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Enterobacter sp. strain Fs-11 adapted to diverse ecological conditions and promoted sunflower achene yield, nutrient uptake and oil contents

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Plant growth-promoting rhizobacteria (PGPR) are under extensive investigation, especially in developing countries, as supplements of chemical fertilizers due to cost-effective and eco-friendly nature. The competence and consistency of PGPR in heterogeneous soil medium and diverse ecological settings are still unclear. The current study presents *in vitro* and field evaluation of a physiologically and genetically characterized PGPR strain *Enterobacter* sp. Fs-11 (GenBank accession # GQ179978) in terms of its potential to thrive in rhizosphere and enhance sunflower crop yield and oil contents under diverse environmental conditions. Under *in vitro* conditions, strain Fs-11 was found to grow optimally at a range of temperature (15 to 40 °C) and pH values (6.5 to 8.5). Extracellular and intracellular localization of the strain Fs-11 in sunflower root cortical cells through transmission electron microscopy confirmed its epiphytic and endophytic root colonization pattern, respectively. In field experiments, conducted at three different agro-climatic locations, inoculation of strain Fs-11 at 50% reduced NP-fertilizer resulted in a significant (Fisher's LSD; $P \leq 0.05$) increase in growth, achene yield, nutrient uptake and oil contents as compared to non-inoculated plants. Inoculation also responded significantly in terms of increase in mono and polyunsaturated fatty acids (oleic and linoleic acids, respectively) without rising saturated fatty acid (palmitic and stearic acid) contents. We concluded that *Enterobacter* sp. Fs-11 is a potential candidate for biofertilizer formulations to supplement chemical fertilizer requirements of sunflower crop under diverse climatic conditions.

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Running title: Ecological adaptation and sunflower oil contents increase by *Enterobacter* sp.

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Acknowledgements

Authors are grateful to Higher Education Commission (HEC), Pakistan for grant of funds for this project under HEC indigenous 5000 PhD Fellowship Program. We are also thankful to Dr. Iftikhar Ali (Deputy Chief Scientist, NIFA, Peshawar) for providing space to conduct field trial and for conducting achene oil and fatty acid analysis. We also appreciate the efforts of Mr. Nasir Rahim (Assistant professor, UPR) for providing resources to conduct field experiment at UPR.

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34 **Abstract**

35 Plant growth-promoting rhizobacteria (PGPR) are under extensive investigation, especially in
36 developing countries, as supplements of chemical fertilizers due to cost-effective and eco-
37 friendly nature. The competence and consistency of PGPR in heterogeneous soil medium and
38 diverse ecological settings are still unclear. The current study presents *in vitro* and field
39 evaluation of a physiologically and genetically characterized PGPR strain *Enterobacter* sp. Fs-11
40 (GenBank accession # GQ179978) in terms of its potential to thrive in rhizosphere and enhance
41 sunflower crop yield and oil contents under diverse environmental conditions. Under *in vitro*
42 conditions, strain Fs-11 was found to grow optimally at a range of temperature (15 to 40 °C) and
43 pH values (6.5 to 8.5). Extracellular and intracellular localization of the strain Fs-11 in sunflower
44 root cortical cells through transmission electron microscopy confirmed its epiphytic and
45 endophytic root colonization pattern, respectively. In field experiments, conducted at three
46 different agro-climatic locations, inoculation of strain Fs-11 at 50% reduced NP-fertilizer
47 resulted in a significant (Fisher's LSD; $P \leq 0.05$) increase in growth, achene yield, nutrient uptake
48 and oil contents as compared to non-inoculated plants. Inoculation also responded significantly
49 in terms of increase in mono and polyunsaturated fatty acids (oleic and linoleic acids,
50 respectively) without rising saturated fatty acid (palmitic and stearic acid) contents. We
51 concluded that *Enterobacter* sp. Fs-11 is a potential candidate for biofertilizer formulations to
52 supplement chemical fertilizer requirements of sunflower crop under diverse climatic conditions.

53 **Keywords:** *Enterobacter* sp**.,** Field evaluation, Growth studies, Transmission electron
54 microscopy, Fatty acids, Agro-climatic locations

55 **1. Introduction**

56 Plant growth-promoting rhizobacteria (PGPR) are able to associate and persist in the dynamic
57 rhizosphere and endosphere environments (Singh, Pandey & Singh, 2011), thereby benefiting
58 plants in terms of nutrient acquisition from soil. Due to its nutritional value, rhizosphere serves
59 as PGPR hotspot driving a complex cross-talk between plants and microbes (Buée et al., 2009).
60 In such nutritionally rich environment, PGPR execute many phytobeneficial functions like
61 atmospheric nitrogen fixation, mineral phosphate solubilization, growth hormone production, 1-
62 aminocyclopropane-1-carboxylate (ACC) deaminase activity, quorum sensing, growth
63 suppression of many plant pathogens and confer tolerance in plants against biotic and abiotic

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64 stresses (Bhattacharyya & Jha, 2012; Pérez-Montaño et al., 2014; Akram et al., 2016). The
65 genera identified from rhizosphere with known PGPR-traits are *Pseudomonas, Azospirillum,*
66 *Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia*, *Bacillus,*
67 *Serratia*, *Acinetobacter*, *Erwinia*, *Flavobacterium*, *Proteus*, *Xanthomonas,Agrobacterium,*
68 *Frankia etc.* (Kaymak, 2011; Bhattacharyya & Jha, 2012; Tailor & Joshi, 2014; Mahmood et al.,
69 2017). In developing and developed countries, there has been a continuous applied research on
70 PGPR inoculation with taxonomically different crop plants like wheat (Majeed et al., 2015), rice
71 (Lucas et al., 2009), sunflower (Shahid et al., 2012; Shahid et al., 2015), maize (Qaisrani et al.,
72 2014), bean (Pérez-Montaño et al., 2014) to establish sustainable agricultural practices.
73 PGPR-based bioinoculants are being commercialized to promote plant growth and control
74 disease causing microorganisms (Shoda, 2000; Ashrafuzzaman et al., 2009). Plant-beneficial
75 bacteria belonging to the genera *Azospirillum*, *Azotobacter*, *Burkholderia*, *Enterobacter*,
76 *Rhizobium*, *Bacillus*, *Erwinia*and *Flavobacterium* have been extensively characterized and used
77 as bioinoculants for crop plants with variable degree of success (Rodriguez & Fraga, 1999;
78 Podile & Kishore, 2006). Such PGPR-based biofertilizers are useful in minimizing the
79 application of environmentally unsafe and economically expensive chemical fertilizers (Kucey,
80 Janzen & Leggett, 1989; Stefan, Mihasan & Dunca, 2008). These bioinoculants can be classified
81 into biofertilizers (mobilizing macro and micro nutrients from environment), phytostimulators
82 (growth hormone producing) and biopesticide (suppressing phytopathogens) (Bhattacharyya &
83 Jha, 2012).
84 There exists a great need to benefit from such promising, renewable and eco-friendly
85 bioinoculants to cope with a continuously decreeing fertilizer use efficiency and food security
86 issues in the technologically advanced world (Pii et al., 2015). Farmers around the globe,
87 especially in developing countries, are bending towards bacterial inoculants due to the increasing
88 costs of synthetic fertilizers in addition to their hazardous environmental impacts and non-
89 availability in peak planting seasons (Bashan et al., 2014). Hence, the global acceptance of
90 biofertilizers may reduce the farmer's dependence on agro-chemicals and make the bioinoculants
91 readily available both in developed and developing countries (Gamalero, Berta & Glick, 2009).
92 Consequently, the synthetic fertilizers are expected to be substituted with bacterial inoculants in
93 future (Ahemad & Kibret, 2014).

