Bacterial endophytes of mangrove propagules elicit early establishment of the natural host and promote growth of cereal crops under salt stress

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ABSTRACT

Mangroves, dominating tropical intertidal zones and estuaries, are among the most salt tolerant plants, and propagate through reproductive units called propagules. Similarly to other plants' seeds, propagules may harbor beneficial bacteria. Our hypothesis was that mangroves, being able to grow into seawater, should harbor bacteria able to interact with the host and to exert positive effects under salt stress, which could be exploited to improve crop production. Therefore, we isolated bacterial endophytes from mangrove propagules with the aim to test whether these bacteria have a beneficial potential on their natural host and on different crops like barley and rice, cultivated under salt stress. The 172 bacterial isolates obtained were screened for plant growth promotion (PGP) activities *in vitro*, and the 12 most promising isolates were tested on barley under non-axenic conditions and salt stress. *Gordonia terrae* KMP456-M40 was the best performing isolate, increasing ear weight by 65%. Basing on the *in vivo* PGP activity and the root colonization ability, investigated by fluorescence *in situ* hybridization and confocal microscopy, three strains were additionally tested on mangrove propagule germination and on rice growth. The most effective strain was again *G. terrae* KMP456-M40, which enhanced the root length of mangrove seedlings and the biomass of salt-stressed rice under axenic conditions up to 65% and 62%, respectively. We demonstrated that propagules, the reproductive units of mangroves, host beneficial bacteria that enhance the potential of mangrove seedlings establishment and confer salt tolerance to cereal crops.

KEYWORDS: mangrove ecosystem; endophytes; salt stress; barley; rice; plant growth-promoting bacteria.

INTRODUCTION

Plants and their associated microorganisms have evolved together to adapt to a given environment (Rodriguez and Redman, 2008). In the Neolithic, plant domestication started and became one of the major drivers of plant selection (Ross-Ibarra et al., 2007), determining unknown consequences on their ancestral microbiome. Plants have maintained the ability to select and enrich beneficial microorganisms in the rhizosphere by releasing root exudates (Berg and Smalla, 2009; Lugtenberg and Kamilova, 2009). Such beneficial microbes can colonize the plant tissues endophytically and may be transmitted to the following generations through the reproductive units (*e.g.* seeds (Johnston-Monje and Raizada, 2011; Truyens et al., 2015) or spores (Bragina et al., 2012). Transgenerational transmission includes bacteria that can be essential for the plant since the very early life stage, allowing the plant host to cope with the adverse conditions occurring in harsh environments (Puente et al., 2009) or derived from sudden or periodic environmental stresses (Rahman et al., 2018).

Coastal ecosystems are subjected to cyclic shifts of different environmental conditions such as nutrient availability and salinity and oxygen concentrations in the soil and sediments (Alongi, 1988; Mitra et al., 2008). The importance of plant growth promoting (PGP) bacteria in coastal ecosystems was largely reported (Gontia et al., 2011; Jha et al., 2012; Mapelli et al., 2013; Marasco et al., 2016; Mesa et al., 2015; Siddikee, 2010), as well as the influence of the tidal regime on the selection of specific bacterial assemblages in the root systems (Marasco et al., 2016; Wang et al., 2015).

Mangroves are evolutionarily adapted to the environmental conditions of tropical intertidal ecosystems and have been defined as ‘true extremophiles’, because they can flourish under high salinity, relative substrate hypoxia and strong tidal flows that are unsuitable for most of the terrestrial plants (Dassanayake et al., 2009; Flowers and

Colmer, 2015; Oh et al., 2012; Parida and Jha, 2010). Mangroves are among the most salt tolerant plants known and play a pivotal ecological role for preservation and productivity of tropical coastal ecosystems (Donato et al., 2011; Ezcurra et al., 2016; Sutton-Grier et al., 2015). As other plants growing in naturally saline environments, mangroves host halotolerant and halophilic bacteria (Castro et al., 2014) and were proposed as a valuable source of PGP bacteria (Bashan and Holguin, 2002). Due to the increasing salinization of soils in many regions of the Earth, as a consequence of intensive agricultural practices and climate change, there is a growing interest in the possible exploitation of microorganisms adapted to high salinity as plant biofertilizers/biostimulants (Cardinale et al., 2015; Cho et al., 2015; Egamberdieva et al., 2008; Egamberdieva et al., 2011; Mapelli et al., 2013; Soussi et al., 2016; Tiwari et al., 2011).

The intimate and potentially inheritable positive association of plants with microoganisms is supported by the finding of endophytic PGP bacteria in the plant seeds (Truyens et al., 2015). The first stages of seedling establishment, characterized by high mortality, influence the distribution and fitness of adult plants upon different abiotic and biotic factors (Rand, 2000). The potential inheritance of PGP microbial partners can indeed be especially important in coastal ecosystems, where the first stages of plant growth are challenged by rapid and continuous shift in the environmental conditions. To counteract these adverse conditions, most of the mangrove tree species evolved vivipary (Hong et al., 2018; Kathiresan and Rajendran, 2002; Osborne and Berjak, 1997) and produce propagules that contains a seedling able to rapidly root once droped on the sediment or to survive long floating periods when dispersed by the tidal currents.

We hypothesize that mangrove propagules harbor beneficial endophytic bacteria capable to enhance the root establishment of mangrove seedlings once fallen from the plants into

the seawater, thus playing a role for the stability of the overall mangrove ecosystem. We also hypothesize that beneficial bacteria selected by mangrove propagules can favor non-host plant species, including crops, potentially contributing to enhance salt tolerance and improve their productivity in arid/saline soils, a major abiotic stress threatening modern agriculture (Chaves et al., 2009; Tester and Danevport, 2003).

