*SYMBIOSIS* (2005) 40, 97–107 ©2005 Balaban, Philadelphia/Rehovot ISSN 0334-5114

# Physiological diversity of rhizobia nodulating promiscuous soyabean in Zimbabwean soils

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*(Received July 12, 2005; Accepted October 12, 2005)*

**Abstract**
Rhizobial isolates were obtained from nodules of promiscuous soyabean varieties Hernon 147 and Magoye and specific Roan grown in a range of Zimbabwean soils. A total of 129 isolates authenticated as true rhizobia were characterized using growth rate, elasticity, colour, size, colony shape, acid/alkali production on YEM and tolerance to low and high pH, elevated temperature and salt concentration. Isolates separated into 2 major clusters at a similarity level (%SSM) of 66%. Cluster I contained isolates forming dry colonies (77%) which separated into 9 groups and Cluster II contained those forming the wet colonies (23%) with 4 groups. Acid and salt tolerance patterns did not differ among the two main clusters (the dry and the wet colony types). More isolates forming wet colonies (47%) survived at 40°C than those forming dry colonies (13%). Salt, temperature and acid pH tolerance were not related to geographic origin of the isolates. The promiscuous soyabean variety Magoye nodulated with the widest range of rhizobia (12 groups) followed by Hernon 147 (11 groups) and then Roan (9 groups). Guruve soils had the most diverse range of isolates belonging to 12 groups followed by those from Chiweshe (9 groups) and then those from Chikomba (8 groups). Our results indicate that soyabean is nodulated by a wide range of indigenous rhizobia in African soils.

**Keywords**: *Rhizobium*, *Bradyrhizobium*, promiscuous soyabean, temperature, salt, pH tolerance

## 1. Introduction

Soyabean nodulating rhizobia have been widely studied, characterized and grouped into different genera (Jordan, 1982, 1984; Chen et al., 1988; Kuykendall et al., 1992; Young and Haukka, 1996). Only two rhizobial genera (*Rhizobium* and *Bradyrhizobium*) and four species were described in the first edition of Bergey's manual of systematic bacteriology (Jordan, 1984). Characterization of new isolates from hosts that had not been previously studied, together with the generalized use of 16S rDNA sequencing and polyphasic taxonomic approaches (Martínez-Romero and Caballero-Mellado, 1996; Vandamme et al., 1996; van Berkum and Eardly, 1998) have led to the description of more than 20 new species and four additional rhizobial genera viz: *Allorhizobium*, *Azorhizobium*, *Mesorhizobium* and *Sinorhizobium* (Sylvester et al., 2001; Young et al., 2001). Soyabean rhizobia fall into the fast growing genera *Rhizobium* (Jordan, 1984) and *Sinorhizobium* (Chen et al., 1988) and the slow growing genus *Bradyrhizobium* (Jordan, 1982, 1984).

Promiscuous legumes have been referred to as 'naturally-nodulating' legumes (Mpepereki et al., 2000) because of their ability to nodulate in some soils without inoculation. Magoye and Hernon 147 are two such promiscuous soyabean varieties that were shown to nodulate without inoculation in many Zambian soils (Mwakalombe, 1998). Magoye is originally a cross between varieties Gilbert and K53 (Mwakalombe, 1998), while Hernon 147 is a cross between the varieties 'Herman' and 'None-shattering' (Mpepereki et al., 2000). Rhizobia nodulating soyabean in African soils are poorly characterized. Some characteristics

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used to define rhizobia into different groups include growth rate and colony morphology such as size, shape, colour, texture and general appearance (Graham et al., 1991; Somasegaran and Hoben, 1994). Most *Bradyrhizobium* strains have been shown to produce an alkaline reaction and *Rhizobium* an acid reaction on various solid media in the laboratory.

Physiological characteristics of rhizobia include tolerance to acid, salt, temperature, aluminium and manganese. Antibiotic sensitivity and the ability to utilize different carbohydrates as sole carbon source for growth (Graham et al., 1991) are all important characteristics for the survival of rhizobia in the soil environment. Abiotic factors such as temperature, moisture status, soil acidity and salinity play an important role in the saprophytic survival of rhizobia as well as in nodule formation and function (Mpepereki, 1994). The slow growing rhizobia generally have lower maximum survival temperatures than the fast growers (Mpepereki et al., 1997). Some slow-growing Sudanese tree rhizobia had maximum growth temperatures below 38°C and the fast-growing types had maximum growth temperatures around 44°C (Zhang et al., 1991). Rhizobia nodulating soyabean also differ in their tolerance to salt concentrations. Graham and Parker (1964) and Batzli et al. (1992) have shown that many rhizobia were unable to grow above 2% NaCl (w/v). Tolerance to high salt concentrations could be important in Zimbabwean soils which have widely fluctuating moisture due to prolonged dry seasons. Generally little information is available on the characteristics of the rhizobia nodulating promiscuous soyabean in African soils (Mwakalombe, 1998; Musiyiwa et al., 2005). Differences observed in morphological and physiological characteristics may eventually be tracked down to the molecular composition of their genetic material. Variations in cultural properties as well as physiology of isolates in a soil population may give a rough indication of the different genotypes of rhizobia present.

Indigenous rhizobia could be better adapted to the Zimbabwean soils making them ideal for use as commercial inoculants in local soils. A large collection of rhizobial isolates was made from nodules of soyabean growing in Zimbabwean soils and evaluated for $N_2$-fixation ability with promiscuous and specific soyabean varieties (Musiyiwa et al., 2005). This study was conducted to determine the morphology and physiology of these indigenous rhizobial isolates and to use these characteristics to assess the diversity of rhizobial isolates able to nodulate soyabean in Zimbabwean soils.

## 2. Materials and Methods

*Site selection*

Ninety two soils were collected from Chiweshe (6), Chikomba (11), Makoni (8), Chikwaka (Goromonzi) (16), Guruve (10), Mhondoro (20) and Mutoko (21). These districts are in the agro-ecological regions (A.E.R) 2 and 3 of Zimbabwe (Table 1), where the environmental conditions such as temperature and rainfall favour the growth of soyabean. In A.E.R 2 the average annual rainfall is between 750–1000 mm and in A.E.R. 3 between 650–800 mm.

