SEED TREATMENT WITH SELECTED PLANT GROWTH PROMOTING RHIZOBACTERIA INCREASES MAIZE YIELD IN THE FIELD

G. Breedt[1]*, T.A. Coutinho[2] and N. Labuschagne[3]

[1]Towoomba ADC, Limpopo Department of Agriculture and Rural development, Private bag X1615, Bela-Bela, 0480[1]

[2]Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Private Bag X20 Hatfield 0028

[3]Department of Plant and Soil Science, University of Pretoria, Private Bag X20 Hatfield 0028

SUMMARY

Maize (*Zea Mays L.*) is the most important grain crop in South Africa and is a staple food in many African countries. The beneficial effects of plant growth promoting rhizobacteria (PGPR) on crop growth and yield have been well documented, but obtaining reproducible results under field conditions is often difficult. In the current study, five selected rhizobacterial strains that showed plant growth promoting activities in pilot studies were evaluated for potential enhancement of maize yield under field conditions. The five strains together with a commercial standard were assessed as seed treatments of maize over three seasons in four different soil types. The strains were identified on the basis of 16S rRNA sequencing as *Lysinibacillus sphaericus* (T19), *Paenibacillus alvei* (T29), *Bacillus safensis* (S7) *Bacillus pumilus* (A26) and *Brevundimonas vesicularis* (A40). The best yield increases in maize were obtained during the 2011/2012 and 2012/13 seasons in the Shortlands ecotope with the rhizobacterial strains T19 , T29 and S7, resulting in yield increases ranging from 24% to 34%. Strain T19 rendered the most consistent yield increases during the three successive field trials amounting to 33% and 24% in Shortlands ecotope and 12% in Clovalley ecotope respectively. During 2013/14 a consortium of 3 strains viz. T19, S7 and

A26 gave a 32 % yield increase in Clovalley ecotope. All the rhizobacterial strains solubilised phosphate *in vitro* except T19. Strain T29 showed the best nitrogen fixing activity *in vitro*, proliferating on a nitrogen free substrate and also producing ammonia. All the strains tested positive for indole acetic acid (IAA) production. The current study demonstrates the ability of rhizobacterial strains T19, T29, S7 and A26 applied as seed treatments to significantly enhance maize yield in the field, making development and commercialization of these strains a viable option.

Keywords: Plant growth promoting rhizobacteria, biofertilizers, maize.

INTRODUCTION

There is a growing volume of evidence for effective application of microbes, especially rhizobacteria, in agriculture for the purpose of enhanced crop yield and health (Zahir *et al*. 2004). Furthermore, there is an increased need to curb the use of synthetic fertilizers due to ecological concerns such as contamination of ground water (Spalding & Exner 1993). The combined use of microbial and chemical fertilizers has been demonstrated as a viable approach to reduce excessive use of chemical fertilizers whilst maintaining yields (Stancheva *et al*. 1992; Dobbelaere *et al*. 2001).

Inoculation of maize with rhizobacterial strains has previously been shown to enhance maize seed germination, seedling growth (Rudolph *et al*. 2015) and yield (Chen *et al*. 1994; Fallik & Okon 1996; Zahir *et al*. 1998). The latter authors reported maize yield increases ranging from 11% to 19 % after treatment with rhizobacterial inoculants. Zahir *et al*. (1998) reported an increase in maize grain yield of 19.8% after seed inoculation with four strains each of *Azotobacter* spp. and *Pseudomonas* spp. under field conditions whilst Vedderweiss *et al.* (1999) reported increased shoot and root weight of maize seedlings inoculated with *Azospirillum* spp.

Although the positive effects of plant growth promoting rhizobacteria (PGPR) inoculation have been well documented, inconsistent results regarding plant growth promotion under field conditions are often experienced (Zahir *et al*. 2004). One reason for this could be competition of the native flora with the introduced rhizobacterial strains (Smith *et al.* 1992). The objective of the current study was to evaluate selected novel rhizobacterial strains applied singly and in a mixture, for enhancement of maize yield under field conditions in various soil types in South Africa and elucidating their *in vitro* plant growth promoting (PGP) traits.

