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VARIATION WITHIN STRAINS OF CLOVER NODULE BACTERIA IN THE SIZE OF NODULE PRODUCED AND IN THE "EFFECTIVITY" OF THE SYMBIOSIS

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Strains of *Rhizobium* may be distinguished in their behavior in symbiosis by their ability to infect a given host plant, as well as by the types and numbers of nodules produced and by the nodules' effectiveness in benefiting the host through nitrogen fixation. Allen and Baldwin (1931) have reviewed the changes which strains of *Rhizobium* have been observed to undergo, and the more important of these may be summarized. Nobbe and Hiltner (1893) and Frank (1899) claimed that growth of *Rhizobium* on gelatin media could induce ineffectivity in nitrogen fixation in the nodules or even complete loss of ability to infect the roots. Other workers have claimed that cultivation on nitrogen-rich media leads to a reduction in effectivity and vice versa, and Simon (1908), Snieszko (1929), and Hutchinson (1924) present evidence that culture in soil restores lost virulence and effectivity. Finally, Wünschik (1925) and Allen and Baldwin (1931) claimed that repeated plant passage led to changes in the effectivity of strains, although neither Stapp (1929) nor Virtanen (1945) was able to confirm Wünschik's results.

In all these cases it is not clear whether the observed changes were due to the selective action of the treatment on an already heterogeneous population or whether new variants had appeared during the experiments. The gradual changes often observed could thus be attributed either (1) to the selective increase of a small number of cells originally present in the culture, and bearing the newly observed character, (2) to the development of a few variants that subsequently increased differentially, or (3) to a general change occurring throughout the bacterial population. Further, the majority of tests were made as pot experiments, and under these conditions the possibility of contamination cannot be excluded.

The object of the present work was to study, with pure culture technique, the effects of plant passage and of various cultural environments in inducing variation in a population of *Rhizobium* known to be homogeneous at the commencement of the tests.

MEDIA AND METHODS

All stock cultures were maintained on a yeast-water agar medium and subcultured at about two-month intervals. The medium used had the following composition: mannitol, 1.00 per cent; K_2HPO_4, 0.05 per cent; $MgSO_4$, 0.02 per cent; NaCl, 0.02 per cent; CaCl, 0.02 per cent; $FeCl_3$, 0.001 per cent; 10 per cent aq. yeast extract, 10 per cent; agar 1.5 per cent.

Throughout the experiments late-flowering Montgomeryshire red clover was used as the test plant. The seed was sterilized externally before planting by being washed for 3 minutes in 80 per cent alcohol, then for 3 minutes in 0.2 per cent mercuric chloride, followed by frequent washings with sterile water over a period of some hours. The sterilized seed was transferred with a wire loop to the surface of a test tube slope of "seedling agar" of the following composition: K_2HPO_4, 0.05 per cent; $MgSO_47H_2O$, 0.02 per cent; NaCl, 0.01 per cent; $Ca_3(PO_4)_2$, 0.2 per cent; $FePO_4$, 0.1 per cent; $FeCl_3$, 0.001 per cent; agar, 1.2 per cent. The tubes containing the clover plants were kept in racks in a glasshouse and partially shaded on cloudless days. Development was best in conditions of good light but not under direct sunlight; the roots were shaded.

The plant cultures were allowed to grow under these conditions for 3 months or longer, depending on the time of year and the object of the experiment. At harvest, observations were made of the general appearance of each plant, whether large and dark green or small and pale yellow-green, and the length of all the nodules on the roots were determined; in some experiments dry weight and total nitrogen were also determined. Isolations of substrains from nodules were made from single colonies picked from platings of the contents of single nodules. Before plating, each nodule used for isolation was sterilized externally with alcohol and mercuric chloride as described for seed sterilization.

ORIGIN OF STRAINS A AND H.K.C.

Two original strains of *Rhizobium trifolii* were chosen for this study; an effective strain, strain A, obtained from Professor Bartel of Stockholm (referred to in some previous publications as strain "Bart. A"), and a local ineffective strain named H.K.C. Before the present investigation was commenced in 1939, the stock cultures of both strains and their early derivatives were under the observation of Dr. H. K. Chen. They were used frequently and for several years in studies in strain competition, effectivity, etc.; they were frequently plated, and during this time no changes in effectivity were observed.

In the course of the work rigid bacteriological control excluded contamination by other nodule bacteria, but at intervals the identity of each strain was checked serologically by Dr. A. Kleczkowski. A large number of substrains derived by plant passage from strain A were examined. These were named systematically on isolation, the name of each being derived from the parent strain by the addition of a digit. Thus substrains A151 and A152 were derived from substrain A15, which in turn was derived from substrain A1, which in turn was derived from strain A. It is realized that "substrain" may be regarded as too high ranking a term to distinguish reisolations which were in most cases indistinguishable, but it is retained for clarity.

The complete scheme of isolations made in the course of the first series of experiments on the stability of strain A is shown in figure 1, in which each circle represents a nodule produced by, and containing, the substrain numbered below it, and in which the diagrammatic section of a petri dish represents the selection of that substrain from a single colony. Thus the lowest circle represents a

nodule containing the original strain A, the contents of which were plated in December, 1938, yielding substrain A1. Six nodules containing this substrain were plated at different times, in different experiments, and from these six platings substrains A11 to A16 were derived. When a number of colonies were picked from the plating of the same nodule, such replicate isolations were all given the same substrain number.

THE STABILITY OF STRAIN A WITH RESPECT TO SIZE OF NODULES PRODUCED

The difference between effective and ineffective strains has been elucidated by Chen and Thornton (1940), who showed that, in the clover, pea, and soybean

