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NITROGEN FIXING TREE

GERMPLASM PACKAGE

Jake Halliday

Symposium on Nitrogen Fixing Trees for the Tropics
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INTEGRATED NITROGEN FIXING TREE GERMPLASM PACKAGE

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Introduction

Nitrogen fixing trees (NFTs) are being acclaimed for their potential role in agricultural development. This acclaim has stimulated research on fast-growing, nitrogen fixing trees. Before any species can be put to effective use, whether it be in agriculture, agroforestry, or reforestation, reliable propagation technology is essential. This is a sobering prospect because of the special problems with propagation of leguminous trees. It behoves the research community to anticipate possible future constraints on emergence of a complete utilization technology and to phase the research to be addressed in a balanced manner. This paper discusses just what constitutes the germplasm unit of an NFT and describes strategies for its propagation.

Nitrogen fixing trees potentially benefit from at least two symbiotic relationships with microorganisms. Association with rhizobia confers nitrogen fixing ability. Infection with vesicular arbuscular (VA) mycorrhizae enhances phosphorus uptake. A vigorous NFT at a particular location is frequently a manifestation of an especially effective match between the tree genotype, its symbiotic partner(s), and its environment. In fact such a tree could be heavily dependent on its microsymbioses for its nutrition. Germplasm explorers need to be aware that later performance of a collected accession introduced to a new location may be below expectation unless a specific effort is made to reconstitute equally effective associations.

Introduced NFT species can fail to encounter fully effective microsymbionts spontaneously in the native microflora, and/or nodulate effectively with available inoculants. Even when the trees are inoculated this may be unsuccessful if inoculant strains do not match the tree's specific requirements. Thus an integrated approach to germplasm exploration, selection and introduction is warranted. Seeds, rhizobia and mycorrhizae can be viewed as inseparable components of the NFT germplasm unit.

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NFT Seed Technology

Relatively few NFT species are self-pollinated. Genetic heterogeneity of most NFT species is problematic at virtually every stage in conventional crop improvement strategies. Heterogeneity complicates germplasm exploration, selection, and multiplication and is confounded by the often lengthy generation times of even the fast-growing NFT species. So formidable is the task that releasing mixtures of seed of tree accessions that are phenotypically similar but which are genetically diverse may be the only practical approach to putting these species to work for development in the tropics.

For self-pollinated NFTs, seed production is relatively straight forward. But in fact very little research has been done on the specific culture of any NFT for seed production. Most seed "production" is actually the result of collection from natural NFT populations or from plantations established for purposes other than seed production. As the more promising NFTs move closer to being utilized on an extensive scale, seed production technology becomes increasingly important.

Research on management of Leucaena leucocephala for optimum seed production has been summarized in a technical manual (1). The manual covers: sources of foundation seed, orchard design, site preparation, inoculation and planting, weed control, pest and disease control, orchard maintenance, harvesting, and seed cleaning and storage.

In the case of leucaena, seed production per tree can be enhanced by specific management practices such as spacing and regular annual pollarding. Typical seed yields of K 8 giant leucaena under Hawaii conditions are 0.48 kg/tree at 1 m x 1 m Spacing, and 1.25 kg/tree at 2 m x 2 m spacing in the establishment year. Older trees in our main orchard are yielding about this same level.

It is difficult to understand why there continues to be a seed shortage of such a prolific seeder as the giant leucaena. A single, three year-old tree at Pukalani, Maui, yielded 16 kg of seed. This amount would be sufficient to plant 500 hectares.

It should be remembered that although a particular NFT might have been selected for its adaptation to a particularly stressful environment, seed production of the species need not be conducted under such conditions. The economics of seed production usually permit seed production to be pursued under conditions that favor seed production. These conditions might be very different indeed from those in which utilization of the tree is proposed.

Seed of cross-pollinated species need to be produced in plantings that are screened off or raised in isolation. The latter is the more practical with NFTs.

In Hawaii, cross-pollination of superior introductions of leucaena species with almost ubiquitous inferior naturalized leucaena is problematic. A novel approach to achieving seed production despite this difficulty has been to exploit leucaena's intolerance of acid soils. Naturalized leucaena is absent in certain acid soil enclaves on the Island of Maui. Thus pure seed of leucaena is being produced in such an area (Kuiaha site, Humoxic Tropohumult, pH 4.5) after liming only the immediate seed orchard to permit growth of the desired leucaena line.

Seed technology is at its soundest when the foundation seed is homogeneous. This is a real problem with many of the NFTs. Vegetative propagation and tissue culture approaches are perhaps overrated as solutions to this problem.

While it is relatively easy to achieve rooting of stem cuttings of some NFT species, noteably those that are used as living fenceposts, survival of the vegetative propagules of most species is highly variable under realistic reforestation circumstances. Also, propagation by stem cuttings has a high water requirement and is labor intensive.

Experiences with clonal propagation through tissue culture of legume species have been largely disappointing. This is especially true of the leguminous trees. There has been only limited research on propagation of NFTs by tissue culture methods. In their work with Hawaii's native *Acacia koa*, Skolmen and Mapes (2) found that only juvenile tissue (tips of root suckers) gave calluses. These were then stimulated to differentiate shoots and roots and were able to grow independently. *Acacia koa* is one of very few tree species to have been propagated by tissue culture.

Rhizobium Technology

Genetic diversity of planting material is just one of the features of NFTs that make it necessary to rethink some of the conventional approaches to selection of *Rhizobium* strains for use in inoculants. This section of this paper deals with rhizobial strain selection and inoculant production procedures.

