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Inoculation of native symbiotic effective *Sinorhizobium* spp. enhanced soybean [*Glycine max* (L.) Merr.] grain yield in Ethiopia

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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. They 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 Duncan's Multiple Range Test at $p \leq 0.05$. Phenotypic variability of the test bacteria was undertaken using PAST4.03 Computer Software.

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 co-inoculated 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.

Keywords: Ethiopia, Native *sinorhizobium*, Soybean, Stress tolerance, Nodulation, Grain yield

Background

Soybean is an annual leguminous crop which was initially domesticated in East Asia, and is principally cultivated in North and Latin America at present time. It has

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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 al1982; Scholla and Elkan 1984; Chen et al. 1988,1995; 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 increasing 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 was 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.

Materials and methods

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 m 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 °C (Somasegaran and Hoben 1994). Isolated bacteria were designated GMR (*Glycine max* rhizobia), checked for purity via subculturing and preserved at 4 °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.

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

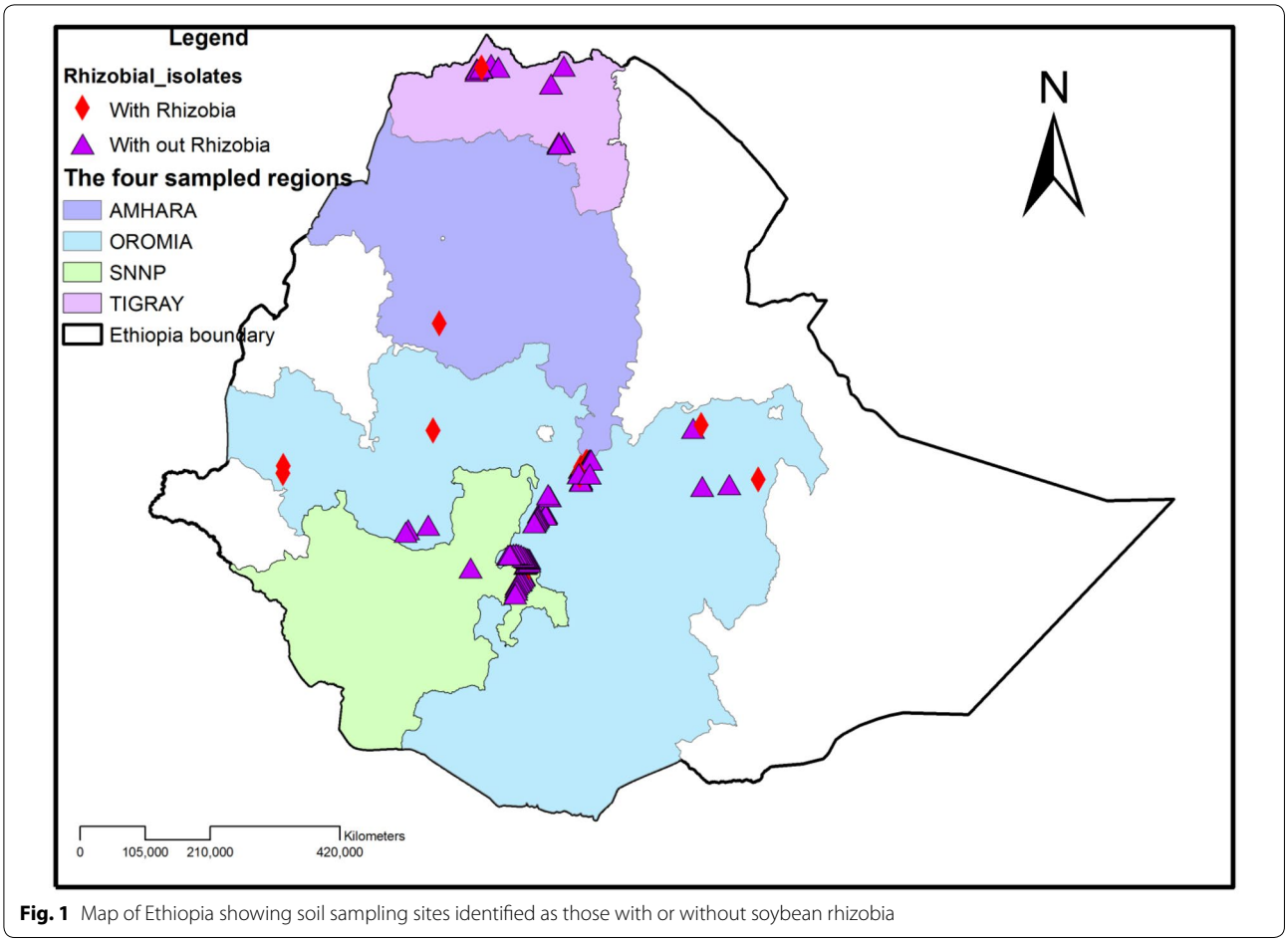

Fig. 1 Map of Ethiopia showing soil sampling sites identified as those with or without soybean rhizobia

