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SYMBIOTIC AND PHENOTYPIC CHARACTERIZATION OF RHIZOBIUM ISOLATES OF FIELD PEA (*PISUM SATIVUM* L.) FABACEAE, FROM CENTRAL AND SOUTHERN ETHIOPIA

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ABSTRACT: A total of 25 rhizobial isolates from field pea (*Pisum sativum* L.) were collected from Shewa, Gojam, Gondar, Wollo and Tigray, and authenticated as root nodule using the trap host. They showed significant variations in their symbiotic effectiveness in nitrogen fixation and enhancing growth of the pea cultivar Markose plants compared to the uninoculated and non-fertilized control plants on sand culture under greenhouse conditions. Although there was inter-site difference in harbouring effective and very effective isolates ranging from 86% (Tigray) and 67% (Gondar) in terms of shoot dry matter accumulation of 50-100%, on the average 76% of the pea rhizobial isolates in the soils of major growing areas in the country were effective and very effective. The inoculated plants also showed differences in total nitrogen contents in the range of 1.72% and 2.93%. These isolates were then characterized based on their cultural, physiological and ecological features in relation to their symbiotic characteristics. The isolates showed diversity in their eco-physiological tolerance (NaCl, pH and temperature) inherent resistance to antibiotics and heavy metals, and heterotrophic competence of utilization of 17 carbon sources. Consequently, isolates NSRlFP1, NSRlFP3, NSRlFP13, NSRlFP17 and NSRlFP18 were the elite rhizobia that can be selected and further tested for their genetic and symbiotic performance in field trials for future bio-inoculant formulation.

Key words/phrases: Antibiotic resistance, Eco-physiological tolerance, Heavy metal resistance, Symbiotic effectiveness.

INTRODUCTION

Pulse crops are grain legumes that are important sources of dietary protein for many people in developing countries. Some of these legumes are known as cool season legumes that include field pea (*Pisum sativum* L.), chickpea (*Cicer arietinum*), faba bean (*Vicia faba* L.), lentil (*Lens cultinaris* Medik), and grass pea (*Lathyrus sativum*) (Jayasundara *et al*., 1998).

These legumes are cultivated in temperate, Mediterranean regions, and at high altitudes in sub-tropical and tropical countries (Jayasundara *et al*.,

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1998). In Ethiopia, the highlands of Gondar, Gojam, northwest Wollo, Tigray and central highlands of Shewa are the major production areas (Asfaw Telaye *et al*., 1994). They occupy 13% of the total cultivated land and 12% of total production of the major crops in the country (CSA, 2004).

Cool season legumes, in general, are used as a break crop in cereal rotations and utilized to restore soil fertility in low-input traditional agriculture (Asfaw Telaye *et al*., 1994), since they are nodulated by, and fix nitrogen with their endosymbiotic root nodulae bacteria known as *Rhizobium leguminosarum* var *viceae*, a common cross inoculation rhizobium that also nodulates many of the cool season legumes. It is estimated that these legumes fix 200-300 kg N $ha^{-1}yr^{-1}$ depending upon their variety, type of endosymbiont and different ecological factors (Sanginga *et al*., 1995).

Field pea (*Pisum sativum*) is the second most important pulse crop in Ethiopia after faba bean in terms of both area coverage and production. It is widely cultivated in the different regions of the country at altitudes between 1,800 and 3,000 m a.s.l. with annual average rainfall of 700-900 mm. According to the Central Statistics Agency (CSA), field pea covers over 254,000 hectares with total production of 230,000 tons that account to 17% of the total grain legume production (CSA, 2004). It is a cash crop and a good source of protein and energy in the diet of the majority of the population.

Field pea production in Ethiopia is generally low and mainly produced for subsistence due to low soil fertility (Desta Beyene and Angaw Tsigie, 1989; Angaw Tsigie and Asnakew Woldeab, 1994) and inefficient traditional agronomic practices (Asfaw Telaye *et al*., 1994).

For many years now, many researchers have undertaken nation-wide studies to improve field pea cultivars (Mussa Jarso *et al*., 2006), and insect pest management (Kemal Ali, 2006). However, many of the hitherto studies were not adequate with quantification of biological nitrogen fixation, and limited to soil plant interaction and fertilizer trials of these legumes in different agricultural research institutes (Desta Beyene and Angaw Tsigie, 1989; Angaw Tsigie and Asnakew Woldeab, 1994; Asfaw Hailemariam and Angaw Tsigie, 2006).

The present study, therefore, was designed with the objective of isolating and characterizing field pea rhizobia from several sampling sites in central and northern parts of the country to evaluate their diversity in symbiotic effectiveness The result will serve as baseline data for future endeavour of

realizing the full potential of biological nitrogen fixing system of field pea into the productivity of low-input agriculture in the country.

