Legumes in Reforestation: Field Screening Trials

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1 INTRODUCTION

Nitrogen is the principal nutrient limiting productivity of agricultural and forest crops. It is also readily lost from soils as a consequence of nitrification, denitrification, and leaching, as well as through biomass removal during harvesting of the crop. In natural ecosystems, nitrogen inputs may be derived from small amounts deposited in rain water and by living organisms, through biological nitrogen fixation. On low nitrogen status soils (such as forest soils following severe fire, or severely disturbed and exposed mineral soils) recolonization by nitrogen-fixing organisms restores fertility over time. In forest and woodland ecosystems, it has been estimated that approximately $40 \times 10^6$ t of nitrogen are fixed annually by leguminous trees and woody actinorrhizal plants (Burns and Hardy 1975).

In intensive agriculture and forestry, demands for increased productivity have been met by the addition of fertilizer nitrogen derived from fossil fuels. As interest in more intensive management of nitrogen fertility in forest ecosystems has increased, so have general concerns over the cost of forest fertilization and the consequences of extensive synthetic fertilizer use on soil and ground water quality. Although immediate benefits from fertilization can sometimes be predicted from agricultural experience and short-term forestry experiments, it may be more appropriate for foresters to determine the long-term management prognosis: Can, or will, the productivity of our forest lands be maintained? How might long-term fertility needs best be met?

1.1 Enhancing Forest Fertility with Legumes

Forest fertilization is used primarily to increase timber yields per unit of time. Economically justified forest fertilization is usually carried out within 10 years of harvest, (Miller and Flight 1979). Nevertheless, the use of synthetic fertilizers will be regulated by the cost of fertilization and value of the wood (Beuter 1979). It is a management practice that can be adjusted to business cycles. Fertilization has been viewed as a strategic investment based on evaluation of short-term benefits and costs. Such an approach may preclude the long-term commitment required to ensure sustained productivity of the forest over extended rotation periods.

The use of nitrogen-fixing organisms to enhance fertility during reforestation may be more amenable than synthetic fertilizers to the natural time scale of forest regeneration. Legume species have been used in reforestation and related research projects in central Europe, Australia, New Zealand, and the USA (Jorgensen 1980). Their primary function has been to ameliorate soil nitrogen deficiencies via biological nitrogen fixation and, through eventual nutrient cycling, to supply the associated tree crop with a form of long-term, slow release available nitrogen. Other benefits of using herbaceous legumes in forestry may include: an economic return in forage where integrated multiple use is practised; competition with weeds; and the establishment of a more suitable micro-climate for tree seedling regeneration and growth.

The actual results and benefits from legume use, however, vary widely. This variability is primarily a consequence of the plant-endophyte combinations used and their interaction with site climate and soil conditions. Satisfactory application of biological nitrogen fixation technology will require the appropriate use of selected legumes and compatible Rhizobium inoculants.

1.2 Screening and Selecting Nitrogen-fixing Species

Agronomically important legumes are an attractive option for the development of screening trials, since some knowledge of their environmental adaptation is available, and commercial seed and inoculants are generally attainable at a relatively low cost. According to Jorgensen's (1980) selection criteria, appropriate candidate legumes should:

1. be able to fix 50-100 kg of N/ha per year for 3-5 years;
2. be able to tolerate infertile, acid soils;
3. be easy to establish despite poor soil conditions and weed competition;
4. be perennials or reseeding annuals;
5. be shade tolerant; and
6. be non-toxic and beneficial as forage.

Such information is not widely available for many appropriate plant species. Nevertheless, enough is known through experience that performance may be reasonably predicted for some species (see Appendix 3).

Compared to Australia, New Zealand, the USA, and Central Europe in the use of biological nitrogen-fixing species in forestry, Canada and British Columbia are about 20-50 years behind in experience. This means the province’s primary effort must be to screen and select appropriate nitrogen-fixing plant species and microbial symbionts. Guidelines for doing so are presented here.

2 OBJECTIVES

This report is intended to be a first step in helping foresters, agrologists, and researchers to develop an effective knowledge base and initial operational recommendations, and to identify research needs. Specifically, the objectives of the report are:

i. to assist operational forest and range managers and research scientists in screening legume species and Rhizobium inoculants for reforestation and integrated resource management use;

ii. to describe a standardized experimental design and methodology for effective trial establishment and assessment and analysis of data; and

iii. to facilitate communication and information exchange for the transfer of this technology to the operational sector.

3 BIOLOGICAL NITROGEN FIXATION

3.1 Legume - Rhizobium Symbiosis

Approximately 80% of the atmosphere surrounding the earth consists of nitrogen gas (N₂). The capability to convert N₂ gas into a usable form (e.g., ammonium [NH₄⁺] nitrogen) is universally restricted to prokaryotic organisms. Nevertheless, the wide variety of nitrogen-fixing systems and their diverse environmental adaptations ensure that most ecological niches are capable of sustaining at least one or two representative species for the replenishment of lost nitrogen (see Appendix 2). For most plants, that vast nitrogen resource is unavailable for growth. However, in one large family of plants a unique relationship has evolved with a class of microorganisms, which permits many of these plants to exploit the gaseous N₂ resource.

Representatives of the legume family (Leguminoseae or Fabaceae) can be infected through their roots by soil micro-organisms of the genus Rhizobium. Within the root nodules that are formed as a result of this infection process, the plant and bacteria develop the complex biological machinery to convert N₂ into NH₄⁺ for use by the plant. This complex symbiosis depends on the specific interaction of the legume host and Rhizobium species, and may be influenced by environmental conditions. Acidic soil, high soil N fertility, drought, water-logging, trace element deficiencies, and low temperatures have all been noted to hinder the symbiosis (Turvey and Smethurst 1983).

