Bacteria culturing is crucial to boost sustainable agriculture

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Abstract

The huge amount of information on microbiomes gained through the advancement of high-throughput sequencing methods is still only partially translated into concrete solutions for societal needs. Here, we highlight that bacteria culturing remains pivotal in microbiology and is crucial for the effective application of plant probiotics in sustainable agriculture.

The importance to secure a plant culturable core microbiota

Plant beneficial bacteria are attracting the interest of growers, consumers, and policy-makers but their potential as plant probiotics is not yet fully exploited, although a net increase of crop productivity by microbial inoculants, coupled with a decreased use of agrochemicals, has recently been demonstrated [1]. Moreover, ongoing research on plant microbiome engineering is generating exciting results about the possibility, for instance, to endow plants with resistance to microbial pathogens through rhizosphere microbiome transplant [2]. This nascent approach is very promising, however its application in agricultural practices is hampered by the limited knowledge of how inocula invasion occurs, of the outcomes in terms of microbiome composition and functioning [3] and of the reproducibility of results between different plant species.

In the current scenario, besides intrinsically uncultivable organisms like mycorrhizal fungi, microbial-based products available for agriculture are largely dependent on culturable strains, including endophytes [4], or consortia that can be produced in bioreactors. Culturing efforts are required also before the industrial production step, to validate *in vivo* the inoculum effects on plant growth and production, and to provide the mechanistic demonstration of their beneficial influence on crops. On the whole, culturing allows to assess the strains technological strength, *e.g.*, interaction with multiple plant species, growth rate, survival to lyophilization or product incorporation, viability maintenance. Recent analyses focused on the improvement of strain delivery, establishment and plant colonization [5], while others identified biotic interactions as a crucial aspect for the rational design of synthetic microbial communities exploitable as bioinoculants [3]. Although these approaches strongly rely on bringing into culture plant-associated microorganisms, the last years were characterized by scarce efforts at developing novel culturing strategies to increase the number of species that could be isolated from the plant-associated microbiota. However, a wider representation of the plant microbiota in culture

collections is crucial for the successful exploitation of microorganisms and/or microbiome engineering approaches in the field. Arif and coauthors [6] recently posed the following outstanding question: "*Can we group core beneficial microbial consortia into categories that work better for common crop–soil–environment combinations*?". Initiatives as the Crop Microbiome Survey (https://www.globalsustainableagriculture.org/the-crop-microbiome-survey/) are moving in this direction, aiming at describing the microbiota associated to primary staple crops on a global scale. In this framework, an efficient access of microbial inoculants to the market requires to downsize the considered plant microbiota diversity to a core set of culturable strains, which can be effective on different crops. We recently identified a core microbiota from literature data on cultured endophytic bacteria, identifying the bacterial genera that are recurrently isolated from different plant species, regardless of the isolation conditions (*e.g.*, growth media) and environmental factors (*e.g.*, soil type) [7]. The detected core culturable plant microbiota overlaps with the results of metataxonomy analyses, thus representing a part of the "true" core plant microbiota [7]. We think that studies focusing on the core culturable plant microbiota, also benefiting from the information gained from metagenomics [2], are pivotal to identify microorganisms that can live in association with several plant species and under a range of environmental/agronomic factors. Such microbes could be the ideal targets to design biofertilizers, biostimulants and biocontrol agents with high efficacy over a wide range of crops. Indeed, some of the core cultured taxa identified by the literature meta-analysis performed by Riva and coauthors [7] (*i.e.*, *Bacillus*, *Pseudomonas, Enterobacter* and *Stenotrophomonas*) are widely associated to plant growth-promoting traits and extensively exploited as effective bioinoculants in field trials [1].

Moreover, a specific focus on the culturable plant core microbiota and its recruitment dynamics by the plant can help developing novel crop varieties better suited for interaction with microbes. A

culturable core microbiota, in fact, allows elucidating at the molecular level the interplay between microbes and plants and among the microbiome members, *i.e.,* by producing *ad hoc* mutant strains and dissecting regulatory pathways. A striking example of the advantages in having a core culturable plant microbiota can be the identification of common gene functions for plant colonization, as recently reported in a large genome survey [8].

We need more culturomics

Culturomics has been designed and largely applied in human microbiology, doubling the number of cultured bacterial species associated to the human gut [9]. The method, specifically exploited for prokaryote isolation, foresees the use of tens of combinations among different culturing conditions, followed by the high-throughput isolation and identification, by MALDI-TOF or 16SrRNA gene sequencing, of thousands of bacterial colonies. This approach is not yet fully exploited by plant microbiologists, but has the potential to bring into culture the long tail of bacterial taxa up to now rarely isolated from the plant [7], possibly due to not optimal culture conditions. In fact, a number of biological reasons can hinder microbial culturability, and their comprehension is crucial to improve the ability to culture novel taxa under laboratory conditions [10]. Among them, the preference for oligotrophic conditions, the requirement of specific signal molecules (e.g., quorum-sensing regulators, resuscitation factors) and the role of microbial interactions in the original habitats should be better addressed to design culturing strategies in plant microbiology. Indeed, an improvement of plant-associated bacteria culturability would be the game changer to develop a wider portfolio of strains (and the derived bio-based products) exploitable for plant growth promotion and protection (Figure 1). Nonetheless, in the last decade only few researches focused on the design of new media and techniques to bring into cultivation novel taxa from the still uncultured fraction of bacterial communities associated to plants [10,11]. The strategies adopted until now include the use of culture media based on plant tissues, instead

