ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

Genetic diversity of Rhizobia and Rhizobacteria from Soybean [*Glycine max* (L) Merr.]: Implication for the Commercial Production and Application to Enhance Soybean Production under Low Input Agriculture in Ethiopia

By

Diriba Temesgen Dagaga

A Thesis Presented to the School of Graduate Studies of the Addis Ababa University in Partial Fulfillment of the Requirements for the PhD Degree in Biology (Applied Microbiology)

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Principal Supervisor: Dr. Fassil Assefa
Co-supervisors: Prof. James, E.K., Dr. Maluk, M., Dr. Iannetta P.P.M.

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Dedication

This work is dedicated to my beloved late father, Temesgen Dagaga, and my beloved mother, Debritu Zegeye, who took me to school and continuously encouraged me to become self confident student when I was not much aware of the value of education.

Declaration

I declare that the thesis hereby submitted by me for the Degree Doctor of Philosophy (PhD) in Biology (Applied Microbiology) to the School of Graduate Studies of Addis Ababa University is my own independent work and has not previously been submitted by me or anybody else where. Any material obtained from other sources is duly acknowledged in the thesis. Signed on May 10, 2017, the School of Graduate Studies, Faculty of Life Sciences, Addis Ababa University.

PhD Candidate _____

Diriba Temesgen

Acknowledgements

I would like to express my gratitude and appreciation first to my advisor, Dr. Fassil Assefa for his unreserved guidance, help, supervision and constructive comments from the identification of the problem of the study to the completion of the work expending his valuable time and energy even on holidays. He also let me use his own car for part of the work and contacted his friends and other institutes to support the study. He treated me friendly and shared with me a lot of his rich research and professional experiences that can contribute a lot to my future career. His critical comments in writing up the paper have really increased my efficiency and confidence.

I would like acknowledge Mada Walabu University for sponsoring me. I also acknowledge the Department of Microbial, Cellular and Molecular Biology and the School of Graduate Studies of Addis Ababa University for funding the study and/or cooperations when requested at different phases of the study.

I would also like to appreciate Bako and Debrezeit Agricultural Research Centers for providing land and technical assistance for the field work. The help of Mr. Zerihun Abebe of Bako Agricultural Research Center in designing the field work is highly appreciable.

I am glad to extend my gratitude to the James Hutton Institute, Scotland, UK for letting me perform the molecular identification of my bacterial isolates. I am grateful to Dr. Pete Iannetta and Prof. Euan James of the institute who allowed me to use their lab and resources, and taught me molecular works. The willingness and help of Suzan (Lab. Manager), Dr. Marta Maluk and Sheena Lamond is also highly acknowledged. The respect and friendly approach of other staff members of the institute and people of the Scotland, UK is unforgettable.

I would like to thank Dr. Zerihun Belay, Dr. Fekadu Shimekit, Mr. Bekele Serbesa and Mr. Girmaye Kenesa for their help in data analysis and other friends including Mr. Getaneh Tesfaye who helped me directly or indirectly during the study.

My deepest appreciation goes to my family; my mother (Debritu Zegeye), my wife (Yenu Getu), my daughter (Sena Diriba) and my son (Umama Diriba), my brothers (Kure Temesgen, Haymanot Awugichewu) and Zenebe Alemayehu) and sisters (Wude Awugichew, Yeshi Bossera and Sukare Alemayehu), my brother-in-law (Tashale Getu) and his wife (Aynalem File), my father-in-law (Ato Getu Higisa), mother-in-law(W/o Yeshi Bekele), and sister-in-law (Sintayo Getu and Zenebech Ejigu) who helped me a lot in different aspects throughout the study. I would be so much happier if my father, Temesgen Dagaga, is alive to see the eventual fruit of what he sowed earlier.

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Acronyms

ACC	aminocyclopropane-1-carboxylic acid
BARC	Bako Agricultural Research Center
BIDCO	Business and Industrial Development Cooperation
BNF	Biological nitrogen fixation
BTB	Bromothymol blue
CV	coefficient of variance
DDARC	Dembi station of Debrezeit Agricultural Research Center
DNA	Deoxyribonucleic acid
FAO	Food and Agriculture Organization of the United Nations
GPS	Global Positioning System
GY	Grain yield
IAA	Indole acetic acid
MEGA7	Molecular Evolutionary Genetics Analysis version 7.0
NBRIP	National Botanical Research Institute's phosphate growth medium
NCBI	National Center for Biotechnology Informational (US)
NDW	nodule dry weight
NN	nodule number
NPP	number of pods per plant
NSPPD	number of seeds per pod
NSPPL	number of seeds per plant
PDA	potato dextrose agar
PGP	Plant Growth Promoting
PGPR	Plant Growth Promoting Rhizobacteria
rpm	Revolutions per minute
rRNA	Ribosomal RNA
SAS	Statistical Analysis System
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SDW	shoot dry weight
SE	symbiotic effectiveness
SL	shoot length

SNB	Soybean nodulating bacteria
SR	Soybean rhizobacteria
TGx	Tropical Glycine Cross
TN	Total nitrogen
TSW	thousands of seed weight
USD	United states dollar
USDA	United States Department of Agriculture
YMA	Yeast extract mannitol agar

Genetic diversity of Rhizobia and Rhizobacteria from Soybean [*Glycine max* (L) Merr.]: Implication for the Commercial Production and Application to Enhance Soybean Production under Low Input Agriculture in Ethiopia

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Abstract

Soybean [*Glycine max* (L) Merr.] is a nutritious crop used as food, feed and a raw material for manufacturing various products. Soybean improves soil fertility due to its association with symbiotic bacterial groups known as *Bradyrhizobium, Rhizobium*/*Sinorhizobium* and *Agrobacterium* species. It is also associated with diverse plant growth promoting rhizobacteria (PGPR) that enhance its health, growth and productivity. Soybean is widely grown in the lowlands regions of Ethiopia with average yield of about 2.0 tons ha^{-1} compared to 2.70 tons ha^{-1} of world average. The low yield of soybean in the country is predominantly attributed to low soil fertility associated with the absence of effective indigenous rhizobia that nodulate and fix enough nitrogen to the host. Attempts to inoculate the crop with exotic rhizobia showed inconsistent and unsatisfactory results that necessitated the search for effective local rhizobia adapted to ecological conditions of the country. To this end, 140 soil samples were collected from various sites of Ethiopia to screen for symbiotically effective soybean rhizobia, and plant growth promoting rhizobacteria (PGPR). The rhzobial isolates were trapped, authenticated and tested for their symbiotic effectiveness using three soybean varieties (Cheri, Ethio-Yugoslavia and Jalele) under greenhouse conditions. The PGPR were screened *in vitro* for their multiple plant growth

promoting traits and potential ecological adaptations. The diversity of the selected rhizobia and PGPR was studied using their phenotypic (numerical taxonomy), and genotypic characters via sequence analysis of 16S rRNA (and some other genes of rhizobia). The most effective rhizobia and the most versatile PGP *Achromobacter* were inoculated on a soybean cultivar (Jalele) to evaluate their effect on nodulation, growth and yield of the crop against a standard soybean inoculant *Bradyrhizobium japonicum* SBTAL379 under field conditions. The result showed that only 18 soil samples (13%) induced nodulation on the host variety from which 21 bacterial isolates were authenticated as soybean rhizobia. The isolates were equally distributed into fast growing (11) and slow growing (10), and grouped under the genus *Rhizobium* and *Bradyrhizobium,* respectively as classified previously. Based on genetic characters, a fast growing isolate (SNB 41) was identified as *Rhizobium*/*Agrobacterium* sp. whereas three slow growing isolates (SNB57B, SNB70 and SNB120A) were identified as *Bradyrhizobium* spp. Likewise, the representative PGPR isolates were also classified into seven genera; six under Proteobacteria (Gram negative): A*chromobacter*, *Acinetobacter*, *Enterobacter*, *Microbacterium*, *Pseudomonas* and *Stenotrophomonas*; and one under the Firmicutes (Gram positive): *Bacillus.* The isolates under the genera *Pseudomonas* and *Stenotrophomonas* were the most diverse group among the PGPR. With regard to their plausible ecological adaptations tested under *in vitro*, the fast growing soybean rhizobia were more tolerant to pesticides, higher temperature and higher NaCl concentrations and more versatile to utilize different carbon and nitrogen sources than the slow growing isolates which were better in their inherent antibiotic resistance (IAR). The majority of the rhizobacteria were grown at 40ºC, 4% NaCl and showed multiple antibiotic and heavy metal resistance. Some of the soybean rhizobia and rhizobacteria also demonstrated multiple PGP traits (2 to 9). The data also showed the overall better performance of gram

negative rhizobacteria and fast growing rhizobia in terms of the number of PGP traits and tolerated stresses. The nodulation and symbiotic effectiveness tests of the rhizobia showed that SNB57B, SNB120A, SNB120C, SNB125A, SNB125B and SNB140 nodulated all the three soybean varieties with prolific nodulation (54-173 nodules plant^{-1}; 1.76-2.33 mg of nodule dry weight plant^{-1}) and shoot dry weight (1.10-2.27 g plant^{-1}) showing highly effective symbiosis (80-100%) in relation to the nitrogen-fertilized control plants under greenhouse experiment. The isolates showed similar pattern of relatively high nodulation parameters and symbiotic performance on Jalele and Cheri varieties compared to the Ethio-Yugoslavia variety. The findings also showed co-inoculation of rhizobia and the PGP *Achromobacterium* significantly increased more growth and yield parameters of soybean at Dembi station of Debrezeit Agricultural Research Center (DDARC) field site with low population of indigenous soybean rhizobia and where maximum nodule number (168 plant^{-1}) and dry matter (1.96 g plant^{-1}), shoot dry matter (25 g plant^{-1}) and total nitrogen (4 %), number of pods (114 plant^{-1}) and seeds (214 plant^{-1}) and grain yield (4.01 tons ha^{-1}) were recorded. There were highly significant ($p \leq 0.05$) effects of the rhizobial isolates on most growth, nodulation and yield parameters. Indigenous soybean rhizobia performed much better than the exotic *Bradyrhizobium japonicum* SBTAL379 and control treatments under greenhouse and field conditions so that they can be further validated and recommended as inoculant (together with the PGP bacterium) to improve growth and productivity of the crop in the country.

Key words: soybean, rhizobia, PGPR, diversity, nodulation, yield

1. Introduction

Soybean (U.S) or Soya bean (UK), *Glycine max* (L) Merr. is an erect, bushy, annual leguminous crop that belongs to the family leguminosae and subfamily Papillinoidae. It is one of the oldest crops domesticated about 4000 years ago in many localities in East Asia including China, Japan, India and Mongolia (Lee *et al*., 2011). Currently, soybean is cultivated worldwide on more than 118.13 millions of hectares of land with an average production of 2.70 metric tons ha^{-1} (USDA, 2015). The major soybean growing countries are the USA, Brazil, Argentina, India and China covering 33.42, 32.10, 19.30, 10.91, and 6.88 millions of hectares of agricultural land respectively, with yield range of 0.83 to 2.96 metric tons ha^{-1}.

In Africa, soybean cultivation had been introduced since 1896; first in Algeria (Shurtleff and Aoyagi, 2009) and later to South Africa, Nigeria, Uganda and Zambia which are currently the major soybean producing African countries covering area ranging from 0.11 million hectares (Zambia) to 0.69 (South Africa) million hectares with average yield of 1.00 metric tons ha^{-1} (Nigeria) to 1.88 metric tons ha^{-1} (Tanzania) (USDA, 2015).

It is a nutritious food and feed containing about 40 % protein, 30% carbohydrate, 21% oil and 5% ash (Scott and Aldrich, 1983). Soybean products are cholesterol free, high in fibre and some minerals, but possess one of the lowest levels of saturated fats among vegetable oils (BIDCO, 2005). Soybean products have a tremendous health benefits in regulating blood glucose in diabetes mellitus (Tsai *et al*., 1987), reduction of postmenopausal osteoporosis (Potter *et al*., 1998), preventing cancer (Messina and Wu, 2009) and lowering serum cholesterol level (Lokuruka, 2010). Consequently, soybean is one of the top international commodities used for

industrial production of soy foods, cosmetics, resin, plastics, biodiesel and fiber (Yi-you, 2004; Ogbemudia *et al*., 2010; Hartman *et al*., 2011).

Soybean is also used as green manure to enhance its productivity, improve soil fertility and benefit other cereal crops in intercropping and crop rotation agricultural systems due to its ability to symbiotically fix up to 450 kg nitrogen ha^{-1} yr^{-1} in association with diverse groups of slow growing *Bradyrhizobium* species (Kuykendall *et al*., 1992; Xu *et al*., 1995; Appunu *et al*., 2008; Yang and Zhou, 2008; Zhang *et al*., 2012), fast growing *Rhizobium*/*Sinorhizobium* species (Keyser *et al.,*1982; Scholla and Elkan, 1984; Chen *et al*., 1988; Chen *et al*., 1995; Saldana *et al*., 2003) and *Agrobacterium* species (Youseif *et al*., 2014).

Soybean is also known for its association with several groups of plant growth promoting rhizobacteria (PGPR) like *Pseudomonas, Bacillus, Enterobacter* and *Microbacterium* that have the ability to fix free-nitrogen (Park *et al*., 2005), produce phytohormones (Masciarelli *et al*., 2014), solubilize inorganic phosphate (Sharma *et al*, 2012), sequester iron (Susilowatl *et al*., 2011) and suppress fungal or viral pathogens (Susilowatl *et al*., 2011; Wahyudi *et al*., 2010 a,b; Khalimi and Suprata, 2011).

Single inoculations/co-inoculations of soybean nodulating bacteria and/or PGPR enhance the health, growth and productivity of the crop. Accordingly, inoculation of soybean with *Bradyrhizobium* improved its growth (Sharma and Kumawat, 2011), its yield by 12-19% (Ulzen *et al*., 2016), by 53% (Tamiru Solomon *et al*., 2012) and by 60-73% (Rechiatu *et al*., 2015) over un-inoculated control. Inoculation of soybean with PGPR increased seedling emergence rate and suppressed damping-off due to *Pythium ultimum* (Le'on *et al*., 2009), and enhanced growth (Stefan *et al*., 2009; Khalimi and Suprata, 2011). Co-inoculation of soybean with

Bradyrhizobium japonicum strains and *Bacillus* sp. (Li and Alexander, 1988; Kravchenko *et a*l., 2013), *Pseudomonas* sp. (Zhang *et al*., 1996; Anteneh Argaw, 2012), *Serratia* sp. (Zhang *et al*., 1996; Dashti *et al.*, 1998) and *Azospirillum* sp. (Aung *et al*., 2013) enhanced its nodulation, N-fixation and seed yield under field conditions.

In Ethiopia, Soybean has been cultivated since 1950 and its production is expanding in different agro-ecologies up to 2,200 meters above sea level (mas) with annual rainfall as low as 500 mm (Fekadu Gurmu, 2007). The demand for soybean is increasing as it is used in traditional or industrial processing of various soy foods (like "Tasty soya" and baby food, called "Faffa"), edible oil and poultry feed production in the country (Zerihun Abebe *et al*., 2015). For the last 30 years, more than 20 soybean varieties differing in their maturity period, yield and compatibility to nodulation with various rhizobial strains have been released (Mekonnen Hailu and Kaleb Kelemu, 2014). The authors further noted that there has been 10 fold and 20 fold increases in the area of cultivation and volume of yield of soybean, respectively from 2002 and 2012. A recent report showed that soybean was cultivated on 30,517.38 hectares of private peasant holdings with average yield of about 2.0 tons ha^{-1} in the country (CSA, 2014).

For a long time, several agronomic studies have been undertaken in the country with inoculation trials using exotic (introduced) commercial rhizobial inoculants; *Bradyrhizobium japonicum* TAL 378 and TAL379. Field inoculation trials have been done using these rhizobia with phosphprus and/or nitrogen applications (Workneh Bekere and Asfaw Hailemariam, 2012; Tamiru Solomon *et al*. 2012; Tekle Yoseph and Walelgn Worku, 2014; Tolera Abera *et al*., 2015; Zerihun Abebe *et al*., 2015) and co-inoculation of rhizobia with plant growth promoting phosphate solubilizing *Pseudomonas* species (Anteneh Argaw, 2012). These studies showed that the yield improvements were not satisfactory and not consistent ranging from less than 1 ton ha^{-1}

to 5.8 tons ha^{-1} across different fields and different varieties. It is not uncommon to find that commercial inoculants often fail to improve growth and yield of pulse crops due to either the existence of highly competitive indigenous rhizobia in the soil and strain-cultivar incompatibility or unfavorable environmental conditions (Hungria *et al*., 2009).

The wide variations and often ineffectiveness in nitrogen fixation and yield improvement of the exotic inoculants necessitates a need for screening symbiotically effective indigenous soybean rhizobia and additional PGPR with multiple PGP traits that are adapted to environmental stresses and compatible to different soybean cultivars in the country. Thus, selection for symbiotically effective and ecologically competent rhizobia and rhizobacteria under *in vitro*, under greenhouse and field conditions is a basis to fully realize the biological nitrogen fixation and growth promotion properties of these microorganisms to enhance the productivity of soybean (Howieson *et al*., 2000; Martínez-Viveros *et al*., 2010)

Although culture collection of rhizobia including soybean rhizobia was started from some parts of Ethiopia in the 1980's (Amare Abebe, 1986), a recent attempt to genetically characterize a few indigenous soybean rhizobia (Aregu Amsalu *et al*., 2012) and a test of their inherent antibiotic resistance (Tolera Abera *et al*., 2015) were carried out. There is still a dearth of information regarding their phenotypic, genotypic and symbiotic features. Obviously, the diversity and effectiveness of indigenous soybean rhizobia and PGPR have not been fully explored while there is a need to screen for their potential nitrogen fixation and other multiple PGP trait, persistence and establishment under plausible soil temperature, pH, salinity, pollutants like heavy metals and pesticides. Moeover, microbial antagonists attract attention in order to exploit their benefits as inoculants for soybean production. Thus, the present study was initiated to explore the phytobeneficial traits of soybean associated native bacteria.

2. Objectives

2.1 General objective

- The general objective of the present study was to screen soybean nodulating rhizobia and other plant growth promoting rhizobacteria from soils of different soybean growing areas of Ethiopia using standard methods for selection of elite inoculants to enhance soybean production.

2.2 Specific objectives

The specific objectives of the current project were

- To isolate indigenous soybean rhizobia and screen for their symbiotic nitrogen fixation effectiveness on different soybean varieties under greenhouse conditions.
- To determine diversity of soybean rhizobia and rhizobacteria using phenotypic and genetic methods
- To screen soybean rhizobia and PGPR for their multiple PGP traits and ecological competitiveness (tolerance to pH, temperature, heavy metal, antibiotic and pesticides) under *in vitro* conditions
- To test the symbiotic effectiveness of selected rhizobia inoculated singly or dually with a PGP rhizobacterium under field conditions

3. Literature review

3.1 Legumes and biological nitrogen fixation (BNF)

Leguminous plants (legumes) are classified in the family Fabaceae (or Leguminosae) with about 650 genera and 20,000 species diversified into three subfamilies; Papilionoideae, Mimosoideae and Caesalpinoideae (Doyle, 1994). The most outstanding feature of legumes is their ability to fix nitrogen in association with root/stem nodule bacteria generally known as rhizobia. Of the

three subfamilies of Leguminosae; 97% of Papilionoideae, 90% of Mimosoideae and 23% of Caesalpinoideae are known to nodulate by rhizobia (de Faria *et al*., 1989). Rao and Rao (1997) suggested that the cumulative effect of inhibitory substances present in the roots/root exudates and predominance of antagonistic microbes lead to low level of *Rhizobium* in the root zone contributing to non-nodulating nature of some legumes.

Legumes are highly diverse in their ecological distribution and growth habits, but contain fruits in the form of pods (called legume) as their principal unifying feature. Based on molecular data and fossil records, Lavin and Schrire (2005) concluded that legumes probably evolved approximately 60 million years ago early in the Tertiary period. However, nodulation evolved about 58 million years ago (Sprent, 2007) and the isolation of root nodule bacteria and their description as N-fixer were carried out in 1888 (Willems, 2006).

Legumes are multipurpose plants serving as sources of foods and feeds, fuel, fiber, oils, fertilizers, timber, chemicals and medicine (Lewis *et al*., 2005). Due to their ability to fix nitrogen in symbiotic association with rhizobia, they colonize barren and marginal lands playing significant role in ecological restoration (Mortier *et al*., 2012). Some legumes such as *Sesbania* and *Tephrosia* are used in agroforestry in tree fallow (Sanchez, 1999) and others like *Sesbania* in alley cropping (Azene Bekele *et al*., 1993).

Biological nitrogen fixation (BNF) via symbiotic association between legumes and their nodulating rhizobia plays a significant role in world agricultural productivity by converting approximately 120 million metric tons of nitrogen into ammonia annually (Freiberg *et al*., 1997), which is equivalent to $ 6.8 billion expenditure on nitrogenous fertilizers (Herridge and Rose, 2000). All the nitrogen fixed by legumes in association with rhizobia is assimilated by plants

with no leaching and negative environmental impact unlike nitrogen fertilizers. However, the amount of N fixed by legumes varies depending upon the host genotype, *Rhizobium* efficiency, edaphic and climatic conditions, and the method of its determination (FAO, 1984).

Techniques used to quantify fixed nitrogen by legumes include nitrogen balance, nitrogen difference, acetylene reduction method and $^{15}N_2$ method (Unkovich *et al*., 2008). In the N-balance method, a net positive N-balance in a soil is attributed to N-fixation if all the possible external inputs except nitrogen fixation and outflows of nitrogen can be accounted and incremental changes in the quantified soil nitrogen. N-difference compares total N of the N-fixing species with that of a neighboring non N_2 -fixing species, with the difference between the two measures assumed to be due to N_2 fixation. The enzyme nitrogenase reduces N_2 to NH_3 and is also capable of reducing acetylene (C_2H_2) to ethylene (C_2H_4). Using C_2H_2 as substrate, roots in air tight vessel are exposed to a C_2H_2-enriched atmosphere (usually 10%) and the rate of C_2H_4 accumulation in gas samples collected over a set interval of time is measured using a gas chromatograph. In the $^{15}N_2$ method, intact or detached roots of plants are placed in a chamber with an atmosphere enriched in $^{15}N_2$. The amount of $^{15}N_2$ in the plant at the end of the incubation period is a direct measure of the rate of N_2 fixation. Table 1 shows the estimated amount of nitrogen fixed by some food legumes.

Table 1. Estimated amount of nitrogen fixed by some food legumes

Legumes	Nitrogen fixed (kg of N/ha/yr)	References
Horse bean/ faba bean (*Vicia faba*)	82-174	Peoples and Griftiths, 2009
Pigeon pea (*Cajanus cajan*)	21-86	Mhango *et al*., 2017
Cowpea (*Vigna unguiculata*)	200	Adjei-Nsiach *et al*., 2008
Soybean (*Glycine max*)	300	Hungria *et al*., 2006
Chick-pea (*Cicer arietinum*)	138	Fatima *et al*., 2008
Mung bean (*Vigna radiata*)	46	Umair *et al*., 2011
Lentil (*Lens esculenta*)	60-110	Peoples and Griftiths, 2009
Groundnut (*Arachis hypogaea*)	21-102	Mhango *et al*., 2017
Pea (*Pisum sativum*)	85-166	Peoples and Griftiths, 2009
Bean (*Phaseolus vulgaris*)	51	Kipe-nolt *et al*., 1993

3.2 Phenology and growth habits of soybean

Soybean is cultivated from latitudes of 0 to 55^{o} and altitude of below sea level to 2000 m (in the tropics) while its major commercial production is confined to between 25^{o} and 45^{o} latitude and altitudes less than1000 m (Whigham, 1983). Photoperiodically, soybean is a short day plant with varying critical values of day length among maturity groups (Purcell *et al*., 2014).

Soybean can be grown on a wide range of soils with pH ranging from 4.5 to 8.5 (Dugje *et al*., 2009), but it is extremely sensitive to deficiency or excessive soil moisture with 60-70% field capacity as optimum soil moisture for nodulation (Balešević-Tubić *et al*., 2011). The ideal rainfall for the crop is between 500 and 1000 mm, but it can grow in areas with as little as 180 mm of rain due its extensive root system (Belfield *et al*., 2011).

Soybean seeds germinate within about 4 days under optimum soil temperature (28-29 ^{o}C) but it takes 2 weeks or more in cold soil (10^{o}C or less) (Purcell *et al*., 2014). The optimum temperature for the growth, nodulation and nitrogen fixation of the crop ranges from 25 to 30 ^{o}C (Stefan *et al*., 2009).

Soybean requires the highest amount of nitrogen among agronomic crops assimilating approximately 100 kg of nitrogen to produce a ton of seeds (Sinclair and Wit, 1975). It utilizes soil mineral nitrogen as NO_3^- or NH_4^+, and atmospheric nitrogen fixed symbiotically in its root nodules. The proportion of N-derived from fixation goes up to 97 % with most estimates falling between 25 and 75%; the lowest values are associated with higher soil nitrate level, ineffective bradyrhizobial strains, and environmental constraints like soil acidity and deficit of soil moisture (Keyser and Li, 1992).

Early studies by Harper (1974) stressed the necessity of both symbiotic N-fixation and application of nitrogen as nitrate for maximum yield of soybean following experimental outcome under hydroponic growth conditions. Plants grown supplied with nitrate showed higher symbiotic N-fixation rate than those grown in the absence of nitrate supply. Moreover, seed yield of soybean was higher for those plants utilizing both nitrate and atmospheric nitrogen than those plants fully dependent on atmospheric nitrogen or those grown at high nitrate level capable of inhibiting symbiotic nitrogen fixation.

Siczek and Lipiec (2011) supposed that soybean plants may show N-deficiency symptoms at two phases: 10 to 12 days after emerging associated with the investment of seed resources in nodulation, and after mid-pod fill associated with translocation of leaves' nitrogen to grain. So,

application of starter dose of N-fertilizer (30-60 kg ha^{-1}) at sowing or at the end of flowering might increase the yield of the crop.

Adeyeye *et al*. (2014) reported a positive grain yield response of soybean to nitrogen-fertilizer application (at 30 and 60 kg ha^{-1}). Similarly, Tekle Yoseph and Walelign Worku (2014) observed significant increase in both nodule number and dry weight of soybean as a result of application of N-fertilizer at 46 kg N ha^{-1} as UREA at Jinka. However, Nishi and Hungria (1996) reported the lack of significant effect of the application of "starter dose" of nitrogen or a dose of N at the onset of pod-filling on soybean grain yield. Dereje Asaminew (2007) also reported the absence of significant effect of N-fertilizer on soybean nodule number, dry weight and volume at Hawassa.

The lack of response of soybean to N-application was assumed to be due to soybean merely substitutes the nitrogen it ordinarly would have derived from BNF with nitrogen from fertilizer (Deibert *et al*., 1979) or due to the translocation of more nitrogen from vegetative reserves following reduced rate of nitrogen fixation as a result of applications of N-fertilizer (Herridge, 1982). Thus, N-fertilization studies do not provide clear evidence as to whether N-fertilization is required to complement the nitrogen supply from BNF to achieve soybean yields that approach yield potential level (Salvagiotti *et al*., 2008).

Soybean possesses two growth phases and two growth habits (Purcell *et al*., 2014). The growth phases are vegetative (V) phase, a phase prior to the onset of flowering; and reproductive (R) phase, a phase from the beginning of flowering onwards. Both vegetative and reproductive phases have different stages (Fig.1). VE is vegetative phase when cotyledons emerge above soil surface, VC is when unifolate leaves unrolled sufficiently, V1 is when leaves fully develop at

unifolate node (first node), V2 is when trifoliate leaf fully develop at node above unifolate node (second node), V3 is when there are three nodes on the main stem with fully developed leaves starting at the unifolate node and Vn is when there are n number of nodes on the main stem with fully developed leaves starting with the unifolate (n^{th} node).

With respect to reproductive (R) growth phase, R1 refers to the start of blooming (one open flower at any node on the main stem), R2 to full bloom (open flower with a fully developed leaf), R3 to beginning of pod (pod 3/16 inch), R4 to full pod (pod 3/4 inch), R5 to beginning seed (seed 1/8 inch), R6 to full seed (seed fills pod cavity), R7 to beginning maturity (one normal pod reaches its matured color on the main stem) and R8 to full maturity (95 % of the pods reaching their matured color).

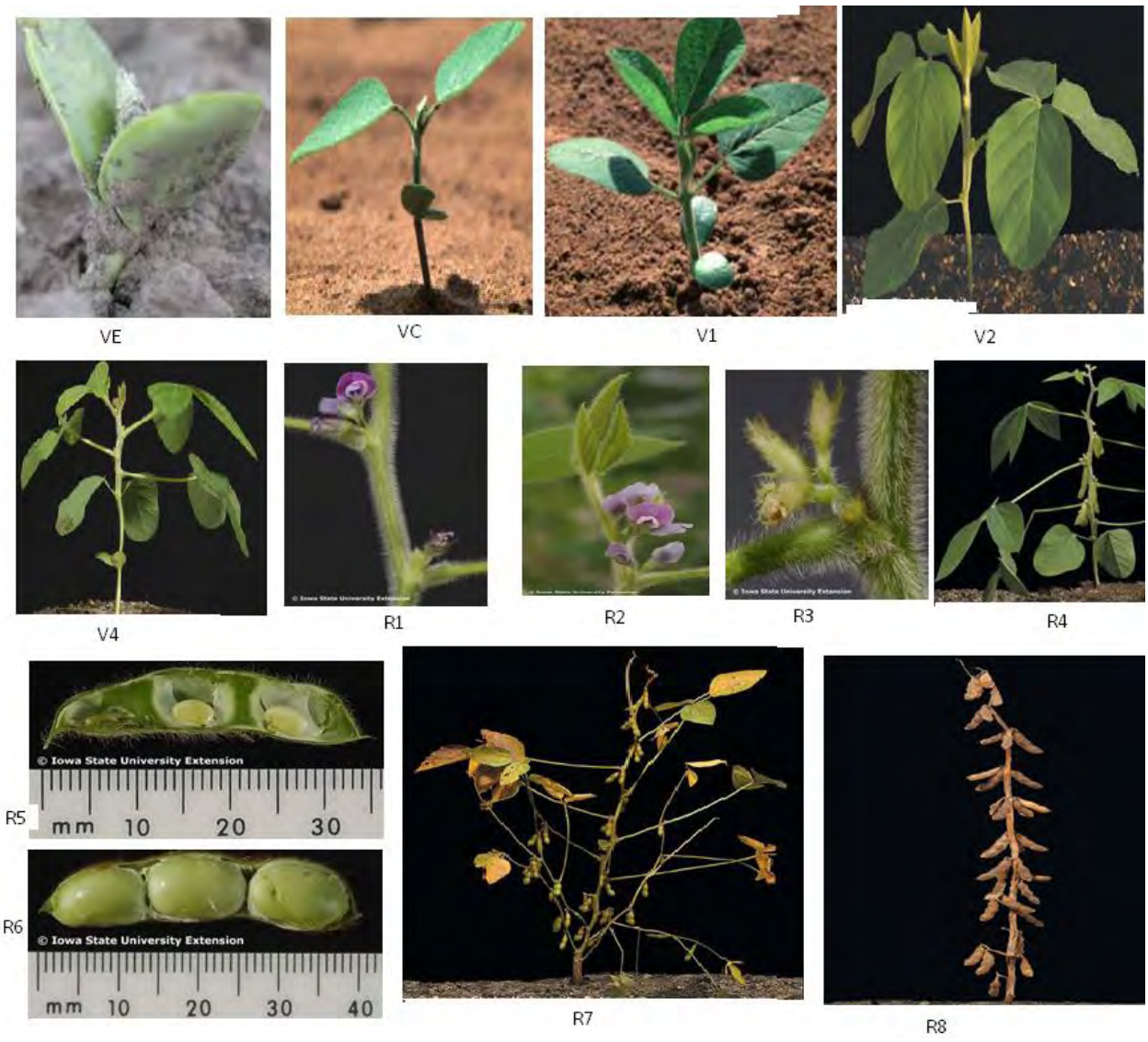

Fig. 1. Growth phases of soybean (www.soybeanmanagement.info, accessed in January, 2015)

The growth habits of soybean are either determinate or indeterminate. Determinate soybean varieties stop vegetative growth and nod production on the main stem soon after flowering begins, but they continue producing nodes on branches until the beginning of seed fill. Indeterminate soybean varieties continue producing nodes on the main stem until the onset of seed fill.

3.3 Soybean nodulation and diversity of its nodulating bacteria

Soybean varieties may be totally incompatible with rhizobia, highly promiscuous or may restrict nodulation by certain groups of soybean nodulating rhizobia (Van *et al*., 2007). The ability of soybean to host rhizobia depends on certain factors like characterstics of Rj-genes (Shiro *et al*., 2013), production of rhizobitoxin (Johnson and Clark, 1958) and inoculum cell density (Waters and Bassler, 2005). Rhizobitoxins are plant metabolites synthesized in roots and translocated to new developing leaves where they produce a chlorosis upon nodulation by certain rhizobial serogroups indicating incompatability. *Rj*-genes are nodulation regulatory genes and soybean cultivars harbouring *Rj*-genes inhibit nodulation or effective nodulation by certain serotypes of rhizobia. Soybean *Rj* genotypes of *rj1*, *Rj2*, *Rj3*, *Rj4*, *Rj5* and *Rj6* and non-*Rj* that lack these genetic phenotypes have been confirmed to exist (*rj* indicates recesive and *Rj* indicates dominant).

Elevated inoculum cell density restricts nodulation of soybean via quorum sensing, which is related to the synthesis and perception of signal molecule called autoinducer (Waters and Bassler, 2005). For instance, an autoinducer (called bradyoxetin) produced by *B. japonicum* when population density becomes higher was found to repress nod genes (Loh and Stacey, 2003). However, evaluation of nodulation potential of 31 Argentinean commercial soybean cultivars by Salvucci *et al*. (2012) revealed that variation in nodulation capacity was not due to response to quorum sensing, bacterial promiscuity or maturity group of the plants but rather due to variation in soybean genotypes.

Non-promiscuous soybean genotypes require either the application of N-fertilizer or inoculation with compatible commercial inocula, commonly strains of *Bradyrhizobium japonicum* in areas like Africa where soybean is not endemic. Soybean breeders at International Institute of Tropical

Agriculture (IITA, in Nigeria) developed new soybean genotype, called Tropical Glycine Cross (TGx) which is nodulated by poplations of *Bradyrhizobium* spp. indigenous to African soils (Abaidoo *et al*., 2000). However, TGx isolates were found to be *Bradyrhizobium* species (like *B. japonicum* and *B. elkanii*) and vary in symbiotic effectiveness explaining why TGx soybean genotypes usually develop N-deficiency symptoms and further suggesting the need to inoculate TGx under such conditions (Abaidoo *et al*., 2000).

Soybean is known to be nodulated by diverse rhizobia of various genera and species having fast (Keyser *et al*., 1982), slow (Jordan, 1982), extra-slow (Xu *et al,* 1995) or variable growth rates (Chen *et al*., 1995). Jordan (1982) placed slow growing rhizobial that change the color of the bromothymol blue-yeast extract mannitol agar (BTB-YMA) medium to blue as a result of alkali production under the genus *Bradyrhizobium* following the approval of the genus name in 1980. Accordingly, the previously known soybean nodulating *Rhizobium japonicum* which was considered as a sole symbiont of soybean was transferred to *Bradyrhizobium japonicum*. Keyser *et al*. (1982) also isolated fast growing rhizobia from root nodules of soybean which were initially included in the genus *Rhizobium.* Since then many fast growing rhizobia and other species of *Bradyrhizobium* have been described from soybean nodules based on numerical taxonomy, DNA G+C content, DNA-DNA hybridization, 16S rRNA sequencing, SDS-PAGE analysis of whole cell protein, and cross-inoculation to other legumes. Consequently, a new soybean symbiont *Bradyrhizobium* sp., *B. elkanii*, was proposed by Kuykedall *et al*. (1992) based on previously documented data and polynucleotide sequence dissimilarity of 14 randomly selected clones from cosmid libraries of *Bradyrhizobium*. Subsequently, Xu *et al*. (1995) isolated extra-slow growing (ESG) strains from nodules of *Glycine max* and *Glycine soja* (wild soybean) grown in Liaoning province of China and proposed a new species as *Bradyrhizobium*

liaoningense with type strain 2281. The ESG strains with generation time of 16.4 to 39.6 hrs form circular, entire, semi translucent, raised, none-mucoid colonies usually with 0.2 to 1 mm diameter within 7 to 14 days on YMA, and utilize a narrow range of carbohydrates, organic acids and aminoacids as sole carbon source and were sensitive to antibiotics.

Vinuesa *et al*. (2005) isolated a slow growing rhizobium from endemic genistoid legumes such as *Adenocarpus, Chamaecytisus, Lupinus and Spartocytisus* sp. and identified it as *Bradyrhizobium canariense*. Later, Yang and Zhou (2008) isolated *Bradyrhizobium canariense* from the nodules of the homologous host soybean grown in China. However, the strains isolated from the heterologous hosts were failed to nodulate soybeans (*Glycine max* and *Glycine soja*). Strains of *B. canariense* sp. nov. form white or creamy colonies of 1-1.5 mm in diameter when incubated at 28^{o}C after 7 days, produce acid reaction, fail to grow at 1% NaCl on YMA and at pH 9, but found to be highly acid tolerant (pH 4.2) and possess symbiotic genes on chromosomes. The type strain for the species is BTA-1 (CFNE 1008). Similarly, a new *Bradyrhizobium* sp. isolated from *Lespedez* spp. and identified as *Bradyrhizobium yuanmingense* sp.nov with type strain CCBAU 10071 by Yao *et al*. (1995) failed to nodulate *Glycine max,* yet the same species was isolated from soybean in India (Appunu *et al*., 2008) indicating that isolates within the same species do not necessarily nodulate soybean depending upon their origin from heterologous or homologous hosts. *Bradyrhizobium yuanmingense* sp. nov. showed colonies less than 1.0 mm in diameter when grown at 28^{o}C after 7 days, absence of growth at pH 5 or 10, sensitive to 1 % NaCl (w/v), possess symbiotic genes on chromosomes, generation time ranging from 9.5 to 16 hrs in peptone yeast extract (PY broth) but 10.2 hrs for the type strain (Yao *et al*., 1995). Recently, Zhang *et al*. (2012) identified a novel soybean bradyrhzobial species, *Bradyrhizobium huanghuaihaiense* from Huang-Huai-Hai, Northern Plain of China.

