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Full Length Research Paper

Factors Affecting Nodulation Efficacy of *Bradyrhizobium japonicum* Inoculant Strain WB74 on Soybean (*Glycine max L. Merrill*)

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Lack of indigenous soil rhizobia that colonize and nodulate soybean (*Glycine max* L. Merrill) roots is a common problem of South African soils. *Bradyrhizobium japonicum* strain WB74 has long been used as an effective commercial inoculant strain for soybean since its introduction in 1998. This paper investigates the major limiting factors involved in the nodulation efficiency of *B. japonicum* WB74 that affect soybean growth and yield in South Africa. Methods including analysis of soil physicochemical properties, farmers' management practices and quality control tests of locally manufactured inoculant products were employed. Inoculant's strain verification was conducted using phylogenetic analysis of the 16S ribosomal RNA of the *Bradyrhizobium* strains in each of the inoculant product. The major findings of this study is that nodulation failure of introduced *B. japonicum* is caused by a combination of several limiting factors such as acidic nature of the soils as well as poor soil nutrition status especially that of phosphorous. Nodulation failure was also prominent in the soybean farms where there was no proper soil management practice. Results of the viability and shelf life studies also indicate that nodulation failure is caused by using sub-standard soybean inoculants products available on the market.

Key words: *Bradyrhizobium japonicum* WB74, inoculants, nodulation, quality control.

INTRODUCTION

Fertilizer nitrogen (N) is one of the major agricultural inputs worldwide to meet the nitrogen requirement of several plants. However, in addition to being expensive for most small scale and subsistence farmers, heavy use of N-fertilizer is both harmful to the environment and results in depletion of fossil fuels needed for the production of nitrogen fertilizers (Bohlool et al.,1992). An alternative and more sustainable process is biological nitrogen fixation (BNF) by a group of symbiotic bacteria called Rhizobia which fix atmospheric nitrogen (N_2) and make it available to plants (Hassen et al., 2012; Zahran, 1999; Bohlool et al., 1992).

Soybean [(*Glycine max* (L.) Merrill.)] is a legume of tropical to subtropical origin and is one of the most

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important sources of food, feed and one of nature's most versatile plants (Keyser and Li, 1992). Inoculation of soybeans with rhizobia throughout the world is estimated to be in the range of 12 - 20 x 10^6 ha/year which results in the establishment of a large rhizobial population in the rhizosphere thereby enhancing improved nodulation and nitrogen fixation (Senevirante et al., 2000). South African soils are largely devoid of rhizobia strains that are able to colonize and nodulate soybean roots and therefore need inoculation of soybeans with effective rhizobia (Bloem, 1998). More than five decades of soybean cultivation in South Africa which involves the use of commercial inoculant strains of rhizobia has resulted in the establishment of populations of *Bradyrhizobium* sp. in the soybean rhizosphere (Botha et al., 2004).

The general principle is that inoculation with rhizobia for subsequent years is often not needed if a legume has a long history of cultivation in that particular area. This is because most of these soils are believed to contain the appropriate rhizobia (Sylvia et al., 2005). However, in areas where acidic soils and high temperature limit rhizobial survival between cropping seasons, re-inoculation is crucially required, which is true for most of the acidic soils in South Africa. Research conducted on biological nitrogen fixation in South Africa resulted in the screening and selection of competitive and high nitrogen fixing soybean inoculant strain *Bradyrhizobium japonicum* WB74 which is a synonym of the Australian strain CB1809. The strain was tested on 30 different soybean cultivars under different geographical locations in the field and proved to be highly competitive and effective in nodulating soybean on South African soils since its introduction in 1998 (Bloem, 1998). *B. japonicum* strain WB74 is currently stored at the South African Rhizobium Culture Collection (SARCC) and is routinely monitored for its viability, purity as well as genetic stability in nodulating soybeans.

Recently, however, there were several concerns as to the nodulation and nitrogen fixation efficiency of this strain due to reports of nodulation failure in various soybeans fields. The main objective of this research was therefore to investigate the major reasons for the reported failure of nodulation in soybeans by introduced commercial strain *B. japonicum* WB74 in South Africa.

