

**Isolation and Characterization of Phosphate Solubilising Rhizobia
Nodulating Wild Field pea (*Pisum sativum* var. *abyssinicum*) from
Southern Tigray, Ethiopia**

Abstract

Phosphorus is the second limiting nutrients next to nitrogen as well as the least mobile element in the soil. This nutrient is one of the major constraint for low productivity of wild field pea in the study site. Hence, the development of environmental friendly and economically accepted to subsistent farmer is undeniably important. Thus, this experiment was initiated to isolate and characterize inorganic phosphate solubilizing rhizobia from root nodules of field pea (*Pisum sativum* var. *abyssinicum*) were characterized for their inorganic phosphate solubilisation ability on Pikovskaya liquid and solid media. Results revealed that all isolates were gram negative, failed to grow on peptone glucose agar, ketolactose test and did not absorb congo-red upon incubation period. Results showed that phosphate solubilisation index of root nodulating bacteria on in vitro Pikovskaya's agar medium varied from 1.54 to 2.70. Inorganic phosphate solubilisation in broth medium dissolved insoluble $\text{Ca}_3(\text{PO}_4)_2$ was within the range of 16.59-23.95 mg plant⁻¹ with pH drop from 7.01 to 5.33. Among the tested rhizobia isolates, HUDRI-8 and HUDRI-25 was found to be highest phosphate solubilisation compared to the remaining isolates, served as efficient phosphate solubilizers, and could be used for further test under field condition. Finally, those isolates effective in N₂ fixation and able to solubilise inorganic P were found to be effective in promoting nodulation and plant growth under greenhouse condition in soil having high and low background rhizobia nodulating wild field pea.

Keywords: Field pea, Phosphate solubilising rhizobia, *Pisum sativum* var. *abyssinicum*, Rhizobium

INTRODUCTION

Phosphorus (P) is a major growth limiting nutrient unlike nitrogen, there is no large atmospheric source that can be made biologically available (Ezawa *et al.*, 2002). In most soils, its content is about 0.05% of which only 0.1% is plant available (Achal *et al.*, 2007). Besides this, inorganic P fertilizer is the main sources of P in the agricultural soils, although 75 to 90% of the added P fertilizer is precipitated by iron, aluminium and calcium complexes present in the soil system (Turan *et al.*, 2006). According to Antoun *et al.*, 1998, report many soil bacteria and fungi have the ability to solubilize phosphorus (P) and make it available to plants. Microorganisms are central point to the soil P cycling and play a significant role in consent the conversion of the element between different inorganic and organic soil P fractions, then releasing available P for plant growth (Oberson, 2001). Phosphorus biofertilizers can help to increase the accessibility of accumulated phosphates for plant growth by solubilization (Gyaneshwar *et al.*, 2002). The involvement of microorganisms in

40 inorganic phosphates solubilization was reported as early as 1903 (Khan *et al.*,
41 2007), and the presence of these microorganisms (PSMs) are everywhere, while
42 their numbers are vary from soil to soil. Among the microbial populations present in
43 the soil, P solubilising bacteria constitute 1-50% and P solubilizing fungi are 0.1 to
44 0.5% (Chen *et al.*, 2006). The most important P solubilizing bacterial genera are
45 *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*,
46 *Micrococcus*, *Aereobacter*, *Flavobacterium* and *Erwinia* (Rodriguez and Fraga,
47 1999). This study found that out of 13 bacterial strains of different genera that
48 screening on different insoluble mineral phosphate substrates were indicated that
49 *Rhizobium*, *Pseudomonas* and *Bacillus* species were the most powerful P
50 solubilizers. Tandon (1987) observed that in 10 out of 37 experiments phosphate
51 solubilizing bacteria (PSB) inoculations resulted in 10-15% increment in crop yields.
52 Khalil (1995) also investigated 10 bacteria and 3 fungi being able to solubilize
53 phosphate on the basis of large clear zone on solid media. *Rhizobium*
54 *leguminosarum* is involving in phosphate solubilization as well as biological nitrogen
55 fixation (BNF) through the root nodules of bacteria (Gyaneshwar *et al.*, 2002). During
56 phosphate solubilization process, 2-ketoglucolnic acid is the most synthesized
57 organic acid (Halder *et al.*, 1990). Phosphate solubilizing rhizobia has been shown to
58 increase the growth of maize and lettuce (Chabot *et al.*, 1996). The multi-
59 functionality exhibited by *R. leguminosarum* makes it important in food production in
60 terms of reducing cost and improving efficiency of P fertilization, especially in P-
61 limited soils (Jia Xie, 2008). So far, phosphate solubilizing of fababean and chickpea
62 nodulating rhizobial isolates from Ethiopian soils have been done by several authors
63 (Girmaye *et al.*, 2014, Assefa *et al.*, 2010 and Mulissa *et al.*, 2016). Feredegn, 2013
64 also assessed the phosphate solubilization of rhizosphere and endophytic bacteria
65 from sugarcane (*Saccharum officinarum* L.). Although the phenotypic and symbiotic
66 effectiveness of rhizobia nodulating field pea (*pisum sativum* var. *sativum*) in
67 Ethiopian soils were studied by Aregu *et al.*, 2012; Fano, 2010 and Kassa *et al.*,
68 2015), the phosphate solubilizing efficacy, symbiotic effectiveness of rhizobia
69 nodulating field pea (*pisum sativum* var. *abyssinicum*) is not well investigated.
70 Therefore, this study was designed to isolate and characterizing indigenous
71 phosphate solubilizing root nodulating bacteria of field pea (*pisum sativum* var.
72 *abyssinicum*) and their effect on converting insoluble P in to soluble P and
73 effectiveness on soil culture.

