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The impact of distillery effluent irrigation on plant-growth-promoting traits and taxonomic composition of bacterial communities in agricultural soil

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1 **The impact of distillery effluent irrigation on plant-growth-promoting traits and**
2 **taxonomic composition of bacterial communities in agricultural soil**

3

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13 **Abstract**

14 Long-term irrigation of agricultural fields with distillery effluent (DE) may alter the physical,
15 chemical and biological properties of the soil. Microorganisms are critical to the maintenance
16 of soil health and productivity. However, the impact of DE irrigation on activity and
17 taxonomy of soil microorganisms is poorly understood. Here we studied plant-growth-
18 promoting (PGP) traits and taxonomic composition of bacterial communities in agricultural
19 soil irrigated with DE in conjugation with irrigation water, using cultivation-dependent and -
20 independent methods. Most of the bacterial isolates obtained from DE irrigated soil were
21 found to display PGP traits (phosphate solubilization, siderophore, indolic compounds and
22 ammonia production). Diverse bacterial taxa were found in both culturable bacterial
23 community and 16S rRNA gene clone library, which belonged to bacterial phyla
24 *Proteobacteria* (Alpha-, Beta- and Gamma- subdivisions), *Firmicutes, Actinobacteria,*
25 *Acidobacteria, Bacteroidetes* and *Gemmatimonadates*. Overall, these results indicate that
26 PGP traits and taxonomic diversity of soil bacterial communities were not severely impacted
27 by DE irrigation.

28

29 *Keywords:* Agriculture, bacterial communities, distillery effluent, plant-growth-promoting
30 traits, 16S rRNA gene

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31 **Introduction**

32 Partially treated or untreated anaerobically digested distillery effluent (DE) is mainly
33 discharged in non-judicious manner on to the agricultural lands and in local waterways in
34 India. Distillery effluent is of brown color with high salt levels and has high biological and
35 chemical oxygen demands (BOD = 40,000-50,000 mg L^{-1} and COD = 80,000-100,000 mg L^{-1}
36) [1]. In India, controlled and judicious application of DE in agriculture is considered as one
37 of the viable option to transform industrial waste to value added resource as it contains
38 considerable amounts of both macro- and micronutrients [2,3,4,5]. However, non-judicious
39 application of DE has also been shown to adversely impact the growth and yield of crop
40 plants [6,7].

41

42 In an agroecosystem, the nutrient accessibility and productivity is largely depends on soil
43 microorganisms, as they play some vital functions such as nutrient cycling [8], soil
44 development [9] and organic matter decomposition [10]. Several studies have shown that DE
45 irrigation alters the soil physico-chemical properties [3,11,12], which, in turn, influence the
46 activity and biomass of soil microorganisms [13,14]. However, the impact of DE irrigation on
47 plant-growth-promoting (PGP) traits and taxonomic composition of soil microbial
48 communities is still poorly understood. Two previous studies have shown that application of
49 industrial effluent to agricultural soils could increase the diversity and catabolic profile of
50 microbial communities [15,16]. However, these studies only considered pulp and paper mill
51 effluent irrigation. Therefore, it is important to study the impact of DE irrigation on PGP
52 traits and taxonomic composition of soil microbial communities.

