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Inorganic chemical fertilizer application reduces putative plant growth-promoting rhizobacteria in wheat

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10 **Abstract**

11 The profound negative effect of inorganic chemical fertilizer application on rhizobacterial diversity
12 has been well documented using 16S rRNA gene amplicon sequencing and predictive metagenomics.
13 We aimed to measure the function and relative abundance of readily culturable putative plant growth-
14 promoting rhizobacterial (PGPR) isolates from wheat root soil samples under contrasting inorganic
15 fertilization regimes. We hypothesized that putative PGPR abundance will be reduced in fertilized
16 relative to unfertilized samples. *Triticum aestivum* cv. Cadenza seeds were sown in a nutrient
17 depleted agricultural soil in pots treated with and without Osmocote® fertilizer containing nitrogen-
18 phosphorous-potassium (NPK). Rhizosphere and rhizoplane samples were collected at flowering
19 stage (10 weeks) and analyzed by culture-independent (CI) amplicon sequence variant (ASV)
20 analysis of rhizobacterial DNA as well as culture -dependent (CD) techniques. Rhizosphere and
21 rhizoplane derived microbiota culture collections were tested for plant growth-promoting traits using
22 functional bioassays. In general, fertilizer addition decreased the proportion of nutrient-solubilizing
23 bacteria (nitrate, phosphate, potassium, iron and, zinc) isolated from rhizocompartments in wheat
24 whereas salt tolerant bacteria were not affected. A 'PGPR' database was created from isolate 16S
25 rRNA gene sequences against which total amplified 16S rRNA soil DNA was searched, identifying
26 1.52% of total community ASVs as culturable PGPR isolates. Bioassays identified a higher
27 proportion of PGPR in non-fertilized samples (rhizosphere (49%) and rhizoplane (91%)) compared to
28 fertilized samples (rhizosphere (21%) and rhizoplane (19%)) which constituted approximately 1.95%
29 and 1.25% in non-fertilized and fertilized total community DNA, respectively. The analyses of 16S
30 rRNA genes and deduced functional profiles provide an in-depth understanding of the responses of
31 bacterial communities to fertilizer; our study suggests that rhizobacteria that potentially benefit plants
32 by mobilizing insoluble nutrients in soil are reduced by chemical fertilizer addition. This knowledge
33 will benefit the development of more targeted biofertilization strategies.

34 **1   Introduction**

35 Since anthropogenic plant domestication began ca.19,000 years ago, edible plants e.g. cereals have
36 been extensively bred (Lev-Yadun et al., 2000, Tanno and Willcox, 2006). However, current high-

37 yielding dwarf crop varieties rely on unsustainable levels of inorganic nitrogen and phosphorous
38 fertilizers, pesticides and other chemical inputs which are environmentally harmful (Rees et al., 2013,
39 Van Grinsven et al., 2013). Wheat is the third most cultivated cereal in the world; the FAO predicts
40 that by 2050 the global population will reach 9.73 billion meaning food production must be
41 accordingly increased by 50 % (FAO, 2017). By 2027, demand for wheat will increase to 833 million
42 tons, which is 10 % above the annual current production (OECD/FAO, 2018).

43 Soil microbial communities influence plant growth, health and resource use efficiency, especially the
44 subset that coexist and are selected to form the root microbiome (Berendson et al., 2012, Mendes et
45 al., 2013, Schlaeppi and Bulgarelli, 2015, Mauchline and Malone, 2017). A gradient of intimacy
46 between plant roots and microbes extends away from the root: plant influence over the microbial
47 community increases nearer the root surface. Root surface microbes are said to inhabit the
48 rhizoplane, and those in soil closely associated with the root, the rhizosphere (Hiltner, 1904, Zhang et
49 al., 2017). Rhizosphere and rhizoplane microorganisms can benefit crop plants in several ways
50 including improved plant nutritional status and protection against biotic and abiotic stresses
51 (Bloemberg and Lugtenberg, 2001, Turner et al., 2013, Choudhary et al., 2016, Ahkami et al., 2017),
52 so are termed plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978).
53 Inoculating plants with PGPR can stimulate crop growth, forming the basis for the biofertilizer
54 industry providing green alternatives to synthetic fertilizers and agrochemicals (Backer et al., 2018).
55 Understanding the effect of agricultural practices, such as fertilization regime, on PGPR populations
56 is essential to optimize microbiome function in the sustainable intensification of agriculture
57 (Hartmann et al., 2015).

58 To date, microbial community studies have focused on taxonomic composition, but the functional
59 potential of the microbiome may be more important to ensure key functions for holobiont fitness (Bai
60 et al., 2015, Lemanceau et al., 2017). With advances in next-generation sequencing technologies, the
61 wheat microbiome has mostly been defined based on CI methods; host genotype (Mahoney et al.,
62 2017), fertilization regime (Kavamura et al., 2018, Chen et al., 2019), land management and seed
63 load (Kavamura et al., 2019), irrigation (Mavrodi et al., 2018), growth stage (Chen et al., 2019) and
64 dwarfing (Kavamura et al., 2020) all affect the rhizosphere community structure. However, little has
65 been done to link taxonomic structure of the wheat microbiome to its functional ability.

66 Using CI and CD methods, we studied the effect of chemical fertilizer on putative PGPR abundance
67 in the commercial wheat variety, Cadenza, from a low input agricultural soil depleted in most
68 nutrients, in which beneficial microorganisms are important to sustain crop production. We
69 hypothesized that the abundance of rhizobacteria with plant growth-promoting traits would be
70 reduced for fertilized relative to unfertilized wheat due to differences in plant nutrient status.
71 Addition of NPK would mean that plants no longer need to interact with beneficial rhizobacteria to
72 provide nutrients to sustain growth. Our aim was to characterize culturable bacteria with plant
73 growth-promoting traits; determine their abundance within culturable communities; and characterize
74 CI and CD 16S rRNA gene DNA to assess the impact of the widely used NPK fertilizer on putative
75 PGPR populations. This was achieved by creating isolate libraries from each soil sample and
76 subjecting them to a range of bioassays which test key traits in nutrient acquisition to establish the
77 abundance of isolates with beneficial traits.16S rRNA gene sequences from isolates were used to
78 create a 'PGPR' database against which total CI amplified 16S rRNA soil DNA was searched in
79 order to determine the relative abundance of culturable PGPR within total community DNA.