Successful biological nitrogen fixation depends on the infectivity and effectiveness of the legume-Rhizobium partnership. Infectivity refers to the recognition of an appropriate host by a Rhizobium species capable of forming a root nodule; effectiveness involves the ability of that nodule to develop the necessary enzymatic machinery for fixing and transporting atmospheric nitrogen.
The members of the Rhizobiaceae, a family of soil bacteria which includes the genus *Rhizobium*, are genetically diverse and physiologically heterogeneous, but unified in classification by the common participation in nitrogen-fixing symbiotic relationships with legume species.

*Rhizobium* species have historically been classified on the basis of their functional behaviour in nodulating particular species of legume. Each cross-inoculation group consisted of that group of legumes nodulated by a specific *Rhizobium* species. Current taxonomic assessment of the family includes two important nitrogen-fixing genera (see Table 1).

**TABLE 1. Genera and species of the Rhizobiaceae and their legume hosts**

<table>
<thead>
<tr>
<th>Genus/Species</th>
<th>Legumes nodulated</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhizobium leguminosarum</em></td>
<td>clovers (<em>Trifolium</em>)</td>
</tr>
<tr>
<td><em>R. leguminosarum</em></td>
<td>beans (<em>Phaseolus</em>)</td>
</tr>
<tr>
<td><em>R. leguminosarum</em></td>
<td>peas (<em>Pisum</em>), vetch (<em>Vicia</em>),</td>
</tr>
<tr>
<td><em>R. leguminosarum</em></td>
<td>lentils (<em>Lens</em>)</td>
</tr>
<tr>
<td><em>R. meliloti</em></td>
<td>alfalfa (<em>Medicago</em>), sweet clover (<em>Melilotus</em>)</td>
</tr>
<tr>
<td><em>R. loti</em></td>
<td>birdsfoot trefoil and other <em>Lotus</em> sp.</td>
</tr>
<tr>
<td><em>Bradyrhizobium japonicum</em></td>
<td>soybean (<em>Glycine</em>)</td>
</tr>
<tr>
<td><em>Bradyrhizobium spp.</em></td>
<td>Many tropical legumes</td>
</tr>
</tbody>
</table>

* Formerly classified as *R. trifolii*, *R. phaseoli*, and *R. leguminosarum*, respectively.

The *Rhizobium* genus includes all fast-growing, acid-producing species: these characteristics are presumptive traits for *Rhizobium* during strain isolation in *vitro*. The *Bradyrhizobium* species are all slow-growing, alkaline producers in *vitro* and include the original soybean bacteria (*B. japonicum*). Effective management of biological nitrogen in forestry will require the use of adapted legumes and the development of appropriate inoculants.

### 3.2 *Rhizobium* Inoculants

An inoculant consists of one or more strains of live bacteria (*Rhizobium* spp.) in a carrier material which supports and protects the bacterial cells. While a variety of carriers have been used commercially, the most common is finely ground peat moss. Because of the natural acidic nature of peat, calcium carbonate (lime) is usually added to ensure a pH near neutral. Peat has both high water-holding capacity and buffering ability; these characteristics contribute to the ability of the carrier to sustain viability of the inoculant bacteria.

Legumes may also be supplied as “pre-inoculated” seeds. Such seed may have been inoculated using the usual peat-based product before shipping, or it may be pre-inoculated using one of several proprietary processes.

All commercial legume inoculants sold in Canada are registered under the federal *Fertilizers Act* administered by Agriculture Canada. Inoculants are regularly sampled and tested by Agriculture Canada to ensure that products meet the quality standards for live bacteria. Federal regulations include a requirement for package labelling which specifies:

- crop species for inoculation
- species of *Rhizobium*
- amount of seed the package will inoculate
federal registration number
- expiry date
- recommended method(s) of application.

There are three important aspects to the successful inoculation of legume seed:

i. selection of the appropriate bacterial strain(s) for the legume species planted;

ii. care in handling of the inoculant and/or pre-inoculated seed; and

iii. careful inoculation of the seed lot.

It is essential to remember that inoculants and inoculated seed contain millions of live *Rhizobium* cells. Proper inoculation requires that the developing target seedling receive the highest possible population of infective and effective bacteria. An inoculation procedure is described in greater detail in Appendix 4.

4 EXPERIMENTAL DESIGN

The design of an experiment is a critical factor in generating experimental data: appropriate experimental design ensures that the data analysis addresses the experimental hypothesis. Although more than one experimental design may be used for a particular experiment, there is usually one which is best suited to the type of investigation and the research resources available for the study.

On a uniform field site, a completely randomized experimental design is often the simplest approach. In field experimentation, however, such uniformity is seldom observed, even under conditions where the site may appear uniform to the eye. To accommodate variation in site and soil properties, a randomized complete block design can be used to partition this spatial variability. In the forested regions of British Columbia, most sites can and should be routinely blocked for experimentation. A typical forest clearcut (Figure 1) will include various slope positions, aspects, and parent material.

![Diagram](image)

**FIGURE 1.** An example of experimental blocking in a forest clearcut.
In the example shown in Figure 1, three blocks might be established on the cutblock: two on morainal parent material on the hillsides (one with a northeast aspect, one with a southwest aspect) and the third on the relatively flat fluvial parent material. These blocks will likely have different ecological attributes such as plant communities, moisture, temperature, and nutrient regimes. It is important to note that the rationale for blocking is to minimize differences within blocks and to maximize differences between blocks. A significant block effect in subsequent data analyses is confirmation that the use of blocks is desirable. The greater the variability among blocks, the more efficient the design becomes in its ability to detect possible treatment differences.

The treatments most often evaluated for legume screening trials are legume species, inoculant strains, and rate of seeding. In the example in Figure 1, assume that a trial will be conducted to assess three legume species using a single inoculant and three seeding rates. A simple factorial arrangement for this experiment would require:


Each block would have nine plots with a species x rate of seeding treatment combination assigned randomly to each plot. A typical layout for one block (replicate) of such an experiment is illustrated in Figure 2.

