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

75 Colmer, 2015; Oh et al., 2012; Parida and Jha, 2010). Mangroves are among the most
76 salt tolerant plants known and play a pivotal ecological role for preservation and
77 productivity of tropical coastal ecosystems (Donato et al., 2011; Ezcurra et al., 2016;
78 Sutton-Grier et al., 2015). As other plants growing in naturally saline environments,
79 mangroves host halotolerant and halophilic bacteria (Castro et al., 2014) and were
80 proposed as a valuable source of PGP bacteria (Bashan and Holguin, 2002). Due to the
81 increasing salinization of soils in many regions of the Earth, as a consequence of intensive
82 agricultural practices and climate change, there is a growing interest in the possible
83 exploitation of microorganisms adapted to high salinity as plant
84 biofertilizers/biostimulants (Cardinale et al., 2015; Cho et al., 2015; Egamberdieva et al.,
85 2008; Egamberdieva et al., 2011; Mapelli et al., 2013; Soussi et al., 2016; Tiwari et al.,
86 2011).
87 The intimate and potentially inheritable positive association of plants with microoganisms
88 is supported by the finding of endophytic PGP bacteria in the plant seeds (Truyens et al.,
89 2015). The first stages of seedling establishment, characterized by high mortality,
90 influence the distribution and fitness of adult plants upon different abiotic and biotic
91 factors (Rand, 2000). The potential inheritance of PGP microbial partners can indeed be
92 especially important in coastal ecosystems, where the first stages of plant growth are
93 challenged by rapid and continuous shift in the environmental conditions. To counteract
94 these adverse conditions, most of the mangrove tree species evolved vivipary (Hong et
95 al., 2018; Kathiresan and Rajendran, 2002; Osborne and Berjak, 1997) and produce
96 propagules that contains a seedling able to rapidly root once droped on the sediment or to
97 survive long floating periods when dispersed by the tidal currents.
98 We hypothesize that mangrove propagules harbor beneficial endophytic bacteria capable
99 to enhance the root establishment of mangrove seedlings once fallen from the plants into

100 the seawater, thus playing a role for the stability of the overall mangrove ecosystem. We
101 also hypothesize that beneficial bacteria selected by mangrove propagules can favor non-
102 host plant species, including crops, potentially contributing to enhance salt tolerance and
103 improve their productivity in arid/saline soils, a major abiotic stress threatening modern
104 agriculture (Chaves et al., 2009; Tester and Danevport, 2003).
105 The aim of this work was to characterize the cultivable bacterial endophytes of *Avicennia*
106 *marina* propagules, assessing their potential to promote plant growth and productivity
107 under salt stress. We therefore evaluated the potential of selected propagule endophytes
108 to mitigate salt stress on two cereal crops with different tolerance to soil salinity, *i.e.*
109 barley (*Hordeum vulgare* L., salt tolerant) and rice (*Oryza sativa* L., salt sensitive) and
110 the effect of the most promising ones on the root establishment of *A. marina* propagules.
111

112 ## 1. MATERIALS AND METHODS

113 *Sampling*

114 *Avicennia marina* mangrove propagules were sampled along the central Red Sea within
115 King Abdullah University of Science and Technology (KAUST) coastline (22.339914°N,
116 39.087972°E, Saudi Arabia) along a 500 meter transect. Mature propagules were
117 randomly collected using sterile tools from nine different plants (one propagule from each
118 plant). Samples were stored at 4°C until the isolation procedure.

119 *Bacteria isolation, genotyping and identification*

120 Propagules were pooled in three groups to perform bacteria isolation on three different
121 media (n=3 per each group). Propagule teguments were surface-disinfected with 70%
122 ethanol for 3 min, 1% sodium hypochlorite for 20 sec and 70% ethanol for 30 sec,
123 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

149 strain per each “ITS group” was selected for subsequent taxonomical identification and
150 physiological characterization. Identification was performed by 16S rRNA gene partial
151 sequencing (Macrogen, Rep. of South Korea), using the universal primers 27F (3’-
152 AGAGTTTGATCMTGGCTCAG-5’) and 1492R (3’-CTACGGCTACCTTGTTACGA-
153 5’) as previously described (Mapelli et al., 2013). 16S rRNA nucleotide sequences were
154 subjected to BLAST search using the blastn program on NCBI database (Altschul et al.,
155 1990) and were deposited in the ENA database under accession numbers LT978404-
156 LT978452. The identification of the twelve selected strains used for the *in vivo* plant
157 growth promotion assays was confirmed by sequencing their entire 16S rRNA gene.

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

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

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

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

173 $MgSO_4$ 0.04 M to obtain a final concentration of ~$5x10^7$ CFUs ml^{-1}. Barley seeds were
174 surface disinfected in ~ 2.5% sodium hypochlorite (Rahman et al., 2018) and then
175 incubated with the bacterial suspension for one hour at 25°C under gentle shaking.
176 Immediately after, 5 coated seeds per each bacterial treatment were placed on sterile
177 germination pouches (Mega International, USA) containing 20 ml of Hogland solution
178 and 10 ml of NaCl solution (final salt concentration 0.17%; electrical conductivity: 4.62
179 dS/m). Pouches were inserted into sterile plastic bags to minimize air contamination.
180 Controls were represented by non-coated seed incubated with 20 ml of sterile 0.04 M
181 $MgSO_4$ and by seeds coated with *Escherichia coli* DSM 6897. The pouches were arranged
182 in a randomized complete block design (RCBD (Clewer and Scarisbrick, 2008) with four
183 blocks. The plants were grown for eight days in a climate chamber (18 h of illumination,
184 22°C during light period and 16°C during dark period, 60% relative humidity). Two
185 plants for each treatment were used for assessing the bacterial root colonization by
186 Fluorescent *In Situ* Hybridization (FISH) and Confocal Laser Scanning Microscopy
187 (CLSM).

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

198 control plants were stained with the Cy3-labelled EUB338MIX probe and the Cy5-
199 labelled LGC354MIX probe. Hybridization was performed at 41°C for two hours in the
200 dark, followed by washing at 42°C. Stained root samples were dipped for 5 seconds into
201 ice-cold water, placed on a glass slide, dried out with soft compressed air, immediately
202 mounted with antifade reagent, covered with a coverslip and finally sealed with nail
203 polish. The occurrence of false positive signals derived from aspecific adhesion of FISH
204 probes or fluorochromes to seed/root structures was checked by staining a subsample with
205 Cy3-, FITC- and Cy5-labelled NONEUB probes (Supplementary Table 1).
206 FISH-stained roots were observed with a confocal laser Leica SP8 (Leica Microsystems
207 GmbH, Mannheim, Germany) (Rahman et al., 2018). Volume-rendering and three-
208 dimensional models of the confocal stacks were created with the software Imaris 8
209 (Bitplane AG, Zürich, Switzerland).

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

211 Twelve isolates, selected on the basis of their PGP-Stress-score, their root colonization
212 ability and their taxonomical broadness, were tested for PGP activity on potted barley
213 plants under non-axenic conditions in greenhouse and under salt stress. Seed inoculation
214 was performed as described above. After the incubation process, ten seeds per bacterial
215 treatment were planted in square plastic pots containing approximately 920 ml (146 g dry
216 weight) of Classic Tonsubstrat ED 73 soil substrate (Einheitserde- und Humuswerke
217 Gebr. Patzer GmbH &Co. KG, Sinntal–Altengronau, Germany), a nutrient rich substrate
218 (Supplementary Table 2). The water capacity (WC) of the moistened substrate was
219 assessed as 120 ml. The pots were irrigated with 100 ml (83% WC) of 125 mM NaCl
220 solution to reach the estimated concentration of 0.5% NaCl (g NaCl $Soil_{dw}^{-1}$). This
221 irrigation allowed the whole substrate to moisten yet avoiding extensive percolation.
222 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 were obtained from single
312 experiments and are available within the Dataverse "madforwater-wp3" created by the
313 University of Milan at the following link: 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
319 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-

345 containing medium which confers osmotic stress (83%). None of the strains demonstrated
346 strictly halophilic habit, since all of them were able to grow in the absence of salt
347 supplement to the medium. Overall, the *Staphylococcus* genus demonstrated the highest
348 levels of abiotic stress tolerance (Supplementary Table 3, Fig. 1).

