A Rapid Method of Selecting Rhizobium Strains

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The efficiency and virulence of Rhizobium strains are generally determined by sterile plant experiments and evaluated on the basis of the yield and N-content of the test plant.

The disadvantage of the plant tests consists in their taking a considerably long time and demanding much work and that the results can be obtained only 6—8 weeks after the start of the experiment.

Therefore there is a world-wide trend to cut the time necessary for the plant tests. In order to solve this problem RABOTNOVA [11] and EBERTOVÁ [2] studied the correlations existing between the redox potential values of the root nodules and effectivity, while WROBEL [18] investigated the relationship between the pH and redox potential values of the root sap of pea. VIRTANEN [15, 16], JORDAN and GARRARD [8] as well as BERGERSEN [1] succeeded in finding a close correlation between the haemoglobin content of the nodules and the N-content fixed by the Rhizobium bacteria. WIERINGA and BAKHUIS [17] found a difference in the free amino-acid compositions of the root saps of pea plants not inoculated, or inoculated with effective and ineffective Rhizobium strains, respectively. With the method developed by these authors the effective strains can be selected 3 weeks after the seeds were sown.

A new trend in research — and this is the subject of this paper — is to develop a rapid method to determine the degree of effectivity that can be used for the selection and the continuous control of a great number of Rhizobium strains.

FEDOROV [3], GUPTA and SEN [4], KERPELY and BAKONDI [10] as well as SHMIDT [14] have tried to find a correlation on the basis of the N-fixing capacity of Rhizobium strains of different activity in liquid cultures, the redox-potential value of the culture medium and the degree of dehydrogenase enzyme activity.

For a diagnostal determination of Rhizobium strains SCHWINGHAMER [12] studied the degree of resistance to antibiotics, GRAHAM and PARKER [7] the degree of thyamine and pantotenate requirements, of pH value, of temperature-dependent growth, of nitrate reduction and of penicillinase production. SILNIKOVA and AGADJANIAN [13] studied the correlation between the effectivity and virulence and the respiration of Rhizobium strains.

A close correlation was found to exist between the glucose consumption (GUPTA and SEN [5]), the phosphatase enzyme activity (ZELEZNA [19]), the degree of phosphate utilization (GUPTA and SEN [6]) and the effectivity of the strains.

In Hungary formerly the effectivity of Rhizobium strains (KERPELY and BAKONDI [10]) was correlated with the change in the redox potential value of the bacterium suspensions and the intensity of cell respiration.

In this study we examine the possibilities of using the amino-acid consumption of the strains of *Rhizobium meliloti*, the measurement of the turbidity of the liquid cultures and the changes in the pH value of the cultures to develop rapid test methods.

Materials and methods

The experiments were carried out with 18 strains of *Rhizobium meliloti* that came partly from the collection of Rhizobium strains of the National Institute for Agricultural Quality Testing, partly from a similar collection of the Phylaxia State Serum Institute, Budapest.

The strains of *Rhizobium meliloti* were tested for effectivity and virulence by plant experiments conducted in sterile test tubes containing nutrient agar. Each test tube contained 8 ml of Nutman's culture medium containing 1 per cent agar. The alfalfa seeds, sterilized with 3 per cent Neomagnol and sterilized distilled water, were allowed to germinate for 24 hours and then two seedlings with 0.5 cm long plant embryos were planted into each test tube. The activity of the control and the various strains was tested 10 times.

The seeds were inoculated with a Rhizobium bacterium suspension washed off an oblique agar surface set to grade 7 of Brown's scale. The plants were grown for 35 days. The evaluation was made on the basis of the green weight and the N-content of the alfalfa plant.

At the same time as the plant experiment was started, the liquid culture medium (a 5 per cent haricot bean brew containing 1 per cent mannite), serving as basis for the rapid laboratory test method, was inoculated with bacteria taken from the same cultures as those used in the plant experiment. 4 ml of the culture medium was put into each test tube and inoculated with 0,1 ml of bacterium suspension. A culture medium that was not inoculated served for control purposes. The tests were repeated 20 times. The liquid cultures were incubated at 28°C and the laboratory tests with the methods described below took place first after 48 hours and then every four days.

The free amino-acid was determined by paper chromatography. We used Schleicher-Schüll paper No 2043/b, a solvent system containing n-butanol, concentrated acetic acid and water (4 : 1 : 1) and ninhydrin containing 0,2 per cent n-butanol. In order to obtain a better separation of the free amino-acids the cartogram was run twice for 16 hours each time.

