Inoculation of Native Symbiotic Effective Sinorhizobium Spp. Enhanced Soybean [Glycine Max (L) Merr.] Grain Yield in Ethiopia

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Research

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Abstract

Background

Soybean [*Glycine max* (L) Merr.] is an annual leguminous crop serving as a source of food and feed, green manure, biodiesel and fiber It is nodulated by diverse slow growing and fast growing rhizobia belonging to the genus *Bradyrhizobium* and *Sinorhizobium*, respectively. In Ethiopia, it has been cultivated since 1950s with lower grain yield history. Yield improvement efforts have been more concentrated on agronomic studies, inoculation of exotic *Bradyrhizobium japonicum* including TAL379 and/or fertilizer application. The results have usually been unsatisfactory and inconsistent. This study was initiated to identify promising indigenous soybean rhizobial inoculant that can enhance yield of the crop in the country.

Methods

Native soybean rhizobia, designated GMR for *Glycine max* rhizobia, were trapped using soybean (cv. Ethio-Yugoslavia) from soils collected across agro-ecologies of Ethiopia. Indigenous soybean rhizobia were screened for *in vitro* tolerance against physico-chemical stresses, plant growth promoting (PGP) traits and symbiotic performances at greenhouse and field levels A reference *B. japonicum* (TAL379) was included in all experiments. A soybean plant growth promoting *Achromobacter* sp. was also included in field trials for co-inoculation. Quantitative data were assessed by analysis of variance (ANOVA) employing SAS computer software package version 9.3. Mean separations were undertaken using the Duncan's Multiple Range Test at $p \leq 0.05$.

Result

GMR that produced acid and grew faster with larger colonies were identified as *Sinorhizobium* spp. and those which produced alkali and grew slowly with smaller colonies were identified as *Bradyrhizobium* spp though further genetic analysis should be performed for verification and identification of their genus and species, respectively. Two *Sinorhizobium* spp (GMR120C and GMR125B) profoundly nodulated different soybean cultivars under greenhouse conditions and significantly improved grain yield ($p \leq 0.05$; maximum 3.98 tons ha^{-1}) compared to 2.41, 2.82 and 2.69 recorded as maximum grain yield (tons ha^{-1}) for TAL379 inoculation, positive control and negative control, respectively in field trials. Higher yield was recorded when GMR125B was coinoculated with *Achromobacter* sp., but when GMR120C was inoculated singly. These GMR also showed efficient utilization of numerous substrates, some PGP traits and potential adaptation to various ecological stresses.

Conclusion

The two *Sinorhizobium* spp. (GMR120C and GMR125B) are promising soybean inoculants that can be used to enhance the productivity of the crop in the country.

1. Background

Soybean is an annual leguminous crop which was initially domesticated in east Asia, but currently cultivated worldwide principally in North and Latin America. It has been cultivated in Africa since 1896 (Shurtleff and Aoyagi 2009). It is one of the top internationally important crops serving as a nutritious food and feed, source of biodiesel and fiber (Scott and Aldrich 1983; Yi-you 2004; Ogbemudia et al. 2010). It is also used as green manure to improve soil fertility due to its ability to symbiotically fix atmospheric nitrogen with different species of *Bradyrhizobium*, a slow growing genus of rhizobia (Kuykendall et al. 1992; Xu et al. 1995; Appunu et al. 2008; Yang and Zhou 2008; Zhang et al. 2012) and species of *Rhizobium, a* fast growing genus of rhizobia (Keyser et al. 1982; Scholla and Elkan 1984; Chen et al. 1988; Chen et al. 1995; Saldana et al. 2003). Chen et al. (1988).reclassified fast growing soybean rhizobia as *Sinorhizobium.* It is also known to be associated with diverse groups of plant growth promoting rhizobacteria (PGPR) (Masciarelli et al. 2014).

Soybean [*Glycine max* (L.) Merrill] establishes effective symbioses with *Bradyrhizobium*, principally with strains belonging to *B. japonicum* and *B. elkanii* (Hungria et al. 2001). Some fast growing strains of soybean rhizobia which were as effective as slow growing counter parts were also reported (Dowdle and Bohlool 1985, Isreal et al. 1986; Hungria et al. 2001). Inoculation of soybean with its rhizobia can improve the growth and yield of the crop (Sharma and Kumawat 2011; Solomon et al. 2012; Rechiatu et al. 2015; Ulzen et al. 2016). PGPR inoculations, usually with *Bradyrhizobium japonicum*, increased seedling emergence rate (Le´ on et al. 2009), nodulation, N-fixation and grain yield of soybean (Dashti et al. 1998; Argaw 2012; Aung et al. 2013; Kravchenko et al. 2013). To achieve better inoculation response, screening of rhizobia is important as they vary in their symbiotic effectiveness, compatibility to various soybean cultivars and ecological adaptation.

In Ethiopia, soybean has been cultivated since 1950s expanding into different agro-ecologies accompanied by increased domestic demand as food and feed yet with low grain yield (Hailu and Kelemu 2014). Recently, Deresse (2019) indicated the availability of 26 released varieties of soybean in the country. The Ethiopian CSA (2019) also reported the production of the crop on 64,720.12 hectares with 149,454.6 tons of grain yield (2.31 tons ha^{-1}) which is low due to poor soil fertility (Argaw 2012) and ineffectiveness of exotic commercial bradyrhizobia (Aserse et al. 2012).

Indigenous soybean rhizobia was first recovered from Ethiopian soil in 1980's (Abebe 1986). However, more attention has been given to agronomic studies, inoculation of exotic *B. japonicum* including TAL379 and/or fertilizer application rather than screening native rhizobia. Genetic characterizations (Aserse et al. 2012; Jaiswal et al. 2016; Fana 2018), evaluation of green house and field symbiotic performance (Argaw 2014) and determination of inherent antibiotic resistance (Abera et al. 2015) are some of the studies that have been carried out on indigenous soybean rhizobia in the country. The studies have indicated predominantly the presence of *Bradyrhizobium* spp. (and few clustering with *Rhizobium*) and good nodulation of some varieties of soybean, but with low grain yield records. The studies have included limited ecological regions and rhizobial traits. Moreover, no promising native soybean inoculant has been established in the country. Therefore, this study was initiated to isolate soybean rhizobia from soils collected from various regions of the country and screen for *in vitro* potential ecological adaptation and for symbiotic performance under green house and field conditions so as to develop better inoculant of the crop.

2. Materials And Methods

2.1 Soil sampling, bacterial isolation and reference strain

Slightly acidic (pH 5.9 to 6.4) composite soil samples with no previous history of inoculation were collected from different agro ecological regions of Ethiopia (Fig. 1) with altitude ranging from 1055 to 1875 meters above sea level (masl). Surface sterilized soybean seeds (cv. Ethio-Yugoslavia; 2% sodium hypochlorite [Lwin, et al. 2012]) were sown into each soil sample in 3 kg capacity disinfected plastic pots (70% ethanol). Plants were grown for 45 days with the provision of distilled sterile water when required and uprooted for nodule collection. Nodules were surface sterilized (4% sodium hypochlorite), crushed and the resulting suspensions were streaked on yeast extract mannitol agar (YMA) plates and incubated for 10 days at 28 ^{o}C (Somasegaran and Hoben 1994). Isolated bacteria were designated GMR (*Glycine max* rhizobia), checked for purity via subculturing and preserved at 4^{o}C on YMA (Vincent 1970). Pure cultures were authenticated on the host plant under greenhouse conditions following standard methods (Somasegaran and Hoben 1994). Authenticated GMR were subjected to determination of various cultural, physiological and symbiotic traits. A *Bradyrhizobium japonicum* strain (TAL379 or USDA 136b), obtained from National Soil Testing Center of Ethiopia, was included in all laboratory, green house and field experiments as a reference.