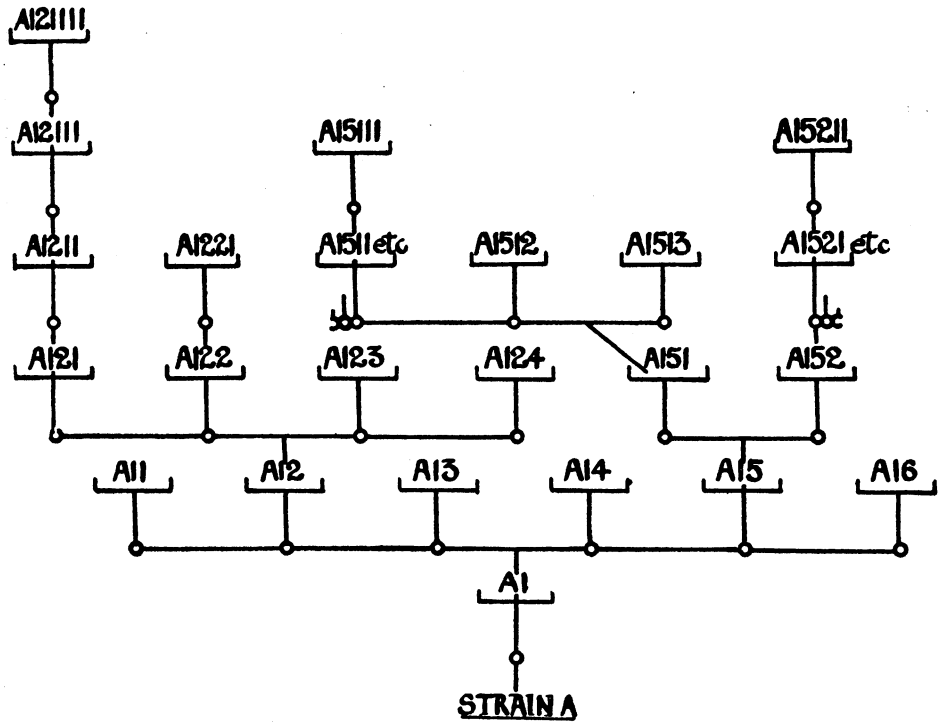

FIG. 1. RELATIONSHIP BETWEEN SUBSTRAINS DERIVED BY PLANT PASSAGE FROM STRAIN A

cross-inoculation groups, the effectivity of a strain does not depend on the efficiency of the nitrogen fixation process itself, but is a function of the mass and duration of the active bacterial tissue of the nodule. It was observed that clover inoculated with the effective strain A had relatively few nodules, ranging widely in size, whereas plants inoculated with the ineffective strain, Coryn, produced a large number of nodules all of which were small and which soon degenerated. Both strains fixed nitrogen at the same rate per unit mass of active bacterial tissue.

The great majority of clover strains conform in their nodulation broadly to the two types which were described by Chen and Thornton, and which are repre-

sented by the strains A and Coryn. Such a generalization does not hold for all strains under all conditions; not only do intermediate strains occur, but under some circumstances relatively large nodules are formed, which are nevertheless ineffective because the bacterial tissue of the nodule is very ephemeral. But any strain, whether or not it conforms to the A or Coryn type, has a characteristic distribution of nodule length when tested on a sufficient number of plants, and a departure from this would almost certainly be reflected in differences in effectivity. The distribution of nodule length was here used as a measure of change in effectivity, not only because it can be based on exact and well-replicated measurements, but also because this criterion has an intrinsic interest quite apart from its relation to effectivity.

Homogeneity tests of principal substrains in respect to nodule length. Studies in variation are apt to be invalidated by failure to ensure that the material under examination is homogeneous; in this instance, a bacteriologically pure line. The early plant passage substrains were therefore tested for their homogeneity in behavior in regard to size of nodules produced.

In the first two experiments the substrains A13 and A15, also A122 and A124, were tested and compared. The population of each was sampled by the plating and picking of about 30 random colonies, each colony being used to inoculate a pair of clover plants grown on an agar slope in a test tube. Each tube was planted with two clover seeds, though sometimes only one plant grew. At harvest a complete record of the nodule length distribution of each plant was made, from which was obtained the mean length of the nodules within each culture tube, i.e., produced from one picked colony. Similarly the average size of nodule produced by a substrain could be calculated as the mean length of all nodules produced by all the colonies picked from a plated nodule containing that substrain.

It was at once evident from an examination of the data of these experiments that the number of nodules and the distribution of nodule size varied greatly from plant to plant, and that the significance of small differences between substrains and between colonies was difficult to assess by simple comparison of mean values, so that recourse was had to statistical analysis. In each experiment independent comparisons were made between the mean nodule lengths in replicate tubes, each infected from a different colony, and between those of individual plants infected from the same colony. These comparisons are illustrated in figure 2. By this means the variance of nodule length within plants, i.e., between nodules on the same plant, was used to determine the significance of differences between duplicate plants in the same tubes; that between plants was utilized for testing differences between colonies, i.e., tubes; and the colony variance was used as an estimate for error in determining the significance of differences between substrains. The variances thus obtained in the second of these first two experiments and in some of the later experiments are given in table 1. In experiment 1, nodules containing substrains A13 and A15 were plated, and 24 random colonies of each substrain were tested on plants. These tests showed no significant difference in mean nodule lengths produced by the two substrains (8.58 ± 0.20,

8.27 ± 0.13 mm per 10, respectively), but complete analysis could not be made here as the individual plant results were not kept separate. In experiment 2, a similar comparison was made between substrains A122 and A124. Here, however, a complete analysis of variance was made, and it showed a significant difference only between duplicate plants in the same tube. The two substrains were similar, and each was homogeneous, no significant difference in behavior showing between isolations from the 69 colonies tested.

This and all later analyses emphasized that there was a significant difference between the nodule length distributions of individual plants in the same tube. This difference may have been due to factors inherent in the plant affecting response to homogeneous bacterial population, or to the development of bacterial

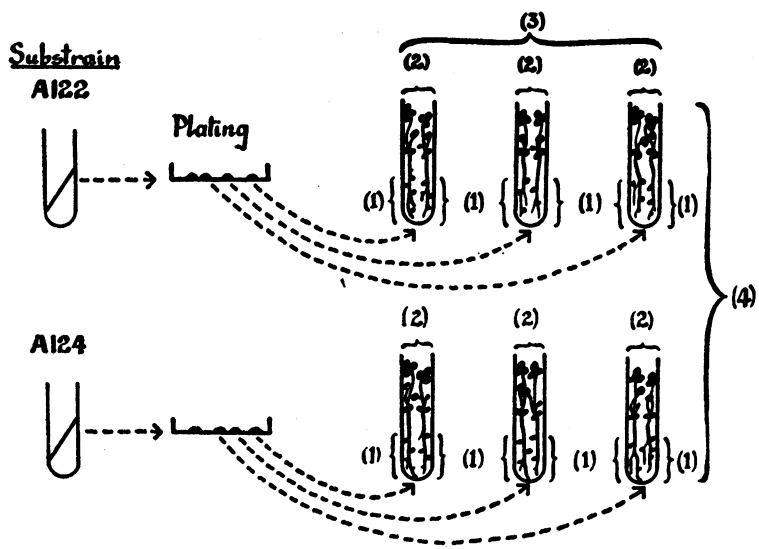

FIG. 2. COMPARISONS MADE IN THE ANALYSIS OF VARIANCE
(1) Variance within plants.
(2) Variance between plants in the same tube.
(3) Variance between colonies (i.e., tubes).
(4) Variance between substrains.

variants in or immediately around the individual plant, or to the selective infection of that plant by bacteria differing from the majority in the mean nodule length which they produce. On the last two views it should be possible to modify the mean nodule length by selection from large and small nodules, respectively, or from plants showing abnormal nodule length distributions. The effect of such selection was therefore tested.

