-Thermus, and Euryarchaeota
Species known in humans but not in the gut	146 bacteria
Species not previously isolated in humans	187 bacteria, 1 archaeon
Potentially new species	197

*Source (Lagier et al. 2015, Lagier et al. 2016)

The dawn of plant-based culture media

Although the results obtained with culturomics of human gut microbiome are immense and represent a success story, it did not draw much attention from research groups of the plant microbiome. Here, the compelling question is "should plant microbiologists follow the steps of human microbiome culturomics and continue using general microbiological media containing nutrients of animal origin (e.g. nutrient agar and R2A, LB)?" The answer from plant endophytes themselves is illustrated in the graphical abstract. Plants, as a holobiont, intimately interplay with their surrounding biota [44–46]. They enter in a number of multiple interactions which are efficiently orchestrated via plant physico-chemical influences, mainly the root system "the Black Box" (Fig. 2). Such complexity of the plant holobiont is amplified when considering the multiplicity of plant interfaces and the high diversity of colonizing dwellers. From the plant side, organs represent multi-layer platforms for docked microorganisms; e.g. the roots constitute, from inward to outward, endorhizosphere, rhizoplane, and ectorhizosphere. Likewise, the leaves incorporate endophyllosphere, phylloplane, ectophyllosphere, as well as caulosphere (stems). Additional compartments develop throughout the plant life, i.e. anthosphere (flowers), carposphere (fruits), and spermosphere (seeds). Correspondingly, the plant microbiome is of great diversity of both prokaryotic (Bacteria, Archaea) and eukaryotic (fungi, oomycetes, and other protistic taxa) endophytes (Rodriguez et al. 2009, Partida-Martinez and Heil 2011). They are able to colonize below- and above-ground plant organs, and exercise profound positive (mutualists), negative (pathogens) and/or neutral/unidentified (commensal endophytes) impacts on plant nutrition and health. The picture is getting more complicated and even fascinated considering interaction between bacterial and fungal groups inside the plant itself, and ability of microbial groups of other environments, e.g. human pathogens,

cross-bordering and adapting to the plant environments (Hardoim et al. 2012, Hardoim et al. 2015, Müller et al. 2015).

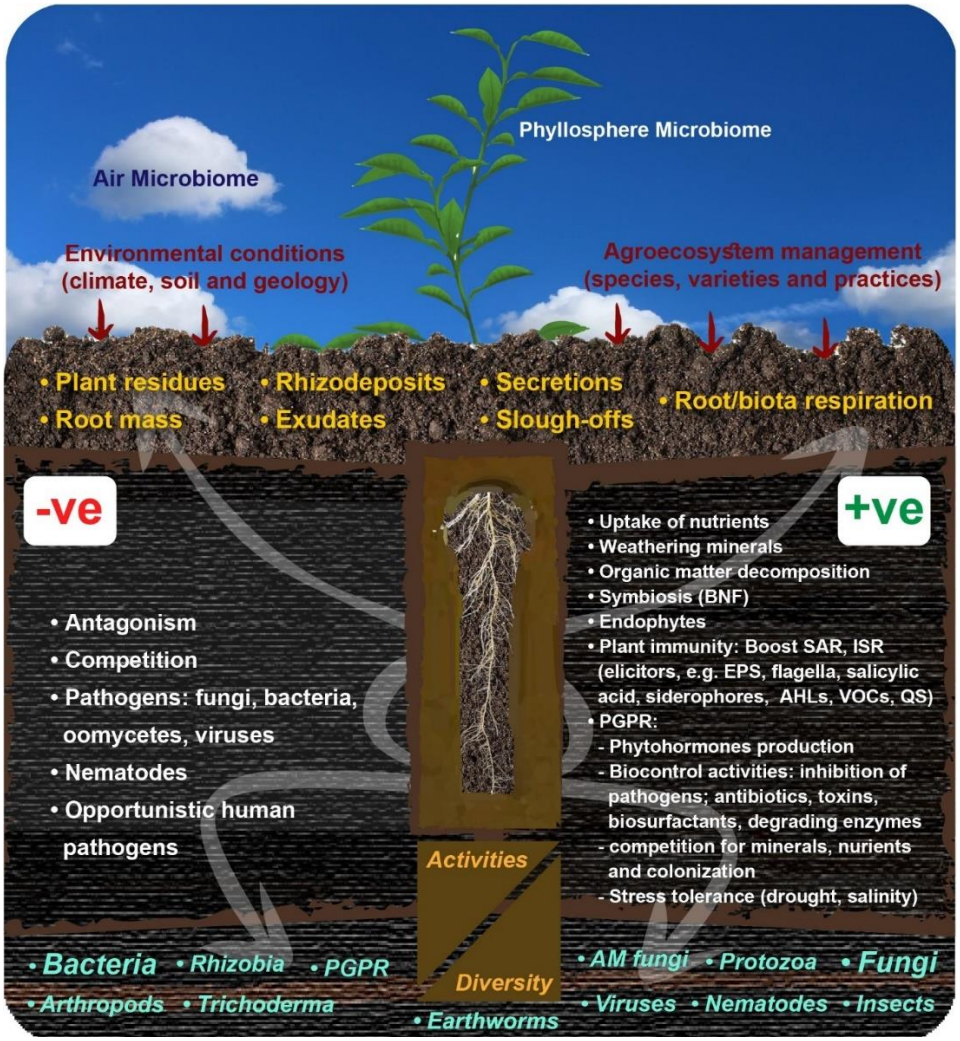


Fig. 2. A black box. A peek through the key slot of the black box, the contained environment of the plant root.

Studies emerged regarding the use of various plant materials as supplements to the general synthetic microbiological culture media, e.g. nutrient agar and R2A (Table 1, Table 4). Chemical analyses of dehydrated powders of fully-grown plants, legumes and non-legumes, illustrate the very rich and complex nutritional/chemical matrix of plants, which is very much imprinted on the root environment (Fig. 3) (Sarhan et al. 2016, Mourad et al. 2018). They contain copious sources of nutritional macromolecules, proteins and carbohydrates, major and minor elements, amino acids and vitamins: a composition that is nearly impossible to tailor in one single or a general synthetic culture medium recommended for common cultivation of the plant microbiota that are used to enjoy such *in situ* nutritional milieu. Therefore, serious efforts were made to introduce and research natural culture media based on the plant, and its inhabiting microbiota, as a sole source of nutrients, in the form of juices, saps and/or dehydrated powders (Cavalcante and Dobereiner 1988, Nour et al.

2012, Sarhan et al. 2016, Youssef et al. 2016, Hegazi et al. 2017, Saleh et al. 2017, Mourad et al. 2018, Sarhan et al. 2018) (Fig. 2). For ease of application and practicability, the packaging of plant powders in teabags was recommended to further be used in the preparation of plant infusions necessary to formulate the plant medium (Sarhan et al. 2016). The nutritional matrix, in terms of complexity, diversity and concentration of the prepared plant-only-based culture media, compared to standard culture media, was rich and compatible enough to satisfy growth of the plant microbiota, i.e. *in vitro* cultivation and *in situ* recovery.

Table 4. Enrichment and/or isolation of previously uncultivated bacterial taxa with the aid of plant materials, used as sole culture media or as supplement to standard culture media.

Bacterial taxa	Type of plant material	Used as sole culture media or as supplements	Isolated in pure culture or enriched *en masse*	Tested environments
Gluconacetobacter diazotrophicus (Cavalcante and Dobereiner 1988)*	Sugarcane shoot	sole	Isolated	Sugarcane
Novosphingobium sp. (Hegazi et al. 2017)	Lucerne shoots powder	sole	Isolated	Lucerne roots
Lysobacter sp. (Hegazi et al. 2017)	Lucerne shoots powder	sole	Isolated	Lucerne roots
Pedobacter sp. (Hegazi et al. 2017)	Lucerne shoots powder	sole	Isolated	Lucerne roots
Verrucomicrobia Subdivision 1 (Da Rocha et al. 2010)	Potato root extracts	supplement	Isolated	Potato roots
Paenibacillus gorilla (Bittar et al. 2014)	Mango juice	sole	Isolated	Gorilla stool
Paenibacillus camerounensis (Bittar et al. 2014)	Mango juice	sole	Isolated	Gorilla stool
Oenococcus oeni (GARVIE 1967)	Tomato juice	supplement	Isolated	Fermented wines
Rhizobacter daucus (Goto and Kuwata 1988)	Potato extract	supplement	Isolated	Carrot roots
BRC1 (Sarhan et al. 2018)	Clover shoot powder	sole	Enriched	Maize roots
Gracilibacteria (GN02) (Sarhan et al. 2018)	Clover shoot powder	sole	Enriched	Maize roots
Omnitrophica (OP3) (Sarhan et al. 2018)	Clover shoot powder	sole	Enriched	Maize roots
Atribacteria (OP9) (Sarhan et al. 2018)	Clover shoot powder	sole	Enriched	Maize roots
Marinimicrobia (SAR406) (Sarhan et al. 2018)	Clover shoot powder	sole	Enriched	Maize roots
Dependentiae (TM6) (Sarhan et al. 2018)	Clover shoot powder	sole	Enriched	Maize roots
Latescibacteria (WS3) (Sarhan et al. 2018)	Clover shoot powder	sole	Enriched	Maize roots
Armatimonadetes (OP10) (Tanaka et al. 2012)	Reed plant roots extract	supplement	Isolated	Reed plant roots

*Numbers between brackets refer to references related.