2.2 Determination of colony morphology, acid/alkali production and generation time

GMR were streaked on YMA plates to determine colony size (mean diameter of five colonies), shape and margin, texture, appearance and gum production according to Lupwayi and Haque (1994). They were also streaked on YMA-BTB (0.5% Bromothymol blue) plates to assess color change as an indication of acid or alkali production. Tests were carried out in triplicates streaking a loopful of active yeast extract mannitol broth (YMB) culture (10 µL; 10^{6} cells mL^{-1}) followed by incubation at 28 ± 0.2 ^{o}C for 7 days.

To determine the generation time (g), each actively growing YMB culture (10 mL) was transferred into 100 mL of sterile YMB in a 250 mL Erlenmeyer flask and shaken at 150 rpm (orbital shaker: Gollen hamp, England; room temperature [20-30^{o}C]; 72 hours). Samples were taken every 2 hours to determine optical density (OD^{540}; Jenway, 6405 Uv/vis spectrophotometer) and colony forming units (cfu) according to Somasegaran and Hoben (1994). Eventually, the generation time (g) was computed from log phase according to White (1995).

2.3 Testing For Tolerance Against Physico-chemical Stresses

Growths of all the GMR at various levels of salt (NaCl), temperature and pH as well as in the presence of antibiotics, heavy metals and pesticides were assessed as follows. YMB cultures were separately streaked on YMA plates (amended with 0.5 to 6% NaCl; w/v) and on Keyser-defined medium plates (pH 4, 4.5, 5, 8.5, 9, 9.5 or 10) and incubated at 28 ± 0.2^{o}C (Lupwayi and Haque 1994). They were also streaked on YMA plates and incubated at 35^{0}C, 37^{0}C, 40^{0}C and 45^{0}C wrapping with parafilm® to minimize moisture lose in evaluating higher temperature tolerance.

YMB cultures of the GMR were streaked on YMA plates containing (µg mL^{-1}) Chloramphenicol (25), Streptomycin sulfate (10), Erythromycin (100), Ampicillin (200), Gentamycin (20), Nalidixic acid (100), Penicillin G (200), Tetracycline (10), Vancomycin (15) and Cifroflaxin (10) to test their inherent antibiotic resistance (IAR) according to Dowdle and Bohlool (1985). They were also streaked on minimal salt agar medium plates (pH 6.8) supplemented with filter sterilized (final concentration, mM) $CoCl_2.6H_2O$ (0.5), $Pb(CH_3COO)_2.3H_2O$ (2.5), $K_2Cr_2O_7$ (0.25), $CuCl_2.2H_2O$, $ZnSO_4.7H_2O$ and $MnSO_4.4H_2O$ (each 0.25) to assess their inherent heavy metal resistance (IHR) according to Hungria et al. (2001). The GMR were also tested for their resistance against 0.2% [w/v] of two fungicides (Mankozeb and curzet; Du Pont de Nemour, France) on YMA plates (Mubeen et al. 2006) and against 0.14% glyphosate (Monsanto Europe S.A, Belgium) on minimal salt agar medium plates (Ahemad and Khan 2010). In all the above stress tests, a loopful of each YMB culture (10 µL; 10^6 cells mL^{-1}) was streaked on a plate and incubated at 28 ± 0.2 °C (except for temperature test) for 5–7 days. Eventually, the presence of growth (+) and absence of growth (-) were recorded as tolerant and sensitive, respectively.

2.4 Evaluation Of Carbon And Nitrogen Substrate Utilizations

A loopful YMB culture of each GMR (10 µL; 10^6 cells mL^{-1}) was tested for utilization of 19 carbon sources (D-glucose, D-fructose, D-galactose, D-arabinose, D-mannose, xylose, maltose, α-lactose, trehalose, D-sucrose, dextrin, insitol, sorbitol, Na-citrate, dextrose, cellobiose, Na-acetate, inulin and sodium propionate) and 13 amino acids (L-Alanine, L-argenine, methionine, DL-phenyl alanine, DL-plolin, leucin, DL-threonine, DL-serine, lysine, DL-tryptophan, glycine, L-tyrosine and DL-glutamic acid), each added to a basal medium according to Amarger et al. (1997). Heat labile carbon sources and amino acids were filter sterilized (0.22 µm) prior to adding into autoclaved basal medium. Presence (+) and absence (-) of GMR growth were recorded as the ability and inability to utilize a substrate, respectively.

2.5 Assessment For Plant Growth Promoting (pgp) Traits

GMR were assessed for *in vitro* antifungal activity, solubilization of three inorganic phosphates (Tri-calcium-, aluminium- and iron-phosphate), production of indole acetic acid (IAA), HCN and some enzymes. Active loopful YMB culture (10 µL; 10^6 cells mL^{-1}) of each GMR was spot inoculated on Pikovskaya's medium plates to evaluate their tri-calcium phosphate solubilization trait. Similar spot inoculations were made on NBRIP (National Botanical Research Institute's Phosphate medium, Lucknow, India) plates to assess for solubilization of aluminum phosphate ($AlPO_4$) and iron-phosphate ($FePO_4$) by substituting $AlPO_4$ or $FePO_4$ for $Ca_3(PO_4)_2$ in NBRIP (Pe'rez et al. 2007). Plates were incubated at 28 ± 0.2°C for 7 days and formation of clear halo around colonies was recorded as phosphate solubilizer. Phosphate solubilization indices (PI) were calculated by dividing the total diameter (halo zone plus colony) to colony diameter (mm).

YMB culture (100 µL) of each GMR was transferred into YMB (5 mL) amended with filter sterilized L-tryptophan (2 g L^{-1}) to detect and quantify IAA production (Parray et al. 2013). Cultures were grown on an orbital shaker (Gollen hamp, England; 150 rpm; room temperature[20-30°C]) for 96 hours, centrifuged (3,000 rpm for 30 minutes) and 2 mL of each supernatant was mixed with 4 mL of Salkowski reagent. The mixtures were kept at room temperature for 25 minutes in darkness to measure absorbance at 530 nm (Jenway, 6405 Uv/vis spectrophotometer). IAA was quantified using a standard curve of known concentrations of pure IAA (HiMedia) in un-inoculated L-tryptophan amended YMB. Uninoculated YMB supplemented with L-tryptophan was used as control.

Four YMB cultures of GMR (each 10 µL; 10^6 cells mL^{-1}) were spotted inoculated equidistantly 2 cm from the center on YMA plates amended with 0.5% sucrose and incubated at 28 ± 0.2°C for 72 hours. A four mm disc of 72 hour potato dextrose agar (PDA) culture of *Fusarium oxyporum* was placed at the center of the Petri dish and further incubated at the same temperature until fungus in the control plates (plates without bacteria) reached the edges of the plates. Eventually, the percentage of inhibition of radial growth (PIRG) of the fungus was calculated according to Siddiqui and Meon (2009).

YMB culture (100 µL; 10^6 cells mL^{-1}) of each GMR was spread on YMA plate amended with 4.4 g L^{-1} of glycine to detect HCN production (Ahemad and Khan 2012**).** Stripe of Whatman filter paper (No.1) was soaked in picric acid solution and fixed to the underside of the lid of plates. Plates were sealed with parafilm® and incubated at 28 ± 0.2°C for 5 days. Change of the filter paper from yellow to light brown, brown or reddish brown was recorded as weak, moderate and strong HCN production, respectively.

YMB cultures (10 µL; 10^6 cells mL^{-1}) of GMR were spot inoculated on nutrient agar plates supplemented with 1.5% skimmed milk powder (Ryden et al. 1973), Carboxymethyl cellulose (CMC) agar plates (Kasana et al. 2008) and chitin agar plates (Bansode and Bajekal 2006) to evaluate protease, cellulase and chitinase activity, respectively. The plates were incubated at 28 ± 0.2 ^oC for 5 days to detect clear zone formation around colonies. CMC plates were flooded with Gram's iodine for 3–5 min in darkness to visualize halo zones.

2.6 Evaluation Of Symbiotic Properties

2.6.1 Green house experiments

All the GMR were authenticated and tested for their symbiotic effectiveness on a soybean cultivar (Ethio –Yugoslavia) used for their trapping. Two more effective GMR (GMR120C and GMR125B) were selected and evaluated on two other soybean cultivars, locally called Jalele and Cheri (Bako Agricultural Research Center, western Ethiopia). A *Bradyrhizobium japonicum* TAL379, which had been commonly used in field experiments in the country, was included as reference in green house and field experiments.

