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QUANTITATIVE EVALUATION OF ACIDITY TOLERANCE OF ROOT NODULE BACTERIA

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ABSTRACT

Quantification of acidity tolerance in the laboratory may be the first step in rhizobial strain selection for the Amazon region. The present method evaluated rhizobia in Petri dishes with YMA medium at pH 6.5 (control) and 4.5, using scores of 1.0 (sensitive, "no visible" growth) to 4.0 (tolerant, maximum growth). Growth evaluations were done at 6, 9, 12, 15 and 18 day periods. This method permits preliminary selection of root nodule bacteria from Amazonian soils with statistical precision. Among the 31 rhizobia strains initially tested, the INPA strains 048, 078, and 671 presented scores of 4.0 at both pHs after 9 days of growth. Strain analyses using a less rigorous criterion (growth scores higher than 3.0) included in this highly tolerant group the INPA strains 511, 565, 576, 632, 649, and 658, which grew on the most diluted zone (zone 4) after 9 days. Tolerant strains still must be tested for nitrogen fixation effectiveness, competitiveness for nodule sites, and soil persistence before their recommendation as inoculants.

Key words: rhizobia, tolerance to pH, Amazon.

INTRODUCTION

The majority of Amazon Basin soils are acid and have low fertility. Soil pH is usually 4.0-4.5, and P, N, Ca, K, Mg are deficient in most plateau soils of the Amazon. It is estimated that P and N are deficient in 90% of the regional soils (4). Agricultural, forestry and agroforestry yields are negatively affected by these constraints. Liming and fertilization are practiced by only a few landowners, because these amendments are expensive and difficult to obtain in the region, especially for small farmers (14).

Nitrogen fixation by legume-rhizobia symbioses may supply nitrogen to the ecosystem, but plants and bacteria must tolerate soil constraints, such as acidity and Al toxicity. Legume nodulation under natural soil fertility is sparse or absent (3,7), except when soil constraints are eliminated (6). *Bradyrhizobium* strains have been selected for their tolerance to low pH and low soil fertility (1,2,16,17) for practical use in agriculture and forest systems. However, these methods do not quantify the rhizobia's tolerance to acidity.

Colony counting of rhizobia grown in acid YM liquid medium is a possibility, but it is very difficult and expensive in terms of material and time for screening a large number of strains or isolates. To facilitate strain selection, a fast and easy method is necessary to evaluate a large number of isolates, as well as to quantify bacterial tolerance/sensitivity to

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these soil constraints. The objective of the present study was to test a laboratory method for this purpose as the first step in selection of root nodule bacteria for acid soils. The goal is to eliminate those strains that are acid sensitive, thus decreasing the number of isolates for other tests, such as root infection, host effectiveness, nodule competitiveness, and soil persistence (5,8,9,10,11), which will be done only for those which present high tolerance to acidity. This procedure may shorten evaluation time and decrease costs in the process of rhizobia selection.

MATERIALS AND METHODS

The present method evaluated several root nodule bacteria strains in Petri dishes with YMA medium (15,18) using an adaptation of a method for streaking rhizobia (15) at two pHs, 4.5 and 6.5 (control). These rhizobia were isolated from different legume plant species and Amazonian soil conditions (Table 1). For adequate solidification, YMA acid medium (pH 4.5) was prepared with 25 g of agar/L, instead of the usual 15 g. The media were not buffered, so as to find strains which are able to modify the pH. This bacterial ability may be important under soil conditions, where all tolerance mechanisms may help their survival and soil colonization. Rhizobia isolates were streaked from a mother Petri dish with YMA (pH 6.5, where bacteria were growing during a five day period) with a platinum loop. Four replicates were used for each rhizobia isolate in each pH medium. Fig. 1 presents the general streaking procedure, which used only one loop per replication from the mother Petri dish. A dilution factor occurs when looping is done from one zone to the other, so zone 4 is the most diluted. Fig. 2 presents the scoring system: 1.00 - "no" growth (no visible growth); 1.25 - some growth only in zone 1; 2.00 - maximum growth in zones 1 and 2; 3.00 - maximum growth in zones 1 to 3; 4.00 - maximum growth in all four zones. Intermediate scores were also given at intervals of 0.25. Growth evaluations were done until score stabilization, which occurred 6-18 days after streaking in the plates. Statistical analyses of growth were done at 6, 9, 12, 15, 18 days after streaking, using the F test and Tukey at 5% for mean comparisons. This method also permits a less rigorous analysis, which consisted of the interpretation given in Table 2, with the best strains presenting scores higher than 3.00. A total of 31 strains were tested by this method. A second test consisted of adding green bromocresol solution to the acid medium to verify possible pH modification during bacterial growth. A visual change may be seen when pH changes from the initial value of 4.5 (bromocresol changes to yellow color at pHs below 4.5, and becomes green/blue at higher pHs).

