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ORIGINAL ARTICLE

Phenotypic, stress tolerance, and plant growth promoting characteristics of rhizobial isolates of grass pea

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Abstract

Grass pea (*Lathyrus sativus* L.) is widely cultivated for food and feed in some developing countries including Ethiopia. However, due to its overexaggerated neuro-lathyrism alkaloid causing paralysis of limbs, it failed to attract attention of the research community and is one of the most neglected orphan crops in the world. But, the crop is considered an insurance crop by resource-poor farmers due to its strong abiotic stress tolerance and ability to produce high yields when all other crops fail due to unfavorable environmental conditions. This study was aimed at screening rhizobial isolates of grass pea and evaluating their symbiotic nitrogen fixation efficiency and tolerance to abiotic stresses. Fifty rhizobial isolates collected from grass pea nodules were isolated, screened, and characterized based on standard microbiological methods. The rhizobial isolates showed diversity in nodulation, symbiotic nitrogen fixation, and nutrient utilization. The 16S rRNA gene sequencing of 14 rhizobial isolates showed that two of them were identified as *Rhizobium leguminosarum* and the remaining twelve as *Rhizobium* species. Based on their overall performance, strains AAUGR-9, AAUGR-11, and AAUGR-14 that performed top and identified as *Rhizobium* species were recommended for field trials. This study screened and identified effective and competitive rhizobial isolates enriched with high nitrogen-fixing and abiotic stress tolerant traits, which contributes much to the application of microbial inoculants as alternative to chemical fertilizers.

Keywords Grass pea · *Rhizobium* · Stress tolerance · Plant growth promotion · 16S rRNA

Introduction

Grass pea is one of the cool-season legume crops within the tribe Viciae including the genera *Vicia*, *Pisum*, and *Lens* that fix inorganic nitrogen in association with root nodule bacteria known as rhizobia. Previous studies on *Vicia*, *Pisum*, *Lathyrus*, and *Lens* (Jordan 1984; Perret et al. 2000; Rivas et al. 2009) showed that the endosymbionts belong to *Rhizobium leguminosarum* biovar *vicieae*. However, recent reports revealed that nodules from *Vicia faba*, *Pisum sativum*, and *Lens esculenta* also harbor other groups of root nodule bacteria other than *Rhizobium leguminosarum* biovar *vicieae* (Santillana et al. 2008; Tena et al. 2017). These include *Rhizobium leguminosarum* biovar trifolli that nodulate trifolium spp. and *Rhizobium etli* that nodulate *Phaseolus vulgaris* (Beyene et al. 2004). Recently, Tena et al. (2017) made a polyphasic study on root nodule from lentil and identified three distinct sub-lineages as *Rhizobium etli*, *Rhizobium leguminosarum*, and *Rhizobium* spp.

Other studies also showed the different levels of symbiotic effectiveness of cross-nodulating rhizobia isolated from one host may not necessarily be effective on another host indicating diversity and specificity even among the *Rhizobium leguminosarum* var. viceae populations (Laguerre et al. 2003; Gebremariam and Assefa 2017). For several years now, extensive studies have been undertaken on phenotypic and symbiotic properties of the tribe Viceae in Ethiopia. Some of these studies were focused on faba bean (Mnalku et al. 2009; Keneni et al. 2010; Belay and Assefa 2011; Legesse

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and Assefa 2014), lentil (Jida and Assefa 2011; Setargie et al. 2015), and field pea (Amsalu et al. 2012; Baye et al. 2015), and cross-inoculation studies on the Viceae tribe (Gebremariam and Assefa 2017).

Grass pea is the fifth most important pulse crop in Ethiopia after faba bean, chickpea, field pea, and red haricot bean (CSA (Central Statistical Authority) 2017) and is used for food, feed, and fodder (Urga et al. 2005). The seed contains proteins, fats, and carbohydrates (about 35% of which is starch), and it also contains glucose, pentosans, phytin, lignin, albumin, prolamine, globulin, guttllin, and several essential amino acids and minerals (Demelash et al. 2015). The protein content of grass pea seeds collected from 15 major production areas of Ethiopia is 27–32% (Urga et al. 2005), which is higher than the average percentage of protein content (21–25%) in other legume seeds (Monsoor and Yusuf 2002). Grass pea also plays an important role as a legume crop in crop rotations, reportedly adding around 67 kg/ha of nitrogen to the soil from symbiosis with *Rhizobium* species in a single season and conferring yield and protein benefits on the subsequent non-legume crop (Jennifer 2003). Apart from biological nitrogen fixation, rhizobia have also plant growth-promoting activities including solubilization of nutrients and production of growth hormones, production of hydrolytic enzymes, production of siderophore, and production of HCN. Such activities directly or indirectly increase plant growth and productivity.

Despite its wide cultivation in Ethiopia due to its ability to grow on marginal lands and reduced moisture requirement, grass pea is one of the neglected orphan crops that failed to attract attention of the scientific research community. This inadvertent neglect of the crop is due to the negative perception of the crop for its alkaloid associated with lathyrism (Moges et al. 2004). Although extensive researches have been undertaken on other related pulse crops, little is known about grass pea contribution to soil fertility and plant health by its rhizobia. The limited studies showed that the root-nodulating rhizobia of grass pea were as diverse as other cross-nodulating hosts such as faba bean, field pea, and lentil (Drouin et al. 1996; Mahdavi et al. 2007; Aoki et al. 2010) contributing to diversity studies on *Rhizobium leguminosarum* var. viceae. The lack of due attention by the scientific community, as it was the case with other orphan crops, precludes the full exploitation of the fifth most important feed and cover crop in the country.

Rhizobial populations are known to vary in their tolerance to major environmental factors (Wei et al. 2008). Consequently, exploiting the enormous potential of symbiotic nitrogen fixation requires the selection and development of efficient inoculant strains compatible to the legume host and the target edaphic environment (Bhargava et al. 2016). To this end, studying the potential of the isolates for physiological versatility is important to select them for their competitiveness in the complex soil environment (Fitouri et al. 2012). Thus, screening and selecting symbiotically highly effective and most tolerant isolates with ecological adaptations that can be introduced as microbial inoculants is key not only for grass pea but also for the cross-nodulation crops such as faba bean, field pea, and lentil.

Materials and methods

Sample collection, isolation, and purification of isolates

Grass pea nodules were collected from the roots of selected standing crops grown in 50 sampling field sites (Table 1); 35 from some districts of South Wollo (Jamma, Woreilu, Tenta, Borena, Kalu, Tehuledere, and Kutaber), 3 from Oromia zone (Artumafursi), and 12 from Western Shoa (A/Gindeberet, Jeldu, and Dendi) (Fig. 1). The nodule samples were collected in vials containing a desiccant (silica gel) covered with 1 cm of cotton wool (Somasegaren and Hoben 1994) and brought to Addis Ababa University. The isolation and purification of the rhizobial isolates were carried out using Yeast Extract Mannitol Agar (YEMA) as described by Vincent (1970). The purified isolates were designated as AAUGR (Addis Ababa University Grasspea Rhizobia) with different numbers representing each isolate and kept at – 20 °C for further work. Colony characteristics including colony diameter, colony texture, acid base reaction on YEMA-BTB media, and mean doubling time were evaluated according to Somasegaren and Hoben (1994) and White (1995). Confirmatory tests that included gram reaction, Congo red absorption, and growth on peptone glucose agar (PGA) media were also carried out (Buck 1982; Somasegaren and Hoben 1994).

