J.Natn.Sci.Foundation Sri Lanka 2017 45 (4): 361 - 366
DOI: http://dx.doi.org/10.4038/jnsfsr.v45i4.8230

RESEARCH ARTICLE

Rhizobial inoculation of *Trifolium repens* L. in Sri Lanka

S.A. Kulasooriya[1*], E.M.H.G.S. Ekanayake[1], R.K.G.K. Kumara[1] and A.M. Sarath Bandara[2]
[1] *National Institute of Fundamental Studies, Hantana Road, Kandy.*
[2] *Ambewela Livestock Company (Private) Limited, Ambewela Farms, Ambewela.*

Revised: 03 March 2017; Accepted: 03 May 2017

Abstract: The objective of this study was to develop an environmental friendly, low cost technology for cultivation of livestock forage legumes so as to minimise the use of nitrogen fertiliser (N-fertiliser) that pollute the pristine highland watersheds. Inoculants were prepared by screening several rhizobial isolates obtained from farm cultivated clover plants and selecting the most efficient strain. In field tests at three separate sites, the average amount of biomass produced by inoculated *Trifolium* plants was higher compared with the plants fertilised with urea. Root nodulation and other plant parameters were assessed on several randomly harvested plants 60 days after planting. The results indicate that root nodulation and plant growth increased significantly in inoculated plants. Average results obtained from nine crop cuts taken during a period of 15 months showed that regeneration and biomass production after crop cutting were better under inoculation than under N-fertiliser application. It is inferred that rhizobial inoculation has the potential to replace N-fertiliser applications to *Trifolium repens* L. in the Sri Lankan highland farming systems.

Keywords: Green technology, highland farming, nodulation, pasture legume, white clover.

INTRODUCTION

Application of rhizobial inoculants to forage legumes is an age old practice to improve nitrogen fixation in pastures (Norris, 1967). It has been successfully practiced mostly in temperate and sub-tropical countries, which maintain large extents of pasture lands to support their dairy industries (Ledgard & Steele, 1992; Herridge *et al*., 2002; Chen *et al*., 2004; Deaker *et al*., 2004). The adoption of this technology in Sub-Saharan Africa has been reviewed recently (Khonje, 2014).

The value of nitrogen fixed annually by inoculated legumes in Australia has been reported to be equivalent to $3 billion worth of N-fertiliser (Howieson & Herridge, 2005).

There are also substantial environmental benefits of substituting symbiotically fixed N for fertiliser N, due to the decrease in contamination of groundwater from leached N and the reduced production of greenhouse gases (Robertson & Vitousek, 2009).

Trifolium repens L. (white clover) is one of the most nutritious and widely grown forage legumes in the world. In infertile and undeveloped hill pastures, the level of nitrogen fixation in white clover has been reported as 17 kgN/ha/year (Grant & Lambert, 1979), whereas in intensively managed pastures the annual nitrogen fixation was reported to be as high as 380 kgN/ha/year (Rumball, 1979).

The Ambewela Farm where the field trials were conducted has an area of 290 ha and is located at 6^{o} 53' 28.907" N and 80^{o} 48' 07.970" E in the Central Hills of Sri Lanka at an elevation of 1847 m above mean sea level. This farm was initially established by the National Livestock Development Board as a government farm in 1942 and there are some records, which show that white and red clovers were test grown in small plots in 1969.

* Corresponding author (ananda@kulasooriya.com; https://orcid.org/0000-0002-3309-1311)

It is most likely that rhizobial inoculants have been used during this introduction although there are no records to confirm it. Once the farm was privatised, both white and red clovers have been re-introduced in the year 2000 and grown on a large scale as forage legumes. No rhizobial inoculation has been done on them and urea is regularly applied to these lands.

The natural soil in this area is of the red-yellow podzolic type with a pH of around 4.5 and an overlying top layer of organic matter. This top layer has been completely eroded due to farming activities since 1942, and dolomite is periodically applied to maintain the soil pH around 5.5 and to make it more suitable for pasture and vegetable cultivations. The average available nitrogen, phosphorus and potassium in this soil have been recorded as 20, 25 and 70 ppm, respectively.