74 **Material and Methods**

75 **Soil sampling site and sample collection**

76 The soil samples for nodule trapping and physico-chemical analysis were collected
 77 from Emba-Alaje and Endamohoni districts of southern Tigray, considering long
 78 history of field pea growing and no history of rhizobium inoculation. The
 79 corresponding GPS data including altitude and soil pH were indicated in Table 1.
 80 Twenty two soil samples were separately collected from the depth of 0-20cm and
 81 stored at 4 °C refrigerator for further experimentation. Soil chemical properties were
 82 done following standard methods compiled in Sahlemedhin and Taye (2001).

83 Table 1. Sampling sites including geographical location and soil pH

District	Kebele	Longitude	Latitude	Elevation (m.a.s.l)	Cropping History	Soil H ₂ O(1:2.5)	pH
Emba-Alaje	Betmera	12°58.787'	039°32.116'	2925	Field pea	6.6	
	Betmera	12°58.822'	039°32.069'	2923	Field pea	7.47	
	Atsela	12°55.615'	039°32.040'	2471	Field pea	7.37	
	Atsela	12°58.408'	039°31.722'	2989	Field pea	7.85	
	Ayba	12°53.589'	039°30.811'	2745	Field pea	6.6	
	Ayba	12°53.660'	039°30.818'	2709	Field pea	6.59	
	Ayba	12°53.611'	039°30.872'	2722	Field pea	5.91	
	Ayba	12°53.973'	039°31.501'	2725	Field pea	6.48	
	Ayba	12°52.584'	039°33.239'	2765	Wheat	7.22	
	Ayba	12°52.614'	039°33.325'	2777	Field pea	6.76	
	Ayba	12°52.077'	039°33.750'	2889	Barley	7.52	
	Tekea	12°54.954'	039°28.254'	2592	Field pea	6.75	
	Tekea	12°55.104'	039°29.343'	2651	Field pea	7.75	
	E/hasti	12°51.481'	039°33.920'	2955	Field pea	7.41	
EndaMohoni	E/hasti	12°51.488'	039°33.899'	2952	Field pea	7.36	
	E/hasti	12°51.477'	039°33.895'	2951	Field pea	7.88	
	E/hasti	12°51.514'	039°33.981'	2944	Field pea	7.75	
	E/hasti	12°50.720'	039°34.006'	2935	Field pea	8.11	
	Tsibet	12°50.549'	039°33.844'	2964	Field pea	7.89	
	Tsibet	12°50.537'	039°33.873'	2965	Fababean	7.58	
	Tsibet	12°50.533'	039°33.856'	2958	Wheat	6.3	
	Sh/gaze	12°50.514'	039°33.383'	2956	Field pea	6.28	

84 Where; E/Alaje= Embaalaje, E/Mohoni= Endamohoni, H/T/hanot=
 85 hazeboteklehaymanot, E/hasti=Enbahasti

86 **Nodule collection and Isolation of Rhizobia**

87 After 45 days of growing period, well grown, large and pink colour nodules were
88 uprooted carefully so as to get intact nodules. The nodules were thoroughly washed
89 with distilled water and surface-sterilized briefly with 70% ethanol and 3% (v/v)
90 solution of hydrogen per oxide (H_2O_2) for 10 sec. and 3 min. respectively (Howieson
91 and Dilworth, 2016). They were then more than 5 times with sterile distilled water,
92 and transferred into sterilized Petri dishes and crushed with flamed glass rod in 0.1 N
93 NaCl. One loop full of the nodule suspension were streaked on freshly prepared
94 Yeast Extract Manitol Agar (YEMA) plates containing 0.0025% Congo red (CR) with
95 pH of 6.8 ± 0.2 , and the plates were incubated at 28 ± 2 °C for 3-5 days. After 5 days
96 of incubation, single colonies were picked and purified by re-streaking on newly
97 prepared YEMA plates. The pure isolates were temporarily preserved at 4 °C on
98 YEMA slants containing 0.3 % (W/V) $CaCO_3$ until further analysis.

99 **Presumptive tests and colony characterization of the isolates**

100 All isolates was examined for presumptive purity using YEMA-CR medium, Gram
101 staining, peptone glucose Agar (PGA) and ketolactose Test (KLT) following the
102 procedures indicated in Somasegaren and Hoben, (1994). The isolates were
103 characterized by colony morphology and acid/base production on YEMA plus
104 $25\mu gml^{-1}$ Bromothymole blue (BTB) media (Ahmed *et al.*, 1984).

105 **Authentication and preliminary screening of symbiotic effectiveness (SE) of** 106 **isolates on sand culture**

107 Seeds of the same variety Raya one (R-1) was surface sterilized as before and five
108 pre-germinated seeds were sown on 1.5 kg surface sterilized capacity pots filled with
109 acid washed sand (95% sulphuric acid). The seedlings were thinned down to three
110 per pot after few days, and inoculated with 1 ml active cells (undiluted cells) grown
111 on YEM broth as the exponential of 10^8 visible cells ml^{-1} . The experimental set up
112 was arranged in a Complete Randomized Block Design (RCBD) with three
113 replications including the positive control (N supplied with 5ml/pot as 1% KNO_3
114 (w/v)) solution once a week, and un-inoculated unfertilized pots as negative control
115 under semi-controlled greenhouse conditions at Haramaya University. All pots were
116 supplied with quarter strength N-free nutrient solution once a week (Somasegaran
117 and Hoben, 1994) and washed with sterilized distilled water as required to control
118 salt accumulation. After 45 days of growing period, all plants were uprooted and

119 washed carefully with tap water. The nodules were cut off from the plant roots to
120 count and then dried at 70 °C for 24hrs until constant weight. The rhizobia
121 infectiveness based on the presence and absence of nodules on seedling root were
122 investigated.