Authentication of the isolates under greenhouse conditions and determining their relative symbiotic effectiveness

All the rhizobial isolates were subjected to pot sand culture to authentication test (definitive test) for 60 days according to Somasegaren and Hoben (1994) (Fig. S1). "Wasse" grass pea variety which was obtained from Debrezeit Agricultural Research Centre was surface-sterilized using 95% ethanol (briefly), bleached with 3% sodium hypochlorite for 4 min, rinsed with five changes of sterile water, and germinated on water agar (0.75 w/v) for 3 days. Five germinated seeds were transferred into 3-kg capacity plastic pots, pre-cleaned with acid (95% H_2SO_4). After 7 days of growth, each seedling was inoculated with 1 ml (10^9cells/ml) of 72-h YEMB-grown culture. The experiment was statistically laid out in a

Table 1 Authentication of the isolates under greenhouse conditions

Isolate code	Collection sites	NN/p (g)	NDW/p (g)	SDW/p (g)	% SE	Effectiveness
AAUGR-1	SW	49 ± 2^{ijk}	0.015 ± 0.000^{pqr}	0.477 ± 0.054^{h}	62.5	E
AAUGR-2	SW	71 ± 6.7^{def}	0.049 ± 0.005^{ghi}	0.778 ± 0.026^{def}	101.2	HE
AAUGR-3	SW	$48. \pm 5.7.6^{ijkl}$	0.039 ± 0.017^{ijk}	0.707 ± 0.047^{efg}	92.7	HE
AAUGR-4	SW	28.7 ± 12.7^{nopq}	0.013 ± 0.001^{qr}	0.275 ± 0.138^{ijk}	36.0	LE
AAUGR-5	SW	98.3 ± 6.1^{c}	0.062 ± 0.006^{ef}	0.898 ± 0.027^{jkl}	117.7	HE
AAUGR-6	SW	103.3 ± 9.7^{abc}	0.066 ± 0.008^{de}	0.988 ± 0.102^{bc}	129.5	HE
AAUGR-7	SW	50 ± 3.6^{hijk}	0.027 ± 0.005^{jklmn}	0.668 ± 0.008^{fg}	87.6	HE
AAUGR-8	SW	50.3 ± 8.1^{hijk}	0.030 ± 0.005^{jklmn}	0.714 ± 0.344^{efg}	93.6	HE
AAUGR-9	SW	102 ± 7.0^{bc}	0.074 ± 0.005^{cd}	1.005 ± 0.088^{bc}	131.7	HE
AAUGR-10	SW	37.7 ± 9.6^{klmno}	0.013 ± 0.001^{qr}	0.438 ± 0.092^{hi}	57.4	E
AAUGR-11	SW	105.7 ± 7^{abc}	0.082 ± 0.005^{bc}	1.013 ± 0.080^{bc}	132.8	HE
AAUGR-12	SW	67 ± 2^{defg}	0.035 ± 0.005^{ijklmn}	0.785 ± 0.103^{def}	102.9	HE
AAUGR 13	SW	31 ± 2^{mnopq}	0.014 ± 0.001^{qr}	0.484 ± 0.068^{h}	63.4	E
AAUGR-14	SW	115.3 ± 8.5^{ab}	0.085 ± 0.006^{b}	1.060 ± 0.089^{ab}	138.9	HE
AAUGR-15	SW	116 ± 9.5^{a}	0.098 ± 0.002^{a}	1.148 ± 0.191^{a}	150.6	HE
AAUGR-16	SW	51 ± 7^{hijk}	0.028 ± 0.006^{ijk}	0.670 ± 0.058^{fg}	87.8	HE
AAUGR-17	SW	53 ± 8.2^{ghij}	0.034 ± 0.012^{ijklmn}	0.653 ± 0.050^{fg}	82.6	HE
AAUGR-18	SW	28.3 ± 3.1^{nopq}	$0.012 \pm 0.00^{qr`}$	0.318 ± 0.024^{ijkl}	41.7	LE
AAUGR-19	SW	60 ± 2.7^{efghi}	0.048 ± 0.006^{ghi}	0.763 ± 0.024^{efg}	100.1	HE
AAUGR-20	SW	80 ± 8.5^{d}	0.056 ± 0.006^{fg}	0.845 ± 0.069^{de}	110.7	HE
AAUGR-21	SW	42.7 ± 7.1^{jklmn}	0.011 ± 0.003^{r}	0.376 ± 0.127^{hijkl}	49.3	LE
AAUGR-22	SW	17.7 ± 2.1^{q}	0.011 ± 0.002^{r}	0.306 ± 0.044^{ijkl}	40.1	LE
AAUGR-23	SW	27 ± 3^{opq}	0.013 ± 0.001^{qr}	0.398 ± 0.038^{hijkl}	52.2	E
AAUGR-24	SW	72.3 ± 8.3^{de}	0.051 ± 0.008^{gh}	0.763 ± 0.015^{defg}	100.1	HE
AAUGR-25	WS	33.7 ± 4.5^{mnopq}	0.020 ± 0.00^{opqr}	0.432 ± 0.202^{hi}	56.6	E
AAUGR-26	WS	64.3 ± 7.2^{efgh}	0.041 ± 0.007^{hij}	0.728 ± 0.031^{efg}	95.4	HE
AAUGR-27	WS	57.7 ± 21.0^{fghij}	0.025 ± 0.017^{nop}	0.429 ± 0.074^{hi}	56.2	E
AAUGR-28	WS	20 ± 3.5^{pq}	0.026 ± 0.005^{lmno}	0.302 ± 0.103^{ijkl}	47.9	LE
AAUGR-29	WS	33.7 ± 6.7^{mnopq}	0.019 ± 0.004^{opqr}	0.363 ± 0.085^{hikjl}	47.6	LE
AAUGR-30	WS	68.3 ± 9.5^{def}	0.041 ± 0.006^{hij}	0.764 ± 0.017^{defg}	100.1	HE
AAUGR-31	WS	26 ± 5.3^{opq}	0.024 ± 0.003^{nopq}	0.395 ± 0.032^{hijkl}	51.8	E
AAUGR-32	WS	28 ± 2.7^{nopq}	0.049 ± 0.006^{klmno}	0.422 ± 0.056^{hij}	55.3	E
AAUGR-33	WS	27.3 ± 4.2^{opq}	0.029 ± 0.004^{klmno}	0.383 ± 0.049^{hijkl}	50.2	E
AAUGR-34	WS	24.7 ± 3.5^{opq}	0.029 ± 0.003^{klmno}	0.456 ± 0.110^{hi}	59.8	E
AAUGR-35	WS	42.7 ± 13.7^{jklmn}	0.029 ± 0.006^{klmno}	0.377 ± 0.063^{hijkl}	49.4	LE
AAUGR-36	WS	26.7 ± 4.5^{opq}	0.028 ± 0.004^{klmno}	0.274 ± 0.030^{jkl}	35.9	LE
AAUGR-37	WS	67 ± 4^{defg}	0.040 ± 0.006^{ijk}	0.680 ± 0.035^{fg}	89.1	HE
AAUGR-38	WS	43.7 ± 3.2^{jklm}	0.034 ± 0.002^{ijklmn}	0.448 ± 0.021^{hi}	58.7	E
AAUGR-39	WS	37.3 ± 8.5^{klmno}	0.010 ± 0.012^{rs}	0.265 ± 0.044^{kl}	34.7	LE
AAUGR 40	WS	51.7 ± 5.0^{hijk}	0.028 ± 0.003^{klmno}	0.436 ± 0.013^{hi}	57.1	E
AAUGR-41	WS	37.3 ± 6^{klmno}	0.025 ± 0.010^{mnop}	0.411 ± 0.032^{hijk}	53.9	E
AAUGR-42	WS	51.5 ± 5.0^{hijk}	0.028 ± 0.002^{klmno}	0.435 ± 0.013^{hi}	80.5	HE
AAUGR-43	WS	45 ± 15.4^{jklm}	0.013 ± 0.001^{qr}	0.273 ± 0.047^{jkl}	35.8	LE
AAUGR-44	WS	$43.7 \pm 14.^{jklm}$	0.030 ± 0.009^{jklmno}	0.371 ± 0.116^{hijkl}	48.6	LE
AAUGR-45	WS	28 ± 4.4^{nopq}	0.013 ± 0.001^{qr}	0.385 ± 0.148^{hijkl}	50.5	E
AAUGR-46	WS	61.3 ± 4.7^{efghi}	0.039 ± 0.006^{ijk}	0.619 ± 0.064^{g}	81.1	HE
AAUGR-48	WS	36.7 ± 5.5^{klmno}	0.013 ± 0.001^{qr}	0.315 ± 0.021^{ijkl}	41.3	LE
AAUGR-49	WS	31 ± 8.9^{mnopq}	0.030 ± 0.004^{jklmno}	0.405 ± 0.079^{hijkl}	53.1	E
AAUGR-50	WS	71 ± 7.8^{def}	0.049 ± 0.007^{ghi}	0.779 ± 0.027^{def}	102.1	HE
+ve		0.0000^{r}	0.000 ± 0.000^{ijk}	0.763 ± 0.011^{defg}	100	
−ve		0.0000^{r}	0.000 ± 0.000^{s}	$0.237 \pm 0.074l^{lm}$		