80 **2   Materials and methods**

81 ## 2.1 Soil collection, experimental setup, and harvesting

82 We evaluated the rhizosphere and rhizoplane soil from wheat grown with and without NPK fertilizer.
83 Soil was collected from Stackyard bare-fallow soil mine (LATLONG 52.000293N, -0.614308E), a
84 well-draining sandy loam soil from the Rothamsted Research experimental farm at Woburn,
85 Bedfordshire (UK). The soil is a Cottenham series (CATT et al., 1980) classified as a Cambric
86 Arenosol (FAO), chosen to reduce the legacy effect of prior cropping systems. Soil was sieved (2
87 mm mesh), mixed thoroughly, and stored at 4 °C in polythene bags prior to use. *Triticum aestivum*
88 cv. Cadenza seeds were surface sterilized (70% ethanol, 10 min; 1.5 % active chlorine, 1 h; 5 x rinse,
89 sterile distilled water (SDW); overnight imbibition, sterile water, 4 °C) before germination on filter
90 paper. Seedlings were planted (1x seedling/pot; 9x9x10 cm pots, ~500 g soil) with and without NPK
91 granules [15% N, 9% P_2O_5, 11% K_2O, 2% MgO with micro-nutrients (B, Cu, Fe, Mn, Mo, Zn);
92 Osmocote, UK] (~2.5 g per pot). Four replicate pots were prepared for each treatment. Plants were
93 grown in a glasshouse (20 °C, 16 h/day light regime) and watered daily with tap water.

94 Pots were harvested at the start of flowering (Zadoks growth stage 61; ten weeks post germination)
95 (Zadoks et al., 1974), resulting in 8 rhizosphere samples and 8 root samples. Height (from soil
96 surface to head of longest stem) was measured, then soil was gently tipped from the pot onto a fresh
97 polythene bag. Loose soil was discarded and non-rhizospheric soil carefully removed. Roots were
98 vigorously shaken in a bag to release tightly attached soil (i.e. rhizosphere) and mixed to
99 homogenize. The root system was excised, cut vertically in half and placed in sterile 10 ml vials for
100 subsequent rhizoplane work. One half was frozen (-20 °C) for soil DNA extraction and the other
101 stored (4 °C) for culture work. Around 5 g of rhizosphere soil and 1 g of root was collected per plant.
102 The remainder of the plant was dried (80 °C, 24 h) and dry foliar plant biomass measured.

103 ## 2.2 Isolation of bacteria

104 To obtain a library of rhizospheric bacteria, 1 g of each rhizosphere soil sample was diluted 10-fold
105 (SDW) and shaken vigorously for 10 min using a shaker. To increase the diversity and number of
106 culturable isolates returned in this study, rhizosphere samples were plated onto both 10TSA and an
107 additional 7 agar types (Supplementary Table S1) (Bai et al., 2015). Soil suspensions were serially
108 diluted and 100 μl of final dilution spread on agar, (6 replicates per agar type). Different dilutions
109 were plated depending on agar type (Supplementary Table S2) and incubated (25 °C) for 4 days.
110 Individual colonies were picked and inoculated in 500 μl tryptone soya broth (TSB) (Oxoid,
111 Basingstoke, UK) (1/10th concentration) in deep well 96-well plates (Supplementary Fig. S1) and
112 incubated (25 °C, 2 days). For rhizoplane-colonizing bacteria, root samples were weighed and 900 μl
113 SDW was added for every 0.1 g root. Samples were shaken vigorously for 10 min using a shaker,
114 serially diluted, plated onto 10TSA and incubated (25 °C, 4-6 days). Individual colonies were picked
115 and inoculated in 500 μl TSB (1/10th conc.) (96-well plates, 25 °C, 2 days) prior to functional
116 analysis.

117 In addition, once colonies had been hand-picked from agar plates the remaining microbial biomass
118 was resuspended in 5 ml SDW using a sterile spreader and transferred to a 50 ml centrifuge tube.
119 Suspensions from technical replicates of the same agar (Supplementary Fig. S2) were combined and
120 each sample vortexed (10 min, high speed) to ensure homogeneity. Sub-samples of each were stored
121 (2 ml Eppendorf tube, -20°C) for genomic DNA extraction.

122 ## 2.3 Bioassays for plant growth-promoting traits

2.3.1 Bioassay inoculation

123
124 Plant growth-promoting functions were tested using previously established bioassays. A sterile 48-
125 prong inoculating manifold was used to spot individual inoculated isolates from the 96-well plate
126 liquid cultures onto agar (2x technical replicates per 96-well plate). Assays were incubated (25 °C, 5-
127 7 days); positive isolates were counted per sample for each functional assay.

2.3.2 Hydrolyzation of casein

128
129 Casein agar is used to detect peptide bond hydrolyzing microorganisms (Frazier and Rupp, 1928).
130 Isolate cultures were spot inoculated onto agar supplemented with skimmed milk powder as the
131 casein source. Casein agar (Hardy Diagnostics, Santa Maria, CA, USA): 5% skimmed milk powder,
132 0.5% pancreatic digest of casein, 0.25% yeast extract, 0.1% D-glucose, 1.25% agar. Hydrolyzing
133 isolates produced a clear halo in the surrounding medium.

2.3.3 Solubilization of insoluble phosphate, potassium and zinc

134
135 Isolate cultures were spot inoculated onto agar plates containing: tricalcium phosphate as an insoluble
136 phosphate source (Pikovskaya, 1948); potash feldspar (Bath potters, UK) as an insoluble potassium
137 source (Zhang and Kong, 2014); and zinc oxide as an insoluble zinc source (HiMedia M2023)
138 (Subba Rao, 1977). Pikovskayas agar (Pikovskaya, 1948): 0.05% yeast extract, 1% D-glucose, 0.5%
139 $Ca_3(PO_4)_2$, 0.05% $(NH_4)_2SO_4$, 0.02% KCl, 0.01% $MgSO_4\cdot7H_2O$, 0.00001% $MnSO_4\cdot H_2O$, 0.00001%
140 $FeSO_4\cdot7H_2O$, 1.5% agar. Aleksandrov agar (Aleksandrov et al., 1967): 0.05% $MgSO_4\cdot7H_2O$, 0.01%
141 $CaCO_3$, 0.2% potash feldspar, 0.5% D-glucose, 0.0005% $FeCl_3\cdot6H_2O$, 0.2% $Ca_3(PO_4)_2$, 2% agar, pH
142 7.0-7.2. Zinc solubilizing agar (Subba Rao, 1977): 1% D-glucose, 0.1% $(NH_4)_2SO_4$, 0.02% KCl,
143 0.01% K_2HPO_4, 0.02% $MgSO_4\cdot7H_2O$, 0.1% ZnO, 1.5% agar. The plates were incubated at 25 °C for
144 5-7 days and observed for the formation of halo zone around the colonies.

2.3.4 Production of siderophores

145
146 Iron solubilization was tested using agar containing chrome azurol S (CAS) and
147 hexadecyltrimethylammonium bromide (HDTMA) which form a blue color complex with ferric iron;
148 a color change to orange is observed when a strong iron chelator such as a siderophore removes iron
149 from the dye complex (Schwyn and Neilands, 1987, Louden et al., 2011). The medium was prepared
150 as outlined in (Louden et al., 2011). Iron solubilization was denoted as either 'positive' or 'negative'
151 based on the presence, or absence of an orange halo surrounding the colony.

