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METHODS PAPER

In situ *similis* culturomic strategies based on vegetable (veggie)-discs extend diversity of in vitro-cultivated microbiota of vegetables

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Abstract

Background and aim Realizing that in vitro cultivation of plant microbiota is crucial to access core resources of the microbial members of the holobiont; culturing strategies are currently advanced based on plant-based culture media. Followed was the introduction of "in situ *similis*" cultivation strategy depending on the use of plant intact organs, e.g. leaves/ roots that finger print plant nutritional composition and expose compartment-affiliated microbiota.

Methods Here, we advance a practical strategy to in vitro cultivation of tomato microbiota, making use of veggie-discs of homologous tomato and heterologous vegetables (potato and taro), as well as plant broth-based culture medium. Colony forming units (CFUs) are well-developed on water agar plates with veggie-discs as such or immersed with over-lay agar technique and/or membrane filters. The culturable bacteria community (CFUs) was analyzed by DGGE, and representative pure isolates were subjected to morpho-physiological studies and 16S rRNA gene sequencing.

Results Veggie-discs acted as compatible natural/nutritional mat developing copious/fully-grown CFUs of bacteria, including actinomycetes, and fungi. The strategy uncovered the highly divergent composition of tomato culturable community, being extended to representatives of Actinomycetota, Bacillota, Bacteroidota and Pseudomonadota. Genuinely, the strategy expanded the diversity of tomato microbiota: brought into cultivation additional 18 genera not previously reported; novel cultivation of unique isolates that showed higher similarity to previously-uncultured clones representing Pseudomonadaceae, Oxalobacteraceae and Sphingomonadaceae.

Conclusion The presented veggie-discs cultivation offers additional tools to in vitro render the hidden compartment-affiliated microbiota (bacteria/

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actinomycetes/fungi) accessible for future application of synthetic community approach (SynCom) and microbiota-target interventions, towards improved vegetables nutrition, health and quality, especially under soilless cultivation.

Keywords Culturomics · In situ *similis* cultivation · Microbiome-mediated strategies · Tomato microbiota · Vegetable microbiota · Veggie-discs

Introduction

Macroorganisms are accompanied by an interdependent complex of microorganisms, and they function in tandem as a collective entity known as the meta-organism (Rausch 2019). It is established that such intimate and reciprocal relationship add to soil biofertility and plant nutrition/ health through increasing nutrients availability and boosting growth under biotic/abiotic stresses. An alliance that is holding promises for eco-friendly solutions that supports future agriculture sustainability and increasing productivity.

Among researchers exists a contrariety regarding the appropriate methodologies to study host-microbe interactions. Historically, physiologic/ genomic information of microbial life was primarily based on information derived from cultivated pure cultures. The breakthrough of metagenomics and single cell genomics significantly impacted our current understanding as it appeared that less than half of the known microbial phyla contained a single cultivated representative, and other phyla composed exclusively of uncultured representatives are referred to as Candidate Phyla (CP) (Solden et al. 2016). In fact, the growing interest in microbiota research has been boosted by the possibility of profiling diverse microbial communities using the next-generation sequencing (NGS). Such culture-independent and high-throughput metagenomic technology enables the identification and comparison of entire microbial communities. The technology encompasses two particular sequencing strategies, the amplicon sequencing of 16S rRNA gene as a phylogenetic marker and shotgun sequencing that captures the complete breadth of DNA within a sample. While the first is limited to taxonomic classification at the genus level, the second offers the advantage of species-/ strain-level classification of bacteria (Rausch 2019).

Does it mean that *"Today cultivation is no longer a requirement for gaining access to information from the uncultivated majority that represents > 99%"* (Solden et al. 2016). The answer is "No", but clearly highlights that in vitro culturability represents a great challenge, and that a deeper knowledge of the microbial world is very much connected with what we are able to in vitro cultivate (Sarhan et al. 2019; Zhang et al. 2021a). Fortunately, the new information generates from metagenomics and single cell genomics can unfold unknown metabolic features of not-yet-cultured bacteria and help their in vitro targeting (Overmann et al. 2017). Along the last two decades, new cultivation concepts have been developed principally based on bettered understanding of the ecology of previously not-cultured bacteria. This resulted in the introduction of improved culture media that simulate the natural makeup of substrates and nutrients. This significantly contributed to the birth of "culturomics" (Lagier et al. 2018), among other omics technologies that are realizing tremendous progress in high-throughput cultivation techniques of microorganisms. Indeed, the complexity of the cellular behavior and its decision-making system may speedily drive the establishment of novel omics and associated techniques. "Microbiomics" is developed for the study of structure, function, and dynamics of a microbial community by integrating multiple omics information, such as genomics, transcriptomic, proteomics and metabolomics. All of the microorganisms of a given environment, called microbiome, are analyzed to study the potential roles of microorganisms in natural environments (Dai and Shen 2022).

Now, it is realized that in vitro cultivation of plant microbiota is crucial to access core resources of the microbial members of the microbiome/holobiont, taking into consideration the functional differentiation among microbiota predominant in different plant-soil systems. Considering that the plant is orchestrating its allied microbiota, we developed genuine culturing strategies employing plant-culture media based on the sole use of host plants in its various forms, e.g. juices, dehydrated powders and broth (Nour et al. 2012; Youssef et al. 2015; Fornefeld et al. 2017; Sarhan et al. 2019; Elsawey et al. 2020; Elsawey 2023). Progressively, we

introduced the leaf-based culture media that intimately simulate real time nutritional/environmental conditions of tested host plants (Nemr et al. 2020). The leaf surfaces acted as natural pads creating "in situ *similis*" environments that extended the diversity of culturable plant microbiota, including genera not commonly reported. Further improvement of the strategy is realized by compatible cultivation of plant microbiota on corresponding leaf strips/root segments-based culture media. As the uncovered culturable community of bacteria was unique and signaled a certain degree of plant organ affinity/compatibility (Nemr et al. 2021). Further evidences were presented to indicate that differential in vitro growth on homologous/ heterologous plant-media enriched/restricted various taxa (Elsawey 2023). A solid proof that such in situ-*similis* culturing strategy simulates/finger prints the nutritional composition of host plants/organs, expands the culturomics of plant microbiota, and identifies preferential pairing of microbiota and related plant organs. This is to support future synthetic community (SynCom) research and assist development of microbiota-target interventions to mitigate adverse limitations of biotic/abiotic stressed environments (Meshram and Adhikari 2024) and of soilless cultivation systems (Tzortzakis 2020).