active yeast extract mannitol broth (YMB) culture (10 µL; 10^6 cells mL^{-1}) followed by incubation at 28 ± 0.2 °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 °C); 72 h]. Samples were taken every 2 h 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).

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 °C (Lupwayi and Haque 1994). They were also streaked on YMA plates and incubated at 35 °C, 37 °C, 40 °C and 45 °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.

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, inositol, 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.

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 h, centrifuged (3,000 rpm for 30 min) and 2 mL of each supernatant was mixed with 4 mL of Salkowski reagent. The mixtures were kept at room temperature for 25 min 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. Un-inoculated 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 h. A 4 mm disc of 72 h 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 °C 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.

Evaluation of symbiotic properties

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 ($\approx -10^8$ 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 h photoperiod; 27 ± 2 °C/17 ± 3 °C day/night temperature) for 60 days. Plants were uprooted followed by nodule collection and enumeration. Shoots and nodules were dried at 70 °C for 48 h 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).

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° 44′ N, 38° 55′ 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 °C (October) to 20 °C (June). The soil temperature of the area (determined at 5, 10 and 20 cm depth) was varied between 22 and 30 °C. Bako ARC is located at 09° 06′ N, 37° 09′ 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 °C (August) to 21.1 °C (October). The soil temperature of the area (determined at the above mentioned depths) was varied from 23 to 25.1 °C. 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.

GMR120C, GMR125B and the reference *B. japonicum* were grown to late log phase (72 h; 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. Seed sowing delay at the latter was due to delay in the beginning of rain though it should be sown in June as at Bako ARC where rain started at the usually time. 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

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

(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).

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). Phenotypic variability of the test rhizobia (GMR and TAL379) was analyzed using multivariate classical cluster analysis and a paired group (UPGMA) algorithm phenotypic dendrogram with Jaccard similarity index was constructed employing PAST4.03 Computer Software based on 79 phenotypic traits.

Result

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 h and from 2.5 to 6 mm, respectively (Table 2). Ten of the GMR displayed fast generation rate (1.5–4 h) with larger colony diameter (3–6 mm) and the remaining two GMR showed slower generation time (5.5–6.6 h) 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.

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 they all managed to grow at pH 9.5. Most of the GMR were able to grow at 40 °C. GMR were also varied in their intrinsic resistance against antibiotics, heavy metals and agrochemicals (Table 3). GMR13, GMR120B, GMR120C and GMR125B combined higher salt (6% NaCl) and higher temperature (40–45 °C.) tolerance traits. 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 when exposed to Erythromycin (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}). GMR13 was sensitive to all the tested antibiotics followed by GMR55 and GMR79 that showed sensitivity against 90% and 80% of the antibiotics. On the contrary, GMR120B resisted 80% of the tested antibiotics. Similarly, all the GMR and

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 characteristics		BTB reaction	Mean g (h)
			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

Table 3 Tolerances of the GMR and the reference *B. japonicum* (TAL379) 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	TAL 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 highest growth values; 2 represents lowest growth value; "+" stands for the presence of growth and "–" stands for the absence of growth in response to the stresses

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. With regard to specific GMR, GMR120C resisted all tested heavy metals followed by GMR57 and GMR125B that resisted 83% of the evaluated heavy metals. All the remaining GMR resisted 67% of the tested heavy metals. 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 sensitive to all the agrochemicals similar to GMR45, GMR46, GMR75 and GMR114.

