Clover Inoculation in New Zealand

Dr Bill Lowther
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Summary

This report discusses the requirement for continued inoculation of white, red and alsike clover seed with rhizobia bacteria.

A cornerstone of New Zealand's internationally competitive pastoral agriculture industry is the dependence on symbiotic nitrogen fixation by clovers. Symbiotic nitrogen fixation is the process where soil bacteria (rhizobia) invade the roots of the legume plant and form root nodules in which gaseous nitrogen is converted into plant available forms. The rhizobia that nodulate clovers were not present in New Zealand soils prior to European settlement but were introduced as contaminants in soil and dust on plants, agricultural equipment, seed and stock hooves. In the early years the natural spread of clover rhizobia in New Zealand soils accompanied pastoral development and allowed nodulation of the sown clover without the need to introduce rhizobia by seed inoculation.

Commercially inoculated and coated seed
With the advent of large scale land clearing and development in the 1950's, particularly on acid soils cleared out of scrub, areas were identified where clovers failed to nodulate because of the absence or low, scattered populations of resident rhizobia. Lack of rhizobia was also identified as a problem when large scale aerial oversowing of tussock grasslands was carried out. To overcome this, clover seed needed to be inoculated with rhizobia prior to sowing. Because of the difficulty of inoculating seed on farms, a factory process was developed with seed inoculated with rhizobia and protected by an adhesive and lime coating (coated seed). Initially the commercially coated seed was of very low quality due to poor commercial inoculants, ineffective adhesives and coating materials. Although normally satisfactory for conventional pasture establishment techniques (i.e. cultivation and lime to correct soil acidity), establishment failures occurred with oversowing.

A cooperative experimental effort between Government and commercial researchers developed an effective commercial process to inoculate and coat clover seed. To ensure the quality of commercially inoculated and coated seed, a voluntary certification scheme was set up with a standard of 300 viable rhizobia per seed after 28 days storage at 20°C. The use of this inoculated and coated (or pelleted) clover seed is critical for the nodulation, and hence establishment of clover in areas devoid of rhizobia (e.g. some tussock grasslands) or where populations may be absent or scattered (e.g. land cleared from scrub).

Where inoculation is needed
Clover rhizobia have spread widely throughout New Zealand, and in the great majority of situations where pasture is being sown, soils naturally contain high levels of resident rhizobia capable of nodulating white, red and alsike clover, and inoculation of clover seed is not required. Rhizobia are capable of surviving many years in the soil even in the absence of clover. If a paddock has a history of clover prior to cropping it is almost certain to contain suitable rhizobia following cropping, and clover seed sown subsequently will not need to be inoculated. Possible exceptions to this are where soil conditions are unsuitable for rhizobia survival (e.g. very acid, pH $\leq$ 5.0) or after long periods in continual maize cropping

(particularly if soil pH is low). However, it has been shown that small populations of rhizobia, either resident or introduced during cultivation, can rapidly increase when lime is applied to correct soil acidity prior to pasture establishment, so it is not possible to conclude with certainty, if paddocks with low populations of rhizobia will respond to inoculation.

Introduction of more effective rhizobia
It has also been suggested that inoculated coated clover seed may improve clover growth through the introduction of more effective rhizobia. A review of the scientific literature suggests that the likelihood of any significant response from doing this is unlikely.

Resident populations of rhizobia in New Zealand pastoral soils can range up to 1,000,000 per gram of soil (or 3,000,000 in a teaspoon of soil). Within each soil there are a wide range of individual rhizobia strains which vary in the amount of nitrogen they fix with white clover, from those that fix very little, to those that fix as much, or more, nitrogen than the inoculant strains (In fact, the inoculant strains in use were all selected from field populations of rhizobia). These resident rhizobia are well adapted to local conditions and compete with the introduced strains for nodule sites. Numbers of rhizobia on commercially inoculated and coated seed are likely to be in the low hundreds, or less, per seed, and results from trials in New Zealand and overseas show that at these rates the inoculant strain(s) forms such a small portion of nodules they will have little or no effect on clover growth.

An additional factor is that cultivars of clovers have all been selected under field situations with the existing populations of rhizobia. This is important as different cultivars can vary in their relationship with different strains of rhizobia and there is no guarantee that the individual strain(s) used in a clover inoculant will be the most effective for any new cultivar of white clover.

Other benefits from seed coating
Although the recommendation for use of inoculated coated clover seed cannot be justified for pasture renewal, the lime coating can provide a localised increase in pH to enhance nodulation when clover is oversown onto low pH soils where it is not economical to apply broadcast lime. There is also considerable evidence to support the use of seed coating to both enhance early seedling growth through targeted application of fertiliser to the germinating seedling (e.g. molybdenum, sulphur) and protecting seedlings from pest damage (e.g. insecticide/nematicide). Research has suggested that seed coating may improve germination by improved seed/soil contact but results have been inconsistent.

