# *Rhizobium* issues affecting the contribution of caucasian clover to New Zealand pastoral agriculture

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## Abstract

Caucasian clover (*Trifolium ambiguum*) is being commercially released for use in New Zealand agriculture. Seed must be inoculated at sowing, as caucasian clover forms a nitrogen-fixing symbiosis only with specific rhizobia that are not native to New Zealand. These rhizobia have the potentially undesirable property of readily forming nodules on white clover that do not fix nitrogen. Caucasian clover inoculant strains available for use in New Zealand were found to be genetically unstable in the laboratory, and must be frequently monitored for their symbiotic properties. A strain of caucasian clover rhizobia that gives better establishment and growth of caucasian clover under oversowing conditions was identified. This strain maintained a higher level of viability under stress conditions in the laboratory and showed enhanced survival on seed in the environment. Growth of caucasian clover results in large populations of caucasian clover rhizobia in the soil. These formed nodules on white clover in the same field environment, although white clover rhizobia dominated nodule formation. Nevertheless it is possible that present caucasian clover inoculants may over time reduce nitrogen fixation and hence the production and persistence of white clover. Further research is required to develop a caucasian clover strain that does not form ineffective nodules on white clover.

**Keywords:** caucasian clover, inoculation, nodulation, rhizobia, *Trifolium ambiguum*, *Trifolium repens,* white clover

## Introduction

Caucasian clover (*Trifolium ambiguum*) is a rhizomatous perennial legume able to grow in a wide range of habitats in its indigenous area, the Caucasus region of the former Union of Soviet Socialist Republics (USSR), the Crimea, the Black Sea coast of the Ukraine, eastern Turkey, and northern Iran (Bryant 1974). Caucasian clover is not a domesticated plant within its indigenous areas and successful introduction of the species from its native habitat has been difficult. This has been in part owing to poor seed production and slow establishment. Development has also been complicated by the existence of natural polyploidy, e.g., diploid (2n = 16), tetraploid (4n = 32) and hexaploid races (6n = 48). However, poor nodulation was the main reason for establishment failure (Hely 1957, Kannenberg & Elliott 1962).

Caucasian clover is being evaluated in New Zealand as an alternative or supplementary legume to white clover (*T. repens*) because it has the potential to be relatively productive and persistent compared with white clover under harsh conditions such as drought. A combination of caucasian clover and white clover may be advantageous in areas where white clover is able to establish quickly to provide ground cover in new pastures, and caucasian clover persists when white clover vigour declines (Watson *et al.* 1996). Although closely related to white clover, caucasian clover is nodulated only by specific strains of rhizobia and is not nodulated by white clover rhizobia. Caucasian clover rhizobia are not found in New Zealand soils and therefore seed must be inoculated at sowing. However, caucasian clover rhizobia have the potentially undesirable property of being able to form nodules on white clover that do not fix nitrogen.

In this paper we review rhizobial problems associated with caucasian clover establishment, and discuss some of our recent unpublished findings.

## Caucasian clover rhizobia

Native or naturalised rhizobia capable of effectively nodulating caucasian clover are not present in soil at sites in the US, Australia or New Zealand (Parker & Allen 1952; Hely 1957). In particular, strains that nodulate white clover do not nodulate caucasian clover. The ability to nodulate caucasian clover is owing to the presence of an additional gene not found in white clover rhizobia (H. McIntyre, unpublished data). The limited number of *Rhizobium leguminosarum* bv. *trifolii* strains available in culture collections that are able to form a nitrogen fixing symbiosis with caucasian clover were all originally isolated from caucasian clover plants and soil from the centre of origin (Zorin *et al.* 1976b). Effectiveness of these strains is variable within each combination of caucasian clover host and bacterial strain. Caucasian clover occurs in several different ploidy forms and it has been suggested that each ploidy level of the plant has specific rhizobial requirements (Zorin *et al.* 1976b).

Poor nodulation of caucasian clover has been overcome by using inoculant strains identified by Zorin and co-workers (Zorin *et al.* 1976a, b). Strain CC283b was found to be a highly effective inoculant with hexaploid caucasian clover (Table 1). Furthermore, it was characterised as greatly superior to strain CC231a which had been used routinely in Australia for inoculation of caucasian clover regardless of ploidy (Zorin *et al.* 1976a). However, in another report, strain CC286a was reported as the most effective strain on all the hexaploid caucasian clover cultivars tested (Zorin *et al.* 1976b). Dear & Zorin (1985) used a mixture of the rhizobial strains CC231a, CC283b and CC286a as the inoculum to ensure nodulation of diploid, tetraploid, and hexaploid caucasian clover. In New Zealand CC231a, CC286a and CC283b are recommended by Manaaki Whenua Landcare Research as the appropriate inoculant strains for the diploid, tetraploid and hexaploid cultivars respectively (Table 1).