123 **Qualitative Phosphate Solubilization Test**

124 The potential of Rhizobium strains for solubilization of insoluble phosphates were
125 checked on the Pikovskaya's agar medium (Pikovskaya, 1948), containing 10g
126 glucose, 0.5g yeast extract, 0.5g NH₂SO₄, 0.1g Magnesium Sulphate (MgSO₄
127 7H₂O), 5g Calcium Phosphate (Ca₃(PO₄)₂), 0.2g NaCl, 0.2g KCl, 0.001g MnSO₄
128 2H₂O, 0.001g FeSO₄ 7H₂O and 15g Agar medium per liter of distilled water. Three
129 days old culture isolates with 10⁸ viable cells ml⁻¹ were streaked on the medium and
130 incubated at 28 ± 2 °C for 7 days. After 7 days of incubation, clear halo zone
131 diameter and colony diameter were measured and microbial phosphorus
132 solubilisation index (SI) was calculated following the formula indicated in Edi-
133 Premono *et al.* (1996)

$$SI = \frac{\text{Colony Diameter} + \text{Halo zone diameter}}{\text{Colony Diameter}}$$

134 **Quantitative Phosphate Solubilisation test**

135 Five pure and best rhizobial isolates were selected based on their solubilization
136 index in Pikovskaya agar medium. 100ml of Pikovskaya broth was prepared without
137 phosphate source and dispensed in 250 ml Erlenmeyer flasks. In each flask, about
138 0.5g of tri-calcium phosphate (Ca₃(PO₄)₂) was added and sterilized at 121 °C at 15
139 psi for 15 minutes. Then 1ml of culture containing about 10⁸ cells ml⁻¹ suspensions of
140 each isolates was inoculated into the medium and kept at 28 ± 2 °C in shaker
141 incubator for about 12 days. All the experiments were carried out in triplicate. 10ml of
142 each isolate was withdrawn at regular intervals of 3 days and was examined for
143 soluble phosphate and pH changes using spectrophotometer and digital pH meter,
144 respectively, following the method cited in Subba Rao (1993).

145 **Screening Effective Isolates Under Soil Pot Experiment**

146 Two bulky soils collected from filed pea growing areas of southern Tigray were
147 grounded, sieved in to 2 mm size particles and filled into 3 kg capacity surface
148 sterilized as before polyethylene plastic pots, and the experiment were set as
149 randomized complete block design (RCBD) in three replications. Five effective
150 rhizobial isolates based on their symbiotic effectiveness on sand culture were
151 selected including N treated pots supplied with 5ml/pot of 1% KNO₃ (w/v) solution
152 once a week as positive control, and un inoculated unfertilized pots as negative
153 control. All pots were treated once a week with stock solutions of 12.5 mg/kg urea,
154 20 mg P₂O₅/kg, 10 mg/kg KCl₂, 5 mg/kg ZnSO₄, 5 mg/kg NaMoO₄ and 5 mg/kg
155 FeSO₄ (Somasegaren and Hoben, 1994). After 45 days of planting shoot and root
156 fraction were separated to determine nodule number and dry weight, shoot dry
157 weight and total nitrogen.

158

159 **Statistical Analysis**

160 The collected data was subjected to analysis of variance (ANOVA) using SAS ver.
161 9.1 (2002) and the differences tested for significance was faced to Fisher method
162 using the least significant differences (LSD) test at 0.05 probability level.

163 **Result and discussion**

164 *Qualitative Phosphate Solubilization*

165 All the tested isolates induced nodulation on the host plant indicating that the tested
166 isolates are the root nodulating bacteria of field pea (*pisum sativum var.*
167 *abyssinicum*).

168 The qualitative phosphate solubilisation showed a clear halo zones around their
169 colonies. The phosphate solubilisation index was ranged from 1.10 to 2.67 and soil
170 pH of moderately acidic to moderately alkaline (5.90 to 8.11) which is the optimum
171 pH for growth of the isolates. Of the tested isolates, five of them showed greater
172 solubilization index (SI) ranging from 1.5 to 2.7 (Table 2). Isolates HUDRI-8, HUDRI-
173 25 and HUDRI-26 were scored the highest solubilisation index at soil pH range of
174 (6.75-7.75) neutral to slightly alkaline.

175 Table 2. Growth of isolates on Pikovaskaya's agar medium

Isolates	Soil pH (1:2.5)	CD (mm)	HD (mm)	SI
HUDRI-8	7.75	3.0	5.0	2.7
HUDRI-18	6.59	9.3	7.0	1.8
HUDRI-25	7.47	4.3	6.7	2.5
HUDRI-26	6.75	3.0	4.0	2.3
HUDRI-30	6.76	5.7	3.0	1.5

176 Key word(s): CD-colony diameter, HD- holo zone, SI- solubilisation index

177

178 This indicates that some rhizobial isolates had the capacity to mobilize phosphates
 179 from in organic tricalcium phosphate (TCP). Similar results were found from *Vicia*
 180 *faba* L. of Ethiopian soils, with soil pH (4.8-6.3) as well as SI in the range of 1.25 to
 181 2.10 (Girmaye *et al.*, 2014). Mulissa *et al.* (2016) also obtained related results from
 182 *Cicer aeritinum* L. in the range of 1.40 to 3.06. Superior solubilisation index was
 183 obtained by Alia *et al.* (2013) from phosphate solubilizing bacteria associated with
 184 roots of vegetables that found within the range of 1.8 - 5.0.