Recommendations

In the great majority of situations where pasture is being sown, soils naturally contain high numbers of resident rhizobia capable of nodulating white, red and alsike clover, and inoculation of clover seed is not required.

The use of inoculated and coated clover seed should be considered in the following situations as an insurance against nodulation failure:-

- *Undeveloped grasslands with no evidence of resident clover (e.g. haresfoot, suckling, white)*
  Although soil in much of the undeveloped grasslands now contains clover rhizobia as a result of natural spread, there are still areas devoid of rhizobia. In addition, even where rhizobia are present their distribution may be patchy. Unless there is a uniform cover of

resident clover, or if the rhizobia status of the area is unclear, the use of inoculated seed would be prudent.

- *Virgin pastoral land cleared directly from scrub*
  This is a precautionary recommendation, as little survey work has been done since the 1980's. Rhizobia may be absent or present in low, scattered populations, particularly if the soil pH is low. As these rhizobia can be expected to multiply during pasture establishment preparations, inoculation responses on clover establishment are unlikely but cannot be ruled out.

- *Paddocks cropped with maize continually for 10^+ years*
  This is also a precautionary recommendation following a clover establishment failure attributed to the absence of rhizobia in a paddock that had been in continuous maize for 13 years. A later survey of paddocks continually in maize detected rhizobia in all paddocks sampled, even after 32 years in maize, although numbers were sometimes low. Although these low populations may increase during pasture establishment preparations it is still possible that inoculation may improve clover establishment.

Introduction

The ability of clover, particularly white clover, to fix atmospheric nitrogen into a plant available form is an important component of the internationally competitive advantage of New Zealand's pastoral industries. This nitrogen fixation process occurs in nodules on the legume root formed by the symbiotic association of rhizobia and the host plant. Rhizobia are soil bacteria that once introduced can survive in favourable conditions, even in the absence of the host, although the numbers may decline to low levels.

The potential to increase nitrogen fixation, and hence pasture production, through manipulation of the symbiotic process has a long research history by both Government and Commercial research organisations. Introduction of rhizobia by inoculating seed prior to sowing is a well recognised technique when sowing clover into soils devoid of rhizobia. However, the technique has also been promoted to introduce more efficient rhizobia (i.e. capable of fixing more nitrogen) into soils with existing populations.

This review summarises the history of inoculation use in New Zealand to reach a conclusion, based on scientific evidence, on the possibility of increasing symbiotic nitrogen fixation by introducing highly effective rhizobia through seed inoculation

NB:

- *Because of the changes that have occurred with the nomenclature of rhizobia, the terminology "clover rhizobia" is used for those rhizobia that form nodules on clover, and unless otherwise stated the clovers referred to are white, red and alsike.*

- *In this document the terms "pelleted clover seed" and "coated clover seed" are used for process where clover seed is inoculated with rhizobia and coated with lime (or like material) usually at a ratio of around 40:30 seed: coating.*

Distribution of rhizobia capable of nodulating clovers

Populations

Rhizobia that nodulate clovers are not native to New Zealand and were introduced accidentally with the onset of European settlement (Greenwood 1965). It is presumed that rhizobia were introduced by chance in soil and dust attached to equipment, plant material, seed and stock hooves. Since then they have spread widely throughout New Zealand by similar means, and possible in windblown dust, and are now widespread in New Zealand soils, sometimes beyond the range of their host clover plants, but are still absent from some areas. The need to inoculate lucerne was recognized very early, and carried out by spreading soil from an existing lucerne stand. This was then superseded by the use of lucerne rhizobia inoculants. However, there is little mention in early literature about any need to inoculate clovers. For example, Saxby (1940) makes no mention of inoculating clover in his article “Grasses and clovers of New Zealand. Clovers are key to high production.” and even his 1956 Bulletin “Pasture Production in New Zealand” makes no mention of inoculation in the very detailed section on pasture establishment. The need for clover inoculation became apparent when large scale land development was initiated on land cleared out of scrub and bush (Sears et al. 1955; Greenwood 1961; During et al. 1962). The results of Greenwood (1961) illustrate the natural spread of clover rhizobia. Rhizobia were undetected in the undisturbed gumland soil, but present after cultivation and the application of lime, and adequate nodulation occurred on the white clover. Similar results were also demonstrated by Gould et al. (1968) where rhizobia were only detected at very low numbers, or not at all, on unimproved acid (pH 4.4-4.8) soils cultivated out of scrub, but increased with application of lime and fertiliser. On the basis of extensive surveys in the North Island, Greenwood (1965) reported a patchy distribution of clover rhizobia in hill country, being undetectable under sod-bound browntop, but plentiful under sheep tracks or sheep camps. On hill country cleared by out of scrub, by either root raking or burning, Macfarlane & Bonish (1986) found resident white clover rhizobia populations were extremely variable within sampling sites (ranging from 40 to 58,000 per gram soil).

