

## Symbiotic relationship between *Rhizobium leguminosarum* biovar *trifolii* and *Trifolium nigrescens*

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**Abstract** Recommended strains of rhizobia for *Trifolium repens* and *T. subterraneum*, and rhizobia isolated from *T. angustifolium* and *T. pratense* root nodules, were used to inoculate seven accessions of *T. nigrescens* and their own macrosymbiont host plants growing in N-free media. When inoculated with the strain of rhizobia recommended for *T. subterraneum*, foliage weights of six of the seven *T. nigrescens* accessions were either the highest, or not significantly less than the highest. This supports the inclusion of *T. nigrescens* in the *T. subterraneum* group when grouping species which respond similarly symbiotically. The response of foliage weights of *T. subterraneum* and *T. repens* plants to inoculation with the strain of rhizobia isolated from *T. angustifolium* was similar to that following inoculation with the recommended strain for *T. repens*, rather than with the recommended strain for *T. subterraneum*. This result indicates that *T. angustifolium* should be included in the *T. repens* group when grouping species which respond similarly in symbiosis.

**Keywords** N-fixation; rhizobia; *Trifolium angustifolium*; *Trifolium nigrescens*; *Trifolium repens*; *Trifolium subterraneum*

## INTRODUCTION

*Trifolium nigrescens* Viv. is an annual, non-stoloniferous clover, native to Turkey, Iraq, Iran, Armenia, Cyprus, the Caucasus region, and the Mediterranean countries of Europe and North Africa (Zohary & Heller 1984; Gillet 1985). In New Zealand, *T. nigrescens* is being investigated for its potential as germplasm for the improvement of *T. repens* through interspecific hybridisation (Hussain & Williams 1997). Zohary (1970) recognised and documented two subspecies of *T. nigrescens*—ssp. *nigrescens* and ssp. *petrisavii*. Williams et al. (2001) have proposed dividing *T. nigrescens* into three subspecies—ssp. *nigrescens*, ssp. *petrisavii*, and ssp. *meneghinianum*—on the basis of hybridisation experiments, rDNA internally transcribed spacer region sequences, and different chromosomal locations of two ribosomal DNA sequences. These traits differ markedly among the *T. nigrescens* subspecies and all three differ from *T. repens* (Williams et al. 2001). Ertekin & Akbayin (2000) described a new variety, *T. nigrescens* ssp. *petrisavii* var. *grandifolium*, that appears to be synonymous with *T. nigrescens* ssp. *petrisavii* var. *meneghinianum* (Hossain 1961), which was the basis of ssp. *meneghinianum* used by Williams et al. (2001).

Conflicting opinions exist in the literature on the symbiotic specificity between *T. nigrescens* and *Rhizobium leguminosarum* bv. *trifolii*. On the basis of species which respond similarly in symbiosis, Nutman (1959) provisionally grouped *T. nigrescens* with *T. subterraneum*. He placed *T. repens* in a different group. However, Nutman (1965) later changed his grouping by placing *T. nigrescens* with *T. repens*. On the basis of Nutman (1965), Burton (1985) also placed *T. nigrescens* with *T. repens*. No experimental results were presented by Nutman (1959, 1965) to support his groupings. These groupings follow the phylogenetic relationship of the species as this is close between *T. nigrescens* and *T. repens* (Williams et al. 2001), with a considerable distance to *T. subterraneum* (Zohary

& Heller 1984). Abberton et al. (1999) found that although a mixture of five strains of rhizobia known to be symbiotically effective on *T. repens* formed nodules on all 24 *T. nigrescens* plants, only four plants exhibited symbiotic N-fixation. This result questions the inclusion of *T. nigrescens* with *T. repens* when grouping species which respond similarly in symbiosis.

This research was initiated to re-examine the symbiotic relationship between *T. nigrescens* and *Rhizobium* strains to identify the most symbiotically effective strain of rhizobia for inoculant use in New Zealand. In addition, symbiotic relationships of the three subspecies proposed by Williams et al. (2001) were investigated.

## MATERIALS AND METHODS

### Rhizobia

Rhizobial strains used included recommended strains for *T. repens* (ICC100), *T. subterraneum* (ICC102), and *T. medium* (ICC106) (Young & Fletcher 1997; Table 1). We also included a strain (ICC125) isolated from a root nodule off a *T. pratense* plant collected by M. Forde in the Caucasus region in November 1989, and a strain (ICC122) isolated from a root nodule from a *T. angustifolium* plant collected on the same expedition. *T. angustifolium* is included in the same symbiotic subgroup as *T. subterraneum* (Nutman 1965; Burton 1985). No strains isolated from, or recommended for, *T. nigrescens* were available in New Zealand. Inoculum treatments were prepared by streaking rhizobia onto yeast mannitol agar plates, incubating for 7 days at 25°C, then suspending in 1/4-strength Jensen's nutrient solution (Vincent 1970).

