Symbiosis, 22 (1997) 275–292
Balaban, Philadelphia/Rehovot

Physiological Characterization of Indigenous Rhizobia Nodulating *Vigna Unguiculata* in Zimbabwean Soils

S. MPEPEREKI[1*], F. MAKONESE [1], and A.G. WOLLUM[2]
[1]*Department of Soil Science and Agricultural Engineering, University of Zimbabwe, P.O. Box MP167, Mt. Pleasant, Harare, Zimbabwe, Tel. +263-4-303211, Fax. +263-4-333407, E-mail. smpepe@zimbix.uz.zw*
[2]*Department of Soil Science, North Carolina State University, P.O. Box 7619, Raleigh, NC 27695-7619, USA*

Received October 8, 1996; Accepted January 30, 1997

Abstract

Cowpea (*Vigna unguiculata* [L.] Walp.) nodule isolates from 14 Zimbabwean soils included similar proportions of both fast- (49%) and slow-growing (51%) rhizobia. Colony morphologies ranged from small (< 1–2 mm) 'dry' or gummy in slow-growing isolates, to large (>3 mm) 'wet' watery/slimy types of fast growing rhizobia. All fast-growing isolates produced an acid reaction while slow-growers produced acid, alkaline or neutral reactions on YEM. The majority of both fast- and slow-growing isolates tolerated high acidity (growth at pH 4.0) and salt (up to 5.5% NaCl). Phosphatase activity ranged from undetectable to 0.02 μmol p-nitrophenol h^{-1} 10^{-6} cells and was generally higher among fast- compared to slow-growing isolates. Intrinsic antibiotic sensitivity was generally higher in fast- compared to slow-growers. The least utilized of 12 sole carbon sources tested were p-hydroxybenzoic acid (31%) and xylose (78%). Despite some similarities with established rhizobial taxa, the indigenous strains showed considerable cultural and physiological diversity and probably include unique types belonging to several, as yet undefined species.

Keywords: Cowpea rhizobia, physiological diversity, stress tolerance, phosphatase activity

*The author to whom correspondence should be sent.

0334-5114/97/$05.50

1. Introduction

Rhizobial populations in tropical soils represent an important reservoir from which superior strains adapted to environmental stresses such as drought, high soil temperatures and extreme pH could be isolated and evaluated for use as legume inoculants. Exploitation of biological nitrogen fixation by symbiotic nodule bacteria to improve the productivity of low-input tropical cropping systems demands in part, that indigenous rhizobial populations be adequately characterized. Minimum criteria for describing new rhizobial species include cultural, morphological, physiological and symbiotic traits in addition to phylogenetic characteristics (Graham et al., 1991). On the basis of growth rate, fast-growing nodule bacteria have been placed in the genus *Rhizobium* and slow-growing ones in the genus *Bradyrhizobium* (Jordan, 1984). Colony morphology (Zhang et al., 1991), substrate utilization (Parker and Ornston, 1984), tolerance to stress factors such as temperature, salinity and acidity (Lindstrom and Lehtomaki, 1988; Martinez-Romero et al., 1991) and antibiotic resistance (Mueller et al., 1988) have all been used as important criteria for distinguishing rhizobia.

African soils harbour a large diversity of rhizobial populations that are only now being described. Cowpea rhizobia indigenous to Nigerian soils are probably the only group that has been studied in some detail (Ahmad et al., 1981; Eaglesham et al., 1987; Sinclair and Eaglesham, 1984; 1990). Research on indigenous fast-growing rhizobia in Senegal, West Africa, has recently led to the description of two fast-growing species, *Sinorhizobium saheli* and *S. teranga* (De Lajudie et al., 1994). Slow-growing indigenous rhizobia nodulating soybean in Zimbabwean soils were recently reported by Davis and Mpepereki, (1995). Their isolates showed cultural and serological similarities to the soybean rhizobia belonging to *Bradyrhizobium elkanii*. While well-known cowpea strains such as CB756, 3G4b20 and TAL677 are of Zimbabwean origin, indigenous rhizobia populations have until recently remained largely uncharacterized. Population levels of rhizobia under field conditions in Zimbabwe have recently been determined (Mpepereki and Makonese, 1995). Host range studies of these populations have shown that the individual rhizobia isolates have rather narrow host ranges (Mpepereki et al., 1996). We present here the first comprehensive study of cultural and physiological characteristics of indigenous rhizobia nodulating cowpea, *Vigna unguiculata* in a wide range of Zimbabwean soils.

2. Materials and Methods

Soil analysis

Soils were sampled at 14 locations representative of the five agro-ecological regions of Zimbabwe, which range from semi-arid Natural Region (NR) 5 (<450 mm rainfall p.a.) to the wet NR 1 (>1000 mm p.a.). Composite samples were sieved to pass through a 2 mm sieve and divided into two. A sub-sample was used to analyze for soil physico-chemical properties including texture, pH, phosphorus and cation exchange capacity (CEC). Soil pH was measured in a 1:5 mixture of soil and 0.01M $CaCl_2$ (Schofield and Taylor, 1955). Soil was dispersed in Calgon (sodium metaphosphate) in a mechanical shaker for 30 minutes. A Bouyoucos hydrometer was used to estimate silt and clay fractions. Soil texture was determined by mechanically separating soil fractions according to size and referring to a soil textural triangle (Thompson and Purves, 1978). Cation exchange capacity was determined by leaching with 0.2 M NH_4Cl and titrating with acid (Russell, 1973). Available phosphorus was determined using anion exchange resin extraction (Saunder and Metelerkamp, 1962).

Isolation of rhizobia

Composite random samples from the surface 30 cm of the soil were mixed thoroughly and used to fill plastic pots each holding about 3.0 kg of soil. Four seeds of cowpea (*Vigna unguiculata* [L.]) cv 'Local Mixed' surface-disinfected in 95% ethanol were sown per pot. Plants were grown for six weeks under natural light in a greenhouse with temperatures ranging from 16 to 34°C and watered with distilled water. Root samples were carefully washed free of soil particles and nodules picked for rhizobial isolation. All nodules were determinate, pinkish-brown and roughly spherical in shape with light and dark bands running from apex to base.