The absence of clover rhizobia from extensive areas of the tussock grasslands (Gaur & Lowther 1980), and hence the failure of clover to nodulate, explains the emphasis on the importance of cocksfoot in early oversowing reports (Sewell 1950, 1952). The fact that Reynolds (1957) obtained excellent clover establishment without inoculation in trial plots laid down close to roads, where rhizobia had obviously established, led him to erroneously conclude that clover inoculation was not required in the Mackenzie Basin. In trials on more isolated areas in the tussock grasslands the essential nature of clover inoculation was soon apparent (Smeatham 1966; Adams 1964) and became a standard recommendation (White 1966). On the basis of very limited sampling, Ludecke & Leamy (1972) concluded that inoculation was not necessary on the drier lower- and mid-altitude tussock grasslands soils as resident rhizobia were present. More extensive sampling showed that the presence of clover rhizobia was not related to the soil but to the presence of haresfoot clover (Gaur & Lowther 1980). Where haresfoot clover occurred, clover rhizobia were always present, resulting in a recommendation that clovers oversown in the tussock grasslands should be inoculated unless there was a uniform cover of haresfoot or other naturalised clovers. However, clover rhizobia populations of up to 4000 per gram of soil were detected in 40-60% of tussock grasslands soils even in the absence of naturalised clovers. This confirms the ability of clover rhizobia to colonise and multiply in the soil even in absence of a host legume (Greenwood 1965).

Populations of >100,000 clover rhizobia per gram of soil have been shown to occur under clover based pastures (Hale 1980, 1981; Gaur & Lowther 1980; Patel & Lambert 1985; Elliot 1997). Once established in the soil, rhizobia can survive in the absence of clover as long as soil conditions, particularly soil pH, are suitable (Greenwood 1965). For example, in Britain, significant numbers of clover rhizobia were still present after 125 years of continuous wheat growing (Nutman 1969). It has been suggested that cropping with maize, particularly with an accompanying drop in pH, could be detrimental to rhizobia survival (Bonish & Steele 1985). In the Poverty Bay, uninoculated white clover failed to establish in a paddock resown to pasture after 15 years of maize cultivation. Tests showed very low numbers of rhizobia (<10 per gram soil) and resowing with inoculated seed resulted in successful establishment. Unfortunately, no soil pH data is available. In an intensive survey of paddocks in maize, Bonish & Steele (1985) failed to find any field where clover rhizobia could not be detected, even after 32 years of continuous maize. Populations of clover rhizobia they considered adequate for clover nodulation (>360 per gram soil) occurred in Manawatu, Bay of Plenty and Waikato soils following 6, 5 and 15 years of continuous maize cropping. In the Bay of Plenty, 4 paddocks had low numbers of clover rhizobia (<100 per gram soil). Three of these paddocks had been in maize for over 15 years. The fourth paddock had only been in maize for 4 years but had a pH of 5.2. Bonish & Steele recommended inoculating clover seed sown into soil that had been in maize for 10 years or more, or in which pH had fallen under 5.5. Caution must be used in interpreting these results and the recommendation to inoculate clover seed sown after a maize crop. Firstly, samples were taken from maize fields before cultivation and fertilisation, particularly lime to raise pH of acid soils, and sowing of clover. Greenwood (1961) demonstrated that even on an acid soil where clover rhizobia could not be detected, the application of lime resulted in the establishment of high populations of rhizobia. Secondly, there is no evidence that even populations as low as 14 rhizobia per gram soil would be too low to ensure nodulation. For example, when Maku lotus was sown into soil with an initial population of 15 rhizobia per gram, all seedlings nodulated (Lowther & Patrick 1993).

Nitrogen fixing effectiveness

In his review of rhizobia in New Zealand soils, Greenwood (1965) identified that clover rhizobia vary in their nitrogen fixing effectiveness with white clover (i.e. the amount of nitrogen a strain of rhizobia fixes in nodules on white clover). Table 1 summarises results from 10 studies investigating numbers and effectiveness of white clover rhizobia in a range of New Zealand soils. There was a wide range of nitrogen fixing effectiveness of the strains on white clover, and apart from one study (Hale 1980), some strains that were similar to, or more effective than the commercial inoculant strains were present. The mean effectiveness of strains from the different experiments was relatively similar over the sites, ranging from 69% to 85% of an inoculant strain. Gaur & Lowther (1980) found one site in the tussock grasslands with a mean effectiveness of 93%, which they attributed to the spread of rhizobia from an adjacent area oversown with inoculated clover seed several years beforehand.

