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Title: Symbiotic Characteristics of Rhizobium leguminosarum bv. trifolii Recovered from Nodules of Field-grown Trifolium subterraneum L.

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Abstract Approved:

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In a series of greenhouse experiments, isolates representing serotypically distinct subpopulations of Rhizobium leguminosarum bv. trifolii recovered from root nodules of field-grown subclover (Trifolium subterraneum cv. "Nangeela"), were evaluated for their symbiotic effectiveness potential, their effect on regrowth potential of subclover, and for their competitive abilities to form nodules. Isolates from the nodule dominant serotypes AS6, AS6-21, and AS21 were shown to be suboptimally effective at fixing nitrogen gas (N_2) relative to RT162X95, a highly effective commercial inoculant strain. Although the majority of isolates representing the minor nodule occupying serotypes AS27, AG4, and AP17 were also shown to be of moderate effectiveness, isolates ADS14 and RW11 of serotype AS27 were equally as effective as RT162X95. Subclover regrowth was influenced by the severity of the first harvest. Plant-isolate combinations

possessing superior N_2-fixing capabilities were more adversely affected by severe clipping than were the less effective combinations. Isolate 88FL3,5, a mediocre N_2-fixing member of the nodule dominant serotype AS6, was not universally more competitive at forming nodules than other isolates. Although 88FL3,5 formed more nodules than many of the other isolates when mixed in equal numbers with each of them separately, isolates ADS14 and ADS16 significantly outcompeted 88FL3,5 even though they represented a minor nodule occupying serotype, AS27. The nodule occupying successes of 88FL3,5 or ADS16 could be overcome if their respective competitor had a numerical advantage ranging between 10:1 and 25:1. Since the soil-borne population of 88FL3,5 has been shown to outnumber other subpopulations of rhizobia in the same soil by at least 10:1, it is not surprising that it can occupy the majority of nodules on field-grown plants even in the presence of more competitive types. Why the highly effective and highly competitive members of serotype AS27 are rarely recovered from nodules of field-grown Trifolium subterraneum L. is a question that merits further study.

Symbiotic Characteristics of *Rhizobium leguminosarum* bv. *trifolii* Recovered from Nodules of Field-grown *Trifolium subterraneum* L.

by

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LITERATURE REVIEW

Introduction

Nitrogen is one of the key elements in plant growth. It is a major constituent of plant protein, chlorophyll, nucleic acids, and other substances. Yet of all the elements needed for plant growth, nitrogen is most often in short supply. This arises from the fact that nitrogen is not a component of the parent material that gives rise to the soil in which the plants grow. Rather, every molecule of nitrogen that comes into the biological realm must be fixed either biologically or by chemical methods.

While the chemical reduction of dinitrogen gas (N_2) to ammonia (NH_3) is not an especially difficult process for modern technological societies, it is very expensive as it requires large inputs of energy to rupture the triple bond holding the two atoms together in the N_2 molecule. Only after these triple bonds are cleaved can the single atoms combine with hydrogen or oxygen to form the compounds ammonium (NH_4^+) or nitrate (NO_3^-) that plants can use (Havelka et al., 1982).

Biological nitrogen fixation

Biological N_2 fixation is a unique property of only a few genera of prokaryotic organisms that contain the genetic information for synthesizing the enzyme system

referred to as nitrogenase. In contrast to the Haber-Bosch process which requires very high temperatures and pressure, the enzyme nitrogenase catalyzes the conversion of N_2 to NH_3 under mild temperatures and normal atmospheric pressure.

Gram-negative soil bacteria belonging to three taxonomically distinct genera, Rhizobium, Bradyrhizobium, and Azorhizobium are capable of forming nitrogen-fixing symbiotic relationships with leguminous plants (Kondorosi, 1989). As a result, cultivated grain and forage legumes can be independent of nitrate or ammonium salts for their N requirements.

Subclover - Short history and importance in Oregon

One agriculturally important legume/Rhizobium symbiotic association is that formed between clovers (Trifolium spp.) and the bacterial species R. leguminosarum bv. trifolii. The importance of forages (including clovers) to agriculture was summarized by Hanson (1974) and includes: improving soil fertility and structure, protecting soil from destructive effects of rainfall, preventing water runoff and soil erosion, reducing pollution in streams and rivers, and increasing animal productivity. Clovers contain from 60 to 80% (w/w) digestible dry matter, and they contribute to improving the nutritional quality of pasture, silage and hay.

Of all annual clovers, subclover (Trifolium subterraneum L.), makes the greatest contribution to livestock feed production and soil improvement world wide (McGuire, 1985). This annual winter forage legume originated from regions surrounding the Mediterranean Sea. It was introduced into the United States from

Australia in 1921 (Leidigh, 1925), and was planted for the first time in Oregon in 1922 (Rampton, 1952). McGuire (1985), estimates there are some 200,000 ha of relatively steep, non-tillable, and shallow soils planted to subclover in Oregon. It has further been estimated that some 800,000 ha of land in western Oregon could be improved for sheep and cattle production with the use of subclover as a forage crop (Knight et al., 1982). Phillips and Bennet (1978) estimated that subclover derives as much as 88% of its N requirement from N_2 gas. Estimates of the total amount of N fixed by subclover range from 58 to 207 kg N ha^{-1} (Phillips and Bennett, 1978; LaRue and Patterson 1981; Brink, 1990). Depending on the management system utilized, some of this N will be returned to the soil as either livestock fecal matter or by death and decomposition of roots and nodules. After mineralization much of this N becomes available to a succeeding crop, or a companion grass.

Rhizobia as a limiting factor in legume production

Legumes may fail to produce profitable yields for a variety of reasons. Among them are factors which specifically limit the quantities of N_2 fixed (Robson and Bottomley, 1990). The absence of symbiotically effective rhizobia can limit the development of legumes in soils with no prior history of the crop and where rhizobial requirements are highly specific (Brockwell et al., 1987; Somasegaran et al., 1988; Sanginga et al., 1989). In addition, introduced rhizobia may fail to persist in the soil leading to poor re-establishment of annual pasture legumes (Chatel et al., 1968; Howieson and Ewing, 1986). Legumes may also fail to

achieve their production potential because of the presence of indigenous populations of rhizobia which are symbiotically ineffective, but are sufficiently competitive to prevent nodulation by introduced inoculant strains (Ireland and Vincent, 1968; Holland, 1970; Jones et al., 1978).

Surveys carried out throughout the world have shown that members of indigenous soil rhizobial populations are not usually ineffective per se but are less effective than inoculant strains that are commercially available (Holding and King, 1963; Holland, 1970; Gibson et al., 1975; Hagedorn, 1978; Rys and Bonish, 1981 Richardson and Simpson, 1989). In the majority of these surveys emphasis was placed on sampling multiple sites. Only a few isolates were screened from each of several sites and attempts were not made to determine the relative abundance of the different effectiveness types in the nodules. Studies from our laboratory have identified the dominant and non-dominant nodule occupants of subclover growing at a field site in western Oregon (Bottomley et al., 1990). However, a comprehensive characterization of their symbiotic effectiveness potential has not been undertaken.

Influence of nodule occupants upon regrowth potential

Forage legumes must undergo periodic defoliation during their life cycle. Rate of growth recovery from defoliation might be influenced by the effectiveness potential of the nodule occupants. Nitrogen fixation is a very energy intensive process, and can utilize as much as 23% of gross photosynthate (Ryle and Powell, 1985a), or 6.5 g C/g N (LaRue and Patterson, 1981). As a consequence, since

defoliation in legumes directly affects the photosynthetic capacity of the legume, its recovery is intimately linked to the process of N_2 fixation. A general relationship between recovery of nitrogen fixation and photosynthetic capacity during regrowth has been demonstrated in alfalfa (Cralle and Heichel, 1981; Fishbeck and Phillips, 1982), white clover (Ryle et al., 1985a) and in subclover (Culvenor et al., 1989). Subclover in particular can be vulnerable to regrowth limitations since it undergoes minimal elongation of branches during vegetative growth, and does not possess the stolons found in perennials such as white clover. Furthermore, subclover does not possess the large reserves of carbohydrates in tap roots that are so important for regrowth in alfalfa (Hodgkinson, 1969; Smith and Silva, 1969). The severity of defoliation in subclover has been demonstrated to be an important factor in the recovery of photosynthetic capacity and nitrogen fixation (Culvenor et al.,1989). In the latter study the plants were inoculated with the highly effective inoculant strain R. leguminosarum bv. trifolii WU95. A question that still remains to be resolved is whether there are differences in the recovery capacity of symbioses driven by isolates of highly effective versus suboptimally effective phenotypes.

