Diversity and plant growth-promoting potential of (un)culturable bacteria in the *Hedera helix* phylloplane

Vincent Stevens (vincent.stevens@uhasselt.be)
Universiteit Hasselt https://orcid.org/0000-0002-3725-1597
Sofie Thijs
Universiteit Hasselt
Jaco Vangronsveld
Universiteit Hasselt

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Abstract

Background

A diverse community of microbes naturally exists on the phylloplane, the surface of leaves. It is one of the most prevalent microbial habitats on earth and bacteria are the most abundant members, living in a community that is highly dynamic. While culture-independent approaches greatly increased our knowledge of microbial communities such as the phylloplane, one of the challenges for microbiologists today remains to develop strategies to culture the vast diversity of microorganisms.

Results

We isolated bacteria from the phylloplane of *Hedera helix* (common ivy), a widespread evergreen that constitutes an excellent model for studying the phylloplane in the field, using the growth media LB, LB01, YMA, YFlour and YEx. We also included a comparison with the uncultured phylloplane, which contained the highest intra-sample (alpha) diversity. Inter-sample (beta) diversity shifts from LB and LB01 containing the highest amount of resources to YMA and YFlour which are more selective, and YEx which is more limited but also more varied in resources. We show the *H. helix* phylloplane is dominated by Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes. Further, all growth media more or less equally favored Actinobacteria and Gammaproteobacteria, whereas Bacteroidetes could only be found on LB01, YEx and YMA. LB and LB01 greatly favored Firmicutes and YFlour was most selective for Betaproteobacteria. At genus level, LB favored the growth of *Bacillus* and *Stenotrophomonas*, while YFlour was most selective for *Burkholderia* and *Curtobacterium*. The *in vitro* plant growth promotion (PGP) profile that we obtained by testing 200 isolates constitutes an important first step to find candidates with advantageous traits within microbe-assisted approaches. Our isolation effort also resulted in a significant collection of bacterial strains underrepresented in public databases, mostly from the phylum Actinobacteria.

Conclusions

This study contributes as a case study of bacterial culturability including an evaluation of five different growth media, a comparison with the uncultured *H. helix* phylloplane community and its relation with functional characteristics such as PGP potential which help us to understand the putative ecological and functional role of microbial members living in the phylloplane.

Background

An abundant and diverse community of microorganisms naturally exists on the surface of above-ground parts of plants, the phyllosphere, which can be further subdivided into the caulosphere (stems), phylloplane (leaves), anthosphere (flowers) and carposphere (fruits). The phyllosphere is one of the most prevalent microbial habitats on earth and bacteria are by far the most abundant and constant members, with a typical cell density of 10^6-10^7 cells cm^{-2} [1, 2]. A majority of phyllosphere studies focusing on

different plant species including *Arabidopsis thaliana* (thale cress), *Lactuca sativa* (lettuce), *Glycine max* (soy bean), *Trifolium repens* (white clover) and *Oryza sativa* (rice), report that leaf communities mainly comprise bacteria belonging to the phyla Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes, where members of Proteobacteria comprise about half of the phyllosphere community, suggesting this composition on higher taxonomic level is similar across various host plant species [2-8]. Increased knowledge of plant–microbe interactions enables a better understanding of their role during plant growth and development [9] and the translation into improved biomass production and microbe-assisted phytotechnologies [10]. In this study, *Hedera helix* (common ivy) was chosen as host plant species. *H. helix* is an evergreen plant, implying it retains its leaves through the year. It is also known for its hardiness and wall/tree climbing ability [11], has a widespread occurrence in natural and urban environments in the northern hemisphere such as private gardens, city centers, municipal parks, nature reserves and forests and therefore constitutes an excellent model for studying the phylloplane in the field.

To enhance our understanding about the diversity and function of microbial communities living in the phylloplane, culture-independent approaches are indispensable. Nevertheless, one of the challenges for microbiologists today remains to develop strategies to culture the vast diversity of microorganisms. Simpler and cheaper alternatives for retrieving a wide diversity of bacteria remain effective, such as varying the diversity of growth media used from complex media rich in macro- and micronutrients to media with very specific carbon and nitrogen sources and low concentrations of mineral salts [12, 13], the addition of (host) plant extracts [14], separate preparation of growth medium components [15], the use of a different solidifying agent [16], and longer incubation periods [13]. Once a collection of bacterial isolates is obtained and maintained in the laboratory, their functional characteristics such as plant growth promotion (PGP) potential can be evaluated including the biosynthesis of PGP hormones and enzymatic activities that can interfere with the plant stress status.

Here, we address some challenges with culturing bacteria by evaluating different growth media that are suitable to grow bacteria associated with the *H. helix* phylloplane using high-throughput sequencing technology, and further compare it with the (total) uncultured bacterial phylloplane community. We used five different growth media solidified with gellan gum, and employed an incubation period of four weeks. The growth media LB [17], LB01 (1/10 dilution of LB), YMA [18], YFlour [19] and YEx were selected to cover a wide variety of bacterial taxa. For each growth medium, representative bacterial colonies were picked and their PGP potential was evaluated. This study gives first insights into which growth media, or combination thereof, are most suitable to grow symbiotic members from the *H. helix* phylloplane. We also provide a culture collection of 200 strains including the characterisation of putative PGP potential which is a fruitful resource for further mechanistic and functional understanding. In addition, we provide a snapshot into the total bacterial community of the *H. helix* phylloplane.

Results And Discussion

Characterization of the uncultured bacterial phylloplane and its culturable fraction

Phylloplane samples from *H. helix* plants were analysed in a culture-dependent and -independent way. Amplicon metagenomics applied to taxonomically identify the phylloplane bacterial diversity of *H. helix* and high-throughput characterization of the culturable fraction using five different growth media resulted in a total of 177,872 high-quality 300 bp V3–V4 16S rRNA gene sequences, representing 1,482 amplicon sequence variants (ASVs). Bacterial intra-sample (alpha) diversity was estimated by rarefaction analysis (**FIGURE A1**) and by calculating three alpha diversity indices: (i) the observed number of ASVs, (ii) Shannon's diversity index and (iii) Simpson's diversity index (**FIGURE 1B**). The uncultured phylloplane samples contained the highest intra-sample diversity, and while diversity in LB01 and YMA was higher compared to LB, YEx and YFlour, diversity in all growth medium samples was low relative to uncultured phylloplane samples, as expected.

