

RESPONSE OF *RHIZOBIUM* STRAINS TO ACID AND ALUMINIUM STRESS

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Summary-Synchronous-culture, enrichment and isolation trials were done to determine effects of simulated soil acidity stress on growth of *Rhizobium* sp. (cowpea group), and to test whether tolerance of a strain is stable.

In synchronous cultures, acidity and Al reduced the frequency of cell division. Non-dividing cells did not die, but those near division showed evidence of heightened sensitivity to Al³⁺ and H⁺.

Differences in tolerance within single-strain populations were evidently not genetically determined. Prolonged culture under stress (72 generations) failed to enrich a strain in putative tolerant variants. And from six strains of different tolerance, isolates from single colonies that had grown on stress medium were no more tolerant than isolates from colonies on non-stress medium. Tolerance is a consistent and stable strain property.

INTRODUCTION

The combination of acidity and aluminium (acid-Al stress) produces in *Rhizobium* a variety of effects (Keyser and Munns, 1978a,b). Reduction in multiplication rate is the effect most important for colonization of soils and roots, and most consistently expressed in defined media. Multiplication is inhibited to different degrees depending on the strain of *Rhizobium*, i.e. strains differ in tolerance. The further possibility that tolerance varies within strains is suggested by some less-consistently observed effects of acid-Al stress. Thus multiplication under stress from large inocula but not small inocula might mean that only a minority of the population is tolerant (H. H. Keyser, unpublished Ph.D. Thesis, University of California, Davis, 1977); and initial mortality or lag followed by population growth (Keyser and Munns, 1978a,b) might be due to death or stasis of a sensitive majority while a tolerant minority multiplies.

We describe experiments to test two hypotheses: first, that reduction in net rate of multiplication under acid-Al stress is partly due to increase in cell death rate; second, that there is genetic variation that would permit within-strain selection for enhanced tolerance. Both hypotheses are rejected; the first by synchronous culture experiments showing a dominant effect of acid-Al on interdivision time; the second by constancy of tolerance despite serial subculture or multiple colony isolations in stress media.

MATERIALS AND METHODS

General

The rhizobia were all from the cowpea group. Their origins were as follows: strain CB756, CSIRO Division of Tropical Crops and Pastures, Brisbane, Queensland; 316n9, USDA, Cell Culture and Nitrogen Fixation Laboratory, Beltsville, MD; TAL171, TAL189, and TAL425, University of Hawaii NifTAL Project, Paia, HI. (TAL171 and TAL189 originated

from Nitragin Co, Milwaukee, WI as strains 176A27 and 176A31 respectively.) Cultures were received and maintained on slants of yeast mannitol agar, and were streaked for purity and tested for effectiveness on mung or cowpea.

Three media were used.

Yeast mannitol agar (YMA) for counting. Each liter contained 0.1 g yeast extract and 20 g agar, 1 mmol CaCl₂, 1 mmol MgSO₄, and as μ mol 100 KCl, 100 KH₂PO₄, 50 FeEDTA, 20 H₃B₃O₃, 2 MnSO₄, 1 ZnSO₄, 0.2 CuSO₄, 0.05 H₂MoO₄, and 0.01 COCl₂. Final pH was adjusted to 6.5.

Low-P liquid defined media (LP and LPAL). To help control pH these had low phosphate to limit growth, arabinose and galactose as carbon source, and high glutamate as buffer. Each litre of LP medium contained 1.8 g glutamic acid, 0.3 g arabinose, 0.3 g galactose, 2.5 μ mol KH₂PO₄, and other mineral salts at half the concentration in YMA (above). Before autoclaving, pH was adjusted to 6.2 with NaOH. An acid version, LPAL, was made from autoclaved and cooled LP by addition of filter-sterilized 600 μ mol AlCl₃ in approximately 10 mm HCl to bring the final solution to pH 4.5, 60 μ mol Al, or to pH 4.6, 50 μ mol Al. Heating Al solutions was avoided; it can cause polymerization of Al ion species and reduce toxicity.

Citrate glutamate agar (CGA and CGAAL) for colony isolation. Each litre of CGA contained 1.8 g glutamic acid, 0.5 g arabinose, 0.5 g galactose, 0.9 g citric acid, mineral salts as in YMA (above), NaOH to bring final pH to 6.4, and bromocresol purple indicator. The CGAAL medium was the same, except that each litre contained 1 g freshly-precipitated Al(OH)₃ as a solid reserve supporting a pH-dependent activity of Al, and pH was raised only to 4.4. To avoid acid hydrolysis, agar solution was autoclaved separately, and not mixed with the autoclaved acid nutrients solution until immediately before pouring into plates. Citrate was added as an Al-mobilizing chelon. Similar mobilization probably occurs in acid soils (Norvell, 1972).

