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Plant powder teabags: a novel and practical approach to resolve culturability and diversity of rhizobacteria

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We have developed teabags packed with dehydrated plant powders, without any supplements, for preparation of plant infusions necessary to develop media for culturing rhizobacteria. These bacteria are efficiently cultivated on such plant teabag culture media, with better progressive in situ recoverability compared to standard chemically synthetic culture media. Combining various plant-based culture media and incubation conditions enabled us to resolve unique denaturing gradient gel electrophoresis (DGGE) bands that were not resolved by tested standard culture media. Based on polymerase chain reaction PCR-DGGE of 16S rDNA fingerprints and sequencing, the plant teabag culture media supported higher diversity and significant increases in the richness of endo-rhizobacteria, namely Gammaproteobacteria (Enterobacteriaceae) and predominantly Alphaproteobacteria (Rhizobiaceae). This culminated in greater retrieval of the rhizobacteria taxa associated with the plant roots. We conclude that the plant teabag culture medium by itself, without any nutritional supplements, is sufficient and efficient for recovering and mirroring the complex and diverse communities of rhizobacteria. Our message to fellow microbial ecologists is: simply dehydrate your plant canopy, teabag it and soak it to prepare your culture media, with no need for any additional supplementary nutrients.

Introduction

With the development of metagenomics, it has become clear that only a small minority of microorganisms, regardless of the environment, can be cultivated in vitro, with the majority of all bacteria still remaining 'unculturable' (Amann et al. 1995, Vartoukian et al. 2010, Pham and Kim 2012). While all of the culture-independent methods have shown that many of these uncultured bacteria are abundant and metabolically active in soil, these methods are not well suited to predict the function or physiology of the cells in their environment. Also, the plant–soil–microbiome will not be fully unraveled until a larger number of the participating prokaryotes are cultured and fully characterized (Zengler 2009).

Some of the previously uncultured bacteria from natural samples can be cultivated if provided with the proper chemical and physical environment (Eilers et al. 2000). Developments over the last decade have led to the recovery of unculturables from diversely populated habitats (Vartoukian et al. 2010, Pham and Kim 2012, Stewart 2012). The use of dilute nutrient media, long-term

Abbreviations – CCM, combined carbon sources medium; CFU, colony forming unit; DGGE, denaturing gradient gel electrophoresis; PCoA, principal coordinates analysis; PCR-DGGE, polymerase chain reaction-denaturing gradient gel electrophoresis; qPCR, quantitative polymerase chain reaction; UPGMA, unweighted pair group method with arithmetic mean.

incubation, diluting the number of bacteria before their encapsulation in gel microdroplets (GMD) (Connon and Giovannoni 2002, Zengler et al. 2002, Davis et al. 2005, Ben-Dov et al. 2009, Song et al. 2009) and co-culturing (Bae et al. 2005, Nichols et al. 2008) has increased the recovery of rarely isolated strains from terrestrial and aquatic environments. Diffusion chambers (Bollmann et al. 2007, Nichols et al. 2008) and soil substrate membrane systems (Ferrari et al. 2008) have been constructed to culture 'unculturable' or rarely cultivated bacteria from marine and soil environments.

The design of culture media for growing a wide range of microorganisms is a real challenge confronting microbial ecologists, and requires comprehensive awareness of the nature and characterization of samples, including all factors that directly affect and mediate growth (Pham and Kim 2012). Culturing methods were partially improved by the use of plant-supplemented culture media, which resulted in significant cultivability for endophytic fungi and bacteria (Zhao et al. 2010, Martyniuk and Oron 2011, Arulanantham et al. 2012, Osman et al. 2012, Murphy et al. 2015). Powders of dehydrated leguminous seeds successfully replaced the beef extract in the selective culture medium of de Man, Rogosa and Sharpe (MRS) and supported better growth of probiotics (Pathak and Martirosyan 2012). Furthermore, microbial biomass and metabolites were productively recovered from culture media based on plant substrates, especially the by-products of agro-industries, including green biorefinery of brown and green juices (Ali et al. 2005, Thomsen 2005).

Our previous publications (Nour et al. 2012, Youssef et al. 2016) provided original results and evidences on the ability of crude plant slurry homogenates, juices and saps, without any supplements, to support culturability of rhizobacteria and retrieval of their in situ populations. For ease of application and practicability, this study introduces dehydrated powders of plants (*Trifolium alexandrinum* and *Paspalum vaginatum*) packed in teabags to prepare liquid infusions rich enough to cultivate endo-rhizobacteria.

We tested the ability of such plant teabag culture media to support in vitro growth of representative isolates of rhizobacteria, and to recover their in situ populations associated to plant roots. The introduced new culture media were compared with standard chemically synthetic ones, not only in quantitative terms of population densities, colony forming units (CFUs), developed on agar plates, but also with respect to their community composition and levels of genetic and taxonomic diversity. For this purpose, total DNA was extracted from all of the culture-dependent communities, i.e. CFUs developed on various agar culture media, followed by 16S rDNA gene amplification, and denaturing gradient gel electrophoresis (DGGE) fingerprinting and sequencing. Culturability of all tested culture media was evaluated by comparing to bacterial copy numbers measured by quantitative polymerase chain reaction (qPCR) of 16S rDNA determined on plant roots.