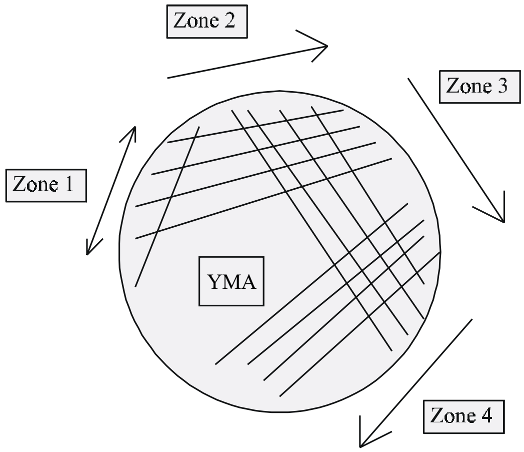
Table 1. Host species and procedence of rhizobia tested on the YMA medium.

STRAINS*	HOST SPECIES	PROCEDENCE
INPA 029	*Vigna unguiculata*	Várzea Ariaú/Solimões
INPA 044	*Vigna unguiculata*	FUCADA/Ultisol
INPA 046	*Vigna unguiculata*	FUCADA/Ultisol
INPA 048	*Vigna unguiculata*	Várzea Ariaú/Solimões
INPA 055	*Vigna unguiculata*	Várzea Ariaú/Solimões
INPA 078	*Vigna unguiculata*	FUCADA/PVA
INPA 511	*Ormosia excelsa*	INPA/CPCA
INPA 520	*Pithecellobium saman*	Maracá/RR
INPA 522	*Clitoria* sp.	Maracá/RR
INPA 526	*Platymiscium paraensis*	Maracá/RR
INPA 550	*Inga edulis*	Calado Lake/AM
INPA 558	*Pithecellobium latifolium*	Anavilhanas/AM
INPA 562	*Pithecellobium latifolium*	Anavilhanas/AM
INPA 563	*Acacia multipinnata*	Maracá/RR
INPA 565	*Rhynchosia minima*	Ariaú/AM
INPA 568	*Dalbergia inundata*	Anavilhanas/AM
INPA 576	*Galactia jussiaeana*	Maracá/RR
INPA 602	*Enterolobium maximum*	Anavilhanas/AM
INPA 609	*Swartzia laevicarpa*	Anavilhanas/AM
INPA 624	*Swartzia laevicarpa*	Anavilhanas/AM
INPA 630	*Pithecellobium latifolium*	INPA/CPCA
INPA 632	*Centrolobium paraensis*	INPA/CPCA
INPA 641	*Pithecellobium inaequale*	INPA/CPCA
INPA 642	*Cassia mimosoides*	Anavilhanas/AM
INPA 649	*Dalbergia inundata*	Anavilhanas/AM
INPA 650	*Clitoria amazonum*	Ponta Negra/AM
INPA 657	*Entada polyphylla*	INPA/CPCA
INPA 658	*Entada polyphylla*	INPA/CPCA
INPA 671	*Inga edulis*	INPA/CPCA
INPA 673	*Abrus tenuiflorus*	Maracá/RR
INPA 678	*Andira riveriana*	INPA/CPCA

* Source: Soil Microbiology Laboratory - CPCA/ INPA

Table 2. Tolerance score ranges for evaluating rhizobia growing on YMA medium.

TOLERANCE	SCORE RANGES
Sensitive	1.00 - 2.00
Moderate tolerance	2.06 - 3.00
Tolerant	3.06 - 4.00



Platinum loop must be sterilized for each streaking zone.
Zone 1. One line. Streaking from a loop, several times in both directions indicated by the arrow.
Zone 2. Four lines streaked in only one direction (indicated by the arrow). Streaking once per line.
Zones 3 and 4. As for zone 2.

Figure 1. Streaking procedure in Petri dishes with YMA medium for rhizobia growth evaluation.

RESULTS AND DISCUSSION

Tolerant rhizobia (INPA 078, Fig. 3A) presented scores of 4.00 at both pHs at 6, 9 or 12 days after streaking in the Petri dishes, while sensitive strain (INPA 641, Fig. 3B) never reached this score. INPA 642 (Fig. 3C) presented a moderate tolerance to pH 4.5.