Rhizobia were isolated from nodules on yeast-extract mannitol (YEM) agar (Somasegaran and Hoben, 1985). Nodules were individually surface-disinfected with 95% ethanol, rinsed with sterile distilled water, crushed in sterile saline (0.85% w/v), and the supernatant aseptically streaked on yeast-extract mannitol (YEM) agar. Plates were incubated at 28°C for up to seven days or until colonies were visible. Single colony isolates were purified by re-streaking on YEM, checked for Gram-staining (negative), growth on YEM with Congo red (little dye uptake) and ability to nodulate the original host (*V. unguiculata*). The ability of isolates to change the pH of their growth medium was scored on YEM agar plates amended with 0.25 mg l^{-1} bromothymol blue. Reference strains included for comparison were *Bradyrhizobium* sp.

CB756, 32H1 and 3G4b20, *Bradyrhizobium elkanii* USDA 31 and USDA 76, *Bradyrhizobium japonicum* USDA 110, *Rhizobium leguminosarum* 127K14 and *Rhizobium tropici* CIAT 899.

Growth rates and colony morphology

Each isolate was raised in broth culture to early log phase, diluted to give approximately 10^5 cells ml^{-1}, and streaked on duplicate YEM plates. Plates were incubated at 28°C for 7–10 days and examined daily to determine the time to first appearance of colonies. Colony diameter in mm was recorded after four days for fast-growing isolates and seven days for slow-growing types. Colony appearance was scored as 'dry' where the surface was smooth and firm, and 'wet' for those strains which were watery or slimy.

Acid tolerance

The lowest pH permitting growth in YEM medium minus KH_2PO_4 (Zablotowicz and Focht, 1981) was determined by inoculating plates at pH 3.0, 3.5, 4.0, 4.5, 5.0 and 5.5 in the acid range and pH 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5 and 11.0 in the alkaline range. A multi-point inoculator designed to fit into a plate was made by pushing hot 3.0 cm-long pins through the bottom of the wells of a microtiter plate. A broth culture of each isolate was diluted to contain approximately 10^5 cells ml^{-1}. Aliquots of 100 µl of each diluted culture were dispensed into sterile microtiter plates. The multi-point inoculator, previously sterilized by dipping in 95% ethanol and flaming, was used to transfer cultures to duplicate plates. Plates were incubated in the dark at 28°C for up to 10 days and scored for growth. For acid tolerance in broth culture, a 1.0 ml aliquot of each of the diluted cultures was inoculated into 25 ml of fresh YEM broth in two replicate Erlenmeyer flasks per pH level. Flasks were incubated at room temperature, (RT) (20–25°C) with shaking and growth monitored visually by comparing turbidity in inoculated and non-inoculated control flasks at the same pH over 10 days.

Salt tolerance

Tolerance to NaCl was determined by checking for growth on YEM agar plates containing 0.1, 0.2, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0% NaCl (w/v). A multi-point inoculator was used to transfer isolates as described above. Replicate plates were incubated at 28°C and examined for growth daily.

Phosphatase activity

Phosphatase activity was determined using the method of Smart et al (1984). Cells were grown in galactose-arabinose glutamate (GAG) medium (Cassman et al., 1981) modified to contain per litre: galactose, 3 g, arabinose 3 g, Na-glutamate, 0.5 g and NH_4Cl, 0.5 g. Other inorganic nutrients were added at the following concentrations (mM): $MgSO_4$, 300; $CaCl_2$, 300; Fe-EDTA, 50; $MnSO_4$, 1; $ZnSO_4$, 0.4; $CuSO_4$, 0.1; Na_2MoO_4, 0.02 and $CoCl_2$, 0.002. Vitamins were included at the following concentrations (mg l^{-1}): thiamine-HCl, 50; Ca-pantothenate, 50; pyridoxine and p-hydroxybenzoic acid, 15 and biotin, 2. The pH was adjusted to 5.6 and buffered by addition of MES (morpholinoethane sulphonic acid) at 2.0 g l^{-1}. The P concentration was adjusted to 1.0 mM for medium containing P (+PGAG) using KH_2PO_4 that was omitted for P-free (–PGAG) medium. Each isolate was grown to turbidity in 20 ml of +PGAG medium at RT with shaking (200 rpm). *Bradyrhizobium elkanii* strain USDA 31 and *B. japonicum* strain 110 were included as the negative and positive controls respectively. A 1.0 ml aliquot was transferred to 20 ml of fresh –PGAG broth and incubated at RT with rotary shaking for 48 h to encourage de-repression of alkaline phosphatase genes. A 1.0 ml aliquot of the 48h-old –PGAG culture was transferred to 5 ml of 0.1 M Tris.Cl buffer, containing p-nitrophenyl phosphate (Sigma 104) (1.0 mg ml^{-1}). Cells were incubated for 1 h at RT with occasional shaking. Activity was indicated by the release of p-nitrophenol (p-Np) which was measured spectrophotometrically at 410 nm (Spectronic 20, Bausch Lomb). Concentration was determined from a standard curve of p-nitrophenol in –PGAG. Cell numbers were determined by dilution drop-plating on +PGAG agar. Phosphatase activity was expressed as µmol p-nitrophenol h^{-1} per million cells (µmol p-Np h^{-1} 10^{-6} cells).

Substrate utilization

Ability to use as sole carbon source each of 12 compounds, L-arabinose, fumarate, D-galactose, D-glucose, inulin, malate, mannitol (reference), p-hydroxybenzoic acid (PHB) neutralized with NaOH, rhamnose, sorbitol, succinate and xylose (all from Sigma) was tested using the defined medium of Lindstrom and Lehtomäki (1988). The C source to be tested was added to a final concentration of 0.1% (w/v). Control plates had no C source. Duplicate plates were inoculated with a multi-point inoculator as previously described, incubated for 7–10 days and examined for growth.