Strains of rhizobia for inoculants

Inoculants available in New Zealand prior to 1955 were mainly for lucerne. However by 1957, clover inoculants were readily available (Cunningham 1957). Trials in 1966 indicated that, although certified by the Department of Scientific Research (Callaghan 1958), the clover inoculant available in New Zealand was ineffective when seed was oversown onto mid-altitude tussock grasslands (Lowther 1967). Improvements were made to the quality of the inoculants by increasing the number of rhizobia in the peat carrier, and more importantly through the selection of strains of rhizobia more adapted to surviving on the seed both before and after oversowing (Lowther & Johnstone 1978).

Table 1: Number and nitrogen fixing effectiveness of clover rhizobia in New Zealand soils (compared to commercial inoculant strains).

Area	Situation	No per g soil	Effectiveness range (as % of inoculant strain)	Mean (as % of inoculant strain)	Authors
Otago	Unimproved No clover	<4 -10^3	-	-	Gaur & Lowther (1980)
Otago	Unimproved Haresfoot clover	10^2 - 10^5	7 - 120	67	Gaur & Lowther (1980)
Otago	Ex pasture	10^6	8 - 117	83	Gaur & Lowther (1980
Manawatu	Ex pasture	10^5	24 -109	70	Pankhurst & Greenwood (1983)
Waikato	Ex scrub	10 - 10^4	0 -120	-	Macfarlane & Bonish (1986)
Taranaki	Pasture	10^4 - 10^6	50 -109	79	Rys & Bonish (1981)
Waikato	Pasture	10^4 - 10^6	31 - 85	69	Hale (1981)
Waikato	Ex pasture	10^3 - 10^4	-	74	Hale (1980)
Manawatu	Pasture	-	3 - 170	76	Patel & Lambert (1985)
Canterbury	Ex pasture	10^5	0 - 120	75	Elliot (1997)

Strains recommended for use in New Zealand manufactured inoculants were PDDCC 2666, 2668 and 2153 while strain CC 275e was recommended for Australian inoculants sold in New Zealand. When oversown, the percentage of seedlings nodulated ranged from 40-49% when inoculated with these strains compared to only 17% with the Australian inoculant strain (TA1) (Lowther & Johnstone 1978). The low seedling nodulation from TA1 was attributed to its poor survival on the inoculated seed before, or on the soil surface after oversowing. Following the demise of the New Zealand inoculant manufacturing industry, strain CC 275e became the strain used for Australian manufactured inoculants used in New Zealand. Although CC275e has been shown to fix more nitrogen with white clover than TA1 (Brockwell & Gibson 1968) and to be more competitive and persistent (Brockwell et al. 1972), TA1 has remained the recommended strain for white clover inoculants in Australia. Brockwell et al. recommended that strains of the serogroup CC275 should be tested for effectiveness under a range of field conditions in Australia, a recommendation that has not been acted on.

The majority of research into strain selection of rhizobia in New Zealand was carried out with Grassland Huia white clover. There is no guarantee that the present inoculant strains are the most symbiotically effective for new cultivars of white clover (Bonish 1980), as clover cultivars vary in their relationship with different strains of rhizobia (Sherwood & Masterson1974; Harrison et al.1989).

Coating materials

Lime coating of inoculated clover seed was developed in Australia to reduce the amount of lime fertiliser required for nodulation and establishment of clover (Loneragan et al. 1955). It also became apparent that lime coating could enhance survival of rhizobia on the seed. Athol

Hasting investigated a range of materials for their effect on rhizobia survival and promoted the use of methyl cellulose as an adhesive and gafsa-phosphate/dolomite as a coating material (e.g. Hastings & Drake 1962, 1963). Unfortunately this work had major evaluation flaws in both laboratory and field evaluations. Rather than measuring the ability of the materials to maintain a high population of rhizobia on the seed in the period after inoculation, the key factor, it measured the ability to keep at least some rhizobia alive for very long periods. Also the technique did not identify the poor quality of the New Zealand inoculants. Although these inoculants and coatings were satisfactory when clover seed was sown into cultivated soil (Greenwood 1961) they were ineffective when seed was oversown (Cullen & Ludecke 1966). Results from oversowing, using commercial seed pelleting based on the recommendations of Hastings, were so bad that farmers were recommended to inoculate their own seed just prior to sowing.

