AN ABSTRACT OF THE THESIS OF

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Title: ___ EVALUATION OF EFFECTIVENESS OF RHIZOBIUM TRIFOLII STRAINS ___

FOR TRIFOLIUM SUBTERRANEUM L. (SUBTERRANEAN CLOVER) IN SOUTHWESTERN ___

OREGON SOILS. _____

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Twelve high effective Rhizobium trifolii strains were evaluated
at four locations in southwest Oregon for the ability to effectively
nodulate Mount Barker subterranean clover. All twelve strains possessed
antibiotic resistance markers to facilitate identification of strains
when extracted from nodules. No significant differences in plant dry
weight means could be observed at two of the sites, while at the re-
maining two sites, plant dry weight means were significantly different.
Nodule occupancy means averaged over these two sites were higher than
those nodule occupancy means for the sites with no significant dif-
ferences in plant dry weights. High variability was demonstrated be-
tween percent nodule occupancy and mean dry plant weight, and some pos-
sible explanations for these results are offered, including a discussion
of the possible role of native strains. Total plant nitrogen values
were found to be significantly different for all sites and the lowest
nitrogen values were found in the site with the greatest plant dry
weights. No one strain of Rhizobium trifolii could be demonstrated to

perform significantly better than all other strains at all sites, and strains isolated in Oregon appeared to have no significant competitive or effective advantage over strains isolated outside of Oregon. The apparent enhancement of seed survivability by planting under the soil surface rather than broadcasting is mentioned.

EVALUATION OF EFFECTIVENESS OF <u>RHIZOBIUM</u> <u>TRIFOLII</u>

STRAINS FOR <u>TRIFOLIUM</u> <u>SUBTERRANEUM</u> L.

(SUBTERRANEAN CLOVER) IN SOUTHWESTERN OREGON SOILS

by

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1. INTRODUCTION

The family Leguminosae is a group of plants that has world-wide distribution. Members of this family have the ability to enter a sym-biotic relationship with bacteria of the genus <u>Rhizobium</u> in which the bacteria reside in nodules formed on the roots of the plant and are provided with nutrients by the plant. The bacteria, when furnished with translocated carbohydrates as an energy supply, fix atmospheric nitrogen which is in turn utilized by the plant. Excess nitrogen that cannot be utilized by the macrosymbiont is excreted into the soil.

The amount of nitrogen fixed in the association between legume and bacterium is variable due to characteristics of the microsymbiont, as well as environmental factors. A legume host can be infected by a <u>Rhizobium</u> strain, produce nodules, and yet no nitrogen is fixed. This can be attributed to the wrong <u>Rhizobium</u> species infecting the wrong host (i.e. cowpea rhizobia infecting soybeans) or to an ineffective (non-nitrogen fixing) strain of the proper species infecting its proper host. The latter situation appears to be a significant problem in im-proving pasture lands in Oregon using subterranean clover and <u>Rhizobium</u> <u>trifolii</u>. Indigenous strains of ineffective <u>R. trifolii</u> can frequently outcompete the rhizobia contained in commercial inoculants, especially if the number of viable cells in the commercial product is low initially. This results in the inability of the subclover stand to become established.

This study was conducted to evaluate selected R. trifolii strains for their ability to effectively nodulate subclover in the diverse soils of southwest Oregon. Any strains found to perform well could possibly be used in commercial inoculum production for this area.

2. LITERATURE REVIEW

2.1 Subterranean Clover

Trifolium subterraneum L., more commonly known as subterranean clover or subclover, is a winter-growing, self-regenerating annual legume. It originated in the Mediterranean region and in parts of western Europe, and through accidental contamination in ryegrass seed (Morley, 1961) or transfer with sheep and fodder (Quinlivan et al.) it was introduced into Australia. It was first identified in Australia in 1899 by a farmer, A.W. Howard, who perceived immediately the important role the legume could play in improving pasture lands. From the initial discovery of the plant until the fifties, subsequent increase in the use of subclover was slow, due to involvement in two world wars and economic recessions, as well as inadequate knowledge of legume nutrition. The fifties and sixties saw a phenomenal rise in the acreage devoted to subclover production. This forage legume is now a species of first order importance in pasture improvement and seed production in Australia and New Zealand.

Western Oregon, because of its Mediterranean-type climate, is another area that is well suited for subclover cultivation. First introduced in Oregon in 1922, it did not draw widespread consideration as a valuable forage species until the late thirties (Rampton, 1945). Since this time, the acreage committed to subclover production has been steadily increasing in western Oregon and northern California. At

the present time approximately 400,000 ha in Oregon are devoted to improved pasture or alfalfa and clover hay, with a potential for 800,000 more hectares to be brought into useful forage production (Hagedorn, in press).

When planting subclover in Oregon for forage or seed production, it is either broadcast or drilled. Drilling the seed to a depth of one inch enhances stand establishment and uniformity (Rampton, 1945). Stand establishment and production is also enhanced by proper inoculation with a good peat inoculum containing high numbers of viable rhizobia (Youngberg, 1972) and application of phosphorous, potassium, sulfur, and molybdenum as needed (Gardner et al., 1973, McGuire et al., 1978, Dawson et al., 1972). Jackson (1972) found liming to be beneficial at some sites where soils were acidic, but lime is expensive, and the more conservative practice of lime pelleting seed before planting seems to be satisfactory in terms of nodulation and stand establishment (McGuire et al., 1978, Roughley et al., 1973).

Subclover, when planted in Oregon in September or October, will germinate following the first precipitation sufficient to wet the seedbed and the seedlings are usually well established by the time the first frost arrives. The legume is slow growing during the winter months, then grows rapidly in April and May as the weather turns warmer. In June and July, as the dry, hot summer weather begins, the subclover flowers, forms seed heads, and then buries these in the soil. The plant then dies and next fall the cycle begins again. Proper management, including well planned grazing by sheep and cattle (Bedell, 1971), of subclover or subclover-grass stands will provide years of production

of highly nutritious forage or high quality seed. Stands approximately
30 years of age that are still highly productive can be found in Ore-
gon today (Hagedorn, personal comm.).

2.2 Effectiveness of Rhizobium

Effectiveness of rhizobia refers to the ability of the bacteria
to fix sufficient quantities of nitrogen to satisfy the needs of its
plant host. Little is known about the physiological and genetic basis
for effectiveness in Rhizobium species. Evaluations of effectiveness
usually involve the inoculation of the legume with the test species
and then waiting an appropriate length of time to see if effective
nodules develop. These are characterized by a pink color and the abil-
ity to reduce acetylene to ethylene, as well as an overall healthy
plant appearance. The degree of effectiveness can be determined by
dry weight evaluations, height measurements, and assays to estimate
the amount of nitrogen being fixed, but as yet the actual inherent
basis for differences in effectiveness among Rhizobium strains is
unknown.