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

352

353 *Barley root colonization ability*

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

367 FISH-CLSM images revealed bacterial root colonization also in non-inoculated control
368 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 the entire plant cycle. No effect of the bacterial inoculation was observed on the fresh and 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,
416 number of leaves and internodes were indeed not significantly different among treatments
417 (ANOVA, $p > 0.08$; Supplementary Table 6).

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

425

426 **DISCUSSION**

427 In this work, we demonstrated that mangrove propagules harbour bacterial endophytes
428 that are beneficial to the root establishment of mangrove plantlets, and/or to the
429 germination and growth or productivity of non-host plant species like rice and barley. The
430 isolate collection established from the endosphere of mangrove propagules included
431 representatives of four bacterial phyla (Proteobacteria, Firmicutes, Actinobacteria,
432 Bacteroidetes) which are largely associated with seeds of a wide range of plants
433 (Johnston-Monje and Raizada, 2011; Nelson, 2017; Truyens et al., 2015). This indicated
434 that the particular reproductive units of *A. marina*, different in the developmental biology
435 from seeds, host microorganisms with the potential of vertical transimission by mangrove
436 plants, suggesting their crucial ecological role for mangrove establishment. These phyla
437 were found to be abundantly present also in other plant tissues (Truyens et al., 2015) and
438 were reported as dominant in soil and acquatic ecosystems (Fierer et al., 2012; Shafi et
439 al., 2017). Furthermore, several genera in our endophyte collection (*e.g. Acineotbacter*,
440 *Bacillus*, *Micrococcus*, *Rhizobium*, *Staphylococcus*) are common in plant seeds
441 (Alibrandi et al., 2017; Truyens et al., 2013; Truyens et al., 2015). Bacterial strains
442 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

493 therefore it is possible that it can provide beneficial effects that we did not measure (e.g.
494 protection from phytopatogens, higher fitness in field conditions or under intense abiotic
495 stresses).
496 Two mangrove endophytes demonstrated the ability to promote the growth of other
497 phylogenetically unrelated plant species. Nonetheless, the PGP effect and the interaction
498 with the competing soil microbiome was strictly depending on the plant host. *Gordonia*
499 *terrae* KMP456-M40 was the unique strain that promoted barley growth (ear dry weight)
500 in not sterile soil, in competition with the autochtonous soil community. Differently, the
501 same strain when inoculated on rice showed PGP effects only in axenic conditions, in the
502 absence of autochtonous competitors. The strain *S. capitis* KMP789-MA55 induced
503 significant beneficial effects only on rice and only under gnotobiotic conditions, resulting
504 in a significantly higher plant dry weight. These results are in accordance with the
505 literature, which indicates how the ability to promote plant growth of different species by
506 a given PGP bacterial strain is highly variable and depends upon each plant-strain pair
507 (Marasco et al., 2013; Rolli et al., 2015).
508 When our selected propagule endophytes were reinoculated on mangroves, no promotion
509 effect was observed on the aerial part after 4 months of growth in potted soil. Possibly,
510 long-term growth experiments would be necessary to measure positive effects on the plant
511 growth and performances. However, propagules inoculated with *G. terrae* KMP456-M40
512 developed significantly longer roots compared to non-inoculated plants during the first
513 26 days after planting. Our results showed that mangrove propagule potentially benefits
514 from the interaction with their own endophytes by improving the seedling fitness for
515 fixing in the sediment against the challenge of the tidal flow. Such finding shows a novel
516 ecological service provided by endophytes to the plant host and indicates a selective force
517 that may drive the process of vertical inheritance of bacteria in mangroves. Despite a large

518 literature body focusing on plant endophytes, there is a gap of knowledge on the vertical
519 inheritance of endophytes in plants living under extreme environmental conditions; to the
520 best of our knowledge, only one study demonstrated so far their importance for the
521 survival and germination of cacti seedling (Osborne and Berjak 1997). From a natural
522 selection perspective, hosting bacteria able to increase root lenght of juvenile plants in
523 the crucial phase of soil colonization represents a competitive advantage under the intense
524 tidal regimes to which *A. marina* and other mangrove species are exposed to (Balke et al.,
525 2011). The capacity to promptly settle in soil is one of the main factors promoting
526 mangrove growth considering that light and space availability are not limiting in their
527 ecosystems, thus decreasing the importance to promote the growth of the aerial parts.
528

529 **CONCLUSIONS**

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

539 Moreover, this work added a further piece of evidence claiming a change in the research
540 pipelines adopted to find new efficient PGP bacteria. *In vivo* primary strain screening
541 should be preferentially adopted since *in vitro* selection of potential PGP candidates can
542 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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770 **Figure Legends**

771

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

781

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

793

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.

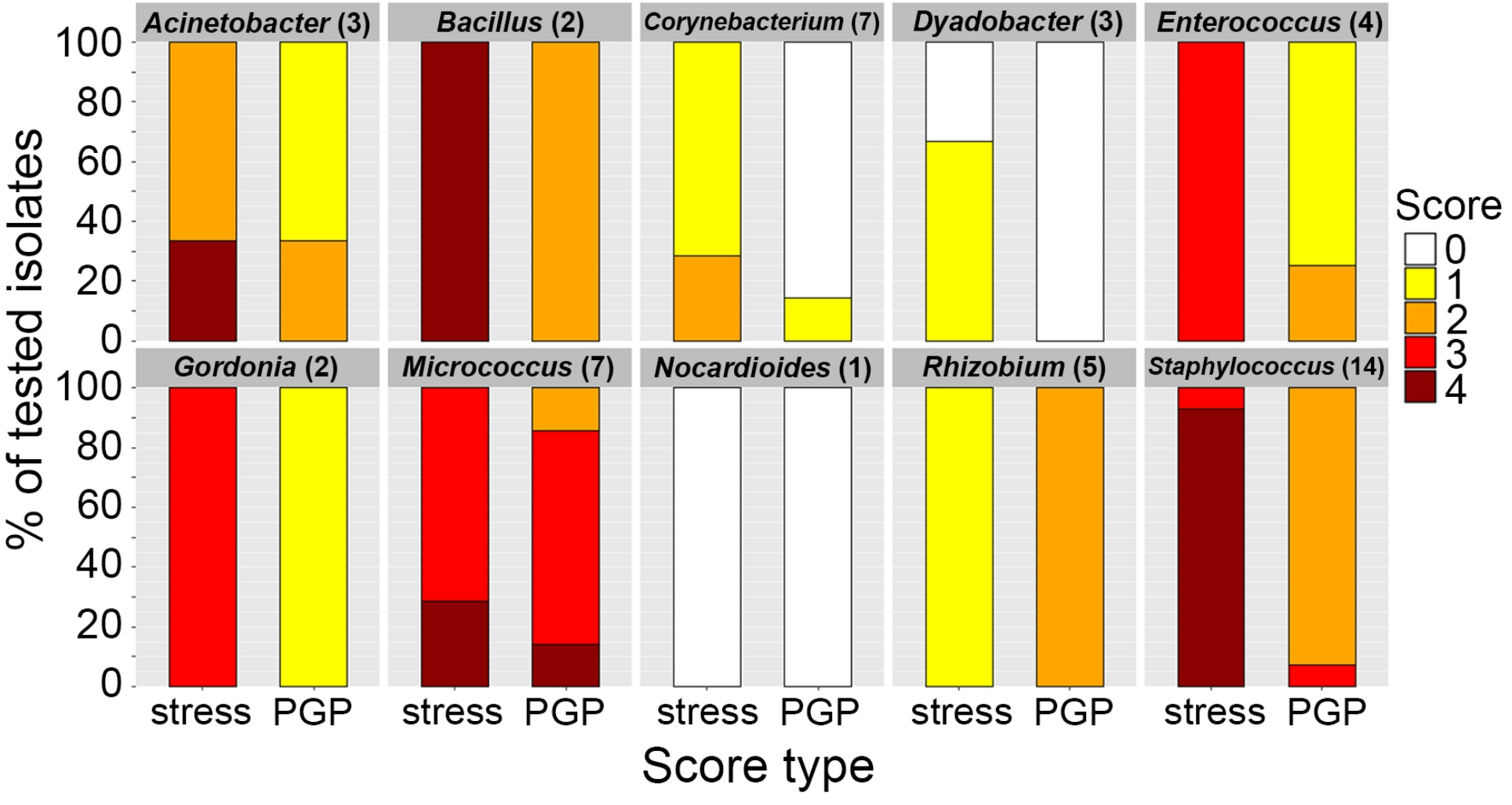
Figure 4. Plant growth promotion of mangrove seedling by *Gordonia terrae* KMP456-M40. Root length of mangrove seedlings inoculated with strain *Gordonia* KMP456-M40 and non-inoculated (S+B-) mangrove seedlings. Illustrative images of inoculated (KMP456-M40) and non-inoculated (S+B-) mangrove propagules 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.

