SYMBIOTIC EFFECTIVENESS IN NODULATED RED CLOVER

I. VARIATION IN HOST AND IN BACTERIA

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1. INTRODUCTION

THE variation to which the symbiosis of the clover root nodule bacteria (*Rhizobium trifolii* Dang.) and the leguminous host plant (*Trifolium pratense*, L.) is subject is partly of bacterial origin and partly of plant origin. It affects all phases of nodule development and function. In this series of papers attention will be directed towards the genetics of the so-called *effectiveness* of the host's response as defined by Stevens (1925) and Thornton (1939). In the effective symbiosis the nodulated plant assimilates fixed nitrogen and is therefore able to make normal growth in the complete absence of combined nitrogen in the root culture solution. In the ineffective symbiosis, on the other hand, no nitrogen is fixed in the host plant. The failure of nitrogen fixation in the ineffective nodule is thought to be due to fundamental incompatibility between bacterium and plant. This leads to premature degeneration of the infected cells of the nodule in which, presumably, nitrogen fixation would normally take place (Chen and Thornton, 1940). Intermediate degrees of effectiveness in strains of clover nodule bacteria have been described (Baldwin and Fred, 1929 and others).

Hitherto the terms effective and ineffective have been used descriptively for *strains of bacteria* according to their average symbiosis with unselected host plants of a particular species or variety. Here they will be used to describe the symbiosis itself irrespective of bacterial strain or plant type unless qualified, as for example in the original use of these terms.

It is the object of this introductory paper to distinguish between effective, intermediate, and ineffective categories of response as clearly as possible from a study of the naturally occurring variation in host plant and bacteria, and also to outline the genetic field of study. Later papers will deal with the genetic analysis of a part of this variation and with the interrelations of strain and host factors. Some of my preliminary results have been briefly reported elsewhere (Nutman, 1946*a*, 1951).

2. MATERIAL AND METHODS

Commercial strains of late-flowering Montgomeryshire red clover were used in all experiments. These will be referred to as unselected clover; they were obtained in the first instance from Sutton's of Reading and later from Messrs Dixon of Ware. Plants were grown in test-tube culture on a medium lacking combined nitrogen and inoculated with nodule bacteria. For details of method, see Thornton (1930) and Nutman (1949).

Response to nitrogen fixation was determined, after 100 days' growth, (1) by a visual comparison of test plants with standard plants, and (2) by taking whole plant dry weights at the end of the experiment. The standard plants for visual grading were selected afresh for each experiment and comprised the following five classes for scoring: 0, completely ineffective, indistinguishable from uninoculated control, leaves remaining small and etiolated; 1, slightly effective, youngest two or three leaves green; 2, intermediate; 3, incompletely effective; 4, normally effective. The relation of these grades to harvest dry weight is given below.

In each experiment inoculation was generally made from a subculture of a single colony picked from a plating of the bacterial strain. Details of the origin of the bacterial strains are given below. The average effectiveness of a particular bacterial strain with unselected clover was estimated by either of the two methods described above using large numbers of replicated plants, each sown singly, so that plant variability could be determined at the same time.

Unpublished data, kindly made available by Dr J. Kleczkowska and Dr H. G. Thornton, of tests of effectiveness of a large number of strains of bacteria freshly isolated from the field will also be referred to below. In these experiments each strain was tested with eight plants grown in pairs.

3. HOST VARIABILITY IN SYMBIOSIS

(a) *With the normally effective strain of* Rhizobium trifolii, *strain A*

This strain of bacteria was originally obtained from Royal Agricultural College in Stockholm. It was effective in fixing nitrogen when first isolated and has retained this characteristic under standard conditions of subculturing in many subsequent tests (see below), so that its general stability and known history make it particularly suitable for studies on host variability. Variants have, however, arisen from time to time. The original strain lost its capacity to induce nodule formation in 1940, and a virulent line was continued in an isolate (A1) from a nodule produced by the original strain. Strain A1, and other re-isolates, have been used for comprehensive tests on strain variation (Nutman, 1946*b*) and has given rise to (i) a completely ineffective mutant (called *f* 12) after being allowed to dry out slowly in sterile soil culture (*ibid.*), and (ii) an intermediately effective variant (AC) following prolonged subculture in liquid media at 1° C. (see below).

