Review

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

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HIGHLIGHTS

- The plant microbiome culturomics is substantially lagging behind the human microbiome.
- Conventional chemically-synthetic culture media recover < 10% of plant-associated microbiota.
- Plant-based culture media (PCM) are introduced as a novel tool for plant microbiome culturomics.
- PCM extended the microbiota culturability to recover unculturable bacterial taxa.
- Streamlined- and large-genomes conspicuously contribute to the dilemma of unculturability.

GRAPHICAL ABSTRACT
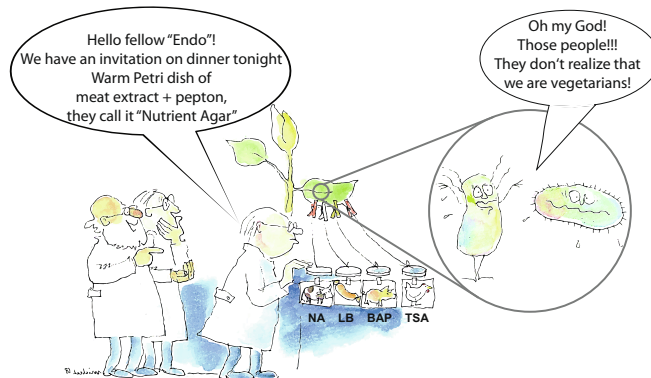
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ABSTRACT

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 inter-actions 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 micro-biologists 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.

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 [1]. 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) [2,3]. 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 [4–6]. 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 [7] was called "the great plate count anomaly" by Staley and Konopka [8], and continued to be researched by microbiologists over the years [9–12]. 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 [13].

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 [14]. Then, Muyzer et al. [15] 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 [16]. 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 [17–19]. 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 [20], 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 [21]. 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 [22].

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 [23]. 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 [24].

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) [25]. Those gains of interest in metagenomic research are 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 [26].

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 composed exclusively of uncultured representatives are referred to as Candidate Phyla (CP) [27,28]. Such uncultivated majority, approx. 90 bacterial candidate phyla, defined as microbial dark matter and exist in various

environmental microbiomes [6,29–31]. 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 [32–34].

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 [27,35]. 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 [27,32,34]. 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 [35]. 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 [36].

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 [37]. 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. [38]: for example, lowering nutrient con-
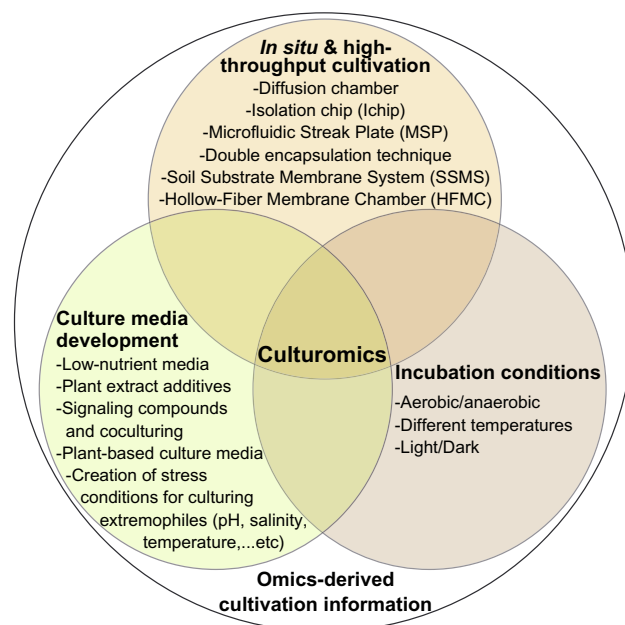

Fig. 1. Toolbox of strategies developed for improving culturability of environmental microbiomes. High throughput culturomics adopt various combinations of the specific methods of the 4 major strategies of *in situ* and high throughput cultivation, culture media development, incubation conditions, and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

centrations in standard media together with longer incubation [39], diluting to extinction to minimize the influence of fast growers and facilitate growth of oligotrophs [40], co-incubating cells individually encapsulated into microdroplets under low flux nutrient conditions [41], adding signaling compounds and/or co-cultivation to trigger microbial growth [42,43].