We have carried out studies to examine the genetic background of the various caucasian clover inoculant strains from culture collections. Molecular characterisation showed that caucasian clover rhizobial strains formed two chromosomal families. The strains originally isolated from plants of unspecified ploidy in Turkey (e.g., strain CC231a) formed a separate group from the strains that originated from hexaploid plants grown in the Caucasus region (e.g., strains CC283b and CC286a), which shared the same chromosomal background (H. McIntyre, unpublished data).

Many rhizobia harbour large extrachromosomal genetic elements known as plasmids, and in clover rhizobia the nodulation genes are found on symbiotic plasmids. Different strains of caucasian clover rhizobia show variation in symbiotic ability and plasmid content that appears to be owing to plasmid rearrangements. Strain ICC103 is a re-isolate of CC286a, the recommended strain for tetraploid caucasian clover. However, Patrick *et al.* (1994) reported very poor nodulation of the tetraploid cv. Treeline when ICC103 was used as the inoculant strain in oversowing trials at a tussock grasslands site. Subsequently, strain ICC103 has been shown to be a growth- and nodulation-deficient derivative of CC286a (Table 1). These defects are due to rearrangements and deletions within the symbiotic plasmid. A number of other isolates, including some which are components of commercial inocula elsewhere in the world, failed to nodulate caucasian clover. This failure was owing to the deletion of a large region of the symbiotic plasmid which included genes essential for nodule formation. The degree of instability observed in the symbiotic plasmid of caucasian clover rhizobia is much greater than that seen in the symbiotic plasmid of rhizobia that nodulate white clover. This is owing to the genetic structure of the symbiotic plasmid found in caucasian clover rhizobia which contains a high proportion of reiterated DNA sequences (H. McIntyre, unpublished data).

The instability of the caucasian clover rhizobial genome was further highlighted by the appearance after culturing in the laboratory of an isolate of strain ICC105 that had a large deletion of DNA from the chromosome. Although this derivative of ICC105 retained the ability to nodulate caucasian clover, its ability to fix nitrogen was greatly reduced (B. Challis, unpublished data).

The instability of the symbiotic plasmid in caucasian clover rhizobia, especially the capacity of strains to

**Table 1** *R. leguminosarum* bv. *trifolii* strains used in New Zealand as caucasian clover inoculants.

| Strains | Source | Derivation[1] | Symbiotic Reaction[2] | Comments |
|---|---|---|---|---|
| ICC105 | Invermay (H. Pryor) | CC283b | +[3] | CC283b is the recommended strain for hexaploid cultivars |
| ICMP4074b | ICMP Landcare Auckland | CC286a | + | CC286a is the recommended strain for tetraploid cultivars |
| ICC103 | Invermay (H. Pryor) | CC286a | poor / late | Required supplementation of defined media with yeast extract for growth |
| ICC148 | University of Otago (H. McIntyre) | Ex-nodule from field grown *T. ambiguum* | + | Field site originally inoculated with CC283b, CC286a, CC231a |
| ICC104 | Invermay (H. Pryor) | CC231a | + | CC231a is the recommended strain for diploid cultivars |

1 CC strains originally from F. Hely Culture Collection, CSIRO Division of Plant Industry Canberra, Australia. Strains CC283b and CC286a were isolated by Zorin and Hely in 1970 from root material of hexaploid caucasian clover plants grown in the Caucasus region. Strain CC231 was isolated by Erdmans and Means in 1955 from a soil sample taken from an area in Eastern Turkey where tetraploid caucasian clover occurs naturally (Zorin *et al.* 1976b).
2 The strains showed the same symbiotic reaction on tetraploid cv. Treeline and hexaploid cultivars.
3 + refers to early and effective nodulation.

change over time from nodulating to non-nodulating, or to alter in effectiveness upon culture in the laboratory, has important implications for use of these strains as inoculants. Regular monitoring of the symbiotic ability and genetic structure of caucasian clover inoculant strains is essential.

To investigate the genetic stability of inoculant strains nodulating caucasian clover in New Zealand field environments, rhizobia were isolated from the nodules of caucasian clover plants growing at three field sites established for 5, 10 and 20 years. Molecular characterisation showed that there was very little genetic diversity among the rhizobia that were recovered (H. McIntyre unpublished data). However, a field isolate that differed from ICC103, ICC104 and ICC105 was identified. This strain was named ICC148 (Table 1) and has subsequently been found to give superior performance as an inoculant strain for hexaploid caucasian clover (Pryor *et al.* 1998).