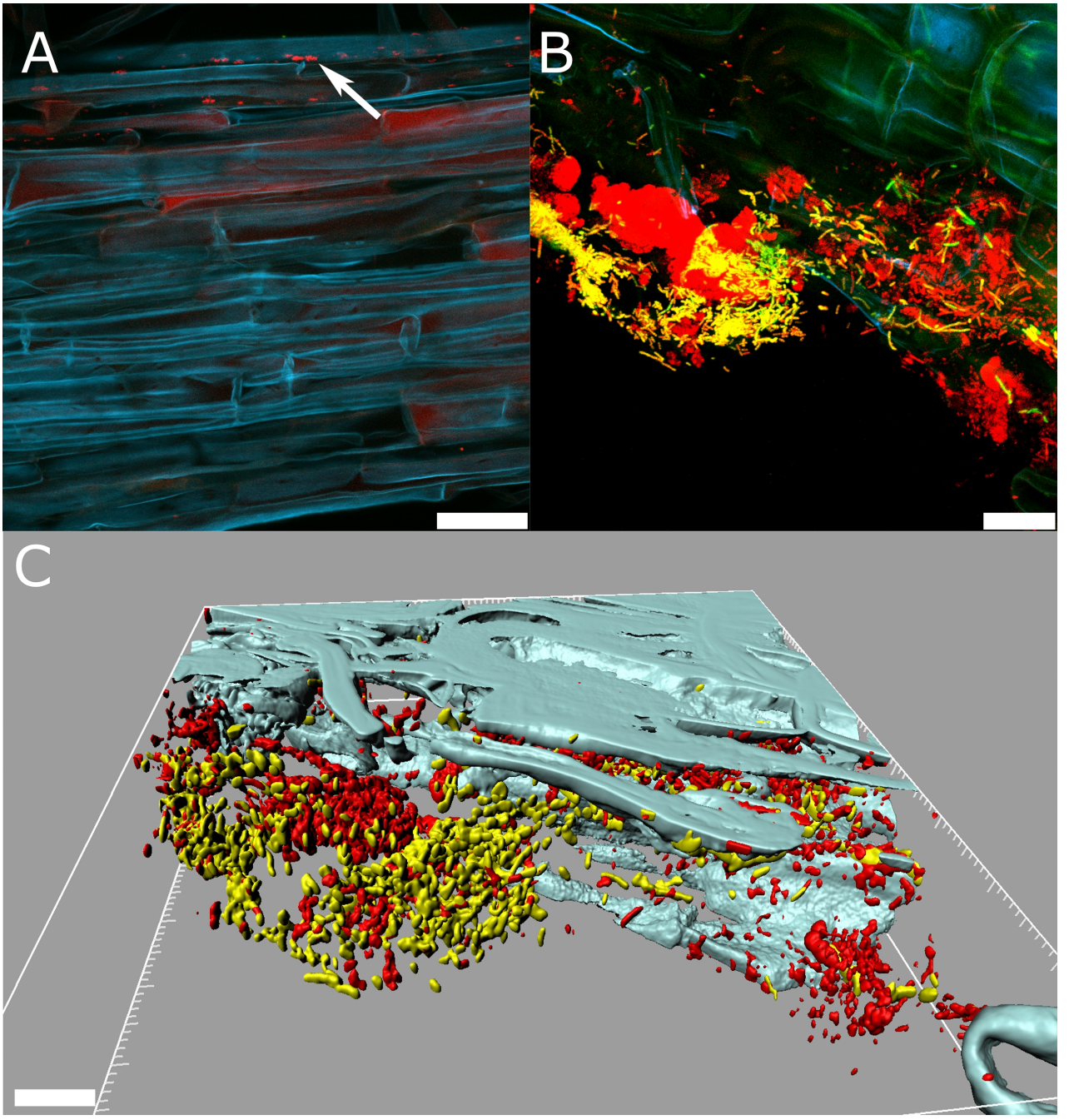
Figure 5. *In vivo* rice growth promotion assay of selected mangrove propagule endophytes under salt stress. Dry weight of rice plantlets i) inoculated with the propagule endophytic isolates KMP789-MA55, KMP123-MS1, KMP456-M40, ii) inoculated the non-PGP strain *Escherichia coli* DSM 6897 and iii) non-inoculated (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.

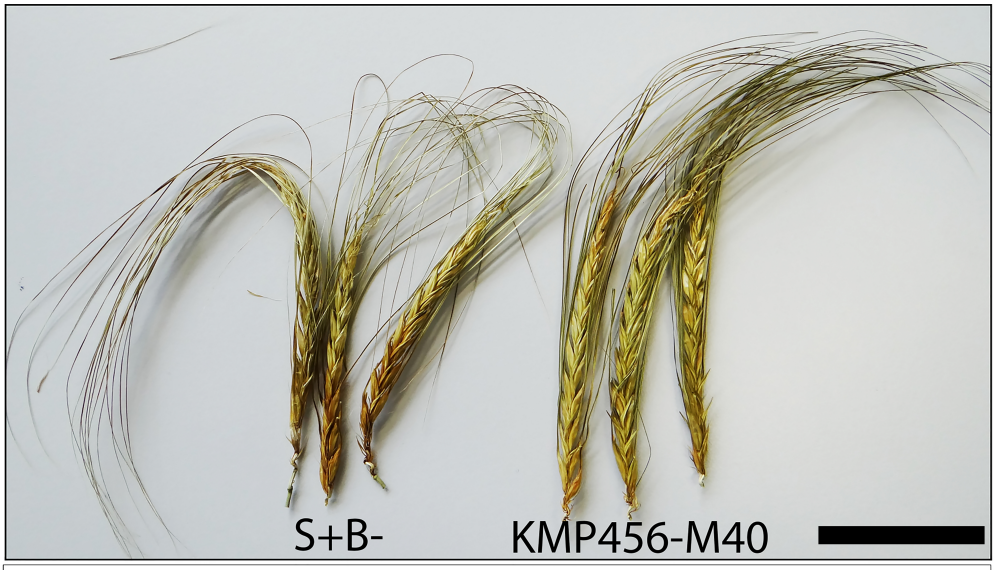
Table 1. Identification, Plant growth promoting (PGP) traits and abiotic stress tolerance of the propagule endophytic strains selected for *in vivo* PGP experiments. The list includes the strain taxonomic classification and the results of the physiological tests performed *in vitro*. Grey boxes indicate 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.