Similarly, Wang *et al.* (2013) described bradyrhizobial isolates of soybean (*Glycine max*) root nodules growing in Danqing City of China as nov. sp.: *Bradyrhizobium daqingense* sp.nov. The type strain for the species is CCBAU 15774, and is closely related to type strain for *Bradyrhizobium yuanmingense* and *Bradyrhizobium liaoningense.*

Although soybean is mainly nodulated by slow growing *Bradyrhizobium* spp, fast growing isolates are also found to nodulate the host (Keyser *et al*., 1982). They were assigned to the genus *Rhizobium* and later identified as *Rhizobium fredii* to differentiate from other species of *Rhizobium* based on molecular and phenotypic data by Scholla and Elkan (1984). Strains of *Rhizobium fredii* form cicular, convex, entire colonies with 1-5 mm diameter within 7 days on YMA, grow at pH 4.5 but inhibited at pH 9.5 or 3% NaCl. Later, Chen *et al*. (1988) proposed a new genus (*Sinorhizobium*) consisting two species: *Sinorhizobium fredii* and *Sinorhizobium xinjiangensis* for fast growing soybean rhizobia. Members of the genus *Rhizobium* and *Sinorhizobium* have many characters in common, acidify the YMA media when grown *in vitro* and carry nodulation and nitrogen fixation genes on symbiotic plasmids.

Two other fast growing rhizobia, *Rhizobium species* NGR234, isolated from *Lablad purpureus* by Trinick (1980), and *Rhizobium meliloti* (strain 042B) isolated from root nodules of alfalfa (Gao and Yang, 1995); both were found to be highly related to *Rhizobium fredii* (Saldana *et al*., 2003; Chen *et al*., 1995) and shown to nodulate soybean (*Glycine max*).

Chen *et al*. (1995) proposed a new species; *Mesorhizobium tianshanese* for soybean nodulating rhizobia having variable generation time (5-15 hrs) and forming cicular, opaque, convex and creamy colonies on YMA usually with 1 to 2 mm diameter after 5 to 7 days. Strains of *Mesorhizobium tianshanese* produce acid in medium containing mannitol grow on YMA with 1

% NaCl and lack plasmids. Recently symbiotically effective strains of the genus *Agrobacterium* were isolated from soybean root nodules by Youseif *et al*. (2014). The authors pointed out the existence of similarities between nifH and nodA of the *Agrobacterium* strains and that of fast growing soybean rhizobia supporting the suggestion of classifying the genus *Agrobacterium* under the genus *Rhizobium* (Farrand *et al*., 2003).

Interestingly, the distributions of soybean microsymbionts are affected by various factors like soil pH, climate, latitude, the host plant varaiety (Rj genotype). Consequently, *Sinrhizobium fredii*, *Sinorhizobium xinjiangense* and *Mesorhizobium tianshanese* are extensively distributed in alkaline-saline soils where as *Bradyrhizobium* species vastly occur and predominate in neutral to acidic soils (Chen *et al*., 1995, Li *et al*., 2011; Zhang *et al*., 2011). Latitudinally, *B.japonicum* dominated temperate locations where as *Bradyrhizobium elkanii*, *Bradyrhizobium yuanmingense* and *Bradyrhizobium liaoningense* dominated tropical localities of Nepal in acidic, moderately acidic and slightly alkaline soils, respectively (Adhikari *et al*., 2012). Similarly, the bradyrhizobial community structure of USA is strongly correlated with latitude where *B .japonicum* and *B. elkanii* are dominant in the northern and the middle to southern regions of USA, respectively (Shiro *et al*., 2013). However, some studies showed that *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* have been isolated from various climates across the world (Adhikari *et al*., 2012).

3.4 PGPR and their plant growth enhancement mechanisms

Plant growth promoting rhizobacteria (PGPR) are soil bacteria that colonize the surface and inner tissues of roots and promote plant growth and health (Drogue *et al*., 2012). Generally, PGPR are defined by three intrinsic features: colonizing the root, surviving and multiplying in

microhabitats associated with root surface, and promoting plant growth (Ahemad and Khan, 2011).

Based on the degree of association with root cells, PGPR may be categorized as intracellular PGPR (iPGPR) or extracellular PGPR (ePGPR) (Sharma *et al*., 2013). ePGPR may exist in rhizosphere, rhizoplane or in intercellular spaces of root cortex. They include different genera of bacteria such as *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Caulobacter*, *Chromobacte*r, *Erwinia*, *Flavobacterium*, *Micrococcus*, *Pseudomonas* and *Serratia*. iPGPR include rhizobia and Frankia that invade roots to form nodules. However, the original definition of rhizobacteria was restricted to free-living bacteria though it was later broadly defined as any root colonizing bacteria including rhizobia and Frankia (Antoun and Prévost, 2005). It is generally accepted now that rhizobia and Frankia should be designated as microbial symbiotic partners (micosymbionts) rather than as symbiotic PGPR or internal PGPR (iPGPR) (Gray and Smith, 2005) though they may behave as PGPR with their non-symbiotic host plants (Sessitsch *et al*., 2002). PGPR that enter and colonize plant interior tissues are known as endophytes (Glick, 2012). They enter plant tissues primarily via roots (and may also enter via aerial structures) and may either remain localised at the point of entry or spread throughout the plant (Hallmann *et al*., 1997).

Mechanisms by which PGPR stimulate plant growth are broadly categorized as direct or indirect, and some traits are considered as direct and at the same time as an indirect mechanism (Glick, 1995).

3.4.1. Direct plant growth enhancement mechanisms of PGPR

The direct plant growth enhancement mechanisms of PGPR include facilitating nutrient acquisition (via N-fixation, phosphate solubilization and siderophore production) and producing phytohormones (Glick, 2012).

Biological nitrogen fixation

Although nitrogen constitutes more than 78% of the atmosphere, it remains unavailable to plants. BNF involves the reduction of atmospheric nitrogen into ammonia which changes into NO_3^- and NH_4^+ that can be absorbed by plants. BNF fixes about 60% of the earth's available nitrogen, and represents an economically beneficial and environmentally sound alternative to chemical fertilizers (Ladha *et al*., 1997).

Non-symbiotic nitogen fixers include free-living bacteria such species of *Azotobacter, Beijerinckia,* and *Clostridium* that living in soil or water deriving energy from chemical subtances (non-photosynthetic) or light (photosynthetic), and associative nitrogen fixers like *Gluconacetobacter diazotrophicus* (formerly *Acetobacter diazotrophicus*), *Azospirillum, Pseudomonas* , *Herbaspirillum*, *Burkholderia*, and *Azoarcus* spp. that reside around the plant roots in the rhizosphere or invade root-intercellular spaces obtaining energy materials from the plants (Ohyama *et al*., 2014).

The rate of BNF is about 3-5 kg/ha/yr for free-living diazotrophs and 15-25 kg/ha/yr for associative diazotrophs compared to up to 600 kg/ha/yr for symbiotic diazotrophs (Chanway *et al*., 2014). The lower BNF efficiency of free living diazotrophs is related to limitation in energy supply, severe oxygen sensitivity of nitrogenase, and antagonistic microbial interactions (Postgate, 1998; Bashan *et al*., 2004).

Phosphate solubilization

Phosphorus is one of the most limiting essential elements for plant growth. Its amount in the soil (organic and inorganic forms) is generally quite high, but its available form is low to support plant growth (Khan *et al.,* 2007). Ahemad and Khan (2011) estimated that 5.7 billion hectares of land contain too little available phosphorus for sustaining optimal crop production world wide. The low bioavailability of phosphorus is attributed to the extreme reactivity of phosphate anions ($H_2PO_4^{-1}$ and HPO_4^{-2}) forming complexes with Fe, Al and Mn between pH 5.5 to 7.00, with silicate mineral between pH 6 and 8, and with Ca between pH 6.5 and 8.5 (Ahemad and Khan, 2011). Under these circumstances, Ca^{+2}, Al^{+3} and Fe^{+3} complexes precipitate about 80% of phosphorus fertilizer added to soil (Goldstein, 1986).

Some rhizobacteria solubilize inorganic phosphates and mineralize organic phosphates to increase available phosphurus content in the soil. Rhizobacterial solubilization of inorganic phosphate is associated with the production of low molecular weight organic acid (Rodriguez *et al*., 2006) such as gluconic acid and citric acid where as the mineralization of organic phosphates occurs via the synthesis of different phosphatases, phosphonatases, phytases and C-P lyases (Lutenberg and Kamilova, 2009).

Siderophore production

Iron plays dominant role in nitogen fixation and assimilation processes being the component of nitrogenase, leghemoglobin, ferredoxin and hydrogenase (Ahemad and Khan, 2011). It is one of the bulk minerals present in plenteous amount on earth, but its availability for assimilaion by plants (and microbes) is extremely low (Ma, 2005). Under aerobic condition, Fe^{+2} (a form required by plants but unstable) is oxidized to form Fe^{+3} as a component structure of insoluble

minerals: goethite (FeO(OH)) and hematite (Fe_3O_2) (Lindsay, 1979) which are poorly soluble in water and so that unavailable for plants. However, some rhizobacteria such as *Bacillus*, *Pseudomonas*, *Geobacter*, *Alcaligenes*, *Clostridium* and *Enterobacter* can reduce Fe^{+3} to Fe^{+2} (Mullen, 1999) and different rhizobacteria produce low molecular weight iron cheletors (known as siderosphores) that sequester Fe^{+3} in the area around the root increasing the rate of its supply to the plant (Singh *et al.*, 2013). Siderosphores have exceptionally high affinity for Fe^{+3} forming a hexa-coordinated Fe-siderophore complex, and many plants can bind, takeup and utilize PGPR iron-siderophore complex (Wang *et al*., 1993). The direct benefits of siderophores on plant growth were demonstrated by inoculating mung bean with siderophore producing *Pseudomonas* strain GRP3 (Glick, 2012).

Phytohormone production

Many PGPR produce phytohormones like cytoknines, gibberellins, auxins and ethylene that can be absorbed by roots affecting their endogenous level and plant response (Libbert and Silhengst, 1970). Salamone *et al*. (2001) reported the production of gibberellins and cytokinins by many PGPR. It has been reported that 80 % of microbes isolated from rhizosphere of different crops possess the ability of synthesizing and releasing auxins as secondary metabolites (Loper and Schroth, 1986). Indole-3-acetic acid (IAA), the main auxin in plants, is usually produced by PGPR from tryptophan in root or seed exudates (Patten and Glick, 2002). Bacterial IAA increases the amount of root exudates via loosening plant cell walls, and it also improves the plant's accessibility to soil nutrients by increasing root length and surface area (Glick, 2012). However, acquisition of PGPR hormones suppresses plant growth if the endogenous level is optimal as plant hormones are required at very low concentration (Glick, 2012). For example,

higher level of IAA induces the activity of ACC synthase that catalyzes ACC production. Rise in ACC level leads to rise in ethylene level which inturn inhibits root growth.

3.4.2. Indirect Growth Enhancement Mechanisms of PGPR

The indirect growth promoting mechanisms of PGPR include their ability to act as biocontrol against phytopathogen through various forms of antagonism like competition, production of antibiotics, siderophores and lytic enzymes (Compant *et al*., 2005; Glick, 2012), production of hydrogen cyanide (Glick, 2012), producing bacteriocin (Glick, 1995; Gupta *et al*., 2000), detoxifying virulent factors (Compant *et al*., 2005), pathogenic signal interference (Lutenberg and Kamilova, 2009) and triggering induced systemic resistance (Glick, 1995; Glick, 2012).

Competition and Production of siderophores

PGPR can compete against phytopathogens for habitable niches of plant surfaces and for available nutrients making it difficult for the pathogen to grow thereby limiting the incidence as well as the severity of diseases. For example, siderophore producing PGPR competitively aquire ferric ion preventing some phytopathogens (like fungi with lower affinity siderophores to Fe^{+3}) from aquiring sufficient amount of iron to proliferate and cause diseases in plants (Kloepper *et al*., 1980). Growth of plants is not generally affected by iron depletion in the rhizosphere caused by biocontrol PGPR since most plants can grow at much lower iron concentrations than most microbes (O'Sullivan and O'Gara, 1992) and many plants can utilize PGPR siderophore-iron complex. Treatment of tomato plant with competitive leaf bacterium *Sphingomonas* sp. prevented *Pseudomonas syringae* from causing pathogenic symptoms (Innerebner *et al*., 2011). However, it is difficult to demonstrate directly how the competition between PGPR and pathogen limit diseases in plants (Glick, 2012).

Production of antibiotics and bacteriocin

Many rhizobacteria are known to produce antibiotics and bacteriocin *in vitro* and under *in vivo* conditions that act against proliferation of phytopathogenes in different ways (Gupta *et al*., 2000). Antibiotics may prevent synthesis of cell wall and proteins of pathogens, and damage their membrane structure (Maksimov *et al*., 2011). For example, 2,4-diacetyl phloroglucino (DAPAG), an antibiotic produced by *Pseudomonas*, is known to work via damaging membrane of *Pythium* spp. (de Souza *et al*., 2003). Utilization of bacterial strains capable of producing HCN and one or more antibiotics are found to be effective in preventing the development of antibiotic resistant pathogens (Glick, 2012).

Bacteriocins are antibacterial metabolites of bacteria that have comparatively narrower killing spectrum and are only toxic to bacteria closely related to the producing strain unlike antibiotics (Riley and Wertz, 2002). But, they are effective and well known mechanisms of minimizing or preventing phytopathogenic proliferation similar to siderophores and antibiotics (Beneduzi *et al*., 2012). Marcescins and megacins are bacteriocins produced by *Serratia marcescens* and *Bacillus megaterium,* respectively (Cascales *et al*., 2007).

Production of lytic enzymes, detoxification of virulent factors and signal interference

Some biocontrol PGPR produce enzymes such as chitinases, cellulases, β-1,3-glucanases and proteases that can lyze cell wall of many pathogenic fungi (Kim *et al*., 2008). For instance, extracellular chitinases produced by *Serratia marcescens* lyzes cell wall of *Sclertium rolfsii* (Ordentlich *et al*., 1988), and β-1,3-glucanase produced by *Paenibacillus* sp. lyzes cell wall of *Fusarium oxysporum* (Singh *et al.,* 1999). PGPR hydrolytic enzymes can hydrolyze and detoxify virulent factors of pathogens such as toxins. For example, *Bacillus cepacia* hydrolyzes fusaric

acid, a phytotoxin produced by *Fusarium species* (Toyoda *et al*., 1988). PGPR may detoxify toxins of phytopathogens through the production of proteins that reversibly bind to the toxin (Compant *et al*., 2005). PGPR lytic enzymes can also degrade autoinducers that mediate quarum-sensing required for the expression of genes for key virulent factors of many pathogenic bacteria (Dong *et al*., 2004). For example: *Bacillus thuringiensis* produces *N*-Acyl homoserine lactone (AHL) lactonases and AHL acylases that hydrolyse AHL, a quorum-sensing molecule required for the synthesis of cell wall degrading enzyme in phytopathogenic *Erwinia carotovora* (Lugtenberg and Kamilova, 2009).

Production of hydrogen cyanide (HCN)

HCN is a volatile secondary metabolite produced by rhizosphere bacteria from glycine by the action of HCN synthetase. It is a PGP mechanism with antifungal (Dowling and O'Gura, 1994), anti-subterranean termite (Devis *et al*., 2007) and anti-nematode activities (Siddiqui *et al*., 2006). HCN producing *Pseudomonas fluorescens* strain CHAO prevented black root rot in tobacco (Stutz *et al*., 1986) and disease caused by *Fusarium oxysporum* in tomato (Duffy *et al*., 2003). However, HCN production may also be associated with deleterious rhizobacteria as it is a powerful inhibitor of several metal enzymes, primarily Cu containing cytochrome C-oxidase (Martinez-Viveros, 2010). HCN first inhibits electron-transport followed by energy supply disruption and eventually death of the organism (Alizadeh *et al*., 2013). The suppression of an auxin responsive gene has led to root growth inhibition by HCN in *Arabidopsis* (Rudrappa *et al*., 2008).

ACC deaminase production

Certain PGPR can modulate hormones produced by plants. In response to different types of environmental stresses such as drought and cold, plants produce ACC (1-aminocyclopropane-1-

carboxylate) from S-adenosyl methionine in the presence of ACC synthase. Since ACC is a precursor of ethylene, the level of ethylene rises in response to a stress. A small rise in ethylene initiates transcription of genes encoding defensive proteins (Glick *et al*., 2007), but higher ethylene level induces defoliation, chlorosis and abscission, inhibition of growth of stem and root and their premature senescence (Li *et al*., 2005; Glick, 2012). However, certain rhizobacteria diminish or prevent the harmful effects of high level ethylene on plants by taking up ACC secreted into rhizosphere and degrading it into readily assimilable ammonia and α-ketobutyrate with the help of their ACC deaminase (Glick *et al*., 1998).

Triggering Induced Systemic Resistance (ISR)

Induced systemic resistance (ISR) is a mechanism in which non-pathogenic rhizobacteria suppress plant diseases through inducing plant structural, physiological and biochemical modifications with out necrotic visible symptoms (Van Loon *et al*., 1998). Structural modifications include cell wall thickening (M'piga *et al*., 1997), strengthening of cell wall and formation of structural barriers like cell wall apposition or papillae (Benhamou *et al*., 1998) and lignifications of cell wall (Anderson and Guerra, 1985). The physiological or biochemical changes include accumulation of pathogenesis-related proteins (PRs) such as β-1, 3-glucanases that degrade fungal cell walls (Maurhofer *et al*., 1994). ISR-positive plants are said to be "primed" or prepared so that they react faster and more strongly to pathogen attack (Glick, 2012).

ISR by selected strains of PGPR against phytopathogens and pest have been proved by spatially separating the pathogen and PGPR in the plant (van Peer *et al*., 1991). For testing foliar protection, ISR inducing PGPR can be applied to root. But, for testing systemic protection

against root pathogen, it can be demonstrated by applying the ISR inducing PGPR on one part and the pathogen on another part of the root system employing split-root system technique to exclude direct microbial antagonism (van Loon *et al*., 1998).

ISR signaling pathway requires jasmonic acid (JA) and ethylene (ET) (Fig. 2) as JA and ET show increased level in infected plants, coordinate the activation of a large set of defense response and found to induce resistance when applied exogenously (Pieterse *et al*., 1998). Moreover, Arabidopsis JA response mutant JAR1 and ET response mutant etr1 failed to develop ISR against *Pseudomonas syringae* pv. upon root colonization by ISR inducing *P.fluorescens* WCS417 (Pieterse *et al*., 1998).

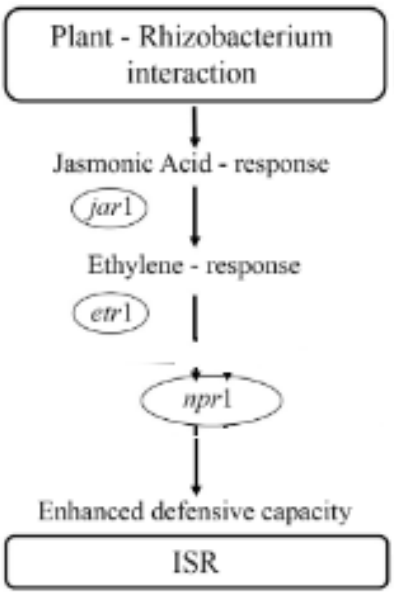

Fig. 2. Siginaling transduction pathways leading to rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis thaliana* (Beneduzi *et al*., 2012).

The transduction of ISR signal requires a regulatory (activator) protein NPR1 (NIM1) functioning in the terminal part of signal pathway (Fig. 2). SIR does not target a specific

pathogen rather it is effective against different pathogens and pests: bacteria, fungi, viruses, nematodes and insects; and it is the activation of latent resistance mechanism which is expressed upon subsequent challenge or inoculation with a pathogen (van Loon, 1998).

3.5 Bacterial strain inoculum selection, formulation and application

Selection and development of elite inoculant strains of root-nodule bacteria that suit both their legume host and the target edaphic environment are required for increased global N-fixation by legumes (Sessitsch *et al*., 2002). The criteria used in selecting strains of rhizobia for inoculant production were outlined by Burton (1984) and Howieson *et al*. (2000). Accordingly, the criteria of selection are; ability to induce nodulation and enhance yield of legume crops under a wide range of soil and climatic conditions and good persistence in the soil against indigenous strains. The inoculants should be also genetically stable and able to grow well in media, survive during manufacturing procedures, in carrier and soil. According to Lupwayi *et al.* (2006), effectiveness is the main criteria due to the cost of strain evaluation in practice. Similarly, the desired characterstics that strains of PGPR inoculum should possess include wide spectrum of plant growth enhancement, high rhizosphere and saprophytic competence ability, compatible to other rhizobacteria, safe to the environment, able to tolerate environmental stresses and easy for mass production.

For easier storage, transportation and application, cell suspensions of selected microbial strains (rhizobia or PGPR) should be immobilized in a certain carrier like peat (decomposed product of plants like mosses), talc (chemically referred to magnesium silicate), vermiculite (a light mica like mineral) and pressmud (a compressed sugar industry waste). Charcoal, soil, lignite and farmyard manure can also be used as carrier material (Mishra and Dadhich, 2010). Some of the important qualities of good carrier material are availability, biodegradability, and non-toxicity.

Once bacterial strains (rhizobia or PGPR) are selected, suitable formulation is required for successful development as inoculant. Formulation typically consists of establishing microorgsanisms in a suitable carrier and it should be cost effective, easy to handle and apply, stabilize and protect microbial strains maintaining the inoculant quality that may be assessed (Xavier *et al*., 2004). The inoculant quality control involves the verification of the identity of the inoculant strain and checking the number of living cells in the inoculant (Teamroong and Boonkerd, 2006). Catroux (2001) also suggested that the nature and content of contaminants should be taken into consideration. However, there is no set of common international standard, some countries like France, Australia and Rwanda have inoculat quality regulations where as countries like US and UK have left inoculant quality control to market forces and the manufacturers' discretion (Smith, 1992).

Microbial inoculant formulations (single or mixed strain based) are available as powder, granular, liquid, slurries, encapsulated and freeze-dried/lyophilized forms. Powder inoculant (generally using peat as carrier) is mixed directly with seeds without using any liquid before sowing (Strijdom and Deschodt, 1976). However, peat powder inoculant can be easily blown away from seeds by air-seeders. Granular inoculants are made by wetting small marble, calcite or silica grains with a good adhesive and inoculating with a powder-type. Granular inoculants contain comparable number of rhizobia as powder inoculants, they are easy to apply (can be placed in furrow) and less dusty, but they are bulkier and have higher storage and transportation costs (Xavier *et al*., 2004).

Liquid formulations are typically aqueous, oil or polymer-based products with additives like glycerol and Arabic gum that provide better adhesion and enhance rhizobial survival (Alvarez *et al*., 2010). Liquid inoculants can be applied either on the seed or in situ, reported to perform

comparable to peat-based products under field conditions (Hynes *et al*., 2001). Slurry inoculants are based on powder-type inoculants suspended in liquid (usually water) that can be directly applied to furrow or the seeds just prior to sowing (Bashan, 1998).

Encapsulated formulation involves immobilization of microbes into polymer matrices (example-alginate) to protect them against soil environment and microbial competition (Alvarez *et al*., 2010). Lyophilized bacteria may be applied to seeds. Lyophilization maintains a high number of viable cells, but the procedure can denature sensitive protein resulting in the death of some cells (Alvarez *et al*., 2010).

Similar to rhizobia, PGPR formulations can be applied to seeds and soil. They can also be applied to vegetative parts like rhizome, to foliar and fruits as spray or in combination of two or more of the methods.

3.6 An overview of soybean inoculation practices

Soybean inoculation (with mainly *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii*) has been practiced over a century where effective rhizobia are absent or insufficient in the soil (Appune *et al*., 2008). Different studies proved the improvement of growth and yield of soybean as a result of inoculating the crop with compatible and effective rhizobial strains. Sharma and Kumawat (2011) found that indigenous *Bradyrhizobium japonicum* strains significantly increased the height and dry weight of the crop even over commercial inoculant (Narmad biofertilizer) in earthern potted soil. Tamiru Solomon *et al*. (2012) reported a 53% yield increase due to inoculation of *Bradyrhizobium japonicum* TAL 379 over un-inoculated control. Workneh Bekere and Asfaw Hailemariam (2012) also reported a positive growth and yield response of soybean to *Bradyrhizobium japonicum* TAL 379 inoculation.

It is well known that rhizobial inoculations do not necessarily improve yield of soybean due to the impact of various biotic and abiotic factors on nodulation of plants (Aung *et al*., 2013). For instance, when indigenous rhizobial strains are dominant in the soil, the plants would not respond to inoculation unless the inoculation is applied at a rate of at least 1000 times greater than the estimated number of indigenous bradyrhizobia to achieve nodule occupancy of over 50 % (Weaver and Frederick, 1974) , which is yet impractical and uneconomical (Keyser and Li, 1992).

Co-inoculation of rhizobia with proper plant growth promoting rhizobacteria (PGPR) is one of the popular approaches to overcome the effect of indigenous rhizobia (Aung *et al*., 2013)**.** Co-inoculation of soybean with *Bacillus* sp. and strains of *Bradyrhizobium japonicum* enhanced the colonization and nodulation of the crop (Li and Alexander, 1988). The authors co-inoculated antibiotic resistant rhizobial strains and antibiotic producing PGP bacteria that suppress rhizosphere inhabitants. Dashiti *et al.* (1998) reported that soybeans co-inoculated with *Bradyrhizobium japonicum* strains and plant growth promoting (PGP) *Serratia* species started fixing nitrogen 2 to 4 days earlier and fixed nitrogen to the tune of 247.9 - 417.4 kg ha^{-1} with 15%-29% increament over the control plants. Co-inoculation of *Bradyrhizobium* (TAL-378) and PGP *Pseudomonas* species increased soybean seed yield by 16% over plants supplied with N and P, each 46 kg ha^{-1} (Anteneh Argaw, 2012). Kravchenko *et al.* (2013) recorded a 10% increase in seed yield for soybean co-inoculated with *Bradyrhizobium japonicum* and a PGP *Bacillus megaterium* over sole inoculation of the *Bradyrhizobium japonicum*.

Under field conditions, *Azospirillum* sp. co-inoculaed with *B. japonicum* CB 1809 resulted in 32%, 27% and 24% increase in nodule number, nodule dry weight and grain yield, respectively over single inoculation of the *B. japonicum* strain; where as co-inoculation of the *Azospirillum*

sp. and *B. japonicum* USDA 110 resulted in 17%, 19% and 35% increase in nodule number, nodule dry weight and grain yield, respectively over single inoculation of *B. japonicum* USDA 110 (Aung *et al*., 2013). It has been suggested that the positive effects of *Azospirillum* are mainly due to its capacity to produce phytohormones rather than BNF (Rodriguez-Navarro *et al*., 2011).

Co-inoculation of rhizobacteria and *Bradyrhizobium japonicum* strains 532C was reported to reduce the negative effect of low root zone temperature (RZT) on nodulation and nitrogen fixation of soybean (*Glycine max*) (Zhang *et al*., 1996). The authors indicated that at 17.5^{o}C (threshold inhibition), co-inoculation of the *Bradyrhizobium japonicum* strain with *Serratia liquefaciens* and *Pseudomonas putida* resulted in up to 72% increase in the number of nodules and 140% in the amount of fixed nitrogen per plant, respectively over those plants inoculated with *Bradyrhizobium japonicum* strain alone in one of the experiments.

Inoculation of soybean with its rhizosphere isolate PGP bacteria alone had also been reported to have some positive impacts on the performance of the crop. Soybean seed inoculation with PGPR (*Pseudomonas fluourescens* and *Bacillus amyloliquefaciens*) increased seedling emergence rate upto 90% and suppressed damping-off caused by *Pythium ultimum* by 74% (Le'on *et al*., 2009). Inoculation of soybean with *Bacillus pumilus* significantly increased plant height (22%) and bean protein content (66%) higher than the non-treated control plants under ecological condition (Stefan *et al*., 2009). Maximum plant height (67 cm) and dry matter (56 g) were recorded in soybean plants inoculated with *Pseudomonas aeruginosa* PaJ indicating 34% and 59% increases in plant height and dry matter yield , respectively compared to untreated plants (Khalimi and Suprata, 2011). The authors also reported 10 to 80% of soybean stunt virus (SSV) incidence in inoculated plants compared to 90% in control plants.

4. Diversity, Ecological and Plant Growth Promoting (PGP) Properties of indigenous Rhizobia nodulating Soybean (*Glycine max* (L) Merr.) from Ethiopian soils

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Abstract

Soybean is a leguminous crop, introduced into Ethiopia in the 1950s. However, its production is low due to lack of nodulation in the field. This situation demands inoculation of soybean seeds with exotic rhizobia that resulted in low and inconsistent yield. To assess their functional and genetic diversity, indigenous soybean rhizobia were recovered using plant induction method from soybean nodules formed in response to inoculation with a diverse range of 140 soils samples across Ethiopia. Only 18 soil samples (13%) induced nodulation from which 21 bacteria were authenticated as rhizobia. Physiological characterisation of the isolates on the basis of growth rate and acidification or alkalisation during growth on YMA medium distinguished them as either acid producing fast growing (52%) or alkali producing slow growing (48%) which were tentatively grouped into *Rhizobium* (and/or related groups) and *Bradyrhizobium* spp., respectively. Numerical taxonomy based on 90 phenotypic features also showed that the isolates were grouped into 3 clusters at about 40% similarity level of which sub-cluster I contained eight *Bradyrhizobium* spp. and sub-clusters IIA, IIB, and cluster III contained nine isolates from *Rhizobium* groups indicating that the majority of the isolates conform to the preliminary categorization based on growth rate. Although it was not conclusive, the phylogenetic study of fewer isolates with sequence analyses of 16S rRNA, nifH, nodA, nodD and recA genes also showed that the fast growing rhizobia were similar to one of the type strains from *Agrobacterium*

or *Rhizobium*; and the slow growing rhizobia were aligned with one of the type strains of *Bradyrhizobium japonicum, B. elkani,* or *B. yauanmingense*. This shows the existence of diverse groups of fast and slow growing soybean rhizobia that needs further analysis using polyphasic approach. Although all isolates showed similarity in pH tolerance and inhernt heavy metal resistance, the fast growing rhizobia were nutritionally more versitile and better tolerant to NaCl (5-6%), high temperature (40^{o}C-45^{o}C) and agrochemicals than the slow growing isolates. which were more resistant to antibiotics. The data also showed that 62% of the isolates acquired multiple (2-5) PGP traits, where all isolates produced IAA (from 4.68-104.73 µM) and fewer isolates (10-40%) showed cellulase and protease activities, antagonistism against *Fusarium oxysporum*, and phosphate solubilization. Generally, the fast growing isolates were better in the overall performance where fast growing (SNB 120C, SNB 125B, SNB 120B, and SNB 41) and the slow growing (SNB 120A, SNB 125A, and SNB75) isolates; could be tested for their symbiotic effectiveness to enhance soybean production in the country.

Key words or phrases: gene sequencing, Phylogenetic analysis, stress tolerance

4.1 Introduction

Soybean is a leguminous crop used in Ethiopia as food and feed. It is nodulated by diverse groups of root nodulating symbiotic bacteria known as rhizobia. Traditionally, the slow growing and alkali-producing *Bradyrhizobium japonicum* was considered as the sole symbiont of the crop (Jordan, 1984). Later, other slow growing rhizobia such as *Bradyrhizobium elkanii* (Kuykedall *et al*., 1992), *Bradyrhizobium liaoningense* (Xu *et al*., 1995), *Bradyrhizobium yuanmingense* (Appunu *et al*., 2008), *Bradyrhizobium canariense* (Yang and Zhou 2008), *Bradyrhizobium huanghuaihaiense* (Zhang *et al*., 2012) and *Bradyrhizobium daqingense* (Wang *et al.,* 2013) were idenified from the crop.

Although *Bradyrhizobium* spp. are the dominant endosymbionts of soybean, it has also been found to be nodulated by *Mesorhizobium tianshanese* with variable generation time, 5-15 hours (Chen *et al*., 1995) and by fast growing *Rhizobium fredii* (Keyser *et al*, 1982; Scholla and Elkan, 1984), *Rhizobium* sp. NGR234 (isolated from *Lablab purpureus*; Trinick, 1980) and *Rhizobium meliloti* (strain 042B) (isolated from alfalfa; Goa and Yang, 1995). These fast growing rhizobia were later grouped into the new genus *Sinorhizobium* (Chen *et al*., 1988). It is also interesting to note that the isolation of symbiotically effective *Agrobacterium* strains from soybean root nodules (Youseif *et al*., 2014), indicating that soybean is a relatively promiscous host nodulated by different types of root nodule bacteria isolated from homologous and heterologous hosts.

Several studies also showed that the geographic distribution of soybean rhizobia is influenced by various factors like soil pH, climate, latitude and host plant variety (Rj genotype). Accordingly, *Sinrhizobium fredii*, *Sinorhizobium xinjiangense* and *Mesorhizobium tianshanese* are widely distributed in alkaline-saline soils where as *Bradyrhizobium* species are predominantly found in neutral to acidic soils (Chen *et al*., 1995, Li *et al*., 2011; Zhang *et al*., 2011). Similarly, community structure of soybean bradyrhizobia showed that *B. japonicum* dominates temperate locations where as *B. elkanii*, *B. yuanmingense* and *B. liaoningense* are widely distributed in tropical localities (Adhikari *et al*., 2012; Shiro *et al*., 2013).

Besides nitrogen fixation, rhizobia isolated from soybean possess plant growth promoting (PGP) properties such as solubilization of inorganic phosphate (Jadhav, 2013), phtohormone production (Chen *et al*., 2002; Boddey and Hungria, 1977), and suppression of phytopathogens (Pawar, 2014). So screening the rhizobia is required for the identification those endowed with such beneficial strains.

In Ethiopia, attempts were made to collect indigenous rhizobia from soybean (Amare Abebe, 1986) for culture collection, genetically characterize (Aregu Amsalu *et al*., 2012) and to evaluate inherent antibiotic resistane (Tolera Abera *et al*., 2015) of a few soybean rhizobia. However, the plant growth promoting and ecological competitive traits have not been comprehensively studied. The objective of this study was therefore to isolate and characterise soybean rhizobia from soils collected from different parts of the country on the basis of genetic and phenotypic diversity to identify potential local soybean inoculant to boost the productivity of the crop.

4. 2. Materials and methods

4.2.1 Description of study sites and rhizobial isolation

The test rhizobia were obtained from soybean nodules (cv. Ethio-Yugoslavia) induced from 140 composite soil samples using plant trapping method (Vincent, 1970). The soil samples were collected from different parts of Amhara, Oromia, SNNP (Southern Nations, Nationalities and Peoples') and Tigray Regional States of Ethiopia (Table 2) under different land management practices: uncultivated or cultivated under different leguminous and non-leguminous crops with no previous inoculation. The collection sites encompassed different agro-ecologies from 1031 to 1913 meters above sea level (mas) with slightly acidic to moderately acidic soils ranging from pH 5.87 to 6.34.