The method was successful for quantitative evaluation of rhizobia tolerance to acidity. It was possible to separate rhizobia strains statistically, and with low variation among the four replicates for each strain at each pH (Table 3). The standard deviations in general were very low, with the majority being between 0.00 and 0.10. Only five of the 31 strains presented standard deviations higher than 0.10. At 18 days of growth, 15 of the 31 strains presented high growth at pH 4.5, being statistically similar or

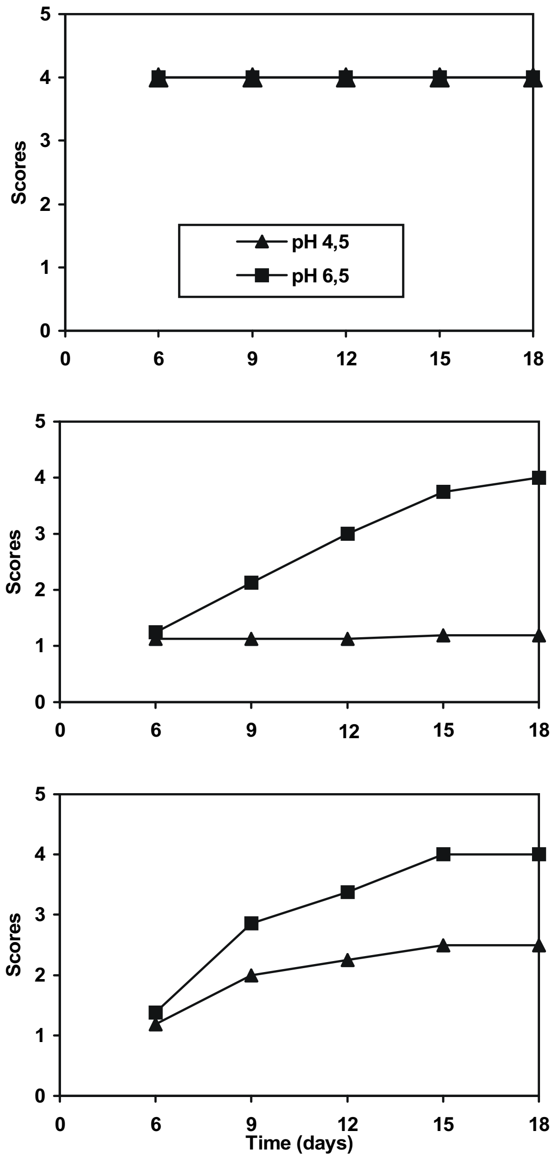


Figure 3. Different degrees of acidity tolerance of rhizobia grown at pH 4.5 and 6.5 during 18 days on YMA medium. A. tolerant strain INPA 078; B. sensitive strain INPA 641; C. moderately tolerant strain INPA 642.

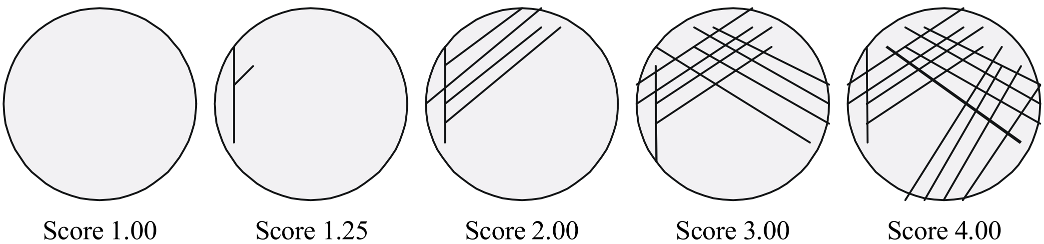


Figure 2. Scores for rhizobia growth in Petri dishes with YMA medium.

Table 3. Mean (sd)[2] growth of root nodule bacteria on YMA medium at pH 4.5 and 6.5. Means of four replications.