![Figure 2](image)

**Treatments**

S1 R1 = species 1 x rate 1
S1 R2 = species 1 x rate 2, etc.

FIGURE 2. A typical randomized layout of treatments in a block.
5 METHODOLOGY

The execution of an experiment is the most critical element in controlling experimental error (Hurlbert 1984). Methods should be well planned and diligently performed. The best-designed experiment will not demonstrate true effects if executed poorly or not maintained and secured during all phases of its existence. This section is presented to aid foresters, agrologists, and researchers in developing methods and records that could be used in experimental legume trials. It is also presented as a guide to developing standards so that all investigators can begin to make meaningful comparisons among such trials being established in British Columbia.

5.1 Site Selection and Plot Location

Selection of experimental sites depends on study objectives, experimental design, and the resources allocated to a project. However, once candidate sites have been selected, a few additional considerations and careful observations are warranted.

Criteria for final selection of the sites usually require that:

i. they best reflect the average conditions that are frequently and extensively encountered given the study objectives;

ii. there are no foreseeable conflicts in land use or management for the life of the study; and

iii. access is reasonable for assessment, extension, demonstration, and public information purposes.

Plot location within a site should ensure that:

i. the plot (and replicate plots) is reasonably uniform in terms of slope, aspect, microtopography, soil moisture and nutrient regime, and vegetation composition; and

ii. areas where soils have been seriously disturbed are avoided (such as landings and skidrows, unless these happen to relate to specific objectives in the design).

5.2 Timing of Assessments and Length of Experiment

Assessments should normally be carried out before treatment and at periodic intervals following the treatments (e.g. 1, 3, and 5 years). The length of the experiment will depend on the study objectives and will be influenced by the growth habits of both the legume and crop tree species, as well as the growing conditions and management regime.

Generally, we anticipate that 5 years will be an absolute minimum for most studies, whereas crown closure (15 - 30 years) may be the optimal period for a legume-reforestation study. The time commitment is an important consideration, as our experience suggests that treatment effects may take several years to demonstrate statistically significant differences. In addition, early effects may disappear later.

5.3 Site and Forest Management History

Reforestation is a management activity that follows harvesting, and is often preceded by one or more site preparation techniques. These precursors to reforestation may influence site environmental conditions to different degrees, effecting changes in the site. It is, therefore, important to document the management history and the general structure of the previous forest stand in order to appreciate and compare the results of different investigations. This historical information is available in the Ministry of Forests (MOF) District or company offices. British Columbia forests are managed under the concept of multiple use, and Forest District and company staff can inform users of any existing or potential conflicts with other users or management plans (e.g., grazing agreements, fish/wildlife concerns, stand tending plans).
5.4 Site and Plot Identification

Once the field experiment has been laid out, it must be marked so that it can be precisely located throughout the life of the experiment. The layout must also be accurately recorded onto field maps and registered as a map reserve with the MOF. Preservative-treated wood posts or metal stakes with signs designating the research site should be placed at critical locations, such as plot corners. Temporary locations (one season to 3 years) can be marked with wire flags or untreated wood stakes, although some critical location points should also be designated with more permanent markers. Paint or coloured flagging is useful in identifying plots or specific treatments, and will help minimize errors in sampling.

Some markers may attract destructive behaviour from animals, and this should be considered in their use and placement. For example, moose and bears often rub and knock over posts; crows pull at small markers attached to seedlings, causing cambium damage; and weather stations are often vandalized. If crop trees are included in the experimental design, they can be used effectively as location points in sampling schemes.

5.5 Environmental Information and Site Assessments

A description of general environmental properties at the experimental site is necessary so that one study can be compared to another, and so that results can be interpreted within and between biogeoclimatic zones and specific ecosystem units. Legume species perform better in some habitats than others, and establishment is often related to climatic conditions at the time of seeding and during germination. For these reasons, the site features, plant community, and soils should be described. It is also important to collect pre-treatment soil samples for a few routine analyses, and to maintain daily weather measurements during legume establishment. These data are essential for the accurate interpretation of establishment problems or failure.

British Columbia has a well-developed and widely used ecological classification system. Each regional office of the MOF has experienced scientists and numerous publications that may be used to describe ecosystems in detail. We strongly recommend that anyone doing a site assessment use these resources.

The steps below should be followed:

- At each site record details of the specific location and topographic features. As well, record the British Columbia Geographic System (BCGS) and National Topographic System (NTS) mapsheet numbers, the latitude and longitude (to the nearest 10 seconds), elevation (m), slope (%), and aspect (° azimuth).

- To describe the soil, choose a representative location outside, but near the plot(s). Record the depth of the forest floor and classify the humus form (e.g., Klinka et al. 1981). Classify the soil according to the Canadian System of Soil Classification (Canada Soil Survey Committee 1987). Estimate the upper profile (0 - 30 cm) soil texture (< 2 mm), and percent volume coarse fragments (> 2 mm). Note any special features such as depth to clay pan or water table.

- At each modal soil pit used for the soil description, collect approximately 1 L each of composite forest floor and mineral soil (0-15 cm) in labeled, double plastic bags. Store the samples in a cool, dark location and transfer these to drying trays within 1 day. The samples should be analysed for total carbon (Leco), total N (Semimicro Kjeldahl), available phosphorus (Bray 1), and pH (CaCl$_2$) (Page et al. 1982).

- Prepare a plant species list and estimate the percent cover for each species in each experimental plot. All of the above information is sufficient to classify the ecosystem(s) and should be incorporated into the reporting process so that the readers may fully appreciate the environment in which the experiment was performed. If you want a more detailed site description, consult Walmsley et al. 1980.