94 On the other hand, the outcome of microbial inoculants is variable especially in field conditions
95 due to their narrow adaptation level under heterogeneous biotic and abiotic factors (Zaidi et al.,
96 2009; Glick, 2012). In addition, adaptability of a particular bioinoculant or bacterial strain to
97 diverse agroclimatic conditions is always challenging (Sharma et al., 2013). Thus, the objective
98 of this research was to evaluate the inoculation effect of a potent and characterized *Enterobacter*
99 sp. strain Fs-11 on various agronomic and physiological attributes of sunflower (*Helianthus*
100 *annuus* L.) under dissimilar ecological conditions of Faisalabad, Peshawar and Rawalakot,
101 Pakistan. Sunflower is an important oil-seed crop carrying ideal combination of saturated and
102 unsaturated fatty acids. The crop has a great potential to bridge up the huge gap between edible
103 oil production and consumption in the country (Khan et al., 2000). We hypothesized that the
104 strain Fs-11 is competent enough to exert its growth-promoting effect when exposed to compete
105 with indigenous rhizobiome and diverse environmental factors.

106 **2. Materials and Methods**

107 **2.1. Background and significance of *Enterobacter*sp. strain Fs-11**

108 *Enterobacter* sp. Fs-11 was originally isolated from sunflower (*Helianthus annuus* L.)
109 rhizosphere soil (EC 0.76 dS m^{-1}, pH 8.02, organic matter 0.79%, total N 0.039% and available P
110 5.6 mg kg^{-1}), sampled from Faisalabad (30° 44′27.10 N, 72°38′18.39 E), Pakistan during a
111 sampling visit in April, 2010. The strain has already been reported as mineral phosphate
112 solubilizing, auxin producing and growth-promoting bacterium (Shahid et al., 2012).

113 **2.2. *In vitro* growth studies**

114 Before inoculating with sunflower seeds in field experiments, strain Fs-11 was investigated *in*
115 *vitro* to thrive at a range of temperature and pH values. A 50-mL culture of strain Fs-11 was
116 grown in Luria-Bertani (LB) broth at150 rpm at a range of temperatures (15, 20, 25, 30, 35 and
117 40°C) and a fixed pH value of 7. In the second experiment, the culture was grown at a range of
118 pH values (6.5, 7.0, 7.5, 8.0 and 8.5) and fixed temperature of 30°C.The culture was harvested
119 after 0.5, 4, 16 and 24 h followed by serial dilution and plating as described by Somasegaran &
120 Hoben (1994) to record bacterial cell density.

121 **2.3. Ultrastructure studies through transmission electron microscopy**

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122 The strain was already reported as efficient root colonizer which was validated through confocal
123 laser scanning microscopic studies and recovering rifampicin-resistant derivatives of strain Fs-11
124 from roots (Shahid et al., 2012). To further investigate whether strain Fs-11 is rhizosphere
125 colonizer or an endophyte, colonization pattern was studied through transmission electron
126 microscopy (TEM). For ultrastructure studies, surface sterilization of *H.annuus* (cv. FH-331)
127 seeds was carried out by immersing in sodium hypochlorite (5 %, w/v) for 10 min. following by
128 rinsing 5 to 6 times with sterilized water. The seeds were inoculated with strain Fs-11 (1×10^8
129 CFU mL^{-1}) and germinated on 1.5 % (w/v) water agar plates. Root hairs of 10-day old
130 gnotobiotically grown *H.annuus* seedlings were cut into pieces (1-3 cm) and processed for
131 observation under transmission electron microscope (TEM, JEOL 1010, Japan) according to the
132 procedure described by Shahid et al. (2015).

133 **2.4. Site selection, soil physico-chemical analysis and meteorological data**

134 Three agro climatically different experimental sites (Fig. 1) of Pakistan i.e. National Institute for
135 Biotechnology and Genetic Engineering (NIBGE) Faisalabad, (31°23'42.13 N, 73°1'37.24 E),
136 Nuclear Institute for Food and Agriculture (NIFA), Peshawar (34°1'13.50 N, 71°28'53.02 E) and
137 The University of Poonch, Rawalakot (UPR), Azad Jammu and Kashmir, Pakistan (33°49'48.04
138 N, 73°48'17.32 E) were selected to evaluate the effect of strain Fs-11 inoculation at reduced
139 fertilizer dose (Ali, Barrientos & Saboor, 2015). Five soil samples from each experimental site
140 were collected for physico-chemical analysis prior to the onset of experiments. Soil physical and
141 chemical properties were presented in table 1. Meteorological data of three sites for entire crop
142 growth period were recorded in collaboration with Pakistan Meteorological Department and
143 presented figure 1.

144 **2.5. Preparation of inoculum and seed surface sterilization**

145 Inoculum of *Enterobacter* sp. Fs-11 was prepared by growing the strain up to 1×10^8 CFU mL^{-1}.
146 The culture was centrifuged at 8,000*g* and washed twice with 0.85% (w/v) saline. The cell were
147 re-suspended in equal volume of saline and diluted to maintain the cell density of 1×10^8 CFU
148 mL^{-1}. Inoculation with completed by dipping the seeds in inoculum for 30 min. Hybrid
149 sunflower (cv. FH-331) seeds were procured from Ayub Agricultural Research Institute (AARI),

150 Faisalabad, Pakistan. The seeds were surface sterilized by 5 % (w/v) sodium hypochlorite as
151 described by Shahid et al. (2012).

152 **2.6. Experimental design, crop husbandry and treatments**

153 Field experiments, at all three experimental sites, were conducted in a randomized complete
154 block design (RCBD) with three replications. A tractor-mounted cultivator was employed to
155 plough the seed-beds 2-3 times followed by planking. Plot size and seed rate was kept as 3×2 m^2
156 and 8 kg ha^{-1}, respectively. Recommended doses of NP fertilizers (N = 50 kg ha^{-1} and P = 90 kg
157 ha^{-1}) were applied in the form of urea and diammonium phosphate (DAP), respectively. All DAP
158 was applied at the time of sowing, while half urea was applied at sowing and remaining half at
159 first irrigation. Manual sowing by the dibbling method was carried out with three seeds per hill
160 and a between-row and between-plant distance of 75 and 25 cm, respectively was maintained.
161 All other standard agronomic and plant protection measures were followed for whole set of
162 experiments. Following three noninoculated treatments were compared with their respective
163 inoculated ones:

164 T_0 = Noninoculated control without fertilizer application
165 T_1 = Noninoculated control with full N and half P fertilizer
166 T_2 = Noninoculated control with full NP fertilizer
167 T_3 = Inoculated with strain Fs-11 without fertilizer application
168 T_4 = Inoculated with strain Fs-11 with full N and half P fertilizer
169 T_5 = Inoculated with strain Fs-11 with full NP fertilizer

170 **2.7. Measurement of growth parameters**

171 Crop was left for five days for sun drying after harvesting and then threshing was carried by
172 physical beating. With the help of scale, height and head diameter of 20 randomly selected plants
173 from each treatment was measured and averaged. Average value of 10 randomly selected plants
174 from each treatment was measured to calculate number of achenes per head and 1000-achene wt.
175 Achene yield was measured per plot basis and then converted to kg ha^{-1}.