For a direct determination of the changes occurring in the quantity of the free amino-acids contained by the liquid cultures another method was also used. This method which is simpler and more rapid than paper chromatography is based on colour reaction. According to this method the 14 day-old liquid cultures were centrifuged at 7000 r.p.m. for 20 minutes to separate the bacteria. To the clear solution free of bacteria ninhydrin containing 0,2 per cent n-butanol was added at a ratio of 5 : 1. The components were mixed by shaking and then the mixture was kept for 20 minutes in a water bath on 80°C. Afterwards the reaction was evaluated by a direct visual inspection of the degree of discolouration of the upper, ninhydrin containing layer.

The liquid cultures of the Rhizobium strains of various activity displayed different degrees of turbidity already on the fourth day of the incubation period. The degree of turbidity was determined on the 14th day partly by nephelometric analysis, by means of a Pulfrich photometer, partly by direct visual evaluation for the purposes of rapid testing.

The pH value of the liquid cultures was measured with an electronic instrument, with a glass electrode.

Results and conclusions

The effectivity and virulence of the 18 strains of *Rhizobium meliloti* were classified on the basis of the results of the vegetation experiments as well as of the total yield and the N-content of the plant (Table 1). As compared to the control 13 strains proved to be effective and 5 strains turned out to be

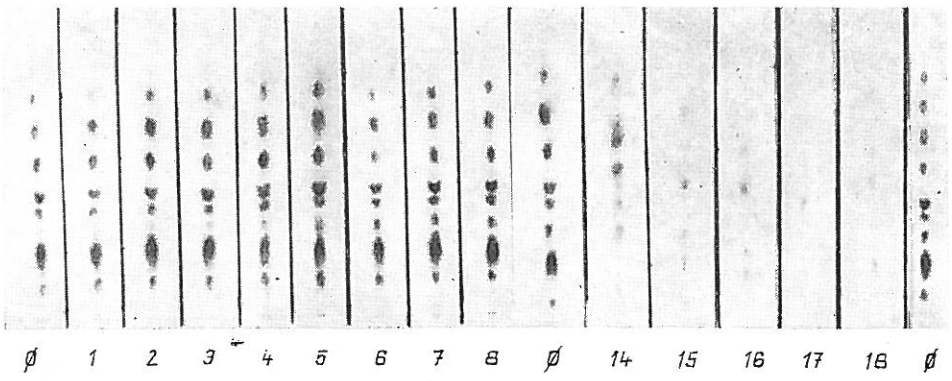

Figure 1

Free amino-acid consumption of strains of Rhizobium meliloti in the liquid culture (culture of 2 weeks)

ineffective. Qualified as ineffective were the Rhizobium strains that displayed a N-content lower than that of the control plants. This difference also manifested itself in the yield. In accordance with the results of the vegetation

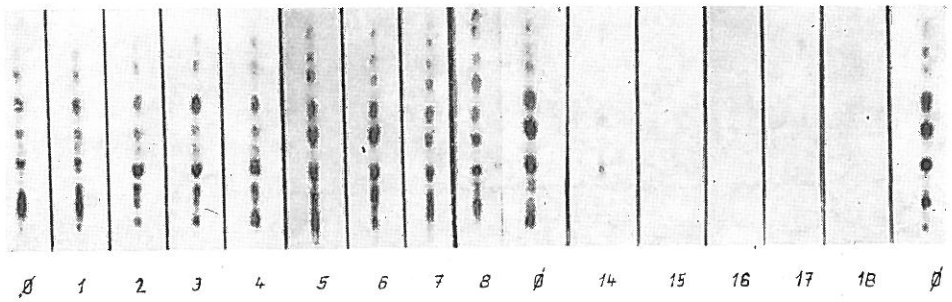

Figure 2

Free amino-acid consumption of strains of Rhizobium meliloti in the liquid culture (culture of 5 weeks)

Comparing the activity of various Rhizobium meliloti strains with a vegetation experiment and a rapid laboratory test of liquid bacterium cultures *Table 1*