Improvements in the standard of New Zealand inoculants (Lowther 1974a), identification of gum arabic (Lowther 1975b) and lime (Lowther 1975b; Lowther & Johnstone 1979) as more effective coating materials, improvements in inoculant formulation (Hale et al 1979), and introduction of vacuum drying in commercial coating process (Taylor & Lloyd 1968) resulted in commercial pellets that gave better establishment of oversown clover than that obtained from freshly inoculated seed (Lowther & McDonald 1973; Lowther & Bonish 1980). Although establishment was often less than that from the small quantities of seed inoculated and lime coated in the laboratory for trials, this was not practical for farmers.

Molybdenum is essential for nitrogen fixation and molybdenum fortified fertilisers are applied where it is deficient. In view of the possible uneven distribution of molybdenum in fertiliser (Lipsett & David 1977), and uneven distribution of aerially applied fertiliser (Scott & Grigg 1970), the addition of molybdenum to seed has been recommended as an effective means of ensuring the application of molybdenum in the vicinity of the establishing seedling (Gault & Brockwell 1980). Gault & Brockwell cautioned the use of soluble molybdenum compounds (e.g. sodium molybdate) in seed coating because of possible reduced survival of rhizobia. However, Lowther (1987) found no detrimental effect on rhizobia survival from applying sodium molybdate to commercially coated seed, which he attributed to the low rate of molybdenum applied. On molybdenum deficient soils the inclusion of molybdenum in commercially coated seed, to ensure an even distribution and placement in the immediate vicinity of the establishing clover seedling, became a standard recommendation (Lowther & Patrick 1994).

Because of the widespread sulphur deficiency in the tussock grasslands, including this in seed coating was suggested as a means of ensuring sulphur in the vicinity of the germinating seedling. In trials where basal superphosphate was applied, establishment from gypsum coating was similar to lime coating on the driest site but consistently lower on the other sites (Lowther 1977; Lowther & Johnstone 1979). In the absence of basal sulphur fertiliser, gypsum coating increased seedling establishment compared to lime coating but the small amount of sulphur in the seed coat had little effect on clover growth. Even though there was only a small effect from sulphur coating, it could be important for seedlings that germinate in areas missed by fertiliser, and Scott & Archie (1978) recommended the application of elemental sulphur to lime coated clover seed to enhance early seedling growth.

Seedling predation by native insects can seriously reduce the establishment of white clover oversown on some tussock grassland soils. This predation can be markedly reduced by the application of systemic insecticide to the seed coat (Barratt et al. 1995) and the use of commercial coated seed with a systemic insecticide incorporated in the coat became a standard recommendation for oversowing (Lowther & Patrick 1994).

White clover seedling vigour can be reduced by clover cyst and root-knot nematodes and this can affect establishment success (Watson, 1990). Pesticides have been formulated as seed coatings to provide seedling protection to white clover from root knot and clover cyst nematodes with variable results (Barker et al. 1993).

Soil Acidity

Soil acidity can have a major effect on white clover through adverse affects on rhizobia survival, the nodulation process and plant growth. These effects can operate directly through pH, and also through calcium deficiency, or aluminium and manganese toxicity. Because of the complexity of the soil acidity complex it is difficult to ascribe precise pH limits for white clover, however to overcome any potential effects a soil pH of 5.8 to 6.0 is recommended (Edmeades et al. 1983).

Early research in Australia by Loneragan & Dowling (1958) demonstrated that the nodulation process was more sensitive to low pH and calcium deficiency than either rhizobia survival or clover growth, and once nodulated plants can fix nitrogen and grow at quite low pH. Seed coating with lime was developed as a means of raising the pH and supplying calcium in the vicinity of the plant root to allow nodulation to occur (Loneragan et al. 1955). In early New Zealand trials, lime coating was ineffective (Cullen & Ludecke 1966; Adams & Lowther 1970) due to the poor quality inoculants and coating materials (Lowther 1975b). Once these problems were overcome, the ability of lime coating to promote nodulation on low pH soils was a critical factor in the development of large areas of acid South Island tussock grasslands where it is not economic to aerially apply lime. In glasshouse trials on tussock grassland soils with a range of pH, lime pelleting was compared with applying lime to the top cm of soil, to simulate aerial application. Lime coating the seed replaced the requirement for lime (2500 kg/ha) on soils with a pH as low as 4.7 (Lowther 1974b), and importantly, lime coating not only increased nodulation on roots in the top 1 cm, but also throughout the pot indicating that once nodulation is initiated it can continue in acid soil. A similar result was obtained with an oversowing trial on an acid (pH 4.8) tussock grassland soil where there was no significant effect of broadcast lime on clover establishment or growth where seed was inoculated and lime coated (Lowther1975a).

Replacing lime applications by the use of lime coating of clover seed in the experiments of (Lowther 1974b, 1975a) should not be extrapolated to cultivated situations. When acid soil conditions were ameliorated by cultivating lime into the soil, clover yields responded to high rates of lime even where seed was lime coated (Lowther unpub.).