Competition for nodulation sites

In most situations rhizobia persist in soil after their introduction. While this should be viewed as one of the success stories of twentieth century agriculture, there is concern that indigenous or naturalized rhizobia will prevent future legume introductions from reaching their N_2-fixing potential. A question that still needs resolution is whether suboptimally effective rhizobia limit biological N_2 fixation and

consequently the yield of current germplasm, and whether or not highly effective and competitive strains can be successfully introduced to correct the situation. On the other hand if they do not limit yield of current germplasm, might they limit that of future improved germplasm? Field studies with soybean [Glycine max (L.), Merrill], show that many strains of Bradyrhizobium japonicum are incapable of supporting maximum yields on improved soybean cultivars (Abel and Erdman, 1964; Caldwell and Vest, 1970). Concern arises from the fact that strains belonging to certain serogroups of B. japonicum have become established as dominant soybean nodule occupants throughout the major production regions of the United States (Damirgi et al., 1967; Ham et al., 1971; Keyser et al., 1984; Weber et al., 1989) and they cannot be displaced with better N_2-fixing strains when current inoculation technologies are practiced (Johnson et al., 1965; Ham et al., 1971; Bohlool and Schmidt, 1973; Weaver and Frederick, 1974).

The term "competition" when applied to Rhizobium spp. usually means competition for nodule formation between different strains from the moment they are together in the same environment until they are inside the nodule (Amarger, 1983). Competition can be regarded as a phenomenon that occurs at several different stages of the free-living and symbiotic states. Strains compete to survive and multiply in the soil prior to seed germination (Chatel and Parker, 1973b; Pinto et al., 1974; Franco and Vincent, 1976). Saprophytic competence is used to refer to this initial level of competition (Chatel et al 1968). The surviving bacteria then compete for available root infection sites. (Nutman, 1962). Since a minority of root

hair infections lead to nodule formation, there might be competitive phenomena occurring during the various stages of nodule development after infection.

Although competitive nodulation has been studied for many years (Dowling and Broughton, 1986; Triplett, 1990; Bottomley, 1992), it has been difficult to gain an understanding of the mechanisms underlying this phenomenon.

Numerous studies have shown that representatives of simple rhizobial mixtures can be distributed in nodules disproportionate to their relative numbers in the inoculant mixture (Bottomley, 1992). In many cases where strain combinations included an effective and an ineffective strain, the former dominated the nodules regardless of relative numbers (Robinson, 1969a; Pinto et. al., 1974; Labandera et. al.,1975;). Other studies, however, showed that effective strains did not always outcompete less effective strains regardless of their population sizes (Amarger, 1981; Demezas and Bottomley, 1986a; de Oliviera and Graham 1990a). In general, when the symbiotic effectiveness potential of the two strains is similar, a positive relationship exists between nodulation success and the relative numbers of the two strains in the inoculant mixture (Franco and Vincent, 1976; Amarger and Lobreau, 1982; Beattie et. al. 1989). In some of these cases, however, poorly competitive strains could only occupy many nodules when they outnumbered the competitor by ratios of 10:1 to >50:1 (Amarger and Lobreau, 1982; Fuhrmann and Wollum, 1989; Mullen and Wollum, 1989). In other cases, occupancy could change significantly by changing strain ratios from 1:1 to 5:1 and even 1:1 to 3:1 (Demezas and Bottomley, 1986). In real soil situations the question to be raised

is whether or not subpopulation densities are significantly different from each other, and are these differences of sufficient magnitude to influence nodulation success or failure. Data collected by Bottomley et. al. (1990) on indigenous subpopulations of R. leguminosarum bv.trifolii from the field indicate that the ratios between five identified subpopulations can range between <2:1 to 10:1 in the nonrhizosphere soil environment, and <2:1 to 32:1 in the clover rhizosphere.

Previous work from this laboratory has shown that serotypes AS6 and AS6-21 are major nodule occupants of field-grown subclover (Leung and Bottomley, unpublished observations). Furthermore their population densities in soil are significantly greater than those of the minor nodule occupants. It was considered important to determine if members of serotype AS6 occupy more nodules because they are inherently more competitive than minor occupants, or whether they rely on their numerical superiority. Prior to this thesis research, competitiveness between members of major and minor nodule occupying serotypes from soil populations had not been evaluated.

OBJECTIVES

The objectives of the experiments described in this thesis were as follows.

1. To evaluate the symbiotic effectiveness potential of isolates of R. leguminosarum bv trifolii representing serotypes that were major and minor occupants of nodules of field-grown subclover Trifolium subterraneum cv. "Nangeela".

2. To determine the regrowth characteristics of subclover when nodulated by isolates of differing symbiotic effectiveness.

3. To determine the outcome of competitiveness between isolates representing the major and minor nodule occupying serotypes.

4. To determine the influence of relative population densities upon the outcome of competition between isolates representing major and minor nodule occupying serotypes.

MATERIALS AND METHODS

Experiment 1. Evaluation of Symbiotic effectiveness of dominant serotypes

(i) Seed source. Seeds of the annual pasture legume subterranean clover Trifolium subterraneum L. cv. "Nangeela" were obtained from LaBrie Ranch and Seed Company, Roseburg, Oregon.

(ii) Seed sterilization. The following procedure was used to surface sterilize the seeds for all the experiments described in this thesis. Ten gram samples of seeds were sieved through a 2-mm mesh and sorted for uniformity and freedom from damage. The seeds were mixed for 30 seconds with 20 ml of 95%(v/v) ethanol in a 250-ml Erlenmeyer flask. After two rinses with sterile distilled water, the seeds were swirled for ten minutes in acidified mercuric chloride (0.2% w/v) followed by rinses of 2 min intervals in six different changes of sterile distilled water.

The seeds were transferred to water agar plates which were then placed in ziplock plastic bags along with damp paper towels. To allow for imbibition of water, the seeds were incubated for one day at 4°C in a refrigerator, then transferred to a drawer and allowed to germinate for two days in the dark at room temperature.

(ii) Bacteria. Eleven isolates of Rhizobium leguminosarum bv. trifolii were chosen to represent the three serotypes commonly found in nodules of subclover growing in a field at the Soap Creek Cattle Facility of Oregon State University. The soil under the pasture represents a silty clay loam of the Abiqua series (fine, mixed, mesic, Cumulic, Ultic, Haploxeroll). The physical and chemical properties of the surface 30-cm have been described in detail elsewhere (Almendras and Bottomley,

1987; Bottomley and Dughri, 1989).

The bacteria were identified by the following trivial codes: 88FL3,5; 88FL3,4; 88FL3,20 and ADS4 (serotype AS6); AS21-1; ADS3; 88FL1,9; and 88FL1,6 (serotype AS21); and 88FL1,1; 1,4; and 88FL1,5 (serotype AS6-21). Strain RT162X95 (Liphatech Co. Milwaukee, WI) was used to place the effectiveness of the soil isolates in perspective since it represents a strain that is highly effective at fixing N_2 in association with subclover.

(iii) Plant growth conditions. A Leonard jar assembly that was composed of a bottomless one quart beer bottle and a wide mouth (800 ml) mason jar was the basic plant growth vessel used for all the experiments described in this thesis. The bottle was inverted into the mason jar and a cheesecloth wick was constructed of sufficient length to reach from the bottom of the mason jar to half way up the beer bottle. The beer bottles were filled to within 3-4 cm of the top with a 1:1 (v/v) mixture of vermiculite and perlite. Nitrogen-free mineral nutrient solution (300 ml) was poured into the mason jars and another 150 ml poured into the vermiculite / perlite mixture held in the beer bottles.

The N-free mineral nutrient solution consisted of the following ingredients (grams per liter): $CaSO_4.2H_2O$ (1.033), K_2SO_4 (0.279), $MgSO_4.7H_2O$ (0.493), K_2HPO_4 (0.145), KH_2PO_4 (0.023), Fe-citrate (0.005), $CaCl_2.2H_2O$ (0.056), and 2.5 ml of a solution containing trace elements (mg per liter): H_3BO_3 (1.43), $MnSO_4.4H_2O$ (1.02), $ZnSO_4.2H_2O$ (0.22), $CuSO_4.5H_2O$ (0.08), $CoCl_2.4H_2O$ (0.10), and $Na_2MoO_4.2H_2O$ (0.05) (Evans,1974). Fe-citrate was dissolved by heating in

2-3 ml of 0.1 M HCl and then added to the nutrient solution. The pH of the solution was adjusted to 6.5 by adding either KOH or HCl dropwise while stirring over a magnetic stirrer. The tops of the jars were covered with aluminum foil, and each assembly was wrapped in a brown paper bag and secured with elastic bands. The assemblies were stood in 5-8 cm of water in metal troughs, autoclaved for 60 minutes, and then left to cool for one day.

