THE EFFECT OF ROOT TEMPERATURE, STRAIN OF LOTUS RHIZOBIA AND SOME HERBICIDES ON THE GROWTH, NITROGEN FIXATION AND NODULATION OF BIRDSFOOT TREFOIL (LOTUS CORNICULATUS L.)

by

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ABSTRACT

The early growth, nitrogen fixation and nodulation of birdsfoot trefoil (Lotus corniculatus L.) as influenced by several factors were the objectives of this study.

The effect of root temperature on the growth and nitrogen fixation of birdsfoot trefoil was studied in growth chamber experiments. Three birdsfoot trefoil varieties, Empire, Leo and Viking, inoculated with each of six Lotus rhizobia strains or provided with NH_4NO_3, were grown in "diSPo" growth pouches, at five root temperatures (9°- 30°C) for 35 days after nodule formation.

Highest dry weights and nitrogen yields per plant were obtained at 18°or 24°C when the plants were dependent on symbiotic nitrogen fixation. Symbiotic combinations (host-strain combinations) differed in their response to root temperatures. At 9° and 12°C, nitrogen fixation was depressed and the growth was poor. The dry weights of plants at 9°C were 19 to 45% of those at 24°C. At 30°C the growth and nitrogen fixation were generally depressed. If NH_4NO_3 was given to plants the growth was superior to plants depending on symbiotic nitrogen fixation at all root temperatures. Significant first and second order interactions were present.

In the field experiment four of these six strains were used in the inoculation of Empire and Leo. Over 90% of the plants bore nodules four weeks after seeding. In seven weeks

practically all plants were nodulated and nitrogen was being fixed. Plant dry weights and dry matter yields were greatest with strain 95C11 which formed large nodules on any host. There were many, small nodules when strain P was used and yields were lower.

When herbicides or a companion crop were used in the establishment of pure birdsfoot trefoil stands, highest dry matter yields were obtained by using benefin, EPTC or 2,4-DB + dalapon. Benefin and EPTC effectively controlled grassy weeds. Percent nodulation and nodule fresh weights of Leo on benefin and EPTC treated plots and those of Empire on benefin treated plots were lower than in the check in the early stages of development. Later nodulation was satisfactory.

2,4-DB + dalapon controlled both grassy and broadleaf weeds. Percent nodulation or nodule fresh weight per plant were not reduced. Paraquat was unsatisfactory as a weed control when sprayed after the emergence of weeds and before the seeding of birdsfoot trefoil. A companion crop depressed the growth of birdsfoot trefoil and nodulation was poorer.

The format for this thesis is outlined below and has been approved by the Council of the Faculty of Graduate Studies and Research of the University of Manitoba.

SECTION 1

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Overall literature review

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INTRODUCTION

As a forage crop birdsfoot trefoil (Lotus corniculatus L.) has several desirable qualities. Trefoil is widely used in the U.S. Under Canadian conditions it has shown potential in many areas as a pasture crop. In Manitoba birdsfoot trefoil has been grown on limited scale. The establishment of new stands has been difficult, mainly due to poor seedling vigor and growth during the first year. This species is sensitive to shading and in competition with other plants the growth and nodulation of birdsfoot trefoil is retarded. Under varying light intensities the seedling growth of birdsfoot trefoil is less than that of alfalfa (Medicago sativa L.) and red clover (Trifolium pratense L.) (40).

Nodulation failures of birdsfoot trefoil have been reported to occur in many areas. Under Manitoba conditions the nodulation of young plants has been poor in some locations. Late in summer root nodules look brownish and are easy to remove. The symbiotic nitrogen fixation is not effective and many plants die.

In the summer of 1967 soil samples were collected from several locations in Manitoba (Appendix 1). It appeared that native Lotus rhizobia effective on L. corniculatus are not present in Manitoba soils or their population is quite small. Only from the experimental areas where the seed had been inoculated prior to seeding could effective Lotus rhizobia be isolated. This clearly indicated the need to

introduce the proper rhizobia through inoculation of seed when new birdsfoot trefoil stands are established.

Soil temperatures are quite low for a relatively long period of time at the beginning and at the end of growing season. During these periods the symbiotic nitrogen fixation and growth of birdsfoot trefoil could be depressed. Possibly symbiotic combinations of birdsfoot trefoil and Lotus rhizobia could be found which work effectively at a wide range of root temperatures. Six Lotus rhizobia strains were tested under controlled environmental conditions at the different root temperatures. Four of these strains were tested later under field conditions.

Because the early growth of birdsfoot trefoil is slow competing species easily eliminate it from the stands. By using herbicides pure trefoil stands can be established. This was studied in a separate experiment. Botanical composition of stands and nodulation of birdsfoot trefoil were specifically investigated.

REVIEW OF LITERATURE

1. Host - rhizobia relationship between *Lotus corniculatus* L. and *Rhizobium* sp.

The root nodule bacteria of birdsfoot trefoil belongs to a specific group of Rhizobium. Fred et al (16) presented a classification of rhizobia in which the root nodule organism of *L. corniculatus* L., *L. uliginosus* L., *L. tetragonolobus* L. and *Anthyllis vulneraria* L. was regarded as one group. The nodulation results obtained in the studies of Erdman and Means (13) showed that the strains of *Rhizobium* isolated from *L. uliginosus* are ineffective on *L. corniculatus* and conversely, strains from *L. corniculatus* are ineffective on *L. uliginosus*. Certain strains appeared to be effective in one experiment, but did not give the same results in another experiment. One strain, 3EOj2, was effective on both host species. This relationship between the rhizobia from *L. corniculatus* and *L. uliginosus* was also noticed by other workers (1, 8).

In addition Gregory and Allen (23) found that certain strains of rhizobia from *Caragana arborescens* L. formed effective nodules on *L. corniculatus*, but did not nodulate *L. uliginosus*. *Rhizobium lupini* and *Lotus-Anthyllis* groups of rhizobia were noticed to overlap considerably in their host-rhizobia relationship (1, 26, 27).

Norris (45) has reported that Rhizobium strains derived from L. corniculatus were acid producers. Strains from L. uliginosus were alkali-producers. He concluded that L. corniculatus represents a more advanced type than L. uliginosus in legume-Rhizobium association. Brockwell et al (5) noticed that effective rhizobia on L. uliginosus were strictly alkali producers. Acid production by Rhizobium from L. corniculatus was less marked and this species fixed nitrogen with rhizobia having a wide range of cultural reactions. This behaviour can be related to distribution of these species, L. uliginosus being confined to acid soils while L. corniculatus is tolerant of both alkaline and acid environments.

2. The growth, nitrogen fixation and nodulation of legumes as influenced by different factors.

2-1 Temperature

Jones and Tisdale (31) were the first scientists to study the effect of soil temperature on legume symbiosis. They found differences among four legumes to tolerate high soil temperatures. Peas were dwarfed at 30°C, clover developed poorly at 36°C, while alfalfa and soybeans grew very well at 36°C. Maximum dry weights per soybean plant were obtained at 24°C. At 30° and 36°C weight of tops was as great as at 24°C while the weight

of nodules declined rapidly. The variability in nitrogen content was greatest in the shoots. The roots were uniform. There was a sudden increase in nitrogen at 21°C as compared with that at 18°C and a sudden fall at 33°C as compared to that at 30°C.

Stalder (59) found the optimum for growth and nodulation of Pisum sativum L. in water culture to lie between 20°C and 24°C with a depression of nodule formation above 27°C.

Meyer and Anderson (44) reported that in agar tube culture in a glasshouse moderately high root temperatures (30°C) exerted a specific inhibitory effect on symbiotic nitrogen fixation of subterranean clover (Trifolium subterraneuym L.). At lower root temperature (20°C) plants inoculated with Rhizobium trifolii fixed nitrogen and grew normally. Uninoculated plants responded normally to nitrogen treatment at both temperatures.

Mes (42,43) noticed that temperate legumes, Vicia sativa L., Pisum sativum L. and Lupinus luteus L., responded differently from tropical legumes Stizolobium deeringianum Bort., Arachis hypogaea L. and Glycine max L. to different temperature conditions. An increase in day temperature from 18°, 19° or 20°C to either 25° or 27°C, generally decreased the nitrogen percentage

and the total nitrogen content of the temperate legumes. An increase in the night temperature from 10° to 21°C generally decreased the total nitrogen content. The growth and nitrogen assimilation of tropical legumes were depressed by the lower range of day temperatures used. At night temperatures below 18°C the nitrogen fixation was poor.

Dart and Mercer (12) studied the nodulation and growth of cowpea plants (Vigna sinensis) under two light intensities, at six temperatures and by using different levels of NH_4NO_3. The optimum temperature for growth and nitrogen fixation was at 27°C. The optimum temperature for primary root nodulation was at 24°C and for secondary root nodulation at 33°C.

Pate (47,48) studied the symbiosis of Medicago tribuloides (barrel medic) and Vicia atropurpurea (purple vetch) over a range of constantly maintained day temperatures at three light intensities and at low night temperature. Total fixation of nitrogen in both species showed a maximum at 24°C with the higher light intensities benefiting fixation in all temperature conditions. At high temperatures (above 27°C) there was a depression of symbiotic activity. The various symbiotic combinations differed in their relative abilities to engage symbiosis under different temperature conditions. Each strain responded in a different manner to the temperature.

According to Possingham et al (52) high root temperature (30°C) has a specific effect on nitrogen fixation and induces nitrogen deficiency in the subterranean clover. There was no evidence of a similar effect with high shoot temperature (30°C) on the nitrogen fixation of the plant. The harmful effect of high root temperatures on nitrogen fixation was similar, regardless of whether the temperature was constant or fluctuating. The decrease in nitrogen fixation was not due to a decrease in the number of nodules. The number of nodules was in fact greater at the high root temperatures, and could well have been a result of the temperature induced nitrogen deficiency. The growth was good when NH_4NO_3 was given to plants.

Philpotts (51) reported that high soil temperature could cause nodulation failure of some legumes. Also nodule formation and development were reduced and fewer large nodules occurred and a higher proportion of the nodules appeared ineffective.

Joffe et al (28) found that symbiotic nitrogen fixation is a thermo-sensitive process in which effective fixation is confined to relatively narrow temperature limits. The differential response of two legumes was evident; low root temperatures inhibited nitrogen fixation more strongly in the case of Arachis hypogaea L. than Trifolium pratense L.

Gibson (19) studied the effect of root temperatures (5° - 30°C) on the growth and symbiotic nitrogen fixation by nodulated plants of four varieties of T. subterraneum L., inoculated with each of two strains of R. trifolii.

Symbiotic nitrogen fixation was reduced at root temperatures below 22°C, and at 5°C was only 10-17% of that achieved at 18°C. At 30°C there was a marked reduction in nitrogen fixation. Some symbiotic combinations achieved levels of growth similar to that made by plants receiving adequate combined nitrogen while the others were consistently less effective.

For both dry weight and nitrogen fixation, there was a significant interaction between the varieties and bacterial strains throughout the temperature range, and above 18°C, the degree of this interaction was influenced by the root temperature.

Earlier it was (18) found that root temperature influenced the relative effectiveness of two strains of R. trifolii on two varieties of subterranean clover.

Nutman studied (46) the symbiotic effectiveness of 15 varieties of subterranean clover in association with several strains of root nodule bacteria, and concluded that the interaction between host varieties and bacterial strains was of minor importance.

At the lower root temperatures (5° and 10°C), the translocation of nitrogen to the shoots was retarded in both nodulated and ammonium nitrate control plants of

Trifolium subterraneum L. and nitrogen was retained in the roots (21). Up to 18°C there was a progressive increase in the proportion of the total nitrogen assimilated and translocated to the shoots. In the nodulated treatments there were both host variety and bacterial strain effects on the distribution of fixed nitrogen. With an increase in root temperature from 8° to 18°C, the rate of increase in the dry weight of the shoots increased more than that of the roots. Up to 18°C the percentage nitrogen level in the roots of the nodulated plants was a function of the strain of nodule bacteria, whereas the percentage nitrogen level in the shoots was a function of the host variety. Above 20°C changes in dry weight increase and its distribution between the roots and shoots, were largely controlled by the effect of root temperature on symbiotic nitrogen fixation although varietal effects were also evident.

In order to minimize differences in the rate of nodule establishment and early nitrogen fixation between varieties and strains, Gibson (20) used relative nitrogen assimilation rates (R_N) and relative growth rates (R_W) to compare different symbiotic combinations. R_N was a more satisfactory parameter than R_W for comparing the varietal and temperature

treatments. This was particularly true for the higher root temperatures (25° - 30°C) which severely reduced nitrogen fixation by three of the eight strains tested. This did not seriously affect the R_W of the plants inoculated with these strains. The strong statistical interactions suggested that the nitrogen fixing ability of varieties depended on both the root temperature and the strain of nodule bacteria.

The effect of temperature on the formation of rhizobial root nodules of beans was investigated by Barrios et al. (3) by using a method of isolated root culture under axenic conditions. Optimum nodulation was obtained at 25°C. At 12° and 33°C, the root growth was reduced and nodulation was nil. At 17° and 30°C the growth was as good as at 25°C but nodulation was reduced an average of 70%. They concluded that decreased nodulation at 17° and 30°C was therefore unrelated to root growth; neither could it be explained on the basis of size of the effective inoculum (number of rhizobia) and abundance of root hairs. Roots held at 30°C nodulated as much as roots held at 25°C, provided they were exposed to 25°C at least for three days after inoculation.

Heinrichs and Nielsen (24) grew alfalfa varieties of diverse genetic origin to determine the effect of soil temperature (5° - 27°C) on herbage and root growth. The air temperature varied between 15° and 32°C. Varieties of Medicago sativa generally yielded more herbage and roots than those of Medicago falcata. The growth of alfalfa was greatly affected by soil temperature; the most herbage was produced at 27°C and most root and nodular tissue at 12°C. Strain x variety interaction was not studied.

Cooper (9) grew birdsfoot trefoil and alfalfa in pots in the greenhouse under three soil temperatures (12°, 18°, 24°C) and three phosphorus fertility levels. The percentage increase in growth was greatest between 12° and 18°C. Top growth of alfalfa increased to about the same degree as the roots, with increasing temperature. In contrast, top growth of birdsfoot trefoil was markedly stimulated with increasing soil temperature levels. At 12°C alfalfa produced a greater yield of tops than birdsfoot trefoil. At 18° and 24°C top yields of birdsfoot trefoil were greater than those of alfalfa.

2-2 Some other environmental factors

Lynch and Sears (37) compared greenhouse and field tests to evaluate several strains of nodule bacteria

with reference to the growth of birdsfoot trefoil and the amount of nitrogen fixed. The field soil was free of effective Lotus rhizobia. Generally the total differences were less marked in the field than in the greenhouse, but within limits, those strains having high nitrogen fixing capacity in the greenhouse also fixed large amounts in the field. The size of nodules was more significant than the number of nodules. There was a constancy of strain response although L. corniculatus is self-sterile and cross-pollinated.