The means followed by different letters are significantly different at $P < 0.01$ (Duncan test)-SPSS 20.0 version. *%SE* percent symbiotic effectiveness, *HE* > 80%, *E* 50–80%, *LE* 35–50%, *IE* < 35%, *NN/P* nodule number per plant, *SDW/P* shoot dry weight per plant, */p* per plant, *g* gram

complete random design (CRD) with three replicates in a greenhouse with a 12-h photoperiod and an average of 28 °C and 15 °C day and night temperature. The sampling included a negative control without treatment and inoculation and a positive control treated with 70 mg/l potassium nitrate (0.05 KNO_3 (w/v)) solution every week. All pots were fertilized with quarter strength of Broughton and Dilworth N-free medium per week and watered every two days as described in Somasegaren and Hoben (1994).

Sixty days after planting (DAP), plants were uprooted to record nodule number, nodule dry weight, and shoot dry weight. The symbiotic effectiveness (SE) of the

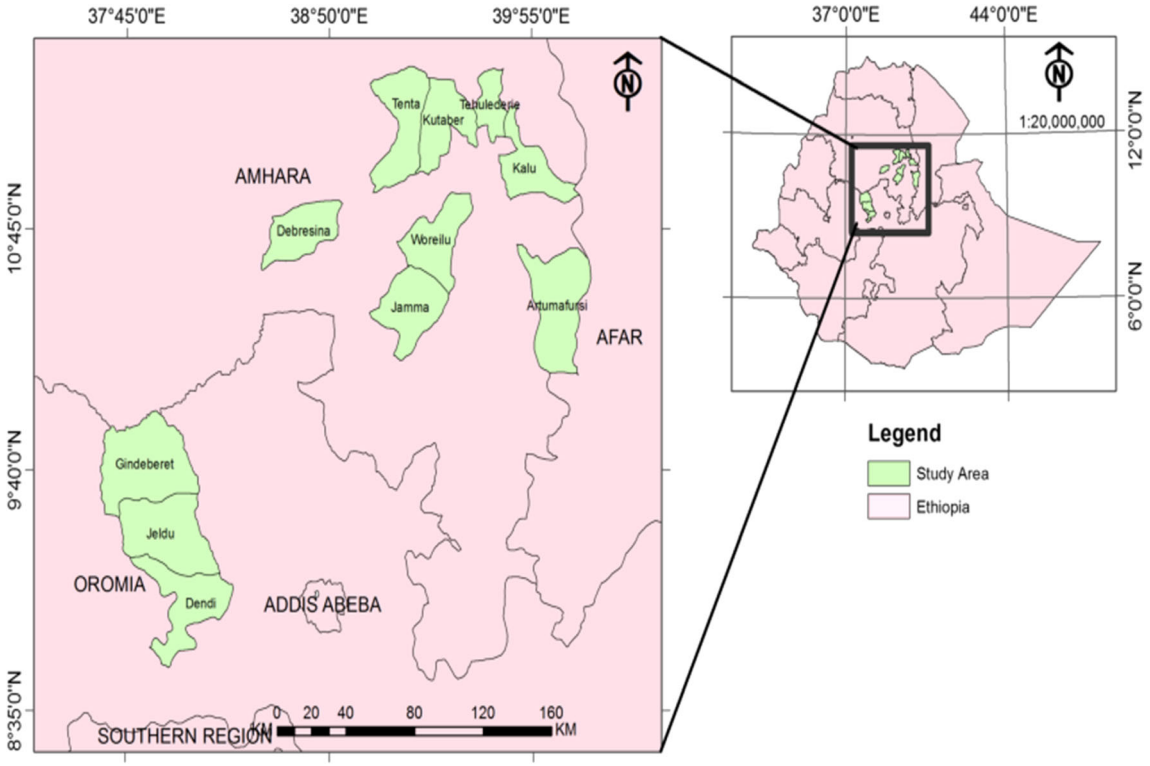

Fig. 1 Map of the study area

isolates was calculated using the formula (Mulongoy 2004):

$$\%SE = \text{Inoculated plant SDW} \times 100N - \text{Fertilized plant SDW}$$

where SDW = shoot dry weight, N = nitrogen, SE = symbiotic effectiveness

The rate of nitrogen-fixing effectiveness was evaluated as: highly effective > 80%, effective 50–80%, lowly effective 35–49%, and ineffective < 35%.

