Increasing symbiotic potentials in white clover

J.R. CRUSH[1] and J.R. CARADUS[2]
[1]*AgResearch Ruakura, Private Bag 3123, Hamilton, New Zealand*
[2]*AgResearch Grasslands, Private Bag 11008, Palmerston North, New Zealand*

Abstract

Lines of white clover with higher and lower numbers of nodules were selected from a mutagenised Huia population, and seed was produced from a polycross of both lines. The progeny showed that the lines tended to have either more but smaller nodules, or fewer and larger nodules. In a further experiment, the line with fewer (larger average size) nodules, fixed more nitrogen (N) and grew bigger. Plants with fewer but larger nodules are a good model for efforts to increase the N-fixing capacity of white clover. An empirical screening method was used to obtain 15 genotypes that showed evidence of high mycotrophy. A second experiment confirmed that 5 of these genotypes gave abnormally high responses to mycorrhizal infection. Examination of the roots of these plants showed that the selections did not differ from their parent lines in terms of root length or root hair cylinder diameter. The results tend to confirm published work with spring wheat, which showed that land races and wild types respond more strongly to mycorrhizal infection than high yielding varieties do. Investigation of the mycorrhizal responses of clover ecotypes that are adapted to low phosphorus soils is a priority for future research.

Keywords: mycorrhizas, nitrogen fixation, nodulation, symbioses, white clover

Introduction

There is evidence from growth cabinet and field experiments that the growth potential of some white clover (*Trifolium repens* L.) cultivars exceeds the capacity of the nodules to supply N (Hoglund & Brock 1974; Brock & Hoglund 1974), so that N fertiliser is required to obtain maximum clover growth rates. The volume of nodule tissue in white clover can be increased by selection (Jones & Burrows 1968; Mytton & Jones 1971), largely through increase in nodule numbers, but as plant yield is not markedly improved, nodule efficiency must be reduced. Nutman (1967) concluded that *T. subterraneum* plants with few nodules are more efficient than those with many, because abundantly nodulated plants divert a disproportionate amount of resources into the meristems and cortical regions of their many nodules.

In pasture soils clover roots are normally infected with mycorrhizal fungi that may improve phosphorus (P) acquisition by the plant when P supply is limiting (Crush 1995). Efforts to introduce elite strains of these fungi into pasture soils have failed. The identification of non-mycorrhizal mutants of *Pisum* and *Vicia* (Duc et al. 1989), suggests that genetic control of the mycorrhizal symbiosis is located at least in part in the host plant. This opens the possibility of screening clover for variation in mycotrophy.

This report describes the selection and testing of white clover populations and genotypes that vary in nodulation and mycotrophy.

Methods

Nodulation

Seedlings of Grasslands Huia white clover that had been mutagenised with ethyl methane sulphonate (EMS) were grown in trays of potting mix in a heat controlled glasshouse. All the trays were inoculated with *Rhizobium leguminosarum bv trifolii* strain NZP 560. Nodulation was checked on each of the 20,000 seedlings, and 50 plants were selected for high and low nodule numbers. Seed was produced from a polycross of each line. Progeny were grown in open pots (500 ml) of steam-sterilised sand inoculated with the same *Rhizobium* strain, in a temperature controlled glasshouse. The pots were irrigated with 100 ml of minus-N nutrient solution (Crush 1995) three times a week. After 100 days root and shoot weights and the number of nodules on each plant were determined.

In another experiment, 30 seedlings of each line were grown in sterile, minus-N sand culture (Crush 1995) inoculated with *Rhizobium leguminosarum bv trifolii* strain ICMP 2668. After 65 days in a glasshouse, the plants were washed free of sand and dried at 60°C. Nodules were rubbed off the dried roots and separated manually from root debris. Each nodule sample was sorted under a dissecting microscope to remove sand grains that had adhered to the roots. Shoots, roots and nodules were weighed and the nodules were counted and their individual areas measured using a PC-based image analysis system and Video-Pro® software. Plant total N was measured on all plants.

Mycotrophy

Ten genotypes from each of 60 lines of white clover were grown in a glasshouse in trays of a P-deficient, non-sterile field soil. The soil was chosen because white clover would make little growth in it unless it was mycorrhizal. All the trays were inoculated with *Rhizobium* NZP 560. Fifteen genotypes that made rapid initial growth without symptoms of P deficiency were selected for further testing. Seven of these were from the cultivar Grasslands Prestige and 4 were from an earlier selection for taprootedness.

