Procedural Manual for Research in Nitrogen-Fixing, Multiple Use Trees (1986)

Pages
67

Size
8.5 x 11

ISBN
0309321859

Committee on Research Grants; Board on Science and Technology for International Development; Office of International Affairs; National Research Council

PROCEDURAL MANUAL FOR RESEARCH IN NITROGEN-FIXING, MULTIPLE-USE TREES

Revised 6 October 1986

INTRODUCTION AND ACKNOWLEDGEMENTS

The Tropical Trees Program of the National Research Council's Committee on Research Grants (CRG) supports high-quality scientific research of wide applicability in developing countries of the Tropics. The program includes research on multipurpose trees, nutrient cycling in tropical forest ecosystems, agroforestry applications in tropical ecosystems and management techniques for both natural and plantation forests.

Through this program the CRG has recognized the importance of, and the need for, international trials of promising tropical tree species conducted in accordance with common silvicultural and data analysis protocols. This view is shared with the international scientific community. The CRG's Tropical Trees Program has adopted the recommendations developed at a recent meeting on the utilization of multipurpose trees held in Kandy, Sri Lanka, organized by the International Union of Forestry Research Organizations (IUFRO). At this meeting research objectives and priorities were established for tropical tree research and published as a monograph, "Increasing Productivity of Multipurpose Tree Species: A Blueprint for Action." These recommendations have subsequently been endorsed by the United Nations Food and Agriculture Organization (UNFAO), the World Bank and other international donor agencies. The information contained within this manual is compatible with the recommendations of the Kandy workshop.

Early in the evolution of the Tropical Trees Program, the need for a procedural manual, which would provide basic protocols common to forestry projects in the Tropics, was identified. A working group was assembled in Washington, D.C., December 14-16, 1983, to prepare a concise manual that would also help to unify the CRG research program. The group was composed of Fredrick Owino (Chairman), Moi University, Kenya; Charles Davey, North Carolina State University, U.S.A.; Yvon Dommergues, Centre Technique Forestier Tropical, France; Carl Gallegos, Agency for International Development, U.S.A.; and Joann Roskoski, NifTAL Project and MIRCEN, University of Hawaii, U.S.A. The manual was reviewed and revised at a coordination meeting of CRG grantees held at Xalapa, Mexico, in October 1984. Rick Van Den Beldt of the International Crops Research Institute for the Semi-Arid Tropics, India, subsequently edited and rewrote parts of the manual. Valuable comments on earlier drafts of the manual were provided by Peter F. Ffolliott of the University of Arizona, U.S.A.; Harold Keyser of the Department of Agriculture, U.S.A.; and John Torrey of Harvard University, U.S.A.. Dr. Davey authored the chapter on soil characterization and monitoring. Drs. Keyser and Torrey authored Appendices 5 and 7 of the manual, respectively.

The manual is divided into six sections, plus appendices:

- Getting Started: Designing the Research Plan
- Tree Germplasm Collection and Handling
- Nursery Operations
- Experimental Designs and Methodology
- Soil Characterization and Monitoring
- Areas of Suggested Field Study: Forestry, Agroforestry, and Microsymbionts

Each section begins with general remarks or suggestions, and then provides detailed methods for the section topic. This is not a comprehensive manual. It does not include, for example, a broad range of experimental designs for field research. The methodologies included were chosen because they are simple, achievable with limited resources and equipment, and reliable. For a more exhaustive treatment of field designs, researchers should consult the following:

P. A. Huxley, ed. Methodology for the Exploration and Assessment of Multipurpose Trees. Nairobi: International Council for Research in Agroforestry, Commonwealth Forestry Institute, and National Research Council (U.S.A.), 1984.

J. Burley and P. J. Wood, comps. A Manual on Species and Provenance Research with Particular Reference to the Tropics. Tropical Forestry Papers, No. 10. Oxford: Commonwealth Forestry Institute, University of Oxford, 1976.

Since the microbial ecology of tropical forest environments is considered an important aspect of research studies, the manual includes detailed methods in the appendices for isolating and managing some of the microsymbionts associated with tropical tree species.

This manual will have two audiences. It is hoped that grantees, who have assisted in the preparation and revision of the material, will find it useful as documentation for procedures commonly agreed upon relating to germplasm collection and field studies. Researchers contemplating preparation of a proposal for a CRG grant on tropical trees may also find the manual a guide for choosing objectives and designing experiments consistent with the work of other grantees. For this reason, the first section deals with the setting of research objectives and collaborative arrangements within the program.

CONTENTS

GETTING STARTED: DESIGNING THE RESEARCH PLAN

Committee on Research Grants projects in tropical forestry originate within the context of a competitive grants program. Successful proposals generally contain a fully developed research plan and experimental procedures designed to achieve explicit, concrete objectives. The proposals will have been reviewed by several technical referees, and the investigator will have had the opportunity to revise the original objectives and research plan in response to the referees' comments. The final version of the proposal is approved by the Committee on Research Grants. The CRG staff prepares a grant document that contains the objectives and most of the details of the research plan. This document must be signed by the institutional administrator. Thus, projects begin with a research plan signed and agreed to by both the principal investigator and the Committee on Research Grants, and the presumption is that the plan will be carried out as described.

Many researchers presenting a proposal are reluctant to prepare a detailed research plan until they have consulted with other experts or until they have seen preliminary research results. They are seemingly caught in a vicious circle, because the proposal cannot be reviewed unless it contains a complete plan. However, the CRG and the referees realize that there are many circumstances that may require modification or updating of the proposed plan, and even an inappropriate plan, if complete and carefully prepared, may be taken as an indication of serious intent and assistance provided for its modification. More details are provided in the document, "Elements of the Successful Scientific Research Proposal," available from the CRG staff.

During the lifetime of the project, and sometimes even before the project commences, it may become necessary to modify the objectives or the research plan. This may be a response to changing circumstances or to unexpected discoveries. This is a natural part of the research process; no one would insist on maintaining the original plan or objectives if better ones have been proposed. Nevertheless, approval of the CRG is required for changes of objectives or the research plan. Generally, an explanation in letter form is sufficient; however, if the modification requires additional funds, the request will be treated as a new proposal. In that case, full details of the change and the justification for it are required, documentation is sent to referees for review, and a decision is made at a scheduled meeting of the Committee on Research Grants.

Almost all CRG projects include visits from international consultants and/or visits of the researchers to laboratories abroad. Exchange and correspondence are the lifeblood of science, and they are particularly important for researchers working in developing countries. If the researchers know with whom they would like to consult, they may include names and other particulars in the proposal. If not, the CRG staff are prepared to assist with the identification of appropriate collaborators or consultants, and to arrange the logistics of international travel.

The consultants visiting the project are contracted by the CRG and have dual responsibilities. They are expected to advise and assist the grantee, and also to advise the CRG and staff how they can be more helpful to the grantees in general. They do not formally evaluate the progress of the project; that is done during a separate visit, usually by a member of the CRG staff.

The CRG Tropical Trees Program involves extensive communication and cooperation among grantees in different countries. Annual reports to CRG, prepared by each grantee, are circulated among all grantees. Grantees meet annually to present and discuss research results, arrange exchanges of germplasm, and plan visits of scientific personnel. The preparation of this manual was recommended in just such a meeting in Nairobi, Kenya, in January 1983. Revised versions of the manual were reviewed at subsequent annual meetings of CRG grantees held in Xalapa, Mexico (1984), Hua Hin, Thailand (1985), and Petersham, Massachusetts, U.S.A. (1986).

TREE GERMPLASM COLLECTION AND HANDLING

GENERAL REMARKS

Two broad types of seed collection are envisaged for most CRG projects, (i) collection of seed for germplasm evaluation, and (ii) collections of bulked seedlots for general forestry and agroforestry research projects. Besides obtaining seed from field collections, seed can often be obtained from the suppliers listed in the following publications:

Peter G. von Carlowitz. Multipurpose Tree and Shrubs Seed Directory Nairobi: International Council for Research in Agroforestry, 1986.

D. G. W. Edwards, comp. World Directory of Tree Seed Workers. Ottawa: Canadian Forest Service for the International Union of Forestry Research Organizations, 1976.

Food and Agriculture Organization of the United Nations. Forest Tree Seed Directory. Rome: FAO, 1975.

Care should be taken when collecting seed in the field to ensure that a good sample of existing germplasm is gathered, and that a range of desirable traits is represented. Important considerations in this regard, from the initiation of the collection trip to the establishment of provenance trials, are discussed below.

PRECOLLECTION CONSIDERATIONS

It is important to limit collecting efforts to a few species. A good rule of thumb is that it is better to collect one species well than collect 20 species badly. Multiple-species collection trips do occur, but participants in these trips are well trained with years of experience in collection methodology.

Before setting out on a collection trip, it is essential to know where to go. Nearly all countries possess national and local herbaria. These institutions should be visited by the collection team to become familiar with the variability in a species and to map out the areas where herbarium specimens were collected. Ideally, all specimens should be located on a map and the trip designed to visit the key spots. Collections should be made from at least five discrete locations. For a widespread species, more sites may be visited.

It is also important to communicate with others working with the species about your intentions to collect provenances. Often, germplasm exchanges may be worked out, greatly increasing the number of accessions available. Such contacts should be aggressively maintained during the entire evaluative program, and collaboration fostered whenever possible.

COLLECTION METHODOLOGY

Generally, most nitrogen-fixing trees are cross-pollinated, although notable exceptions do occur. Leucaena leucocephala is one such highly selfing exception. Because the species is genetically homozygous, more emphasis should be made on collecting types from wide geographical locations than from individual trees in stands.

For out-crossing species, it is important to capture variability present in a stand, and care should be taken to sample a number of trees. Ideally, as many as 30 nonneighboring trees should be sampled, but this can be difficult. Many seed collectors insist on keeping separate seedlots for each tree, but the practice of bulking seedlots within a contiguous population is widely practiced. Maintaining records for each tree sampled is time-consuming, and if this attitude is carried on in the accession trials, it can be expensive in terms of land and funds. So long as genetic variability of the population is represented in a bulked collection, there is little probability of serious loss in germplasm by bulking. A notable exception to this situation is the occasional mother tree that stands out by itself. In this case, it is wise to collect a separate seed sample and evaluate it in the accession trials.

When collecting, it is important to keep careful records of the location and major site features of each collection. Some collectors take detailed site data, including soil samples, for laboratory analysis. For the purpose of this program, however, data forms currently used by other institutions are recommended. Two such forms, from the Oxford Forestry Institute (formerly Commonwealth Forestry Institute) and the Danish/FAO Tree Seed Centre, are presented in Figures 1 and 2. They are both useful and comprehensive, and are recommended where expertise and information exist to fill them out completely.

TABLE I. - Examples of seed collection data sheets

A. Oxford Forestry Institute

Species: Pinus oocarpa Schiede Seed No: K31 Store No: 1/71

Country: Nicaragua Department: Nueva Segovia

District: Dipilto Site: El Junquillo

Latitude: 13° 42'N Longitude: 86° 35'W Altitude (m): 1,000

Situation: At the western end and on the southern slopes of the Cordillera de Dipilto, which forms the northern boundary of Nicaragua at this point. The stands, which lie about 5 kms north of Macualizo and 8 kms west of Dipilto, form part of an area of about 150,000 hectares of generally open pine forest which stretches more than 70 kms roughly east-west along the cordillera and extends northwards to the pine forests of Honduras. Rainfall increases progressively eastwards and in this direction the lower slopes below 800 metres elevation are increasingly occupied by P. caribaea and the higher slopes above 1,500 metres by P. pseudostrobus and broadleaf forest. At the western end, in the Macuelizo area, the lower slopes and valleys support only a dry thorn scrub and the only pine species is P. oocarpa which in fact forms most of the pine forest throughout the cordillera.

Soil: Very freely-draining sandy or gravelly soil with abundant quartz, derived in situ from decaying granitic rocks, outcropping extensively on the steeper slopes and ridges. Erosion is active. The soils are generally very shallow, except in hollows or valleys, and have very little humus content. pH 5.7.