(v) Planting and Inoculation. On October 18, 1989, the pregerminated seeds were aseptically planted to a depth of about 5-6 mm in the vermiculite / perlite mixture. Six seedlings were planted per jar and four replicate jars were inoculated with each of the field isolates of R. leguminosarum bv trifolii and strain RT162X95. Each of the bacterial cultures had been grown for 3 days at 28°C in 25 ml portions of yeast extract mannitol (YEM) broth. Four jars were left uninoculated to serve as contamination checks. Each jar was covered with a petri dish lid (100 x 15 mm) and transferred to a growth chamber. Illumination was provided by eight F30T12-D-RS fluorescent lamps (Lifeline/Sylvania), and twelve 25 watt incandescent bulbs (General Electric). A daylength of 14 hours, and an air temperature of 22 to 27°C were provided.

Three days after planting (DAP) the petri dishes were removed, the seedlings thinned to two per jar, and sterile paraffinized sand applied to the top of the jars to a depth of about 2-2.5 cm. The sand formed a hydrophobic layer that reduced evaporation and helped to safeguard against aerial contamination by rhizobia.

Two weeks after planting, the seedlings were transferred to a greenhouse, thinned to one per assembly, and arranged on the benches in four blocks. Within each block the replicates were randomly distributed. The conditions in the greenhouse are described elsewhere (Bottomley and Jenkins, 1983; Demezas and Bottomley, 1986.) All subsequent experiments described in this thesis were thus arranged.

(vi) Plant harvesting. On December 19, 1989, sixty two DAP, shoots were harvested. The herbage was placed into envelopes, dried in a 55°C oven for 14 days, and then weighed to determine the dry matter yields.

(vii) Statistical analysis. An analysis of variance and a separation of means were performed on the data using the general linear model of the SAS (Statistical Analysis System Institute, Inc. 1988) software.

Experiment 2. Evaluation of Symbiotic effectiveness of non-dominant serotypes.

After evaluating the symbiotic effectiveness potential of nodule-dominant serotypes, a similar experiment was planted on Dec.19, 1989 with the objective of comparing the effectiveness potential of isolates representing serotypes that are consistently minor occupants of nodules on field-grown subclover.

(i) Bacteria. The following 12 isolates were used: MS10; MS13; RW3 and ADS13 (serotype AP17); ADS16; ADS14; RW11; and RW10 (serotype AS27); ADS19; ADS2; FL2,48; and FL2,40 (serotype AG4). The commercial inoculant RT162X95 was again included for comparison. Preparation of seedlings, bacterial cultures,

and plant growth conditions were the same as described above.

(ii) Shoot harvest. Herbage was harvested on Feb.19, 1990, sixty two days after planting, dried in an oven for 14 days at 55°C, and the dry matter yields determined. Analysis of variance on the dry matter yields was carried out as described in experiment one.

Experiment 3. Influence of plant-isolate combinations of differing symbiotic growth potential upon subclover regrowth characteristics.

(i) Seeds. The design of regrowth experiments was similar to that used in the effectiveness trials with the exception that seeds of Nangeela subclover were obtained from Dr. G. Evers, Texas A & M University, Agricultural Experiment Station, Angleton, Texas.

(ii) Bacteria. Most of the isolates used in the first of these regrowth experiments were the same as those used in experiment 1 and belonged to serotypes AS6, AS6-21, and AS21. Isolate 88FL1,5 (AS6-21) was replaced with isolate 88FL1,11(AS6-21), and isolate 88FL1,9 with AS6-1(AS6). The surface-sterilized, pregerminated seedlings were planted and inoculated with the appropriate bacterial cultures on April 3, 1990. After 7 days the seedlings were thinned to two per jar, and 14 days after planting (DAP) they were thinned to one per jar and transferred to the greenhouse.

(iii) Shoot harvest. The first harvest of the herbage was carried out on June 4, 1990, sixty- two days after planting. A severe clipping was undertaken whereby all expanded leaves and stems were excised from the crown of the plants.

Herbage was dried at 55°C for 16 days and weighed. Regrowth of plants was allowed to develop for 30 days and the shoots harvested again.

(iv) Total nitrogen analysis. Replicates samples of oven dry herbage were composited by treatment, ground in a blender, and digested in a combination of Kjeldahl catalyst and concentrated H_2SO_4. Ammonium was determined in the extracts using an Alpkem auto analyzer (Clackamas, OR) following the method of Bremner and Mulvaney (1982).

(v) Statistical analysis. An analysis of variance and separation of means were carried out on dry matter, nitrogen concentration, and total nitrogen data as in the other two experiments.

Experiment 4. Second Regrowth Experiment.

(i) Bacteria. Since there was no evidence for serotype membership per se differentially influencing the symbiotic effectiveness ranking, isolates used in the second regrowth experiment were selected because of their effectiveness traits measured in experiments 1 and 2. Eleven isolates were chosen to cover effectiveness traits ranging from very effective, moderately effective, and poorly effective. The highly effective isolates were ADS14(AS27), RW11(AS27), AS6-1(AS6), and ADS3(AS21). The moderately effective isolates were ADS4(AS21), AS21-1(AS21), and ADS16(AS27). The poor isolates were 88FL3,20(AS6), 88FL3,4(AS6), 88FL1,11(AS6-21), and 88FL1,6(AS21). The commercial isolate RT162X95 was also included.

The seedlings were planted and inoculated on Feb. 19, 1991, thinned to two

plants per jar seven days later, and 14 DAP thinned to one plant per jar and transferred to the greenhouse. The shoots were harvested on April 13, 1991, fifty four DAP. The second harvest was carried out on May 10, 1991, after 28 days of regrowth. A major difference between experiment 4 and experiment 3 was that three fully expanded leaves were left on each plant at the first harvest. After drying, the shoot samples were ground, digested, and analyzed for total N as described earlier.

(ii) Statistical analysis. In addition to the analysis of variance and separation of means which were carried out on the data as in the preceding experiments, paired t tests were also carried out to compare yield parameters from the first harvest with those of the second harvest.

COMPETITION EXPERIMENTS

(i) Competitors. Having established the nitrogen fixing characteristics of the isolates representing dominant and non-dominant serotypes, the next phase of inquiry was to determine their relative competitive abilities when challenged to the plants as simple mixtures. Isolate 88FL3,5 a mediocre N_2-fixing isolate was chosen to represent the nodule dominating serotype, AS6. 88FL3,5 was competed one-on-one against eight isolates representing four serotypes: ADS3 and 88FL1,6(AS21), ADS14 and ADS16(AS27), MS13 and ADS13(AP17), ADS19 and FL2,48(AG4). Peat-based inoculants of each isolate were used to formulate the paired combinations of competitors.

(ii) Peat inoculant preparation. Each of the isolates was grown as a pure culture

for three days at 28°C in 50 ml of yeast extract mannitol (YEM) broth. Fifty gram portions of pre-packaged, pre-neutralized ($CaCO_3$), and radiation-sterilized peat were obtained from Dr. P. Singleton of the NifTAL project, University of Hawaii, Maui, HI. Following the procedure of Somasegaran and Hoben (1985), a 40 ml portion from each of the 3-day old cultures (ca.10^9 cells / ml) was aseptically injected into a bag of peat. After hand kneading each bag to ensure uniform distribution of the liquid culture throughout the peat, the peat cultures were incubated at 28°C for 14 days.

(iii) Subdivision and storage of inoculants. After 14 days each of the bags was surface sterilized with 95% ethanol and opened under a laminar flow hood. The peat inoculant was spread onto a 95% ethanol-sterilized sheet of aluminum foil, divided into 10 approximately equal portions, and each of the latter was transferred into a sterile 20 ml scintillation vial (Owen-Illinois, Toledo OH). The vials were labelled and stored at 4°C.

(iv) Determination of the population density of the peat inoculants. One gram portions of peat cultures were transferred to sterile 100 ml screw cap bottles containing 49 ml of a mineral salts solution (pH 6.5) composed of the following (grams per liter): $MgSO_4.7H_2O$ (0.2), NaCl (0.1) and K_2HPO_4 (0.5). After 3 min of vigorous shaking, 2.0 ml portions of the suspensions were mixed with 18 ml portions of the mineral salt solution in 50 ml screw cap tubes. Using a different sterile pipette for each transfer, the inoculant suspensions were serially diluted through a 10-fold series by drawing 2.0 ml from the preceding tube and adding to

another 18 ml until a final dilution of $1:10^6$ was obtained. One-tenth milliliter portions of the $1:10^5$ and the $1:10^6$ dilutions were spread onto YEM agar plates. The plates were incubated at 28°C for 3-5 days, and the number of colony forming units per gram of peat determined. Satisfactory peat cultures were produced that contained approximately 10^9 viable cells per gram of peat. The results are shown in table A.1. (See appendix)

(v) Determination of the number of rhizobial cells per subclover seed. Five grams of subclover seed were surface sterilized as described previously. Excess moisture was removed from the seeds by spreading them on a sheet of alcohol-sterilized aluminum foil under a laminar flow hood. All subsequent manipulations of seed were carried out under the flow hood and using aseptic techniques. Using an alcohol-flame-sterilized spatula, the seeds were turned occasionally to facilitate moisture removal. Seeds were placed into a sterile 250 ml Erlenmeyer flask, covered with aluminum foil and placed on ice. Gum arabic solution (4.8 g in 10 ml distilled water) was prepared in another 250 ml Erlenmeyer flask and placed on ice. A portion of the gum arabic solution (0.5 ml) was added to the five-gram portion of seeds in a 200 ml sterile beaker. Both seed and gum were stirred thoroughly with an alcohol-flame-sterilized glass rod. One gram of the peat inoculant was added to the gum-coated seeds and stirred until all the seeds were completely covered. An additional 0.5 g of peat inoculant was added to saturate the excess gum arabic solution. This procedure gave satisfactory results since all of the peat was picked up by the seeds. The seeds did not clump together, were

easily handled, and were fully coated with the inoculant.