53

54 It is now well documented that only a small proportion (1–5%) of the total soil

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55 microorganisms can be cultured on currently known growth media [17]. The introduction of
56 cultivation-independent methods has provided a more thorough insight into changes
57 occurring in composition and function of microbial communities [18,19]. This study was
58 designed to investigate the PGP traits and taxonomic composition of soil bacterial community
59 from agricultural fields receiving DE irrigation from more than a decade. For assessing PGP
60 traits, phosphate solubilization, siderophore, indolic compounds and ammonia production
61 were measured for bacterial isolates, and taxonomic characterization of bacterial
62 communities was performed using 16S rRNA gene amplicon sequencing of DNA extracted
63 from bacterial isolates and of DNA directly extracted from soil.
64
65 **Materials and methods**
66 **Sampling site and soil collection**
67 The agricultural fields around Gajraula, Uttar Pradesh, India were selected as sampling site,
68 which are being irrigated with DE released from secondary treatment plant of Jubilant
69 Organosys distillery industry for more than a decade. The chemical composition of DE used
70 for irrigation is provided in Table 1. In March 2010, soil samples were randomly collected
71 from a depth of approximately 15 cm using a soil auger along zigzag paths (Zigzag sampling).
72 The samples were transported to the laboratory in an ice-box at 4 ˚C, and there stored at -20
73 ˚C for microbiological analyses. The soil was sandy loam in texture with pH 7.64; electrical
74 conductivity (EC), 0.37 dSm^{-1}; organic carbon (OC), 0.64%; total N, 0.08%; Olsen P, 15.86
75 Kg ha^{-1}, extractable K, 367.62 Kg ha^{-1} and, Na^{+}, 132 Kg ha^{-1}.
76
77 **Cultivation of bacterial isolates**

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78 Triplicate soil samples (1 g) were taken from each core subsample, homogenized in 10 ml of
79 0.85% saline, and serially diluted (10-fold dilution) in the same saline. Aliquots (100 µl) were
80 spread on tryptic soy broth agar medium (TSBA; Difco, USA) and Reasoner's 2A medium
81 (R2A; Difco, USA) plates. Based on morphological differences like shape, size, colour and
82 margin, single colonies were picked at random from the culture plates, purified and
83 maintained on the respective media for further analyses.
84

85 ***In vitro* screening of bacterial isolates for PGP traits**

86 All the bacterial isolates were screened for phosphate solubilization on Pikovskaya's agar
87 plates [20], siderophore production on chrome-Azurol-S agar medium [21], indolic
88 compounds production by Salkowski colorimetric method (indole-3-acetic acid, indole
89 pyruvic acid and indole acetamide) [22] and ammonia production in peptone water [23].
90

91 **Taxonomic identification of bacterial isolates**

92 Genomic DNA was extracted from all the bacterial isolates using the method described by
93 Pospiech and Neumann [24], and stored at -20 ˚C prior to PCR amplification. The 16S rRNA
94 gene was PCR amplified using universal primer pair pA (5′-
95 AGAGTTTGATCCTGGCTCAG-3′) and pH (5′-AAGGAGGTGATCCAGCCGCA-3′) by
96 following the conditions described by Edwards et al. [25]. Approximately 1 µg of PCR
97 products were restricted with endonucleases *Dde* I, and *Taq* I (Fermentas, USA) separately at
98 37 ˚C for overnight. The restricted PCR products were resolved by electrophoresis in 2.5%
99 agarose gels and banding pattern was visualized in a gel documentation and analysis system
100 (Alphaimager, USA) using ethidium bromide staining. Strong DNA bands were scored for
101 similarity and clustering analysis using NTSYS pc2.0 program (Applied Biostatistics Inc.,

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102 USA). The purified PCR products (16S rRNA gene) of representative isolates from each
103 dendrogram cluster was used as template in cycle sequencing reactions using both pA and pH
104 primers with fluorescent dye-labelled terminators (Applied Biosystems, USA). The
105 sequencing was performed in a 3130xl Genetic Analyzer (Applied Biosystems, USA).
106
107 The resulting sequences were compared with 16S rRNA gene sequences available in the
108 NCBI GenBank database by BLASTn search (https://blast.ncbi.nlm.nih.gov). The 16S rRNA
109 gene sequences was aligned using CLUSTALW algorithm implemented in MEGA 6.0 [26],
110 and a phylogenetic tree was also constructed in MEGA 6.0 using the neighbor-joining
111 method with 1000 bootstraps. The 16S rRNA gene sequences of bacterial isolates were
112 submitted to NCBI GenBank database under accession numbers HM480326 to HM480328,
113 HM480330 to HM480333, HM480335 and HM480337.
114
115 **Soil DNA extraction and clone library construction**
116 Soil DNA was extracted (0.25 g of each sample in duplicate) using MoBio Powersoil^{TM} DNA
117 extraction kit (MoBio Laboratories, USA) according to the manufacturer's instructions. To
118 remove humic acid contaminations, extracted soil DNA was further purified by using Wizard
119 DNA clean up system (Promega, USA). The 16S rRNA gene was PCR amplified from soil
120 DNA using the same universal primer set (pA and pH) and conditions as used for the DNA of
121 bacterial isolates. All PCR products were purified using QIAEX II Gel Extraction Kit
122 (Qiagen, Germany) and ligated into the plasmid vector pCRII-TOPO (Invitrogen, USA)
123 following the manufacturer's instructions. The plasmid vectors containing the 16S rRNA
124 gene fragments were transformed into electrocompetent *Escherichia coli* TOP10 cells
125 (Invitrogen, USA). Standard blue/white selection method was used to screen positive clones

