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Consistent associations with beneficial bacteria in the seed endosphere of barley (*Hordeum vulgare* L.)

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ABSTRACT

The importance of the plant microbiome for host fitness has led to the concept of the "plant holobiont". Seeds are reservoirs and vectors for beneficial microbes, which are very intimate partners of higher plants with the potential to connect plant generations. In this study, the endophytic seed microbiota of numerous barley samples, representing different cultivars, geographical sites and harvest years, was investigated. Cultivation-dependent and -independent analyses, microscopy, functional plate assays, greenhouse assays and functional prediction were used, with the aim of assessing the composition, stability and function of the barley seed endophytic bacterial microbiota. Associations were consistently detected in the seed endosphere with *Paenibacillus*, *Pantoea* and *Pseudomonas* spp., which were able to colonize the root with a notable rhizocompetence after seed germination. In greenhouse assays, enrichment with these bacteria promoted barley growth, improved mineral nutrition and induced resistance against the fungal pathogen *Blumeria graminis*. We demonstrated here that barley, an important crop plant, was consistently associated with beneficial bacteria inside the seeds. The results have relevant implications for plant microbiome ecology and for the holobiont concept, as well as opening up new possibilities for research and application of seed endophytes as bioinoculants in sustainable agriculture.

Introduction

From the microbiological point of view, a plant is a heterogeneous mosaic of microhabitats harboring specific microbiomes, which play fundamental roles in host fitness [6,25,37,46]. These unique microbiomes are integrated together with the host, thus forming the so-called holobiont [48,51]. The seed microhabitat has received attention recently for its potential as a reservoir and vector for beneficial microbes, although it remains one of the less-investigated plant microhabitats [27]. Seed endophytes, mainly belonging to *Proteobacteria* and *Firmicutes*, were detected in surface-sterilized seeds of various plants, including important crops such as legumes and cereals (reviewed by Truyens et al. [45]), and they showed biocontrol and plant growth promotion (PGP) activities [3,12,16,19,21,49]. Seed-associated microbes should be regarded as very intimate microbial partners of higher plants, with the potential to connect successive plant generations [18,35].

In this study, the seed endophytic microbiota of barley (*Hordeum vulgare* L.) was investigated with the aim of assessing the composition, stability and function of the bacterial seed endophytes. The hypothesis was that specific and beneficial plant-microbe interactions would result in stable associations in the context of the plant genetic variability, and at the spatial and temporal scale investigated. In fact, as a general concept for higher plants, those individuals able to store beneficial bacteria in their seeds could have the possibility of transmitting them to the next generations, which could then already profit from their presence in the early growth stages. Eventually, these beneficial associations might be turned into holobiont traits.

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Table 1
Barley (*Hordeum vulgare* L.) seed samples analysed in this study.

Sample ID	Cultivar	Provided by	Abbreviation used in the text	Geographical origin	Harvest year
Morex_pb	Morex	Plant Breeding Institute (JLU–Giessen)	Mx_pb	Giessen (Germany)	2012
Propino_s	Propino	Syngenta Company	P_s	Unknown location (Germany)	2013
Propino_s2	Propino	Syngenta Company	P_s2	Ziesendorf (Germany)	2016
Overture_cs	Overture	Crop Science Institute (JLU–Giessen)	O_cs	Giessen (Germany)	2015
Montoya_cs	Montoya	Crop Science Institute (JLU–Giessen)	M_cs	Giessen (Germany)	2015
Overtue_li	Overture	Limagrain Company	O_li	Rosenthal bei Peine (Germany)	2016
Montoya_ac	Montoya	Ackermann Company	M_ac	Niederbayern (Germany)	2016

In the framework of this study, a large spectrum of methods, including isolation, high-throughput sequencing, microscopy, PGP assay, biocontrol assay and functional prediction were applied to seven barley seed samples (Supplementary Fig. S1) that represented different cultivars, geographical sites and collection years. With this multifaceted approach of complementary methods, we intended to show the existence of stable and beneficial bacterial associations in the seed endosphere of the barley holobiont.

Materials and methods

Seed samples

Barley seeds of the cultivars Propino, Morex, Overture and Montoya were collected from different locations in Germany in different years (2012–2016) by different providers, and were kindly donated to our laboratory. In total, seven different samples were analysed, according to the combination of cultivar, origin and year (Table 1). Each sample consisted of a seed batch, stored in an individual paper bag at 4 °C until analysis.

Isolation and identification of barley seed endophytes

One gram of each barley seed sample (composed of 18–22 seeds) was surface-sterilized by immersion for two hours in a 1:1 mixture of commercial hypochlorite (approximately 2.5% ClO^-) and a sterile solution containing 1 g Na_2CO_3, 30 g NaCl and 1.5 g NaOH, per liter of distilled water [20], at room temperature and with hand-shaking five times during incubation. Then, the sterilisation solution was removed and the seeds were washed five times, 30 min each, in sterile distilled water, at 25 °C with shaking at 100 rpm, to remove the hypochlorite completely. The surface-sterilized seeds were crushed with a sterile mortar and pestle in 10 mL filter-sterilized 0.18% sodium pyrophosphate ($Na_2H_2P_2O_7$). Dilutions 10^0–10^{-3} were plated onto both AC and CASO solid media (Carl Roth GmbH + Co., Karlsruhe, Germany) and incubated at 25 °C.

The polymorphism of the 16S–23S intergenic transcribed spacers (ITS) was used to group the isolates into phylotypes ("ITS groups"; Supplementary Fig. S2). This DNA region is hypervariable and regarded as strain-specific [10,13,47], thus the ITS groups are expected to represent either one single strain or closely related strains of the same species. One or more representative isolates of each ITS group (see Table 2) were identified by sequencing the 16S rRNA gene. Three isolates of ITS group 2 (belonging to the genus *Pantoea*) were additionally characterized by phylogenetic analysis of the *gyrB* gene, using the newly designed primer set gyrB-for (5′-AAGTGCATCAGCAGATTTACGTCCA-3′)/gyrB-rev (5′-TCACGGGCACGGGCA-3′). More details are provided in the Supplementary Methods.

Cultivation-independent analysis of barley seed endophytes

Total DNA was extracted from ~0.5 g of surface-sterilized ground seeds (six seed samples) and from three replicates of non-sterilized seeds (sample Morex_pb), according to Bürgmann et al. [8] with modifications. Three independent runs were performed in order to obtain a sufficient number of sequences, and an independent DNA extraction was performed for each sample replicate. Amplicon libraries of the hypervariable regions (V4 + V5) of the 16S rRNA, obtained with universal bacterial primers, were sequenced with the Ion Torrent technique. The Ion Torrent data were analysed with QIIME version 1.9 [9] and the SILVA123 reference database. For each seed sample, the sequences obtained from the different replicates were combined, which gave one representative sequence pool per seed sample. More details are provided in the Supplementary Methods.

Plate functional assays

Representative isolates of ITS groups 1, 2, 3, 4 and 11 were evaluated for the following growth promoting traits: growth on nitrogen-free medium, using a modified NFB medium [23]; inorganic phosphate solubilisation, using $AlPO_4$ (AP), $Ca_3O_8P_2$ (CP) and $FePO_4$ (FP) as substrates, according to Bashan et al. [5]; organic phosphate solubilisation using inositol hexaphosphate (IHP) agar medium [33]; siderophore production, using liquid King's medium B [36] and 2 mM chrome azurol S (CAS) solution [2,38]; ACC-deaminase production, using DF medium amended with 1-aminocyclopropane-1-carboxylate (ACC) as the only nitrogen source [38]; potassim solubilisation, using Aleksandrov medium [1] amended with mica powder [29]; and indole acetic acid (IAA) production (quantitative), according to Bric et al. [7], using the Salkowski reagent [17]. Bacterial tolerance was tested on AC medium adjusted with 2.5%, 5% or 7.5% (w/v) NaCl (salt stress), and with 10 or 20% polyethylene glycol (PEG; drought stress). Heat and cold stress were tested at 45 °C and 4 °C, respectively. More details are provided in the Supplementary Methods.