*Soil sampling*

All equipment used for sampling the soils was first disinfected using 95% ethanol. At each site 10 sub-samples of soil were collected from the top 30 cm where microbial activity is highest, into a plastic bag, and mixed thoroughly. The samples were kept away from direct light during transportation and stored in a cool dark storage room to reduce microbial mortality.

*Soil characteristics*

The soils were ground to pass through a 2 mm sieve. The pH was determined in 0.01 M $CaCl_2$ (1:5, w/v) The soil texture was determined using the hydrometer method. The organic matter content (%C) was measured using the Walkley-Black method (Nelson and Sommers, 1996). Total soil N was estimated using the Kjeldahl method (Bremner, 1996). Cation exchange capacity (CEC) and exchangeable bases were determined using ammonium acetate (Sumner and Miller, 1996) as the extracting agent.

*Nodule collection, isolation of rhizobia and authentication*

Two promiscuous soyabean varieties, Hernon 147 and Magoye and a specific variety, Roan were grown in 92 different soils potted in 300 ml plastic containers. The positive control was inoculated with *Bradyrhizobium japonicum* strain MAR 1491 (= USDA 110) while the negative (no inoculation) controls were grown in sterile vermiculite. Seeds were surface disinfected with 95% ethanol for about 30 seconds and rinsed with sterile water 5 times prior to planting. Nitrogen-free nutrient solution (Mclure and Israel, 1979) was alternated with distilled water for watering the plants. After seven weeks, the plants were uprooted and their roots were washed with running tap water. The nodules were separated carefully using a pair of scissors leaving a small portion of the root system attached. Isolations were done from both fresh and dried nodules (dried in silica gel). The dried nodules were left to imbibe distilled water overnight prior to isolation of rhizobia after which each single nodule was surface disinfected in 95% ethanol, and rinsed with five changes of sterile water. Sterile blunt forceps were used to squash the nodules and a small amount of the fluid from each nodule was then streaked onto a separate plate of yeast extract mannitol (YEM) agar with Congo Red (25 $\mu g\ ml^{-1}$). Plates were incubated at 28°C for 8 days.

Re-streaking was done from single colonies which were opaque, milky or watery-translucent, and which did not absorb Congo Red, until uniform colonies of each isolate were obtained. The authenticity of the pure cultures as rhizobia was tested by inoculating soyabean plants in the greenhouse and observing them for nodulation.

Pure cultures of the isolates were inoculated into YEM broth and incubated on a rotating shaker at 200 rotations per minute at room temperature (~25°C) for 3 days or until turbidity was observed. Plastic pots, 300 ml in volume, were filled with sterile horticultural vermiculite which was then watered with distilled water. Each culture was used to inoculate Magoye seeds planted in two pots each with three seeds. Each seed was inoculated with about 0.3 ml of broth culture before covering with vermiculite. Pots were watered with distilled water alternated with N-free nutrient solution and the soyabean harvested at 7 weeks after planting. Nodulation on the crown and the tap roots proved the authenticity of the isolates as rhizobia. Isolates authenticated as true rhizobia were stored on agar slants at 4°C in the refrigerator and characterized in terms of their cultural morphology and tolerance to salt, temperature and pH.

*Cultural properties*

Each isolate was cultured in YEM broth on a rotating shaker at 200 rpm at room temperature (~25°C) for 3 days or until the media was turbid. Serial dilutions of the cultures up to $10^{-5}$ were prepared. A 25 µl aliquot of each culture was then plated onto the YEM agar and spread using a glass rod. The plates were examined for growth daily for 8 days and colonies were described according to shape, colour and texture, number of days to visible colony formation and size of colonies. Production of acid or alkali on mannitol was tested by using bromothymol blue (BTB) as an indicator (CIAT, 1988). The BTB was incorporated into YEM agar at a concentration of 25 µg $ml^{-1}$ just before sterilization of the media. The YEM agar incorporated with the BTB was then poured into plates and 25 µl of broth cultures of the isolates were spread-plated. The plates were incubated at 28°C and were observed for colour changes in the media for 8 days.

*Physiological properties*

*Acid tolerance*

Yeast extract mannitol medium minus $K_2HPO_4$ which is a buffer (Zablotowicz and Focht, 1981), was prepared and sterilized at 121°C for 15 minutes. The pH of YEM media was then adjusted to pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 by adding HCl and to pH 7.5, 8.0, 8.5, 9.0, 10.0 and 11.0 by adding NaOH. Each isolate diluted up to $10^{-5}$ was then spread-plated onto the media at all the pH values and the plates incubated at 28°C for 8 days. Growth or no growth of colonies was recorded as positive and negative, respectively.

*Salt tolerance*

Yeast extract mannitol agar with different NaCl concentrations was prepared and poured into plates. The concentrations were 0.1, 0.2, 0.5, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6% NaCl. Each plate was divided into 8 equal sectors. Aliquots of 25 µl for each isolate, diluted to $10^{-5}$, were drop-plated in duplicate onto 2 opposite sectors using a micro-titre pipette. The plates were incubated at 28°C for 8 days and scored for presence or absence of colonies.

*Temperature tolerance*

Plates with YEM agar were each divided into 8 equal sectors. Aliquots of 25 µl for each isolate were drop plated in duplicate onto 2 opposite sectors using a micro-titre pipette. Each broth culture had been diluted to give approximately $10^5$ cells $ml^{-1}$. The plates were incubated at the following temperatures: 20°C, 28°C, 32°C, 35°C, 37°C, 40°C and 45°C. The presence or absence of colonies was recorded after 8 days of incubation at the respective temperatures as positive (+) and negative (–) respectively. Plates in which no growth was observed were re-incubated at 28°C for a further 8 days and presence or absence of colonies recorded.