Intrinsic antibiotic sensitivity

Sensitivity of rhizobial isolates to 14 antibiotics was tested using Mastring-S antibiotic discs (Mast Laboratories, Merseyside, UK). The antibiotics tested and their concentrations (ml^{-1}) were: ampicillin (Amp), 25 mg; cephalothin (Cep), 30 mg; chloramphenicol (Chl), 25 mg; cotrimoxazole (Cot), 25 mg; erythromycin (Ery), 5 mg; gentamicin (Gen), 10 mg; nalidixic acid (Nal), 30 mg; nitrofurantoin (Nit), 50 mg; penicillin G (Pen), 1 unit; streptomycin (Str), 10 mg; sulphacotrimazole (Smx), 50 mg; sulphatriad (Sut), 200 mg; tetracycline (Tet), 25 mg and trimethoprim (Tri), 2.5 mg. Plates of YEM agar were inoculated by spreading a 0.1 ml aliquot containing approximately 10^8 cells ml^{-1} on the surface of each, allowing the liquid to soak in and then placing discs impregnated with different antibiotics on the plate. Plates were replicated twice and incubated at 28°C for 7–10 days. Sensitivity was scored on the basis of presence or absence of an inhibition zone around the disc.

3. Results

Growth rates, colony morphology and pH reaction

Gram-negative isolates which nodulated the original trap host, *V. unguiculata* (cowpea) or *M. atropurpureum* (siratro) were retained for characterization. Of the 140 cowpea nodule isolates evaluated for growth rate, 49% were slow-growing and 51% fast-growing (Table 1). Although on average there were similar proportions of fast- and slow-growing isolates across all sites, half of the sites were dominated by either of the two types. Colony sizes ranged from less than 1.0 mm diameter in 6.4% of the isolates and reference strains 32H1, USDA 31 and USDA 76 (all slow-growing) to over 3.0 mm in 22% of the isolates and CIAT 899 (all fast-growing) (Table 2). Fast-growing isolates formed colonies of the 'wet' type while slow-growing types produced mostly 'dry' colony types (Table 2).

The majority of the cowpea isolates (69%) gave an acid reaction in YEM agar; including all 71 fast-growers. The pH reaction among slow-growing isolates ranged from acid to alkaline (Fig. 1A). Reference strains, 32H1 and CB756 and the fast-growing *R. tropici* CIAT 899 all gave an acid reaction.

Acid tolerance

The lowest pH at which isolates could grow in YEM ranged from pH 3.5–5.5 for both fast- and slow-growing types (Fig. 1B). The largest proportion of both fast- and slow-growing isolates grew at pH 4.0. The lowest pH values per-

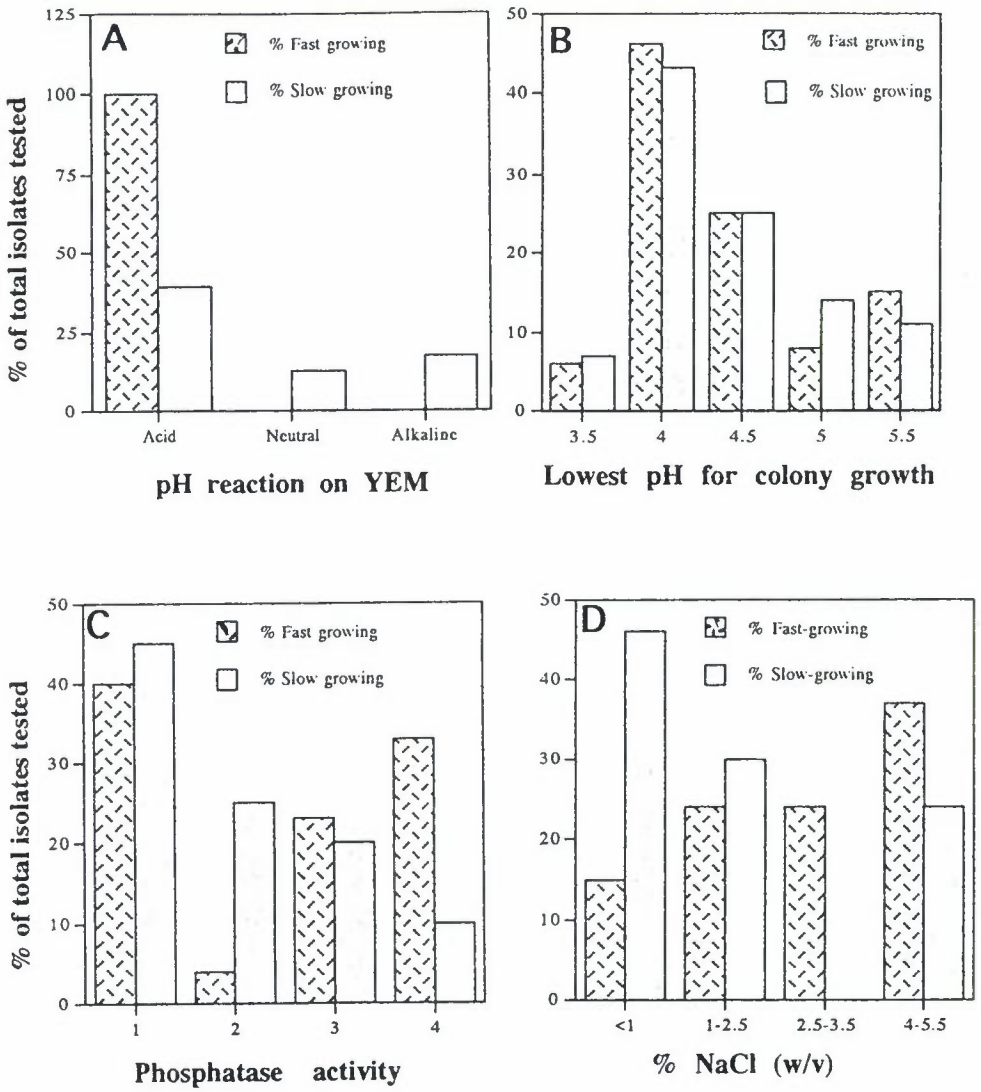

Figure 1. Physiological characteristics of 71 fast- and 69 slow-growing rhizobial isolates from *V. unguiculata*. **A**: pH reaction on YEM agar; **B**: lowest pH for colony growth; **C**: phosphatase activity (1 = 0–0.0004; 2 = 0.0004–0.0013; 3 = 0.0013–0.002; 4 = >0.002 µmol p-nitrophenol h^{-1} 10^{-6} cells); **D**: tolareance to NaCl concentration.

