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EXPERIMENTS ON THE CULTIVATION OF RHIZOBIUM IN LIQUID MEDIA FOR USE ON THE ZUIDERZEE POLDERS

by

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For 15 years the rhizobia, which were used for the inoculation of the Zuiderzee-polder soils, were grown on agar plates in large Petri-dishes. For legumes with small seeds (clover, lucerne) 3 to 6 g of the original undiluted slimy bacterial substance were used per kg of seed, for big seeds (beans, peas, vetch and lupines) 1 g per kg (HARMSEN, 1935).

During the inoculation-campaign contamination of the plates by air-borne micro-organisms often increased enormously, with the result that the cultures on a great percentage of the plates were valueless and had to be discarded (HARMSEN, 1936).

Some data on growing of rhizobia in a liquid medium have already been published (VAN SCHREVEN, 1951). In 1932 and 1933 tests had been made to determine whether growing of rhizobium in a liquid medium is preferable to growing it on agar. As the growth in a liquid medium in these experiments did not give satisfactory results, this method was abandoned for a while, but in October 1945 the use of rhizobium cultures in solution was again studied.

For this purpose an aerated culture solution of 100 ml in an Erlenmeyer flask of 300 ml was inoculated with *Rhizobium trifolii* and *R. meliloti*, respectively.

The composition of the solution in 90 parts by volume of tap water and 10 parts by volume of neutral yeast extract was as follows: 1.5 % mannitol, 0.2 % $CaCO_3$, 0.05 % K_2HPO_4, 0.02 % $MgSO_4$, 0.02 % NaCl.

The development of the bacteria was very satisfactory. After it had become evident that a good growth could also be obtained with several species of rhizobia in 6 l Erlenmeyer flasks with 5 l of aerated culture solution, an experiment was made in 1947 with a solution of 20 l in a milk-can with a soldered lid. In the lid were two apertures, one for the aeration tube and one for letting out the air and for introducing the culture solution. At the bottom a drain-cock had been inserted, through which samples could be taken without contaminating the solution. The composition of the solution was the same as above, but 1.15 % glucose + 0.35 % mannitol were used instead of 1.5 % mannitol. To this solution 5 ml paraffinum liquidum were added to reduce the development of foam. The solution was inoculated with *R. trifolii*. After 9 days it contained 1500 million bacteria per ml. The numbers were counted directly in a counting chamber of FUCHS-ROSENTHAL with a depth of 0.01 mm. As it was impossible to count the small bacteria in the ordinary squares, they were counted in the small squares formed by the double dividing-lines.

The numbers of *R. leguminosarum* (strain A 2), which developed in the same solution in a milk-can are given in table 1.

TABLE 1.

Numbers of *R. leguminosarum* in millions per ml solution.

After . . . . hours	Direct count	Plate count
0	not yet possible	1
24	300	180
48	900	240
72	1200	130
96	850	180
120	750	340
144	750	120

The maximum number of bacteria was found by direct counts after 3 days growth.

In another experiment, pH determinations (with a glass-electrode) were made of the solutions in two milk-cans inoculated with *R. phaseoli* and the numbers of bacteria were determined by direct counts (table 2). Evidently a pH correction is not necessary.

TABLE 2.

Influence of *R. phaseoli* on the pH of the culture solution.

After .... hours	Numbers of bacteria in millions per ml		pH	
	I	II	I	II
0	8	4	7.0	7.0
16	110	30	6.9	6.9
24	420	300	—	—
40	540	300	6.8	6.8
48	250	360	6.8	6.6
64	330	450	6.7	6.7

The influence of the rate of aeration was studied in two milk-cans inoculated with *R. meliloti*. The solutions of these milk-cans were respectively aerated with 10 (A) and 100 l air per hour (B).

The higher rate of aeration greatly increased the numbers of bacteria but reduced the average cell length (table 3).

TABLE 3.

Influence of the rate of aeration on the numbers of rhizobia in liquid medium in millions per ml.

After .... hours	Direct count		pH		Average length in μ	
	A	B	A	B	A	B
0	—	—	7.1	7.2	—	—
8	—	—	7.2	7.1	—	—
23	320	540	7.0	7.0	3.5—5	2.5—3
32	400	800	6.8	6.9	3—4	—
48	800	2410	6.9	6.8	—	—
56	860	2500	6.9	6.7	—	—
72	890	3320	6.7	6.5	—	—
80	670	2490	6.7	6.4	—	—
96	400	3260	—	—	3—4	1.5—2.5

It is known that rhizobium can use glucose as a source of carbon (MÜLLER and STAPP, 1925; FOOTE *et al.*, 1929) and since glucose is much cheaper than mannitol, it was important to know whether it could replace mannitol. For this purpose the following two culture solutions were compared:

A: a solution with 1.15 % glucose and 0.35 % mannitol,
B: a solution with 1.5% glucose without mannitol.