Nodule selection experiments. Two plants infected with substrain A15 were chosen for the first test, experiment 3 (table 1). From the first plant having nodules of a mean length of 1.09 mm, a nodule 1.7 mm long was selected and plated, and from this plating 35 random colonies were picked (substrain A151), and each was separately tested on clover. The second plant bore nodules with

a mean length of 0.59 mm, from which a nodule of 0.5 mm was plated. Thirty-eight colonies were picked (substrain A152) and each was tested on clover. Analysis of variance showed no significant difference between the mean nodule lengths produced by the substrains isolated from the large and the small nodule, respectively. The comparison of replicate colonies showed that the bacterial contents of each nodule from which the substrains were isolated were also homogeneous (table 1, experiment 3).

In the second test (experiment 4), isolations were made from nodules on plants in experiment 3. Seven plants, each having few and large nodules produced by substrain A151, were selected, and from these 11 nodules ranging in length from 1.7 to 2.6 mm in length were plated. Thirty-one random colonies were picked from these plates and tested on clover (substrains A1511, A1512, etc). Two plants having many small nodules produced by substrain A152 were also selected, and from these plants 12 nodules 0.6 to 0.7 mm long were taken from the older roots, to avoid using young nodules that may later have grown larger. These nodules were plated and 27 colonies tested (substrains A1521, A1522, etc.). The mean nodule length produced by isolations from the large nodules was 0.845 mm; that produced by isolations from the small nodules was 0.802 mm. Analysis showed that these means were not significantly different, but the variance between isolates from different nodules on plants inoculated with the same substrain was less than that between replicate colony isolates from the same nodule. Thus, selection over two plant passages of large and small nodules borne on plants bearing, respectively, few large and many small nodules failed to alter the mean nodule length produced by the selected substrains.

However, the mean nodule lengths produced by replicate *colonies* from the same nodule in experiment 4 did show a variance significantly exceeding that between duplicate plants in the same tubes (table 1). This was attributable to two tubes in which both plants bore unusually large nodules. The colonies from which these tubes were inoculated came from different nodules inoculated with substrain A151, and the remaining colonies from the same two nodules produced nodules of normal length. Nevertheless, a large nodule from one of these tubes was plated, and 36 random colonies obtained from it were compared with 33 random colonies from a plating of substrain A1221, whose parent substrain, A122, had been shown in experiment 2 to produce a normal nodule length distribution. The isolates from this large nodule (substrain A15111) produced a mean nodule length of 1.079 $\pm$ 0.031 mm, which was not significantly different from that produced by substrain A1221, i.e., 1.107 $\pm$ 0.017 mm. There was thus no evidence that the abnormality in the two tubes of experiment 4 was due to any difference in the bacteria.

Similar negative results were also obtained in experiment 6, in which the contents of an abnormally large nodule containing substrain A1211 were plated and 35 colony isolates (A12111) compared in nodule formation with 35 from the ancestral substrain A1 (table 1).

The selection from large and small nodules, described above, took no account

TABLE 1

Analysis of variance of nodule length

EXPERIMENT	STRAINS	MEAN NODULE LENGTH (in 0.1 mm)	SOURCE OF VARIANCE	D.F.	SUMS OF SQUARES	MEAN SQUARE	F
2	A122	10.38	Between substrains	1	12.13	12.13	0.14
	A124	10.57	Between colonies	67	5,727.25	85.48	1.36
			Between plants	79	4,963.54	62.83	4.72 xxx
			Within plants	1,092	14,549.60	13.32	
Total				1,239	25,252.52	20.38	
6	A1	8.37	Between substrains	1	22.91	22.91	0.50
	A12111	8.13	Between colonies	60	2,746.34	45.77	1.75 x
			Between plants	62	1,617.78	26.09	3.03 xxx
			Within plants	1,397	12,029.39	8.61	
Total				1,520	16,416.42	10.80	
3	A151	8.06	Between substrains	1	16.76	16.76	0.59
	A152	7.89	Between colonies	71	2,021.58	28.47	0.99
			Between plants	100	2,879.99	28.80	3.59 xxx
			Within plants	1,961	15,731.57	8.02	
Total				2,133	20,649.90	9.68	
4	A1511 etc	8.45	Between A151 and A152 lines	1	73.81	73.81	2.57
	A1521 etc	8.02	Between nodule isolates sister substrains	21	603.13	28.72	0.19
			Between colonies	35	5,344.87	152.42	5.81 xxx
			Between plants	58	1,520.78	26.22	3.42 xxx
			Within plants	1,445	11,089.73	7.67	
Total				1,560	18,622.32	11.94	
10	A121111	10.50	Between substrains	1	253.60	253.60	0.81
	A121111W	9.58	Between colonies and between plants	28	8,738.73	312.09	20.25 xxx
			Within plants	1,193	18,378.83	15.41	
Total				1,222	27,371.16	22.40	
11	A121111	10.67	Between substrains	1	1,878.20	1,878.20	343.36 xxx
	A121111W[1]	7.43	Between colonies	16	87.51	5.47	0.04
			Between plants	6	898.38	149.73	55.66 xxx
			Within plants	371	998.09	2.69	
Total				394	3,862.18	9.80	

of the degree of benefit shown by the plant; the plants from which the small nodules were selected did bear evidence of some nitrogen fixation. Occasionally, however, a plant was observed which showed the dwarf size, etiolation, red petioles, and the many small nodules characteristically produced by an ineffective bacterial strain. In one typical instance the mean nodule lengths of the aberrant and of the remaining normal plants in an experiment were found to be 0.533 $\pm$ 0.009 mm and 0.840 $\pm$ 0.010 mm, and in a second case 0.584 $\pm$ 0.010 mm and 1.124 $\pm$ 0.014 mm. In these and in other cases plants showing effective and ineffective responses appeared together in the same tube. From the first of these ineffective plants 34 colony isolations (substrain A16) were compared with the parent substrain A1 and with isolations from a large nodule (substrain A121111). The mean nodule lengths produced by each substrain were: A16, 1.045 $\pm$ 0.014 mm; A1, 1.025 $\pm$ 0.031 mm; and A121111, 1.092 $\pm$ 0.019 mm. They are not significantly different.