- Collect on-site, daily weather information for air temperature, relative humidity, and precipitation during the first complete growing season. If the legumes are fall-seeded, collect the same data from the time of seeding until snowfall remains for the winter, or well into the dormancy period of other on-site herbaceous plants, and for the next growing season.
5.6 Treatment Response Variables

In many cases, the trial will focus primarily on the establishment and growth of the legumes and crop tree species, and on an evaluation of the infectivity and effectiveness of the Rhizobium-host plant symbiosis. Thus, not only will specific estimates of the effectiveness of the symbioses have to be made, but the question of how much and how fast newly fixed nitrogen may be made available for crop tree use will have to be answered. Furthermore, you may want to assess the capability of the legume component to compete with weedy species on the site. These and other questions will be stated in your experimental objectives and will influence the choice of appropriate experimental design, sampling schemes, and statistical tests. We suggest the following response variables and measurements, which are often appropriate for tests of hypotheses related to the above questions.

5.6.1 Establishment and growth

- For legume growth, estimate the percent cover/unit area and average height (nearest 1.0 cm) at the peak of the herbaceous growing season. For the establishment phase, estimate the number of emergents/unit area (ranges within classes may be sufficient) at the end of the first full growing season and at intervals when significant changes may be anticipated to have occurred.

- Measure the crop tree seedling height (nearest 0.1 cm) and basal diameter (nearest 0.1 mm) from and at the ground surface, respectively. These measurements should be made immediately after planting and at scheduled periodic times according to the study plan at the end of the growing seasons.

- If the plantation is already established, measure the previous years' leader growth prior to the establishment of the trial. The precision of these measurements will depend on the age and growth in plantations. In young plantations (less than 10 years age or 4 m height), height can usually be measured to the nearest 1.0 cm and diameter to the nearest 0.1 cm.

5.6.2 Infectivity and effectiveness

Qualitative estimates of the parameters for most trials are appropriate. The presence and abundance of nodules on the roots will indicate the success and degree of Rhizobium infection in the host plant.

- Carefully excavate the soil around the plant beginning several centimetres from the crown. Remove the entire soil-root mass and gently agitate it in water to expose the nodules. After some initial reconnaissance sampling, you should be able to form classes of abundance (e.g., none, few, many, abundant) that reflect the success of infection.

- Cut open several nodules on each sample plant and observe whether a pink to red pigmentation exists in the cortical nodule tissue. The pigmentation is due to the presence of leghemoglobin, a protein required during active nitrogen fixation in legumes. Record the presence or absence of pigmentation; presence indicates that fixation is occurring (effectively), absence indicates infectivity only. A green or brown coloration of the cortical tissue is usually a reflection of nodule senescence which may be part of the natural seasonal cycle, a function of a relatively ineffective inoculant strain, or a response to environmental stress.

Quantitative field measurements for amounts of fixed nitrogen have commonly used the acetylene reduction assay (Hardy et al. 1968). More recently, the 15N/14N natural abundance ratio has been used in some experiments (Rennie 1984; Binkley et al. 1985). These methods have controversial strengths and weaknesses (Hauck and Weaver 1986) and therefore we do not recommend them for standard screening trials.

5.6.3 Recycled fixed-nitrogen

Legumes add readily decomposable organic matter, enriched with fixed nitrogen, to the soil. The fixed nitrogen effect may be evaluated indirectly by the chemical analysis of plant tissue and soil material.
The least variable property, and thus the least expensive to sample, is likely to be associated with foliar N (total percent nitrogen per dry gram sample) of the crop trees (Jorgensen 1980).

Our field experience suggests that adequate amounts of foliage from trees 5 years of age and older can be taken without harmful damage. However, for younger trees, destructive sampling could be harmful. When sampling of young seedling foliage is considered, extra seedlings should be included in the initial planting specifically for destructive sampling.

- Foliage should be sampled when the trees have finished growing for the season. Take samples from current year’s growth in the upper two or three whorls. Keep these samples frozen until they can be oven dried and prepared for analysis.

- When sampling for soil N, concentrate on the upper profile. This includes the forest floor (if present) and the 0-15 cm mineral layer. Soils are living ecosystems and therefore have significant seasonal variability in their chemistry. Always sample for treatment effect comparisons at the same time in any one year, and at the same time in succeeding sampling years. For samples to be consistent and biologically relevant, we recommend late spring sampling when the soil is at, or just declining from, field capacity.

Bulk density of the soil sampling layers must also be measured so that percent N can be converted to kilograms of N per hectare.

Follow collection and storage procedures from Section 5.5. Total N (Kieldahl) and mineralizable N (anaerobic) are common analyses for estimating soil N status.

5.7 Photography

A photographic record complements traditional data collection, is extremely useful in communicating results, and can be used to assess competing vegetation.

- To achieve continuity throughout the life of the experiment, take the photos from fixed positions and at the same stage of vegetative development (e.g., at flowering or seed dispersal).

6 DATA ANALYSIS

The type of analysis will be determined by the experimental design and sampling scheme(s). It should be done while the experiment is being planned, in consultation with a statistician. Generally, the use of computerized statistical analysis will be performed. The data must be entered into a computer file in a format compatible with the software being used. The particular software will dictate how the desired analysis should be executed. The results will be interpreted by the investigator(s). In this section, we provide an example of a common design, and an analysis of variance (ANOV) table associated with it.

6.1 A Sample Analysis

An example of an efficient and practical legume screening trial would be designed to replicate the experimental units within a biogeoclimatic subzone. This would allow the results to be analysed and interpreted with predicted confidence for that subzone.

Several sites (cutblocks) that represent typical and extensive features for that subzone would be selected. Subzones have complexes of ecosystem units that are operationally managed as treatment units. Typically, these treatment units will have a similar range of soil moisture and nutrient regimes (MNR’s).