Climate: Mean annual rainfall at Macuelizo (5 km S) is 904 mm (35 ins) with the following distribution:-

J	F	M	A	M	J	J	A	S	O	N	D
4	1	21	16	113	180	110	85	149	163	49	13

No temperature data are available from Macuelizo but at Ocotal, about 20 kms east and 400 metres below the level of El Junquillo mean monthly maximum temperatures in the dry season range from 28°C to 32°C.

Description of stand: Very open pine forest on steep slopes (20° to 35°) with only thin grass cover including Andropogon sp. and Pennisetum sp. A few Quercus sp. occur in some moister valleys. The largest pines are over 30 metres (100 ft) high and up to 80 cms d.b.h. Pine regeneration is generally sparse although good in patches and the area shows evidence of frequent fierce fires over a long period of years. Growth rings are confused and difficult to interpret but growth appears to be slow, with 3 to 5 rings per cm.

Seed bearers: B.H. diam (cms): 40 to 60 Height (m): 25 to 30
Branching: 70° to 80° Boles: Straight, cylindrical, sound.

Methods of collection: From selected trees in felling sites (36 trees)

Date of collection: January, 1971.

TABLE 2. Examples of seed collection data sheets

B. Danish/FAO Tree Seed Centre, Humlebaek

DAN/FAO NO:

SEED COLLECTION DATA - DAN/FAO FOREST TREE SEED CENTRE

Botanical Name: Provisional No:

Vernacular Name: Provenance:

LOCALITY

Latitude: Country:

Longitude: Province/State:

Elevation: Region and/or adm. unit:

Map Ref:

Detailed Location:

SITE

Soil Type:

Slope: Aspect:

Drainage: Ann. rainfall:

Monthly rainfall distrb.:

Nearest Weather Station:

STAND

Association:

Density: open.... patchy.... dense.... Establishment

Height: Age:

Diameter: Stem Form:

State of Stand:

Remarks:

COLLECTION

Method: Date collected:

Number of Trees: Spacing of Trees:

Quantity of seed/cones: Condition of seed/cones:

Possibility of commercial coll.:

Remarks:

SEED

Extraction Method: Treatment:

Yield per unit volume: Germination:

Remarks:

Description by: Collector:

SEED STORAGE AND HANDLING

Collected seed should be cleaned of insects, twigs, detritus, and seed pods as soon as possible. It should be dried thoroughly, treated with an appropriate insecticide (if necessary), and stored in airtight, insect-proof containers. Most hard-seeded legumes will store for extended periods, but other soft-coated or small seeds may deteriorate rapidly. Literature on the properties and storage capabilities of your seedlots should be consulted, and germination tests performed on suspicious seedlots often. Seed is best stored under conditions of controlled temperature and relative humidity. These conditions, however, are often impossible to obtain in the tropics. The rule of thumb, "store in an environment where relative humidity and temperature in degrees Fahrenheit (°F) sum to 100," is worthwhile in most cases (for example, 30 percent humidity, 70°F; 50 percent humidity, 50°F).

PROVENANCE TRIALS

Provenance trials are best established from seedlings to ensure optimum use of the seed, which may be exceedingly scarce for some accessions. Common nursery practices, including seed scarification and the use of suitable containers or other establishment techniques, should be carefully observed.

The best provenance trials are those that can handle great numbers of accessions easily and may be added to yearly as new accessions are gathered. Experimental designs for such trials are discussed in the next two subsections. Plots should consist of two rows of trees spaced at between 1 m x 1 m to 2 m x 2 m, depending on climatic conditions. Since plots will be close together, there is usually little need for border rows; however, these may be added if space permits. Plots should consist of between 10 and 20 plants, depending on the variability inherent in the species. Each accession should be represented in the tree bank by two, five-tree row plots with 1 meter spacing within rows and 1.5 meter spacing between rows.

NURSERY OPERATIONS
(after Burley and Wood, 1976)

THE NURSERY STAGE IN TRIALS

The nursery stage of a species or provenance trial fulfills three main functions:

(1) The provision of suitable planting stock for the field stage
(2) Evaluation of juvenile genetic differences
(3) Evaluation of juvenile/mature correlations and of the possibility of selecting promising populations based on juvenile characters.

THE LOCATION OF NURSERIES

Experience has shown that differences in nursery treatment can produce effects that are noticeable in plantations for many years. It is therefore important that nursery treatments and conditions such as light, drainage, and aspect be kept as uniform as possible.

EXPERIMENTAL DESIGN IN NURSERIES

It is desirable to use replicated, randomized designs to assess the magnitude of differences that arise from nursery practices, and it is similarly desirable to use identical experimental designs in the nursery and in the field. In this way, the two sets of replication effects will be confounded and will not reduce the precision with which population differences can be estimated. A different situation exists where species have widely differing growth rates in the nursery, and it is less important where large differences between populations are expected later in the rotation.

Edge effects are sometimes noted with nursery beds. If growth differences are considerable, the edge plants should be discarded or used as surrounds. If edge effects appear in blocks of pots, they can be minimized by periodic rearrangement of the pots.

Poor germination may sometimes result in a shortage of planting stock for some populations. This may necessitate adjustments in the field design of the trial. For instance, if a lattice were planned and one or two populations only were affected by poor germination, it might be best to continue with a randomized complete block design, provided that three replications could be completed. Serious deficiencies will necessitate drawing up a fresh design for the field stage. This should be done, if possible, with the advice of a biometrician.

NURSERY PRACTICE

It is not possible to legislate for all types of trials and climatic regimes. Nevertheless, the following might be taken as a checklist of some important points. Whatever practice is used, its features and results must always be carefully recorded.

Germination

- Seedlots should be divided and sown one replication at a time.
- Different species and provenances must be rigorously separated--by separator boards (without causing shading) or by sheets if sowing is done in a bed.
- Sowing should be done quickly.
- Pregermination in moist sand, vermiculite, or blotting paper may be a useful way of ensuring rapid and uniform germination, and of keeping populations separate. It also avoids "pricking out" at a later stage.

Pricking Out or Transplanting

If this is done, it should be done early. Plants should be pricked out when ready, regardless of differences in growth between populations. Estimates of numbers required should take account of mortality after pricking out, and of replacements required later in the field. At this stage there is a particular risk of mixing populations, and no plants should be moved without adequate labels. Potted stock, which in dry or unreliable climates often has better rates of survival, is especially prone to this.

Culling

The removal of very poor or deformed plants is part of normal nursery practice, but especially where "wild" or untried seed sources are being used, a record of plants rejected should be kept, as this may be an important characteristic of some species or provenances. Normal nursery records should provide information on the number of sound planting stock produced per unit of seed sown.

Size of Plants

The development of nursery techniques is usually carried out concurrently with species and provenance work. If, however, markedly different growth rates are apparent in different provenances, it may not be possible to have all plants ready together, and in this case it may be better to re-sow the trial at different dates, provided seed is available, to have all stock ready at the same time.

Inoculation (See Appendix 5)

PRIORITIES IN NURSERY ASSESSMENT

The following program of assessments is given in order of priority. Sampling intensity should ideally be related to the actual variability observed in each case.

Assessments at the nursery stage are time-consuming, and a minimum of information should cover germination rates and percentage, age, and the size of the seedlings when transferred to the field.

The nursery stage of species and provenance trials presents opportunities for assessing differences in populations at an early stage. Similarly, trials undertaken in greenhouses or other controlled environments can also yield important information. There are three main objectives: biosystematic studies, juvenile-mature correlation, and seed source identification.

Biosystematic Studies

The taxonomic and genecological relationships between populations are often poorly known, and the relatively uniform conditions in nurseries, etc., permit more precise evaluation of genetic similarities and differences than is possible in the natural habitat, or in field trials. Measurements are made easily, and destructive sampling is more convenient in these trials. The complex relationships between populations can therefore be studied through multivariate analysis, based on characteristics that are probably under more direct genetic control than are the usual field measurements of height and diameter.

Seed Source Identification

Large areas of plantations, and of trials, have been established in many parts of the world from seed whose origin is inadequately known. It is possible to distinguish different populations by the morphological and chemical characters of seeds and seedlings, and a provenance trial is thus an opportunity to test a wide variety of populations in this way.

Studies in Controlled Environments

The use of accurately defined and controlled environments in growth chambers, phytotrons, and greenhouses is particularly valuable for testing physiological responses, or the interaction of population differences with the effects of simple environmental factors such as temperature or photoperiod.

For further information regarding nursery establishment and management, consult J. Burley and P. J. Wood, comps., A Manual on Species and Provenance Research with Particular Reference to the Tropics, or Fred R. Weber, Reforestation in Arid Lands (Mt. Rainier, Maryland: Volunteers in Technical Assistance, 1977).

EXPERIMENTAL DESIGNS AND METHODOLOGY

GENERAL REMARKS

Investigators should follow the general guidelines for design of field studies outlined in J. Burley and P. J. Wood, comps., A Manual of Species and Provenance Research with Particular Reference to the Tropics. It is recommended that the investigator seek the advice of a statistician familiar with agricultural and forestry field studies when planning field studies, and contact the CRG staff for suggestions regarding technical consultation if such assistance is desired. Some consultation during the initial planning stages may facilitate the statistical analysis of trial results, and may actually save the experimenter some time and resources by simplifying the design.

In this section no attempt is made to provide either a comprehensive or definitive compilation of experimental methodology and data analysis. Instead, a rather modest attempt is made to synthesize what is known about the various techniques, and list some of the pitfalls common in field research of this type.

PLANNING, DESIGN, AND LAYOUT OF THE EXPERIMENT

Siting the Experiment

Fields designated for forestry and agroforestry research are often more variable and of poorer quality than those available to agricultural researchers. Such land may be recently cleared from the forest, or be "waste land" with poor soils and climate. This choice is sometimes made deliberately to ensure that trial data reflect "real world" conditions. In such situations, greater care must be given to the siting and layout of the experiment in order to avoid the incorporation of large experimental errors. It is far better to select a site and then design the experiment than to fit a given experimental design to a site.

The first step in siting the experiment is to obtain, to the extent possible, a history of the site. A recently cleared site will be quite variable, containing areas of former clearing, fires, stumps, etc. Fields in which trials are established sometimes have fertility gradients that may introduce unanticipated and undesirable complications.

Other hazards should be noticed. The presence of foot and animal paths, waterlogged depressions, rock outcrops, and wide variations in soil color or depth are all factors that can affect plot yield and should therefore be avoided.

It is helpful to obtain as much detailed information about the site as time and money will allow. Attention to these details at the outset of the project, when there is relatively little work to do, will facilitate the report-writing stage, when personnel are often removed from the project for other duties. Such information should include:

- Date of information collection
- Locational data (latitude, longitude, altitude, slope, aspect)
- Climatic data (monthly rainfall and temperatures)
- Soil data (standard fertility data: N, P, K, Ca, Fe, Al, etc.; cation-exchange capacity [CEC]; water-holding capacity; soil classification [use FAO/Unesco or other internationally recognized guidelines])
- Ecology (pests, diseases, vegetation and weeds; abiotic hazards [typhoons, fires, etc.]; other factors [stoniness, waterlogging, etc.])

Current records should be kept on meteorological and ecological data, as they can often be used to explain experimental trends. Soil data are often taken throughout an experiment to monitor site changes and determine the long-term effects of the treatment on the soil.

Experimental Blocks

A block is a set of items or experimental units, under treatment or observation, that have been grouped to minimize environmental effects or initial differences between items or units with respect to the variables being studied.

It is essential to remember that there should be as little field variability within a block as possible. At the same time, field differences between blocks probably will exist. Thus, if an experiment is located on a slope with a soil fertility gradient running from top to bottom, it is unwise to locate blocks adjacent to each other running down the slope. A better choice would be to make the blocks long rectangles along the slope contour, each block therefore incorporating its own characteristic fertility. In this manner, Block I would be at

the top of the hill, and Block X located parallel to I and at the bottom. Differences in fertility from the top to the bottom of the hill would simply become block effects in the analysis of variance. Within each block, rows should run up and down the slope, to sample variation uniformly.

Blanket treatments (such as irrigation), which must often be done in several operations, should be applied by blocks and not across blocks. Similarly, during harvesting or data collection, it is best to proceed by blocks, rather than across blocks, lest methodology differ slighty within a block.