Table 1. Sampling locations and proportions of fast- and slow-growing cowpea nodule isolates from Zimbabwean soils.

Location	Agro-ecology region[1]	Soil texture	Soil pH[2] ($CaCl_2$)	P (resin) (µg/g) dwt	CEC (meq/ 100 g)	Cropping, rhizobial inoculant use history	Total isolates	Fast-growing (%)	Slow-growing (%)
Chabwino	2	SCL	5.1	21	2.7	Maize, tobacco, soya inoculated; irrigated, CM	15	27	73
Chikwaka	2	S	4.3	16	1.5	Maize, sunflower; sweet potato, CA	14	21	79
Chisumbanje	5	C	7.9	30	63.9	Wheat , cotton, soya inoculated; irrigated, CM	8	38	62
Gobera	5	LS	4.2	7	1.1	Maize, sorghum, peanut, cowpea intercrop, CA	10	60	40
Manjonjo	2	S	4.3	8	3.6	Maize, peanut, sunflower CA	7	100	0
Mawabeni	4	SC	5.2	13	4.6	Pasture grasses, wild legumes, CA	10	100	0
Mhondoro	2	S	4.7	17	1.8	Maize, peanut, cowpea intercrop, CA	8	75	25
Middle Save	5	SCL	6.4	70	12.1	Cotton, wheat, soya inoculated, irrigated, CM	10	60	40
Musami	2	S	4.3	38	2.9	Maize, peanut, curcubits intercrop, CA	12	25	75
Musiiwa	2	SCL	4.8	7	2.2	Maize, sunflower, peanut CA	16	56	44
Nyanga	1	SL	4.2	29	2.1	Hops, seed potatoes; irrigated, CM	10	30	70
Plumtree	4	S	4.3	6	2.2	Maize, sorghum, bambarra nut, CA	9	33	67
Serima, Gutu	3	S	6.1	6	1.2	Maize, sunflower, sorghum, CA	6	67	33
Thornpark, Harare	2	C	4.6	11	11.2	Maize, wheat, soya inoculated, irrigated, CM	5	0	100

[1]Agro-ecological regions: 1 = >1000 mm rainfall; 2 = 750–1000 mm; 3 = 680–800 mm; 4 = 450–650 mm; 5 = <450 mm. [2]pH in a 1:5 mixture of soil and 0.01M $CaCl_2$. CM = commercially managed farm; CA = communal area smallholding. Texture: C = clay; S = sand; L = loam.

Table 2. Cultural characteristics of indigenous rhizobia nodulating cowpea, *Vigna unguiculata* in Zimbabwean soils.

Growth rate category	Time to visible colony size (days)	Colony diameter (mm)	Colony characteristics	% of total isolates
Slow	5–7	small (<1 mm)	'dry', no gum, off white, opaque	6.4
Slow	5–7	medium (1–2 mm)	'dry', gummy, convex, firm surface, regular	42.6
Fast	3–5	large (2–3 mm)	'wet', irregular, translucent, ±granular with age	29.3
Fast	2–3	very large (>3 mm)	'wet'', translucent, with opaque centre, copious extracellular slime	21.7

Total isolates tested = 140.

mitting growth were identical in liquid and solid media for 65% of the isolates tested (data not shown). For about 25% of the isolates, the lowest pH for growth was higher by 0.5–1.0 pH unit in liquid compared to solid medium. The two slow-growing reference strains 32H1 and CB756 grew at a minimum pH of 4.5 while USDA 31 and USDA 110 grew at minimum pH of 5.0. The fast-growing *R. tropici* CIAT 899 grew at pH 4.0. In the alkaline range, about 50% of all isolates tested, 70% of them fast-growing, grew at pH 11.0 (data not shown). Slow-growing reference strains grew at a maximum pH of 9.0.

Phosphatase activity

Phosphatase activity among the isolates ranged from undetectable to as high as 0.02 µmol p-Np h^{-1} 10^{-6} cells (Fig. 1C). The largest proportion of cowpea isolates showing little or no phosphatase activity was slow-growing, while those with activities in excess of 0.002 µmol p-Np h^{-1} 10^{-6} cells were all fast-growing (Fig. 1C). Phosphatase activity was undetectable in strain USDA 31 used as the negative control and low (0.0008 µmol p-Np h^{-1} 10^{-6} cells) for USDA 110, the positive control.

Salt (NaCl) tolerance

Maximum NaCl concentrations at which isolates could grow ranged from

<1%–5.5% (w/v) NaCl. None of the isolates tested grew in 6.0% NaCl. The isolates appeared to segregate into two broad categories; those of low to moderate salt tolerance able to grow below 3.5% NaCl and a significant proportion tolerating 4% and higher (Fig. 1D). The largest proportion of fast-growing isolates (37%) tolerated NaCl concentrations above 4%, while the largest proportion of slow growers (46%) could not grow above 1% NaCl (Fig. 1D). The slow-growing cowpea reference strains 32H1 and CB756 and the fast-growing CIAT 899 had maximum tolerance levels below 2% NaCl.

