SYMBIOTIC EFFECTIVENESS IN NODULATED RED CLOVER

IV. THE INFLUENCE OF THE HOST FACTORS i_1 AND ie UPON NODULE STRUCTURE AND CYTOLOGY

F. J. BERGERSEN and P. S. NUTMAN
Division of Plant Industry, C.S.I.R.O., Canberra, Australia

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1. INTRODUCTION

In previous papers Nutman (1954*b*, 1957) has described two independent recessive host genes (i_1 and ie) each of which causes ineffective symbiosis

TABLE 1
Symbiosis with bacterial strain

Plant lines	Bacteria strains					
	Related strains			Unrelated strains		
	A	A211	f_{12}	C1F	SU297	Haldon 1
Controls : unselected or heterozygous plants	E	E	I	E	EI	EI
$i_1 i_1$ (fam. 148) *	I	E	I	E	EI	EI
$ie ie$ (fam. 167) †	I	I	I	E and I	I	I

E = effective response

I = ineffective response

EI = intermediate response

* Nutman, 1954.

† Nutman, 1957.

between red clover plants and the normally effective strain of *Rhizobium trifolii*, Rothamsted strain A. These symbioses are characterised by failure in nitrogen fixation. This paper gives the result of cytological investigations of the structure of nodules on plants homozygous for each of these genes. Comparison is also made between these nodules and normal ones produced by other bacterial strains upon the same plant material, and by the effective strain A upon unselected clover.

Plants were grown in test tubes as described previously (Nutman, 1949) using several families of red clover homozygous for i_1 and for ie as well as heterozygotes and unselected families as controls. Plants were inoculated soon after germination with one or other of a number of known strains of nodule bacteria giving the responses shown in the table. As the plants developed the position and date of appearance

of each nodule was noted on drawings of the root systems. Plants were taken at weekly intervals and nodules of known age were fixed in Flemming's solution and embedded in paraffin wax. Serial microtome sections ($5-7\mu$) were cut and stained with Heidenhain's hæmatoxylin or with rose bengale and light green (Brenchley and Thornton, 1925).

2. STRUCTURE AND CYTOLOGY OF CONTROL EFFECTIVE NODULES

The structure and cytology of control effective nodules studied in this investigation agree in every respect with earlier published descriptions; these may be summarised as follows: The effective clover nodule (plate I fig. 1) is an elongate cylindrical outgrowth of the root consisting of an uninfected cortex of diploid cells enclosing an inner tetraploid tissue which contains the bacteria, (Wipf and Cooper, 1938). An endodermal sheath surrounds the infected tissue and separate endodermi enclose each vascular strand in the cortex. The nodule grows at its tip which is meristematic and the progressive infection of new cells produced by the meristem takes place distally from infection threads extending from the already infected cells. The threads consist of minute tubes of cellulose laid down by the plant; these contain the bacteria embedded in slime. The bacteria are discharged into the host cells (usually near the nucleus) from vesicles which arise upon the thread. After their release, the bacteria (rod form) multiply in the host cytoplasm and then enlarge until they completely occupy the cytoplasm of the enlarged host cell or leave only a small vacuole. Bacteria in this form constitute the so called "bacteroids" presumed to be the active agents in fixation (plate I, fig. 4). Bacteroids isolated from the host cell are unable to grow on artificial media (Müller and Stapp, 1925; Almon, 1933), although they react with vital stains (Spicher, 1954). The host nucleus of the infected cell first enlarges and then becomes amœboid, the nucleolus losing its

Plate I

- FIG. 1.—A section of an effective nodule produced by strain ClF bacteria on an i_1 plant. m = meristem; v = vascular strand; b = bacteroid containing tissue. $\times 300$.
- FIG. 2.—A section of an ineffective nodule produced by strain f_{12} on a heterozygous plant. m = meristem; v = vascular strand; tb = transient bacteroid containing tissue; db = degenerating bacterial tissue. $\times 300$.
- FIG. 3.—Bacteroid containing cells in a section of an effective nodule produced by strain A bacteria on a heterozygous plant. $\times 1600$.
- FIG. 4.—Bacteroid containing cells in a section of an effective nodule produced by strain ClF bacteria on an ie plant. $\times 1600$.
- FIG. 5.—A section of an ineffective nodule produced by strain A bacteria on an i_1 plant. m = meristem; v = vascular strand; b = cells containing rod-shaped bacteria. $\times 300$.
- FIG. 6.—A high-power photograph of an i_1 /strain A nodule showing rod-shaped bacteria in the cytoplasm. i = infection thread; b = bacteria in the cytoplasm. $\times 1600$.

m

m

v

tb

v

FIG. 1.

FIG. 2.

b

db

FIG. 3.