**Table 1** Strains of *Rhizobium leguminosarum* bv. *trifolii*, their background, and the host from which they were isolated.

ICC no.	Host plant	Reisolate of
100	<i>T. repens</i>	ICMP2153b*
102	<i>T. subterraneum</i>	WU290*
106	<i>T. medium</i>	ICMP8378b*
122	<i>T. angustifolium</i>	DSIR6737†
125	<i>T. pratense</i>	DSIR6816†

\*Recommended inoculant strains (Young & Fletcher 1997).

†Collected by M. Forde in 1989 in the Caucasus region.

### Plant material

Seed of accessions of *T. nigrescens* and *T. angustifolium* was supplied by the Margot Forde Germplasm Centre (Table 2), with the number depending on availability. Cultivars of *T. pratense*, *T. repens*, and *T. subterraneum* were purchased commercially. Seeds were scarified by light rubbing with fine sandpaper, sterilised for 5 min in 1.5% sodium hypochlorite, rinsed in sterile water, and germinated on inverted water agar plates at 25°C. When radicles reached 0.5 mm, seedlings were aseptically planted onto N-free agar slopes (Vincent 1970) in 20 × 150 mm test tubes. Five days after planting, seedlings were inspected and only those with normal cotyledon and root growth were selected for use.

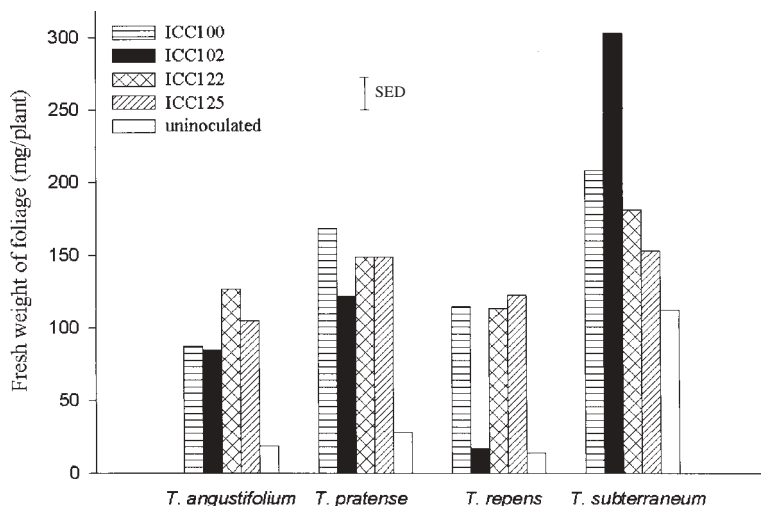
### Inoculation treatments

In Experiment 1, the symbiotic effectiveness of ICC100, ICC102, ICC122, and ICC125 on their host, and their interaction with the other legumes, were determined by inoculating *T. repens*, *T. subterraneum*, *T. angustifolium*, and *T. pratense* plants with each of the strains and measuring foliage growth. Uninoculated plants of each species were included as controls. In Experiment 2, the host-strain relationships between five strains of rhizobia (Table 1) and the *T. nigrescens* plant accessions

**Table 2** Legume species and accessions of three *T. nigrescens* taxa.

Host plant	No. of seedlings in each inoculation treatment for each strain of rhizobia (= replicates)
<b>Experiment 1</b>	
<i>T. angustifolium</i> AZ 154 (CPI 21877)	10
<i>T. pratense</i> cv. Montgomery	8
<i>T. repens</i> cv. Huia	10
<i>T. subterraneum</i> cv. Woogenellup	14
<b>Experiment 2</b>	
<i>T. repens</i> cv. Huia	6
<i>T. nigrescens</i> ssp. <i>nigrescens</i>	
AZ 2225	11
AZ 3281	3
AZ 3290	4
ssp. <i>petrisavii</i> var. <i>petrisavii</i>	
AZ 3257	13
AZ 3276	7
AZ 3287	7
ssp. <i>petrisavii</i> var. <i>meneghinianum</i>	
AZ 1308	6