MATERIALS AND METHODS

Survey of the major soybean growing farms and onsite observation

In January 2012, selected soybean growing farms in nine soybean farms located in three provinces in South Africa including the Free State, KwaZulu Natal and Mpumalanga were surveyed three months after planting. In these farms where the plant growth stage was sufficient enough to start nodulation, plants were carefully dug out to evaluate the effectiveness of the nodules visually. The visual classification and scoring system was done using a modification of the scheme used by Corbin et al. (1977) for chickpea. The nodule score for soybean is determined based on the number, size, colour and position of nodules across the root. Nodule colour scoring was made using a rating scale of 0-3, where 0 = no nodule, 1 = white nodules, 2 = green nodules, 3 = pink nodules; Nodule number was scored as 3 = many nodules, 2 = average, 1 = few nodules and 0 = no nodule; Nodule position was scored as 4 = on the crown, 3 = side and tap root, 2 = side root and 1 = on root tip; Size of nodules was scored as 3 = large, 2 = intermediate, 1 = small, 0 = too small to none.

Management practices and soil sample analysis

Farmers at each soybean farm were questioned about the various management practices they followed as these could affect the expected yield to be obtained after using the rhizobium inoculant. These include the practices starting from land preparation to planting, fertilizer and fungicide/herbicide application, conservation agricultural practices and handling of the rhizobium inoculants. Soil samples were collected from different sites of the plots in each soybean farm and major physical and chemical analyses were conducted at the Institute for Soil, Climate and Water (ISCW) laboratory of the ARC in Pretoria which included particle size analysis (%), chemical analysis and cation exchange capacity (CEC).

Inoculants pH, moisture content and plate count determination of rhizobia

The moisture content and the pH of the soybean inoculants obtained from the local manufacturers were determined using standard protocols every month (Somasegaran and Hoben, 1994). Likewise, randomly chosen sachet of the inoculant from each of the four manufacturers was chosen and tested each month for a period of five months to determine the colony forming unit (counts) of the *Bradyrhizobium.* strain per gram of the inoculant (cfu g^{-1}) over the shelf life period. Ten grams of the perlite inoculant was mixed in 90 ml sterile distilled water and mixed well to give a 10^{-1} dilution. For the inoculant purchased from manufacturer IV, 10 ml of the inoculant was transferred into 90 ml sterile distilled water. The mixture was then incubated at 30°C for 45 min on a rotary shaker at 100 rpm. A tenfold serial dilution (up to 10^{-6}) was made by transferring 1 ml of this mixture into 9 ml sterile distilled water. Aliquot (1 ml) from the 10^{-3} - 10^{-5} dilution series was spread plated on sterile Yeast Mannitol Agar (YMA) containing (g L^{-1}): Mannitol (10), KH_2P0_4 (0.5), $MgSO_4$ (0.2), yeast extract (0.4), NaCl (0.1), distilled water (1L). The YMA is supplemented with 10 ml L^{-1} Congo red solution. The plates were incubated at 28°C for 3-8 days and the resulting colonies were counted to determine the cfu g^{-1} or cfu ml^{-1} of the *B. japonicum* WB74 like colonies.

Inoculants strain verification by 16S rRNA sequence analysis

Randomly selected pure colonies of the rhizobia from YMA plates were subjected to DNA extraction to amplify the conserved 16S rRNA genes by the polymerase chain reaction (PCR) using forward primer 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse primer 5'-TAC CTT GTT ACG ACT TCA CCC CA-3' (Lane, 1991). The amplification was performed in a 25 µl reaction volume containing 6 µl of the template DNA, 5 µl Flexi buffer, 2.5 µl MgCl, 0.5 µl dNTPs, 0.5 µl of each of the foreword and reverse primers, 0.5 µl Taq polymerase and 9 µl of nuclease free water. Amplifications were carried out in an Eppendorf Master cycler Gradient apparatus (Applied Biosystems, USA) with an initial denaturation at 94°C for 4 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min and a final extension at 72°C for 1 min. PCR amplified DNAs were visualized by electrophoresis on a 1% agarose gel in TBE buffer containing

Table 1. On farm evaluation of the various management practices used by farmers and observation of the nodulation pattern and scoring.