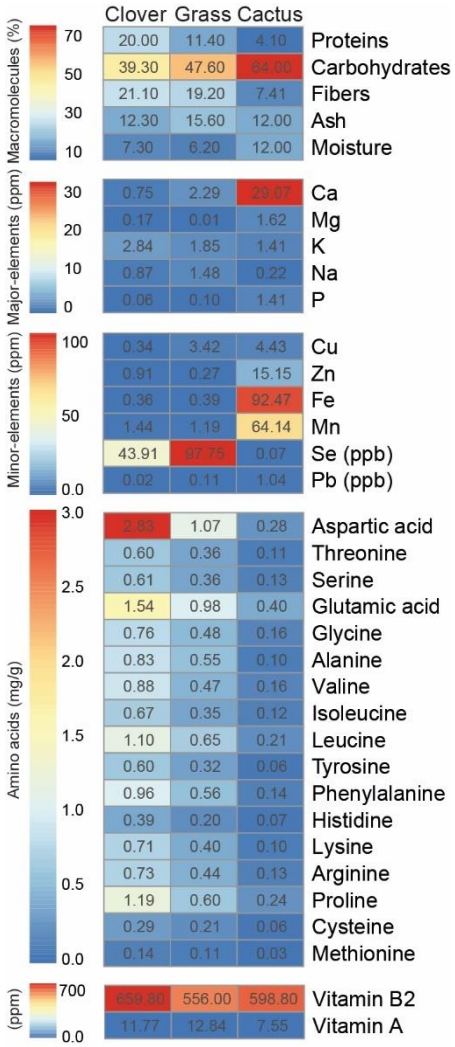


Fig. 3. Plant-based culture media. General chemical analyses of dehydrated powders of plants used to prepare plant-based culture media. The analyses included legume (*Trifolium alexandrinum*, Berseem clover), non-legume (*Paspalum vaginatum*, turfgrass), as well as the common desert cactus (*Opuntia ficus-indica*, prickly pear), and represents the mosaic of nutritional matrices of diversified macro-molecules, major and minor elements, amino acids, and vitamins. Source (Sarhan et al. 2016, Youssef et al. 2016, Mourad et al. 2018)

The various forms of plant-only-based culture media supported excellent *in vitro* growth of hundreds of tested bacterial isolates (Nour et al. 2012, Sarhan et al. 2016, Youssef et al. 2016, Hegazi et al. 2017, Saleh et al. 2017, Mourad et al. 2018, Sarhan et al. 2018) (Fig. 4). They represented 89 species of 23 families belonging to the big four phyla of Proteobacteria, Firmicutes, Bacteriodetes, and Actinobacteria (Fig. 4, Table 5). In addition, batch cultures of liquid culture media based on various plant materials, slurry homogenates, juices and/or dehydrated powders of various cultivated and desert plants, supported excellent biomass production (*ca.* > 10^8 cells ml^{-1}) of a number of plant growth-promoting bacteria (PGPB). The doubling times of tested *Klebsiella oxytoca*, *Enterobacter agglomerans*, and *Azospirillum brasilense* were comparable to standard culture media, if not shorter (Nour et al. 2012, Youssef et al. 2016, Mourad et al. 2018). Interestingly, cell

survivability in such batch cultures of plant media was maintained for longer times compared to standard culture media.

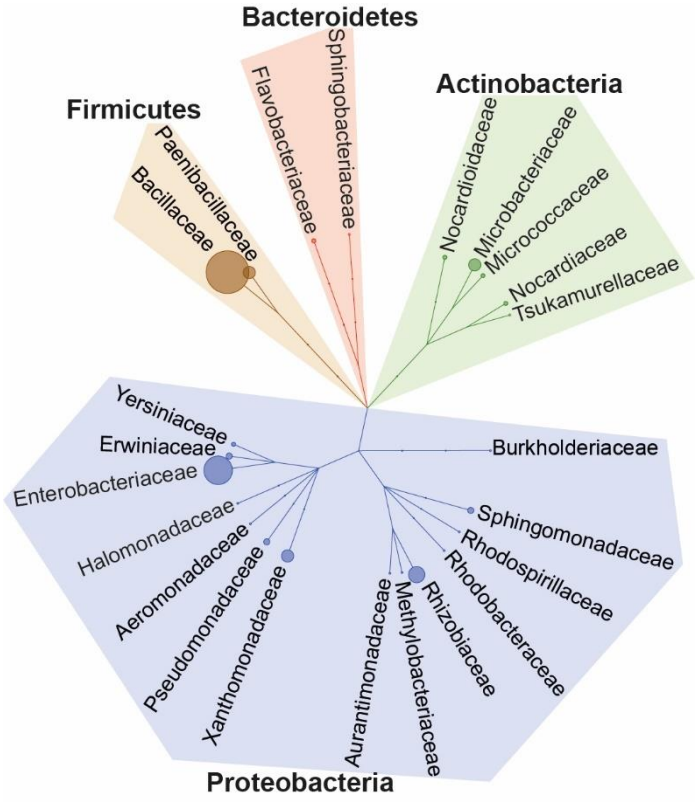


Fig. 4. Plant-only-based culture media supported growth. Phylogenetic relationships at the family level of 298 pure isolates in total tested and successfully grown on plant-only-based culture media, in their various formulations. The isolates represented 89 species and 23 families of the four big phyla (Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria). The tree is based on the NCBI taxonomy database (ncbi.nlm.nih.gov/Taxonomy/CommonTree/wwwcmt.cgi). For further details about the tested species, please refer to Table (5). Source (Sarhan et al. 2016, Youssef et al. 2016, Saleh et al. 2017, Mourad et al. 2018).

Table 5. Taxonomic affiliation of the pure isolates tested and confirmed good growth on plant-only-based culture media with their various formulations

Phylum: Actinobacteria	Family: Aurantimonadaceae
Family: Nocardioidaceae	*Aureimonas altamirensis*
Nocardioides zeicaulis	Family: Rhodobacteraceae
Nocardioides endophyticus	*Paracoccus yeei*
Family: Nocardiaceae	Family: Yersiniaceae
Rhodococcus enclensis	*Serratia rubidaea*
Rhodococcus cercidiphylli	*Serratia ficaria*
Family: Micrococcaceae	Family: Xanthomonadaceae
Kocuria marina	*Lysobacter* sp.
Kocuria rhizophila	*Stenotrophomonas* sp.
Family: Tsukamurellaceae	*Stenotrophomonas maltophilia*
Tsukamurella tyrosinosolvens	Family: Methylobacteriaceae
Family: Microbacteriaceae	*Methylobacterium mesophilicum*
Agreia sp.	Family: Sphingomonadaceae
Herbiconiux flava	*Novosphingobium* sp.
Plantibacter flavus	*Sphingomonas* sp.
Curtobacterium herbarum	*Sphingomonas paucimobilis*
Microbacterium sp.	Family: Aeromonadaceae
Curtobacterium flaccumfaciens	*Aeromonas hydrophila*
Phylum: Firmicutes	Family: Erwiniaceae
Family: Paenibacillaceae	*Pantoea* sp.
Brevibacillus sp.	*Pantoea agglomerans*

Brevibacillus nitrificans	*Erwinia* sp.
Paenibacillus timonensis	Family: Enterobacteriaceae
Paenibacillus sp.	*Cronobacter* sp.
Paenibacillus macerans	*Cronobacter sakazakii*
Paenibacillus polymyxa	*Cronobacter dublinensis*
Family: Bacillaceae	*Kosakonia oryzae*
Bacillus safensis	*Kosakonia radicincitans*
Bacillus velezensis	*Kosakonia cowanii*
Bacillus aryabhattai	*Enterobacter cloacae*
Bacillus aerophilus	*Enterobacter ludwigii*
Bacillus stratosphericus	*Enterobacter* sp.
Bacillus tequilensis	*Escherichia* sp.
Bacillus endophyticus	*Klebsiella* sp.
Bacillus flexus	*Klebsiella pneumoniae*
Bacillus vallismortis	*Klebsiella oxytoca*
Bacillus mojavensis	*Citrobacter freundii*
Bacillus smithii	Family: Rhizobiaceae
Bacillus lentus	*Rhizobium aegyptiacum*
Bacillus subtilis	*Rhizobium rosettiformans*
Bacillus subtilis subsp. subtilis	*Rhizobium binae*
Bacillus subtilis subsp. spizizenii	*Rhizobium cellulosilyticum*
Bacillus sp.	*Rhizobium etli*
Bacillus pumilus	*Rhizobium* sp.
Bacillus megaterium	*Sinorhizobium meliloti*
Bacillus licheniformis	*Agrobacterium tumefaciens*
Bacillus circulans	Family: Burkholderiaceae
Bacillus cereus	*Burkholderia cepacia*
Bacillus amyloliquefaciens	Family: Pseudomonadaceae
Phylum: Bacteroidetes	*Pseudomonas luteola*
Family: Sphingobacteriaceae	*Pseudomonas oryzihabitans*
Pedobacter sp.	*Azotobacter chroococcum*
Family: Flavobacteriaceae	*Pseudomonas* sp.
Chryseobacterium lathyri	*Pseudomonas fluorescens*
Chryseobacterium indologenes	*Pseudomonas aeruginosa*
Phylum: Proteobacteria	Family: Rhodospirillaceae
Family: Halomonadaceae	*Azospirillum brasilense*
Halomonas sp.	

