

SYMBIOTIC EFFECTIVENESS IN NODULATED RED CLOVER

V. THE n AND d FACTORS FOR INEFFECTIVENESS

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Received 12.i.68

1. INTRODUCTION

NUTMAN (1954*b*, 1957) and Bergersen and Nutman (1957) described two independent recessive host genes (i_1 and i_2) each of which prevents nitrogen fixation in the nodules formed by red clover infected with a particular bacterial strain that is usually effective (fixes nitrogen) with this host. This paper presents evidence for two other recessive genes that cause ineffective responses in plants nodulated by this strain (*Rhizobium trifolii* strain 0403 (Bascomb 1965), formerly called A121111.)

2. METHODS

Plants were grown from surface-sterilised seed in test tubes on an agar medium without combined nitrogen but inoculated with nodule bacteria, as described in earlier papers. In addition to strain 0403 the following strains were also used in some tests: Nos. 1, 5, 6, 30, 32, 35, 47, 212 and 220 (Rothamsted collection numbers). Nitrogen fixation was followed during growth by grading the plants for size against representative plants selected in each experiment, using five standards in some experiments (grades 0-4 in tables) and nine in others. Notes were also made of the seedling's age when nodules first appeared and nodules were counted at harvest (60 or 80 days). In some experiments plants were weighed dry at harvest, and nitrogen contents determined and nodule lengths measured. Ineffectively responding plants selected for breeding at harvest were given one or two milligram doses of potassium nitrate and left for three or four weeks before transferring into John Innes compost No. 1; effectively responding plants were transferred without prior feeding. Plants were shaded until established and were then regularly given John Innes liquid feed. Pollinations were made using the procedures of Williams (1925).

When determining genetic ratios, the intermediately effective plants with gradings of two or more on the 0-4 scale, or of three or more on the 0-8 scale, were assigned to the effective category. The few plants graded 1 on the 0-4 scale were regarded as half effective and half ineffective. The rationale of this procedure is given in the first paper of this series (Nutman, 1954*a*, p. 41). In the tables and text, the following abbreviations will be used: crosses between effectively responding plants, $E \times E$; crosses between effectively and ineffectively responding plants, $E \times I$; etc.

3. THE n FACTOR—BREEDING RESULTS

The evidence for the recessive factor n came from a study of the descendants of two plants M and N) of late-flowering Montgomeryshire

red clover (M.R.C.) both of which responded effectively with *Rhizobium trifolii* strain 0403. These plants when crossed produced the ineffective F_1 plant P_4 (table 1, family 222) and segregated a few ineffectives in the F_2 generation (families 222, 227). The number of ineffectives segregating in family 227 suggested single factor inheritance and this was amply confirmed by further breeding, although in certain pedigrees as detailed below, the segregation ratios diverged widely from expected values. Plant M was also crossed with the two effectively responding plants O and U and the plant N with U. Only the cross $M \times U$ (family 228) yielded more ineffectives than unselected material, but none of the F_2 families derived from these crosses (families 231-235) segregated ineffectives.

TABLE 1

*Response of family $M \times N$ and collateral families, inoculated with *Rhizobium trifolii* strain 0403.
Numbers of plants in each category of response*

Family No.	Cross type	Progeny response					Ratio I: E†
		0	1	2	3	4*	
222	$M \times N$	$P_4 = 1$	1	1	2	32	—
223-226	F_2 of $M \times N$ ($E \times E$)	1	—	1	1	14	—
227	F_2 of $M \times N$	1	1	—	1	3	1.5: 4.5
228	$M \times U$	1	1	—	2	14	—
229	$M \times O$	—	—	—	2	17	—
230	$U \times N$	—	—	—	—	8	—
231-235	other F_2 s	—	1	11	22	69	—
Unselected control; late flowering Montgomeryshire red clover		—	1	6	19	34	—

* Grade 0 completely ineffective, Grade 4 fully effective.

† Half of plants in Grade 1 assigned to effective category and half to ineffective category.

Selection of clearly ineffectively responding plants was made difficult by the late development of a partially effective response in plants that were at first ineffective. Plants showing a changing response are indicated in table 2 by the symbol *Ie*. Table 2 shows that these indeterminant effectives did not breed true and when crossed with effectives they gave results that could not be easily interpreted. Thus the $I \times Ie$ crosses in the MN line gave two-thirds ineffectives and the $Ie \times E$ crosses one-third ineffectives. Crosses among effective plants were either mostly effective (family 245) or segregated one-quarter ineffectives, suggesting simple inheritance (families 246, 247). Full effectiveness was restored on outcrossing and in the next generation approximately one-quarter of the progeny were ineffective (families 250-253).

The P_4 ineffective was also crossed with an effective plant of known antecedents. One parent (plant H) responded ineffectively with strain 0403, and the other (plant J) responded effectively. However, the F_2 of the $H \times J$ cross contained very few ineffectives (3 in 96), indicating that the ineffectiveness of plant H was not simply inherited. The outcross of P_4 with $H \times J$ was almost wholly effective (table 2, family 254), and in four F_2 families rather fewer than one-quarter responded ineffectively. The deficit of ineffectives was in one family only (family 258); other families segregated to give almost exactly one-quarter ineffectives. In the F_3 families the dis-

TABLE 2

*Response of families of plants derived from P₄, inoculated with Rhizobium trifolii strain 0403.**Number of plants in each response category*