MATERIALS AND METHODS

In vitro assays for PGPR traits

Bacterial Cultures

All rhizobacterial cultures were obtained from the University of Pretoria's PGPR culture collection. Strains were maintained using Microbank™ beads (Pro-Lab Diagnostics) stored at -70°C and streaked onto nutrient agar (Biolab, Wadeville) as needed.

Rhizobacterial Identification and phylogenetic tree construction

For identification, a pure culture of each PGPR strain was sent to Inqaba Biotechnical Industries (Hatfield, Gauteng, South Africa) for sequencing of the 16S rRNA gene region. The strains were identified based on species relatedness to other strains based on BLASTN searches in the NCBI data libraries. At Inqaba the DNA was extracted with Zymo Fungal/Bacterial DNA extraction kit (Zymo Research Corp.), the PCR performed using DreamTaq (Fermentas Life Sciences, DreamTacTM Green PCR Master Mix) and the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') used. The sequencing reaction was performed with ABI Big Dye v3.1 and the clean-up performed with the Zymo Sequencing Clean-up kit (ZR-96, DNA Sequencing Clean-up KitTM).

The phylogenetic tree was constructed using the strains 16S rRNA sequences and comparing the strains to the nucleotide sequences of the PGPR species listed in Gupta *et al.* (2015) and Vejan *et al.* (2016). The 16s rRNA sequences were aligned and a neighbour joining phylogenetic tree constructed using Mega 6 (Tamura *et al.* 2013) and the sequences submitted to the NCBI database for accession number allocation.

In vitro assays

Mineral phosphate solubilization

Phosphate solubilization was evaluated according to the procedures described by Nautiyal (1999) in Pikovskaya amended medium. The agar medium was prepared by amending bacteriological agar (Biolab, Wadeville) with 10g/l glucose, 5g/l NH_4Cl, 1g/l $MgSO_4.7H_2O$ and 5mg/ml $Ca_3(PO_4)_2$ and adjusting the pH to 7.2. The media was then autoclaved at 121^0C for 20 minutes and left to cool to handling temperature before pouring into sterile 90mm Petri-dishes in a laminar flow cabinet.

The rhizobacterial strains were stab-inoculated into the Pikovskaya amended media with a flame sterilised inoculation needle. Four strains were inoculated at a 90^0 angle per plate using five replicates per strain. The plates were incubated for 5 days at 25^oC. A positive reaction for phosphate solubilization was recorded when a clear halo developed around the rhizobacterial colony in the Pikovskaya medium.

Assessment of atmospheric nitrogen fixing ability

The rhizobacterial strains were evaluated for asymbiotic nitrogen fixation and ammonia production. The Nessler's reagent test (Dye 1962) was used to test for nitrogen production in nitrogen free media in order to test for nitrogen leakage.

Nitrogen fixation – growth in N free medium

Winogradsky nitrogen free medium was prepared as described by Tchan & New (1984). The Winogradsky semi-solid medium in test tubes and solid agar plates were prepared by adding 1.5g or 7g bacteriological agar to 500ml distilled water respectively.

The rhizobacterial strains were transferred to the agar plates by means of a flame sterilised inoculation loop and subsequently stab inoculated into the semi-solid media by means of a flamed inoculation needle. All treatments were replicated twice. The agar plates

and semi-solid media containing test tubes were incubated for 10 days at 25^{o}C before evaluating colony growth on the solid medium and pellicle formation in the test tubes (Baldani & Dobereiner 1980; Caceres, 1982).

Detection of ammonia production with Nessler's reagent

Ammonia production was assessed as described by Rana *et al.* (2012). One millilitre sterile peptone water medium (Biolab, Wadeville) was added to each sterile test tube and inoculated with the respective rhizobacterial strains using a flamed inoculation loop, sealed with Parafilm and labelled. This was done in triplicate for all treatments before incubating for 3 days at 25^{o}C on a rotary shaker. A brown/yellow colour change in the peptone water medium indicated a positive reaction for ammonia (Dye 1962).