The aim of this work was to characterize the cultivable bacterial endophytes of *Avicennia marina* propagules, assessing their potential to promote plant growth and productivity under salt stress. We therefore evaluated the potential of selected propagule endophytes to mitigate salt stress on two cereal crops with different tolerance to soil salinity, *i.e.* barley (*Hordeum vulgare* L., salt tolerant) and rice (*Oryza sativa* L., salt sensitive) and the effect of the most promising ones on the root establishment of *A. marina* propagules.

1. MATERIALS AND METHODS

Sampling

Avicennia marina mangrove propagules were sampled along the central Red Sea within King Abdullah University of Science and Technology (KAUST) coastline (22.339914°N, 39.087972°E, Saudi Arabia) along a 500 meter transect. Mature propagules were randomly collected using sterile tools from nine different plants (one propagule from each plant). Samples were stored at 4°C until the isolation procedure.

Bacteria isolation, genotyping and identification

Propagules were pooled in three groups to perform bacteria isolation on three different media (n=3 per each group). Propagule teguments were surface-disinfected with 70% ethanol for 3 min, 1% sodium hypochlorite for 20 sec and 70% ethanol for 30 sec, followed by rinsing five times with sterile distilled water for 2 min and finally for 1 hour

(Cherif et al., 2015). The effectiveness of the disinfection procedure was evaluated by plating the last washing water on Trypic Soy Agar (TSA) plates. No colonies were obtained from all the control plates after 6-days incubation at 30°C. After disinfection, the propagule teguments (3 mm thick) were aseptically removed, the internal tissues were smashed in physiological solution (0.9% NaCl) using sterile mortar and pestle, and the obtained suspension was shaken at room temperature under rotation for 1 hour. One mL of the resulting suspension was 10-folds serially diluted in physiological solution and plated in triplicate onto different media, widely used for the selection of halotolerant/halophilic or endophytic bacteria: i) Marine Agar (Conda, Spain), ii) medium 869 1:10 (Barac et al., 2004) and iii) a mixture 1:1 (vol/vol) of Sea Salt (Sigma-Aldrich, St. Louis, MO, USA) and medium 869 1:10. After 72 hours of incubation at 30°C, colonies with different morphology were picked and streaked three successive times on the same medium to obtain pure bacterial cultures. A collection of 172 endophytic bacterial strains was established and cryopreserved in 25% glycerol at -80°C. Strain codes include different numbers according to the plant of origin (1/2/3: propagules collected from mangrove specimens 1-2-3; 4/5/6: propagules collected from mangrove specimens 4-5-6; 7/8/9: propagules collected from mangrove specimens 7-8-9) and indicate the medium used for isolation (MA: Marine Agar; M: medium 869 1:10; MS: 1:1 Sea Salt and 869 1:10).

The genomic DNA of each isolate was extracted by boiling cell lysis (Marasco et al., 2012). The bacteria collection was dereplicated by ITS-PCR (16S-23S rRNA Internal Transcribed Spacer-PCR) fingerprinting using the primers ITS-F (5'-GTCGTAACAAGGTAGCCGTA-3') and ITS-R (5'- GCCAAGGCATCCACC -3') as previously described (Cardinale et al., 2004; Mapelli et al., 2013). Isolates were grouped according to their identical ITS-PCR fingerprint profile and at least one representative

strain per each “ITS group” was selected for subsequent taxonomical identification and physiological characterization. Identification was performed by 16S rRNA gene partial sequencing (Macrogen, Rep. of South Korea), using the universal primers 27F (3’-AGAGTTTGATCMTGGCTCAG-5’) and 1492R (3’-CTACGGCTACCTTGTTACGA-5’) as previously described (Mapelli et al., 2013). 16S rRNA nucleotide sequences were subjected to BLAST search using the blastn program on NCBI database (Altschul et al., 1990) and were deposited in the ENA database under accession numbers LT978404-LT978452. The identification of the twelve selected strains used for the *in vivo* plant growth promotion assays was confirmed by sequencing their entire 16S rRNA gene.

In vitro characterization of bacterial isolates for PGP traits and abiotic stress tolerance

In vitro screening of PGP activities was performed on one representative strain for each polymorphic ITS-group, for a total of 48 strains. Inorganic phosphate solubilization and the production of indole-3-acetic acid (IAA), ammonia, protease and exopolysaccharides (EPS) were assessed as previously described (Cherif et al., 2015). Strains were also tested for abiotic stress tolerance, namely their ability to grow at 42°C (heat stress), in presence of 5% and 10% NaCl (salt stress) and 20% polyethylene glycol (PEG) (drought stress) (Mapelli et al., 2013). The isolates were ranked according to their PGP- and Stress-score (each positive-resulting test = 1 score point).