Birdsfoot trefoil is known to survive and grow under a wide range of soil conditions. It can persist on poorly drained and droughty soils. It grows on low fertility and acid soils. The growth responses of trefoil are also quite different from those of alfalfa (57,58).

McKee (39,40) reported that inadequate nodulation or lack of nodulation is a problem in the establishment of birdsfoot trefoil under unfavorable conditions. Nodulation could occur within a pH range of 4.5 to 7.9 but the growth and survival of the seedlings and nodulation were satisfactory only in a pH range of 6.2 to 7.5. Mineral deficiences or imbalances were reflected more in depressed and retarded growth and

nodulation than in the appearance of the deficiency symptoms. Added nitrogen did not prevent formation of nodules but did reduce their number and size. Low soil moisture also depressed and retarded nodulation more than plant growth. The use of heavier rates of inoculation (100x and 1000x) did not alter the nodulation responses reported above.

Lynch and Sears (38) noticed that the strains of rhizobia from L. corniculatus responded differently to fertilizer treatments. The nitrogen content of plants was higher when the plants were inoculated with one of the strains and limestone alone or in combination with phosphorus and/or potassium. Soil treatment had no pronounced effect on the nitrogen content of plants nodulated by one of three other strains of rhizobia.

The growth and nodulation of birdsfoot trefoil was more sensitive to shading than that of red clover or alfalfa (40). Also with increasing plant competition nodulation declined. Satisfactory nodulation resulted when competing species were eliminated. Seedlings of birdsfoot trefoil nodulated under shading equivalent to 25% of daylight but required at least 50% for the nodules to be functional.

Shading and defoliation of L. uliginosus induced a rapid fading of pink colour of the nodules (7). Subsequently the nodules became brown, as did the roots, and many of the laterals and some main roots died. Decreasing light or increasing temperatures from 60°to 90°F affected the root growth more than the top growth of birdsfoot trefoil (22).

McKee (41) found that most satisfactory nodulation based on nodule size, number, colour and mass occurred on plants grown under natural photoperiod (12-15 hours) and the poorest on plants grown under conditions of the 9 to 24 hour photoperiod. Empire was less adequately nodulated than Viking.

The behaviour of strains of rhizobia has been noticed to vary. Abel and Erdman (2) found that in fields free or essentially free of soybean rhizobia some strains were more effective on Lee soybeans than others in several characteristics. One strain was effective in one year and ineffective in another.

Johnson et al. (30) studied the success of strains of Rhizobium japonicum applied as inoculum in different amounts and by different methods in producing nodules on soybeans. Some strains were more successful as inoculum than others.

According to Burton and Curley (6) at least 200,000 rhizobia per seed are required to bring about effective nodulation of soybeans under good planting conditions.

2-3 Companion crop and certain herbicides

In the establishment of pure trefoil stands selective herbicides have been used to eliminate competition by other species. Successful results have been obtained by using different chemicals alone or in combination with cultural practices.

Scholl and Staniford (55) found that pre-emergence applications of trichloroacetic acid (TCA) or post-emergence, 2,2-dichloropropionic acid (dalapon) gave almost complete control of grassy weeds with little or no injury to the legume. A combination of dalapon and 4-(2,4-dichlorophenoxy) butyric acid 4(2,4-DB) was superior to dalapon alone since the 4(2,4-DB) reduced broadleaf weeds significantly. Companion crops significantly reduced stands and yields of birdsfoot trefoil under all managements studied.

Scholl and Brunk (54) reported that dalapon was very effective in controlling competition from annual grasses, and dalapon combined with 4(2,4-DB) gave almost complete control of both grasses and broadleaf weeds.

Peters and Davis (50) found that yields of birdsfoot trefoil were increased when broad leaf weeds were controlled with 4(2,4-DB). When dalapon was used with 4(2,4-DB) the yields were slightly improved.

Kerr and Klingman (33) studied the effect of mowing, dalapon and TCA on weed control, establishment and growth of birdsfoot trefoil. Three mowings in combination with two dalapon applications resulted in the highest trefoil yields. Dalapon did not inhibit nodulation when it was used at rates of 6 lbs. per acre or less.

In field trials Garcia and Jordan (17) showed that 2,4-DB, alone or in combination with dalapon, reduced nodulation and tended to decrease the efficiency of nitrogen fixation in birdsfoot trefoil. Dalapon appeared to enhance the inhibitory action of 2,4-DB on nodulation. No obvious cytological differences could be detected in the nodules or in the isolated bacteroids of field treated and untreated plants. Under growth chamber conditions, 2,4-DB drastically reduced trefoil growth and nodulation particularly in treatments where the herbicide came directly in contact with the plants. It appeared that the reduction in nodulation and nitrogen fixation was a result of plant damage and abnormal root growth caused by 2,4-DB application.

Peters (49) reported that ethyl N,N-di-n-propyl-thiolcarbamate (EPTC) controlled broadleaf weeds effectively. Also grassy weeds decreased when rates

of EPTC increased. Best control of broadleaf weeds and grasses was obtained by using 2,4-DB and dalapon. Apparently 2,4-DB injured birdsfoot trefoil to some extent. Serious losses of birdsfoot trefoil occurred on plots having oats as a companion crop.

According to Wakefield and Skaland (60) pre-emergence weed control with EPTC was very successful. Results with 2,4-DB combined with dalapon were comparatively poor as weed control was not achieved and yields of birdsfoot trefoil were depressed. The legume growth was retarded by the herbicide combination although the symptoms of injury were not evident.

Schreiber (56) noticed that EPTC as a surface application at 8 lb/acre did not give satisfactory broadleaf or grass weed control but was non-injurious to both alfalfa and birdsfoot trefoil.

Linscott and Hagin (35) and Linscott et al. (36) found that EPTC applied at planting time effectively controlled annual grassy weeds in seedings of birdsfoot trefoil. Control of annual broadleaf weeds by EPTC was incomplete. A post emergence application of 2,4-DB in addition to the EPTC treatment resulted in excellent overall weed control.

Fletcher (14) investigated the effects of several herbicides on the growth of Rhizobium trifolii. The herbicides had no effect on the growth of R. trifolii

on agar cultures at concentrations up to 25 ppm. The herbicide concentration in the soil is much below this value under normal agricultural practices.

In aseptic agar test tube and soil culture experiments it was noticed that 2,4-DB was relatively non-toxic as a herbicide when the growth and nodulation of white clover (Trifolium repens L.) was studied (15).

Jordan and Garcia (32) reported that growth of Lotus nodule bacteria was temporarily suppressed, in a yeast extract mineral salt, medium, containing 2,4-DB at 50 and 100 mg/ml. The 2,4-DB concentration needed to completely suppress the growth of bacteria appeared to be above 500 mg/ml. When added during the logarithmic phase of growth 2,4-DB at 50 and 100 mg/ml did not inhibit the growth of cells, so that the growth inhibiting effect of the herbicide apparently was directed primarily against lag phase cells. Pre-incubation of rhizobia in a medium containing 2,4-DB at concentrations up to 10 µg/ml did not affect the capacity of the bacteria to effectively nodulate their host plant.

SECTION 2

Results of research in publication form

RESULTS OF RESEARCH

1. The influence of root temperature on the early growth and symbiotic nitrogen fixation of nodulated Lotus corniculatus L. plants.

ABSTRACT

Three birdsfoot trefoil (Lotus corniculatus L.) varieties inoculated with one of six Lotus rhizobia strains or dependent on ammonium nitrate, were grown in diSPo growth pouches under controlled environmental conditions at five root temperatures (9° - 30°C) for 35 days after nodule formation.

When the plants were dependent on symbiotic nitrogen fixation the highest dry weights and nitrogen yields per plant were obtained at 18° or 24°C depending on symbiotic combination. At 9° and 12°C nitrogen fixation was depressed and the growth was poor. The dry weights of plants at 9°C were 19 to 45% of those at 24°C. At 30°C the growth and nitrogen fixation were generally depressed. If combined nitrogen as NH_4NO_3 was given the growth was superior to plants depending on symbiotic nitrogen fixation at all root temperatures.

Significant interactions indicate that the nitrogen fixing ability of varieties was dependent on both root temperature and the strain of Lotus rhizobia.

INTRODUCTION

The growth and nitrogen fixation of many legume species are specifically influenced by temperature. The optimum night and day temperature depended on the legume species (42,43). Pate (47,48) observed that Medicago tribuloides Desr. and Vicia atropurpurea Desf. had maximum nitrogen fixation at 24°C. Each strain of Rhizobium seemed to respond differently at various root temperatures. The optimum temperature for the growth and nitrogen fixation of Vigna sinensis L. was found to be at 27°C (12).

Jones and Tisdale (31) found that four legumes differed in their response to root temperature. According to Meyer and Anderson (44) and Possingham et al. (52) high root temperature (30°C) had a specific effect on nitrogen fixation and induced nitrogen deficiency in subterranean clover (Trifolium subterraneum L.). The uninoculated plants responded normally to nitrogen treatment at all root temperatures. High shoot temperatures did not have a similar effect (52). Gibson (18,19,20) found that the optimum root temperature for the symbiotic nitrogen fixation and growth of subterranean clover was between 22°and 26°C. The nitrogen fixing ability of varieties depended on both the root temperature and the strain of nodule bacteria.

The early growth and development of birdsfoot trefoil is poor and it is influenced by several factors. This study was conducted under controlled environmental conditions

to determine the effects of root temperature on the early growth of birdsfoot trefoil (Lotus corniculatus L.) plants which were dependent on symbiotic nitrogen fixation or combined nitrogen (NH_4NO_3).

MATERIALS AND METHODS

The seed of birdsfoot trefoil varieties Empire, Leo and Viking was obtained from the Department of Plant Science, University of Manitoba. The seeds were selected for uniformity of size and weight, both within and between varieties and surface sterilized by immersion in 80% ethanol for one minute, in 2.5% hypochloride ($HClO^-$) for two minutes and rinsed 10 times with sterile, distilled water. The seeds were germinated on 1.5% water agar for 6-7 days (Empire 7, Leo and Viking 6 days) in Petri dishes in darkness at room temperature. Qualls and Cooper (53) found that speed of germination and rate of elongation follow this order during the non-photosynthetic stage of development. The seedlings were kept in a growth cabinet in light for two days before transplanting, to strengthen the seedlings.

The plants were grown in diSPo growth pouches (Fig.1) (16.5 x 17.5 cm) developed by Northrup, King & Co., Minneapolis, Minnesota, U.S.A. The pouches were sealed leaving 4 cm unsealed in the center of each pouch. 25 ml of nutrient solution was added to pouches and sterilized at 121^{o}C and 1 atm pressure for 20 minutes. One seedling was transplanted through the opening which was closed with paper clips to prevent contamination.

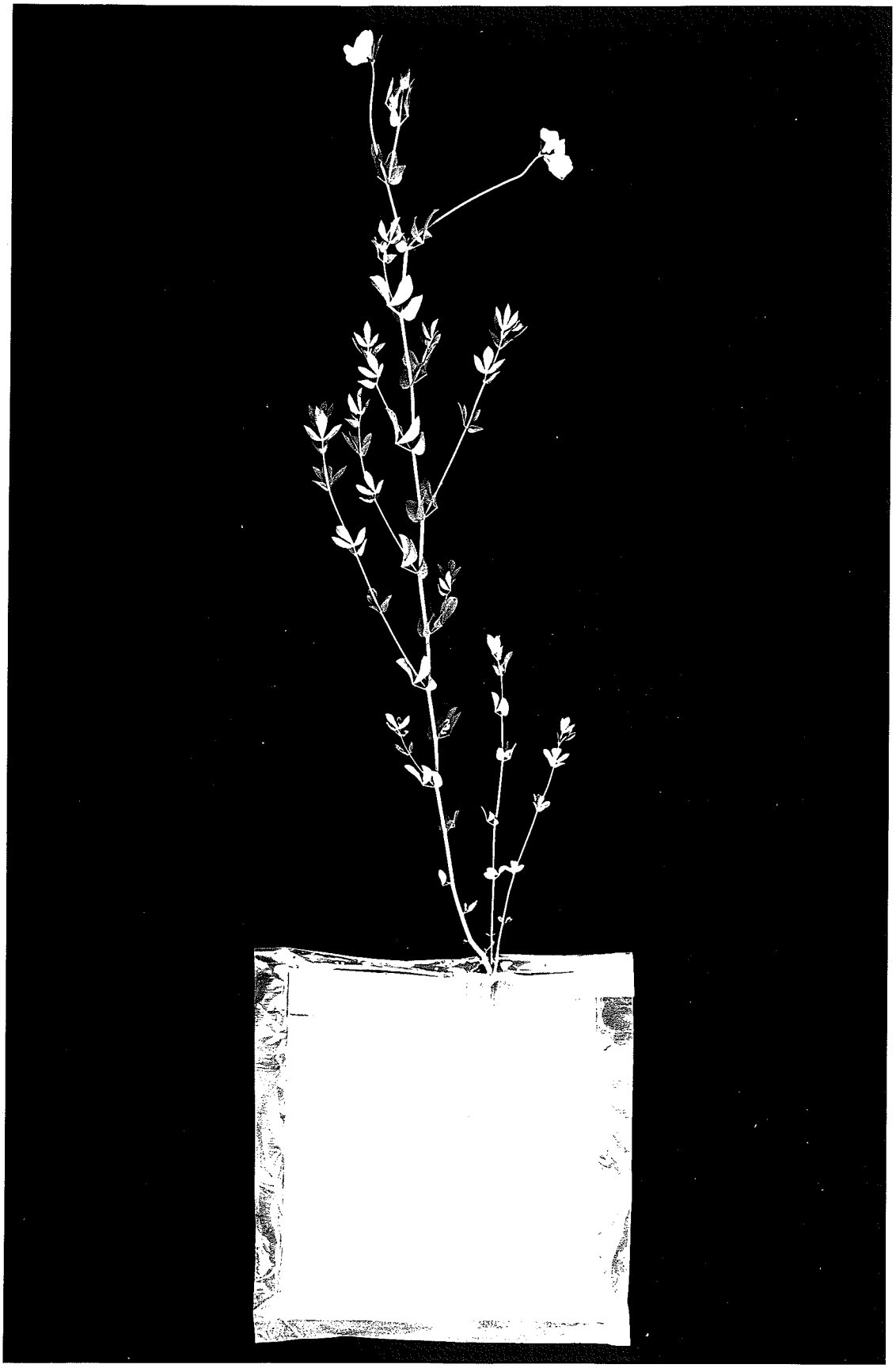

Figure 1. DiSPo growth pouch used in root temperature studies.

Nitrogen free nutrient solution developed by Hely (25) was used for plant cultures. One ml of trace element solution (19) was used with 5 p.p.m. of Fe as FeEDDA and distilled water was added to make up 1000 ml. After autoclaving the pH was 6.7 in the pouches. When the plants were harvested pH was around 6.5. This was checked by adding 20 ml distilled water to pouches before reading the pH value.