IAA production

IAA production was tested according to the S2/1 method as described by Glickmann & Dessaux (1995). Sterile nutrient broth (100ml) was inoculated with each rhizobacterial strain respectively and placed on a rotary shaker for 48 hours at 25°C and 150rpm. The cultures were then transferred to sterile 50ml conical tubes and centrifuged at 3000 x g for 10 minutes. The supernatant (1 ml) was transferred to a test tube containing 2 ml of Salkowski's reagent. The Salkowski reagent was prepared by slowly adding 4.5g $FeCl_3$ to 1litre of 10.8 M (67%) H_2SO_4. As a control only Salkowski reagent was added to the sterile nutrient broth. A colour change to yellow-brown in the solution indicated the presence of IAA.

Field trials

Rhizobacterial strains were selected from the University of Pretoria's PGPR culture collection, based on their performance in previous greenhouse trials on wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) (data not shown). The commercial product Brus® (Stimuplant®, Gauteng, South Africa), was also included in the study for comparative

purposes. The rhizobacterial strains were grown for 48h in sterile nutrient broth (Biolab, Wadeville, South Africa) at 25^{o}C in a shake incubator. Subsequently 200g quantities of sterile Perlite® powder in sealed autoclavable plastic pouches were inoculated with 21ml of the 48h old nutrient broth culture of the respective rhizobacterial strains and incubated for 14 days at ambient temperature. Maize seed (cultivar P1615R, Pioneer®, Rosslyn, Gauteng, South Africa) was treated with the respective inoculants at a rate of 200g Perlite® powder inoculum per 50kg of maize seed (Stimuplant® commercial recommendations). For the control treatment the seed was coated with sterile Perlite® powder before planting (200g powder per 50kg of maize seed).

Three sets of maize field trials were conducted over three consecutive seasons from 2011 to 2013 on the Southern part of the Springbok flats, south east of Bela Bela in the Limpopo Province (28°21'E, 24°25'S; 1 184 m above sea level), South Africa. The trial areas are situated in the summer rainfall area with a long-term average annual rainfall (60 year average) of 627 mm per annum (Towoomba Agricultural Development Centre weather station data). The long-term daily average maximum and minimum temperatures at Towoomba ADC vary between 29.7°C and 16.5°C for December and 20.8°C and 3.0°C for July respectively (Towoomba ADC weather station data).

The trials were planted in four different soil types namely Arcadian, Clovalley, Huttons and Shortlands ecotope, identified according to the Soil Classification Working Group (1991). The trials were conducted under dry-land conditions except for the trial in the Shortlands ecotope that received supplementary irrigation during the 2012/13 season when drought stress was observed in the crop. During the 2012/13 season insufficient rainfall and high prevailing temperatures caused crop failure in the dryland trials in Huttons and Arcadian ecotopes. The trial conducted in Shortlands ecotope received supplementary irrigation and was successfully completed. In the trials conducted during the 2011/12 and 2012/13 seasons

in Arcadian, Huttons and Shortlands ecotopes, the treatments were as follows: a) Untreated control, b) individually applied bacterial strains T19, A40, A-29, S7, A26 and the commercial product Brus® was included as a standard. The trial in the Clovalley ecotope was only conducted during the 2013 season and the treatments consisted of the best performing rhizobacterial strains from the previous trials namely T19, a mixture of T19, S7 and A26 . The commercial product Brus® was again included for comparative purposes.

The maize seed cultivar P1615R (Roundup ready®, Pioneer®,Gauteng, South Africa) was selected for all the trials based on its suitability to hot climates and high yield potential of 13.52t/ha under irrigation.

The row spacing for all trial sites was 0.9 m and 0.5 m inter and intra row spacing respectively as prescribed for dryland conditions (Du Plessis 2003). The trial layout was a completely randomized design (CRD) and each treatment consisted of four 200 m long rows that were replicated 18 times per treatment. Based on the soil analyses conducted by the University of Pretoria's Soil Science Laboratory, soil nitrogen and phosphate levels were amended to 100kg N/ha and 75kg P/ha as per commercial production recommendations (Du Plessis 2003) at planting, using ammonium nitrate (280 g/kg LAN) and superphosphate (10.5%) (Omnia©, Bryanston, South Africa).