Tolerance to pH, temperature, and salt

Each isolate was grown on YEMB media adjusted at different pH (4.0, 4.5, and 5.0) and on YEMA media adjusted at pH 8.5, 9.0, 9.5, and 10.0 and incubated at 28 ± 2 °C for 3–5 days. Similarly, the isolates were grown at different salt concentrations of 1%, 2%, 3%, 4%, 5%, 6%, 7%, and 8%, of NaCl (Bernal and Graham 2001) and incubated at 28 ± 2 °C for 3–5 days. The isolates' potential to grow at different incubation temperatures was also evaluated by incubating at 4 °C, 10 °C, 15 °C, 35 °C, 40 °C, and 45 °C for 3–5 days as indicated in Lupwayi and Haque (1994). Growth of bacterial colonies was recorded as an indication of resistance to these environmental stresses.

Inherent antibiotic resistance and heavy metal tolerance of the isolates

Inherent antibiotic resistance (IAR) of the rhizobial isolates was determined by inoculating (10^6cells ml^{-1}) on solid YEMA medium containing filter-sterilized (0. 22 mm Millipore filters) antibiotics (μg/ml): ampicillin (30), chloramphinicol (40), erythromycin (30), nalidixic acid (20), neomycin (20), streptomycin (10), and tetracycline (30). The isolates (10^6 cells/ml) grown for 48 h were inoculated to the same medium containing filter-sterilized heavy metals (HM) at concentrations of (μg/ml) Hg ($HgCl_2$) (5), Kr ($K_2Kr_2O_7$) (50), Mn ($MnCl_2$) (200), Ni ($NiCl_2$) (200), Pb ($Pb(CH_3COO)_2$) (50), and Zn ($ZnCl_2$) (100) culture (Mohamed et al. 2012). All plates were incubated at 28 ± 2 °C for 3–5 days. Growth of bacterial colonies was recorded as an indication of resistance.

Heterotrophic utilization of the isolates

Carbon utilization of isolates was determined on a basal medium (1 g of KH_2PO_4; 1 g K_2HPO_4; 0.01 g $FeCl_3 \cdot 6H_2O$; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.1 g $CaCl_2$; 15 g agar) containing each of the 12 carbohydrate starch, cellobiose, dextrin, lactose, sucrose, galactose, maltose, fructose, glucose, arabinose, xylose, and sorbitol as 10% (w/v) by reducing the yeast extract to 0.05 gl^{-1}

liter following the method of Somasegaren and Hoben (1994). Similarly, the ability of isolates to utilize different nitrogen sources was tested on the same basal medium containing each of the 7 amino acids L-lysine, L-leucine, L-alanine, glycine, L-aspargine and L-cystine, and L-diphenylamine (Amargar et al. 1997). The plates were incubated at 28 ± 2 °C for 3–5 days. Growth of bacterial colonies was recorded as an indication of nutrient utilization.

Molecular characterization

Molecular characterization of some selected rhizobial isolates for the presence of 16S rRNA gene was conducted at the Institute for Agricultural Biosciences, Oklahoma State University, as previously described (Adal et al. 2018).

Colony PCR amplification of 16S rRNA

Fourteen rhizobial isolates that showed better performance in greenhouse and stress tolerance tests and for which amplicons were obtained were selected for 16S rRNA gene characterization. A single colony from a 48-h grown rhizobial culture was taken using a sterile pipette tip, added into PCR mix (10 μl 5× Phusion HF buffer, 1 μl 10 mM dNTPs, 2.5 μl 10 μM forward primer, 2.5 μl 10 μM reverse primer, 0.5 μl Phusion DNA polymerase, DNA template (small piece of single colony), and 33.5 μl Nuclease-Free Water), and well mixed through pipetting up and down for several times. PCR amplification of 16S rRNA genes was carried out using the primers 8.27F 5′ AGA GTT TGA TCC TGG CTC AG 3′ and rD1-5′ ACGGCTAC CTTGTTA CGACTT 3′ (Weisburg et al. 1991; Eardly et al. 1996; and Bontemps et al. 2010). The PCR conditions were preheating at 98 °C for 30 s″, 35 cycles of 98 °C for 10 s″ denaturing, 56 °C for 30 s″ annealing, 72 °C for 30 s″ elongation with a final extension of 72 °C for 10 min′ (Weisburg et al. 1991).

Purification of PCR products and sequencing of 16S rRNA genes

The colony PCR products were run on 1% agarose gel, and appropriate bands were excised from the gel and purified using PCR purification kit (Qiagen, Chicago, IL, USA) following the standard protocol recommended by the manufacturer. Five microliters of each purified PCR product was used for Sanger sequencing at the Oklahoma State University Core Facility. PCR products were sequenced using the same primers used in the PCR amplification. Sequence reads were edited using Bioedit software and compared for homology with sequences of other organisms deposited in the National Center for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST) to determine their identity to known sequences (Shayne et al. 2003).

Clustering analysis

The isolates's phenotypic variability was analyzed with a computer cluster analysis using similarity coefficient (Hammer et al. 2001). The ecophysiological and heterotrophic utilization that included resistance to temperature, pH, salt, antibiotics, and heavy metals and utilizations of carbohydrates and amino acids were used to construct a phenogram. Traits were coded 1 for positive and 0 for negative. The final matrix contained 22 isolates and 59 traits. A computer cluster analysis of 59 phenotypic variables was carried out using Ward's linkage and Squared Euclidian distance as a measure of dissimilarity coefficient (Ward 1963), and a dendrogram was constructed PAST ver.2.17c (Hammer et al. 2001).

Data analysis

The data recoded were analyzed and interpreted using ANOVA. The experimental treatments were compared and contrasted against their control following Duncan's multiple range test (DMRT). The correlation between different greenhouse data was evaluated by Pearson correlation coefficient using SPSS v.20.

Result

Isolation, presumptive, and cultural characterization of the isolates

A total of 50 legume-nodulating bacteria were isolated from grass pea–growing areas of South Wollo (35 isolates), Artumafursi (3 isolates), and West Shewa (12 isolates). The determination of the confirmatory tests and cultural characterization of the isolates are shown in Table 1. The confirmatory test result showed that all isolates but isolate AAUGR-47 were Gram-negative and failed to grow on PGA media and to absorb Congo red. Similarly, all the isolates except AAUGR-47 changed the YEMA-BTB to yellow and showed growth with doubling time ranging from 1.27 to 4.73 h. The isolates showed variations in colony size in which 7 (14 %) and 10 (20.4 %) displayed the highest and smallest colony diameter of 5. 5 mm and 2.0 mm. The colony diameter of the remaining isolates was in between this range. The isolates exhibited large watery (LW) (45%) and large mucoid (LM) (55%) colony textures. All these colony and growth features accompanied by acid production (yellow on YEMA-BTB) are characteristics of fast-growing and acid-producing rhizobia.