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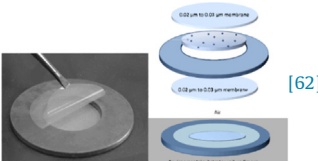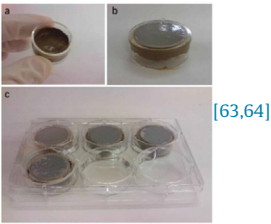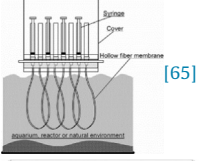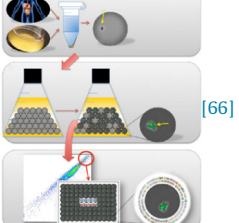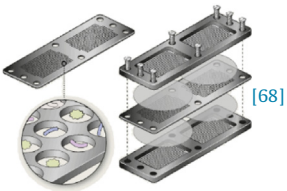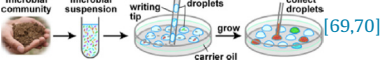
Table 1
Progressive supplements of culture media to improve culturability of environmental microbiomes.

Culture media supplementation	Recovered taxa
Basal medium supplemented with isoleucine and yeast extract [44][a]	*Aminobacterium mobile*
Basal medium supplemented with yeast extract [45]	*Acidilobus aceticus*
Nitrogen-free LGI-P medium supplemented with sugarcane juice [46]	*Burkholderia tropica*
10-fold-diluted Difco marine broth 2216 supplemented with yeast extract [47]	*Hoeflea phototrophica*
Postgate's medium B supplemented with yeast extract [48]	*Desulfitibacter alkalitolerans*
MPN soil solution equivalent (SSE) supplemented with pectin, chitin, soluble starch, cellulose, xylan, and curdlan as carbon sources [49]	*Edaphobacter modestus* and *Edaphobacter aggregans*
Basal medium supplemented with humic acid and vitamin B (HV medium) [50]	*Pseudonocardia eucalypti*
TSA, casein-starch, and 869 culture media supplemented with plant extracts [51]	*Kaistia* sp. and *Varivorax* sp.
Peptone-Yeast extract-Glucose medium (PYG) supplemented with Resuscitation-promoting factors (Rpf) [52]	*Arthrobacter liuii*
Modified Biebl and Pfennig's medium [53]	*Thiorhodococcus fuscus*
Culture media based on extracts of potato, onions, green beans, black beans, sweet corn, sweet potato, or lentils [54]	Biomass production of *Pseudomonas fluorescence*
Selective King's B medium supplemented with lichens extract [55]	Resulted in higher endo-lichenic and ecto-lichenic bacterial CFU counts
Basal medium supplemented with sugarcane bagasse [56]	Higher CFU recovery compared with other standard media
Fastidious anaerobic agar and blood agar media supplemented with siderophores-like molecules [57]	*Prevotella* sp., *Fretibacterium fastidiosum*, *Dialister* sp., and *Megasphaera* sp.
Minimal medium supplemented with peels of orange, potato, or banana [58]	Biomass production of *Bacillus subtilis*
PBS buffer supplemented with pig fecal slurry or dried grass hay as carbon sources [59]	*Streptococcus caviae*
MRS and TSB supplemented with Titania (TiO$_2$) nanoparticles [60]	Enhanced biocontrol performance of PGPR strains against *Fusarium culmorum*
Modified 80% ethanol soil extract culture media [61]	18 novel species including isolates belonging to *Verrucomicrobia* and *Elusimicrobia*

[a] 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 [62][a]	Deltaproteobacteria, Verrucomicrobia, Spirochaetes, and Acidobacteria	[62]
Soil substrate membrane system (SSMS) [63,64]	Enrichment of uncultured Proteobacteria and TM7, as well as isolation of *Leifsonia xyli* sp. nov.	[63,64]
Hollow-Fiber Membrane Chamber (HFMC) [65]	Enrichment of uncultured Alphaproteobacteria, Gammaproteobacteria, Betaproteobacteria, Actinobacteria, Spirochaetes, and Bacteroidetes	[65]
Single cell encapsulation in gel microdroplets (GMD) [66]	Enrichment of uncultured Gammaproteobacteria, Betaproteobacteria, Alphaproteobacteria, Bacteroidetes, and Planctomycetes [67]	[66]
Isolation chip (Ichip) [68]	Enrichment of Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria, Gammaproteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Planctomycetes, and Verrucomicrobia	[68]
Single-Cell Cultivation on Microfluidic Streak Plates [69,70]	Enrichment of uncultured Proteobacteria, Firmicutes, Actinobacteria, Bacteroides, Acidobacteria, Planctomycetes, and Verrucomicrobia, in addition to isolation of novel *Dysgonomonas* sp.	[69,70]

[a] Numbers between brackets refer to references related.

supplied by neighboring species. This allowed the cultivation of variants that otherwise would not grow *ex situ* [12]. Some of the resulting chamber-reared populations were spontaneously lab-domesticated to acquire the ability to grow *in vitro* [65]. 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 [12].