**Fig. 1** Interactions between the host legumes and rhizobia from *T. repens* (ICC100), *T. subterraneum* (ICC102), *T. angustifolium* (ICC122), and *T. pratense* (ICC125). SED is the standard error of the difference for comparing rhizobia treatments within individual plant accessions.



listed in Table 2 were examined by inoculating all accessions with each of the strains. Because of the limited number of seed of some accessions, the number of suitable seedlings for each inoculation treatment varied (Table 2). Seedlings were inoculated with 1 ml of inoculum treatment containing  $>10^8$  rhizobia. No *T. nigrescens* accessions were available for uninoculated controls. However, all plants inoculated with ICC106 failed to nodulate, and their foliage weights are presented as unnodulated plants.

After inoculation, tubes were placed in constant temperature water baths at 25°C in a glasshouse with supplementary lighting to give a 16-h day.

### Experimental design

Tubes were arranged in three 11 × 12 cell wire frames. The experiments were set up as a split-plot design, with four blocks per frame for Experiment 1 and two blocks per frame for Experiment 2. Sets of four (Experiment 1) or five (Experiment 2) adjacent cells constituted main plots, to which plant accessions were randomly allocated. In some cases multiple main plots of plant accessions were used because replicates ranged from 8 to 14 (Experiment 1) and from 3 to 13 (Experiment 2) plants for each rhizobia treatment (Table 2). Rhizobia were randomised to cells within main plots.

### Assessments

Plants were harvested 36 (Experiment 1) and 40 (Experiment 2) days after inoculation when visual

differences in growth were evident. Plants were removed from the tubes, the presence or absence of nodules assessed, then plants were cut at the cotyledonary node and the fresh weight of the foliage determined. The weights of unnodulated plants were not included in the statistical analyses. When available N is the main limiting factor, plant fresh weight and total N are highly correlated, allowing an assessment of nitrogen fixing effectiveness of each strain of rhizobia (Vincent 1970).

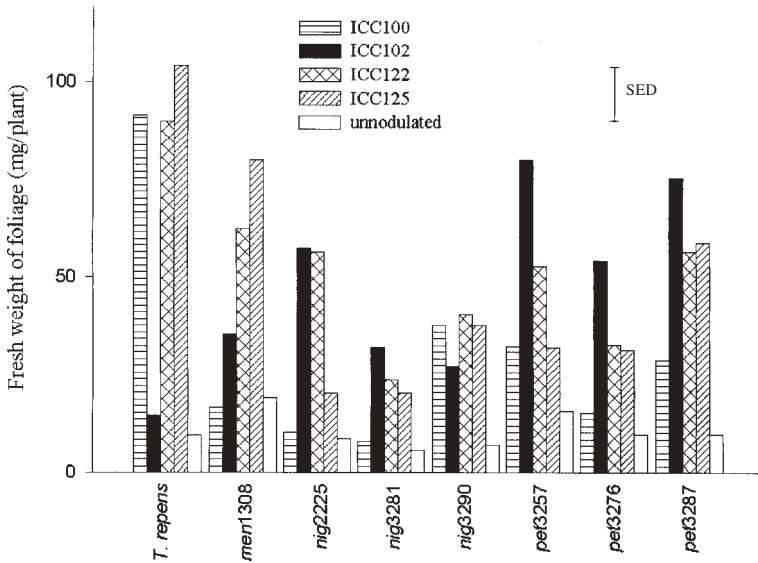
Foliage weight for each experiment was analysed (GenStat 2000) by residual maximum likelihood (Patterson & Thompson 1971), a generalisation of ANOVA for unbalanced designs (with the random effects given by cell within main plot within block within frame), and the fixed effects by plant accession, rhizobia, and their interaction.

## RESULTS AND DISCUSSION

### Experiment 1

The four strains of rhizobia nodulated all plant lines, with the exception of *T. angustifolium* where only 80 and 90% of plants were nodulated by strains ICC102 and ICC125, respectively. This suggests there may have been other species as contaminants in this seed line.

Foliage weight of *T. subterraneum* plants inoculated with the recommended strain of rhizobia for *T. subterraneum* (ICC102) was significantly



**Fig. 2** Interactions between *T. nigrescens*—ssp. *nigrescens*, ssp. *petrisavii* var. *petrisavii*, ssp. *petrisavii* var. *meneghinianum*—and rhizobia from *T. repens* (ICC100), *T. subterraneum* (ICC102), *T. angustifolium* (ICC122), *T. pratense* (ICC125), and unnodulated plants. SED is the standard error of the difference for comparing rhizobia treatments within individual plant accessions.