From the second ineffective plant infected with substrain A151, 20 colony isolations (substrain A1512) were compared with 20 from a large nodule on a normal plant (substrain A1513). The mean nodule lengths for A1512 were 1.108 $\pm$ 0.026 mm, and for A1513, 1.081 $\pm$ 0.026. In both these experiments, therefore, the bacteria isolated from the plants that showed ineffective responses produced the mean nodule size and type of response typical of an effective bacterial substrain.

From these results it was concluded that the substrains derived from strain A since 1939 were very stable with respect to effectivity response and size of nodules. During the experiments outlined above 28 plant passage substrains were examined, involving the testing of about 600 colonies, the response of about 1,200 plants, and the measurement of more than 16,000 nodules. In this extensive series of tests no persistent change in the bacteria with respect to mean nodule length appeared, either during normal growth in the plant or as a result of deliberate selection.

Plant passage. The experiments summarized in figure 1 yielded some data on the absence of an influence of plant passage on effectivity, since most of these comparisons were made between substrains which had been pased through the plant for different numbers of times before testing. In these experiments the effects of 2, 4, 5 plant passages were tested, and in no case was any effect evident, a result contrary to the reports of Allen and Baldwin (1931), who described changes from effectivity to ineffectivity, and vice versa, on plant passage. It was thought that differences in experimentation might have been responsible for this disagreement, in that each plant passage culture was made in these experiments from a colony on a plating of the contents of a single nodule, so that an infrequent dissociative change would almost certainly have been overlooked. To test this possibility further experiments were undertaken using the stable effective substrain A121111 and the ineffective strain H.K.C., in an endeavor to follow Allen and Baldwin's technique and in addition to compare the effects of intervening plating and agar culture. The substrain A121111 was selected, not only because of its stability under laboratory conditions, but also because

there was no doubt of its bacteriological purity. The strain H.K.C. had not undergone so rigorous an examination as A121111 but had been under observation in the laboratory for a considerable time and was not known to have produced any forms differing in effectivity.

The transfers were conducted under sterile conditions using the agar tube technique of plant culture, and the isolates were tested in controlled test tube experiments. The series of plant passage transfers was begun in November, 1940, by inoculating seedling agar slopes, planted with red clover, with the two

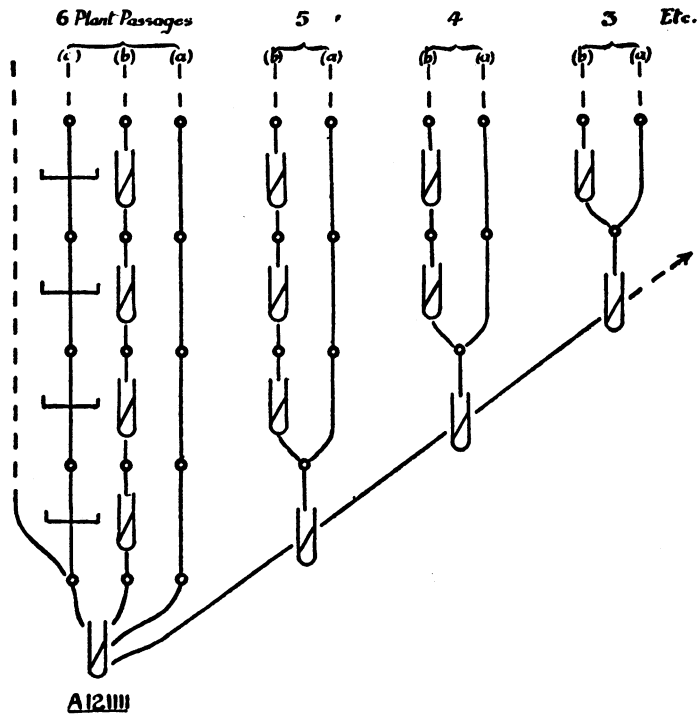

FIG. 3. SCHEME OF ISOLATION, PLATING, AND SUBCULTURE FOLLOWED IN PLANT PASSAGE EXPERIMENTS

strains. The scheme of isolations then followed will be made clear by reference to figure 3.

Three methods of plant passage were employed. (a) Six random nodules from the previous plant passage were crushed in saline, and the resulting suspension was used directly to infect the next plant passage. (b) A suspension from six crushed nodules was grown on an agar slope, incubated for 1 week, and then used as inoculum. (c) A similar nodule suspension was plated and a colony isolation used as inoculum. At each plant passage, fresh series using methods (a) and (b) were started from the stock cultures and continued to the end of the experiment. Thus, by September, 1941, substrains had been obtained that had been passed through the plant from 1 to 6 times by methods (a) and (b), including

one that had been passed 6 times with a plating between each passage. In addition, an isolation was made in September, 1941, from the original plant inoculated with the stock culture of A121111, which had been kept since November, 1940. This scheme was designed to test whether changes in the bacteria were produced by plant passage and whether these were lost during growth in agar or through the effects of plating.

The final isolates were each tested in 4 replicate agar tube cultures, each of which contained from 1 to 3 plants. After a period of 5 months of growth the nodules were measured as in previous experiments of this type, and, in addition, the dry weights of the individual plants and the total nitrogen content of half of each treatment (2 replicates) were determined. The results summarized in table 2 show no significant difference between the treatments. The control tubes remained without nodules.

In view of these negative results a further plant passage experiment was conducted; this was a continuation of the one described above in that the isolation of further plant passage inocula was made from the agar experiment just described. The number of plant passages was extended to eight, but final tests were only made on the fourth and eighth plant passage, and on the stock culture. The final effectivity test consisted of 10 replicate test tube cultures of seedling agar medium for each treatment, each tube planted singly with red clover. The seeds were sown in June and the experiment was concluded in October. The results are shown in table 2. Again no significant effects were produced by any of the methods of plant passage either as regards nodule length, plant dry weight, or nitrogen fixation. The culture obtained after 8 plant passages with intervening plating did not differ significantly in nodule length produced from the stock substrain A121111, which itself, after 5 previous similar passages, had been found not to differ significantly from its parent substrain A1. Thus 13 passages with intervening platings failed to alter the strain in respect to the character measured.