Plant growth promotion assay

The isolate P_s_AC_13b, which was representative of the dominant ITS group 2 (*Pantoea agglomerans*-related), was tested for plant growth-promotion activity on barley (cultivar Propino) in the greenhouse. A full-factorial experimental design was employed, which included three factors: substrate (two levels), substrate sterilization (two levels) and bacterial inoculation (two levels), thus resulting in eight treatments (Supplementary Fig. S3). The two substrates used were the nutrient rich Einheitserde, a fertilized peat-clay soil (type "Classic Tonsubstrat ED73"; Einheitserde-und Humuswerke Gebr. Patzer GmbH Co. KG, Sinntal-Altengronau, Germany) and the nutrient poor "Unterboden" (a manually collected B-horizon soil [42], mixed 1:1 with perlite) (Supplementary Table S1). Surface-sterilized seeds were inoculated by incubation for 1/2 h in a 0.03 M $MgSO_4$ bacterial suspension containing $\sim 5 \times 10^7$ CFU mL^{-1}. Uninoculated seeds were incubated in sterile 0.03 M $MgSO_4$. (Supplementary Fig. S3). Height, chlorophyll content, fresh weight, dry weight, water content and element concentrations were used as plant growth and quality parameters. More details are provided in the Supplementary Methods.

Table 2
Frequency and tentative identification of the phylotypes (ITS groups) isolated from the barley seed endosphere. P_s: cultivar Propino from Syngenta, harvest year 2013; P_s2: cultivar Propino from Syngenta, harvest year 2016; Mx_pb: cultivar Morex from the Institute of Plant Breeding (Giessen); M_ac: cultivar Montoya from Ackermann; M_cs: cultivar Montoya from the Institute of Crop Sciences (Giessen); O_li: cultivar Overture from Limagrain; O_cs: cultivar Overture from the Institute of Crop Sciences (Giessen).

ITS group	Barley seed sample							No. of sequenced isolates (size of the longest sequence)	Best BLAST hit(s) of the longest sequence	Acc. no.	Similarity (%)	Best EZ-Taxon hit of the longest sequence	Acc. no.	Similarity (%)
	P_s	P_s2	Mx_pb	O_li	M_ac	M_cs	O_cs							
1	5	1	1	2				3 (1392)	*Paenibacillus nicotianae* 3Cp1	NR_134783	98.7	*Paenibacillus kyungheensis* DCY88	KF793934	99.1
2	11	1	2	1	6			4 (1333)	*Pantoea agglomerans* (vs[a])		100	*Pantoea vagans* LMG 24199	EF688012	99.8
3	9		2					2 (1341)	*Plantibacter flavus* (vs)		100	*Plantibacter flavus* VKM Ac 2504	jgi.1118344	100
4	4			1		1		1 (1371)	*Bacillus cereus* (vs)		100	*Bacillus cereus* ATCC 14579	AE016877	100
5	2						1	1 (1310)	*Chryseobacterium indoltheticum* (vs)		100	*Chryseobacterium indoltheticum* DSM 16778	jgi.1096611	100
6	1		1					1 (1363)	*Rhodococcus fascians* PDD-29b-2	HQ256815	99.7	*Rhodococcus fascians* LMG 3623	JMEN01000010	99.6
7	1			1		1	1	3 (1371)	*Staphylococcus haemoliticus* (vs)		99.9	*Staphylococcus haemolyticus* MTCC 3383	LILF01000056	99.9
8	1			1				1 (1364)	*Bacillus safensis* (vs) *Bacillus pumilus (vs)*		100	*Bacillus safensis* FO 36b	ASJD01000027	100
9	1							1 (1383)	*Xanthomonadaceae* bacterium PDD-60b-2	KR922181	99.9	*Luteimonas terrae* THG MD21	KJ769177	99.6
10				1				1 (1386)	*Staphylococcus warneri* (vs)		99.9	*Staphylococcus warneri* ATCC 27836	L37603	100
11	2							2 (1382)	*Pseudomonas trivialis* PDD-32b-53	HQ256851	100	*Pseudomonas trivialis* DSM 14937	JYLK01000002	99.9
12	1							1 (1337)	*Staphylococcus pasteuri* (vs) *Staphylococcus warneri (vs)*		99.9	*Staphylococcus pasteuri* ATCC 51129	AF041361	99.9
13			1					1 (1319)	*Pseudomonas* sp. (vs) *Pseudomonas lurida (vs)*		100	*Pseudomonas lurida* DSM 15835	AJ581999	100
14	2							2 (1379)	*Microbacterium* sp. (vs)		100	*Microbacterium testaceum* DSM 20166	X77445	99.9
15						3		3 (1377)	*Xanthomonas translucens* (vs)		100	*Xanthomonas translucens* DSM 18974	CAPJ01000550	100
16	1							1 (1358)	*Terrabacter terrae* C2-4c-14	JX517277	99.6	*Sanguibacter keddieii* DSM 10542	CP001819	99.6
17			5					3 (1364)	*Erwinia persicina* (vs) *Erwinia rhapontici (vs)*		99.9	*Erwinia persicina* NBRC 102418	BCTN01000053	100
18	1		2					2 (1352)	*Staphylococcus epidermidis* (vs) *Staphylococcus caprae (vs)*		100	*Staphylococcus epidermidis* ATCC 14990	L37605	100
19	1							1 (1302)	*Pseudoclavibacter terrae* (vs) *Pseudoclavibacter helvolus* CJ-G-TSA2		100	*Pseudoclavibacter terrae* THG MD12	KJ769174	100

Italic numbers indicate ITS groups.
[a] vs: various strains.

Biocontrol assay

The barley rhizosphere was inoculated three times with ITS groups 1, 2 and 11 (representative isolates O_li_AC_3, P_s_AC_13b and P_s_CA_4b, respectively) or with an unidentified isolate from barley stem (EST-M DE ms2) at OD_{600} = 0.1 or with 10 mM $MgCl_2$ (negative control), by drenching during two weeks post germination and prior to challenge with *Blumeria graminis* f. sp. *hordei*. Three days after the last treatment with endophyte strains or $MgCl_2$, barley leaves (cv. Golden Promise) were inoculated with *B. graminis* f. sp. *hordei* by blowing fresh spores originated from infected barley leaves (~100 conidia/cm^2). The inoculated leaves were kept on 1% water-agar plates at room temperature under low-light conditions for five days. The number of pustules was assessed per cm^2. The experiment was performed in three independent biological replications and with n = 10 plants for each experimental point.

Functional prediction of shared vs. *unshared seed endophytic microbiota*

To assess the functional potential of the total microbiota, the Ion Torrent data were analysed by the software Tax4fun [4], which allows reliable functional inference based on the taxonomic identity and the corresponding known functional pathways available in the KEGG orthologs database [22]. Since the aim was to highlight potentially enriched functions in the shared fraction of the barley seed endophytic microbiota, the OTU table obtained by NGS was divided into two separate OTU tables, composed of only the shared or the non-shared genera, respectively. More details are provided in the Supplementary Methods.

Fluorescent in-situ *hybridization and confocal laser scanning microscopy (FISH-CLSM)*

Barley seeds, as well as young roots of plantlets germinated on sterile agar-H_2O plates from surface-sterilized seeds, were used for FISH analysis. Some seeds were inoculated with the plant growth-promoting rhizobacterium (PGPR) *Hartmannibacter diazotrophicus* [44] before germination in order to test the rhizosphere competence of the seed endophytes in presence of a strong competitor. Seeds or young roots were fixed with paraformaldehyde, cut into 30 μm-thick sections (seeds only) and stained according to Cardinale et al. [11], using the Cy3-labelled universal bacterial probe EUB338MIX together with either the Cy5-labelled *Gammaproteobacteria*-specific Gam42a probe or the FITC-labelled *H. diazotrophicus*-specific E19-2 probe. FISH-negative samples were stained with NONEUB probes labelled with the same fluorochromes (Supplementary Table S2).

FISH-stained samples were observed with the confocal laser scanning system Leica SP8 or SP5 (Leica Microsystems GmbH, Mannheim, Germany). Confocal stacks were visualized with the software Imaris 8.3 (Bitplane AG, Zürich, Switzerland). More details are provided in the Supplementary Methods.

