An Agar Plate Method for Rapid Screening of *Rhizobium* for Tolerance to Acid-Aluminum Stress¹

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ABSTRACT

An agar plate method has been developed for the rapid, preliminary screening of large numbers of rhizobia for tolerance to acidity and aluminum (acid-Al). Media containing pH indicators are used, and distinguish true tolerance from cases where growth depends on a previous pH shift. Strains which were highly tolerant or sensitive to acid Al on agar behaved similarly in defined liquid media. Strains that formed dry, pinpoint colonies were more sensitive to acid-AI than those that formed large, gummy colonies.

Additional Index Words. acidity, aluminum, cowpea rhizobia, pH indicators, Rhizobium japonicum.

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METHODS have been developed recently for the preselection of *Rhizobium* strains tolerant of the soil acidity factors: aluminum, phosphate, and acidity (Date and Halliday, 1978; Keyser and Munns, 1979) and so of possible value as inoculant strains in the tropics. In these studies test strains are added to the stress media, incubated, and then examined for turbidity. In more refined tests, incubated cultures are monitored for cell multiplication prior to detectable pH changes.

Screening for tolerance of acidity and Al can be vitiated by shifts in pH, which rhizobia sometimes induce depending on the buffering and particular composition of the medium (Date and Halliday, 1978). In agar media, local neutralization at the point of inoculation could become specially important, especially with large inocula. We report on an agar plate method that permits rapid screening of large numbers of rhizobia for tolerance to

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an acid-A1 stress; and the application of this method to the screening of 224 rhizobia belonging to *Rhizobium japonicum* and *Rhizobium* sp.

MATERIALS AND METHODS

Media

Two media containing the basal solution of Keyser and Munns (1979) but with galactose and arabinose, at 5 g/L each, instead of mannitol were used to test the response of rhizobia to acid-Al stress. The basal solution contained (µM): MgSO₄, 300; CaCl₂, 300; FeEDTA, 10; KCI, 10; MnCl₂, 1; ZnSO₄, 0.4; CuC1₂, 0.1; Na₂MoO₄, 0.02; Co(N0₃)₂, 0.001. In addition, the media contained 1.8 g/L of sodium glutamate, 5 µM KH₂PO₄, 1.5 mM KCl and 20 g/L of agar. The control (LP) medium contained the modified basal medium plus 0.005% bromcresol purple (BCP) indicator (Conn, 1957). The pH was adjusted to 7.0 with 1N NaOH before autoclaving and fell to 6.0 after autoclaving. The stress (AL) medium contained the modified basal medium, 0.005% bromcresol green (BCG) indicator (Conn, 1957), and 50 µM Al. The Al, as 5 mM $KAl(SO_4)_2$, was filtersterilized before addition to agar held at 47°C. The pH was adjusted to 4.7 with 1N HCl before autoclaving; the final pH was 4.6 after the addition of Al. The agar media were poured into square petri plates (100 by 15 mm) at 20 mL/plate and allowed to solidify. Plates were dried at 40°C for 3 h before inoculation.

Liquid forms of LP and AL media were similarly prepared, but without agar and indicator. They were dispensed at 20 mL per 50-mL conical flask prior to autoclaving. Cooled, liquid AL medium received aluminum to provide 50 μM Al.

Rhizobia

Rhizobia from the culture collection of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, were used in the tests. All nodulate cowpea (*Vigna unguiculata*) cultivar TVu 1190 and some, in addition, nodulate soybean (*Glycine max*) cultivar TGm 344.

Determining the Inoculum Level

Before inoculation, 16 equal squares were marked on the bottoms of triplicate petri plates of AL agar media. A loop calibrated to deliver 0.38 ± 0.04 AL was used to inoculate squares with tenfold dilutions of the test strains, containing

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from about 10^2 to 10^8 rhizobia/mL. The LP agar plates were similarly inoculated. The plates were incubated at 30 ° C and examined daily for growth and change in the colors of indicators over a 10-d period.

Plate Screening for Tolerance of Acid-Al Stress

The plate method was used to screen 224 rhizobia of diverse origins. Triplicate plates of LP and AL media were inoculated with dilute suspensions (ca. 10^6 to 10^7 cells/mL) of each strain, prepared from yeast extract-mannitol (YEM) slant cultures. The plates were incubated at 30 ° C and examined daily for growth and change of pH of the media over a 10-d period.

Correspondence Between Methods

Two experiments were conducted to evaluate the correspondence between acid-Al tolerance on agar plates and in liquid media, and to examine the relationship between ammonia production and pH changes in the growth media.