FIG. 4.

m

v

b

i

FIG. 5.

FIG. 6.

b

identity and being replaced by a number of particles of chromatinic material. Parallel with the hypertrophy of the host cell nucleus, changes occur in the nuclei of the bacteroids (Bergersen, 1955). The infected cells of the nodule contain hæmoglobin, the function of which is uncertain ; this imparts a pink colour to the nodule. Starch does not accumulate to a marked degree in effective nodules.

As the nodule becomes older, bacteria from the infection threads remaining at the base of the nodule invade the intercellular spaces and with the breakdown of the bacteroids ultimate degeneration of the tissue occurs.

The commonest kind of ineffectiveness in the nodule has been ascribed to bacterial strain ; for example in nodules formed by strains of bacteria from another species or sub-group of the host (Vincent, 1954), or in nodules formed by originally compatible bacteria which have lost the ability to fix nitrogen (Nutman, 1946). Such nodules are usually characterised by earlier decay of the bacteroid containing tissue as with the ineffective variant of strain A (strain *f*₁₂) on control plant material (plate I, fig. 2) although other types of ineffectiveness are known (Bergersen, 1955, and unpublished observations). Thus in general the amount of nitrogen fixed is directly related to the amount of bacteroid containing tissue found within the nodule (Chen and Thornton, 1940).

3. THE NODULES OF *i*₁ PLANTS

The structure and cytology of ineffective nodules produced on *i*₁ plants by bacterial strain A differed strikingly from the above description, although infection and the initial differentiation of the tissues of the nodule proceeded in a normal manner. The infection of the individual host cell from the infection thread was also normal as was the multiplication of the rods within the cytoplasm. However, instead of giving rise to bacteroids following initial multiplication, the bacteria remained in the rod form (plate I, figs. 5 and 6). Degeneration of the infected tissue occurred earlier than in normal effective nodules (20-25 days) but later than in nodules produced by typically ineffective strains such as *f*₁₂ (7-10 days). The growth of the *i*₁ strain A nodule ceased at an early stage and as a result further infection of the root continued at a high rate (Nutman, 1954). The reason for the cessation of growth is not known but does not appear to be due to the ultimate invasion of the meristem by infection threads as occurs with *f*₁₂ nodules. Changes in the host nucleus prior to cell degeneration were the same as in effective nodules, *viz.* enlargement followed by change in shape and fragmentation of nucleoli. Starch granules were abundant throughout older nodules.

The specificity of the action of the gene *i*₁ was manifest also at a cytological level. With either strains ClF or A211, a variant of A with which *i*₁ homozygotes responded effectively, bacteroid formation

was normal, the actual shape of the bacteroids being characteristic for these strains (plate I, figs. 3 and 4).

With strain *f*₁₂ (a mutant of strain A, ineffective with all plant material, Nutman, 1946) early decay of bacterial tissue was characteristic of the strain, but no bacteroid formation occurred.

In none of these ineffective associations was hæmoglobin found in amounts which could be detected spectroscopically. From these observations it was concluded that failure to fix nitrogen in *i*₁ plants inoculated with strain A bacteria was due solely to a host-bacterial interaction which prevents bacteroid formation, the small size of the nodule being secondary.

4. THE INEFFECTIVE NODULES OF *ie* PLANTS

On plants of *ie/ie* genetical constitution inoculated with strain A, nodule structure external to the infected tissue was normal and nodules were initiated in the normal way. The central infected tissues, however, consisted largely of small irregularly shaped cells and within these neither the normal invasion and multiplication of the rod form of the bacteria nor bacteroid formation was found. Examination showed that the abnormal cell pattern within the nodule was a result of renewed cell division following immediately upon the release of the bacteria from the infection threads (plate II, figs. 7-11). In a minority of infected cells which did not divide, bacterial multiplication ceased and no further changes occurred in the host nucleus. Where divisions occurred both infected cells and nearby uninfected cells were often involved. In most cases infected cells divided whilst the bacteria were still localised within the cell so that the first division separated the infected portion of the cell from the rest. The uninfected cell thus formed continued to divide a number of times whereas no further divisions generally occurred in the infected cell within which the bacteria seemed to parasitise the nucleus. When, however, first division failed to localise the bacterial infection, further division of the infected cell occurred to give rise to a group of small infected cells. Except in special instances, to be noted below, the bacteria in these cells remained in the rod form, and did not multiply to any great extent. The infected host cells showed a reduced affinity for the light green stain (plate II, fig. 7). These reactions of the host to the release of the bacteria from the infection thread were initiated near the nodule meristem, but the meristem itself was quite unaffected by them and continued its development in spite of an increasing degree of nitrogen starvation in the host. In contrast therefore to most ineffective nodules, *ie* nodules were large structures. Presumably because of the continued activity of the meristem, further infection of the root was inhibited and nodules were relatively sparse (Nutman, 1956).