2.3.5 Salt tolerance

152
153 *In vitro* screening of the isolates tolerance to salt stress was tested by culturing strains on 10TSA
154 supplemented with 5% (w/v) sodium chloride (NaCl). 10TSA with no additional NaCl was included
155 as a control.

2.3.6 Statistical analyses for culture-dependent work

156
157 Statistical differences in the frequency of positive vs. negative isolates (n=376) between non-
158 fertilized and fertilized wheat were performed in R 3.6.1 (http://www.r-project.org) using the
159 'chisq.test' function. Box plots were created in GraphPad Prism version 8 for Mac (San Diego, CA:
160 GraphPad Software, Inc). This software was also used to calculate two-way analysis of variance
161 (ANOVA) and pair-wise t-tests with Šidák correction for bacterial abundance and absolute
162 abundance of nutrient-solubilizing isolates. Data were first normalized by logarithmic transformation.
163 Normality was confirmed by quantile-quantile normality plots and Shapiro-Wilk test; homogeneity of
164 variances was confirmed by residuals versus fits plots and Spearman's test for heteroscedasticity.

165 **2.4   DNA analysis**

166 **2.4.1 Mixed culture DNA extraction and quantitation**

167 Each mixed culture sample was subjected to Sigma GenElute Bacterial Genomic DNA extraction kit
168 using the lysozyme utilizing Gram-positive bacterial preparation method to ensure lysis of both
169 Gram-positive and Gram-negative cells, according to the manufacturer's instructions. DNA purity
170 and concentrations were established by NanoDrop spectrophotometry (Thermo Scientific,
171 Wilmington, DE, United States), and a Qubit 2.0 Fluorimeter using the dsDNA HS assay kit (Thermo
172 Scientific), respectively.

173 **2.4.2 Soil DNA Extraction and Quantitation**

174 For each sample, total soil DNA was extracted from approximately 0.25 g homogenized soil (Qiagen
175 DNeasy PowerSoil DNA isolation kit (Venlo, Netherlands)), according to the manufacturer's
176 instructions using the MP Biomedicals FastPrep-24 machine twice (30 s, 5.5 m s^{-1}). DNA
177 concentrations and purity were determined as above.

178 **2.4.3 Amplicon library preparation and sequencing**

179 To assess the impact of fertilizer on microbial community composition, bacterial 16S rRNA gene
180 amplicons were subjected to Illumina® sequencing using the MiSeq platform. Amplicons (~460 bp)
181 spanning the V3-V4 hypervariable region of the 16S rRNA gene were produced using primers 341F
182 (5'-CCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3')
183 (Klindworth et al., 2013). Rhizosphere DNA was sent to Novogene (HK) (Wan Chai, Hong Kong)
184 for 2 x 250 bp paired-end sequencing on a MiSeq instrument. Rhizoplane and bacterial samples were
185 sequenced in-house (2 x 300 bp paired-end sequencing) on a MiSeq instrument; see Supplementary
186 Methods for full details. Amplicon preparation followed the protocol of Kozich et al. (Kozich et al.,
187 2013).

188 **2.4.4 Processing of 16S rRNA gene amplicon sequence data**

189 Demultiplexing of raw sequences was performed by CASAVA data analysis software (Illumina).
190 Paired-end sequences were merged using the vsearch merge_pairs function (Rognes et al., 2016) then
191 filtered, de-replicated, and denoised to identify ASVs using the DADA2 1.2 (Callahan et al., 2016)
192 pipeline with Quantitative Insights into Microbial Ecology (QIIME2) (version 2018.11.0) default
193 parameters (Bolyen et al., 2019). The resulting ASV table retained high quality nonchimeric reads
194 and was used to build a phylogenetic tree using the align-to-tree-mafft-fasttree command in QIIME2.
195 Taxonomy was assigned using the SILVA132 database (Quast et al., 2012, Yilmaz et al., 2013). All
196 non-bacterial ASVs were removed for further analysis.

197 **2.4.5 Colony PCR for identification of rhizobacterial isolates**

198 To identify individual rhizobacterial isolates, amplicons (~1500 bp) spanning almost the full length
199 of the 16S rRNA gene were produced using primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3')
200 and rD1 (5'-AAGGAGGTGATCCAGCC-3'). From a total of 1504 rhizobacterial isolates (94 isolates
201 per rhizosphere sample; 94 isolates per rhizoplane sample), 541 isolates were sequenced to gain a
202 representative population of species present in non-fertilized and fertilized wheat. Amplicons were
203 produced by colony PCR and sent to Eurofins Genomics Germany for purification and Sanger
204 sequencing; see Supplementary Methods for full details. Amplicon sequences were processed in
205 Geneious Prime version 2020.1.1; taxonomically assigned using the SILVA Alignment,

206   Classification and Tree (ACT) service (www.arb-silva.de/act); and deposited in NCBI GenBank (for
207   individual accession numbers see Supplementary Data).

2.4.6 Isolate database creation

209   The resulting 541 16S rRNA gene sequences and corresponding taxonomy were used to create
210   QIIME2 taxonomy and ASV files to identify whether isolates matched major ASVs within the 16S
211   rRNA gene amplicon dataset. The classify-consensus-blast command in QIIME2 was used at 100%
212   sequence identity to search the 16S rRNA gene amplicon sequences against the isolate database.
213   Secondly, a 'PGPR' database was created which only included isolate sequences identified as
214   'putative PGPR' (as they were found to be positive for at least one functional trait) to identify the
215   relative abundance of potentially plant beneficial isolates within CI and CD amplicon datasets. ASVs
216   identified as 'Bacteria' were considered rhizobacterial isolates.

2.5   Data visualization and statistical analyses

2.5.1 Total 16S rRNA gene dataset

219   The resulting ASV table was analyzed in R 3.6.1 using Phyloseq (v1.30.0) (available at
220   https://joey711.github.io/phyloseq/) (McMurdie and Holmes, 2013). Amplicon sequencing data were
221   normalized using DESeq2 to account for differences in sequencing bias (Love et al., 2014), except
222   for alpha diversity analysis, which was calculated by normalizing sequence number to minimum
223   sample size (8554) by random subsampling. The subsampling of sequences still yielded sufficient
224   resolution of bacterial communities, as suggested by rarefaction curve analysis (Fig. S3). Two-way
225   type III ANOVA was performed using the R function 'aov' with fertilizer and rhizocompartment as
226   factors, to determine the dominant factor contributing to variation in means for alpha diversity data.
227   Normality was confirmed by quantile-quantile normality plots and the Shapiro-Wilk test;
228   homogeneity of variances was confirmed by residuals versus fits plots and the Levene's test (R
229   package: car (v3.0-6)). Beta diversity was determined by principle Coordinate Analysis (PCoA)
230   which was employed on weighted UniFrac distance matrices using the 'ordinate' function in the
231   Phyloseq package; significantly different clusters were determined using 'adonis' with the
232   'betadisper' test to check for equal variance (R package: vegan (v2.5.6)) (Oksanen et al., 2019).
233   Phylum level community composition was investigated by relative abundance of normalized data,
234   after removal of bacterial ASVs only classified to Kingdom level and ASVs assigned as
235   'environmental samples' for visualization purposes. Unique and shared ASVs were determined in
236   Excel. To identify ASVs preferentially associated with fertilization and vice versa, the differential
237   relative abundances (fold changes) of ASVs between the different groups were determined. Low
238   abundance ASVs with less than 3 counts in <20% of the samples were removed. This was performed
239   individually for each data set with the DeSeq2 package, using the Wald significance test and the
240   Benjamini-Hochberg *p* value correction. All graphs were rendered in Prism 8.