Soybean farm	Inoculants* used	Use of** Sticker	Application of various external inputs†					Nodulation scoring (0-4)				Yield ha^{-1} (tone)¥
			Mo	P	KCl	N	Surface lime	Color	Size	Number	Position	
Bothaville 1	Peat (MI)	-	+	-	-	+	+	0	0	0	0	2.00
Bothaville 2	Peat (MI)	+	+	-	+	-	-	1	2	2	2	2.80
Viljenskroon	Peat (M III)	-	+	-	-	-	-	1	2	2	3	1.30
Reitz	Peat (MI)	-	+	+	+	-	-	1	2	2	2	0.62
Piet Retief I	Peat (MI)	-	+	+	+	-	+	3	3	4	4	3.60
Piet Retief II	Peat (MI)	-	+	-	-	-	-	3	2	3	3	2.80
Middleburg	Peat (MIII)	-	+	+	+	-	+	3	2	3	3	2.60
Leandra	Peat (M II)	+	+	-	-	-	+	3	3	4	3	1.78
Bergville	Peat (MIII Liquid (MIV)	-	+	-	+	-	-	3	3	3	3	3.80

*Inoculants purchased by farmers from manufacturers (M I, II and III) are all peat formulated while those from manufacturer IV are liquid formulation. **Stickers are mixed with peat inoculants in water to help the inoculants stick to the seeds to prevent being washed away while watering. †Mo= Molybdenum, P = phosphorous, KCl = potassium chloride, N = starter nitrogen, + = applied, - = not applied; ¥ The yield data shown is provided by each farmer after harvest.

0.5 mg/ml ethidium bromide. Sequencing of the 16S PCR products was performed at Inqaba Biotech (Pretoria, South Africa) and the resulting sequences were blast searched on the NCBI data library. After edition and proper alignments of the nucleotide sequences of the rhizobia from the inoculants and reference strains obtained from the NCBI data base library, phylogenetic tree was constructed using both the Neighbor Joining (NJ) and Un-weighted Pair Group Method (UPGMA) in MEGA5 program (Tamura et al., 2007).

RESULTS

On farm observation of soybean plants and management practices

Most of the farmers planted in November except the farm in Piet Retief, Mpumalanga and another farm in Bergville that was planted in October (data not shown). The majority of the farmers used the commonly known Pannar cultivar PAN1666R, while two farmers, one in Bothaville and another one in Leandra planted more than six soybean cultivars (data not shown). Soil surface lime application before planting was made by farmers in the Free State (Bothaville1) and Mpumalanga (Piet Retief1, Middleburg and Leandra) (Table 1). However, no surface lime application was made by the other farmers even if it was necessary to do so. The farmers also vary in their usage of fertilizer and other chemicals input except in the application of molybdenum (Mo) in which most of them applied molybdenum as a seed treatment before planting (Table 1).

Farmers in all the visited farms inoculated their soybeans with Rhizobium inoculants prepared either in the form of peat or liquid formulation supplied by local manufacturers. Among the farms surveyed in this study, one farmer in Bergville, KwaZulu Natal had also used a rhizobium inoculant called 'Rhizoliq' imported from abroad by a local distributor company (data not shown). Results of on farm observation of the nodules formation and scoring of their effectiveness based on color, size position and number is presented in Table 1 and Figure 1.

Soil physical and chemical analysis

Generally, samples collected from three soybean farms in the Free State and three farms in Mpumalanga had high sand content (Table 2). Only Bergville in Kwazulu Natal and Piet Retief in Mpumalanga had higher proportion of clay (Table 2). Two farms in the Free State and one in KwaZulu Natal were characterized by a strongly acidic soil (pH = 5.25 to 5.42), while four other soybean farms (three in Mpumalanga and one in Free State) had a moderately acidic soil (pH= 5.7 - 6.0) (Table 2). The soils in the soybean farms in

Figure 1. On farm evaluation of randomly uprooted nodules (average of three) for three soybean farms six to eight weeks after planting and inoculation. The scoring scheme of Corbin et al. (1977) was used to evaluate the effectiveness of the nodules based on color (0-3), size (0-3), number (0-4) and position (0-4) of nodules. a) Piet Retief 1 nodules with a scoring of 3, 3, 3, 4; b) Reitz nodules with a score of 1, 2, 2, 2; c) Bothaville 1 with score of 0, 0, 0, 0 (without nodules).