Soybean seeds were surface sterilized as mentioned earlier, pre-germinated on 1.5% (w/v) water-agar plates and three seedlings were transplanted into sterile potted river sand (3 kg pot^{-1}). Each seedling was pipetted at its base with 1 mL log phase YMB culture (≈ 10^8 cells, $0.91OD^{540}$). N-fertilized (0.05% KNO_3 every week) and un-inoculated (non-nitrogen fertilized) pots were included as positive and negative control, respectively. All pots were watered (distilled H_2O) as required and weekly supplied with quarter strength N-free nutrient solution for grain legumes (Broughton and Dilworth 1970). Treatments were done in triplicate and arranged in a randomized complete block design in a greenhouse (12 hour photoperiod; 27 ± 2^oC/17 ± 3^oC day/night temperature) for 60 days. Plants were uprooted followed by nodule collection and enumeration. Shoots and nodules were dried at 70^0C for 48 hours for dry weight determination (Somasegaran and Hoben 1994). Shoot total nitrogen was determined following Kjeldahl method and the symbiotic effectiveness (SE) of the GMR was calculated according to Purchino et al. (2000).

2.6.2 Field Experiment

Symbiotic performance of GMR120C and GMR125B was also evaluated under rain fed field conditions. A soybean rhizosphere PGP *Achromobacter* sp. SR20A (Temesgen et al. 2019) was included for co-inoculation tests. An early maturing soybean cultivar (Jalele), which also showed well nodulation and growth under greenhouse conditions, was selected principally due to inconsistency in the duration of rainy season. Early maturing soybean varieties have short growing season and suitable for moisture stressed agro ecology of Ethiopia (Deresse 2019).

Field experiments were conducted at two agricultural research centers (ARC): Bako ARC and Dembi Station of Debrezeit ARC during the main cropping seasons (June to October). Dembi station of Debrezeit ARC is located at $8^o44'$ N, $38^o55'$ E and at 1930 masl in East Shoa Zone of Oromia Regional State, central Ethiopia. The average rainfall during the cropping seasons was 61, 161, 215, 101 and 9.7 mm for June, July, August, September and October, respectively. The mean monthly temperature varied from 16^oC (October) to 20^oC (June). The soil temperature of the area (determined at 5, 10 and 20 cm depth) was varied between 22 and 30^oC. Bako ARC is located at $09^o06'$ N, $37^o09'$ E and at 1650 masl in West Shoa Zone of Oromia Regional State. The average rainfall during the cropping seasons was 260.1, 222.4, 135.3, 136.5 and 71.3 mm for June, July, August, September and October, respectively. The mean monthly temperature was within the range of 19.6^oC (August) to 21.1^oC (October). The soil temperature of the area (determined at the above mentioned depths) was varied from 23^oC to 25.1^oC. Climatic data of the field sites were obtained from their respective research centers. The most probable number (MPN) of indigenous soybean rhizobia (determined by plant infection method according to Somasegaran and Hoben (1994) and the properties of composite soil samples of the field sites (determined at Nekemte Soil Laboratory and Testing Center, Western Ethiopia, following standard methods) are shown in Table 1.

Table 1
MPN of indigenous soybean rhizobial and some properties of soils of the experimental sites.

Field site	pH1:2.5	OC%	TN%	AP(ppm)	OM%	Particle size %			Texture	MPN
						sand	silt	clay		
BARC	5.02	2.17	0.175	12.85	4.21	47	33	30	Sand clay loam	6.3×10^3
DDARC	6.15	3.63	0.313	31.85	6.26	27	47	26	Loam soil	2.2×10^1

OC = organic carbon, TN = total nitrogen, AP = available phosphate, ppm = parts per million, OM = organic matter, MPN = most probable number; pH1:2.5 = pH determined by 1:2.5 Soil:H_2O ratio method; Values of soil properties are means of three replicates.

GMR120C, GMR125B and the reference *B. japonicum* were grown to late log phase (72 hours; 10^9 cells mL^{-1} YMB) and applied to surface sterilized seeds in two step method according to Singleton et al. (1990). For co-inoculation, cultures of each GMR (and reference strain) and the rhizobacterium (SR20A; $\approx 10^9$ cells mL^{-1} nutrient broth) were mixed in a 1:1 (v/v) ratio according to Yadav et al. (2011) prior to mixing with peat, a carrier.

There were 8 treatments: three separate rhizobia inoculations (GMR120C, GMR125B and TAL379), these three rhizobia each co-inoculated with SR20A, a urea applied control and a control without any input. Treatments were designed in a randomized complete block design with three replications. Urea (for N-supplied plots) and tri-superphosphate, P_2O_5 (to all plots) were applied to the soil at the rate of 46 kg ha^{-1} as recommended by conventional farmers' fertilizer recommendation level before sowing according to Argaw (2012).

Seeds were sown 10 cm apart from each other in a row (8 rows per plot; 40 cm between successive rows) at 4 cm depth on June 24 at Bako ARC, but on July 17 at Dembi station of Debrezeit ARC due to late on set of rainy season. Sixty days after planting (60 DAP), ten plants were randomly uprooted from the third border rows of each plot to count nodules, measure shoot height and determine shoot and nodule dry weights. Shoot nitrogen content was determined using Kjeldahl method (Sertsu and Bekele 2000). At physiological maturity, ten other plants were randomly collected from the two central rows of each plot to determine the number of pods per plant, number of seeds per pod and per plant, grain yield and weight of 1000 seeds at 10% seed moisture (Grain Moisture Meter Draminski®, Poland).

2.7 Data Analysis

Data were assessed by analysis of variance (ANOVA) employing SAS computer software package version 9.3 (SAS, 2000–2004). Mean separations were undertaken using the Duncan's Multiple Range Test at $p \leq 0.05$. All quantitative data sets were tested for normality using Kolmogorov Simirnov test and for variance homogeneity by Bartlett's test before being pooled for combined ANOVA (Gomez and Gomez 1984).

3. Result

3.1 Cultural characteristics of GMR

Twelve GMR that were recovered from nodulated plants showed generation time (g) and colony diameter varying from 1.5 to 6.6 hour and from 2.5 to 6 mm, respectively (Table 2). Ten of the GMR displayed fast generation rate (1.5–4 hour) with larger colony diameter (3–6 mm) and the remaining two GMR showed slower generation time (5.5–6.6 hour) with smaller colony diameter (2.5 or 3 mm). GMR with fast and slow generation time turned BTB-YMA medium into yellow and blue, respectively. Most of the GMR produced circular-gummy colonies with entire margin (CGE) except a slow growing GMR (GMR75) and two fast growing GMR (GMR120C and GMR125B) which formed filamentous-dry(less gummy) colonies with irregular margin (FDI). Three *Sinorhizobium* spp. (GMR55, GMR102 and GMR114) and the reference TAL 379 showed shinny translucent colonies with entire margin.