Changes in effectiveness may also arise in culture on standard media but they do not present serious practical difficulty. I find that heterogeneity in stock cultures can be avoided by periodic plating. In this way strain A has retained the same average degree of effectiveness with unselected clover throughout. The strain A re-isolate used here has been described in previous publications as A121111.

The variation in the response of clover inoculated with strain A is shown in fig. 1 as a frequency diagram of dry weight of individual plants. This diagram combines the results of five experiments carried out at different times but under similar conditions. For plotting as a single histogram the individual dry weights for each of the five experiments were adjusted to a common mean calculated from the combined experiments.*

This diagram illustrates the very variable nature of the response of unselected clover with a pure line effective strain of nodule bacteria. The majority of plants are normally effective with a dry weight ranging from about 100 mgm. down to about 65 mgm. Plants between 65 mgm. and 30 mgm. dry weight are intermediately effective and those below 30 mgm. are wholly ineffective and are indistinguishable from uninoculated controls. The ineffective class comprises about 2 per cent. of the total population. The distribution is markedly skew with a long tail extending into classes of smaller and smaller dry weight, and appears here to be fairly continuous.

A very similar distribution has been found in a single large experiment on host-strain adaptation in which 50 strains of effective nodule bacteria were tested on nine samples of clover seed of different origin (Nutman and Read, 1952). Analysis showed that in the whole experiment average bacterial strain differences were insignificant and plant strain differences small. The histogram of the distribution of dry weight in this experiment as a whole without distinction of host or bacterial types is reproduced in fig. 2 for comparison with fig. 1. Here also the distribution is positively skew and extends over about the same range of dry weight, but it differs from fig. 1 in clearly showing a secondary mode in the ineffective class. The ineffective plants in this group were of very mixed origin and inoculated with a variety of strains, suggesting that they comprised a discrete class of plants distinguished from the remainder in giving rise to a completely ineffective symbiosis with normally effective bacterial strains.

(b) The response of unselected clover with the intermediately effective strains AC and 39B

The strain AC was isolated as a variant of strain A after subculture at low temperature ; details of its origin are as follows :— Two replicated cultures maintained in liquid yeast-water mannitol medium at 1° C. from March to October 1942 gave rise on plating to colonies of two types—a large normal watery colony and a small white butyrous colony. The normal colony was much more abundant

* In figs. 1-4 the horizontal scale is divided into larger or smaller class intervals according to the amount of data available. To facilitate comparison between figures vertical scales are adjusted to give histograms of about the same maximum height.

than the variant and appeared later on the plates. The picked colonies of the normal type on re-plating gave rise at first to colonies of both types ; a second plating gave no admixture. At both platings picked colonies were tested on pairs of plants with the results shown in table 1.

Both tests showed that the parental colony type was normally effective and that the small colony variant gave rise to a highly variable response with an intermediate average. This result may be due either to an accentuation of normal host differences by the variant strain, or it may be due to further dissociation of the variant into effective and ineffective types, as has since been shown by Kleczkowska (1950) to occur in some phage resistant mutants of *Rhizobium trifolii*.

TABLE 1

Response of trifolium plants inoculated with normal (A) and variant (AC) strains of bacteria. (Results shown in terms of number of plants in each response grade (0-4))

Bacterial culture	Number of plants in each grade				
	0	1	2	3	4
A	...	1	2	6	42
AC (1st plating)	3	3	...	2	8
AC (2nd plating)	24	...	2	4	4
AC1 large nodule isolates . . .	10	4	3	1	6
AC2 small nodule isolates . .	3	2	3	3	1

The differences between the proportions of the contrasted types of response in the two platings recorded in table 1 suggest admixture but the fact that plants of like response did not usually occur together in the same tube favoured the first view.