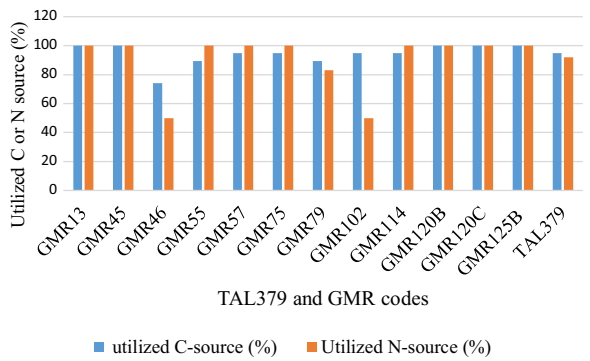

Fig. 2 Percentage of carbon and nitrogen sources utilized by the GMR and the reference TAL379

Utilization of carbon and nitrogen substrates

Six fast growing GMR (GMR13, GMR45, GMR120B, GMR120C and GMR125B) were able to utilized the entire tested carbon sources (19) and nitrogen sources (12) (Fig. 2).Three other fast growing GMR (GMR55, GMR57 and GMR114) and a slow growing GMR (GMR75) were also able to utilize all the tested nitrogen sources. However, a slow growing GMR (GMR46) utilized the least percentage of carbon source (74%). It also utilized the least percentage of nitrogen sources (50%) together with a fast growing GMR102. Na-citrate and lysine were the carbon and nitrogen sources, respectively, that were utilized relatively by less number of bacteria

than the other carbon or nitrogen sources. The reference *B. japonicum* (TAL379) utilized over 90% of the carbon and nitrogen sources.

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*) (Fig. 3). Twenty five percent 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). Fifty eight and fifty percent of the GMR showed protease and cellulase activity, respectively. Two (17%) of the GMR; GMR79 and GMR102 showed anti-*Fusarium oxysporum* activity with 33% and 17% inhibition of radial growth (PIRG), respectively. However, no GMR showed iron-phosphate ($FePO_4$) solubilization, chitinase and HCN production activities (Fig. 3). The reference *B. japonicum* (TAL379) solubilized aluminium phosphate with SI of 1.4 and recorded a lower level of IAA (10.23 µM).

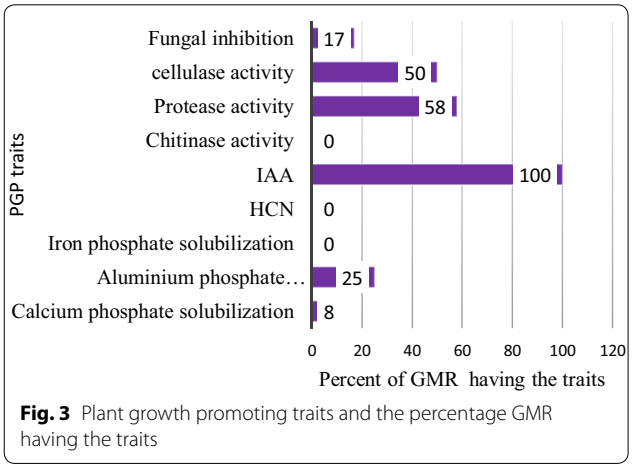

Fig. 3 Plant growth promoting traits and the percentage GMR having the traits

Phenotypic variability

In the dendrogram constructed based on phenotypic similarities of the GMR plus TAL379, a *Bradyrhizobium* sp. (GMR46) was separated from the rest at about 57% level of similarity (Fig. 4). GMR46 was distinguished from the rest bacteria mainly due to its ability to utilize narrower range of substrates and its sensitivity against salt and pesticides. The remaining rhizobia were separated into a major cluster (GMR13 and two sub clusters [II and III]) and a minor cluster (I) at 75% similarity level. Cluster I consisted two *Sinorhizobium* spp. (GMR57 and GMR114) and a *Bradyrhizobium* sp. (GMR75). Members this cluster were characterized

