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Phenotypic, Host Range and Symbiotic Characteristics of Indigenous Soybean Nodulating Rhizobia from Ethiopian Soils

Yifru Abera[1], Cargele Masso[2] and Fassil Assefa[3]

[1]*Ethiopian Institute of Agricultural Research, Debrezeit Agricultural Research Center, Debrezeit, Ethiopia,*
[2]*Natural Resource Management, International Institute of Tropical Agriculrture (IITA), Nairobi, Kenya,*
[3] *Department of Cellular, Microbial and Molecular Biology, Addis Ababa University, Addis Ababa, Ethiopia*

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አኩሪ አተር ከተለያዩ የራይዞብያ ዝርያዎች ጋር ርክበዛካላዊ ጥምረት በመፍጠር በስባቢ አየር ውስጥ የሚገኘውን የናይትሮጂን ውህድ ህይወት ላላቸው ነገሮች ተስማሚ ወደሆነው የንጥረ ነገር ይዘት በመቀየርና በመጠቀም እንዲሁም የአፈርን ለምነት በማሻሻል ከሚታወቁ የጥራጥሬ ሰብሎች አንዱ ነው:: ሆኖም አኩሪ አተር በአንፃራዊነት ለኢትዮጵያ አዲስ ሰብል እንደመሆኑ የራሱ የሆነ የራይዞቢያ ዝርያ በአፈር ውስጥ ላይኖር ይችላል ተብሎ ቢገመትም ለአንዳንድ ተዛማጅ የጥራጥሬ ሰብሎች (ለምሳሌ የላም አተር) ተስማሚ ከሆኑ የራይዞብያ ዝርያዎች ጋር ውጤታማ ርክበዛካላዊ ጥምረት መፍጠር ይችላል:: የዚህ ጥናት ዋና ዓላማም ከሀገራችን የአፈር ፀባይና አየር ንብረት ጋር የተላመዱ ከተዘማጅ ጥራጥሬ ሰብሎች ጋር የቀድሞ ጥምረት ካላቸው ራይዞቢያዎች ውስጥ ለአኩሪ አተር የሚስማሙ ሀገር በቀል የሆኑ የራይዞብያ ዝርያዎች ለማግኘት ነው:: ይህንን ጥናት ለማካሄድ ከሀገራችን የተለያዩ አኩሪ አተር አብቃይ አካባቢዎች አኩሪ አተር ያለ ህያው ማዳበሪያ ከሚበቅልባቸው ቦታዎች ወካይ የአፈር ናሙናዎች በመሰብሰብ፤ በናሙናዎቹም ላይ ሁለት የአኩሪ አተር ዝርያዎች (አዋሳ-95 እና ክላርክ-63K) እና አንድ የላም አተር ዝርያ (Bole) በግሪንሀውስ ውስጥ በማሳደግ 67 ራይዞብያዎችን በማጥመድ ልዩነታቸውና ውጤታማነታቸው ተፈትሿል:: በጥናቱም መሰረት አብዛኞቹ (93 በመቶ) ዘገምተኛ እድገት ያላቸው የ 'Bradyrhizobium' ዝርያዎች እንደሆኑ የተቀሩት (7 በመቶ) ደግሞ ፈጣን እድገት ያላቸው ራይዞብያዎች መሆናቸው ታውቋል:: ከዚህም በተጨማሪ በላቦራቶሪ በተደረገው ጥናት መሰረት የተገኙት ራይዞብያዎች የተለያዩ ካርበኖችና የናይትሮጂን ምንጮችን እንደምግብነት በመጠቀም፣ አሲዳማነትንና ጨዋማነትን በመቋቋም፣ እንዲሁም በተፈጥሮ የተለያዩ ፀረ-ባክቴሪያዎችንና ክቡድ ብረታ ብረቶችን በመቋቋም አቅማቸው እና ሙቀትን በመቋቋም አቅማቸው የተለያዩ መሆናቸው በጥናቱ ተረጋግጧል:: ሁሉም የተገኙት ራይዞብያዎች ክላርክ-63K ከተባለዉ የአኩሪ አተር ዝርያ ጋር በተለያየ የውጠ ጋታማነት ደረጃ ርክበዛካላዊ ጥምረት መፍጠር እንደሚችሉም ጥናቱ አሳይቷል:: ከእነዚህም ውስጥ 12 በመቶ የሚሆኑት በጣም ውጤታማ ጥምረት ፈጣሪዎች ሲሆኑ የተቀሩት 88 በመቶ የሚሆኑት በመካከለኛ ደረጃ ውጤታማ ጥምረት ፈጣሪዎች ሆነው ተገኝተዋል:: በተመረጡ 15 የሚሆኑ ራይዞብያዎች ላይ የተደረገው ተጨማሪ የግሪንሀውስ ጥናት እንደሚያመለክተው አስራ ሁለቱ (80 በመቶዎቹ) ከሶስት የተለያዩ ተዛማጅ ጥራጥሬ ሰብሎች (ማሾ፣የርግብ አተርና የላም አተር) ጋር በተለያየ የውጤታማነት ደረጃ፤ ከለውዝ ጋር ደግሞ አንዱ ብቻ ርክበዛካላዊ ጥምረት የሚፈጥሩ መሆናቸው ጥናቱ አረጋግጧል::

Abstract

Soybean is an exotic crop to Ethiopia and may not necessarily have a specific endosymbiont in the soil. However, since it is a promiscuous host, nodulated by cross nodulating rhizobia, it is likely that some compatible endosymbionts exist from heterologous hosts that could nodulate it with effective nitrogen fixation. This necessitated the search for effective indigenous rhizobia isolates and/or compatible and effective cross-inoculating rhizobia that are already adapted to local conditions. To this end, a total of 67 bacterial isolates were trapped from different soil samples using two soybean varieties (Clark-63K and Awassa-95) and one cowpea variety (Bole), to evaluate their diversity and screen for their symbiotic effectiveness. Accordingly, the majority of isolates (93%) were tentatively categorized into alkali producing slow growing Bradyrhiobium spp. and the others (7%) were fast growing and acid producing rhizobia. The isolates showed differences in utilizing various carbon and nitrogen sources and tolerance to acidity, salinity and temperature. The isolates were also diverse in their inherent antibiotic and heavy metal resistance. All the isolates were able to nodulate soybean variety Clark-63K with significant difference in their capacity to infect and effectively fix nitrogen evidenced from variations in nodulation parameters and shoot dry weights. Accordingly, the isolates induced nodulation with nodule number ranging from 2 to 49 nodules plant^{-1}; nodule dry weight of 16 mg plant^{-1} to 94 mg plant^{-1} and shoot dry weight between 585 and 1012 mg plant^{-1}. Using shoot dry weight as an indicator of the relative effectiveness of the isolates, 12% of the isolates were highly effective (SE $\geq$ 80%) and 88% were effective (SE from 50 to 80%) on soybean. Furthermore, the isolates showed narrow and broad host ranges on four legume species viz., cowpea, mung bean, pigeon pea, and peanut. Accordingly, many isolates (67%) formed nodules with effective nitrogen fixation with cowpea (Vigna unguiculata), pigeon pea (Cajanus cajan) (47%), and on few cases with mung bean (Vigna radiata) showing different level of effectiveness. However, the data showed very narrow host range on peanut (Arachis hypogaea) where, only one isolate formed effective nodules.