Commercial seed coating

There is little published information on the current standard of commercially coated seed in New Zealand. Inoculation of white clover has always been recognised as difficult as once applied to the seed, rhizobia can die rapidly, particularly by desiccation during the first few hours on the seed. After this rapid death there is a continual slow decline. Death rate is particularly affected by temperature both during manufacture and storage.

Initially, commercial coated seeds had low numbers of rhizobia. Identification of more suitable adhesives and better adapted strains of rhizobia improved survival, but a major breakthrough appears to come from the use of vacuum drying to reduce heat stress on the rhizobia.

As a result of laboratory and field trials in the 1970's, the Government set up a voluntary testing service (The Inoculant and Coated Seed testing Service [ICSTS]), and seed purchased by Government Departments had to pass the required standard (Anon 1979a & b). Commercially inoculated and coated seed had to have a population of at least 300 viable rhizobia per seed after storage at 20°C for 28 days after manufacture. This standard was based on extensive laboratory and field tests of commercially coated seed (Lowther & Bonish 1980; Bonish et al. 1980).

Table 2 presents results from 1 year's evaluation of commercially coated clover seed. Results illustrate the difficultly some manufacturers have had in commercially coating clover seed. Manufactures A & C produced coated seed with few viable rhizobia after storage periods of only 11 and 23 days. Coated seed from Manufacturer A was tested for 4 years and populations were always low. Although the numbers of viable rhizobia on coated seed from Manufacturer B were often below the 300 per seed required for certification, good field results were obtained with seed stored for up to 49 days. Longer storage periods (e.g. 72-81 days) resulted in the death of most rhizobia and low levels of seedling nodulation.

Table 2: Comparison of number of rhizobia per seed and % of seedling surviving following oversowing of commercially coated clover seed onto a tussock grassland site with no resident rhizobia. Laboratory inoculated and coated seed as standard (Lowther & Bonish 1980)

Manufacturer	Age of sample (days)	Rhizobia per* seed	% seedling nodulated **
A	11	23	6
A	?	4	3
B	21	90	51
B	22	900	52
B	28	90	43
B	28	20	43
B	30	230	52
B	35	230	53
B	36	90	48
B	46	400	52
B	49	150	68
B	55	230	30
B	72	9	13
B	81	4	10
C	23	9	4
C	23	4	6
C	23	4	10
C	23	4	4
Inoculated	1	42	14
I+ lime coat	1	230	44

* *The variability in number of rhizobia per seed may be due to the imprecise nature of the plant infection technique used to estimate rhizobia numbers.*

** *Seedlings that fail to nodulate do not survive*

This testing was undertaken in the 1970's and I am not aware of any later published values for viable rhizobia on commercially coated white clover in New Zealand. It would appear valid to assume that the number of viable rhizobia in the low hundreds or even lower, for commercially coated seed even shortly after inoculation.

Australian results where batches of commercially coated white clover were sampled from seed retailers also demonstrate the difficulty in maintaining sufficient numbers of rhizobia on white clover seed under practical farming conditions. The majority of samples had low populations; 66% had less than 250 rhizobia per seed and half of the samples had less than 100 rhizobia per seed. The reason for this was illustrated by the rapid decline in the number of rhizobia on commercially coated seed stored at 15^{o}C, from around 1000 per seed to less than 100 per seed in 3 weeks (Brockwell et al. 1995).

Introduction of Effective Rhizobia

The use of inoculated, coated clover seed to introduce rhizobia with a higher nitrogen fixing ability than resident rhizobia has been promoted. Although increased nitrogen fixation has been achieved under experimental conditions, published results suggest that it is unlikely to be achieved in normal farm situations with commercially available inoculated coated clover seed.

Overseas

In their review "Recent advances in inoculant technology and prospects for the future" Brockwell & Bottomley (1995) concluded that with conventional seed inoculation techniques, it is not possible to introduce more effective strains of rhizobia unless the resident population is very low (<1000 per gram soil) and ineffective on clover. Even in this situation effects can be expected to be transitory.

Successful introductions of effective strains on soils with low populations of resident rhizobia have been achieved when the inoculant strains outnumber the resident rhizobia by around 10 (Weaver & Frederick 1974) to 100-fold (Ireland & Vincent 1968; Materon & Hagedorn 1982). However, the interaction between rhizobia and host is complex (Brockwell & Bottomley 1995). Effective rhizobia have been successfully introduced into low-fertility peat or peaty podzol soils in the UK where strains ineffective on white clover are widespread (Holding & King 1963). However, even on these soils the response to inoculation was not consistent and only occurred where the inoculant strains form >50% of the nodules (Newbould et al. 1982). Different strains of rhizobia vary in their initial competitive ability (Roughley et al. 1976; Materon & Hagedorn 1982: Ames-Gottfred & Cristie 1989) and persistence (Gibson et al. 1976). The competitive ability between strains can also be affected by host cultivar (Roughley at al. 1976) and site (Roughley et al. 1976).