An experiment was carried out to determine whether a mixture of effective and ineffective strains was present by re-isolating from large and from small nodules on plants in cultures containing (1) two effective plants, (2) an effective and an ineffective plant, (3) two ineffective plants. With large nodule isolates (AC1) and with small nodule isolates (AC2) similar mixtures of responses were found.

Many later tests confirmed the average intermediately effective nature of this strain ; the wide divergence in individual plant response being unrelated to further bacterial variation.

The symbiotic behaviour of this strain in terms of plant dry weight is summarised in fig. 3. This distribution differs from figs. 1 and 2 in being negatively skew, and shows a maximum frequency in the ineffective class and possibly a secondary maximum in the effective class.

A further example of an intermediate distribution, given in fig. 4, relates to plants inoculated with strain 39B. This strain was isolated from a hill pasture on Mynydd Llangattwg, Brecknockshire, in 1943, by Dr H. G. Thornton and was shown by Dr J. Kleczkowska to be of average intermediate effectiveness in tests made in 1944 and 1945 (unpublished). The results summarised in fig. 4 confirm these findings and show further that the strain 39B in contrast to AC has a true intermediate mode at about 60 mgm., the tail of the distribution in the ineffective class containing a higher proportion of plants than with an effective strain but relatively fewer than with strain AC. The experiments made with AC and 39B summarised in figs. 3 and 4 were carried out at the same time and with the same sample of seed.

(c) *Response of unselected clover with the ineffective bacterial strains HKC and f12*

The frequency diagram, fig. 5, shows the results of tests made with a naturally occurring ineffective strain (HKC) and a stable ineffective mutant of strain A called *f* 12, both strains previously described (Nutman, 1946*b*).

These histograms are similar in showing negative skewness with a single mode at about 14 mgm. and no secondary modes in intermediate or effective weight classes. Absolute variability is much less than with intermediate or effective strains, and probably reflects differences in the amounts or efficiency of utilisation of the original reserves of nitrogen in the seed.

4. BACTERIAL STRAIN VARIABILITY

Further data on bacterial strain variation is provided by extensive studies by Kleczkowska and Thornton (unpublished) on the geographical distribution in Britain of effectiveness in clover nodule bacteria, isolated from both red and white clover. In these experiments eight replicated plants of Montgomeryshire red clover were sown in pairs for each strain tested, using the agar culture technique described above. The average plant dry weight (x) for each strain was related to the mean dry weight of plants inoculated with the standard strain A (A) and to that of uninoculated control plants (C). The statistic $\frac{x-C}{A-C} \times 100$ enabled comparisons to be made of strains tested at different times. With the permission of the authors a histogram of the distribution of this ratio is given in fig. 6 for a large population of strains. The general form of this distribution resembles the distribution of individual dry weights of plants inoculated with effective strains of bacteria (figs. 1 and 2), in showing positive skewness and a marked secondary mode in the ineffective class. The long tail of high values is probably due to the chance occurrence of abnormally low estimates of A; the effectiveness of A is undoubtedly exceeded by some strains but not to the extent suggested by values up to +350 in fig. 6.

It was noted in the course of this survey that ineffective strains are more prevalent in the hill pastures of the west and north of Britain

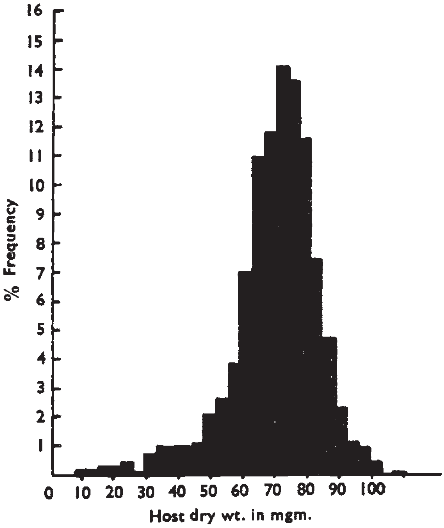

Fig. 1.—Distribution of the response of 1355 unselected clover plants inoculated with Strain A.