To infer bacterial inter-sample (beta) diversity, we employed PCoA on a Bray–Curtis dissimilarity matrix (**FIGURE 1A**). Statistical analysis revealed that the choice of growth medium is a significantly contributing diversity-determining factor (R^2 = 0.2925, p < 0.001). Moreover, visual examination of the PCoA plot shows that inter-sample bacterial diversity shifts when considering LB and to a lesser extent LB01, which contain the highest amount of resources (especially nitrogen sources), to YMA and YFlour which are more selective and to YEx which is more limited but also most varied in resources. Differences in carbon/nitrogen ratio and carbon sources between the growth media likely contribute to the respective biodiversity and prevailing taxonomic groups observed, as it is the case in other bacterial (culture) systems [20-22].

Bacterial diversity of the uncultured phylloplane appears to be different from the cultured bacterial diversity; these differences are further illustrated in **FIGURE 2**. At the phylum level (**FIGURE 2A**), for uncultured phylloplane samples on average 90.7% of ASVs could be taxonomically classified within the four major phyla with following relative abundances: Proteobacteria (51.1%; subdivided as 30.9% Alphaproteobacteria, 14.9% Gammaproteobacteria and 5.9% Betaproteobacteria), Actinobacteria (15.5%), Bacteroidetes (19.2%) and Firmicutes (4.9%). 5.6% of ASVs was classified within 12 other phyla (Acidobacteria, Armatimonadetes, Chlamydiae, Cyanobacteria, Deinococcus–Thermus, Fusobacteria, Gemmatimonadetes, Nitrospirae, Planctomycetes, Saccharibacteria, Verrucomicrobia and candidate phylum WPS-1) and the remaining 3.7% could not be classified at phylum level. It was previously reported for different plant species, including *A. thaliana*, *L. sativa*, *G. max*, *T. repens* and *O. sativa*, that the phyllosphere community mainly comprises bacteria belonging to phylum Proteobacteria (with classes Alphaproteobacteria and Gammaproteobacteria in particular), Actinobacteria, Bacteroidetes and Firmicutes, where members of Proteobacteria constitute ~50% of the community composition [2-8]. Here we show that this holds also true for *H. helix*, and it further strengthens the finding that the composition of the phyllosphere microbiome on higher taxonomic level is similar across various host plant species.

For growth medium samples, 100% of ASVs could be taxonomically classified within these the phyla Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes. This could be expected, given the taxonomic structure of the *H. helix* phylloplane and the general finding that the vast majority of cultured bacteria are affiliated with these phyla [23]. All selected growth media more or less equally favored Actinobacteria and Gammaproteobacteria, with average relative abundances of 19.0% and 34.7%, respectively. Bacteroidetes however were only found on LB01, YEx and YMA. LB and LB01 greatly favored Firmicutes compared to the other growth media with an average relative abundance of 36.1% and 27.1%, respectively, and YFlour was most selective for Betaproteobacteria (20.6%). **FIGURE 2B** illustrates the relative abundances of the 10 most abundant genera across all growth medium samples and their relation with the uncultured bacterial phylloplane. LB favored the growth of *Bacillus* and *Stenotrophomonas*, with average relative abundances of 19.2% and 16.3%, respectively, while YFlour was most selective for *Burkholderia* (13.9%) and *Curtobacterium* (14.7%). On the other hand, *Rhizobium* could be found on all growth media except on LB. Also, in the uncultured phylloplane on average 18.1% of ASVs could not be classified at genus level. For growth medium samples this was 4.7% for LB, 5.4% for LB01, 9.0% for YEx, 11.0% for YFlour and 6.8% for YMA. In other words, most potentially novel bacterial species were cultured on YFlour, while LB and its 1/10 diluted version LB01 had the highest abundance of known bacteria.

The highest proportion of ASVs (76.3%) is unique for the growth media, 21.9% is shared between at least two of the growth media and only 1.8% is shared between all growth media (**FIGURE 3**). This highlights the importance of the use of varied growth media in the context of capturing most of the bacterial diversity. However, it is important to note that ASV abundance is not considered in this picture. The 50 most abundant ASVs in the uncultured bacterial phylloplane samples and their phylogenetic relationship are shown in **FIGURE 4**. Culturing was successful with at least one of the selected growth media for 18 of these top 50 ASVs. All top ASVs were classified within the phyla Proteobacteria, Actinobacteria, Bacteroidetes or Firmicutes except one (ASV 49) that was classified as *Fusobacterium* within the phylum Fusobacteria.

A comparison of the viable count of phylloplane bacteria growing on the growth media, expressed in colony-forming units (CFU) per gram of fresh leaf material, is shown in **FIGURE 5B**. LB and LB01 comprised a significantly higher number of bacterial colonies compared to YEx, YFlour and YMA ($p < 0.05$). From the selected growth media, LB and LB01 contain the highest amount of (nitrogen) resources thereby making it easier for *r*-selected species that are notably emphasized by high growth rates to grow, likely explaining the higher viable count on these growth media.

Functional and taxonomical characteristics of isolates from the selected growth media

Evaluation of PGP potential, determined as indole-3-acetic acid (IAA), 3-hydroxy-2-butanone (acetoin) and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase production by bacterial isolates cultured with