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126 and checked for right insert size by PCR. All the positive PCR products were restricted with
127 endonucleases *Dde*I and *Hha*I, and restricted PCR fragments were resolved by
128 electrophoresis on 2.5% agarose gels.
129

130 **Sequence processing**

131 Representatives of each unique restriction pattern of 16S rRNA gene clones were sequenced
132 on 3130xl Genetic Analyzer (Applied Biosystems, USA) using vector specific M13 forward
133 and reverse primers. All the resulting sequences were analysed using mothur program [27].
134 First, a set of unique sequences was generated by binning identical 16S rRNA gene
135 sequences. Next, the chimeric sequences were removed using the mothur implementation of
136 UCHIME algorithm [28] in denovo mode. Finally, the sequences were clustered into
137 operational taxonomic units (OTUs) at a threshold of ≥97% sequence similarity using the
138 average neighbor clustering algorithm [29]. Phylogenetic analysis of representatives of each
139 OTU was performed in similar manner as described for bacterial isolates. Representative
140 sequences of each OTU were submitted to GenBank under accession numbers HQ450123 to
141 HQ450150.
142

143 **Results and discussion**

144 **Cultivation of bacterial isolates and analysis of PGP traits**

145 A total of 87 bacterial isolates were obtained on TSBA and R2A media. Of these, 45 bacterial
146 isolates were found positive for the PGP traits, including phosphate solubilization (57.3%
147 isolates), ammonia production (54.0% isolates), indolic compounds production (52.4%
148 isolates) and siderophore production (47.5% isolates) (Table 2). Phosphorus availability in
149 soil is vital for growth and development of plants. Phosphate-solubilizing bacteria increase

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150 phosphorus availability in soil through solubilization and mineralization of inorganic
151 phosphates, such as $Ca_3(PO_4)_2$, $AlPO_4$, and $FePO_4$ [30,31,32]. A high percentage of bacterial
152 isolates recovered form DE irrigated soils showed phosphate solubilization activity, which
153 indicates that DE irrigation did not suppress the growth of phosphate-solubilizing bacterial
154 community. An increase in population size of phosphate-solubilizing bacterial is also
155 reported earlier in agricultural soils irrigated with textile effluent [33]. We have also observed
156 a higher proportion of bacterial isolates positive for ammonia, siderophore and indolic
157 compounds production. Similar to our results, Tripathi et al. [15] found a higher fraction of
158 *Streptomyces* isolates displaying siderophore and indolic compounds production in pulp and
159 paper mill effluent irrigated agricultural soils. Microorganisms produce siderophore to
160 chelate various metals that could be inhibitory to their growth [34], and it has also been
161 reported that production of siderophore enhances production of indolic compounds [35],
162 which possibly explains why we recovered a high percentage of bacterial isolates showing
163 production of both siderophore and indolic compounds.
164

165 **Culturable bacterial community**

166 Cluster analysis based on 16S rRNA gene restriction pattern, grouped bacterial isolates into
167 nine clusters. The 16S rRNA gene sequences of one representative isolate from each cluster
168 were identified as *Bacillus megaterium*, *Bacillus simplex, Bacillus thuingiensis, Bacillus*
169 *subtilis*, *Paenibacillus pabuli*, *Arthrobacter crystallopoites*, *Sinorhizobium fredii*, *Mitsuaria*
170 *chitosanitabida* and *Lysobacter yangpyeongensis* (Table 3). A phylogenetic reconstruction of
171 16S rRNA gene sequences of these isolates together with sequences of their nearest relatives
172 is shown in Fig. 1. The collection of bacterial isolates was dominated by *Bacillus* and
173 *Bacillus* derived genera belonged to phylum *Firmicutes* (61%) (Fig. 2a), followed by