*Cluster analysis of results*

Results were scored as 1 for the positive results and 0 for negative for the morphological as well as for the physiological characteristics, i.e. all NaCl concentrations, pH and temperatures used. Analysis of results was done using the SPSS program. Similarities were compared using simple matching coefficient and clustering was done using average linkage between groups.

## 3. Results

*Soil characteristics*

At least 50% of soils from Chikomba, Chikwaka, Mhondoro and Mutoko had pH values below 5.0 while only one soil from Guruve had a pH below 5.0 (Table 1). None of the soil characteristics were related and all had correlation coefficients below 0.1 except for TEB and CEC with a correlation coefficient of 0.6. Of the 25 soils randomly selected for analysis four were sandy clay loam, six were sand, two were sandy clay, four loamy sand and the rest sandy loam in texture. Seven soils tested had organic matter content above 1% while the rest had below 1% organic matter content. The %N content of the soils tested was very low across all the districts ranging from 0.04% to 0.38%. The CEC ranged from 1.00 to 11.23 $cmol_c\ kg^{-1}$ of soil. More soils from Guruve and Mutoko had relatively higher CEC as compared with soils from the other districts. The exchangeable bases Na and K were mostly below 1 $cmol_c$

$kg^{-1}$ of soil while Ca and Mg were below 5 except for Ca at Hwariyesango in Guruve (8 $cmol_c$ $kg^{-1}$ of soil) and Musonza in Chikwaka (5.3 $cmol_c$ $kg^{-1}$ of soil). Soil P was relatively higher in soils from Chiweshe than in soils from the other districts. The soil from Hwariyesango had the highest P content of 70 $\mu g$ $g^{-1}$ of soil. Fifty-three of the 92 soils had pH values below 5.0.

*Morphology and physiology of isolates*

Many fast-growing isolates were obtained, but none of these formed nodules on the promiscuous soyabean variety Magoye used as the test legume, seven weeks after planting and were discarded. A total of 129 slow growing authenticated isolates and the reference strain MAR 1491 were characterized and clustered into different groups (Table 2, Fig. 1). All the isolates were broadly similar in their morphological and physiological properties joining at a similarity (%$S_{SM}$) value of 66%. These then formed two major clusters (Fig. 1) based on colony morphology. The larger Cluster I contained 77% of the isolates with an 84% similarity and could be further separated into 9 major groups (groups 1–9). Cluster I isolates formed mainly dry, domed, milky and elastic colonies with only three isolates in group 4 forming flat colonies. The isolates in Cluster II (23%) were about 83% similar based on their morphological and physiological properties. They formed the wet, inelastic and flat colony types which produced alkali on YEM agar. These were further separated into four major groups designated as 10, 11, 12 and 13 (Fig. 1). The separation of the isolates into the two clusters was not based on host plant or geographical origin. The distinguishing features of the 13 groups are given in Table 2.

*Cluster I*

Group 1 had the largest number of isolates (47) and included the reference strain MAR 1491 (Fig. 1). Groups 1, 2 and 3 isolates were closely related in their colony and physiological properties. They all produced alkali, and were not very tolerant to NaCl with most growing at concentrations of 0.1–0.2% NaCl and less than 25% in each group growing at 0.5% NaCl (Table 2). They were weakly tolerant to low and high pH. Most grew between pH 4.5–9.0 with a few isolates tolerating pH 4.0 and pH 9.5. Most isolates in Groups 1, 2 and 3 were not tolerant to high temperature growing at 32–35°C with few growing at 37°C and surviving 32–37°C and very few 40°C. The isolates in these three groups could be differentiated when grown on YEM by colony size, where those in Group 1 formed colonies of about 1 mm, while those in Group 2 were more than 1 mm and those in Group 3 less than 1 mm in diameter. The isolates in Groups 1–3 were originally isolated from all the three soyabean varieties, Magoye, Hernon 147 and Roan, and were mainly from Chikomba, Chiweshe and Guruve districts (Table 2).

Groups 4 and 5 constituted isolates forming colonies ranging in size from less than 1 mm to 2 mm in diameter. Group 4 isolates formed flat, elastic colonies and produced alkali while Group 5 isolates produced domed, elastic colonies and produced acid on YEM (Table 2). Group 4 isolates were more tolerant to NaCl than Group 1, 2 and 3 isolates, most (67%) tolerating 0.1–0.5% with a few (33%) tolerating 1.0–1.5% NaCl. Group 5 isolates were even more tolerant to NaCl than Group 4 isolates with most (67%) growing between 0.1–3.5% and 33% of the isolates growing at 5% NaCl. Isolates in Groups 4 and 5 were more tolerant to a wide pH range than those in Groups 1–3. Most could grow in pH range 4.5–9.0 and one strain in each group could grow at pH 4.0 and 9.5. Group 4 isolates were similar to Groups 1–3 isolates in temperature tolerance and survival while Group 5 isolates were more tolerant to temperature with one strain growing and surviving up to 40°C. Group 4 isolates were from Magoye and Roan while those in Group 5 were from Magoye and Hernon 147. Group 4 isolates were from Chiweshe, Guruve and Mhondoro districts while Group 5 isolates were from Chikomba, Chiweshe and Guruve (Table 2).

Isolates in Group 6 formed colonies greater than 1 mm in diameter and were alkali producers on YEM while those in Group 7 formed colonies about 1 mm in diameter and produced acid on YEM (Table 2). Both Group 6 and 7 isolates were more tolerant to NaCl than those in Groups 1–3 with more than half of the Group 6 isolates growing up to 1% NaCl and some from both groups growing in the whole range of NaCl concentration of 0.1–5%. These two groups were also more tolerant to a wide range of pH with some of the isolates growing between pH 4.0–11. Some of the isolates from Group 7 could grow at 40°C while Group 6 isolates were less tolerant to high growth temperature with the majority only growing at 32°C and less than half at 35°C and even fewer at 37°C. Isolates from the two groups however could survive exposure to a similar range of temperature of 32–40°C. The isolates in Group 6 were from all the three soyabean host varieties while those in Group 7 were from the promiscuous varieties Magoye and Hernon 147. The isolates in Group 6 were from Chikomba, Chiweshe, Guruve, Mhondoro and Mutoko districts while those in Group 7 were from Chikomba, Chiweshe and Guruve (Table 2).