the selected growth media, is shown in **FIGURE 5A**. IAA is the most common phytohormone of the auxin class, and induces cell elongation and division with all subsequent results for plant growth and development [24]. The volatile phytohormone acetoin was already shown to promote growth and induce systemic resistance in *Arabidopsis thaliana* [25, 26], and ACC deaminase reduces ethylene levels, which is related to plant growth promotion [27]. In our study regarding phylloplane bacteria, those capable of IAA production were high-abundant on LB and LB01 and nearly absent on YFlour. YEx is characterized by bacterial isolates showing relatively high production of acetoin. Isolates producing ACC deaminase were low-abundant on all growth media. The bacterial 16S rRNA gene of all 200 isolates was partially sequenced and these sequences could be taxonomically assigned to genus level (**FIGURE 5C**). Most isolates were assigned to the genera *Curtobacterium* (41) and *Methylobacterium* (37). *Frigoribacterium* (16), *Bacillu*s (13), *Rathayibacter* (11) and *Sphingomonas* (10) and *Pantoea* (9) were also common. That one-fifth of cultured bacteria are classified within the genus *Curtobacterium* may not be surprising, as this genus is ubiquitously reported to be associated within phyllosphere habitats [28-31]. In one comprehensive isolation study comprising 200 leaf samples of soybean and corn plants, *Curtobacterium* species could be isolated from every sample [32]. Also, previous culture-independent phyllosphere studies paired with isolation have allowed the identification of representative bacterial strains from various genera, including *Methylobacterium* [33], *Frigoribacterium* [29]*, Sphingomonas* [34, 35] and *Pantoea* [36]. Most isolated *Curtobacterium* and *Methylobacterium* species in this study were able to produce IAA and acetoin, while regarding ACC deaminase such correlations are not that clear. This PGP profile can help to select bacterial isolates with specific PGP traits that can be exploited in microbe-assisted approaches, such as improved biomass production, plant protection or phytoremediation [10]. However, it is important to note that evaluating PGP traits based on *in vitro* experiments solely has its caveats [37]. For example, it is possible that the production of phytohormones does not occur in the natural plant–microbe partnership or that this production occurs in a pathogenic context [38]. Follow-up *in vivo* inoculation experiments are necessary to conclusively evaluate PGP potential, but nevertheless *in vitro* PGP screening remains an important first step.

Our isolation of phylloplane bacteria on a variety of growth media resulted in a significant collection of bacterial strains underrepresented in public databases. Most isolates (104/200) were taxonomically classified within the phylum Actinobacteria, which represented 20 out of a total of 37 genera, including *Curtobacterium* (41), *Frigoribacterium* (16), *Rathayibacter* (11), *Glaciibacter* (5), *Cellulomonas* (4), *Frondihabitans* (4), *Microbacterium* (4), *Nocardioides* (3), *Cellulosimicrobium* (2), *Leifsonia* (2), *Nocardia* (2), *Sediminihabitans* (2), *Arthrobacter* (1), *Brevibacterium* (1), *Flexivirga* (1), *Gordonia* (1), *Herbiconiux* (1), *Micrococcus* (1), *Patulibacter* (1) and *Rhodococcus* (1). This is interesting given the fact that Actinobacteria members are well-known for their secondary metabolite production [39] and abundant occurrence in extreme environments, characterized by acidic/alkaline pH, low or high temperatures, salinity and radiation, and low levels of moisture and resources [40]. For example, *Frigoribacterium* and *Glaciibacter* are typical psychrophilic genera containing a rare group of B-type peptidoglycan [41, 42], and also *Frondihabitans* species are well-adapted to colder and ultraviolet light-exposed environments such as the phylloplane [43].

In light of the coordinated efforts to expand our understanding about plant-associated bacteria and life in general, several strains from this study were selected for whole-genome sequencing in the framework of the U.S. Department of Energy (DOE) Joint Genome Institute (JGI) project the “Genomic Encyclopedia of Bacteria and Archaea (GEBA)” [44]. A further pangenomic study including a comparison with all publicly available genomes to understand which properties are specific to the phylloplane is ongoing and planned to be further elaborated on in the nearby future.

Conclusions

The uncultured phylloplane samples contained the highest intra-sample diversity, and inter-sample bacterial diversity shifts from LB and LB01 containing the highest amount of (nitrogen) resources to YMA and YFlour which are more selective, and YEx which is more limited but also more varied in resources. The majority of ASVs of uncultured *H. helix* phylloplane samples could be taxonomically classified within the phyla Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes in a distribution that is consistent with previous phyllosphere studies which further strengthens the finding that the composition of the phyllosphere microbiome on higher taxonomic level is similar across various host plant species. These four major phyla were fully represented within growth medium samples. All growth media more or less equally favored Actinobacteria and Gammaproteobacteria, whereas Bacteroidetes could only be found on LB01, YEx and YMA. LB and LB01 greatly favored Firmicutes and YFlour was most selective for Betaproteobacteria. At genus level, LB favored the growth of *Bacillus* and *Stenotrophomonas*, while YFlour was most selective for *Burkholderia* and *Curtobacterium*. The highest proportion of ASVs was found to be unique for the growth media, which further highlights the importance of the use of varied growth media in the context of capturing most of the bacterial diversity. The *in vitro* PGP profile that we obtained by testing 200 isolates constitutes an important first step to find candidates with advantageous traits within microbe-assisted approaches, such as improved biomass production, plant protection or phytoremediation. Our isolation efforts also resulted in a significant collection of bacterial strains underrepresented in public databases, mostly from the phylum Actinobacteria, which are currently under further investigation in a pangenomic approach aiming to expand our understanding of bacteria living in the phylloplane.

Methods

Collection and preparation of phylloplane samples

Leaves (n = 80, 20 per site) of *H. helix* of three to six months old growing in the wild were collected at four sites around Hasselt, Belgium (coordinates in WGS84 format: 50.936546, 5.317226 (A); 50.928680, 5.332674 (B); 50.940104, 5.438675 (C); 50.921694, 5.433951 (D)). The distances in km between the sites are: A–B, 1.4; A–C, 8.5; A–D, 8.4; B–C, 7.6; B–D, 7.2 and C–D, 2.1. The soil type at the four sites was sandy loam with an average pH of 6.4 ± 0.1 (A: 6.41 ± 0.02, B: 6.56 ± 0.02, C: 6.42 ± 0.01, D: 6.37 ± 0.02) and average soil organic matter content of 958 ± 154 mg kg^{-1} (A: 766 ± 61 mg kg^{-1}, B: 932 ± 28 mg kg^{-1},

C: 1169 ± 35 mg kg^{-1}, D: 965 ± 26 mg kg^{-1}). Permission for sampling was obtained by local legislation and performed in accordance with institutional and international guidelines. Plant leaves were identified as specimens belonging to *H. helix* by the first author, and verified by all co-authors; voucher specimens are available from Hasselt University. Leaves were cut from the plants at shoulder height using sterile forceps, put in sterile tubes (five leaves per tube) filled with autoclaved phosphate buffer (50 mM $Na_2HPO_4•7H_2O$, 50 mM $NaH_2PO_4•H_2O$, 0.8 mM Tween 80, pH 7.0) and immediately transferred to the laboratory. Leaf weight was determined gravimetrically and microbial cells were detached from the leaf surface by sonication (100 W, 42 kHz, 3 min), followed by shaking on an orbital shaker (240 rpm, 30 min). Next, 16 resulting leaf wash suspensions (four per site, each suspension resulted from five leaves) were centrifuged (4000 rpm, 15 min) and the resuspended pellets were randomly pooled into four samples. For each sample, an aliquot was immediately stored at -80°C until DNA isolation; another aliquot was stored overnight at 4°C for culturing of phylloplane bacteria.