Lie (1974) reviewed a number of environmental factors that contri-
bute to alterations in effectiveness. Nitrogen, oxygen and carbon
dioxide availability, the presence of ethylene, soil moisture status,
soil acidity, presence of combined nitrogen and the temperature of
the root environment, as well as other factors, all play a part in
determining the effectiveness of Rhizobium. He indicated that the
results of trials using Rhizobium strains subjected to the above condi-
tions could prove useful in selecting specialized strains for use in

certain environments. Hagedorn (1978), working in southwest Oregon soils, reported that mean site effectiveness for subclover of indigenous Rhizobium trifolii was positively correlated with the percent base saturation and total exchangeable bases. Negative correlations were obtained with effectiveness versus exchangeable acidity and nitrate nitrogen plus ammonium nitrogen. Holding et al. (1963) also reported a positive correlation between mean site effectiveness and percent base saturation in indigenous populations of R. trifolii in Scotland.

Effectiveness evaluation of newly isolated R. trifolii strains, or of strains originating in an area different from that where it is being considered for use as an inoculum strain, is an ongoing process. Such testing is important in discovering new strains that are highly effective under different environmental conditions. In greenhouse studies comparing R. trifolii strains isolated from self-established white clover in acid soils in New Zealand with proven effective strains such as TA1 and NZ6, Blair (1967) found that the proven strains were significantly more effective than the native strains at pH 6.8. One of the three native strains was more tolerant of low pH (pH 5.0-6.0) than the proven strains while still retaining its effectiveness.

Law and Strijdom (1975) tested 19 R. trifolii strains isolated in South Africa for their effectiveness on two indigenous clover species, Trifolium africanum and T. burchellianum. Nodulation frequency by all strains was high (none less than 80 percent), but effectiveness measurements varied greatly. Effectiveness appeared to have no relation to the type of clover the strains were isolated from, or to the region of Africa from which the rhizobia were originally isolated.

Six of the strains were found to be highly effective and comparable to strains previously recommended for clover inoculation.

Field study results reported by Chatel et al. (1973), using three Trifolium species and recent isolates of R. trifolii from healthy plants growing in poor pastures, suggested that these isolates were more effective than those found in commercial inoculants. Plots were established at three sites representing soil types in which pasture failures were commonplace. These plots were inoculated and planted using the new strains (WU112 and WU290) and the commercial strains (TA1, UNZ29 and WA7). The new strains outperformed the old proven strains in terms of establishment, persistence and effectiveness. This agrees with work done by Hagedorn (1979, in press), who also found that, in certain soils, newly isolated strains from Oregon could outperform the proven Australian commercian strain TA1.

2.3 Competition Among Rhizobium Species and Strains

In the search for highly effective Rhizobium strains, it must be remembered that those strains which are most effective are not always those which are most competitive although effectiveness and competitiveness do seem to coincide in a majority of cases. An instance in which these two properties did not coincide was reported by Johnston and Beringer (1976). While working with strains of R. leguminosarum they found that when two strains were introduced onto the same pea plant, an ineffective strain (924) could outcompete an effective strain (846) and eighty percent of the nodules formed were found to contain the ineffective strain.

In contrast to the Johnston and Beringer study, Robinson (1969) reported that effective strains of R. trifolii occupied the majority of nodules in mixed inoculations with a number of ineffective strains. He hypothesized that the host plant can distinguish the strains early in the infection process. Brockwell and Katznelson (1976) also found that some type of host legume specificity was in operation to allow the plant to exercise a preferential selection of effective over ineffective strains.

In a laboratory study by Pinto et al. (1974), it was found that competitiveness did not correlate with the relative speed with which the strains produced nodules. R. meliloti strain SU51 was found to be superior in terms of competition over strains SU126 and SU27, both of which were equally effective to the first strain. R. trifolii strain TA1 was superior to SU254 on three varieties of clover. Mixed infections occurred, but were usually less than 10 percent of the nodules examined.

Ireland and Vincent (1968) investigated the competition of R. trifolii strains TA1 and UNZ29 with native populations found in red basaltic soils in New South Wales. On Yarloop and Bacchus Marsh subclover varieties they found that the inoculum strains, and especially strain TA1, were highly successful in outcompeting the native ineffective rhizobia. In direct contrast to this report is a study by Holland (1970). He investigated the reason why subterranean, rose and crimson clovers inoculated with commercial inocula failed to establish a stand on some northern California range soils. He found a highly ineffective native population of R. trifolii that could outcompete the inoculum

strains. This problem was overcome by applying four times the recommended rate of the commercial inoculum. He did not establish, however, the optimum numbers of rhizobia per seed required for successful stand establishment.

A very thorough field study was executed by Roughley et al. (1976). They tested the ability of four R. trifolii strains to compete with indigenous Rhizobium populations at five sites with pH values ranging from 5.4 to 6.2. Woogenellup and Mount Barker were the subclover test varieties used. The R. trifolii populations in these soils ranged from 0 to 4.20 x 10^6 cells per gram of soil. At the site with no naturalized rhizobia, all nodules were formed by introduced rhizobia. At the site with 4.20 x 10^6 cells per gram approximately 1/3 of the nodules formed were due to the inoculum strains and the rest of the sites fell between these two extremes. Success of any particular strain of bacteria, whether inoculated singly or in mixed culture, was not consistent from site to site and it appeared that soil conditions, clover variety and influence on native strains all affected which strain could compete best with the natives. In addition, one site was monitored at 8, 10, 14, and 22 weeks to determine if the percentage of nodules from which the inoculum strains could be isolated changed with time. It was found that no significant changes occurred in the distribution of strains. Also, inoculant strains were isolated at a higher frequency from Mount Barker subclover than from Woogenellup subclover (host mean 58 percent vs. 39 percent, respectively). This trend was found to be consistent with three of the four strains.

Gibson et al. (1976) performed a study using Woogenellup subclover and five strains of R. trifolii found to be effective for this cultivar. Two soils with high populations of indigenous rhizobia were chosen for test site locations and seed was incoulated with a mixture of all five strains. Strain WU95 was found to be most competitive against both the natives and the other inoculum strains, occupying 63 percent of the nodules (strains were identified serologically). Native rhizobia occupied only 11 percent of the nodules tested and the results were strikingly similar for both sites. Single strain inoculation was also performed and good results were obtained with 69 to 100 percent of the nodules examined containing the inoculum strains.

