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ISOLATION AND CULTIVATION OF *Rhizobium* STRAINS FOR TROPICAL FORAGE LEGUMES USING ACID MEDIA

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Surface sterilized seeds of *Desmodium ovalifolium* CIAT 350, *Stylosanthes capitata* 1019, *Stylosanthes guianensis* 136 and *Macroptilium atropurpureum* (Siratro) were planted in pots containing a sand-grit mixture and inoculated with soil collected from Amazonas, Pará and Roraima in Amazonia, Brazil. The plants were watered with acid (pH 4.5) or neutral (pH 6.8) nutrient solution. .Three crushed nodules from each plant were streaked onto yeast mannitol agar (YMA) a) with bromocresol green indicator (pH 4.5) and b) with bromothymol blue indicator (pH 6.8). In most cases the isolates grew equally well on the two media or better on the acidified medium. Five out of 139 isolates grew better on neutral medium. However, further studies comparing isolates in acidified media containing either arabinose or mannitol as a carbon source showed that they all produced alkali in the mannitol medium,whereas only some did so in the arabinose medium. Growth of most isolates was not as good in either of these media as in neutral YMA. The possible use of acidified medium containing arabinose as a carbon source for isolation and cultivation of rhizobia for tropical forage legumes is discussed.

INTRODUCTION

Date and Halliday (1979) showed that when a rhizobium strain (CIAT, 1460) isolated from S. guianensis was inoculated into acid liquid growth medium (pH 4.5) containing mannitol as the carbon source the pH rose during growth, whereas when arabinose was used instead of mannitol, the pH stayed low. The same strain was found to grow better at pH 4.5 than 6.8. This suggests that if conventional neutral yeast mannitol agar (YMA) were used for isolation of rhizobia from plants growing in acid soils, some strains would fail to grow and therefore not be isolated. It is also suggested that the ability of rhizobia to grow in acid media may be related to their ability to persist when inoculated into acid soils. Keyser and Munns (1979) used an acid medium containing high Al, low P and mannitol as carbon source to screen rhizobium strains. 65% of strains which were ineffective when tested on cowpeas in acid soils had also failed to grow in the screening medium. Only one strain nodulated effectively in acid soil and failed to grow in the screening medium.

In the experiments described here, rhizobium strains were isolated from acid soils on acidified YMA and then compared with other rhizobia for their ability to grow and modify the pH in various acid media. The aim of these experiments was to design an acid medium in which rhizobia from acid soils could be isolated and also evaluated for their ability to grow under acid conditions.

MATERIAL & METHODS

Three surface soil samples were collected from each of three regions of Amazonia in Brazil: Roraima (natural savanna); a heavy yellow latosol cleared of primary forest and planted with unfertilized cover crop legumes near Manaus (Instituto Nacional de Pesquisas da Amazonia, Estaçao Experimental de Silvicultura Tropical: INPA, EEST) and sandy latosol used for introductions of pasture legumes at the Centro de Pesquisa Agropecuária do Tropico Umido (CPATU), Belem.

At Rothamsted Experimental Station, England, polypropylene pots containing a 1:1 sand grit mixture were autoclaved and watered with either acid or neutral nitrogen-free nutrient solution (composition: 0.001 MKCl; 62.5 uM K_2HPO_4, 0.001 M $MgSO_4.7H_2O$, 400 uM $CaSO_4.2H_2O$, 0.01 ppm Cu, 0.025 ppm Zn, 0.25 ppm Mn, 0.0025 ppm Mo, 0.125 ppm B, 1.966 mg Fe EDTA per litre of distilled water). The pH of this solution was adjusted after autoclaving to 4.5 or 6.8.

Seeds of Stylosanthes guianensis CIAT 136, Stylosanthes capitata CIAT 1019, Desmodium ovalifolium CIAT 350 and Macroptilium atropurpureum "Siratro" were surface sterilized and planted in nine acid and nine neutral pots. About 1 g. of the same soil type was placed on top of the seeds of the four legumes in one acid and one neutral pot. The 18 pots were placed in a greenhouse where the maximum temperature was 35°C, and were watered periodically with the appropriate sterilized nutrient solution. After approximately eight weeks the roots of the plants were placed in sterile petri dishes. Three nodules from each plant (if present) were surface sterilized, crushed and streaked onto acid neutral yeast mannitol agar plates (YMA: composition 5 g K_2HPO_4, 2g $MgSO_4 7H_2O$, IgNaCl, 10 g mannitol, 0.4 g powdered yeast extract, 15 g agar and 10 ml 0.04% aqueous solution of bromothymol blue (BTB) or bromocresol green (BCG). The pH was adjusted after autoclaving with sterile HCl to give a green colour in both media). The plates were incubated at 28°C and a single colony was selected from the best grown of each pair of acid and neutral plates and streaked onto another pair of the same two media. Growth of the isolates was evaluated on the two media. If the colonies were larger and more numerous growth was considered to be better in that medium.