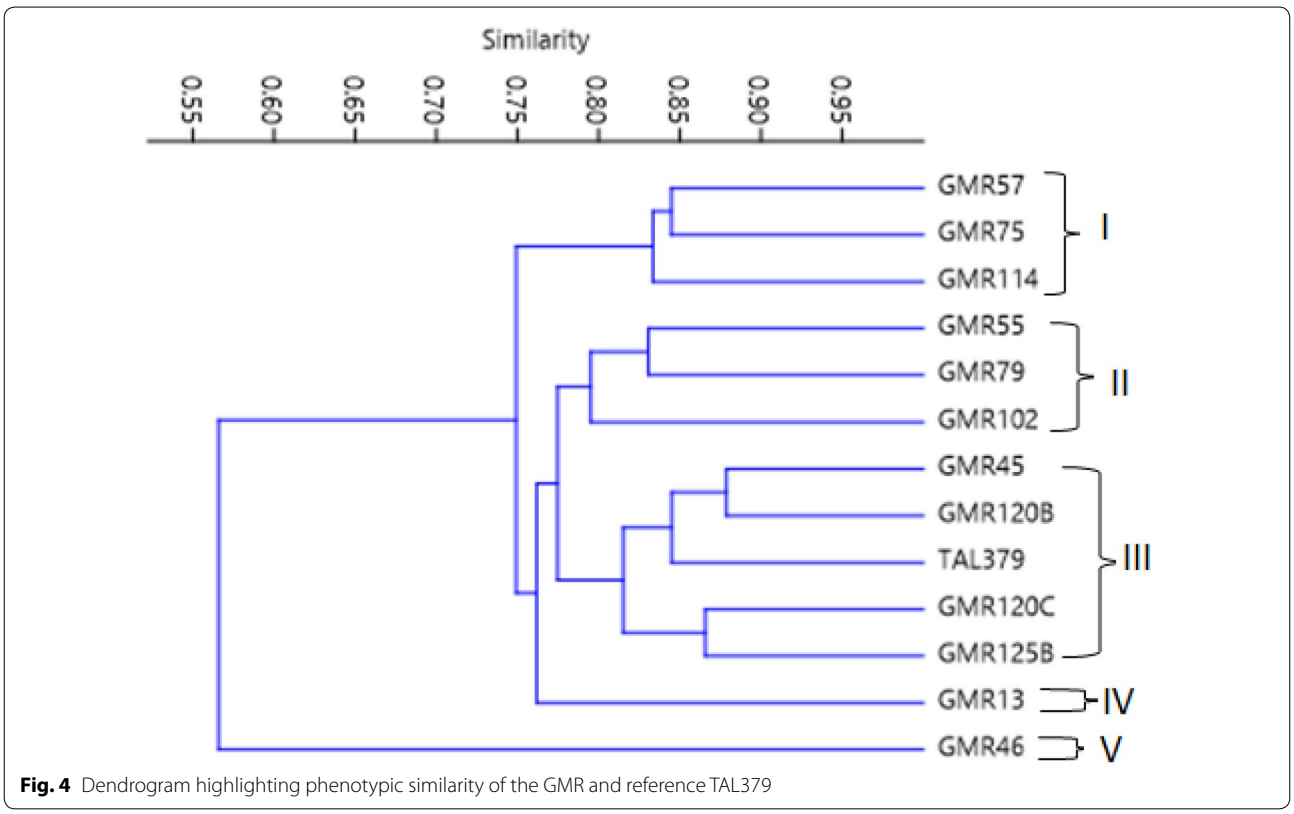

Fig. 4 Dendrogram highlighting phenotypic similarity of the GMR and reference TAL379

by efficient utilization of substrates and with relatively higher antibiotic sensitivity and resistance against heavy metals. Cluster II comprised three *Sinorhizobium* spp. (MRR55, GMR79 and GMR102) which were characterized by antibiotic sensitivity and tolerance against higher salt concentration and heat level traits. Cluster III consisted four *Sinorhizobium* spp. and TAL379 which shared features like utilization of wide range of substrates and relatively good tolerance against antibiotics and higher salt concentration. Symbiotic effective *Sinorhizobium* spp (GMR120C and GMR125B) were also included in cluster III and separated from each other at about 87% similarity level. The test rhizobia formed no cluster beyond about 88% similarity level. GMR13 was separated from all the test bacteria at about 76% similarity level and it was unique in being sensitivity to all the tested antibiotics.

Symbiotic properties

Green house experiments

Plants inoculated with GMR120C and GMR125B showed good growth with greener leaves (Fig. 5). On the other hand, TAL379 inoculated or negative control plants showed stunted growth with yellowish leaves. The positive control (N-fertilized) plants showed medium growth and in the greenness of their leaves.