For example, we might decide that the blocks in Figure 1 are MNR’s. In this case Block 1 is dry and poor, Block 2 is medium in moisture and nutrients, and Block 3 is wet and rich. These three MNR’s make up one block
on a site. After viewing other sites, we might select four cutblocks in total, having a range of similar MNR’s. We might further decide to screen three clover species at three rates of seeding (i.e., control [0], 10, and 20 kg seed/ha) using one commercial *Rhizobium* inoculant. The factorial arrangement would therefore be: site (4) x MNR (3) x block (1) x species (3) x rate (3) x inoculant (1) = 108 experimental units (plots).

This is a split-plot experimental design. The sites are split into MNR’s of one block (whole plot) each; blocks in this case are not replicated within site. There are two error terms, one for the main effects of site and MNR, and another for species, rate, and important interactions (see Table 2). This design is very sensitive to species and rate effects (large error term), but less sensitive to site and MNR effects (small error term). Since we expect a significant effect for MNR’s, we do not require a powerful design to detect it. This example suggests that we are especially interested in detecting effects of species and rate, and possible interactions between the two including any interaction with MNR’s.

**TABLE 2. An example of an ANOV table for a split-plot design in a legume screening trial**

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>df</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site (S = 4)</td>
<td>S-1</td>
<td>3</td>
</tr>
<tr>
<td>Regime (R = 3)</td>
<td>R-1</td>
<td>2</td>
</tr>
<tr>
<td>Block (B = 1)</td>
<td>(B-1) (SR)</td>
<td>0</td>
</tr>
<tr>
<td>Error A</td>
<td>(subtotal)</td>
<td>6</td>
</tr>
<tr>
<td>Species (Sp = 3)</td>
<td>Sp-1</td>
<td>2</td>
</tr>
<tr>
<td>Rate (Rt = 3)</td>
<td>Rt-1</td>
<td>2</td>
</tr>
<tr>
<td>Inoculant (I = 1)</td>
<td>I-1</td>
<td>0</td>
</tr>
<tr>
<td>Species x Rate</td>
<td>(Sp-1) (Rt-1)</td>
<td>4</td>
</tr>
<tr>
<td>Regime x Species</td>
<td>(R-1) (Sp-1)</td>
<td>4</td>
</tr>
<tr>
<td>Regime x Rate</td>
<td>(R-1) (Rt-1)</td>
<td>4</td>
</tr>
<tr>
<td>Regime x Species x Rate</td>
<td>(R-1) (Sp-1) (Rt-1)</td>
<td>8</td>
</tr>
<tr>
<td>Error B</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>107</td>
<td></td>
</tr>
</tbody>
</table>

7 REPORTING, EXTENSION, AND DEMONSTRATION

There are several ways to communicate results; usually a combination of techniques is most effective. First, however, the results and interpretations should be reviewed by experts in the field. If you work for an agency or company (as an employee or on contract), or are a graduate student, there are likely to be set standards and procedures for this review process. If you require assistance, however, contact the authors.

The MOF has several types of publications suitable for various levels of experimentation and directed at different audiences. There are also many scientific and technical journals that accept manuscripts for publication. These are the main sources for publishing results of forest research in British Columbia. However, there are many other public news media and organizations that may wish to communicate your results to their readers or members. This kind of reporting (extension and public information) includes newspapers, topical magazines, radio, television, as well as presentations at meetings, workshops, and seminars. It may also be appropriate for you to provide training opportunities based on experience from the research; for example, you may want to hold a workshop on methods of seed inoculation.
8 RECORDS

Complete, accurate, and secure records of all experimental designs, field plans, procedures, and data are mandatory in any experiment. Appendix 5 provides sample forms for the minimum information and data collection suggested in this report. For security, duplicate copies of all original records should be stored at a location removed from the normal work station.
9 LITERATURE CITED


B.C. Ministry of Agriculture and Fisheries. 1988. Forage crop recommendations for central British Columbia. Agdex. 120.32.


APPENDIX 1. Glossary of terms

Acid-producing The fast-growing _Rhizobium_ species show a characteristic acidic reaction when grown on yeast mannitol agar containing the pH indicator bromothymol blue.

Actinorhizal The adjective to describe those woody plant species nodulated by the actinomycete _Frankia_. Actinorhizal species include _Alnus_, _Eleagnus_, and _Ceanothus_.

Alkaline-producing The slow-growing _Bradyrhizobium_ species show a characteristic alkaline reaction when grown on yeast mannitol agar containing the pH indicator bromothymol blue.

Carrier material An inert material used to protect and deliver live _Rhizobium_ to the legume seed. Carrier materials should be non-toxic, and have good moisture absorption capacity, near neutral pH, and small particle size (70-100% through a 200 mesh screen). It should also be easily and economically available. A sterile carrier is preferable for the highest numbers of live _Rhizobium_ and prolonged survival in the carrier. Non-sterile finely ground peat is the most common carrier currently used in North American inoculants.

Cortical tissue (cortex) The interior tissue in the cortex of the plant root. When the infection thread penetrates this tissue, cortical cells are stimulated to divide and initiate nodule formation. The interior of the nodule may also be referred to as the nodule cortex.

Cross inoculation group A classification of legume species with respect to _Rhizobium_ response. Leguminous species mutually susceptible to nodulation by a specific kind of _Rhizobium_ contribute a cross-inoculation group, e.g., _Medicago_, _Melilotus_, and _Trigonella_ belong to the same cross-inoculation group and are nodulated by _R. meliloti_ strains.

Denitrification The gaseous loss of nitrogen by either biological or chemical mechanisms, but exclusive of ammonia volatilization.

Ecological niche The functional role of a species in a community.

Effectivity The nitrogen-fixing capability of a _Rhizobium_ strain once a root nodule has been formed. More effective strains show higher rates of nitrogen fixation. Ineffective strains may form nodules, but they are not active.

Endophyte Term used to describe the infecting agent in a plant-microbial relationship. In nitrogen fixation, the endophyte is the _Rhizobium_ spp. for legumes or _Frankia_ in the case of actinorhizal associations.

