Studies on Induced Variation in the Rhizobia

I. Defined Media and Nodulation Test Techniques

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The rhizobial bacteria can be readily grown on a variety of media containing complex nutrient extracts of microbial, mineral, or plant origin, but growth on nonsupplemented carbohydrate-inorganic salts media is poor for most species. Only a minor part of the extensive research devoted to *Rhizobium* has dealt with the minimum requirements for cultural growth (Wilson, 1940; Wilson and Wilson, 1942; Allen and Allen, 1950). Establishment of minimal media is prerequisite to the isolation of nutritional mutants, potentially useful in the study of the biochemical basis of nodulation (infectiveness) and nitrogen fixation (effectiveness). Besides establishing a need for media adapted to specific cultural experiments, the induction of such cultural marker characters requires periodic testing of variants for retention of infectiveness and effectiveness.

This paper gives a brief description of the defined media and modified nodulation testing techniques developed specifically for experiments with rhizobial strains which are variant for characters such as antibiotics reaction, phage reaction, or nutritional requirements. The work described represents a part of the procedure-developing phase of a research program involving the marking of cultures for attempted inter-specific manipulation of infectiveness and effectiveness in *Rhizobium*.

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MATERIALS AND METHODS

Formulation of the minimal medium was based on approximate growth comparisons (colony size), performed in the following sequence: (1) comparison of several widely used media and the stepwise elimination of macroelements which did not appear essential for growth; (2) comparison of the glucose-salts (GS) medium (macroelements group listed in table 1) at varying levels of each salt, kept below the level of precipitation of insoluble compounds like the calcium phosphates; (3) substitution of deionized water for tap water and comparisons of several trace element formulations with contrasting high and low levels of individual elements.

In the organic supplementing tests, concentrated aqueous solutions of 21 amino acids and 10 vitamins were spotted on preplated bacteria in combinations designed to detect interaction of two amino acids and two or more vitamins. Confirmation of any apparent growth stimulatory response was obtained by incorporation of the growth factors into the minimal medium at a range of concentrations. Final quantitative measurements of growth in liquid media were made with a Coleman[2] nephelometer. The surface-layering method of plate inoculation was used throughout these experi-

[2] Coleman Instruments, Inc., Maywood, Illinois.

TABLE 1

Inorganic components of media

Macroelements A* (ME-A)	Amt	Macroelements B (ME-B)	Amt	Trace Elements (TE)	Amt
	g/L		*g/L*		*mg/L*
NH_4NO_3	1.0	KCl	0.5	H_3BO_3	1.0
KH_2PO_4	0.3	KH_2PO_4	0.2	$ZnSO_4 \cdot 7H_2O$	1.0
K_2HPO_4	0.3	$MgSO_4 \cdot nH_2O$	0.2	$CuSO_4 \cdot 5H_2O$	0.5
$MgSO_4 \cdot nH_2O$	0.1	$CaSO_4 \cdot 2H_2O$	0.2	$MnCl_2 \cdot 4H_2O$	0.5
$Ca(NO_3)_2 \cdot 4H_2O$	0.05			$Na_2MoO_4 \cdot 2H_2O$	0.1
				Fe-EDTA†	1.0

* Demineralized water used. Individual macroelement stock solutions and bulked trace element stock solutions (excepting Fe-EDTA) prepared at a concentration of 100 times the above working concentrations. Amounts for all elements, excepting iron, given on a compound basis.

† Iron chelated by the EDTA (ethylenediaminetetraacetic acid) compound, Sequestrene® AA (Geigy Industrial Chemicals, Ardsley, New York). Prepared by addition of $FeSO_4$ to Sequestrene AA (tetra-acid) and KOH, followed by aeration to pH 5.5; maintained in stock at a concentration of 5000× (Fe^{+++} basis).