The experiment was made in duplicate in Erlenmeyer flasks. The solutions were inoculated with *R. meliloti*, strain A 15. Counts at intervals up to 65 hours showed no difference in growth in the two media (table 4).

TABLE 4.

Numbers of *R. meliloti* in millions per ml in a glucose solution and without mannitol (direct count).

After . . . . hours	A (with mannitol)	pH	B (without mannitol)	pH
16	880		950	
40	3840		3500	
47	3160		3410	
65	3480	6.3	3600	6.2

In another experiment milk-cans were filled with different culture solutions and inoculated respectively with *R. trifolii* (strain A 8) and *R. meliloti* (strains A 15 and A 18). The solutions were incubated at 29—30° C. The following media were used:

1. The original culture solution with 1.15% glucose and 0.35% mannitol, inoculated with strain A 8.
2. The same solution as 1, but inoculated with strain A 15.
3. The same solution as 1, but with 2% solid yeast, replacing 10% yeast extract, and 2% milk powder replacing mannitol-glucose, inoculated with strain A 15 (in duplicate).
4. The same solution as 1, inoculated with strain A 18.
5. As 4, but 2% solid yeast in place of 10% yeast extract and 1.15% sucrose in place of glucose.

The numbers of rhizobia estimated by common plate counts [1]) (average of 10 replicates) are given in table 5.

[1]) For the agar plates generally the following medium is used: 90 parts by volume of tap water and 10 parts by volume of yeast extract with 1.5% mannitol, 0.2% $CaCO_3$, 0.05% K_2HPO_4, 0.02% $MgSO_4$, 0.02% NaCl, 1.5% agar.

TABLE 5.

Numbers of rhizobia in millions per ml, grown in milk-cans.

Treatment	After . . . . days				
	2	3	4	5	7
1) Original solution, *R. trifolii*, A 8	143	273	316	539	376
2) ,, ,, , *R. meliloti*, A 15	633	1033	857	811	903
3) As 1), but with 2% solid yeast and 2% milk powder, *R. meliloti*, A 15	856	1280	2154	1223	2031
4) Original solution, *R. meliloti*, A 18	1553	2557	2212	2256	1108
5) As 4), but 2% yeast and 1.15% sucrose, *R. meliloti*, A 18	1209	2224	2748	3480	3428

The following conclusions can be drawn from this experiment:

1) A greater number of rhizobia per ml was obtained with *R. meliloti*, strain A 18 than with strain A 15. Still smaller numbers were obtained with *R. trifolii*, strain A 8.
2) 2 % solid yeast and milk powder gave better growth than a 10 % solution of neutral yeast extract and mannitol-glucose.
3) Sucrose can be used in place of glucose.
4) After 5 days the numbers of rhizobia in the milk-cans vary from ± 500 to 3500 millions per ml, depending on the rhizobium strain and on the medium [1]).

The development of foam on aeration is greater with solid yeast than by yeast extract. For that reason yeast extract is more often used. Recently sucrose has been used in place of mannitol-glucose and 5 ml paraffinum liquidum is added to check foaming. After boiling, the hot nutrient solution is poured into the milk-can and immediately sterilized for 1 hour at 120° C. The preparation of the inoculum in liquid medium is now carried out as follows:

1) A diluted suspension of rhizobium is sown out in Petri-dishes with 10 ml agar medium.
2) A colony is picked and an agar slant culture made.
3) A suspension from this slant culture, after 2—7 days incubation at 30° C., is made in 10 ml sterile water.

[1]) If the bacteria have a mean length of 3 μ and the solution contains 3000 million bacteria per ml, all the bacteria of a milk-can with 20 l solution, when laid behind each other in a row, form a distance corresponding with 4.5 × the circumference of the earth, or almost half the distance between earth and moon!

4) Three l of a culture solution in a container are inoculated with this suspension. The medium in this flask is incubated at 29—30° C. and aerated with sterile air.
5) After two days this solution can be used as inoculum for a series of milk-cans. Before using the inoculum from the container a small sample is siphoned over and examined by means of a phase contrast microscope. Then 150 ml of the inoculum are siphoned over into the milk-can through a sterile hollow needle which is attached to the end of the rubber tube both of which have been sterilized previously. The needle is inserted between the cotton-wool and the wall of the air exit tube in the lid of the milk-can. Thus it is not necessary to remove the cotton-wool during the inoculation of the culture solution, so that an important source of infection is eliminated. The flow of inoculum from the receiver is regulated by means of a clip fixed on the rubber tube and by using a stop-watch.
6) The inoculated medium of the milk-cans is incubated at 29—30° C. and aerated for three days with sterile air (100 l per hour). Before using the inoculum for the Zuiderzee-polder soils, a small sample of each milk-can is examined microscopically.