Data collection

In each trial a total of 280 plants were harvested per replicate, per treatment, from only the two inner rows at approximately 12 % grain moisture. After harvesting, three individual grain samples were taken per replicate and the average grain moisture content determined in order to re-calculate the grain yield mass of all samples according to a standard of 12 % moisture content.

Statistical analysis

All data were analysed using proc GLM procedures of SAS 9.4 at P=0.05. Means were separated using Fishers Least significant test.

RESULTS

Rhizobacterial identifications

The bacterial strains were identified as T19 *Lysinibacillus sphearicus* (accession number: KY575152), S7 as *Bacillus safensis* (accession number: KY575342), A40 as *Brevundimonas vesicularis* (accession number: KY575154), A26 as *Bacillus pumilus* (accession number: KY575343) and T29 as *Paenibacillus sp.* (accession number: KY575153).

In vitro PGPR traits

Mineral phosphate solubilization

All the rhizobacterial strains except T19 and the commercial product Brus® were able to solubilise phosphate (Table 1). Strain A40 showed the greatest phosphate solubilization activity, producing a halo of more than 3mm radius.

Assessment of atmospheric nitrogen fixing ability

Rhizobacterial strain T29 had the best atmospheric nitrogen fixing ability, testing positive in the assays on N-free semi-solid and solid media (Table 1). All the strains tested positive for ammonia production with the exception of S7, although this strain did grow in the nitrogen free semi-solid and solid media.

IAA production

All the strains tested positive for production of the plant growth hormone IAA except for the commercial inoculant Brus® (Table 1).

Field trials

In the field trial conducted during 2011/2012 in Huttons ecotope , the best results were obtained with the product Brus® and A26 as seed treatments, resulting in significant yield increases of 19.88% and 19.17 % respectively, followed by treatments with A40 (13.82%), T19 (13.26%) and T29 (7.33%) (Table 1). However, in the Arcadian ecotope all the rhizobacterial strains caused a reduction in yield. In the Shortlands ecotope trial treatments with T19 and T29 resulted in significant yield increases of 33.69% and 30.36% respectively, followed by treatments with Brus® (16.65% increase) and S7 (8.4% increase) (Table 1). In comparison with the untreated control yielding 8.54t/ha, this amounted to actual increases in terms of tonnes per hectare of 2.88t/ha; 2.5t/ha; 1.42t/ha and 0.72t/ha for T19, T29, Brus and S7, respectively. In contrast, seed treatment with strain A26 caused a 11% reduction in yield.

During the 2012/13 season, in the Shortlands ecotope (trails receiving supplementary irrigation), significant yield increases were recorded for maize treated with S7 (34.14%), T19 (24.63%), A26 (17.93%) and T29 (13.80%) (Table 2). In comparison to the untreated control that yielded 3.8t/ha, the actual yield increases amounted to 4.7t/ha; 5.11t/ha; 4.5t/ha and 4.3t/ha for S7; T19; A26 and T29, respectively. Treatment with the commercial product Brus® did not result in a significant yield increase whereas treatment with A40 resulted in a 5.9% reduction in yield. The rhizobacterial strain that gave the most consistent results over the two seasons was T19 rendering a 33.6% yield increase during the 2011/2012 season and a 24.6% increase during the 2012/13 season.

In the trial conducted during the 2013/2014 season in the Clovalley ecotope the treatment with the mixture of 3 strains as well as the treatment with the single strain T19,

significantly increased maize yield by 24.72 % and 11.13 % respectively (Table 3). In terms of tonnes per hectare aforementioned yield increases amounted to 3.71t/ha and 3.15t/ha compared to the control.

DISCUSSION

In the current study, the selected rhizobacterial strains exhibited a variety of direct PGP traits including IAA production, phosphate solubilization and N-fixation (Table 4). All 5 strains produced IAA during the *in vitro* assays. Indole-3-acetic acid is a phytohormone known to be involved in root initiation, cell division, and cell enlargement (Salisbury 1994). IAA-producing PGPR are commonly believed to cause an increase in root growth and root length, resulting in greater root surface area, thereby enabling the plant to access more nutrients from soil (Vessey 2003).