The above culturing strategies were mainly experimented with field crops, and demonstrated the superiority of cultivation of plant microbiota on their natural plant-based culture media. Such media based on the sole use of vegetative parts of the plants (stems/leaves/roots) in the form of plant broth (Elsawey et al. 2020), plant powders (Sarhan et al. 2016) and intact leave strips and root segments (Nemr et al. 2020; Nemr et al. 2021). It remains to test the possibility of cultivation of microbiota for the first time on intact veggie-discs of their homologous/heterologous fruits/tubers/corms. Once the method is appropriated, this will pave the way to be further applied to compartment-affiliated microbiota of vegetable crops.. For this purpose, the cultivability of tomato plant microbiota was tested on veggie-discs prepared from homologous tomato compared to those of heterologous vegetables (potato and taro). In addition, the tomato plant broth-based culture medium was prepared and included in the study (Elsawey et al. 2020). The culturable community developed on agar plates in the form of CFUs was monitored. First, the community composition/diversity of such culturable bacterial community was analyzed by Denaturing Gradient Gel Electrophoresis (DGGE). Second, the single/secluded colonies were picked up to obtain multiple pure isolates representing all of the tested cultivation methods. The resulting pure isolates were subjected to morpho-physiological studies and 16S rRNA gene sequencing and taxa identification.

Materials and Methods

Host plant and sampling

The tested host plant is tomato (*Solanum lycopersicum*, TGRC LA 2662 and LA3003, Tomato Genetic Resource Center, UCDavis, USA). Plants were grown under protected field cultivation at the experimental farm of Faculty of Agriculture, Cairo University, Giza-Egypt (30.0131°N, 31.2089°E). The vegetative parts of plants at the flowering stage were sampled in two biological replicates. Plant shoots were first inserted and separated in plastic bags, and then roots were gently uprooted and packed in respective plastic bags. Samples were transferred to the lab and stored at 4 °C until microbiological analysis was conducted within 24 h after sampling.

Preparation of rhizosphere and phyllosphere samples for analysis

The phyllosphere samples (epi-/endo-phytic) were prepared by gently washing the plant shoots in tap water followed by sterilized distilled water, then ca.10 g were transferred to 90 ml of the basal salt of CCM culture medium (Hegazi et al. 1998) as buffered diluent. For the endo-rhizosphere,*ca.*1 0 g of the root system were surface sterilized and sterility check was carried out by placing segments of roots on the surface of prepared nutrient agar plates. The root segments were transferred to 90 ml of the basal salt of CCM culture medium (Youssef et al. 2004). Such initial shoot/root suspensions were carefully shacked for 30 min., and further serial dilutions were prepared.

Culture media and microbiological analysis

The tomato plant-broth culture medium was prepared from the vegetative part of the plants in the form of semi-solid culture tubes and agar plates according to

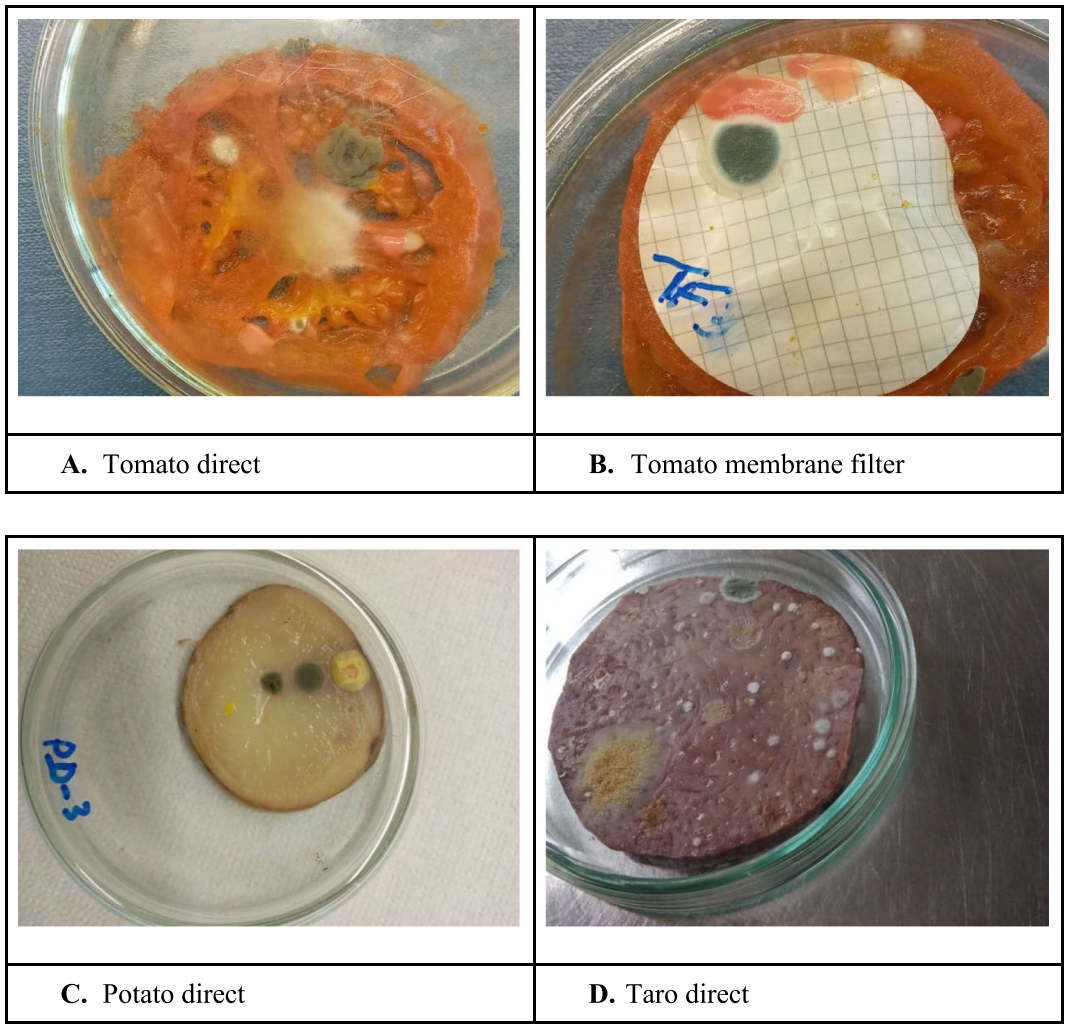

Fig. 1 Colonies of bacteria, including actinomycetes, and fungi developed on veggie-discs prepared from various vegetables. Inocula of serial dilutions prepared for the phyllosphere/endo-rhizosphere of tomato plants were either directly administered on top of veggie-discs (tomato, **A**; potato, **C**; taro, **D**) and/ or filtered through membrane filters that were placed on the surface of veggie-discs of tomato (**B**). (measurements in mm with 0.6–1.0 × magnification)

the protocol described by (Elsawey et al. 2020). With the aid of sharp knife, the veggie-discs (< 0.5 cm dia) were cut from fresh and thoroughly-washed tomato fruits, potato tubers and taro corms, and distributed among Petri dishes, one each. All prepared culture media, tubes of semi-solid plant broth and dishes of veggie- discs, were autoclaved for 20 min at 121 °C, and then kept overnight at 25 °C for sterility check before use.