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174 bacterial genera from Alpha- Beta and Gamma- subdivisions of *Proteobacteria* (31%), and
175 *Actinobacteria* (8%) (Fig. 2a). Members of the phyla *Firmicutes*, *Proteobacteria* and
176 *Actinobacteria* are commonly reported to dominate in culturable soil bacterial communities
177 [16,36,37,38,39].
178
179 **Bacterial 16S rRNA gene clone library**
180 A total of 107 bacterial 16S rRNA gene clones were picked, and after restriction analysis
181 these were grouped in to 57 different clusters. Sequence analysis of these 57 clones indicated
182 presence of 6 chimeric 16S rRNA gene sequences, which were removed prior to further
183 analysis. Chimeric sequences are generated in PCR amplification of environmental DNA by
184 sequence hybridization between closely related microbial taxa [40]. A total of 28 OTUs were
185 detected based on ≥97% sequence similarity. These OTUs were affiliated with six bacterial
186 phyla, including *Proteobacteria* (Alpha-, Beta-, Gamma- and Delta- subdivisions) (46%),
187 *Acidobacteria* (14%), *Firmicutes* (14%), *Actinobacteria* (11%), *Bacteroidetes* (4%) and
188 *Gemmatimonadates* (4%) (Fig. 2b). Phylogenetic analysis of 16S rRNA gene sequences of
189 these OTUs together with sequences of their nearest relatives is shown in Fig. 3. Bacterial
190 OTUs corresponding to the phylum *Proteobacteria* were most abundant in clone library (Fig.
191 2b). *Proteobacteria* is the most abundant bacterial phylum in soil clone libraries [41], which
192 is known to contain a great level of physiological and metabolic diversity, and play a crucial
193 role in cycling of carbon, nitrogen and sulfur [42]. *Proteobacteria* is also designated as
194 copiotrophic bacterial taxa [43], which are highly responsive to nutrient amendment [44], and
195 their dominance in clone library could be the result of increased nutrient status of DE
196 irrigated soil. The OTUs belonging to *Acidobacteria*, *Firmicutes* and *Actinobacteria* were the
197 other abundant bacterial phyla in clone library (Fig. 2b). *Acidobacteria* is one of the most

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198 dominant soil taxa [45,46], however, due to difficulties associated with cultivation of these
199 taxa, very little is known about their physiology and potential functions [47]. Recent
200 comparative genomic studies have suggested that *Acidobacteria* may play an important role
201 in organic matter decomposition [48]. The proportion of *Firmicutes* OTUs was lower in clone
202 library compared to culturable bacterial community (Fig. 2). This discrepancy is also reported
203 earlier [49,50], and there have been several reasons put forth to explain this difference, which
204 include difficulties associated with lysing endospores during soil DNA extraction and bias in
205 PCR amplification [51]. The members of *Actinobacteria* processes cellulolytic activities
206 which enable them to degrade a wide range of soil organic matter [52]. The increase in
207 actinobacterial population has been reported earlier in soils receiving industrial effluent
208 irrigation [15,53].
209
210 In summary, DE irrigation did not seem to have an adverse effect on PGP traits of culturable
211 bacterial community, therefore using DE in conjugation with irrigation water could be a
212 viable water reuse method in regions facing water scarcity. The cultivation-dependent and -
213 independent methods provided a holistic picture of the bacterial community composition in
214 DE irrigated soil. Further studies focusing on more extensive sampling of DE irrigated
215 agricultural soils in different regions and times of year are necessary to gain better
216 understanding of structure and function of microbial communities.
217
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222

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370 **Figure legends**

371 **Fig. 1** Phylogenetic tree based on the 16S rRNA gene sequences of bacterial isolates
372 cultivated from DE irrigated agricultural soil. The tree was created by the neighbour-joining
373 method. The numbers on the tree indicate the percentage of bootstrap sampling derived from
374 1000 replicates. 16S rRNA gene sequence of *Ferroplasma thermophilum* used as an out-
375 group.

