NITROGEN FROM MICROBES

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by

J. H. VINCENT

On this occasion we once again commemorate the work
of William James Farrer who, probably more than any other man,
laid the foundations of Australia's wheat industry. However,
there are good reasons for believing that his interest in
Australia's rural industries were even wider. It is apparent,
for example, that as a result of his stay at Duntroon with
George Campbell, shortly after he arrived in this country, he
was acutely aware of the needs of our pastoral industries. Indeed
in 1873 Farrer expressed the hope that pastoral science would one
day be taught in the colony because it had "an interest as great
and a sphere as important as that which agricultural science already
possessed". It is fitting therefore that amongst Farrer
Medalists there have been those who have been concerned with
pastures, particularly with the legume component and the contribution
it makes towards production and soil fertility. The essential
nutrient role of nitrogen has also been dealt with, but I seem
to be the first, in this series of lectures, to speak for the vast
population of microbes which are basically responsible for the
fertility of the soil, particularly in the matter of its nitrogen
supply.

In this account I shall be considering in fairly broad
terms all the main points where microorganisms are significant but
I shall be giving rather more attention to the question of
biological nitrogen fixation particularly where this involves the
symbiosis between leguminous host and rhizobia.

1. Distribution and Forms of Nitrogen

The nitrogen available for biological use is an extremely small part of the total amount of the element on Earth (Stevenson). Most (98%) is locked up in rocks, although at very low concentrations (50 p.p.m.). Almost all of the remainder is atmospheric dinitrogen (N_2), which, on an equal area basis, is about 10,000 times as much as all that in the soil, the ocean and in living organisms. Life then has available a capital amount of only 0.0007 of one percent of all planetary nitrogen, and very little of this (a few kg/ha) is available for use at one time. The role of microorganisms as major agents for the recirculation and augmentation of usable nitrogen, and the part they can play in its immobilisation or loss, will be the subject of this account.

We are chiefly concerned with three inorganic forms of nitrogen: wholly reduced (NH_3 or NH_4^+); wholly oxidised (NO_3^-); elemental (N_2). The first two serve as the usual source of nitrogen for plants and microorganisms; the last is utilisable only by some bacteria and blue-green algae.

However, practically all soil nitrogen is in organic combination. Its concentration varies from about 0.02% - 2.5%, as one ranges from a very sandy soil to peat. Values like 0.08% - 0.4% are more representative. The detailed chemistry of soil organic nitrogen is complex; only about half of it has been characterised, as bound amino-acid (20 - 40%) and hexosamines (5 - 10%)(Bremner). It has been calculated (Bartholemew) that there is about 3 times as much N immobilised in soil humus as there is in living plants (Bartholemew) .

2. Nitrogen Re-circulation

Amino-acids can be used as source of N directly by micro-organisms, and some by plants, but the re-circulation of nitrogen chiefly depends on the conversion of part of the soil's organic-N to ammonia. This is brought about by the action of many diverse

microorganisms and is termed mineralisation. In appropriate
circumstances two-stage oxidation of ammonia yields nitrate as
final product. This is nitrification. Either form is available
for assimilation by plants and, of course, by microbes. A
fertile soil will regularly be in the process of having some of its
organic-N converted to a utilisable form but the rate of liberation
will depend on the quantity and nature of soil organic matter and
the extent to which conditions favour microbial activity. The lift
in fertility following periods of fallow and cultivation results
from the liberation and accumulation of the ammonium and nitrate
ions favoured by these practices.

What happens when fresh organic material is put into the
soil requires further consideration. First there will be greatly
increased microbial activity; fungi, actinomycetes and bacteria,
and ultimately protozoa, will grow very rapidly because of the
fresh energy supply put at their disposal. This new microbial
substance will necessitate nitrogen input, which will have to be
supplied, if not from the added organic matter then from what is
available in the soil. Because of this a crop sown shortly after
the addition of low-nitrogen organic matter finds itself short of
this element because of competition with the actively growing microbes.

1.5% N in organic matter is the approximate critical point.
Below this, soil N is likely to be immobilised in microbial
substance (assimilation), above nett liberation (mineralisation) is
possible· Table 1 gives an example to show the difference between
turning in a low nitrogen crop residue and one rich in this element,
such as a green legume. The values given in this table are
illustrative only; many factors will influence the actual balance.
However the principle is established that, unless additional N is
supplied as fertilizer, the turning in of low-N residues will create
a condition of acute shortage of this element (Table 1). When the
amount of N in added organic matter is greater than the proportion
needed for microbial substance, the excess will become available for

plant use although some will be temporarily immobilised in the
bodies of microorganisms. The latter will be available for
mineralisation in succeeding years but at a slow rate (1 - 4%
recoverable yearly). Consequently the direct benefit of turning
in nitrogen-rich green manure will be greatest in the first year.

These considerations affect practice. It is better to
get low %N residues into the soil earlier, high %N residues
closer to the time nitrogen is needed. The slow release of
organic N after the first year explains the relatively small N
response obtainable from compost, as well as very good nodulation
and evident N-fixation by clover growing out of old dung pats.

3. Nitrification

Nitrification merits further brief consideration. Its
practical use goes back to the manufacture of nitre in dung heaps
during Napoleonic times and its recognition as a biological
phenomenon to observations on nitrate production in sewage
(Schoesing & Muntz, 1877). The classical organisms responsible for
the multi-step oxidation from fully reduced to fully oxidised N,
are Nitrosomonas ($NH_4^+ \rightarrow NO_2^-$) and Nitrobacter ($NO_2^- \rightarrow NO_3^-$).
Each process results in a nett energy gain to the oxidising organism,
which, coupled to that organism's biosynthetic system, enables it
to reduce atmospheric CO_2 to suitable organic form. Other, more
complicated, pathways have been demonstrated, by which a range of
microorganisms (bacteria, actinomycetes and fungi) can raise the
oxidative level of N with inorganic or organic N substrates (Alexander).