With preliminary experiments, the inocula of 200 µl of suitable dilutions, 10^{-1}- 10^{-3} and 10^{-2} –10^{-5} for phyllosphere and endo-rhizosphere respectively, were directly administered on the top of each of the prepared veggie-discs, and /or passed through a membrane filter (0.45 µm dia) that were straight placed on the surface of the prepared veggie-discs (Fig. 1). For further improvement, two major experiments were carried out for phyllosphere as follows: a) each of the autoclaved veggie- discs plates was evenly covered with *ca.* 20 ml of sterilized/molten semi-solid water agar (10 g agar L^{-1}) to contain the leaked veggie juices, as of heat/autoclavation treatment, within the plates as well as to create more spacious surfaces for inocula spreading and for developing colonies; b)

the inoculum was dispensed / homogenized in tubes of 2.0 ml of sterilized/molten semi-solid water agar (1.75 g agar L^{-1}) prior to overlaying on the surfaces of the previously prepared veggie- discs water agar plates (Fig. 2). A third experiment was carried out for the endo-rhizosphere applying both modifications. Incubation took place at 25 °C for 2–10 days, and developed CFUs were carefully macro-/micro-scopically examined. All colonies developed on veggie-discs agar plates were single colony-purified to obtain representative pure isolates.

Representative isolates and 16S rRNA gene sequences for phylogenetic affiliation

All colonies developed on agar plates and representing various culture media were further sub-cultured on their corresponding semi-solid culture tubes (Table S1). Isolates that were successfully sub-cultured were subjected to DNA extraction and 16S rRNA gene sequencing (Sarhan et al. 2016; Elsawey et al. 2020; Nemr et al. 2021). The purified PCR products were sequenced by Eurofins MWG Operon (Ebersberg, Germany). Partial 16S rRNA gene sequences are deposited in the GenBank database under their respective accession numbers (Table S2). The 16S rRNA gene sequences were taxonomically assigned by comparison with those available in GenBank using Blast N, and phylogenetic trees were constructed by using the neighbor-joining methods and Bootstrapping on 1,000 replicates. The constructed trees were saved in Newick format and visualized with iTol (itol.embl.de) (Elsawey et al. 2020; Nemr et al. 2021).

Total community DNA extraction and denaturing gradient gel electrophoresis (DGGE) analysis

For DNA extraction of the culturable community, and according to (Sarhan et al. 2016), all CFUs developed on representative agar plates of the tested culture media were harvested using 0.05 M Na Cl solution. DNA quality was assessed using a Nano Photometer (Nano Photometer NP80 Touch, Implen GmbH, Munich, Germany). The total extracted community DNA (TC-DNA) was used to amplify the whole 16S rRNA gene using the 9bfm (GAG TTTGATYHTGGCTCAG) and 1512r (ACG-GHTACCTTGTTACGACTT) primers (MüHling et al. 2008; Nemr et al. 2021). Related procedures and protocols are those well-described by (Elsawey et al. 2023). To obtain the PCR product of the V3 region, 2 µL of the purified 16S rRNA PCR product (10 ng μL^{-1}) were re-amplified using the 341fGC (CGCCCGCCGCGCGCGGCGGGCGGGGCG GGGGCACGGGGCCTACGGGAGGCAGCAG) and 518r (ATTACCGCGGCTGCTGG) primers; the reaction conditions and thermal cycling program were used as described by (Elsawey et al. 2023). DGGE was performed using the VS20WAVE-DGGE Mutation Detection System (Cleaver Scientific, United Kingdom). The gels were stained for 30 min with SYBR Gold stain and recorded with a UV Trans illuminator (Cleaver Scientific, United Kingdom). The DGGE fingerprints were analyzed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) analysis based on the Euclidean distance matrix derived from DGGE fingerprints. The resulting dendrogram was visualized using GelJ software v.2.0 (Heras et al. 2015).

Data analyses

STATISTICA (Statsoft, Inc. Tusla, USA, Version 10.0 http://www.statsoft.com) was used for the analysis of variance (ANOVA) to examine the significant effects. The phylogenetic trees were annotated using the online tool Interactive Tree of Life (iTOL) (http://itol.embl.de). R version 4.0.2 (https://www.r-project.org/), R-studio (https://www.rstudio.com/), R-project packages (cran.r-project.org), "ggplot2" and "scales" were used for constructing pie charts and stalked columns bars. The DGGE fingerprints were analyzed using GelJ software v.2.0 (Heras et al. 2015).

Results and Discussion

It is observed that veggie-discs naturally acted as in situ-*similis* nutritional mat that directly furnished the development of copious and fully-grown CFUs of microbiota, including bacteria, actinomycetes and fungi (Fig. 1). Inclusion of veggie-discs in semi-solid agar was advantageous via incorporating the nutritional juices leaked out during heat treatment by autoclavation as well as stretching CFUs growth all over the surfaces of Petri dishes (Fig. 2). As to the microbiota of tested tomato phyllosphere, CFUs counts