Table 2. Geographic distribution of soybean rhizobial isolates with their respective Soil pH

Serial No.	Rhizobial isolate	Sampling regions	GPS of Sampling sites				Soil pH
			Latitude	Longitude	Altitude	Regional State	
1	SNB13	Hawassa area	37N0442062	0768336	1875	SNNP	6.34
2	SNB41	Awash town area	37P0535514	0946779	1517	Oromia	6.20
3	SNB43	Awash town area	37P0538912	0949647	1482	Oromia	6.18
4	SNB45	Adama	37P0543983	0953974	1469	Oromia	6.29
5	SNB46	Adama	37P0545278	0955408	1464	Oromia	6.22
6	SNB57B	Adami Tulu	37P0532529	0936696	1657	Oromia	6.00
7	SNB 57C	Adami Tulu	37P0532529	0936696	1657	Oromia	6.00
8	SNB70	T/Adyabo	37P0375216	1593079	1061	Tigray	6.18
9	SNB71	T/Adyabo	37P0374900	1593081	1061	Tigray	6.06
10	SNB79	T/Adyabo	37P0374896	1593103	1055	Tigray	5.95
11	SNB101	T/Adyabo	37p0375219	1592974	1057	Tigray	6.04
12	SNB102	Bako town area	37º09’E	09º06’	1653	Oromia	6.40
13	SNB114	Awash town area	37P053999	0948998	1581	Oromia	6.23
14	SNB 55	Adami Tulu	37P053299	0936993	1628	Oromia	6.06
15	SNB120A	Bako town area	37º09’1”E	09º06’	1651	Oromia	5.87
16	SNB120B	Bako town area	37º09’1”E	09º06’	1651	Oromia	5.87
17	SNB120C	Bako town area	37º09’1”E	09º06’	1651	Oromia	5.87
18	SNB125A	Dembecha	37º13’56.3”	10º39’58.6”N	1859	Amhara	6.18
19	SNB125B	Dembecha	37º13’56.3”	10º39’58.6”N	1859	Amhara	6.18
20	SNB140	Luba Dakab	37p 0730561	UTM 1014840	1779	Oromia	5.89
21	SNB 75	Wutete	36p 0822828	UTM 0926865	1632	Oromia	5.99

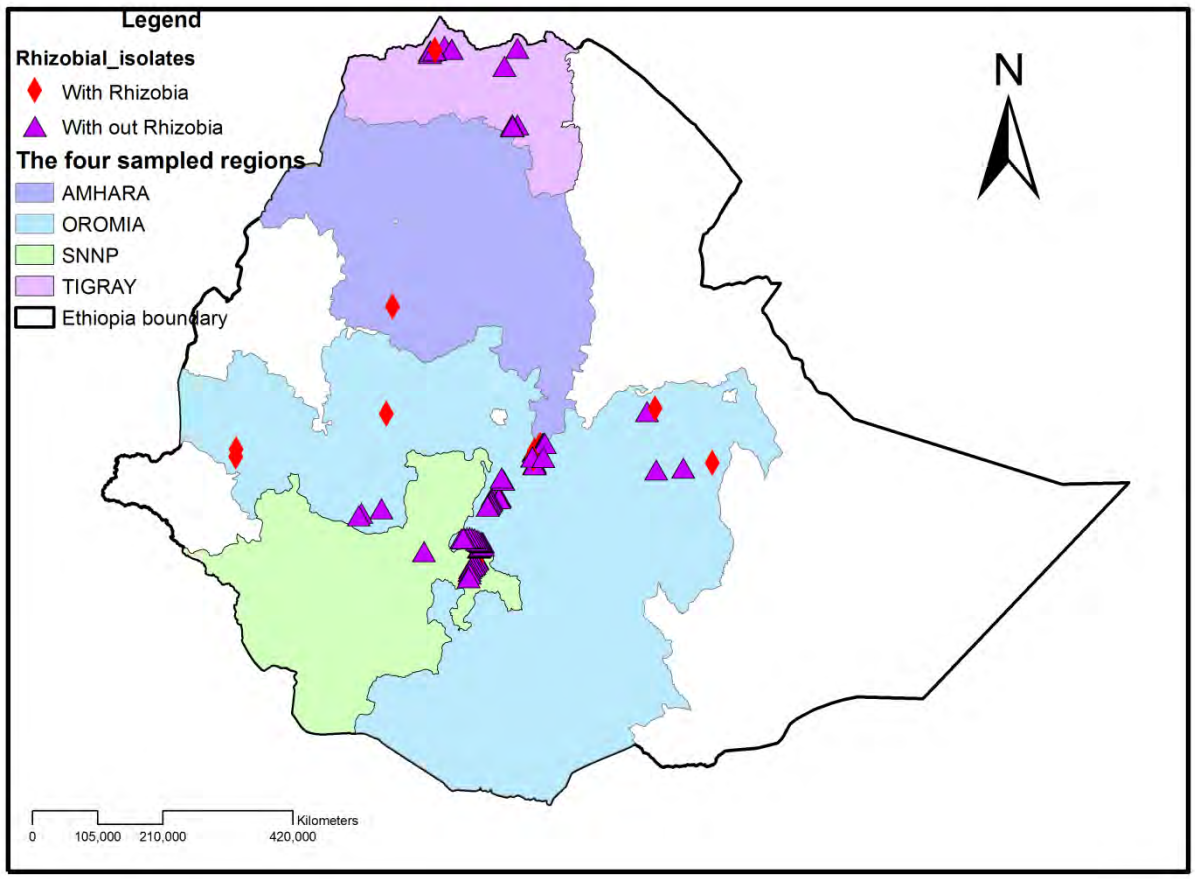

Fig. 3. Study areas showing the presence and absence of soybean rhizobia

The nodules were surface sterilized (4% sodium hypochlorite), crushed, and the nodule suspensions were inoculated into yeast extract mannitol agar (YMA) medium containing (g L^{-1}) KH_2PO_4 (0.5), $MgSO_4.H_2O$ (0.2), NaCl (0.1), yeast extract (0.2), mannitol (10) and agar (15). They were sub-cultured, purified and eventually authenticated on the host variety on sand culture under greenhouse conditions using standard methods (Somasegaran and Hoben, 1994). The isolates were designated as SNB (Soybean Nodulationg Bacteria), and preserved at 4ºC on a YMA slant containing $CaCO_3$ (Vincent, 1970). A standard soybean *Bradyrhizobium japonicum* (SBTAL 379), obtained from the Ethiopian National Soil Testing Center, was included as reference for all tests.

4.2.2 Cultural characterstics

Experimental conditions

All tests were carried out in triplicates on YMA medium inoculated with a loopful culture (10 µL; 10^6 cells mL^{-1}) and incubated at 28±0.2 ^oC for 5-7 days unless stated otherwise. In all cases, positive control plates were included and rhizobial growth was scored qualitatively by visual inspection as (+) for positive growth and as (-) for no growth.

Colony characteristics

The rhizobial isolates were streaked on YMA plates to evaluate colony size (mean diameter of five colonies), shape and margin, texture (elastic or buttery), appearance (shiny/translucent or opaque) and gum production (dry or gelatinous) according to Lupwayi and Haque (1994).

Acid/alkali production

All the isolates were inoculated into BTB (0.5 % Bromothymol blue)-YMA medium to detect color change due to acid or alkali production (Jordan, 1984).

Determination of generation time

Growth rate was determined by inoculating 10 mL of active cells into 100 mL sterilized YEM broth in 250 mL Erlenmeyer flask and grown on orbital shaker (Gollen hamp, England) at 150 rpm at room temperature for 72 hours. Samples were taken every two hours to measure optical density (OD^{540}; Jenway, 6405 Uv/vis spectrophotometer) and to determine viable colony forming units (cfu) on YMA according to Somasegaran and Hoben (1994). Finally, the generation time (g) was calculated from the logarithmic phase according to White (1995) using the formula

$g = [\log 2 (t)]/[\log X - \log Xo]$ where t is time elapsed, Xo is first OD reading and X is second OD reading in logarithmic phase.

4.2.3 Test for Salt (NaCl), temperature and pH tolerances

The isolates (a loopful;10µL; 10^6 cells mL^{-1}) were inoculated into YMA containing 0.5 to 6% NaCl (w/v) and into Keyser-defined medium (composition; Appendix 1) adjusted to pH 4, 4.5, 5, 8.5, 9, 9.5 and 10 and incubated at 28±0.2ºC according to Lupwayi and Haque (1994). The isolates were also grown on YMA and incubated at temperatures of 35^0C, 37^0C; 40^0C and 45^0C by wrapping the plates with parafilm® to minimize moisture lose.

4.2.4 Intrinsic resistance to antibiotics (IAR), heavy metals (IHM) and pesticides

Different antibiotics, heavy metals and pesticides were filter sterilised (0.22 µm), streaked plates (a loopful;10µL; 10^6 cells mL^{-1}) were incubated at 28 ± 0.2 ºC for 5-7 days and growth of the rhizobial isolates was qualitatively evaluated as (-) for no growth and as (+) for positive growth in all cases. All the isolates (twenty one) and the reference strain were inoculated 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). The isolates were also tested for their inherent heavy metal resistance (IHM) by growing them on minimal salt agar medium plates according to Hungria *et al*. (2001). The medium contained (g L^{-1}) KH_2PO_4 (1), K_2HPO_4 (1), NH_4NO_3 (1), $MgSO_4.7H_2O$ (0.2), $CaCl_2.2H_2O$ (0.02), $FeSO_4.7H_2O$ (0.01) and agar (15) supplemented with 10 g of mannitol and 0.7 g of NH_4Cl, adjusting its pH to 6.8 and adding the heavy metals at final concentrations of (mM or milli molar); $CoCl_2.6H_2O$ (0.5 mM), $Pb(CH_3COO)_2.3H_2O$ (2.5 mM), $K_2Cr_2O_7$ (0.25 mM), and $CuCl_2.2H_2O$, $ZnSO_4.7H_2O$ and $MnSO_4.4H_2O$ (each 0.25 mM).

The isolates were also tested for their resistance to 0.2% [w/v] of commercial formulations of two fungicides (Mankozeb and curzet; Du Pont de Nemour, France) on YMA plates according to Mubeen *et al*. (2006) and resistance to 0.14% glyphosate (a herbicide; Monsanto Europe S.A, Belgium) on minimal salt agar medium plates (Ahemad and Khan, 2010) with composition indicated above.

4.2.5 Nutritional versatility of isolates on carbon and nitrogen substrates

Isolates (a loopful;10µL; 10^6 cells mL^{-1}) were grown separately on 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 nitrogen substrates (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) added to a basal medium to evaluate their nutritional diversity according to Amarger *et al*. (1997). The basal medium contained (L^{-1}) 1 g of K_2HPO_4, 1 g of KH_2PO_4, 0.01 g of $FeCl_3.6H_2O$, 0.2 g of $MgSO_4.7H_2O$, 0.1 g of $CaCl_2$, 1 g of $(NH_4)_2SO_4$, and 15 g of agar. Heat labile carbon sources (D-arabinose, D-mannose, sorbitol, D-galactose, maltose, Na-citrate, xylose, trehalose, and cellobiose) and amino acid (DL-tryptophan and DL-glutamic acid) were filter sterilized (0.22 µm) and included into autoclaved basal medium. Each carbon and nitrogen substrate was separately added as 1% and 0.5% (w/v), respectively by substituting ammonium sulfate with the same quantity of mannitol in testing for nitrogen substrate. Growth of the rhizobial isolates was qualitatively evaluated as (-) for no growth and as (+) for positive growth or indicative of substrate utilization in all cases.

4.2.6 Plant growth promoting (PGP) characteristics of soybean rhizobia

4.2.6.1 Solubilization of Inorganic phosphates

The isolates were tested for their ability to solubilize three inorganic phosphate sources (Tri-calcium-, aluminium- and iron- Phosphate). A loopful young rhizobial culture (10µL; 10^6 cells mL^{-1}) was spot inoculated on Pikovskaya's medium to determine their tri-calcium phosphate solubilization ability. The medium contained (g L^{-1}) glucose (10), $Ca_3(PO_4)_2$ (5), $(NH_4)_2SO_4$ (0.5), NaCl (0.2), $MgSO_4.7H_2O$ (0.1), KCl (0.2), yeast extract (0.2), $MnSO_4.7H_2O$ and $FeSO_4.7H_2O$ (0.0003 each) and agar (15). The cultures were also spot inoculated into NBRIP (National Botanical Research Institute's Phosphate) medium to detect their solubilization of aluminum phosphate ($AlPO_4$) and iron-phosphate ($FePO_4$) by substituting $AlPO_4$ or $FePO_4$ for $Ca_3(PO_4)_2$. NBRIP medium contains (g L^{-1}) glucose (10), $Ca_3(PO_4)_2$ (5), $MgCl_2.6H_2O$ (5), $(NH_4)_2SO_4$ (0.1), $MgSO_4.7H_2O$ (0.25), KCl (0.2) and), agar (15) according to Pe'rez *et al*. (2007). Isolates that formed clear halo around their colonies were identified as phosphate solubilizers. Phosphate solubilization index (PI) was calculated by dividing the total diameter (halo zone and colony) to colony diameter (in mm) according to Prashar *et al.* (2012).

4.2.6.2 IAA ($C_{10}H_9NO_2$) production detection and quantification

Each isolate (100 µL) was inoculated into 5 mL YEM broth amended with filter sterilized L-tryptophan (2 g L^{-1}) to detect and quantify indole acetic acid (IAA) production (Parray *et al*., 2013). The culture was grown on a shaker (Gollen hamp, England) at 150 rpm at room temperature for four days, centrifuged at 3,000 rpm for 30 minutes from which 2 mL of the supernatant was mixed with 4 mL of Salkowski reagent (7.5 mL of 0.5M $FeCl_3.6H_2O$ + 150 mL of 95-98 % H_2SO_4 + 250 mL of distilled H_2O). The mixture was kept at room temperature for 25

minutes in darkness to develop pink color and measure the absorbance at 530 nm (Jenway, 6405 Uv/vis spectrophotometer). The IAA was quantified using a standard curve of known concentrations of pure IAA (HiMedia; 5, 10, 20, 50, 80 and 100 µg $mL^{-1)}$ in un-inoculated L-tryptophan amended YEM broth. Non-inoculated L-tryptophan supplemented YEM broth medium was used as control.

4.2.6.3 *In vitro* antagonistic activity against pathogenic fungus

The soybean nodulating bacterial isolates (a loopful;10µL; 10^6 cells mL^{-1}) were spotted on YMA plates amended with 0.5% sucrose at a distance of 2.0 cm from the center at four equidistant points (12, 3, 9 and 6 o'clock positions) and incubated at 28 ± 0.2ºC for 72 hrs (Chandra *et al*., 2007). Then, a 4 mm disc of 72 hrs potato dextrose agar (PDA) culture of *Fusarium oxyporum* was placed at the center of the Petri dishes and 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 as PIRG = [(R1-R2)100]/[R1], where R1 was radial growth of fungus in control plate, and R2 was radial growth of the fungus in the co-inoculated plates (Siddiqui and Meon, 2009).

4.2.6.4 Production of hydrogen cyanide

The rhizobial isolates (100 µL; 10^6 cells mL^{-1}) were inoculated into YMA plates amended with 4.4 gL^{-1} of glycine to detect HCN production (Ahemad and Khan, 2012**).** Stripes of filter paper (Whatman filter paper No.1) were soaked in the yellow picric acid solution (2.5 g of picric acid and 12.5 g of Na_2CO_3 dissolved in 1L of distilled water) and fixed to the underside of the upper lids and sealed with parafilm® (to avoid the escaping of volatiles like HCN), and incubated at 28

± 0.2°C for 5 days. A color change of the yellow filter paper to light brown, brown or reddish brown was recorded as weak, moderate and strong production of HCN, respectively.

4.2.6.5 Tests for protease, chitinase and cellulase activities

Protease activity was assessed by spot inoculating the rhizobial isolates on nutrient agar plates supplemented with 1.5% skimmed milk powder (Ryden *et al*., 1973). The soybean rhizobial isolates were also spot inoculated on Carboxymethyl cellulose (CMC) agar plates containing (g L^{-1}) $NaNO_3$ (2), K_2HPO_4 (1), $MgSO_4$ (0.5), KCl (0.5), CMC sodium salt (2), peptone (0.2), and agar (17) to evaluate their cellulase activity according to Kasana *et al*. (2008). The cultures were also inoculated into chitin agar constituting (g L^{-1}) colloidal chitin (4), $MgSO_4.7H_2O$ (0.5), K_2HPO_4 (0.7), KH_2PO_4 (0.3), $FeSO_4.7H_2O$ (0.01), $MnCl_2$ (0.001), NaCl (0.3), yeast extract (0.2) and agar (20) as described by Bansode and Bajekal (2006). In all cases, plates were spot inoculated with a loopful of broth culture (10µL; 10^6 cells mL^{-1}) and incubated at 28 ± 0.2 °C for 3-5 days to detect clear zone formation around their colonies.

4.2.7 Numerical Analysis

Phenotypic variability of the soybean rhizobial isolates was analyzed by a computer cluster analysis using a similarity coefficient. A phenotypic dendrogram was constructed by Ori Past Computer Software (Hammer *et al*., 2001) with multivar cluster analysis based on 90 phenotypic characteristics.

4.2.8 Gene sequencing and phylogenetic analyses

Sequencing of the 16S rRNA genes of four selected rhizobial isolates (SNB41, SNB57B, SNB70, SNB120A) was undertaken at James Hutton Institute, Scotland, UK. Sequencing of

nifH, nodA, nodD, and recA, and phylogenetic analyses based on these genes were performed only for those isolates for which these genes were successfully amplified.

4.2.8.1 DNA extraction

A single colony of each rhizobial isolate was inoculated into 5 mL of YEM broth and incubated on a rotary C24KC Refrigerated Incubator shaker (Edison, NJ, USA) at 28 ºC and 150 rpm for not more than 24 hrs. A total of 4 mL (2 x 2 mL) of log phase liquid cultures (10^9 cells mL^{-1}) was harvested by centrifugation (14,000 rpm; 10 min; 4°C) in a 2 mL microfuge tube.

Rhizobial pellets were re-suspended in 400 µL sterile Tris-EDTA (TE) buffer and lyzed with 10 µL of 20% (w/v) sodium dodecyl sulfate (SDS; final concentration of 0.5% [w/v]) to which 10 µL (>800 u mL^{-1}) proteinase K (Sigma #P4850) was added and mixed by vortexing for 5 seconds. The mixture was incubated at 37 ºC in water bath for 1 hour followed by the addition of 420 µL of phenol:chloroform:isoamyl alcohol (25:24:1 [v/v/v]; Sigma #P2069), vortexing for 1 minute and centrifugation (14,000 rpm; 10 min; 4°C). The aqueous phase or the upper layer (≈175 µL) was recovered, and mixed well with 1/10 x volume (17.5 µL) of 3 M Sodium acetate (pH 5.2) and 3x volume (655 µL) of isopropanol (Sigma #I9030).

DNA was precipitated by incubating the mixture at -80°C for 15 minutes (or at -20°C over night) and pelleted by centrifugation (14,000 rpm; 15 min; 4°C). DNA pellet was washed with 200 µL of 70% [v/v] ethanol, centrifuged (14,000 rpm; 1 min; 4°C), oven dried (micofuge tubes opened) at 37ºC for 15 minutes, re-suspended in TE buffer (25 µL each) and one µL of each DNA sample was assessed for its quality (absorbance 260:280 ratio) and quantity (µg mL^{-1}) using a ND -1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, US).

4.2.8.2 Amplification, purification and sequencing of 16S rRNA, nifH, nodA, nodD and recA genes

Amplification of a portion of 16S ribosomal RNA (rRNA), nitrogenase iron protein (*nifH*), N-acyl transferase nodulation protein A (*nodA)*, the regulatory *nodD* genes (*nodD*) and DNA recombinase A (*recA*) was performed using a G-Storm GS1 thermal cycler (GRI Ltd, Braintree, UK) employing specific primers for each gene (Table 3). For nodD, primer combinations NBA12xY6, NBF12'xY5, NBA12xNBF12' and Y5xY6 were used for the first round PCR (PCR1). One µL NBA12xNBF12' products of PCR1 were also used in the second round PCR with Y5xY6 **(**Zeze *et al*., 2001). The PCR reaction mixture (50 µL for each sample) was prepared according to the manufacturer's recommendations and constituted of milliQ-water (33.75 µL), 5X clear Go Taq® G2 Buffer (Promega, 10 µL), 10 mM dNTPs (Invitrogen, 1 µL), forward and reverse primers (2 µL each), Go Taq polymerase (Promega #M3175, 0.25 µL) and DNA template (1 µL). PCR reaction mixture containing 1µL sterile distilled water instead of DNA templates was included as negative control.

The successful amplification of PCR products (8 µL each) was resolved by electrophoresis (80 mV, 20 min or 100 mV , 15 min #E844 Consort Electrophoresis Power Supply; Sigma-Aldrich) on a 1% [w/v] agarose gel (for 16S rRNA) or 2% [w/v] agarose gel (for the remaining genes) containing 1µL SYBR-SAFE (Invitrogen) followed by UV-illumination (FluorChem® Imager, Alpha Innotech). A 1 kb ladder and a 100 kb ladder were run adjacent to the test samples for16S rRNA gene and for the rest of the genes, respectively. The PCR products were purified as stated by the manufacturer using QIAquick Spin columns (Qiagen, Inc., Chatsworth, Calif.). The purified product was eluted using 30 µL Buffer EB (10 mM Tris-Cl, pH 8) and cleaned on QIAquick membrane according to the manufacturer's recommendations and eventually stored at

Table 3. Primers used in the study

Target gene	Primer code	Primer sequence	Thermal profile	References
16S	8-27F	AGAGTTTGATCCTGGCTCAG	2min 95°C, 35x(30s 56 °C, 1.5min 72°C), 15min 72°C	Weisburg *et al*. (1991).
	rD1	AAG GAG GTG ATC CAG CC		
nifH	PolF	TACGGNAARGGSGGNATCGGCAA	2min 95°C, 35x(30s 60 °C, 1min 72°C), 15min 72°C	Bontemps *et al*.(2010), Haukka *et al*. (1998)
	PolR	AGCATGTCYTCSAGYTCNTCCA		
nodA	Fnod A1	TGCRGTGGAARNTRNNCTGGGAAA	2min 95°C, 40x(30s 55 °C, 1min 72°C), 15min 72°C	Haukka *et al*. (1998), Youseif *et al*. (2014)
	RnodA2	GGNCCGTCRTCRAAWGTCARGTA		
nodD	NBA12	GGATSGCAATCATCTAYRGMRTC	2min 95°C, 35x(30s 55 °C, 1min 72°C), 15min 72°C	Zeze *et al*. (2001)
	NBF12'	GGATCRAAAGCATCCRCASTAT		
	Y6	CGCAWCCANATRTTYCCNGGRTC		
	Y5	ATGCGKTTYARRGGMCTNGATCT		
recA	63	ATCGAG CGGTCGTTCGGCAAGGG	2min 95°C, 35x(30s 60 °C, 1min 72°C), 15min 72°C	Gaunt *et al*. (2001)
	504	TTGCGCAGCGCCTGGCTCAT		

-20 ºC for sequencing. Sequencing of all the genes was performed at 40 ng μL^{-1} with primers mentioned earlier using an ABI3730 DNA analyzer having a 36 cm x 48 cm capillary array. The isolates were identified using the sequence analysis with the software BioEdit Sequence Alignment Editor version 7.2 and screened against databases using the nucleotide basic local alignment tool (BLASTN) queuing system (Altschul *et al*., 1997) 2.2.28 on the NCBI website (www.ncbi.nlm.nih.gov)

4. 2.8.3 Phylogenetic analysis

The evolutionary history of the rhizobial isolates was inferred based on 16S rRNA, *nifH*, *nodA*, *nodD* and *recA* gene sequencing using the Maximum Likelihood method based on the Tamura and Nei (1993) model. Phylogenetic trees for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with

superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions *per* site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016) in each case.

4.3. Results and Discussion

4.3.1 Rhizobial trapping and authentication

Out of the 140 soil samples collected from various regions of the country, only 18 soil samples (13%) induced nodules on the soybean variety Ethio-Yugoslavia (data not shown) indicating that most of the soil samples did not harbor rhizobia compatible to the host. Some studies in the past also showed that soil samples from Hawassa and Ziway (South Central Ethiopia) (Bayou Bunkura Allito, 2015), Bako (Western Ethiopia) (Tamiru Solomon *et al*., 2012) and Shinille (Eastern Ethiopia) (Anteneh Argaw, 2014) failed to nodulate soybean even the promiscuos soybean genotype TGx-1336424 as reported by the latter author.

From the nodules, 23 bacteria were isolated of which 21 isolates (91%) were authenticated as soybean rhizobia (Table 4). The fact that the isolates were collected from soils without any history of inoculation may suggest that the soybean variety was either promiscuously infected by indigenous rhizobia from heterlogous host legumes, or the presence of local rhizobia specific to soybean as previously reported from Ethiopia (Aregu Amsalu *et al*., 2012), Egypt (Youseif *et al.*, 2014) and Brazil (Hungria *et al*., 2001). The close taxonomic similarities of indigenous soybean B*radyrhizobium* spp. with those isolated from *Crotalaria incana* and *Indigofera arrecta* from Ethiopia (Aregu Amsalu *et al*., 2012) indicated that native legumes could be a source of novel ecologically adapted symbiotically effective soybean rhizobia (Tang *et al*., 2012).

4.3.2 Cultural characteristics of soybean rhizobia

Colony size varied from 2 - 6 mm in diameter (average 3.60) after 5-7 days, and generation time of 1.5 - 6.6 h (Table 4). Accordingly, a number of the isolates displayed fast growth (1.5-4 hour generation time) with larger colony diameter of 3-6 mm similar to fast growing soybean rhizobia; *Rhizobium fredii* (from Taiwan) with generation time of 1.7 h (Young *et al*., 1988) and with generation time varying from 1.4 to 4 h from Brazil (Hungria *et al*. 2001). Other indigenous soybean rhizobial isolates displayed slow growth with generation time of 5.2-6.6 h with smaller colony diameter (2-3 mm). The slow growing soybean rhizobial isolates reported here showed more rapid cycling than previously characterised ones; 13 h (Singh *et al*., 2013) and 9.5-16 h for *Bradyrhizobium yuanmingense* (Yao *et al*., 1995; Appunu *et al*., 2008).

The isolates also changed the color of the BTB-YMA medium either into yellow or blue (Table 3) showing a distinct dichotomy of fast growth/yellow color and slow growth/blue color described by Jordan (1984). Consequently, based on fast growth (generation time of 2-4 h) and slow growth (>4 h) (Jordan, 1984), and large colony diameter; up to 5 mm for fast and below 2 mm for slow growers (Scholla and Elkan, 1984; Sadowsky *et al*., 1983; Singh *et al*., 2013), and acid or alkali production on the BTB-YMA medium within seven days of incubation, 52% of the isolates were tentatively classified into fast growing and acid producing *Rhizobium* or other fast growiing groups, whereas the remaining 48% of the isolates were slow growing and alkali producing *Bradyrhizobium* spp. (Table 4).

The isolates produced circular and mucilagenous colonies with entire margin except SNB75 (*Bradyrhizobium*), and SNB120C and SNB125B (*Rhizobium*) that formed colonies having filamentous shape and margin with little mucilage production. Similarly, slow growing soybean rhizobia (Fuhrmann, 1990) and fast growing soybean rhizobia (Sadowsky *et al*., 1983) displayed

irregular colony margins with less exopolysaccharides. Moreover, a number of isolates: SNB55 (*Rhizobium*), SNB102 (*Rhizobium*), SNB114 (*Rhizobium*), SNB101 (*Bradyrhizobium*), SNB 120A (*Bradyrhizobium*), SNB140 (*Bradyrhizobium*), and the reference strain SBTAL 379 (*Bradyrhizobium*) showed shinny translucent colonies similar to both fast growing *Sinorhizobium fredii* of soybean (Chen *et al*., 1988) and slow growing soybean *Bradyrhizobium* spp. (Zhang *et al*., 2012). In general, the data showed that the isolates exhibited differet types of colony texture and shape irrespective of their taxonomic affiliations.

Table 4. Preliminary taxonomic classification of soybean root nodule bacteria based on growth and cultural characteristics after growing on YMA medium, at 28±2°C for 5-7 days.

Sr.No	Isolate	Colony characterstics		BTB reaction	Mean g (hr)	Taxonomic group
		Size (mm)	Colony texture and shape*			
1	SNB 13	5	CGE	Yellow	2.0	*Rhizobium* /others
2	SNB 41	6	CGE	Yellow	2.0	*Rhizobium* /others.
3	SNB43	2	CGE	Blue	5.5	*Bradyrhizobium* sp.
4	SNB45	6	CGE	Yellow	1.5	*Rhizobium* /others
5	SNB46	2.5	CGE	Blue	5.5	*Bradyrhizobium* sp.
6	SNB57B	2	CGE	Blue	5.3	*Bradyrhizobium* sp.
7	SNB57C	3	CGE	Yellow	3.7	*Rhizobium* /others
8	SNB70	2.5	CGE	Blue	5.4	*Bradyrhizobium* sp.
9	SNB71	2	CGE	Blue	5.9	*Bradyrhizobium* sp.
10	SNB79	4	CGE	Yellow	3.3	*Rhizobium* /others
11	SNB101	2.5	CGES	Blue	5.2	*Bradyrhizobium* sp.
12	SNB102	4	CGES	Yellow	3.6	*Rhizobium* /others
13	SNB114	5	CGES	Yellow	3.8	*Rhizobium* /others
14	SNB55	3	CGES	Yellow	3.2	*Rhizobium* /others
15	SNB120A	3	CGES	Blue	6.1	*Bradyrhizobium* sp.
16	SNB120B	5	CGE	Yellow	4.0	*Rhizobium* /others
17	SNB120C	3	IFLG	Yellow	3.6	*Rhizobium* /others
18	SNB125A	3	CGE	Blue	6.2	*Bradyrhizobium* sp.
19	SNB125B	6	IFLG	Yellow	3.5	*Rhizobium* /others
20	SNB140	3	CGES	Blue	6.4	*Bradyrhizobium* sp.
21	SNB75	3	IFLG	blue	6.6	*Bradyrhizobium* sp.
22	SBTAL 379^{X}	3	CGES	Blue	4.0	*Bradyrhizobium japonicum*

* CGE; Circular, gummy colonies with entire margin; S; shiny transluscent texture; IFLG; Filamentous colonies with irregular margin and less gum production, x reference strain

4.3.3 Salt, pH and higher temperature tolerance

The isolates varied in tolerance to NaCl concentrations ranging from 0.5% - 6% [w/v] (Table 5), and the result showed a clear difference between the NaCl-sensitive slow-growing isolates (0.5-2%) and the relatively NaCl-tolerant fast growing isolates (1.5-6%) as observed for other fast growing and slow growing soybean rhizobia strains (Sadowsky *et al*., 1983; Chen *et al*., 1988 Youseif *et al*., 2014).

Table 5. Salt, pH and higher temperature tolerance of the soybean rhizobial isolates (and the reference SBTAL 379) grown on YMA and incubated at 28±2ºC for 5-7 days.

Growth rate	Ser No.	Isolate	% NaCl [w/v]	Temperature (ºC)	pH range
Fast growing	1	SNB 13	6	45	5.5 – 10
	2	SNB 41	4.5	40	5 – 10
	3	SNB45	5	37	5 – 10
	4	SNB57C	2	40	5 – 9.5
	5	SNB79	4	40	5 – 10
	6	SNB102	4	40	5 – 10
	7	SNB114	1.5	40	5 – 9.5
	8	SNB55	4	40	5.5 – 10
	9	SNB120B	6	40	5 – 10
	10	SNB120C	6	45	5 – 10
	11	SNB125B	6	40	5.5 – 10
Slow growing	12	SNB43	0.5	37	5 – 9.5
	13	SNB46	0.5	37	5.5 – 9.5
	14	SNB57B	0.5	37	5.5 – 9.5
	15	SNB70	0.5	37	5 – 9.5
	16	SNB71	0.5	37	5.5 – 9.5
	17	SNB101	0.5	37	5 – 10
	18	SNB120A	0.5	40	5 – 10
	19	SNB125A	2	40	5 – 10
	20	SNB140	0.5	40	5.5 – 9.5
	21	SNB75	1.5	40	5.5– 10
	22	SBTAL 379	3	37	5 – 10

Many isolates (43%) managed to grow over wider pH range (5.0-10) (data not shown) whereas none cultured well at pH's 4.5, or less. The pattern of pH tolerance did not correlate with fast or

slow growth characters, but appeared as a general character. However, the isolates were relatively sensitive to lower pH (4) that was tolerated by other soybean *Bradyrhizobium* strains from India (Appunu and Dhar, 2006) and fast growing soybean rhizobia from Brazil (Hungria *et al*., 2001) and from Egypt (Youseif *et al*., 2014).

All soybean rhizobial isolates were able to grow at 37 ºC (data not shown), but only SNB13 and SNB120C were able to grow at the higher temperature (45ºC; Table 5). Previous studies also showed the tolerance of some soybean rhizobia to 40-45 ºC irrespective of their growth rate or taxonomic status (Hungria *et al*., 2001; Chen *et al*., 2002; Singh *et al*., 2013; Youseif *et al*., 2014).

4.3.4 Intrinsic resistance to antibiotics, heavy metals and pesticides

The soybean rhizobia differed in their IAR (Table 6). All isolates were sensitive to erythromycin (100 μg mL^{-1}) but the tolerance to other antibiotics was varied from 10-80% of the tested antibiotics (Table 6) except for SNB13. Accordingly, some fast growing isolates (SNB41 and SNB120B) and the slow growing isolates (SNB101, SNB120A, and SNB125A) were resistant to 80% of the tested antibiotics (Table 6). Many of the isolates (76%) were resistant to chloramphenicol, penicillin G, or vancomycin, whereas over 50% of the isolates were susceptible to streptomycin sulfate, cifroflaxin or gentamycin. The data showed that IAR by slow growing isolates was more pronounced than that of the fast growing isolates (Table 6). Generally, slow growing isolates were more tolerant to cifroflaxin, gentamycin, nalidixic acid, vancomycin, tetracycline and chloramphenicol than fast growers; whereas fast growing isolates were more resistant to ampicillin and streptomycin than the slow growing group.

The pattern of resistance to chloramphenicol by slow growing soybean rhizobia (Dowdle and Bohlool, 1985; Sharma *et al*., 2010) and streptomycin (Dowdle and Bohlool, 1985) was previously reported. Although all (100%) and a large number (66%) of the isolates were sensitive to erythromycin (100 μg mL^{-1}) and streptomycin sulfate (10 μg mL^{-1}), respectively, Young and Chao (1989) reported the resistance of fast growing and slow growing strains of soybean rhizobia to 200 μg mL^{-1} of erythromycin and 100 μg mL^{-1} of streptomycin showing IAR may be dependent upon previous exposure of the isolates to the antibiotics produced by indigenous soil bacteria in their habitat rather than the taxonomic status of the rhizobia.

All of the soybean rhizobial isolates were resistant to lead acetate (2.5 mM), zinc sulfate and manganese sulfate (0.25 mM each) (Table 6). However, over 80% of them were sensitive to potassium dichromate or copper chloride (0.25 mM each) (Table 5). However, soybean rhizobia from Brazil failed to grow on a medium containing 2.5 mM lead acetate but were tolerant to 0.25 mM potassium chromate as reported by Hungria *et al*. (2001). The data did not show distinctive pattern in heavy metal resistance/sensitivity between the slow and fast growing groups of soybean rhizobia.

All rhizobial isolates were sensitive to Curzet, but 24% and 57% were tolerant to Mancozeb and Glyphosate, respectively (Table 6). The data also showed that fast growing isolates were more resistant to the tested agrochemicals implying their better survival chance upon exposure to the chemicals.

Table 6. Intrinsic resistance to antibiotics, heavy metals and pesticides by rhizobial isolates grown on YMA (antibiotics and pesticides tests) and on minimal salt agar medium (heavy metals test) at 28±2ºC for 5-7 days

Features	Fast Growing											Slow growing										
	SNB 13	SNB 41	SNB 45	SNB 57C	SNB 79	SNB 102	SNB 114	SNB 55	SNB 120B	SNB 1120C	SNB 125B	SNB 43	SNB 46	SNB 57B	SNB 70	SNB 71	SNB 101	SBTAL 379	SNB 120A	SNB 125A	SNB 140	SNB 75
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 meatal (mM)																						
$Pb(CH_3COO)_2.3H_2O$ (2.5)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
$CoCl_2.6H_2O$(0.5)	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+	+	+	+
$K_2Cr_2O_7$ (0.25)	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
$ZnSO_4.7H_2O$ (0.25)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
$MnSO_4.H_2O$(0.25)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
$CuCl_2.2H_2O$(0.25)	-	-	-	+	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-
Pesticides																						
Mancozeb (2g L^{-1})	+	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-
Curzet (2g L^{-1})	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-
Glyphosate (1444μg mL^{1})	-	+	-	+	+	+	-	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+

+ stands for resistance and – stands for sensitive

4.3.5 Utilization of carbon and nitrogen substrates

Soybean rhizobial isolates were able to catabolize most of the carbon and nitrogen substrates tested (Table 7). However, fewer isolates assimilated sodium citrate and tri-sodium propionate

(57% each) as carbon source, and DL-threonine and DL-serine (52% each) as nitrogen source. Fast growing soybean rhizobia were more effective than slow growing types in their capacity to utilise a wider spectrum of C-sources (90-100%) and amino acids (54-100%) (Table 7). Slow growing isolates, with few exceptions like SNB 120A and SNB75, demonstrate the capacity to use only a narrow range of C-sources (as low as 54%) and amino acid (as low as 38%). Similar pattern of C-utilization between the two taxonomic groups was previously reported (Sadowsky *et al*., 1983; Young *et al*., 1988; Ansari and Rao, 2014). Other studies also showed that fast and slow growing soybean rhizobia utilized different types of amino acids (Chen *et al*., 1988; Hungria *et al*., 2001). It is interesting to note that 90% and 50% of the slow growing isolates were as versatile as the fast growing isolates in utilizing inositol and citrate, respectively. This pattern was different from the report of Xu *et al*. (1995) where 79 % and 10% of soybean rhizobia (*Bradyrhizobium japonicum* strains) utilized sodium citrate and inositol, respectively. Although most of the fast growing isolates (80%) were able to utilize threonine, only 27% of the slow growing isolates were able to utilize it (Table 7).This was similar to the number of (22%) slow growing soybean rhizobia that utilized the same amino acid as a sole N-source (Zhang *et al*., 2012).