RANDOMIZATION

One of the basic tenets of the analysis of variance is the randomization of plots. However, it is surprising how often randomization is done casually or arbitrarily. The use of random number tables or hand calculators with built-in random number functions is advised for all sites. Even the systematic designs (noted below) are capable of some degree of randomization.

The question of what to do when randomization fails--for example, when a given treatment occurs four times where four blocks border each other--is a much argued one. It is recommmended that in such cases the randomization procedure be completely redone.

EXPERIMENTAL DESIGNS

General Considerations

Prior to a discussion of the various experimental designs useful in the CRG program, topics of a more general nature might be discussed regarding the development of an experimental design. These include plot size, the number of replications, and data collection.

Plot Size

The overriding concern with the establishment of plots in a field experiment is that the plots incorporate as little site variability as possible. Often, in an effort to ensure that field trials reflect "real world" conditions, plots are made too big, and inherent variability creates large experimental errors that may mask true treatment effects.

At the same time, particularly in tree trials, if plots are too small, inherent genetic effects may do the same thing. A good rule of thumb for most tree species is that eight rows by nine rows will be small enough to avoid intra-plot variability, but large enough to ensure a good sample. A large proportion of a plot area is composed of border rows, which are crucial in forestry experiments. In trials where inter-plot competition is expected, two, and sometimes three, border rows should be established.

Number of Replications

Increase in the number of replications concomitantly decreases residual experimental error. Although rules do exist that help to determine the number of replications, most field trials include four. In variable fields, however, it will always pay to put in an extra replication, although, in such cases, advice should be sought from a qualified biometrician. There is a practical "break-even" point to increasing numbers of replications. As the number of replications increases, so does the necessary land area and, in most instances, the site variability.

Data Collection

A regular program of what to measure and how often to measure should be decided on before the installation of the trial and adhered to throughout the trial. Data should be recorded in a permanent form at the outset, if possible, and copied as little as possible, as serious mistakes have been known to occur in even the most carefully transcribed data. It is important for the project leader to closely supervise data collection teams, and when feasible, participate in data collection activities.

Multilocational Testing

It is a good idea to plan to install the same experiment in at least two widely different locations, incorporating different site variables such as elevation, rainfall, and soil types. Treatment effects are often highly site-dependent.

Reporting

The goal of the CRG program is to develop a body of knowledge on tropical trees, particularly nitrogen-fixing trees. It cannot be stressed enough that it is expected that project results will be published in appropriate journals to share data with colleagues elsewhere. The nature and form of data that will be most useful and suitable for publication should be kept in mind by the investigator at all stages of experimentation. Publication may be in informal journals, such as Nitrogen-Fixing Tree Research Reports, or in more formal journals, such as Agroforestry Systems or Unasylva, as well as in local professional journals.

SELECTED DESIGNS

There are many different experimental designs that can be used in plantation trials. Each design has its advantages and disadvantages and is chosen for a particular purpose. Some of these designs are described

briefly below. Since choice of design is important to the success of the trial, considerable care and advice should be sought before selecting an experimental design. A useful reference in this regard is J. Burley and P.J. Wood, comps., A Manual on Species and Provenance Research with Particular Reference to the Tropics.

Completely Randomized Designs

These designs, although making use of replicated treatments, randomize them throughout the experiment rather than establish them in blocks. Such designs are feasible when the site is homogeneous, as may be the case on the laboratory bench or in the greenhouse. Many nursery studies and microsymbiont work lend themselves to this type of experimental design. However, even greenhouse conditions can be variable with respect to shading, watering, and other factors, and such designs should be used only when it is clearly determined that no such heterogeneity exists.

Randomized Complete-Block Designs

In a randomized complete-block (RCB) design, treatments are replicated and placed in blocks of one full set of treatments per block (see Figures 3 and 4 below).

Such RCB designs are widely used in agricultural research, and justifiably so. These designs are suitable for a wide variety of experimental objectives. Analysis is simple and is understood around the world. Also, they are amazingly resilient, and a failed treatment will rarely seriously affect the rest of the experiment. Some forestry trials--for example, those involving provenance or species comparisons--have large numbers of treatments and may not be suitable for RCB designs. In such cases, large block size may incorporate site variability, resulting in large experimental residual error.

FIGURE 3

A possible randomization of four populations in a randomized complete-block design with four replicates.

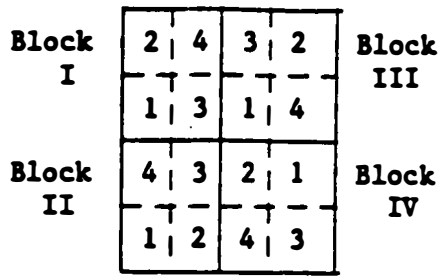
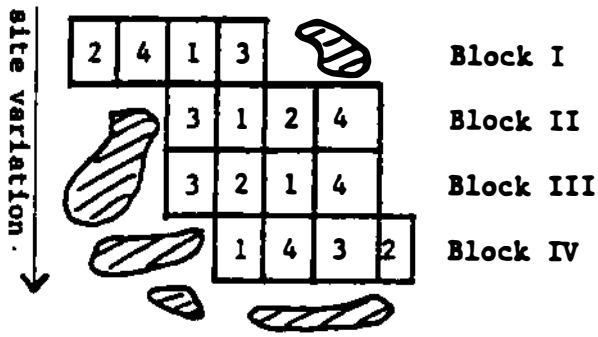


FIGURE 4

Plot layout for a field trial of four complete blocks of four populations. The site varies systematically in the direction of the arrow, and there are rock outcrops as indicated.



Split-Plot Designs

Such designs are ideal when treatments are factorialized; for example, when lime and phosphorus are applied in various combinations. It is important to consider carefully which of the treatments will be designated as main plots, and which will be established as subplots. Subplots are statistically tested with greater precision--thus, they should be composed of the variable to be studied in depth. Variables that are difficult to keep straight--for example, spacing--are easiest to work with when installed as main plots.

It is always wise, even for experienced field researchers, to talk over the design of a split-plot experiment with a knowledgeable colleague or a biometrician.

Augmented-Block Design

The augmented-block design consists of two sets of treatments: (1) "core" or replicated treatments that occur once in every block, and (2) "augmented" or unreplicated treatments that occur just once in the entire experiment. Treatments are randomized within blocks. Core treatments are replicated in every block, and the augmented treatments are divided equally among the blocks. Any number of augmented treatments may be selected, however, the maximum number of augmented treatments is determined by the following formula:

$$\#\text{ augmented treatments} = (\#\text{ core treatments} \times \#\text{ blocks}) / 2$$

In this manner, a wide number of treatments may be examined. Such trials have been used to look at a range of spacings, and the augmented treatments are used to "fill the gaps." Another demonstrated use of

the design is in species trials where the experimenter chooses five or so "core" species to examine closely, and a large number of augmented species to examine casually.

Latin Squares

A Latin square design permits systematic environmental variation to be estimated in two directions. The plots are arranged in rows and columns as shown, for example, in Figure 5. Each population occurs once in each row and once in each column, and the number of replicates of each population is equal to the number of rows or columns. The designs are most suitable for moderate numbers of populations. If there are many populations, a Latin square is too large, while for very small experiments (for example, three or four populations) residual variation may be imprecisely estimated.

Latin square designs may be particularly useful in greenhouse experiments where the sources of systematic variation are obvious. A set of Latin squares for varying numbers of populations is given in the following: R. A. Fisher and F. Yates. Statistical Tables for Biological, Agricultural and Medical Research. 6th Edition. Edinburgh and London: Oliver and Boyd, 1963.

FIGURE 5

A Latin square design for five populations.

	Column 1	2	3	4	5
Row 1	A	C	B	E	D
2	B	D	A	C	E
3	C	A	E	D	B
4	D	E	C	B	A
5	E	B	D	A	C

Systematic Designs

Systematic designs have been in use in forestry and agricultural research since the publications of J.A. Nelder in 1962. These designs, widely used by the Centre Technique Forestier Tropical, have recently been proposed by the International Council for Research in Agroforestry to have considerable potential in agroforestry research. For further information, see P. A. Huxley, ed., Methodology for the Exploration and Assessment of Multipurpose Trees, Part 4F.

For additional information concerning statistical analysis, see Frank Freese. Elementary Statistical Methods for Foresters. Agricultural Handbook 317. Washington, D.C.: Forest Service, U.S. Department of Agriculture, 1967.

SOIL CHARACTERIZATION AND MONITORING

GENERAL REMARKS

The soil, climate, and associated biota comprise the environment from which we select candidate N-fixing trees and into which we place them for testing and evaluation. It is as important that we document several of the soil characteristics as it is that the climate and location be documented. This is true for both the site of origin and the site of evaluation. Unless the evaluation site is described in some detail, it will not be possible to extrapolate results from one site to another.

Certain soil characteristics can be observed and recorded in the field. These are mostly physical properties and include such things as soil horizon thickness, color, and structure. Other important, but unseen, features such as soil acidity or nutrient levels must be determined in the laboratory. These are mostly chemical properties of the soil. The soil biota include the n-fixing microbes and the mycorrhizal fungi. They are discussed in detail elsewhere in this manual.

Some soil characteristics are relatively stable and need to be determined only once. Other soil characteristics are more changeable. For the site of origin they may be recorded once or seasonally. For the site of evaluation, they will need to be determined at least annually. If determined annually, it is important that the soil samples be collected at the same time each year.

SOIL CLASSIFICATION

In order for people in various parts of the world to understand something about the soil from which a species is collected and in order to compare their soils with those involved with the species evaluation, it is necessary that the soils involved be classified in a system that is widely understood. Two such systems exist. They are similar in many respects and generally a soil classifed under one system may be classified under the other. Thus soils should be classified according to either the system described in Soil Taxonomy (Soil Survey Staff, 1975) or by FAO (Dudal, 1968, 1970). For a comparison of the systems and other related information, see Buol, et al. (1980). Classification will tell the reader much about the soil's origin and general characteristics. Specific details concerning the present condition of the soil must be determined by soil analysis.

SOIL ANALYSIS

Soil properties to be determined initially

Texture is one of the most stable characteristics of the soil. It is significantly affected only by erosion. In that case, the soil particles are physically moved by water (or wind) and deposited elsewhere. Thus, in the absence of significant erosion, texture may be determined once and then assumed to remain unchanged for a very long time.

Soil texture should be determined by the Bouyoucos hydrometer method (Day, 1965). The percent sand, silt, and clay and the textural classification (e.g., silty clay loam) should be determined for the following depths in the soil: 0-15, 15-30, and 30-50 cm. In some cases, it will be necessary to determine texture by horizon (e.g., separation of ultisols from oxisols) and this may require sampling below the 50 cm level.

Soil properties to be determined more than once

The following table lists the soil properties to be measured, the units of expression, and a notation regarding the method to be used. These properties are usually determined on the 0-15 cm soil depth. Other depths (15-30 and 30-50 cm) are optional.

From the information on the table, which must be determined in a laboratory, the following two values may be calculated. The first should be done for all soils while the second is needed only for those soils that are more acidic than pH 5.5.

Effective cation exchange capacity (ECEC):

To determine the ECEC, the meq/100 g of Ca, Mg, K, and Al are summed. If Na is found to be a significant cation, it should also be added to this sum.

Percent Al saturation:

In those soils that are more acidic than pH 5.5, Al often occupies much of the nutrient-holding capacity (ECEC) of the soil. In addition, Al is quite toxic to many plants and may be directly responsible for the failure of introduced plants to survive and grow in a new environment. To determine the % AL saturation, divide the exchangeable Al (meq/100 g) by the ECEC and express the results as a percent. Soils that are less that 40% Al saturated will not cause trouble except for very sensitive plants.