The mean annual rainfall in this area has been recorded as 179.4 mm ranging from a minimum of 20.8 mm (February) to a maximum of 515.6 mm (December). The daytime ambient temperature in this area has been recorded as a mean of 20.5 ^{o}C ranging between 16 – 25 ^{o}C.

This farm is located in close proximity to the Horton Plains National Park with its highland grasslands (wet patanas) and cloud forests adjacent to the Hakgala, Kirigalpoththa and Kikiliyamana mountains. These ecosystems possess valuable natural vegetations with a high endemicity of both flora and fauna. Regular application of mineral nitrogen fertilisers to these pasture lands, which are the water catchment areas to three major rivers of Sri Lanka, results in pollution that is posing a threat to the vegetations.

The present study commenced with two principal objectives: (a) to examine whether rhizobial inoculation can be adopted to improve biomass production in white clover and (b) to reduce the application of urea and minimise environmental pollution caused by the regular addition of such fertilisers to these pristine highlands.

METHODOLOGY

Isolation of rhizobia from white clover nodules

Rhizobial strains were isolated from nodules of white clover plants growing at the Ambewela Farm. Isolation and purification of rhizobia was done initially on congo red yeast mannitol agar (CRYMA) followed by purification and maintenance on yeast mannitol agar (YMA) (Somesegaran & Hoben, 1994). Nodules were surface sterilised by immersing in 70 % (v/v) ethanol for 1 – 2 min followed by about 3 min in a 4 % (v/v) solution of sodium hypochlorite. They were rinsed six times in fresh changes of sterilised water. Each nodule was aseptically crushed in a drop of sterile water in a sterilised Petri dish using a sterilised wooden applicator stick. A loopful of the nodule crush was streaked on CRYMA. Pure rhizobial colonies were obtained by successive streaking on CRYMA, and the purified isolates were finally cultured on plain YMA and stored in Eppendorf tubes under glycerol in a refrigerator.

Preparation of inoculants

Dilution streak plates were prepared on separate CRYMA plates from the glycerol stock cultures of the isolates and incubated at room temperature (30 ^{o}C daytime and 23 ^{o}C night time) for 5 ds under observation checking for the purity of the cultures. One loopful from a pure colony was transferred to 20 mL of yeast mannitol broth and incubated at room temperature for 5 ds under continuous shaking on an orbital shaker. These cultures were used to inoculate pre-germinated seeds planted in pots.

Screening and selection of rhizobia under greenhouse conditions

Isolates were subsequently screened for effectiveness of nodulation on clover plants in greenhouse pot experiments. These investigations were done in a naturally lit greenhouse at the National Institute of Fundamental Studies in which the light intensity varied within a range of 182 to 1902 lux and the ambient temperatures varied within a range of 23 ^{o}C (night time) to 32 ^{o}C (daytime).

Preparation of pots and potting medium

The screening of the isolates was done in river sand culture in pots (11.5 cm radius holding 1.5 kg of sand). The pots were sterilised by soaking overnight in 4 % hypochlorite solution and thoroughly rinsing with boiled water. River sand was washed and sterilised by autoclaving. The bases of the pots were layered with autoclaved paper towels to close the bottom holes and were then filled with sand. A sterilised polyvinyl chloride watering tube was inserted into the centre of each pot.

Surface sterilisation of seeds

White clover seeds were rinsed in 95 % ethanol for 10 s and immersed in a hydrogen peroxide solution for 5 min with gentle swirling. Then the seeds were rinsed with six changes of sterilised water and imbibed overnight.

Planting of seeds and seedling inoculation

Six pre-germinated seeds were planted in each pot under aseptic conditions and each seed was inoculated using 1 mL of broth inoculants, which had an approximate density of 10^8 cells/mL. The pot was then covered with a clear plastic wrap. When the seedlings emerged the wrap was removed and the surface of the pots was covered with sterilised alkathene beads. The mouth of the watering tube was covered with a piece of aluminium foil. Water and nutrients as required were provided through the watering tube.