Family No.	Cross type	Progeny response					Ratio I: E	χ^2	P
		0	1	2	3	4			
<i>The MN line</i>									
236-240	F ₃ , I _c × I	44	10	9	9	5	—	—	—
241-244	I _c × E	22	11	16	30	1	—	—	—
245	E × E	2	1	14	8	1	—	—	—
246, 247	E × E	8	9	11	21	3	12.5: 39.5	1.41	0.5
248-249	F ₃ ineffectives × E, outcrosses	—	—	1	14	8	—	—	—
250-253	F ₂ s of crosses 248, 249	25	6	13	15	19	28.0: 50.0	2.98	0.7-0.5
<i>The MN HJ line</i>									
254	P ₄ × HJ, outcross	1	—	—	4	34	—	—	—
255-257	F ₂ of cross 254	13	9	16	25	26	17.5: 71.5	1.48	0.7
258	F ₂ of cross 254	1	2	5	8	3	2.0: 17.0	3.68	0.1-0.05
259-266	F ₂ × F ₃ , I × E	31	12	16	33	132	—	—	—
267, 268	F ₃ × F ₂ , E × E	2	—	—	—	1	—	—	—
269, 270	F ₄ , I × I	14	2	5	12	15	—	—	—
271	F ₄ ineffective × E, outcross	—	—	—	12	15	—	—	—
<i>The MU and O lines</i>									
272-275	F ₃ , I × I _c	23	4	4	20	24	25.0: 50.0	—	—
276-279	I _c × E	—	—	—	9	47	—	—	—
280-282	I _c × E	8	—	4	15	5	8.0: 24.0	2.99	0.5-0.3
283	I _c × E	9	1	5	3	1	9.5: 9.5	—	—
284-287	E × E	1	—	1	15	58	—	—	—
288, 289	F ₄ , I × I	65	10	—	—	1	—	—	—
290	F ₃ ineffective × E, outcross	—	—	1	11	10	—	—	—
291-295	F ₂ of cross 290	66	15	46	90	77	73.0: 220.0	5.47	0.5-0.3
296-299	Backcrosses I × E	37	5	3	32	7	39.5: 44.5	4.15	0.5-0.3
300, 301	F ₃ , E × I	—	—	10	28	21	—	—	—
302, 303	E × I	18	—	3	11	7	18.0: 21.0	0.31	0.9-0.8
304-306	F ₃ , E × E	3	2	12	21	34	—	—	—
307, 308	E × E	17	1	5	15	8	17.5: 28.5	5.24	0.1-0.05
291 etc. and 266 etc.	Mean plant dry weights in mg.	12.2	23.1	35.9	43.2	47.7			
291 etc. and 266 etc.	Mean N content per plant in mg.	0.13	0.29	0.55	0.54	—			

tribution of effectives and ineffectives was erratic. Crosses between differently responding types did not give the expected equal numbers of effectives and ineffectives, nor did the ineffectives breed true; one family was almost wholly effective and the other segregated to give equal numbers of effectives and ineffectives. A possible explanation would be interference from factors derived from the ineffective plant H or misclassification of ineffectives because of late response. This material was not used for further work.

The mixed M, U and O material gave results which were less influenced by delayed effective responses. In about half the families the proportions of ineffectives did not differ significantly from those expected on a simple factor hypothesis, and in the two families with completely ineffective parents all plants but one were ineffective (families 288, 289). Outcrossing restored full effectiveness, and in F_2 one-quarter of the progeny was wholly ineffective (families 291-295). Four backcrosses made to ineffective plants gave equal numbers of effectives and ineffectives.

Corroborative evidence of simple inheritance for ineffectiveness was obtained in further breeding. The responses of the F_3 families were of two kinds: normally effective (300, 301, 304-306) and segregating (302, 303, 307 and 308). When one parent was ineffective, about equal numbers of effective and ineffective progeny were recorded. When both parents were effective, the number of ineffectives in the segregating families was somewhat greater than expected but not significantly so (families 307, 308). All F_3 families contained a few albino plants, the symbiotic response of which could not be determined.

The results therefore show that ineffectiveness in families 288 and 289 was caused by a simple recessive host gene, which will be designated *n*. The results from the MN line and from the $P_4 \times HJ$ line make it probable that the gene *n* was derived from plant M and present in plant P_4 . Such ineffectives weighed about one-quarter that of fully effective plants and contained little nitrogen (table 2).

Of the many ineffectives in families 236-308 transplanted into soil only a few survived, and it is for this reason that crosses involving ineffectives were few. Those that survived in soil did not flower freely but otherwise grew normally on mineral nitrogen. When combined nitrogen was withheld they quickly became nitrogen deficient with pale foliage (see plate 1, fig. a).

(i) *Time of first nodule formation*

The time nodules first form is much affected by growing conditions such as temperature, and for this reason only broad comparison was possible between experiments. The original P_4 ineffectives nodulated at about 32 days, which is 3 weeks later than nodules usually first form in red clover. A study of nodulation times in families segregating for *n* showed that late nodulation was not characteristic of *n* ineffectives. Factors for earlier nodulation were evidently introduced from the outcross parents, O, U and HJ and the effective parent of family 270.

Late nodulation was correlated with the delayed development of the effective response already noted, and was the likely cause of the irregular segregations in certain families shown in table 2.

The tendency for *n* ineffectives to form their first nodules slightly later than effectively responding plants probably reflects residual linkage with

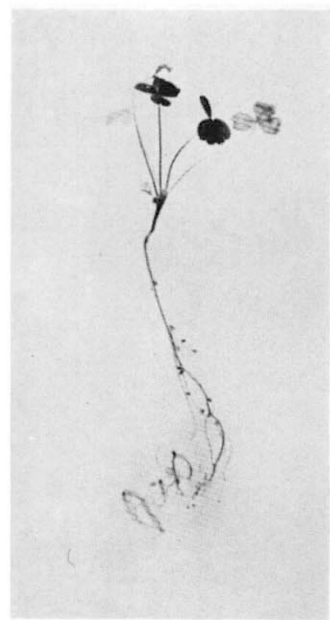
Plate I

FIG. *a*.—*nn* and *Nn* plants growing in nitrogen deficient soil.