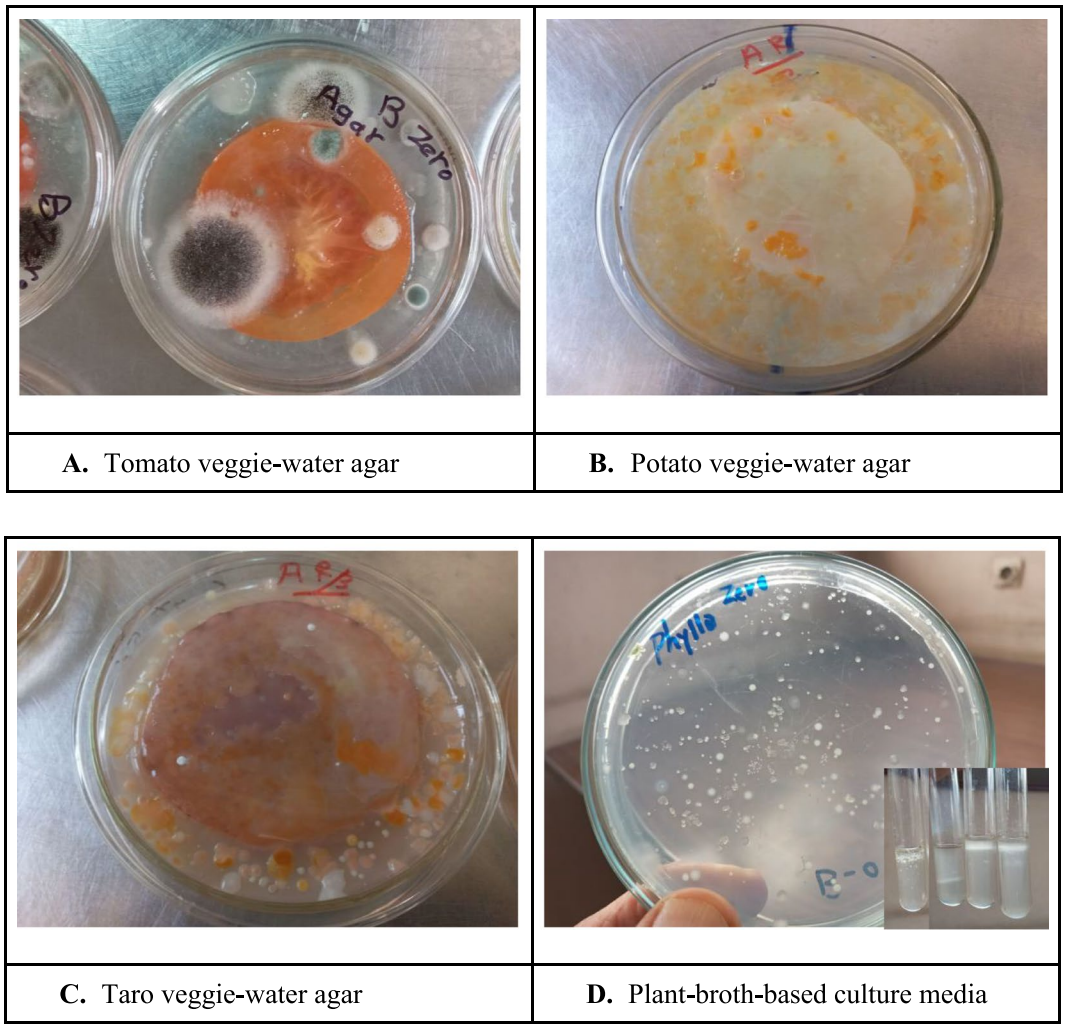

Fig. 2 Colonies of bacteria, including actinomycetes, and fungi developed on veggie-discs immersed in semi-solid water agar layer. Inocula of serial dilutions prepared for tomato phyllosphere/endo-rhizosphere were dispensed in semi-solid water agar just prior homogenously-overlaid on the prepared veggie-discs water agar plates: Veggie-discs of tomato (**A**), Potato (**B**) and taro (**C**). And (**D**): Discrete colonies developed on plant (tomato) broth-based agar medium; inserted are the good growth (pellicle formation) of pure isolates in corresponding plant broth-based semi-solid culture medium. (measurements in mm with 0.5–0.9 × magnification)

were in the range of > log 2.5 – 5.0 g^{-1} root/shoot. ANOVA analysis indicated significant differences attributed to the single effects of biological replicates and the type of culture media (Table 1A). Analogue to the plant broth-based culture medium, the veggie-discs of various tested vegetables supported a good population of CFUs; though yielding lower CFU counts. Irrespective of the type of vegetable discs, the modification of using veggie-discs immersed in thin water agar layer rendered more nutrients and extra space for the growing colonies that resulted in significant increases (> 30%) in CFUs counts. Compared to the phyllosphere, the endo-rhizosphere of tomato harbored much higher counts of endophytic bacteria in the range of > log 3.7 – 9.0 g^{-1} (Table 1B). ANOVA analysis indicated significant differences attributed to the single effects of the type of culture media. As well and comparable to the plant broth-based culture medium, the veggie-discs-water agar of tested vegetables supported good growth of endophytic

Table 1A ANOVA analysis of log numbers of CFUs of bacteria of tomato phyllosphere developed on various culture media: Veggie-discs, veggie-discs water agar and plant broth-based culture medium, (data are log means, n =8)

I- *The phyllosphere: Epi- and endo-phytic populations*	
Experiment 1: Two-factor ANOVA analysis	Log No. CFUs g^{-1} DW.
Treatments	
Factor A: Biological replicates (tomato lines)	
Line 1 (TGRC LA 2662)	3.153[b*]
Line 1 (TGRC LA 3003)	3.592[a]
LSD (p value ≤ 0.05)	**0.096**
Factor B: Culture media	
Plant broth	4.901[a]
Tomato veggie-discs	2.271[e]
Potato veggie-discs	3.655[b]
Taro veggie-discs	2.183[e]
Tomato veggie-discs water agar	2.876[c]
Potato veggie discs water agar	5.078[a]
Taro veggie-discs water agar	2.645[d]
LSD (p value ≤ 0.05)	**0.214**
Experiment 2: Three- factor ANOVA analysis	
Treatments	
Factor A: Biological replicates (tomato lines)	
Line 1 (TGRC LA 2662)	2.849[b]
Line 1 (TGRC LA 3003)	3.391[a]
LSD (p value ≤ 0.05)	**0.110**
Factor B: Culture media	
Veggie-discs	2.703[b]
Veggie-discs water agar	3.537[a]
LSD (p value ≤ 0.05)	**0.110**
Factor C: Veggie discs, homologous/heterologous	
Tomato veggie-discs	2.580[b]
Potato veggie-discs	4.367[a]
Taro veggie-discs	2.414[c]
LSD (p value ≤ 0.05)	**0.162**

Statistically significant differences are designated by different letters ($p \leq 0.05$, $n = 8$)

Each figure represents the average of CFUs developed on four replicates of agar plates

*Means followed by the same letter are not significantly different ($p < 0.05$)

rhizobacteria. Differences were significant among all tested culture media, being highest for the plant broth-based culture medium followed by veggie-discs-water agar of taro, potato and tomato respectively. Obviously, this is reflecting the differences in the nutritional make up of tested homologous and heterologous veggie-discs. PCR-DGGE fingerprinting of the 16S rRNA gene segment recovered from total CFUs developed on all tested culture media was performed. This is to have a broad idea on the composition of the cultivable communities of bacteria as affected by the tested culture media. In case of the endo-rhizosphere of tomato, the UPGMA analysis resulted in clear banding patterns and clustering of the produced DGGE bands. Based on the analysis of distance scores, the UPGMA clustering was differentiated into two main clusters at a cluster cutoff value of 0.61. The first one contained the bacterial communities developed on all of the replicates of the homologous veggie-discs of tomato. The second cluster of

Table 1B ANOVA analysis of log numbers of CFUs of bacteria of tomato endo-rhizosphere developed on various culture media: Veggie-discs, veggie-discs water agar and plant broth-based culture medium, (data are log means, $n=8$)

II-*The endo-rhizosphere populations*		
Experiment 3: Two- factor ANOVA analysis	Log No. CFUs g^{-1} DW.	
Factor A: biological replicates (tomato lines)		
Line 1 (TGRC LA 2662)	6.686[a]	
Line 1 (TGRC LA 3003)	6.641[a]	
LSD (p value ≤ 0.05)	**0.268**	
Factor B- Culture media		
Plant broth	9.309[a]	
Tomato veggie-discs water agar	3.726[d]	
Potato veggie-discs water agar	6.294[c]	
Taro veggie-discs water agar	7.324[b]	
LSD (p value ≤ 0.05)	**0.457**	
Interaction A X B	Line 1	Line 2
Plant broth	10.011[a]	8.607[b]
Tomato veggie-discs water agar	3.596[e]	3.857[e]
Potato veggie-discs water agar	5.761[d]	6.828[c]
Taro veggie-discs water agar	7.375[c]	7.273[c]
LSD (p value ≤ 0.05)	**0.646**	Line 2

Statistically significant differences are designated by different letters ($p \leq 0.05$, $n=8$)

Each figure represents the average of CFUs developed on four replicates of agar plates

*Means followed by the same letter are not significantly different ($p<0.05$)

heterologous cultivation was further sub-clustered at cutoff value of 0.71, with tendency to separate bacterial communities developed on veggie-discs of potato, taro and plant broth-based culture medium (Fig. 3). Variability among tested replicates might be attributed to the densities of culturabale CFUs developed on tested veggie-discs that require including higher number of replicates for better interpretation of results.