376

377 **Fig. 2** Relative abundance of bacterial phyla in (a) culturable bacterial community and (b)
378 16S rDNA gene clone library.

379

380 **Fig. 3** Neighbour-hood joining phylogenetic tree of bacterial OTUs recovered from 16S
381 rRNA gene clone library of DE irrigated soil.

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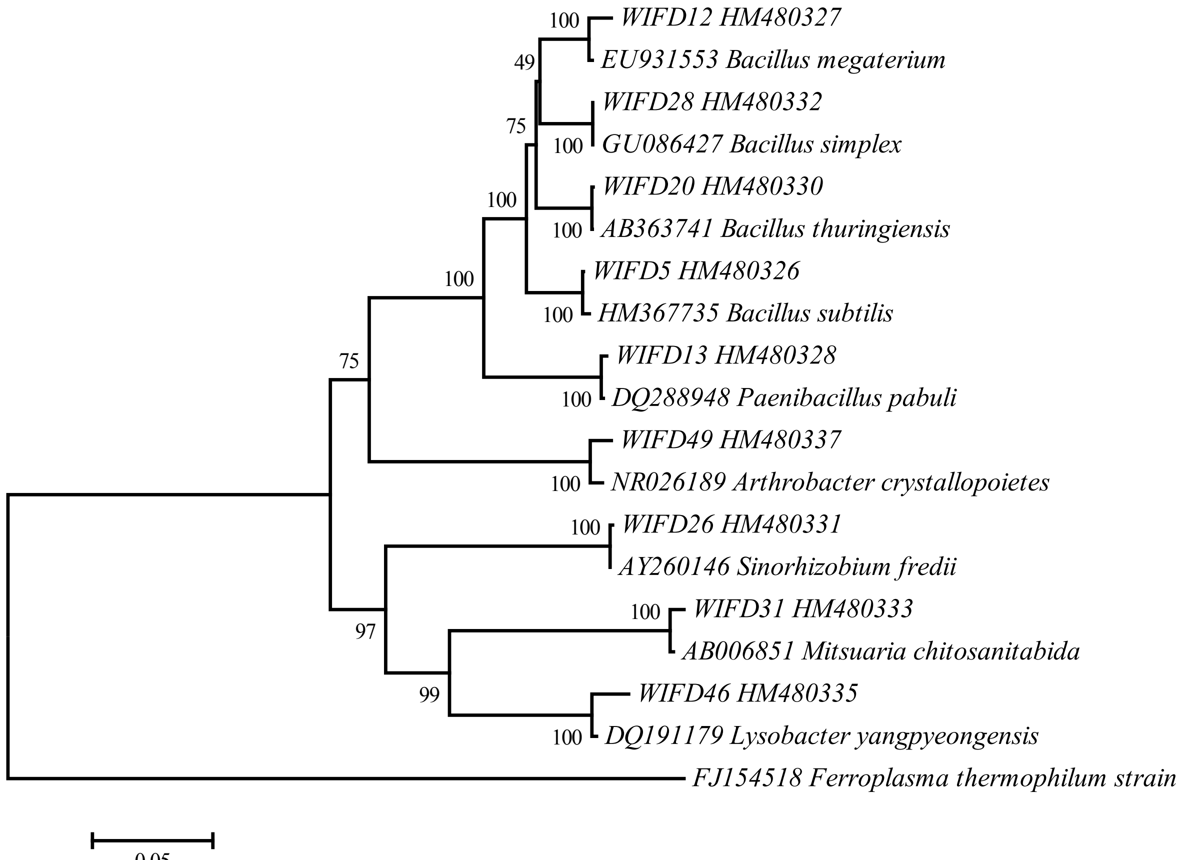