185 *Quantitative Phosphate Solubilisation*

186 The quantitative phosphate solubilisation efficacy of selected rhizobial isolates were
 187 further evaluated by measuring the soluble P (mg L^{-1}) and the changes in pH as
 188 presented in Table 3. Accordingly, the amount of solubilised P released by the
 189 isolates exhibited wide variation ranging from 16.59 to 23.95 mg L^{-1} , with a
 190 significant drop in pH from 7.13 to 5.23. Similar results were obtained by Assefa *et al.*
 191 (2010), all bacterial isolates of faba bean (*vicea faba*) were solubilized TCP in the
 192 range of 5-39 $\text{mg}/50\text{ml}$ with a drop in pH ranging from 6.8-4 after 20 days of
 193 incubation. Various phosphate solubilization values were obtained by incubating
 194 them at different incubation period.

195 The ANOVA result showed a significant difference ($P < 0.05$) at the first 3 days
 196 incubation. The highest phosphate solubilizations were recorded from treatments
 197 inoculated with HUDRI-30 (21.84 mg L^{-1}) followed by HUDRI-25 (21.72 mg L^{-1}), and
 198 the lowest P solubilizations (3.43 mg L^{-1}) were recorded from un-inoculated
 199 treatment (Table 3). Phosphorus solubilization in the inoculated treatment was 537%

200 higher than the un-inoculated one, which is seven fold. The same treatments
201 incubated for the next 6 days had also significantly higher P discharge over the un-
202 inoculated one by 413%. The highest amount of P discharge 23.95, 23.48 and 23.00
203 mg L⁻¹ were recorded by isolates HUDRI-8, HUDRI-25 and HUDRI-26, respectively.
204 After 9 days of incubation, the highest P solubilizations (22.83 mg L⁻¹) were recorded
205 by HUDRI-26. Incubation of isolates for uninterrupted 12 days, the highest P
206 solubilization was found by inoculating HUDRI-8 (23.32 mg L⁻¹) followed by HUDRI-
207 26 (22.02 mg L⁻¹); resulting in 354.58% and 329.24% over the un-inoculated. With
208 regard to the incubation period, the highest P solubilisation (23.95 and 23.48 mg L⁻¹)
209 was found at the sixth day, while the lowest P discharge (16.59 mg L⁻¹) was recorded
210 at the first 3 days of incubation. The current result was significantly lower than the
211 results obtained by Assefa *et al.* (2010) (39 mg/50ml). Other researches were done
212 by Sharma *et al.* (2012), isolates from tea rhizosphere, Qian *et al.* (2010) from
213 shallow eutrophic lake and Feredegn (2013), isolates from rhizosphere and
214 endophytic of sugarcane solubilized TCP within the range of 40.62-136.73 mg L⁻¹, 4-
215 170 mg L⁻¹ and 45.12- 88.41 mg L⁻¹, respectively.

216 The pattern of interaction between phosphate discharge and pH at different
217 incubation period had a strong negative correlation ($r = -0.613$ and $r = -0.542$) from
218 day 6 and 9, respectively, followed by day 3 and 12 with $r = -0.517$ and $r = -0.202$
219 (Table 4). This result was corresponding to Assefa *et al.* (2010), inverse correlation
220 between the amounts of P solubilize and reduction in pH ($r \geq -0.93$). Alia *et al.* (2013)
221 also found negative correlation ($r = -0.862$), ($r = -0.94$) correlation from bacterial
222 growth on mung bean by Buddhi and Min-Ho (2013) also found similar trend.

223

224

225

226 Table 3. Tri-calcium phosphate solubilization efficiency of selected isolates

Isolates	3 days		6 days		9 days		12 days	
	pH	P (mg L ⁻¹)	pH	P (mg L ⁻¹)	pH	P (mg L ⁻¹)	pH	P (mg L ⁻¹)
HUDRI-8	5.93±0.214 ^{bc}	16.59±7.123 ^b	5.54±0.015 ^b	23.95±0.767 ^a	5.37±0.164 ^b	20.41±8.911 ^a	5.25±0.069 ^b	23.32±8.100 ^a
HUDRI-18	5.93±0.263 ^{bc}	16.81±0.966 ^b	5.27±0.136 ^c	22.77±2.915 ^a	5.53±0.045 ^b	20.72±1.015 ^a	5.38±0.217 ^{ab}	19.76±1.127 ^b
HUDRI-25	5.61±0.063 ^{bc}	21.72±0.981 ^{ab}	5.49±0.029 ^b	23.48±0.214 ^a	5.59±0.017 ^b	20.67±0.563 ^a	5.97±0.351 ^a	21.41±0.374 ^{ab}
HUDRI-26	6.21±0.316 ^b	19.17±2.072 ^a	5.45±0.051 ^{bc}	23.00±2.951 ^a	5.40±0.220 ^b	22.83±6.639 ^a	5.23±0.261 ^b	22.02±9.374 ^{ab}
HUDRI-30	5.31±0.144 ^c	21.84±2.302 ^a	5.26±0.058 ^c	21.18±1.128 ^a	5.24±0.089 ^b	20.17±0.893 ^a	5.23±0.031 ^b	21.27±0.225 ^{ab}
Control	7.01±0.00 ^a	3.43±0.00 ^c	6.97±0.00 ^a	4.67±0.00 ^b	7.21±0.00 ^a	5.49±0.00 ^b	7.13±0.00 ^b	5.13±0.00 ^c
G mean	6.00	16.59	5.66	19.84	5.72	18.38	5.69	18.82
CV (%)	5.77	14.74	2.01	8.11	3.62	8.63	6.49	8.07
LSD(0.05)	0.62	4.35	0.20	2.86	0.37	2.82	0.62	2.70

227 Where; Means followed by the same letters are not significantly different at p< 0.05 (Fisher's LSD test)

228

229 Table 4. Correlation coefficients of P and pH parameters on phosphate solubilizing
230 bacteria

	Day 3		Day 6		Day 9		Day 12	
	pH	P	pH	P	pH	P	pH	P
pH		-0.52*		-0.6**		-0.54*		-0.20*
P (<0.05)		0.03		0.01		0.02		0.42
P	-0.52*		-0.61**		-0.54*		-0.20*	
P (<0.05)	0.03		0.01		0.02		0.42	