Results

Isolation and identification of barley seed endophytes

A total of 79 pure bacterial cultures, selected as morphologically different colonies grown on each plate, were obtained from the surface-sterilized seed samples. The CFU concentrations ranged from 10^2 for Overture_cs to 10^6 for Morex_pb (Supplementary Fig. S4). Control plates inoculated with the last washing water did not show bacterial growth, except for Montoya_ac and Morex_pb, which showed a few colonies with an identical morphology to the most frequent morphotypes observed on the isolation plates. The isolates were grouped into 19 phylotypes (ITS groups) according to their 16S–23S ITS profile (Table 2). The most frequent phylotype was ITS group 2, which occurred in five of the seven seed samples (including all four barley cultivars) and represented almost half of the colonies grown on all plates. It was followed by ITS group 1, which was found in four seed samples (including three barley cultivars), and by ITS group 4 found in three seed samples. ITS group 3 was abundant but not widespread, while ITS group 7 was isolated from four seed samples, but represented only 2% of all the colonies (Table 2).

For each ITS group, one or more representative isolates were identified by near-full length 16S rRNA gene sequencing (34 isolates out of 79), followed by both BLAST and EzTaxon alignment (Table 2). The 16S rRNA gene sequences of the isolates belonging to the same ITS group were identical.

All isolates were identified at the genus level by phylogenetic analysis, and for most of them the closest species was identified (Fig. 1). In total, four phyla were isolated (*Firmicutes*, *Actinobacteria*, *Proteobacteria* and *Bacteroidetes*) (Table 2; Fig. 1). For the most abundant phylotype (ITS group 2), the *gyrB* gene of three representative isolates, originating from the seeds of three different barley cultivars, was amplified and sequenced. The sequences clustered together with *P. agglomerans* LMG 1286^T (Supplementary Fig. S5). ITS groups 1 and 4 (the second and third most abundant phylotypes, respectively) were most closely related to *Paenibacillus kyungheensis* and *Bacillus cereus*, respectively (Table 2; Fig. 1). Interestingly, the typical pathogen *Xanthomonas translucens*, which causes black chaff and bacterial streak in wheat [43], also appeared among the isolates of one seed sample (ITS group 15; Table 2 and Fig. 1). Other interesting, although less frequent phylotypes, included two *Pseudomonas* spp. (ITS groups 11 and 13) and four different *Staphylococcus* spp. (ITS groups 7, 10, 12 and 18), as well as an unusual *Bacteroidetes* isolate closely related to *Chryseobacterium indoltheticum*, which was isolated from two seed samples (ITS group 5; Table 2 and Fig. 1).

Bacteria identified as ITS groups 1, 2 and 17 were also isolated from the rhizoplane of young plants germinated on Petri dishes from surface-sterilized seeds. These seedlings also harboured *Pseudomonas* bacteria similar to ITS 11/13 in both the rhizoplane and the endosphere, as well as other *Pseudomonas* spp. in the rhizoplane.

The 16S rRNA gene and the *gyrB* sequences obtained in this study were submitted to NCBI GenBank under the accession numbers MF664180–MF664219, and MF615386–MF615388, respectively.

Cultivation-independent analysis of barley seed endophytes

Six of the seven seed samples were analysed by high-throughput sequencing. A total of 1,790,006 non-chimeric sequences were obtained (average length 311.6 nucleotides), of which 2328 were of bacterial origin grouped into 254 OTUs (Overture.cs: 126 sequences, Overture.li: 131, Montoya.ac: 230, Montoya.cs: 424, Propino.s: 456, Morex.pb: 961; between 2 and 740 sequences per technical replicate). Interestingly, the number of sequences obtained by high-throughput sequencing correlated very well with the CFU concentrations (Pearson $r^2 = 0.97$, $p < 0.001$) (Supplementary Table S3; Supplementary Fig. S4). Sequences were submitted to EMBL (www.ebi.ac.uk/ena/submit/sra/) under the project number PRJEB22108.

No unclassified reads were found and each OTU was identified at least to the class level. Six bacterial phyla were detected: *Proteobacteria* (94.8% relative abundance), *Actinobacteria* (3.4%), *Firmicutes* (1.2%), *Bacteroidetes* (0.39%), *Acidobacteria* (0.17%) and *Deinococcus–Thermus* (0.09%). *Gammaproteobacteria* was by far the most dominant class (90.21%), followed by *Actinobacteria* (3.4%) and *Alphaproteobacteria* (2.9%) (Fig. 2a). At the family level, *Enterobacteriaceae*, *Pseudomonadaceae* and *Xanthomonadaceae* dominated,

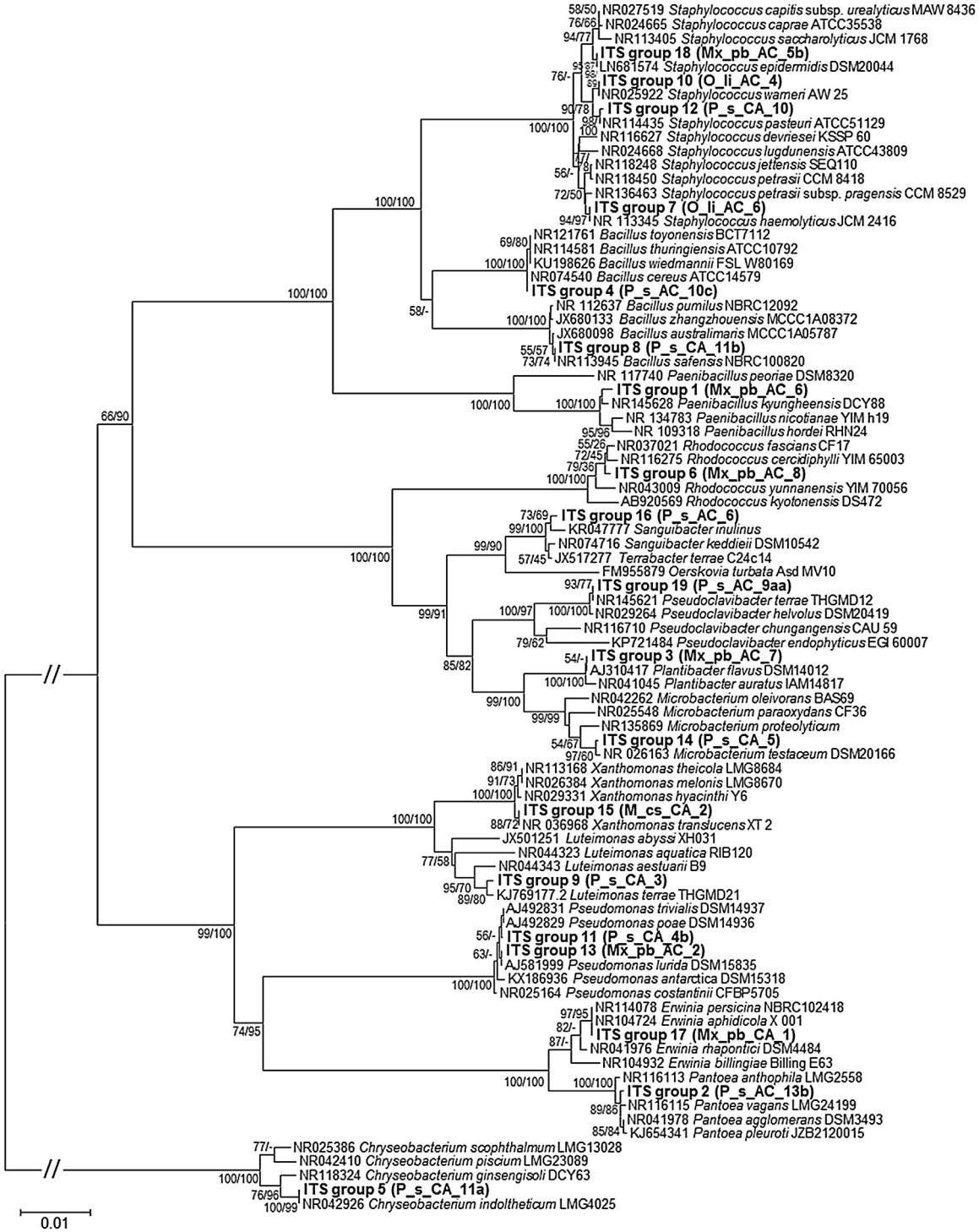

Fig. 1. Phylogenetic analysis of the 16S rRNA gene sequences representative of the 19 ITS groups isolated from the barley seed endosphere and the four closest sequences in the GenBank database, as retrieved by BLAST alignment (type material only). The tree topology was obtained with the neighbor-joining method based on a multialignment of 1335 nucleotide positions. Numbers at the nodes represent the percentage bootstrap values obtained with both the neighbor-joining and the maximum likelihood methods (1000 and 100 bootstrap re-samplings, respectively). Only bootstrap values ≥50% for at least one method are shown. - indicates absence of the corresponding node in the maximum likelihood tree. Scale bar indicates substitutions per site.