MATERIALS AND METHODS

Study sites

The nodule samples were collected from farmers' fields of representative field pea-growing highlands of Shewa, Gojam, Gondar, Wollo and Tigray regions of Ethiopia (Fig. 1).

Sampling and isolation

Nodules of field pea were collected at the stage of flowering from mid-August to mid-October and rhizobia were isolated from nodules using standard methods (Vincent, 1970).

Cultural and growth characterization of the isolates

Rhizobial isolates were routinely isolated using YMA, purified on YMA-Congo red plate media and peptone glucose agar and preserved into YMA-$CaCO_3$ slant according to Lupway and Haque (1994). Acid or alkaline production of isolates was verified by growing them on Bromothymole Blue (0.05%) (BTB)-YMA medium (Jordan, 1984). Cultural characteristics were also examined based on their colony size and texture on YMA medium (Ahmed *et al*., 1984). Growth and eco-properties of isolates were undertaken in triplicates on YMA medium adjusted to pH 7 by inoculating 106/ml into the medium, and incubating at 28°C for 5-7 days, unless stated otherwise. Results were recorded qualitatively either as + for growth or – for no growth.

Growth rate was monitored by inoculating isolates on YM liquid medium and growing them for 48 hours. Growth rate was measured by taking samples every 4 hours and measuring optical density at 540 nm using spectrophotometer (UV-7804C-Ultraviolet-Visible spectrophotometer) against uninoculated YM broth media as a blank. The generation time was calculated from the logarithmic phase (White, 1995) based on the following formula:

$$g = \frac{\log^2 (t)}{\log^X - \log^{Xo}}$$

where g = generation time, t = time elapsed, XO = first OD, X = second OD reading and OD= optical density. Generation time (g) = t/n where n = number of generations and t = time elapsed.