Introduction

Soybean [*Glycine max* (L.)] is one of the grain legumes that can fix atmospheric nitrogen in symbiosis with diverse root nodule bacteria belonging to different genera and species. Most of soybean nodulating rhizobia are slow growing *Bradyrhizobium* species: *B. japonicum, B. elkanii, B. liaoningense* and *B. yuanmingense* (Biate *et al.,* 2014). The other symbionts include fast growers classified into *Sinorhizobium fredii* and *Sinorhizobium xinjiangense* while moderately slow-growing rhizobia belong to *Mesorhizobium tianshanense* (Man *et al.,* 2008). However, other slow- and fast-growing rhizobia genetically distinct from those mentioned above were also reported to nodulate soybean (Appunu *et al*. 2008).

It is reported that soils outside the center of origin and domestication of soybean harbor indigenous rhizobia that promiscuously nodulate soybean cultivars with high symbiotic effectiveness in different African countries (Klogo *et al.,* 2015;

Gyogluu *et al*., 2016) suggesting that effective, competitive and locally adapted strains can be selected for use as inoculants for soybean.

In Ethiopia, some studies indicated that indigenous rhizobia are capable of establishing symbiosis with different soybean cultivars (Aregu *et al.,* 2012, Jaiswal *et al*., 2016, Diriba, 2017). According to Aregu *et al.* (2012) soybean might be either promiscuously nodulated by indigenous symbionts of other heterologous legume hosts, or by local rhizobia specific to soybean that are found in the Ethiopian soils. The authors observed the close taxonomic similarities of the endosymbionts of soybean with slow growing *Bradyrhizobium* spp isolated from the two legumes; *Crotalaria incana* and *Indigofera arrecta.* Diriba (2017) also isolated and characterized both fast and slow growing rhizobia that effectively nodulate the host implying that native legumes could be a source of novel ecologically adapted symbiotically effective rhizobia for the recently introduced soybean crop in Ethiopia.

However, there is a dearth of information about the distribution of rhizobia nodulating soybean from a wider area, and their host range with other cross inoculation hosts. Identification of effective locally adapted strains with wide host ranges could be useful in the development of inoculant strains which can survive longer in agricultural soils and hence reduce the need for inoculant application every growing season (Musiyiwa *et al*., 2005).

In this study, rhizobia nodulating soybean were isolated from major prospective soybean growing areas of Ethiopia to evaluate their phenotypic, host range and symbiotic characters under laboratory and greenhouse conditions so as to determine their potenitial for future inoculant production.

Materials and Methods

Collection of soil samples and Isolation of rhizobia

Fifty-nine geo-referenced soil samples at a depth of 0-20 cm were randomly collected from soybean fields from different agro-ecology of Oromia, Benishangul-Gumuz and Southern Nations, Nationalities and Peoples' Region (SNNPR) regional states of Ethiopia (figure 1). The collection sites are located within an elevational range of 1071 to 1854 meters and were extremely acidic to neutral soils ranging from pH 4.2 to 6.9. The soils were used to trap rhizobia by planting seeds of two soybean varieties, Awassa-95 and Clark-63K, and one cowpea variety, Bole, in the greenhouse for 45 days following standard methods (Somasegaran and Hoben, 1994). The seeds of soybean varieties were obtained from Pawe Agricultural Research Center and cowpea seed was obtained from Melkassa research center

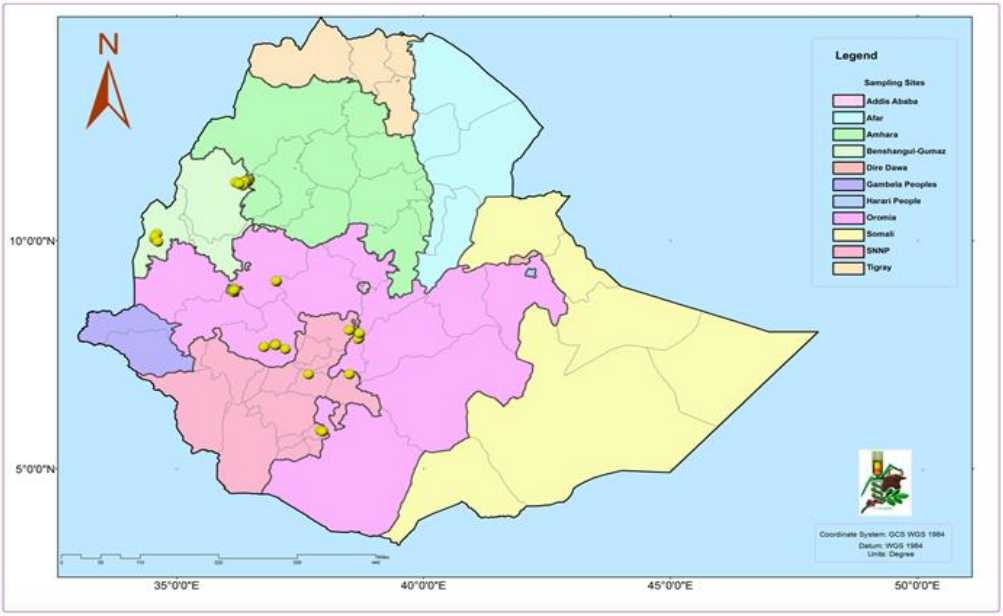

Figure1. Geographic distribution of soil sampling sites (dots indicate sampling points)

After 45 days of planting, nodules were collected, sterilized using 70% ethanol and 3% sodium hypochlorite, crushed, suspended in water, and streaked on yeast extract mannitol agar (YEMA) medium (Somasegaran and Hoben, 1994) and incubated at 28°C for 7-10 days. YEMA contained 0.5 g K_2HPO_4; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.1 g NaCl; 0.5 g yeast extract; 10 g mannitol; 15 g agar; per liter. Single colonies were picked up and re-streaked on YEMA medium to obtain pure colonies, gram stained, and preserved on YEMA medium slants containing 0.3 % (w/v) $CaCO_3$ at 4°C for further studies.

Growth and cultural characteristics

Isolates were grown on YEMA for 3-7 days to study their cultural characteristics (colony size and texture), and on YEMA containing 25 µg ml^{-1} BTB for acid/base reaction (Jordan, 1984). Growth rate was determined by inoculating 1 ml of active culture (24 h old cells) into 100 ml sterilized YEM broth in 250 ml Erlenmeyer flask and grown on orbital shaker at 150 rpm at room temperature for 72 hours. Samples were taken just at the time of inoculation (0hr) and every 6 hours interval to measure optical density (OD^{540}; Jenway 6405 UV/Visible spectrophotometer) and to determine viable colony forming units (cfu) on YEMA. Finally, mean generation time was calculated from the exponential phase of the growth curve (Somasegaran and Hoben, 1994).

Eco-physiological and biochemical characteristics

All the following tests were carried out in triplicates on YEMA plates divided into 10 equal squares of which each square was spot-inoculated with10 µl of the culture previously grown on YEM broth to exponential phase (10^8 cells ml^{-1}). The ability of isolates to grow on acidic or basic media was determined on YEM agar plates adjusted to pH 4, 5, 6.5, 8 and 9.5 (Hungria *et al*., 2001) by using 1 N HCl or NaOH. They were also inoculated to YEMA solid medium supplemented with NaCl at concentrations of 0.1, 0.5, 0.8, 1, 2, 3, and 4% (w/v) and incubated at 30, 37, 40 and 45°C to evaluate their salt and temperature tolerance, respectively (Youseif *et al*., 2014).

