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Variation in Acid-Al Tolerance of *Bradyrhizobium japonicum* Strains from African Soils

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Seventy-six strains of *Bradyrhizobium japonicum* (54 African and 22 exotic) were examined for their tolerance to acidity (pH 4.5), low P (5 μM) and high Al (50 and 100 μM) levels by using an agar plate method. Forty-four strains were tolerant to acidity regardless of P levels (1,000 or 5 μM) of the medium and 22 were sensitive. The remaining 10 strains differed in their tolerance to acidity depending on the level of P of the medium; nine required a low P (5 μM) level for being tolerant but one required a high P level (1,000 μM). All of the 21 strains which could grow under the most severe stress conditions used, consisting of low P and high Al (100 μM) levels at pH 4.5, showed tolerance to acidity at both levels of P. Isolates from a highly acidic soil (Ultisol, pH(H₂O) 4.3) of Onne, south-eastern Nigeria, showed different levels of acid-Al tolerance but tended to be more tolerant than those from a slightly acidic soil (Alfisol, pH (H₂O) 6.4) of Ibadan, south-western Nigeria. However, 20% of the Onne isolates and 30% of the Ibadan isolates were sensitive to acidity, respectively. Thus, the tolerance of the strains was not necessarily determined by the acidity of the bulk soils in which they occurred. Extracellular polysaccharide production by the strains which was observed at low P level did not show any distinct relation to acid-Al tolerance. The great variation in acid-Al tolerance observed among the strains of *B. japonicum* suggests that it may be possible to select strains for use as inoculants in acid soils.

Key Words: acidity, African rhizobia, aluminum, *Bradyrhizobium japonicum*.

Performance of inoculated strains of *Bradyrhizobium japonicum* into a highly acidic soil (Ultisol, pH(H₂O) 4.6) of Onne, south-eastern Nigeria, was very high in terms of nodulation of soybeans (*Glycine max* (L.) Merr.) in the first year but was poor in the second year (Bromfield and Ayanaba 1980; International Institute of Tropical Agriculture 1981). It is suggested that some acid soil factors may adversely affect the ecological behavior of introduced rhizobial strains and that the use of strains tolerant to such acid soil factors would be more beneficial for the utilization of biological nitrogen fixation.

Several common chemical factors in acid soils are known to affect, independently or in association with, the growth of legumes and rhizobia: acidity itself, deficiency in Ca, K, P,

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or Mo and toxicity of Mn or Al (Munns 1986). A great variation in the tolerance to such acid soil factors has been shown among strains of *Rhizobium leguminosarum* biovar *trifoli* (Thornton and Davey 1983; Thurman et al. 1985), *R. leguminosarum* biovar *phaseoli* (Munns et al. 1979; Graham et al. 1982; Lowendorf and Alexander 1983), *R. meliloti* (Howieson et al. 1988), *R. loti* and *Bradyrhizobium* sp. (lotus) (Cooper 1982), *Bradyrhizobium* sp. (cowpea group) (Date and Halliday 1979; Keyser and Munns 1979a, b; Keyser et al. 1979; De Carvalho et al. 1981; Munns and Keyser 1981; Ayanaba et al. 1983), and *B. japonicum* (Keyser and Munns 1979a, b; Ayanaba et al. 1983). Little information, however, is available on African native strains of *B. japonicum*. Ayanaba et al. (1983) reported that some African rhizobial isolates nodulating both cowpea (*Vigna unguiculata* (L.) Walp.) and the soybean cultivar TGm344 showed wide variations in tolerance.

Tolerance of strains of rhizobia to acid soil factors is generally tested by growth in defined liquid media (Date and Halliday 1979; Keyser and Munns 1979a, b; Howieson 1985). Keyser and Munns (1979a) reported that in the test of tolerance to acid soil factors using *Bradyrhizobium* sp. (cowpea group) and *B. japonicum*, Al (25 to 50 μM) induced probably a more severe stress than low Ca (50 μM), and/or high Mn levels (200 μM). Thornton and Davey (1983) showed that in liquid culture media, the growth of *R. trifoli* was more limited by high acidity (pH 4.2 to 4.6) or high Al level (15 to 40 μM) than by low P level (1 to 6 μM). Ayanaba et al. (1983) demonstrated that screening of *Rhizobium* for tolerance to acid-Al stress could be achieved by using an agar plate method and that this method enabled researchers to test large numbers of rhizobia.