Two Australian experiments are relevant to New Zealand. When laboratory inoculated and coated white clover seed was sown onto three soils with different populations of resident rhizobia, strain CC275e (recommended for use in New Zealand) was more competitive and persistent than TA1 (Australian recommended strain) (Brockwell et al. 1972). On the 2 sites with low populations of resident rhizobia (400 or less per gram soil), strain CC275e still occupied at least 50% of nodules after 3 years. In contrast, the highest occupancy recorded for TA1 was 21% a year after sowing. No data is given for nodule occupancy in the first year on the site with the highest population of resident rhizobia (14,500 per gram soil), but in the second year the nodule occupancy was only 27% for CC275e and TA1 was not detected. Brockwell et al. (1975) demonstrated that the level of resident rhizobia affected the competitiveness of strain TA1 on 10 day old commercially coated white clover seed (135 rhizobia per seed at sowing). On a soil with few resident rhizobia (40 per gram), almost all nodules were occupied by TA1. However, on a soil with 10,000 resident rhizobia per gram, less than 10% of the nodules were formed by TA1.

New Zealand

In view of the existing international research evidence it is not surprising that there are few reported experiments in New Zealand on the effect of inoculation in soils with resident populations of rhizobia.

Hale (1980, 1981) compared inoculating rhizobia directly into the soil and inoculating seed. In short term pot trials he demonstrated that clover growth could be increased if **high concentrations** of effective rhizobia were inoculated into the soil just prior to sowing. With soil inoculation, the introduced rhizobia were competitive against the resident population forming 49-87% of the nodules on the clover roots, which increased clover growth. Using seed inoculation, only 3-18% (Hale 1980) and 7-38% (Hale 1981) of the nodules on the clover roots were formed by the inoculant strain and there was no indication of any increase in plant growth.

Macfarlane & Bonish (1986) recommended inoculation of white clover sown into land cleared out of scrub because of the scattered and often low populations of rhizobia. They found 2 out of 7 sites where inoculation had a significant effect because resident population were absent or so low that inoculation was probably essential for nodulation and hence seedling survival. There was a rapid multiplication of resident rhizobia in the presence of the seedling white clover roots which resulted in a low recovery of inoculant rhizobia in the nodules.

Gaur & Lowther (1982a, 1982b) found a complex interaction of sowing method, inoculation rate, seed pelleting and strain of rhizobia on percentage of nodules occupied by the inoculant strain. Strains performed differently when inoculated seed was sown into cultivated soil or oversown. In a cultivated situation, strain PDDCC 2163 (a New Zealand re-isolate of the strain recommended for Australian Inoculants marketed in New Zealand) had no apparent effect on nitrogen uptake or clover growth and nodule occupancy was only 8 to 32%. The most competitive and persistent strain was PDD 4144 (a New Zealand reisolate of TA1). This contrasts with overseas trials which have shown TA1 to be a poor competitor when inoculated onto white clover (Brockwell et al. 1972) and red clover (Materon & Hagedorn 1982). When oversown on to a site with a resident population of haresfoot clover there was a significant increase in clover nitrogen uptake and clover growth after 15 months from seed inoculation with strain PDDCC 2163. The increase was significant even with seed slurry inoculated at the normal recommended rate (600 rhizobia per seed at sowing). This is surprising as the strain was detected in only 38% of the nodules in this treatment 6 months after sowing. There was only a small further increase in clover growth from increasing the inoculation level to 10-times the recommended rate and no apparent effect from lime pelleting even though the nodule occupancy at 6 months increased from 38% to 57%. Strain occupancy after 15 months was still 55%. Caution must be used in attributing all the effect to competition with resident rhizobia as even though the area had an average resident population of 4.9 x $10^{5,}$ these may have been unevenly distributed. As suggested by Macfarlane & Bonish (1986), inoculant rhizobia would be essential for nodulation of seedlings germinating in areas where rhizobia were absent or in very low numbers. Strain PDDCC 2163 was recommended for use in inoculants used in New Zealand because of its superior performance when inoculated seed was oversown onto tussock grasslands devoid of rhizobia (Lowther & Johnstone 1978). There is no logical explanation for the enormous difference in nodule occupancy from PDDCC 2163 on the oversown and cultivated sites. Because of this, and the contrast with the consensus of other published results, I have serious reservations about these results, even though I am a co-author. In addition, these results were obtained with laboratory inoculated and coated seed that had populations of rhizobia at sowing considerably higher than would be expected on commercially inoculated and coated seed.

