

DEVELOPING LUCERNE AND RHIZOBIA WITH IMPROVED TOLERANCE TO ACIDIC SOILS: A NOVEL APPROACH

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SUMMARY

A multidisciplinary approach to improving the tolerance of lucerne and rhizobia to acid soils is being pursued, involving the selection of plants, rhizobia and the symbiosis between plants and rhizobia.

Lucerne plants have had 4 cycles of selection for improved root length in solution culture at low pH with toxic levels of aluminium. In greenhouse trials, lucerne selections grown in acidic soils, collected from Victoria and NSW, showed an increase in root mass of between 0 and 225% and an increase in shoot mass of 0 to 26% compared with commercial cultivars.

Two hundred and twenty strains of rhizobia were isolated from acidic soils under mature lucerne stands in NSW. Solution screening has shown that at least 4 of these strains are able to nodulate 50% of lucerne plants at pH 4.8 compared with less than 10% of plants nodulated with the commercial strain (RRI 128). One of these strains (SRDI 675) almost doubled plant nodulation compared to the commercial strain when it was used to inoculate lucerne plants grown in an acid soil collected from Inman Valley, SA.

Solution culture screening has also shown variation among lucerne populations for their ability to nodulate at pH 5. Plants with pink nodules after 4 weeks of growth in this system have been recovered in a nitrogen free environment and then crossed to generate the 1st cycle of lucerne population with improved nodulation.

This paper summarises the most recent results following almost 10 years of collaborative research to develop successful screening systems and subsequent selection for these traits and will discuss the strategies used to progress their development.

Key words: *Medicago sativa*, alfalfa, nodulation, acidity tolerance, aluminium, *SinoRhizobium*.

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INTRODUCTION

Lucerne or alfalfa (*Medicago sativa*) is a summer active perennial pasture legume, often described as the queen of forages and grown on over 33 M ha worldwide. Poor tolerance to acidic soils is a major factor restricting further adoption around the world (Humphries and Auricht 2001).

The sensitivity of lucerne to low pH has been recognised for decades (Munns 1965; White 1967). Lucerne is ideally established on soils in the pH_{Ca} range of 5.5 to 8.0 (Stanley *et al.* 2002), and although it is grown on more acidic soils, lucerne yield on these soils is often suboptimal (Pinkerton and Simpson 1986). Nodulation in lucerne is impaired at pH_{Ca} <5.5 (Munns 1968) and soluble aluminium restricts root growth (Campbell *et al.* 1988; Munns 1965) and nodulation (Bordeleau and Prevost 1994) in many soils with a pH_{Ca} <5. Reductions in nodulation and root growth combine to severely inhibit establishment, production, nitrogen fixation and survival on very acidic soils.

Previous efforts to identify a lucerne population tolerant to aluminium toxicity have reported a narrow range of tolerance between populations, but all have identified variability within populations (Bouton 1996; Buss *et al.* 1975; Campbell *et al.* 1989; Zhang *et al.* 2004). Screening systems that utilise soil initially looked promising for plant selection and breeding, but in most cases the tolerance is too closely linked to specific properties of the particular soil chosen (Narasimhamoorthy *et al.* 2007). Solution culture screening systems have been developed to select plants with tolerance to aluminium at pH 4.5 (Garnett *et al.* 2003; Humphries *et al.* 2006; Scott *et al.* 2008). The systems have produced repeatable results that demonstrate positive response to recurrent mass selection (Zhang *et al.* 2007) and a good correlation with field rankings has been reported for annual *Medicago* species (Scott *et al.* 2008).

A solution culture system maintained at pH 5.0 was developed by Charman *et al.* (2008) to evaluate the ability of rhizobia strains to form nodules at low pH, leading to the identification of strain 'SRDI 291'. This system also makes it possible to identify and select individual lucerne plants that are better able to nodulate with rhizobia at low pH.

In this paper, the recent development of acid tolerant lucerne plants and selection of rhizobia is discussed. Preliminary results using promising strains are presented from pot experiments using field soil. The progress in developing lucerne plants tolerant to aluminium toxicity at low pH is reported, including validation experiments conducted in the greenhouse with a range of field soils.