Table 2
Genus, colony features, acid/alkali production and generation time (g) of the GMR (*Glycine max* rhizobia)

Sr.No	GMR code	Suggested genus	Colony characterstics		BTB reaction	Mean g (hr)
			Size (mm)	Colony texture and shape[1]		
1	GMR 13	*Sinorhizobium*	5	CGE	Yellow	2.0
2	GMR45	*Sinorhizobium*	6	CGE	Yellow	1.5
3	GMR46	*Bradyrhizobium*	2.5	CGE	Blue	5.5
4	GMR55	*Sinorhizobium*	3	CG(S)E	Yellow	3.2
5	GMR57	*Sinorhizobium*	3	CGE	Yellow	3.7
6	GMR75	*Bradyrhizobium*	3	FDI	blue	6.6
7	GMR79	*Sinorhizobium*	4	CGE	Yellow	3.3
8	GMR102	*Sinorhizobium*	4	CG(S)E	Yellow	3.6
9	GMR114	*Sinorhizobium*	5	CG(S)E	Yellow	3.8
10	GMR120B	*Sinorhizobium*	5	CGE	Yellow	4.0
11	GMR120C	*Sinorhizobium*	3	FDI	Yellow	3.6
12	GMR125B	*Sinorhizobium*	6	FDI	Yellow	3.5

1 = circular-gummy colonies with entire margin, CG(S)E = circular-gummy (shinny colonies) with entire margin; FDI: Filamentous, dry (less gummy) colonies with irregular margin

3.2 Tolerance against stresses

Most fast growing GMR tolerated 4 to 6% NaCl whereas slow growing GMR tolerated ≤ 1.5% NaCl (Table 3). None of the GMR could grow at pH lower than 5, but all managed to grow at pH 9.5. Most of the GMR were able to grow at 40 ^{o}C. GMR were also varied in their intrinsic resistance against antibiotics, heavy metals and agrochemicals (Table 3). Ampicillin (200 µg mL^{-1}), Chloramphenicol (25 µg mL^{-1}), Penicillin G (200 µg mL^{-1}) and Tetracycline (10 µg mL^{-1}) were resisted by at least 50% of the GMR. However, none of the GMR grew in the presence of Erythromycine (100 µg mL^{-1}). Similarly, 75% of the GMR were sensitive to Gentamycin (20 µg mL^{-1}), Streptomycin sulfate (10 µg mL^{-1}) and Cifroflaxin (10 µg mL^{-1}). On the other hand, all the GMR and the reference *B. japonicum* resisted $ZnSO_4.7H_2O$ and $MnSO_4.H_2O$ (0.25 mM each) and $Pb(CH_3COO)_2.3H_2O$ (2.5 mM) (data not shown). However, $CuCl_2.2H_2O$ and $K_2Cr_2O_7$ (0.25 mM each) inhibited the growth of 83% and 75% of the GMR, respectively. Mancozeb (2 g L^{-1}) and curzet (2 g L^{-1}) inhibited the growth of 67 and 100% of the GMR, respectively.. Glyphosate (0.14%) also inhibited the growth of 42% of the GMR. The reference *B. japonicum* was also sensitive to the agrochemicals.

Table 3
Tolerances of the GMR and the reference *B. japonicum* SBTAL 379 against various stresses

Stress	Fast growing										Slow growing		
	GMR 13	GMR 45	GMR 55	GMR 57	GMR 79	GMR 102	GMR 114	GMR 120B	GMR 120C	GMR 125B	GMR 46	GMR 75	SBTAL 379
%NaCl* (w/v)	6	5	4	2	4	4	1.5	6	6	6	0.5	1.5	3
[1]Temperature(°C)	45	37	40	40	40	40	40	40	45	40	37	40	37
[2]pH	5.5	5	5.5	5	5	5	5	5	5	5.5	5.5	5.5	5
Antibiotics (µg mL^{-1})													
Ampicillin(200)	-	+	-	+	-	-	+	+	+	+	-	+	+
Chloramphenicol(25)	-	+	-	+	-	-	+	+	+	+	+	+	+
Gentamycin (20)	-	-	-	-	-	-	-	+	-	+	+	-	+
Naldixic acid (100)	-	+	-	-	-	-	-	+	+	-	+	-	+
Penicillin G (200)	-	+	+	+	+	+	+	+	+	+	+	+	+
Streptomycin – sulfate (10)	-	+	-	-	-	+	-	+	-	-	-	-	+
Tetracycline (10)	-	-	-	-	-	+	-	+	+	+	+	+	-
Vancomycin (15)	-	+	-	+	+	-	-	+	+	+	+	-	+
Erythromycine (100)	-	-	-	-	-	-	-	-	-	-	-	-	-
Cifroflaxin (10)	-	-	-	-	-	-	-	-	+	+	+	-	-
Heavy metals (mM)													
$CoCl_2.6H_2O$(0.5)	-	+	+	+	+	+	+	+	+	+	+	+	-
$K_2Cr_2O_7$ (0.25)	+	-	-	-	-	-	-	-	+	+	-	-	-
$CuCl_2$.2H2O(0.25)	-	-	-	+	-	-	-	-	+	-	-	-	-
Agrochemicals													
Mancozeb (2 g L^{-1})	+	-	+	-	-	-	-	-	+	+	-	-	-
Curzet (2 g L^{-1})	-	-	-	-	-	-	-	-	-	-	-	-	-
Glyphosate (1444 µg mL^{1})	-	-	+	+	+	+	-	+	+	+	-	-	-

1 represents higher growth values; 2 represent lower growth value; "+" stands for the presence of growth and "–"stands for the absence of growth in response to the stresses

3.3 Utilization Of Carbon And Nitrogen Substrates

Eleven of the 19 carbon and 6 of the 12 nitrogen sources were utilized by all of the GMR and the reference *B. japonicum.* Na-citrate was utilized relatively by fewer (58%) GMR than the rest carbon sources (Table 4). Fifty percent of the fast growing GMR were capable of metabolizing all the tested carbon and nitrogen sources where as a slow growing GMR46 failed to utilize 32% and 50% of the carbon and nitrogen sources, respectively.

Table 4
Carbon and nitrogen substrate utilization pattern of the GMR (*Glycine max* rhizobia) and TAL379

Substrates	Fast growing										Slow growing		
	GMR 13	GMR 45	GMR 55	GMR 57C	GMR 79	GMR 102	GMR 114	GMR 120B	GMR 120C	GMR 125B	GMR 46	GMR 75	TAL 379
Carbon source													
Tri-sodium -propionate	+	+	+	+	+	+	+	+	+	+	-	-	+
Maltose	+	+	-	+	+	+	+	+	+	+	-	+	+
Dextrin	+	+	+	+	+	+	+	+	+	+	-	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	-	+	+
Na-acetate	+	+	+	+	+	+	+	+	+	+	-	+	+
Na-citrate	+	+	-	+	-	-	-	+	+	+	-	+	-
Galactose	+	+	+	+	-	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	-	+	+	+	+	+	+	+	+	+
Nitrogen sources													
DL-Phenyl -alanine	+	+	+	+	+	-	+	+	+	+	-	+	+
DL-threonine	+	+	+	+	+	-	+	+	+	+	-	+	+
DL-serine	+	+	+	+	+	-	+	+	+	+	-	+	+
Lysine	+	+	+	+	-	-	+	+	+	+	-	+	-
DL-tryptophan	+	+	+	+	-	-	+	+	+	+	-	+	+
Glycine	+	+	+	+	+	-	+	+	+	+	-	+	+

+ represent utilization, - represent failure of utilization, Carbon and nitrogen sources (material and method section) utilized by all of the bacteria are excluded from the table.

3.4 Plant Growth Promoting (pgp) Traits

All the GMR and the reference *B. japonicum* produced IAA which significantly varied ($p \leq 0.05$) from 2.5 µM for GMR46 (*Bradyrhizobium*) to 116 µM for GMR45 (*Sinorhizobium*) (Table 5). Three of the GMR solubilized aluminium phosphate (SI: 1.4–1.6) with no significant variation ($p \leq 0.05$) whereas tri-calcium phosphate was solubilized by GMR13 alone (SI = 1.5) (Table 5). Fifty percent of the GMR showed protease (or cellulase) activity whereas anti-*Fusarium oxysporum* activity was demonstrated by GMR79 and GMR102 with 33% and 17% PIRG, respectively. No GMR showed iron-phosphate ($FePO_4$) solubilization, chitinase and HCN production activities .