Root colonization analysis by fluorescence in situ *hybridization-confocal laser scanning microscopy (FISH-CLSM)*

The twelve selected isolates were tested for their root colonization efficiency on barley plants (*H. vulgare* cv. Propino) cultivated in growth chamber and under axenic, hydroponic conditions. The isolates were grown in liquid Tryptic Soy Broth (TSB) medium. Cells were harvested by centrifugation (15 min, 4000 rpm) and resuspended in

$MgSO_4$ 0.04 M to obtain a final concentration of ~$5x10^7$ CFUs ml^{-1}. Barley seeds were surface disinfected in~ 2.5% sodium hypochlorite (Rahman et al., 2018) and then incubated with the bacterial suspension for one hour at 25°C under gentle shaking. Immediately after, 5 coated seeds per each bacterial treatment were placed on sterile germination pouches (Mega International, USA) containing 20 ml of Hogland solution and 10 ml of NaCl solution (final salt concentration 0.17%; electrical conductivity: 4.62 dS/m). Pouches were inserted into sterile plastic bags to minimize air contamination. Controls were represented by non-coated seed incubated with 20 ml of sterile 0.04 M $MgSO_4$ and by seeds coated with *Escherichia coli* DSM 6897. The pouches were arranged in a randomized complete block design (RCBD (Clewer and Scarisbrick, 2008) with four blocks. The plants were grown for eight days in a climate chamber (18 h of illumination, 22°C during light period and 16°C during dark period, 60% relative humidity). Two plants for each treatment were used for assessing the bacterial root colonization by Fluorescent *In Situ* Hybridization (FISH) and Confocal Laser Scanning Microscopy (CLSM).

Barley roots inoculated with Gram negative isolates and uninoculated roots were fixed with a 3:1 mixture of 4% paraformaldehyde and ice–cold 1× Phosphate Buffered Saline (PBS), by incubation at 4°C for eight hours. The samples were then washed four times with ice–cold PBS, and then stored in 99.8% ethanol:PBS (1:1) at -20°C. Barley roots inoculated with Gram positive isolates were fixed directly in 99.8% ethanol:PBS (1:1) and stored at -20°C. Root segments of inoculated plants of about 0.5 cm length were stained by in tube–FISH (Cardinale et al., 2008), using the Cy3-labeled EUB338MIX probe (the equimolar mixture of EUB338, EUB338II and EUB338III probes) to stain all bacteria, and a Cy5- or FITC-labelled specific probe corresponding to the class or the phylum of the inoculated bacterium (Supplementary Table 1). Roots of uninoculated

control plants were stained with the Cy3-labelled EUB338MIX probe and the Cy5-labelled LGC354MIX probe. Hybridization was performed at 41°C for two hours in the dark, followed by washing at 42°C. Stained root samples were dipped for 5 seconds into ice-cold water, placed on a glass slide, dried out with soft compressed air, immediately mounted with antifade reagent, covered with a coverslip and finally sealed with nail polish. The occurrence of false positive signals derived from aspecific adhesion of FISH probes or fluorochromes to seed/root structures was checked by staining a subsample with Cy3-, FITC- and Cy5-labelled NONEUB probes (Supplementary Table 1).

FISH-stained roots were observed with a confocal laser Leica SP8 (Leica Microsystems GmbH, Mannheim, Germany) (Rahman et al., 2018). Volume-rendering and three-dimensional models of the confocal stacks were created with the software Imaris 8 (Bitplane AG, Zürich, Switzerland).

*PGP test on barley (*Hordeum vulgare*) under non-axenic conditions and salt stress*

Twelve isolates, selected on the basis of their PGP-Stress-score, their root colonization ability and their taxonomical broadness, were tested for PGP activity on potted barley plants under non-axenic conditions in greenhouse and under salt stress. Seed inoculation was performed as described above. After the incubation process, ten seeds per bacterial treatment were planted in square plastic pots containing approximately 920 ml (146 g dry weight) of Classic Tonsubstrat ED 73 soil substrate (Einheitserde- und Humuswerke Gebr. Patzer GmbH &Co. KG, Sinntal–Altengronau, Germany), a nutrient rich substrate (Supplementary Table 2). The water capacity (WC) of the moistened substrate was assessed as 120 ml. The pots were irrigated with 100 ml (83% WC) of 125 mM NaCl solution to reach the estimated concentration of 0.5% NaCl (g NaCl $Soil_{dw}^{-1}$). This irrigation allowed the whole substrate to moisten yet avoiding extensive percolation. Seeds were covered with 1 cm layer of moistened substrate and pots were arranged

223 according to a RCBD with 5 blocks. Controls were represented by non-inoculated seeds
224 (S+B-, where "S" indicates salinity and "B" bacteria) and by seeds coated with *E. coli*
225 DSM 6897. Besides the twelve bacterial isolates, an additional treatment was included,
226 namely the mixture of three isolates (treatment "MIX"), *Staphylococcus capitis* KMP789-
227 MA55, *Bacillus pumilus* KMP123-MS1 and *Gordonia terrae* KMP456-M40 (Table 1).
228 Plants were grown for 60 days in greenhouse with daylight of 18 hours (artificial light
229 switched off when natural light exceeded 10 Klx), and temperature of 20/18°C
230 (day/night). After eleven days, germination was considered complete and each pot was
231 rarefied to four plants. Immediately after rarefaction, each pot soil was inoculated with
232 50 ml of the respective bacterial suspension in 0.04 M $MgSO_4$ (10^8 CFUs ml^{-1}), to an
233 estimated final concentration of 3.4×10^7 cell g^{-1} soil (dw). At germination and
234 rarefaction, pots were irrigated with 100 ml of NaCl solutions (250 mM) to reach a final
235 salt concentration in the soil of 2.5%. Thereafter, tap water was used for irrigation, two
236 times per week. Every ten days, the plant height was recorded and, nine weeks after
237 sowing, the stems and the ears of the four plants were separately collected from each pot,
238 and their fresh weight was recorded ($g\,pot^{-1}$). Stems and ears were then dried at 80°C for
239 48 h before assessing the dry weight.