231

232 *Symbiotic Effectiveness of Isolates on Unsterilized Soil:*

233 The physico-chemical properties of the soils are presented in Table 5. The textural
234 class of the districts were classified as sandy clay loam. Similar results were found
235 by Amanuel *et al.*, 2015, from Tekea and Shimta kebeles with particle size
236 distribution of 50-54% sand, 18-17% silt and 35-30% clay fractions, respectively. The
237 pH of the two districts was slightly acidic (6.38-6.42) according to the ratings of
238 Tekalign (1991), which is the optimum pH range for bacterial growth. Low organic
239 matter (1.7-2%) and low to medium total nitrogen (0.01-0.14%) was found according
240 to Murphy (1968). This lower soil organic matter could be due to the presence of
241 continuous cropping system, cultivation and intensive tillage practice.

242 Table 5. The soil physico-chemical properties

Parameters	E/Alaje	E/Mohoni	Status	Reference
OM (%)	1.72	1.96	Low	Murphy (1968)
Available P (mg/kg)	18.78	17.7	high	Olsen <i>et al.</i> (1954)
Total N (%)	0.09	0.14	low to medium	Murphy (1968)
pH	6.42	6.38	slightly acidic	Tekalign (1991)
EC(mhos/cm)	0.09	0.09	low	Horneck <i>et al.</i> (2011)
CEC (meq/100g soil)	40.20	43.40	very high	Landon (1991)
Textural Class	Sand 52% Silt 18% Clay 30%	Sand 59% Silt 16% Clay 30%	Sandy clay loam	

243

244 High available P (18-19 mg kg⁻¹) and very high CEC (40.2-43.4 meq/100gsoil) was
245 found from the study area according to the ratings of Olsen *et al.* (1954) and Landon
246 (1991), respectively. This is in agreement with the findings of (Amanuel *et al.*, 2015)
247 who reported the characterization of agricultural soils of southern Tigray, in capacity
248 building for scaling up of evidence-based best practice in Ethiopia (CASCAPE)
249 intervention woredas. According to Horneck *et al.* (2011), soil test interpretation
250 guide the electrical conductivity was low.

251 After nodulation test on sand culture, five symbiotically effective isolates (HUDRI-15,
252 26, 28, 43 and 44) were selected and further tested for their performance on a soil
253 pot culture. The data showed that the inoculated plants produced significantly
254 ($P < 0.05$) higher nodule number (NN), nodule dry weight (NDW), shoot dry weight
255 (SDW) and total plant nitrogen (TN) (Table 6). The highest nodule numbers (156 and
256 145) were found from HUDRI-15 and HUDRI-28 isolated from E/Alaje and E/mohoni
257 soils, respectively. The current result was higher than the number of nodules found
258 by Asrat (2017) (112 NN/plant) for field pea treated with commercial strain 1018. The
259 lowest nodule number per plant was recorded from un-inoculated plants (31
260 NN/plant) (Table 5). N treated plants also reduced nodule number per plant by 36%
261 (156-100 NN/plant) and 42% (145-84 NN/plant) compared to other treatments from
262 the two soils, respectively. This result indicates that application of nitrogen somehow
263 inhibited nodule development in field pea. Anteneh and Abere (2017) also reported
264 that application of N reduced nodule number (62 NN/Plant and 20.00NN/Plant) in
265 2012 and 2013 cropping season.

266 Inoculation of the host plant also significantly ($P < 0.05$) affected nodule dry weight.
267 The highest nodule dry weight (NDW) was recorded from HUDRI-15 (0.189 g plant⁻¹)
268 and HUDRI-28 (0.117 g plant⁻¹) relative to the other inoculants and control
269 treatments on both soils (Table 6). This result was in agreement with Asrat (2017)
270 (0.094 and 0.009 g plant⁻¹) of field pea *rhizobium* inoculation. However, it was slightly
271 lower than the results obtained by Anteneh and Abere (2017) (0.552 and 0.140 g
272 plant⁻¹) two years report. This might be due to the ecological factors, which are
273 tested on field condition.

274 The effect of inoculation on shoot dry weight (SDW) was found significant ($P < 0.05$)
275 and values were superior to the positive and negative control. Isolates HUDRI-15

276 and HUDRI-28 gave the highest shoot dry weight ($1.64 \text{ g plant}^{-1}$) and ($1.42 \text{ g plant}^{-1}$)
277 on both soils, and it was advanced by 43 and 25% over the negative control (Table
278 6). In contrary to this result Asrat (2017) was found higher shoot dry weight in the
279 range of 14 to 29 g plant^{-1} . Anteneh and Abere (2017) also reported that field pea
280 rhizobium inoculation increased shoot dry weight on the range of 57 to 87 g plant^{-1} .

281 A significant effect of *Rhizobium* inoculation on the plant N accumulation of field pea
282 was observed among the treatments including N treated and un-inoculated (Table 6).
283 The highest total N accumulation was obtained from plants treated with HUDRI-15
284 (3.67%) and HUDRI-15 (3.53%) on the two districts, respectively. This result was in
285 agreement with Asrat (2017) found in the range of 3.5-4.1% total N from inoculated
286 field pea. The total N accumulation was found to be 70% and 89% increment over
287 the negative control.