The ability of isolates to utilize different carbon sources was determined by growing them on a basal medium containing different carbohydrates at 1% (w/v): D-arabinose, D-mannose, Raffinose, D-galactose, maltose, xylose, Na-citrate, trehalose, cellobiose, dextrin, glucose, sucrose, fructose, and lactose. The basal medium contained; (g/L): K_2HPO_4, 1; KH_2PO_4, 1; $FeCl_3.6H_2O$, 0.01; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.1; $(NH_4)_2SO_4$, 1; and 15 g of agar. Heat labile carbohydrate sources (D-arabinose, D-mannose, Raffinose, D-galactose, maltose, xylose, Na-citrate, trehalose and cellobiose) were sterilized by membrane filtration using millipore with pore size of 0.22 micron and added to the autoclaved carbohydrate free basal medium. The heat-stable carbohydrates (dextrin, glucose, sucrose, fructose and lactose) were autoclaved together with the medium. To test N utilization, isolates were grown on the same medium by adding 0.5 g/l of L-lysine, L-asperginine, L-cystine, L-leucine, L-phenylalanine, L-Methionine and L-Glutamate after replacing ammonium sulfate by 1 g/liter of mannitol (Amarger *et al*., 1997).

The intrinsic antibiotic and heavy metal resistance of isolates was determined on solid YEMA medium containing the following filter-sterilized antibiotics and heavy metals in µg ml^{-1}: kanamycin (100), streptomycin (100), chloramphenicol (100), gentamycin (250), nalidixic acid (50 µg/ml), erythromycin (100), ampicillin (50) and tetracycline (20); $ZnCl_2$ (50), $CoCl_2$ (25), $AlCl_3$ (250), $CuCl_2$ (50), $MnCl_2$ (500) and $FeCl_3.6H_2O$ (750). Growth was recorded as (+) for presence of growth and (-) for no growth after incubation at 28°C for 10 days.

Authentication and preliminary screening of isolates for symbiotic effectiveness

This test was conducted on sand culture at Debre Zeit Agricultural Research Center greenhouse. Seeds of soybean variety Clark-63K were surface-sterilized by immersion in 70% ethanol for 1 minute, followed by treatment with 3% sodium hypochlorite solution for 3 min and washed thoroughly six times with sterilized distilled water. Seeds were germinated on 1% (w/v) water-agar at 28°C and transplanted after three days into surface sterilized 3-kg pots filled with acid

washed and sterilized sand (Somasegaran and Hoben, 1994). The seedlings were allowed to grow for 3 days in the greenhouse and later thinned down to three before inoculation with a specific rhizobial isolate. For inoculation, each seedling received 1.0 ml culture of the appropriate rhizobial isolate (approximately 10^9 cells) which had been grown on YEM broth for 7 days at 28°C. Treatments without inoculation, inoculated with reference strain (MAR 1495) and a +N treatment at a rate of 70 µg N ml^{-1} applied as KNO_3 solution once a week were included as controls.

Each treatment was replicated trice and pots were arranged in a completely randomized design in the greenhouse. The plants were irrigated weekly with N-free nutrient solution prepared according to Broughton and Dilworth (1971) cited in Somasegaran and Hoben (1994) and with sterilized distilled water as necessary. Plants were harvested after 45 days of planting to determine number of nodules (NN), nodule dry weight (NDW) and dry weight of shoots (SDW). The relative efficiency (RE) of each isolate was calculated using the methods of Purcino *et al*. (2000). Accordingly, RE = (inoculated SDW/SDW with N) x 100, where inoculated SDW is the dry weight of shoots after inoculation with the respective isolates, and SDW with N is the dry weight of shoots in the treatment that received KNO_3. RE values of the isolates were rated as highly effective (HE) >80%; effective (E), 50-80%; slightly effective (SE), 35-50%; or ineffective (I) <35%.

Host range of soybean rhizobia isolates

Fifteen randomly selected isolates, authenticated on soybean, were tested for their ability to nodulate four legumes that were reported to form symbiosis with the genus *Bradyrhizobium* viz., *Arachis hypogaea* (peanut), *Cajanus cajan* (pigeon pea), *Vigna radiata* (mung bean) and *Vigna unguiculata* (cowpea). Seeds of each crops were surface sterilized, pre-germinated and seedlings were transplanted aseptically into surface sterilized pots as before. Similarly, all greenhouse activities including experimental design, number of replications, thinning, inoculation, watering and N-free nutrient application were as described above for authentication and preliminary screening of soybean rhizobia. Treatments without inoculation and a +N treatment at a rate of 70 µg N ml^{-1} applied as KNO_3 solution once a week were included as controls for each legume species. Plants were grown for 45 days in the greenhouse and harvested for determination of nodule number, nodule dry weight and shoot dry weight. The relative efficiency (RE) of each isolate was calculated as described before using the methods of Purcino *et al*. (2000).

Data analysis

Analysis of variance (ANOVA) for comparisons between the treatments for the recorded parameters was conducted using GenStat Version 17. Mean separations was undertaken using the Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$ probability level.

Results and Discussion

Isolation, morphological and cultural characteristics of the bacterial isolates

In this study, 100% and 80% of the soil samples from different sampling sites induced nodulation on one cowpea and two soybean varieties, respectively; indicating that the soils harbor compatible nodulating rhizobia for both crops. This may also indicate that rhizobia may originally evolve from cowpeas that cross nodulate the recently introduced soybean crop. A total of 67 rhizobial isolates were collected of which 47 isolates (70%) were obtained using; Awassa-95 and Clark-63K soybean varieties as trap hosts while the remaining 20 isolates were obtained from cowpea variety 'Bole' as a trap host.

The result showed that soybean nodulating rhizobia might have been widely distributed than previously thought by Diriba (2017) who reported that only 13% of the soil samples collected from different parts of Ethiopia harbored root nodule bacteria nodulating soybean. This variation could be due to differences in soybean genotypes used for trapping, cropping history, and land use of sampling areas.

The isolates showed variations in colony size (0.5 to 5.0 mm in diameter), generation time of 3.0–13.6 h, and the majority of the isolates except As-7-Bo, Jm-9-Cl, Jm-5-Aw, As-5-Bo and Pw-5-Aw changed the color of YEMA to blue indicating that they were alkali producers (Table 1). Accordingly, a number of the isolates displayed slow growth (generation time of 6.3-13.6 hour) with smaller colony size (0.5 to 2.0 mm diameter). Other indigenous soybean rhizobial isolates displayed fast growth with generation time of 3.0 to 4.2 hours and colony size ranging from 2.0-5.0 mm. The majority of the isolates showed watery to milky translucent texture (80% of the isolates) and few (20%) were opaque (Table 1).

According to Amarger (2001), rhizobial strains having generation time below 6 h are called fast growers, while those with doubling times more than 6 h were designated as slow growers. Jordan (1984) showed that alkali production is a characteristic of mainly slow growing rhizobia. Thus, sixty-two isolates (93% of the isolates) were characterized by alkaline reaction and slow growth (colonies visible only after 5 days and generation time 6.3-13.6) similar to the growth rate of slow growing, *Bradyrhizobium*, soybean rhizobial isolates (Appunu *et al*., 2008; Singh *et al*., 2013). The 5 isolates (7%) (Jm-9-Cl, As-7-Bo, As-5-Bo, Jm-5-Aw and

Pw-5-Aw) showed an acid reaction in YEMA and were fast growers with visible growth after 3 days and displayed generation time of 3.0 to 4.2 hours, similar to the generation time of fast growers with generation time of 1.4 to 4 h from Brazil (Hungria *et al*. 2001) and from Ethiopia (Diriba, 2017).

Although the fast growers tend to have larger colony size (≥2 mm), some isolates exhibited different colony size irrespective of their growth rate. Similarly, Chen *et al* (2002) in Paraguay reported that local soybean isolates formed different colony size regardless of their growth rate and taxonomic grouping.