2.5.2 Isolate and PGPR 16S rRNA gene dataset

242   The resulting isolate ASV table and PGPR ASV table were analyzed using Phyloseq (as described
243   above), normalized using DESeq2 and transformed to relative abundances. Heatmaps were created in
244   Excel. ASVs identified as 'Bacteria' were considered rhizobacterial isolates or putative PGPR; the
245   remaining ASVs were unassigned. To identify whether isolates matched ASVs preferentially
246   associated with fertilization and vice versa, the differential relative abundances (fold changes) of
247   ASVs between the different groups were determined, as described above. Statistical differences
248   between mean putative PGPR abundance between treatments were assessed using two-way ANOVA
249   and pair-wise t-tests with Šidák correction. Normality was confirmed by quantile-quantile normality

250   plots and Shapiro-Wilk test; homogeneity of variances was confirmed by residuals versus fits plots
251   and Spearman's test for heteroscedasticity. Box plots were created in Prism 8.

3   Results

3.1   Plant phenotypical data

254   Fertilizer addition increased aerial biomass of wheat from 1.27 ± 0.32 g to 4.11 ± 0.95 (Welch's two-
255   sample t-test: t=-5.645, df=3.67, p=0.006) (Supplementary Fig. S4). There were no statistical
256   differences in mean height of non-fertilized vs. fertilized wheat plants (p>0.05) (Supplementary Fig.
257   S4).

3.2   Rhizobacterial community composition in culture -independent and -dependent
          communities

260   Amplicon-based analysis of the V3-V4 region of the 16S rRNA gene generated a total of 902,187
261   sequences from 16 CI samples (median 50,658 sequences per sample) and 279,558 sequences from
262   16 CD samples (median 17,235 sequences per sample). Filtering, denoising, and removal of chimeras
263   resulted in 521,098 (CI) and 206,960 (CD) high-quality sequences, retaining 58% and 70% of initial
264   reads, respectively. The number of sequences per sample ranged from 20,809 to 49,203 (CI) and
265   12,623 to 24,165 (CD). One CD sample was removed due to low sequence number (<100). ASV
266   analysis using the SILVA132 database generated 4,879 ASVs from CI DNA and 426 ASVs from CD
267   DNA (after removal of ASVs that were not classified as bacteria (archaeal, eukaryote, metagenome,
268   unassigned)). The CD approach retrieved 4.9% of ASVs from the CI total community; 244 (4.9%)
269   ASVs were found using both CI and CD methods indicating that 182 (3.6%) ASVs detected by the
270   CD method, were absent from the CI DNA dataset (Fig. 1).

3.3   Fertilizer treatment and niche effects on rhizobacterial community diversity

272   Rhizobacterial community beta-diversity, as measured by weighted UniFrac distances, was
273   influenced by both rhizocompartment and fertilizer. PCoA of CI communities showed clustering of
274   samples by rhizocompartment along the first principle coordinate axis (PC1) and clustering by
275   fertilization along the second principle coordinate axis (PC2) (Fig. 2A, CI). Two-way permutational
276   analysis of variance (PERMANOVA) indicated that both rhizocompartment and fertilizer effect were
277   significant in CI rhizobacterial communities, as well as their interaction (Supplementary Table S3).
278   Rhizocompartment accounted for 49% of the variance (p = 0.001) and fertilizer accounted for 24% of
279   the variance (p = 0.001). The two-way PERMANOVA of CD rhizobacterial community beta
280   diversity indicated a significant effect of rhizocompartment (20% of variance, p = 0.002), whereas
281   the effect of fertilization was weaker, although still statistically significant (13% of variance, p =
282   0.035), but the interaction was not significant (Supplementary Table S4). PCoA of the CD
283   rhizobacterial community revealed that samples also clustered by rhizocompartment along PC1 (Fig.
284   2A, CD), whereas there was some overlap between treatment groups along PC2, consistent with the
285   small effect size in the PERMANOVA analysis.

286   Rhizobacterial CI community alpha diversity, as measured by the Shannon diversity index, was
287   consistent across rhizocompartments but lower in samples from fertilized wheat (Fig. 2B, CI). Two-
288   way type III ANOVA indicated that the effect of fertilizer was significant (p = 0.0024), while the
289   effects of rhizocompartment and interaction were not (Supplementary Table S5). Rhizobacterial CD
290   communities showed lower alpha diversity compared to CI communities (Fig. 2B, CD), with
291   fertilizer and rhizocompartment causing less change in variation (p>0.05) (Supplementary Table S6).

3.4 Comparison of isolation media in assessing the influence of fertilizer on rhizosphere bacterial communities

In total, 894 bacterial ASVs were identified from 63 mixed culture samples (4 biological replicates from 8 agar-types for non-fertilized and fertilized wheat rhizosphere samples (1 outlier removed)). The use of 7 additional agar types, as well as 10TSA, retrieved 8.6% of CI ASVs compared to 4.2% from just 10TSA.

Effect of fertilizer was more apparent when a larger number of agar types were used to isolate rhizobacteria. Overall, mean alpha diversity was greater in samples from non-fertilized wheat (2.95 ± 0.31) compared to fertilized wheat (2.66 ± 0.31) (d.f=1, F=21, p<0.0001) however, there were no statistical differences between individual means for each agar-type (d.f=7, F=3.6, p>0.05) (Fig. 3A). Furthermore, fertilizer was the dominant factor contributing to variation in beta diversity (PERMANOVA, F=40, r^2=0.35. p=0.001), accounting for 61.7% variation along PC1 compared to 16.7% variation along PC2 as caused by agar-type (PERMANOVA, F=2.6, r^2=0.16. p=0.004) (Fig. 3B), with no significant interaction. The relative abundance of different taxonomic groups in mixed cultures shifted from non-fertilized wheat plants and fertilized wheat plants (Fig. 3C). Most notable, was the higher abundance of Actinobacteria in the rhizosphere of fertilized wheat plants across all media types compared to a higher abundance of Proteobacteria in the rhizosphere of non-fertilized wheat plants.