The inoculant-coated seeds were spread onto sterile aluminum foil, and 100 seeds transferred to a screwcap bottle containing 100 ml of the mineral salts solution described above. After vigorously shaking for two minutes to wash off the peat culture into the solution, the suspension was serially diluted through a ten-fold series to a final dilution of $1:10^5$ by taking 10 ml volumes and mixing with 90 ml portions of the mineral salts solution. The last two dilutions($1:10^4$ and $1:10^5$) were plated in triplicate on YEM agar and incubated at 28°C for 3-5 days. The colonies were counted on each plate and the number of rhizobial cells per seed were calculated. Excellent seed inoculation was obtained with 1.8 and 3.3 x 10^6 cells per seed obtained for isolates 88FL1,6 and MS13 respectively.

(vi) Determining the actual ratios of the population mixtures prepared on the seeds. A preliminary experiment was carried out to determine if a 1:1 population mixture of two isolates could be obtained on seeds using peat inoculants.

A mixture of the peat inoculants of isolate 88FL1,6 (AS21) [$1.95x10^9$ cells per g], and isolate MS13(AP17) [$2.85x10^9$ cells per g] was prepared. One gram of the MS13 peat inoculant was mixed with 1.45 g of the 88FL1,6 inoculant in a 50 ml sterile beaker. A spatula was used to stir and turn the peat for 4-5 min. During the mixing process an effort was made to crush the peat mixture against the sides of the beaker to ensure thorough mixing.

Clover seeds which had been previously surface sterilized and air-dried were coated with the mixed inoculant as described earlier. After coating, 100

seeds were transferred into 100 ml mineral salts solution, shaken vigorously, and the suspension serially diluted. The $1:10^5$ and $1:10^6$ dilutions were plated in triplicate on YMA plates and incubated at 28°C for 3-5 days.

The colonies that appeared were labeled numerically on the underside of the petri dish and a loopful of the cells from each colony transferred into 1 ml phosphate buffer (pH 7.2) in a 1.7 ml microfuge tube (National Scientific Supply, Inc. San Rafael, CA). The cells were suspended by vortexing on a Bronwill mixer (Lab-line Instruments, Inc., Melrose Park, IL.) and then centrifuged in a microfuge (Spinco Division, Beckman Instruments, Inc., Palo Alto, CA) at 13,750 rpm for 5 min. The supernatants were discarded, cells resuspended in 1.0 ml phosphate buffer and centrifuged for 5 min. After decanting the supernatant, cells were resuspended in 100 ul of sterile distilled water.

Using a micropipetter (P200), portions (5 ul) of the suspended cells were used to produce duplicate smears on microscope slides. A total of 36 slides representing 36 colonies were prepared. Eighteen of the slides were labeled for staining with a fluoresecein-labeled immunoglobin conjugate FA (AP17) (specific for MS13). The other 18 were labeled for staining with FA (AS21), a conjugate that is specific for 88FL1,6). Another set of 10 slides was also prepared this time with 3 smears per slide, for a total of 30 colonies, and labeled as before. After air drying the smears were heat fixed by passing each slide through a flame thrice, and each smear was circled with indelible felt pen marker on the underside of the slide.

The smears were stained with a rhodamine-gelatin conjugate (Demezas and Bottomley, 1986) and incubated at 55°C for 10 min. One duplicate of the slides/smears was stained with FA (AS21). The other set was stained with the FA (AP17). The slides were incubated in subdued light at room temperature for 15 min. Excess FA was washed off with 0.02M (pH 7.2) sodium phosphate buffer. After drying the slides, FA mounting fluid (2-3 ul) was added to each of the smears and covered with a 22x60 mm no.1 cover slip. A drop of immersion oil was then placed on top of each smear. The smears were viewed at 1000X magnification with a Zeiss epifluorescent microscope equipped with a fluorescein filter set.

The cells that reacted with the specific FA fluoresced a bright yellowish green. Fluorescence was scored on a scale of 0 to +4 with the most brilliant scored as +4 and the negative smears 0. Of the 66 smears examined 33 were positive to the AS21 FA and negative to the AP17 FA. Thus a satisfactory procedure was available to produce a 1:1 ratio of the isolates on seeds.

Experiment 5. To determine the competitiveness of the isolates representing dominant and non-dominant serotypes.

(i) Preparation of 1:1 mixtures of competitors.

Five months after preparing the peat inoculants, the population densities were determined, following the same procedure as outlined earlier. The results are presented on table A.2. These densities were used to determine the amounts of each peat inoculant needed to produce 1:1 mixtures of various isolate combinations. An example of how the amounts were determined is described

below.

The peat inoculant of isolate 88FL3,5 (AS6) contained a population of $6.3x10^8$ cells g^{-1} and 88FL1,6(AS21) a population of $8.5x10^8$ cells g^{-1}. Disregarding the 10^8 factor (it is the same for both isolates), add the populations together. Thus, 6.3+8.5 = 14.8. The amount of 88FL3,5 peat inoculant required in the 1.5 g mixture for seed coating is given by: 8.5x1.5 divided by 14.8 which equals 0.8615 g (0.8615g x $6.3x10^8$ cells/g = $5.4x10^8$ cells). The amount of 88FL1,6 peat inoculant needed is given by: 6.3x1.5 divided by 14.8, which equals 0.6385 g. (0.6385g x $8.5x10^8$ cells/g = $5.4x10^8$ cells). Mixing 0.8615 g and 0.6385 g of peat inoculants 88FL3,5 and 88FL1,6, respectively produced 1.5 g of peat containing $5.4x10^8$ cells of each isolate per gram.

Eight inoculant-pair combinations were evaluated. Isolate 88FL3,5 was competed against ADS3(AS21), 88FL1,6(AS21), ADS14(AS27), ADS16(AS27), MS13(AP17), ADS13(AP17), ADS19(AG4), and FL2,48(AG4). The symbiotic effectiveness of 88FL3,5 was evaluated by inoculating it alone onto seedlings without competitors. Nitrogen supplemented (5 mM KNO_3) and uninoculated control plants were also included. Seeds from the Texas lot were used in this experiment. The sterilization and inoculation procedures are described elsewhere. Planting and inoculation were carried out between Dec.19, 1990 and Dec.24, 1990. The plants were transferred to the greenhouse on Dec.31, 1990 and harvested on Feb.24, 1991 (62 DAP). Herbage was dried for three days at 55°C and weighed.

(ii) Nodule harvest. The Leonard jar assemblies were disassembled and the

vermiculite/perlite mixture gently washed off the roots. Nodules were randomly recovered from each plant and 21 were chosen at random from each replicate (a maximum total of 84 per treatment). Nodules were placed in small glass tubes (10 x 60 mm) covered on both ends with nylon pantyhose secured with rubber bands. Each tube was marked with a letter and number to represent the treatment and replicate from which the nodules were recovered. During nodule sampling it was noted that many of the crown region nodules had already senesced. As a consequence, the number of nodules obtained from some of the treatments was less than 84.

Nodules were surface sterilized by placing the nodule containing glass tubes in a 2 L plastic beaker containing 0.1% Tween 80 (Scientific Supply Co.Seattle WA). The tubes were swirled for 3 min followed by four 1-min rinses with sterile distilled water. A rinse of 30 seconds in 95% ethyl alcohol was followed by two rinses of 1 min duration with sterile distilled water. This was followed by 5 min in 20% (v/v) Chlorox bleach and six 2-min rinses in sterile distilled water. The nodules were placed individually in microtiter wells and crushed in 20 to 100 ul of sterile distilled water depending on the size of the nodule. Tweezers used to crush the nodules were alcohol-flame sterilized between each well.