All of the colonies developed on agar plates of veggie-discs were picked for single colony isolation of representative isolates. More than 160 pure isolates were obtained, among which 109 were successfully further sub-cultured. A total of 88 isolates were successfully 16S rRNA gene sequenced, 39 represented the phyllosphere (epi-and endophytic) and 49 represented the endo-rhizosphere (Table S1). Based on 16S rRNA gene sequencing, a total of 24 bacterial genera were distinguished. This is in addition to 7 isolates that were broadly identified as only "Bacterium strain" and 6 isolates as previously uncultured bacterial clones (Table 2; Tables S2, S3).

The tested plant-based culturing methods exceptionally resulted in a highly variable and heterogeneous composition of the in vitro culturable community. In general and at the phylum level, culturability of tomato microbiota was extended to include representatives of four phyla. In general, Bacillota and Pseudomonadota were common among the culturable communities of both phyllosphere and endo-rhizosphere, while Actinomycetota were distinguished for the former and Bacteroidota for the latter (Table 2). With veggie-discs cultivation of bacteria residing the phyllosphere, representative isolates of Pseudomonadota and Bacillota were shared with the homologous veggie-discs of tomato and plant broth-based culture media. With the latter culture medium, isolates of Actinomycetota were additionally resolved. With endo-rhizosphere, cultivation on various homo-/hetero-logous veggie-discs supported differential patterns of phyla distribution, with the additional and exceptional recovery of representatives of Bacteroidota on homologous plant broth-based culture medium.

Table 2 Genera of bacterial isolates cultured by the tested plant-based culturing methods compared to those previously reported in literature (using culture-dependent and culture-independent methods) for tomato microbiota

Bacterial Phyla/Genera	Plant spheres		Culture media			*Literature survey*	
	Phyllosphere	Endo-rhizos-phere	Plant broth	Veggie-discs homologous	Veggie–discs heterologous	*Culture – dependent methods*	*Culture – independent methods*
Pseudomonadota							
Achromobacter	-	Yes	-	Yes	-	-	Yes[12,16,20]
Acinetobacter	Yes	-	Yes	-	-	Yes[2]	Yes [10,12,13,15,17,20]
Agrobacterium	-	Yes	-	Yes	Yes	-	Yes[18]
Caulobacter	-	Yes	-	-	Yes	-	Yes [16,19,20]
Cupriavidus(Ralstonia)	-	Yes	-	Yes	-	-	Yes [10,19,20]
Enterobacter	Yes	-	-	Yes	-	Yes[1,2,4]	Yes[10,16,20]
Massilia	Yes	-	-	-	Yes	-	Yes[8,19,20]
Moraxella	Yes	-	-	-	Yes	-	-
Pantoea	Yes	-	Yes	Yes	Yes	Yes[2]	Yes [10,12,13,15]
Pseudomonas	Yes	Yes	Yes	-	Yes	Yes[1,2,6,11]	Yes [10,12,15,16,18,19,20]
Rhizobium	-	Yes	-	Yes	Yes	-	Yes [10,15,16,18,19,20]
Sphingomonas	Yes	-	-	-	Yes	-	Yes[8,10,12,13,16,18,19,20]
Variovorax	-	Yes	-	-	Yes	-	Yes[16]
Actinomycetota							
Arthrobacter	Yes	-	-	-	Yes	-	Yes[8,15,16,20]
Curtobacterium	Yes	-	-	-	Yes	-	Yes[12]
Modestobacter	Yes	-	Yes	-	-	-	-
Mycobacterium	Yes	-	-	-	Yes	-	Yes[14,16,19,20]
Microbacterium	-	Yes	-	-	Yes	Yes[3,6]	Yes[16,18,19,20]
Streptomyces	Yes	-	Yes	-	Yes	-	Yes[10,12,14,15,16,19,20]
Bacillota							
Bacillus	Yes	Yes	Yes	-	Yes	Yes[2,3,4,5,6,11]	Yes [10,12,13,14,15,16,17,19, 20]
Paenibacillus	Yes	-	Yes	Yes	Yes	-	Yes[12,16,19,20]
Priestia	Yes	-	-	-	Yes	-	-
Bacteroidota							
Dyadobacter	-	Yes	Yes	-	-	-	Yes[16,20]
Flavobacterium	-	Yes	Yes	-	-	-	Yes[12,16,19,20]
Bacterium strain	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	-	**-**
Uncultured bacterium	**Yes**	**Yes**	**Yes**	-	**Yes**	**-**	**-**

1-(Pérez-Rodriguez 2020), 2-(Anzalone et al. 2021), 3-(Basumatary et al. 2021), 4-(Chaouachi et al. 2021), 5-(Sharma et al. 2021), 6-(Cochard et al. 2022), 7-(Chialva et al. 2020), 8-(Cheng et al. 2020), 9-(Lee et al. 2016), 10-(Dong et al. 2019), 11-(Tian et al. 2017), 12-(Abdulsalam et al. 2023), 13-(Sumbula et al. 2020), 14-(Chen et al. 2022), 15-(Zhang et al. 2021b), 16-(Lee et al. 2019), 17- Romero et al. 2014, 18-(Ottesen et al. 2013), 19-(Zhang et al. 2023), 20-(Anzalone et al. 2022)

At genera level, the highest diversity of culturable microbiota was reported in the phyllosphere (Fig. 4) compared to the endo-rhizosphere (Fig. 5). Out of total 24 genera resolved, 15 (63%) were reported in the phyllosphere compared to 11 (46%) in the endo-rhizosphere (Fig. 6). In both compartments, common were the genera of *Bacillus* and *Pseudomonas,* representing 8%. Other genera distinguished either the phyllosphere (13 genera) or the endo-rhizosphere (9 genera) (Table 2; Fig. 6A). For either plant compartments, variability in the diversity of culturable bacteria is clearly distinguished in response to the