The plants were watered later with ¼-strength nutrient solution when needed by using an automatic syringe. The plants were inoculated 6 (Leo, Viking) or 7 (Empire) days after transplanting. The number of rhizobia per ml was 10^6 - 10^7 as checked by plate count.

Six Lotus rhizobia strains were used to inoculate the host plants. The strains were 867, 868 from Microbiology Research Institute, Ottawa, Ontario; 95C11, 95C13 from Dr. J. L. Burton, Nitragin Sales Corporation, Milwaukee, Wisconsin, U.S.A., and L and P from Dr. H. L. Jensen, State Laboratory for Soil and Crop Research, Lyngby, Denmark.

The Rhizobium strains were maintained on 1.5% nutrient agar. A culture medium by Johnson et al. (29) was used with 3.0 g mannitol per liter. The pH was adjusted to 6.8. The cultures were grown at 28°C and stored at 4°C. Stock cultures were transferred at monthly intervals.

Figure 2. Waterbath used in root temperature studies. Plants in one basket formed a replicate. Experimental unit consisted of two separate plants (pouches) which were pooled together for dry weight and nitrogen determinations.

After inoculation the plants were kept in a Coldstream growth cabinet. Temperature was 21°C during the 18-hour day and 14°C during the night. Illumination was provided by 32 Sylvania Electric fluorescent lamps supplemented with eight 60 Watt incandescent lamps. Light intensity was 17,200 lux (1600 f.cl.) above the growth pouches as measured with Weston Illumination Meter, Model 756.

Nitrogen was given to plants according to the following scheme:

Temp.°C	I*	II**	III***	Total N mg/plant
9	3	3	-	6
12	3	5	3	11
18	3	5	7	15
24	3	5	7	15
30	3	5	7	15

* mg N/plant 1 day before temperature treatment started

** mg N/plant 11 days after temperature treatment had started

*** mg N/plant 26 days after temperature treatment had started

Due to accumulation of nitrogen in plants less N was given at lower root temperatures (9°, 12°C). When the plants were well nodulated and the nitrogen fixation had begun (19 days after inoculation) plants were arranged in 10 replicates and root temperature treatment was started by transferring growth pouches in test tube baskets into water baths (Fig. 2). The experimental design was a split-split-plot randomized block with root temperatures in main

plots and varieties and Lotus rhizobia strains in sub-plots. The analysis of variance was calculated after log transformations and Duncan's new multiple range test (5% level) was used to detect the differences between the treatments. Separate cooling unit and coils were used when root temperature was below ambient. The root temperatures were 9°, 12°, 18°, 24° and 30°C. The error range in Brownwill Thermomix II constant temperature regulator was less than $\pm$ 0.5°C.

The plants were harvested after 35 day root temperature treatment and dried at 95°C for 18 hours. The dry weights of plants were measured and nitrogen contents were determined by using a micro-Kjeldahl method.

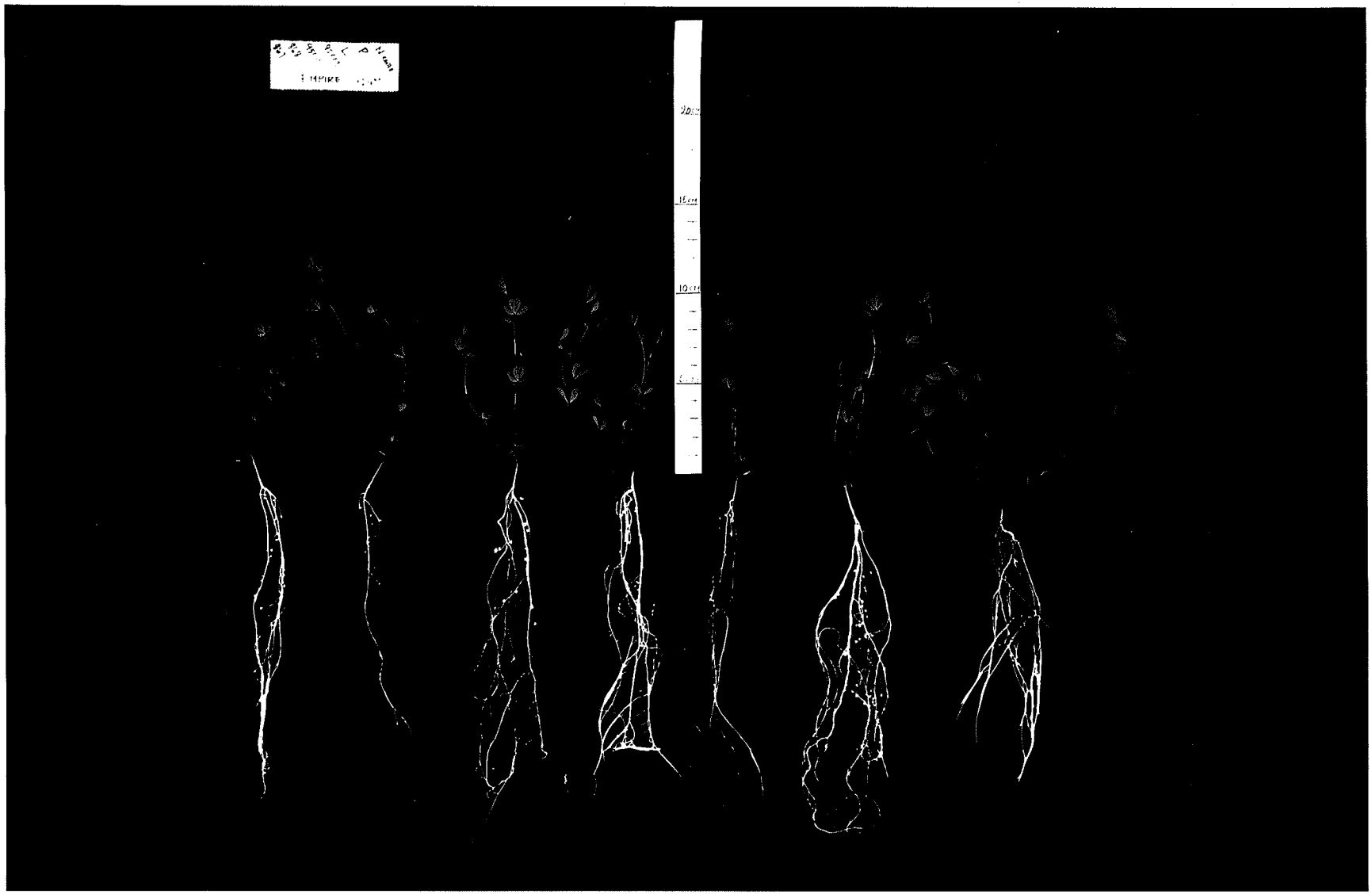

Figure 3. Plants of Empire after 35 days of growth at root temperature 24°C. Plants inoculated with Lotus rhizobia strains (left to right); 867, 868, 95C11, 95C13, L, P, and N-control.

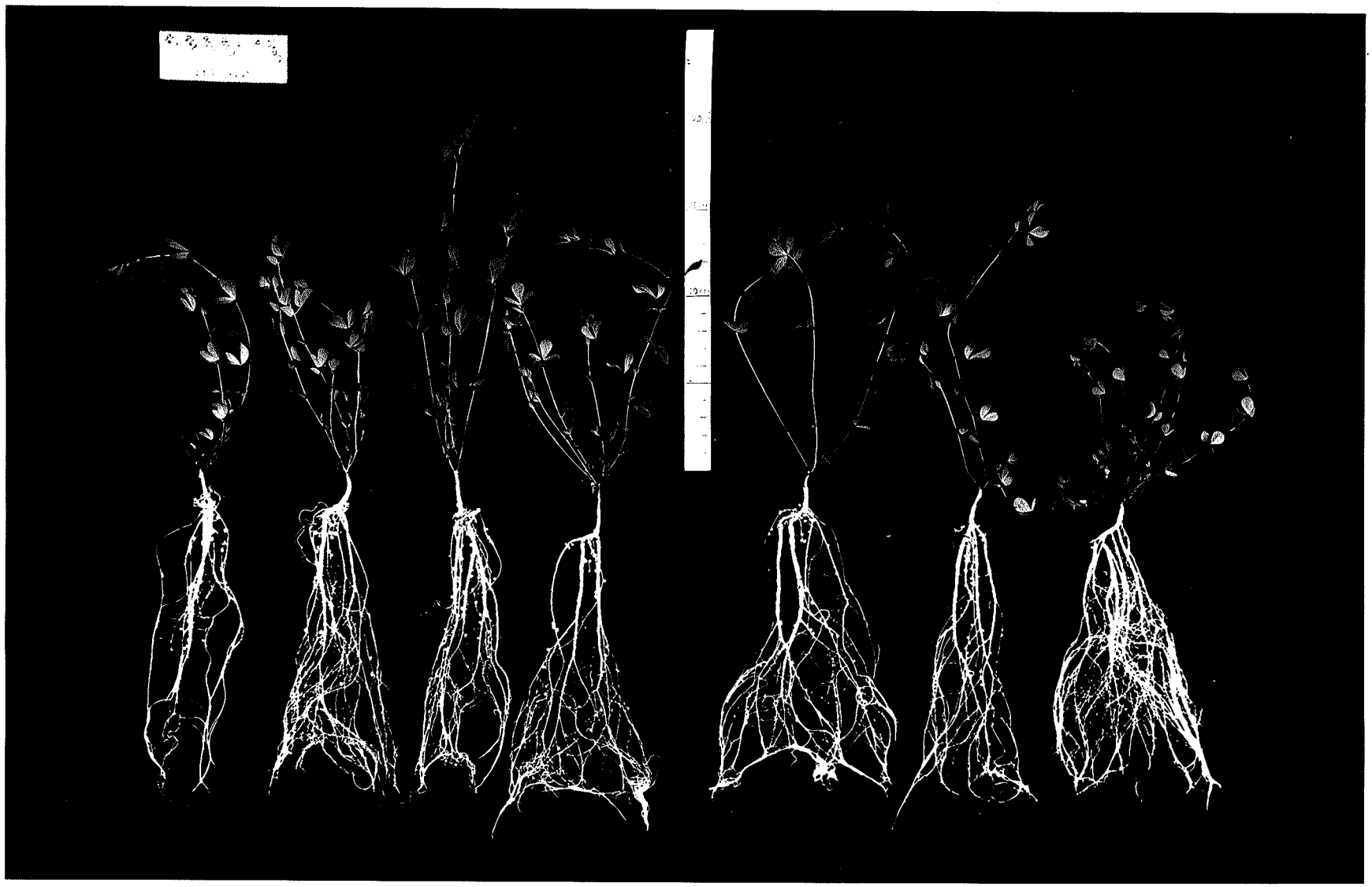

Figure 4. Plants of Leo after 35 days of growth at root temperature 24°C. See figure 3 caption for details.

Figure 5. Plants of Viking 35 days of growth at root temperature 24°C. See figure 3 caption for details.

RESULTS

Three birdsfoot trefoil varieties, Empire, Leo and Viking, were grown at five root temperatures 9°,12°, 18°, 24°, and 30°C. The plants were inoculated with one of six Lotus rhizobia strains or combined nitrogen (NH_4NO_3) was given to plants. Nitrogen controls were included as a strain in the statistical analysis of dry weight data. This was not done with the nitrogen content data because of the accumulation of nitrogen in the control plants.

The dry weights and nitrogen contents of three varieties were highest at root temperatures 18°or 24°C when the plants were dependent on symbiotic nitrogen fixation (Tables 1 and 2, Appendices 2 and 3). At 18°C all varieties with the strain 867 had the best growth and nitrogen contents were also high. Differences between the best and poorest strains within varieties with respect to the dry weight and nitrogen content of plants were significant (5% level) at this root temperature.

At 24°C Empire/P (=Empire inoculated with P) was the best symbiotic combination within this variety. Leo/868 was the best symbiotic combination with Leo but with the other strains the growth and nitrogen fixation were also satisfactory. With Viking the strains 95C11, L and 867 were considerably better than strain P. In figures 3-5 the three varieties inoculated with each of six strains of Lotus rhizobia and N-control plants can be seen after 35 day temperature treatment.

Table 1. Total dry weights of plants (mg/plant) at different root temperatures. 1/

Variety	Strain	Root temperature °C					
		9	12	18	24	30	Mean
Empire	867	22.1	32.2	82.5	75.5	25.6	47.6
	868	26.2	36.3	64.0	57.8	31.9	43.3
	95C11	28.1	32.6	60.4	66.3	26.4	42.8
	95C13	31.1	29.1	72.2	80.2	33.2	49.1
	L	24.1	35.8	61.0	91.5	29.5	48.4
	P	23.1	42.2	41.8	110.9	28.0	49.2
	N-Control	86.4	87.0	289.8	244.6	365.4	214.6
	Mean	34.4	42.2	96.0	103.8	77.1	70.7
Leo	867	49.5	60.4	231.5	202.5	66.6	122.1
	868	55.0	57.3	137.7	272.7	125.8	129.7
	95C11	40.7	64.4	169.0	208.3	68.3	110.1
	95C13	60.6	56.5	119.2	196.7	176.1	121.8
	L	48.2	51.4	165.7	247.4	118.3	126.2
	P	45.9	51.5	141.9	213.0	93.1	109.1
	N-control	143.2	137.5	522.5	476.0	612.8	376.6
	Mean	63.3	68.4	212.5	259.5	180.1	156.5
Viking	867	51.1	58.4	193.5	165.9	57.5	105.3
	868	34.5	43.4	152.1	132.4	84.7	89.4
	95C11	42.7	46.8	110.9	182.2	56.9	87.9
	95C13	49.5	53.0	129.4	110.4	83.6	85.2
	L	40.4	45.1	115.3	173.4	77.9	90.4
	P	46.1	49.8	121.7	105.2	60.5	76.7
	N-control	140.6	173.5	565.0	539.7	602.7	404.3
	Mean	57.8	67.1	198.7	201.3	146.3	134.2

1/ For comparisons between treatments see appendix 16

The dry weights and nitrogen contents of Leo at 24°C were usually considerably higher than at 18°C (Tables 1 and 2, Appendices 2 and 3). The yields of Empire and Viking were somewhat lower at 18° than at 24°C. At 24°C Empire/L, Empire/P, Viking/95C11 and Viking/L grew much better than at 18°C.

At both root temperatures (18°, 24°C) with the same strains Leo had significantly higher dry weights and nitrogen contents than Empire. Viking had also higher yields than Empire although not so many significant differences were present. Leo had generally higher yields than Viking and some symbiotic combinations of Leo significantly outyielded those of Viking. At 24°C Leo had considerably higher dry weights and nitrogen contents than Viking when the same strain was used. At 18°C the differences between Leo and Viking were smaller.

At 9° and 12°C the differences in the dry weights and nitrogen contents of plants between strains within varieties were non-significant. At 12°C the yields were slightly higher than at 9°C. The symbiotic combinations of Empire performed less well than those of Leo and Viking at these root temperatures. Differences between Leo and Viking were small.