By this method several milk-cans can be inoculated with a bacterial suspension by starting from one separate colony. With this procedure practically no contamination takes place.

Field experiments.

The amount of liquid inoculum to be used per kg of seed was studied in two field trials in 1947. These were laid out in an early reclaimed part of the North-Eastern polder, situated in the centre of the polder, on heavy loam.

The first experimental field was on the polder section 0 13 and was seeded on May 29th in a warm dry period. There was no rain for a week after sowing. The weather conditions were thus very unfavourable for the inoculum, as it had also been dry for some time before the test plots were laid out.

A second experimental field was laid out on June 10th on polder section 0 11.

Black medic (*Medicago lupulina*) and lucerne (*M. sativa*) were sown at the rate of 25 kg/ha in four replicates on plots of 4 × 6 m². The objects were treated as follows:

1 = not inoculated
2 = 5 ml
3 = 10 ml
4 = 20 ml
5 = 50 ml
} liquid inoculum per kg of seed
6 = 4 g inoculum grown on agar plates per kg of seed.

The crop yields and nitrogen content of the green material are shown in table 6. Increased doses of inoculum above 5 ml gave no increase in yield. It was however considered safer to apply 20 ml in practice to ensure success. The effect of the liquid inoculum proved to be very satisfactory and when applied at the latter rate there is no difference in effect with the agar-inoculum used at the rate of 4 g per kg of seed.

TABLE 6.

Yield data of the test trial on parcel 0 11 in 1947 (after 80 days).

Treatment	Black medic				Lucerne			
	Fresh weight of green material kg/are	Dry weight of green material kg/are	N of dry material %	Weight of N kg/are	Fresh weight of green material kg/are	Dry weight of green material kg/are	N of dry material %	Weight of N kg/are
Not inoculated	59	19.0	2.65	0.504	53	16.2	3.26	0.528
5 ml/kg	146	45.8	3.77	1.727	86	27.9	3.44	0.960
10 ml/kg	147	48.3	3.85	1.860	68	24.9	3.31	0.824
20 ml/kg	136	46.5	3.87	1.800	83	28.7	3.50	1.005
50 ml/kg	130	43.4	3.77	1.636	84	25.9	3.57	0.925
4 g/kg	140	47.2	3.81	1.798	81	25.0	3.54	0.885

Treatment	Yield data of the test trial on parcel 0 13 in 1947 (after 92 days) Black medic			
Not inoculated	123	37.2	3.64	1.354
5 ml/kg	212	62.4	3.73	2.328
10 ml/kg	198	58.9	5.44 [1])	3.204
20 ml/kg	236	68.0	3.46	2.353
50 ml/kg	238	69.0	3.52	2.429
4 g/kg	263	80.0	3.29	2.642

[1]) This content is probably incorrect and should be 3.44, in which case the weight of N is 2.026 kg.

The question, whether the development of nodules by clover, lucerne and black medic would be influenced if the seed was inoculated 3, 7 or 14 days respectively before sowing, was examined in 1948 in a field experiment. Control plots were sown with uninoculated seed and with seed that was inoculated on the day of sowing. In the lucerne and black medic the differences of the inoculated and uninoculated plots were very clear. The clover plots did not show such differences, nodulation also being profuse on the uninoculated plots. The lucerne and black medic seed storage after inoculation retarded development of nodules and growth of the plant, the effect increasing with the period of storage. Retardation, however, was slight and no longer noticeable after 2.5 months growth. If the weather conditions are so unfavorable at the moment of the delivery of the inoculum that sowing is impossible — and the seed may have been inoculated and sown a week later when the weather conditions have improved — it is necessary to store the seed in a fairly dry condition to exclude heating.

During 1947 to 1951 4840, 3060, 3620, 1620 and 1640 l of liquid inoculum were made for the North-Eastern polder.

For big seeds 1 l of inoculum is mixed with 100 kg of seed, for medium-sized seeds 1,5 l per 100 kg are used and for small seeds 2 l per 100 kg. Thus one milk-can with 20 l inoculum is sufficient for 50 ha lucerne or black medic. Applied in these quantities the inoculum has given complete satisfaction. The production of the inoculum by this method is about four times as cheap as by the agar plate method previously used, and can be carried out by only a few persons. On a large scale, however, the process is only practicable when the inoculum need not be kept for a long time and need not be transported for long distances.

Summary.

A description is given of a method by which rhizobium can be grown in milk-cans in an aerated liquid medium. The application of the procedure on a big scale is only practicable when the inoculum need not be kept for a long time and need not be transported for long distances.

Literature.

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