The mycorrhizal responses of the 15 genotypes were compared with that of their parent lines. Stolon tips from the parent lines and the selections were rooted in sterile sand, before being transplanted into pots of a non-sterile hill soil with an Olsen P content of 9 µg P/g soil. Five replicates were left to be infected naturally with the resident mycorrhizal fungi, and another 5 replicates were maintained as non-mycorrhizal controls by drenching the pots with benomyl (Carey et al. 1992). The pots were kept in randomised, replicated blocks in a temperature controlled glasshouse for 64 days. Shoot and root fresh weights were recorded and the presence or absence of mycorrhizal infections in the roots was confirmed microscopically following Phillips and Hayman (1970). Stolon tips from five genotypes that showed exceptional response to mycorrhizal infection, compared with the response of their parent lines, were grown on in low ionic strength solution culture (Blamey et al. 1991) together with stolon tips from each parent line. Root lengths were measured on the preserved roots (formalin, acetic acid, alcohol) of these plants, using a Mark 2, Delta-T root area meter with Root, version 3 software. Diameters of the 'root plus root hair' cylinders were measured on 10 lateral root segments of each plant, using a dissecting microscope fitted with an eyepiece micrometer.

Results

Nodulation

In the first evaluation the low nodule number selection had significantly fewer nodules than the high nodule number line (Table 1) but plant size was unchanged. Nodule numbers ranged from 4 to 105 nodules per plant in the low nodule number selection, and from 4 to 290 in the high nodule number selection. When the plants were grown in bacteriologically controlled minus-N sand culture, the low nodule number plants had heavier shoots, and larger nodules, than the high nodule number selection (Table 2). The average number of nodules per plant did not vary significantly between the selections but results from the image analysis showed that the high nodule number selection had more small nodules, while the low nodule number selection had more large nodules (Figure 1). Plant N concentration did not vary between the selections, but the low nodule number plants contained significantly more N, and fixed more N per nodule (Table 2).

Table 1: Initial comparison of lines derived from white clover genotypes selected for low and high nodule number. n = number of plants evaluated.

Line	n	Shoot DM (g)	Root DM (g)	Nodule no.	Nodules per g root DM
Low nodule seln.	200	0.62	0.18	43	319
High nodule seln.	199	0.67	0.19	71	465
P		ns	ns	***	***

Table 2: Growth and nitrogen fixation in the high- and low-nodule number selections of white clover grown in minus-nitrogen sand culture.

	High nodule seln.	Low nodule seln.	*P*
mg shoot DM/plant	178	251	0.01
mg root DM/plant	107	121	ns
mg nodules/plant	10.7	14.2	0.01
nodules/plant	37.6	32.8	ns
plant % N	3.26	3.28	ns
mg N/plant	9.3	12.1	0.05
mg N/mg nodule DM	0.84	0.85	ns
mg N/ nodule	0.27	0.39	0.01

Mycotrophy

Mycorrhizal responses [{(DM mycorrhizal plant minus DM non-mycorrhizal plant)/DM non-mycorrhizal plant} × 100] for the 15 clover genotypes selected in the stage 1 screening, are shown in Table 3 (the 5 genotypes selected for further testing are asterisked). Examination of the roots showed that the benomyl treatment was effective in keeping plants non-mycorrhizal and that untreated plants were mycorrhizal. The high P response clover parent line also gave a large response to mycorrhizal infection, although this was exceeded by one of the genotypes selected from this material.

Root length per g shoot DM did not vary between parent lines and the selections from each parent. There were no significant differences in mean root length between any of the clovers with an overall mean value of 1.46 m per g shoot DM. Average root hair cylinder diameters were the same in parents and the selections.

Discussion

The results for the high and low nodule number lines confirm earlier work (Jones & Burrows 1968; Mytton & Jones 1971) that showed it was possible in white clover to select for variation in nodulation. Dried nodules were measured because it is easier to separate dried nodules from the roots than picking off live nodules. We

Figure 1: Frequency distribution (%) of nodule size classes for the high nodule number selection (open bars) and the low nodule number selection (solid bars).