Table 5. Various plant growth promoting (PGP) properties of GMR and the reference *B. Japonicum* TAL379

No	Isolate	$Ca_3(PO_4)_2$ SI[1]	$AlPO_4$ SI	IAA (µM)	Protease	Cellulase	PIRG[2]
1	GMR13	1.5	1.6[a]	52.62[b]	+	+	-
2	GMR45	-	-	116[a]	-	-	-
3	GMR46	-	-	2.50[e]	-	-	-
4	GMR55	-	-	38.84[c]	+	-	-
5	GMR 57	-	1.4[a]	26.42[d]	-	-	-
6	GMR79	-	-	40.02[c]	+	+	33%
7	GMR102	-	-	9.52[e]	+	-	17%
8	GMR114	-	-	25.85[d]	-	+	-
9	GMR120B	-	-	14.14[d]	+	+	-
10	GMR120C	-	1.4[a]	26.91[d]	+	+	-
11	GMR125B	-	-	23.78[d]	+	+	-
12	GMR 75	-	-	60.33[b]	-	-	-
13	TAL379	-	1.4[a]	10.23[e]	-	-	-

1 = solubilization index, **2** = percentage of inhibition of radial growth of fungus, number raised to the same letter in a column are not statistically different at $p \leq 0.05$

3.5 Symbiotic Properties

3.5.1 Green house experiments

The nodule number and dry weight (mg) induced by GMR ranged from 2 to 106 and from 17 to 201 $plant^{-1}$, respectively (Table 6). Two *Sinorhizobium* spp.(GMR120C and GMR125B) induced higher number of nodules associated with higher nodule dry weight, shoot total nitrogen percent and symbiotic effectiveness than the other GMR and the reference TAL379 on all the three cultivars of the crop. The accumulated shoot total nitrogen percent these *Sinorhizobium* spp. inoculated plants was in the range of 3.14 to 3.33 whereas their symbiotic effectiveness was in the range of 100 to 170% on their three soybean cultivars. They also improved shoot height over uninoculated negative control plants though the differences were not statistically significant among all treatments ($p \leq 0.05$). On the contrary, the other GMR and the reference *B. japonicum* showed poor nodulation (nodule number: 2 to 14) and symbiotic performance (58.5 to 100%) on all the three cultivars of soybean (Table 6).

Table 6
Greenhouse inoculation effects of the GMR and TAL379 on the three cultivars of soybean

Cultivar	Treatment	NN	NDW(mg)	SL(cm)	%TN	SDW(g)	SE(%)
Ethio-Yugslovia	GMR13	10^{c}	50^{bc}	18^{abc}	1.7^{b}	0.8^{c}	60.24^{bc}
	GMR46	15^{c}	31^{e}	17^{abc}	1.8^{b}	0.9^{c}	64.88^{bc}
	GMR55	2^{c}	27^{e}	19^{abc}	1.9^{b}	1.1^{bc}	82.92^{ab}
	GMR57	3^{c}	75^{ab}	18^{abc}	1.8^{b}	1.1^{bc}	78.05^{ab}
	GMR75	2.3^{c}	40^{cd}	16^{cd}	2.9^{a}	0.8^{c}	58.54^{bc}
	GMR79	2^{c}	17^{e}	20^{abc}	1.6^{b}	1.3^{bc}	97.56^{a}
	GMR102	3.0^{c}	29^{e}	16^{abc}	1.9^{b}	1.2^{bc}	90.24^{a}
	GMR114	14^{c}	23^{e}	14^{cd}	1.9^{b}	0.8^{c}	61.22^{bc}
	GMR120B	3^{c}	36^{e}	20^{abc}	1.9^{b}	1.2^{bc}	100.00^{a}
	GMR120C	103^{a}	61^{ab}	20^{abc}	3.3^{a}	1.4^{bc}	104.63^{a}
	GMR125B	70^{b}	87^{ab}	21^{ab}	3.2^{a}	1.4^{bc}	100.00^{a}
	TAL379	2^{c}	29^{e}	17^{abc}	1.7^{b}	0.5^{c}	39.02^{c}
	N+	0^{c}	0^{f}	21^{ab}	2.8^{a}	1.4^{bc}	100.0^{a}
	N-	0^{c}	0^{f}	19^{ab}	1.4^{b}	0.6^{c}	41.46^{bc}
Cheri	GMR120C	80^{a}	110^{b}	17^{abc}	3.14^{a}	1.8^{b}	139.5^{a}
	GMR125B	94^{a}	201^{a}	22^{ab}	3.27^{a}	2.0^{a}	160.52^{a}
	TAL379	2.3^{b}	46^{c}	20^{ab}	1.67^{b}	0.6^{c}	44.90^{c}
	N+	0^{c}	0^{d}	17^{abc}	2.93^{a}	1.3^{bc}	100.0^{abc}
	N-	0^{c}	0^{d}	12^{d}	1.40^{b}	0.8^{c}	57.89^{bc}
Jalele	GMR120C	89^{a}	146^{a}	27.0^{a}	3.30^{a}	2.3^{a}	170.0^{a}
	GMR125B	106^{a}	130^{a}	25.3^{a}	3.33^{a}	2.1^{a}	160.0^{ab}
	TAL379	2^{c}	44^{b}	20^{abc}	1.63^{b}	1.1^{bc}	85.0^{cd}
	N+	0^{c}	0^{c}	21^{ab}	2.87^{a}	1.3^{bc}	100.0^{cd}
	N-	0^{c}	0^{c}	18^{abc}	1.43^{b}	0.9^{c}	70.0^{d}

NN = nodule number, NDW = nodule dry weight, SL = shoot length, SDW = shoot dry weight and SE = symbiotic effectiveness. Numbers raised to different letters are significantly different (at $p \leq 0.05$) for each cultivar (Comparisons were made within columns among inoculants for each cultivar).

3.5.2 Field Experiments

3.5.2.1 Nodulation and growth responses of soybean

Inoculation of selected GMR120C and GMR125B singly or dually with the plant growth promoting *Achromobacter* sp. (SR20A) induced significantly higher nodule number than control treatments and reference *B. japonicum* at Dembi station of Debrezeit ARC (Table 7). At Bako ARC field site, only un-inoculated control plants and GMR120C-SR20A co-inoculated plants produced significantly different nodule

number among the treatments. The nodule number and nodule dry weight of GMR inoculated plants varied from 32–131 and from 0.45 to 1.27 (g plant^{-1}), respectively. At both field sites, N-fertilized plants produced the least number of nodules. The exotic *B. japonicum* TAL379 showed poor nodulation as revealed even under field condition with fewer MPN background soybean rhizobia. Significant variations in shoot height and total nitrogen were recorded only among few treatments even though shoot total nitrogen percent was consistently higher for GMR inoculations.

Table 7
Effects of different treatments on nodulation and growth of soybean at the two field sites

Treatment	NN	NDW (g)	SH (cm)	TN%	SDW (g)
Bako ARC field site					
GMR120C	46.11^{bc}	0.45^{a}	34.13^{a}	3.57^{a}	15.6^{abc}
GMR125B	46.22^{bc}	0.63^{a}	38.56^{a}	3.48^{a}	24.2^{a}
TAL379	42.78^{bc}	0.57^{a}	36.00^{a}	2.93^{b}	16.0^{abc}
N+	28.44^{c}	0.33^{a}	38.33^{a}	3.10^{b}	22.1^{ab}
N-	55.45^{a}	0.60^{a}	38.56^{a}	2.40^{b}	14.7^{bc}
GMR120C*SR20A	58.00^{a}	0.58^{a}	35.78^{a}	3.88^{a}	13.5^{bc}
GMR125B*SR20A	32.00^{c}	0.48^{a}	34.44^{a}	3.51^{a}	14.5^{bc}
TAL379*SR20A	43.0^{bc}	0.51^{a}	34.33^{a}	3.00^{b}	12.0^{c}
Dembi station of Debrezeit ARC field site					
GMR120C	69.67^{bc}	1.08^{ac}	40.33^{a}	3.63^{ab}	22.1^{abc}
GMR125B	116.67^{ab}	1.27^{ac}	36.33^{abc}	3.48^{abc}	15.^{ab}
TAL379	10.0^{d}	0.16^{de}	35.33^{abc}	2.90 ^{cd}	8.4^{c}
N+	4.56^{d}	0.02^{de}	25.67^{c}	3.13^{bcd}	16.8^{bc}
N-	10.37^{d}	0.08^{de}	27.00^{bc}	2.20^{d}	7.4^{c}
GMR120C*SR20A	33.0^{c}	0.88^{ac}	34.67^{abc}	3.88^{a}	15.1^{ab}
GMR125B*SR20A	131.67^{ab}	1.67^{ac}	39.33ab	3.58^{ab}	25.0^{a}
TAL379*SR20A	10.35^{d}	0.91^{de}	29.67^{abc}	2.97^{dc}	7.4^{c}

NN = nodule number, NDW = nodule dry weight, SH = shoot height, TN%= shoot total nitrogen percent, SDW = shoot dry weight. Values raised to different letters in a column are significantly different at $p \leq 0.05$.