Examples of efficient production of microbial biomass and metabolites from culture media based on plant substrates and by-products of agro-industries exist in the literature, e.g. green biorefinery of brown and green juices (Ali et al. 2005, Thomsen 2005). Recently, the development of “plant pellets” for instant preparation of plant-based culture media for cultivation and biomass production of rhizobia, in terms of dry weight and optical density was successfully proceeded (data under review). Formulations of plant pellets were based on mixtures of Egyptian clover powder (*Trifolium alexandrinum* L.) together with supplements of agro-byproducts, glycerol and molasses. Such plant pellets are considered a cost and labor-effective scheme for lab and industrial use, satisfying requirements for production of agro-biopreparates.

The tested plant-only-based culture media supported *in situ* recovery of plant microbiota colonizing the ecto- and endorhizospheres. Reproducible results were obtained with all of the tested cultivated maize, clover, barley, as well as desert plants, ice plant and cacti (Nour et al. 2012, Sarhan et al. 2016, Youssef et al. 2016, Hegazi et al. 2017, Saleh et

al. 2017, Mourad et al. 2018, Sarhan et al. 2018). Remarkably, the plant-based culture media supported higher percentages of culturability of endophytes (Nour et al. 2012, Sarhan et al. 2016, Youssef et al. 2016, Hegazi et al. 2017, Saleh et al. 2017, Mourad et al. 2018, Sarhan et al. 2018). The CFUs that developed were well-defined and distinct macro- and microcolonies, compared to the bigger, undefined, slimy and creeping colonies grown on standard nutrient and soil extract agar media (Nour et al. 2012, Sarhan et al. 2016, Youssef et al. 2016, Hegazi et al. 2017, Saleh et al. 2017, Mourad et al. 2018, Sarhan et al. 2018). Compared with the total bacterial numbers, based on qPCR analysis using the universal primers of Lane (Lane 1991), and calculations of Klappenbach et al. (Klappenbach et al. 2000) and Schippers et al. (Schippers et al. 2005), the culturable population, in terms of total CFUs, were higher on plant-only-based culture media (20-70%) than on standard culture media (2-18%) (Sarhan et al. 2016, Hegazi et al. 2017, Saleh et al. 2017, Mourad et al. 2018, Sarhan et al. 2018). Such obvious increases in culturability are probably attributed to the distinguished development of microcolonies, percentages exceeded 30% of the total colonies, together with prolonged incubation time. This resembles other cultivation strategies reported to boost the development of such microcolonies, e.g. the use of over-lay agar plating techniques, diffusion chamber-based technique, encapsulation of cells in gel microdroplets and soil slurry membrane systems (Zengler et al. 2002, Ferrari et al. 2005, Stewart 2012).

Culture-dependent DGGE fingerprinting of 16S rRNA gene of endophytes, grown on agar plates, clearly clustered the group of band profiles of plant-based culture media away from the tested standard culture media, and in the case of maize and barley joined with culture-independent bacterial communities of intact plant roots (Sarhan et al. 2016, Mourad et al. 2018). The plant-only-based-culture media with its unique diversity and complexity of nutrients supported higher values of alpha diversity, an observation that was confirmed earlier by supplementing culture media with natural nutrients, e.g. soil extract (Lutton et al. 2013). This provides clear evidence on the highly relatedness/closeness of the culturable population developed on the plant-only-based culture media to the *in situ* population of endo-rhizosphere of clover and maize.

Furthermore, Saleh et al. (Saleh et al. 2017) introduced specific plant-based-seawater culture media for successful recovery of the microbiome of halophytic plants grown in salt-affected environments of the Mediterranean basin. This culture medium increased culturability (> 15.0-20.0%) compared to the conventional chemically-synthetic culture medium supplemented with (11.2%) or without (3.8%) NaCl. Based on 16S rRNA gene

sequencing, representative isolates of prevalent halotolerant bacteria were closely related to *Bacillus* spp., *Halomonas* spp., and *Kocuria* spp. Remarkable improvement in culturability of endophytic fungi and bacteria was also reported by the use of plant-supplemented culture media (Zhao et al. 2010, Martyniuk and Oron 2011, Arulanantham et al. 2012, Osman et al. 2012, Murphy et al. 2015). Moreover, dehydrated powders of leguminous seeds successfully replaced the beef extract in the selective MRS culture medium, and supported better growth of probiotic bacteria of *Lactobacillus casei* and *Lactobacillus lactis* (Pathak and Martirosyan 2012).

It was evident that the use of plant-only-based culture media successfully extended the range of cultivability among rhizobacteria of Lucerne. Such plant-based culture media enabled the successful recovery of its specific micro-symbiont, namely *Sinorhizobium meliloti*, which require multiple growth factors, e.g. amino acids/vitamins (Watson et al. 2001), naturally present with balanced amounts in the plant medium, compared to obscure quantities in the yeast extract added to the standard culture media of YEM, LB, and TY (Watson et al. 2001). Cultivability was further extended to fastidious and hard-to-grow and/or not-yet-cultivated members. This included non-rhizobia isolates whose cultivation require very rich media supplemented with a variety of growth factors, e.g. *Novosphingobium*, requiring casein hydrolysate, nicotinic acid, pyridoxine, thiamine, glycine, asparagine and glutamine (Sharma 2003); *Pedobacter*, requiring tryptone, yeast extract, and NH_4Cl (Ong et al. 2016), and *Lysobacte*r, requiring yeast extract, in addition to antibacterial and antifungal drugs inhibiting other microorganisms (Yin 2010).

Unculturability and candidate phyla in the plant microbiome

The main reason behind unculturability of certain microorganisms is their own genetic make-up that confers the metabolic, physiological, and ecological potentials. In that sense, unculturability might be attributed to two main reasons: first, the auxotrophic nature of microbes with minimal genomes and restricted anabolic capacities (Hug et al. 2016). This auxotrophy may range from minimal levels, lacking single or a few critical elements, e.g. vitamins, co-enzymes, a few amino acids, to maximal levels, e.g. absence of entire metabolic pathways such as biosynthesis of amino acids and nucleotides. Assuming that a bacterial strain lacks only one gene (or gene cluster) for synthesizing a particular organic compound, this particular compound may be added to the culture medium to enable growth. However, the number of genes lacking, i.e. the degree of auxotrophy of a bacterium, determines the possibility of generating a strain-supplementing culture medium. Second, the oligo-/prototrophic nature, where microbes with large genomes and complex metabolism,

are capable of synthesizing the majority of their nutritional needs but have restricted replication mechanisms, i.e. maintain single or double rRNA operons (*rrn*). It is reported that *rrn* copy number is a reliable and generalized proxy for bacterial adaptation to resource availability (Nemergut et al. 2016, Roller et al. 2016).

Sarhan et al. (Sarhan et al. 2018) analyzed the overall phyla abundance of the culturable maize root microbiome developed on plant-only-based culture media. They demonstrated significant enrichment of the candidate phyla BRC1, Omnitrophica (OP3), Atribacteria (OP9), Dependentiae (TM6), Latescibacteria (WS3), and Marinimicrobia (SAR406), on mixed agar plates (Fig. 4 in Sarhan et al. (Sarhan et al. 2018)). This is in addition to the enrichment of some representative OTUs belonging to AC1, FBP, Gracilibacteria (GN02), Hydrogenedentes (NKB19), Parcubacteria (OD1), Aminicenantes (OP8), Armatimonadetes (OP10), Microgenomates (OP11), Ignavibacteriae (ZB3), WPS-2, and WS2 (Fig. S5 in Sarhan et al. (Sarhan et al. 2018)). The significant enrichment of all of such candidate phyla and diverse OTUs on the plant-based culture media, even as mixed cultures, is a strong indication of the complexity and diversity of nutrients in such media that most likely fulfill the nutritional requirements, and mimic conditions that prevail in their natural habitat, as symbionts (Armanhi et al. 2017). This is also confirmed by the successful isolation and recovery of some taxa of candidate phyla radiation (CPR), *Candidatus* Phytoplasma, and TM7, by tedious efforts to construct a complex culture media to satisfy their nutritional requirements (Soro et al. 2014, Contaldo et al. 2016).

