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The functional characterisation of soybean (*Glycine max* L.) rhizospheric bacteria indigenous to Ethiopian soils

Diriba Temesgen[1, 2*], Marta Maluk[3], Euan K. James[3], Pietro P. M. Iannetta[3] and Fassil Assefa[1]

[1]Department of Microbial, Cellular and Molecular Biology, College of Natural Sciences, Addis Ababa University, Cellular and Molecular Biology, P. O. Box 1176, Ethiopia.
[2]Department of Biology, Natural and Computational Sciences, Mada Walabu University, P. O. Box 247, Bale-Robe, Ethiopia.
[3]James Hutton Institute, Invergowrie, Ecological Sciences, Dundee DD2 5DA, Scotland, UK.

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Ethiopia remains a net importer of soybean partly due to low average yields which may be improved by inoculation with rhizobia and/or plant growth promoting rhizobacteria (PGPR). The functional characterisation of 231 rhizobacteria isolated from the rhizosphere of soybean grown in 102 soils collected from different pedo-climatic regions of Ethiopia was carried out. Isolates were initially characterised by Gram staining and then functionally for: indole-3-acetic acid production; phosphate solubilisation; growth on a nitrogen-free medium; and, resistance to the pathogenic fungus *Fusarium oxysporum*. A sub-set of 72 of the best performing isolates were tested *in vitro* for: production of bioprotectants; polysaccharide degradation; and their relative capacity to maintain growth in response to extremes of: temperature; pH; salinity; antibiotics; pesticides; and, heavy metals. Twenty isolates with the best PGPR potential were identified *via 16S rRNA* gene sequencing. Seventeen isolates were Gram-negative: *Pseudomonas* (7); *Stenotrophomonas* (5); *Acinetobacter* (3); *Enterobacter* (1); and *Achromobacter* (1). Gram-positive types were: *Bacillus* (2); and, *Microbacterium* (1). Of the six of the most promising PGPR tested on soybean plants, *Achromobacter* and *Acinetobacter* significantly enhanced soybean seed germination, seedling growth and plant vigour index compared to non-inoculated plants.

Key words: Soybean, Ethiopia, plant growth promoting rhizobacteria, plant growth promoting rhizobacteria (PGPR), seedling vigour.

INTRODUCTION

Soybean (*Glycine max* L.) is an important source of high protein grain in Ethiopia, having been introduced in the early 1950 s (Shurtleff and Aoyagi, 2009). Soybean has mainly been used for processing into baby food, edible oils, common cultural dishes and animal feeds (Abebe et al., 2015). Due to poor soil fertility (Argaw, 2012), the

*Corresponding author. E-mail: direteme@gmail.com. Tel: +251 911731114.

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average yield of soybean in Ethiopia is less than 2 t ha^{-1}; which is significantly below the yield potential of the crop (Cooper, 2003). Consequently, Ethiopia imports 15 kt of soybean products, costing 11 M$ (US) y^{-1} to fulfil its demand (Hailu and Kelemu, 2014).

Soybean can satisfy its entire nitrogen (N) demand from atmospheric di-nitrogen gas *via* biological nitrogen fixation (BNF), a process which is mediated by symbiotic rhizospheric bacteria, referred to collectively as rhizobia (Gyaneshwar et al., 2011; Peix et al., 2015). The principal rhizobial symbionts of soybean are species of *Bradyrhizobium*, *Ensifer* and *Sinorhizobium* (Jordan, 1982; Scholla and Elkan, 1984; Kuykendall et al., 1992; Chen et al., 1995; Gao and Yang, 1995; Appunu et al., 2008). Soybean also forms symbiotic relationships with other soil microbes including *Agrobacterium* (Youseif et al., 2014) and mycorrhizas (Sylvia et al., 1993; Troeh, 2006) which may also improve its fitness and yield.

Various free-living plant growth promoting rhizobacteria (PGPR) were also reported in association with soybean. Among the most common PGPR genera isolated from soybean rhizosphere are *Pseudomonas*, *Bacillus*, *Enterobacter* and *Microbacterium*. PGPR isolates have been applied exogenously as seed-borne inoculants to improve seed germination, seedling fitness and crop performance (Malviya and Singh, 2012) *via* a variety of mechanisms. The mechanisms include increasing the availability of nutrients such as: N via BNF (Park et al., 2005); phosphorus and zinc, via solubilisation of inorganic zinc phosphate (Sharma et al., 2012); iron chelation using siderophore (Susilowati et al., 2011); production or modulation of phytohormones (Masciarelli et al., 2014); and, disease resistance through induction of host induced systemic resistance (Glick, 2012), and production of anti-fungal enzymes and antibiotic compounds (Susilowati et al., 2011). Such traits may explain the potential of PGPR to suppress root-rot pathogens (Leon et al., 2009), viruses (Khalimi and Suprapta, 2011), pathogenic fungi (Wahyudi et al., 2011a; Wahyudi et al., 2011b) and parasitic nematodes (Kloepper et al., 1992).

PGPR have been isolated from grain legume crops cultivated in Ethiopian soils, such as lentil (Midekssa et al., 2015), chickpea (Midekssa et al., 2016) and faba bean (Keneni et al., 2010), but also from non-fabaceous crops such as Teff (Woyessa and Assefa, 2011b; Woyessa and Assefa, 2011a) and coffee (Muleta et al., 2007, 2009, 2013). There are no peer-reviewed reports detailing the functional diversity of soybean specific PGPR isolated from Ethiopian soils, except that of Argaw (2012), who reported the effect of phosphate-solubilising Pseudomonas species isolated directly from Ethiopian soil upon phosphorus uptake of soybean. Therefore, the objective of the present study was to isolate and characterise indigenous soybean PGPR for traits which may potentially improve the performance and yield of the crop. Initial screening was used to identify and test the most promising isolates as PGPR, and which were tested as seed inoculants for effects on soybean seed germination and seedling growth.

MATERIALS AND METHODS

Soil sampling and rhizobacteria isolation

Soybean cv. Ethio-Yugoslavia (Bako Agricultural Research Center), was cultivated under greenhouse conditions (25±2/17±3°C day/night temperature, 12 h photoperiod, watering every two days in 3 L pots) in soils gathered from 102 different locations across three regional states of Ethiopia (Figure 1). After 45 d of growth, the plants were carefully uprooted, and 10 g of roots plus adhering soil were washed-off into 90 ml of sterile saline [0.9% (w/v) NaCl]. The resultant soil suspension was then subject to a 10-fold serial dilution and 100 µl spread on 'nutrient agar' (NA, Code TM341; Titan Biotech Ltd., India) plates. Colonies were selected and re-cultured to ensure purity. Next, 231 soybean rhizobacteria (SR) isolates were grown in nutrient broth (NB, Code 350; Titan Biotech Ltd, India) to log-phase (about 10^9 cells mL^{-1}; OD_{540} 0.91), in order to prepare 25% glycerol stocks for long-term storage at -80°C.