accounting for 39.2%, 25.7% and 24.5% of the total sequences, respectively (Fig. 2b). At the genus level, *Pantoea* showed the highest relative abundance (27.1%), followed by *Pseudomonas*, *Xanthomonas* and *Stenotrophomonas* (25.7%, 14.6% and 8.7%, respectively). *Pantoea* and *Pseudomonas* were detected in all six seed samples, *Xanthomonas* in five and *Stenotrophomonas* in four. Further low-abundant genera occurring in all six samples were *Caulobacter* and a *Bradyrhizobiaceae*-related taxon (0.9% and 0.5% relative abundance, respectively; Supplementary Table S3). The coverage of the analysis was suboptimal for the two Overture seed samples, while for the other samples >96% of the diversity at the genus level was revealed (Supplementary Fig. S6). All the isolated genera were also retrieved by Ion Torrent sequencing, with the exception of the genera *Paenibacillus*, *Pseudoclavibac-*

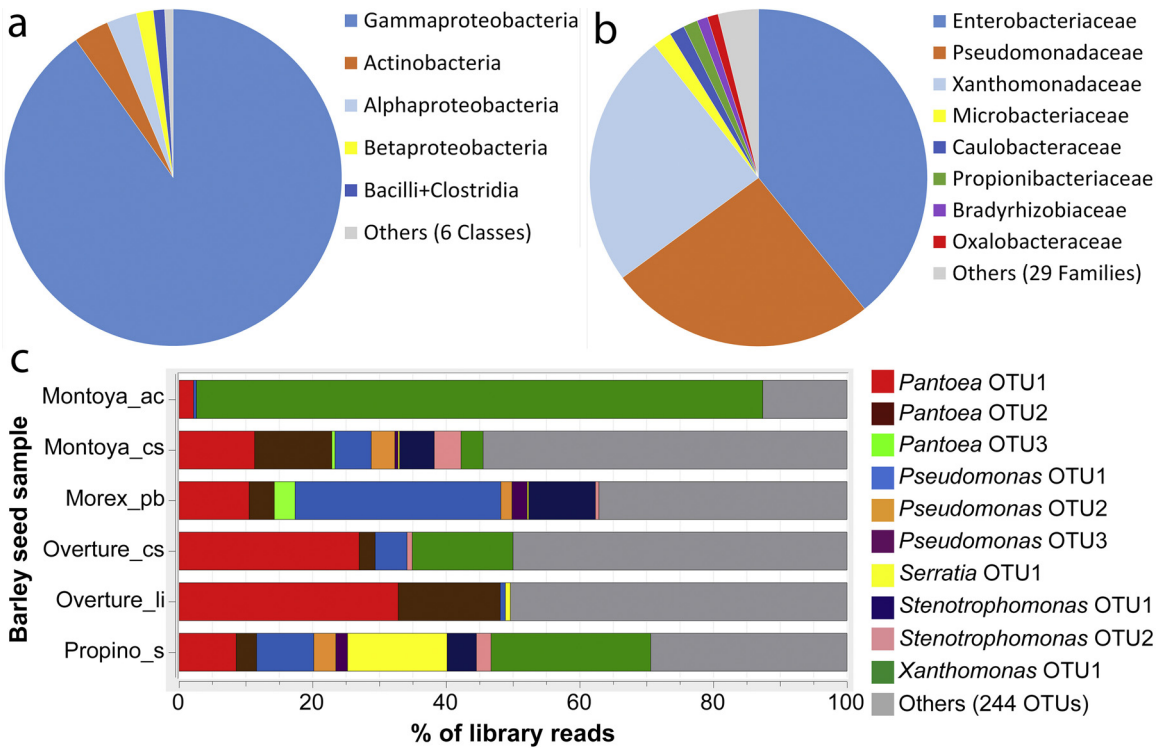

Fig. 2. Taxonomic structure of barley seed endophytic microbiota derived from cultivation-independent 16S rRNA gene-sequencing. (a) Major classes detected in the seed endosphere of barley (all samples); (b) Major families detected in the seed endosphere of barley (all samples); (c) Relative abundance of the 10 most abundant OTUs in the endosphere of the six barley seed samples analysed.

ter, *Staphylococcus* and *Luteimonas* (Table 2; Supplementary Table S3).

Among the ten most abundant OTUs, there were two OTUs identified as *Pantoea*: the first one ("*Pantoea* OTU1" in Fig. 2c) was detected in all seed samples, while the second one ("*Pantoea* OTU2" in Fig. 2c) occurred in five out of the six samples. These two *Pantoea* OTUs showed 100% identical representative sequences. This representative sequence was also 100% identical to the 16S rRNA gene sequences of the *Pantoea* isolates (ITS group 2) over the whole length of the high-throughput sequencing amplicons (329 nucleotides), thus suggesting that the dominant *Pantoea* isolated on plates was actually the most abundant organism of the total seed endophytic microbiota of barley. A common *Pseudomonas* OTU was also found in all seed samples (Fig. 2c), and its 16S rRNA gene sequence was identical to that of the isolated *Pseudomonas* (ITS groups 11 and 13) over the whole length of the high-throughput sequencing amplicons (329 nucleotides), suggesting that these phylotypes (closely related to *Pseudomonas trivialis*/*poae*/*lurida*; Fig. 1) were actually as widespread as *P. agglomerans* in the barley seeds, but more difficult to isolate.

To the best of our knowledge, twelve genera were detected in this study for the first time as seed endophytes: *Alistipes*, *Saccharibacillus*, *Blautia*, *Faecalibacterium*, *Candidatus* Brownia, *Epilithonimonas*, *Truepera*, *Thermus*, *Pseudobutyrivibrio*, *Ruminiclostridium*, *Rubritepida* and *Sutterella* (Supplementary Table S3). All of them belonged to the less-abundant taxa and, interestingly, most of them were typical gut or rumen bacteria.

From non-sterilized seeds (cultivar Morex), a much higher number of bacterial sequences was obtained (14,864 total reads from three replicates) in comparison to surface-sterilized Morex seeds (961 total reads from five replicates), which indicated a low abundance of seed endophytes compared to those inhabiting the seed surface. However, the composition of the two bacterial communities differed only for less abundant taxa, while the major bacterial populations were the same (Supplementary Fig. S7). The genus *Pseudomonas* appeared to be enriched in the endosphere, while *Pantoea* abundance was similar in both surface-sterilized and non-sterilized seeds. Interestingly, *Paenibacillus* (abundant and widespread among the isolates), which was not found in the total seed endophytic microbiota, appeared in all three non-sterilized seed samples (Supplementary Fig. S7).

Plate functional assays

To assess the PGP potential, functional tests were performed with the isolates representative of the most abundant ITS groups 1 and 2 (*Paenibacillus* and *Pantoea*, respectively, both most-widespread), 3 and 4 (*Plantibacter* and *Bacillus*, respectively, both less widespread), as well as ITS group 11 (*Pseudomonas*, not frequent among isolates but indicated by high-throughput sequencing as abundant and shared across all barley seed samples). *Pantoea* ITS group 2 performed best, showing an ability to solubilize potassium (mica), produce auxin and hydroxamate type siderophores, and grow in the absence of nitrogen sources; moreover, it tolerated salinity and cold stresses (Table 3). The second and third best performing phylotypes were *Pseudomonas* ITS group 11 and *Paenibacillus* ITS group 1, respectively. Interestingly, *Pseudomonas* ITS group 11 showed functional traits complementary to those of *Pantoea* ITS Group 2, such as production of catechol-type siderophores and phosphate solubilisation (Table 3). The performance of *Plantibacter* ITS group 3 (less frequent and less abundant) was very weak, while *Bacillus* ITS group 4 performed well only in the tolerance tests (probably because it is a spore-forming bacterium). These results indicated that the consistent barley seed endophytes (*Pantoea*, *Pseudomonas* and *Paenibacillus*) possessed the greatest potential for PGP, whereas less widespread phylotypes (*Plantibacter* and *Bacillus*) did not show notable PGP potential. The representative isolate of the best performing *Pantoea* ITS group 2 (P_s_AC_13b)

Table 3
Plate assays of plant growth promotion traits and tolerance of the most frequent phylotypes isolated from the barley seed endosphere. Representative isolates tested were: O_li_AC_3 (ITS group 1), P_s_AC_13b (ITS group 2), Mx_AC_7 (ITS group 3), M_cs_CA_3 (ITS group 4) and P_pb_CA_4b (ITS group 11). N-free: growth on nitrogen-free medium; ACC: growth on 1-aminocyclopropane-1-carboxylate as the sole nitrogen source; Sid: siderophore synthesis; K: potassium solubilisation; IAA: auxin synthesis. PEG: growth on medium amended with polyethylene glycol.