The isolates in the last groups in Cluster I, Groups 8 and 9, both produced alkali on YEM plates. In Group 8 which comprised only two strains, one produced colonies less than 1 mm in diameter while colonies of the other strain were greater than 1 mm on YEM. The 13 strains in Group 9 all formed colonies about 1 mm in diameter. Group 8 isolates were more tolerant to NaCl mostly growing between 0.1 and 3.5% NaCl while the majority of isolates in Group 9 grew at 0.2% with only a few growing up to 2% NaCl. Group 8 strains tolerated between pH 4.5–11 while in Group 9 some isolates tolerated the whole range of pH tested, between 3.5–11. In Group 8 only one strain was

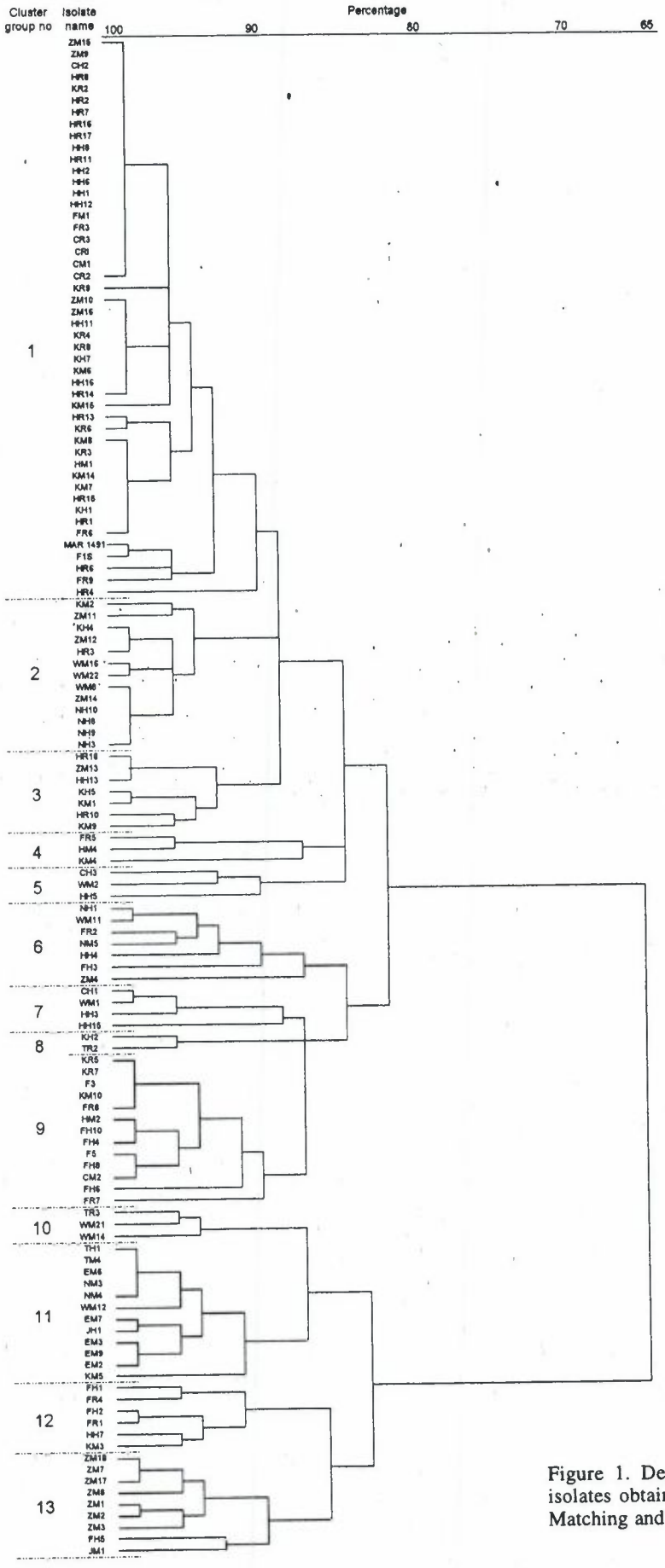


Figure 1. Dendrogram showing clusters of rhizobial isolates obtained from Zimbabwean soils using Simple Matching and Average Linkage (Between Groups).

Table 1. Agro-ecological regions and soil $pH_{CaCl_2}$ at the sites from which the rhizobia were isolated.

| District | Agro-ecological region[1] | No. of soils tested | No. of soils with $pH_{CaCl_2}$ below 5 | No. of soils with soyabean history |
|---|---|---|---|---|
| Chikomba | 3 | 11 | 11 | 2 |
| Chikwaka | 2 | 16 | 13 | 0 |
| Chiweshe | 2 | 6 | 2 | 2 |
| Guruve | 3 | 10 | 1 | 6 |
| Makoni | 2 | 8 | 3 | 0 |
| Mhondoro | 2 | 20 | 13 | 1 |
| Mutoko | 3 | 21 | 10 | 0 |

[1]Agro-ecological regions: 2 = 750–1000 mm rainfall per annum; 3 = 650–800 mm.