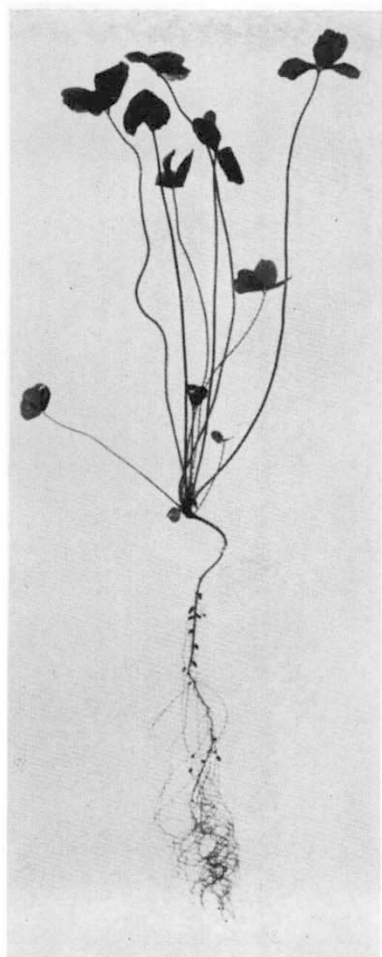
FIGS. *b*, *c* and *d*.—*nn*, *Dd* and *dd* plants grown in an agar medium without combined nitrogen and inoculated with *Rhizobium trifolii* strain 0403.



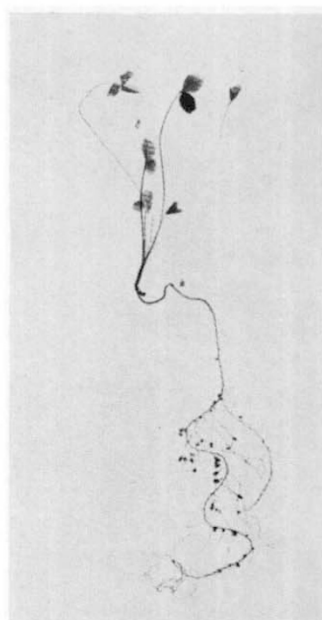
a



b



c



d

late factors from family 222; initial nodulation is known to be affected by many genes (Nutman 1946, 1949 and 1953).

(ii) *Nodule number*

Nodule number, like nodulation time, is affected by experimental conditions so that detailed comparisons could not be made between experiments. Nevertheless, the results showed that the gene *n* has little if any effect on nodule number; ineffectives had fewer nodules in some families and more in others. The outcross parent strongly influenced the nodule number of the progeny. For example, the parents of family 222 bore 6 and 7 nodules only, and this is reflected in the relatively sparse nodulation of their progeny and near descendants (mean nodule numbers of 0 and 4 grade plants were respectively 37 and 34). On the other hand, the effective parent of cross 254 was abundantly nodulated and was itself the progeny of abundantly nodulating plants, having respectively 83 and 154 nodules. The F_2 and backcross families of this cross, which had many nodules, also showed a very wide range in nodule number, from 6 to 227. Intermediately effective plants tended to have more nodules than others, otherwise nodule number seemed not to be correlated with response.