Experimental effect (as in significant effect) The result at a specific significance level that accepts a hypothesis.

Factorial experiment An experiment in which all levels of a given factor are combined with all levels of every other factor in the experiment.

Host The plant (legume) which is infected by the soil or inoculant _Rhizobium_ strain.

Hypothesis (as in statistical hypothesis) An assumption about the population being studied.

Infection The complex process of recognition and entry of the _Rhizobium_ strain into the root of the legume host, leading to root nodule development and nitrogen-fixing activity.

Infectivity The ability of a _Rhizobium_ strain to form a root nodule. In the soil, infectivity may be a complex response involving the competitive ability of the strain with other strains, as well as the basic capability to recognize and infect the host legume root.

Inoculant The combination of _Rhizobium_ culture and inert carrier material (e.g., peat) used to deliver _Rhizobium_ to the developing legume seedling.

_In vitro_ Literally - "in glass", used with reference to the study of biological processes outside the living organism, e.g., measurement of enzyme activity in a cell extract in a test tube.
APPENDIX 1. (Continued)

Leaching The removal from the soil of materials in solution.

Leghemoglobin A nodule-specific soluble protein responsible for oxygen transport and maintenance of a low free oxygen tension in the nodule. Leghemoglobin gives the active N₂-fixing nodule its typical pink-red coloration and is a useful diagnostic aid for an active symbiosis.

Leguminous Having to do with the legume plant.

Nitrogen fixation The enzymatic conversion of gaseous N₂ to NH₄⁺ by nitrogenase.

Nodule The structure developed in response to infection of the legume host by a nitrogen-fixing soil bacterium. While most nodules are formed on legume roots, some tropical species are also able to form nitrogen-fixing nodules on stems. The nodule is the site of active conversion of nitrogen to ammonia, from which the fixed N is transported to the plant.

Nitriﬁcation The biochemical oxidation of ammonium (NH₄⁺) to nitrate (NO₃⁻).

Pigmentation Coloration of the root nodule. Usually used with reference to color changes associated with the presence or absence of leghemoglobin.

Power of the design The ability of an experimental design to detect significant differences.

Prokaryotes Organisms that lack a nuclear membrane, endoplasmic reticulum, and mitochondria — includes the bacteria and cyanobacteria (formerly blue-green algae).

Qualitative variable A variable whose values vary in kind, not degrees, e.g., categories or classes.

Quantitative variable A variable that can assume specific values, e.g., tonnes per hectare or percent nitrogen per gram sample.

Randomization (in experimental design) The assignment of treatments to experimental units so that all units considered have an equal chance of receiving treatment.

Replication (in experimental design) Repeating a treatment two or more times. Its function is to provide an estimate of experimental error and to provide a more precise measure of treatment effects.

Rhizobium Aerobic, gram-negative, motile, rod-shaped soil bacteria. Characterized by the ability to infect a legume host and form a nitrogen-fixing symbiosis.

Sampling error The variability associated with the measurement differences among samples.

Senescence Aging. The deterioration of a root nodule as a result of the normal developmental cycle, or prematurely induced as a consequence of environmental stress. Often detected by visual changes in nodule pigmentation.

Symbiosis The biological situation in which organisms live together in an intimate association. Traditional use of this term with reference to biological nitrogen fixation has also included the assumption that the association is mutually beneficial to the participants.

Treatment An independent variable whose effect is measured on one or more dependent variables, e.g., nitrogen fertilization (independent variable) effects on yield (dependent variable).
APPENDIX 2. Nitrogen-fixing systems in British Columbia

Legume - *Rhizobium* Systems

- *Astragalus* spp. (milk-vetch)
- *Cytisus* (scotch broom)
- *Glycyrrhiza* spp. (wild licorice)
- *Hedysarum* (hedysarum)
- *Lathyrus* spp. (peavine)
- *Lotus* spp. (trefoil)
- *Lupinus* spp. (lupine)
- *Medicago* spp. (medic, alfalfa)
- *Melilotus* spp. (sweet clover)
- *Onobrychis* (sainfoin)
- *Oxytropis* spp. (locoweed)
- *Pisum* (garden pea)
- *Psoralea* (California tea)
- *Robinia* (black locust)
- *Teline* (trench broom)
- *Thermopsis* spp. (golden bean)
- *Trifolium* spp. (clover)
- *Trigonella* (blue fenugreek)
- *Ulex* (common gorse)
- *Vicia* spp. (vetch)

Non-Legume Nitrogen-Fixing Systems

Vascular plants/Alnus-type (actinorrhizal) nodule systems

- *Dryas* spp. (mountain-ovens)
  
  *(D. dummonndii, D. integrifolia, D. octopetala)*
- *Alnus* spp. (alders)
  
  *(A. incana, A. rubra, A. viridis)*
- *Ceanothus* spp. (redstem, snowbush)
  
  *(C. sanguineus, C. velutinus)*
- *Elaeagnus* spp. (Russian-olive, silverberry, cherry)
  
  *(E. angustifolia, E. commutata, E. multiflora)*
- *Shepherdia* spp. (thorny-buffaloberry, soopolallie)
  
  *(S. argenta, S. canadensis)*
- *Myrica* spp. (bayberry, sweet gale)
  
  *(M. californica, M. gale)*
- *Purshia* (antelopebush)
  
  *(P. tridentata)*

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1 Genera are cited from Fabaceae family in R.L. Taylor and B. MacBryde, 1977, *Vascular Plants of British Columbia: A Descriptive Resource Inventory*, Tech. Bull. No. 4, The Botanical Garden, Univ. B.C., Vancouver, B.C., 754 p. (Not all members of this family are capable of being infected with *Rhizobium* and fixing N₂ although the symbiosis is widespread in the common agricultural species.)

APPENDIX 2. (Continued)

Non-Legume - *Rhizobium*

*Trichodes terrestris* (puncture vine)*

Liverwort and *Sphagnum* - *Nostoc* (a cyanobacteria) Systems

*Blasia puggilla, Phaeoceros laevis, Sphagnum* spp.