Materials and methods

Tested plants

Samples representing the whole root system together with the adjacent soil of fully grown clover (*T. alexandrinum*) and maize (*Zea mays*) plants were obtained from the experimental fields of the Faculty of Agriculture, Cairo University, Giza, Egypt.

Culture media

Plant teabag culture media

The vegetative parts of fully grown clover (*T. alexandrinum*) and mowed blades of turfgrass (*P. vaginatum*) were dehydrated in the sun for 24 h, and then oven dried at 70°C for 1–2 days. The dehydrated plant materials were mechanically ground to pass through a 2 mm sieve to obtain fine dehydrated powder. Teabags were prepared by packing 2 g of the dehydrated powder into each bag and sealing by stapling. Plant powders alone (4 g) or two teabags (each containing 2 g) were added to 1 l of distilled water to obtain the liquid plant infusion (Fig. S1I, Supporting information). Agar culture media were prepared by adding agar (2% w/v), pH adjusted to 7.0, then autoclaved for 20 min at 121°C. The teabags were left in the culture media during autoclaving for further plant extraction. Media were tested to ensure sterility before use.

Recommended standard culture media

Rich nutrient agar (Jensen 1962), soil extract agar (Parkinson et al. 1971) and N-deficient combined carbon sources medium (CCM) (Hegazi et al. 1998) were also used. Nutrient agar contains (Atlas 1979) ($g\,l^{-1}$): beef extract, 3.0; peptone, 5.0; glucose, 1.0; yeast extract, 0.5; agar, 15; pH 7.2. Soil extract agar contains ($g\,l^{-1}$): glucose, 1.0; peptone, 1.0; yeast extract, 1.0; K_2HPO_4, 1.0; soil extract, 400 ml; tap water, up to 1 l; agar, 15; pH 7.2.

N-deficient CCM comprises ($g\,l^{-1}$): glucose, 2.0; malic acid, 2.0; mannitol, 2.0; sucrose, 1.0; K_2HPO_4, 0.4; KH_2PO_4, 0.6; $MgSO_4$, 0.2; NaCl, 0.1; $MnSO_4$, 0.01; yeast extract, 0.2; fermentol (a local product of corn-steep liquor), 0.2; KOH, 1.5; $CaCl_2$, 0.02; $FeCl_3$,

0.015; Na_2 MoO_4, 0.002. In addition, $CuSO_4$, 0.08 mg; $ZnSO_4$, 0.25 mg; sodium lactate (50% v/v), 0.6 ml were added. Agar was added (15 g l^{-1}) and pH adjusted to 7.2.

Pure isolates of rhizobacteria

Eight representative pure isolates of endo-rhizobacteria (*Azotobacter chroococcum*, *Bacillus macerans*, *Bacillus circulans*, *Burkholderia cepacia*, *Enterobacter clocae*, *Enterobacter sakazakii*, *Klebsiella oxytoca* and *Pseudomonas luteola*) were obtained from the culture collection of the Department of Microbiology, Faculty of Agriculture, Cairo University, Giza, Egypt (Othman et al. 2003, 2004). They were initially inoculated into semisolid CCM culture medium, and aliquots of 100 µl from the resulting broth cultures were evenly spread on the surface of agar plates of the various tested culture media. The inoculated plates were incubated at 30°C for 2–7 days, and the resulting growth was examined visually and microscopically. The growth index recorded as follows: 1 = scant (discontinuous bacterial lawn, with scattered colonies); 2–3 = good (continuous bacterial lawn); and 4–5 = very good (continuous and denser bacterial lawn).

Total bacterial quantification using quantitative real-time PCR

Detection and quantification of bacterial 16S rDNA copy numbers were performed by quantitative real-time PCR (qRT-PCR) using the CFX96 Touch™ Detection System (Bio-Rad Inc., CA) in optical grade 96-well plates. DNA isolated from roots was diluted 1:10 (v/v) and analyzed in duplicates. The PCR reaction was performed in a total volume of 25 µl using SYBR® green master mix (Bio-Rad) containing 2 µl DNA (ca. 3–15 ng), 2.5 µl of 3.3 pmol of both the universal forward 519f (CAGCMGCCGCGGTAANWC) and reverse 907r (CCGTCAATTCMTTTRAGTT) primers (Lane 1991), and 5.5 µl PCR water. The standard curve was constructed using a 407 bp length fragment of a purified PCR product from the *Escherichia coli* 16S rDNA gene, securing 10-fold dilutions in the range of 2.5E+2 to 2.5E+7 (Fig. S2). The amplification of DNA was performed according to the thermal amplification cycling program: 3 min initial denaturing at 95°C, 40 thermal cycles of denaturation at 95°C for 15 s, annealing at 53°C for 30 s and extension at 72°C for 42 s, followed by melting curve construction by increasing the temperature from 53 to 95°C with fluorescence detection every 0.5°C to verify the PCR quality (Fig. S2). The bacterial cell numbers were obtained indirectly by assuming 3.6 is the average number of rDNA operons (Klappenbach et al. 2000, Schippers et al. 2005).