At 9° and 12°C the dry weights and nitrogen contents of varieties were significantly lower than at 18° or 24°C with the same strains of Lotus rhizobia.

Table 2. Nitrogen contents of plants (mg/plant) at different root temperatures. 1/

Variety	Strain	Root temperature °C					
		9	12	18	24	30	Mean
Empire	867	0.44	0.70	2.17	2.52	0.39	1.24
	868	0.50	0.93	1.79	1.86	0.52	1.12
	95C11	0.54	0.93	1.70	2.17	0.51	1.17
	95C13	0.61	0.75	2.26	2.72	0.71	1.41
	L	0.41	0.85	1.71	2.89	0.45	1.26
	P	0.42	0.98	1.09	3.63	0.42	1.31
	Mean	0.49	0.86	1.79	2.63	0.50	2.15
Leo	867	1.04	1.71	7.76	8.11	1.32	3.99
	868	1.19	1.58	4.89	10.82	2.41	4.18
	95C11	0.89	1.66	5.70	7.98	1.57	3.56
	95C13	1.25	1.47	4.09	8.22	4.60	3.93
	L	0.92	1.26	5.26	8.85	2.13	3.69
	P	0.91	1.43	4.49	7.09	1.62	3.11
	Mean	1.03	1.52	5.37	8.51	2.28	4.62
Viking	867	1.02	1.41	5.51	5.21	1.10	2.85
	868	0.66	0.99	4.69	4.64	1.51	2.50
	95C11	0.88	1.14	3.66	6.51	1.37	2.71
	95C13	0.97	1.22	3.98	4.10	2.04	2.46
	L	0.77	0.99	3.50	5.19	1.16	2.32
	P	0.94	1.08	3.93	3.36	0.88	2.04
	Mean	0.87	1.14	4.21	4.83	1.34	3.53

1/ For comparisons between treatments see appendix 17.

At 30°C strain 95C13 was best in combination with all varieties. There were no significant differences in dry weights and nitrogen contents of plants when different strains were used with Empire. Leo/95C13 was superior to the other symbiotic combinations of Leo. With Viking strains 868 and 95C13 were the best.

Leo and Viking at 30°C were significantly better than Empire in combination with the same strain in terms of dry weights and amount nitrogen fixed. The difference between Leo/95C13 and Viking/95C13 was also significant. At 30°C the dry weights and nitrogen contents of plants were significantly smaller than at 24°C. Only Leo/95C13 was not significantly different at these root temperatures.

The uninoculated nitrogen control plants had significantly higher dry weights than plants which were dependent on symbiotic nitrogen fixation at all root temperatures. Highest dry weights of nitrogen control plants were obtained at 30°C. At 18° and 24°C the dry weights were smaller than at 30°C but the differences were non-significant within varieties. At 9° and 12°C the dry weights were significantly smaller than at 18°, 24° or 30°C. At all root temperatures Leo and Viking were superior to Empire when combined nitrogen was given to plants.

The interactions strain x variety, temperature x strain and temperature x variety were significant. Also the second order interaction was significant (Appendices 2 and 3).

DISCUSSION

The dry weights and nitrogen contents of plants were highest at root temperatures 24° and 18°C (optimum temperature) when the plants were dependent on symbiotic nitrogen fixation. This has been also noticed with other species (18, 19, 20, 31, 44, 52). There were significant differences between the symbiotic combinations at these root temperatures. Strain 867 was most effective with the three varieties at lower root temperatures (12°, 18°C) whereas at 30°C it was as effective as other strains. Empire/P, Leo/868 and Viking/95C11 grew very well at 24°C. Leo seemed to perform better at 24°C than at 18°C whereas Empire and Viking did equally well with many strains at both temperatures. Pate (47,48) reported that various strains act differently depending on temperature.

At 30°C nitrogen fixation of plants was reduced and dry weights were 25 to 89% below yields at 24°C. All symbiotic combinations of Empire showed poor growth and nitrogen fixation at 30°C. Within Viking greater variability was noticed with different strains. Leo had significantly better nitrogen fixation and growth with strains 95C13, P and L than with strains 95C11 and 867. Strain 95C13 seemed to work effectively at 30°C with all varieties. Gibson (19) found that some symbiotic combinations of subterranean clover were more effective at high root temperature (30°C) than others.

At 9° and 12°C nitrogen fixation was significantly less than at 18° or 24°C. The dry weights of plants were only 19 to 45% at 9°C compared with yields at 24°C. At 12°C the nitrogen contents and dry weights of plants were slightly greater than at 9°C. Although no significant differences in the nitrogen contents of plants within varieties could be found at 9° and 12°C some symbiotic combinations of varieties could fix nitrogen up to 1.5 times more than the poorest combinations. Possibly by careful selection of strain and variety the early growth of birdsfoot trefoil could be improved. This was also noticed in a study with subterranean clover by Gibson (19).

At all root temperatures the symbiotic combinations of Empire seemed to be much poorer than those of Leo and Viking. Leo was usually somewhat better than Viking when the same strain of Lotus rhizobia was used.

The nitrogen control plants of these three varieties grew significantly better than those depending on symbiotic nitrogen fixation at all root temperatures. Earlier reports indicate that the best symbiotic combinations of T. subterraneum L. can have equal growth to nitrogen control plants (18, 19,20). The dry weights of nitrogen control plants were greatest at 30°C where nitrogen fixation was usually reduced.

All interactions were significant. This would suggest that the early growth of varieties depended to a large extent on both Rhizobium strain and root temperature.

The temperature x variety interaction was mainly due to a smaller increase for Empire between low (9°, 12°C) and optimum temperatures (18°, 24°C) than Leo and Viking. Also between 18°and 24°C Leo showed a considerable increase whereas Empire and Viking had smaller increases between these temperatures.

The temperature x strain interaction was due to temperature sensitivity of most of the strains. At 9° and 12°C differences between strains were small. Strain 867 performed considerably better at 18°C than other strains. Again at 30°C strain responses varied greatly 95C13 being the best one and L and 867 the poorest ones. Significant interaction was also present between Lotus rhizobia strain and trefoil variety. Within each variety there was at least one symbiotic combination which seemed to differ from others. This was also reported by Gibson (18,19,21). He demonstrated a significant interaction between R. trifolii and T. subterraneum. He states that this is a common phenomenon in this species.

2 The effect of Lotus rhizobia strains on the growth and nodulation of Lotus corniculatus L. under field conditions.

ABSTRACT

The growth and nodulation of two birdsfoot trefoil (Lotus corniculatus L.) varieties, Empire and Leo, during the year of establishment was studied in the field. Plants were inoculated with each of four Lotus rhizobia strains. Four weeks after seeding about 92% of the plants bore nodules. After seven weeks practically all plants were nodulated and nitrogen was being fixed. Plant dry weights and dry matter yields were greatest with Rhizobium strain 95C11 which formed large nodules on each host. With strain P many small nodules were formed and yields were lower.

INTRODUCTION

The early growth and development of birdsfoot trefoil (Lotus corniculatus L.) is slow. Seedling vigor is poor and establishment of new stands is difficult. Nodulation of this forage legume has been unsatisfactory in many areas. The root nodule bacteria symbiotic with birdsfoot trefoil belong to a specific group of Rhizobium (1,8,13). The effect of several environmental factors on the growth and nodulation of birdsfoot trefoil was studied by McKee (39,40,41). Lynch and Sears (37,38) reported variation in the nitrogen fixing efficiency between strains of Lotus rhizobia and in their response to fertilizer treatments (Ca,Mg,P,K). Kunelius and Clark (34) found that the early growth and nitrogen fixation of birdsfoot trefoil is dependent on variety, strain of Lotus rhizobia and root temperature. The present study was conducted to test the effect of some of these Lotus rhizobia strains on the early growth and nodulation of birdsfoot trefoil under field conditions.

MATERIALS AND METHODS

The experiment was conducted at MacGregor, Manitoba on an Almasippi fine sandy loam with a pH of 7.7. The experimental area was fertilized with 62 kg/ha of K_2O as muriate potash (0-0-60) and 62 kg/ha of P_2O_5 as triple superp hosphate (0-45-0) by broadcasting on plots. The nitrogen content of the soil was < 15 ppm NO_3-N . The birdsfoot trefoil varieties used were Empire and Leo sown at 7.0 kg/ha. The seed was surface sterilized with 95% ethanol for five minutes and rinsed ten times with sterile, distilled water before inoculation.

Four Lotus rhizobia strains, 867, 95C11, 95C13, and P were used. Each strain of rhizobia was grown separately in 500 ml flasks as shake culture by using 100 mls of nutrient solution: K_2HPO_4 0.5 g/l, $MgSO_4$ $7H_2O$ 0.2 g/l, $CaCl_2$ 0.1 g/l, $FeCl_3$ 6.0 mg/l, mannitol 10.0 g/l, yeast extract 1.0 g/l. One ml/l of trace elements were added (19).

One ml of each Rhizobium culture in log phase was transferred to these flasks. Shake cultures were grown for 7 days at 28°C in darkness. After this 35 mls of uncontaminated broth was added to each 100 grams of sterile (gamma irradiated) peat which was neutralized with a fine grade of $CaCO_3$. The treated peat was kept for two weeks at room temperature after which plate count and a plant infection technique (4) indicated

that the number of rhizobia in the peat was 10^9/gram. The acid production of each strain was checked by the Norris method (45).

Before inoculation the seeds were made sticky by mixing them with 45% (w/v) gum arabic suspension. The peat was mixed with the seed one day prior to seeding with a V-belt seeder on June 3, 1969. Seeding depth was about 1 cm. All equipment was sterilized with 95% ethanol prior to use. The experimental design was a split-plot randomized block, replicated 5 times, with Rhizobium strains as main plots (4.8 x 2.1 m) and varieties as subplots. There were four rows in a sub-plot with a 30 cm space between rows. The distance between main plots was 1.2 m. To check for the presence of Lotus rhizobia uninoculated Leo seed was grown in pots containing this soil in the greenhouse.

The plots were sampled 4, 7 and 10 weeks after seeding, with 30, 30 and 25 plants respectively being the number dug from inner rows. The percent nodulation, number of nodules per plant, weight of nodules per plant and dry weight of shoots were recorded. The experimental area was handweeded three times during the summer. Percent dry matter was determined after drying 800 grams green material at 80°C for 24 hours. Two inner rows of each sub-plot were cut Sept. 8, 1969 to determine dry matter yields (kg/ha).

Protein content of the tops was determined by the Kjeldahl method (N x 6.25). The data was subjected to an analysis of variance and Duncan's new multiple range test was applied to measure the differences between the treatments.

RESULTS

Four weeks after seeding 86 to 97% of plants bore nodules or nodule formation had been initiated (Table 3). No significant differences in percent nodulation between strains of rhizobia or varieties could be found (Appendix 4). However, a higher proportion of the total plants of both varieties had no nodules and fewer plants bore 3 or more nodules when 95C13 was used than with other strains (Fig. 6). Most plants had nodules only on main root (Table 4) and some of these were pink in color.

After seven weeks of growth all plants were well nodulated. No significant differences in percent nodulation could be found (Table 3). At this stage many nodules were pink in color and plant leaves were dark green indicating that nitrogen was adequate. More than half of the plants had nodules on laterals as well as on the main root (Table 4). Over 75% of Leo plants had 5 or more nodules while for Empire this value was about 50% (Fig.7). Both varieties with strain P had 100% nodulation. More plants bore 5 or more nodules when inoculated with P than with other strains.

In ten weeks all host plants bore nodules i.e. percent nodulation was 100% (Table 3). There were no significant differences in the number of nodules per plant between the symbiotic combinations (Table 5).

Table 3. Percent nodulation of varieties Empire and Leo inoculated with strains of Lotus rhizobia

Strain	Sampling time weeks after seeding				
	4		7		10
	Empire	Leo	Empire	Leo	Empire & Leo
867	94.1 a 1/	94.3 a	98.5 a	99.4 a	100.0
95C11	91.5 a	92.7 a	97.6 a	96.9 a	100.0
95C13	87.0 a	86.1 a	93.9 a	98.0 a	100.0
P	97.4 a	92.0 a	100.0 a	100.0 a	100.0
Mean	92.5	91.1	97.5	98.6	100.0

1/ Duncan's new multiple range test: within each sampling values with the same letter are not significantly different (P = .05).

Table 4. Percentage of plants having nodules on laterals

Strain	Sampling time - weeks after seeding			
	4		7	
	Empire	Leo	Empire	Leo
867	2.3	1.7	53.6	76.6
95C11	1.1	2.4	46.0	78.0
95C13	0.7	2.4	57.0	76.9
P	1.1	0.9	57.6	89.5
Mean	1.3	1.9	53.6	80.3

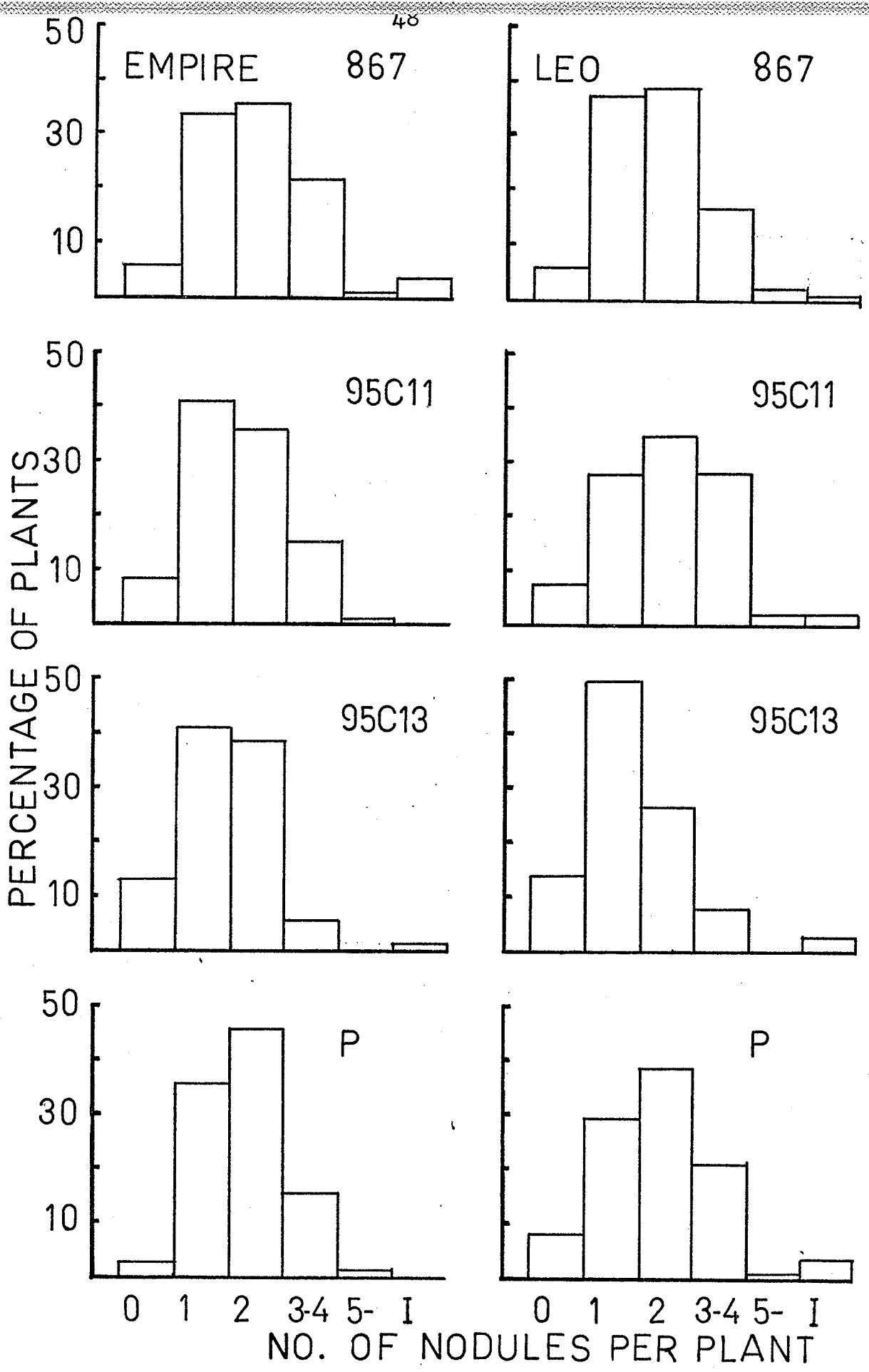

Figure 6. Percentage of plants having different number of nodules four weeks after seeding. 867, 95C11, 95C13 and P are the strains of Lotus rhizobia. I=initiating nodule(s) (visible, non-functional).