2.4 Use of Antibiotic Resistance in Studying the Ecology of Rhizobium

When undertaking studies of Rhizobium ecology, several options for identifying the rhizobia are open to the researcher. Techniques such as immunodiffusion and immunofluorescence are suitable, but both are time consuming and costly. The use of antibiotic resistant mutants still appears to be the most popular due to its swiftness, low cost, and reliability. However, not all antibiotics or rhizobia are suitable for use, as will be discussed.

Schwinghamer (1964) was one of the first to develop antibiotic resistant mutants of Rhizobium for use in ecological studies. He developed mutants of effective strains of R. leguminosarum, R. meliloti and R. trifolii in a stepwise fashion (increasing concentration of antibiotic as organism adjusted) using a gradient plate technique described by Szybalski and Bryson (1952). In this technique, the antibiotic containing medium is poured into a petri plate, which is then tilted

such that the agar hardens at a slant. An overlay of plain agar medium is then added, resulting in a plate that has a much higher concentration of antibiotics at one end.

Once mutants were obtained they were tested for effectiveness in greenhouse trials. It was found that all three species, when resistant to streptomycin, kanamycin or polymyxin, retained their effectiveness in all but very few instances. In contrast, twenty-nine of thirty-three viomycin mutant clones were ineffective, and fifteen of sixteen neomycin mutants were ineffective. This agrees with work by Hendry and Jordan (1969), who also found that R. meliloti strains labeled with viomycin-resistance became uniformly ineffective.

Schwinghamer (1967) extended this work to include a much wider range of antibiotics. On the basis of this study, he placed the antibiotics into three groups; Group I being those which alter effectiveness little or not at all, Group II altering effectiveness moderately (1/2 of mutants showing partial or complete loss of effectiveness), and Group III showing complete loss of effectiveness in 3/4 of the mutants. These groupings are suspect, however, since they are not based on consideration of the mode of action of the antibiotics. Also, some of the results reported by Schwinghamer (1964, 1967) conflict with a later report (Schwinghamer, 1973) in that the earlier papers mention little loss of effectiveness associated with conferred streptomycin resistance, while in his later paper he reports a loss of effectiveness in at least twenty percent of the streptomycin resistant cultures. Schwinghamer also reported loss of effectiveness in R. trifolii at very low levels of penicillin-resistance (4.4 ug/ml average), while

Abdel-Wahab et al. (1976) reported no appreciable loss of effectiveness in R. trifolii mutants resistant to 400-1000 I.U./ml (240-600 ug/ml).

Pankhurst (1977), working with Lotus rhizobia, reported that antibiotics inhibiting protein synthesis, such as streptomycin and chloramphenicol, produced little loss of effectiveness, while antibiotics inhibiting nucleic acid synthesis (nalidixic acid and rifampicin) and cell wall synthesis (novobiocin and penicillin) caused significant loss of effectiveness in 20-100 percent of strains. He also found a more pronounced loss of effectiveness in mutants of fast-growing strains than in slow-growing strains. He suggested that resistance to some antibiotics may inhibit the development of bacteria into bacteroids, thus possibly explaining the reason for loss of nitrogen fixing ability. This same hypothesis was offered by Hendry and Jordan (1969). Schwinghamer (1967) suggested that, in the case of neomycin, viomycin and D-cycloserine, perhaps an alteration in the cell wall is responsible for the induced ineffectiveness.

Some workers have used resistant strains to study competition with indigenous populations of soil rhizobia. Obaton (1971) developed stepwise mutants of R. meliloti by transferring cultures to media containing progressively higher concentrations of streptomycin and kanamycin. Two mutants were obtained, both resistant to 740 ug/ml streptomycin and one with an additional marker of resistance to 79 ug/ml kanamycin. With these markers, the bacteria could easily be reisolated from nodules. Experiments using a Medicago sp. and the mutants showed that, in three years of growth in non-sterile soil, the number of nodules inhabited by the mutant strains decreased only eight percent, from

100 percent to 92 percent. Obaton concluded that no reverse mutations occurred, and that this method for studying Rhizobium ecology was fast and reliable. Like Obaton, Schwinghamer (1973) also suggests that the use of a double marker on strains is advisable to avoid the need for more difficult and expensive means of identification of reisolated srains.

Brockwell et al. (1977) used strains of R. trifolii resistant to 120 ug/ml streptomycin and antigenically marked to compare these two characteristics as well as the competitiveness of these strains against native Rhizobium populations in soil. These marked strains were fully effective. Seed of two subclover varieties was inoculated and sown, and plants were harvested and evaluated once a year for four successive years. Strains were found to compete well with the natives in the first year, but gradually declined (from 100 percent nodule occupancy by one strain to 40 percent) over the four-year period. This phenomenon has been reported frequently in the Australian literature. However, the comparison between the streptomycin markers and the anti-genic markers showed good agreement between these two methods, and little evidence for spontaneous loss or transfer of either type of marker once in the environment.

Other investigators (Danso et al., 1973 and Danso and Alexander, 1974) used mutants to study the viability of rhizobia in soil when not associated with a legume. They developed stepwise mutants of R. trifolii and R. meliloti resistant to 1000 µg/ml streptomycin. When introduced into non-sterile soil, these mutants could easily be re-isolated using a nutrient medium containing 1000 µg/ml streptomycin

and 150 μg/ml actidione (to inhibit overgrowth of plate by fungi).

The strain numbers of rhizobia were found to decline slowly, with increased soil temperature and dryness speeding this decline. They reported that the use of antibiotic resistant mutants was a fast, reliable and inexpensive means of following the fate of rhizobia in soils. Imshentskii et al. (1970, 1976), also studying the fate of Rhizobium when introduced into soil, agrees that the antibiotic resistance method is highly suitable for use in studying Rhizobium ecology. His explanation of an initial large drop in mutant Rhizobium numbers when introduced into soil being due to grazing protozoans agrees with the results of Danso et al. (1975).

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3. EVALUATION OF EFFECTIVENESS OF <u>RHIZOBIUM</u> <u>TRIFOLII</u> STRAINS FOR <u>TRIFOLIUM</u> <u>SUBTERRANEUM</u> L. (SUBTERRANEAN CLOVER) IN SOUTHWESTERN OREGON SOILS

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3.1 ABSTRACT

Twelve highly effective Rhizobium trifolii strains were evaluated at four locations in southwest Oregon for the ability to effectively nodulate Mount Barker subterranean clover. All twelve strains possessed antibiotic resistance markers to facilitate identification of strains when extracted from nodules. No significant differences in plant dry weight means could be observed at two of the sites, while at the remaining two sites, plant dry weight means were significantly different. Nodule occupancy means averaged over these two sites were higher than those nodule occupancy means for the sites with no significant differences in plant dry weights. High variability was demonstrated between percent nodule occupancy and mean plant dry weight, and some possible explanations for these results are offered, including a discussion of the possible role of native Rhizobium strains. Total plant nitrogen values were found to be significantly different for all sites and the lowest nitrogen values were found in the sites with the greatest plant dry weights. No one strain of R. trifolii could be demonstrated to perform significantly better than all other strains at all sites, and strains isolated in Oregon appeared to have no significant competitive or effective advantage over strains isolated outside of Oregon. The apparent enhancement of seed survival by planting under the soil surface rather than broadcasting is mentioned.