Amplicon metagenomics of the bacterial phylloplane

Leaf wash suspensions (*n* = 4) were centrifuged (13200 rpm, 20 min, 4°C) and genomic DNA was isolated using the NucleoSpin Soil kit (Macherey–Nagel, Düren, Germany). The V3–V4 hypervariable region of the bacterial 16S rRNA gene was PCR-amplified using 341F (5'-CCTACGGGNGGCWGCAG-3') and 785R (5'-GACTACHVGGGTATCTAATCC-3') primers with attached GS FLX Titanium adaptors, the sequencing key TCAG, and a sample-specific multiplex identifier. PCR products were purified by gel electrophoresis (1.5% agarose gel, 90 V, 45 min) and the 514 bp bacterial amplicon was excised and further purified using the UltraClean GelSpin DNA extraction kit (Mo Bio Laboratories, Carlsbad, CA, USA). Samples were brought to an equimolar concentration (10^{10} molecules μL^{-1}) using the Quant-iT PicoGreen dsDNA assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Correct amplicon size and integrity were checked on an Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA), followed by sequencing on a Genome Sequencer FLX system (Roche Applied Science, Penzberg, Germany) with GS FLX Titanium series reagents by Macrogen Europe (Amsterdam, The Netherlands).

High-throughput characterization of cultured phylloplane bacteria

Leaf wash suspension aliquots of 10 µL (*n* = 4) were pooled, diluted 1/100 and inoculated on 120 x 120 mm square Petri dishes containing LB [17], LB01 (1/10 dilution of LB), YMA [18], YFlour [19] or YEx (this study) and incubated at 30°C (10 replicates per growth medium). Gellan gum was used as solidifying agent because of its thermal stability and related resistance to drying out, which makes it possible to incubate at 30°C for a much longer time compared to agar [45]. Phosphate-containing components were separately autoclaved to prevent the formation of growth-inhibiting molecules such as H_2O_2 [15]. The composition of the growth media is summarized in **TABLE 1**. Four weeks after inoculation, the number of CFU per gram of fresh leaf material on the growth media was determined on eight replicates per growth

medium and subsequently all present colonies were suspended in autoclaved 10 mM $MgSO_4$ followed by centrifugation (4000 rpm, 15 min). Resuspended pellets were pooled into four samples per growth medium. Pellets were immediately stored at -80°C until DNA isolation. Similar as described previously, genomic DNA was isolated and the V3–V4 hypervariable regions of the bacterial 16S rRNA genes were PCR-amplified, purified and prepared for sequencing.

Evaluation of plant growth promotion potential

Bacterial phylloplane colonies were randomly picked from two replicates per growth medium. Isolated colonies (*n* = 200, 40 per growth medium) were checked for purity by streaking and grown for 24 h in their respective liquid growth medium at 30°C on a shaker (150 rpm), washed and resuspended in 2 mL 10 mM MgSO4 solution to obtain suspensions containing bacteria in mid-exponential phase ($OD_{600\ nm}$ = 0.4). Next, 20 µL of this bacterial suspension was used for the detection of IAA production using the Salkowski's reagent method [46], for the detection of acetoin production using the Voges–Proskauer test [47], and for assessing ACC deaminase activity by monitoring the amount of α-ketobutyrate that was generated by the enzymatic hydrolysis of ACC [48]. Genomic DNA of all isolates was extracted using the MagMAX DNA Multi-Sample Kit (Life Technologies, Carlsbad, CA, USA) and a MagMAX Express-96 Deep Well Magnetic Particle Processor (Life Technologies, Carlsbad, CA, USA). The bacterial 16S rRNA gene was partially PCR-amplified using 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') primers and 20 µL of the PCR product was used for unidirectional Sanger sequencing using the 27F primer by Macrogen Europe (Amsterdam, The Netherlands).

Processing of sequencing data

Sequencing data were received in FASTQ format with GS FLX Titanium adaptors and the sequencing key TCAG trimmed from all sequences, demultiplexed based on the sample-specific multiplex identifier and further processed with DADA2 1.12.1 [49] for single-end analysis. All reads were quality-filtered by truncation to 340 bp (discarding all reads with less than 340 bp) and subsequently trimming 40 bp from the 5'-side with maxEE = 2 (maximum number of expected errors), resulting in a data set consisting only of high-quality V3–V4 16S rRNA gene sequences of exactly 300 bp with all irrelevant sequence parts removed. For error model learning, dereplication, sample inference and chimera removal default parameter settings were used. Taxonomy was assigned to each resulting ASV with IDTAXA [50] using the RDP 16S rRNA training set v16 [51]. The ASV table with assigned taxonomy was imported into phyloseq 1.28.0 [52] for making phylogenetic bar charts and rarefaction and diversity analysis. Intra-sample diversity was assessed with ASV observations, Shannon diversity and Simpson diversity. Inter-sample diversity was measured with principal coordinates analysis (PCoA) on a Bray–Curtis dissimilarity matrix, and different outcomes were tested using permutational multivariate analysis of variance (PERMANOVA, 999 permutations). Phylogenetic tree construction was done with PhyML 3.1 [53] after alignment of the sequences with MUSCLE 3.8.31 [54]. Differences in CFU between growth media were tested with a

Kruskal–Wallis test followed by pairwise comparisons using a Wilcoxon rank-sum test with Benjamini–Hochberg correction. MicEco 0.9.11 was used for making a Venn diagram. Sanger sequencing data were processed using sangerseqR 1.20.0 and sangeranalyseR 0.1.0 [55], and resulting high-quality sequences were taxonomically assigned with BLAST+ 2.9.0 [56] using the RDP 16S rRNA training set v16 [51]. All data handling was done within R 3.6.3 [57].

Abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
ASV	amplicon sequence variant
CFU	colony-forming unit
IAA	indole-3-acetic acid
PGP	plant growth promotion

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The metagenomic libraries of the bacterial phylloplane communities and sequences of the cultured bacteria on all growth media are available from the Short Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under project accession number PRJNA626008 and individual FASTQ sample identifiers SAMN14614763–86. All partial 16S rRNA gene sequences obtained by Sanger sequencing are available from NCBI under accession numbers MT360054–253.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

VS and ST conceived the study. VS performed the experiments, analysed the data, prepared the figures and wrote the manuscript. ST and JV made suggestions to improve the manuscript. All authors approved the final draft of the manuscript.

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Tables

TABLE 1 Composition of the growth media. Products are given in g L^{-1} distilled water. Products marked in grey were filter-sterilized before being added to the other autoclaved components. Phosphate-containing components (underlined) were separately autoclaved. All growth media have pH 7.0.

	LB [17]	LB01 (1/10 dilution of LB)	YEx (this study)	YMA [18]	YFlour [19]
$CaCO_3$					0.3
$CoCl_2$			1.9×10^{-4}		
$CuCl_2$			1.7×10^{-5}		
Fe(III)NH_4 citrate			4.8×10^{-3}		
H_3BO_3			6.2×10^{-5}		
K_2HPO_4			_	0.1	
KH_2PO_4			_	0.4	
$MgSO_4 \cdot 7H_2O$	1	0.1	1	1	1
$MnCl_2$			1×10^{-4}		
$Na_2HPO_4 \cdot 2H_2O$			0.04		
NaCl	10	1	0.1	0.1	
$NaMoO_4$			3.6×10^{-5}		
$NiCl_2$			2.4×10^{-5}		
Tris			6.1		
$ZnSO_4 \cdot 7H_2O$			1.4×10^{-4}		
D-Fructose			0.5		
D-Glucose			0.5		
Gluconate			0.7		
Lactate			0.4		
Mannitol				10	
Succinate			0.8		
Sucrose					0.3
Plain flour					6
Tryptone	10	1			
Yeast extract	5	0.5	0.4	0.4	0.3
Gellan gum	7.5	7.5	7.5	7.5	7.5

Figures

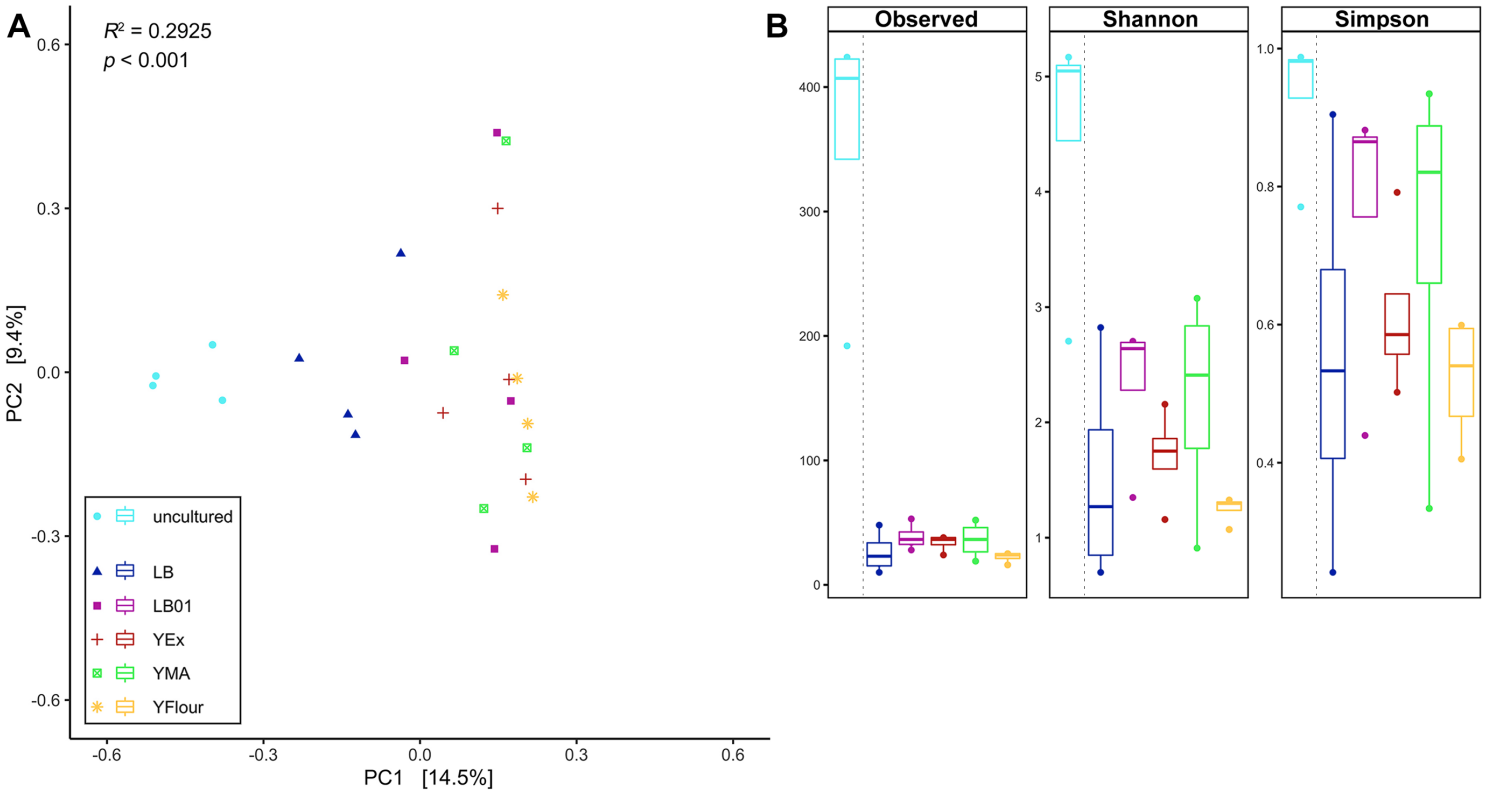

Figure 1

Intra- and inter-diversity of growth medium and phylloplane samples. Intra-sample diversity was assessed with amplicon sequence variant (ASV) observations, Shannon diversity and Simpson diversity (B; n = 24, 4 per growth medium and 4 uncultured phylloplane samples). Inter-sample diversity was measured with principal coordinates analysis (PCoA) on a Bray–Curtis dissimilarity matrix (A). The x- and y-axes are indicated by the first and second principal coordinate (PC), respectively, and the values in parentheses show the percentages of the variation explained.