Experiment 1

Twenty rhizobia identified as either tolerant or sensitive to acid-Al stress on agar media were used. Duplicate flasks of LP and AL media were inoculated with 0.04 mL of suspensions of the rhizobia in a diluent (Keyser and Munns, 1979) so as to provide 10^2 to 10^3 cells/mL. The LP and AL media without arabinose and galactose and in which sodium glutamate served as the source of carbon and nitrogen for growth were similarly inoculated. Flasks were incubated on a reciprocal shaker operating at 110 strokes/min at room temperature. The shaker was automatically switched on for 1 h every other hour. After 10 d (15 d for very slow growing strains), the optical density of the suspensions were determined at 470 nm. The suspensions were centrifuged in the cold at 4,342 X g for 20 min, and the ammonium-N content (Mitchell, 1972) and pH of the supernatant fluid were determined.

Experiment 2

Two cowpea rhizobia, one (IRc 381) sensitive to and the other (IRc 353) tolerant of the acid-AI stress on agar media, were used. Eighteen flasks each of LP and AL media were inoculated with each strain and incubated as in Experiment 1. Initially and after 5, 10, 15, 20, and 25 d, three flasks of each medium inoculated with each strain were removed from the shaker and the optical density, pH, and ammonium-N content of each suspension were determined, as in Experiment 1.

RESULTS AND DISCUSSION

The rates of BCP and BCG used here are far below the 0.04% recommended for bacteriological work (Conn, 1957). Nonetheless, color changes of these indicators permitted early visual detection of pH shifts during rhizobial growth on agar plates.

Rhizobia that tolerate acid-Al grew and could be detected easily by the agar plate method. An inoculum of $0.4 \ \mu\text{L}$ from a suspension of 10^6 to 10^7 rhizobia/mL was best for distinguishing between acid-Al tolerance and sensitivity (Table 1). Growth of acid-Al tolerant strains was usually detected prior to a pH change. In some instances, growth and color change of the medium were observed on the same day. In such cases, more frequent observation than on a daily basis was required to ascertain whether growth preceded color change. By the 10th day when the incubation was usually terminated, all acid-Al tolerant strains showed appreciable growth, and several strains had rendered the AL medium sufficiently more alkaline or more acid than it originally was, producing

Table 1—Effect of cell concentration on the response of rhizobia to acid-Al by the agar plate method.

<i>Rhizobium</i> Strain‡	No. of rhizobia/mL (× 10 ⁸)	Response‡ in AL medium when indicated dilutions were sources of inoculum							
		0	۱0-۱	10-2	10-3	10-4	10-5	10-	
IRc 353	5.12	+	+	+	+	+	+		
381	7.92	±		_	_	_	_		
516A	4.74	+	+	+	±	±	±	_	
IRj 2144B	6.30	+	+	±		_		_	
2147B	0.84	±	±	_	_	-	_	_	
2163	6.84	ŧ	-	-	-	-	-	-	

† Strains designated as IRc were isolated from cowpeas; those designated as IRj were isolated from soybeans.

 \ddagger Growth response by comparison with growth in LP medium: + = good growth; $\pm = \text{fairly good growth}$; $\pm = \text{poor growth}$; - = no growth.

color halos around colonies. If plates were incubated for 25 d, many of the strains sensitive to acid-Al still had not grown in the AL medium, even though growth in the LP medium was abundant. Proximity to acid-Al tolerant strains that altered the pH of the medium did not affect the response of sensitive strains. However, a few sensitive strains grew scantily in the AL medium, usually, after the 20th day. In any case, strains that are highly tolerant to acid-Al always showed significant growth within 10 d, before pH change in the AL medium. Graham et al. (1982) used an agar plate method without pH indicators to preselect *Rhizobium phaseoli*, assuming that growth of single colonies was indicative of acid or Al tolerance.

Acid-Al tolerance of a large, diverse collection of rhizobia was readily detected using the agar plate method (Table 2). Tolerance of the acid-Al stress was found among both cowpea and soybean rhizobia. Rhizobial origin appeared to influence the response, as described in a previous study (Ayanaba and Wong, 1982), but on closer examination it appeared that colony type mattered more. Thus, strains that formed dry, pinpoint colonies on YEM agar were extremely sensitive to the acid-Al stress. When the proportion of "dry" strains from a particular source was high, the proportion of the total number of strains which tolerated the acid-Al stress was low. By contrast, most strains with large, gummy "wet" colonies tolerated the stress more.

When strains identified as tolerant or sensitive to acidAl on agar plates were subbed in liquid media, growth, as determined by optical density, was severely curtailed by the acid-Al stress among rhizobia previously identified on agar media as sensitive, but not those considered tolerant (Table 3). Some strains, e.g., IRc 353 and IRj

Table 2—Response of cowpea and soybean rhizobia to acid-Al stress on agar plates.

Host of isolation	Location/ Source†	Number o	f rhizobia	Growth in acid-Al		
		In test	Dry‡	No.§	%	
Cowpea	Onne	46	8	27(13)	58.7	
	Maradi	36	35	0	0	
	Other	16	7	6(4)	37.5	
Soybean	Onne	50	23	27(19)	54.0	
	Maradi	38	36	9(2)	23.7	
	Other	14	0	13(12)	92.9	
	Culture collection	24	13	5(2)	20.8	

[†] Onne is in Nigeria; soil has a pH of 4.6 and is highly leached. Maradi is in Niger Republic; soil has a pH of 6.1 and is subjected to desiccation and high temperatures.