Previous publications by the author (3,4) have defined step-wise screening procedures for selecting strains of *Rhizobium* to use in legume inoculants. Most selection procedures for crop legumes stress the matching of specific rhizobial strains with the host genotype. Such a procedure is valid for certain NFTs, but is inappropriate, at least in the short-term, for most NFTs that may be less defined genetically. Hopefully, in the long-term the host germplasm would be more homogeneous and inoculants could be developed on the basis of matched specific strains.

A second complication for inoculation of NFTs is that vegetative propagation may be necessary for some species. Conventional inoculant methods involve application of rhizobia directly to the propagule or indirectly to the soil. Modifications have not been validated for use with vegetative propagation. Such validation is necessary because delay time between planting and root emergence is much longer with vegetative propagules than in the case of seed germination. The period during which rhizobia are vulnerable to adverse factors is prolonged. There may not be survival of adequate numbers to effectively nodulate the root when it finally emerges.

The following account outlines an accepted approach to the selection of Rhizobium strains for use in legume seed inoculants. The procedures described were used successfully in a specific program concerned with the selection of appropriate rhizobia for forage legume introductions in acid, infertile soils of tropical Latin America (3). The principles underlying the approach apply equally well to strain selection for NFTs and some examples of alternative methodologies are mentioned in the text. Individual investigators can modify the techniques and improvise with equipment to suit their own purposes and the facilities available to them provided they take account of the underlying principles of Rhizobium strain selection stressed here.

Strain selection is performed to ensure that a legume seed inoculant contains a strain, or strains, of Rhizobium capable of forming fully effective, nitrogen-fixing nodules on the legume species for which it is recommended and under the conditions of soil and climate in which the legume crop is grown.

Some characteristics of strains of Rhizobium to be used as legume inoculants can be regarded as "essential" whereas others are "desirable" depending on the specific selection objective.

One esssential characteristic is the ability to nodulate the NFT of interest in the field conditions under which it is grown. Such strains are referred to as infective. Strains of Rhizobium which are infective in the field will usually have exhibited competitive ability if they displaced nodulation by native strains present at the site. They will also have been stress tolerant if they successfully nodulated legumes in soils with excesses or deficiencies in their physical/chemical composition.

A second essential characteristic is that the strain be able to fix sufficient nitrogen to sustain a level of legume production close to, or surpassing, the production possible if the legume were supplied with nitrogenous fertilizers. Such strains are referred to as effective. Strains which are fully effective are usually carbon efficient and hydrogen efficient as well. The "efficiency" of a Rhizobium strain is seldom measured during strain selection and use of the term in this context should be avoided. Effectiveness is usually what is meant.

A third essential character of an ideotypic Rhizobium strain is that it should perform satisfactorily when subjected to the component processes of commercial-scale inoculant production systems. Inoculant strains must multiply well in bulk culture and be able to mature to high populations in the carrier material.

A fourth essential character is ability to survive well during distribution to, and use by, farmers. Strains should be tolerant to the anticipated maximum temperature that they will encounter. They must also survive well during the seed/soil inoculation procedures used by farmers. Additionally, they must survive on seed in soil from the time of their application until the emerging legume radicle is susceptible to infection (usually at least seven days). Strains for NFTs will need to survive for even longer periods if they are used with vegetative propagules, and/or to cope with delayed germination. Characteristics which are in the "desirable" category are long-term persistence and fungicide/insecticide tolerance.

Long-term persistence is expected of strains of Rhizobium used to inoculate perennial species. Implicit in the concept of persistence is saprophytic competence, a summary term for all those traits that permit a Rhizobium strain to live as a stable member of the soil microflora, even in the absence of its legume host. Persistence of strains for annual crop legumes from season to season may be considered a desirable trait in some circumstances, as it obviates the need for inoculation in subsequent years. But there may be cropping systems in which carry-over strains from a previous crop may nodulate a following crop relatively ineffectively and even out-compete effective introduced strains. This can occur in rotations of soybean with peanut and cowpea that nodulate with the cowpea miscellany.

Fungicide or insecticide resistance may be desirable traits when normal practice is to sow legume seeds pre-treated with these substances, some of which are toxic to most stains of Rhizobium.

Rhizobium strains do vary widely in the characteristics listed above. Some strains nodulate some genera, or species, or varieties of legumes and not others. This has given rise to the durable, but highly criticized, taxonomy of rhizobia based on their cross-inoculation affinities. Among the strains capable of infecting and nodulating a particular legume, there is great variation in the amount of nitrogen they fix, i.e., variation in effectiveness. There is considerable strain variation in the other listed traits as well and thus an opportunity exists to select superior strains. Unlike higher plants which can be improved through breeding and hybridization, Rhizobium improvement is currently practical only by selection from natural populations.

As will be appreciated from the following procedures, the selection of superior Rhizobium strains is a lengthy undertaking.

Several years of study may be necessary to complete characterization and testing. Given that strains of Rhizobium for many legumes, including some NFTs, have already been developed at research labs around the world, it makes sense to obtain and use these, rather than initiate an extensive selection program (5). Selection of rhizobia is only really justified when the specific selection objective cannot be satisfied by strains held in existing collections. Examples of circumstances under which strain selection may be required are as follows:

(1) When the legume of interest is an uncommon species for which there is no recommended inoculant strain. This is the "state-of-the-art" for the majority of NFTs.