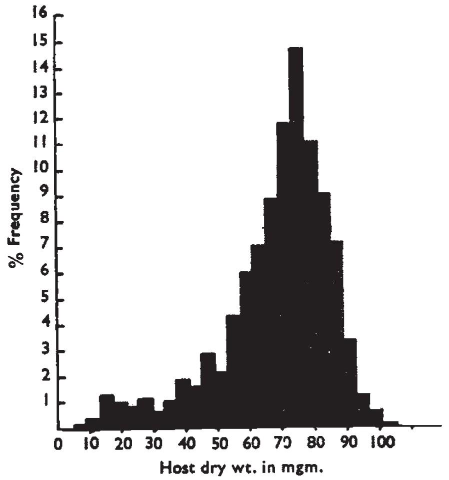

Fig. 2.—Distribution of the response of six local races of red clover inoculated with effective strains of bacteria.

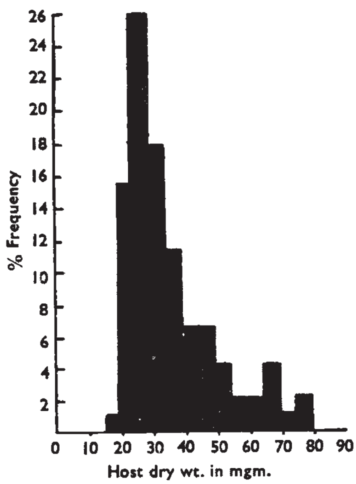

Fig. 3.—Distribution of response of red clover inoculated with the Intermediate Strain AC.

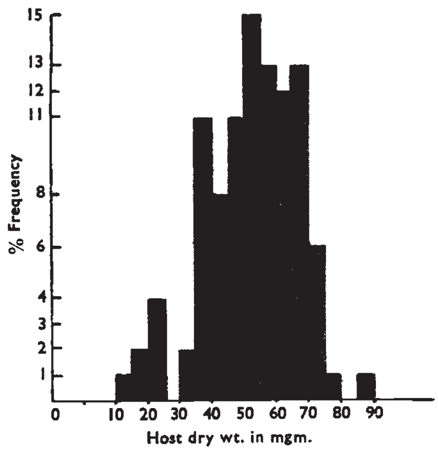

Fig. 4.—Distribution of response of red clover inoculated with the Intermediate Strain 39 B.

(Thornton, 1952) and because of the interest of these areas a large number of strains from hill pastures were examined. This has accentuated the secondary ineffective maximum. The correspondence between the histograms 1, 2 and 6 illustrates the complementary nature of the variation in host and bacteria.

The typically effective and typically ineffective types of response in symbiosis appear therefore to be separated in these diagrams by an indefinite number of intermediate grades. These may be regarded as parts of the normal distributions of effective or ineffective populations or as modal grades of a number of true intermediate distributions.

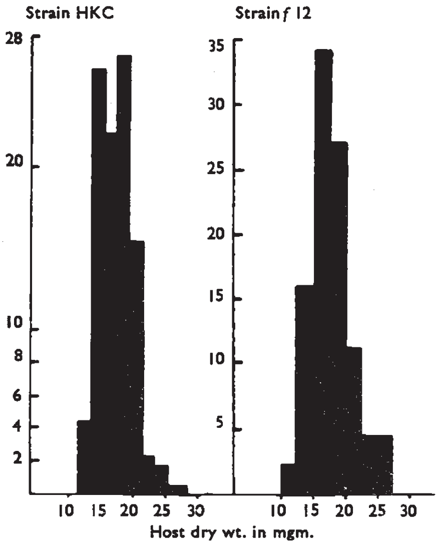

FIG. 5.—Distribution of response among clover plants inoculated with the Ineffective Strains HKC and *f* 12.