‡ Rhizobia that form dry, pinpoint colonies on YEM agar.

§ Numbers in parentheses are of highly tolerant strains; these grow to the same extent in AL and LP media.

Acid-Al pН µg NH, ∙N/mL Optical density Rhizobium response on agar LP AL LP LP strain AL AL IRc 80 Sensitive 1.316 0.027 8.10 4.61 0.100 0.138 109 Tolerant 1.310 1.000 4.28 4.90 0.100 0.138 130 Sensitive 0.363 0.022 6.20 4.65 0.100 0.100 326B Tolerant 1.318 1.300 4.223.90 0.100 0.103 342 Tolerant 1.2570.742 3.75 4.32 0.758 0.100 352 Tolerant 1.314 1.323 4.16 4.25 0.100 0.799 353 Tolerant 1.3121.322 4.31 3.88 0.100 0.100 356B Tolerant 1.1310.643 4.52 4.80 0.100 0.100 360 Tolerant 0.691 0.246 5.65 4.680.100 0.100 381 Sensitive 1.307 0.027 8.18 4.68 0.100 0.135 1.250 405 Sensitive 0.259 7.85 4.70 0.100 0.100 516A Tolerant 0.270 0.063 6.08 4.65 0.100 0.100 IRj2005 Tolerant 1.314 1.312 4.35 3.92 0.100 0.100 2137A Tolerant 0.634 0.537 4.70 5.820.100 0.100 2138A Tolerant 0.998 0.5644.950.100 0.100 4.752140B Tolerant 1.323 1.2697.623.950.199 0.1322144B Tolerant 1.317 1.283 8.20 4.65 0.414 0.1252150A Tolerant 1.126 1.143 4.25 4.32 0.142 0.100 2151A Tolerant 1.2967.38 1.2897.48 0.100 0.100 2163 Sensitive 1.082 0.044 7.40 4.60 0.100 0.100 2164B Sensitive 0.369 0.0325.523.65 0.100 0.100 LSD 0.05 (same medium): 0.5520.437 0.212 LSD 0.05 (different 0.446 0.424 0.207 medium):

Table 3—Response of 21 rhizobial strains to acid-Al stress in liquid media.

Table 4—Growth, pH alteration, and ammonia production by two cowpea strains in media containing sodium glutamate as the sole source of carbon and nitrogen.

Rhizohium	Acid-Al	Optical density		pH		μg NH₄••N/mL	
strain	on agar	LP	AL	LP	AL	LP	AL
IRc 353 IRc 381	Tolerant Sensitive	0.768 1.308	1.257 0.066	8.15 8.68	8.40 4.75	0.11 41.46	2.63 0.10
LSD 0.05 (same medium): LSD 0.05 (different media):		0.050 0.035		0.076 0.150		1.302 3.301	

2005, were highly tolerant, growing equally well in both media. Others, e.g., IRc 356B and IRc 360, were probably moderately tolerant because they were only partly inhibited by the acid-Al stress. The response of rhizobia did not depend on their species.

Growth preceded pH changes of the liquid media, just as on agar plates (Table 3). This pH change is a consequence of the production of acidity (H ions) or ammonia. Physiological concentrations of glutamate are known to favor ammonia production, whereas higher levels, as used in the media of this investigation, favor aspartate synthesis (Jones, 1965).

Ammonia accumulated in small amounts but this did not appear to be related to acid tolerance because ammonia was detected in media containing either tolerant or sensitive strains (Table 3). Ammonia accumulated also in both LP and AL media. If sodium glutamate served as the sole source of carbon and nitrogen, tolerant strain IRc 353 grew in and raised the pH of both media, but ammonia accumulated in AL medium only, whereas the sensitive strain IRc 381 grew in, raised the pH of and accumulated ammonia in LP medium (Table 4).



Fig. 1—Growth, pH shifts, and ammonia production by an acid-Al sensitive (IRc 381) and tolerant (IRc 353) cowpea rhizobia in the AL and LP media. Bars represent LSD values (5% level) for comparisons among rhizobia and media.

The same two strains were employed in an experiment to examine further their ability to grow, alter the pH, and produce ammonia in LP and AL liquid media over a 25-d period (Fig. 1). The Al-tolerant strain IRc 353 behaved as it did previously (Table 4), i.e., it grew and produced large quantities of ammonia in AL but not LP medium. The pH of both media increased: significant growth occurred before pH change in the AL medium. This observation of a significant pH change of the defined medium containing arabinose and galactose as carbon sources differs from that of Date and Halliday (1978), who observed no significant pH change by strain CIAT 1460 growing in a similar medium. The sensitive strain IRc 381 grew in and raised the pH of the AL medium after 15 d, but no ammonia accumulated in the medium. More work is required to explain whether ammonia production influences acid-Al tolerance of glutamate-grown rhizobia.

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