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Genetics of symbiosis and nitrogen fixation in legumes

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1. Introduction

Soon after the isolation of nodule bacteria in 1888, differences were recognized in the ability of bacterial strains to form nodules on particular host plants and in the nitrogen-fixing ability of the nodules so formed. These and other symbiotic heterogeneities were attributed, sometimes correctly, to bacterial strain differences, not then thought to be open to formal genetic analysis. The realization that the host plant was an essential component of this variability came only gradually, stimulated by observations of host varietal differences and by the demand for reliable and homogeneous material for experimental work. Only within the last two decades has host variability been studied by plant breeding, and bacterial strain differences by some of the methods of microbial genetics. This review, except for a brief reference to earlier work of some historic interest, will consider only genetic problems open to investigation by these methods.

The developmental sequence in all legume nodules is broadly similar. The initial infection phases are followed by the induction of the nodule, the invasion of part of the nodular tissue and culminate in bacteroid formation and nitrogen fixation; the genetics of symbiosis will be considered in this context.

2. Host varietal differences

Voorhees (1915) was the first to record differences in the nodulation of varieties of a legume and to speculate on their probable genetic origin. He grew six varieties of soybean in field trials and pot experiments and noted differences in the number, size and distribution of nodules on the roots.

Interest in varietal effects then appears to have lapsed until Bjälfve's (1933) report on the differential response of four vetches (*Vicia sativa*) and two clovers (*Trifolium repens*) varieties. Since then many reports have appeared on varietal differences in nodulation sometimes affecting yield and reference to these will be found in the following: For *Pisum* or *Vicia* Bjälfve (1933), Razumovskaia (1937), Federov & Svitych (1959), Krasilnikov & Melkumova (1963), Gelin & Blixt (1964); for *Medicago* and *Melilotus* Gibson (1962), Blair (1967); for *Glycine* or *Arachis* Duggar (1935), Boyes & Bond (1942), Johnson & Means (1960), De Mooy (1965), Hamatová (1965), Döbereiner, Arruda & Penteado (1966), Döbereiner & Arruda (1967); for *Phaseolus vulgaris* Franco & Döbereiner (1967), and for *Trifolium* Nutman & Read (1952), Nutman (1961*b*), Gibson (1964).

3. The genetics of host resistance (absence of nodule formation)

3.1. *Resistance to infection by non-specific bacteria*

Wilson (1939*a*) considered that hereditary factors in the host determine its susceptibility to nodule formation by strains of bacteria isolated from other legumes (i.e. from other 'cross-inoculation' groups). Using aseptic conditions he examined the cross-infection of a wide range of genera and species of legumes by strains of bacteria of very diverse origin, and found many exceptions to the cross-inoculation grouping then recognized and also observed large differences in the nodulation of individual seedlings of many species, which he attributed to genetic variation.

Because self-pollinating species appeared to nodulate with fewer strains of bacteria than those which were cross-pollinating, he concluded that the self-pollinating plants possessed fewer hereditary 'susceptibility factors' (Wilson 1939*b*). To test this concept he collected seed from individual seedlings of cross-pollinating species of legumes growing in isolated localities and tested them against a range of bacterial strains (Wilson 1946). Differences in susceptibility within a host species were thereby demonstrated.

Although the broad correlation between cross-infection and a cross-pollinating habit found by Wilson was probably fortuitous, his conclusion that hereditable host factors determine nodule formation by particular strains of bacteria was correct and has been confirmed by later work.

3.2. *Resistance to infection by specific bacteria*

The problem of susceptibility has also been approached through a study of individual non-nodulating or resistant plants that are occasionally found in species generally nodulating readily, such as red clover (*Trifolium pratense*) and soybean (*Glycine max*), and by experiments on interspecific hybridization.

The author selected a number of such resistant plants of red clover for breeding but only three survived to maturity. From one of these a resistant line was raised in which resistance was attributable to a recessive factor (r) acting in conjunction with a cytoplasmically transmitted component (σ) (Nutman 1949*a*). Breeding experiments indicated that the cytoplasmic factor was produced in r homozygotes, but only if some of the factor was already present in the embryosac; zygotes homozygous for r but not containing σ cytoplasm did not survive. The gene r and possibly also similar factors interacting with σ to give resistance, were shown to be fairly common in the wild population, the rarity of resistant plants being due to the rarity of σ cytoplasm. Resistance plants remained without nodules when inoculated with a range of strains of nodule bacteria or when planted in unsterile soil.

In addition to preventing nodule formation, the $rr\sigma$ genotype reduced plant vigour, caused some chlorosis and increased leaf hairiness. The r gene did not appear to be linked with sterility (S) alleles or with factors affecting other aspects of symbiosis. Neither resistance nor susceptibility were transmissable by grafting. The roots of resistant plants stimulated the multiplication of nodule bacteria in the rhizosphere, and the hairs of resistant plants were curled by bacterial secretions, but no infection threads were observed in them. Resistance attributable to *rrσ* therefore occurs at the root hair surface and may be caused either by the production of some factor conferring resistance or by the failure of the host to produce something necessary for infection to take place.