Recovery of in situ endo-rhizosphere bacterial populations

To prepare the endo-rhizosphere samples, plant roots were initially washed with tap water to remove loose soil particles. They were then surface sterilized with ethanol (70% for 30 s) and sodium hypochlorite (3% for 30 min) (Youssef et al. 2004), then washed with sterilized water before crushing in a previously autoclaved Waring blender with adequate volumes of the basal salts of CCM medium as a diluent. From this original root suspension (ca. 5 g roots in 45 ml diluent, referred to as the mother culture), further serial dilutions were prepared, and aliquots (100 µl) of suitable dilutions were surface inoculated on agar plates, with three replicates, prepared from all of the tested culture media. Incubation took place at 30°C for 2–7 days and CFUs, including microcolonies (µCo, <1 mm diameter discriminated with 40× magnification), were counted throughout. Dry weights of roots were obtained by drying the original root suspension at 70°C for 1–2 days.

Harvesting of CFUs and DNA extraction

To allow the development of as many individuals of the rhizobacteria community as possible, agar plates of tested culture media were kept incubated for 7 days. For DNA extraction, all CFUs grown on each of the representative agar plates were harvested by using 0.05 *M* NaCl. DNA was also extracted from the initial root suspension (referred to as the mother culture) originally prepared for CFU counting. Aliquots of 2–5 ml of both harvested CFUs and root suspensions were centrifuged for 10 min at 9500 *g*. Bacterial genomic DNA extraction from the resulting pellets was performed using the method adapted from the JGI protocol (William and Feil 2012). In the case of maize samples, DNA was extracted using the QIAGEN DNeasy plant mini kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions.

16S rDNA-based PCR-DGGE

First, the 16S rDNA fraction was amplified with the Bio-Rad C1000 Thermal Cycler (Bio-Rad, Hercules, CA). Bacterial DNA was used as a template, and the primers 9bfm (GAGTTTGATYHTGGCTCAG) and 1512r (ACGGHTACCTTGTTACGACTT) were used to amplify the whole 16S rDNA gene (Mühling et al. 2008). The reaction was performed in a total volume of

25 µl with 2 µl template DNA (ca. 2–18 ng μl^{-1}), 12.5 µl of QIAGEN TopTaq master mix (Qiagen Inc.), 5.5 µl PCR water and 2.5 µl of 3.3 pmol of both primers. The thermal cycling program was adjusted as follows: 4 min initial denaturation at 95°C, 30 thermal cycles of 1 min denaturation at 95°C, 1 min annealing at 56°C and 1 min of extension at 74°C; PCR was completed by a final extension step at 74°C for 10 min. QIAquick PCR Purification Kit (Qiagen Inc.) was used to purify the PCR product, according to the manufacturer's instructions. Two microliter of the purified 16S rDNA PCR product (10 ng μl^{-1}) were re-amplified using the 341f-GC (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGC ACGGGGCCTACGGGAGGCAGCAG) and 518r (ATTA CCGCGGCTGCTGG) primers (Muyzer et al. 1993, Mühling et al. 2008) for obtaining PCR products of the V3 region. PCR reaction conditions and thermal cycling programs were the same as those used for 16S rDNA amplification. PCR products of the V3 region were heated at 95°C for 5 min and stored at 65°C until loading onto the gradient gel.

DGGE analysis

This was performed using Dcode Mutation Detection System (Bio-Rad Inc., CA). PCR products were electrophoresed on 8% polyacrylamide gels containing a 30–70% denaturing gradient of formamide and urea with 1× TAE buffer. DGGE was conducted at 60°C for 20 h at 50 V. The gel was stained with SYBR® Gold Nucleic Acid Gel Stain (Life Technologies Inc., Darmstadt, Germany) and photographed with a UV gel documentation system (Biometra GmbH, Goettingen, Germany). A self-created standard fingerprint of mixed PCR products from six pure bacterial strains (*Pectobacterium carotovorum* DSM 30168, *Pseudomonas fluorescens* DSM 50090, *Listeria innocua* DSM 20649, *Arthrobacter globiformis* DSM 20124, *Lactobacillus plantarum* DSM 20174 and *Bifidobacterium breve* DSM 20213) was included in every DGGE run. All of these strains were obtained from DSMZ-Germany (https://www.dsmz.de/) and revived according to the provider's instructions.

Numerical analysis of the DGGE fingerprints

The DGGE fingerprints were analyzed using Phoretix 1D pro software. Principal coordinates analysis (PCoA) was performed using GenALEx 6.5 (provided as a Microsoft Excel add-in) (Peakall and Smouse 2012). The total number of DGGE bands was used to represent 16S rDNA richness. The diversity indices, Shannon index (H′) and Simpson's index (D) were calculated using PAST software v 3.0 (Hammer et al. 2001).

Sequencing of DGGE bands

Dominant DGGE bands were cut off with sterile 200 µl tips and eluted in 10 µl of PCR water at 4°C overnight. The supernatants were used as template for PCR re-amplification. Re-amplification of DNA fragments was performed using bacterial primers 314f without the GC clamp and 518r. Amplification was verified by electrophoresis on 1.5% agarose gels. The PCR products were purified using QIAGEN PCR purification kit (Qiagen Inc.) and sequenced by the Sanger method (Eurofins MWG Operon, Ebersberg, Germany).