It used to be the fashion to promote nitrification as an
altogether desirable, and even essential part of the soil N cycle.
However plants differ in their preference for NH_4^+ and NO_3^-, and
there are circumstances when the latter form is agronomically the
less desirable. Nitrification changes the slowly leached, readily
adsorbed, cationic form to the readily leached anion. Nitrification
also provides substrates for N-loss by denitrification in that NO_2^-

and NO_3^- act as alternative electron acceptors if soil conditions become anaerobic. Lowered pH, consequent on nitrification may lead to toxic symptoms due to solubilising of Al but the same effect with nutrient elements such as K, Ca, Mg, P, Mn, can be advantageous. Some agricultural practice is designed to control nitrification, particularly in order to avoid leaching and denitrification losses.

4. Binding of Nitrogen

The ammonium ion has the useful property of being able to combine as an exchangeable base with organic and inorganic negatively charged surfaces in the soil complex. However firmer, less advantageous bonding, can also occur (Nommik; Mortland & Wolcott) and reactions of this kind might well become more important with increased use of anhydrous ammonia and NH_3-liberating fertilizers, such as urea.

5. Losses of Nitrogen from the Soil

Loss of soil nitrogen may occur into the air, by volatilization or denitrification, or into groundwater, rivers and the ocean, by leaching. Nitrogen balance experiments that attempt to account for residual soil nitrogen, losses due to crops and leaching and gains by fixation or deposit from the atmosphere almost always show a significant unaccounted for deficit. This is attributed to loss of volatile N; which has ranged through all levels up to half the N applied in fertilizer (Broadbent & Clark)   .

Loss of ammonia can occur with surface applications of fertilizer, and from urine particularly if the soil has poor capacity to adsorb NH_4^- and if pH is above 8. The hydrolysis of urea both raises pH and provides NH_3, so that this risk is greater with this form of fertilizer. It has been calculated that 5 - 20% fertilizer N can be lost in this way and it certainly constitutes a significant source of loss with grazed pasture.

Denitrification involves the reduction of nitrate and

nitrite and results in nitrous oxide and dinitrogen, both of which
are volatile and lost to the atmosphere. It is favoured by
conditions (such as suitable temperature, pH and decomposable organic
substrate) which suit the growth of microorganisms, able, under
anaerobic conditions to use nitrate, or nitrite, as alternative
terminal acceptors of electrons. The stepwise reduction of nitrate
at least includes the steps:

nitrate → nitrite → nitrous oxide → dinitrogen.
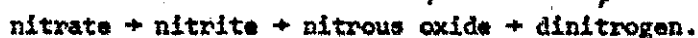
In almost any soil there are likely to be times and zones where
oxygen is sufficiently short for some denitrification to occur. It
obviously becomes more serious when soils are flooded or very wet;
it would be worsened by the use of nitrate fertilizers under such
conditions, or the nitrification of ammoniacal fertilizers before a
flooded phase. In any case denitrification is likely to occur in
localised pockets in the soil and may account for 10 - 15% losses of
applied fertilizer.

An alternative, as yet undefined denitrification route,
seems to involve aerobic oxidation of ammonia to nitrite and production
of dinitrogen from the latter, consequent on reaction with some soil
fraction (Broadbent & Clark)   .

The main routes for the loss of nitrogen from the soil are
summarised in Fig. 1.

6. Nitrogen Gains

 (a) Non-biological gains of N

Most measurements show that any gain to soil by precipitation
from the atmosphere (due to electrical or photochemical reduction of N_2)
amounts to only a few kg/ha, and could only be of significance in a
practically closed ecosystem such as a natural plant : animal
community (Stevenson).   Higher estimates seem attributable to local
industrial, dust or ocean contamination and can hardly have general

significance, otherwise it would be much more difficult than it is to demonstrate nitrogen responses in field experiments.

(b) Biological gains

The reduction of dinitrogen by biological agents is certainly the more significant means by which soil and water add to the supply of usable nitrogen. The organisms able to do this are all microorganisms, in fact, the simplest (procaryotic) forms: bacteria, higher bacteria and blue-green algae. Mostly they can do this in pure culture; a few function in the form of a N-fixing symbiosis with a higher plant. According to the organism and the conditions the nett gain may range from a few kg/ha. to several hundreds. In the latter case the N_2-fixed provides a considerable increment in the level of soil nitrogen.

The free-living and symbiotic systems will be dealt with separately in the succeeding sections; the latter, quantitatively the most significant, will be considered in rather more detail.

7. Dinitrogen fixation in free-living systems (Jensen)

(a) Organotrophic bacteria

The discovery of microorganisms able to use dinitrogen as sole source of this element goes back to the early history of microbiology, when this capacity was demonstrated in an anaerobic spore-former (Clostridium butyricum) and, most strikingly, the vigorous aerobic genus, Azotobacter. Both of these groups of bacteria have been subject to intensive research into their natural occurrence, the conditions affecting growth and dinitrogen fixation, the biochemical mechanism of the process, their actual or potential practical significance. The availability of newer more sensitive techniques ($^{15}N_2$, detection of acetylene reduction by means of gas liquid chromatography has led to the positive identification of dinitrogen utilisation in a number of other, taxonomically quite unrelated bacteria, notably Bacillus polymyxa, Klebsiella (Aerobacter), Pseudomonas,

Achromobacter, Arthrobacter. Additionally some bacteria, once
labelled Azotobacter, have been judged worthy of generic rank
(Beijerinckia, Derxia).