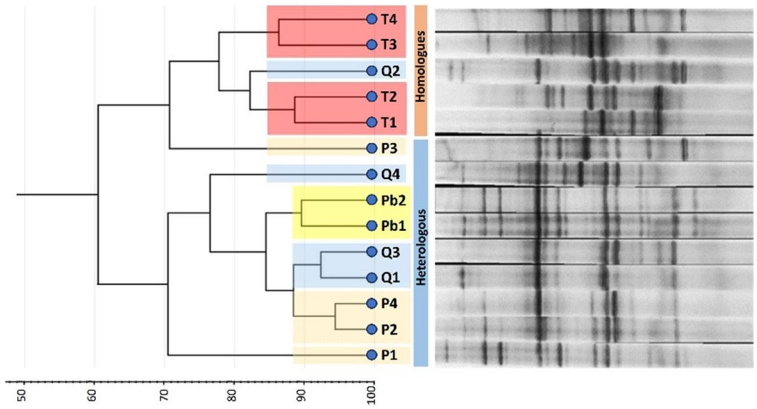

Fig. 3 UPGMA clustering of Euclidean distances of DGGE fingerprints of culturable bacterial communities of tomato endo-rhizosphere. Each culture medium is represented by four replicates (plates 1–4), except for 2 samples for plant broth (Pb): Cultivation on homologous tomato veggie-discs (T), heterologous veggie-discs of potato (P) and taro (Q), and on tomato plant broth-based culture medium (Pb)

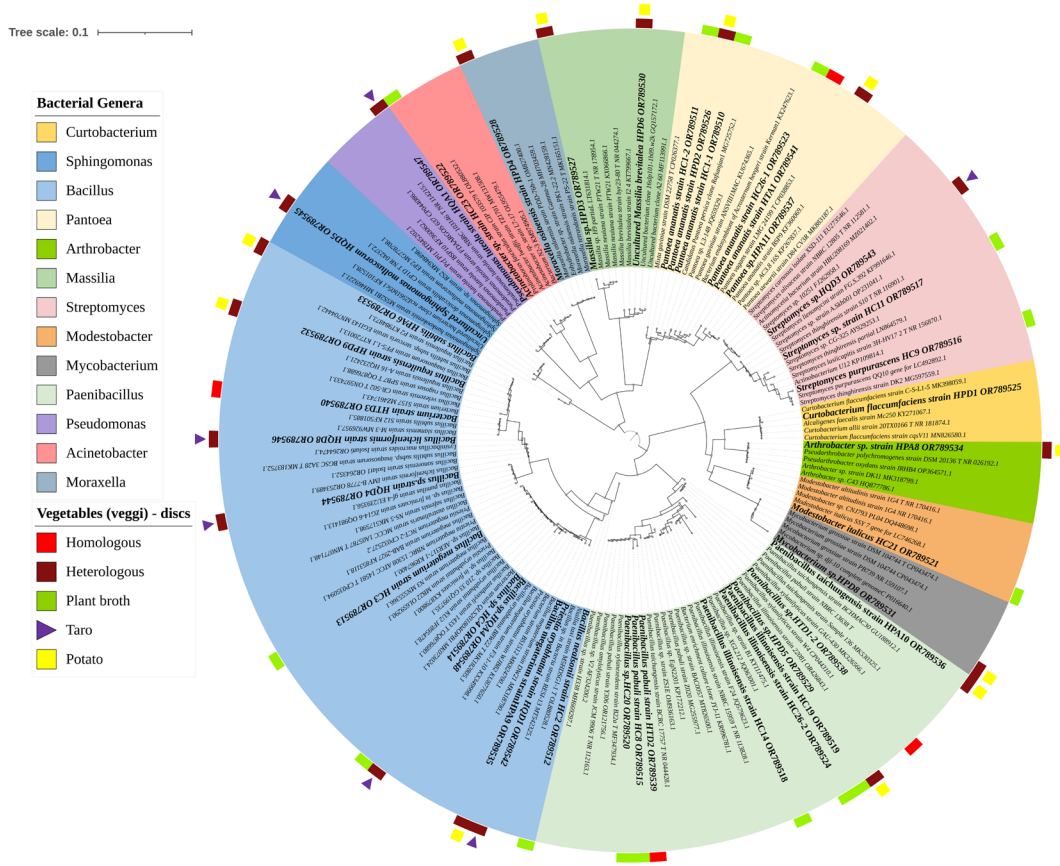

Fig. 4 Phylogenetic tree of 16S rRNA gene sequences of the 39 isolates representing phyllosphere of tomato plants. The colored labels indicate the taxonomic groups on genera level and culture media (plant broth, homologous and heterologous veggie-discs). Respective isolates identifier followed by their accession number are highlighted in bold and larger font style. The closely- related strains are written in normal style, while type strains indicated by a T followed by the accession number. The phylogenetic tree was interfered using the neighbor-joining method. The bootstrap values for 1,000 replicates are adorning the branches as cycles