240 *Plant growth promoting assays on mangrove (*Avicennia marina*)*

241 *PGP test on mangrove* (A. marina) *under non-axenic conditions and salt stress*

242 Approximately 450 mature propagules of similar size, shape and color were collected
243 from *Avicennia marina* trees located in the sampling site described above and placed in
244 six germination beds (0.8m x 0.3m) containing 60% silver sand (playpit sand, Hanson
245 HeidelbergCement Group) and 40% substrate (Metromix 200). Pericarp was removed
246 from the propagules to facilitate the germination process and each germination bed was

watered with ~1.5 L solution composed by 50% Red Sea and 50% tap water (~2% final salinity). After two weeks, 200 germinated propagules were selected based on their size-homogeneity and transplanted in 50 plastic pots (four seeds per pot) containing 3 L of substrate (60% silver sand and 40% substrate Metromix 360) and arranged according to a RCBD with 5 blocks. Each propagule was inoculated with 3 ml of a bacterial suspension (*S. capitis* KMP789-MA55, *B. pumilus* KMP123-MS1 or *G. terrae* KMP456-M40, respectively; Table 1) in 0.04 M $MgSO_4$ to an estimated final concentration of 10^8 cells g^{-1} soil. Controls were setup as for the barley plant assay. Pots were watered with 700 ml of 1:1 Read Sea water and tap water into flower pot holders once a week. After two weeks, propagules were inoculated for the second time in the same way as the first inoculation. Plant height along with the number of leaves and internodes was recorded every 7 days for a total of 63 days.

Mangrove root establishment test and salt stress

Two hundred propagules were collected as described above and placed in four separated germination beds (0.8 m × 0.3 m) containing 60% silver sand (playpit sand, Hanson HeidelbergCement Group) and 40% substrate (Metromix 360). Propagules prepared in the different germination beds were separately treated with the three selected bacterial strains (*S. capitis* KMP789-MA55, *B. pumilus* KMP123-MS1 and *G. terrae* KMP456-M40; Table 1). Three milliliters of bacterial cells suspended in 0.04 M $MgSO_4$ were pipetted directly onto the root apical meristem of propagules (50 per treatment) and the surrounding soil to an estimated final concentration of 10^8 cells g^{-1} soil. Control propagules (50) were treated only with sterile 0.04 M $MgSO_4$. Substrate was watered once a week with 700 ml saline solution (2:1 Red Sea water and tap water). After 26 days, the root length of treated propagules was measured and compared with the non-inoculated controls.

Plant growth promotion assay on rice (O. sativa*)*

PGP test on rice under axenic conditions and salt stress

Rice seeds (*Oryza sativa* cv. Carnaroli) were surface disinfected with 2.5% bleach for 2.5 hours plus 5% bleach for 5 seconds at 25°C. Seeds were washed five times with sterile water before the imbibition period of 24 hours in sterile water. Three selected isolates, namely *S. capitis* KMP789-MA55, *B. pumilus* KMP123-MS1 and *G. terrae* KMP456-M40 (Table 1), were inoculated on rice seeds in the same way as the barley plant assay. Controls were prepared as in colonization assays on barley plants. Fifteen seeds per treatment were placed in Petri dishes containing 10 ml of MS solution at 0.10% NaCl (EC: 5.6 dS/m). Plants were grown for five days in a growth chamber (26°C; 12 hours of light/12 hours of darkness; 60% relative humidity). Stems and roots were harvested and then dried at 105°C for 24 hours before assessing the dry weight.

PGP test on rice under non axenic conditions and salt stress

Rice seeds were surface disinfected and inoculated with the same three selected strains used under axenic conditions (Table 1). Ten seeds per treatment were planted into plastic pots containing 3 L of substrate composed of 40% organic substrate (Florastar, ASDCO Fert), 30% silver sand (playpit sand, Hanson HeidelbergCement Group) and 10% vermiculite (Turface MVP, Turface Athletics). The WC of the moistened substrate was estimated to be 220 ml. Each pot was irrigated with 200 ml of solution (83% WC) composed by 69 ml sterile Red Sea water (3.8% salinity), 111 ml tap water and 20 ml NPK fertilising solution (200 gL^{-1} NO_3^{2-}; 200 gL^{-1} PO_4^{3-}; 200 gL^{-1} K^+). Seeds were covered with 1 cm layer of moistened substrate and pots were arranged according to a RCBD with 5 blocks. Negative controls were prepared as in colonization assays on barley plants. After two weeks, germination was considered complete and each pot was rarefied

296 to four plants. Immediately after rarefaction, each pot was inoculated with 50 ml of
297 bacterial suspension in 0.04 M $MgSO_4$ (10^8 CFUs ml^{-1}) directly onto the soil, to an
298 estimated final concentration of 10^7 cell g^{-1} substrate (dw). At rarefaction, pots were
299 watered with 200 ml solution composed by 50 ml sterile Red Sea water, 140 ml tap water,
300 10 ml NPK fertilising solution and 0.2 g iron chelate. Plants were watered three times a
301 week to maintain the substarte at constant WC. After 19 weeks of growth in greenhouse
302 (25°C; 70% UR; natural illumination), the stems and the ears of the four plants from each
303 pot were separately collected, and after 24 h at 105°C their dry weight was recorded (g
304 $plant^{-1}$).

305 *Statistical analyses*

306 Statistical differences of plant growth parameters were assessed between treatments by
307 ANOVA followed by Tukey Post-hoc test at $p < 0.05$, using the software SPSS 20 (IBM
308 Corporation, USA). Normality of distribution and omogeneity of variance were assessed
309 with Shapiro-Wilk and Levene's test respectively. Student's t-test was used to compare
310 the growth parameters of bacterized plants *vs.* non-inoculated negative controls. All
311 original data related to the PGP tests reported in this work are available within the
312 Dataverse "madforwater-wp3" created by the University of Milan at the following link:
313 https://doi.org/10.5072/FK2/AJALUQ.