Smears were made on microscope slides with 4 ul portions of the bacteroid-containing extracts from each well. A duplicate set of seven slides was prepared for each replicate (each slide contained three nodule smears). After air drying, heat fixing, and circling each smear, the slides were placed in slide boxes until

examined by immunofluorescence microscopy.

(iii) Indirect immunofluorescence procedure for representatives of serotype AS27. During the staining process, smears of ADS14 and ADS16 were found not to react with the AS27 FA. These isolates did, however, react with unconjugated AS27 antiserum in immunodiffusion plates indicating that antibody had been lost during preparation of the FA.

An indirect FA method (Somasegaran and Hoben, 1985) was used to analyze the smears. Fluorescein-labeled goat anti-rabbit immunoglobin (Sigma Chemical Co., St. Louis, MO) was used in conjunction with the whole AS27 antiserum for staining the ADS14 and ADS16 smears. Smears of ADS14 and ADS16 were stained with rhodamine-gelatin conjugate followed by a 15 min incubation with AS27 antiserum (diluted 1:100 and adsorbed three times with AS6-1 cells to remove antibodies cross reactive with members of serotype AS6) (see below). Excess antiserum was washed off the smears with 0.02 M sodium phosphate buffer (pH 7.2) and the smears restained with the manufacturer's recommended dilution (1:80) of goat anti-rabbit immunoglobin (in 0.02 M sodium phosphate buffer pH 7.2). After incubating for 15 min, smears were rinsed, air-dried, and examined by immunofluorescence as described earlier.

(iv) Adsorption of antiserum AS27. Adsorption of AS27 antiserum was accomplished as follows. Isolate AS6-1 was cultured for three days at 28°C in 50 ml quantities of a glutamate-mannitol defined medium (Leung and Bottomley, 1987). The cells were harvested by centrifugation at 12000 x g for 10 min, and the

supernatant decanted. The cells were washed twice in 0.15 M phosphate buffer, pH 7.2, and centrifuged at 12,000 x g. Cells were resuspended in 1 ml phosphate buffer, transferred into preweighed microfuge tubes, centrifuged at 12,500 x g for 5 min, and the wet weight of the cells determined.

Antiserum AS27 was diluted one-hundred fold in 0.02M phosphate buffer pH 7.2 and mixed with the AS6 cells at a ratio of 0.4 mg (wet wt) cells to 1.0 ml antiserum. The mixture was incubated at room temperature for 1 to 2 h with intermittent gentle shaking. The cell-antiserum suspension was centrifuged at 12,000 x g for 10 min and the antiserum filtered through a 0.2-um pore size membrane (Nuclepore Corp., Pleasanton, CA). The adsorbed antiserum was mixed with a fresh sample of AS6 cells and the process repeated. Three adsorptions were required to completely eliminate the cross reaction of AS27 antiserum with 88FL3,5 (AS6) that was detectable by indirect immunofluorescence.

Experiment 6. To determine if superiority or inferiority in numbers influences the outcome of competitive nodulation.

Based upon the results of experiment 5, a follow-up experiment was designed to determine if numerical superiority of another competitor would neutralize the inherent competitiveness of isolate ADS16(AS27), and isolate 88FL3,5(AS6).

The following isolate combinations and ratios were used in this experiment. Isolate 88FL3,5(AS6) was mixed with varying amounts of peat inoculants of either FL2,48(AG4), or ADS16(AS27) in an attempt to achieve ratios of 25:1, 10:1, 1:1,

1:10, and 1:25. To address the impact of relative nodule occupancy by the two isolates on plant yield, the symbiotic effectiveness of each of the three microsymbionts was measured as well as growth of nitrate supplemented and uninoculated (-N) control plants.

(i) Mixing Procedure. Using the procedures described earlier, the population densities of the peat inoculants 88FL3,5, ADS16, and FI2,48 were checked between May 24, and May 28, 1991. It was found that they had not changed significantly from the earlier experiments. Densities of isolate 88FL3,5, ADS16, and FL2,48 were 8.24 ∓ 1.7 $x10^8$, 1.33 ∓ 0.03 x 10^9 and 7.90 ∓ 0.14 x 10^8 cells g^{-1} respectively.

To produce the various inoculant ratios, the following procedure was followed. Amounts of each of the two individual inoculants required to produce the 1.5 g total were determined as described previously. The amounts required for each inoculant were weighed in triplicate. One replicate portion of each inoculant were mixed together to produce the 1:1 combination and stored in a 4°C refrigerator. The second and third replicates were used to prepare the 1:10 and 10:1 combinations. An example of how this was done is described below.

The amounts of the peat inoculants required to produce a 1:1 mixture of isolate 88FL3,5 and ADS16 were 0.922 g and 0.578 g respectively. To dilute the ADS16 inoculant 1:10, 0.5 g of peat inoculant was thoroughly mixed with a 4.5 g portion of sterile uninoculated peat. From this ten-fold dilution, a 0.578 g portion was mixed with 0.922 g of the original inoculant of isolate 88FL3,5. This mixture

resulted in the desired 10:1 ratio of the 88FL3,5(AS6):ADS16(AS27) combination.

To prepare the reciprocal mixture, 0.5 g of the 88FL3,5 inoculant was mixed with 4.5 g sterile peat. From this 1:10 diluted mixture of 88FL3,5, 0.922 g were mixed with 0.578 g of the ADS16 inoculant. The same procedure was used to prepare the 1:25 and 25:1 ratios, with the dilution being achieved by mixing 0.4 g quantities of the primary inoculants with 9.6 g sterile peat.

(ii) Planting and inoculation. The seeds were inoculated and planted between June 1 - June 4, 1991, thinned to one plant per jar on June 7, 1991 and transferred to the greenhouse on June 13, 1991.

(iii) Shoot and nodule harvests. The shoots and nodules were harvested between July 16 - July 25, 1991, 47 - 52 DAP. Nodule occupancy was determined using the procedures previously described. After oven drying, the shoot materials were composited by treatment, ground in a blender, digested and analyzed for total N as described earlier.

(iv) Statistical analysis. Analysis of variance, separation of means, and paired t tests were carried out on arcsine √% transformed nodule occupancy data.

RESULTS

Experiment 1. Symbiotic effectiveness of nodule dominating serotypes

Although plants nodulated by isolates representing nodule-dominant serotypes produced significantly more dry matter than the uninoculated controls, (Table 1), all isolates showed suboptimal symbiotic effectiveness capabilities on subclover cv. "Nangeela". None of the isolates yielded the equivalent dry matter of the commercial inoculant strain, RT162X95. Furthermore, the isolates were not uniformly effective and were subdivided into two groups. The less effective group was comprised of six isolates that included three of the four AS6 isolates and two of the four AS6-21 isolates.

Experiment 2. Symbiotic effectiveness of non-dominating serotypes

A similar experiment was conducted to assess the symbiotic effectiveness of the isolates that represent serotypes rarely recovered from field nodules (Table 2). The non-dominant serotype representatives showed a range of effectiveness values and were, in general, significantly less effective than RT162X95. However, whereas no isolates from the nodule dominant serotypes were as effective as RT162X95 (Table 1), two isolates representing a minor serotype AS27 were as effective as RT162X95. Comparisons of the effectiveness of isolates represented in table 1 with those in table 2 are not possible because plant growth conditions were superior in the latter experiment (RT162X95, 1.28 g versus 1.81 g plant^{-1}).

Experiment 3. Influence of plant-isolate combination upon regrowth potential of "Nangeela" subclover.

Since the majority of the isolates representing nodule dominant serotypes are sub-optimally effective, an experiment was conducted to assess the effectiveness potential of different plant-isolate combinations under regrowth conditions.

(i) Effectiveness rankings after first harvest.

Based upon the dry matter yields of the first harvest the isolates could be separated into four different effectiveness groups (Table 3). Isolates ADS3(AS21), and AS6-1(AS6) produced as much herbage as did strain RT162X95 and N-fertilized plants (3.0 to 3.5 g plant^{-1}). The second group (II) contained only one isolate (88FL1,1) whose yield (2.43 g plant^{-1}) was significantly different from any other isolate. The third group (III) was composed of five isolates that produced mediocre plant yields ranging between 0.6 and 1.1 g plant^{-1}. The fourth group (IV) contained isolates that were poorly effective (0.25 to 0.38 g plant^{-1}). With the exception of isolate 88FL1,6, the ranking status was quite similar to that found in experiment 1. Again, it should be noted that plant growth conditions were superior in this experiment relative to experiments 1 and 2. (RT162X95, 2.99 versus 1.81, and 1.28 g plant^{-1}).

In general, the total N fixed by the plant-isolate combinations followed the same trend as the dry matter yield. In harvest 1 there was a twenty-fold difference in the amount of N_2 fixed by the best and the worst plant-isolate

combinations (Table 3). In addition to fixing lower amounts of N_2, plants nodulated by group IV isolates had a substantially lower concentration of N in their herbage (mean = 29.7 mg N g^{-1}) than plants nodulated by isolates from groups I, II, or III (average = 40.7, 40.3, and 37.0 mg N g^{-1}, respectively)(Table 4).