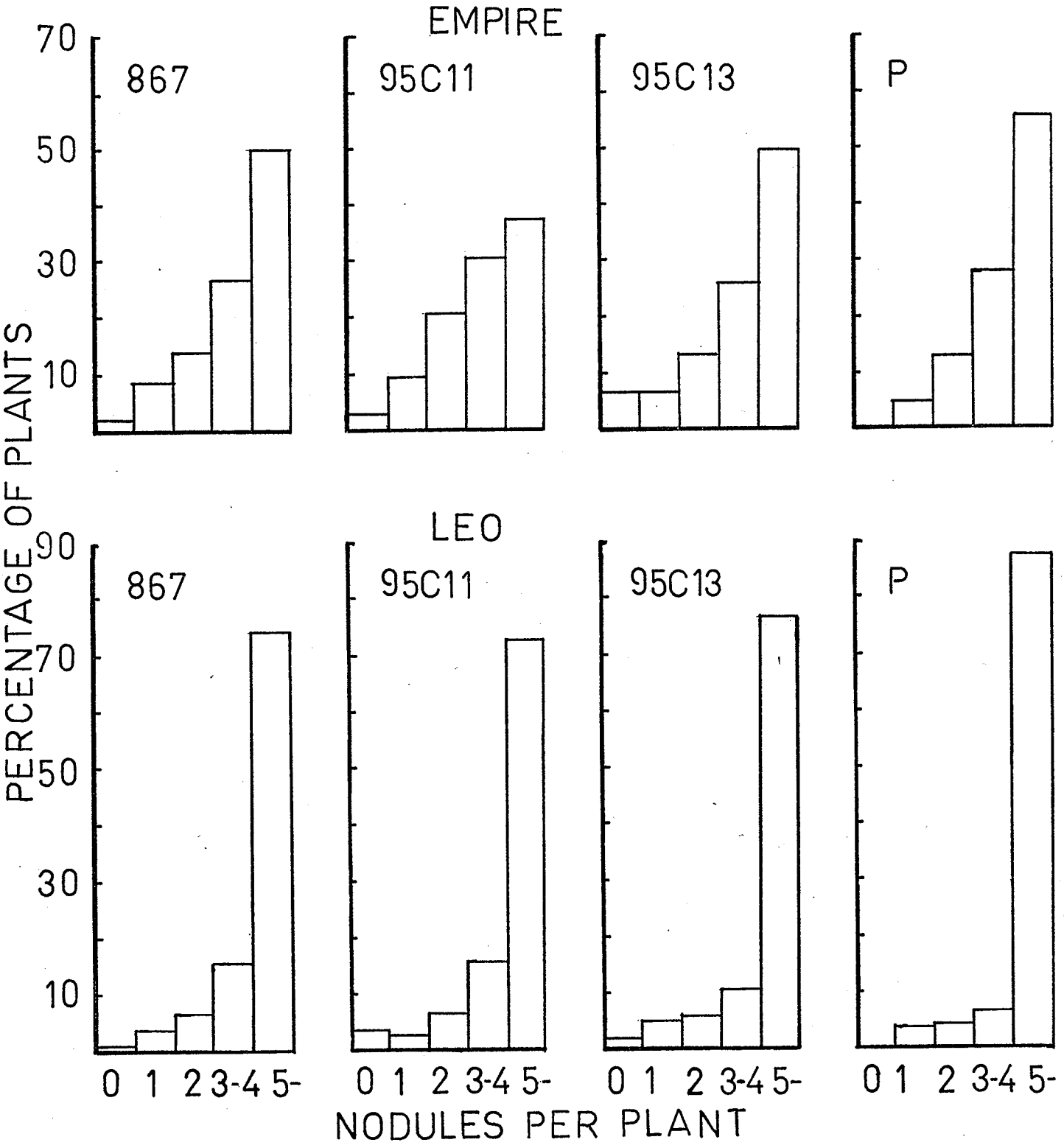

Figure 7. Percentage of plants having different number of nodules seven weeks after seeding. 867, 95C11, 95C13 and P strains of Lotus rhizobia.

However, Leo with strains 95C13 or P had more nodules per plant than with strains 867 or 95C11. Fresh weight per nodule varied significantly between the symbiotic combinations. Strain 95C11 with Leo had significantly bigger nodules than other strains with Empire or Leo (Table 5). The interaction between the strain of Lotus rhizobia and variety was significant (Appendix 5).

Significant differences between varieties in average nodule weight per plant were noted (Appendix 6). Seven weeks after seeding the average nodule weight per plant of Empire was only 36% that of nodules on Leo. Two strains, 95C11 and 95C13 , with Leo produced significantly more nodule mass than any strain with Empire (Table 6). Within Empire or Leo no strain appeared to be superior to any other after seven weeks of growth.

Ten weeks after seeding the nodule mass of Empire plants was about half of that of Leo (Table 6). In combination with 95C11 Leo had a significantly higher nodule weight per plant than Empire with any strain. Within each variety strain effects were not significantly different. The greater nodule weight per plant of Leo was due to a higher nodule number and larger nodules.

Table 5. Number of nodules per plant and fresh weight per nodule 10 weeks after seeding

Strain	Nodules per plant		Fresh weight mg per nodule	
	Empire	Leo	Empire	Leo
867	18.6 a 1/	21.5 a	2.11 bc	2.45 bc
95C11	18.5 a	19.3 a	2.06 bc	4.81 a
95C13	22.0 a	27.6 a	1.23 c	2.46 bc
P	21.8 a	29.2 a	1.64 c	2.23 bc
Mean	20.2	24.4	1.76	2.99

1/ Duncan's new multiple range test: values with the same letter are not significantly different (P = .05).

Table 6. Average nodule weight mg/plant 7 and 10 weeks after seeding.

Strain	Sampling time weeks after seeding			
	7		10	
	Empire	Leo	Empire	Leo
867	4.8 b 1/	12.2 ab	39.4 b	57.0 ab
95C11	5.8 b	18.0 a	36.3 b	97.1 a
95C13	5.6 b	16.9 a	26.7 b	66.5 ab
P	5.6 b	13.4 ab	36.0 b	62.4 ab
Mean	5.5	15.1	34.6	70.7

1/ Duncan's new multiple range test: within each sampling values with the same letter are not significantly different (P = .05).

The dry weights of Leo shoots four weeks after seeding were greater than those of Empire (Table 7, Appendix 7). The shoot dry weights of Leo plants at the second sampling (7 weeks) were considerably greater than those of Empire (Table 7, Appendix 7). Leo inoculated with strains 95C11 and 95C13 had significantly heavier shoots than did Empire in combination with any strain. Strain effects within varieties were not significantly different. Similarly ten weeks after seeding the growth of Leo was superior to that of Empire (Table 7, Appendix 8). Although both varieties had the highest shoot dry weights when they were inoculated with 95C11 differences within varieties were not significant.

The variety Leo produced significantly more dry matter (kg/ha) than did Empire (Table 8, Appendix 9) in the fall of year of seeding. Strains of Lotus rhizobia did not significantly alter dry matter production within varieties. However, strain responses varied with variety; Leo in combination with 867 or 95C11 produced significantly higher dry matter yields than did Empire inoculated with P or 95C13.

The average protein content of shoots of Empire and Leo was not significantly different (Table 8). Strains were by contrast significantly different (Appendix 9).

Table 7. Dry weights of shoots in mg 4, 7, and 10 weeks after seeding

Strain	Sampling time weeks after seeding					
	4		7		10	
	Empire	Leo	Empire	Leo	Empire	Leo
867	4.5 a 1/	6.7 a	26.4 b	45.8 ab	247.4 b	302.0 ab
95C11	7.0 a	7.8 a	33.2 b	58.0 a	271.0 ab	421.5 a
95C13	3.7 a	7.9 a	27.3 b	57.4 a	214.1 b	318.1 ab
P	4.4 a	5.1 a	26.9 b	43.9 ab	208.3 b	337.9 ab
Mean	4.9	6.9	28.5	51.3	235.2	344.9

1/ Duncan's new multiple range test: within each sampling values with the same letter are not significantly different (P = .05).

Table 8. Dry matter yields (kg/ha) and protein content of shoots [in percent (N x 6.25)] of two varieties of birdsfoot trefoil in the fall of year of seeding.

Strain	Dry Matter (kg/ha)		Protein content (%)	
	Empire	Leo	Empire	Leo
867	1611 abc 1/	2366 a	20.2 a	20.0 ab
95C11	1876 abc	2455 a	17.8 b	18.6 ab
95C13	1424 bc	2328 ab	20.3 a	19.1 ab
P	1343 c	2160 abc	18.3 ab	19.5 ab
Mean	1563	2327	19.2	19.2

1/ Duncan's new multiple range test: values with the same letter are not significantly different (P = .05).

Within Empire shoots of plants inoculated with 867 and 95C13 contained significantly more protein than shoots from plants inoculated with 95C11. Within Leo differences were not significant.

DISCUSSION

Most (86-97%) of the plants of the varieties Empire and Leo had 1-4 nodules on each main root four weeks after seeding. Although initially nodulation of both varieties was slowest with strain 95C13 dry weight per plant was not decreased significantly. Seven weeks after seeding all plants were equally nodulated regardless of strain of Lotus rhizobia used. Variety differences in nodulation were large; seven weeks after seeding 72-87% of plants of Leo but only 37-55% of Empire, had 5 or more nodules on lateral roots. For Empire nodule fresh weight was smaller than that of Leo. With strain P both varieties tended to have many small nodules and the growth of plants was poor. Fewer but larger nodules were formed with strain 95C11 and this coincided with higher plant dry weights and dry matter yields. Large nodules have been found to be most effective in terms of nitrogen fixation (37).

From the beginning Leo appeared to grow faster than Empire. Dry weight per shoot and total dry matter production were both considerably higher for Leo than for Empire. Within varieties strain effects were not significantly different in terms of dry matter production. However, the protein content of shoots of Empire varied significantly in combination with different strains of

Lotus rhizobia. This seems to support our earlier finding (34) when under controlled conditions considerable variation was noted between strains. A hail storm eight weeks after seeding damaged the plants a little but the recovery was rapid and results were not influenced.

In this experiment the soil pH was 7.7. All four strains were acid producers and formed effective nodules on host plants. This observation supports the work of Brockwell et al. (5) who found that Rhizobium from L. corniculatus is suited to acid and alkali conditions. McKee (39,40) also found that good nodulation was obtained at pH range of 6.2 to 7.5. The absence of a native competing population of Lotus rhizobia plus a heavy rate of inoculum probably contributed to the high incidence of nodulation.

3. The effects of some herbicides and a companion crop on the growth and nodulation of Lotus corniculatus L. under field conditions.

ABSTRACT

The highest birdsfoot trefoil (Lotus corniculatus L.) dry matter yields were obtained when benefin, EPTC or 2,4-DB + dalapon were used as weed control. Benefin and EPTC effectively controlled grassy weeds. For Leo treated with EPTC or benefin and for Empire treated with benefin percent nodulation and nodule fresh weights per plant were lower than in the check in the early stages of development. Later nodulation was satisfactory.

2,4-DB + dalapon controlled both grassy and broadleaf weeds. Percent nodulation or nodule fresh weight per plant were not reduced. Paraquat was unsatisfactory as a weed control when sprayed after the emergence of weeds before the seeding of trefoil. A companion crop depressed the growth of birdsfoot trefoil and nodulation was poorer.

INTRODUCTION

In the establishment of pure birdsfoot trefoil (Lotus corniculatus L.) stands selective herbicides have been used to control competing weeds. Satisfactory results have been obtained by using chemicals alone or in combination with cultural practices.

Dalapon alone gave almost complete control of grassy weeds with little or no injury to the legume (54,55). A combination of 2,4-DB + dalapon has been used to control both grasses and broadleaf weeds (49,54,55). The nodulation of birdsfoot trefoil was not reduced by dalapon at the rates of 6 lbs/acre (6.6kg/ha) or less (33). Garcia and Jordan (17) showed that 2,4-DB alone or in combination with dalapon reduced nodulation and decreased the efficiency of nitrogen fixation in birdsfoot trefoil. This was a result of plant damage and abnormal root growth caused by 2,4-DB. Some injury to birdsfoot trefoil was caused by2,4-DB (49) or by 2,4-DB + dalapon (60). EPTC has been noticed to control effectively annual grassy weeds (35,36) or grassy and broadleaf weeds (60).

Companion crops significantly reduced stands and yields of birdsfoot trefoil (54,55). The depressing effect of shading or plant competition on the growth and nodulation was greater in birdsfoot trefoil than in alfalfa and red clover (11,40).

In this experiment the effect of using herbicides or an oat companion crop on the establishment and nodulation in pure trefoil stands was studied.

MATERIALS AND METHODS

The experiment was conducted on an Almasippi sandy soil at MacGregor, Manitoba. Plots were fertilized with a broadcast application of 62 kg/ha of P_2O_5 as superphosphate (0-45-0) and 62 kg/ha of K_2O as muriate of potash (0-0-60). The soil had a pH of 7.7 and a nitrogen content 11 ppm NO_3-N. The birdsfoot trefoil varieties Leo and Empire were seeded with a Planet Junior on May 28, 1969. Seeding rate was 7.6 kg/ha with 30 cm between rows. Seeding depth was approximately 1 cm. Seed was inoculated with a commercial inoculum at ten times the recommended rate. The seed was made sticky with a 46% (w/v) gum arabic water suspension before mixing with the inoculum.

