Symbiotic Interactions of Geographically Diverse Annual and Perennial *Trifolium* spp. with *Rhizobium leguminosarum* bv. *trifolii*

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ABSTRACT

Perennial clovers are being evaluated for their potential to reduce groundwater levels in Australian cropping zones where many soils are considered too acidic for reliable lucerne nodulation. However, the release of effective inocula for perennial clovers into such areas where sub clover is the predominant legume, could potentially compromise nitrogen fixation from this valuable annual clover if the symbiosis between the new inoculants and sub clover is not optimal. Studies were therefore designed to increase our understanding of these symbiotic interactions to optimise the management of legume-rhizobia interactions to extend (rather than restrict) the use of legumes in new environments.

To assist the understanding of interactions between clovers and their micro-symbionts, a glasshouse-based study of the cross-inoculation characteristics of 38 strains of *Rhizobium leguminosarum* biovar *trifolii* (*R. l. trifolii*) associated with 38 genotypes of annual and perennial *Trifolium* spp*.* from world centres of diversity was undertaken. Rhizobial isolates and clovers were assembled from South and equatorial Africa, North and South America and the Euro-Mediterranean regions. There was substantial specificity amongst the African clovers for effective nodulation. No strain of rhizobia from the South American perennial *T. polymorphum,* or from the Ethiopian clovers, was able to nodulate sub clover effectively, whilst less than 33% of the 18 strains from these regions could form nodules with the less promiscuous Mediterranean annual *T. glanduliferum*. Seventy of 476 cross-inoculation treatments examined did not nodulate, whilst 81 treatments clearly demonstrated effective nodulation. The remainder of the cross-inoculation pairings revealed only partially effective or ineffective nodulation. Two barriers to effective nodulation were identified from the cross-inoculation study: a geographic barrier representing the broad centres of clover diversity, across which

few host-strain combinations were effective; and within each region, a significant barrier to effective nodulation between an isolate from an annual host on a perennial host, or *vice versa*. Clovers and their rhizobia from within the Euro-Mediterranean region of diversity were more able to overlap the annual/perennial barrier than genotypes from the other regions. The data indicate that it will be a substantial challenge to develop inocula for perennial clovers that do not adversely affect nitrogen fixation by sub clover and other annual clovers in commerce, especially if the perennial clovers originate from Africa or America.

To investigate the management of legume-rhizobia interactions when introducing legumes into new environments, a study was initiated in Uruguay (Mediterranean annual clovers were introduced into a predominantly perennial clover setting) that could be considered opposite to the situation emerging within southern Australia (perennial clovers evaluated in a predominantly annual clover setting). The Uruguayan grasslands contain populations of indigenous *R. l. trifolii* that nodulate endemic *T. polymorphum* but form ineffective nodules on clovers originating from the Mediterranean region. Importantly in the Uruguayan setting, Government policy has facilitated the introduction of numerous varieties of annual Mediterranean clovers with the aim of improving overall winter production in their naturally managed grasslands. In an attempt to understand the rhizobial ecology of this scenario, a cross-row experiment was set-up in 1999 in a basaltic, acid soil in Glencoe, Uruguay, to follow the survival and symbiotic performance of nine exotic strains of *R. l. trifolii*. In this thesis I report on the ability of the introduced strains to compete for nodule occupancy of Mediterranean clover hosts and show the impacts of the introduced strains on the productivity of the indigenous Uruguayan clover, *T. polymorphum.* Of the introduced strains, WSM1325 was a superior inoculant and remained highly persistent and competitive in forming effective symbioses with the Mediterranean hosts, *T. purpureum* and *T. repens*, in the

Uruguayan environment over a 3 year period. *T. purpureum* and *T. repens*, when inoculated with the introduced strains, did not nodulate with any indigenous *R. l. trifolii* as typed from nodules of *T. polymorphum.* Conversely, there were no nodules on the Uruguayan host *T. polymorphum* that contained the introduced *R. l. trifolii*. These results revealed that there were effective symbioses between strains of *R. l. trifolii* and clovers, even though the soil contained ineffective *R. l. trifolii* for all hosts. This represents the first reported example of selective nodulation for an effective symbiosis *in situ* with annual and perennial clovers in acid soils. This phenomenon raised the question of whether this was restricted to the particular edaphic scenario in Glencoe, Uruguay.

Glasshouse-based experiments in Australia were conducted to further understand the selection phenomenon. Two strains were selected for comparisons; strain WSM1325 isolated from an annual clover in the Mediterranean and WSM2304 isolated from the perennial clover *T. polymorphum* in Uruguay, South America. Variables that may have been specific to Glencoe were investigated. Thus, the effect of cell density and strain ratio at the time of inoculation, as well as soil pH, were examined on the two hosts (*T. purpureum* and *T. polymorphum*). Each was exposed to the same effective and ineffective micro-symbionts. In co-inoculation experiments at a cell density of 10^4 cells mL^{-1}, each host nodulated solely with its effective strain, even when this strain was out-numbered 100:1 by the ineffective strain. However, the selection process ceased when the effective strain was out-numbered 1000:1. At higher basal cell concentrations of 10^5 - 10^8 cells mL^{-1}, selection for WSM1325 to form effective nodules on *T. purpureum* was evident, but was significantly reduced as the ratio of ineffective cells in the inoculum increased above 4-fold. These results indicate that the selection mechanism is highly dependent upon the basal rhizobial cell density. Soil pH did not significantly

alter the process, which could not be simply explained by the rate of strain growth, or extent of nodulation.

Greater precision was sought in the terminology applied to nodulation outcomes where legumes have a choice of micro-symbiotic partners from within the same species of root-nodule bacteria. The nominated preferred terms are “non-selective”, “exclusive”, and “selective” nodulation.

In view of the difference in host range between WSM1325 and WSM2304 and the selective nodulation process, a preliminary investigation into the genetic backgrounds of WSM1325 and WSM2304 was conducted. A selected range of gene regions were amplified by PCR from each strain and sequenced. Comparative analysis of the nucleotide sequences revealed that although the 16S rRNA sequences were identical, the *atpD*, *GSII* and *nodD* sequences contained distinct differences revealing disparity between the pSym replicons and between the chromosomal replicons of these strains. Of the genes sequenced, the highest degree of divergence was noted for the symbiotic NodD protein products, which are known to be critical determinants in the nodulation of specific hosts. An examination of the *nodD* gene region of WSM1325 and WSM2304 revealed a further contrasting feature; the regulatory gene *nodR* was present in the *nodD* gene region of WSM1325 but absent in WSM2304. Since NodR is known to be required for adding highly unsaturated fatty acyl groups onto the Nod-factor backbone, I could now hypothesise that the nodulation incompatibility observed between *Trifolium* hosts and micro-symbionts obtained from different geographical locations may result from differences in Nod-factor decoration. With the full genome sequence of the two strains WSM1325 and WSM2304 soon to be available, the role of *nodR* and any link to the selection phenomenon described in this thesis can be addressed.

DECLARATION

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

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