Other North Island trials provide further information on nodule occupancy. In field trials on four different soils, the slurry inoculation of seed at the recommended rate resulted in only 1-9% of nodules being formed by the inoculant strain 4 months after sowing (Hale 1980). Pankhurst & Greenwood (1983) found that the inoculant strain formed 27% of nodules five months after seed was sown into cultivated soil, but the percentage declined with time and no nodules containing the inoculant strain were detected 24 months after sowing. Pankhurst & Greenwood also provide a good illustration of the problems with trying to introduce effective rhizobia. Nodules <3 cm from the crown of the plant had 30% occupancy of the inoculant strain, nodules on lower roots had only 3% occupancy, while nodules on stolons had 7% occupancy.

There appears to be only one experiment where long term response to seed inoculation has been investigated (Brown 1996). This compared 2 paddocks in the Te Anau Basin that were resown with either bare seed or commercially inoculated and coated seed. Three years after sowing there was no apparent difference in the distribution of symbiotic effectiveness of the rhizobia or in the proportion of strains genetically similar to the inoculant strain between the two paddocks.

Discussion

The aim of this review was to provide definitive recommendations, based on scientific evidence, for the need to inoculated white, red and alsike clover in New Zealand. Because of its agricultural importance the majority of the research has been carried out on white clover but the same inoculant is used for these three clovers.

The ability of rhizobia to survive in soils in the absence of the host legume means that inoculation is not necessary for clover nodulation in soils that have been in pasture, even after going through crop rotations (except possibly long periods of maize).

There are some “grey” areas where it is prudent to err on the side of caution and include some “precautionary recommendations”. Although clover rhizobia have spread throughout most New Zealand soils there are some areas they have not fully colonised (e.g. acid, higher altitude tussock grassland soils and acid soil cleared from scrub and bush) and inoculation of sown clover is recommended. Although the presence of existing clover (e.g. haresfoot, suckling, white clover) indicates the presence of clover rhizobia, it is not possible to accurately identify the rhizobia status of undeveloped country where with no resident clover, so in these situations inoculation is recommended for clover oversowing.

Where pasture is established into soil with few resident rhizobia by conventional cultivation, with fertiliser application, particularly lime, numbers can increase by rapid multiplication, of rhizobia, either resident or introduced, during preparation for sowing. However, because this cannot be guaranteed to result in a uniform distribution of rhizobia throughout a paddock, particularly where there is only a short interval before pasture establishment, inoculation responses may occur.

The recommendation to inoculate clover after 10 years of continual maize cropping is based on one reputed failure of clover establishment due to the absence of rhizobia, and a survey showing low numbers of rhizobia in some paddocks after multiple maize crops. Interpretation of reasons for the clover establishment failure is difficult due to lack of information on soil properties. In the survey, samples were taken before paddocks were cultivated and fertilised, prior to pasture establishment. Evidence from other studies suggests that multiplication of the existing low populations, simply due to cultivation and fertiliser application, would likely provide sufficient rhizobia for the nodulation of germinating clover seedlings.

The first commercially produced inoculated and coated clover seed had very low numbers of rhizobia and although satisfactory when sown into cultivated soil, nodulation failures occurred when seed was oversown. Following improvements in technology, commercially inoculated and coated seed became an important component of the extensive land development that occurred in New Zealand. However, survival of rhizobia on the seed remains a problem and numbers at sowing are often less than 100 per seed, under the storage periods experienced through the rural distribution chain, and on farms prior to sowing. When inoculated and coated seed is oversown, higher seed rhizobia levels are needed to obtain the best clover nodulation and survival.

It has been suggested that inoculated coated clover seed may improve clover growth through introducing more effective rhizobia. A review of the scientific literature suggests that any significant response from this is unlikely. Resident populations of rhizobia in New Zealand pastoral soils are high, up to 1,000,000 per gram of soil (or 3,000,000 in a teaspoon of soil). Within each soil there are a wide range of individual rhizobia strains varying in the amount of nitrogen they can fix, from those that fix little to those that fix as much, or more, nitrogen than the inoculant strains. Numbers of rhizobia on commercially inoculated and coated seed are likely to be in the low hundreds, or less, per seed, and results from trials in New Zealand and overseas show that at these rates the inoculant strain(s) forms such a small portion of nodules they will have little or no effect on clover growth. Even if a process was developed to supply higher populations of rhizobia on coated seed at sowing (i.e. > 1000) it is unlikely that consistent significant increases in clover growth would be achieved because of the complexity of the nodulation process.

The development of commercial inoculation and coating for clover seed has been important in pastoral development in New Zealand. However, rhizobia inoculation now has only limited importance in a few defined areas where rhizobia are absent.

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