Soil property	Units	Methods
Acidity	pH	Soil: water, 1:2.5 (V:V), pH meter
Available nutrients		
P	ppm	Modified Olson soil extractant (Hunter, 1979). See below for details of method.
Cu		
Fe		
Mn		
Zn		
Exchangeable elements		
K	meq/100 g	Soil to be extracted with 1 M NaCl. Potassium to be determined by flame phometry and Ca and Mg by atomic absorption spectrophotometry. Hydrogen and Al to be determined by by titration (Thomas, 1982).
Ca		
Mg		
Al		
H		
Nitrogen		
Total	%	Kjeldahl (Bremner and Mulvaney, 1982)
NH_4	ppm	(Keeney and Nelson, 1982)
NO_3	ppm	(Keeney and Nelson, 1982)
Organic carbon	%	Chromatic acid oxidation of Walkley and Black (Nelson and Sommers, 1982)
The remaining tests are optional or are required in specific areas.		
Bulk density	g/ml	Oven-dry weight of undisturbed core of known volume (Blake, 1965). Sample usually taken from the surface of mineral soil, mineral soil, but may be taken from any depth(s) of interest.
Field capacity	%	Weight of water lost during oven-drying, divided by the oven dry weight of sample taken from 5-15 cm depth after gravitational water has drained from thoroughly wetted soil. Usually requires one day in sands, two days in loams, and three days in clays.
Electrical capacity	mmhos/cm	Use conductivity meter. Test only required where there is reason to suspect high soluble salt content of the soil.
Exchangeable Na	meq/100 g	See other exchangeable elements (above) for method. Only required where there is reason to suspect high soluble salt content of the soil.

Modified Olson extracting solution (Hunter, 1979)

Hunter's (1979) modification of the Olson extracting solution adds EDTA to the basic $NaHCO_3$ solution. This allows the determination of additional elements. Details of the preparation and use of the extracting solution are as follows:

Preparation:

1. Dissolve 420 g of $NaHCO_3$ in distilled water.

2. Dissolve 37.2 g of di-sodium EDTA in distilled water.

3. Mix the above two solutions in distilled water and bring the total volume to 10 liters. This final solution will be 0.5N $NaHCO_3$ and 0.01M EDTA.

4. Adjust final solution to pH 8.5 with NaOH.

5. Store in a polyethylene bottle.

Procedure for use:

1. Add 2.5 ml of dried, seived (2 mm) soil and 25 ml of the extracting solution to a beaker or bottle.

2. Stir or shake for 10 minutes.

3. Filter until solution is clear.

4. Analyze for the appropriate elements (see above table).

AREAS OF SUGGESTED FIELD STUDY: FORESTRY, AGROFORESTRY, AND MICROSYMBIONTS

GENERAL REMARKS

Grants in the CRG program have been awarded on the basis of proposals submitted to the Committee. This section has been prepared to assist the researcher in deciding exactly what to study under the purview of the grant. Three broad areas are discussed: forestry, agroforestry, and microsymbiont research. Of these, only the microsymbiont section is discussed in depth, because there is a wealth of information on methodology in the literature for the other two topics. Also, members of the working group feel that the effort to standardize methodology in microsymbiont research will be particularly worthwhile, as standard methods do not currently exist.

Unforeseen circumstances, such as staff changes, disruptive climatic events, or changes in administrative policy, can always occur, requiring the reassessment or modification of some areas of investigation, even after the proposal has been approved. The areas of interest listed here should assist the researcher in modifying study objectives.

FORESTRY RESEARCH

It is hoped that the CRG grants will contribute greatly to the knowledge concerning fast-growing tropical trees, particularly those of use to small farmers as sources of food, fuel, fodder, and other products. The following areas are among those of interest to the program.

Species Trials

It is always valuable to have an opportunity to evaluate recommended exotic species in association with locally preferred ones. In general, randomized complete-block designs are best when few species are being studied; the augmented-block design is perhaps best when many species must be studied.

Spacing Studies

Increasing the population of trees per hectare serves to reduce diameter growth markedly and height development somewhat. Yet, close spacings often yield the greatest biomass per unit area. The exact relationships of this phenomenon have not been worked out for many of the species of interest. Augmented-block designs and systematic "fan" designs are best for screening a large array of populations directed toward the development of response curves.

Fertilization

Fertilization, known to pay in most forestry schemes, is an attractive area of study for the CRG grants. Split-plot and related designs are best employed for factorial studies involving a few species and a few rates of fertilizer or other soil amendment. It is not advisable to include too many treatments, as block size can become quite large, exacerbating already heterogeneous conditions in most fields.

Admixture Trials

The role of tree legumes as a source of nitrogen fertility in forestry plantations is little understood, although it appears to be a promising practice where it has been employed. Typically, such experiments utilize the randomized-block design, although systematic arrangements could be of use. A timber or pulp species is interplanted at varying rates with a suitable nitrogen-fixing tree that grows at about the same rate. It is easy to make profound errors in judgement when planning such experiments. Hence, the advice of experts and a thorough review of the literature are recommended.

Allometric Relationships

Such studies involve the development of predictive relationships among trunk volume or weight, branch weight, caloric value, leaf yield, etc., and independent measurements such as tree height, diameter breast height, and spacing. Such relationships are most precise when trees are destructively harvested. Thus, it is best to include such studies as part of some of the other topics presented above. An extra replication could be established for this purpose with little extra time and effort.

Germplasm Conservation

As described in "Tree Germplasm Collection and Handling," germplasm collection and evaluation is a major thrust of the program. Such collections are usually ongoing over a period of years; hence, it is wise to design evaluation trials that can be enlarged as more seed is collected.

Breeding and Breeding Systems

Little is known of the breeding systems of most nitrogen-fixing species. This is unfortunate, as little progress in breeding superior types can occur until this information becomes available. Much can be learned by simple bagging and the hand-pollination of the flowers of these species. Breeding work *per se* is not advised, as it often entails a commitment far beyond the scope of most CRG grants.

AGROFORESTRY RESEARCH

The role of nitrogen-fixing trees in agroforestry research has been demonstrated in numerous casual observations of field plantings and traditional cropping systems. Yet, surprisingly little hard data exist on agroforestry systems. There is a great need to quantify yields and devise sound management methods in agroforestry research. The use of appropriate experimental designs and strict adherence to scientific principles are essential when approaching agroforestry research. The role of the CRG grants program is to critically assess, not casually interpret, agroforestry systems.

Species Screening

Outside of *Leucaena*, *Gliricidia*, and a few *Prosopis* and *Acacia* species, not much is known of the potential of tree species in agroforestry systems, particularly nitrogen-fixing species. Fodder analysis, combustion characteristics, coppicing ability, etc., are possible areas of screening for agroforestry potential. Accepted laboratory techniques, which are not listed here, should be used.

Hedgerow Management

Most agroforestry systems currently being advanced involve the use of tree hedgerows under intensive management. Randomized complete-block designs are most appropriate for this type of research. Major variables typically include harvesting interval, height of cutting, species selection, plant population, and the like.

Cropping Systems

A thorough understanding of the relationship between crops and trees in agroforestry systems is a crucial need. Extremely little work has been done on this problem. Three major areas are identified:

- Alley cropping: Work at the International Institute of Tropical Agriculture at Ibadan, Nigeria, has demonstrated the efficacy of planting rows of crops between relatively closely spaced rows of trees. Either net biomass production (and economic yield) or the use of tree prunings as green manure may be studied.

- Stabilization of shifting cultivation: Similar to alley cropping, this research is usually confined to rolling uplands. Trees are planted on contours for soil conservation, fodder, and green manure, at various spacings, in association with crops.

- Field tree establishment: Trees are allowed to grow in cropped fields at variable, but usually very low, populations per hectare. The effect of nitrogen input, shading, water competition, etc., may all be studied. There are numerous traditional systems that may be studied scientifically, *in situ*, potentially saving much time. These studies are of great value in arid areas.

MICROSYMBIONT RESEARCH

Generally speaking, most species of fast-growing, nitrogen-fixing trees are nodulated by *Rhizobium* bacteria, while some nonleguminous genera, such as *Casuarina* and *Alnus*, are nodulated by an actinomycete, *Frankia*. Because many of the CRG grants involve work with these nitrogen-fixing bacteria, procedures for working with *Frankia* and rhizobia have been specified in this manual. These procedures are listed in the appendices and are referred to in the text that follows. Also included are several procedures involving vesicular-arbuscular (VA) mycorrhizal fungi, which establish a symbiotic relationship with the roots of many tree species, facilitating uptake of mineral nutrients, especially phosphorus. In some cases, these fungal associations have been shown to be required for successful tree establishment and growth.

International Sources of Cultures

Rhizobium and *Frankia* are soil bacteria that form root nodules on susceptible host plants. Methods for their isolation and growth in pure culture have been developed. Thus far, VA fungi have not been grown successfully in pure culture.

The isolation of *Rhizobium* and *Frankia* is time-consuming, and investigators not specifically studying these microsymbionts are urged to obtain authenticated *Rhizobium* and *Frankia* strains for use as inocula from the sources listed in Table 1. It may be a better use of time and resources to obtain bacterial cultures from outside sources rather than attempt their isolation and culture within the purview of most grants. Many cultures from these sources have been characterized as to their environmental tolerances. Vesicular-arbuscular mycorrhizae are available for inocula only from infected plants maintained in pot culture.

It is important that the institutions listed below be contacted and told of your work with microsymbionts, as most are interested in and have facilities to handle the exchange of *Rhizobium*, *Frankia*, or VA mycorrhizae.

Table 1. Sources of *Rhizobium*, *Frankia*, and VA Mycorrhizal Fungi for Inocula

***Rhizobium*:**

Dr. Rosemary Bradley
Pasture Legume Improvement Program
Centro Internacional de Agricultura Tropical
AA 67-13
Cali
COLOMBIA

Prof. J. R. Jardim Freire
Rhizobium MIRCEN
IPAGRO
Caixa Postal 776
90000 Porto Allegre
Rio Grande do Sul
BRAZIL

Dr. S. O. Keya
Departments of Soil, Science and Botany
University of Nairobi
P.O. Box 30197
Nairobi
KENYA

Dr. H. H. Keyser
Nitrogen Fixation and Soybean Genetics Laboratory
U.S. Department of Agriculture
Building 011A, HH 19, BARC-W
Beltsville, Maryland 20705
U.S.A.

Dr. P. Somesegran
NifTAL Project and MIRCEN
University of Hawaii
P.O. Box O
Paia, Maui, Hawaii 96779
U.S.A.

***Frankia*:**

Dr. Dwight Baker
Battelle-Kettering Laboratory
150 East South College Street
Yellow Springs, Ohio 45387-0268
U.S.A.

Dr. Yvon Dommergues
BSSFT (CTFT/CNRS/ORSTOM)
45 bis Avenue de la Belle Gabrielle
94160 Nogent-sur-Marne
FRANCE

Dr. Maurice Lalonde
Department of Ecology and Pedology
Faculty of Forestry
Laval University
Sainte-Foy, Quebec G1K 7P4
CANADA

Mrs. Mary P. Lechevalier
Waksman Institute
Rutgers University
P.O. Box 759
Piscataway, NJ 08854-0759
U.S.A.

VA Fungi: Dr. M. Dye
Soil Microbiology Section
Rothamsted Experimental Station
Harpenden, Hertshire, AL5 2JQ
UNITED KINGDOM

Dr. Norman Schenck
Plant Pathology Department
University of Florida
Gainesville, Florida 32611
U.S.A.

Dr. Tim Wood
NPI Institute
417 Wakara Way
Salt Lake City, Utah 84108
U.S.A.

Possible Areas of Microsymbiont Studies

Before embarking on studies of microsymbionts, it is important to critically assess the capacity of existing staff and equipment to handle this difficult, time-consuming, and precise research. The work, as important as it is, will produce few useful results unless the experimental procedures given below are closely followed, particularly those steps involving sterile techniques.

In some microsymbiont research, it may be appropriate to start with the isolation of native rhizobia and then compare them with the most effective strains from the above sources for the particular legume of interest. However, the handling of microorganisms requires a person with some microbiological training, a means of sterilizing media and materials, and a sterile transfer hood. Since it is often difficult to identify the source of nodules collected in mixed species stands, a "trap" plant method is recommended. This method, outlined in full in Appendix 1, involves growing seedlings of a desired species in soil taken from around that species. After a period of time, nodules may then be taken from the "trap" plant.