Table 7. Pattern of utilization of carbon and nitrogen substrates by soybean rhizobia grown on minimal salt medium at 28±2ºC for 5-7 days. Tested carbon and nitrogen sources (material and method section) not indicated in the table are those utilized by all of the isolates

Feature	Fast growing											Slow growing										
	SNB 13	SNB 41	SNB 45	SNB 57C	SNB 79	SNB 102	SNB 114	SNB 55	SNB 120B	SNB 1120C	SNB 125B	SNB 43	SNB 46	SNB 57B	SNB 70	SNB 71	SNB 101	SNB 120A	SNB 125A	SNB 140	SNB 75	SBTAL 379
Sole C-source																						
Tri-sodium - propionate	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	-	-	-	+
Maltose	+	+	+	+	+	+	+	-	+	+	+	-	-	-	+	+	+	+	+	+	+	+
Dextrin	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
Inositol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Na-acetate	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	+	+	-	+	+	+
Na-citrate	+	+	+	+	-	-	-	-	+	+	+	-	-	-	+	-	-	+	+	+	+	-
galactose	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
trehalose	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+
cellobiose	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Sole N-source																						
Methionineee	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	+	-	-	+	+
DL-Phenyl - alanine	+	+	+	+	+	-	+	+	+	+	+	-	-	+	-	-	-	+	-	+	+	+
DL-threonine	+	+	+	+	+	-	+	+	+	-	+	-	-	-	-	-	-	+	-	-	+	+
DL-serine	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	+	-	-	+	+
Lysine	+	+	+	+	-	-	+	+	+	+	+	-	-	-	+	+	-	+	-	-	+	-
DL-tryptophan	+	+	+	+	-	-	+	+	+	+	+	-	-	-	+	-	-	+	-	-	+	+
Glycine	+	+	+	+	+	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	+	+
L-tyrosine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+
DL- glutamic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+

+ = utilized, - = not utilized

4.3.6 Numerical Analysis

Cluster analysis of phenotypic data grouped the indigenous soybean rhizobial isolates into two sub-clusters at about 32% similarity level and further into three sub-clusters; I, II and III at 40% similarity level (Fig. 3). Cluster I included entirely slow growing; salt, chloramphenicol and naldixic acid sensitive eight isolates. At about 56% similarity level, sub-cluster II split into three; IIA consisting three fast growing isolates, IIB consisting three fast and the slow growing reference SBTAL 379 which separated from them at around 65% similarity level, and IIC constituting two fast growing (SNB57C and SNB114) and two slow growing (SNB75 and SNB 120A) isolates. Members of group IIA are salt tolerant but sensitive to antibiotics whereas that of group IIB are nutritionally highly versatile capable of utilizing all the tested C-sources except the reference SBTAL379. Both fast and slow growing members of cluster IIC catabolized all the N-sources, tolerated ampicillin and penicillin G, but sensitive to gentamycin. Members of cluster III are all fast growing, catabolized all the tested C-sources, highly salt tolerant (6%) and were able to grow at least at 40^{o}C. Clustering at 40% similarity level, almost overlapped the categorization of the isolates into slow growing (*Bradyrhizobium* spp.) and fast growing (*Rhizobium*/other fast growing groups) based on growth rate and acid or alkali production. No cluster of rhizobial isolates with each other or the reference strain was formed beyond 88% similarity level indicating their phenotypic diversity which could used for identification and selection of good inoculants.

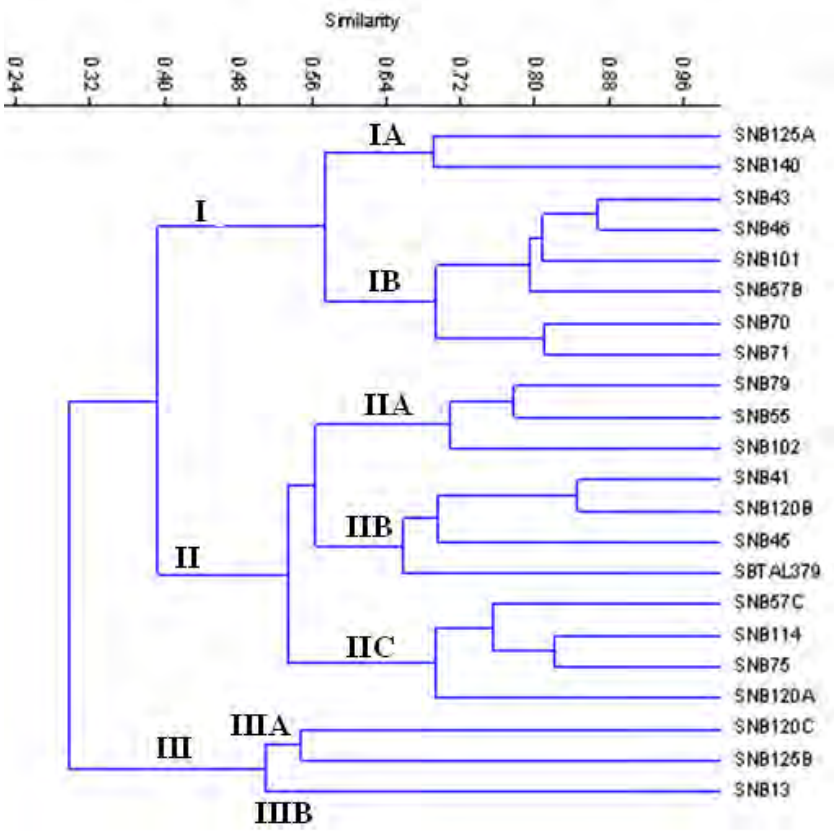

Fig. 4. Dendrogram highlighting phenotypic similarity of the indigenous soybean rhizobial isolates and reference strain.

4.3.7 Sequencing and phylogenetic analyses of 16S rRNA, nifH, nodA, nodD, and recA

Based on 16S rRNA gene sequencing, the fast growing isolate (SNB41) was identified as *Agrobacterium tumefaciens,* where as the three slow growing isolates (SNB57B, SNB70 and SNB120A) were identified as *Bradyrhizobium* spp. Showing 99% sequence similarity with NCBI references except for SNB120A (Table 8). The sequence similarity of SNB120A (88%) with uncultured *Bradyrhizobium* sp. clone G16-1D02 is less than 97%, the threshold percent of 16S rRNA similarity for a species definition (Stackebrandt and Goebel, 1994). SNB57B was identified as *Bradyrhizobium yuanmingense* which was described from soybean by Appunu *et al*.

(2008). Similarly, *Agrobacterium* strains were described as soybean symbionts by Yuseif *et al*. (2014).

In the 16S rRNA gene phylogenetic tree (Fig. 4), the three slow growing isolates were clustered with *Bradyrhizobium* species. SNB57B and SNB70 showed closer similarity with *Bradyrhizobium japonicum* USDA6^{T}, whereas SNB120A was located distantly near the group containing *Bradyrhizobium elkanii* USDA76^{T}. The fast growing isolate (SNB41) was also clustered with *Agrobacterium*, closer to *Agrobacterium fabrum* strain C58 rather than with fast growing soybean rhizobial genera such as *Rhizobium*/*Ensifer fredii* previously decribed from the crop (Scholla and Elkan, 1984).

Table 8. Identity of some of the rhizobial isolate based on sequencing analyses of different genes

SN	Sample Code	Length of query (bp)	Identity	Best Match ID (NCBI ref)	Cover (%)	Identity (%)
16S						
1	SNB41	1377	*Agrobacterium tumefaciens* ORS3405	EF054875.1	100	99
2	SNB70	1352	*Bradyrhizobium sp.* JNVU TW14	KR071009.1	99	99
3	SNB120A	1330	Uncultured *Bradyrhizobium sp.* Clone G16-1-D02	FJ193226.1	100	88
4	SNB57B	1311	*Bradyrhizobium yuanmingense* Rc-391-01	LN896395.1	100	99
nifH						
5	SNB57B	346	*Bradyrhizobium yuanmingense* CIR24	JQ810085.1	96	99
6	SNB70	352	Uncultured bacterium GYMB-32B	AJ716252.1	96	97
7	SNB120A	363	*Bradyrhizobium sp.* TUTRAB5B1	KR491977.1	95	99
nodA						
8	SNB57B	567	*Bradyrhizobium sp.* ORS305	FJ150399.1	98	97
9	SNB120A	566	*Bradyrhizobium elkanii* USDA76	AM117554.1	99	100
	NodD					
10	SNB41	765	*Rhizobium leguminosarum* bv. Viciae P-2.69	GQ358097.1	100	99
	SNB57B	745	*Bradyrhizobium japonicum* E109	CP010313.1	96	91
11	SNB120A	747	*Bradyrhizobium elkanii* USDA94	U04609.1	99	93
recA						
12	SNB41	454	*Rhizobium pusense* LMG 25623	LN812155.1	99	99
13	SNB120A	454	*Bradyrhizobium sp.* UFLA06-31	KJ740102.1	99	99

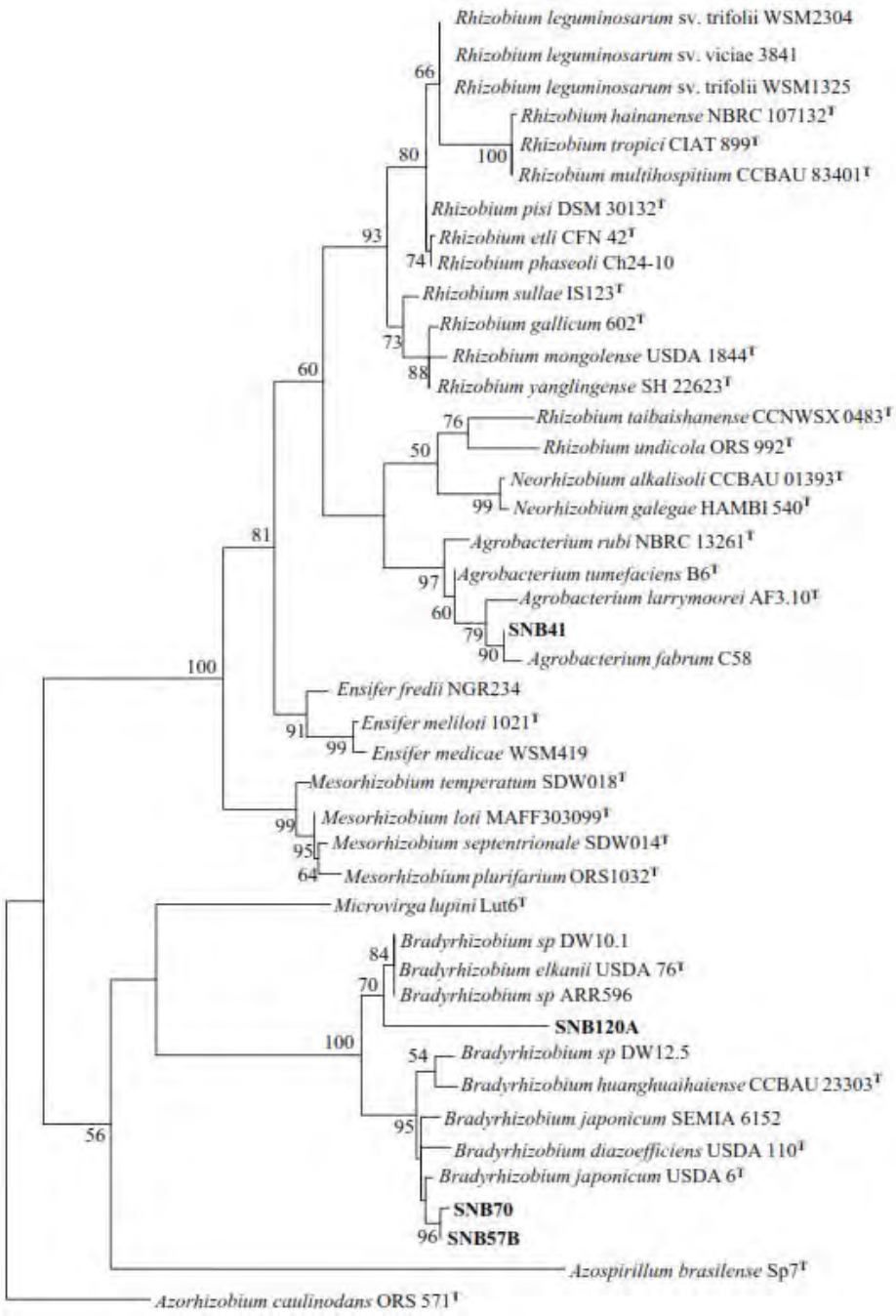

Fig. 5. Phylogenetic tree of the 16S rRNA genes of rhizobial isolates constructed using the maximum likelihood method (1000 bootstrap replicates), only bootstrap values >50% are shown. The type strains are shown by a "T" at the end of each strain code. Tree are rooted with *Azorhizobium caulinodans* ORS 571T and the tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches.

NifH gene sequencing analysis identified SNB57B as a strain of *Bradyrhizobium yuanmingense* similar to 16S rRNA gene sequencing analysis and SNB120A as *Bradyrhizobium* sp. sharing 99 % sequence similarity with their respective NCBI references (Table 8). SNB70 shared 97 % *nifH* gene sequence similarity with uncultured bacterium GYMB-32B (Table 8) and 96 % *nifH* gene sequence similarity with *Bradyrhizobium* sp. M12 having NCBI accession number KF113074.1 (data not shown). All the three slow growing isolates (SNB57B, SNB70 and SNB120A) were clustered within the clade containing various species of *Bradyrhizobium*, but some what distantly from each other in the phylogenetic tree constructed based on *nifH* gene sequencing (Fig. 5) showing their diversity.

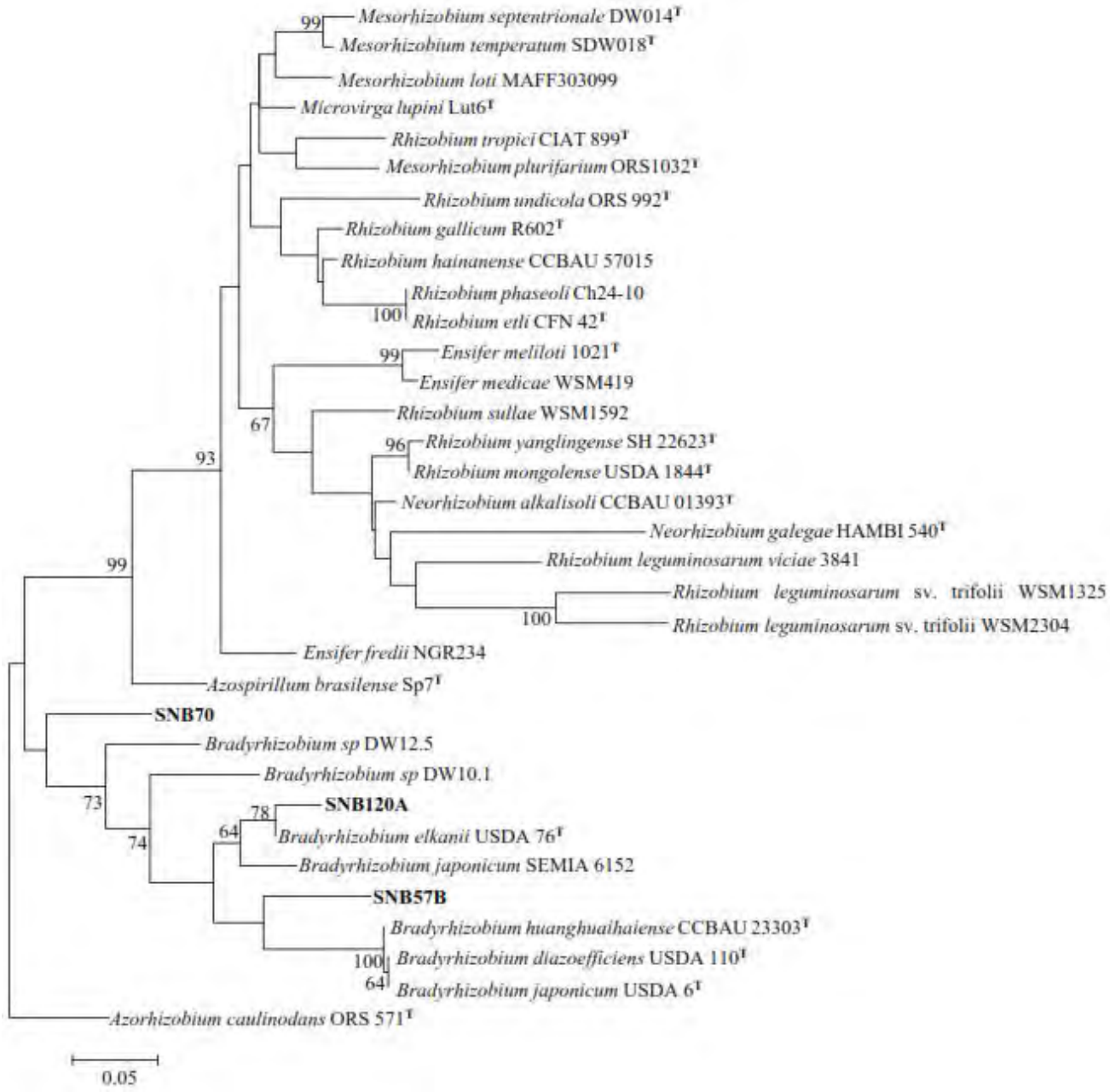

Fig. 6. Phylogenetic tree of the *nifH* genes constructed using the maximum likelihood method (1000 bootstrap replicates), only bootstrap values >50% are shown. The type strains are shown by a "T" at the end of each strain code. Tree are rooted with *Azorhizobium caulinodans* ORS 571T and the tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches.

Two slow growing isolates; SNB120A and SNB57B were identified as *Bradyrhizobium* on the basis of *nodA* sequence analyses sharing 100% and 97 % sequence similarity with *Bradyrhizobium elkanii* USDA76 and *Bradyrhizobium* sp. ORS305, respectively (Table 8). SNB57B was clustered close to a group containing *B. japonicum* USDA6^{T} where as SNB120A was clustered with *B. elkanii* 76^{T} in *nodA* phylogenetic tree (Fig. 6) similar to that of *nifH*.

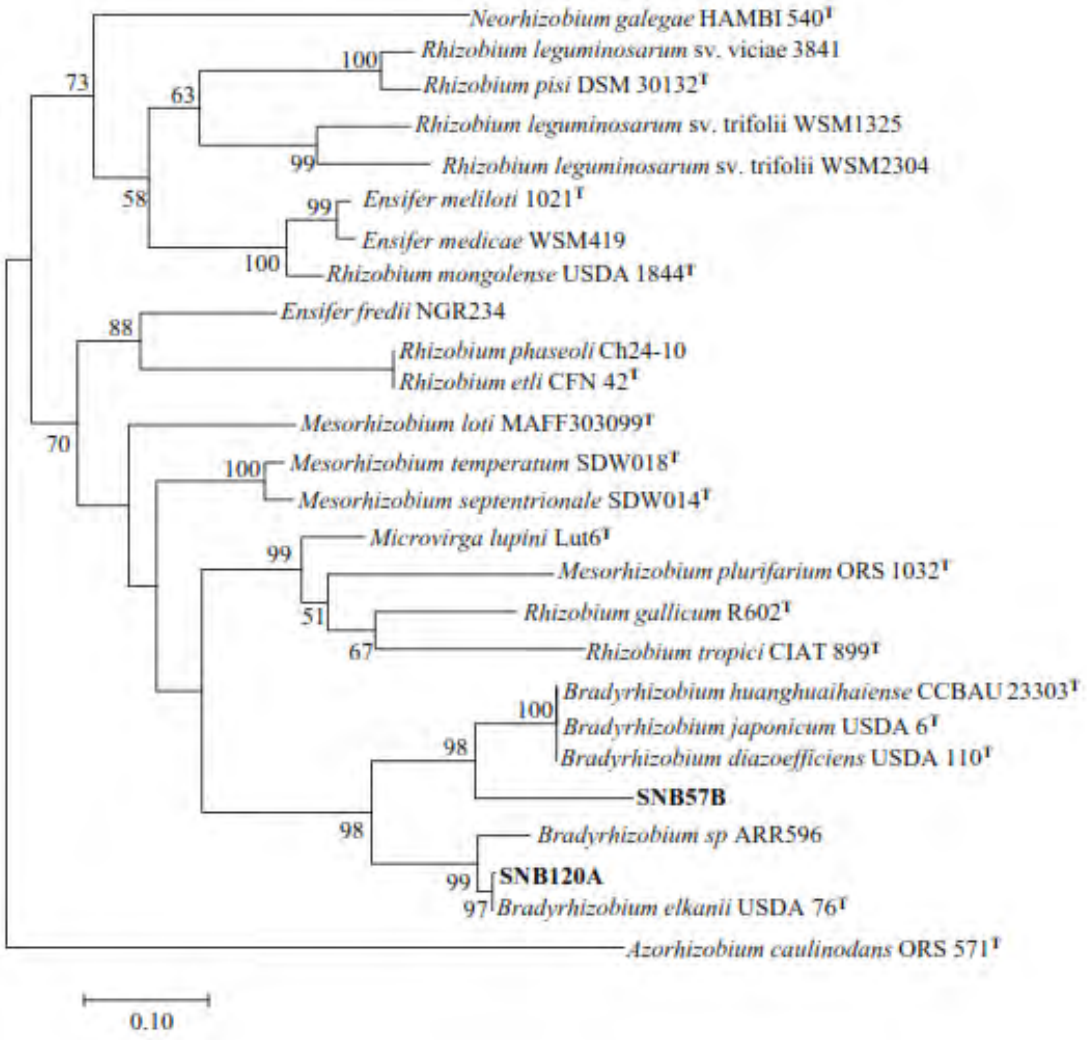

Fig. 7. Phylogenetic tree of the *nodA* gene constructed using the maximum likelihood method (1000 bootstrap replicates), only bootstrap values >50% are shown. The type strains are shown by a "T" at the end of each strain code. Tree are rooted with *Azorhizobium caulinodans* ORS 571T and the tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches.

The fast growing isolate (SNB41) showed 99% *nodD* gene sequence similarity with *Rhizobium leguminosarum* bv. Viciae P-2.69, whereas SNB57B and SNB120A shared 91% and 93% *nodD* gene sequence similarity with *Bradyrhizobium japonicum* E109 and *Bradyrhizobium elkanii* USDA94, respectively (Table 8). SNB120A was also identified as strain of *B. elkanii* in *nodA* gene sequencing analysis. In the phylogenetic tree constructed with *nodD* gene sequence (Fig. 7), SNB41 was closely clustered with *Rhizobium pisi* DSM30132^{T}, previously called *R. leguminosarum* DSM 30132 nodulating *Pisum sativum*, *Trifolium repens* and *Phaseolus vulgaris* (Rami´rez-Bahena *et al*., 2008). The slow growing SNB57B and SNB120A were clustered with *Bradyrhizobium* species in the *nodD* gene based phylogenetic tree.

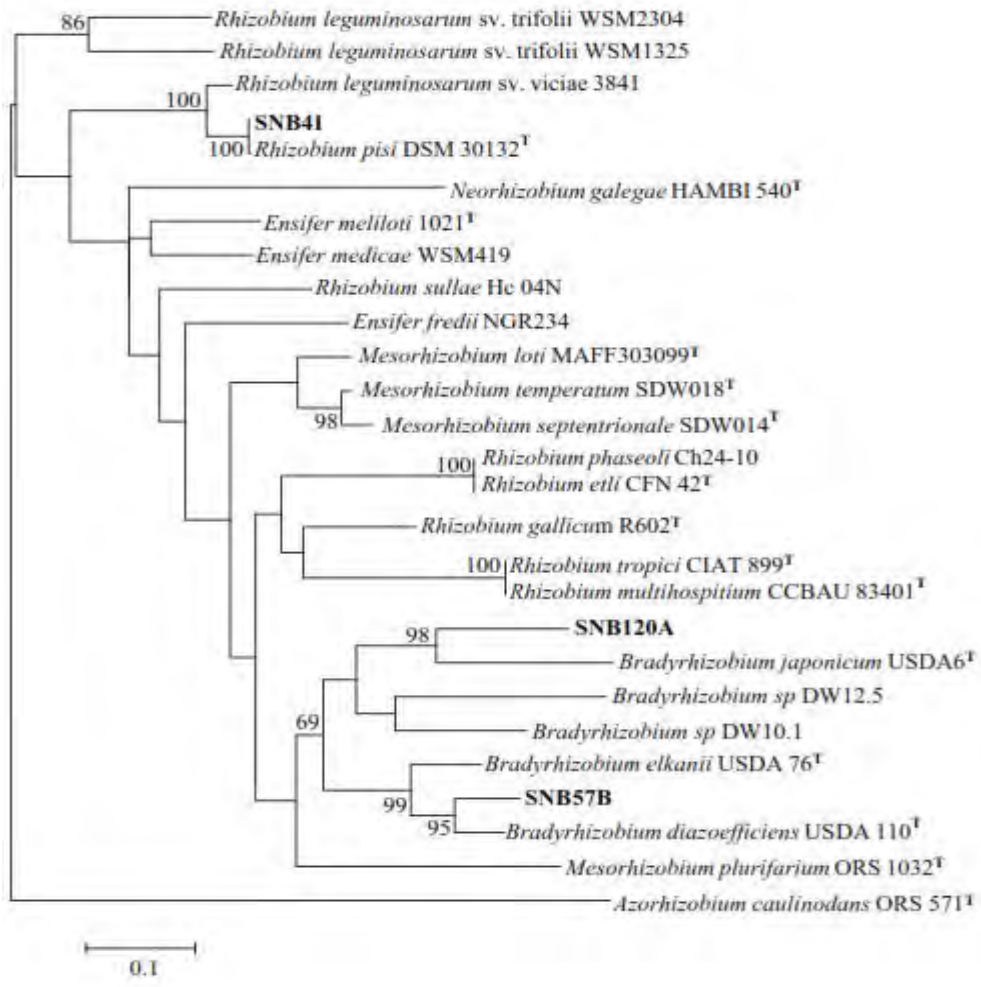

Fig. 8. Phylogenetic tree of the *nodD* gene constructed using the maximum likelihood method (1000 bootstrap replicates), only bootstrap values >50% are shown. The type strains are shown by a "T" at the end of each strain code. Tree are rooted with *Azorhizobium caulinodans* ORS 571T and the tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches.

The fast growing isolate (SNB41) shared 99% *recA* gene sequence similarity with *Rhizobium pusense* LMG25623; the type strain for *Rhizobium pusense* isolated from the rhizosphere of chickpea (*Cicer arietinum* L.) by Panday *et al*. (2011). SNB120A was identified as *Bradyrhizobium* sp. sharing 99% *recA* gene sequence similarity with *Bradyrhizobium* sp. UFLA0631 (Table 8). In the *recA* phylogenetic tree (Fig. 8), SNB41 was clustered with *Agrobacterium* and SNB120A was also clustered close to *B. elkanii* USDA76^T similar to the 16S

rRNA phylogenetic tree supporting the consistency of *recA* and 16S rDNA phylogenies in numerous bacteria (Gaunt *et al*., 2001). SNB120A was also clustered with *B. elkanii* USDA76^T both in *nifH* and *nodA* phylogenetic trees.

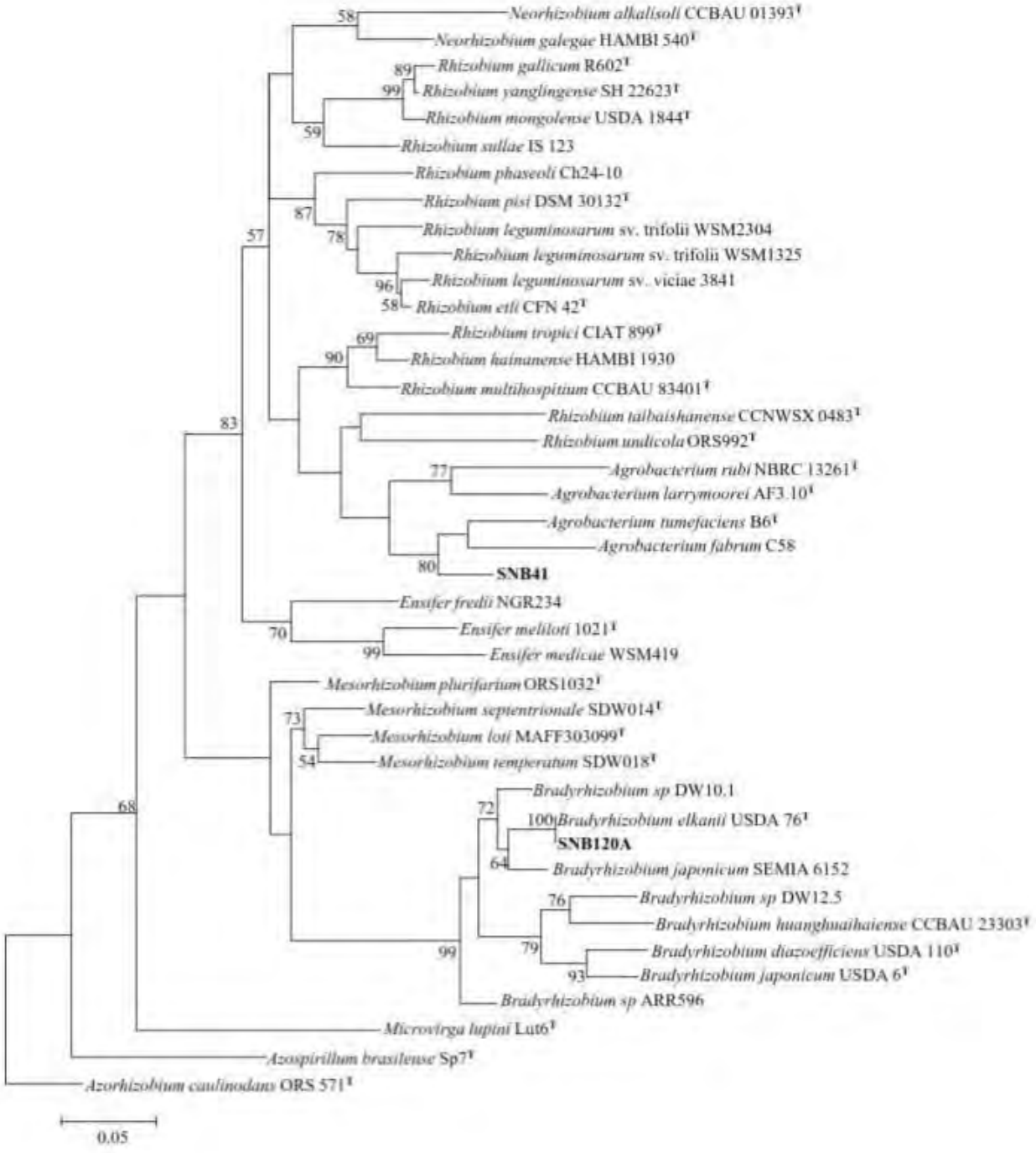

Fig. 9. Phylogenetic tree of the *recA* genes constructed using the maximum likelihood method (1000 bootstrap replicates), only bootstrap values >50% are shown. The type strains are shown

by a "T" at the end of each strain code. Tree are rooted with *Azorhizobium caulinodans* ORS 571T and the tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches.

In summary, the sequence analyses 16S rRNA, *nodD* and *recA* genes indicated that SNB41 could be a species of *Agrobacterium* or *Rhizobium* but not likely to be a *Sinorhizobium* (*Ensifer*) *fredii*. Based on the analyzed gene sequences, the three slow growing isolates (SNB57B, SNB70 and SNB120A) were placed under the genus *Bradyrhizobium*; SNB57B and SNB 120A are more likely to be *Bradyrhizobium yuanmigense* and as *Bradyrhizobium elkanii*, respectively.

4.3.8 PGP properties of rhizobial isolates

4.3.8.1 Phosphate solubilization (PS)

All the isolates failed to solubilize iron phosphate where as two isolates (10%; SNB13 and SNB57B) and five isolates (24%; SNB13, SNB120C, SNB57B, SNB57C, SNB71) were able to solubilize tri-calcium phosphate and aluminium phosphate with solubilization indices (SI) of 1.3-1.5, and 1.4-1.7, respectively (Table 9). Only one isolate (SNB57B) solubilzed both aluminium and tricalcium phosphates. The data showed that fewer indigenous rhizobia (10%) had the capacity of solubilizing tri-calcium phosphate with SI 1.3-1.5 than the number (30%) and more efficient (SI of 1.0-3.2) soybean rhizobial isolates reported by Jadhav (2013). This implies a need for their co-inoculation with good phosphate solubilizing rhizobacteria.

4.3.8.2 IAA ($C_{10}H_9NO_2$) production

The indigenous soybean rhizobia showed great difference in their efficiency of IAA production ranging from 2.5 (SNB43) to 116 µM (SNB45) (Table 9). The finding showed the higher efficiency of the fast growing soybean rhizobia (9.52-116 µM; average 44 µM) than slow

growing isolates (2.5-97.5 µM; average 29 µM) (data not shown). The IAA production efficiency of the isolates (2.5-116 µM) was similar to IAA production by slow growing and fast growing strains of soybean rhizobia (1.02-130 µM) (Chen *et a*l, 2002); but higher than that of IAA production (4.88 µM - of 44.68 µM) reported for soybean *Bradyrhizobium* strains (Boddey and Hungria,1997). These rhizobia with different efficiency of IAA production affect the growth of the crop differently as the effect of the hormone depends on its concentration.

4.3.8.3 *In vitro* evaluation of the isolates for theit inhibitory enzyme, HCN production and suppression of fusarium

In this study, 33% and 38% of the soybean rhizobial isolates showed protease and cellulase activities, respectively (Table 9). However, none of them produced halo zones around their colonies on chitin agar indicating lack of chitinase activity. HCN was released by a single isolate (SNB41). Two fast growing isolates (SNB79 and SNB102) inhibited *Fusarium oxysporum* with fungal radial growth inhibition (PIRG) of 33% (SNB79) and 17% (SNB102). Similarly, Pawar *et al*. (2014) reported the inhibition of *Fusarium oxysporum* by soybean nodule *Rhizobium* with zone of inhibition of 0.4±0.2 cm.

In general, more than half of the soybean rhizobia showed multiple PGP properties (2-5 out of 8 characters: 25-63%), of which 5 isolates (24%) displayed IAA production together with protease and cellulase activities. Among the isolates, SNB13 showed the maximum number of PGP characters (63%), folowed by isolates SNB79, and SNb120C that exhibited 50% of the tsted PGP characters. The reference B. japonicum SBTAL379 showed IAA production (10.33µM) and AlPO4 solubilization with SI os 1.4 (data not sown). Some sample *in vitro* plant growth promoting traits are indicated in Appendix 9.

Table 9. Rhizobial isolates with two or more plant growth promoting (PGP) properties.

No	Isolate	$Ca_2(PO4)$ (SI*)	$AlPO_4$ (SI*)	IAA (µM)	Protease	Cellulase	HCN	PIRG**	%PGP@
1	SNB13	1.5	1.6	52.62	+	+	-	-	63
2	SNB41	-	-	104.73	-	-	+	-	25
3	SNB43	-	-	4.68	-	+	-	-	25
4	SNB57B	1.3	1.5	23.47	-	-	-	-	38
5	SNB 57C	-	1.4	26.42	-	-	-	-	25
6	SNB79	-	-	40.02	+	+	-	33%	50
7	SNB101	-	1.7	38.84	-	+	-	-	38
8	SNB102	-	-	9.52	+	-	-	17%	38
9	SNB114	-	-	25.85	-	+	-	-	25
10	SNB 55	-	-	38.84	+	-	-	-	25
11	SNB120B	-	-	14.14	+	+	-	-	38
12	SNB120C	-	1.4	26.91	+	+	-	-	50
13	SNB125B	-	-	23.78	+	+	-	-	38
%	62%	10%	24%	100%	33%	38%	5%	10%	

*****SI=solubilization index**, ****PIRG= percentage of inhibition of radial growth of fungus, **@** percentage of PGP traits demonstrated out of the tested eight PGP traits (solubilization of iron phosphate is excluded from the table since none of the isolates solubilzed it)

Based on multiple PGP properties, and wide range of ecological and nutritional characters, the isolates were ranked to be used for selecting them for further studies in the future (Table 10). Accordingly, the fast growing isolates such SNB120C, SNB125B, SNB120B, SNB41 and SNB13 showed better performance than the other isolates, including the moderate slow growing SNB120A and the reference *Bradyrhizobium* SBTAL379 (Table 10). These isolates can be potential inoculant candidates following evaluation of their symbiotic effectiveness under greenhouse and field conditions.

Table 10. Performance of the isolates based on their inherent ecophysiological, nutritional and PGP traits tested under *in vitro* conditions.

Growth rate	Serial No.	Rhizobia	C-sources (19)*	N-sources (13)	Pesticides (3)	Temperature (4)	NaCl (12)	Antibiotic (10)	Heavy metals (6)	PH (8)	PGP traits (8)	Total (out of 83)	Rank
Fast	1	SNB120C	5	5	4	5	5	4	5	4	2	39	1
Fast	2	SNB125B	5	5	4	4	5	4	5	3	2	37	2
Fast	3	SNB120B	5	5	2	4	5	5	4	4	2	36	3
Fast	4	SNB 41	5	5	2	4	4	5	4	4	2	35	4
Fast	5	SNB 13	5	5	2	5	5	0	4	4	4	34	5
Fast	6	SNB57C	5	5	4	4	2	3	5	4	1	33	6
Fast	7	SNB79	5	5	2	4	4	2	4	4	3	33	6
Fast	8	SNB45	5	5	0	3	5	4	5	4	1	32	8
Fast	9	SNB55	5	5	2	4	4	1	5	4	2	32	8
Fast	10	SNB102	5	3	2	4	4	2	4	4	2	30	10
Slow	11	SBTAL 379	5	5	2	3	3	4	3	4	1	30	10
Slow	12	SNB120A	5	5	2	4	1	5	4	4	0	30	10
Slow	13	SNB75	5	5	2	4	2	3	4	4	0	29	13
Slow	14	SNB125A	5	2	2	4	2	5	4	4	1	29	13
Slow	15	SNB140	5	3	4	4	1	4	4	3	0	28	15
Fast	16	SNB114	5	5	0	4	2	2	4	4	1	27	16
Slow	17	SNB101	4	3	0	3	1	5	4	4	2	26	17
Slow	18	SNB70	5	4	0	3	1	4	4	4	0	25	18
Slow	19	SNB57B	4	4	0	3	1	3	4	3	2	24	19
Slow	20	SNB71	5	3	0	3	1	4	4	3	0	23	20
Slow	21	SNB43	3	3	0	3	1	4	4	4	1	23	20
Slow	22	SNB46	4	3	0	3	1	4	4	3	0	22	22

*Numbers in the parenthesis represent the number of testes made for each character, and number 5, 4, 3, 2, 1, and 0 represented that isolates demonstrated 80-100%=5, 60-79%=4, 40-59%=3, 20-39%=2, 1-19%=1 and 0%=0 of the tested traits.