Furthermore, all the rhizobacterial strains except T19 and Brus® (tested positive for phosphate solubilisation. Phosphorus is known to be the second most limiting nutrient after nitrogen i.t.o. plant growth. This is mainly due to the low availability thereof in soils even though large reserves may exist in the soil (Stevenson and Cole 1999). Phosphate solubilizing rhizobacteria enhance the availability of phosphorus by secretion of organic acids and phosphatases thereby converting phosphate to plant-available forms (Kim *et al.* 1998).

Although all 5 rhizobacterial strains tested positive for N-fixation in one or more of the assays, there is still little evidence that this would lead to agronomically significant levels of biological N-fixation (BNF) as PGPR's mode of action for the stimulation of plant growth is rarely credited to BNF (Vessey 2003).

The increase in maize yield obtained as a results of seed treatment with rhizobacterial strains in the current study is consistent with findings by other researchers, for example, Chen *et al*. (1994); Fallik & Okon (1996) and Zahir *et al*. (1998), who reported maize yield

increases ranging from 11% to 19 %. The large yield increases of 33.69 % and 30.36% obtained with T19 and T29 treatments, respectively, during the 2011/12 field trial in the current study are remarkable compared to the lower yield increases reported in the abovementioned studies. Strains T19 and S7 were previously demonstrated to enhance the early growth parameters of maize such as seed germination and seedling growth (Rudolph *et al.* 2015). This early growth enhancement will most probably be reflected in increased yields as was seen in the current field trials.

Bacterial treatments applied in the current study show a clear tendency to enhance crop yield in the lighter, nutrient poor Shortlands ecotope whereas yield increases were less pronounced in the Huttons ecotope or even reduced in the heavier, more fertile Arcadian ecotopes. Our observations of poor PGPR performance in heavier, more fertile soils and better performance in lighter, poorer soils are in agreement with similar findings by other researchers e.g. Fallik & Okon (1996) and Egamberdiyeva (2007). It was found by Paglia & De Nobili (1993) that soil porosity has a dramatic effect on plant enzyme activity and root development. They showed that urease activity dramatically decreased when soil pore size decreased while phosphatase activity was not significantly affected by pore size. These observations could explain the poor yields obtained in the Arcadian ecotopes with their high clay content, in the current study.

According to Babalola (2010) variability in PGPR results under field conditions can mainly be attributed to climatic conditions such as soil type, soil texture, temperature, water etc. Nevertheless, Gosling *et al.* (2006) were of the opinion that, because PGPR inoculants do enhance crop yield in comparison with untreated controls in natural soil, it is an indication that they are more efficient in promoting plant growth than the native rhizobacteria present in the controls. Our findings concur with those of Gosling *et al*. (2006) and Babalola (2010).

Various authors mentioned the difficulty of achieving consistent field performance of PGPR related to the heterogeneity of abiotic and biotic factors and competition with the indigenous micro-organisms (Nelson 2004; Wu *et al.* 2012). In our study, during the 2013/2014 field trial, the consortium of strains A26, T19 and S7 resulted in significantly better results than T19 applied on its own. The restrictions encountered under field conditions can therefore be overcome by applying a mixture of PGPR strains with a variety of plant growth promoting traits. The benefit of using mixtures of PGPR strains has been demonstrated in previous studies (e.g. Kannan & Sureendar 2009; Nain *et al.* 2010; Reddy *et al.* 2012.)

The current study demonstrates the ability of rhizobacterial strains T19, T29, S7 and A26, applied as seed treatments, to enhance maize yield in the field, thus making development and commercialization of these strains a viable option.