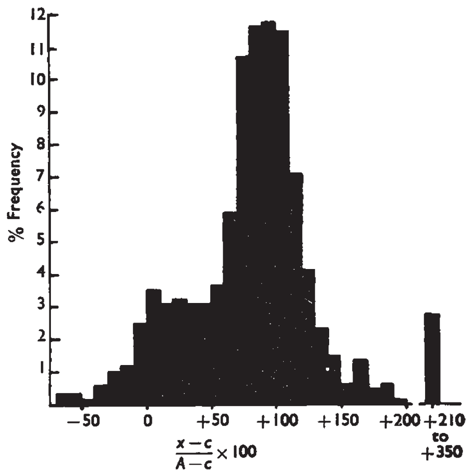

FIG. 6.—The distribution of Effectiveness among 677 strains of *Rhizobium Trifolii*.

5. HOST SELECTION EXPERIMENTS

Although the response of a plant may be conveniently measured by its dry weight or N content, these criteria cannot be used in breeding experiments and recourse has to be made to the visual classification of plant response into five grades (0-4) noted above. In the application of a grading method to genetic analysis of material showing segregation of effective and ineffective plants, however, a difficulty arises in classifying intermediately responding plants. This difficulty applies in particular to the assignment of plants placed in grades " 1 " and " 2 ".

If dry weight is used as the criterion, a separation of effectively from ineffectively responding plants can be fairly accurately made in figs. 2, 4 and 5 at about 30 mgm. ; plants above this weight belong to the effective population and those below to the ineffective. It was found that 95 per cent. of the plants in grade 2 and 55 per cent. of plants in grade 1 weighed more than 30 mgm. Since small numbers are usually involved, the following simple correction has been applied to the numbers of plants placed in these categories : grade 1 plants are regarded as half effective and half ineffective and grade 2 plants as wholly effective.

(a) Selections and tests made with strain A

The responses of families raised from selected plants are summarised in table 2 assuming half the responses classified in grade 1 to be effective. The parent plants' responses are also indicated in the table; two of these were exceptionally effective and vigorous and were designated 4+ on the scale used.

TABLE 2

Percentage of ineffective plants in crosses among original selections

(Inoculated strain A throughout)

Family number	Cross type	No. of plants	Per cent. ineffectives (and range)
	1. *Effective × effective*		
1	4+×4+	19	5·3
2-5	4+×4	114	2·0 (0-11·5)
6-25	4×4	385	1·0 (0-9·1)
26-31	4×3	225	1·5 (0-4·9)
32	4×3	25	16·2
	2. *Effectives × ineffectives*		
33, 34	4+×0	60	0·9 (0-1·5)
35-49	4×0	405	2·1 (0-9·4)
50	4×0	43	17·4
	3. *Ineffectives × ineffectives*		
	(i) Parent plants unrelated		
51-78	all 0×0	720	3·6 (0-9·1)
79		16	18·5
80		54	30·6
81		27	98·2
	(ii) Parent plants related		
82-84	all 0×0	58	0·9 (0-2·2)
85		44	18·2
86		4	25·0
87		28	39·2
88		31	43·5
89		22	50·0
90		27	66·6
91		15	83·3

The results show that progeny of crosses among effectively responding plants (subsequently referred to as effectives) of grades 3, 4 or 4+ as well as crosses between effectives and ineffectively responding plants (subsequently referred to as ineffectives) were generally effective. With the exception of families No. 32 and No. 50 only 2 per cent. of

Response of clover inoculated with nodule bacteria, strain A :
(*a*) plant homozygous for i_1 ; (*b*) heterozygote.

a total of 1208 plants distributed in 48 families responded ineffectively. The much higher proportion of ineffectives in the two exceptional families suggest genetic segregation of recessive factors for ineffectiveness.