ITS group	Plant growth promotion tests[a]									Tolerance tests						
	Phosphate solubilisation				N-free	ACC	Sid	K	IAA (μg mL^{-1})	NaCl (%)			Heat (45 °C)	Cold (4 °C)	PEG (%)	
	Inorganic			Organic						2.5	5.0	7.5			10	20
	Ca–P	Al–P	Fe–P	IHP												
1	++	+	+	+	++	+	-	+	3.42	++	+	–	–	+/–	+	–
2	++ (c)	+ (c)	+ (c)	+	++	+	++ (v)	++ (h)	24.84	++	++	++	–	+	++	–
3	–	–	–	–	–	–	+/– (v)	–	−0.62	++	+	–	–	+/–	–	–
4	–	–	-	+/–	–	–	–	–	−3.08	++	+	+/–	++	–	++	–
11	++ (c, h)	+ (c)	+ (c)	++	–	–	++ (y)	–	10.56	++	+	–	–	+	–	–

Italic numbers indicate ITS groups.
[a] ++ normal growth (single colonies of typical size); + less growth (single colonies of smaller size); +/– very little growth (no single colonies, only growth in the inoculation area); – no growth; (c) colour change of the medium; (h) solubilisation halo visible; (v) violet; (y) yellow.

was then chosen for the PGP assay in the greenhouse, while all three widespread and well-performing endophytes (*Pantoea*, *Paenibacillus* and *Pseudomonas*) were tested in the biocontrol assay.

Plant growth promotion assay

The effect of the most abundant and widespread phylotype (ITS group 2, phylogenetically close to *P. agglomerans*) was tested on barley in an inoculation assay in the greenhouse (Supplementary Fig. S3), using the representative isolate P_s_AC_13b and two different substrates (one nutrient-rich, "Einheitserde", and one nutrient-poor, "Unterboden"; Supplementary Table S1). Analysis of 16S amplicon libraries demonstrated at a depth of 67,969 (substrate "Einheitserde") and 31,478 reads (substrate "Unterboden") that no *Pantoea*-related sequence was detected. Ten days after transplanting in the greenhouse (DAT) (14 day-old plants), the inoculation increased the plant height significantly in both sterilized and unsterilized nutrient-poor soil, and in the sterilized nutrient-rich soil (main effect ANOVA, $F_{1,32} = 7.42$, $P = 0.010$; partial-$\eta^2 = 0.19$; 1-$\beta = 0.75$; N = 5) (Fig. 3a; Supplementary Fig. S8). Chlorophyll content showed the same 14 DAT trend (main effect ANOVA, $F_{1,32} = 5.6$, $P = 0.024$; partial-$\eta^2 = 0.15$; 1-$\beta = 0.63$; N = 5) (Fig. 3b). At 60 DAT, the effect of inoculation was still significant (main effect ANOVA, $F_{1,32} = 6.94$, $P = 0.013$; partial-$\eta^2 = 0.18$; 1-$\beta = 0.73$ N = 5), since all *Pantoea*-inoculated plants were higher than the corresponding uninoculated plants, especially in the sterilized nutrient-poor soil (Fig. 3c; Supplementary Fig. S9). It is worth noting that all the significant differences showed a large effect size (partial-$\eta^2 > 0.14$), despite the slightly suboptimal analysis power (1-$\beta < 0.8$). These results demonstrated that *P. agglomerans*, which was consistently associated with barley seeds, supported plant growth, especially under harsh conditions (in nutrient deficit or dysbiotic soil, or both) during the critical early stages of growth. At 42 DAT, no effect of the inoculum was observed on the weight and water content of the plants grown on the nutrient-rich substrate Einheitserde (Fig. 3d). However, a significant effect of the inoculum was still observed on both the average fresh weight (Student's *t*-test, $T_8 = 2.51$, $P = 0.037$; Cohen's d test = 1.58; N = 5) and the water content (Student's *t*-test, $T_8 = 2.32$, $P = 0.049$; Cohen's d test = 1.47; N = 5) of the barley plants grown on the sterilized, nutrient-poor substrate Unterboden (Fig. 3e, asterisks). The same plants showed a significantly higher concentration of K and Mg (Mann–Whitney U test, $p = 0.037$ and 0.036, respectively) (Fig. 3f, asterisks). Interestingly, Mg is linked to chlorophyll in plants and, moreover, these results were coherent with the plate K-solubilisation assay.

Biocontrol assay

In order to study the impact of the isolated seed endophytes on barley plants even further, ITS groups 1, 2 and 11 (related to *Paenibacillus*, *Pantoea* and *Pseudomonas*; representative isolates O_li_AC_3, P_s_AC_13b, and P_s_CA_4b, respectively) were tested in an *in planta* biocontrol assay. The protective abilities against the barley fungal pathogen *B. graminis* f. sp. *hordei* were assessed by inoculating the rhizosphere of barley plants with the selected endophytes prior to challenge with the powdery mildew-causing fungus. Inoculation with all three bacteria significantly lowered the number of fungal pustules from 32 per cm^{-2} to 21 per cm^{-2} on average, which represented a large size effect (ANOVA, $F_{4,128} = 5.22$, $P < 0.001$; partial-$\eta^2 = 0.14$; 1-$\beta = 0.97$; N = 30). The inoculation with an isolate obtained from barley stem (EST-M DE ms2) had no impact (Fig. 4). These results indicated that the three most common barley seed endophytes, in addition to the growth promoting capacity observed for *P. agglomerans* (Fig. 3), could also induce resistance to a phytopathogen.

Functional prediction of shared vs. *unshared seed endophytic microbiota*

According to the KEGG functional pathways, the shared fraction of the seed endophytic microbiota (as identified by high-throughput sequencing results) showed a significantly higher potential for energy metabolism (sugar transport, sugar metabolism and nitrogen metabolism; Supplementary Fig. S10). The sample coverage of the analysis (i.e. the fraction of the taxa within each sample that found matches to KEGG organisms, the so-called FTU metric) was in the range 78–99% for the shared fraction and 68–97% for the unshared fraction.

Fluorescent in-situ *hybridisation and confocal laser scanning microscopy (FISH-CLSM)*

FISH staining of non-sterilized seeds showed a dense colonization of the seed coat, across its whole thickness (Fig. 5a–c). After surface sterilization (two hours in 1.3% ClO^-), bacterial cells were detected in the seed endosperm, no cells were detected in the aleuronic layer and only a few cells were detected in the innermost part of the seed coat (Fig. 5d). These cells were apparently resistant to the sterilization treatment, which was coherent with the growth of a few colonies from the last washing water in some of the control plates. Detection of seed endophytes was difficult due to strong seed autofluorescence and low bacterial density, which was coherent with the results of both isolation and high-