The two other original resistant selections described by Nutman (1961*a*) were sister plants which had no seed when crossed together, but when crossed with sister nodulating plants produced twenty-one nodulating plants, two resistant plants and a high proportion of abortive seeds. Nodulating plants of the same family when inter-crossed also segregated a few resistant plants, none of which, however, survived for breeding, so that the nature of their hereditary resistance could not be determined.

Aughtry (1948) examined susceptibility and resistance in medics by hybridizing *Medicago sativa* and *M. falcata* (both autotetraploid species) and compared the nodulation of the F_1 and F_2 clones with the parent plants (using stem cuttings grown under sterile conditions). Strains of bacteria were employed that gave contrasted nodulation (presence or absence) on the parent material. The technique was not wholly satisfactory because no clone gave 100% nodulation with any strain of bacteria. Nevertheless, large, consistent and statistically significant effects were found between clones. Nodule formation was dominant over the absence of nodules, clones of F_2 plants derived from nodulated parents were more often nodulated than those of F_2 plants from parents without nodules, and nodulation differences between host families were affected significantly by the bacterial strains used in the tests. Aughtry's results gave no indication of major genes controlling susceptibility in these species.

Williams & Lynch (1954) described a non-nodulating mutant of soybean (*Glycine max*) in a backcross involving the varieties 'Lincoln' and 'Richmond'. Failure to form nodules was caused by a single recessive factor, called *no*. Plants homozygous for this factor formed nodules very rarely when grown in soil, but occasionally produced some nodules when inoculated with certain strains of *Rhizobium japonicum* or grown in water culture (Clark 1957). Research is now directed towards elucidating the function of the *no* gene, and its dominant allele, so far without success (Hubbell & Elkan 1967). Induction of polyploidy in resistant and susceptible soybean lines indicated that resistant roots were not deficient in the tetraploid cells (Tanner & Anderson 1963).

The *no* factor has been introduced into other soybean varieties and a number of isogenic lines differing only in the possession of this factor are now available and have proved of agronomic value as control material for assessing nitrogen fixation. Resistant soybean lines given nitrogen fertilizer produce yields equivalent to that of nodulated lines without fertilizer (Weber 1966*a*, *b*).

Resistance has also been studied by grafting experiments. Reciprocal grafts of resistant (*no*) and susceptible scions and roots of soybean showed that nodulation is determined by the genotype of the root. This result is similar to Nutman's (1949*a*) with susceptible and resistant red clover plants and Richmond's (1926) with *Phaseolus vulgaris* and *P. lunatus*, which require different strains for nodulation. Rudin, Popapov & Germanova (1953) using inter-family grafts of legumes and non-legumes, obtained nodules on roots of peas (*Pisum*) grafted with buckwheat (*Fagopyrum*) and horse bean (*Vicia*) grafted with *Nasturtium*, the number of nodules depending on the vigour of the grafted scion. Nodules were formed only on roots of leguminous plants. Bonnier, Hely & Manil's (1952) report of nodule formation on soybean roots by *Rhizobium trifolii*, *R. leguminosarum* etc. grafted to the corresponding scion (*Trifolium pratense*, *Pisum sativum*, etc.) was obtained in experiments without bacteriological control and is therefore open to question. Hely, Bonnier & Manil (1953) and Evans & Jones (1964) also obtained some effect of scion on the nodulation of stock using grafts of *Trifolium hybridum* and *T. ambiguum*.

Attempts to introduce the Caucasian clover, *Trifolium ambiguum*, to European and American agriculture failed because it did not nodulate satisfactorily with local strains of *Rhizobium trifolii* (Keim 1954, and others). There are many similar instances of specificity within a cross-inoculation group, but the *T. ambiguum* example is of particular interest because the clover group is the best defined of the cross-inoculation groups, being restricted to a single genus—*Trifolium*.

Hely (1957) investigated the nodulation of diploid and polyploid races of *T. ambiguum* in aseptic culture using a wide range of bacterial strains isolated from Turkey and New Zealand, and although no breeding experiments were done the correlations he established are of genetic interest. Seedlings of hexaploid races of *T. ambiguum*, when inoculated with strains of *R. trifolii* from Turkey, formed their first nodules mostly between the first and third week after germination, a few plants nodulated later than this and a smaller number remained unnodulated. Diploid races nodulated considerably later, no nodules forming before two weeks, about one fifth of the plants nodulating after 3 weeks and a similar proportion remaining without nodules.

Strains of bacteria from New Zealand selected for their ability to form some nodules on *T. ambiguum*, nodulated the hexaploid races, but much later than the Turkish strains and about half the inoculated plants were without nodules at eleven weeks, i.e. were resistant. The diploid race of *T. ambiguum* was completely resistant to the New Zealand strains.

Similar relationships were found for the number of nodules formed on roots of young plants. Hexaploid races inoculated with Turkish strains produced the greatest number and diploid races inoculated with New Zealand strains the fewest nodules, or none at all. These results suggest that resistance in this species may be caused by the accumulation of factors for lateness and sparseness of nodulation. Hely noted that 'resistant' plants transferred into soil sometimes formed a few nodules after a further considerable lapse of time. If sparseness and lateness are recessive characteristics they would tend to accumulate in partially inbred lines derived from small populations of introduced seed, with which this work was done, and more so in diploids than in hexaploids. Evans & Jones (1964) showed that sexual hybrids between *T. ambiguum* and *T. hybridium* (raised by embryo culture) nodulated satisfactorily.