Ultra-small bacterial and archaeal cells

Some groups of Bacteria and Archaea produce ultra-small cells (also called ultramicrobacteria, UMB) with diameters < 0.5 µm (often < 0.3 µm) and genomes < 1 Mb (Baker et al. 2010, Luef et al. 2015). Such UMB recently showed considerable overlap with bacterial Candidate Phyla Radiation (CPR) (Hug et al. 2016, Proctor et al. 2018). These prokaryotes have lost many genes underlying the biosynthesis of such metabolites that can be easily taken up, depending on either symbiotic partners or freely available compounds in the surrounding community. These uptake abilities can compensate for missing nucleotides, lipids, and amino acid biosynthesis pathways (Konstantinidis and Tiedje 2004, Solden et al. 2016). Although this minimization of genomes and cell sizes appears to contradict the “rational” of evolution, it can provide several benefits to bacteria, such as evading host immunity of animals or plants, and Rhizophagy (Dalia and Weiser 2011, White et al. 2018). It is also reported that the smaller the cell the easier the transit through plant cell walls, e.g. *Candidatus* Phytoplasma (Oshima et al. 2013).

Remarkably, free-living organisms have been found to be among the ultra-small prokaryotes, but there is evidence that many of them are ectosymbionts or reliant on amoebal hosts (Solden et al. 2016). Consistently, UMB were found to express abundant pili, which may be necessary for interacting with other organisms or the environment via adhesion to extracellular surfaces (Solden et al. 2016). Another important feature of UMB, that hinder their cultivation, is the low numbers of ribosomes, which in turn allow only low growth rates (Luef et al. 2015). Due to such slow growth rates, UMB cannot compete with fast growing bacteria on nutrient-rich media. In general, the likelihood of isolating and culturing UMB can be considered to be low for strains that rely mainly on host or microbial community metabolism. However, alternative cultivation approaches have successfully been applied for culturing a few strains that were previously thought to be unculturable. Interestingly, plant-only-based culture media were able to enrich such UMB phyla (Dependentiae (TM6), Gracilibacteria (GN02), Omnitrophica (OP3), Parcubacteria (OD1), and Saccharibacteria (TM7)) among the maize root microbiome (Fig. 4 in Sarhan et al. (Sarhan et al. 2018)). Such a group of phyla were reported among the low abundance bacterial groups in various environments (Proctor et al. 2018).

Large genome sizes and culturability

On the contrary, a large genome size does not inevitably imply easily culturing, but rather, possibly complicate the cultivation demands. Various genomic and physiological characterization studies of candidate phyla revealed examples of large genomes with comprehensive metabolic capabilities. Such capabilities are contrary to recently analyzed genomes of several candidate bacterial phyla, which have restricted anabolic capacities, small genome size, and depend on syntrophic interactions for growth (Youssef et al. 2015). In contrast, these large genomes possess single or limited copy numbers of *rrn*, which in turn is reflected in slow cell growth rates. It is also reported that the number of *rrn* in bacterial genomes predicts two important components of reproduction: growth rate and growth efficiency (Roller et al. 2016). This implies that the growth rate of bacteria positively correlates with *rrn* copy numbers, i.e. bacteria that possess multiple *rrn* have higher growth rates and shorter doubling times than those with single or double operons (Klappenbach et al. 2000, Roller et al. 2016). An example is the candidate phylum OP10 “Armatimonadetes”, which have a genome of ~5.2 Mb and the majority of metabolic pathways involved in biosynthesis of fatty acids, purines, and pyrimidines, but lack some TCA and histidine biosynthesis enzymes. Despite this relatively large genome size, it possesses a single split *rrn* (Hu et al. 2014).

Successfully, the first isolate of OP10 was cultivated on one hundred-fold diluted Trypticase Soy Agar (TSA) culture media (Tamaki et al. 2011). Another OP10 isolate was enriched and isolated from reed plants using minimal media supplemented with ground plant roots as a carbon source (Table 4) (Tanaka et al. 2012). In general, OP10 isolates do not require any unique substrate for their cultivation, but only prolonging cultivation (~30 days) and low-nutrient media. Hence, colonies of OP10 fail to grow on high-strength nutrients (higher than 1.5 g of total organic carbon per liter) such as nutrient agar, TSA, or LB media (Im et al. 2012).

Another striking example is the candidate phylum WS3 “Latescibacteria”, which maintains a relatively large genome of ~7.7 Mb, and encodes numerous biosynthetic capabilities and a rich repertoire of catabolic enzymes and transporters, with the potential to utilize a variety of substrates (Youssef et al. 2015). This bacterial phylum lacks a single representative isolate, and has anaerobic nature and predicted slow growth rate due to possessing a relatively large genome and a single *rrn*. However, OTUs of such phyla have been enriched *in vitro* among the bacterial phyla of maize roots using plant-only-based culture media for cultivation (Fig. 4 in Sarhan et al. (Sarhan et al. 2018)). Another situation is the phylum Verrucomicrobia, which have been isolated on oligotrophic culture media containing potato rhizosphere extracts. Such plant-enriched culture media recovered the highest CFU counts in general, and microcolonies in particular, at least seven-fold more effectively than recovery observed on R2A (Da Rocha et al. 2010). Moreover, *Akkermansia muciniphila*, the previously unculturable human gut bacterial strain, has been enriched among the plant microbiome of maize roots on plant-only-based culture media (Sarhan et al. 2018). In general, such phylum were reported to require prolonged incubation periods, since their doubling time ranges from 7 to14 hours, and analysis of their genome, ~5.2 Mb, revealed anaerobic metabolism as well as a single *rrn* (Wertz et al. 2012).

Conclusions and future perspectives

Specific culturomics strategies based on the plant-based culture media and multi-omics-derived information are the future keys to discover novel members of plant microbiomes, and hidden secrets of their multi-interactions with host plants. These proposed strategies would lead to recovering novel taxa of critical ecological niches, i.e. plant-beneficial microbes and plant-pathogens, revealing mechanisms of plant-microbiome adaptation and co-evolution, and help to understand complex microbe-microbe network interactions. This is not only to enable cultivation of the not-yet-cultured highly abundant core microbial

members, but also to mine for less abundant species, which can empower and facilitate plant microbiome engineering for future improvement of plant fitness and yield production.

Acknowledgments

The present work was funded by the German-Egyptian Research Fund (GERF-STDF 5032). Hegazi acknowledges the support of Alexander von Humboldt Foundation, for equipment subsidies and financing his research stays in Germany and at IGZ in particular, and of the German Academic Exchange Service (DAAD) for funding Cairo University student trainings at IGZ, Germany. Our gratitude is extended to Prof. Eckhard George, the IGZ research director, for his continuous support and cooperation. We are grateful to Mr. Michael Becker for depicting our idea of the vegetarian nature of the plant endophytes in the cartoon drawing presented as the graphical abstract, and to our undergraduate student Abdul Karim Noah for his excellent help in graphical designs.

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Chapter 6: General discussion and conclusions

General discussion and conclusions

Potentials of plant-based culture media

Plant roots are highly dynamic ecosystems, colonized by millions of microorganisms that contribute significantly in maintaining plant fitness and vitality. However, the majority of those microorganisms are not-yet cultured, and therefore cannot be deployed in any agricultural practices or applications. Therefore, exploring those microorganisms *in vitro* is an eminent task for microbiologists and represent a real microbiological challenge, mainly due to the lack of critical knowledge on their nutritional requirements as well as their physiological and ecological characteristics (Pham and Kim 2012, Stewart 2012). Recently, the plant-based culture media were introduced as competent candidates for resolving this microbiological challenge. As they were shown to support biomass production of rhizobacteria and proved to be efficient in recovery of plant-associated microbiota (Nour et al. 2012, Osman et al. 2012, Sarhan et al. 2016, Youssef et al. 2016, Mourad et al. 2018). However, there were two insistent questions remained to be answered: 1) how far these plant-based culture media can exclusively recover bacterial groups that cannot be recovered elsehow, and had never been yet-recovered?, 2) Which concentrations are best suited for recovery of the most of the plant-associated bacteria?

Culture media of very low nutrient concentrations recovered novel bacterial species

In chapter 2 of this study, we investigated very low concentrations of plant-based culture media, namely 4.00, 1.00, 0.50, and 0.25 g of dehydrated plant powders L^{-1}, to culture the alfalfa rhizobacteria. All the tested concentrations of the plant-based culture media significantly excelled over the artificial culture media, in terms of culturability percentages, i.e. CFU counts compared with total bacterial counts measured by quantitative real-time PCR. Additionally, among the different plant-based culture media, the concentrations of 1.00, 0.50, and 0.25 g of dehydrated plant powders L^{-1} displayed higher culturability percentages compared to the concentration of 4.00 g L^{-1}. Furthermore, we used the denaturing gradient gel electrophoresis (DGGE) to compare the culturable community composition recovered on each culture medium, which revealed distinct clustering patterns according to the culture media concentrations. In other words, artificial culture media clustered apart from the plant-based culture media, which were furtherly separated into 2 distinct clusters, representing the high concentration (i.e. 4.0 g dehydrated plant powders L^{-1}) and the low concentrations (i.e. 1.0, 0.5, and 0.25 dehydrated plant powders g L^{-1}). Interestingly, we recovered 13 isolates that could not grow on the tested artificial culture

media; three of which represented previously unculturable bacterial species, namely *Lysobacter* sp. (KR911855.1), *Novosphingobium* sp. (KR911852.1 and KR911854.1) and *Pedobacter* sp. (KR911853.1). Consistent with other bacterial culturability studies (Davis et al. 2005, Medina et al. 2017), such results can imply that low nutrient concentrations can significantly improve and extend culturability of plant microbiota.