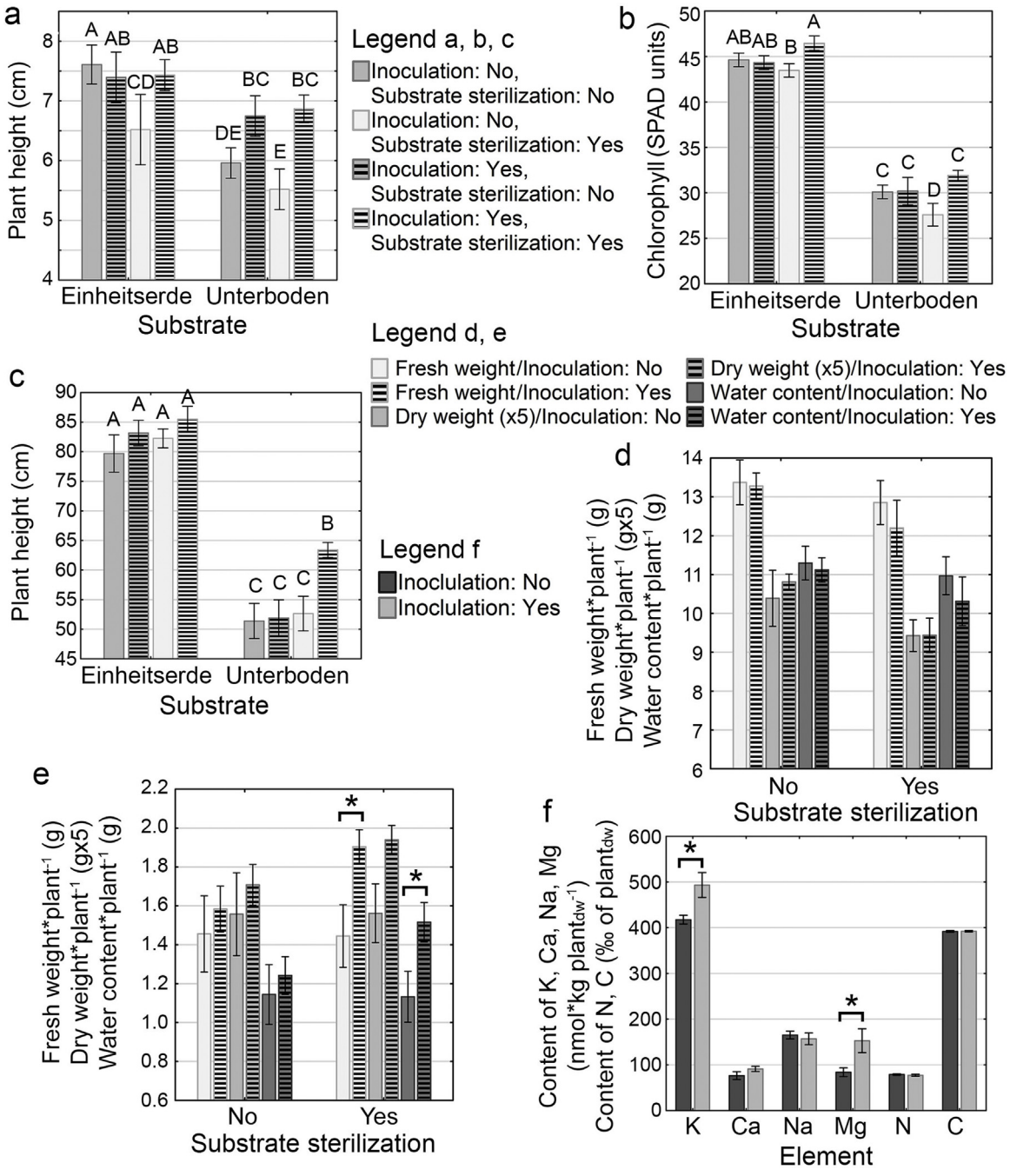

Fig. 3. Plant growth promotion assay with the common *Pantoea agglomerans*-related seed endophyte (ITS group 2, representative isolate: P_s_AC_13b). (a) Plant height ten days after transplantation (DAT). (b) Average relative chlorophyll content of the plants at 14 DAT. (c) Average plant height at 60 DAT. Different letters in panels a, c and d indicate significantly different means (LSD post-hoc test, $p < 0.05$; N = 5). (d) and (e) Average fresh weight, dry weight and water content at 42 DAT of the plants grown on nutrient-rich Einheitserde (d) or nutrient-poor Unterboden (e) substrate. Asterisks indicate significantly different means (Student's *t*-test, $p < 0.05$; N = 5). (f) Element content at 42 DAT of the plants grown on Unterboden. Asterisks indicate significantly different means (Mann–Whitney U test, $p < 0.05$; N = 5).

throughput sequencing. No bacterial signal was detected in seed sections stained with non-sense FISH probes (Fig. 5e). The single channel images of all panels in Fig. 5 are shown in Supplementary Fig. S11.

FISH staining of young roots of plants grown on sterile agar plates from surface-sterilized seeds showed dense bacterial colonization from the root tip to the maturation zone of the root (Fig. 6a), which appeared clearly in the rhizoplane (Fig. 6b and d). Dominant *Gammaproteobacteria*, as well as other bacteria, already colonized the root hairs in the differentiation zone (Fig. 6b). In the root cap, the detached border cells were especially densely colonized (Fig. 6c and d). Interestingly, *Firmicutes*, one of the rarest phyla detected (Fig. 2a; Supplementary Table S3), was also observed in the roots (Supplementary Fig. S12).

To test the rhizocompetence of seed endophytes in presence of a strong competitor, surface-sterilized seeds were inoculated with the PGPR *H. diazotrophicus* and then germinated on sterile agar plates. The roots of these plants showed colonization by both the inoculated bacterium and the seed endophytes (Fig. 6e), thus demonstrating their notable rhizocompetence. Interestingly, physical interactions between the inoculated *H. diazotrophicus* and the seed endophytes were observed (mixed colonies, Fig. 6f). The single

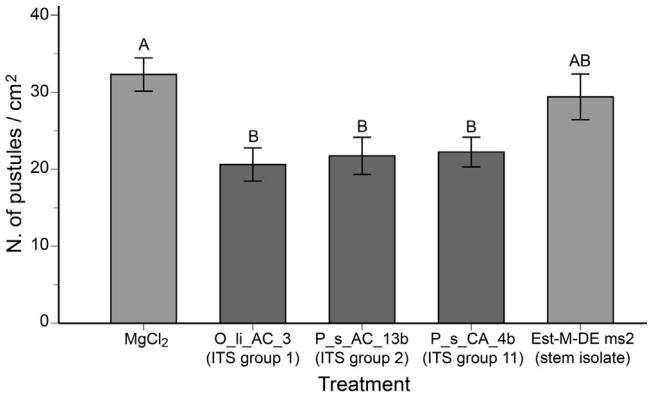

Fig. 4. Biocontrol assay with the common seed endophytes (ITS group 1, 2 and 11; representative isolates O_li_AC_3, P_s_AC_13b and P_s_CA_4b, respectively) against the fungal pathogen *Blumeria graminis*. 10 mM $MgCl_2$ and another isolate obtained from barley stem (EST-M DE ms2) served as controls. Different letters indicate significantly different means (Tukey's post-hoc test, $p < 0.05$; N = 30).

channel images of all panels in Fig. 6 are shown in Supplementary Fig. S13. No bacterial signal was detected in seed sections stained with non-sense FISH probes (Supplementary Fig. S14).

Discussion

Seed-associated microbes are of high interest, due to their potential of being vertically transmitted to the next generation. In the case of beneficial microbes, the positive effects could be displayed immediately after germination. In this study, it was demonstrated that the seeds of the important crop plant barley were stably associated with beneficial bacterial taxa, in the context of the plant genotypes, origin and harvest years investigated. The integration of cultivation-dependent and -independent methods identified the taxa as: (i) *Paenibacillus* (*kyungheensis*-related), which was widespread among the isolates but did not occur in the total endophytic microbiomes, since its real abundance was very likely below the detection level or there was bias in the primer specificity; (ii) *Pseudomonas* (*trivialis*-related) that was widespread in the total endophytic microbiomes but rare among the isolates, which could be explained by the typical difficulty of certain environmental bacteria to grow on nutrient media; and (iii) *Pantoea* (*agglomerans*-related) that was abundant and widespread among both the isolates and the total microbiomes.