Lichen Associations (cyanobacteria, commonly *Nostoc*)

*Collema, lobaria, Pelligera, Leptogium, Stereocaulon*

*Phyllosphere* (Leaf Surface) and Rhizosphere Systems

Free living or symbiotic nitrogen-fixing bacteria and cyanobacteria may be present on leaves, roots, and leaf-mould (forest floor); evidence obtained by acetylene reduction and 15N assays.

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* Genera reported elsewhere to nodulate (Bond 1982) have not been documented in British Columbia to the authors' knowledge.
APPENDIX 3. Cultivated legumes recommended for use in forest silviculture management

*Astragalus cicer* L.  
milk-vetch  
perennial  
- medium height, 50-74 cm (1,3)  
- difficult to establish, persists once established (3,6)  
- slightly acid to moderately alkaline soils (1,3)  
- frost hardiness is excellent, better than alfalfa (3)  
- drought tolerant (2)  
- requires 33-100 cm/yr of water (2)  
- low to medium fertility requirements (2)  
- prefers moderately coarse, medium to heavy soil (2); grows well on calcareous soils (6)  
- does well in high altitudes (2)  
- inoculant available (8)

*Lotus corniculatus* L.  
bird's-foot trefoil  
perennial  
- height 35-49 cm, moderately deep rooted, short broad leaves (2,3)  
- aggressiveness is weak, stand persistence is a problem, does not like competition; do not use in mixes, long-lived (3,9)  
- moderately acid to slightly alkaline (3)  
- good frost hardiness, cold tolerant (2,3)  
- requires 45-152 cm/yr of water (3)  
- fertility requirements - low to medium (3)  
- does well on imperfectly drained, shallow soils. Also on deep or moderately deep, medium to heavy soil and sandy loam to poorly drained clay (2,3,5,6)  
- inoculant available (8)

*Lotus uliginosus*  
Schkuhr  
big trefoil  
perennial  
- seedling vigor is only fair, short-lived (6)  
- adapted to strongly acid soils (2,6)  
- moderately cold hardy (2)  
- high moisture requirement - 60 MAP (2)  
- requires P and K for good agricultural yield (6)  
- adapted to poorly drained soils (2,6)  
- does well on the coastal zone of the Pacific Northwest  
- inoculant available (8)

*Lupinus perrenne*  
perennial lupine  
perennial  
- tall 75-99 cm, deep rooted (3)  
- medium aggressiveness, long-lived (3)  
- highly acid to neutral soils (3)  
- good frost hardiness (3)  
- requires 38-130 cm/yr of water (3)  
- low fertility requirements (3)  
- light to medium soils, good on loams and loam sands (3,6)  
- inoculant available but not always stocked (8)

*Medicago falcata* L.  
Sickle medic  
- medium to tall, 70-80 cm, strong lateral root system (3)

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1 Numbers in parentheses refer to reference source listed at the end of this appendix.
APPENDIX 3. (Continued)

*Medicago falcata* L. (Continued)

- weak aggressiveness (3)
- sensitive to soil acidity, slightly acid to moderately alkaline, limited below pH 5.0 (1.3)
- excellent frost hardiness (3)
- rainfall required 38-60 cm/yr (3)
- medium to high levels of phosphate and potash plus minor elements (3)
- light to heavy soils (3)
- inoculant available (8)

*Melilotus* spp. Mill.
common, purple, variegated alfalfa

- medium to tall, 60-90 cm (3)
- weak aggressiveness (3)
- slightly acid to moderately alkaline soil, forms nodules at pH 4.9 - 5.0 but nitrogen fixation is reduced (2,3,6)
- fair to good frost hardiness, depends on the variety (2,3)
- 38 to 75 cm/yr rainfall required (3,6)
- medium to high levels of phosphate and potash plus minor elements; requires N for establishment (3)
- medium, well-drained loams to clays (2,3,5,6)
- inoculant available (8)

*Onobrychis vicifolia* Scop.

sainfoin

- medium to tall, 50-100 cm (1,3)
- weak aggressiveness (3)
- mildly acid to moderately alkaline (1,3)
- good frost hardiness (3,6)
- drought resistant (6)
- requires 35-100 cm/yr rainfall (3)
- low to medium fertility requirements; requires phosphorus at establishment (3,9)
- medium to heavy soil, deep with good moisture holding capacity (3)
- requires good drainage (1)
- grows in interior Washington and Oregon (6)
- inoculant available (8)
- seeds germinate quickly on moist soils (3)
APPENDIX 3. (Continued)

*Trifolium hybridum* L.
alsike clover

- perennial
- short to medium, 35-65 cm, up to 1 m long taproot (3)
- quick establishment, weak aggressiveness, short-lived (3)
- moderately acid to mildly alkaline, acid tolerant (1,3,7)
- excellent winter hardiness, cv “Aurora” is more frost hardy than “Tetra” (2,7)
- intolerant of drought and high temperatures (1,6)
- requires 45-130 cm/yr rainfall (3)
- medium levels of phosphate and potassium (3)
- medium to heavy soil, silt loam to muck (3)
- found in temperate to subarctic areas (6)
- inoculant available (8)
- shade tolerance is poor (1)
- used in local seed mixtures

*Trifolium pratense* L.
Red clover

- biennial to short-lived perennial
- low growing (2)
- short-lived (1,2)
- yields reduced if soil pH is less than 5.0; nodulation occurs at pH 4.9 - 5.0, best yield at pH 6.0 - 6.8 (1,6)
- cold tolerant, single-cut varieties are recommended for northern latitudes as it is frost resistant and persists up to 7 years (6,7)
- intolerant of lengthy periods of drought (1,7)
- requires at least 60 cm/yr rainfall (2)
- adapted to drained or poorly drained soils (2)
- humid areas with moderate temperatures (1)
- inoculant available (8)
- shade tolerant (2)