3.5.2.2 Yield And Yield Related Responses Of Soybean

GMR inoculated plants produced higher number of pods per plant (51 to 102) but lower number of seeds per pod (1.64 to 2.08) at Dembi station of Debrezeit ARC compared to Bakoo ARC field site with 37 to 44 pods plant^{-1} and 2.07 to 2.08 seeds pod $^{-1}$. (Table 8). However, the mean number of seeds per plant (NSPPL) was higher at Dembi station of Debrezeit ARC corresponding to the higher number of pods per plant (NPP). Grain yield for GMR120C and GMR125B inoculation was varied from 3.16 to 3.98 tons ha^{-1} at the field sites. Single GMR120C inoculation resulted in higher grain yield (3.81–3.97 tons ha^{-1}) than its co-inoculation with SR20A (3.35–3.51 tons ha^{-1}) at both field sites. On the contrary, GMR125B-SR20A co-inoculations increased the grain yield from 3.16 to 3.70 and from 3.24 to 3.98 tons ha^{-1} at Bako ARC and Dembi station of Debrezeit ARC, respectively. The increase was over a half ton ha^{-1} at each field site. GMR120C

and GMR125A inoculations (individually or each with SR20A) outperformed nitrogen fertilization and the reference *B. japonicum* inoculation at both field sites. GMR inoculation increased 12–35% (Bako ARC) and 53–87% (Dembi station of Debrezeit ARC) grain yield over nitrogen fertilization. GMR inoculation also increased 30–57% (Bako ARC) and 103–149%% (Dembi station of Debrezeit ARC) grain yield over inoculation of the reference *B. japonicum* .

Table.8 Effects of different treatments on yield and related traits of soybean at the two field sites

Treatment	NPP	NSPPD	NSPPL	TSW(g)	GY (tons ha^{-1})
Bako ARC field Site					
GMR120C	37^{a}	2.07^{ab}	77^{a}	212.2^{a}	3.81^{a}
GMR125B	44^{a}	2.28^{a}	102^{a}	175.08^{a}	3.16^{a}
TAL379	39^{a}	2.17^{ab}	86^{a}	165.74^{a}	2.41^{a}
N+	39^{a}	1.91^{b}	74^{a}	180.01^{a}	2.82^{a}
N-	32^{a}	2.16^{ab}	70^{a}	169.29^{a}	2.69^{a}
GMR120C*SR20A	42^{a}	2.08^{ab}	88^{a}	171.23^{a}	3.51^{a}
GMR125B*SR20A	40^{a}	2.08^{ab}	76^{a}	191.30^{a}	3.70^{a}
TAL379*SR20A	32^{a}	2.09^{ab}	67^{a}	168.00^{a}	2.43^{a}
Dembi station of DARC field site					
GMR120C	102.0^{a}	1.64^{ad}	$159.^{ab}$	173.85^{a}	3.97^{a}
GMR125B	72^{ac}	1.87^{ac}	133^{abc}	175.35^{a}	3.24^{ab}
TAL379	35^{c}	1.54^{bd}	54^{d}	156.52^{a}	1.30^{c}
N+	64^{abc}	1.25^{d}	71^{cd}	173.0^{a}	2.11^{bc}
N-	55^{bc}	1.44^{cd}	81^{d}	162.0a	1.37^{c}
GMR120C*SR20A	51^{bc}	1.82^{ac}	96^{ac}	183.50^{a}	3.35^{ab}
GMR125B*SR20A	85^{ab}	2.08^{a}	176^{ab}	192.95^{a}	3.98^{a}
TAL379*SR20A	39^{c}	1.55^{bd}	60^{c}	163.0^{a}	1.60^{c}

NPP = number of pods per plant, NSPPD = number of pods per plant, NSPPL = number of seeds per plant, TSW = thousands of seed weight, GY = grain yield. Values raised to different letters in a column are significantly different ($p \leq 0.05$).

3.5.2.3 Mean Comparisons Of The Two Field Sites

The overall mean nodule number and mean nodule dry weight of all treatments were significantly higher at Dembi station of Debrezeit ARC than that of Bako ARC at $p \leq 0.05$ (Table 9). Statistically insignificant differences were observed in shoot height, shoot dry weight and shoot total nitrogen percent between the two field sites.

Table 9
Mean comparison of various parameters recorded for all treatments at the two field sites

Field sites	Nodulation and growth parameters				
	SH (cm)	NN	NDW (g)	SDW (g)	TN%
BARC	36.3[a]	44[b]	0.519[b]	16.575[a]	3.23[a]
DDARC	34.9[a]	81.91[a]	1.094[a]	17.308[a]	3.42[a]
	Yield and yield related parameters				
	NPP	NSPPD	NSPPL	TSW (g)	GY(tons ha^{-1})
BARC	38.13[b]	2.11[a]	80.00[b]	179.11[a]	3.066[a]
DDARC	66.67[a]	1.74[b]	127.94[a]	182.38[a]	3.076[a]

NN = nodule number, NDW = nodule dry weight, SH = shoot height, TN%= shoot total nitrogen percent, SDW = shoot dry weight, NPP = number of pods per plant, NSPPD = number of pods per plant, NSPPL = number of seeds per plant, TSW = thousands of seed weight, GY = grain yield. Values raised to different letters in a column are significantly ($p \leq 0.05$) different.

The overall mean number of pods per plant (NPP) and number of seeds per plant (NSPPL) were also significantly higher at Dembi station of Debrezeit ARC than Bako ARC ($p \leq 0.05$) corresponding to the higher nodule number and dry weight. However, significantly higher number of seeds per pod was recorded at Bako ARC than Dembi station of Debrezeit ARC ($p \leq 0.05$). No significant variations were recorded in terms of grain yield and thousand grain weight between the two field sites (Table 9).

3.5.2.4 Effect Of Replications, Gmr, Location And Gmr*location Interaction

Replication (rep) showed significant effect on shoot height, shoot TN%, on the number of seeds per plant (NSPPl) and thousands of seed weight (TSW) whereas GMR showed highly significant effect on nodule number (NN) and shoot TN% at Bako ARC field site ($p \leq 0.05$) (Table 10). On the other hand, GMR imposed highly significant effect on nodule number (NN) and nodule dry weight (NDW), shoot TN%, number of seeds per plant (NSPPL) and grain yield (GY) at Dembi station of Debrezeit ARC field site with lower MPN (2.2×10^1) of soil soybean rhizobia compared to BARC (6.3×10^3) (Table 10).. However, replication (rep) showed no significant effect on all the nodulation, growth, yield and yield related parameters at the Dembi station of Debrezeit ARC field site.

Combined ANOVA of the two field sites revealed the effect of the GMR on more nodulation, growth and yield traits of the crop than location (Loc), replication (Rep) and GMR*loc(location) interaction. GMR showed highly significant effect on NN, NDW, SDW, shoot TN%, NSPPD, NSPPL and GY (Table 10). Location showed highly significant effect on NPP and NSPPD (number of seeds per pod) whereas GMR *location interaction showed highly significant effect on NN and NDW alone. Replication (rep) showed a significant effect only on shoot height (SH).

Table 10
Effects of rep, GMR, loc and GMR*loc interactions on various parameters of soybean at the field sites.