(2) When inoculation of the particular legume with recommended strains of Rhizobium under field conditions fails to give adequate nodulation and nitrogen fixation. This can occur if the legume variety is different from that with which the inoculant strain was developed, or if the soil and climatic conditions vary from those under which the inoculant was developed.

A step-wise selection procedure will be described for the development of a Rhizobium strain recommendation for legumes planted under a particular soil condition. This approach is unconventional in the sense that strains of Rhizobium in current use as legume seed inoculants are developed for the species of legume with which they will be used, rather than the soil type in which the legume will be grown.

In the technologically advanced countries, it is normal farm practice to modify soil conditions to be suitable for a particular crop. It is not unreasonable, therefore, to expect a rhizobial inoculant for a legume species to perform well wherever that legume is grown. In the developing nations, however, soil amendment is minimal or not practiced at all, and crop plants are often grown under stresses of adverse soil factors that cannot be economically alleviated. For most utilizations, e.g., reforestation, NFTs will be introduced to unamended sois. It may be unreasonable to expect that a single strain of Rhizobium will perform equally well as an inoculant in the wide array of soil types under which its host legume is grown in the tropics. One reason that legume inoculation is not widely successful in developing countries is that available inoculants obtained from the U.S., Australia, or elsewhere do not have strains selected for, and adpated to, the extremes of soil stress encountered in the tropics (6).

There is a widely held view that strain selection and legume inoculation have little potential for improving yields of tropical legumes since tropical legumes are not specific in their Rhizobium strain requirements, and because suitable rhizobia occur universally in tropical soils. Spontaneous nodulation of NFT species in their natural environment creates an impression that specific inoculation is not called for (Appendix III).

There are a few notable exceptions, such as soybeans and leucaena, and thus two categories of tropical legumes were recognized. The promiscuous (P) group can be nodulated by a wide array of strains of tropical rhizobia. The specific (S) group requires specific rhizobial strains for nodulation. The majority of tropical legumes were judged to belong to the P group and it has been generalized that it is unnecessary to inoculate these legumes with rhizobia, as no benefit would be expected.

The grouping of tropical legumes simply as S or P types is no longer tenable nor useful. Many tropical legumes previously placed in the P group are now known to form fully effective (i.e., high nitrogen-fixing) symbioses with only a few strains out of the diverse array of rhizobia that can nodulate them. Thus a distinction is drawn between this promiscuous-ineffective (PI) group of legumes and the promiscuous-effective (PE) group (7). Studies of the Rhizobium affinities of the tropical forage legumes, for example, reveal that a majority of them are in the PI group, suggesting a potential for increasing their production by providing appropriate strains of rhizobia.

The important role played by stress factors of tropical soils as modifiers of symbiotic performance is now well recognized (6). Thus tropical legumes can and do benefit from inoculation when strains are selected specifically for the particular variety of legume being planted and for tolerance of the soil conditions in which that legume is to be grown.

No strain selection program should be undertaken without clear definition of the specific selection objective(s). The methods of selection employed may need to be modified to suit the objective. The specific selection objective for which the procedures that follow were developed was to select strains of Rhizobium able to nodulate and fix nitrogen in association with acid tolerant legume accessions being introduced to the acid, infertile soils of Latin America.

Successful selection of superior rhizobia is favored if the number of strains from which the selection is made is large and diverse. The most meaningful test of Rhizobium performance is field evaluation since this is an integrated appraisal of the various traits that make up a successful inoculant strain. However, the management of field trials to select rhizobia is difficult and costly, even when the number of strains under test is small. Multi-stage screening procedures that progressively eliminate undesirable strains from an initially high number of contenders yielding a relatively small number of promising strains for testing at the field level. This is one way to reconcile the requirements that selection be from a diverse genetic base, and that strains also be assessed under field conditions.

It is advisable to include in the screening procedure strain of Rhizobium that originated from a diverse array of host plant germplasm and that are representative of diverse geographic regions. But some reduction of the number of strains can be made

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based on what is known from other selection programs. In general, rhizobia isolated originally from the same genus, and sometimes species, as the legume for which a superior strain is being sought emerge from selection programs as the best strains for use in legume inoculants. Also, when the specific selection objective includes tolerance to a particular soil stress or climatic condition, rhizobia isolated from legumes growing under those conditions are the most likely to be rated highly in the selection process. Hopefully, there is a Rhizobium collection or collections of authenticated strains of known origin available to the investigator. Otherwise, a suite of strains has to be assembled. Present status of recent strain acquisition for NFTs at NifTAL is reflected in Appendix I. Only after checking whether likely strains are available from existing Rhizobium collections, such as the Rhizobium Germplasm Resource at NifTAL, should collection and isolation of new strains be contemplated. Detailed procedures for the collection, isolation, purification, authentication, characterization, and preservation of strains of Rhizobium are described elsewhere (3,4). Pre-selection of strains with suitable background should aim to generate a cluster of 50-100 rhizobia that will feed into Stage I of the strain selection procedure.