More definite evidence for hereditary influences is given by the progeny tests of crosses among ineffectives. These are of two kinds : between unrelated plants and between related plants. Ten of these families, 7 of them with related parents, show varying proportions of ineffectives much in excess of the few per cent. found in the unselected populations. About equal numbers of effectives and ineffectives occur in families 88 and 89 ; about three-quarters of ineffectives in families 90 and 91.

The simple proportions of ineffectives in these families suggest simple modes of inheritance ; the genotypes of the parent plants of family 81 and probably also of 91 may be similar.

The further breeding behaviour of some of these families will be discussed in later papers of this series, but it is evident from these preliminary results that, with an effective strain of bacteria, the proportion of plants showing an ineffective response may be raised by simple selection and breeding from ineffectives.

(b) *Selections and tests made with intermediate bacterial strains*

A few selections were made of plants inoculated with an intermediate strain C13R (isolated by J. Kleczkowska from clover-sick Woburn soil in 1941). This strain unfortunately became avirulent before progeny tests of the host could be made with it, and was therefore substituted in these tests by strain AC. The results are shown in table 3 (1) with the response of unselected clover for comparison.

In contrast to the results with strain A, plants raised from effectively responding parents inoculated with an intermediate strain are themselves considerably more effective than those of ineffective or mixed parentage. In this experiment unselected clover has given the least effective response ; no significant difference appears in the response of families with either one or both parents ineffective.

In view of the different results obtained with intermediate and effective strains a number of families selected with strain A were re-examined with the intermediate strains AC and 39B. These families had previously been shown to respond almost wholly effectively with strain A although their parental responses were diverse (table 2). The results are given in table 3 (ii) and show that host selection against strain A has little or no effect on the average response of plants inoculated with the intermediate strains. With strain 39B there appears to be a reduction in the number of grade 4 responding plants but without an increase of ineffectively responding plants. The numbers of plants in these tests are insufficient to distinguish small trends.

TABLE 3

Progeny tests of selected Trifolium *families with intermediate strains of bacteria*

Family number	Cross type (parental responses)	No. of plants	Response (per cent.)				
			0	1	2	3	4
1. *Selected with strain C1 3R : progeny test with strain AC*							
01-03	4×4	60	12	2	13	32	41
04-05	4×0	68	28	12	22	23	15
06	0×0	26	15	16	23	27	19
Unselected clover		48	40	21	17	14	8
2. *Selected with strain A*							
(i) Progeny test with strain A							
6-9	4×4	17	6	6	...	6	82
35-40	4×0	103	4	1	8	15	72
51-54	0×0	49	2	...	2	22	73
(ii) Progeny test with strain AC							
6-9	4×4	18	39	33	1	1	6
35-40	4×0	52	54	25	15	4	2
51-54	0×0	16	38	25	25	12	...
(iii) Progeny test with strain 39B							
6-9	4×4	17	...	...	18	35	47
35-40	4×0	58	7	10	12	40	31
51-54	0×0	20	...	...	45	50	5

6. CONCLUSIONS

The above examination of naturally occurring variation in the symbiosis of *Rhizobium trifolii* and *Trifolium pratense* shows that effectiveness in N-fixation in the root nodules is determined by the interaction of bacterial strain and host plant factors which are independently subject to genetic variation. Moreover, different combinations of host and strain factors may have identical expression in symbiosis. Thus the ineffective symbiosis of family 81 with strain A is phenotypically indistinguishable from that of unselected clover with the *f*12 mutant of strain A. The complementary nature of the relations of plant and bacteria is also evident in the similar distributions of effectiveness in host and strain populations (figs. 1-6).

The range of genetic variation under the influence of these factors is represented diagrammatically in fig. 7. This figure also shows the genetic interrelations in the host, in an abbreviated form, the width of the lines connecting progeny and parent indicating their approximate frequency.