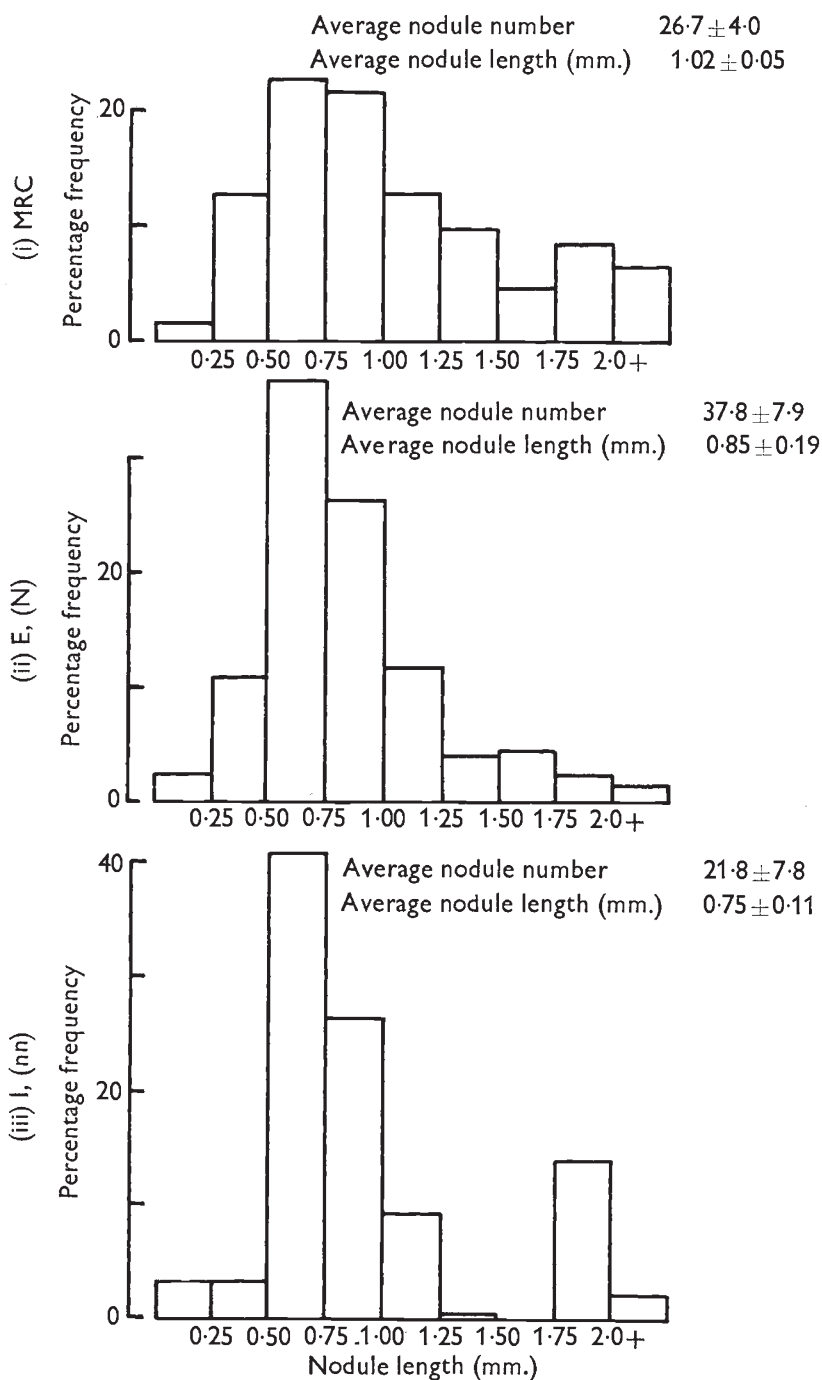
(iii) *Nodule size*

Nodules on P_4 were rather large, measuring 1.3, 1.4, 1.6, 1.9 and 2.0 mm. in length. All nodules were measured in one experiment inoculated with strain 0403 and planted with the segregating families (259-270 and 291-295). At the same time nodules were measured on plants of unselected clover. Fig. 1, i and ii, shows that effective plants of *n*-segregating families bore more but smaller nodules than unselected clover, and that *n*-type ineffective nodules of the particular families used in this experiment were both slightly fewer and slightly smaller than effective nodules on sister plants, although a few ineffective nodules became quite large (fig. 1, iii). Plate 1, fig. b, shows the nodulation of an *n* ineffective.

4. THE *d* FACTOR—BREEDING RESULTS

The recessive factor *d* was found in a plant (P_5) of late flowering Montgomeryshire red clover that gave an ineffective response with an intermediately effective bacterial strain (CI3R), which became avirulent and was then lost. The 5 F_2 families were re-examined with the normally effective strain 0403 and the results in table 3 show that one-quarter of the progeny were ineffective, indicating single-factor inheritance, with the effective response dominant over the ineffective response (families 309-313). Five ineffectives were selected for further breeding, but only two survived. In the F_3 generation both crosses (families 314, 315) between effectives gave effective progeny and of the two small families with unlike parents one was normally effective (family 316) and the other segregated ineffectives (family 317). The $I \times I$ cross was sterile.

The P_5 plant and ineffectively responding sibs when outcrossed gave fully effective progeny (families 318-320), and these intercrossed segregated the expected proportion of one-quarter ineffectives (families 321-323). The two F_3 crosses between ineffectives did not breed true but gave rise to about equal numbers of effectives and ineffectives (families 324, 325). Backcrosses of presumed heterozygotes to the ineffective parent gave very variable



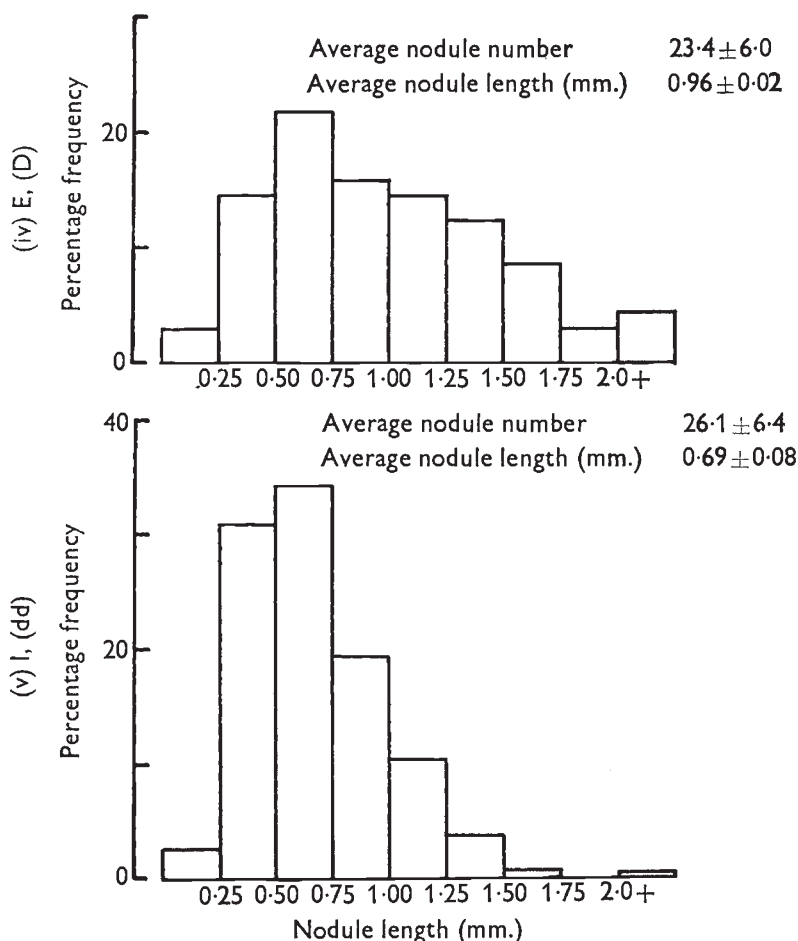


FIG. 1.—Distribution of nodule size on M.R.C. and effectively and ineffectively responding plants in *n*-segregating and *d*-segregating lines, inoculated *Rhizobium trifolii* strain 0403.