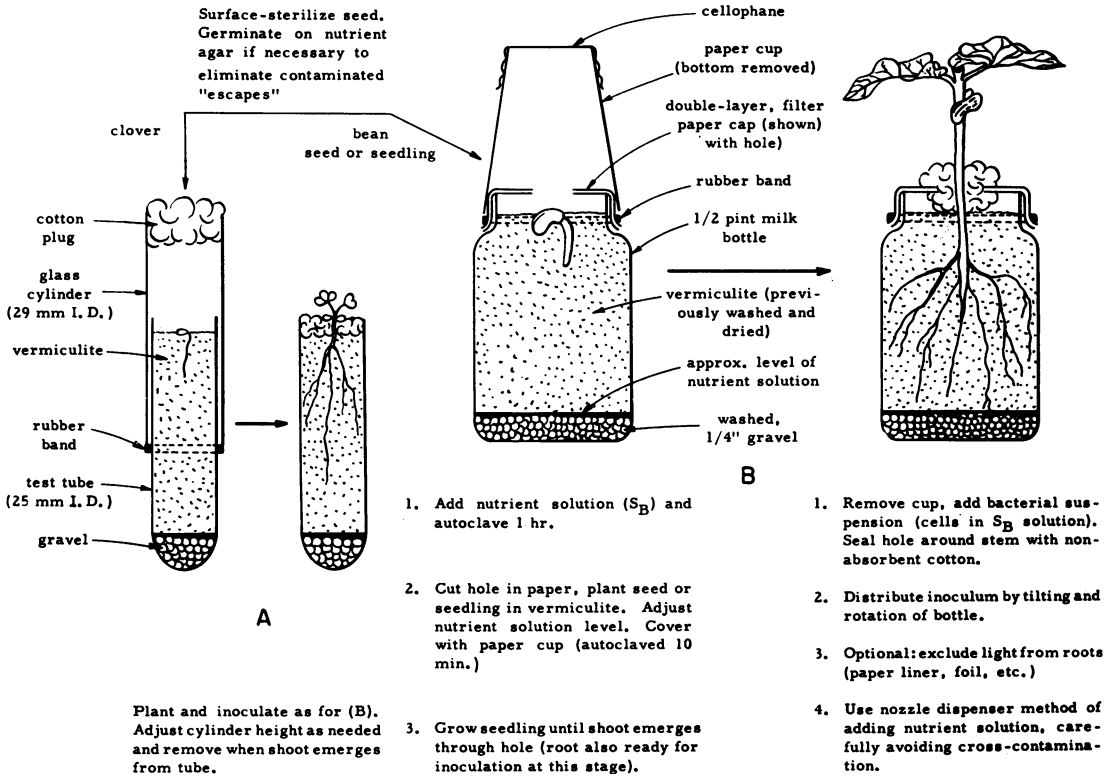

Figure 1. Procedure used in nodulation tests of cultures of *Rhizobium* on (*A*) small-seedling plants and (*B*) large-seedling plants.

TABLE 2

Some characteristics of media used for culture of Rhizobium

Designation of Medium	Composition	Use	Comparative Growth of Cultures of 4 *Rhizobium* Species*			
			L41	P42	T311	M4
			%	%	%	%
GS†	Glucose (0.5%), salts (ME-A + TE)	Growth factor tests‡	17	27	28	32
GS-minimal No. 1	GS + vitamin supplement (biotin, pantothenate)	Biochemical mutant screening (L41, T311)	67	36	59	35
GS-minimal No. 2	GS + vitamin supplement (thiamine, pantothenate)	Biochemical mutant screening (P42, M4)	33	52	55	59
GS-Y	GS + yeast extract (0.1%)	General culture growth	91	110	97	106
G_1S-Y	GS-Y with low glucose (maximum of 0.1%)	Streptomycin tests and phage culture	98	101	101	97
GS-YC	GS-Y + casein acid hydrolysate (0.1%)	Biochemical mutant screening	100	100	100	100
Other media:§						
Wilson and Wilson's	See reference, 1942		6	25	8	19
Campbell and Hofer's	See reference, 1943	Phage culture	70	158	77	106
Medium "79"	Allen, 1951		100	110	83	133

* Cultures (left to right) represent *Rhizobium leguminosarum* strain L41, *R. phaseoli* strain P42, *R. trifolii* strain T311, and *R. meliloti* strain M4. Growth comparisons are based on nephelometer measurements (expressed as a per cent of the GS-YC complete medium) of 18-hr log-phase cultures grown on a shaker at 28 C. The lowest value (6 per cent) given above corresponds to the base level of the inoculum.

† Difco Noble agar or washed Bacto agar (Difco Laboratories, Inc., Detroit, Michigan) used for all plating involving GS or minimal media. Substitution of mannitol for glucose gave no significant growth difference.

‡ GS solution also used as the "saline" solution for cell washing or dilution procedures where GS-Y broth cannot be employed.