176 **2.8. Measurement of physiological parameters**

177 Aachenephosphorus (P) contents were measured by the vanadium phospho-molybdate yellow
178 colour method (Yoshida, Forno & Cock, 1976) using using UV-visible spectrophotometer
179 (JENWAY6305, UK), while nitrogen (N) contents were determined by wet digestion with H_2SO_4
180 using micro-Kjeldahl method as described by Sparks et al. (1996). Furthermore, achene oil and
181 fatty acid contents were recorded through gas chromatography as described by Erickson (1980).

182 **2.9. Statistical analysis**

183 Data were analyzed by one- and two-way analysis of variance (Steel, Torrie & Dickey, 1997),
184 and presented as the mean of three replications (n =3) using Statistix ver. 8.1 software
185 (Tallahassee, Florida). Values that differ significantly ($P \leq 0.05$) according to Fisher's least
186 signficant difference (LSD) method are shown with different low-case letters. To determine the
187 effect of locations on various parameters, principal component analysis (PCA) was conducted
188 using SPSS 17 software (SPSS Inc., USA).

189 **3. Results**

190 **3.1. *In vitro* growth studies**

191 Growth curves constructed after *in vitro* experimentation resulted in optimal cell density of
192 *Enterobacter* sp. Fs-11 at the tested temperature and pH values (Fig. 2). After 24 h of incubation,
193 cell density at 30°C (10.21 ± 0.39 CFU mL^{-1}) and pH 7.5 (10.34 ± 0.41 CFU mL^{-1}) was found
194 maximum. The minimum cell density of strain Fs-11 after same incubation period was
195 determined at 15°C (7.97 ± 0.38 CFU mL^{-1}) and pH 6.5 (8.47 ± 0.34 CFU mL^{-1}). On an average,
196 the growth of strain Fs-11 at variable temperature and pH settings was found optimum which
197 confirmed its potential to evaluate the strain as inoculum under diverse ecological conditions.

198 **3.2. Ultra-structure studies through TEM**

199 Cells of *Enterobacter* sp. Fs-11 were localized in rhizoplane of sunflower (Fig. 3). Some
200 bacterial cells were found embedded in an extracellular matrix of root hair cells (Fig. 2b). Strain
201 Fs-11 was also clearly spotted inside the root cortical cells (Fig. 2c,d). Thus, *Enterobacter* sp. Fs-
202 11 was found to be a rhizocompetent strain with substantial ability to colonize sunflower
203 rhizosphere and to demonstrate both ecto- and endophytic behaviour.

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204 **3.3. Soil physico-chemical analysis**

205 The soil textures of three experimental locations NIBGE, NIFA and UPR were sandy loam, clay
206 loam and loam with 8.3, 8.2 and 7.3 pH values, respectively. Similarly, organic matter
207 percentage was higher in UPR (1.78) than NIBGE and NIFA (0.68 and 1.12, respectively). The
208 total N was calculated as 0.55, 0.44 and 0.76 g kg^{-1}. The available P (6.54, 4.74 and 6.1 mg kg^{-1})
209 and K (203, 164, 224 mg kg^{-1}) was measured at NIBGE, NIFA and UPR, respectively (Table 1).
210 Thus the soil conditions were quite heterogeneous for field experiments.

211 **3.4. Yield response of sunflower to inoculation**

212 Inoculation of strain Fs-11 along with half dose of recommended NP-fertilizer (T_4) significantly
213 (Fisher's LSD; $P \leq 0.05$) boosted sunflower plant height (ca. 149, 112 and 140 cm), head diameter
214 (ca. 38, 35 and 40 cm), number of achenes per head (ca. 917, 709 and 282) as compared to non-
215 inoculated plants with 50% reduced P fertilizer (T_1) at NIBGE, NIFA and UPR, respectively
216 (Table 2). The inoculation response of sunflower in terms of growth parameters was even
217 statistically at par with non-inoculated plants with full dose of NP-fertilizer (T_2). A non-
218 significant effect of T4 was recorded at all locations in terms of 1000-achene weight (ca 69, 57
219 and 69 g, respectvely) as compared to T_2. Subsequently, achene achene yield (2804, 2138 and
220 3182 kg ha^{-1}) in terms of locations NIBGE, NIFA and UPR, respectively was significantly high
221 as compared to T_1 and statistically at with T_2. Treatment T_5 (inoculation with full dose of
222 recommend fertilizer) did not enhance sunflower growth and yield significantly as compared to
223 T_4, suggesting that strain Fs-11 showed plant growth promoting potential under reduced NP
224 fertilizer application.

225 **3.5. Effect of inoculation on sunflower nutrient uptake**

226 Inoculation affected non-significantly in terms of achene and leaf N contents at all three
227 locations. The achene N contents (37.33, 35.39 and 38.98 g kg^{-1}) and P contents (36.83, 34.81
228 and 35.15 g kg^{-1}) of T_4 at NIBGE, NIFA and UPR, respectively were found statistically at par
229 with T_1 and even T_2 (Table 3). On the other hand, inoculation of *Enterobacter* sp. Fs-11 at 50%
230 reduced P-fertilizer (T_4) significantly promoted P-uptake of achenes (14.94, 11.99 and 15.37 g
231 kg^{-1}) and leaves (12.65, 14.20 and 16.32 g kg^{-1}) at NIBGE, NIFA and UPR, respectively as
232 compared its corresponding non-inoculated treatment (T_1). Achene and leaf P contents after

233 inoculation at 50% reduced P fertilizer were measured statistically at par with non-inoculated
234 plant with full dose of NP-fertilizer.

235 **3.6. Effect of inoculation of sunflower achene oil and fatty acid contents**

236 Moreover, inoculation with strain Fs-11 at half P-fertilizer enhanced achene oil contents
237 significantly at NIBGE (41.05%) and UPR (42.72%) as compared to non-inoculated treatment
238 (T_1). At UPR the oil contents of T_4 (43.24%) were found statistically similar to that of T_1
239 (41.27%). Similarly, mono and polyunsaturated fatty acids (oleic and linoleic acids, respectively)
240 were also affected non-significantly after inoculation at 50% reduced P fertilizer as compared to
241 non-inoculated plants with half P fertilizer dose. In case of saturated fatty acids (Palmitic acid
242 and stearic acid) a similar non-significant effect was recorded after inoculation at all locations.
243 On the other hand, a comparative decreasing trend was calculated in case of palmitic acid in
244 inoculated treatments as compared to non-inoculated ones (Table 4).

245 In principal component analysis, all the variables responded to inoculation of strain Fs-11 in
246 similar pattern at NIBGE and UPR, although these locations have diverse ecological conditions.
247 Only oleic acid C18:1 and linoleic acid C18:2 were influenced positively by NIFA, while all
248 other yield and physiological parameters were favored by the climatic conditions of NIBGE and
249 UPR (Fig. 4).