3.2 INTRODUCTION

In southwest Oregon there are vast tracts of hill lands that have been converted to pastures. The soils in these areas are generally acidic and of marginal fertility, and the growth of native grasses is poor. Presently, a program is under way to improve these pastures through the cultivation of subterranean clover. These efforts, however, have met with mixed success. In some cases, subclover stands have been established with relative ease, and are productive for forage year after year. At other locations, the clover has not established at all, or it has produced stands of widely varying quality (Hagedorn, 1978 and 1979). This can be due in part to soil conditions such as temperature, moisture status and percent base saturation, or it can be attributed to a problem with the <u>Rhizobium</u> commercial innoculum. Low numbers of viable cells, or strains of rhizobia unable to compete with indigenous soil populations could contribute to failure of stand establishment.

The study was conducted to evaluate selected <u>R. trifolii</u> strains for their ability to effectively nodulate subclover in the diverse soils of southwest Oregon.

3.3 MATERIALS AND METHODS

Experimental plots were established at four sites (Figure 1) and were designated as follows: site 1 - McKenzie, site 2 - Geaney, site 3 - Carman, and site 4 -Metz Hill. All sites were located in hilly pasture lands except site 4, which was located adjacent to a wheat field in an interior valley. Ground slopes at sites 1, 3, and 4 were less than 3 percent, and site 2 had a 15 percent slope. Soil characteristics (Table 1) were determined from samples obtained at each site prior to sowing and analyzed by the Oregon State University Soil Testing Laboratory as outlined in a laboratory circular by Berg and Gardner, 1978. Each site was subsequently fertilized as recommended by the Oregon State University Fertilizer Guide for subclover with 7.36 kg of P per hectare as concentrated superphosphate (45% P_2O_5), 18.4 kg of K per hectare as potash (60% K_2O_5), 3.6 kg S per hectare as gypsum, and Mo as molybdenum sulfate at a rate of 0.28 kg/ha.

Duplicate plots 1.8 m by 2.4 m were established for each strain treatment, with 0.9 m buffer zones between duplicates and neighboring plots. The design was not randomized so that cross-contamination by adjacent treatments could be kept at a minimum. Mount Barker subclover was planted in 6 rows with 30 cm between rows at a seeding rate of 11.0 kg/ha. Planting was done with disposable plastic gloves to keep strain treatments separate. Controls consisting of fertilizer plus 5.12 kg of N per hectare as urea and fertilizer plus no nitrogen were also established. Planting was done in early October, 1978.

Plant tube assays (Vincent, 1970) were performed prior to sowing on soil from each site. Numbers of rhizobia per gram are reported in Table 1. A higher proportion of ineffective to effective strains was isolated from the assay at each site, except at site 3, where the opposite was true.

Twelve strains of R. trifolii previously found to be effective on subclover in greenhouse trials were chosen for use as treatments. Four strains were provided by Dr. J.C. Burton of Nitragin Company, and the other eight strains were isolated from soils in various locations in western Oregon. Through the use of a stepwise gradient plate technique (Szybalski and Bryson, 1952), strains were labeled with either single or double resistance markers with antibiotics at 100 ug/ml. This conferred resistance facilitates identification of the strains upon subsequent extraction from the nodules. Strains were retested using the plant tube assay to insure that the antibiotic-induced alterations had not modified the effectiveness of the strain. Strain designations are as follows, with the first number in each set representing the strain number reported in this paper. Antibiotics are abbreviated str for streptomycin, cap for chloramphenicol, neo for neomycin, ery for erythromycin, and nov for novobiocin: 1 - 16268 str and neo, 2 - 16278 ery, 3 - 16247 ery and nov, 4 - 16293 ery and cap, 5 - J-6 ery and cap, 6 - C-6 str and ery, 7 - J-8 nov, 8 - C-1 str, 9 - D-1 str, 10 - D-6 cap, 11 - J-14-2 ery and cap, and 12 - D2ASO ery and str. Strains 1-4 were the commercial strains and 5-12 were the native Oregon strains. Each strain was grown separately in yeast mannitol broth (YM) and then mixed with peat sterilized by gamma irradiation (Vincent,

1970). The mixture was allowed to mature at 25 C for two weeks. The
inoculum was then applied to the seed using gum arabic as an adhesive,
and lime pelleted according to methods of Vincent, 1970. Due to an
error in laboratory peat production (pH of peat too low), a three week
dry, hot period following planting, and then the onset of a record
cold winter, it was necessary to reinoculate the plots to insure the
presence of sufficient numbers of rhizobia. Large broth cultures were
produced for each strain, and contained at least 5.0×10^8 rhizobia/ml.
These were taken to the field sites, diluted one part culture to four
parts YM salts, and one liter of suspension (containing at least 1.25
$\times 10^8$ cells/ml) was distributed over each appropriate plot. This proce-
dure is routinely employed in Australia when the area to be inoculated
is too small to justify the production of commercial peat inoculum
(Roughley, 1970).

Samplings of the plants were done at 20, 27, and 32 weeks from
the time of planting. Ten groups of approximately two or more plants
per group were removed from each plot. Plants were excised 0.5 cm
below the lower leaf junction, washed, dried at 60 C for 72 hours,
and weighed in groups of two (total of 20 plants per plot sampled).
The 20 week sampling (third week of February) was performed soon after
the unusually cold weather (Figure 2) had ended. Weather conditions
were still cool, and rainfall in February had been up to 82 percent
greater than normal. The plants were sparsely nodulated and still stunted
from the severe cold period. Under these conditions, any differences
in plant dry weight observed were not due to the action of the inoculum
strains, and therefore the first sampling was not considered in the results.

At the 27 week sampling, nodule occupancy tests were done in addition to the dry weight analyses. Three nodules per plant were removed, two from the crown region and one from the distal regions of the roots. The nodules were surface-sterilized with 0.2 percent acidified $HgCl_2$, crushed and spotted on plates of YM agar containing the appropriate antibiotics. A control plate of plain YM was also used. Plates were incubated for 5 days at 25 C before observation.