The isolates were first distinguished by their Gram status using the KOH method of Buck (1982). In short, a 24 h old colony of each SR isolate was selected and suspended in 50 µl of 3% [w/v] KOH on clean microscope slide to assess the gelling. The isolates were then characterised and screened for their relative PGP potential using a two-stage process. Firstly, for traits of: phosphate solubilisation, indole-3-acetic acid (IAA) production, pathogenic *Fusarium oxysporium* inhibition, and, capacity to survive on N-free growth media, indicative of capacity for N scavenging and perhaps BNF potential. Secondly, a subset of 72 rhizobacteria isolates that demonstrated a higher level for at least one of the four tested potential PGP traits were selected and tested to discern their relative potential to enable plant protection *via* 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 (ampicillin, chloramphenicol, gentamycin, neomycin, streptomycin sulphate, naldixic acid, penicillin G, vancomycin and erythromycin), pesticides (glyphosate, mancozeb and curzet), salt (NaCl) and heavy metals ($CoCl_2*6H_2O$, $Pb(CH_3COO)_2*3H_2O$, $K_2Cr_2O_7$, $CuCl_2*2H_2O$, $ZnSO_4*5H_2O$ and $MnSO_4*4H_2O$).

Functional characterisation of the rhizobacteria isolates

All the functional characterisation tests were carried out in sterile conditions in triplicate for each isolate, using 100 µL of liquid inoculum (single colony grown overnight at 28°C and adjusted to 10^6 cells ml^{-1}), unless otherwise stated.

Indole-3-acetic acid (IAA) Production

Rhizobacterial IAA production was tested by transferring a single colony of each isolate into 5 ml sterile nutrient broth amended with filter sterilised L-tryptophan (2 g L^{-1}) and incubating at 30°C for 3 days. Each culture was then centrifuged at 5000 ×*g* for 15 min (Sigma 4K15) and 1 ml of supernatant was mixed with 2 ml of Salkowski reagent (Acuña et al., 2011). The mixture was incubated in dark at room temperature for 25 min. Development of pink colour indicated IAA production. IAA was quantified immediately after the incubation period by measuring the absorbance at 530 nm with spectrophotometer (Jenway, 6405 UV/VIS spectrophotometer). IAA

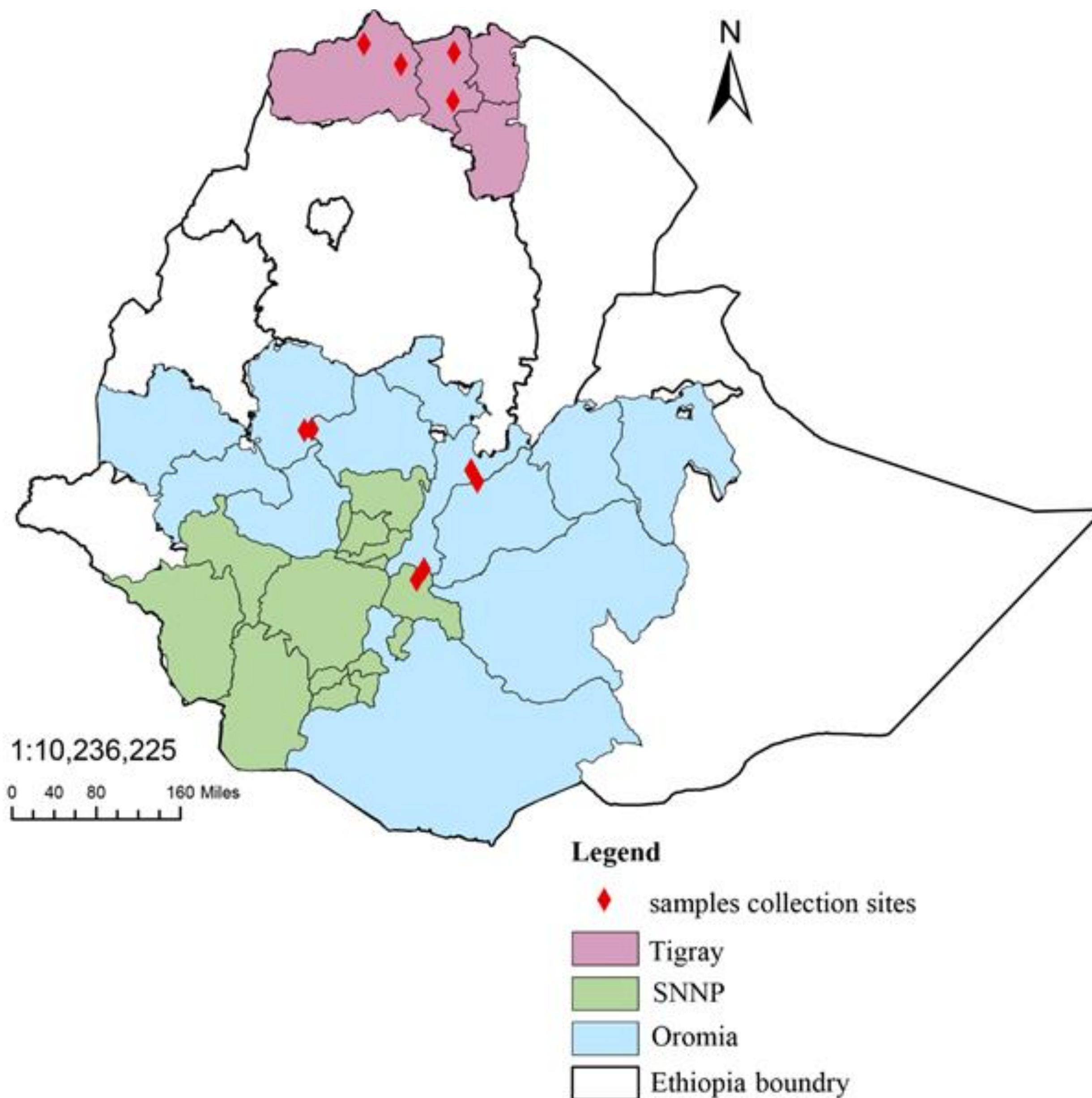

Figure 1. A map of Ethiopia showing the location of soil sampling sites (black diamonds) within the three regional states of Ethiopia; Oromia, SNNP (South Nation Nationalities and People), and Tigray.

concentration values were obtained by preparing a standard curve with increasing IAA (HiMedia; PCT0803) concentration (5, 10, 20, 50, 80 and 100 μg ml^{-1}) in nutrient broth amended with L-tryptophan as stated above.

Solubilisation of Al, Fe and Tri-calcium phosphates

The isolates were evaluated for their solubilisation of tricalcium, iron and aluminium phosphates by spot-inoculation onto plates of National Botanical Research Institute's Phosphate growth medium (NBRIP) containing 20 mM $Ca_3(PO_4)_2$ or 40 mM $AlPO_4$ or 30 mM $FePO_4$, and 60 mM glucose, 20 mM $MgCl_2$*6H_2O, 1 mM $(NH_4)_{2\,s}O_4$, 1 mM $MgSO_4$*7H_2O, 3 mM KCl and 1.5% [w/v] agar (Nautiyal, 1999). Phosphate solubilisation index (PSI) for each inorganic phosphate source was calculated as PSI [mm] = (colony diameter + halo zone diameter)/colony diameter.

Nitrogen fixation

The isolates were tested for their potential capacity for BNF by stabbing into Burk's N-free semi- solid medium with sterile inoculating needle (24 Gauge Wire) which was first immersed in liquid culture of rhizobacteria at log phase. Growth (pellicle formation) was assessed after incubation at 30°C for 5 days, visually recorded and evident was interpreted as proof of the potential capacity for BNF, and/or the capacity to scavenge and

grow on very low level of nitrogen (Laskar and Sharma, 2013).