4.4. Conclusion and recommendations

Soybean nodulating bacteria are not widely distributed in Ethiopia for they were isolated from only 13% of the soil samples. The isolates constituted almost equal proportion of fast growing (52%) and slow growing (48%) members. The tentative grouping of the isolates into fast growing (*Rhizobium* and related taxa) and slow growing (*Bradyrhizobium*) was supported by phenotypic cluster analysis at 40% similarity level and also by gene sequencing and phylogenetic analyses. The fast growing isolate (SNB41) was identified as *Agrobacterium*/*Rhizobium* sp. and slow growing isolates (SNB57B, SNB70 and SNB120A) as *Bradyrhizobium* spp. SNB57B and SNB 120A are more likely to be *Bradyrhizobium yuanmigense* and as *Bradyrhizobium elkanii*, respectively.

The soybean rhizobia showed some pattern of stress tolerance, substrate utilization and PGP traits according to their growth rate. Fast growers were more tolerant to salt (5-6% NaCl), temprtature (40-45^{o}C), and to agro-chemicals. They also utilized wider range of carbon and nitrogen substrates, and many of them (62%) acquired multiple PGP properties (≥2). The slow growing isolates showed resistance to a wider range of antibiotics. However, both groups of the isolates were sensitive to pH 4.5. Fast growing isolates were better in the overall performance where fast growing (SNB 120C, SNB 125B, SNB 120B, and SNB 41) and the slow growing (SNB 120A, SNB 125A, and SNB75) isolates can be qualified as potential inoculant candidates to enhance soybean production in the country provided that their symbiotic effectiveness and competitiveness are validated under field conditions. Since the true taxonomic status of all of the isolates is not clearly established, it requires subsequent studies in that direction.

5. Diversity of plant growth promoting rhizobacteria of soybean [*Glycine max* (L) Merr.] from Ethiopia

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Abstract

Plant Growth Promoting Rhizobacteria (PGPR) naturally colonize root system, enhance growth and health of plants. Several studies showed that growth and productivity of plants could be improved by inoculating them with PGPR that are ecologically tolerant and possess multyiple PGP properties. Therefore, 231 rhizobacteria were isolated from rhizosphere of soybean grown in soils (102) from different pedoclimatic regions of Ethiopia. All the isolates were initially tested for their basic Gram reaction and potential to enhance plant growth via indole-3-acetic acid (IAA) production, phosphate solubilisation, biological nitrogen fixation (BNF) and growth inhibition of pathogenic *Fusarium oxysporum* trait. Accordingly, seventy two isolates with at least one higher level of these traits were selected for further *in vitro* plant growth promoting traits and tolerances to pH, higher temperature, antibiotics, salt (NaCl), pesticides and heavy metals and for phenotypic cluster analysis with Ori Past computer software. Twenty isolates with multiple PGP traits, of which six were assayed for *in vitro* effect on soybean seeds germination and seedlings growth. The potentent isolates, were identified via 16S rRNA gene sequencing. The 20 PGPR were classified into seven genera *Pseudomonas* (7), *Stenotrophomonas* (5), *Acinetobacter* (3), *Bacillus* (2), 1 species each of the genera A*chromobacter*, *Enterobacter* and *Microbacterium.* The result showed that some of the soybean rhizobacteria such as *Pseudomona*s

(SR99B) and *Stenotrophomonas* (SR29A and SR43B) demonstrated multiple PGP traits (3 to 9). The majority of the isolates were grown at 40^{o}C, 4% NaCl, and resistant to some antibiotics and heavy metals. Gram negative rhizobacteria occupied top positions in ranking the isolates based on their number of PGP traits and stress tolerance. The soybean rhizobacteria were diverse in their phenotypic and genotypic features where *Pseudomonas* and *Stenotrophomonas* species were the dominant groups among genetically identified isolates, and SR20A (*Achromobacter* sp.) and SR20B (*Acinetobacter* sp.) were the most effective ones that enhanced *in vitro* soybean seed germination (%) and seedlings growth over the control among the tested rhizobacteria. Rhizo-competence and compatibility to symbiotically effective soybean rhizobia of SR20A and SR20B should be evaluated prior to their recommendation as inoculant.

Key words or phrases: Plant growth promoting rhizobacteria, PGP traits, stress tolerance, 16SrRNA gene sequencing

5.1. Introduction

Plant Growth Promoting Rhizobacteria (PGPR) are bacteria which are closely associated with roots and promote growth in both leguminous and non-leguminous plants. Soybean is one of the leguminous crops that have been extensively researched for its symbiotic relationship with root nodulating bacteria (Jordan, 1982; Scholla and Elan, 1984; Kuykedall *et al*., 1992; Chen *et al*., 1995; Gao and Yong, 1995; Appunu *et al*., 2008; Youseif *et al*., 2014) and mycorrhiza (Sylvia *et al*., 1992; Troeh, 2006). However, the interest and focus of research on PGPR of the crop has been intensified since the 1990s (Kloepper *et al*., 1992; Park *et al*., 2005; Leon *et al*., 2009; Stefan *et al*., 2010; Wahyudi *et al*, 2010; Khalimi and Suprata, 2011; Susilowatl *et al*., 2011; Wahyudi *et al*, 2011a, 2011b; Malviya and Singh, 2012).

Several PGPR were isolated and characterized from soybean of which the genera *Pseudomonas, Bacillus, Enterobacter* and *Microbacterium* are the dominant. Many of these microorganisms were endowed with multiple plant growth promoting traits and able to enhance soybean seed germination (Malviya and Singh, 2012), fix nitrogen (Park *et al*., 2005), solubilize inorganic phosphate (Sharma *et al*, 2012), sequester iron with siderophore and produce chitinase and HCN to suppress fungal root pathogen (Susilowatl *et al*., 2011), produce phytohormones (Masciarelli *et al*., 2014) and reduce soybean stunt virus (SSV) (Khalimi and Suprata, 2011).

It is known that PGPR are also diverse in their resistance to the prevailing environmental stresses such as salinity, pH, heavy metals, pesticides, and microbial antagonisms in the soil (Glick, 2012). This indicates that practical application of PGPR as biofertilizer requires isolation and screening them for multiple PGP traits and potential adaptation to various environmental stresses.

In Ethiopia, PGPRs have been isolated from crops of fabaceous species lentil (*Lens culnaris Medik;* Mulisa Jida *et al*., 2015), chickpea (*Cicer aeritinum* L; Mulisa Jida *et al*., 2016) and from faba bean (*Vicia faba* L.; Gemechu Keneni *et al.,* 2010). Similar isolations were also made from non-fabaceous crops such as Tef (*Eragrostis tef* Zucc. Trotter; Delelegn Weyesa and Fassil Assefa, 2011a, 2011b), and coffee (*Coffea arabica* L; Diriba Muleta *et al*, 2007, 2009, 2013). These studies showed that diverse group of rhizobacteria with multiple PGP traits colonize the rhizosphere and significantly enhance plant health and productivity under greenhouse conditions. However, there is a gap of information on soybean specific PGPRs isolated from Ethiopian soils except few studies on phosphate solubilisation and/or uptake by soybean (Asfaw Hailemariam, 2003; Anteneh Argaw, 2012). Hence, this study was carried out to isolate indigenous soybean rhizobateria to determine their phenotypic and genotypic diversities, PGPR properties, and to

assess stress tolerances with the objective of selecting elite isolates to be part of Integrated Soil Fertility Management (ISFM) and Integerated Pest management (IPM) for soybean production.

5.2. Materials and Methods

5. 2.1. Source of PGP Rhizobacteria

A total of 231 rhizobacterial isolates were retrieved from roots of soybean (cv. Ethio-Yugoslavia; obtained from Bako Agricultural Research Center), grown on 102 composite soil samples collected from different parts of Ethiopia (Fig. 9 and Table 11) under different land management systems including uncultivated land, cultivated land under various leguminous and non-leguminous crops including soybean but with no history of inoculation.

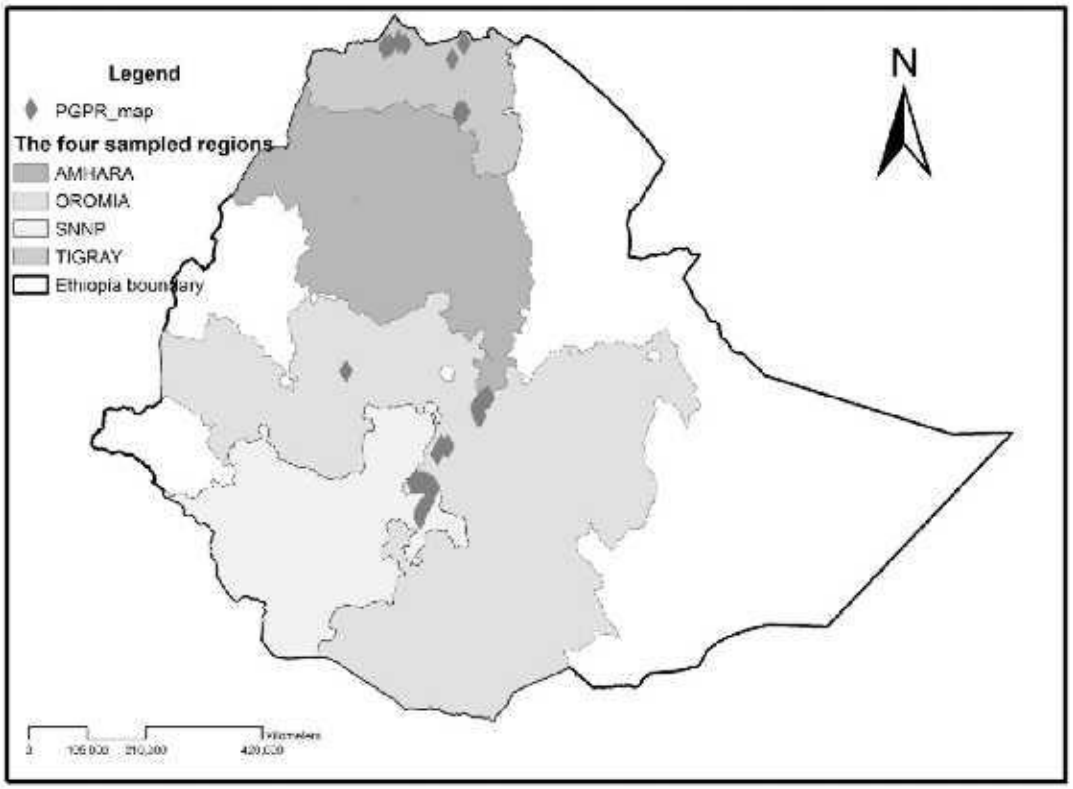

Fig. 10. Map of Ethiopia showing soil sampling sites

Table 11. Isolation sites of some of the rhizobacterial isolates

Isolates	Longitude	Latitude	Altitude	Regional state
SR3A	37N047872	0870838	1644	SNNP
SR6A	37N046573	0865425	1661	SNNP
SR7A	37N0464371	0863379	1656	SNNP
SR10A	37N046029	0857264	1620	SNNP
SR14	37N0440644	0767363	1913	SNNP
SR20A	37N0431107	0747592	1724	Oromia
SR20B	37N0431107	0747592	1724	Oromia
SR29A	37N0446078	0800682	1784	Oromia
SR29B	37N0446078	0800682	1784	Oromia
SR40	37P0534214	0945155	1534	Oromia
SR41B	37P0535514	946779	1517	Oromia
SR43B	37P0538912	0949647	1482	Oromia
SR44B	37P0541396	0951779	1469	Oromia
SR45B	37P0543983	0953974	1469	Oromia
SR47B	37P0549280	0958307	1450	Oromia
SR48B	37P0550765	0958877	1420	Oromia
SR50	37P0536433	09266818	1549	Oromia
SR69B	37P0499944	1669738	1572	Tigray
SR77	37P0487481	1566750	1927	Tigray
SR99B	37P0500378	1469660	1561	Tigray

The plants were maintained in pot culture for 45 days under greenhouse conditions (25±2/17±3 ^{o}C day/night temperature, 12 hour photoperiod, watering every two days in 3 L pots). Plants were carefully uprooted and ten grams of roots and adhering soil were suspended in 90 mL sterile normal saline (0.9% NaCl) to make 10 fold serial dilution. The rhizobacteria were isolated by spreading 0.1 mL of each dilution on nutrient agar plates, subsequently purified, and preserved at 4^{o}C on nutrient agar slants and in nutrient broth with 15% glycerol at -80°C (Karakurt and Aslantas, 2010). The isolates were designated as SR, representing Soybean Rhizobacteria.

5.2.2 Characterization of isolates

5.2.2.1 Gram reaction

The isolates were tested for their Gram reaction using KOH method (Buck, 1982). A 24 hour old colony of each rhizobacterial isolate was picked and mixed with 3% KOH on clean microscope slide. The mixture was lifted with inoculating loop to about 1 cm from the slide and the presence and absence of obvious stringiness (viscosity) were recorded as gram negative and gram positive bacteria, respectively.

5.2.3 Screening isolates for PGP properties

The isolates were characterized and screened for their relative PGP potential using a two stage process. Firstly, all the 231 isolates were evaluated for traits of phosphate solubilisation, indole-3-acetic acid (IAA) production; inhibition of pathogenic *Fusarium oxysporium* and for capacity to survive on N-free growth media as indicative of biological nitrogen fixation (BNF) potential. Secondly, a sub-set of 72 rhizobacteria isolates that demonstrated good potential of at least one of the four tested PGP traits were selected and tested further to discern their relative potential to

enhance plant health by evaluating the capacity to produce hydrogen cyanide, protease, chitinase and cellulase. The 72 isolates were also screened for their relative tolerance against abiotic stresses including: temperature, pH, antibiotics, pesticides, salt (NaCl) and heavy metals. All the tests listed below were carried out under sterile conditions in triplicates for each isolate using an active culture (100 µL of liquid inoculum from a single colony after overnight growth at 28ºC± 0.2 adjusted to 10^6 cells mL^{-1} for 5-7 days unless otherwise stated

5.2.3.1 IAA Production

Indole acetic acid (IAA) production was tested by transferring a single colony of each isolate into 5 mL sterile nutrient broth amended with filter sterilized (0.22 µm) L-tryptophan (at a final concentration of 2 g L^{-1}) and incubating at 30ºC for three days. Each culture was centrifuged at 3,000 rpm for 30 min from which 1mL of the supernatant was mixed with 2 mL of Salkowski reagent (Acuña, *et al*., 2011). The mixture was kept at room temperature for 25 min in the dark to observe the development of pink color as indicative for IAA production. IAA was quantified by measuring the absorbance using spectrophotometer (Jenway, 6405 Uv/vis spectrophotometer) at 530 nm against a standard curve constructed from known concentrations (5, 10, 20, 50, 80 and 100 µg mL^{-1}) of pure IAA (HiMedia, India).

5.2.3.2 Solubilization of Al, Fe and Tri-calcium phosphates

The isolates were evaluated for their solubilization of tricalcium, iron and aluminium phosphates by spot-inoculating (10 µL; 10^6 cells mL^{-1}) them on plates of NBRIP (National Botanical Research Institute's Phosphate) containing (g L^{-1}) glucose (10), $Ca_3(PO4)_2$/$AlPO_4$/$FePO_4$ (5), $MgCl_2.6H_2O$ (5), $(NH_4)_2SO_4$ (0.1), $MgSO_4.7H_2O$ (0.25), KCl (0.2) and agar (15) according to

Pe'rez *et al*. (2007). Phosphate solubilization index (SI) was calculated by dividing the total diameter (halo zone and colony) by colony diameter (mm).

5.2.3.3 Nitrogen fixation

The isolates were tested for their BNF capacity by stabbing in and assessing their capacity to grow and form pellicle in Burk's N-free semi-solid medium incubated at 30ºC for 5 days (Laskar and Sharma 2013). Burk's N-free semi-solid medium contains (g L^{-1}) glucose (10), KH_2PO_4 (0.41), Na_2SO_4 (0.52), $CaCl_2$ (0.2), $MgSO_4.7H_2O$ (0.1), $FeSO_4.7H_2O$ (0.005), $Na_2MoO_4.2H_2$ (0.0025) and agar (1.8). Relative sizes of pellicles were recorded to relate to the relative BNF potential of the isolates.

5.2.3.4 *In vitro* antifungal activity

The antifungal activity of all the 231 isolates was tested using a dual culture method. Ten micro litres (10^6 cells mL^{-1}) of each rhizobacterial isolate was spot-inoculated onto the surface of potato dextrose agar (PDA)-nutrient agar (1:1[w/w]; Saraf *et al*., 2007) petri dishes (90 mm diameter) at a distance of 3 cm from the centre and at four equidistant points (12, 3, 9 and 6 o'clock positions). The plate was then incubated at 28ºC for 48h. A 4 mm disc from 48 h/28 ºC PDA grown culture of the test pathogen *Fusarium oxysporum* (obtained from EIAR, Ethiopian Institute of Agricultural Research), was placed at the centre of each plate (including rhizobacterial free control plates) and incubation resumed under the same conditions until the fungus had grown to the outer-edge of the control plates. Percentage inhibition of radial growth (PIRG) was calculated as, [(C-T)/C] ×100, where: C is radial growth (mm) of fungus on control plates; and, T is the radial growth of the fungus in the dual culture.

At this stage, 72 isolates that demonstrated multiple PGP traits or good potential of at least one of the above tested PGP traits were selected for further characterisation for HCN production, enzymatic activity (protease, cellulase and chitinase) and potential tolerance to ecological factors using standard methods. All tests were undertaken aseptically in triplicates by inoculating 100 µL of overnight active culture adjusted to 10^6 cells mL^{-1} and incubated at 28 ± 0.2ºC for 5-7 days unless stated otherwise.

5.2.3.5 Production of hydrogen cyanide

Each of the 72 sub-set of rhizobacteria isolate (100µL; 10^6 cells mL^{-1}) was spread on nutrient agar plate (90 mm diameter) amended with 4.4 g L^{-1} glycine (Bharucha *et al*., 2013). Whatmann™ filter paper No.1 was soaked in a solution of 2.5 g picric acid and 12.5 g Na_2CO_3 dissolved in a liter of distilled water. This was fixed to the underside of the lid of each plate and sealed with Parafilm™ and incubated at 28ºC± 0.2 for 3-5 days. Cultures were assessed for their relative capacity to change the filter paper over 3 stage categorical colour scale, defined as: light brown, brown and reddish brown recorded as weak, moderate and strong HCN production, respectively.

5.2.3.6 Protease, Cellulase and Chitinase activities of the isolates

Each rhizobacterial isolate (10 µL; 10^6 cells mL^{-1}) was spot-inoculated on nutrient agar plates supplemented with 1.5 % skimmed milk powder to test for protease activity (Ryden *et al*., 1973). Cellulose production was tested on Carboxymethyl cellulose (CMC) agar plates (Kasana *et al*., 2008). Chitinase activity was evaluated on chitin-agar plates (Bansode and Bajekal, 2006) (Media composition; section 4.2.6.8). The inoculated plates were all incubated at 28 ± 0.2ºC for 72 h for clear zone formation around their colonies as indication of enzyme activity. For, plus-

CMC plates, clearing was visualised after flooding the plates with Gram's iodine (prepared by dissolving 2 g of KI and 1 g of iodine in 300 mL distilled water) for 3-5 min in the dark. Chitinase activity indices were calculated as the ratio of the diameter of the colony plus clear zone/colony diameter (in mm; Ahmed *et al*., 2014).

5.2.4 Physico-chemical stress tolerances

5.2.4.1 Temperature, pH, Salt (NaCl) and pesticide tolerance

Colony growth for each of the 72 rhizobacteria sub-set was assessed at different incubation temperatures (35, 37, 40 and 45^0C). Due to failure of nutrient agar to solidify well at pH 4 and 4.5 tolerance to these pH values was tested in nutrient broth (100 µL; 10^6 cells mL^{-1} culture inoculated into 5 mL nutrient broth and shaked at room temperature for 72 hours) whereas tolerances to pH 5, 5.5, 6, 8, 8.5 and 9; to NaCl (1-7% [w/v]; Damodaran *et al*., 2013) and to fungicides [curzet and mankozeb; Du Pont de Nemour, France; 2 g L^{-1} (Mubeen *et al*., 2006)] were evaluated on nutrient agar plates. Tolerance to herbicide [glyphosate^{TM}, Monsanto Europe S.A, Belgium; 1444 µg L^{-1} was tested on minimal salt agar plates (Ahemad and Khan, 2010)]. A loopful of overnight nutrient broth culture (10µL;10^6 cells mL^{-1}) of each rhizobacteria isolates was streaked on nutrient agar plates and incubated at 28 ± 0.2ºC for 72 h to test pH and salt tolerance. Turbidity of nutrient broth (for pH 4 and 4.5) and appearance of bacterial colonies on nutrient agar plates were recorded as tolerance against the controls.

5.2.4.2 Intrinsic resistance to antibiotics and heavy metals

Actively growing rhizobacteria (10 µL; 10^6 cells mL^{-1}) of the 72 isolates were spot inoculated individually onto nutrient agar plates supplemented with filter-sterilized (0.22 µm) (µg mL^{-1}) of ampicillin (100), chloramphenicol (5), gentamycin (5), neomycin (50), streptomycin sulphate

(20), nalidixic acid (50), penicillin G (20), vancomycin (5) and erythromycin (100) according to Wang *et al*. (2009). The isolates were also spot-inoculated onto minimal-salt agar plates to test for their inherent tolerance to heavy metals: $CoCl_2.6H_2O$ (0.5 mM); $Pb(CH_3COO)_2.3H_2O$ (2.5 mM); $K_2Cr_2O_7$ (0.25 mM); $CuCl_2.2H_2O$, $ZnSO_4.5H_2O$; and, $MnSO_4.4H_2O$ (0.25 mM each) according to Hungria *et al*. (2001). Inoculated plates were incubated at 28ºC ± 0.2 for 72 h in both testes. Visible colony growth showing isolates were recorded as tolerant.

5.2.5 Identification of the isolates using analyses of phenotypic features and 16S rRNA sequences

5.2.5.1 Phenotypic characters

Multivariate cluster analysis of the phenotypic variability among the 72 fully-characterised isolates and construction of dendrogram were carried out using Ori PAST™ software (Hammer *et al.* 2001) based on 49 phenotypic traits.

5.2.5.2 16S rRNA sequence analyses

5.2.5.2.a DNA extraction

Twenty rhizobacteria with better potential PGP traits and ecological tolerance were selected from the phenotypic experiments for the 16S rRNA sequencing and phylogenetic analysis. A single colony of each rhizobacterium was inoculated into 5 mL of nutrient broth and incubated at 28 ºC with 150 rpm for not more than 24 h. A total of 4 mL (2 x 2 mL) of log phase liquid cultures were harvested by centrifugation (14,000 rpm; 10 min; 4°C) in a 2 mL microfuge. The pellets were re-suspended in 400 µL of sterile Tris-EDTA (TE) buffer and lyzed with 10 µL of 20% (w/v) sodium dodecyl sulfate (SDS, final concentration of 0.5% [w/v]) to which 10 µL (>800 u mL^{-1}) proteinase K (Sigma #P4850) was added and thoroughly mixed. The mixture was

incubated at 37ºC in water bath for 1 hour, thoroughly mixed with 420 µL of phenol:chloroform:isoamyl alcohol (25:24:1 [v/v/v]; Sigma #P2069) and centrifuged (14,000 rpm; 10 min; 4°C). The upper aqueous layer (≈175 µL) was recovered, and subsequently mixed well with 1/10 x volume (17.5 µL) of 3 M Sodium acetate (pH 5.2) and 3x volume (655 µL) of isopropanol (Sigma #I9030). DNA was precipitated at -80 °C for 15 minutes , pelleted by centrifugation (14,000 rpm; 15 min; 4°C), washed with 200 µL of 70% [v/v] ethanol, centrifuged (14,000 rpm; 1 min; 4°C), oven dried at 37ºC for 15 minutes and re-suspended in TE buffer (25 µL each). The quality (absorbance at 260:280 ratio) and quantity (µg uL^{-1} value) of DNA were determined using a ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, US).

5.2.5.2.b Amplification and sequencing of 16S rRNA

A portion of 16S ribosomal RNA (rRNA) of each rhizobacterial isolate was amplified using forward primer 8-27F (AGAGTTTGATCCTGGCTCAG) and reverse primer rD1 (AAG GAG GTG ATC CAG CC) (Weisburg *et al*., 1991). The PCR reaction mixture (50 µL for each sample) was prepared according to the manufacturer's recommendations and constituted of milliQ-water (33.75 µL), 5X clear Go Taq® G2 Buffer (Promega, 10 µL), 10 mM dNTPs (Invitrogen, 1 µL), forward and reverse primers (2 µL each), Go Taq polymerase (Promega #M3175, 0.25 µL) and DNA template (1 µL). The PCR thermal profile comprised one time denaturation at 95°C for 2 min, amplification using 35 cycles (30s 94°C; 30s 56°C, 1.5 min 72°C), and a final elongation step at 72°C for 15 min using a G-Storm GS1 thermal cycler (GRI Ltd, Braintree, UK).

Successful amplifications of the 16S rRNA PCR products were resolved by electrophoresis on a 1% [w/v] agarose gel containing 1µL SYBR-SAFE (Invitrogen) followed by UV-illumination

(FluorChem® Imager, Alpha Innotech). The PCR products (~1,400 bp) were purified as stated by the manufacturer using QIAquick Spin columns (Qiagen, Inc., Chatsworth, Calif.), eluted using Buffer EB (10 mM Tris-Cl, pH 8) and cleaned on QIAquick membrane according to the manufacturer's recommendations. Cleaned 16S rRNA's were stored at -20^{o}C until sequencing using the earlier primers and an ABI3730 DNA analyzer having a 36 cm x 48 cm capillary array.

5.2.5.2.c Phylogenetic analysis

The likely genus of each isolate was identified via analysis of the 16S rRNA sequence with BioEdit Sequence Alignment Editor Version 7.2 software to remove primer sequences. The sequences were then screened against databases using the nucleotide basic local alignment tool (BLASTN) queuing system (Altschul *et al*., 1997) 2.2.28 on the NCBI website.

The diversity of the rhizobacterial isolates were assessed on the basis of their relative 16S-rRNA gene sequence information using the Maximum Likelihood method (Tamura and Nei, 1993) and MEGA7 software (Kumar *et al*., 2016). Initial tree(s) for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and selecting the topology with superior log likelihood value. The tree obtained (see results ad discussion section) was drawn to scale with branch lengths measured in the number of substitutions *per* site. All positions containing gaps and missing data were eliminated.

5.2.6 Seed germination and seedlings growth assays

Six rhizobacterial isolates (SR7A, SR10A, SR20A, SR20B, SR43B and SR69B) having multiple PGP traits (3 to 7) such as highest zone of pathogenic fungal inhibition and maximum amount of IAA production. All associated with good stress tolerance traits were selected to evaluate their

effect on soybean seed germination and seedlings growth. Soybean seeds were surface sterilised in 2% sodium hypochlorite for three min (Lwin *et al*., 2012) and dipped into each rhizobacterial nutrient broth culture (approximately 10^9 cells mL^{-1}) for 5 hours according to Malviya and Singh (2012). Surface sterilised seeds dipped into sterile nutrient broth for 5 h were also included as control. All seeds were plated on Petri dishes (90 mm; 10 seeds per Petri dish) with moist Whatman filter paper moistened with sterile distilled water and incubated at 28 ± 0.2^oC for five days before measures were taken for % Germination of total seeds with germination scored as radicle protrusion (mm), and Vigour Index (VI), ((% germination) x average total seedling length (mm; root length plus shoot length); Agrawal and Agrawal (2013).

5.2.7 Data analyses

One way ANOVA of seed germination assay data was done using SPSS version 15. Significance differences were determined using Tukey HSD at 0.05 levels after one way ANOVA.

5.3. Results and Discussion

5.3.1 Screening for PGP properties and preliminary taxonomic status of the Rhizobacteria

Of the total 231 soybean rhizobacterial isolates screened for Indole acetic acid (IAA) production, phosphate solubilization, N-fixation and anti-fungal activity, 87 % produced IAA, 16% survived on N-free medium, 13% inhibited *Fusarium oxysporum* and again 13% soulubilized tri-calcium phosphate (Table 13). Out of the 231 rhizobacteria, 72 isolates (31%) of which 58% and 42% were gram negative and gram positive bacteria, respectively.

Based on the analysis of 16S-rRNA gene sequencing, 18 of the selected 20 soybean PGPR were identified under seven genera (*Achromobacter, Acinetobacter, Bacillus, Enterobacter, Microbacterium, Pseudomonas* and *Stenotrophomonas*) distributed in 4 clades:

Gammaproteobacteria (Gram negative), Bacilli (Gram positive), Betaproteobacteria (Gram negative), and Actinobacteria (Gram positive) comprising 14, 2, 1 and 1 of the isolates, respectively (Table 12; Fig. 10). Two rhizobacteria, SR44B and SR50 that were assigned to uncultured bacterial clones using 16S rRNA sequencing also clustered within Gamaproteobacteria clade making a total of 16 rhizobacteria under the clade.

The clade *Gammaproteobacteria* included 4 genera: *Pseudomonas, Acinetobacter, Stenotrophomonas* and *Enterobacter*. The genus *Pseudomonas* contained six isolates: SR3A, SR41B, SR45B, SR47B, SR77 and SR99B with 99% sequence similarity with NCBI reference strains of *Pseudomonas* sp. AF32, *Pseudomonas lini* P2, *Pseudomonas menteilii, Pseudomonas* sp. AS2, *Pseudomonas* sp. R01 and *Pseudomonas* sp. AF32, respectively (Table 12).

Phylogenetic analysis indicated the close relatedness of SR3A and SR99B to *Pseudomonas lini* H2P2; SR41B to *Pseudomonas lini* N5; SR45B and SR47B to *Pseudomonas plecoglossicida* E3.NA; and SR77 to *Pseudomonas clemoncea* PE22. SR41B is indicated as strains of *Pseudomonas lini*. SR50 was related to *Pseudomonas putida* S28 (supported by the bootstrap value of 77%) though it shared 99% rRNA sequence similarity with uncultured bacterium clone CT14C1C603 having NCBI accession number JQ426964.1.

The genus *Stenotrophomonas* included four of the isolates of which SR14, SR29A and S43B were identified into different strains of *Stenotrophomonas maltophilia,* and SR29A into a *Stenotrophomonas* sp. with 97- 99% sequence homology (Table 12). SR44B was placed closely with *S. maltophilia* 81(bootstrap value 99%) in the phylogenetic tree though it shared 99% sequence similarity with uncultured bacterium clone CN2-80, NCBI accession number HQ218839.1. The genus *Acinetobacter* included three isolates (SR10A, SR20B and SR40).

SR20A shared 99% 16S rRNA sequence similarity with *Achromobacter mucicolens* OZK37 (Table 12) but clustered with *Achromobacter* sp. DP147B (bootstrap value 100%) within Betaproteobacteria clade (Fig. 10).

The clade Actinobacteria and Bacilli included one (SR7A) and two (SR6A and SR69B) isolates, respectively. SR7A possessed 99% 16S rRNA gene sequence similarity with *Microbacterium oxydan* M90 NCBI accession number LN890176.1 (Table 12) and closely clustered with *Microbacterium martipicum* ST5 (bootstrap value 97%) (Fig.10). SR6A shared 100 % 16S rRNA sequence similarity with *Bacillus thuringiences* CTC strain (NCBI accession number CP013274.1) whereas SR69B shared 99% sequence similarity with *Enterobacter cloacae* strain Y219 (Table 12) and *Bacillus thuringensis* strain XL6 NCBI accession number CP013000.1 (data not shown). But, both SR6 and SR69B were closely clustered with *Bacillus cereus* AR156 and *Bacillus cereus* BM4-1 (Fig. 10) since both were identified to be gram positive.

Table 12. Genetic identity of selected rhizobacterial isolates based on 16S rRNA sequencing analysis

SN.	Isolate	Length of query (bp)	Identity	Best Match ID (NCBI ref)	Cover (%)	Identity (%)
1	SR3A	1423	*Pseudomonas sp* AF32	EU680973.1	100	99
2	SR6A	1405	*Bacillus thuringiensis* CTC	CP013274.1	100	100
3	SR7A	1189	*Microbacterium oxydans* M90	LN890176.1	100	99
4	SR10A	1387	*Acinetobacter sp* ST5	KJ867437.1	99	99
5	SR14	1313	*Stenotrophomonas maltophilia* 81	KM114935.1	39	97
6	SR20A	1369	*Achromobacter mucicolens* OZK37	KT716268.1	100	99
7	SR20B	1423	*Acinetobacter sp.* WP19	KU523563.1	100	99
8	SR29A	1413	*Stenotrophomonas maltophilia* T25	KT719219.1	99	98
9	SR29B	1422	*Stenotrophomonas sp* CanR-73	KT580665.1	98	98
10	SR40	1412	*Acinetobacter sp.* MSG8	FJ848381.2	100	99
11	SR41B	1386	*Pseudomonas lini* P2	KM349418.1	100	99
12	SR43B	1326	*Stenotrophomonas maltophilia* 81	KM114935.1	98	99
13	SR44B	1425	*Uncultured bacterium clone* CN2-80	HQ218839.1	100	99
14	SR45B	1431	*Pseudomonas monteilii*	LC015566.1	99	99
15	SR47B	1419	*Pseudomonas sp.* AS2	KP233816.1	99	99
16	SR48B	1413	*Enterobacter sp.* WS05	JN210900.1	99	95
17	SR50	1420	*Uncultured bacterium clone* CT14C1C603	JQ426964.1	100	99
18	SR69B	1442	*Bacillus thuringensis* strain XL6	CP013000.1	100	99
19	5R77	1415	*Pseudomonas sp.* R01	KT890299.1	100	99
20	SR99B	1428	*Pseudomonas sp.* AF32	EU680973.1	99	99

In previous studies, the dominant soybean rhizobacteria with PGP traits were *Pseudomonas* (Park *et al*., 2005; Leon *et al*., 2009; Wahyudi *et al*., 2010; Wahyudi *et* al., 2011a; Susilowatl *et al*., 2011; Bagalkar, 2013), *Stenotrophomonas* (Park *et al*., 2005; Ma *et al*., 2010; Sugiyama *et al*., 2014), *Bacillus* (Kloepper *et al*., 1992; Park *et al*., 2005; Peterson *et al*., 2006; Leon *et al*., 2009; Stefan *et al*., 2010; Wahyudi *et al*., 2010; Wahyudi *et* al., 2011b; Sharma *et al*., 2012; Masciarelli *et al*., 2014; Sugiyama *et al*., 2014), *Enterobacter* (Bagalkar, 2013; Ramesh *et al*., 2014; Sugiyama *et al*., 2014), *Acinetobacter* (Bagalkar, 2013) and *Microbacterium* (Kloepper *et al*., 1992**).** Although *Achromobacter* species with PGP traits were identified from rhizosphere of maize (Bumunang and Babalola, 2004), *Brassica juncea* (Ma *et al*., 2010) and tobacco (Huang *et al*., 2015), they were rarely reported from soybean rhizosphere. Egamberdieva *et al*. (2016) reported *Achromobacter* species from soybean rhizosphere grown in soil amended with 2% hydrochar rather than from those plants grown in control soil under greenhouse condition and the authors emphasised the shifting effect of the hydrochar on plant growth promoting rhizobacterial community.

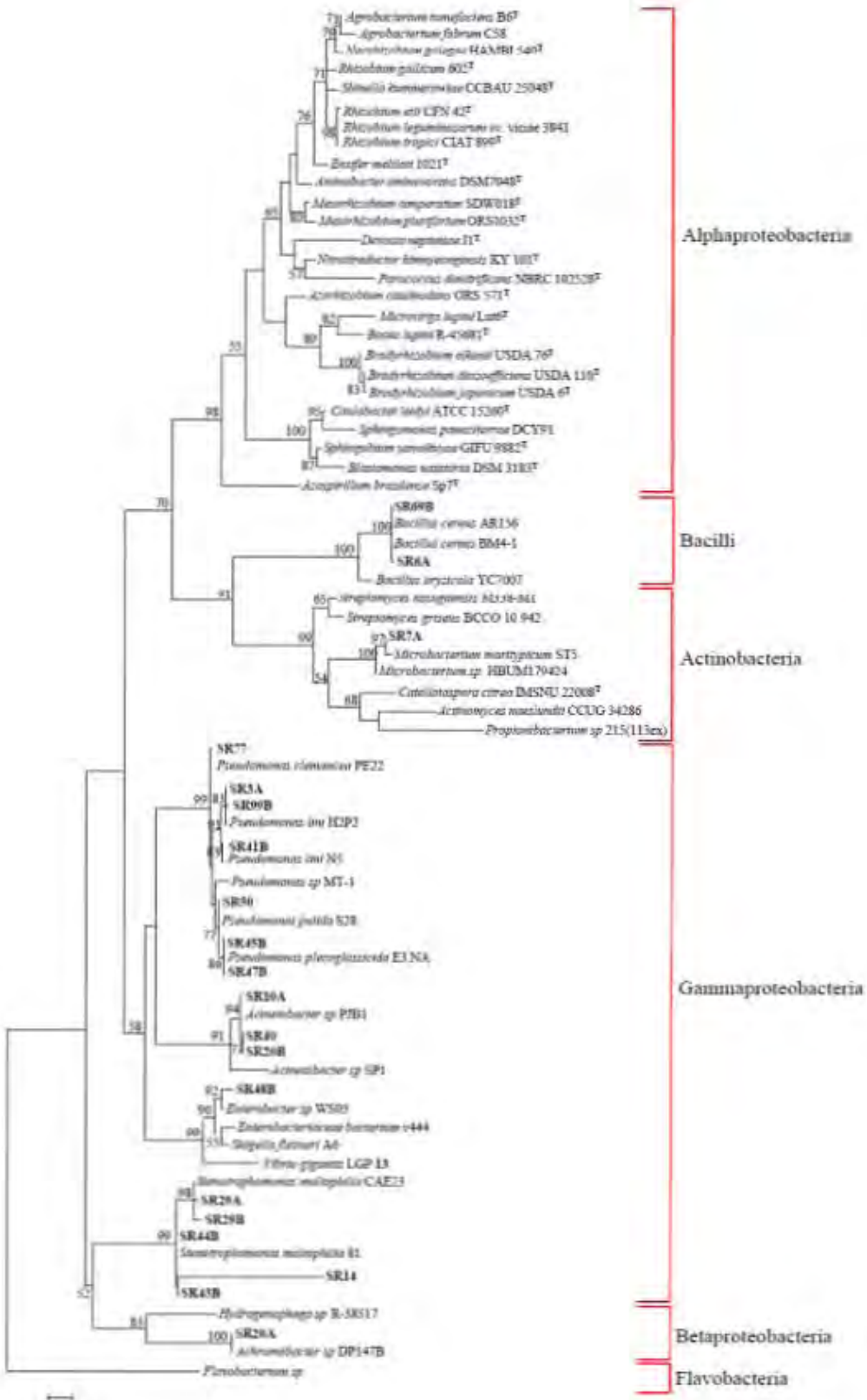

Fig. 11. Phylogenetic trees showing similarity based upon 16S-rRNA PCR product sequences obtained from the 20 selected rhizobacterial isolates of soybean relative to sequence information for the same gene region for other rhizobacteria (obtained from the from the database).