Substrate utilization

Most of the isolates used all the 12 carbon compounds except p-hydroxybenzoic acid (PHB) which was metabolized by only 31% of the isolates tested (data not shown). Only strain USDA 31 among three reference strains tested could grow on PHB. Xylose was used by 78% of 41 cowpea isolates tested with six slow-growing and three fast-growing isolates unable to use it as sole carbon source.

Intrinsic antibiotic sensitivity

Fast-growing isolates were generally more sensitive to the antibiotics tested compared to slow-growing types (Table 3). Antibiotics to which slow-growing cowpea isolates showed the greatest sensitivities were in order from most to least potent: tetracycline > streptomycin >sulphatriad > gentamicin > chloramphenicol > ampicillin > cephalothin = sulphamethoxazole > erythromycin > cotrimoxazole > nalidixic acid > nitrofurantoin > penicillin G > trimethoprim. The order was slightly different with fast-growing isolates, with 100% sensitivity to tetracycline, sulfatriad, gentamicin and chloramphenicol. Cowpea reference strains were on average less sensitive than indigenous isolates.

4. Discussion

Although the cowpea *V. unguiculata* is generally thought to associate with slow-growing rhizobia, our results are significant in demonstrating that fast- and slow-growing isolates were equally represented in nodules across locations, an indication of the host legume's permissiveness (Table 1). Some sites were however dominated by one type, e.g. fast-growers at Mawabeni and Manjonjo and slow-growers at Thornpark and Chikwaka (Table 1). The reasons for this apparent location effect on distribution of types are unclear but probably

Table 3. Antibiotic sensitivity of indigenous rhizobia nodulating cowpea, *Vigna unguiculata* in Zimbabwean soils.

Antibiotic[1]	Concentr. (μg/ml)	Slow growers		Fast growers	
		No. tested	Sensitive (%)	No. tested	Sensitive (%)
Tetracycline	25	30	83.3	26	100
Streptomycin	10	30	77.0	26	84.6
Sulfatriad	200	25	74.0	20	100
Gentamicin	10	21	71.4	23	100
Chloramphenicol	25	30	67.0	28	100
Ampicillin	25	32	59.4	28	71.4
Cephalothin	30	26	50.0	22	45.5
Sulfamethoxazole	50	22	50.0	22	40.9
Erythromycin	5	25	40.0	24	37.5
Cotrimazole	25	24	33.3	22	45.5
Nalidixic acid	30	21	28.6	23	30.4
Nitrofurantoin	50	22	27.3	22	54.5
Penicillin G	1 U	30	23.3	27	14.8
Trimethoprim	2.5	22	22.7	21	28.8

[1]Antibiotic discs (Mastring S) were used on inoculated YEM plates. Sensitivity based on presence/absence of inhibition zone.

include cropping history and edapho-climatic factors. There was no record of inoculation with commercial cowpea-type rhizobia at any of the sampling locations.

Fast-growing isolates produced colony morphologies characteristic of species in the genus *Rhizobium* (Jordan, 1984) (Table 2). About 75% of fast-growing isolates produced large amounts of extracellular polysaccharide slime (EPS), a trait they shared with *R. tropici* strain CIAT 899 while the rest produced small to moderate amounts of EPS giving them a 'wet' or glistening look. Our observations are consistent with variations in colony characteristics and extracellular polysaccharide production previously reported among the fast-growing rhizobia (Zevenhuizen and Bertocchi, 1989; Gil-Serrano et al., 1990). The quantity of the C source appeared to influence the extent of extracellular polysaccharide production by some isolates. For example some fast-growing isolates and strain CIAT 899 produced large amounts of slime in

GAG, but very little when the C substrate concentration in that medium was reduced to 10%. Among the slow-growing isolates, colony morphologies ranged from the pinpoint-sized 'dry' type similar to reference *Bradyrhizobium* sp. strains 32H1 and CB756 to the larger 'gummy' type similar to that of *Bradyrhizobium* sp. (Arachis) strain 3G4b20. A few colonies were irregular and had a glistening 'wet' look. La Favre et al. (1991) also refer to 'wet' slow-growing (brady)rhizobia from Nigerian soils.

All 71 fast-growing indigenous isolates produced an acid reaction on YEM agar (Fig. 1A) as did *R. tropici* strain CIAT 899. In contrast, slow-growing isolates were more diverse, producing acid, neutral and alkaline reactions. In comparison, reference slow-growing cowpea strains 32H1 and CB756 produced an acid reaction while soybean strains USDA 31, USDA 76 and USDA 110 all produced alkaline reactions. Our results confirm the invalidity of linking slow growth to alkali production (Hernandez and Focht, 1984) and are in agreement with similar observations made on cowpea rhizobia from Nigerian soils (Eaglesham et al., 1987).

Cowpea rhizobia have been reported to be acid-tolerant (Keyser et al., 1979; Lowendorf, 1980; Zablotowicz and Focht, 1981). Our study showed that significant proportions of both slow- and fast-growing cowpea-nodulating rhizobia had similar, surprisingly high levels of acid-tolerance, with most able to grow at pH 4.0 (Fig. 1B). Graham et al. (1994) tested 45 strains from the groups *Rhizobium, Azorhizobium* and *Bradyrhizobium* for pH tolerance and found that only *Rhizobium tropici* (strain CIAT 899) grew at pH 4.0 in unbuffered medium. This acid-tolerance may be indicative of adaptation to acid soil environments. Nine of the 14 soils from which our isolates originated had pH values lower than 5.0 (Table 1).