Agar type had minimal effect on bacterial diversity within treatment groups. There were no statistical differences in mean alpha diversity between different agar types for a given sample from either non-fertilized or fertilized wheat. Furthermore, PCoA showed no distinct groupings between agar type for non-fertilized wheat (Supplementary Fig. S5A) (PERMANOVA, F=1.1, r^2=0.24, p=0.38) however, fertilized wheat samples from TWYE agar formed a clear grouping compared to the other agar types (Supplementary Fig. S5B). With TWYE samples present, media type significantly affected bacterial community structure (PERMANOVA, F=11.9, r^2=0.29, p=0.001). However, when TWYE samples were removed from PCoA, agar type had no significant effect on bacterial community structure (PERMANOVA, F=0.86, r^2=0.056, p=0.59).

3.5 Effect of fertilizer on abundance of culturable rhizobacteria with plant growth-promoting traits

Based on previous evidence of fertilization altering the structure of wheat rhizosphere bacterial communities (Kavamura et al., 2018), we hypothesized that addition of fertilizer would reduce, specifically, the presence of putative plant growth-promoting rhizobacteria.

In general, rhizobacteria that tested positive for solubilization of plant macronutrients: organic N (casein), inorganic phosphate ($Ca_3(PO_4)_2$) and potassium (potash feldspar), and plant micronutrients: iron ($FeCl_3 \bullet 6H_2O$) and zinc (ZnO), had a statistically higher relative abundance in isolate libraries cultured from non-fertilized wheat samples compared to fertilized wheat samples (Fig. 4A and 4B) (Supplementary Table S9). However, there was no significant difference in the relative abundance of salt-tolerant rhizobacteria in non-fertilized wheat samples compared to fertilized wheat samples (Fig. 4B) (Supplementary Table S9). Relative abundance of nutrient solubilizing isolates were greater in rhizosphere ($49.2 \pm 13\%$; two-tailed chi-square test: $X^2 = 116$, p<0.0001, d.f = 1) and rhizoplane ($90.7 \pm 9\%$; $X^2 = 389$, p<0.0001, d.f = 1) samples from non-fertilized wheat compared to fertilized wheat ($21.5 \pm 2\%$ and $19.1 \pm 14\%$ from rhizosphere and rhizoplane, respectively) (Fig. 4C).

334   Absolute abundance of bacteria isolated from the rhizoplane (mean log CFU counts.g soil^{-1}: 8.84 ±
335   0.34) was higher compared to the rhizosphere of wheat (mean log CFU counts.g soil^{-1}: 6.70 ± 0.05)
336   (two-way ANOVA: F=18.3, d.f=1, p<0.0001) whereas fertilizer addition had no significant effect on
337   rhizobacterial abundance (p>0.05) (Fig. 5A). In comparison, absolute abundance of nutrient-
338   solubilizing bacteria isolated from rhizocompartments in wheat were statistically higher from
339   rhizoplane samples (F=113, d.f=1, p<0.0001) and were also statistically higher in non-fertilized
340   compared to fertilized wheat samples (F=20.7, d.f=1, p=0.0007) (Fig. 5B). Post-hoc multiple
341   comparison tests with Šidák correction showed differences in means in rhizosphere (log CFU
342   counts.g soil^{-1}: 8.18 ± 0.08 and 7.24 ± 0.67 in non-fertilized and fertilized wheat, respectively)
343   (p=0.0289) and rhizoplane (mean log CFU counts.g soil^{-1}: 6.12 ± 0.09 and 5.37 ± 0.27 in non-
344   fertilized and fertilized wheat, respectively) (p=0.0075) samples. However, there were no statistical
345   differences between individual assays for absolute abundance of isolates from non-fertilized vs.
346   fertilized wheat (Supplementary Fig. S6).

347   **3.6   Identification of culturable isolates within culture-independent and -dependent amplicon**
348   **datasets**

349   An isolate database was curated from 541 isolates (275 from non-fertilized wheat; 266 from fertilized
350   wheat) which consisted of a total of 27 genera (Fig. 6) (Supplementary data S1). Rhizobacterial
351   isolates that displayed growth-promoting abilities were identified as being mostly *Bacillus* species
352   (48%) in the rhizosphere and *Pseudomonas* (50%) in the rhizoplane of non-fertilized wheat (Fig.
353   6A). For fertilized wheat plants the majority of isolates were also identified as *Bacillus* (52%) in the
354   rhizosphere and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (48%) in the rhizoplane
355   (Fig. 6A).

356   In total, 96 (1.92%) isolate bacterial ASVs were also identified as being present in the total
357   community amplicon 16S rRNA gene dataset from this work. The remaining 4,906 ASVs could not
358   be assigned to any culturable isolates from this work; 88 (1.84%) bacterial ASVs were identified in
359   the CI amplicon dataset and 58 (13.6%) bacterial ASVs were identified in the CD amplicon dataset
360   (Fig. 6B). Comparatively, at ninety-nine percent sequence identity, 246 (4.5%) isolate ASVs were
361   classified as bacteria from 5456 ASVs. However, to increase accuracy of isolate identification, one
362   hundred percent sequence identity was used. Overall, 23 genera were identified in the CI and CD
363   amplicon datasets, when searched against the isolate database; no ASVs were classified as
364   *Rhodococcus, Fictibacillus, Paraburkholderia* and *Agrobacterium.*

365   The PGPR isolate database identified 76 ASVs (1.52%) as PGPR within the CI and CD communities
366   (Fig. 6C). Overall, relative abundance of PGPR isolates were relatively low (<5%) however, there
367   was a statistically higher mean abundance of PGPR in non-fertilized CI samples (0.019 ± 0.006)
368   compared to fertilized CI samples (0.012 ± 0.002) (two-way ANOVA: F=22.7, df=1, p=0.0005) (Fig.
369   7). Compartment also contributed to difference in means and was statistically higher in the
370   rhizosphere (0.020 ± 0.006) compared to the rhizoplane (0.013 ± 0.003) (two-way ANOVA: F=14.7,
371   df=1, p=0.0024). There was no significant interaction with fertilization regime. Post-hoc multiple
372   comparison tests with Šidák correction showed differences in means in rhizosphere (0.024 ± 0.002
373   and 0.016 ± 0.004 in non-fertilized and fertilized wheat, respectively) (p=0.0009) but not in
374   rhizoplane (0.015 ± 0.003 and 0.011 ± 0.003 in non-fertilized and fertilized wheat, respectively)
375   (p>0.05) samples.