Stage I - Screening for Genetic Compatibility: In this stage, strains of Rhizobium are screened for ability to nodulate the legume of interest. The test used involves a high degree of bacteriological control and is suited to handling large numbers of strains. The system most commonly used is based on growth tubes in which seedlings are raised in a solid, nutrient medium under artificial illumination. Seeds must be surface sterilized, usually with concentrated sulphuric acid, hypochlorite, or acidified mercuric chloride. They are pre-germinated in inverted, sterile perti dishes of water agar. When the radicles are 3-5 mm long, uniform seedlings are transferred aseptically to tubes containing agar deeps (or slants). Tubes are routinely 2.5 x 25 cm, capped with a plug of muslin-wrapped cotton wool. Aliquots of 1 ml of suspension of the test strains are added to each tube either at transplanting or 3-5 days later. At least three replications of each strain treatment are essential and five are preferred. Roots of seedlings should be shielded from light. Alternatively, tubes may be wrapped in aluminum foil. Two control treatments are required. In one case the plants are "inoculated" with sterile water only (uninoculated control) and in the other case they are provided with 70 ppm nitrogen as ammonium nitrate (or potassium nitrate) solution (plus nitrogen control). Tubes are scored at intervals for the presence or absence of nodules. With many tropical legumes, tumor- or callus-like outgrowths can occur on roots of seedlings raised in growth tubes. These outgrowths occur in the presence or absence of rhizobia and are not nodules. They cannot be distinguished from nodules by eye. Plants should be harvested from tubes and checked under a binocular microscope for real nodules. "Apparent" nodules lack structural organization and leghemoglobin. Timing of the harvest varies depending on legume species but will usually be about 35 days after sowing.

Some investigators place significance on other data taken on plants grown in growth tubes. "Earliness to nodulation" may be of some value. It is inappropriate, however, to attribute relative nitrogen fixation effectiveness to strains based on nitrogen accumulation in plants raised under such artificial conditions. The root medium and atmospheric composition within plugged test-tubes differ from those which the plants require for optimum performance and may constrain expression of nitrogen-fixing potential.

Alternate methodologies are required for large-seeded species that quickly become cramped in growth tubes. These include the use of growth pouches or "Gibson" tubes. Growth pouches are made of autoclavable plastic and have an absorbent towel insert. Seedlings germinate in a fold (or are pre-germinated and transplanted into the fold) at the upper rim of the pouch. Roots develop within the pouch nourished by a nutrient medium, and plant tops grow in the open air. The method offers the advantage that effective nodulation can be reliably determined, but caution in attributing relative effectiveness of strains on a pouch test basis is necessary. Modifications of the method include subdividing the pouches with heat bonding to permit a single pouch to be used for several strain treatments, or replications of the same treatment.

In the case of "Gibson" tubes, the tube contains a long agar slant that reaches to the upper rim of the tube, and are filled to the rim with liquid medium or sterile water. They are capped with aluminum foil. Radicles of pre-germinated seedlings are entered through a small orifice in the aluminum. The roots develop inside the tube and the plant tops grow outside the tube. The method offers similar advantages to those of pouches, namely that effective nodulation shows up readily. Modifications of "Gibson" tubes include omission of the liquid phase or half filling the tubes.

Obviously nodulation in the uninoculated control treatments in Stage I raises concern about inadequate bacteriological control and invalidates the experiment.

Some texts advocate dedication of entire light rooms for the culture of plants in growth tubes. Most workers will find a low cost system of racks and portable fluorescent tubes more than adequate for their needs. Such a system is highly flexible and can be readily modified to serve for pouches or "Gibson" tubes that require overhead illumination. The issue of light quality has been overplayed. Regular domestic fluorescent lamps have served satisfactorily in the screening procedure described here.

Stage II - Screening for Nitrogen Fixation Effectiveness: In this stage the objective is to rank infective strains from Stage I in order of potential nitrogen fixation effectiveness with the legume species/cultivar of interest. Theoretically, in this test there should not be any factors limiting growth of the legume except nitrogen, so that full expression of each strain's

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nitrogen fixation effectiveness is possible. In practice it is assumed that the nutrient regime and other aspects of growth conditions are not limiting, even though there are known examples of legumes for which standard conditions are not non-limiting. Sand jar assemblies are used in this test because they permit more realistic growth conditions than tubes, pouches, etc., but retain the high degree of the bacteriological control which is still essential if valid results are to be expected.

The Leonard jar is one example of such a sand jar assembly. Watering is the most common source of contamination in Rhizobium strain testing in pots and in the field. Leonard-type sand jars greatly reduce the frequency of watering and are, therefore, less prone to contamination. Sand jars are easily constructed from locally available materials, but have the disadvantage that sterilizing them requires a very large autoclave.

As with growth tubes, surface sterilized pre-germinated seeds are sown in the sand jars. Four seedlings are allowed to establish and thinned later to two by snipping off the tops. Drops (standardized rate) of suspensions of strains of Rhizobium are added to seedlings in the jars five days after sowing (one strain per jar). Plants are harvested destructively at a time after sowing that depends on the legume species under test. Usually 60 days after sowing is appropriate.

Data taken on sand jar experiments vary from investigation to investigation and include the following:

- nodule number
- nodule dry weight and/or fresh weight
- nodule color
- nodule distribution
- total plant fresh/dry weight
- top weight (fresh/dry)
- root weight (fresh/dry)
- acetylene reduction rate
- percentage N in tissues
- total N produced

Of these, total N produced is the most meaningful integration of nitrogen fixation effectiveness over time and as this is highly correlated with total plant dry weight, a reliable measure of relative effectiveness of strains of Rhizobium is possible with nothing more sophisticated nor costly than a common balance.

The main problem encountered with this test relates to overheating in greenhouses or growth rooms where the experiments are performed. Most of the sand jar trials observed by this author in the tropics are, in fact, selecting high temperature tolerant rhizobia at the same time! Other problems relate to the occasional failure of the irrigation from beneath which depends on capillary rise, and breakage of glass components in autoclaving and handling.