Nodules are also a source of plant-specific rhizobia; thus, it is important to consider extracting the symbionts from intact nodules. Also, nodules from the "trap" plant method may be used to obtain pure strains of rhizobia by isolation techniques. A method of isolating rhizobia from nodules is given in Appendix 2. Extreme care must be taken to ensure sterile conditions when using this procedure. Briefly, the procedure involves surface-sterilizing the nodules, crushing them, and incubating the exudate on a series of yeast-extract mannitol (YEM) plates. The colonies are then transferred to agar slants and stored in an incubator until used.

Suggestions for the isolation and culture of Frankia are given in Appendix 7. These bacteria are more difficult to isolate than Rhizobium, but successful culture, once isolated, is not difficult. Sources of available cultures are given in Table 1, above.

VA mycorrhizae have not yet been successfully grown in pure culture, and one must rely on pot-culture propagation as a means of maintaining sporulating organisms in soil for use as inoculum.

The next step in the research chain is to screen microsymbiont isolates. The purpose of preliminary screening is to compare several rhizobia, Frankia, or VA fungi for infectivity and effectivity on the tree species to be used in the field trials. This initial screening should greatly reduce the number of field plots required by ensuring that only the most effective microsymbionts are used to inoculate seedlings for field trials. The procedures, detailed in Appendices 5 (rhizobia), 6 (mycorrhizae), and 7 (Frankia), can be done in pots or plastic bags. If followed properly, these procedures will allow an investigator to simultaneously determine which isolates promote best growth in a particular soil and in which field test sites inoculation will be necessary because the required microsymbiont is not present.

Another valuable area of experimentation is the role of microsymbionts in the nursery, where their presence or absence can make a substantial difference in the health and growth of seedlings. If one objective of your study is to assess the effect of inoculation on the growth or productivity of nitrogen-fixing trees, it will be necessary to set up nursery stock with and without inoculum. The following procedure, by class of symbiont, is used for this type of study.

Rhizobia:

1. Make a mixture of washed sand and soil from the field trial sites at a 1:1 ratio.

2. Add 25 ppm P, using either simple or triple superphosphate.

3. Add 100 ppm K (as KCl or K_2SO_4).

4. Sterilize this nursery mixture with methyl bromide. This can be done by digging a pit 1 m x 1 m x 0.5 m and lining the pit with plastic. Fill the pit with the nursery mixture and add some water to moisten. For greatest effect, the air temperature should be at least 15°C. Cover the soil in the pit with plastic. Inject the fumigant under the plastic, using 1 g methyl bromide to every 10 kg soil. If the field soil (step 1) has a high level of native rhizobia, then step 4 may not be necessary (especially if they are effective N_2-fixers). Whether inoculation is necessary here is best determined by leaving some soil unsterilized and then comparing inoculated and uninoculated treatments with treatments in sterilized soil. Note: Methyl bromide is toxic and must be used with care.

5. Wait two days.

6. Divide the soil in half. To one half add 100 ppm N (as urea, NH_4, NO_3).

7. Fill plastic bags (10 cm wide by 20 cm deep with drainage perforations at the bottom) with either the plus or minus nitrogen soil. Do not fill to the top of the bag.

8. Place three surface-sterilized seeds on the top of the soil in each bag. (Use hypochlorite solution; see Appendix 2.)

9. Add 1 ml of the liquid inoculum derived from the YEM broth culture (see rhizobia screening study) to the seeds in those bags that did not receive nitrogen fertilizer.

10. When choosing an inoculum, use the strains that the screening study indicated were the best for a particular species and soil.

11. Place the bags on a bed of gravel or on a raised platform off the ground.

12. Water when necessary with sterilized water.

    Make sure that water is applied until a few drops come out of the drainage holes in the bags. This will ensure that all of the soil in the bags is moistened, and not just the surface soil.

13. Separate the inoculated and uninoculated bags by at least a 1 m buffer strip. Protect bags from damage by animals, insects, and man.

14. Seedlings are ready to transplant in the field when they are 30 cm+ in height. Try to time nursery establishment so that the seedlings are transplanted to the field at the beginning of the rainy season.

Mycorrhizae and Rhizobia:

If the objective of the study is to compare the growth of nitrogen-fixing trees plus and minus rhizobia and VA fungi, then prepare the soil as for rhizobia (steps 1 through 6 above). Then:

1. Divide the fumigated nursery mixture into three parts: one part receives nitrogen, one part receives rhizobia, and the third part receives rhizobia and VA inoculants.

2. Mix VA fungal inoculum at the same rate as in the screening study (see Appendix 6 for this rate, as well as for the procedure to prepare VA inoculum), but prior to putting the soil in the bags.

3. Once the soil is in the bags, inoculate the surface-sterilized seeds with the rhizobium inoculum (1 ml/seed) as was done in step 9 above.

Frankia:

The procedure for Frankia is the same as for rhizobia with respect to the division of soil and fertilizer additions, with the exception that the Frankia should be mixed into the soil (see Appendix 7 for details).

Assessment of Nitrogen Fixation in Field Plantations

Assessment of nitrogen fixation in the field is an important part of research with nitrogen-fixing trees. Two methods for assessing nitrogen fixation are generally used. The first is the ^{15}N method, which requires costly reagents (^{15}N) and equipment (a mass spectrometer). The second is the nitrogen-balance method, which requires simple equipment and is both low-cost and labor-intensive.

The nitrogen-balance method may be the most appropriate in many circumstances. It is particularly suited to sandy soils, but can be used in any soil. The method consists of estimating the total nitrogen content of the tree stand (including the soil) by the Kjeldahl method, and comparing it with the nitrogen content of unplanted soil or with an

uninoculated stand of the tree species of interest. Some errors arise because of inputs of nitrogen in dry fallout and rain and losses of nitrogen *via* denitrification and leaching, which are difficult to measure. However, if one maintains control plots, these errors should be minimized and, at worst, will lead to a conservative estimate of nitrogen fixation. However, because environmental variability is often large and the Kjeldahl method cannot detect small nitrogen differences between treatments, the nitrogen-balance method should only be used when it can be assumed that a species is fixing at least 50 kg nitrogen/ha/yr and when the study period is 5 years or more.

1. Establish four plots/species and four control plots for each sampling time. It is recommended that sampling be done several times; for example, for *Casuarina*, plots can be harvested at 0, 3, 6, and 9 years.

2. Plot size can vary according to the species.

3. Treatments are allocated to plots randomly. Control plots may contain natural vegetation or uninoculated individuals of tree species of interest. If uninoculated individuals are used, first establish that microsymbionts for this species do not occur naturally in the control plots.

4. Establish at least a 4-meter buffer strip between plots, and trench around plot perimeters, if possible, to prevent roots from growing from one plot to another.

5. Make regular collections of litter, using mesh litter traps raised off the soil surface and emptied weekly, and analyze litter for nitrogen content.

6. At each sampling period, four treatments and four control plots are harvested, and the following measurements are made:

   a. Total nitrogen in the above- and below-ground plant parts (weight x % nitrogen). (Roots should be extracted to a depth of 1 meter and subsampled by size class for biomass and nitrogen determinations.)

   b. Total nitrogen in the soil profile at 0-10 cm, 10-20 cm, and 20-30 cm. The number of samples required will depend on the heterogeneity of the soil.

7. The amount of nitrogen fixed can be estimated by subtracting the nitrogen content of the stand without nitrogen-fixing trees from the stands with nitrogen-fixing trees.

REFERENCES

Burley, J., and P. J. Wood, comps. A Manual on Species and Provenance Research with Particular Reference to the Tropics. Tropical Forestry Papers, No. 10. Oxford: Commonwealth Forestry Institute, University of Oxford, 1976.

Blake, G.R. "Bulk density" in: Methods of Soil Analysis. Part 1., C.A. Black, D.D. Evans, J.L. White, L.E. Ensminger and F.E. Clark (eds.) Madison, Wisconsin, U.S.A.: American Society of Agronomy, 1965.

Bremner, J.M. and C.S. Mulvaney. "Nitrogen - total" in: Methods of Soil Analysis. Part 2. second edition, A.L. Page (ed.) Madison, Wisconsin, U.S.A.: American Society of Agronomy, 1982.

Buol, S.W., F.D. Hole, and R.J. McCracken. Soil Genesis and Classification. second edition. Ames, Iowa, U.S.A.: Iowa State University Press, 1980.

Carlowitz, Peter G. von. Multipurpose Tree Seed and Shrub Directory. Nairobi: International Council for Research in Agroforestry, 1986.

Day, P.R. "Particle fractionation and particle-size analysis" in: Methods of Soil Analysis. Part 1, C.A. Black, D.D. Evans, J.L. White, L.E. Ensminger and F.E. Clark (eds.) Madison, Wisconsin, U.S.A.: American Society of Agronomy, 1965.

Dudal, R. "Definitions of soil units for the soil map of the world" in: World Soil Resources Report 33. Rome: FAO, 1968.

Dudal, R. "Key to the soil units for the soil map of the world" in: Soil Res. Dev. and Can. Ser., Land and Water Dev. Div. Rome: FAO, 1970.

Edwards, D. G. W., comp. World Directory of Tree Seed Workers. Ottawa: Canadian Forest Service for the International Union of Forestry Research Organizations, 1976.

Fisher, R. A., and F. Yates. Statistical Tables for Biological, Agricultural and Medical Research. 6th Edition. Edinburgh and London: Oliver and Boyd, 1963.

Food and Agriculture Organization of the United Nations. Forest Tree Seed Directory. Rome: FAO, 1975.

Freese, F. Elementary Statistical Methods for Foresters. Agricultural Handbook 317. Washington, D.C.: Forest Service, U.S. Department of Agriculture, 1967.

Hunter, A.H. Suggested soil and plant analytical techniques for tropical soils research program laboratories. Orange City, Florida, U.S.A.: Agro Services International, 1979.

Huxley, P. A., ed. Methodology for the Exploration and Assessment of Multipurpose Trees. Nairobi: International Council for Research in Agroforestry, Commonwealth Forestry Institute, and National Research Council (U.S.A.), 1984.

Keeney, D.R. and D.W. Nelson. "Nitrogen - total" in: Methods of Soil Analysis. Part 2. second edition, A.L. Page (ed.) Madison, Wisconsin, U.S.A.: American Society of Agronomy, 1982.

Nelder, J. A. "New Kinds of Systematic Designs for Spacing Experiments." Biometrics, 18 (1962), 183-307.

Nelson, D.W. and L.E. Sommers. "Total carbon, organic carbon, and organic matter" in: Methods of Soil Analysis. part 2. second edition, A.L. Page (ed.) Madison, Wisconsin, U.S.A.: American Society of Agronomy, 1982.

Soil Survey Staff. "Soil Taxonomy" in Handbook 436 of the U.S. Dept. of Agriculture. Washington, D.C., U.S.A.: U.S. Government Printing Office, 1975.

Thomas, G.W. "Exchangeable cations" in: Methods of Soil Analysis. Part 2. second edition. A.L. Page (ed.) Madison, Wisconsin, U.S.A.: American Society of Agronomy, 1982.

Weber, Fred R. Reforestation in Arid Lands. Mt. Rainier, Maryland: Volunteers in Technical Assistance, 1977.

APPENDIX 1

Isolation of Rhizobia

"Trap"-Plant Method for Obtaining Rhizobia

1. For each site from which isolates of a particular species are desired, collect 5 kg of soil from the 0-20 cm layer. If the tree species under investigation is present in the site, collect the soil from under individuals of this species.

2. Mix each soil sample with 5 kg of sterilized sand, vermiculite, or perlite.

3. Place the mixture in five (4-inch) clay pots, or in plastic bags with holes punched in the bottom for drainage.

4. Plant three scarified and surface-sterilized seeds per pot; the scarification method will depend on the type of seed.

5. Thin to one plant per pot when the first true leaves appear.

6. Place a half-inch layer of sterile sand over the soil in the pot to prevent airborne contamination.

7. Water as often as needed with sterilized tap water.

8. Place the pots in a glasshouse. If none is available, the plants can be placed outside on a table raised off the ground, but they must be protected from animals and adverse weather.

9. Harvest two pots after 2 months and check for nodules. If none are present, repeat the harvest at 4 months. If no nodules have formed after 4 months, assume that the species will not nodulate in that particular unamended soil.