376   **3.7   Major ASVs within wheat rhizobacterial communities**

377 Differential abundance analysis identified ASVs that were highly associated with non-fertilized
378 wheat compared to fertilized wheat in rhizosphere and the rhizoplane samples. Overall: 54 ASVs
379 (3.5% of all ASVs identified in CI rhizosphere samples); 115 ASVs (3.5% of all ASVs identified in
380 CI rhizoplane samples); 17 ASVs (6.4% of all ASVs identified in CD rhizosphere samples); and 16
381 ASVs (6.0% of all ASVs identified in CD rhizoplane samples) were differentially abundant between
382 non-fertilized and fertilized samples (Supplementary Table S7 and S8). Six genera (15 ASVs) were
383 enriched in non-fertilized samples (p<0.01) and they included members of the Proteobacteria
384 (46.97%), Firmicutes (9.09%) and Actinobacteria (7.58%) (Fig. 8). Conversely, nineteen genera (36
385 ASVs) were enriched in fertilized samples and they included members of the Proteobacteria
386 (49.26%), Actinobacteria (31.62%), Bacteroidetes (10.29%) and Firmicutes (4.41%) (Fig. 8). In
387 particular 9 ASVs identified as *Pseudomonas* were enriched in non-fertilized samples compared to
388 fertilized samples where 4 ASVs identified as *Catenulispora, Leifsonia* and *Rhodanobacter* were
389 enriched (Fig. 8).

390 Key ASVs that were differentially abundant in both the amplicon dataset and isolate dataset were
391 identified (p<0.01). They included: *ASV_3448_Pseudomonas*, *ASV_1275_Pseudomonas*, *and*
392 *ASV_4785_Pseudomonas* in non-fertilized wheat samples and *ASV_1051_Rhizobium*,
393 *ASV_4593_Rhizobium*, *ASV_4049_Pedobacter*, *ASV_1557_Leifsonia*, *ASV_1818_Rhodanobacter*,
394 *ASV_1979_Rhodanobacter*, *ASV_4187_Paraburkholderia* and *ASV_2089_Lysobacter* in fertilized
395 wheat samples (Fig. 8). Four ASVs (ASV_4593, ASV_4049, ASV_1979, ASV_4187) were
396 differentially more abundant in fertilized wheat samples in CI and CD amplicon datasets and CI and
397 CD isolate datasets.

398 **4   Discussion**

399 We hypothesized that the addition of chemical fertilizer would reduce putative PGPR populations in
400 wheat. We found that the abundance of rhizobacteria with acquisitional traits for key plant nutrients
401 (endogenous nitrogen, phosphate, potassium, iron and zinc mobilization) were significantly reduced
402 in wheat grown in soils treated with NPK fertilizer.

403 We combined both culture-independent and -dependent methods to study the impact of fertilizer on
404 microbiome community composition and diversity. Our CI results confirmed previous studies
405 showing that fertilizer alters community structure and reduces bacterial alpha diversity in the root
406 environment (Jorquera et al., 2014, Zhu et al., 2016, Cui et al., 2018, Kavamura et al., 2018, Lian et
407 al., 2018, Chen et al., 2019, Pagé et al., 2019, Liang et al., 2020). Our CD results support this as beta
408 diversity was influenced and alpha diversity reduced by fertilizer. We decided to analyze CI
409 rhizosphere and rhizoplane datasets together despite their having been sequenced using different
410 approaches because, whilst inter-sequencing center variation can be significant (Schloss et al., 2011),
411 treatment effect consistently outweighs run variation (Wen et al., 2017). Thus, we acknowledge that
412 compartment effect (in CI samples) could be partially due to bias in sequencing run; however, it is
413 unlikely only due to this given the high percentage variability in beta-diversity, and previous
414 evidence that different soil compartments have distinct microbial compositions (van der Heijden and
415 Schlaeppi, 2015). Additionally, our main aim was to study the effect fertilizer had on rhizobacterial
416 diversity which is distinct in both compartments. Finally, we have previously found that fertilizer
417 application reduces microbial species richness in the rhizosphere (Kavamura et al., 2018) and when
418 re-examining the dataset from this study there is also a reduction in species richness in the bulk soil
419 of plots receiving high levels of inorganic N fertilizer, but this effect is exacerbated in the
420 rhizosphere. Therefore, we conclude that despite fertilizer also influencing bacterial community

421   structure in bulk soil (Chen et al., 2016, Ding et al., 2016, Francioli et al., 2016, Soman et al., 2017,
422   Dai et al., 2020, Kumar et al., 2018) it also has a profound effect on the rhizo-microbiome.

423   We tested how much the use of multiple agar types increased the percentage of total community
424   DNA retrieved when compared to commonly used 10TSA only. The agar types were selected from
425   Bai et al. (2015) which characterized extensive culture collections isolated from *Arabidopsis* leaf and
426   root microbiomes and demonstrated that the majority of leaf- and root-dwelling microbes were
427   amenable to culture. Whilst Bai et al. used colony picking, as well as limiting dilution and cell
428   sorting to purify bacterial isolates we were more interested in mixed culture taxonomy that resulted
429   from the use of different isolation media, similar to the method used in Kavamura et al. (2019). In
430   fact, the use of seven additional agar types increased the percentage of ASVs retrieved twofold
431   compared to 10TSA only which, considering 10TSA retrieved half the ASVs from all agar types
432   combined, supports its representability as an isolation medium. We reported a higher percentage of
433   culturable ASVs (4.2%) retrieved in total community DNA, in contrast to Stefani et al. (2015) and
434   Kavamura et al. (2019) who retrieved 2.4% and 2.2%, respectively, using similar isolation methods.
435   Both studies analyzed 16S rRNA gene datasets by clustering sequences into operational taxonomic
436   units; the increased percentage retrievability which we report could be due to improved taxonomic
437   resolution from ASV analysis (Fierer et al., 2017). Additionally, we found that agar type had little
438   effect on the diversity and composition of rhizobacterial isolates cultured from soil. We conclude that
439   this is likely due to the agar types used being less selective, not genera-specific and similar in
440   composition which would explain the lack of diversity yielded. It would be interesting in future work
441   to test microbial composition isolated from agar with similar conditions as found in soil, such as soil
442   extract, root exudate and plant extract media.