§ Used here mainly for purposes of growth comparisons. Wilson and Wilson's medium is an inorganic macrosalts medium; the other two are organic supplemented "complete" media.

ments, in preference to the surface-spreading technique which is complicated by cell motility and variables of agar surface drying.

Plant inoculation procedures used in nodulation and nitrogen-fixation tests are illustrated in figure 1 and described under Results and Discussion. The cultures used in this study were: *Rhizobium leguminosarum* strain L41, *Rhizobium phaseoli* strain P42, *Rhizobium trifolii* strain T311, and *Rhizobium meliloti* strain M4. The nitrogen-free nutrient salts solution (S_B solution) used for growing the plants contains the trace elements (TE) and the B group of macroelements (ME-B) given in table 1.

Results and Discussion

Media and growth. The media formulations are given in tables 1 and 2. Growth of all cultures on the GS base is inadequate for practical purposes of a minimal medium, thus necessitating partial supplementation with organic growth factors. Supplementing with 21 amino acids, although done only empirically by spot test methods on three cultures, did not produce sufficient growth stimulation to warrant more extensive study of amino acids. Several amino acids, notably cystine, inhibited growth when used at high concentrations. Stimulation of rhizobial growth commonly occurred in zones surrounding some contaminant bacterial colonies of undetermined identity. No attempt was made to fractionate the exometabolites.

As anticipated on the basis of earlier reports (Wilson, 1940; Wilson and Wilson, 1942) of vitamin responses in the rhizobia, sufficient growth enhancement was obtained from vitamin addition to make the practical use of a vitamin-supplemented GS medium feasible as a minimal medium, although the level of growth is still well below that of the "complete" glucose salts-yeast extract (GS-Y) or glucose salts-yeast extract-casein acid hydrolyzate (GS-YC) media. It is necessary, however, to determine the specific supplements for each culture to be used in biochemical mutation work, so that a given vitamin-supplemented medium will be "minimal" only with respect to cultures responding to the particular supplement. Of the 4 cultures (see table 2) evaluated in this study, *R. leguminosarum* L41 responded to biotin (0.05 μg per ml) plus calcium pantothenate (0.2 μg per ml); *R. phaseoli* P42 and *R. meliloti* M4 responded to thiamine (0.2 μg per ml) plus calcium pantothenate; and *R. trifolii* T311 was stimulated by either combination. In each instance, biotin or thiamine alone produced the major single-vitamin effect, but a further complementary effect, equivalent to the stimulation of the complete vitamin mixture (10 vitamins), resulted from the addition of the pantothenate. The minimal media thus permit screening for induced deficiencies for all but two of the vitamins, as well as for amino acids. None of the above media supported good growth of a culture of *R. japonicum*, generally recognized as a "slow-growing" species.

It should be noted that the same trace element mixture is used for both the bacterial media and the plant nutrient solution. No precipitation or pH adjustment problems were encountered in any of the media at the inorganic levels given in table 1. A lower glucose content in the G_1S-Y medium is used for reasons of reducing glucose inactivation of streptomycin in antibiotic experiments (unpublished) and decreasing gum formation in bacteriophage experiments.

Attempts to induce biochemical mutants. No nutritionally deficient mutants were found in preliminary experiments in which X-rays were used as the mutagen and three methods of mutant enrichment (Davis, 1949; Adelberg and Myers, 1953; Lubin, 1959) were employed for screening purposes. The major emphasis in these experiments was on manipulation of variables in the enrichment procedures to give more effective elimination of wild type cells. In the penicillin methods of