In vitro antifungal activity

The antifungal activity of all the 231 isolates was tested using a dual culture method (Saraf et al., 2007). Ten microliter of liquid inoculum (10^6 cells ml^{-1}) of each SR isolate was spot-inoculated onto the surface of 1:1 (w/w) nutrient agar and potato dextrose agar (NA-PDA, Code CM003 and CM139) respectively, Oxoid LTD, England) 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 plates were then incubated at 28°C for 48 h. A 4 mm disc from 48 h/28°C PDA grown culture of the test pathogen *F. oxysporum* (EIAR, Ethiopian Institute of Agricultural Research) was placed at the centre of each plate (including rhizobacteria 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, PIRG = [(radial growth of fungus on control plates – radial growth of the fungus in the dual)/ radial growth of fungus on control plates]*100.

Hydrogen cyanide (HCN) production

Each of the 72 subset SR isolates (100 µl; 10^6 cells ml^{-1}) were spread on NA 90 mm diameter Petri dishes amended with 4.4 g L^{-1} glycine (Bharucha et al., 2013). Strip of Whatman™ filter paper No.1 (Sigma #WHA1001500) was soaked in picric acid solution (2.5 g picric acid and 12.5 g Na_2CO_3 dissolved in 1 L of distilled water) and fixed to underside of the lid of each plate. Dishes were sealed with Parafilm™ and incubated at 28°C for 3 to 5 days. Cultures were assessed for hydrogen cyanide production by their relative capacity to change the yellow colour of the filter paper. Reactions were scored as weak, moderate and strong HCN production if the filter paper turned light brown, brown or reddish brown, respectively.

Protease, cellulase and chitinase activities

To test for cellulase, chitinase and protease activity, each rhizobacterial isolate (10 µl; 10^6 cells ml^{-1}) was spot-inoculated on Carboxymethyl cellulose (CMC) agar plates (Kasana et al., 2008), chitin agar (Bansode and Bajekal, 2006) and NA plates supplemented with 1.5% [w/v] skimmed milk powder (Ryden et al., 1973), respectively. The plates were incubated at 28°C for 72 h and the "clear" zone formation of their colonies was recorded as indication of enzyme activity. For CMC agar plates, clear zones were visualised after flooding the plates with Gram's iodine (2 g of KI and 1 g of iodine in 300 ml distilled water) for 3 to 5 min in the dark. Chitinase activity indices (SI) were calculated according to Ahmed et al. (2014) as SI = (colony diameter + halo zone diameter)/colony diameter.

Characterisation of stress tolerance traits

Tolerance to temperature stress was evaluated by colony growth incubated at a series of different temperatures (35, 37, 40 and 45°C). Tolerance to pH stress was evaluated by growth in nutrient broth (at pH 4 and 4.5, as NA did not solidify well at that pH) or on NA plates (at pH 5, 5.5, 6, 8, 8.5 and 9). Liquid cultures were prepared by inoculation of 100 µl of 10^6 cells ml^{-1} into 5 ml nutrient broth and shaking at room temperature for 72 h. Growth of cultures was measured in terms of turbidity or OD. Plates were prepared by streaking 10 µl of 10^6 cells ml^{-1} of each rhizobacteria isolates onto NA plates and colony growth was examined after incubating at 28°C for 72 h.

Salt (NaCl; 1-7% [w/v]) tolerance was tested as described by Damodaran et al. (2013) on nutrient agar plates to examine colony growth. The plates were prepared by streaking 10 µl of 10^6 cells ml^{-1} of each rhizobacteria isolates onto NA plates supplemented with the indicated salt concentration and incubating at 28°C for 72 h.

Pesticides tolerance was assessed on NA plates supplemented with 2 g L^{-1} CURZATE®MWG (Du Pont (UK) Limited, PCS NO 04723) containing 4.5% [w/w] cymoxanil-cyanoacetamide oxime, and also with 2 g L^{-1} of 68% [w/w] mancozeb-ethylene (bis) dithiocarbamate (Mubeen et al., 2006) and 1444 µg L^{-1} Roundup® (Monsanto; containing glyphosate) was supplemented to minimal salt agar plates at 0.014% to test the tolerance of the isolates (Ahemad and Khan, 2010). All the plates were streaked with a 10 µl of 10^6 cells ml^{-1} of each rhizobacterial isolate and incubated at 28°C for 72 h to examine colony growth.

Antibiotic tolerance was tested according to Wang et al. (2009). Actively growing rhizobacteria were spot inoculated (10 µl; 10^6 cells ml^{-1}) onto NA plates supplemented with the following filter-sterilised antibiotics at concentrations (µg ml^{-1}); ampicillin (100), chloramphenicol (5), gentamycin (5), neomycin (50), streptomycin sulphate (20), nalidixic acid (50), penicillin G (20), vancomycin (5) and erythromycin (100). Rhizobacteria isolates were also spot-inoculated onto minimal-salt agar plates to test for their 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 28°C for 72 h in both antibiotic and heavy metals tests. Growth response was scored as presence (+) or absence (-) of growth.

Functional classification

Multivariate cluster analysis of the phenotypic variability among the 72 fully-characterised subset of SR isolates was carried out using Ori PAST™ software (Hammer et al., 2001).

Phylogenetic analysis

Based on their potential to be PGPR and tolerance to environmental stress, 20 rhizobacteria isolates were chosen to be also characterised by *16S rRNA* gene sequencing to identify their genus. The *16S rRNA* gene sequence data also allowed an assessment of sequence similarity among these 20 isolates and relative to other similar bacterial data held in genetic databases.

DNA isolation

To isolate DNA, a single colony of each rhizobacterium was inoculated into 5 ml of NB and incubated at 28°C shaking at 120 ×*g* over-night on a rotary C24KC Refrigerated Incubator Shaker (Edison, NJ, USA). A total of 4 ml of culture at log phase was harvested by centrifugation (14,000 ×*g*; 10 min; 4°C; Sigma 1–1 5 PK). Rhizobacteria pellets were re-suspended in a mixture of 400 µl of Tris-EDTA (TE) buffer, 10 µL proteinase K (Sigma #P4850) and 10 µl of 20% (w/v) sodium dodecyl sulfate (SDS). The mixture was incubated at 37°C for 1 h before mixing with 420 µL of phenol:chloroform:isoamyl alcohol (25:24:1 [v/v/v]; Sigma #P2069) and then centrifuged (14,000 ×*g*; 10 min; 4°C; Sigma 1–1 5 PK). The upper aqueous layer (175 µl) was recovered and combined with 1/10x volume (17.5 µl) of 3M sodium acetate (pH 5.2) and 3.74 x volume (655 µl) of isopropanol (Sigma #I9030). DNA was precipitated at -80°C for 15 min, pelleted by centrifugation (14,000 ×*g*; 15 min; 4°C; Sigma 1–1 5 PK), washed with 200 µl of 70% [v/v] ethanol, then centrifuged (14,000 ×*g*; 1 min; 4°C; Sigma 1–1 5 PK), dried at 37°C for 15 min and re-suspended in TE buffer (25 µl). The

quality and quantity of DNA were determined using a ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, USA).

PCR amplification

A 1473 bp portion of *16S* ribosomal DNA (*rrs*) gene of each rhizobacterial isolate was amplified using forward primer 8–27F (AGAGTTTGATCCTGGCTCAG) and reverse primer rD1 (AAGGAGGTGATCCAGCC) (Weisburg et al., 1991). Standard polymerase chain reaction (PCR) was performed using a G-Storm GS1 thermal cycler (GRI Ltd, Braintree, UK) in a 50 µL reaction mixture consisting of 33.75 µl milliQ-water, 10 µl 5X clear Go Taq® G2 Buffer, 1 µl 10 mM dNTPs (Invitrogen), 2 µl 10 mM of each primer, 0.25 µl Go Taq polymerase (Promega #M3175) and 1 µl DNA template. PCR amplifications were performed with a 2-min denaturing step at 95°C, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1.5 min with a final elongation step at 72°C for 15 min. Products were run on a 1% [w/v] agarose gel containing SYBR® Safe DNA Gel Stain (Invitrogen S33102) and visualised using UV-illumination (FluorChem® Imager, Alpha Innotech). PCR products were purified using QIAquick-spin columns (Qiagen, Inc., Chatsworth, CA) and sequenced using an ABI3730 DNA analyser. The 16S data was submitted to GenBank under accession numbers: MG557785-MG557802.