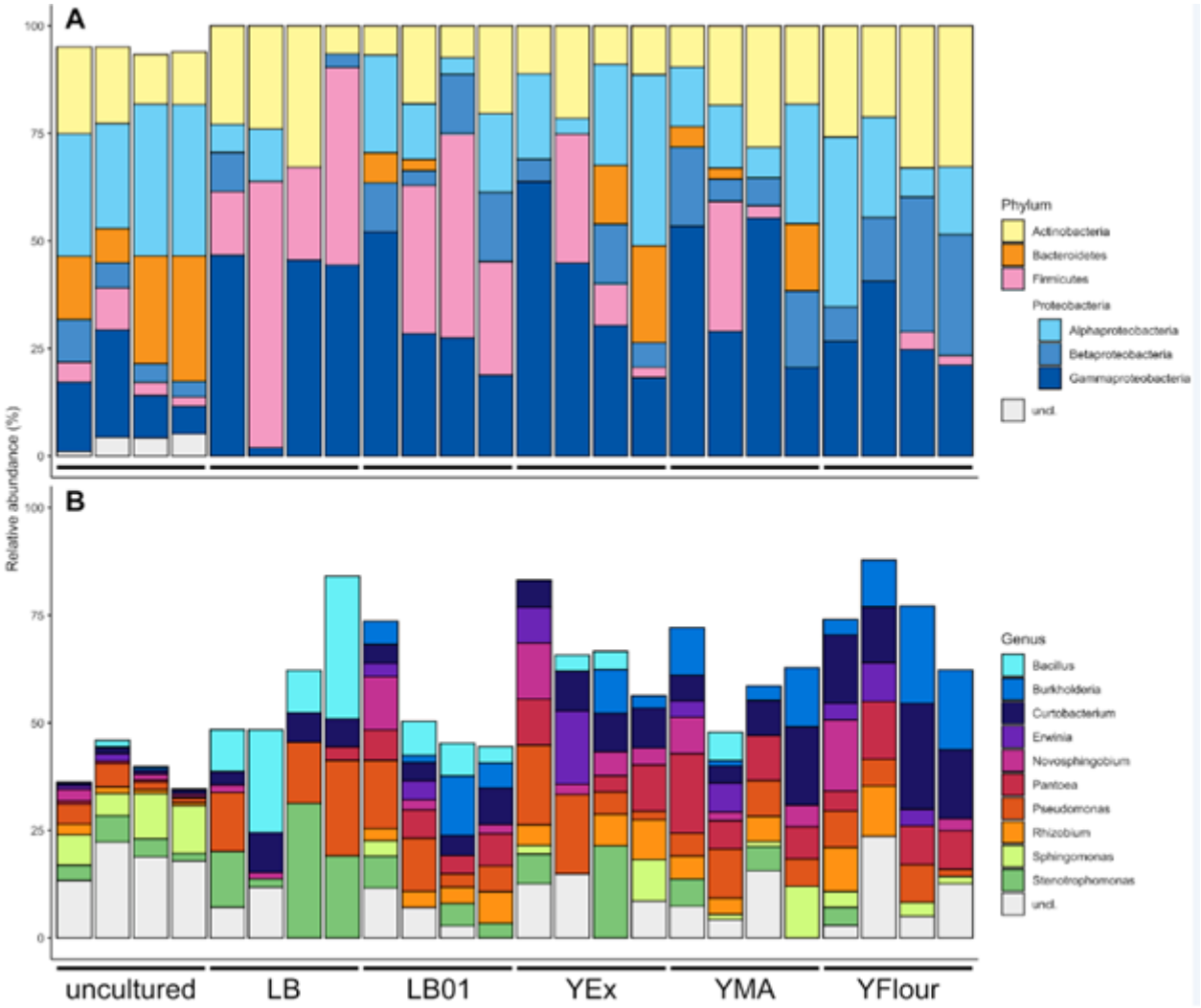

Figure 2

Taxonomical diversity of cultured phylloplane bacteria on the selected growth media and relation with the uncultured phylloplane. Relative abundances of the four major phyla Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes (A) across all samples (n = 24, 4 per growth medium and 4 uncultured phylloplane samples). The relative abundances of the top 10 genera across all growth medium samples and their relation with the uncultured bacterial phylloplane is also shown (B). uncl.: unclassified.