At the 32 week sampling, total plant nitrogen analyses were done in addition to the dry weights. The weighed plants from each plot (all plants in one plot bulked together for the anlaysis) were analyzed at the Oregon State University Soil Testing Laboratory using the macro-Kjeldahl method.

At site 3, the plants in some plots were receiving some competition from thistles, but could still be found around the perimeters of the thistle region. At the third sampling, this site was covered by a confluent growth of subclover that even grew in areas that had not been seeded. The rancher was questioned, and it was found that subclover had been planted in the area seven years earlier, and that the stand had never established itself. On a much smaller scale, this phenomenon was observed at other sites in that several large clover plants (usually white or subclover) could be found growing within each plot even when the experimental clover was still very small. It appears that the growth of large quantities of subclover may greatly stimulate the growth of clovers already present in small quantities, perhaps through the transfer of growth factors in root exudates, or through the stimulation of the effective portion of the indigenous Rhizobium

population (Brockwell and Katznelson, 1976). The experimental plots were still distinguishable, and were sampled normally. The second site was grazed by elk prior to the 32 week sampling, and sampling was more selective in that plants that had not been grazed were chosen preferentially over those that had been. At times it was difficult to determine if the plant had been grazed, and this is doubtless a source of error in the later analysis of the plot.

The data were subjected to an analysis of variance within each sampling. If significance due to the F test was found, the data were then subjected to single degree of freedom contrasts using Duncan's Multiple Range Test. Correlation coefficients were used as measures of association among variables.

3.4 RESULTS

Subterrenean clover established well at all sites. Plants were
dark green and healthy. Significant differences in dry weights due
to site were observed in the 27 week sampling at sites 3 and 4 (Table
2), site 3 having the largest significant site mean dry weight. No
differences in mean plant dry weight could be shown between sites 1
and 2. Differences due to strain treatments within a site were also
found in the 27 week sampling at sites 3 and 4, but not at sites 1
and 2, as shown in Table 3. These differences will be discussed in
conjunction with nodule occupancy results, as it would be inappropriate
to discuss the merits of any one strain over another without knowing
if it actually inhabited a large proportion of the plant nodules.

Nodule occupancy results for the 27 week sampling (Table 6) varied
from treatment to treatment and from site to site, although some strains,
such as 1 and 12 showed a definite pattern of low percent occupancy
for all sites. Occupancy data for sites 1 and 2 have been included,
even though there were no significant differences in dry weights between
treatments at these sites. When means for total percent occupancy for
sites 1 and 2 were compared to sites 3 and 4, the means for sites 1
and 2 were, at a minimum, 12 percent lower than those for sites 3 and
4. The same trends were observed when the treatment means for percent
nodule occupancy at sites 3 and 4 were averaged. Seven out of the
twelve means were larger than the average of the occupancy means across
all sites, with five out of twelve means 17 percent to 30 percent greater
than the overall averages. This indicates that a greater proportion

of known effective nodules were located on plants in sites 3 and 4,

again the sites with significantly greater plant dry weight averages.

However, comparing individual percent nodule occupancy values to individual

mean plant dry weights for strain treatments to try and elucidate those

strains which were superior in both respects was more difficult than

the above comparison. For example, at site 3, strains 4, 7, and 1

had the highest plant dry weights, respectively, and were not signifi-

cantly different from each other. The percent nodule occupancy scores

for these were 98.3 percent, 86.5 percent and 10.8 percent respectively.

So, even though strain 1 had a plant dry weight comparable to the others,

it had a nodule occupancy that was at least 75.7 percent less than

the other two means. Incidences of low percent nodule occupancy and

high mean plant dry weight and the converse were seen repeatedly

throughout the data for the 27 week sampling period, as is shown by

the lack of correlation between dry weight and percent nodule occupancy

(r = .2153). One exception, strain number 3 at site 4, had a mean

plant dry weight significantly different from all others at that site,

and it also had a mean percent nodule occupancy of 100 percent. Treat-

ment means across sites showed some significant differences (Table

4) between strains, but again the categories for significance overlapped

to such an extent that there is little value in attempting to designate

any of the strains superior to the others in terms of both high nodule

occupancy and high mean dry weight at all sites. It can also be ob-

served from Table 3 that attempts to compare mean plant dry weights

for the individual strains against the fertilizer no N and fertilizer

plus N controls were largely futile, possibly due to the profuse

nodulation of the controls by effective native strains. Dry weights for both control treatments were typically located in the center of the distribution, and were not significantly different from any but the very highest, and sometimes lowest, means.

The 32 week sampling was the most important sampling period. The plants were reaching maturity and setting seed. Although no nodule occupancy testing was done, the nodule occupancy results of the 27 week sampling were utilized following precedents set by other investigators (Hagedorn, 1979, Roughly et al., 1976), which show that the percent nodule occupancy by a strain does not fluctuate significantly over the same season. As with the 27 week sampling, significant differences in plant dry weights due to sites were observed only at sites 3 and 4 (Table 2), with site 4 possessing the largest significant difference in dry weight. Differences due to strain treatments within a site were also observed at sites 3 and 4 (Table 5).

Reflecting the results of 27 weeks, the 32 week comparison of strain treatment plant dry weight means and nodule occupancy was variable. Plant dry weight means showing no significant differences had variable nodule occupancy results (dry weight versus nodule occupancy correlation coefficient r = .0882). No individual strain of R. trifolii could be chosen as outstanding in terms of dry weight at the 27 week sampling also were among those that had significantly higher dry weights at the 32 week sampling period. Strains 3 and 7 are examples. However, strains in common at both the 27 and 32 week samplings yielding high plant dry weights and low percent nodule occupancy were also observed (strains 1 and 6, for example). Treatment means across sites at this

sampling did not show any significant differences between dry weights for strain treatments as were seen at the 27 week sampling. In the 32 week sampling, as in the 27 week sampling, comparison of strain treatments to the control treatments was largely unsatisfactory due to the overlap of significance intervals, probably resulting from heavy nodulation of the controls by effective native rhizobia.

Percent total nitrogen values for the 32 week sampling were determined to be significantly different from site to site (Table 7) but differences among strain treatments within each site proved insignificant. Sites with the highest mean plant dry weights were lowest in total nitrogen. This observation is supported by the dry weight versus total nitrogen correlation coefficient or $r = -.6253$.