Table 1. Growth and colony characteristics of some indigenous soybean rhizobial isolates grown on YEMA, YEMA-BTB medium at 28±2°C for 3-7 days from Ethiopia

Isolate	Isolation region	Trap host	Colony size (mm)	Colony texture	MGT	Acid/Base reaction	Group
Jm-1-Bo	Oromia	Cowpea	1.0	Watery translucent	8.1	Blue	SG
Jm-7-Cl	Oromia	Soybean	2.0	White opaque	6.8	Blue	SG
Jm-8-Cl	Oromia	Soybean	1.0	Milky translucent	8.2	Blue	SG
Jm-9-Cl	Oromia	Soybean	4.0	White opaque	3.4	Yellow	FG
Jm-4-Cl	Oromia	Soybean	1.0	Milky translucent	6.6	Blue	SG
Jm-5-Bo	Oromia	Cowpea	1.0	Watery translucent	10.8	Blue	SG
Jm-5-Aw	Oromia	Soybean	2.0	Opaque	4.0	Yellow	FG
Bk-2-Aw	Oromia	Soybean	1.0	Watery translucent	8.4	Blue	SG
Bk-3-Aw	Oromia	Soybean	1.0	Watery translucent	8.0	Blue	SG
Cw-2-Cl	Oromia	Soybean	1.0	Watery translucent	12.4	Blue	SG
Cw-3-Aw	Oromia	Soybean	0.5	Milky translucent	8.5	Blue	SG
Cw-6-Aw	Oromia	Soybean	0.5	White opaque	11.2	Blue	SG
AD-1-Bo	Oromia	Cowpea	1.0	Watery translucent	11.4	Blue	SG
AD-2-Aw	Oromia	Soybean	0.5	Watery translucent	10.7	Blue	SG
As-1-Aw	B/Gumuz	Soybean	0.5	Milky translucent	13.2	Blue	SG
As-5-Bo	B/Gumuz	Cowpea	5.0	Watery translucent	3.0	Yellow	FG
As-5-Aw	B/Gumuz	Soybean	0.5	Watery translucent	7.4	Blue	SG
As-6-Bo	B/Gumuz	Cowpea	2.0	Milky translucent	7.5	Blue	SG
As-6-Aw	B/Gumuz	Soybean	1.0	White opaque	7.2	Blue	SG
As-7-Cl	B/Gumuz	Soybean	2.0	Milky translucent	7.4	Blue	SG
As-7-Bo	B/Gumuz	Cowpea	2.0	Milky translucent	3.2	Yellow	FG
Pw-2-Aw	B/Gumuz	Soybean	1.0	Watery translucent	6.8	Blue	SG
Pw-3-Cl	B/Gumuz	Soybean	2.0	Milky translucent	6.6	Blue	SG
Pw-5-Aw	B/Gumuz	Soybean	2.0	Milky translucent	4.2	Yellow	FG
Pw-11-Aw	B/Gumuz	Soybean	2.0	Milky translucent	7.2	Blue	SG
Aw-1-Aw	SNNPR	Soybean	0.5	Watery translucent	13.2	Blue	SG
Aw-2-Aw	SNNPR	Soybean	0.5	Milky translucent	10.6	Blue	SG
Aw-6-Cl	SNNPR	Soybean	2.0	Milky translucent	10.8	Blue	SG

SG: Slow Growing, FG: Fast Growing, SNNPR: Southern Nations Nationalities and Peoples' Region, MGT: Mean generation time (growth rate)

Most of the previous studies on soybean rhizobia in Ethiopia (Aregu *et al*., 2012; Gyogluu *et al*. 2014; Jaiswal *et al*. 2016) and other African countries (Gyogluu *et al*. 2014; Herrmann *et al*., 2014; Chibeba *et al*., 2017) showed the dominance of slow growers over fast growers. However, Diriba (2017) obtained an equal proportion of fast and slow growing soybean nodulating rhizobia in Ethiopian soils. Youseif *et al*. (2014) from Egypt on the other hand reported the dominance of fast growing soybean nodulating rhizobia (75%) over slow growing (25%).

The results of the present study also indicated that slow growers are the dominant rhizobia population nodulating soybean in Ethiopian soils which tend to be acidic or neutral, corrborating the findings of some authors who argued that *Bradyrhizobium* spp are dominant in neutral to acidic soil (Li *et al*. 2011; Zhang *et al*. 2011) while in contradictory to that of Diriba (2017). Since the isolates were obtained from soils without any history of inoculation, the isolates represented the indigenous soybean rhizobia that are adapted to the local environmental conditions.

Authentication and symbiotic effectiveness of the isolates on sand culture

All isolates were able to nodulate soybean variety Clark-63K with variations in their symbiotic effectiveness. Accordingly, the mean nodule number of the inoculated plants ranged from 2 nodules plant^{-1} for isolates AD-1-Bo to 49 nodules plant^{-1} for isolate Pw-11-Cl with mean of 26 nodules plant^{-1} (Table 2).

The isolates also showed differences in nodule dry weight with lowest and highest of 16 mg plant^{-1} and 94 mg plant^{-1} **(**Mean = 61 mg plant^{-1}) for isolates AD-1-Bo and Pw-2-Aw, respectively. Eight isolates (Pw-11-Cl, Cw-1-Cl, As-6-Bo, As-4-Bo, As-7-Aw, As-7-Cl, Cw-3-Aw and Pw-8-cl) displayed significantly higher ($P < 0.05$) nodule number than the commercial reference strain, MAR 1495. On the other hand, a number of the indigenous isolates (63%) produced significantly higher ($P < 0.05$) nodule dry weight than MAR 1495 (Table 2).

The inoculated plants also showed variations in shoot dry weight within the range of 585 and 1012 mg plant^{-1} for isolates Bk-1-Aw and Bk-3-Aw, respectively (table 2). Eight isolates (12%) were highly effective on the basis of shoot dry weight accumulation of more than 80% in relation to the N-fertilized control plants. The remaining 88% were categorized as symbiotically effective accumulating 50-80% of the dry weight of the N-fertilized control plants. The relative effectiveness data revealed that Bk-3-Aw was the most effective isolate followed by Aw-2-Aw with 88% and 85% of the shoot dry weight of the nitrogen supplied control treatment (TN), and the commercial reference strain also showed 78% symbiotic effectiveness on the host plant (table 2). In contrast, isolate Bk-1-Aw was the least effective isolate with about 51% of the shoot dry weight of the TN control.