Table 2. Differentiating characteristics among groups of rhizobial isolates.

| Group no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | Total isol. | Clust.I isol. | Clust.II isol. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. of isolates in group | 48 | 13 | 7 | 3 | 3 | 7 | 4 | 2 | 13 | 3 | 12 | 6 | 9 | 130 | 100 | 30 |
| **Colony characteristics** | | | | | | | | | | | | | | | | |
| Slow growing | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Colony size | | | | | | | | | | | | | | | | |
| Less than 1 mm | 0 | 0 | 100 | 33 | 33 | 0 | 0 | 50 | 0 | 0 | 0 | 0 | 0 | 8 | 10 | 0 |
| 1 mm | 100 | 0 | 0 | 67 | 67 | 0 | 100 | 0 | 100 | 0 | 0 | 100 | 100 | 65 | 69 | 50 |
| Greater than 1mm | 0 | 100 | 0 | 0 | 0 | 100 | 0 | 50 | 0 | 100 | 100 | 0 | 0 | 28 | 21 | 50 |
| Alkali production | 100 | 100 | 100 | 100 | 0 | 100 | 0 | 100 | 100 | 100 | 100 | 100 | 100 | 95 | 93 | 100 |
| Acid production | 0 | 0 | 0 | 0 | 100 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 7 | 0 |
| Dry colony type | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 0 | 0 | 0 | 0 | 77 | 100 | 0 |
| Wetcolony type | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 23 | 0 | 100 |
| Dome shaped | 100 | 100 | 100 | 0 | 100 | 100 | 100 | 100 | 92 | 0 | 0 | 0 | 0 | 76 | 99 | 0 |
| Flat colonies | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 8 | 100 | 100 | 100 | 100 | 25 | 4 | 100 |
| Milky | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 0 | 0 | 0 | 0 | 77 | 100 | 0 |
| Watery translucent | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 23 | 0 | 100 |
| White | 0 | 0 | 0 | 0 | 0 | 14 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| Elastic | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 0 | 0 | 0 | 0 | 77 | 100 | 0 |
| Non elastic | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 23 | 0 | 100 |
| **NaCl tolerance** | | | | | | | | | | | | | | | | |
| 0.1% NaCl | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 0.2% NaCl | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 0.5% NaCl | 21 | 23 | 14 | 67 | 67 | 57 | 25 | 100 | 38 | 100 | 92 | 83 | 44 | 41 | 30 | 77 |
| 1.0% NaCl | 4 | 0 | 0 | 33 | 67 | 57 | 25 | 100 | 23 | 100 | 8 | 0 | 0 | 15 | 15 | 13 |
| 1.5% NaCl | 4 | 0 | 0 | 33 | 67 | 43 | 25 | 100 | 23 | 100 | 8 | 0 | 0 | 14 | 14 | 13 |
| 2% NaCl | 2 | 0 | 0 | 0 | 67 | 29 | 25 | 50 | 8 | 67 | 0 | 0 | 0 | 8 | 8 | 7 |
| 3.5% NaCl | 2 | 0 | 0 | 0 | 67 | 29 | 25 | 50 | 0 | 33 | 0 | 0 | 0 | 6 | 7 | 3 |
| 5% NaCl | 0 | 0 | 0 | 0 | 33 | 14 | 25 | 0 | 0 | 33 | 0 | 0 | 0 | 3 | 3 | 3 |
| **pH tolerance growth at** | | | | | | | | | | | | | | | | |
| pH 3.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| pH 4.0 | 4 | 8 | 0 | 33 | 33 | 29 | 50 | 0 | 46 | 33 | 17 | 33 | 11 | 16 | 15 | 20 |
| pH 4.5 | 75 | 85 | 29 | 100 | 67 | 100 | 100 | 50 | 100 | 67 | 92 | 83 | 100 | 82 | 79 | 90 |

Table 2. Continued.

| Group no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | Total isol. | Clust.I isol. | Clust.II isol. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. of isolates in group | 48 | 13 | 7 | 3 | 3 | 7 | 4 | 2 | 13 | 3 | 12 | 6 | 9 | 130 | 100 | 30 |
| **pH tolerance growth at** | | | | | | | | | | | | | | | | |
| pH 5.0 | 98 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 99 | 99 | 100 |
| pH 5.5 | 98 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 99 | 99 | 100 |
| pH 8.0 | 98 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 99 | 99 | 100 |
| pH 8.5 | 98 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 89 | 98 | 99 | 97 |
| pH 9.0 | 60 | 69 | 57 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 56 | 77 | 74 | 87 |
| pH 9.5 | 10 | 0 | 14 | 33 | 33 | 100 | 100 | 100 | 100 | 100 | 42 | 67 | 22 | 37 | 34 | 47 |
| pH 10 | 0 | 0 | 0 | 0 | 0 | 86 | 100 | 100 | 100 | 100 | 0 | 17 | 11 | 23 | 25 | 17 |
| pH 11 | 0 | 0 | 0 | 0 | 0 | 29 | 100 | 100 | 69 | 100 | 0 | 0 | 0 | 15 | 17 | 10 |
| nd | 2 | | | | | | | | | | | | | 1 | 1 | 0 |
| **Temperature growth at** | | | | | | | | | | | | | | | | |
| 32°C | 98 | 85 | 100 | 67 | 100 | 86 | 75 | 50 | 92 | 100 | 100 | 100 | 78 | 92 | 92 | 93 |
| 35°C | 42 | 46 | 57 | 33 | 33 | 43 | 75 | 0 | 69 | 67 | 50 | 50 | 44 | 48 | 47 | 50 |
| 37°C | 4 | 0 | 0 | 0 | 33 | 14 | 50 | 0 | 23 | 67 | 8 | 0 | 22 | 11 | 9 | 17 |
| 40°C | 0 | 0 | 0 | 0 | 33 | 0 | 50 | 0 | 15 | 67 | 8 | 0 | 0 | 6 | 5 | 10 |
| nd | 2 | 15 | 0 | 0 | 0 | 14 | 25 | 50 | 8 | 0 | 0 | 0 | 22 | 7 | 7 | 7 |
| **Survival at** | | | | | | | | | | | | | | | | |
| 32°C | 44 | 85 | 100 | 67 | 100 | 71 | 50 | 50 | 85 | 100 | 92 | 100 | 78 | 69 | 63 | 90 |
| 35°C | 44 | 85 | 100 | 67 | 100 | 71 | 50 | 50 | 85 | 100 | 92 | 100 | 78 | 69 | 63 | 90 |
| 37°C | 23 | 54 | 43 | 67 | 100 | 71 | 50 | 0 | 85 | 100 | 92 | 67 | 78 | 53 | 44 | 83 |
| 40°C | 2 | 0 | 29 | 0 | 67 | 29 | 50 | 0 | 31 | 100 | 50 | 0 | 56 | 21 | 13 | 47 |
| 45°C | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8 | 67 | 8 | 0 | 22 | 5 | 1 | 17 |
| nd | 0 | 31 | 0 | 0 | 0 | 29 | 50 | 50 | 15 | 0 | 8 | 0 | 22 | 11 | 11 | 10 |
| **Host plant** | | | | | | | | | | | | | | | | |
| Magoye | 25 | 54 | 43 | 67 | 33 | 43 | 25 | 0 | 23 | 67 | 83 | 17 | 89 | 41 | 32 | 70 |
| Hernon147 | 21 | 38 | 29 | 0 | 67 | 43 | 75 | 50 | 31 | 0 | 17 | 50 | 11 | 28 | 30 | 20 |
| Roan | 50 | 8 | 29 | 33 | 0 | 14 | 0 | 50 | 31 | 33 | 0 | 33 | 0 | 28 | 34 | 10 |
| nd | 2 | | | | | | | | 15 | | | | | | | |
| Ref. strain | 2 | | | | | | | | | | | | | | | |
| **Place of origin** | | | | | | | | | | | | | | | | |
| Chikomba | 19 | 23 | 14 | 0 | 33 | 14 | 25 | 0 | 8 | 0 | 0 | 0 | 89 | 19 | 17 | 27 |
| Chiweshe | 44 | 8 | 43 | 33 | 33 | 14 | 50 | 0 | 8 | 0 | 0 | 17 | 0 | 25 | 31 | 3 |
| Guruve | 25 | 38 | 43 | 33 | 33 | 14 | 25 | 50 | 23 | 67 | 17 | 17 | 0 | 25 | 28 | 17 |
| Hurungwe | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 50 | 0 | 33 | 17 | 0 | 0 | 3 | 1 | 10 |
| Mhondoro | 8 | 0 | 0 | 33 | 0 | 29 | 0 | 0 | 46 | 0 | 0 | 67 | 0 | 13 | 13 | 13 |
| Mutoko | 0 | 31 | 0 | 0 | 0 | 29 | 0 | 0 | 0 | 0 | 25 | 0 | 11 | 8 | 6 | 13 |
| Wedza | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 42 | 0 | 0 | 4 | 0 | 17 |
| Unnknown | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 15 | 0 | 0 | 0 | 0 | 2 | 3 | 0 |
| Ref. strain | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |

tested and could grow at 32°C and survive at 32 and 35°C. Group 9 isolates were more tolerant to high temperature with most growing at between 32–35°C but some could grow up to 40°C. Most Group 9 isolates survived 32–37°C but a few could survive temperatures as high as 45°C. Group 8 isolates were from Hernon 147 and Roan while Group 9 isolates were from all the three host varieties,. Group 8 isolates were from Guruve and Hurungwe while Group 9 isolates were mostly from Mhondoro, Guruve, Chikomba and Chiweshe (Table 2).

*Cluster II*

Isolates in Cluster II were separated into four groups 10–13 (Fig. 1). They were all alkali producers (Table 2). Occurrence of two distinct colony types from a single isolate was almost always associated with the isolates in Cluster II. This phenomenon was observed even when the isolates were further purified by dilution and re-streaking, thus reducing the chances of mixed cultures arising from cohabitation of different strains of rhizobia in one nodule.

Isolates in Groups 10 and 11 formed colonies greater

than 1 mm while those in Groups 12 and 13 formed colonies about 1 mm in diameter on YEM after 7 days. Most of the isolates in Groups 12 and 13 were not very tolerant to NaCl as they only tolerated between 0.1–0.5% NaCl. In Group 11 only a few tolerated up to 1.5% NaCl while Group 10 isolates were more tolerant with some tolerating the whole range of NaCl, 0.1–5% (Table 2).

Group 10 isolates were also more tolerant to a wider range of pH 4–11 particularly alkali tolerance where all the isolates could grow at pH 11. Isolates in the other three groups were mostly tolerant to pH 4.5–9 with a few growing at pH 9.5–10. Group 10 isolates exhibited more tolerance to temperature with some growing between 32–40°C and surviving exposure to 32–45°C. Groups 11 and 13 had similar tolerance to high temperature, but none of the strains could grow at 40°C. Group 12 was less tolerant to elevated temperature with isolates only growing at between 32–35°C and surviving exposure to 32–37°C (Table 2).

Group 10 isolates were from Magoye and Roan, group 11 and 13 from Magoye and Hernon 147 while Group 12 isolates were from all the three host species. Group 10 isolates were from Hurungwe and Guruve, Group 11 from Mutoko, Wedza, Guruve and Hurungwe, Group 12 from Mhondoro, Chiweshe and Guruve and Group 13 from Chikomba and Mutoko.

The largest number of isolates from the specific soyabean variety Roan were in Group 1 (Table 2). These composed 50% (25) of Group 1 isolates and included the reference strain MAR 1491. Group 5, 7, 11 and 13 isolates were from promiscuous varieties Magoye and Hernon 147. More than 50% of the isolates in Cluster II which formed the wet colony type were from Magoye (Table 2). Isolates in the other groups were a mixture, obtained either from nodules of the specific or the promiscuous soyabean varieties. Isolates from Guruve belonged to 12 of the different groups, those from Chiweshe and Chikomba belonged to 9 and 8 different groups, respectively. Four groups of isolates were common in at least four districts while the rest of the groups were isolated from two to three districts.

## 4. Discussion

Occurrence of fast growing, soyabean nodulating rhizobia has been reported in some African soils (de Lajudie et al., 1994), including isolates from Zimbabwe (Mpepereki et al., 1997), as well as in other soils of the world (Chen et al., 1988). *Agrobacterium* isolates have been shown to co-habit with rhizobia in the nodules of soyabean (van Rensburg and Strijdom, 1971, 1972). In this study the fast growing isolates from all locations failed to form nodules on the test soyabean variety Magoye and although not verified, could possibly be strains of *Agrobacterium*. Results similar to those of this study have been reported in West African soils where fast growing rhizobia obtained from nodules of a single soyabean plant could not induce nodulation when they were tested on the original promiscuous host soyabean variety TGX 1660-19F (Ayanu et al., 1998). All the authenticated isolates formed colonies after 5–7 days of incubation, which is typical of the *Bradyrhizobium* genus (Graham et al., 1991).