Extendedly, the previous results triggered another question, which is "*how low is low?*"; the question we tried to answer in chapter 3 of this study. Since 0.25 g of dehydrated plant powders were enough to recover bacterial CFU counts, we went far more to completely eliminate nutrients in the culture media by cultivating plant microbiota on water agar only without any nutritional supplements. Surprisingly, such water agar recovered bacterial CFU counts of the rhizosphere and phyllosphere of maize (*Zea mays* L.) and sunflower (*Helianthus annuus* L.), comparable to other standard culture media. We proved that the only nutritional source that could be utilized to promote development of those CFUs on water agar was the nutrients present in the inoculum suspension used for initial microbial serial dilutions. Therefore, such inoculum effect is highly recommended to be taken into consideration when cultivating plant-associated microbiota. This is the first time in literature, to the best of our knowledge, to report culturing plant-associated microbiota on nutrients-free culture media, i.e. water agar (Figure 6.1).

Furthermore, we subcultured some of the colonies recovered on water agar and another highly diluted plant-based culture medium and sequenced their 16S rRNA genes. The sequencing results revealed predominance of the phylum Actinobacteria, whose members characterized by low competitiveness, compared with other surrounding microbes (Singh and Dubey 2018). Such remarkable abundance of Actinobacteria is possibly attributed to the very low nutrient concentrations in the culture media, i.e. inoculum nutrients, which provides nutrients perfectly synchronized with the environments of the microbes *in situ* (Figure 6.1). Further, we isolated two novel actinobacterial species, namely *Herbiconiux* sp. (MK100487) and *Agreia* sp. (MK100485), using such highly-diluted plant-juice culture media and the inoculum effect.

The previous results, of chapter 2 and chapter 3, exposed the potentiality of the plant-based culture media, particularly of very-low concentrations, in culturing the plant-associated microbiota. However, there is a major concern regarding the ordinary use of those plant-based culture media, which is their reproducibility. As plants undergo several developmental stages throughout their lifetime, which is correlated with severe fluctuation in their gene expressions and thus their biochemical and nutritional composition. Therefore,

formulating a standardized defined or even semi-defined culture medium, based on plant materials is a challenging task. However, using nutrient-free culture media based only on the nutrients of the inoculum (Figure 6.1), can provide empirical, spatial, and temporal specificity for the *in-situ* plant microbiota, i.e. in a certain place at a specific time (Youssef et al. 2016, Hegazi et al. 2017).

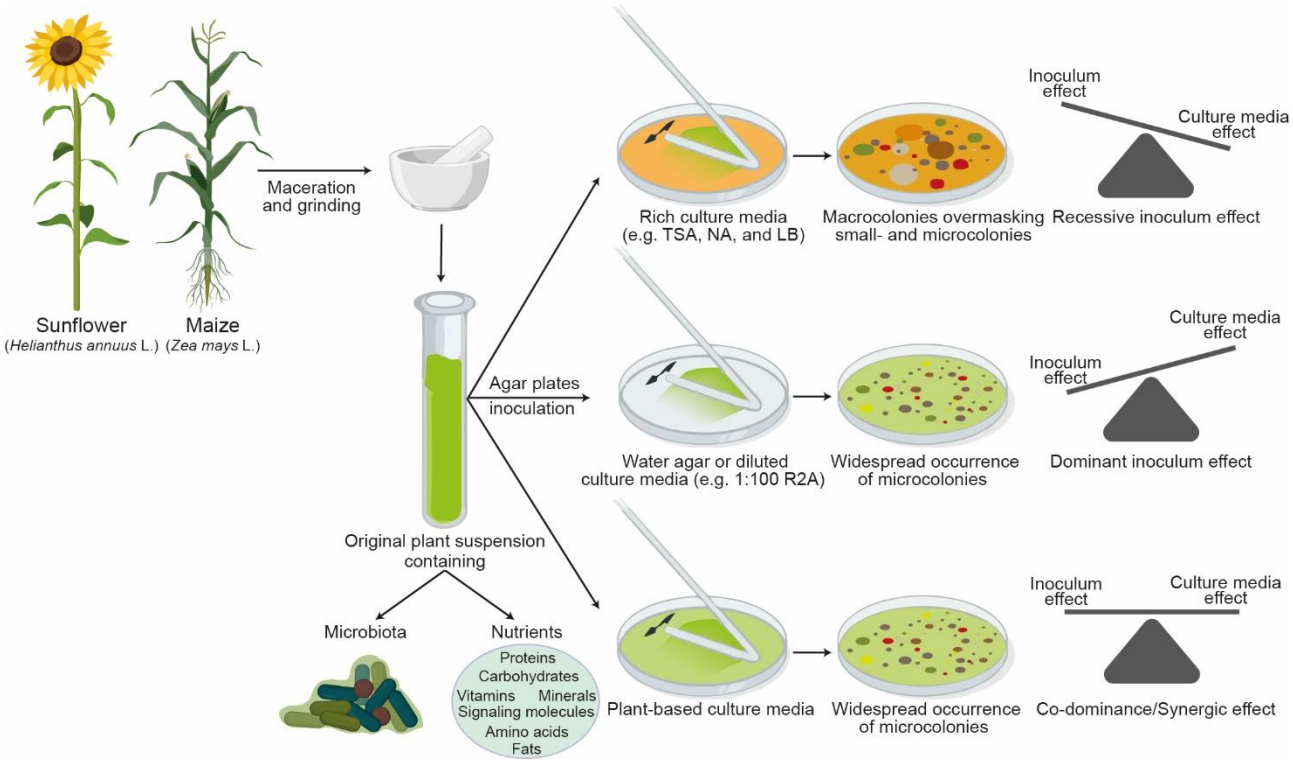

Figure 6.1: Flowchart of the rationale behind the experimental procedures of chapter (3), showing the power of inoculum-effect on recovering microcolonies and thus less-abundant bacterial groups.

G3 PhyloChip microarrays revealed an unprecedented enrichment of the less abundant- and unculturable bacterial taxa on plant-based culture media

To gain a comprehensive insight on the overall maize root microbiome diversity as affected by different culture media, we used the high throughput G3 PhyloChip microarray method to characterize the microbiome composition as recovered by artificial- and plant-based culture media (culture-dependent), in addition to the original root microbiome (culture-independent). In general, the used standard artificial culture media displayed high affinity towards enriching the most abundant bacteria taxa, e.g. Firmicutes, Gammaproteobacteria, Betaproteobacteria, and Flavobacteria, whereas the plant-based culture media were highly favoring the other less-abundant bacterial taxa, e.g. Actinobacteria, Chloroflexi, Elusimicrobia, Gemmatimonadetes, Planctomycetes, Tenericutes, Deltaproteobacteria, Bacteroidia, as well as the unculturable phyla Atribacteria (OP9), BRC1, Dependentiae

(TM6), Latescibacteria (WS3), Marinimicrobia (SAR406), Omnitrophica (OP3), and Saccharibacteria (TM7).

The very simple explanation for such differences in reflecting microbiome diversity is the performance of bacteria, in terms of competitiveness, in and out of their natural environments. Recently, Li et al. (2019) demonstrated that bacterial growth rates in rich culture media are strongly correlated with the copy numbers of 16S rRNA gene, while those growth rates were significantly less correlated with 16S rRNA numbers in natural soil. Many ecological studies demonstrated relative low abundance of fast-growing microbes that have high 16S rRNA gene copy numbers and full metabolic capacities in natural environments, e.g. Firmicutes and Gammaproteobacteria (Peiffer et al. 2013, Hardoim et al. 2015, Youssef et al. 2016). In other words, the fast-growers *in vitro* are slow-growers *in situ*, and *vice versa*.

Conclusively, and to a high extent, the plant-based culture media imitated the natural plant environments which resulted in better bacterial recovery, resembling to the original bacterial diversity, compared with the standard used culture media. Such improved recovery is likely due to: i) the natural nutritional composition of the plant-based culture media, which encourage recovery of more bacterial groups; and/or ii) the very low nutrient concentrations of the plant-based culture media, which limit the creeping growth of fast-growing bacteria, which allows development of bacterial microcolonies (Howell et al. 2005, O'May et al. 2016).