Harvesting of plants

Plant growth was assessed by visual rating and they were harvested at flower initiation (35 – 40 days after seeding). The number of nodules and nodule dry weight per plant, and shoot and root dry weights of the uprooted plants were recorded. The rhizobium strain that performed best during the greenhouse screening experiment was labelled as *SRL Clov 1* and used for the field trials at Ambewela Farm.

Preparation of coir dust based solid inoculants for field experiments

Aged, raw coir dust was collected from coir fibre factories. Coir fibres were first removed and the dust was cleaned, powdered and sieved through a fine sieve of 0.25 mm mesh and the pH of the powder was adjusted to 6.0 (Seneviratne *et al*., 1999). The rhizobium broth having a cell density of 10^8 cells/mL was diluted 10 times with sterile distilled water and injected into the seasoned coir dust powder sealed in polypropylene packets and sterilised by autoclaving for 15 min at 120 ^oC under 1 kg/cm^2 pressure. These packets were incubated to mature at ambient room temperature (23 to 30 ^oC) for 1 wk prior to field application.

Field experiments

Field testing was done at the Ambewela Farm by applying rhizobial inoculants. The soils of this farm fall into the category of red-yellow podzolic type and the average soil pH is maintained around 5.5 by periodic addition of dolomite. Treatments were given in 2 m × 3 m field plots in a randomised complete block design. Three such blocks were set up in different locations taking the topography of the land into consideration.

The following treatments were given to *Trifolium repens* (white clover):

T1: Basal dressing of urea fertiliser at the rate of 40 kg/ha followed by top dresses at the rate of 25 kg/ha of urea applied 5 ds after each crop cut.
T2: Basal dressing of urea fertiliser followed by spraying of liquid rhizobial inoculants 5 ds after each crop cut.
T3: Basal dressing of urea fertiliser and no top dressings.
T4: Basal dressing of coir based (solid) rhizobial inoculants, followed by spraying of liquid rhizobial inoculants 5 ds after each crop cut.
T5: Basal dressing of coir based solid inoculants and no top dressings.
T6: Basal dressing of liquid inoculants, followed by spraying of liquid inoculants 5 ds after each crop cut.
T7: Basal dressing of liquid inoculants and no top dressings.
T8: No basal dressing but top dress of liquid inoculants sprayed 5 ds after each crop cut.
T9: Control, without the application of urea or rhizobial inoculants.

Nodulation and dry matter production under the different basal treatments

The customary practice at this farm is to start harvesting the aboveground biomass of clover for feed preparations 75 ds after seeding. In this experiment, recording of experimental readings commenced after an initial 60-day period of growth when the plants appeared to be fully grown. Three randomly selected plants were carefully uprooted with soil from plots that received the basal treatments of urea, solid inoculants, liquid inoculants and control without urea application or inoculation. Nine plants per treatment from the 3 blocks were removed for analyses of nodulation.

Regeneration of clover after crop cutting

Regeneration of the aboveground biomass was assessed by taking crop cuts after periods of re-growth ranging from 30 to 48 ds based upon visual observation of plant growth. Triplicate crop cuts per plot were taken using a 0.5 m^2 wooden frame. Accordingly 9 crop cuts were taken per treatment from each block working out to a total of 27 crop cuts per treatment from the 3 blocks. After taking the crop cuts the above ground biomass of the entire plot was harvested and removed. N-fertiliser additions and spraying of liquid rhizobial inoculants to the harvested plots were done 5 ds after harvesting. The crop cut samples were air dried and then oven dried at 65 ^oC to a constant weight.

RESULTS AND DISCUSSION

Isolation of rhizobia

A total of 12 rhizobial strains obtained during the initial isolations from field collected nodules on CRYMA were purified and grown on YMA. Five strains selected on the basis of colony morphology on YMA were screened in the greenhouse for nodulation and plant growth. The strain that produced the best growth and nodulation was labelled as *SRL Clov 1* and used for field testing at the Ambewela Farm. This strain was characterised as a Gram negative, non-sporing, motile bacillus and was tentatively identified as *Rhizobium leguminosarum* bv. *trifolii* but no molecular characterisations were performed.