The common weeds were:

Green foxtail - Setaria viridis

Red root pigweed - Amaranthus retroflexus

Lamb's quarters - Chenopodium album

Wild radish - Raphanus raphanistrum

Wild buckwheat - Polygonum convolvulus

Mustard - Brassica sp.

The following treatments were used:

(1) Check, where no herbicides were applied

(2) Companion crop, Sioux oats 50 kg/ha, sown between the rows. This was harvested in August 2½ months after seeding by pulling the plants from the soil.

(3) EPTC (ethyl N, N-dipropylthiolcarbamate) applied at 4.4 kg/ha active ingredient five days before seeding and incorporated with the soil immediately by using a rotavator.

(4) Benefin (N-butyl-N-ethyl-α,α,α-trifluoro-2, 6-dinitro-P-toluidine) at 1.3 l/ha active ingredients. This was also incorporated with soil.

(5) Paraquat (1,1'-dimethyl-4,4'-bipyridinium) was sprayed eight days after seeding of other plots at the rate of 7.0 kg/ha. The weeds were at the two to four leaf stage. Seeding was done two hours later.

(6) 2,4-DB (4(2,4-dichlorophenoxy) butyric acid) + dalapon (2,2-dichloropropionic acid) applied 23 days after seeding when weeds were at the 3-5 leaf stage. The rate of application was 1.43 kg/ha 2,4-DB + 1.67 kg/ha dalapon active ingredient. All herbicides were applied at 450 liters of water/ha.

The experimental design was a randomized, 4 replicate split-plot with varieties as main plot and herbicides as sub-plots. The sub-plot size was 2.1 x 4.8 m.

Initial establishment of Empire was poor due in part to damping-off. Stand counts were taken on July 31, 1969. During the summer several heavy rains as well as a hail storm on July 30 damaged stands but the plants recovered well.

Sampling was done 6, 9 and 12 weeks after seeding. Respectively 50, 25 and 10 plants were taken from the five inner rows of each sub-plot. Samples were later examined in laboratory. Two center rows were harvested (0.6 x 4.5 m) September 8 at which time Leo had reached the full bloom stage and Empire was in vegetative stage. Dry matter percentages were computed from an 800 gram subsample. Additional subsamples of 600 to 800 g were hand separated to determine botanical composition.

RESULTS

After six weeks of growth there were significant differences in percent nodulation between the treatments (Table 9, Appendix 10). In particular within the variety Leo plants treated with paraquat had significantly more nodules than did other treatments. The differences between other treatments were not significant. Where herbicides were incorporated with soil (EPTC and benefin) nodulation appeared to be poorer than in the check. Similarly, Empire on benefin treated plots was also poorly nodulated.

Nine weeks after seeding Leo showed considerably greater nodulation than did Empire (Table 9, Appendix 10). Differences among treatments were not significant although the percent nodulation again appeared to be lower when EPTC or benefin were applied and highest where paraquat was used. Twelve weeks after seeding all plants had nodules.

Nine weeks after seeding the nodule fresh weights per plant of Leo were considerably higher than those of Empire (Table 10, Appendix 11). Within varieties differences between treatments were not significant. For the variety Leo nodule fresh weights were highest in the check, paraquat or 2,4-DB + dalapon treated plots. Within Empire differences between treatments were small.

Table 9. Percent nodulation of birdsfoot trefoil varieties Empire and Leo 6, 9 and 12 weeks after seeding

TREATMENT	Sampling time weeks after seeding				
	6		9		12
	Empire	Leo	Empire	Leo	Empire & Leo
	1/				
Check	27.7 b	37.1 b	61.9 a	90.2 a	100.0
Companion crop (oats)	28.6 b	46.3 ab	65.5 a	90.6 a	100.0
EPTC	33.8 b	26.8 b	61.0 a	78.7 a	100.0
Benefin	18.7 b	30.8 b	62.4 a	76.8 a	100.0
Paraquat	46.5 ab	72.6 a	78.6 a	92.8 a	100.0
2,4-DB-Dalapon	36.0 b	40.9 b	70.5 a	84.5 a	100.0
Mean	31.9	42.4	66.6	85.6	100.0

1/ Duncan's new multiple range test: within each sampling values with the same letter are not significantly different (P = .05).

Table 10. Nodule fresh weight mg per plant 9 and 12 weeks after seeding

Treatment	Sampling time weeks after seeding			
	9		12	
	Empire	Leo	Empire	Leo
	1/			
Check	3.3 b	23.5 a	137.2 b	238.2 ab
Companion crop (oats)	3.6 b	15.2 ab	132.2 b	214.5 ab
EPTC	3.5 b	11.6 ab	225.0 ab	280.7 ab
Benefin	4.4 b	12.7 ab	249.4 ab	253.9 ab
Paraquat	5.1 b	19.3 a	201.4 ab	393.8 a
2,4-DB-Dalapon	4.7 b	19.0 a	203.0 ab	275.5 ab
Mean	4.1	16.9	191.4	276.1

1/ Duncan's new multiple range test: within each sampling values with the same letter are not significantly different (P = .05).

Twelve weeks after seeding nodule mass per plant was greater for Leo than for Empire (Table 10). Nodule fresh weights per plant were smallest when a companion crop or no herbicide (check) was used with Empire. On plots where birdsfoot trefoil growth was vigorous due to effective weed control (EPTC, benefin or 2,4-DB + dalapon) nodule mass per plant was high.

Differences in nodule fresh weights between varieties and treatments twelve weeks after seeding were not significant (Table 11). The interaction between variety and treatment was, however, significant (Appendix 12).

The number of nodules per plant varied considerably but no statistically significant differences were observed at this time (Table 11, Appendix 12). The number of nodules per plant of Leo in EPTC and paraquat treated plots was higher than in other plots. Empire had the smallest number of nodules per plant when established with a companion crop.

Although the number of viable seeds sown per meter was same for both varieties there were fewer plants per meter of row in plots of Empire (Table 11). Obviously damping off in the seedling stage was more serious in this variety than in comparable plots of Leo. The survival of seedlings of this slower growing variety could also have been influenced by the dry conditions of early summer. The number of plants per

Table 11. Fresh weight per nodule (mg) and number of nodules per plant 12 weeks and number of plants per meter of row 9 weeks after seeding.

Treatment	Fresh weight mg per nodule		Number of nodules per plant		Number of plants per meter of row	
	Empire	Leo	Empire	Leo	Empire	Leo
	1/					
Check	3.0 a	4.0 a	46.8 a	59.9 a	70.3 abcde	104.4 a
Companion crop (oats)	3.6 a	3.3 a	37.7 a	66.6 a	56.1 cde	97.0 a
EPTC	4.1 a	3.2 a	54.5 a	91.7 a	61.6 bcde	85.3 abcd
Benefin	3.9 a	4.1 a	64.1 a	61.7 a	52.4 de	94.0 ab
Paraquat	3.2 a	4.6 a	62.2 a	85.8 a	38.4 e	42.5 e
2,4-DB-dalapon	3.2 a	4.4 a	62.7 a	62.6 a	61.3 bcde	89.9 abc
Mean	3.5	3.9	54.6	71.4	56.7	85.5

1/ Duncan's new multiple range test: values with the same letter are not significantly different (P = .05).

meter of row was greatest within both varieties where no herbicide (check) was used. By contrast the number of plants was lowest in those plots treated with paraquat where damping off was most serious.

The per plant dry weight of Leo was greater than that of Empire after six weeks of growth (Table 12). Differences between the treatments were also significant (Appendix 13). The dry weights per plant were highest on check plots and lowest on EPTC and paraquat treated plots. The growth of both varieties was vigorous on benefin treated plots. Competition from the companion crop or weeds did not affect the growth of trefoil at this stage.

Similarly after nine weeks Leo had higher dry weights per plant than did Empire (Table 12.). These dry weights per plant were highest where EPTC, benefin or 2,4-DB + dalapon were used. The competing weeds in check and paraquat treated plots as well as the companion crop reduced the growth of trefoil.

Leo produced significantly higher dry matter yields than Empire under all treatments (Table 13, App. 13). For both varieties yields from EPTC, benefin and 2,4-DB + dalapon treated plots were considerably higher than from the three other treated plots. Companion crop

Table 12. Dry weights of plants (mg) 6 and 9 weeks after seeding and protein content of shoots (%) in the fall of year of establishment.

Treatment	Dry weight mg/plant				Protein content of shoots	
	Sampling time weeks after seeding					
	6		9			
	Empire	Leo	Empire	Leo	Empire	Leo
	1/					
Check	11.6 bcde	20.6 a	37.6 de	101.7 abc	20.2 ab	17.9 ab
Companion crop (oats)	10.0 de	19.0 ab	29.2 e	70.3 bcde	21.2 a	19.8 ab
EPTC	9.5 de	15.5 abcd	72.2 abcde	126.8 a	17.7 ab	18.1 ab
Benefin	10.3 cde	20.0 a	74.6 abcde	107.3 ab	17.3 ab	16.6 b
Paraquat	7.1 e	13.8 abcde	33.7 e	93.3 abcd	19.8 ab	19.0 ab
2,4-DB-dalapon	9.9 de	17.7 abc	49.5 cde	112.8 ab	19.2 ab	17.7 ab
Mean	9.7	17.8	49.4	102.0	19.2	18.2

1/ Duncan's new multiple range test: within each sampling values with the same letter are not significantly different (P = .05).

reduced dry matter yields considerably. The significant interaction between variety and treatment (Appendix 15) indicated that the ability of herbicides to control weeds was also related to the variety growth pattern. The effect of the herbicides was minimal in the late summer and weeds could take over unless the host variety was vigorously competitive.

Broadleaf weeds were controlled most effectively in both varieties with the addition of 2,4-DB + dalapon (Table 13). Other herbicides did not control broadleaf weeds very well. Generally less broadleaf weeds were present in the Leo than in Empire stands. This would indicate that Leo was competitively more successful than Empire. Grassy weeds were controlled totally by EPTC and benefin. 2,4-DB + dalapon was also satisfactory although in plots of Empire control was not complete.

Paraquat applied after the emergence of weeds but before the seeding of trefoil was not a satisfactory weed control method. Oat companion crop was effective in controlling grassy weeds only.

The protein contents of the shoots were highest in those plots where an oat companion crop was used (Table 12). Since these plants were generally weaker and at a less advanced stage of growth it would seem

Table 13. Dry matter yields (kg/ha) of birdsfoot trefoil, broadleaf and grassy weeds in the fall of year of establishment

Treatment	Birdsfoot trefoil		Broadleaf weeds in the stands of		Grassy weeds in the stands of	
	Empire	Leo	Empire	Leo	Empire	Leo
	1/					
Check	519 ef	1815 abc	797 a	929 a	512	227
Companion crop (oats)	424 f	1341 cdef	890 a	460 a	90	55
EPTC	1579 bcd	2667 a	954 a	660 a	0	0
Benefin	1472 cde	2530 a	947 a	810 a	36	0
Paraquat	654 def	1275 cdef	1227 a	954 a	681	660
2,4-DB-dalapon	1394 cde	2420 ab	524 a	372 a	355	71
Mean	1007	2008	890	698	279	169

1/ Duncan's new multiple range test: values with the same letter are not significantly different (P = .05).

that the observed differences in protein content are more a reflection on variety and stage of development and less a consequence of treatment per se.

DISCUSSION

In many regions successful results have been obtained when certain herbicides have been used in the establishment of pure birdsfoot trefoil stands. The main emphasis in these studies has been on botanical composition and control of specific weeds. It has been shown that under certain conditions nodulation of birdsfoot trefoil could be affected by the herbicide (17). This experiment studied the influence of several herbicides on the nodulation and establishment of pure trefoil stands under field conditions.

2,4-DB + dalapon effectively controlled both grassy and broadleaf weeds. After six weeks of growth the dry weights per plant were equal to the check and no visible symptoms of damage were noticed. Some injury has been reported in the trefoil stands treated with 2,4-DB (49) or with 2,4-DB + dalapon (60). In this experiment, however, birdsfoot trefoil grew well and dry matter yields were high.

Percent nodulation after six and nine weeks and nodule fresh weight per plant after nine and twelve weeks of growth were not reduced by the herbicide combination used in this experiment. Garcia and Jordan (17) noticed some reduction in nodulation and nitrogen fixation of birdsfoot trefoil by 2,4-DB

alone or in combination with dalapon.

EPTC and benefin controlled grassy weeds effectively but broadleaf weeds were not eliminated. However, in the early summer all weeds were under control and trefoil could grow without competition from other species. Percent nodulation six and nine weeks and nodule fresh weight per plant of Leo nine weeks after seeding on EPTC and benefin plots were lower than on the other plots. This would indicate slower early nodulation of Leo when EPTC and benefin were used. After twelve weeks percent nodulation was 100% within all treatments. Nodule fresh weight and number of nodules per plant were high.

The highest dry matter yields were obtained from EPTC and benefin treated plots. The growing conditions on these plots were good from the beginning because the weeds were not present. Later broadleaf weeds did not disturb the growth of birdsfoot trefoil seriously because the stands were well established. Under these conditions vigorous birdsfoot trefoil stands can be established by using EPTC, benefin or 2,4-DB + dalapon and inoculating the seed with many effective Lotus rhizobia.

Paraquat did not give satisfactory weed control in trefoil stands when this herbicide was applied

after the emergence of weeds in the spring. Weed control was poor and the trefoil yields were low. Good nodulation was possibly due to more favourable environmental conditions because seeding was done a week later than on the other plots.

The companion crop did not affect the growth of birdsfoot trefoil during the early stages of development. Later companion crop controlled grassy weeds quite effectively but oats and broadleaf weeds were competing with trefoil, plants were weak and yields were small. It has been shown previously that a companion crop will reduce trefoil stands (10,54,55). The nodulation of birdsfoot trefoil seemed to be adequate although later in the season it was poorer than on plots where good control of weeds was obtained. McKee (40) noticed that birdsfoot trefoil was not adequately nodulated if oats, weeds or orchardgrass were competing with the host plant. Under all treatments Leo grew more rapidly than Empire. This was indicated by higher plant dry weights and dry matter yields.

There were differences in protein contents of shoots between varieties and treatments. These were presumably partially due to differences in the stage

of development. Leo had started blooming and Empire was in vegetative stage. Where a companion crop or weeds provided competition birdsfoot trefoil was weaker in an earlier stage of development.

SECTION 3

Discussion of entire research program reported in Section 2

GENERAL DISCUSSION

The early growth and development of birdsfoot trefoil has been studied extensively. It has been observed that the seedling vigor of this species is poor. The establishment of new stands is difficult; however, once established, birdsfoot trefoil will survive for several years.

Nodulation failures of birdsfoot trefoil have been reported to be frequent. When soil samples were collected from several Manitoba locations (Appendix 1) and plants were grown in pots in the greenhouse effective nodulation of birdsfoot trefoil was not obtained. Only experimental areas seemed to contain some effective Lotus rhizobia on birdsfoot trefoil probably due to inoculation of seed.