MATERIALS AND METHODS

Breeding lucerne for tolerance to aluminium toxicity in solution culture

Lucerne seedlings were grown in hydroponic solution consisting of 120 L of 1 mM CaCl₂ solution pumped through 15 L containers with floating seed holders. The system capacity was 4000 seedlings held in 10 containers with 5 replications. Seeds were surface sterilised and pre-germinated in petri dishes, before being planted with a uniform 5 mm radical length. The solution culture was maintained at pH 4.5 with

daily adjustments with 0.1 M HCl throughout the experiment. Aluminium chloride was added on day 3 of the experiment as 3uM AlCl₃ 6H₂O, and root length was measured 14 days later. Plants with roots that continued to grow in the presence of aluminium were selected as tolerant individuals. Data on root length was collected from individual seedlings and then analysed using an analysis of variance on a replicate mean basis over the 20 seedlings.

Seedlings from 32 breeder's lines (including cultivar SARDI Seven) were selected from solution culture and used to develop populations through 4 cycles of recurrent mass selection. Genetic diversity was maintained by using paired crosses in each generation. TA24 is the fourth cycle of selection and was developed by crossing plants with high root length from TA22 population.

Screening lucerne for low pH and Aluminium toxicities in pot experiments

Results from a range of pot studies using field soils are reported. A list of the soils and their chemical and physical properties are presented in Table 1. Experiments had a minimum of 6 replications and a randomised complete block design was used. Pots were watered up to 70% field capacity and monitored daily to ensure soil moisture never fell below 50% field capacity. The aim of the irrigation regime was to minimise the incidence of root disease.

Table 1. Chemical and physical properties of soils used in pot validation studies. Chemical analysis by CSPB, WA. Disease risk assessment based on quantification of pathogen DNA levels, tests provided by SARDI.

Soil	pH (CaCl ₂)	Al _{Ca} (ppm)	CEC (meq/100g)	Al (% CEC)	Disease risk	Texture
Warrnambool, Vic.	4.4	7	6	12	Low	Clay loam
Benalla, Vic.	4.4	8	6.6	5	High <i>Pythium</i>	Sandy loam
Seymour, Vic.	4.3	20	5.1	27	High <i>Pythium</i>	Clay loam
Howlong, NSW	4.5	2.2	4.2	5.5	Low	Clay loam
Inman Valley, SA	4.9	1.7	1.2	9	Low	Sand

Screening rhizobia for tolerance to low pH in solution culture

The method for evaluating the pH tolerance of lucerne rhizobia in solution culture has been previously described by Charman *et al.* (2008). The strains used in experiments in this paper were isolated from mature lucerne stands growing on a range of moderately acidic soils in NSW (Table 2). The same solution culture was used in the current experiment but maintained pH at 4.8, instead of 5.0. No aluminium was added to solution culture experiments with rhizobia. Rhizobia strains were evaluated in a randomised complete block with 4 replications, and analysed with an analysis of variance.

The method has also been adapted to enable plant selection work. Twenty one days after inoculation, individual plants with pink nodules and dark green leaves (indicating effective nitrogen fixation), were selected and recovered in soil for use in subsequent breeding activities.

Table 2. Description of strains of *Sinorhizobium meliloti* used in greenhouse solution culture and soil experiments. Chemical analysis by CSPB, WA

Strain	Nearest town	Soil pH _{Ca}	Al _{Ca} (ppm)	Organic C (%)
SRDI 291	Bordertown, SA	5.0	Not known	1.7
SRDI 593	Crookwell, NSW	5.3	1.5	1.1
SRDI 643	Manton, NSW	5.4	2.7	1.1
SRDI 675	Bookham, NSW	5.5	1.7	1.1
SRDI 733	Book Book, NSW	5.3	0.7	0.9
RRI 128	Mallee region, VICT	7.6	Not known	Not known

RESULTS

Breeding lucerne for tolerance to aluminium toxicity

Breeding for improved root length in a low pH solution containing toxic levels of aluminium was successful, with a 3-fold increase in root length after 14 days following 3 cycles of selection (Figure 1). The mean root length increased from 2.4 cm in parent cultivar SARDI Seven to 7.2 cm in TA22 population.