Field experiments

The mean values on nodulation obtained from the 27 plants per treatment harvested after the initial growth of 60 days are presented as nodule number and nodule dry weight (Figure 1), and shoot and root dry weight (Figure 2). Inoculation has enhanced root nodulation and there was very little nodulation in plants uprooted from the uninoculated plots (Figure 3). Overall growth and nodulation in the experimental block in the lowest location among the three blocks was very poor at this stage. Accordingly data from this block were not included in the statistical analyses.

The results on initial root nodulation and growth of the plants show that inoculation has increased nodulation (Figure 1), and such increases have produced better plant growth (Figure 2). Similar results have been reported by several researchers overseas (Norris, 1967; Ledgard & Steele, 1992; Herridge *et al*., 2002; Chen *et al*., 2004; Deaker *et al*., 2004), and rhizobial inoculation of forage legumes is a routine practice in all large scale dairy producing countries. Among the four treatments examined, best nodulation and plant growth have been recorded under basal seed inoculation with coir dust based solid inoculants. The application of coir dust based rhizobial inoculants has been successful in increased nodulation and enhanced crop growth and yield of a number of food legumes in Sri Lanka (Kulasooriya *et al*., 2014).

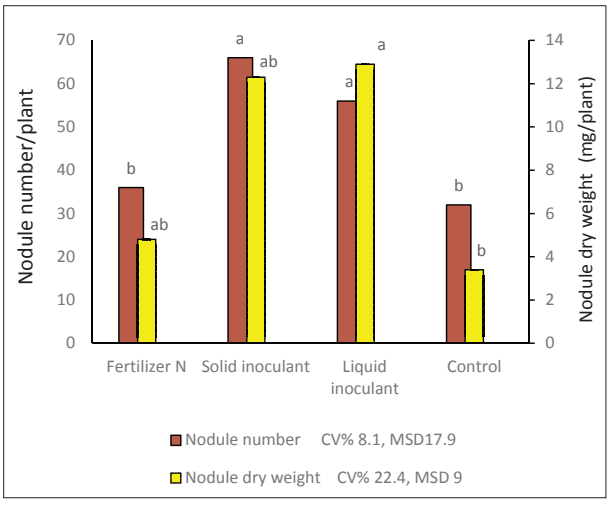

Figure 1: Nodulation in 60 day old white clover plants

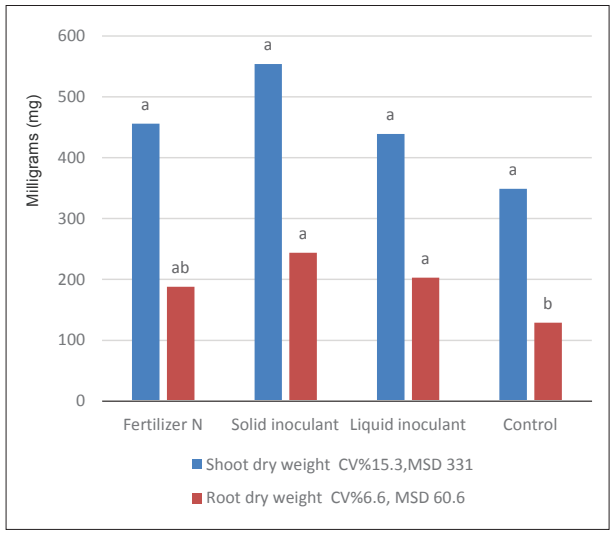

Figure 2: Average shoot and root dry weights (mg/plant) in 60 day old white clover plants

Figure 3: Root nodulation in uninoculated (left) and inoculated (right) white clover plants

In this farm once a clover cultivation is harvested, it is allowed to regenerate for periods ranging from 30 to 48 days (depending upon the weather and crop re-growth) before the next harvest is obtained. A similar practice was adopted in the case of the experimental plots in obtaining crop cuts to evaluate regeneration of clover under the nine treatments given.