Strains are ranked on the basis of their yields in Stage II. The demarcation of effectiveness categories is somewhat subjective, but nevertheless useful. Strains are assessed relative to the uninoculated control and the nitrogen control and described as (in ascending order of merit) parasitic, ineffective, partially effective, moderately effective, or fully effective.

Ordinarily about 30-50 strains would be evaluated at Stage II in Leonard jars. Three replications are essential and five are preferred. The top ten strains are chosen for further screening at Stage III.

The principal merit of Leonard jar trials are that data on the potential effectiveness of strains of Rhizobium with a particular legume tend to be upheld in independent screening trials by other investigators. Thus, researchers can exchange information that is stable and demonstrable on the nitrogen-fixing potential of strains. Pot and field trials, on the other hand, give information of the plant/Rhizobium soil interaction that may or may not be repeatable at other locations.

Stage III - Screening for Symbiotic Effectiveness Under Physical, Chemical and Biological Stresses of Site Soils: The fully effective nitrogen fixation effectiveness expressed under Stage II conditions will not necessarily be upheld under real field conditions. Thus, before selecting a final cluster of three strains of Rhizobium for field evaluation, it is advisable to subject a larger group (ten) of potentially effective strains to some of the physical chemical and biological stresses of soils for the inoculant is being developed. This stage is particularly useful if the specific selection objective(s) includes adaptation to a particular stress, such as soil acidity. Stage III also has a value in selection programs for "non-stress" soils. In Stage II sand jar evaluation, test strains did not have to compete against native rhizobia.

This third stage involves a pot experiment in which strains are tested with the host plant and production related to that of uninoculated control plants and nitrogen fertilized plants. Soil is collected from the plough layer and mixed to uniformity to produce a homogeneous experimental material. Unsterilized soil is used. Soil may be amended at fertilizer rates equivalent to field practice, but only the nitrogen control plants receive nitrogen (equivalent to 100 kg N/ha). Procedures for calculating the fertilizer additions are detailed elsewhere. Not all soils behave satisfactorily in pot experiments and other amendments may be necessary, particularly with heavier soils. The following should be considered:

(1) sieving to remove large soil aggregates and stones.

(2) addition of high carbon ratio residues such as bagasse at 1-2% (dry weight basis) to counter balance excessive mineralization of nitrogen resulting from soil handling.

(3) addition of volcanic cinder, vermiculite, or other materials to improve soil aeration and drainage.

Sowing procedure and inoculation is the same as for sand jars in Stage II. About 6-8 seedlings are planted and thinned to 2-4 plants/pot, depending on the species. Thinning is by snipping off the plant tops, rather than pulling entire plants from the soil. Size is optional, but 20-25 cm in diameter is usual. Six replications of each treatment are required.

Precautions against cross-contamination in this stage are essential. Watering, which in greenhouses in the tropics is needed daily, is the primary source of contamination. It can be minimized by:

(1) filling pots so that the soil level is 3 cm below the pot rim.

(2) watering gently to avoid splashing.

(3) using grid or mesh benches instead of solid benches, so that pots can drip through onto the floor.

(4) raising pots on supports (such as petri dish lids) so that there can be no water flow on the bench surface from the emergent roots from one pot to those of another.

(5) assigning watering to a single, informed individual.

Other precautions include avoidance of overheating of the roots and nodules in pots and minimizing non-treatment effects. Pots should be set up in a randomized, complete block design but not re-randomized thereafter because of the overriding problem of contamination through handling.

As with sand jars, plant dry matter production is the most meaningful parameter to be determined and is the basis for ranking strains. The top three strains are promoted to Stage IV.

Stage IV: - Single Location Evaluation of Strains of Rhizobium and Inoculation Methodology under Field Conditions: Strains emerging from Stage III are evaluated for nodulation and nitrogen fixation under field conditions. Although the preferred measure of the response by a legume to inoculation with the test strains of Rhizobium is grain yield (dry matter production in the case of forages), there are many factors which, under field conditions, can prevent differences in nitrogen fixed by the strains being translated into differences in yield. Therefore, field trials should include a mid-season harvest to determine dry matter production. Plot size should be sufficient to house two fully bordered harvest areas. The standard plot layout used in the International Network of Legume Inoculation Trials (6,7) is recommended.

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When the specific selection objective includes overcoming soil stress, the field trial at Stage IV can amalgamate the strain selection approach and other strategies for overcoming the stress. In this case, several inoculation methods were appraised for their ability to overcome the effect of acid soil stress on nodulation. Simple seed inoculation with an aqueous suspension of peat-based inoculant containing the test strains was one treatment. Others involved pelletting the inoculated seeds with finely-milled lime or rock phosphate. These treatments were compared to control plots of uninoculated plots and plots fertilized with nitrogen. The comparison between these treatments is most valid, in a scientific sense, when there are no other factors limiting plant growth. But the comparison is most realistic when the level of agronomic inputs is economically feasible and similar to that used by farmers in the region where the legume is grown. In the procedure adopted, the scientific validity was considered of lesser importance than the need to be realistic and a minimal blanket fertilization of elements other than nitrogen was applied. Three replications of the treatments were established in a randomized complete block design. Experience has indicated that four replications are desirable.

Precautions against cross contamination are of paramount importance. Common pathways of contamination are:

(1) careless handling of inoculated seed at planting time.