P. agglomerans is a plant endophyte [34] with known beneficial effects [14,39]. It was shown as one of the common seed endophytes in maize [21,40], as well as other plants such as *Eucalyptus*, where it is transmitted from seeds to seedlings [15]. Although *P. agglomerans* has been assigned to risk group 2 (potentially human pathogenic

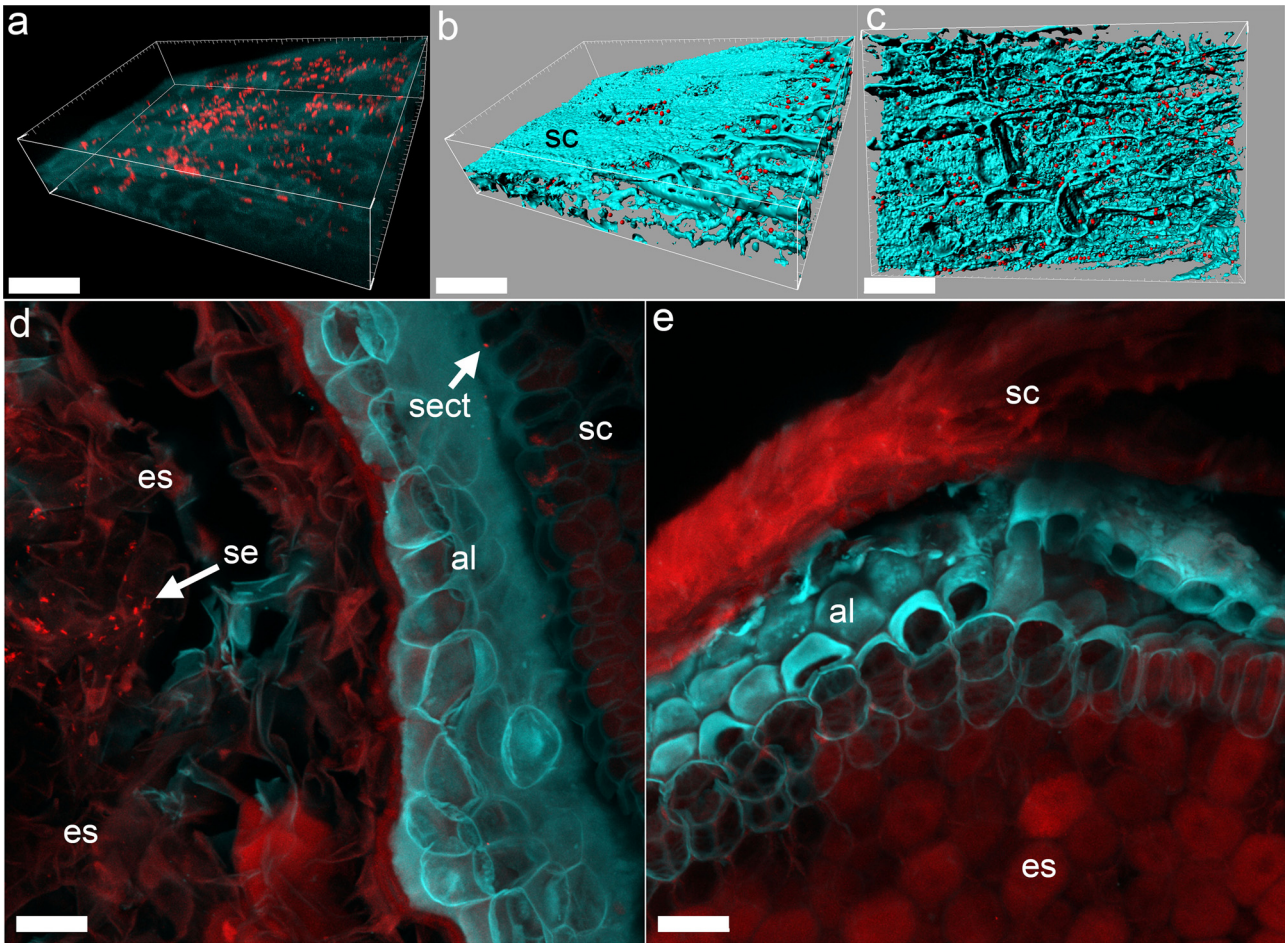

Fig. 5. Confocal laser scanning microscopy images showing the bacterial colonization of barley seeds. (a) Volume rendering of a confocal series, showing the seed coat of untreated barley seeds; red: bacteria (stained with the Cy3-labelled bacterial probe EUB338MIX); cyan: seed coat tissue (autofluorescence). (b) and (c) Three-dimensional models (upper- and under-side, respectively) of (a), composed of red spheres (bacteria) and cyan iso-surfaces (seed coat tissue). (d) Volume rendering of a confocal series, showing the transversal section of a surface-sterilized barley seed; red: bacteria; cyan: seed tissues. (e) Volume rendering of a confocal series showing the negative control for FISH staining, using the Cy3-labelled non-sense probe NONEUB. sc = seed coat; es = endosperm; al = aleurone cells; se = seed endophytic bacteria; sect = seed ectophytic bacteria. Scale bars = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

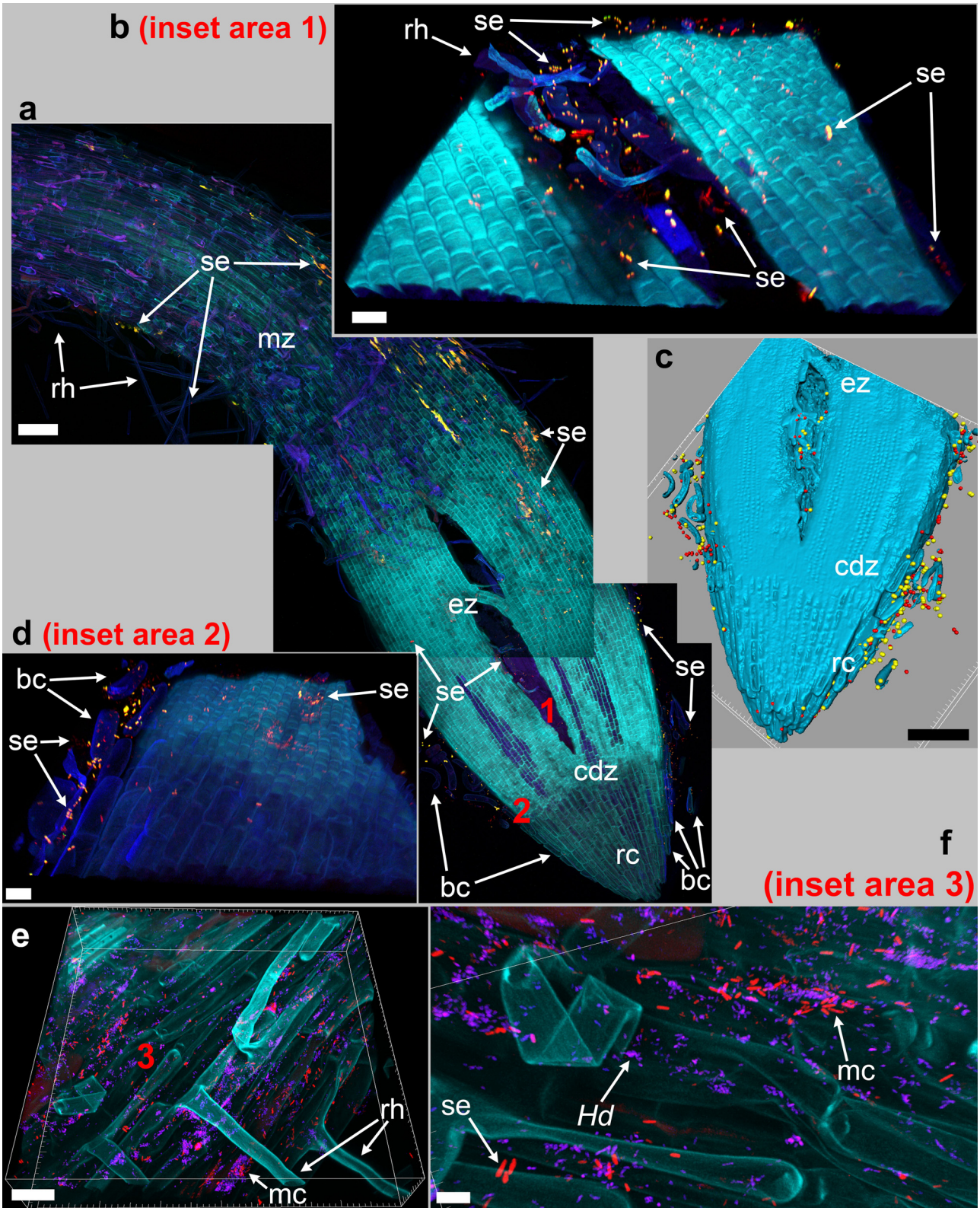

Fig. 6. Confocal laser scanning microscopy images showing the bacterial colonization of young barley roots. Bacteria were stained by fluorescent *in situ* hybridization with the Cy3-labelled bacterial probe EUB338MIX, the *Gammaproteobacteria*-specific Cy5-labelled Gam42a probe and the *Hartmannibacter diazotrophicus*-specific FITC-labelled E19-2 probe. (a) Composite image of three manually aligned maximum projections, showing the bacterial colonization of a barley root tip by seed endophytes after germination in a closed, sterile environment; yellow: *Gammaproteobacteria*; red: other bacteria; autofluorescence of root tissues appears in blue (prevalently root hairs and border cells) and cyan; (b) Volume rendering of a confocal series showing area 1 in panel A; (c) Three-dimensional model composed of yellow spheres (*Gammaproteobacteria*), red spheres (other bacteria) and cyan iso-surfaces (root tissues), showing the root cap, cell division and elongation zones; (d) Volume rendering of a confocal series showing area 2 in panel A. (e) Volume rendering of a confocal series showing the colonization of barley roots by seed endophytes (red) and *Hartmannibacter diazotrophicus* (purple); the latter was inoculated on the seeds after surface sterilization and before germination (see Materials and Methods for details). (f) Volume rendering of a confocal series showing the magnification of area 3 in panel e. rh = root hairs; bc = border cells; rc = root cap; cdz = cell division zone; ez = elongation zone; mz = maturation zone; Hd = *Hartmannibacter diazotrophicus*; se = seed endophytes; mc = mixed colonies. Scale bars: a and c = 100 μm; b and d = 20 μm; e = 30 μm; f = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