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 [5,71–73]. 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. [72]; 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. [71] identified > 1000 prokaryotic species, thereby adding > 500 species that represent > 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 [74].

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

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

1. Out of 70 culturing 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 to 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

[a] Source [71,72].

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 [43–45]. They enter in a number of multiple interactions which are efficiently orchestrated via plant physicochemical 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 [75,76]. 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 [77–79].

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) [80,81]. 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 [80–87] (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 [81]. 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.

The various forms of plant-only-based culture media supported excellent *in vitro* growth of hundreds of tested bacterial isolates [80–82,84–87] (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 [80,86,87]. Interestingly, cell survivability in such batch cultures of plant media was maintained for longer times compared to standard culture media.

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 [92,93]. Recently, the development of "plant pellets" for instant preparation of plant-based
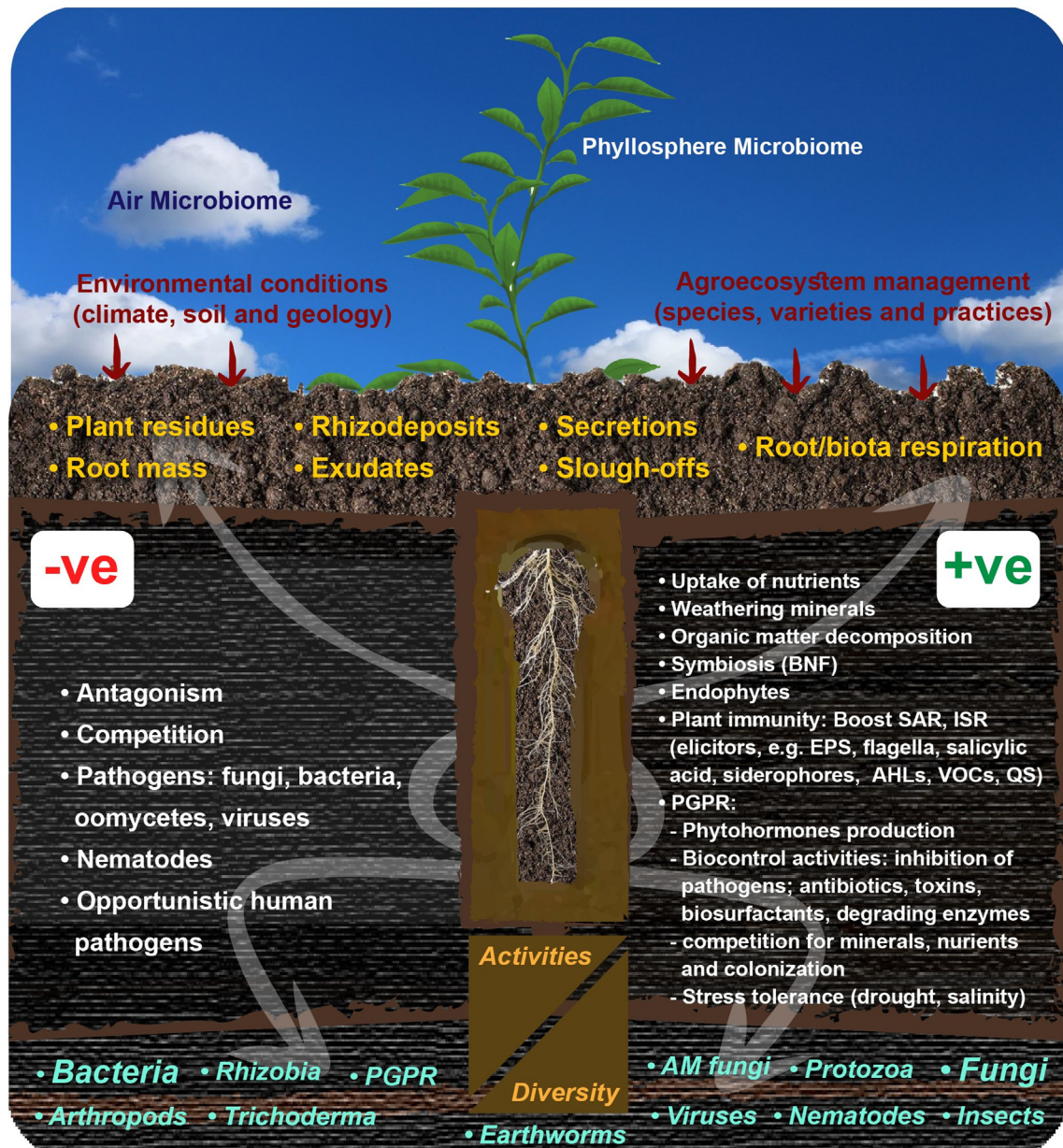
Fig. 2. The black box. A peek through the key slot of the black box, the contained environment of the plant root.