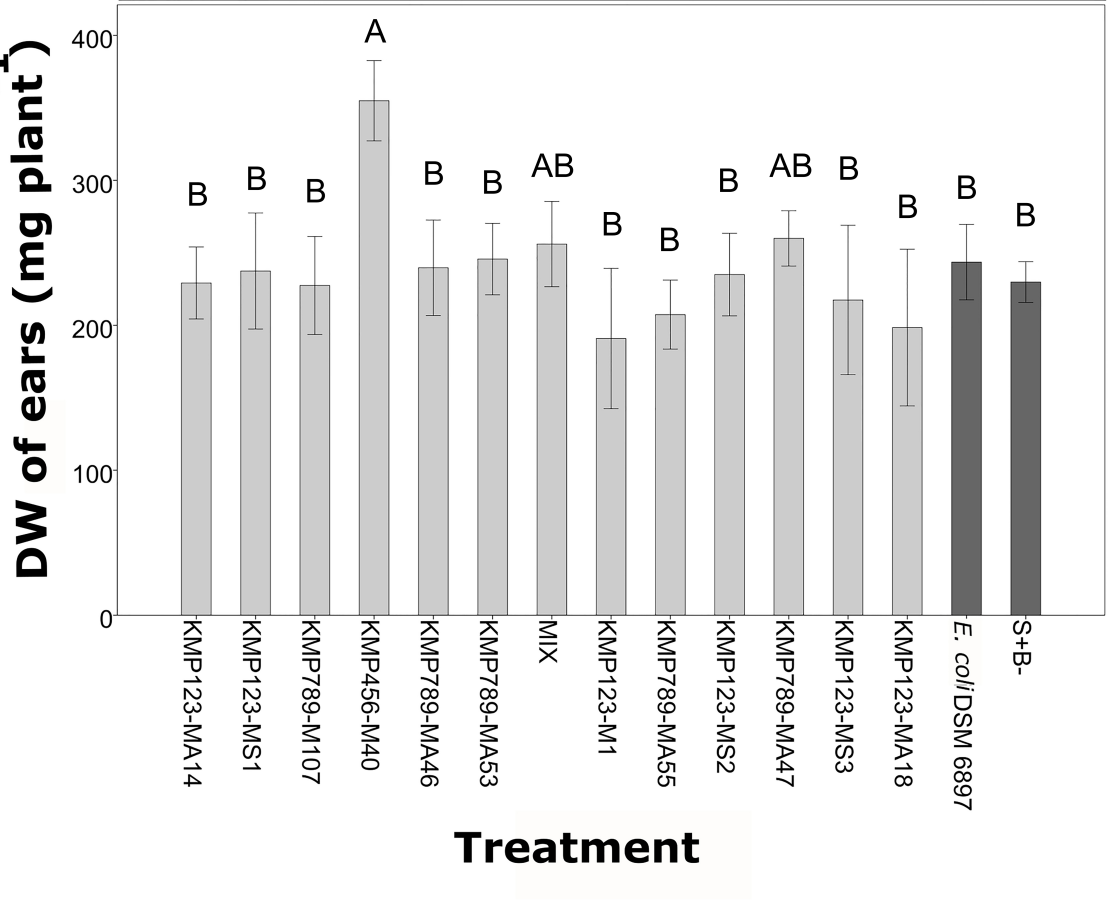
Isolate name	Closest relative species (% of 16SrRNA identity)*	PGP activity						PGP score	Abiotic stress tolerance				Stress score	Total score
		IAA	P Sol	NH_3	Sid	Prot	EPS		42°C	5%NaCl	10%NaCl	PEG		
KMP123-MA14	*Acinetobacter ursingii* (98)	■		■				2	■	■	■	■	4	6
KMP123-MS1	*Bacillus pumilus* (100)	■		■				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

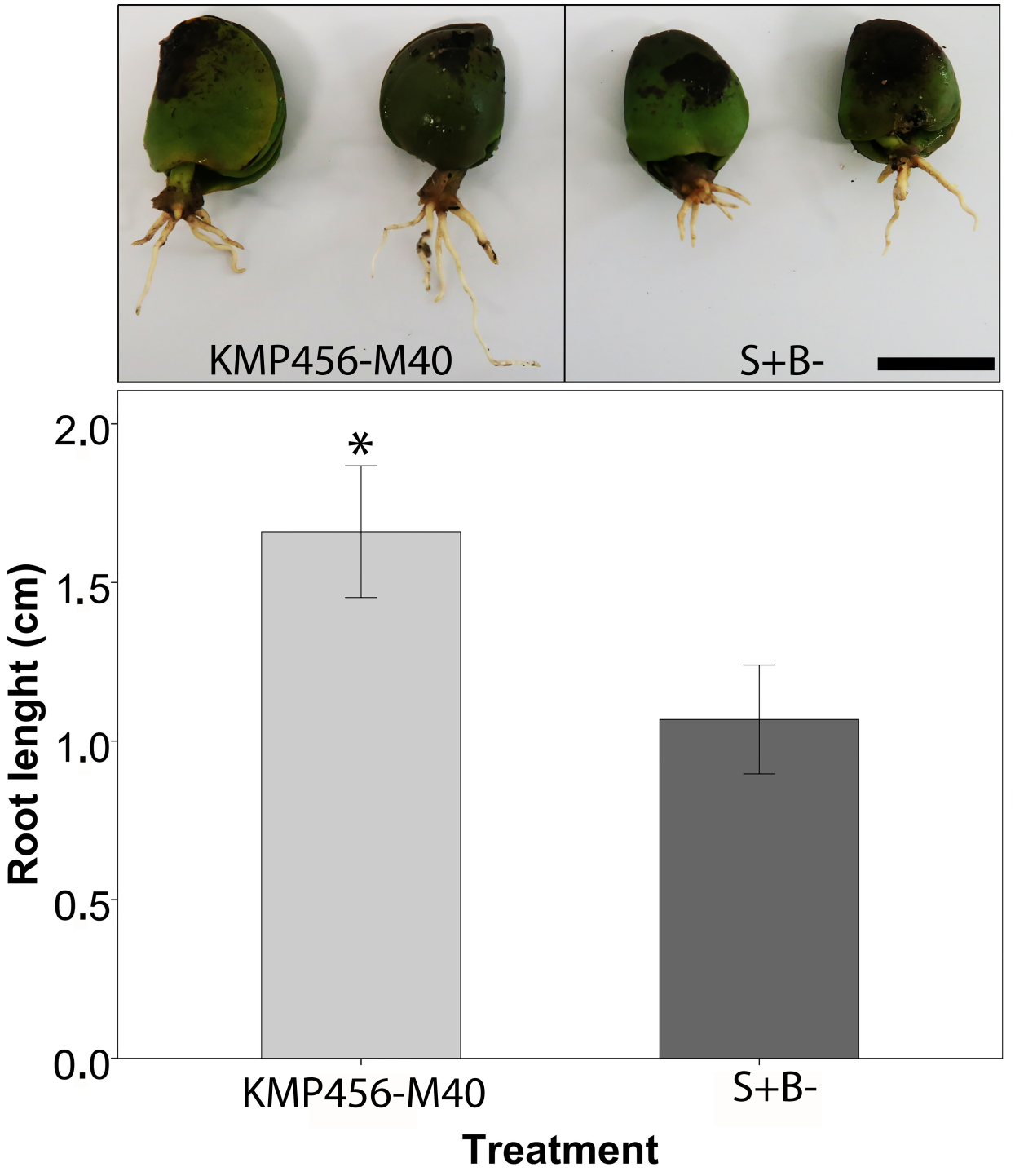
* According to BLAST alignment of the full 16S rRNA gene sequence