organisms), a re-evaluation of many clinical isolates demonstrated that most of the strains were erroneously identified [32]. In fact, one strain is already used in a commercial biocontrol product in New Zealand [41].

Interestingly, the *P. kyungheensis*-related bacterium isolated in this study (ITS group 1) had an identical 16S sequence to a bacterium isolated from lucerne seeds in Japan (sequence accession number: KP852522) that is an antagonist against *Fusarium oxysporum*. This suggests the existence of ubiquitous beneficial bacteria associated with the seeds of very different plant species. The recent description of a new *Paenibacillus* species isolated from maize seeds [26], very closely related to *P. kyungheensis* and *P. nicotianae*, further supports this hypothesis.

This current study focused on bacteria colonizing the seed endosphere because endophytes are considered to have a higher adaptation to the plant habitat. However, it was found that the composition of the total seed-associated microbiota did not differ consistently from that of only the seed endophytes, therefore, for future research, the seed ectophytes could also be considered.

Quite different CFU values were obtained from the different seed samples, perhaps as an effect of the cultivar (both Overture samples had lower values) and storage time (the new Propino seeds from 2016 had smaller values than Propino seeds from 2013). The low number of sequences obtained by high-throughput sequencing was likely due to the low fraction of bacterial DNA compared to plant DNA, as previously shown for another plant species [3]. Therefore, in this study, cultivation-independent analysis was used to complement and confirm the results of the isolation, and to reveal additional uncultivated species. In one seed sample (cultivar Montoya from the Ackermann company), *Xanthomonas* was most abundant, whereas in the other samples *Pantoea* and *Pseudomonas* were the dominant taxa, and this difference might be due to the different soil/environmental conditions where the plants were growing. Interestingly, Yang et al. [50] did not detect *Xanthomonas* in barley seeds but found it in the roots of axenically-grown plants.

The results with barley seed microbiota were in agreement with other studies on seed-associated microbes [18,21,35,49], and suggested that the association with beneficial microbes inside the seeds was a common feature of the plant holobionts. Although some microorganisms might just be highly adapted to endophytic life styles without necessarily being beneficial, the specificity and stability shown by these associations, which are likely to be the result of long lasting co-evolution processes, resemble those of symbiotic relationships, in which both partners receive benefits: the bacteria will be sheltered in the seeds and spread together with them, thus gaining priority access to the new root habitat immediately after germination. In turn, the plant will already benefit from the bacterial effects at the very early growth stages. Early growth stages are critical for all organisms, and the plants could use seed-stored bacteria to guarantee important ecological services (nutrient scavenging, growth stimulation, protection against pathogens, etc.) to their progeny. Interestingly, a complementarity of beneficial traits was observed among the three widespread phylotypes, especially *Pantoea* ITS group 2 and *Pseudomonas* ITS group 11 (Table 3), which was in agreement with our previous observation on *Anadenanthera colubrina* seed endophytes [3] and supports the hypothesis of a function-driven assemblage of the seed microbiome.

The greenhouse assays with barley under different conditions demonstrated that the shared barley seed endophytes (*Paenibacillus*, *Pantoea* and *Pseudomonas*, respectively ITS groups 1, 2 and 11) had positive effects especially under challenging conditions, such as poor and dysbiotic soil (Fig. 3) and biotic stress (Fig. 4). This was coherent with the principle of the conditional outcomes of plant-microbe interactions: non-optimal conditions reveal higher beneficial effects exerted by associated microbes [30]. Considering the variable and unpredictable conditions in nature, such associations with beneficial bacteria can represent a great advantage for the plant, enhancing the adaptation ability and thus the survival rate. The growth promotion and biocontrol effects exerted by the common barley seed endophytes suggest the existence of a "minimal functional microbiome" able to provide beneficial services to the new-born plants in case of germination under harsh conditions.

A key factor for expression of the beneficial effects on the plant is good rhizosphere competence, which allows a rapid bacterial colonization of the roots. The results of the present study showed that barley seed endophytes were indeed able to exit the seed and efficiently colonize the root system (Fig. 6a–d), even in the presence of abundant microbial competitors (Fig. 6e–f). The efficient energy metabolism of the shared seed endophytes, as shown by functional prediction (Supplementary Fig. S10), could determine fast growth, which might be responsible in part for the high rhizocompetence observed.

It was previously shown that seed endophytes, as well as other plant-associated microbes, possessed beneficial traits, and promoted plant growth and health [16,31,45,49]. The current study showed the occurrence of specific, beneficial bacteria stably associated with barley seeds. This evidence led to the hypothesis that the seed endophytes might be more than transiently associated bacteria selected from the surrounding environment. In an evolutionary framework, these beneficial associations might have been positively selected, eventually resulting in a stable barley holobiont feature. In this study, seeds were analysed from Germany (Table 1), which poses the question of whether barley seeds from other countries/continents would harbour the same bacteria. Further analysis on additional barley cultivars from other regions and continents are therefore necessary in order to address the actual extent and significance of these associations. However, previous studies identified *P. agglomerans* as a barley endophyte in the stem [24] and in the seeds [28,50]. These studies and the present results supported the conclusions that the association between barley and certain bacterial taxa (including *P. agglomerans*) seems to be stable, although the interaction mechanisms allowing such stability remain to be elucidated. It is possible that such seed associations are widespread in plants and, if so, they represent one of the main mechanisms of holobiont adaptation and evolution. This partnership between plant and microbes has developed during long-term natural selection processes, and was optimized by adjustment to biotic and abiotic stresses. Considering the high rhizocompetence of the seed endophytes, such a close interaction offers new perspectives for optimizing plant development and enhancing resistance in agricultural and forest ecosystems.

Conclusions

In this study, a polyphasic approach was used to study the ecology of the bacterial seed endophytes of barley. In the context of the cultivars, geographic origins and harvest years investigated, a consistent association was found with species related to *Pantoea*, *Pseudomonas* and *Paenibacillus*. These seed endophytes showed beneficial effects for the host, especially under harsh conditions (abiotic and biotic stress), as well as an exceptional rhizocompetence, possibly supported by their efficient energy metabolism. This multifaceted study indicated that seed endophytes should be considered as key partners of the barley holobiont, and opens up new perspectives for research of the plant microbiome and evolution. The results also suggested that seed-associated microbes deserve priority attention as bioinoculants in targeted sustainable agriculture.

Author contributions

MD M.R. performed FISH-CLSM on seed sections, germination of seeds on membranes and the Ion Torrent sequencing. E.F. characterized and identified the isolates. C.S. produced the preliminary data that gave the idea for the study and produced the plants inoculated with *H. diazotrophicus*. H.-W.K. contributed to the greenhouse experiment by providing the space and by giving advice, as well as performing plant element analysis and contributing to writing the manuscript. Z.A. performed plant element analysis, chlorophyll measurement, gave advice for the greenhouse experiment and revised the manuscript. A.S. performed the biocontrol assay. S.S. contributed to designing the study and revised the manuscript. M.C. designed and coordinated the study, performed FISH-CLSM on roots, the greenhouse experiment, functional prediction, analysis of Ion Torrent sequencing data, phylogenetic analysis of the isolates and statistical analysis, and wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.syapm.2018.02.003.

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