Table 2. Soil chemical and physical properties from nine soybean farms in three provinces in South Africa.

Soybean	Chemical analysis				Extractable cations (cmol (+)/kg						Particle size† (%)			
farms	C (%)	P*	N-NO_3*	Al*	Mn*	Na	K	Ca	Mg	CEC	Sand	Silt	Clay	Soil pH
Bothaville 1	0.31	4.94	1.23	62.26	14.15	0.116	0.362	2.18	1.359	5.755	79	2	19	5.98
Bothaville 2	0.29	5.19	0.47	71.66	11.49	0.070	0.240	0.670	0.572	3.081	86	3	11	5.40
Viljenskroon	0.45	72.4	0.36	65.80	17.80	0.099	0.453	5.760	0.688	3.789	84	6	10	6.93
Reitz	0.51	2.95	2.96	117.21	25.48	0.068	0.243	0.756	1.568	3.80	81	5	14	5.25
Piet Retief 1	2.18	2.40	0.21	290.00	35.91	0.147	0.445	6.491	1.943	13.06	29	17	54	6.77
Piet Retief 2	2.26	2.57	0.61	236.22	11.85	0.088	0.302	2.371	0.629	6.499	76	8	16	5.70
Middleburg	1.1	15.1	1.58	117.7	13.81	0.076	0.233	1.675	0.799	4.362	76	6	18	6.01
Leandra	1.62	2.84	0.28	131.76	43.65	0.079	0.543	3.048	1.276	7.978	66	12	24	5.80
Bergville	2.27	2.34	1.23	265.72	20.51	0.131	0.198	4.214	1.678	12.55	34	19	47	5.42

*mg/kg; CEC = cation exchange capacity†, Sand = 0-0.0 mm; Silt = 0.05-0.00 mm; Clay = <0.002 mm.

Piet Retief I (Mpumalanga) and Viljenskroon (Free State) had pH which is close to neutral (pH = 6.77 - 6.93) (Table 2). Summary of the comparison of soil physical characteristics and chemical properties including soil nitrate and aluminum level, cation exchange capacity (CEC) and extractable cations for all the soybean farms is presented in Table 1.

plate count of Rhizobia

The initial count of the *B. japonicum* WB74 like colonies in the inoculants from the three local manufacturers I, II, and III was > 10^8 cfu g^{-1} and was equivalent to a log transformed value of 8.29, 8.33 and 8.06, respectively (Table 3). During the second month, the count slightly decreased by only 0.35 log units for both manufacturer I and II and by 0.36 log units for manufacturer III (Table 3).

The decrease in the counts became very significant during the fourth month in which count from manufacturer II was less by 1.8 log units, manufacturer I by 1.82 log units and manufacturer III by 2.49 log units. The initial *Bradyrhizobium* count for manufacturer IV was 1.21 x 10^8 cfu ml^{-1} in the first month and decreased to 9.2 x 10^5 cfu ml^{-1} in the fourth month (Table 3).

Strain verification by 16S rRNA sequence analysis

The amplified product of the 16S rRNA gene of each of the *Bradyrhizobium* strain retrieved from the four soybean inoculants was subjected to sequencing reaction. After appropriate edition of the sequences using both the Bio-edit and Chromas lite program, a 930 base pair of nucleotides was generated for each sequence and aligned online using MAFFT nucleotide alignment tool. Neighbour Joining (NJ) and UPGMA (data for UPGMA tree not shown) phylogenetic trees constructed from the aligned sequences revealed that the strains from the four different soybean inoculants fall into three separate

Table 3. Plate count of *Bradyrhizo bium* strain WB74 like colonies per gram of ea ch soybean in oculants and determination of pH and moisture content over a period of five months to determine the viability of the rhizobia and the inoculant's quality in long term storage.