Table 2. Symbiotic traits of the rhizobial isolates under greenhouse condition

Isolate	NNPP	NDWPP (mg)	SDWPP (mg)	SE (%)	SE rating
Cw-2-Cl	28.0h-p	67.0c-k	714.89h-p	62	E
Aw-6-Bo	12.3s-x	65.6c-l	749.33d-p	65	E
Jm-5-Bo	32.0e-n	72.2b-i	800.11c-o	70	E
Jm-4-Cl	32.7e-m	75.4a-g	852.72b-l	74	E
As-1-Aw	9.0u-y	41.1n	619.56o-q	54	E
As-6-Bo	45.3a-c	63.9c-m	770.67d-p	67	E
Aw-3-Cl	36.3c-j	81.8a-d	836.94b-m	73	E
Cw-6-Cl	16.0q-w	63.7c-m	735.89f-p	64	E
As-4-Bo	44.7a-c	71.2b-i	801.61c-o	70	E
Pw-4-Aw	7.0w-y	49.5j-n	683.17j-q	59	E
As-6-Aw	36.7c-i	67.3c-k	690.67j-q	60	E
Cw-3-Cl	30.0g-p	67.4c-k	780.28c-p	68	E
Aw-2-Cl	36.0c-j	53.1h-n	627.89n-q	55	E
Pw-13-Cl	7.7v-y	52.1i-n	808.33c-o	70	E
Pw-6-Cl	21.7m-t	71.8b-i	817.33b-o	71	E
As-5-Aw	26.3h-r	67.1c-k	877.78b-j	76	E
Cw-3-Bo	26.0i-r	64.0c-m	762.89d-p	66	E
Aw-4-Bo	32.0e-n	69.0b-j	835.72b-m	73	E
Pw-10-Bo	20.0o-u	82.2a-d	823.50b-n	72	E
Jm-14-Cl	15.3r-w	49.0j-n	720.22g-p	63	E
Aw-4-Cl	31.3f-o	76.7a-f	855.94b-l	74	E
Jm-13-Cl	33.0e-m	70.4b-i	662.83k-q	58	E
Bk-3-Aw	36.7c-i	88.6ab	1011.78ab	88	HE
MAR-1495	27.2h-q	41.6n	894.28b-i	78	E
Aw-1-Cl	15.3r-w	43.3m-n	681.33j-q	59	E
As-8-Cl	37.7b-h	70.9b-i	833.00b-m	72	E
Cw-3-Aw	39.7a-g	54.4h-n	663.33k-q	58	E
Cw-1-Cl	48.0ab	82.4a-c	687.17j-q	60	E
Jm-12-Cl	28.0h-p	60.2e-n	796.44c-o	69	E
As-7-Aw	41.7a-f	79.8a-e	871.33b-j	76	E
Bk-2-Aw	33.0e-m	69.8b-j	939.45b-e	82	HE
Jm-1-Bo	32.0e-n	72.0b-i	847.50b-l	74	E
Aw-6-Cl	26.2i-r	63.7c-m	834.56b-m	73	E
As-8-Aw	34.0d-l	63.8c-m	772.78d-p	67	E
As-7-Cl	39.7a-g	78.1a-f	931.44b-f	81	HE
AD-2-Bo	9.0u-y	44.2m-n	818.83b-o	71	E
As-5-Cl	27.0h-q	54.0h-n	792.39c-o	69	E
Cw-6-Aw	33.7d-l	68.9b-j	949.17b-d	83	HE
Jm-5-Cl	29.7g-p	48.9j-n	742.33e-p	65	E
Pw-14-Bo	8.3v-y	52.9h-n	637.67m-q	55	E
As-7-Bo	23.3l-r	77.4a-f	806.50c-o	70	E
Bk-4-Bo	11.7t-x	72.3b-i	880.61b-j	77	E
Jm-8-Cl	28.0h-p	69.1b-j	864.89b-k	75	E
Pw-3-Cl	23.0l-s	62.4c-m	930.056b-f	81	HE
Pw-4-Bo	7.3w-y	53.5h-n	894.61b-i	78	E
Pw-7-Bo	33.0e-m	54.7g-n	772.39d-p	67	E
Pw-8-Cl	43.0a-e	73.2b-h	864.06b-k	75	E
Pw-12-Aw	32.3e-n	63.4c-m	919.00b-g	80	HE
As-5-Bo	21.0n-t	78.9a-f	912.44b-h	79	E

Aw-1-Aw	32.0e-n	75.4a-g	901.39b-h	78	E
AD-2-Aw	25.1j-r	45.3l-n	698.89i-p	61	E
Pw-3-Bo	35.0c-k	47.3k-n	735.83f-p	64	E
Bk-1-Aw	18.7p-v	44.6m-n	584.67p-q	51	E
Pw-9-Bo	30.0g-p	60.2e-n	801.00c-o	70	E
Jm-5-Aw	31.0f-o	80.3a-e	845.44b-l	74	E
Aw-2-Aw	37.0c-i	62.3c-m	977.89bc	85	HE
Jm-9-Cl	3.7x-y	23.7o	696.11i-p	61	E
Jm-7-Cl	18.7p-v	63.7c-m	863.83b-k	75	E
Pw-12-Bo	24.0k-r	44.8m-n	661.06l-q	57	E
Pw-2-Aw	28.8g-p	93.8a	826.11b-n	72	E
Pw-11-Cl	49.0a	69.1b-j	727.72g-p	63	E
Jm-6-Cl	9.7u-y	41.3n	741.89e-p	65	E
Pw-11-Bo	12.0s-x	66.8c-k	775.28d-p	67	E
Jm-3-Aw	30.7f-o	69.3b-j	838.89b-m	73	E
Pw-11-Aw	18.7p-v	53.0h-n	946.11b-d	82	HE
Cw-5-Cl	33.0e-m	61.2d-n	747.78d-p	65	E
AD-1-Bo	1.7x-y	16.0op	786.11c-o	68	E
Pw-5-Aw	12.0s-x	58.4f-n	637.83m-q	55	E
+ve control	0.0y	0.0p	1149.78a		
_ve control	0.0y	0.0p	501.17q		
CV (%)	21.98	16.72	12.25		
Significance	**	**	**		

It is interesting to note that all highly effective isolates were from slow growing rhizobia. This effectiveness of the isolates was clearly noticeable from their green to deep green color of the leaves; compared to yellowish or light green colors for the un-inoculated plants (data not shown). Similarly, Diriba (2017) reported that selected isolates from Ethiopian soils were either effective or highly effective, indicating indigenous rhizobia were capable of establishing highly effective symbiosis with soybean similar to the indigenous rhizobia in other African countries (Youseif *et al*., 2014; Klogo *et al*., 2015; Gyogluu *et al*., 2016).

Stress tolerance of isolates

The data in Figures 1-3 show that soybean rhizobia exhibited variation in their stress tolerance. The majority of the isolates grew in YEMA supplied with 0.5%, almost half of them were able to grew at 0.8% NaCl and only a few isolates were tolerant to 1-4% NaCl (Figure 1). The isolates showed a pattern in that few slow growers (Jm-14-Cl, Jm-6-Cl and Jm-7-Cl) survived 1% NaCl; whereas fast growing isolates were tolerant to 1-4% NaCl, indicating that fast growing isolates were more tolerant to salt than the slow growers (data not shown).

Similarly, Youseif *et al*. (2014) and Diriba (2017) reported that local slow growing soybean isolates from Egypt and Ethiopia could barely tolerate 0.5% NaCl, respectively. The poor tolerance of the indigenous isolates to salt stress may be due to the fact that they are originated from non-saline soils, since it is often believed that saline soils naturally select strains more tolerant to salinity.