Molecular evolutionary relationship analysis

The BioEdit Sequence Alignment Editor Version 7.2 software (http://www.mbio.ncsu.edu/BioEdit/BioDoc.pdf) was used for initial analysis of the *16S rRNA* gene sequences. The sequences were than screened against Gene Bank databases (http://www.ncbi.nlm.nih.gov/) using the nucleotide basic local alignment tool (BLASTN) queuing system (Altschul et al., 1997). The evolutionary history was inferred using the Maximum Likelihood method (Tamura and Nei, 1993) and MEGA7 software (Kumar et al., 2016). Initial trees for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pair-wise distances estimated using the Maximum Composite Likelihood (MCL) approach and selecting the topology with superior log likelihood value. The obtained 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.

Assessment of rhizobacteria PGP potential via seed germination assay

Six SR isolates (SR7A, SR10A, SR20A, SR20B, SR43B and SR69B) were selected to evaluate their effect on soybean seed germination and seedling growth. These were selected on the basis of their multiple (3-7) potential PGP traits, with: SR7A, SR10A, SR20A, SR20B showing greatest capacity to solubilise phosphate; SR43B showing greatest area of pathogenic fungal exclusion and SR69B showing greatest IAA production.

Soybean seeds were surface sterilised in 2% sodium hypochlorite for 3 min (Lwin et al., 2012) and dipped into rhizobacterial NB cultures (approximately 10^9 cells ml^{-1}) for 5 h according to Malviya and Singh (2012). Surface sterilised seeds dipped into sterile NB for 5 h were included as control. All seeds were cultured on Petri dishes (90 mm; 10 seeds per Petri dish) with 3 MM Whatman filter paper moistened with sterile distilled water at 28°C for 5 days. Seedling length (mm), determination of percentage of seed germination (%) and Vigour Index (VI) were recorded according to Agrawal and Agrawal (2013). One-way ANOVA test on seed germination assay data was done using SPSS (SPSS Inc., SPSS for Windows, Version 15.0, Chicago: SPSS Inc.). Significant differences were determined using Tukey HSD at 0.05 level.

RESULTS AND DISCUSSION

Functional PGP traits

Of the 231 soybean rhizobacterial isolates initially screened for their potential PGP traits, 198 isolates produced IAA, 30 isolates solubilised bound calcium phosphate, 38 isolates survived on N-free medium (assumedly as a function of BNF and/or N- scavenge capacity) and 29 isolates inhibited growth of the fungal pathogen *F. oxysporum* (Table 1 and Supplementary Table 1).

IAA production

The concentration of IAA production ranged from 8 to 143 µg ml^{-1} (15 fold) with mean value of 29 µg ml^{-1} (Table 1). The IAA levels are comparable to other reports for soybean PGPR such as *Bacillus fusiformis* PM-5 (140.9 µg ml^{-1}) (Park et al., 2005) and *Enterobacter cloacae* MDSR9 (125 µg ml^{-1}) (Ramesh et al., 2014), though less than that of *Bacillus subtilis* PRBS^{-1} (310 µg ml^{-1}) (Araújo et al., 2005).

Solubilisation of phosphates

Solubilisation of $Ca_3(PO_4)_2$ and $AlPO_4$ was achieved by 30 and 28 isolates, with PSI for $Ca_3(PO_4)_2$ varying from 1.1 to 2.8 and from 1.2 to 2.6 for $AlPO_4$ (Table 1). Of the twenty *16S rRNA* gene sequenced isolates, the highest $Ca_3(PO_4)_2$ PSIs were recorded for SR40 (*Acinetobacter* sp.), SR99B (*Pseudomonas* sp.), SR20B (*Acinetobacter* sp.), SR10A (*Acinetobacter* sp.) and SR20A (*Achromobacter mucicolens*) with values varying from 2.8 to 2.2 (Table 2). These high $Ca_3(PO_4)_2$ PSI values are comparable to those reported by Bagalkar (2013) for soybean *Pseudomonas*, *Enterobacter* and *Acinetobacter* rhizobacteria species. Maximum of 2.6 for $AlPO_4$ PSIs was recorded for the *16S rRNA* gene sequenced isolates: SR10A (*Acinetobacter* sp.) and SR20A (*A. mucicolens*); whereas *Acinetobacter* sp.: SR8, SR20B, SR7A and SR40 showed $AlPO_4$ PSIs of 2.5, 2.4, 2.3 and 2.2, respectively. One isolate, SR20A (*A. mucicolens*) showed high capacity to solubilise phosphorous from both $Ca_3(PO_4)_2$ and $AlPO_4$. This contrasts with an *Achromobacter* sp. isolated from soybean rhizosphere amended with 2% hydrochar which failed to liberate phosphate from $Ca_3(PO_4)_2$ in Pikovskaya medium (Egamberdieva et al., 2016).

Table 1. Data acquired for potential plant growth promoting (PGP) traits characterised from rhizobacteria isolated from the rhizosphere of soybean (*Glycine max* L.). The strains characterised were isolated from the roots of plants grown in soil gathered from different pedoclimatic regions of Ethiopia (Figure 1). Results are expressed from a two-stage process, with 231 strains characterised at stage-one. A sub-set of 72 (highlighted in grey) were selected on the basis of their PGP potential and additional traits were assessed at the second characterisation stage. Data are expressed relative to the % of the total number of Gram-positive and -negative strains assessed, which for the 231 isolates screened at stage one was 96 and 135, respectively. For the 72 isolates screened at stage 2, this was 30 and 42, respectively.

PGP trait	Value	Total No. isolates tested	No. strains with PGP potential (% of total)	Gram-type (% of total)	
				Positive	Negative
IAA production	8–143 (µg ml^{-1})	231	86	93	81
$(Ca)_3PO_4$ solubilisation	1.11–2.75 (SI[a])		13	5	19
$AlPO_4$ solubilisation	1.08–2.57 (SI)		12	1.0	20
$(Ca)_3PO_4$ and $AlPO_4$	1.08–2.75 (SI)		10	1.0	16
BNF potential	+/-		16	16	17
Antifungal activity	19–82 (PIRG[b])		13	7	16
Cellulase	+/-	72	38	24	57
Chitinase	1.11–2.75 (SI[a])		43	55	27
Protease	+/-		60	69	47
HCN	+/-		21	31	7

[a], solubilisation index. [b], % inhibition of fungal radial growth.

Table 2. The identity of soybean rhizobacteria (SR) denoted by their isolate 'SR' Code and genus and/or species, the latter determined from NCBI-BLAST similarity with the isolates 16S-rRNA gene sequence data. Plant growth promoting (PGP) traits are indicated in number values or "+" and "-" entries denote traits where no functional capacity was evident.