All of these bacteria depend on organic carbon
compounds for their metabolism, and a great deal of attention
has been given to the relationship between the amount of carbon
compound consumed and the amount of dinitrogen fixed. The
suitability of any available carbon source has also to be
considered. Significant associations between the classical
dinitrogen-fixing bacteria and organisms able to decompose
cellulose and hemicellulose provide the means of making use of
these major constituents of plant residues   . However the
total supply of such residues and the relative sparse occurrence
of the dinitrogen-fixing bacteria in most cultivated soils cast
considerable doubt on the generally quantitative significance of
this source of additional nitrogen. Exceptions are to be found
where large amounts of plant residues are allowed to decay under
partially or periodic anaerobic conditions, such as rice fields
and the heavy textured loams of the Nile delta and Northern
Africa more generally   . Very high Azotobacter counts have been
recorded in such circumstances leading to the possibility of an
agronomically significant gain of nitrogen to the soil. A most
interesting set of nitrogen balances has been assembled from
Egyptian experience,   which can be taken as indicative of the
benefit that can arise from non-symbiotic fixation under the most
promising conditions. These results (Fig. 2) confirm the prediction
that significant gains of N by free-living soil microorganisms
depends on the return of a considerable amount of organic material
as crop residues. They also show the quantitative superiority of
the legume (Beans) as a provider of this element. Losses were
chiefly due to drainage in irrigation water, and part at least of
the plant residue effect may have been to lessen this by N-
immobilisation. Similar conditions may apply to pasture soils and

leys, provided there is sufficient accumulation of organic matter
suitable for the promotion of fixation (about 50 kg decomposable
carbon for each kg dinitrogen fixed). Fixation at the rate of
18 kg/ha has also been estimated for Azotobacter paspali,
specifically associated with a variety of Paspalum notatum .

(b) Photosynthetic microorganisms

The question of energy supply is different when one
considers dinitrogen-fixing microorganisms which are also
photosynthetic, and which therefore have the advantage that they
are self-contained in this respect, provided other conditions are
favourable for their growth and CO_2 utilisation.

Dinitrogen fixation has been demonstrated in photosynthetic
bacteria but the agricultural significance of this is reduced by
the restricted habitats suitable for these organisms. There has been
a recent report of significant fixation due to photosynthetic
bacteria in a beach sand in Sweden, as well as a favourable appraisal
of their role in association with rice . In the latter case
association with other, heterotrophic bacteria permitted aerobic
growth and fixation of N_2, apparently due to excretion of pyruvic
acid by the associated heterotrophs. Usefulness in waste and sewage
purification is also indicated in pilot trials.

The blue-green algae are effective pioneers of new and
relatively inhospitable soil surfaces, including low rainfall regions
where dried algal crusts persist between the odd favourable occasions
when the rain (or condensed dew) permits a period of rapid growth .
They also have a contribution to make (although not always specially
welcome) by their abundant growth in shallow bodies of water.

The agricultural role of blue-green algae is quantitatively
significant in the case of flooded rice-fields where major gains of N
have been recorded: in some cases sufficient to provide for the needs
of the associated rice crop. A recent review quotes gains commonly
in the order of 10 - 20% (sometimes two or three fold) as a result of

inoculation with selected algal cultures. Some field results with Swedish soils estimate yearly fixation by blue-green algae in the order of 4 to 50 kg/ha. Extremely high rates of fixation under puddles in a clay soil indicated the possibility of contributions by these organisms in temperate regions . Estimates extrapolated from laboratory experiments with flooded rice soils gave very high values of 180 - 240 kg N/ha/p.a. .

(c) Assessment of significance

In summary, Jensen, who did so much in his monumental study on the Australian scene to shatter any illusions as to reliance on the free-living bacteria to make good nitrogen deficiencies being created by the bare - fallow - wheat rotation, concludes as follows. Non-symbiotic N_2-fixation has to be regarded generally as a long term factor in the economy of nature; able to compensate in part for losses of nitrogen from the biological cycle, slowly building up organic nitrogen in bare and poor soils and helping to maintain reserves Cultivation and the release of inorganic nitrogen associated with destruction of organic matter, leads to the potentially disastrous situation where the organic-carbon-demanding dinitrogen-fixers are unable to cover more than a fraction of the crop's requirements for nitrogen. Moreover the fraction so supplied will diminish as increasing population pressure demands more protein from nature's biological nitrogen cycle.

8. Symbiotic Systems

The logistics of N_2-fixation are much better when it comes to active symbiotic associations between bacteria and terrestrial plants because the latter act as more versatile providers of carbon-compound energy, as well as providing a structured habitat for the microsymbiont. The latter generally takes the form of a mass of specialised root tissue (nodule) within which the bacterial partner (microsymbiont) has a close and relatively enduring association with

the host plant (macrosymbiont). When such a partnership is
functioning efficiently, the bacteria are close to an abundant
supply of C compounds which provide needed energy, as well as
the residues used for the production of amino-acids by combination
with the reduced nitrogen (NH_3) contributed by the microsymbiont.

Such a symbiosis between plant and bacterium has been
achieved at several levels of the evolutionary process. Blue-
green algae : fungal associations in lichens permit these forms
to act as prime colonisers. In the Gymnosperms, cycads, such as
Macrozamia, have a nodule tissue inhabited by blue-green algae .
The conifer, Podocarpus, carries nodule-like structures which
seem capable of fixation in one case, although in other experience
any fixation was marginal . It is however with Angiosperms that
the symbiotic association has been most developed. These cases
are conveniently treated as first those which belong to families
other than the Leguminosae, and finally the nodulated legumes.

9. Non-leguminous Angiosperms

> The alder, whose fat shadow nourisheth
>
> Each plant set neare to him long flourisheth"
>
> - William Broune (1630, cit Tarrant & Trappe).

Root nodulation has been recorded amongst members of 7
orders of dicotyledonous angiosperms in addition to the Family
Leguminosae. Most of the genera belong to the Northern Hemisphere
but some also occur in the Southern Hemisphere (Myrica in southern
Africa). Casuarina is the genus representative of nodulated non-
legumes in Australia; it also extends widely into the sub-tropics
and tropics outside of Australia.