314

315 **RESULTS**

316 *Bacterial isolation, identification and* in vitro *screening of PGP activities*

317 A total of 172 bacterial isolates were obtained from propagule internal tissue of *A. marina*
318 mangroves. The isolates clustered into 48 polymorphic ITS groups phylogenetically

affiliated to 18 species distributed in 10 genera (Supplementary Table 3) and 4 phyla (42% Proteobacteria, 37% Firmicutes, 17% Actinobacteria and 3.5% Bacteroidetes). Overall, the majority of the bacterial isolates belonged to the genera *Acinetobacter* (Proteobacteria) and *Staphylococcus* (Firmicutes) (Supplementary Table 3). Medium 869 1:10, not saline and largerly used to isolate plant endophytes (Barac et al., 2004), allowed the isolation of bacterial strains from all the samples, for a total of 9 different species. The same medium with the addition of sea-salts to simulate the marine environment, led to isolate bacteria only from one of the pool propagule samples, all affiliated to three species of the Firmicutes phylum (Supplementary Table 3, Supplementary Table 4). The conventional marine medium Marine Agar allowed the isolation of 8 bacterial species, generating a phylogenetic diversity similar to Medium 869 1:10.

One strain from each ITS group (n=48) was tested *in vitro* for traits related to PGP activity. The most widespread activities within the selected isolate collection were IAA and ammonium production, whereas none of the strains produced EPS or solubilized phosphate (Supplementary Table 3). The results of the PGP activity tests were computed for each strain in a "PGP score", reporting the total number of positive activities. Isolates belonging to the genus *Micrococcus* (7 strains of 3 different species, 8% of the collection) showed the highest PGP score, being positive for 3-4 of the tested potential PGP traits. All the strains belonging to *Staphylococcus* and *Rhizobium* genera showed a PGP score of 2, except the strain *S. capitis* KMP789-MA55 that was positive to 3 PGP traits (Supplementary Table 3, Fig. 1). Aiming to test the ability of the isolates to thrive in the mangrove ecosystem, they were also tested for the tolerance to abiotic stresses typical of this environment: high temperature, salt and osmotic stress. Overall, propagule endophytes showed a high tolerance toward abiotic stresses (Supplementary Table 3, Fig. 1). The majority of the strains was indeed able to grow at 42°C (81% of the tested strains),

in growth medium supplemented by 5% NaCl (62%), 10% NaCl (39%) and in PEG-containing medium which confers osmotic stress (83%). None of the strains demonstrated strictly halophilic habit, since all of them were able to grow in the absence of salt supplement to the medium. Overall, the *Staphylococcus* genus demonstrated the highest levels of abiotic stress tolerance (Supplementary Table 3, Fig. 1).

Twelve isolates were selected for the *in vivo* plant growth promotion test on barley (Table 1) based on i) high PGP score, ii) broad taxonomic affiliation and iii) rapid growth rate (data not shown).

Barley root colonization ability

Barley seeds coated with bacteria were cultivated under salt stress in hydroponic axenic conditions, with the aim to analyse by FISH-CLSM the bacterial root colonization ability. These information were used, together with the results of the *in vivo* barley PGP assay, to select the best candidates to be further tested *in vivo* on mangrove and rice. The twelve tested isolates showed different root colonization abilities. Seed inoculation with *Gordonia* KMP456-M40, *Enterococcus* KMP789-M107, *Micrococcus* KMP789-MA53, *Staphylococcus* KMP123-MS2 and –MS3, *Acinetobacter* KMP123-MA14 and *Bacillus* KMP123-MS1 resulted in extensive root colonization, as demonstrated by the observation of dense bacterial microcolonies on the root surface (Fig. 2B-C; Fig. S1A-G). The other isolates tested, on the contrary, did not show evident root colonization ability (Fig. S1H-I). The preferential site of colonization was the surface of the roots, especially the root hairs in the developing zone (Fig. 2; Fig. S1); the root autofluorescence was intense enough to allow identification of the root tissues.

FISH-CLSM images revealed bacterial root colonization also in non-inoculated control plants stained with the universal bacterial EUB338MIX probe (Fig. 2A), reasonably conferred by native seed endophytes. Similarly, the bacterial cells stained only by the EUB338MIX probe in the inoculated roots, also should be considered as native seed endophytes (Fig. 2; Fig. S1 D-H). The finding of native root endophytes was expected, since it is known that barley seeds host an endophytic bacterial community which can colonize the root habitat upon seed germination (Rahman et al., 2018). Barley roots inoculated with *Staphylococcus* KMP123-MS2, *Acinetobacter* KMP123-MA14 and *Bacillus* KMP123-MS1, showed a higher level of colonization by native endophytes (red cells, Fig. 2B-C; Fig. S1D-F) compared to non-inoculated roots (Fig. 2A), suggesting a possible stimulating effect of the inoculated bacteria on the native seed microbiota. Interestingly, it appeared that indigenous endophytes were able to interact with these isolates, ending up with the formation of mixed micro-colonies (Fig. 2B-C; Fig. S1D-F). It must be considered that some seed endophytes might also belong to the the same taxonomical group of the inoculated bacterium, resulting in a double staining and potentially leading to an overestimation of the inoculants. However, roots of uninoculated plants, when stained with the probe LGC354MIX specific for Firmicutes, did not show any double-stained bacterial cell (Fig. 2A), thus confirming that all the LGC354MIX-stained cells on the roots of plants inoculated with *Bacillus* KMP123-MS1 were belonging to the inoculant rather than to endogenous endophytes of the same phylogenetic group (Fig. 2B-C; Fig. S1F).