These results point to the possibility under certain conditions of breeding for increased nitrogen fixation in the host. Previous studies on varietal relationships (Wilson, Burton and Bond, 1932 ; Bjälfve, 1935, etc.) and on adaptation between local races of red clover and nodule bacteria (Nutman and Read, 1952) suggest the same possibility.

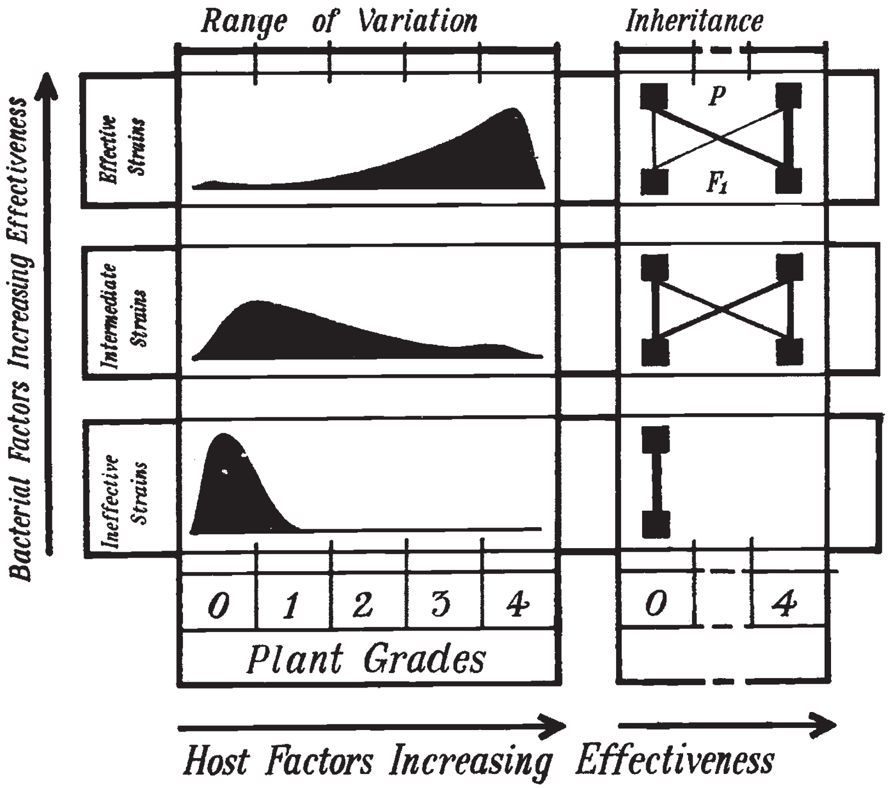

FIG. 7.—Diagram showing the genetic interrelations of host and bacteria in symbiosis. Each horizontal panel includes responses found with a single strain of bacteria. The superimposed columns show variation and inheritance in the host. The true breeding of ineffectively responding plants inoculated with ineffective strains of bacteria is inferred ; no crosses were made.

Most scope for improvement appears to lie with intermediate type symbiosis and least with those which, on the one hand, involve an ineffective bacterial strain or, on the other hand, a normally effective strain. Experiments have shown that with the latter, effectiveness is not increased by selection for a single host generation ; further experimentation on selection over a number of generations is required.

7. SUMMARY

From a comparative study in red clover of variation in symbiotic effectiveness, the following results were obtained :—

(1) With effective or partially effective strains of bacteria, the

response of individual plants in a variety, in terms of amount of growth made on a nitrogen-free medium showed a skew distribution.

(2) In general, distribution curves of host response with such strains show a primary mode of effectively, and a secondary mode of ineffectively responding plants.

(3) The relative sizes but not usually the positions of these modes vary with bacterial strain.

(4) With ineffective strains of bacteria the distribution curve is negatively skew and shows a single mode only.

(5) Preliminary breeding experiments show that the host plant differences are inherited.

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