The nodule number and dry weight (mg) induced by GMR ranged from 2 to 106 and from 17 to 201 plant^{-1}, respectively with statistically significant variation at $p \leq 0.05$ (Table 4). The minimum nodule number

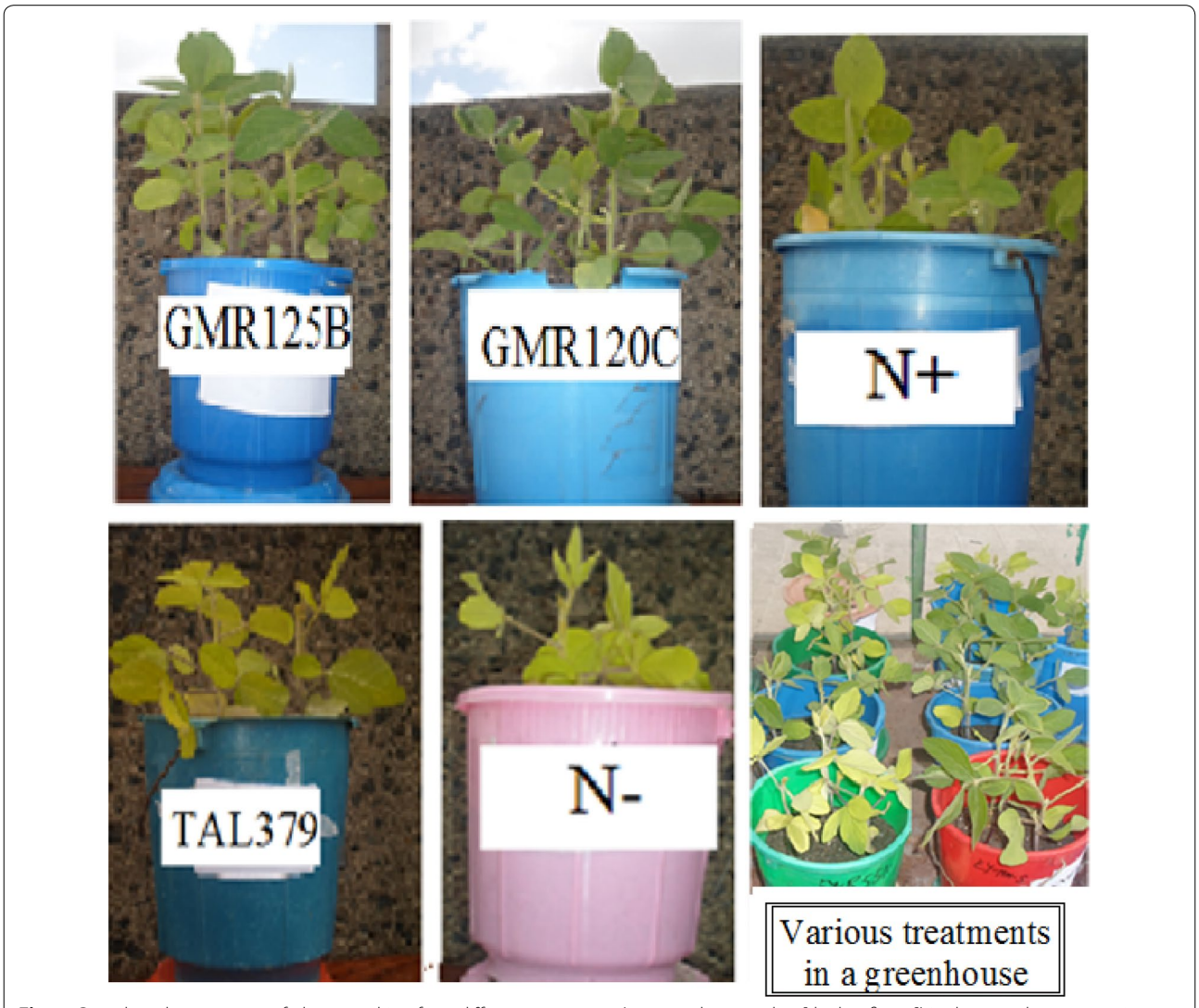

Fig. 5 Growth and appearance of plants resulting from different treatments (separated or together [the last figure]) in the greenhouse experiments

Table 4 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, *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)

and nodule dry weight was recorded for GMR79. 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 of 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 the three soybean cultivars. The symbiotic effectiveness was higher on cultivar Jalele and Cheri than Ethio-Yugslovia. The *Sinorhizobium* spp 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) with symbiotic performance ranging from 58.5 to 100% on the three cultivars of soybean (Table 4). As expected, un-inoculated control plants produced no nodules.

Field experiments

Nodulation and growth responses of soybean GMR120C and GMR125 inoculated plants produced abundant nodules with pinkish interior (Fig. 6b) grew well with greener leaves and more branches (Fig. 6a). Positive and negative control plants showed relatively poor growth with yellower leaves (Fig. 6c, e). Similarly TAL379 inoculated plants showed less growth with fewer small and white nodules (Fig. 6d; white arrows).