Inoculant/	Viable plate count (cfu g^{-1})*					Inoculant *pH**				Moisture content (%)*			
Manufacturer	I	II	III	IV	V**	I	II	III	IV	I	II	III	IV
Manufacturer I (Perlite)	1.98 x 10^9	8.8 x 10^8	4.8 x 10 8	3.0x 10^7	>10^6	7.72	7.50	7.51	7.61	33	33	33	30
Manufacturer II (Perlite)	2.14 x 10^9	9.7x 10^8	5.7x 10^8	3.4x 10^7	>10^6	7.72	7.83	7.64	7.48	33	32	33	30
Manufacturer III (Perlite)	1.15 x 10^9	5.1x 10^8	3.2 x 10^8	3.8 x 10^6	>10^6	8.43	8.19	8.06	8.12	35	33	34	30
Manufacturer IV (Liq uid)	1.21x 10^9	9.4 x 10^8	7.8 x 10^8	9.2 x 10^6	>10^6	7.47	7.52	7.60	5.58	NA	NA	NA	NA

*The viable plate counting was determined over a period of five months (I - V) and pH and moisture content determination was conducted over a period of four months (I-IV). **contaminants were detected at the fifth month from the 10^{-6} dilution series of the NA plates, therefore the YMA plates containing the *Bradyrhizobia* were not counted due t o the presence of contaminants after the fourth month.