4. Inheritance of factors affecting nodule initiation and growth

4.1. *Early and late nodule formation*

In most of the work outlined above, the cause of nodules failing to form was not studied; nodules were recorded only when seen with the unaided eye. Nodule formation is the consequence of a complex sequence of events starting outside the root, and this can fail at different stages of development.

Many legumes are initially infected through their root hairs, but the numbers of hairs that become infected differ greatly in different seedlings, as also does the proportion of such infections that develop into nodules. This aspect of infection has not been studied genetically although Nutman (1967) and P. J. Dart, P. S. Nutman, C. Roderigo-Barreuco & R. Roughley (unpublished) have shown differences in numbers of root hairs infected between certain varieties and hybrids of subterranean clover.

Only a small proportion of infection threads usually continue growth into the cortex and become associated with a meristematic centre to form a nodule; the rest abort at different stages in development. The time nodules first appear as macroscopic organs on the root therefore depends on: (1) the time elapsing before the hairs become infected, (2) the delay caused by the first formed infection threads aborting, (3) the time taken for the infection thread to grow from the hair to the cortical cells from which the nodule develops, and (4) the rate the young nodule grows.

Stages 1 and 2 last longer and possibly vary more in duration than stages 3 and 4, although genetic work has so far considered only the whole process. Earliness or lateness of nodule formation is usually assessed by the age of the seedling when the first nodule forms (initial nodule formation). This has been shown to be hereditable in *Trifolium pratense*, probably multifactorily controlled, although major genes may also influence the time of initial nodulation as in a dwarf form of *Trifolium subterraneum* found in a cross between the varieties 'Tallarook' and 'Morocco' which nodulates later than normal plants (Nutman 1967). Variability in time of initial nodulation is large in *T. ambiguum* (Hely 1957, 1963), *T. pratense* (Nutman 1946*a*, 1949*b*, 1953) and *T. repens* (Jones 1963) which are cross-pollinated and self-sterile species, and small in *T. subterraneum* (Nutman 1967) which is cleistogamous, and *T. glomeratum* (Darbyshire 1966) which is self-pollinating. Most differences in time of initial nodulation in subterranean clover are independent of differences between bacterial strains in the promptness with which they nodulate (Nutman 1967). Initial nodulation is the same in diploid and autotetraploid races of *T. subterraneum*, whereas in *T. ambiguum*, as noted above, it is later in diploid than hexaploid lines, and in *T. pratense* diploid lines nodulate earlier than tetraploids (Nilsson & Rydin 1954). Early and late lines of red clover equally stimulate the multiplication of nodule bacteria in the rhizosphere (Purchase & Nutman 1957). Clover plants when grown in pairs in a small volume of medium form fewer nodules than when grown singly. This inhibitory effect is stronger with early than late nodulating plants (Nutman 1953). The presence of small amounts of nitrate or nitrite in the root medium slightly delays the initiation of the first nodule, encourages hair infection (Gibson & Nutman 1960; Darbyshire 1966) and lessens the difference observed between the nodulation of early and late lines (Nutman 1957*a*). But if the inoculation of the young seedling is appreciably delayed the difference between the nodulation of 'early' and 'late' lines disappears.

These results suggest that selection for earliness is for either prompt primary hair infection, for continued infection thread growth from the hair into the cortex, for early and rapid nodule primordial development, or for a combination of these effects. An early nodulating habit is advantageous particularly for annual species grown in nitrogen-starved soils.

4.2. *Host control of nodule number*

Some of the first records of host varietal differences refer to nodule number although this characteristic is influenced by many factors of strain and environment. Woodworth & Sears (1936) showed that the soybean variety 'Peking' formed fewer nodules than 'Illini' and by crossing and selection they demonstrated that nodule number is inherited. Similar conclusions were drawn from varietal comparisons or from breeding experiments for *Lespedeza* by Woodworth & Sears (1937), for varieties of *Glycine max* by Shulyndin (1953), Döbereiner *et al.* (1966), and Weber (1966*a*), for *Pisum sativa* by Gelin & Blixt (1964), and Federova (1966), for *Centrosoma pubescens* and *C. plumeri* by Bowen (1959) and Bowen & Kennedy (1961), for *Trifolium repens* by Jones (1962, 1963), for *Trifolium pratense* by Nutman (1946*a*, 1948, 1953, etc.) and for *Trifolium subterraneum* by Nutman (1967).

Polyploidy affects nodule number differently in different species. In *T. ambiguum* (Hely 1957), *T. repens* (Nilsson & Rydin 1954), *Glycine max* (Oinuma 1952) and with certain strains of *Rhizobium trifolii* with *T. repens* (Weir 1961*a*) polyloids formed more nodules, but in *T. subterraneum* (Nutman 1967) autotetraploids formed fewer nodules than diploids. Weir's (1961*b*) work on $2n$ and $4n$ races of *T. pratense* and *T. repens* suggests that the influence of ploidy on nodule number also varies with conditions of growth.