Symbiotic effectiveness of the isolates on sand culture under greenhouse conditions

The preliminary screening for symbiotic effectiveness of the isolates showed variations in nodulation and growth characters (Table 1, Figs. S2-S4). Accordingly, the rhizobia-induced nodules on the host plant with nodule number (NN) ranging from 17/plant (AAUGR-22) to 116/plant (AAUGR-15) and mean nodule dry weight (NDW) ranging from 0.011 g/p (AAUGR 22) to 0.098 g/p (AAUGR-15), indicating a 7-fold difference in nodule number and a 9-fold difference in nodule dry weight between the highest and lowest nodulating isolates.

Among the inoculated plants, all of them accumulated a higher shoot dry weight than the negative control and nearly half of them accumulated a higher shoot dry weight than the positive control plants ($P < 0.05$). The amount of shoot dry matter accumulated by the host plants was in the range of 0.27 g/p (AAUGR-39) to 1.15 g/p (AAUGR-15), suggesting that the most effective isolate helped to accumulate approximately 5-fold more biomass than the least effective isolate. The same host plants inoculated by the same isolates of AAUGR-15, AAUGR-14, AAUGR-11, AAUGR-6, and AAUGR-5 showed similar pattern in NN, NDW, and SDW, implying high correlation between nodule dry weight and shoot dry weight ($r = 0.864$ and 0.817). The data also showed a strong positive relationship at $p < 0.01$ among the parameters evaluated. The correlation between shoot dry weight and percent symbiotic effectiveness was found to form a relatively more direct and perfect linear relationship than the other parameters ($r = 0.988$). Based on the relative plant dry matter accumulation of the inoculated plants with uninoculated and nitrogen-fertilized control (positive control), 22 (45%) of the isolates were highly effective in nitrogen fixation with > 80% of shoot dry matter accumulation, whereas 20 (41%) of them accumulated 50–80% of shoot dry matter compared with the nitrogen-fertilized positive controls. In general, 86% of the isolates performed well in their symbiotic effectiveness.

Physiological tolerance of the isolates

All the isolates showed a significant difference in physiological tolerance to pH, salt, temperature, antibiotics, heavy metals, and utilization of carbohydrates and amino acids as substrates (Table 2). Nearly half of the isolates (45.5 %) tolerated both lower and higher pH and temperature ranges. Moreover, the isolates also showed variation in resistance to the tested antibiotics and toxic heavy metals. However, some exhibited similar resistance to both antibiotics and heavy metals.

Tolerance to salt, pH, and temperature

The potential of the isolates to tolerate a wide range of salt, pH, and temperature was determined (Table 2). All isolates displayed growth at 1–3% salt concentrations, 20–35 °C temperatures and a pH range of 6–9. Almost half of the isolates were tolerant to 40 °C, 6% NaCl, and pH of 4. However, fewer isolates were tolerant to 10 °C and 8% NaCl. Few isolates (18%) that included AAUGR-2, AAUGR-6, AAUGR-9, and AAUGR-24 were tolerant to a wide pH range of 4–10, whereas 9 isolates (40.9%) grew at a wide temperature range of 10–40 °C.

Intrinsic antibiotic and heavy metal resistance

The isolates also showed variations in IAR and HMR tolerance (Table 2). Many isolates were resistant to nalidixic acid, erythromycin, and neomycin, whereas fewer isolates were resistant to streptomycin and tetracycline. Among the isolates, AAUGR-6, AAUGR-9, AAUGR-24, AAUGR-2, AAUGR-11, AAUGR-14, AAUGR-15, AAUGR-19, and AAUGR-30 grew and tolerated the majority of the tested antibiotics. The isolates were resistant to manganese (96%), lead (82%), and Zn (77%) and fairly resistant to nickel (68%) and chromium (55%), but sensitive to mercury (23%), and 40% of the isolates including AAUGR-9, AAUGR-50, AAUGR-5, AAUGR-6, AAUGR-11, AAUGR-14, AAUGR-24, and AAUGR-46 were highly tolerant to many of the tested heavy metals. Isolates AAUGR-2, AAUGR-6, AAUGR-9, AAUGR-11, AAUGR-14, and AAUGR-24 showed the highest resistance to all or most of the tested antibiotics and heavy metals.

Pattern of carbohydrate and amino acid utilization of isolates

The potential of the isolates to utilize a wide range of carbohydrates and amino acids is shown in Table 2. All isolates utilized most of the monosaccharides and disaccharides except trehalose (63.6) and Na-citrate (41%) that were utilized by fewer isolates. Isolates AAUGR-2, AAUGR-9, AAUGR-11, AAUGR-12, AAUGR-24, and AAUGR-42 utilized all the tested carbohydrates, and the other isolates catabolized 80–90% of the carbon sources, indicating the versatility of the isolate to utilize carbohydrate sources. Isolates also showed versatility (46–100%) in utilizing different amino acid sources (Table 2), and 18 % of the isolates including AAUGR-2, AAUGR-5, AAUGR-11, and AAUGR-24 catabolized all nitrogen sources. The amino acids utilized the least were leucine (77.3%) and lysine (81.8%).

Clustering analysis

The numerical analysis conducted based on 59 phenotypic features showed the grouping of the 22 isolates into three distinct clusters at 25% dissimilarity level (Fig. 2). Cluster I was further divided into two sub-clusters comprising of three isolates in sub-clusters IA (AAUGR-37, AAUGR-42,

Table 2 Tolerance to abiotic stresses and utilization to carbohydrates and amino acids