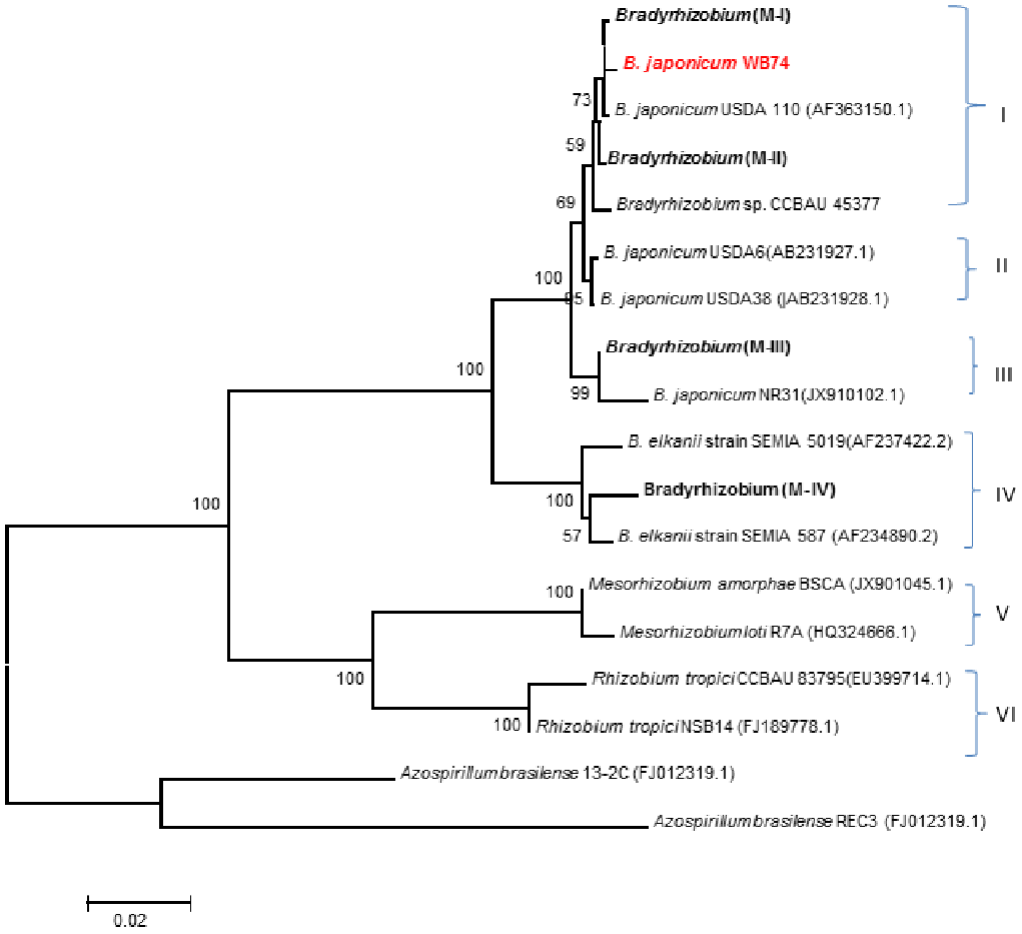

Figure 2. Neighbor-Joining tree to show the phylogenetic relationship of *Bradyrhizobium* strains used in four soybean inoculants in South Africa with *B. japonicum* strain WB 74 and selected reference strains obtain ed from the NCBI data base. The evolutionary distances were computed using the Jukes-Cantor method and scale bars represent substitution of 2 bases per 100 nucleotide positions. The *B. japonicum* strain WB 74 and the *Bradyrhizobium* strains recovered from the four inoculants were designated in bold and letters in parenthesis represent Manufacturer I - IV. The tree is rooted with *Azosprillum brasilense* strains. NB. O nly strains from inoculant manufacturers I and II have grouped together with *B. japonicum* WB74 strain.

clusters (Figure 2). In both the NJ and UPGMA trees, only strains from manufacturer I and manufacturer II clustered with *B. Japonicum* WB74 (Cluster1) (Figure 2). Inoculant strain from manufacturer III clustered in a separate group with *B. japonicum* strainNR31 (JX910102.1) using the Neighbour Joining method (Figure 2) and with *B. japonicum* USDA strains in the UPGMA method (data not shown). The *Bradyrhizobium* strain from manufacturer IV clustered with *Bradyrhizobium elkani* SEMIA 5019 and *B. elkani* SEMIA 587 (Figure 2).

DISCUSSION

The major limiting factors that contributed to the failure of nodulation of soybean by introduced *B. japonicum* strain WB74 in South Africa was investigated in this study. In our survey to investigate the management practices used by the farmers, there is indication of lack of consistency among the different soybean farms. For instance, although most of the surveyed farmers have treated their seeds with molybdenum before planting, failure of legumes to nodulate under acidic soil conditions of Bothaville2, Reitz and Bergville farms was observed. In all the three farms where the soil pH is strongly acidic (5.2 - 5.4), farmers did not intend to use surface lime application before planting. Zahran (1999) reported that legumes usually fail to nodulate under acidic soil conditions leading to impaired symbiotic efficiency and reduced yield. Inoculant strains of rhizobia, no matter how competitive they are, do not express their full capacity for N_2 fixation if other limiting factors such as unfavourable soil pH, mineral toxicity,and nutrient deficiency and plant diseases impose limitations on the vigour of the host legume (Brockwell et al., 1995; Peoples et al., 1995; Thies et al., 1995).

One of the principal yield limiting nutrients in many regions in the world is phosphorous (P) which is very essential for both nodulation and nitrogen fixation (Zahran, 1999). Soils in the seven soybean farms with very low P levels ranging from 2 - 7.0mg/kg may not sufficiently support the process of BNF by rhizobia. Except for the soybean fields in Piet Retief, Middleburg and Retz, no management practices were made by the other farmers to treat the soils with added phosphorous even if the amount was too low. It should be noted that field grown soybean has a high 'P' requirement when it is dependent on BNF for its nitrogen supply (Keyser and Fudi, 1992). The unavailability of P may be caused by higher level of soil exchangeable Aluminium (Al). In many acidic soils, aluminium and manganese toxicities are responsible for limiting plant growth (Yang et al., 2009; Liao et al., 2006).

The low P availability in acidic soils results when free Al-oxides bind native and applied P into a form unavailable to plants (Liao et al., 2006). In the current study, the level of soil exchangeable Al in the soils of the different soybean fields was observed to be high ranging from 62.3 to 290 mg/kg. According to this study, soils from soybean fields in Mpumalanga (Piet Retief, Leandra) and KwaZulu Natal (Bergville) contained the highest amount of soil aluminium as compared to those in the Free State (Bothaville, Viljenskroon, Reitz) which showed very low nodulation. The fact that these soybean genotypes thrived well under conditions of high exchangeable Al concentration in the soil (>100mg/kg) make them highly tolerant to aluminium toxicity. It is therefore clear that 'P' deficiency could be one of the limiting factors for the difference in yield/ha between Piet Retief and the other soybean farms.