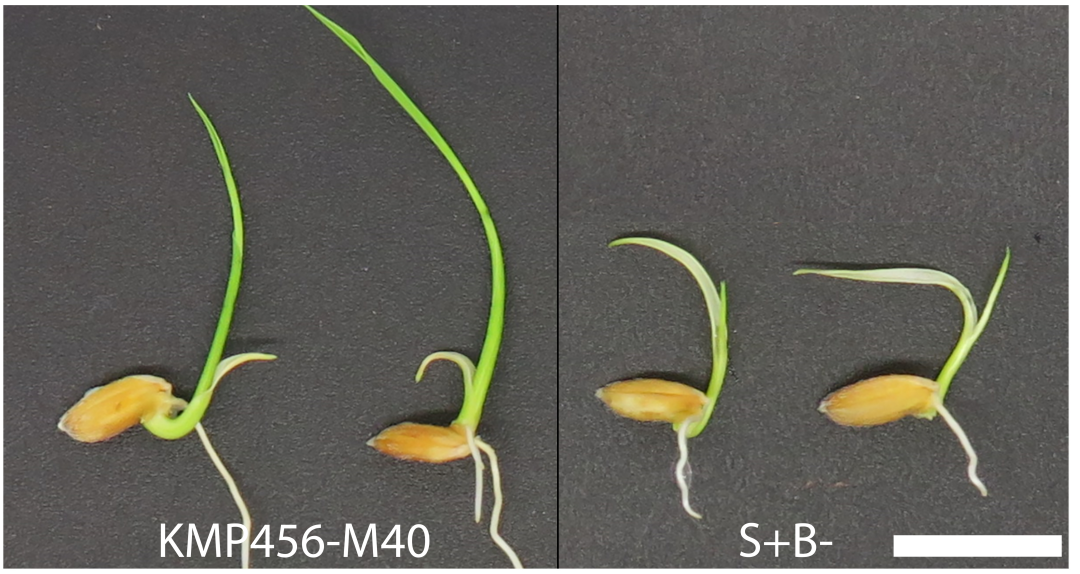

Acinetobacter (3)
Bacillus (2)
Corynebacterium (7)
Dyadobacter (3)
Enterococcus (4)
Gordonia (2)
Micrococcus (7)
Nocardioides (1)
Rhizobium (5)
Staphylococcus (14)
% of tested isolates
100
80
60
40
20
0
stress
PGP
Score type
Score
0
1
2
3
4

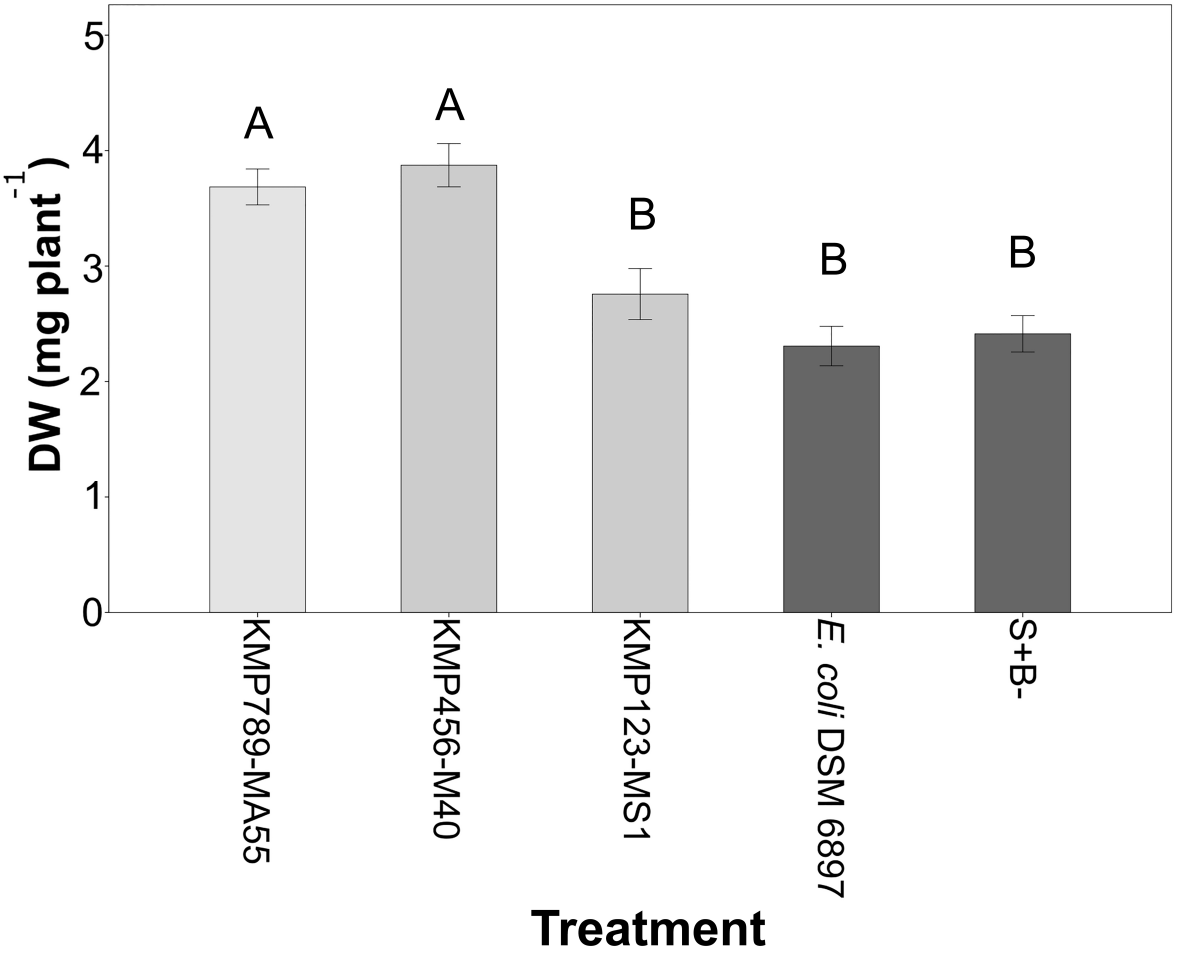

A
B
C


S+B-
KMP456-M40


DW of ears (mg plant-1)
400
300
200
100
0
A
B
B
B
B
B
B
AB
B
B
B
AB
B
B
B
B
KMP123-MA14
KMP123-MS1
KMP789-M107
KMP456-M40
KMP789-MA46
KMP789-MA53
MIX
KMP123-M1
KMP789-MA55
KMP123-MS2
KMP789-MA47
KMP123-MS3
KMP123-MA18
E. coli DSM 6897
S+B-
Treatment


KMP456-M40
S+B-
2.0
1.5
1.0
0.5
0.0
*
Root lenght (cm)
KMP456-M40
S+B-
Treatment


KMP456-M40
S+B-


DW (mg plant-1)
0
1
2
3
4
5
A
A
B
B
B
KMP789-MA55
KMP456-M40
KMP123-MS1
E. coli DSM 6897
S+B-
Treatment

SUPPLEMENTARY INFORMATION TO:

Mangrove propagule endophytes: Plant growth promoting potential toward mangrove seedlings and cereal crops

Riccardo Soldan, Francesca Mapelli, Elena Crotti, Sylvia Schnell, Daniele Daffonchio, Ramona Marasco, Marco Fusi, Sara Borin and Massimiliano Cardinale