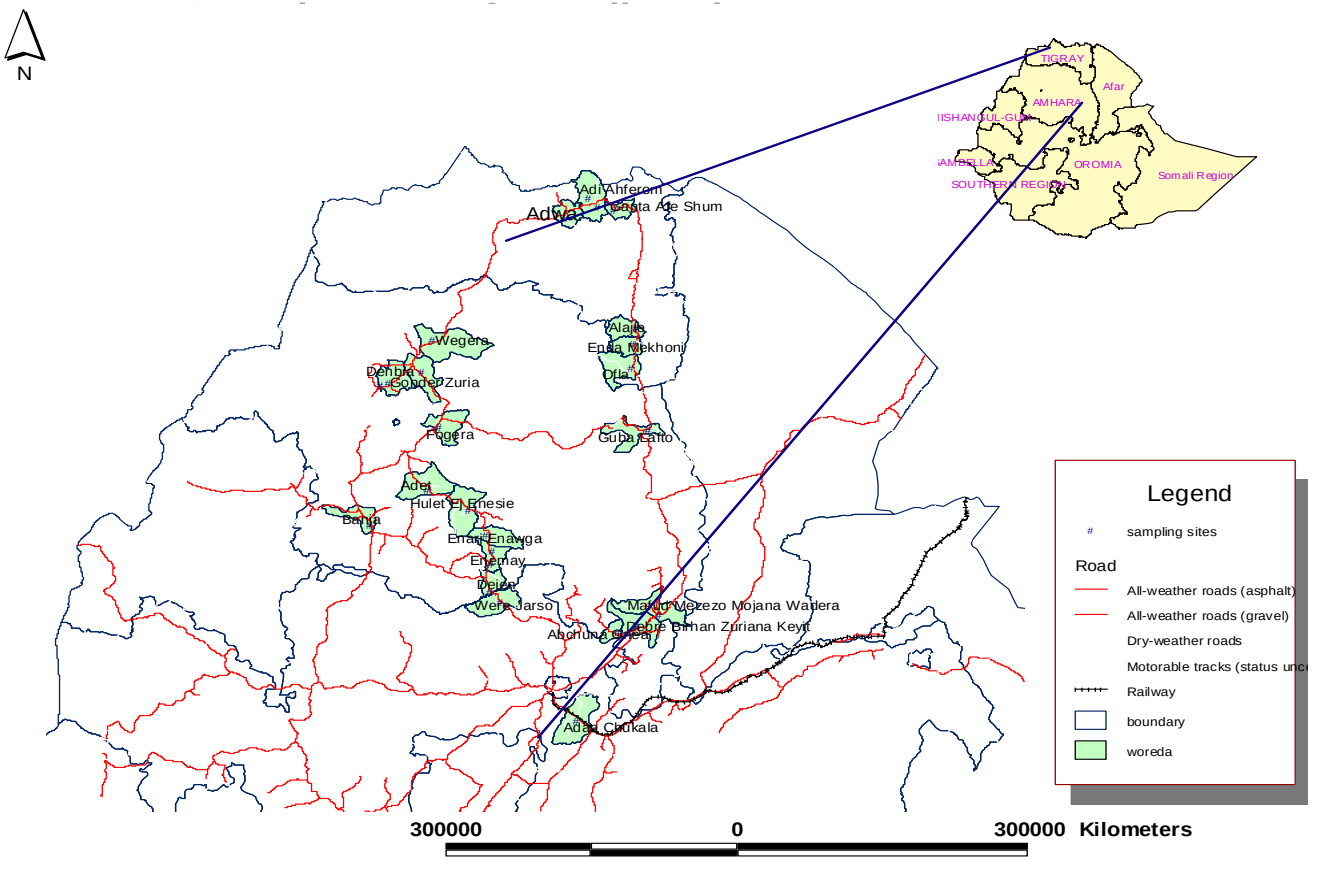

Fig.1. Location of sampling sites.

Authentication and evaluation of symbiotic efficiency

Isolates were tested for their infectivity (nodule formation) and effectivity in nitrogen fixation ability using sterile sand culture with *Pisum sativum*, cultivar Markose. River sand was soaked in $1NH_2SO_4$ for 24 hours and extensively washed with tap water several times, and filled into alcohol-sterilized (70%) 3 kg capacity plastic pots. The seeds were surface sterilized with 95% ethanol for 3 minutes followed by 0.2% acidified mercuric chloride and subsequently washed with 5 changes of sterile distilled water, and germinated in sterile petri dishes before planting (Vincent, 1970). Pots were planted with three germinated seedlings at a depth of 1 cm and each seedling was inoculated with 1 ml (109 cells ml^{-1}) of the test rhizobia. The experiment was statistically laid out in a randomized block design with a total of 25 treatments with three replications. Two uninoculated treatments, one with nitrogen fertilizer (positive control, 0.05%) and other without nitrogen (negative control) were included (Somasegaran and Hoben, 1994).

Plants were grown in a greenhouse with a 12/12 hour light/dark cycles and a maximum day and night time temperature of 27 ±1, 20 ±1°C, respectively. All pots were fertilized with Jensen's nitrogen-free nutrient media (Jensen, 1985) once a week, and watered every two days with tap water. Plants were harvested after 45 days of planting to record nodule number, and colour, and shoot dry matter. Plant total nitrogen content was determined by modified Kjeldhal method from plant dry matter according to Bremer (1965).

Percentage of effectiveness of the isolates was calculated based on the relative plant shoot dry matter accumulation of inoculated plants in relation to nitrogen-fertilized control plants according to Date (1993) as cited in Purchino *et al*. (2000).

$$SE = \frac{\text{Inoculated plant DM X 100\%}}{\text{N-fertilized plant DM}}$$

where DM = dry matter and SE = symbiotic effectiveness. Nitrogen fixing effectiveness was classified as ineffective, <35%; lowly-effective, 35-50%; effective, 50-80%; and highly effective, >80%.

One way analysis of variance (ANOVA) was performed on the symbiotic efficiency data using SPSS 11.5 version of statistical program. Mean separation of nodule number, shoot dry matter and total nitrogen data were calculated using Tukey's HSD values when the F-test was significant at $p<0.05$.

Tests for ecological characteristics (pH, temperature and salt tolerance)

Nineteen isolates were tested to pH using Keyser defined plate media which is modified by CIATs according to Lupway and Haque (1994). The pH of the media was adjusted after autoclaving to 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9 and 9.5 with sterile 0.1N HCl or 1N NaOH. Growth of bacteria at different temperatures was investigated by incubating cultures on YEMA plate at 5, 10, 25, 30, 35, 38 and 40°C (Lupway and Haque, 1994). The effect of different salt concentrations on the growth of isolates was investigated by inoculating cultures on YEMA-NaCl plate adjusted at NaCl concentration of 0.05, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0 (w/v) (Maatallah *et al*., 2002).

Tests for intrinsic antibiotic and heavy metal resistance

The intrinsic antibiotic resistance of isolates to different antibiotics was evaluated according to Zhang *et al*. (1991). The antibiotics were supplemented after being filter-sterilized (0.2 µm) on YMA media at two concentrations of 5µg ml^{-1} and 10µg ml^{-1}. The antibiotics were chloramphenicol, streptomycin, nalidixic acid, rifampicilin, neomycin, kanamycin and novobiocin. Most of these were dissolved in water except chloramphenicol, and rifampicilin that were prepared in absolute alcohol (95%) and diluted to the required volume with distilled water. Resistance to heavy metals was also determined on solid YMA media containing the following filter-sterilized heavy metals at the concentration of (µg ml^{-1}): $MnCl_2$ 250, 500; $HgCl_2$ 5, 10; $ZnCl_2$ 50,100 and $CuCl_2$ 50, 100 (Maatallah *et al*., 2002).