Sequence analysis

16S rDNA sequences were compared with their closest matches in both the GenBank (www.ncbi.nlm.nih.gov/BLAST) and ribosomal database project (www.rdp.cme.msu.edu) to obtain the nearest phylogenetic neighbors. Sequence alignments and phylogenetic trees were constructed by ClustalW1 (www.genome.jp/tools/clustalw) using the unweighted pair group method with arithmetic mean (UPGMA) method (Thompson et al. 1994). The 16S rDNA sequences identified in this study have been deposited in the GenBank database under the accession numbers KT123284 to KT123298 and KT304798 to KT304809.

Statistical analysis

Statistical analysis was carried out using STATISTICA v10 (StatSoft Inc., 2011).

Results

The tested plant dehydrated powders are nutritionally rich enough

Chemical analyses of the tested dehydrated plant powders indicated their richness in carbohydrates, proteins, fibers, macro- and micronutrients, amino acids and vitamins (Table S1). Such nutrients of natural origin and concentrations are comparable to the nutrient matrix in the root environment, governed by the dynamic flux of plant root exudates.

Plant teabag culture media increased the culturability of rhizobacteria

Preliminary experiments demonstrated that representative pure strains of the genera *Azotobacter* spp., *Bacillus* spp., *Burkholderia* spp., *Enterobacter* spp., *Klebsiella* spp. and *Pseudomonas* spp., developed very well on agar plates of the tested plant teabag culture media. Their

growth indices were highly comparable to the standard chemically synthetic culture media (nutrient agar and CCM) (data not shown).

The tested plant teabag culture media supported the development of well-defined and distinct macro- and microcolonies of endo-rhizobacteria associated with the clover roots (Fig. S1I). In contrast, colonies grown on standard nutrient agar and soil extract agar were undefined, slimy and spread over the agar (Fig. S1II). Such spreading nature of colony growth may camouflage the underlined microcolonies resulting in obscuring CFU quantitative evaluation. Statistical analysis indicated significant differences ($P < 0.05$) attributed to the single effects of culture media and incubation time, as well as two-way interaction (culture media × incubation time). Longer incubation resulted in further development of microcolonies, particularly on plant teabag culture media, that culminated in the highest CFUs recovery (Table 1).

The bacterial 16S rDNA gene copy numbers per gram of dry clover root were determined by qPCR (Fig. S2), and the mean log of calculated bacterial cell numbers obtained for four replicates was 9.35 ± 0.067. With longer incubation, the culture-dependent method, in terms of CFUs numbers developed on agar plates, represented 16–35% of qPCR bacterial cell numbers; the highest was on plant teabag culture media (28–35%) compared with standard nutrient agar (18%), soil extract (25%) and CCM (16%) (Table 1). Bearing in mind the limitations of primers used (http://dnaresearch.oxfordjournals.org/content/early/2013/11/25/dnares.dst052.full.pdf+html), results obtained indicated the ability of the plant teabag culture media to significantly increase the relative culturability of in situ endo-rhizobacteria populations.

DGGE analysis revealed higher diversity of endo-rhizobacteria developed on plant teabag culture media

DGGE fingerprinting of the 16S rDNA, recovered from total bacterial colonies grown on different agar culture media, was used to characterize the composition of endo-rhizobacteria communities associated with clover roots. A specific comparison was carried out among populations developed on plant teabag culture media and the commonly used standard nutrient agar and soil extract agar (Fig. 1 and Fig. S3). DGGE analysis resulted in clear banding patterns of sufficient complexity (11–24 major bands) and reproducibility to investigate differences in bacterial communities among the different culture media. Based on the analysis of distance scores by Phoretix 1D pro software, the constructed dendrogram (Fig. 1) distinguished two clusters at a similarity level of 58%. The first cluster contained the band profiles of the standard nutrient agar medium, while the second one included all those of the plant-based culture media prepared from either plant powders or teabags. At a higher similarity level (87%), subclustering occurred that was attributed to the type of plant powder (clover or grass) and its preparation (powders or teabags). Taking into consideration the DGGE profile of soil extract agar population, distinguished clustering was also shown (Fig. S3). While DGGE banding profiles of CCM were solitary separated at 62% similarity level, soil extract agar profiles joined those of the plant-based culture media, but later separated at a level of >80%.

For endo-rhizobacteria associated with maize roots, the UPGMA cluster analysis illustrated, at a similarity level of 0.40, the remoteness of the nutrient agar cluster away from those of both root samples and plant teabag culture media (Fig. 2A). This is a further indication of the ability of plant teabag culture media to correctly recover the in situ population of endo-rhizobacteria associated with root samples, being clustered with the plant roots and distant from the nutrient agar. Furthermore, PCoA of bacterial DGGE banding profiles (Fig. 2B) showed clear separation of three main groups along the PCoA-1 axis, which reflects 35.4% variation, representing plant roots, plant teabag culture media and standard nutrient agar. Whereas the PCoA-2 coordinates, which reflect 25.3% variation, showed two main groups along the axis, the group of plant teabag culture media joined with the plant roots, away from the nutrient agar group.