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 [83][a]	Sugarcane shoot	Sole	Isolated	Sugarcane
Novosphingobium sp. [82]	Lucerne shoots powder	Sole	Isolated	Lucerne roots
Lysobacter sp. [82]	Lucerne shoots powder	Sole	Isolated	Lucerne roots
Pedobacter sp. [82]	Lucerne shoots powder	Sole	Isolated	Lucerne roots
Verrucomicrobia Subdivision 1 [88]	Potato root extracts	Supplement	Isolated	Potato roots
Paenibacillus gorilla [6]	Mango juice	Sole	Isolated	Gorilla stool
Paenibacillus camerounensis [6]	Mango juice	Sole	Isolated	Gorilla stool
Oenococcus oeni [89]	Tomato juice	Supplement	Isolated	Fermented wines
Rhizobacter daucus [90]	Potato extract	Supplement	Isolated	Carrot roots
BRC1 [85]	Clover shoot powder	Sole	Enriched	Maize roots
Gracilibacteria (GN02) [85]	Clover shoot powder	Sole	Enriched	Maize roots
Omnitrophica (OP3) [85]	Clover shoot powder	Sole	Enriched	Maize roots
Atribacteria (OP9) [85]	Clover shoot powder	Sole	Enriched	Maize roots
Marinimicrobia (SAR406) [85]	Clover shoot powder	Sole	Enriched	Maize roots
Dependentiae (TM6) [85]	Clover shoot powder	Sole	Enriched	Maize roots
Latescibacteria (WS3) [85]	Clover shoot powder	Sole	Enriched	Maize roots
Armatimonadetes (OP10) [91]	Reed plant roots extract	Supplement	Isolated	Reed plant roots

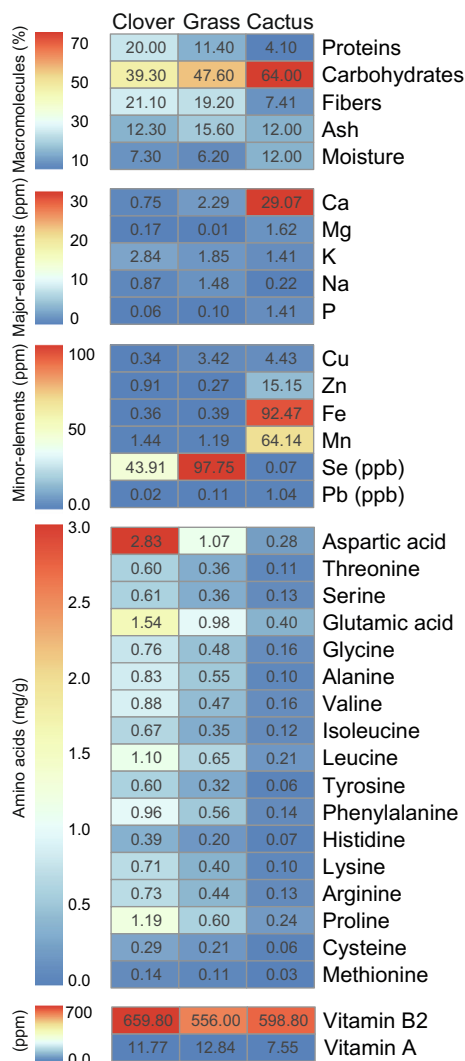[a] Numbers between brackets refer to references related.