Inoculation of the two selected *Sinorhizobium* spp. (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 5). At Bako ARC field site, only un-inoculated and N-fertilized unsupplied control plants and GMR120C-SR20A co-inoculated plants produced significantly different or higher nodule number among the treatments. The nodule number and nodule dry weight of GMR inoculated plants was in the range of 32–132 and 0.45–1.67 (g plant^{-1}), respectively. The

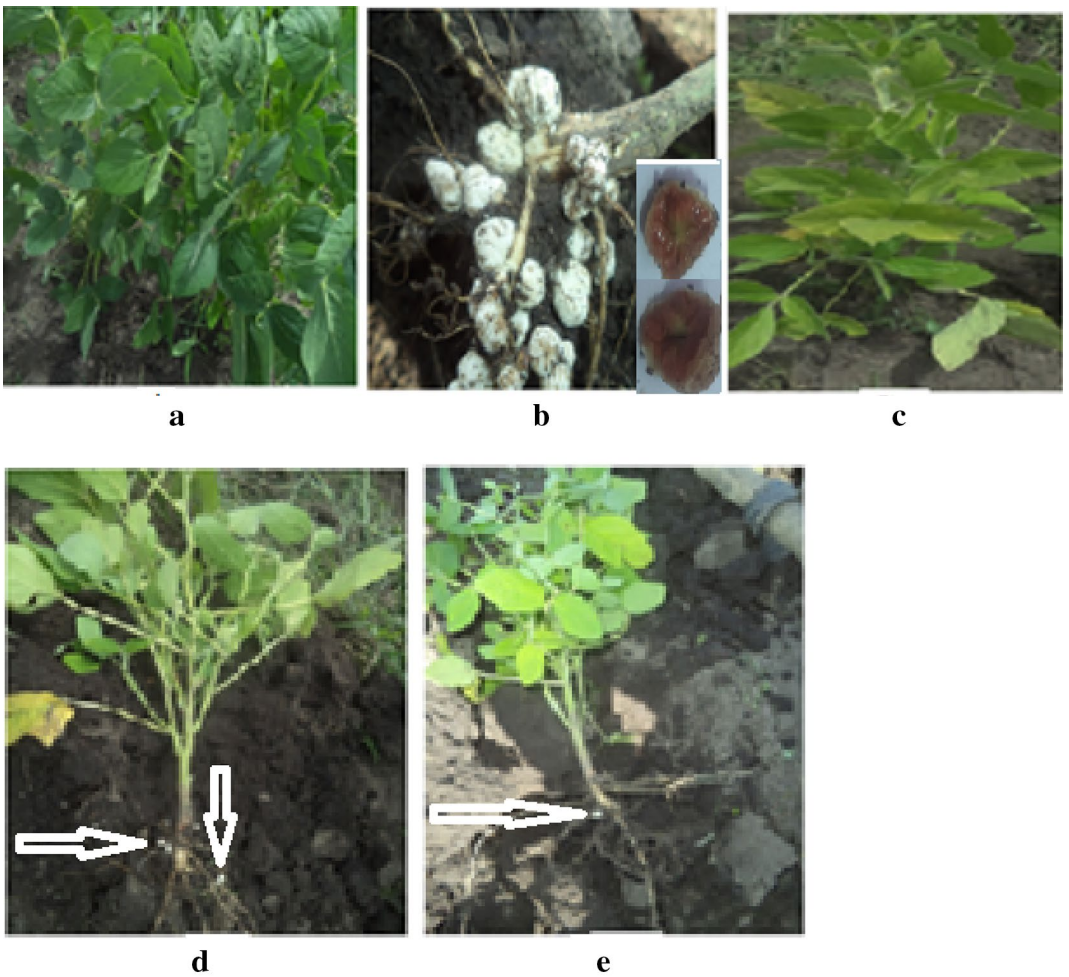

Fig. 6 Appearance of selected GMR inoculated plants (**a**) and their nodules including interior sections (**b**), N-supplied plants (**c**), uprooted plants inoculated with TAL379 (**d**) and uprooted negative control plants (**e**) under field condition. The white arrows pointed towards small white nodules

maximum nodule number associated with maximum nodule dry weight was recorded for plants co-inoculated with GMR125B and SR20A. At both field sites, N-fertilized plants produced the least number of nodules. Variations were also recorded in shoot height, total nitrogen percent and dry weight among the treatments at both field sites, but the variations were statistically significant ($p \leq 0.05$) only in few cases though shoot total nitrogen percent was consistently higher for GMR inoculations. The exotic *B. japonicum* TAL379 showed poor nodulation as revealed under field condition with fewer MPN background soybean rhizobia. Under such field site, TAL379 also accumulated lower shoot total nitrogen percent and shoot dry weight than GMR inoculated singly or dually with the PGP *Achromobacter* sp.