3.5 DISCUSSION

The observation that the experimental plots of subterranean clo-
ver became well established was particularly important at the Geaney
site (site 2). The rancher had broadcast subclover seed on his estab-
lished pastures and in the cultivated areas adjacent to the experimental
plots. A total lack of establishment was observed in his newly seeded
areas. This result is best attributed to the severely cold weather
in Oregon during the winter of 1978-79 (mean January temperature was
the coldest on record). The experimental plots survived due to the
planting of the seed one inch below the soil surface, and this has
been shown to enhance seed survival and stand establishment (Morley,
1961 and Rampton, 1945). The seedlings were able to survive night
time temperatures as low as -13 C.

The consistently higher values for nodule occupancy percentages
and plant dry weights at sites 3 and 4 as compared to sites 1 and 2
are not easily explained in terms of 1 or 2 factors, but rather involve
a series of factors including soil properties, native Rhizobium popula-
tions, and characteristics of the inoculum strains. Sies 3 and 4 had
very low indigenous R. trifolii populations, and thus less competition
for the inoculum strains. However, the proportion of native effective
strains was greater than the ineffective strains at site 3. These
two sites also had the highest values for percent base saturation.
High base saturation is conducive to good plant growth, and Hagedorn
(1978) showed that, for southwest Oregon, the base saturation was signi-
ficantly correlated with the effectiveness of the indigenous Rhizobium

populations. This would increase the chances of an effective Rhizobium strain from the soil to nodulate plants in a site where the inoculum strain could not. There are several possible explanations for the inconsistencies evident at 27 and 32 weeks for many treatments at sites 3 and 4 where plant dry weight was not proportional to percent nodule occupancy by the inoculum strain:

1. The inoculum strain lost its antibiotic resistance, and is really present in the nodules even though it can no longer be identified.

2. Perhaps the few nodules of the strain are so effective they can take care of all the plant's nitrogen needs, regardless of whether the rest of the nodules present are ineffective.

3. Although the strains were infective and effective in the plant tube assay after mutation with the antibiotics, perhaps other parts of the cells' physiology were affected making them more susceptible to stress in the field.

4. Perhaps strains of highly effective indigenous rhizobia that were not detected with the initial soil dilution were stimulated and nodulated the plants, outcompeting the inoculum strains.

The first alternative is not very likely based on the work of Brockwell et al., 1976, among others. The resistance characteristic has been shown to be very stable if the strains are carefully selected and mutated. The second alternative is probably not significant, as very large nodules were encountered infrequently at the 27 week sampling when the assay for nodule occupancy was done. Each test plant had at

least 6-10 effective pink nodules, so it is not likely that only 1 or 2 were doing all of the fixation. The third alternative is a reasonable possibility. The strains were tested in the greenhouse after mutation, and there is a good possibility that other aspects of the cell, such as stress resistance, could have been effected, as well as any inherent stress intolerance characteristics present in the cell before mutation. The fourth alternative is also a very likely one. Gibson et al., 1976, found this to be true in field experiments performed in Australia. If the inoculum strains are stressed, the natives, adapted to the specific soil conditions, could invade the clover roots and form effective nodules. This could also explain the abundant effective nodulation of the fertilizer no N and fertilizer plus N controls at all sites, and why there were no significant differences between the controls and the majority of strain treatments.

The reversed site order for the nitrogen values (sites with largest plants having least nitrogen) is readily explained by the following information. As a subclover plant matures, the nitrogen content decreases. Some of this nitrogen is translocated from the leaves to the developing seed heads. When sampling, some of these seed heads are lost because they are buried in the soil and break off when the plant is removed. Nitrogen is lost in root exudates, also (Morley, 1961). Additionally, the weather was warmer in the spring at the fourth site than at the other three sites, and the plants matured faster there, also contributing to the overall low mean nitrogen percentage of the plants at this site.

Due to some of the reasons already discussed, trying to select the single strain that performed better than the rest is not possible.

The best evaluation that can be made is of groups of strains that performed well in terms of both high nodule occupancy and high dry weight. Strains 3 and 7, a Nitragin strain and an Oregon strain, respectively, were best in this respect. However, a larger group of strains having low treatment strain nodule occupancy and high dry weights could also be demonstrated. Strains 1 (Nitragin) and 6, 9, and 12 (Oregon isolates) are examples of this. In this study, the strains isolated in Oregon soils did not appear to have any advantage over the Nitragin strains in terms of competitiveness or effectiveness. However, it must be remembered that these were not purchased inoculum preparations that were applied, and therefore no comparisons between the commercially prepared inoculum and these strains can be made, except to say that under the conditions of the experiment these strains performed as well as the Oregon strains.

Studies of longer duration will be essential to determine whether or not these R. trifolii strains can survive the hot, dry summer weather conditions in sufficient numbers to effectively nodulate the subclover when it germinates again in the fall. Hopefully, the results presented in this paper will stimulate further interest not only in the microbial aspects of subclover nodulation, but also in the importance of an optimum fertilizer program (as employed here) coupled with a somewhat higher seeding rate than is normally used and the drilling of seed in the successful establishment of subclover.

ACKNOWLEDGEMENT

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LEGEND

FIGURE 1. Map of Oregon showing location of experimental sites in
the southwest portion of the state.