In the present paper we report on the variations in the tolerance to acid-Al stress among strains of *B. japonicum* isolated mostly from African soils. Acidity, low P and high Al levels were factors examined using an agar plate method.

MATERIALS AND METHODS

Rhizobial strains. Seventy-six strains of *B. japonicum* from the culture collection of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria were used. Fifty-four of them were isolates obtained from African soils, 20 of which were derived from either the soybean promiscuous cultivars (Malayan, Orba or 3H/149/1) or non-promiscuous ones (TGm294 or Bossier) grown in a highly acidic soil of Onne (Ultisol, pH(H₂O) 4.3, Al saturation rate 63.3%) and another 20 of which were derived from either cultivar grown in a slightly acidic soil (Alfisol, pH(H₂O) 6.4) of Ibadan, south-western Nigeria (Table 1). Some of the African isolates nodulated cowpeas (IITA 1979). Three wild-type strains and their antibiotic-resistant mutants were included. One of them was resistant to spectinomycin and the other two to streptomycin. All the strains were pre-cultured on slopes of yeast extract-mannitol agar medium (YEM agar) (Vincent 1970).

Test agar media. Six media prepared according to the method of Keyser and Munns (1979b): full-defined; full-defined, acid; low P level; low P level, acid, and two different high Al levels (50 and 100 μM) each of which was supplemented with ionagar (15 g/liter) (Oxoid Co., Ltd., England) were used for testing. Each medium contained the basal solution: mannitol 10 g/liter; salts (μM) MgSO₄ 300, CaCl₂ 300, ferric EDTA 100, KCl 10, MnCl₂ 1, ZnSO₄ 0.4, CuCl₂ 0.1, Na₂MoO₄ 0.02, and Co(NO₃)₂ 0.002. Five hundred micromolar of each KH₂PO₄ and K₂HPO₄ and Na-glutamate (1.1 g/liter) were added to the two full-defined media, whereas 5 μM KH₂PO₄, 1.5 mM KCl, and Na-glutamate (1.1 g/liter) were added to the other four media. All the media contained growth factors: thiamine (1 ppm),

Table 1. Chemical properties of surface soils.

	Onne soil	Ibadan soil ^a
pH (1 : 1 aqueous suspension)	4.3	6.4
Organic C (%)	1.58	0.71
Total N (%)	0.12	0.09
Available P (Bray 1) ($\mu\text{g/g}$)	95.5	2.0
C.E.C. (meq/100 g)	2.78	4.55
ex. Ca	0.37	2.48
ex. Mg	0.25	0.88
ex. K	0.08	0.06
ex. Na	0.07	0.19
ex. Al	1.76	0.00

^a Soil sample was collected from newly cleared land of IITA.

Table 2. Designation for the degree of response to acid-Al stress by *Bradyrhizobium* isolates from Onne and Ibadan soils.

pH	P (μM)	Al (μM)	Tolerance ^a						
			I	II	III	IV	V	VI	VII
4.5	1,000	0	S	S	S	T	T	T	T
4.5	5	0	S	T	T	S	T	T	T
4.5	5	50	S	S	T	S	S	T	T
4.5	5	100	S	S	S	S	S	S	T
Designation									

^a Growth on each medium was examined by comparison with that on full-defined medium (pH 6.8, P 1,000 μM and Al 0 μM) and the isolates were designated as T (tolerant) or S (sensitive), accordingly.