Yield is correlated with the formation of many nodules in soybeans and in pea varieties that had similar ancestry. Of the 25 varieties of soybean examined by Döbereiner *et al.* (1966), four lines bred for commercial use gave poor yields in nitrogen-deficient soil because of their poor nodulation. Sparsely nodulating plants of white clover grown in aseptic culture yielded more than those with many nodules but sparsely and abundantly nodulating selections of red clover and of subterranean clover yielded the same (Nutman 1948, 1967), but Rubenchik, Bershova & Yurchenko (1967) showed that strains of bacteria isolated from tetraploid red clover formed more nodules and were more effective on tetraploid than diploid lines.

Gelin & Blixt (1964) suggested that nodule number in the pea varieties studied may be controlled by two major genes, abundant nodulation being recessive over sparse nodulation. A single recessive gene in red clover causing early nodule degeneration (see next section) also resulted in the production of many nodules (Nutman 1954*b*). In subterranean clover two independent and simply inherited genetic factors affecting nodule number or size have been described. One is the dwarfing factor, mentioned above which causes sparse as well as late nodulation. The other, for which the genetic evidence is incomplete, affects nodule size with a particular bacterial symbiont but does not affect nodule number, contrary to the general relation between nodule size and number (Nutman 1967).

Crosses between sparsely and abundantly nodulating plants of red clover bear intermediate numbers of nodules (Nutman 1948), whereas in subterranean clover the habit of abundant nodule formation in hybrids is dominant over sparse nodulation, and nodule number usually ranges more in F_2 than in the parents. Further selection for nodule abundance in subterranean clover usually increased nodule number above that of the parent variety with most nodules, but selection for nodule sparseness did not reduce nodule number below that of the sparse parent (Nutman 1967). All work with other species also suggests that nodule number is inherited in a complex manner. Sparsely and abundantly nodulating selections of red and subterranean clovers have been of value in physiological studies of nodule formation on young seedlings, and in investigating the factors that control the total amount of nitrogen-fixing tissue formed by the mature nodulated plant. In red clover, but not in subterranean clover, nodule formation and lateral root formation appear to be controlled by the same or linked genetic factors, sparsely nodulating selections forming few lateral roots and abundantly nodulating selections many lateral roots (Nutman 1948). In both clovers uninoculated young seedlings at first produce more lateral roots than inoculated seedlings, suggesting that nodules initially arise at foci that otherwise form lateral roots. Further work with red clover employing delayed inoculation and nodule excision showed that the continued formation of infectable foci was inhibited by the formation and growth of nodules and roots already present, the large nodules of sparsely nodulating plants having a greater inhibitory effect than the small nodules of abundantly nodulating plants. It was suggested that this constituted a self-regulatory mechanism controlling nodule number and size and as a result, the amount of nitrogen fixed (Nutman 1949*b*, 1952).

This hypothesis was further investigated in sparsely and abundantly nodulating plant selections of subterranean clover which, though differing several-fold in the number of nodules formed, were indistinguishable in yield. Nodule number and nodule length were inversely related such that aggregate nodule length tended towards a constant value independently of the number of nodules present. A study of the anatomy of nodules from both abundantly and sparsely nodulating plants showed that the whole nodulated plant formed substantially the same amount of active nitrogen-fixing tissue irrespective of the number of nodules formed; the average rate of nitrogen fixation per unit of volume of 'bacteroid tissue' including uninfected cells, was also the same, namely, about 22 $\mu g\ N\ mm^{-3}\ d^{-1}$.

A controlling mechanism to ensure the efficient balance of supply and use of fixed nitrogen is 'advantageous', to the host, otherwise either insufficient or an excessive amount of nodule tissue might be formed. Similar relationships concerning nodule number and size were found with ineffective (non-N-fixing) strains of bacteria and with partially effective symbioses, the actual number of nodules depending on how soon the nodule meristems ceased to grow. The process of control suggested would have greatest adaptive effect for fully effective (N-fixing) symbiosis and least for ineffective symbiosis.

5. The genetics of symbiotic effectiveness

In spite of the theoretical interest and practical importance of the efficiency of the nitrogen-fixing process within the nodule, the scope of genetic work on this aspect is limited. So far work is reported only for four species of the genus *Trifolium* and two species of *Lotus*.

5.1. Lotus *species hybrids*

Strains of *Rhizobium japonicum* effective with *L. corniculatus* are usually ineffective with *L. uliginosus* and vice versa (Erdman & Means 1949; Jensen 1967). Gershon (1961) has investigated this relationship by interspecific hybridization using diploid and tetraploid lines tested with bacterial strains effective on one or other species, but not both. Most hybrids and selected lines responded effectively with strains effective with *L. uliginosus*, but the results indicated that several genes in different linkage groups may determine nitrogen-fixing specificity. A chromosomal dose effect was also observed; the triploid hybrids nodulated effectively with fewer bacterial strains than those involving tetraploids.

5.2. *Trifolium ambiguum*

Though not confirmed by breeding experiments, Hely's (1957, 1963) studies on $2n$, $4n$ and $6n$ lines of *T. ambiguum* are relevant for the correlations he established between effectiveness and other characteristics. As with nodule formation already discussed, individual plants differed greatly in their ability to fix nitrogen. Even with the best bacterial strains many plants in each ploidy class responded ineffectively or in a poorly effective manner; the diploids showed the least and the hexaploids the greatest effectiveness. Good effectiveness was correlated with early nodule formation and with an increase in nodule number and total nodule volume. Late nodulation, caused by delayed inoculation made the response still less effective on this species, as in red clover (Nutman 1949*b*).