Type strains are denoted by ^{T}, and the tree with the highest log likelihood (-7016.5) is shown, and rooted using *Flavobacterium* sp. Trees were generated using the Maximum Likelihood method (1000 bootstraps), and only bootstrap values >50 are given. These values appear at branch-points as %'s (of total bootstraps/trees), for which the isolates shown clustered together

5.3.1.1 Phenotypic clustering analysis

The 72 rhizobacterial isolates were divided into two clusters around 36% similarity level, and into further six clusters at about 45% similarity level (Fig 11). No phenotypic clusters were formed beyond 96 % similarity level implying the phenotypic diversity of the isolates.

Phenotypic clustering analysis of the rhizobacterial isolates showed some similarity with their 16S rRNA sequencing identification (indicated in brackets) and phylogenetic analysis. Four *Stenotrophomonas* species (SR14, SR29A, SR29B and SR43B) were clustered together and the two *Bacillus* species (SR6A and SR69B) were also clustered together at about 48 and 75% similarity level, respectively (Fig. 11). Similarly, all the three *Acinetabacter* species were clustered together at around 85% similarity level. Members of the *Pseudomonas* species were clustered with different groups of the rhizobacteria except three of them that clustered together at about 72% similarity level. However, genetically different groups of rhizobacteria such as SR7A (*Microbacterium*) and SR40 (*Acinetobacter*) were clustered together at 96% phenotypic similarity level (Fig. 11) showing phentypically similar bacterial groups may or may not be similar genotypically.

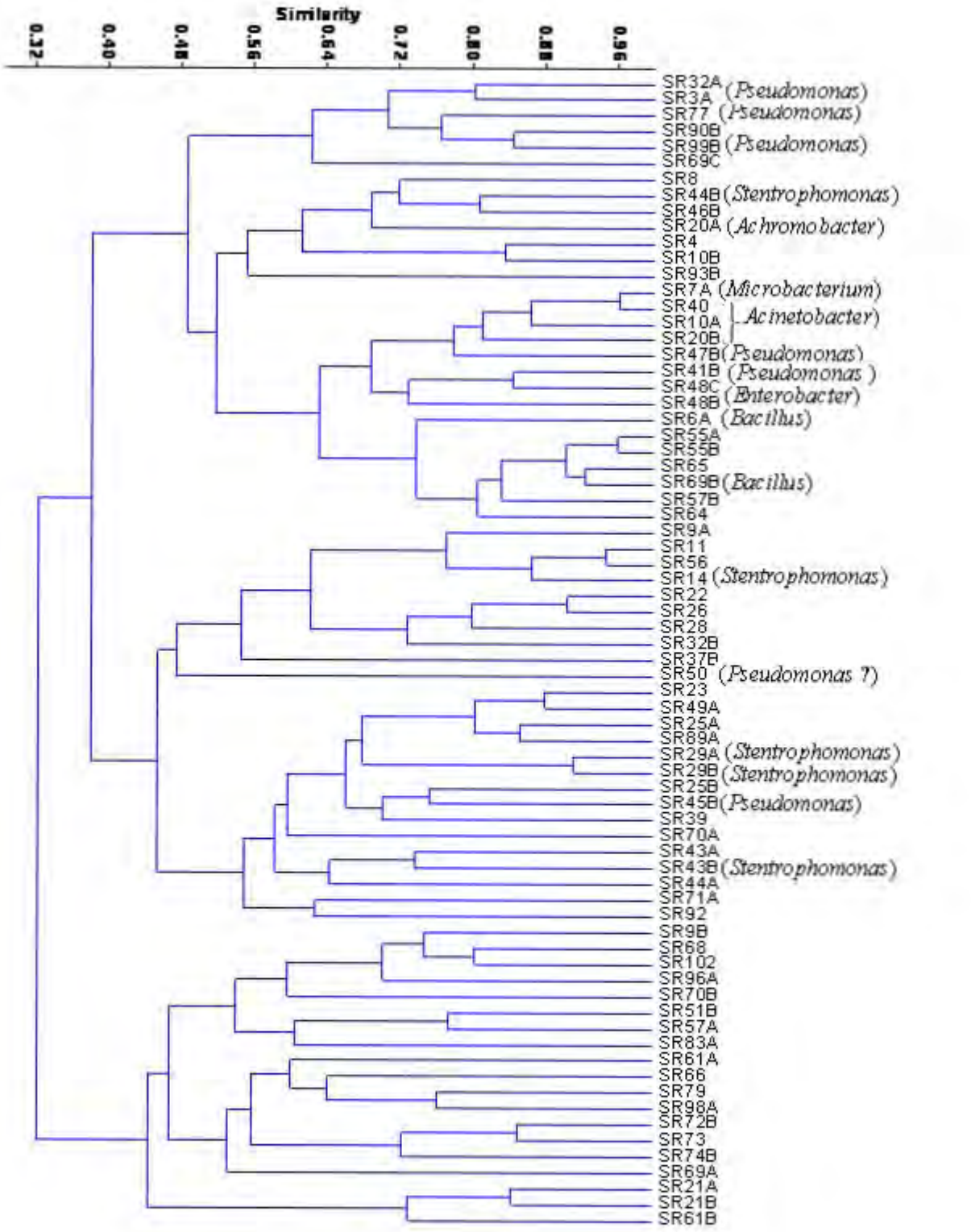

Fig. 12. Dendrogram highlightenig the phenotypic similarity of the 72 rhizobacteria. Isolates identified via 16S sequencing are indicated in paranthesis.

5.3.1.2 PGP properties

Indole acetic acid (IAA) production

Most of the Gram positive (93%) and Gram negative isolates (81%) produced IAA ranging from 8-143.3 µg mL^{-1} with average production of 29 µg mL^{-1} , and a median of 15 µg mL^{-1} (Table 13). The most efficient isolate, SR69B (*Bacillus thuringensis*) produced 143.3 µg mL^{-1} followed by few isolates that released 101.3-135.5 µg mL^{-1} IAA- (Table 13).

The most effective isolates could produce almost 10-20 fold times more IAA than the less effective ones. The data also showed that the Ethiopian isolates produced IAA at concentrations which are comparable to that reported for other PGPRs isolated from soybean roots such as *Bacillus fusiformis* PM-5 (140.9 µg mL^{-1}, Republic of Korea; Park *et al*., 2005), and *Enterobacter cloacae* MDSR9 (125 µg mL^{-1}, India; Ramesh *et al*., 2014) though less than *Bacillus subtilis* PRBS-1 (310 µg mL^{-1}, Brazil; Arau'jo *et al*., 2005).

Table 13. Detection of PGP traits of the rhizobacterial isolates in relation to their gram reaction (groups) tested on their respective media at 30°C at differnt incubation time.

PGP trait	Value	Total N°. of Isolates Tested	% of total exhibiting PGP trait	% of total N°. isolates for each Gram type	
				positive	negative
IAA[x] production	8 -143 (µg mL^{-1})	231	86	93	81
$(Ca)_3PO_4$ solubilisation	1.11-2.75 (SI[*])	231	13	5	19
$AlPO_4$ solubilisation	1.08-2.57 (SI)	231	12	1.0	20
$(Ca)_3PO_4$ and $AlPO_4$	1.08-2.75 (SI)	231	10	1.0	16
BNF[xx] potential	+/-	231	16	16	17
Antifungal activity	19-82 (PIRG**)	231	13	7	16
Cellulase activity	+/-	72	38	24	57
Chitinase activity	1.11-2.75 (SI[*])	72	43	55	27
Protease activity	+/-	72	60	69	47
HCN production	+/-	72	21	31	7
The range in the total number of PGP traits of the 72 potential PGPR isolates				2-6	2-9
The average number of PGP traits of the 72 potential PGPR isolates				3	5

*SI= solubilization index**, **** PIRG=percentage of inhibition of radial growth, x= indole-3-acetic acd, BNF= biological nitrogen fixation, + (showed the trait), - (not showed the trait)

Phosphate solubilization

The data showed 13% and 12% of the isolates solubilized tri-calcium phosphate (TCP) and aluminium phosphate, respectively (Table 13) whereas none of the isolates was able to solubilize iron phosphate. The data also showed more percentage of gram negative isolates (16%) solubilized both inorganic phosphate sources compared to gram positive isolares (1%) tested (Table 13). The isolates varied in their efficency of phosphate solubulization reflected from their solubilization indices (SI) ranging from 1.08 to 2.75 (Table 13).

Of the 16S-sequenced isolates highest tricalcium phosphate SIs were achieved by SR40 (*Acinetobacter sp.*), SR99B (*Pseudomonas sp.*), SR20B (*Acinetobacter sp.*), SR10A (*Acinetobacter sp*) and SR20A (*Achromobacter mucicolens*) with SIs varying from 2.8 to 2.2 (Table 14). These high indices are comparable to those reported for soybean rhizobacteria *Pseudomonas, Enterobacter* and *Acinetobacter* species (1.3 - 2.8) reported from India (Bagalkar, 2013). Maximum $AlPO_4$ SI (2.6) was achieved by isolates SR10A (*Acinetobacter sp*) and SR20A (*Achromobacter mucicolens*), whereas SR8, SR20B (*Acinetobacter sp.*), SR7A and SR40 (*Acinetobacter sp*) showed phosphate SIs of 2.5, 2.4, 2.3 and 2.2, respectively. SR20A (*Achromobacter mucicolens*) solubilized both TCP and $AlPO_4$, whereas *Achromobacte*r sp. isolated from soybean rhizosphere soil (Müncheberg, Germany) amended with 2% hydrochar lacked TCP solubilization trait in Pikovskaya medium (Egamberdieva *et al*., 2016)

Nitrogen fixation

Of the 38 isolates (16%), which grew in Burk's N-free semi-solid medium, 12 produced conspicuous pellicles indicating their better growth and BNF efficiency (Table 13; Appendix 3). Among the 16S-rRNA gene sequenced isolates, SR3A (*Pseudomonas sp.*), SR6A (*Bacillus*

thuringiensis), SR29A (*Stenotrophomonas maltophilia*) and SR29B (*Stenotrophomonas sp.*) were the best biological nitrogen fixers (Table 14) The N-fixing ability of *Bacillus* species isolated from soybean rhizosphere was previously reported (Park *et al*., 2005; Masciarelli *et al*., 2014). Biological nitrogen fixing trait is important to provide useable form of nitrogen to the associated host plant when soil available form of nitrogen is limited as has been reported for soils of several regions of the world.

Table 14. In vitro qualitative and/or quantitaive evaluation of PGPR properties of the rhizobacterial taxonomic groups grown under different cultural conditions

No	Isolate	$Ca_3(PO4)_2$ SI*	$AlPO_4$- SI	IAA µg/ml	N_2-fixation	PIRG**	HCN production	Protease	Cellulase	Chitinase SI	Gram reaction
1	SR3A (*Pseudomonas sp)*	1.5	1.22	74	+++	49	+++	+	-	-	-ve
2	SR6A (*Bacillus thuringiensis*)	1.25	-	94.4	+++	-	-	-	-	-	+ve
3	SR7A (*Microbacterium oxydans*)	2.1	2.25	59	-	-	-	-	-	-	+ve
4	SR10A (*Acinetobacter sp.*)	2.25	2.57	41	+	-	-	-	-	-	-ve
5	SR14 (*Stenotrophomonas maltophilia*)	1.11	1.08	71.1	++	48	-	+	-	2.75	-ve
6	SR20A (*Achromobacter mucicolens*)	2.22	2.57	47	+	-	-	-	-	-	-ve
7	SR20B (*Acinetobacter sp.*)	2.33	2.43	46.8	+	-	-	-	-	-	-ve
8	SR29A (*Stenotrophomonas maltophilia*)	1.43	1.73	50.4	+++	51	-	+	-	2.0	-ve
9	SR29B (*Stenotrophomonas sp.*)	1.57	1.73	58.5	+++	37	-	+	-	2.5	-ve
10	SR40 (*Acinetobacter sp.*)	2.75	2.22	43	-	-					-ve
11	SR41B (*Pseudomonas lini*)	1.22	1.44	51.9	++	-		+	-	-	-ve
12	SR43B (*Stenotrophomonas maltophilia*)	1.23	-	36.9	-	82	+++	+	+	2.75	-ve
13	SR44B ***	1.38	1.5	60	-	-	-	-	-	-	-ve
14	SR45B (*Pseudomonas monteilii*)	-	1.33	101.3	+	-	-	-	+	2.5	-ve
15	SR47B (*Pseudomonas sp.*)	1.9	1.5	59.2	-	-	-	-	-	-	-ve
16	SR48B (*Enterobacter sp.*)	1.63	1.13	36.3	+	19	-	+	-	-	-ve
17	SR50 ***	1.11	2	51.5	++	62	+	+	+	2.2	-ve
18	SR69B (*Bacillus thuringensis*)	1.33	-	143	-	-	-	-	-	-	+ve
19	SR77 (*Pseudomonas sp.*)	1.8	2	39.5	-	27	+++	+	-	-	-ve
20	SR99B (*Pseudomonas sp.*)	2.36	1.83	8.0	-	44	+	+	+	-	-ve

*SI = solubilization index, **PIRG = percent of inhibition of radial growth of fungus, SI= solubilization index, ***identified as clones of uncultured bacteria via 16S rRNA sequencing

Antifungal activity

Twenty nine of the 231 rhizobacterial isolates (13%) showed antagonistic activity against the fungal pathogen *Fusarium oxysporum*, which causes soybean root-rot. PIRG values ranged from 82 % (SR43B, *Stenotrophomonas maltophilia*) to 19% (SR48B, *Enterobacter sp.*) (Table 13; Appendix 3). SR50 demonstrated the second highest PIRG value (62%) beside having all the other tested PGP traits. Fifteen (52%), thirteen (45%) and 7 (24%) of the 29 rhizobacteria isolates with anti-*F. oxysporum* activity showed chitinase, HCN, and both chitinase and HCN activity, respectively (Appendix 3). The PRIG values reported here are similar to those reported for *Baccilus* (30-40%; Wahyudi *et al*., 2011b) and *Pseudomonas* (11- 60%; Susilowatl *et al*., 2011) isolated from soybean rhizospheres.

HCN production

Of the 72 isolate tested, 15 (21%) produced HCN (Table 13; Appendix 4). Some of the HCN producing bacteria are indicated in Table 14. Of the 15 cyanogenic rhizobacterial isolates, five (33%) isolates [SR3A (*Pseudomonas* sp.), SR32A, SR43B (*Stenotrophomonas maltophilia*), SR77 (*Pseudomonas* sp.) and SR90B], two (13%) isolates (SR48C and SR69C) and the remaining 8 (53%) cyanogenic rhizobacterial isolates including SR99B (*Pseudomonas* sp.) showed higher, moderate, and lower level of HCN production as indicated by the change in the color of picric acid impregnated yellow filter paper into reddish brown, brown, and light brown, respectively. Similarly, Susilowatl *et al*. (2011) indicated the production HCN by 36% of *Pseudomonas* species of soybean rhizosphere indicating it some what a common trait of rhizosphere bacteria though it can be considered as both plant growth promoting or inhibiting trait.

Production of fungal cell wall degrading enzymes

Screening of the 72 isolates for different enzymatic activity related to pathogen inhibition showed that 43 (60%) and 31 (43%) of the isolates possessed protease and chitinase activity, respectively (Table 13; Appendix 4). Seven (10%) rhizobacterial isolates: SR9B, SR43B (*Stenotrophomonas maltophilia*), SR50, SR69A, SR70A, SR70B and SR102) produced the two types of enzymes; SR43B (*Stenotrophomonas maltophilia*), SR50, SR70B and SR102 also inhibited growth of *Fusarium oxysporum*. Among the isolates SR11, SR14 (*Stenotrophomonas maltophilia*), SR25B and SR43B (*Stenotrophomonas maltophilia*) demonstrated higher chitin solubilization index (2.75 mm) on chitin agar implying their higher level of chitinase activity. Similarly, Leon *et al*. (2009) reported the production of chitinase and protease by six soybean rhizobacteria belonging to *Bacillus, Pseudomonas* and *Burkholderia* species. Choudhary (2011) reported the production of protease and chitinase by two selected strains of *Pseudomonas* species from soybean rhizosphere; whereas Susilowatl *et al*. (2011) and Wahyudi *et al*. (2011a) reported chitinase activity for 20% and 43% of soybean rhizosphere *Pseudomonas* sp, respectively. Similarly, Masciarelli *et al*. (2014) reported the production of protease by a soybean rhizobacterium *Bacillus amyloliquefaciens* strain LL2012**.**

5.3.2 Tolerance of Rhizobacteria to different ecological factors

The selected isolates (72 rhizobacteria) were tested for their inherent tolerance to different ecological factors under *in vitro* condition (Table 15, Appendix 5). Most of the rhizobacteria were resistant to incubation temperature of 40ºC (67%), but sensitive to 45ºC (17%), lower pH 4.5 (15%) and 7% NaCl (19%).

Table 15. The effect of selected physico-chemical parameters on the growth of the rhizobacterial isolates

Parameter	values	Percentage resistance
pH	4.5	15
	5	29
Temperature	40^{o}C	67
	45 ^{o}C	17
NaCl	6%	22
	7%	19
$CoCl_2.6H_2O$	0.5 mM	29
$K_2Cr_2O_7$	0.25 mM	18
$CuCl_2.2H_2O$	0.25 mM	5.5
Erythromycin	100 µg mL^{-1}	53
Streptomycin sulfate	20 µg mL^{-1}	50
Gentamycin	50 µg mL^{-1}	46
Naldixic acid	50 µg mL^{-1}	42
Neomycin	50 µg mL^{-1}	36
Glyphosate	1444 µg mL^{-1}	49
Mankozeb	2 g L^{-1}	14

Alarge number of rhizobacterial isolates (over 85%) were resistant to antibiotics **(**µg mL^{-1}**)**; Ampicillin (100), Chloramphenicol (5), Penicillin G (20) and Vancomycin (5) (data not shown). But, 50 to 64% of them were sensitive to Neomycin (50), nalidixic acid (50), Gentamycin (50) and streptomycin (20) (Table 15; Appendix 6). Although a few rhizobacteria failed to resist all the antibiotics, SR22, SR25A, SR25B, SR26, SR29A (*Stenotrophomonas maltophilia*), SR43A, SR43B (*Stenotrophomonas maltophilia*), SR45B (*Pseudomonas monteilii*) and SR49A were able to resist all of the tested antibiotics (Appendix 6). Similar multiple antibiotic resistant soybean

rhizobiobacteria were also previously reported (Wang *et al*., 2009; Madhaiyan *et al*., 2010). Antibiotic resistance trait is useful for survival in soil where various antibiotic producing microbes co-exist.

The rhizobacterial isolates (80-90%) were resistant to heavy metals; $Pb(CH_3COO)_2.3H_2O$ (2.5 mM); $ZnSO_4.5H_2O$; and, $MnSO_4.4H_2O$ (0.25 mM each) (data not shown), but 94%, 82% and 71% of the isolates were sensitive to $CuCl_2.2H_2O$ (0.25 mM), $K_2Cr_2O_7$ (0.25 mM) and $CoCl_2.6H_2O$ (0.5 mM), respectively (Table 15) . A few isolates totally failed to resist all the heavy metals whereas SR73 was resistant to all the tested heavy metals except copper chloride (Appendix 5). Similarly, the isolates were relatively sensitive to agrochemicals where 49% were tolerant to glyphosate. However, only 14% of the isolates were resistant to mankozeb and none of them grew in the presence of the fungicide, curzet (Table 15). Six rhizobacteria (9%): SR8, SR20A (*Achromobacter mucicolens*), SR39, SR44B, SR46B and SR50 were tolerant to both mankozeb and glyphosate (Appendix 5) implying their better survival chance in case these chemicals are applied in pest management. Although the pesticides were applied at the recommended dose, the sensivity of these non-target organisms to the chemicals indicate the need to use environmenmentally friendly biocontrol agents.

The overall perforrmance of 20 rhizobacteria in terms of their potential ecological tolernce and PGP properties is summarized (Table 16).

Table 16. Pattern (%) of PGP traits and stress tolerances of the plant growth promoting rhizobacteria (number of PGP traits or stress tolerances present divided by the total number of parameters tested x 100)

isolates	PGP	pH	Temp	NaCl	Heavy metals	Antibiotics	Pesticides	Total *	Rank
SR50 (Unidentified)	91	88	75	100	67	100	67	588	1
SR14 (*Stenotrophomonas)*	55	88	100	100	67	89	0	499	2
SR20A(*Achromobacter)*	55	75	100	71	50	56	67	474	3
SR29A (*Stenotrophomonas)*	73	63	75	71	50	100	33	465	4
SR45B (*Pseudomonas)*	64	63	75	71	50	100	33	456	5
SR29B (*Stenotrophomonas)*	73	63	75	71	50	89	33	454	6
SR44B (Unidentified)	36	63	50	86	50	67	67	419	7
SR43B (*Stenotrophomonas)*	73	63	75	4	50	100	33	398	8
SR3A (*Pseudomonas)*	64	50	75	57	50	56	33	385	9
SR20B (*Acinetobacter)*	55	63	100	43	67	44	0	372	10
SR48B (*Enterobacter)*	64	63	50	71	67	56	0	371	11
SR6A (*Bacillus)***	36	63	75	57	67	67	0	365	12
SR99B (*Pseudomonas)*	82	50	50	57	33	56	33	361	13
SR69B (*Bacillus)***	27	63	100	29	67	67	0	353	14
SR41B (*Pseudomonas)*	55	63	75	43	50	33	33	352	15
SR40 (*Acinetobacter)*	36	75	75	43	50	44	0	323	16
SR7A (*Microbacterium)***	36	75	75	43	50	44	0	323	16
SR77 (*Pseudomonas)*	64	50	25	43	50	56	33	321	18
SR10A (*Acinetobacter)*	45	63	75	43	50	44	0	320	19
SR47B (*Pseudomonas)*	36	63	50	29	67	44	0	289	20

*Sum of percentages of PGP traits (11), pH tolerance (8), temperature tolerance (4), NaCl tolerance (7), heavy metal resistance (6), antibiotic resistance (9) and pesticide resistance (3) exhibited by each rhizobacterium, **(Gram positive)

The 20 rhizobacterial isolates, identified via 16S rRNA sequencing, varied in most of their potential PGP properties and tolerances to several environmental factors (Table 17) Accordingly, members of the Proteobacteria (Gram negative bacteria) showed more versatility in PGP and ecophysiolgical traits than isolates from the firmicutes (Gram positive): *Bacillus* and *Microbacterium*. Of all the isolates, SR50 (showed 16S rRNA gene sequence similarity with uncultured bacterial clone but phylogenetically related to *Pseudomonas*) showed the best performance followed by *Stenotrophomonas maltophilla* (SR14), *Achromobacter mucicolens*)

(SR20A), *Stenotrophomonas* (SR29A), SR 44B (showed 16S rRNA gene sequence similarity with uncultured bacterial clone but phylogenetically related to *Stenotrophomonas*), *Stenotrophomonas* (SR43B), and *Pseudomonas* sp (SR3A). However, isolates with a set of several weak PGP traits may not be good inoculants and a given PGP trait may be preferred based on the prevailing environmental condition(s).

5.3.3 Seed germination assay

Seed germination assay with selected rhizobacterial isolates showed significant differences in the soybean seed gemination and seedling growth (Table 17). Accordingly, SR20A (*Achromobacter mucicolens*) impacted the highest effect on almost all seed germination and seedling growth parameters ranging from 18% (germination percent) to 86% (vigor index), followed by SR20B (*Acinetobacter sp.*) on all parameters varying from 14% (germination percent) to 58% (vigor index) compared to the control (Table 18). Likewise, SR10A (*Acinetobacter*) showed moderate effect on all germination and growth parameters with 9-34% increase compared to the control group. It is intersing to note that SR69B (*Bacillus thuringensis*) produced the largest quantity of IAA (143 μg mL^{-1}), but did not show any effect on the germination and growth parameters except a 12% increase in shoot length of soybean compared to the control (Table 17). Vigor index (VI) was positively correlated with germination index, shoot length, germination percent and root length with Person's r of 0.189, 0.672, 0.816 and 0.936, respectively at 0.01significance level.

Table 17. Effects of different treatments on seed germination and seedling growth of soybean

Treatment	Germination %	Root length (cm)	Shoot length (cm)	Vigor index
Control	73.33[b]	4.47[b]	1.47[c]	435.58b
SR7A	83.33[a] (14%)*	4.08[b]	1.77[ab] (20%)	487.48b
SR10A	80.00[a] (9%)	5.58[a] (25%)	1.73[ab] (18%)	585[a] (34%)
SR20A	86.67[a] (18%)	7.20[a] (61%)	2.15 [a] (46%)	810.4[a] (86%)
SR20B	83.33[a] (14 %)	6.27[a b] (40)	2.00 [a] (36%)	689..14[a](58%)
SR43B	73.33[b]	4.80[b]	1.57[c]	467b
SR69B	66.67[b]	4.92[b]	1.64[bc] (12%)	437.35b

Data are means from three replications and values followed by different letter(s) in a column indicate significant differences ($p<0.05$). Numbers in parenthesis showed the % increases of the germination and growth parameters compared to the control groups.

In general, inoculation of soybean seeds with the local rhizobacteria increased percentage of seed germination by 10-18%, and shoot length by 18-46% compared to the uninoculated control. This was similar to the increase in germination and seedling growth by 6-20% and upto 14%, respectivly by culture filtrate of some unidentified soybean rhizobacterial isolates within five days after germination (Melnykova *et al.*, 2013). Increased seed germination may be due to increased synthesis of hormones like gibberellins that would trigger the activity of enzymes such as amylase that increase in the availability of assimilable forms of seed content (Gholami *et al*., 2009).

5.4. Conclusion and Recommendation

The rhizobacterial isolates varied in their potential PGP traits and tolerances to ecological factors. Some of them possessed multiple PGP traits (up to 9). IAA production was being

demonstrated by the majority of the isolates (86%) where as biological nitrogen fixation (BNF), solubilization of inorganic phosphates and antifungal activity were exhibited by limited number of the isolates (≤16%). HCN was also produced by less proportion of the isolates (21%) compared to cellulase, chitinase or protease activity (38-60% of the isolates).

Potential tolerances of the isolates to ecological factors varied from tolerances to almost all of- to none of- the factors. The isolates showed good tolerance to temperature of 40^{o}C and 4% NaCl which were tolerated by 67% and 65% of the isolates, respectively. Most of the isolates (80-100%) resisted some antibiotics (Ampicillin, Chloramphenicol and Penicillin G) and heavy metals [$Pb(CH_3COO)_2.3H_2O$, $ZnSO_4.5H_2O$ and $MnSO_4.4H_2O$]. Sensitive to some pesticides (glyphosate, mankozeb and curzet), antibiotics (Neomycin, naldixic acid, gentamycin and streptomycin sulphate), heavy metals [$CoCl_2.6H_2O$, $K_2Cr_2O_7$ and $CuCl_2.2H_2O$] as demonstrated by 50 to 100% of the isolates. Higher sensitivity was also shown against acidity; pH5 was tolerated by less than 30% of the isolates.

Members of the Gammaproteobacteria; *Pseudomona*s and *Stenotrophomonas* dominated the 20 rhizobacteria identified via 16S rRNA sequencing. Most bacteria of the same genus, identified via 16S rRNA sequencing, were clustered together at about 50% similarity level in the phenotypic cluster analysis.

Most inoculations resulted in significant effects on soybean seed germination and seedling growth parameters over the control; SR20A (*Achromobacter mucicolens*) and SR20B (*Acinetobacter* sp.) promoted 18-86% and 14-58% of the parameters, respectively over the control. Compatibility to effective soybean rhizobia and rhizocompetence SR20A and SR20B should be evaluated prior to their application as inoculant.

6. Symbiotic effectiveness of indigenous soybean rhizobia of Ethiopia

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Abstract

Soybean is a leguminous crop which is being used as a nutritious food and feed, and as input in various industrial processings. Due to its low productivity and limited area of production, Ethiopia has been forced to import soybean and its products. Efforts to enhance soybean yield via rhizobial inoculation in Ethiopia has principally involved few exotic strains, but the yield improvement is not satisfactory and consistent. To evaluate their effect on nodulation and growth of three varieties of soybean (Ethio-Yugoslavia, Cheri and Jalele), six indigenous soybean rhizobia (SNB57B, SNB120A, SNB120C, SNB125A, SNB120B and SNB140) and an exotic reference *Bradyrhizobium japonicum* SBTAL379 were inoculated on sand culture in a greenhouse. SNB120A, SNB120C, SNB125SA and SNB125B and the reference strain were further tested on Jalele for their effect on nodulation, growth and yield under field conditions at two sites [DDARC (Dembi Station of Debrezeit Agricultural Research Center) and Bako Agricultural Research Center (BARC)] inoculated singly or dually with a soybean isolate PGP *Achromobacter* species. The indigenous rhizobia well nodulated all the three cultivars of soybean with higher symbiotic effectiveness. Inoculation of indigenous soybean rhizobia improved shoot growth and dry matter, nodule number and dry matter, number of pods and number of seeds per pod at DDARC field site as well as shoot total nitrogen, number of seeds

per plant and grain yield over the un-inoculated controls at both field sites. Maximum nodule number (168 plant^{-1}) and dry matter (1.96 g plant^{-1}), shoot dry matter (25 g plant^{-1}) and total nitrogen (4%), number of pods (114 plant^{-1}) and seeds (214 plant^{-1}), and grain yield (4.01 tons ha^{-1}) were recorded at DDARC field site due to co-inoculation of rhizobial isolates and the PGP bacterium. A highly significant ($P \leq 0.05$) effect of the rhizobial isolates on most growth, nodulation and yield parameters recorded. The indigenous soybean rhizobia performed much better than the exotic strain under field and greenhouse conditions, each leading to yield of over 3 tons ha^{-1}. So, they can be used as inoculant together with the PGP bacterium to boost soybean yield in the country after some further evaluation under varied field conditions.

Key words: Soybean, rhizobia, inoclation, nodulation, growth, yield

6. 1. Introduction

Soybean is a multipurpose leguminous crop that serves as source food and feed, and raw material various industrial products. It was introduced to Ethiopia in 1950s and has been widely cultivated in different agroecologies from sea level up to 2,200 meters above sea level (mas) (Fekadu Gurmu, 2007). Attempts had been made to release more than twenty soybean varieties with improved agronomic characters to improve yield, and to increase the area of cultivation and volume of soybean production by 10 fold and 20 fold, respectively from 2002 to 2012 with productivity level of 1.06 tons ha^{-1} (Mekonnen Hailu and Kaleb Kelemu, 2014). The Central Statistical Agency (CSA, 2014) reported the production of soybean on 30,517.38 hectares of private peasant holdings with average yield of about 2.0 tons ha^{-1} during the main rainy season of 2013/14. The yield was lower than the world average (2.7 tons ha^{-1}) of the 2013/14 (USDA, 2016) and much lower than its potential yield of 6-8 metric tons ha^{-1} (Cooper, 2003) so that the

country imports 15 million Kg of soy bean/product expending 11 million US dollars year^{-1} (Mekonnen Hailu and Kaleb Kelemu, 2014).

Soil nitrogen and phosphorus deficiency is the most important factor that limits productivity of soybean and other crops in Ethiopia (Anteneh Argaw, 2012). According to Keyser and Li (1992), soybean requires approximately 100 kg of N for production of a ton of seeds, and can derive up to 97% of its N-requirement from its symbionts if it is inoculated with symbiotically effective and environmentally resistant rhizobial inoculants with compatible varieties under suitable conditions.

Soybean inoculation with rhizobia (some times with rhizobia and plant growth promoting rhizobacteria) has been practiced for over a century into soils where the crop had been introduced and/or in the presence of insufficient effective indigenous rhizobia (Appune *et al*., 2008). Inoculation of soybean with *Bradyrhizobium* improved its growth (Sharma and Kumawat, 2011) and yield by 60-73% (Rechiatu *et al*., 2015) over un-inoculated control. Co-inoculation of soybean with *Bradyrhizobium japonicum* and PGPR enhanced its nodulation, N-fixation and seed yield under field conditions (Zhang *et al*., 1996; Dashti *et al.*, 1998; Anteneh Argaw, 2012; Aung *et al*., 2013; Kravchenko *et a*l., 2013).

Commercial inoculants often fail to improve growth and yield of pulse crops in its geographic area due to the existence of highly competitive and ineffective indigenous rhizobia in the soil, strain-cultivar incompatibility or adverse environmental conditions. However, co-inoculation of effective rhizobia and plant growth promoting (PGP) microorganisms is known to overcome the negative effect of indigenous rhizobia (Aung *et al*., 2013) and to modulate some stress factors in the soil (Zhang *et al*., 1996).

Attempts to improve the yield of the crop in the Ethiopia include improving some agronomic practices, fertilizer applications and/or microbial inoculations. The effects of variety and planting date (Abe Shergo *et al*., 2010), planting pattern and plant density (Daniel Markos *et al*., 2011) and row spacing (Mohammed Worku and Tessema Astatkie, 2015) were investigated and recommended for some varieties at the study areas. However, many of the hitherto studies focused on inoculation, co-inoculation and fertilizer trials using exotic rhizobia; TAL378 and TAL379 on few varieties rather than well screened indigenous rhizobia for their symbiotic effectiveness, potential adaptations to environmental stresses and compatibility to different soybean cultivars in the country (Anteneh Argaw, 2012; Tamiru Solomon *et al*., 2012; Workneh Bekere and Asfaw Hailemariam, 2012; Workneh Bekere *et al*. 2013; Tekle Yoseph and Walelgn Worku, 2014). The inconsistent and often unsatisfactory results of these trials coupled with the dearth of information on the diversity and symbiotic effectiveness of indigenous soybean rhizobia necessitated screening of indigenous rhizobia potentially adapted to environmental stresses for their compatibility and symbiotic performance with different soybean cultivars in the country. Thus, the current study was initiated to test the inoculation effects of selected indigenous soybean rhizobia on three soybean varieties (Ethio-Yugoslavia, Cheri and Jalele) under greenhouse conditions and a co-inoculation trial of a few elite isolates together with a soybean plant growth promoting *Achromobacter* species on the growth and yield of the most receptive soybean cultivar (Jalele) under field conditions.

6.2. Materials and Methods

6.2.1. Green house experiments

6.2.1.1 Microbial inoculants

Rhizobial isolates (SNB57B, SNB120A, SNB120C, SNB125A, SNB125B and SNB140) were selected for greenhouse screening out of which SNB120A, SNB120C, SNB125A, and SNB125B; and one *Achromobacte*r sp; soybean rhizobacterium with multiple PGP traits was selected for co-inoculation trial under field conditions. A *Bradyrhizobium japonicum* strain SBTAL379 was included as a reference soybean rhizobium in both greenhouse and field experiments.

6.2.1.2. Soybean cultivars

Three soybean cultivars: Ethio-Yugoslavia (late maturing; 145-154 days), Cheri (a medium maturing; 135 days) and Jalele (early maturing; 120 days) (all obtained from BARC) were selected for the greenhouse experiment. These cultivars are good yielding and resistant to the common diseases according to the BARC.

6.2.1.3 Inoculation, Experimental Design and Conditions

Soybean seeds were surface sterilized with 2% sodium hypochlorite according to Lwin *et al*. (2012) and germinated on 1.5% (w/v) water-agar plates. Five pre-germinated seeds from each cultivar were transplanted into 3 kg capacity pots filled with acid washed sand and thinned down to three five days after planting. One mL of active rhizobial yeast extract mannitol (YEM) culture (about 10^8 cells, $0.91OD^{540}$) of each isolate (SNB57B, SNB120, SNB120C, SNB125A, snb125B, SNB140 and the reference SBTAL379) was inoculated into the base of each seedling.