## Effective inoculation of caucasian clover for oversowing

Under oversowing conditions, establishment of hexaploid caucasian clover has been reported to be inferior to that of white clover (Lucas *et al.* 1981). This low establishment has since been attributed to nodulation problems owing to the use of a seed inoculation treatment inappropriate to oversowing conditions (Patrick *et al.* 1994). Vincent *et al.* (1962) indicated that temperature and water loss are two key factors affecting the survival of clover rhizobia. In some environments where oversowing is carried out, this stress is compounded by freeze thaw cycles in the top soil layers. The germination of oversown seeds is often delayed owing to low rainfall or frosts during this period. It is therefore crucial for inoculant bacteria to maintain a viable state for some time after seed is oversown.

Inoculation of seed with strain ICC148 resulted in 56% of seedlings nodulated in field trials compared with 44% with ICC105, a re-isolate of the recommended strain (Pryor *et al.* 1998). Increased percentage nodulation from strain ICC148 was consistent over seven sites in the Otago and Canterbury tussock grasslands, and after different storage intervals from inoculation to sowing. The advantage of ICC148 was not related to higher numbers at sowing (Pryor *et al.* 1998) and must therefore be owing to enhanced survival on the seed following sowing, enhanced survival on the soil surface, and/or the ability to multiply in the soil and nodulate the host. Strain ICC148 is now available on caucasian clover seed that has been commercially Prillcote® pelleted.

In order to understand the basis of the superior performance of strain ICC148 to ICC105, we undertook detailed studies comparing the survival of the strains in the field under oversowing conditions. Caucasian clover seeds were inoculated with either ICC105 or ICC148 and sown on the soil surface at two locations in Central Otago, Naseby and Hawkdun. Bacterial viability was determined on individual seeds recovered from the field. Numbers of viable rhizobia on the seed declined rapidly, but strain ICC148 maintained a significantly higher bacterial viability than ICC105, with 57% of the seeds inoculated with ICC148 versus 18% of the seeds inoculated with ICC105 having a viable rhizobial population 3 weeks after sowing (B. Challis, unpublished data).

Laboratory studies comparing the tolerance of strains ICC105 and ICC148 to storage at various relative humidities and to transfer between relative humidities were also performed. Strain ICC148 maintained a higher level of bacterial viability at all relative humidities. These differences were accentuated when the strains were alternated between 0% and 100% relative humidity (B. Challis, unpublished data). This difference between the strains in the ability to survive under stress may explain the superior performance of ICC148 as an inoculant. In addition, it appears that laboratory assays involving switches in humidity may offer a cheap pre-screening of inoculant strain suitability, reducing the number of strains that need to be trialed in expensive field experiments.

In their review, Taylor & Smith (1998) stated that low seedling vigour was a major limitation to the establishment of caucasian clover. However, selection of more effective rhizobia has the potential to overcome this limitation (Pryor *et al.* 1998). In addition to increasing the percentage of seedlings nodulated, strain ICC148 improved caucasian clover growth with seedling growth after 4 months being 70% higher with ICC148 than with ICC105. In the second year, productivity of caucasian clover swards was 86% higher with ICC148 through both increased plant numbers and increased individual plant growth (Pryor *et al.* 1998).

To date, all studies of caucasian clover rhizobia have been carried out with derivatives of just two different strains. There is the opportunity to screen further isolates from the centre of origin for strains that are genetically stable and highly effective.

## Ineffective nodulation of white clover

Caucasian clover rhizobial strains also nodulate white clover, but the nodules do not fix nitrogen. Establishment of caucasian clover in New Zealand pastures will lead to a build-up of a population of its specific rhizobia, which is of potential concern because ineffective nodulation of white clover is likely to reduce nitrogen fixation. Once rhizobia for a particular legume become established in the soil, it is difficult to eradicate them; hence it is important to determine whether these rhizobia do nodulate white clover under field conditions.

We investigated a 5-year-old field plot of caucasian clover (inoculated with ICC105) containing volunteer white clover plants. The soil and white clover roots contained $10^5$ rhizobia/g soil and $10^6$ rhizobia/g root material able to nodulate both white clover and caucasian clover. In order to determine the impact that this high population of caucasian clover rhizobia had on white clover nodule occupancy, nodules were removed from the white clover plants and the nodule isolates were characterised. Strain ICC105 formed 11% of the nodules on these white clover plants (R. Elliot, PhD thesis, University of Otago), a surprising finding, as inoculant strains are not usually able to establish in such high numbers over a short period of time, or compete with naturalised rhizobia for the nodulation of a host legume. No information is available on the effect that this is likely to have in the field, but laboratory studies showed reduced nitrogen fixation by white clover as the proportion of nodules occupied by strain ICC105 increased compared to occupation by an effective white clover strain.