Soils from the soybean fields for which the exchangeable Al was high were characterized by lower level of available P. For example, the available soil P for Piet Retief soil with exchangeable Al level of 290 mg/kg was only 2.4 mg/kg, whereas Viljenskroon soil with exchangeable Al level of 65.8 mg/kg contains 72.4 mg/kg available P. A similar contrast can be made between the soils in Bergville (KZN) and Bothaville (Free State) for the relationship between exchangeable Al and available P in the soil. Under such extreme conditions, farmers' management practices play a vital role to ameliorate the hazardous effect of aluminium toxicity in acidic soils. In general, Al and Mn toxicity becomes severe in soils with pH < 5, but can also occur at pH levels as high as 5.5 and limit plant growth (Rout et al., 2001).

One possible explanation for a rather high yield in Piet Retief soil with the highest level of exchangeable Al and low P level is that the soybean growing in these soils might have evolved adaptive mechanisms to grow in such low-P soils. This includes exudation of organic acids, phosphatase and other compounds that could mobilize 'P' from bound 'P' pools in the soil such as Al-P and also reduce its toxicity (Liao et al., 2006). The surface lime treatment of the soils in Piet Retief, Retz, Middleburg, Leandra and Bothaville might also have reduced the toxic effect of the high Al content in these soils. Amelioration of acidic soils rich in Al helps to increase the soil pH and decrease the concentration of the extractable Al and Mn thereby improving N_2 fixation and growth (Obiri-Nyarko, 2012; Fageria and Baligar, 2003).

The high yield obtained in Bergville with high acidic soils and low P level could be explained in terms of other features of the soil such as high CEC and soil organic matter (C%). The soil in Bergville with high organic carbon (C%= 2.27) and the high CEC probably gives it a good test level which offers a large nutrient reserve for the survival and functioning of the rhizobia and their symbiont. Moreover, the high clay content and cation exchange capacity of soils in Piet Retief and Bergville renders a high water holding capacity. This observation is supported by previous investigation which showed that the population kinetics of introduced strains of rhizobia is

a function of soil organic carbon, water holding capacity and CEC (McInns and Haq, 2007).

Bradyrhizobium strains nodulating soybeans are generally sensitive to acid soils. Thus one cause of nodulation failure in legumes is the inability of the rhizobia to persist under such conditions. In the current study, soils of Bergville, Retz and Bothaville farms with a pH range of 5.2 - 5.4 are in the strongly acidic category and are not suitable for the survival of the rhizobia without proper management to reduce the acidity. Acidity has more severe effects on rhizobia multiplication than Al stress and low-P conditions (Taylor et al., 1991). In this study for instance, Piet Retief soil with high Al level and low P condition has a near neutral pH which is conducive for the survival of the soybean *Bradyrhizobium* in the soil. The farmer's proper management of this soil especially surface lime application and treatment of the soil with added P could have contributed to the success of the inoculated rhizobia and the attainment of the high yield.

Soil NO_3^- has a negative effect on the activity of the nitrogen fixing rhizobia by inhibiting the functioning of the enzyme nitrogenase and legheamoglobin. By doing so, soil NO_3^- in general inhibits nodule formation and nitrogen fixation(*Zahran*, 1999). An interesting correlation was observed in this study (data not shown) with regard to yield and soil NO_3^- level where soybeans growing in soil with the lowest NO_3^- level in Piet Retief had the highest yield (3.6 tone ha^{-1}) and Reitz soil with the highest NO_3^-level had a yield of only 0.6 tone ha^{-1}. In a study to investigate the inhibitory effect of NO_3^- on nodulation, nodule growth was completely stopped when young soybean plants growing in hydroponic culture were supplied with 5 mM nitrate (NO_3^-) solution (Takuji et al., 2011).