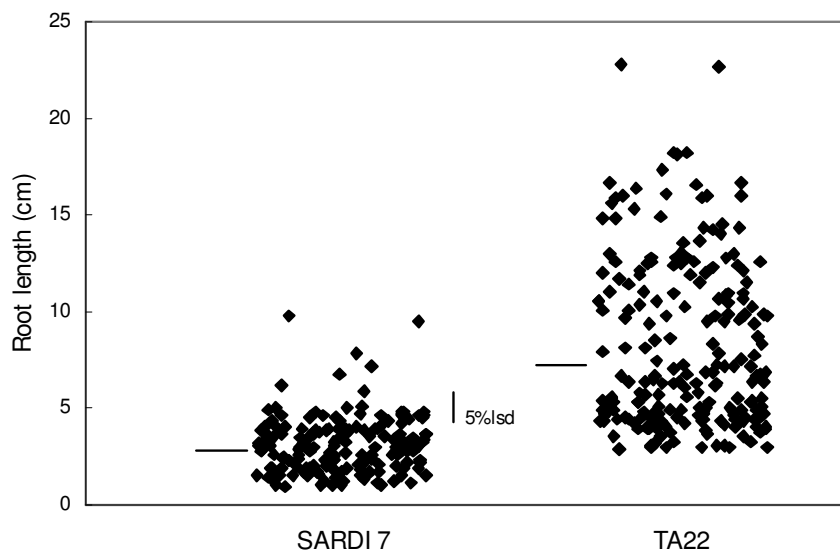


Figure 1. Progress made with 3 cycles of recurrent mass selection for root elongation in solution culture. Each point represents the root length of an individual lucerne plant 14 days after aluminium is added. Data points are separated horizontally for clarity. The horizontal line indicates the mean root length of each genotype, and the vertical line represents the 5% lsd for comparison of genotype means.

TA24 (selected from TA22) has also shown increased root weight over SARDI Seven in all soils tested, except for Benalla, and improved shoot weight at Benalla and Seymour (Figures 2 and 3). The greatest difference recorded was in the no-lime treatment in the Warrnambool soil, where TA24 showed more than twice the root weight of SARDI Seven. The smallest differences in root weight were in the Benalla and Howlong soils, with 0 and 14 % increases respectively.

The response to lime was greater in SARDI Seven than TA24 (Figure 2), with root weight more than doubling for SARDI Seven. The root-weight response to lime was not significant in TA24.

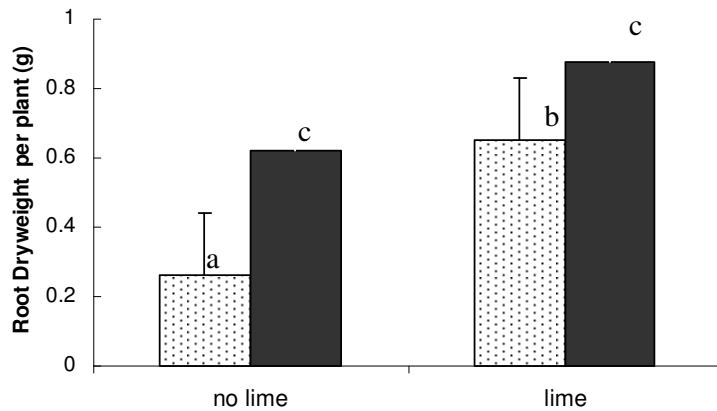


Figure 2. Response of root weight in SARDI Seven (dotted) and TA24 (black) to the addition of 1 t/ha to an acidic soil from Warrnambool with aluminium toxicity. Error bar shows the 5% lsd for effect of variety. Letters indicate significant differences at P<0.05 level for effect of lime.

Smaller differences were observed in shoot-weight than root weight on acidic soils, with 16 and 26% increases in shoot-weight in the Benalla and Seymour soils respectively, and no difference in the Howlong soil (with a trend for higher shoot-weight in SARDI Seven).