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TABLES

Table 1 *Effect of seed treatment with selected PGPR strains on maize yield under field conditions during the 2011/2012 season*

Treatments*	Yield (kg/ha) ‡		
	Huttons	Arcadia	Shortlands
T19	3421.94 ±181.59[c]	4743.75 ±616.73 [c]	11413.92 ±375.65 [e]
T29	3242.69 ±237.02 [b]	4187.07 ±368.79 [ab]	11132.34 ±494.88 [e]
S7	2971.84 ±446.75 [a]	4901.32 ±310.98 [d]	9254.66 ±748.09 [c]
A40	3438.74 ±81.82 [c]	4239.55 ±148.03 [b]	8742.95 ±1067.94 [b]
A26	3600.44 ±339.48 [d]	4075.96 ±236.88 [a]	7550.38 ±777.92 [a]
Brus®	3621.95 ±136.49 [d]	4293.58 ±95.45 [b]	9958.83 ±1073.60 [d]
Control	3021.29 ±232.25 [a]	5046.71 ±414.23 [d]	8537.51 ±851.86 [b]
P- Value	P < 0.001	P < 0.001	P < 0.001
LSD (0.05)	11.53	79.26	41.75

*T19, S7 A40 and T29 are rhizobacterial strains from the University of Pretoria's PGPR culture collection. Brus® is a commercial product of Stimuplant (Gauteng, South Africa).
‡ Yield determined at a moisture content of 12%.
§Treatment means followed by the same letter within the same column do not differ significantly, (P=0.05) according to the Least Significant Difference (LSD) test.

Table 2 *Effect of seed treatment with selected PGPR strains on maize yield under field conditions during the 2012/2013 season.*

Treatment*	Yield (kg/ha)‡
	Shortlands ecotope
T19	4755.75 [e]±420.20
T29	4342.61 [c]±368.38
S7	5118.78 [f]±417.48
A40	3595.18 [a]±364.78
A26	4500.34 [d]±435.69
Brus®	3870.44 [b]±507.54
Control	3816.00 [b]±578.01
P – Value	P < 0.001
LSD (0.05)	129.90

*T19, S7, A40, A26 and T29 are rhizobacterial strains from the University of Pretoria's PGPR culture collection whereas Brus® is a commercial product from Stimuplant© (Gauteng, South Africa).
‡ Yield determined at a moisture content of 12%.
§Treatment means within the same column followed by the same letter do not differ significantly, (P=0.05) according to the Least Significant Difference (LSD) test.

Table 3 *Effect of seed treatment with selected PGPR strains on yield of maize under field conditions during the 2013/2014 season.*

	Yield (kg/ha) ‡
Treatment*	Clovalley ecotope
T19	3150.88±201.58 [b]
Mix§	3711.64±180.38 [c]
Control	2794.24±57.93 [a]
P – Value	P < 0.001
LSD (0.05)	431.10

*T19 and mix are strains from the University of Pretoria's PGPR culture collection.
‡ Yield determined at a moisture content of 12%.
§ Mixture of the strains A26, T19 and S7.
§§Treatment means within the same column followed by the same letter do not differ significantly, (P=0.05) according to the Least Significant Difference (LSD) test.

Table 4 *In vitro PGPR traits exhibited by the rhizobacterial strains*

Rhizobacterial strain*	Identification (16srRNA)	Phosphate* solubilization	Nitrogen fixation†			IAA production§
			Semi-solid medium	Solid medium	Nessler's reagent‡	
T19	*Lysinibacillus sphearicus*	-	+	0	+	+
T29	*Paenibacillus sp.*	+	+	2	+	+
S7	*Bacillus safensis*	+	+	1	-	+
A40	*Brevundimonas vesicularis*	+++	-	1	+	+
A26	*Bacillus pumilus*	+	+	1	+	+
Brus®	Commercial standard	-	-	-	-	-

* Phosphate solubilization was assessed on Pikovskaya medium where a clearing zone constituted a positive reaction : – = no clearing zone, + = 0-1 mm, zone, ++ = 1-2 mm zone, +++ = 2-3mm zone.
† Nitrogen fixation was determined by colony formation in the solid media and pellicle formation in the semi-solid media by rhizobacterial strains inoculated in N-free media;0= no colony formation, 1= small colony formation, 2= profuse colony formation, - = no pellicle; + = presence of pellicle.
‡ Production of ammonia was indicated by a colour change to yellow brown +=positive for ammonia production -= no ammonia production
§ IAA production was indicated by a colour change to yellow-brown +=IAA produced, -= no IAA production