Plants (pots) fertilized with nitrogen (0.05% KNO_3) every week, and other pots without inoculation and N-fertilization were included as positive and negative controls, respectively. All experiments were done in triplicate and the pots were arranged in a randomized complete block design in a greenhouse with 12 h photoperiod, day temperature (25±2^oC) and night temperature (17±3^oC). All the pots were watered as required and provided weekly with quarter strength N-free nutrient solution for grain legumes (Broughton and Dilworth, 1970; composition indicated in Appendix 7). Sixty days after planting, plants were uprooted and roots were washed with a gentle tap water to collect and count nodules. Shoots and nodules were dried at 70^0C for 48 hours for dry matter determination (Somasegaran and Hobenm, 1994). Dried shoot samples were finely ground to determine nitrogen content using Kjeldahl method (Sahlemedhin Sertsu and Taye Bekele, 2000). The symbiotic effectiveness (SE) of the rhizobial isolates was calculated according to Purchino *et al.* (2000) as

$$SE = \left[\frac{\text{Shoot dry weight of plants inoculated with a rhizobial isolate}}{\text{[Shoot dry weight of N-fertilized plants]}} \right] 100$$

6.2.2. Field experiments

6.2.2.1 Field experimental sites and their descriptions

Field experiments were conducted at two different sites: Bako Agricultural Research Center (BARC) and Dembi Station of Debrezeit Agricultural Research Center (DDARC) during 2013/2014 cropping seasons (June-October). BARC is located at 09º06' N, 37º09' E and at 1650 meters above sea level 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

19.6ºC (August) to 21.1ºC (October) whereas the relative humidity (RH %) ranged from 49 (June) to 54 (for both August and September). The soil temperature of the area (determined at 5, 10 and 20 cm depth) was varied from 23ºC to 25.1ºC.

DDARC is located at 8º44' N, 38º55' E and at 1930 meters above sea level in East Shoa Zone of Oromia Regional State. 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 was varied from 15.9ºC (October) to 20ºC (June), and RH% was also varied from 54 (October) to 80 (June). The soil temperature of the area (determined at 5, 10 and 20 cm depth) was between 22 and 30ºC. All climatic data of each field site were obtained from their respective research centers records.

6.2.2.2 Soybean Variety Selection and its brief description

Jalele (Pedigree, AGS-217), an early maturing soybean cultivar (54 days to flowering and 120 days to maturity), was selected for both experimental field sites principally due to the inconsistency in the duration of rainy season, and its well nodulation and growth under greenhouse conditions. It is adapted to zones with 900-1300 mm rain fall and 1300 to 1850 meters above sea level. The cultivar is reported by BARC to yield 22 Qt/ha at research station (and 15 Qt/ha on farm) wth 170 g of 1000 seed weight.

6.2.2.3 Bacterial selection

Two fast growing (SBTAL 120C and SNB 125B) and two slow growing (SNB120A and SNB125A) indigenous soybean rhizobia as well as a soybean rhizosphere *Achromobacterium* (SR20A) were selected for the field experiments. The soybean rhizobia were selected based on their high symbiotic effectiveness on sand culture (greenhouse), ability to catabolize different

carbon and nitrogen substrates, and resistance to various physico-chemical stresses. The soybean rhizosphere bacterial isolate *Achromobacter* sp. (SR20A) was selected due to its multiple PGP properties, its tolerance to various physico-chemical stresses and its compatibility to the selected rhizobia being tested by disc plate diffusion method according to Navprabhjot and Poonam (2013). A *Bradyrhizobium japonicum* SBTAL379, which had been used in some field experiments in the country, was included as reference strain.

6.2.2.4 Inoculum preparation and seed inoculation

Each rhizobial isolate was grown to late exponential phase (72 hrs) and adjusted to 10^9 cells mL^{-1} yeast extract mannitol broth. The inoculants were mixed with peat to coat seeds using 20% sucrose as sticker in the two step method according to Singleton *et al*. (1990). One litre culture of each bacterial inoculant was thoroughly mixed with a kg of sterile fine peat powder and cured at 28 ± 0.2ºC for one week. For combined inoculation, a rhizobial culture and the rhizobacterium (SR20A; adjusted to 10^9 cells mL^{-1} of nutrient broth) culture were mixed in 1:1 (v/v) ratio according to Yadav *et al*. (2011) prior to mixing with peat. Twenty mL of the sucrose solution was added to a plastic bag containing 1 kg of surface sterilized soybean seeds. The plastic bag was twist shut to trap as much air as possible and swirled gently until all the seeds were evenly wet. The plastic bag was opened to add 50 g of inoculant and inflated again and swirled gently until all the seeds were coated evenly with about 2.0×10^6 rhizobia per seed, which is higher than 1×10^5 rhizobia per a seed of soybean enough for good nodulation and nitrogen fixation (Singleton *et al*., 1990). Coated seeds were spread on a clean plastic sheet under tree shade and allowed to air dry for a few minutes.

6.2.2.5 Soil properties and MPN of indigenous soybean rhizobia of the field sites

The texture of soil of DDARC and BARC was loam and sand clay loam, respectively (Table 18). The pH of BARC soil (5.02) is strongly acidic where as that of DDARC soil (6.15) is slightly acidic according to Bruce and Rayment (1982). The organic carbon of DDARC soil was 3.63% and that of BARC soil was 2.17%, both falling in very low range (FAO, 1990). However, the organic matter content of DDARC soil (6.26%) and BARC (4.21%) could be rated as very high and high, respectively as described by Charman and Roper (2007). The percentage of total nitrogen of soil of DDARC (0.313%) and BARC (0.18%) can also be categorized as high and moderate, respectively (Bruce and Rayment, 1982). The available phosphorus (AP) of DDARC soil was 31.85 parts per mellion (ppm), whereas that of BARC soil was 12.85 ppm falling in the very high and moderate range, respectively according to Holford and Cullis (1985). The population counts of indigenous soybean rhizobia in the soil of the field site of BARC and DDARC were $6.3x10^3$ and $2.2X10^1$ g^{-1} of soil, respectivly (Table 18). The MPN of indigenous soybean rhizobia in the soil of BARC is higher than soil rhizobial population (10^3 g^{-1} of soil) normally considered to be high and enough for optimum nodulation of many tropical legumes (Danso and Owiredu, 1988). The physical properties of soil were determined at Nekemte sol laboratory and testing center where as the MPN of indigenous rhizobia was determined at Green house of Applied Microbilogy, Addis Ababa Uinversity. Descriptions of soil pH and compositions are indicated in Appendix 9 for reference.

Table 18. 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	$6.3 X10^3$
DDARC	6.15	3.63	0.313	31.85	6.26	27	47	26	Loam soil	$2.2 X10^1$

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.

6.2.2.6 Land preparation and sowing

Both field sites were ploughed and prepared according to the standard agricultural practices. At BARC site, a total area of 391.2 m^2 (12 m x 32.6 m) land was divided into three blocks separated by a canal with a width of 1.5 m. Each block was sub-divided into eight plots (each 3 by 3.2 m separated by 1 m canal) corresponding to eight treatments: three separate rhizobia inoculations (SNB120C, SNB120B and SBTAL379), these three rhizobia each co-inoculated with SR20A (SNB120C + SR20A, SNB120B + SR20A and SBTAL379 + SR20A), a urea fertilizer applied control and a control without any input. Again at DDARC site, a total area of 592.8 m^2 (49.4 m x12 m) was divided into three blocks each separated by 1.5 m wide canal. Each block was sub-divided into 12 plots (3 by 3.2 m each) separated by 1 m canal corresponding to 12 treatments: five separate rhizobia inoculations (SNB120C, SNB120B, SNB120A, SNB125A and SBTAL379), these five rhizobia each co-inoculated with SR20A (SNB120C + SR20A, SNB120B + SR20A, SNB120A + SR20A, SNB125A + SR20A and SBTAL379 + SR20A), a urea applied control and a control without any input. 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 Anteneh Argaw (2012). Treatments were designed in a randomized complete block design with three replications. Eight rows were made on each plot and seeds (rhizobia coated and untreated) 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, 2014 at BARC, but on July 17, 2014 at DDARC due to late on set of rainy season. Twenty centimetres of land was left between the external rows and the edge of each plot. SNB120A and SNB125A were excluded from BARC field site due to lack of the required area of land safe from animal pest threat.

6.2.2.7 Sampling plants

Plants were sampled at two different stages; 60 days after planting and at physiological maturity. Sixty days after planting (60 DAP), ten plants were randomly uprooted from the third boarder rows of each plot and washed to retrieve intact nodules from roots. Nodules were collected to count and determine their dry weight. Shoot height and dry matter were recorded. Dried shoot samples were finely ground to determine nitrogen content using Kjeldahl method (Sahlemedhin Sertsu and Taye Bekele, 2000) described as follows. For shoot nitrogen content determination, 0.2 g finely grounded shoot material was thoroughly mixed with K_2SO_4, $CuSO_4$ and selenium powders in 10:1:0.1 ratio in a Macro-Kjeldahl flask (750 mL) followed by the addition of 7 mL of H_2SO_4(98%) and 3 mL of H_2O_2 (30%).. The mixture was consecutively heated at 100 ^{0}C for 30 minutes, at 75^{0}C for 20 minutes and at 50^{0}C for 20 minutes using Turbothermo digester (Gerhardt, German). The mixture was cooled and fitted into Vapodest distiller (Gerhardt, German), programmed to add 75 mL of 40% NaOH, 25 mL of 2% boric acid indicator (containing methyl red and bromocresol green) and 100 mL of distilled water. One hundred fifty milli litres of the distillate was received in 250 mL Erlenmeyer flask fitted into the Vapodest distiller. The amount of ammonia released due to acid digestion was determined by titrating the

distillate with 0.1 N H_2SO_4 until the color changes from green to pink.The percentage of nitrogen content of the samples was calculated after correcting for the blank as:

$$\% N = \frac{[(A-B) \times N \times 0.014 \times mcf]}{[S]} \times 100,$$

where A = mL of H_2SO_4 required for titration of a sample, B = mL of H_2SO_4 required for titration of blank, S = air-dry sample weight in grams, N = normality of H_2SO_4 (0.1N), 0.014 = meq weight of nitrogen in g, and mcf = moisture correction factor.

At physiological maturity (120 DAP), ten other plants were randomly taken from the two central rows of each plot to determine the number of pods per plant, number of seeds per pod and per plant, seed yield and weight of 1000 seeds at 10% seed moisture, measured using Grain Moisture Meter (Draminski®, Poland) . Number of plants per plot was recorded at harvesting time from the central six rows, and the mean was computed to determine the final stand.

6.2.3 Data analyses

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 $p \leq 0.05$ probability level. All quantitative data sets were tested for normality using Kolmogorov Simirnov test and for variance homogeneity by Bartelett's test before being pooled for combined ANOVA (Gomez and Gomez, 1984). Accordingly**,** Log(NN+10) and square root of (NDW+0.5) for both greenhouse and field, Log(SDW+1), LogNPP, LogNSPPL and LogGY were computed for field data transformations where, NN= nodule number, NDW= nodule dry weight, SDW=shoot dry weight, NPP=number of pods per plant, NSPPL= number of seeds per plant and GY=grain yield.

6.3. Results and Discussion

6.3.1 Green house experiment

6.3.1.1 Growth and nodulation of the soybean cultivars

The data showed soybean rhizobial inoculants induced nodules on the three cultivars ranging from 34 (SNB57B) to 173 (SNB125A) on cultivar Cheri with nodule dry weight (NDW) of 60 (SNB57B on Ethio-Yugslovia) to 205 mg/plant (SMB125A on Cheri (Table 19). The overall pattern of nodulation of the inoculants showed average number of nodules plant^{-1} of 104, 90 and 78 on Cheri, Eth-Yougoslavia, and Jalele varieties, respectively (Table 19). Although Ethio-Yugoslavia variety was as good as the the other varieties in nodulation, (90 nodules plant^{-1}), its average NDW (84 mg plant^{-1}) was much lower than NDW 153, and 125 mg plant^{-1} recorded from Cheri and Jalele variety, respectively. Among the inoculants, SNB125A induced the highest NN and NDW on all varieties except the Jalele.

The isolates also showed significant difference in shoot dry weight (0.8-2.27g plant^{-1}) with high symbiotic effectiveness (80-100%), except SNB57B on Cheri (SE 63%)) in relation to that of nitrogen fertilized control according to Purchino *et al*. (2000). Although almost all the isolates were highly effective, SNB120C resulted in almost 2 times in SDW on Cheri and Jalele varieties compared to SNB57B inoculations indicating the existence of variation in the effectiveness of the isolates (Table 19). The mean SDW (g plant^{-1}) obtained from inoculation of the isolates was 1.68 (Cheri), 1.70 (Jalele), and 1.30 (Eth-Yugoslavia).

Shoot length (SL) of the inoculated plants varied from 15.66 to 27 cm plant^{-1} with average values of 22 cm (Jalele), 20cm (Ethio-Yugoslavia) and 19cm (Cheri). Total Nitrogen (TN) also varied from 2.67% to 3.37% without showing significant ($P \leq 0.05$) difference amongst the local

inoculants and varieties (Table 19). Apperance of plants resulting from various treatments in the greenhouse experiments is shown in Appendix 10.

In almost all the cases, the selected indigenous rhizobial inoculants performed well over *Bradyrhizobium japonicum* refence SBTAL379 and N-deprived plants in both nodulation and growth parameters with significant (P≤0.05) differences tested under greenhouse conditions.

Table 19. Inoculation trial of soybean rhizobia on three soybean cultivars under greenhouse conditions.

Cultivar	Treatment	NN	NDW(mg)	SL(cm)	%TN	SDW	SE(%)
Ethio-Yugslovia	SNB57B	1.81(54)^{d}	1.84(60)^{ab}	19.33^{ab}	2.67^{a}	1.10^{ab}	80.5^{ab}
	SNB120A	1.98(85)^{bc}	1.89(69)^{a}	17.33^{b}	3.17^{a}	1.10^{ab}	80.48^{ab}
	SNB120C	2.05(103)^{ab}	1.76(61)^{ab}	19.50^{ab}	3.33^{a}	1.43^{a}	104.64^{a}
	SMB125A	3.09(118) ^{a}	2.08(117)^{a}	21.67^{a}	3.30^{a}	1.50^{a}	109.76^{a}
	SNB125B	1.89(70)^{cd}	1.97(87)^{a}	21.33^{a}	3.20^{a}	1.37^{a}	100^{a}
	SNB140	2.07(108)^{ab}	2.03(113)^{a}	22.0^{a}	2.93^{a}	1.30^{a}	95.12^{a}
	Average	89.67	84.39	20.20	3.1	1.30	95.0
	SBTAL379	1.09(2.3)^{e}	1.55(28)^{b}	17.33^{b}	1.60^{b}	0.53^{c}	39.03^{c}
	N+	1.00(0)^{e}	1.00(0)^{c}	21.33^{a}	2.87^{a}	1.37^{a}	100.0^{a}
	N-	1.00(0)^{e}	1.00(0)^{c}	19.67^{ab}	1.40^{b}	0.57^{b}	41.46^{bc}
Cheri	SNB57B	1.63(34)c	1.94(79)^{d}	15.67^{d}	2.83^{a}	0.80^{bc}	63.16^{bc}
	SNB120A	2.02(96)^{b}	2.26(173)^{ab}	19.67^{abc}	3.17^{a}	1.93^{a}	152.6^{a}
	SNB120C	1.94(80)^{b}	2.08(110)^{c}	17.0^{cd}	3.14^{a}	1.77^{a}	139.5^{a}
	SMB125A	2.26(173)a	2.33(205)^{a}	20.67^{ab}	3.37^{a}	2.10^{a}	163.2^{a}
	SNB125B	2.01(94)^{b}	2.32(201)^{a}	21.83^{a}	3.27^{a}	2.0^{a}	160.52^{a}
	SNB140	2.19(150)^{a}	2.19(150)^{bc}	17.67^{bcd}	3.10^{a}	1.50^{ab}	118.4^{ab}
	Average	104.44	152.78	18.75	3.19	1.68	132.89
	SBTAL379	1.10(2.3)^{d}	1.75(46)^{e}	19.67^{abc}	1.67^{b}	0.57^{c}	44.90^{c}
	N+	1.0(0)^{d}	1.0(0)^{e}	16.67^{cd}	2.93^{a}	1.27^{abc}	100.0^{abc}
	N-	1.0(0)^{d}	1.0(0)^{e}	12.33^{e}	1.40^{b}	0.73^{bc}	57.89^{bc}
Jalele	SNB57B	1.95(80)^{a}	2.09(150)^{a}	22.0^{bc}	2.77^{a}	1.11^{cd}	84.14^{cd}
	SNB120A	1.87(66)^{a}	2.10(116)^{a}	18.0^{cd}	2.77^{a}	1.97^{ab}	147.5^{ab}
	SNB120C	1.98(89)^{a}	2.19(146)^{a}	27.0^{a}	3.30^{a}	2.27^{a}	170.0^{a}
	SMB125A	1.86(64)^{a}	2.05(103)^{a}	15.67^{d}	3.33^{a}	1.6b^{c}	120.0^{bc}
	SNB125B	2.1(106)^{a}	2.14(130)^{a}	25.3^{ab}	3.33^{a}	2.14^{ab}	160.0^{ab}
	SNB140	1.82(66)^{a}	2.02(104)^{a}	21.3^{bc}	3.0^{a}	1.10^{cd}	82.5^{cd}
	Average	78.22	124.78	21.56	3.17	1.70	127.40
	SBTAL379	1.08(2)^{b}	1.73(44)^{b}	19.67^{cd}	1.63^{b}	1.13^{cd}	85.0^{cd}
	N+	1.00(0)^{b}	1.00(0)^{c}	20.67^{c}	2.87^{a}	1.33^{cd}	100.0^{cd}
	N-	1.00(0)^{b}	1.00(0)^{c}	18.0^{cd}	1.43^{b}	0.93^{d}	70.0^{d}

NN= nodule number, NDW= nodule dry weight, SL= shoot length, SDW= shoot dry weight and SE=

symbiotic effectiveness. *Numbers in the parenthesis indicate values before data transformation and values raised to different letters are significantly different (at p≤0.05) for each cultivar (Comparisons were made within columns among inoculants for each cultivar).*

6.3.2 Field experiments

6.3.2.1 Effects of different treatments on nodulation and growth of soybean

The different treatments showed significant (P≤0.05) differences in the number of nodules at each field site, and between the two field sites (Table 20). Appearance and nodules of some of the treatments are indicated in Fig. 12. The inoculated plants showed significant (P≤0.05) variations in the number of nodules (plant $^{-1}$) between the two sites; BARC (32-58) and DDARC (10-168) irrespective of single or co-inoculation treatments. The number of nodules at BARC was relatively consistent compared to a very wider range recorded from plants grown at DDARC site. The exotic reference *Bradyrhizobiaum* SBTAL379 induced fewer nodules (10 plant^{-1}) at DDARC field site under low native rhizobial density, contrary to numerous nodules (168 plant^{-1}) it induced on the same soybean cultivar on a soil harboring no native soybean rhizobia (Tamiru Solomon *et al.*, 2012). The relatively poor capacity of SBTAL379 to nodulate different soybean cultivars relative to a "local" soybean rhizobial isolate was also described by Anteneh Argaw (2014). This may be attributed to change in the nodulation capacity of the strain through time. At both field sites, nitrogen fertilized plants produced the least number of nodules which could be due to the negative effect of soil nitrogen on nodulation as previously explained (Kinkema *et al*., 2006). The data also showed significant difference in nodule dry weight between BARC and DDARC corresponding to their pattern of nodule number; higher for DDARC (Table 22).

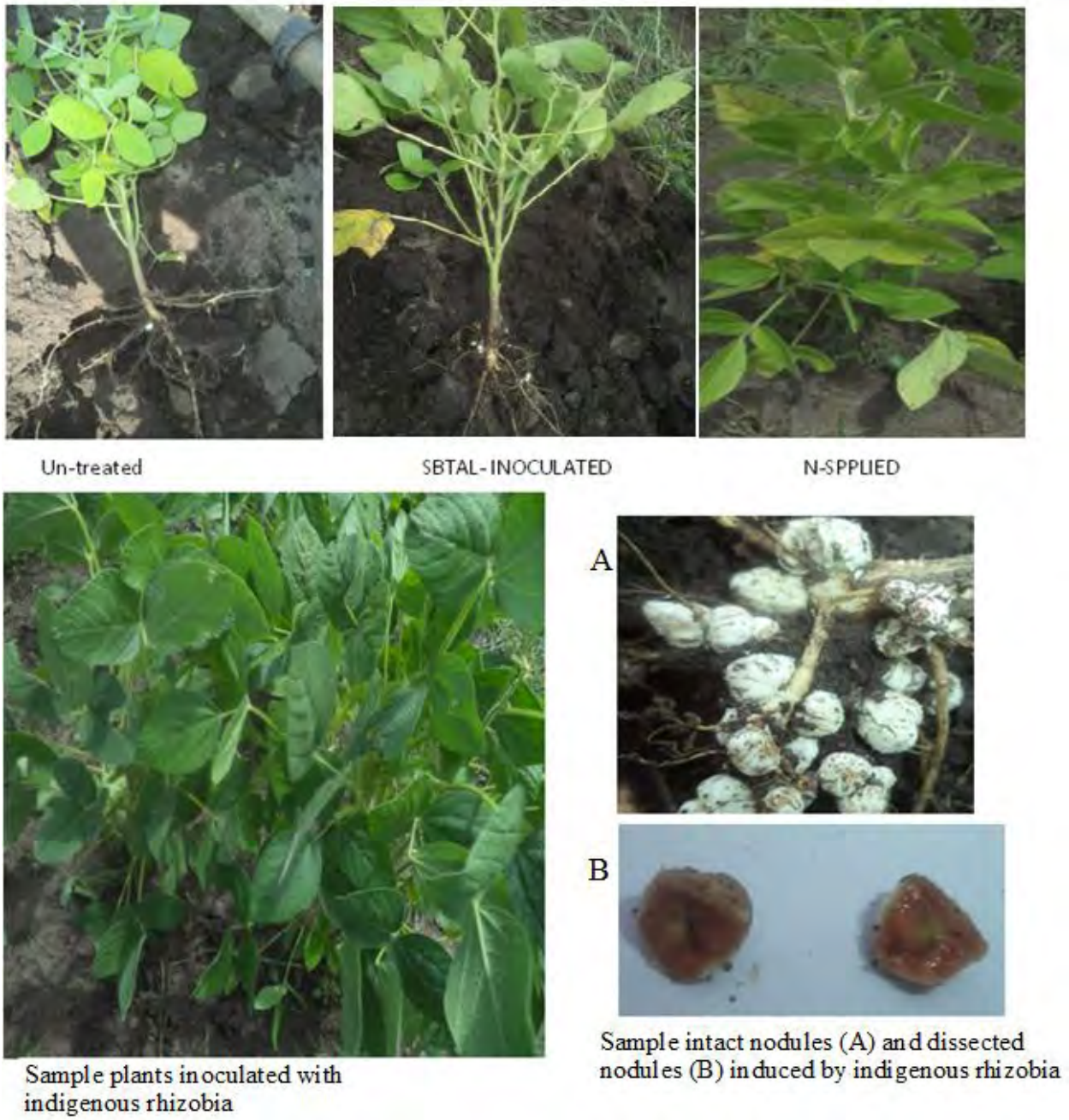

Fig. 13. Appearance of plants and nodules with different treatments in the field experiment

Table 20. Effects of different treatments (8 at BARC and 12 at DDARC) on nodulation and growth of soybean at the field sites

Treatment	NN	NDW	SH	TN%	SDW
BARC field site					
SNB120C	1.74(46.11)^{bc}	0.97(0.45)^{a}	34.13^{a}	3.57^{a}	1.19(15.6)^{abc}
SNB125B	1.75(46.22)^{bc}	1.06 0.63)^{a}	38.56^{a}	3.48^{a}	1.38(24.2)^{a}
SBTAL379	1.71(42.78)^{bc}	1.03 (0.57)^{a}	36.00^{a}	2.93^{b}	1.20(16.0)^{abc}
N+	1.56(28.44)^{c}	0.91(0.33)^{a}	38.33^{a}	3.10^{b}	1.34(22.1)^{ab}
N-	1.79(55.45)^{a}	1.04 (0.60)^{a}	38.56^{a}	2.40^{b}	1.16(14.7)^{bc}
SNB120C*SR20A	1.80(58.00)^{a}	1.04 (0.58)^{a}	35.78^{a}	3.88^{a}	1.13(13.5)^{bc}
SNB125B*SR20A	1.61(32.00)^{c}	0.98 (0.48)^{a}	34.44^{a}	3.51^{a}	1.11(14.5)^{bc}
SBTAL379*SR20A	1.70(43.0)^{bc}	1.00 (0.51)^{a}	34.33^{a}	3.00^{b}	1.07(12.0)^{c}
DDARC field site					
SNB120C	1.90(69.67)^{bc}	1.26(1.08)^{ac}	40.33^{a}	3.63^{ab}	1.34(22.1)^{abc}
SNB125B	2.05(116.67)^{ab}	1.31(1.27)^{ac}	36.33^{abc}	3.48^{abc}	1.18(15.)^{ab}
SBTAL379	1.27(10.0)^{d}	0.81(0.16)^{de}	35.33^{abc}	2.90^{cd}	0.89(8.4)^{c}
N+	1.14(4.56)^{d}	0.72(0.02)d^{e}	25.67^{c}	3.13^{bcd}	0.87(16.8)^{bc}
N-	1.20(10.37)^{d}	0.76(0.08)^{de}	27.00^{bc}	2.20^{d}	0.72(7.4)^{c}
SNB120C*SR20A	1.63(33.0)^{c}	1.17(0.88)^{ac}	34.67^{abc}	3.88^{a}	1.14(15.1)^{ab}
SNB125B*SR20A	2.15(131.67)^{ab}	1.47(1.67)^{ac}	39.33ab	3.58^{ab}	1.39(25.0)^{a}
SBTAL379*SR20A	1.27(10.35)^{d}	0.83(0.91)^{de}	29.67^{abc}	2.97^{dc}	0.79(7.4)^{c}
SNB120A	2.20(153.67)^{ab}	1.50(1.81)^{a}	38.33^{ab}	3.8^{a}	1.31(24)^{a}
SNB125A	2.13(139.67)^{ab}	1.33(1.37)^{ac}	32.00^{abc}	3.64^{ab}	1.18(16.5)^{ab}
SNB120A*SR20A	2.21(135.33)^{ab}	1.56(1.92)^{a}	46.67^{a}	4.00^{a}	1.39(25)^{a}
SNB125A*SR20A	2.25(168.00)^{a}	1.56(1.96)^{a}	38.33^{ab}	3.86^{a}	1.39(25.0)^{a}

NN= nodule number, NDW= nodule dry weight, SH= shoot height, TN%= shoot total nitrogen percent, SDW= shhot dry weight. Values raised to different letters in a column are significantly different at $p \leq 0.05$ and values in parentheses indicate the data before transformation

The soybean plants were also differed in shoot length (25.67-46.67cm) without showing significant differences among treatments at BARC field site and with significance difference among few treatments at DDARC site. Though the difference was insignificant, the shoot length was lower at DDARC than BARCsite (Table 22). The data also showed significant ($p \leq 0.05$) differences at times with a discrepancy in shoot dry matter (7.4g-25g plant^{-1}) among the different treatments at both field sites. Although the inoculants SNB120C and SNB125B did not show

significantly different impact on nodulation parameters of the soybean plant, the latter induced 55% SDW comparable the former at the BARC site.

At DDARC site, single inoculation with 120C increased 47% more SDW of the soybean than the 125B inoculation contrary to the result from BARC site. Similarly, inoculation by SNB120A increased SDW by 45% over SNB125A inoculation. However, co-inoculation of SNB125A and SNB125B with the SR 20A showed much improvement in plant SDW where that of co-inculation of SR20A and SNB120A showed almost no change indicating variations in co-inoculation effects among different rhizobia. In general, the data showed that all co-inoculated plants showed a reduction in SDW, at the BARC site compared to the single inoculated cohorts. On the contrary, most of the co-inoculated plants showed significant increase in SDW upon co-inoculation (except 120C+SR20A) at the DDARC sites.

The response of soybean plants to the different treatments in terms of shoot total nitrogen content (2.20-4.00%) is shown in Table 20. Accordingly, plants inoculated with rhizobial isolates (singly or dually with SR20A) increased shoot total nitrogen (mean value of 3.67%) showing significant difference compared to the SBTAL 379 inoculation (mean value of 2.95%), un-ionoculated N-fertilized and non-fertiliized control plants (mean value of 2.7) with few exception at both BARC and DDARC field sites (data not shown). The nitrogen fertilized plants contained less nitrogen content (3.1%) than most of rhizobial inoculations at both field sites. Similarly, Anteneh Argaw (2012) reported the accumulation of significantly higher shoot nitrogen (4.05%) by soybean plants inoculated with rhizobia and phosphate solubilizing bacteria compared to nitrogen supplied plants (3.14%). This could be related to the non-responsive character of soybean to the application of fertilizer nitrogen (Scott and Aldrich, 1983) and dependency of the crop on its symbionts for a larger part of nitrogen requirement for growth and dry matter

production as previously described (Appunu and Dhar, 2006; Appunu *et al*., 2008; Girgis *et al*., 2007).

6.3.2.2 Effects of different treatments on soybean yield and yield related parameters

Number of pods per plant (NPP) varied from 32 to 44 with mean value of 38 at BARC site, with out showing significant ($p \leq 0.05$) difference amongst the treatments. However, soybean plants displayed higher NPP from 35 to 114 with mean value of 73 at DDARC site showing significant ($p \leq 0.05$) differnce among some of the treatments (Table 21). It is interesting to note that co-inoculation with SNB120A + SR20A, single inoculation with SNB 120C, and co-inoculation with SNB125A + SR20 increased NPP by 107, 85, and 69%, respectively over none nitrogen fertilized- uninoculated control at DDARC site. Under low level of indigenous soil soybean rhizobia at DDARC site, most inoculations of the rhizobial isolates with or without the PGP (SR20A) produced higher NPP (85-114) than the NPP (77) recorded for the same soybean cultivar due to inoculation with *Bradyrhizobium japonicu*m strains (SBTAL379) under field conditions lacking native soybean rhizobia (Tamiru Solomon *et al*., 2012). However, inoculation of SBTAL 379 singly or with the PGPR (SR20A) resulted in reduced NPP (below 40) in the current study.

The other yield related parameter; number of seeds per pod (NSPPD) was in the range of 1.91 and 2.28 with mean value of 2.10 (BARC field site) without showing significant ($p \leq 0.05$) differnce among the inoculation treatments, except SNB125B inoculated and N-fertilized plants (Table 21). The data showed a lower pattern of NSPPD; ranging from 1.25-2.08 with mean value of 1.74 in the case of DDARC site. The lowest NSPPD were recorded from the nitrogen fertilized plants at both field sites. Tamiru Solomon *et al*. (2012) and Tolera Abera *et al*. (2015) reported a 2.21 and 2.0 number of seeds per pod, respectively for the currently used soybean

cultivar inoculations which is similar to this report. Tairo and Ndakidemi (2013) reported mean NSPPD of 2.1 to 2.3 from Tanzania whereas Oya *et al*. (2004) reported mean NSPPD ranging from 1.5 to 2.38 from Brazil though the crop varieties are different from the present one.

The number of seeds per plant (NSPPL) varied from 67-102 (with mean of 80) at BARC field site and from 60-214 at DDARC site with mean value of 128, which is significantly ($p \leq 0.05$) higher than that of BARC site (Table 22). In general, there was no significant ($p \leq 0.05$) difference between single inoculation and co-inoculation of the indigenous and the PGP SR20A treatments in NSPPL although these inoculations showed significant ($p \leq 0.05$) difference from the the non-fertilized, the N-fertilized and refernce SBTAL inoculum treatments.

In general, the NSPPD of plants at BARC field site was significantly higher than DDARC field site which could be attributed to the late onset of rainy season at DDARC that forced the seeds to be sown in the third week of July almost a month after the BARC site. Moreover there was also early cessation of rainfall at DDARC field site (materials and method section) which might influence seed development. However, the number of seeds per plant (NSPPL) was significantly higher for DDARC field site than BARC field site (Table 22) corresponding to the higher number of pods per plant (NPP) recorded at the site.

Mean thousands of seed weight (TSW) was 179 g (BARC field site) and 182 (DDARC field site) (Table 22), whereas Tamiru Solomon *et al*. (2012) and Tolera Abera *et al*. (2015) reported 213 and 330 g, respectively as TSW for the crop compared to 170 g recorded by BARC upon releasing the cultivar, Jalele. No significant differences in TSW were observed among different treatments at each field site, and between means of the two field sites.

Maximum seed yield (GY) (3.81 tons ha^{-1}) was recorded for SNB120C single inoculation at BARC field site and for SNB120A co-inoculated with SR20A (4.01 tons ha^{-1}) at DDARC field site (Table 21). Inoculation of the indigenous soybean isolates singly or dually with the PGP SR20 resulted in 12-35% (BARC) and 54-90% (DDARC) yield increase over the N-fertilized treatment, whereas they resulted in 17-42% (BARC) and 136-193 (DDARC) yield increase over the none N-fertilized controls. The mean grain yield of the two field sites (3.1 tons ha^{-1}) is higher than 2.56 tons ha^{-1} (Tamiru Solomon *et al*., 2012) and 2.52 tons ha^{-1} (Tolera Abera *et al*., 2015) reported for field inoculation of the cultivar. Fitsum Merkeb *et al*. (2016) inoculated four commercial soybean rhizobia on soybean cultivar named Belessa-95 at Pawe district (North West part of Ethiopia) and reported even a lower maximum yield (2.17 tons ha^{-1}) than this study. However, the GY recorded in the present study was comparable to the soybean yield (tons ha^{-1}) in major producing countries like Argentina (3.17) and USA (3.2); and was better than the world average (2.7) reported for 2015/2016 (USDA, 2016).

Co-inoculation of rhizobial isolates with the plant growth promoting rhizobacterium, SR20A (*Achromobacter* species) showed improvements in nodulation, growth and yield of soybean showing differences with field sites in some cases. For instance, SNB120C co-inoculated with SR20A enhanced most of the tested parameters than its single inoculation at BARC field site where as SNB125B co-inoculated with the same PGPR enhanced all the nodulation, growth and yield parameters over its single inoculation at DDARC field site. Co-inoculation improved shoot total nitrogen for all inoculated strains at both field sites, the number of nodules except for SNB125B (BARC field site) and SNB120C (DDARC field site) and grain yield except for SNB120C at both field sites. Improvements of soybean shoot height, number of pods and seeds

per plant, plant total nitrogen (Anteneh Argaw, 2012), grain yield (Anteneh Argaw, 2012; Hungria *et al*., 2015) and nodulation (Masciarelli *et al*., 2014) due to co-inoculation of bradyrhizobial strains and PGPR were also reported.

Table 21. Effects of different treatments on soybean yield and yield related parameters

Treatment	NPP	NSPPD	NSPPL	TSW	GY
BARC field Site					
SNB120C	1.56(37)^{a}	2.07^{ab}	1.87(77)^{a}	212.2^{a}	0.651(3.81)^{a}
SNB125B	1.61(44)^{a}	2.28^{a}	1.96(102)^{a}	175.08^{a}	0.60(3.16)^{a}
SBTAL379	1.58(39)^{a}	2.17^{ab}	1.92(86)^{a}	165.74^{a}	0.50(2.41)^{a}
N+	1.58(39)^{a}	1.91^{b}	1.86(74)^{a}	180.01^{a}	0.58(2.82)^{a}
N-	1.50(32)^{a}	2.16^{ab}	1.83(70)^{a}	169.29^{a}	0.57(2.69)^{a}
SNB120C*SR20A	1.62(42)^{a}	2.08^{ab}	1.94(88)^{a}	171.23^{a}	0.65(3.51)^{a}
SNB125B*SR20A	1.58(40)^{a}	2.08^{ab}	1.87(76)^{a}	191.30^{a}	0.66(3.70)^{a}
SBTAL379*SR20A	1.49(32)^{a}	2.09^{ab}	1.81(67)^{a}	168.00^{a}	0.52(2.43)^{a}
DDARC field site					
SNB120C	2.00(102.0)^{a}	1.64^{ad}	2.20(159.33)^{ab}	173.85^{a}	0.69(3.97)^{a}
SNB125B	1.83(72)^{ac}	1.87^{ac}	2.10(133)^{abc}	175.35^{a}	0.62(3.24)^{ab}
SBTAL379	1.55(35)^{c}	1.54^{bd}	1.73(54)^{d}	156.52^{a}	0.35(1.30)^{c}
N+	1.75(64)^{abc}	1.25^{d}	1.83(71)^{cd}	173.0^{a}	0.49(2.11)^{bc}
N-	1.61(55)^{bc}	1.44^{cd}	1.77(81)^{d}	162.0a	0.35(1.37)^{c}
SNB120C*SR20A	1.68(51)^{bc}	1.82^{ac}	1.93(96)^{ac}	183.50^{a}	0.63(3.35)^{ab}
SNB125B*SR20A	1.90(85)^{ab}	2.08^{a}	2.22(176)^{ab}	192.95^{a}	0.70(3.98)^{a}
SBTAL379*SR20A	1.48(39)^{c}	1.55^{bd}	1.67(60)^{c}	163.0^{a}	0.39(1.60)^{c}
SNB120A	1.94(88)^{ab}	2.07^{a}	2.25(177)^{a}	205.9^{a}	0.70(4.0)^{a}
SNB125A	1.82(74)^{ac}	1.94^{ab}	2.11(143)^{ab}c	170.79^{a}	0.70(3.98)^{a}
SNB120A*SR20A	2.05(114)^{a}	1.87^{ac}	2.32(214)^{a}	218.87^{a}	0.70(4.01)^{a}
SNB125A*SR20A	1.93(93)^{ab}	1.86^{ac}	2.20(171)^{ab}	215.85^{a}	0.70(4.0)^{a}

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 and values in parentheses indicate the data before transformation.