The negative results obtained by three different methods of plant passage make it difficult to explain the discrepancy between these results and those of Allen and Baldwin (1931) on grounds of technique. The difference may be in the greater homogeneity of the bacterial strains used in the present work. The evidence here presented shows that an originally homogeneous strain of *Rhizobium* does not undergo modification in its symbiotic behavior on repeated plant passage.

EFFECT OF STORING SUBSTRAIN A121111 IN SOIL

Appearance of an ineffective variant. The first part of this work provided no evidence that either plant passage or nodule selection could modify a genetically uniform strain, but it did provide, in the later plant passage derivatives of strain A, some material most thoroughly tested as to genetical homogeneity. It was decided to test the stability of this strain outside the plant. The first series of tests were made after storing substrain A121111 in Woburn soil.

Woburn sandy soil passed through a 0.5-mm sieve was distributed in 60-mg amounts in large (2-inch diameter) test tubes, which were plugged and sterilized

TABLE 2

Test of plant passage by three methods

METHOD OF PASSAGE	NUMBER OF PLANT PASSAGES	MEAN NODULE NUMBERS PER PLANT	MEAN NODULE LENGTHS PER PLANT	MEAN DRY WEIGHT PER PLANT	MG N PER PLANT
Test made Sept., 1941, to Feb., 1942. Substrain A121111					
			10/mm	*mg*	
(a) Direct plant passage	(Stock) 0	15.3	12.2	30.3	1.26
	2	26.6 ±7.90	9.4 ±0.98	30.3	1.25
	3	20.8 ±4.30	10.1	30.0	0.93
	4	13.9	11.0	29.1	1.00
	5	13.6	11.3	26.4	0.85
	6	14.4	14.3 ±2.05	35.7 ±4.16	1.19
(b) Agar slope	2	21.0	11.4	33.6	1.05
	4	13.2 ±2.74	11.0	25.6 ±4.28	0.80
	6	17.6	9.5	27.6	0.86
(c) Plating	6	13.2	13.1	29.9	0.92
Test made June to October, 1942. Substrain A121111					
(a) Direct plant passage	(Stock) 0	49.2 ± 6.3		85.76 ±11.6	2.23
	4	60.0		100.70	2.47
	8	50.8		105.96	2.66
(b) Agar slope	8	70.8 ±10.2		88.0	2.34
(c) Plating	8	56.7		110.84	2.86
Test made Sept., 1941, to February, 1942. Strain H.K.C.					
(a) Direct plant passage	(Stock) 0	41.7	9.0	8.85	0.17
	2	38.8	8.7 ±0.54	9.21	0.16
	4	42.4	9.8	8.60 ±1.98	0.16
	6	43.6 ±6.1	8.7 ±1.68	9.48	0.18
(b) Agar slope	2	42.6	8.7 ± 0.55	8.58	0.17
	6	40.6	9.4	8.88	0.16
(c) Plating	6	36.3 ±4.4	10.0 ±0.50	9.75 ±0.87	0.16
Test made June to October, 1942. Strain H.K.C.					
(a) Direct plant passage	(Stock) 0	103.6		30.42 ±1.99	0.42
	4	failed			
	8	failed			
(b) Agar slope	8	142.5 ±14.5		42.30 ±3.12	0.50 ±0.04
(c) Plating	8	102.7 ±13.6		33.04 ±3.93	0.44

in an autoclave by heating to 15 pounds for 1 hour on 2 successive days. Three of the tubes were given equal quantities of a thick suspension in sterile water of strain A121111 taken from a yeast agar slope, and three similar tubes of sterilized soil were kept without inoculation. These remained sterile. The tubes with soil were stored at room temperature in the dark for 6 months, by which time the soil had become dry and crumbly. Platings of the three soil samples were then made on yeast-water agar. The colonies that developed had an appearance similar to those typical of substrain A121111, and all appeared alike.

TABLE 3

Effectivity response and average nodule length of plants inoculated with isolations from soil cultures of substrain A121111

Test made May to July, 1941

Colonies showing effective response are placed above the line in the table and those showing ineffective response below

REPLICATE COLONY DESIGNATION	SOIL CULTURE NO. 1	SOIL CULTURE NO. 2	SOIL CULTURE NO. 3
	Average nodule length per plant	Average nodule length per plant	Average nodule length per plant
a	9.99	15.56	14.50
b	11.73	14.96	10.06
c	11.26	13.26	12.80
d	14.43	17.20	16.47
e	9.70	6.27	13.23
f	13.19	5.83	10.39
g	13.29	6.35	14.44
h	10.00	5.34	11.14
i	14.92	6.73	8.13
j	8.69	6.71	11.46
k	15.15	6.03	11.13
l	13.20	6.11	13.48
m	7.66	6.64	15.90
n	6.29	6.34	14.68
o	6.26	5.63	16.86
p			11.93
q			5.55

The average number of nodules on plants showing an effective response was 11.98.
The average number of nodules on plants showing an ineffective response was 55.48.

About 15 colonies derived from each soil sample were selected at random from each plate, and from each colony a loopful of bacterial growth was transferred to a tube containing red clover seedlings grown aseptically on the usual nitrogen-deficient agar medium. These tests on clover were kept from May to July, by which time the plants could be classified readily by eye as showing either an effective or an ineffective response. The plants fell into two clearly separate groups. Measurements of the nodules were made and these confirmed the clear-cut separation. Table 3 gives the mean nodule length found on plants

inoculated from every tested colony, the values below the horizontal line in each column referring to tubes showing ineffective responses. It is evident that clear-cut dissociation had taken place in the substrain A121111 during soil storage, giving rise to markedly ineffective variants. These appeared in all three tubes, though different proportions of variant to parent were found in each. Tests were then made of the contents of nodules on these plants. Subsequent investigation was most thorough in the case of soil culture no. 2, and description, illustrated by figure 4, will be confined to substrains from this soil, although data from soil cultures no. 1 and no. 3, as far as they went, confirmed the general conclusions.