Ca-pantothenate (1 ppm), and biotin (0.1 ppm). Full-defined and low P level media were adjusted to pH 6.8 with 1 N NaOH and the others to pH 4.5 with 1 N HCl before addition of AlK(SO₄)₂ and the growth factors. Furthermore, methyl red in ethanol was added as a pH indicator to make a final concentration of 0.002%. The media were autoclaved (121°C, 15 min), then Al and the growth factors were added through filter (0.22 μm) sterilization before being dispensed into plates (30 ml/plate). Autoclaving and addition of these substances slightly changed the pH values of the media but did not affect the testing.

Testing of acid-Al tolerance. A loopful of rhizobia grown on the slope of YEM agar was suspended in sterilized 0.85% saline to give a similar turbidity among strains. Rhizobia were inoculated with sterilized cotton swabs on the six different agar media pre-dried for 3 h at 45°C. Twenty strains were tested in one plate and the experiment was duplicated. Observation was made daily during a 10-day-incubation at 28°C. Tolerance of rhizobia to acidity or acid-Al stress was evaluated by comparing their growth on the stress media with that on the full-defined medium. Tolerance of Onne and Ibadan isolates was designated as shown in Table 2, based on their response to various levels of the stress.

RESULTS AND DISCUSSION

Production of alkaline substances

Most rhizobia grown on laboratory media are known to produce either acid or alkaline substances (Norris 1965; Jones and Burrows 1969; Cooper 1982; Hernandez and Focht 1984), depending on the preferential use of sugars or organic nitrogen compounds, respectively, as their source of energy (Parker et al. 1977). For the evaluation of the growth of rhizobia on

Table 3. Effect of P levels on growth of *B. japonicum* at pH 6.8 and 4.5.^a

pH	Growth in 1,000 μM P compared to that in 5 μM P		
	Larger	Similar	Smaller
6.8	2 (2.6)	70 (92.1)	4 (5.3)
4.5	2 (2.6)	55 (72.4)	19 (25.0)

^a Growth of strains was compared after 10-day incubation by visual readings (0-5) of the size of colonies and the differences of the minimum 2-point readings were considered significant. Results are indicated as the numbers of strains and their proportions in percent in parentheses.

Table 4. Effect of pH on growth of *B. japonicum* at two different levels of P.^a

P (μM)	Growth at pH 6.8 compared to that at pH 4.5		
	Larger	Similar	Smaller
1,000	42 (55.3)	31 (40.8)	3 (3.9)
5	32 (42.1)	44 (57.9)	0 (0.0)

^a See the footnote in Table 3.

such media, therefore, changes in pH had to be detected in order to monitor the response of the rhizobia to certain pH values. Methyl red employed in the present experiment could be used for such purpose. About half of the strains tested produced alkaline substances when grown on the agar media, but it was observed that their growth preceded the shift of pH of the media. Ayanaba et al. (1983) reported that a strain of rhizobia tolerant to acid-Al stress on agar medium grew well in liquid stress medium before change of pH. Thus, it appeared that the tolerance of rhizobia to acid-Al stress may not be directly related to their alkali production.

Phosphorus and pH effects on growth and gum production of rhizobia

Table 3 shows the effect of P levels on the growth of the rhizobia under two pH conditions (6.8 and 4.5). Over 90% of the strains grew similarly at pH 6.8 regardless of P levels. On the contrary, at pH 4.5, in 25% or 19 strains, the growth was less profuse at the high P level than at the low one. Thus, this observation indicates that the tolerance of some rhizobia to acidity seemed to differ depending on the level of phosphorus of the medium and that low P stress was more severe at pH 4.5 than at pH 6.8. Uptake and utilization efficiency of phosphorus were reported to be markedly different among strains of rhizobia, which accounted for the difference in the low P tolerance of rhizobia (Cassman et al. 1981b; Beck and Munns 1984). The data shown in Table 3 suggest that the efficiency of phosphorus uptake and utilization might change depending on the level of pH surrounding the rhizobia.

Growth of rhizobia at pH 6.8 and 4.5 was also compared at different P levels (1,000 and 5 μM) (Table 4). The number of rhizobia with more profuse growth at pH 6.8 was larger when the phosphorus level was high while the number of rhizobia showing similar growth at both pH 6.8 and 4.5 was smaller under the same P conditions. This observation suggests that the growth of the rhizobia at pH 4.5 was more severely affected when the phosphorus level was high, indicating that the low pH stress was more clearly revealed at higher P level. Phosphorus level may have to be high when the effect of low pH stress on the growth of rhizobia is tested.