The mechanisms for rhizobia tolerance to low pH are not well understood but plasmids (Chen et al., 1993) and EPS (Cunningham and Munns, 1984) have been implicated. A significant correlation between EPS production and acid tolerance has been reported in the fast-growing *Rhizobium* (Cunningham and Munns, 1984). Both acid tolerance and EPS production have been associated with genes on a megaplasmid in *R. leguminosarum* bv *trifolii* (Chen et al., 1993). Our results suggest that EPS production may not be the mechanism for acid tolerance, since the latter characteristic was present even in isolates not producing EPS. Our findings partly support the conclusions of a study by Graham et al (1994) who found that in *R. tropici* (strain UMR 1899) acid tolerance was not an adaptive response, nor was it plasmid-mediated, correlated with EPS production or related to synthesis of polyamines. Graham et al. (1994) suggested that pH tolerance may be associated with outer membrane composition and structure after observing that strain UMR 1899 cells

accumulated glutamate under acid stress, becoming more hydrophobic and resistant to crystal violet.

Slow-growing cowpea isolates appeared to be more sensitive to alkaline conditions than fast-growing types. The majority of those that could grow at pH 11.0 were fast-growing while most slow-growers including reference strains 32H1, 3G4b20 and CB756 and soybean strains USDA 76 and USDA 110 could only tolerate a maximum pH of 9.5 (data not shown). Fast-growing pigeon pea rhizobia have also been reported to grow at pH 11.5 in culture media (Rao, 1994). Our results confirm the observations of Zablotowicz and Focht (1981) that in general slow-growing cowpea rhizobia appear to be more sensitive to alkaline growth conditions compared to fast-growers.

We found considerable diversity and rather high salt tolerance among rhizobia indigenous to Zimbabwean soils in contrast to reports of low and narrow salt tolerance ranges among rhizobia populations from other regions. Fast-growing isolates showed diversity in tolerance to salt, with the largest proportion able to grow between 4–5.5% NaCl (Fig. 1D). However, in contrast to our findings, fast-growing strains of the species *R. leguminosarum*, *R. meliloti*, *R. galegae* and *R. loti*, failed to grow in 1.0% NaCl (Martinez-Romero et. al., 1991). Indeed *R. tropici* strain CIAT 899 that shared morphological characteristics with some of our salt-tolerant fast-growing isolates could not grow above 1% NaCl. Batzli et al. (1992) also reported that fast-growing rhizobia nodulating *Robinia pseudoacacia* failed to grow in media with >1.5% NaCl. In Nigeria, 79 cowpea isolates which included both fast- and slow-growing types failed to grow at 2% NaCl while only one grew at 1% NaCl (Eaglesham et al., 1987).

Slow-growing isolates were just as diverse as fast-growing types in their salt tolerance characteristics (Fig. 1D). Interestingly, no slow-growing isolates were identified to grow between 2.5–3.5% NaCl concentration. The slow-growers thus divided neatly into two distinct salt tolerance groups, about 25% able to grow above 4% NaCl and the rest unable to grow above 2.5% NaCl. Of the latter group most (nearly 50%) could not grow above 1% NaCl.

The occurrence of significant numbers of salt-tolerant rhizobia in local soils is unusual. Soils at sampling locations were not saline (data not shown). In Zimbabwe the rainfall is unimodal and characterized by frequent mid-season dry spells which subject the soils to fluctuating moisture levels. This could increase relative solute concentrations and impose cycles of osmotic stress on the rhizobia populations. The preponderance of salt-tolerant isolates in our collection could thus be indicative of adaptation to osmotic stress. It is also possible that the unusual salt tolerance characteristics observed in our isolates indicate the existence of unique, as yet unexplored genotypes, which could represent new species.

Fast-growing isolates divided roughly into two large groups, one with little or no phosphatase activity (<0.0004 µmol p-nitrophenol h^{-1} 10^{-6} cells) and another with relatively high phosphatase activity (0.0004 - >0.002 µmol p-nitrophenol h^{-1} 10^{-6} cells) (Fig. 1C). In the latter group, the greatest proportion of isolates had activities in excess of 0.002 µmol p-nitrophenol h^{-1} 10^{-6} cells. The wide range in phosphatase activity is evidence of the heterogeneity of this group. The majority of slow-growing cowpea isolates showed little or no phosphatase activity (Fig. 1C). This may partly explain the erroneous conclusion by Smart et al. (1984) who, after testing three strains of *Bradyrhizobium japonicum* concluded that slow-growing rhizobia have no phosphatase activity.

Observed enzyme activities were not correlated to soil P levels. For example Gobera, with extremely low P had three isolates with very high phosphatase activities. Chabwino with six isolates had low soil P but phosphatase activity was spread over the whole range, an indication that P status may not be directly influencing observed enzyme activity. Phosphatase activity may thus be an intrinsic property of rhizobial cells. From an ecological standpoint, strains possessing phosphatase activity may have a competitive advantage in exploiting organic P in soil environments where the nutrient is limiting.

Ability to utilize as sole carbon sources large numbers of compounds is an important trait in numerical taxonomy (De Lajudie et al., 1994). Fast- but not slow-growing rhizobia are reported to be able to utilize as sole carbon sources certain mono-, di- and trisaccharides (Elkan, 1992). However, we found little nutritional diversity among either the fast- or slow-growing isolates for the relatively small number (12) of carbon sources tested. Most of the isolates could not grow on PHB which contains a substituted benzene ring that many rhizobia are unable to cleave (Dreyfus et al., 1988).

Antibiotic sensitivity of rhizobial strains has been used extensively both for general characterization and for identification of marked strains (Beynon and Josey, 1980; Rupela et al., 1982; Eaglesham, 1987; Mueller et al., 1988; Date and Hurse, 1991; Batzli et al., 1992). In this study indigenous isolates varied in the number and type of antibiotics they were sensitive to and the level of sensitivity (Table 3). Fast-growing isolates were generally more sensitive than slow-growing types. Reference slow-growing strains were on average less sensitive to the antibiotic levels tested compared to the indigenous slow-growing isolates. This observation agrees with results of other studies that showed slow-growing rhizobia to be less sensitive to antibiotics (Elkan, 1992). Mueller et al. (1988) observed that sensitivity of rhizobia may be low for those antibiotics that may be routinely produced by other soil organisms.