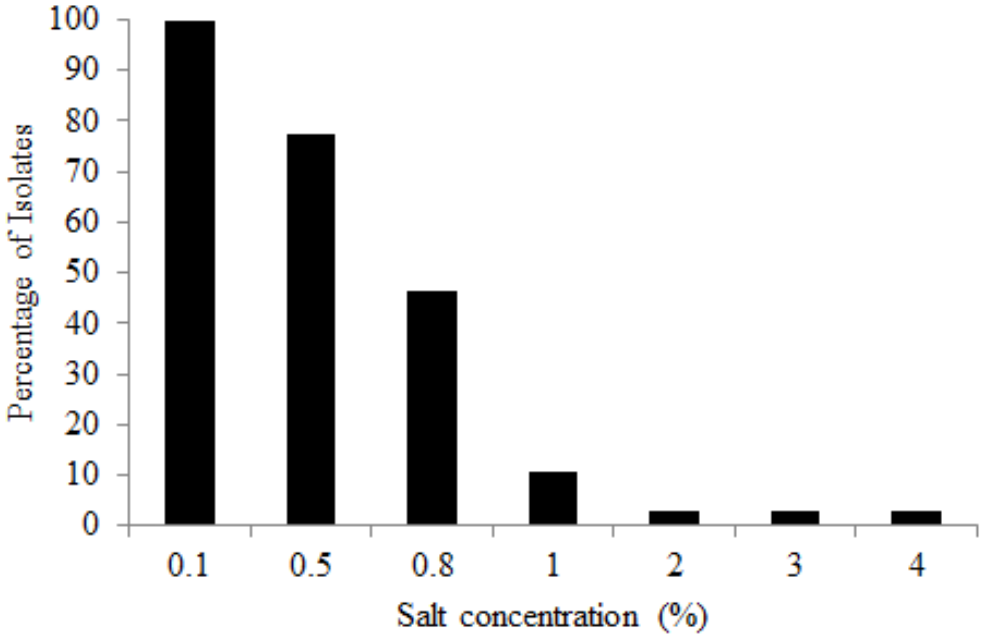

Figure 1. Tolerance of soybean rhizobia isolates to different salt concentration

Contrary to a narrow range of salt tolerance, the isolates were versatile in their tolerance to pH extremes (Figure 2). Almost all isolates grew on YEMA at pH values of 5.0 to 9.5 irrespective of their taxonomic grouping similar to both slow and fast-growing soybean isolates from Egypt which tolerate pH 5 to 1l (Youseif *et al*., 2014) and soybean isolates from Ethiopia which tolerate a wide range of NaCl (5-10) (Dirbia, 2017). Chen *et al* (2002) from Paraguay also reported that all soybean rhizobial isolates were tolerant to a wide pH range (6.5 to 9).

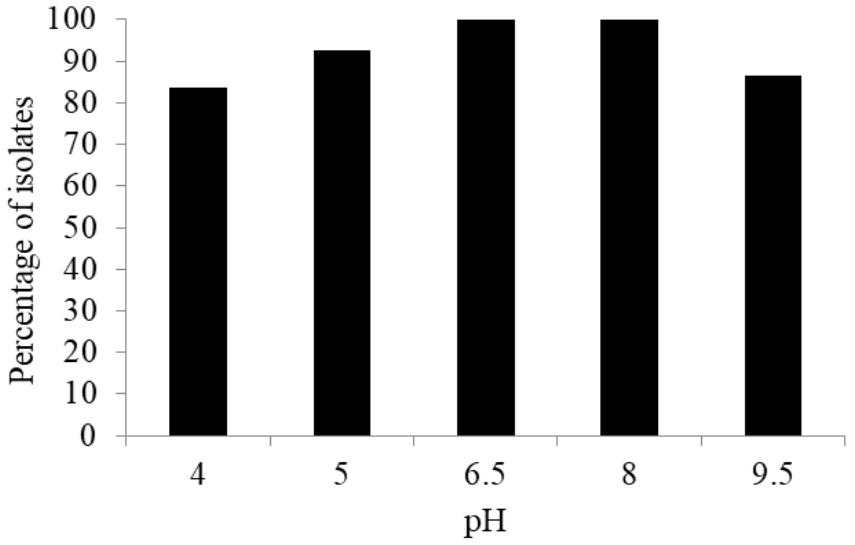

Figure 2. Tolerance of soybean rhizobia isolates to pH

It is interesting to note that many of the isolates (84%) in this study were tolerant to pH 4 (Figure 2). This tolerance to acidic conditions may reflect the fact that the majority of the isolates were collected from very strongly and moderately acidic soils and hence were adapted to such conditions. Other studies also showed the same pattern of tolerance to acidity by some soybean rhizobial strains from India (Appunu and Dhar, 2006) and Thailand (Nuntagij *et al*. 1997) and some fast-growing soybean rhizobia from Brazil (Hungria *et al*., 2001) and from Egypt (Youseif *et al*., 2014). All isolates were able to grow well at 30^{0}C which was considered as the optimum temperature for growth of root nodulating bacteria (Harwani, 2006). Almost half of the isolates were tolerant to 37^{o}C, but barely 10% of the isolates (AD-2-Aw, As-5-Bo, Bk-2-Aw, Bk-3-Aw, Cw-5-Cl and Jm-9-Cl) were tolerant to temperatures of 40^{o}C, but none of them were able to grow at 45^{o}C (data not shown), similar to reports of weak growth of *Bradyrhizobium* spp at this temperature (Chen *et al*., 2002; Youseif *et al*., 2014; Chibeba *et al*., 2017; Diriba, 2017). The soybean rhizobia also differed in their IAR pattern (Figure 3). Almost all of the isolates were resistant to nalidixic acid, tetracycline, ampicillin, chloramphenicol but sensitive to gentamycin, kanamycin, streptomycin, and erythromycin. Similarly, Ansari and Rao (2014) reported that all slow and fast-growing soybean rhizobia were sensitive to gentamicin but showed maximum resistance to nalidixic acid and Diriba (2017) reported that 76% of local rhizobial isolates from Ethiopia were resistant to chloramphenicol. However, there was no clear trend on IAR pattern between the taxonomic groups.

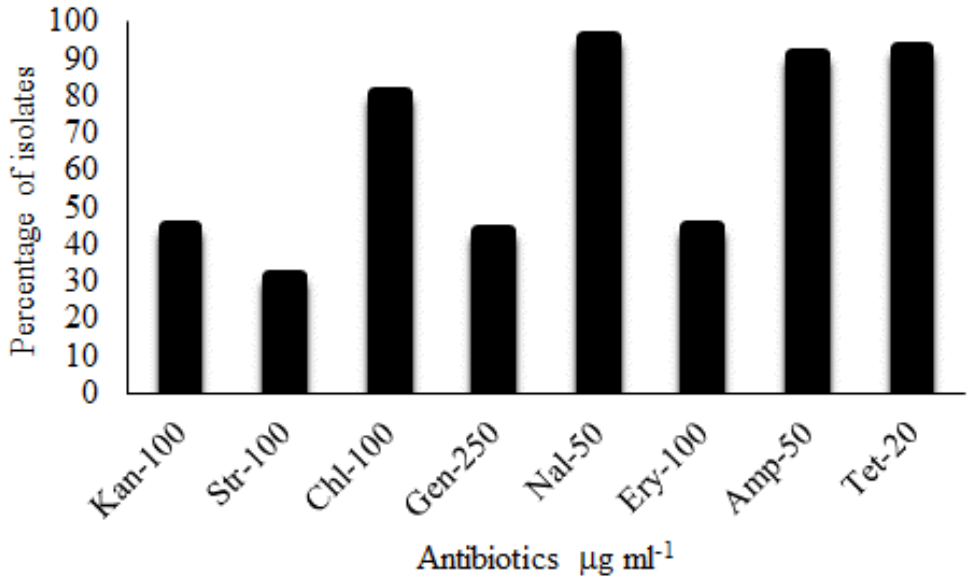

Figure 3. Effect of different antibiotics on growth of soybean rhizobia.

The isolates also showed resistance to heavy metals of Mn, Al, Zn and Co but less so to Cu (62.7%) and highly sensitive to Fe (100%) (data not shown). The resistance of local soybean rhizobial isolates to Mn and Zn were previously reported in Ethiopia (Diriba, 2017). However, the data did not show distinctive pattern in heavy metal resistance/sensitivity between the slow and fast-growing groups of soybean rhizobia.

Utilization of carbon and nitrogen substrates

Soybean rhizobial isolates varied in their ability to metabolize different carbon and nitrogen substrates tested (Figures 4 and 5). All of the tested isolates utilized the monosaccharides D-mannose, xylose and glucose. On the other hand, none of the isolates utilized Na-citrate and dextrin (data not shown) similar to previous reports on dextrin (Sadowsky *et al*., 1983) and Na-citrate (Hungria *et al*., 2001). However, Diriba (2017) reported that a number of slow growers and the fast growers utilized citrate and dextrin. Although all the slow growers utilized the remaining monosaccharides (D-arabinose, D-galactose and fructose), only 60% of the fast growers utilized these monosaccharides; implying the poor growth of some fast growers on these carbon sources. Hungria *et al*. (2001) previously reported that only 67% of fast growing soybean isolates from Brazil utilized fructose and galactose. On the other hand, the utilization of the majority of disaccharides was limited to fast growers, except the utilization of sucrose and lactose by some slow growers (Figure 4).