( $P < 0.001$ ) higher than foliage weights of plants inoculated with the other three strains of rhizobia (Fig. 1). The foliage weight of *T. repens* plants inoculated with strain ICC102 was significantly ( $P < 0.001$ ) lower than the foliage weights of plants inoculated with the other three strains of rhizobia.

There was no significant difference ( $P > 0.05$ ) between foliage weights of *T. angustifolium* plants inoculated with the different strains of rhizobia. When *T. subterraneum* plants were inoculated with their recommended strain of rhizobia (ICC102) the mean foliage weight was significantly ( $P < 0.001$ ) higher than plants inoculated with the strain of rhizobia from *T. angustifolium* (ICC122). However, the mean foliage weight of *T. repens* plants inoculated with the recommended strain of rhizobia (ICC100) did not differ significantly from plants inoculated with ICC122. This pattern of foliage weight response of *T. subterraneum* and *T. repens* plants to the strain of rhizobia isolated from *T. angustifolium* (ICC122) is consistent with *T. angustifolium* belonging to the same symbiotic grouping as *T. repens*, not the *T. subterraneum* group as suggested by Burton (1985).

## Experiment 2

The recommended strain of rhizobia for *T. medium* (ICC106) failed to nodulate any plants, including *T. repens*. This was unexpected as the source culture (ICMP8378b) is classified as symbiotically

effective on *T. repens* (Young & Fletcher 1997) indicating that ICC106 is either a non-nodulating variant of ICMP8378b or a contaminant. Foliage weights of unnodulated plants inoculated with ICC106 are presented in Fig. 2 but have not been included in the statistical analyses.

With two exceptions, all other strains of rhizobia nodulated all the plant accessions. These exceptions were strains ICC100 and ICC125, which nodulated 72 and 86% of the var. *petrisavii* AZ 3276 plants, respectively.

Foliage weights of six of the seven *T. nigrescens* accessions inoculated with the strain of rhizobia recommended for *T. subterraneum* (ICC102) were either the highest, or not significantly different from the highest (Fig. 2). Mean foliage weight over all *T. nigrescens* accessions inoculated with the strain of rhizobia recommended for *T. repens* (ICC100) was 21.3 mg/plant, significantly ( $P < 0.001$ ) lower than the 51.7 (SED = 5.1) mg/plant for those inoculated with the strain of rhizobia recommended for *T. subterraneum* (ICC102). This result is consistent with the inclusion of *T. nigrescens* in the same symbiotic grouping as *T. subterraneum* (Nutman 1959) rather than the *T. repens* grouping (Burton 1985). This is surprising given the close phylogenetic relationship between *T. nigrescens* and *T. repens* (Williams et al. 2001). *T. subterraneum* on the other hand is classified taxonomically at a considerable distance from *T. nigrescens* and *T. repens* (Zohary & Heller 1984). Rhizobial/host

plant symbiotic affinities in these species clearly do not reflect plant species ancestry.

There was an apparent differentiation between two of the subspecies of *T. nigrescens* in their growth response to the different strains of rhizobia (Fig. 2). The mean foliage weight of the three ssp. *petrisavii* accessions inoculated with ICC102 was significantly higher ( $P < 0.001$ ) than ssp. *petrisavii* accessions inoculated with the other strains. This was consistent with the three ssp. *petrisavii* accessions. However, this response to ICC102 was not apparent with the ssp. *nigrescens* and ssp. *meneghinianum* accessions. Further research with var. *meneghinianum* is required as five of the AZ 1308 plants inoculated with ICC102 had small, white nodules and low foliage weights (17–32 mg/plant). The other plant had large pink nodules and foliage weight of 98 mg/plant. The large variability of plant growth within some accessions inoculated with the same strain of rhizobia was a feature of these experiments. As few as three seedlings of some accessions were inoculated with a strain of rhizobia, therefore, caution is required in comparing the results of these accessions.

In general, this research supports the inclusion of *T. nigrescens* in the same symbiotic grouping as *T. subterraneum* (Nutman 1959) rather than the *T. repens* grouping (Burton 1985).

## ACKNOWLEDGMENTS

We thank Warren Williams for supply of seed and the inspiration for this research, and Roger Littlejohn for statistical design and analysis. This research was funded by the New Zealand Foundation for Research, Science and Technology.

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