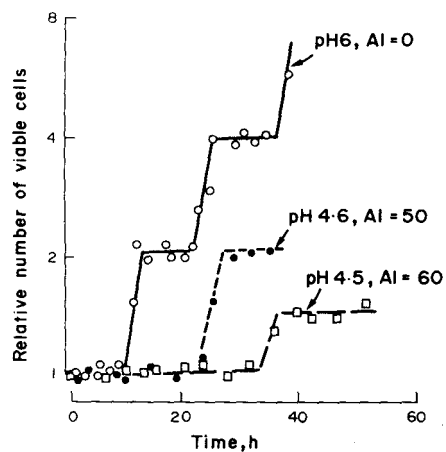


Fig. 1. Synchronous cultures of CB746 with and without acid-AI stress. Conditions were imposed 3 days before establishment of synchrony, and maintained throughout. Lines fitted subjectively.

Synchronous cultures

Synchronous growth of strain CB756 was compared in LP and LPAL media to identify whether acid-AI stress reduces division frequency, causes division failure, or causes death between division. Helmstetter and Cummings' (1964) procedure was modified to produce cultures of low population density and suit available equipment. About 20 ml of a culture grown to 5×10^5 cells ml⁻¹ in the appropriate medium (LP or LPAL) was syringed through a 25 mm Millipore GS (0.22 μ m pore) filter in a plastic holder ("Swinnex"). So loaded, the filter was aseptically reversed in the holder for backwashing with fresh medium from the syringe at mean flow rate of 1 ml min⁻¹. After backwashing for 25 min, daughter cells released from the filter were collected for 2-6 s in 10 ml medium, to start the synchronous culture. (A hypodermic needle on the filter outlet allowed small drop size and could be flamed and cooled before the collection.) The cultures were counted periodically (Fig. 1) by removing replicate drops (41 μ l) and plating them on YMA without dilution (Vincent, 1970).

Data points in Fig. 1 are means for triplicate cultures of each treatment, i.e. each medium. Half of our attempts at this procedure produced cultures that were synchronous and at the right density for the counting procedure.

Serial subculture

Strain TAL189, which grows in acid-AI media after considerable initial mortality (Keyser and Munns, 1978b), was serially subcultured nine times to test whether it could be enriched in putative tolerant variants by prolonged culture under stress. To begin, triplicate 10-ml cultures were set up in each medium, and then serially subcultured every 25-days by transferring 40 pi samples into new 10-ml lots of the appropriate medium. At the 3rd and 7th transfer the old cultures had less than 5×10^6 cells ml⁻¹ and pH between 6.0 and 6.3 (LP) or 4.5 and 4.7 (LPAL). From the 8th set of subcultures, and from the first set which had been stored in the refrigerator, further sets were run at the same time to establish comparability. And these were used to inoculate a final set that yielded the growth curves of Fig. 2.

Colony isolates on solid media

Colony isolates from YMA, CGA and CGAAL were compared to see whether they differed in ability to colonize CGAAL. To begin, serial 10-fold dilutions of suspensions from YMA were spread onto plates of CGA and CGAAL. Colonies were counted under a dissecting microscope after 21 days. Five randomly chosen colonies from each medium were again serially diluted, spread onto CGA and CGAAL, and counted. The experiment was repeated with six strains. Always, fewer colonies grew on CGAAL than expected from the count on CGA. Ratios of these two counts were calculated for each isolate and analyzed after log₁₀ transformation (Table 1).

RESULTS

Synchronous culture

The sole effect of 50 μ m AI at pH 4.6 was to prolong the time between divisions (Fig. 1). Slightly more severe stress, 60 μ m AI at pH 4.5, prolonged the interdivision time even further, and caused incomplete

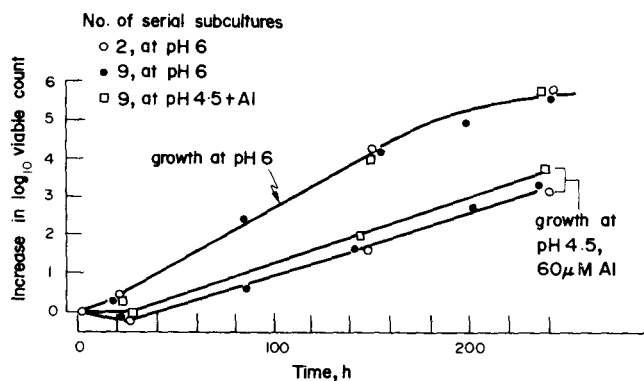


Fig. 2. Similarity of growth response to acid-AI stress regardless of prior exposure to the stress. Cultures of TAL189 had been previously subcultured in either acid-AI or no-stress media, to a maximum of 9 serial transfers (190 days, estimated 72 generations).