Strains INPA	Scores[1]/days of growth: 6 days		9 days		12 days		15 days		18 days	
	pH 4.5	pH 6.5	pH 4.5	pH 6.5	pH 4.5	pH 6.5	PH 4.5	pH 6.5	pH 4.5	pH 6.5
29	1.25(.00)a	1.25(.00)a	2.00(.00)a	2.00(.00)a	3.50(.00)b	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
44	1.75(.00)a	1.75(.00)a	2.00(.00)b	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
46	1.25(.00)a	1.31(.02)a	1.63(.02)b	2.56(.10)a	2.06(.02)b	2.56(.10)a	3.00(.00)b	3.19(.06)a	3.50(.00)b	3.75(.00)a
48	2.00(.00)a	2.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
55	1.00(.00)b	2.00(.00)a	2.75(.00)b	3.00(.00)a	2.25(.00)b	3.50(.00)a	3.25(.00)b	4.00(.00)a	4.00(.00)a	4.00(.00)a
78	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
511	2.00(.00)a	2.00(.00)a	2.25(.00)a	3.19(.02)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
520	1.00(.00)a	1.00(.00)a	1.25(.00)b	2.00(.00)a	2.00(.00)b	3.50(.00)a	2.75(.00)b	4.00(.00)a	3.50(.00)b	4.00(.00)a
522	1.25(.00)b	1.75(.00)a	2.00(.00)b	3.00(.00)a	3.25(.00)b	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
526	1.50(.00)b	2.00(.00)a	1.75(.00)b	2.75(.13)a	2.06(.02)b	3.38(.02)a	3.88(.06)b	4.00(.00)a	2.88(.06)b	4.00(.00)a
550	1.38(.06)b	1.75(.04)a	2.44(.14)a	2.56(.06)a	3.19(.02)b	3.56(.06)a	3.25(.00)b	4.00(.00)a	3.75(.00)b	4.00(.00)a
558	1.00(.00)b	1.75(.00)a	1.94(.02)b	2.44(.10)a	2.75(.04)b	3.38(.06)a	3.25(.00)b	3.75(.00)a	3.25(.00)b	3.75(.00)a
562	1.25(.00)b	1.56(.02)a	1.31(.02)b	2.00(.00)a	2.56(.14)b	2.75(.00)a	3.00(.00)b	3.75(.00)a	3.00(.00)b	3.75(.00)a
563	1.25(.00)b	2.00(.04)a	2.13(.02)b	2.48(.02)a	3.25(.00)b	4.00(.00)a	3.25(.00)b	4.00(.00)a	3.25(.00)b	4.00(.00)a
565	2.00(.00)b	2.19(.02)a	3.13(.02)a	3.25(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
568	1.81(.02)a	1.50(.08)b	2.00(.00)b	2.63(.02)a	2.00(.00)b	2.63(.02)a	2.00(.00)b	3.13(.02)a	2.00(.00)b	3.38(.02)a
576	2.25(.00)a	2.25(.00)a	3.25(.00)b	3.69(.02)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
602	1.00(.00)a	1.00(.00)a	1.00(.00)b	1.50(.00)a	2.25(.00)b	2.75(.00)a	2.75(.00)b	3.50(.00)a	3.50(.00)b	3.75(.00)a
609	1.00(.00)b	1.25(.00)a	2.00(.00)b	3.00(.00)a	2.75(.00)b	4.00(.00)a	3.25(.00)b	4.00(.00)a	3.75(.00)b	4.00(.00)a
624	1.25(.00)a	1.25(.00)a	1.75(.00)b	2.75(.00)a	2.25(.00)b	3.00(.00)a	3.00(.00)b	3.50(.00)a	3.50(.00)a	3.50(.00)a
630	1.19(.02)b	1.44(.02)a	1.38(.02)b	2.00(.00)a	1.98(.02)b	2.75(.00)a	2.13(.02)b	3.94(.02)a	2.13(.02)b	4.00(.00)a
632	2.25(.00)a	1.88(.02)b	3.25(.00)a	2.63(.02)b	4.00(.00)a	3.50(.00)b	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
641	1.13(.02)a	1.25(.00)a	1.13(.02)b	2.13(.10)a	1.13(.02)b	3.00(.00)a	1.19(.06)b	3.75(.00)a	1.19(.06)b	4.00(.00)a
642	1.81(.02)a	1.38(.02)b	2.00(.00)b	2,86(.02)a	2.25(.00)b	3.38(.02)a	2.50(.00)b	4.00(.00)a	2.50(.00)b	4.00(.00)a
649	2.25(.00)b	3.06(.02)a	3.25(.00)b	4.00(.00)a	3.25(.00)b	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
650	1.25(.00)b	3.00(.00)a	2.50(.08)b	3.25(.00)a	3.25(.00)b	3.75(.00)a	3.75(.00)b	4.00(.00)a	3.75(.00)b	4.00(.00)a
657	2.00(.00)a	2.00(.00)a	3.00(.00)a	3.00(.00)a	3.50(.00)b	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
658	2.00(.00)a	2.00(.00)a	3.25(.00)a	3.13(.02)a	3.75(.00)a	3.88(.02)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
671	2.00(.00)a	2.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
673	1.00(.00)a	1.00(.00)a	1.75(.00)a	1.88(.10)a	2.31(.31)b	3.69(.02)a	2.25(.08)b	3.88(.02)a	2.25(.08)b	3.88(.02)a
678	2.19(.64)b	2.69(.02)a	2.69(.43)b	3.75(.04)a	3.50(.08)b	4.00(.00)a	3.63(.19)b	4.00(.00)a	3.63(.19)b	4.00(.00)a