5. Conclusions

Although our cowpea nodule isolates shared cultural and physiological characteristics with strains of defined species, their diversity points to the possible existence of several unique as yet unidentified species. Significant proportions of both fast- and slow-growing isolates were unique in their high tolerance to acid pH and salt, high phosphatase activity and intrinsic antibiotic sensitivity. Also, fast- and slow-growing isolates were equally represented across sites, providing further evidence to dismiss the myth that all 'cowpea rhizobia' are slow-growing. Additional characterization studies which include sequencing of 16S rRNA genes are currently underway to define the taxonomic status of the rhizobia indigenous to Zimbabwean soils.

Acknowledgements

We gratefully acknowledge funding support for this work from the University of Zimbabwe Research Board and the Rockefeller Foundation through a support grant to the Faculty of Agriculture, University of Zimbabwe.

REFERENCES

Batzli, J.M., Graves, W.R., and van Berkum, P. 1992. Diversity among rhizobia effective with *Robinia pseudoacacia* [L.]. *Applied Environmental Microbiology* **58**: 2137–2143.

Beynon, J.L. and Josey, D.P. 1980. Demonstration of heterogeneity in a natural population of *Rhizobium phaseoli* using variation in intrinsic antibiotic resistance. *Journal of General Microbiology* **118**: 437–442.

Cassman, K.G., Whitney, A.S., and Fox, R.L. 1981. Phosphorus requirements of soybean and cowpea as affected by the mode of nutrition. *Agronomy Journal* **73**: 17–22.

Chen, W.X., Yan, G.H., and Li, J.L. 1988. Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen nov. *International Journal of Systematic Bacteriology* **38**: 392–397.

Chen, H., Gartner, E., and Rolfe, B.G. 1993. Involvement of genes on a megaplsmid in the acid tolerant phenotype of *Rhizobium leguminosarum* biovar *trifolii*. *Applied Environmental Microbiology* **59**: 1058–1064.

Corby, H.D.L. 1988. Types of rhizobial nodules and their distribution among the Leguminosae. *Kirkia* **13**: 53–123.

Cunningham S.D. and Munns, D.D. 1984. The correlation between extracellular polysaccharide production and acid tolerance in *Rhizobium*. *Soil Science Society of America Journal* **48**: 1273–1276.

Dakora, F. and Vincent, J.M. 1984. Fast-growing bacteria from nodules of cowpea (*Vigna unguiculata* (L.) Walp.). *Journal of Applied Bacteriology* **56**: 327–330.

Date, R.A. and Hurse, L.S. 1991. Intrinsic antibiotic resistance and serological characterization of populations of indigenous *Bradyrhizobium* isolated from nodules of *Desmodium intortum* and *Macroptilium atropurpureum* in three soils of S.E. Queensland. *Soil Biology and Biochemistry* **23**: 551–561.

Davis, P. and Mpepereki, S. Symbiotic and serological characterization of soybean rhizobia from Zimbabwean soils. *Zimbabwe Journal of Agricultural Research* **33** (1): in press.

Eaglesham, A.R.J., Stowers, M.D., Sinclair, M.J., Ayanaba, A., and Goldman, B.J. 1984. Patterns of diversity of cowpea miscellany rhizobia from three West African locations. In: Ghai, B.S., ed. *Symbiotic Nitrogen Fixation*. USG, Ludhiana, pp. 115–125.

Eaglesham, A.R.J., Stowers, M.D., Maina, M.L., Goldman, B.J., Sinclair, M.J., and Ayanaba, A. 1987. Physiological and biochemical aspects of diversity of *Bradyrhizobium* sp. (*Vigna*) from three West African soils. *Soil Biology and Biochemistry* **19**: 575–581.

Eaglesham, A.J.R. 1987. The use of intrinsic antibiotic resistance for *Rhizobium* study. In: G.H. Elkan, ed. *Symbiotic Nitrogen Fixation Technology*. Marcel Dekker, New York, pp. 185–204.

Elkan, G.H. 1992. Taxonomy of the rhizobia. *Canadian Journal of Microbiology* **38**: 446–450.

Gil-Serrano, A.A., del Junco, S., Tejero Mateo, P., Megias, M. and Caviedes, M.A. 1990. Structure of the extracellular polysaccharide secreted by *Rhizobium leguminosarum* bv. *phaseoli* CIAT 899. *Carbohydrate Research* **204**: 103–107.

Graham, P.H., Sadowsky, M.J., Keyser, H.H., Barnet, Y.M., Bradley, R.S., Cooper, J.E., De Ley, D.J., Jarvis, B.D.W., Roslycky, E.B., Strijdom, B.W., and Young, J.P.W. 1991. Proposed minimal standards for the description of new genera and species of root-and stem-nodulating bacteria. *International Journal Systematic Bacteriology* **41**: 582–587.

Hernandez, B.S. and Focht, D.D. 1984. Invalidity of the concept of slow growth and alkali production in cowpea rhizobia. *Applied Environmental Microbiology* **48**: 206–210.

Jordan, D.C. 1984. Family III. Rhizobiaceae Conn. 1938. In: Krieg N.R. and J.G. Holt, eds. *Bergey's Manual of Systematic Bacteriology*. Vol 1. Williams and Wilkins, Baltimore, MD, pp. 234–254.

Keyser, H.H., Munns, D.N., and Hohenberg, J.S. 1979. Acid tolerance of rhizobia in culture and in symbiosis with cowpea. *Soil Science Society of America Journal* **43**: 719–722.

Kuykendall, L.D., Saxena, B., Devine. T.E., and Udell, S.E. 1992. Genetic diversity in *Bradyrhizobium japonicum* (Jordan 1982) and a proposal for *Bradyrhizobium elkanii* sp. nov. *Canadian Journal of Microbiology* **38**: 501–505.

Lahda, J.K., Pareek, R.P., So, R., and Becker, M. 1990. Stem-nodule symbiosis and its unusual properties. In: Gresshoff P.M., ed. *Nitrogen Fixation: Achievements and Objectives*. Chapman and Hall, London, pp. 633–640.