The results of clover biomass regeneration under the different treatments are presented as rates of regeneration ($g/m^2/day$) in order to make comparisons among the crop cuts obtained after different time periods (Table 1). These results show that seed inoculation with coir based solid inoculants have given better rates of regeneration than those of N-fertiliser applications.

Table 1: Regeneration of white clover plants after crop harvest

Treatment	Rate of regeneration ($g/m^2/day$)
T1: N-fertiliser (basal + t.d.*)	1.46 ^{abcd}
T2: N-fertiliser (basal + t.d.* liquid inoculation)	1.51 ^{abc}
T3: N-fertiliser (basal + no t.d.*)	1.57 ^{ab}
T4: Seed inoculation (basal coir based + t.d.* liquid inoculation)	1.63 ^{a}
T5: Seed inoculation (basal coir based + no t.d.*)	1.60 ^{a}
T6: Seed inoculation (basal liquid + t.d.* liquid inoculation)	1.56 ^{ab}
T7: Seed inoculation (basal liquid + no t.d.*)	1.42 ^{bcd}
T8: No seed inoculation (t.d.* liquid inoculation)	1.36 ^{cd}
T9: Control (no N-fertiliser + no inoculation)	1.33 ^{d}

t.d.*: top dress with urea or liquid inoculation

Mean values superscripted by the same letters are not significantly different at 5 % probability

Note: The entire plot was harvested after crop cuts were taken and allowed to regenerate.

Either urea fertiliser or rhizobial inoculants were applied 5 days after harvesting.

Increased rates of regeneration after crop cutting were recorded under rhizobial inoculation than with N-fertiliser applications (Table 1). This shows that nitrogen supplied directly to the host plants through fixation is better utilised than that provided by chemical fertiliser applications, part of which could be lost and also be available for associated weeds. This was also evident by the visual observation of higher weed growth in the N-fertilised experimental plots.

The overall biomass production (total of all the crop cuts taken) under the 9 different treatments are shown in Table 2. Expressed as percentage increases over the control treatment, the highest increase of 21 % has been recorded for coir dust based solid inoculation followed by spraying of liquid inoculants after each crop cut. The next highest of 19 % was recorded for coir dust based basal treatment without any top dressing. Basal liquid inoculants followed by spraying of liquid inoculants after each crop cut have given a 16 % increase, which is the same as that obtained with basal N-fertiliser additions. All these treatments have recorded significant biomass increases above that of the unfertilised, uninoculated control treatment.

According to the results presented in Table 2 all the treatments including N-fertiliser applications have given significantly higher biomass increases over the control treatment, which did not receive either chemical N-fertiliser or rhizobial inoculation. This showed that clover plants have responded positively to N nutrient supply. The highest average biomass harvested during the 12 month period was obtained in the treatment with coir based inoculation as a basal dressing followed by top dressings with liquid inoculation after each crop cut, and this was statistically significant.

Higher nodulation, better growth (biomass production) and regeneration of white clover were obtained with rhizobial inoculation and these were either above or equal to those obtained with urea fertiliser application.

CONCLUSION

It is concluded that urea application to the pastures of white clover at the Ambewela Farm can be replaced by rhizobial inoculation without any reduction in biomass production and this should reduce the cost of clover cultivation and minimise environmental pollution.

Table 2: Total dry matter production (total of all the crop cuts)

Treatment	Mean dry weight (g/m^2)	% increase over control
T1: N-fertiliser (basal + t.d.*)	502 [abc]	9
T2: N-fertiliser (basal + t.d.* liquid inoculation)	520 [abc]	13
T3: N-fertiliser (basal + no t.d.*)	534 [abc]	16
T4: Seed inoculation (basal coir based + t.d.* liquid inoculation)	556 [a]	21
T5: Seed inoculation (basal coir based + no t.d.*)	544 [ab]	19
T6: Seed inoculation (basal liquid + t.d.* liquid inoculation)	534 [abc]	16
T7: Seed inoculation (basal liquid + no t.d.*)	484 [abc]	6
T8: No seed inoculation (t.d.* liquid inoculation)	464 [bc]	1
T9: Control (no N-fertiliser + no inoculation)	459 [c]	–

t.d.*: top dress with urea or liquid inoculation

Mean values superscripted by the same letters are not significantly different at 5 % probability

Note: Three random crop cuts per plot were taken using a ½ m^2 wooden frame.