Symbol of strain of Rhizobium meliloti	Vegetation experiment in test tube culture				Rapid laboratory tests in liquid culture					
					Degree of free amino-acid consumption			Measurement of turbidity		
					Paper chromatographic test		Ninhydrin reaction (culture of 2 weeks)	Subjective judgement	Nephelo-metric test	pH-value (culture of 2 weeks)
					Culture of			culture of 2 weeks		
	Green yield g/20 plants	Percentage	N mg/20 plants	Number of nodules/20 plants	2 weeks	5 weeks				
Control	1,34	100	3,50	0	+++	+++	++++	∅	0,45	6,6
1. Lu 3/67	2,06	153	6,77	45	+++	+++	++++	∅	0,20	6,5
2. Lu 2/67	2,01	150	6,01	66	+++	++++	++++	+	0,11	6,6
3. Lu 10/60	1,99	147	5,60	42	+++	+++	++++	∅	0,39	6,7
4. Lu 171/64	1,98	147	5,40	37	+++	++++	++++	∅	0,05	6,5
5. Lu B	1,89	141	5,12	45	++++	++++	++++	∅	0,37	6,7
6. Lu K_1	1,85	138	5,67	58	++++	++++	++++	+	0,34	6,8
7. Lu 5/12	1,83	136	4,94	57	+++	++++	+++	++	0,25	6,6
8. Lu 411/64	1,80	134	4,93	67	++++	++++	++	++	0,22	6,4
9. Lu 18/63	1,78	132	5,66	64	++++	++++	++	—	—	6,6
10. Lu 14/63	1,74	129	5,06	61	++++	++++	+++	++	0,05	7,0
11. Lu 1/67	1,69	126	5,18	43	++++	++++	++++	++	0,38	6,5
12. Lu 12	1,67	124	5,60	58	++++	++++	++	∅	0,28	7,0
13. Lu 12/62	1,66	123	5,32	41	+++	++++	++	∅	0,11	7,2
14. Lu 7/7	1,58	118	3,31	4	+++	++	+	+++++	0,80	8,8
15. Lu T	1,49	111	3,20	6	+	∅	∅	+++++	0,77	7,8
16. Lu 26a/67	1,57	117	2,97	0	++	+	∅	+++++	0,85	7,8
17. Lu D	1,46	108	2,64	0	++	∅	∅	+++++	0,80	8,1
18. Lu N	1,37	102	2,44	6	++	∅	∅	+++++	0,82	7,8

Paper chromatography. Amino-acid spots:
∅ = completely vanished
+ = very slightly visible
++ = slightly visible
+++ = moderately visible
++++ = complying with the control

Ninhydrin colour reaction:
∅ = completely colourless
+ = very slight violet
++ = clearly visible violet
+++ = moderate violet
++++ = very strong violet

Turbidity measurement by visual inspection:
∅ = complete clearness
+ = very slight turbidity
++ = slight turbidity
+++ = moderate turbidity
++++ = major turbidity
+++++ = very great turbidity (jelly-like state)

experiment, no change could be observed in the free amino-acid content of the liquid culture as compared to the control in the case of effective strains even after an incubation time of 5 weeks. As a contrast, the ineffective strains considerably reduced the free amino-acid content of the liquid culture already after 2 weeks, and after the 5th week they used up practically the whole quantity for their development. The chromatograms of the most effective (1 to 8) and ineffective (14 to 18) strains of *Rhizobium meliloti* taken at the end of the second and fifth week, respectively, are presented in Figures 1 and 2.

The quick colour reaction brought about in the test tube containing ninhydrin showed, like the chromatographic tests, evaluable differences in the amino-acid quantities used up by the strains even after an incubation time of 2 weeks (Figure 3).

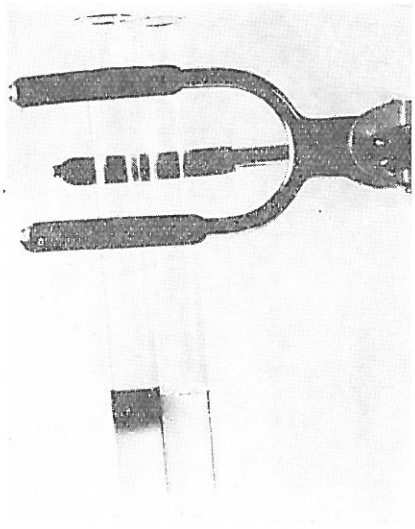

Figure 3

Detection of aminoacid by the rapid ninhydrin colour reaction. — On the left: Effective strain Lu 3/67 (1), On the right: Ineffective strain Lu N (18)

In the liquid cultures of the *Rhizobium meliloti* strains of various activity, the nutrient solution displayed different degrees of turbidity. With the effective strains a slight opalization could be observed only in some cases even after 2 weeks, while with the ineffective strains considerable turbidity presented itself after the first 48 hours and resulted in a jelly-like state after 2 weeks. The results of the visual inspection and the nephelometric analysis are given in Table 1. The fact that the results obtained by the nephelometric analysis show a considerable scattering can be explained by that, that the culture medium which became gelatinous in the case of increased turbidity could not be measured with the nephelometer with the necessary accuracy.