Based on the number and pattern of DGGE bands of maize endo-rhizobacteria, diversity indices were computed to bacterial communities recovered from all tested culture media compared with those found on plant roots. The values of the richness (S) as well as the Shannon–Wiener index (H′) were positively related to the diversity of the endo-rhizobacteria community, while the Simpson's dominance index (D) was negatively related (Table 2). The highest levels of diversity were reported for the maize roots [20 of Richness and 2.968 of Shannon–Wiener indices (H′)] followed by the plant teabag culture media (14–15 and 2.662–2.685), and the lowest for the nutrient agar (11 and 2.398). On the contrary was the order of Simpson's dominance indices (D), where nutrient agar exhibited the highest indices (0.0909) compared with either roots (0.0520) or plant teabag culture media (0.0683–0.0698). Furthermore, non-paired heteroscedastic Student's *t*-test showed highly significant levels ($P = 3.178E\text{-}08$) between the

Table 1. Log numbers of CFUs [data are log means ± standard error (SE), n = 3] including microcolonies (μCo, < 1 mm diameter, discriminated with 40× magnification) of culturable rhizobacteria developed on various culture media, and bacterial quantification using real-time PCR of clover roots. The mean value of qPCR cell numbers (obtained indirectly assuming that the average 16S rDNA copy number per cell is 3.6) is log 9.35 ± 0.067 g^{-1} root dry weight obtained for four replicates. Statistical significant differences are indicated by different letters (*P* value ≤ 0.05, n = 3).

Culture media	Log culturable CFUs at 36 h incubation (% of culturability)	Log culturable CFUs at 84 h incubation (% of culturability)	Log microcolonies CFUs at 84 h incubation (% of culturability)	% of microcolonies of total CFUs at 84 h incubation
Nutrient agar	7.71 ± 0.013 (2)[a]	8.61 ± 0.072 (18)[b]	7.75 ± 0.121 (2)	14
Soil extract	7.98 ± 0.030 (4)[c]	8.75 ± 0.027 (25)[d]	7.94 ± 0.42 (4)	16
CCM	7.55 ± 0.012 (2)[ef]	8.56 ± 0.034 (16)[b]	8.05 ± 0.027 (5)	31
Grass powder	7.87 ± 0.027 (3)[c]	8.91 ± 0.029 (35)[gh]	8.54 ± 0.116 (15)	43
Clover powder	7.55 ± 0.020 (2)[ef]	8.80 ± 0.025 (28)[gh]	8.69 ± 0.095 (21)	77
Grass teabags	7.65 ± 0.018 (2)[ae]	8.86 ± 0.022 (32)[gh]	8.36 ± 0.023 (10)	32
Clover teabags	7.46 ± 0.072 (1)[f]	8.80 ± 0.038 (28)[g]	8.67 ± 0.129 (20)	73

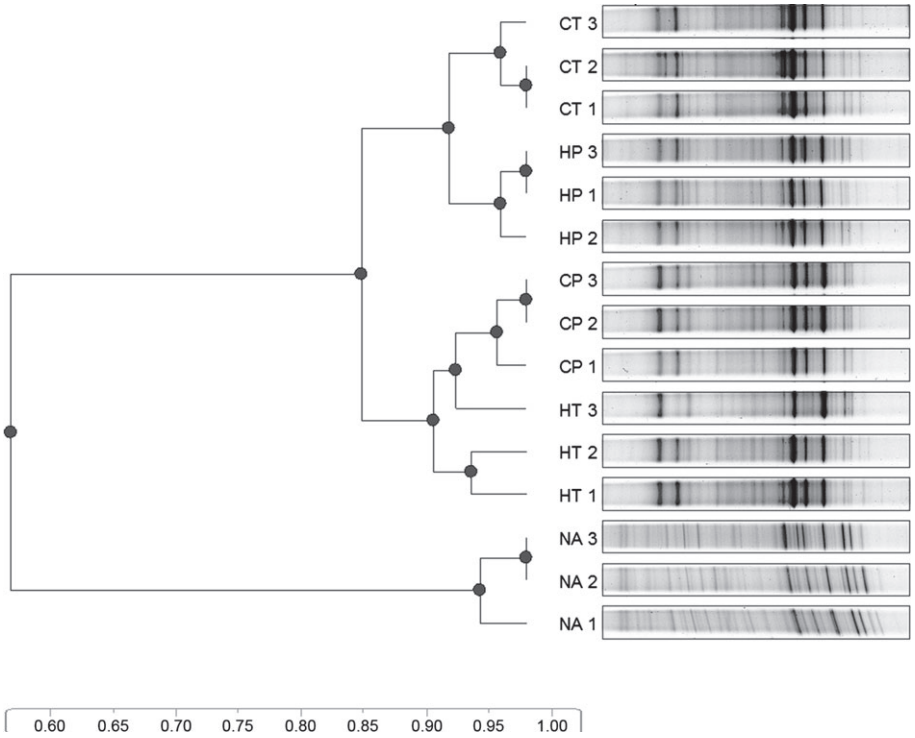

Fig. 1. DGGE bands obtained for harvested CFUs of clover rhizobacteria developed on all tested culture media; UPGMA cluster analysis based on Euclidean distances of DGGE lanes. Each lane represents the CFUs harvested from one agar plate, and each culture medium is represented by three replicates (plates 1–3). NA, nutrient agar; HT, grass teabags; CP, clover powder; HP, grass powder; CT, clover teabags.

beta diversity distances of nutrient agar vs maize roots, and plant teabag culture media vs maize roots (Fig. 3).