The above observations relate to a field site sampled 5 years after the establishment of caucasian clover, and the longer-term implications of widespread use of caucasian clover are unknown. However the current work does point to the possibility that the use of present caucasian clover rhizobial inoculants may over time reduce nitrogen fixation and hence the establishment, production and persistence of white clover. To pre-empt any such problems, it would be useful to develop a caucasian clover rhizobial inoculant strain that is not detrimental to white clover nitrogen fixation.

## Summary

Establishment of caucasian clover has been hampered by nodulation failure, though use of more adapted strains of caucasian clover rhizobia has ameliorated this problem. However, nodulation and hence establishment when seed is oversown are still not optimal. Available inoculant strains are genetically unstable and must be monitored on a regular basis to ensure that effective isolates are always used. Caucasian clover rhizobia form ineffective nodules on white clover. A better understanding of the ecology of naturalised rhizobial populations is required in order to be able to predict the consequences to white clover of introducing these rhizobial strains to New Zealand pastures. We recommend that a caucasian clover rhizobial inoculant strain that is not detrimental to white clover nitrogen fixation should be developed as soon as possible.

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**REFERENCES**

Bryant, W.G. 1974. Caucasian clover (*Trifolium ambiguum* Bieb.). *Journal of the Australian Institute of Agricultural Science 40*: 11–18.

Dear, B.S.; Zorin, M. 1985. Persistence and productivity of *Trifolium ambiguum* M. Bieb. (Caucasian clover) in a high altitude region of south-eastern Australia. *Australian journal of experimental agriculture 25*: 124–132.

Hely, F.W. 1957. Symbiotic variation in *Trifolium ambiguum* M. Bieb. with special reference to the nature of resistance. *Australian journal of biological science 10*: 1–16.

Kannenberg, L.W.; Elliott, F.C. 1962. Ploidy in *Trifolium ambiguum* M Bieb. in relation to some morphological and physiological characters. *Crop science 2*: 378–381.

Lucas, R.J.; White, J.G.H.; Daly, G.T.; Jarvis, P.; Meijer, G. 1981. Lotus, white clover and Caucasian clover oversowing, Mesopotamia Station, South Canterbury. *Proceedings of the New Zealand Grassland Association 42*: 142–151.

Parker, D.T.; Allen, O.N. 1952. The nodulation status of *Trifolium ambiguum*. *Proceedings of the Soil Science Society of America 16*: 350–353.

Patrick, H.N.; Lowther, W.L.; Trainor, K.D. 1994. Inoculation for successful establishment of Caucasian clover. *Proceedings of the New Zealand Grassland Association 56*: 101–105.

Pryor, H.N.; Lowther, W.L.; McIntyre, H.J.; Ronson, C.W. 1998. An inoculant *Rhizobium* strain for improved establishment and growth of hexaploid Caucasian clover (*Trifolium ambiguum*). *New Zealand journal of agricultural research 41*: 179–189.

Taylor, N.L.; Smith, R.R. 1998. Kura clover *(Trifolium ambiguum* M. Bieb) breeding, culture, and utilisation. pp. 153–178. *In:* Advances in agronomy, D.L. Sparks (ed.) Academic Press, Newark, Delaware.

Vincent, J.M.; Thompson, J.A.; Donovan, K.O. 1962. Death of root-nodule bacteria on drying. *Australian journal of agricultural research 13*: 258–270.

Watson, R.N.; Neville, F.J.; Bell, N.L.; Harris, S.L. 1996. Caucasian clover as a pasture legume for dryland dairying in the coastal Bay of Plenty. *Proceedings of the New Zealand Grassland Association 58*: 183–188.

Zorin, M.; Brockwell, J.; Müller, W.J. 1976a. Symbiotic characteristics of *Trifolium ambiguum* seedlings grown in tube culture as affecting subsequent symbiotic vigour. *Australian journal of experimental agriculture and animal husbandry 16*: 854–862.

Zorin, M.; Dear, B.S.; Hely, F.W. 1976b. Young plant vigour and nodulation studies in diploid forms of *Trifolium ambiguum* Bieb. *Australian CSIRO Division of Plant Industry Field Station Records 15*: 35–40.

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