Plant-based culture media as a perfect milieu for microbe-microbe interactions

Although chemical analyses of the plant materials, used for preparation of the plant-based culture media, revealed deficiency in some amino acids, namely L-tryptophan, L-asparagine, and L-glutamine, they exhibited good potentials in enriching not-yet-cultured bacteria of the maize root microbiome, particularly members of bacterial candidate phyla radiation (CPR). Interestingly, genome-based metabolic analyses of those CPR displayed their incapability to synthesize most of the amino acids (Hug et al. 2016, Castelle and Banfield 2018, Parks et al. 2018), which implies that their enrichment is attributed to other factors beyond the constituents of the culture medium. One of these factors can be deduced by addressing one particular group such as Saccharibacteria (TM7), which was among the enriched phyla on the plant-based culture media (Figure 6.2), and considered to be the first reported cocultured member of the candidate phyla radiation (Soro et al. 2014, He et al. 2015). Saccharibacteria is a ubiquitous bacterial group which has been found in various environmental ecosystems, e.g. in human oral (He et al. 2015), soil (Ferrari et al. 2014), plants (Sarhan et al. 2018), and marine microbiomes (Glasl et al. 2019). Using microfluidic

chips, for single-cell isolation, and genome amplification, the genome of Saccharibacteria was partially sequenced and extensively analyzed to unveil the potential physiological and ecological properties of this bacterial group (Hugenholtz et al. 2001, Marcy et al. 2007). Analysis of their 16S rRNA gene sequences suggested resistance to antibiotic streptomycin, the key information which later enabled the first culture of the Saccharibacteria. However, such culture was accompanied physically by another previously uncultured Actinobacterium (Soro et al. 2014). Later studies unveiled the obligate epibiotic nature of the cultivated Saccharibacteria phylotypes TM7x, AC001, and PM004 on the Actinobacterial species *Actinomyces odontolyticus* strain XH001, *Pseudopropionibacterium propionicum* F0230a or F0700, respectively (He et al. 2015, Bernstein et al. 2019). Furthermore, the complete genome sequences of the Saccharibacteria displayed lack of biosynthetic capacities for amino acids and other co-factors and enzymes.

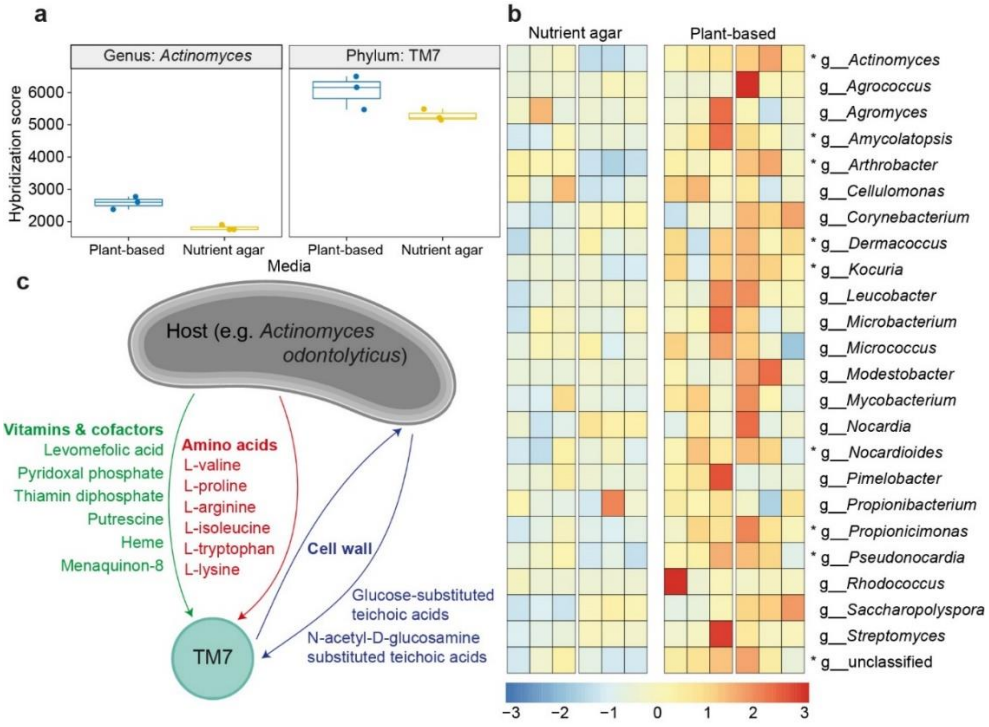

Figure 6.2: Co-culturing of Saccharibacteria (TM7) and *Actinomyces odontolyticus*: a, boxplots comparing the overall abundance of the genus *Actinomyces* and phylum TM7 on nutrient agar and plant-based culture media, based on analysis of maize root microbiome using G3 PhyloChip microarrays; b, Heatmap comparing the overall abundance of different Actinobacterial genera detected by PhyloChip analysis; c, Proposed metabolic-exchange between TM7 and *Actinomyces odontolyticus,* adapted from Bernstein et al. (2019).

Recently, Bernstein et al. (2019) developed a probabilistic model to computationally quantify the potential of a genome-based metabolic network an organism, to produce certain set of metabolites, without any given information about the surrounding environmental conditions. This model can predict inter-microbial metabolic distances as well as microbial co-occurrences. Applying this model on TM7x and its host *Actinomyces odontolyticus* XH001, unveiled further evidences on the dependence of TM7x on its host, based on the

following model: i) TM7x is able to synthesize several cell-wall components which the host is unable to synthesize, while *A. odontolyticus* experimentally displayed overexpression of N-acetyl-D-glucosamine during co-culturing; ii) Metabolomics experiments on this co-culture identified L-Pro-L-Val as a potential signaling molecule between TM7x and its host, in addition to the L-arginine, L-isoleucine, L-tryptophan, and L-lysine that could be exchanged to be catabolized by Saccharibacteria; and iii) Other vitamins and cofactors can be synthesized and exported to TM7x (Bernstein et al. 2019) (Figure 6.2c).

Based on the previous information, it is highly suggested that the plant-based culture media supported culturability of members of the bacterial candidate phyla, not because of their nutritional richness but rather due to their excellent potential in enhancing other microbial groups, e.g. Actinobacteria. Such Actinobacteria can be recruited by other parasitic bacteria, i.e. members of the CPR, and establish inter-microbial metabolic exchange relationships, like the one between TM7x and *A. odontolyticus.* This can be demonstrated by the significant higher enrichment of bacterial candidate phylum Saccharibacteria and Actinobacteria on the plant-based culture medium (i.e. co-occurrence), compared with the other artificial culture medium (Figure 6.2a, b). Following this model, CPR can complement their auxotrophic metabolism, and maintain their survival on the plant-based culture media.

In addition to Saccharibacteria, the recently cultured and intensively studied CPR model, other entirely unculturable bacterial phyla displayed significant higher enrichment on plant-based culture media, such as Atribacteria (OP9), BRC1, Dependentiae (TM6), Latescibacteria (WS3), Marinimicrobia (SAR406), and Omnitrophica (OP3). Genomic studies of some of them predicted dependent lifestyles, either via parasitism, e.g. Dependentiae (TM6) (Yeoh et al. 2016), or via association, e.g. Latescibacteria (WS3) (Youssef et al. 2015). Furthermore, Actinobacteria are well-known for their rich production of several active- and secondary metabolites, which probably are involved in such microbial interactions, and represent an attractive repertoire for other auxotrophic unculturable bacteria. Therefore, plant-based culture media can represent an excellent theatre for such inter-microbial communication and interactions, and they are highly recommended to be implemented in future plant-microbe-microbe interaction studies.

Culturomics and plant-based culture media

At the beginning of the study, we aimed to optimize the nutrient concentrations of plant-based culture media to achieve a generic medium that can be used for recovery of as

much as possible of the *in-situ* plant-associated bacterial taxa. Based on the generated results, the best approach to maximize the benefits of the plant-based culture media is to integrate them with other high throughput cultivation methods, and formulation of group-specific culture media based on genome composition of the targeted bacterial groups (Figure 6.3).