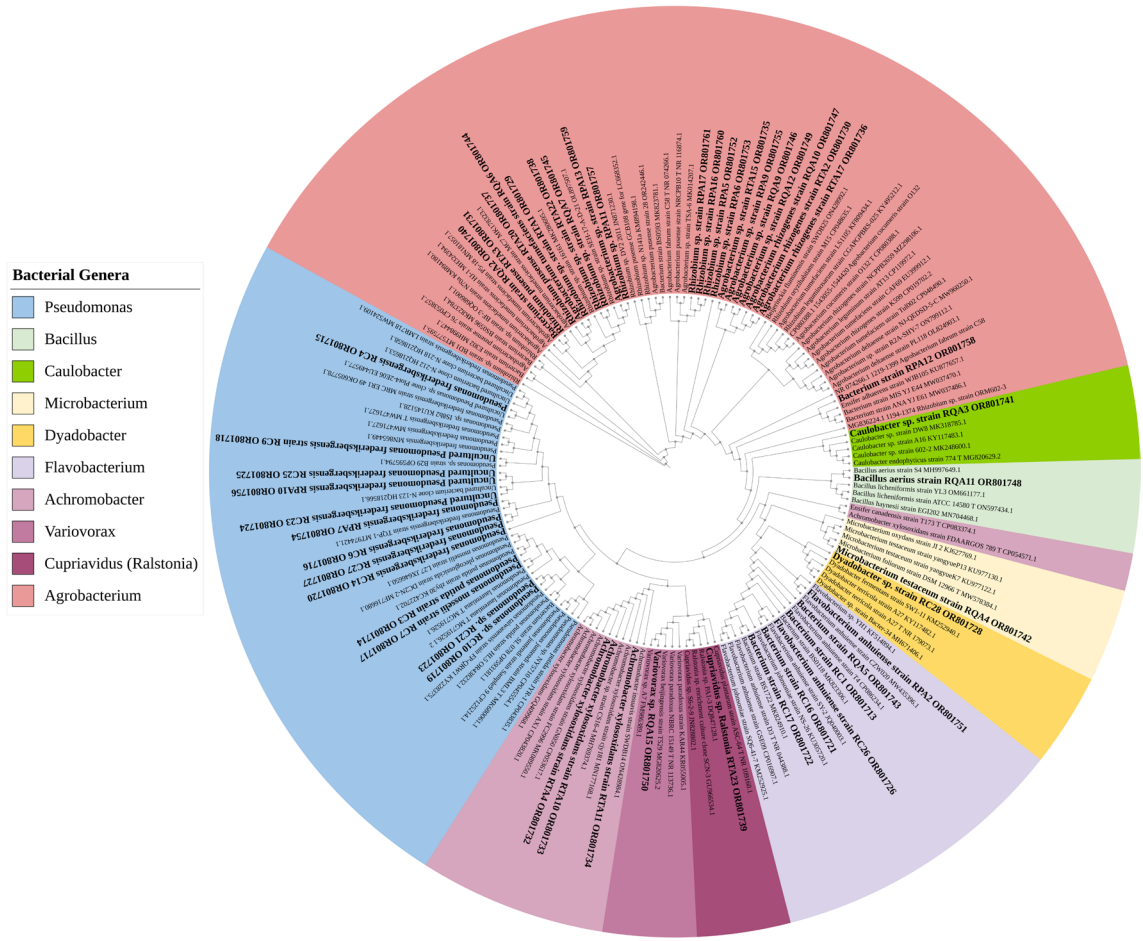

Fig. 5 Phylogenetic tree of 16S rRNA gene sequences of the 49 isolates representing the endo-rhizosphere of tested tomato plants. The colored labels indicate the taxonomic groups on genera level. Respective isolates identifier followed by their accession number are highlighted in bold and larger font style. The closely- related strains are written in normal style, while type strains indicated by a T followed by the accession number. The phylogenetic tree was interfered using the neighbor-joining method. The bootstrap values for 1,000 replicates are adorning the branches as cycles

tested culture media. As an example, within phyllosphere community, homologous cultivation on both tomato plant broth-based culture medium and tomato veggie-discs culture medium shared the genera of *Pantoea* and *Paenibacillus*. Independently, tomato veggie-discs recovered representatives of Pseudomonadota (*Enterobacter*) and broadly-identified Bacterium strain, while the plant broth supported the growth of the genera of Bacillota (*Bacillus*), Pseudomonadota (*Acinetobacter*) and Actinomycetota (*Streptomyces, Modestobacter*) (Fig. 6B). The varying nutritional makeup of the heterologous veggie-discs of potato/ taro did stamp on the diversity and richness of the culturable community (Fig. 6D and E). For phyllosphere, diversity was extended on potato veggie-discs (8 genera) and taro (5 genera) compared to the homologous tomato veggie-discs (3 genera) (Table S3). Likewise, such changeful in diversity of reported genera is reported for the bacterial community of the endo-rhizosphere (Tables S2, S3).

Reviewing related literature of in vitro cultivation of tomato microbiota, in both phyllosphere/ rhizosphere, demonstrated the common use of chemically-synthetic culture media (e.g. nutrient agar, King's medium B agar, Luria broth) as such or supplemented with plant extracts/juices of potato/tomato (potato glucose agar, ATTC medium 965 with tomato juice). The Six genera previously reported in literature were

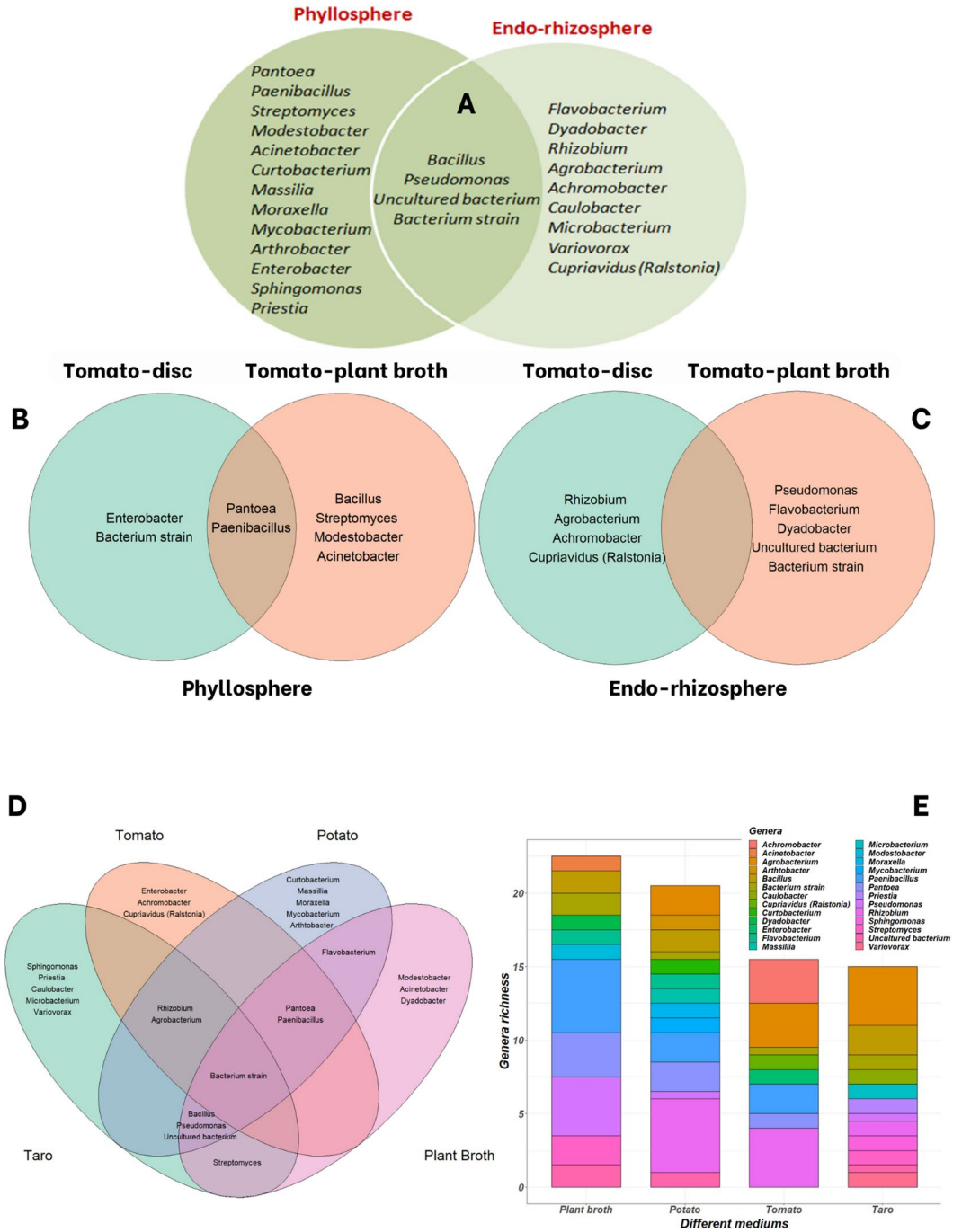

Fig. 6 Venn diagrams representing the distribution of genera of representative isolates developed on tested plant-based culture media: Plant compartment effect, phyllosphere /endo-rhizosphere (**A**); homologous cultivation on veggie-discs and plant broth-based culture medium of phyllosphere (**B**) and endo-rhizosphere (**C**); diversity on various cultivation methods irrespective of plant compartments (**D**). Over all richness of detected genera in both phyllosphere and endo-rhizosphere (**E**)

successfully developed on the tested plant-based culture media, namely *Acinetobacter*, *Bacillus*, *Enterobacter*, *Microbacterium*, *Pantoea* and *Pseudomonas*. Genuinely, the presented plant-based culturing strategies significantly extended the diversity of tomato microbiota. Additionally, they brought into