Lindstrom, K. and Lehtomaki, S. 1988. Metabolic properties, maximum growth temperature and phage-typing as means of distinguishing *Rhizobium* sp. (Galega) from other fast-growing rhizobia. *FEMS Microbiology Letters* **50**: 277–287.

Lindstrom, K., Lipsanen, P., and Kaijalainen, S. 1990. Stability markers used for identification of two *Rhizobium galegae* inoculant strains after five years in the field. *Applied Environmental Microbiology* **56**: 444–450.

Lowendorf, H.S. 1980. Factors affecting survival of *Rhizobium* in soil. *Advances in Microbial Ecology* **4**: 87–123.

Martinez-Romero, E., Segovia, L., Mercante, F.M., Franco, A.A., Graham, P., and Pardo, M.A. 1991. *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* [L.] beans and *Leucena* sp. trees. *International Journal Systematic Bacteriology* **41**: 417–426.

McClure, P.R. and Israel, D.W. 1979. Transport of nitrogen in the xylem of soybean plants. *Plant Physiology* **64**: 411–416.

Mpepereki, S. and Makonese, F. 1995. Prevalence of cowpea and soybean rhizobia in Zimbabwean soils. *Zimbabwe Journal of Agricultural Research* **33** (2): in press.

Mpepereki, S., Wollum II, A.G., and Makonese, F. 1996. Diversity in symbiotic specificity of cowpea rhizobia indigenous to Zimbabwean soils. *Plant and Soil* **186**: 167–171.

Mueller, J.G., Skipper, H.D., Shipe, E.R., Grimes, L.W., and Wagner, S.C. 1988. Intrinsic antibiotic resistance in *Bradyrhizobium japonicum*. *Soil Biology and Biochemistry* **20**: 879–882.

Padmanabhan, S., Hirtz, R.D., and Broughton, W.J. 1990. Rhizobia in tropical legumes: cultural characteristics of *Bradyrhizobium* and *Rhizobium* sp. *Soil Biology and Biochemistry* **22**: 23–28.

Parker, D. and Ornston, L.N. 1984. Nutritional diversity in Rhizobiaceae revealed by auxanography. *Journal of General Microbiology* **130**: 1743–1750.

Pinero, D., Martinez, E., and Selander, R.K. 1988. Genetic diversity and relationships among isolates of *Rhizobium leguminosarum* biovar *phaseoli*. *Applied Environmental Microbiology* **54**: 2825–2832.

Rao, D.L.N. 1994. Microbiology of salt-affected soils. In: Rao, D.L.N., N.T. Singh, R.K. Gupta, and N.K. Tyagi, eds. *Salinity Management for Sustainable Agriculture*. CSSRI, Karnal, India, pp. 41–64.

Russel, J.R.M. 1973. Cation exchange capacity measurements on some non-calcareous Rhodesian subsoils. *Rhodesian Journal of Agricultural Research* **11**: 77–82.

Rupela, O.P., Josey, D.P., Toomsan, B., Miltal, S., Dart, P.J., and Thompson, J.A. 1982. Application of inherent antibiotic resistance to ecological studies of rhizobia. In: Graham P.H. and S.C. Harris, eds. *Biological Nitrogen Fixation Technology for Tropical Agriculture*. Centro International de Agricultura Tropical, Cali, pp. 625–639.

Saunder, D.H. and Metelerkamp, H.R.R. 1962. Use of anion-exchange resins for determination of available phosphorus. *Transactions of the International Society of Soil Science Commisions* **IV** and **V**: 847–849.

Schofield, R.K. and Taylor, A.M. 1955. The measurement of soil pH. *Soil Science Society of America Proceedings* **19**: 164–167.

Sinclair, M.J. and Eaglesham, A.R.J. 1984. Intrinsic antibiotic resistance in relation to colony morphology in three populations of West African cowpea rhizobia. *Soil Biology and Biochemistry* **16**: 247–251.

Smart, J.B., Dilworth, M.J., and Robson, A.D. 1984. Effect of phosphorus supply on phosphate uptake and alkaline phosphatase activity in rhizobia. *Archives of Microbiology* **140**: 281–286.

Somasegaran, P. and Hoben, H. 1985. *Methods in Legume-Rhizobium Technology*. NifTAL Project, University of Hawaii, Paia.

Slyvester-Bradley, R., Thornton, P., and Josey, P. 1988. Colony dimorphism in *Bradyrhizobium* strains. *Applied Environmental Microbiology* **54**: 1033–1038.

Thompson, J.G. and Purves, W.P. 1978. *A Guide to the Soils of Zimbabwe*. Chemistry and Soil Research Institute, Department of Research and Specialist Services, Harare.

Trinick, M.J. 1980. Relationships among the fast-growing rhizobia of *Lablab purpureus, Leucena leucocephala, Mimosa* sp., *Acacia farnesiana* and *Sesbania grandiflora* and their affinities with other rhizobial groups. *Journal of Applied Bacteriology* **49**: 39–53.

Wedlock, D.N. and Jarvis, B.D.W. 1986. DNA homologies between rhizobia that nodulate *Galega* and other *Rhizobium* and *Bradyrhizobium* species. *International Journal of Systematic Bacteriology* **36**: 550–558.

Zablotowicz, R.M. and Focht, D.D. 1981. Physiological characteristics of cowpea rhizobia: evaluation of symbiotic efficiency in *Vigna unguiculata*. *Applied Environmental Microbiology* **41**: 679–685.

Zevenhuizen, L.P.T. and Bertocchi, C. 1989. Polysaccharide production by *Rhizobium phaseoli* and the typing of their excreted anionic polysaccharides. *FEMS Microbiology Letters* **65**: 211–218.

Zhang, X., Harper, R., Karsists, M., and Lindstrom, K. 1991. Diversity of *Rhizobium* bacteria isolated from the root nodules of leguminous trees. *International Journal of Systematic Bacteriology* **41**: 104–113.