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

MD Mahafizur Rahman[a], Elias Flory[a], Hans-Werner Koyro[b], Zainul Abideen[b,c], Adam Schikora[d], Christian Suarez[a], Sylvia Schnell[a], Massimiliano Cardinale[a,*]

[a] *Institute of Applied Microbiology, Research Center for BioSystems, Land Use, and Nutrition (IFZ), Justus-Liebig-University Giessen, Heinrich-Buff-Ring 26-32, D-35392 Giessen, Germany*
[b] *Institute of Plant Ecology, Research Center for BioSystems, Land Use, and Nutrition (IFZ), Justus-Liebig-University Giessen, Heinrich-Buff-Ring 26-32, D-35392 Giessen, Germany*
[c] *Institute of Sustainable Halophyte Utilization, University of Karachi, Karachi 75270, Pakistan*
[d] *Institute for Epidemiology and Pathogen Diagnostics, Julius Kühn-Institut Federal Research Centre for Cultivated Plants (JKI), Messeweg 11/12, D-38104 Brauschweig, Germany*

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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.

* Corresponding author.
E-mail address: massimiliano.cardinale@umwelt.uni-giessen.de (M. Cardinale).

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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.

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