Figure 4 shows, diagrammatically, the plant passage tests made with colony isolates from soil culture no. 2. A circle again represents the selection of a

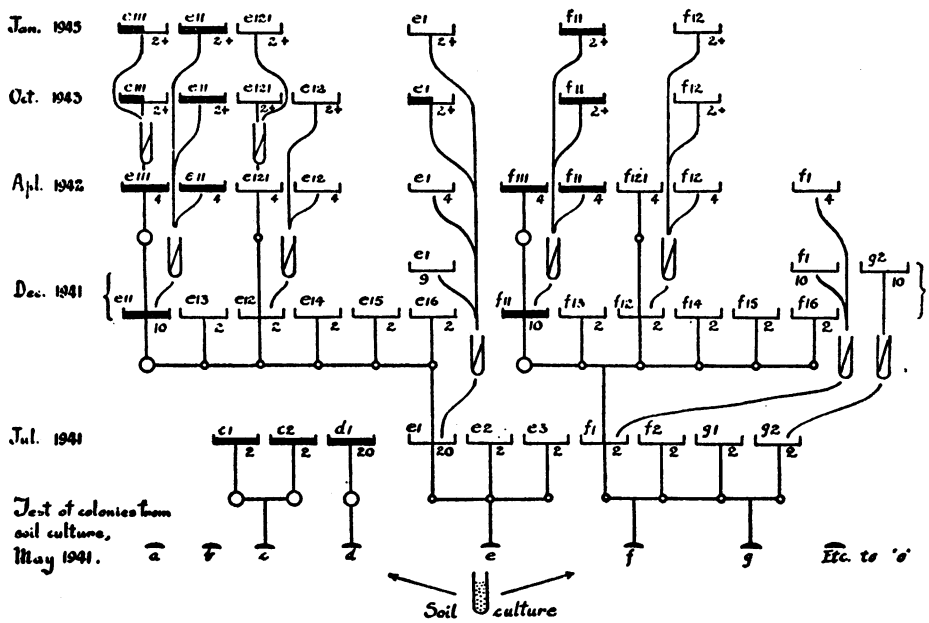

FIG. 4. THE DERIVATION OF SUBSTRAINS FROM A121111 AFTER STORAGE IN SOIL

nodule for plating, and the diagrammatic section of a petri dish indicates a test of replicate random colonies from a plating, the number of colonies tested being shown below. A thick base to the petri dish indicates that all the colonies tested gave effective responses, and a half-thick base, that the colonies differed in this respect. The designations of substrains bear the letter showing the soil culture colony from which they came, the numerals showing by the number of integers the number of subsequent plant passages. A diagrammatic agar slope indicates a stock culture.

Of the 15 colonies from soil no. 2 that were tested, 4, labeled *a* to *d*, produced effective nodules. Two nodules derived from colony *c* were plated, and 2 colonies from each nodule plating were tested, each on duplicate tubes of clover (table 4). One nodule from the colony *d* was also plated, and 20 colonies from this plating

were separately tested on clover; all these 24 colonies gave effective responses. The remaining 11 colonies from soil produced ineffective responses on the plants tested with them and also gave nodule length distributions that were typical of ineffective strains. Two nodules from plants inoculated from each colony from *e* to *m* were plated, and from each plating two colonies were picked and tested on clover (figure 4). An additional nodule from the tube inoculated with colony *e* was plated, and 20 colonies from this plating were separately tested on clover. Every one of these 54 colonies derived from 18 ineffective nodules gave a typical ineffective response when tested on the plant. These rather

TABLE 4

Tests of colony isolates from nodules of the first plant passage from soil culture no. 2 of substrain A121111

Test made July to November, 1941

SUBSTRAIN TESTED	NUMBER OF COLONIES PICKED AND TESTED	EFFECTIVITY	MEAN NODULE LENGTH PER PLANT	±
c1	2	E	10.97	0.90
c2	2	E	11.08	0.67
d1	20	E	11.43	0.58
e2	2	I	6.23	0.37
e3	2	I	5.52	0.02
e1	20	I	6.04	0.32
h1	2	I	5.71	0.16
i1	2	I	5.62	0.07
i2	2	I	5.57	0.24
j1	2	I	6.36	0.06
j2	2	I	5.85	0.15
k1	2	I	5.65	0.11
f2	2	I	5.79	0.54
f1	2	I	5.83	0.07
g2	2	I	6.14	0.46
g1	2	I	5.89	0.19
k2	2	I	5.90	0.08
l1	2	I	5.69	0.25
l2	2	I	6.93	0.51
m1	2	I	5.69	0.11
m2	2	I	5.56	0.21

extensive tests thus gave no indication of any mixture of effective parent and ineffective variant, either in the original colonies from soil or in the nodules produced when these original colonies were tested.

Serological identity of parent and ineffective variant. Subcultures made from the 54 colonies mentioned above, as well as from 16 colonies isolated from effective and ineffective plants inoculated from soils no. 1 and no. 3, were all tested serologically by Dr. A. Kleczkowski, who used an antiserum obtained against the original strain A. Equivalent agglutination occurred to a titer of 1:1,600 with all colonies, and with the strains A and A121111 used as controls. No agglutination occurred in any saline control. Cross-adsorption tests were also made

with the original effective strain and the ineffective dissociant, and no detectable differences were found. Full details of these experiments have been published (Kleczkowski and Thornton, 1944). These tests, together with the sterility of the original control soil cultures, completely excluded the possibility that the ineffective variant was a contaminant.

TABLE 5

Test of replated substrains from the first plant passage and of nodule isolates from the second plant passage after storing substrain A121111 in soil

Tests made December, 1941, to March 1942

SUBSTRAIN	NUMBER OF COLONIES TESTED	MEAN NODULE LENGTH PER PLANT	±	OBSERVED EFFECTIVITY
Tested Dec., 1941				
First passage Substrain (stock)				
*e*1	9	6.21	0.186	All ineffective
*f*1	10	6.79	0.158	All ineffective
*g*1	10	6.26	0.115	All ineffective
Second passage Nodule isolate				
*e*11	10	8.94	0.381	All effective
*e*12	2	5.85	0.070	All ineffective
*e*13	2	6.32	0.089	All ineffective
*e*14	2	6.79	0.708	All ineffective
*e*15	2	5.92	0.240	All ineffective
*e*16	2	5.97	0.290	All ineffective
*f*11	10	8.38	0.985	All effective
*f*12	2	6.57	0.195	All ineffective
*f*13	2	6.07	0.195	All ineffective
*f*14	2	6.36	0.220	All ineffective
*f*15	2	6.31	0.046	All ineffective
*f*16	2	5.84	0.261	All ineffective
Tests made April, 1942, to July, 1942				
First and second passage Substrains (stock)				
*e*1	4	6.57	0.29	Ineffective
*e*11	4	11.70	1.36	Effective
*e*12	4	6.49	0.19	Ineffective
Third passage Nodule isolates				
*e*111	4	11.53	0.98	Effective
*e*121	4	6.97	0.99	Ineffective