Isolates	Sampling sites	Utilization (%)		Tolerance to			IAR resistance		HM tolerance	
		CHOs	Aas	Temp. (°C)	pH	%Salt	%IAR	Antibiotics	%	HM cpds resisted
AAUGR-2	Tewa	100	100	10–40	4–10	1–7	85.7	AM, ER, CL, NA, NE, ST	100	Hg, Cr, Mn, Ni, Pb, Zn
AAUGR-3	D/Senbo	80	72.7	15–40	5–9	1–4	57.1	AM, ER, NA, NE	50	Mn, Pb, Zn
AAUGR-5	Chefebelo	93.3	100	10–40	5–10	1–7	71.4	AM, ER, NA, NE, TE	83.3	Cr, Mn, Ni, Pb, Zn
AAUGR-6	Hulagosh	93.3	81.8	10–40	4–10	1–8	100	AM, ER, CL, NA, NE, ST, TE	83.3	Cr, Mn, Ni, Pb, Zn
AAUGR-7	Yamed	60	54.5	20–35	6–9	1–5	42.9	ER, NA, NE	33.3	Mn, Pb
AAUGR-8	Ababor	53.3	45.5	20–35	6–9	1–5	57.1	ER, NA, NE, TE	33.3	Mn, Zn
AAUGR-9	Chelemie	100	81.8	10–40	4–10	1–8	100	AM, ER, CL, NA, NE, ST, TE	100	Hg, Cr, Mn, Ni, Pb, Zn
AAUGR-11	Qul/Amba	100	100	10–40	5–10	1–7	85.7	AM, ER, CL, NA, NE, TE	83.3	Cr, Mn, Ni, Pb, Zn
AAUGR-12	Degnu	66.7	72.7	20–40	6–9	1–5	57.1	ER, CL, NA, NE	33.3	Mn, Ni
AAUGR-14	Mariam	100	90.9	10–40	6–10	1–7	85.7	AM, ER, CL, NA, NE, TE	83.3	Cr, Mn, Ni, Pb, Zn
AAUGR-15	T/sertsu	93.3	90.9	10–40	5–9	1–7	85.7	AM, ER, CL, NA, NE, ST	66.7	Cr, Mn, Ni, Pb
AAUGR-16	Geshober	86.7	72.7	15–35	6–9	1–3	57.1	ER, CL, NA, TE	50	Cr, Pb, Zn
AAUGR-17	Abajale	93.3	72.7	20–35	6–9	1–4	57.1	AM, ER, NA, NE	33.3	Mn, Zn
AAUGR-19	D/ afaf	93.3	81.8	20–40	5–10	1–3	85.7	AM, ER, CL, NA, NE, TE	66.7	Mn, Ni, Pb, Zn
AAUGR-20	D/guracha	86.7	81.8	15–35	4–9	1–6	71.4	AM, ER, NA, NE, TE	66.7	Cr, Mn, Ni, Pb
AAUGR-24	Jerjero	100	100	10–40	4–10	1–8	100	AM, ER, CL, NA, NE, ST, TE	83.3	Cr, Mn, Ni, Pb, Zn
AAUGR-26	Adame	66.7	63.6	20–35	6–9	1–5	42.9	ER, NA, TE	33.3	Mn, Zn
AAUGR-30	Gobeya	93.3	72.7	10–40	6–9	1–6	85.7	AM, ER, CL, NA, NE, ST	66.7	Hg, Mn, Ni, Pb
AAUGR-37	Chireti	80	45.5	15–35	5–9	1–5	42.9	ER, CL, NA	66.7	Mn, Ni, Pb, Zn
AAUGR-42	T. bultuma	80	45.5	15–35	5–9	1–5	42.9	ER, CL, NA	66.7	Mn, Ni, Pb, Zn
AAUGR-46	A. Bolto	100	63.6	15–35	6–9	1–4	42.9	CL, NA, NE	83.3	Hg, Cr, Mn, Pb, Zn
AAUGR-50	Tulu meda	93.3	72.7	15–40	5–10	1–6	57.1	AM, ER, NA, NE	100	Hg, Cr, Mn, Ni, Pb, Zn

CHos carbohydrates, *Aas* amino acids, *IAR* intrinsic antibiotic resistance, *HM* heavy metal, *cpds* compounds, *AM* ampcillin, *ER* erythromycin, *CL* chloroamphenicol, *NA* nalidixic acid, *NE* neomycin, *ST* stryptomycin, *TE* tetracycline

and AAUGR-16) and four isolates in sub-cluster IB (AAUGR-3, AAUGR-19, AAUGR-17, and AAUGR-46). Cluster II was also further divided into two sub-clusters with four isolates consisting of AAUGR-7, AAUGR-8, and AAUGR-26 in sub-cluster IIA and AAUGR-12 in sub-cluster IIB. Cluster III was further classified into three sub-clusters comprising of eleven isolates that included isolates AAUGR-2, AAUGR-5, AAUGR-6, AAUGR-9, AAUGR-11, AAUGR-14, AAUGR-15, AAUGR-20, AAUGR-24, AAUGR-30, and AAUGR-50. Clustering decreased below 5% level of dissimilarity, indicating their phenotypic diversity. Taken together, the clusters indicated high diversity among grass pea rhizobia.

16S rRNA gene sequence analysis

Among the rhizobial isolates characterized for 16S rRNA genes, only 2 (14.3%) of the sequenced isolates AAUGR-24 and AAUGR-42 matched with *Rhizobium leguminosarum* with 100% sequence similarity, whereas the majority 12 (85.7%) were grouped with *Rhizobium* species with 99.8% similarity (Table 3).

Discussion

The conventional presumptive and authentication tests together with cultural and growth characteristics showed that 98% of the isolates were authenticated as fast-growing root nodule bacteria (Vincent 1970). Thus, according to the plant host specificity for infection and nodulation as recorded in the cool-season legume crops of tribe Viceae, the isolates were categorized as *Rhizobium leguminosarum* bv. *viciae* (Berkum et al. 1995). The findings of this study were similar to the rhizobial phenotypic features recorded from rhizobia-nodulating cross-inoculating hosts: faba bean (Mnalku et al. 2009; Belay and Assefa 2011; Legesse and Assefa 2013), field pea (Amsalu et al. 2012; Baye et al. 2015), and lentil (Jida and Assefa 2011; Rashid et al. 2012; Setargie et al. 2015).

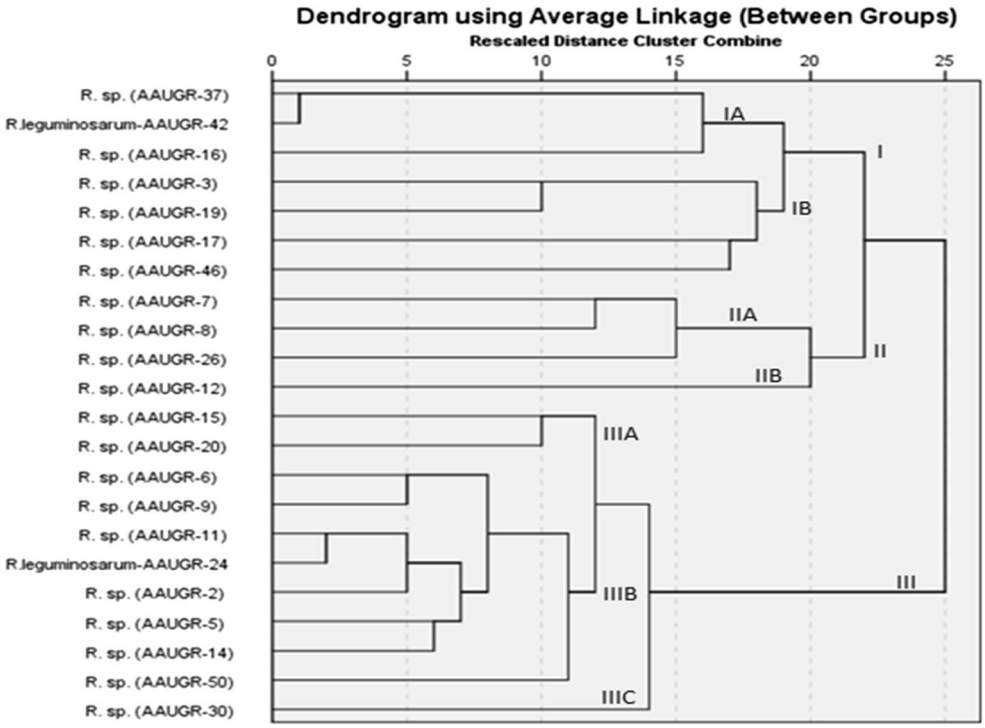

Fig. 2 Dendrogram highlighting the phenotypic diversity of grass pea rhizobial strains