Isolate SR Code	Genus/ species (as determined by NCBI-BLAST similarity)	Gram-type	$Ca_3(PO4)_2$ (SI[a])	$AlPO_4$ (SI[a])	IAA (µg ml^{-1})	BNF potential[d]	PIRG[b]	HCN[e]	Protease	Cellulase	Chitinase
3A	*Pseudomonas* sp.	-	1.5	1.2	74	+++	49	+++	+	-	-
6A	*Bacillus thuringiensis*	+	1.3	-	94	+++	-	-	-	-	-
7A	*Microbacterium oxydans*	+	2.1	2.3	59	-	-	-	-	-	-
10A	*Acinetobacter* sp.	-	2.3	2.6	41	+	-	-	-	-	-
14	*Stenotrophomonas maltophilia*	-	1.1	1.1	71	++	48	-	+	-	2.8
20A	*Achromobacter mucicolens*	-	2.2	2.6	47	+	-	-	-	-	-
20B	*Acinetobacter* sp.	-	2.3	2.4	47	+	-	-	-	-	-
29A	*Stenotrophomonas maltophilia*	-	1.4	1.7	50	+++	51	-	+	-	2.0
29B	*Stenotrophomonas* sp.	-	1.6	1.7	59	+++	37	-	+	-	2.5
40	*Acinetobacter* sp.	-	2.8	2.2	43	-	-	-	-	-	-
41B	*Pseudomonas lini*	-	1.2	1.4	52	++	-	-	+	-	-
43B	*Stenotrophomonas maltophilia*	-	1.2	-	37	-	82	+++	+	+	2.8
44B	*Stenotrophomonas* sp.	-	1.4	1.5	60	-	-	-	-	-	-
45B	*Pseudomonas monteilii*	-	-	1.3	101	+	-	-	-	+	2.5
47B	*Pseudomonas* sp.	-	1.9	1.5	59	-	-	-	-	-	-
48B	*Enterobacter* sp.	-	1.6	1.1	36	+	19	-	+	-	-
50	*Pseudomonas* sp.	-	1.1	2	52	++	62	+	+	+	2.2
69B	*Bacillus* sp.	+	1.3	-	143	-	-	-	-	-	-
77	*Pseudomonas* sp.	-	1.8	2	40	-	27	+++	+	-	-
99B	*Pseudomonas* sp.	-	2.4	1.8	8	-	44	+	+	+	-

[a], solubilisation index. [b], % inhibition of fungal radial growth. [d]BNF potential and [e]Capacity of HCN production scored as either; +, ++ or +++, indicating slight, moderate or high growth, respectively.

Nitrogen fixation

Of the 38 isolates which could grow in Burk's N-free medium, 12 isolates (including: *Pseudomonas* sp. SR3A, *Bacillus thuringiensis* SR6A, *Stenotrophomonas maltophilia* SR29A and *Stenotrophomonas* sp. SR29B) produced conspicuous pellicles indicating their better potential for BNF and/or for N uptake- and use-efficiency (Table 2). The BNF potential capacity of *Bacillus* species isolated from soybean rhizosphere was previously reported (Park et al., 2005; Masciarelli et al., 2014), however the presence of key *nif* genes, indicative of BNF, remains to be carried out for the isolates identified here.

Anti-fungal activity

Twenty nine of the 231 rhizobacteria isolates showed *in vitro* antagonistic activity against the fungal pathogen *Fusarium oxysporum*, which causes soybean root-rot. PIRG values ranged from 82% (*Stenotrophomonas maltophilia* SR43B) to 19% (*Enterobacter* sp. SR48B) (Table 2 and Supplementary Table 1). Fifteen, 13 and 7 isolates with antifungal activities produced chitinase, HCN, and both chitinase and HCN, respectively which could contribute to fungal inhibition. The PIRG values reported here are similar to those 30 to 40% reported for *Baccilus* (Wahyudi et al., 2011b) and 11 to 60% reported for *Pseudomonas* (Susilowati et al., 2011) isolated from soybean rhizosphere.

HCN production

Of the 72 isolates tested, 15 demonstrated high, moderate or low levels of HCN production (Table 1 and Supplementary Table 2). HCN production was evident as indicated by the depth of colour change in the control (yellow) picric acid impregnated filter paper to dark-brown, brown or light-brown, respectively. These three classes were evident as: five dark brown/high HCN producing isolates: SR3A (*Pseudomonas* sp.), SR43B (*S. maltophilia*), SR77 (*Pseudomonas* sp.), SR32A and SR90B; two brown/moderate HCN producing isolates: SR48C and SR69C; eight light-brown/low HCN producing isolates (including *Pseudomonas* sp. SR99B). The ratio of HCN evolving rhizobacteria found here is less than the 36% for *Pseudomonas* species isolated from soybean rhizosphere and reported by Susilowati et al. (2011).

Production of hydrolytic enzymes: proteases, chitinase and cellulose

Among the 72 SR isolates selected for secondary screening, 43, 31 and 27 exhibited protease, chitinase and cellulase activities, respectively (Table 1 and Supplementary Table 2). Seven isolates: SR9B, SR43B, SR50, SR69A, SR70A, SR70B and SR102 showed activity for all the three enzymes. Of these isolates, SR43B, SR50, SR70B and SR102 also inhibited growth of *F. oxysporum* with PRIG values from 28 to 82. Four isolates: SR11, SR14, SR25B and SR43B demonstrated the highest chitin solubilization index (2.8). Soybean rhizobacteria showing chitinase activity (Wahyudi et al., 2011a) and/or cellulase activity (Leon et al., 2009; Masciarelli et al., 2014) has also been reported.

Tolerance of SR to various stress factors

Twenty five of the 72 selected isolates were tolerant to elevated temperature (40°C), wider pH range (5.5 to 8.5) and 4% [w/v] NaCl. However, higher temperature, NaCl concentration and lower pH limited the growth of isolates as only 9, 13 and 14 of them were able to grow at pH 4.5, 45°C and 7% NaCl (w/v), respectively (Table 3 and Supplementary Table 3). Interestingly, all the isolates which grew at pH 4.5 also grew at pH 9.0 indicating their wider tolerance to this factor. One isolate: SR14 (*Stenotrophomonas maltophilia*) showed higher salt (7% NaCl [w/v]) and high temperature (45ºC) tolerance traits combination which is similar to *Pantoea agglomerans* R-42 soybean rhizosphere isolate (Son et al., 2006).

Of the 72 SR isolates tested, 62 were tolerant to antibiotics: ampicillin, chloramphenicol, penicillin G and vancomycin. However, the majority of the isolates (50-64%) were sensitive to streptomycin, gentamycin, nalidixic acid and neomycin (Table 3 and Supplementary Table 4). A few SR (SR22, SR25A, SR25B, SR26, SR29A, SR43A, SR43B, SR45B, SR49A and SR50) were able to resist all of the tested antibiotics. Multiple antibiotic resistances among soybean rhizobacteria have been reported previously (Wang et al., 2009; Madhaiyan et al., 2010). Rhizobacterial antibiotic resistance is a trait of ecological significance as it may facilitate survival in soils where they may be exposed to various antibiotic producing microbes such as actinomycetes from which over 60% of the naturally occurring antibiotics were recorded (Huck et al., 1991).

Many SR isolates: 66, 65 and 60 were tolerant to compounds containing Pb (2.5 mM), Mn (0.25 mM), and Zn (0.25 mM), respectively (Table 3). However, only 21, 13 or 4, of the 72 SR screened, managed to tolerate compounds containing Co (0.5 mM), Cr (0.25 mM) and Cu (0.25 mM), respectively. Only 5 SR isolates were not tolerant to any of the heavy metal containing compounds to which they were exposed (Supplementary Table 3).