(2) use of field implements without sterilizing them between plots.

(3) tramping from plot to plot (by laborers, animals, visitors, etc.)

(4) run-off and other drainage problems caused by poor site selection.

The best Rhizobium/inoculation method combination is then selected and subjected to further testing in Stage V. It could be justified to produce and use legume inoculant based on Stage IV evidence, but there remains the risk that the selected strain will be a successful inoculant only in the specific soil and climatic conditions under which it is was selected. A further stage is essential to determine the range of suitability of inoculant developed for a single location in Stage IV.

Stage V: - Multi-location Testing of the Response to Inoculation with Selected Rhizobium Strains: A standard design developed for the International Network of Legume Inoculation Trials (INLIT) is available for those contemplating multi-location trials on the response of legumes to inoculation with selected strains of Rhizobium (8). One of the major constraints to fuller utilization of legume inoculation in the tropics is that there has not been convincing demonstration on a wide scale that yield increases will result with local legume varieties under local soil and climatic conditions (7). Stage V trials can assist in

deriving the data necessary for predicting more reliably whether a legume will respond to inoculation or not.

The trial has three basic treatments: plants inoculated with Rhizobium; plants not inoculated; and plants not inoculated but fertilized with nitrogen. The comparison is made at two fertility levels which, for convenience, shall be referred to as "farm fertility" and "maximal fertility." Fertilizer levels are determined on the basis of information available locally.

With three treatments at two fertility levels replicated four times, a 24 plot, randomized, complete block design results. The treatments in the first replication can be deliberately arranged to serve as a demonstration in which the treatments that are most frequently compared are located side-by-side to facilitate visual observation of treatment differences. Plot arrangement is the same as for Stage IV (Figure 4). Experimental layout is as in the International Network of Legume Inoculation Trials (8). Row spacing, planting distance, and seed depend on the legume in question and plot size will necessarily be bigger for NFT inoculation trials.

The plus nitrogen control plots receive 100 kg N/ha but in two doses. At planting, 25 kg N/ha are applied and 75 kg N/ha added 4-5 weeks later in the case of grain legumes. With forages the 25 kg/ha is applied at planting and 25 kg N/ha applied after each cut (approximately 3 month intervals).

It is best to sow the "uninoculated" and "nitrogen fertilized" plot first. Only after the seeds in these plots have been covered are the inoculated seeds prepared for sowing in the remaining plots. This minimizes the risk of contamination of the plots that are not to receive rhizobia.

Stage V trials can be used to characterize selected strains for competition and persistence if the inoculant strain is "marked" serologically or with antibiotic resistance. Such strains of Rhizobium can be detected in the nodule population and their ability to compete against strains native to the site determined. These strains can also be detected, if present, in the soil in following seasons, or in the nodule populations of subsequent legume crops sown uninoculated.

The International Network of Legume Inoculation Trials (INLIT) coordinated by the University of Hawaii NifTAL Project are available for 17 agriculturally important tropical legumes including the NFT Leucaena leucocephala. Inoculants developed for INLIT each contain three serogically distinct, effective strains of Rhizobium from diverse geographic and host germplasm backgrounds (8). Each INLIT is potentially an ecological study of the relative performance of the three exotic strains between themselves and in competition with indigenous soil strains. It is also a long-term persistence trial. A mixed inoculant of 6 marked strains is now offered for NFT research.

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For some specific selection objectives, the development of rapid screening procedures may reduce the time taken to develop a reliable inoculant strain, or may greatly increase the likelihood of successful inoculant strains emerging from the step-wise screening previously described. For example, in the case of selection of rhizobia for acid, infertile soils, a laboratory prescreening that preceded the Stage I test greatly increased the range and numbers of strains that could be addressed. It eliminated effective strains predestined to fail in the field but which would have passed through Stages I, II, and possibly III consuming time and resources. The prescreening test was based on the reasonable assumption that for a strain of Rhizobium to be a successful inoculant for legumes grown in acid soils, ability to multiply well at low pH is an essential trait. Synthetic media were developed that tested ability to multipy at low pH, and only those strains passing the test were fed into the step-wise screening program (9). Investigators may find it useful to adopt rapid prescreening steps for their own objective(s).

As with any screening program, there is always the risk that discarded materials that could not be accommodated in the later stages would have performed well in the field. In the procedure described, the stage-to-stage transition that is most problematic is that from Stage II to Stage III. Rankings of strains in sand jars do not necessarily hold up when subjected to the stresses of site soils. Although 10 fully effective strains are passed across from II to III, examples have occurred in which as few as three of the strains could nodulate at Stage III and only one of these was effective.

When dealing with uncommon legume species, such as NFTs, an investigator should be concerned about whether the routine media used in Stage I and Stage II are, in fact, non-limiting on growth of the legume plant so that Rhizobium characters can be expressed. As an example of this, it was found that Stylosanthes capitata, a legume with high tolerance to soil acidity factors and native only to acid soil regions of South America, could not be nodulated by any one of more than 100 Stylosanthes isolates (including many specifically from S. capitata) tested at Stage I. Nor would S. capita grow in Stage II. Only when the growth medium was acidified to a pH lower than 5.0 and the Ca and P levels lowered ten-fold would the plant nodulate and grow.

Even though the screening procedure is lengthy, attempts to short-cut the sequence are ill-advised. Recommendation of strains of Rhizobium for NFT inoculation without first performing field trials similar to those described in Stage IV and Stage V is risky in the face of accumulating data that indicate that site variation in performance of selected strains is common (10).