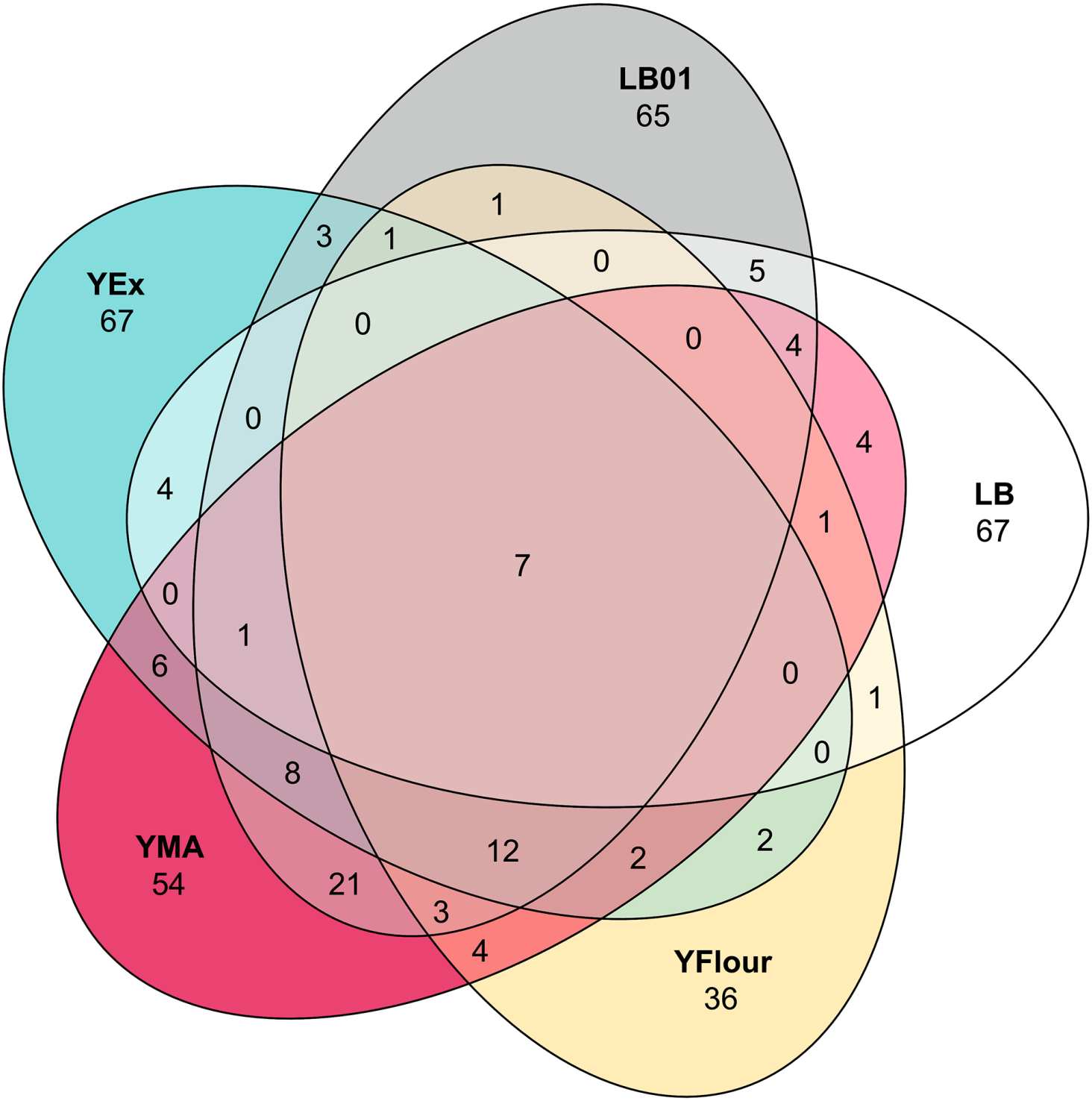

Figure 3

Venn diagram showing shared and unique amplicons sequence variants (ASVs) for the selected growth media. All 379 ASVs that were obtained by culturing are depicted according the selected growth media. The highest proportion of ASVs (76.3%) is unique for the growth media, 21.9% is shared between at least two of the growth media and only 1.8% is shared between all growth media. Note that ASV abundance is not considered in this picture.