10. On the first date that nodules are found, remove the nodules from the roots and isolate the rhizobia as quickly as possible; preferably the same day. If nodules must be stored before isolation, place nodules in test tubes that are half filled with silica gel, anhydrous $CaCl_2$, or some other dessicant. Cover the dessicant with a layer of cotton or glass wool, and place clean nodules on top of this layer. Stopper the tube tightly, and store under refrigeration. Rhizobia can be successfully isolated from young nodules stored in this manner for up to 1 month.

11. Before isolation, rehydrate the stored nodules by soaking in sterilized tap water.

APPENDIX 2

Extraction of Rhizobia from Nodules[a]

Materials Required

1. Clean muslin or nylon mosquito netting.
2. Clean (not necessarily sterile) test tubes (15 mm x 15 cm is best, but any size will do).
3. Potassium or sodium hypochlorite (available as commercial or household bleach).
4. Sterile tap water.
5. A glass stirring rod.
6. An alcohol lamp or Bunsen burner.
7. A platinum wire inoculating loop.
8. 95% ethanol.
9. Poured 10 cm petri plates of yeast-extract mannitol agar (see Appendix 5 for formula); two to four plates for each nodule.

Procedure

1. Place one nodule in a test tube
2. Fill the tube one-quarter full with sterile tap water and shake vigorously by tapping the tube against the palm of the hand several times.
3. Secure the mosquito netting over the open end of the tube.
4. Pour out the water in the tube through the netting.
5. Repeat steps 2 through 4 until the water poured out of the tube is clear.
6. Add 95% ethanol to the tube to just cover the nodules.

NOTE: [a]Modified from J. M. Vincent. A Manual for the Practical Study of Root-Nodule Bacteria. IBP Handbook No. 15. Oxford: Blackwell Scientific Publications, 1970.

7. Immediately pour out the ethanol.

8. Pour in enough sterilizing agent to cover the nodules and rotate the tube to coat the sides with it.

9. Pour off the sterilizing agent.

10. Fill the tube halfway with sterile water and swirl the tube to rinse the nodules.

11. Repeat step 10 at least ten times.

12. Dip the stirring rod in the ethanol and pass through a flame.

13. Use the end of the stirring rod to crush the nodules in the sterilizing tube.

14. Spread a loop of the fluid from the crushed nodule over the surface of a YEM plate. Label the plate with the date and other identifying information. Make two plates from the tube.

15. Repeat steps 1 through 14 to obtain the number of isolates desired for each species/soil combination. (Note: Five isolates from each species/soil combination is a reasonable number of isolates if several species or soils are being studied.)

16. Incubate the plates at 26°C and examine them periodically for the growth of isolated colonies. Rhizobia from some trees are fast-growing (for example, Leucaena, Prosopis, and Gliricidia spp.) and will produce colonies within 3 to 5 days. Rhizobia from other species (such as Enterolobium and Albizia spp.) are slower growing, and will take 10 days or longer to produce much smaller colonies. Most rhizobia colonies will be moist, translucent, or whitely opaque, and some will be gummy. Be sure to wait at least 2 weeks before discarding plates that show no growth, since some slow-growing rhizobia are very slow to develop visible colonies.

17. Using the transfer loop, pick up some material from an isolated colony and restreak this material onto a new YEM agar plate.

18. When growth occurs on the new plate, obtain some material from an isolated colony and perform a gram stain.

19. If the gram stain is gram negative, transfer some of the material from the colony from which the gram stain was made to a YEM agar slant.

20. Immediately label the slant and record the following information in a notebook:

    a. Host plant from which the isolate was obtained.

    b. Location where the host plant was growing, and whether it was grown in the field, in the greenhouse, etc.

    c. Soil type where the host was growing (if known).

    d. Soil pH where the host plant was grown (if known).

    e. Colony morphology (translucent, opaque, gummy).

    f. Colony size (diameter in mm) at 3-4 days for fast-growing rhizobia, and at 10-15 days for slow-growing rhizobia.

    g. Whether the isolate was fast- or slow-growing.

    h. Date of isolation.

    i. Name of person who made the isolation.

    j. Name of the laboratory.

21. Store slants in the refrigerator and transfer the isolates to new slants at least every 4 months. (Note: Some cultures--for example, some Sesbania rhizobia--may have to be transferred every 2 weeks.)

APPENDIX 3

Authentication of Rhizobia

1. Take a loopful of material from a slant and transfer it aseptically to a 250 ml flask containing 100 ml of YEM broth (see Appendix 8 for formula).

2. If possible, place the flasks on a rotary shaker until the liquid is quite cloudy. Another way to obtain material for the authentication test, if a rotary shaker is not available, is to wash the bacteria off the surface of several slants using 5 ml of sterile distilled water per slant. If the latter method is used, be sure to first make a new slant for the permanent culture collection before washing off the old slant.

3. For small-seeded species (Leucaena size or smaller), the authentication test is performed in test tubes with seedling agar (see Appendix 8 for formula). For large-seeded species, Leonard jars may be used.

4. Place one surface-sterilized seed in each test tube (see Appendix 8 for methods to surface-sterilize seeds). Set up three tubes for each species/isolate combination.

5. Pipette 0.1 ml of the broth culture or 0.1 ml of the material washed from the slant over the seed.

6. Place the tubes in an area where they can receive light (for example, in the greenhouse or near a laboratory window).

7. When needed, add nitrogen-free plant nutrient solution (see Appendix 8 for formula) to the large test tube assemblies through a glass tube or a drinking straw.

8. Periodically check for the presence of nodules.

9. When the leaves of the seedlings in the tubes hit the cotton plug at the top of the tube, aseptically remove the cotton to allow the leaves to protrude from the tube and replace the cotton around the stem to reform the sterile barrier.

10. If no nodules are present after 1 month, discard the assemblies and conclude that the isolate was either not a Rhizobium or not a Rhizobium capable of nodulating the species of interest.

APPENDIX 4

Screening of Rhizobia

1. Collect enough soil from the 0-20 cm horizon in each proposed field test site to fill ten thick plastic bags (6 cm x 20 cm) for an uninoculated control, and ten bags for each isolate to be evaluated. Punch holes in the bottom of the bags for drainage. Mix the soil very well and dispense into the pots or bags.

2. Place three scarified seeds of the test species on the surface of the soil in each pot or bag.

3. Inoculate each seed by pipetting 1 ml of a broth culture of the Rhizobium to be tested (use YEM broth that was previously inoculated with a loopful of material from the stock slant, and then grown up until the broth just turned visibly turbid).

4. Cover the seeds with the same soil that is in the bags, so that the thickness of the soil above the seeds equals the diameter of the seed.

5. Place the bags in a glasshouse or, if none is available, on a table outside that is raised off the ground. Shade, if necessary, and protect the bags from animals, pests, and weather extremes.

6. Group all of the bags for each isolate together and separate groups by at least 1 meter. This prevents cross-contamination from one group of pots or bags to another. When watering the bags, use sterile tap water or sterile nutrient solution lacking nitrogen (see Appendix 8), and take care not to touch the surface of the soil in the bags with the watering device.

7. Thin to one plant per bag when the first true leaves appear. Harvest when the tallest plant is 30 cm tall.

8. Remove the entire soil mass from the bag and carefully extract the roots and nodules from the soil. Examine the soil for nodules that may have sloughed off the roots during harvest. Use water if necessary to wash the soil away from the roots and nodules.

9. Cut the top portion of the plant from the roots 1 cm above the root collar.

10. Separate the nodules from the roots, dry at 70°C for 48 hours, and then weigh the dried nodules. Separate leaves from stems, and dry and weigh them in the same way.

11. Grind stems and leaves separately and analyze each for total nitrogen content using the Kjeldahl or other similar method.

12. Calculate the effect of the different isolates on the growth and nitrogen content of the test species by subtracting the total nitrogen content of the uninoculated control plants from that of the inoculated plants.

APPENDIX 5

Preparation and Use of Legume Inoculants

This appendix describes the preparation and use of legume inoculants containing the nodule bacteria of the family Rhizobiaceae. A good quality inoculant is a prerequisite for adequate nodulation and nitrogen fixation. Inoculant preparation requires aseptic procedures and access to microbiological facilities. If inoculant preparation is not feasible at your facility, specific inoculants (cultures or a peat-based carrier) can be obtained from the NifTAL Project and MIRCEN or from the Rhizobium Study and Collection Center at the U.S. Department of Agriculture.

Inoculation of legumes in the greenhouse or nursery can be accomplished with liquid broth culture (yeast-extract mannitol) or with a peat carrier. For use in the field with the direct seeding of legumes, the peat inoculant is recommended.

Preparation of Inoculum

While peat is the most common carrier for inoculants, bagasse, filter mud, charcoal, and lignite have also been used. The procedure described below relates specifically to peat as the carrier, and it would apply with minor variations to the others.

1. Particle size of the peat.

   The peat should be milled to particles of 200 mesh or finer (pass through a 0.075 mm sieve) for use as a seed coating.

2. Neutralization of the peat.

   Most peats are too acid for use as a carrier for rhizobia without neutralization with lime or dolomite. Finely divided $CaCO_3$ gives rapid reaction. Adjust pH to 7 ± 0.3.

3. Sterilization of the peat.

   If possible, the peat should be autoclaved (120°C) for 2 hours, then allowed to cool for 24 hours, with provision for gas exchange.

4. Inoculation of the peat.

a. Culturing of the rhizobia.

The strain(s) of rhizobia should be grown in yeast-extract mannitol (YEM) broth, the composition of which follows:

K_2HPO_4	0.5 g
$MgSO_4$	0.2 g
NaCl	0.1 g
Mannitol	10.0 g
Yeast extract	0.4 g
Distilled water	1.0 l (adjust pH to 6.8 - 7.0)

Autoclave at 120°C for 15 minutes. After cooling to room temperature, 1 liter of broth can be inoculated with a loopful of culture from an agar slant. Fast-growing rhizobia will reach maximum cell density (about 10^9 cells per milliliter) in 3 to 5 days at 25°C, while slow growers require 7 to 10 days.

b. Addition of rhizobial broth to the peat.

When the strains have reached maximum density, the YEM broth is added to the peat at a volume to obtain 35-40 percent moisture in the peat on a wet weight basis. The author routinely adds 100 ml of mature YEM broth to 180 g peat (36 percent moisture). With the wet peat in a plastic bag, gently mix the contents to ensure good wetting of the peat, which is initially hydrophobic.

5. Incubation and storage of peat inoculant.

Though the peat inoculant can be used after adequate mixing (step 4b), best results are obtained by allowing the peat to incubate at room temperature (25-30°C) for 2 to 3 weeks. This permits the growth of rhizobia in the peat to high population levels. During this period, the bag should not be closed tight, as exchange of gases is important for adequate growth of the rhizobia. Do not allow the moisture content to fall below 30 percent. If it should, add sterile YEM broth to a moisture level of 35 percent. The inoculant should not be kept at room temperature for more than 4 weeks, but should then be kept in a refrigerator (4°C). When stored at 4°C the peat can be used as an inoculant for up to 12 months. However, it is best to use the inoculant as soon as possible after the 2-3 week incubation.

Use of Inoculum

1. Inoculating directly with YEM broth culture in the greenhouse or nursery.

   Under favorable greenhouse and nursery conditions, legume seed inoculation can be achieved with YEM broth culture. From step 4a above, the mature broth should contain about 10^9 cells per ml. The addition of 0.1 ml directly to each seed would provide 10^8 cells of rhizobia, which is more than adequate. At this rate, 1 liter of broth will permit the inoculation of 10,000 seeds. After the seed is placed in a hole or furrow, drip the 0.1 ml directly onto the seed, and cover before the broth dries.

2. Inoculation of legume seed with peat.

   The inoculation of seed with peat is best accomplished by the use of an adhesive. Excellent results are obtained with a 40% solution of gum arabic (=gum acacia). A 5% methyl ethyl cellulose solution can also be used. The gum arabic solution is prepared by heating 100 ml water and slowly adding 40 g with constant stirring. Neutralization of the solution may be needed.