In the absence of adequate number of highly effective rhizobia in many soils, the need arises to use rhizobial inoculants. It is advisable to inoculate the soil with high number of effective rhizobia to out-compete the population of ineffective native rhizobia (Deaker et al., 2004). For instance increasing the number of effective rhizobia applied to the seeds between 10-100 fold will improve nodulation and grain yield (Herridge et al., 2002). According to legume inoculants quality control procedures set for the registration of inoculant products in South Africa, any inoculant which does not comply with the standards will be rejected for marketing. The inoculants will be rejected if the number of rhizobia is < 5 x 10^8 cfu g^{-1} (for peat), < 6.5 x 10^8cfu g^{-1}(for perlite) and < 2 x 10^9cfu ml^{-1} (for liquid inoculants); contaminants present on the 10^{-5} dilution plates; pH of carrier < 6.5 or > 7.5; rhizobial strain is doubtful (Strijdom and van-Rensburg, 1981).

The near alkaline pH of the perlite and liquid inoculants detected in this study are not favourable for prolonged survival and multiplication of the *Bradyrhizobium.* Strains of *B.japonicum* are found to be less tolerant to alkaline pH and have lower survival as the pH of the inoculant increases (Gomez et al., 1997). In addition to pH, the low level of moisture content in the three perlite inoculants from the local suppliers (30- 35%) could have subjected the rhizobia in the perlite to desiccation stress and a decrease in the number of rhizobia from the required 6.5 x 10^9 to 1.15 x 10^8 per gram of the inoculant. Low inoculant moisture content will lead to a high mortality of the bacteria when they are inoculated to the seeds. Up on seed treatment, it will result in the average decrease of 3 log units (about 1000 rhizobia $seed^{-1}$) (Cartroux et al., 2001).

In this study, molecular characterization using sequence analysis of 16S rRNA revealed that all soybean inoculant strains from the four inoculant manufacturers fall under the *B. japonicum* cluster .However, only strains from two manufacturers were closely related to the desired *B. japonicum* WB74 strain. The inoculant strain from manufacturer III was more closely related to a different strain, that is, *B. japonicum* strain NR31 (JX910102.1) rather than with *B. japonicum* WB74, while inoculant strain from manufacturer IV clustered with *B. elkani* SEMIA 5019 (AF237422.2) and *B. elkani* SEMIA 587 (AF234890.2). Strain SEMIA 5019 is initially isolated from a high manganese soil in Reo de Janerio and has been used as an inoculum since 1979 whereas strain SEMIA 587 was isolated from Rio Grande de Sol in Brazil (Santos et al., 1999).

The recommended registered strain to be used as soybean inoculant in South Africa is *B. japonicum* strain WB74 and no other strain of rhizobia, other bacteria or fungi is allowed to be present in the inoculant without being registered (personal communication with office of the Registrar, Pretoria). Our study shows that a wide variety of soybean inoculants with strains of *Bradyrhizobium* other than the recommended WB74 strain are being manufactured and/or imported by different companies to be sold to the farmers. However, there is no research to date that indicates the adaptation of these new strains of *Bradyrhizobium* to the South African soil conditions, nor is their efficacy determined under different geographical and soil condition for at least two seasons before being registered.

In summary, it has been noted in this study that the efficacy of the inoculant strain *B. japonicum* WB74 in the nodulation of soybean on South African soils is largely dependent on the intrinsic characteristics of the inoculants, farmers' management practices and other soil variables all of which affect the nodulation process and the entire symbiotic interaction. It is therefore recommended that before introducing rhizobial inoculants into the soil, sufficient soil analysis and proper management practices be conducted to improve any limiting factors that hamper the nodulation efficiency of the rhizobia. Moreover, with the increasing production and the need to market legume inoculants in South Africa, selection of strains for effective nodulation and nitrogen fixation must be conducted under field condition. The inoculant pro-

ducts manufactured using the elite rhizobial strains should comply with the standards by undergoing at least a six month quality control test which involves, number of rhizobia, inoculant consistency (pH, moisture content), rapid strain verification and shelf life determination.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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