Site		NN	NDW	SH	SDW	TN%	NPP	NSPPD	NSPPL	TSW	GY
Effects of replication (rep) and GMR **on growth, nodulation and yield**											
BARC	rep	NS	NS	*	NS	*	NS	NS	*	*	NS
	GMR	**	NS	NS	NS	**	NS	NS	NS	NS	NS
	Cv	9	12	8	10	17	7	9	7	16	22
DDARC	rep	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	GMR	**	**	NS	*	**	*	NS	**	NS	**
	cv	15	18	18	21	15	13	17	11	21	19
Combined ANOVA: Effects of location, rep, and GMR**-location interaction**											
Loc		NS	NS	NS	*	NS	**	**	NS	NS	NS
Rep		NS	NS	*	NS	NS	NS	NS	NS	NS	NS
GMR		**	**	NS	**	**	NS	**	**	NS	**
GMR *loc		**	**	*	*	NS	NS	NS	NS	NS	NS
CV		10	15	15	18	15	12	11	10	21	19

NS = not significant, *(significant), ** (highly significant) at $p \leq 0.05$

NN = nodule number, NDW = nodule dry weight, SH = shoot height, SDW = shoot dry weight, TN%=total nitrogen percent of shoot, NPP = number of pods per plant, NSPPD = number of seeds per pod, NSPPL = number of seeds per plant, TSW = thousands of seed weight, GY = grain yield, rep = replication, GMR = *Glycine max* rhizobiia, loc = location, GMR *loc = GMR-location interaction, Cv = coefficient of variance, BARC = Bako Agricultural Research Center, DDARC = Dembi Station of Debrezeit Agricultural Research Center.

3.5.2.5 Correlation Test

A significant or highly significant positive correlations were recorded among many variables (Table 10). Number of pods per plant (NPP) showed a highly significant positive correlation with nodule number, nodule dry matter, shoot total nitrogen and shoot dry matter with Person's $r \geq 0.59$. Number of seeds per pod was highly significantly correlated with number of nodules per plant and number of pods per plant whereas number of seeds per plant was significantly correlated with shoot height (Table 11). There was also a highly significant positive correlation between grain yield (GY) and nodule number, nodule dry matter, number of pods per plant and number of seeds per pod with Person's $r \geq 0.63$. Insignificant negative correlations were encountered in few cases.

Table 11. Correlations among different variables in the field trials

	SH	NN	NDW	TN	SDW	NPP	NSPPD	NSPPL	TSW	GY
SH	1									
NN	0.2	1								
NDW	-0.09	0.57*	1							
TN	-0.24	0.42	0.76**	1						
SDW	-0.23	0.42	0.77**	0.9997**	1					
NPP	0.13	0.59**	0.68**	0.76**	0.77**	1				
NSPPD	0.13	0.61**	0.43	0.56*	0.57*	0.60**	1			
NSPPL	0.57*	0.16	-0.05	0.09	0.1	0.13	0.39	1		
TSW	0.11	0.79**	0.70**	0.67**	0.67**	0.71**	0.76**	0.2	1	
GY	0.17	0.99**	0.63**	0.47*	0.48*	0.64**	0.63**	0.14	0.82**	1

*=significant correlation, **=highly significant correlation, NN=nodule number, NDW=nodule dry weight, SH=shoot height, SDW=shoot dry weight, TN%=total nitrogen percent of shoot, NPP= number of pods per plant, NSPPD= number of seeds per pod, NSPPL= number of seeds per plant, TSW= thousands of seed weight, GY= grain yield,

Discussion

Based on generation time (g), colony diameter and BTB-YMA color changes (Jordan, 1982), ten GMR are fast growing soybean rhizobia similar to earlier reports (Scholla and Elkan 1984; Young et al. 1988; Chen et al. 1988; Hungria et al. 2001) whereas the two remaining GMR are slow in line with previous reports (Sadowsky et al. 1983; Singh et al. 2013). Thus, the fast growing GMR and slow growing GMR belong to the genus *Sinorhizobium and Bradyrhizobium*, respectively (Table-2) according to the current taxonomy of soybean rhizobia though further characterizations involving genetic method should be employed to verify genus and further identify the species to which they belong.

Colony features of the GMR reported here are also revealed earlier. Irregular colonies with less exopolysaccharides (Sadowsky et al. 1983) and shiny translucent colonies with entire margin (Chen et al. 1988) for fast growing soybean rhizobia as well as irregular colonies and less exopolysaccharides (Fuhrmann 1990) and shiny translucent colonies with entire margin. (Zhang et al. 2012) for slow growing soybean rhizobia were also reported previously.

Fast growing GMR showed better salt (NaCl) tolerance than slow growers similar to most previous reports on soybean rhizobia (Sadowsky et al. 1983, Chen et al. 1988; Youseif et al. 2014). They also showed higher temperature tolerance regardless of their taxonomic position as identified by Hungria et al.(2001) and Singh et al. (2013). The whole GMR were acid sensitive failing to grow at pH lower than 5, but they are potentially adapted to alkaline environment as all grew at pH 9.5 though they originated from slightly acidic soil (pH 5.9 to 6.4). Similarly, GMR resisted some antibiotics, and heavy metals, but they were sensitive to some other antibiotics, heavy metals and recommended doses of agrochemicals. Tolerance against these chemicals impart them survival advantages in the soil as several soil microbes produce antibiotics (Huck et al. 1991), various natural and anthropogenic processes release heavy metals into soil, water, air and their interface (Masindi and Muedi 2018) and fungicide seed treatments and post seedling emergence application of herbicide like glyphosate is common in soybean production (Bierman et al. 2006). Sensitivity against agrochemicals implies the potential harmful effects of the agrochemicals on non-target soil microbial populations.

The GMR efficiently utilized most of the carbon and nitrogen sources as demonstrated via their growth. This makes possible to grow them on alternative available substrates in laboratory and it contributes to their survival in soils where such substrates may be exudated from roots or released as various microbes act on complex organic matter. Fast growers generally demonstrated utilization of wider ranges of carbon and nitrogen sources in line with other reports (Sadowsky et al. 1983; Young et al. 1988; Ansari and Rao 2014). However, a fast growing GMR102 failed to utilize 50% of the amino acids.

IAA production varied significantly among the GMR ($p \leq 0.05$) being as low as 2.5 µM for a *Bradyrhizobium* sp. (GMR46) and as high as 116 µM for a *Sinorhizobium* sp. (GMR45) falling within the range previously reported by Chen et al. (2002) for soybean rhizobia. Bacterial IAA improves the plant's accessibility to soil nutrients by increasing root length and surface area, but it suppresses plant growth when the endogenous level is optimal as plant hormones are required at very low concentration (Glick 2012). Maximum aluminium

phosphate and tri-calcium phosphate solubilization index of the GMR (1.6) was lower than previous value (3.2) reported by Jadhav (2013). Phosphate solubilizing bacteria liberate phosphorus from insoluble complex formed among phosphate anions ($H_2PO_4^{-1}$ and HPO_4^{-2}) and Fe, Al or Mn between pH 5.5 to 7.00 (Ahemad and Khan 2011). However, no GMR managed to solubilized $FePO_4$. Protease (or cellulase) activity demonstrated by 50% of the GMR might have contributed to anti-*Fusarium oxysporum* activity recorded for GMR79 (33% PIRG) and GMR102 (17% PIRG) as described by Kim et al. (2008). However, these fungal antagonistic GMR did not demonstrate chitinase and HCN production which are also known to be fungal inhibitors (Ordentlich et al. 1988; Dowling and O'Gura 1994).

GMR varied in their nodulation and associated effectiveness in which two *Sinorhizobium* spp. (GMR120C and GMR125B) were superior. The greenhouse symbiotic effectiveness (100 to 170%) recorded for these two GMR was rated as highly effective according to (Lalande et al. 1990). They were also compatible to tested cultivars of soybean, efficient in utilizing different carbon and nitrogen substrates and good in stress tolerances showing their potential ecological fitness. All of these have been considered as desirable features of inoculants (Howieson et al. 2000).