- i. M.R.C.
- ii. Effective plants in families segregating for the *n* gene.
- iii. Ineffective plants homozygous for *n*.
- iv. Effective plants in families segregating for the *d* gene.
- v. Ineffective plants homozygous for *d*.

results; two families segregated with expected equal numbers of plants showing the two types of response (families 326, 327), and two families were predominantly ineffective (families 328, 329).

Further outcrosses of ineffectives to unrelated effectives produced effective progeny (families 330-331); the F_2 again contained the expected one-quarter ineffectives, but in F_3 results were irregular in lines that were expected to be pure breeding for ineffectiveness. One small family consisted mostly of effective plants (family 355) and eight families were largely ineffective (families 356-363).

These results generally support a simple mode of inheritance for ineffectiveness. The deviations from the expected ratios, which occurred in both

directions, may be caused by interference from other genetic factors. Possible modifiers in such families as 316, 324 or 325 were not investigated by further breeding.

Further genetic evidence for the *n* and *d* factors is given below in a section on hybridizing ineffectives.

TABLE 3

Response of families derived from the P₅ ineffective plant and related plants inoculated with Rhizobium trifolii strain 0403. Numbers of plants in each response category

Family No.	Cross type	Progeny Response					Ratio I: E	χ^2	P
		0	1	2	3	4			
309-313	F ₂ derived from family 03 (Nutman, 1954a)	32	8	7	38	56	36: 105	3.74	0.7-0.5
314, 315	F ₃ , E \times E	1	3	3	14	40	—	—	—
316	I \times E	—	—	7	28	7	—	—	—
317	I \times E	1	1	—	2	—	1.5: 2.5	—	—
318-320	P ₅ (and sibs) \times E, outcrosses	—	—	4	4	21	—	—	—
321-323	F ₂ of crosses 318-320	13	—	16	14	12	13.0: 42.0	0.28	0.95-0.98
324-325	F _{3s} , I \times I	13	3	3	4	10	—	—	—
326, 327	Backcrosses E \times I	15	3	6	6	5	16.5: 18.5	13.03	<0.01
328, 329	Backcrosses E \times I	28	4	4	2	1	—	—	—
330, 331	F ₃ ineffective \times E, outcrosses	—	—	1	11	5	—	—	—
332-354	F ₂ of cross 330	52	9	52	43	79	56.5: 158.5	12.06	0.7-0.5
355	F ₃ , I \times I	—	3	—	2	3	—	—	—
356-363	I \times I	58	8	5	2	1	—	—	—
332 etc.	Mean plant dry weight in mg.	12.0	18.3	21.7	24.2	31.3	—	—	—
332 etc.	Mean N content per plant in mg.	0.17	0.37	0.41	0.87	0.72	—	—	—

(i) *The time of first nodule formation*

The relation between the time of first nodule formation and symbiotic response was examined in F₂ families segregating *d* ineffectives and in outcrosses. The original *d* segregants nodulated only a few days later than sister effective plants, and after each successive outcross this difference became smaller until in families 332 and 354 it was non-significant (2 days).

(ii) *Nodule number*

The relation between plant response and nodule number was examined in the main groups of families segregating *d* ineffectives. The ineffectives segregating in the original F₂ (families 309-313) had many more nodules than plants of effective categories, and those selected for breeding bore an average of 95 nodules per plant. Plants showing a very slightly effective response in these families were also abundantly nodulated.

The two outcross parent plants of families 318-320 formed 92 and 27 nodules respectively and their progeny formed an intermediate number of nodules with fewest in the most effective class. The ineffective plants selected for breeding bore 11, 17, 18 and 20 nodules. In F₂ families (families 321-323) intermediate numbers of nodules were formed with fewest in the effective class (average 29 nodules) and most in plants of intermediate effectiveness (average 46 nodules). The tendency for intermediately effective plants to form most nodules was also evident in F₂, F₃ and backcross families; the

ineffectives formed appreciably more nodules than plants of the most effective class (35 nodules compared with 24 nodules).

The two effectives used for further outcrosses (families 330-331) bore few nodules (12 and 19 respectively) and this further diminished the numbers of nodules formed in the F_2 and F_3 generations. The d homozygotes, unlike the n -type ineffectives, in general formed more nodules than fully effective heterozygotes, the results suggesting that this tendency is greater when factors for abundant nodulation are also present.

(iii) *Nodule size*

Fig. 1, iv and v, shows distributions of nodule length for plants of the 0 and 4 response categories in the d line. The number of nodules formed on each group of plants was about the same, and covered the same range in nodule size. However, the d -type ineffective nodules were, on average, smaller than nodules on either sister effective plants or on unselected red clover (fig. 1, i). These differences are illustrated in plate 1 (fig. *c* shows an effective dD heterozygote for comparison with a d -type ineffective, fig. *d*).