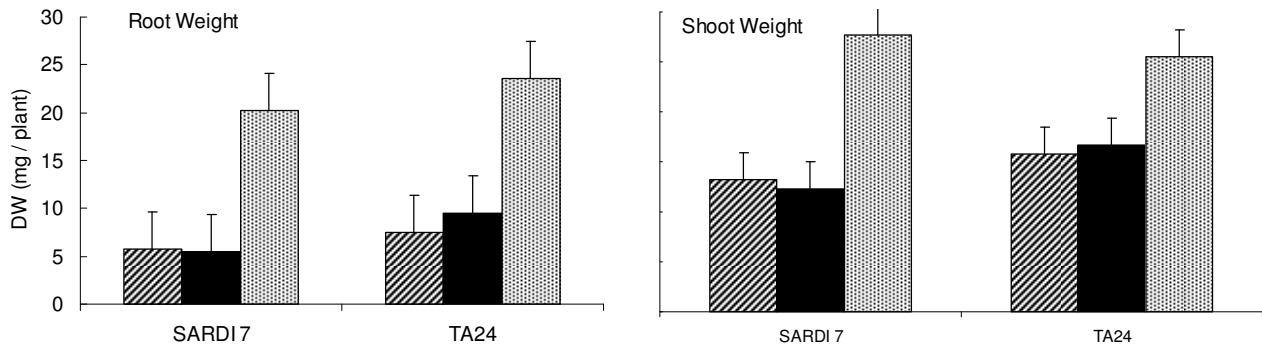


Figure 3. Root and shoot weight of acid-aluminium tolerant strain TA24 and control cultivar SARDI Seven in greenhouse pot studies using field soil collected from Benalla (hatched) and Seymour (solid) in Victoria and Howlong (dotted) in NSW. Error bar shows the 5% lsd for effect of variety.

Selecting an acid tolerant rhizobia

In a solution culture maintained at pH 4.8 the current commercial *Rhizobium* strain (RRI 128) nodulated a low percentage of lucerne plants (Figure 4). By contrast, Strains SRDI 675, SRDI 643, SRDI 291, SRDI 733 nodulated approximately 50% of lucerne plants. Lucerne plants that nodulate with the commercial *Rhizobium* strain have been selected (from several experiments) for crossing to form a population with enhanced capacity to nodulate with rhizobia at low pH.

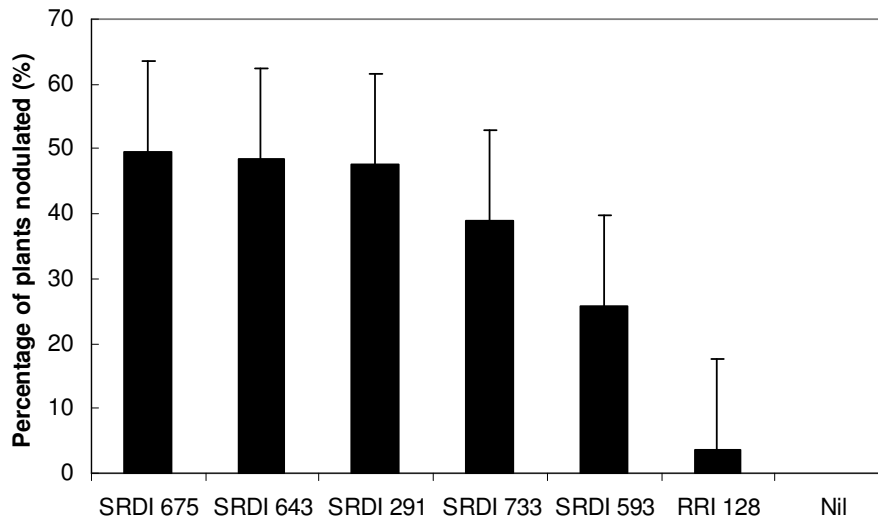


Figure 4. Nodulation by strains of *Sinorhizobium meliloti* isolated from NSW soils compared to commercial strain RRI 128, in solution culture at pH 4.8. Nil represents control with no inoculum. Error bar shows the 5% lsd for effect of strain.