Bacteria mediated plant growth promotion on barley cultivated under salt stress

The twelve selected strains were applied, separately or in mixture, to barley seeds subsequently planted in potted soil and cultivated under saline stress in greenhouse for

392 the entire plant cycle. No effect of the bacterial inoculation was observed on the fresh and
393 dry shoot weight for any of the strains (ANOVA, $p > 0.05$; Supplementary Table 5).
394 However, the strain *Gordonia terrae* KMP456-M40 demonstrated a PGP activity by
395 significantly increasing the ear dry weight by 65%, when compared with control non-
396 inoculated plants and also with plants inoculated with a non-PGP *E. coli* strain (ANOVA,
397 $p = 0.006$) (Fig. 3). The beneficial effect of this strain on barley was also confirmed in
398 axenic conditions, obtaining a significant increase in root and shoot dry weight in
399 comparison with control plants (Student's t-test, $p < 0.001$ and $p = 0.019$ respectively;
400 data not shown).

401

402 *Bacteria mediated plant growth promotion on mangrove propagules and rice cultivated*
403 *under salt stress*

404 The PGP effect on mangrove and rice plants was evaluated for the following strains: *G.*
405 *terrae* KMP456-M40, chosen because it showed a significant positive effect on barley,
406 *B. pumilus* KMP123-MS1, chosen because it was the best barley root colonizer and
407 appeared to interact synergistically with the native seed endophytes, and *S. capitis*
408 KMP789-MA55, chosen because it had the highest *in vitro* PGP potential among the
409 *Staphylococcus* spp. abundantly present in the collection.

410 In a mangrove propagule germination assay, *G. terrae* KMP456-M40 significantly
411 affected root establishment, inducing the development of longer roots, compared to the
412 non-inoculated propagules, during the first weeks of growth (Student's t-test, $p = 0.03$;
413 Fig. 4). However, in the following growth stage neither KMP456-M40 nor the other tested
414 strains further improved the growth parameters of *A. marina* plantlets developed from
415 propagules growing in non-sterile substrate over a period of 4 months. Plant height,

number of leaves and internodes were indeed not significantly different among treatments (ANOVA, $p > 0.08$; Supplementary Table 6).

A significant PGP effect induced by the selected propagule endophytes was observed in rice cultivated under axenic salty-hydroponic condition (Fig. 5). Two out of the three tested strains induced a significant increase of the dry weight of plants: *G. terrae* KMP456-M40 and *S. capitis* KMP789-MA55 significantly increased rice biomass of 62 and 65%, respectively (ANOVA, $p < 0.001$; Fig. 5). Such positive PGP effect was nevertheless not observed when rice was cultivated in non-sterile soil (ANOVA, $p > 0.5$) (Supplementary Table 7).

DISCUSSION

In this work, we demonstrated that mangrove propagules harbour bacterial endophytes that are beneficial to the root establishment of mangrove plantlets, and/or to the germination and growth or productivity of non-host plant species like rice and barley. The isolate collection established from the endosphere of mangrove propagules included representatives of four bacterial phyla (Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes) which are largely associated with seeds of a wide range of plants (Johnston-Monje and Raizada, 2011; Nelson, 2017; Truyens et al., 2015). This indicated that the particular reproductive units of *A. marina*, different in the developmental biology from seeds, host microorganisms with the potential of vertical transimission by mangrove plants, suggesting their crucial ecological role for mangrove establishment. These phyla were found to be abundantly present also in other plant tissues (Truyens et al., 2015) and were reported as dominant in soil and acquatic ecosystems (Fierer et al., 2012; Shafi et al., 2017). Furthermore, several genera in our endophyte collection (*e.g. Acineotbacter*,

Bacillus, *Micrococcus*, *Rhizobium*, *Staphylococcus*) are common in plant seeds (Alibrandi et al., 2017; Truyens et al., 2013; Truyens et al., 2015). Bacterial strains isolated from propagules belonged to taxa commonly found in plant/soil habitats and not typical of the marine environment. We therefore speculate that the mangrove plant, despite its tidal habitat, mainly recruits bacterial endophytes from the soil environment rather than from the seawater. The presence of *Staphylococcus* isolates, commonly found in association with humans (Kloos and Musselwhite, 1975), may be interpreted as signature of anthropization of the ecosystem where mangrove propagules were collected, hypothesizing their uptake from the water/sediment through the root system. *Staphylococcus* isolates were, however, recently found consistently associated with plant tissues (Ali et al., 2010), including seeds (Alibrandi et al., 2017; Sánchez-López et al., 2018). Our isolates showed indeed a considerable tolerance to a wide range of abiotic stresses typical of the coastal mangrove environment, like high temperature, osmotic stress and high NaCl concentration, indicating that they are adaptated to this specific habitat. High-throughput sequencing-based studies specifically designed to the evaluation of the overall microbiota structure and diversity in mangrove propagules and surrounding water and sediments could further support these observations.

The majority (80%) of the 48 isolates tested *in vitro* for PGP traits showed a potential to benefit the plant through several different mechanisms comprising auxin production (71% of the strains) in accordance to their endophytic lifestyle (Hardoim et al., 2015), thus indicating a role in sustaining mangrove growth. The finding of such potentially beneficial bacteria in the tissues of the mangrove early juveniles, the propagules, can lead to hypothesise their vertical transmission to the new plant generation and a consequent key role on the plant fitness, a possibility that should be verified by specific dedicated experiments.