The isolates induced nodules on the host plant with nodule number (NN) ranging from 17 to 116/plant and mean nodule dry weight ranging from 0.010 to 0.098 g/plant, indicating 7- and 9-fold difference between the highest and lowest nodulating isolates, respectively. Likewise, other authors that worked on cross-inoculating group rhizobia also reported similar variations ranging from 2.5 to 9 times and from 2.5 to 15 times for nodule number/plant and nodule dry weight/plant, respectively (Belay and Assefa 2011; Mnalku et al. 2009; Argaw 2012a, b; Legesse and Assefa 2013; Tsegaye et al. 2015). This indicated the existence of variations in rhizobial effectiveness which accounts to low rhizobial density, incompatibility of the rhizobia, and difference in rhizobial strain and host plant type (Vincent 1970). The host plants inoculated with AAUGR (*Rhizobium* sp. AAUGR-15, AAUGR-14, AAUGR-11, and AAUGR-6) and isolate AAUGR-5 that displayed similar pattern in NN and NDW showed high correlation between nodule dry weight and shoot dry weight (r = 0.864 and 0.817).

The difference in shoot dry matter accumulated between the highest (1.15 g/p) and the least (0.27 g/p) inoculated plants was greater than fourfold which coincided with the findings of Argaw (2012a, b) and Belay and Assefa (2011) who reported 4.5 times and 5.8 times difference between the highest and least faba bean rhizobia-inoculated plants, indicating the existence of variations in rhizobial effectiveness (Vincent 1970). This could be due to difference in agro-ecological zones of Ethiopia (Berkum et al. 1995; Wolde-Meskel 2007) and variations in rhizobial strains and plant species (or cultivars) (Neelawan 2012). Among the isolates, 86% of them were found highly effective and effective in their symbiotic effectiveness similar to other reports in which authors reported 55–100% effective and highly effective rhizobial isolates compared with the positive control (Mnalku et al. 2009; Argaw 2012a, b; Legesse and Assefa 2013; Tsegaye et al. 2015). The correlation between shoot dry weight and percent symbiotic effectiveness showed a relatively more direct and perfect linear relationship than the other parameters (r = 0.988), suggesting that shoot dry matter is a good indicator of relative effectiveness revealing sound correlation between the nitrogen-fixing capacity of legumes and their shoot dry matter accumulation (Mulongoy 2004). Among the isolates, AAUGR-15, AAUGR-14, AAUGR-11, AAUGR-9, AAUGR-6, and AAUGR-5 promoted grass pea growth under greenhouse conditions, indicating their potential for application at field conditions.

Grass pea rhizobia showed variations in the tested physiological parameters. All isolates were tolerant to 6–9, whereas 5 (23 %) grew at pH 4 and 9 (40 %) grew on the medium adjusted at pH 10, indicating that the isolates were more tolerant to alkaline than acidic pH. Lebrazi and Benbrahim (2014) reported that rhizobia can grow at high pH since alkalinity is less harmful to the survival of rhizobia. Most isolates grew at higher salt concentrations similar to rhizobial isolates from *Vicia faba*, *Lens culinaris*, and *Pisum sativum* (Mohammad et al. 1991; Zahran et al. 2003; Keneni et al. 2010; Legesse and Assefa 2013; Setargie et al. 2015) showing that fast-growing rhizobia are more salt-tolerant than slow-growing rhizobia (Zahran 1999) and could be used as a remedy in areas where 40% of the world's land surface is

Table 3 16S rRNA gene partial sequencing of selected *Rhizobium* strains

Isolate code	Target organism	Closest gene bank similarity	Best match ID. (NCBI)	Query cover (%)	Identity(%)
R-2	*Rhizobium* species	*Rhizobium* sp. strainM3P42AN	MN597993.1	100	99.8
R-6	*Rhizobium* species	*Rhizobium* sp. strain M3P38N2	MN597991.1	100	99.8
R-9	*Rhizobium* species	*Rhizobium* sp. strain BOP20N3	MN597982.1	100	99.8
R-11	*Rhizobium* species	*Rhizobium* sp. strainBOP1N2	MN597975.1	100	99.8
R-14	*Rhizobium* species	*Rhizobium* sp. strainLS1.11	MN429014.1	100	99.8
R-19	*Rhizobium* species	*Rhizobium* sp. strain RhOF57A	MN006388.1	100	99.8
R-20	*Rhizobium* species	*Rhizobium* sp. Am4-4	AY210707.1	100	99.8
R-24	*Rhizobium leguminosarum*	*Rhizobium leguminosarum* strain KsHCa3	MK809177.1	100	99.8
R-30	*Rhizobium* species	*Rhizobium* sp. STM 4O44	EF152481.1	100	99.8
R-37	*Rhizobium* species	*Rhizobium* sp. FB2405	JN558656.1	100	99.8
R-38	*Rhizobium* species	*Rhizobium* sp. LDj5	KM999134.1	100	99.8
R-40	*Rhizobium* species	*Rhizobium* sp. L17	KT387836.1	100	99.8
R-42	*Rhizobium leguminosarum*	*Rhizobium leguminosarum* bv. *viciae* strain GH	DQ660318.1	100	100
R-49	*Rhizobium* species	*Rhizobium* sp. strain EC3	MF624033.1	100	99.8

categorized as having potential salinity problems (Zahran 1999). The isolates of this study showed growth at a temperature range of 10–45 °C that was similar to the findings of Workalemahu (2009) and Legesse and Assefa (2013) who reported faba bean rhizobial isolates that showed growth at similar temperature range, implying that rhizobia are tolerant to heat (Michiels et al. 1994) and tolerant to freezing temperatures as low as – 10 °C (Cloutier et al. 1992). Among the isolates of this study, AAUGPR-6, AAUGPR-9, AAUGPR-11, AAUGPR-14, and AAUGPR-24 grew better in the tested tolerance parameters which could be considered potential candidates for use as inoculants in areas where pH, salinity, and temperature are persistent problems.