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 Bako ARC field site with 37 to 44 pods plant^{-1} and 2.07 to 2.08 seeds pod $^{-1}$. (Table 6). 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). The maximum number of pods plant^{-1} was recorded for GMR120C inoculated plants at Dembi station of Debrezeit ARC field site. But, the maximum number of seeds pod^{-1} and number of seeds plant^{-1} was recorded for GMR125 inoculated plants at Bako ARC field site and for GMR125B-SR20A co-inoculated plants at Dembi station of Debrezeit ARC field site, respectively. 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,

Table 5 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 $p \leq 0.05$

Table 6 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}	162.0^{a}	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 ($p \leq 0.05$)

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.* Statistically insignificant variations were also encountered in thousands of seed weight among different treatments at both field sites (Table 6).

Mean comparisons of various parameters recorded at 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 7). Statistically insignificant differences were observed in shoot height, shoot dry weight and shoot total nitrogen percent between the two field sites.

The overall mean number of pods plant^{-1} (NPP) and number of seeds plant^{-1} (NSPPL) were 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 7).

*Effects of replications, GMR, location and GMR*location interaction* Replication (rep) showed significant effect on shoot height, shoot TN%, on the number of seeds plant^{-1} (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 8). On the other hand, GMR imposed highly significant effect on nodule number (NN) and nodule dry weight (NDW), shoot TN%, number of seeds plant^{-1} (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 Bako ARC with 6.3 $X10^{3}$ MPN of soil soybean rhizobia (Tables 1 and 8). However, replication (rep) showed no significant effect on all the nodulation, growth, yield and yield related parameters at 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),

Table 7 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

Table 8 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

replication (Rep) and GMR*Loc interaction. GMR showed highly significant effect on NN, NDW, SDW, shoot TN%, NSPPD, NSPPL and GY (Table 8). Location showed highly significant effect on NPP and NSPPD whereas GMR*Loc interaction showed highly significant effect on NN and NDW alone. Replication (rep) showed a significant effect only on shoot height (SH).

Correlation test A significant or highly significant positive correlations were recorded among many variables (Table 9). Number of pods $plant^{-1}$ (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 pod^{-1} was highly significantly correlated with number of nodules $plant^{-1}$ and number of pods $plant^{-1}$ whereas number of seeds $plant^{-1}$ was significantly correlated with shoot height (Table 9). There was also a highly significant positive correlation between grain yield (GY) and nodule number, nodule dry matter, number of pods $plant^{-1}$ and number of seeds pod^{-1} with Person's $r \geq 0.63$. Insignificant negative correlations were encountered in few cases.

Table 9 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 authentication tests, generation time (g), colony diameter and BTB-YMA color changes (Jordan, 1982), ten GMR were identified as 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 were identified as slow growing soybean rhizobia 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 the genera and further identify the species to which they belong.

Features of the GMR like 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. This shows GMR reported here and previously have diverse colony features.

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). GMR also showed higher temperature tolerance regardless of their taxonomic position as identified by Hungria et al. (2001) and Singh et al. (2013) though the highest growth temperature (45 °C) was recorded for two fast growers. Mechanisms which enhance salt (NaCl) tolerance in bacteria include up take $K^{\div}$, synthesis and increase in intracellular glutamate, proline and glycine betaine (Rudulie, and Bernard 1986). Increased levels of chaperones was also indicated as a method of rhizobial adaptation to higher temperature as higher amounts of chaperones prevent protein denaturation and permit proper protein folding and mRNA stability (Alexandre and Oliveira 2010).. 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). pH impacts microbial cell growth and functions via influencing protein and nucleic acid syntheses, nutrient uptake, degradation and utilization (Guan et al. 2013). Similarly, GMR resisted some antibiotics and heavy metals, but they were sensitive to some other antibiotics, heavy metals and recommended doses of agrochemicals. Rhizobia heavy metal tolerance mechanisms encompass heavy metal adsorption and accumulation, and release of some enzymes and bioactive metabolites to facilitate their removal (Hao et al 2014) Antibiotic modification, antibiotic efflux and target modification constitutes bacterial antibiotic resistance mechanism (Peterson and Kaur 2018) Degradation of some fungicides by rhizobia was also reported by Moawad et al (2014). 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). Generally, GMR were sensitivity to the tested agrochemicals implying the potential harmful effects of the chemicals on non-target soil microbial populations.