We screened SR isolates for their tolerance to pesticides. After exposing them to recommended pesticide doses, the SR isolates were sensitive to the pesticides tested as none of them tolerated the fungicide Curzate®, and only 35 and 10 of the isolates were tolerant to glyphosate and mancozeb, respectively (Table 3). A

Table 3. Percentage data for isolates from second stage characterisation that were able to tolerate, survive and grow when exposed to a range of various environmental and biophysical factors including: temperature; pH; osmotic and ionic stress, due to salt (NaCl); toxic heavy metals; antibiotics; and pesticides.

Temperature (ºC)			pH						% NaCl [w/v]			
37	**40**	**45**	**4.5**	**5**	**5.5**	**8.5**	**9**	**3**	**4**	**5**	**6**	**7**
97	67	18	13	29	75	99	92	83	65	44	22	19

Antibiotic (µg mL^{-1})[a]									Pesticides (g L^{-1})		
Amp. (100)	**Chl. (5)**	**Pen. (20)**	**Van. (5)**	**Ery. (100)**	**Str. (20)**	**Gen. (50)**	**Nal. (50)**	**Neo. (50)**	**Glyphosate (1.4)**	**Mancozeb (2)**	**Curzet (2)**
89	88	86	86	53	50	46	42	36	49	14	0

Heavy metals					
$Pb(CH_3COO)_2*3H_2O$ (2.5 mM)	**$MnSO_4*4H_2O$ (0.25 mM)**	**$ZnSO_4*5H_2O$ (0.25 mM)**	**$CoCl_2*6H_2O$ (0.5 mM)**	**$K_2Cr_2O_7$ (0.25 mM)**	**$CuCl_2*2H_2O$ (0.25 mM)**
92	90	83	29	18	6

[a] The acronyms for the various antibiotics tested are defined as follows: Amp., ampicillin; Chl., chloramphenicol; Pen., penicillin G; Van., vancomycin; Ery., erythromycin; Str., streptomycin sulfate; Gen., gentamycin; Nal., naldixic acid; and, Neo., neomycin.

small proportion of the isolates (11%), including SR8, SR20A, SR32A, SR32B, SR39, SR44B, SR46B and SR50 were tolerant to both mancozeb and glyphosate. Intensive use of pesticides with long half-life may accumulate in the soil and impact negatively on PGPR diversity. The 20 isolates selected for genetic identification on the basis of the number of their potential PGP traits and number of stresses they could tolerate, were predominated by Gram-negative though a general purpose medium (NA) was used for their isolation.

Multivariate cluster analysis of SR functional traits

Multivariate cluster analysis of functional trait data of the 72 SR isolates distinguished them into two broad groups (A and B) with around 36% similarity level, and into further four groups at about 44% similarity level (Figure 2). No phenotypic clusters were formed beyond 96% similarity level. The phenotype clusters aligned with identity of some of the isolates distinguished by their *16S rRNA* gene sequence data (Figure 3). All the 20 potential PGP SR isolates appeared in the clade A (Figure 2). 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 (Figure 2). Similarly, all the three *Acinetabacter* species were clustered together at around 85% similarity level. *Pseudomonas* species were clustered within different groups/clades of the rhizobacteria except three of them that clustered together at about 72% similarity level (Figure 2). However, genetically different groups of rhizobacteria such as SR7A (*Microbacterium*) and SR40 (*Acinetobacter*) were clustered together at 96% similarity level (Figure 2). Such overlapping of phenotypic traits of genetically distinct rhizobacteria may account for compensatory effects when soil rhizosphere diversity may be limiting (Paul and Clark, 1989).

Genetic characterisation of SR identified as potentially PGP

Based on the *16S rRNA* gene sequence data, the 20 potential PGP SR were identified as members of the bacterial genera *Achromobacter*, *Acinetobacter*, *Bacillus*, *Enterobacter*, *Microbacterium*, *Pseudomonas* and *Stenotrophomonas* distributed in 4 clades (I–IV), the majority (16) being within *Gammaproteobacteria* (Figure 3; Table 4; Supplementary Table 5). The *Gammaproteobacteria* included 4 genera of SR

Table 4. The 20 selected soybean rhizobacteria (SR) isolates ranked according to their percentage of number of their plant growth promoting (PGP) traits and percentage of number of tolerated stresses. Numbers under the column PGP indicate percentage of number PGP traits demonstrated out of the total tested (9); tricalcium phosphate solubilisation, $AlPO_4$ solubilisation, $FePO_4$ solubilisation, IAA production, BNF potential, PIRG, HCN, protease, cellulase and chitinase activity). Numbers in columns under pH to pesticides indicate the percentage of number of stresses tolerated out of the total tested for each stress given in brackets; pH (8), temperature (4), NaCl (7), heavy metals (6), antibiotics (9) and pesticides (3).

Isolate SR Code	Genus/ species (as determined by NCBI-BLAST similarity)	Clade*	Gram-Type	PGP traits	pH	Temperature	NaCl	Heavy metals	Antibiotics	Pesticides	Total [a]	Rank
50	*Pseudomonas* sp.	III	-	90	88	75	100	67	100	67	587	1
14	*Stenotrophomonas maltophilia*	III	-	70	88	100	100	67	89	33	547	2
43B	*Stenotrophomonas maltophilia*	IV	-	70	63	75	57	67	100	33	465	3
29A	*Stenotrophomonas maltophilia*	IV	-	70	63	75	71	50	100	33	462	4
20A	*Achromobacter mucicolens*	I	-	40	75	100	71	50	56	67	459	5
29B	*Stenotrophomonas* sp.	IV	-	70	63	75	71	50	89	33	451	6
44B	*Stenotrophomonas* sp.	I	-	60	63	50	86	50	67	67	443	7
45B	*Pseudomonas monteilii*	IV	-	30	63	75	71	50	100	33	422	8
3A	*Pseudomonas* sp.	I	-	70	50	75	57	50	56	33	391	9
48B	*Enterobacter* sp.	II	-	60	63	50	71	67	56	0	367	10
6A	*Bacillus thuringiensis*	II	+	30	63	75	57	67	67	0	359	11
20B	*Acinetobacter* sp.	II	-	40	63	100	43	67	44	0	357	12
41B	*Pseudomonas lini*	II	-	40	63	75	43	50	33	33	357	12
99B	*Pseudomonas* sp.	I	-	70	50	50	57	33	56	33	349	14
69B	*Bacillus thuringensis*	II	+	20	63	100	29	67	67	0	346	15
7A	*Microbacterium oxydans*	II	+	30	75	75	43	50	44	0	317	16
40	*Acinetobacter* sp.	II	-	30	75	75	43	50	44	0	317	16
77	*Pseudomonas* sp.	I	-	60	50	25	43	50	56	33	317	16
10A	*Acinetobacter* sp.	II	-	40	63	75	43	50	44	0	315	19
47B	*Pseudomonas* sp.	II	-	30	63	50	29	67	44	0	283	20

[a] Total refers to sum of percentages of demonstrated PGP traits (11) and tolerated pH (8), temperature (4), NaCl (7), heavy metals (6), antibiotics (9) and pesticides (3);* clades in the dendrogam generated by the analysis of phenotypic traits.

isolates, such as *Pseudomonas*, *Acinetobacter*, *Stenotrophomonas* and *Enterobacter. Pseudomonas* SR isolates were: SR3A, SR41B, SR45B, SR47B, SR50, SR77 and SR99 – all with 99% *16S rRNA* gene sequence similarity to NCBI reference strains (Supplementary Table 5).