There were strains forming watery and translucent colonies irrespective of P levels (1,000 or 5 μM) in the full-defined medium during the 10-day-incubation period. On the contrary, P levels affected the appearance of colonies of some other strains; some strains formed white colonies on the same medium when the level of phosphorus was high while watery and translucent ones when the phosphorus level was low (Table 5). The latter type of colonies were formed presumably due to the large production of gummy substances which

Table 5. *Bradyrhizobium japonicum* strains tolerant to acid-Al (pH 4.5, 100 μM Al) stress.

IITA accession No.	Origin or source	Alkali production ^a	Colony type	
			High P (1,000 μM)	Low P (5 μM)
IRj 2001	cv. Malayan, Onne	—	nd ^b	nd
2002	cv. Malayan, Onne	+	white	watery
2004	cv. Malayan, Onne	+	white	watery
2007	cv. Orba, Onne	+	white	watery
2013	cv. Malayan, Ibadan	+	white	watery
2019	cv. Orba, Ibadan	+	white	watery
2020	cv. Orba, Ibadan	—	nd	nd
2025	cv. TGm294, Ibadan	+	white	watery
2033	cv. Orba, Yandev	—	white	watery
2035	cv. Orba, Yandev	—	nd	nd
2038	cv. Malayan, Samaru	—	nd	nd
2039	cv. Malayan, Samaru	+	nd	nd
2048	cv. TGm294, Samaru	—	white	watery
2049	cv. TGm294, Samaru	—	nd	nd
2052	cv. Bossier, Samaru	nd	nd	nd
2109	Univ. of Wisconsin, U.S.A. (SM 61A76)	—	nd	nd
2119	ENSA, Ivory Coast (G3)	+	nd	nd
2120	ENSA, Ivory Coast (G8)	nd	nd	nd
2123	USDA (311b138)	+	nd	nd
2128	Thailand (THA2)	+	white	not watery
2129	Thailand (THA5)	+	white	not watery

^a Alkali production: +, producing; —, not producing; nd, not determined. ^b nd, not determined.

occurred when the level of P of the medium was low. This gum production was considerably depressed on the stress media. Cassman et al. (1981a) also observed an abundant production of external gum by strains of *B. japonicum* in defined liquid medium with low- and moderate-P levels, but not with the high P concentrations routinely supplied in laboratory media. These results suggest that gum production of certain rhizobia may be enhanced when the level of P of the medium decreases, and raises a question about the physiological relatedness of rhizobia grown on high P media to those in field soils.

In the study of cowpea rhizobia isolated from three locations in West Africa, two types of colonies, dry pinpoint or large and gummy, were frequently observed when grown on YEM agar (Ahmad et al. 1981; International Institute of Tropical Agriculture 1981) and Ayanaba et al. (1983) noted that rhizobial strains forming the former type of colonies were more sensitive to acid-Al stress than those forming the latter ones. Cunningham and Munns (1984) showed that the high production of extracellular polysaccharide is positively correlated with the acid tolerance of rhizobia. In this study, the relation between gum production and acid-Al tolerance of rhizobia was not clear. In this connection, Howieson et al. (1988) also reported that in *R. trifoli*, there was no distinct link between polysaccharide production and acid tolerance.

Response of rhizobia to acid-Al stress

Rhizobial strains showed a great variation in their tolerance to acid-Al stress (Fig. 1). Most strains tolerated the acidity stress (pH 4.5) equally well regardless of P levels (1,000 or 5 μM) of the medium; 44 strains (57.9% of total) were tolerant and 22 (28.9%) were sensitive.