(ii) Effectiveness ranking after second harvest.

With the exception of isolate 88FL3,20, the ranking of the plant-isolate combinations remained in the same groups at the second harvest. There was, however, a noticeable trend for a negative impact of superior growth at first harvest upon the regrowth potential of subclover. For plants nodulated by isolates in group I the dry matter yield of harvests 2 relative to harvest 1 ranged between 0.67 and 0.80. In contrast, the yield ratio for isolates in group III ranged between 0.88 and 1.08 (excluding 88FL3,20). The same trend was seen in the total amount of N_2 fixed. The harvest 2 : harvest 1 ratio for the N_2 fixed by plant-isolate combinations in group I ranged between 0.69 to 0.78 whereas in groups III and IV the ratio ranged between 0.97 to 1.28. The greater impact of harvests upon regrowth of superior plant-isolate combinations was not simply due to the greater sensitivity of more vigorous plants. The nitrate supplemented plants were not influenced in a negative manner by harvest and they were the largest plants at harvest 1.

Experiment 4. Influence of plant-isolate combination on regrowth potential.

(i) Effectiveness rankings after first harvest.

Based on statistical analysis of the first harvest dry matter yields, the isolates were separated into four effectiveness groups (Table 5). The first group (I) consisted of two isolates, ADS14 and RW11, from serotype AS27. As in experiment 2, they produced the most dry matter and fixed significantly more N_2 (56.3 to 61.6 mg plant^{-1}) than any other isolate. Three other groups were delineated. Group II contained four isolates (38 to 45 mg N plant^{-1}) and group III contained five isolates in which the amount of N_2 fixed ranged between 12.8 and 22.2 mg N plant^{-1}. The N concentrations of herbage in group III and IV plant-isolate combinations were substantially lower than for isolate combinations in groups I and II (31.5 and 26.0 mg g^{-1} versus 38.4 and 37.5mg g^{-1}) (Table 6).

(ii) Effectiveness rankings after second harvest.

In contrast to the previous regrowth experiment (Table 3), 10 of the 11 plant-isolate combinations produced substantially more dry matter at the second harvest than for the first. In addition, there was evidence for differential growth responses among the plant-isolate combinations in groups II and III. The dry matter yield increase of isolate AS6-1 was sufficiently low for it to become part of group III (42 to 71 mg N plant^{-1}). Isolate 88FL1,11 dropped into group IV as a result of its performance in harvest 2. Along with the overall increase in dry weights and amounts of N_2 fixed, there was a substantial increase in herbage N concentration. This increase was especially pronounced in groups III and IV (31.5 to 42.4 mg N g^{-1} and 26.0 to 37.7 mg N g^{-1} respectively) (Table 6).

Experiment 5. Competitive nodulating ability of isolates from serotype AS6.

The mediocre N_2-fixing isolate 88FL3,5 outcompeted both isolates representing serotype AP17 and one isolate from each of serotypes AG4 and AS21 (Table 7). In contrast, isolates representing serotype AS27 outcompeted 88FL3,5 for nodule occupancy. Significantly fewer nodules were occupied by 88FL3,5 in these two treatments than in any other treatment. Erratic nodule occupancy was observed among the replicates of the ADS19(AG4) treatment.

Experiment 6.A. Influence of relative numbers upon the nodule occupancy by 88FL3,5 and FL2,48 and on plant yield.

In agreement with data presented in Table 7, isolate 88FL3,5 completely dominated nodule occupancy when its population was either numerically equivalent or greater than FL2,48 (Table 8). In contrast, when FL2,48 outnumbered 88FL3,5 by ratios of 10:1 and 25:1, the situation was reversed and FL2,48 dominated the nodules in just as complete a manner. Not surprisingly, at ratios of 25:1, 10:1, and 1:1, where 88FL3,5 dominated the nodule occupancy, both dry matter yield and total N_2 fixed were not significantly different from those of plants nodulated by 88FL3,5 alone (Table 9). In contrast, when FL2,48 dominated the occupancy, the values for herbage yield and N_2 fixed were similar to those achieved by plants nodulated by FL2,48 alone.

Experiment 6.B. Influence of relative numbers upon the nodule occupancy by 88FL3,5 and ADS16 and on plant yield.

Isolate ADS16 completely dominated nodule occupancy when its population was either numerically equivalent to, or greater than 88FL3,5 (Table 10). Significantly more nodules were occupied by 88FL3,5 as it gained numerical advantage over ADS16. In contrast to the situation with FL2,48, occupancy by 88FL3,5 significantly increased between each of the 1:1, 10:1, and 25:1 ratios. Nevertheless, the increase in co-occupancy along with the variance among replicates resulted in occupancy by either isolate being not significantly different from each other.

The yield data reflected upon the occupancy. Where occupancy by ADS16 was 100 %, plant yields were equivalent to that of plants supplemented with nitrate or nodulated with ADS16 alone (Table 11). As occupancy of 88FL3,5 increased progressively from zero to 72.9%, plant yield progressively decreased. When the occupancy by AS6 reached 72.9% the yield and N_2 fixed were not significantly different from those values for plants nodulated by 88FL3,5 alone. Interestingly, the proportion of nodules occupied by the highly effective ADS16 was still substantial (42.5%) despite the mediocrely effective plant phenotype.

Table 1. Dry matter yield of " Nangeela" subclover nodulated with isolates representing nodule-dominant serotypes AS6, AS6-21, and AS21 of R. leguminosarum bv. trifolii.

Isolate	Serotype	g plant^{-1}
RT162X95		1.28**
88FL1,1	AS6-21	0.95*
ADS3	AS21	0.87*
88FL1,6	AS21	0.78*
ADS4	AS6	0.76*
AS21-1	AS21	0.75*
88FL1,9	AS21	0.69
88FL3,4	AS6	0.68
88FL3,20	AS6	0.65
1,4	AS6-21	0.60
88FL1,5	AS6-21	0.59
88FL3,5	AS6	0.50
Uninoculated		0.03
LSD(0.05)		0.24
CV(%)		21.50

** Denotes that RT162X95, the commercial inoculant strain, is significantly more effective ($P < 0.05$) than any other strain.

* Denotes those isolates not significantly less effective ($P < 0.05$) than isolate 88FL1,1.

Table 2. Dry matter yield of " Nangeela" subclover nodulated with isolates representing minor nodule occupying serotypes (AG4, AP17, AS27) of R. leguminosarum bv. trifolii

Isolate	Serotype	g plant^{-1}
RW11	AS27	2.10**
ADS14	AS27	1.95**
RT162X95		1.81**
MS10	AP17	1.45*
ADS19	AG4	1.43*
FL2,48	AG4	1.36*
RW10	AS27	1.32*
RW3	AP17	1.31*
ADS2	AG4	1.18*
MS13	AP17	1.13*
FL2,48	AG4	1.12*
ADS16	AS27	1.08
ADS13	AP17	1.03
Uninoculated		0.02
LSD(0.05)		0.29
CV(%)		16.06

** Denotes isolates that were not significantly less effective ($P < 0.05$) than RT162X95, the commercial inoculant strain.

* Denotes isolates that were not significantly less effective ($P < 0.05$) than isolate MS10.

Table 3. Influence of plant-isolate combination upon the yield and N_2 fixed by "Nangeela" subclover over successive harvests (Expt. 3).

Eff grp†	Isolate (serotype)	harvest 1		harvest 2	
		g plant^{-1}	N** mg plant^{-1}	g plant^{-1}	N mg plant^{-1}
I	ADS3(AS21)	3.48	150.3	2.34	104.1
	AS6-1(AS6)	3.18	123.1	2.55	92.0
	RT 162X95	2.99	120.5	2.14	94.9
II	88FL1,1(6-21)	2.43	97.9	2.12	92.0
III	88FL3,20(AS6)	1.09	41.2	0.37	15.2
	AS21-1(AS21)	1.02	37.9	0.97	36.6
	ADS4(AS6)	0.92	35.6	0.92	35.8
	88FL3,4(AS6)	0.66	24.4	0.71	31.2
	1,4(AS6-21)	0.60	20.5	0.53	22.5
IV	88FL1,11(AS6-21)	0.38	11.5	0.33	11.0
	88FL1,6(AS21)	0.33	9.5	0.26	8.5
	88FL3,5(AS6)	0.25	7.6	0.30	9.6
	N-supplemented‡	3.48	127.0	4.20	186.0
	Uninoculated	0.03	N.D.	-----	-----
	LSD(0.05)	0.52		0.48	
	CV(%)	26.39		27.31	

† Eff grp. Denotes effectiveness group defined on the basis of first harvest dry matter yields.