In order to get a good nodulation of birdsfoot trefoil under these conditions effective Lotus rhizobia must be introduced into the soil with the seed. L. corniculatus and L. uliginosus require specific strains of Lotus rhizobia (1,8,13). The growth chamber experiments, as reported in part one, indicated that all six strains of Lotus rhizobia used were effective on L. corniculatus.

The symbiotic nitrogen fixation had an optimum root temperature from 18° to 24°C. Below 18°C and above 24°C there was generally a decrease in nitrogen fixation and growth of birdsfoot trefoil. With other species the same kind of sensitivity has been noticed (18,19,20,31,44,52).

At 18°, 24° and 30°C there were considerable differences between varieties and strains of Lotus rhizobia. Empire was slow growing, whereas Leo and Viking grew faster at all root temperatures. At 9° and 12°C differences were small between the strains of Lotus rhizobia and birdsfoot trefoil varieties.

In appendix 18 soil temperatures at Glenlea Research Station, Glenlea, Manitoba are given for May - September, 1968 and 1969. In spring and early summer soil temperatures are relatively low for a long period of time. The behaviour of strains of Lotus was not studied at root temperatures between 18° and 12°C. Possibly at this temperature range differences could be found i.e. some strains could fix more nitrogen at lower root temperatures than other strains. More effective symbiotic combinations could hasten the early growth of birdsfoot trefoil under these conditions.

Before the symbiotic nitrogen fixation had started young seedlings showed symptoms of nitrogen deficiency. The seed reserves were small and time to the first active nodules was quite long (15-18 days). This was a critical period and many plants suffered or died from an apparent deficiency in nitrogen.

Later plants receiving combined nitrogen grew much better than the ones dependent on symbiotic nitrogen fixation at all root temperatures. This indicates

that symbiotic nitrogen was not sufficient for optimum growth of trefoil under these conditions. Particularly under temperature stress at high (30°C) and low (9°, 12°C) root temperatures in general little nitrogen was fixed and the plants showed symptoms of nitrogen deficiency. Nitrogen control plants grew quite well at these temperatures. The interactions between the three factors were significant. See discussion on page 39.

In paper 2 results are reported when four of the six strains of Lotus rhizobia were tested under field conditions. Again Leo grew faster than Empire. The strain effects within varieties did not show significant variation in terms of dry matter yields although there was a considerable difference in protein content of shoots of Empire. Nodulation was satisfactory with any of the four strains. Only strain 95C13 showed somewhat slower nodulation at the early stages of growth but this did not affect the growth of plants. Although the nitrogen content of soil was low the colour of leaves was deep green and plants were vigorous from the start indicating that nitrogen was being fixed. Eight weeks after seeding, a hailstorm damaged the plants slightly but they recovered rapidly.

The number of rhizobia which is introduced into the soil with the seed has been noticed to be important.

Where the native population of rhizobia is high many organisms per seed are needed to assure good nodulation of soybeans by desired strain(s) (6). Under circumstances where the native population of rhizobia is low or there are no effective strains present, introduced rhizobia may have to survive in the new environment possibly for a long period of time before the infection and nodulation of host can happen. If a high number of rhizobia is introduced or they are protected against harmful factors, then likely more viable organisms will be present at the time of infection. In this experiment environmental conditions were favourable for plant growth, high numbers of effective rhizobia were introduced and host plants bore effective nodules. Possibly in this way the nodulation failures of birdsfoot trefoil can be reduced.

Paper 3 reports results of different practices of weed control in birdsfoot trefoil stands. Earlier reports indicated that 2,4-DB (49) or 2,4-DB + dalapon (60) caused some damage to birdsfoot trefoil at its early stages of development. Nodulation and nitrogen fixation could also be depressed (17). After six weeks of growth no damage to birdsfoot trefoil was visible at the rates of herbicides used. Dry weights of plants and nodulation were similar to the check.

2,4-DB + dalapon, EPTC and benefin provided a good weed control and yields were highest on these plots. However, some reduction in nodulation occurred when EPTC or benefin were used. This could be due to the harmful effect of EPTC or benefin and/or a loose seedbed, because one week prior to seeding the herbicides were incorporated by using a rotavator. Later in summer the growth and nodulation of these plants was excellent. Because weather conditions were favorable EPTC and benefin also controlled broadleaved weeds in the early summer. If these weeds are not controlled possibly 2,4-DB can be used in combination with EPTC and benefin. Although some broadleaf weeds were present in stands in late summer they did not seriously affect the growth of birdsfoot trefoil.

Surprisingly paraquat controlled weeds quite poorly. At the time of spraying, weeds were small (2-6 cm) and obviously many plants started growing afterwards.

In an environment where other species were not competing good growth of birdsfoot trefoil could be obtained during the year of establishment. If weeds were not controlled or a companion crop was used the growth of birdsfoot trefoil was retarded. Birdsfoot trefoil is a poor competitor and shading will drastically depress the growth and nodulation of the legume (11,40,54,55).

By using herbicides, inoculating the seed with many effective rhizobia (10x recommended rate) and maintaining satisfactory fertility level of soil, good growth and nodulation of birdsfoot trefoil can be obtained during the year of establishment under Manitoba conditions.

SECTION 4

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Appendices

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APPENDIX 1

Isolation of rhizobia effective on *Lotus corniculatus* from Manitoba soils

Location	Crop	Result[x]
At the beginning of June, 1967		
1/ Dauphin	summerfallow	-
2/ Gladstone	summerfallow	-
3/ Lundar	experimental area (*Lotus corniculatus*)	-
4/ The Pas	natural meadow (*Phragmites* sp.)	-
5/ The Pas	experimental area (Medicago sp.)	-
6/ Swan River	summerfallow	-
7/ Wabowden	experimental area (*Trifolium* sp.)	-
October 11-15, 1967.		
1/ Carberry	potato	-
2/ Eriksdale	experimental area (*L. corniculatus*)	-
3/ Eriksdale	oats	-
4/ Grosse Isle	wheat	-
5/ Kenora, Ontario	*Lupinus* sp.	-

6/ Lundar	experimental area (L. Corniculatus)	+
7/ Lundar	barley	-
8/ MacGregor	Medicago sp.	-
9/ Portage la Prairie	wheat	-
10/ Selkirk	summerfallow	-
11/ Stonewall	Medicago sp.	-
12/ Sylven	newly broken land	-

x - = no nodulation of L. corniculatus

\+ = effective nodulation of L. corniculatus

APPENDIX 2

Analysis of variance of total dry weights of plants in root temperature experiment

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Level of Significance	
Total	1049	151.146				
Blocks	9	0.156	0.017			
Temperature (T)	4	47.494	11.874	368.29	1%	
Error I	36	1.161	0.032			CV=9.5%
Variety (V)	2	25.504	12.752	509.62	1%	
T x V	8	2.079	0.260	10.39	1%	
Error II	90	2.252	0.025			CV=8.3%
Rhizobium (R)	6	45.823	7.637	366.82	1%	
T x R	24	6.529	0.272	13.07	1%	
V x R	12	0.831	0.069	3.33	1%	
T x V x R	48	2.461	0.051	2.46	1%	
Error III	810	16.864	0.021			CV-7.6%

APPENDIX 3

Analysis of variance of total nitrogen content of plants in root temperature experiment

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Level of Significance	
Total	899	59.141				
Blocks	9	0.098	0.011			
Temperature (T)	4	34.791	8.698	578.15	1%	
Error I	36	0.542	0.015			CV=26.8%
Variety (V)	2	11.027	5.514	549.30	1%	
T x V	8	2.279	0.285	28.38	1%	
Error II	90	0.903	0.010			CV=21.9%
Rhizobium (R)	5	0.385	0.077	7.96	1%	
T x R	20	1.087	0.054	5.61	1%	
V x R	10	0.188	0.019	1.95	5%	
T x V x R	40	1.312	0.033	3.39	1%	
Error III	675	6.537	0.010			CV=21.5%

APPENDIX 4

Analysis of variance of percent nodulation of varieties Empire and Leo inoculated with strains of Lotus rhizobia 4 and 7 weeks after seeding

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Level of Significance
1/					
Total	39	3145.16			
Blocks	4	350.60	87.65		
Strains	3	451.27	150.42	2.50	N.S.
Error I	12	722.34	60.20		CV-8.5%
Varieties	1	0.91	0.91	0.01	N.S.
Strains x Var.	3	137.86	45.95	0.50	N.S.
Error II	16	1482.18	92.64		CV-10.5%
2/					
Total	39	468.46			
Blocks	4	20.76	5.19		
Strains	3	96.21	32.07	3.76	5%
Error I	12	102.31	8.53		CV-3.0%
Varieties	1	12.77	12.77	1.01	N.S.
Strains x Var.	3	33.72	11.24	0.89	N.S.
Error II	16	202.69	12.67		CV-3.6%

1/ 4 weeks after seeding

2/ 7 weeks after seeding

APPENDIX 5

Analysis of variance of the number of nodules per plant and fresh weight per nodule in Lotus rhizobia strain test 10 weeks after seeding

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Level of Significance
1/					
Total	39	1594.87			
Blocks	4	82.52	20.63		
Strains	3	329.94	109.98	2.47	N.S.
Error I	12	534.77	44.56		CV=29.9%
Varieties	1	175.98	175.98	6.91	5%
Strains x Var.	3	64.24	21.42	0.84	N.S.
Error II	16	407.41	25.46		CV=22.6%
2/					
Total	39	69.23			
Blocks	4	11.19	2.80		
Strains	3	16.08	5.36	10.33	1%
Error I	12	6.23	0.52		CV=30.3%
Varieties	1	15.09	15.09	20.27	1%
Strains x Var.	3	8.73	2.91	3.91	5%
Error II	16	11.92	0.74		CV=36.4%

1/ Number of nodules per plant

2/ Fresh weight per nodule

APPENDIX 6

Analysis of variance of average nodule weight per plant in Lotus rhizobia strain test 7 and 10 weeks after seeding

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Level of Significance
1/					
Total	39	1935.33			
Blocks	4	163.17	40.79		
Strains	3	73.15	24.38	0.69	N.S.
Error I	12	424.20	35.35		CV=57.7%
Varieties	1	939.94	939.94	51.82	1%
Strains x Var.	3	44.65	14.88	0.82	N.S.
Error II	16	290.21	18.14		CV=41.4%
2/					
Total	39	38114.56			
Blocks	4	7578.77	1894.69		
Strains	3	2665.38	888.46	1.61	N.S.
Error I	12	6623.24	551.94		CV=44.6%
Varieties	1	13057.32	13057.32	37.68	1%
Strains x Var.	3	2644.59	881.53	2.54	N.S.
Error II	16	5545.26	346.58		CV-35.3%

1/ 7 weeks after seeding

2/ 10 weeks after seeding

APPENDIX 7

Analysis of variance of dry weights of shoots (mg) in Lotus rhizobia strain test 4 and 7 weeks after weeding

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Level of Significance
1/					
Total	39	238.87			
Blocks	4	24.55	6.14		
Strains	3	38.37	12.79	3.21	N.S.
Error I	12	47.80	3.98		CV=33.8%
Varieties	1	38.22	38.22	8.77	1%
Strains x Var.	3	20.23	6.74	1.55	N.S.
Error II	16	69.70	4.36		CV=35.4%
2/					
Total	39	11503.66			
Blocks	4	2911.01	727.75		
Strains	3	730.48	243.49	2.69	N.S.
Error I	12	1085.82	90.49		CV=23.9%
Varieties	1	5207.50	5207.50	63.47	1%
Strain x Var.	3	256.09	85.36	1.04	N.S.
Error II	16	1312.75	82.05		CV=22.3%

1/ 4 weeks after seeding 2/ 7 weeks after seeding

APPENDIX 8

Analysis of variance of dry weights of shoots (mg) in Lotus rhizobia strain test 10 weeks after seeding

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Level of Significance
Total	39	508321.81			
Blocks	4	154212.56	38553.14		
Strains	3	42524.00	14174.66	1.66	N.S.
Error I	12	102569.44	8547.45		CV=31.9%
Varieties	1	120307.81	120307.81	25.37	1%
Strains x Var.	3	12829.19	4276.39	0.90	N.S.
Error II	16	75878.88	4742.42		CV=23.7%

APPENDIX 9

Analysis of variance of dry matter yields (kg/ha) and protein content of shoots (%) in Lotus rhizobia strain test in the fall of year of seeding

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Level of Significance
1/					
Total	39	20975824.00			
Blocks	4	9285101.00	2321275.00		
Strains	3	929072.00	309690.63	0.93	N.S.
Error I	12	4015808.00	334650.63		CV=29.7%
Varieties	1	5835436.00	5835436.00	121.46	1%
Strains x Var.	3	141700.00	47233.33	0.98	N.S.
Error II	16	768691.00	48043.19		CV=11.3%
2/					
Total	39	98.23			
Blocks	4	25.60	6.40		
Strains	3	20.86	6.95	4.00	5%
Error I	12	20.85	1.74		CV=6.9%
Varieties	1	0.17	0.17	0.13	N.S.
Strains x Var.	3	9.05	3.02	2.22	N.S.
Error II	16	21.70	1.36		CV=6.1%

1/ Dry matter yields

2/ Protein content

APPENDIX 10

Analysis of variance of percent nodulation in herbicide experiment

6 and 9 weeks after seeding

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Level of Significance
1/					
Total	47	15822.50			
Blocks	3	1908.02	636.01		
Variety	1	1330.36	1330.36	2.69	N.S.
Error I	3	1486.54	495.51		CV-60%
Treatment	5	5797.29	1159.46	8.63	1%
Var. x Treatment	5	1267.83	253.57	1.89	N.S.
Error II	30	4032.44	134.41		CV-31%
2/					
Total	47	13220.87			
Blocks	3	1466.96	488.99		
Variety	1	4303.54	4303.54	12.99	5%
Error I	3	993.18	331.06		CV-24%
Treatment	5	1440.75	288.15	1.87	N.S.
Var. x Treatment	5	393.78	78.76	0.51	N.S.
Error II	30	4622.63	154.09		CV=16%

1/ 6 weeks after seeding

2/ 9 weeks after seeding

APPENDIX 11

Analysis of variance of nodule fresh weight (mg) per plant 9 and 12 weeks after seeding in herbicide experiment

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Level of Significance
1/					
Total	47	3971.56			
Blocks	3	464.69	154.90		
Variety	1	1960.94	1960.93	27.02	5%
Error I	3	217.69	72.56		CV=81%
Treatment	5	214.38	42.88	1.42	N.S.
Var x Treatment	5	206.81	41.36	1.37	N.S.
Error II	30	907.25	30.24		CV=52%
2/					
Total	47	481527.13			
Blocks	3	36824.67	12274.87		
Variety	1	86148.06	86148.06	4.40	N.S.
Error I	3	58693.13	19564.38		CV=60%
Treatment	5	84472.31	16894.46	2.87	5%
Var x Treatment	5	38619.50	7723.90	1.31	N.S.
Error II	30	176769.38	5892.31		CV=33%