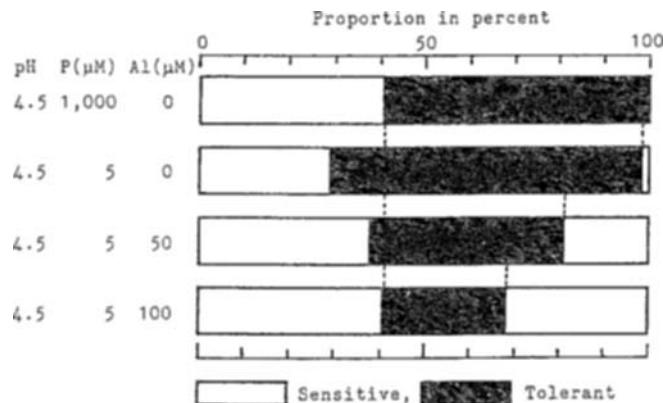


Fig. 1. Effects of acidity and P and Al addition at various levels on growth of *B. japonicum* inoculated on agar media. Growth on each medium was examined by comparison with that on full-defined medium (pH 6.8, P 1,000 μM and Al 0 μM) and the isolates were designated as sensitive or tolerant, accordingly.

The remaining 10 strains, however, differed in their tolerance to acidity depending on the level of P; 9 (11.8%) required a low P level (5 μM) for being tolerant while 1 (1.3%) required a high P level (1,000 μM). The latter strain may have failed to take up and utilize P when the phosphorus concentration was low under acid conditions. The reason for the lack of tolerance to acidity of the former 9 strains at high P level remains to be elucidated.

Thirty-three of the 53 strains that tolerated acidity at the low P level were able to grow under acid-Al (pH 4.5, 50 μM) stress, 21 of those 33 strains or 27.6% were further tolerant to a higher level of Al (100 μM), the most severe stress of all tested. Thus, the tolerance to acidity was not always associated with a tolerance to acid-Al, as reported by Thornton and Davey (1983), indicating that the acidity stress for rhizobia may be enhanced by the presence of aluminum. Similar results were obtained by Keyser and Munns (1979b) who noted that 3 out of 8 *B. japonicum* strains that were tolerant to pH 4.5 tolerated the acid-Al (pH 4.5, 50 μM) stress. These most tolerant strains (Table 5) either originated from several locations in Africa or were obtained from other *Rhizobium* sources than Africa.

Soil microenvironment and acid-Al tolerance of rhizobia

Does the acidity of the bulk soil determine the acid-Al tolerance of rhizobia isolated from that soil? To investigate this problem, the acid-Al tolerance of the isolates from a highly acidic Onne or slightly acidic Ibadan soil (pH(H₂O) 4.3 and 6.4, respectively) was compared (Fig. 2). The Onne isolates tended to be more tolerant than the Ibadan isolates. Some Onne isolates, however, were sensitive to the least severe acid stress (pH 4.5), whereas some Ibadan isolates were tolerant to the most severe acid-Al stress employed. Thus, the acidity of bulk soil appeared to affect to some extent the acid-Al tolerance of rhizobia residing in that soil. However, it did not seem to determine the tolerance of rhizobia. Jones and Burrows (1969) also reported the absence of correlation between the final pH of the culture media in which isolates of *R. trifoli* grew and the pH of the soils from which they originated.

Rhizobia generally reside in microsites in soil. Accordingly, as Munns (1986) suggested, the acidity or acid-Al conditions of the micro-environment may affect the acid-Al tolerance of the inhabitants in the microsites. Figure 2 shows that there was less variation in such conditions in the Onne soil than in the Ibadan soil. Further research needs to be carried out in this connection.

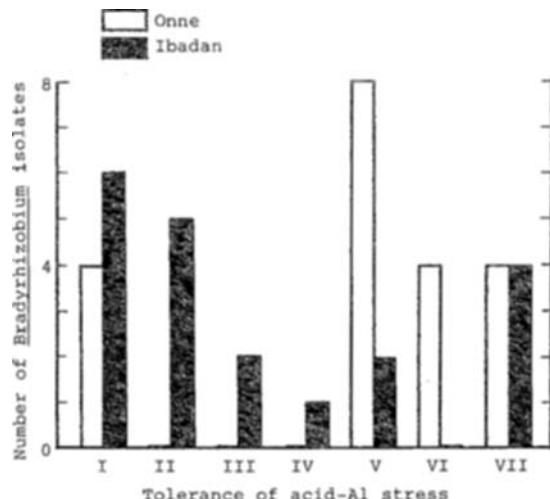


Fig. 2. Variation in the tolerance to acid-Al stress shown by *Bradyrhizobium* isolates from Onne and Ibadan soils. Twenty isolates each were tested. I-VII: see Table 2.