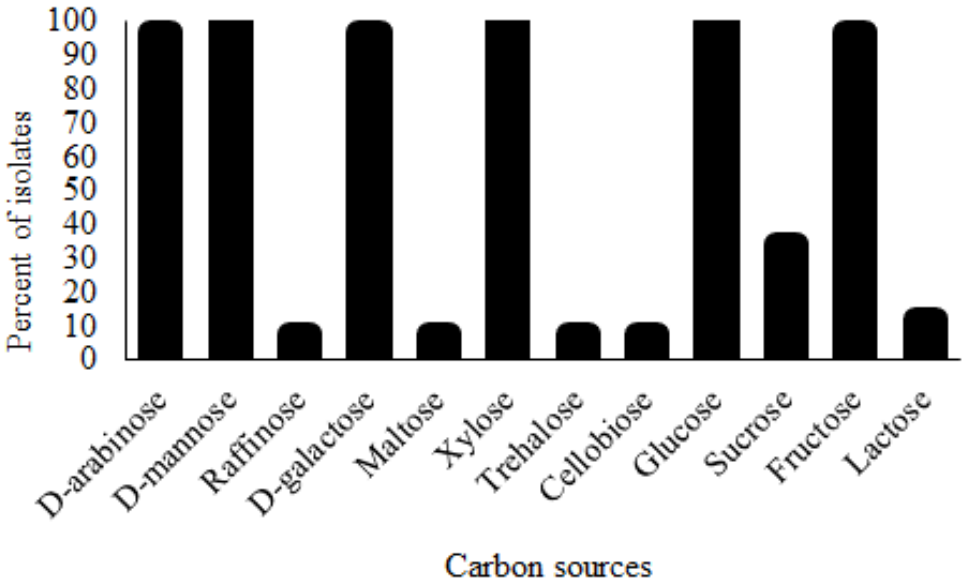

Figure 4. Pattern of carbon utilization by indigenous soybean isolates

The data showed different trend in carbon utilization by the different taxonomic groups. Accordingly, the fast-growing soybean rhizobia were more versatile than slow growing ones in that the fast growers utilized 60-90% whereas the slow growers utilized 40-50% of the carbon sources (data not shown). There were conflicting reports on the pattern of sugar utilization by fast growing and slow growing soybean rhizobia. Although different authors (Sadowsky *et al*., 1983; Young *et al*., 1988; Hungria *et al*., 2001; Diriba, 2017) showed that fast growing soybean rhizobia were more versatile in sugar utilization, Ansari and Rao (2014) showed that the slow growers had greater catabolic versatility than the fast growers.

In earlier studies one of the distinguishing features of soybean rhizobia was the inability of the slow growers to utilize disaccharides. However, in this study, few slow growers utilized sucrose and lactose similar to the findings of some of recent studies (Ansari and Rao, 2014; Diriba, 2017).

The isolates showed difference in the utilization of nitrogen sources (Figure 5). Accordingly, the fast-growing soybean rhizobia were more versatile than slow growing ones in that the fast growers utilized all N sources, whereas the slow growers with few exceptions like As-7-Cl, Pw-3-Cl, Pw-12-Aw, Pw-11-Cl and Jm-4-Cl utilized only 57-86% of the N sources (data not shown). Similar pattern of N-utilization between the two taxonomic groups was previously reported (Diriba, 2017).

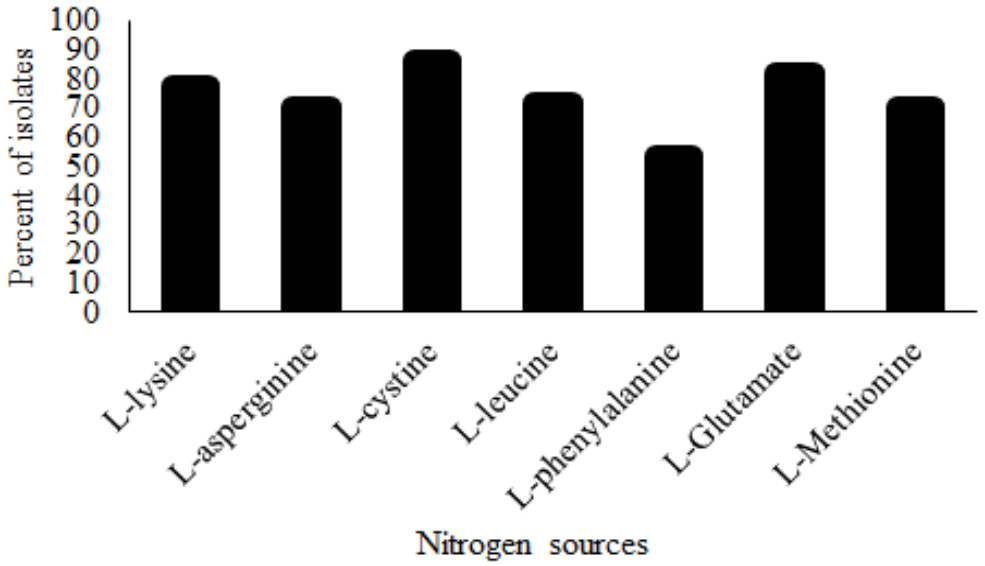

Figure. 5. Utilization of different nitrogen sources by indigenous soybean isolates

In general, isolates Jm-5-Aw, Jm-9-Cl, Bk-3-Aw, Aw-2-Aw, and Jm-4-Cl were the most nutritionally versatile and eco-physiologically tolerant in vitro, and symbiotically effective under greenhouse conditions.

Host range of soybean rhizobia isolates

The ability of 15 selected rhizobial isolates to form nodules and effectively fix nitrogen was determined on other cross-nodulating hosts, of which 12 isolates (80%) nodulated either or all cowpea, pigeon pea and mung bean hosts (table 3). The data showed that 75% and 58% of these isolates displayed effective and very effective symbiosis with their heterologous cowpea and pigeon pea hosts, respectively. However, half of them formed slightly effective and effective nodules on mung bean. Musiyiwa *et al*. (2005) reported that all soybean isolates tested nodulated cowpea with 58% being moderately effective to very effective and about 82% nodulated pigeon pea 36% of which were effective, but all failed to form nodules on peanut. Appunu *et al*. (2009) also reported that all rhizobia isolated from soybean nodulated cowpea, 67% nodulated mung bean and 22% formed nodules with pigeon pea and peanut. Yang and Zhou (2008) showed that two *Bradyrhizobium* strains isolated from soybean could also nodulate peanut growing in the same ecological niche that may have phylogenetic connection with one another. Contrary to the present finding, Ansari and Rao (2013) indicated that soybean rhizobia did not nodulate mung bean at all. The variation could be due to differences in the type of the soybean isolates.

The study showed that with respect to nodulation ability; cowpea, pigeon pea and mung bean were equally promiscuous by forming nodules with 80% of the tested isolates (table 3). However, in terms of effectiveness, cowpea was the most promiscuous, forming effective symbiosis with 67% of the isolates tested, followed by pigeon pea (47%) and mung bean (40%). Peanut on the other hand, was the most specific of the hosts examined, with about 93% of the isolates unable to nodulate or forming ineffective symbiosis. Several studies in the past also showed that cowpea is one of the most promiscuous legume hosts in the so-called 'cowpea' miscellany group of rhizobia (Musiyiwa *et al*., 2005; Pule-Meulenberg *et al*., 2010). The promiscuity of their group was previously showed on the closest relative mung bean (Pueppke and Broughton, 1999; Risal *et al*., 2012) and distantly associated pigeon pea (Coutinho *et al*., 1999).