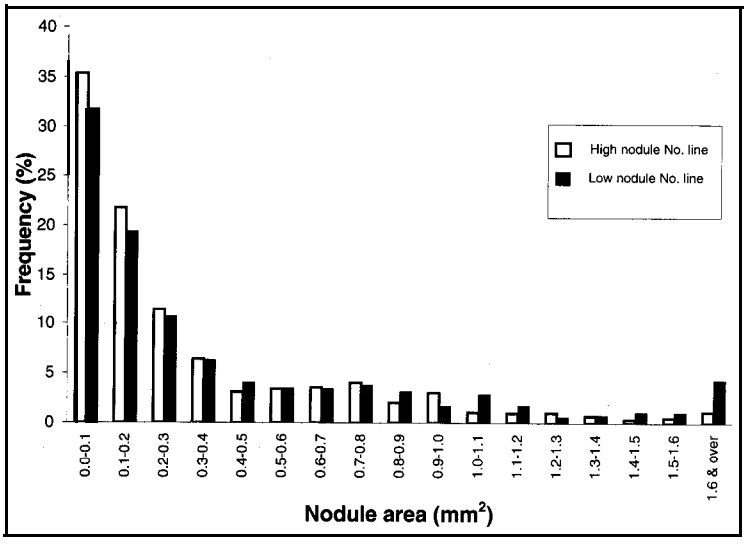

Table 3: Mycorrhizal response [{ (DM mycorrhizal plants minus DM non-mycorrhizal plants)/DM non-mycorrhizal plants} x 100] of parent clover lines and putative high mycotrophy selections.

Clover lines	Mycorrhizal response (%)
Prestige	0
Selections ex Prestige	4, 33, 33, 20, 6, 6, 57*
High P response	109
Selections ex High P response	30,291'
Low P response	28
Selection ex Low P response	199'
Al-susceptible	56
Selection ex Al-susceptible	41
Tap-rooted	38
Selections ex Tao-rooted	0, 24, 70*, 174'

* Plants selected for further testing

have assumed that the extent of nodule shrinkage during drying is similar for both populations. In many legumes there is an inverse relationship between nodule number and nodule size and Nutman (1967) concluded that fewer (i.e. larger) nodules should be more efficient because large nodules have a greater ratio of bacteroid to non N-fixing tissue.The increases in total N fixed, N fixed per nodule, and shoot DM in the low nodule number line, confirm Nutman's prediction. Plants with fewer, but larger nodules are good models for breeding programmes to improve the N-fixation capacity of white clover.

Mytton & Jones (1971) found both average nodule size and plant size declined between the first and third cycle of selection for nodule mass. The decrease in nodule size probably explains the reduction in plant growth, but these experiments do not distinguish between the reciprocal influences of inherent plant vigour, and nodule growth and function. In alfalfa and crimson clover, selection for nodulation and/or acetylene reduction resulted in lines with heritable differences in nitrogenase activity and plant growth (Seetin & Barnes 1977; Smith et al. 1982). However, none of this work distinguished between the effects of inherent plant vigour, and nodule size/nodule number relationships. Small differences in seedling vigour, and hence nodulation, may have influenced our initial selection process, and been carried over into the subsequent experiments. This issue is currently under investigation.

Nodules are only a small part of the total plant weight (3.8% in the current experiments), so differences in carbon partitioning between bacteroid and non-fixing tissue in selections varying in average nodule size, seem unlikely to have much impact on plant growth. However, the cumulative effect in our second experiment was a 30% increase in plant growth and N fixed. Since white clover nodules have an apical meristem and are capable of considerable growth and longevity, there may be substantial scope for conserving plant resources by having fewer, but larger nodules. There was substantial overlap in the frequency distribution of nodule size classes for the two experimental populations. Current research is attempting to obtain more discrete poulation types for nodule size and number.

Empirical screening for mycotrophy, which was based on an assumed relationship between mycotrophy and seedling growth rate, provided plants that gave a variable response to mycorrhizal infection in the controlled experiment. It is not known what plant factors determined this variation in mycorrhizal response but it was extreme in Prestige, resulting on average in no apparent benefit from mycorrhizal infection in the parent genotypes. However the early stages of these empirical screening techniques always include anomalous results. The degrees of root subdivision and root hair development are key determinants of mycorrhizal dependence (Baylis 1970). Root length and root hair data showed that the genotypes with the greatest mycorrhizal response had not been accidently selected for coarse roots.

Cultivar x mycorrhizal response interactions are rare in white clover. When they have occurred, the interaction seems to have been generated by the presence of an unimproved clover ecotype in the experiment (Crush 1978; Crush & Caradus 1980). Similar results

have been reported in spring wheat (Manske 1990) where mycorrhizas stimulated P uptake and growth of land races and wild forms, much more than in high yielding varieties. In our initial screening, 7 of the 15 clover genotypes selected for further testing came from Prestige. This cultivar was bred from ecotypes persisting in old Northland pastures (Cooper & Chapman 1993), and may retain more adaptation to low fertility soils than is found in high yielding cultivars.