*Trifolium repens* L.
white clover

- perennial
- very short, 15-35 cm, spreads by natural reseeding or by running root stocks along the soil surface (1,2,3)
- medium aggressiveness (3)
- nodulates at pH 4.8 but pH 5.4 is necessary for appreciable nitrogen fixation (1,3,6)
- frost hardy, Ladino type is less hardy than the Dutch type (1,3)
- low tolerance to drought, goes dormant (1,6)
- requires 45-152 cm/yr of rainfall (3)
- medium fertility requirements, good nitrogen fixer (3)
- light to heavy soils, best for fine sand to clay (3)
- good for hard climates (4)
- inoculant available (8)
APPENDIX 3. (Concluded)

*Trifolium subterraneum* L. annual
*subterraneum clover*
  - difficult to start, persistent once established (2,6)
  - moderate acidity, nodulation satisfactory in pH range of 4.9 - 5.0, best at pH greater than 5.5 (4,5,6)
  - not cold tolerant (2) dieback will occur
  - well-drained sandy to loamy soils (5)
  - good in dry climate, adapted to warm winters and dry summers (4,6)
  - inoculant available (8)
  - self-perpetuating (2)

Reference sources (complete citation is Section 9):

1. Smoliak and Bjorge 1981
2. Carr 1980
3. Buckerfield's 1986
4. Eichel 1979
5. Haines and DeBell 1979
6. Lacelle 1971
7. B.C. Ministry of Agriculture and Fisheries 1988
8. Nitragin Ltd. 1980
9. Personal communication from Richardson Seed Company Limited, Vancouver, B.C. March 1986
APPENDIX 4. Inoculant procedure

For the legume-Rhizobium symbiosis to work, the Rhizobium bacteria must be present in the soil, and it must be able to infect the specific legume and be capable of effectively fixing nitrogen. To ensure this, all legume seed should be inoculated with the correct bacteria with the use of a proper method. Several methods of inoculation are available; we have provided one stick solution method that has proven successful in our recent experimental trials and has subsequently been used by several range managers in the Prince Rupert Forest Region.

Throughout the storage, inoculation, and seeding, remember that you are caring for a population of living organisms. Excessive heat, drying, or other extreme environmental conditions will damage or kill the bacteria. A useful publication, Inoculation of Legume Crops (Agdex 100/23-1 1985), can be obtained by request from the Print Media Branch, Alberta Agriculture, 7000-113 Street, Edmonton, Alta., T6H 5T6.

Powdered Milk Sticker Solution Method

Materials needed:
- 1 bag (25kg) seed
- 300 mesh lime† (amount depends on seed size)
- one [weight will vary depending on source] appropriate package of inoculant specific to legume seed
- 50 g powdered milk mixed in 500 ml water (10% weight/volume)
- cake mixer or blender
- cement mixer

Procedure:
Keep inoculant refrigerated or frozen until needed, then thaw if necessary and combine with powdered milk solution. Mix thoroughly with an electric cake mixer or blender. The latter implement should be used in short bursts (up to 15 sec.) to avoid localized heating or physical damage to the bacteria. Empty the bag of seeds into the cement mixer, pour the sticker/inoculant solution onto the seed, and mix until all the seed is covered and slightly wet (approximately 10 min.). In the cement mixer, begin to add the lime until all seeds are coated and resemble small granular pellets (about 4-375 g coffee tins of lime for alsike clover). This step will take approximately 10 more minutes. Use the inoculated seed within 2 days, preferably just before sufficient precipitation to wash the inoculant into the soil and moisten the seed for germination.

† Using coarser lime (smaller mesh size) will not coat the seed as required. This lime (300 mesh) must usually be ordered from a wholesaler, as it is not commonly available at local garden/farm suppliers.
APPENDIX 5. Sample assessment forms

The following are suggested formats and information that may be modified and added to depending on the assessment requirements. A project identification and site information form need only be completed once; the response variable form(s) would be completed at each sampling period.

PROJECT IDENTIFICATION AND SITE INFORMATION

Project name and reference code:
Project leader:
Forest Region, District, contact person:
Company name and contact person:

BCGS no.: NTS no.: Latitude: Longitude:
Biogeoclimatic zonation and reference:
Directions to site from nearest community:

Attach to this form:
1. portion of NTS sheet with site location identified; and
2. detailed project layout to scale, including blocks, plots, sampling locations, and other important features.

SOIL DESCRIPTION

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<th>Aspect:</th>
<th>Slope:</th>
<th>Date:</th>
<th>Recorded by:</th>
</tr>
</thead>
</table>

| Soil horizon | Thickness cm | Texture | Coarse fragment % volume | PH % | C % | N % |

(The soil profile should be described to a depth of approximately 50 cm or restrictive layer, and special features such as seepage should be noted here; this description should be repeated at least once in each block).

VEGETATION DESCRIPTION

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<thead>
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</table>

<table>
<thead>
<tr>
<th>Shrub layer:</th>
<th>Herb layer:</th>
<th>Moss/lichen layer:</th>
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</thead>
<tbody>
<tr>
<td>Spp. cover %</td>
<td>Spp. cover %</td>
<td>Spp. cover %</td>
</tr>
</tbody>
</table>

(The major indicator species as well as the dominant species should be recorded for each layer in each block. If the experimental unit has natural regeneration, or is an established plantation, then the tree species must also be listed).
APPENDIX 5. (Continued)

RESPONSE VARIABLES

Most of these data would be recorded on computer forms or directly to a computer file using a field data recorder. The following are provided as example formats intended to be modified by the investigator(s). Laboratory analyses might be recorded in a similar fashion.

**Page 1 - Legume**

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<td>Subplot</td>
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**Page 2 - Tree seedling**

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**Page 3 - Nodulation**

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