1/ 9 weeks after seeding

2/ 12 weeks after seeding

APPENDIX 12

Analysis of variance of fresh weight per nodule (mg) and number of nodules per plant 12 weeks after seeding in herbicide experiment

	Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Level of Significance
1/						
	Total	47	31.97			
	Blocks	3	2.34	0.78		
	Variety	1	2.09	2.09	1.24	N.S.
	Error I	3	5.04	1.68		CV=35%
	Treatment	5	2.02	0.40	1.04	N.S.
	Var x Treatment	5	8.85	1.77	4.57	1%
	Error II	30	11.62	0.39		CV=17%
2/						
	Total	47	30589.20			
	Blocks	3	3069.48	1023.16		
	Variety	1	3358.37	3358.37	1.71	N.S.
	Error I	3	5898.87	1966.29		CV=70%
	Treatment	5	3471.82	694.36	1.70	N.S.
	Var x Treatment	5	2555.70	511.14	1.25	N.S.
	Error II	30	12234.95	407.83		CV=32%

1/ Fresh weight / nodule

2/ Number of nodules / plant

APPENDIX 13

Analysis of variance of plant dry weights (mg) in herbicide experiment

6 and 9 weeks after seeding

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Level of Significance
1/					
Total	47	1490.61			
Blocks	3	281.34	93.78		
Variety	1	774.41	774.41	17.20	5%
Error I	3	135.04	45.02		CV=49%
Treatment	5	163.83	32.77	8.53	1%
Var x Treatment	5	20.75	4.15	1.08	N.S.
Error II	30	115.24	3.84		CV=14%
2/					
Total	47	64906.56			
Blocks	3	1396.40	465.47		
Variety	1	33174.81	33174.81	17.85	5%
Error I	3	5574.21	1858.09		CV=57%
Treatment	5	13489.45	2697.89	8.41	1%
Var x Treatment	5	1652.53	330.51	1.03	N.S.
Error II	30	9619.52	320.65		CV=24%

1/ 6 weeks after seeding

2/ 9 weeks after seeding

APPENDIX 14

Analysis of variance of number of plants per meter of row 9 weeks and protein content of shoots in the fall of year of establishment in herbicide experiment.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Level of Significance
1/					
Total	47	30184.61			
Blocks	3	1085.61	361.87		
Variety	1	9982.12	9982.12	24.82	5%
Error I	3	1206.69	402.23		CV=28%
Treatment	5	10102.94	2020.59	10.35	1%
Var x Treatment	5	1949.61	389.92	1.99	N.S.
Error II	30	5857.67	195.26		CV=20%
2/					
Total	47	180.87			
Blocks	3	5.22	1.74		
Variety	1	13.13	13.13	5.28	N.S.
Error I	3	7.45	2.48		CV=8%
Treatment	5	62.97	12.59	4.51	1%
Var x Treatment	5	8.31	1.66	0.60	N.S.
Error II	30	83.79	2.79		CV=9%

1/ Number of plants per meter of row

2/ Protein content of shoots

APPENDIX 15

Analysis of variance of birdsfoot trefoil and broadleaf weed dry matter yields in herbicide experiment (Log_{10} x transformed).

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Level of Significance	
1/						
Total	47	4.939				
Blocks	3	0.244	0.081			
Variety	1	1.609	1.609	34.4	1%	
Error I	3	0.140	0.047			CV=7%
Treatment	5	1.745	0.349	12.7	1%	
Var x Treatment	5	0.374	0.075	2.7	5%	
Error II	30	0.827	0.028			CV=5%
2/						
Total	47	9.374				
Blocks	3	0.378	0.126			
Variety	1	0.338	0.338	0.4	N.S.	
Error I	3	2.358	0.786			CV=32%
Treatment	5	2.056	0.411	3.3	5%	
Var x Treatment	5	0.546	0.109	0.9	N.S.	
Error II	30	3.698	0.123			CV=13%

1/ Birdsfoot Trefoil

2/ Broadleaf Weeds

Appendix 16. Total dry weights of plants (mg/plant) at different root temperatures (log_{10}x transformed) 1/

Variety	Strain	Root temperature °C 9	12	18	24	30	Mean
Empire	867	1.328	1.495	1.873	1.869	1.365	1.586
	868	1.410	1.515	1.763	1.728	1.479	1.579
	95C11	1.440	1.490	1.749	1.799	1.381	1.572
	95C13	1.471	1.452	1.832	1.886	1.497	1.628
	L	1.376	1.538	1.738	1.938	1.463	1.611
	P	1.351	1.592	1.594	2.032	1.423	1.598
	N-contr.	1.928	1.899	2.450	2.367	2.473	2.223
	Mean	1.472	1.568	1.857	1.946	1.583	1.685
Leo	867	1.685	1.777	2.355	2.282	1.808	1.981
	868	1.733	1.726	2.119	2.414	2.075	2.013
	95C11	1.595	1.766	2.207	2.290	1.821	1.936
	95C13	1.758	1.736	2.055	2.288	2.202	2.008
	L	1.678	1.693	2.193	2.374	2.051	1.998
	P	1.652	1.695	2.127	2.290	1.921	1.937
	N-contr.	2.151	2.122	2.702	2.656	2.774	2.481
	Mean	1.750	1.788	2.251	2.371	2.093	2.051
Viking	867	1.693	1.741	2.257	2.210	1.700	1.920
	868	1.526	1.620	2.159	2.101	1.915	1.864
	95C11	1.616	1.648	2.038	2.255	1.733	1.858
	95C13	1.683	1.716	2.103	2.031	1.851	1.877
	L	1.595	1.641	2.041	2.217	1.871	1.873
	P	1.626	1.680	2.065	1.998	1.759	1.826
	N-contr.	2.143	2.225	2.746	2.727	2.776	2.523
	Mean	1.697	1.753	2.201	2.220	1.944	1.963

1/ Standard error of difference between

Two varieties	0.012
Two root temperatures with the same or different varieties	0.028
Two varieties with the same or different strains of Lotus rhizobia	0.029
Two varieties with the same root temperature and strain of Lotus rhizobia	0.065
Two root temperatures with the same variety and strain of Lotus rhizobia	0.066

Appendix 17. Nitrogen contents of plants (mg/plant) at different root temperatures ($\log_{10}(x + 1)$ transformed) 1/

Variety	Strain	Root temperature °C					
		9	12	18	24	30	Mean
Empire	867	0.156	0.222	0.488	0.540	0.138	0.309
	868	0.176	0.275	0.429	0.441	0.178	0.300
	95C11	0.187	0.280	0.418	0.487	0.176	0.309
	95C13	0.205	0.240	0.497	0.558	0.229	0.346
	L	0.150	0.264	0.406	0.573	0.160	0.310
	P	0.149	0.289	0.306	0.653	0.151	0.309
	Mean	0.170	0.262	0.424	0.542	0.172	0.314
Leo	867	0.306	0.429	0.936	0.938	0.360	0.594
	868	0.336	0.397	0.755	1.051	0.520	0.611
	95C11	0.272	0.410	0.807	0.929	0.403	0.564
	95C13	0.342	0.390	0.693	0.959	0.731	0.623
	L	0.281	0.348	0.777	0.975	0.484	0.573
	P	0.278	0.383	0.719	0.874	0.398	0.530
	Mean	0.303	0.393	0.781	0.954	0.483	0.583
Viking	867	0.301	0.374	0.798	0.782	0.297	0.510
	868	0.218	0.295	0.738	0.738	0.393	0.476
	95C11	0.271	0.325	0.662	0.871	0.360	0.498
	95C13	0.291	0.341	0.691	0.700	0.439	0.492
	L	0.248	0.295	0.640	0.777	0.326	0.457
	P	0.273	0.309	0.678	0.619	0.269	0.430
	Mean	0.267	0.323	0.701	0.748	0.347	0.477

1/ Standard error of difference between

Two varieties	0.008
Two root temperatures with the same or different varieties	0.020
Two varieties with the same or different strains of *Lotus* rhizobia	0.020
Two varieties with the same root temperature and strain of *Lotus* rhizobia	0.044
Two root temperatures with the same variety and strain of *Lotus* rhizobia	0.045

Appendix 18. Soil temperatures (°C) at Glenlea Research Station, Glenlea, Manitoba. May-September 1968

Date	May		June		July		August		September	
	Depth in cm									
	5	10	5	10	5	10	5	10	5	10
1	7.8	6.7	11.7	10.6	13.4	12.2	15.6	15.0	13.9	13.9
2	8.9	7.2	10.7	9.5	11.1	11.1	16.7	16.1	12.8	12.8
3	6.1	6.1	13.4	11.7	14.5	13.4	17.2	16.1	13.4	12.8
4	5.0	4.5	15.6	13.9	14.5	13.9	17.2	16.1	12.4	12.2
5	2.5	2.9	14.5	12.8	13.9	13.4	18.4	17.2	12.8	12.2
6	6.7	5.6	13.9	12.2	16.7	15.6	19.5	18.4	11.7	11.7
7	6.1	5.0	12.8	11.7	18.4	16.7	17.8	16.7	12.2	11.7
8	7.2	5.6	12.2	11.1	17.2	16.7	17.2	16.7	11.7	11.1
9	2.9	3.4	12.8	11.7	13.9	13.4	15.6	15.6	10.6	10.6
10	5.0	5.0	12.8	11.7	14.5	13.9	14.5	14.5	12.8	12.2
11	3.4	3.9	12.2	11.1	17.2	16.1	14.5	13.9	13.4	12.8
12	7.2	6.1	10.0	10.0	16.1	15.0	16.1	15.6	14.5	13.4
13	7.8	6.7	13.9	12.2	19.5	17.8	15.6	15.0	15.6	14.5
14	10.6	8.9	11.1	11.1	17.2	16.7	12.2	12.8	15.0	14.5
15	8.9	8.9	10.0	10.0	17.8	17.2	13.9	13.4	16.7	15.6
16	4.5	4.5	11.7	11.1	18.4	17.2	15.0	13.9	16.7	15.0
17	4.5	4.5	12.8	12.2	18.4	16.7	11.7	12.2	15.6	15.0
18	3.4	2.9	13.4	12.8	17.2	16.7	14.5	13.4	13.4	12.8
19	2.9	2.9	12.8	12.2	16.7	16.1	15.0	13.9	14.5	13.4
20	3.4	3.4	10.0	13.4	18.9	17.8	15.0	13.9	14.5	13.9
21	5.0	5.0	15.0	13.9	17.8	17.2	13.9	13.4	14.5	13.9
22	6.7	6.7	13.9	13.4	15.6	15.0	15.6	14.5	15.0	13.9
23	6.7	6.7	14.5	13.4	17.8	16.7	16.1	15.0	13.9	13.9
24	9.5	8.9	13.9	13.4	15.6	15.6	15.6	14.5	12.8	12.8
25	10.6	9.5	12.8	12.2	16.7	16.1	13.9	13.4	11.7	11.1
26	10.6	9.5	12.8	12.2	17.2	16.7	13.4	12.8	10.0	10.0
27	10.0	8.9	14.5	13.4	15.0	15.0	13.4	12.8	10.0	10.0
28	10.0	8.9	15.0	13.9	13.9	13.9	15.0	13.9	10.0	9.5
29	10.0	8.9	14.5	13.9	15.6	15.0	17.2	15.6	9.5	9.5
30	11.1	10.0	14.5	13.9	15.6	15.0	16.7	15.0	11.1	10.6
31	11.7	10.6			14.5	14.5	15.0	14.5		

Appendix 18. Soil temperatures (°C) at Glenlea Research Station, Glenlea, Manitoba. May-September 1969

Date	May		June		July		August		September	
	Depth in cm									
	5	10	5	10	5	10	5	10	5	10
1	5.6	4.5	8.4	8.4	12.8	11.7	16.1	16.1	15.0	15.0
2	5.0	4.5	6.7	6.7	12.2	11.7	17.2	16.7	16.7	15.6
3	5.0	4.5	8.4	7.8	13.9	12.8	18.9	17.8	17.2	16.1
4	6.1	5.0	9.5	8.4	14.5	13.4	18.9	17.8	17.8	16.7
5	8.4	6.7	11.7	10.9	13.4	12.8	18.4	17.8	18.4	17.2
6	7.2	6.7	10.6	10.0	13.9	13.4	19.5	17.8	17.2	16.7
7	6.7	5.6	10.0	9.5	13.9	12.8	17.8	17.2	16.7	16.1
8	6.1	5.0	8.9	8.9	15.6	13.9	17.2	16.7	15.6	15.0
9	4.5	3.9	11.1	10.0	16.1	15.0	18.4	17.2	13.9	13.9
10	3.9	3.4	12.2	11.7	16.7	15.6	18.9	17.8	15.6	14.5
11	2.8	2.8	8.9	8.9	17.2	16.1	19.5	18.9	13.4	13.4
12	2.8	2.8	8.4	7.8	18.9	17.8	20.5	19.5	14.5	13.9
13	4.5	3.9	7.2	7.2	20.0	18.9	20.0	18.9	15.0	14.5
14	6.7	5.6	9.5	8.9	18.9	18.4	18.4	17.8	17.2	15.6
15	8.4	7.2	9.5	8.9	18.9	17.8	17.8	17.2	18.4	16.1
16	6.1	5.6	11.7	11.1	17.2	16.7	17.8	17.2	13.9	13.9
17	3.9	4.5	10.6	10.0	17.2	16.7	18.9	17.8	12.2	12.2
18	5.6	5.0	12.2	11.1	17.2	16.7	17.2	16.7	12.8	12.2
19	5.6	5.6	10.0	10.0	17.2	16.1	16.7	16.1	13.9	14.3
20	3.9	3.9	8.4	8.4	16.1	15.6	17.2	16.7	15.0	13.9
21	4.5	4.5	10.6	9.5	17.2	16.1	17.8	17.2	15.6	14.5
22	5.6	5.0	11.7	10.6	17.8	16.7	19.5	18.4	15.0	14.5
23	7.2	6.7	12.2	11.1	17.2	16.1	19.5	18.4	12.2	12.2
24	7.2	6.7	13.4	12.2	16.7	16.1	20.0	18.9	11.7	11.7
25	6.1	5.6	14.5	12.8	16.1	16.1	20.0	18.9	11.7	11.1
26	8.9	7.2	13.9	12.8	17.2	16.1	20.0	18.9	11.7	11.1
27	13.4	11.1	14.5	13.4	17.2	16.1	18.9	17.8	10.0	10.0
28	13.4	11.7	11.7	11.1	16.7	16.1	18.4	17.8	9.5	9.5
29	11.1	10.6	13.9	12.8	17.2	16.1	19.5	18.4	10.6	10.0
30	11.1	10.6	12.2	12.2	18.9	17.8	18.4	17.8	8.4	8.9
31	12.2	11.1			17.2	16.7	16.7	16.7		