250 **4. Discussion**

251 Potential PGPR strains have been isolated, characterized and commercialized as bioinoculants in
252 various parts of the world, especially in developing countries. The consistency of these bacterial
253 bioinoculants in terms of performance has always been a question mark mainly due their
254 adaptability in diverse climatic conditions, heterogeneous nature of soil and competition of
255 PGPR strains with indigenous microbiota. Thus, success of biofertilizer lies in its adaptation to
256 local environment and its ability to complete with various biotic and abiotic factors. This study
257 report here environmental adaptability and plant growth-promotion potential of *Enterobacter* sp.
258 Fs-11, which was isolated, characterized reported earlier (Shahid et al., 2012) as mineral
259 phosphate-solubilizing strain with considerable ability to synthesize indole-3-acetic acid *in vitro*.
260 The strain also survived in rhizosphere of sunflower and enhanced its growth significantly in a
261 pot experiment (Shahid et al., 2012). Looking at the PGPR potential of strain FS-11, it was

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262 planned to evaluate the strain under diverse field conditions. Before field experiments, the strain
263 was characterized for its adaptability to a range of temperature and pH values *in vitro*. The
264 purpose of *in vitro* growth studies was to confirm the ability of the strain FS-11 to thrive under
265 variable temperature and pH values. The optimal growth of strain Fs-11 (8-10 CFU mL^{-1}) at a
266 range of temperature and pH values after 24 h of incubation made it a prospect for inoculation
267 under variable agroclimatic conditions (Fig. 2). *In vitro* growth studies made it feasible select
268 agro-ecologically different zones for its field inoculation experiments. Consistent to our study,
269 Pandey et al. (2014) isolated some bacterial strains from Uttarakhand, India with wide range of
270 temperature and pH tolerance ability. In vitro studies also confirmed its epiphytic and endophytic
271 nature through TEM. Localization of the strain Fs-11 in sunflower rhizosphere and endosphere
272 after its inoculation under gnotobiotic conditions reaffirmed the hypothesis that the bacterium is
273 a potential root colonizer (Fig. 3). Despite the fact that colonization studies were conducted in
274 the absence of any indigenous bacterial competition, its colonization in heterogeneous soil is
275 already reported (Shahid et al., 2012). Localization of various plant growth-promoting
276 rhizobacteria (PGPR) in rhizosphere, rhizoplane and endosphere of plant roots and nodules
277 through TEM have already been reported by various researchers (Schloter et al., 1997; Hameed
278 et al., 2005; Jeun et al., 2008; Yasmeen et al., 2012; Shahid et al., 2015).

279 Selection of sites for field experiments is made after studying the agro-ecological locations of
280 Pakistan. Pakistan is divided into ten agro-climatic zones based on climate, water availability,
281 land use and geography (Chaudhry & Rasul, 2004). After inoculation of strain Fs-11, asignificant
282 (Fisher's LSD; $P \leq 0.05$) increase in growth and yield parameters as compared to noninoculated
283 control plants (T_1) at NIBGE, NIFA and UPR may be attributed to its rhizosphere adaptation
284 under variable temperatures and soil types, mineral phosphate solubilisation and IAA synthesis
285 (Table 2). Auxin synthesis and mineral phosphate solubilisation potential of PGPR has already
286 been reported to enhance plant growth and nutrient acquisition from the soil (Igual et al., 2001;
287 Chen et al., 2006; Shirmardi et al., 2010; Akram et al., 2016). Andreote et al. (2009) and Shankar
288 et al. (2011) reported the increased root colonization and plant growth promotion after PGPR
289 inoculation. Plant growth-promoting rhizobacteria are also reported to enhance wheat, lentil and
290 cotton growth under variable temperature and soil conditions (Egamberdiyeva & Höflich, 2003;
291 Zafar et al., 2012). Similarly, significant increase of achene P contents at 50% reduced P
292 fertilizer dose (T_4) in comparison with noninoculated plants at NIBGE, NIFA and UPR was due

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293 to export of P from soil to sunflower achenes and leaves after inoculation (Table 3). The possible
294 mechanism of achene P contents increase by strain Fs-11 inoculation may be transport of P from
295 soil to plant by intense root colonization, organic acid production and making unavailable soil
296 phosphate available by lowering the pH of the surroundings (Shahid et al., 2012; Hanif et al.,
297 2015). On the other hand, achene and leaf N contents were not affected significantly after
298 inoculation. This non-significant effect might be due to inability of strain Fs-11 to fix
299 atmospheric nitrogen. Thus, significant growth and physiological modulations in sunflower plant
300 after strain Fs-11 inoculation made the strain competitive and potent in diverse environmental
301 conditions.

302 Being an oilseed crop sunflower is globally grown for its seed oil yields. After inoculation at
303 NIBGE and NIFA, a significant increase in achene oil contents as compared to non-inoculated
304 plants with 50% reduced P and no NP fertilizer doses (T_0 and T_1) might be due to better vigor of
305 inoculated plants and better acquisition of soil nutrients. These results are contrary to the
306 findings of Akbari et al. (2011), who reported that PGPR inoculation do not affect the achene oil
307 contents. On the other hand, (Ekin, 2010) reported a significant increase in sunflower achene oil
308 contents after inoculation with phosphate solubilizing bacteria M-13. Increase in oil contents of
309 inoculated plants might be attributed to the better P nutrient acquisition from soil as P is directly
310 responsible for oil yield increase in plants. Unsaturated fatty acids (linoleic acid and oleic acid)
311 were remained statistically at par in terms of inoculated and non-inoculated treatments at all
312 experimental sites. This might be due to unknown ecological and physiological factors
313 responsible to accumulate these oils in sunflower seeds. Palmitic acid C16:0 was comparatively
314 less-accumulated in inoculated plants, which was a positive dietary sign for humans and animals
315 feeding on sunflower seeds. According to the World Health Organization, palmitic acid
316 consumption in human diet increases the risk of developing cardiovascular diseases. At the same
317 time, non-inoculated treatment with full fertilizer (T_2) was also found statistically at par with Fs-
318 11 inoculated treatment (T_4) in key parameters like achene yield and oil contents at NIBGE,
319 NIFA and UPR. This suggested that inoculation of strain Fs-11 at 50% reduced P fertilizer dose
320 competed with application of full dose of recommended fertilizer. Comparatively better effect of
321 inoculation at UPR and NIBGE than at NIFA may be due to the better soil and climatic
322 conditions throughout the crop growth period.

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323 **Conclusion**

324 Current study proved that *Enterobacter* sp. Fs-11 is a rhizocompetent strain that can boost
325 nutrient acquisition under diverse soil conditions after adhering with plant roots. The strain is
326 adaptable to variable temperature and soil pH conditions and thus a potent candidate to enhance
327 sunflower yield and oil contents. The strain Fs-11 can cutoff P fertilizer requirements to half
328 without compromising achene yield and oil contents. Hence, it is a potential candidate for
329 biofertilizer of sunflower crop for sustainable yield in a cost-effective manner.