289 Table 6. Evaluation of symbiotic effectiveness of isolates on soil culture

Treatment	Nodule number		Nodule dry weight(g plant ⁻¹)		Shoot dry weight (g plant ⁻¹)		Total Nitrogen (%)	
	E/Alaje soil	E/Mohoni soil	E/Alaje soil	E/Mohoni soil	E/Alaje soil	E/Mohoni soil	E/Alaje soil	E/Mohoni soil
HUDRI-15	156.00±3.46 ^a	103.33±2.40 ^c	0.189±0.03 ^{ab}	0.089±0.03 ^c	1.64±0.13 ^a	1.41±0.17 ^{ab}	3.67±0.135 ^a	3.53±0.098 ^a
HUDRI-26	111.67±3.84 ^d	86.00±3.46 ^d	0.097±0.01 ^{bc}	0.092±0.00 ^{bc}	1.31±0.14 ^{ab}	1.28±0.16 ^{ab}	3.36±0.120 ^{ab}	3.05±0.034 ^{ab}
HUDRI-28	138.67±1.76 ^b	145.33±2.91 ^a	0.109±0.03 ^a	0.117±0.00 ^a	1.51±0.17 ^{ab}	1.42±0.11 ^{ab}	2.48±0.057 ^c	3.08±0.045 ^{ab}
HUDRI-43	150.00±7.64 ^{ab}	126.00±3.46 ^b	0.097±0.00 ^{bc}	0.108±0.00 ^{ab}	1.61±0.17 ^a	1.28±0.23 ^{ab}	3.40±0.038 ^{ab}	2.84±0.038 ^b
HUDRI-44	125.67±3.48 ^c	96.00±2.08 ^c	0.121±0.01 ^{ab}	0.100±0.00 ^{abc}	1.53±0.05 ^a	1.34±0.21 ^{ab}	3.48±0.038 ^{ab}	3.07±0.070 ^{ab}
N ⁺	100.00±3.06 ^d	84.33±1.66 ^d	0.046±0.00 ^{bc}	0.005±0.00 ^{bc}	1.22±0.01 ^a	1.24±0.02 ^{ab}	2.60±0.027 ^c	2.42±0.039 ^c
N ⁻	31.00±1.15 ^e	36.33±2.40 ^e	0.065±0.02 ^c	0.014±0.02 ^d	1.15±0.09 ^b	1.14±0.04 ^{ab}	2.16±0.05 ^d	1.87±0.226 ^d
CV (%)	5.875	5.045	34.85	10.87	14.30	19.84	4.65	5.51
LSD (0.05)	11.95	8.51	0.071	0.017	0.37	0.47	0.21	0.27

290 Where: CV= coefficient of variation, LSD= least significant difference, values are ±SE, numbers in the same column followed by the
 291 same letter(s) are not significantly different at $\alpha < 0.05$

292 Conclusion

293 It can concluded that the phosphate solubilizing rhizobia exhibited a broad range of
294 ability of solubilizing TCP *in vitro*. Most of the isolates originated from Emba-alaje are
295 generally able to solubilise inorganic TCP. Among all the isolates, maximum
296 potential to solubilize tri-calcium phosphates are HUDRI-8 and HUDRI-25. Results
297 found an inverse correlation between amount of solubilized phosphate and pH of the
298 culture medium. Isolate that are effective in N₂ fixation and able to solubilise TCP are
299 found to be effective in improving nodulation and plant growth under greenhouse
300 condition. Further research is recommended to investigate its efficacy under field
301 trials in diverse soil types having different amount of soil P.

302 References

- 303 Abere M., Heluf G. and Fassil, A. 2009. Symbiotic Effectiveness and
304 Characterization of Rhizobium Strains of Faba Bean (*Vicia faba* L.). Collected
305 from Eastern and Western Hararghe Highlands of Ethiopia. *Ethiopian Journal*
306 *of Natural Resources*, 11 (2): 223-244.
- 307 Achal, V.; Savant, V.V. & Sudhakara Reddy, M. (2007). Phosphate Solubilization by
308 Wide
309 Type Strain and UV-induced Mutants of *Aspergillus tubingensis*. *Soil Biology*
310 *and*
311 *Biochemistry*, Vol. 39, No.2, (February 2007),pp. 695-699,ISSN 0038-0717
- 312 Ahmed, M.H., Rafique U., M. and McLaughlin, W. 1984. Characterization of indigenous
313 rhizobia from wild legumes. *FEMS Microbiology Letters* 24:197-203.
- 314 Alemayehu W. 2009. The effect of indigenous root nodulating bacteria on nodulation
315 and growth of Faba bean (*Vicia faba*) in the low input agricultural systems of
316 Tigray highlands, Northern Ethiopia *Middle East Journal of Science*, 1:30-43.
- 317 Alia, A.A., Shahida, N. Khokhar, B. J., Saeed, A. and Asad. 2013. Phosphate
318 solubilizing bacteria associated with vegetables roots in different ecologies.
319 *Pakistan Journal of Biotechnology*, 45: 535-544.
- 320 Amanuel Z., Girmay G. and Atkilt G. 2015. Characterisation of Agricultural Soils in
321 CASCAPE Intervention Woredas in Tigray Region.
- 322 Amargaer N., Macheret V., Aguerre G. 1997. *Rhizobium gallicum* sp. Nov, and
323 *Rhizobium giardinii* sp. Nov. from *Phaseolus vulgaris* nodules. *International*
324 *Journal of systemic and Bacteriology*, 47: 996-1006.
- 325 Anteneh A. and Abere M. 2017. Symbiotic effectiveness of *Rhizobium*
326 *leguminosarum* *bv. viciae* isolated from major highland pulses on field pea
327 (*Pisum sativum* L.) in soil with abundant rhizobial population, *Annals of*
328 *Agrarian Science*, [http:// dx.doi.org/10.1016/j.aasci](http://dx.doi.org/10.1016/j.aasci).
- 329 Antoun, H., C.L. Beauchamp, N. Goussard, R. Chabot, and L. Roger. 1998. Potential
330 of
331 *Rhizobium* and *Bradyrhizobium* species as plant growth promoting
332 rhizobacteria on non-legumes: Effect on radishes (*Raphanus sativus* L.).
333 *Plant Soil* 204:57-67.