An effect of *Rhizobium* strain on effective nodulation was also measured on plants grown in the Inman Valley soil amended with 100 kg.ha⁻¹ lime. The number of nodules per plant increased from 1.2 from a commercial strain RRI 128 to 2.0 from strain SRDI 675 (Figure 5). All other strains performed similarly to RRI 128. After 4 weeks, non-nodulated plants were chlorotic with smaller leaves; however no differences in herbage yield were measured (results not presented).

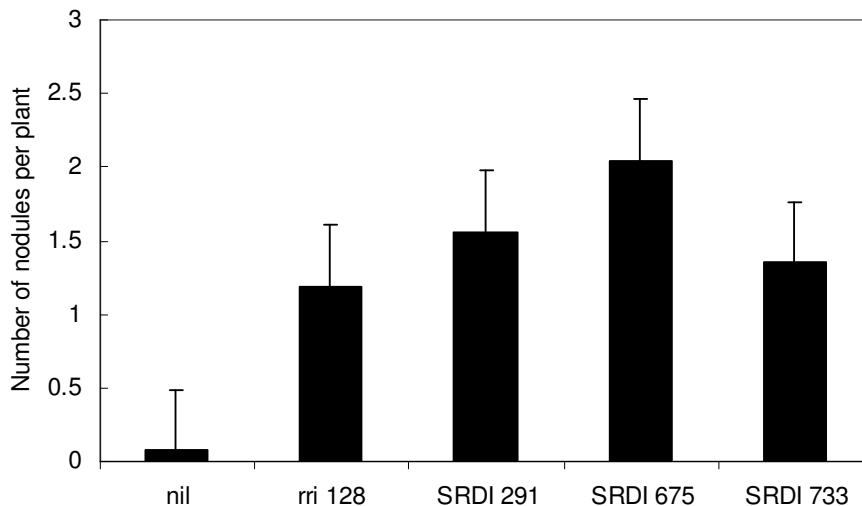


Figure 5. Nodulation of lucerne cultivar SARDI Ten with *Rhizobium* strains in greenhouse study after 4 weeks using field soil collected from Inman Valley, SA and amended with 100kg.ha⁻¹ of laboratory grade lime. The pH_{Ca} of amended soil at the completion of the experiment was 5.5. Error bar shows the 5% lsd for effect of strain.

DISCUSSION

A multidisciplinary effort to develop both lucerne populations and rhizobia that are tolerant to acidic soils using solution culture systems appears to be successful following a range of greenhouse experiments that validated the performance of the selected genotypes in field soils.

Breeding lucerne tolerant to toxic levels of aluminium at low pH

The solution culture system initially developed by Garnett *et al.* (2003) and modified by Humphries *et al.* (2006) has been used to develop lucerne populations with a 3-fold improvement in root length following 3 cycles of recurrent selection. The solution culture system has the advantage of being very uniform in comparison with screening for aluminium tolerance in soil where there may be other confounding effects (Bouton 1996 and Dall'Agnol *et al.* 1996). It is also efficient from a plant breeding perspective because larger number of plants can be evaluated in a short time frame in a small amount of space, with tight control of aluminium and nutrient concentrations, which allow for repeatable results. In soil, complex genotype by soil interactions can confound the effects of aluminium toxicity (Narasimhamoorthy *et al.* 2007). The advantage of a soil assay, however, is that it best mimics field conditions (Narasimhamoorthy *et al.* 2007). It is therefore encouraging to present results from greenhouse experiments using field soils that validate the progress made in improving aluminium tolerance of TA24 population in solution culture.

In a study using inbred annual *Medicago* species where genotypes were replicated, Narasimhamoorthy *et al.* (2007) report that the solution culture system produces some false positives in comparison to a soil assay. This suggests that it may be useful to develop a sequential screening system where seedlings are initially grown for 2 weeks in solution culture are selected and then refined following regrowth when transplanted into field soil(s).