330 **Conflict of interest**

331 The authors declare that the research was conducted without any conflict of interest.

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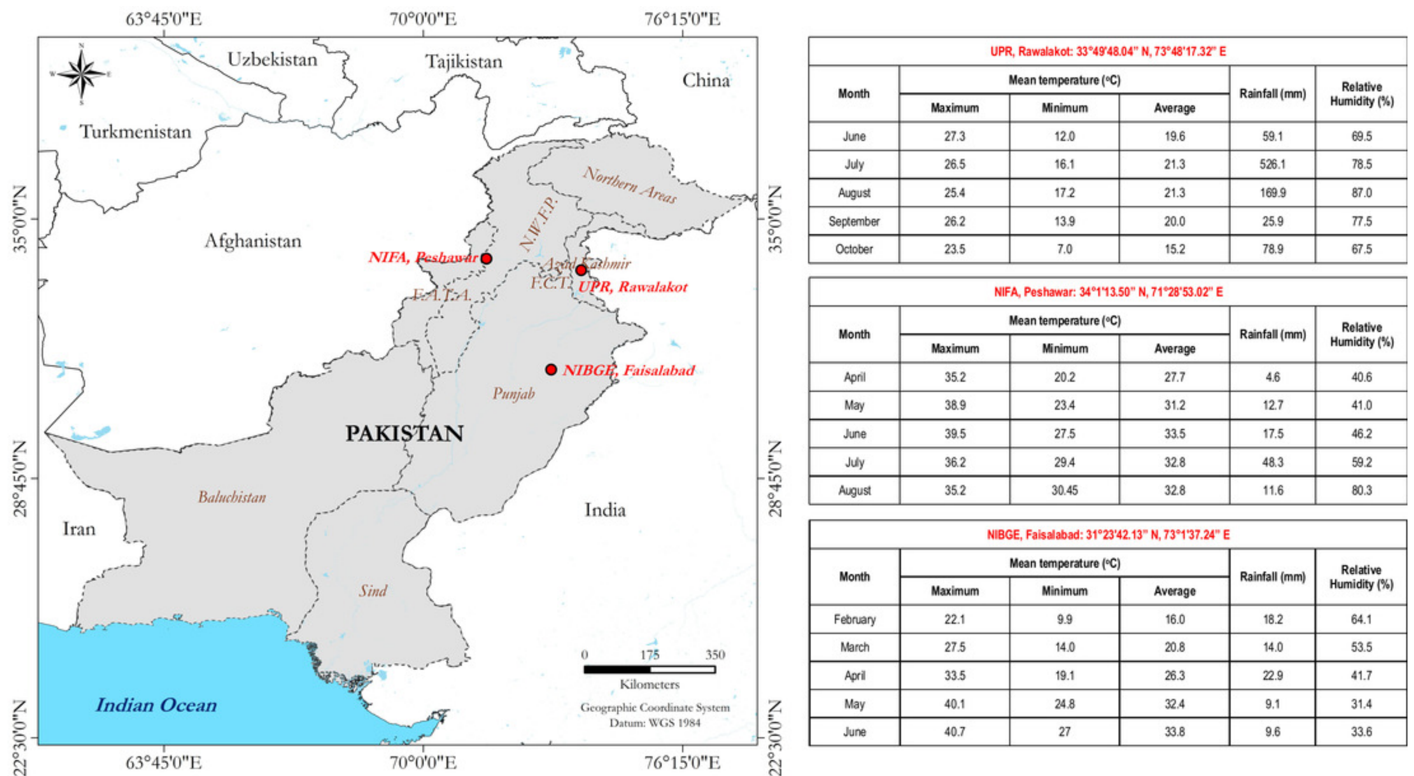
Figure 1

Experimental sites located in three different agro-climatic zones of Pakistan showing the meteorological data collected separately for each location during the crop growth period



UPR, Rawalakot: 33°49'48.04" N, 73°48'17.32" E					
Month	Mean temperature (°C)			Rainfall (mm)	Relative Humidity (%)
	Maximum	Minimum	Average		
June	27.3	12.0	19.6	59.1	69.5
July	26.5	16.1	21.3	526.1	78.5
August	25.4	17.2	21.3	169.9	87.0
September	26.2	13.9	20.0	25.9	77.5
October	23.5	7.0	15.2	78.9	67.5

NIFA, Peshawar: 34°1'13.50" N, 71°28'53.02" E					
Month	Mean temperature (°C)			Rainfall (mm)	Relative Humidity (%)
	Maximum	Minimum	Average		
April	35.2	20.2	27.7	4.6	40.6
May	38.9	23.4	31.2	12.7	41.0
June	39.5	27.5	33.5	17.5	46.2
July	36.2	29.4	32.8	48.3	59.2
August	35.2	30.45	32.8	11.6	80.3

NIBGE, Faisalabad: 31°23'42.13" N, 73°1'37.24" E					
Month	Mean temperature (°C)			Rainfall (mm)	Relative Humidity (%)
	Maximum	Minimum	Average		
February	22.1	9.9	16.0	18.2	64.1
March	27.5	14.0	20.8	14.0	53.5
April	33.5	19.1	26.3	22.9	41.7
May	40.1	24.8	32.4	9.1	31.4
June	40.7	27	33.8	9.6	33.6

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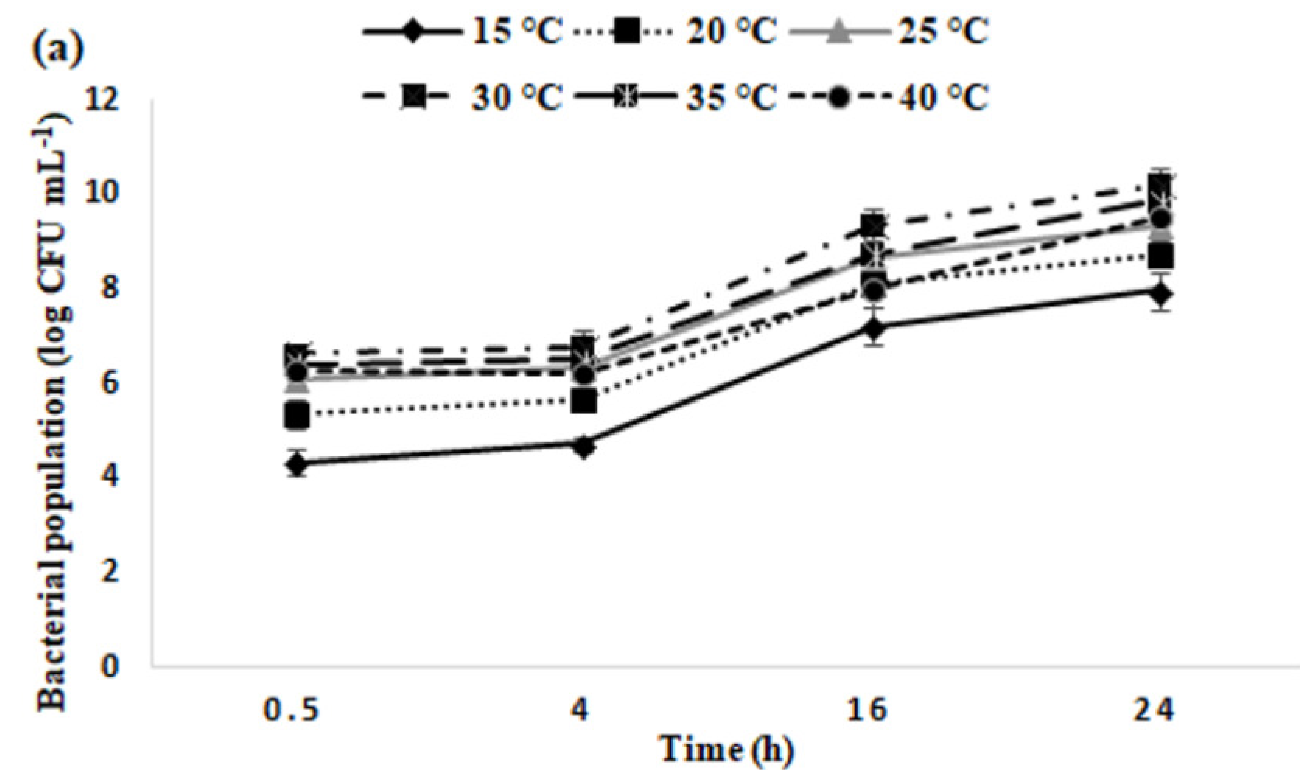
Figure 2