Supplementary Table 1. List of the probes used for FISH-CLSM in this study. Details and reference are included for each probe. FA: formamide

Name	Sequence (5'- -3')	Fluorescent dye	Target	% FA (41 °C)	Reference
EUB338*	GCTGCCTCCCGTAGGAGT	Cy3	Universal (most Bacteria)	15	Amann R. I., Binder B. J., Olson R. J., Chisholm S. W., Devereux R. and Stahl D. A. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56: 1919−1925.
EUB338II*	GCAGCCACCCGTAGGTGT	Cy3	Planctomycetales	15	Daims H., Brühl A., Amann R., Schleifer K.-H. and Wagner M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. 22: 434−444.
EUB338III*	GCTGCCACCCGTAGGTGT	Cy3	Verrucomicrobiales	15	Daims H., Brühl A., Amann R., Schleifer K.-H. and Wagner M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. 22: 434−444.
ALF968	GGTAAGGTTCTGCGCGTT	Cy5	Alphaproteobacteria, except of Rickettsiales (79% coverage)	35	Neef A. (1997). Anwendung der in situ Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in komplexen mikrobiellen Biozönosen. Doctoral thesis (Technische Universität München).
LGC354A**	TGGAAGATTCCCTACTGC	Cy5	Part of Firmicutes (Low G+C Gram positive bacteria)	35	Meier, H., Amann, R., Ludwig, W., and Schleifer, K.H. (1999) Specific oligonucleotide probes for in situ detection of a major group of gram-positive bacteria with low DNA G+C content. Syst Appl Microbiol 22: 186–196.
LGC354B**	CGGAAGATTCCCTACTGC	Cy5	Part of Firmicutes (Low G+C Gram positive bacteria)	35	Meier, H., Amann, R., Ludwig, W., and Schleifer, K.H. (1999) Specific oligonucleotide probes for in situ detection of a major group of gram-positive bacteria with low DNA G+C content. Syst Appl Microbiol 22: 186–196.
LGC354C**	CCGAAGATTCCCTACTGC	Cy5	Part of Firmicutes (Low G+C Gram positive bacteria)	35	Meier, H., Amann, R., Ludwig, W., and Schleifer, K.H. (1999) Specific oligonucleotide probes for in situ detection of a major group of gram-positive bacteria with low DNA G+C content. Syst Appl Microbiol 22: 186–196.
HGC236	AACAAGCTGATAGGCCGC	FITC	High G+C Gram positive Bacteria	15	Erhart, R., D. Bradford, R. J. Seviour, R. Amann, and L. L. Blackall (1997). Development and use of fluorescent in situ hybridization probes for the detection and identification of Microthrix parvicella in activated sludge. Syst. Appl. Microbiol. 20:310-318:

GAM42a***	GCCTTCCCACATCGTTT	FITC	Gammaproteobacteria	35	Manz W., Amann R., Ludwig W., Wagner M. and Schleifer K.-H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. Syst. Appl. Microbiol.15: 593 - 600.
GAM42a comp***	GCCTTCCCACTTCGTTT	/	Betaproteobateria	35	Manz W., Amann R., Ludwig W., Wagner M. and Schleifer K.-H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. Syst. Appl. Microbiol.15: 593 - 600.
CF319a/b	TGGTCCGTRTCTCAGTAC	ATTO488	Bacteroidetes	35	Manz W., Amann R., Ludwig W., Vancanneyt M. and Schleifer K.-H. (1996). Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. Microbiol. 142: 1097-1106
NONEUB-Cy5	ACTCCTACGGGAGGCAGC	Cy5	/	****	Wallner G., Amann R. and Beisker W. (1993). Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry. 14: 136−143.
NONEUB-Rhodamine	ACTCCTACGGGAGGCAGC	Rhodamine	/	****	Wallner G., Amann R. and Beisker W. (1993). Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry. 14: 136−143.
NONEUB-FITC	ACTCCTACGGGAGGCAGC	FITC	/	****	Wallner G., Amann R. and Beisker W. (1993). Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry. 14: 136-143.

* used mixed in equimolar concentration

** used mixed in equimolar concentration

*** used mixed in equimolar concentration

**** the same formamide concentration used for the positive FISH probes in the same experiment

Supplementary Table 2. Physical and chemical properties of sterile Classic Tonsubstrat ED 73 substrate (Einheitserde- und Humuswerke Gebr. Patzer GmbH &Co. KG, Sinntal–Altengronau, Germany), as provided by the manufacturer. CAL: calcium acetate lactate.

Substrate physical and chemical properties	Values
pH ($CaCl_2$)	5.8
KCl	2.5 gl^{-1}
EC	0.3-0.9 mS cm^{-1}
N ($CaCl_2$)	250 mg l^{-1}
P (CAL)	300 mg l^{-1}
K (CAL)	400 mg l^{-1}
S (fresh weight)	200 mg l^{-1}
Mg (fresh weight)	700 mg l^{-1}

Supplementary Table 3. Identification, PGP traits and abiotic stress tolerance of selected endophytic strains isolated from the mangrove propagules (one isolate for each ITS group). The list includes the taxonomic classification of the strains and the results of the physiological tests performed. 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. 1/0 = positive/negative.

Isolate name	Representative isolate*	N of isolates**	PGP activity						PGP score	Abiotic stress tolerance				Stress score
			IAA	P Sol	NH_3	Sid	Prot	EPS		42°C	5%NaCl	10%NaCl	PEG	
KMP456-M46	*Acinetobacter ursingii* (KM281506)	4	1	0	0	0	0	0	1	1	0	0	1	2
KMP456-M50	*Acinetobacter ursingii* (KJ585678)	56	1	0	0	0	0	0	1	1	0	0	1	2
KMP123-MA14	*Acinetobacter ursingii* (MF984402)	1	1	0	1	0	0	0	2	1	1	1	1	4
KMP123-MS1	*Bacillus pumilus* (MG576190)	4	1	0	1	0	0	0	2	1	1	1	1	4
KMP123-MA1	*Corynebacterium durum* (AF543285)	1	0	0	0	0	0	0	0	1	0	0	0	1
KMP123-MA3	*Corynebacterium durum* (AF543285)	3	0	0	0	0	0	0	0	1	0	0	1	2
KMP123-MA7	*Corynebacterium durum* (AF543285)	1	0	0	0	0	0	0	0	1	0	0	1	2
KMP123-MA8	*Corynebacterium durum* (AF543285)	1	0	0	0	0	0	0	0	1	0	0	0	1
KMP123-MA9	*Corynebacterium durum* (GU561325)	2	0	0	0	0	0	0	0	1	0	0	0	1
KMP123-MA11	*Corynebacterium durum* (AF543285)	2	1	0	0	0	0	0	1	1	0	0	0	1
KMP123-MA2	*Corynebacterium durum* (AF543285)	2	0	0	0	0	0	0	0	1	0	0	0	1
KMP456-M36	*Dyadobacter ginsengisoli* (NR_041372)	2	0	0	0	0	0	0	0	0	0	0	1	1
KMP456-M41	*Dyadobacter ginsengisoli* (NR_041372)	3	0	0	0	0	0	0	0	0	0	0	0	0
KMP456-M38	*Gordonia terrae* (KR476419)	1	0	0	1	0	0	0	1	1	1	0	1	3
KMP456-M40	*Gordonia terrae* (KR476419)	1	1	0	0	0	0	0	1	1	1	0	1	3
KMP456-M39	*Dyadobacter koreensis* (NR_113977)	1	0	0	0	0	0	0	0	0	0	0	1	1
KMP789-M123	*Enterococcus casseliflavus* (MF925491)	3	1	0	0	0	0	0	1	1	1	0	1	3
KMP789-M107	*Enterococcus casseliflavus* (MF925491)	8	1	0	0	0	1	0	2	1	1	0	1	3
KMP789-M116	*Enterococcus casseliflavus* (MF925491)	1	1	0	0	0	0	0	1	1	1	0	1	3
KMP789-M117	*Enterococcus casseliflavus* (MF959774)	3	1	0	0	0	0	0	1	1	1	0	1	3
KMP789-MA46	*Micrococcus luteus* (HM355595)	1	1	0	1	0	1	0	3	1	1	1	1	4
KMP789-MA60	*Micrococcus yunnanensis* (KR476438)	4	1	0	1	0	1	0	3	1	1	0	1	3
KMP789-MA45	*Micrococcus aloeverae* (MG661749)	1	1	0	1	0	0	0	2	1	1	0	1	3
KMP789-MA41	*Micrococcus aloeverae* (MG661749)	4	1	0	1	0	1	0	3	1	1	0	1	3