The microsymbionts of non-leguminous angiosperms have
resisted all attempts at isolation in pure culture; consequently
this symbiosis is difficult to study so far as the specific relation-
ship between the two partners is concerned. Short of the absolute
proof that requires pure culture isolation it seems most likely that

the nodule of the non-leguminous angiosperm contains an
actinomycete as its bacterial partner. There appears to be some
degree of cross-invasiveness, but also some degree of specificity,
between different host species and genera   .

Undoubtedly the nodulated non-legumes have a significant
ecological role in certain natural plant communites. The praise
given the alder above receives factual support in evidence as to
its usefulness in improving a forest environment when N-gains in
leaf fall, excretion and root and nodule decomposition can lead to
an annual accretion of soil N from 12 to 300 kg/ha/p.a. - the
equivalent of about $100 in fertilizer equivalence . Myrica has
also been used as an undercover forestry crop in Japan   , and
Casuarina has had an accepted place as a coffee-shade tree, based
on empirical observation of its usefulness in Papua-New Guinea.

10. The legume-rhizobium symbiosis

   (a) Brief history

   "Then Jacob gave Esau bread and pottage of

      lentiles ..... Genesis 25, 34.

   The use of legumes can be traced back to the earliest
history of Man: possibly as early as 6,000 B.C. Jacob's mess
of pottage seems to have been the red lentil (Ervum lens); David's
provisions for his army include beans (Vicia faba), lentils and
probably peas (II Samuel, 17, 28). Lucerne was cultivated in
Babylonia (about 700 B.C.) and its title "hay of the Medes" was
responsible for the generic name Medicago. It seems to have come
into Italy from Central Asia via Greece and/or into Spain with
the Moors (Nutman) . The Greeks were aware of the legume's
agricultural virtues: "Beans .... are not a burdensome crop to
the ground; they even seem to manure it ......" (Theophrastus,
370 - 285 B.C.). They also had a place in Roman crop husbandry:
"Legumes should be planted on light soils, not so much for their
own crops as for the good they do to subsequent crops" (Varro, 37 B.C.)

The inclusion of legumes in crop rotations declined in Europe in early mediaeval times when rough natural vegetation and bare fallow took its place. Later they found their way back into conservational rotations of European agriculture, and spilled over by accident or design into new agricultural areas.

The scientific appreciation of the legume-rhizobium symbiosis stems from the observation by Hellriegel and Wilfarth (1889) of the significance of the legume's nodules in making the host independent of combined-N. At practically the same time Beijerinck isolated the bacterial microsymbiont (Rhizobium).

Inoculation by the transfer of soil taken from an area supporting growth of selected legume, the inoculation of seed with pure-culture rhizobia and the use of inoculated peat as a vehicle for the carriage of the bacteria followed in succession - each subject to difficulties of one kind or another, each demanding a large amount of investigation. The amount of early effort put into the study of rhizobia, and the phenomenon of legume nodulation, can be gauged by the 1932 classic monograph by Fred, Baldwin and McCoy where the bibliography listed more than a thousand articles produced in a period of some forty years.

By that time most of the aspects that interest us to-day had in some measure been touched on, and most of the problems needing solution had been recognised. Work had in fact gone just about as far as the microbiological and biochemical techniques of the day would permit. Further progress depended partly on a sharpening of the analytical tools at our disposal partly on the exploitation of new techniques such as electron microscopy, chromatography, gel-diffusion and the use of labelled antibody, heavy-N_2 and use of acetylene as a convenient substitute for N_2 in studying the fixation process.

(b) The legume scene in Australia

There are many members of the family Leguminosae in

Australia which, so far as they have been studied, appear to be
nodulated with their own truly indigenous rhizobia. With the
exception of some, which can be used as browse shrubs or cut for
drought fodder, and Trigonella suavissima in parts of Queensland
and New South Wales, native Australian legumes have little
agricultural significance.

Lucerne and white clover would have been the first
introductions brought in by the English settlers by the 1850's.
Separately, and accidentally, subterranean clover and many species
of annual medics came in from the Mediterranean, the first at least
80 years ago, and advanced northwards from their points of arrival
in the south, selecting, or being selected by, the soil and climatic
conditions to which they proved so well suited . "Townsville
stylo" (Stylosanthes humilis) found its way into Queensland over 60
years ago; others potentially useful as tropical or sub-tropical
species have since been introduced, selected and in some cases used
for the production of new varieties.

It is apparent that the early introductions to the
temperate parts of Australia carried fortuitously rhizobia able to
nodulate and, in many cases, effectively fix N_2 with their hosts.
Conditions needed for rhizobial survival no doubt played their part
in determining the natural spread, or the restriction, of the
corresponding legumes. With the possible exception of rhizobia from
native Trigonella, which forms an association, generally inadequate,
with some of the introduced species of Medicago, there were no truly
native rhizobia for the introduced temperate legumes.

Legumes like soybean, cowpea, lupin and the tropical species
present quite a different problem. Many of them can in fact be
nodulated by native rhizobia, though the association is often sub-
optimal or completely ineffective. The hosts' rhizobial requirements
range from extreme invasibility (as in Phaseolus atropurpureus) to
marked specificity (Centrosema, Lotononis, Leucaena).

(c) Potential gains by nodulated legumes

Accurate determination of the amount of N_2 fixed by the nodulated legume under field conditions is generally difficult. Moreover any such estimate expresses the result in a particular situation: an interaction involving host, bacterium and the environment. Recorded gains can therefore be recorded only as a range, with the extreme values in both directions likely to be relatively unusual. Table 2 is however a fair statement of the position.