Effectiveness in this species seems to be determined by many factors. Its extreme variability is probably related to its obligatory cross-breading habit as noted above.

5.3. *Trifolium subterraneum*

Nutman (1961*b*) examined the symbiotic effectiveness of fifteen varieties and hybrids of subterranean clover with the effective strain *Rhizobium trifolii* SU 297, using aseptic plant culture. Of five diploid and autotetraploid lines, only one tetraploid variety ($4n$ 'Dwalganup') yielded more than the diploid. Individual plant variation, which was very small within varieties, increased slightly in hybrids. Slight hybrid vigour affecting symbiotic effectiveness was observed in hybrids and F_2 families, but all attempts to breed higher yielding lines failed. No ineffectively responding plants segregated in any F_2 and of the many thousands of plants examined in this study only two were completely ineffective in their response (both in varieties, not in hybrids). These plants on selfing gave normal progeny over several generations.

As mentioned in an earlier section, the 'Tallarook' × 'Morocco' cross gave dwarf plants which nodulated late and formed few nodules. Dwarfs yielded less than normal plants but there was no evidence that symbiotic factors are concerned in the small yield; nodule anatomy and bacteroid morphology were normal. Some tests were done with several strains of bacteria varying from fully effective to completely ineffective. No significant host × strain interactions were found.

Gibson (1964) found that hybrids of the subterranean clover variety 'Northam First Early' with certain other varieties produced segregants in F_2 that responded ineffectively with the effective bacterial strain NA 30, not used by Nutman. Gibson ascribed this ineffectiveness to a major gene (recessive); the ineffective plants were otherwise normal.

The general lack of variability and the highly effective response shown by this species with effective strains of bacteria may, like its early nodulating habit, be a result of natural selection for effectiveness in a species growing in soils deficient in nitrogen, either in the region of its natural distribution or as an agricultural pasture legume.

5.4. *Trifolium repens*

Jones (1962, 1963) crossed highly effective selections of S100 'No Mark' and found that certain F_1 families yielded about 10% more in test tube culture than the original variety. This increase disappeared in later generations probably because of inbreeding depression. Further selection again gave increases in F_1.

5.5. *Trifolium pratense*

Red clover like white clover, is extremely variable and Nutman (1954*a*, etc.) examined the basis of this heterogeneity by selecting for high effectiveness, by examining the influence of inbreeding and by selection for ineffectiveness.

Nutman & Mareckova (1967) compared many crosses between plants which were normally effective with crosses between highly effective selections, and found that some but not all crosses among highly effective plants contained significantly more plants in the high yielding classes and appreciably fewer in the low yielding classes, with about equal numbers in the modal classes. The average difference in yield between the two main categories was 5 % over all crosses.

The effect of inbreeding in lessening symbiotic effectiveness was examined in three pedigrees which did not segregate simple factors for ineffectiveness (Nutman 1957*b*). The decline in effectiveness was correlated with the degree of inbreeding, and showed as an increase in the proportion of plants responding in an intermediately effective manner.

The complex genetics of symbiotic effectiveness in this species is also shown by studies on the adaptation of local varieties of host to local races or strains of bacteria, and by records of changes over a period of time in the response of a variety to a particular strain of bacteria. Nutman & Read (1952) examined the response of several local varieties of British and Swedish red clover with nodule bacteria isolated from the localities from which the plant varieties were obtained. All combinations of plant type and bacterial isolate were examined in glasshouse conditions at Rothamsted, England. The Swedish (but not the British) material showed a small but significant adaptation associated with locality. Each local variety of host responded most effectively with bacteria isolated from its own locality (5 % yield difference). The differences within the Swedish material were attributed to the very long-standing use of clearly defined local varieties of clover; in the U.K. the local varieties were of comparatively recent origin.

The history of the effectiveness of the bacterial strain 'Coryn' also provides evidence of hereditary changes in the host. This strain originally isolated in 1933 (Chen & Thornton 1940) is a stable ineffective strain on red and white clover that has been used in a great variety of investigations. Kleczkowska (1958) reported that this strain occasionally responded partially effectively with the red clover variety 'Late-flowering Montgomeryshire' (L.F.M.). All attempts to isolate effective mutants failed but tests made with the Coryn strain from the Rothamsted Collection, and Coryn strains recovered from other collections, on other varieties and on old seed of L.F.M. suggested that the host variety had changed over the years so as to give some degree of nitrogen fixation with Coryn. Examination of records of response of L.F.M. to Coryn showed that effectiveness first appeared in this variety in about 1954. Some Swedish varieties were shown to possess the factor, or factor complex that allows effective symbiosis with 'Coryn' and others did not (Kleczkowska 1959). The inheritance of the partially effective resonse of the L.F.M. variety was confirmed by breeding. Ineffectively responding plants of L.F.M. crossed *inter-se* gave a mostly ineffective progeny, crosses between effectives and ineffectives were mixed in their response and crosses among effectives gave effective response in some families but not in others.

5.5.1. *Major genes determining ineffectives in* Trifolium pratense (*factors ie*, i_1, *n and d and their interrelation*).