Inoculated soybean plants showed response in terms of nodulation growth, yield and yield related parameters. Inoculation of the two selected *Sinorhizobium* spp. (GMR120C and GMR125B) singly or each with the plant growth promoting *Achromobacter* sp. (SR20A) induced significantly higher nodules than the control treatments and reference *B. japonicum* at Dembi station of Debrezeit ARC. with lesser MPN of background soybean rhizobia (22) than that of Bako ARC field site soil (6.3×10^3) which can related to the higher interference of higher number of background rhizobia in the latter case. The least number of nodules recorded for N-fertilized plants at both field sites could be due to the nodulation inhibitive effect of soil nitrogen (Kinkema et al. 2006). The exotic *B. japonicum* (TAL379) showed poor nodulation as revealed even under field condition with fewer MPN background soybean rhizobia contrary to the report of Solomon et al. (2012) on the current soybean variety under field condition with no indigenous soybean rhizobia. The nodule number and nodule dry weight of GMR inoculated plants reported here were greater than those reported from India by Jaiswal et al. (2017) as a result of inoculating soybean with different rhizobia. Shoot total nitrogen percent was consistently higher for GMR inoculations unlike shoot height and dry matter.

The grain yielded due to *Sinorhizobium* spp. (GMR120C and GMR125A) inoculation reported here are much better than 2.17, 2.52 and 2.56 tons ha^{-1} reported by Merkeb et al. (2016), Abera et al. (2015) and Sisay et al. (2019), respectively. Jaiswal et al. (2017) reported even a lower grain yield ranging from 1.31 to 1.59 **tons** ha^{-1} for soybean plants inoculated with different rhizobial strains. However, Leggett et al. (2017) reported a higher grain yield (3.70 tons ha^{-1}) for several soybean inoculation under different field conditions in USA which is also comparable to the present report. Grain yield improvement is related to the establishment of effective symbiosis by the fast growing GMR (*Sinorhizobium* spp.) in line with previous reports (Dowdle and Bohlool. 1985, Isreal et al. 1986; Hungria et al. 2001) though *Bradyrhizobium* has commonly been used as soybean inoculant. Hungria et al. (2001) also stressed the great commercial interest in fast growing soybean rhizobia as it saves time for their mass production compared to slow growers.

The overall significantly lower mean nodule number ($p \leq 0.05$) accompanied by lower mean nodule dry weight recorded at Bako ARC with higher MPN of soil soybean rhizobia could be due to the interference of the higher background rhizobia as described by Thies et al. (1991), It also indicates the lower nodulating traits of the higher native soil rhizobia at Bako ARC field site. Relatively higher seed yield was recorded at Dembi station of Debrezeit ARC than Bako ARC ($p \leq 0.05$) due to higher number of pods per plant (NPP) and number of seeds per plant (NSPPL) at the former field site as pointed out by Board et al. (2003). The means of various parameters (Table 9) represent means of all treatments including controls and the ineffective reference *B. japonicum* inoculation for which lower results were recorded. So the means would be higher than the indicated values if they are computed for the effective GMR inoculations excluding values for controls and the reference *B. japonicum* inoculation.

GMR imposed significant or highly significant effect ($p \leq 0.05$) on 70% of the tested nodulation, growth, yield and yield related parameters at Dembi station of Debrezeit ARC field site compared to 20% in the case of Bako ARC site with higher MPN of indigenous soybean rhizobia. Singleton and Tavares (1986) indicated that statistically significant inoculation responses can be avoided in the presence of as few as 20 indigenous rhizobia g^{-1} of soil with some effective strains under greenhouse experimental conditions. At both field sites GMR inoculation showed highly significant effect on the number of nodules similar to the report of Samudin and Kuswantoro (2018).

Combined ANOVA of the two field sites revealed highly significant effect of the GMR inoculation on 70% of the tested nodulation, growth, yield and yield related traits of the crop in contrast to replication (Rep), location (Loc), and GMR*loc(location) interaction that showed significant or highly significant effect on 10%, 30% and 40% of the tested parameters, respectively showing inoculation of GMR was a

more important factor. Solomon et al. (2012) reported significant effect of rhizobial inoculation on the number and dry matter of nodules, plant dry matter and total nitrogen, number of seeds per pod and per plant, and on grain yield similar to the current report. However, the authors reported highly significant effect of the rhizobial strains on the thousands of seed weight (TSW) contrary to the current report.

The higher nodulation trait of the two *Sinorhizobium* spp. (GMR120C and GMR125B) had contributed to yield improvement as the number of nodules was highly significant correlated with the number of pods plant^{-1} (r = 0.59), number of seeds per pod (r = 0.61) and grain yield (r = 0.99) implying they have better infection trait to induce nodules and better symbiotic effeteness to fix nitrogen.

Conclusion

The indigenous *Glycine max* [L] Merr. rhizobia (GMR) were diverse as revealed by cultural, stress tolerance and symbiotic features. Most of them grew faster and produced acid with larger colonies and hence identified as *Sinorhizobium* spp. members of the *Sinorhizobium* spp. metabolized wider range of substrates, showed better tolerance to higher salt (NaCl) concentration and elevated temperature. However, only two *Sinorhizobium* spp. (GMR120C and GMR125B) well nodulated different cultivars of soybean (green house) and improved grain yield under field conditions showing their potential to be used as inoculant. For better outcome, GMR120C should be inoculated singly whereas GMR125B should be co-inoculated with the PGP *Achromobacterium* sp. Sequence analysis of genes like 16S rRNA should be performed to verify the suggested taxonomic placement of the GMR.

List Of Abbreviations

ARC – Agricultural Research Center

BTB – Bromothymol Blue

GMR – *Glycine max* rhizobia

IAA – Indol 3- Acetic Acid

MPN – Most Probable Number

PGP – Plant growth promoting

PIRG – Percentage of Inhibition of Radial Growth

YMA – Yeast extract manitol agar

YMB - Yeast extract manitol broth

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The datasets generated and/or analysed during the current study are available in the Addis Ababa

University repository. The study soybean rhizobia are deposited in Soil and Environmental

Microbiology Laboratory, College of Natural Sciences, Addis Ababa, Ethiopia

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Both authors identified the research problem and prepared the research design. DT performed laboratory and field experimental works under the supervision of FA. Both authors wrote, read and approved the manuscript..

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Figures

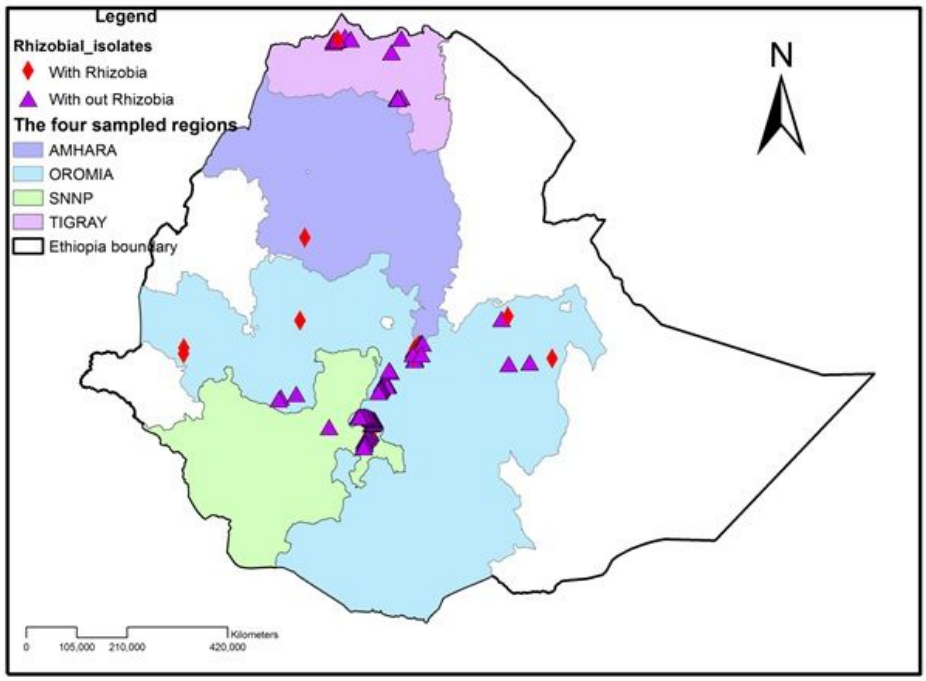

Figure 1

Map of Ethiopia showing soil sampling sites identified as those with or without soybean rhizobia.