Carbon source utilization (Heterotrophic competence)

Isolates were tested for their abilities to utilize 17 different carbon sources, according to Somasegaran and Hoben (1994). The carbon sources were arabinose, cellobiose, citrate, dextrin, fructose, galactose, glucose, glycerol, lactose, maltose mannose, mannitol, sorbitol, starch, sucrose, trehalose and xylose. Ten percent of the sugar solutions were filter-sterilized (0.2 µm) and separately added to the autoclaved basal YMA medium (without mannitol) aseptically.

RESULTS

Isolation, cultural and colony morphology of the isolates

A total of 25 field pea rhizobia were isolated from Gojam (7), Shewa (5), Tigray (7), Gondar (5) and Wollo (1) (Table 1). None of the isolates absorbed Congo red from YMA + CR (Congo red) media, and grew on peptone glucose agar (PGA) medium except NSRlFP24, that failed to form nodules on the host upon reinoculation. All, but isolate NSRlFP33 (Gondar) changed the colour of BTB-YMA from green to yellow (data not shown).

Table 1. Cultural and growth characteristics of authenticated rhizobia isolates from different sampling sites in central and northern parts of Ethiopia after 5-7 days of growth on YMA medium.

Isolate	Sampling areas	Sampling woreda	Generation time	Colony size (mm)	Colony textute
NSRIF2	Gojam	Dejen	2.3	3.0	LM
NSRIF3	"	**Enemai**	**1.8**	**5.0**	**LW**
NSRIF4	"	**Enarj Enawga**	3.8	4.0	LM
NSRIF5	"	**Enarj Enawga**	3.6	5.0	LW
NSRIF6	"	Hulet Ejen Nesae	4.1	3.0	LM
NSRIF7	"	Adet	2.7	3.0	LM
NSRIF8	Gondar	Gondar	2.2	3.0	LM
NSRIF9	"	Denbia	2.1	3.0	LM
NSRIF10	"	Denbia	2.4	5.0	LW
NSRIF24	"	Wereta/Fogera	1.8	3.8	LM
NSRIF33	"	Ambaghiorgis	7.6	2.0	LM
NSRIF18	Wollo	Guba Lafto	3.6	4.0	LM
NSRIF11	Tigray	Adwa	**2.6**	4.0	LM
NSRIF12	"	Adi Aheferom	**2.4**	3.0	LM
NSRIF13	"	Adi Aheferom	3.5	3.8	LM
NSRIF14	"	Adigrat	3.7	4.8	LM
NSRIF15	"	Amablaggie	3.0	4.5	LM
NSRIF16	"	Maichew	3.7	4.0	LM
NSRIF17	"	Korem/Ofla	**2.6**	4.1	LW
NSRIF1	Shewa	GhohaTsion	2.3	3.0	LM
NSRIF19	"	Tarma Ber	4.1/	4.0	LW
NSRIF20	"	Keyt	11.5	1.0	SD
NSRIF21	"	Mendida	4.5	2.0	LM

LM (large mucoid colonies with opaque, gummy and buttery characteristics); LW (large watery colonies with transparent texture, and production of copious amount of exo-polysaccharides); SD (small dry colonies with flat surface with little or no mucoid production)

Almost all of the isolates were fast-growing with short doubling time (generation time). All isolates displayed generation time between 2-4 hours, except isolates NSRIFP 20 and NSRIFP21 with slower generation times of

4.5 hours and 11.53 hours, respectively. They also displayed colony sizes of 2.0-5.0 mm, with large mucoid (LM) colonies on YMA medium with opaque, gummy and buttery texture whereas a few isolates assumed larger watery (LW) colonies with transparent texture, and production of copious amount of exo-polysaccharides upon 5-7 days of incubation. However, isolate NSRIFP20 was characterized by small dry (SD) texture with flat surface with little or no mucoid production, and with the smallest diameter of 1 mm (Table 1).

Authentication and symbiotic effectiveness

All isolates, but NSRIFP24, were able to form nodules on field pea (*Pisum sativum*), though they showed diversity in their symbiotic properties (Table 2). The inoculated plants showed deeper green leaves with tall and branched shoots that were different from the plants grown in the negative control pots.