(Table 2)

11. Conditions Affecting the Legume-rhizobium Symbiosis

These can be considered in terms of compatibility between the symbionts, operating at the pre-invasion stage , controlling infection and determing effectiveness of the nodular association (Nutman) , and in relation to the operation of environmental factors (Vincent) .

(a) Compatibility between Rhizobium and host

Host invasibility and rhizobial invasiveness are gene controlled and can be modified by variety selection or breeding, on the one hand, or lost by rhizobial mutation, on the other. Nodulation within accepted inoculation groups need not occur for all host x rhizobial combinations (e.g. within the clovers with species of Medicago and within soybeans (and Corby, pers. comm.). There may also be problems of reduced compatibility restricting nodulation, at least under some, if not under all conditions (e.g. lines of 'non-nodulating' soybean and difficulties with the Woogenellup (= Marrar) cultivar of subterranean clover .

Compatibility also operates within inoculant groups and often imposes severe agronomic restrictions as, for example, subterranean v white clover , host genotype within red clover

(Nutman)   , subterranean clover   , species of Medicago   ,
Medicago falcata crosses with lucerne   , soybean   and
Centrosema   . Again genotypic change in the Rhizobium can lead
to lost effectiveness   .

In the case of legumes that have spread naturally it is
likely that processes of field selection will have already achieved
a fair match between rhizobium and host. If a new species or
variety of legume is being introduced into a soil where a
compatible rhizobium is absent or sparse, attention needs to be
given to the concomitant introduction of the correct bacterial
partner. Such rhizobial introduction is likely to be a good deal
easier where there is complete or virtual absence of rhizobia able
to invade the host, but much more difficult, particularly in the
long term, when invasive but ineffective rhizobia are already present.
In these circumstances the agronomist (and the specialist providing
the introduced material) must take into account the rhizobial
component as part of the total environment, and be assured that the
problems created by the introduction are justified by the advantage
likely to accrue.

To a less, but still considerable extent thought needs
to be given to problems attending any demand at the commerical level
for new specialised inoculants. This not only creates difficulty at
manufacture, distribution and farmer use; it also involves time-
consuming steps in the testing and selection of new inoculant
strains.

The rhizobiologist has, in the past, been presented with
several rather shattering examples of the "fait accompli": by
the agronomist, the plant introduction officer and the plant breeder.
A symbiosis at that level is also much to be desired.

(b) Environmental conditions

A great deal of the spectacular benefit resulting from
the use of legumes has been directly the result of providing better

conditions for the functioning of the symbiosis established between a natural or sown legume and the rhizobia already in the soil. This is particularly the case where there has been a reasonable opportunity for colonisation over a good many years, so that fairly good rhizobial strains are already there ready to respond to any improvement in conditions. Similar ameliorative steps have often to be taken when a new host and its rhizobia are being introduced particularly when it is aimed to have both persist in succeeding years. Such action has been concerned with survival of rhizobia, nodulation and/or nodule functioning (Vincent) .

. Acid conditions can affect rhizobial survival and multiplication and, due to problems of nutrition and toxicity, the functioning of the nodule. The calcium ion seems to play a specific and practical role in connection with nodulation and, to some extent fixation . Various disorders due to trace element deficiencies affect the growth of legumes; some have a more specific role in connection with nodule functioning. In practical terms Mo, as essential part of the nitrogen-fixing enzyme complex, is the most important and has, of course been the subject of intensive investigation and application. Sulphur deficiency has also been defined: it operates, not directly in connection with N_2-fixation but for completed protein synthesis. The element most often deficient under Australian conditions is however phosphorus and generally the effect of its application is to cause a very great improvement in yields both of established pasture and at the time of establishment . The deficiency evidently operates through the host plant, but causes problems in both nodule formation and function. For these reasons application of phosphate can be responsible for a roughly equivalent increase in fixed N_2 .

Combined nitrogen, except very light dressings under some circumstances, is likely to depress both nodule formation and nodule functioning. The degree of inhibition caused by the application of combined nitrogen will be markedly affected by growing conditions and the demand therefore made on this element. Competition by the

N-stimulated non-leguminous pasture component may also put the associated legume at a serious disadvantage. A great deal more needs to be known about the long and short-term effects of the application of combined nitrogen to legume-containing pasture, particularly the circumstances and management procedures that might maximise production whilst allowing sufficient legume recovery for the maintenance of a balanced sward . Greater tolerance to combined N may be achievable in the symbiotic situation. The same difficulty does not occur if the fertilized crop (e.g. winter oats) is grown separately .

12. Practicalities of the Rhizobial Situation

At this stage it can be appropriately asked: What can be done to assess and improve the rhizobial side of the legume-bacterium symbiosis as it operates under field conditions?

These specific points seem to merit consideration:

(a) In respect of existing legumes,

(i) Can production be lifted by soil
amelioration, if necessary with re-seeding?

(ii) Are the associated rhizobia good, bad or
indifferent with these legumes?

(iii) Would an introduced inoculant sown with the
same legumes improve the situation?

(b) For an introduced legume,

(i) Are there rhizobia already in the soil which
are able to invade the introduced host?

(ii) Will nodules formed by such rhizobia be effective,
ineffective or intermediate in N_2-fixation?

(iii)   If invasive rhizobia are absent, can an

effective rhizobial strain be used so as

to provide satisfactory nodulation in

the first and in succeeding years?

(iv)   If invasive but ineffective rhizobia are

present can an effective introduction

compete for the production of nodules?

Will it retain adequate representation

in succeeding years.

(v)   Is the introduced legume therefore likely

to be a practical proposition?

13. Legume inoculants (Vincent)

Seed "inoculation", the introduction of appropriate
rhizobia with sown legume seed, has been found necessary in many
parts of the world, particularly in Australia.