16S rDNA band sequencing displayed more taxa richness on plant teabag culture media than on standard nutrient agar

DGGE bands (OTUs) obtained for endo-rhizobacteria developed on nutrient agar as well as clover teabags were subjected to sequencing (Fig. 4). As far as the 16S rDNA-V3 region is concerned, DGGE band patterns resolved for both nutrient agar and clover teabags were carefully examined to pinpoint those easily distinguishable and obtainable for re-amplification. From a total of 29 bands obtained, two (En2 and En26) were short enough sequences to be well classified. The remaining 27 bands were successfully sequenced; 9 were found common to both culture media, while 11 and 7 bands were confined to clover teabags and nutrient agar, respectively (Fig. 4A, C). A phylogenetic tree was

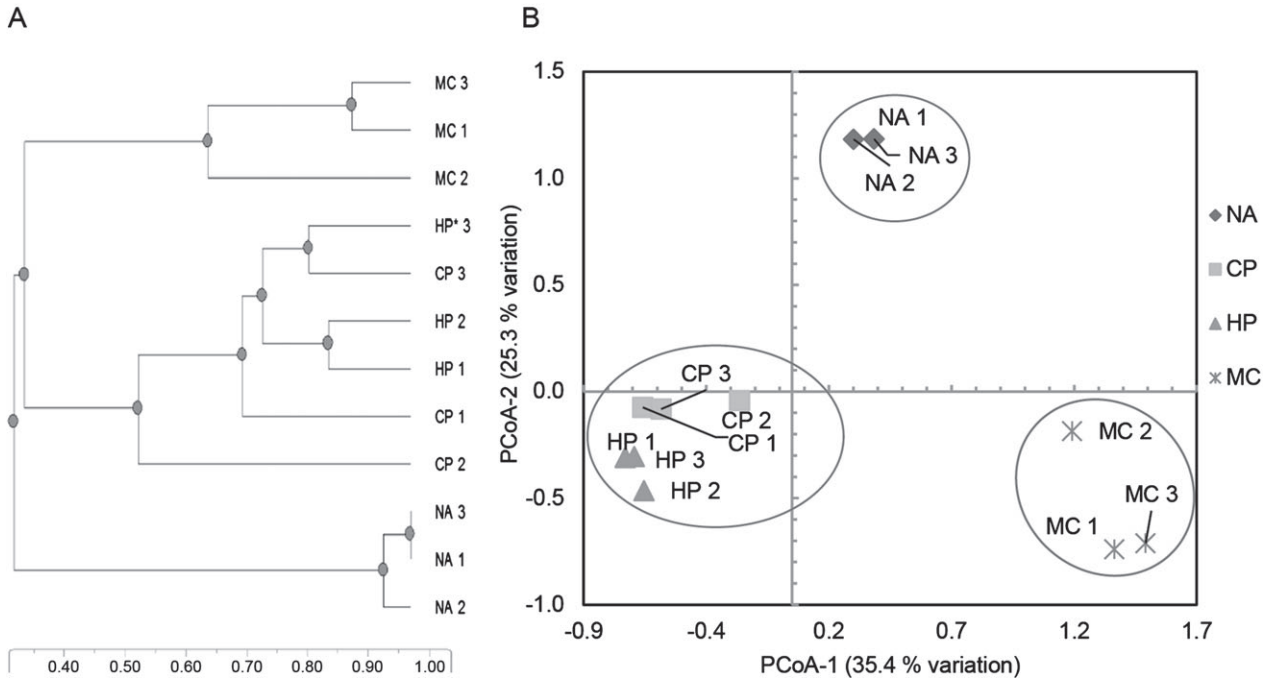

Fig. 2. PCR-DGGE cluster analyses (based on Euclidean distance metrics) of endo-rhizobacteria communities associated with maize roots and CFUs harvested from different culture media agar plates. (A) UPGMA phylogenetic cluster analysis; (B) PCoA generated from unweighted DGGE banding data, with triplicate samples. NA, nutrient agar; CP, clover teabags; HP, grass teabags; MC, maize roots.

Table 2. Richness (S), Simpson's dominance (D) and Shannon–Wiener (H′) diversity indices of maize endo-rhizobacteria populations associated with maize roots and those cultured on various culture media. Means ± standard error (SE), n = 3.

Media	Richness (S)	Dominance (D)	Shannon (H′)
Nutrient agar	11	0.0909 ± 0.0000	2.398 ± 0.000
Clover teabags	14	0.0698 ± 0.0016	2.662 ± 0.023
Grass teabags	15	0.0683 ± 0.0016	2.685 ± 0.023
Maize roots	20	0.0520 ± 0.0056	2.968 ± 0.105

constructed (Fig. 4B). According to the identification results (Table S2), all of the OTUs recovered on standard nutrient agar were confined to the class of Gammaproteobacteria, family Enterobacteriaceae, except for one OTU (En28), which belonged to the class of Alphaproteobacteria, family Rhizobiaceae. In contrast, OTUs obtained from clover teabags represent the class of Gammaproteobacteria, family Enterobacteriaceae, and predominantly the class of Alphaproteobacteria, family Rhizobiaceae. The phylogenetic analysis indicated that the unique bands of the clover teabag culture media occupied a distinct cluster apart of both the nutrient agar unique bands and those common to both culture media (Fig. 4B).