Table 1. Effect of source of isolates on their ability to colonize citrate glutamate agar with $\text{Al}(\text{OH})_3$ at pH 4.5 (CGAAL)

Strain	\log_{10} (colonies produced/cells inoculated)			
	from YMA pH 6.5	from CGA pH 6.2	from CGAAL pH 4.9	from CGAAL pH 4.5
316n9	< -7	< -6	< -6	ND
TAL189	-2.0	-2.2	ND	-2.7
CB756	-1.0	-1.0	ND	-0.7
TAL425	-1.5	-0.6	ND	-0.5
TAL171	-0.8	ND	ND	-0.6
LSD $P = 0.05$ (316n9 excluded from analysis of variance)			1.05	

ND = not determined.

doubling as well. There was no death between divisions.

Serial subculture

Control of population and pH were successful. The population did not exceed 7×10^6 cells ml^{-1} and pH rose less than 0.2 units. Cultures newly exposed to acid-Al stress suffered some initial mortality so that the viable count halved (Fig. 2). Otherwise, previous culturing treatment had no effect. Growth rates were less in LPAL than in LP, and were unaffected by previous culturing treatment.

Colonization of solid medium under stress

The fraction of inoculant cells that produced colonies within 21 days on CGAAL medium varied between strains; but it did not depend on whether the inoculant cells came from a stress medium (CGAAL) or not (CGA or YMA) (Table 1).

The CGAAL medium evidently maintained stress reliably only during early colony development, after which colonies of some strains developed bromocresol purple halos indicating a local pH substantially above 5.

DISCUSSION

In evaluations of effects of pH and Al on growth, the most obviously important experimental requirement is control of pH. We substituted galactose and arabinose for mannitol following Date and Halliday's (1978) report that this prevents alkali formation. We also used glutamate (pK = 4.6) at buffer concentrations. Despite these measures, some strains locally neutralized the acid gel medium. But pH did not rise in liquid media so long as population density remained low, as we found previously (Keyser and Munns, 1978a). Population growth was effectively limited simply by supplying P at initial concentrations resembling those in soil solutions.

The nature of Al toxicity in bacteria has received little or no attention. In plant root cells, the main effect is on cell division (Foy *et al.*, 1978). This is also true of *Rhizobium* as shown by Fig. 1, and by the doubling of cell length we observed in acid-Al media. It is not clear how Al stops or delays cell division. There is evidence that in plant cells it causes failure of DNA synthesis, failure of chromatids to separate (incomplete anaphase), and lack of division despite

replication of the genome (binucleate cells and many anaphase figures) (Foy *et al.*, 1978).

Acid-Al stress did not kill non-dividing cells, consistent with evidence that similar rhizobia can survive exposure to millimolar concentrations of Al salts (B. Perkasem, unpublished Ph.D. Thesis, University of Western Australia, Perth, 1977). But some cells might have been killed at or about the time of division. This effect, tentatively confirmed by adding Al to synchronous cultures at different times (unpublished data), could cause the less-than-twofold multiplication observed in synchronous culture (Fig. 1). Age-dependent variation in stress tolerance has been shown in *Escherichia coli*, where additions of NaCl at high concentration are most lethal during rapid growth but less lethal during stationary phase (Doudoroff, 1940).

Clearly, cells within a population of a given strain differ in ability to divide or form a colony under acid-Al stress; but such variability in stress tolerance need not imply genetic variation, and is probably commonplace (e.g. Doudoroff, 1940; Steinborn and Roughley, 1975). It becomes important here because of interest in selecting rhizobial strains for tolerance of soil acidity. Such selection would not be rational if a strain's tolerance is variable and unstable.

Thus our most important conclusion is that neither prolonged culture under stress nor isolation of successful colonies on stress medium altered the tolerance of strains established and maintained in normal fashion. A strain's tolerance might be improvable by selection after artificial mutagenesis. But it appears that spontaneous mutation towards tolerance is not abnormally frequent, that naturally-tolerant variants within strains are unlikely, and that a strain's degree of tolerance is a stable property.

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Rhizobium response to acid and

Al

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		-2.2		
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