The history of the use of legume inoculants in Australia
is briefly recapitulated in Fig. 3 from the issue of a few cultures
by the New South Wales Department of Agriculture in 1914. By 1938
most states were issuing cultures but the rising demand after World
War II placed a heavy burden on such agencies and necessitated
handing over to commercial manufacture. In New South Wales (in 1954)
the Department vacated the field in favour of commercial manufacture
and the one Victorian supplier, already in business with agar
cultures, changed over to the use of peat which had become the general
method in use in this State. Recent usage of legume inoculants in
Australia by host group and state is shown in Figs. 4 and 5.

The Australian inoculant industry had a difficult beginning
in that a large proportion of the first batches of cultures were
found to be unsatisfactory, at a time when a general awareness of the
need for effective nodulation, and ability to recognise the signs of
failure, highlighted cases of poor quality in the product. It became

apparent   that there was an urgent need for better production
methods and adequate supervision and control. Out of this situation
there evolved a cooperative advisory and control service, involving
the University of Sydney and the New South Wales Department of
Agriculture (U-DALS) which, supported by manufacturers and primary
industry funds, served all manufacturers and resulted in considerable
improvement in the quality of legume inoculants produced in Australia
Standards set by this body were accepted by all State authorities
which virtually ceased production on their own account. Since 1970
the informal organisation has been replaced by the official Australian
Inoculants Research and Control Service (A.I.R.C.S) located at the
Biological and Chemical Research Institute, Rydalmere.

In addition to control measures per se, a good deal of the
work that has gone into studying the root-nodule bacteria and their
symbiosis with the host has been aimed at securing better results with
the practice of seed inoculation.

Requirements for the successful manufacture and use of
inoculants can now be considered.

(a) Strain selection and maintenance

Strains must be selected critically, maintained carefully
and tested regularly to minimize the chance of undetected variation
in symbiotic properties. These are the properties that are looked
for:

(i)   capacity to colonise, and competitively form

effective N_2-fixing nodules with the host,

or hosts, for which it is recommended.

(ii) adaptability to field conditions: survival

("saprophytic competence")   and, if possible,

a degree of tolerance to sub-optimal conditions

(such as acidity, low or elevated temperature,

desiccation, microbial antagonists)   .

(iii) good, or superior performance with a wide
range of host varieties, and if possible,
species, ("wide spectrum" strain).

(iv) good laboratory qualities, such as relatively
fast growth rate (within the limits set by
the species) and genetic stability; an
uncommon antigenic constitution is also
advantageous for "tracking" purposes.

The intrinsic capacity to form effective nodules with a
range of hosts can be quickly assessed in a relative fashion by
one of several methods suitable to light room or greenhouse   .
Those that pass this qualifying test can be subjected to the
vicissitudes of field tests in locations chosen to provide a
reasonable range of conditions. Field tests are essential both in
the original selection of strains and as a regular check on
maintenance of the desirable properties on which selection was
first based. They are, of course, expensive and, to be done well,
must include as few strains as possible. More field tests of
nodule-forming competitiveness are needed to supplement information
obtained in simpler more defined situations.

A lot of work has gone into the selection of strains for use
as legume inoculants in programmes developed by U-DALS, the state
departments of agriculture and C.S.I.R.O. This has provided valuable
background to strain recommendations. However no one who has been
associated with such programmes, would claim that they have been
entirely satisfactory. Manpower and financial resources have always
been too restricted and essential interstate cooperation, in some
cases, either insufficient or non-existent. The testing programme
needs to be adequately planned and coordinated, and there needs to
be sufficient liaison between official bodies, and between agricultural
sub-disciplines, to bring to light, where there is yet time, problems
likely to be encountered in new localities or with new hosts.

Unfortunately some rhizobia are very unstable genetically and the changes consequent on the appearance of mutants often mean a decline in symbiotic capacity. In the course of strain recommendations for the clover rhizobia over the past 20 years there have been at least 3 recommended strains which have become less invasive or completely non-invasive and 6 where ineffective mutants have become prominent in the population. Two points emerge:

(i) Lyophilized reference cultures should be maintained with sufficient replication to enable regular return to this "stabilised" material.

(ii) Cultures currently in use commercially must be regularly checked for the maintenance of symbiotic properties. In this connection it is vital to realise that a light room or greenhouse test with inoculum taken from a massed culture can fail to detect a majority of non-invasive or even of invasive but ineffective rhizobia. A simple quantitative test of a diluted series would on the other hand provide a quick means of detecting both conditions.

(b) The manufacture and quality of inoculants

Although it was harsh medicine at the time one can see, in retrospect, that it was fortunate that the early failures in Australian commercial production of legume inoculants were made so apparent by the demanding circumstances associated with their use. Such failures were followed by dramatic improvement, although those concerned with the operation (whether production or control) were never short of a problem. This area of applied microbiology still issues its challenges. Besides cases of strain instability and

complications arising from the introduction of new host varieties, difficulties have at various times included:

(i) contamination during growth in broth, resulting in partial or complete replacement of rhizobia (need for improved aseptic manufacturing technique).

(ii) insufficient growth at the broth stage (attention to temperature and aeration; nutrient requirements).

(iii) poor multiplication and survival in non-sterile peat carrier (due to unsuitable peat, toxic products of high temperature drying, excess salinity, microbiological antagonism; high storage temperature).

(iv) re-growth of surviving non-rhizobia, or of post-treatment contaminants, in "sterilized" peat, (inadequate autoclave treatment; incomplete sterilisation by γ-irradiation; faulty aseptic technique).

(c) Seed Inoculation

Granted an inoculum is satisfactory, i.e. with enough viable specifically effective rhizobia at the time of use, what are the factors that determine satisfactory nodulation in the year of sowing? This can be considered in terms of survival on and around the sown seed, and of success in forming nodules, perhaps competitively.