1 - Scores: 1.00 = “no visible” growth; 4.00 = maximum growth.
2 - (sd) = standard deviation.
3 - Means with the same letter in a line (inside each date) are not statistically different (Tukey test at 5%).

equal to growth at pH 6.5: INPA 029, 044, 048, 055, 078, 511, 522, 565, 576, 624, 632, 649, 657, 658, and 671.

This method also allows selection of strains without statistical analysis, by choosing those which grow well on the medium (scores > 3.00). Using this less rigorous criterion of evaluation (without statistical analysis, Table 2), it is possible to include in this tolerance group the strains INPA 046, 520, 550, 558, 563, 602, 609, 650, and 678, since they presented scores higher than 3.00 at pH 4.5 at 18 days of growth.

Growth stabilization occurred from day 6 to day 15 after streaking the plates. Consequently the time of growth may also be used for selection, since those strains that present higher scores with less time of growth indicate they are more tolerant to low pH. Thus, at 9 days of growth, it was possible to verify, statistically, that the strains INPA 048, 078, and 671 presented 4.00 scores at both pHs. The less rigorous evaluation, using the Table 2 as reference, also includes in this highly tolerant group the strains INPA 511, 565, 576, 632, 649, and 658, which presented scores higher than 3.00 at this time of evaluation.

When bromocresol green was used to evaluate visual changes of medium pH, only strain INPA 046 presented the ability to decrease medium pH. All the other strains presented no visual pH changes in the medium. The ability to decrease the pH may have affected in some way the tolerance of the strain INPA 046, which reached scores higher than 3.00 only after 18 days of growth. None of the strains presented a visual change of the medium pH, which could be a mechanism to neutralize the acidity, as observed by other authors (2, 13, 19).

The method proved useful for quantitative evaluation of rhizobia tolerance at low pH. It is very easy and fast for screening a large number of rhizobia isolates under laboratory conditions. Statistical analysis may be used when accuracy is necessary. This method also evaluated fastgrown rhizobia (*Rhizobium leguminosarum* bv. *phaseoli*) (Oliveira, L.A. and Graham, P.H., unpublished results) for this purpose, as well as for evaluating tolerance to other soil constraints, such as aluminum toxicity (12). However, it is only the first step in rhizobia selection. Tests for nitrogen fixation effectiveness, competitiveness for nodule sites and soil persistence (1,5,8,9,10,11) must be done, before recommendation of these tolerant strains as inoculants.

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RESUMO

Avaliação quantitativa da tolerância de rizóbios à acidez.

A quantificação da tolerância à acidez em testes de laboratório pode ser o primeiro passo na seleção de estirpes de rizóbios para a Amazônia. O presente método avaliou isolamentos de rizóbios em placas de Petri contendo meio YMA com pHs 6,5 (controle) e 4,5, usando notas de 1,0 (sensíveis, sem crescimento visual), até 4,0 (tolerantes, máximo crescimento). As avaliações foram realizadas aos 6, 9, 12, 15 e 18 dias de crescimento. O método permite selecionar preliminarmente, rizóbios isolados de solos da Amazônia, com precisão estatística. Entre as 31 estirpes inicialmente testadas, as estirpes INPA 048, 078 e 671 apresentaram notas iguais a 4,0 em ambos os pHs testados após os 9 dias de crescimento. Ao se analisar as estirpes usando um sistema menos rigoroso (nota de crescimento acima de 3,0), foi possível incluir também neste grupo, as estirpes INPA 511, 565, 576, 632, 649 e 658, que cresceram na zona mais diluída (zona 4) após 9 dias. As estirpes tolerantes devem ser testadas para eficácia na fixação de nitrogênio, competição por sítios de nódulos e persistência no solo antes de serem recomendadas para o uso em inoculantes comerciais.

Palavras-chave*:* Rhizobia, tolerância ao pH, Amazônia

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