Clustering, using simple matching coefficient separated the isolates into two main clusters, based on colony morphology, forming the dry, milky, elastic, mostly dome shaped (Cluster I) and the wet, flat, watery translucent, inelastic, alkali producing colony types (Cluster II). These dry and wet colony types have also been previously reported for some soyabean and cowpea isolates from Zimbabwean soils (Mpepereki, 1994) and in Zambia (Mwakalombe, 1998). The isolates forming dry colony types were more diverse as these were separated into nine major groups based on colony size and stress tolerance while those forming wet colonies separated into four groups. Isolates in Cluster II formed colonies equal to or greater than 1 mm in diameter, while some in Cluster I formed very small colonies (>1 mm in diameter) on YEM after 7 days. Of the isolates in Cluster I, 32%, 30% and 34% were from promiscuous Magoye, Hernon 147 and specific Roan, respectively. In Cluster II, 70%, 20% and 10% were from promiscuous Magoye, Hernon 147 and specific Roan, respectively. In a similar study the isolates forming wet colonies were from the promiscuous soyabean varieties Magoye and Hernon 147 while no wet colony producers were obtained from the specific variety Kaleya (Mwakalombe, 1998).

Isolates with high salt tolerance belonged to seven of the groups and included isolates WM2, ZM4, NH1, TR3 and CH3. Tolerance to NaCl was not based on colony form as both wet and dry colony types had high salt tolerance. Three percent of these isolates could tolerate NaCl concentrations as high as 5%. Similar tolerance to NaCl has been previously observed in a similar study where 39% of 23 isolates from specific soyabean varieties were able to grow at 5% NaCl (Mpepereki, 1994). Sodium chloride tolerance by Sudanese tree rhizobia has also been reported with some isolates growing at 3% NaCl (Zhang et al., 1991). Previously most rhizobia species had been reported to be unable to grow above 2% NaCl (Batzli et al., 1992; Graham and Parker, 1964). Very few soils used for agriculture in Zimbabwe are sodic or have high salt concentrations. Therefore the relevance of tolerance to the wide range of NaCl by the isolates is not clear, although this is a useful physiological test for discriminating rhizobial isolates. Salt tolerance also could be an adaptation for surviving soil environments with fluctuating moisture levels as is common in Zimbabwean soils.

The isolates tolerant to high salt concentrations were obtained from the three soyabean varieties and from all the different districts except for Wedza. Tolerance of isolates to high NaCl was therefore not associated with geographical origin of isolates or the host soyabean variety. Nine of the

13 groups contained isolates with tolerance to low pH. These isolates belonged to both clusters forming wet and dry colonies with all the groups in Cluster II (wet colony types) and seven of the nine groups in Cluster I having some isolates tolerant to pH 4. In other studies, isolates forming the 'wet colony' types have been reported to be more tolerant to acid-aluminium stress than the isolates forming dry pinpoint colonies (Ayanaba et al., 1983). Ability of isolates to produce acid on YEM did not seem to be related to their tolerance to low pH. Ayanaba et al. (1983) showed that growth preceded pH changes in liquid media and on agar plates containing galactose and arabinose instead of mannitol.

Acid tolerance was not related to host of origin as the isolates tolerant to low pH were from both the specific and the promiscuous soyabean varieties. Most of the soils used in this study had $pH_{CaCl2}$ values above 4.5 but some isolates were able to form colonies at pH 4.0 and pH 3.5. Isolates forming colonies at pH 4.0 and 4.5 were also obtained from districts with soils having pH above and below 5.0. Acid tolerance was therefore not related to the pH of the soil of origin of the bacteria. Similar observations were also made in Nigerian soils where acid tolerance of some 54 soyabean isolates was not related to the pH of soils of origin (Asanuma and Ayanaba, 1990). Variability of pH in the micro-environment especially the rhizosphere where microbial activity is highest could account for the apparent non-relatedness of pH of the soils and acid tolerance of the resident rhizobia. Activities of rhizosphere microorganisms can alter the acidity and alkalinity of the immediate environment for rhizobia (Wild, 1993). At least some strains from all the 13 groups could grow at pH 9 with isolates from five of the groups growing up to pH 11. There were therefore fewer groups of the isolates adapted to extremely high pH than to low pH. This could be related to the inherent low pH of the soils in most of the Zimbabwean soils from which these rhizobia were isolated.

Isolates in four of the groups could grow at a wider range of temperature while those in six of the groups could grow at a narrow range of temperature. More groups of the isolates (9) could however survive exposure to high temperature (40°C) than those able to grow at high temperature (5). Some isolates could therefore not grow at high temperature although their viability at lower temperatures was not affected by exposure to the higher temperatures. Generally wet colony type isolates appeared to be more temperature tolerant than the isolates forming dry colonies. However at high temperature values of 35°C and 40°C these wet colony types formed dry colonies while at low temperature the plates almost always had a mixture of some partly dry and wet colonies. This indicates colony dimorphism, a phenomenon which has been observed in rhizobia strains such as USDA 76 (Sylvester-Bradley et al., 1988). This switching of colonies from one form to another could be a strategy to survive adverse conditions although the mechanism is not clear. Fewer groups (3) of rhizobia with isolates from the specific soyabean varieties could survive high temperatures than those with isolates from promiscuous soyabean variety Magoye (7 groups). The promiscuous soyabean variety Magoye was associated with strains of rhizobia with a wider range of temperature tolerance including those that can survive temperatures of 45°C. This may partly explain why Magoye is said to be promiscuous. The isolates tolerant to a wide range of temperature were distributed across the whole range of districts. These results indicate that geographical origin may not be related to temperature tolerance of rhizobial isolates.