of synthetic formulations, and the simulation of environmental conditions, while others, still not used in plant microbiology, could be applied (Box 1). A long-term *in situ* cultivation approach, based on the use of 'isolation microwell chambers' incubated in rhizosphere samples, allowed the isolation of hundreds of strains [12], including *Delftia* and *Herbaspirillum* representatives (about 5% of the entire collection) that were rarely reported in the recently established repertoire of cultured endophytic strains [7]. Such discrepancy can be ascribed to different biological materials used for bacteria isolation (*i.e.*, rhizosphere versus endosphere samples), but it also suggests that culturomics can allow the enrichment and isolation of taxa that are still uncultured or underrepresented in the cultured fraction of the plant microbiome. The recent application to wheat rhizosphere [12] shows that miniaturized systems (*i.e.*, microfluidic systems like 'I-Chip' and 'Soil-on-a-Chip' [11,13]) incubated under *in-situ* conditions could allow the isolation of novel strains. While specific technological adaptations would be required to apply microfluidics on the plant endosphere, researchers are exploring the use of natural substrates, like leaves, to build '*in situ similis*' conditions [14].On the other side, the replacement of synthetic growth media by those exclusively based on plant tissues seems promising to catch out of the so-called "microbial dark matter"a part of the still uncultured microorganisms. For example, the use of powder teabags obtained from plant tissues successfully improved bacteria culturability, allowing the isolation of putative novel rhizobacterial species that were not cultured using an artificial medium [15].

Noteworthy, the practical need to get a reliable core of culturable bacteria joins both plant and human microbiology, mostly for the development of effective probiotics [9], and can act as a driving force to accelerate the technical advancements and the scientific efforts oriented towards culturomics.

Concluding remarks

While food security is hampered by global problems (*e.g.*, drought, scarcity of fertilizers) the demand for more sustainable agricultural practices is urgent. Nowadays, there are promising examples of strains that are able to promote crop productivity under adverse conditions [1] and belong to the currently identified core of culturable plant microbiota. To develop microbial-based products of great utility for sustainable food production, we need to combine soon culturomics to the screening of the beneficial effect played by the culturable core of plant-associated bacteria on different plant species. We believe that bacteria culturability remains crucial both for fundamental research and for applied microbiology, and the urgent need of its improvement should not be overlooked neither in educational courses nor by funding agencies when planning research grant opportunities.

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Figure caption

Figure 1. The potential of culturable plant-associated bacteria for sustainable agriculture. On the left: the inner circle represents the core of culturable plant-associated bacteria (blue cells) while the outer circle includes those bacteria that are rarely reported in plant-derived culture collections (red cells). On the right: improved culturing strategies (*e.g.*, those based on the use of novel media, microfluidic technologies, and cell sorters for single-cell culturing) are needed to widen the

current portfolio of strains exploitable for plant growth promotion and protection (upper panel), to identify a core culturable microbiota and to define the best targets for industrial production of plant probiotics (lower panel).

Box 1 – Bacteria culturability limits and how to overcome them: a brief outlook

In the last two decades, the progress in the ability to culture and isolate novel microbial taxa has been slow. Nonetheless, the advancement and implementation of novel culturing strategies led to increase the number of environmental cultured bacteria, especially from soil and marine ecosystems, bringing into culture representatives of taxa previously detected exclusively by molecular analyses [10]. To date, only some of the developed culturing strategies have been applied to plant microbiology (Table I), providing promising results on the possibility to gain into culture an ever-increasing number of strains of potential interest for biotechnological application.

Table I. Summary of the main developed strategies employed to culture environmental bacteria

Culturing strategy	**Methods to improve bacteria culturing**	**Application in plant microbiology**
Oligotrophic conditions	Use of low nutritional input media, *e.g.*, plant-only based media	*Medicago sativa* rhizospheric soil samples [15]
Simulated natural environment	*In situ* cultivation using microwell chambers	*Triticum aestivum* rhizospheric soil samples [12]
Simulated natural environment	Use of intact leaves to establish supportive substrates	Root and leaf endosphere, and leaf surface [14]
Simulated natural environment	Use of microfluidic technologies (Lab-on-a-Chip)	Interaction studies performed on bipartite/tripartite interactions between fungal hyphae, bacterial cells and roots [11]
Single-cell separation/ High-throughput dilution-to-extinction culturing	Use of cell sorter and/or robotics for single-cell culturing	-
Cultivation in co-culture	Use of helper strains and/or their spent growth media	-
Cultivation of slow-growing bacteria	Extended incubation time to allow the detection of visible colonies	-

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