The underlying objective of inoculation technology is to place such high numbers of preselected strains of rhizobia in the vicinity of the emerging root that they have a competitive advantage over any indigenous soil strains with lesser N-fixing ability in the formation of root-nodules.

Inoculation technology involves: selection of strains of rhizobia that are compatible and effective N-fixers with particular legumes; multiplying selected strains to high population densities in bulk cultures; incorporating the liquid rhizobial cultures into a carrier material (usually finely milled peat) for packaging and distribution; and finally, coating the seeds of legumes with the carrier or implanting the soil with the inoculant directly into the seed drill.

In addition to the selection criteria already described, inoculant strains need an ability to grow and survive in peat inoculants.

The host genotype interacts with the infecting strain of Rhizobium in determining the level of nitrogen fixation with the host playing the dominant role. Thus two sources of variation (plant and Rhizobium strain) can be exploited in selection programs. Most commonly, though, the plant is selected independently and a suitable strain sought thereafter, thus allowing only for exploitation of strain variability. The range of specificities of host genotype interactions is well illustrated by soybean and the African clovers.

Such specificities give three options in the approach to selection of strains for inoculants: numerous inoculants, each with a highly effective strain for individual species; "wide-spectrum" strains that vary from good to excellent in nitrogen fixation with a range of legumes; or multiple-strain inoculants containing the best strain for each host species. There may be a conflict between the option that would be chosen for commercial expediency and that which is scientifically excellent. In Australia "wide-spectrum" strains are used when these are available, but there is increasing use of specialized inoculants with specific strains for individual hosts. Despite findings which suggest that multi-strain inoculant should be avoided because of possible antagonistic and competitive effects in culture and the likelihood of competition in nodule formation from the less effective strains, this is the approach used successfully by the U.S. inoculant industry.

The number of NFT species to be addressed exceeds 1,000 (Appendix II). An expert group reduced the list of NFTs of highest priority to 44 species. Inoculant for these is needed even before development of specialized inoculants can be completed, and NifTAL advocates a multi-strain inoculant incorporating wide-spectrum, fast- and slow-growing rhizobia. Results to date with this inoculant vindicate this approach (11).

Most legume inoculants are prepared by adding liquid cultures of Rhizobium to a finely-ground carrier material such as peat. Although mixtures of peat with soil or compost mixtures, lignite, coir dust and some other organic materials have been used, peat has proven to be the most acceptable carrier worldwide. Agar, broth and lyophylized cultures are not recommended because of the very poor survival of these forms of the inoculum on seed.

Peat cultures can be prepared in two ways. Either ground (milled) peat is mixed with a high variable count (more than 10^9 rhizobia/ml) broth culture in sufficient volume to provide the minimum number of Rhizobium acceptable for use, or sterilized peat is inoculated with a small volume of culture and incubated to allow multiplication of the rhizobia in the carrier (12). The choice of method will depend on two main factors: the survival of the rhizobia in peat in numbers high enough to meet a minimum standard of quality; and the availability of suitable, sterilizable containers and sterilizing facilities. The two factors that most affect survival of rhizobia in peat are temperature of storage and sterility of the peat. There are differences among species and also between strains of the same species of Rhizobium in their ability to survive well in peat.

Like all biological products, legume inoculants are prone to loss of quality owing to variation in the organism concerned and from unforeseen factors affecting some aspect of growth or survival. Quality control is an indispensable component of inoculant technology. In Australia, large scale manufacture of legume inoculants is by private enterprise, and an independent (government) control laboratory maintains and supplies recommended strains of Rhizobium to the industry. This laboratory checks strains annually for ability to fix nitrogen, assesses quality of cultures during and after manufacture, and conducts such research as may be necessary to overcome problems associated with production and survival in the final product. In the U.S., the industry is free to select its own strains and official control ensures only that the product can form nodules on the legume for which it is recommended.

Although control of quality of inoculants is primarily in the manufacturer's interest and therefore his responsibility, power of control by external bodies provides protection from less scrupulous operators and genuine failure of a strain beyond the manufacturer's control. Not all countries back their control labs with legislation. In Australia, this control extends to holding stocks of the strains used in inoculants. This is not the case in the U.S.

In addition to assessment of quality throughout manufacture, it is important to monitor quality of product in retail outlets. Standards acceptable at this level may vary from that at manufacture and between countries. It is important that standards be realistic and within the capability of manufacturers, yet ensure that sufficient viable rhizobia are applied to the seed to provide a satisfactory inoculation. This can be as few as 100 rhizobia per seed but in cases of severe environmental stress as high as 10,000 or even 500,000. Despite several attempts, it has not been possible to gain acceptance of a universal set of standards for inoculant products.

The first attempts at inoculation involved the transfer of soil from one field to the next, but with the isolation of the organisms responsible for nodule formation, artificial cultures

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soon replaced the laborious soil transfer technique. The usual inoculation technique is to treat seed just before sowing either with a dust or with a slurry in water or adhesive solution. Adhesives such as gum arabic and substituted celluloses not only ensure that all the inoculum adheres to the seed surface but also provides a more favorable environment for survival of the inoculant. Pelleting of seed with finely ground coating materials such as lime, bentonite, rock phosphate and even bauxite have been used to protect rhizobia during their time on the seed coat. Pelleting is a simple on-farm technique but custom-pelleted (by seedsmen at farmer's request) and preinoculated seed is now more popular. This latter procedure is potentially able to provide high populations of rhizobia on the seed for long periods of time (one growing season to the next) but has not yet been fully developed or exploited. Most preinoculation procedures are based on multiple coatings, alternately of adhesive and finely ground pelleting materials as used in simple pelleting. The peat inoculant is included as one (or more) of these coating layers.