   The recommended rate of inoculation is about 10 g of peat for each kilogram of seed. While seed size varies, the object is to obtain seed that is visibly coated with peat. For each kilogram of seed, addition of 20-30 ml of gum arabic solution should coat the seed thoroughly. This is followed by the addition of the peat, with adequate mixing to give uniform coating of the seeds. Inoculated seed should be planted within 24 hours. In the field, the inoculated seed should not be exposed to direct sunlight for long periods of time. Once the inoculated seed is placed in the furrow or hole, it should be quickly covered.

Useful References with Details of Legume Inoculant Preparation and Use

[J. Burton.] Legume Inoculants and Their Use. Rome: Food and Agriculture Organization of the United Nations, 1984.

R. J. Roughley, "The Preparation and Use of Legume Seed Inoculants." Plant and Soil, 32(1970), 675-701.

P. Somasegaran and H. J. Hoben. Methods in Legume-Rhizobium Technology. Paia: University of Hawaii NifTAL Project and MIRCEN, 1985.

J. M. Vincent. A Manual for the Practical Study of Root-Nodule Bacteria. IBP Handbook No. 15. Oxford: Blackwell Scientific Publications, 1970.

Sources of Rhizobium Cultures and Peat Inoculants

Refer to page 28, Table 1: Sources of Rhizobium, Frankia, and VA Mycorrhizal Fungi for Inocula.

APPENDIX 6

Propagation and Screening of VA Mycorrhizal Fungi[a]

None of the many species of vesicular-arbuscular (VA) mycorrhizal fungi have been successfully grown in pure culture. Therefore, one must culture the microorganisms by infecting susceptible host plants and multiplying the fungi through growth and sporulation on the root system--a method commonly called pot culture. A starter culture or inoculum must be obtained from a preexisting pot-culture source.

1. Sterilize substrate (washed sand, perlite or vermiculite or, preferably, soil with P content less that 10 ppm) in an autoclave or with methyl bromide (Dow Fume MC33 or MC2). For methyl bromide sterilization, use 2 g fumigant per 10 kg substrate. Place substrate in a large plastic bag and add the methyl bromide. Rotate the bag so that the methyl bromide is evenly distributed throughout the substrate. Follow the instructions listed by the manufacturer for using methyl bromide, as it is a toxic substance.

3. Fill large plastic dish pans (approximately 40 cm x 40 cm x 20 cm) with the sterilized medium. Punch holes in the bottom for drainage.

4. If a large amount of starter culture has been obtained from one of the sources listed previously, then mix the starter evenly throughout the entire pan. If only a small amount of culture is on hand, place the starter culture in strips across the pan and cover with 2 cm of sterile sand.

5. Place surface-sterilized seeds of sudan or bahia grass or sorghum over the inoculum strips, or at 10 cm x 10 cm spacing, when the inoculum has been evenly mixed throughout the pan. These grasses serve as suitable host plants for the VA fungi.

6. Water the plants when necessary. Do not overwater.

7. Feed weekly with a plant-nutrient solution (see Appendix 8) with low phosphorus. Keep plants in full sunlight, if possible.

NOTE: [a]Modified from Ferguson, J. J. and S. H. Woodhead. 1982. Production of endomycorrhizal inoculum. A. Increase and maintenance of vesicular-arbuscular mycorrhizal fungi. In: Methods and Principles of Mycorrhizal Research (N. C. Schenk, ed.), American Phytopathological Society, St. Paul, Minn. pp. 47-54.

8. After 2 months, pull out a few plants from one of the pans and examine the roots to see if they are infected with VA fungi (see Appendix 8 for formula for mycorrhizal stains). A dissecting scope is adequate to see VA fungi in roots. Mycorrhizae inside the stained roots will appear as blue hyphae with or without globular vesicles.

9. If roots from the test plants are infected, harvest the rest of the plants in the pans.

10. Cut off the plant shoots and discard.

11. Chop or cut the roots with a knife or scissors and mix the chopped roots back into the sand in the pans.

12. The mixture of chopped roots and sand containing spores is the mycorrhizal inoculum. It may be divided, air dried, and put into plastic bags that are subsequently sealed. Inoculum prepared in this way and stored at 5°C has been found to remain viable for as long as 4 years.

13. To compare the efficacy of different mycorrhizae on nitrogen-fixing trees, add 5% vol/vol fresh weight inoculum/soil from the field trial sites. Fill ten bags or pots (6 cm x 20 cm) with soil+inoculum for each VA fungus to be evaluated, and an additional ten bags with soil only to serve as controls.

14. The rest of the experimental procedure is identical with that described in Appendix 4 above (steps 2 through 12, omitting those steps that relate to the introduction of rhizobia if mycorrhizal fungi are to be evaluated alone). If VA fungi are being screened in the presence of rhizobia, nitrogen as well as phosphorus should be omitted from the nutrient solution.

15. At the end of the experiment, phosphorus content of seedlings should be assessed as well as nitrogen content.

APPENDIX 7

Isolation, Culture, and Inoculation with *Frankia*

Frankia is a filamentous bacterium of the Actinomycetales that occurs generally in the soil. Filaments enter the roots of susceptible woody dicotyledonous plants (collectively referred to as actinorhizal plants), inducing nodules derived from branched and modified lateral roots. Within these root nodules, *Frankia* fixes dinitrogen (N_2), converting it *via* ammonia (NH_3) to amino acids and proteins. The organic nitrogen becomes available to the host plant and is used for its growth and development.

Both in nodules and in pure culture, *Frankia* forms filaments that branch and ramify. In most host plants, the filaments terminate in swollen endings called vesicles within which the enzyme nitrogenase is formed. *Casuarina* and other members of the Casuarinaceae are exceptions, in that no vesicles are formed and *Frankia* remains filamentous, although capable of nitrogen fixation. (Protection from oxygen denaturation is provided by modification of the host cells.) In addition to vesicles, *Frankia* often sporulates in the nodule or in pure culture. Sporangia up to 60 um may form in culture, although the spores formed within tend to be spherical and only 1.0-1.5 um in diameter.

Isolation and Culture of *Frankia*

Since root nodules contain enriched colonies of *Frankia*, isolation of *Frankia* for establishing pure cultures is most easily performed with surface-sterilized root nodules. The following procedure is a simple method that has been used successfully on nodules of a number of different host plants.

Select freshly collected nodules. Ideally, it is useful to do an acetylene reduction test to demonstrate their activity in N_2 fixation. Otherwise, take nodules from healthy green plants, select young nodules that are turgid and not excessively suberized or browned. Rinse the nodules with tap water to remove soil particles. Excise individual nodule lobes, removing nodule roots, if present. Rinse in distilled water several times. Surface-sterilize the nodule lobes by immersing them for 20 minutes in commercial bleach solution (sodium hypochlorite solution (5.25%) diluted 3:10 in water and amended with a trace of detergent). Rinse several times with sterile distilled water. After repeated rinsing, aseptically place each separate nodule lobe into a test tube containing 5 ml of sterile yeast-dextrose broth and plugged with a cotton plug. Incubate the tubes at 28°C for 7-10 days to test for surface-contaminating bacteria. If the tubes are cloudy or contain fungal growth, discard them by autoclaving.

Nodule lobes demonstrably free of contaminating bacteria are cut in half with a scalpel or broken open with tweezers to release Frankia filamentous growth, and each nodule is transferred aseptically to a test tube containing an appropriate nutrient medium for the growth of Frankia (for example, QMOD medium, or BAP medium, or M6B medium; see Appendix 8). Incubate the tubes at 28°C.

Outgrowth of Frankia filaments usually occurs from pieces of the nodule lobe and can form small fluffy colonies in the medium in the tube. When the growth of Frankia becomes apparent, the material in the tube can be homogenized or the fluffy mat transferred to fresh medium in a flask (20 ml medium in a 50 ml Erlenmeyer flask) and allowed to increase as a fluffy filamentous mat. Subculture by homogenizing, and then subdivision can be made thereafter in liquid culture (use 50 ml medium in a 125 ml Erlenmeyer flask) every 4-6 weeks. Homogenization is most easily done with a sterile mortar and pestle, or with a Potter-Elvehjem glass homogenizer. The culture may also be broken into tiny filaments by drawing up the filamentous material repeatedly through a large-bore needle attached to a sterile glass syringe.

If you are provided with a living culture of Frankia in a small sterile tube in liquid medium, transfer the culture, using sterile techniques, to a flask containing Frankia medium (see above). The liquid medium should remain clear with a filamentous colony of Frankia forming and growing at the bottom of the flask. If the medium turns cloudy, contaminating bacteria have entered the culture. Fungal contamination is probable if filamontous colonies with visible dark spores are seen floating in the medium. Fungal contaminants may be controlled or prevented by adding cycloheximide (100 ug/ml) to the medium before autoclaving.

Preparation of Frankia Inoculum

If pure cultures of Frankia are not available, it is still possible to prepare inocula for seedlings from freshly collected nodules or even from dried nodules. The basic procedure involves grinding up nodules in a mortar and pestle and applying the ground-nodule suspension to the roots of seedlings or to soil around the seedling roots.

For each gram (fresh weight) of nodules, add 20 ml of sterile distilled water, and grind it up in a mortar and pestle. If care is needed to exclude other Frankia strains as contaminants, one can surface-sterilize the nodules as described above, and rinse the mortar and pestle repeatedly with 70% ethyl alcohol.

The inoculum can be applied to seedling roots at the time of seedling transfer by root-dipping in the suspension or inoculum, or by adding 0.5 ml of inoculum suspension to the base of each seedling in the soil in which it is to grow.

If pure cultures of Frankia are available, one can obtain effective inocula from flask cultures grown for 4 weeks in liquid medium. The filamentous mat should be removed from the nutrient medium by decanting the medium or by centrifugation. Repeated rinses of the filamentous mat with sterile distilled water are desirable. Then the Frankia filaments and sporangia, and spores which are also probably present, should be homogenized to a preparation of finely divided filaments. One can easily measure the packed cell volume (pcv) of this homogenized culture. Such a preparation makes an excellent inoculum. On average, 0.1 ml pcv is applied per seedling in plants grown in water culture or seedlings planted in sand or soil in containers. Application may be by root dipping or injection into the soil around planted seedlings.

Seedlings should show macroscopically visible nodules within 2-3 weeks, and their presence is accompanied by greening and new growth at the tips of the young plants after 4-8 weeks.

APPENDIX 8

Special Formulae and Apparatus

A. Yeast-Extract Mannitol (YEM) Broth and Agar[a]

K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
Mannitol[b]	10.0 g
Yeast Water[c]	100.0 ml
Distilled water	900.0 ml

Autoclave at 120°C for 15 minutes.[d] The YEM agar contains 15 g agar per liter.

NOTES: [a]Modified from J. M. Vincent. A Manual for the Practical Study of Root-Nodule Bacteria. IBP Handbook No. 15. Oxford: Blackwell Scientific Publications, 1970.

[b]Can be substituted by using cheaper carbon compounds.

[c]Fresh yeast extract is prepared from 100 g baker's compressed yeast mixed with 1 liter of cold water. Allow it to stand at room temperature for 1 to 2 hours, autoclave for 40 to 60 minutes, allow it to settle (or centrifuge), and use the clear supernatant as yeast water (the pH of the yeast water should be 6.8, if not, adjust before adding it to the medium). Commercially available yeast extract can also be used; 0.4 g/l is satisfactory.

[d]Repeated heating should be avoided. Therefore, make the agar medium in quantities that can be used with only one melting or preferably use the sterilization process itself to melt the agar.

B. Seedling Agar[a] and Small Seed Test Tube Assembly for Rhizobia Authentication Test

$CaHPO_4$	1.0 g
K_2HPO_4	0.2 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.2 g
$FeCl_3$	0.1 g
Water	1.0 l
Agar	15.0 g
Micronutrients[b]	1.0 ml

Autoclave at 120°C for 15 minutes and dispense equal volumes into sterile test tubes with cotton plugs. The size of the tubes will depend on the size of the plant species. The depth of the agar in the tube should be about 5 cm.

NOTES: [a]Modified from H. L. Jensen. "Nitrogen Fixation in Leguminous Plants. I. General Characters of Root-Nodule Bacteria Isolated from Species of Medicago and Trifolium in Australia." Proceedings of the Linnean Society of New South Wales, 66 (1942), 98-108.