At the Centro Internacional de Agricultura Tropical (CIAT) Colombia, preliminary experiments were carried out on 16 of the above isolates and some other Brazilian and Colombian Rhizobium strains. The composition of the basic medium used was 68 mg KH_2PO_4, 87 mg K_2HPO_4 7.35 mg $CaCl_2H_2O$, 29.2 mg EDTA, 27.0 mg $FeCl_3 6H_2O$, 73.9 mg $MgSO_4 7H_2O$

55.9 mgKCl, 0.252 mg $MnCl_2 4H_2O$, 0.114 mg $ZnSO_4 7H_2O$, 0.017 mg $CuCl_2 2H_2O$, 0.0041 mg $NaMoO_4 2H_2O$, 0.1 mg biotin (filter sterilized) and 20 g agar. To this basic medium either 5 g arabinose or 10 mannitol, and either 650 mg KNO_3 or 220 mg Na glutamate and 4 ml 0.4% aqueous solution of BCG were added. The carbon substrates were either autoclaved or filter sterilized. Growth and the production of acid (yellow) or alkali (blue) were evaluated at various intervals during incubation at 28°C.

In a further experiment the growth and pH changes of 109 isolates were compared on the same basic medium as described above containing 5g autoclaved arabinose as the carbon substrate, with an initial pH of 4.5 and 8 ml 0.4 % aqueous solution of bromocresol green.

RESULTS & DISCUSSION

Table 1 shows that of the 139 isolates from the four legumes, 109 (78%) grew better on the acidified YMA than on YMA which was initially neutral. However, the final colour of the acidified YMA was blue (alkali was produced) in all cases. Therefore, the fact that the isolates grew better in this medium does not necessarily mean that they grow better under acid conditions.

When the growth of 16 isolates was compared on mannitol and arabinose media with the same initial pH (4.5) it was found that the mannitol media rapidly turned blue with all the tested strains. The arabinonse media turned blue in some cases but only after a longer incubation period. Initial growth was the same with the two substrates although growth improved in the mannitol medium as it became more alkaline. Growth was better with KNO_3 than with Na glutamate as a nitrogen source, and there was no difference between autoclaved and filter sterilized media. In no case was the growth as good as in neutral YMA. These results imply that strains vary in their ability to produce alkali in acid medium containing arabinose as the carbon source.

A larger number of isolates (109) including some from nodules on leguminous trees collected near Manaus and some from the CIAT culture collection were tested on acid arabinose medium with KNO_3 as a nitrogen source. The isolates were assigned to groups according to the final pH of the medium. 58% (63 isolates) either produced acid or the pH did not change. Some of these produced alkali during the early stages of growth but later the medium became acid (i.e. pH $<$ 4.5).

Thirty one isolates (28%) produced alkali. All the isolates from tree legumes fell into this group, as did those from Leucaena leucocephala. Some of the isolates in this group grew better in the acid medium than in neutral YMA.

A further group of strains (14%) produced mixed reactions, with the older part of the culture producing acid and the younger part alkali or viceversa.

Clearly this medium is not ideal for testing growth of rhizobium strains in acid medium, since many of the isolates were able to modify the pH even when using arabinose as a carbon source. It would

also not be very suitable as the sole medium for isolation or rhizobium strains which grow in acid media since most of the strains grew much more scantily on it than on neutral YMA. However, an acid medium is necessary for the strains which do not grow well on neutral YMA. A medium containing NH_4Cl instead of KNO_3 as a nitrogen source might supply the right conditions for minimum pH change. (See table 2.). Yeast extract would result in an alkaline reaction (Graham, 1964), and should not therefore be included. When making new nodule isolations both this medium and conventional neutral YMA could be used. It is possible that more acid tolerant rhizobia would be selected by this means.

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TABLE 1: Comparison of growth of nodule isolates from four tropical forage legumes on neutral and acidified yeast mannitol agar. "Better" growth signifies that colonies were larger and more numerous on that medium.

	Stylosanthes guianensis CIAT 136	*Stylosanthes capitata* CIAT 1019	*Desmodium ovalifolium* CIAT 350	*Macroptilium atropurpureum* "Siratro"	Total Isolates
Total number of isolates	41	13	42	43	139
Better on acidified YMA	29	6	33	41	109
Equal on acidified and neutral YMA	10	5	9	0	24
Better on neutral YMA	2	1	0	2	5

TABLE 2

SUGGESTED ACID MEDIUM FOR ISOLATING AND SCREENING RHIZOBIUM

	mg/L		ug/L
KH_2PO_4	68	$MnCl_24H_2O$	252
K_2HPO_4	87	$ZnSO_47H_2O$	114
$CaCl_2.H_2O$	7.35	$CuCl_22H_2O$	17
EDTA	29.2	$NaMoO_42H_2O$	4.1
$FeCl_36H_2O$	27.0		
$MgSO_4.7H_2O$	73.9		
Biotin (filter sterilized)	0.1		
	g/L		
NH_4Cl or	1.2	Bromocresol green (0.4%	
KNO_3	0.65	aqueous solution)	4 ml/L
Arabinose	5.0	Final pH 4.5 corrected	
Agar	20.0	with HCl after autoclaving	