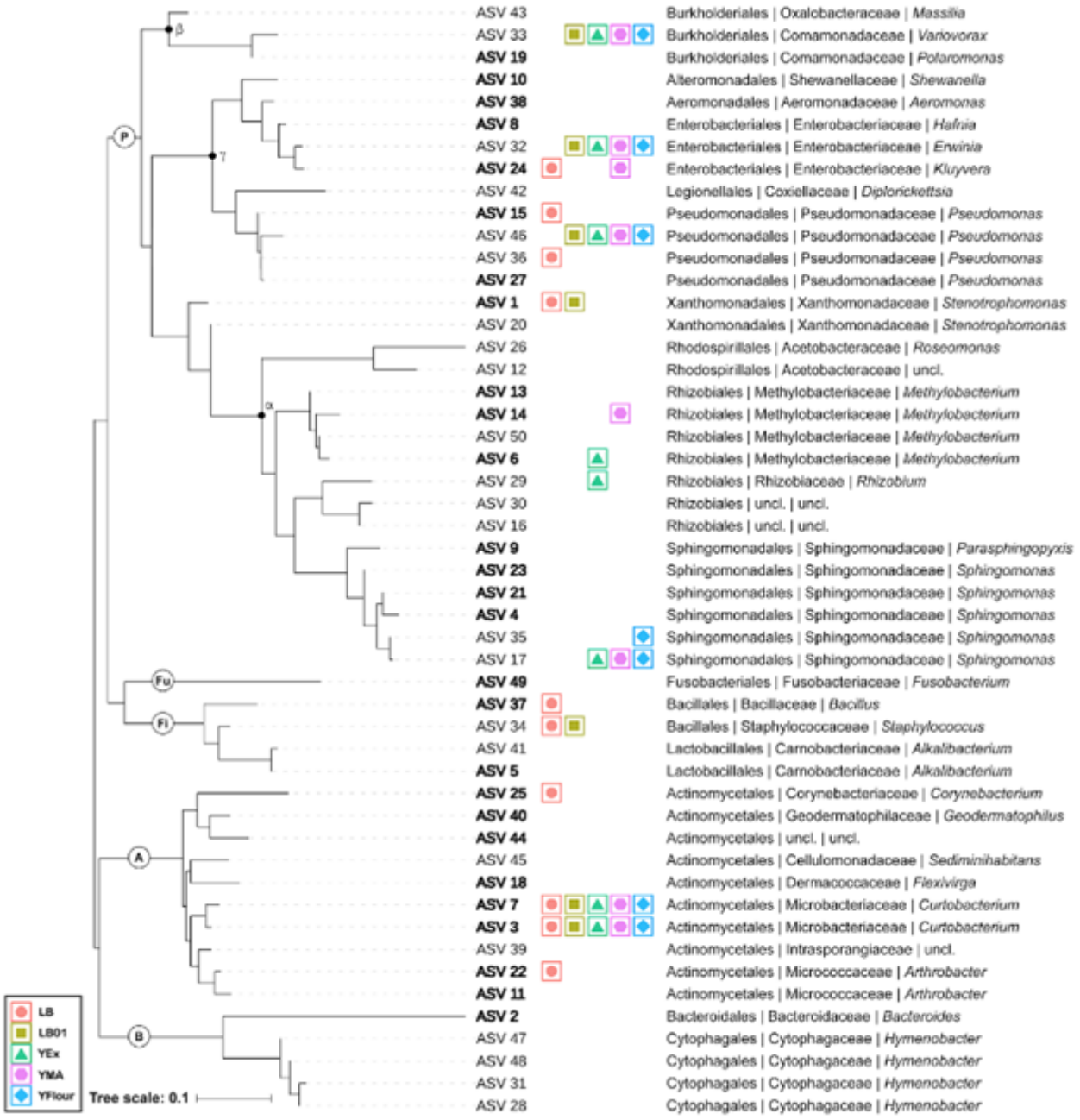

Figure 4

Most abundant amplicons sequence variants (ASVs) and relation with the selected growth media. The top 50 ASVs in uncultured bacterial phylloplane samples are given with their phylogenetic relationship and taxonomic classification (order | family | genus). ASVs depicted in bold are part of the core microbiome, here defined by presence in all phylloplane samples. For each ASV, it is indicated if culturing was successful on the selected growth media with a corresponding symbol. P: Proteobacteria, α: Alphaproteobacteria, β: Betaproteobacteria, γ: Gammaproteobacteria, A: Actinobacteria, B: Bacteroidetes, Fi: Firmicutes, Fu: Fusobacteria, uncl.: unclassified.

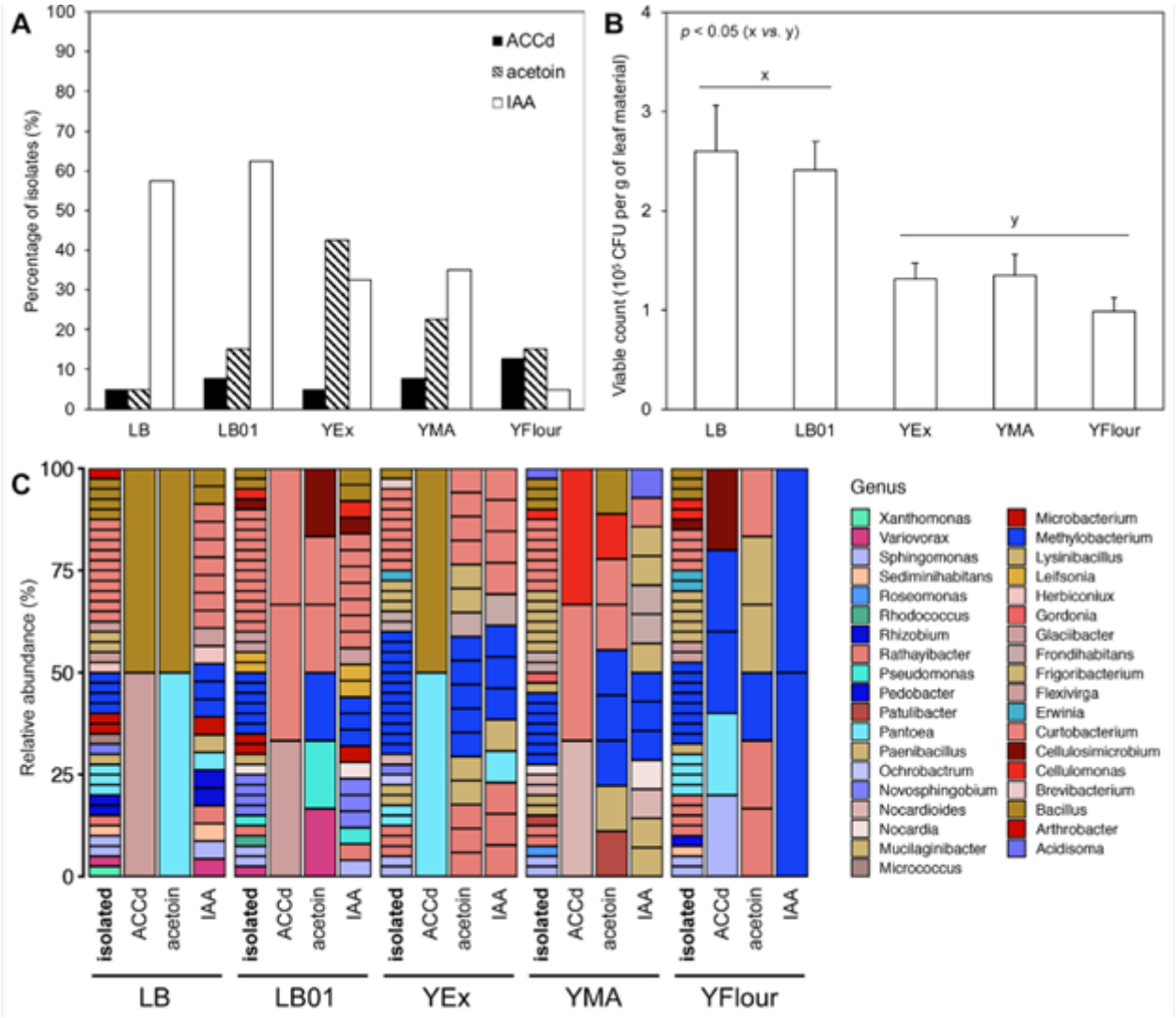

Figure 5

Plant growth promotion potential and viable count of the selected growth media. Four weeks after inoculation, the number of colony-forming units (CFU) per gram of fresh leaf material on the growth media was determined (B; n = 40, 8 per growth medium), with “x” and “y” indicating two significantly different groups ($p < 0.05$). Plant growth promotion (PGP) potential was evaluated for isolates from each growth medium (A; n = 200, 40 per growth medium). The taxonomical classification on genus level of these 200 isolates tested for PGP potential is also shown (C). ACCd: 1-aminocyclopropane-1-carboxylic acid deaminase, acetoin: 3-hydroxy-2-butanone, IAA: indole-3-acetic acid.

Supplementary Files

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