The remaining work with this species has been directed towards a study of the genetics of the ineffective response. Nutman (1954*a*) showed that 2 to 4% of individual plants of common varieties formed nodules that did not fix nitrogen with the particular effective strain employed in the work-strain 0403 formerly A121111 (Bascomb 1965). A further 20% responded poorly or suboptimally. In these experiments plants scored as ineffectives were visually normal in other respects.

Altogether more than fifty ineffective plants were selected for study of which twenty survived for crossing. Evidence for simple inheritance of the ineffective response was found in five pedigrees of which four were studied intensively and are discussed separately below. In all pedigrees the effective condition was dominant.

The ie gene

Nodules formed readily on *ie* homozygotes, their numbers and sizes covering the same ranges as on heterozygotes or dominant homozygotes. The development of ineffective *ie* nodules diverged from the effective pattern at the stage when bacteria are released from infection threads. Infection threads were abundant and more frequently branched and vesicular than in effective nodules. The bacteria in ineffective nodules multiplied little and were not transformed into bacteroids. Concurrently the infected host cell, and often neighbouring uninfected cells, were stimulated to divide repeatedly to produce an irregular small-celled 'tumourized' tissue, which was tetraploid as in effective nodules. Leghaemoglobin was not formed and much carbohydrate accumulated in the nodule.

All attempts to alter the response of strain 0403 with *ie* homozygotes by plant passage and by isolation from large nodules failed. Tests with other strains of bacteria showed that the *ie* line responded in a partially or fully effective manner with certain other effective strains. In nodules formed by such strains, in which the effect of the *ie* factor is partially or totally suppressed, the central region of the nodule contained mixtures of 'tumourized tissue' and normal bacteroid tissue in varying proportions (Bergersen & Nutman 1957).

Although Nutman (1957*b*) gives extensive evidence for the existence of the *ie* factor for ineffectiveness, not all F_2 and backcross families segregating *ie* gave the expected proportion of effective and ineffective plants, the deficit of ineffectives being attributed to suppressor or modifying genes.

The i_1 gene

Nodules of i_1 plants at first developed normally but degenerated prematurely and the invaded cells did not form bacteroids. Plants homozygous for i_1 formed more than twice as many nodules, but much smaller ones, than effective plants. Leghaemoglobin was not formed and carbohydrate accumulated (Bergersen & Nutman 1957). This gene, unlike *ie*, was only occasionally obscured by other hereditary host factors. One such factor was a simple recessive (*m*), the double recessive *mm*, $i_1 i_1$ responding effectively. The genes i_1 and *r* (resistance factor) segregated independently (Nutman 1954*b*).

Tests of i_1 plants with a wide range of bacterial strains showed that the ineffective response was confined to strain 0403; with all other strains i_1 plants responded like the parent variety.

Repeated passage of strain 0403 through i_1 plants failed to alter the response, but two kinds of stable mutants of this strain, which responded differently with i_1 plants, were isolated from nodules. One mutant was wholly effective with i_1 plants and another responded effectively with some i_1 plants and not others, the plant breeding results indicating that this effective response was also controlled by a single gene.

The n gene

The central zone of the small nodules of *n* ineffectives is sparsely infected by bacteria, and the proportion of uninfected cells is much less than in normal nodules. Ineffectives homozygous for *n* form nodules at about the same time and of the same size as those on sister effective plants. Neither bacteroids nor leghaemoglobin forms, and storage carbohydrate is abundant. Among the families segregating for this factor were some characterized by the late development of an effective response in a proportion of plants homozygous for the *n* factor. The genetics of this modification of the expression of the *n* gene was not further studied. In contrast to the *ie* and i_1 ineffectives, *n* homozygotes responded ineffectively with three of eight strains unrelated to 0403 and showed a range of effective response with the remaining five strains similar to that given by unselected clover (Nutman 1968).

The d gene

Ineffectives homozygous for *d* formed their first nodules at the same time as effective plants. These were rather fewer and smaller than on effective plants. Cells of the interior of nodules on *d* homozygotes formed by strain 0403 were mostly uninfected, some were tumourized. Leghaemoglobin was not formed and nodules contained much carbohydrate. Ineffectiveness caused by the *d* factor was infrequently affected by other host factors, although there was good evidence for modifying genes in certain families that were not further investigated (Nutman 1968).

The response of plants homozygous for the *d* gene was examined with seven strains of bacteria unrelated to strain 0403. Ineffectiveness was shown with three strains, two of which showed similar specificity when tested on *n* homozygotes the other strains responded effectively with *n* homozygotes.

The interrelation of the genes ie, i_1, n and d

Plants homozygous for each of the above factors were crossed together in most combinations and the response of their progenies and F_2s examined with strain 0403. The crosses between ineffectives gave normal effective progeny, and the F_2s all segregated in the proportion seven ineffectives: nine effectives, showing that the four factors were non-allelic and independent. Experiments designed to study the linkage relationships between certain pairs of these factors failed because none of the ineffectives selected in F_2s was found to be homozygous for two recessive factors; the double recessives were probably non-viable.

Failure to establish an effective symbiosis in red clover can thus be ascribed to at least four simply inherited and independent recessive host factors, which prevent normal effective development at different stages, the sequence of gene action probably being $d \rightarrow n \rightarrow ie \rightarrow i_1$. Nothing is known of the underlying metabolic events caused by these genes.