The PGP potential of the cultured bacterial endophytes was further tested *in vivo* on phylogenetically distant plants of pivotal agricultural interest, the cereals barley and rice having different salt sensitivity.

The strain *G. terrae* KMP456-M40, which significantly improved root establishment of mangrove propagules, also positively affected the growth of rice and the growth and productivity of barley cultivated under salt stress. The genus *Gordonia* has recently attracted great interest for biotechnological applications due to the high potential of some species to degrade xenobiotics and environmental pollutants (Arenskötter et al., 2004). Kayasth *et al.* (2014) isolated a *Gordonia* strain from the rhizosphere of the halophyte *Chenopodium murale*, which showed nitrogen fixing and other PGP activities when inoculated on pearl millet. Here we showed that strain *G. terrae* KMP456-M40 improved rice dry mass by 62% under saline and gnotobiotic conditions and the dry weight of barley ears by about 65% under non-axenic conditions. These results largely exceed the performances previously described for any PGP bacteria on barley grain yield that was enhanced maximum up to 27% (Baris et al., 2014). The growth promotion effects of *G. terrae* KMP456-M40 on barley ears could be driven by the supply of auxins, which the strain was capable to produce in *in vitro* conditions. However, we cannot exclude either that other non-tested PGP activities may play a role since *G. terrae* KMP456-M40 was, among the twelve isolates tested on barley, the strain exhibiting the lowest PGP-score *in vitro* (Table 1). This observation confirms previous works, which demonstrated that bacteria with scarse PGP-related traits *in vitro* can perform better *in vivo* (Cardinale et al., 2015).

The strain *B. pumilus* KMP123-MS1 demonstrated to be an efficient root colonizer of the barley seeds and to be capable to interact with the seed indigenous microbiota as indicated by the formation of mixed micro-colonies on root tissues. Despite the excellent root

colonization capacity, *B. pumilus* KMP123-MS1 did not promote growth or productivity of the three tested plant species, mangrove, barley and rice. Plant tissue colonization by strain *B. pumilus* KMP123-MS1 was nevertheless not detrimental to the plants and therefore it is possible that it can provide beneficial effects that we did not measure (e.g. protection from phytopatogens, higher fitness in field conditions or under intense abiotic stresses).

Two mangrove endophytes demonstrated the ability to promote the growth of other phylogenetically unrelated plant species. Nonetheless, the PGP effect and the interaction with the competing soil microbiome was strictly depending on the plant host. *Gordonia terrae* KMP456-M40 was the unique strain that promoted barley growth (ear dry weight) in not sterile soil, in competition with the autochtonous soil community. Differently, the same strain when inoculated on rice showed PGP effects only in axenic conditions, in the absence of autochtonous competitors. The strain *S. capitis* KMP789-MA55 induced significant beneficial effects only on rice and only under gnotobiotic conditions, resulting in a significantly higher plant dry weight. These results are in accordance with the literature, which indicates how the ability to promote plant growth of different species by a given PGP bacterial strain is highly variable and depends upon each plant-strain pair (Marasco et al., 2013; Rolli et al., 2015).

When our selected propagule endophytes were reinoculated on mangroves, no promotion effect was observed on the aerial part after 4 months of growth in potted soil. Possibly, long-term growth experiments would be necessary to measure positive effects on the plant growth and performances. However, propagules inoculated with *G. terrae* KMP456-M40 developed significantly longer roots compared to non-inoculated plants during the first 26 days after planting. Our results showed that mangrove propagule potentially benefits from the interaction with their own endophytes by improving the seedling fitness for

fixing in the sediment against the challenge of the tidal flow. Such finding shows a novel ecological service provided by endophytes to the plant host and indicates a selective force that may drive the process of vertical inheritance of bacteria in mangroves. Despite a large literature body focusing on plant endophytes, there is a gap of knowledge on the vertical inheritance of endophytes in plants living under extreme environmental conditions; to the best of our knowledge, only one study demonstrated so far their importance for the survival and germination of cacti seedling (Osborne and Berjak 1997). From a natural selection perspective, hosting bacteria able to increase root lenght of juvenile plants in the crucial phase of soil colonization represents a competitive advantage under the intense tidal regimes to which *A. marina* and other mangrove species are exposed to (Balke et al., 2011). The capacity to promptly settle in soil is one of the main factors promoting mangrove growth considering that light and space availability are not limiting in their ecosystems, thus decreasing the importance to promote the growth of the aerial parts.

CONCLUSIONS

Our results reveal the existence of an endophytic beneficial microbiome in the mangrove inheritance organs, the propagules, which are capable to promote plant establishment in the critical early growth phase of newborn plants. This finding highlights the importance of plant-bacteria association under extreme environmental condition and suggests a relevant role of the plant microbiota for the protection of coastal ecosystems. Some of the cultured endophytes, in particular the strain *Gordonia terrae* KMP456-M40, demonstrated to be able to enhance the growth of two cereal crops (barley and rice, largely used as staple food) under salt stress, thus being promising candidates for a sustainable agricultural production in salt-affected soils.

Moreover, this work added a further piece of evidence claiming a change in the research pipelines adopted to find new efficient PGP bacteria. *In vivo* primary strain screening should be preferentially adopted since *in vitro* selection of potential PGP candidates can lead to overlook the active ones. Selection of PGP bacterial strains needs to be, moreover, tailored for the plant species of interest, since the *in vivo* beneficial effect is hardly predictable basing on data obtained on different species.

The screening for the best candidates is neverhtless only the first step towards the establishment of PGP bacterial culture collections. Future works must focus on the mechanisms of interactions, which will shed light on the molecular basis of the growth promotion. This will in turn act as a positive feedback to improve the efficiency of both isolation and selection strategies.