5. THE INFLUENCE OF BACTERIAL STRAIN ON THE RESPONSE OF n AND d HOMOZYGOTES

The effect of bacterial strain was examined in four experiments comparing unselected red clover (M.R.C.) with F_2 and F_3 lines segregating for the n and d factors. By using segregating material the known effect of inbreeding in decreasing the effectiveness of response is lessened and can be allowed for, and the effects of the n and d homozygotes is assessed against a heterogeneous genetic background, more comparable with unselected material. Altogether 36 segregating families were used in these experiments with individual plants distributed at random between the inoculation treatments, and the results of the four experiments are combined. Table 4 shows the results in terms of the percentage of plants in each of the five classes of symbiotic response. The results for strain 0403 are placed at the top of the table and the remaining strains (32-6) are arranged in descending order of effectiveness with unselected clover. With strain 0403, the n and d lines gave the expected proportion of 25 per cent. ineffectively responding plants and in order to facilitate comparison with M.R.C. a second distribution of response is given in the table where the numbers of ineffectives in the n - or d -segregating lines exceeds appreciably the number found in M.R.C. This is calculated after subtracting one-quarter of the ineffectively responding plants from the observed distribution.

Strains 32, 5 and 1 are of outstanding effectiveness; strains 220-212 are of intermediate effectiveness and strain 6 is largely ineffective with unselected clover. The following effects appear in the results for the segregating families.

(1) There is a general loss of effectiveness because of inbreeding. This occurs with all combination of strains and hosts, except possibly in the d -segregating line with strain 212.

(2) The proportions of ineffective plants in n -segregating families are considerably more than in unselected clover with strains 0403, 30 and 212 and somewhat more with strain 220, even after allowing for the effect of inbreeding.

(3) The proportion of ineffectives in the d -segregating families is greater

than for unselected clover with strains 0403, 220, 30 and 35, after allowing for inbreeding.

For strain 0403 the increases in the proportion of ineffectives in the two kinds of segregating line almost exactly corresponds to the expected segregation of the *n* and *d* recessives. For the *d*-segregating line with strains 220 and 30 and for the *n*-segregating line with 220, the excess number of ineffectives is too few to exclude simple specificity as an explanation of the distributions of the response for these strains, as for strain 0403. For the other strains the numbers of additional ineffectives greatly exceeds one-quarter. The

TABLE 4

Responses of n-segregating families, d-segregating families and unselected red clover with different strains of bacteria
Percentages of plants in each response category

Bacterial strain	I Montgomeryshire red clover					II <i>n</i> -segregating families					III <i>d</i> -segregating families					Total number of plants
	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4	
0403	2	—	2	33	63	26	6	21	29	18	29	5	14	12	40	213
						1	8	28	39	24†	5	7	19	16	53†	—
32	—	—	—	11	89	—	—	11	31	58	—	—	17	—	83	86
5	—	—	—	16	84	—	—	2	16	82	—	—	3	18	78	127
1	—	—	—	20	80	—	—	25	34	41	4	—	11	—	85	88
47	—	—	—	37	63	—	3	23	32	42	—	—	—	—	—	61
220	—	3	13	53	30	10	20	27	33	10	38	7	31	—	24	89
						—	7	36	44	13†	14	9	41	—	32†	—
30	2	2	11	40	45	53	6	15	23	4	39	23	7	13	19	131
						37	8	20	30	5†	17	30	9	17	26†	—
35	31	3	7	28	31	35	3	23	10	29	60	4	20	4	12	85
											47	5	26	5	16†	—
212	36	13	31	21	—	86	10	4	—	—	30	17	30	4	17	114
						82	13	5	—	—†	—	—	—	—	—	—
6	78	2	12	5	2	100	—	—	—	—	89	7	4	—	—	125

Figures in heavy type indicate significant excess of ineffectives compared with MRC.

† Second distribution calculated by omitting 25 per cent. of ineffectives (from class 0 only except for *n*-segregants with strain 220).

results could be interpreted for the strains in question (strain 30 and 212 with *n*-segregating families and strain 35 with the *d*-segregating families) as specificities involving genetic factors other than *n* or *d* that are possibly segregating in these families. Alternatively, because these excess ineffectives occur only with poorly effective strains of bacteria, such strains might respond ineffectively with *d* or *n* heterozygotes. Extensive breeding experiments would be needed to sort out these interactions.

The *n* or *d* interactions with the bacterial strains that lead to ineffectiveness are highly specific (cf. particularly strains 35 and 212 with the two groups of families) and seem to occur only with strains of average or poor effectiveness. With very poorly effective strains such as strain 6, inbreeding has the effect of eliminating the effective response and obscures any specific interaction there may be between host and strain.

6. THE RELATION BETWEEN THE FACTORS *i*₁, *i*₂, *n* AND *d*

Nodule morphology and response to bacterial strains are affected

differently by the genes i_1 and ie , and breeding tests confirmed that these factors were non-allelic and independent (Nutman, 1954*b*).

Table 5 shows the response of other crosses involving pairs of the four factors i_1 , ie , n and d , and the F_2 families raised therefrom. Crosses between all pairs of ineffective homozygotes gave mostly effectively responding plants, except in the $ie \times n$ cross where three families (Nos. 370-372) had a small proportion of ineffectives and one family (No. 373) segregated equal numbers of effectives and ineffectives. This may indicate a genetic relation between ie and n , or more probably the intrusion of other factors affecting effectiveness in these families. Only one successful cross (family 368) was made between plants homozygous respectively for the factors i_1 and d , and to supplement this a cross was made using an i_1 heterozygote. This also gave wholly effective progeny.