(i) Survival on the seed

Australian workers have necessarily been greatly concerned with this question which involves aspects such as death due to

desiccation, acid soil, or contact with acid superphosphate, and
in some cases a toxic seed coat factor or an applied pesticide .

Death during drying, and over a longer period is affected
by the form of inoculum, additives and a covering pellet and, of
course, temperature. A short period of acceptance of commercial
lyophilised cultures ended when it was found that their survival on
inoculated seed was disastrously short (Vincent) and strikingly in
contrast to long bench life as judged by direct plating . Peat
cultures were very much superior to cultures washed off from agar,
although the gap could to some extent be narrowed by a suitable
additive (such as sucrose, maltose or gum arabic). Even peat culture
could be improved by the last of these. The interaction between these
factors, temperature and kind of rhizobium has been well studied on
a quantitative basis and there is no doubt that when an initially
heavy inoculum is applied with these conditions optimal, satisfactory
nodulation can be obtained with average field plantings.

Properly peat-inoculated pelleted seed has considerable
staying power, sufficient to ensure the agronomic advantages that
flow from this practice. However the Australian viewpoint has been
against putting a lot of rhizobia on the seed only to allow most of
them to die by holding the inoculated seed a long time before sowing.
It has seemed better to give the seedling the advantage of extra number
rhizobia, an advantage often needed under our conditions. On the other
hand the inoculant industry in the United States has been plagued by a
widely promoted scheme for vacuum-processed preinoculated seed. To
the best of my knowledge there has been no adequate independent evidence
that this process permits reasonable survival on such seed, and any
attempt to introduce the same, or a similar process, to this country,
without convincing direct supporting evidence would be a retrograde
step. Ability to check the quality of any such pre-inoculated seed,
and to take advisory or stronger action as might seem necessary, could
constitute the critical test of the safeguards which have been
established to protect the farmer consumer.

(ii) Colonisation of the root surface

The rhizobia sown with the seed must be able to multiply and spread over the root surface so as to be available when the plant becomes susceptible to invasion. A period in dry, warm (or hot), soil awaiting germination, an acid environment or associated microbial antagonists can variously exercise an unfavourable influence at this point . Additives and pelleting can be advantageous, as can the neutralisation of a band of soil in the vicinity of the sown rows. Experimentally, favourable response has been obtained by the use of soil fumigants when there appeared to be biological antagonism.

(iii) Competition for nodule formation

Soils, carrying a considerable population of rhizobia ineffective with a sown legume provide a considerable challenge to the successful introduction of a new effective strain. A close study of such a situation showed the importance of the chosen inoculum strain and the great influence of the numerical relationship between the competitors .

Experiments of this kind are of considerable practical importance but the complexity of the system involved means that some basic questions are left unanswered. A second approach is to provide a relatively simple model and make the comparison between competitors against the background of their known representation on the root surface. This has permitted the relative competitiveness of paired strains to be quantitatively expressed and related to the important symbiotic properties of each strain assessed in separate pure culture. Results with a good many comparisons with different rhizobial pairs and different hosts certainly showed the fortunate fact that the effective N_2-fixing strain had under those conditions a distinct competitive advantage over an ineffective. However similar large differences in competitiveness that have been between equally effective strains and the extent to which the same relative competitiveness operates in the soil has yet to be determined.

14. **Rhizobia in the Longer Term**

If conditions are such that the effectively nodulated plant
has a better opportunity to survive than one which is ineffectively
nodulated then one can expect most of a natural population of rhizobia
to be at least moderately effective with the common legume of the
area. Exceptions may occur if factors other than N limit plant
response, and thereby remove any selective advantage favouring the
effective association. Such a situation might partly explain the
high incidence of ineffective strains in some upland white clover
pastures in the United Kingdom. This rather simplified view would
however have to be developed to pay more attention to the possibility
of competitive advantage by the more effective strain in nodule
formation, being affected by general ability to survive, in association
with the host and in its absence. Away from the host, the most
important antagonists may be non-rhizobial; on the root any direct
inter-rhizobial antagonism could become more operative   .

If one regards a surviving population - even one which is
extremely sparse - as having persisted in a hostile environment
because of a suitable combination of tolerances to the conditions
of that environment, then it would be expected that most introductions,
not selected for the same combination of circumstances, would be at
a relative disadvantage in the years following a successful initial
establishment. In fact a good deal of the evidence suggests such an
explanation, in that recovery of a generally satisfactory inoculum
strain becomes very difficult in even the second year, at the same
time as what appear to have been sparsely represented strains come
to dominate the population associated with a steadily improving
legume representation   . However many selecting factors can be
expected to operate under field conditions and if their relative
significance varies, or is modified, conditions of fluctuating
dominance and recession could result. A strain selected for
"saprophytic competence"   , by one set of circumstances might prove
saprophytically incompetent" in another environment, where different

limiting factors were operative.

This is an area where speculation can certainly outrun
facts but qualitative and quantitative techniques now at our
disposal, and the mounting interest of investigators in many
countries holds out considerable promise of useful progress.
Important factors exercising an influence appear to be the persistent
or fluctuating presence of a suitable host ; presence of other
vegetation ; tolerance of strains to high temperature ;
interaction between rhizobia and clay particles .

15. Future of the Nodulated Legume

As this account has probably made quite clear, there are
often difficulties in establishing and maintaining the N_2-fixing
symbiotic system, due to factors associated with survival of the
bacteria, the specificity which determines whether a particular
symbiosis will function effectively and the rather more exacting
demands made by the symbiotic system compared with the situation with
combined N supplied at will. Not surprisingly then the agronomist
and the farmer often look to advantages that might be apparent in the
latter.

Is there then a future for the nodulated legume in the
farm economy?