As a result of the above sequence of events within the nodule the proportion of infected cells was less than in an effective nodule of

comparable size, but the total number of cells was larger. With the exception of "parasitised" cells no degeneration of the host nuclei occurred, and even in these cells normal resting nuclei were sometimes found. No hæmoglobin could be detected in these nodules.

The apparent tumourisation of regions of the nodule about centres of infection also occurred where bacteria multiplied to any extent in the intercellular spaces. An example of this reaction in the host

TEXT-FIG. 1.—A diagrammatic representation of the division of cells in an *ie* ineffective nodule.

1. The initially infected cell.
 2. An infected cell which has undergone division after the release of bacteria from the infection thread.
 3. Further division of the uninfected cell resulting from the first division: the bacteria are parasitizing the nucleus of the infected cell.
 4. The final stage in which the infected cell is surrounded by dividing cells.
- n* = nucleus; *c* = uninfected dividing cells; *inf.* = infection thread.

is shown in Plate II, fig. 11. The intercellular mass of bacteria was here very considerable and cells immediately adjoining the site of intercellular invasion were observed to be unaffected. The stimulus to division occurred at some distance from the infection and has given rise to a complete cambial layer isolating the invasion. This type of reaction occurred most frequently in nodules produced by the strain A211.

Because of the great range in size of nuclei found within the nodule it was at first thought that the secondary cell divisions were associated with a breakdown of the tetraploid condition. Observation showed that mitoses were normal, no abnormal anaphase or telophase patterns were seen and chromosome counts of metaphase plates confirmed the tetraploid condition of these cells (Plate II, figs. 9 and 10).

The ineffectiveness in *ie* homozygotes was previously shown not to be confined to strain A, but was found also with a number of other effective and intermediate strains shown in table 1. In every case the nodule pattern of ineffectiveness in *ie* plants with these strains was found to be similar to that described for strain A.

The action of the *ie* gene may thus be visualised as predisposing the host cells to division, the actual stimulus to division being the release of the bacteria from the infection threads into either the host cell itself or the intercellular spaces. In both situations the bacteria were in contact with the host protoplast either directly or through plasmodesmata. No reaction occurred in cells penetrated by unbroken threads.

5. EFFECTIVE AND PARTIALLY EFFECTIVE NODULES ON *ie* PLANTS

An effective response was found with strain A on occasional plants of the *ie* line, with the formation of more nodules than occur on ineffectives. Such associations were either effective or partially effective from the outset or showed a change in response from ineffectiveness in the younger seedlings to full effectiveness in older plants. In some seedlings the change in response in the top growth of the plant corresponded to a marked change in the pattern of

Plate II

FIG. 7.—A section of an *ie*/strain A nodule showing regions of tumourisation. $\times 330$.

FIG. 8.—A cell wall tracing of the section shown in fig. 7. The dark-shaded areas represent groups of released bacteria.

FIG. 9.—A high-power photograph of part of a section of an ineffective, *ie*/strain A nodule, showing an infection thread crossing a cell whose nucleus is in metaphase. $\times 1700$.

FIG. 10.—As in fig. 9 but the metaphase plate is perpendicular to the plane of the section. The bacteria released from the infection thread are clearly seen. $\times 1700$.

FIG. 11.—A section of an ineffective nodule produced by strain A211 bacteria on an *ie* plant. *m* = meristem; *i* = intercellular space filled with bacteria and surrounded by uninfected dividing cells. $\times 330$.

nodulation on the roots as shown in the previous paper (Nutman, 1957, plate I, fig. C). Normal or delayed effectiveness were not due to bacterial change and were thought to be due to the action of modifying factors in the host.

Inoculation of *ie* plants with strains such as ClF (known to be of outstanding effectiveness in the unselected host) gave a symbiosis which was largely effective. Wherever a fully effective response occurred in *ie* plants, the nodule structure was normal, the bacterial rods released from the infection threads giving rise to normal bacteroid tissue. Elsewhere nodules were of mixed structure consisting of parts which were normal and parts which showed tumourisation characteristic of *ie* ineffectiveness. The normal and tumourised regions were distributed as a mosaic in some nodules (text fig. 2*a*); in others, particularly in those plants showing a change in response and corresponding change in nodule pattern, the normal tissue was confined to the distal end of the nodule (text fig. 2*b*). Even with wholly ineffectively responding plants occasional isolated cells or small groups of cells escaped tumourisation and within these bacteroid formation took place.