Instability of the ineffective variant. In the two tests of the soil derivatives so far described the division into effective and ineffective types also showed as a clear distinction in the average nodule length per plant, but one plant infected with substrain *e*1 and one infected with *f*1 each bore one large nodule (2.9 mm and 2.4 mm in length) as well as numerous small nodules, with none of inter-

mediate size (figure 5). From both of these plants, platings were made from the large nodule and from five of the small nodules (figure 4). Twenty random colonies from each large nodule and two colonies from each small nodule were tested (substrains *e*11 to *e*16, and *f*11 to *f*16). Colonies from plated stock cultures of the parent substrains *e*1 and *f*1 were tested at the same time. The results are summarized in table 5. The parent substrains and all those derived from the small nodules (*e*12 to *e*16 and *f*12 to *f*16) produced ineffective responses. All the 20 colonies derived from the two large nodules produced effective responses (*e*11 and *f*11). A further test was then made by plating a large nodule containing substrain *e*11 and a small nodule containing substrain *e*12. Four colonies were tested from each plate (*e*111 and *e*121) as well as from stock cultures of the parental substrains. This test (table 5) confirmed the purity of both the large and the small nodule derivatives, both in regard to effectivenes and size of nodules produced. Nevertheless, when stock cultures of these five *e* substrains were retested in October, 1943, after a year's cultivation *in vitro*, substrain *e*111, originally effective, and *e*1, originally ineffective, then produced, on plating, mixtures of effective and ineffective colonies (figure 4). After a further long interval a test begun in January, 1945, using stock culture substrains of *e*1, *e*11, *e*111, and *e*121, as well as *f*11 and *f*12, confirmed these results by showing the liability of the variant substrains to revert in agar culture (figure 4).

INSTABILITY OF THE SUBSTRAIN A121111 STOCK CULTURE ON AGAR

The stock culture of A121111 was maintained on yeast-water agar and subcultured at intervals of 2 to 3 months. Throughout 1941–42 it was frequently used in experiments of all kinds, and it maintained its effectivity.

During this period, in April, 1942, a white colony dissociant appeared, the surface colonies of which differed from the normal type in presenting a very convex and often umbilicate or lobed surface, and instead of having a more or less mucilaginous texture, they were butyrous and could readily be lifted entire from the surface of the agar with an inoculating needle. The surface of the colony was smooth, but not so smooth as the normal type. The dissociant type was morphologically indistinguishable from the normal type. Israilsky and Starygin (1930) have described what are probably similar rough variants, and Almon and Baldwin (1933) have also described variants in pigmentation and gumminess, but no studies of the effectivity of these variants were made.

The two types described above remained distinct when subcultured and later purified by plating twice. The new substrain was designated A121111W. It was found to be serologically identical with the parent substrain A121111.

Both strains were then tested on red clover in the usual way. A comparison of the mean nodule size produced by this variant and by its parent strain is given in table 1 (experiment 10). Analysis of the variance showed no significant difference between the behavior of the two strains. The mean dry weight of the plants inoculated with A121111 was 49.11 $\pm$ 2.86 mg, and that of the plants inoculated with A121111W was 44.00 $\pm$ 2.90 mg, a difference which again was

not significant. Thus in terms of dry weight and nodule size the effectivity of the dissociant was the same as that of the parent culture. The replated parent, A121111, was returned to stock, but again in November, 1943, it was observed on plating to consist of two colony types; the normal type and a dissociant type similar in all respects to A121111W. This was designated A121111W[1] and on serological examination was found to be indistinguishable from A121111, but on testing on clover was found to be ineffective (table 1, experiment 11).

A further ineffective variant appeared in the stock culture of A121111 early in 1944, but the behavior of this variant has not been studied in much detail. The relative numbers of effective and ineffective bacteria in the stock cultures of substrain A121111 were determined at intervals by testing random colonies. The proportion of the latter rose to about half by November 5, 1943, increasing during November and December and then disappearing. Simultaneous tests on plant passage isolates derived from substrain A121111 did not reveal any ineffective variants. Subsequent tests of the stock culture made at frequent intervals during 1945 have revealed no further ineffective variants.

DISCUSSION

The changes in character occurring in bacteria fall into two main classes. First there are those that are cyclical, as changes in cell form and structure appearing in such an order as to constitute a life cycle. These have been described in *Rhizobium* (Bewley and Hutchinson, 1920; Thornton and Gangulee, 1926) and can in fact be seen in the pleomorphism of all cultures of strain A and its derivatives. They are clearly distinguishable from the kind of changes here considered, which are of the type that persist for an appreciable time after the variant has been isolated and after it has been grown under the same conditions as its parent form.

Persistent changes again appear to fall into two groups. There are those in which a strain shows a progressive change, as when pathogenic virulence increases on host passage or is reduced by culture on laboratory media; and there are those sudden, and more or less permanent, changes in character which may appear either following a stimulus or without evident cause. It is to these sudden changes that the term "dissociation" is usually applied.

The apparent distinction between these last two types of variation is reminiscent of that formerly drawn between continuous variation and discontinuous variation or mutation in higher organisms, and it seems likely that an appearance of a gradual change in a bacterial population may be due to the selective increase of mutant forms in that population. Indeed, the work of Lincoln (1940), Gowen (1941), and Gowen and Lincoln (1942) on the rate of spontaneous and X-ray-induced dissociation in *Phytomonas stewartii* strongly suggests that bacterial dissociation in general is strictly analogous to mutation in higher organisms.

The genetical study of either progressive or dissociative variation is greatly complicated by the fact that the variant is usually observed not when it first occurs but only after it has grown for many cell generations in competition with

the parent form. The effects of plant passage upon a strain of *Rhizobium* recorded by Allen and Baldwin (1931) had the appearance of slow adaptive change, and because of the localized method of infection seemed to offer a chance of analyzing this type of variation.