Figures

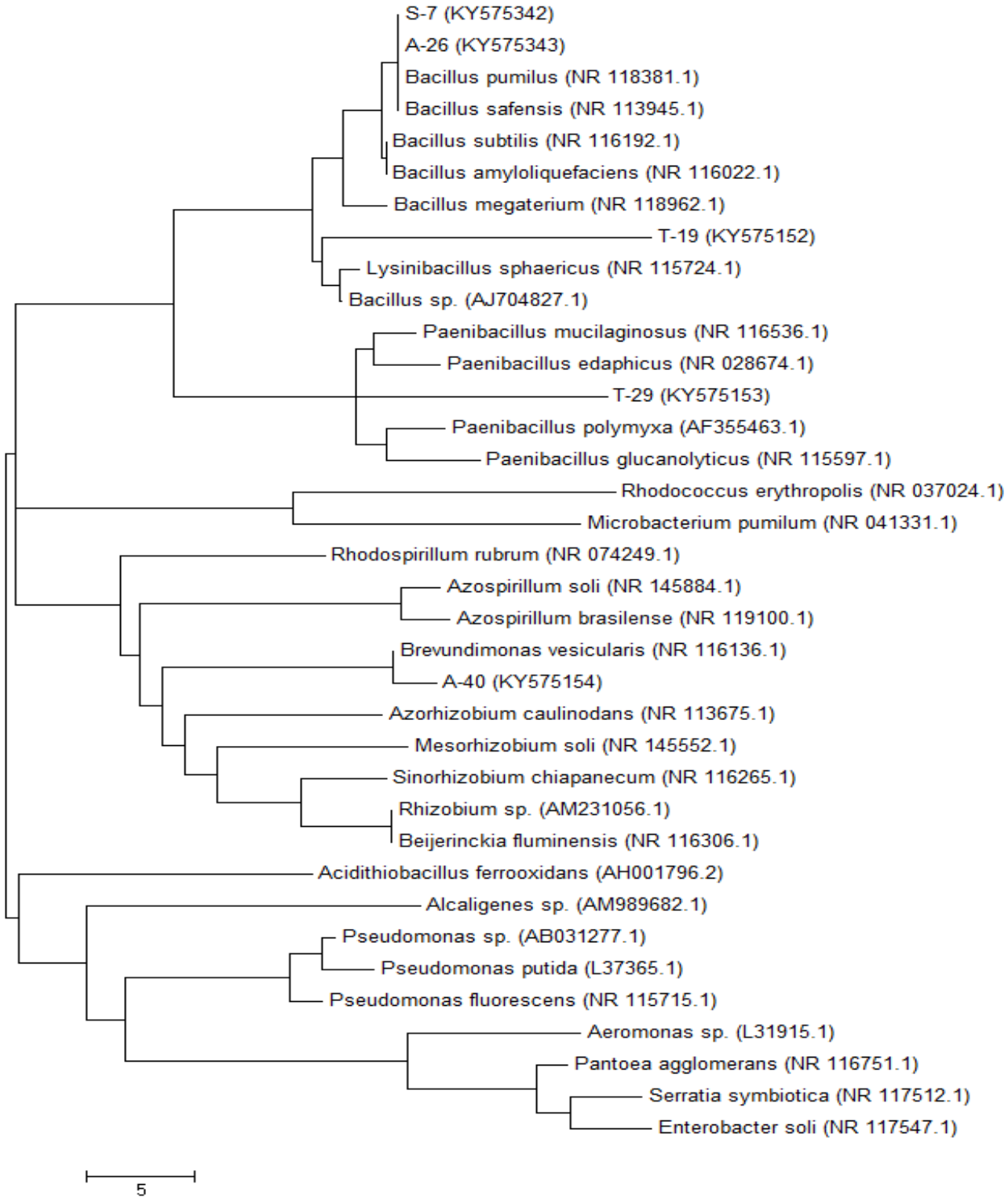

Figure 1: Phylogenetic tree of the rhizobacterial strains (current study) and known PGPR species listed in Gupta *et al.* (2015) and Vejan *et al*. (2016).