6. DISCUSSION

Chen and Thornton (1940) ascribed ineffectiveness in symbiosis to the transient nature of the bacteroid containing tissue of the nodule and were able to show that the amount of nitrogen fixed was a direct function of the volume of bacterial tissue and its duration in time. This explanation was found also to apply to ineffectiveness due to boron deficiency where there is failure in vascular development as well as in bacteroid formation (Brenchley and Thornton, 1925). Again, in plants homozygous for the host factors i_1 or *ie*, ineffectiveness and bacteroid absence are correlated. Moreover the development of varying degrees of effectiveness in certain symbioses involving *ie* plants is closely related to the amount of bacteroid tissue present. These facts underline the primary role of the bacteroid in fixation.

From the point of view of development these results are of interest in showing that the sequence of changes leading to eventual bacteroid formation are determined by inherent factors in the host which operate in different ways and at different times. Thus the host gene *ie* acts in this sequence before the host gene i_1 and may cause a radical re-orientation in the development of the nodules. This takes place on contact between the bacteria and host cytoplasm and leads to formation of wound periderm presumably due to the production of some substance, possibly of a hormone nature. In this way the bacteria are confined in much the same way as are the pathogens in necrotic lesions produced by plant parasites (Gäumann, 1950, p. 456). This reaction of the host is very variable, depending upon the amount of development of bacterial rods taking place outside the infection thread. At one extreme the bacteria appear to be arrested in development immediately upon release into the cell and no divisions or other

TEXT-FIG. 2.—Cell wall tracings of *ie*/ClF nodules.

(a) A mixed response : both bacteroid-containing and tumourised tissue present in the same nodule.

(b) A changed response : tumourised tissue at the base and normal effective tissue at the tip of the nodule.

Released bacteria are shown as darkly shaded areas and bacteroid-containing cells are stippled.

FIG. 9.

FIG. 7.

FIG. 10.

FIG. 8.

FIG. 11.

i

m

changes ensue. With increasing bacterial proliferation the isolation of the infection takes place by an increasing number of secondary divisions of the original infected cell and its neighbours. With extensive secondary divisions the spindles become orientated radially to give rise to a spheroid periderm.

Under certain conditions the above *ie*/bacterial interactions do not take place and the symbiosis proceeds to completion. This appears to be determined by the genetic background in the host or by a change in the metabolism of the host either within the single nodule or progressively affecting the whole plant.

The irregular proliferation of the cells of *ie* nodules suggests a comparison with crown gall. Klein and Klein (1953) have demonstrated the close relationship between *Agrobacterium tumefaciens* and *Rhizobium* by transforming a strain of *Rhizobium* to an apparently normal virulent crown-gall producing organism by treatment with desoxyribonucleic acid from tumour-producing bacteria. In a later paper Klein (1954) summarised the known facts of crown-gall formation and concluded that wound substance, polymerised desoxyribonucleic acid produced by the bacteria, and auxin were all involved. In *ie* ineffectiveness the hypertrophy of the inner part of the nodule into a largely disorganised tumour-like growth, incapable of carrying out the symbiotic reactions of nitrogen fixation resembles crown gall to a remarkable degree. Even the normal root nodule may be regarded as an organised tumour of crown-gall type.

The i_1 gene acts at a later stage than *ie* and its effect is to prevent the final morphological stage in symbiosis—bacteroid formation. This gene inhibits bacteroid formation only with strain A, with other effective strains normal bacteroids are formed. Not only is the ineffectiveness due to the i_1 gene more specific than *ie* ineffectiveness but also it can only be revoked by specific mutation in the bacteria or by the action of supplementary host factors and in each case the reversal of the response in the host is complete.

As nothing is known of the nature of the stimulus leading to bacteroid formation no speculation can be offered regarding their absence in i_1 nodules. It is clear, however, that bacteroid formation is not conditioned solely by the host or by the bacteria.

7. SUMMARY

1. The structure of nodules produced by certain strains of *Rhizobium trifolii* on the roots of red clover plants homozygous for two independent recessive genes (i_1 and *ie*), has been examined. These genes cause failure in nitrogen fixation.

2. In one type (i_1) ineffectiveness is due to failure of the infecting bacteria to produce bacteroids; this is found only with one particular strain of bacteria.

3. In the other type (*ie*) abnormal divisions of the host tissue take place in the vicinity of bacteria which have been released from infection

threads. This gives rise to a disorganised tumour-like growth in which no bacteroids are formed.

4. Tumourisation in *ie* nodules is not restricted to symbiosis with strain A, but occurs with a number of other effective strains of bacteria. With strains of bacteria exceptionally effective on unselected red clover varying degrees of effectiveness are found. These are associated with corresponding amounts of bacteroid formation. In certain nodules both tumourisation and normal bacteroid formation occur side by side.

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