** N=Kjeldahl N determined on composite samples of herbage drawn from four replicates of each treatment.

‡ Plants were grown in nutrient solution supplemented with 5 mM KNO_3.

Table 4. Influence of plant-isolate combination upon nitrogen concentration in herbage of subclover "Nangeela" over successive harvests (Expt. 3).

Eff grp†	Isolate (serotype)	Kjeldahl N (mg g^{-1})	
		Harvest 1	Harvest 2
I	ADS3(AS21)	43.2	44.5
	AS6-1(AS6)	38.7	40.8
	RT 162X95	40.3	44.3
	Mean	40.7	43.2
II	88FL1,1(AS6-21)	40.3	43.4
	Mean	40.3	43.4
III	88FL3,20(AS6)	37.8	41.2
	AS21-1(AS21)	37.2	37.7
	ADS4(AS6)	38.7	38.8
	88FL3,4(AS6)	37.0	44.2
	1,4(AS6-21)	34.1	42.5
	Mean	37.0	40.9
IV	88FL1,11(AS6-21)	30.2	33.4
	88FL1,6(AS21)	28.8	32.8
	88FL3,5(AS6)	30.2	31.9
	Mean	29.7	32.7

† Eff grp. Denotes effectiveness group defined on the basis of first harvest dry matter yields (same as table 3).

Table 5. Influence of plant-isolate combination upon the yield and N_2 fixed by "Nangeela" subclover over successive harvests (Expt. 4).

Eff grp†	Isolate (serotype)	harvest 1		harvest 2	
		g plant^{-1}	N mg plant^{-1}	g plant^{-1}	N mg plant^{-1}
I	RW11(AS27)	1.54	61.6	3.38*	152.8*
	ADS14(AS27)	1.53	56.3	2.97	132.2*
II	RT162X95	1.22	44.3	2.46	113.5*
	ADS3(AS21)	1.17	45.1	2.77	128.1
	ADS16(AS27)	1.16	43.5	2.59*	117.0*
	AS6-1(AS6)	1.07	38.0	1.55	69.8*
III	ADS4(AS6)	0.76	22.2	1.52	65.5*
	88FL3,20(AS6)	0.58	18.4	0.97*	41.6*
	AS21-1(AS21)	0.56	19.0	1.41*	58.8*
	88FL3,4(AS6)	0.53	16.6	1.62*	70.6*
	88FL1,11(AS21)	0.40	12.8	0.50	20.6
IV	88FL1,6(AS21)	0.21	5.4	0.25	9.5
	LSD(0.05)	0.31	12.8	0.32	15.08
	CV(%)	26.38	26.63	12.69	13.12

† Eff grp. Denotes effectiveness group defined on the basis of the first harvest dry matter yield.

* Denotes that the second harvest dry matter yield and Kjeldahl N values were significantly greater ($P < 0.05$) than the corresponding first harvest values as determined by a paired t test.

Table 6. Influence of plant-isolate combination upon nitrogen concentration in herbage of subclover"Nangeela" over successive harvests (Expt. 4).

Eff grp†	Isolate (Serotype)	Kjeldahl N (mg g^{-1}) Harvest 1	Harvest 2
I	RW11(AS27)	40.0	45.2 N.S.
	ADS14(AS27)	36.8	44.6 N.S.
	Mean	38.4	44.9
II	RT162X95	36.3	46.2*
	ADS3(AS21)	38.9	46.2 N.S.
	ADS16(AS27)	37.3	45.3 N.S.
	AS6-1(AS6)	37.3	45.0 N.S.
	Mean	37.5	45.7
III	ADS4(AS6)	28.6	43.2*
	88FL3,20(AS6)	31.6	42.7*
	AS21-1(AS21)	33.9	41.6 N.S.
	88FL3,4(AS6)	31.3	43.6*
	88FL1,11(AS21)	32.2	40.9 N.S.
	Mean	31.5	42.4
IV	88FL1,6(AS21)	26.0	37.7*
	LSD(0.05)	3.6	2.7
	CV(%)	7.2	4.3

† Eff grp. Denotes effectiveness group defined on the basis of the first harvest dry matter yields (same as Table 5).

N.S. Denotes that second harvest N concentration values are not significantly ($P < 0.05$) greater than first harvest values as determined by a paired t test.

* Denotes that second harvest N concentration values are significantly greater ($P < 0.05$) than first harvest values as determined by a paired t test.

Table 7. Nodule occupancy by isolate 88FL3,5(AS6) in competition with representatives of serotypes AS21, AS27, AG4 and AP17 of R. leguminosarum bv. trifolii.

88FL3,5 Competitor	88FL3,5 nodule occupancy (%)†	occupancy (range)‡	P¶
ADS13(AP17)	86.8 [98.8]	(95-100)	*
FL2,48(AG4)	83.0 [97.8]	(95-100)	*
MS13(AP17)	82.8 [97.1]	(93-100)	*
ADS3(AS21)	77.8 [91.0]	(73-100)	*
88FL1,6(AS21)	62.5 [76.7]	(52-93)	N.S.
ADS19(AG4)	47.6 [52.8]	(0-67)	N.S.
ADS16(AS27)	30.6 [26.4]	(14-31)	N.S.
ADS14(AS27)	14.2 [6.1]	(5-10)	*
LSD(0.05)	25.1		
C.V.(%)	28.4		

† Nodule occupancy values by 88FL3,5 include both singly and co-occupied nodules and are presented as arcsine √% transformed values. Values in brackets are the actual percentage means. Analysis of variance was carried out on arcsine √% transformed data. Occupancy values represent the means of four replicates. Eighteen to twenty one nodules per replicate were analyzed by immunofluorescence.

‡ Range values represent the highest and lowest occupancy values obtained from four replicates of each treatment.

¶ P Denotes the results of data analysis by a paired t test to determine if occupancy by one isolate was significantly different ($P < 0.05$) from the other. Comparisons were determined on arcsine √% transformed data.

N.S. Not significant.

Table 8. Influence of different inoculum ratios on nodule occupancy by isolates 88FL3,5 and FL2,48(AG4).

Ratio 88FL3,5: FL2,48	Nodule numbers†	Nodule occupancy (%)‡ 88FL3,5	(range)ƒ	FL2,48	(range)ƒ	mixedff	P¶
25:1	55.0	85.7 [98.3]	(95-100)	6.1 [3.3]	(0-10)	4.8	*
10:1	51.8	90.0 [100.0]	(100)	8.9 [4.9]	(0-14)	4.9	*
1:1	52.3	76.5 [92.5]	(84-100)	26.7 [21.3]	(9-42)	14.9	N.S.
1:10	45.8	3.2 [1.2]	(0-5)	90.0 [100]	(100)	1.2	*
1:25	76.0	6.4 [2.4]	(0-5)	90.0 [100]	(100)	2.4	*
LSD(0.05)	19.3	10.2		11.5			
C.V.(%)	22.8	13.0		17.2			

† Nodule number values represent the means of four replicates. When inoculated alone, isolates FL2,48 and 88FL3,5 formed 77 and 64 nodules per plant, respectively.

‡ Nodule occupancy values for 88FL3,5 and FL2,48 include both singly and co-occupied nodules and are presented as arcsine √% transformed values. Values in brackets are the actual percentage means. Analysis of variance was carried out on arcsine √% transformed data. Occupancy values represent the means of four replicates.

ƒ Range values in parenthesis represent the lowest and highest occupancy values of four replicates.

ff Mixed occupancy values represents the actual percentages of nodules co-occupied by both isolates.

¶ P Denotes the results of data analysis by a paired t test to determine if occupancy by one isolate was significantly different ($P < 0.05$) from the other. Comparisons were determined on arcsine √% transformed data.

N.S. Not significant.

Table 9. Influence of nodule occupancy by isolates 88FL3,5 and FL2,48 of R. leguminosarum bv. trifolii on subclover yield parameters.

Ratio 88FL3,5:FL2,48	g plant^{-1}	N conc† mg g^{-1}	Total N mg plant^{-1}
88FL3,5 alone	0.12	21.1	2.53
25:1	0.20	30.1	6.02
10:1	0.12	27.1	3.25
1:1	0.27	33.4	9.02
1:10	0.81	32.6	26.11
1:25	0.65	35.5	23.08
FL2,48 alone	0.90	35.7	32.11
N-supplemented	0.88	40.2	35.38
LSD(0.05)	0.18		
CV(%)	28.08		

† N concentration values were determined on a composite sample from each treatment.

Table 10. Influence of different inoculum ratios on nodule occupancy by isolate 88FL3,5 and ADS16(AS27).