The inoculated plants were found to be significantly different in the number of nodules/ plant, shoot dry matter and total nitrogen content at $p = 0.05$ (Table 2). The plants acquired nodules ranging from 29 nodules/plant inoculated with isolate NSRIFP33 (Gondar) to 108 nodules/plant inoculated with isolate NSRlFP2 (Gojam). Almost all of the tested isolates showed higher plant shoot dry matter compared to uninoculated and non-nitrogen fertilized control (negative control). The highest shoot dry matter of 3.35 g/plant was obtained from the inoculated plants with isolates of NSRlFP1 (Shewa) and NSRlFP5 (Gojam) followed by NSRlFP2 and NSRlFP4 (Gojam) that accumulated 2.91 g/plant and 2.60 g/plant, respectively. These values were slightly, but not significantly higher than the shoot dry weight of N-fertilized positive control plants (2.52 g/plant). The lowest shoot dry weight of 0.68 g/plant was recorded from the host plants inoculated with isolate NSRlFP32 (Shewa) which was similar to the shoot dry weight of negative control plants (0.58 g/plant) (Table 2).

Based on the relative plant shoot dry matter accumulation of inoculated plants with nitrogen-fertilized control, the rate of effectiveness of the isolates ranged from 133% dry matter accumulation by NSRlFP1 (Shewa) and NSRlFP5 (Gojam), to lowest rate of effectiveness of 27% dry matter accumulation by isolate NSRlFP32 (Shewa) (Table 2). Six isolates from Tigray (86%), 4 isolates from Shewa (80%), 5 isolates (71%) from Gojam, 4 isolates (67%) from Gondar and Wollo were very effective and effective, with the mean effectiveness of 76% (19 isolates); whereas 24% (6 isolates) showed lowly effective and ineffective fixation on the host plant.

Table 2. Authentication and symbiotic properties of rhizobial isolates on the host pea plant (Variety Markose) after 45 days of growth under greenhouse conditions.

Treatment	Sampling areas	Nodule number/plant	Total Nitrogen (%)	Shoot dry weight (g/plant)	%Effectiveness	Rate of effectiveness
NSRIFP2	Gojam	108 [a]	2.93[a]	2.91[ab]	116	Very effective
NSRIFP 3	"	**65[a-d]**	**2.57[a-f]**	**2.05[a-e]**	**81**	"
NSRIFP 4	"	**75[a-d]**	**2.68[ab]**	**2.60[a-c]**	**103**	"
NSRIFP 5	"	98[ab]	2.88[ab]	3.35[a]	133	"
NSRIFP 6	"	48[ad]	1.71[g-l]	1.23[b-e]	45	Lowly effective
NSRIFP 7	"	68[ad]	1.72[f-l]	1.45[b-e]	56	Effective
NSRIFP 31	"	40[bcd]	2.12[a-k]	1.24[b-e]	46	Lowly effective
NSRIFP 8	Gondar	74[ad]	2.51[ag]	2.40[a-e]	95	Very effective
NSRIFP 9	"	**73[ad]**	2.34[a-i]	**2.11[a-e]**	84	"
NSRIFP 10	"	**78[ad]**	2.39[a-h]	2.45[b-e]	97	"
NSRIFP 33	"	**29[d]**	**1.88[d-l]**	**0.97[cde]**	**38**	**Lowly effective**
NSRIFP 18	Wollo	55[a-d]	2.39[a-h]	**2.02[a-e]**	81	**Very effective**
NSRIFP 11	Tigray	78[a-d]	2.32[a-j]	2.29[a-e]	90	"
NSRIFP 12	"	**74[a-d]**	**2.50[a-g]**	**2.59[abc]**	**103**	"
NSRIFP 13	"	73[a-d]	2.39[a-j]	**2.34[a-d]**	**93**	"
NSRIFP 14	"	95[abc]	**2.82[ab]**	2.41[a-e]	96	"
NSRIFP 15	"	78[a-d]	2.49[a-h]	1.80[a-e]	71	"
NSRIFP 16	"	33[cd]	1.52[k-l]	1.11[b-e]	44	Lowly effective
NSRIFP 17	"	56[a-d]	2.47[a-h]	2.16[a-e]	86	Very effective
NSRIFP 1	Shewa	106 [a]	2.93[a]	3.35[a]	133	"
NSRIFP 19	"	62[a-d]	2.59[a-e]	2.12[a-e]	84	"
NSRIFP 20	"	66[a-d]	2.49[a-k]	2.08[a-e]	83	"
NSRIFP 21	"	47[a-d]	2.00[c-k]	1.85[a-e]	73	Effective
NSRIFP 32	"	47[a-d]	1.76[g-l]	0.68[de]	27	Ineffective
+control			2.47[a-h]	2.52[a-d]	-	-
-control			1.56[i-l]	0.58[e]	-	-

Numbers in the same column followed by different letters are significantly different at 5% level (Tukey HSD); letters in the columns (abc..) are rank of the means

The total nitrogen (TN) contents of the inoculated plants was within the range of 1.52% inoculated by isolate NSRlFP16 (Tigray), to the highest value of 2.93% inoculated by isolates NSRlFP1 (Shewa) and NSRlFP2 (Gojam), followed by isolate NSRlFP5 (Gojam) with TN of 2.88%.