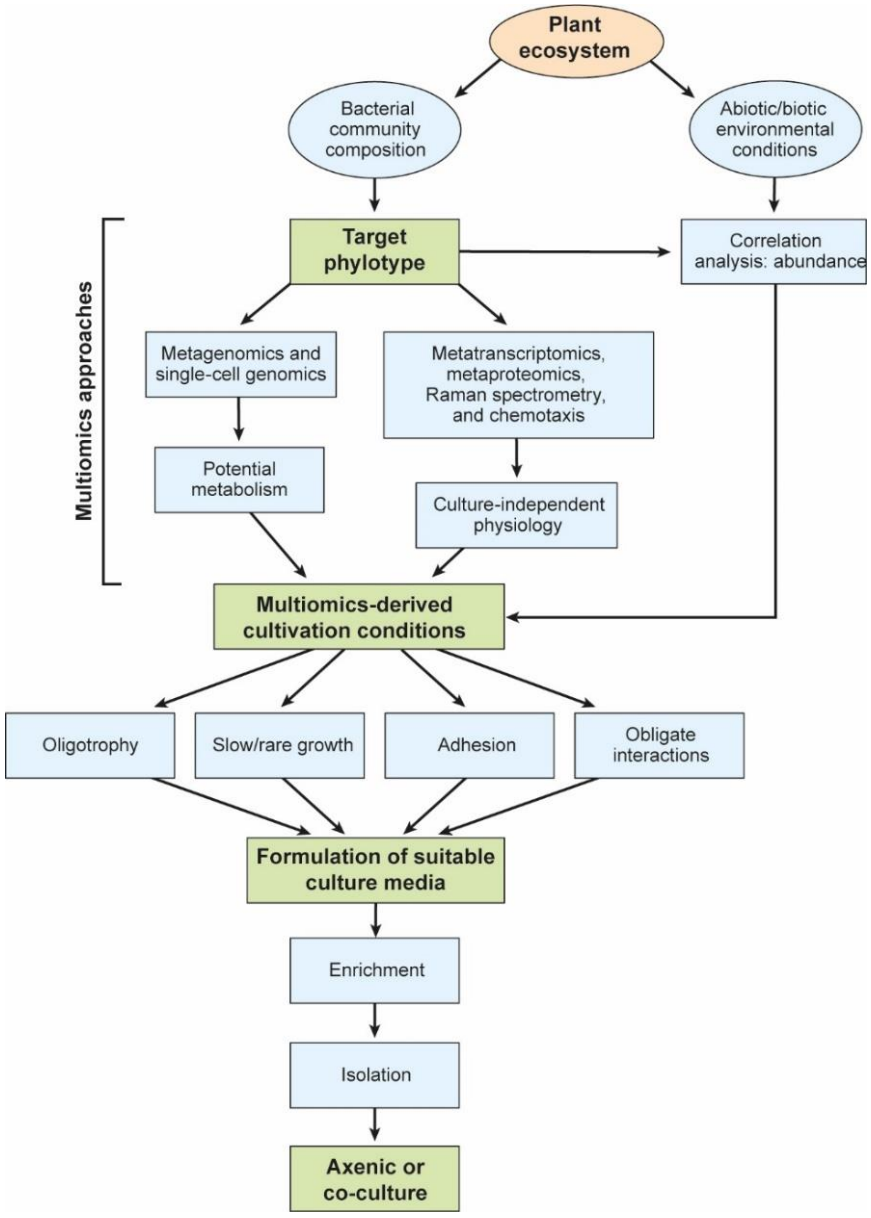

Figure 6.3: Proposed flowchart for successful targeted cultivation of plant-associated unculturable bacteria, adapted from Overmann et al. (2017). Green boxes indicate the decisive steps.

Summary and the new findings

This study aimed to optimize the use of plant-based culture media for exploration of the plant-associated microbiota and extend the culturability borders to isolate novel bacterial taxa. The study aimed also to meticulously characterize the culture-dependent and culture-independent microbiomes in view of the developed plant-based culture media.

In chapter 2 and based on previous studies that suggested the use of plant-materials as substrates for cultivating plant-associated bacteria, different concentrations of the plant materials, i.e. dehydrated plant powders, were experimented to culture the alfalfa roots-associated bacteria. Those concentrations were 4.00, 1.00, 0.50, and 0.25 g plant powders L^{-1}. Culturable bacterial CFU counts were compared with total bacterial counts measured by quantitative real-time PCR (qPCR) to assess the culturability on each of the tested culture media. Compared with standard culture media, all the tested concentrations of plant-based culture media resulted in significant higher culturability percentages than the total bacterial counts measured with qPCR. Furthermore, replica plating technique was used to cross-test the culturability of isolates recovered on the plant-based culture media on artificial culture media and *vice versa*. Moreover, these isolates which exclusively developed on plant-based culture media and failed to grow on artificial culture media were selected to identify their taxonomic affiliations. Four isolates of which were classified as previously unculturable bacteria or as novel species.

In chapter 3, cultivation of rhizosphere- and phyllosphere-associated bacteria of maize and sunflower were experimented on nutrients-free water agar and compared with highly diluted culture media, either artificial of plant-based. Denaturing Gradient Gel Electrophoresis (DGGE) was used to compare the culturable bacterial communities on each of the tested culture media and plant parts (phyllosphere and rhizosphere). Comparable CFU counts and DGGE profiles were obtained on all tested culture media with clear separation between phyllosphere and rhizosphere culturable communities. Moreover, some microcolonies (< 1 mm dia.) were picked randomly and used for 16S rRNA gene sequencing to determine their taxonomic affiliation. Majority of them were classified as Actinobacteria, and two of which were classified, most likely, as novel species.

In chapter 4, the high throughput microbiome characterization tool G3 PhyloChip was used to characterize the entire culturable and unculturable prokaryotic communities of the maize roots. Particular attention was devoted to the candidate bacterial phyla and lineages that are spread throughout the tree of life. It was evident that the plant-based culture media

significantly enriched the less abundant and rare-to-culture bacterial taxa, excelling over the artificial culture media used. Most importantly and for the first time in literature, significant *in vitro* enrichment of the uncultured bacterial candidate phyla Atribacteria (OP9), BRC1, Dependentiae (TM6), Latescibacteria (WS3), Marinimicrobia (SAR406), Omnitrophica (OP3), and Saccharibacteria (TM7) were detected on the plant-based culture media.

In chapter 5, the chronological development of culture media was reviewed, together with the recent attempts to improving culture media performance. The recent trends on human microbiome culturomics and the potential opportunities to adopting similar trend on plant microbiome were discussed. Also, a toolbox for plant-microbiome culturomics was proposed, combining plant-based culture media, high throughput culturing techniques, and different incubation and physical conditions. Finally, we discussed how genome composition affects culturability of bacteria, and represents an inevitable information for formulating a proper culture media for particular bacterial groups. Different cultivation strategies of the unculturable bacterial taxa were proposed according to their genome size and rRNA operon copy numbers.

Future perspectives: Microbiome engineering and reproduction

Current and future agriculture are facing serious challenges, in respect of producing enough food to feed the escalating world population. Several biotic and abiotic stresses are facing agricultural production worldwide, causing severe losses in annual yields (fao.org). Therefore, enhancing plant fitness to those stresses deserves extensive investigation to facilitate improvement of crop production and rehabilitation of stress-affected and disease-infested areas.

Studies on plant-microbe interactions presented several microbial strains that were shown to improve crop productivity and confer resistance against diseases (Compant et al. 2010, Buchholz et al. 2018, Sarwar et al. 2018). Additionally, various microbial mechanisms were shown to enhance plant adaptation to such stresses (Waller et al. 2005, Ruppel et al. 2013). To this end, microbial ecologists have attempted to better understand microbiome diversity, functionally classify key bacterial families and species and unearth mechanisms involved in promoting plant health. These approaches have culminated in industrial-level production of identified plant growth promoting (PGP) bacteria and their application on soil systems to increase fertility and improve crop yield and quality (Vassilev et al. 2012, Berger et al. 2018). They represent a cost-effective strategy that yields substantial outputs from low inputs. Single organism inoculation was a successful strategy for decades, and got improved by time through mixed inoculations of various bacterial species for multiple plant growth-promoting as well as biocontrol functions (O'Callaghan 2016). However, microbial populations introduced to farmlands often decline rapidly and they may occasionally get outcompeted by indigenous microbiomes, potentially having unexpected deleterious effects on ecosystem functions (Ruppel et al. 2006).

Therefore, the concept of microbiome transplantation, which is now well-established as a human medical procedure (Bakken et al. 2011, Ooijevaar et al. 2019), is adapted by plant microbiome researchers to enhance plant adaptation to certain biotic and abiotic stresses (Vorholt et al. 2017, Oyserman et al. 2018, Toju et al. 2018a). Harnessing microbiomes to increase plant nutrient uptake and resistance to biotic and abiotic stresses offers one of the few untapped reservoirs of opportunities to confront sustainability issues in agriculture. However, optimization of plant–microbial partnerships is an overwhelming task given the complexity of the plant-microbe and the microbe–microbe interactions, and the dependence of those interactions on different environmental conditions. Therefore,

underpinning key microbial hub species and taxa as well as understanding core functions and interactions associated to plant growth has merited strong attention from plant microbiome researchers for agro-sustainability practices (Agler et al. 2016, Toju et al. 2018b, Toju et al. 2018a).

The approach of microbiome transplantation can be achieved through two main strategies: i) microbiome transplantation, i.e. transplanting microbiome of resistant plant to another susceptible plant (Kwak et al. 2018); and ii) microbiome assembly, using bottom-up approach, i.e. assembling as many as possible of the microbiome members *in vitro* and formulating microbial consortia or synthetic communities (Agler et al. 2016, Vannier et al. 2019, Zhang et al. 2019).

The emergent issue now is the reproducibility and maintenance of the microbiomes for possible large-scale applications. The core of such issue is the ability to maintain the unculturable bacteria in vitro; failing to do so will result in a defective microbiome and thus will omit important community functions (Toju et al. 2018b). However, and based on the results of this study, plant-based culture media represent a possible solution for this issue, as they displayed high performance in either supporting cultivation of not-yet-cultured species, or through *en masse* cultivation of plant microbiome, i.e. enriching the whole microbiome at once, which allowed enrichment of very rare taxa and unculturable candidate phyla, as reported in chapter (4). In conclusion, establishing strategy of culturomics based on plant-based culture media is a promising approach to achieve the best outcome of the microbiome transplantation approach.

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Erklärung/Declaration

I declare, that I have written the present thesis for doctorate and without help of others. Other than the presented references were not used and quoted results were always marked with the relevant reference. I have shown my own contribution of the publications on which the cumulative dissertation is based. The present thesis was never either abroad or in Germany submitted for examination in the present or a similar version.

Ich erkläre, dass ich die eingereichte Dissertation selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe. Den eigenen Anteil an den dieser kumulativen Dissertation zugrunde liegenden Publikationen habe ich dargestellt. Die vorgelegte Dissertation wurde bisher weder im Ausland noch im Inland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

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Bolzano, 17.08.2020

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Acknowledgements

I would like to express my deep thanks and appreciation to those people without whom this thesis could not be present. I owe every one of them a lot. Those people are:

Prof. Dr. Bettina Eichler-Löbermann who accepted to supervise my PhD without even knowing me. She gave me an exceptional opportunity to get to this achievement.