In the present work, in which a bacterial strain of carefully tested phenotypic purity was used, neither plant passage nor selection was able to modify either the effectivity of the strain or the related character of mean nodule length. This would seem to exclude, at least in regard to the strains used, the occurrence of a slow progressive change in the bacterial population during passage through the host plant. But it does not necessarily exclude the occurrence of gene mutation in the bacteria giving rise to variants differing from the parent form in the size and effectivity of the nodule produced; and a closer examination of this possibility may be made on the basis of the results presented above.

It is evident that the readiness with which mutants which affect nodule size or effectivity can be detected differs greatly according to whether the mutation is in the direction of greater size and effectivity, or in the direction of smaller size and of ineffectivity. In the latter case, a parent strain normally producing effective nodules of large adult size will also produce nodules which are small because still young, so that an individual small nodule produced by a mutant can neither be distinguished at sight from a small young nodule produced by the parent strain, nor will its ineffectivity perceptibly influence the growth of the host plant. Such a mutation occurring in a nodule will thus be found only if the nodule in which it took place is selected for plating, and then only if the mutant form is selected in one or more of the resulting colonies picked and tested on the plant, upon which it will produce a smaller mean nodule size and give an ineffective response. Hence the chance of finding a mutation producing smaller nodules is limited by the number of colonies separately tested on the plant. During the experiments on plant passage and substrain selection, commencing with the effective strain A, more than 600 colonies were tested in this way. With any reasonable mutation rate, it is unlikely that gene mutations producing smaller nodules would have been detected, unless the mutant type increased considerably in relation to the parent type either in the colony or in the nodule. There was no evidence of such selective growth.

The likelihood of finding a variant in the direction of larger nodule size and effectiveness is, on the contrary, very much greater because it has a probability of being detected if it occurs either in the colony picked and tested, or in the nodule, since the presence of even a single nodule of abnormally large size will be readily seen among the small nodules produced by the parent strain, and a single effective nodule may perceptibly improve the growth of the plant (figure 5). Hence the chance of detecting an effective variant does not depend upon the number of colonies tested, but on the number of nodules produced and examined.

In the plant passage experiment using the ineffective strain H.K.C. a total of 2,896 nodules were examined. This number may well have been insufficient to chance upon an effective variant, even supposing that a mutant change in a

single factor would render effective the strain H.K.C. When, however, the ineffective variants produced by storing substrain A121111 in soil were passed through the plant, some 13,400 resulting nodules were examined, and among these two large nodules which were shown to contain effective organisms were observed. With a reasonable mutation rate, such a result would not be unexpected, and it may be legitimate to assume that here a reversion occurred in a single hereditable factor in the bacteria.

The plant passage experiments as a whole are thus consistent with the view that bacterial mutants affecting nodule size and effectiveness occur during plant

FIG. 5. CLOVER PLANTS INOCULATED WITH THE EFFECTIVE SUBSTRAIN *d*1 AND THE INEFFECTIVE VARIANTS *e*1 AND *e*2

The single large nodule produced by substrain *e*1, from which the effective reversion form was recovered, is indicated with an arrow.

passage, though there is no evidence that the plant environment selects bacterial variants in either direction. Plant passage is not a means of altering the efficiency of the bacteria. An absence of selection among the bacteria during plant passage may be related to their separation into microscopic colonies within the individual cells of the nodule, combined with the absence of suitable conditions for bacterial growth on the agar in which the plants were grown.

On the other hand, the high percentage of ineffective variant types producing small nodules, which were found after storing the effective substrain A121111 in soil, can best be explained by supposing that in the soil environment a strong selection occurred in favor of the ineffective variant.

It will be recalled that the ineffective form appeared in all three soil cultures, and from this it might be deduced that the conditions in the soil caused the dissociation. This is not necessarily the case. Each tube of soil was inoculated from the same mass inoculum from a slope culture of A121111, and it is probable that these enormous populations already contained numbers of mutant forms, some of which may have been relatively at an advantage in the new environment.

The storage in soil resulted in desiccation, which, like other unfavorable conditions, is a recognized "incitant" to dissociation insofar as it permits the survival of minority sections of the population. On yeast-water agar, even moderate desiccation leads to the death of bacteria. Their persistence in desiccated soil is probably due to the protective action of the soil colloids (Heller, 1941).

Changes in effectivity also occurred sporadically in culture on agar and possibly in the medium in the neighborhood of the plant root and in the nodule itself, but the similarity of the results of the tests made in 1943 and 1945 of stock cultures of *e*1, etc., show that the factors influencing the multiplication of the variant do not necessarily cause it to supplant completely the parent forms, or even to become dominant. Information is needed on the nature of the equilibrium existing in such heterogeneous populations, as well as on the particular soil conditions apt to encourage the emergence of ineffective variants, and on the relative stability in soil of different strains of *Rhizobium*.

These results have an important bearing on the practice of legume seed inoculation in emphasizing the need for frequent tests of the effectivity of cultures issued for this purpose in order to guard against the development of undesirable variants.

It will also be recalled that statistical analysis of the data revealed great variation in the response of individual clover plants to the same strain of bacteria, in some cases to an extent involving complete ineffectivity in the response of individual plants with the normally effective strain A. This plant variability is being investigated on genetical lines.

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SUMMARY

The paper describes experiments on the influence of passage through the host legume and of storage in soil and on agar medium upon the symbiotic behavior of two strains of *Rhizobium*. The results refer to mean length and "effectivity" of nodules on red clover grown under bacteriologically controlled conditions.

The original culture of the effective strain A was found to be uniform in the

behavior of isolations from replicate colonies, although considerable variation was found between the responses of individual plants infected from the same colony.

Neither plant passage with or without intervening plating or short-time culture on agar nor selection from large or small nodules had any effect on the mean size or effectivity of the nodules produced by strain A.

Plant passage similarly failed to modify the behavior of the ineffective strain H.K.C.

On the other hand, after the storage of strain A in sterilized Woburn sandy soil ineffective variants were found to constitute a considerable proportion of the bacterial population. These variants resembled the parent type in cultural and serological characters.

After the passage of these ineffective variants through the plant, two reversions to the effective parent type were found among the 13,400 nodules examined. These remained effective on further plant passage.

Stock cultures on agar slopes, both of the effective parent type and of the ineffective variant, showed an occasional tendency to produce new variants in effectivity. Variants in type of growth on agar also appeared under these conditions.

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