GMR efficiently utilized most of the carbon and nitrogen sources as demonstrated via their growth. This is important to grow them on alternative available substrates in laboratory and can contribute to their survival in soils where such substrates may be exudated from roots or released as a result of decomposition of complex soil 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. Variation in bacterial utilization of organic substrates relies on their physiological capacities and substrate complexity (Koranda et al. 2014).

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). GHR were not good phosphate solubilizers as the 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) and no GMR managed to solubilize $FePO_4$. Phosphate solubilizing bacteria liberate phosphorus from insoluble complexes 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). Bacterial solubilization of inorganic phosphate is associated with the production of low molecular weight organic acid (Rodriguez et al. 2006). Protease activity demonstrated by 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).

Cluster analysis based on phenotypic traits separated a *Bradyrhizobium* sp. (GMR46) from the rest rhizobia at lower level percent of similarity level as it was unique in being sensitive to all the test antibiotics. *Bradyrhizobium* spp. and *Sinorhizobium* spp were also clustered together implying that taxonomically distinct bacteria can share large percentage of phenotypic similarities. No clustering was formed above about 87% similarity level indicating the existence of phenotypic distinctiveness among the GMR.

GMR varied in their nodulation and associated effectiveness in which two *Sinorhizobium* spp. (GMR120C and GMR125B) were superior. Abundant nodulation of the two selected *Sinorhizobium* spp. (GMR120C and GMR125) inoculated plants indicates their higher infective traits. Their inoculation resulted in well growth of plants with greener leaves indicating their promising symbiotic performance. 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 the three experimental cultivars of soybean and the co-inoculated PGP *Achromobacterium* sp. (SR20A), 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).

Field 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) resulted in good growth of plants with greener leaves and abundant branches implying their effectiveness. Their single or co-inoculation 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 (2.2×10^1) than that of Bako ARC field site soil with 6.3 $X10^3$ MPN of soybean rhizobia. This is likely due to the higher interference of higher number of background soybean rhizobia in the latter case as previously described by Singleton and Tavares (1986). The nodules induced by GMR120C and GMR125B were well developed and pink indicating they are Effective nodules according to Kukkamalla1 and Vardhan (2016). The least number of nodules recorded for N-fertilized plants at both field sites could be due to the nodulation inhibitive effect of soil available nitrogen (Kinkema et al. 2006). 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 exotic *B. japonicum* (TAL379) showed poor nodulation as revealed even under field condition with fewer MPN background soybean rhizobia. Moreover, TAL379 induced small white nodules indicating, the strain is not only less infective but also ineffective under the given field conditions. However, Solomon et al. (2012) reported the formation of abundant nodules on the currently reported soybean cultivar under field conditions but having no indigenous soybean rhizobia. Exotic

rhizobial inoculants often fail to improve growth and yield leguminous crops as a result of highly competitive indigenous rhizobia in the soil, strain-cultivar incompatibility or unfavorable environmental conditions (Hungria et al., 2009).

The grain yielded due to *Sinorhizobium* spp. (GMR120C and GMR125A) inoculation reported here (3.16–3.97 tons ha^{-1}) 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 soybean 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 showed 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 impact 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 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 7) 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 were 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). Replication (rep) showed significant effect on few (Bako ARC) and on none of (Dmbi Station of DAR) the tested nodulation, growth, yield and related parameters indicating it was not as important factor as GMR inoculation.

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 interaction that showed significant or highly significant effect on 10%, 30% and 40% of the tested parameters, respectively implying 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 pod^{-1} and $plant^{-1}$, 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, the overall phenotypic variability analysis 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 temperature levels. 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. as shown in the field experiment. Sequence analysis of genes like 16S rRNA should be performed to verify the suggested taxonomic placement of the GMR.

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 mannitol agar; YMB: Yeast extract mannitol broth.

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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 the manuscript. Both authors read and approved the final manuscript.

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Availability of data and materials
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.

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