In the long term, sustainable agriculture will require white clovers with maximum symbiotic capacities. An immediate task is to investigate mycorrhizal responses in ecotypes adapted to low P soils, and high yielding cultivars across a range of soil P levels. This knowledge and information about the heritability of mycotrophy, would allow development of stategies for clover breeding for different levels of per hectare production. There are no international precedents for this type of work, but Graham and Eissenstat (1994) suggest the potential value of breeding plants for greater susceptibility to mycorrhizal colonisation will depend on the carbon cost/P benefit of mycorrhiza for the specific crop, soil and environmental conditions. The responsiveness of clovers in New Zealand pastures to both N and P fertilisers, suggests that energy is unlikely to limit increased symbiotic activity. There are enough reports on variation in nodulation in forage legumes to suggest a breeding programme would be successful. Sand culture from which roots can be readily extracted, and image analysis for rapid counting and measuring of nodules may be useful techniques in searching for optimum nodulation types.

Acknowledgements

J.P.M. Evans, T. Dunn and M.J. Mills for technical assistance; financial support from the Foundation of Research, Science and Technology.

References

Baylis, G.T.S. 1970. Root hairs and phycomycetous mycorrhizas in phosphorus deficient soil. *Plant and Soil 33*: 713–716.

Blamey, F.P.C.; Edmeades, D.C.; Asher, C.J.; Edwards, D.G.; Wheeler, D.M. 1991. Evaluation of solution culture techniques for studying aluminium toxicity in plants. pp 905–912. *In:* Wright, R.J. et al. (eds). *Plant-soil interactions at low pH*. Kluwer Academic Publishers, Dordrecht.

Brock, J.L.; Hoglund, J.H. 1974. Growth of 'Grasslands Huia' and 'Grasslands 4700' white clovers II. Effects of nitrogen and phosphorus. *New Zealand Journal of Agricultural Research 17*: 47–53.

Carey, P.D.; Fitter, A.H.; Watkinson, A.R. 1992. A field study using the fungicide benomyl to investigate the effect of mycorrhizal fungi on plant fitness. *Oecologia 90*: 550–555.

Cooper, B.M.; Chapman, D.F. 1993. Grasslands Prestige (G39), a white clover cultivar originating from northern New Zealand. *Proceedings International Grassland Congress 17*: 458–459.

Crush, J.R. 1978. Changes in effectiveness of soil endomycorrhizal fungal populations during pasture development. *New Zealand Journal of Agricultural Research 21*: 683–685.

Crush, J.R.; Caradus, J.R. 1980. Effect of mycorrhizas on growth of some white clovers. *New Zealand Journal of Agricultural Research 23*: 233–237.

Crush, J.R. 1995. Effect of VA mycorrhizas on phosphorus uptake and growth of white clover (*Trifolium repens* L.) growing in association with ryegrass (*Lolium perenne* L.). *New Zealand Journal of Agricultural Research 38*: 303–306.

Duc, G.; Trouvelot, A.; Gianinazzi-Pearson, V.; Gianinazzi, S. 1989. First report of non-mycorrhizal plant mutants (myc-) obtained in pea (*Pisum sativum* L.) and faba bean (*Vicia faba* L.). *Plant Science 60*: 215–222.

Graham, J.H.; Eissenstat, D.M. 1994. Host genotype and the formation and function of VA mycorrhizae. *Plant and Soil 159*: 179–185.

Hoglund, J.H.; Brock, J.L. 1974. Growth of 'Grasslands Huia' and 'Grasslands 4700' white clovers I. Effects of temperature and nitrogen. *New Zealand Journal of Agricultural Research 17*: 41–45.

Jones, D.G.; Burrows, A.C. 1968. Breeding for increased nodule tissue in white clover (*Trifolium repens* L.). *Journal of Agricultural Science, Cambridge, 71*: 73–79.

Manske, G.G.B. 1990. Genetical analysis of the efficiency of VA mycorrhiza with spring wheat I. Genotypical differences and a reciprocal cross between an efficient and non-efficient variety. pp. 397–405. *In:* El Bassam, N. et al. (eds). *Genetic aspects of plant mineral nutrition*. Kluwer Academic Publishers, Dordrecht.

Mytton, L.R.; Jones, D.G. 1971. The response to selection for increased nodule tissue in white clover (*Trifolium repens* L.). *Plant and Soil, special volume*: 17–25.

Nutman, P.S. 1967. Varietal differences in the nodulation of subterranean clover. *Australian Journal of Agricultural Research 18*: 381–425.

Phillips, J.M.; Hayman, D.S. 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society 55*: 158–161.

Seetin, M.W.; Barnes, D.K. 1977. Variation among alfalfa genotypes for rate of acetylene reduction. *Crop Science 17*: 783–787.

Smith, G.R.; Knight, W.E.; Peterson, H.H. 1982. Variation among inbred lines of crimson clover for N_2 fixation (C_2H_2) efficiency. *Crop Science 22*: 716–719.

■