- 334 Aregu A., Fassil A. and Asfaw H. 2012. Symbiotic and phenotypic characterization of
 335 Rhizobium isolates of field pea (*Pisum sativum* L.) from central and southern
 336 Ethiopia. *Ethiopian Journal of Biological Sciences* 11(2): 163-179.
- 337 Asrat M. 2017. Competitiveness and symbiotic effectiveness of rhizobial inoculants
 338 on field pea (*Pisum sativum*) under greenhouse and field conditions, MSc
 339 Thesis, Addis Ababa University, Addis Ababa, Ethiopia.
- 340 Assefa K., Fassil A. and P.C. Prabu, 2010. Characterization of acid and salt tolerant
 341 rhizobial strains isolated from faba bean fields of Wello, Northern Ethiopia.
 342 *Journal of Agricultural Science and Technology*, 12: 365-376.
- 343 Assefa K., Fassil A. and P.C., Prabu. 2010. Isolation of phosphate solubilizing
 344 bacteria from the rhizosphere of faba bean of Ethiopia and their abilities on
 345 solubilizing insoluble phosphates. *Journal of Agricultural Science and
 346 Technology*, 12: 79-89.
- 347 Bernal, G. and Graham, P.H. 2001. Diversity in the rhizobia associated with
 348 *Phaseolus vulgaris* L. in Ecuador and comparisons with Mexican bean
 349 rhizobia. *Journal of Microbiology*, 47: 526-534.
- 350 Buddhi C. W. and Min-Ho Y.. 2013. Phosphate solubilizing bacteria: Assessment of
 351 their effect on growth promotion and phosphorous uptake of mung bean
 352 (*Vigna radiate* L.R. Wilczek, *Chilean journal of agricultural research* 73:3.
- 353 Chabot, R., H. Antoun and M.P. Cesas. 1996. Growth promotion of maize and
 354 lettuce by phosphate solubilizing *Rhizobium leguminosarum* biovar phaseoli.
 355 *Plant Soil*, 184: 311-321.
- 356 Chen, Y.P.; Rekha, P.D.; Arunshen, A.B.; Lai, W.A. & Young, C.C. (2006).
 357 Phosphate
 358 Solubilizing Bacteria from Subtropical Soil and their Tricalcium Phosphate
 359 Solubilizing Abilities. *Applied Soil ecology*, Vol. 34, No.1, (November 2006),
 360 pp. 33-4, ISSN 0929-1393
- 361 Ezawa, T., S. E. Smith and F. A. Smith. 2002. P metabolism and transport in AM
 362 fungi. *Plant Soil* 244:221-230.
- 363 Fano B. 2010. Phenotypic and Symbiotic characteristics of Rhizobia nodulating field
 364 Pea (*Pisum sativum* L.) in southern Tigray, Ethiopia. An MSc Thesis, School
 365 of Graduate Studies, Adiss Abeba University.
- 366 Feredegn D. 2013. Isolation of Rhizosphere and Endophytic Bacteria from
 367 Sugarcane (*Saccharum officinarum* L.) with Nitrogen Fixing and Phosphate
 368 Solubilizing Characteristics from Wonji-Shoa Sugar Estate and Farmers
 369 Landraces of Ethiopia. MSc Thesis, Submitted to Haramaya University,
 370 Haramaya, Ethiopia.
- 371 Girmaye K., Mulissa J., Fassil A. 2014. Characterization of Phosphate Solubilizing
 372 Faba Bean (*Vicia faba* L.) Nodulating Rhizobia Isolated from Acidic Soils of
 373 Wollega, Ethiopia *Sci. Technol. Arts Res. J.* 3: 11-17.
- 374 Gyaneshwar, P., G. Naresh Kumar and L.J. Parekh. 2002. Effect of buffering on the
 375 phosphate solubilizing ability of microorganisms. *World J. Microbiol. Biotech.*,
 376 14: 669-673.
- 377 Halder A.K., Mishra A.K., Bhattacharya P., Chakrabarthy P.K. 1990. Solubilization of
 378 rock phosphate by Rhizobium and Bradyrhizobium. *Journal General Applied
 379 Microbiology*, 36: 81-92.
- 380 Horneck, D.A. D.M. Sullivan, J.S. Owen, J.M. and Hart. 2011. *Soil test interpretation
 381 guide*.
- 382 Howieson, J.G., Dilworth, M.J. (Eds.). 2016. Working with rhizobia. *Australian Centre
 383 for International Agricultural Research: Canberra*, 173: 15-19.