443   Putative PGPR were characterized using a variety of functional bioassays to test a representative
444   population of the culturable rhizo-microbiome. We found a clear difference in relative abundance of
445   nutrient-solubilizing bacteria isolated from rhizosphere and rhizoplane samples between fertilized
446   and non-fertilized wheat. There was a marked reduction in both relative and absolute abundance of
447   nutrient solubilizing bacteria in fertilized samples. We hypothesized that this would be the case since
448   fertilized plants and microorganisms can utilize easily available NPK source and do not need to
449   solubilize NPK. Indeed, key enzymes involved in microbial solubilization of P, alkaline
450   phosphatases, were shown to be strongly decreased by P fertilization in the rhizosphere (Spohn and
451   Kuzyakov, 2013) which suggests that fertilizer reduces hydrolytic enzyme-producing
452   microorganisms or enzyme production in organisms capable of such function. The role of
453   fertilization on soil enzymatic activities has been investigated in detail over the last 40 years
454   (Bautista-Cruz and Ortiz Hernandez, 2015) where increasing evidence suggests that chemical
455   fertilization can inhibit or slow down synthesis of hydrolytic enzymes. However, linking enzyme
456   production to individual microbiome members or groups is particularly challenging (Sergaki et al.,
457   2018). Our study suggests that genera such as *Bacillus, Pseudomonas, Rhizobium* and *Streptomyces*
458   are producers of hydrolytic enzymes in soil. Of course, this study does not exclusively show that the
459   culturable rhizobacteria with plant growth-promoting traits contributed to plant growth, and it is
460   possible that the bacteria were releasing nutrients for their own consumption. However, spatial
461   differentiation of microbes in the rhizosphere has been demonstrated to reduce plant-microbe
462   competition as much as possible (Marschner et al., 2011). Additionally, taxonomically and
463   functionally similar bacteria to those we isolated in this study have been shown to increase plant
464   growth when inoculated in soils (Assainar et al., 2018, Masters-Clark et al., 2020, Wang et al., 2020,
465   Wilkinson, 2020).

466   We hypothesized that there is a host selection process for nutrient-solubilizing bacteria, driven by
467   poor nutrient availability conditions and that fertilizer reduces this selection as wheat can utilize the
468   readily available nutrients in soil and no longer need to interact with beneficial bacteria. Our results
469   show a higher abundance of nutrient-solubilizing rhizobacteria in the rhizoplane, a compartment
470   more intimately associated with the plant host than the rhizosphere, which could suggest a plant
471   mediated selection process. Alternatively, the competition for nutrients might be heightened in the
472   rhizoplane as compared to the rhizosphere, and nutrient-solubilizing organisms could be under a
473   stronger selection when nutrients are depleted. We also found bacteria with known plant growth-
474   promoting properties such as *Paenibacillus, Streptomyces* and *Pseudomonas* to be more abundant in
475   root-associated soil from non-fertilized wheat (Vejan et al., 2016, Liu et al., 2019). However, it is
476   unclear why fertilizer addition would inhibit root colonization by these bacteria. It is possible that
477   rhizobacteria are less able to metabolize primary nutrients in the form presented in agricultural
478   fertilizers than other members of the soil microbiome. If this is the case, it would follow that they are
479   also less competitive in this environment and this would be reflected in their lower abundance.
480   *Pseudomonas*, for example, colonize plants through chemotaxis into the rhizosphere along a gradient
481   of root exudates, followed by surface association and migration on the rhizoplane to ultimately form
482   a bacterial biofilm, which would explain their increased abundance in the rhizoplane. In addition,
483   many *Pseudomonas* spp. produce enzymes and other signaling molecules such as lipopolysaccharides
484   to successfully colonize the plant rhizosphere and manipulate plants by activating symbiotic
485   pathways (Oldroyd, 2013). It has been shown that nutrient starvation triggers plants to activate
486   symbiotic pathways with bacteria (Zipfel and Oldroyd, 2017), so addition of fertilizer could mean
487   plants no longer need to activate such pathways and thus no longer interact with beneficial bacteria,
488   such as *Pseudomonas.* Chen et al. 2019 showed that the order Pseudomonadales in rhizosphere
489   samples responded negatively to organic acid released by wheat in response to N fertilization (Chen
490   et al., 2019) which correlate with our results that show a reduced abundance of *Pseudomonas* in soil
491   samples from fertilized wheat and suggest that organic acid release by wheat as a response to
492   fertilizer addition indirectly inhibits selection and colonization of *Pseudomonas* of the root system.
493   The higher abundance of pseudomonads was more apparent in culturable rhizobacterial communities.
494   Other non-culturable species may play just as important a role but are not as amenable to study.

495   Reducing environmental and financial costs of conventional agriculture requires novel management
496   and breeding strategies to shift current high input to more sustainable biological methods. Soil
497   microbiome manipulation promises to contribute to more environmentally benign agriculture by
498   promoting plant growth and suppressing pathogenic microorganisms (Mauchline and Malone, 2017)
499   and show promise in reducing chemical fertilizer application without influencing crop growth
500   (Assainar et al., 2018, Wang et al., 2020). Our work provides a binary method to determine
501   abundance of bacteria with beneficial traits in soil samples. Furthermore, we endeavored to link
502   individual isolates cultured from soil samples to total soil community DNA. Culturable bacteria with
503   plant growth-promoting traits are invaluable to the commercialization of biostimulants for sustainable
504   agriculture (Backer et al., 2018). Therefore, establishing their abundance within total soil DNA will
505   be useful to determine the overall impact that bioinoculants have on the microbiome as well as to
506   determine optimal inoculation dose. However, first we must understand plant host evolutionary and
507   domestication history to identify plant traits linked to microbial recruitment by wild relatives (Perez-
508   Jaramillo et al., 2016). Significant knowledge gaps limit our understanding of the soil microbiome.
509   The field needs to move beyond simple descriptions of community diversity to identify patterns in
510   this complexity and recognize when that complexity is important (Lemanceau et al., 2017). This will
511   enable us to make soil microbiome research of practical utility to human undertakings, towards a
512   cooperative relationship with terrestrial ecosystems instead of our current one-sided and often self-
513   destructive relationship.

514　This study contributes to our understanding of the impact of fertilizer on wheat rhizobacteria and
515　supports previous studies showing the deleterious effect of chemical fertilizer on plant rhizobacteria,
516　particularly through highlighting the greater abundance of putative PGPR in unfertilized plants. It is
517　assumed that wild relatives have co-evolved with the microbial community of native soils, selecting
518　microbes beneficial to growth and health. Here, we show the probability that wheat plants can select
519　growth-promoting bacteria to their roots to establish mutually beneficial associations and that
520　chemical fertilizer reduces this selection. We propose a culture-dependent method to characterize the
521　functional ability of the microbiome, and link this to culture-independent total soil DNA. We hope
522　that this work contributes to the shift from simple microbiome studies on taxonomic characterization
523　to conceptual framework analysis identifying and explaining patterns in the soil microbiome in
524　agriculture and natural systems (Harris, 2009).

525　**5　Conflict of Interest**

526　The authors declare that the research was conducted in the absence of any commercial or financial
527　relationships that could be construed as a potential conflict of interest.

528　**6　Data Availability Statement**

529　The raw sequences analyzed for this study can be found in the NCBI Sequence Read Archive (SRA),
530　accession PRJNA625513 and in NCBI Genbank, accessions MT354024 - MT354564.