FIGURE 2. Weather data for the McKenzie site (site 1) showing the
difference between the 1978-79 winter season (solid
circles) and a thirty year average (open circles, 1 -
January, 2 - February, 3 - March, etc.).
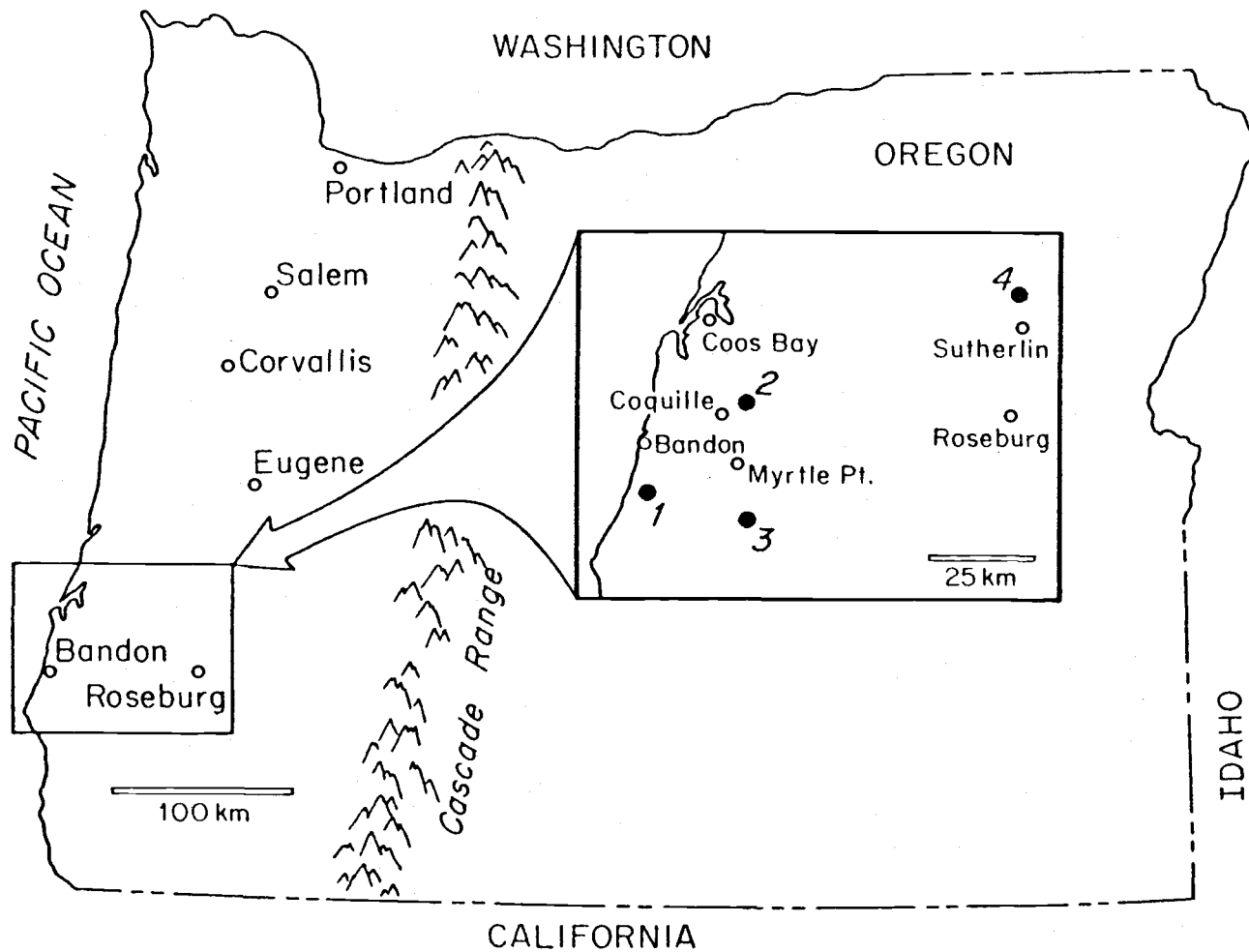
FIGURE 1.
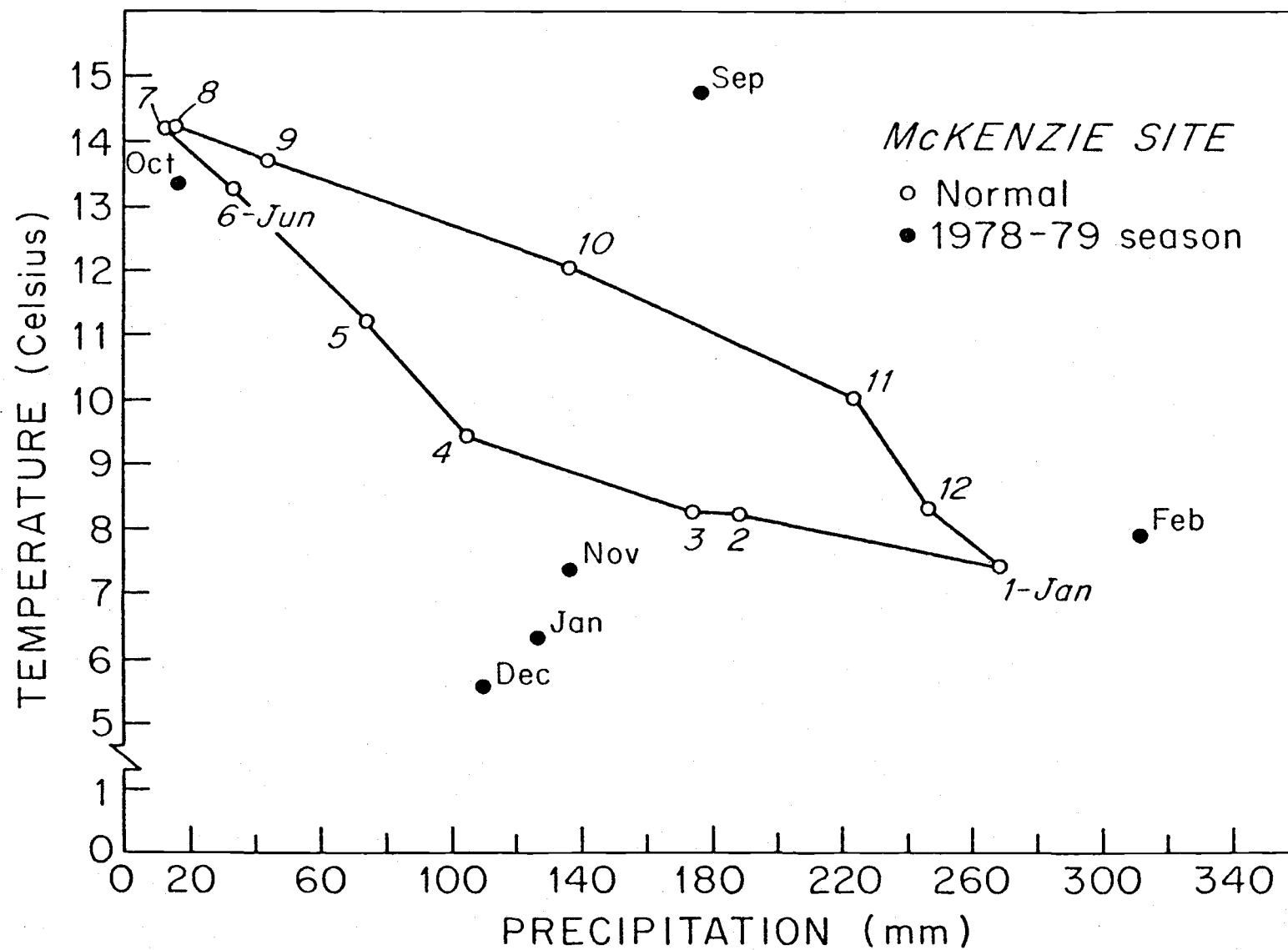
FIGURE 2.

Table 1. Soil characteristics of experimental sites at sowing.