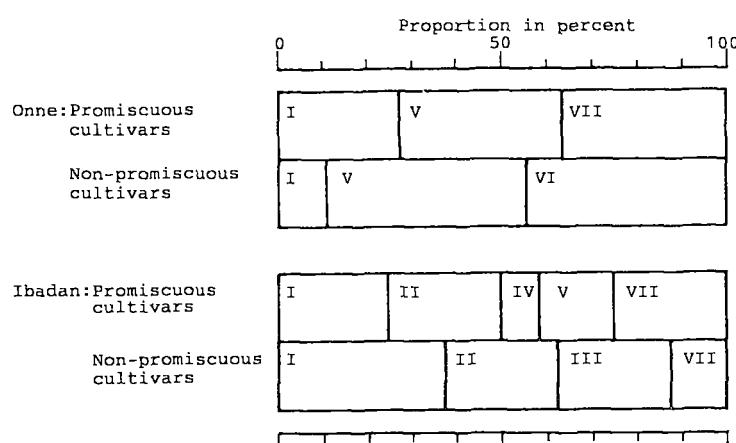


Fig. 3. Comparison of acid-Al stress tolerance between *Bradyrhizobium* isolates from promiscuous and non-promiscuous cultivars of soybean grown in Onne and Ibadan soils. Eleven and 9 isolates were tested for the promiscuous and non-promiscuous cultivars, respectively, in Onne soil and 12 and 8 isolates, respectively, in Ibadan soil. I-VII: see Table 2.

Effects of antibiotic mutation and host promiscuity on acid-Al tolerance of rhizobia

Three wild type strains and their antibiotic-resistant mutants were included in this study. Mutation induced by the two antibiotics employed did not seem to affect the acid-Al tolerance of the rhizobia; all of them were as sensitive or tolerant as their wild types were (data not shown). These results support the observation made by Ayanaba and Wong (1982) that the mutants resistant to antibiotics were as tolerant to acidity as their parents were.

Rhizobia nodulating non-promiscuous soybean cultivars are known to nodulate promiscuous ones, too, but the reverse is not always true (International Institute of Tropical Agriculture 1979; Bromfield and Roughley 1980), suggesting that there are differences among strains of rhizobia in terms of compatibility with host cultivars. The promiscuous soybean cultivars (Nangju 1980; Pulver et al. 1985) from which the Onne and Ibadan isolates were

obtained and the acid-Al tolerance of these isolates are shown in Fig. 3. Acid-Al tolerance of the isolates was not distinctly related to the promiscuity of the host plants from which they originated.

Selection of rhizobia for use under acid soil conditions in Africa

The consistent and stable properties of the tolerance of rhizobia to acidity and aluminum stresses (Bromfield and Jones 1980; Munns and Keyser 1981; Munns 1986) and the great variation in the acid-Al tolerance among the strains of *B. japonicum* observed in this study suggest that it may be possible to select strains for use as soybean inoculants in acid soils. It is obvious, however, that there are some other adverse abiotic environmental stresses under tropical African conditions, like high temperature and/or desiccation (Osa-Afiana and Alexander 1982) to which rhizobial inocula are probably exposed, particularly during the fallow period. Therefore, it is important to test the effect of those stresses on the saprophytic competence of rhizobia (Thornton and Davey 1984) and their symbiotic effectiveness under acid field conditions in which the inoculation of rhizobia is necessary or recommendable to grow soybeans. At the same time, screening or breeding of soybean cultivars adapted to acid soil conditions should be promoted (Munns et al. 1981).

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