Time-lapse in vitro growth of Enterobacter sp. Fs-11 at different temperature (a) and pH (b) levels. Error bars represent standard deviation (n = 3)


(a)
15 °C
20 °C
25 °C
30 °C
35 °C
40 °C
Bacterial population (log CFU mL-1)
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10
8
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4
2
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16
24
Time (h)

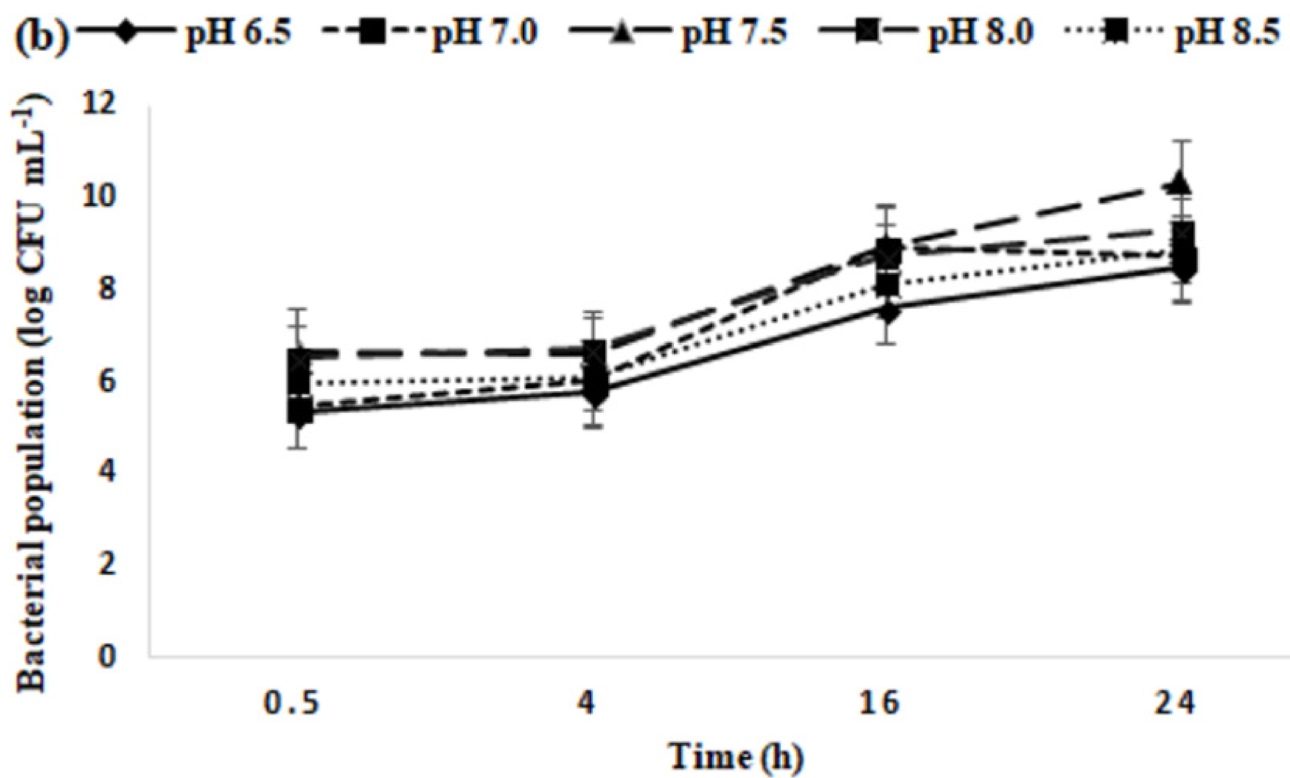
(b)
pH 6.5
pH 7.0
pH 7.5
pH 8.0
pH 8.5
Bacterial population (log CFU mL-1)
12
10
8
6
4
2
0
0.5
4
16
24
Time (h)

Figure 3

Localization of Enterobacter sp. Fs-11 with gnotobiotically-grown sunflower rhizoplane (a), extracellular matrix of root hair cells (b) rhizosphere (c,d) and inside root cortical cells (c,d).B Bacteria, CW Cell wall, RC Root cell, EM Extracellular matrix,

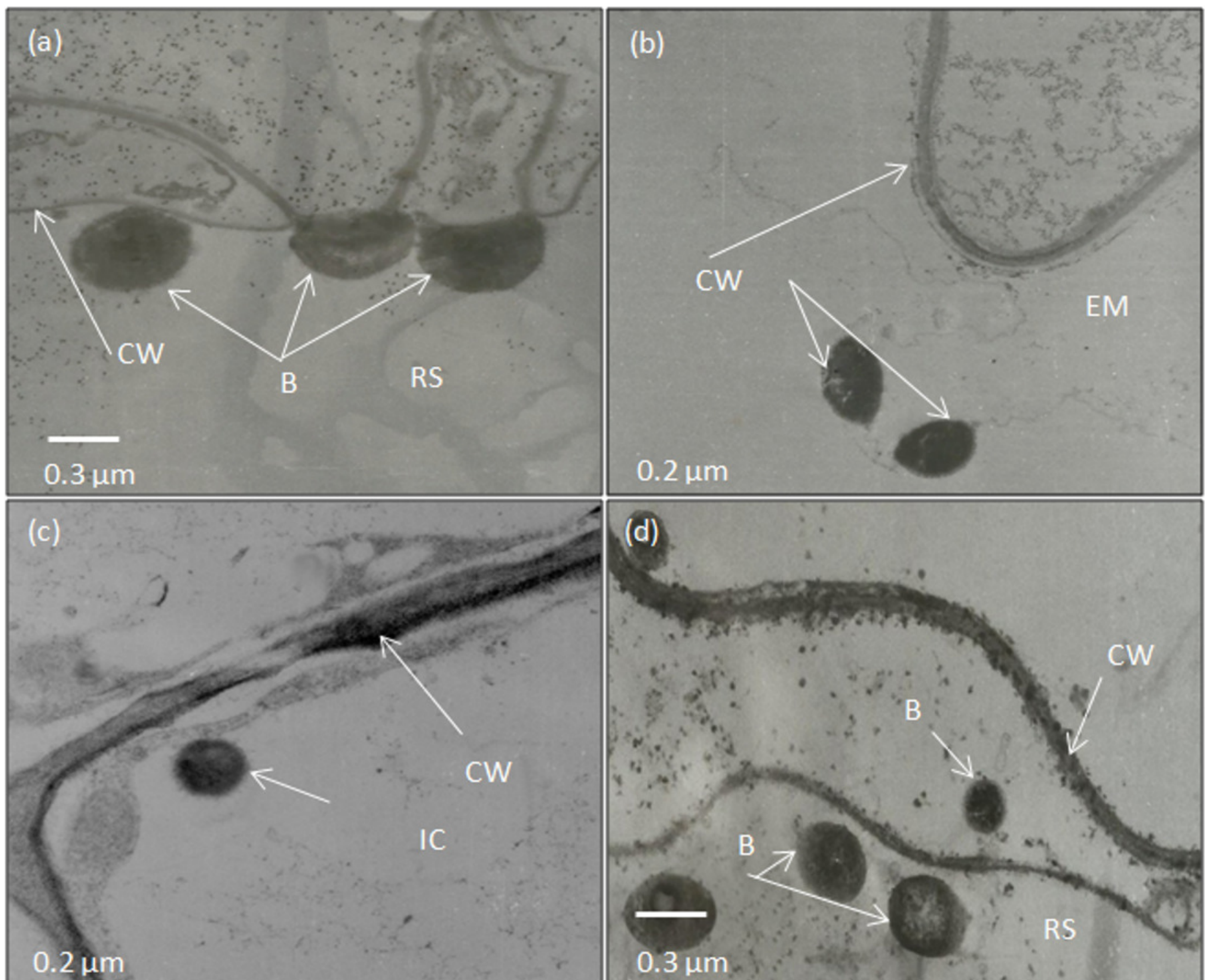

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Figure 4

Comparative effect of different locations on agronomic and physiological parameters of sunflower as determined by principal component analysis (PCA). Input data were first analyzed by two-way analysis of variance (Steel, Torrie & Dickey, 1997), are presen