Eco-physiological characteristics of rhizobial isolates

The nineteen very effective and effective isolates were tested for their tolerance and resistance to different eco-physiological factors (Table 3). The isolates showed variations in their tolerance to pH, salt (NaCl), and

temperature. The pattern of pH tolerance of the isolates was within pH 5.0- and pH 9.5. Many isolates (40%) were tolerant to a narrow acidic and neutral pH range of 5 and 7.0; whereas a number of isolates (30%) were capable of growing between neutral and basic pH range of 7.0 and 9.5. The relatively pH tolerant isolates were NSRIFP3 (Gojam), isolates NSRIFP11, 13, 17 (Tigray), NSRIFP18 (Wollo) and NSRIFP 8 (Gondar) that were capable of growing at wider pH range of 5.0-9.5 (Table 3). Most of the isolates (80%) were relatively sensitive to higher than 0.1% NaCl concentration in the medium.

Table 3. Eco-physiological tolerance and heterotrophic competence of the symbiotically very effective and effective rhizobial isolates of field pea.

Isolate	**Tolerance (range)**			**Resistant isolates tested for**		**Sugar utilized by isolates (%)**
	pH	**Salt (%)**	**Temperature**	**Antibiotics**	**Heavy metal**	
NSRIFP1	5-7	0.1	20-35°C	-	Mn	94
NSRIFP2	5-7	0.1	20-35°C	chl, nal	Mn	94
NSRIFP3	**5-9**	**5.0**	**5-38°C**	**nal**	**Mn, Cu, Hg, Zn**	**88**
NSRIFP4	**7-9**	0.1	**10-35°C**	**chl, kan, nal, rif, str**	**Mn**	**82**
NSRIFP5	7-8	0.1	20-3 °C	nal, rif	-	88
NSRIFP7	6-7	0.1	20-35°C	nal	Mn	82
NSRIFP8	5.5-8	0.1	20-35°C	nal	Mn	88
NSRIFP9	5-5.7	0.1	20-35°C	nal, str	Mn	88
NSRIFP10	**7-9**	**2.0**	20-35°C	**cep, nal**	Mn	**94**
NSRIFP11	**5.5-9.5**	**2.0**	20-35°C	nal	Mn	88
NSRIFP12	**5-5.8**	0.1	20-35°C	**cep, chl, nal**	**Mn, Hg**	**100**
NSRIFP13	**5.5-9.5**	0.1	**5-38°C**	**rif, str**	**Mn, Hg**	**88**
NSRIFP14	7.0	0.1	10-35°C	**chl, nal, rif, str**	Mn	**94**
NSRIFP15	7.0	0.1	20-35°C	nal,	Mn	**88**
NSRIFP17	**5-9.5**	**6.0**	**5-35°C**	**cep, nov, rif**	**Mn, Hg**	**82**
NSRIFP18	5-9	0.1	20-35°C	**chl, nal, neo, rif**	**Mn, Zn**	**94**
NSRIFP19	7-8	0.1	**10-38°C**	cep, chl, nal, nov, rif, str	Mn	100
NSRIFP20	7.0	0.1	20-35°C	chl, nal	Mn	76
NSRIFP21	7-9.5	0.1	5-35°C	-	Mn	76

Antibiotics: cep - cephalosporium; chl - chloramphenicol; kan - kanamycin; nal - nalidixin; neo - neomycin; nov - novobiocin; rif - rifampicin; str - streptomycin
Heavy metal: Cu - copper; Hg - mercury; Mn - manganese; Zn - zinc

The most resistant isolate was isolate NSRIFP17 that was able to grow at 6% NaCl, followed by isolates NSRIFP3, NSRIFP10 and NSRIFP11 that were resistant to grow in the medium containing 5% and 2% NaCl, respectively.

The optimum growth temperature range for all the isolates was 20-35°C (Table 3). However, the number of isolates decreased at lower and higher incubation temperatures, where only 24% of the isolates were found to grow beyond the two ends. Consequently, the most tolerant isolates; NSRIFP3, NSRIFP13, NSRIFP17 were capable of growing at incubation temperatures of 5-38°C, followed by isolates NSRIFP4, NSRIFP14, and NSRFIP19 that were resistant to the lower temperature range of 10-38°C.

Intrinsic antibiotic and heavy metal resistance

Isolates showed wide variations in resistance to different antibiotics (Table 3). However, most isolates (20) were resistant to nalidixic acid, followed by the number of isolates resistant to chloramphenicol (10), and rifampicin (10). The isolates, in general were sensitive to novobiocin, kanamycin, and neomycin.