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Figure Legends

Figure 1. Plant growth promotion (PGP) traits and stress tolerance score observed within the propagule endophytic bacteria collection. One representative isolate for each ITS group (N = 48) was characterized *in vitro* for PGP activities (production of ammonium, indole-3-acetic acid, proteases and siderophores, release of exopolysaccharides and solubilization ohinorganic phosphate). Results are represented according to the taxonomic identification of the strains at the genus level (number of the isolates tested for each phylogenetic group is reported), expressed as "PGP score" and "stress score" accounted as the number of positive tests obtained by each representative strain. The percentage of strains exhibiting each score value are reported for each genus.

Figure 2. Barley root colonization by *Bacillus pumilus* KMP123-MS1. Fluorescence *in situ* hybridization-confocal laser scanning microscopy (FISH-CLSM) images of barley root colonized by isolate KMP123-MS1 through seed coating. Yellow: *Firmicutes* (double stained by both the Cy5-labeled LGC345-MIX and the Cy3-labeled EUB338-MIX probes); red: other bacteria (stained by the EUBMIX probe only); cyan: root autofluorescence **(A)** Maximum projection of non-inoculated barley root with cells of native seed endophytes (arrow). **(B)** Maximum projection of a barley root after seed inoculation with *Bacillus pumilus* KMP123-MS1. Mixed colonies between native barley seed endophytes (red) and *B. pumilus* KMP123-MS1 (yellow) suggest an interaction during root development. **(C)** Three-dimensional model of panel B. Scale bars: A, 50 µm; B, 25 µm; C, 20 µm.

Figure 3. Plant growth promotion assay on barley under saline condition. Dry weight of barley ears obtained from plants inoculated separately with each of the twelve endophytic strains. Plants inoculated by the non-PGP *Escherichia coli* strain DSM 6897 were included as additional control. Significant differences (ANOVA, $p < 0.01$, followed by Tukey test, $p < 0.05$) were indicated by letters. Error bar: ± 1 SE. An illustrative image of ears from inoculated (*Gordonia terrae* KMP456-M40) and non-inoculated (S+B-) barley plants is included on the top of the graph. Scale bar: 4 cm.

Figure 4. Plant growth promotion of mangrove seedling by *Gordonia terrae* KMP456-M40. Root length of mangrove seedlings inoculated with strain *Gordonia* KMP456-M40 and non-inoculated (S+B-) mangrove seedlings. Illustrative images of inoculated (KMP456-M40) and non-inoculated (S+B-) mangrove propagules after root emission are included on the top of the graph. Significant differences (Student's T-test, $p < 0.05$) were indicated by asterisk. Error bar: ±1 SE. Scale bar: 1 cm.

Figure 5. *In vivo* rice growth promotion assay of selected mangrove propagule endophytes under salt stress. Dry weight of rice plantlets i) inoculated with the propagule endophytic isolates KMP789-MA55, KMP123-MS1, KMP456-M40, ii) inoculated the non-PGP strain *Escherichia coli* DSM 6897 and iii) non-inoculated (S+B-). An illustrative image of inoculated (by the strain KMP456-M40) and non-inoculated (S+B-) rice plantlets is included on the top of the graph. Significant differences (ANOVA, $p < 0.01$, followed by Tukey test, $p < 0.05$) were indicated by letters. Error bar: ± 1 SE. Scale bar: 1 cm.

Table 1. Identification, Plant growth promoting (PGP) traits and abiotic stress tolerance of the propagule endophytic strains selected for *in vivo* PGP experiments. The list includes the strain taxonomic classification and the results of the physiological tests performed *in vitro*. Grey boxes indicate a positive screening result. IAA = indole-3-acetic acid production; P Sol = inorganic phosphate solubilization; NH_3 = ammonium production; Sid = siderophore production; Prot = protease production; EPS = exopolysaccharides release; PEG = 20% polyethylene glycol.

Isolate name	Closest relative species (% of 16SrRNA identity)*	PGP activity						PGP score	Abiotic stress tolerance				Stress score	Total score
		IAA	P Sol	NH_3	Sid	Prot	EPS		42°C	5%NaCl	10%NaCl	PEG		
KMP123-MA14	*Acinetobacter ursingii* (98)	■		■				2	■	■	■	■	4	6
KMP123-MS1	*Bacillus pumilus* (100)	■		■				2	■	■	■	■	4	6
KMP456-M40	*Gordonia terrae* (99)	■						1	■	■		■	3	4
KMP789-M107	*Enterococcus casseliflavus* (97)	■				■		2	■	■		■	3	5
KMP789-MA46	*Micrococcus luteus* (99)	■		■		■		3	■	■	■	■	4	7
KMP789-MA53	*Micrococcus yunnanensis* (99)	■		■	■	■		4	■	■		■	3	7
KMP123-M1	*Rhizobium huautlense* (99)	■		■				2				■	1	3
KMP789-MA55	*Staphylococcus capitis* (99)	■		■	■			3	■	■	■	■	4	7
KMP789-MA47	*Staphylococcus epidermidis* (100)	■			■			2	■	■	■	■	4	6
KMP123-MS3	*Staphylococcus massiliensis* (99)	■		■				2	■	■	■	■	4	6
KMP123-MS2	*Staphylococcus cohnii* (100)	■		■				2	■	■	■	■	4	6
KMP123-MA18	*Staphylococcus saprophyticus* (99)	■		■				2	■	■	■	■	4	6

***** According to BLAST alignment of the full 16S rRNA gene sequence