In the phylogenetic analysis (Figure 3), SR3A and SR99B were closely clustered with *Pseudomonas lini* H2P2; SR41B with *Pseudomonas lini* N5; SR45B and SR47B with *Pseudomonas plecoglossicida* E3.NA; SR77 with *Pseudomonas clemoncea* PE22; and SR50 with *Pseudomonas putida* S28. The genus *Stenotrophomonas* included five of the SR isolates: SR14, SR29A, SR29B, SR43B and SR44B having *16S rRNA* gene sequence homology (97 to 99%) with *Stenotrophomonas maltophilia* 81, *S. maltophilia* T25, *Stenotrophomonas* sp. CanR-73, *S. maltophilia* 81 and *Stenotrophomonas* sp. DNPA8, respectively (Supplementary Table 5). The genus *Acinetobacter* included three isolates (SR10A, SR20B and SR40; Supplementary Table 5).

Actinobacteria, Bacilli and Betaproteobacteria included one (SR7A), two (SR6A and SR69B) and one (SR20A) of the isolates, respectively (Figure 3). Isolate SR7A possessed 99% *16S rRNA* gene sequence similarity with *Microbacterium oxydan* M90 (Supplementary Table 5) and closely clustered with *Microbacterium martipicum* ST5 (Figure 3). Isolate SR6A showed 100% *16S rRNA* gene sequence similarity with *Bacillus thuringiensis* CTC strain (Supplementary Table 5), whereas SR69B shared 99% *16S rRNA* gene sequence similarity with *Bacillus thuringiensis* XL6 (Supplementary Table 5) and both isolates (SR6A and SR69B) clustered with *Bacillus cereus* strains (Figure 3). SR20A shared 99% *16S rRNA* gene sequence similarity with *Achromobacter mucicolens* OZK37 (Supplementary Table 5), and clustered with *Achromobacter* sp. DP147B within Betaproteobacteria

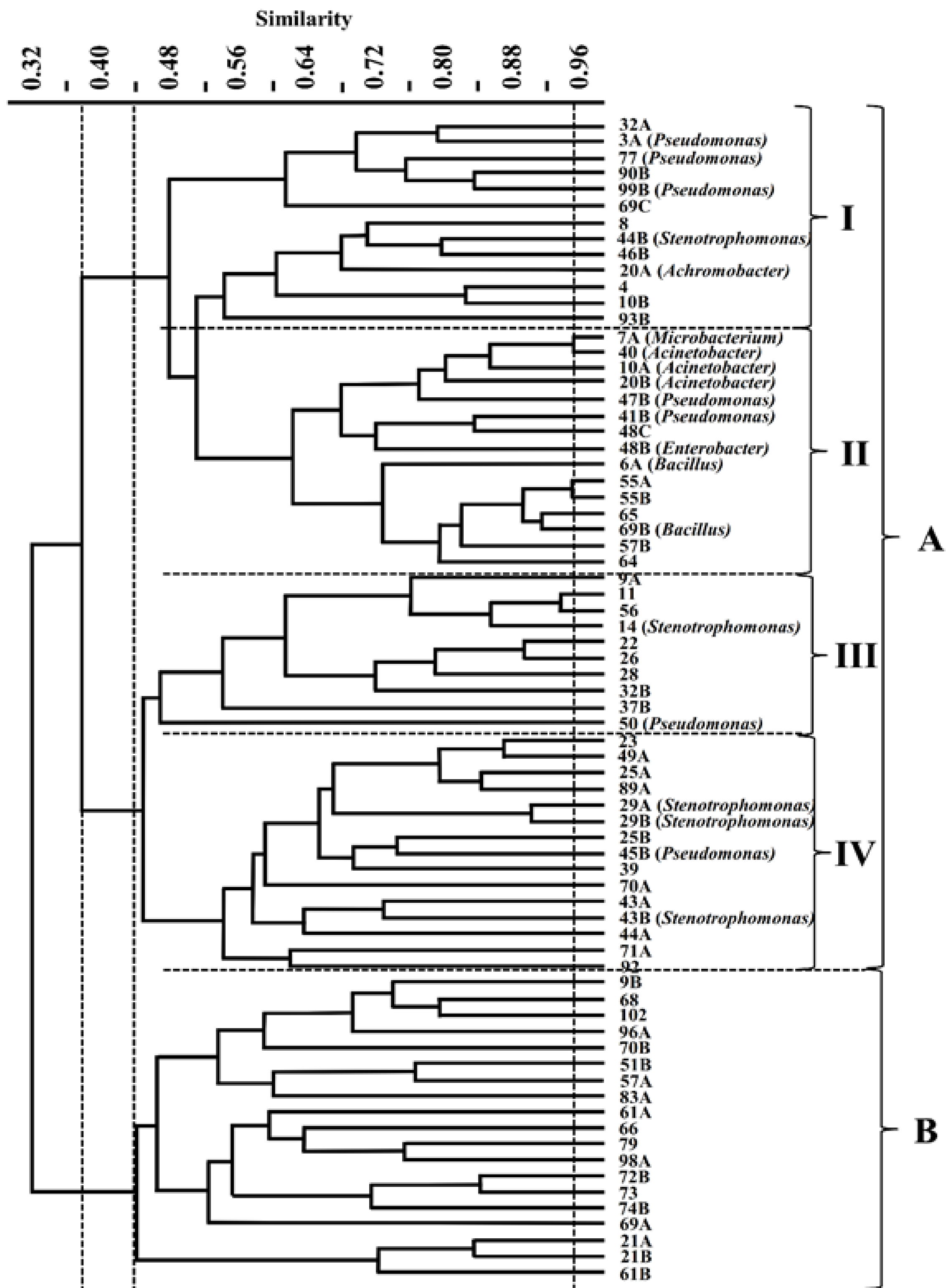

Figure 2. A functional diversity phylogram generated from the multivariate cluster analysis of data for potential plant growth promoting (PGP; stage 1 screening) and stress tolerance-traits (and some PGP traits; stage 2 screening), for 72 soybean rhizobacteria (SR). The image shown illustrates the functional similarity of SR, which are identified by their code number. For 20 potential PGP SR (Table 4), their genus (in parenthesis) as determined by 16S-rRNA gene sequence data is also shown. Characterisation in this way demonstrates two broad functional groups 'A' and 'B', discriminated at the 38% similarity level (dotted line). Also, within group A are four functionally distinct clades (I–IV), which were discriminated at the 44% similarity level (dotted lines).