382

383 **Fig. 1**

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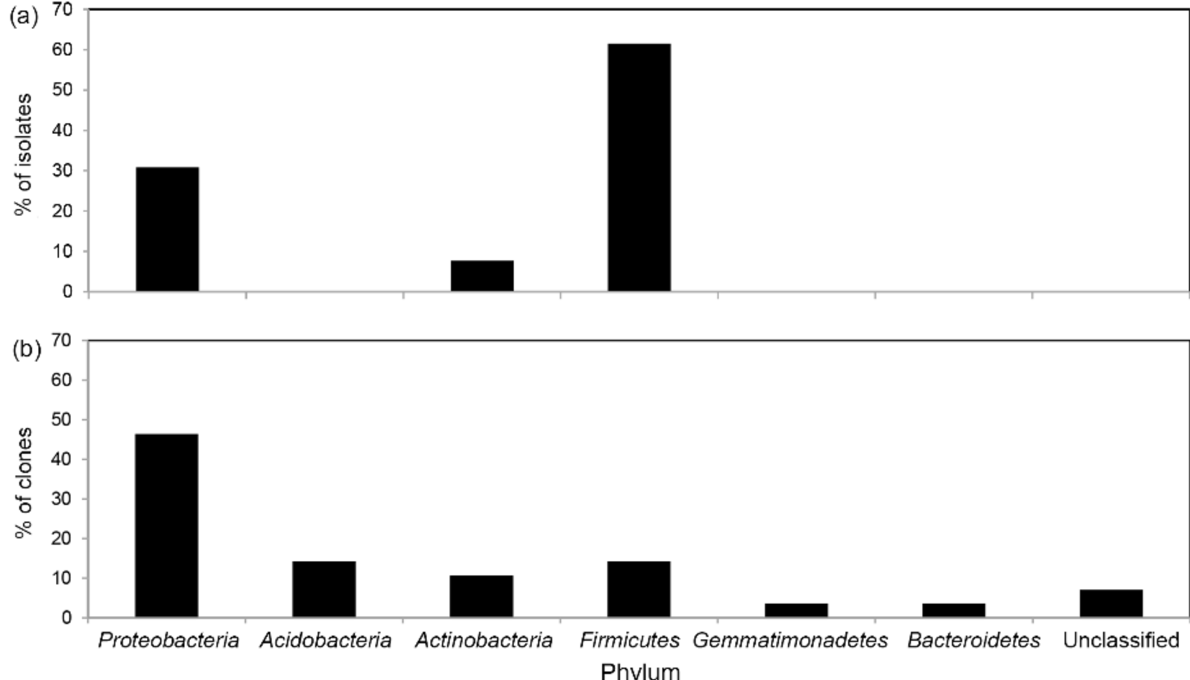