Soaking seeds in a broth suspension and then exposing them to either high pressure or vacuum to impregnate the rhizobia into or below the seed coat has not proven successful. Theoretically, rhizobia introduced in this way would be protected from drying and other adverse environmental conditions, but the quality of products produced commercially has been variable to very poor. It is, in fact, an indictment of the research workers in such inoculant methods that 25 years has yielded so little progress in an area that has so much to offer for those concerned with the practical aspects of agricultural microbiology. The preinoculation technique is particularly applicable in a development setting because a high quality and reliable product could be marketed by a manufacturer or seed distributor without the need for farmer involvement in legume inoculation.

An alternative to pelleting and preinoculation in recent years has been the use of concentrated liquid or solid granular peat culture. These are sprayed or drilled directly into the soil with the seed during planting. Suspensions of rhizobia either as reconstituted frozen concentrates or suspensions of peat inoculant can be applied with conventional equipment. Similarly, granulated peat inoculants can be drilled in from separate hoppers on the drilling equipment. These methods have been especially successful for introducing inoculant strains into situations where there are large populations of competing naturally occurring soil rhizobia or in cases of adverse conditions such as hot-dry soils and where insecticide or fungicide seed treatment precludes direct seed inoculation. Solid inoculant, also known as granular or "soil implant" inoculant is advantageous also where seeding rates for crop legumes of 70-100 kg/ha make on-the-farm inoculation logistically impractical. It is these granular inoculants that would appear most appropriate also for use with NFTs.

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Vesicular Arbuscular Mycorrhizal Technology

Mycorrhizal infection of many NFTs occurs spontaneously in field soils in Hawaii. Thirty species of NFT from the NFT Germplasm Resource held at NifTAL (Appendix III) were sown in Hamakuapoko soil (Typic Haplustoll, ph 6.9) on Maui. Naturalized vegetation at the site includes spiny amaranth (Amaranthus spinosa), some wild Cruciferae, and the legumes Indigofera fructicosa and Leucaena leucocephala. All but one of the introduced species were observed to be heavily infected with VA mycorrhizae by 12-16 weeks after planting (Table 1). This suggests that specific inoculation of NFT seeds with VA mycorrhizae may be unnecessary.

Table 1. Observations on presence or absence of nodules and the degree of VA mycorrhizal infection on roots of leguminous trees introduced to Hamakuapoko soil.

NFT No.	Species	Nodulation	VA Mycorrhizal Infection
101	Acacia albida	yes	90 %
106	Acacia holoserica	yes	42 %
171	Acacia mangium	yes	80 %
152	Acacia mellifera	yes	76 %
103	Acacia nilotica	yes	91 %
154	Acacia nubica	yes	88 %
157	Acacia seyal var. seyal	yes	99 %
338	Albizzia chinensis	yes	96 %
181	Albizzia falcataria	yes	94 %
185	Albizzia julibrissin	yes	91 %
182	Albizzia moluccanna	yes	97 %
161	Calliandra calothyrsus	yes	96 %
321	Cassia siamea	no	100 %
320	Enterolobium cyclocarpum	yes	98 %
127	Julbernardia globiflora	no	28 %
569	Leucaena leucocephala	yes	95 %
114	Prosopis africana	yes	90 %
116	Prosopis juliflora	yes	94 %
323	Samanea saman	yes	100 %
303	Sesbania grandiflora	yes	86 %
120	Tamarindus indica	no	98 %

(from Halliday and Nakao, 1982)

It has been shown that leucaena seedlings raised under nursery conditions did not become infected spontaneously in a peat moss/vermiculite rooting medium. The medium had not been sterilized, but it is presumed that the source materials were largely free of mycorrhizal spores. Following transplanting to Hamakuapoko field soil, seedlings became progressingly infected with VA mycorrhizae and after 8 weeks attained a level of infection (95%) typical of field grown leucaena (Table 2).

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Table 2. VA Mycorrhizal infection of *Leucaena leucocephala* established by direct seeding or by transplanting. (Data of P. Nakao, unpublished)

Plant age (days)	VA Mycorrhizal infection (as percentage)			
	direct seeded		transplanted	
21	51	in field	0	nursery
49	74		0	
56	82		0	
63	95	in field	2	in field
70	>95		43	
84	>95		61	
112	>95		95	

-- nursery plants raised in dibbling tubes in a non-sterile peat moss-vermiculite mixture (3:5 ratio by volume) and transplanted to the field on day 60.

-- Typic Haplustoll, pH 6.9, Hamakuapoko, Maui, Hawaii.

Further research is necessary to determine whether other species are readily infected with native VA mycorrhizae and whether VA mycorrhizae are ubiquitous in tropical soils.

None of the above considerations precludes the possibility that at some point in the future, mycorrhizal inoculant technology might emerge based on displacement of relatively ineffective native strains by selected strains that are more highly effective phosphorus absorbers. But for the present, inoculation of NFTs with mycorrhizae seems unnecessary. This is perhaps just as well because inability to raise VA mycorrhizae in the absence of a host plant remains a serious obstacle to large-scale production of VA mycorrhizal inoculants.

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