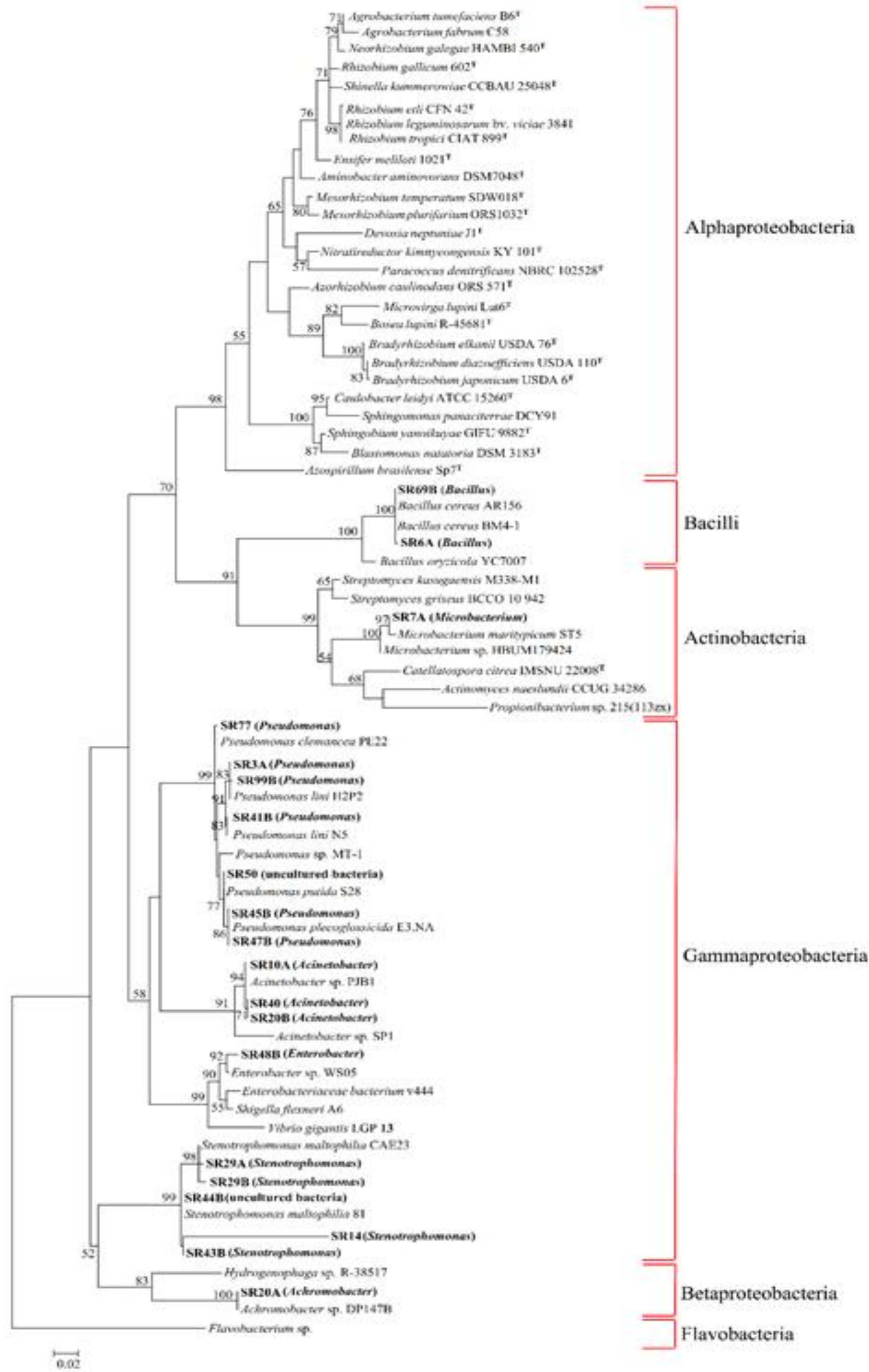

Figure 3. Phylogenetic trees showing similarity based upon 16S rRNA gene PCR product sequences obtained from the 20 selected soybean rhizobacterial (SR) isolates. The phylgenetic tree shows the localisation of the SR isolate derived data, relative to sequence information for the same gene region for other rhizobacteria (obtained from the NCBI database). Type strains are denoted ^{T}. The tree shown gave the highest log likelihood (-7016.5), and is rooted using *Flavobacterium* sp. All trees were generated using the Maximum Likelihood method (1000 bootstraps), and only bootstrap values >50 are given. The values shown at branch-points give % (of total bootstraps/trees) at which the isolates shown clustered together.

Table 5. The effects of six different soybean rhizobacteria (SR) with plant growth promoting (PGP) potential upon seed % germination and seedling traits. The values shown are means ± SE (n=3). Statistically significant differences are noted in grey shaded cells, where P<0.01. **NS** denotes parameters for which to statistical differences could not be found (p<0.05) over the control.

Isolate SR code	Germination % [NS]	Root length (cm) [NS]	Shoot length (cm)	Vigor index [NS]
Control	73 ± 21	4.5 ± 1.8	1.5^{c} ± 0.3	435 ± 221
7A	83 ± 16	4.1 ± 1.9	1.8^{abc} ± 0.1	487 ± 21
10A	80 ± 0	5.6 ± 1.0	1.7^{abc}± 0.2	585 ± 12
20A	87 ± 6	7.2 ± 1.0	2.2^{a} ± 0.2	810 ± 115
20B	83 ± 15	6.3 ± 1.1	2.0^{ab} ± 0.0	689 ± 207
43B	73± 6	4.8 ± 1.2	1.6^{bc} ± 0.2	467 ± 120
69B	67 ± 15	4.9 ± 0.9	1.6^{bc} ± 0.2	437 ± 136

(Figure 3).

In previous studies, the dominant soybean rhizobacteria with potential PGP traits were *Pseudomonas* (Park et al., 2005; Leon et al., 2009; Wahyudi et al., 2010; Susilowati et al., 2011; Wahyudi et al., 2011a; 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). *Achromobacter* species with PGP traits were identified from rhizosphere of maize (Bumunang and Babalola, 2014), *Brassica juncea* (Ma et al., 2010) and tobacco (Huang et al., 2015), but rarely reported as SR isolates. Egamberdieva et al. (2016) reported *Achromobacter* species from the soybean rhizosphere grown in soil amended with 2% hydrochar (HTC) from maize silage rather than from those plants grown in non-amended soil under greenhouse condition – here the authors argued that the HTC had a positive effect on PGPR diversity.

Seed germination assay

Seed germination assay with selected rhizobacterial isolates showed differences (Table 5). Accordingly, SR20A (*Achromobacter mucicolens*) impacted the highest effect on 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) over the control, though the differences were statistically significant only with respect to shoot length (Table 5). Likewise, SR7A (*Microbacterium oxydans*) and SR10A (*Acinetobacter*) showed moderate effect on most of the germination and growth parameters. It is interesting to note that SR69B (*Bacillus* sp.) produced the largest quantity of IAA (143 $\mu g{*}ml^{-1}$) 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. 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 the 0.01 significance level (data not shown).

Similarly, the enhancement of soybean seed germination (68 to 82% compared to 62% for the control) and seedling growth (up to 8.2 cm compared to 7.2 cm for the control within five days after germination) by culture filtrate of some unidentified soybean rhizobacterial isolates was reported by 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 covert seed starch to metabolisable sugars (Gholami et al., 2009).

Concluding remarks

Fifty-eight (81%) of the selected 72 SR isolates demonstrated multiple potential PGP (3-9) and stress tolerance traits. Predominated by *Pseudomonas* and *Stenotrophomonas*, Gram-negative SR constituted 85% of the 20-potential soybean PGPR identified via *16S rRNA* gene sequencing. Tests to discern whether the SR isolates described here possess PGP potential that extends to improvement of yield and yield qualities remains to be shown. Nevertheless, there is proven potential demonstrated here that specific PGP rhizobacteria can enhance germination and seedling development. The impact of this may confer a functional advantage at agroecologically important early and vulnerable life history stages. Also, we highlight that the exact mechanism(s) underpinning such benefits also remains to be demonstrated. For example, the question

should be posed: *to what extent is any improvement in plant fitness the direct result of an applied microbe - as opposed to the action of other factors, such as a facilitative effects other soil microorganism, which have occurred as synergistic response to the applied rhizobacteria?* Such considerations also highlight the importance of testing PGP potential of inoculants in sterile rooting media, as well as soil. Such future research foci could provide impetus towards the development of PGPR, or/and exploitation of the underpinning mechanisms, as a relatively inexpensive alternatives to current dependencies on expensive agrochemicals, including fertilisers and pesticides.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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