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 [80,81,86]
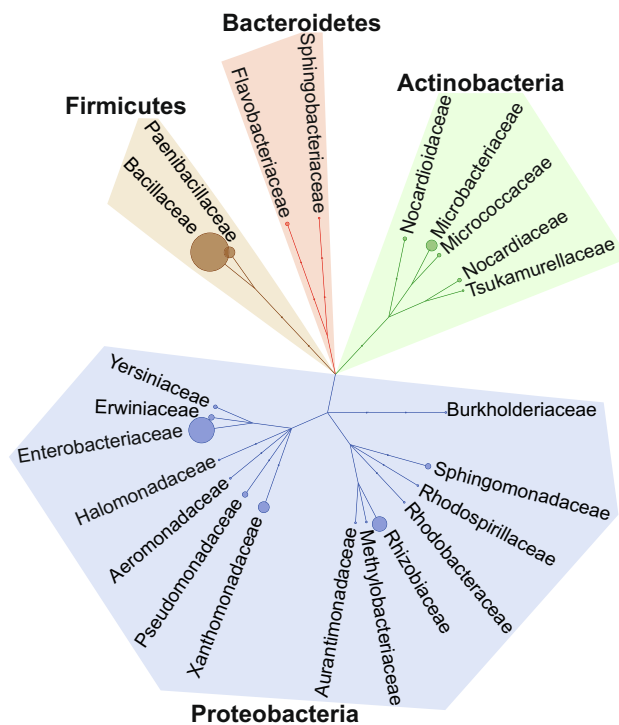

Fig. 4. Plant-only-based culture media supported *in vitro* growth of hundreds of tested bacterial plant microbiota isolates. Phylogenetic relationships at the family level of 298 pure isolates in total tested and successfully grown on plant-only-based culture media, in their various formulations. The 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 [80,81,84,86]

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 [80–82,84–87]. Remarkably, the plant-based culture media supported higher percentages of culturability of endophytes [80–82,84–87]. 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 [80–82,84–87]. Compared with the total bacterial numbers, based on qPCR analysis using the universal primers of Lane [94], and calculations of Klappenbach et al. [95]

and Schippers et al. [96], 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%) [80–82,84,85]. 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 [41,63,97].

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 [80,81]. The plant-only-based culture media with their 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 [98]. 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 endorhizosphere of clover and maize.

Furthermore, Saleh et al. [84] 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

Table 5
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
Nocardio ides 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.	

(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 [99–103]. 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* [104].

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 [105], 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 [105]. 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 [106]; *Pedobacter*, requiring tryptone, yeast extract, and NH_4Cl [107], and *Lysobacter*, requiring yeast extract, in addition to antibacterial and antifungal drugs inhibiting other microorganisms [108].

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 [32]. 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 [109,110].

Sarhan et al. [85] 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. [85]). 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. [85]). 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 [111]. 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 [112,113].

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 [114,115]. Such UMB

recently showed considerable overlap with bacterial Candidate Phyla Radiation (CPR) [32,116]. 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 [27,117]. Although this minimization of genomes and cell sizes appears to contradict the "rationale" of evolution, it can provide several benefits to bacteria, such as evading host immunity of animals or plants, and Rhizophagy [118,119]. It is also reported that the smaller the cell the easier the transit through plant cell walls, e.g. *Candidatus* Phytoplasma [120].

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 [27]. 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 [27]. Another important feature of UMB, that hinder their cultivation, is the low numbers of ribosomes, which in turn allow only low growth rates [114]. 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 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 [85]. Such a group of phyla were reported among the low abundance bacterial groups in various environments [116].

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 [121]. 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 [110]. 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 [95,110]. 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* [122].

Successfully, the first isolate of OP10 was cultivated on one hundred-fold diluted Trypticase Soy Agar (TSA) culture media [123]. Another OP10 isolate was enriched and isolated from reed plants using minimal media supplemented with ground plant roots as a carbon source (Table 4) [91]. 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 [124].

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 [121]. This bacterial phylum lacks a single representative isolate, and has an 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. [85]). 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 [88]. 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 [85]. 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* [125].

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.

Conflict of interest

The authors have declared no conflict of interest.

Compliance with Ethics Requirements

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

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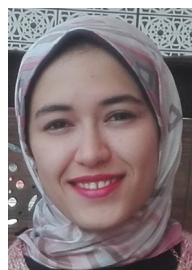
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