TABLE 5

*The relation between the factors i_1 , ie , n and d . Plants inoculated with strain 0403.
Number of plants in each grade*

Family No.	Cross	Response of progeny					Corrected ratio	χ^2	P
		0	1	2	3	4			
364-367	$i_1 i_1 \times nn$	—	—	—	15	55	—	—	—
368	$i_1 i_1 \times dd$	—	—	—	6	22	—	—	—
369	$i_1 I_1 \times dd$	—	—	—	9	3	—	—	—
370-372	$ie ie \times nn$	3	2	8	26	37	4.0: 72.0	—	—
373	$ie ie \times nn$	8	—	7	3	—	8.0: 10.0	—	—
374	$ie ie \times dd$	—	—	7	16	11	—	—	—
375, 376	$nn \times dd$	—	—	1	10	5	—	—	—
377-380	$F_2, (i_1 \times n)$	65	—	4	14	67	65.0: 85.0	2.08 (7: 9)	0.7
380-383	$F_2, (ie \times n)$ of fams. 370-372	52	4	4	26	28	54.0: 60.0	5.46 (7: 9)	0.3-0.2
384-391	$F_2, (ie \times d)$	62	4	14	49	57	64.0: 122.0	12.34 (7: 9)	0.2-0.1
392-394	$F_2, (n \times d)$	39	4	7	13	32	41.0: 54.0	2.48 (7: 9)	0.5
377-394	—	218	12	29	102	184	224.0: 321.0	38.98 (1: 1)	< 0.01**

Most F_2 crosses contained about equal numbers of effective and ineffective plants, with the ratios not differing significantly from the 7: 9 expected of independent segregation of the two factors. In this respect the $ie \times n$ F_2 did not differ from the others. Although segregation in most individual families was not significantly different from equality, the total χ^2 calculated on this hypothesis was highly significant, indicating that the F_2 parent plants were not homozygous for any of the factors concerned.

In certain of these F_2 families an attempt was made to identify the doubly recessive plants by back crossing. The results (table 6) provide additional evidence for the identity and independent inheritance of these factors and for the different viabilities of the recessive homozygotes. In these experiments, about six plants per family were grown only for as long as necessary to classify them as effective or ineffective. An arbitrary grouping was used for those families with only one wholly or partially effective plant and only one ineffective plant; the former was classified as ineffective and the latter as effective.

Given equal survival of the homozygotes, four-sevenths of the backcrosses

should be wholly ineffective, one-seventh wholly effective and two-sevenths should segregate equal numbers of effective and ineffective. These three classes were found in each group of crosses, but the number of ineffectively responding families was fewer than expected in all groups, but particularly in the backcrosses of F_2 ($i_1 \times n$) ineffective and F_2 ($ie \times n$) ineffective, to i_1 and ie homozygotes respectively. This is probably because the i_1 and ie homozygotes are less viable than n homozygotes. In the segregating families a good agreement was found with the expectation of equal numbers of effective and ineffective plants.

TABLE 6

*Backcrosses of ineffectives of mixed origin to plants homozygous for one or other of the recessive factors i_1 , n or ie
Plants inoculated with strain 0403*

Family No.	Description of backcross (1×1)	Number of families		Response of progeny (Number of plants.)			
		Observed	Expected	All ineffective	Segregating		All effective
					I	E	
395-396	$F_2, (i_1 \times n) \times i_1 i_1$	2	18	12	—	—	—
397-413	$F_2, (i_1 \times n) \times i_1 i_1$	17	5	—	40	54	—
414-426	$F_2, (i_1 \times n) \times i_1 i_1$	13	9	—	—	—	74
427-437	$F_2, (i_1 \times n) \times nn$	11	15	86	—	—	—
438-451	$F_2, (i_1 \times n) \times nn$	14	8	—	63	54	—
452, 453	$F_2, (i_1 \times n) \times nn$	2	4	—	—	—	12
454-461	$F_2, (ie \times n) \times ie ie$	8	21	43	—	—	—
462-468	$F_2, (ie \times n) \times ie ie$	7	10	—	23	22	—
469-489	$F_2, (ie \times n) \times ie ie$	21	5	—	—	—	126
490-506	$F_2, (ie \times n) \times nn$	17	27	87	—	—	—
507-516	$F_2, (ie \times n) \times nn$	10	14	—	30	38	—
517-537	$F_2, (ie \times n) \times nn$	21	7	—	—	—	115

$$\chi^2 \text{ 1.87; } P = 0.8-0.7.$$

In each backcross one ineffective in seven of the F_2 progeny should have been recessive for both factors, but none was identified, although many more than this number was used in the appropriate test crosses. This suggests that the double recessive is a lethal or is much less viable than plants recessive for one factor only. This interpretation is supported by the results in table 5, showing that fewer ineffectives segregated in F_2 than expected (224 instead of 238).

7. DISCUSSION

This paper extends earlier work on the effect of major genes controlling the development of effective symbiosis in red clover by describing two further recessive factors n and d each of which prevents nitrogen fixation in nodules formed by *Rhizobium trifolii* strain 0403.

As with the factor ie (Nutman, 1957), difficulty was experienced in breeding wholly ineffectively responding lines, homozygous for the factor n . Because aberrant results were found more in some host families than others, this was attributed to genetic interactions, and there was no evidence of

bacterial strain mutation affecting the results. The eventual success in obtaining nearly pure-breeding *n*-type ineffective lines probably owed much to the elimination by frequent outcrossing and re-selection, of modifying and suppressor genes. The *d* factor, in contrast to the *n* factor, segregated normally in most lines, although there was evidence of genetic interactions in a few. Factors modifying the expression of the *n* and *d* genes were not studied by further breeding.