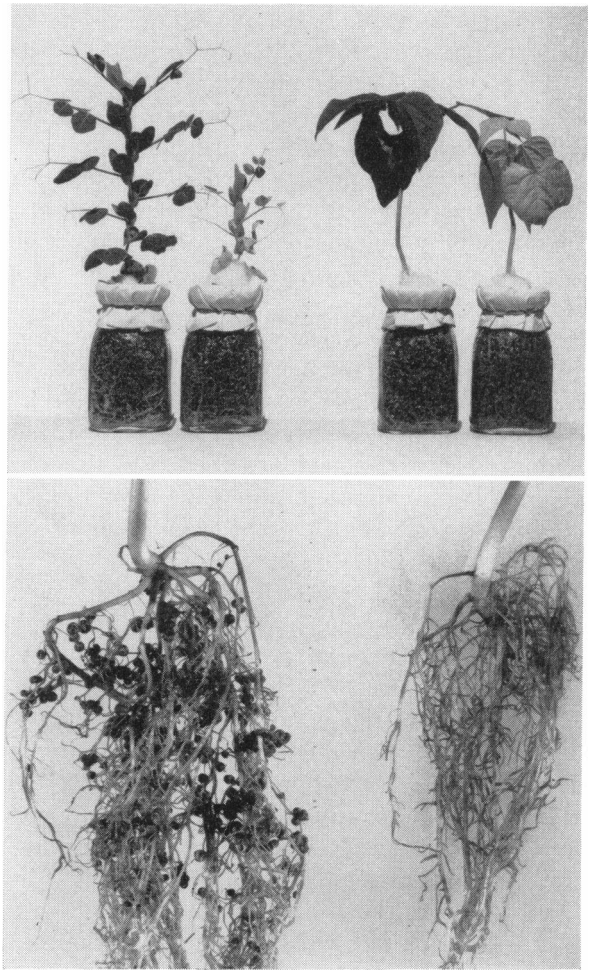

Figure 2. (*Top*) Pea (*left*) and bean (*right*) plants tested for nodulation by the method of figure 1*B*. Uninoculated control plants (*right*, each set) are not nodulated and show nitrogen starvation symptoms. (*Bottom*) Nodulated and nodule-free roots of inoculated and control bean plants.

enrichment the relatively high penicillin tolerance of the rhizobia necessitates the use of high doses (concentration and time) of the antibiotic to give a significant bactericidal effect rather than mere bacteriostasis. Since only about 900 randomly selected (replica plating not used) colonies from "enriched" populations were directly tested for inability to grow on minimal media, it is premature to speculate whether *Rhizobium* may be uniquely more refractory to biochemical mutation than many other bacterial species. Further attempts to induce biochemical mutants have been deferred in favor of antibiotics resistance and phage resistance as marker characters.

Technique for nodulation and nitrogen fixation tests. The procedure outlined in figure 1 and illustrated in figure 2 is a modification of widely used methods in which small seedlings (*e.g.*, alfalfa, clover) are grown on agar in test tubes, and large-seedling plants (*e.g.*, beans, peas) are grown on a sterile sand culture in glazed jars (Erdman and Burton, 1938; Allen, 1951). The method of figure 1*B* fulfills the requirement for replicated single-plant tests (seedborne or airborne contamination, when present, confined to a single plant) and is designed primarily to grow the bulky shoot of the large-seedling plants outside the container while confining only the roots to a small, closed system which is relatively simple to prepare, inoculate, and maintain. Confinement of the root system (kept at the optimum nutrient solution level) to the small volume does not seriously limit normal development of the plants through the stage of growth when observations on nitrogen fixation efficiency can be made. As far as simplicity of method and economy of space is concerned, the extensible-tube method (figure 1*A*) for testing of alfalfa or clover seedlings offers no particular advantage over the commonly used agar-tube technique, but it provides a means of growing the shoot externally, under more favorable conditions of aeration and light.

The above method, although adapted to the requirement of simply detecting infectiveness in a pure culture or uniform bacterial population, would not be suitable for detection of infrequently occurring infective cells in a population of noninfective cells. Thus, the detection of induced nodulation in a noninfective culture would require massive inoculation of a larger number of plants, grown under nutrient culture conditions. Use of adequately marked cultures, as planned for such experiments, and the use of low levels of antibiotics (assuming the use of antibiotic-resistant cultures) in the substrate would lessen the stringent requirement of excluding airborne rhizobial contamination.

Acknowledgments

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Summary

A defined inorganic medium, developed for nutritional studies with the rhizobial bacteria, supported only a low level of growth of these bacteria. Combinations of two or more vitamins stimulated growth sufficiently to permit use of the vitamin-supplemented medium as a minimal medium for cultures responding to a particular vitamin combination, although in no case was growth comparable to that obtained on the yeast extract medium. Amino acids tested were not stimulatory. No biochemical mutants were isolated in preliminary experiments involving X-rays as a mutagen.

A plant nutrient solution, with the same trace element composition as the bacterial media, was formulated for plant nodulation tests. Modified methods of culturing plants are described as used for routine testing of bacterial strains for nodulation and nitrogen fixing ability.

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