Table 22. Mean comparison of nodulation, growth and yield of soybean of the two field sites

Site	Parameters									
	SH	NN	NDW	SDW	TN%	NPP	NSPPD	NSPPL	TSW	GY
BARC	36.3[a]	1.7[b]	1.0[b]	1.2[a]	3.23[a]	1.6[b]	2.1[a]	1.9[b]	179.11[a]	0.6[a]
DDARC	34.9[a]	1.8[a]	1.2[a]	1.1[a]	3.42[a]	1.8[a]	1.7[b]	2.0[a]	182.38[a]	0.6[a]

NN= nodule number, NDW= nodule dry weight, SH= shoot height, TN%= shoot total nitrogen percent, SDW= shhot 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 are significantly ($p \leq 0.05$) different.

6. 3.2.3 Effect of replications (rep), isolates, location and isolate-location interaction on nodulation, growth and grain yield

Replication showed significant effect on shoot height and TN%, on the number of seeds per pod (NSPPD) and thousands of seed weight (TSW) at BARC (Table 23). Isolates showed highly significant ($p \leq 0.05$) effect on nodule number (NN) and shoot TN% at both field sites and on nodule dry matter (NDW), number of seeds per plant (NSPPL) and grain yield (GY) at DDARC field site alone. Isolates also showed significant ($p \leq 0.05$) effect on shoot dry weight (SDW) and number of pods per plant (NPP) at DDARC field site. The significant ($p \leq 0.05$) effects of the isolates on more parameters at DDARC site than BARC site might be related to the lower native soybean rhizobial population of the former. Singleton and Tavares (1986) indicated that statistically significant inoculation responses can be eliminated when there are as few as 20 indigenous rhizobia per g of soil with some effective strains in greenhouse experiments.

The effect of location was significant on SDW and highly significant ($p \leq 0.05$) on the NPP and NSPPD as revealed by combined ANOVA of different parameters of the two field sites (Table 23). Replication (rep) showed insignificant ($p \leq 0.05$) effect on all parameters except shoot height (SH), whereas isolates showed highly significant ($p \leq 0.05$) effect on nodule number (NN) and dry weight (NDW), SDW and TN%, NSPPD, NSPPL and GY. Isolate-location interaction showed significant effect on SH and SDW, but highly significant ($p \leq 0.05$) effect on NN and NDW. Tamiru Solomon *et al.* (2012) reported significant effect of isolates 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 study. However, the authors reported highly significant effect of the rhizobial strains on the number of pods per plant (NPP) and thousands of seed weight (TSW) contrary to the current study.

Table 23. Effects of replication (rep), isolates (iso), location (loc) and isolate-location (iso*loc) interactions on growth, nodulation and yield for inoculations common to both field sites.

Site		NN	NDW	SH	SDW	TN%	NPP	NSPPD	NSPPL	TSW	GY
Effects of replication (rep) and isolates (iso) on growth, nodulation and yield											
BARC	rep	NS	NS	*	NS	*	NS	NS	*	*	NS
	iso	**	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
	iso	**	**	NS	*	**	*	NS	**	NS	**
	cv	15	18	18	21	15	13	17	11	21	19
Combined ANOVA: Effects of location, rep, and isolate-location interaction											
Loc		NS	NS	NS	*	NS	**	**	NS	NS	NS
Rep		NS	NS	*	NS	NS	NS	NS	NS	NS	NS
Iso		**	**	NS	**	**	NS	**	**	NS	**
Iso*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, iso=isolate, loc=location, iso*loc= isolate-location interaction, Cv= coefficient of variance, BARC= Bako Agricultural Research Center, DDARC= Dembi Station of Debrezeit Agricultural Research Center.

Correlation test results indicated a significant or highly significant positive correlation among most parameters (Table 24). Number of pods per plant (NPP) showed a highly significant positive correlation with nodule number (Person's r=0.59), nodule dry matter (Person's r=0.68),

shoot total nitrogen (Person's r=0.76) and shoot dry matter (Person's r=0.77). There was also a highly significant positive correlation between grain yield (GY) and nodule number (Person's r=0.99), nodule dry matter (Person's r=0.63), number of pods per plant (Person's r=0.64) and number of seeds per pod (Person's r=0.63). Insignificant negative correlations were encountered in few cases.

Table 24. Correlations among different variables

	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,

6.4. Conclusion and recommendations

6.4.1 Conclusion

- The three soybean cultivars (Ethio-Yugoslavia, Cheri and Jalale) vary in their growth and nodulation responses to inoculations with similar rhizobia, Cheri being tended to form more nodules but comparable with Jalele in symbiotic effectiveness.
- The mean nodule number and dry matter, number of pods and seeds per plant were significantly higher at DDARC field site having much lower indigenous soybean rhizobia than BARC field site.

- Rhizobial strains significantly affected more number of growth, nodulation and yield parameters of soybean compared to rhizobia-location interaction, location alone or replications at both field sites.

- The majority of selected indigenous soybean rhizobia were symbiotically highly effective at greenhouse level accompanied by much better yield improvement over the exotic bradyrhizobial strain and un-inoculated controls at both field sites indicating their suitability to be used as inoculants for the production of the crop in the country.

- Co-inoculation of rhizobial strains and a PGPR enhanced nodulation, growth and yield of soybean varying with field sites in some cases.

6.4.2 Recommendations

- Indigenous soybean nodulating bacteria (SNB120A, SNB120C, SNB125A, SNB125B and SNB140) are good inoculants candidates for all the three soybean varieties (Ethio-Yugslovia, Cheri and Jalele) as they are compatible to them revealed by well nodulation with high symbiotic effectiveness (≥80%)

- Under higher indigenous soybean rhizobia like soil of Bako Agricultural Research Center (BARC), single inoculation of SNB120C or dual inoculation of SNB125B and SR20A are better inoculant candidates. However, under low indigenous soybean rhizobia co-inoculation of SR20A and SNB120A or SNB125A should be applied for better grain yield improvement.

- Under more stressful coditions like higher soil temperature and salinity, SNB120C and SNB125B should be applied rather than the relatively sensitive SNB120A and SNB125A

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List of Appendices

Appendix 1. composition of Keyser-defined medium (Lupwayi and Haque, 1994)

A. Micronutrient	gram per litre
$MnCl_2.4H_2O$	504
$ZnSO_4.7H_2O$	0.227
$CuCl_2.2H_2O$	0.034
$NaMoO_4.2H_2O$	0.008
B. Phosphate stock solution	
KH_2PO_4	1.36
C. Vitamin stock solution	
Thiamine HCl	0.4000
d-Pantothenic acid (Ca)	0.4000
Biotin	0.0001

Medium	**per litre**
Glycerol	5 mL
K_2SO_4	0.131 g
Na glutamate	0.220 g
$MgSO_4.7H_2O$	0.074 g
$CaCl_2.2H_2O$	0.007 g
Fe-EDTA	0.035 g
Solution A	0.5 mL
Solution B	1.0 mL
Solution C	1.0 mL
Agar	20 g

Appendix 2. Plant growth promoting (PGP) properties of rhizobial isolates

No	Isolate	$Ca_2(PO4)$ SI*	$AlPO_4$ SI	IAA (µM)	Protease	Cellulase	HCN	PIRG**
1	**SNB13**	1.5	1.6	52.62	+	+	-	-
2	**SNB41**	-	-	104.73	-	-	+	-
3	**SNB43**	-	-	4.68	-	+	-	-
4	**SNB45**	-	-	116	-	-	-	-
5	**SNB46**	-	-	2.5	-	-	-	-
6	**SNB57B**	1.3	1.5	23.47	-	-	-	-
7	**SNB 57C**	-	1.4	26.42	-	-	-	-
8	**SNB70**	-	-	11.22	-	-	-	-
9	**SNB71**	-	-	3.59	-	-	-	-
10	**SNB79**	-	-	40.02	+	+	-	33%
11	**SNB101**	-	1.7	38.84	-	+	-	-
12	**SNB102**	-	-	9.52	+	-	-	17%
13	**SNB114**	-	-	25.85	-	+	-	-
14	**SNB 55**	-	-	38.84	+	-	-	-
15	**SNB120A**	-	-	18.64	-	-	-	-
16	**SNB120B**	-	-	14.14	+	+	-	-
17	**SNB120C**	-	1.4	26.91	+	+	-	-
18	**SNB125A**	-	-	97.51	-	-	-	-
19	**SNB125B**	-	-	23.78	+	+	-	-
20	**SNB140**	-	-	27.44	-	-	-	-
21	**SNB 75**	-	-	60.33	-	-	-	-
22	**SBTAL379**	-	1.4	10.23	-	-	-	-

*****SI=solubilization index**, ****PIRG= percentage of inhibition of radial growth of fungus

Appendix 3. Tricalcium and aluminium phosphate solubilisation indices, IAA production, N-fixation, percent of inhibition of radial growth fungus and Gram reaction of the initial 231 rhizobacteial isolates

Serial No	Isolate	TCP-SI	$AlPO_4$- SI	IAA µg/ml	N_2-fixation	PIRG	Gram rxn	Serial No	Isolate	TCP-SI	$AlPO_4$- SI	IAA µg/ml	N-fixation	PIRG	Gram rxn
1	SR1A	-	-	-	-	-	-ve	41	SR20A	2.22	2.57	47	+	-	-ve
2	SR1B	-	-	28	-	-	+ve	42	SR20B	2.33	2.43	46.8	+	-	-ve
3	SR2A	-	-	30	-	-	+ve	43	SR20C	-	-	30	-	-	-ve
4	SR2B			12	-	-	+ve	44	SR21A	-	-	60.3	-	36	+ve
5	SR3A	1.5	1.22	74	+++	49	-ve	45	SR21B	-	-	-	++	39	+ve
6	SR3B	-	-	36	-	-	-ve	46	SR21C	-	-	25	-	-	+ve
7	SR3C	-	-	22	-	-	-ve	47	SR22	-	-	60.3	-	-	-ve
8	SR4	-	1.36	85.1	-	-	-ve	48	SR23	-	-	43	+	-	-ve
9	SR5A	-	-	34	-	-	-ve	49	SR24A	-	-	16	-	-	+ve
10	SR5B	-	-	-	-	-	-ve	50	SR24B	-	-	33	-	-	-ve
11	SR5C	-	-	31	-	-	-ve	51	SR25A	-	-	56.2	+	-	-ve
12	SR6A	1.25	-	94.4	+++	-	+ve	52	SR25B	-	-	46.5	+	-	-ve
13	SR6B	-	-	14	-	-	+ve	53	SR25C	-	-	19	-	-	+ve
14	SR6C	-	-	21	-	-	-ve	54	SR25D	-	-	-	-	-	-ve
15	SR7A	2.1	2.25	59	-	-	+ve	55	SR25E	-	-	11	-	-	-ve
16	SR7B	-	-	31	-	-	+ve	56	SR26	-	-	55	-	-	-ve
17	SR7C	-	-	16	-	-	-ve	57	SR27A	-	-	33	-	-	+ve
18	SR8	1.78	2.5	123.1	-	-	-ve	58	SR27B	-	-	9	-	-	-ve
19	SR9A	1.14	-	51	-	26	-ve	59	SR27C	-	-	13	-	-	+ve
20	SR9B	-	-	39	++	-	+ve	60	SR28	-	1.42	-	-	54	-ve
21	SR9C	-	-	22	-	-	+ve	61	SR29A	1.43	1.73	50.4	+++	51	-ve
22	SR9D	-	-	-	-	-	-ve	62	SR29B	1.57	1.73	58.5	+++	37	-ve
23	SR10A	2.25	257	41	+	-	-ve	63	SR29C	-	-	25	-	-	+ve
24	SR10B	1.13	1.36	70	-	24	-ve	64	SR29D	-	-	35	-	-	-ve
25	SR10C	-	-	28	-	-	+ve	65	SR30A	-	-	30	-	-	-ve
26	SR11	1.2	1.25	54.4	-	51	-ve	66	SR30B	-	-	-	-	-	-ve
27	SR12A	-	-	32			-ve	67	SR31A	-	-	13	-	-	-ve
28	SR12B	-	-	19	-	-	-ve	68	SR31B	-	-	16	-	-	+ve
29	SR12C	-	-	14	-	-	-ve	69	SR32A	-	1.4	47.9	+	24	-ve
30	SR13A	-	-	-	-	-	+ve	70	SR32B	-	-	47.8	+	58	-ve
31	SR13B	-	-	28	-	-	-ve	71	SR32C	-	-	28	-	-	+ve
32	SR14	1.11	1.08	71.1	++	48	-ve	72	SR33A	-	-	-	-	-	-ve
33	SR15A			14	-	-	+ve	73	SR33B	-	-	30	-	-	+ve
34	SR15B	-	-	28	-	-	+ve	74	SR34A	-	-	24	-	-	-ve
35	SR16A	-	-	-	-	-	-vve	75	SR34B	-	-	-	-	-	+ve
36	SR16B	-	-	36	-	-	+ve	76	SR35A	-	-	30	-	-	-ve
37	SR17A	-	-	32	-	-	-ve	77	SR35B	-	-	16	-	-	-ve
38	SR1AB	-	-	25	-	-	-ve	78	SR36A	-	-	-	-	-	-ve
39	SR18	-	-	17	-	-	-ve	79	SR36B	-	-	26	-	-	-ve
40	SR19	-	-	-	-	-	-ve	80	37A	-	-	34	-	-	-ve

erial No	Isolate	TCP-SI	AlPO4- SI	IAA µg/ml	N2-fixation	PIRG	Gram rxn	Serial No	Isolate	TCP-SI	AlPO4- SI	IAA µg/ml	N-fixation	PIRG	Gram rxn
81	SR37B	-	1.55	-	++	37	-ve	119	SR51C	-	-	10	-	-	+ve
82	SR37C	-	-	34	-	-	+ve	120	SR52A	-	-	12	-	-	-ve
83	SR38A	-	-	-	-	-	-ve	121	SR52B	-	-	13	-	-	+ve
84	SR38B	-	-	36	-	-	-ve	122	SR53A	-	-	-	-	-	-ve
85	SR39	-	-	44	-	-	-ve	123	SR53B	-	-	26	-	-	-ve
86	SR40	2.75	2.22	43	-	-	-ve	124	SR54A	-	-	19	-	-	-ve
87	SR41A	-	-	32			+ve	125	SR54B	-	-	-	-	-	-ve
88	SR41B	1.22	1.44	51.9	++	-	-ve	126	SR55A	-	-	59.2	-	-	+ve
89	SR41C	-	-	15	-	-	+ve	127	SR55B	-	-	71.1	-	-	+ve
90	SR41D	-	-	13	-	-	-ve	128	SR55C	-	-	-	-	-	+ve
91	SR41E	-	-	25	-	-	-ve	129	SR56	1.4	1.21	59.3	-	59	-ve
92	SR42A	-	-	34	-	-	+ve	130	SR57A	-	-	60.6	-	74	-ve
93	SR42B	-	-	-	-	-	-ve	131	SR57B	-	-	135.5	+++	-	+ve
94	SR43A	-	-	66.4	-	41	-ve	132	SR57C	-	-	35			+ve
95	SR43B	1.23	-	36.9	-	82	-ve	133	SR58A	-	-	34	-	-	-ve
96	SR43C	-	-	29	-	-	+ve	134	SR58B	-	-	32	-	-	+ve
97	SR44A	-	-	36.6	-	37	-ve	135	SR58C	-	-	-	-	-	-ve
98	SR44B	1.38	1.5	60	-	-	-ve	136	SR59A	-	-	34	-	-	+ve
99	SR44C	-	-	32	-	-	-ve	137	SR59B	-	-	23	-	-	-ve
100	SR45A	-	-	13	-	-	+ve	138	SR60A	-	-	-	-	-	-ve
101	SR45B	-	1.33	101.3	+	-	-ve	139	SR60B	-	-	10	-	-	-ve
102	SR45C	-	-	30	-	-	+ve	140	SR61A	-	-	25.3	-	-	+ve
103	SR46A	-	-	31	-	-	-ve	141	SR61B	-	-	28	++	47	+ve
104	SR46B	1.25	-	53.8	-	-	-ve	142	SR61C	-	-	-	-	-	+ve
105	SR46C	-	-	26	-	-	+ve	143	SR62A	-	-	9	-	-	-ve
106	SR47A	-	-	17	-	-	+ve	144	SR62B	-	-	8	-	-	-ve
107	SR47B	1.9	1.5	59.2	-	-	-ve	145	SR63A	-	-	-	-	-	-ve
108	SR47C	-	-	-	-	-	+ve	146	SR63B	-	-	14	-	-	-ve
109	SR48A	-	-	31	-	-	+ve	147	SR64	-	-	114	+	-	+ve
110	SR48B	1.63	1.13	36.3	+	19	-ve	148	SR65	-	-	105	-	-	+ve
111	SR48C	2.1	1.25	49.2	+++	-	-ve	149	SR66	-	-	37.4	+++	-	+ve
112	SR48D	-	-	35	-	-	+ve	150	SR67A	-	-	21	-	-	-ve
113	SR49A	-	-	34.2	+	-	-ve	151	SR67B	-	-	33	-	-	-ve
114	SR49B	-	-	26	-	-	+ve	152	SR67C	-	-	15	-	-	-ve
115	SR49C	-	-	-	-	-	-ve	153	SR68	-	-	27.5	+	59	+ve
116	SR50	1.00	2	51.5	++	62	-ve	154	SR69A	-	-	19.3	+	-	+ve
117	SR51A	-	-	18			+ve	155	SR69B	1.33	-	143	-	-	+ve
118	SR51B	-	-	59.3	+	46	-ve	156	SR69C	1.47	-	66.6	+++	45	+ve

Serial No	Isolate	TCP-SI	$AlPO_4$- SI	IAA µg/ml	N_2-fixation	PIRG	Gram rxn	Serial No	Isolate	TCP-SI	$AlPO_4$- SI	IAA µg/ml	N-fixation	PIRG	Gram rxn
157	SR69D	-	-	29	-	-	-ve	197	SR87B	-	-	21	-	-	-ve
158	SR69E	-	-	30	-	-	-ve	198	SR88A	-	-	-	-	-	-ve
159	SR70A	-	-	23.6	+	-	+ve	199	SR88B	-	-	22	-	-	+ve
160	SR70B	1.29	-	46.8	-	56	+ve	200	SR89A	-	-	0	+++	-	-ve
161	SR70C	-	-	-	-	-	-ve	201	SR89B	-	-	31	-	-	+ve
152	SR70D	-	-	18.5	-	-	-ve	202	SR89C	-	-	-	-	-	+ve
163	SR71A	-	-	47	+	-	-ve	203	SR90A	-	-	26	-	-	+ve
164	SR71B	-	-	22.7	-	-	+ve	204	SR90B	1.9	1.8	21.5	+++	25.6	-ve
165	SR71C	-	-	23	-	-	+ve	205	SR90C	-	-	24	-	-	+ve
166	SR72A	-	-	-	-	-	-ve	206	SR91A	-	-	15	-	-	+ve
167	SR72B	-	-	51.2	+	-	+ve	207	SR91B	-	-	33	-	-	+ve
168	SR72C	-	-	29	-	-	-ve	208	SR92	-	-	41	-	-	-ve
169	SR73	-	-	76	-	-	+ve	209	SR93A	-	-	14	-	-	+ve
170	SR74A	-	-	23			-ve	210	SR93B	1.37	1.43	17.4	-	-	-ve
171	SR74B	-	-	33.1	+	-	+ve	211	SR93C	-	-	30	-	-	+ve
172	SR74C	-	-	11	-	-	-ve	212	SR94A	-	-	34	-	-	-ve
173	SR75A	-	-	25.6	-	-	-ve	213	SR94B	-	-	26	-	-	+ve
172	SR75B	-	-	-	-	-	-ve	214	SR95A	-	-	24	-	-	+ve
175	SR76A	-	-	30	-	-	+ve	215	SR95B	-	-	36	-	-	-ve
176	SR76B	-	-	29	-	-	+ve	216	SR96A	-	-	10.1	+++		+ve
177	SR77	1.8	2	39.5	-	27	-ve	217	SR96B	-	-	9	-	-	+ve
178	SR78A	-	-	22	-	-	-ve	218	SR96C	-	-	17	-	-	-ve
179	SR78B	-	-	17	-	-	-ve	219	SR97A	-	-	13	-	-	-ve
180	SR78C	-	-	28.5	-	-	-ve	220	SR97B	-	-	14.6	-	-	+ve
181	SR79	-	-	56	-	-	+ve	221	SR98A	-	-	13.5	+++	-	+ve
182	SR80A	-	-	18	-	-	+ve	222	SR98B	-	-	30	-	-	+ve
183	SR80B	-	-	16	-	-	-ve	223	SR98C	-	-	21	-	-	-ve
184	SR81A	-	-	-	-	-	-ve	224	SR99A	-	-	25.8	-	-	+ve
185	SR81B	-	-	33	-	-	-ve	225	SR99B	2.36	1.83	8.0	-	43.7	-ve
186	SR82A	-	-	26	-	-	-ve	226	SR99C	-	-	13	-	-	+ve
187	SR82B	-	-	17	-	-	+ve	227	SR100A	-	-	16	-	-	-ve
188	SR83A	-	-	48	-	-	-ve	228	SR100B	-	-	28	-	-	-ve
189	SR83B	-	-	38	-	-	+ve	229	SR101A	-	-	30	-	-	+ve
190	SR84A	-	-	14	-	-	-ve	230	SR101B	-	-	25	-	-	+ve
191	SR84B	-	-	27	-	-	+ve	231	SR102	-	-	11.4	-	28	+ve
192	SR85A	-	-	15	-	-	-ve								
193	SR85B	-	-	13	-	-	+ve								
194	SR86A	-	-	-	-	-	-ve								
195	SR86B	-	-	18	-	-	+ve								
196	SR87A	-	-	12	-	-	-ve								

Appendix 4. HCN production and hydrolytic enzyme activities of the selected 72 rhizobacteria

Serial No.	Isolate	HCN	Potease	Cellulase	Chitinase	Serial No.	Isolate	HCN	Potease	Cellulase	Chitinase
1	SR3A	+++	+	-	-	37	SR48B	-	+	-	-
2	SR4	-	-	-	-	38	SR48C	++	+	-	-
3	SR6A	-	-	-	-	39	SR49A	-	+	-	1.8
4	SR7A	-	-	-	-	40	SR50	+	+	+	2.2
5	SR8	-	-	+	-	41	SR51B	-	+	+	-
6	SR9A	-	+	-	-	42	SR55A	-	-	-	-
7	SR9B	-	+	+	1.71	43	SR55B	-	-	-	-
8	SR10A	-	-	-	-	44	SR56	-	+	-	2.5
9	SR10B	-	-	-	-	45	SR57A	-	+	+	-
10	SR11	-	+	-	2.75	46	SR57B	-	-	-	-
11	SR14	-	+	-	2.75	47	SR61A	-	+	+	-
12	SR20A	-	-	-	-	48	SR61B	-	+	+	-
13	SR20B	-	-	-	-	49	SR64	-	-	-	-
14	SR21A	-	+	+	-	50	SR65	-	-	-	-
15	SR21B	-	+	+	-	51	SR66	-	+	-	-
16	SR22	+	+	-	2.2	52	SR68	-	-	+	1.67
17	SR23	-	+	-		53	SR69A	-	+	+	1.94
18	SR25A	-	+	-	2.0	54	SR69B	-	-	-	-
19	SR25B	-	+	-	2.75	55	SR69C	++	-	+	-
20	SR26	-	+	-	2.4	56	SR70A	-	+	+	1.67
21	SR28	+	+	-	2.4	57	SR70B	-	+	+	1.77
22	SR29A	-	+	-	2.0	58	SR71A	-	+	-	1.67
23	SR29B	-	+	-	2.5	59	SR72B	-	-	+	2.15
24	SR32A	+++	+	-	-	60	SR73	-	-	+	-
25	SR32B	+	-	-	2.0	61	SR74B	-	-	+	1.17
26	SR37B	+	-	+	2.2	62	SR77	+++	+	-	-
27	SR39	-	-	-	2.0	63	SR79	-	+	+	-
28	SR40					64	SR83A	-	+	+	-
29	SR41B		+	-	-	65	SR89A	-	+	-	1.67
30	SR43A	+	+	-	2.5	66	SR90B	+++	+	-	-
31	SR43B	+++	+	+	2.75	67	SR92	-	+	-	1.9
32	SR44A	-	-	+	1.86	68	SR93B	-	-	-	-
33	SR44B	-	-	-	-	69	SR96A	-	+	+	-
34	SR45B	-	-	+	2.5	70	SR98A	-	+	+	-
35	SR46B	-	-	-	-	71	SR99B	+	+	+	-
36	SR47B	-	-	-	-	72	SR102	+	+	+	1.11

HCN; +, ++, and +++ indicate small, moderate and large amount, respectively

Appendix 5. Growth pH range, resisted heavy metals, pesticides, temperature and NaCl concentration of the 72 rhizobacteria

No	Isolate	pH range	Temp. (°C)	NaCl (%)	Heavy metals*	pesticides
1	SR3A	5.5-8.5	40	4	Pb, Zn, Mn	Glyphosate
2	SR4	4.5-9	40	7	Pb, Zn, Mn	Glyphosate
3	SR6A	5.5-9	40	4	Pb, Co, Zn, Mn	-
4	SR7A	5-9	40	3	Pb, Zn, Mn	-
5	SR8	5-9	40	7	Pb, Zn, Mn	Glyphosate, mankozeb
6	SR9A	4.5-9	40	7	Pb, Zn, Mn	Glyphosate
7	SR9B	5-9	40	4	Pb, Co, Zn, Mn	-
8	SR10A	5.5-9	40	3	Pb, Zn, Mn	-
9	SR10B	4.5-9	37	7	Pb, Zn, Mn	Glyphosate
10	SR11	5.5-9	40	7	Pb, Co, Zn, Mn	Glyphosate
11	SR14	5.5-9	45	7	Pb, Co, Zn, Mn	Glyphosate
12	SR20A	5-9	45	5	Pb, Zn, Mn	Glyphosate, mankozeb
13	SR20B	5.5-9	45	3	Pb, Co, Zn, Mn	-
14	SR21A	5-9	45	2	Pb, Cr, Zn, Mn	Mankozeb
15	SR21B	5-8.5	45	2	Pb, Cr, Mn	-
16	SR22	4.5-9	40	7	Pb, Co, Zn, Mn	Glyphosate
17	SR23	5.5-9	40	5	-	Glyphosate
18	SR25A	5.5-9	45	4	-	Glyphosate
19	SR25B	5-9	40	5	Pb, Mn	Glyphosate
20	SR26	4.5-9	40	7	Pb, Zn, Mn	Glyphosate
21	SR28	4.5-9	40	7	Pb, Zn, Mn	Glyphosate
22	SR29A	5-9	40	5	Pb, Zn, Mn	Glyphosate
23	SR29B	5-9	40	5	Pb, Zn, Mn	Glyphosate
24	SR32A	5-8.5	35	5	Pb, Zn, Mn	Glyphosate, mankozeb
25	SR32B	4.5-9	40	7	Pb, Zn, Mn	Glyphosate, mankozeb
26	SR37B	5-9	40	7	Pb, Co, Zn, Mn	Glyphosate
27	SR39	5-9	40	5	Pb, Zn, Mn	Glyphosate, mankozeb
28	SR40	5-9	40	3	Pb, Zn, Mn	-
29	SR41B	5.5-9	40	3	Pb, Zn, Mn	Glyphosate
30	SR43A	5.5-9	40	4	Pb, Co, Zn, Mn	-
31	SR43B	5.5-9	40	4	Pb, Co, Zn, Mn	Glyphosate
32	SR44A	5.5-9	40	3	Pb, Co	Glyphosate
33	SR44B	5.5-9	37	6	Pb, Zn, Mn	Glyphosate, mankozeb
34	SR45B	5.5-9	40	5	Pb, Zn, Mn	Glyphosate
35	SR46B	5.5-9	37	5	Pb, Zn, Mn	Glyphosate, mankozeb
36	SR47B	5.5-9	37	2	Pb, Co, Zn, Mn	-

No	Isolate	pH range	Temp. (ºC)	NaCl (%)	Heavy metals	Pesticides
37	SR48B	5.5-9	37	5	Pb, Co, Zn, Mn	-
38	SR48C	5.5-9	37	5	Pb, Zn, Mn	Glyphosate
39	SR49A	5.5-9	40	5	Zn	Glyphosate
40	SR50	4.5-9	40	7	Pb, Zn, Mn, Cu	Glyphosate, mankozeb
41	SR51B	5.5-9	37	2	Pb, Zn, Mn	-
42	SR55A	5.5-9	37	2	Pb, Co, Zn, Mn	-
43	SR55B	5.5-9	37	2	Pb, Co, Zn, Mn	-
44	SR56	4.5-9	37	7	Pb, Co, Zn, Mn	Glyphosate
45	SR57A	5.5-9	37	2	Pb, Mn	-
46	SR57B	5.5-9	37	2	Pb, Zn, Mn	-
47	SR61A	5.5-9	37	5	Pb, Cr, Zn, Mn	-
48	SR61B	5.5-9	45	5	Pb, Cr, Zn, Mn	Mankozeb
49	SR64	5.5-9	45	3	Pb, Co, Zn, Mn	-
50	SR65	5.5-9	45	2	Pb, Co, Zn, Mn	-
51	SR66	6-9	45	5	Pb, Cr, Zn, Mn	-
52	SR68	6-9	45	4	Pb, Co, Zn, Mn	-
53	SR69A	6-9	45	6	Pb, Cr, Zn, Mn	Mankozeb
54	SR69B	5.5-9	45	2	Pb, Co, Zn, Mn	-
55	SR69C	5.5-8	37	4	Pb, Zn, Mn	Glyphosate
56	SR70A	5.5-9	40	4	Pb, Zn, Mn	-
57	SR70B	6-9	37	3	Pb, Zn, Mn	Glyphosate
58	SR71A	6-9	40	3	Pb, Cr, Zn, Mn	-
59	SR72B	6-9	40	3	Pb, Co, Cr, Zn, Mn	-
60	SR73	6-9	40	3	Pb, Co, Cr, Zn, Mn	-
61	SR74B	6-9	40	4	Pb, Cr, Zn, Mn	-
62	SR77	6-9	35	3	Pb, Zn, Mn	Glyphosate
63	SR79	6-9	40	3	Pb, Cr, Zn, Mn	-
64	SR83A	6-9	40	1	-	-
65	SR89A	5.5-9	37	4	-	Glyphosate
66	SR90B	6-9	37	4	Pb, Mn	Glyphosate
67	SR92	6-9	37	2	-	Glyphosate
68	SR93B	6-8.5	37	7	Pb, Zn, Mn, Cu	Glyphosate
69	SR96A	6-8.5	40	4	Pb, Cr, Zn, Mn	-
70	SR98A	6-9	37	3	Pb, Cr, Zn, Mn	-
71	SR99B	6-9	37	4	Pb, Mn	Glyphosate
72	SR102	6-9	40	4	Pb, Zn, Mn	-

Appendix 6. Antibiotic resistance of the 72 rhizobacterial isolates

Serial number	Isolate (SR) Code	Ampicillin (100 µg mL^{-1})	Chloramphenicol (5 µg mL^{-1})	Gentamycin (5 µg mL^{-1})	Neomycin (50 µg mL^{-1})	Streptomycin (20 µg mL^{-1})	Naldixic acd (50 µg mL^{-1})	Penicillin G (20 µg mL^{-1})	Vancomycin (5 µg mL^{-1})	Erythromycin (5 µg mL^{-1})
1	3A	+	+	-	-	-	-	+	+	+
2	4	+	+	-	-	-	-	+	+	+
3	6A	+	+	+	-	+	-	+	+	-
4	7A	+	+	-	-	-	-	+	+	-
5	8	+	+	-	-	-	+	+	+	+
6	9A	+	+	+	+	+	-	+	+	+
7	9B	+	+	-	-	-	-	+	+	-
8	10A	+	+	-	-	-	-	+	+	-
9	10B	+	+	-	-	-	-	+	+	+
10	11	+	+	+	+	+	-	+	+	+
11	14	+	+	+	+	+	-	+	+	+
12	20A	+	+	-	-	-	+	+	+	-
13	20B	+	+	-	-	-	-	+	+	-
14	21A	-	-	-	-	-	-	-	-	-
15	21B	-	-	-	-	-	-	-	-	-
16	22	+	+	+	+	+	+	+	+	+
17	23	+	+	+	+	+	-	+	+	+
18	25A	+	+	+	+	+	+	+	+	+
19	25B	+	+	+	+	+	+	+	+	+
20	26	+	+	+	+	+	+	+	+	+
21	28	+	+	+	+	+	-	+	+	+
22	29A	+	+	+	+	+	+	+	+	+
23	29B	+	+	+	+	+	-	+	+	+
24	32A	+	+	-	-	-	-	+	+	+
25	32B	+	+	+	+	+	-	+	+	+
26	37B	+	+	+	+	+	-	+	+	+
27	39	+	+	+	+	+	-	+	+	+
28	40	+	+	-	-	-	-	+	+	-
29	41B	+	-	-	-	-	-	+	+	-
30	43A	+	+	+	+	+	+	+	+	+

31	43B	+	+	+	+	+	+	+	+	+
32	44A	+	+	+	+	+	-	+	+	+
33	44B	+	+	-	-	-	+	+	+	+
34	45B	+	+	+	+	+	+	+	+	+
35	46B	+	+	-	-	-	+	+	+	+
36	47B	+	+	-	-	-	-	+	+	-
37	48B	+	+	-	-	-	+	+	+	-
38	48C	+	-	-	-	-	-	+	+	-
39	49A	+	+	+	+	+	+	+	+	+
40	50	+	+	+	+	+	+	+	+	+
41	51B	+	+	+	-	+	-	+	+	+
42	55A	+	+	-	-	+	+	+	+	-
43	55B	+	+	-	-	+	+	+	+	-
44	56	+	+	+	+	+	-	+	+	+
45	57A	+	+	+	-	-	-	+	+	-
46	57B	+	+	-	-	+	-	+	+	-
47	61A	-	+	+	-	+	+	-	-	+
48	61B	-	-	-	-	-	-	-	-	-
49	64	+	+	+	-	+	+	+	+	-
50	65	+	+	-	-	-	+	+	+	-
51	66	-	-	-	-	-	+	-	-	-
52	68	+	+	-	-	-	-	+	-	-
53	69A	+	+	+	+	-	+	+	-	-
54	69B	+	+	-	-	+	+	+	+	-
55	69C	+	-	-	-	-	-	-	+	+
56	70A	+	+	+	+	+	-	+	+	+
57	70B	+	+	-	-	+	-	+	+	-
58	71A	-	+	+	+	+	+	+	+	-
59	72B	+	+	+	-	+	+	+	+	+
60	73	+	+	-	-	+	+	+	+	+
61	74B	+	+	+	-	-	+	-	-	+
62	77	+	+	-	-	-	-	+	+	+
63	79	-	-	-	-	-	+	-	+	-
64	83A	+	+	-	-	-	-	-	+	-
65	89A	+	+	+	+	+	-	+	+	+
66	90B	+	+	-	-	-	-	+	+	-
67	92	+	+	+	+	+	+	+	+	-
68	93B	+	-	-	-	-	-	+	+	-
69	96A	+	+	-	-	-	-	+	-	-
70	98A	-	+	-	-	-	+	-	-	-
71	99B	+	+	-	-	-	-	+	+	+
72	102	+	+	-	-	-	-	+	+	-

Appendix 7. Composition of N-free nutrient solution for grain legumes (Broughton and Dilworth, 1970)

Stock solution	Chemical	g/liter
1	$CaCl_2.2H_2O$	294
2	KH_2PO_4	136.1
3	Fe $C_6H_5O_7.3H_2O$	6.7
	$MgSO_4.7H_2O$	123.3
	K_2SO4	87.0
	$MnSO_4.H_2O$	0.338
4	H_3BO_3	0.247
	$ZnSO_4.7H_2O$	0.288
	$CuSO_4.5H_2O$	0.100
	$CoSO_4.7H_2O$	0.056
	$Na_2MoO_2.2H_2O$	0.048

Appendix 8. Rating some soil properties

A. Rating for soil pH (Bruce and Rayment, 1982)

pH	Rating
> 9	Very strongly alkaline
9.0-8.5	Strongly alkaline
8.4-7.9	Moderately alkaline
7.8-7.4	Mildly alkaline
7.3-6.6	Neutral
6.5-6.1	Slightly acidic
6.0-5.6	Moderately acidic
5.5-5.1	Strongly acidic
5.0-4.5	Very strongly acidic

B. Rating for soil OM, total N, and available P

OM (%)*	total N (%)**	available P (mg kg^{-1}) ***	Rating
< 0.70	---	---	Extremely low
0.70-1.0	< 0.05	< 5	Very Low
1.0-1.70	0.05-0.15	5-10	Low
1.70-3.0	0.15-0.25	10-17	Moderate
3.0-5.15	0.25-0.50	17-25	High
> 5.15	> 0.5	> 25	Very high
---	---	---	Extremely high

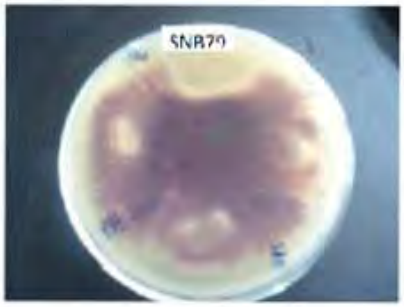
Source: Charman and Roper (2007) *, Bruce and Rayment (1982) **, Holford and Cullis (1985) ****

Appendix 9. Some sample *in vitro* plant growth promoting traits

Phosphate solubilization

HCN production

Indole acetic acid production



Pathogenic fungal inhibition

Appendix 10. Apperance of plants resulting from various treatments in the greenhouse experiments