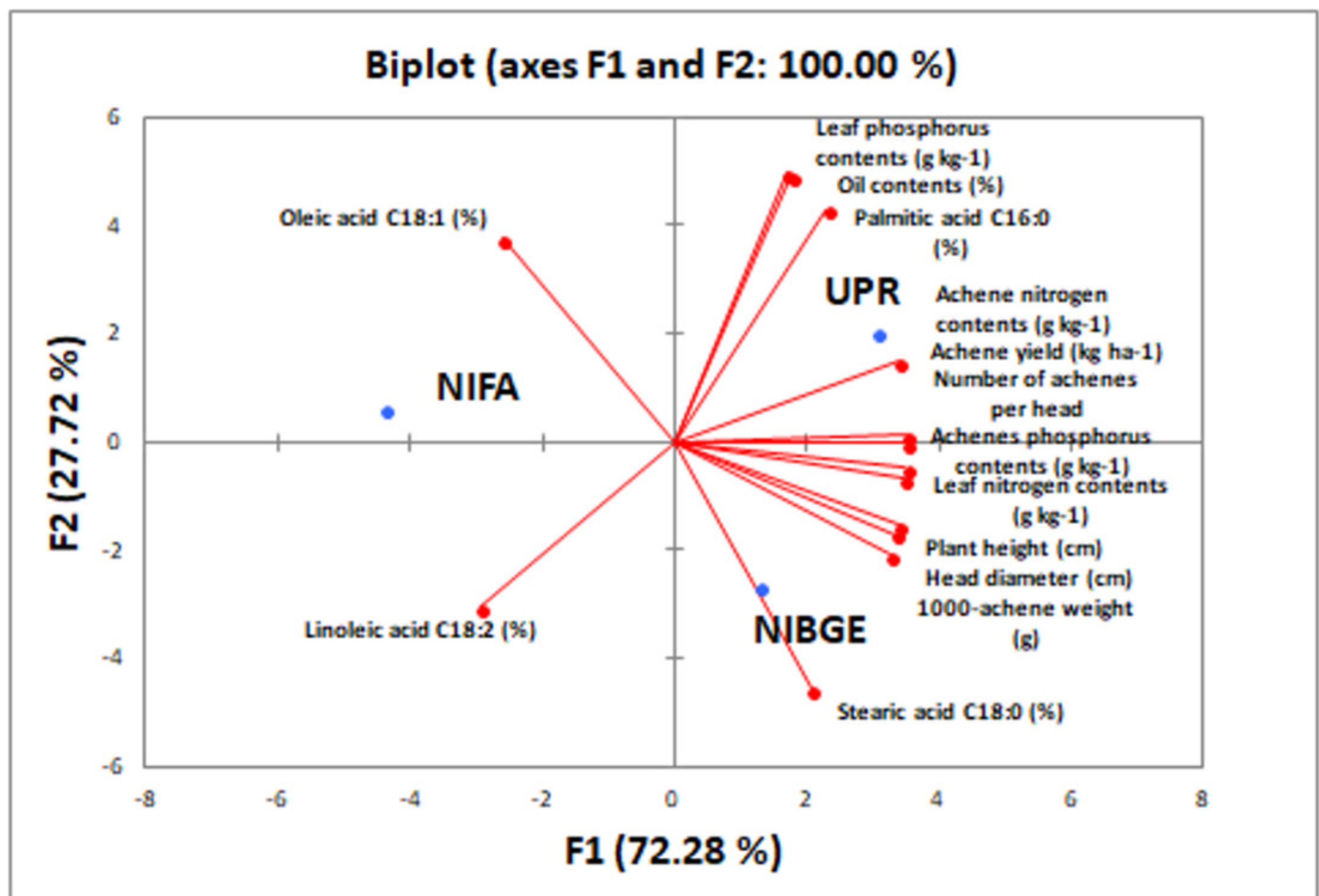

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Table 1 (on next page)

Physico-chemical properties of soil samples collected from experimental locations of NIBGE, NIFA and UPR

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1 **Table 1** Physico-chemical properties of soil samples collected from experimental locations of NIBGE, NIFA and
2 UPR

Parameters	Locations		
	NIBGE	**NIFA**	**UPR**
EC (dS m^{-1})	0.75	0.81	0.82
Soil pH	8.3	8.2	7.3
Soil texture	Sandy loam	Clay loam	Loam
Bulk density (mg m^{-3})	1.37	1.59	1.51
Organic matter (%)	0.68	1.12	1.78
Organic C (g kg^{-1})	3.8	4.4	4.5
Total N (g kg^{-1})	0.55	0.44	0.76
C:N ratio	6.9	10	5.9
Available P (mg kg^{-1})	6.54	4.74	6.1
Available K (mg kg^{-1})	203	164	224

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Table 2 (on next page)

Effect of seed inoculation of *Enterobacter* sp. Fs-11 on sunflower growth and yield

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1 **Table 2** Effect of seed inoculation of *Enterobacter* sp. Fs-11 on sunflower growth and yield

Treatments	Plant height (cm)	Head diameter (cm)	Number of achenes head^{-1}	1000-achene weight (g)	Achene yield (kg ha^{-1})
NIBGE, Faisalabad					
T_0	114.67 C	30.33 C	550.71 B	58.02 C	2321.1 C
T_1	123.11 C	33.17 BC	604.67 B	66.63 ABC	2665.3 AB
T_2	139.23 AB	38.33 AB	704 AB	72.57 A	2902.0 A
T_3	126 BC	34 ABC	628.34 B	62.55 BC	2449.0 BC
T_4	149.32 A	37.67 AB	916.67 A	67.60 AB	2804.1 A
T_5	142.33 A	39.33 A	901.12 A	69.25 AB	2854.5 A
LSD ($P \leq 0.05$)	15.55	5.33	240.79	9.24	238.07
NIFA, Peshawar					
T_0	87.18 C	23.67 C	402.76 B	40.61 C	1493.3 C
T_1	94.14 C	30.67 BC	482.33 B	51.60 B	1806.2 B
T_2	120.66 AB	35.69 AB	787.44 A	63.99 A	2218.7 A
T_3	94.00 C	26.33 C	434.70 B	50.61 B	1656.3 BC
T_4	111.67 B	34.89 AB	708.67 A	57.09 AB	2138.0 A
T_5	129.48 A	39.25 A	811.00 A	62.25 A	2154.3 A
LSD ($P \leq 0.05$)	9	7.08	108.78	7.94	292.96
UPR, Rawalakot					
T_0	108.37 C	25.73 C	490.11 C	51.09 D	2549.7 B
T_1	124.19 B	33.93 B	696.46 B	61.70 BC	2738.2 B
T_2	152.39 A	42.17 A	986.67 A	72.83 A	3140.6 A
T_3	119.01 BC	28.68 BC	570 BC	57.83 CD	2739.4 B
T_4	139.67 A	40.26 A	882.33 A	68.73 AB	3182.3 A
T_5	143.35 A	39.87 A	901 A	73.91 A	3254.3 A
LSD ($P \leq 0.05$)	15.03	5.53	177.09	10.15	312.37

2 Data analyzed by one-way analysis of variance (Steel, Torrie & Dickey, 1997), are presented as the mean of three
3 replications (n=3). Values that differ significantly ($P \leq 0.05$) according to Fisher's least signficant difference (LSD)
4 method are presented with different letters.