Nine such crop cuts were obtained during a period of 12 months. CV: 5.3 % MSD: 79.1

Acknowledgement

Technical assistance provided by Ms. A.H.M.C.D. Abeyrathne and field assistance provided by Mr. A.H.M.A.K. Tennekoon of the National Institute of Fundamental Studies and Mr. S.S. Bandara and Mr. Weerakone Bandara of Ambewela Farms are gratefully acknowledged.

REFERENCES

1. Chen W., McCaughey W.P. & Grant C.A. (2004). Pasture type and fertilization effects on N_2 fixation, N budgets and external energy inputs in Western Canada. *Soil Biology and Biochemistry* **36**(8): 1205 – 1212. DOI: https://doi.org/10.1016/j.soilbio.2004.04.003
2. Deaker R., Roughley R.J. & Kennedy I.R. (2004). Legume seed inoculation technology - a review. *Soil Biology and Biochemistry* **36**(8): 1275 – 1288. DOI: https://doi.org/10.1016/j.soilbio.2004.04.009
3. Grant D.A. & Lambert M.G. (1979). Nitrogen fixation in pasture V. Unimproved North Island hill country, 'Ballantrae'. *New Zealand Journal of Experimental Agriculture* **7**: 19 – 22. DOI: https://doi.org/10.1080/03015521.1979.10426155
4. Herridge D., Gemell G.H.E. & Hartley E. (2002). Legume inoculants and quality control. In: Inoculants and nitrogen fixation of legumes in Vietnam. *ACIAR Proceedings* (ed. D. Herridge), 17 – 18 February, Hanoi, Vietnam, pp. 105 – 115.
5. Howeison J. & Herridge D. (2005). 'Foreword' special issue. *Australian Journal of Experimental Agriculture* **56**: 45.
6. Khonje D.J. (2014). Adoption of the rhizobium inoculants technology for pasture improvement in sub-Saharan Africa. *Trypanotolerant Livestock in West and Central Africa, volume 2, Country Studies*. FAO Corporate Document Repository, p. 13.
7. Kulasooriya S.A., Ekanayake E.M.H.G.S., Kosala Kumara R.G. & Gunarathna A.H.M.C. (2014). Use of rhizobial inoculants could minimize environmental health problems in Sri Lanka. *Industrial and Environmental Biotechnology* (eds. K. Pramanik & J.K. Patra), pp. 433 – 442. Studium Press, New Delhi, India.
8. Ledgard S.F. & Steele K.W. (1992). Biological nitrogen fixation in mixed legume/grass pastures. *Plant and Soil* **141**: 137 – 153. DOI: https://doi.org/10.1007/BF00011314
9. Norris D.O. (1967). The intelligent use of inoculants and lime pelleting for tropical legumes. *Tropical Grasslands* **1**(2): 107 – 121.
10. Robertson G.P. & Vitousek P.M. (2009). Nitrogen in agriculture: balancing the cost of an essential resource. *Annual Review of Environmental Resources* **34**: 97 – 125.
11. Rumball P.J. (1979). Nitrogen fixation in pasture II. Northland warm temperate, Kaikohe. *New Zealand Journal of Experimental Agriculture* **7**: 7 – 9. DOI: https://doi.org/10.1080/03015521.1979.10426152
12. Seneviratne G., Van Holm L.H.J. & Ekanayake E.M.H.G.S. (1999). Effect of peat and coir-dust based rhizobial inoculants on the nodulation, plant growth and yield of soybean [*Glycine max* (L) Mettil] Cv PB 1. *Tropical Agricultural Research and Extension* **2**: 136 – 139.
13. Somesegaran P. & Hoben H.J. (1994). *The Handbook for Rhizobia-Methods in Legume-Rhizobium Technology*, pp. 450. Springer-Verlag, New York, USA.