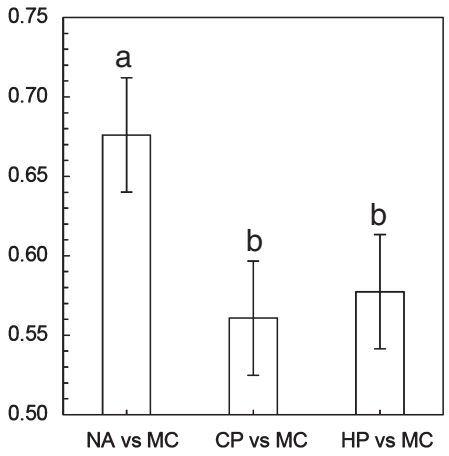

Fig. 3. Average values of beta diversity distances comparing maize plant roots (MC) with tested culture media (CP, clover teabags; HP, grass teabags; NA, nutrient agar). Statistically significant differences are indicated by different letters (P value ≤ 0.05).

Discussion

Molecular tools of microbial ecology have discovered more than 85 novel bacterial phyla since 1987; most of which have not yet been cultured, nor even one representative isolate or single genome sequence recovered (Achtman and Wagner 2008). Therefore, the

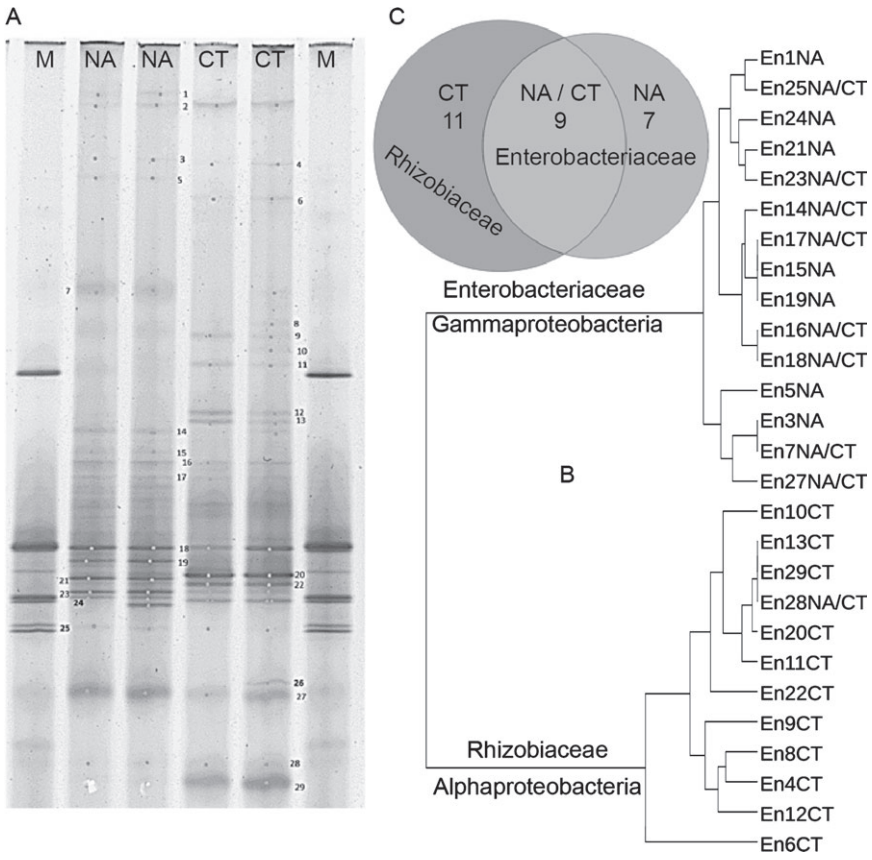

Fig. 4. Culture-dependent DGGE analyses of endo-rhizobacteria associated with clover roots. (A) DGGE profile of culturable clover endo-rhizobacteria communities (En) developed on nutrient agar (NA) and clover teabags culture media (CT); (B) UPGMA cluster analysis based on V3-16S rDNA sequences analysis from selected common and unique bands; (C) Venn diagram representing the common and different bands among the two tested culture media of NA and clover teabags.

culturable community does not represent the total phylogenetic diversity, and the huge biotechnological potential of such unexplored populations is still concealed within unculturable populations. Clearly there is a compelling need to secure culturability of new bacterial entities that play significant environmental roles (Pham and Kim 2012, Doty et al. 2013).

Consistent with our previous findings, the tested plant teabag culture media remarkably supported recovery of in situ endo-rhizobacteria associated with roots of clover and maize (Nour et al. 2012, Youssef et al. 2016). The significant increase in culturability was most likely related to the development of microcolonies with prolonged incubation time, representing 32–77% of the total CFUs developed on plant teabags compared with 14–31% on standard chemically synthetic agar media. Counts of microcolonies were really higher (2–5-fold) when using homologous plant dehydrated powders.