384
385 **Fig. 2**

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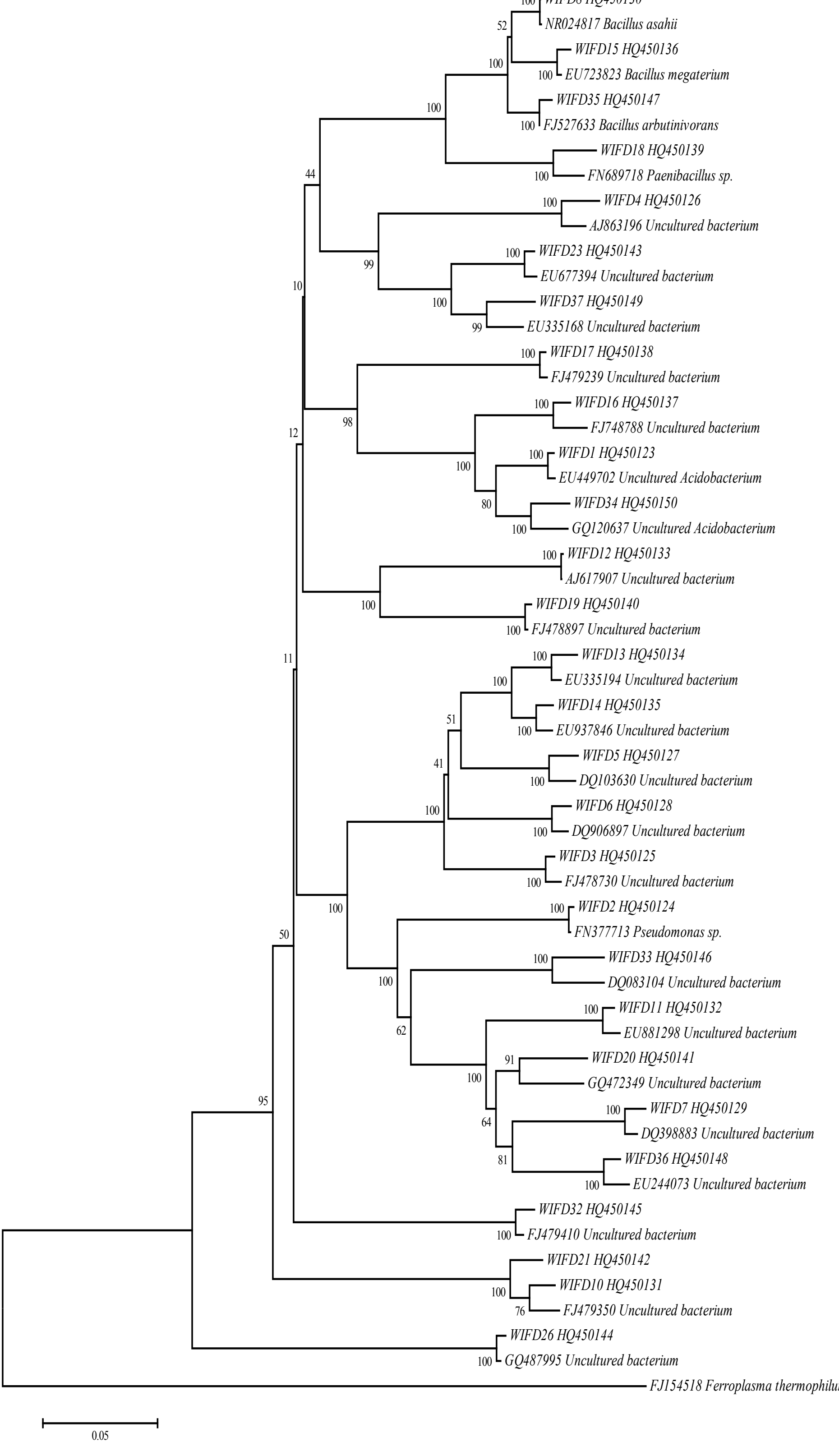

386

387 **Fig. 3**

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388 **Table 1.** Chemical nature of DE used for irrigation.

Compositions	values
pH	7.8
EC (dS m^{-1})	8.2
TDS (mg L^{-1})	5370.0
BOD (mg L^{-1})	4760.0
COD (mg L^{-1})	28000.0
Organic carbon (mg L^{-1})	154000.0
N (mg L^{-1})	1900.0
P (mg L^{-1})	43.0
K (mg L^{-1})	5736.0
Na (mg L^{-1})	525.0

389

390 **Table 2.** Phylogenetic affiliations of representative bacterial isolates.

Representative isolate	16S rRNA sequence homology		
	Species identified	NCBI accession number	% homology
WIFD5	*Bacillus subtilis*	HM480326	99
WIFD12	*Bacillus megaterium*	HM480327	97
WIFD13	*Paenibacillus pabuli*	HM480328	98
WIFD20	*Bacillus thuringiensis.*	HM480330	98
WIFD26	*Sinorhizobium freddi*	HM480331	99
WIFD28	*Bacillus simplex*	HM480332	99
WIFD31	*Mitsuaria chitosanitabida*	HM480333	98
WIFD46	*Lysobacter yangpyeongensis*	HM480335	97
WIFD49	*Arthrobacter crystallopoites*	HM480337	97

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391 **Table 3.** Plant-growth-promoting traits of bacterial isolates cultivated from DE irrigated soil.