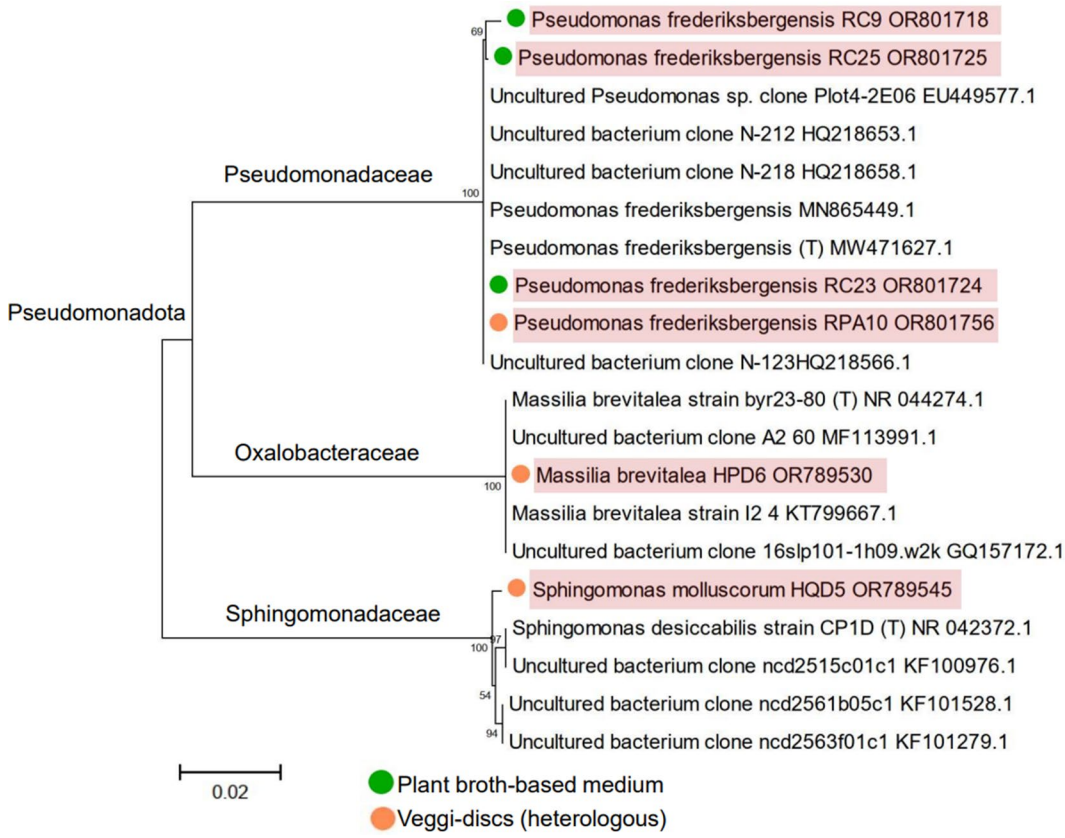

Fig. 7 Based on 16S rRNA gene sequences, a neighbor-joining phylogenetic tree of unique bacterial isolates showing higher similarity to previously uncultured clones, and recovered by cultivation on various plant-based culture media. Bootstrapping was performed with 1000 replicates; the percentage of trees in which the associated taxa clustered together is shown next to the branches. Phylogenetic analysis was conducted in MEGA X

cultivation more 18 genera that were not reported earlier in literature, and describe novel members of the culturable tomato microbiota (Table 2). Such genera represent both plant phyllosphere (10 genera) and endo-rhizosphere (8). Among these genera, the phyllosphere differentially harbored representatives of Actinomycetota followed by Pseudomonadota and Bacillota; while the endo-rhizosphere accommodated members of Pseudomonadota followed by Bacteroidota. Of importance is that members of these particular genera are reported to efficiently combat soil-borne diseases in tomatoes (Hu 2016; French et al. 2020; Hassan 2020; Meshram and Adhikari 2024) that encouraged the increasing application of various microbial inoculants in agriculture, e.g. biopesticides, bioprotectants, biostimulants and biofetilizers, to support plant growth and suppress pathogens (Souza et al. 2015).

Veggie-discs in particular eased the culturing of a number of distinguished genera, either on the homologous tomato discs (5 genera of *Achromobacter*, *Agrobacterium*, *Cupriavidus*, *Paenibacillus* and *Rhizobium*) or on heterologous discs of potato/taro (11 genera, Table 2). Of great interest is that three of such genera were enriched on the tested plant-based culture media, *Moraxella, Modestobacter* and *Priestia*, were not previously reported using culture-independent analysis (Table 2). Among such genera, there are members which are reported to possess genes encoding for properties that help with stress alleviation, and the production of small molecules like vitamin B12, over polymers like polyhydroxybutyrate (PHB) and synthesis of multiple proteins/metabolites that possibly contribute to the nutrition and quality of the fruits (Escobar Rodríguez et al. 2021). In this respect, it is reported

that the microbial communities of tomato plant in hydroponics is less diverse than of those cultivated in soil that could directly play a crucial role in the flavor of tomato fruit (Escobar Rodríguez et al. 2021).

In general, the introduced plant-based culturing strategies not only recovered all of the genera that are commonly reported for tomato microbiota but also preferentially detected more isolates that belonged to the genera *Rhizobium* and *Cupriavidus* (*Ralstonia*) from the endo- rhizosphere (Table 2). Strikingly, our results strongly suggest the novel cultivation of 13 isolates that represent seven genera. Seven of such isolates showed higher similarity to strains of broad-identification (Bacterium strain) in NCBI GenBank (Table 2, Tables S2, S3). The remaining six isolates showed higher similarity to previously uncultured clones representing the families of Pseudomonadaceae, Oxalobacteraceae and Sphingomonadaceae of the phylum Pseudomonadota (Fig. 7).

Conclusions

In conclusion, the introduced culturing method of veggie-discs is proved to be reliable by itself. The strategy is to be further extended to cultivate the microbiota of compatible organs of the vegetable plants, e.g. fruits (carposphere), flowers (anthrosphere) and seeds (spermosphere). The ultimate goal is to significantly expand the culturability of compartment- affiliated microbiota of vegetable crops. It is advanced as an additional culturomic tool for more in vitro screening and bringing into acquisition new microbiota resources. This is to best service the application of microbiota-target interventions of vegetables growing, especially under soilless cultivation, towards higher productivity and better quality products.

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Declarations

Conflict of interest The authors have declared no conflict of interest.

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