In the solution culture system employed in this experiment, plant genetic variation was more pronounced than in soil, which was also observed by Campbell *et al.* (1989) but not by Narasimhamoorthy *et al.* (2007). Root extension rates in our solution culture are sensitive to small increases in Al, and a discriminating concentration has been used that that maximises the expression of plant genetic variability. A range of field soils were used in this study to help identify chemical and physical properties that may affect or mask improvements in aluminium tolerance. Soils with moderate to high saturation of aluminium on the cation exchange, such as Warrnambool and Seymour, were more responsive than those with a low concentration of Al, such as Benalla and Howlong. The Seymour and Benalla soils were identified by a PCR diagnostic test as having a high probability of *Pythium* infection. Disease symptoms (necrotic lesions, inhibition of lateral root formation) were frequently observed on plants growing in these soils, and this is likely to have complicated the expression of Al tolerance in these soils.

Selecting an acid tolerant rhizobia

The results in this study offer further encouragement that lucerne and its rhizobia can be selected to form an effective symbiosis at or below pH 5.0. The improved nodulation in cultivar SARDI Ten (Figure 4) with *Rhizobium* strains isolated from

NSW soils suggest that quite large gains could potentially be made by changing the *Rhizobium* strain. This change could be implemented quickly, and the benefits would likely extend to many of the existing lucerne cultivars sown in acidic soils.

Rhizobium strain SRDI 291, identified by Charman *et al.* (2008), performed well in solution culture, but was not better at nodulating lucerne than the current commercial strain (RRI 128) in soil. Strain SRDI 291 was isolated from a mildly acidic sand (pH_{Ca} 5.0) in the south east of South Australia. The collection of isolates from acidic soils in a zone where most lucerne expansion is anticipated has produced a number of strains with putative acidity tolerance, including SRDI 675. Strain SRDI 675 improved nodulation in solution culture and soil, and therefore replaces SRDI 291 as the most promising strain to date. The program is yet to complete evaluation of strains isolated from field collections, so it is expected that additional strains could be added to the cohort of strains identified with putative tolerance to acidic soils.

Additional greenhouse experiments are planned that will evaluate the twelve most promising rhizobia strains in the Inman Valley soil (amended with 50 kg/ha lime), and a second red-brown earth from Howlong, NSW. The use of the two soils that differ in their cation exchange capacities may remove the effect of any specific interactions between rhizobia and Ca and K deficiency. The greenhouse soil experiments, where temperature and soil moisture were controlled, provide an intermediate step between solution culture and field experiments. Using the field soils in pots as part of the *Rhizobium* strain selection process will hopefully reduce problems encountered by (Howieson *et al.* 1988) where rhizobia performance in the laboratory differed markedly to that in field evaluation.

Once greenhouse evaluation is completed, the most promising 5 strains will be evaluated in field trials on acidic to mildly acidic soils. Saprophytic competence of these strains will also be measured in these trials. The ability of rhizobia to survive in soil over summer and re-nodulate new cohorts of lateral roots produced during growth periods is believed to be important despite the perennial nature of lucerne providing some capacity to support longer lived indeterminate nodules in the subsoil.

A strain selected for commercialisation will also need to be effective at fixing nitrogen, and survive on seed, since the large majority of lucerne sold is pre-inoculated. The current strain, RRI 128, is very effective at fixing nitrogen with lucerne (Ballard *et al.* 2005) and initial observations indicate that the strains showing promise are close to RRI128 in their symbiotic capacity.

Bringing it all together: Lucerne varieties that can tolerate aluminium at low pH and nodulate with acid tolerant rhizobia

A tandem based method of selection is now underway where plants that nodulate with acid tolerant rhizobia at low pH are being selected from within our aluminium tolerant breeders lines. The aim is to integrate the two plant traits and combine this with a better strain of *Rhizobium*. Our ability to achieve this outcome may define the success of the program. That is, all three targets; aluminium tolerance, plant nodulation capacity and better rhizobia may all need to be present to deliver success in the field. It is this type of a novel, cohesive and collaborative approach that is most likely to succeed in finding solutions to the complex problem of developing lucerne and

rhizobia with acid soil/aluminium toxicities, which has eluded scientific fraternity for the past 30 years.

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