Isolate name	Representative isolate*	N of	PGP activity						PGP	Abiotic stress tolerance			Stress
KMP789-MA63	*Micrococcus aloeverae* (KX082870)	2	1	0	1	0	1	0	3	1	1	0	1	3
KMP123-M29	*Micrococcus yunnanensis* (KX866674)	1	1	0	1	0	1	0	3	1	1	1	1	4
KMP789-MA53	*Micrococcus yunnanensis* (KR476438)	1	1	0	1	1	1	0	4	1	1	0	1	3
KMP456-M37	*Nocardioides hwasunensis* (KF424714)	1	0	0	0	0	0	0	0	0	0	0	0	0
KMP123-M1	*Rhizobium huautlense* (AM237359)	6	1	0	1	0	0	0	2	0	0	0	1	1
KMP123-M6	*Rhizobium huautlense* (AM237359)	1	1	0	1	0	0	0	2	0	0	0	1	1
KMP123-M7	*Rhizobium huautlense* (AM237359)	3	1	0	1	0	0	0	2	0	0	0	1	1
KMP123-M8	*Rhizobium huautlense* (AM237359)	1	1	0	1	0	0	0	2	0	0	0	1	1
KMP123-M11	*Rhizobium huautlense* (AM237359)	1	1	0	1	0	0	0	2	0	0	0	1	1
KMP123-MA33	*Staphylococcus capitis* (FJ380955)	1	1	0	1	0	0	0	2	1	1	1	0	3
KMP789-MA35	*Staphylococcus capitis* (FJ380955)	3	1	0	1	0	0	0	2	1	1	1	1	4
KMP789-MA38	*Staphylococcus capitis* (FJ380955)	2	0	0	1	1	0	0	2	1	1	1	1	4
KMP789-MA54	*Staphylococcus capitis* (FJ380955)	3	0	0	1	1	0	0	2	1	1	1	1	4
KMP789-MA55	*Staphylococcus capitis* (FJ380955)	2	1	0	1	1	0	0	3	1	1	1	1	4
KMP789-MA40	*Staphylococcus capitis* (FJ380956)	1	0	0	1	1	0	0	2	1	1	1	1	4
KMP123-MS2	*Staphylococcus cohnii* (LN774351)	4	1	0	1	0	0	0	2	1	1	1	1	4
KMP789-MA47	*Staphylococcus epidermidis* (MF286538)	5	1	0	0	1	0	0	2	1	1	1	1	4
KMP789-MA52	*Staphylococcus epidermidis* (MF286538)	1	1	0	0	1	0	0	2	1	1	1	1	4
KMP123-MS3	*Staphylococcus massiliensis* (NR_116422)	2	1	0	1	0	0	0	2	1	1	1	1	4
KMP123-MA15	*Staphylococcus saprophyticus* (EU162006)	3	1	0	1	0	0	0	2	1	1	1	1	4
KMP123-MA18	*Staphylococcus saprophyticus* (KU579262)	15	1	0	1	0	0	0	2	1	1	1	1	4
KMP123-MA13	*Staphylococcus saprophyticus* (LN774580)	1	1	0	1	0	0	0	2	1	1	1	1	4
KMP123-MA34	*Staphylococcus saprophyticus* (KT986100)	1	1	0	1	0	0	0	2	1	1	1	1	4
KMP456-M35	*Bacillus flexus* (LC189347)	1	1	0	1	0	0	0	2	1	1	1	1	4

33 * Isolates have been identified according to 16S rRNA gene sequencing: the best BLAST match indicates the closest described relative species

34 ** Number of isolates belonging to the same ITS group of the characterized one

Supplementary Table 4. Overall information on the propagule endophyte collection established in this study. The sample of origin and the medium used for the strain isolation are reported below as well as the number of isolates and ITS groups obtained from each medium/sample.

	Isolation medium					
	Marine Agar (MA)		869 1:10 (M)		869 1:10 + Sea Salt (MS)	
Propagule Sample	N. of isolates	N. of ITS groups	N. of isolates	N. of ITS groups	N. of isolates	N. of ITS groups
KMP-123	33	15	12	6	10	3
KMP-456	0	0	70	9	0	0
KMP-789	30	11	17	4	0	0
Total	63	26	99	19	10	3

Supplementary Table 5. Plant growth promotion assay with barley. Mean (g plant^{-1}) and standard deviation of inoculated barley plants under non-axenic conditions.

	Treatment	Mean (g plant^{-1})	Std. deviation
FW_shoots	*A. ursingii KMP123-MA14*	4.347	0.368
	B. pumilus KMP123-MS1	4.504	0.228
	E. casseliflavus KMP789-M107	4.672	0.101
	G. terrae KMP456-M40	4.697	0.582
	M. luteus KMP789-MA46	5.115	0.999
	M. yunnanensis KMP789-MA53	4.548	0.324
	R. huautlense KMP123-M1	4.477	0.565
	S. capitis KMP789-MA55	4.937	0.481
	S. cohnii KMP123-MS2	4.538	0.269
	S. epidermidis KMP789-MA47	4.784	0.089
	S. massiliensis KMP123-MS3	4.513	0.253
	S. saprophyticus KMP123-MA18	4.828	0.543
	MIX	4.603	0.603
	E. coli	5.142	0.855
	S+B-	4.482	0.173
DW_shoots	*A. ursingii KMP123-MA14*	0.940	0.132
	B. pumilus KMP123-MS1	0.995	0.143
	E. casseliflavus KMP789-M107	1.051	0.119
	G. terrae KMP456-M40	1.098	0.076
	M. luteus KMP789-MA46	1.151	0.219
	M. yunnanensis KMP789-MA53	1.008	0.099
	R. huautlense KMP123-M1	1.029	0.214
	S. capitis KMP789-MA55	1.074	0.073
	S. cohnii KMP123-MS2	0.994	0.179
	S. epidermidis KMP789-MA47	1.069	0.082
	S. massiliensis KMP123-MS3	0.974	0.161
	S. saprophyticus KMP123-MA18	1.047	0.094
	MIX	1.005	0.154
	E. coli	1.176	0.228
	S+B-	1.016	0.126

Supplementary Table 6. Mean (cm plant^{-1}) and standard deviation of mangrove height, from week 1 to week 9.