The most resistant isolates to the different antibiotics were; NSRIFP19 (6 antibiotics), NSRIFP4 (5 antibiotics), NSRIFP14 (4 antibiotics), and NSRIFP12 (3 antibiotics). On the contrary, isolates NSRIFP1 and NSRIFP21 did not show any growth in the presence of any antibiotic (Table 3). The heavy metal resistance of the isolates showed that they were generally tolerant to $MnCl_2$, and sensitive to $HgCl_2$, $ZnCl_2$ and $CuCl_3$. Only isolate NSRlFP3 (Gojam) was relatively tolerant to several concentrations of the tested heavy metals, whereas isolate NSRIFP5 was the most sensitive (Table 3).

Carbon source utilization (Heterotrophic competence)

All isolates almost utilized 60% of the tested sugars that included mannitol, dextrin, maltose, glycerol, lactose, galactose, mannose, and only isolates NSRIF12 and NSRIF19 utilized citrate and starch. In general, the isolates were capable of utilizing 76% up to 100% of the sugars as carbon and energy sources.

Resistance and tolerance of isolates to several of the abovementioned eco-physiological factors indicated that NSRIFP3, NSRIFP4 (Gojam) NSRIFP17, NSRIFP12, NSRIFP13 (Tigray), NSRIFP10 (Gondar), NSRIFP18 (Wollo), NSRIFP19 (Shewa) showed a wider range of resistance to a combination of one or more of the tested eco-physiological factors (Table 3).

DISCUSSION

The data showed that 24 root nodule bacterial isolates (96%) of field pea collected from major field pea-growing areas of the country were authenticated as rhizobia (Vincent, 1970). The results on their colony size, colony texture, and acid production in the growth medium were typical of the characteristics of fast-growing rhizobia similar to field pea rhizobia isolated from southern Tigray (Fano Berhe, 2010) and eastern and western Hararghe (Kassa Bayeh, 2011).

Although the tested isolates were able to infect their host plant to fix atmospheric nitrogen leading to higher plant growth than the non-inoculated and nitrogen-free controls, they varied in the number of nodules they induced, and the shoot dry weight they helped the host to accumulate due to their effectiveness in nitrogen fixation. The wide range of nodulation amongst the isolates showed that the high-nodulating isolate NSIRFP2 (108 nodules/plant) induced 4 times more nodules than the poor-nodulating isolate NSRIFP33 (29 nodules/plant) (Table 2). This is almost similar to the one recorded from pea rhizobia (25-93 nodules/plant) (Fano Berhe, 2010). This pattern of induction by the high and low-nodulating endosymbionts was also similar to number of nodules induced by pea rhizobial isolates from eastern and western Hararghe, although they profusely nodulated pea plant to the tune of 84-246 nodules/plant (Kassa Bayeh, 2011).

Based on the relative shoot dry matter accumulation of the inoculated plants to nitrogen-fertilized control (Date, 1993 cited in Purchino *et al*., 2000), 76% of the isolates displayed symbiotic efficiency of very effective and effective level that were able to accumulate 50-100% of shoot dry weight compared to the nitrogen-fertilized control (Table 2).

Similar pattern of effectiveness of 67% was also recorded from pea rhizobia from southern Tigray (Fano Berhe, 2010) and relatively higher effectiveness of 90% from western Hararghe (Kassa Bayeh, 2011). Ballard *et al*. (2004) also reported 61-98% effectiveness of inoculated field pea plants compared to the control plants. A study on the same cross-inoculating endosymbiont from faba bean also showed that soil samples from North Gondar harboured more than 80% effective and very effective rhizobia (Zerihun Belay and Fassil Assefa, 2011).

The mean comparison based on percentage of total nitrogen content and mean nodule number was also different among treatments at the same level of significance. Most isolates with higher shoot dry matter accumulation were also found to have high nodule number whereas total nitrogen (TN)

content (2.49-2.93) did not necessarily corroborate with shoot dry matter (Table 2).

The responses of the selected rhizobial isolates to different stress factors that presumably existed in the soil were variable (Table 3). Most of the isolates were characterized by a narrow tolerance to either slightly acidic or slightly basic pH of the medium (6-8) (70%), whereas a few were tolerant to pH 5-9.5. This shows that the rhizobial isolates in this study were slightly sensitive to acidity compared to field pea rhizobia isolates that were tolerant to pH 4.5-9.5 from southern Tigray (Fano Berhe, 2010), and eastern and western Hararghe (Kassa Bayeh, 2011), and faba bean rhizobia from Wello (Asefa Keneni *et al*., 2010).