The pH value of the 2 week-old liquid cultures — as a function of the activity of the strains — underwent a considerable change. The change of the pH value, as compared to the control, was $\pm 0{,}1-0{,}5$ with the effective strains and $+1{,}2-2{,}2$ in the case of the ineffective ones (Table 1).

A good agreement was observed to exist between the results of the vegetation experiment and the laboratory tests of the liquid cultures. The strains of *Rhizobium meliloti* that proved to be effective and ineffective, respectively, on the basis of the vegetation experiment, showed consequently distinct differences also in the course of the laboratory tests covering the amino-acid consumption, the degree of turbidity of the cultures and the change in the pH value.

To support the above statement, strains Lu K_1, Lu 5/12 and Lu 7/7 were selected from the 18 strains of *Rhizobium meliloti* examined and a pot experiment (in semi-sterile washed quartz-sand) was conducted with them using the method described in one of our former papers [9] (Figure 4).

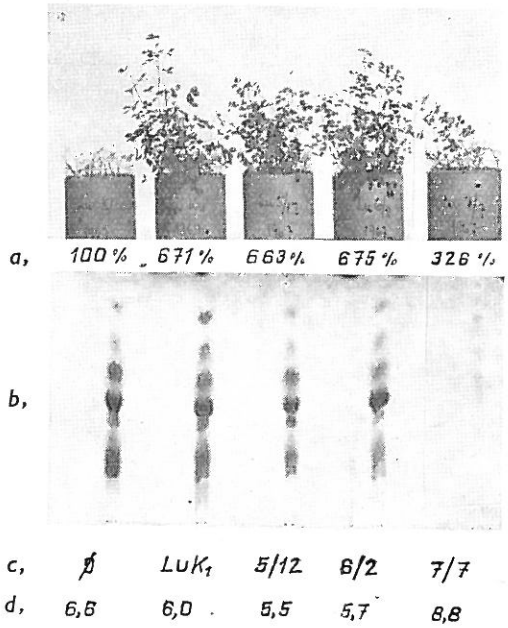

Figure 4

Determination of the effectivity of strains of Rhizobium meliloti with a sterile plant experiment and with the rapid test method. (a, Ratios of dry matter production of the plant tests, in per cent; b, amino-acid consumption; c, symbol of strains; d, change of pH value in liquid cultures)

The results obtained in this test confirmed the data supplied by the plant experiment carried out in the test tube containing nutrient agar. In the laboratory tests conducted with the strains that were found to be effective in the plant experiment no change occurred in the free amino-acid contents of the liquid cultures, and the pH values were between 5,5—6,0 while in the case of the slightly active strain the free amino-acid spots of the liquid culture disappeared almost entirely and the pH value was as high as 8,8.

According to the analytical results the determination of the effectivity of Rhizobium strains made with rapid laboratory methods allows the number of strains involved in the vegetation experiment to be reduced essentially.

With the laboratory method the ineffective strains can be sorted out with full certainty on the basis of the amino-acid consumption, the degree of turbidity and the pH value.

With the highly active and the ineffective strains, respectively, dependable results can be obtained even by applying only one test method, while with the strains of medium activity the results yielded by the three methods are not consequently unequivocal so the simultaneous application of the three methods is advisable. In this way not only the strains with the two extreme values can be selected, but also statements concerning strains of medium activity can be made.

Consequently, with the rapid laboratory test the Rhizobium strains can be selected in maximum two weeks, after a preliminary test consisting of the amino-acid detection, the rapid ninhydrin colour reaction, the subjective determination of the degree of turbidity by visual inspection and the determination of the pH value was performed.

Summary

A rapid laboratory test method has been developed for the selection of a great number of Rhizobium strains. With this method the number of the Rhizobium strains involved in plant experiments can be considerably reduced.

It was found that there was a good agreement between the results obtained in the vegetation experiment carried out to determine the effectivity of the strains of *Rhizobium meliloti* and those supplied by the rapid laboratory test.

With the rapid laboratory test the preliminary selection of the strains of *Rhizobium meliloti* can be performed in two weeks on the basis of the consequent differences presenting themselves in the amino-acid consumption of the strains in the liquid culture, the degree of turbidity of the cultures and the change in the pH value.

As regards the effective strains of *Rhizobium meliloti*, no free amino-acid consumption takes place in the liquid culture, this latter does not become turbid but remains clear and the pH value does not exceed 7,2.

As to the ineffective strains of *Rhizobium meliloti* a large quantity of the free amino-acid is used up in the liquid culture, this latter becomes considerably turbid and jelly-like and the pH value is above 7,2.

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