6. Effect of colchicine and mutagenic agents

The nuclei of nodule cells are largely tetraploid (or of higher ploidy (Mitchell 1965; Funke 1957; Kodama 1967)), and the infected polyploid tissue may originate from tetraploid cells present in the roots before infection or may arise subsequently (Bhaskaran & Swaminathan 1954). Pretreatment of roots with small concentrations of colchicine is reported to stimulate the formation of nodules in *Medicago sativa*, in several species of *Trifolium* and in *Ornithopus sativus*, including $2n$ and $4n$ lines (Bonnier 1954; Migahid, Elnady & Rahman 1959; Weir 1961*b*; Trolldenier 1959), but van der Starre, van der Molen, Bossink & Quispel (1967) sometimes failed to stimulate nodulation of *Trifolium pratense* with colchicine. It is not known whether colchicine stimulates nodulation by increasing the number of tetraploid cells in the root cortex or acts in some other way.

Migahid *et al.* (1959) investigated the effect of radiation of the seed on the nodulation of *Trifolium alexandrinum* and *Trigonella foenum-graecum*. A total dose of about 800 r (over a period of 7 days) had an optimal effect in doubling the numbers of nodules produced and in increasing lateral rooting; nodule number also varied more after irradiation. N. S. Subba Rao (unpublished) found little effect on nodule number of irradiation up to 100 kr of seed of *Trifolium glomeratum*. Strong irradiation with γ-rays decreased nodulation and inhibited nitrogen fixation in *Pisum sativum*, and led to the bacteria mutating within the nodule (Hamatová 1966).

Changes observed in nodulation in the field or in controlled cultural conditions have sometimes been ascribed to an effect of the resistance of the bacteria within the tissues of the host. Wünschik (1925), Allen & Baldwin (1931) recorded regular changes in effectiveness of the bacteria in plant passage. Virtanen (1945) and Nutman (1946*b*), using pure lines of bacteria, were unable to confirm any regular effect of plant passage but recorded rare mutational changes in effectiveness.

Bonnier (1950) and Hamatová (1963) with soybean, Hamatová-Hlávačková (1963) with lucerne and Saric (1956) with cowpea (*Vigna*), etc., growing in soil not containing the appropriate bacteria, observed slow change in the effectiveness of nodulation thought to be adaptive under the influence of the host plant. An alternative explanation could be the selective multiplication of the very few bacteria that may have been present originally or introduced on the seed or in some other way. Vanderveken (1964) showed that symbiotic effectiveness in white clover is destroyed by infecting the plant with clover phyllody virus, but that bacteria from nodules on infected plants possessed the characteristics of the parent strain when isolated and inoculated to virus-free hosts. In contrast Joshi, Carr & Jones (1967) and Joshi & Carr (1967) claimed that the bacteria were altered by virus infection of the plant. Richmond (1926) also claimed an effect of host plant on bacteria in experiments with grafted plants in which, although the scion did not affect the susceptibility of the root, seed from grafted plants were nodulated by strains specific to both scion and root stock. Joshi *et al.* and Richmond's results are difficult to interpret and await confirmation.

7. Rhizobium genetics

Because of the importance of non-symbiotic marker genes in the microbial techniques of recombinations, transformation and transduction, it is appropriate to introduce this section with a brief account of some general genetical researches in *Rhizobium*.

Markers most employed are antibiotic resistance and biochemical mutants obtained by selective procedures with or without the aid of mutagenic agents. Balassa (1954, 1957) and Balassa & Gabor (1961) transformed streptomycin resistance, and Gadre, Mazumdar, Modi & Parekh (1967) penicillin resistance between species of *Rhizobium* and in some of this work the transforming *DNA* of the donor strain was shown to be inactivated by *DNA*-ase. Balassa (1960) transformed cysteine independence and Gadre *et al.* (1967) transformed fructose dependence between species. Schwinghamer (1960) used X-rays to produce a range of biochemical mutants suitable for genetic work. *Rhizobium* has been used as an acceptor bacterium for successful transformation of tumour-inducing capacity from *Agrobacterium tumefaciens* (Klein & Klein 1953; Kern 1964).

Lysogeny has been recorded in *Rhizobium trifolii* by Marshall (1956), Takahashi & Quadling (1961), Kleczkowska (1965*a*) and Schwinghamer & Reinhardt (1963), in *Rhizobium meliloti* by Szende & Ordögh (1960) and Kowalski (1966) and in *Rhizobium leguminosarum* by Schwinghamer & Reinhardt (1963), and in strains from three cross-inoculation groups by Davies (1958). Bacteriocins giving rise to lysis in *Rhizobium* have been found in strains from all cross-inoculation groups (Roslycky 1967). In none of these studies have induced phages been sufficiently long-lived or stable for genetic studies, and as yet there is no authenticated record of transduction in *Rhizobium*.

Genetic studies on the symbiotic properties of *Rhizobium* are therefore limited to simple mutational records, to the study of direct action of phage and other selective agents on symbiotic properties or to studies on transformation.