5 T_0 = Noninoculated control without fertilizer application
6 T_1 = Noninoculated control with full N and half P fertilizer
7 T_2 = Noninoculated control with full NP fertilizer
8 T_3 = Inoculated with strain Fs-11 without fertilizer application
9 T_4 = Inoculated with strain Fs-11 with full N and half P fertilizer
10 T_5 = Inoculated with strain Fs-11 with full NP fertilizer

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Table 3(on next page)

Effect of seed inoculation of *Enterobacter* sp. Fs-11 on nitrogen and phosphorus uptake New Roman

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1 **Table 3** Effect of seed inoculation of *Enterobacter* sp. Fs-11 on nitrogen and phosphorus uptake

Treatments	Achene N contents (g kg^{-1})	Leaf N contents (g kg^{-1})	Achene P contents (g kg^{-1})	Leaf P contents (g kg^{-1})
NIBGE, Faisalabad				
T_0	30.54 C	27.47 C	11.39 B	7.66 B
T_1	35.33 AB	34.68 AB	11.13 B	8.26 B
T_2	37.39 A	35.72 A	14.71 A	11.65 A
T_3	32.92 BC	30.36 BC	11.66 B	7.37 B
T_4	37.33 A	36.83 A	14.94 A	12.65 A
T_5	37.69 A	35.27 A	15.39 A	12.98 A
LSD ($P \leq 0.05$)	3.85	4.44	1.32	1.69
NIFA, Peshawar				
T_0	26.83 C	23.62 B	10.03 C	7.66 D
T_1	31.38 BC	29.57 AB	10.61 BC	8.26 CD
T_2	39.47 A	32.63 A	14.46 A	11.41 BC
T_3	31.12 BC	29.18 AB	11.33 ABC	8.37 CD
T_4	35.39 AB	34.81 A	11.99 ABC	14.20 AB
T_5	35.66 AB	34.36 A	13.72 AB	14.98 A
LSD ($P \leq 0.05$)	5.78	6.07	3.59	3.56
UPR, Rawalakot				
T_0	32.38 C	28.86 B	9.69 B	9.03 C
T_1	36.09 BC	36.33 A	11.80 B	10.78 BC
T_2	40.37 A	35.31 A	15.88 A	13.57 AB
T_3	34.77 C	29.08 B	12.17 B	10.64 BC
T_4	38.98 AB	35.15 A	15.37 A	16.32 A
T_5	38.34 AB	36.27 A	15.26 A	16.65 A
LSD ($P \leq 0.05$)	3.72	5.68	2.89	3.19

2 Data analyzed by one-way analysis of variance (Steel, Torrie & Dickey, 1997), are presented as
3 the mean of three replications (n=3). Values that differ significantly ($P \leq 0.05$) according to
4 Fisher's least signficant difference (LSD) method are presented with different letters

5 T_0 = Noninoculated control without fertilizer application
6 T_1 = Noninoculated control with full N and half P fertilizer
7 T_2 = Noninoculated control with full NP fertilizer
8 T_3 = Inoculated with strain Fs-11 without fertilizer application
9 T_4 = Inoculated with strain Fs-11 with full N and half P fertilizer
10 T_5 = Inoculated with strain Fs-11 with full NP fertilizer

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Table 4(on next page)

Effect of seed inoculation of *Enterobacter* sp. Fs-11 on oil and fatty acid contents

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1 **Table 4** Effect of seed inoculation of *Enterobacter* sp. Fs-11 on oil and fatty acid contents

Treatments	**Oil contents (%)**	**Oleic acid C18:1 (%)**	**Linoleic acid C18:2 (%)**	**Palmitic acid C16:0 (%)**	**Stearic acid C18:0 (%)**
NIBGE, Faisalabad					
T_0	36.65 C	27.74 C	35.84 C	6.03 A	1.20 C
T_1	38.84 B	27.99 BC	33.17 C	5.63 AB	1.37 BC
T_2	40.50 A	36.95 A	43.87 A	5.21 BC	1.83 A
T_3	38.87 B	31.98 ABC	40.01 B	5.28 BC	1.60 AB
T_4	41.05 A	33.71 ABC	43.44 AB	4.78 C	1.53 AB
T_5	40.74 A	34.17 AB	42.45 AB	4.89 C	1.38 BC
LSD ($P \leq 0.05$)	1.42	6.34	3.58	0.68	0.32
NIFA, Peshawar					
T_0	37.34 D	31.09 C	36.20 B	6.35 A	1.23 B
T_1	39.47 BCD	33.49 BC	40.37 AB	5.36 BC	1.41 AB
T_2	40.28 ABC	40.78 A	43.99 A	4.76 CD	1.77 A
T_3	38.84 CD	32.05 BC	36.04 B	5.83 AB	1.15 B
T_4	42.72 A	35.57 B	42.12 A	5.41 BC	1.31 AB
T_5	41.96 AB	39.59 A	42.21 A	4.22 D	1.46 AB
LSD ($P \leq 0.05$)	2.59	3.96	5.49	0.93	0.48
UPR, Rawalakot					
T_0	38.23 C	29.57 C	34.59 C	6.83 A	1.19 B
T_1	41.27 BC	30.17 C	36.94 BC	5.77 B	1.40 AB
T_2	44.79 A	33.67 ABC	42.36 A	5.40 B	1.69 A
T_3	42.24 AB	33.16 BC	35.29 C	5.72 B	1.40 AB
T_4	43.24 AB	37.49 AB	41.30 AB	5.43 B	1.41 AB
T_5	43.34 AB	38.09 A	42.13 A	4.99 B	1.42 AB
LSD ($P \leq 0.05$)	3.37	4.82	4.78	0.89	0.44

2 Data analyzed by one-way analysis of variance (Steel, Torrie & Dickey, 1997), are presented as the mean of three
3 replications (n=3). Values that differ significantly ($P \leq 0.05$) according to Fisher's least signficant difference (LSD)
4 method are presented with different letters

5 T_0 = Noninoculated control without fertilizer application
6 T_1 = Noninoculated control with full N and half P fertilizer
7 T_2 = Noninoculated control with full NP fertilizer
8 T_3 = Inoculated with strain Fs-11 without fertilizer application
9 T_4 = Inoculated with strain Fs-11 with full N and half P fertilizer
10 T_5 = Inoculated with strain Fs-11 with full NP fertilizer

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