- 384 Jia Xie. 2008. Screening for calcium Phosphate Solubilizing *Rhizobium*
385 *leguminosarum*
- 386 Jordan, D.C. (1984). Family III. Rhizobiaceae. In: Bergey's Manual of Systematic
387 Bacteriology, Vol.1, pp. 234-254, (Krieg, N.R., Holt, J.G). The Williams and
388 Wilkins, Baltimore.
- 389 Kassa B., Ameha K., Fassil A. 2015. Isolation and Phenotypic Characterization of
390 Field Pea Nodulating Rhizobia from Eastern Ethiopia Soils. *World Applied*
391 *Sciences Journal* 33 (12): 1815-1821.
- 392 Khalil, S. 1995. Direct application of phosphate rock and appropriate technology
393 Fertilizers in Pakistan. In: *Direct Application of Phosphate Rock and*
394 *Appropriate Technology Fertilizers in Asia– What Hinders Acceptance and*
395 *Growth, Proc. Int.Workshop*, (Eds.): K. Dahanayake, S.J. Vankau Wenbergh
396 and D.T. Hellums. Feb: 20-25. Int. Fertilizer Devel. Centre, Kandy, Srilanka.
397 pp. 231-36.
- 398 Khan, M.S.; Zaidi, A. & Wani, P.A. (2007). Role of Phosphate-Solubilizing
399 Microorganisms
400 in Sustainable Agriculture-A Review. *Agronomy for Sustainable*
401 *Development*, Vol. 27, No. 1, (March 2007), pp. 29-43, ISSN 1774-0746
- 402 Landon J.R. 1991. Booker Tropical Soil Manual: *A hand book for soil survey and*
403 *agricultural land Evaluation in the tropics and subtropics*. New York.
- 404 Lupwayi, N and Haque, I. 1994. Legume *Rhizobium* Technology Manual.
405 Environmental Sciences Division International Livestock Center for Africa.
406 Addis Ababa, Ethiopia. Pp.1-93.
- 407 Mulissa J. M., Carolin R. Löscher, Ruth A. Schmitz, Fassil A. 2016. Phosphate
408 solubilization and multiple plant growth promoting properties of rhizobacteria
409 isolated from chickpea (*Cicer aeritimum* L.) producing areas of Ethiopia.
410 *African Journal of Biotechnology*, 15(35): 1899-1912.
- 411 Murphy, H.F., 1968. A report on fertility status and other data on some soils of
412 Ethiopia. Collage of Agriculture HSIU. Experimental Station Bulletin No. 44,
413 Collage of Agriculture: 551p.
- 414 Oberson, A., D.K. Friesen, I.M. Rao, S. Buhler, and E. Frossard. 2001. Phosphorus
415 transformations in an oxisol under contrasting land-use system: The role of
416 the microbial biomass. *Plant Soil* 237:197-210.
- 417 Olsen, R. Cole, S. Watanabe, F. and Dean, L. 1954. Estimation of available
418 phosphorus: in soils by extraction with sodium bicarbonate. United states
419 department of agriculture, 939: 1- 19.
- 420 Peoples, M.B., K.E. Giller, D.F. Herridge and J.K. Vessey. 2002. Limitations to
421 biological nitrogen fixation as a renewable source of nitrogen for agriculture:
422 *Nitrogen Fixation Global Perspectives*. pp. 356-360. In: T. Finan, M. O'Brain,
423 M.R. Lagzell, D.B. Vessey and W. Newton (eds.). ABI Publishing, New York.
- 424 Pikovskaya, R.I. 1948. Mobilization of phosphorus in soil in connection with the vital
425 activity of some microbial species. *Microbiology* 17, 362-370.
- 426 Purcino, H. M. A., Festin, P. M. and Elkan, G. H. 2000. Identification of
427 effective strains of *Bradyrhizobium* for *Archis Pinto*. *Trop. Agric.* 77:226-231.
- 428 Qian, Y., J. Shi, Y. Chen, L. Lou, X. Cui, R. Cao, P. Li and J. Tang. 2010.
429 Characterization of phosphate solubilizing bacteria in sediments from a
430 shallow eutrophic lake and a wetland: Isolation, Molecular Identification and
431 Phosphorus Release Ability Determination. *Molecules*, 15:8518-8533;
432 doi:10.3390 /molecules15118518.

433 Rodriguez, H. and R. Fraga. 1999. Phosphate solubilizing bacteria and their role in
434 plant growth promotion. *Biotechnol. Adv.* 17:319-339

435 Sahlemedhin S. and Taye B. 2001. Soil and plant analysis

436 SAS. 2002. SAS/STAT User's Guide, Version 9.1.3. SAS Inc., Cary, NC.

437 Sharma, B.C., R. Subba, A. Saha. 2012. In *vitro* solubilization of tricalcium
438 phosphate and production of IAA by phosphate solubilizing bacteria isolated
439 from tea rhizosphere of Darjeeling Himalaya. *Plant Sciences Feed*, 2(6): 96-
440 99.

441 Solomon L. and Fassil A. 2014. Symbiotic and Phenotypic Characteristics of
442 Rhizobia Nodulating Faba Bean (*Vicia Faba*) from Tahtay Koraro, North
443 western Zone of Tigray Regional State, Ethiopia, *International journal of*
444 *technology enhancements and emerging engineering research*, 2:2347-4289.

445 Somasegaran P, Hoben HJ (1994) Hand book for rhizobia methods in Legume-
446 Rhizobium technology. Springer-verlag, Heidelberg, Germany

447 Subba Rao, N.S. 1993. Biofertilizers in agriculture and forestry. 3rd Edition. Oxford
448 and IBH publishing Co. Pvt. LTD., New Delhi. PP: 129-135.

449 Tandon, H.L. 1987. Phosphorus research and production in India. Fertilizer
450 Development and Consultation Organisation New Delhi, pp. 160.

451 Tekalign T. 1991. Soil, plant, water, fertilizer, animal manure and compost analysis.
452 Working Document No. 13. *International Livestock Research Center for*
453 *Africa*, Addis Ababa.

454 Turan, M., N. Ataoglu, and F. Sahin. 2006. Evaluation of the capacity of phosphate
455 solubilizing bacteria and fungi on different forms of phosphorus in liquid
456 culture. *J. Sustainable Agri.* 28:99-108.

457 Vishal, K., Deshwal A., Chaubey. 2014. Isolation and Characterization of Rhizobium
458 leguminosarum from Root nodule of Pisum sativum L. *Journal of Academia*
459 *and Industrial Research*, 464: 2278-5213.

460 Zerihun B. and Fassil A. 2010. Symbiotic and phenotypic diversity of *Rhizobium*
461 *leguminosarum* bv. *viciae* from Northern Gondar, Ethiopia. *African Journal of*
462 *Biotechnology* 10(21): 4372-4379.

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464