Krasilnikov (1941*a*, 1945, 1958), by growing *Rhizobium trifolii*, *R. meliloti* and *R. leguminosarum* separately in each other's culture-filtrates over long periods of time, was able to transfer nodule forming ability from one strain to another, and also it is claimed to a species of soil *Pseudomonas*. These changes may have been caused by the presence of *DNA* in the culture filtrates, although the filtrates were not prepared with this end in view. Izrail'skii (1954, 1957), using similar techniques, was unable to induce *Rhizobium trifolii* to form nodules on lucerne after prolonged culture in culture filtrates of *R. meliloti* or vice versa. Using *DNA* preparations some transformations of nodulating capacity between *Rhizobium* species have been reported; *Rhizobium japonicum* to nodulate lucerne (Balassa 1956) and *R. meliloti* to nodulate lupin (Balassa 1960). Using *Rhizobium* polysaccharide preparations, Ljunggren (1960) restored virulence to an avirulent mutant of *R. trifolii*, and Lange & Alexander (1960) transformed strains of *R. japonicum*, *R. meliloti* and *R. trifolii* to nodulate their unaccustomed hosts. In both these studies the activities of the polysaccharide preparations were destroyed by treatment with *DNA*-ase, indicating that the active principle in the extracts was *DNA*. In Ljunggren's experiments the transformed virulent strains, like the donor virulent strains, caused the plant root to secrete polygalacturonase and Ljunggren & Fåhraeus (1961) showed that the amount of polygalacturonase produced was not related to quantitative differences in strain infectiveness or host susceptibility.

The donor strain used by Ljunggren and the strain from which the recipient avirulent mutant was derived were both effective in fixing nitrogen on the test clover used, and the nodules produced by the transformed strain were also effective. In all other instances the nodules produced by the transformed strain were ineffective. However, Balassa (1960) observed with his interspecific transformations that reisolated transformants when repeatedly re-transformed by *DNA* from the original donor strain eventually produced a few effectively nodulated test plants, suggesting that strain effectiveness is controlled by several factors that are not all transferred at one time. Wagenbreth (1965) recorded very infrequent transformations of strain specificity and warns against the possibility of some donor cells surviving the *DNA* extraction.

Kleczkowska (1965*b*) studied the *DNA*-mediated transformation of symbiotic effectiveness in *Rhizobium trifolii* using related and unrelated effective and ineffective strains. The ineffective characteristic was transferred fairly frequently but ineffectives were not transformed into effectives.

The selective or mutagenic action of bacteriophage was first studied by Krasilnikov (1941*b*), who showed that phage-resistant forms of *R. trifolii* differed from parent strains in morphology and in their capacity to nodulate clover. Kleczkowska (1950, 1964) showed that phage-resistant mutants tended to be ineffective, and that wild-type ineffective strains treated with phage did not give rise to effective variants but that ineffective mutants occasionally reverted to effectiveness when treated with phage. Kleczkowska (1950, 1964) also examined the effect of phage, of *DNA* from an ineffective donor, and both phage and *DNA* together, on the response of an effective recipient strain. Phage had the smallest effect and the effect of phage and *DNA* together was approximately additive.

8. Discussion

Because the genetic evidence is often lacking, certain categories of bacterial variation have been omitted from this account or mentioned only briefly, and for this reason the conspectus of variation that emerges is biased in favour of the host. However, even a cursory examination of the extent of symbiotic variation shows that host and bacterial differences cover similar characteristics and range. Thus hosts vary in cross-infection susceptibility, strains vary in cross-infection virulence; hosts are infected early or late or bear few or many nodules, strains infect early or late and nodulate sparsely or abundantly; host factors determine whether or not nitrogen is fixed in nodules, and strains are effective or ineffective. Many other minor features of nodule morphology and activity could be added to this list. The study of each facet of variation therefore poses the problem of its source, and even when definite genetic factors have been implicated in one symbiont, these have meaning only in relation to the symbiosis.

Variation in the initial infection of the root and the induction of the nodule are predominantly quantitative in their expression and are commonly associated with polygenetic inheritance. An exception might be the inheritance of initial susceptibility, which in the two examples so far studied is inherited simply. Major genes can affect any stage of development but have been more often described as influencing the later intracellular stages, causing radical changes in development, or even its complete arrest, resembling incompatability or hypersensitivity reactions in pathology. Such deleterious effects are associated with recessive host factors and seem in red clover to be rather readily affected by modifying and suppressor genes. The four independent factors for ineffectiveness described in red clover, although affecting very closely related processes in normal development, can be assigned to a definite sequence of activity, and it is to be hoped that further work will elucidate the metabolism and biochemistry of the events controlled by these factors.

Whether bacterial genes will show parallel features and behaviour remains to be discovered; certainly the patterns of host-strain specificity are complex, and the work on red clover suggests that some major host and bacterial genes are complementary, the expression of one being wholly dependent on the other. These generalizations are tentative because they are based on only a few studies in which the choice of material was governed more by technical and physiological than genetic considerations.

Correlations between symbiotic and non-symbiotic characteristics have been little studied, but the work on clovers indicates a close genetic and morphogenetic connexion between nodule formation and lateral root formation and an outline is suggested of the mechanism that regulates root and nodule development and the amount of nitrogen fixed.

There is not substantial evidence that the genotype of one symbiont is directly affected by the other, although there are good reasons for thinking that evolutionary processes act on the combined symbiotic phenotype. More work is needed before variation can be systematically exploited by plant breeders and microbial geneticists to improve legumes as nitrogen fixers but indications are that eventual benefits will be worthwhile.

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