Most of the isolates (80%) were sensitive to salt for they were limited to grow at 0.1% NaCl, whereas only 20% of the isolates were resistant to 0.5-2% salt concentration (Table 3). Sensitivity pattern of the isolates in this study was similar to the report of Kassa Bayeh (2011), where most of the rhizobia isolated from pea plants from eastern and western Hararghe were sensitive to NaCl concentration of more than 0.1%. A slightly different result from southern Tigray showed that all isolates from field pea were tolerant to 1% NaCl, and a few isolates were very tolerant up to 7% salt concentration. Sensitivity of isolates (*Rhizobium leguminosarum* var *viceae*) from the same cross-inoculation faba bean host to salt was also reported by Asefa Keneni *et al*. (2010) and Zerihun Belay and Fassil Assefa (2011). Lindstrom and Lehtomaki (1988) reported that only 31% and 23% of the tested *R. leguminosarum* isolates were grown at 1 and 2% NaCl, respectively.

All isolates were found to grow within 20-35°C, which is the optimum growth temperature for most strains of rhizobia (Somasegaran and Hoben, 1994). However, only 20% of the isolates grew at 5-38°C, which was similar to the maximum temperature resistance pattern of *R. leguminosarum* reported by Jordan (1984). Fano Berhe (2010) and Kassa Bayeh (2011) also reported the same pattern of temperature tolerance of pea rhizobial isolates within the range of 4-35°C and 5-40°C, respectively. Zerihun Belay and Fassil Assefa (2011) also reported that isolates from faba bean can grow within the temperature range of 15-35°C.

The general pattern of carbon utilization showed that the isolates were capable of utilizing 76% and 100% of carbon sources (Table 3), indicating that the isolates were versatile in assimilating different carbon sources and heterotrophically competent that ensured their survival in the soil. Fano

Berhe (2010) also reported that rhizobial isolates from southern Tigray utilized 87% of 13 carbon sources. Stowers (1985) previously reported that fast-growing and free-living rhizobia have the ability to utilize a wide variety of carbon sources for growth and energy with several pathways available for carbon catabolism. However, the limited catabolic activity of *R. leguminosarum* on citrate and starch was also reported by several workers (Jordan, 1984; Lindstrom and Lehtomaki, 1988; Fano Berhe, 2010; Zerihun Belay and Fassil Assefa, 2011).

With regard to antibiotic and heavy metal resistance, field pea rhizobial isolates exhibited a random and wide range of susceptibility to these parameters (Table 3). Most of the isolates were found to be sensitive to kanamycin, neomycin, and novobiocin, and resistant to higher concentration of nialidixic acid and chloramphenicol. Other workers have also reported resistance to nalidixic acid by *R. leguminosarum* isolates (Hagedorn, 1979), to chloramphenicol and nalidixic acid by rhizobial isolates from field pea (Fano Berhe, 2010), and faba bean (Zerihun Belay and Fassil Assefa, 2011). The isolates were also found to be resistant to manganese, and sensitive to copper chloride, zinc chloride and mercuric chloride (Table 3). This was slightly different from the report of Kassa Bayeh (2011) where pea rhizobia showed resistance to zinc and copper chloride.

It is established that effectiveness of isolates under controlled environment may not necessarily be concurrent to their performance in the field because of the environmental conditions that govern the process of nitrogen fixation (Sanginga *et al*., 1995). It is therefore, important to evaluate the effectiveness of the different isolates in relation to their *in vitro* resistance and tolerance to different eco-physiological factors, if they are to be used as microbial inoculants. Although most of the rhizobial isolates (76%) from the different sampling sites were effective and very effective in nitrogen fixation of field pea, all of them may not be competitive if they are inoculated in the soil under different environmental stresses.

The data showed that NSRIF1 and NSRIF5 were the most effective isolates in relation to their symbiotic effectiveness (100%) under greenhouse conditions; their competitiveness in the field was questionable for they were very sensitive to antibiotics and heavy metals (Table 3). However, out of the symbiotically effective and very effective isolates, NSRlFP3 and NSRlFP4 (Gojam), isolates NSRlFP12, NSRlFP13, NSRlFP14 and NSRlFP17 (Tigray), isolate NSRlFP18 (Wollo), and NSRlFP10 (Gondar), NSRIFP19 (Shewa) were symbiotically very effective and simultaneously resistant to

different antibiotics, and heavy metals, and tolerant to several of the eco-physiological characters (Table 3).

Although the study was not exhaustive and very limited to a number of isolates, dependent on one host variety (Markose), and to a few sampling sites of the northern and central parts of the country, it gave an insight that most of the soils in the major field pea-growing areas in the country harboured symbiotically effective rhizobia that nodulate field pea. For the soils that do not have effective endosymbionts for pea production, some of the abovementioned symbiotically effective and eco-physiologically tolerant isolates could be further tested on several pea varieties in field trials. The best performing isolates can then be recommended to enhance biological nitrogen fixation and field pea production. In general, if the selection of rhizobial isolates from pea were properly followed with the appropriate genetic, competitiveness, and environmental studies, the isolates can partly contribute to integrated soil fertility management in the low-input agriculture in the country.

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