At least on the Australian scene it seems generally
conceded that pasture legumes will remain the prime form of N supply
in the foreseeable future . Complementary or replacing roles
have been indicated for fertilizer N at strategic periods e.g. to
obtain quick growth in the Spring) and for winter cereal crops .
The main potential use of N on pasture seems to be in dairying in
favourable situations .

It is interesting to look at some of the implications of
using fertilizer N in place of the legume as the main provider of
pasture nitrogen (Table 3).

The present legume N-equivalent was obtained by updating

Donalds 1960 estimate of one million tons p.a. (based on 10 million
hectares) to 1.6 million tons (based on the 60% increased in area
since 1960) . The application of fertilizer-N to achieve this
N-increment could be expected to be associated with significant
loss, and the figure has been rounded off at 2.5 million tons
to allow for this (36% loss). At 20¢ Kg-N this would cost the
farmer $500 million p.a. and the government $200 in bounty. Sixteen
manufacturing plants (each with a capacity of 160,000 tons p.a.)
would be entirely given over to this purpose.

If one takes the estimated potential for improved pasture
quoted by Hutton   , representing a nine-fold increase over the
present, then one arrives at the almost astronomic figures given in
the lower line of the table.

Between these two figures we could probably expect at
least a twofold increase by the end of the century by which time
pasture legume-N would be worth $1,000 million to the farmer (in
to-day's currency) and a saving of $400 million to the government.

We would have to add to these considerations the problems
involved in the substitution of geological fuel energy for sunlight
utilised by the N-fixing legume; the centralised location of
fertilizer-N plants engendering localised pollution problems and
involving transportation and labour costs, and the risk of
eutrophication in run-off and leached water from any excessive or
unfortunately timed application of fertilizer.

This is not to doubt that fertilizer-N has its own
valuable place in agricultural practice. So far as a legume pasture
is concerned the problem that most needs solution is how to use
the two sources of nitrogen so as to give a pasture the lift it might
need at critical times without finishing up entirely with N-dependent
grass. The legume and its associated rhizobium need to be better
understood and sometimes pampered a little, but I think it will remain
a major feature of the Australian agricultural scene for many years
yet.

In conclusion I should like to express my sincere thanks to the Chairman and members of the Farrer Memorial Trust for the honour they have done me personally, and for the recognition this award accords to Australian work done on Rhizobium and the nodulated legume. It is indeed a privilege to have been one of this nation-wide team for more than 30 exciting years of research and practical application.

I also crave your indulgence to put on record my thanks to my own teachers at Sydney University, particularly Walter Lawrie Waterhouse, twice Farrer Medallist, who introduced me to the joys of research and helped, with the modest means then at our disposal, to get my work started. My thanks too to all of those who, as students and colleagues, have contributed so much over many years, and to my agronomic colleagues at Sydney University who so readily recognised a rhizobial problem for what it was, and got the applied side of the work well and truly launched. I am glad too to acknowledge generous financial support by industry, bank and government research funds. Finally may I thank all those who in various other capacities have helped, not least by putting up with the enthusiasms, as well as the occasional frustrations, of an agricultural researcher.

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Table 1

Example[a] of N-Balance between Assimilation

and Mineralisation

| Plant Residues | Nitrogen (kg) | | | |
	In Microorganisms	From Organic Matter	Assimilated[b]	Mineralised[c]
Cereal (0.5%N)	85	25	60	—
Legume (3%N)	85	150	—	65

[a]Calculation based on Alexander p. 260 ff[6], taking 1.2 parts of N

required for the microbial substance produced by the decomposition of

each 100 parts of plant residue decomposed.

[b]Supplied by soil inorganic-N.

[c]Made available as inorganic-N.

Table 2

Mid-range

Host	N_2-fixed* kg/ha	Value in terms of Fertilizer N**$
Lucerne	50 - 350	52
Clovers	50 - 200	33
Lotus	190	49
Pasture legumes	10 - 170	23
Peas	30 - 140	22
Soybean	40 - 600	82
Tropical legumes	20 - 260	36

*Based on Alexander[6], Nutman[5], Vincent[5], Henzell[30], Williams[8], Bell & Nutman[31], Sundara Rao[32].

**approx. mean figure at 20¢/kg.

Table 3

Implications of Substitution of Legume-N by

Fertilizer-N[*]

	Legume N-equivalent	Value[†]	Bounty[**]	Factory Requirement
	Tons x 10^3	$ x 10^6	$ x 10^6	
Present	2,500	500	200	16
Potential	18,500	3,700	1,500	120

[*]Based on calculated N-fixed p.a. by pasture legumes.

[†] at $200 per ton N.

[**]at $80 per ton N.

Fig. 1

Main Causes of N-loss from the Soil

(a) as volatile ammonia.

(b) as result of denitrification.

(c) by leaching of soluble nitrate.

Fig. 2

Nitrogen Balance in Nile Delta

Columns above line : nett gain; below nett loss.

Residues of previous crop in each set of 3 (L → R):

    0, normal, 5x normal.

Specified crop in year of experiment.

Data from Abd-el-Malek[13].

Fig. 3

History of Legume Inoculant Use in Australia

Ordinate: estimated culture sales.

Abscissa: .   year.

N.S.W. etc.:   year of first cultures.

UDALS: Control by University-Department Laboratory

                Service.

AIRCS: Control by Australian Inoculant Research

                and Control Service.

Data supplied by Dr. R.J. Roughley.

Fig. 4

Inoculant Usage by Host

Height of columns : percentage in each host group

(L.H.S. 1969 - 1970; R.H.S. 1971 - 1972).

Width of column adjusted to show relative usage in the

two years.

Based on data supplied by Dr. R.J. Roughley.

Fig. 5

Inoculant Usage by States

Height of each column (and numeral) indicates proportion of

Australian cultures used in each state;   L.H.S., 1969 - 1971;

R.H.S., 1971 - 1972.

Based on data supplied by Dr. R.J. Roughley.