531　**7　Author Contributions**

532　TR, TM, IC, and VK: designed the experiments. TR, VK, and MA performed the experiments and
533　collected the data. TR analyzed the data with input from AT, VK, and MA for bioinformatic
534　analyses. TR wrote the manuscript. All co-authors edited and commented on the manuscript.

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544　**10　Supplementary Material**

545　The Supplementary Material for this article can be found online

546　**11　References**

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Figure 1. Rhizobacterial taxonomic composition in culture-independent and -dependent communities of wheat grown in soils with and without fertilizer addition. Venn diagram showing the number and proportion of unique ASVs (at 97% similarity) in **(A)** culture-independent and **(B)**

18

758   culture-dependent communities and **(C)** shared ASVs detected with both methods. Pie charts
759   correspond to the percentage of bacterial phyla and classes of Proteobacteria assigned to each ASV.

760   **Figure 2. Culture-independent (CI) and culture-dependent (CD) bacterial community diversity**
761   **in the rhizosphere and rhizoplane of wheat grown in soils with and without fertilizer addition.**
762   **(A)** PCoA plots of bacterial composition based on weighted UniFrac distances for CI and CD
763   communities at ASV level. The percentage shown in each axis corresponds to the proportion of
764   variation explained. **(B)** Alpha diversity estimates at ASV level for rhizosphere and rhizoplane
765   bacterial communities in non-fertilized and fertilized wheat; with median (line) and hinges at first
766   and third quartiles (25th and 75th percentiles). Significant differences as determined by two-way type
767   III ANOVA are shown by '**' for $p<0.01$ between treatment groups. Symbols represent rhizosphere
768   (open circles) and rhizoplane (closed circles) samples from non-fertilized (red) and fertilized (blue)
769   wheat.

770   **Figure 3. Influence of different culture-agar on bacterial community diversity and structure in**
771   **the rhizosphere of wheat grown in soils with and without fertilizer.** **(A)** Alpha diversity estimates
772   at ASV level for rhizosphere bacterial communities grown on 8 agar types with median (line) and
773   hinges at first and third quartiles (25th and 75th percentiles). Significant differences as determined by
774   two-way type III ANOVA are shown by '**' for $p<0.01$ between treatment groups. **(B)** PCoA plot of
775   bacterial community composition based on weighted UniFrac distances at ASV level, colored by
776   fertilizer treatment. Symbols represent the isolation media that bacteria were cultured on from
777   rhizosphere samples: TSA (10th conc.) (circles), TSA (squares), TYG (up-triangles), YEM (down-
778   triangles), M408 (diamond), M715 (hexagon), FM (plus), TWYE (cross). **(C)** Average relative
779   abundances of bacterial phyla within the bacterial 16S V3-V4 sequences. Identifications are based on
780   the SILVA database for prokaryotes.

781   **Figure 4. Relative abundance of culturable rhizobacteria with plant growth-promoting traits**
782   **isolated from wheat grown in soils with and without fertilizer.** **(A)** Nitrate-, phosphate-,
783   potassium-, iron-, and zinc-solubilization as determined by casein, PVK, AVK, CAS, and zinc
784   carbonate agar, respectively. Casein, phosphate, potassium and zinc solubilization are indicated by
785   clear halos surrounding the colony whereas orange halos indicate iron solubilization. Salt tolerance as
786   determined by 10TSA supplemented with 5% NaCl. Box plots show relative abundance of **(B)**
787   solubilizing isolates and salt tolerant isolates and **(C)** relative abundance of PGPR isolates, as
788   determined by functional bioassays; with median (line) and hinges at first and third quartiles (25th
789   and 75th percentiles). Each point represents an isolate library (n=94) derived from rhizosphere (open
790   circles) and rhizoplane (closed circles) samples. Single colonies were isolated from different non-
791   selective agar and 10TSA respectively and inoculated in TSB (1/10th conc.) in a 96-well plate; sterile
792   TSB (1/10th conc.) in 2x wells were left uninoculated to act as a negative control. Significant
793   differences as determined by chi-squared tests are shown by '*' and '****' for $p<0.05$ and $p<0.0001$

794   **Figure 5. Effect of chemical fertilizer (NPK) on absolute abundance of culturable bacteria**
795   **isolated from the rhizosphere and rhizoplane of wheat grown in low nutrient soil.** **(A)** Absolute
796   abundance (log CFU counts.g soil-1; CFU is colony-forming units) of culturable rhizobacteria
797   isolated from soil samples on 10TSA. **(B)** Absolute abundance (log CFU counts.g soil-1) of nutrient
798   solubilizing rhizobacteria as determined by functional bioassays. Significant differences as
799   determined by post-hoc multiple comparison tests with Šidák correction are shown by '*' and '**'
800   for $p<0.05$ and $p<0.01$, respectively.

801 **Figure 6. Heatmaps representing abundance and associated functional ability of bacterial**
802 **isolates from rhizosphere (RS) and rhizoplane (RP) samples from non-fertilized (NF) and**
803 **fertilized (F) wheat. (A)** The full region of the 16S rRNA gene was amplified and sequenced for
804 each rhizobacterial isolate. Identifications are based on the SILVA database for prokaryotes. Grey
805 indicates relative abundance of genera in each sample (no. of isolates per sample shown below
806 columns). Red indicates relative abundance of positively solubilizing isolates from total number of
807 isolates sequenced (541). 'n' shows number of genera identified from total number of isolates
808 sequenced. **(B)** All culturable rhizobacterial isolate sequences and **(C)** plant growth-promoting
809 rhizobacteria (PGPR) isolate sequences were used to create a database with which the culture-
810 independent (CI) and culture-dependent (CD) amplicon datasets were searched against using blast at
811 100 percent sequence identity.

812 **Figure 7. Box plots showing relative abundance of ASVs identified as putative plant growth-**
813 **promoting rhizobacteria (PGPR) in culture-independent and -dependent isolate datasets.** A
814 PGPR database was created from 16S rRNA gene sequences from culturable bacterial isolates with
815 acquisitional traits for key plant nutrients. Each point represents the proportion of ASVs that were
816 identified as Bacteria in CI and CD datasets, with median (line) and hinges at first and third quartiles
817 (25th and 75th percentiles).. Significant differences as determined by post-hoc multiple comparison
818 tests with Šidák correction are shown by '*' and '**' for $p<0.05$ and $p<0.01$, respectively.

819 **Figure 8. ASVs that significantly differ in abundance in non-fertilized wheat vs. fertilized**
820 **wheat.** Differentially abundant ASVs detected by DESeq2 at a significance level of $p_{adjusted} < 0.05$
821 which were found to be enriched in (A) culture-independent (CI) and (B) culture-dependent (CD)
822 rhizosphere (open circles) and rhizoplane (closed circles) communities in non-fertilized and fertilized
823 wheat. ASVs depicted in bold were also detected in the isolate dataset. Only ASVs classified to
824 genus level are pictured; for full list see supplementary material.