Site No.	Site Name	Rhizobium trifolii (cells/g)	Soil classification	pH	P	K	Ca	Mg	Na	CEC	% B.S.	Total N (%)	NH_4-N plus NO_3-N (µg/g)
					ppm			meq/100 g					
1	McKenzie	170	clayey, mixed, mesic Typic Haplohumults*	5.1	24	208	4.5	.99	.39	27.62[+]	23.21	.49	9.5
2	Geaney	580	fine, mixed, mesic Ultic Dystrochrepts	5.5	9	368	8.8	6.9	.28	34.16	49.53	.25	13.6
3	Carman	170	fine, mixed mesic Typic Dystrochrepts	5.6	53	604	13.5	4.4	.17	25.18	77.92	.28	12.3
4	Metz Hill	2	fine, mixed, mesic Ultic Haploxeralfs	5.3	19	298	6.2	2.6	.64	19.40	52.58	.26	14.6

*All soils in table developed from sedimentary parent material.

[+]CEC determined at pH 7.0 using ammonium acetate method.

Table 2. Comparison of plant dry weight means (per 2 plants) among sites

Sampling 2 (27 weeks)

Site	Site mean
3	.36*
4	.30
2	.22a
1	.20a

Sampling 3 (32 weeks)

Site	Site mean
4	3.72*
3	2.15
2	1.12a
1	1.05a

*values within a column not followed by letters in common are significantly different at th 5% level of probability.

Table 3. Plant mean dry weights (per 2 plants) and percent nodule occupancy for strain treatments within sites for sampling 2 (27 weeks).

Site 1		Site 2		Site 3			Site 4		
Strain	Mean, in g	Strain	Mean, in g	Strain	Mean, in g	%Nod. occ.	Strain	Mean, in g	% Nod. occ.
Fert plus N	.29a*	3	.26a	4	.56a	98.3	3	.60	100.0
5	.26a	11	.26a	7	.51ab	87.5	5	.41a	54.2
4	.25a	4	.26a	1	.45abc	10.8	6	.40ab	5.8
1	.23a	7	.25a	11	.39bcd	20.8	8	.35abc	65.0
2	.22a	Fert plus N	.24a	3	.38bcde	90.8	9	.33abcd	7.5
3	.21a	9	.24a	9	.38bcdef	12.5	1	.32abcd	4.2
6	.21a	10	.23a	12	.35bcdefg	4.4	7	.28abcd	96.7
7	.19a	12	.23a	2	.35bcdefgh	100.0	Fert plus N	.25abcd	---
Fert No N	.17a	6	.21a	10	.35bcdefghi	42.9	10	.25abcd	99.2
12	.16a	2	.20a	Fert plus N	.33cdefghij	---	Fert no N	.25abcd	---
8	.16a	1	.19a	8	.32cdefghij	71.7	4	.23cd	37.5
9	.16a	8	.18a	Fert no N	.24defghij	---	2	.23cd	92.5
10	.15a	5	.17a	6	.20ghij	.93	12	.21cd	5.0
11	.14a	Fert no N	.16a	5	.18j	90.0	11	.17d	48.3

*Values within a column not followed by letters in common are significantly different at the 5% level of probability.

Table 4. Plant dry weight means (per 2 plants) for strain treatments
across sites for sampling 2 (27 weeks).

Strain	Mean, in g.
3	.36a*
4	.32ab
7	.31abc
1	.30abcd
Fert plus N	.28bcde
9	.28bcde
6	.25bcde
5	.25bcde
8	.25bcde
2	.25bcde
10	.25bcde
11	.24bcde
12	.23cde
Fert no N	.20e

*values within a column not followed by letters in common are signifi-
cantly different at the 5% level of probability.

Table 5. Plant mean dry weights (per 2 plants) and percent nodule occupancy for strain treatments within sites for sampling 3 (32 weeks).

Site 1		Site 2		Site 3			Site 4		
Strain	Mean, in g	Strain	Mean, in g	Strain	Mean, in g	% Nod. occ.	Strain	Mean, in g	% Nod. occ.
2	1.61a*	11	1.69a	7	3.69a	86.5	5	6.30a	54.2
1	1.45a	6	1.64a	10	3.05ab	42.9	1	5.43ab	4.2
11	1.17a	1	1.49a	3	2.91abc	90.8	9	5.28abc	7.5
4	1.16a	Fert plus N	1.35a	12	2.81abcd	4.4	3	5.23abcd	100.0
5	1.06a	10	1.29a	8	2.64abcde	71.7	6	4.58bcde	5.8
Fert plus N	1.05a	Fert no N	1.10a	4	2.04bcdef	98.3	Fert plus N	3.76cdef	---
9	1.03a	8	1.05a	6	1.96bcdef	.93	10	3.35fg	99.2
8	.98a	7	1.04a	11	1.94bcdef	20.8	8	3.28fgh	65.0
Fert no N	.95a	2	.98a	5	1.93bcdef	90.0	Fert no N	3.16fghi	---
7	.91a	12	.98a	9	1.86bcdef	12.5	2	2.97fghij	92.5
6	.90a	3	.90a	2	1.43ef	100.0	7	2.55fghijk	96.7
12	.88a	9	.81a	Fert plus N	1.31f	---	4	2.49ghijk	37.5
3	.81a	4	.75a	Fert no N	1.30f	---	11	1.92jk	48.3
10	.78a	5	.67a	1	1.11f	10.8	12	1.73k	5.0

*Values within a column not followed by letters in common are significantly different at the 5% level of probability.

Table 6. Means of percent nodule occupancy by strain treatments for each site.

| Site | Strain treatment | | | | | | | | | | | | Site mean across treatments |
	1	2	3	4	5	6	7	8	9	10	11	12	
1	0.8	63.3	61.7	62.5	60.8	3.3	45.8	39.2	.80	73.3	49.2	5.8	38.9
2	0.0	6.7	34.2	3.3	14.2	10.0	70.8	100.1	58.3	70.0	11.7	28.3	34.0
3	10.8	100.0	90.8	98.3	90.0	.93	86.5	71.7	12.5	42.9	20.8	4.4	52.5
4	4.2	92.5	100.0	37.5	54.2	5.8	96.7	65.0	7.5	99.2	48.3	5.0	51.3
Treatment mean across all 4 sites	4.0	65.6	71.7	50.4	54.8	5.0	75.0	69.0	19.8	71.4	32.5	10.9	
Treatment means across sites 3 and 4 only	7.5	96.3	95.4	67.9	72.1	3.4	91.6	68.4	10.0	71.1	34.5	4.7	

Table 7. Mean percent nitrogen content of plants among sites.

Site	Mean, in g.
2	2.58*
1	2.48
3	2.31
4	1.83

*All values in column are significantly different at the 5% level of probability.