[b]From stock containing: 0.05% B; 0.05% Mn; 0.005% Zn; 0.005% Mo; and 0.002% Cu.

C. Large Test Tube Assemblies for Authentication of Large Seeded Species[a]

1. Place a glass pipette, glass tube, or plastic drinking straw in a large test tube. Make sure the top of the tube protrudes at least several centimeters above the top of the test tube.

2. Place a 2 cm layer of gravel in the bottom of the test tube.

3. Fill the rest of the test tube with vermiculite or washed sand, leaving a few centimeters clearance at the top.

4. Cap the glass tube or pipette or bend over the straw. Plug the test tube with cotton. Autoclave the assembly at 120°C for 15 minutes. The straw or tube will be used to add nutrient solution to the seedlings during incubation.

D. Surface Sterilization of Seeds

1. Seeds that have been scarified using concentrated sulfuric acid may also be considered surface-sterilized. However, to maintain sterility, the acid should be washed off with at least ten rinses of sterile distilled water.

2. Seeds scarified by using mechanical methods (rasping with a file or puncturing the seed coat with a needle), soaking in hot or cold water, or by passage through the gut of animals, should be soaked for 15 minutes in a 4% household bleach solution. It may be necessary to do some preliminary tests to determine if a 15-minute soaking in household bleach is sufficient. Rinse the seeds well (at least ten times in sterile distilled water) before placing the seeds in the authentication tubes.

NOTE: [a]Modified from J. M. Vincent. A Manual for the Practical Study of Root-Nodule Bacteria. IPB Handbook No. 15. Oxford: Blackwell Scientific Publications, 1970.

E. Staining of Roots for Vesicular-Arbuscular Mycorrhizal Fungi[a]

Solution 1:	lactic acid	500 ml
	glycerine	360 ml
	H_2O	325 ml

Solution 2: Same as solution 1, but add 0.05% trypan blue.

Procedure:

1. Clear roots in 10% KOH for one hour at 90°C. (It may be necessary to repeat this step several times if the roots are highly colored. For highly colored tree roots, it may be necessary to add H_2O_2 to clear the roots.)
2. Rinse the roots three times with tap water.
3. Place the roots in 1% HCl for 3 minutes.
4. Stain the roots by placing them in solution 2 (above) for 10 minutes at 90°C.
5. Drain off the excess stain.
6. Rinse the roots twice with tap water.
7. Destain further by rinsing the roots in solution 1, which can also be used to store the roots until they can be examined microscopically. (Note: Many tree roots take up the dye very strongly, so it may be necessary to soak the roots in several fresh changes of solution 1 until the roots are destained sufficiently to see the mycorrhizae. One way to minimize this problem is to always choose the finest roots for staining).

NOTE: [a]Modified from J. M. Phillips and D. S. Hayman. "Improved Procedure for Cleaning Roots and Staining Parasitic and Vesicular-Arbuscular Mycorrhizal Fungi for Rapid Assessment of Infection." Transactions of the British Microbiological Society, 55 (1970), 159-161.

F. Plant Nutrient Solution[a, b]

Solution	Element	Form	M.W.	g/l	M
1	Ca	$CaCl_2.2H_2O$	147.03	294.1	2.0
2	P	KH_2PO_4	136.09	136.1	1.0
3	Fe	Fe Citrate	355.04	6.7	0.02
4	Mg	$MgSO_4.7H_2O$	246.5	123.3	0.5
	K	K_2SO_4	174.06	87.0	0.5
	Trace Elements				
	Mn	$MnSO_4.7H_2O$	169.02	0.338	0.002
	B	H_3BO_3	61.84	0.247	0.004
	Zn	$ZnSO_4.7H_2O$	287.56	0.288	0.001
	Cu	$CuSO_4.5H_2O$	249.69	0.100	0.0004
	Co	$CoSO_4.7H_2O$	281.12	0.056	0.0002
	Mo	$Na_2MoO_4.2H_2O$	241.98	0.048	0.0002

Make up the four solutions in 0.5 liter quantities and store. To make 10.0 liters of complete plant nutrient solution, add 5.0 ml of solutions 1 through 4.0 to 5.0 liters of water, add 4.0 more liters of water and adjust the pH to 6.8. Make up to final volume of 10 liters.

NOTES: [a]Modified from W. J. Broughton and M. J. Dilworth. "Control of Leghaemoglobin Synthesis in Snake Beans." Biochemistry Journal, 125 (1971), 1075-1080.

[b]The nutrient solution described above lacks nitrogen and is suitable for testing for effective N_2-fixing strains of Rhizobium. If used in tests for VA mycorrhizae, the solution should be made up without added P.

G. Modified OMOD Medium[a]

Component	Final concentration (in distilled water)
K_2HPO_4	0.3 g/l
NaH_2PO_4	0.2 g/l
$MgSO_4.7H_2O$	0.2 g/l
KCE	0.2 g/l
Yeast Extract (Difco)	0.5 g/l
Bactopeptone (Difco)	0.5 g/l
Glucose	10.0 g/l
Ferric Citrate (1% soln.)	1.0 ml/l
Minor Salts[b]	1.0 ml/l
Lipid Supplement[c]	1.0 ml (5 mg/l)

Adjust pH to 6.8 - 7.0 and add 0.1 g/l $CaCO_3$.

NOTES: [a]From M. Lalonde and H. E. Calvert. "Production of Frankia Hyphae and Spores as an Infective Inoculant for Alnus Species." In Symbiotic Nitrogen Fixation in the Management of Temperature Forests. Edited by J. C. Gordon, C. T. Wheeler, and D. A. Perry. Corvallis: Forest Research Laboratory, Oregon State University, 1979.

[b]Minor Salts Stock Solution (g/l): H_3BO_3, 1.5; $MnSO_4.7H_2O$, 0.8: $ZnSO_4.7H_2O$, 0.6; $CuSO_4.5H_2O$, 0.1; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.2; $CoSO_4.7H_2O$, 0.01.

[c]Lipid Supplement: Dissolve 500 mg of L- -lecithin (commercial grade from soybeans, 22% phosphatidyl choline, P-5638 from Sigma Chemical Co., St. Louis, MO) in 50 ml of absolute ethanol, and add 50 ml of distilled water.

H. Revised BAP Medium for Frankia[a]

Autoclave Together	Final Conc.	Stock	Use/per Liter
$MgSO_4.7H_2O$	0.2 mM	0.08 M (19.72 g/l)	2.5 ml
$CaCl_2.2H_2O$	0.07 mM	0.07 M (10.3 g/l)	1.0 ml
NH_4Cl	5.0 mM	1.0 M (53.5 g/l)	5.0 ml
FeNa EDTA[b]	0.02 mM	0.02 M	1.0 ml
Micronutrients[c]	--		1.0 ml
Vitamins[d]	--		1.0 ml

Autoclave (A) or filter-sterilize (F) individually, then add to autoclaved solution.

(A) Phosphate buffer[e]	10 mM	1 M	10.0 ml	
(F) Carbon source Na propionate	5 mM	1 M (96.1 g/l)	5.0 ml	pH 6.7
(or: Na pyruvate	10 mM	1 M (110.0 g/l)	10.0 ml	pH 6.3)

Use distilled water for all stocks and final solution.

Adjust pH of all stock solutions (except vitamins and micronutrients) to pH 6.7 or to optimum pH for a particular isolate (for example, pH 6.3 for CcI3).

NOTES: [a]Medium modified from M. A. Murry, M. S. Fontaine, and J. G. Torrey. "Growth Kinetics and Nitrogenase Induction in Frankia sp. HFP ArI5 Grown in Batch Culture." Plant and Soil, 78 (1984), 61-78.

[b]FeNa EDTA: $FeSO_4.7H_2O$, 5.56 g/l + Na_2EDTA, 7.45 g/l. Adjust pH to 6.3 with 5 N NaOH.

[c]Micronutrients Stock: (g/l) H_3BO_3: 2.86, $MnCl_2.4H_2O$: 1.81, $ZnSO_4.7H_2O$: 0.22, $CuSO_4.5H_2O$: 0.08, $Na_2MoO_4.2H_2O$: 0.025, $CoSO_4.7H_2O$: 0.001.

[d]Vitamin Stock: (mg/100 ml) thiamin HCl: 10, pyridoxin HCl: 50, nicotinic acid: 50, biotin: 22.5, folic acid: 10, Ca pantothenate: 10, riboflavin: 10.

[e]Phosphate buffer: Titrate 1M KH_2PO_4 (136.09 g/l) with 1M K_2HPO_4 (174.18 g/l) to appropriate pH (about 7/4 vol/vol for pH 6.7).

Final solution pH: using propionate 6.7
using pyruvate 6.3

I. M6B-Plus Medium

Component	Final Concentration (in double distilled water)
Yeast Extract (Difco)	5.0 g/l
Dextrose	10.0 g/l
Casamino Acids (Difco)	5.0 g/l
KH_2PO_4	1.0 g/l
$MgSO_4.7H_2O$	0.1 g/l
$CaCl_2.2H_2O$	0.01 g/l
$CoCl_2$	0.0001 g/l
Hoagland's Trace Element Solution[a]	1.0 ml/l
B-D Vitamin Solution[b]	1.0 ml/l
FeNa EDTA[c]	10.0 mg/l
Adjust pH to 6.5	

NOTES: [a]Hoagland's Trace Element Stock Solution

H_3BO_3	2.86 g/l
$MnCl_2.4H_2O$	1.81 g/l
$ZnSO_4.7H_2O$	0.22 g/l
$CuSO_4.5H_2O$	0.08 g/l
$Na_2MoO_4.2H_2O$	0.025 g/l

[b]B-D Vitamin Stock Solution

Thiamin HCl	10 mg/100 ml
Nicotinic Acid	50 mg/100 ml
Pyridoxin HCl	50 mg/100 ml

[c]Alternative: Use Stock Solution

$FeSO_4.7H_2O$	7.56 gm/l	combine.
Na_2EDTA	7.45 gm/l	Use 1 ml/l

BIBLIOGRAPHY

Broughton, W. J., and M. J. Dilworth. "Control of Leghaemoglobin Synthesis in Snake Beans." Biochemistry Journal, 125 (1971), 1075-1080.

[Burton, J.] Legume Inoculants and Their Use. Rome: Food and Agriculture Organization of the United Nations, 1984.

Ferguson J. J. and S. H. Woodhead. 1982. Production of endomyxorrhizal inoculum. A. Increase and maintence of vesicular-arbuscular mycorrhizal fungi. In: Methods and Principles of Mycorrhizal Research (N. C. Schenck, ed.), American Phytopathological Society, St. Paul, Minn. pp. 47-54.

Jensen, H. L. "Nitrogen Fixation in Leguminous Plants. I. General Characters of Root-Nodule Bacteria Isolated from Species of Medicago and Trifolium in Australia." Proceedings of the Linnean Society of New South Wales, 66 (1942), 98-108.

Lalonde, M., and H. E. Calvert. "Production of Frankia Hyphae and Spores as an Infective Inoculant for Alnus Species." In Symbiotic Nitrogen Fixation in the Management of Temperate Forests. Edited by J. C. Gordon, C. T. Wheeler, and D. A. Perry. Corvallis: Forest Research Laboratory, Oregon State University, 1979.

Murry, M. A., M. S. Fontaine, and J. G. Torrey. "Growth Kinetics and Nitrogenase Induction in Frankia sp. HFP ArI3 Grown in Batch Culture." Plant and Soil, 78 (1984), 61-78.

Phillips, J. M., and D. S. Hayman. "Improved Procedure for Cleaning Roots and Staining Parasitic and Vesicular-Arbuscular Mycorrhizal Fungi for Rapid Assessment of Infection." Transactions of the British Microbiological Society, 55 (1970), 158-161.

Roughley, R. J. "The Preparation and Use of Legume Seed Inoculants." Plant and Soil, 32 (1970), 675-701.

Somasegaran, P., and H. J. Hoben. Methods in Legume-Rhizobium Technology. Paia: University of Hawaii NifTAL Project and MIRCEN, 1985.

Vincent, J. M. A Manual for the Practical Study of Root-Nodule Bacteria. IBP Handbook No. 15. Oxford: Blackwell Scientific Publications, 1970.