The original *n* and *d* ineffective plants differed in the time their first nodules formed and in nodule number, characteristics known to be controlled by many genetic factors. A study of the effects of outcrossing showed that both these characteristics (and nodule size) are unaffected by the *n* factor but that the *d* factor increases nodule number and makes nodules smaller; in this the effect of the *d* factor resembles that of the *i*₁ factor, which, however, has more influence on nodule number and size.

Experiments showed that the four factors *i*₁, *ie*, *n* and *d* are inherited independently, but further work on their interrelation was prevented by failure to select and identify, by backcrossing, the plants doubly recessive for pairs of factors; the breeding results indicate that such plants did not survive.

Tests with strains of bacteria of different origin and effectiveness on unselected clover showed similar but not identical patterns of specificity with respect to the *n* and *d* genes. Highly effective bacterial strains gave a fully effective response with both *n* and *d* homozygotes, whereas less effective strains responded either effectively or ineffectively with *n* and *d* homozygotes. It is of particular interest that some intermediate strains that responded ineffectively with *n* homozygotes, responded effectively with *d* homozygotes, and vice versa. This pattern of specificity therefore is intermediate between that described for the *i*₁ factor, which is restricted to strain 0403, and the slight specificity of the *ie* factor.

Table 7 summarises the host-strain relationships so far known, for these four factors. Each interaction between host factor and strain leading to ineffectiveness is shown by a + sign. With ineffective or nearly ineffective strains the possible effect of plant factors cannot be distinguished and such ineffective interactions are put within brackets. The \pm entries refer to heterogeneity in the response of recessive homozygotes, which in some pedigrees are thought to be caused by other genetic factors (Nutman, 1954*b*, p. 58).

Studies on mutation in strain 0403 (Nutman, 1954*b*) suggested that the *i*₁ factor interacts with a simple heritable property of the bacterial strain 0403 to prevent normal nodule development. Studies of the development of *i*₁ and *ie* nodules also showed that failure or incompatibility occurs at particular points in nodule ontogeny (Bergersen and Nutman, 1957).

On this interpretation the two highly effective strains (Nos. 5 and 32) possess factors for incompatibility with respect to the gene *ie*, but not to genes *i*₁, *n* or *d*. Less effective strains may or may not possess a particular factor necessary for the expression of plant ineffectiveness; for example, the strain 35 possesses the complementary ineffective factor for the gene *d* but not *n*, whereas the strain 212 possesses the factor complementing gene *n* but not *d*. Among the 16 strains tested at least 10 different patterns of interaction are shown.

The existence of specific strain factors was inferred from table 7 by

comparing the numbers of ineffectives in segregating lines with those in unselected red clover, after allowing for inbreeding. In about half of these comparisons the numbers of ineffectives in segregating lines exceeded expectation, in some by a large margin. This may be because such lines also segregate for other factors that interact with poorly effective strains, or that the heterozygotes (n , N , etc.) are also ineffective with poor strains.

TABLE 7
Simply inherited host factors and bacterial strain factors that interact to cause ineffective symbiosis

	Bacterial strain	Host gene			
		i_1	ie	n	d
	7	—	.	.	.
	32	—	±	—	—
	210	—	.	.	.
	5	.	±	—	—
	1	.	.	—	—
	47	.	.	—	—
	0403	+	+	+	+
	0403211	—	+	.	.
	0408	±	+	.	.
	220	—	—	+	+
					and (+)
	30	—	+	+	+
				and (+)	and (+)
	35	.	.	—	+
					and (+)
	212	.	+	+	—
				and (+)	
	6	(+)	(+)	±	(+)
	33	(+)	(+)	(+)	(+)
	0404	(+)	(+)	.	.

+ Ineffective response with all plants homozygous for factor indicated.

± Ineffective response with a proportion of plants homozygous for the factor indicated.

(+) Unspecified ineffective responses, viz. those not known to be caused by interaction with the host factor indicated.

— Effective or partially effective response with all plants homozygous for the factor indicated.

Not tested.

This study reinforces Fergus and Hollowell's (1950) opinion that "red clover probably has the most adaptable and plastic genotype of any agricultural plant", and the complete genetic elucidation of its symbiosis may be impracticable.

A further paper will consider the morphogenesis and fine structure of n - and d -type ineffective nodules.

8. SUMMARY

1. Genetic evidence is presented for two recessive factors (n and d) either of which causes ineffective symbiosis in red clover.

2. The factor n causes failure in nitrogen fixation in nodules produced by

Rhizobium trifolii strains 0403, 30, 212 and probably also strain 220, but does not affect fixation in nodules produced by strains 1, 5, 32, 35 and 47. Nodule formation, number and size are unaffected by the *n* gene.

3. The recessive factor *d* causes failure in nitrogen fixation in nodules of red clover produced by strains 0403, 30, 35 and 220, but does not affect fixation in nodules produced by strains 1, 5, 32 and 212.

4. Plants homozygous for *d* bear more but smaller nodules than heterozygotes, but this factor does not affect the time the first nodules appear on the roots.

5. The factors *n* and *d* (and the factors *i*₁ and *ie*—Nutman, 1954*b*, 1957) are independent and non-allelic, and breeding results indicate that plants homozygous for more than one of these factors have much reduced viability or are non-viable.

Acknowledgments.—I thank Mrs Elinor McVey and Mrs Penny Coshier for able technical assistance and present and past colleagues, particularly Sir Gerard Thornton, F.R.S., for helpful discussion and criticism.

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