Ratio 88FL3,5:ADS16	Nodule numbers†	Nodule occupancy (%)‡ 88FL3,5	(range)ƒ	ADS16	(range)ƒ	mixedff	P¶
25:1	38.5	59.1 [72.9]	(58-86)	40.3 [42.5]	(49-68)	13.7	N.S.
10:1	54.8	34.8 [32.8]	(24-43)	63.0 [77.1]	(57-95)	13.2	N.S.
1:1	42.0	13.1 [9.8]	(0-20)	90.0 [100]	(100)	9.8	*
1:10	43.0	0 [0]	(0)	90.0 [100]	(100)	0	*
1:25	40.8	7.8 [3.7]	(0-10)	90.0 [100]	(100)	3.7	*
LSD(0.05)	16.9	13.4		11.3			
C.V.(%)	25.6	38.7		10.0			

† Nodule number values represent the means of four replicates per treatment. When inoculated alone, isolates 88FL3,5 and ADS16 formed 64 and 36 nodules per plant, respectively.

‡ Nodule occupancy values for 88FL3,5 and ADS16 represents the means of four replicates, include both singly and co-occupied nodules and are presented as arcsine √% transformed values. Values in brackets are the actual percentage means. Analysis of variance was carried out on arcsine √% transformed data.

ƒ Range values in parentheses represent the lowest and the highest occupancy values of four replicates.

ff Mixed occupancy values represent the actual percentages of nodules co-occupied by both isolates.

¶ P Denotes the results of data analysis by a paired t test to determine if occupancy by one isolate was significantly different ($P < 0.05$) from the other. Comparisons were determined on arcsine √% transformed data.

N.S. Not significant.

Table 11. Influence of nodule occupancy by isolates 88FL3,5(AS6) and ADS16(AS27) of R. leguminosarum bv. trifolii on subclover yield parameters.

Ratio 88FL3,5:ADS16	g plant^{-1}	N conc.† mg g^{-1}	Kjeldahl N mg plant^{-1}
88FL3,5 alone	0.12	21.1	2.53
25:1	0.22	32.0	7.04
10:1	0.61	37.0	22.57
1:1	0.74	37.5	27.75
1:10	1.14	34.9	39.79
1:25	n.a.	40.3	n.a.
ADS16 alone	0.98	40.0	39.20
N-supplemented	0.88	40.2	35.38
LSD(0.05)	0.15		
CV(%)	20.49		

† N concentration values were determined on a composite sample from each treatment.

n.a. Not available.

DISCUSSION

The findings presented in this thesis have a bearing upon a number of unresolved aspects about dinitrogen fixation in subclover. Furthermore they are of significance to the phenomenon of competition for nodulation sites between rhizobial strains.

There are several reports in the literature that show field-grown subclover both in California (Holland, 1970; Jones et al., 1978), and in Oregon (Hagedorn, 1978, Demezas and Bottomley, 1984), can be nodulated primarily by rhizobia of limited symbiotic effectiveness. While growth responses to inoculation of seed with highly effective inoculant strains has been reported on several occasions, invariably these occurred under conditions where an effective soil rhizobial population was absent (Holland, 1970; Jones et al., 1978; McGuire et al., 1978; Hagedorn, 1979). Most situations that exist today are probably more similar to the one described in this work, i.e. the rhizobial population occupying nodules of subclover in improved pastures is of sub-optimum symbiotic effectiveness. Since the data presented in this thesis show that the majority of isolates that actually dominate field nodules (AS6 and AS6-21) are suboptimally effective at fixing dinitrogen, an opportunity now presents itself for me to determine directly at a specific site if such nodule occupants can limit the yield and /or N content of field-grown subclover.

The results of herbage analyses from this study also provide circumstantial evidence that some of the subclover growing in the western United States might

be N limited due to rhizobia of suboptimum effectiveness. The N content of subclover herbage grown in Oregon (Hagedorn, 1979; Drlica and Jackson, 1979; Almendras and Bottomley, 1987; Jackson T.L., unpublished data) and in California (Jones et al., 1982; Shock et al., 1983; Phillips et al., 1983; Boursier et al., 1989), can range from values as low as 2 to 3%, to as high as 4 to 4.5%. This range of values is similar to that obtained from the most mediocre to the most effective plant-isolate combination used in this study (Tables 4 and 6). However, it should be remembered that a low concentration of N in herbage of soil-grown subclover may be due to other reasons such as the stage of plant maturity at sampling time, or to other mineral nutrient limitations (Jackson et al., 1964; Dawson and Bhella, 1972; Drlica and Jackson, 1979).

The two regrowth experiments described in this thesis reveal the sensitivity of subclover regrowth to the harvest strategy utilized. Culvenor et al. (1989) found that the rate of shoot regrowth in subclover declined with increasing severity of defoliation. The results of the regrowth experiments in this thesis are similar. In the second experiment where the clipping was not as severe as the first one, recovery and regrowth was better. Since severe clipping had a greater negative effect upon the regrowth of plant-isolate combinations showing superior effectiveness, any plant-isolate combination considered to be of superior effectiveness should be evaluated over more than one harvest and under more than one clipping regime.

The findings about competition for nodulation can be discussed relative to

other published works on the subject. To my knowledge, these are the first competition studies in which rhizobial isolates known to represent either nodule-dominant or non-dominant serotypes indigenous to a particular site have been used.

Although 88FL3,5 was generally a superior competitor against most of the soil isolates when tested in 1:1 mixtures, the results with isolate FL2,48 showed that the nodulating success of 88FL3,5 could not be guaranteed regardless of population ratio. Similarly, 88FL3,5 could only nodulate successfully in the presence of ADS16 if it had the numerical advantage (10 to 25:1). Using simple mixture studies such as the ones described in this thesis, others have shown that nodulating success can be strongly influenced by the relative numbers of different strains in the inocula (Franco and Vincent, 1975; Amarger and Lobreau, 1982; Demezas and Bottomley, 1987; Beattie et al., 1989; Lochner et al., 1989; Fuhrmann and Wollum, 1989). The question that remains unanswered, however,is whether or not the densities of rhizobial subpopulations in a soil are significantly different from each other, and whether such differences contribute to nodulating success or failure. Schmidt and co-workers found that soil populations of B. japonicum serogroups were numerically equivalent and concluded that the nodulating success of serogroup 123 of B. japonicum on soybeans was not due to a numerical advantage (Schmidt and Robert, 1985; Bottomley, 1992). Unfortunately, no studies were done to compare the competitive abilities of members of serogroup 123 from that soil with those of minor nodule occupants.

The results presented in this thesis support the hypothesis that the nodulating dominance of field-grown subclover by members of serotype AS6 is probably the result of a combination of superior competitive and saprophytic traits. Since the soil population of the 88FL3,5 subpopulation is at least ten-fold greater than any other serotype in this soil (Bottomley and Dughri, 1989; Leung and Bottomley, unpublished observations), it is reasonable to believe that the 88FL3,5 subpopulation depends upon its numerical advantage to form a significant portion of the nodules in the presence of superior competitors such as members of serotype AS27. In other cases, a combination of superior competitive ability and numerical dominance probably combine to achieve the observed results. However, it is essential that further studies be carried out to determine if other members of the same clonal line as 88FL3,5 show similar competitive characteristics. From such activities it is anticipated that a standard isolate will be chosen as a model of the 88FL3,5 population for use in competition studies.

No simple explanation can be given to account for the fact that members of serotype AS27 are rarely found in nodules of field-grown plants (Leung and Bottomley, unpublished observations). Obviously, neither superior dinitrogen-fixing nor competitive abilities are adequate to guarantee nodulating success under field conditions. Either the soil population of these organisms is very small relative to 88FL3,5 types, or is not uniformly distributed throughout the field site. It is also possible that environment x genotype interactions play an important role in neutralizing the inherent competitiveness of AS27 members.

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APPENDIX

Table A.1. Population density of isolates of R. leguminosarum bv. trifolii in peat cultures after fourteen days of incubation at 28°C.

Isolate(serotype)	Cells(g^{-1} of peat) x 10^9
ADS14(AS27)	3.00
ADS16(AS27)	2.90
ADS13(AP17)	3.15
MS13(AP17)	2.85
88FL1,6(AS21)	1.95
ADS3(AS21)	1.55
88FL3,5(AS6)	0.74
AS6-1(AS6)	3.00
FL2,48(AG4)	1.50
ADS19(AG4)	1.00

Table A.2. Population densities of isolates of R. leguminosarum bv. trifolii in peat cultures after 5 months of storage at 4°C.

Isolate(serotype)	Cells (g^{-1} of peat) x 10^9
ADS14(AS27)	1.07
ADS16(AS27)	0.82
ADS13(AP17)	0.77
MS13(AP17)	0.85
88FL1,6(AS21)	0.66
ADS3(AS21)	1.60
88FL3,5(AS6)	0.63
AS6-1(AS6)	0.21
FL2,48(AG4)	0.79
ADS19(AG4)	0.42