The nutrient complexity and diversity provided by the plant teabag culture media will probably stimulate growth of physio-nutritional groups other than the viable and culturable communities, e.g. non-replicating, starving and dormant physiological groups (Davis 2014). The development of microcolonies has also been significantly enhanced by other new methods for culturing uncultured bacterial population, e.g. the use of overlay agar techniques for plating (Nour et al. 2012), diffusion chamber-based techniques, encapsulation of cells in gel microdroplets and soil slurry membrane systems. Several of the microcolonies developed by such techniques, although determined as unculturable bacteria, have indeed been isolated in pure cultures (Zengler et al. 2002, Ferrari et al. 2005, Stewart 2012).

Taking into consideration the limitations of DGGE fingerprinting of the 16S rDNA (Muyzer et al. 1993), the technique is still of practical use to study microbial community structure and diversity (Hu et al. 2015, Peng et al. 2015). Therefore we used culture-dependent DGGE fingerprinting of the 16S rDNA (Edenborn and Sexstone 2007) to characterize the bacterial

communities, CFUs, developed on agar plates representing all of the tested culture media. Clover teabags supported the recovery of both Gammaproteobacteria (family Enterobacteriaceae) and Alphaproteobacteria (family Rhizobiaceae). It is reported (Hardoim et al. 2015) that most of the prokaryotic endophyte 16S rDNA sequences in the GenBank database belong to the Gammaproteobacteria group (26%), followed by the Alphaproteobacteria group (18%). In contrast, the standard nutrient agar enriched the class of Gammaproteobacteria. This agrees with the findings of Pereira et al. (2011) who failed to isolate any Alphaproteobacteria members from maize roots using standard nutrient agar medium.

It appeared that the nutrient multiplicity provided by the plant teabag culture media most likely supports the diversity of endo-rhizobacteria by increasing the abundance of available food resources, which is expressed by alpha and beta diversity (Whittaker 1960, 1972, Tuomisto 2010). The plant teabag culture media supported higher alpha diversity. This is similar to Lutton et al. (2013) who reported higher alpha diversity values for culture media from natural sources containing a more natural nutrients balance (soil extract agar) than the standard nutrient agar, as such or even diluted (1% nutrient agar). With respect to beta diversity, the highest, and most significant distances from maize roots were reported for the standard nutrient agar, and the lowest for the plant teabag culture media. This provides further evidence that endo-rhizobacteria populations cultured on the plant teabag culture media are closely correlated to the in situ endo-rhizobacteria populations on maize roots.

The above findings are currently being examined and evaluated by G3 PhyloChip microarray (unpublished data, manuscript in preparation). It is observed that on maize roots the top nine classes represented were Gammaproteobacteria, Actinobacteria, Alphaproteobacteria, Bacilli, Betaproteobacteria, Bacteroidia, Clostridia, Planctomycetia and Flavobacteriia. On average, Gammaproteobacteria and Alphaproteobacteria comprised the largest proportion, i.e. 13.8 and 5.7% of the overall Hybscores, respectively. Plant teabag culture media gave comparable respective percentages of 16.4 and 4.3. In contrast, the nutrient agar further enriched Gammaproteobacteria (20.7%) and down-graded Alphaproteobacteria (1.7%). The predominance of Rhizobiales followed the descending order of 9.9% on plant roots, 5.6% on clover teabags and 2.6% on nutrient agar culture media.

In conclusion, plant teabag culture media, without any amendment, are sufficient and efficient for culturing rhizobacteria, and progressively recovering their in situ populations. We were able to resolve unique DGGE bands that were not detected after culturing on standard culture media. Results of 16S rDNA-DGGE fingerprints and diversity indices confirmed that plant teabag culture media supported higher diversity as well as significant increases in the richness of endo-rhizobacteria. Our findings recommend the wide application of this novel approach, and potentially open up new horizons for culturing unculturable bacteria. Concomitantly, this highlights the need to revise long-established information on the ecology of rhizobacteria that has resulted from the sole use of chemically synthetic culture media. The new methodology, currently under patent procedure (Egyptian patent office – No. 506/2015; http://www.egpto.eg.wipo.net/), is considered not only as a methodological breakthrough to explore the plant–soil–microbiome, but is also ideal for 'low tech' laboratories.

Authors' contributions

N. A. H introduced the idea. N. A. H, M. S. S and S. R. developed the experimental design. M. S. S. performed the PCR, qPCR, DGGE experiments. M. S. S. and A. C. S. carried out the result analyses. M. T. performed the chemical analyses of plant powders. M. S. S., M. A. H., H. H. Y. and E. F. M. carried out the in vitro and in situ CFUs counting of rhizobacteria. M. S. S., M. F. and N. A. H. wrote the manuscript. All the authors reviewed and approved the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Chemical analyses of the dehydrated powders of clover (*Trifolium alexandrinum*) and turfgrass (*Paspalum vaginatum*).

Table S2. Results of DGGE band sequencing, accession numbers and their phylogenetic affiliation.

Fig. S1. Development of rhizobacteria on culture media prepared from plant teabags and standard culture media.

Fig. S2. Quantitative real-time PCR for quantification of endo-rhizobacteria associated with clover roots.

Fig. S3. UPGMA cluster analysis of DGGE bands obtained for culturable rhizobacteria of clover on various tested culture media.

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