The isolates of this study showed variations in their tolerance and sensitivity to the tested antibiotics (Keneni et al. 2010; Zahran et al. 2012; Hewedy et al. 2014; Abera et al. 2010) with a pattern of ST > TE > CL > AM > NE > ER > NA which is adopted as a useful complementary tool for characterization and discrimination of rhizobial isolates (Zahran et al. 2012). Similarly, the isolates showed variability in heavy metal tolerance (Jida and Assefa 2011; Baye et al. 2015) with sensitivity pattern of $HgCl_2$ > $K_2Cr_2O_7$ > $NiCl_2$ > $ZnCl_2$ > $Pb(CH_3COO)_2$ > $MnCl_2$. Most of the isolates of this study showed the ability to grow at high metal concentrations which is also found in many rhizospheric microorganisms (Lakzian et al. 2002) and may be the result of intrinsic or induced mechanisms (Giller et al. 1998). It can be concluded that no significant difference was observed in the ability of isolates to tolerate antibiotics and heavy metals. Isolates AAUGR-2, AAUGR-6, AAUGR-9, AAUGR-11, AAUGR-14, and AAUGR-24 showed the highest resistance both to the tested antibiotics and heavy metals, indicating correlation between metal and antibiotic tolerance in bacteria because of the likelihood that resistance genes to both antibiotics and heavy metals may be located closely together on the same plasmid in bacteria and are thus more likely to be transferred together in the environment (Elizabeth et al. 2012).

Grass pea rhizobia showed wide range of utilization to the tested carbohydrate and nitrogen sources as similarly reported by Maâtallah et al. 2002; Wielbo et al. 2012; Zahran et al. 2012; Legesse and Assefa 2013; Tena et al. 2016 which may be advantageous for survival in soil (Gauri et al. 2011) and be used as a marker for rhizobial classification (Zabaloy and Gomez 2005). The types of carbohydrates tested in this study showed variations in their degree of utilization by the isolates monosaccharides > disaccharides > polysaccharides similar with the findings of Jida and Assefa 2011; Zahran et al. 2012 reported a broad range carbohydrate utilization ability of fast-growing rhizobial isolates. Variability among the isolates in utilizing the amino acid sources was observed, indicating that utilization of amino acids is one of the phenotypic characteristics for possible taxonomic diversity of the study isolates (Zahran et al. 2012. The inconsistency observed in substrate utilization among rhizobial isolates may have ecological relevance which could be important to live as a saprophyte in soil or colonize plant roots and live symbiotically (Charabarti et al. 1981).

The ecological stresses and adaptations to nutritional utilization tests conducted showed that isolates AAUGR-2, AAUGR-5, AAUGR-6, AAUGR-9, AAUGR-11, AAUGR-14, AAUGR-15, AAUGR-24, and AAUGR-50 were the most tolerant and considered potential candidates to relieve environmental stresses including soil pH, salinity, and temperature that affect the survival, metabolism, and functioning of rhizobia (Abdullah 2002), which in turn limit crop productivity through affecting legume-rhizobia symbiosis (Andres et al.

2012). Consequently, isolating and screening rhizobial inoculants that are compatible to legume host and the target edaphic environment is important since rhizobial populations are known to vary in their tolerance to major environmental factors (Wei et al. 2008). These isolates that exhibited physiological versatility in all the tested physiological assays could be used as an important candidates of microbial inoculants in competitive and complex soil environments (Somasagaren and Hoben 1985).

In this study, isolates AAUGR-2, AAUGR-6, AAUGR-9, AAUGR-11, and AAUGR-24 showed better performances in the tested parameters which were followed by isolates AAUGR-5, AAUGR-14, AAUGR-15, AAUGR-19, AAUGR-20, AAUGR-30, and AAUGR-50 and were regarded as an important inoculants at soil where fluctuations in pH, nutrient availability, temperature, and salinity among other factors greatly influence the growth, survival, and metabolic activity of nitrogen-fixing bacteria and plants and their ability to enter into symbiotic interactions (Werner and Newton 2005). Screening isolates on the basis of their stress tolerance level in culture media is essential for the selection of microbial inoculants for field applications (Fitouri et al. 2012) to improve symbiotic efficiency and crop productivity in agricultural systems (Broughton et al. 2003). Isolates AAUGR-2, AAUGR-6, AAUGR-9, AAUGR-11, AAUGR-14, AAUGR-20, and AAUGR-30 showed similar diversity in both phenotypic functional traits and 16S rRNA sequence analysis, whereas the remaining isolates failed to show similarity which may be due to activity inconsistency at in vitro conditions.

The 16S rRNA gene sequencing result showed that out of the 14 rhizobial isolates, 12 (85.7%) of them were found to be closely related to *Rhizobium* species with 99.8% identity and 2 (14.3%) isolates to *Rhizobium leguminosarum* with100% identity which corroborated with the findings of (Berrada et al. 2012) who reported the identification of 2 *Rhizobium leguminosarum* and 12 *Rhizobium* species from bean plants with 98–100% identity. Rivas et al. (2009) reported *Rhizobium leguminosarum* bv. *viciae* is the specific microsymbiont of the legumes of the tribe Vicieae, which comprises the genera *Vicia*, *Pisum*, *Lens*, and *Lathyrus*. On the other hand, Aoki et al. (2010) reported 97–100% sequence identity of 16S rRNA gene of rhizobia isolated from *Lathyrus japonicus* nodules with 16S rRNA gene sequences of *Rhizobium* species, *Rhizobium phaseoli*, *Rhizobium pisi*, *Rhizobium multihospitum*, *Rhizobium tropici*, and *Rhizobium radiobacter* which is nearly similar with the findings of this study. Moreover, Drouin et al. (1996) reported 100% sequence identity of two *Lathyrus japonicus* and *Lathyrus pratensis* 16S rRNA gene with *Rhizobium* species, *Rhizobium etli*, *Rhizobium meliloti*, and *Rhizobium leguminosarum* bv. phaseoli. Similarly, Tena et al. (2017) also reported genotypically diverse rhizobia-nodulating lentil (*Lens culinaris* Medik.), implying that the phylogenetic positions of grass pea–nodulating rhizobia to *Rhizobium* species was not unexpected. Further, molecular identification using housekeeping genes such as *recA*, *glnII*, *atpD*, and *ropB* and other symbiotic genes needs to be conducted for these 12 *Rhizobium* species to specify as *Rhizobium leguminosarum* which are more informative for phylogenetic analysis and identification of closely related species than 16S rRNA (Rashid et al. 2012).

In a nutshell, the result of this study confirmed that grass pea rhizobia showed the potential of tolerating a wide range of abiotic stresses, broad range of nutrient versatility, and nitrogen fixation. This enhanced the screening of elite rhizobial strains having better symbiotic and ecophysiological potential that could help tolerate abiotic stresses, increase soil fertility, and thereby improve the growth and yield of rhizobia-associated crop plants. Some rhizobial isolates including AAUGR-5, AAUGR-6, AAUGR-9, AAUGR-11, and AAUGR-14 exhibited consistent performance in all the tested parameters indicating their effective and competitive ability. The 16S rRNA gene sequence analysis conducted on some selected rhizobial isolates showed the identification of *Rhizobium* species and *Rhizobium leguminosarum* with 99.8–99% and 100% identity, respectively. In general, the rhizobial strains AAUGR-6, AAUGR-9, AAUGR-11, AAUGR-14, and AAUGR-24 that were identified as *Rhizobium* species and *Rhizobium leguminosarum* and ranked top in all the tested symbiotic and stress tolerance parameters were recommended as microbial inoculants for field applications.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest to this work.

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