Isolate no.	PGP traits			
	P-solubilization[a]	Siderophore production	NH_3 production	Indolic compounds (μg mg^{-1} protein)
WIFD1	4.6 ± 0.9	8.1 ± 0.7	+	78.60 ± 29.45
WIFD2	5.1 ± 0.9	6.2 ± 0.7	+	79.89 ± 29.45
WIFD4	ND	ND	+	60.61 ± 29.45
WIFD5	4.3 ± 0.9	7.1 ± 0.7	+	139.73 ± 29.45
WIFD6	5.3 ± 0.9	5.8 ± 0.7	ND	76.25 ± 29.45
WIFD9	6.8 ± 0.9	6.3 ± 0.7	+	102.40 ± 29.45
WIFD10	5.1 ± 0.9	ND	+	97.58 ± 29.45
WIFD11	4.2 ± 0.9	6.5 ± 0.7	+	108.80 ± 29.45
WIFD14	5.7 ± 0.9	ND	ND	98.66 ± 29.45
WIFD15	ND	7.2 ± 0.7	+	128.43 ± 29.45
WIFD16	5.1 ± 0.9	7.1 ± 0.7	+	128.75 ± 29.45
WIFD17	4.4 ± 0.9	6.7 ± 0.7	ND	ND
WIFD18	4.1 ± 0.9	ND	+	ND
WIFD19	4.7 ± 0.9	ND	+	117.92 ± 29.45
WIFD21	6.9 ± 0.9	6.1 ± 0.7	ND	ND
WIFD23	ND	ND	+	90.57 ± 29.45
WIFD24	5.3 ± 0.9	7.3 ± 0.7	ND	130.45 ± 29.45
WIFD25	4.9 ± 0.9	6.1 ± 0.7	+	ND
WIFD26	4.1 ± 0.9	5.5 ± 0.7	+	108.07 ± 29.45
WIFD27	ND	ND	+	92.63 ± 29.45
WIFD28	4.4 ± 0.9	7.2 ± 0.7	ND	137.23 ± 29.45
WIFD31	6.1 ± 0.9	ND	+	ND
WIFD32	5.4 ± 0.9	ND	ND	ND
WIFD34	6.4 ± 0.9	6.7 ± 0.7	+	126.24 ± 29.45
WIFD35	6.1 ± 0.9	7.4 ± 0.7	+	113.02 ± 29.45
WIFD36	ND	7.2 ± 0.7	ND	149.66 ± 29.45
WIFD37	ND	ND	+	82.36 ± 29.45
WIFD38	5.5 ± 0.9	ND	+	ND
WIFD39	4.2 ± 0.9	ND	+	ND
WIFD40	5.3 ± 0.9	7.4 ± 0.7	ND	150.62 ± 29.45
WIFD41	ND	5.3 ± 0.7	+	126.22 ± 29.45
WIFD42	3.7 ± 0.9	6.4 ± 0.7	+	132.83 ± 29.45
WIFD43	7.3 ± 0.9	7.2 ± 0.7	+	128.13 ± 29.45
WIFD44	ND	ND	ND	51.71 ± 29.45
WIFD45	6.1 ± 0.9	ND	+	133.11 ± 29.45

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WIFD46	5.5 ± 0.9	ND	ND	ND
WIFD48	7.1 ± 0.9	6.8 ± 0.7	+	ND
WIFD49	6.2 ± 0.9	6.3 ± 0.7	+	117.99 ± 29.45
WIFD50	5.8 ± 0.9	5.8 ± 0.7	+	ND
WIFD53	4.1 ± 0.9	7.3 ± 0.7	+	135.64 ± 29.45
WIFD54	4.1 ± 0.9	6.4 ± 0.7	ND	118.87 ± 29.45
WIFD58	7.2 ± 0.9	6.6 ± 0.7	+	ND
WIFD59	ND	7.3 ± 0.7	+	60.38 ± 29.45
WIFD60	ND	ND	+	83.39 ± 29.45
WIFD61	4.2 ± 0.9	7.8 ± 0.7	+	ND

392

393 [a] Radius of halo zone in mm

394 (–) Zone observed not observed, (ND) not detected, (+) positive for particular traits