	Treatment	Mean (cm plant^{-1})	Std. deviation
Plant height 1	*B. pumilis KMP123-MS1*	5.913	1.977
	E.coli	5.475	1.344
	G. terrae KMP456-M40	5.705	1.731
	S. capitis KMP789-MA55	5.275	1.276
	Non-inoculated	5.038	1.484
Plant height 2	*B. pumilis KMP123-MS1*	9.737	2.626
	E.coli	9.237	1.585
	G. terrae KMP456-M40	9.923	2.448
	S. capitis KMP789-MA55	9.063	1.762
	Non-inoculated	8.808	2.079
Plant height 3	*B. pumilis KMP123-MS1*	12.938	3.305
	E.coli	12.275	1.593
	G. terrae KMP456-M40	13.051	3.079
	S. capitis KMP789-MA55	12.200	2.391
	Non-inoculated	12.167	2.627
Plant height 4	*B. pumilis KMP123-MS1*	15.812	3.460
	E.coli	15.137	2.006
	G. terrae KMP456-M40	16.090	3.530
	S. capitis KMP789-MA55	15.250	2.803
	Non-inoculated	14.910	3.067
Plant height 5	*B. pumilis KMP123-MS1*	17.663	3.893
	E.coli	16.363	2.236
	G. terrae KMP456-M40	17.731	3.715
	S. capitis KMP789-MA55	16.363	3.059
	Non-inoculated	16.474	3.517
Plant height 6	*B. pumilis KMP123-MS1*	18.887	4.321
	E.coli	17.275	2.935
	G. terrae KMP456-M40	18.692	4.301
	S. capitis KMP789-MA55	16.988	3.710
	Non-inoculated	17.410	4.055
Plant height 7	*B. pumilis KMP123-MS1*	19.675	4.559
	E.coli	17.900	3.146
	G. terrae KMP456-M40	19.282	4.594
	S. capitis KMP789-MA55	17.575	3.99i3
	Non-inoculated	18.103	4.272
Plant height 8	*B. pumilis KMP123-MS1*	19.918	4.584
	E.coli	18.125	3.271
	G. terrae KMP456-M40	19.513	4.595
	S. capitis KMP789-MA55	17.795	3.992
	Non-inoculated	18.572	4.343
Plamt height 9	*B. pumilis KMP123-MS1*	21.157	4.655
	E.coli	19.450	3.702
	G. terrae KMP456-M40	20.605	4.851
	S. capitis KMP789-MA55	18.820	4.278
	Non-inoculated	20.043	4.749

Supplementary Table 7. Plant growth promotion assay with rice. Mean (g plant^{-1}) and standard deviation of dry weight of inoculated rice plants under non-axenic conditions.

	Treatment	Mean (g plant^{-1})	Std. deviation
DW_ears	*B. pumilus KMP123-MS1*	5.341	0.228
	E. coli	6.000	0.214
	G. terrae KMP456-M40	5.650	0.119
	S. capitis KMP789-MA55	5.050	0.132
	Non-inoculated	5.550	0.076
DW_stems	*B. pumilus KMP123-MS1*	9.000	0.371
	E. coli	8.800	0.371
	G. terrae KMP456-M40	8.000	0.371
	S. capitis KMP789-MA55	8.800	0.371
	Non-inoculated	8.200	0.371

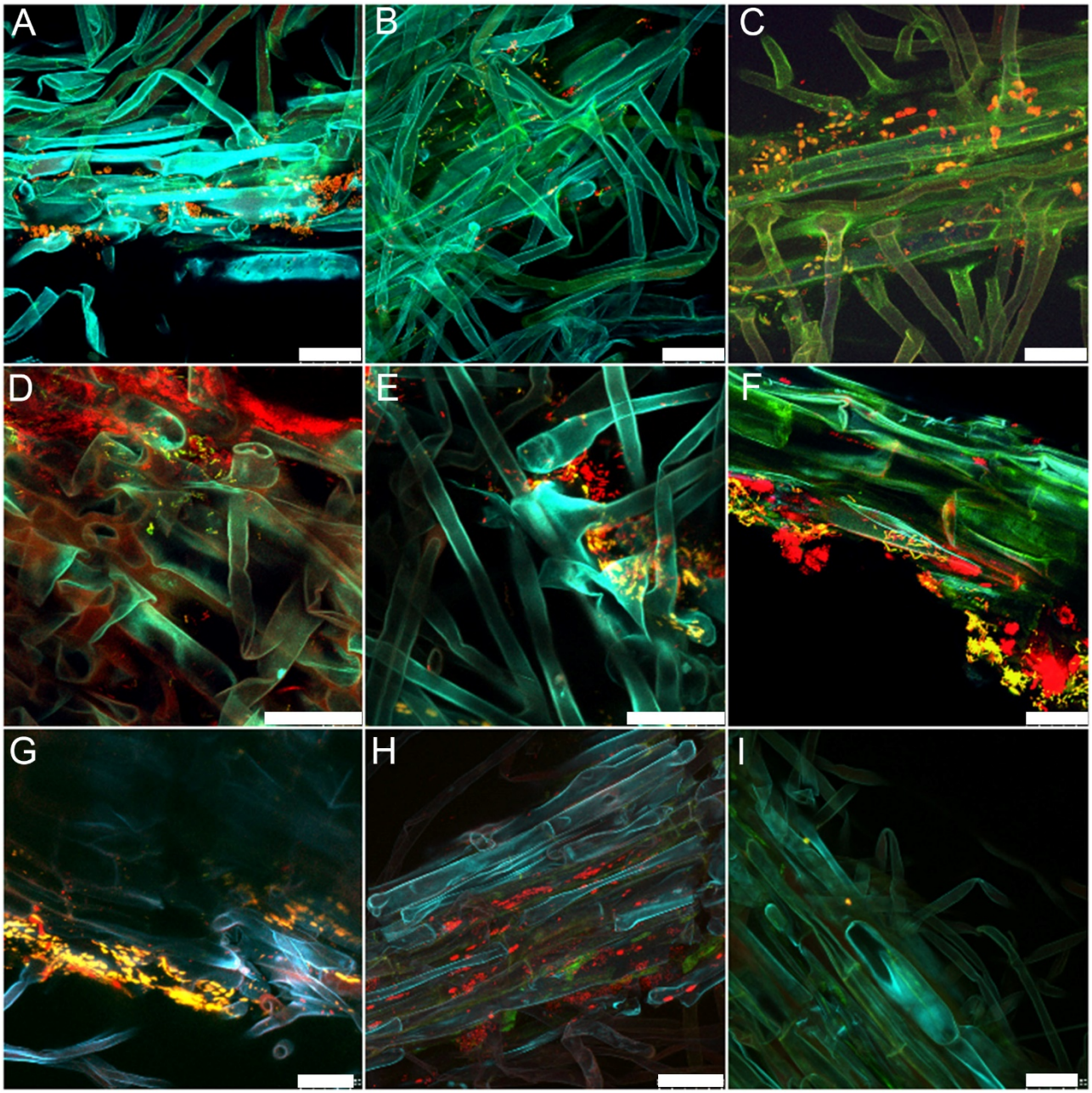


Fig. S1. **Confocal laser scanning microscopy images (maximum projections) showing fluorescence *in situ* hybridization (FISH)-stained bacteria colonizing barley roots grown in germination pouches**. Barley plants were inoculated with the following mangrove propagule isolates: A) *Gordonia* KMP456-M40; B) *Enterococcus* KMP789-M107; C) *Micrococcus* KMP789-MA53; D) *Staphylococcus* KMP123-MS2; E) *Acinetobacter* KMP123-MA14; F) *Bacillus* KMP123-MS1; G) *Staphylococcus* KMP123-MS3; H) *Micrococcus* KMP789-MA46; I) *Staphylococcus* KMP789-MA55. The roots were stained with the Cy3-labeled EUB338MIX bacterial probe and one of the following group-specific probes: A, C) FITC-labeled HGC236 – specific for Actinobacteria; B, D, F, G, I) Cy5-labeled LGC254MIX – specific for Firmicutes; E) FITC-labeled Gam42a – specific for Gammaproteobacteria. Yellow: inoculated bacteria (signal of the EUB338MIX probe overlapped with the signal of the group specific probe); red: bacteria only stained by the EUB338MIX probe. Scale bars: A, B, F-I (50µm); C, D, E (25 µm).