The study also revealed that isolate Jm-4-Cl had broad host ranges nodulating all the four legumes tested, whereas As-6-Aw, Aw-6-Cl and Pw-5-Aw had restricted host and could not nodulate all the legumes tested (Table 3).

The inoculated plants showed variations in their nodulation properties (Table 3). Accordingly, variations in number of nodules ranged from 12-29 plant^{-1} (Mean = 13 nodules plant^{-1}) on cowpea, 27-44 plant^{-1} (Mean = 24 nodules plant^{-1}) on pigeon pea and 8-38 plant^{-1} (Mean= 23 nodules plant^{-1}) on mung bean. They also displayed difference in nodule dry weight which ranged from 82-192 mg plant^{-1} (Mean = 91.8 mg plant^{-1}) on cowpea, 15-109 mg plant^{-1} (Mean = 48.4 mg plant^{-1}) on pigeon pea and 25-140 mg plant^{-1} (Mean = 87 mg plant^{-1}) on mung bean. This indicates that cowpea was more permissible to effective nodulation than pigeon pea.

The response of the host legumes to inoculation in terms of shoot dry weight also varied greatly (table 3). It ranged from 1.02-1.93 g plant^{-1} (mean = 1.55 g plant^{-1}) for cowpea, 0.36-0.98 g plant^{-1} (0.78 g plant^{-1}) for pigeon pea and 0.36-1.25 g plant^{-1} (mean= 0.73 g plant^{-1}) for mung bean. On cowpea, six isolates (AD-2-Aw, Aw-2-Aw, Bk-3-Aw, Cw-2-Cl, Cw-6-Aw and Jm-4-Cl) effectively nodulated the host plants displaying highly effective nitrogen fixation (RE >80%). Similarly, these isolates and As-5-Aw also displayed highly effective nitrogen fixation on pigeon pea. Therefore, isolates As-5-Aw, AD-2-Aw, Aw-2-Aw, Bk-3-Aw, Cw-2-Cl, Cw-6-Aw and Jm-4-Cl can be considered as a candidate for inoculant production for cowpea and pigeon pea after testing their competitiveness in the presence of other indigenous and/ exotic strains under field conditions. However, only two isolates (Bk-3-Aw and Aw-2-Aw) were categorized as symbiotically effective on

Table 3. Nodulation and symbiotic characteristics of 15 selected soybean rhizobial isolates on different legume species

Treatment	Cowpea				Pigeon pea				Mung bean			
	NNPP	NDWPP (mg)	SDWPP (g)	SE	NNPP	NDWPP (mg)	SDWPP (g)	SE (%)	NNPP	NDWPP (mg)	SDWPP (g)	SE (%)
AD-2-AW	29.3a	155.78a-c	1.86a	96	26.8e	102.67a	0.92b	81	29.2b	126.80a	0.78c	37
As-5-Aw	12.2f	172.87ab	1.29b	66	35.0a-e	106.68a	0.95b	83	29.0b	97.68b	0.77c	36
As-7-Cl	27.3ab	140.72bc	0.878c-e	45	43.8a	40.40c	0.38c	34	12.0ef	44.50cd	0.43d	20
Aw-2-Aw	27.2ab	159.23a-c	1.85a	95	29.9c-e	97.98a	0.93b	82	37.8a	140.33a	1.21b	57
Bk-3-Aw	19.2cd	123.98cd	1.91a	98	27.9de	71.30b	0.97b	85	19.3cd	138.67a	1.25b	59
Cw-2-Cl	18.2de	159.10a-c	1.72a	88	26.6e	87.84ab	0.97b	85	10.3f	38.67c-e	0.45d	21
Cw-3-Aw	28.0ab	192.20a	1.22bc	63	30.2b-e	15.47de	0.36c	32	8.0f	24.92e	0.36d	17
Cw-6-Aw	23.5bc	163.82a-c	1.67a	86	40.1abc	109.03a	0.92b	81	30.7b	106.20b	0.78c	37
Jm-4-Cl	13.7ef	134.25bc	1.93a	99	38.6abc	89.10ab	0.98b	86	23.0c	95.28b	0.77c	36
Pw-13-Cl	13.8ef	82.33de	1.01b-d	52	37.6a-d	22.21c-e	0.37c	32	30.5b	52.067c	0.47c	22
Pw-11-Cl	3.0g	12.27f	0.525e	27	40.5a	35.01cd	0.37c	32	9.5f	27.57de	0.43d	20
Pw-3-Cl	12.0f	63.22f	0.667de	34	36.1a-e	45.37c	0.34c	30	15.8de	27.45de	0.41d	19
As-6-Aw	0.0g	0.00f	0.535e	28	0.0f	0.0e	0.43c	38	0.0g	0.0f	0.36d	17
Pw-5-Aw	0.0g	0.00f	0.528e	27	0.0f	0.0e	0.36c	32	0.0g	0.0f	0.40d	19
Aw-6-Cl	0.0g	0.00f	0.487e	25	0.0f	0.0e	0.37c	33	0.0g	0.0f	0.41d	20
Unfertilized	0.0g	0.00f	0.552e		0.0f	0.0e	0.38c		0.0g	0.0f	0.37d	
+ N	0.0g	0.00f	1.94a		0.0f	0.0e	1.14a		0.0g	0.0f	2.11a	
CV (%)	22.35	27.86	18.56		22.33	27.91	18.56		17.7	19.4	12.5	
Significance	***	***	***		***	***	***		***	***	***	

Mean values followed by the same letters in each column are not significantly different according to DMRT ($P \leq 0.05$); +N= with optimum amount of nitrogen; NN= number of nodules plant^{-1}, NDW=nodule dry weight plant^{-1}, SDW=shoot dry weight plant^{1}; Significance: ** showed significant difference at $P < 0.001$

mung bean accumulating 50-80% of the dry weight of the N-fertilized control plants. This indicates that soybean isolates in this study were more compatible with cowpea and pigeon pea than mung bean.

In general, out of the 12 isolates that nodulated the tested legumes, Bk-3-Aw and Aw-2-Aw nodulated all the three cross-nodulating legumes with highly effective nodulation (HE) on cowpea and pigeon pea and effective nodulation (E) with mung bean.

Conclusion and Recommendations

Soybean is an exotic crop to Ethiopia, despite this, it was found to be effectively nodulated by indigenous root nodule bacteria mainly slow growing *Bradyrhizobium* spp commonly nodulating of a cross nodulation cowpea and pigeon hosts. The isolates were diverse in their growth and cultural characteristics, nutritional versatility to utilize different carbon and nitrogen sources. Their difference in tolerance to grow at different pH, NaCl concentration incubated at various temperatures, and resistance to several antibiotics and heavy metal implied their taxonomic diversity and plausible ability of their eco-physiological tolerance to persist in the soil. Since the present study relied on phenotypic characteristics for assessing soybean rhizobia diversity, future research should include the use of genetic methods to reveal a more accurate picture on the diversity of soybean rhizobia.

The study conducted using selected isolates, indicated that some of the isolates have wide host ranges and are effective on most of the legumes. Isolates Aw-2-Aw and Bk-3-Aw were relatively effective on the three legumes; cowpea, pigeon pea, and mung bean indicative of their potential to be used as inoculant strains for these legumes after testing their symbiotic effectiveness and competitiveness under field condition in the presence of other indigenous and/or exotic strains.

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