Some isolates from this study could grow at 40°C, while others could survive incubation at 45°C. Temperatures in the low altitude areas of Zimbabwe sometimes exceed 40°C (Meteorological Consultancy Services, 1987) and hence soil surface temperatures above 40°C are possible (Mpepereki, 1994). These isolates with high tolerance to temperature could therefore be adapted to these high soil temperatures in some Zimbabwean soils. Isolates from specific soyabean varieties surviving high temperatures above 40°C have also been reported in some Zimbabwean soils (Mpepereki et al., 1996b) and in Sudanese soils (Zhang et al., 1991). The proportion of isolates surviving 40°C and above under laboratory conditions was however small.

Rhizobia that can grow and survive wider temperature fluctuations may have a competitive advantage in the nodulation of legumes. Temperature changes may markedly change the nodule occupancy by competing strains (McDermott and Graham, 1990) and this may be related to the activity and ability of the rhizobia to grow and survive at extreme temperatures. Some strains of *Bradyrhizobium* spp. were found to be poor competitors with *B. japonicum* on promiscuous *Glycine max* cv. Malayan between 24°C and 33°C but formed more nodules than *B. japonicum* at 36°C (Roughley et al., 1980). This could be related to a strain's ability to tolerate and grow at high temperature. Isolates which could survive higher temperatures such as those in groups 7, 10, 11 and 13 such as CH1, FH6, TR3, WM21, EM7 and KM10 are therefore more likely to occupy a higher percentage of nodules on soyabean in hot areas. A desirable strain for tropical soils should be able to tolerate high temperatures and also have a high N fixing potential otherwise occupation of nodules by ineffective rhizobia will reduce the amount of $N_2$ fixed.

Isolates from the promiscuous soyabean variety Magoye were present in 12 of the 13 groups, those from Hernon 147 in 11 groups and those from the specific variety Roan in 9 groups. In a similar study the strains indigenous to Zambian soils were grouped into 8 classes for promiscuous Magoye compared with four, three and two classes for cowpea, Hernon 147 and specific Kaleya respectively, based on physiological parameters (Mwakalombe, 1998). This ability to interact with a wider range of rhizobia is consistent with the promiscuity trait of the Magoye variety. The property also explains the readiness of Magoye to

nodulate in many soils that were tested for the presence and potential effectiveness of rhizobia (Musiyiwa et al., 2005).

Guruve soils had the most diverse range of isolates (12 groups) followed by those from Chiweshe (9 groups) and then those from Chikomba (8 groups). Guruve soils were generally richer in clay content with a high pH and the district has a history of soyabean growing without inoculation in most soils. The other districts generally have sandy soils, which are poorer in nutrient status and very few of these soils have ever been cropped to soyabean in the past. Rainfall is also higher in the northern regions of Zimbabwe such as Guruve and decreases to the south. Some Chiweshe soils also had a history of soyabean growing. These factors may have contributed to the survival and occurrence of a wider range of rhizobia in these than other districts.

Most of the isolates (24/37) from the specific soyabean variety Roan were in group 1 in which the reference inoculant strain MAR 1491 also belonged. This probably indicates that Roan is more compatible with this group of isolates as opposed to the other groups. These isolates in group 1 as well as 2 and 3, apparently are less tolerant to high pH, salt and temperature extremes than the other isolates in Cluster I. Forty seven isolates in group 1 were about 90% similar to the reference strain in their morphological and physiological properties. This indicates a large number of indigenous strains which are similar to or that are not very different from the exotic strain *B. japonicum* MAR 1491 in some Zimbabwean soils.

Tolerance to acidity may be desirable for inoculant strains for soyabean to be grown in acidic soils. If such an acid tolerant strain is required then it may be selected from groups 4, 5, 6, 7, 9, 10, 11, 12 and 13 which had some isolates which were tolerant to low pH. Isolates tolerant to wide range of pH included KR5, KR7, FR7 and HH4. Those tolerant to high temperatures may be more suitable for use in very hot areas as opposed to the less tolerant strains. Isolates tolerant to high temperature were in groups 7, 10, 11 and 13 and included CH1, FH6, TR3, WM21, EM7 and KM10 which could grow or survive at 40°C and above. Some isolates appeared to be tolerant to temperature as well as pH or salt and these included TR3, WM14, and KR7. These isolates could be more suitable as inoculants in environments with harsh conditions. The survival of rhizobia under extreme physiological conditions are some of the properties that are desirable for an inoculant strain. The inoculant strain should be able to adjust to the field conditions in which soyabean or other legumes are being grown for effective nodulation and hence increased yield. The performance of the rhizobia-legume symbiosis will, however always be determined by a combination of factors. The isolates should not only be able to survive adverse environmental conditions but must also be more competitive for nodulation than other indigenous rhizobia which could be less effective and fix insufficient nitrogen for soyabean in the field. Hence the more tolerant isolates from this study could be further screened for symbiotic effectiveness and competitiveness.

## 5. Conclusions

The classification using cultural and physiological properties demonstrated that a wide range of rhizobial types are present in Zimbabwean soils. Isolates tolerant to stress factors such as salt which include WM2, ZM4, NH1, TR3 and CH3, to temperature such as CH1, FH6, TR3, WM21, EM7 and KM10 and to a wide pH range such as KR5, KR7, FR7 and HH4 are present in Zimbabwean soils. Some isolates were tolerant to temperature as well as a wide range of pH or salt and these included TR3, WM14, and KR7. The isolates forming wet colony types appeared to be more tolerant to temperature than those forming the dry colonies. Tolerance to salt and acidity of the isolates was not related to colony morphology, variety or geographical origin. The isolates from Guruve were the most diverse in terms of colony morphology and physiological properties followed by those from Chiweshe. Isolates which were similar to the inoculant strain MAR 1491 were abundant in some Zimbabwean soils where soyabean has been previously grown without inoculation.

The study has shown that some soyabean rhizobial isolates tolerant to stress factors are present in Zimbabwean soils. These could be developed as inoculants. There is need for further physiological characterization including effectiveness and competitiveness of the isolates. Molecular characterization of the wide variety of isolates is necessary to ascertain which species of rhizobia are able to nodulate soyabean in African soils.

## Acknowledgements

We thank the Forum for Agricultural Husbandry of the Rockefeller Foundation for funding.

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