PD. Dr. habil. Silke Ruppel who believed in me from the beginning of my research career, and exerted tremendous efforts supporting my MSc and PhD paths. You always had kind and warm words to advise and criticize me.

Prof. Dr. Nabil Hegazi who adopted me in his research group, and did everything in his capacity as my direct supervisor to introduce me the international scientific community and advance my career path. Special thanks for your open-hearted personality, and for the great life lesson that will be inherited to my colleagues as well as my future students ... You are something special!

Prof. Dr. Mohamed Fayez who did an excellent supervision and assistance during my study/research at Cairo University, and his availability to give me important contributions for the improvement of my manuscript.

Dr. Sascha Patz for his excellent bioinformatics and programming assistance and guidance throughout the study.

Mr. Elhussein Mourad, my long-life colleague with whom I started my research journey. He is the one who invented the idea of the second manuscript (using water agar).

Dr. Mervat Hamza, Dr. Hanan Youssef, Frau. Birgit Wernitz, Dr. Ann-Christine Scherwinski, and Ms. Awatef for their wonderful technical assistance during my research at Cairo University and IGZ.

My colleagues Mohamed R. Abdelfadeel, Ahmed T. Morsi, Rahma Nemr, Hend Elsawey, and Saif Khodary - I would like to thank them for sharing their time, enriching discussions, providing every lab support in their capacity.

I would like also to extend my thanks and appreciation to the institutes and organizations that hosted, financed, and/or supported my research and study paths

To Cairo University, my *alma mater* where I worked and studied in Egypt - I hope to have enough time in my life to reward with my knowledge.

To Leibniz-IGZ, the German institute that hosted my Alexander von Humboldt research stay, and funded my research logistics.

To Alexander von Humboldt Foundation, which supported my early-research career as a graduate student, and financed my research stay in Germany

To the Mediterranean Agronomic Institute of Bari (CIHEAM-MAIB) who provided very exceptional support of one-year of professional courses related to plant pathology and entomology, that are highly impacted on my research ideas and discussions, specially chapter 4.

CV

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On-campus Education

Jan 2019 - Now	**PhD student, Agronomy** Faculty of Agricultural and Environmental Sciences, Rostock University, Rostock, Germany
Oct 2016 – Jun 2017	**MSc Diploma, Integrated Pest Management (IPM), (60 ECTS)** Mediterranean Agronomic Institute of Bari / Libera Università Mediterranea (LUM) Jean Monnet, Casamassima, Italy
Jan 2015 – May 2017	**MSc, Microbiology (120 ECTS)** Faculty of Agriculture, Cairo University, Giza, Egypt
Sep 2009 – Jun 2013	**BSc, Biotechnology (280 ECTS)** "The International Biotechnology English Program", Faculty of Agriculture, Cairo University, Giza, Egypt

Online Education

Dec 2018	**Quantitative Biology Workshop (7.QBWx)** edX | Massachusetts Institute of Technology - MITx (verified certificate)
Jul 2017 – Aug 2018	**MicroMasters, Bioinformatics (30 ECTS)** edX | University System of Maryland & University of Maryland University College, Hyattsville-Maryland, USA (verified certificate, MicroMasters record)
May 2017	Finding Hidden Messages in DNA (Bioinformatics I) by UCSD on Coursera (verified certificate)
Mar 2017	Biology Meets Programming, by UCSD on Coursera (verified certificate)

Research experience

Jul 2018 - Now Jan 2014 – Oct 2016	**Research Assistant** Environmental Studies & Research Unit (ESRU), Cairo University, Giza, Egypt
Jan 2015 – Dec 2017	**Research Assistant** German-Egyptian funded project “Sprout MO”: Risk assessment and improving seeds, sprouts and herbs' microbiological quality
Oct 2014 – Dec 2014	**Research Fellow** PD.Dr. Silke Ruppel research group, Leibniz-Institute of Vegetables & ornamental Crops (IGZ), Großbeeren, Germany
Sep 2013 – Dec 2013	**Technical Assistant** Biotechnology Laboratories, Cairo University Research Park (CURP), Egypt

Honors & Scholarships

Oct 2016 – Jun 2017	**Mediterranean Agronomic Institute of Bari (MAIB) MSc Scholarship**
Oct 2014 – Dec 2014	**Alexander von Humboldt (AvH) Fellowship**: The Professor and his student research stay; accompanying Prof. Nabil Hegazi through a research stay of 3 months at IGZ, Großbeeren, Germany
Jun 2013	**Ranked as 2nd** out of 16 in Biotechnology program, during the 4-years BSc university study

Skills & Trainings

Nov 2018	**International Metagenomics Workshop** Bioinformatics workshop organized by Tübingen University and Leibniz-Institute IGZ, Germany; and Cairo University and Nile University, Egypt
Jun 2017	**International Plant Protection Convention (IPPC)/CIHEAM training course** "Developing Phytosanitary Capacity", CIHEAM-IAMB, Bari, Italy
Mar 2016	**Technical Assistance** Assisted in workshop for post- and under-graduate students in molecular microbial ecology techniques, IGZ/Cairo University, Giza, Egypt
Sep 2014 – Oct 2014	**DAAD-funded training** Training in Molecular Microbial Ecology techniques, Leibniz-Institute of Vegetables & Ornamental Crops (IGZ), Großbeeren, Germany
Aug 2012	**Graduation training** The Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Giza, Egypt The training included plant transformation using different methods, detection of virus-infected plants, plant tissue culture and bioinformatics
Apr 2012	**DAAD study trip to Germany** The trip included visits to organic farms (Naturland members), universities (TU Braunschweig), and research institutes (HZI, ZALF, JKI-PB, and DSMZ)
Dec 2011 – Jan 2011	**Lab-Field training** Joined training at Environmental Studies & Research Unit (ESRU), Giza, Egypt Training on basic microbiological techniques; cultivation, isolation and biomass production of PGPR and bio-fungicides

Computer skills

OS	Windows, UNIX
Programming Languages	Python, R, MATLAB, Bash, and SQL

Languages

Arabic	Mother tongue
English	Advanced
German	Elementary: A2
Italian	Elementary: A1

Publications

- **Sarhan, MS**, Mourad, EF, Nemr, RA et al. (2019). An inoculum-dependent culturing strategy (IDC) for the cultivation of environmental microbiomes and the isolation of novel endophytic Actinobacteria. The Journal of antibiotics, 1-6. DOI. 10.1038/s41429-019-0226-4
- **Sarhan, MS**; Hamza, MA; Youssef, HH; Patz, S; Becker, M; et al. (2019). Culturomics of the plant prokaryotic microbiome and the dawn of plant-based culture media–A review. ***Journal of Advanced Research***. DOI: 10.1016/j.jare.2019.04.002
- **Sarhan, MS**; Patz, S; Murphy, B; Ruppel, S; Hegazi, NA. (2018) G3 PhyloChip analysis confirms the promise of plant-based culture media for unlocking the composition and diversity of the maize root microbiome and for recovering unculturable candidate divisions/phyla. ***Microbes & Environments***, 33(3):317-325. DOI: 10.1264/jsme2.ME18023
- Mourad, EF; **Sarhan, MS**; ….& Ruppel, S; Hegazi, NA. (2018) Plant Materials are Sustainable Substrates Supporting New Technologies of Plant-Only-Based Culture Media for in vitro Culturing of the Plant Microbiota. ***Microbes and environments***, 33(1), 40-49. DOI: 10.1264/jsme2.ME17135
- Hegazi NA, **Sarhan MS**, Fayez M, Patz S, Murphy BR, Ruppel S (2017) Plant-fed versus chemicals-fed rhizobacteria of Lucerne: Plant-only teabags culture media not only increase culturability of rhizobacteria but also recover a previously uncultured Lysobacter sp., Novosphingobium sp. and Pedobacter sp. ***PLoS ONE*** 12(7): e0180424. DOI:10.1371/journal.pone.0180424
- Saleh, MY; **Sarhan, MS**; Mourad, EF; Hamza, MA; Abbas, M; Youssef, HH;…. & Hegazi, NA (2017) A novel plant-based-sea water culture media for in vitro cultivation and in situ recovery of the halophyte microbiome. ***Journal of advanced research***. DOI:10.1016/j.jare.2017.06.007
- **Sarhan, MS**, Mourad, EF, Hamza, MA, Youssef, HH, Scherwinski, AC, El-Tahan, M, ... & Hegazi, NA (2016). Plant powder teabags: a novel and practical approach to resolve culturability and diversity of rhizobacteria. ***Physiologia plantarum***, 157(4), 403-413.DOI:10.1111/ppl.12469
- Youssef, HH; Hamza, MA; Fayez, M; Mourad, EF; Saleh, MY; **Sarhan, MS**, …. & Hegazi, NA (2016). Plant-based culture media: Efficiently support culturing rhizobacteria and correctly mirror their *in-situ* diversity. ***Journal of advanced research***, 7(2), 305-316. DOI:10.1016/j.jare.2015.07.005